The mannans of bakers' yeast (Saccharomyces cerevisiae) were fractionated on a column of diethylaminoethyl-Sephadex into five subfractions. Phosphate content of these mannans subfractions was proportional to the concentration of NaCl solutions used in the chromatographic separation. Quantitative precipitin reactions showed that the serological reactivities of the subfractions were proportional to the content of phosphate. The result of acetolysis study showed that the amounts of mannoligotetraose and phosphate-containing oligosaccharides increased proportionally to the acidity, whereas the amount of mannose decreased inversely. The results from quantitative precipitin reaction tests and acetolysis study demonstrated that both phosphate contents and multiplicity of branching moieties of mannans subfractions increased proportionally, i.e., micro-heterogeneity concerning the acidity comprised in the parent bulk mannan is not attributable merely to the coexistence of molecular species containing different amounts of phosphate but also to the presence of more of the branching moieties.

**MATERIALS AND METHODS**

**Materials.** The fresh whole cells of bakers' yeast, supplied by the Oriental Yeast Co. Ltd., Tokyo, Japan, were from a single-strain culture, the same as used in a preceding study (9). The following manno-oligosaccharides were also the same as those used in a preceding study (9): \(O\alpha-D\)-mannopyranosyl-(1 → 3)-\(O\alpha-D\)-mannopyranosyl-(1 → 2)-\(O\alpha-D\)-mannose (Man\(_3\)); a mixture of \(O\alpha-D\)-mannopyranosyl-(1 → 3)-\(O\alpha-D\)-mannopyranosyl-(1 → 2)-\(O\alpha-D\)-mannose and \(O\alpha-D\)-mannopyranosyl-(1 → 2)-\(O\alpha-D\)-mannose (Man\(_2\)); \(O\alpha-D\)-mannopyranosyl-(1 → 2)-\(O\alpha-D\)-mannose (Man); \(O\alpha-D\)-mannopyranosyl-(1 → 2)-\(O\alpha-D\)-mannose (Man\(_3\)); and d-mannose (Man), used for the standards in thin-layer chromatography (TLC). Unless otherwise stated, all other reagents used in the present study were the commercially available guaranteed-class reagents.

**Preparation of the bulk mannan from bakers' yeast.** The preparation of the bulk mannan was carried out by the method of Peat et al. (10), modified by us as follows. A suspension of acetone-dried whole cells of bakers' yeast (300 g) in water (1.2 liters) was heated in an autoclave for 2 h at 140°C. After being cooled, the mixture was centrifuged for 20 min at 7,000 rpm, and the residue was stirred with water (1 liter) and centrifuged again for 20 min at 7,000 rpm. The combined filtrates were concentrated in vacuo to small volume (about 200 ml), dialyzed overnight against running tap water, and concentrated to about 200 ml. To the solution, under vigorous stirring, was added 800 ml of absolute ethanol containing 0.1% CH\(_3\)COONa. After being left for 30 min, the precipitate was collected by centrifugation, then dehydrated with...
absolute ethanol, and dried in vacuo over P$_2$O$_5$ to yield a light-brown amorphous powder (37 g).

The above crude extract (30 g) was dissolved in 300 ml of water, and the cloudy solution was stirred for 2 h with 50 ml of a 4:1 (vol/vol) mixture of CHCl$_3$-n-butanol. Then the mixture was centrifuged at 2,500 rpm for 15 min, and the supernatant collected was repeatedly treated by the same procedure until a completely clear solution was obtained. The supernatant was concentrated to about 150 ml and then was poured into 800 ml of absolute ethanol containing 0.1% CH$_3$COONa. The precipitate was collected by centrifugation, washed with ethanol, and dried in vacuo over P$_2$O$_5$ to give a tan-yellow amorphous powder (16 g).

To an aqueous solution of the above crude polysaccharide fraction (10 g in 200 ml) was added 200 ml of Fehling solution (a 1:1 [vol/vol] mixture of aqueous solutions of 3.5% CuSO$_4$·5H$_2$O, 17.3% Rochelle salt, and 5.0% NaOH), and the resultant precipitate was collected by centrifugation for 15 min at 3,000 rpm and then washed with 400 ml of hot water (70°C). The copper-mannan complex contained in a centrifuge tube was admixed with wet amberlite IR-120 (H$^+$) resin, and then stirring was continued until the complex dissolved and the blue color of the copper ions 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% Na$_2$CO$_3$ solution, then concentrated in vacuo to 50 ml, and dialyzed overnight against running tap water. The retentate was again concentrated in vacuo to about 50 ml, and the solution was poured into absolute ethanol containing 0.1% CH$_3$COONa. After washing with ethanol by centrifugation, the resultant precipitate was dried over P$_2$O$_5$, under diminished pressure to give the crude mannann fraction (5 g; 3.3%, on a weight basis, of the acetylated cells).

**Chromatographic fractionation of the bulk mannann.** An aqueous solution of the bulk mannann (3 g in 40 ml) was applied to a column (4 by 25 cm) of DEAE-Sephadex A-50 (acetate), and the elution was effected in a stepwise manner with water and 0.025, 0.05, 0.1, and 0.25 M NaCl. The flow rate was 0.5 ml/min, and a 10-μl sample of each fraction was assayed for carbohydrate content with phenol-sulfuric acid reagent (3). Subfractions A, B, C, D, and E (Fig. 1) were evaporated in vacuo to dryness, and, after dissolving in a minimum amount of water, the solutions were dialyzed overnight against running tap water. Each mannann subfraction was again concentrated to 5 ml and then poured into 25 ml of absolute ethanol containing 0.1% CH$_3$COONa, and the precipitated polysaccharide was collected by centrifugation at 2,500 rpm for 15 min. After being washed with absolute ethanol, the mannann subfractions were dried in vacuo over P$_2$O$_5$. The yields of fractions A, B, C, D, and E were 0.27, 0.75, 0.56, 0.68, and 0.17 g, respectively; total recovery was 81%.

**Acetolysis study of the mannann subfraction.** Acetolysis study of the mannann subfraction was carried out as described in a preceding paper (9). To isolate sufficient amounts of the phosphorus-containing manno-oligosaccharide fractions, each mannann subfraction (100 mg) was subjected to acetolysis. The de-O-acetylated manno-oligosaccharide mixture was then fractionated on a column (2 by 100 cm) of Bio-Gel P-2. Peaks at the void volume of the four elution profiles corresponding to fractions B, C, D, and E were shown to contain phosphorus and were designated fractions B-Vo, C-Vo, D-Vo, and E-Vo, respectively. The four common peaks of neutral carbohydrate in the elution profiles of five acetolyses were designated fractions I, II, III, and IV and identified with authentic specimens as Man$_4$, Man$_3$, Man$_2$, and Man, respectively, by TLC. Eluates corresponding to the peak of each Vo fraction were evaporated in vacuo to dryness, and weighed after drying over P$_2$O$_5$ in vacuo. On the other hand, the amounts of fractions I, II, III, and IV corresponding to each mannann subfraction were computed from the peak dimensions of the elution profile and then converted into a molecular ratio.

**Partial purification of fraction E-Vo by DEAE-Sephadex chromatography.** An aqueous solution of 10 mg of fraction E-Vo in 1.0 ml was applied to a column (2.5 by 7 cm) of DEAE-Sephadex A-50 (acetate). Elution was effected by the passing through of 200 ml of water, followed by 200 ml of 0.5 M NH$_4$HCO$_3$. The results of quantitative determination of carbohydrate and phosphorus showed that the amounts of

![Fig. 1. Elution profile of 3 g of bulk mannann on a DEAE-Sephadex A-50 column (acetate; 4 by 25 cm) by stepwise elution with water and NaCl solutions. Samples (10 μl) of fractions were assayed for carbohydrate content with phenol-sulfuric acid reagent (3). O.D., Optical density.](http://jb.asm.org/doi/suppl/10.1128/JB.155.1.64-74.1975/suppl.pdf)
carbohydrate in the former and the latter eluates were less than 4.5 and 82%, respectively. The eluate was evaporated in vacuo to dryness and further desiccated over P₂O₅. The results of chemical analyses showed that the ratio of mannose and phosphate groups (Man/P) ratio of this purified fraction E-Vo was 3.4. When developed twice on TLC, this fraction gave a tailing spot with a significantly lower Rᵢ value (0.25) than did any neutral acetylation fragment and showed positive reactions to both anisaldehyde and ammonium molybdate-perchloric acid reagents.

Immunochemical methods. Antiserum was prepared by immunizing a rabbit with heat-killed whole cells of bakers' yeast. The antiserum had an agglutination titer of 1:2,560 against the immunizing cell suspension and showed a high precipitin activity against the homologous yeast mannan. Quantitative precipitin reaction was carried out by the method of Sunayama (14), as follows. To 0.1 ml of antiserum in small test tubes (1.6 by 10.4 mm) was added 0.5 ml of saline solution of serial amounts of the mannan. After incubation at 37°C for 1 h, the mixtures were allowed to stand at 4°C for 16 h and then centrifuged at 2,500 rpm for 10 min. Each precipitate was washed twice carefully with 0.5 ml of ice-cold saline. The amounts of protein precipitated were determined by the Folin method of Lowry et al. (7). Precipitin inhibition assay was performed by the following method. Antisera (0.1 ml) were preincubated with a known quantity of inhibitor (0.1 ml) for 1 h at 37°C. To the solution was added 0.5 ml of saline solution containing 6.3 μg of each mannan subfraction, an amount corresponding to the equivalent points of the quantitative precipitin curves (Fig. 2). The inhibition ratio was calculated by the following formula: percent inhibition = (1 - A/B) x 100, where A and B are the amounts of precipitated protein with and without inhibitor, respectively.

Other methods. Specific rotations were determined in a 1-dm semimicrowell with an Applied Electric automatic polarimeter. Unless otherwise stated, all evaporation were carried out below 40°C. Pre-coated silica gel plates (TLC plates silica gel 60, without fluorescent indicator; 0.25-mm thickness; 5 by 20 cm, E. Merck AG, Darmstadt, Germany) were used for TLC. The solvent used for TLC was 5:3:2 (vol/vol) n-butanol-ethanol-water. Sugars and phosphates on the silica gel plates were detected by spraying with the anisaldehyde reagent (18) and the ammonium molybdate-perchloric acid reagent (17), respectively. Gel filtration chromatography of manno-oligosaccharides was carried out by using a column (2 by 100 cm) of Bio-Gel P-2 (400 mesh; Bio-Rad Laboratories, Richmond, Calif.). Total carbohydrate and total phosphate were determined with the phenol-sulfuric acid reagent (3) and by the method of Ames and Dubin (1), respectively. Total protein was determined by the Folin method of Lowry et al. (7).

RESULTS AND DISCUSSION

It has been shown by many workers that mannans of a few species of yeast are heterogeneous due to the presence of phosphate groups (2, 5, 11, 13, 16). For the mannan of K. brevis, Stewart and Ballou reported that this polysaccharide can be resolved into at least five subfractions by DEAE-Sephadex chromatography (13). They further provided the following findings: that the Man/P ratio varies among the mannans of many species of yeasts; and that the phosphate linkages in the original cell wall mannan are probably in the diesterified form, which is cleaved by the action of acid or alkali during the isolation procedure, leaving corresponding monoeesterified polysaccharide(s) (13). The bulk mannan specimen employed in the present study was shown to contain monoesterified phosphate groups, because the titration curve of this mannan with a 0.004 N NaOH solution showed inflection points at pH 4.5 and 7.5. Yield of the bulk mannan in the present study was 3.3%, on a weight basis, of the acetylene-dried cells. It is also worthy of note that the possibility of the presence of the other phosphorus compounds, such as inorganic phosphate, phospholipids, and nucleic acids, in the bulk mannan can be excluded, because the mannan gave a completely negative reaction of inorganic phosphate, and fatty acid, ribose, and 2-deoxyribose were not detected in the acid hydrolysate of the mannan. This mannan was fractionated on a column of DEAE-Sephadex to give five mannan subfractions, designated A, B, C, D, and E as shown in Fig. 1. These subfractions were shown to contain increasing amounts of phosphate with increasing concentrations of NaCl solution used as eluants (Table 1). However, it is unreasonable to consider that the weight ratio of these mannan subfractions exactly corresponds to that of the original phosphate-containing mannan moieties.

![Fig. 2. Quantitative precipitin curves of five mannan subfractions against homologous anti-whole-cell serum of parent bakers' yeast: Symbols: ○, fraction A; ⊙, fraction B; △, fraction C; ▲, fraction D; and □, fraction E. O.D., Optical density.](http://jb.asm.org/)
consisting of the parent cell wall. For the mannan of *K. brevis*, Raschke and Ballou reported that the branching moieties containing phosphate groups display a strong serological activity against the homologous anti-*K. brevis* whole-cell serum (11). Therefore, we carried out a series of quantitative precipitin tests between five mannan subfractions and homologous anti-*S. cerevisiae* whole-cell serum (Fig. 2). The order of the antibody-precipitating activities of these mannans was A < B < C < D < E, which was proportional to their phosphate content. To analyze structural differences, these mannans were investigated by the controlled acetolysis method (4). Figure 3 shows the acetolysis fingerprints of these mannan subfractions. Fractions I, II, III, and IV, which contained no phosphorus and which were identified as Man₄, Man₃, Man₂, and Man by comparison with authentic samples on TLC, were produced in all cases of acetolysis. The oligosaccharide containing phosphate from all of the fractions except A was eluted at void volume region, and amounts of phosphate and carbohydrate in this region increased in proportion to the phosphate content of the starting mannan subfractions. Table 2 shows the recov-

![Acetolysis fingerprints of subfractions A (A), B (B), C (C), D (D), and E (E). Each acetolysate, obtained from 100 mg of the mannan subfractions, was applied onto a column (2 by 100 cm) of Bio-Gel P-2 and eluted with water (7 ml/h). 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. Symbols: ○, carbohydrate at 490 nm; ×, phosphorus at 820 nm. O.D., Optical density.](http://jb.asm.org/)
Table 1. Chemical composition of mannan subfractions

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>% Composition</th>
<th>Optical rotation, specific</th>
<th>Man/P molar ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbohydrate*</td>
<td>Protein*</td>
<td>Phosphate*</td>
</tr>
<tr>
<td>A</td>
<td>99</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>B</td>
<td>96</td>
<td>0.1</td>
<td>0.23</td>
</tr>
<tr>
<td>C</td>
<td>98</td>
<td>0.1</td>
<td>0.57</td>
</tr>
<tr>
<td>D</td>
<td>94</td>
<td>0.1</td>
<td>0.99</td>
</tr>
<tr>
<td>E</td>
<td>93</td>
<td>0.3</td>
<td>2.09</td>
</tr>
</tbody>
</table>

* Determined by the phenol-sulfuric acid method (3).

** Determined by the Folin method of Lowry et al. (7).

a Quantitated by the method of Ames and Dubin (1) as -PO₃H₂.

b c 1.0, l 1.0, water.

c The ratio of mannos residues to phosphate groups.

The series of fractions Vo, I, II, III, and IV, on a weight basis, and the molecular ratios of four neutral acetolysis products, Man₄, Man₃, Man₂, and Man, calculated from the peak dimensions in each acetolysis fingerprint. A precipitin inhibition test of the mannan and the homologous anti-whole-cell serum was carried out by using Man₄ as an inhibitory hapten which has been determined to correspond to the antigenic determinant groups (15) of this bakers' yeast mannan (Fig. 4A). The amount (in micrograms) of Man₄, required for 50% inhibition was also found to be proportional to the phosphate contents of five mannan subfractions. This result appears to provide evidence substantiating the findings obtained by the acetolysis study.

For the phosphate-containing manno-oligosaccharides corresponding to fraction Vo of the present study, Thieme and Ballou reported that this fraction consists of a mixture of phosphates of Man₄ and Man₃ (16). To investigate the precipitin-inhibitory power of the phosphate-containing manno-oligosaccharides, fraction E-Vo was first purified by the method of Thieme and Ballou (16) by DEAE-Sephadex chromatography, using water and 0.5 M NH₄HCO₃ as the eluants. Figure 4B shows the result of the precipitin inhibition assay. Similar to Man₄, the purified acidic oligosaccharide fraction possessing a Man/P ratio of 3.4, a mixture of Man₄-P and Man₃-P, was found to reveal a strong precipitin-inhibitory activity against all antigen-antibody systems, and the amounts (in micrograms) required for 50% inhibition were also proportional to the phosphate contents of the parent mannan subfractions. However, the precipitin-inhibitory power of the purified fraction E-Vo appeared slightly weaker than that of Man₄.

Table 2. Manno-oligosaccharide phosphate(s), neutral manno-oligosaccharides, and mannose produced by acetolysis of five mannan subfractions

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>% Recovery in fraction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vo</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
</tr>
<tr>
<td>E</td>
<td>12</td>
</tr>
</tbody>
</table>

* Calculated on the basis of carbohydrate weight. Figures in parentheses indicate the molecular ratios of Man₄, Man₃, Man₂, and Man in each acetolysate, with Man₄ representing unity. Fractions I, II, III, and IV were identified by TLC as Man₄, Man₃, Man₂, and Man, respectively.

From the results obtained in the present study, it is reasonable to conclude that, with the increase of the amount of phosphates of the parent mannan subfractions, the amounts of Man₄, and phosphate-containing oligosaccharide also increased proportionally, whereas mannose decreased inversely in five acetolyses. Such microheterogeneity is attributable to the existence of various molecular species containing different amounts of phosphate groups which appear to dominate complexity of the branching moieties.

To provide similar examples substantiating the above assumptions, a series of immunochemical studies using several mannanas of the other genera of yeast are now in progress. Because the present work was done on mannan which was isolated by a procedure using strongly alkaline and acidic reagents, Fehling solution and cation-exchange resin, respectively, the degradation of some labile antigenic determinants removed from the parent mannan should be considered. An immunochemical study dealing with the comparison of antibody-precipitating activities between several mannan-protein conjugates prepared by the Cetavlon (cetyl trimethyl ammonium bromide) precipitation method (6, 8) and their degradation products with alkali and acid is also in progress.
ACKNOWLEDGMENTS

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LITERATURE CITED


