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Studies on Fungicides. XI.[1] Biosyntheses of β-Glucan and Citin-like Substance of Cell Wall from *Cochliobolus miyabeanus*. (2)

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The biosynthesis of the β-glucan and the chitin-like substance, main components of the cell wall of *Cochliobolus miyabeanus*, were studied. The chemical structures of the synthesized polysaccharides by incubating with mycelial enzyme (soluble enzyme, particulate enzyme, crude enzyme), UDP-^{14}C-glucose or UDP-^{14}C-N-acetylglucosamine and glucan or chitodextrin were examined. Judging from these results, possible synthetic pathways were presented. N-acetylglucosaminyl residues were transfered to the non reducing terminals of chitodextrin from UDP-sugar molecules by incubating with the particulate enzyme, forming a straight chain of β-1,4 linked N-acetylglucosamine polymer. A part of the UDP-N-acetylglucosamine was converted to UDP-N-acetylgalactosamine with the soluble enzyme, followed by forming branched unit of N-acetylgalactosamine connected through C-1 by α-glycosidic linkage with the particulate enzyme. In the glucan synthesis glucosyl residued were transfered to the non reducing terminal and the internal glucosyl residues of β-glucan from UDP-glucose molecules by the particulate enzyme, forming the β-1,3 glucan having branched units connected through C-6 and C-1.

Introduction

In the previous paper,[3,4] the authors reported the presence of the β-glucan and the chitin-like substance (Fig. 1) in the cell wall of *Cochliobolus miyabeanus* as main components, and that particulate enzymes obtained from the mycelia stimulate the incorporation of ^{14}C-glucose

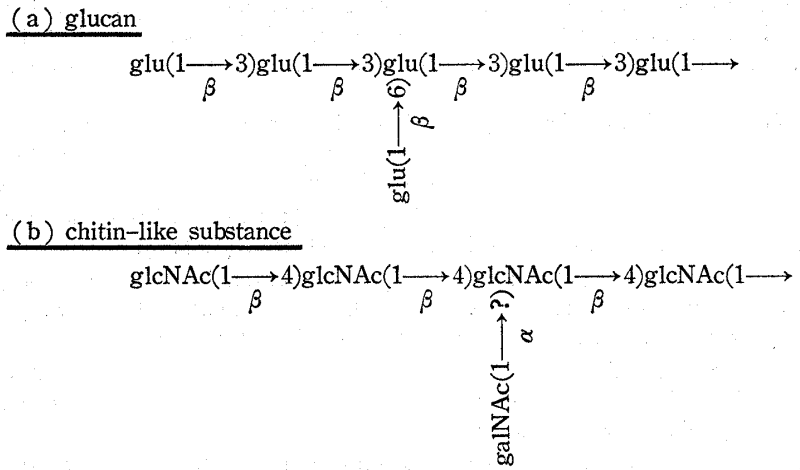

Fig. 1. Chemical Structures of Glucan and Chitin-like Substance in the Cell Wall of *Cochliobolus miyabeanus*

glu: glucose glcNAc: N-acetylglucosamine galNAc: N-acetylgalactosamine

1) This work was presented in the part at the Annual Meeting of the Phytopathological Society of Japan, 1973.
2) Location: *Motoyama-Kitamachi, Higashinada-ku, Kobe.*
3) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **19**, 448 (1971).
4) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **19**, 1402 (1971).

or ^{14}C-N-acetylglucosamine from the UDP-derivertives into the β-glucan or the chitin-like substance.[5]

The present paper deals with the chemical structures of these compounds synthesized by the enzymes obtained from mycelia. Although many reports have been presented on the cell free system of the cell wall chitin biosynthesis,[6,7] little is known on the β-glucan[8,9] which is one of the main components same as to the chitin-like substance.

Materials and Methods

1) **Preparation of the Enzyme Solution**——The preparation of the enzyme solution (crude enzyme, particulate enzyme and soluble enzyme) from mycelia was described in early paper.[5]

2) **Preparation of ^{14}C-Polysaccharides by Mycelial Enzymes**——The experimental conditions have already been described.[5] The typical reaction systems as shown in Table I were incubated at 27° (for glucan synthetase) or 25° (for chitin synthetase) for 60 min, followed by adding EtOH still standing for 15 hr at 4°, the EtOH solution was centrifuged at 3000 rpm for 5 min and the isolated precipitate (polysaccharide) and supernatant were used for further analyses.

Table I. Reaction Systems for Syntheses of Glucan or Chitin-like Substance by Mycelial Particulate Enzyme

For glucan		For chitin-like substance	
0.08M Tris-HCl buffer pH 8.2 (contained 0.01M $MgCl_2$, 0.001M EDTA)	0.5 ml	0.08M Tris-HCl buffer pH 7.53 (contained 0.01M $MgCl_2$, 0.001M EDTA)	0.5 ml
Cell wall glucan (20 mg/ml)	0.5 ml	Cell wall chitodextrin	0.5 ml
0.08M UDP-^{14}C-glucose (5 μCi/ml)	0.1 ml	0.08M UDP-^{14}C-N-acetylglucosamine (5 μCi/ml)	0.1 ml
Enzyme solution[a]	0.5 ml	Enzyme solution	0.5 ml

a) The preparation of enzyme solution was described in early paper.[5]

3) **Hydrolyses of Synthesized Polysaccharides**——The synthesized polysaccharides by mycelial enzymes were hydrolyzed by acid or enzymes under the following conditions. The glucan was hydrolyzed by heating with 4N HCl at 100° for 4 hr or by incubating with α or β-glucosidase (100 mg/ml, Sigma, St. Louis) in 0.01M McIlvaine buffer pH 6.15 at 30° for 24 hr. The chitin-like substance was hydrolyzed by heating with 6N HCl at 100° for 6 hr or by incubating with lysozyme (100 mg/ml, Sigma, St. Louis) in 0.01M phosphate buffer pH 7.25 at 37° for 24 hr, and resulted hydrolysates were submitted to thin layer chromatographic or Paper chromatographic analyses.

4) **Methylation, Methanolysis and Acid Hydrolysis of the Synthesized β-Glucan**——Methylation of the β-glucan was performed according to the method of Hakomori,[10] and the experimental conditions on methanolysis and acid hydrolysis of the methylated polysaccharide have already been described.[3]

5) **Chromatography**——a) Paper Chromatography (PPC): PPC analysis was carried out on Toyo Roshi paper No. 50 with a following solvent, pyridine–*n*-BuOH–H_2O (4: 6: 3).

b) Thin-Layer Chromatography (TLC): TLC analysis was carried out on cellulose powder plate (0.25 mm thick) or Kiesel gel G containing 0.1M AcONa plate (0.25 mm thick) with following solvents. Solvent A, $CHCl_3$–1%NH_4OH (6: 4); Solvent B, AcOEt–propane 2-ol–H_2O (32: 12: 6).

c) Determination of Separated Sugars: The separated sugars on chromatograms obtained by PPC and TLC were detected with aniline hydrogen phthalate reagent or alkaline silver nitrate reagent for neutral sugars and with Elson-Morgan's reagent for amino sugars.

6) **Measurement of the Radioactivity**——The determination of radioactivity in aqueous solution with liquid scintilation counter was described in early paper,[5] and the radioactivities on paper or thin layer chromatograms were counted with a radio chromatoscanner (Aloka Co. JTC-202 B type).

5) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **22**, 610 (1974).
6) L. Claster and D.H. Brown, *J. Biol. Chem.*, **228**, 729 (1957).
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8) M.C. Wang and S. Bartnicki-Garcia, *Biochem. Biophys. Res. Commun.*, **24**, 832 (1966).
9) M.C. Wang and S. Bartnicki-Garcia, *Annal. Biochem.*, **26**, 412 (1968).
10) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

Results and Disucussion

The main components of the cell wall of *Cochliobolus miyabeanus* were recently characterized as β-glucan and chitin-like substance shown in Fig. 1.[3,4] and mycelial enzymes stimulated incorporation of glucosyl residue or N-acetylglucosaminyl residue into β-glucan or chitin-like substance of mycelial cell wall from UDP-glucose or UDP-N-acetylglucosamine.[5] Therefore, the fine structures of these biosynthesized polysaccharides were studied in detail. As described previously,[5] the labelled polysaccharides were prepared by the reaction systems indicated in Table I and precipitated polysaccharides or supernatants resulted by adding EtOH to reaction systems were submitted to clarify the sugar components, the isolated polysaccharides were hydrolyzed in 4N HCl at 100° for 4 hr (for glucan) or in 6N HCl at 100° for 6 hr (for chitin-like substance) and the hydrolysates were separated by TLC as described in materials and methods and the radioactivities on thin-layer chromatograms were counted in a radio chromato scanner. The results presented in Fig. 2 (a—b) indicate that both synthesized ^{14}C-polysaccharides were found at original point of TLC before hydrolysis and in addition to these polysaccharide only ^{14}C-glucose or ^{14}C-glucosamine was found after acid hydrolysis.

In the TLC analysis on the supernatant of reaction system for glucan synthesis (Fig. 3a), ^{14}C-polysaccharide, UDP-^{14}C-glucose, ^{14}C-glucose and two unidentified sugars showing low *Rf* values which were presumed as ^{14}C-laminaritriose and ^{14}C-laminaribiose were detected. In the case of chitin-like substance synthesis (Fig. 3b), ^{14}C-polysaccharide, UDP-^{14}C-N-acetylglucosamine and ^{14}C-acetylglucosamine were detected in the supernatant of reaction system.

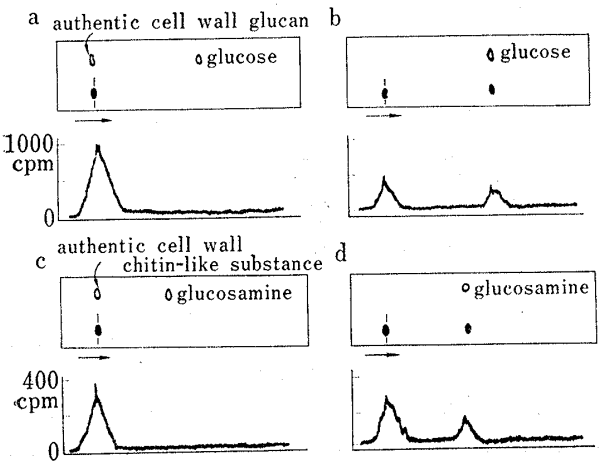

Fig. 2. Thin-Layer Chromatograms of Synthesized ^{14}C-Polysaccharides before or after Acid-Hydrolyses

a: synthesized ^{14}C-glucan before acid hydrolysis
b: acid hydrolysate of ^{14}C-glucan with 4N HCl for 4 hr
c: synthesized ^{14}C-chitin-like substance before acid hydrolysis
d: acid hydrolysate of ^{14}C-chitin-like substance with 6N HCl for 6 hr
The synthesized polysaccharide was precipitated by adding EtOH to 80% saturation into the reaction system and aqueous solution of the separated polysaccharide was submitted to TCL analysis.

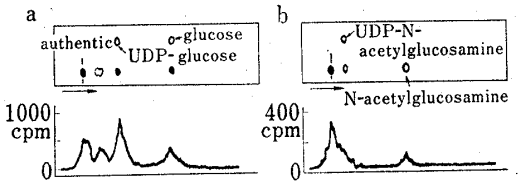

Fig. 3. Thin-Layer Chromatograms of Supernatants of the Reaction Systems

a: supernatant of the reaction system for glucan synthesis
b: supernatant of the reaction system for chitin-like substance synthesis
The synthesized polysaccharide was removed by adding EtOH to 80% saturation into the incubated reaction system and the resultant supernatant was submitted to TLC analysis.

Fig. 4(a—c) shows the results of PPC analyses on enzymic hydrolysate of the synthesized polysaccharides. Any ^{14}C-glucose was not released by α-glucosidase treatment, while it was found by β-glucosidase treatment (Fig. 4a, b). On the chitin-like substance, the paper chromatogram of the enzymic hydrolysate was cut into 1 cm sections and counted in toluene scintilation fluid. As shownin Fig. 4c, ^{14}C-N-acetylglucosamine was released by lysozyme treatment on the chitin-like substance. These results of Fig. 3, 4 indicate that glucosyl or N-acetylglucosaminyl residue was transported into the cell wall glucan or chitin-like substance without undergoing any modification from the added UDP-derivertives by mycelial particulate enzymes, and the both newly formed polysaccharide contained β-type linkages. In Fig. 4b, c only 40% or 51% of radioactivities to total incorporated radioactivities were found as glucose

or N-acetylglucosamine and the rests were found as polycaccharides even in the hydrolysates incubated for 24 hr. The reason why the enzymes we used failes to digest completely the newly formed polysaccharides is being studied. Since the glucan in this fungal cell wall was composed of β-1,3 linked glucan having branched units connected through C-6 and C-1 (Fig. 1), in order to confirm the existence of β-1,3 glucosyl linkage and branched units connected through C-6 and C-1, the newly formed glucan was methylated. After repeated methylation by checking with infrared spectrum, the completely methylated compound was submitted to methanolysis and the product was used for TLC analysis after acid hydrolysis. On TLC analysis (Fig. 5)

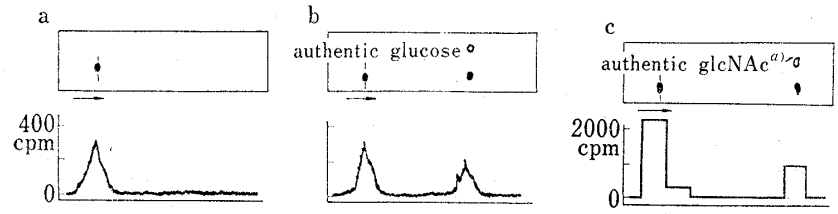

Fig. 4. Paper Chromatograms of Enzymic Hydrolysates of Synthesized Polysaccharides

a: α-glucosidase treated glucan
b: β-glucosidase treated glucan
c: lysozyme treated chitin-like substance
a) N-acetylglucosamine

The synthesized glucan was incubated with α or β-glucosidase (100 mg/ml) in 0.01M McIlavaine buffer pH 6.15 at 30° for 24 hr. The synthesized chitin-like substance was incubated with lysozyme (100 mg/ml) in 0.01M phosphate buffer pH 7.25 at 37° for 24 hr. The hydrolysates were deproteinized by shaking with $CHCl_3$, MeOH (9:1) mixture and the concentrated aqueous solutions were submitted to PPC analyses.

^{14}C-2,4-dimethylglucose, ^{14}C-2,4,6-trimethyl-glucose and ^{14}C-2,3,4,6-tetramethylglucose were found in the methylated products. The molar ratio of each of the methylated sugars was determined by counting the radioactivities of them and given in Table II. The presence of ^{14}C-2,4,6-trimethylglucose and ^{14}C-2,4-dimethylglucose in the methylated product indicated the presence of 1,3 disubstituted glucopyranose and 1,3,6 trisubstituted glucopyranose in the newly formed glucan and 2,3,4,6-tetramethylglucose is derived from non-reducing terminal glucopyranose.

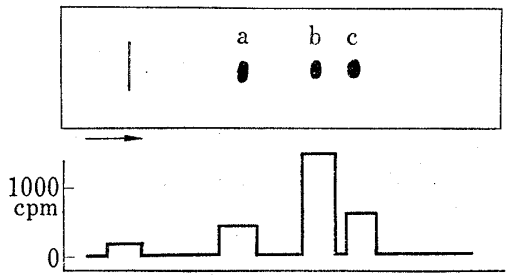

Fig. 5. Thin-Layer Chromatogram of Methylated Sugar Derived from the Synthesized Glucan

a: 2,4-dimethylglucose
b: 2,4,6-trimethylglucose
c: 2,3,4,6-tetramethylglucose

The spots corresponded to methylated sugars were scratched up from the plate and extracted with H_2O followed by evaporating. The residue was counted in toluene scintilation fluid.

These results demonstrate that the fine structure of the newly formed glucan by mycelial particulate enzyme in cell free system is completely same as to the β-glucan in the present fungal cell wall (Fig. 1). Although the chitin-like substance in cell wall is composed of β-1,4 linked N-acetyl-glucosamine having branched units of N-acetylgalactosamine connected through C-1 by α-glycosidic linkage as shown in Fig. 1 and the branched units of N-acetylgalactosamine are hydrolyzed by refluxing in 2N HCl,[4] no galactosamine derivertive was detected in the hydrolysate of newly formed chitin-like substance and the supernatant of reaction system (Fig. 3, 4).

These results suggested that the newly formed polysaccharide by particulate enzyme has no branched units of N-acetylgalactosamine. To make clear the mechanism on connecting the branched units, UDP-^{14}C-acetylglucosamine and chitodextrin were incubated

Table II. Molar Ratio of Methylated ^{14}C-glucose Derived from the Synthesized Glucan

Compound	cpm	molar ratio[a)]
2,4-Dimethylglucose	426	2.1
2,4,6-Trimethylglucose	2156	9.7
2,3,4,6-Tetramethylglucose	642	3.0

a) determined by the radioactivities

with soluble, particulate or crude enzyme solution in 0.08M Tris-HCl buffer pH 7.53 at 25° for 60 min respectively and the precipitated polysaccharides by adding EtOH to reaction systems were hydrolyzed in 2N HCl. The results of PPC analyses on the hydrolysates of newly formed polysaccharides and the supernatants of reaction systems are shown in Fig. 6. In the case of incubating with the soluble enzyme, no hexosamine derivertive was found on the hydrolysate of precipitated polysaccharide with the exception of a small amount of ^{14}C-compound remained at the original point (Fig. 6b), while in addition to ^{14}C-polysaccharide, ^{14}C-N-acetylgalactosamine and ^{14}C-N-acetylglucosamine were detected on the supernatant (Fig. 6a). Since the *Rf* values of N-acetylglucosamine and N-acetylgalactosamine were very similar and it was difficult to distinguish between these two amino sugars by PPC analyses, the spot of corresponded to these amino sugars was eluted by H_2O and identified by gas–liquid chromatography under the condition described previously.[11)] When the particulate enzyme was used as synthetase, ^{14}C-polysaccharides were found in PPC analyses on both the hydrolysate of precipitated polysaccharide and the supernatant, while any ^{14}C-hexosamine derivertive was not detected (Fig. 6c, d). Fig. 6e—g indicate the results when the crude enzyme was used as synthetase. As shown in Fig. 6e, ^{14}C-polysaccharide and ^{14}C-N-acetylgalactosamine were found in PPC analysis on the supernatant. Since one spot indicated by an arrow in Fig. 6e remained unclarified, the spot was eluted by H_2O and hydrolyzed in 0.5N HCl at 100° for 30 min. On PPC analysis of this hydrolysate, ^{14}C-galactosamine and ^{14}C-glucosamine were detected (Fig. 6f). This result suggests that the unclarified spot corresponds to the mixture of UDP-^{14}C-N-acetylgalactosamine and UDP-^{14}C-N-acetylglucosamine or analogous compounds. These results are summarized in Table III.

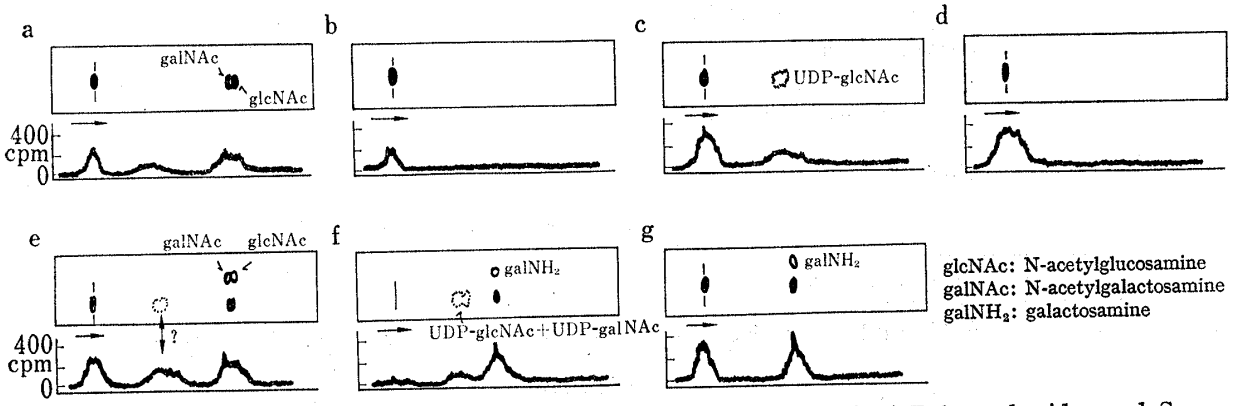

Fig. 6. Paper Chromatograms of the Acid Hydrolysates of Synthesized Polysaccharides and Supernatants of Reaction Systems obtained by Incubation with Fractionated Enzymes

a: supernatant of the reaction system with soluble enzyme
b: acid hydrolysate of synthesized chitin-like substance with soluble enzyme
c: supernatant of the reaction system with particulate enzyme
d: acid hydrolysate of synthesized chitin-like substance with particulate enzyme
e: supernatant of the reaction system with crude enzyme
f: acid hydrolysate of the eluate from the spot indicated by an arrow in (e)
g: acid hydrolysate of synthesized chitin-like substance with crude enzyme

11) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **19**, 252 (1971).

Table III. Detection of Galactosamine Derivertives from the Hydrolysates of Synthesized Polysaccharides or the Supernatants of Reaction Systems Incubated with Fractionated Enzyme

Enzyme	Hydrolysate of polysaccharide[a)]	Supernatant[b)]
Crude enzyme	+	−
Soluble enzyme	−	+
Particulate enzyme	−	−

a) EtOH was added to the reaction system incubated with fractionated enzyme to give 80% alcohol solution, and precipitated polysaccharide was hydrolyzed in 2 N HCl at 100° for 120 min. After removal of acid, the residue was submitted to PPC analysis for the detection of galactosamine derivertives.
b) After removal of precipitated polysaccharide by adding EtOH and successive centrifugation, the supernatant of the reaction system was submitted to PPC analysis for the detection of galactosamine derivertives.

In the reaction system with the particulate enzyme, the precipitated polysaccharide has no branched units of N-acetylgalactosamine which are hydrolyzed in 2N HCl and any ^{14}C-galactosamine derivertive is not detected in the supernatant. On the contrary, when the crude enzyme (the mixture of particulate enzyme and soluble enzyme) is used as synthetase the precipitated polysaccharide has branched units of ^{14}C-N-acetylgalactosamine and in the supernatant some ^{14}C-galactosamine derivertives are found. When the soluble enzyme is used, ^{14}C-N-acetylgalactosamine is found in only the supernatant and the precipitated polysaccharide has no branched units. All experimental results demonstrate that the biosynthetic pathway of polysaccharide in cell wall is expressed as shown in Chart 1.

N-acetylglucosaminyl residues are transfered to the non reducing terminals of chitodextrin from some UDP-N-acetylglucosamine molecules which are added to the reaction system by

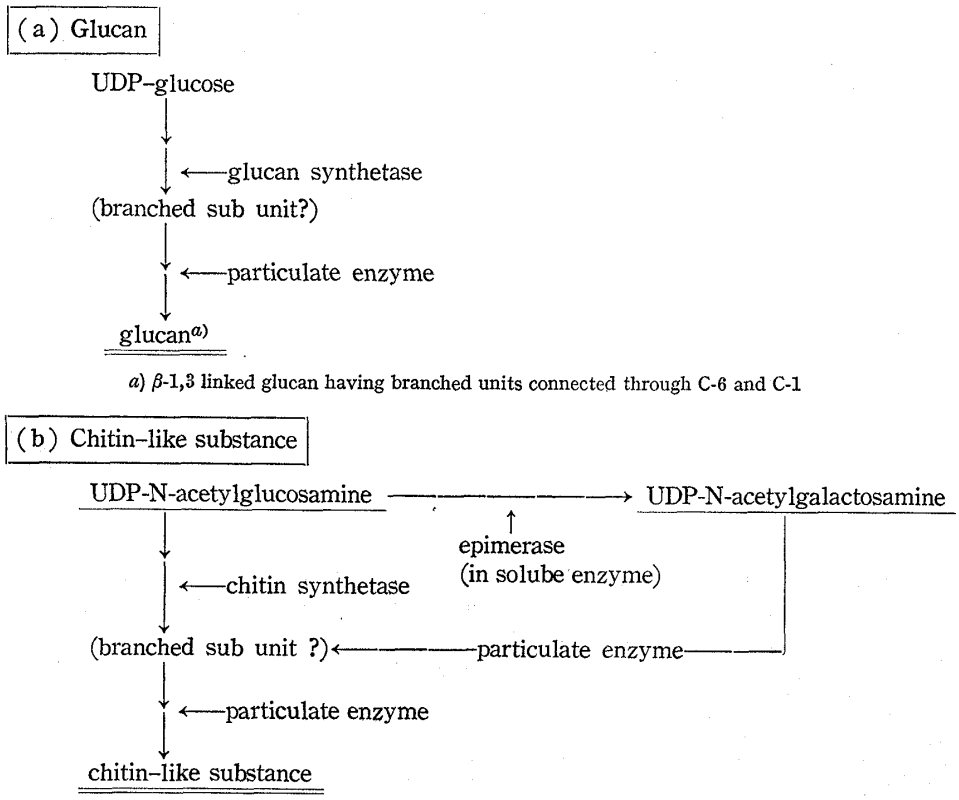

Chart 1. Possible Biosynthetic Pathways of Polysaccharides in Cell Wall of *Cochliobolus miyabeanus*

incubating with the particulate enzyme forming a straight chain of β-1,4-linked N-acetylglucosamine polymer. A part of the UDP-N-acetylglucosamine is converted to UDP-N-acetylgalactosamine with the soluble enzyme, followd by forming branched unit of N-acetylgalactosamine connected through C-1 by α-glycosidic linkage with the pariculate enzyme. In the β-glucan synthesis, glucosyl residues are transfered to the non reducing terminals of oligo β-glucan from some UDP-glucose molecules which are added to the reaction system with the particulate enzyme, forming the β-1,3 glucan and a part of glucosyl residue is connected through C-6 and C-1 forming a branched unit with the particulate enzyme. Although in these cases, the particulate enzymes act on two different functions, that is, the one function is the elongation of the polysaccharide chain and the other function is formation of the branched unit. It is not clear either these functions are participated by the same enzyme or not. Lipid intermediates, formed by transfer of sugar derivartive from nucleotide precursors to a lipid phosphate acceptor (undeca prenol derivertive) have been shown to be involved in the biosynthesis of bacterial wall polymers (for instance, peptidoglycan,[12] O-antigenic side chain,[13] teichoic acid,[14] capsular polysaccharide[15] *etc.*). Further research is planned to purify the particulate enzyme(s) and to make clear the possibility of the presence of such lipid intermediates in the biosynthesis of the fungal cell wall glucan or chitin-like substance.

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