Immunoochemical Studies of the Mannans of *Saccharomyces cerevisiae* X2180-1A-5 and *Saccharomyces cerevisiae* 4484-24D-1 Mutant Strains, with Special Reference to Their Phosphate Content

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The mannans from *Saccharomyces cerevisiae* mutant strains X2180-1A-5 and 4484-24D-1, both of which were shown to contain small amounts of phosphate (less than 0.2%), were fractionated on a column of diethylaminoethyl-Sephadex into five subfractions designated as fractions I to V. These subfractions contain different amounts of phosphate, ranging from 0.03 to 0.09 (strain X2180-1A-5) and from 0.01 to 0.17% (strain 4484-24D-1). Fractions I to IV from strain X2180-1A-5 showed nearly identical precipitin activities against the homologous anti-whole cell serum, whereas fraction V, containing the largest amount of phosphate and protein among this mannan subfraction series, showed unexpectedly weaker precipitin activity than those of the other fractions. A synthetic mannan consisting of consecutive α-1→6-linked D-mannopyranosyl residues was found to be cross-reactive with all the mannan subfractions of strain X2180-1A-5 against anti-X2180-1A-5 serum. On the other hand, antibody-precipitating activities of the mannan subfractions of the latter strain were proportional to their phosphate content, although the increments of precipitated antibody nitrogen among the subfractions were quite small. However, fraction V of this mannan subfraction series, containing the largest amounts of phosphate and protein, showed lower precipitin activity than did the other four fractions. These findings indicate that mannans containing no phosphate or relatively small amounts of phosphate, such as those investigated in the present study, are less heterogeneous in the densities of the branching moieties than are highly phosphorylated mannans. These findings suggest that the transfer step of mannosyl-1-phosphate into the precursor(s) of the wild-type strain mannans during the biosynthetic process corresponds to the key reaction responsible for the anionic heterogeneity due to the density heterogeneity of the antigenic determinants.

It has been demonstrated by Ballou and his co-workers that phosphate groups are important constituents of the antigenic determinant groups of yeast mannans. They reported that the phosphomannan of *Kloeckera brevis* could be fractionated by DEAE-Sephadex chromatography into five subfractions containing different amounts of phosphate. They also assumed that the mannosyl-1-phosphate group in this mannan, as well as the longest branching moieties consisting solely of α-1→2-linked mannopyranosyl residues, acted as an antigenic determinant (17).

Recently, Okubo et al. have shown that the mannan of *Saccharomyces cerevisiae* (bakers' yeast) can also be fractionated on a column of DEAE-Sephadex into five subfractions containing different amounts of phosphate, and the antibody-precipitating activities of these mannan subfractions against the homologous antiserum are proportional to their phosphate content (11). These workers further demonstrated that the mannan subfractions of *Candida albicans* NIH A-207, NIH B-792, and J-1012 strains also showed a similar relationship between phosphate content and serological activities (10). To obtain additional evidence to confirm the above relationship, immunochemical properties of mannans of very low phosphate content, such as those of *S. cerevisiae* mutant strains X2180-1A-5 and *S. cerevisiae* 4484-24D-1 (abbreviated as A- and D-strains, respectively) developed by Ballou and his co-workers (3) must be investigated.

The present paper reports the results from an immunochemical study of the mannans of A-
and D-strains. Immunochemical properties of these mannans were compared with those of the mannan of an S. cerevisiae wild-type strain, which had been used in the previous study (11).

MATERIALS AND METHODS

Strain used. The mutant strains of S. cerevisiae (X2180-1A-5 and 4484-24D-1) were kindly supplied by C. E. Ballou, Department of Biochemistry, University of California, Berkeley, via T. Nakajima, Department of Agricultural Chemistry, Tohoku University, Sendai, Japan. These strains were maintained on agar slants as described by Raschke et al. (13). The results of a morphological examination of both mutant strains using the Gram-staining method indicated that the whole cells of these strains showed a deep violet color. However, both A- and D-strains showed very weak absorption of the dye when stained with Alcian blue (6).

Cultivation of strains. Each strain was cultured in a 500-ml flask containing a liquid medium described by Nakajima and Ballou (8) on a reciprocal shaker at 26 to 28°C for 48 h. The cells were harvested by centrifugation, washed thoroughly with saline, and dehydrated with a large volume of acetone.

Preparation of the bulk mannans. Preparation of mannans was completed in accordance with the description given in the preceding study (11) by means of Fehling solution. Yields of the bulk mannans of A- and D-strains were 3.0 and 3.3%, respectively, based on the dry weight of the whole cells.

Chromatographic fractionation of the bulk mannans. The method adopted for chromatographic fractionation of bulk mannans was similar to that of the preceding study (11). An aqueous solution of each bulk mannan (5 g in 40 ml) was applied to a column of DEAE-Sephadex A-50 (acetate, 4 by 25 cm), and the elution was effected in a stepwise manner using water and 0.025, 0.05, 0.1, and 0.25 M NaCl solutions subsequently. The elution profiles are shown in Fig. 1. Eluate corresponding to each mannan subfraction was separately collected and recovered by a procedure similar to that described previously (11). The mannan subfractions of A- and D-strains were designated as fractions A-I, -II, -III, -IV, and -V and D-I, -II, -III, -IV, and -V, respectively. Yields of the two mannan subfraction series are given in Table 1.

Other mannans. Five mannan subfractions (W-I, -II, -III, -IV, and -V) of an S. cerevisiae wild-type strain (W-strain, a single culture of commercially available bakers' yeast supplied by Oriental Yeast Co. Ltd., Tokyo) were the same as the specimens used in the previous study (11). The synthetic mannan consisting of consecutive α-1→6-linked D-mannopyranosyl residues was identical with the specimen described by Frechet and Schuerch (5).

![FIG. 1. Elution profiles of the bulk mannans of S. cerevisiae X2180-1A-5 (A) and 4484-24D-1 (B) by DEAE-Sephadex chromatography (A-50, acetate, 4 by 25 cm) by stepwise elution with water and NaCl solutions.](http://jb.asm.org/)

### Table 1. Chemical composition of mannan subfractions from S. cerevisiae X2180-1A-5 and 4484-24D-1

<table>
<thead>
<tr>
<th>Mannan fraction</th>
<th>Carbohydrate (%)</th>
<th>Protein (%)</th>
<th>Phosphate (%)</th>
<th>Specific optical rotation (°)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-bulk</td>
<td>86</td>
<td>5.9</td>
<td>0.05</td>
<td>+74°</td>
<td>49</td>
</tr>
<tr>
<td>A-I</td>
<td>87</td>
<td>4.7</td>
<td>0.03</td>
<td>+87°</td>
<td>25</td>
</tr>
<tr>
<td>A-II</td>
<td>85</td>
<td>4.8</td>
<td>0.06</td>
<td>+78°</td>
<td>14</td>
</tr>
<tr>
<td>A-III</td>
<td>83</td>
<td>8.1</td>
<td>0.08</td>
<td>+60°</td>
<td>11</td>
</tr>
<tr>
<td>A-IV</td>
<td>78</td>
<td>8.4</td>
<td>0.08</td>
<td>+57°</td>
<td></td>
</tr>
<tr>
<td>A-V</td>
<td>62</td>
<td>22.0</td>
<td>0.09</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>D-bulk</td>
<td>88</td>
<td>2.6</td>
<td>0.09</td>
<td>+68°</td>
<td>31</td>
</tr>
<tr>
<td>D-I</td>
<td>91</td>
<td>1.6</td>
<td>0.01</td>
<td>+71°</td>
<td>39</td>
</tr>
<tr>
<td>D-II</td>
<td>90</td>
<td>2.2</td>
<td>0.09</td>
<td>+55°</td>
<td>12</td>
</tr>
<tr>
<td>D-III</td>
<td>87</td>
<td>3.2</td>
<td>0.14</td>
<td>+47°</td>
<td>14</td>
</tr>
<tr>
<td>D-IV</td>
<td>85</td>
<td>4.7</td>
<td>0.16</td>
<td>+34°</td>
<td></td>
</tr>
<tr>
<td>D-V</td>
<td>80</td>
<td>9.7</td>
<td>0.17</td>
<td>+17°</td>
<td>4</td>
</tr>
</tbody>
</table>

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

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

c Quantitated by the method of Ames and Dubin as -PO₄H₂ (1).

d c, 1.0 l; f, 1.0 water.

* Weight basis of the corresponding bulk mannan.
Antisera. Antisera to A-, D-, and W-strains were prepared by immunizing three groups of three rabbits, each with heat-killed cells of each strain; antisera obtained from each group of rabbits were combined. All antisera had agglutinin titer of more than 1:1,280 against the immunizing cell suspensions. Comparison of the cross-agglutinability of antisera used in the present study against whole cells of the other strains gave the following results. (i) Anti-A-strain serum agglutinated W-strain cells very weakly, but did not agglutinate D-strain cells at all. (ii) Anti-D-strain serum was able to agglutinate W-strain cells strongly, but no reactivity was observed with A-strain cells. (iii) Anti-W-strain serum did not agglutinate D-strain cells at all, but exhibited only a weak cross-reactivity with A-strain cells. These results were coincident with those reported by Ballou et al. (3).

Immunochemical methods. The quantitative precipitin reaction and agar gel double diffusion analysis were carried out according to the methods of Suna- yama (18) and Ouchterlony (12), respectively.

Other methods. Specific rotations were determined in a 1-dm semimicrotube with an Applied Electric automatic polarimeter. All evaporation was carried out below 40°C. Total carbohydrate and total phosphate were determined with phenol-sulfuric acid reagent (4) 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

In the present study, we investigated the relationship between carbohydrate content and immunochemical properties of the mannans of A- and D-strains. These strains are the mannans mutants developed by Ballou and his co-workers by mutagenesis of the corresponding wild-type strains, S. cerevisiae X2180-1A and S. cerevisiae 4484-24D (2). With regard to the structural features of the former mutant strain mannan, Raschke et al. have revealed that this mannan consists of a backbone moiety composed of consecutive α-1→6-linked D-mannopyranosyl residues and very small amounts of branching moieties corresponding to tetraosyl, triosyl, and biosyl units (mn2 strain) (13). On the other hand, it has been indicated that the chemical structure of the latter mutant strain mannan lacks either nonreducing terminal α-1→3-linked D-mannopyranosyl residue or a mannosyl-1-phosphate group (mn1, mn4 strain) (3). They have further pointed out that these mutant mannans can be distinguished by the cross-precipitin reaction using antisera corresponding to the whole cells of the A- and D-strains of the former mutant, and the corresponding wild-type strain S. cerevisiae X2180-1A, since these antisera were shown to be strictly specific for α-1→6, α-1→2, and α-1→3-linked D-mannopyranosyl residues, respectively (13).

As the phosphate contents of the bulk mannans of A- and D-strains were shown to be smaller than those of the corresponding wild-type strains and appear to be proportional to the average lengths of the branching moieties of these three mannans, they assumed that the elongation step of branching moieties of the wild-type mannan during biosynthesis was dominated by phosphate groups (14). Therefore, it seemed of interest to determine whether these mutant mannans containing small amounts of phosphate show anionic heterogeneity similar to that observed in the wild-type mannan (11). The bulk mannans of A- and D-strains prepared by the Feilih solution method in the present study were shown to contain small amounts of phosphate (0.05 and 0.09%, respectively). Each bulk mannan was fractionated into five subfractions, designated as fractions A-I to V or D-I to -V, by DEAE-Sephadex chromatography employing the stepwise elution system described in the previous studies (10, 11). As noted in Fig. 1, the bulk mannans of both mutant strains gave larger amounts of neutral and weakly acidic subfractions, fractions I and II. The amounts of the strongly acidic subfractions, fractions III to V, were relatively small as compared with the corresponding fractions from the W-strain mannan reported previously (11). Table 1 summarizes the analytical data for these mannan subfractions together with those for the parent bulk mannans. It is obvious that the two series of mannan subfractions of both A- and D-strains contain increasing amounts of phosphate, ranging from 0.03 to 0.09 and 0.01 to 0.17%, respectively, although these values are lower than those of the W-strain mannan fraction series, from 0.00 to 2.09% (11). The protein content of these mutant mannan subfractions, however, was also proportional to the concentration of NaCl solutions used for the elution. Fraction A-V was found to contain a large amount of protein, about 20%, and the least amount of carbohydrate, 60%. Contribution of the protein moieties to the anionic behavior of the parent mannans should be considered, because these moieties in yeast mannan were shown to contain small but significant amounts of acidic amino acid residues (15). Participation of the protein moieties in the anionic behavior of both mutant mannans used in the present study cannot also be ruled out, since both phosphate and protein contents in the A- and D-mannan subfraction series were found to increase proportionally to the concentration of NaCl solutions used for elution. However, the mannan subfraction series of both mutant strains were used for further study without removal of their protein moieties.
The results of quantitative precipitin reactions between the mannan subfractions and antisera of the A-, D-, and W-strains are depicted in Fig. 2A, B, and C. Against anti-A-strain serum, fractions A-I to -IV gave nearly identical amounts of antibody nitrogen, whereas fraction A-V displayed low precipitin activity. This suggests that the existence of a large amount of protein, which does not participate in the serological activities, is attributable to low serological activity. Against anti-D-strain serum, fractions D-I to -IV were found to precipitate antibody nitrogen proportionally to their phosphate content. However, the difference in precipitin activities of the four mannan subfractions was quite small. Fraction D-V was shown to be less reactive with this serum than were the other subfractions having lower phosphate contents. The fact that fraction D-V, containing the largest amount of phosphate, was unable to precipitate the largest amount of antibody nitrogen may be attributable to its lower carbohydrate content.

Fractions W-I to -V showed considerably high cross-reactivities with anti-D-strain serum, precipitating nearly equal amounts of antibody nitrogen among all the mannan subfractions. On the other hand, all of the mannan subfractions of A-strain, fractions I to V, were unable to show any cross-reactivity with anti-D-strain serum. These results indicate that the densities of the cross-reactive antigenic determinant groups in fractions W-I to -V, corresponding to the branching moieties consisting of α-1→2-linked D-mannopyranosyl residues, are similar.

Against anti-W-strain serum, the homologous mannan subfractions precipitated antibody nitrogen in amounts proportional to their phosphate content, and, with the exception of fractions A-IV and -V, all the mannan subfractions from the two mutant strains did not show any cross-reactivity with this antisera. The fact that fractions A-IV and -V showed weak positive precipitin activities against anti-W-strain serum suggests that small amounts of cross-reactive manno-oligosaccharide moieties, of which the existence in the corresponding bulk mannan has been proposed by Nakajima and Ballou (9), may be located in these subfractions.

The foregoing results from the precipitin study of the mannans of three S. cerevisiae strains seem to substantiate the concept that the mannan subfractions obtained from the corresponding bulk mannan of low phosphate content show quite similar precipitin activities, i.e., the difference of precipitin activities of the mannan subfractions is small when the phosphate content of the parent bulk mannan is low.

The synthetic D-mannan, composed of consecutive α-1→6-linked D-mannopyranosyl residues, having a molecular weight of about 180,000 (5), showed a strong cross-reactivity against only anti-A-strain serum, which is nearly identical to those of fractions A-I to -IV, providing direct evidence that anti-A-strain serum contains antibody(ies) corresponding to the above structure.

The results of the agar gel double diffusion reactions between fraction V of three strains and the corresponding antisera were consistent with those obtained in the quantitative precipitin reactions (Fig. 3). Anti-A-strain serum cross-reacted with fractions W-V and A-V, and anti-D-strain serum was shown to be cross-reactive only with fraction W-V, affording completely fused precipitin lines. Anti-W-strain serum was unable

![Fig. 2. Quantitative precipitin curves of each five mannan subfractions of three S. cerevisiae strains against anti-whole cell sera of the same strains. (A) Anti-A-strain serum; (B) anti-D-strain serum; (C) anti-W-strain serum. Mannan subfraction of A-strain (○), D-strain (●), W-strain (△), and synthetic mannan (▲).](http://jb.asm.org/6)
to cross-react with any of the mannan subfractions from A- and D-strains, except for fraction A-V.

Results obtained in the previous (10, 11) and present studies can be summarized as follows. Regardless of the amount of phosphate contained, mannans of many species of yeasts can be fractionated by anion-exchange chromatography into a number of subfractions containing different amounts of phosphate. Moreover, the difference in the amounts of antibody nitrogen precipitated by the mannan subfractions which have been obtained from the same bulk mannan is proportional to the difference in their phosphate content. It is also noteworthy that mannans containing very small amounts of phosphate, such as A- and D-strain mannans, are less heterogeneous in the densities of antigenic determinant groups than are highly phosphorylated mannans. In other words, the transfer step of mannosyl-1-phosphate into the precursor(s) of highly phosphorylated mannan corresponds to the key reaction responsible for the anionic heterogeneities due to the heterogeneity of the density of phosphate-containing antigenic determinant groups, as observed previously on the mannans of K. brevis and W-strain by Thieme and Ballou (17) and Okubo et al. (11), respectively.

ACKNOWLEDGMENTS

We thank C. E. Ballou, Department of Biochemistry, University of California, Berkeley, and T. Nakajima, Department of Agricultural Chemistry, Tohoku University, for supplying the S. cerevisiae X2180-1A-5 and 4484-24D-1 mutant strains. This work was supported in part by a grant-in-aid from the Ministry of Education, Science, and Culture, Japan.

LITERATURE CITED