Subunit Structure of the Phosphomannan from *Kloeckera brevis* Yeast Cell Wall†

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ABSTRACT: The cell wall phosphomannan of the yeast *Kloeckera brevis* has been fractionated by chromatography on DEAE-Sephadex into four components of differing phosphate content (Thieme, T. R., and Ballou, C. E. (1971), *Biochemistry 10*, 4121). Each of these four phosphomannan fractions has now been resolved by gel filtration on Bio-Gel A-5m, giving up to three components of different molecular size. The phosphomannans, isolated from log-phase cells, gave three broad peaks which contained material with molecular weights of approximately 500,000, 100,000, and 25,000, as determined by sedimentation equilibrium. After Pronase digestion of the phosphomannans, only one species was obtained with a molecular weight of 25,000–35,000. Thus, mannan "subunits" appear to be cross-linked by protein to form higher molecular weight complexes. Each of the phosphomannans contained about one glucosamine per subunit, which may serve to link the mannan to the protein component as in other glycoproteins. Mannan, isolated from cells in stationary phase, had a low phosphate content and consisted almost entirely of subunits with a molecular weight of 25,000. Thus, the composition and the extent of cross-linking of mannan in the cell wall seems to vary with the growth phase of the yeast.

The cell wall of many yeasts is composed of a rigid glucan layer surrounded by a thick mannan–protein coat. The exact structure and organization of both layers are still largely undetermined. Since several hydrolytic enzymes have been isolated from such cell walls, the function of at least a part of the protein is apparent. For example, invertase obtained from *Saccharomyces cerevisiae* cell wall has been shown to be a glycoprotein containing approximately 50% mannan (Gascon *et al.*, 1968). On the other hand, "purified" mannan from yeast cell walls usually contains only about 6% covalently linked protein (Phaff, 1971). Since such preparations may have molecular weights less than 100,000 (Sentandreu and Northcote, 1968), it is unlikely that the protein they contain (which would have a molecular weight less than 6000) is derived from enzymes. In fact, it has been suggested that the protein component of mannan may also be involved in anchoring the polysaccharide to the cell surface, since it was found that papain digestion of cell walls led to release of the mannan in soluble form (Eddy, 1958). In agreement with this view, mannan solubilized by the action of glycolytic enzymes is further reduced in size on treatment with alkali (Eddy and Longton, 1969). This observation led to the proposal that the mannan molecules were attached to protein which was cross-linked by alkali-labile disulfide bonds. Treatment of some yeast cells with mercaptoethanol is known to release enzymes from the cell wall and to increase the susceptibility of the wall to attack by glycolytic enzymes (Kidby and Davies, 1970).

In the work reported here, we have investigated the macromolecular nature of the mannan–protein complex of the phosphomannan from the cell wall of the yeast *Kloeckera brevis*. It was found that a portion of the phosphomannan consisted of polysaccharide units which were cross-linked by protein. Although not dissociated by mercaptoethanol, this glycoprotein fraction was degraded to small subunits through the action of Pronase. The degree of cross-linking varied with the phosphate content of the mannan and the growth phase of the yeast. A significant part of the mannan occurred as glycopeptide subunits (mol wt 25,000–35,000) that were not reduced in size by Pronase digestion. Our results suggest that the *K. brevis* cell wall mannan contains several macromolecular species composed of polysaccharide subunits, containing about 150 mannose units, attached to polypeptides ranging from 15 amino acids in the smallest form, to 60 in the intermediate form and 300 in the largest. A reasonable interpretation is that the polypeptides serve to cross-link and anchor the mannan on the cell surface, forming a matrix in which mannan–protein enzymes and other structures are embedded. Burger *et al.* (1961) have expressed this idea as involving a membrane-like structure in which the external invertase may be held. Additional cross-linking of the protein component by disulfide bonds would provide a further refinement of the basic structure (Nickerson and Falcone, 1956).

Experimental Section

Materials. Phosphomannan was isolated from *K. brevis* cells in log phase (6-hr growth) and stationary phase (70-hr growth) as described by Thieme and Ballou (1971). Four phosphomannan fractions were obtained by stepwise elution with KCl from a DEAE-Sephadex column and are referred to as phosphomannan-20, phosphomannan-13.6, phosphomannan-8.6, and phosphomannan-6.5, according to their mannose to phosphate ratios (Figure 1). Contaminating protein was removed from each preparation by chromatography on a Sephadex SE-50 column (2 × 40 cm) which was eluted with 0.05 M phosphoric acid at pH 2.0. The phosphomannans were eluted in the void volume of this column.

Pronase (grade B) came from Calbiochem, Bio-Gel A-5m (200–400 mesh) and Dowex AG 50W-X-8 from the Bio-Rad Corp., and Sephadex SE-50 and DEAE-Sephadex (A-25) from Pharmacia.

General Procedures. Published analytical procedures were employed for carbohydrate (Stewart *et al.*, 1968), phosphate (Bartlett, 1959), nitrogen (Long and Staples, 1961), and protein (Lowry *et al.*, 1951; Hirs, 1967). Bovine serum albumin

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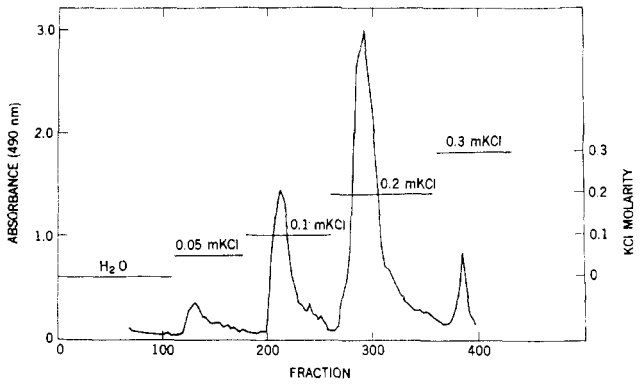

FIGURE 1: Elution of phosphomannan fractions from DEAE-Sephadex with KCl. The four peaks from left to right, detected by their carbohydrate content, are designated phosphomannan-20, phosphomannan-13.6, phosphomannan-8.6, and phosphomannan-6.5.

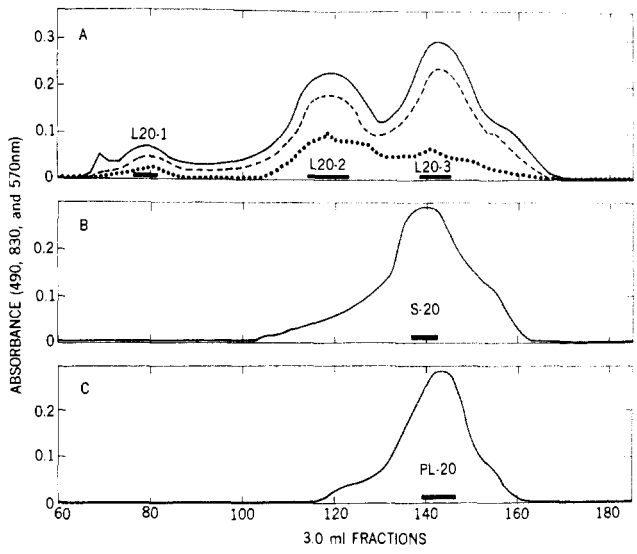

FIGURE 2: Gel filtration of phosphomannan-20. (A) Log-phase mannan, (B) stationary-phase mannan, and (C) Pronase-digested log-phase mannan. Assays were performed for carbohydrate (solid line), ninhydrin-positive protein (dotted line), and phosphate (dashed line). The peaks are labeled as follows: L is log-phase mannan, S is stationary-phase mannan, and P is Pronase-digested mannan. The number following this letter stands for the phosphate content of the mannan, and the number after the dash (if any) is the peak number in the chromatogram. Fractions which are marked with a heavy line were pooled, dialyzed against distilled water, lyophilized, and the molecular weights were measured by sedimentation equilibrium.

(Pentex, crystallized) was used as a standard in protein determinations.

While several gel filtration materials were tested (Sephadex G-100, Sephadex G-200, and Bio-Gel P-150), Bio-Gel A-5m gave the best separation of *K. brevis* mannan into its components of different size. A 2 × 200 cm column was used and elution was effected with 0.2 M KCl at a flow rate of 10 ml/hr, the effluent being collected in 3.0-ml fractions.

A Spinco Model E analytical ultracentrifuge was used for measurements of sedimentation velocity which were made with both the schlieren and the 280-nm photoelectric scanning systems. Molecular weights were determined in 0.20 M KCl by high-speed sedimentation equilibrium using interference optics according to Yphantis (1964). The partial specific volume of sucrose (0.618) was used in calculations. Photographic plates were analyzed with a Nikon Model 6C microcomparator.

For Pronase digestion, about 50 mg of phosphomannan was dissolved in 25 ml of 0.1 M Tris buffer at pH 7.8 containing 1 mM Ca^{2+}, and four additions of Pronase (0.5 mg each) were made during a 48-hr period. Incubation was carried out at 40°, except for phosphomannan-8.6 which was incubated at 60°. After removal of Pronase by treatment with Dowex 50 (H^+), the products were chromatographed on Bio-Gel A-5m.

The amino acid content of mannans was measured with a Beckman Model 120C amino acid analyzer. Amino acid analysis, in the presence of large amounts of carbohydrate, is complicated because of the reaction between amines and sugars (Gottschalk, 1966). To minimize this reaction, hydrolysis of mannan was done at high dilution. About 9 mg of mannan was dissolved in 50 ml of 6 N HCl in a 100-ml Kjeldahl flask which was then sealed under vacuum. Hydrolysis was performed at 110° for 10–50 hr to calculate the rate of destruction of individual amino acids and the final results were corrected accordingly. Methionine appeared in minor amounts in the mannan but was not quantitated. Because of its high rate of destruction, the glucosamine content was checked by performing the hydrolysis with 4 N HCl for 17 hr, when very little destruction was found. The glucosamine content of mannans was found to be the same using this method or by doing the hydrolysis in 6 N HCl and assuming a 55% destruction after 30 hr. The cysteine content was measured by estimation of cysteic acid after performic acid oxidation of the mannan according to Moore (1963).

Results

Gel Filtration and Sedimentation Studies of K. brevis Mannan. Log-phase phosphomannan-20 gave three broad peaks on gel filtration (Figure 2),[1] all of which contained protein. While each mannan fraction was polydisperse, the pattern indicates that certain molecular weight ranges predominated. Sedimentation equilibrium studies, on the material in the narrow range marked under the top of a peak, gave a plot of log Δy *vs.* x^2 with a straight line (Figure 3) indicating some homogeneity (Schachman, 1959). The peak which was eluted in the void volume (L20-1)[1] had a molecular weight of about 500,000, while L20-2 and L20-3 had molecular weights of 100,000 and 23,000, respectively (Table I).

Digestion of log-phase phosphomannan-20 with Pronase converted all of the mannan to material with a molecular weight of 23,000 (part C, Figure 2), which suggests that mannans L20-1 and L20-2 were cross-linked by peptide bonds. Fraction L20-2 was about four times the size of the product resulting from Pronase digestion, suggesting that it was composed of four mannan subunits of 23,000 molecular weight. Mannan fraction L20-1 was about 20 times the size of the basic unit.

In stationary-phase cells (part B of Figure 2), only a low molecular weight peak (S20) was observed, with a molecular weight of about 20,000. The gel filtration patterns of phosphomannans were not altered if cells were frozen and then allowed to remain at room temperature for several hours before isolation of the mannan. This makes it unlikely that endogenous proteases produced the low molecular weight mannans. Moreover, the smaller units were not artifacts of the

[1] Refer to the legend of Figure 2 for definition of the terminology used in indentifying the different mannan fractions.

TABLE I: Molecular Weights and Sedimentation Coefficients of Phosphomannans.[a]

Log-Phase Phosphomannans						Stationary-Phase Phosphomannans	
Native			Pronase Digested				
Mannan	Mol Wt	$s_{20,w}$ (S)	Mannan	Mol Wt	$s_{20,w}$ (S)	Mannan	Mol Wt
L20-1	550,000	7.09					
L20-2	100,000	4.64					
L20-3	23,000	2.35	PL20	23,000	2.37	S20	19,000
L13.6-1	82,000	4.41					
L13.6-2	22,000	2.70	PL13.6	22,000	2.30	S13.6	28,000[b]
L8.6-1	*c*	*c*				S8.6-1	485,000
L8.6-2	93,000	3.64				S8.6-2	115,000
L8.6-3	33,500	*c*	PL8.6	36,500	2.87	S8.6-3	30,000

[a] Molecular weights, measured in 0.2 M KCl with a mannan concentration of 1 mg/ml, were independent of concentration. [b] Pronase digestion of this mannan gave material with a molecular weight of 29,000. [c] Not determined.

isolation procedure since the patterns were not changed if the phosphomannans were autoclaved for 2 hr.

Log-phase phosphomannan-13.6 was similar to phosphomannan-20, except that there was less of the very high molecular weight component (Figure 4). Again Pronase digestion produced a low molecular weight unit (PL13.6), one-fourth the size of the larger material (L13.6-1). Stationary-phase cells gave a mannan with only the low molecular weight peak. This material (S13.6) was not reduced in size by Pronase digestion (Table I, footnote *b*).

Log-phase phosphomannan-8.6 gave three peaks (Figure 5), including some very high molecular weight mannan (L8.6-1). In contrast to phosphomannan-20 and phosphomannan-13.6, mannan fraction L8.6-2, with a molecular weight of 93,000, was only about three times the size of the subunit obtained by Pronase treatment. Pronase digestion of phosphomannan-8.6 was slow, perhaps because of its high phosphate content. When digestion was carried out at 40°, as for the other mannans, peak L8.6-1 was eliminated while peaks L8.6-2 and L8.6-3 were unchanged. After digestion at 60°, peak PL8.6 was produced with a molecular weight of 36,500, and a plot of the sedimentation equilibrium data showed some

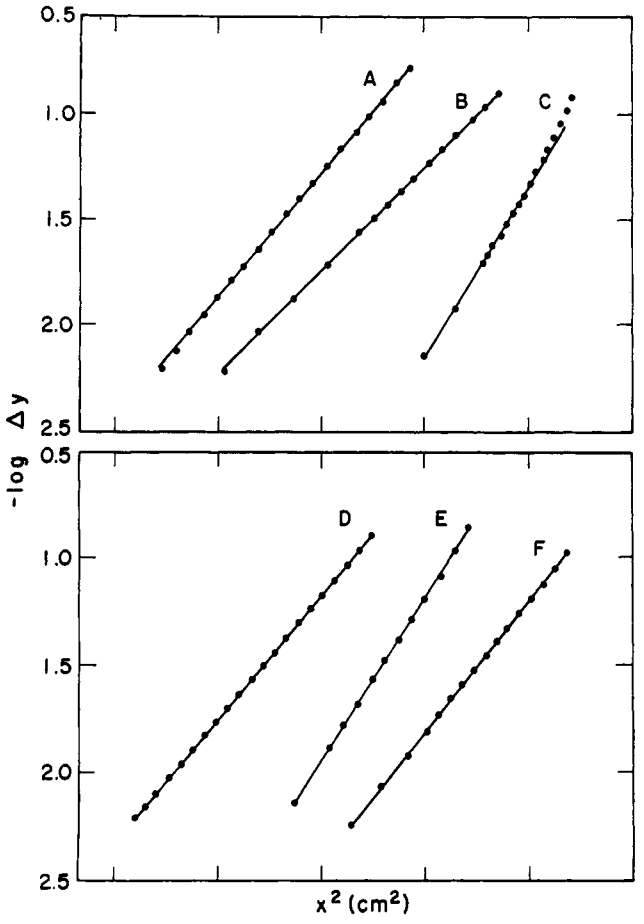

FIGURE 3: Sedimentation equilibrium data for L8.6-2 (A), S8.6-1 (B), PL8.6 (C), L13.6-2 (D), S13.6 (E), and PL13.6 (F). Δy is the fringe displacement and is proportional to the solute concentration. x is the distance from the axis of rotation.

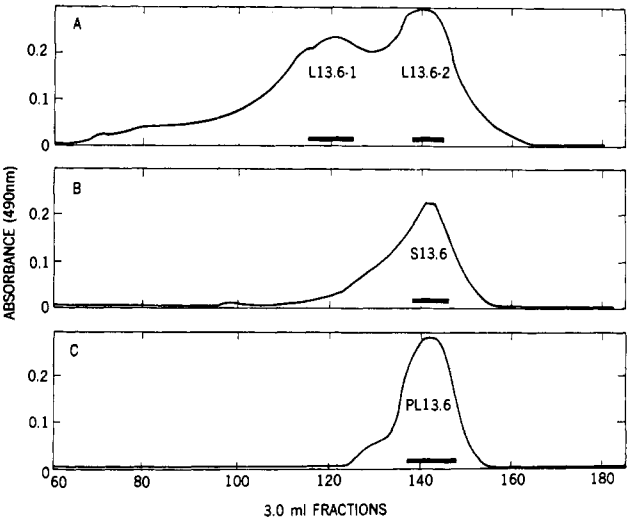

FIGURE 4: Gel filtration of phosphomannan-13.6. (A) Log-phase mannan, (B) stationary-phase mannan, and (C) Pronase-digested log-phase mannan. Peak designations follow the system employed in Figure 2.

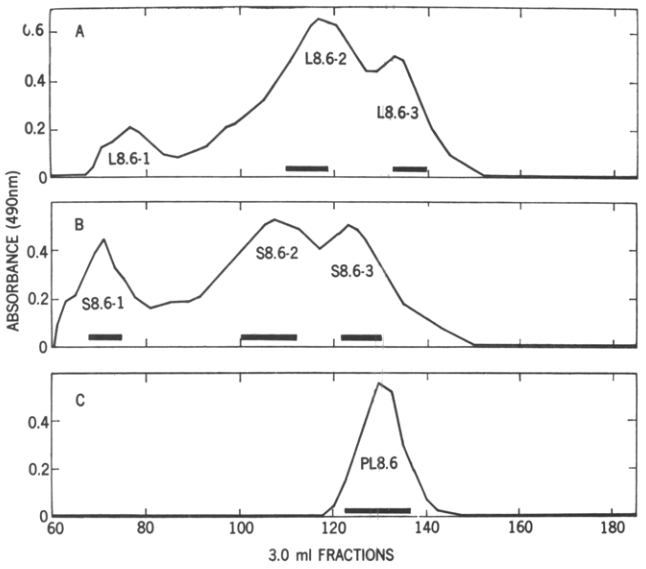

FIGURE 5: Gel filtration of phosphomannan-8.6. (A) Log-phase mannan, (B) stationary-phase mannan, (C) Pronase-digested (60°), and log-phase mannan. Refer to Figure 2 for the system followed in labeling the peaks.

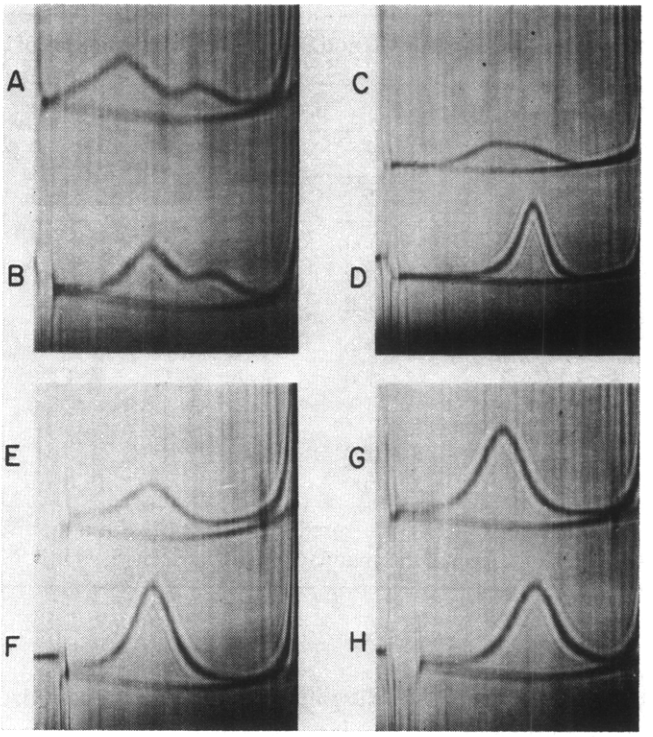

FIGURE 7: Sedimentation velocity patterns of *K. brevis* phosphomannans before and after Pronase digestion. The centrifugal force was 330,800*g* at 67,000 rpm with a titanium rotor. The phosphomannan concentration was 10 mg/ml in 0.2 M KCl. Sedimentation coefficients are listed in Table I. Figures A, B, C, and D are the patterns for phosphomannan-20, -13.6, -8.6, and -6.5, respectively. Figures E, F, G, and H are for the Pronase-digested forms of the same phosphomannans.

inhomogeneity. Unlike stationary-phase phosphomannan-20 and phosphomannan-13.6, stationary-phase phosphomannan-8.6 still gave three peaks. In fact, the material in peak S8.6-1 was somewhat larger than the corresponding fraction from log-phase cells. Digestion of stationary-phase phosphomannan-8.6 with Pronase at 40° eliminated only peak S8.6-1 (Figure 6A), as was observed for log-phase phosphomannan-8.6 when treated similarly. Pronase digestion of isolated S8.6-1 at 40° yielded a new mannan (molecular weight of 119,000) similar in size to S8.6-2 which had a molecular weight of 115,000. This indicates that S8.6-1 consisted of four units of a molecule the size of S8.6-2.

Figure 7 shows the sedimentation velocity patterns given by the log-phase phosphomannan preparations and the products they yielded after Pronase digestion, while the sedimentation cofficients are reported in Table I. Phosphomannan-6.5, which was not studied by gel filtration, gave one symmetrical peak on sedimentation, the coefficient of which was reduced after Pronase digestion. The sedimentation velocity patterns of the four phosphomannans were not changed in 10 mM mercaptoethanol.

Amino Acid Composition of K. brevis Phosphomannans. The purest mannan preparations contained about 6% protein as measured by the Lowry method, quantitative amino acid analysis, and total nitrogen determination. When sedimentation velocity patterns of phosphomannan-8.6 were examined with the 280-nm photoelectric scanning system, the ultraviolet-absorbing material had the same sedimentation rate as the phosphomannan measured by schlieren optics (Figure 8).

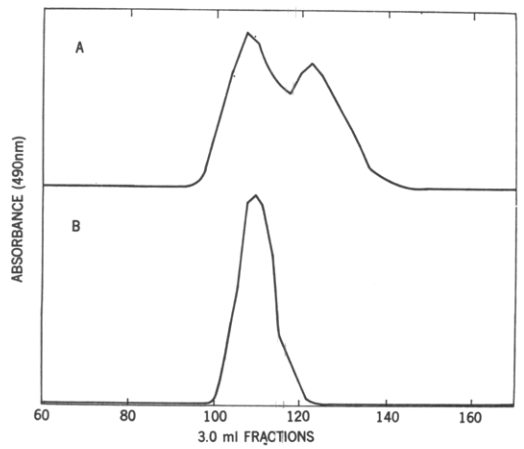

FIGURE 6: Gel filtration of (A) Pronase-digested (40°) stationary-phase phosphomannan-8.6 and (B) Pronase-digested (40°) peak S8.6-1 (Figure 5B).

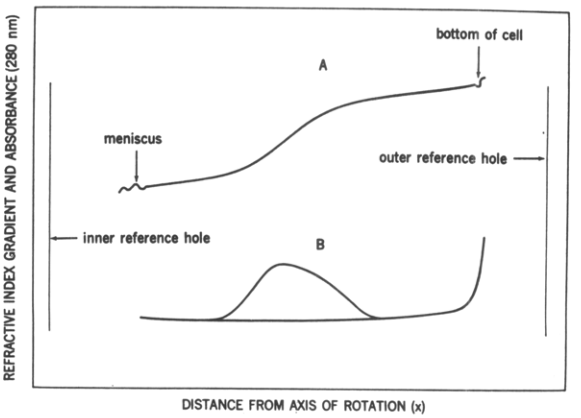

FIGURE 8: Sedimentation velocity data of log-phase phosphomannan-8.6 as measured by the photoelectric scanning system measuring absorbance at 280 nm (A) and by the schlieren optical system (B).

TABLE II: Amino Acid Composition of Total Phosphomannans from Log-Phase Cells.[a]

Amino Acid	Phospho-mannan-20	Phospho-mannan-13.6	Phospho-mannan-8.6	Phospho-mannan-6.5
Lysine	0.60	0.18	0.23	0.35
Histidine	0.15	0.05	0.08	0.19
Arginine	0.20	0.02	0.03	0.07
Cysteic acid	*b*	*b*	0.34	*b*
Aspartic acid	1.39	0.97	1.28	1.60
Threonine	2.20	1.83	2.50	2.91
Serine	2.90	1.68	1.91	2.42
Glutamic acid	1.50	0.60	0.77	1.17
Proline	0.59	0.26	0.49	0.62
Glycine	2.60	0.45	0.59	0.72
Alanine	1.50	1.21	1.30	1.71
Valine	0.92	0.55	0.87	1.05
Isoleucine	0.40	0.36	0.43	0.54
Leucine	0.45	0.27	0.56	0.78
Tyrosine	0.30	0.21	0.33	0.44
Phenylalanine	0.21	0.08	0.14	0.24
Glucosamine	1.03	0.84	0.96	1.05

[a] Moles of amino acid per gram-mole of mannan subunit. The following subunit molecular weights were used in the calculations: phosphomannan-20, 23,000; phosphomannan-13.6, 22,000; phosphomannan-8.6, 36,500; phosphomannan-6.5, 36,500.

TABLE III: Amino Acid Content of Individual Phosphomannan Fractions.[a]

	Phosphomannan				
Amino Acid	L20-1	L20-2	L20-3	S8.6[b]	S8.6-1
Lysine	0.73	0.60	0.46	0.31	2.73
Histidine	0.15	0.25	0.13	0.11	0.58
Arginine	0.18	0.25	0.13	0.04	0
Aspartic acid	2.00	1.50	1.47	1.75	5.47
Threonine	4.50	1.80	0.96	3.31	13.7
Serine	3.50	2.70	1.75	2.48	9.27
Glutamic acid	1.95	1.50	0.94	1.01	5.39
Glycine	1.67	2.60	1.10	0.63	4.87
Alanine	2.35	1.30	1.20	1.87	6.65
Valine	1.58	0.75	0.36	1.16	4.02
Isoleucine	1.04	0.30	0.18	0.56	1.73
Leucine	0.94	0.40	0.19	0.73	2.56
Tyrosine	0.60	0.29	0	0.43	1.66
Phenylalanine	0.37	0.22	0	0.19	0
Glucosamine	1.00	1.00	1.00	1.00	1.00

[a] Molar ratios relative to glucosamine. [b] Stationary-phase phosphomannan-8.6.

This is evidence that all of the protein was covalently linked to the polysaccharide. The amino acid analyses of the log-phase phosphomannans are given in Table II. All four preparations contained approximately one glucosamine per Pronase-formed subunit. Except for phosphomannan-6.5, there was approximately one aspartic acid per glucosamine.

As seen in Table II, the other amino acids occurred in nonintegral ratios, and in many cases (*e.g.*, lysine, histidine, and isoleucine) there was less than 1 mole of amino acid/subunit. Table III shows that the different mannan fractions separated by gel filtration had different amino acid compositions.

Discussion

Each of the four *K. brevis* cell wall phosphomannan fractions obtained by DEAE-Sephadex chromatography contained about 6% protein. During fractionation by gel filtration, the protein and carbohydrate were found in nearly constant relative amounts over a large molecular weight range. Thus each mannan subfraction appears to have the same amount of protein. Assuming every mannan molecule is attached to protein, it is unlikely that the bulk of the protein component is enzymatic in nature since only the very large mannan fractions with a molecular weight of 500,000 would have a protein moiety as large as the usual enzyme. In contrast to the phosphomannan–protein complexes studied here, the mannan–protein enzyme invertase contains about 50% protein (Gascon *et al.*, 1968).

The present work suggests that at least some of the protein in the mannan preparations performs the specific function of cross-linking mannan subunits. Each phosphomannan from log-phase cells consisted of three species with molecular weights in the regions of 500,000, 100,000, and 25,000. On Pronase digestion, the larger forms yielded subunits of 25,000–35,000 molecular weight, suggesting that they had been cross-linked by protein.

Each of the mannan subunits contained about 15 amino acids as well as 1 mole of glucosamine and 1 of aspartic acid. This is suggestive that the linkage between carbohydrate and protein in *K. brevis* phosphomannan involves the latter two constituents, as has been proposed for *S. cerevisiae* mannan (Sentandreu and Northcote, 1968) and clearly established for other glycoproteins (Gottschalk, 1966). Since the other amino acids occurred in nonintegral and even fractional ratios per subunit, there does not appear to be an exact repeating sequence of amino acids as might be expected for a "structural" peptide. A protein chain without repeating sequences could have several aspartic acid residues, most of which were linked to mannan subunits through glucosamine. Alternatively, there could be a repeating sequence of amino acids in any one mannan–protein complex, but several varieties of proteins could be present. The three mannan fractions separated by gel filtration showed different amino acid analyses, indicating such protein heterogeneity.

The function of the cross-linking of mannan subunits by protein is unknown, although it obviously may help to maintain the integrity of the cell wall. Another possibility is that cross-linking is involved as a step in the secretion of the mannan. Eddy and Longton (1969) have shown that mannan associated with intracellular membranes (mannan presumably in the process of being secreted) has a higher molecular weight than cell wall mannan. It would be interesting to know whether a precursor–product relationship exists between the mannan–protein complexes of different molecular size.

An additional function of the protein component may be to regulate the transport of mannans, either within the cell during biosynthesis, or outside in the cell wall. Sentandreu and Lampen (1970) have demonstrated that the biosynthesis of the protein and mannan moieties of *S. cerevisiae* mannan are

interdependent. In the present case, it has been found that the amino acid composition of phosphomannans varied both as a function of molecular weight and of phosphate content. A similar observation was made with *S. cerevisiae* mannan by Jones (1968). The amino acid sequence of the protein moiety may determine where the mannan of a particular molecular weight and charge is placed in the cell wall. Thus, mannans could have different structures at bud scars or at different levels within the cell wall. An important observation of the current investigation is that the molecular weight distribution of the phosphomannans varied with the growth phase of the cell. In stationary-phase cells, the phosphomannan low in phosphate content was not cross-linked by protein (mol wt 25,000), while the phosphomannan with a high phosphate content contained a greater proportion of the larger complex (mol wt 500,000). This may indicate that a different population of mannans is formed as the cell ages and the wall performs a new set of functions. Significant changes in the nature of the phosphomannan might also result from changes in the phosphate content of the medium which occur during cell growth (Slodki *et al.*, 1970).

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Equations for the Equilibrium Constants of Nonideal Systems Derived from the Multinomial Theory Using the z-Average Molecular Weight†

Moisés Derechin

ABSTRACT: Equations for the calculation of equilibrium constant of self-associating systems are derived using $M_{z,app}$.

Equations for the calculation of mass equilibrium constants of self-association reactions for ideal systems using the z-average molecular weight were given before (Derechin, 1969b). In this paper equations for nonideal systems using the z-average molecular weight are derived.

Theory

Various definitions and derivations made before for ideal systems (eq 4–11 of paper III of this series, Derechin, 1969b) can be applied to nonideal systems simply by replacing $M_{z,app}$ for M_z thus leading to eq 1, where $M_{z,app}$ is the z-average molecular weight as calculated from experimental data.

$$\sum_{r=1}^{\xi}\left(\frac{d^{(r-1)}\frac{M_{z,app}}{M_1}}{dc^{r-1}}\right)_{c=0}\times\left[\sum_{\alpha_2=0}^{r}\cdots\sum_{\alpha_m=0}^{r}{}_{(\alpha_1,\,\alpha_2,\,\ldots,\,\alpha_m)}{}^{1}\prod_{i=2}^{m}K_i^{\alpha i}\right]=\xi K_\xi \quad (1)$$

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