

Fungal Polysaccharides

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FOREWORD

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PREFACE

Most fungi produce some type of polysaccharide that can be found in cell membranes or walls and as exocellular components. The role of these polysaccharides is not well understood, but in some cases they function as structural components of the fungal cell wall. This does not mean that fungal polysaccharides do not have important roles. For example, fungal polysaccharides are involved directly in host-pathogen interactions between fungi in mammals and plants. Also, surface exocellular polysaccharides of industrially useful fungi (yeast in particular) have been studied in great detail in order to determine their role in various specific applications.

Like bacterial polysaccharides, fungal polysaccharides of several types have been discovered that vary widely in their sugar composition, linkage types, molecular parameters, and physical properties.

This volume focuses on various aspects of fungal polysaccharide research that are currently being carried on throughout the world. Therefore, topics in this volume vary from the genetics of polysaccharide biosynthesis to host-pathogen interactions and from immunochemistry to the chemical and physical characterization of specific and interesting fungal polysaccharides that have industrial utility.

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October 23, 1979

KAZUO MATSUDA

Genetics of Yeast Mannoprotein Biosynthesis

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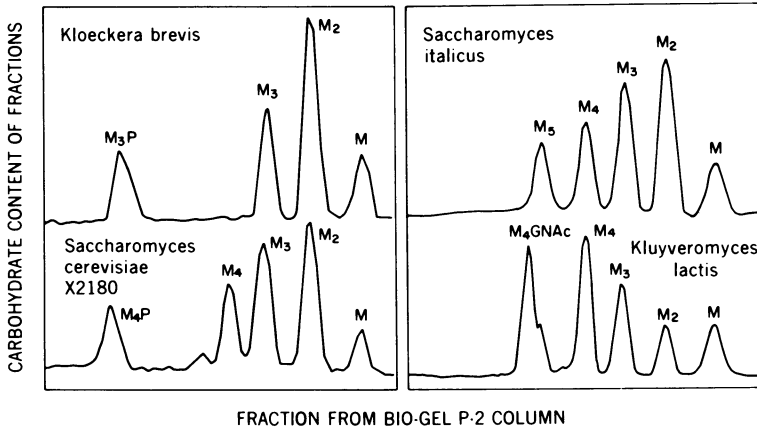
In this paper I intend to summarize what we know about yeast mannoprotein structure, what features of the biosynthetic pathway have been defined, how genetic techniques have been useful in gaining this information, and what is known about the genetic control of mannoprotein biosynthesis.

Yeast Mannoprotein Structure

Prior to 1965, the limited information available was due mainly to the methylation studies of Haworth *et al.* (1) which indicated that yeast mannan was a highly-branched polysaccharide with 1→2, 1→3 and 1→6 linkages, in no specific order. Dramatic developments have occurred during the last 10 years that have revolutionized our understanding of the structure of this very complex glycoprotein we now call a mannoprotein. The first important development was the acetolysis procedure, first applied by Gorin and Perlin (2) and then extended in a series of papers in our laboratory (3-10), that allowed selective cleavage of the 1→6 linkages in the mannan component of the glycoprotein and facilitated the isolation of oligosaccharide fragments whose structures could then be established by classical methods. From a comparison of the acetolysis oligosaccharide patterns (Figure 1) from different mannoproteins (6), it was clear that the mannan structures were species-specific (11).

The second important step in elucidation of mannoprotein structure was the discovery of an enzyme with the ability to remove the mannan sidechains selectively so that the backbone structure could be studied (12). As a consequence, several yeast mannans, although not all, have been shown to have a linear α 1→6-linked backbone (13, 14, 15). As a corollary, it follows that the acetolysis oligosaccharides represent sidechain fragments of the mannan, and by their characterization we were able to arrive at schematic structures for the mannan chains (Figure 2).

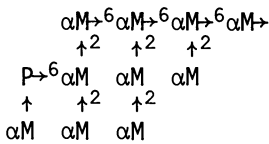
These two developments facilitated a third; namely, the elucidation of the immunochemistry of the yeast mannoproteins.



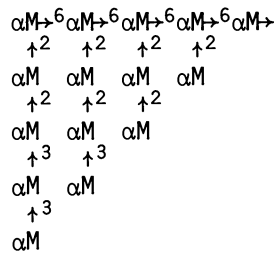
Science

Figure 1. Acetolysis oligosaccharide patterns from different yeast mannoproteins (11). The products from the reaction, which was carried out according to Ref. 6, were separated on a Bio-Gel P-2 column (2 × 200 cm) by elution with 0.1M acetic acid. M to M₅ are mannose to mannopentaose, M₃P is mannotriose phosphate, M₄P is mannotetraose phosphate, and M₄GNac is N-acetylglucosamine linked to mannotetraose.

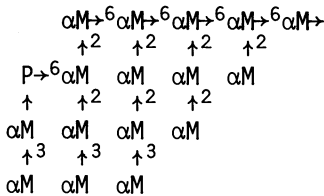
Kloeckera brevis



Saccharomyces italicus



Saccharomyces cerevisiae



Kluyveromyces lactis

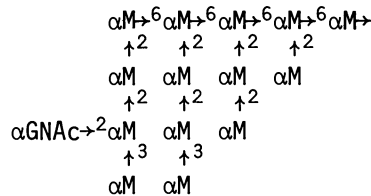


Figure 2. Structures for the mannoproteins whose acetolysis patterns are shown in Figure 1. The structures illustrate the nature of the backbone and sidechains but they are not intended to indicate the order or number of sidechains.

Rabbit antisera raised against intravenously injected whole yeast cells give strong precipitin reactions with isolated mannoproteins (16) and the homologous reactions can usually be inhibited by acetolysis fragments from the mannan (17, 18, 19, 20). Such studies revealed that the immunodominant structures of the yeast cell surface are the mannoprotein sidechains, and that some sidechains are more immunogenic than others (Figure 3).

The discovery that the mannan component of the yeast cell was the major surface determinant produced important dividends, for it suggested a procedure by which mutants with altered mannoproteins might be obtained. The procedure involved the selection, from a mutagenized yeast culture, of variants that no longer were agglutinated by antiserum prepared against the parent cells (21). Because the only phenotypic expression of such mutants is a change in the carbohydrate component of the mannoproteins, characterization of the mutants required extensive structural analysis of the cell walls (22). The screening for cell surface mutants, as contrasted with selection or enrichment, can be done with antisera of different specificities (23) or with the Alcian Blue dye binding assay (24) that will detect the presence or absence of phosphate in the mannan. A recent improved procedure, reported at this meeting (25), describes the use of a fluorescent lectin for the selection and enrichment of yeast mannoprotein mutants by a fluorescence-activated cell sorter. This procedure should be of general use if fluorescent antibodies can be employed in place of lectin.

The various mannoprotein mutants will be discussed in a later section of this report, but one mutant must be considered now because it has played an extremely important role in elucidating the structure of the mannoproteins. This is the *mnn2* mutant, which is characterized by an acetolysis pattern that reveals mostly mannose and indicates that the mannoprotein has a predominantly unbranched α 1-6-linked backbone structure (21). Studies by Dr. Tasuku Nakajima, while a postdoctoral visitor in my laboratory, demonstrated that the small amount of branching still detected in the mannoprotein represented the presence of a core structure that linked the main mannan chain to the protein (Figure 4) (26). A very important conclusion of this study is that yeast mannoproteins and mammalian and plant glycoproteins have identical asparagine-linked core carbohydrate structures (27), and that this core structure is modified in yeast by the addition of a polymannose outer chain. A final point of interest is the fact that the yeast core structure, even in a homogeneous glycoprotein, is heterogeneous (28).

Yeast Mannoprotein Biosynthesis

A number of early studies on mannan biosynthesis (29, 30) were done without a clear idea of the structure of the substance and, consequently, were difficult to interpret. With the

advantage of hindsight, we can now predict that the process should be divided into two parts: (1) the synthesis of the serine- and threonine-linked oligosaccharides and (2) the synthesis of the asparagine-linked units (Figure 5). Each of these can be visualized as having three or more phases, namely, *initiation*, *elongation* and *termination*, with the possibility of a *terminal modification* step for the asparagine-linked units. From studies primarily in the laboratory of Widmar Tanner, at Regensburg, the initiation step both for the oligosaccharide chains linked to hydroxy amino acids (31) and the polysaccharide chains linked to asparagine (32) are known to involve lipid carriers. Dolichyl mannosylphosphate is the donor for addition of the first mannose to serine and threonine, whereas the subsequent mannoses are derived from guanosine diphosphate mannose. Biosynthesis of the polysaccharide units linked to asparagine is much more complex; the core unit is first constructed as a dolichyl-linked derivative, apparently similar to that involved in synthesis of mammalian glycoproteins (33), this is transferred to asparagine in the protein, and the outer chain of the polysaccharide is formed by sequential addition of mannose units from guanosine diphosphate mannose. Modifications of the outer chain are observed in many mannoproteins, and in two instances that have been studied they include addition of mannosylphosphate units (34) and N-acetylglucosamine (35) to the mannan side chains.

Now that the general aspects of mannoprotein structure and biosynthesis are manifest, significant new advances will depend on the purification of the various glycosyltransferases and a study of their reaction parameters with exogenous acceptors (36). An important aim should be to delineate the steps in the assembly of the lipid-linked core unit, including any processing of the molecule (37) that may occur prior to its transfer to the protein. Whether the $\alpha 1 \rightarrow 6$ linkage in the core is made by the same enzyme that makes this linkage in the outer chain must be answered, and the transferase that adds mannose in $\alpha 1 \rightarrow 3$ linkage to the backbone of the core must be distinguished from the $\alpha 1 \rightarrow 3$ -mannosyltransferase that adds this unit in terminal positions elsewhere in the mannoprotein (36).

Genetic Analysis of Yeast Mannoproteins

In this section, I consider the variants in *Saccharomyces* mannan structure that occur naturally and those that have been produced by directed mutation. From a comparison of their acetylation patterns, three interfertile wild-type *Saccharomyces* species have been distinguished. These differ only in the lengths of their longest side chains that, in turn, reflect the activities of two presumably different $\alpha 1 \rightarrow 3$ -mannosyltransferases (Figure 6). Diploids of strains A and B have the B phenotype, whereas diploids of either A or B with C have the C phenotype, which suggests that

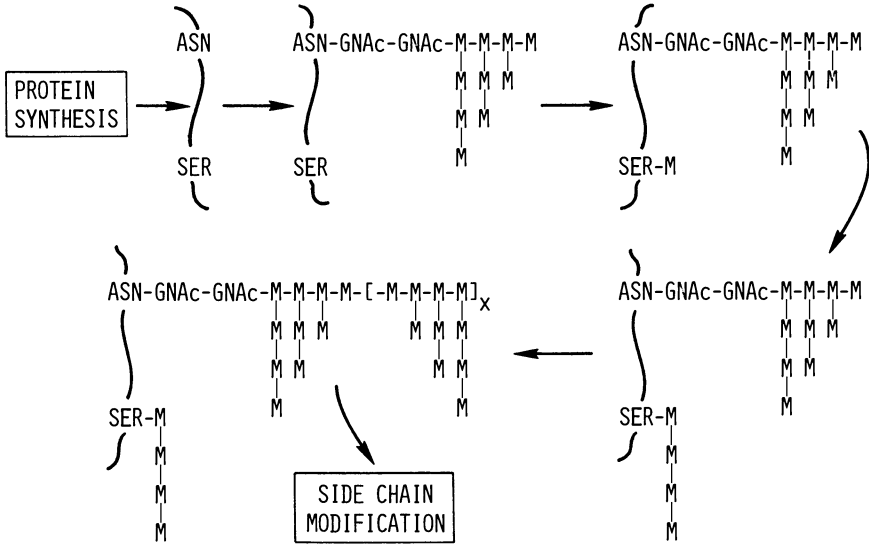


Figure 5. Postulated glycosylation pathway in mannoprotein biosynthesis. The core unit attached to asparagine is derived from Dolichol-P₂-GNac-Man_y, where y is about 15, whereas the first mannose attached to serine is derived from Dolichol-P-Man. In the above figure, x is about 3 to 5.

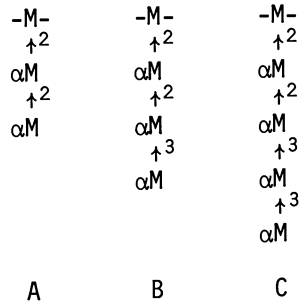


Figure 6. Structures of the longest side-chains in three wild-type interfertile Saccharomyces species. These structures support the existence of a natural polymorphism within the species and they are explained by two independently segregating α1 → 3-mannosyltransferases along with inactive forms of each locus.

two different and dominant $\alpha 1 \rightarrow 3$ -mannosyltransferases are involved in determining these structures (11).

Several mannan mutants of *S. cerevisiae* have been obtained by treatment with ethyl methane sulfonate (Figure 7) (38). The *mn1* mutant has the phenotype of strain A and does not complement it, which indicates that they involve the same locus. This mutation is of particular interest because of its pleiotropic nature; that is, the addition of mannose is affected in several structurally different locations. In contrast, the *mn2* and *mn5* (39) mutations seem to affect only the outer chain, as do the *mn4* and *mn6* (34) mutations, since mannosylphosphate units occur only in the outer chain. The *mn3* mutation is similar to *mn5*, although it also affects the serine-linked units. No mutant with a defect in forming the $\alpha 1 \rightarrow 6$ -backbone has been observed.

Several of the mannan mutants have been placed on the genetic map (Figure 8), and it is notable that the first two mapped were centromere-linked (23, 40). Centromere linkage is postulated to favor retention of a gene by minimizing recombination.

Genetic Control of Mannoprotein Biosynthesis

Several levels of control must be involved in mannoprotein biosynthesis (Table I). Because these are glycoproteins, the

Table I

Levels of Control in Mannoprotein Biosynthesis

1. Protein synthesis: transcription & translation.
2. Glycosyltransferase activity and localization.
3. Amino acid acceptor sequences.
4. Synthesis and processing of mannan core.
5. Translocation of glycosylated protein.
6. Addition of outer chain to mannan core.
7. Termination and modification of outer chain.

first and most fundamental control is at the level of protein biosynthesis. Several cell wall enzymes, such as invertase and acid phosphatase, are subject to catabolite repression (41), and the synthesis of these mannoproteins is presumably controlled by regulation of transcription and translation. Sexual agglutinins, glycoproteins controlled by the mating type locus, are probably under similar regulation (42).

The second level of control of mannoprotein biosynthesis involves the glycosyltransferases that construct the precursors and add them to the protein. We have already seen that the distribution of such enzyme activities in the wild population of *Saccharomyces* species varies in a fashion reminiscent of the enzymes

involved in formation of the blood group substances in mammals. The wide distribution on the yeast chromosomes of the genes controlling the known mannosyltransferases (23) shows that they do not occur in any operon-like arrangement.

A third level of control is probably exerted by the amino acid sequences that determine glycosylation sites in the protein. In mammalian glycoproteins, all asparagine glycosylation sites occur in a sequence -Asn-X-Ser(Thr)-, although all such sites are not glycosylated (43). Moreover, in some instances the same protein may be synthesized with or without the carbohydrate component being added; for example, ribonuclease A and B. As yet, no published study of yeast mannoprotein biosynthesis has dealt with this matter, nor has the structural requirement for glycosylation at serine and threonine residues in a protein been investigated. An interesting example of the latter type of mannoprotein is the sexual agglutinin from *Hansenula wingei* type 5-cells. This substance contains 10% protein that is 55% serine and 9% threonine, and at least 90% of these hydroxy amino acids are glycosylated (44). Thus, about two-thirds of the amino acid residues in the protein chain carry attached oligosaccharides.

A fourth level of control in mannoprotein biosynthesis concerns the structure of the core oligosaccharide that is linked to asparagine. This unit has been shown to be heterogeneous whether isolated from a bulk cell wall mannoprotein preparation (45), from a pure enzyme such as external invertase (46), or even from the intracellular mannoprotein carboxypeptidase Y (28). It is not known whether this heterogeneity is real or is an artifact of mixing of the isolated oligosaccharides that are individually homogeneous at a particular glycosylation site in the protein. Some support for the latter possibility is the fact that carboxypeptidase Y, which contains four carbohydrate chains, gives four oligosaccharides in approximately equimolar amounts when digested with endo- β -N-acetylglucosaminidase. It is also possible that the yeast mannoprotein core is made as a homogeneous substance, which then undergoes a processing reaction to yield various homologs in a manner similar to that already documented for mammalian glycoprotein chains (37).

A fifth, and perhaps one of the most important levels of control, concerns the factors that determine whether a glycoprotein to which the core oligosaccharides have been added is retained inside the cell, as carboxypeptidase Y is, or is further glycosylated to give a mannoprotein that is secreted, as external invertase is (47). Based on what has been learned in other systems, the primary signal that determines ultimate destination probably will be found in the protein component, and it seems unlikely that the carbohydrate will play a determining role (48, 49). The critical step may well be the type of packaging that occurs at the time of glycosylation, and that the molecules destined for the periplasm are secreted into vesicles of the Golgi

system whereas those to be retained in the cell are secreted into the vacuolar system (50).

A sixth level of control must determine which of the core units added to a mannoprotein are elongated and which are not. Thus, external invertase has 9-10 carbohydrate chains per protein subunit (51), and over half of them appear to consist solely of the core unit (46). A simple explanation could be that these units are sequestered inside when the invertase folds into its native conformation, and only those core units still exposed on the surface of the protein have the outer chain added.

As a seventh, and perhaps final, level of control, I come to those factors that may determine the extent of elaboration and modification of the mannan outer chains. The acetolysis patterns suggest that each yeast makes mannoproteins with a characteristic degree of branching (6), and it is possible that the side chains occur in some loosely repeating order (10). This would not require outside information, such as that supplied by a template, but could result from the characteristic affinities of the different glycosyltransferases for the changing structure at the growing end of the chain (36). Thus, the $\alpha 1 \rightarrow 6$ -mannosyltransferase could have a high affinity for a chain terminated by a branch of $\alpha 1 \rightarrow 2$ -linked mannose units, but a very low affinity for a chain terminated by two or more unsubstituted $\alpha 1 \rightarrow 6$ -linked units. If the $\alpha 1 \rightarrow 2$ -mannosyltransferase that initiates branching had the reverse specificity, this would assure that branching and backbone synthesis could proceed with some regularity.

Other factors could regulate the length of the mannan chains. Because guanosine diphosphate mannose is the donor, its availability would be critical and is possibly controlled by the energy charge of the cell (52). It appears that mannoprotein molecules are synthesized and processed at a fairly constant rate, and that the molecules made in a cell with limited mannose donor would be secreted with less than the normal amount of carbohydrate. Alternatively, in the *mn2* mutant that makes mannan without sidechains, the molecules appear to be made with a somewhat longer backbone, which could result from the availability of excess mannose donor that was not used to form the sidechains. Thus, the elongation process may operate like an assembly line, except that the line does not stop if the cell is unable to add a particular sugar, or the model may be changed if the usual parts are not available.

The cellular localization of the different glycosyltransferases will probably turn out to be of very great importance in this respect. These enzymes are membrane-bound and may be distributed in a nonrandom fashion in the endoplasmic reticulum and the Golgi apparatus, perhaps in some relation to the order in which the different processes are carried out (53). A type of mutant this predicts is one that secretes an altered mannan even though it possesses the apparently normal glycosyltransferase activity inside the cell. We have obtained such a mutant in

Kluyveromyces lactis that secretes mannan lacking the terminal $\alpha 1 \rightarrow 2$ -linked N-acetylglucosamine units even though cell extracts show the wild-type transferase activity when assayed with an exogenous acceptor (35). Alternative explanations for this observation have been considered (Table II), and it still may develop

Table II
Possible Defects in *Kluyveromyces lactis*
Mannoprotein *mnn2-2* Mutant

-
1. Temperature sensitive enzyme.
 2. Enzyme with altered K_m .
 3. Altered donor availability.
 4. Altered acceptor structure.
 5. Over production of an inhibitor.
 6. Under production of an activator.
 7. Altered localization of enzyme.
-

that a defect in enzyme localization is involved (54). Such defects have been noted in other systems in which processes dependent on membrane-bound enzymes are disrupted because the enzyme fails to bind to the membrane or is incorrectly inserted (55).

In *S. cerevisiae*, a similar mannan modification reaction occurs that involves the addition of mannosylphosphate units to the trisaccharide sidechains (34). The function of these units is unknown but they could obviously control the charge on the cell surface, which may be important in some environmental situations. We have obtained two types of mutants that regulate this process. They segregate independently, but both result in a mannan without phosphate. One mutation (*mnn4*) (22) is dominant and probably affects a locus that regulates synthesis of the mannosylphosphate transferase (Table III); the other is recessive

Table III
Possible Defects in *Saccharomyces cerevisiae*
Mannoprotein *mnn4* Mutant

-
1. Over production of an inhibitor.
 2. Over production of a phosphodiesterase.
 3. Formation of an altered subunit leading to negative complementation.
 4. Formation of an altered repressor.
-

(*mnn6*) (34) and could be at the structural gene locus for this enzyme. A third locus that regulates the amount of phosphate in

the mannan has been reported (24). It also is dominant and maps on the same chromosome as *mmm4*, although the two segregate independently (23). The characterization of these three genetic loci may help to elucidate the mechanism by which this step in manno-protein biosynthesis is regulated.

Concluding Remarks

This brief summary of the structure, biosynthesis and genetic control of yeast mannoproteins should, if anything, point out the complexity of the problem with which we are dealing. Our present knowledge of the structure of *Saccharomyces cerevisiae* mannoproteins makes possible a logical study of its biosynthesis. The mannan mutants have facilitated this study, but, more importantly, they have suggested probable mechanisms for the overall regulation of its biosynthesis, translocation and secretion. It is obvious that the isolation and characterization of additional mutants will be useful in furthering this work, and it is an approach that is strongly recommended.

Acknowledgements

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Structural Studies on the Cell Wall Polysaccharides from *Pyricularia oryzae*

A Pathogenic Fungus of Rice Blast Disease

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Various biochemical approaches to the pathogenicity of *Pyricularia oryzae*, a rice blast fungus, have been studied in our laboratory from the stand points of toxins (1,2) and glycosidases (3,4) produced by the pathogens. Recently, our interest in host-pathogen interaction has focused on the cell wall polysaccharides of this phytopathogenic fungus (5,6,7). The cell wall of *P. oryzae* was found to be constituted of at least three different polysaccharides; proteo-galactoglucomannan, β -1,3-D-glucan containing β -1,6 linkages and chitin (8). In this paper we describe the characterization of the two main polysaccharide components, a galactoglucomannan and an alkali insoluble β -D-glucan, by using methylation, Smith degradation, fragmentation by acids and enzymes, and immunochemical methods (9,10,11). We also discuss about the organization of the polysaccharide components of the *P. oryzae* cell walls and the relation between cell wall antigenicity and species specificity (8, 12).

An alkali insoluble β -D-glucan (9).

An alkali insoluble β -D-glucan was isolated from the mycelium of *Pyricularia oryzae* P-2 which was harvested in late logarithmic phase. Protein, glyco-gen and heteroglycan were removed by exhaustive extraction with hot water in an autoclave at 120° and 1 N sodium hydroxide. Chitin was removed by treatment with

chitinase from Streptomyces sp. (7). After freeze-drying and grinding, the sample was obtained as a white powder (Glucose: 97.3 %, Nitrogen: 0.06 %) which was insoluble in water and alkali, but slightly soluble in dimethyl sulfoxide. The molecular weight of the glucan was determined by the procedure of Jones and Ballou (13), which involves sodium borotritide reduction of the glucan and the specific activities of a standard oligosaccharide and the reduced glucan. The results of analysis are shown in Table 1. From the specific activities of gentiotetraitol and the reduced glucan, a number average molecular weight of about 38,500 was calculated for the P. oryzae cell wall β -D-glucan. Dextran T-20 was used as a control polysaccharide.

TABLE 1. Incorporation of tritium from sodium borotritide into reducing ends of gentiotetraose and cell wall glucan of Pyricularia oryzae (9)

Compound reduced	Tritium incorporated cpm	Specific activity cpm / 100 μ g	Molecular weight
Gentiotetraose	53,981	5,742	666
Dextran T-20 ^{a)} (M.W.15,000)	2,780	241	15,811
Cell wall Glucan	496	99	38,491

a) Pharmacia Catalogue, Uppsala, Sweden. (Dextran T-20 Mn 15,000, Mw 22,300)

Agricultural and Biological Chemistry

Methylation of the cell wall glucan first with dimethyl sulfate and alkali (14), and then with methyl iodide in the presence of methylsulfinyl carbanion (15) yielded the fully methylated glucan (OCH₃: 45.4%). The methylated glucan showed $[\alpha]_D^{+47}$ (c=1 in chloroform) and a single peak on ultracentrifugation analysis.

Hydrolysis of the methylated glucan with sulfuric acid (16) afforded a mixture of methylated sugars which were shown by paper chromatography (9) to consist of 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-O-methyl-D-glucose. The quantitative estimation of these methyl derivatives was carried out by separating

When the cell wall glucan was treated with periodate at 5°, 0.36 mole of periodate was consumed and 0.18 mole of formic acid was produced per anhydroglucose unit as shown in Table 3.

TABLE 3. Periodate oxidation of the cell wall glucan of Pyricularia oryzae.

	Time (days)				
	5	10	20	30	40
Periodate consumption (mole/mole)	0.17	0.25	0.32	0.35	0.36
Formic acid production (mole/mole)	0.07	0.12	0.16	0.18	0.18

This indicates that two molar proportions of periodate were consumed with the simultaneous liberation of one molar proportion of formic acid, for an average of six residues of D-glucose. The periodate oxidation results were in good agreement with the methylation studies.

Fragmentation analysis also supported methylation and periodate oxidation results. The controlled acetylation of the cell wall glucan afforded a homologous series of laminaridextrins, such as glucose, laminaribiose and laminaritriose. On the other hand, enzymatic digestion of the glucan with an exo-type β -1,3-D-glucanase from Basidiomycete sp. QM 806 (18) afforded a mixture of gentiobiose and gentiotriose.

The simplest possible structure proposed for the cell wall glucan of Pyricularia oryzae, based on the results of methylation, periodate oxidation and fragmentation analysis is shown in Fig. 1.

The proposed structures are in harmony with our previous results of the selective enzymatic degradation of Pyricularia oryzae cell wall by lytic enzymes from Bacillus circulans (7). The cell wall was digested by endo- β -1,3-glucanase with a substantial decrease in turbidity but slight liberation of reducing sugar, while the action of β -1,6-glucanase resulted in liberation of a considerable amount of reducing sugar although the concomitant decrease in turbidity was only slight. These results suggest that the cell wall of Pyricularia oryzae is constituted of either a

net work of β -1,3-D-glucose units interspersed with β -1,6-D-glucose units, or the latter β -1,6-D-glucose units are short side chains on the backbone of a β -1,3-D-glucan.

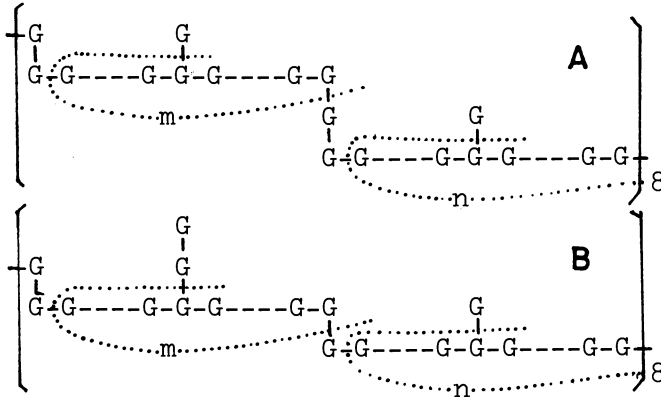
Our present results strongly support the latter idea, although the presence of a glucan of different types cannot be completely neglected.

A proteo-heteroglycan (10,11).

A proteo-heteroglycan (PHG) was obtained from *Pyricularia oryzae* mycelia by hot water extraction followed by DEAE-cellulose chromatography and gel filtration. The isolated and purified heteropolymer from *P. oryzae* mycelia was a white powder, easily soluble in water and had $[\alpha]_D^{20} +72.5^\circ$. Sedimentation analysis with an analytical ultracentrifuge gave a single peak and the sedimentation coefficient was calculated to be 6.1 S. The heteropolymer was composed of 91 % of carbohydrate and 9 % of protein. The phosphorus content of PHG was negligible, the molar ratio of mannose to phosphorus is about 280. High voltage electrophoresis showed a single spot of carbohydrate and protein. The proton magnetic resonance spectrum of PHG showed several H-1 proton signals at 5.0-5.5 ppm. These results indicate that the polymer contains mainly α -linkages. The molecular weight of the PHG was determined to be 320,000 by gel filtration on a Sepharose 6-B column. Alkali or enzymatic treatment reduced the molecular size of the PHG (Fig.2). The β -elimination reaction caused a slight modification of the structure of the PHG and the molecular weight was reduced to 280,000 with the liberation of oligosaccharides. On the other hand, digestion with *exo*- α -D-mannanase caused considerable breakdown of the molecule and the degraded molecule was measured to be 50,000 daltons.

The PHG was hydrolyzed and the component sugars of the PHG were analyzed (Fig.3). The ratio of the sugar components was found to be mannose (6): glucose (2): galactose (1). When this PHG was hydrolyzed under milder conditions (0.05 N sulfuric acid), only galactose was released from the original polymer after 2 hour hydrolysis (Fig.3 : lower) indicating that all of the galactose residues in the PHG are in the furanose form.

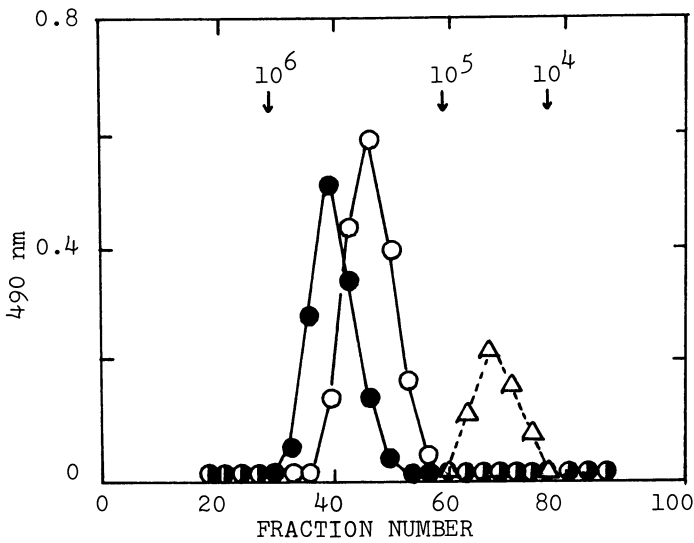
The PHG was hydrolyzed to an extent of 38 % by *Arthrobacter* *exo*- α -D-mannanase (13) and the liberated sugar was only D-mannose. As this enzyme is capable



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Figure 1. Possible structures proposed for the cell wall glucan of *Pyricularia oryzae*: G → G, β-1,3-glucosidic linkage; ↓; β-1,6-glucosidic linkage; m + n = 22.

(9).



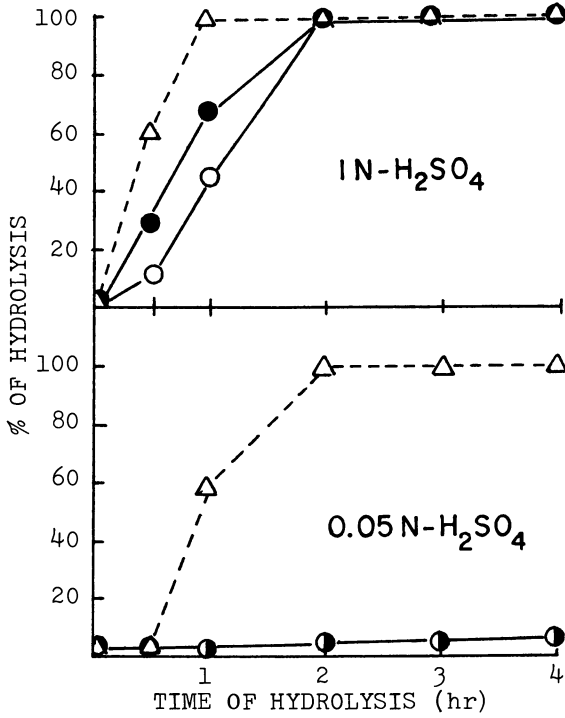
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Figure 2. Elution pattern from Sepharose 6-B columns of the various PHG preparations: 1, (●) original PHG; 2, (○) alkali-treated PHG; 3, (△) *exo-α-D-mannanase* digested PHG (10).

of splitting only (1→2) or (1→3) mannosidic linkages, the remaining polysaccharide (62 % of the total sugar) should consist of α-1,6 mannosidic and/or of the glycosidic linkages other than the mannosidic one.

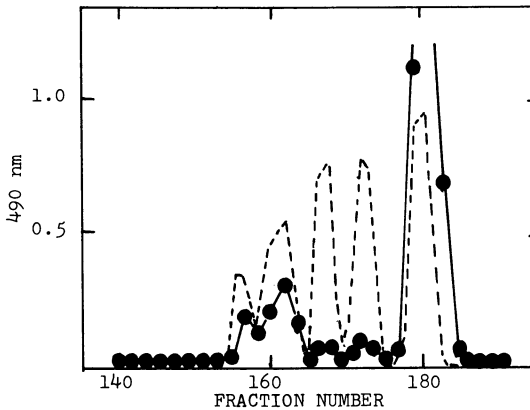
Methylation of the samples was done by Hakomori method (15) and the hydrolysis products of the methylated samples were converted to alditol acetate derivatives and methyl glycosides. The alditol acetates were analysed by GLC on a column of ECNSS-M and the methyl glycosides were separated on a column of polyDEGS. The results of the methylation analysis of the carbohydrate moiety of the original PHG, the degraded PHG prepared by partial acid hydrolysis and the α-mannanase resistant core are shown in Table 4. Glucose and galactose were obtained only as tetramethyl derivatives indicating that these two sugar components are located at non-reducing termini. The absence of galactose derivatives on the chromatogram of the degraded PHG supports the result obtained by partial acid hydrolysis of the PHG. After liberation of the galactose residue from the PHG, a slight increase of the ratio of 2,3,4-tri-O-methyl mannose derivatives was observed with a concomitant decrease of 3,4-di-O-methyl mannose derivatives. This indicates that a part of the galactose residues is attached directly to the backbone mannose units. Analysis of the α-mannanase resistant core showed a dramatic increase of 2,3,4-tri-O-methyl mannose derivatives and a decrease of 2,4,6-, 3,4,6-tri-, and 3,4-di-O-methyl mannose derivatives. The ratio of tetramethyl galactose and glucose were slightly increased after enzymolysis. The heteroglycan has three different mannosidic linkages, namely (1→2), (1→3) and (1→6), and the branch occurs at 2 and/or 6 of the backbone mannose units. The average chain length was calculated to be about 5.

Controlled acetolysis which cleaves the (1→6) linkages preferentially, gives finger prints for the yeast mannan, the structure of which consists of a (1→6) linked backbone with (1→2) or (1→3) linked short side chains (19). We have tried to use this method for the *Pyricularia oryzae* heteroglycan. The PHG and the *Arthrobacter* exo-α-D-mannanase resistant PHG core were acetolyzed for 12 h at 40°. The deacetylated acetolysis products were separated on a column of Bio-Gel P-2 (Fig. 4). Typical acetolysis patterns were obtained from the original PHG. It could be seen from the elution profile that the original PHG has either a (1→6) linked backbone with (1→2) or (1→3) linked side chains, or (1→2) or (1→3) linked oligosaccharide blocks interspersed in a molecule and these were link-



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Figure 3. Release of sugars from the PHG by acid hydrolysis: (●) mannose, (○) glucose, (△) galactose (10).



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Figure 4. Comparison by gel filtration on Bio-Gel P-2 of the acetolysis patterns of (---) the intact PHG and (●—●) the *exo*- α -D-mannanase digested PHG core (11).

TABLE 4. Molar ratios of the hydrolysis products of methylated original, acid degraded, and α -mannanase digested proteoheteroglycan from *P. oryzae*.

O-methyl hexose derivatives Linkages	Molar ratios ^{a, b)}		
	Original	Acid degraded	Enzyme digested
2,3,4,6-tetra-O-methyl mannose M ¹ -	1.0	1.0	0.1
2,3,4,6-tetra-O-methyl glucose G ¹ -	1.8	1.8	1.8
2,3,5,6-tetra-O-methyl galactose Gal ¹ -	0.7	0	0.9
2,4,6-tri-O-methyl mannose - ³ M ¹ -	0.9	0.8	0
2,3,4-tri-O-methyl mannose - ⁶ M ¹ -	4.7	4.9	9.9
3,4,6-tri-O-methyl mannose - ² M ¹ -	5.2	5.3	3.2
3,4-di-O-methyl mannose - ⁶ M ¹ - ₂	3.3	2.7	1.7

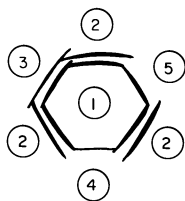
a) Determined by the combined use of GLC and PPC.

b) Relative to the molar ratio of 2,3,4,6-tetra-O-methyl glucose.

ed through (1→6)-mannosidic bonds. The latter structure must be unlikely, since the acetolysis pattern from the mannanase resistant core showed a remarkable decrease of oligosaccharide fractions indicating that exo- α -D-mannanase splits off the α (1→2) or α (1→3) linked side chains from the (1→6) linked backbone. If the latter is true, the acetolysis pattern would be essentially unchanged before and after enzymolysis. Methylation analysis of the mannanase-resistant core, described above also supported the former structure.

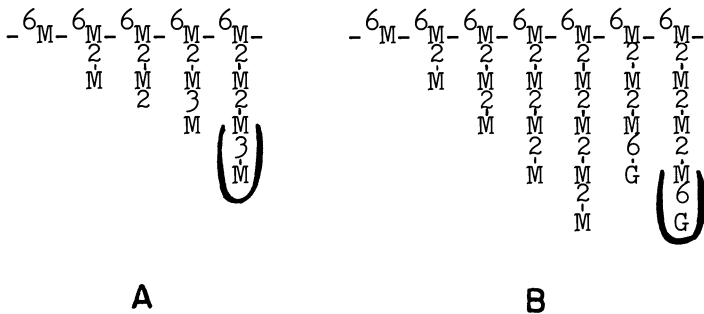
The elution profile also shows that the maximum length of the branches is 5 hexose units and the molar ratio of the branches of various length was found to be mono (8): di (6): tri (4): tetra (2): penta (1). Penta and tetrasaccharide branches (containing one mannose unit which came from the (1→6) mannan backbone) of the PHG were resistant to exo- α -D-mannanase digestion. This indicates that these side chains must be terminated by sugar residues other than mannose or must have mannosidic linkages other than α (1→2) or α (1→3) at the nonreducing termini. Paper chromatography of the monosaccharide fractions showed three kinds of sugars, namely mannose, glucose, and galactose, suggesting that part of the side chains were degraded during acetolysis. Especially, the attachment of the galactofuranose must be labile on acetolysis. Penta and tetrasaccharide fractions have glucose as well as mannose. This supports the results from acetolysis of the mannanase resistant PHG core. The chemical studies of the PHG described above were not able to elucidate the structure of the side chain containing glucose. This problem could be solved by the use of immunological methods.

As shown in Fig.5, the PHG strongly cross reacted with Candida utilis glucomannan (20) and weakly with Saccharomyces cerevisiae mannan (21) whose structures have been established (Fig.6). Using these mannans and chemically modified PHG, quantitative precipitation reactions with anti-Pyricularia oryzae serum were carried out (Fig. 7). Candida utilis mannan reacted with the serum more strongly than Saccharomyces cerevisiae mannan did. Partial acid hydrolysis caused the lowering of the precipitation activity of the PHG indicating that the galactofuranose residue is one of the important immunodeterminants. Treatment of the PHG with exo- α -D-mannanase also caused a slight loss of the precipitation activity. To confirm the above observation, the inhibition test was performed using oligosaccharides from Candida utilis glucomannan and from Saccharomyces cerevisiae mannan



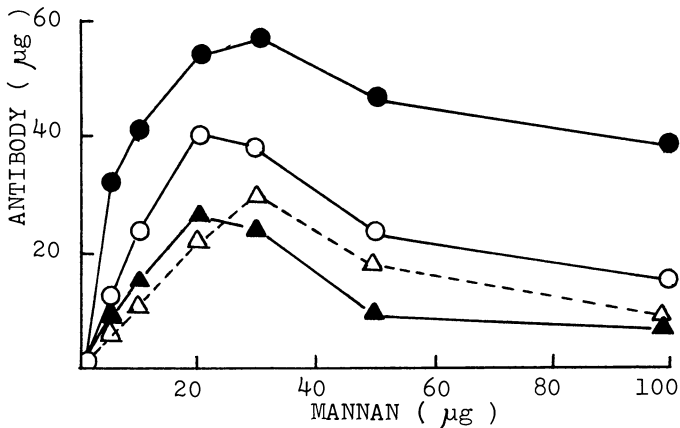
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Figure 5. Representation of agar gel double diffusion patterns of (1) anti *P. oryzae* serum with (2) PHG, (3) *C. utilis* glucomannan, (4) *S. cerevisiae* mannan, and (5) *Hansenula anomara* water-soluble polysaccharide (11).



Journal of Biochemistry

Figure 6. Immunodominant sidechain structures illustrating the mannans from A, *S. cerevisiae* wild-type (25); B, *C. utilis* mutant (20). The curved lines indicate the presumed binding sites (11).



Journal of Biochemistry

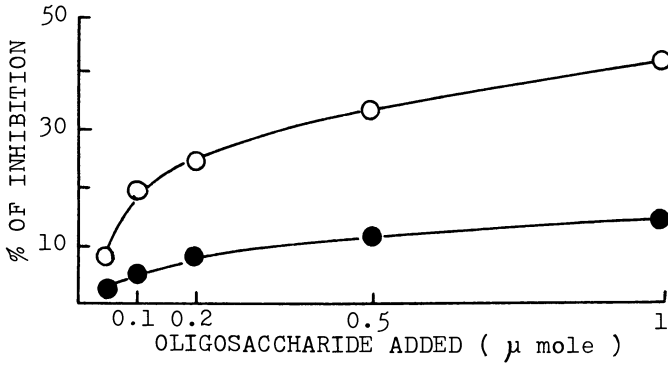
Figure 7. Quantitative precipitation curves for *P. oryzae* antiserum with (●) PHG, (△) partial acid hydrolyzed PHG, (○) *C. utilis* glucomannan, (▲) *S. cerevisiae* wild-type mannan (11).

(Fig.8). The glucosyl mannotriose, $\alpha\text{G}(1\rightarrow6)\alpha\text{M}(1\rightarrow2)\alpha\text{M}(1\rightarrow2)\text{M}$ from Candida utilis glucomannan, was shown to be the effective inhibitor for the homologous precipitation reaction, whereas mannotetraose, $\alpha\text{M}(1\rightarrow3)\alpha\text{M}(1\rightarrow2)\alpha\text{M}(1\rightarrow2)\text{M}$ from Saccharomyces cerevisiae mannan showed only 15 % inhibition in the same system. These results suggest that both oligosaccharides are structural analogues of Pyricularia oryzae heteroglycan side chains. Though we have not tested galactofuranose or manno oligosaccharides containing galactofuranose termini, these sugars should exhibit significant inhibition of the reaction, since we were not able to get 100 % inhibition by the single use of the oligosaccharides described above. This is also supported by the fact that the partially hydrolyzed PHG showed weaker precipitation activity when compared with the original PHG. Thus, it appears that Pyricularia oryzae galactoglucomannan contains three different kinds of immunodeterminants, namely $\alpha\text{G}(1\rightarrow6)\text{M}$, $\alpha\text{M}(1\rightarrow3)\text{M}$, and $\alpha\text{Gal}(1\rightarrow2)\text{M}$. Among them, the most dominant immunodeterminant group of the PHG molecule must be a tetrasaccharide containing an $\alpha\text{G}(1\rightarrow6)\text{M}$ group. The results obtained from the present work now enable us to discuss the side chain structure of the PHG. Methylation studies have shown that the carbohydrate moiety of the PHG consists of (1 \rightarrow 6), (1 \rightarrow 2), and (1 \rightarrow 3) mannosidic linkages and branches occur at C-2 and/or C-6. The average chain length is about 5 hexose units and three kinds of nonreducing terminal sugars, namely mannopyranose, glucopyranose, and galactofuranose are found. Among them, glucopyranose and galactofuranose residues are present exclusively as nonreducing termini. The results from partial acid hydrolysis substantiate the methylation results. All of the galactose residues in the molecule were selectively removed by mild acid hydrolysis indicating that the galactose exists in the furanose form and as a nonreducing terminal sugar. Controlled acetolysis of yeast mannan and analysis of its fragments by gel filtration described by Ballou and his coworkers (19) might be a useful method for the structural analysis of a fungal heteroglycan which has $\alpha(1\rightarrow6)$ -mannan backbone structure. The gel filtration of the Pyricularia oryzae galactoglucomannan gave a sharp acetolysis pattern indicating that the PHG might have the yeast mannan type structure. In addition, the results from methylation analysis and acetolysis of the exo- α -D-mannanase resistant PHG core strongly supported the structure which has a (1 \rightarrow 6)-mannan backbone with side chains of mono to tetrasaccharide units. However, by PPC, mannose, glucose, and

galactose were detected in the monosaccharide fractions eluted from the Bio-Gel P-2 column indicating that a part of the glucose and galactose were liberated from the side chain during acetolysis. A reasonable explanation for this is that glucose is attached to the nonreducing end of the side chain through an $\alpha(1\rightarrow6)$ -linkage as indicated by the inhibition experiment with glucosyl mannotriose from *Candida utilis* glucomannan and the linkage involving galactofuranose may also be labile on acetolysis. As shown in Fig. 4, the exo- α -D-mannanase digestion combined with acetolysis has shown that the tri- and tetrasaccharide side chains are resistant against mannose hydrolysis indicating the presence of terminal glucose in these branches. On the basis of chemical, enzymatic and immunochemical results described here, the side chain structure of *Pyricularia oryzae* galactoglucomannan is proposed as illustrated in Fig. 9.

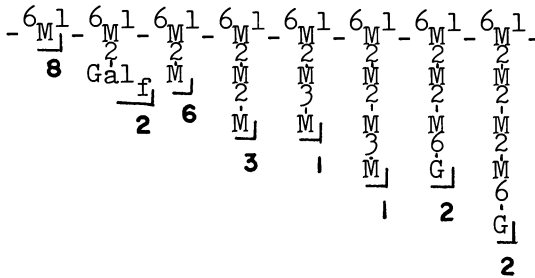
Organization of the polysaccharide components of the cell walls (7,8).

We have described two main polysaccharide components in the cell wall of *Pyricularia oryzae* in the former sections. However, the *P. oryzae* cell wall must be constituted of other types of polysaccharides. When the purified cell walls were fractionated with ethylenediamine according to the method of Korn and Northcote (22), four different polysaccharide fractions were obtained as shown in Table 5. The fractionated components were slightly mixed with each other. The results of the fractionation studies indicates that the cell wall of *Pyricularia oryzae* consists of at least three different polysaccharides, namely, proteogalactoglucomannan, β -1,3-D-glucan containing β -1,6-linkage and chitin. Microscopic observation of fluorescent-antibody labeled mycelia have shown that the proteo-galactoglucomannan was located in outer layer but it did not cover completely the inner layer, probably β -D-glucan (23). Chitin must be present in inner most part of mycelia because most of the β -D-glucan was extractable from the cell wall by ethylenediamine, alkali, hot DMSO treatment but chitin was not. The β -D-glucan may be very heterogeneous or may be present in a single macromolecule which can partially be broken by alkali.



Journal of Biochemistry

Figure 8. Inhibition of anti *P. oryzae* serum by glucosyl mannotriose, (○) αG-(1 → 6)αM(1 → 2)αM(1 → 2)M from *C. utilis* glucomannan and mannotetraose, (●) αM(1 → 3)αM(1 → 2)αM(1 → 2)M from *S. cerevisiae* mannan (11).



Journal of Biochemistry

Figure 9. Proposed structure for the carbohydrate part of the proteo-heteroglycan from *P. oryzae*. All sugar residues are in the α-configuration. The subscripts outside the brackets indicate the average molecular proportion of the various types of sidechains. The order of the sidechains along the backbone is unknown. M indicates an α-D-mannopyranosyl residue, G is an α-D-glucopyranosyl residue, and Gal₁ is an α-D-galactofuranosyl residue (11).

TABLE 5. Chemical characteristics of each cell wall fraction of Pyricularia oryzae

Fractions	Hydrolysis Products ^{a)}	Types of Polysaccharides
I	Glc, Man, Gal, Amino acids	Proteo-Heteroglycan
II	Glc, Man, Gal, GlcN, Laminari-oligo, Gentiobiose	Proteo-Heteroglycan, β -1,3-Glucan
III	Glc, GlcN, Laminari-oligo, Gentiobiose	β -1,3-Glucan
IV	GlcN, Glc,	Chitin

a) Hydrolysis was performed with 2N H₂SO₄, 6N HCl, endo- β -1,3-glucanase from Bacillus₂ circulans WL-12 and exo- β -1,3-glucanase from Basidiomycete QM 806.

Serological properties of the cell surface (12).

As described in the former sections, a serologically active heteroglycan has been characterized. We thought the antigenicity of the cell wall of Pyricularia oryzae might be a probe for the specific recognition on the various strains of this rice blast fungus. The presence of antigenic polysaccharide on the cell wall of microorganisms is well known. Particularly, bacterial lipopolysaccharide O-antigen (24) and yeast mannan (25) are well characterized. Usually, cell surface antigens are species specific. However, it is not clear whether the cell surface antigenicity is different from strain to strain in one species. Recently, Fukazawa et al. (26) have investigated on Candida sake using chemical and serological techniques and showed that Candida sake are divided into four groups. Pyricularia oryzae is tentatively classified into three groups (races), namely N, C, and T according to their pathogenecities for rice plants. N group has a pathogenic specificity for Japonica varieties, C group is pathogenic to Chinese rice varieties and T group

TABLE 6. Test tube agglutination with various dilution of *P. oryzae* H-373 cell wall polysaccharide antigen against antibodies obtained from the representative *P. oryzae* strains of each of four races, N-1; H373, C-1; THU62-132, T-1; Ken53-33, 0; THU65-01

Antibody	Dilution of antigen (x 10,000)							
	x2	x4	x8	x16	x32	x64	x128	
N; H373	+++ ^{a)}	+++	+++	++	+	+	+	†
C; THU62-132	+++	+++	+++	+	+	+	-	-
T; Ken53-33	+++	+++	+++	+	+	+	-	-
0; THU65-01	+++	+++	+++	+	+	+	-	-

a) +++; strong precipitation, ++; positive precipitation, +; weak precipitation, †; precipitation is not clear, -; negative precipitation

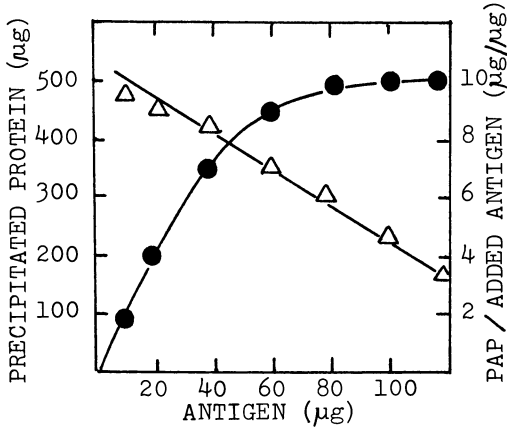


Figure 10. Quantitative precipitation curve for anti *P. oryzae* H-373 serum with H-373 cell wall antigen: (●—●) PAP, precipitated antibody protein, (△—△) PAP/added antigen.

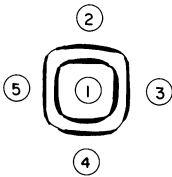


Figure 11. Representation of agar gel double diffusion pattern of (1) anti *P. oryzae* H-373 serum with (2) the cell wall antigen from H-373, (3) THU 62-132, (4) Ken 53-33, and (5) THU 65-01 (11).

is specific for foreign varieties other than Chinese, for instance, Indica varieties. In addition to the above three races, nonpathogenic strains are also isolated and classified into 0 group. The strains of Pyricularia oryzae used in this study were representative strains of each of four races, namely, T-1 (Ken 53-33), C-1 (THU 62-132), N-1 (H 373), 0 (THU 65-01). All strains were grown at 28° for 3 days on Vogel N-yeast extract medium (28). To obtain antibody against P. oryzae cells, male albinorabbits weighing approximately over 2.5 Kg each were immunized by the intramuscular injection of acetone-dried P. oryzae cells with Freund's complete adjuvant (29) three times at two weeks intervals. Antigen polysaccharides were extracted from Pyricularia oryzae N-1 (H 373) cell walls with ethylenediamine followed by DEAE-cellulose chromatography (8). The quantitative precipitation curve (Fig.10), obtained by plotting precipitated antibody protein versus added antigen, gave a straight line indicating that the antigen of P. oryzae cell wall is well purified. This antigenic fraction was further purified and characterized as a proteo-galactoglucomannan as described in the section II. In the present study, we used a partially purified antigen fraction. The serological relationship of various strains of Pyricularia oryzae was examined by micro tube agglutination tests with various dilution of a standard cell wall polysaccharide fraction versus antibodies from the representative Pyricularia oryzae strains of each of four races (Table 6). The result indicated that no difference in agglutination titer is found among the various races from Pyricularia oryzae. The specificity of antigens of each group of Pyricularia oryzae was also examined by the gel diffusion tests (Fig.11). Two precipitation bands were obtained with all antigens from Pyricularia oryzae races and both bands were completely fused with the ones from the neighbor.

The results obtained above indicate that all of the antigen from various strains of Pyricularia oryzae should be serologically the same or very close. Therefore, it seems difficult to recognize the variety of the races of Pyricularia oryzae by the serological method using cell surface antigens.

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Toward Understanding the Structure, Biosynthesis, and Function of a Membrane-Bound Fungal Glycopeptide

Biosynthetic Studies

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The reaction sequences by which glycoproteins of yeasts and fungi are synthesized are not completely understood. A portion of the work on the biosynthesis of glycoproteins in yeast conducted in Ballou's laboratory is discussed elsewhere in this volume (1) and therefore will not be considered here.

UDP-N-acetyl-D-glucosamine and GDP-D-mannose were first isolated from yeasts (2) and may serve as glycosyl donors in glycoproteins containing both N-acetyl-D-glucosamine and D-mannose. Evidence is accumulating that lipid linked sugars, such as mannosyl phosphoryl dolichol and other dolichol-containing saccharides, are important intermediates in the biosynthesis of fungal glycoproteins (3-6). *Aspergillus niger* contains a polyprenol phosphate (exo-methylene-hexahydropolyprenol phosphate) which accepts mannose from GDP-D-mannose and transfers the mannosyl residue to a mannan (5,6). Dolichol occurs naturally in yeasts. The mannosyl residue attached to seryl or threonyl residues of the glycoprotein are derived from mannosyl phosphoryl dolichol and the other mannosyl residues of mannose-containing oligosaccharides are derived directly from GDP-D-mannose (7). Phosphomannan from *Hansenula holstii* contains some mannosyl residues attached by phosphodiester to the C-6 atom of other mannosyl residues (8-10). The phosphomannosyl residue of GDP-D-mannose is transferred to form 1-O-D-mannosyl 6'-phosphoryl mannan.

Studies on the biosynthesis of complex glycoproteins such as the ethanolamine/N,N'-dimethylaminoethanol-containing peptidophosphogalactomannan of *Penicillium charlesii* are limited. Uridine 5'(α -D-galactofuranosyl pyrophosphate) was isolated from extracts of *P. charlesii* and tentatively characterized by Trejo *et al.* (11). They claimed that UDP-Gal_f was the galactofuranosyl donor in an *in vitro* system composed of cofactors and the supernatant solution resulting from centrifugation of a cell homogenate of *P.*

charlesii at 500xg. They presented no evidence to show that all unbroken cells were removed by centrifugation.

This paper reviews and extends our work on the biosynthesis of peptidophosphogalactomannan in P. charlesii.

Experimental

Isolation and Fractionation of Membranes. Membranes were obtained from P. charlesii and fractionated by isopycnic sucrose gradient ultracentrifugation (12) and the membranes ($\rho = 1.1$ g/cc) were solubilized in Triton X-100 and partially purified on an affinity column with peptidophosphogalactomannan as the ligand (13).

b) Quantification of ^{14}C -, ^{32}P and ^3H -labelled substances. Radioactively labelled substances were quantified by liquid scintillation spectrometry (12,14). Radioactivity in paper chromatograms was quantified by determining the radioactivity on the paper strips subsequent to sectioning the chromatogram into 0.5 x 2 cm strips (12).

c) Isolation, purification and fractionation of peptidophosphogalactomannan and its degradation products. Peptidophosphogalactomannan was isolated and purified as described previously (15) and the various degradation products following treatment with alkali, acid, or the acetolysis procedure were fractionated on Bio-Gel P-2 (15).

Results

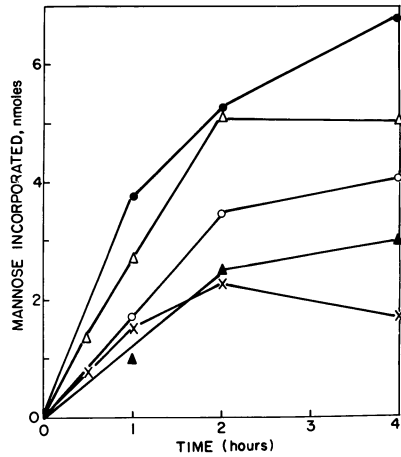
Studies in vivo. Penicillium charlesii releases peptidophosphogalactomannan into the growth medium starting soon after the culture becomes starved for NH_4^+ , as shown previously (16). Also it is apparent that 48 hr cultures contain both lipopeptidophosphogalactomannan and cytoplasmic-peptidophosphogalactomannan (Table VII) (17). Separate experiments have been conducted to determine the time of onset of synthesis of extracellular peptidophosphogalactomannan using radio-labeled precursors (16). Approximately 78% of the total polymer is synthesized between day 2.5 and 9 based on the dilution of radio-labeled D-glucose or phosphate added at day 2.5. In companion experiments, in which the organism was first grown on radio-labeled substrate for 2.5 days and then transferred to growth medium of the same age but without radiolabeled substances in it, the specific activity of the isolated hexoses and phosphate were 22% of that of the initial growth medium. Similar experiments were carried out with L-(U- ^{14}C)-threonine. The ratio of the specific activities of the dehydroaminoacyl residues obtained from peptidophosphogalactomannan obtained from cells cultured in labeled threonine

between 2.5 and 9 days was 4-fold larger than that of cells cultured in labeled threonine for only the first 2.5 days. These data suggest that about 31 μ moles of peptidophosphogalactomannan were formed during the first 30 hr after germination, that an additional 110 μ moles of carbohydrate was synthesized in the 132 hr after 2.5 days. The rate of release of peptidophosphogalactomannan was linear up to about 7 days and the medium is nearly depleted of glucose by then. Using 6.7 days and 125 μ moles of hexose, a value of 95 μ moles of hexose was incorporated into peptidophosphogalactomannan during the 100.8 hr starting at 2.5 days. This value of 0.95 μ mole per hr compares well with that estimated from the total quantity synthesized during the first 48 hr (17).

Studies with a mutant (*P. charlesii* 17D) of *P. charlesii* which is incapable of growing in D-galactose showed that the glycopeptide which it secretes into the growth medium contains only about 2 galactofuranosyl residues per molecule and 1 atom of phosphorus (18). A second mutant (*P. charlesii* 17B) which contained about 5 galactofuranosyl residues per molecule of glycopeptide also contained only 1 or 2 atoms of phosphorus. These results suggest that the incorporation of phosphorus in whatever form it takes may require the presence of a galactofuranosyl residue in that region of the mannan before the phosphorus containing residue can be transferred. The glycopeptide from the 17D mutant contained about one mole of ethanolamine per mole of glycopeptide.

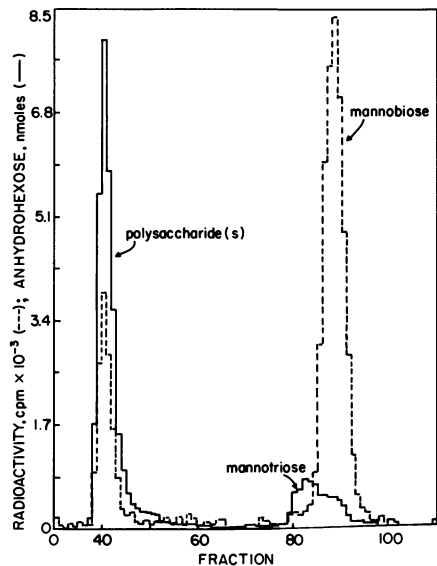
Several investigators have shown that 2-deoxy-D-glucose inhibits the synthesis of glyco-enzymes (19-22). Higher concentrations of 2-deoxy-D-glucose are required to inhibit cell wall synthesis than are required to inhibit glyco-enzyme synthesis (22). The active inhibitor is probably GDP-2-deoxy-D-glucose, an analogue of GDP-D-mannose. We have shown that 15 mM 2-deoxy-D-glucose added to *P. charlesii* cultures inhibits synthesis and secretion of galactofuranosidase as shown by the isolation of peptidophosphogalactomannan containing a ratio of galactose:mannose of 3:2 and approximately 10-fold decrease in galactofuranosidase activity in the culture filtrate (23). The presence of 2-deoxy-D-glucose in the growth medium appeared to have no effect on the composition of peptidophosphogalactomannan other than the quantity of ethanolamine found was increased to 2.06 moles per mole of peptidophosphogalactomannan. This increase by one residue greater than that usually found in the polymer may not be significant. However, it is apparent that 2-deoxy-D-glucose is not an effective inhibitor of synthesis of peptidophosphogalactomannan even though galactofuranosidase synthesis was inhibited. Although the reason for this difference in inhibition is not known, it may be that UDP-2-deoxy-D-glucose is a more effective inhibitor of the metabolism of UDP-N-acetyl-D-glucosamine than it is of that of GDP-D-mannose and that galactofuranosidase contains N-acetyl-D-glucosamine residues.

Figure 1. Time course of ^{14}C -mannose incorporation into acceptors. The reaction mixtures were composed as described in (12) with the indicated mannosyl acceptor. The acceptors added to the system were: (●) peptidophosphogalactomannan; (Δ) 0.01N HCl-treated peptidophosphogalactomannan; (○) 0.5N NaOH treated peptidophosphogalactomannan; (\blacktriangle) 2,4-dinitrophenyl-peptidophosphogalactomannan; (×) none. The reaction was terminated and the products separated as described (12).



Journal of Biological Chemistry

Figure 2. Distribution of carbohydrate and ^{14}C in fractions from Bio-Gel P-2 following treatment of ^{14}C -labeled peptidophosphogalactomannan in which the ^{14}C was derived from GDP-D- ^{14}C mannose. A reaction mixture containing GDP-D- ^{14}C mannose, peptidophosphogalactomannan, and membrane preparation was incubated for 2 hr and the peptidophosphogalactomannan reisolated as described (12). The peptidophosphogalactomannan was treated with 0.5N NaOH (34) followed by neutralizing the reaction mixture and passing it over a Bio-Gel P-2 column. The (—) carbohydrate concentration and (---) ^{14}C are shown for each fraction.



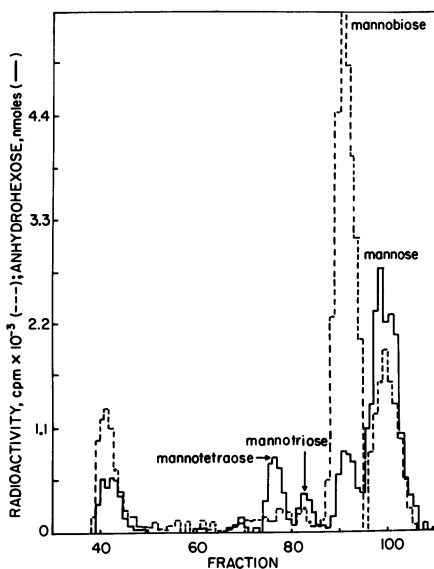
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Biosynthesis of peptidophosphogalactomannan in vitro. Man-
nose incorporation. Studies on the incorporation of hexosyl
residues into peptidophosphogalactomannan in this laboratory
have been confined to systems which transfer mannosyl residues
from GDP-D-mannose on to a polypeptide-(seryl/threonyl)-mannosyl
acceptor to form the mannosyl-containing peptidophosphogalac-
tomannan (12) and also on to a polypeptide-(phosphogalactomannan)
to extend the mannan chain by one or two mannosyl residues (13).

Membranes with a density of about 1.1 g/cc were used as the
source of the crude mannosyl-transferase (12). Mn^{2+} was required
for mannosyl-transferase activity, and the addition of peptido-
phosphogalactomannan increased about 3-fold the quantity of
mannose incorporated into glycopeptide-like substances (Fig. 1).
Substitution of phosphogalactomannan for peptidophosphogalacto-
mannan resulted in little increase in mannose incorporation
above that observed in controls having no added mannose (12).
This suggests that the mannose was incorporated into one of the
oligosaccharides rather than into the phosphogalactomannan
region. (^{14}C)Mannosyl-containing peptidophosphogalactomannan
was isolated from a reaction mixture following a 2-hr incubation
period, and the polymer purified (15). The purified glycopep-
tide was treated with 0.5 N NaOH to release phosphogalactomannan
and the oligosaccharides. The reaction mixture was neutralized
and passed over a Bio-Gel P-2 column to resolve the saccharides
(Fig. 2). About 10% of the radioactivity was found in the
phosphogalactomannan region and the remainder in fractions
coincident with mannobiose. The ^{14}C -mannosyl-containing polymer
was subjected to acetolysis and the acetolysis products were
chromatographed on Bio-Gel P-2 (Fig. 3). About 80% of the
radioactivity was located in the fraction coincident with manno-
biose. Radioactivity was also obtained in the monosaccharide
fraction which contained all of the galactose as well as a small
portion of the mannose. The time course of incorporation of
mannose from GDP-D-mannose into the oligosaccharide and phos-
phogalactomannan regions of peptidophosphogalactomannan was
followed. Incorporation into phosphogalactomannan occurred
rapidly while that into the oligosaccharide region continued up
to 4 hr (Table I). However, the extent of incorporation into the
oligosaccharide was much greater than that into phosphogalacto-
mannan. From these data it is not possible to determine if
separate enzymes transfer mannose to the two regions or if there
is an intramolecular mannosyl transfer. We were not able to
obtain evidence for participation of a mannosyl-lipid inter-
mediate in either of these reactions (Fig. 4).

Preliminary experiments established that about 10% of the
mannosyltransferase activity was solubilized by Triton X-100, and
that most of this activity was directed toward mannosylating
phosphogalactomannan (Fig. 5) (13). The mannosyltransferase
activity which remained in the pellet also retained its activity.
Mannosyltransferase activity was purified partially by passing

Figure 3. Distribution of carbohydrate and ^{14}C in fractions from Bio-Gel P-2 following acetolysis of ^{14}C -mannose-labeled peptidophosphogalactomannan. The reaction was conducted as described (12) and the products following acetolysis were fractionated on a column of Bio-Gel P-2. The (—) carbohydrate concentration and (---) ^{14}C are shown for each fraction.



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Table I

Time Course of Mannose Incorporation into Oligosaccharide and Polysaccharide Regions of Peptidophosphogalactomannan

Incubation Time	Polysaccharide	Oligosaccharide
hr	nmole	nmole
1	0.73	2.5
2	0.60	5.4
4	0.67	7.6
6	1.04	7.0

Each reaction mixture contained: Tris-maleate, (pH 7.0), 5 μmol ; GDP-D-(^{14}C) mannose, 25 nmole; MnCl_2 , 1.5 μmol ; 2-mercaptoethanol, 0.3 μmol ; peptidophosphogalactomannan, 3 mg; and membrane preparation, 150 μg . Incubations were carried out at 25°C for indicated interval, and the reaction stopped by heating at 100°C for 2 min. The low molecular weight substances were removed by dialysis. Each sample was treated with 0.5 M NaOH for 24 hr, neutralized and chromatographed on paper and the radioactivity in the polysaccharide and oligosaccharide peaks determined. (Taken from *J. Biol. Chem.* 1977, 252, 2187; Table IV).

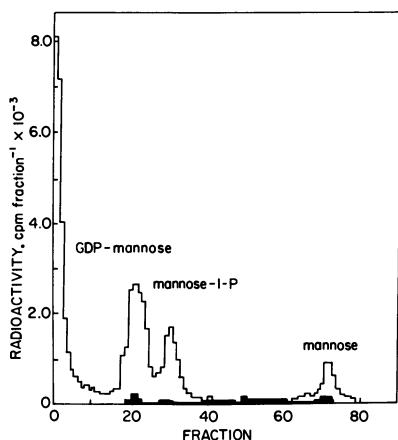
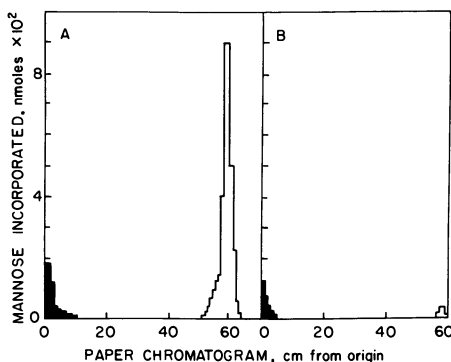
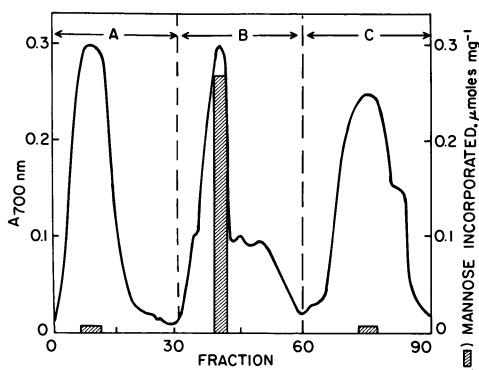


Figure 4. Distribution of ^{14}C -mannose-containing saccharides on paper chromatogram in a system containing GDP-D- ^{14}C mannose, peptidophosphogalactomannan, and components of mannosyltransferase system. The reaction mixture (12) was incubated at 25°C for 1 hr and the reaction stopped by heating for 2 min at 100°C and the reaction mixture was extracted with CHCl_3 :methanol, 2:1 (v/v) and the organic and aqueous layers chromatographed (12). The chromatograms were removed from the tank, dried, and cut into 0.5-cm strips and the ^{14}C in each strip determined.



Journal of Supramolecular Structure

Figure 5. Distribution of ^{14}C -mannose-containing saccharides on paper chromatograms after treating the peptidophosphogalactomannan with 0.5N NaOH. The complete system, in a volume of $60\ \mu\text{L}$, contained Tris-maleate, pH 7.0, in 5mM 2-mercaptoethanol; GDP-D- ^{14}C mannose, 5 nmol; MnCl_2 , 1.5 μmol ; 2-mercaptoethanol, 0.3 μmol ; peptidophosphogalactomannan, 3 mg; and membrane preparation (150 μg protein, Panel A) or 49 μg of protein from membranes treated with 1% Triton X-100, Panel B. The reaction mixture was incubated for 2 hr, the reaction was stopped by heating at 100°C for 2 min, and it was dialyzed against distilled deionized water to remove low-molecular-weight substances. The dialyzed reaction mixture was treated with 0.5N NaOH (34) and was neutralized, concentrated, and chromatographed (12). The chromatograms were sectioned and the ^{14}C determined (13).



Journal of Supramolecular Structure

Figure 6. Partial purification of soluble-mannosyltransferase by affinity chromatography on peptidophosphogalactomannan-Sepharose 4B (13). Membranes were prepared (12) and were extracted with 1% Triton X-100. The supernatant obtained following centrifuging the mixture at $120,000 \times g$ for 30 min was made 25mM in $MnCl_2$ and applied to a column containing peptidophosphogalactomannan-Sepharose 4B pre-equilibrated with 50mM Tris-maleate-5mM 2-mercaptoethanol-25mM $MnCl_2$. The adsorbent was washed with this buffer until the protein concentration in the effluent was negligible (Fraction 30), then the buffer was changed to one which contained Tris-maleate-mercaptoethanol as above, but no $MnCl_2$ (Fractions 30 to 60), and finally the adsorbent was washed with Tris-maleate-2-mercaptoethanol buffer containing 0.5M NaCl (Panel C). Each fraction contained approximately 1 mL. Samples were removed from each and (—) protein and (▨) mannose incorporated determined using the system described previously (12).

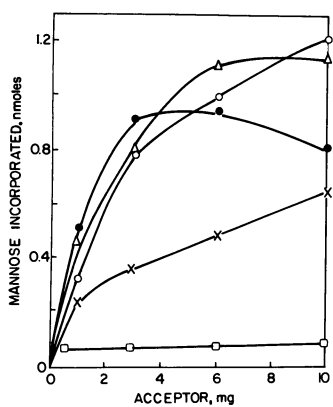


Figure 7. Influence of acceptor concentration on mannosyltransferase activity (13). Crude, Triton X-100 solubilized enzyme (26 μg protein) was incubated in a standard reaction mixture (52) with the indicated concentration of the acceptors, and the reaction was stopped and the quantity of ^{14}C -mannose that was incorporated into peptidophosphogalactomannan determined (12). (X) Peptidophosphomannan, (●) phosphogalactomannan, (○) phosphomannan, (Δ) mannan, and (■) α -mannosidase-treated peptidophosphogalactomannan were tested as mannosyl acceptors.

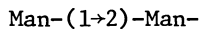
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the Triton X-100 soluble extract containing added MnCl_2 over a peptidophosphogalactomannan-Sepharose 4B affinity column and the activity released from the column by omitting MnCl_2 from the buffer system (Fig. 6). The enzyme showed a dependence on Mn^{2+} for activity and the addition of Mg^{2+} did not enhance activity nor could Mg^{2+} replace Mn^{2+} . The optimum concentration of Mn^{2+} was about 20 mM. However, this high concentration of Mn^{2+} required for maximal activity probably reflects the chelating effect of maleate buffer used in the assay system.

In this system mannose incorporation continued for 2 hr and the activity of the enzyme was increased in a hyperbolic fashion with increase in the concentration of GDP-D-mannose.

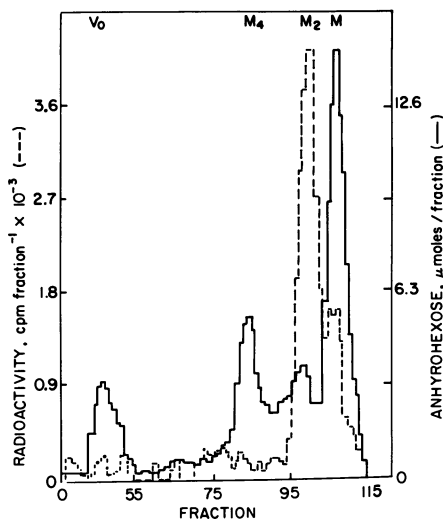
Various partial degradation products of peptidophosphogalactomannan were prepared and each was tested in the system to determine if it was a suitable acceptor of mannosyl residues (Fig. 7). Mannan, phosphomannan and phosphogalactomannan were all excellent acceptors in the system and 6-10 mg of mannan or phosphomannan accepted 1 nmole of mannose in 2 hr catalyzed by 26 μg of protein. Peptidophosphogalactomannan was not as effective as an acceptor. Possibly the mannosyltransferase binds to some of the mannose-containing oligosaccharides which would cause this region of the polymer to serve as an inhibitor of the transfer of mannose to phosphogalactomannan region. The most striking observation was afforded by the inability of α -mannosidase treated peptidophosphogalactomannan to serve as a mannosyl acceptor in this system. This experiment was repeated several times, each time the same result was obtained.

^{14}C -Mannosyl-containing phosphogalactomannan was prepared with the soluble mannosyltransferase using phosphogalactomannan as the mannosyl acceptor. Following the 2 hr incubation period, phosphogalactomannan was isolated in the usual manner (15) and the polymer subjected to acetolysis. The products were chromatographed on Bio-Gel P-2 (Fig. 8). This figure shows that ^{14}C -mannose was incorporated into positions in which either a (1 \rightarrow 6) or a (1 \rightarrow 2) glycosidic linkage was formed; that is, both ^{14}C -mannose and ^{14}C -mannobiose were obtained from the acetolysis products. This suggests that the mannosyltransferase was either transferring two mannosyl residues to form the mannobiose or, as is more likely, the phosphogalactomannan acceptor was heterogeneous at the non-reducing terminal end of the mannan with at least two types of termini which serve as suitable acceptors in this system:



We currently know little about the heterogeneity of the nonreducing terminal region of the mannan. However, α -mannosidase degrades about 15% of the mannan and the product is not a

Figure 8. Location of ^{14}C -mannosyl residues derived from ^{14}C -phosphogalactomannan following acetolysis (13). A reaction mixture containing 30 mg phosphogalactomannan, 125 μg of crude mannosyl-transferase, and other components of the mannosyl-transferase system (13) were incubated for 2 hr and the reaction stopped and phosphogalactomannan reisolated. Twenty mg of phosphogalactomannan was obtained. The polymer was subjected to acetolysis and the products were deacetylated and fractionated on Bio-Gel P-2 as described (13). The (—) anhydrohexose and (---) ^{14}C were determined in each fraction. The void volume, v_0 , and the position of elution of mannose, M; mannobiose, M_2 ; and mannotetraose, M_4 are given on the figure.



Journal of Supramolecular Structure

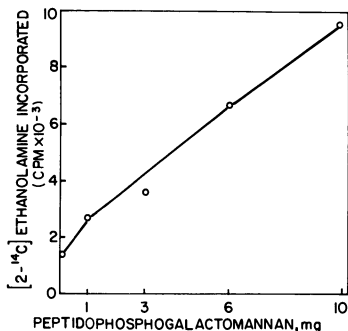
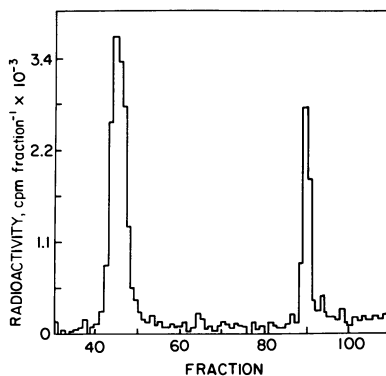


Figure 10. Fractionation on Bio-Gel P-2 of the products formed by treatment of ^{14}C -ethanolamine-containing phosphogalactomannan with 0.5N NaOH. A reaction mixture containing 50 mg phosphogalactomannan and the same final concentrations of other reactants as described in Figure 2 in a total volume of 0.3 mL was incubated and the reaction stopped as in Figure 9. Phosphogalactomannan was isolated and purified as described previously (15). The polymer was treated with 0.5N NaOH for 24 hr, the reaction mixture neutralized, and passed onto a column of Bio-Gel P-2. The products were eluted with distilled, deionized water and the ^{14}C was determined in each fraction. The void and inclusion volumes of the column occur at 42–45 and 100–105, respectively.

Figure 9. Influence of peptidophosphogalactomannan concentration on ^{14}C -ethanolamine incorporation into polymers. The reaction mixture contained membrane fraction M_5 ($\rho = 1.18 \text{ g/mL}$ as described) (12), Tris-maleate, pH 7.5, 5 μmol ; 2-mercaptoethanol, 0.3 μmol ; CDP-(2- ^{14}C)-ethanolamine, 3.33 nmol; MnCl_2 , 1.5 μmol ; peptidophosphogalactomannan in the indicated quantity, all in a total volume of 60 μL . The reaction mixture was incubated for 2 hr at 25°C and the reaction stopped by heating at 100°C for 2 min. The products were separated by paper chromatography (12). ^{14}C -Tthanolamine-containing polymers remain at the origin.



suitable acceptor in this system (Fig. 7). This treatment may have degraded the mannan chain down to either the first galactan chain or to phosphoethanolamine/phospho-N,N'-dimethylamino-ethanol residue, and the specificity of the mannosyltransferase is sufficiently great that it will no longer transfer mannosyl residues to the nonreducing terminal end. Unlike some mannosyltransferases, this enzyme has little or no activity when mannose, mannobiose or mannotetraose is used as the potential mannosyl acceptor.

Ethanolamine incorporation. Preliminary studies have shown that membrane fraction V ($\rho = 1.2$ g/cc) catalyzes a peptidophosphogalactomannan-dependent transfer of ethanolamine, or more likely phosphoethanolamine, from CDP-ethanolamine (Fig. 9). Approximately 4-fold more ethanolamine was transferred into peptidophosphogalactomannan when α -mannosidase-treated peptidophosphogalactomannan was used as the acceptor than occurred with untreated peptidophosphogalactomannan. The ^{14}C -ethanolamine-containing peptidophosphogalactomannan was isolated and treated with 0.5 N NaOH, and the products of the neutralized reaction mixture were passed over a Bio-Gel P-2 column. This treatment released about 35% of the ^{14}C into a low molecular weight fraction and the remainder was not released (Fig. 10). These results are similar to those obtained with ^{14}C -ethanolamine-containing peptidophosphogalactomannan synthesized *in vivo* (14,17).

The activity of the ethanolaminetransferase is almost linearly dependent on the concentration of peptidophosphogalactomannan. In the system used, approximately 0.2-0.8 nmole of ethanolamine was transferred per hr. We know little about the substrate specificity of the enzyme except that α -mannosidase treated peptidophosphogalactomannan was a better acceptor than untreated polymer. This observation suggests that processing the nonreducing terminal end of the mannan may be an important process in the biosynthesis of peptidophosphogalactomannan.

Possible functions of peptidophosphogalactomannan. The evidence presented in this paper coupled with that presented previously (24) suggests that peptidophosphogalactomannan does not have a structural function in the cell wall. The evidence suggests that peptidophosphogalactomannan is derived from a membrane-bound lipopeptidophosphogalactomannan. This amphipathic substance appears to be tightly associated with a membrane fraction of density 1.18 g/cc and the membrane is particularly rich in acid phosphatase activity. This membrane fraction may be derived from organelles which contain the acid-hydrolases. The lipo-peptidophosphogalactomannan is likely a precursor of peptidophosphogalactomannan found in the extracellular culture filtrate.

We anticipate that the polypeptide of lipo-peptidophosphogalactomannan will also be heterogenous like that of the

extracellular polymer. Heterogeneity in the sequence of amino acyl residues of the polypeptide suggests that the polypeptide portion of peptidophosphogalactomannan is derived from several different proteins. It is known that yeasts and fungi produce glyco-enzymes containing multiple oligosaccharides attached to the polypeptide (25-27). Some enzymes, such as the exocellular (wall-bound or mural) invertase of yeast, contain as much as 50% by weight of carbohydrate (28), other enzymes such as the glucamylases contain less than 10% carbohydrate (25). Smith and Ballou (29) have suggested that the carbohydrate portion of yeast invertase functions in concert with disulfide bonds to anchor the glycoenzyme to the yeast cell wall.

It is possible that lipo-peptidophosphogalactomannan represents a membrane-bound form of the exocellular enzyme with the lipo-phosphogalactomannan region serving to anchor the protein to the plasma membrane with the protein located between the plasma membrane and the cell wall. However, there is no role for soluble "cytoplasmic" peptidophosphogalactomannan in this model. An alternative model, which is more consistent with our data, is in part an extension of the model of Blobel (30). We propose that phosphogalactomannan may have two physiological functions. The first function is to protect the mural enzymes from premature proteolytic cleavage of the peptide chain as the chain is transported from the ribosome into the lumen of the organelle which will later fuse with the plasma membrane. The second function is to react with charged groups of the plasma membrane and cell wall thus serving to anchor the enzyme in the periplasmic space. According to this model lipo-peptidophosphogalactomannan is obtained from the membrane-bound mural enzymes which are being protected from lysis by the galactomannan; soluble "cytoplasmic" peptidophosphogalactomannan is obtained from the mural enzymes in the periplasmic space and from those species from which the hydrophobic residue(s) have been removed. We have observed that peptidophosphogalactomannan binds several proteins in addition to galactofuranosidase and that harsh conditions are required to release some of these proteins. Braatz and Heath have suggested that during secretion exocellular enzymes are protected from wall-bound proteases by a glucosamine-containing "protective" polysaccharide (31). Phosphogalactomannan may serve a similar function in the Ascomycetes.

If phosphogalactomannan does serve a protective function during synthesis of mural proteins, then it may be necessary to add the mannosyl residues to the mannan in a stepwise fashion from GDP-D-mannose rather than in a block from a lipid-linked intermediate mannosyl-containing oligosaccharide. The role of phosphoethanolamine in the proposed protective function may be similar in type to that reported by Holtje and Tomasz for ethanolamine and choline in cell wall teichoic acid of Diplococcus pneumoniae; that is, ethanolamine-containing cell wall teichoic

acid blocked muramidase activity while choline-containing cell wall teichoic acid did not interfere with muramidase activity (32).

Summary

Investigations on the in vivo and in vitro formation of peptidophosphogalactomannan have been reviewed and new work presented. It was shown that α -mannosidase treated phosphogalactomannan was an excellent ethanolamine acceptor from CDP-ethanolamine catalyzed by membrane bound enzymes. However, this modified phosphogalactomannan would not accept mannosyl residues from GDP-D-mannose in a partially purified system.

Possible functions for peptidophosphogalactomannan and the new lipopeptidophosphogalactomannan were suggested. It was proposed that phosphogalactomannan may protect, at the time of synthesis as well as later, those enzymes which are destined to become cell wall bound or extracellular. The relationship between structure, function and biosynthesis are discussed.

Acknowledgement

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Toward Understanding the Structure, Biosynthesis, and Function of a Membrane-Bound Fungal Glycopeptide

Structural Studies

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Fungi secrete numerous glycopeptides, polysaccharides, oligosaccharides and a host of lytic enzymes which degrade the extracellular polymers. Galactocarolose, a 5-0- β -D-galactofuranosyl-containing decasaccharide, and mannocarolose, a mannapyranosyl-containing nonasaccharide, were among the first fungal extracellular saccharides to be examined extensively by methylation techniques (1). Both of these polymers were obtained from the filtrates of 28-day stationary cultures of *P. charlesii* grown on the Raulin-Thom medium (2). We have extended the early work of Haworth and colleagues (1,2,3) and that of Gorin and Spencer (4), and Hough and Perry (5,6) to show that galactocarolose and mannocarolose of *P. charlesii* are degradation products of a larger ethanalamine-/N,N'-dimethylaminoethanol-containing peptidophosphogalactomannan (7-12). We have also shown that *P. chrysogenum*, *Penicillium claviforme*, *Penicillium patulum* and *Penicillium raistrickii* secrete galactofuranosyl-containing polymers into their growth media (13). Peptidophosphogalactomannans have been obtained from *Cladosporium werneckii* (14,15) and peptidogalactomannans have been obtained from numerous species of *Aspergillus* (16,17,18) and *Penicillium* (18). The data suggests that peptidogalactomannans, or peptidophosphogalactomannans, may be common to several genera of Ascomycetes.

Peptidogalactomannans and peptidophosphogalactomannans have been extracted from powdered fungal cells and they also accumulate in the growth medium. It has been assumed by many investigators that these polymers represent cell wall degradation products. However, the cell walls of 14-day stationary cultures of *P. charlesii* contain no galactofuranosyl residues (19) and the alkali-soluble, alcohol-insoluble fraction of cell walls of 3-day aerated cultures of *P. charlesii* contain only 1.6% galactofuranosyl residues and no detectable mannapyranosyl residues (20). This fraction also contained glucosyl residues and ethanalamine. The results from both laboratories suggest that the cell walls of *P. charlesii* contain few galactofuranosyl residues and that the

galactofuranosyl residues present are not released by alkali as a galactomannan or phosphogalactomannan as is expected if peptidophosphogalactomannan is an integral part of the fungal cell wall. It is also unlikely that putative cell wall galactofuranosyl residues were removed by the action of exo- β -D-galactofuranosidase because neither the growth medium nor cell extracts of 3-day aerated cultures contain measurable galactofuranosidase activity (21,22), and galactofuranosidase is not secreted into the growth medium until several days later (21). Furthermore, it has been shown that P. charlesii starts synthesizing peptidophosphogalactomannan, or possibly a precursor polymer, soon after it germinates (23) but the peptidophosphogalactomannan is not secreted until the growth medium is depleted of NH_4^+ . Because of this dilemma we initiated a search for intracellular galactofuranosyl-containing polymers.

This paper reviews and extends our work on structural characterization of the extracellular ethanolamine-/N,N'-dimethylaminoethanol-containing peptidophosphogalactomannan of P. charlesii, and describes our studies on isolation, purification and partial characterization of a membrane-bound galactofuranosyl-containing polymer which we shall refer to as lipopeptidophosphogalactomannan.

Experimental

Penicillium charlesii G. Smith ATCC 1887 was maintained (7) and cultured (24) at 20°C with constant aeration as described previously.

Isolation and Fractionation of Membranes. Mycelia from 48 hr cultures were separated from the growth medium by filtration and the mycelia were washed with several volumes of distilled, deionized H_2O at 4°C and the water removed from the mycelia by pressing between paper towels. The mycelia were pulverized at 4°C in 50 mM Tris-HCl, pH 7.5 buffer with Al_2O_3 ; ratio of mycelia:alumina:buffer, 1:0.3:2 (w/w/v). Membranes were isolated from the homogenate essentially as described previously (25). The supernatant solutions from the initial centrifugation and the membrane wash were pooled, dialyzed against distilled deionized water for at least 24 hr at 4°C, and used as a source of "cytoplasmic" peptidophosphogalactomannan.

The membranes were separated by isopycnic sucrose gradient ultracentrifugation (25). The individual membrane fractions were pelleted by centrifugation at 120,000xg in a Ti-75 rotor of a Beckman L2-65B ultracentrifuge and the pellet resuspended in 50 mM Tris-HCl, pH 7.5 buffer.

Chromatography

Gel permeation. Bio-Gel A-5m (57 x 2.5 cm column) was equilibrated in the appropriate buffer and packed in a column. Three ml sample of membranes was applied to the top of the column and the column irrigated with 40 mM Tris-HCl-40 mM NaCl-0.24% deoxycholate (DOC) at a flow rate of 10 drops min⁻¹. Fractions containing 100 drops (approximately 1.7 ml) were collected.

Ion exchange. The borate complexes of various glycopeptide preparations were fractionated on Whatman DE-23 (10).

The polypeptides obtained by treating the glycopeptide(s) with anhydrous HF were fractionated on Whatman DE-23 in the acetate form. The sample was applied in 0.02 M pyridinium acetate, pH 5 and the column was washed with 70 ml of 0.02 M pyridinium acetate (pH 5.0) followed by a gradient from 0.03 to 1.0 M acetic acid. Each fraction contained 3.5 ml.

Affinity chromatography. Concanavalin A was linked to cyanogen bromide activated Sepharose 4B by standard procedures (26). The remaining active groups on the cyanogen bromide activated Sepharose 4B were blocked by treatment with 1 M ethanolamine, pH 7.0 for 2 hr at 4°C. Approximately 90% of the concanavalin A was linked covalently to Sepharose 4B. Immobilized concanavalin A was activated by incubation overnight at 4°C in 40 mM Tris-HCl-40 mM NaCl-1 mM MgCl₂-1 mM MnCl₂-1 mM CaCl₂. Before use the concanavalin A-Sepharose 4B preparation was equilibrated with 40 mM Tris-HCl-40 mM NaCl-0.24% DOC.

Fractions containing DOC-soluble glycopeptide obtained from the column of Bio-Gel A-5m were applied to a column of concanavalin A-Sepharose 4B and the column was washed with 40 mM Tris-HCl-40 mM NaCl-0.24% DOC until the UV-absorbing material was removed. The DOC-soluble glycopeptide was eluted with 40 mM Tris-HCl-40 mM NaCl-0.24% DOC-1% methyl- α -D-mannopyranoside.

Thin layer chromatography. Chromatography on Brinkman silica gel plates was conducted with the solvent systems given in the appropriate tables. After development of the chromatogram, the position of ¹⁴C was determined by scraping the silica gel in 2 cm sections into scintillation vials. Scintillation fluid was added and the ¹⁴C was determined in a liquid scintillation spectrometer.

Hydrogen fluorolysis. Carbohydrate was removed from the glycopeptide with anhydrous HF (27) without cleavage of the polypeptide. The products were dissolved in pyridine-H₂O, the anisoleglycosides were precipitated with water, and the polypeptides were resolved by ion exchange chromatography.

Carbon-13 NMR spectroscopy. Natural abundance carbon-13 NMR spectra were taken on a Varian XL-100 spectrometer operated in the Fourier transform mode at a frequency of 25.2 MHz. Samples of phosphogalactomannan were usually in excess of 100 mg ml⁻¹ of ²H₂O. The field was locked on the deuterium signal and the spectra were recorded with proton noise decoupled. Chemical shifts are reported in ppm from an external reference solution of 5% sodium (trimethylsilyl)-1-propane sulfonate (TSP) in a coaxial tube. The spectrum shown represents data from 64,000 transients.

Enzymic and chemical assays. a) Galactofuranosyl-containing chains. Exo-β-D-galactofuranosidase (21) was used to release galactofuranosyl residues from galactan chains and the quantity of galactose released was quantified (28). Controls without substrate or galactofuranosidase or with inactivated enzyme were carried out and appropriate corrections were made.

b) Carbohydrate. Total carbohydrate was determined by the phenol-sulfuric acid method (29).

c) Total phosphate. Samples were ashed according to the procedure of Ames and Dubin (30) and phosphate was determined by the method of Fiske et al. (31).

d) Protein. Protein was determined by the procedure of Lowry et al. (32) with bovine serum albumin as a reference.

e) Free amino groups. The free amino groups were reacted with 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) (33) and the low molecular weight substances were removed. The derivatized glycopeptides were hydrolyzed with 6 N HCl for 22 hr at 110°C in sealed, evacuated tubes, and the dansyl-amino acids were chromatographed on polyamide layer sheets (11).

f) Low molecular weight polypeptides. The low molecular weight polypeptides were quantified by reacting with fluorescamine and measuring the fluorescence emission at 460 nm following irradiation at 390 nm on a Farrand spectrofluorometer. The relative fluorescence emission was compared to leucine as a reference.

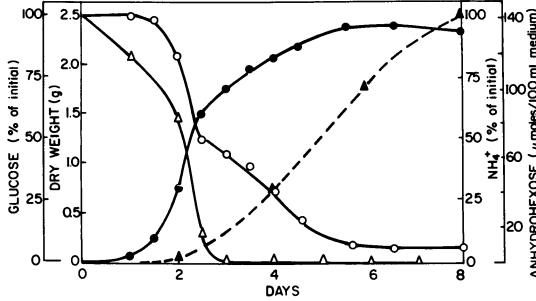
g) Carbon-14 labeled substances. Carbon-14 was quantified with a Beckman LS-235 liquid scintillation spectrometer (25).

Results

Extracellular peptidophosphogalactomannan. The growth medium of aerated cultures of *P. charlesii* becomes depleted of NH₄⁺ after about 2.5 days but glucose is not depleted until about the eighth day (Fig. 1). The growth medium used (34) is unbalanced

with an excess of carbohydrate with respect to NH_4^+ . At the onset of NH_4^+ starvation the rate of glucose uptake decreases as does the rate of increase in dry weight, and the organism starts secreting a non-dialyzable hexosyl-containing polymer. This polymer accumulates in the growth medium until the medium is depleted of glucose. The polymer from 8- to 10-day aerated cultures was isolated by precipitation from 50 mM borate at pH 9.5 as the cetyltrimethyl ammonium-borate complex (35) and purified by ion-exchange chromatography (7). Nearly all of the carbohydrate was eluted from DEAE-cellulose (borate) with 0.01 N HCl-0.06 N LiCl; the same conditions which eluted the glucosyl-, galactofuranosyl-, mannopyranosyl-containing polymers obtained from 28-day stationary cultures of *P. charlesii* (7). Preliminary chemical analyses showed that the aerated cultures contained a saccharide composed of galactofuranosyl and mannosyl residues. The saccharides obtained from the aerated culture filtrates was further purified by gel-permeation chromatography. The partially purified polymer was subjected to isoelectric focusing and over 90% of the polymer showed an isoelectric point near pH 2 (10). However, the fractions obtained from gel-permeation chromatography showed considerable heterogeneity with respect to the molar ratios of hexose:phosphate and percentage of galactose in the saccharides (Fig. 2) (21). The polymers with the largest mass (fraction 13, Fig. 2) contained 70% galactose and a hexose:phosphate ratio of greater than 30 which is in contrast to that (fraction 20) containing less than 30% galactose and a hexose:phosphate ratio of 12. These and other results showed a proportional increase in mass with increase in percentage of galactose. Furthermore, we showed that the polymer from 6-day cultures had a weight average molecular weight of about 68,000 and 69.9% galactose as compared to the polymer from 12-day cultures with a weight average molecular weight of 22,600 and 12.1% galactose (Fig. 3). The percentage of galactose in extracellular saccharides decreased from about 70% in 6-day cultures to 30% in 12-day cultures. This decrease in mass and percentage galactose coincided with the appearance of *exo*- β -D-galactofuranosidase in the growth medium (21). Galactofuranosidase degrades the galactomannan rapidly until galactose accounts for only about 15% of the total saccharide. Galactomannans from other species of fungi also contain approximately 15% galactose. We have obtained galactofuranosidase activity from culture filtrates of several *Penicillium* species and one species of *Aspergillus* (36).

Treatment of the phosphogalactomannan with either 0.1 N HCl for 90 min at 100°C or with *exo*- β -D-galactofuranosidase for 2 days removed essentially all of the galactosyl residues. The acid treatment also removed some of the phosphorus, but treatment with galactofuranosidase removed only the galactosyl residues. These results suggest that the galactosyl residues occur in the furanosyl form and that there are no galactofuranosyl residues within the mannan backbone. Gel permeation chromatography



Archives of Microbiology

Figure 1. Time course of D-glucose and NH₄⁺ uptake from the growth medium and increase in dry weight of mycelia of *P. charlesii* cultures. *Penicillium charlesii* was cultured in a modified Raulin-Thom medium (24). The contents of the flasks were removed at intervals, filtered and the filtrate was assayed for (○—○) total carbohydrate, (△—△) NH₄⁺, (●—●) dry weight, and (▲—▲) pPGM (23). The initial concentrations of D-glucose and NH₄⁺ were 278 and 36.3mM, respectively.

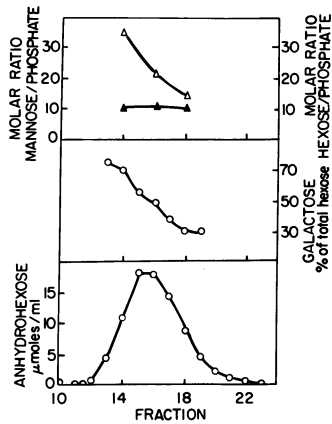


Figure 2. Evidence for heterogeneity in peptidophosfogalactomannan (21). A 50-mg sample was fractionated on Bio-Gel P-60 column (1.5 × 82 cm). The elution profile is shown in the bottom panel. The percentage of galactose as a function of total carbohydrate is given in the middle panel. The (▲) mannose:phosphate ratio and (△) hexose:phosphate ratio are shown in the top panel.

Journal of Biological Chemistry

showed that the phosphomannan had a mass of about 20,000 daltons.

Proton nuclear magnetic resonance spectrum of a 5% solution of phosphomannan was taken in $^2\text{H}_2\text{O}$ at 29°C and the spectrum of the protons attached to the anomeric carbon atoms show resonance signals at 5.04, 5.10 and 5.22 p.p.m. (Fig. 4). The signal at 5.04 p.p.m. likely represents an $\alpha(1\rightarrow6)$ linkage and those at 5.10 and 5.22 represent $\alpha(1\rightarrow2)$ linkages similar to those observed for yeast mannan oligosaccharide (37). Integration of the area under these peaks suggests a ratio of mannosyl(1 \rightarrow 6):mannosyl(1 \rightarrow 2) of 1:3.

Treatment of the galactomannan with alkali resulted in a time-dependent increase in absorbance at 241 nm, typical of that observed when glycosyl residues undergo β -elimination from seryl or threonyl groups of a protein (38). It was shown that the galactomannan was attached to a polypeptide containing about 30 amino acyl residues with a mass of about 3,000 daltons (11). The polymer has the chemical composition of a peptidophosphogalactomannan. A reasonably homogenous preparation containing 15% galactose is composed of 110 mannosyl residues, 18 galactosyl residues, 10 atoms of phosphorus and 30 amino acyl residues per molecule of peptidophosphogalactomannan. Treatment of the peptidophosphogalactomannan with 0.4 N NaOH changes the molar ratios of galactose:mannose and P:mannose from 1:5.4 to 1:4.6 and 1:10.1 to 1:8.6, respectively. However, there was no change in the ratio of P:galactose. This suggests that the phosphogalactomannan released from the polypeptide contained 90 mannosyl residues; a loss of about 20 mannosyl residues from that calculated for peptidophosphogalactomannan.

Phosphogalactomannan. Yeast glycopeptide has mannosyl-containing oligosaccharides attached by O-glycosidic linkage to seryl and threonyl residues of the polypeptide (39). However, the phosphomannan is attached to an asparaginyl group of the polypeptide through an N-glycosidic linkage. Therefore, the oligosaccharides are readily released by 0.4 N NaOH at 25°C while the N-glycosidic linkage is stable under these conditions. Peptidophosphogalactomannan from *P. charlesii* was treated with NaB^3H_4 in alkali followed by neutralizing the reaction mixture and dialysis to separate the low molecular weight substances from the non-dialyzable ones. The non-dialyzable fraction was treated with 2 N H_2SO_4 at 100°C for 3 hr, the reaction mixture was neutralized, deionized by passage over Amberlite MB-3 resin, and analyzed by paper chromatography for the presence of ^3H -sugar alcohols. All of the ^3H was found in the region of the chromatogram corresponding to mannitol (11). We conclude that the phosphogalactomannan region is attached to the polypeptide by an O-glycosidic linkage which when treated with alkali resulted in the release of a polysaccharide with a reducing terminal mannose residue.

Figure 3. Sedimentation equilibrium ultracentrifugation of peptidophosphogalactomannan (21). Solutions containing 0.05% peptidophosphogalactomannan in 0.1M NaCl were centrifuged for 24 hr at 20.6°C in an AnD rotor. Galactose accounted for (○) 69.6% and (●) 12.1% of the total carbohydrate in the two samples. Centrifugation was conducted at 30,000 rpm in a Spinco Model E analytical ultracentrifuge.

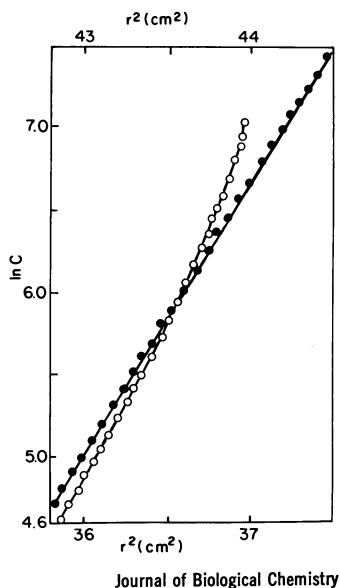
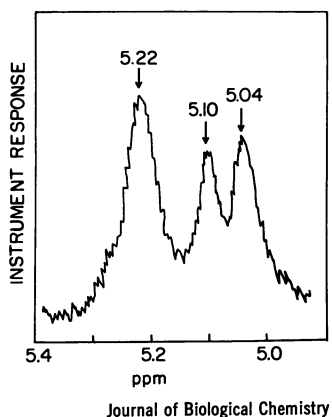


Figure 4. Proton magnetic resonance spectroscopy of the anomeric proton region of peptidophosphomannan (10). Fifty mg of peptidophosphomannan was dissolved in $^2\text{H}_2\text{O}$ and held at room temperature for 1 hr. The solvent was removed under reduced pressure and the residue redissolved in 1 mL $^2\text{H}_2\text{O}$. The NMR spectrum was taken on a Varian XL-100 MHz spectrometer at 29°C with the instrument locked on deuterium and referenced to an internal standard of sodium 2,2-dimethyl-2-silapentane-5-sulfonate.



Chemical analysis of phosphogalactomannan showed that each molecule contained about 9 galactan chains and 9 atoms of phosphorus. Phosphogalactomannan from peptidophosphogalactomannan containing 15% galactose has a ratio of P:mannose of 1:8.6 and 18% galactose. Each galactan chain contains about 2-galactosyl residues.

In a separate experiment the saccharides were β -eliminated in the presence of NaOH and phosphogalactomannan separated from the low molecular weight substances by gel permeation chromatography. The low molecular weight substances which were refractionated on Bio-Gel P-2, were resolved into fractions containing mannose, manno-*biose*, and manno-*triose* with small quantities of larger saccharides (Fig. 5) (10). The linkages of manno-*biose* and manno-*triose* have not been determined, but preliminary experiments suggest that the manno-*triose* fraction contains primarily D-mannopyranosyl-D-mannopyranosyl-(1 \rightarrow 2)-D-mannose (36).

Methylation analysis. Peptidophosphogalactomannan and its degradation products were quantitatively methylated and the methylated polymers were converted to their permethylated alditol acetates in an attempt to determine the types of linkages in the polymer. The permethylated alditol acetates were separated and quantified by gas-liquid chromatography and they were identified by their fragmentation patterns in a mass spectrometer. A quantitative recovery of galactose was obtained, but 10 to 15 mannosyl residues were lost during the chemical manipulations. This probably occurred because of the alkali lability of some of the mannose-containing oligosaccharides. Thus, only 2 to 3 non-reducing terminal mannosyl residues were observed in contrast to the 12 to 14 expected based on the quantity of oligosaccharides released with alkali. Therefore, the data on the phosphogalactomannan, and phosphomannan are probably the most reliable. The data (Table I) show 8-9 moles of nonreducing terminal galactosyl residues and variable quantities of internal galactosyl residues per mole of phosphogalactomannan. The mannan appears to contain about 3-fold more (1 \rightarrow 2) than (1 \rightarrow 6)-linked mannosyl residues. In addition, the occurrence of approximately 9 moles of 4,6-dimethyl mannose, a number equivalent to the number of nonreducing terminal galactosyl residues, and the loss of 4,6-dimethyl mannose when the phosphogalactomannan is treated with dilute mineral acid or peptidophosphogalactomannan is treated with galactofuranosidase, suggests that the galactan chains are attached by (1 \rightarrow 3)-linkage to a mannosyl residue which also has another mannosyl residue attached to it by a (1 \rightarrow 2)-linkage.

^{13}C -NMR spectroscopy. We have now examined the ^{13}C NMR spectrum of phosphogalactomannan. The polymer was first partially degraded with galactofuranosidase to increase the prominence of the resonance signals from the mannosyl residues and to

Table I
 PERMETHYLATED ALDITOL ACETATES IN PEPTIDOPHOSPHOGALACTOMANNAN AND ITS DERIVATIVES

Parent Sugar	Position of O-Methyl Group	pPGM*2		PGM ³		pPM ³	
		Galactofuranosidase Treatment	+	B	A	A	A
Mannose	2,3,4,6	-					
Galactose	2,3,5,6	2	1	2	2	10	
Galactose	2,3,6	12	1	8	8-9	-	
Mannose	2,3,4	38	11	60	114	-	
Mannose	3,4,6	22	26	17	18	26	
Mannose	4,6	62	75	45	51	61	
Mannose	3,4	14	4	11	9	-	
		8	4	3	4	4	

*Abbreviations: pPGM, peptidophosphogalactomannan; PGM, phosphogalactomannan; pPM, peptidophosphomannan.

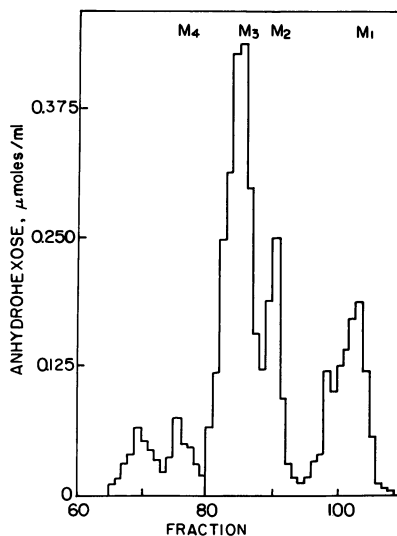
1. Samples under heading A and B were derived from pPGM which contained 53% and 40% galactose, respectively. PGM was calculated to contain 90 mannosyl residues. The number of hexosyl residues per mole is calculated on the basis of 90 mannosyl residues and 20 residues in the phosphogalactomannan and oligosaccharide regions respectively.
2. The permethylated alditol acetates were separated on Chromosorb W with 3% polyphenyl ether (6 rings) as the liquid phase.
3. The permethylated alditol acetates were separated on ECNSS-M and OV-225.

decrease the signals from the internal galactosyl residues. The spectrum (Fig. 6) contains four signals (A through D) in the region which is characteristic of anomeric carbon atoms (90 to 110 ppm), numerous signals (E through M) in the region of 65 to 85 ppm, and the doublet at 42.7 ppm which is coincident with the C(2') methylene group of L- α -glycerophosphorylethanolamine. The spectra of reference compounds consisting of the α - and β -anomers of methyl galactofuranoside and D-mannopyranosyl- α (1 \rightarrow 2)-D-mannopyranosyl- α (1 \rightarrow 2)-D-mannose were compared to that of phosphogalactomannan (Fig. 6) and to that of phosphomannan (not shown). The resonance signal (A) at 109.5 ppm is that from the anomeric carbon of the internal 5-O- β -D-galactofuranosyl residues and that (A') at 110.4 ppm was probably from the anomeric carbon of the 9 nonreducing terminal galactofuranosyl residues. The large signal (E) at 84.0 ppm is derived from C(2) and C(4) of the 5-O- β -D-galactofuranosyl residues. The resonance signal of the C(5) atom of methyl- β -D-galactofuranoside is at 73.2 ppm and the signal at 78.2 ppm shifted downfield by 5 ppm is strong evidence for the occurrence of a glycosidic attachment to C(5) of the galactosyl residue. The resonance signals at 79.1 (G) and 63.7 ppm (M) are those from the C(3) and C(6) carbon atoms, respectively. Treatment of the polymer with dilute mineral acid causes the loss of signals at positions A', A, E, G and H, and the signal (M) at 63.7 ppm is decreased to the same peak height as signal K. The signal at 63.7 ppm represents the C(6) atom of the free hydroxymethyl groups of both mannopyranosyl and galactofuranosyl residues.

The resonance signals (B-D) at 104.9, 103.3 and 100.9 ppm represent those from the anomeric carbon of three species of pyranosides. The signals at 104.9 and 103.3 coincide with the nonreducing terminal mannopyranosyl and internal α (1 \rightarrow 2)-D-mannopyranosyl residues, respectively. The signal at 100.9 ppm has not been identified, but it is known to be eliminated upon removal of the phosphorus at pH 3.5. However, the chemical shift of the anomeric carbon atom of an α -D-mannopyranosyl-1-phosphoryl residue should be around 97.1 ppm (40) which seems to eliminate an α -D-mannopyranosyl-1-phosphoryl group as being attached to the mannan backbone as occurs in the glycopeptide from *C. werneckii* (15).

The resonance signals (F, I-M) all coincide well with resonance signals from internal mannopyranosyl residues. The signal from the C(3) atom of the mannopyranosyl residues to which the galactan chains are attached should appear at about 5 ppm downfield from unsubstituted C(3) atom of internal mannopyranosyl residues; that is at about 78 ppm. The signal from this C(3) atom may be buried in that of the C(5) signal from 5-O- β -D-galactofuranosyl residues (signal H). The signal (L) at 65.7 ppm and the unlettered one which appear as shoulders on other major resonance species may represent some of the species to which the

Figure 5. Release of mono- and oligosaccharides from the glycopeptide in 0.4N NaOH (10). Approximately 20 mg of peptidophosphogalactomannan were treated in the dark in 0.4N NaOH in an atmosphere of N_2 for 72 hr. The saccharides were fractionated, after neutralization, on a Bio-Gel P-2 column (3×92 cm). Reference substances of glucose, maltose, raffinose, and stachyose showed maximum concentrations in Fractions 103, 81, 85, and 79, respectively. Carbohydrate was quantified by the phenol-sulfuric acid assay (29)



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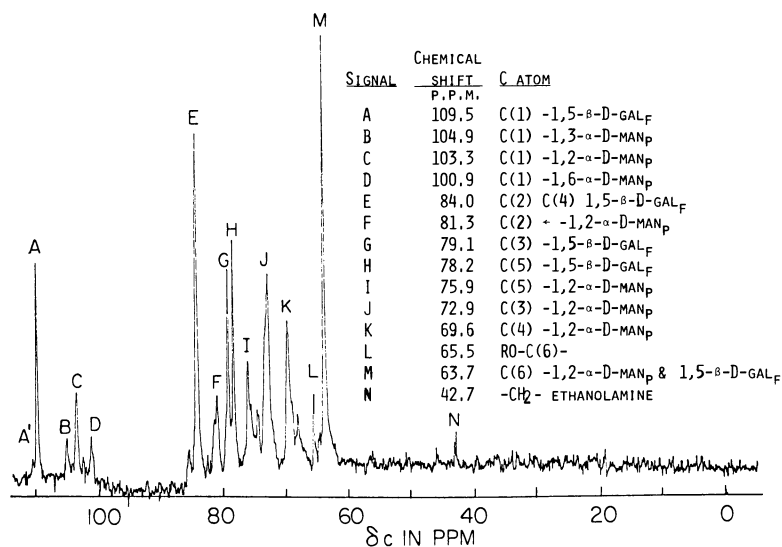


Figure 6. Proton decoupled natural abundance ^{13}C -NMR spectrum of galactofuranosidase treated phosphogalactomannan. Pictured is the spectrum, taken on a Varian XL-100 spectrometer, from 0 to 3000 Hz downfield from an external reference of 5% TSP. The spectrum of a sample (100 mg mL^{-1}) was taken at 37°C in a 5-mm diameter NMR tube. The spectrum shown represents data accumulated from 64,000 transients.

phosphoryl groups are attached. Resonance signals of some C atoms from $\alpha(1\rightarrow6)$ mannopyranosyl residues may be nearly the same as those from some C atoms of $\alpha(1\rightarrow2)$ mannopyranosyl units. For instance the J signal probably represents two unresolved resonance signals as do signals at F and K.

Acetolysis of the Glycopeptide. Conditions of acetolysis developed in Ballou's laboratory (41,42) provide selective cleavage of $(1\rightarrow6)$ mannopyranosidic linkage in mannans containing both $(1\rightarrow6)$ and $(1\rightarrow2)$ glycosidic linkages. The galactofuranosyl residues will be cleaved also because of their acid lability. Samples of peptidophosphogalactomannan, phosphogalactomannan and phosphomannan were acetolyzed, the acetylated sugars were extracted into methanol, and deacetylated with metallic sodium in methanol and the products of the reaction were fractionated on Bio-Gel P-2. A typical elution pattern shows five saccharide containing peaks. The moles of oligosaccharide per mole of polymer was calculated for each preparation. The data show that a preparation containing 53% galactose released 12 moles of mannotetraose, 6 moles of mannotriose, 14 moles of mannobiose and 17 moles of mannose during acetolysis (Table II). Acetolysis of the phosphogalactomannan gave similar results except that this polymer contained 2, 9 and 6 moles less of mannotriose, mannobiose and mannose, respectively, than peptidophosphogalactomannan. This suggests that acetolysis also cleaves the O-glycosidic linkages between the mannopyranosyl residues and the seryl and threonyl residues. The 17 points of cleavage between the oligosaccharides and the polypeptide estimated by this indirect procedure is in reasonably agreement with the number of residues estimated by β -elimination. It was shown in separate experiments with ^{32}P labeled peptidophosphogalactomannan that the ^{32}P was extracted into the aqueous phase following acetolysis. There was negligible quantity of carbohydrate in this phase.

Polypeptide. The release of phosphogalactomannan, mannose, mannobiose and mannotriose from the polymer by alkali suggests that these saccharides are attached to a polypeptide (11). The amino acid composition of a peptidophosphogalactomannan preparation was determined (Table III). This table shows that approximately one-half of the amino acyl residues of the polypeptide are either seryl or threonyl residues and that the polypeptide has no aromatic or sulfur-containing amino acids. Treatment of the polymer with alkali followed by reduction of the α,β -dehydroaminoacyl residues formed resulted in a loss of all but 2 of the seryl residues and essentially all of the threonyl residues (Table IV). Furthermore, the number of alanyl residues increased from 4 to 8 and 4 residues of α -aminobutyric acid were obtained. These were derived from the reduction products of the α,β -dehydroserine (α -aminoacrylic acid) and α,β -dehydrothreonine (α -aminocrotonic acid), respectively, following β -elimination of saccharides from seryl

Table II

Oligosaccharides Derived from Peptidophosphogalactomannan
and its Derivatives

Glycan Acetylated	Moles/mole Glycan				
	M ₅	M ₄	M ₃	M ₂	M
pPGM*	1.9	11.7	5.6	13.8	17.6
PGM	1.9	10.1	3.7	4.7	11.8
PM	1.6	8.8	4.8	7.1	14

*Abbreviations: pPGM, peptidophosphogalactomannan; PGM, phosphogalactomannan; PM, phosphomannan; M₅, mannopentaose; M₄, mannotetraose; M₃, mannotriose; M₂, mannobiose; M, mannose.

Acetolysis was carried out on pPGM and PGM which contained 53% and 62% galactose, respectively. A value of 110 mannosyl residues/mole is used for pPGM and 90 mannosyl residues/mole is used for both PGM and PM.

Table III
Amino Acid Composition of *P. charlesii* Glycopeptide

Amino Acid	Amino Acid Residues ^a				Maximal or Extrapolated Values	Integral Number
	4 hrs ^b	11 hrs ^b	22 hrs ^c	44 hrs ^b		
Ser	8.47	7.54	7.19	5.93	8.60	9
Thr	5.80	6.32	5.62	5.63	6.32	6
Ala	2.62	3.22	3.87	4.05	4.06	4
Gly	1.96	2.40	2.52	2.76	2.76	3
Val	0.79	1.21	1.66	1.48	1.66	2
Pro	1.21	1.54	2.21	1.39	2.21	2
Asx	0.76	0.88	1.01	1.08	1.08	1
Glx	0.83	1.07	1.43	1.04	1.43	1
His	0.24	0.77	-	0.68	0.77	1
Ile	0.21	0.45	0.53	0.45	0.53	0-1
Leu	0.29	0.38	0.53	0.36	0.53	0-1
Lys	0.25	0.26	0.36	0.25	0.36	0-1
Arg	Trace	Trace	Trace	Trace	Trace	Trace
Trp ^d	-	-	-	-	-	Trace
Phe	Trace	Trace	Trace	Trace	Trace	Trace
Met	Trace	Trace	Trace	Trace	Trace	Trace
Tyrd	-	-	-	-	-	Trace

a) Based on a molecular weight of 26,500.

b) Average of two determinations.

c) Average of three determinations.

d) Determined by the spectrophotometric method.

Table IV

Changes in Amino Acid Composition Following Alkaline Borohydride Treatment of the Glycopeptide

Amino Acid	Residues ^a /mole ^b		
	Untreated polymer	Alkaline Borohydride Treated polymer	Increase or Decrease
Serine	7.28	1.92	(-) 5.36
Threonine	5.66	0.30	(-) 5.36
Alanine	3.82	7.82	(+) 4.00
α -Aminobutyric Acid	0	4.02	(+) 4.02

- a) Quantity present after 22 hours hydrolysis in constant boiling HCl at 110° in a sealed evacuated tube.
 b) Based on a molecular weight of 26,500.

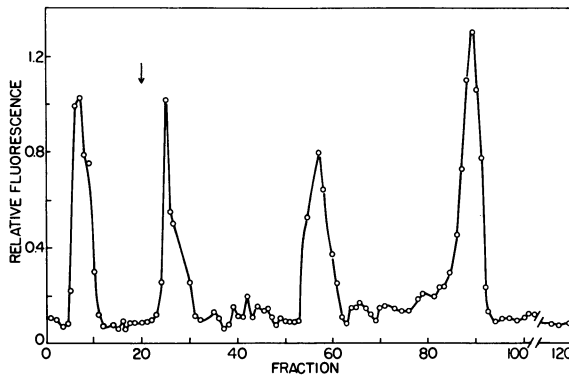


Figure 7. Separation of polypeptides, derived from peptidophosphogalactomannan by treatment with anhydrous HF, by anion exchange chromatography. Peptidophosphogalactomannan was treated with anhydrous HF, the polypeptide that remained was passed through a Bio-Gel P-6 column and the fraction containing the polypeptide was lyophilized and was dissolved in pyridinium acetate (pH 5.0). The sample was applied to a Whatman DE cellulose (DEAE cellulose) that had been pre-equilibrated in 0.02M pyridinium acetate buffer. The DEAE cellulose was washed with 0.02M pyridinium acetate buffer until the first peak came off, then a gradient of 0.02M pyridinium acetate (pH 5)-1M acetic acid was started. About 20-30% of the material that was applied to the column was not eluted from the column.

and threonyl residues. The amino acyl composition of the polypeptide obtained from *P. charlesii* peptidophosphogalactomannan is unique in containing such a large proportion of seryl and threonyl residues, and in containing essentially no aromatic or sulfur containing amino acids. However, several of the peptidogalactomannans from fungi (14,43) contain polypeptides with a similar amino acyl composition.

We noted that isoleucine, leucine and lysine occurred in quantities which are far below one residue per mole of polypeptide (Table III). This suggests that either the molecular weight of peptidophosphogalactomannan is twice as large as we calculated from equilibrium ultracentrifugation experiments, or that the polypeptide is heterogeneous. This heterogeneity could result from the preparation containing polypeptides of slightly different number of residues, with some species containing a his-ile-leu-lys or his-leu-ile-lys on one end. Carboxy-terminal amino acid analysis of the polypeptide showed that none of the above amino acids was at the C-terminal end. Amino-terminal amino acid determinations were made using dansyl chloride as a reagent to derivatize the free amino groups. The derivatized amino acids were separated on polyamide layers and serine, aspartate, glutamate and glycine residues were shown to be derivatized (11). A fifth fluorescent area was observed which was identified as dansyl-ethanolamine. Treatment of peptidophosphogalactomannan with pronase served to modify the polypeptide so that it contained a serine residue on both the C-terminal and N-terminal ends, but the number of isoleucyl, leucyl and lysyl residues per mole of polypeptide remained considerably less than one. We have extended this work by treating the peptidophosphogalactomannan with anhydrous HF which cleaves O-glycosidically linked residues at 0°C without cleaving the peptide bond (27). The mass of this polypeptide preparation is about 3,000 daltons and elutes as one sharp peak from a Bio-Gel column (not shown). The preparation was chromatographed on a weak anion exchange resin and the polypeptide was resolved into 4 fractions (Fig. 7). Attempts at sequencing these individual fractions suggest that none are completely homogenous (44). Thus we conclude that the peptidophosphogalactomannan is derived from at least 4, and possibly from many more, polypeptides. The implications with respect to function will be discussed in a later section.

Non-carbohydrate constituents. We have shown that all of the phosphorus occurs in diester linkage (10) yet neither the methylation analysis nor the ¹³C NMR spectroscopy provided any evidence for the occurrence of a glycosyl-1-phosphoryl residue. Finding dansyl-ethanolamine following derivatization of peptidophosphogalactomannan with dansyl groups, suggested that phosphoethanolamine might be attached to the mannan. However, a quantitative determination of ethanolamine revealed only about one residue per mole of peptidophosphogalactomannan (11). It was also

demonstrated that radiolabeled ethanolamine in the growth medium was incorporated into peptidophosphogalactomannan and that treatment of the polymer released about 40% of the radioactivity with the remainder still covalent attached to the polysaccharide (12). This suggests that ethanolamine or its derivatives occur in two environments within the peptidophosphogalactomannan. However, essentially all of the ^{32}P of ^{32}P -labeled peptidophosphogalactomannan remained associated with the phosphogalactomannan. The small amount of ^{32}P which was released was eluted from Bio-Gel P-2 in fractions (68-73) coincident with those which also contain ^{14}C from ethanolamine (Figs. 8,9) as well as carbohydrate. This fraction may represent a mannotetraose, or possibly a mannopentaose, unit containing covalently bound phosphorus and ethanolamine, or a derivative of ethanolamine. Peptidophosphogalactomannan containing ^{14}C from L-(^{14}C - CH_3)methionine was prepared and the polymer was treated with alkali. As occurred with the ^{14}C -ethanolamine-labeled glycopeptide, approximately 40% of the ^{14}C was released as a low molecular weight substance and the remainder was covalent associated with the phosphogalactomannan. Nearly all of the ^{14}C which was released was eluted in fractions 95-101 from the Bio-Gel P-2 column similar to that when the polymer was labeled with ^{14}C from ethanolamine (Fig. 9) (12). It was shown (12) that fractions 38-45, 65-75 and 95-105 contained N,N'-dimethylaminoethanol with the largest quantity in fractions 65-75. The occurrence of N,N'-dimethylaminoethanol in the peptidophosphogalactomannan was demonstrated by gas-liquid chromatography of the hydrolysis products of the Smith degradation reactions (12). Doubly labeled peptidophosphogalactomannan containing ^{14}C from L-(CH_3 - ^{14}C)methionine and ^3H from (1- ^3H)ethanolamine was prepared and the polymer was subjected to Smith degradation procedure. This treatment resulted in the formation of two ^{14}C -, ^3H -labeled substances (Fig. 10). These substances also contained phosphorus as shown by the ^{32}P in these fractions as demonstrated in separate experiments with ^{32}P -labeled peptidophosphogalactomannan (not shown). Acetolysis of ^{32}P , ^3H -ethanolamine, or L- ^{14}C -methionine-labeled peptidophosphogalactomannan showed that all of the radioactivity extracted into the aqueous phase. Thus the acetolysis procedure cleaved the phosphoethanolamine/phospho-N,N'-dimethylaminoethanol residues from the mannan and negligible carbohydrate was associated with these fractions. These results, when taken collectively, strongly suggest that phosphoethanolamine/phospho-N,N'-dimethylaminoethanol occurs in two separate environments in peptidophosphogalactomannan. The galactofuranosyl residues are removed enzymically from the peptidophosphogalactomannan without loss of phosphorus or ethanolamine. The methylation data presented in another section suggests that phosphorus may be attached to residues which have free hydroxyl groups at carbon atoms 3 and 4. Preliminary ^{31}P NMR spectroscopy also shows that the ^{31}P nucleus is located in two environments; one with a phosphoethanolamine-/phospho-N,N'-dimethylaminoethanol adjacent to a free hydroxyl

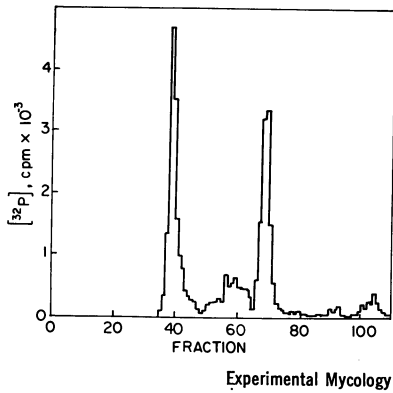


Figure 8. Distribution of ^{32}P following chromatography of the low-molecular-weight substances from ^{32}P -labeled glycopeptide on Bio-Gel P2 (12). The low-molecular-weight substances from alkali-treated peptidophosphogalactomannan that separated from the high-molecular-weight substances on Sephadex G-50 were chromatographed on a column of Bio-Gel P-2, minus 400 mesh (93×2.5 cm) with distilled deionized water as the eluent. Fractions (3.4 mL) were analyzed for ^{32}P and the data are given as cpm per sample.

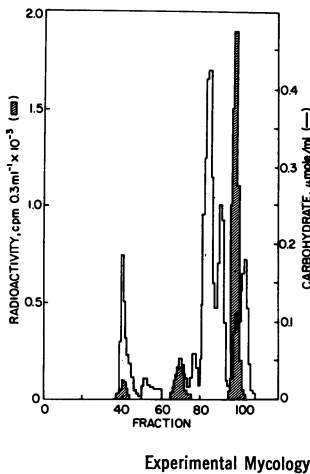


Figure 9. Distribution of ^{14}C (shaded region) and carbohydrate following chromatography of the low-molecular weight substances from alkali-treated peptidophosphogalactomannan from ^{14}C -ethanolamine-labeled peptidophosphogalactomannan on Bio-Gel P-2. The low-molecular-weight substances were obtained and treated as described in Figure 13 and (12).

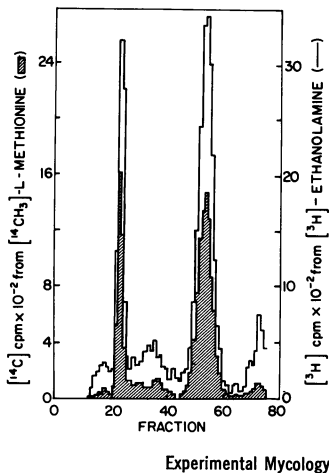


Figure 10. Separation of Smith degradation products of double-labeled peptidophosphogalactomannan on Dowex-2-formate (12). Double-labeled peptidophosphogalactomannan was prepared containing ^3H and ^{14}C from (1- ^3H)ethanolamine (shaded region) (500 μCi) and L-(CH_3 - ^{14}C)methionine (100 μCi) added to a culture (150 mL) 2.5 days after adding approximately 10^8 P. charlesii spores. The growth conditions and growth media are described elsewhere (12). Ten mg of peptidophosphogalactomannan obtained from this culture filtrate after 9 days were subjected to Smith degradation and the products were fractionated on Dowex-2-formate according to the procedure of Wells and Dittmer (47).

group and the other with the phospho-compounds not adjacent to a free hydroxyl group.

Cytoplasmic Peptidophosphogalactomannan. Fungal peptidogalactomannans have been extracted from powdered preparations (43). However, we were unable to obtain phosphogalactomannan from the cell walls of 3-day cultures of P. charlesii (20). When we examined extracts of P. charlesii cells we found galactofuranosyl residues both in a soluble cytoplasmic and a membrane-bound cytoplasmic fraction.

The soluble cytoplasmic fraction was separated from the membranes by centrifugation at about 100,000 x g and the glycopeptides isolated by a procedure described previously (10). The galactofuranosyl-containing glycopeptide(s) which were isolated had chromatographic properties which are similar to those of the extracellular peptidophosphogalactomannan except that the "cytoplasmic" soluble-glycopeptide may be somewhat larger. The polypeptide was estimated to contain 61-64 amino acyl residues of which 27 were seryl and threonyl. Treatment of the glycopeptide with alkali resulted in the release of carbohydrate from approximately 14 seryl and threonyl residues. The oligosaccharides which were released were fractionated on Bio-Gel P-2 and mannose-containing saccharides eluting in the positions of mannotriose and mannobiose were obtained. The polysaccharide which eluted in the void volume contained galactose and mannose. Galactose was released by either 0.01 N HCl or galactofuranosidase.

The glycopeptide was derivatized with dansyl chloride and the dansyl-glycopeptide was hydrolyzed and three fluorescence areas coincident with dansyl-glycine, dansyl-serine and dansyl-ethanolamine were observed on the sheets following two dimensional chromatography on sheets of polyamide. The occurrence of ethanolamine was confirmed by its position of elution from a column of the amino acid analyzer, and it was quantified by comparison to reference solution of ethanolamine. A 22 hr hydrolysate of glycopeptide in 6 N HCl contained about 0.3 moles of ethanolamine per mole of glycopeptide. Our estimate of the molecular weight of the soluble "cytoplasmic" glycopeptide is at present based on its position of elution from a Sephadex column, and SDS disc polyacrylamide gel electrophoresis, which give a value between 80,000 and 90,000.

The glycopeptide was shown to contain phosphorus and when subjected to isoelectric focusing the glycopeptide banded in the region of pH 2-3. This evidence suggests that the polymer may be a precursor of the exocellular peptidophosphogalactomannan which has been characterized more extensively.

Membrane-bound Peptidophosphogalactomannan. The membranes obtained following centrifugation at about 100,000 x g were fractionated by isopycnic sucrose gradient ultracentrifugation. Six

membrane-containing bands were visible and each band was removed and the membranes pelleted. The enzyme activities associated with each band and the occurrence of galactofuranosyl residues based on the release of galactose with galactofuranosidase or with 0.01 N HCl at 100°C for 90 min was determined for each band. It was found that membrane band V ($\rho = 1.18$ g/cc) was the only one which contained galactofuranosyl residues. This fraction was also particularly rich in acid phosphatase although it contained only about 5% of the total protein applied to the gradient. The specific activity of acid phosphatase was 4-fold greater in membrane fraction V than that in the crude membrane preparation. In contrast, the specific activities of Mg-ATPase, Na/K-Mg-ATPase, glucose-6-phosphate phosphatase, 5'-nucleotidase and alkaline phosphatase in membrane fraction V were either equal to that in the crude membrane preparation or several fold less than that of the crude membrane preparation.

Membrane fraction V was treated with a series of detergents, chaotropic agents, trypsin, or alkali in an attempt to solubilize the galactofuranosyl-containing substances. Although a number of such treatments partially solubilized the galactofuranosyl-containing substance(s), the insoluble fraction also contained galactofuranosyl residues. Treatment of fraction V with CHCl_3 :methanol 2:1 (v/v) resulted in concentrating all of the galactofuranosyl-containing substances at the interface. These substances were solubilized by making the suspension 1 M with KCl followed by adjusting the solution to 2% in sodium deoxycholate (Table V). Although this treatment solubilized all of the galactofuranosyl-containing substances, it only solubilized 11% of the protein in the membrane fraction.

The galactofuranosyl-containing substances were purified by combining gel permeation chromatography on Bio-Gel A-5m followed by rechromatography on concanavalin A-Sepharose 4B. The KCl-DOC-soluble substances were applied to Bio-Gel A-5m in 40 mM Tris-HCl-40 mM NaCl-0.24% DOC, pH 7.5 buffer (Tris-NaCl-DOC buffer) and the column irrigated with this buffer system. The galactofuranosyl-containing substances were eluted in fractions 105-115 which contained less than 10% of the protein. These fractions were pooled and applied to a concanavalin A-Sepharose 4B column. The adsorbent was washed with the Tris-NaCl-DOC buffer until the eluate coming through had negligible absorbance at 280 nm. The adsorbent was then irrigated with Tris-NaCl-DOC buffer containing 1% methyl- α -D-mannopyranoside. Galactofuranosyl-containing substances were obtained in fractions 31-36 (Fig. 11).

The DOC-soluble galactofuranosyl-containing polymer was electrophoresed on SDS disc polyacrylamide gels. A broad band which was heavily stained with Coomassie blue migrated at an average R_f of 0.19 (not shown). Carbohydrate containing polymers which stained with periodate Schiff reagent also migrated in a broad band in this region. Two faint bands which stained with Coomassie blue migrated with R_f 's 0.79 and 0.82. A molecular

Table V

Solubilization of Galactofuranosyl-containing
Substance(s) from Interface^a

Treatment ^b	Galactofuranosyl Residues ^c	
	Soluble ^d	Insoluble
1 M KCl	-	+
1 M TCA, pH 7.2	+	+
1 mM phosphotungstic acid, pH 6.8	+	+
2% DOC	+	+
1 M KCl + 2% DOC	+	-

- a) Membranes were treated with 10 volumes $\text{CHCl}_3:\text{MeOH}$, 2:1 (v/v) and the material accumulating at the interface was collected.
 b) Treated for two hours at 4°C.
 c) Measured with galactose oxidase after treatment with galactofuranosidase.
 d) Soluble after centrifugation at 100,000 x g for 1 hour.

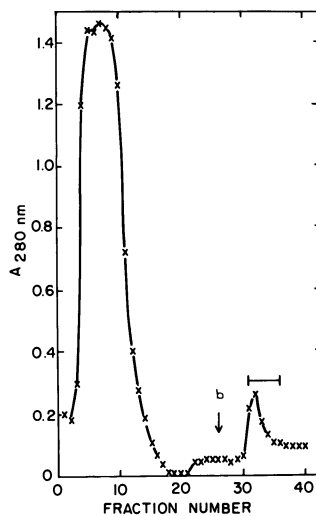


Figure 11. Fractionation of polymer on a concanavalin A-Sepharose 4B column. Galactofuranosyl-containing fractions from a Bio-Gel A-5m column were pooled and applied to a column of concanavalin A-Sepharose 4B. The adsorbent was washed successively with 40mM Tris-HCl-40mM NaCl-0.24% deoxycholate, pH 7.5, followed by the same buffer containing 1% methyl- α -D-mannopyranoside starting with Fraction 27. Fractions containing 100 drops were collected and 0.5 mL samples were assayed for galactofuranosyl residues (21). Fractions containing galactofuranosyl residues are indicated with a bar.

weight of 105,000 was estimated for the material at R_f 0.19. However, it is apparent that the polymer is very heterogenous.

Amino acid analyses were performed on the glycopeptide preparation following hydrolysis of the polymer(s) in 6 N HCl for 22 hr. Approximately 38% of the amino acids were represented by seryl and threonyl residues (Table VI). In addition, the amino acid composition is similar to that obtained for both the extracellular and soluble "cytoplasmic" peptidophosphogalactomannans. The polypeptides contained only trace quantities of aromatic amino acyl residues, no detectable quantities of sulfur-containing amino acyl residues, and were rich in serine, threonine and alanine. The percentage of proline decreased as the glycopeptide size increased. Thus, the average number of amino acyl residues is calculated on the basis of a polymer containing two prolyl residues.

Treatment of the galactofuranosyl-containing polymer(s) with alkali resulted in a decrease of 11 seryl and 9 threonyl residues, based on a polypeptide of 110 amino acyl residues. Mannobiose was the major low molecular weight oligosaccharide released by alkali (not shown). However, only 49% of the carbohydrate applied to the column was eluted.

Experiments were conducted to determine the free amino groups available for reaction with dansyl chloride. Only one faint fluorescent area in the region of dansyl-lysine was observed. No dansyl-ethanolamine was observed. However, if the polymer was first treated with 6 N HCl to hydrolyze the amino acids, dansyl amino acids were obtained. Quantitative analysis for ethanolamine from the amino acid analyzer gave 1.3 ethanolamine residues for 2 proline residues. The low solubility of the polymer in aqueous solution may have been the primary factor leading to lack of derivatization of the intact glycopeptide.

The composition of peptidophosphogalactomannan from 2-day culture filtrates was compared to that of the cytoplasmic soluble- and membrane-bound galactofuranosyl-containing substances (Table VI). Galactose released by dilute acid accounts for 60-65% of the total carbohydrate. These data when coupled with that obtained with galactofuranosidase, suggests that the three glycopeptides contain about the same percentage of galactofuranosyl residues. The DOC-soluble polymer appeared to contain about 26% protein as compared to about 15% polypeptide in the soluble glycopeptides. The ratio of hexose:P of approximately 15:1 is considerably less than the 30:1 value which we routinely obtain for extracellular peptidophosphogalactomannan containing a large percentage of galactofuranosyl residues. At present we have no explanation for this observation.

Table VII shows that 2-day cultures have produced in all three forms of the peptidophosphogalactomannan a quantity equivalent to 11 μ moles of hexose/100 ml of culture. The average germination time is 36 hr. We calculate that a quantity of peptidophosphogalactomannan equivalent to $0.9 \mu\text{mole hr}^{-1} 100 \text{ ml}^{-1}$

Table VI
Amino Acid Composition of Peptidophosphogalactomannans

Amino Acid	Exocellular	Peptidophosphogalactomannans	
		Soluble-cytoplasmic	Membrane bound
Serine	9	16	24
Threonine	6	11	19
Alanine	4	7	14
Glycine	3	8	13
Valine	2	4	8
Proline	2	2	2
Aspartate/Asparagine	1	4	10
Glutamate/Glutamine	1	5	8
Histidine	1	1-2	5
Isoleucine	0.5	1	5
Leucine	0.5	1-2	5
Lysine	0.4	1	3-4
Arginine	T	T	T
Phenylalanine	T	T	T
Tyrosine	T	T	T
Methionine	T	-	-

The number of moles of each amino acid per mole of peptidophosphogalactomannan is based on 2 prolyl residues per mole.

Table VII
Chemical Characterization of Glycopeptides

Source of Glycopeptide	μ moles An-Hex ^a	An-Hex ^a	An-Hex ^a	Galactose ^b
	flask	phosphate	protein	An-Hex ^a
Growth medium	2.5	16	6.7	0.66
Supernatant	10.4	15	7.0	0.64
DOC-soluble	4.0	12	3.8	0.60

^aAnhydrohexose.

Polymer treated with 0.05 N HCl for 90 minutes at 110°C. Galactose released measured by the coupled galactose oxidase-horse-radish peroxidase assay.

of culture is formed. Therefore, 8-day cultures should produce a quantity of peptidophosphogalactomannan equivalent to about 140 μ moles of hexose. Fig. 2 shows that culture filtrates from 8-day cultures contain approximately this quantity of peptidophosphogalactomannan.

The physical properties of membrane-bound peptidophosphogalactomannan suggest that it is amphipathic which is in contrast to those of soluble peptidophosphogalactomannans. Furthermore, only a portion of the phosphogalactomannan region was solubilized by treating membrane-bound peptidophosphogalactomannan with alkali. This suggests that the phosphogalactomannan contains hydrophobic substances attached by covalent linkages which are stable to 0.5 N NaOH. No evidence was obtained for fatty acyl residues attached to the mannan. However, membrane-bound peptidophosphogalactomannan contained approximately 13-fold more ^{14}C per mole of hexosyl residues than soluble cytoplasmic peptidophosphogalactomannan when the peptidophosphogalactomannans were isolated from 48-hr cultures to which (1- ^{14}C)acetate had been added after 24 hr (Table VIII). Essentially all of the radioactivity was removed from the growth medium within 6 hr after the addition of (1- ^{14}C)acetate (not shown). Essentially none of the ^{14}C was incorporated into either the galactofuranosyl or mannopyranosyl residues. A small amount of the ^{14}C was incorporated into the amino acids. Hydrolysis of the ^{14}C -labeled peptidophosphogalactomannan in 2 N HCl for 4 hr at 110°C followed by thin layer chromatography in appropriate solvents showed that the ^{14}C was not incorporated primarily into fatty acids or cholesterol. Treatment of ^{14}C -labeled peptidophosphogalactomannan with 4 N KOH for 5 hr at 110°C released ^{14}C (13%) which was soluble in CHCl_3 :methanol 2:1 (v/v). The radioactivity in the organic solvent migrated coincident with sphingosine in two solvent systems (Figs. 12,13) but somewhat different than sphingosine in two other solvent systems (Fig. 14,15). An aliquot of the organic extract was treated with IO_4^- (45) and the reaction mixture was extracted with methylene chloride. The methylene chloride extract was chromatographed. Periodate treatment caused about 40% of the radioactivity to be displaced from the origin where sphingosine is located, to an R_f of 0.5 (Fig. 16). Similar treatment of sphingosine resulted in a product which had an R_f of 0.61.

These experiments show that membrane-bound peptidophosphogalactomannan contains several fold more ^{14}C derived from acetate than does the soluble peptidophosphogalactomannans. This suggests that the amphipathic nature of the membrane-bound peptidophosphogalactomannan may be a result of hydrophobic substance(s) derived from acetate which are attached to the phosphogalactomannan. No evidence was obtained for fatty acyl residues or sterols being attached by ester linkage to the phosphogalactomannan. Some of the ^{14}C was released by 4 N NaOH and the substance(s) released has properties similar to a sphingosine derivative. The 4 N alkali should have released any fatty acyl residues in amide linkage

Table VIII

^{14}C from $[1-^{14}\text{C}]$ Acetate Incorporated into DOC-soluble Glycopeptide

Source of Glycopeptide ^a	$^{14}\text{C}/\mu\text{mole}$ Anhydro-hexose
	cpm x 10^{-3}
Growth medium	1.9
Supernatant	2.7
DOC-soluble	35.3

- a) Glycopeptides isolated and purified from cultures grown in four flasks under standard conditions. Twenty four hours after inoculation 1 mCi ($1-^{14}\text{C}$) acetic acid (58 mCi/mmmole) was added to each of two flasks.

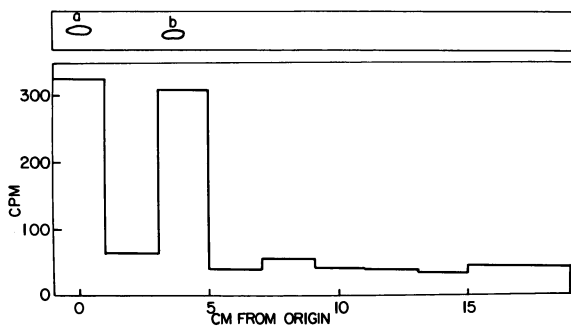


Figure 12. Thin layer chromatography of base-hydrolyzed ^{14}C -labeled deoxycholate-soluble peptidophosphogalactomannan. ^{14}C -Labeled deoxycholate-soluble peptidophosphogalactomannan was treated with 4N KOH for 5 hr at 110°C in a sealed evacuated tube. The solution was extracted with CHCl_3 :methanol, 2:1 (v/v). Fifty μL sample was applied to a silica-gel, thin layer plate and chromatographed in benzene:diethyl ether:ethyl acetate:acetic acid, 80:10:10:2 (v/v/v/v). Reference compounds lysine, ethanalamine, monomethylethanalamine, dimethylethanalamine, and choline were chromatographed in a similar manner. The mobility of sphingosine is depicted in the upper lane.

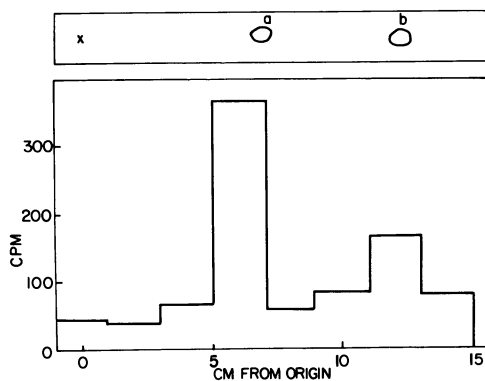


Figure 13. Thin layer chromatography of base-hydrolyzed ^{14}C -labeled deoxycholate-soluble peptidophosphogalactomannan. The ^{14}C -labeled peptidophosphogalactomannan was treated as described in Figure 12. A $50\text{-}\mu\text{L}$ sample was applied to a thin layer of silica gel and chromatographed in CHCl_3 :methanol: H_2O , 100:42:6 (v/v/v). The mobility of sphingosine is depicted in the upper lane.

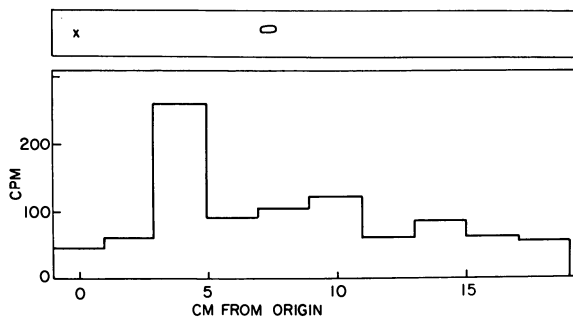


Figure 14. Thin layer chromatography of base-hydrolyzed ^{14}C -labeled deoxycholate-soluble peptidophosphogalactomannan. The ^{14}C -labeled peptidophosphogalactomannan was treated as described in Figure 12. A $50\text{-}\mu\text{L}$ sample was applied to a thin layer of silica gel and chromatographed in CHCl_3 :methanol:2N NH_4OH , 80:20:2 (v/v/v). The mobility of sphingosine is depicted in the upper lane.

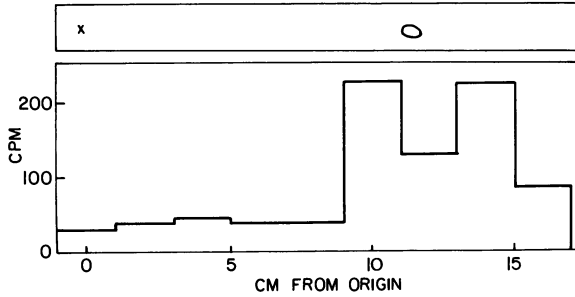


Figure 15. Thin layer chromatography of base-hydrolyzed ^{14}C -labeled deoxy-cholate-soluble peptidophosphogalactomannan. The ^{14}C -labeled lipo-peptidophosphogalactomannan was treated as described in Figure 12. A $50\text{-}\mu\text{L}$ sample was applied to a thin layer of silica gel and chromatographed in CHCl_3 :methanol: H_2O , 49:4:2 (v/v/v). The mobility of sphingosine is depicted in the upper lane.

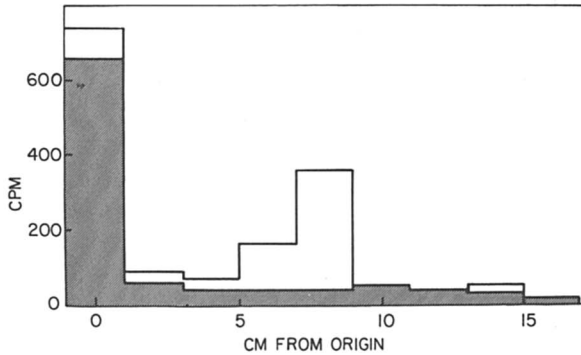


Figure 16. Thin layer chromatography of periodate-treated base-hydrolyzed ^{14}C -labeled lipo-peptidophosphogalactomannan. The ^{14}C -labeled lipo-peptidophosphogalactomannan was treated as described in Figure 12. A $50\text{-}\mu\text{L}$ sample was applied to a thin layer of silicic acid and chromatographed in hexane:diethylether, 9:1 (v/v). The ^{14}C remained at the origin as shown in the cross hatched area. A $100\text{-}\mu\text{L}$ sample was treated with periodate according to the procedure of Sweeley and Moscatelli (45), and the sample applied to a thin layer silica gel plate and chromatographed in hexane:diethylether. Sphingosine remained at the origin, and sphingosine treated with periodate migrated with an R_f of 0.61.

with the amino group of the sphingosine-like portion of the molecule. No ^{14}C -labeled fatty acids were found. Treatment of the methylene chloride-soluble substance(s) with periodate released a substance that was only slightly more polar than the fatty aldehyde released when sphingosine is treated with periodate. Preliminary gas chromatography-mass spectrometer of the substance solubilized by treatment with 4 N alkali suggests that the long chain base is dehydrosphingosine. Phytosphingosine and dehydrophytosphingosine are the predominant long chain bases present in fungi and plants. Dihydrosphingosine composes 3% of the sphingolipid bases in yeast (45). Triacyldihydrosphingosine has been obtained from the growth medium of *Hansenula ciferrii* (46).

We tentatively conclude that the membrane-bound amphipathic peptidophosphogalactomannan contains one or more lipophilic residues attached to the mannan. The amphipathic polymer will be designated lipo-peptidophosphogalactomannan to distinguish it from the soluble peptidophosphogalactomannans.

It was noted that storage of membrane fraction V even at -20°C resulted in a decrease and sometimes a complete loss in lipo-peptidophosphogalactomannan. This membrane fraction may contain enzymes which convert lipo-peptidophosphogalactomannan to peptidophosphogalactomannan.

Summary

Investigations on the structure of a peptidophosphogalactomannan of *Penicillium charlesii* has been reviewed and new work presented. Carbon-13 NMR spectroscopy has been used to confirm earlier structural studies. Structural studies on the polypeptide show that the polypeptide is derived from several species of proteins. We also report the occurrence in *P. charlesii* extracts of a soluble "cytoplasmic" peptidophosphogalactomannan and a membrane-bound species which because of a hydrophobic region we have termed lipo-peptidophosphogalactomannan. The two new galactomannan derivatives have been partially characterized and have been shown to have amino acid composition which is similar to that of the extracellular peptidophosphogalactomannan except that the polypeptide portions are considerably greater. The hydrophobic region, unlike the galactomannan, derives its carbons from acetate. Preliminary results suggest that the hydrophobic character is given by a long chain base with properties similar to sphingosine and dehydrosphingosine. This lipo-peptidophosphogalactomannan has been isolated and purified by gel-permeation and affinity chromatography.

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Immunochemical Examination of the Polysaccharides of Mucorales

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An increasing interest is being shown in the polysaccharides of fungi. In particular, chemistry and biochemistry of the intra- or exo-cellular polysaccharides have been studied by several workers. The relationship between taxonomy and chemical structure of fungal polysaccharides is an interesting subject. Cell wall chemistry and taxonomy of fungi were reviewed by Bartnicki-Garcia (1). In yeasts particularly, chemotaxonomy by means of application of proton magnetic resonance spectroscopy was described by Gorin and Spencer(2), and the acetolysis fingerprinting technique by Kocourek and Ballou(3) can be also used to determine the taxonomy of yeasts. These methods are based on the differences in the chemical structure of their mannan components. Similarly, serological clasification of yeasts by the use of slide agglutinin test was reported by Tsuchiya, Fukazawa and Kawakita(4).

During the last twenty years or so, various types of fungal polysaccharides have been examined in our laboratory. In the early nineteen seventies, we have examined Mucorales polysaccharides(5,6,7,8), which was expected to be a good example of chemotaxonomy, because it had been known that these fungi contain conspicuous quantities of uronic acid and fucose(9). According to our experimental results, the main exocellular polysaccharides of these fungi were highly complicated glycans composed of fucose, mannose, galactose, 2-acetamido-2-deoxy-glucose and 2-acetamido-2-deoxy-galactose, and the fucose contents of Absidia cylindrospora and Mucor mucedo were extremely higher than that of Rhizopus nigricans. Partial acid hydrolysis of these polysaccharides gave fucose and fucose-free mannose-rich fragment. Results of the periodate oxidation and Smith degradation showed that large portions of fucose and mannose were oxidized, and large quantities of glycerol and tetrahydric alcohols were detected. Minor exocellular polysaccharides were also isolated from these fungi. These contained glucuronic acid as an additional component. As major cell wall components, acidic heteroglycans composed of fucose, galactose and glucuronic acid were also isolated.

It has been known that some species belonging to Mucorales

cause Mucormycosis. However, immunochemical examination of the fungi has not been performed in detail. If a highly specific antigenic substance is available, this must be useful for the diagnosis of Mucormycosis, and if the specific antigenic substance is a polysaccharide fraction, it is of interest in connection with chemotaxonomy. As the first step in our investigation, isolation and immunochemical examination of serologically active substances for rabbit of A. cylindrospora, Mucor hiemalis and Rhizopus nigricans, which are members of Mucorales, were carried out.

Preparation (10,11)

The spore suspension of A. cylindrospora IFO 4000 was inoculated into a modified Sabouraud's liquid medium which was composed of 4%(w/v) glucose and 1%(w/v) dialyzable peptone. The culture was incubated for 30 days at 25°C with reciprocal shaking until the stationary phase was attained. The collected mycelium was suspended in cold saline and homogenized by a Waring blender and then disrupted mechanically with a French press(400kg/cm², 3-times). The disrupted cell fraction, ACWC, was separated into the supernatant fluid and a residual pellet. The dialyzed supernatant was centrifuged at 45000 rpm for 60 min. at 4°C, and the supernatant was lyophilized and designated as ACI. The residual pellet including cell walls was washed with 0.5% sodium dodecyl sulfate and distilled water to remove cytoplasmic components. The collected cell wall by centrifugation, ACW, was microscopically free from cytoplasmic contaminant. The washed mycelium was extracted with 45% phenol by the method of Westphal and Jahn(12). The aqueous layer of the supernatant was lyophilized after extensive dialysis. The resulting powder was designated as PHW. The nondialyzable fraction of the culture filtrate was lyophilized. The resulting powder was designated as ACE.

A part of the cell wall fraction was extracted with 0.25M sodium hydroxide at 4°C for 24hr and the extract was then subjected to pronase digestion to remove protein. The digested extract was chromatographed on a DEAE cellulose column by stepwise elution with water, sodium bicarbonate and sodium hydroxide. The yield of the major fraction, which was eluted with 0.5M sodium bicarbonate, SE, was 20.3% of the cell wall by weight. Another part of ACW was extracted successively with 45% phenol-water(PE), then with hot water in an autoclave(HE), and finally with 1% potassium carbonate at room temperature for 24hr(PCE). The yields of these fractions, PE, HE and PCE were 0.47%, 0.27% and 4.4%, respectively.

Antisera

ACW and ACE were suspended in saline to make the concentration 20mg/ml and 10mg/ml, respectively. The suspension of ACW was homogenized in a Teflon homogenizer. Emulsions of ACW and ACE

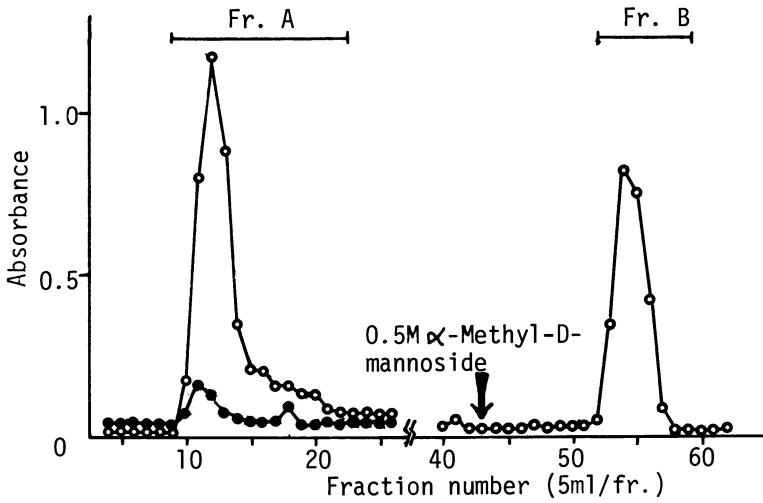
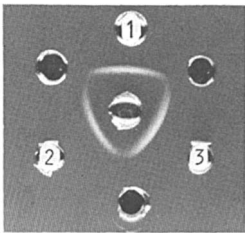


Figure 1. Affinity chromatography of ACI with Con A-sepharose



Journal of General Microbiology

Figure 2. Immunodiffusion pattern of the serologically active fractions with anti-ACE serum: (1) ACI-B; (2) PHW-B; (3) ACE-B (11)

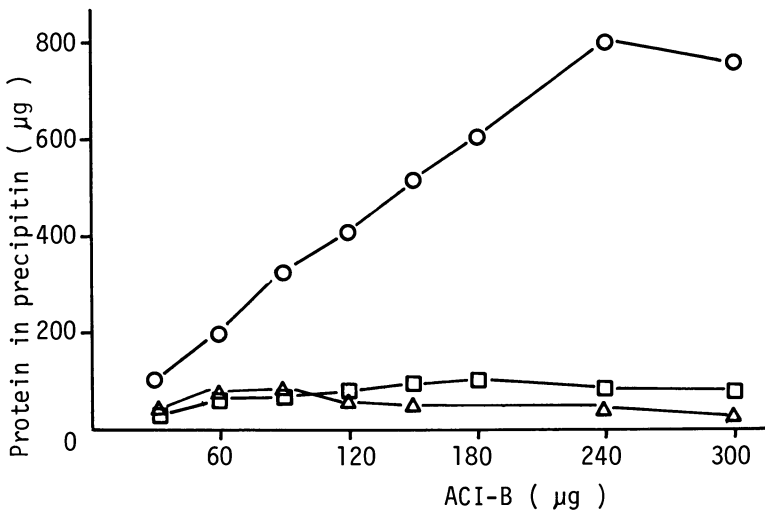


Figure 3. Quantitative precipitin curves of (O) anti-ACE, (\square) anti-ACWC, and (Δ) anti-ACW sera against ACI-B

were prepared by adding an equal volume of Freund's complete adjuvant (Difco). Male New Zealand white rabbits, each weighing 2.5 to 3.0 kg were immunized with 0.5ml of the emulsions by intracutaneous injection once a week for 3 or 6 weeks. One or two weeks after the last injection, the rabbits were bled and the separated sera were stored in a frozen state. These antisera were designated anti ACW serum and anti ACE serum. The antiserum, designated as anti ACWC serum, was prepared by immunizing rabbits with 0.5ml of a homogenate of the whole cell in saline (100mg wet wt/ml) once a week for 7 weeks. This antiserum was also kept frozen.

In the test of double diffusion in agar gel according to the method of Ouchterlony (13), minor fractions, PE, HE and the supernatant fraction of the disrupted cells, ACI, gave a precipitin arc against to anti ACE serum, respectively, and the immunodiffusion patterns suggested their serological similarity. However, the major fractions, SE and PCE did not give precipitin arc.

Isolation of Antigenic fractions (11)

It is well known that many fungal mannans and mannose-containing polysaccharides serve as antigens (14,15,16). In recent years, lectins have been increasingly used for fractionating complex carbohydrates. For the isolation of mannan or mannose-containing polysaccharides, application of concanavalin A seems to be effective. From our previous results (5,6,7,8), since the presence of mannose was expected in the antigenic substances of Mucorales, we tried concanavalin A sepharose column chromatography. The fraction ACI was dissolved in 0.15M phosphate buffer (pH 7.0) and the solution was heated in a boiling water bath for 15 min. After cooling, the solution was applied to a column of con A sepharose 4B and eluted with the same buffer, to give fraction ACI-A, and then with 0.5M α -methyl-D-mannoside in the same buffer to give fraction ACI-B (Figure 1). In the both cases of PHW and ACE, quite similar elution patterns were also obtained. In the immunodiffusion patterns of ACI-B, PHW-B and ACE-B with anti ACE serum, clear precipitin lines which fused with each other were observed (Figure 2). The results of immunoelectrophoresis showed that these fractions gave precipitin arcs which were similar to each other in their mobilities with anti ACE serum. Quantitative precipitin curves of anti ACE serum showed the highest activity against ACI-B. About 8-times of the activity was shown comparing to anti ACW and anti ACWC sera (Figure 3), and ACI-B-treated sera did not react with ACE-B and PHW-B. Therefore, anti ACE serum was used for the subsequent experiment. The other fractions, which were not absorbed on the con A sepharose column, ACI-A, ACE-A and PHW-A, gave different weak precipitin arcs (Figure 4).

Chemical analysis of ACI-B, ACE-B and PHW-B are shown in Table 1. Major constituents were neutral sugars (fucose and mannose in Mol. ratios of 1:2.3-2.5) and proteins. Phosphate was

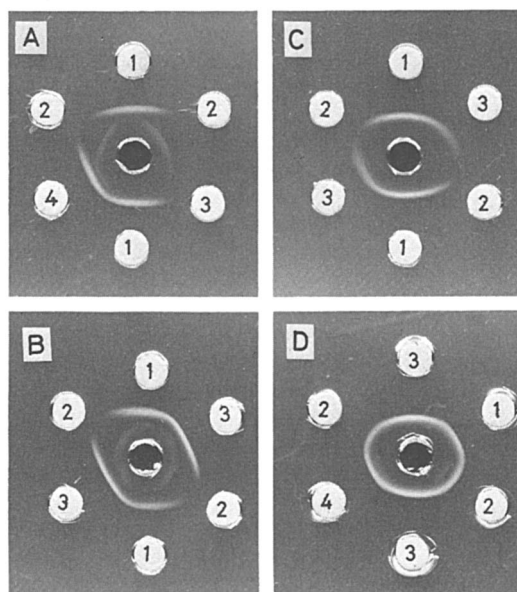


Figure 4. Immunodiffusion patterns of each fraction of antigenic substances with anti-ACE serum: A: (1) ACI-B, (2) ACI-A₁, (3) ACI-A₂, (4) ACI; B: (1) ACE-B, (2) ACE-A, (3) ACE; C: (1) PHW-B, (2) PHW-A, (3) PHW; D: (1) ACI-B, (2) ACE-B, (3) PHW-B, (4) ACI

Table 1. Chemical analysis of ACI-B, ACE-B, and PHW-B

	Neutral sugar content (%,w/w)	Molar ratio of Fuc and Man	Protein content (%,w/w)	Phosphate content (%,w/w)	Total (%,w/w)
ACI-B	63.0	1 : 2.5	22.7	7.3	93.0
ACE-B	55.6	1 : 2.5	7.7	15.8	79.1
PHW-B	80.7	1 : 2.3	8.9	1.9	91.5

Traces of galactose, glucosamine, galactosamine and glucuronic acid were also detected in all fractions.

also found.

To elucidate whether the serologically reactive site is a carbohydrate or a protein moiety, ACI-B was subjected to periodate oxidation and digestion with proteolytic enzymes. Portions of periodate oxidized ACI-B and pronase/trypsin-digested ACI-B were examined by immunodiffusion with anti ACE serum. Periodate-oxidized ACI-B and its serial dilutions in saline were also used for the precipitin reaction in a fine glass tube. Periodate-oxidized ACI-B did not react with the antiserum, but digestion of ACI-B with pronase and trypsin did not affect its serological reactivity. The immunodiffusion pattern fused completely. From these results, it is estimated that the intracellular mannan-rich fraction possesses serological activity.

Serological cross-reaction (17)

As described above, fungal mannans or mannose-containing polysaccharides such as galactomannan or rhamnomannan are known as antigenic substances. It is interesting to examine the cross-reactivity between Mucorales and other fungi. The test antigens were prepared according to the procedure of the case of A. cylindrospora antigens. They were cultured in a Sabouraud's liquid medium with shaking for 2 weeks at 27°C.

The cross-reactivity of the antigenic fractions from four Mucor species, Rhizopus nigricans and six different genera of fungi belonging to Oomycetes or Fungi imperfecti against anti ACE serum were carried out by the immunodiffusion method.

Anti ACE serum reacted with exo(E)- and intracellular(I)-fractions from M. hiemalis, M. javanicus, M. lamprosporus, M. mucedo and R. nigricans as well as those of from A. cylindrospora. Samples prepared from Alternaria solani, Fusarium solani, Aspergillus spp., Cladosporium herbarum and Penicillium isolandicum did not react with anti ACE serum, in spite of the fact that these species produce mannose-containing polysaccharides (18,19,20), (Table 2). Antiserum against whole cells of Ab. lichtheimii gave a weak cross-reaction with a phenol extract of Candida albicans (21), but the serological cross-reactivities have not been elucidated in detail. In our experiments, neither mannans from Saccharomyces cerevisiae (22) and Candida albicans (Duke strain) (23) nor galactomannan from Penicillium chrysogenum (24) reacted with the anti serum to A. cylindrospora. These findings suggest that the anti ACE serum has the specificity only for Mucoraceae or Mucorales.

In the immunodiffusion analysis of E-fractions from four Mucor species and R. nigricans against anti ACE serum, each of these active fractions gave a precipitin band which was identical with that of A. cylindrospora (Figure 5). The results of immunodiffusion analysis and immunoelectrophoresis of MHI-B and RNI-B, which were isolated from M. hiemalis IFO 5303 and R. nigricans IAM 6070, also suggest that these fractions contained a common

Table 2. Cross reactivities of E- and I-fractions from various fungi against anti-ACE serum

	E-fraction		I-fraction	
	5 mg.ml ⁻¹	0.5 mg.ml ⁻¹	5 mg.ml ⁻¹	0.5 mg.ml ⁻¹
<u>Absidia cylindrospora</u> IFO 4000	+	+	+	+
<u>Mucor hiemalis</u> IFO 5303	+	+	+	+
<u>M.javanicus</u> IFO 4569	+	+	+	+
<u>M.lamprosporus</u> IFO 6337	+	+	+	+
<u>M.mucedo</u> IFO 5776	+	+	+	+
<u>Rhizopus nigricans</u> IAM 6070	+	+	+	+
<u>Pythium debaryanum</u> IFO 5919	-	-	-	-
<u>Alternaria solani</u> IFO 5924	-	-	-	-
<u>Aspergillus niger</u> IFO 4043	-	-	-	-
<u>A.parasiticus</u> IFO 4301	-	-	-	-
<u>Cladosporium herbarum</u> IFO 4459	-	-	-	-
<u>Fusarium solani</u> IFO 5232	-	-	-	-
<u>Penicillium islandicum</u> IFO 5234	-	-	-	-
Mannan of <u>Candida albicans</u> (Duke)	/	/	-	-
Mannan of <u>Saccharomyces cerevisiae</u>	/	/	-	-
Galactomannan of <u>Penicillium chrysogenum</u>	/	/	-	-

Precipitin band was found(+) and not found(-)

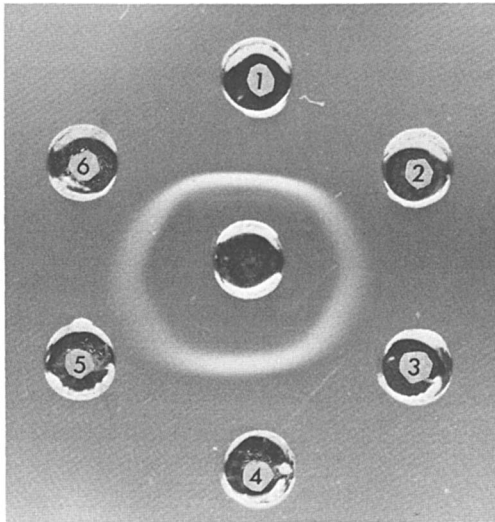
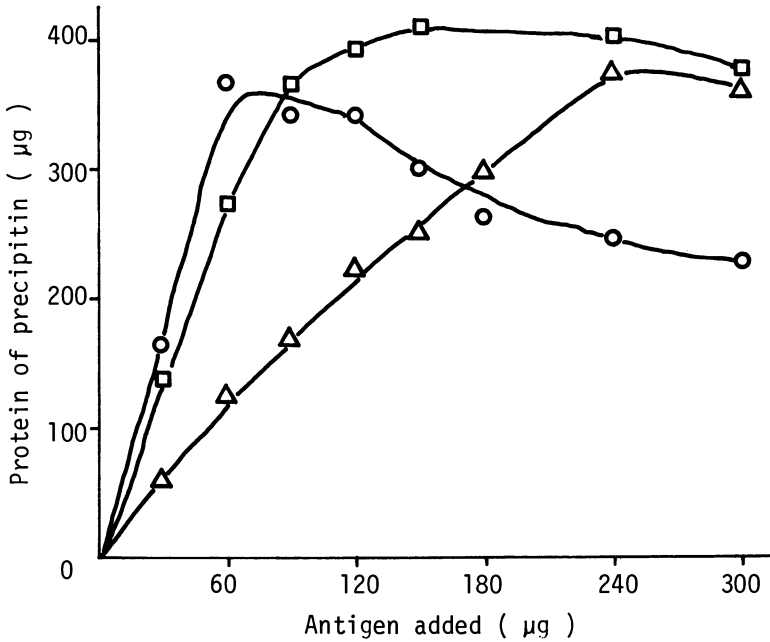


Figure 5. Immunodiffusion pattern of the E-fractions from *A. cylindrospora*, *R. nigricans*, and four *Mucor* species: (1) *A. cylindrospora*, (2) *R. nigricans*, (3) *M. hiemalis*, (4) *M. javanicus*, (5) *M. lamprosporus*, (6) *M. mucedo*, center is anti-ACE serum



Journal of General Microbiology

Figure 6. Quantitative precipitin curves of (○) ACI-B, (△) MHI-B, and (□) RNI-B against anti-ACE serum (17)

Table 3. Methylated alditol acetates derived from the methylated manno-oligosaccharides

0-acetyl-0-methyl alditol	Relative retention time		Molar ratio in			
	Observed ^{a)}	Literature ^{b)}	Oligo-1	-2	-3	-6
1,5-di-0-acetyl-2,3,4,6-tetra-0-methyl mannitol	1.00	1.00	1.0	1.0	1.0	1.0
1,5,6-tri-0-acetyl-2,3,4-tri-0-methyl mannitol	2.44	2.48	1.8	2.6	3.5	6.5

a) Retention times of alditol acetate derivatives were expressed relative to the mobility of 1,5-di-0-acetyl-2,3,4,6-tetra-0-methyl glucitol.

b) The data from literature (Lindberg, 1972) were quoted.

c) Molar ratios of the alditol acetates were estimated from relative peak areas of gas chromatogram. Oligo-4 and -5 were not estimated.

serological determinant. Quantitative precipitin curves of MHI-B, RNI-B and ACI-B against anti ACE serum are shown in Figure 6. These patterns suggest that each antigenic fraction may not be homogeneous.

Inhibition tests (25)

In order to estimate the serological determinant of ACI-B, which is a fucomannan-peptide fraction, the inhibition tests of ACI-B, MHI-B and RNI-B with anti ACE serum by purified manno-oligosaccharides were carried out.

ACE-II fraction, which was treated with pronase and partially purified by gel filtration using a sephadex G-200 column, was dissolved in 0.05M sulfuric acid at the concentration of 1%(w/v), and the mixture was heated in a boiling water bath for 4hr. After cooling and neutralization, gel filtration on a column of Biogel P-2 was carried out by water elution. Oligosaccharide fractions thus obtained were purified by descending paper chromatography using a Whatman 3MM paper and a solvent system of ethyl acetate-pyridine-acetic acid-water 5:5:1:3(v/v) for 30 to 80hr. Each separated oligosaccharide on the paper chromatogram was extracted with distilled water, and was passed through a Dowex 50 (H) column for removal of traces of ionic contaminants and then lyophilized. Sugar component of the oligosaccharides was determined by thin layer and GC analysis of the acid hydrolysate. Structural determination of these oligosaccharides was carried out by methylation analysis of its alditol acetate derivatives (Table 3), and by the estimations of R_{mannose} values, optical rotations and degree of polymerizations (Table 4).

These results show that all of the six oligosaccharides consist of α -(1 \rightarrow 6)-linked linear mannopyranosyl residues, and are tri- to octa-saccharides.

As is shown in the Figure 7, the precipitation reaction of ACI-B with anti ACE serum was inhibited by penta-, hexa-, hepta-, and octa-saccharides. These higher molecular weight oligosaccharides also inhibited the reaction of MHI-B and RNI-B with anti ACE serum. The inhibition percents by 2 μ M addition of each oligosaccharide were shown in Table 5.

These facts suggest that at least the α -(1 \rightarrow 6)-linked mannopyranosyl residues might play a significant role as a part of the common determinant of the serologically active substances of *A. cyrindrospora*, *M. hiemalis* and *R. nigricans*. However, the inhibition by these oligosaccharides was less than the case of the inhibition by α -D-mannopyranosyl-(1 \rightarrow 3)- α -D-mannopyranosyl-(1 \rightarrow 2)- α -D-mannopyranosyl-(1 \rightarrow 2)-D-mannose of the precipitin reaction between mannan and anti whole cell serum of *Saccharomyces cerevisiae* (26). This fact suggests a possibility of the presence of a stronger inhibitor. It is also possible that ACI-B is not homogeneous.

Table 4. Chemical properties of the purified manno-oligosaccharides (25)

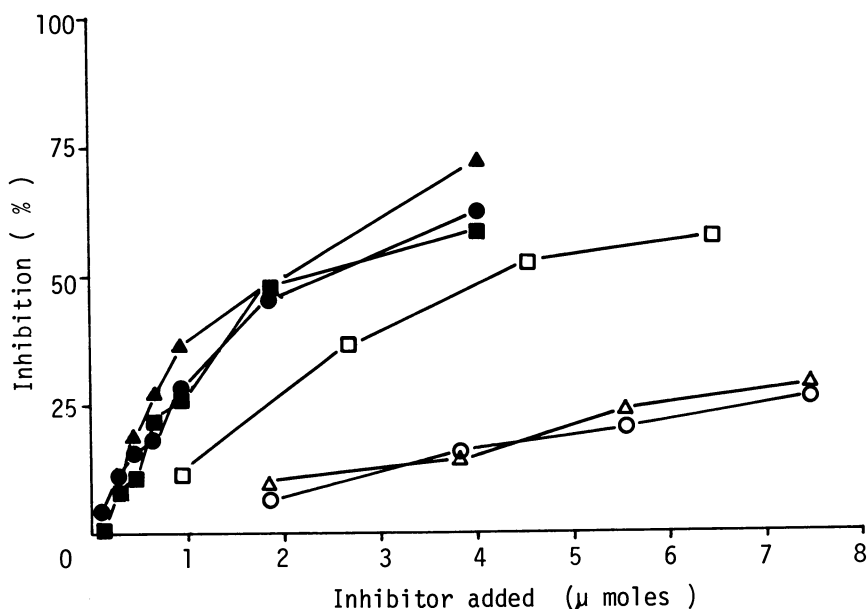
	Sugar content (% w/w)	R _{mannose}	[α] _D ²⁰ (in water)	Degree of polymerization		
				Somogyi	NaB ³ H ₄	Glc ^{**}
Oligo-1	97.9	0.39	+64.7	3.04	ND	2.8
Oligo-2	91.2	0.24	+70.8	3.83	ND	3.6
Oligo-3	95.9	0.14	+71.0	5.02	ND	4.5
Oligo-4	95.9	0.09	+71.8	5.88	6.38	ND
Oligo-5	98.2	0.06	+81.1	6.38	7.13	ND
Oligo-6	96.6	0.03	+81.2	7.84	8.60	7.5

ND, Not determined.

* Calculated as anhydrous mannose.

** Calculated from the molar ratios of tetra- and tri-O-methyl alditol acetates derived from the methylated oligosaccharides.

Journal of General Microbiology



Journal of General Microbiology

Figure 7. Inhibition by oligosaccharides of the precipitin reaction between ACI-B and anti-ACE serum: (○) trisaccharides, (△) tetrasaccharides, (□) pentasaccharides, (●) hexasaccharides, (▲) heptasaccharides, (■) octasaccharides. Reaction system: 200 μ L antiserum (two-fold diluted), 150 μ g ACI-B, and 200 μ L inhibitor were mixed; total volume of reaction mixture was 500 μ L. Maximum addition for the hexa-, hepta-, and octasaccharides was 4 μ mol because they were in limited quantities. (25)

Chemical analysis of ACI-B, MHI-B and RNI-B are shown in Table 6. It is of interest that fucose content of RNI-B is less than that of the others.

Fluorescent antibody staining

Previously, Hawker and Abbot showed that the inner cell walls, which were newly formed during germination of the sporangiospores of *R. nigricans* and *R. sexualis*, resembled the cell walls of the vegetative hyphae in the electron microscopic study (27). Almost all fungal hyphae including Mucorales grew by apical growth and cell synthesis occurred in hyphal apex (28). In our observation by a fluorescent antibody technique using fluorescein isothiocyanate conjugated anti ACE serum (29), the germinating hyphae and the sporangiospores of *A. cylindrospora* showed uniform fluorescence as the vegetative hyphae did. The fluorescence was not limited to the apex. From these facts, it is estimated that some part of the antigenic substance of *A. cylindrospora* may be uniformly distributed on the surfaces of the younger and older hyphae. With the fluorescent antibody treatment, paraffin sections of *M. hiemalis* and *R. nigricans* exhibited fluorescence as *A. cylindrospora* did (Hayashi, Yadomae and Miyazaki, unpublished data).

Conclusion

Results of the preliminary examination of the antigenic fractions of Mucorales were described. The antigenic active fractions, ACI-B, MHI-B and RNI-B, have been purified partially. Analytical data of these fractions suggest that they are phosphate-containing fucomannan-peptide fractions. Serological activity of these fractions with anti ACE serum is inhibited by α -(1 \rightarrow 6)-linked mannopyranosyl oligosaccharides. This suggests that the mannose portion should participate in the antigenic determinant. However, the precipitin titers are low, and the heterogeneity of these fractions still unresolved. Detailed chemical and immunochemical examinations are now in progress. The antigenic substance, ACI-B, can be isolated from the supernatant of the disrupted mycelium of the fungus. This indicated that some part of the serologically active fraction of *A. cylindrospora* may be loosely bound on the surface of the mycelium and easily released from the surface.

Abstract

Partially purified serologically active fractions, ACI-B, MHI-B and RNI-B, were separated by affinity chromatography with a con A sepharose column from the supernatant of disrupted mycelium fluid of *A. cylindrospora*, *M. hiemalis* and *R. nigricans*, respectively. These active fractions contained mannose as their

Table 5. Inhibition of the precipitation reactions of ACI-B, MHI-B and RNI-B with anti-ACE serum by the oligosaccharides

Inhibitor	Inhibition (%)		
	ACI-B	MHI-B	RNI-B
Tri-saccharide	7.0	15.2	15.0
Tetra- "	8.9	12.6	10.2
Penta- "	27.0	33.0	22.1
Hexa- "	46.0	67.0	62.7
Hepta- "	46.3	79.3	70.1
Octa- "	46.9	63.8	59.4

Reaction mixture contained 2 μmol oligosaccharide, twofold diluted anti-ACE serum and 150 μg ACI-B or RNI-B or 300 μg MHI-B.

Table 6. Chemical analysis of ACI-B, MHI-B, and RNI-B

	Neutral sugar content (%w/w)	Molar ratio of Fuc and Man	Protein content (%w/w)	Phosphate content (%w/w)	Total (%w/w)
ACI-B	63.0	1 : 2.5	22.7	7.3	93.0
MHI-B	58.7	1 : 2.9	7.1	2.2	68.0
RNI-B	55.7	1 : 25.7	19.7	1.3	77.7

Traces of galactose, hexosamine, and uronic acid were detected in all of these fractions.

main common component.

Six manno-oligosaccharides, which were composed of linear α -(1+6)-linked mannopyranosyl residues, were isolated from the partial acid hydrolysate of pronase-treated exo-cellular glycan of *A. cylindrospora*. The penta-saccharide and the higher molecular weight oligosaccharides inhibited the precipitin reaction of ACI-B, MHI-B and RNI-B with anti ACE serum.

Cross reactivity of anti ACE serum among some Mucorales, the exo-cellular and disrupted supernatant fractions prepared from other fungi was observed only in Mucorales. This suggests that a common serological determinant is present in Mucorales.

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Immunochemistry of *Candida albicans* Mannan

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Many species of yeasts are known to produce mannans containing various amounts of phosphate, which can be fractionated by anion-exchange chromatography (1, 2, 3, 4). Recently, we revealed that the mannan of *Saccharomyces cerevisiae* (bakers' yeast) can be fractionated on a column of diethylaminoethyl (DEAE)-Sephadex into five subfractions containing different amounts of phosphate, and that the antibody-precipitating activities of these mannan subfractions against their homologous antiserum were proportional to their phosphate content (5). The above findings suggest that many yeast mannans are composed of heterogeneous molecular species in which different number of phosphate groups exist, and that the mannan subfraction containing the largest amount of phosphate corresponds to the strongest antigen of the parent yeast. In the present study, the mannans from three *C. albicans* strains of different serotype, NIH A-207 (serotype A) (A-strain) (6, 7), NIH B-792 (serotype B) (B-strain) (6, 7), and J-1012 (serotype C) (J-strain) (8) were investigated to determine whether a similar relationship between phosphate content and serological activity could be observed. Also, cross-reactivity between the fractionated mannans of the *C. albicans* strains and anti-*S. cerevisiae* serum, the latter of which has been shown to be cross-reactive with the mannan of B-strain (9, 10), was investigated in order to assess density of the cross-reactive antigenic determinant groups in the fractionated mannan of B-strain. A brief communication dealing with the phosphate content and serological activity of the mannan subfractions of A-, B-, and C-strains appeared recently (11).

Materials and Methods

The *C. albicans* strains used. Three strains of *C. albicans*, NIH A-207, NIH B-792, and J-1012, were kindly provided by Dr. Y. Fukazawa, Meiji College of Pharmacy, Tokyo.

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Cultivation of the strain. Each strain was cultivated in a 500-ml flask containing 0.5%-yeast extract-added Sabouraud liquid medium on a reciprocal shaker at 26 to 28°C for 60h. The cells were harvested by centrifugation, washed thoroughly with saline, and dehydrated with a large volume of acetone.

Preparation of the bulk mannans. Two batches of the bulk mannan of each strain were prepared by the method of Peat et al. (12), modified by us as follows: A suspension of acetone-dried whole cells of each strain (300g) in water (3 liter) was heated in an autoclave for 2h at 140°C. After being cooled, the mixture was centrifuged for 20min at 7,000rpm, and the residue was stirred with water (1 liter) and centrifuged again for 20min at 7,000rpm. The combined filtrates were concentrated in vacuo to small volume (ca. 300ml), dialyzed overnight against running tap water, and concentrated in vacuo to ca. 200ml. To the solution was added 800ml of absolute ethanol containing 0.1% sodium acetate. After being left for 30min, the precipitate was collected by centrifugation, then dehydrated with absolute ethanol, and dried in vacuo over P₂O₅ to yield an amorphous powder. The above crude extract (15g) was dissolved in water (150ml), and the cloudy solution was stirred for 2h with a 4 : 1 (v/v) mixture of chloroform and n-butanol (200ml). Then the mixture was centrifuged at 2,500rpm for 15min, and the supernatant collected was repeatedly treated by the same procedure until a completely clear solution was obtained (For preparation of the bulk mannans of Batch II, this deproteinization procedure was omitted). The supernatant was concentrated in vacuo to ca. 100ml and then was poured into 600ml of absolute ethanol containing 0.1% sodium acetate. The precipitate was washed with ethanol, and dried in vacuo over P₂O₅. To an aqueous solution of the above crude polysaccharide fraction (10g in 100ml) was added 200ml of Fehling solution (a 1 : 1 (v/v) mixture of aqueous solution of 3.5% CuSO₄·5H₂O and 17.3% Rochelle salt containing 5.0% NaOH), and the mixture was stirred for 12h at room temperature (Batch I) or for 5min (Batch II). The resultant precipitate was collected by centrifugation for 15min at 3,000rpm and then washed with 400ml of hot water (70°C). The copper-mannan complex contained in a centrifuge tube was admixed with wet Amberlite IR 120 (H⁺) resin, stirring was then continued until the complex dissolved and the blue color of the copper ion disappeared completely. The mixture was filtered with a glass filter, and the residue was thoroughly washed with water. The combined filtrate and washing were neutralized with 10% Na₂CO₃ solution, then concentrated in vacuo to ca. 50ml, and the solution was poured into absolute ethanol containing 0.1% sodium acetate. After washing with ethanol by centrifugation, the resultant precipitate was dried over P₂O₅ under diminished pressure to give a bulk mannan fraction. Yields of the mannans of Batches I and II of A-, B-, and J-strains were 6.4, 7.8, and 6.7%, and 7.5, 8.2, and 6.9%, respectively, of the

dry weight of the parent whole cells.

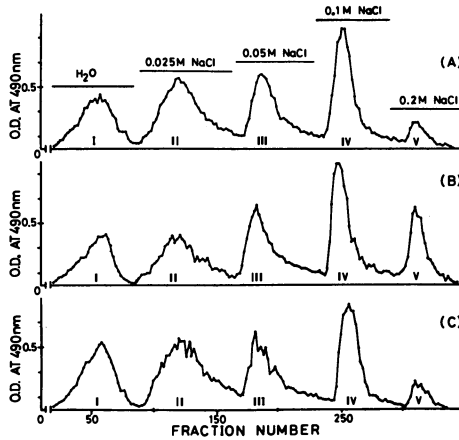
Chromatographic fractionation of the bulk mannans. An aqueous solution of the bulk mannan (3g in 40ml) was applied to a column (4 by 25cm) of DEAE-Sephadex A-50 (acetate), and the elution was effected in a stepwise manner with water, 0.025, 0.05, 0.1, and 0.2M NaCl. The flow rate was 0.5ml/min, and a 10 μ l sample of each fraction was assayed for carbohydrate content with phenol-sulfuric acid reagent (13). Subfractions I, II, III, IV, and V (Figs. 1 and 2) were evaporated in vacuo to dryness, and, after dissolving in a minimum amount of water, the solution was dialyzed against running tap water. Each mannan subfraction was again concentrated to 5ml and then poured into 25ml of absolute ethanol containing 0.1% sodium acetate, and the precipitated polysaccharide was collected by centrifugation at 2,500rpm for 15min. After being washed with absolute ethanol, the mannan subfractions were dried in vacuo over P₂O₅. The yields of Subfractions I, II, III, IV, and V from the bulk mannans of Batch I and II are given in Tables I and II.

Antisera. Antisera to the *C. albicans* A- and B-strains of Batch I were prepared by immunizing two groups of three rabbits each with the whole cells of each strain, and antisera obtained from each group of rabbits were combined. Anti-J-strain serum, Batch I, was kindly supplied by Dr. Y. Fukazawa. All antisera of Batch I had agglutinin titers of more than 1 : 1,280 against the immunizing cell suspensions. Antisera of A-, B-, and J-strains of Batch II were prepared by the same procedure as that for Batch I. Agglutinin titers of antisera of A- and J-strains were shown to be more than 1 : 1,280, whereas that of anti-B-serum was considerably low, 1 : 320.

Anti-*S. cerevisiae* whole cell serum, Batch I, was the same specimen as that used in the previous study (5). It had a very high agglutinin titer, more than 5,120. On the other hand, that of anti-*S. cerevisiae* serum, Batch II, was 1 : 1,280.

Mannan subfractions of *S. cerevisiae*. The mannan subfractions of *S. cerevisiae*, Batch I, were the same specimens as those employed in the previous study (5), and those of Batch II were prepared by the same procedure as the mannan subfractions of three *C. albicans* strains of Batch II used for the present study. Analytical data for the mannan subfractions of *S. cerevisiae*, Batch II, are shown in Table II.

Immunochemical methods. The quantitative precipitin reaction was carried out according to the previous description (5) as follows: To 0.1ml of antiserum in a small test tube (1.6 by 10.4cm) was added 0.5ml of saline solution of serial amounts of the mannan. After incubation at 37°C for 1h, the mixture was allowed to stand at 4°C for 16h and then centrifuged at 2,500rpm



Journal of Bacteriology

Figure 1. Elution profiles of the bulk mannans of three *C. albicans* strains (Batch I) by DEAE-Sephadex chromatography (A-50, acetate, 4×25 cm) using a stepwise elution system consisting of water and NaCl solutions. Ten- μ L aliquots of fractions were assayed for carbohydrate content with phenol-sulfuric acid reagent (13): (A) *C. albicans* NIH A-207 (A-strain); (B) *C. albicans* NIH B-792 (B-strain); (C) *C. albicans* J-1012 (J-strain) (11).

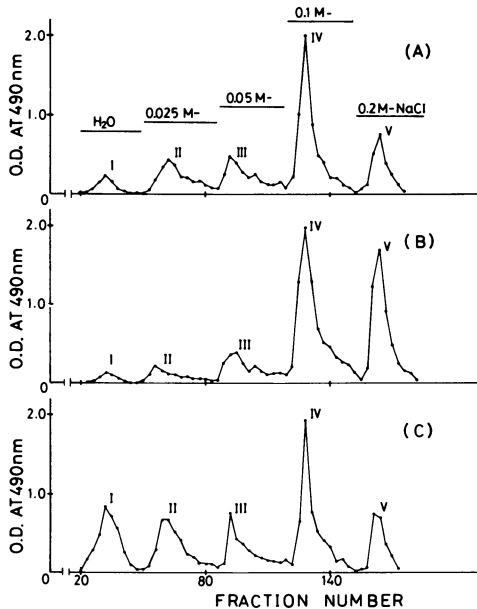


Figure 2. Elution profiles of the bulk mannans (Batch II) of three *C. albicans* strains by DEAE-Sephadex chromatography (A-50, acetate, 4×25 cm) using a stepwise elution system consisting of water and NaCl solutions. Ten- μ L aliquots of fractions were assayed for carbohydrate content with phenol-sulfuric acid reagent (13): (A) *C. albicans* NIH A-207 (A-strain); (B) *C. albicans* NIH B-792 (B-strain); (C) *C. albicans* J-1012 (J-strain).

for 10min. Each precipitate was carefully washed twice with ice-chilled saline. The amounts of protein in the precipitate were determined by the Folin method of Lowry et al. (14).

Chemical analyses. Total carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (13), and total phosphorus was quantitated by the method of Ames and Dubin (15), respectively.

Results

Fractionation of the bulk mannans of three *C. albicans* strains by anion-exchange chromatography. Figures 1-A, -B, and -C shows the elution profiles of DEAE-Sephadex chromatography of the bulk mannans of Batch I using a stepwise elution system consisting of water, 0.025-, 0.05-, 0.1-, and 0.2M NaCl solutions subsequently. Under the above condition, all bulk mannans were shown to be resolved into each five subfractions designating as Subfractions I, II, III, IV, and V, respectively. The amounts of Subfractions I and II from A- and B-strains were relatively smaller than those of the other subfractions, III, IV, and V, while the bulk mannan of C-strain gave almost the same amounts of Subfraction I, II, III, and IV. Elution profiles of the bulk mannans of Batch II prepared by short-term treatment with Fehling solution are essentially similar to those of Batch I (Figs. 2-A, -B, and -C). However, the most significant difference between the bulk mannans of Batch I and II is that of the amounts of Subfraction V of B-strain, suggesting that the cause is attributable to the different conditions of treatment with Fehling solution, as pointed out by Thieme and Ballou on the phosphomannan of *Kloeckera brevis* (16).

Chemical composition of the mannan subfractions of three *C. albicans* strains. Table I shows the analytical data for each five mannan subfractions, Batch I, obtained from the corresponding bulk mannans of three *C. albicans* strains. It is clear that three series of mannan subfractions contain increasing amounts of phosphate. With the exception of B-strain mannan subfraction series, protein contents of the subfractions are also proportional to the NaCl concentration used in the elution. Among the three mannan subfraction series, those of the J-strain show the largest Man/P ratio in comparison with those of each corresponding subfractions of the two other series. The mannan subfractions of B-strain series exhibited the smallest Man/P ratio. Furthermore, the order of the sum of the yields of Subfractions I and II can be represented as $J \geq A > B$, indicating that the parent bulk mannan of J-strain consists of relatively larger amounts of molecular species containing fewer phosphate groups. On the other hand, the order of the sum of Subfractions IV and V is shown to be $B > A \geq J$, demonstrating that the mannan of the

Table I. Chemical composition of mannan subfractions (Batch I) isolated from *C. albicans* NIH A-207 (A-strain), *C. albicans* NIH B-792 (B-strain), and *C. albicans* J-1012 (J-strain). (11)

Mannan sub-fractions	Carbo-hydrate ^a (%)	Protein ^b (%)	Phosphate ^c (%)	Man/P molar ratio	Yield ^d (%)
A-I	96	0.8	0.00	>1000	14.5
A-II	94	0.8	0.18	224	23.5
A-III	96	1.1	0.44	94	19.0
A-IV	93	1.4	0.84	48	19.5
A-V	92	2.3	1.20	34	3.5
B-I	97	1.3	0.00	>1000	12.5
B-II	95	1.1	0.21	198	17.0
B-III	95	1.3	0.60	69	19.5
B-IV	96	1.4	1.15	36	21.0
B-V	93	1.4	1.83	22	12.0
J-I	95	1.0	0.00	>1000	16.6
J-II	94	1.3	0.10	392	24.8
J-III	92	2.2	0.29	139	17.6
J-IV	92	2.6	0.57	70	16.7
J-V	91	4.1	0.63	63	3.2

a Determined by the phenol-sulfuric acid method (13).

b Determined by the Lowry-Folin method (14).

c Quantitated by the Ames-Dubin method (15) as PO_3H_2 .

d Weight basis of the corresponding bulk mannan.

first strain contains relatively larger amounts of the highly phosphorylated mannans. Table II summarizes the results of chemical analysis of the mannan subfraction series of Batch II. Although considerable differences are observed between the yields of the mannan subfractions of the two batches, especially in Subfractions V of the B-strain series, the existence of an essentially consistent relationship between their phosphate contents and the concentrations of NaCl solutions used for elution can be discerned.

Quantitative precipitin reactions between the mannan subfractions and antisera of three *C. albicans* strains. The results of the quantitative precipitin reaction between the mannan subfractions of Batch I and their corresponding antisera are depicted in Figs. 3-A, -B, and -C. In the homologous antigen-antibody systems, the amounts of antibody precipitated with each mannan subfraction of A- and B-strains are found to be proportional to the phosphate content of the mannan. These results are obviously similar to those obtained with the mannan subfractions and the homologous antiserum of *S. cerevisiae* (5). On the other hand, the quantitative precipitin curves of the J-strain mannan subfractions and anti-J-serum significantly differ from those of A- and B-strains, *i.e.*, Subfraction J-III shows nearly identical precipitin activity with that of Subfractions J-IV and -V, although subfractions J-I, -II, and -III afforded proportional amounts of antibody nitrogen to their phosphate contents. Furthermore, the difference in the maximal amounts of precipitated nitrogen by J-I and -V appears to be significantly smaller than those of the corresponding two mannan subfractions of A- and B-strains, reflecting a smaller difference in the densities of the antigenic determinants in the mannan of J-strain. The larger differences in antibody-precipitating activities of Subfraction I and V in both the A- and B-strain series than that observed in the J-strain series can also be regarded to correlate with their large differences in phosphate content, suggesting the presence of a larger difference in the density of the antigenic determinant groups.

In the cross-precipitin reaction against antisera of the other serotype strains, all mannan subfractions of A- and J-strains were found to be capable of displaying strong reactivities to the antisera of different serotype strains, affording the amounts of antibody nitrogen proportional to their phosphate contents. This finding indicates that the densities of the cross-reactive antigenic determinant groups in each series of mannan subfractions are also proportional to their phosphate contents. The mannans of B-strain are shown to be cross-reactive to relatively weaker extents with antisera of A- and J-strains; *i.e.*, only Subfractions B-IV and -V are cross-reactive with antisera of the A- and J-strains. Subfraction B-III can be regarded to be a highly specific antigen fraction of B-strain because this subfraction is devoid of any cross-reactive constituent and shows a

Table II. Chemical composition of mannan subfractions (Batch II) isolated from C. albicans NIH A-207 (A-strain), C. albicans NIH B-792 (B-strain), C. albicans J-1012 (J-strain), and S. cerevisiae.

Mannan subfrac-tions	Carbo-hydrate ^a (%)	Protein ^b (%)	Phosphate ^c (%)	Man/P molar ratio	Yield ^d (%)
A-I	94	1.9	0.03	>1000	3.9
A-II	92	2.5	0.35	114	14.3
A-III	92	2.6	0.72	56	13.7
A-IV	91	2.5	1.23	32	30.3
A-V	90	3.0	1.76	22	12.7
B-I	95	2.2	0.06	688	2.1
B-II	92	2.5	0.38	105	5.1
B-III	93	2.6	0.76	53	11.4
B-IV	92	2.6	1.38	29	31.9
B-V	90	2.3	2.36	17	24.9
J-I	94	2.1	0.03	>1000	15.1
J-II	91	3.0	0.20	202	17.5
J-III	91	3.5	0.50	81	13.7
J-IV	90	4.1	0.95	42	22.1
J-V	89	4.3	1.70	23	9.7
<u>S. cerevisiae</u>					
A	96	1.5	0.04	>1000	4.8
B	94	1.9	0.22	186	8.7
C	92	2.3	0.47	85	12.0
D	91	3.1	0.78	51	23.4
E	90	3.9	1.45	27	25.5

^a Determined by the phenol-sulfuric acid method (13).

^b Determined by the Lowry-Folin method (14).

^c Quantitated by the Ames-Dubin method (15) as $-PO_3H_2$.

^d Weight basis of the corresponding bulk mannan.

considerably strong reactivity to its homologous antiserum. Thus, the results of the quantitative precipitin assay against anti-B-strain serum indicate that the antibody-precipitating activities of two mannan subfraction series of A- and J-strains are closely similar to those of B-strain, while the last latter showed distinctly lower cross-reactivities against antisera of the two former strains, A and B. The mannan subfractions and antisera of Batch II gave the consistent results with those obtained in the reaction of the mannans and antisera of Batch I (Figs. 4-A, -B, and -C).

Cross-precipitin reaction between the mannan subfractions of *S. cerevisiae* and three *C. albicans* strains against their antisera, vice versa. As depicted in Fig. 3-A, none of the mannan subfractions of *S. cerevisiae* (Batch I) were shown to cross-react with anti-A-strain serum. All heterologous mannan subfractions, including those of *S. cerevisiae*, were found to be cross-reactive with anti-B-strain serum in the extents proportional to their phosphate contents (Fig. 3-B), indicating that the densities of the cross-reactive antigenic determinant groups in these mannan subfractions are also proportional to their phosphate contents, and further indicating that the densities of the cross-reactive antigenic determinant groups in these mannan subfractions are also proportional to their phosphate contents. The order of the extents of cross-reactivities of three heterologous mannan subfraction series can be represented as $A \doteq J > S. cerevisiae$, providing a finding that the cross-reactivities between the mannans of *S. cerevisiae* and anti-B-strain serum are significantly lower than those observed between antigens and antisera of *C. albicans*. Against anti-*S. cerevisiae* serum, the mannan subfractions of B- and J-strains were found to be cross-reactive in low extents, and those of A-strain were completely unreactive to this serum as shown in Fig. 3-D. The above findings also indicate that the extent of cross-reactivity between the mannans of *C. albicans* and anti-*S. cerevisiae* serum is also lower than those of antigen and antisera of *C. albicans*. Additionally, the difference between the amounts of precipitated antibody with Subfractions I and V of B- and J-strains was not significant, thus indicating that the densities of the cross-reactive antigenic determinant groups in these mannan subfractions, of which Hasenclever and his co-workers (9, 10) assumed to be the $\alpha 1 \rightarrow 3$ linked D-mannopyranosyl residues, do not correlate with their phosphate contents. The results of cross-precipitin reactions between the mannans of *S. cerevisiae* and antisera of three *C. albicans* strains of Batch II gave essentially similar results to those of the corresponding antigens and antisera of Batch I, excepting the fact that the anti-*S. cerevisiae* serum of Batch II did not show any cross-reactivity with all mannan subfractions of three *C. albicans* strains of Batch II (Figs. 4-A, -B, -C and -D).

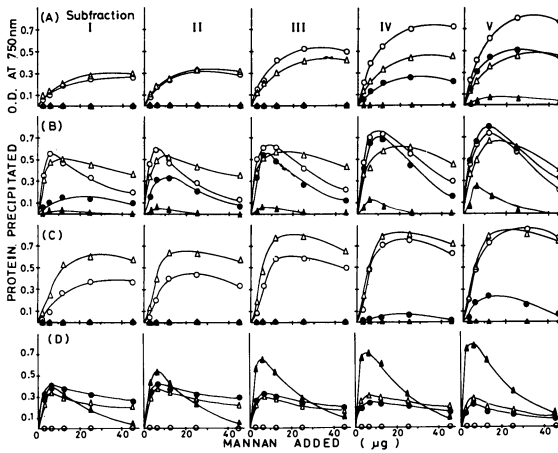


Figure 3. Quantitative precipitin reactions between the mannan subfractions (Batch I) and antisera of three *C. albicans* strains and *S. cerevisiae*: (A) anti-*C. albicans* NIH A-207 (A-strain) serum; (B) anti-*C. albicans* NIH B-792 (B-strain) serum; (C) anti-*C. albicans* J-1012 (J-strain) serum; (D) anti-*S. cerevisiae* serum. Mannan subfractions are of: (○) A-strain; (●) B-strain; (△) J-strain; (▲) *S. cerevisiae*.

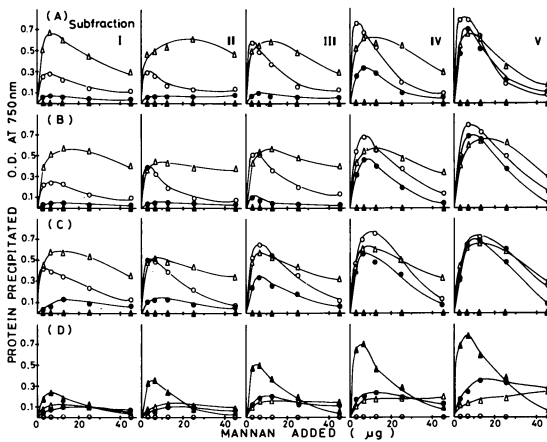


Figure 4. Quantitative precipitin reactions between the mannan subfractions (Batch II) and antisera of three *C. albicans* strains and *S. cerevisiae*: (A) anti-*C. albicans* NIH A-207 (A-strain) serum; (B) anti-*C. albicans* NIH B-792 (B-strain) serum; (C) anti-*C. albicans* J-1012 (J-strain) serum; (D) anti-*S. cerevisiae* serum. Mannan subfractions are of: (○) A-strain; (●) B-strain; (△) J-strain; (▲) *S. cerevisiae*. Dilutions of anti-*S. cerevisiae* serum are homologous system, $\times 6$; heterologous system, $\times 1.5$.

Discussion

It is well-known that C. albicans is one of the most important pathogenic fungi causing a serious infectious disease, Candidiasis, in humans. In order to provide a serological classification system for yeasts including genus Candida, antigen structure analyses of C. albicans and its closely related species have been conducted by several workers, Martin et al. (17), Tsuchiya et al. (18), Pospíšil (19), and Biguet et al. (20), respectively, although assignment of symbols in the antigen formulae proposed by these workers to the chemical structures of the isolated antigen has not been achieved as yet.

In 1961, Hasenclever and Mitchell (6, 7) reported that species C. albicans can be classified into two groups exhibiting distinctly different serotypes designated A and B, respectively. Hasenclever and his co-workers also provided the following findings that the antigenicity of yeasts including genera Candida, Saccharomyces, and Hansenula, is attributable to their cell wall mannans (9, 10). On the structural feature of the antigenic determinant groups of the mannans of B-strain, they assumed that $\alpha 1 \rightarrow 3$ linked D-mannopyranosyl residues occupying the non-reducing terminal groups in this mannan dominate the serotype specificity, because the mannan of S. cerevisiae, in which the existence of the above type of linkages had been confirmed by Peat et al. (21), was shown to be cross-reactive with the mannan of B-strain against anti-S. cerevisiae serum (10).

Thereafter, the presence of an additional serotype, C, was proposed by Tsuchiya and his co-workers in the C. albicans strains isolated from patients of Candidiasis in Japan (8).

Analysis of the chemical structure of the mannan of a C. albicans strain, first achieved by Bishop and his co-workers, provided evidence that this mannan possesses a highly branched structure consisting of D-mannopyranosyl residues connecting with $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ linkages (22).

In 1965, an important modification of the chemical structure of bakers' yeast mannan was made by Ballou and his co-workers. They pointed out that the longest branching moieties of this mannan correspond to a tetraose, O- α -D-mannopyranosyl (1 \rightarrow 3) O- α -D-mannopyranosyl (1 \rightarrow 2) O- α -D-mannopyranosyl (1 \rightarrow 2) mannose (23). In 1967, the Canadian workers and Hasenclever published the modified findings of structural analysis of the mannan of several Candida strains including those of C. albicans serotype A, C. albicans serotype B, C. tropicalis, C. stellatoidea, and C. parapsilosis, affording evidence that all mannans contain small amounts of $\alpha 1 \rightarrow 3$ linked D-mannopyranosyl residues in addition to the large amounts of $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ linkages (24).

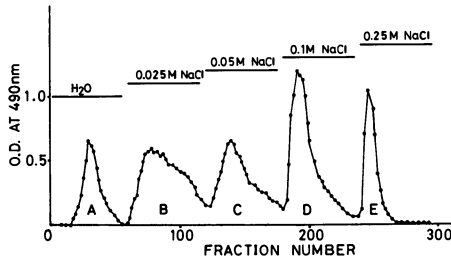
Basing on the above findings provided by the workers above mentioned, we started a series of immunochemical studies of yeast mannans. In 1968, we reported analysis of the antigenic determinant groups of the mannan of S. cerevisiae, in which the mannan

was fragmented by two methods, acetolysis and partial acid-hydrolysis, separately, and the resultant oligosaccharides were assayed for their precipitin-inhibitory activities to an antigen antibody system consisting of the parent mannan and anti-S. cerevisiae whole cell serum. The result of this assay clearly indicated that a mannotetraose, $\text{Man } \alpha 1 \rightarrow 3\text{Man} \alpha 1 \rightarrow 2\text{Man} \alpha 1 \rightarrow 2\text{Man}$, isolated from the acetolysate of the parent mannan showed the strongest precipitin-inhibitory activity, providing evidence that the tetraose moieties in the mannan correspond to the antigenic determinant groups (25). Correctness of this finding was confirmed by Ballou (26).

As subsequent work in this series, we have analyzed the antigenic determinant groups of the mannan of a C. albicans strain isolated in Japan by means of similar procedure to that of the analysis of the antigenic determinants of the mannan of S. cerevisiae, and a mannohexaose consisting of one $\alpha 1 \rightarrow 3$ and four $\alpha 1 \rightarrow 2$ linkages obtained from the acetolysate of the parent mannan was shown to reveal the strongest precipitin-inhibition against the homologous mannan-anti-C. albicans whole cell serum system (27, 28). Further analytical studies of the antigenic determinant groups were conducted on the mannans of A- and B-strains, respectively, to obtain the following findings; 1) a mannoheptaose connecting solely with $1 \rightarrow 2$ linkages isolated from the acetolysate of the parent mannan corresponds to the antigenic determinant groups of the mannan of C. albicans NIH A-207 (A-strain) (29) and 2) a hexaose fraction containing $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 3$ linkages in a ratio of 10 : 1 revealed the strongest inhibitory activity against the homologous mannan-antiserum system of the B-strain (30).

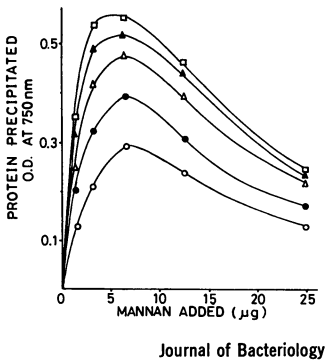
However, the mannans of S. cerevisiae and C. albicans so far used in our previous study were the neutral fractions excluded from phosphorus and protein, consisting of minor parts of the parent bulk mannans, and relatively little information was available regarding the immunochemical properties of the acidic mannan fractions, the major part of the bulk mannans, despite various important findings on the structural feature of the location site of phosphate groups in several yeast mannans accumulated through the efforts of Ballou and his co-workers (31, 32, 33).

Recently, we published the results of an immunochemical study of the bulk mannan of S. cerevisiae with special reference to the serological activity of the acidic fraction (5). Namely, the bulk mannan of S. cerevisiae was fractionated on a column of DEAE-Sephadex into five subfractions containing different amounts of phosphate groups according to the method described by Thieme and Ballou on the fractionation of the phosphomannan of Kloeckera brevis (16) (Fig. 5), and these mannan subfractions were assayed for their antibody-precipitating activities against the homologous anti-whole cell serum (Fig. 6). From the result of the quantitative precipitin reaction, it was evident that the antibody-precipitating activity of these mannan subfractions was



Journal of Bacteriology

Figure 5. Elution profile of 3 g of bulk mannan on a DEAE-Sephadex A-50 column (acetate, 4×25 cm) by stepwise elution with water and NaCl solutions. Samples ($10 \mu\text{g}$) of fractions were assayed for carbohydrate content with phenol-sulfuric acid reagent (13); O.D., optical density (5).



Journal of Bacteriology

Figure 6. Quantitative precipitin curves of five mannan subfractions against homologous anti-whole-cell serum of parent baker's yeast: (○) Fraction A; (●) Fraction B; (△) Fraction C; (▲) Fraction D; (□) Fraction E; O.D., optical density (5).

proportional to their phosphate contents (Table III). Therefore, it is reasonable to mention that the mannan subfraction containing the largest amount of phosphate groups corresponds to the strongest antigen of the parent yeast. The structural analysis of five mannan subfractions by the controlled acetolysis technique afforded a finding that the density of the longest branching moiety corresponding to the antigenic determinant group in these subfractions was proportional to their phosphate content (Fig. 7). Contribution of phosphate groups to the serological activity as an antigenic determinant group is deniable, because inorganic phosphate was shown to be completely inactive to inhibit the homologous antigen-antibody system. These findings indicate that two types of microheterogeneities, relating to phosphate content and to density of the branching moieties, coexist in a bulk mannan, and the function of phosphate groups can therefore be assumed to act as a marker evoking structural complexity by elongation of the branching moieties, or terminating the extending branching moieties, or both.

In the present study, we revealed the existence of a microheterogeneity in three *C. albicans* strains of different serotypes, A, B, and C, similar to that observed in the bulk mannan of *S. cerevisiae* (5). From the results obtained in the previous (5) and present studies, the following findings can be obtained: 1) Microheterogeneity residing in the bulk mannans of *S. cerevisiae* and *C. albicans* strains can be regarded to correlate to their phosphate contents which dominate the densities of the branching moieties including the antigenic determinant groups. 2) The fact that the reactivities of the mannan subfractions of three *C. albicans* strains against anti-A-strain serum were strong and proportional to their phosphate contents indicates that the densities of the cross-reactive antigenic determinant groups, corresponding to a mannoheptaose moiety consisting solely of $\alpha 1 \rightarrow 2$ linked D-mannopyranosyl residues (29), are correlative with the phosphate content of the mannan subfractions of three *C. albicans* strains. 3) On the other hand, the finding that cross-reactivities between antigens and antisera of three *C. albicans* strains and *S. cerevisiae* were weak and not necessarily proportional to the phosphate contents of the antigens indicates that the densities of the cross-reactive antigenic determinant groups, corresponding to the branching moieties possessing non-reducing terminal $\alpha 1 \rightarrow 3$ linked D-mannopyranosyl residues (25, 26, 30) in the mannan subfractions of *C. albicans* are not closely correlate to their phosphate contents. 4) It will also be worthy to note that the mannan subfraction of each strain containing the largest amount of phosphate corresponds to the strongest antigen of the parent yeast, allowing an assumption that such a molecular species displaying antibody response in the strongest extent should locate the utmost surface of the cell wall of the parent yeast.

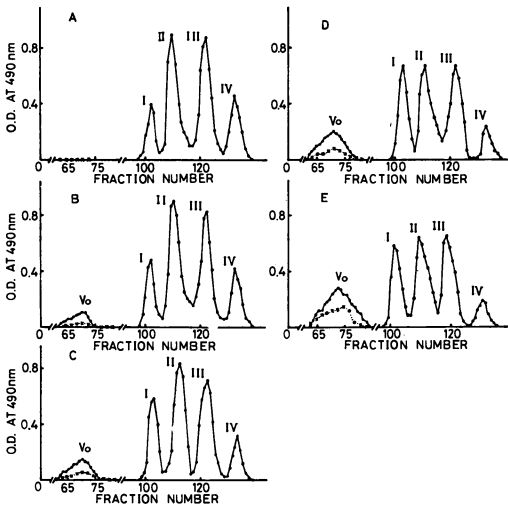
Further immunochemical investigation of yeast mannans relating to their anionic microheterogeneity can be expected to

Table III. Chemical composition of mannan subfractions (5)

Subfrac- tion	% Composition			Optical rotation, specific ^d	Man/P molar ratio ^e
	Carbo- hydrate ^a	Protein ^b	Phosphate ^c		
A	99	0.0	0.00	+80°	>1,000
B	96	0.1	0.23	+78°	178
C	98	0.1	0.57	+77°	73
D	94	0.1	0.99	+76°	41
E	93	0.3	2.09	+72°	19

- a Determined by the phenol-sulfuric acid method (13).
- b Determined by the Folin method of Lowry et al. (14).
- c Quantitated by the method of Ames and Dubin (15) as PO₃H₂.
- d c1.0, 1l.0, water.
- e The ratio of mannose residues to phosphate groups.

Journal of Bacteriology



Journal of Bacteriology

Figure 7. Acetolysis fingerprints of Subfractions A (A), B (B), C (C), D (D), and E (E) (5). Each acetolysate, obtained from 100 mg of the mannan subfractions, was applied onto a column (2 × 100 cm) of Bio-Gel P-2 and eluted with water (7 mL/hr). Samples (20 μL) of eluates corresponding to Fractions I, II, III, and IV were assayed for the amounts of carbohydrate, and 50 μL of Fraction Vo was used for the determination of carbohydrate and phosphorus in the void volume regions: (●) carbohydrate at 490 nm; (×) phosphorus at 820 nm; O.D., optical density.

provide much interesting information on the architecture of yeast cell walls and the function of phosphate groups existing in many polysaccharides.

Abstract

The mannans isolated from Candida albicans strains, NIH A-207 (serotype A), NIH B-792 (serotype B), and J-1012 (serotype C), were fractionated on a column of DEAE-Sephadex into five subfractions containing different amounts of phosphate.

Antibody-precipitating activities of the mannan subfractions of strains NIH A-207 and NIH B-792 were proportional to their phosphate content, while those of strain J-1012 did not show regularly proportional precipitin activity.

A similar tendency was also observed in the cross-reaction between the mannan subfractions of strains NIH A-207 and J-1012 and their heterologous antisera. The mannans of strain NIH B-792 showed lower cross-reactivities against antisera of strains NIH A-207 and J-1012, *i.e.*, only two subfractions containing larger amounts of phosphate were able to cross-react with these antisera.

The mannan subfractions of Saccharomyces cerevisiae, prepared by the similar chromatographic fractionation of the corresponding bulk mannan, showed no or very weak cross-reactivity with antisera of strains NIH A-207 and NIH B-792, while the mannan subfractions of strains NIH B-792 and J-1012 were weakly cross-reactive with anti-S. cerevisiae serum, affording almost identical amounts of precipitated antibody nitrogen regardless of the phosphate content of these mannan subfractions.

From the above findings, it is reasonable to conclude that mannan molecular species containing the largest amount of phosphate groups corresponds to the strongest antigen of the parent yeast, and that such a molecular species is assumed to occupy the utmost surface layer of the cell wall of the parent yeast.

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β -Glucans and β -Glucan Hydrolases in Plant Pathogenesis with Special Reference to Wilt-Inducing Toxins from *Phytophthora* Species

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The interaction between plants and microbial pathogens can be described in molecular, physiological, morphological and ecological terms. Some of the molecular events in host-pathogen interactions are summarised in Table I. Many of these events involve carbohydrates or carbohydrate-containing molecules. Soil-borne pathogens may be attracted chemotactically to the root surface; chemotactic agents known include ethanol, amino acids and sugars. Adhesion of the invading organism to the plant surface is in some cases through a carbohydrate ligand-lectin interaction. Penetration of the host surface cell walls and subsequent invasion of the underlying tissues may involve hydrolytic enzymes secreted by the pathogen which can degrade wall components such as polysaccharides and cutin. Invasion of the host may be facilitated by secretion of microbial toxins, some of which are polysaccharide or glycoprotein in nature. These toxins may cause symptoms such as wilting and cellular necrosis through interference with the host's metabolism.

Following penetration by micro-organisms a number of resistance mechanisms may be initiated by the plant. These resistance mechanisms appear to be triggered by the plant's ability to recognize the pathogen as "non-self", a characteristic also shown by plant mating systems and somatic cell interactions (35). Among the numerous resistance phenomena are localised metabolic responses or hypersensitive reactions which can lead to cell death, browning and collapse of cells around the site of infection. If the plant responds with sufficient speed, the invasion is not sustained and the interaction is said to be incompatible. One response is modification of the cell wall either to provide material which may encapsulate the micro-organism or to form a barrier through lignification or callose deposition. Another mechanism which may enable the plant to resist infection is the production of fungistatic compounds known as phytoalexins, which are elicited by both chemical and physical stimuli. Among the chemical elicitors are microbial polysaccharides and glycoproteins. A further defence may involve

TABLE I: Molecular Events in Host-pathogen Interactions

<u>Events in Infection and Symptom Induction</u>	<u>Agents</u>
Chemotactic attraction of soil organisms to root surfaces. (<u>1,2</u>)	Ethanol, amino acids, sugars.
Recognition and binding of micro-organisms to root surfaces. (<u>3,4,5,6,7</u>)	Carbohydrate ligands on surfaces of micro-organisms or roots (mucilage), carbohydrate-binding proteins on surfaces of micro-organisms or roots.
Penetration of root surfaces, intercellular or intracellular invasion. (<u>8,9,10,11</u>)	Microbial polysaccharide hydrolases specific for cellulose, xylan, pectin, 1,3- β -glucans, etc.
Injury or killing of host cells. Interference with primary metabolism, local necrosis, release of phytohormones, systemic effects, e.g. wilting. Membrane leakage and disruption. (<u>12,17</u>)	Low mol. wt. microbial toxins, microbial polysaccharides and glycoproteins, microbial enzymes.
Inhibition of host enzymes. (<u>18,19</u>)	Microbial inhibitors.
<u>Events in Host Resistance</u>	<u>Agents</u>
Immobilisation and encapsulation of infecting agents. (<u>20,21,22</u>)	Plant cell wall polysaccharides and lectins.
Hypersensitive reactions. (<u>23,24</u>)	Microbial polysaccharides.
Production of phytoalexins (post-infection, anti-microbial agents) formed <i>de novo</i> from remote precursors. (<u>3,23,25,26,27</u>)	Chemical and physical stimuli including microbial products, polysaccharides, glycoproteins and peptides.
Production of anti-microbial substances from pre-existing substrates. (<u>17</u>)	?
Increases in levels of glycan hydrolases and other enzymes. (<u>11,28</u>)	Microbial metabolites e.g. 3,6- β -glucans as inducers.
Production of ethylene. (<u>29,109,110</u>)	Microbial polysaccharides.
Deposition of callose, gums, phenolics and polyphenolics in and around infected cells. Increases in hydroxyproline-rich glycoproteins in cell walls (<u>30,31,32,33,34</u>)	?

enhancement of the level of the plant's own complement of polysaccharide hydrolases which are believed to be involved in depolymerising the cell walls of invading micro-organisms. Recently it has been shown by Kuć (36) that localised host reaction to the pathogen may confer resistance on other parts of the plant. This implies a transfer of information through the plant. The nature of the transmitted signal has not been resolved but in the related phenomenon of insect-induced resistance a wound hormone is produced at the site of interaction of insect and plant. This hormone is believed to be carbohydrate in nature and structurally related to plant cell walls (37).

It is the purpose of this paper to summarise the present state of information concerning those aspects of plant-microbial interactions in which β -glucans and β -glucan hydrolases are involved and to make special reference to their role in wilt-induction by *Phytophthora* species. It has not been possible within the framework of this paper to discuss the extent to which these phenomena may be generally representative of molecular events in plant pathogenesis, nor should it be inferred that all or any of the events involving β -glucans and β -glucan hydrolases are necessarily encountered in any particular plant-pathogen interaction.

2. The Role of β -Glucans and β -Glucan Hydrolases of Pathogens in Infection and Symptom Induction in Host Plants

2.1 Microbial β -Glucan Hydrolases and Lysis of Plant Cell Wall Polymers. Many soil and aerial pathogens gain access to host tissues by enzymic lysis of epidermal cell walls of roots, leaves, stems, etc.. Phytopathogenic organisms possess an array of inducible polysaccharide hydrolases capable of degrading the complex polysaccharides of the plant cell wall (8, 9, 10). The enzymes include hydrolases for 1,4- and 1,3- β -glucans (11, 38). The hydrolases are of general importance in the penetration and spread of the pathogen in plant tissues but are not determinants of varietal specificity (23, 25).

2.2 Wilt-inducing β -Glucans from Micro-organisms. Some pathogens are known to produce toxins which interfere with the host's metabolism and growth, and may eventually lead to the plant's death. The chemistry of microbial toxins and their effects on the plant have been reviewed (13, 14, 15, 16, 17). Wilting is a common symptom in many plant diseases and some toxins isolated from pathogens are able to induce wilting experimentally. Among these wilt-inducing toxins are high molecular-weight polysaccharides and glycoproteins. Table II lists those studied and the symptoms they induce.

Table II
SOME HIGH MOLECULAR WEIGHT
FUNGAL AND BACTERIAL
PHYTOTOXINS

COMPOUND	SOURCE	SYMPTOMS INDUCED
1,2- β -Glucan	<u>Agrobacterium tumefaciens</u>	Wilting (39)
1,3,1,6- β -Glucan (Mycolaminarin)	<u>Phytophthora</u> spp.	Wilting (40)
Culture filtrate	<u>P. cryptogea</u>	Veinal necrosis, collapse and de- hydration of lamina (41)
Glycopeptide (98% galactose, 0.4% protein)	<u>Erwinia amylovora</u>	Wilting (42)
Glycopeptide (Mainly 1,6- α - mannosyl substituted by 3-linked rhamnose)	<u>Ceratocystis ulmi</u>	Wilting (15)
Glycopeptide (Highly branched, mainly mannose and glucose)	<u>Corynebacterium sepedonicum</u>	Wilting (43, 44)
Glycopeptide (Mostly L-fucose, mannose, galactose, glucose)	<u>Corynebacterium insidiosum</u>	Wilting (45)

2.2.1 Wilt-inducing β -Glucans from *Phytophthora* Species.

Wilting is a symptom of many diseases induced by species of Oomycetes. For example *Phytophthora cinnamomi*, the causal organism of "die-back" in horticultural plants such as fruit trees as well as trees, shrubs and wildflowers of Australian native forests (46, 47), produces wilting and shrivelling of cotyledons and leaves of *Eucalyptus* seedlings (48, 49).

We have studied the wilt-inducing effect of macromolecular products of three species of *Phytophthora* chosen on the basis of their pathogenicity to the indicator plant *Eucalyptus sieberi* (50). These were the extremely pathogenic *P. cinnamomi*, the less pathogenic *P. cryptogea* and the non-pathogenic *P. nicotianae*.

Fungi were grown in batch culture in a liquid medium (51) and the ethanol-precipitable compounds collected from the culture filtrate after 5-7 days growth. The biological activity of this material on *E. sieberi* seedlings is shown in Figure 1. Similar results were obtained with seedlings of *E. cypellocarpa*, which is tolerant to *P. cinnamomi* (48).

Fractionation of the crude *P. cinnamomi* preparation was achieved on DEAE-cellulose as shown in Figure 2a. The unbound fraction I was much more active in wilt-induction on a carbohydrate basis than the bound fraction II. The unbound fraction (I) was rechromatographed on DEAE-cellulose (Figure 2b) to give a component rich in carbohydrate but containing some protein. Chromatography of this component on CM-cellulose (Figure 2c) yielded an unbound carbohydrate fraction containing 1.2% protein.

Gel filtration chromatography indicated that the carbohydrate from the *P. cinnamomi* cultures purified by ion-exchange chromatography was a polysaccharide which was polydisperse with respect to apparent molecular weight in the range 30,000 to 200,000 daltons.

Chromatography of *P. cryptogea* and *P. nicotianae* preparations on DEAE-cellulose gave elution profiles similar to that of *P. cinnamomi*. The unbound fractions were predominantly polysaccharide: the fraction from *P. nicotianae* contained 0.2% protein and that from *P. cryptogea* 0.4% protein. The wilt-inducing activity of the purified polysaccharides is shown in Figure 3.

The monosaccharide composition and structure of the *P. cinnamomi* polysaccharide was investigated by acid hydrolysis, methylation and specific enzymic degradation (50). Each of the polysaccharides was composed predominantly of glucose (see Table III).

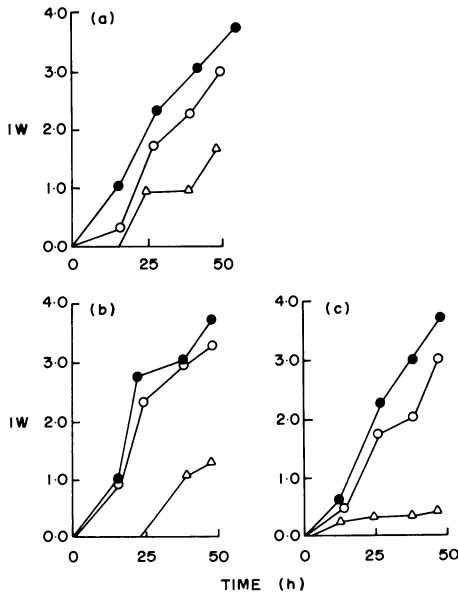


Figure 1. *E. sieberi* wilting bio-assay of unfractionated ethanol-insoluble polymers from the culture filtrates of (a) *P. cinnamomi*, (b) *cryptogea*, and (c) *P. nicotianae*; polymer concentration: (●) 500 µg/mL; (○) 50 µg/mL; (△) 5 µg/mL. The assessment of wilting was performed by introducing substances to be tested through the cut ends of the main rootlets of 1–2 month old *E. sieberi* seedlings immersed in solutions of test material. Wilt assays were conducted under controlled conditions in the presence of nystatin (50 units/mL) and tetracycline (50 µg/mL). The degree of wilting was assessed at regular intervals during 40-hr tests. An arbitrary score of wilting severity (index of wilting, IW) was used to record the seedlings' response: 1, slight wilting; 2, pronounced wilting; 3, severe wilting; and 4, death. Each compound was tested in triplicate and the IW was the mean score.

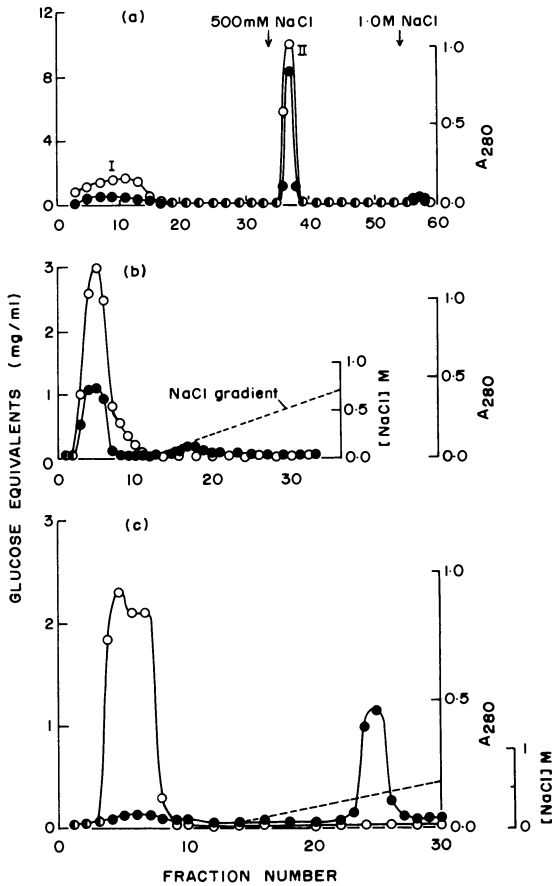


Figure 2. Fractionation of *Phytophthora cinnamomi* extracellular polymers:

(○) glucose equivalents; (●) absorbance at 280 nm.

(a) Stepwise elution of components of the ethanol-insoluble polymers from culture filtrates of *P. cinnamomi* on DEAE-cellulose. The column was loaded with a preparation containing 280 mg carbohydrate and 218 mg protein in 104 mL of 10mM tris-acetate (pH 8) and washed successively in 250 mL of 10mM tris-acetate (pH 8), 200 mL of same buffer 0.5m NaCl and 100 mL of the starting buffer 1.0M NaCl. 9-mL fractions were collected. (b) Gradient elution chromatography of Peak I (Figure 2a) on DEAE-cellulose. Fractions 1-22 were pooled, concentrated on a rotary film evaporator under reduced pressure at 40°C, and dialyzed against 10mM tris-acetate (pH 8). The concentrated fractions (33 mL) containing 109 mg carbohydrate and 75 mg protein were loaded onto the column that was washed with 40 mL starting buffer followed by a 300-mL gradient (0-0.75M NaCl in starting buffer). (c) Gradient elution chromatography of Peak I from DEAE-cellulose. Fractions 2-11 (Figure 2b) were pooled, concentrated, and dialyzed against distilled water and then against 10mM acetic acid-sodium acetate buffer, pH 5.0. The concentrated fraction (32 mL) containing 88 mg carbohydrate and 53 mg protein was loaded onto a column and washed with 100 mL starting buffer followed by a 200-mL gradient (0-0.75M NaCl in starting buffer).

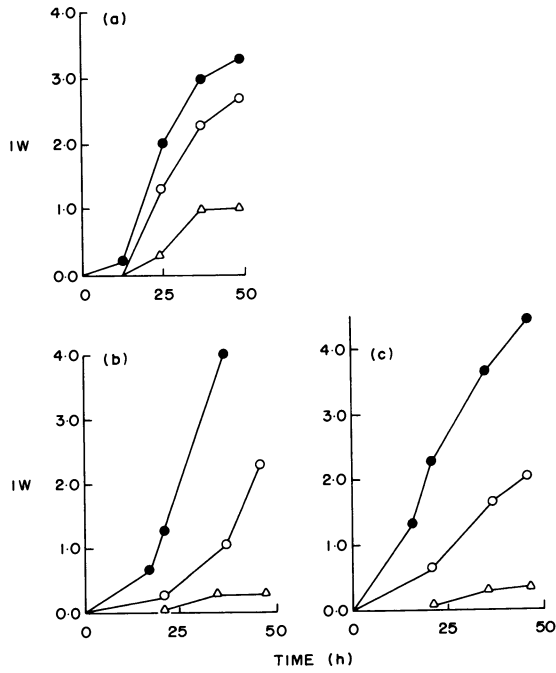


Figure 3. *E. seiberi* wilting bio-assay of purified *Phytophthora* polysaccharides; polymer concentration: (●) 500 µg/mL; (○) 50 µg/mL; (△) 5 µg/mL. (a) *P. cinnamomi* polysaccharide from successive DEAE-cellulose and CM-cellulose chromatography. (b) *P. cryptogea* polysaccharide from DEAE-cellulose chromatography. (c) *P. nicotianae* polysaccharide from DEAE-cellulose chromatography.

TABLE III. Monosaccharide Composition of the Purified Polysaccharides from *Phytophthora* Culture Filtrates (50)

	Arabinose %	Mannose %	Glucose %
<i>P. cinnamomi</i>	-	4	96
<i>P. cryptogea</i>	trace	trace	100
<i>P. nicotianae</i>	trace	trace	100

Methylation analysis indicated that there were 3-linked glucosyl residues in the molecule, as well as glucosyl residues substituted at both 3- and 6-positions (see Table IV).

TABLE IV. Glucosidic Linkage Composition of the *Phytophthora* Polysaccharides as Determined by g.l.c./m.s. of their Partially Methylated Alditol Acetates (50)

Deduced Glucosyl Component	T*	Molar Percentage		
		<i>P. cinnamomi</i>	<i>P. cryptogea</i>	<i>P. nicotianae</i>
Terminal glucosyl	1.00	27	34	34
1,3-linked glucosyl	1.87	44	23	26
1,6-linked glucosyl	2.30	7	6	8
1,3,6-linked glucosyl	4.55	22	37	32

* Retention times relative to 1,5-di-O-acetyl-2,3,4,6-tetramethyl-D-glucitol.

The polysaccharides were β -linked-glucans as judged by their susceptibility to hydrolysis by specific β -glucan hydrolases and from their infra-red spectra. Hydrolysis of the *P. cinnamomi* polysaccharide by a series of β -glucan hydrolases with defined specificity gave further structural information. The *Euglena* 1,3- β -glucan exo-hydrolase (EC 3.2.1.58) (52) only partially degraded the polysaccharide, releasing mainly glucose and smaller amounts of a compound with the R_{glc} of gentiobiose; while the *Rhizopus arrhizus* 1,3- β -glucan endo-hydrolase (EC 3.2.1.6) (53) produced more complete degradation, releasing glucose and a series of oligo-glucosides. The *Bacillus subtilis* 1,3:1,4- β -glucan endo-hydrolase (EC 3.2.1.73) (54) did not degrade the polysaccharide. Hydrolysis by the exo-hydrolase suggested that some 1,3-linked portions of the polysaccharide were located at terminal non-reducing ends. In addition, the extensive cleavage by the *Rhizopus* endo-hydrolase indicated that there are susceptible 1,3-linkages throughout the polymer. The methylation and enzymic data do not distinguish between a molecule having a 1,3-linked backbone and branch points at C-6 and one having a 1,6-linked backbone and branching at C-3.

When the enzyme-treated preparations were tested for biological activity in the wilt induction assay it was found that the *Rhizopus* enzyme-treated preparation had lost its biological activity (Figure 4). The *Bacillus* enzyme-treated preparation retained its activity as might be expected from the inability of this enzyme to depolymerise the preparation. After treatment with the *Euglena* exo-hydrolase, which caused only partial degradation, the polymer retained most of its wilt-inducing activity.

2.2.2 Relationship of Wilt-inducing 3,6- β -Glucans to Hyphal Wall Polysaccharides. The wilt-inducing 3,6- β -glucans are comparable in structure to soluble 3,6- β -glucan components of hyphal walls and culture filtrates of other species of Oomycetes. Related polysaccharides have also been found in higher fungi (see Table V). Typically they have a linear 1,3- β -glucan backbone and are substituted by lateral side chains consisting of single, glucosyl residues linked through C-6 to main chain residues. The methylation and enzymic data for the *P. cinnamomi* polysaccharide are consistent with such a structure although other structures are also compatible with the data.

The hyphae of *Phytophthora parasitica* have been shown to have bilayered cell walls, the innermost layer consisting of cellulose microfibrils in a matrix of 3,6- β -glucan and protein and an outermost layer of water-soluble 3,6- β -glucan (71). In *Schizophyllum commune* a filamentous material containing 3,6- β -glucan has been shown to cover the surface of the hyphae (72). It is presumably this layer which is shed into the culture medium during cultivation of these fungi in shaken flasks. *Phytophthora* spp. and other species of Oomycetes also have cytoplasmic 3,6- β -glucans (mycolaminarins) and their phosphate esters (73). These polysaccharides also induce wilting in avocado (*Persea indica*) and other plants (40) (see Table II).

2.2.3 Wilting Response Induced by Various Polysaccharides in *E. sieberi* Seedlings. We have compared the wilt-inducing capacities of the *Phytophthora* preparations and a number of polysaccharides of known structure (50). The results are shown in Table VI. At comparable concentrations the degree and type of wilting response induced by neutral β -linked polysaccharides was indistinguishable from that induced by the *Phytophthora* polysaccharide preparations. The charged polysaccharides, CM-pachyman and hyaluronic acid, had only slight activity in the wilting assay. Of the two α -glucans tested, soluble starch was without effect and a dextran, 1,6- α -glucan, caused severe wilting of the cotyledons only, a response quite different from that found with the β -linked polysaccharides. Differences between the wilting response given by starch, dextran and pectin and that given by β -glucans have been observed in other studies (40) (see Table VI).

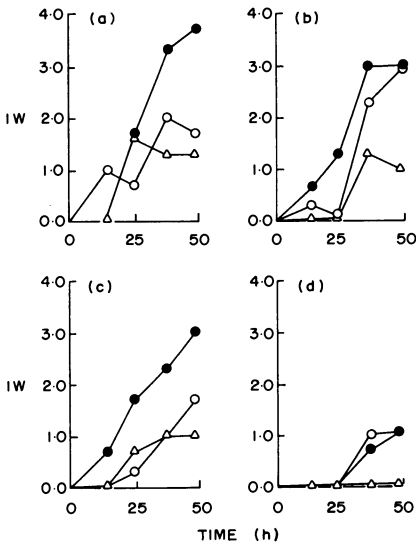


Figure 4. *E. sieberi* wilting bio-assay of the purified and enzymically treated *P. cinnamomi* polysaccharide; (●) 500 µg/mL; (○) 50 µg/mL; (△) 5 g/mL; (a) untreated control; (b) after treatment with *B. subtilis* 1,3,1,4-β-glucan endohydrolase; (c) after treatment with *E. gracilis* 1,3-β-glucan exohydrolase; (d) after treatment with *R. arrhizus* 1,3-β-glucan endohydrolase.

TABLE V: Some Water-soluble 1,3- β -Glucans from Fungi

Structure	Other Monomers or Substituents	Term	Structural Linkages (% or ratio)			References
			1,3-	1,6-	1,3,6- 1,4-	
<i>DEUTEROMYCOTINA</i>						
<i>Microsporium quinckeanum</i> (Mycelium)	Term Man	10	22	58	8	(55, 56)
<i>Trichophyton granulorum</i>		21	25	35	19	(56)
<i>OOMYCETES</i>						
<i>Phytophthora megasperma</i> (Mycelium)		13	56	-	13	(57)
(Culture filtrate)	Large extra-cellular fraction	24	32	18	27	(57)
	Small extra-cellular fraction	8	72	12	9	(57)
<i>Pythium debaryanum</i> (Culture filtrate)		10	18	3	10	(58)
<i>ASCOMYCOTINA</i>						
<i>Saccharomyces cerevisiae</i> (Autolysate)	Term Ara 1,5 Ara	21	16	43	15	1 (59)
(Hot water extract)		16	5	65	14	(60)
<i>Monilinia fructicola</i> (Culture filtrate)		1.1	2	-	-	(1,3:1,4) (61) 10

BASIDIOMYCOTINA

<i>Schizophyllum commune</i> (Culture filtrate)	SCB	25	49	-	25	1	(62, 65)
<i>Ustilago zeae</i>	B	1	1.5	4.7	0.3		(63, 64)
<i>Polyporus fomentarius</i> (Basidiocarp)	SCB	9	11	26	19		(66)
<i>Polyporus ignarius</i> (Basidiocarp)	SCB	9	13	26	21		(66)
<i>Fomes annosus</i> (Basidiocarp)	B	20	24	36	20		(67)
<i>Lentinus edodes</i> (Degraded lentinan)	SCB	5	17	1	5		(68, 69)
<i>Uromyces phaseoli</i> (Germ tube cell walls)	SCB	1	13	1	1		(70)

Abbreviations : Man, mannose; Ara, arabinose; GlcA, glucuronic acid;
 B, branched; SCB, side chain branched; Term, terminal.

Table VI
WILTING RESPONSE OF *E. SIEBERI* SEEDLINGS
TO VARIOUS POLYSACCHARIDES

POLYSACCHARIDE	MAIN LINKAGES	STRUCTURE	INDEX OF [¶] WILTING (<i>E. SIEBERI</i>) (50)	WILT INDUCTION (<i>PERSEA INDICA</i>) [†] (40)
<u>P. CINNAMOMI</u>	3-, 6- and 3,6-β-	SCB	3.0	-
LAMINARIN (from <u>Laminaria hyperborea</u>)	3- and 3,6-β-	SCB	3.0	+ (1 mg/ml)
MYCOLAMINARIN (phosphate)	3, 6- and 3,6-β-	SCB	ND	+ (10μg/ml and 50μg/ml)
LAMINARIN (from <u>Eisenia bicyclis</u>)	3- and 6-β-	L	2.3	ND
CM-PACHYMAN	3-β-	L	1.0	ND
BARLEY GLUCAN	3- and 4-β-	L	2.7	ND
HYALURONIC ACID	3- and 4-β-	L	0.3	ND
LUTEAN	6-β-	L	2.3	ND
PUSTULAN	6-β-	L	2.3	+ (1mg/ml)
STARCH (soluble)	4- and 4,6-α-	B	0	+ ^{††}
DEXTRAN	6-α-	L	3.0 ^{**}	+ ^{††}
CM-CELLULOSE	4-β-	L	ND	+ (1mg/ml)
PECTIN	4-α-GALUA	L	ND	+ (1mg/ml) ^{***}

¶For explanation of index of wilting see caption to Figure 1

* [Polysaccharide] 500μg/ml

† Also tested on glycine max L and Theobroma cacao L

**Only cotyledons affected

***Only leaves affected

††Caused marginal necrosis in leaves

L Linear

SCB Side chain branching

B Branched

ND Not determined

2.2.4 Mechanism of Wilt-induction by β -Glucans. In general, plant wilting may be induced by toxins in one of several ways - by prevention of water uptake, by increase in water loss through impairment of stomatal function, by prevention of water transport within the plant or by alteration of membrane permeability (12, 16). Light microscopy of *E. sieberi* seedlings in which wilting had been induced by *P. cinnamomi* 3,6- β -glucan failed to reveal any physical blockage such as tyloses or vascular gels in the root or stem vessels (50) nor were major blockages due to mycelium or tyloses seen in the vascular tissue of seedlings directly inoculated with *P. cinnamomi* (74). However, it has been suggested (12) that high molecular weight polysaccharides might block the petiolar veinlets and "the ultra-filters of the pit membrane". Ultrastructural examination would be needed to test this possibility.

Low molecular weight toxins from *Fusicoccum amygdali* (75) and *Helminthosporium maydis* (76) are known to affect stomatal function through their action on guard cells but no polymeric toxins have been shown to act at this site.

A primary effect of wilt-inducing toxins of low molecular weight may be through plasma membrane damage, resulting in increased water permeability (14, 16). The macromolecular wilt-inducing toxins may also interact with membrane components and have similar effects on water permeability. Thus glycopeptides from culture filtrates of *Fulvia fulva* (syn. *Cladosporium fulvum*) cause leakage of electrolytes from isolated tomato mesophyll cells. It is suggested that the glycopeptides may bind reversibly to plasma membranes (77).

Although there is no direct evidence for alteration of membrane permeability by *Phytophthora* cell wall β -glucans, there is evidence that low molecular weight (<3400 daltons) water-soluble polysaccharides from enzymically degraded *Phytophthora infestans* cell walls can interact with potato protoplasts (78). Protoplasts were agglutinated by 21 μ g/ml polysaccharide and at higher concentrations were killed. Although no other polysaccharide tested caused agglutination, the 3,6- β -glucan, laminarin, inhibited this reaction but could not reverse it once it had occurred. The nature of the receptor sites for the degraded *P. infestans* polysaccharide is not known. However plant protoplasts do bind β -glucosyl Yariv antigens, indicating that the protoplast membrane has the capacity to interact with phenyl β -glucoside ligands (79, 80).

No clear-cut description of the molecular basis of initial events in the experimental, polysaccharide-induced wilting response can be given at present and its relationship to wilting induced by pathogens *in vivo* has not been established.

3. The Role of β -Glucans and β -Glucan Hydrolases in Host Resistance

Invasion by micro-organisms may induce physiological

responses in the plant which limit colonization. The metabolic changes underlying these responses are triggered by microbial products which include polysaccharides and glycoproteins. The hypersensitive reaction, production of phytoalexins, 1,3- β -glucan hydrolases and 1,3- β -glucans are such host responses.

3.1 β -Glucans and the Induction of the Hypersensitive Reaction. The hypersensitive reaction associated with incompatible infections is characterised by localised browning and death of infected cells. This reaction has been reproduced in tuber tissue from resistant and susceptible potato varieties by a heat-stable component isolated from *P. infestans* mycelium (24). The active agent is thought to be related to the glucan or glucose-containing glycoprotein reported (81) from *P. infestans* mycelial extracts. The ability of laminaribiose and methyl- β -D-glucopyranoside but not cellobiose, gentiobiose, lactose, α -glucosides, α -mannoside or tri-N-acetyl glucosamine, to inhibit the hypersensitive reaction led to the conclusion that terminal 1,3- β -linked glucose residues were determinants in the interaction. There is preliminary evidence (82) that a β -glucose-specific potato lectin is the receptor for the hypersensitivity-inducing polysaccharides. This lectin is not the same as the receptor for the β -glucosyl Yariv antigen shown to be present on many protoplast membranes (79, 80). An apparently contradictory observation that a low molecular weight 1,3- β -glucan (D.P. 18-23) from the mycelium of *P. infestans* inhibits hypersensitivity (83) may be explained in terms of competition for the glucose-specific lectin.

3.2 β -Glucan Elicitors of Phytoalexin Production. Phytoalexins are post-infection toxins formed *de novo* by the host. These compounds, which are diverse in chemical structure, are fungistatic but generally not phytotoxic (3, 23, 25, 26, 27).

Phytoalexin formation can be induced by both physical and chemical stimuli. Thus freezing, bruising or scratching as well as fungal, bacterial and viral infection may increase phytoalexin levels (26). Chemicals such as heavy metal ions and a wide range of organic molecules including the microbial polysaccharides, glycoproteins and polypeptides listed in Table VII, also cause phytoalexin accumulation. Increases in phytoalexin levels may result from an increase in phytoalexin synthesis elicited for example by a fungal polysaccharide or glycoprotein, or by inhibition of the phytoalexin degrading system, for example by metal ions (91).

Among the polysaccharide elicitors are 3,6- β -glucans from *Phytophthora megasperma* (84, 85) and yeast cell walls (59) and the algal glucan, laminarin (84), which have structures comparable to those described for the wilt-inducing *P. cinnamomi*, *P. cryptogea* and *P. nicotianae* polysaccharides (see Table VII). The *P. megasperma* 3,6- β -glucan elicitor was depolymerised by the

Table VII
POLYSACCHARIDES AND GLYCOPROTEINS
WHICH ELICIT PHYTOALEXIN PRODUCTION

SOURCE OF ELICITOR	STRUCTURE OF ELICITOR	PHYTOALEXIN ASSAY SYSTEM	REFERENCE
<u>Phytophthora megasperma</u> var. <u>sojiae</u> (culture filtrate and cell wall)	3,6- β -glucan	Glyceollin (<u>Glycine max</u>)	27,84,85
<u>Phytophthora infestans</u> (mycelial extracts)	Unidentified glucans, gluco- and manno-glycoproteins	Rishitin (<u>Solanum tuberosum</u>)	80,86
<u>Colletotrichum lindemuthianum</u> (culture filtrate)	3,4- β -glucan traces of man and gal	Phaseollin (<u>Phaseolus vulgaris</u>)	18,87
<u>Colletotrichum trifolii</u> and <u>C. destructivum</u> (culture filtrates)	Neutral polysaccharides -glc, gal, man and rha	Phaseollin (<u>Phaseolus vulgaris</u>)	88
<u>Saccharomyces cerevisiae</u> (water soluble component from autolysate)	3,6- β -glucan	Glyceollin (<u>Glycine max</u>)	59
<u>Rhizopus stolonifera</u> (culture filtrate)	Glycoprotein (33% glc equivs. 67% protein)	Casbene (<u>Ricinus communis</u>)	89
<u>Laminaria hyperborea</u> (intracellular)	Laminarin (3,6- β -glucan)	Glyceollin (<u>Glycine max</u>)	84
<u>Aspergillus niger</u> (cell walls)	Nigeran (3,4- α -glucan)	Glyceollin (<u>Glycine max</u>)	90

Euglena 1,3- β -glucan exo-hydrolase but the enzyme-resistant residue was still active in eliciting the response (57). The enzyme-resistant residue is an oligosaccharide (mol. wt. 10,000) with 3-linked, 3,6-linked and terminal glucosyl residues. It is highly-branched with over half the glucosyl residues in the back-bone substituted with glucosyl side chains, compared with one in every eight in the undigested elicitor (27). Acid hydrolysis of *P. megasperma* cell walls produced a series of glucose oligosaccharides from which a biologically active nona-saccharide was isolated. This has been tentatively suggested to be a 1,6- β -oligoglucoside bearing two laminaribiosyl residues linked to the backbone at C-3 on adjacent residues (27). The *P. megasperma* elicitor resembles the *P. cinnamomi* wilt-inducing 3,6- β -glucan (50) in both linkage composition and in its retention of biological activity after *Euglena* exo-hydrolase action. The possibility that the same molecular determinants evoke the two different responses is suggested but further comparative structural information is required.

The observations that the phytoalexins phaseollin (92) and pisastin (93) have injurious effects on plant plasma membranes suggests that wilting observed in some plant diseases could be an indirect result of phytoalexin production. Whether this occurs in the case of β -glucan-induced wilting of *Eucalyptus* species is not known since phytoalexins have not been reported from eucalypts. However the dose of polysaccharide needed to produce the maximum phytoalexin response in the soya bean hypocotyl test systems (84) is an order of magnitude lower than that required for wilt-induction in the seedling assays (50).

Little is known about the molecular events which initiate the biosynthesis of phytoalexins but a membrane-elicitor interaction seems likely (26).

3.3 Host β -Glucan Hydrolase Induction. A further response to microbial invasion of host tissues involves an increase in activity of certain host enzymes, especially the glycan hydrolases which are potentially able to depolymerise microbial polysaccharides.

Lysis of fungal hyphae within plant tissues has been observed for a number of different host-symbiont combinations (11). Although lysis could result either by autolysis of the fungus or through the action of host enzymes, the weight of evidence (11) favours the latter. The plant enzymes include the ubiquitous 1,3- β -glucan hydrolases (94, 95, 96, 97, 98), chitinase (96, 99) and lysozyme (100, 101, 102). Direct evidence for *in vivo* activity of 1,3- β -glucan hydrolases is given by the observation (40) that ^{14}C -mycolaminarin introduced into avocados (*Persea indica*) is metabolised to both higher and lower molecular weight polymers which become distributed throughout the plant.

Infection of *Nicotiana glutinosa* plants by tobacco mosaic virus, tomato spotted wilt virus and broad bean wilt virus

evoked an increase in 1,3- β -glucan hydrolase activity (103). Similar, though smaller, responses have been found in certain other viral infections of indicator plants (104). Infection of tomato plants by pathogens with different cell wall compositions e.g. *P. cryptogea* (cellulose and 3,6- β -glucan), *Verticillium albo-atrum* (chitin and 3,6- β -glucan) and the bacterium *Pseudomonas solanacearum* (murein and lipopolysaccharide-protein) leads to an increase in activity of plant 1,3- β -glucan hydrolase and chitinase (99). Similarly musk melon (*Cucumis melonis*) inoculated with *Fusarium oxysporum* (chitin and 3,6- β -glucan) showed an increase in 1,3- β -glucan hydrolase levels (28). After 13 days the activity of the enzyme was twice as great in the resistant compared with the susceptible variety. Extracts of plant tissue showed comparable changes in their ability to hydrolyse a *Fusarium* cell wall preparation.

The increases in activities of both 1,3- β -glucan hydrolases and chitinase in response to infection appears to be part of a general plant resistance mechanism. The nature of the inducers of the enzymes is not clear but 3,6- β -glucan components of the cell walls of the invading fungi may be involved. This is supported by the observation that when melon seedlings were placed in a solution (0.08%) of the 3,6- β -glucan laminarin for 18h at 15°C there was a subsequent increase in the level of 1,3- β -glucan hydrolase in the test plants (28). Furthermore, pretreatment in this way depressed *Fusarium* disease symptoms in plants up to 21d after inoculation, whereas all controls wilted within 14d. The implied ability of laminarin or its metabolic products to induce a 1,3- β -glucan hydrolase in higher plants, and thereby a resistance to fungal attack, is an observation of theoretical and practical importance.

Ethylene treatment of plants increases levels of 1,3- β -glucan hydrolase and chitinase in bean leaves (105, 106) but not in pea epicotyls (97). The effect of increased hydrolase activities is correlated with retardation in the rate of development of bean rust (*Uromyces phaseoli*) infections (106). Pretreatment of tomato plants with ethylene (5.0 ppm, 48h) induced resistance to *Verticillium* and lysis of the mycelium was seen in roots and stem internodes (107).

The phytohormone ethylene is thus a possible linking factor between the infection process and 1,3- β -glucan hydrolase induction. Ethylene production is dependent on a "highly structured and delicately poised enzyme complex" which is probably located in the plasma membrane (108). Fungal and viral infections are associated with an increased production of ethylene (109, 110) although the mechanism of activation of the ethylene synthesizing system is not clear. However, the recent observation that ethylene formation is induced in bean hypocotyl segments by preparations isolated from germ tube cell walls of *Uromyces phaseoli* (29) suggests the possibility of activation of the membrane-bound ethylene generating system by fungal cell

wall polysaccharides. 1,3- β -Glucans are known to be present in *Uromyces phaseoli* cell walls (70) (see Table V).

It is not easy to rationalise the conflicting possibilities which may arise from the action of plant 1,3- β -glucan hydrolases during microbial infection. In assessing the biological role of these hydrolases it will be important to understand not only the mechanism of their induction and their mode of action on β -glucan substrates but also their sub-cellular location and their *in vivo* rates of action in relation to the infection process. These factors will ultimately determine the concentration and size of β -glucans and their hydrolysis products available at particular tissue and cell sites for biological activity.

Apart from contributing to the host's defence by lysing and so killing the hyphae of the invading pathogen, 1,3- β -glucan endo- and exo-hydrolases of the plant could exert their effects in other ways. It has been shown that soya bean 1,3- β -glucan exo-hydrolase, together with uncharacterised enzymes of the cell wall, can depolymerise and inactivate the 3,6- β -glucan elicitors of phytoalexin formation (98) and in other situations they may also inactivate the 3,6- β -glucan inducers of the hypersensitive response. These effects would appear to be counter-productive from the point of view of the plant's resistance system. However, depolymerisation to an inactive form might have the advantage of localising the phytoalexin response (98) and other biological effects such as the hypersensitive reaction to the immediate region of the attack. Enzymic depolymerisation of wilt-inducing 3,6- β -glucans would presumably be a useful part of the plant's resistance mechanism.

On the other hand the action of the 1,3- β -glucan hydrolases, especially the exo-hydrolases, would be to reduce the size of the 3,6- β -glucans to a point where they might more readily diffuse through the plant but still retain their biological activity (27, 50). Systemic distribution of the β -glucan or its active low mol. wt. derivatives through the host plant could be of significance in initiation of general wilting and induction of 1,3- β -glucan hydrolases in parts of the plant removed from the site of infection.

The possibility of modulation of the activity of the plant 1,3- β -glucan hydrolases by the pathogen is raised by the observation that the *Phaseolus* 1,3- β -glucan endo-hydrolase is inhibited by proteins from the fungal pathogen *Colletotrichum lindemuthianum* (18).

3.4 Callose Formation. Deposits or papillae between host plasma membranes and cell walls are seen in plant tissues infected by fungi, nematodes and viruses (30). These deposits often react positively with the aniline blue fluorochrome and are believed to be composed of callose, a polysaccharide complex which may include β -glucans containing 1,3- and 1,4-linkages (111).

The deposits may function in plugging or sealing the wound caused by injury due to the pathogen and may restrict the loss of molecules and ions and the movement of toxic substances into the tissues. Little is known about induction of the synthesis of the callose although membrane-bound plant 1,3- β -glucan synthetases have been described (112). Callose formation is induced by various chemical and physical stimuli (113, 114, 115) and there is evidence that membrane disruption (116, 117), such as may result from microbial infection, can initiate callose deposition. Whether the stimulus is provided by a specific binding to plasma membranes of microbial metabolites, such as 3,6- β -glucans is not known, although it is significant that ethylene can also induce callose formation (116).

3.5 β -Glucans and Acquired Resistance. A low molecular weight 1,3- β -glucan, resembling mycolaminarin, extracted from the leaves and stems of potato (*Solanum tuberosum*) plants infected with *P. infestans* (118) has been shown to inhibit lesion formation due to potato virus X in potato and several other viruses in tobacco (119, 120). The molecular basis of the acquired resistance to the viruses is not known but in view of the recent observations of Kuć (36) on acquired resistance to fungal infection its elucidation will be of great significance.

4. Conclusion

The data summarised above have focused on the involvement of 3,6- β -glucans from the walls and cytoplasm of fungal hyphae in evoking a number of physiological changes which form part of the host-pathogen interaction.

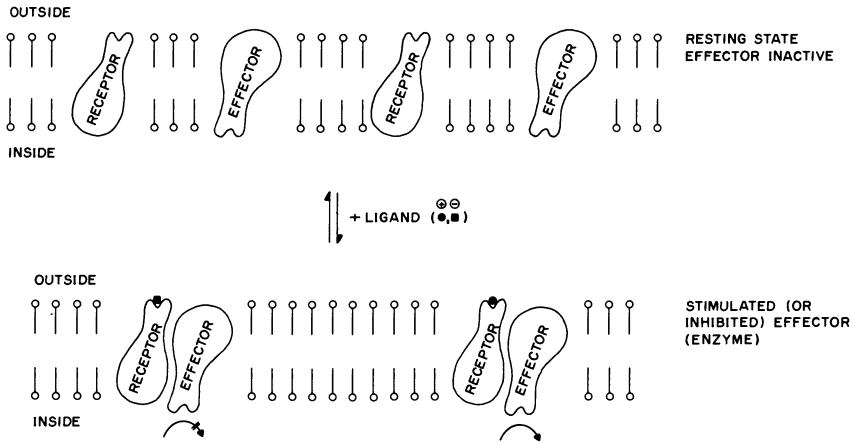
Wilting, hypersensitivity and accumulation of phytoalexins and 1,3- β -glucan hydrolases can be induced experimentally by administration of 3,6- β -glucans to the plant. It is likely that such glucans contribute to similar host reactions associated with fungal infection, but in most cases direct demonstration of their involvement is lacking. None of the effects of 3,6- β -glucans appear to be related to the pathogenicity of microbial races or species to host plants but appear to be secondary to pathogenicity determining events or mechanisms.

A feature of the biological activity of the 3,6- β -glucans is their apparent interaction with plant cells through recognition by specific carbohydrate-binding molecules, or lectins, located in the plasma membrane (78, 82). Presumably binding could occur either following access of micro-organisms to the protoplast surface during the initial invasive phases of infection, or following depolymerization of fungal 3,6- β -glucans by host hydrolases to biologically active fragments which are small enough to diffuse through cell walls. How binding of

3,6- β -glucans to membranes could initiate intracellular events such as phytoalexin and 1,3- β -glucan hydrolase accumulation, or induction of hypersensitive reactions, has not been demonstrated. Specific structural features are required for β -glucans to be biologically active, but β -glucans are not the only initiators of host responses: the bean phytoalexin, phaseollin, is elicited not only by 3,4- β -glucans from *Colletotrichum spp.* (18, 87, 88) but also by the polypeptide, monilicolin A, from *Monilinia fructicola* (121) and by physical stimuli (23); wilting in avocados is induced for example by both 3,6- β -glucans and pectins (40). Any model for the interactions must take these observations into account.

The plasma membrane offers a logical site for the receipt and translation of diverse external physical and chemical stimuli including those originating from a pathogen. General concepts of membrane transduction of information have been elaborated, largely on the basis of experimental information from mammalian systems on the regulation of lymphocyte activation, and the control of cellular metabolism by hormone receptors on adipocytes and hepatocytes (122, 123, 124). The transduction system is envisaged as a series of recognition elements (receptors) which bind soluble molecules or cell surface components. The receptors receive signals and initiate the cellular response by interaction with the effector which produces the intracellular messenger. In the case of the adipocyte, the adenylyl cyclase effector catalyses the synthesis of the intracellular messenger, cyclic-3',5'-AMP. In this way cell surface signals are transmitted to the cytoplasm. The receptors are conceived as regulatory molecules capable of exercising influence over alternative conformations of a particular effector molecule through physical interactions. In the case of B and T lymphocytes the receptors are believed to be membrane-bound immunoglobulins (IgGs). The ligand-receptor complex stabilises the conformation of the effector in a form which permits its biological activity to be expressed. This concept has been extended in the mobile receptor hypothesis of Cuatrecasas (124) (Figure 5) in which the receptor and the effector are physically separate and discrete structures, but after binding of the ligand, which provides the external signal, combine to give the active effector.

These concepts of information transduction by membranes may be used to integrate the scattered observations on the biological effects of interactions of 3,6- β -glucans with plant cells. The observation that chemically different groups of compounds may elicit the production of the same response such as wilting or formation of a phytoalexin, may be explained in terms of multiple receptors coupled to a single effector system which initiates the intracellular response. This would be analogous to the adenylyl cyclase of the adipocyte which is stimulated by seven different hormones and inhibited by at least three others (124). In the



Annual Review of Biochemistry

Figure 5. General two-step fluidity hypothesis for the mechanism of modulation of effectors in cell membranes by ligands. The central feature is that the receptors and the effectors are discrete and separate structures that acquire specificity and affinity for complex formation only after the receptor has been occupied by the ligand. These structures can combine after binding of the ligand because of the fluidity of cell membranes. The ligand binding sites of the receptor are on the external face, exposed to the aqueous medium, and the active site of the effector (enzyme) is facing inward toward the cytoplasm of the cell. After Cuatrecasas (124).

adipocyte system, independent membrane functions e.g. ion transport, adenylate cyclase and ATPase may be activated by a single ligand acting through a receptor which can complex with and modify separate effectors. In the plant system this mechanism might apply to the systems controlling, for example, phytoalexin production and ion permeability.

The extension of the membrane transduction concept to plant-pathogen interaction requires the participation of an intracellular messenger. The involvement of cyclic-3',5'-AMP as an intracellular messenger in plants is unproven, although possible elements of a cyclic-3',5'-AMP regulatory system are established (125, 126). Several observations suggest that ethylene may be considered in this role. Thus ethylene is generated following wounding of tissues in infection (109, 110) and has been implicated in phytoalexin production (26), enhancement of hydrolase activity (105, 106, 107) and callose formation (116). If ethylene acts as the intracellular messenger, the membrane-bound ethylene generating system would be the effector which is coupled, for example, to a 3,6- β -glucan receptor. The involvement of ethylene is not likely to be simple and would probably be related to the level of other hormones e.g. auxins, cytokinins, etc. in the tissues (108).

While the current state of knowledge does not permit much more than speculation concerning the molecular events at plant cell membranes in host-pathogen interactions, the application of the concepts derived from the mammalian membrane receptor-effector systems provides a unifying model which allows rationalisation of the available data and may suggest useful experimental approaches.

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Studies on the Synthesis, Organization, and Conformation of Crystalline Nigeran in Hyphal Walls

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The structure, function and assembly of fungal cell wall polysaccharides are important areas of research for several reasons. They serve as useful models for understanding the structural organization of similar molecules in plant cell walls. Fungi are responsible for the great majority of plant diseases and certain of their wall polysaccharides have been implicated in processes related to disease resistance (i.e., as elicitors of phytoalexin production⁽¹⁾). Well characterized wall polysaccharides have been used to monitor developmental processes, two examples being regulation of chitin synthesis during yeast bud scar formation⁽²⁾ and the interrelationship of (1→3)- α -glucan synthesis and fruiting body formation in *Aspergillus*.⁽³⁾ Wall polysaccharides also serve as taxonomic aids^(4,5) in classification of fungi. This report reviews some of the more recent work aimed at understanding the synthesis, structure and organization of one of the polysaccharides found in the *Aspergillus* cell wall: nigeran, a regular copolysaccharide with alternating (1→3)- α and (1→4)- α linkages⁽⁶⁾ between glucopyranosyl units (Figure 1).

Nigeran (α)_D + 250⁰ (C, 1 NaOH) is isolated from the hyphae of certain groups of *Aspergillus* and *Penicillium* species.^(5,7) While it can represent 40% to 50% of the wall dry weight under certain conditions,⁽⁸⁾ it does not appear to occur in spores.⁽⁹⁾ The polysaccharide is unusual in that it is readily soluble in water at 95⁰C but is quite insoluble at room temperature. This property is exploited to extract and purify nigeran from mycelia.

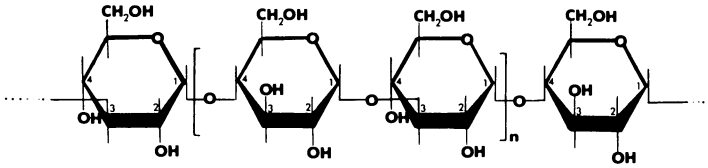


Figure 1. The repeating unit of nigeran shown in the Haworth projection.

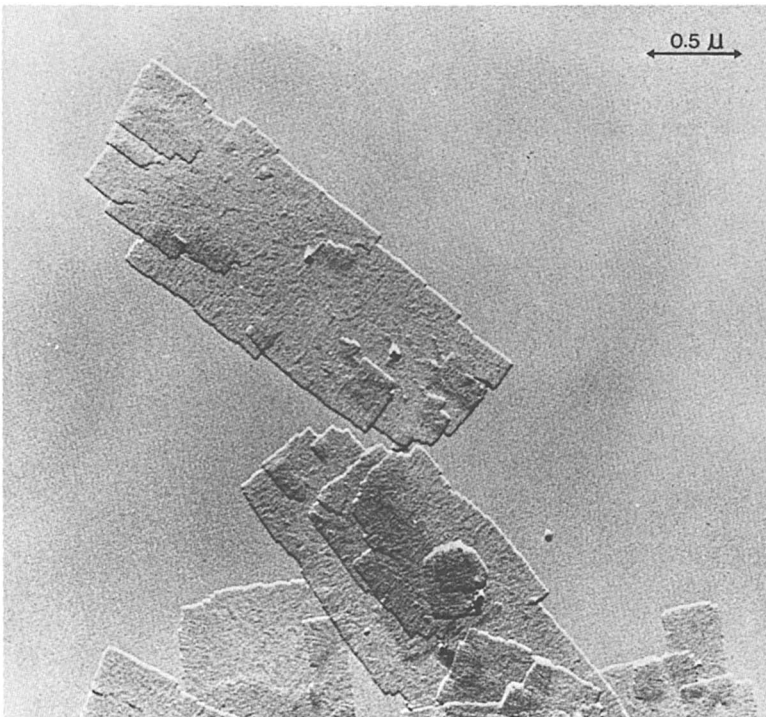


Figure 2. Shadowed lamellar single crystals of nigeran grown from dilute aqueous solution, the sample was solvent exchanged from water to methanol before drying (15).

Cell Wall Location of Nigeran

Experiments utilizing the (1→4)- α glucanase, mycodextranase, which is highly specific for nigeran, were conducted with mechanically treated hyphae walls of *A. niger* and the results suggested that the polymer is a constituent of the wall matrix rather than a cytoplasmic component.⁽¹⁰⁾ This conclusion is based on the fact that susceptibility of the polymer to enzymatic hydrolysis *in situ* is greatly facilitated by heating aqueous suspensions of the walls to 100°C prior to digestion at 40°C. About 6 to 8 times as much polymer is converted to specific hydrolysis products in the heated walls as compared to the amounts liberated from those not given such treatment. Two alternative explanations have been proposed: a buried (or inaccessible) wall location for nigeran in the wall or the existence of the polymer in some crystalline array that confers resistance to hydrolysis.⁽¹⁰⁾

Additional evidence for a wall location has been presented by Gold, who utilized autoradiography and electron microscopy of thin sections prepared from *A. aculeatus* hyphae to locate the polymer.⁽¹¹⁾ Their studies showed that following uptake of (³H) glucose by cells actively synthesizing nigeran, radioactivity was localized primarily around the hyphal perimeter. Control experiments utilizing cells not depositing the polymer yielded hyphae which showed a rather uniform distribution of label throughout the thin sections.

Preparation and Analysis of Nigeran Single Crystals

Since nigeran is a chiral polymer, it is hardly surprising to find that it can occur in crystalline form.⁽²⁾ Being water soluble at elevated temperatures, the polysaccharide can be obtained as rather perfect single crystals on slow cooling of dilute solutions. A full description of the preparation and characterization of these thin lamellar substrates has been reported.^(13,14) The appearance of the crystals is shown in Figure 2 as observed by transmission electron microscopy. Depending on the method of drying, a "smooth" or "cracked" crystalline surface is observed. The system of parallel cracks derives from dehydration that occurs due to the effect of the vacuum of the electron microscope while solvent exchange drying prevents this.⁽¹⁵⁾

The "dry" and "hydrate" forms of nigeran correspond to specific crystallographic states with and without water of hydration in the crystal lattice, respectively.^(13,14) X-ray and electron diffraction studies have established the unit cell and chain conformation of these two polymorphs.^(13,14)

Nigeran Biosynthesis

Gold⁽⁸⁾ found that high levels of nitrogen in the culture medium prevented the accumulation of nigeran in *A. aculeatus* mycelia. However, after the cells were shifted to a nitrogen deficient medium (pH = 5.0) for a few hours, nigeran deposition occurred and reached levels equivalent to 30% of the dry weight of the cells within about 50 hours. No significant cell growth was observed during this period, and it was demonstrated that the polymer is not utilized by the organism as a carbon source during starvation, furthermore its incorporation into the wall was blocked by cycloheximide.⁽⁸⁾

Studies conducted in our laboratory extended the original observations by Gold, et al.,⁽⁸⁾ regarding the effect of nitrogen on nigeran deposition. Two dozen species of *Aspergillus* and *Penicillium* exhibited the same behaviour showing that this is not a unique effect for only one organism.⁽⁵⁾ However, there are also many species of these genera that do not synthesize nigeran under conditions of nitrogen deprivation. Thus, the species fall into two distinct taxonomic groupings and the presence or absence of nigeran in certain previously assigned taxa suggests some of these taxa may not be natural.⁽⁵⁾ The species examined in this study display a wide range of maximum nigeran levels in their walls and only *P. melinii* was found capable of degrading its endogenous polysaccharide.

Crystallinity of Nigeran in Vivo

When hyphal wall preparations of either *A. awamori* or *A. niger* are shadowed and observed in the electron microscope, there is a strong correlation between their electron opacity and their nigeran content. Removal of nigeran from the wall either by heating aqueous suspensions, digestion with mycodextranase, or extraction with cold 0.5N NaOH was found to decrease this electron opacity⁽¹⁶⁾ (Figures 3 and 4). Analysis of thin sections, revealed that the deposition is not accompanied by any significant increase in wall thickness and results of enzyme digestion experiments with intact hyphae also argue that much of the nigeran is located toward the external side of the wall.⁽¹⁶⁾

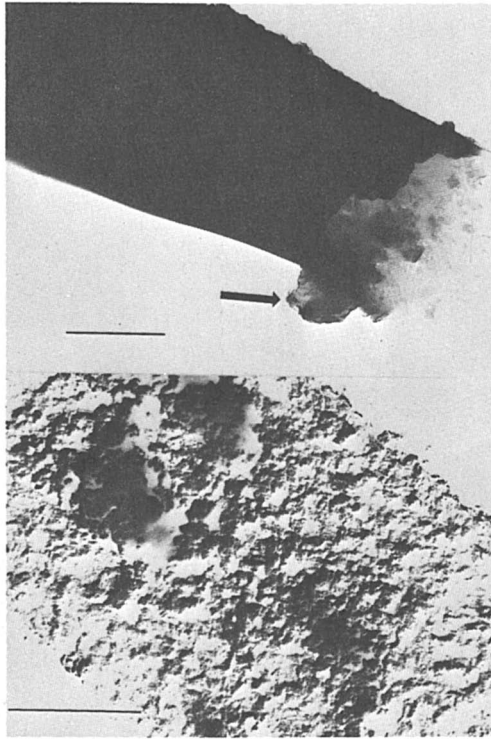
The organization of nigeran revealed by the above studies suggested the possibility that it is tightly packed in the wall, possibly in a crystalline arrangement. X-ray diffraction data recorded for samples of intact hyphae as well as walls of *A. niger* and *A. awamori* were compared with the x-ray reflections from single crystals of pure polymer. Table I gives some representative data and shows that nigeran exists as a distinct crystalline phase in the wall. Some of the observed reflections were due to the presence of chitin. Sample E

TABLE I: Interplanar Distances for Hydrated Cell Wall Samples of *A. awamori*

Samples ^a	Interplanar Distances (nm)											
Nigeran (Calculated)	0.880	0.736	0.685	0.495	0.477	0.418	0.360	0.330	0.311	0.288	0.257	0.223
Nigeran (Observed)	0.882	0.739	0.608		0.481	0.421	0.358	0.339	0.309	0.288	0.258	0.222
-Chitin (Calculated)	0.942			0.496		0.421		0.337		0.267	0.257	
A	0.927	0.750	0.608	0.489		0.420		0.338	0.317	0.270	0.257	
B	0.967	0.737	0.616	0.499		0.418		0.337	0.320	0.268	0.257	
C	0.956	0.744	0.616	0.507		0.423	0.356			0.270		
D	0.988			0.505		0.420		0.338	0.312	0.275	0.251	
E	1.295 ^b		0.595	0.492		0.418		0.343			0.257	

^a Aqueous suspensions of Samples A through D were freeze-dried after treatments and Sample E was kept in aqueous suspension. Starting, A-D contain approximately 37% and Sample E 8% dry weight as nigeran. Sample A, no treatment; B, heated at the boiling point for 15 minutes, centrifuged while hot and walls washed with hot water. C, dry heated 15 minutes at 105°C, D heated in a Soxhlet extractor for 36 hours with water at the boiling point and washed with hot water. For details of X-ray diffraction methodology see (16).

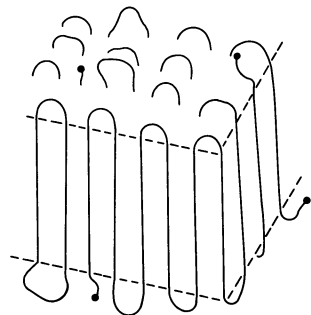
^b (1→3)- α -D-Glucan.



Journal of Bacteriology

Figures 3 and 4. Electron micrographs of *A. awamori* hyphal wall preparations shadowed with gold-palladium 60:40; bar length = 1 μm . (Figure 3, top) Hyphal wall containing approximately 37% of its dry weight as nigeran. The electron beam does not penetrate the hyphal wall and the appearance of the broken end (arrow) suggests that the increased opacity is associated with its external surface. (Figure 4, bottom) Hyphal wall from same preparation as above after enzymatic digestion to partially remove nigeran. As suspension of 10 mg (dry weight) of walls was incubated in 1 mL of acetate buffer, pH 4.5, at 40°C for 1 hr with 0.3 unit (20) of mycodextranase. See Ref. 16 for details.

Figure 5. Schematic of chain organization in lamellar single crystals. The "chain folds" are shown as regular with adjacent reentry although larger loops at the folds are also a possibility. Based on the known degree of polymerization of the nigeran (ca. 1000–2000 anhydroglucose units), the chain orientation in the crystals and crystals' dimensions, chain folding must occur (14).



was a 10% cell suspension of A. niger which contained only 6% nigeran (based on the dry weight of the sample) and detection of the crystalline nigeran pattern shows the sensitivity of the method and the highly developed crystallinity of the hyphal nigeran. These observations clearly indicate that at least some of the nigeran in the wall crystallizes in situ and since biosynthesis occurs below the solubilization temperature of the polysaccharide, "simultaneous" or "successive" polymerization and crystallization processes must take place. To what extent the nascent morphology resembles the lamellar model with chain folding, as shown in Figure 5, is not yet firmly established. Nevertheless, some notions concerning the influence of the crystalline state on enzyme accessibility can be obtained by using the single crystals as model substrates⁽¹⁷⁾ (see also below).

One very interesting observation made in the course of the x-ray experiments was that prolonged heating of wall or hyphal suspensions in water or treatment with ice cold 0.5N NaOH failed to remove all of the nigeran as evidenced by the retention of the characteristic diffraction pattern after these treatments (Table I, Sample D). That portion of nigeran which is inaccessible to boiling water extraction retains its crystallinity (Table I) and when extracted with hot alkali is removed along with a glucan fraction.⁽¹⁸⁾ This stability of the crystal lattice is quite remarkable considering the ease of solubility of the free polysaccharide in hot water. Treatment with hot 0.5N NaOH was required to remove all of the nigeran (detectable either by x-ray diffraction or by enzyme digestion) from the wall. While cold alkali or mycodextranase treatments of the hyphae diminished the intensity of the x-ray pattern, they did not eliminate it. These results suggest that nigeran is present in at least 2 domains in the hyphal wall in both A. niger and A. awamori.

Addition of exogenous mycodextranase to cells or cell walls gives partial digestion of the total nigeran pool, the amount being dependent on the species and the reaction temperature.⁽¹⁶⁾ The extent to which each domain is susceptible to enzyme degradation has not been determined directly, but data in Table II shows that it differs with species and/or culture conditions. P. melinii which also accumulates nigeran⁽⁵⁾ produces an endogenous enzyme activity (presumably mycodextranase) which can degrade up to 96% of its nigeran when placed in an environment lacking a carbon source. In addition, treatment of cells with boiling water extracts all of the nigeran.⁽⁹⁾ Therefore, in contrast to A. niger this species does not have a domain that is resistant to boiling water extraction. The foregoing indicates that

TABLE II: Distribution of Nigeran in A. Niger and A. Awamori Hyphal Walls^a

Organism	Nigeran Content (mg/100 mg of wall)	Total Nigeran Removed by (%)		
		Hot 1N NaOH	Boiling Water	Mycodextranase
<u>A. awamori</u>	39	100	72	78
<u>A. niger</u>	11	100	54	39

^a A. awamori walls were prepared from cells which were grown in submerged culture and then shifted to nitrogen-deficient medium for 96 hours. A. niger walls were prepared from cells grown in surface culture on complete medium. For details see (16).

incorporation of nigeran chains into hyphal walls is accomplished so that variations in organization and location are obtained while basic crystallinity is retained.

Enzymatic Depolymerization of Lamellar Single Crystals of Nigeran

Because the crystalline structure of nigeran in Aspergillus and Penicillium hyphal walls is identical to that of the lamellar single crystals, they afford a unique model system for studying nigeran chain organization in the wall as well as for understanding the nature of heterogeneous enzymatic degradation of crystalline polysaccharides with folded chain conformation. Viscometry, electron microscopy and permeation chromatography have been used to study the effects of mycodextranase on digestion of single crystals of nigeran.⁽¹⁷⁾ Buffered suspensions of crystals were subjected to attack by the enzyme and the extent of degradation was estimated by measuring changes in turbidity due to light scattering at 600 nm. The absorbance at this wavelength (A660) is directly proportional to concentrations of the insoluble polysaccharide within the range employed. Concentrations were also determined colorimetrically using the phenol-sulfuric acid assay.⁽¹⁹⁾

Table III shows the results obtained with two crystalline preparations of the polysaccharide. Single crystals and a "quench precipitate" (prepared by quickly chilling a polymer solution in ice to precipitate the polysaccharide with minimal crystallinity) were compared. The extent of degradation of lamellar crystals is highly temperature dependent, with the total fraction of polymer digested at any temperature being finite and quite reproducible from one batch of crystals to another. Incubation up to 20 hours causes no additional decrease in turbidity nor do the crystals inactivate the enzyme. The plateau in absorbance is evidence of a "two region" model of crystal morphology, i.e., one with both accessible and inaccessible zones. The extent of digestion at 20°C of the lamellar crystalline material is to be compared with that of the "quench precipitate" form at the same temperature. X-ray and electron microscope examination of the "quench precipitate" material confirmed that it is crystalline but that classical single crystal organization, where each unit cell is in register with its neighbour, is absent. Percentages of nigeran solubilized during digestions were also measured by the phenol-sulfuric acid assay method⁽¹⁹⁾ and there was excellent agreement between the two procedures (data not shown). Thermal effect on the crystalline morphology are reversible as evidenced by the fact that pre-incubation of crystals at 50°C followed by cooling to 20°C and incubation with enzyme gives the same decrease in A660 as a sample not previously pre-incubated.

TABLE III: Percentages of Initial Absorbance at 600 nm of Various Nigeran Preparations Subjected to Digestion with Mycodextranase^a

Sample Time (min)	Incubation Temperature (°C)				
	20 ^o	20 ^o	20 ^o	40 ^o	50 ^o
	Single Crystals	"Quench" Crystals	Nigeran Protein Complex	Single Crystals	Single Crystals
0	100	100	100	100	100
30	98	30	80	80	60
60	95	20	60	75	40
90	92	18	48	75	37

^a The various nigeran preparations were suspended at a concentration of approximately 0.19 mg/ml in 0.1 M acetate buffer pH 4.5. The initial absorbance due to light scattering of each suspension was measured at 600 nm with a Gilford Model 240 spectrophotometer. They were then incubated at the indicated temperatures with 0.3 unit of mycodextranase and the A₆₀₀ values determined periodically. The quench precipitate form of nigeran was prepared by heating a suspension of crystals at 100°C for 15 minutes and immediately cooling the solution in an ice bath prior to taking the initial absorbance reading.

Intrinsic viscosities of dimethyl sulfoxide (DMSO) solutions prepared from undigested nigeran single crystals ($(\eta)_{\text{DMSO}} = 6.23 \text{ dl/g}$) and from those subjected to the action of mycodextranase at 20°C ($(\eta)_{\text{DMSO}} = 3.40 \text{ dl/g}$) shows that glycosidic bonds are broken in regions that cause a significant drop in \overline{DP} . However, electron microscopy⁽¹⁷⁾ and light scattering studies (cf. Table III) show that there is very little effect on overall crystal size and shape and only a small fraction of the total polymer material is being solubilized at this temperature. Gel filtration chromatography of DMSO solutions prepared from control and digested crystals (washed prior to solubilization) indicate a broadening of the elution profile after digestion which confirms that products of a lower \overline{DP} are produced by such treatment.⁽¹⁷⁾ Furthermore, since the areas under the two elution curves were about the same, this supports the conclusion that most of the single crystal material remains insoluble after digestion at 20°C .

Since mycodextranase is an endo-glucanase which requires at least 6 contiguous glucopyranosyl residues for a productive enzyme-substrate complex (20), it is proposed that sections of exposed chains form extended regions or "loops" outside the crystal lattice (Figure 5) which are large enough to be cleaved by the glucanase and only a minimum of these cleavages produce soluble products. Electron micrographs (data not shown) confirm that overall shape and dimensions of the crystals is maintained after the 20°C digestion. The above results lead to the following conclusions: since the base areas of the crystal does not change significantly during digestion at 20°C , a surface attack is implied. At higher digestion temperatures there is more breakdown of the lamellae, suggesting chain segments become more mobile and accessible to the enzyme.

Whether the folded lamellar configuration of nigeran is present in the hyphal matrix is still conjectural. Since digestion of hyphal nigeran *in situ* by mycodextranase is quite limited at 20°C in both A. niger and A. awamori, it may be argued that a folded arrangement of the chains protects the polymer (and hence the wall) against attack by foreign glycanases. Also, its highly crystalline structure may serve to maintain integrity of hyphal structure during periods of environmental stress.

These studies add to our understanding of the relationship between fine structure of polysaccharides in cell walls and their accessibility to enzymes. An important consideration in accessibility must obviously be the textural organization of crystallites and whether their packing provides interstices large enough to permit an enzyme to bind.

A Nigeran-Protein Complex Isolated from Culture Filtrates

Utilizing conditions which rapidly deplete available nitrogen (e.g., high cell density) an insoluble carbohydrate-protein complex can be isolated from culture filtrates of several Aspergillus species following centrifugation, washing and dialysis. The carbohydrate portion of the complex was confirmed to be nigeran by its reactivity in the phenol-sulfuric acid assay, solubility properties in water, susceptibility to complete digestion by mycodextranase, and analysis of the total acid hydrolysate which yields glucose as the sole sugar. Based on both chemical and gravimetric analyses, the complex is approximately 96% to 98% glucose and 2% to 4% protein. (9)

When an aqueous suspension of the complex is placed in a boiling water bath, a portion remains insoluble. This residue can be obtained free of associated carbohydrate by repeated treatment with water at 95°C and centrifugation. Therefore, the protein is not covalently bonded to nigeran. The hot water insoluble fraction reacts positively in both the Folin-Lowry (21) and Coomassie blue (22) assays for protein. While no significant amount of it is solubilized in the hot water used to extract the nigeran, it dissolves in 0.1% SDS. Typical preparations of the protein fraction contain less than 4 mg of carbohydrate (expressed as glucose) per gram. Essentially, identical values are obtained following removal of nigeran enzymatically. The complete digestibility of the associated polysaccharide indicates that the protein does not protect it from degradation. It should be noted that extraction of nigeran from cells or cell walls of those species that secrete this complex affords a product devoid of any detectable protein.

X-ray analysis of the complex gives reflections indicating that at least some of the polysaccharide is crystalline. This fact is confirmed by the kinetics of enzymatic hydrolysis of the complex at 20°C which are intermediate in rate between the "quench precipitate" and that of a lamellar crystalline preparation of pure nigeran (Table III). SDS polyacrylamide gel electrophoresis of the solubilized protein fraction indicates it is composed of four components. The major one constituting probably 80% or more of the staining material has an apparent molecular weight of approximately 12,000; the 3 minor species present have molecular weights of 16,000; 22,500 and 30,000. (9) Their relationship to another (if any) is unknown at this time.

The kinetics of production of nigeran and the nigeran-protein complex by A. awamori are detailed in Figure 6. The nigeran content in the cell walls at "zero" time is elevated relative to the levels usually noted when cells

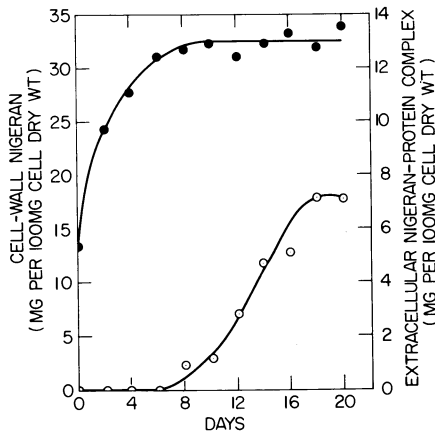


Figure 6. Time course of production of (●) cell wall nigeran and (○) extracellular nigeran protein complex by *A. awamori* on incomplete medium. After growth on complete medium, washed cells were transferred to medium lacking nitrogen source (16). Aliquots of the culture were withdrawn periodically and cells were separated by filtration. The nigeran-protein complex was isolated from the filtrate by centrifugation at $30,000 \times g$ and washed repeatedly with water.

are shifted to nitrogen deficient medium. In this particular experiment, a high cell density depleted the nitrogen in the medium resulting in some accumulation prior to shifting the cells to the nitrogen deficient medium. However, it is evident that the nigeran-protein complex does not begin to accumulate until after maximum amounts of nigeran have been deposited in the wall.

The origin and function of the protein fraction associated with nigeran is unknown. It is possible that control of the processes related to secretion and deposition of nigeran is lost when certain levels of polymer occur in the walls and that this protein fraction arises from vesicle or plasma membrane components closely associated with the polymer in situ during synthesis and movement to its point of deposition. It is also possible that the proteins are normally present in the wall, but are not complexed with nigeran. Alternatively, it may be a portion of the enzyme system involved in nigeran biosynthesis.

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Structural Studies on Fungal Polysaccharides Using Carbon-13 and Proton NMR Spectroscopy

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P.M.R. spectroscopy is complementary to the more recent ^{13}C technique in that much smaller samples can be used for structural and quantitative determinations on a given polysaccharide. However, Allerhand (1) has pointed out the amount of information given by a proton spectrum decreases with the size and complexity of the molecule and the "resonance density" is comparatively high. In the case of polysaccharides marked broadening of the signals occurs, so that individual resonances can only be detected in the H-1 region. However in the case of the mannan from Trichosporon aculeatum, purified via Fehling solution, the H-1 signals serve as a fingerprint (Fig. 1) and the high signal portion shows that β -D-mannopyranose units are present (2). In some other mannose-containing polysaccharides from yeasts NHAc groups of N-acetyl-D-glucosamine units may be identified through the methyl signals at δ 2.6 (3).

Such H-1 signals can be used as a fingerprint using known mannans as references (Table I) in order to identify parent yeasts isolated from rivers and lakes. This is aided by the versatility of yeasts in producing mannose-containing polysaccharides of different chemical structure (3). In cases where spectra cannot be obtained the component sugars can sometimes be used to define the polysaccharide.

One of the most striking features of ^{13}C spectroscopy is in elucidating structural order in polysaccharides. For example, a six signal spectrum is given by linear amylose (Fig. 2; 4) [or with amylopectin which shows a very minor sharp non-reducing end unit signal of C-4, which has much greater segmental motion than C-6 of the branch-unit, which should be at δ 67 but is so broad to be invisible (5)]. A 12 signal spectrum is obtained from the mannan from Rhodotorula glutinis, which contains alternating, rather than consecutive, (1 \rightarrow 3)- and (1 \rightarrow 4)-substituted β -D-mannopyranose units (Fig. 3; 6). Although pullulan, which contains a three-unit repeating sequence, gives only 14 signals (Fig. 4), these may be interpreted in terms of 18 signals some of which overlap (7,8). The shifts of the signals may be estimated

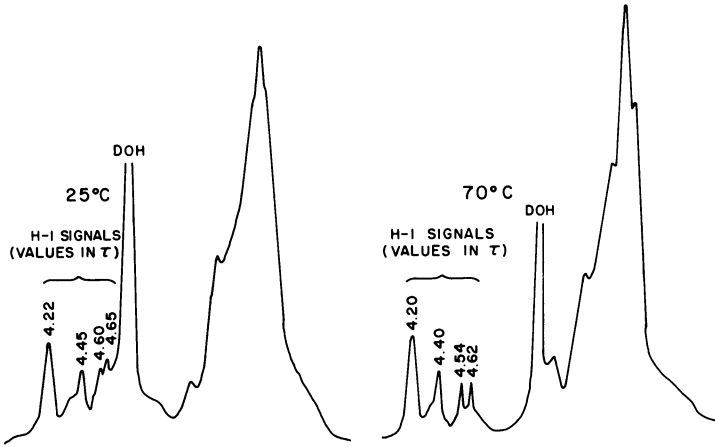


Figure 1. ^1H NMR spectrum of mannan from *Trichosporon aculeatum* (D_2O , 25°C , and 70°C)

Table I. Differentiation of 450 Yeast Species by ^1H NMR Spectroscopy of Component Mannose-Containing Polysaccharides

- A. 410 WHICH GIVE MANNOSE-CONTAINING POLYSACCHARIDE (VIA FEHLING PRECIPITATE)
-- 150 DIFFERENT TYPES BY P.M.R.
- B. 21 GIVE FEHLING PRECIPITATE, BUT POLYSACCHARIDES GIVE TOO VISCOUS A SOLUTION FOR P.M.R.
- C. 19 PRODUCE POLYSACCHARIDES NOT GIVING A PRECIPITATE WITH FEHLING SOLUTION.

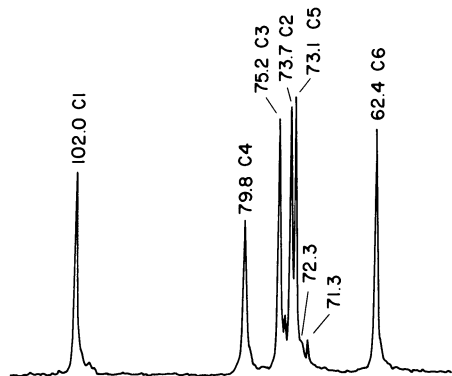


Figure 2. ^{13}C NMR spectrum of amylopectin

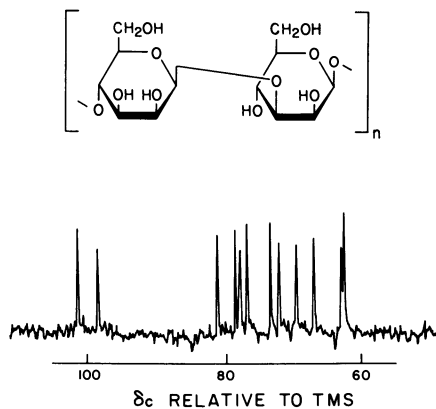


Figure 3. Chemical structure ^{13}C NMR spectrum of the mannan from *Rhodotorula glutinis*

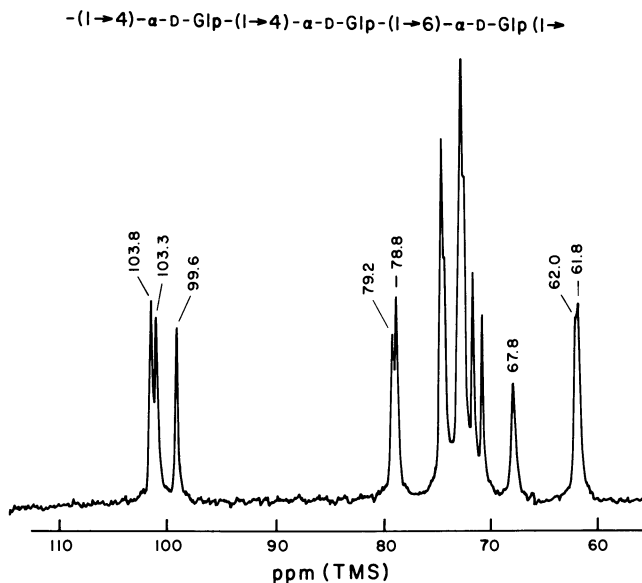


Figure 4. ^{13}C NMR spectrum and structure of pullulan from *Tremella mesenterica*, pD 7.0

approximately by considering those of α -D-(1 \rightarrow 4)- and α -D-(1 \rightarrow 6)-linked D-glucopyranans and various related oligosaccharides. It can be seen that ^{13}C spectroscopy is useful in gauging the purity of a polysaccharide when the base-line noise is low. This is possible when a large sample is used as with 20 mm diameter n.m.r. tubes (9) or with a high field spectrometer. Also spectra can be used as fingerprints as in the identification by Colson et al. (8) of pullulan from *Tremella mesenterica*. Often poorly resolved signals can be resolved by change of pH or pD, for example the C-6 signals of pullulan are better distinguished at pD 14. Also the C-2 and C-5 signals of amylose are better separated under alkaline conditions (4). These shifts are sometimes relatively minor compared with those of carbons concerned in the glycosidic linkage. Dorman and Roberts (4) have explained this phenomenon in the case of amylose by changes in hydrogen bonding leading to modification of the dihedral torsion angles ϕ and ψ of the glycosidic bonds. However use of LiBr, which modifies the specific rotation, did not cause shift changes.

As in p.m.r. spectra the presence of ^{13}C signals with characteristic shifts can be used to identify certain structures. One common structure in fungal polysaccharides is β -D-galactofuranose, which gives very low field signals at δ_{C} 107-109 and distinctive signals at δ_{C} 78-85. Such structures appear to be impurities in glucomannans from *Ceratocystis* spp. (Fig. 5; 10). Although not depicted, the polysaccharide obtained from *C. paradoxa* 102-J grown at 37° gives signals at δ_{C} 175.6 and 23.4 which correspond respectively to CH_3 and carbonyl carbons of N-acetyl groups, respectively.

The detection of other unusual features, such as malonic acid esters and phosphorodiester is discussed later.

Two ^{13}C methods are available for determination of glycosidic configuration. The most commonly used, which is applicable to glucopyranose, xylopyranose, galactopyranose and arabinopyranose residues and various furanose forms is direct observation of the C-1 chemical shift which is configurationally dependent (11). For example the alkali soluble glucan(s) of *Sporothrix schenckii* give C-1 signals at δ_{C} 103.8 and 104.0, typical of β -D-linkages (Fig. 6), but at much lower field than δ_{C} 100-102 expected from α -D-linkages (12). Thus the C-4 signal at δ_{C} 80.3, indicates the presence of unexpected (1 \rightarrow 4)-O-substituted β -D-glucopyranose units, by analogy with the similar signal of barley glucan (13) and of lichenan (14). The glucan from *Pleurotus ostretus* was shown to contain both β -D-(1 \rightarrow 3)-linked and 4-O-substituted α -D-glucopyranose units by the presence of signals at δ_{C} 104 and 102, respectively (15). On the other hand C-1 shifts of mannopyranose units are not sensitive to configuration, in contrast to H-1 signals in proton spectra (3). However, configuration can be determined in all cases in the pyranose series from their $J_{13\text{C}-1, \text{H}-1}$ coupling constants, which should be 169 Hz for the α and 160 Hz for β -anomers (16).

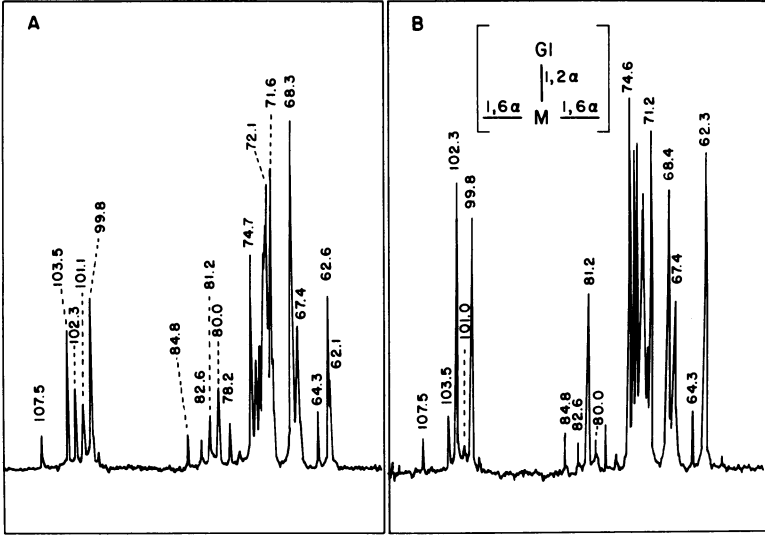


Figure 5. Partial ^{13}C NMR spectra of galactofuranose-containing polysaccharides in (A) glucomannan and mannan from *Ceratocystis paradoxa* and (B) glucomannan from *Ceratocystis brunnea*

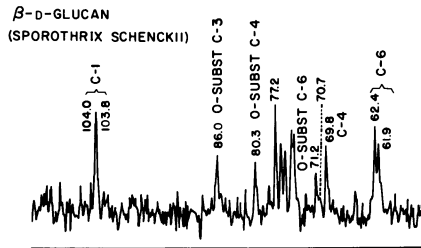


Figure 6. ^{13}C NMR spectrum of (1 \rightarrow 4), (1 \rightarrow 3), and (1 \rightarrow 6) linked β -D-glucopyranan(s) from *Sporothrix schenckii*

Identification of ^{13}C n.m.r. signals of polysaccharides is often based on the spectrum of the monosaccharide component and taking into account two shifts that occur on O-glycosylation, the α -effect where the substituted resonance is shifted downfield by 4-9 ppm and the β -effect where the adjacent resonances are shifted upfield by ~ 1 ppm. Longer range shifts, although often observed, are less than 1 ppm. The α and β -effects are apparent in the spectrum of bakers' yeast mannan (Fig. 7; 17) except that the β -effect at C-1 is quite strong as the result of O-glycosylation adjacent to an axial C-O bond.

The signals in the O-substituted region were identified by reference to spectra of homologous series of oligosaccharides obtained on partial acetolysis. For example on going up a series of α -D-(1 \rightarrow 2)-linked mannose oligosaccharides the size of the signal at δ_{C} 102.2 of internal units increases in an irregular fashion compared with those at δ_{C} 103.7, corresponding to non-reducing end units. The signal at δ_{C} 100.2 was assigned to C-1 of 2,6-di-O-substituted units by a process of elimination and by virtue of the upfield β -shift from the C-1 signal of (1 \rightarrow 6)-linked α -D-mannopyranan. The signals at $\sim \delta_{\text{C}}$ 80 were identified in a similar way from (1 \rightarrow 2)- and (1 \rightarrow 3)-linked oligosaccharides.

The α -shifts observed on O-glycosylation are mostly less than that caused on O-methylation. A similar related effect was observed by Koch et al. (18) who found that C-1 shifts of isopropyl and tert-butyl α -D-glucopyranosides were at 4 and 7.7 ppm high field than that of the methyl glycoside (Fig. 8). This was assumed to be due entirely to the gauche effect between the CH_3 groups of the aglycone and the anomeric center. Thus, the isopropyl group is analogous to the glycosyl unit in that the gauche effect occurs in 2 of the 3 depicted rotamers.

The strong β -effect that occurs with O-glycosylation adjacent to an axial C-O group can be explained using methyl ethers of inositols as models (Fig. 9; 19). In the first example the O-methylated OH group is equatorial and one of the the adjacent C-O bonds is axial and it can be seen that in the rotamer of lowest free energy a gauche interaction takes place with a resulting upfield shift. Similarly in a 1,2-diaxial system the preferred rotamer undergoes a gauche interaction.

Deuterium labelling has been used in fungal polysaccharides to identify signals. For example relatively direct incorporation of deuterium into a yeast mannan from *Rhodotorula glutinis* containing alternate (1 \rightarrow 3) and (1 \rightarrow 4)-linked β -D-mannopyranose units was reported when [6- $^2\text{H}_2$], [5- ^2H]- and [3- ^2H]-derivatives of glucose were used as precursors (20). The signals of carbons attached to deuterium were diminished in size due to the α -deuterium effect and in one case of C-6 labelling β -upfield deuterium shift of 0.12 ppm (3Hz) was sufficiently large compared with line width (6Hz) to be observable (Fig. 10). Thus the C-5 assignments were confirmed. These data were consistent with assignments based on α and β -methylation shifts arising with β -D-mannose and

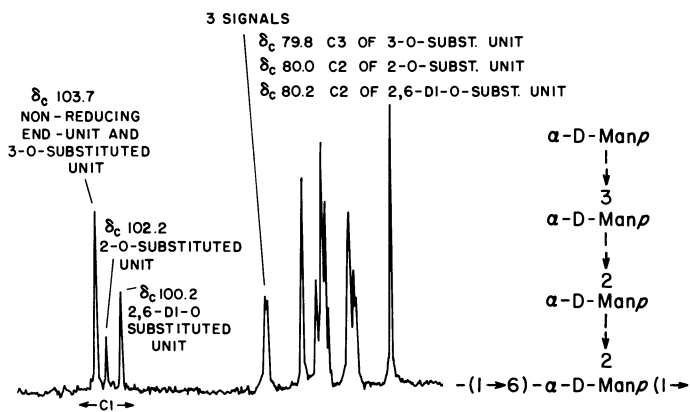


Figure 7. ^{13}C NMR spectrum of baker's yeast mannan

α -D-GLUCOPYRANOSIDE DERIVATIVE	δ_c OF C-1, COMPARED WITH METHYL GLUCOSIDE, PPM
ISOPROPYL	-4.0
TERT-BUTYL	-7.7

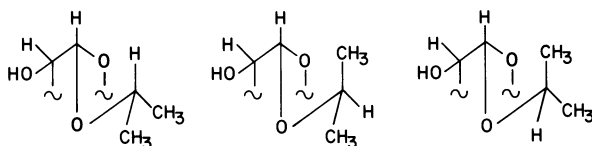


Figure 8. Rotamers of isopropyl group of isopropyl α -D-glucopyranoside about C-1: shift comparisons with other glycosides

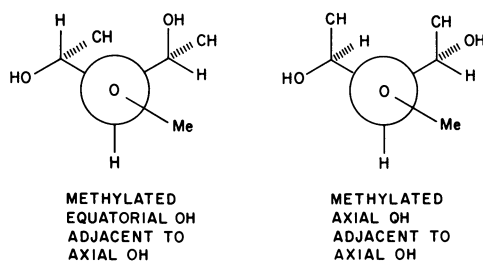


Figure 9. *Gauche* effects on methylation of OH group adjacent to axial OH

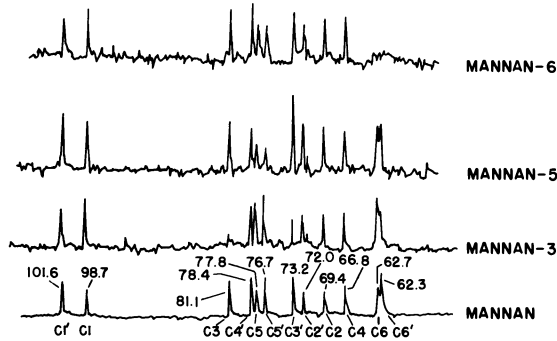


Figure 10. ¹³C NMR spectra of mannans of *Rhodotorula glutinis* obtained from deuterium-labelled glucose precursors (6-²H₂, 5-²H, and 3-²H derivatives)

Table II. Summary of Structures of Mannan Isolated from *S. schenckii* under Different Conditions

STRAIN	CULTURE MEDIUM	TEMP. (°C)	MORPHOLOGY	MAJOR STRUCTURE
1099.12	YCV	37	MIXED (YEASTS PREDOMINATING)	MONORHAMNOSYLMANNAN
1099.12	M, BHI	37	100% YEASTS	MONORHAMNOSYLMANNAN
1099.12	SAB	37	90% YEASTS	MONORHAMNOSYLMANNAN
1099.12	BHI, SAB	25	MIXED (MYCELIUM PLUS CONIDIA PREDOMINATING)	MAINLY DIRHAMNOSYLMANNAN
1099.18	M	37	100% YEASTS	MONORHAMNOSYLMANNAN
1099.18	M	25	100% YEASTS	MONORHAMNOSYLMANNAN WITH MINOR AMOUNTS OF 4-O- AND 2,4-DI-O-SUBSTITUTED α-D-MANp UNITS
1099.12	YCV	25	CONIDIA	MONORHAMNOSYLMANNAN
1099.12	BHI	25	UNSPORULATED MYCELIUM	GALACTOMANNAN
1099.18	B	25	UNSPORULATED MYCELIUM	DIRHAMNOSYLMANNAN

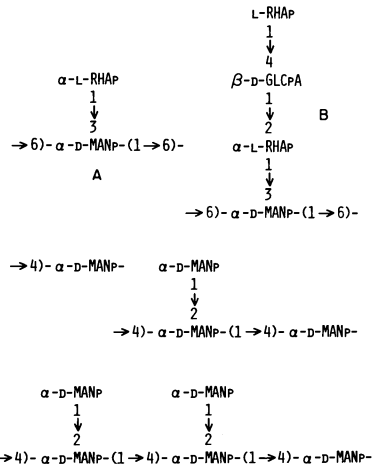


Figure 11. Some of the structures associated with polysaccharides from *Sporothrix schenckii* and *Ceratocystis stenoceras*

those of a structurally related β -D-(1 \rightarrow 4)-linked D-mannopyranan. It was fortunate in this case that marked randomization of label did not occur. Less success was observed with the rhamnomannan from *Sporothrix schenckii* and it was necessary to use [1- 13 C] and [2- 13 C] derivatives of glucose as precursors to effect identification of some of the signals (21).

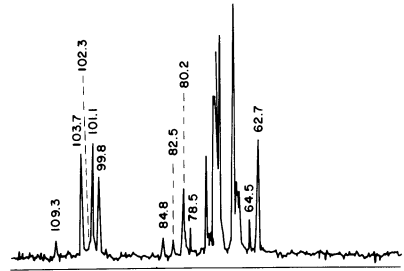
Rapid identification of components of polysaccharide mixtures and quantitation of polysaccharides can be carried out once characteristic signals are identified. One such example concerns *Sporothrix schenckii*, the causative agent of the disease sporotrichosis, which is dimorphic and can give rise to a number of polysaccharides whose production is influenced by its morphology, strain of organism, medium of growth and temperature (Table II). With the aid of conventional analytical procedures it was found that monorhamnosylmannan is associated with the yeast form, although minor amounts of 4-O- and 2,4-di-O- substituted α -D-mannopyranose structures are formed in combination with other structures at lower temperatures (22). It was found that the mycelial form gives a dirhamnosylmannan with two unit side-chains and this is the main antigenic determinant, as determined by oligosaccharide inhibition studies with rabbit antisera and by the reaction of polysaccharides with some of patients with sporotrichosis (23). The formation of hyphae in vivo is suggested from the 13 C n.m.r. spectrum of conidial polysaccharide, which contains monorhamnomannan as distinct from the dirhamnosylmannan of hyphae (24). Previously only the yeast form was identified in lesions. A fourth polysaccharide, a galactomannan was produced using a brain heart infusion medium.

Depicted structures are formed from *S. schenckii* in addition to a galactomannan and a dirhamnosylmannan which is similar to A except for an additional O- α -L-rhamnopyranosyl-(1 \rightarrow 2)-unit in the side chain (Fig. 11). Structure B is a structure produced from *Ceratocystis stenoceras*, a yeast that is often found associated with *S. schenckii* in its natural habitat of vegetation. Signal differences indicate that it is not the perfect stage of *S. schenckii* (25), as previously suggested.

The structure of the galactomannan can be partly elucidated from its 13 C spectrum (22) since a minor β -D-galactofuranose structure is indicated and the chemical shifts of the small signals at δ_c 84.8, 82.5, 78.5 and 64.5 correspond to those of C-4, C-3 and C-5 of methyl β -D-galactofuranoside (Fig. 12). The similarity in chemical shift shows that 2-O-, 3-O-, 5-O- and 6-O-substitution, if present, is minor and that the galactofuranose units exist mainly as single unit non-reducing ends and not consecutively.

Figure 13 depicts spectra of polysaccharide preparations from various strains of *Sporothrix schenckii*, and are examples of the multitude of 13 C signals that may be detected in the O-glycosylated carbon regions of the spectra (25). Excluding the β -D-galactofuranose signals 10 characteristic signals are

Figure 12. ^{13}C NMR spectrum of galactofuranose-containing polysaccharide from *S. schenckii*



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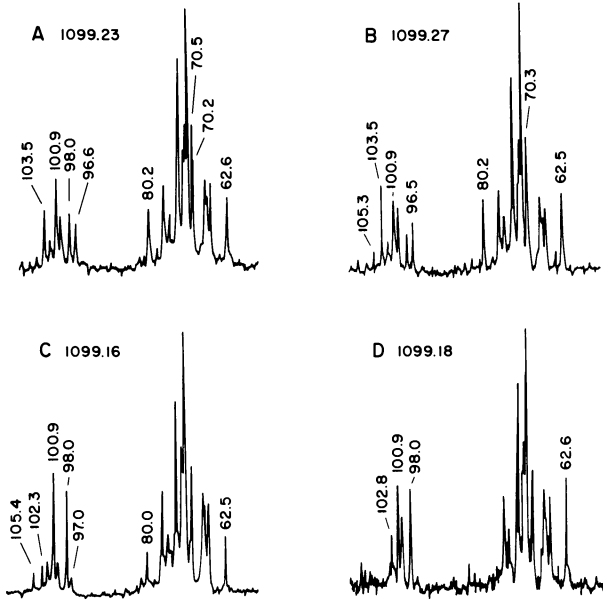


Figure 13. ^{13}C NMR spectra of polysaccharide preparations from various strains of *S. schenckii*

present corresponding to possible structures in the main chain and side chains (Table III). Most of the assignments were based on those of oligosaccharides formed on partial hydrolysis or acetolysis, but some were deduced, such as the 2,4-di-O-substituted α -D-mannopyranose structure whose signal at $\delta_C 100.3$ has to be at approximately 2 ppm higher field than that of the 4-O-substituted α -D-mannopyranose units, because of the 1,2-diaxial β -effect (26).

Other signals such as those at $\delta_C 102.3$ and $\delta_C 80.3$ can be attributed to the C-1' and C-4 portions of the L-rhamnopyranosyl non reducing end-unit of the 3-unit side chain of the *Stenoceras* polysaccharide (Fig. 11; structure B). These are removed on partial hydrolysis (Fig. 14), the side-chain signals at $\delta_C 81.9, 97.4, 97.9$ and 105.4 remaining. The latter corresponds to C-1 of β -D-glucopyranosyluronic acid residues (21).

Few examples occur in the literature of fungal polysaccharides which contain esters whose position can be determined by ^{13}C n.m.r. spectroscopy, but oddly both examples concern structures that should be revised.

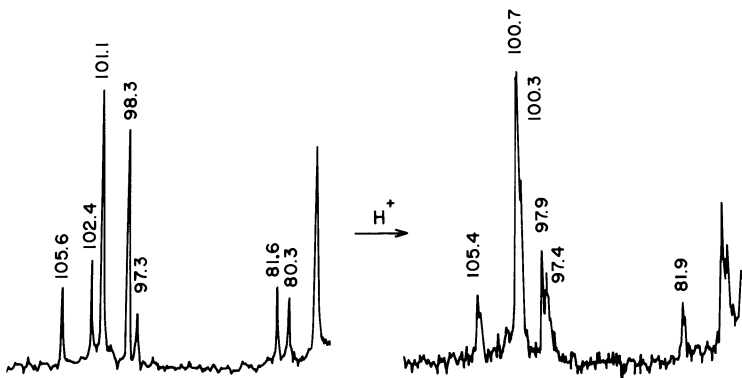
Experiments have been carried out to determine the position of the malonate substituent in the β -D-(1 \rightarrow 5)-linked D-galactofuranan of *Penicillium citrinum*. Kohama et al. (27) proposed that the malonate was at position 2 or 3 since the units were resistant to periodate oxidation. Since the signal at $\delta_C 77.5$ was less prominent in the malonylgalactan than in the galactan (Fig. 15) it appeared that the signal belonged to C-3 and malonic ester at this position shifted downfield being superimposed with the C-2 and C-4 signals at $\delta_C 83$. The new signals at $\delta_C 66$ and 76 were ascribed to diester and ester forms of malonic acid (28). The present proposal is different and takes into account assignments attributed to a β -D-(1 \rightarrow 5)-linked galactofuranose tetrasaccharide from *Penicillium charlesii* galactomannan, which was based in turn of assignments for methyl β -D-galactofuranoside and its 5-O-methyl derivative with its attendant α - and β -shifts (29). It has been known for a long time that in the aliphatic series acetylation of hydroxyl groups causes strong downfield α -shifts and upfield β -shifts (4). This is reflected in the shifts occurring on 3-O-acetylation of α and β -anomers of D-glucose (30) and N-acetyl-D-glucosamine (Table IV; 31). Thus the malonogalactan spectrum is consistent with a malonate ester at C-6 with β -shift of +4.5 ppm and a α -shift of -1.5 ppm. The resistance of some of the units to periodate oxidation could be due to the relative resistance of a trans-vicinal diol group to the reagent.

The shifts occurring on O-phosphorylation differ somewhat from those of acetates and if anything their interpretation is simpler. Phosphorylation of simple sugar derivatives results in downfield shifts of the substituted resonance, with little effect on other signals, except for ^{13}C - ^{31}P coupling (Table V; 31, 32). Two bond ^{13}C -O- ^{31}P coupling of 6-8Hz occurs with the phosphory-

Table III. Assignment of ^{13}C Signals of Nuclei in Polysaccharides from *S. schenckii* and *C. stenoceras*

SIGNAL $\delta_{\text{C}} \pm 0.2$ PPM	ASSIGNMENT
105.5	C-1, L-RHAP-(1 \rightarrow 4)- β -D-GLUPA-(1 \rightarrow 2)- α -L-RHAP-
103.7	C-1, α -L-RHAP-(1 \rightarrow 2)- α -L-RHAP-
103.7	C-1, α -D-MANP-(1 \rightarrow 2)- α -D-MANP-
102.3	C-1, -(1 \rightarrow 4)- α -D-MANP-(1 \rightarrow 4)- α -D-MANP C-1, L-RHAP-(1 \rightarrow 4)- β -D-GLUPA-(1 \rightarrow 2)-
101.1	C-1, (1 \rightarrow 3), (1 \rightarrow 6) DI-O-SUBST α -D-MANP
100.3	C-1, (1 \rightarrow 2), (1 \rightarrow 4) DI-O-SUBST α -D-MANP

SIGNAL $\delta_{\text{C}} \pm 0.2$ PPM	ASSIGNMENT
98.2	C-1, α -L-RHAP-(1 \rightarrow 3)- α -D-MANP-
97.2	C-1, β -D-GLUPA-(1 \rightarrow 2)- α -L-RHAP-(1 \rightarrow 3)- α -D-MANP-
96.8	C-1, α -L-RHAP-(1 \rightarrow 2)- α -L-RHAP-(1 \rightarrow 3)- α -D-MANP-
81.4	C-2, β -D-GLUPA-(1 \rightarrow 2)- α -L-RHAP-(1 \rightarrow 3)- α -D-MANP-
80.3	C-2, α -L-RHAP-(1 \rightarrow 2)- α -L-RHAP-(1 \rightarrow 3)- α -D-MANP-
	C-4, L-RHAP-(1 \rightarrow 4)- β -D-GLUPA-(1 \rightarrow 2)- α -L-RHAP-



*Figure 14. ^{13}C NMR spectra of *C. stenoceras* polysaccharide and its partial hydrolysis product*

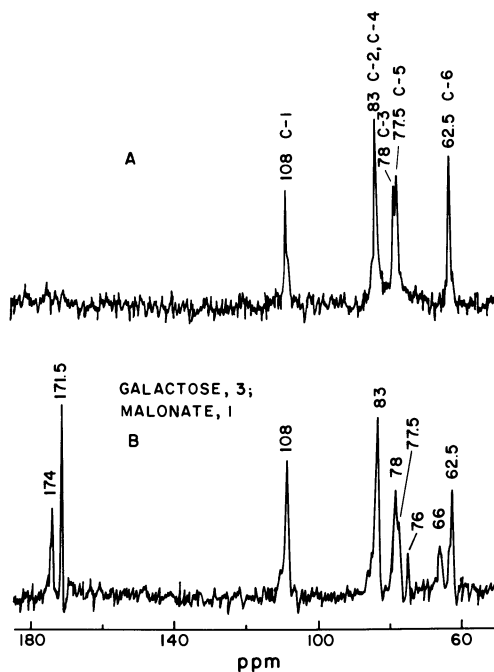


Figure 15. ^{13}C NMR spectra of (B) malonate-containing galactan of *Penicillium citrinum* Thom 1131 and (A) derived $\beta\text{-D-(1}\rightarrow\text{5)}$ -linked D-galactofuranan

Table IV. ^{13}C Signal Displacements Occurring on 3-O-acetylation

STARTING MATERIAL	SIGNAL DISPLACEMENT PPM					
	C-1	C-2	C-3	C-4	C-5	C-6
$\alpha\text{-D-GLUCOSE}^\dagger$	-0.1	<u>-1.8</u>	<u>+2.5</u>	<u>-2.0</u>	-0.3	-0.4
$\beta\text{-D-GLUCOSE}^\dagger$	-0.2	<u>-1.8</u>	<u>+1.5</u>	<u>-1.8</u>	-0.2	-0.3
$\alpha\text{-D-GLcNAc}^*$	+0.1	<u>-1.9</u>	<u>+3.1</u>	<u>-2.3</u>	-0.2	-0.2
$\beta\text{-D-GLcNAc}^*$	-0.6	<u>-1.7</u>	<u>+1.8</u>	<u>-2.1</u>	-0.2	-0.2

† VIGNON AND VOTTERO, 1976

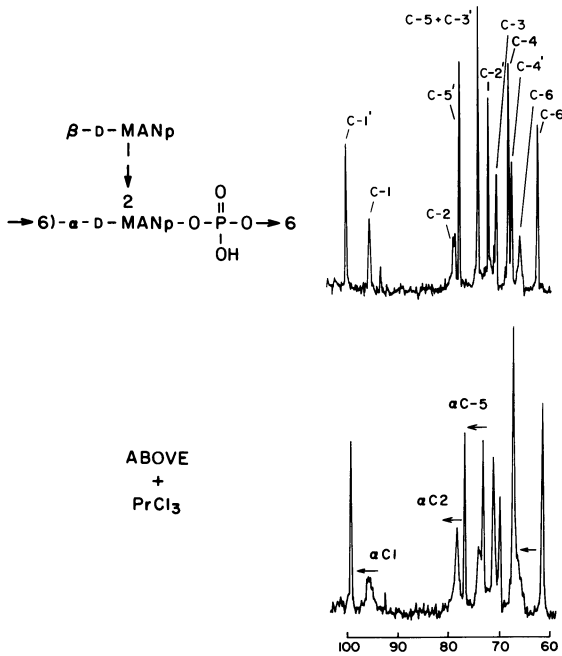
* BUNDLE ET AL., 1973

Table V. ^{13}C Signal Displacements Occurring on O-phosphorylation

REACTION	SIGNAL DISPLACEMENT PPM					
	C-1	C-2	C-3	C-4	C-5	C-6
α -D-GlcNAc → 1-PHOSPHATE †	<u>+1.8</u>	0	+0.6	-0.2	+0.4	0
β -D-GlcNAc → 1-PHOSPHATE †	<u>+0.5</u>	-0.3	+0.1	0	+0.2	+0.3
α -D-MAN → 1-PHOSPHATE *	<u>+2.6</u>	+0.3	+0.2	+0.1	-	+1.9
α -D-MAN → 6-PHOSPHATE *	+0.7	+0.5	+0.4	0	-0.5	<u>+3.9</u>
β -D-MAN → 6-PHOSPHATE *	+0.7	+0.4	+0.4	-0.2	-0.7	<u>+3.9</u>

† BUNDLE ET AL., 1973

* GORIN AND MAZUREK, 1974

Figure 16. Effect of PrCl_3 on the ^{13}C NMR spectrum of phosphonomannan from *Hansenula capsulata*

lated carbon with three bond $^{13}\text{C}-\text{C}-\text{O}-^{31}\text{P}$ coupling of the adjacent carbon(s). The value is from 2-10Hz depending on the dihedral angle of the $^{13}\text{C}-\text{C}$ and $\text{O}-^{31}\text{P}$ bonds (33). Such considerations have been used to determine the positions of phosphodiester substitution in many phosphorylated polysaccharides from bacteria (34). In the phosphomannan of *Hansenula capsulata* (Fig. 16) coupling cannot be observed since the signals are wider than 7Hz, but of the four possible coupled signals, three of them C-1, C-2 and C-6 could be recognized by their relative (35). However the position of the hidden C-5 signal was all-important since it would indicate whether the phosphomannan had the structure proposed by Slodki and coworkers where the 6-phosphorylated unit had the β -configuration (Fig. 18; 36) or another one (Fig. 17; 32) where, on the basis of ^{13}C n.m.r. evidence obtained on the mannobiose phosphate, the 6-phosphorylated unit has the α -configuration. The C-5 signal was detected by use of lanthanide shift reagents (35). Using known monosaccharide derivatives it was found that praseodymium chloride caused a strong downfield shift of the phosphorylated resonance and a weaker one of the adjacent resonance. The shifts observed for europium chloride were equal for each carbon and in the opposite direction (Table VI).

It was found that an addition of PrCl_3 one broad signal emerged from a large one and this was in the region of C-5 of α -D-mannopyranose rather than that of the β -anomer (which is farther downfield), thus lending support to our previously prepared structure.

The parameter arising from molecular motion, spin lattice relaxation time T_1 , spin-spin lattice relaxation time T_2 and the nuclear Overhauser enhancement (nOe) are of prime importance in ^{13}C n.m.r. spectroscopy of polysaccharides. In Figure 18 the dependence of T_1 and T_2 on correlation time τ_c , assuming isotropic motion, is based on complex mathematical equations presented by Komoroski and colleagues (37). The most critical is T_2 since it governs signal width, whereas even with a line width of 150Hz, the T_1 was observed at 0.06 sec which is ideal in terms of collecting spectral data by Fourier transform.

With high molecular weight polysaccharides with $\tau_c \sim 10^{-8}$ sec there is a lack of sensitivity due to nOe values approaching 1, leading to loss in spectral sensitivity.

Increasing the magnetic field (Fig. 19) does not effect T_2 's and nOe values markedly, although the pronounced increase of T_1 values (38) may effect collection of data from sugar units with a higher degree of segmental motion and negate the increased sensitivity of the spectrometer. However no experiments have yet been carried out to determine T_1 at high field. But, for example, if T_1 became more than 0.5 sec it would be inconvenient collecting quantitative data in an antigated experiment (39,40) where nOe is suppressed, and when data accumulation is made when the decoupler is on, but off during a delay at least 5 times the T_1 of the

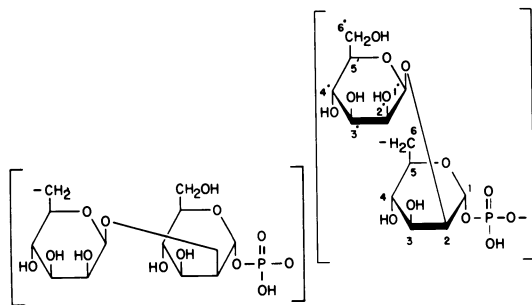


Figure 17. Possible structures of *H. capsulata* phosphomannan

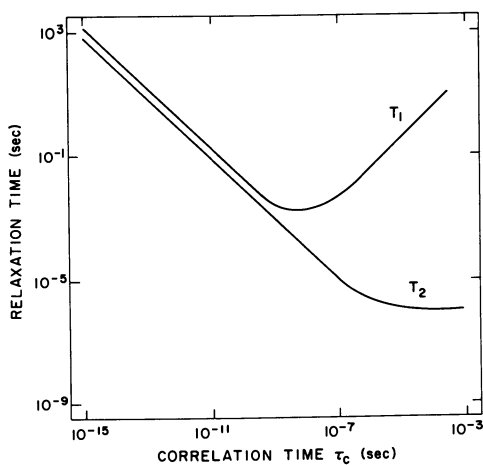


Figure 18. Relation of T_1 and T_2 values with correlation time τ_c

Table VI. ^{13}C Signal Displacements Occurring on Addition of Lanthanide Ion to Mannose Phosphates in the Acid Forms

COMPOUND 65MG IN D_2O (0.8ML)	HYDRATED SHIFT REAGENT $\sim 8\text{MG}$	SIGNAL DISPLACEMENT PPM					
		C-1	C-2	C-3	C-4	C-5	C-6
α -D-MAN-6-P	PrCl_3	+0.04	+0.08	0	+0.16	<u>+0.40</u>	<u>+1.19</u>
β -D-MAN-6-P	PrCl_3	-0.16	-0.08	-0.12	+0.16	<u>+0.40</u>	<u>+1.19</u>
α -D-MAN-1-P	PrCl_3	<u>+0.79</u>	<u>+0.32</u>	+0.16	+0.04	0	-0.28
α -D-MAN-6-P	EuCl_3	-0.04	-0.08	-0.04	-0.08	<u>-0.28</u>	<u>-0.32</u>
β -D-MAN-6-P	EuCl_3	0	0	-0.12	-0.08	<u>-0.28</u>	<u>-0.32</u>
α -D-MAN-1-P	EuCl_3	<u>-0.28</u>	<u>-0.28</u>	-0.08	-0.12	-0.04	+0.28

observed resonance (90° pulse).

Rees and coworkers (41) have shown that in the ι-carrageenan gel at 15° the segmental motion of all portions of the double helix form is so little that the resulting T_2 's are too low for signal observation. On increase of temperature the gel dissociates so that a normal ^{13}C n.m.r. spectrum is obtained (Fig. 20).

On going from the solution of β-D-glucan of *Lentinus edodes* to the gel the 0-glycosylated C-3 signal (Fig. 21) disappears, thus showing that junction zones occur in the β-(1→3) linked portion of the glucan (15). Probably if the resonance was observable it would be shifted as in the gel of the β-(1→3)-linked D-glucan of *Alcaligenes faecalis* (42), a phenomenon attributable to differences of the dihedral torsional angles of the glycosidic bond, ϕ and ψ .

T_1 values are of interest in polysaccharides since they reflect the chemical structure of the molecule. In stachyose (Fig. 22), the values reflect greater segmental motion at the ends of the molecule (43). However in a trisaccharide of a ganglioside the N-acetyl-neuraminic acid acts as an anchor (44), similar to the bulky aglycone portion of the κ-strophanthoside where the T_1 values of the cymarose portion are lower than those of successive β-D-glucopyranose units (45).

In a linear (1→2)-, (1→6)-linked α-D-mannopyranan of *H. capsulata* the values [Freeman Hill modification of the inversion recovery method (46)] of each of T_1 's of the C-1's and the 0-substituted C-2 are the same, being 0.14 sec (Fig. 23; 47).

However in the branched chain mannan of *S. fragilis* the T_1 values of main-chain nuclei are 0.09 sec (70°, 25.2 MHz), less than 0.16 sec for the C-1 of the side chain (Fig. 24). Although there was no correspondence of ρ values to segmental motion it was noticeable at 30° that the main chain C-1 signal (12.3 Hz) was broader than that of the side chain signal (8.6 Hz) but this difference decreased on increase of temperature.

The effect was paralleled in the rhamnomannan of *Sporothrix schenckii* whose ^{13}C spectrum has signals already assigned (21). Whereas nuclei of main chain units of mannose had T_1 's of 0.10-0.19 sec those of the rhamnose side chain are from 0.12-0.18 sec, values that indicate that the method may be of value in signal assignment (Fig. 25).

It would be of interest to determine T_1 values of polysaccharide nuclei at high field in order to measure their increase and thus gauge the advantage of using large field spectrometers in obtaining ^{13}C n.m.r. spectra of polysaccharides. No data is yet available, although Levy et al. (48) investigated poly(n-butylmethacrylate) which contains a main chain and side chain with the depicted numbering system (Fig. 26).

It is noticeable (Table VII) that T_1 's of the side chains increase with temperature, the effect being more marked approaching the end of the side chain. Also on going from 22.6 MHz to

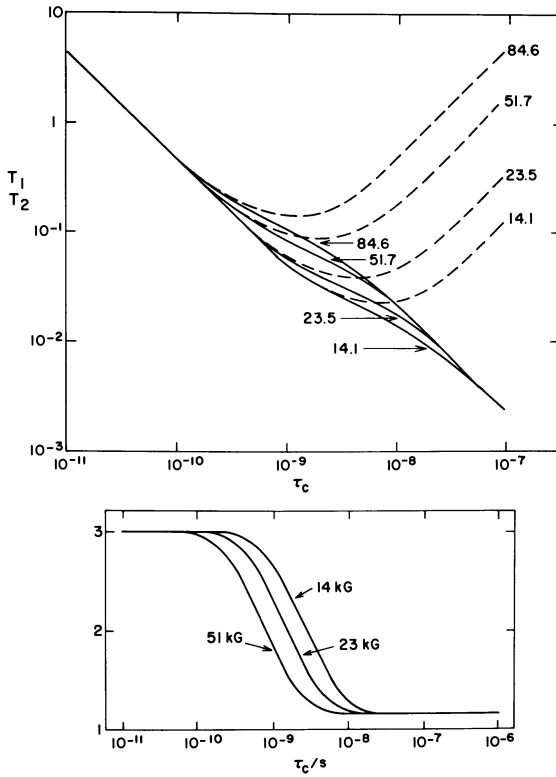


Figure 19. Relation of (---) T_1 , (—) T_2 , and nOe values with applied magnetic field

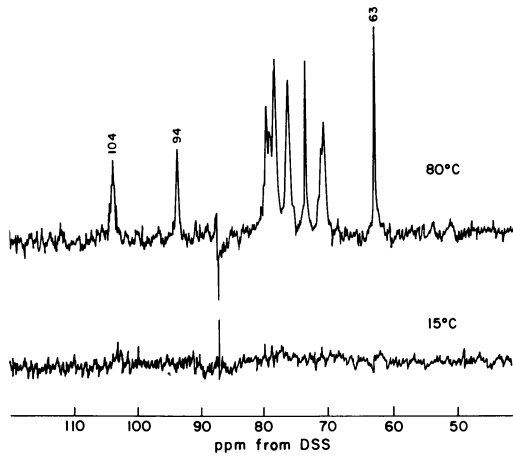


Figure 20. ^{13}C NMR spectra of i-carrageenan of 15°C and 80°C

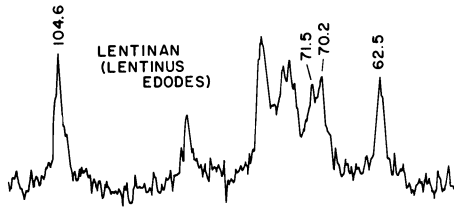


Figure 21. ^{13}C NMR spectrum of (1 \rightarrow 3), (1 \rightarrow 6) linked β -D-glucopyranan ($\text{Me}_2\text{SO}-d_6$) of Lentinus edodes

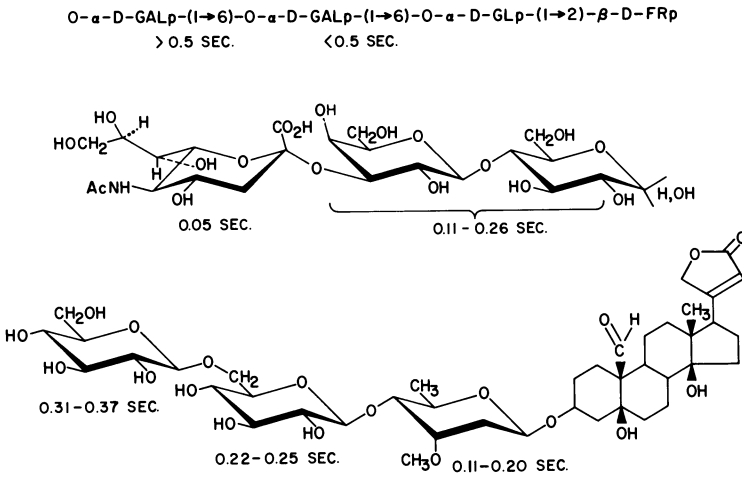


Figure 22. T_1 values of ^{13}C nuclei in oligomeric compounds

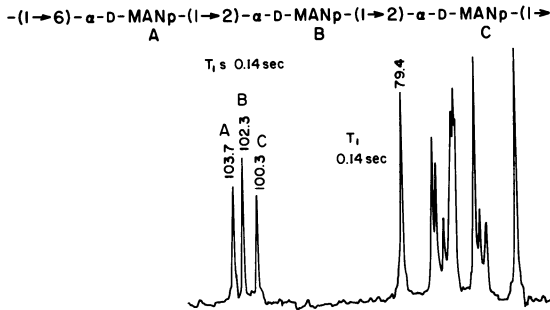


Figure 23. ^{13}C NMR spectrum and structure of linear mannan from *H. capsulata*

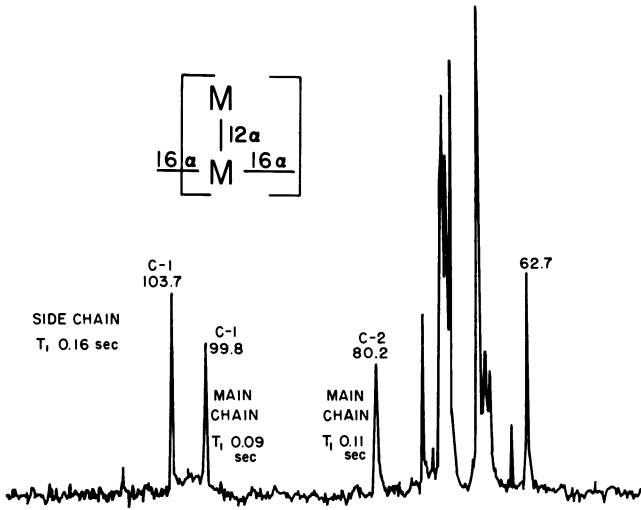


Figure 24. T_1 values of nuclei in main chain and side chains of mannan of *Saccharomyces fragilis*

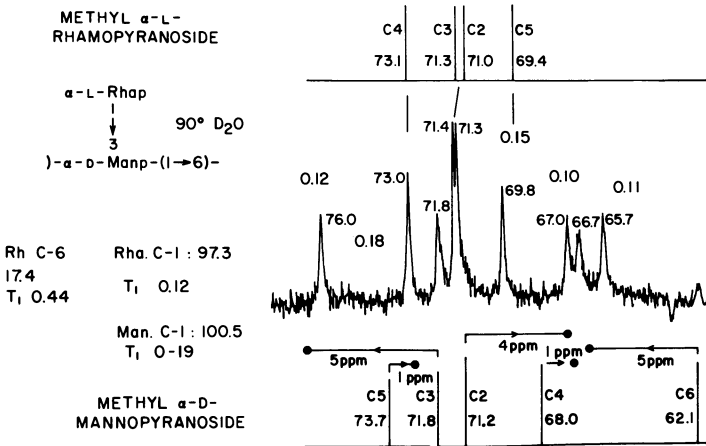


Figure 25. Partial ^{13}C NMR spectrum and T_1 values of nuclei of rhamnomanan of *S. schenckii*

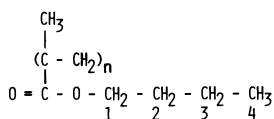
Figure 26. Poly(*n*-butylmethacrylate)

Table VII. ^{13}C Spin-Lattice Relaxation Times and $n\text{Oe}$ Values of Resonances of Poly(*n*-butylmethacrylate) as 50% (w/w) Solution in Toluene- $^2\text{H}_6$

TEMP. °C	SIDE CHAIN				MAIN CHAIN	
	C-1	C-2	C-3	C-4	CH ₂	C
	T_1 (67.9 MHz) SEC.					
6	0.35	0.23	0.39	1.0	0.21	2.3
111	0.35	1.4	3.4	6.2	0.11	1.6
	T_1 (22.6 MHz) SEC.					
10	0.13	0.20	0.45	1.1	--	0.69
105	0.15	0.67	1.4	2.8	0.04	0.70
	NOE (67.9 MHz)					
14	2.4	2.7	--	2.8	1.67	1.47
101	1.42	1.98	--	2.3	1.36	1.59
	NOE (22.6 MHz)					
11	1.47	2.5	--	2.6	--	1.89
104	2.1	2.3	--	2.4	1.59	2.1

67.9 MHz there is more than twofold increase of T_1 values of both the main chain and the side-chain nuclei.

Abstract

Carbon-13 nuclear magnetic resonance spectroscopy is useful in polysaccharide chemistry for, (1) determining regularity or irregularity of structural sequences, (2) elucidating chemical structures, such as ring size, configuration of glycosidic linkage, position of glycosidic substitution, and position of ester substitution, (3) monitoring polysaccharide production in a multicomponent system, and (4) interpreting molecular motion, which is sometimes of aid in signal assignment.

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Structural Aspects of Exocellular Yeast Polysaccharides

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Exocellular is a term employed restrictively in this review to describe polysaccharides elaborated into the medium, away from the cells, during submerged cultivation. It excludes cell-bound, capsular or cellular polysaccharides which may be liberated, often in small amounts, through physical and autolytic processes. The exocellular biopolymers from yeast contain remarkable examples of biochemical diversity. They have been of some significance to yeast taxonomy and immunology and could well have application in industry and agriculture. There has been no overall review in English of new structures since the beginning of this decade (1,2).

The known types of yeast polysaccharides are anionic, wherein the anion is either glycuronate or phosphate diester, and neutral. Because they are, for the most part, distinctive for the yeast genera that produce them, the polysaccharides will be subdivided accordingly in the discussion that follows.

Glycuronate Anionic Polysaccharides

Tremella/Cryptococcus. The first definitive compositional and structural studies (3,4) on a member of this family of polysaccharides were done on the heteropolysaccharide produced by C. laurentii var. flavescens NRRL Y-1401. Because haploid, yeast-like species of the basidiomycetous genus Tremella formed glucuronoxylomannans (GXM) similar to those from species of Cryptococcus, a taxonomic relationship was suggested between the genera (5,6). Basidiomycetous perfect states were subsequently discovered for C. laurentii (7) and C. neoformans (8).

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Table I
 Glucuronoxylomannans: Cryptococcus/Tremella

Organism	Molar ratios relative to <u>D</u> -glucuronic acid				Reference
	$[\alpha]_D$	<u>D</u> -Xyl	<u>D</u> -Man	OAc	
<u>C. laurentii</u>	{ +21°	2	5	1.5	(3)
	{ +38°	1	4		(4)
<u>C. neoformans</u>	{	2	5 ^a		(10)
		2	3 ^b		
<u>T. mesenterica</u>	{ -26°	4	4	0.6	(5)
	{ -27°	7	5	0.7	(9)
<u>T. fusiformis</u>		2	8		(11)

^a Serotype A (E. Reiss, unpublished).

^b Serotype B; also contains 0.5 mole D-galactose.

Selected data on composition and optical rotations are listed in Table I in order to illustrate some pertinent factors. Molar ratios of sugar components cited for T. mesenterica GXM are from work with the same strain (NRRL Y-6158) in different laboratories (5,9). A similar comparison is made for work on GXM from C. laurentii Y-1401 (3,4). Apparently, variation in composition can occur due to different conditions of production. The C. neoformans polysaccharides are from serotypes A (E. Reiss, unpublished) and B (10). Data on T. fusiformis GXM is from recent work by Sone and Misaki (11) on material extracted from the outer cell wall of the haploid form. Similar polysaccharides have been isolated from fruiting bodies (12) and cell walls (13,14) of the perfect stages of T. mesenterica and other Heterobasidiæ.

Table I further illustrates that the exocellular acidic polysaccharides from Tremella are generally more highly xylosylated than those from Cryptococcus and display more negative optical rotations (5). Positive increases in specific rotations of partially dextrosylated T. mesenterica GXM (6,9) suggest that D-xylosyl residues occur as β -linked outer chains.

Table II summarizes methylation analyses carried out on certain GXM listed in Table I. Analysis of the carboxy-reduced C. neoformans serotype A polysaccharide was recently performed at NRRC in cooperation with Dr. Errol Reiss of the Center for Disease Control, Atlanta. The results are in accord with a structure consisting of a main chain of (1 \rightarrow 3)-linked D-mannosyl residues, two-thirds of which are also (1 \rightarrow 2)-linked by single residue branches of D-xylose and D-glucuronic acid. (All sugar residues referred to in text and figures are in the pyranose form.) The polysaccharide from the cell walls of T. fusiformis has a related structure. It appears, however, that there are also backbone

(1→3)-linked D-mannosyl residues (1→2)-linked by single or short side-chains of D-mannose (11).

A more complex, highly branched structure emerged (Table II and Figure 1) from methylation studies on the GXM of T. mesenterica Y-6158 (9). To account for the percentage of mannosyl residues not involved in branching, D-xylose was considered to occur as the indicated (1→2)-linked side chain sequences. Although a majority of the backbone D-mannosyl residues are involved in branching at C-2, there is also some branching at C-4. The linkage involving D-glucuronic acid was confirmed by complete characterization of the aldoburonic acid, 2-O-β-(D-glucopyranosyluronic acid)-D-mannose, liberated by acid hydrolysis. This disaccharide had previously been obtained from T. mesenterica Y-6151 (6).

Further interesting structural features of GXM were revealed in a following publication (15). It had been previously observed (16) that, in contrast to the heteropolysaccharide from C. laurentii Y-1401, that from T. mesenterica Y-6151 did not cross-react with antisera to Type II pneumococcal capsular polysaccharide even after partial dexylosylation by mild acid hydrolysis. Fraser and coworkers (15) extended this observation to the polysaccharide from strain Y-6158 and demonstrated that, even through the majority of the glucuronic acid residues in the T. mesenterica polysaccharides were protected from periodate oxidation by 3-O-acetylation, deacetylation did not bring about an increase in precipitability with a Type II antiserum. Smith degradation, with simultaneous deacetylation and removal of all the xylose residues, left glucuronomannans that were precipitable by the antiserum. Consequently, it was concluded that, in the Tremella polysaccharides, the xylose side chains are situated so as to sterically hinder interaction of the glucuronic acid determinants with the antiserum. It is noteworthy that the periodate-resistant mannan remaining after a single Smith degradation of polysaccharide Y-1401 and successive degradations of polysaccharide Y-6158 had respective $[\alpha]_D$ of +44° and +89°. The lower rotation suggests incomplete degradation to an α-(1→3)-linked, polymannose, main chain.

Other questions to be resolved for the Tremella/Cryptococcus class of polysaccharides include sequence and spacing of substituent side-chains along the mannose backbone. Knowledge of such details could, perhaps, explain the low antigenicity of C. neoformans (17).

Lipomyces. When component analyses were first carried out on heteropolysaccharides from these soil yeasts, it was recognized that the compositional differences had taxonomic value (18). As shown in Table III, polysaccharides from L. lipofer are glucuronomannans while those from strains of L. starkeyi also contain residues of D-galactose. Both types are O-acetylated. It was further noted that acid hydrolysis liberated from L. starkeyi

Table II
Methylation of Glucuronoxylomannans
O-Methyl ethers of

Organism	D-Xyl		D-Man				D-GlcU
	2,3,4-	3,4-	2,3,4,6-	2,4,6-	2,6-	4,6-	2,3,4-
<i>C. neoformans</i>	2			2		3	1 ^a
<i>T. mesenterica</i>	3	3	0.5	1	1	3	1
<i>T. fusiformis</i>	2	tr	2	5		5	1

^a Determined as a derivative of 2,3,4,6-tetra-O-methyl-D-glucose from the carboxy-reduced polysaccharide.

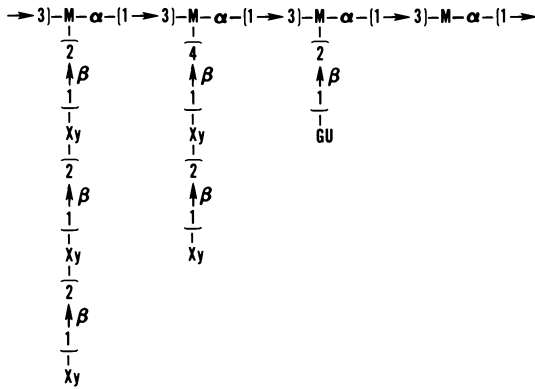


Figure 1. Schematic average repeat unit of exocellular polysaccharide from *Tremella mesenterica* NRRL Y-6158: GU, D-glucuronic acid; M, D-mannose; Xy, D-xylose (9)

polysaccharide a trisaccharide composed of \underline{D} -mannose and \underline{D} -glucuronic acid in the molar ratio 1:2.

Detailed structural investigations of *Lipomyces* polysaccharides have been carried out in Moscow by Kochetkov and coworkers. Initial examination (19) of a glucuronomannan from *L. lipofer* included isolation and characterization of the aldoburonic acid 3-O- α -(\underline{D} -glucopyranosyluronic acid)- \underline{D} -mannose, methylation analysis, and Hoffmann degradation of the amide of the methylated polysaccharide. The latter procedure yielded a methylated form of a (1 \rightarrow 4)-linked mannobiose. A later investigation (20), which included oxidation of the reduced polysaccharide acetate by CrO_3 and alkaline degradation of the methylated native polysaccharide, established the linear glucuronomannan repeat until structure depicted in Figure 2.

The Hoffmann degradation was also employed (21) to investigate the galactosylated polysaccharide from a strain of *L. tetrasporus* (Table III and Figure 2). This decomposition gave a methylated derivative of 4-O- β - \underline{D} -mannopyranosyl- \underline{D} -mannose. Oxidation of the reduced polysaccharide acetate with CrO_3 in acetic acid and subsequent Hakomori methylation of the reduced di- and trisaccharide products led to their identification as 2-O- α - \underline{D} -glucopyranosyl- \underline{D} -glucose and 2-O- α - \underline{D} -glucopyranosyl(3-O- \underline{D} -galactopyranosyl)- \underline{D} -glucose. The presence of adjacent glucosyl residues derived from reduction of the glucuronic acid residues is reminiscent of the trisaccharide from *L. starkeyi* polysaccharide. Analyses of glycosyl polyols also present in reduced mixture of oxidation products provided further evidence for a proposed repeat unit (Figure 2) that was modified by more recent work (22) in which a β -anomeric configuration was assigned to the \underline{D} -galactopyranosyl end group on the basis of ^{13}C -n.m.r. spectroscopy and reevaluated conditions for oxidation with CrO_3 . The point of branching was confirmed by identification of 4,6-di-O-methyl-1,2,3,5-tetra-O-acetyl- \underline{D} -sorbitol as the sole di-O-methyl product of methylation analysis of the carboxy-reduced polysaccharide.

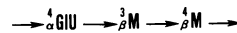
A further note: It had been observed (23) that deacetylation of an *L. starkeyi* galactoglucuronomannan abolished its cross-reactivity to type XVIII antipneumococcal serum. Earlier work had suggested (24) that an O-acetylated α - \underline{D} -galactosyl residue was the immunodominant sugar in type XVIII pneumococcal polysaccharide. We recently found that prior de-O-acetylation is required for full susceptibility of all *L. starkeyi* polysaccharides tested to oxidation by \underline{D} -galactose: O_2 oxidoreductase.

Rhinocladia Glucosaminuronans. Two black yeast-like fungi and their unusual acidic extracellular polysaccharides were studied at NRRC. *R. mansonii* Y-6272 forms a heteropolysaccharide, $[\alpha]_{\underline{D}}^{-6^\circ}$, composed of residues of 2-acetamido-2-deoxy- \underline{D} -glucose and 2-acetamido-2-deoxy- \underline{D} -glucuronic acid in a molar ratio of approximately 2:1 (25,26). *R. elatior* NRRL YB-4163 was later found to produce an apparent homopolysaccharide, $[\alpha]_{\underline{D}}^{-75^\circ}$, consisting of 2-

Table III
Exopolysaccharides of Lipomyces

Yeast	Molar ratios of				Reference
	<u>D</u> -Gal	<u>D</u> -Man	<u>D</u> -GlcU	OAc	
<u>L. lipofer</u>	--	2	1	1	(23)
<u>L. starkeyi</u>	2	1	2	1	(23)
<u>L. starkeyi</u>	1	2	2	1	(23)
<u>L. tetrasporus</u>	0.4	2	2		(21)

Lipomyces lipofer: Glucuronomannan

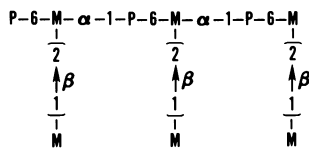


L. tetrasporus: Galactoglucuronomannan



Figure 2. Repeat unit structures of *Lipomyces polysaccharides*; Gal, D-galactose; GIU, D-glucuronic acid; M, D-mannose (20, 54)

Hansenula capsulata



Hansenula holstii

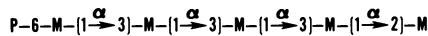


Figure 3. Repeat units of poly(phosphoric diester) O-phosphonomannans; the unit depicted for *Hansenula capsulata* is a trimer

acetamido-2-deoxy-D-glucuronic acid residues (27). Complete inertness of both polysaccharides to periodate at 20°C indicated linkage through 3- or 4-positions, or both. The optical rotations indicated primarily β -linkages in the homopolysaccharide and possibly mixed α and β in the heteropolymer. Recent methylation and ^{13}C -n.m.r. studies by Lindberg and coworkers (55) reveal a linear, β -(1 \rightarrow 4)-linked structure for the homopolysaccharide and a simple, regular, β -(1 \rightarrow 3)-linked structure for the heteropolysaccharide. As for the low levorotation of the latter, it should be noted that the analogous curdian, primarily a β -(1 \rightarrow 3)-D-glucan, gives $[\alpha]_D^{18^\circ}$ in alkali (56).

O-Phosphonohexoglycans

Exocellular yeast O-phosphonomannans (formerly, "phosphomannans") were the first shown to contain the phosphodiester structure D-mannose 6-(D-mannosyl phosphate) (28,29). It was subsequently found that exocellular (30) and cell wall (31) O-phosphonomannans of yeast include a variety of structural types which have in common the D-mannose 6-phosphate moiety of the phosphoric diesters. The results of ^{31}P -n.m.r. spectroscopy on the various O-phosphonoglycans have been interpreted as indicating that within a given polymer all phosphoric diester groups are in a similar electronic environment and, considering those examples of known configuration, that the anomeric sugar phosphate linkages are likely all α (32). O-Phosphonomannans are elaborated into the culture medium, often in very high yields, by yeasts belonging to the genus *Hansenula* and related genera (33). Strains of *Sporobolomyces* are also known that produce O-acetyl-O-phosphonoglucogalactans (34,35).

Two types of O-phosphonomannan have been distinguished. In the first type, macromolecular structure is primarily dependent upon phosphoric diester linkages between mannose oligosaccharides. The second type are polysaccharides in which the glycosyl phosphate residues occur exclusively as nonreducing end groups.

Figure 3 depicts repeat units that have been isolated from autohydrolyzates of those O-phosphonomannans which are, for the most part, poly(phosphoric diesters) of D-mannose oligosaccharides. These repeat units constitute $\sim 90\%$ of the macromolecular structure. The remaining portions, although quite different in each example shown, are high molecular weight; i.e., mannan-like. Partial autohydrolysis of *H. capsulata* O-phosphonomannan (mannose:P ~ 2.5) selectively cleaves hemiacetal phosphate linkages and liberates a β -(1 \rightarrow 2)-linked disaccharide monophosphate (36) together with lesser amounts of a dimer, trimer (shown in Figure 3), and a lightly phosphorylated α -D-mannan (30). The true structure of the monomer, 6-O-phosphono-(2-O- β -D-mannopyranosyl-)D-mannose, is suggested by Gorin on the basis of ^{13}C -n.m.r. spectroscopy (37). The structure depicted for the trimer is compatible with Gorin's data. A model mannose phosphodiester has been synthesized (38).

Its acid and alkaline hydrolytic behavior is the same as that displayed by the known O-phosphomannans.

Autohydrolysis of H. holstii O-phosphomannan (mannose:P ~5) liberates a pentasaccharide monophosphate of the apparent structure given in Figure 3. The structure of the dephosphorylated oligosaccharide was deduced from a methylation analysis (39) and accords with earlier Smith degradations of the intact biopolymer (40). Of particular current interest is the high-molecular-weight coproduct of autohydrolysis. It is a highly phosphorylated (mannose:P ~6) monoester (32) and, as such, must represent the "core" or backbone of the native biopolymer (41). Supporting this notion is the observation that concanavalin A strongly interacts with the core, but not at all with the intact O-phosphomannan produced by H. holstii Y-2448 (41). Apparently, both the core and the oligosaccharide α -(1 \rightarrow 2)-linked mannosyl residue involved in hemiacetal phosphate are shielded by succeeding α -(1 \rightarrow 3)-linked residues in the side chains. The nature of possible nonreducing end group(s) in the evidently highly branched biopolymer is not known. Recent interest by several laboratories in the high-molecular-weight phosphomonoester fragment is occasioned by its extraordinary potency as an inhibitor of pinocytosis of human β -glucuronidase by human fibroblasts (42). There is a growing body of evidence that relatively acidic "high uptake" forms of such lysosomal hydrolases bear monoester phosphate markers which are recognized by fibroblast receptors (43).

Table IV lists those yeasts (other than the aforementioned Sporobolomyces sp.) that synthesize polysaccharide-like O-phosphoglycans along with the various α -D-glycose 1-phosphate end groups found thus far (30). A new aspect of the "phosphomannan" story is introduced in this table: When grown in the absence of added orthophosphate, almost all such yeasts alternatively synthesize neutral α -D-mannans and glucomannans. Common biogenetic origin of the neutral and phosphorylated mannans and glucomannans is evidenced by similar amounts of D-glucose or its absence in the biopolymers produced by a given strain of yeast.

Neutral Polysaccharides

α -D-Mannans. Table V summarizes the results of methylation analyses performed (44) on the series of α -D-mannans produced by yeasts that, with the exception of Pichia mucosa YB-1344, can also make O-phosphomannans. Mannans of low degree of branching are synthesized by H. capsulata and H. holstii. The former produces a mannan with an approximately 2:1 ratio of (1 \rightarrow 2)-:(1 \rightarrow 6)-linked residues; the latter forms a mannan comprising (1 \rightarrow 2)- and (1 \rightarrow 3)-linked residues in nearly 1:1 ratio. As might be expected for a mannan with only a small percentage of residues linked (1 \rightarrow 6), H. holstii mannan was resistant to mild acetolysis, a procedure that preferentially cleaves (1 \rightarrow 6) linkages.

Table IV
Highly Branched Neutral Mannans; Corresponding O-Phosphonoglycans

Yeast	Neutral/ <u>O</u> -phosphono-	α -Glycosyl-P-
<u>Hansenula minuta</u>	α - <u>D</u> -Glucomanan	<u>D</u> -Mannosyl
<u>Hansenula henricii</u>	α - <u>D</u> -Glucomanan	<u>D</u> -Glucosyl
<u>Pichia pinus</u>	α - <u>D</u> -Glucomanan	<u>D</u> -Glucosyl
<u>Pichia</u> sp.	α - <u>D</u> -Mannan	{ <u>D</u> -Mannosyl <u>D</u> -Mannobiosyl
<u>Torulopsis pinus</u>	α - <u>D</u> -Mannan	{ <u>D</u> -Glucosyl <u>D</u> -Mannanosyl- <u>D</u> -glucosyl

Table V
Methylation of Yeast Mannans

Yeast type	Percentages of <u>O</u> -methyl ethers					
	Tetra	3,4,6	2,4,6	2,3,4	3,4	2,4
<u>Hansenula capsulata</u>	2	58	--	38	2	--
<u>Hansenula holstii</u>	4	48	44	--	4	--
<u>Pichia</u> sp.	28	20	25	--	--	24
<u>Torulopsis pinus</u>	19	54	--	10	17	--
<u>Pichia mucosa</u>	23	4	48	3	2	20

Structures of the highly branched mannans were considered in terms of the (1→6)-linked backbone commonly found in mannans extracted from the cell walls of yeasts (31). Distinctive differences are seen both in the types of linkages connecting side-chain mannosyl residues and in those at points of branching from the main chains. Acetolysis revealed, for all such mannans, heterogeneous distribution of side-chain lengths (44). An attempt was made to assess the composition of side chains by partial degradations with α -D-mannosidase as described by Jones and Ballou (45). The various mannans were subjected to methylation analyses after they had undergone 30-70% degradation (M. E. Slodki, M. C. Cadmus, F. R. Seymour, R. M. Stodola, unpublished results). Only minor changes were noted for the more linear mannans, which suggest that they comprise regular, repeat-unit sequences rather than block sequences of mannosyl residues in the same linkage.

More extensive changes in patterns of methylated fragments occurred in the mannans from *P. mucosa* and *Torulopsis pinus* Y-2023. The major change in *P. mucosa* mannan after 29% degradation consists in losses of (1→3)-linked side-chains and emergence of unbranched (1→6)-linked backbone residues. Acetolysis, which yields the non-(1→6)-linked side-chain plus the backbone residue to which it is attached, had shown an average side-chain length of 2.8 residues (45). The most prominent oligosaccharide present was a tetrasaccharide. Professor Ballou (personal communication) has recently isolated a fully (1→3)-linked linear tetrasaccharide from an acetolyzate of the mannan. The greatest change in methylation pattern as a result of enzymic degradation occurred in *T. pinus* mannan. Here, α -(1→2)-linked side-chains were rapidly eroded down to the backbone mannosyl residues.

α -D-Glucomannans. Unlike the neutral α -D-mannans, complete methylation of the α -D-glucomannans has been difficult to achieve. Our most recent methylation-fragmentation analyses have been successful and indicate that the three polysaccharides examined are quite similar. D-Glucose occurs exclusively as nonreducing end groups; only a minor percentage of such end groups is D-mannose. This result explains the resistance of these polysaccharides to degradation by the exo- α -mannosidase (30). According to the (1→6)-linked backbone hypothesis, the results suggest that (1→2)-linked side-chains terminated by D-glucosyl end groups are attached through (1→3)-linkages to mannosyl residues of the main chain. The glucomannans give similar HPL chromatograms (M. E. Slodki and F. R. Seymour, unpublished results) of oligosaccharides resulting from acetolysis. A pentasaccharide predominates in all three acetolyzates; i.e., the major side-chain consists of four residues.

Pentosylmannans. Gorin and Spencer (46) found that three of seven *Trichosporon* species examined formed pentosyl mannans. That from *T. cutaneum* contained L-arabinose in addition to D-xylose and

D-mannose. Smith degradation destroyed the side-chain residues and left an α -(1 \rightarrow 3)-linked mannan backbone, $[\alpha]_D^{+43^\circ}$. Partial acid hydrolysis liberated D-xylose and the disaccharide 4-O- α -L-arabinopyranosyl-D-xylose. These results, together with those of methylation analyses, suggested a branched structure wherein some of the (1 \rightarrow 3)-linked mannosyl backbone residues are substituted with D-xylopyranosyl, D-mannopyranosyl and 4-O- α -L-arabinopyranosyl-D-xylopyranosyl units.

β -D-Mannans. Species of *Rhodotorula* give similar exocellular β -D-mannans (47). The most extensive structural investigations were carried out on the mannan from the best producer, *R. glutinis*. An alternating arrangement of β -(1 \rightarrow 3)- and β -(1 \rightarrow 4)-linked residues was deduced from methylation analysis and characterization of di- and trisaccharides liberated by partial acid hydrolysis. Crystalline 4-O- β -D-mannopyranosyl-D-mannose was isolated in 67% yield. The trisaccharide was identified as 3-O- β -D-mannopyranosyl-4-O- β -D-mannopyranosyl-D-mannose. Many species of *Rhodotorula* (48,49) and also one of *Sporobolomyces* (50) have since been found to produce this type of polysaccharide.

Fucogalactan. Fukagawa and coworkers described production (51) and structural characterization (52) of this polysaccharide. It is produced by a soil isolate classified as a strain of *Rhodotorula glutinis*. The polysaccharide, $[\alpha]_D^{-38^\circ}$, contains L-fucose:D-galactose:0-acetyl in respective molar ratios of 1:1:0.6. Partial acid hydrolysis removed L-fucose residues and afforded a residual D-galactan of $[\alpha]_D^{+81^\circ}$. Smith degradation of the deacetylated fucogalactan gave a polysaccharide composed of galactose and fucose in approximate 4:1 molar ratio. A second such degradation destroyed the remaining fucose and gave an insoluble galactan. Analysis of the low-molecular-weight products of Smith degradation indicated that galactose residues present in side-chains, likely in (1 \rightarrow 6)-linkage, were oxidized by periodate in the deacetylated fucogalactan but were protected in the native polysaccharide. Partial acetolysis gave disaccharide products in which almost all of the fucose and some of the galactose residues were (1 \rightarrow 2)-linked. Since over half of the acetolysis product was predominantly free fucose, it appears that almost all of these residues are either nonreducing end groups or are linked at C-2.

The preceding discussion has reviewed the extent of recently acquired knowledge about the structures of exocellular yeast polysaccharides and to point up some of the gaps that remain. Much of the work described has provided useful chemotaxonomic information. We have further seen that structural features discerned for a given polysaccharide can lead to more rapid understanding of a related one. In this process, the work of the structure chemist often leads the biologist to explore hitherto unperceived relationships among organisms. A good example to watch is the recent work (53,54) which has uncovered remarkable

similarity between the polysaccharides of Lipomyces lipofer and those of several mycelial Ascomycetes that bear little apparent morphological similarity to the former.

Abstract

Like other microorganisms, yeasts that produce exocellular polysaccharides belong to taxonomically related groups whose members are characteristically mucoid. Within each group, similar polysaccharides are formed which differ with regard to molar ratios and linkages of component sugars as well as degree of O-acylation. Sufficient information is now available to permit review and comparison of structural details of polysaccharides elaborated by yeasts and yeast phases of certain fungi which either belong to or are related to the genera Hansenula, Rhodotorula, Cryptococcus, Lipomyces, and Rhinocladiella.

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Structure and Enzymatic Degradation of Elsinan, a New α -D-Glucan Produced by *Elsinoe leucospila*

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Many microorganisms are known to accumulate significant amounts of extracellular polysaccharides during their growth under suitable cultural conditions. Currently, however, commercial production of the microbial polysaccharides are limited to dextran, produced by *Leuconostoc mescenseoides*, scleroglucan (polytran) by *Sclerotium* species and the related fungi, xanthan by *Xanthomonas campestris* (1), and pullulan by *Aureobasidium pullulans* (2). A few other polysaccharides, such as β -(1 \rightarrow 3)-D-glucan, like curdlan produced by a mutant of *Alcaligenes faecalis* (3), appear to be under commercial exploitation. These polysaccharides possess unique properties, useful in food and pharmaceutical industries, as plasma expanders, food additives, edible packing films and other applications.

Apart from above industrial utilization, the water insoluble, and adherent α -(1 \rightarrow 3)-linked D-glucans, elaborated by cariogenic *Streptococcus mutans*, have recently attracted much attention, in relation to the role in the formation of dental caries (4).

In the course of searches of new microbial polysaccharides having unique structures and useful properties, we became aware that a fungus isolated from a spot of the white scab of the tea leaves, identified as *Elsinoe leucospila* (5), produces a mucous layer, when grown on sucrose-potato extract agar. The chemical investigations of this polysaccharide indicated it to be a new type of α -D-glucan containing both (1 \rightarrow 4)- and (1 \rightarrow 3)-D-glucosidic linkages. The glucan was designated as Elsinan, since other fungi belonging to *Elsinoe* species were found to produce similar glucans.

In this paper, we describe the production, detailed structure, enzymatic degradation and isolation of novel oligosaccharides, and also derivatization, such as 3,6-anhydro-elsinan. Some of rheological properties and potential utilization of this glucan will also be discussed.

Production of Elsinan

In a preliminary study, the conidia of *E. leucospila* strain CS-1 were suspended in sterilized water and inoculated on a cellophane sheet which covers the agar-plate containing 2% sucrose and potato-extract. After growing at 24° for 4 - 5 days, the slimy colonies on the cellophane sheet were carefully collected, suspended in water, centrifuged, and the polysaccharide in the supernatant was precipitated by addition of 3 volumes of ethanol. After purification by dialysis followed by repeated precipitations with ethanol, the polysaccharide was shown to comprise solely D-glucose. The glucan did not give any color when reacted with iodine, although structural analyses showed the presence of a high proportion of α -(1+4)-D-glucosidic linkages. These findings prompted us to investigate the detailed structure and properties of the polysaccharide.

For the production of elsinan by submerged culture, the fungus was grown in a medium containing 5% sucrose, either potato-extract (dialyzate of hot water extract of fresh potato (300 g per 1 liter medium)) or 0.5% corn steep liquor (CSL) plus 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.05% KCl. The fermentation was carried out in shaking flasks in a small fermentor (10 liter volume) with aeration (equal volume to that of medium per min), at 24° for 4 - 6 days.

The time course of the production of elsinan by the submerged culture is shown in Figure 1. The glucan formation reaches the maximum after 5 - 6 days with rapid consumption of sucrose. The reducing sugars released during the fermentation contained both glucose and fructose at the early stage, and are gradually utilized. The viscous cultural broth was clarified by centrifugation and the crude polysaccharide was precipitated from the supernatant by addition of 3 volumes of ethanol or acetone. It was dissolved in water, dialyzed and precipitated again with ethanol, and then lyophilized; yield 23 - 25 g per liter of broth.

Figure 2 shows the effect of various carbon-sources (each 5%) on the production of elsinan, when CSL and NaNO₃ were used as nitrogen source. Among the different carbohydrates or polyhydroxy alcohols tried, sucrose and fructose seem to be most effectively utilized for production of elsinan (2.6 and 2.3 g, respectively, from 100 ml medium). Glucose was the best carbon source for growth of the fungus, but the yield of the glucan was less than from sucrose. Galactose and glucitol were very poor carbon sources.

Chemical Properties of Elsinan (6)

The polysaccharide purified from the cultural filtrate is essentially free from nitrogen compound (N < 0.1%), and is composed solely of D-glucose, as revealed by paper- and

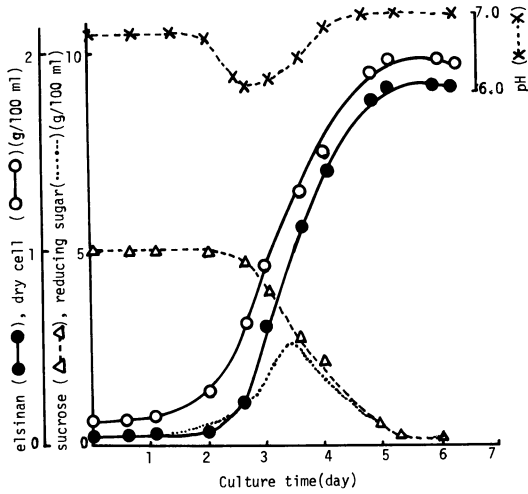


Figure 1. Time course of production of elsinan. Five liter medium containing sucrose was fermented with aeration, 5–6.5 L/min and agitation, 300 rpm, at 24°C: (●) elsinan; (○) dry cell; (–△–) sucrose; (–×–) pH; (· · ·) reducing sugar.

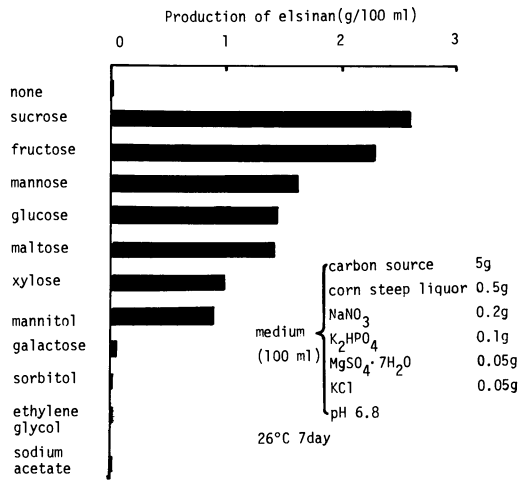


Figure 2. Effects of carbon sources on the production of elsinan

gas-liquid chromatography (g.l.c.), after complete hydrolysis by heating with M-sulfuric acid for 5 h. The homogeneity of elsinan was assessed by ultracentrifugal analysis ($S_{20,w} = 5.92 \times 10^{-13}$). The molecular

weight of the native glucan was in a range of $2 - 6 \times 10^6$, as estimated by gel exclusion chromatography on a Sepharose column. The high specific rotation, $[\alpha]_D + 243^\circ$ (c,0.8, water) and $+ 239^\circ$ (c,0.8, M NaOH), and the characteristic absorbance at 840 cm^{-1} in i.r. spectrum indicate that the D-glucosidic linkages are of α -configuration.

The glucan is readily soluble in warm water to give a highly viscous solution, having an intrinsic viscosity, $[\eta] 1.86$ at 25° . Although its aqueous solutions are stable at low concentrations, they tend to form gels at higher concentration. Some viscosity properties will be discussed later.

Structure of Elsinan (6)

Mode of D-glucosidic linkages For the linkage analysis, the glucan synthesized from sucrose was methylated by the method of Hakomori (7), and the methylated glucan was hydrolyzed with acid. The partially methylated sugars were analyzed by g.l.c.-m.s. at 180° using a ECNSS-M column, after conversion into their corresponding alditol acetates. The result showed the presence of 2,3,6-tri-(70.8%) and 2,4,6-tri-O-methyl-D-glucose (28%), together with small proportions of 2,3,4,6-tetra-(0.7%) and 2,4-di-O-methyl-D-glucose (0.5). Although the retention times of 2,3,6- and 2,3,4-tri-O-methyl-D-glucose were very close to each other, under the conditions employed, the 2,3,6-tri-O-methylglucose was identified as the methyl glucoside by g.l.c..

All the glucan preparations elaborated from different carbon sources were found by methylation analysis to have essentially the same structures (Table I). The glucan produced from a medium containing glucose appears to have a slightly higher content of (1 \rightarrow 4)-linkages than the polysaccharides from other carbohydrate sources. However, there may be no essential structural difference between the glucans prepared from various sources.

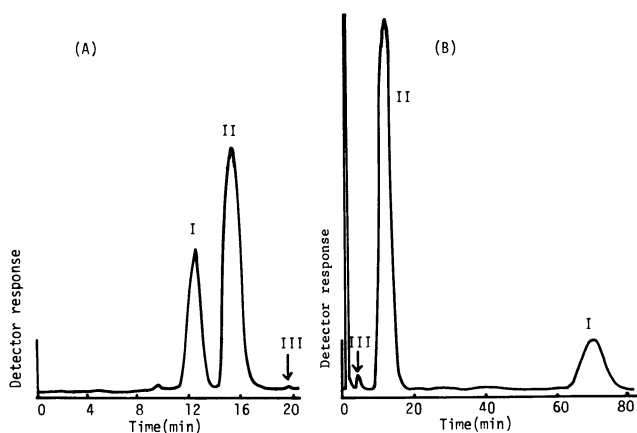
These glucosidic linkages assigned were also supported by the results of periodate oxidation and Smith degradation. Elsinan was oxidized with 0.03 M sodium periodate at 4° , and after complete oxidation (5 days; periodate consumption, 0.80 mole, and formic acid production, 0.07 mole per glucose residue), the oxidized glucan was reduced with sodium borohydride. Erythritol, glucose and a trace of glycerol in the hydrolysis product were quantitatively analyzed by high performance liquid chromatography (h.p.l.c.), and also by g.l.c., after reduction with borohydride followed by acetylation (Figure 3). Their molar proportions were

TABLE I
 YIELDS AND METHYLATION ANALYSES OF ELSINAN^a PRODUCED FROM VARIOUS
 CARBON SOURCES

Yield of elsinan (g dry weight/100 ml of broth)	Carbon source						
	2.6	2.3	1.6	1.5	1.4	1.0	0.9
0-methyl-D-glucose(%)	2.6	2.3	1.6	1.5	1.4	1.0	0.9
2,3,4,6-tetra-	0.4	0.7	0.4	1.6	0.4	0.4	0.4
2,4,6-tri-	27.9	27.4	27.3	21.4	27.6	28.0	27.6
2,3,6-tri-	71.7	71.9	72.1	76.1	72.0	71.6	72.0
2,4-di-	(trace)	(trace)	0.2	0.9	(trace)	(trace)	(trace)

^aElsinan was produced by shaking culture in a medium containing 5% of the carbon source and 0.5% of corn steep-liquor.

Carbohydr. Res., **66** (1978) 53.



Carbohydrate Research

Figure 3. Identification of the products of complete Smith degradation of elsinan. (A) analyzed with a Yanaco liquid chromatograph Model L-1030 fitted with a refractive-index indicator, on a column of SCX-1001 (6×500 mm), with water as carrier, at 25°C ; (B) analyzed by gas-liquid chromatography, on a column of 3% of ECNSS-M, programed from $100^\circ\text{--}190^\circ\text{C}$ ($6^\circ\text{C}/\text{min}$): I, glucose; II, erythritol; III, glycerol (6).

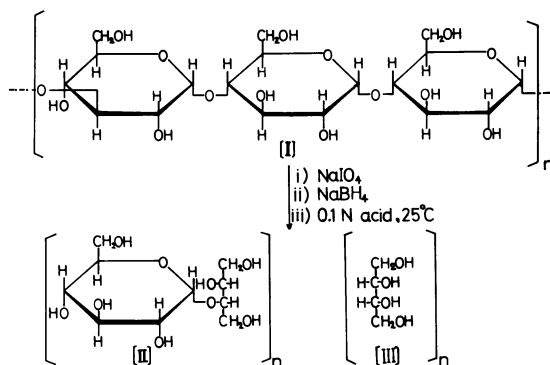


Figure 4. Smith degradation of elsinan

68.4%, 30.1% and 1.5%, respectively, as estimated by g.l.c. The erythritol and glucose should have arisen from (1→4)- and (1→3)-linked D-glucose residues, and the trace of glycerol from the non-reducing terminal ends of the slightly branched glucan. Thus, both methylation and periodate oxidation studies clearly indicate that elsinan is an essentially linear molecule consisting mainly of α -(1→4)- and (1→3)-D-glucosidic linkages in a molar ratio of 2.3 - 2.5 : 1.0. The presence of very small proportions of 2,4- and 2,3,4,6-tetra-O-methyl-D-glucose may be due to a limited proportion of branching at 0 - 6 positions of the (1→3)-linked D-glucose residues. There may be one branching point per ~140 sugar residues.

Sequence of the D-glucosidic linkages — Since elsinan contains both (1→3)- and (1→4)-linkages, the glucan was subjected to mild Smith degradation. Paper and gas liquid chromatography (as trimethylsilyl derivatives) showed the presence of erythritol and 2-O- α -D-glucosyl-D-erythritol and trace of glycerol (Figures 3 and 4). Their molar ratio was 1.44 : 1.00 : 0.04, as estimated by g.l.c.. It is evident that the erythritol arises from consecutive α -(1→4)-linked D-glucose residues, such as →4)-Glc-(1→4)-Glc-(1→, whereas 2-O- α -D-glucosyl-D-erythritol must arise from a single, α -(1→3)-linked D-glucose residue flanked by (1→4)-linkages. When the mild hydrolyzate from the glucan-polyalcohol was applied to a column of Bio-gel P-2, no appreciable peak corresponding to a polysaccharide or oligosaccharide emerged. This result suggests the essential absence of consecutive α -(1→3)-linked D-glucose residues.

Fragmentation of elsinan by partial acid hydrolysis — For examination of detailed structural features, elsinan was subjected to partial, acid hydrolysis with 0.5 M sulfuric acid for 4 h at 85°. The degradation products were fractionated by a Charcoal column, followed by preparative, paper chromatography. Table II shows the results of the fractionation.

Disaccharide — Two disaccharides were detected by paper chromatography. By methylation analysis, they were identified as nigerose and maltose, respectively.

Trisaccharide — Two trisaccharide components were separated by paper chromatography. One component ($[\alpha]_D + 152^\circ$) was identified as maltotriose. The other trisaccharide components (trisaccharide A), which gave a single spot on a paper chromatogram (R_f 0.53; butanol-pyridine-water, 6:4:3) were further fractionated by preparative liquid chromatography with borate buffer, according to Torii *et al.* (8). This procedure gave two distinct components, trisaccharide A-1 (M_{Glc} 0.28) and A-2 (M_{Glc} 0.60).

TABLE II
 YIELDS OF OLIGOSACCHARIDE FRACTIONS^a FROM PARTIAL ACID HYDROLYSIS OF ELSINAN

Fraction	Eluent(% ethanol)	Weight (mg)	Components
1	water	1160	glucose
2	4,7, and 9%(2 liters)	400	disaccharides
3	9(liters), 11, and 13%(2 liters)	208	trisaccharides
4	13(2 liters) and 15%(2 liters)	55	tri- and tetra-saccharides
5	15%(2 liters)	28	tetrasaccharides
6	20%	90	tetra- and penta-saccharides
7	25%	59	penta-, hexa-, and hepta-saccharides
8	warm 50%	22	higher oligosaccharides

^aOligosaccharides were separated by a charcoal column (4.2 X 57 cm) with stepwise elution by 4-liter portions of aqueous ethanol.

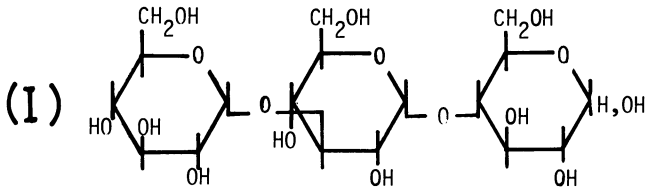
From the results of methylation analysis and the action of yeast α -D-glucosidase, trisaccharide A-1 was identified as $\text{O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-D-glucose}$ (I). Trisaccharide A-2 was characterized in a similar manner, as $\text{O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-glucose}$ (II).

Tetrasaccharide — At least, two tetrasaccharides were detected by paper chromatography, one having R_g 0.30 and the other R_g which corresponds to maltotetraose. They were separated from each other on a filter-paper sheet. The first component (R_g 0.30), which consists of (1 \rightarrow 3)- and (1 \rightarrow 4)-D-glucosidic linkages in the ratio of 1 : 2, was found by paper electrophoresis to be a mixture of two tetrasaccharides. One (M_{G1c} 0.22) was identical to $\text{O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-D-glucose}$ (III), which has been isolated from a digest of elsinan with human salivary α -amylase. The other component (M_{G1c} 0.51) may be either $\text{O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-D-glucose}$ or $\text{O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-D-glucose}$.

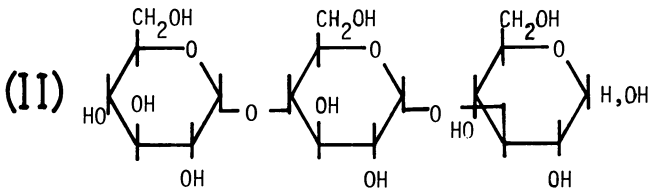
The other tetrasaccharide component (R_g 0.26) had the same R_g value as that of maltotetraose, which was identified by methylation and the action of beta amylase.

Acetolysis — Acetolysis of elsinan was performed according to the method of Matsuda *et al.* (9). The glucan (3 g) was added to a mixture of acetic anhydride (14.4 ml), acetic acid (9.6 ml), and sulfuric acid (1.8 ml) at 25°. After stirring for 7 days at 25°, the acetolysis product was obtained by pouring into iced water, and then extraction with chloroform (yield, 4.5 g). The syrup was deacetylated with sodium methoxide in methanol (yield 2.1 g). The mixture of oligosaccharides was fractionated on a charcoal column followed by paper chromatography.

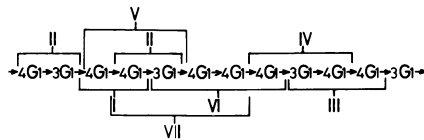
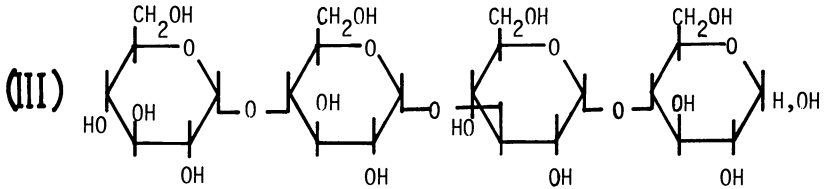
Among various oligosaccharides, nigerose and maltose were identified as disaccharides. The trisaccharide fraction contained at least two components, maltotriose, $\text{O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-D-glucose}$, and $\text{O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-D-glucose}$. The tetrasaccharide fraction appeared to be a mixture of maltotetraose and a tetrasaccharide composed of one α -(1 \rightarrow 3)- and two α -(1 \rightarrow 4)-linked D-glucose residues. Thus, the methylation and fragmentation data establish that elsinan consists of three consecutive α -(1 \rightarrow 4)-linked D-glucose residues, joined by α -(1 \rightarrow 3)-linkages. However, the ratio of (1 \rightarrow 4)- to (1 \rightarrow 3)-linkages is 2.3 - 2.5 :1, suggesting the presence of a minor proportion of maltotetraose residues in the glucan molecule. This supposition was confirmed by the isolation of maltotetraose in the partial, acid hydrolyzate. Acetolysis of elsinan also gave oligosaccharide products similar to those obtained from the partial, acid hydrolysis. The proposed structure of elsinan, and the formation of oligosaccharides by partial, acid hydrolysis is illustrated in Figure 5.



TRISACCHARIDE A-1, 3-O- α -D-GLUCOSYL-MALTOSE



TRISACCHARIDE A-2, 4-O- α -D-GLUCOSYL-NIGEROSE



Carbohydrate Research

Figure 5. Repeating unit of elsinan: I, maltose; II, nigerose; III, maltotriose; IV, 3-O- α -D-glucosylmaltose; V, 4-O- α -D-glycosylnigerose; VI, maltotetraose (6).

Elsinan has some points of structural resemblance with pullulan, an extracellular α -D-glucan of a black yeast, *Audiodbasidium pullulans* (2), and also with nigeran, the α -glucan of *Aspergillus niger* (10). Pullulan is a water-soluble, linear polymer composed of maltotriose joined by α -(1 \rightarrow 6)-linkages, instead of the (1 \rightarrow 3)-linkages found in elsinan. Furthermore, the structure of pullulan, which contains about 6% of maltotetraose (11), is again reminiscent of the presence of maltotetraose in elsinan, although further structural analysis would be necessary to determine the exact ratio of maltotetraose to maltotriose residues in elsinan. Nigeran, obtained by hot-water extraction from the mycelia of *Aspergillus niger*, contains both α -(1 \rightarrow 3)- and (1 \rightarrow 4)-D-glucosidic linkages, in an alternating sequence. Interestingly, the solubility of elsinan differs from that of nigeran. It may be noted that lichenan, a water-soluble β -D-glucan extracted from Iceland moss (*Cetraria islandica*), is a linear polysaccharide having almost the same structural sequences as elsinan, except for the opposite anomeric configurations, namely cellotriose and cellotetraose residues joined by (1 \rightarrow 3)- β -D-glucosidic linkages.

In relation to chemical taxonomy, other plant pathogenic fungi belonging to the *Elsinoe* species, such as *E. fawetti*, responsible for citrus scab, have also been found to produce extracellular α -D-glucans similar to the elsinan of *E. leucospila*.

Enzymatic Degadation of Elsinan (12)

Since elsinan has a unique structural feature, its digestability and also methanism of the enzymatic degradation have attracted our attention. In the searches of enzymes capable of degrading this glucan, we became aware of susceptibility of elsinan to particular type of α -amylase, such as salivary and pancreas amylase. This prompted us to examine various amyolytic enzymes, and isolation of the degradation products.

Enzymes — Salivary α -amylase was purified from the human saliva of adult males by ammonium sulfate precipitation (25 - 80% saturation), followed by gel-filtration on Sephadex G-200 column, and reprecipitation with ammonium sulfate (0-45% saturation). The enzyme was dissolved in 0.05 M phosphate buffer, pH 6.8, containing 0.05 M sodium chloride. Other enzymes used were hog pancreas α -amylase (type I-A; Sigma Co.), *Bacillus subtilis* α -amylase (liquefying type), *Aspergillus oryzae* α -amylase (Take amylase, purified), Termamyl amylase (Novo Ind. Co.), isoamylase of *Pseudomonas amyloclavata*, Pullulanase of *Aerobacter aerogenes*, *Bacillus subtilis* α -amylase (saccharifying type, type II-A, Sigma Co.), glucoamylase of *Rhizopus niveous* and sweet potato β -amylase.

TABLE III
ACTION OF SEVERAL ENZYME ON ELSINAN

Enzyme	Hydrolysis (%) elsinan soluble starch	Velocity (soluble starch =100)
α -amylase (saliva)	29	4
α -amylase (porcine pancreas, Sigma)	33	5.5
α -amylase (<u>Bacillus subtilis</u> , saccharifying, Seikagaku Kogyo)	35	66
α -amylase (<u>Bacillus subtilis</u> , liquefying, Seikagaku Kogyo)	0	0
α -amylase (<u>Bacillus subtilis</u> , Sigma)	0	0
α -amylase (<u>Aspergillus oryzae</u> , Sankyo)	8	4
α -amylase (Termamyl, Novo)	0	0
glucoamylase (<u>Rhizopus niveus</u> , Seikagaku Kogyo)	0	0
β -amylase (Sweet potato, Sigma)	0	0
isoamylase (<u>Pseudomonas amylocleramosa</u> , Hayashibara)	0	0
pullulanase (<u>Aerobacter aerogenes</u> , Hayashibara)	0	0

Activity of salivary α -amylase was measured by incubation of the enzyme with 0.5% soluble starch in 0.05 M phosphate buffer (0.2 ml), pH 6.8, containing 0.005 M sodium chloride at 37° for 20 min, and the reducing sugars were measured by the method of Nelson-Somogyi. One unit was defined as the amount of enzyme which produces one μ mole of reducing group (as glucose) per min. Activities of other enzymes were assayed by similar methods.

Action of Starch-degrading Enzymes — Susceptibilities of elsinan toward several amylolytic enzymes were examined, and the degrees of hydrolysis at the final stages and the initial velocities of hydrolysis of elsinan and of soluble starch are compared (Table III). It is apparent that elsinan is degraded by some α -amylase, e.g., salivary, pancreas, *B. subtilis* (saccharifying), and Taka amylase, but not with other enzymes tested here. Among these enzymes, bacterial saccharifying amylase appears to be most effective in terms of both initial hydrolysis velocity and the degree of hydrolysis (35% estimated as glucose). Nevertheless, the apparent hydrolysis rate of elsinan by this enzyme is one half toward the soluble starch.

The distribution pattern of the enzymatic digestion products after 24 h incubation with several α -amylases are compared in Table IV.

These oligosaccharides were analyzed by h.p.l.c. It must be noted that the main products by the actions of salivary, hog pancreas and bacterial saccharifying α -amylase appear to be identical to each other. It was characterized as 4-O- α -nigerosyl-D-glucose. The mode of action with Taka amylase seems to differ from those with salivary and pancreas amylases.

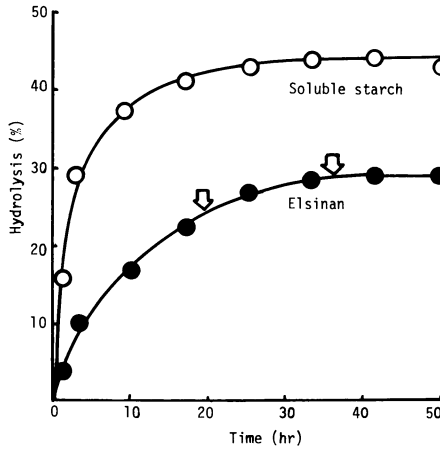
Degradation of Elsinan with Human Salivary Amylase — When the partially purified salivary amylase was acted on elsinan, it was gradually hydrolyzed as shown in Figure 6. The hydrolysis of soluble starch reaches the maximum after 5 h, with apparent hydrolysis of 44%(as glucose), while elsinan was more slowly hydrolyzed (apparent hydrolysis, 29%, after 25 h). K_m value and V of salivary amylase with elsinan were 6.9×10^{-2} M (glucose unit) and 0.12 μ mole/min. unit enzyme, respectively. Under the same condition, K_m 6.8×10^{-13} M and V_{max} 0.96 μ mole were obtained for soluble starch.

The enzyme digest from elsinan was shown by paper chromatography to contain glucose, disaccharide, trisaccharide and tetrasaccharide. Figure 7 shows the profile of h.p.l.c. of these oligosaccharides.

For characterization of major oligosaccharides, the enzyme digest was fractionated using a charcoal column, and each sugar fraction eluted with aqueous ethanol (0 to 25%) was purified by preparative paper chromatography (Whatman 3 MM, butanol-pyridine-water, 6:4:3).

TABLE IV
REACTION PRODUCT OF ELSINAN WITH SEVERAL α -AMYLASE

Enzyme	Hydrolysis (%)	Reaction product(%)						
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	G ₇
Saliva	29	12.0	0.6	75.3	12.1	0	0	0
Porcine pancreas	33	12.4	0.4	85.2	2.0	0	0	0
<u>Bacillus subtilis</u> (saccharifying)	35	7.5	0	92.5	0	0	0	0
<u>Aspergillus oryzae</u>	8	0	0	0	56.6	0	0	43.4



Journal of Applied Biochemistry

Figure 6. Time course of enzymatic hydrolysis of elsinan with human salivary α -amylase: (↙); enzyme was supplemented (12)

The disaccharide fraction ($[\alpha]_D + 130^\circ$) was identified as maltose. The trisaccharide ($[\alpha]_D + 164^\circ$, R_g 0.9 and M_{Glc} 0.25) was shown by methylation analysis to contain equal parts of (1 \rightarrow 3)- and (1 \rightarrow 4)-D-glucosidic linkages. Since the methylation analysis of the borohydride-reduced trisaccharide indicated that the reducing terminal residue is joined by (1 \rightarrow 4)-linkage, this trisaccharide was characterized as $O\text{-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-D-glucose [4-O-}\alpha\text{-nigerosyl-D-glucose]}$ (see, formula I).

The tetrasaccharide showed $[\alpha]_D + 184^\circ$, R_g 0.28 and M_{Glc} 0.23. Although chromatographic migration was close to that of maltotetraose, it consists of one mole of (1 \rightarrow 3)- and two moles of (1 \rightarrow 4)-D-glucosidic linkages per non-reducing terminal residue. Reduction followed by methylation indicated that the reducing terminal is joined by (1 \rightarrow 4)-bond. By the action of α -D-glucosidase, glucose and 4-O- α -nitrosyl-D-glucose were produced. Thus, the tetrasaccharide was characterized as $O\text{-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{3)-O-}\alpha\text{-D-glucosyl-(1}\rightarrow\text{4)-D-glucose (3-maltosyl-maltose)}$ (see, formula III).

From the isolation of 4-O- α -nigerosyl-D-glucose (74.3%) and 3-maltosyl-maltose (12.1%), salivary amylase may attack, preferentially, the glucosidic bonds between the α -(1 \rightarrow 4)-linked sugar residues, as indicated in Figure 8. The formation of 3-maltosyl-maltose can be explained by the cleavage of α -(1 \rightarrow 4)-D-glucosidic linkages in the maltotetraose units. The glucose and maltose, in addition to the above oligosaccharides might be attributed to further hydrolysis of the higher saccharides.

The action pattern of salivary, hog pancreas and *B. Subtilis* (saccharifying) enzyme seem to be very similar in respect to their initial velocities, the extents of hydrolysis and the oligosaccharides formed by the enzymatic actions.

Action of Taka Amylase — Taka amylase has a lower activity on elsinan, compared with other mammalian and bacterial α -amylases, and forms tetra- and heptasaccharide, suggesting that the enzyme possesses different specificity from other amylases toward elsinan, probably, it requires certain length of the consecutive α -(1 \rightarrow 4)-linked D-glucose residues. The Taka amylase digest of elsinan (2 g) was centrifuged, and the higher saccharides in the supernatant was precipitated with ethanol (800 mg), leaving a mixture of oligosaccharides (976 mg) in the solution. The oligosaccharide mixture was fractionated by paper chromatography, which gave tetrasaccharide and heptasaccharide fractions.

Tetrasaccharide; $[\alpha]_D + 185^\circ$ (water), R_g 0.34 and M_{Glc} 0.25

The tetrasaccharide was shown by methylation analysis to consist of (1 \rightarrow 4)- and (1 \rightarrow 3)-D-glucosidic linkages in the ratio of 2:1, and after borohydride-reduction the ratio was changed to 1:1, indicating that the reducing terminal is joined by (1 \rightarrow 4)-linkages. This tetrasaccharide yielded on partial, acid hydrolysis, maltotriose and 4-O- α -nigerosyl-D-glucose. In addition, the

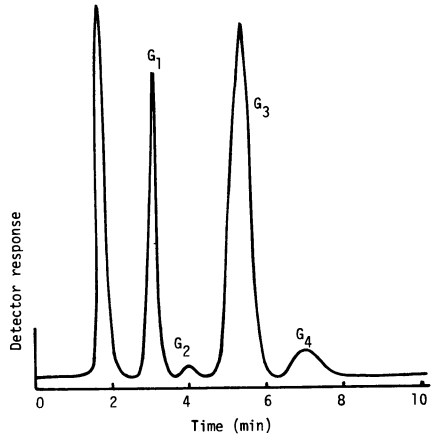
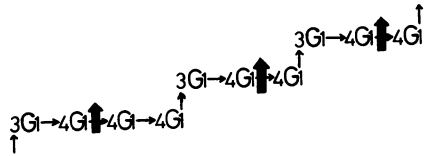


Figure 7. High performance liquid chromatography of reaction product of elsinan with human salivary α -amylase.

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Figure 8. Action pattern of human salivary α -amylase on elsinan: (\blacktriangledup) represents the sites cleaved by the enzyme; G, α -D-glucopyranosyl residue



Journal of Applied Biochemistry

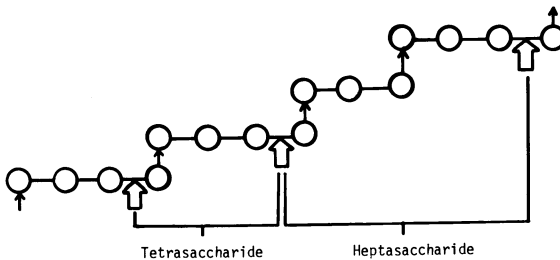
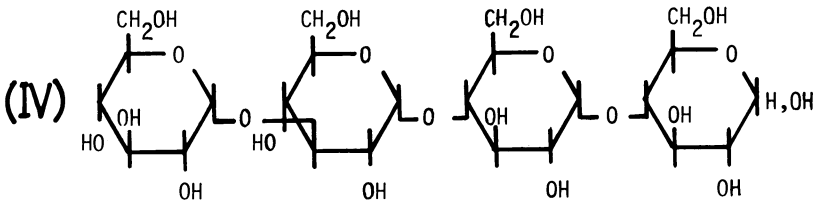


Figure 9. Possible action pattern of Taka amylase, and release of tetra- and heptasaccharide: (\blacktriangleup), site cleaved by Taka amylase; (\circ), α -D-glucopyranosyl unit; (—), (1 \rightarrow 4)-D-glucosidic linkage; (---), (1 \rightarrow 3)-D-glucosidic linkage.

tetrasaccharide was found to be resistant to α -D-glucosidase. Therefore, it was characterized as O - α -D-glucosyl-(1 \rightarrow 3)- O - α -D-glucosyl-(1 \rightarrow 4)- O - α -D-glucosyl-(1 \rightarrow 4)-D-glucose (see, formula IV).

Heptasaccharide; R_g 0.11 and M_{GlC} 0.23.

Acid hydrolysis of the methylated heptasaccharide gave 2,3-, 4,6-tetra-, 2,4,6-tri- and 2,3,6-tri- O -methyl-D-glucose, in the molar ratio of 1 : 2 : 4. The action of salivary α -amylase resulted in the formation of 4- O - α -nigerosyl-D-glucose and glucose (approximate ratio, 2 : 1). From these findings the heptasaccharide is most probably made up of the sequences shown in Figure 9. The fact that the structure of the tetrasaccharide released by Taka amylase differs from that produced by the action of salivary amylase, and the heptasaccharide may have the sequence shown in Figure 9, strongly suggest that Taka amylase attacks, selectively, α -(1 \rightarrow 4)-D-glucosidic linkages, adjacent to the terminal end in the maltotetraose units which are probably located as short blocks (see, Figure 9).

Thus, the variability of the actions of several α -amylases from different origins on elsinan is consistent with the conventional classification of amylases. The enzymes having high activities on the relatively lower maltosaccharides, such as maltotetraose and maltopentaose, are likely to hydrolyze elsinan. On the other hand, the incapability of certain α -amylases, e.g., the liquefying type amylases, may be due to their affinities to higher maltosaccharides. This can be supported by the fact that Taka amylase shows a lower activity to yield particular tetrasaccharide and heptasaccharide.

As regards the enzymatic degradation of elsinan, it may be interesting to examine the susceptibility of this glucan to myco-dextranase, which has been known to be specific to nigeran. The investigations on the action of different enzymes on elsinan should provide more knowledges on the substrate specificities of these enzymes.

Physical Properties of Elsinan

As described already, elsinan is a high molecular, linear polysaccharide consisting of the regular arrangements of α -(1 \rightarrow 4)- and (1 \rightarrow 3)-D-glucosidic linkages. This glucan was found to have a high viscosity, much greater than that of pullulan, and to form a strong and acid-resistant film. These properties may be useful for applications of this polysaccharide to the fields of food and pharmaceutical industries.

Viscosity ——— Elsinan is readily soluble in hot water and gives a high viscous solution, at a low concentration, approximately 10 times than that of pullulan. For instance, 3% aqueous

elsinan solution gives 100 CP. Figure 10 shows the relationship between concentration and viscosity of aqueous solution, measured at 30°, after dissolved at 80°. Unlike pullulan, elsinan tends to form a gel, at 5% or higher concentration. Elsinan solutions are highly pseudoplastic, and viscosity decreases rapidly with increasing the shear rate (Figure 11).

With regard to the effect of temperature on viscosity, when a 2% aqueous solution of elsinan is dispersed at 30°, and the solution is gradually heated, 5° per min, a slight decrease in the viscosity is seen at the temperature up to 45°, after which the viscosity increased to reach a maximum at 60°, then decreased rapidly with increasing temperature (Figure 12). However, when the elsinan dispersion is preheated at 90° for 30 min, the viscosity is lower than that without preheating; there is no viscosity peak. These viscosity characteristics might be due, at least, to irreversible changes in the inter-molecular association of the elsinan chains.

The effects of pH and electrolytes on the viscosity of elsinan are shown in Figure 13 and 14. The viscosity (2% solution) of elsinan appears not to be altered in a wide range of pH (3 - 11). The compatibility with salts is exhibited over a wide range of salt concentration. The non-ionic, neutral nature of the elsinan molecule may be responsible for its stability to salts and pH.

Film Formation ——— Elsinin forms strong and resilient films on evaporation of its aqueous solution. Some of the properties of the film are listed in Table V. It was found that, like pullulan film, elsinan film is impervious to oxygen, suitable as coating or packing film of food. For instance, when oleic acid was packed in the elsinan film, there was no coloring observed, even after three months. In another experiment, when fresh sardines were coated with elsinan films and then air-dried, no coloration due to auto-oxidation was observed over four months. Another characteristic property of the elsinan film may be relatively stable in a dilute acidic solution (pH 1 - 4), probably due to its linear structure consisting of α -(1 \rightarrow 4)- and (1 \rightarrow 3)-D-gluco-

Chemical Modification of Elsinan (13)

Recently various chemical modifications and derivatizations of polysaccharides have been developed to meet new needs. However, introduction of 3,6-anhydro-linkages to the polysaccharides appear to be limited to 3,6-anhydro-amylose (14). Our interest has been drawn to the introduction of 3,6-anhydro-linkages into α -(1 \rightarrow 4)-linked D-glucose units of elsinan, whereby some alteration of the physical properties would be expected.

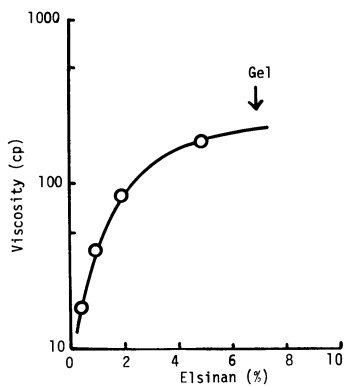


Figure 10. Effect of concentration on viscosity of elsinan dispersed at 100°C, measured with a rotational, shear-type viscometer at 2.5 rpm; cp represents centipoise.

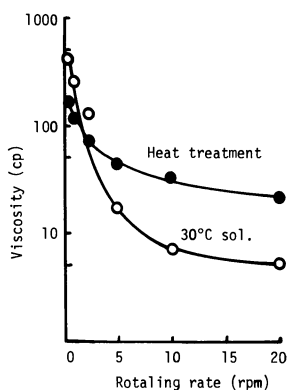


Figure 11. Pseudoplasticity of elsinan solution

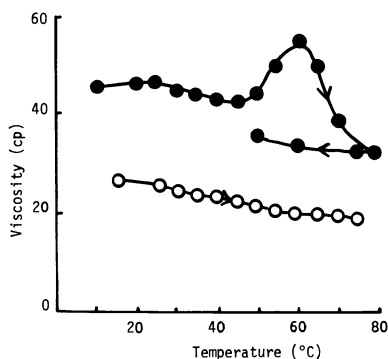


Figure 12. Effect of temperature on viscosity of elsinan (2% w/w) dispersed at (●) 30°C or at (○) 100°C, measured at 20 rpm, programmed from 10° to 80°C (5°/min).

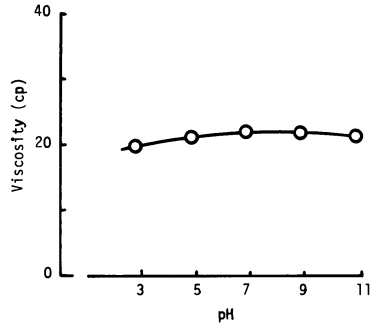


Figure 13. Effect of pH on the viscosity of elsinan (2% w/w) dispersed at 100°C, measured at 20°C

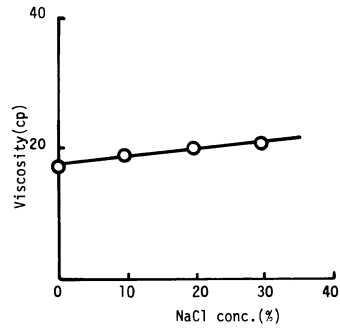


Figure 14. Effect of ionic strength (NaCl) on the viscosity of elsinan (2% w/w) dispersed at 100°C, measured at 20°C

TABLE V
PROPERTIES OF ELSINAN FILM

Permeability of oxygen	1.0 ml/m ² , 24 h, atm
Bending strength	973 times
Tensile strength	950 kg/cm ²
Hygroscopicity	11.1% (Relative humidity 33%)
	15.2% (Relative humidity 65%)
	19.5% (Relative humidity 90%)
Transparency	Excellent
Heat-sealing property	Excellent
Digestibility	Partially degraded by salivary and pancreas α-amylases

The synthesis of 3,6-anhydro-elsinan was achieved by a simple method, which involves mild sulfation possibly at 0-6 positions by treatment with dimethylsulfoxide-SO₃, and then alkali treatment of the partially sulfated glucan with 2 N sodium hydroxide at 80°. The desulfation and simultaneous anhydro-ring formation were checked by measurement of the change in the optical rotation. Scheme I shows a series of the reactions.

Table VI summarizes the properties of the native, partially sulfated, and 3,6-anhydro- derivative of elsinan. The 3,6-anhydro-elsinan contained 3,6-anhydro-glucose residues, approximately a half of the original (1→4)-linked glucose residues. The anhydro-elsinan showed a low optical rotation. In i.r. spectrum the formation of a new absorption band at 895 cm⁻¹ was recognized. These results suggest the changes in the conformation of α-(1→4)-linked glucose residues from C-1 to 1-C forms. The introduction of 3,6-anhydroring to elsinan gives very low viscosity, and also the resistance to the action of salivary amylase.

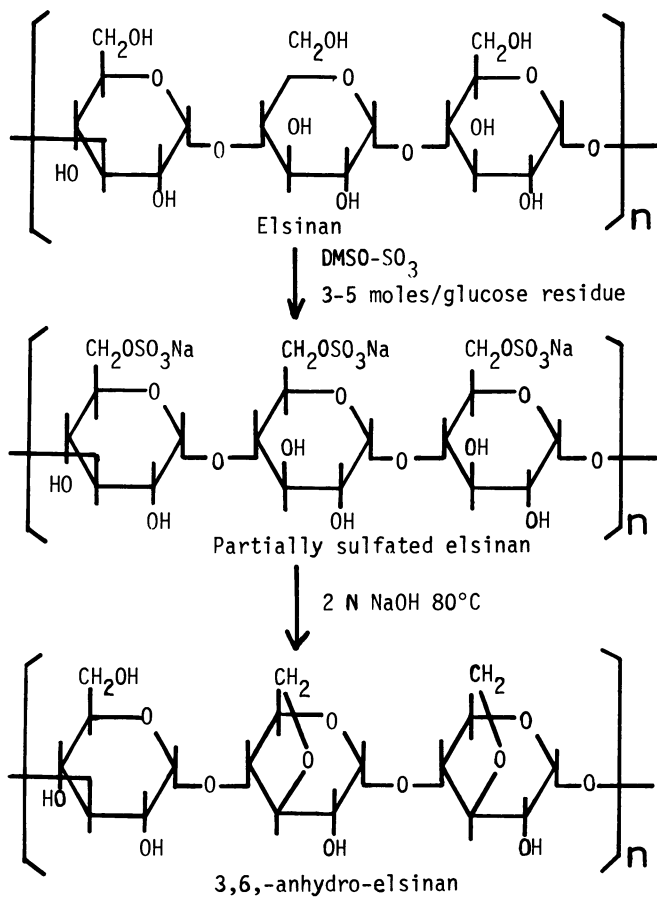
The formation of 3,6-anhydro-glucose residues was confirmed by analysis of the acid hydrolysis products. Paper chromatography as well as g.l.c. revealed the presence of 3,6-anhydro glucosyl- and glucose. It was found that the glycosidic linkages of 3,6-anhydro glucose residues are sensitive to acid. For instance, when 3,6-anhydro elsinan was heated with 0.1 N sulfuric acid at 80° for 30 min, considerable hydrolysis occurred, with formation of anhydro glucose, 4-O-glucosyl-3,6-anhydro-D-glucose, 4-O-maltosyl-3,6-anhydro-D-glucose, O-α-D-glucosyl-(1→3)-O-α-D-glucosyl-(1→4)-3,6-anhydro-D-glucose etc.

Although our primary purpose to obtain somewhat agar-like physical property failed, better control of the reaction conditions and distribution of 3,6-anhydro-linkages, may yet provide a unique derivative of elsinan or other polysaccharides.

Abstract

Elsinan is an extracellular α-D-glucan produced by a strain of *Elsinoe leucospila* from sucrose or other carbohydrates in an aerobic condition. Methylation studies, Smith degradation, partial acid hydrolysis and acetolysis established that elsinan is composed mainly of maltotriose residues and maltotetraose residues (minor) joined by α-(1→3)-linkages.

Elsinan is partially hydrolyzed by certain α-amylases, e.g., salivary, pancreas and bacterial (saccharifying) α-amylase to release mainly 4-O-α-nigerosyl-D-glucose. The action of Taka amylase, which hydrolyzes elsinan more slowly, results in the formation of a tetrasaccharide, O-α-D-glucosyl-(1→3)-O-α-D-glucosyl-(1→4)-O-α-D-glucosyl-(1→4)-D-glucose, and a heptasaccharide composed of (1→4)- and (1→3)-linked D-glucose residues.



Scheme 1. Synthesis of 3,6,-anhydro-elsinan

TABLE VI
COMPARISON OF PROPERTIES OF NATIVE AND MODIFIED ELSINAN

	Elsinan	Partially sulfated elsinan	3,6-anhydro- elsinan
Optical rotation	+243°	+126.3°	+71.7°
Degree of substitution		0.61 ^a (S, 8.39%)	0.38 (0.53/(1+4)-linkage)
Intrinsic viscosity (25°C)	1.86	0.052	0.029
Molecular weight	3 X 10 ⁵	2 X 10 ⁵	2 X 10 ⁵
Action of salivary α -amylase	†		—

a) Presumably substituted at O-6 position.

Alpha-(1→4)-linked D-glucose residues in the elsinan molecule can be converted partially into 3,6-anhydro-D-glucose residues through preferential sulfation at the O-6-position, and subsequent alkali treatment. The introduction of 3,6-anhydro groups was confirmed by methylation analysis and also by isolation of oligosaccharides from mild, acid hydrolyzate.

Solutions of elsinan gives high viscosity and pseudo-plasticity. The viscosity exhibits excellent pH stability, and compatibility with the presence of salts. Elsinan forms strong and resilient films that are impervious to oxygen. These properties and partial digestibility by α -amylases may propose wide utilization of elsinan in food, pharmaceutical and other industries.

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Texture and Crystal Structure of Fungal Polysaccharides

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The study of crystalline conformation and cell wall morphology of polysaccharides is helpful in the search for structure-function relationships. For example, in the materials system composing fungal cell walls one finds polysaccharides such as: chitin, (1 \rightarrow 3)- β -D-glucan, nigeran and (1 \rightarrow 3)- α -D-glucan, whose known physical properties are quite different. The first two are only slightly crystalline in fungi, but fibrous and water insoluble; chitin(1) is a microfibrillar entity, while (1 \rightarrow 3)- β -D-glucan(2) is a triple helix "network forming" skeletal substance. Nigeran(3) on the other hand, is highly crystalline, but it is soluble in warm water and is considered to play a space-filling role in the cell wall. Its propensity to "chainfold" when crystallizing from dilute solution and its high level of crystallinity can provide it with resistance to enzymatic attack (3). The water insoluble (1 \rightarrow 3)- α -D-glucan has only recently been studied in the cell walls of different fungi (4). This polysaccharide seems to have a structural function in those organisms.

Extracellular fungal polysaccharides are also produced by some fungi(5), however, very little information is available about the crystal structure of that class of polysaccharides (6).

The crystalline conformations and cell wall morphologies of typical fungal polysaccharides have been derived from x-ray fiber diffraction studies and microdiffraction analysis in the electron microscope. The recording of suitable diffraction data requires deincrustation and crystallization inducing treatments (7). From the recorded data it is possible to gain some idea of the intimate relation of various crystalline polysaccharides in the cell wall, although it is sometimes necessary to complement these studies with

in-depth analysis of a material from a pure non-fungal source for a precise determination of the crystal structure.

Almost all the linkage types are encountered in the fungal polysaccharides and several combinations of different linkages are possible leading to a large number of polysaccharides with different properties. Figure 1 shows two glucose residues which can be linked in eight different ways. The anomeric oxygen, O(1), can be linked to four different oxygen atoms located on the next residue. When one considers that each disaccharide can be linked to the next glucose with again eight possible linkages and that different disaccharides can be used, the number of possible polysaccharides is very great.

This report is a general survey of the principal structural and morphological characteristics of the most common fungal polysaccharides. The four polysaccharides already mentioned generally are the most abundant and extensive information is generally available. Some other less characterized polysaccharides which however have interesting properties, will also be described. The focus of our review will be the correlation of crystalline structure and molecular conformation with morphological and functional features.

The X-Ray Diffraction Approach

X-ray diffraction techniques are the only way of determining the crystal structure of natural and synthetic polymers, although the x-ray data itself obtained from a crystalline polymeric fiber or film is not sufficient to allow complete refinement of the structure. Conformational analysis and electron diffraction represent complementary methods which will facilitate the determination of the structure. The necessary requirements for the x-ray approach are: crystallinity and orientation. X-ray data cannot be obtained from an amorphous sample which means that a non-crystalline polymeric material must be treated in order to induce or improve crystallinity. Some polymers, such as cellulose and chitin, are crystalline and oriented in the native state.(1)

In order to crystallize, a polymer must possess regularity in the chemical structure along the chain. The monomeric unit can be very simple as in polyethylene or very complex as it is in the pneumococcal polysaccharides(8), but as long as it is regularly repeated, the polymer will be suitable for crystallization. An irregularly branched chain

crystallizes with difficulty, whereas a randomly occurring single sugar substituent on a homopolysaccharide backbone only prevents crystallization if the substitution is too frequent. Wood xylans, with their 4-O-methyl- β -D-glucuronic acid moieties are examples of the latter.⁽³⁰⁾ If the substituent residue or other functional group occurs with regular or near regular periodicity, crystallinity should be found at all degrees of substitution; galactomannans having α -D-galactose residues appended to a D-mannose in the main chain are an example.⁽⁹⁾ Another example is the secondary cellulose acetate which crystallizes even though all hydroxyls are not acetylated.⁽¹⁰⁾

Orientation of the chains along the fiber axis is required for x-ray structure determination. As contrasted with small molecules which yield perfect single crystals, a film or a fiber obtained from a polymer material consist of local areas of order called crystallites embedded in amorphous domains. Once the chains are oriented, the crystallites have a common orientation for one unit cell axis, namely the "fiber axis" and the other two unit cell dimensions have cylindrical symmetry about this axis. Figure 2 shows the different levels of organization from the oriented chain, to the anisotropic crystallite with its unique orientation inside the fiber: the chain axis parallel to the fiber axis, but all possible rotations of the crystallite about the chain axis are present. Accordingly, an oriented fiber yields a single crystal rotation pattern without rotating the fiber, since the x-ray beam "sees" a large number of crystallites at once, each rotated differently about the fiber axis.

Figure 3 shows the different steps involved in the crystal structure determination of a polysaccharide starting from the natural sources. Once the polysaccharide has been isolated and its chemical structure is well defined, crystal structure determination can proceed provided that the polysaccharide can be made to crystallize.

When a natural polysaccharide is amorphous, a film or a fiber must be cast, starting from a solution; subsequent annealing and stretching provides a crystalline and oriented sample. The treatment sequence usually involves stretching the sample at high humidity (or relatively high temperature for a thermoplastic), followed by annealing treatment with the sample kept under tension to maintain orientation.

The x-ray diffraction pattern is then recorded from which information on the unit cell of the polysaccharide

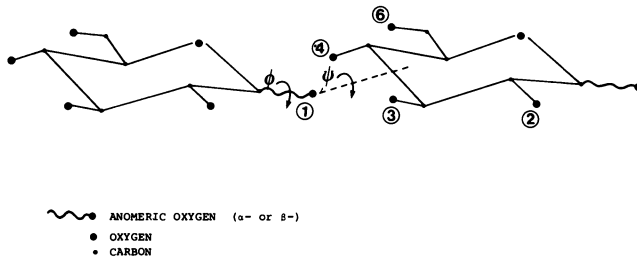


Figure 1. Two glucose residues can be linked in eight different ways leading to different polysaccharides with different properties. Since it is possible to have different types of linkages and different sugar residues, the number of possible polysaccharides is very large.

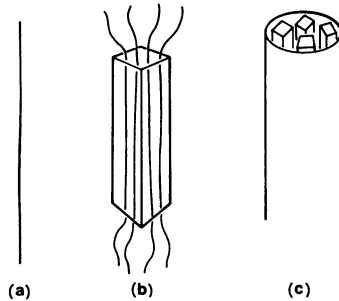


Figure 2. Hierarchy of structural organization: (a) single chain in crystalline conformation; (b) chain in crystallite; (c) crystallites oriented inside fiber

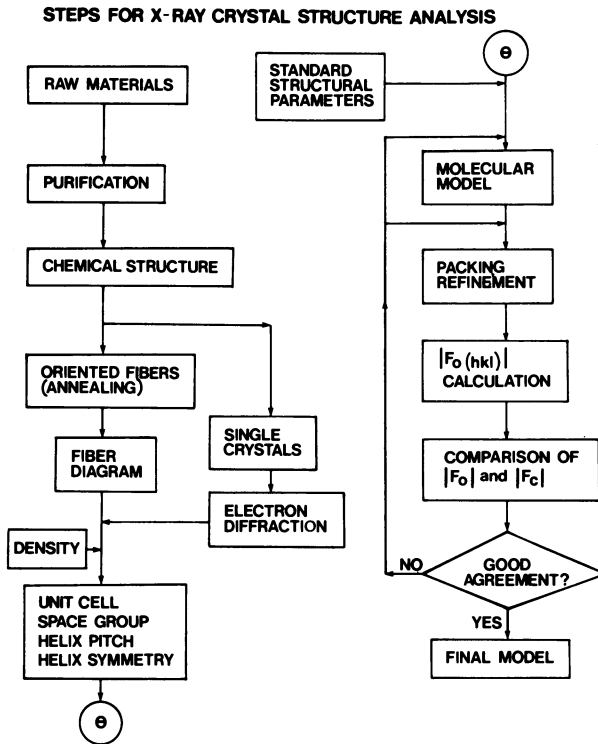


Figure 3. Flowchart of operations involved in evaluation of x-ray fiber diagram for polysaccharide crystal structure analysis: left, experimental procedure and evaluation of fiber diagram; right, computational refinement

as well as on the symmetry and pitch of a single chain helix are obtained. A fortunate fact is that the molecular axis of symmetry, that is, the long chain axis generally coincides with that unit cell axis which has a common orientation along the fiber direction, namely the fiber axis. Unit cell periodicity in this direction, often referred to as the "fiber repeat", is directly related to the conformation and symmetry of the monomer residues in the direction of the chain. Experimentally, the fiber repeat is directly derived from the layer line spacing of a fiber diagram.(11)

The parameters of the unit cell and the space group can be determined solely from the x-ray fiber diagram although electron diffraction recorded from single crystals of the polysaccharide are very helpful since they give much more precise information about the base plane.(12) Once the unit cell dimensions are known, density measurements will tell how many asymmetric units are present in the unit cell. Moreover, once the symmetry and the advance per monomer of the helix (obtained from the fiber diagram) are known, the number of chains present in the unit cell can be deduced.

In a first step, the three dimensional structure of a single helix is generated starting with the known structure of a single sugar residue for which the crystal structure is well known. The helix symmetry and advance per monomer, derived from the fiber diagram, are the basic helix determinants. If no steric hindrance or short contacts are present, the "packing" of the helices can be performed with the chains disposed according to the space group requirements. The best "packing" model (the one with the minimum energy) will be used for comparison of theoretically predicted x-ray diffraction intensities with those observed. If the structure presumed is correct, there will be good agreement between the two sets of intensities. On the other hand, if the model postulated is wrong, the whole procedure will have to be repeated after adjustments have been made in the original model.

High-speed computers and versatile programs allow one to refine the structure by simultaneously varying many parameters. This leads to a model in agreement with the experimental data. Unfortunately, in a fiber diagram, there are not enough x-ray reflections to permit the use of Fourier synthesis methods. However, prior knowledge of the chemical structure coupled with conformational and packing approaches, based on minimum energy considerations, allow a reasonable approach to finding a very probably correct crystal structure.

In the next section, the determination of the structure of (1→3)- β -D-glucan will be described in detail since it represents a complex structure solved with the help of x-ray diffraction and computer-based conformational and packing analysis.

(1→3)- β -D-Glucan

Although encountered in many yeasts and fungi, this polysaccharide is ubiquitous in the biological world. It fills a variety of functions (Table I) and is known under several different names. For example, paramylon is a highly crystalline granular (1→3)- β -D-glucan which is a reserve polysaccharide in Euglena gracilis.(13) Curdlan(2), also a pure (1→3)- β -D-glucan, its synthesized extracellularly by a bacteria (Alcaligenes faecalis var. myxogenes) and is poorly crystalline.(14) It is found in other algae(15,16) and also in more advanced organisms such as cotton fibers(17) and in the pollen tubes of Lilium longiflorum(18) where it is fibrillar and associated with cellulose.

This glucan is almost always present in yeasts and fungal species and the most well known are listed in Table I. In those organisms(7,19,20,21,22) the polysaccharide is believed to play a structural function in the cell wall.(20)

Crystal Structure. Curdlan powder was the source of (1→3)- β -D-glucan used in our study since it is commercially available and has been demonstrated to be a linear chain constituted almost exclusively of (1→3)- β -D-glucose residues. The polysaccharide was obtained from Takeda Chemicals Company in Japan and its morphology has been previously described.(2,11) It is received as a spray-dried powder which is very poorly crystalline.

Fibers were obtained by extrusion of a 10% dimethylsulfoxide (DMSO) solution of curdlan into a methanol bath followed by washing of the gel fibers in water and drying at constant length. The x-ray fiber patterns recorded(23) for these fibers show poor crystallinity but good orientation. Only a few broad diffraction spots can be measured, leading to a unit cell determination (Table II). The diffraction data is not extensive enough to allow one to propose a definite structure for this polymorph. However, Takeda, et al.(24), have been able to improve this x-ray pattern and they proposed a structure consisting of a single helix with a 7_1 symmetry.

TABLE I
 Natural Sources
 and Functions of $(1\rightarrow3)\text{-}\beta\text{-D-Glucans}$

STORAGE	Unicellular Algae	<u>Euglena crocilis</u> (13)
	Brown Algae	<u>Cladophona rupestris</u> (15)
	Green Algae	<u>Laminaria</u> (16)
EXOCELLULAR	Bacteria	<u>Alcaligenes faecalis</u> var. <u>myxogenes</u> (2)
STRUCTURAL	Plant	<u>Lilium Longiflorum</u> (18)
		<u>Gossypium arboreum</u> (cotton) (17)
	Yeast	<u>Saccharomyces cerevisiae</u> (19,20) <u>Schizosaccharomyces octosporus</u> (21)
	Fungi	<u>Armillaria mellea</u> (7) <u>Penicillium notatum</u> (22) <u>Lentinus elodes</u> (7)

TABLE II
Unit Cell Parameters of (1+3)- β -D-Glucan Polymorphs

Sample	a=b* (Å)	c (Å)	Density** (Observed) (g/cm)	Density (Calculated) (g/cm)	Number of Glucose per Cell	Number of Water Molecules per Cell	Number of Chains per Cell
"As Spun"	16.8	22.8	1.45	1.59	30	-	4-6
After Annealing (Hydrated Form)	15.6	18.8	1.47	1.48	18	36	3
After Annealing (Dry Form)	14.3	5.872	1.49	1.54	6	0	3

* Hexagonal unit cell.

** Measured by flotation in a p-xylene / toluene mixture.

In order to improve the crystallinity, the fibers were annealed under tension in a sealed bomb in the presence of water at 145°C. This treatment greatly improved the crystallinity and the diffraction data so obtained and provided enough information to allow a complete structure determination.

Depending on the relative humidity (R.H.), two different x-ray diagrams are obtained, from which two different unit cells can be derived. Table II shows that both unit cells are hexagonal with the values of a and b slightly different depending on the relative humidity. However, the c value (the fiber repeat) changes radically being about three times larger in the "hydrated" form than in the "dry" form.

To account for all the observations, a triple helix structure was proposed for the (1 \rightarrow 3)- β -D-glucan. It consists of three intertwinning 6_1 helices (see figure 4) with a fiber repeat of 17.31 Å for the "dry" form and 5.6 Å for the "hydrated" form. The three intertwinced strands form a cylinder stabilized by an interstrand hydrogen bonding "triad" in the middle of the triple helix. Figure 5 illustrates such a scheme where one O(2) oxygen of each strand is part of the triad. This hydrogen bonding pattern was first proposed by Atkins(25) for the (1 \rightarrow 3)- β -D-xylan which was also interpreted as a crystalline triple helical structure.

This model can explain the large change in the c value which is observed in transforming from "hydrate" to "dry" crystal form. Since no apparent physical modification of the fiber is observed, the chains do not significantly modify their relative positions. Therefore, this transformation was explained as due to a change in the symmetry present in each unit cell when going from one form to the other. The three-fold symmetry between the three helices in the dry form allows the c value to be 1/3 of the repeat of one individual helix. When hydration takes place, it destroys the three-fold symmetry and the single helix pitch becomes the fiber repeat in the hydrated form.

Density measurements indicate that there are no water molecules in the "dry" form, whereas in the "hydrate" form, two water molecules per each glucose residue are found. One can anticipate that the hydrated form will be present in the cell wall of the fungi although the dry form was detected in isolated paramylon granules.(14)

Figure 5 represents the projection of the triple helix of the dry form in the XZ plane.(26) A single chain consists of a helix with six glucose residues per turn

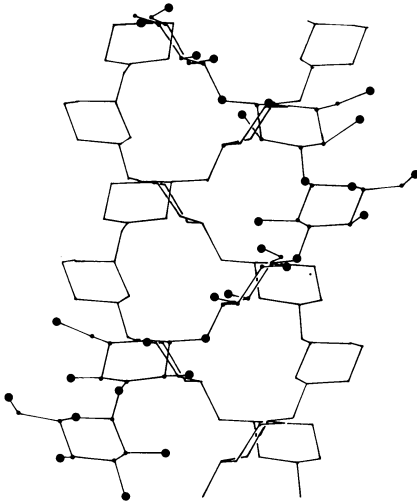


Figure 4. XZ projection of a triple helix. For clarity purposes, only one of the three helices is detailed. The other helices show only the backbone. The single helix has six glucose units per turn and an advance per unit of 2.93 Å.

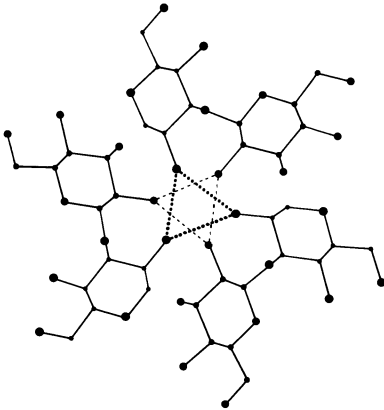


Figure 5. XY projection of a triple helix showing the hydrogen bonds formed in the middle of the triple helix.

and an advance per monomer of 2.94 Å. No intramolecular hydrogen bonds along a single helix are found, but interstrand bonds as described earlier are present.

Packing of the rigid chains, based on minimum energy analysis coupled with x-ray diffraction data, was performed for the "dry" form and the base plane projection of the final model is presented in Figure 6. The unit cell is hexagonal and the space group is $P6_3$. In addition to the interstrand hydrogen bond (not shown in Figure 6), inter triple-helix hydrogen bonds are observed between O(4)---O(6) and O(6)---O(6), the distance being 2.80 Å for both cases.

The packing analysis of the "hydrate" form is underway(27), but this polymorph should be very similar to the "dry" form. Although the lattice parameters are different, due to the presence of two water molecules per glucose unit and the loss of the three-fold symmetry, the basic triple helix structure is believed to be retained.

Morphology. Kreger and Meuse(28) were the first to report x-ray powder diagrams of a purified (1→3)-β-D-glucan (which they called "hydroglucan") in the cell walls of baker yeasts. X-ray powder diagram of this polysaccharide was almost identical to that of paramylon powder. They also observed small differences in the x-ray diagrams and suggested the possibility of two polymorphs. They also noted the presence of chitin and yeast mannan as part of the cell walls of yeast and fungi.(22)

The microfibrillar morphology of (1→3)-β-D-glucan was demonstrated by Jelsma and Kreger(7) when fragments of purified hyphal walls consisting of aggregates of short microfibrillar particles were observed in the electron microscope. The authors proposed that the original glucan in the native wall is probably involved in a molecular network consisting of lipids, polysaccharides, chitin and even protein and mineral material. Indeed, since in the native wall the glucan is poorly crystalline, the microfibrils observed after treatment may be a consequence of the purification process and perhaps are not present in the same form in the fungal cell walls.

It has been shown that microfibrils can be obtained when a (1→3)-β-D-glucan is precipitated from an NaOH solution(14) (see Figure 7). One of the features of those microfibrils is a near absence of crystallinity. Thus, even if the cell walls of the fungi do not show crystallinity, it is possible that the microfibrils may be already present as such in the native wall. The

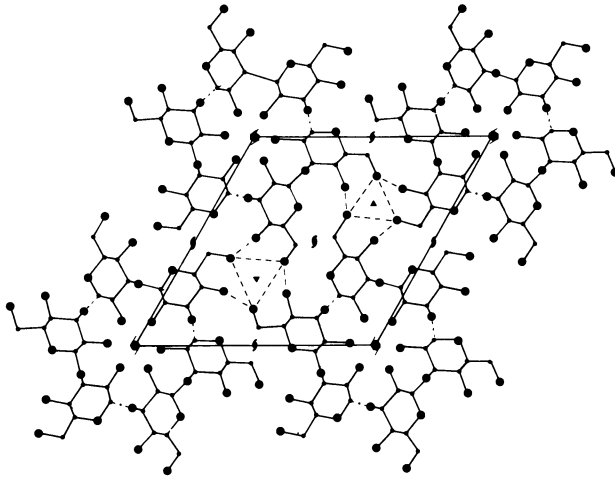


Figure 6. AB projection of the unit cell of the dry form of (1 \rightarrow 3)- β -D-glucan showing the inter triple helix hydrogen bonds. The intrachain hydrogen bonds of Figure 2 are not drawn.

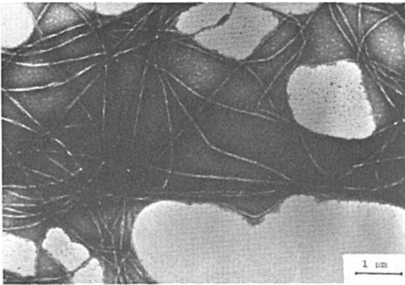
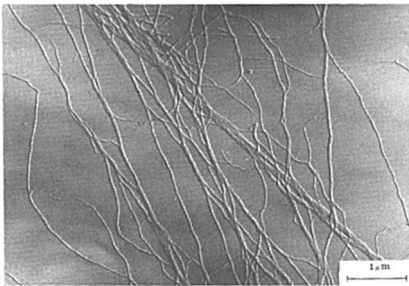


Figure 7. Scanning electron micrographs of curdlan microfibrils (14) from slow neutralization of dilute NaOH solution: upper, shadowed with tungsten; lower, stained with uranyl acetate

treatment with HCl causes an enhancement of the crystallinity. Moreover, the presence of pure (1→3)-β-D-glucan fibrils in the native form of fungi has been demonstrated in certain species.(20)

Chitin

Chitin is a substance of considerable biological importance. It consists of (1→4)-β-D-glucose chains with every C(2) substituted by an acetyl amino group.(15) It is found, as a component of fungal and bacterial cell walls, in insect cuticles and in the shell of crustaceans.(1) Like cellulose, it occurs in more than one crystal form and α-chitin, which is more common than β-chitin, is the form encountered in fungi.

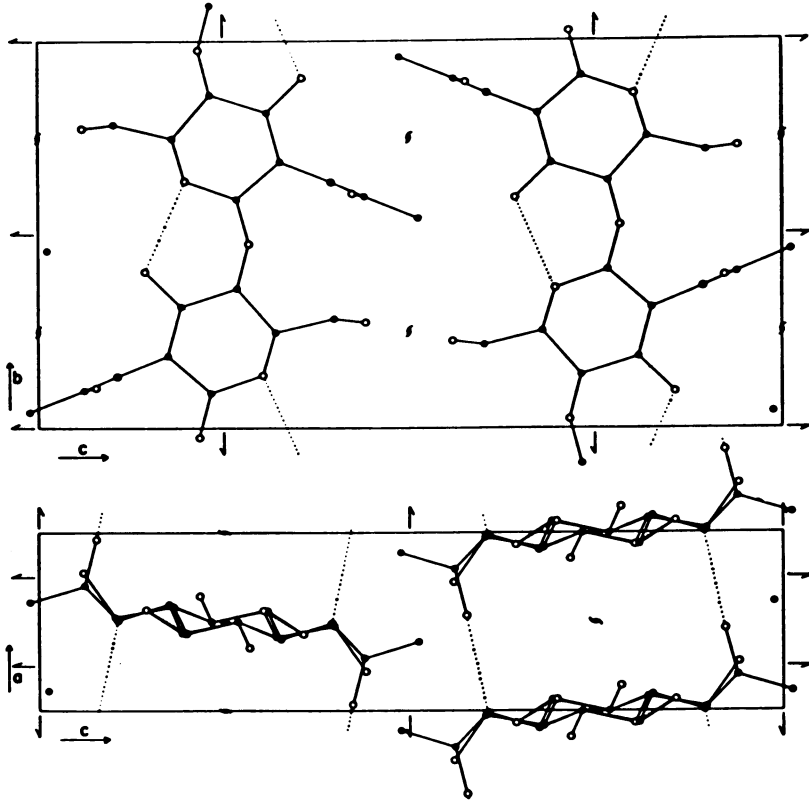
The most acceptable structure for α-chitin is the unit cell proposed by Carlstrom(29) shown in Figure 8. The fiber repeat is identical with that of cellulose, the space group is $P2_1 2_1 2_1$ and the unit cell contains two antiparallel chains.

There are two principal features in the hydrogen bonding scheme: (i) the presence of an intramolecular O(3')---O(5) bond, and (ii) the bonding in the plane of the amide group which is almost perpendicular to the fiber axis giving rise to planes of N-H...O=C hydrogen bonds. The latter provide a strong intermolecular force between adjacent parallel chains in one direction, while the neighbouring antiparallel chains seem to be held by hydrophobic bonds between methyls in the acetamido group or by cross-chain hydrogen bonds involving the hydroxymethyl groups.(30)

Chitin is almost always found as crystalline microfibrils usually embedded in a matrix constituted of other polysaccharides. For example, in Saccharomyces cerevisiae(19), chitin is found in the form of microfibrils along with fibrillar (1→3)-β-D-glucan. The latter is also found in other species as a matrix polysaccharide embedding the chitin microfibrils as in Schizophyllum commune.(31) This composite material is also associated with (1→3)-β-D-glucan to constitute the final cell wall.

(1→3)-α-D-Glucan

As mentioned earlier (1→3)-α-D-glucan is often found in the walls of fungal cells(32) along with chitin and (1→3)-β-D-glucan. The polysaccharide from Schizophyllum commune is soluble in dilute alkali solution and is constituted solely of D-glucose linked (1→3)-α.(33) A



Journal of Biophysical and Biochemical Cytology

Figure 8. The unit cell proposed by Carlström (29) for α -chitin. The cell is orthorhombic and has two antiparallel chains. Chains having the same polarity are hydrogen-bonded through planes of $C=O \cdots H-N$ running perpendicular to the fiber axis.

pure (1→3)- α -glucan has also been isolated from the fungus *Polyporus tumulosus* (34) and Jelsma and Kreger studying different fungal (1→3)- α -D-glucans, observed the presence of several polymorphs based on x-ray powder diffraction of cell walls. (4)

Recently, Ogawa, et al. (35), obtained an x-ray fiber diagram of a "regenerated" pure (1→3)- β -D-glucan. The polysaccharide which was extracted from a bacterial medium (*Streptococcus salivarius*) contained some glucose residues joined by (1→4) and (1→6) linkages as side chains, but these could be removed by a Smith degradation. Since it was impossible to obtain oriented fiber with the pure polysaccharide, they had to use an acetylated derivative soluble in chloroform to be able to heat, soften and stretch the sample. The oriented fibers were then deacetylated to a regenerated pure (1→3)- α -D-glucan and after annealing in a sealed bomb at 140°C in the presence of water, a satisfactory fiber diagram was obtained.

The diffraction data obtained from this fiber led Ogawa, et al. (35) to propose a 2_1 helix conformation with a fiber repeat of 8.44 Å for a single chain. The unit cell is monoclinic with $a=8.21$ Å, $b=9.56$ Å, $c=8.44$ Å and $\gamma=90^\circ$. No water molecules and four glucose residues are present in the unit cell.

In order to compare the structure of this polysaccharide to its glycosidic isomer polysaccharide, Ogawa's data was used to generate a model for the conformation of a single chain. (36)

Figure 9 illustrates the best model according to the analysis. The chain is almost completely extended, since a virtual bond length of 4.25 Å is almost equal to the advance per monomer of 4.22 Å. Intramolecular hydrogen bonding between O(3)---O(5') with an oxygen to oxygen distance of 2.80 Å is proposed. Three different positions, tg, gg and gt (37) are possible since the side group does not affect the conformation of the chain.

The proposed ribbon-like conformation for the crystalline polysaccharide is very similar to that of the cellulose chain which is also almost completely extended. Although (1→3)- α -D-glucan is soluble in aqueous alkali, hydrazine and N-methylmorpholine-N-oxide, it is insoluble in water or common organic solvents. It is probably a good structural material for cell walls since its mechanical properties will not be seriously affected by water.

It should be noted that this conformation corresponds to the regenerated form of (1→3)- α -D-glucan, and it is possible that the conformation is different in

the cell walls, although Jelsma(38) suggested the same interpretation for the native polysaccharide from a fungal source. Three minimum energy positions are available for the (1→3)- α -D-glucan in the conformation energy map shown in Figure 10, as calculated by Sathyanarayanan and Rao.(39) It would thus be possible to encounter other conformations for this polysaccharide in the cell walls.

An energy based packing analysis was performed(40) for this polysaccharide by considering two independent chains, which implies the possibility of having parallel or antiparallel chains. Results based on these calculations show that the antiparallel structure seems to be the more probable model for this compound, although an x-ray refinement has not yet been performed. Interchain hydrogen bonds are readily formed between the chains and are probably responsible for the water insolubility of the compound.

The (1→3)- α -D-glucan has been observed in microcrystalline condition, both in native walls and in precipitates from alkaline solution.(22,41,42) It is found in the outer layer of the cell walls of Schizophyllum commune directly adjacent to the chitin microfibrils and covered by a mucilage polysaccharide.(22) In this organism it acts as a microcrystalline material coating the chitin microfibril layer.(32) Thick irregular (1→3)- α -D-glucan fibers of similar width and appearance as those of S. Commune have also been observed in wall preparations of other fungi.(43,44,45)

Nigeran

Nigeran, also called mycodextran, is a hot water soluble linear, alternating (1→3), (1→4)- α -D-glucan(46,47) and was first isolated by Dox and Niedig(48,49) from Penicillium expansum and Aspergillus niger. In this latter organism, it is a wall component(50,51) and might occupy a buried location within the wall. Its role, however, is not clearly defined.

In order to better understand the behaviour of this polysaccharide, the proposed crystalline structures should be examined. The "dry" and "hydrate" form of this polysaccharide have been studied by x-ray crystallography(52) and electron diffraction techniques. Table III gives a resume of the data available for this compound. In both polymorphs, the unit cell is orthorhombic.

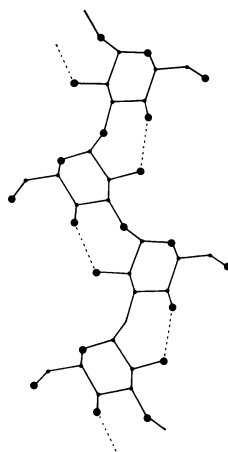


Figure 9. Chain conformation of (1→3)- α -D-glucan. The helix has a two-fold symmetry with an advance per monomer of 4.22 Å.

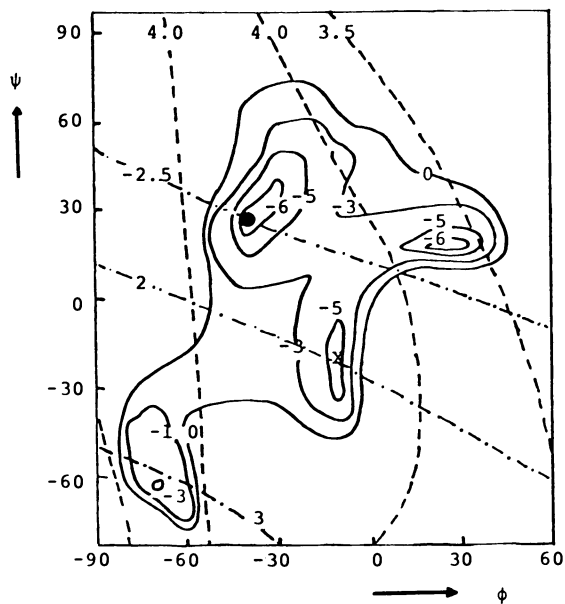


Figure 10. Energy map (ϕ, ψ) in kcal/mol per residue for (1→3)- α -D-glucan. The (· · ·) iso-n and (- - -) iso-h curves are superimposed; (○) energy minimum position; (×) position of our model.

TABLE III

Unit Cell Dimensions for Nigeran
Polymorphs (Both Unit Cells are Orthorhombic)

	<u>a (Å)</u>	<u>b (Å)</u>	<u>c (Å)</u>
"Dry" Form	17.75	6.0	14.6
"Hydrated" Form	17.60	7.35	13.4

When going from one polymorph to the other, only the b dimension of the base plane changes, while a is almost identical in both structures. In the hydrated polymorphs, it has been proposed that sheets of chains are separated by a layer of water in the unit cell.(53) According to the model, water causes the unit cell to expand in the b direction while no effect is observed in the "a" direction. However, the precise localization of the water molecules, which could help to understand the hydration details, is not yet accomplished.

A precise model of the "dry" form has been proposed(54) based on x-ray diffraction, electron diffraction and conformational analysis. Figure 11 represents the conformation of a single chain in the "dry" state. It is a two-fold helix with an advance per monomer of 7.31 Å. A hydrogen bond between O(2)---O(3') is present. The overall conformation is similar to a "corrugated ribbon", as first proposed by Sundararajan *et al.* for the hydrated form.(55) Each unit cell contains two chains and they are antiparallel, which is in agreement with the chain folding proposed in the lamellar single crystals of this macromolecule. Intermolecular hydrogen bonds are observed between the central chain and its antiparallel neighbours.

Nigeran extracted with hot water represents about 5% of the wall's dry weight(51,56) but studies by Bobbitt *et al.*(3), have shown that this treatment fails to extract all the nigeran. They conclude that nigeran chains occupy at least three distinct domains in the hyphal wall and that the polymer's organization in situ is highly crystalline. While it cannot be ruled out that a certain proportion of non-crystalline nigeran may be present in the wall, all three domains contain the crystalline polymer. The first fraction is directly exposed at the wall's outer surface. Beneath this exposed fraction is a second crystalline domain inaccessible to enzyme but removable with boiling water.

A third portion detectable only by x-ray diffraction was associated with other components and could not be extracted, even with prolonged boiling. It was removed by hot dilute alkali and was associated in the wall with another glucan fraction.

The role that nigeran plays within the fungal wall is unknown. It has been suggested(57) that the nigeran may function in maintaining integrity of the cell surface under adverse environmental conditions. Another possible function is as an overflow product that accumulates when nitrogen metabolism ceases.(58) Bobbitt *et al.*(3), suggested the possibility that nigeran may be a storage product, utilized during cleistothecium development, but that the enzyme necessary for its breakdown has been lost by mutation. The loss of such an enzyme would explain the findings of Dox(48) and Gold *et al.*(57), that nigeran is not broken down during starvation.

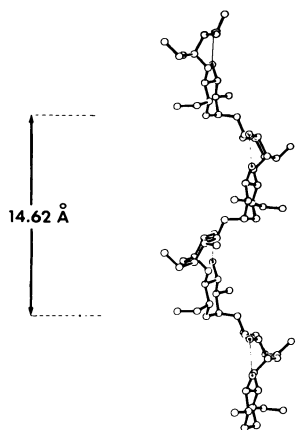
Lichenan

It is of considerable interest to examine this glucan since it is a regular copolymer containing both the (1→3)- β and the (1→4)- β linkages. Structural studies(59) on the product from Iceland moss have established the structure illustrated in Figure 12 which can be described as a poly(1→3)- β -cellotriase. It has to be noted that this 33% structural perturbation of cellulose leads to a drastically modified solubility since lichenan is water soluble.

Although it is sometimes referred to as a "white amorphous powder", there is a published record of x-ray diffraction from lichenan as early as 1930.(60) No crystal structures were proposed but at that time, it was recorded as a powder pattern and interpreted as similar to β -cellulose, an early terminology for regenerated cellulose.

X-ray diagrams of lichenan were recorded and conformational analysis(61) allows the proposal of a model for the lichenan chain. The analogy between lichenan and regenerated cellulose was found to be valid, although it would have been more precise to refer to "water" cellulose. The fiber diagram reveals that the equatorial 101 and 002 reflections of "water" cellulose are on the first layer line in lichenan, an observation which led to the proposal of a "pleated sheet" structure.(61,62)

The best chain conformation(61) corresponds to a right-handed three-fold helix with an advance per



Journal of Molecular Biology

Figure 11. Conformation of a single chain of nigeran in the dry state. It is a two-fold helix with an advance per monomer of 7.31 Å (71).

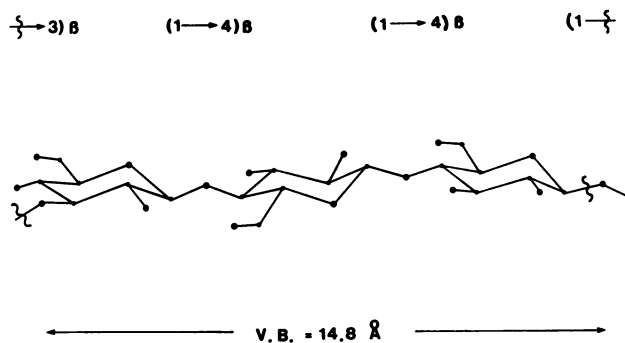


Figure 12. Lichenan asymmetric unit. The chemical analysis shows it consists of a regular (1→3)-β-celotriose.

monomer of 14.01 Å (Figure 13). The unit cell of the "hydrate" form is trigonal with $a=b=9.9$ Å and $c=42.03$ Å. Packing analysis and x-ray refinement are underway to further confirm this structure.

Intermolecular forces are weak, judging from interchain distance, the overall structure is reminiscent of a paracrystalline organization. By contrast, the cellulose II structure, which lichenan resembles, has strong inter-molecular hydrogen bonds of slightly unequal strength in two nearly perpendicular directions. Since both (1→3)-β and (1→4)-β linkages are used as cell wall skeletal materials in fungi, it is interesting to find this hybrid molecule in a cell wall role. However, the structural requirements of a moss being clearly minimal compared to trees, it is not surprising to find this hybrid molecule which is bound to be more water sensitive than cellulose or chitin.

Other Polysaccharides

There are many other polysaccharides found in fungi and it is noteworthy that they are mainly composed of glucose. One exception is the yeast mannan family, which is highly branched and possesses (1→2)-α, (1→6)-α and sometimes (1→3)-α linkages (15) except for Rhodotorula glutinis (63) which is an alternating, linear, β-linked (1→3), (1→4) D-mannan. Interestingly, heteropolysaccharides are not usually found in substantial quantity in fungi.

Other glucans encountered in fungi are elsinan (64) from Elsinoe leucospila which is constituted of maltotriose units linked by (1→3)-α linkages. It is water soluble and gels at concentrations greater than 2%. No x-ray diffraction study or morphological information is available at this time.

Pullulan (65) is a linear polysaccharide of ca. 250 α-D-glucopyranose residues with (1→4) and (1→6) linkages regularly distributed in the proportion of 2:1. There are not many works done on the crystal structure of this polysaccharide probably because the (1→6) linkage renders it difficult to crystallize. The same is true for pustulan, a (1→6)-β glucan isolated from a lichen: Umbilicaria pustulata. (66)

Finally, there are (1→3)-β-D-glucans with glucose residues branched with a (1→6) linkage about every fourth residue of the main chain. (67) Those polysaccharides named scleroglucan, are secreted by certain fungi, particularly the genus Sclerotium and have useful and diverse applications. (68)

Conclusion

In the case of cellulose and starch, the structural and storage roles are so well defined that their characteristics may be taken as the starting point to systematize in terms of morphology: structural materials are fibrous and storage polysaccharides are granular. In addition, one can state that structural materials are characterized by high-tenacity and storage granules are probably of a fine structural design as to permit controlled and localized physiological transformation.

Figure 14 shows four homoglucons in their crystalline conformation (single chain only) and the double arrows join those which are conformationally related. Cellulose (similar to chitin) and (1→3)- α -D-glucan are ribbon-like and nearly fully extended; amylose and (1→3)- β -D-glucan form large amplitude helices, i.e., the virtual bond length is much greater than the observed advance per monomer. In the case of (1→3)- β -D-glucan, the advance per monomer is 4.22 Å and the virtual bond length is 4.25 Å. The same is true for cellulose.

The comparison between the two chain pairs is also consistent with the physical properties. Paramylon and amylose are known for their gel forming properties and their carbon reserve function in plants.^(13,69) Although (1→3)- β -D-glucan also plays a structural role when it occurs in the cell walls of yeast⁽⁷⁾, it is probably more of a matrix substance than anything else. The ability to form multiple helices probably accounts for unusual solubility phenomena such as retrogradation⁽⁶⁹⁾ and irreversible gel formation on heating.⁽¹³⁾

It seems that the kind of linkage is extremely important in determining the conformation of the chain which in turn will be responsible for the secondary and tertiary structure adopted by the polysaccharide. Obviously, the ribbon-like units of chitin can pack readily into dense, rectangular cross-sectioned microfibrils ideal as the skeleton material of cell walls. Therefore, helical conformation, secondary and tertiary structure are dictated by well-defined variations in the main chain chemistry.

The structural role of chitin is quite well established since it crystallizes and electron microscopy shows the presence of microfibrils. However, the role of nigeran, lichenan, (1→3)- α -D-glucan and (1→3)- β -D-glucan is not precisely known. The latter has

Figure 13. Helical conformation of lichenan is derived from x-ray data and conformational analysis. It is a right-handed, three-fold helix with an advance per monomer of 14.01 Å.

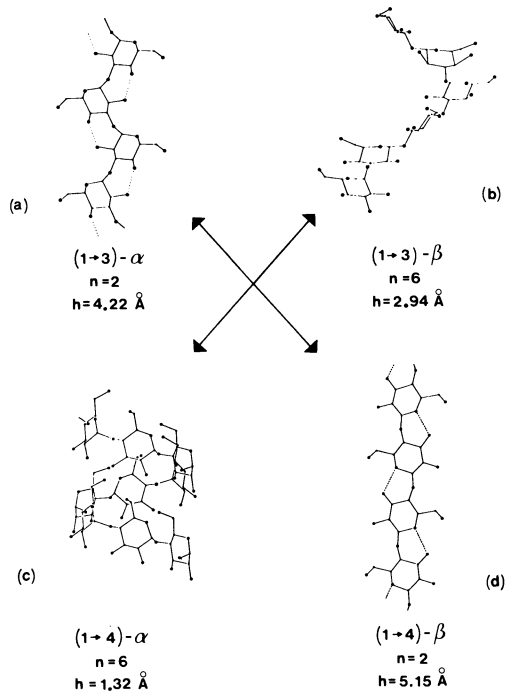
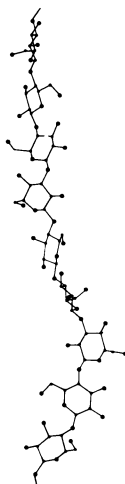


Figure 14. Crystalline conformation of a single chain for glucans: (a) $(1 \rightarrow 3)\text{-}\alpha\text{-D}$ -glucan; (b) $(1 \rightarrow 3)\text{-}\beta\text{-D}$ -glucan; (c) V-amylose; (d) cellulose. The number of residues per turn (n) and the advance per monomer (h) are given. The arrows indicate the polysaccharides that show a similarity in their conformation.

been observed in paramylon(13) as disk-like granules and as fibrils in cell walls.(7) In fungal cell walls, this material is believed to form a matrix embedding the chitin microfibrils. It can be compared to the hemicelluloses in wood where the cellulose microfibrils play the role of fiber reinforcing agent and the hemicelluloses provide the matrix. Moreover, (1→3)-β-D-glucan, because of its gelling properties, can be used as a "water storage" polysaccharide. It also adds flexibility to the cell wall, while chitin is present for its strength-giving properties.

A more recent challenge is to interpret structure-function for (1→3)-α-D-glucan. Its structure and conformational resemblance to cellulose makes us think it is a structural polysaccharide. However, observations so far indicate that it is a protective coating, itself covered by a mucilage(31); it may add strength and flexibility to the wall since it is crystalline.(31,41,42) Freeze-etched replicas of S-glucan, the common name for this polysaccharide, suggest that a liquid crystalline organization is present in micron-sized domains at the surface layer of hyphae of *Schizophyllum commune*.(42) The layer closest to the plasmalemma is a composite of chitin microfibrils embedded in a matrix polysaccharide which is branched glucan with a high proportion of (1→3)-β-D-glucan linkages.

Nigeran is a space filling polysaccharide since it is not a suitable structural material due to its warm-water solubility. Although its role is now known, its high level of crystallinity at room temperature could confer a protective property especially when it is the outer layer of the cell.(70)

Although the x-ray method has been very useful in the determination of crystal structure of polysaccharides, additional methods such as electron diffraction and electron microscopy coupled with selective enzymology are needed for understanding the behaviour of fungal polysaccharides in the native cell walls. Because the cell wall components are often crystalline, diffraction contrast methods of electron microscopy could prove valuable in future fine structure studies.(70)

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Industrial Potential of Fungal and Bacterial Polysaccharides

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Polysaccharides, or gums as they are known in industry, have utility because they thicken, suspend, or stabilize aqueous systems. Some gums produce gels, or act as emulsifiers, flocculants, binders, film-formers, lubricants, and friction reducers. Traditionally, these gums have been derived from algal and botanical sources, but more recently, some gums have been derived from microbial sources. These gums are shown in Table I.

Table I. Gums Derived From Algal, Botanical, and Microbial Sources

<u>Algal</u>	<u>Botanical</u>	<u>Microbial</u>
agar	guar gum	dextran
algin	gum arabic	xanthan gum
carrageenan	gum ghatti	
furcellaran	gum tragacanth	
	karaya gum	
	locust bean gum	
	pectin	

The major objective of this article is to describe the diversity of properties associated with polysaccharides derived from microbial, i.e., fungal and bacterial sources. Also I will provide details of some of the new bacterial polysaccharides originating from our laboratories. I will place emphasis on the key property or properties of each polysaccharide that I discuss. I will not discuss properties which are not related to the potential utility of the polysaccharides. I have also deliberately omitted discussion of the economic aspects of these polysaccharides.

For the past thirty to forty years, considerable research has been devoted to the study of polysaccharides produced by

Table II. Polysaccharides Produced by Fungi

<u>Polysaccharide</u>	<u>Organism</u>	<u>Structure/ Composition</u>
Pullulan	<u>Aureobasidium</u> (<u>Pullularia</u>)	1,4 α - <u>D</u> -glucose
	- <u>pullulans</u>	1,6 α - <u>D</u> -glucose
Scleroglucan	<u>Sclerotium glucaenicum</u>	1,3 β - <u>D</u> -glucose
		1,6 β - <u>D</u> -glucose

Table III. Polysaccharides Produced by Bacteria

<u>Polysaccharide</u>	<u>Organism</u>	<u>Structure/ Composition</u>
Xanthan gum	<u>Xanthomonas campestris</u>	<u>D</u> -glucose, <u>D</u> -mannose, <u>D</u> -glucuronic acid
Curdlan	<u>Alcaligenes faecalis</u> var. <u>myxogenes</u>	1,3 β - <u>D</u> -glucose
Dextran	<u>Leuconostoc</u> species	1,6 α - <u>D</u> -glucose 1,2; 1,3; 1,4 α - <u>D</u> -glucose
PS-7 Gum	<u>Beijerinckia indica</u> var. <u>myxogenes</u>	glucose (73%), rham- nose (16%), gluco- ronic acid (11%)
PS-10 Gum	Soil bacterium	glucose (39%), galactose (29%), fucose (13%), gluco- ronic acid (19%)
PS-21 Gum	Soil bacterium	mannose (33%), glucose (29%), galactose (21%), glucuronic acid (17%)
PS-53 Gum	Soil bacterium	glucose (41%), fucose (40%), glucuronic acid (19%)
PS-60 Gum	<u>Pseudomonas</u> species	glucose (41%), rhamnose (30%), uronic acid (29%)

microorganisms in order to determine their industrial potential.(1) During this period, several microorganisms have been shown to produce polysaccharides which have interesting properties. These polysaccharides are listed in Tables II and III.

These tables provide a summary of the major polysaccharides produced by microorganisms which either have the potential, based on their properties, to become commercial products or which have already become products of commerce.

Pullulan (2)

Pullulan is the extracellular polysaccharide produced by Aureobasidium pullulans. The polysaccharide is composed of malto-tetraose units linked together through the 1 and 6 positions on adjacent triose or tetraose units.

Purified pullulan is a white, non-hygroscopic powder which dissolves in hot or cold water. Pullulan does not provide high viscosity solutions at low concentration. The most important property of pullulan is the ability to form films. The films are prepared by dissolving pullulan in water at 5-10% concentration and continually drying the solution applied to a smooth surface. The major advantage of pullulan films is the very low oxygen permeability. The oxygen permeability of pullulan films and three films of commerce are shown in Table IV. (3)

Table IV. Oxygen Permeability of Pullulan Films

<u>Sample</u>	<u>Oxygen Permeability (cc/m², 24 h, atm., 25°C)</u>
Pullulan films	0.60 - 2.50
Cellophane	4.70
Cellophane (moisture proof)	8.58
Polypropylene	1100

Pullulan films have several other advantages. The films are colorless, tasteless, odorless, transparent, resistant to oil and grease, and heat sealable. The properties of the films can be modified by chemical modification of pullulan, blending with polyvinylalcohol, gelatin, or amylose, and by addition of plasticizers. These properties indicate that pullulan can be used as a coating or packaging for foods to prevent their oxidation. This is the only apparent outstanding application for pullulan.

Scleroglucan (4)

Scleroglucan is a capsular polysaccharide produced by species of the genus Sclerotium. The polysaccharide produced by Sclerotium gluconicum consists of a linear chain of glucopyranosyl units

linked β -D-(1 \rightarrow 3) with single glucopyranosyl units linked β -D-(1 \rightarrow 6) to every third glucose unit of the main chain.

Purified scleroglucan dissolves in hot or cold water to produce high viscosity solutions, whereas the crude isolate from the fermentation broth produces low viscosity solutions. These data are shown in Table V.

Table V. Viscosity of Crude and Purified Scleroglucan

	<u>Crude Scleroglucan</u>	<u>Purified Scleroglucan</u>
Viscosity* (1% conc.)	20 - 80 cP	1600 cP
Viscosity* (0.5% conc.)	--	500 cP
Gum Content	46 - 50%	90%

*Brookfield LVT Viscometer, 60 rpm.

Scleroglucan solutions are pseudoplastic or shear thinning. The relationship between the viscosity of solutions of crude and purified scleroglucan and shear rate is shown in Figure 1.

Based on these curves, scleroglucan does not have a yield value, although the very high viscosity at low shear rate indicates that scleroglucan may have suspending properties.

The viscosity of scleroglucan at 0.5% concentration and 2% concentration is essentially constant over the range 10° to 90°C. This relationship is shown in Table VI.

Table VI. Effect of Temperature on the Viscosity of
Crude Scleroglucan Solutions

<u>Temp. (°C)</u>	<u>Viscosity at 2% Conc.</u>	<u>Viscosity at 0.5% Conc.</u>
15	1500	160
25	1400	--
40	1400	--
60	1400	--
80	1400	--
90	1400	140

Brookfield LVT Viscometer, 30 rpm

The viscosity of solutions of scleroglucan is not changed by changes in pH over the range 1 to 11. Prehydrated scleroglucan is compatible with electrolytes such as 5% sodium chloride, 20% calcium chloride, 5% sodium sulfate, and 10% disodium hydrogen

phosphate.

Based on these properties, it has been suggested that scleroglucan has utility in porcelain and ceramic glazes, extruded refractory products, paints, inks, pesticide sprays, secondary oil recovery, drilling muds, and as a binder for ceramics.

Xanthan Gum (5,6,7,8)

Xanthan gum is the extracellular polysaccharide produced by *X. campestris*. The repeating-unit structure of xanthan gum is shown in Figure 2. (9) As shown in the figure, each repeating unit contains two glucose units, two mannose units, and one glucuronic acid unit. The main chain is built up of β -D-glucose units linked through the 1- and 4- positions; i.e., the chemical structure of the main chain is identical to that of cellulose. The sidechain consists of a terminal β -D-mannose unit glycosidically linked to the 4-position of the β -D-glucuronic acid unit, which in turn is glycosidically linked to the 2-position of α -D-mannose. This three-sugar side-chain is linked to the 3-position of every other glucose residue in the main chain. Also, approximately half of the terminal D-mannose residues carry a pyruvic acid ketalically linked to the 4- and 6- positions. The nonterminal D-mannose unit contains an acetyl group at the 6 position.

Xanthan gum dissolves in either hot or cold water to produce high viscosity solutions at low concentration. The relationship between viscosity and concentration is shown in Figure 3. Xanthan gum has a viscosity of approximately 300 cP at a concentration of 0.5% and 1400 cP at a concentration of 1.0% when measured at 60 rpm with a Brookfield Model LVF viscometer at $\approx 25^{\circ}\text{C}$.

Xanthan gum solutions have unique rheological properties. Aqueous solutions of xanthan gum are highly pseudoplastic; that is, the viscosity decreases rapidly as the shear rate is increased. This decrease is instantaneous and reversible. This aspect of the rheological properties of xanthan gum solutions is shown in Fig. 4. The property of pseudoplasticity is also referred to as shear-thinning. Xanthan gum solutions also have a rheological yield point that is apparent at concentrations greater than 0.75%. The shear stress, shear rate curve is shown in Figure 5. These data were obtained by use of the Wells-Micro Brookfield viscometer and the relaxation technique of Patton (1966). The working yield point is defined as the shear stress at a shear rate of 0.01 sec^{-1} . As can be seen from the figure, a 1.0% xanthan gum solution has a working yield point of 50 dynes cm^{-2} , whereas guar has essentially no working yield point.

Xanthan gum solutions are remarkably resistant to thermal degradation.

Exposure to temperatures as high as 80°C for extended periods has little effect on the viscosity of xanthan gum solutions. This resistance to thermal degradation is enhanced by the presence of

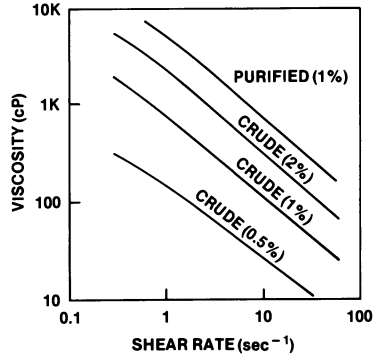


Figure 1. Viscosity of crude and purified Scleroglucan vs. shear rate

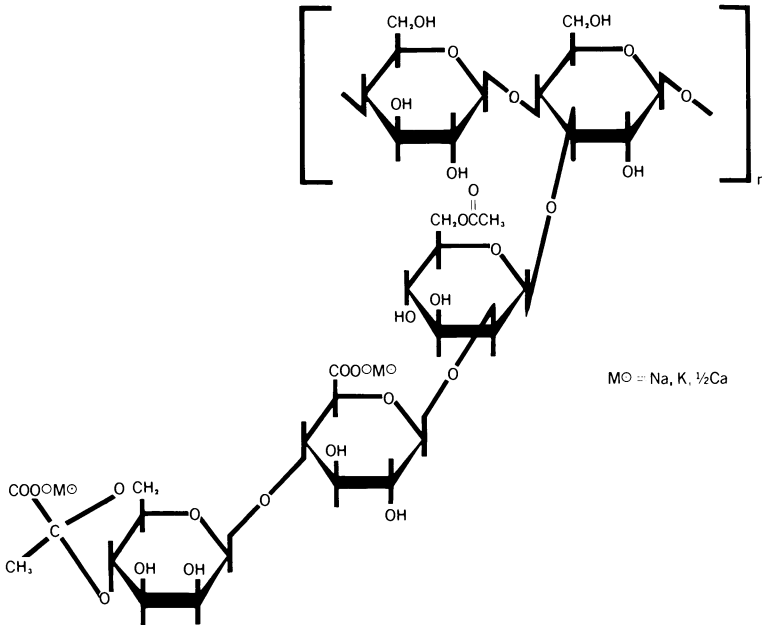


Figure 2. Xanthan gum structure

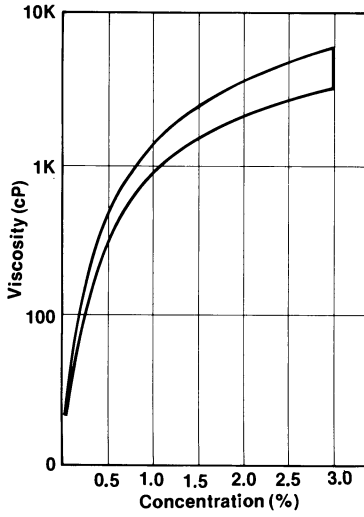


Figure 3. Relationship between viscosity and concentration for xanthan gum with 0.1% NaCl

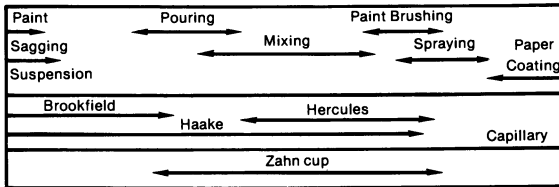
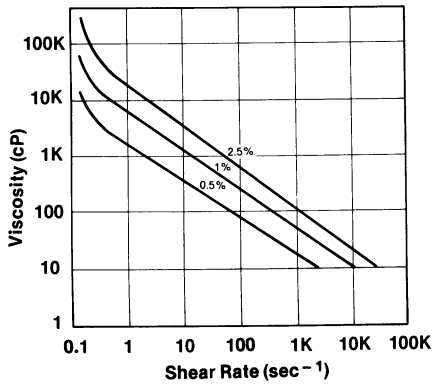


Figure 4. Relationship between shear rate and viscosity for xanthan gum

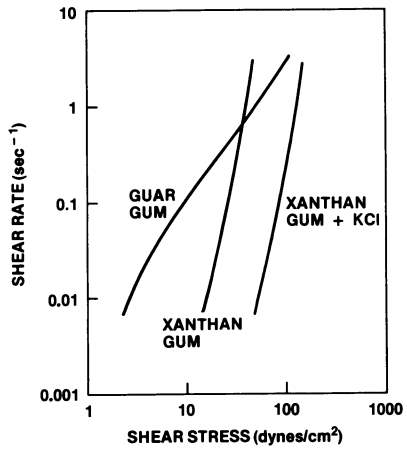


Figure 5. Relationship between shear rate and shear stress for xanthan gum and guar; all gums at 1% concentration

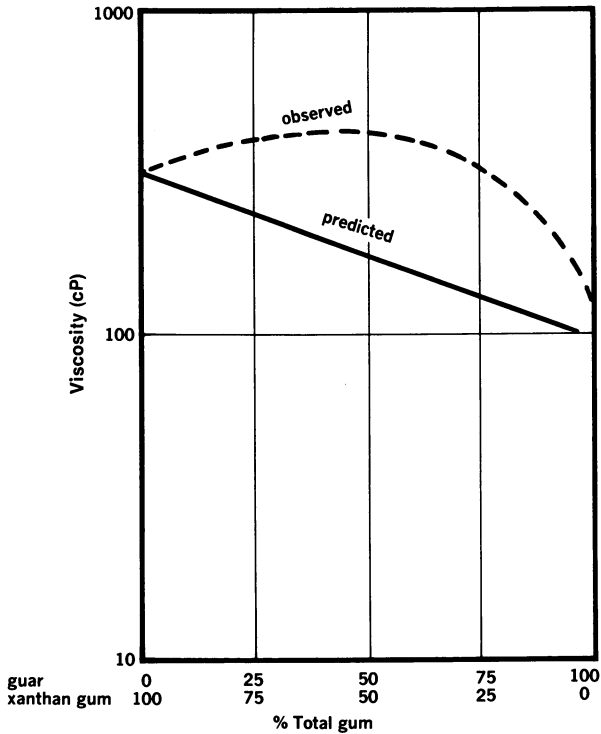


Figure 6. Viscosity of xanthan gum, guar gum solutions (0.5% total gum concentration); Brookfield LVT Viscometer, 60 rpm

salts such as potassium chloride. Also, the viscosity of xanthan gum solutions is essentially constant between -18°C (25°F) and 79°C (200°F).

The viscosity of aqueous solutions of xanthan gum is essentially independent of pH between pH 6 and pH 9, and shows only small changes in viscosity over the pH range of pH 1 to pH 11.

Xanthan gum has excellent stability and compatibility with high concentrations of many salts; for example, it is compatible with 15% solutions of sodium chloride and 25% solutions of calcium chloride.

Xanthan gum will dissolve in acids such as 5% acetic acid and remain stable for several months unless the temperature is elevated. Also, xanthan gum is soluble directly in 5% sodium hydroxide solutions. Sodium hydroxide solutions of 0-15% can be thickened, provided the gum is pre-dissolved in water prior to adding the sodium hydroxide solution. These highly alkaline, thickened solutions have exceptional viscosity stability. Water miscible compounds such as ethanol are compatible with xanthan gum solutions up to concentrations of 50%. Solutions, emulsions, and gels that contain xanthan gum have excellent freeze-thaw resistance and many applications in the food industry.

Perhaps the most outstanding and unusual property of xanthan gum is the reactivity with galactomannans such as locust bean gum, (10) and guar gum (11).

When xanthan gum is combined with guar gum, a synergistic viscosity increase occurs (Fig. 6). As can be seen, the viscosity of a mixture of xanthan gum and guar gum has a higher viscosity than expected, especially at a ratio of approximately equal parts of guar and xanthan gum. (12)

The xanthan gum, locust bean gum combination shows a large viscosity increase (Fig. 7) even at low colloid concentration. At high colloid concentrations, a thermoreversible and cohesive gel is produced. This is shown in Fig. 8, which also provides the relationship between gel strength and colloid ratio. As was the case with guar, the maximum synergism as measured by gel strength occurs at approximately equal parts of locust bean gum and xanthan gum.

Because of these unique properties, xanthan gum has utility in a wide range of applications. In the petroleum area, xanthan gum is used to thicken oil-well drilling fluids and to provide viscous aqueous solutions for enhanced oil recovery. In the industrial area, xanthan gum finds utility in thickening textile printing pastes, acid and alkaline cleaners, slurry explosive formulations, and in a wide range of other industrial applications.

Xanthan gum has utility in a wide range of food applications because of its unique properties. Examples of these applications are bakery jellies, sauces and gravies, salad dressings, and dairy products.

Xanthan gum has utility in agricultural applications. Xanthan

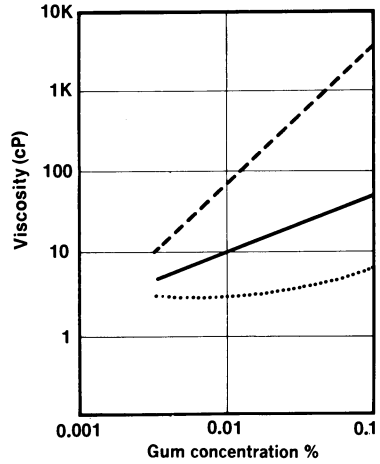


Figure 7. Viscosity of xanthan gum, locust bean gum solutions (< 0.1% gum concentration): (---) xanthan gum-locust bean gum; (—) xanthan gum; (· · ·) locust bean gum

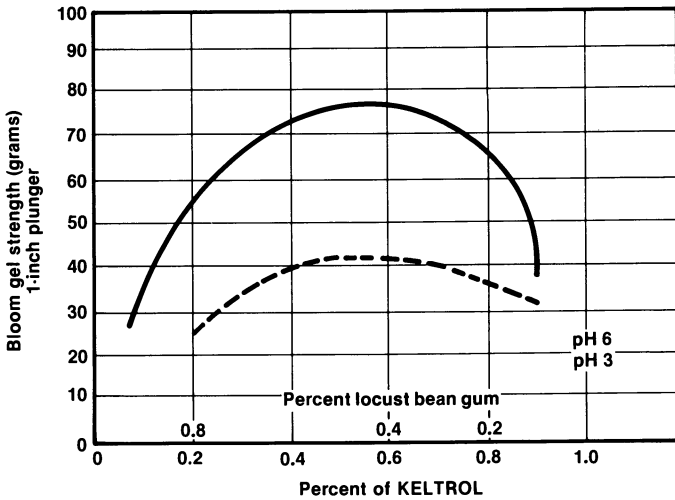


Figure 8. Gel strength of xanthan gum, locust bean gum gels (1% gum concentration)

gum will suspend minerals and vitamins in molasses used for animal liquid feed supplements, and it will also suspend protein in calf milk replacers. The unique rheological properties of xanthan gum improve sprayability, reduce drift, and increase the cling to vegetation of fungicides, herbicides, and insecticides.

Dextran (2)

Dextran is the polysaccharide produced by several species of the genus Leuconostoc. Structurally, dextrans are D-glucans in which α -1,6-linkages are predominant.

Dextran is perhaps one of the most extensively investigated polysaccharides, yet it has not achieved significant commercial success. It dissolves in cold water to produce viscous solutions, but the viscosity is low in comparison to the viscosity of many other polysaccharides used industrially. Viscosity data for a high molecular weight (5 to 40 x 10⁶ Daltons) dextran and xanthan gum are shown in Table VII.

Table VII. Viscosity Data for Dextran and Xanthan Gum

<u>Concentration (%)</u>	<u>Viscosity (cP)</u>	
	<u>Dextran</u>	<u>Xanthan Gum</u>
5	350	Very high
2	32	6000
1	--	1400
0.5	--	600

Brookfield LVT Viscometer, 60 rpm

Based on these data, it is apparent that dextran does not have good thickening efficiency. Dextran is used because selected fractions or derivatives can be prepared which have a specific molecular size and shape. This is necessary in certain pharmaceutical applications where physiological compatibility is required. For example, dextran with a high percentage of 1,6-linkages and a molecular weight of $75 \pm 25 \times 10^6$ Daltons is substantially less antigenic when used as a blood plasma extender than other polysaccharides or other dextrans. Also the ability to control the size and shape has allowed the preparation on a commercial scale of gel permeation chromatography supports. Products are available which effect separation on the basis of size and/or charge. These products are sold under the tradename Sephadex. Dextran can complex with large amounts of metallic ions such as ferric ion and calcium ion. The iron complex has been reported to be useful in combating anemia.

Curdlan (13)

Curdlan is the extracellular polysaccharide produced by Alcaligenes faecalis var. myxogenes [10C3 (mutant K)]. Structurally, curdlan is a linear polysaccharide composed entirely of β -1,3-glucose units.

Curdlan is insoluble in cold water, but upon heating, a firm gel is formed. A 2% suspension of the gum produces an elastic and resilient gel when heated at 90°C for ten minutes. The strength of the gel is dependent on the temperature alone and not the time of heating. The gel strength of a 3% suspension of curdlan heated at different temperatures is shown in Table VIII.

Table VIII. Gel Strength of Curdlan

<u>Temp. (°C)</u>	<u>(Gel Strength (g cm⁻²))</u>		
	<u>3%</u>	<u>2%</u>	<u>1%</u>
55	10	--	--
60	500	--	--
70	500	--	--
80	550	--	--
90	1300	--	--
100	1700	700	250

Data of Maeda, et al. (14)

The properties of the curdlan gel have been shown to be intermediate between those of the elastic gelatin gel and the brittle agar gel. The relationship between the breaking strength and the elastic modulus of agar, curdlan, and gelatin is shown in Figure 9.

Also it has been suggested that curdlan would have utility in foods such as gelled desserts, spaghetti, noodles, dietetic foods, hamburgers, and sausages.

PS-7 Gum (16)

PS-7 gum is the extracellular polysaccharide produced by Beijerinckia indica var. myxogenes. The structure of the polysaccharide is not known, but the composition consists of glucose (73%), rhamnose (16%), and glucuronic acid (11%).

PS-7 gum dissolves in hot or cold water to produce high viscosity solutions at low concentration. The relationship between viscosity and concentration is shown in Figure 10. For comparison, the same relationship for xanthan gum is included in the figure.

PS-7 gum solutions are highly pseudoplastic. The pseudoplasticity of PS-7 gum at 0.21% concentration is essentially

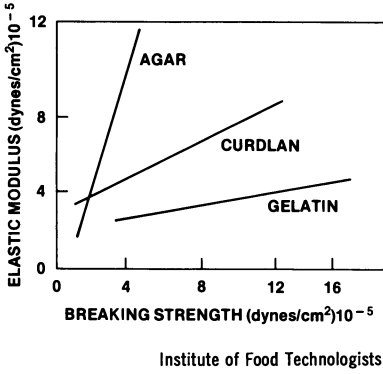


Figure 9. Gel properties of agar, curdlan, and gelatin, data of Kimura, et al. (15)

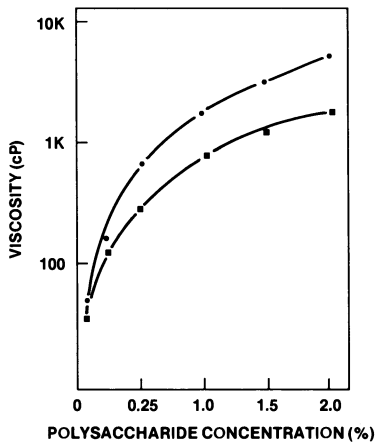


Figure 10. Viscosity vs. concentration relationship of PS-7: (●) PS-7; (■) xanthan gum; Brookfield LVT Viscometer, 60 rpm

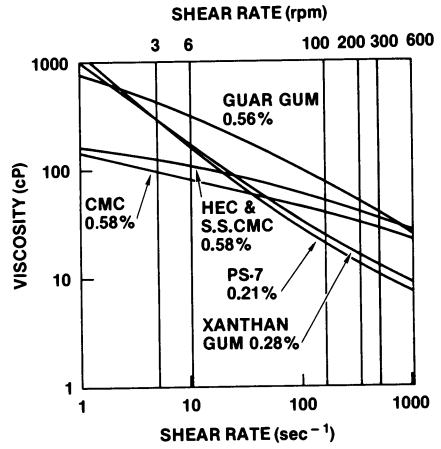


Figure 11. Viscosity vs. shear rate relationship of PS-7: Fann Viscometer Model 35A; 0.28% conc \equiv 1 lb/bbl

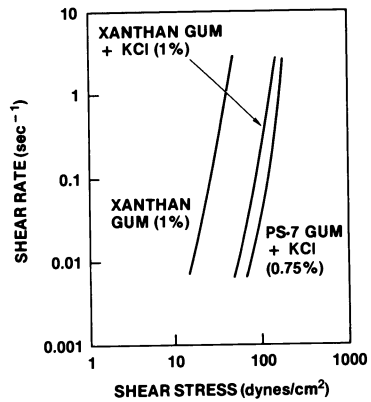


Figure 12. Low shear rate rheological properties of PS-7 gum

identical to the pseudoplasticity of xanthan gum at 0.28% concentration. This relationship is shown in Figure 11.

PS-7 gum solutions have a rheological yield point. The shear stress, shear rate curve is shown in Figure 12.

As can be seen from the figure, PS-7 gum at 0.75% concentration has a working yield value of 80 dynes cm^{-2} . This is compared to xanthan gum, which has a working yield value of 50 dynes cm^{-2} at 1% concentration. These data indicate that PS-7 gum will be a more effective suspending agent than xanthan gum.

The viscosity of PS-7 gum solutions is constant between 25°F and 200°F. The viscosity of aqueous solutions of PS-7 gum is constant over the pH range of pH 3.0 to pH 12.

PS-7 gum has excellent stability and compatibility with high concentrations of many salts. It is compatible, for example, with 26% solutions of sodium chloride and 32% calcium chloride.

The properties of PS-7 gum described above indicate that PS-7 gum would be effective to control the rheological properties of oil well drilling muds.

A drilling mud must be able to suspend the cuttings, carry them to the surface, and lubricate the drill bit. The pseudoplasticity and yield point of PS-7 gum provide these properties. Also the compatibility with high levels of salt allow drilling muds to be prepared using brines or seawater.

Other potential applications for PS-7 gum include dripless water-based latex paint, wall joint cement adhesives, and textile printing.

PS-10, (17) PS-21 Gum, and PS-53 Gum

These three polysaccharides are produced by bacteria which were isolated from soil samples taken from the rhizosphere of plants growing in Tahiti (PS-10) and the Canal Zone (PS-21, PS-53). The polysaccharides are considered together because they have utility in imparting excellent properties to water-based latex paints. The structure of these polysaccharides has not yet been elucidated, but the compositions are shown in Table IX.

These polysaccharides dissolve in cold water to produce high viscosity solutions at low concentration. The viscosity of each of the gums is shown in Table X.

The polysaccharides were evaluated in a semi-gloss latex paint formulation (Table XI).

The thickening efficiency, 60° gloss, and flow and leveling of PS-10, PS-21, and PS-53 in comparison to a hydroxyethylcellulose which is widely used in the paint industry are shown in Table XII.

These data indicate that PS-10, PS-21, and PS-53 have superior flow and leveling and gloss than the hydroxyethylcellulose, and that PS-21 and PS-53 have better thickening efficiency.

Table IX. Composition of PS-10, PS-21, and PS-53

	<u>PS-10</u>	<u>PS-21</u>	<u>PS-53</u>
Glucose (%)	37	26	37
Galactose (%)	28	19	--
Mannose (%)	--	29	--
Fucose (%)	12	--	35
Rhamnose (%)	--	--	--
Glucuronic acid (%)	18	15	16
Acetyl (%)	4.5	5.7	7.0
Pyruvate (%)	--	4.9	4.8

Table X. Viscosity of PS-10, PS-21, and PS-53

<u>Gum Concentration</u>	<u>PS-10</u>	<u>PS-21</u>	<u>PS-53</u>	<u>Hydroxyethyl Cellulose</u>
1%	1400 cP	2100 cP	3000 cP	1450 cP
0.5%	200 cP	600 cP	--	--

Table XII. Properties of PS-10, PS-21, and PS-53 in a Semi-gloss Latex Paint

	<u>Pounds of Thickener/ 100 gal.</u>	<u>Vis. (KU) (24 h)</u>	<u>60° Gloss</u>	<u>Flow/Leveling*</u>			
				<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>
PS-10	5.0	83	68	7	12	14	16
PS-21	3.5	81	64	8	13	14	17
PS-53	3.0	82	67	9	13	14	18
Hydroxyethyl cellulose	3.5	79	56	7	14	17	18+

* Determined using the method described in Kelco Technical Bulletin I-21. The lower the number, the better the flow and leveling.

Table XI. Semi-Gloss Latex

<u>Material</u>	<u>Pounds</u>	<u>Gallons</u>
<u>Grind</u>		
Water	72.5	8.7
DOWICIL 75	2.0	0.17
TAMOL 731 (25%)	9.0	7.00
Propylene Glycol	60.0	1.00
Ethylene Glycol	22.0	2.25
CARBITOL Solvent	18.0	2.25
DREW Y281	2.0	0.25
Titanium Dioxide R-900	270.0	8.00
Hexylene Glycol	10.0	1.31
Aerosol OT 75% (Aq)	2.0	0.50
Grind to 7+ N.S.		
<u>Letdown</u>		
RHOPLEX AC-490	433.0	49.50
DREW Y281	3.0	0.39
Thickener Solution*	178.0	20.75
	<hr/>	<hr/>
Total	1080.5	102.68

* Amount adjusted for each thickener tested to obtain approximately equal paint viscosity.

% Pigment Volume 28.0

% Total Volume Solids 30.0

PS-60 Gum (18)

PS-60 gum is the extracellular polysaccharide produced by an unnamed *Pseudomonas* species. The structure of the polysaccharide has not been elucidated, but it contains rhamnose, glucose, and uronic acid. The polysaccharide also contains 3 - 4.5% acetyl groups.

The polysaccharide dissolves in cold water to produce highly viscous solutions (3000 to 7000 cP at 1% concentration). After heating and cooling of the solution, a weak, very elastic gel is formed.

The most interesting feature of PS-60 is the ability to form firm, non-elastic, brittle gels after deacetylation. These gels are thermoreversible, and the setting and melting point data in comparison to kappa carrageenan and agar are shown in Table XIII.

Table XIII. PS-60 Gel Properties

	<u>Gel Nature</u>	<u>M.Pt. (°C)</u>	<u>Setting Pt. (°C)</u>	<u>Hysteresis (°C)</u>
Native PS-60	Very elastic	65-70	65-70	None
Deacetylated PS-60				
K ⁺ form	Brittle	90	31-46	45-60
Ca ⁺⁺ form	Brittle	90	45-50	45-50
Kappa carrageenan	Brittle	40-95	25-75	12-20
Agar	Brittle	60-97	32-39	<65

These data indicate that deacetylated PS-60 has gel properties similar to those of agar and K-carrageenan. Therefore, it is to be expected that this polysaccharide will find utility in those industrial applications where agar and K-carrageenan are now used.

In addition, I anticipate that this new polysaccharide will have general utility for: thickening, suspending, emulsifying, stabilizing, lubricating, film-forming, and binding. In particular, I expect that the polysaccharide will have uses in the following applications or products: adhesives, wall-joint cements water-retentive grouts and mortars, spackling compounds, can sealing, boiler compounds, latex creaming, welding-rod fluxes, brazing pastes, ceramic glazes and extrusions, cleaners and polishes, toys, emulsions (latex, asphalt, silicone), silver recovery, seed coatings, spray control for pesticides, emulsifiable concentrated and flowable pesticides, tobacco binders, water-based inks, lithographic fountain solutions, leather finishes, hydro-mulching and hydroseeding, textile printing and finishing, wet-end paper additives, wet-end paper retention and formation aid, anti-stick compounds, mold-release agents, liquid resins, slurry and packaged explosives, petroleum and water-well drilling muds,

petroleum workover and completion fluids, petroleum stimulation fluids, cosmetics, pharmaceutical suspensions and emulsions.

Also this gum will have utility in food systems such as jellies and other high sugar systems, beverages including citric acid based drinks, dairy products including ice cream and yogurt, dressings, dry mixes, icings, and glazes, syrups, farinaceous foods, canned and retorted foods, and bakery fillings. It is expected that this new gum will function as an agar, carrageenan, and pectin substitute in food systems.

Summary

It was my original intention to compare and contrast the properties of fungal and bacterial polysaccharides, but this would not have been fruitful based on the limited data base.

This presentation has shown that fungi and bacteria produce polysaccharides which have a diversity of interesting properties. These properties have either resulted in the polysaccharide being used in commerce, e.g., xanthan gum, or indicated that the polysaccharides have potential utility in commerce. However, in spite of my earlier statement that I would not discuss economics, it would be remiss of me if I did not point out that the most difficult task in the field of microbial polysaccharides is to produce them on a large scale economically.

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INDEX

A

- Absidia cylindrospora*, immunodiffusion pattern of E-fractions 87f
- Acetolysis patterns of yeast mannoproteins 2f
- Agar, gel properties 263f
- Alditol acetates in peptidophosphogalactomannan, permethylated 58t
- Amino acid composition
after alkaline borohydride treatment of glycopeptide 64t
of *P. charlesii* glycopeptide 63t
of peptidophosphogalactomannans 72t
- Amylopectin, ¹³C NMR spectrum 160f
- Anionic polysaccharides, glycuronate 183-189
- Antigenic
determinant groups of mannans of *C. albicans* strains, analysis of .. 106
fraction ACI-B and anti-ACE serum, inhibition of precipitin reaction between 90f
fractions ACI-B, MHI-B, and RNI-B against anti-ACE serum, precipitin curves of 88f
- Antigenicity of yeasts relationship to cell wall mannans 105
- Antisera of *C. albicans* strains and *S. cerevisiae*, precipitin reactions between mannans and 104f
- Aspergillus awamori* hyphal wall preparations, electron micrographs 148f

B

- Bacterial polysaccharides, industrial potential 251-269
- Baker's yeast (*see Saccharomyces cerevisiae*)
- Biosynthesis
of *S. cerevisiae* mannoprotein 8f
chromosome locations of genetic loci 8f
genetics of yeast mannoprotein 1-12
glycosylation pathway in mannoprotein 6f
nigeran 146

Biosynthesis (*continued*)

- of peptidophosphogalactomannan in *P. charlesii* 36
- of peptidophosphogalactomannan in vitro 39
- ethanolamine incorporation 45
- ethanolaminetransferase
activity 45
- mannose incorporation 39
- mannosylation of phosphogalactomannan 39
- mannosyltransferase activity 39
- mannosyltransferase, effect of Mn(II) and Mg(II) 43
- peptidophosphogalactomannan degradation products as mannosyl residue acceptors 43-45
time course 40t
- studies of *P. charlesii*, in vivo 36
- yeast mannoprotein 3-5
- asparagine-linked units 5f
- glycosyltransferases 5
- serine- and threonine-linked oligosaccharides 5
- ¹³C spin-lattice relaxation times and nOe values of resonances of 179t
- (*n*-Butylmethacrylate), poly 179f

C

- ¹³C (*see* Carbon-13) 159-180
- Callose formation 132-133
- Candida albicans*
antisera 97
bulk mannans 96-97
mannan, immunochemistry of 95-110
serotypes 105
strain(s)
cross-precipitin reactions between mannans of *S. cerevisiae* and antisera of 103
cultivation of 96
mannan(s) of 99, 100t, 102t
analysis of antigenic determinant groups of 106
chemical structure of 105
elution profiles of bulk 98f
fractionation of bulk 99

Candida albicans (continued)

- microheterogeneity in (see Microheterogeneity in *C. albicans* strains)
- precipitin reactions between mannan subfractions and antisera of 101-104

Candida utilis, immunodominant sidechain mannan structures 25f

Carbon-13

- determination of glycosidic configuration 162
- nuclear magnetic resonance signals of polysaccharides, identification of 164
- β -effect 164
- in *O*-substituted region 164
- α -shifts 164
- spectrum (a)
 - of baker's yeast mannan 165f
 - of *Ceratocystis stenoceras* polysaccharide 170f
 - of galactofuranose-containing polysaccharides 163f
 - of galactofuranose-containing polysaccharide from *S. schenckii* 168f
 - of β -D-glucopyranan from *S. schenckii* 163f
 - of β -D-(1 \rightarrow 5) linked D-galactofuranan 171f
 - of malonate-containing galactan of *P. citrinum* 171f
 - of mannans of *Rhodotorula glutinis* 166f
 - chemical structure and 161f
 - of phosphomannan from *H. capsulata*, effect of PrCl₃ on 172f
 - of polysaccharide preparations from *S. schenckii* strains .. 168f
 - and spin-lattice relaxation time values of nuclei of rhamnannan of *S. schenckii* 178f
 - and structure of linear mannan from *H. capsulata* 177f
 - and structure of pullulan from *Tremella mesenterica* 161f
- spectroscopy
 - determination of ester position in fungal polysaccharides by 169
 - of polysaccharides influencing factors 173-180
 - shifts occurring on *O*-phosphorylation 169-173
 - parameter arising from molecular motion 173

Carbon-13 (continued)

- spectroscopy (continued)
 - shifts occurring on *O*-phosphorylation (continued) polysaccharides influencing factors 173-180
 - spin-lattice relaxation times .. 173
 - structural studies on fungal polysaccharides 159-180
- nuclei in oligomeric compounds, spin-lattice relaxation times 177f
- signal(s)
 - displacements occurring
 - on 3-*O*-acetylation 171t
 - on addition of lanthanide ion to mannose phosphates 174t
 - on *O*-phosphorylation 172t
 - of nuclei in polysaccharides from *S. schenckii* and *C. stenoceras* 170t
 - shifts of structures identification 162
- spectra of ι -carrageenan at 175f
- spin-lattice relaxation times and nOe values of resonances of poly(*n*-butylmethacrylate) 179t

Carbon-14

- ethanolamine-containing phosphogalactomannan, fractionation of products 44f
- ethanolamine incorporation into polymers 44f
- mannose
 - containing saccharides, distribution of 41f
 - incorporation into acceptors, time course 38f
 - labeled peptidophosphogalactomannan, distribution after acetolysis 40f
 - mannosyl residues, location of 44f
 - phosphogalactomannan after acetolysis 44f
- ι -Carrageenan, ¹³C spectra of 175f

Cell-wall

- glucan of *P. oryzae*, structures 20f
- location of nigeran 145
- mannans, antigenicity of yeasts 105
- nigeran, time course of production 155f
- polymers, plant, microbial β -glucan hydrolases and lysis 115
- polysaccharides
 - from *P. oryzae*, structural studies 15
- Centromere linkage 7
- Chain conformation of (1 \rightarrow 3)-D-glucan 238f
- Chain organization in lamellar single crystals 148f

Chitin 234
 crystalline microfibrillarity 234
 hydrogen-bonding scheme 234
 occurrence 234
 structure 234
 unit cell, proposed 235f

Chitinase, activity in response to infection 131

Correlation time, spin-lattice relaxation time values with 174f

Cross-precipitin reaction between mannans of *C. albicans* and *S. cerevisiae* antiserum 103

Cryptococcus, glucuronoxylomannans 184t

Cryptococcus, taxonomic relationship between *Tremella* and 183

Crystal structure, determination steps 223

Crystal structure of fungal polysaccharides 221

Crystalline
 conformation of single chain for glucans 244f
 nigeran in hyphal walls 143-156
 structure, x-ray diffraction determination of polymeric 222-227

Crystallinity of nigeran 146-151
 cell-wall samples of *A. awamori*, interplanar distances for hydrated 147t
 crystal lattice stability 149
 distribution in hyphal walls of *A. niger* and *A. awamori* 150t
 electron opacity of cell-wall, correlation with 146
 x-ray diffraction data 146

Crystallization, structural requirements for polymeric 222-223

Curdlan 262
 applications, potential 262
 gel properties 263f
 gel strength 262t
 microfibrils, scanning electron micrographs 233f
 properties 262

Cytoplasmic peptidophosphogalactomannan characterization 68

D

Deuterium labeling signal identification 164

Dextran 261
 applications 261
 viscosity data 261t

E

Effectors modulation in cell membranes, mechanism of 135f

Elsinan 242
 action pattern of human salivary α -amylase 212f
 chemical modification 214
 comparison of properties of native and modified 219t
 introduction of 3,6-anhydro-linkages into α -(1 \rightarrow 4)-linked D-glucose units 214-217
 chemical properties 198-200
 effect(s)
 of carbon sources on production 199f
 of concentration on viscosity 215f
 of ionic strength on viscosity 216f
 of pH on viscosity 216f
 of temperature on viscosity 215f
 enzymatic degradation 197-220
 enzymes used 207-209
 human salivary amylase, degradation with 209-211
 disaccharide fraction 211
 trisaccharide fraction 211
 starch-degrading enzymes, action 208t, 209, 210t
 Taka amylase action 211
 heptasaccharide fraction 213
 tetrasaccharide fraction 211
 film, properties 216t
 with human salivary α -amylase, reaction product 212f
 with human salivary α -amylase, time course of enzymatic hydrolysis 210f
 identification of Smith degradation products 202f
 physical properties 213-214
 film formation 214
 viscosity 213
 effects of pH, temperatures, and electrolytes 214
 possible action pattern of Taka amylase on 212f
 production 198
 repeating unit 206f
 Smith degradation 202f
 solution, pseudoplasticity 215f
 structural similarities to nigeran and pullulan 207
 structure 200-207
 acetolysis products 205
 fragmentation by partial acid hydrolysis 203, 204t
 D-glucosidic linkages, mode 200
 D-glucosidic linkages, sequence .. 203
 yields and methylation analyses .. 201t
 disaccharide components 203
 tetrasaccharide components 205
 trisaccharide components 203
 structure degradation 197-220

Elsinan (*continued*)

synthesis of 3,6-anhydro-	218f
time course of production	199f
<i>Elsinoe leucospila</i> , α -D-glucan	197-220
Enzymatic	
degradation of elsinan	197-220
depolymerization of nigeran	151-153
mycodextranase attack	151
temperature dependence	151
hydrolysis of elsinan with human	
salivary α -amylase, time course	210f
Enzymic depolymerization of wilt-	
inducing 3,6- β -glucans	132
Ethylene phytohormone link between	
infection process and 1,3- β -glucan	
hydrolase induction	131
<i>Eucalyptus sieberi</i> wilting bioassay	
of purified <i>Phytophthora</i> polysac-	
charides	120f, 123f
of unfractionated ethanol-insoluble	
<i>Phytophthora</i> polymers	118f
Exocellular	
definition of	183
nigeran-protein complex, produc-	
tion time course	155f
polysaccharide from <i>Tremella</i>	
<i>mesenterica</i> , repeat unit	186f
yeast O-phosphonomannans	189
yeast polysaccharides, structural	
aspects	183-194
Exopolysaccharides of lipomyces	188t
Extracellular peptidophosphogalacto-	
mannan	52
Extracellular saccharides, fungal	49
F	
Fucogalactan	193
Fungal	
cell-wall polysaccharides	143
extracellular saccharides	49
glycopeptide biosynthetic studies,	
membrane-bound	35-47
glycopeptide, structural studies,	
membrane-bound	49
hyphae, lysis	130
polysaccharides	
carbon-13 NMR determination	
of ester position in	169
chemotaxonomy	81-82
crystal structure	221
deuterium labeling signal	
identification	164
industrial potential	251-269
texture structure	221
using ^{13}C NMR spectroscopy,	
structural studies	159-180
using proton NMR spectroscopy,	
structural studies	159-180
Fungi, water-soluble 1,3- β -glucans	
from	124-125t

G

Galactan of <i>P. citrinium</i> , ^{13}C -NMR	
spectra of malonate-containing ..	171f
D-Galactofuranan, ^{13}C -NMR spectra	
of β -D-(1 \rightarrow 5) linked	171f
Galactofuranosidase-treated phospho-	
galactomannan, ^{13}C -NMR spectrum of	60f
Galactofuranosyl	
-containing polymer, fractionation ..	70f
-containing substance(s) from inter-	
face, solubilization	70t
residues	49-77
GPD-D-(^{14}C)mannose-peptidophospho-	
galactomannan-mannosyltrans-	
ferase components, distribution	
of ^{14}C -mannose-containing sac-	
charides	41f
GDP-D-mannose, glycosyl donor in	
glycoproteins	35
Gelatin, gel properties	263f
Genetic(s)	
analysis of yeast mannoproteins	5-7
control of mannoprotein biosyn-	
thesis	7-12
control levels	7t
addition of outer chain to	
mannan core	10t
aminoacid acceptor sequences	9t
glycosyltransferase activity and	
localization	7t-9t
protein synthesis	7t
synthesis and processing of	
mannan core	9t
termination and modification of	
outer chain	10t
translocation of glycosylated	
protein	9t-10t
of yeast mannoprotein biosynthesis	1-12
Glucan(s)	
crystalline conformation of single	
chain	244f
α -D- of <i>Elsinoe leucospila</i>	197-220
of <i>Pyricularia oryzae</i> , structure of	
cell-wall	20f
(1 \rightarrow 3)- α -D-	234-237
energy map	238f
microcrystallinity	237
occurrence	234-236
structural model	236
energy-based packing analysis	237
2_1 helix conformation proposal	236
β -	
acquired resistance	133
elicitors of phytoalexin	
production	128-130
hydrolase(s)	
induction, host	130-132
and lysis of plant cell-wall	
polymers, microbial	115

Glucan(s) (*continued*)

in plant pathogenesis113-136
 role of in host resistance 127
 induction of hypersensitive
 reaction by 128
 mechanism of wilt-induction by .. 127
 from microorganisms, wilt-
 inducing 115
 from *Phytophthora* species,
 analytical procedure, wilt-
 inducing 117
 in plant pathogenesis113-136
 role of in host resistance 127
 β -D- of *P. oryzae* mycelium15-19
 isolation15-16
 structural determination16-19
 fragmentation analysis 18
 hydrolysis products of
 methylated 17t
 molecular weight determination 16t
 periodate oxidation 18t
 1,3- β - from fungi, water-soluble 124-125t
 1,3- β -hydrolase(s)
 activity increase in response to
 infection 131
 induction, ethylene phytohor-
 mone link between infection
 process and 131
 pathogenic modulation of activity
 reduction in size of 3,6- β -glucans
 (1 \rightarrow 3)- β -D-227-234
 AB projection of unit cell 233f
 crystal structure 227
 density measurements 230
 effect of relative humidity on
 x-ray diagrams 230
 packing analysis of "hydrate"
 form 232
 packing of rigid chains, dry
 form 232
 triple helix structure proposal .. 230
 morphology232-234
 microfibrillarity 232
 natural sources and functions 228t
 occurrence 227
 polymorphs, unit-cell parameters 229t
 3,6- β -, 1,3- β -glucan hydrolases,
 reduction in size of 132
 3,6- β , relationship of wilt-inducing
 to hyphal-wall polysaccharides 122
 α -D-Glucomannans 192
 β -D-Glucopyranan of *Lentinus edodes*,
¹³C-NMR spectrum of 177f
 β -D-Glucopyranan from *Sporothrix*
schenkii, ¹³C-NMR spectrum of 163f
 Glucosaminuronans, *Rhinoctadiella* 187-189
 Glucose residue linkages, formation
 of 224f
 Glucuronomannan from *L. lipofer*,
 structure 187

Glucuronoxylomannans

cryptococcus 184t
 methylation 186t
 methylation analyses 184-185
 structural features 185
Tremella 184t
 Glycopeptide(s)
 acetolysis 61
 amino acid composition of *P.*
charlesii 63t
 biosynthetic studies, membrane-
 bound fungal 35-47
 carbon-14 from [1-¹⁴C] acetate
 incorporated into 74t
 changes in amino acid composition
 after alkaline borohydride
 treatment 64t
 chemical characterization 72t
 distribution of ³²P after chroma-
 tography-labeled 67f
 release of saccharides from sodium-
 hydroxide treated 60f
 structural studies, membrane-bound
 fungal 49
 Glycosidic configuration, ¹³C methods
 for determination of 162
 Glycosyl donor in glycoproteins 35
 Glycosylation pathway in manno-
 protein biosynthesis 6f
 Glycosyltransferases, cellular
 localization 10-11
 Glycuronate anionic polysac-
 charides183-189
 Guar gum solutions, viscosity of
 xanthan gum- 258f
 Guar, relationship between shear rate
 and shear stress 258f
 Gums, derived 251t
 GXM (glucuronoxylomannans) 184-185

H

Hansenula holstii O-phosphono-
 mannan, autohydrolysis 190
 Helical conformation of lichenan 244f
 Helix, XY projection of triple 231f-232f
 Heterogeneity in peptidophospho-
 galactomannan 54f
 Host
 β -glucan hydrolase induction130-132
 -pathogen interactions, molecular
 events 114t
 resistance, β -glucans 127
 hydrolases 127
 Human salivary α -amylase, action
 pattern on elsinan 212f
 Human salivary α -amylase, time
 course of enzymatic hydrolysis
 of elsinan with 210f

- Hypersensitive reaction, induction
by β -glucans 128
- Hypchal wall(s)
polysaccharides, relationship of
wilt-inducing 3,6- β -glucans to 122
- preparations, electron micrographs
of *A. awamori* 148f
- synthesis, organization and con-
formation of crystalline
nigeran 143-156

I

- Immunochemical
examination of the polysaccharides
of mucorales 81-93
- methods for mannan evaluation 97
- study of bulk mannan of *S. cerevisiae* 106
- serological activity of acidic
fraction 106
- studies of yeast mannans 105-106
- Immunochemistry of *C. albicans*
mannan 95-110
- Immunochemistry of yeast manno-
proteins 1-3
- Immunodominant sidechains in yeast
mannoproteins 4f
- Industrial
potential of bacterial polysac-
charides 251-269
- potential of fungal polysac-
charides 215-269

K

- Kluyveromyces lactis* mannanprotein
mutant, possible defects 11t

L

- Lamellar
configuration of *nigeran*, folded 153
- single crystals, chain organization .. 148f
- single crystals of *nigeran* 144f
- enzymatic depolymerization 151-153
- Lichenan 207, 240
- asymmetric unit 241f
- helical conformation 244f
- structural model 240-242
- Linkage, centromere 7
- Lipomyces*
exopolysaccharides 188t
- heteropolysaccharides, taxonomic
value of compositional
differences 185-187
- polysaccharides, repeat unit
structures 188f

- Lipomyces (continued)*
tetrasporus strain, galactosylated
polysaccharide structure 187
- Lipopeptidophosphogalactomannan
function 45-46
- Lipopeptidophosphogalactomannan,
thin layer chromatography of 76f
- Locust bean gum gels, gel strength
and viscosity of xanthan gum- 260f
- Lysis of fungal hyphae 130
- Lysis of plant cell-wall polymers,
microbial β -glucan hydrolases
and 115

M

- Mannan(s)
antigenicity of yeasts relationship
cell-wall 105
- of *Candida albicans* and *S. cere-
visiae* antiserum, cross-precipi-
tin reaction between 103
- of *Candida albicans* strain(s)
analysis of antigenic determinant
groups 106
- chemical structure 105
- elution profiles of bulk 98f
- fractionation of bulk 99
- carbon-13 NMR spectrum of
baker's yeast 165f
- chemical structure of *S. cerevisiae* .. 105
- chromatographic fractionation of
C. albicans bulk 97
- elution profile of bulk 107f
- evaluation, immunochemical
methods for 97
- from *Hansenula capsulata*, ^{13}C -
NMR spectrum and structure
of linear 177f
- highly branched neutral 191t
- immunochemical studies of yeast 105-106
- immunochemistry of *C. albicans* ..95-110
- isolated from *S. schenckii*,
structures 166t
- methylation of yeast 191t
- mutants of *S. cerevisiae* 7
- preparation of *C. albicans* bulk ..96-97
- of *Rhodotorula glutinis*, ^{13}C -NMR
spectra 166f
- from *Rhodotorula glutinis*, chemical
structure and ^{13}C -NMR
spectrum 161f
- of *Saccharomyces cerevisiae* and
antisera of *C. albicans* strains,
cross-precipitin reactions
between 103
- of *Saccharomyces cerevisiae*,
immunochemical study of bulk
(see Immunochemical study of
bulk mannan of *S. cerevisiae*)

Mannan (*continued*)
of *Saccharomyces fragilis*, spin-lattice relaxation time values of nuclei 178f
structure, *Saccharomyces cerevisiae*, wild-type immunodominant sidechain 25f
subfractions
acetolysis fingerprints 109f
against homologous anti-whole-cell serum of *S. cerevisiae*, quantitative precipitin curve and antisera of *C. albicans* strains, quantitative precipitin reactions between 101-103
and antisera of *C. albicans* strains and *S. cerevisiae*, quantitative precipitin reactions between 104f
of *Candida albicans* strains, chemical composition 99, 100t, 102t
chemical composition 109t
of *Saccharomyces cerevisiae* 97
from *Trichosporon aculeatum*, proton NMR spectrum 160f
yeast 242
 α -D-Mannans, methylation analyses 190-192
 β -D-Mannans 193
Mannanphosphate content and serological activity, relationship between yeast 95-110
Mannoprotein(s)
acetolysis oligosaccharide patterns of yeast 2f
biosynthesis of carbohydrate part of *S. cerevisiae* (*see* Biosynthesis of carbohydrate part of *S. cerevisiae* mannoprotein)
genetic control of (*see* Genetic control of mannoprotein biosynthesis)
genetics of yeast 1-12
glycosylation pathway 6f
yeast 3-5
from direct mutation, alterations in *S. cerevisiae* 8f
genetic analysis of yeast 5-7
immunochimistry of yeast 1-3
immunodominant sidechains in yeast 4f
mutant(s)
fluorescent lectin for selection and enrichment of yeast 3
mnn2 3
mnn2-2, possible defects in *Kluyveromyces lactis* 11t
mnn4, possible defects in *S. cerevisiae* 11t
schematic structures 2f
structure, elucidation of yeast 1-3

Mannoprotein(s) (*continued*)
structures of protein-linked carbohydrate chains in *S. cerevisiae* mannoprotein 4f
Mannosyltransferase activity, influence of acceptor concentration 42f
components, distribution of ¹⁴C-mannose-containing saccharides in GDP-D-(¹⁴C)mannose-peptidophosphogalactomannan- 41f
purification of soluble 42f
Membrane-bound fungal glycopeptide biosynthetic studies 35-47
Membrane-bound fungal glycopeptide, structural studies 49
Microbial pathogens, interaction between plants and 113
Microbial pathogen resistance mechanisms, plant 113-115
Microheterogeneity in *C. albicans* strains 108
Monosaccharide composition of purified *Phytophthora* polysaccharides 120t
Monosaccharides from sodium-hydroxide treated glycopeptide, release of 60f
Mucor species, immunodiffusion pattern of E-fractions 87f
Mucorales
antigenic fraction ACI-B, quantitative precipitin curves of antisera against 83f
antigenic substances with anti-ACE serum, immunodiffusion patterns of fractions 85f
fraction ACI with Con A-sepharose, affinity chromatography 83f
immunochemical examination of polysaccharides 81-93
serologically active fractions with anti-ACE serum, immunodiffusion pattern 83f
serologically active substances 82
antisera preparation 82-84
fluorescent antibody straining 91
inhibition test 89
ACI-B, MHI-B, and RNI-B, chemical analysis 92t
manno-oligosaccharides, chemical properties of purified .. 90t
manno-oligosaccharides, methylated alditol acetates from methylated 88t
precipitation reactions of ACI-B, MHI-B and RNI-B with anti-ACE serum by oligosaccharides, inhibition 92t

- Mucorales (*continued*)
 serologically active substances (*continued*)
 isolation of antigenic fractions 84-86
 fractions ACI-B, ACE-B, and PHW-B, chemical analysis 85t
 serologically reactive site, elucidation 86
 mycelial preparation 82
 serological cross-reaction 86
 of E- and I-fractions from various fungi against anti-ACE serum 87t
- Mutant(s)
 fluorescent lectin for selection and enrichment of yeast manno-protein 3
mnn2, mannoprotein 3
 possible defects in mannoprotein 11t
 of *S. cerevisiae*, mannan of 7
- Mutation, direct, alterations in *S. cerevisiae* mannoproteins 8f
- N
- Nigeran 237-240
 biosynthesis 146
 nitrogen effect on deposition 146
 cell-wall location 145
 crystallinity 239
 in dry state, conformation of single chain 241f
 elsinan structural similarities 207
 enzymatic depolymerization of lamellar single crystals 151-153
 folded lamellar configuration 153
 function in cell wall 240
 in hyphal walls, synthesis, organization, and conformation of crystalline 143-156
 in vivo, crystallinity 146-151
 lamellar single crystals 144f
 occurrence 237-240
 properties 143
 -protein complex isolated from culture filtrates 154-156
 kinetics of production 154
 structural analysis 154
 -protein complex, time course of production of exocellular 155f
 repeating unit 144f
 single crystals, preparation and analysis 145
 structural model 237
 time course of production of cell-wall 155f
 unit cell dimensions for polymorphs 239t
- NMR (nuclear magnetic resonance) 159-180
- Nuclear Overhauser enhancement (nOe), relation of spin-lattice relaxation times 175f, 179t
- O
- Oligomeric compounds, spin-lattice relaxation time values of ¹³C nuclei 177f
- Oligosaccharides
 derived from peptidophosphogalactomannan and derivatives of precipitin reaction between antigenic fraction ACI-B and anti-ACE serum, inhibition 90f
 from sodium-hydroxide treated glycopeptide, release 60f
- P
- Paint, composition 267t
- Pathogen(s)
 interaction between plants and microbial 113
 interactions, molecular events in host- 114t
 resistance mechanisms plant, microbial 113-115
- Pathogenesis β -glucans in plants 113-136
 hydrolases 113-136
- Pathogenic modulation of activity of 1,3- β -glucan hydrolases 132
- Pathogenicity of *P. oryzae* 15
- Penicillium charlesii*
 biosynthesis of peptidophosphogalactomannan 36
 biosynthetic in vivo studies of (*see* Biosynthetic in vivo studies of *P. charlesii*)
 cultures, increase in dry weight of mycelia 54f
 cultures, time course of D-glucose and NH₄⁺ uptake from growth medium 54f
 glycopeptide, amino acid composition 63t
 peptidophosphogalactomannan 50
 carbon-13 NMR spectroscopy 52
 chromatography 51
 composition of polypeptide from 61-65
 enzymic and chemical assays 52
 hydrogen fluorolysis 51
 isolation and fractionation of membranes 50
- Pentosylmannans 192
- Peptidophosphogalactomannans 49-77
 amino acid composition 72t
 characterization, cytoplasmic 68

- Peptidophosphogalactomannans
(*continued*)
- characterization, membrane-bound 68-77
 - composition of polypeptide from *P. charlesii* 61-65
 - concentration on ¹⁴C-ethanolamine incorporation into polymers, influence of 44f
 - and derivatives, oligosaccharides derived from 62f
 - and derivatives, permethylated alditol acetates 58f
 - distribution
 - of ¹⁴C and carbohydrate after chromatography of low-molecular-weight substances from alkali-treated 67f
 - of ¹⁴C-mannose-containing saccharides after sodium-hydroxide treatment of 41f
 - of carbohydrate and ¹⁴C after acetolysis of ¹⁴C-mannose-labeled 40f
 - of carbohydrate and ¹⁴C after treatment of ¹⁴C-labeled 38f
 - extracellular galactomannan with alkali, treatment 55
 - galactosyl residues removal 53
 - phosphomannan, proton NMR spectrum 55
 - functions 45-47
 - heterogeneity 54f
 - in vitro, biosynthesis of (*see* Biosynthesis of peptidophosphogalactomannan in vitro)
 - mannosyltransferase components, distribution of ¹⁴C-mannose-containing saccharides in GDP-D-(¹⁴C)mannose- 41f
 - methylation analysis 57
 - noncarbohydrate constituents 65-68
 - Penicillium charlesii* (*see* *Penicillium charlesii* peptidophosphogalactomannan)
 - in *Penicillium charlesii*, biosynthesis of (*see* Biosynthesis of peptidophosphogalactomannan in *P. charlesii*)
 - sedimentation equilibrium ultracentrifugation 56f
 - separation of polypeptides derived from 64f
 - separation of Smith degradation products of double-labeled 67f
 - thin layer chromatography 74f
- Peptidophosphomannan, proton NMR of anomeric proton region 56f
- Phosphate content and serological activity, relationship between yeast mannan- 95-110
- Phosphoethanolamine 46
- Phosphogalactomannan 55
- chemical analysis 57
 - C-NMR spectroscopic analysis 57-61
 - ¹³C-NMR spectrum of galactofuranosidase-treated 60f
 - fractionation of products formed by sodium-hydroxide treatment of ¹⁴C-ethanolamine-containing 44f
 - physiological functions 46
- O-Phosphonohexoglycans 189-190, 191t
- Phosphonomannan, *H. capsulata* ¹³C-NMR spectrum 172f
- possible structures 174f
- O-Phosphonomannan
- autohydrolysis of *H. holstii* 190
 - exocellular yeast 189
 - repeat units of poly(phosphoric diester) 188
 - types of 189
- Phytoalexin production, β -glucan elicitors 128-130
- Phytophthora*
- cinnamomi* extracellular polymers, fractionation 119f
 - polymers, *E. sieberi* wilting bioassay of ethanol-insoluble 118f
 - polysaccharide(s)
 - Eucalyptus sieberi* wilting bioassay of purified 120f
 - and enzymically treated 123f
 - glucosidic linkage composition 121t
 - monosaccharide composition of purified 120t
 - species, analytical procedure, wilt-inducing β -glucans from 117
 - species, wilt-inducing toxins from 113-136
- Plant(s)
- cell-wall polymers, microbial β -glucan hydrolases and lysis .. 115
 - and microbial pathogens, interaction between 113
 - microbial pathogen resistance mechanisms 113-115
 - pathogenesis, β -glucans 113-136
 - hydrolases 113-136
- Polymeric crystalline structure, x-ray diffraction determination 222-227
- Polymeric crystallization, structural requirements 222-223
- Polypeptides, derived from peptidophosphogalactomannan, separation 64f
- Polypeptide from *P. charlesii* peptidophosphogalactomannan, composition 61-65

- Polysaccharide(s)
 carbon-13 NMR
 spectra of *C. stenoceras* 170f
 spectroscopy, determination of
 ester position in fungal 169
 spectroscopy, elucidation of
 structural order 159
 commercial production of
 microbial 197
 component identification 167
 components of *P. oryzae* cell walls,
 organization 27-29
 polysaccharide fractions 29
 differentiation of yeast species by
 proton NMR spectroscopy of
 component mannose-containing 160t
Eucalyptus sieberi wilting bioassay
 of *Phytophthora cinna-*
momi 120f, 123f
 fungal
 cell-wall 143
 chemotaxonomy 81-82
 crystal structure 221
 deuterium labeling signal
 identification 164
 industrial potential 251-269
 texture structure 221
 using NMR spectroscopy, struc-
 tural studies 159-180
 glucose residue linkages, formation 224f
 glucosidic linkage composition of
Phytophthora 121t
 glycuronate anionic 183-189
 gums 262-269
 identification of ¹³C NMR signals .. 164
 industrial potential of bacterial .. 251-269
 with industrial use or potential 252t
 bacterial 252t
 fungal 252t
 mixtures of polysaccharides,
 components identification 167
 monosaccharide composition of
 purified *Phytophthora* 120t
 of mucorales, immunochemical
 examination 81-93
 neutral 190-194
 partial ¹³C-NMR spectra of galacto-
 furanose-containing 163f
 preparations from *S. schenckii*
 strains, ¹³C-NMR spectra 168f
 from *Pyricularia oryzae*, structural
 studies on cell-wall 15
 relationship of wilt-inducing 3,6-β-
 glucans to hyphal-wall 122
 repeat unit structures of *Lipomyces*
 from *Sporothrix schenckii*, ¹³C-NMR
 spectrum of galactofuranose-
 containing 168f
- Polysaccharide(s) (*continued*)
 from *Sporothrix schenckii* and *C.*
stenoceras, ¹³C signals of nuclei 170t
 strain of *L. tetrasporus*, structure
 of galactosylated 187
 structural aspects of exocellular
 yeast 183-194
 structures from *S. schenckii* and
C. stenoceras 166f
 from *Tremella mesenterica*, repeat
 unit of exocellular 186f
 wilting response induced by 122
 Precipitin reaction between antigenic
 fraction ACI-B and anti-ACE
 serum, inhibition by oligosac-
 charides 90f
 Protein complex isolated from culture
 filtrates, nigeran- 154-156
 Protein complex, time course of pro-
 duction of exocellular nigeran- 155f
 Proteoheteroglycan
 acetolysis patterns of 22f
 by acid hydrolysis, release of sugars 22f
 exo-α-D-mannanase-digested core,
 acetolysis patterns 22f
oryzae anti-serum, diffusion
 patterns 25f
 preparations, elution patterns 20f
 of *Pyricularia oryzae* mycelium 19
 isolation 19
 structural determination 19
 acetolysis 21
 elution profile 24
 hydrolysis 19-21
 products 23t
 methylation 21
 molecular weight deter-
 mination 19
 quantitative precipitation
 reactions 24-26
 sidechain structure deter-
 mination 26-27
 from *Pyricularia oryzae*, proposed
 structure for 28f
 Proton NMR spectrum of mannan
 from *Trichosporon aculeatum* ... 160f
 Proton NMR spectroscopy, structural
 studies on fungal polysac-
 charides 159-180
 Pullulan 242, 253
 elsinan structural similarities 207
 films, advantages 253
 films, oxygen permeability 253t
 properties 253
 from *Tremella mesenterica*, ¹³C
 NMR spectrum and structure .. 161f
Pyricularia oryzae
 antiserum, diffusion pattern 31f
 antiserum, inhibition 28f

Pyricularia oryzae (continued)
 antiserum, quantitative precipitation curves 25f
 cell surface, serological properties of (see Serological properties of *P. oryzae* cell surface)
 cell walls, organization of polysaccharide components 27-29
 mycelium, proteoheteroglycan (see Proteoheteroglycan of *P. oryzae* mycelium)
 pathogenicity 15
 proposed structure for proteoheteroglycan from 28f
 structural studies on cell-wall polysaccharides 15
 structures of cell-wall glucan 20f

Q

Quantitative precipitin curves
 of antigenic fractions ACI-B, MHI-B, and RNI-B against anti-ACE serum 88f
 of antisera against Mucorales antigenic fraction ACI-B .. 83f
 of mannan subfractions against homologous anti-whole-cell serum of *S. cerevisiae* for *P. oryzae* antiserum 25f, 31f
 reactions between mannan subfractions and antisera of *C. albicans* strains 101-103
S. cerevisiae 104f

R

Resistance mechanisms, plant, microbial pathogen 113-115
 Rhamnonannan of *S. schenckii*, ¹³C-NMR spectrum and spin-lattice relaxation times 178f
Rhinochladiella glucosaminuronans .. 187-189
Rhizopus nigricans, immunodiffusion pattern of E-fractions from 87f

S

Saccharides
 after sodium-hydroxide treatment of peptidophosphogalactomannan, distribution of ¹⁴C-mannose-containing 41f
 fungal extracellular 49

Saccharides (continued)
 in GDP-D-(¹⁴C)mannose-peptidophosphogalactomannan-mannosyltransferase components, distribution of ¹⁴C-mannose-containing 41f
Saccharomyces cerevisiae
 antiserum (a) 97
 of *Candida albicans* strains, cross-precipitin reactions between mannans of 103f
 cross-precipitin reaction between mannans of *C. albicans* and .. 103
 immunochemical study of bulk mannan of (see Immunochemical study of bulk mannan of *S. cerevisiae*)
 mannan
 chemical structure 105
 mutants 7
 structure 5
 subfractions 97
 mannoprotein(s)
 biosynthesis of carbohydrate part of (see Biosynthesis of carbohydrate part of *S. cerevisiae* mannoprotein)
 from direct mutation, alterations 8f
 mutant, possible defects 11t
 structures of protein-linked carbohydrate chains 4f
 quantitative precipitin curves of mannan subfractions against anti-serum of 107f
 quantitative precipitin reactions between mannan subfractions and antisera of *C. albicans* strains and 104f
 wild-type *C. utilis*, immunodominant sidechain mannans structures from 25f
Saccharomyces species, longest sidechains in three wild-type infertile 6f
 Scleroglucan 242, 253-255
 shear rate, viscosity of crude and purified 256f
 solutions, effect of temperature on viscosity of crude 254t
 uses 255
 viscosity of crude and purified 254t
 Serological
 activity, relationship between yeast mannan phosphate content and 95-110
 classification system for yeasts 105
 properties of *P. oryzae* cell surface .. 29-32
 antigen-antibody agglutination tests 30t

Spin-lattice relaxation time(s) and nuclear Overhauser enhance- ment (nOe), relation of	175f
and nuclear Overhauser enhance- ment values of poly(<i>n</i> -butyl- methacrylate), ¹³ C	179t
values of carbon-13 nuclei in oligomeric compounds	177f
of nuclei in main chain and side chains of mannan of <i>S.</i> <i>fragilis</i>	178f
of nuclei of rhamnonannan of <i>S.</i> <i>schneckii</i> , partial ¹³ C-NMR spectrum and	178f
with correlation time	174f
Structural organization	224f

T

Taka amylase on elsinan, possible action pattern	212f
Tetrasaccharide fraction	211
Toxins from <i>Phytophthora</i> species, wilt-inducing	113-136
<i>Tremella</i> and <i>Cryptococcus</i> , a taxonomic relationship between	183
glucuronoxylomannans	184t
<i>mesenterica</i> , repeat unit of exo- cellular polysaccharide from	186f

U

UDP- <i>N</i> -acetyl- <i>D</i> -glucosamine, glycosyl donor in glycoproteins	35
--	----

W

Wilt-inducing 3,6- β -glucans relationship to hyphal-wall polysaccharides	122
β -glucans from microorganisms	115
β -glucans from <i>Phytophthora</i> species, analytical procedure ..	117
toxins from <i>Phytophthora</i> species	113-136
Wilt induction by β -glucans, mecha- nism of	127
Wilting bioassay of purified and enzymically treated <i>Phytophthora cinnamomi</i> poly- saccharide, <i>E. sieberi</i>	123f
of purified <i>Phytophthora</i> polysac- charides, <i>E. sieberi</i>	120f
of unfractionated ethanol-insoluble <i>Phytophthora</i> polymers, <i>E. sie-</i> <i>berii</i>	118f
Wilting response induced by poly- saccharides	122

X

Xanthan gum	255-261
applications	259-261
-guar gum solutions, viscosity	258f
and guar, relationship between shear rate and shear stress	258f
-locust bean gum gels, gel strength and viscosity	260f
properties	255
pseudoplasticity of aqueous solutions	255
reactivity with galactomannans ..	259
resistance of solutions degradation	255
stability	259
viscosity of aqueous solutions	259
relationship between shear rate and viscosity	257f
relationship between viscosity and concentration	257f
structure	255, 256f
viscosity data	261t
X-ray diffraction determination of poly- meric crystalline structure	222-227

Y

Yeast(s) mannans	242
immunochemical studies	105-106
methylation	191t
mannanphosphate content and sero- logical activity, relationship between	95-110
mannoprotein(s) acetolysis oligosaccharide patterns	2f
biosynthesis	3-5
genetics	1-12
genetic analysis	5-7
immunochemistry	1-3
immunodominant sidechains	4f
mutants, fluorescent lectin for selection and enrichment	3
structure	1-3
<i>O</i> -phosphomannans, exocellular	189
polysaccharides, structural aspects of exocellular	183-194
relationship to cell-wall mannans, antigenicity	105
serological classification system	105
species by proton NMR spectroscopy of component mannose- containing polysaccharides, differentiation	160t