Studies on the Antigenic Activities of Yeasts

V. Effect of α-mannosidase Digestion on the Immunochemical Properties of the Mannan of Saccharomyces cerevisiae

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ABSTRACT

In order to provide further information on the chemical nature of the antigenic determinants of the mannan of Saccharomyces cerevisiae, the mannan was digested by Arthrobacter α-mannosidase, and 9, 21, 35, 59 and 62%-partially degraded mannans were prepared in the present study. Acetolysis of each degraded mannan showed that only a small amount of the tetrasaccharide was detectable in the 35%-digested mannan, whereas the predominant product of the 59 and 62%-digested mannan was mannose. The result of a quantitative precipitation reaction with the degraded mannans showed that the precipitation activities were partially or completely destroyed by the action of the enzyme. The lack of the tetrasaccharide moieties of the mannan were noticeable by a decrease in the precipitating ability. It was observed that the decreasing ratio of either the maximum amount of the antibody N precipitable by the mannan or percent degradation of the mannan were essentially equal and yielded nearly a straight relationship between 0 and 2.0 hr digestion. However, the 59 and 62%-digested mannans, containing trace amounts of di- and trisaccharides in the branching parts, showed no significant antigenic activities. Furthermore, the molar ratio of the tetrasaccharide relative to the trisaccharide also gradually decreased. These observations confirm that the tetrasaccharide moiety, Manα1→3Manα1→2Manα1→2Man, plays an important role as the antigenic determinant. The core mannan moiety completely lost both the precipitating ability and inhibitory activity in ranges employed up to 1500 µg. These findings offer a direct proof that the core mannan moiety of mannan is not responsible for antigenic activity, and functions merely as the “carrier” of the antigenic determinants which dominate the immunological specificity.

In the first paper of this series, the authors reported that the antigenic determinants of the mannan from Saccharomyces cerevisiae, baker’s yeast, were a tetrasaccharide moiety, Manα1→3Manα1→2Manα1→2Man, which corresponded to the longest

Manα1→3Manα1→2Man : O-α-D-mannopyranosyl-(1→3)-O-α-D-mannopyranosyl-(1→2)-D-mannose.

Manα1→3Manα1→2Man : O-α-D-mannopyranosyl-(1→3)-O-α-D-mannopyranosyl-(1→2)-D-mannose.
branching parts of parent polysaccharide. In addition to this description it was found that the core-moiety of this mannan joined through α1→6 linkages, did not participate directly to govern antigenic activity on the homologous antigen-antibody system [24].

Recently, Jones and Ballou isolated an Arthrobacter species, which was capable of growing on the mannan of S. cerevisiae as a sole carbon source, and producing an extracellular α-mannosidase into the culture medium [7]. This α-mannosidase is an inducible enzyme and splits most α1→2 and α1→3 linkages in the branching parts of the mannan, leaving a resistant polymer containing mainly α1→6 linkages [8, 9].

Therefore, studies on the relationships between the antigenic activities and structure of the mannan by the treatment of α-mannosidase are of interest. The present paper deals with the effect of α-mannosidase digestion on the antigenic activities of the mannan of S. cerevisiae.

MATERIALS AND METHODS

Antigenic mannan and oligosaccharides. The baker’s yeast was supplied by the Oriental Yeast Co. Ltd., Tokyo. The purified neutral mannan was prepared by the procedure employed previously [17]. This was homogeneous in both ultracentrifugal analysis and free boundary electrophoresis. Properties of the purified mannan are shown in Table 1. Oligosaccharides, Manα1→2Man, Manα1→2Manα1→2Man (plus Manα1→3Manα1→2Man) and Manα1→3Manα1→2Man prepared from the mannan by acetolysis, were the same specimens as described previously [24].

Assay of the enzyme activity. Enzyme assay was carried out essentially by the method of Jones and Ballou [8]. The amount of reducing sugar was determined by the method of Somogyi-Nelson [19] with mannose as the standard material.

Preparation of α-mannosidase. Arthrobacter species GJM-1 was kindly donated by Dr. C. E. Ballou, Department of Biochemistry, University of California. The preparation of enzyme was carried out by a method essentially similar to that described by Jones and Ballou [7], employing a mannan of S. cerevisiae as a carbon source in 0.1% concentration. Two liters of medium divided into 4 Fernbach flasks were inoculated and shaken at 37°C for 48 hours. After removal of cells by centrifugation at 9000 rpm for 30 min at 4°C, the enzyme was isolated from the culture filtrate by the addition of solid ammonium sulfate. After 12 hours, the resultant precipitate was collected by centrifugation and dissolved in 0.1 M-potassium phosphate buffer (pH 6.8) containing 0.1 μmole of CaCl₂, and dialyzed

<table>
<thead>
<tr>
<th>Table 1. Properties of the mannan of S. cerevisiae</th>
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<tr>
<td><strong>Total carbohydrate</strong> (%)</td>
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<tr>
<td>99.8</td>
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</table>

a) Determined as mannose by modified Molisch method.
b) Micro Dumas method.
c) Allen’s modified method.
d) C=1.0, I=1.0 in water.
e) Sample 1% in water, 59780 rpm at 20°C, Spinco model E type.
f) Arsenite titration method.
g) Alkaline hypoiodite method.
against the same buffer. Ammonium sulfate-precipitation was repeated until the enzyme solution no longer gave a positive reaction with Molisch’s reagent. Further purification was not done in the present study. The final volume was 6 ml from 21 of the culture medium, and protein concentration was 1.93 mg per ml. One of enzyme protein contained approximately 51 units of activity. One unit of activity was defined as that amount of enzyme which released 1 µmole of mannose under the assay condition [8].

From the dialysate of the culture filtrate after ammonium sulfate-precipitation, an α-mannosidase-resistant coremannan moiety was recovered by the addition of Fehling’s solution.

Preparation of the partially degraded mannan by the treatment with α-mannosidase. Five hundred mg of the purified mannan in 250 ml of 0.05 M-potassium phosphate buffer (pH 6.8) containing 0.1 µmole of CaCl₂, were incubated with 6 ml of the enzyme solution in a water bath at 37 C. Aliquots of the incubation mixture (each 50 ml) were withdrawn at various times; 0.5, 1.0, 2.0, 5.0 and 23 hr after addition of enzyme. The mannose content released at each interval was estimated by the method of Somogyi-Nelson [19]. Enzymatic hydrolysis pattern of mannan is shown in Fig. 1; 9% of mannan was digested after 0.5 hr, 21% after 1.0 hr, 35% after 2.0 hr, 59% after 5.0 hr and 62% after 23 hr, respectively.

Each reaction mixture was concentrated to a small volume under reduced pressure and the enzyme protein was removed by Sevag’s method. Then inorganic salts were removed through columns of Amberlite IR-410 (OH⁻ form) and IR-120 (H⁺ form). After concentration, a deionizing solution was applied on a column (2×100 cm) of Sephadex G-25 (super fine) to separate the degraded mannan moiety and the mannose. The results are shown in Fig. 2. The partially degraded mannan fractions, a high molecular weight residue, were collected and concentrated to a small volume. They were then precipitated with ethanol and dried in vacuo over phosphorus pentoxide. The yields of each high molecular fraction were 85, 71, 54, 38 and 18 mg respectively. Appropriate molecular weights for each degraded mannan, M, was calculated from the following formula: \( M = B - \frac{B}{100} \times C \), where \( B \) is molecular weight of intact mannan (M.W. = 22 000) [8] and \( C \) is per cent of degradation.

Quantitative precipitation reaction and agar gel diffusion. Antiserum was prepared by the method described previously [24], and the antibody titer was 1:1280 by the agglutination test. Quantitative precipitation reactions and inhibition tests were carried out by the methods described previously [22].

Agar-gel diffusion reactions were carried
General procedures. Acetolysis of intact and enzyme-digested mannans was carried out according to the method described previously [24]. Descending paper chromatography was performed on Toyo Roshi No. 51 A paper using the following solvent: n-butanol, ethanol, water, 2:1:1 (v/v). Sugars were detected by a silver nitrate-sodium hydroxide reagent [25]. Quantitative paper chromatography was carried out by the following procedure; after spraying a guide strip, the filter paper containing sugars was cut out and eluted with water, then the solution was made up to 10 ml. One ml of each eluate was tested for quantitative carbohydrate content by the phenol-sulfuric acid method [3]. The Carbohydrate content of the eluate from the column of Sephadex G-25 was assayed by the modified Molisch method [2]. The total protein in the enzyme preparation and the precipitate from the quantitative precipitation reaction were determined by a modified Lowry's method [4].

RESULTS

Degradation of the Mannan by α-Mannosidase

By degradation of antigenic mannan with α-mannosidase, five kinds of partially de-
graded mannan fractions, i.e., 9, 21, 35, 59 and 62%-degraded mannans, were obtained in the present study. The elution patterns of each reaction mixture on a Sephadex G-25 column are shown in Fig. 2. Mannose was detected as a single low molecular weight compound by paper chromatography, and no oligosaccharides were detected.

**Acetolysis of the Degraded Mannans and Core Mannan Moiety**

Each 10 mg of the degraded mannans

![Fig. 3. Paper chromatogram of the acetolysis products of the partially degraded mannans by α-mannosidase. Chromatogram developed with n-butanol, ethanol, water, 2:1:1(v/v) by descending method for 20 hr. Standard materials are di-, tri- and tetrasaccharides isolated from the mannans by acetolysis. a. Man. b. Biose. c. Triose. d. Tetraose.]

**Table 2. Molar ratio of the acetolysis products of the partially degraded mannans**

<table>
<thead>
<tr>
<th>Digestion time (hr)</th>
<th>Mannose</th>
<th>Biose</th>
<th>Triose</th>
<th>Tetraose</th>
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<tr>
<td>0 hr (intact mannans)</td>
<td>0.42</td>
<td>0.85</td>
<td>1.0</td>
<td>0.48</td>
</tr>
<tr>
<td>0.5 hr (9%)</td>
<td>0.88</td>
<td>1.50</td>
<td>1.0</td>
<td>0.37</td>
</tr>
<tr>
<td>1.0 hr (21%)</td>
<td>0.86</td>
<td>1.80</td>
<td>1.0</td>
<td>0.28</td>
</tr>
<tr>
<td>2.0 hr (35%)</td>
<td>8.20</td>
<td>2.0</td>
<td>1.0</td>
<td>0.10</td>
</tr>
<tr>
<td>5.0 hr (59%)(b)</td>
<td>Main product</td>
<td>Trace</td>
<td>Trace</td>
<td>0.0</td>
</tr>
<tr>
<td>23.0 hr (62%)(b)</td>
<td>Main product</td>
<td>Trace</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Core mannans</td>
<td>Main product</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
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\(a\) Molar ratios were calculated relative to trisaccharide.

\(b\) Molar ratios of the acetolysis product in these fractions were not determined, since the amounts of the oligosaccharides were quite small.
was subjected to acetolysis for 13 hr at room temperature. The products were subsequently de-acetylated by a sodium methoxide method and desalted by an ion-exchanger, Amberlite IR-410 (OH⁻ form) and IR-120 (H⁺ form). After concentration to a small volume, they were applied to paper chromatography. Paper chromatograms of the acetolysates of each degraded mannan are shown in Fig. 3. Table 2 shows the results of the molar ratios of the oligosaccharides obtained by the quantitative paper chromatography. The molar ratios of these products were calculated relative to the trisaccharide. Acetolysis of the intact mannan (0 hr) produced mannose and three kinds of oligosaccharides, di- to tetrasaccharide. It can be seen from Fig. 3 and Table 2 that the tetrasaccharide gradually disappeared by increasing the incubation time and only a small amount was detectable at 2.0 hr, whereas the mannose increased rapidly. After 5.0 and 23 hrs digestion, the predominant product was mannose and small amount of disaccharide was observed, but the tetrasaccharide had disappeared from the acetolysate. The core mannan moiety afforded only mannose in the above procedure.

Antigenic Activities of Partially Degraded Mannans

The result of the quantitative precipitation reaction is shown in Fig. 4. Precipitation activities of the mannan decreased gradually with an increase in the incubation time with the enzyme. The amount of intact mannan which was required to afford a maximum amount of nitrogen in quantitative precipitation reaction was 15 µg, and those of three degraded mannan fractions, 0.5, 1.0 and 2.0 hr-hydrolysates, were 19, 25 and 30 µg respectively. However, the other two fractions which were isolated from the 5.0 and 23 hr-hydrolysates, showed no significant antigenic activities, even when they were tested in amounts up to 500 µg respectively. The core mannan moiety, containing only α1→6 linkages recovered from culture filtrate, was ineffective in its ability to precipitate antibody N. Fig. 5 shows three straight lines which represent the relationships between incubation-time and the amounts of maximum antibody N precipitable in the quantitative precipitation reaction with the degradation products, per cent degradation of the parent mannan and the molar ratio of the tetrasaccharide relative to the trisaccharide in

![Antigenic Activities of Partially Degraded Mannans](image-url)
the acetolysate of each degradation product. It was clearly that the precipitation activity decreased gradually according to the length of incubation time, and the degree of degradation of the mannan was inversely proportional to the former. The change of molar ratio of the tetrasaccharide to trisaccharide was proportional to that of precipitation activity. Fig. 6 shows the double gel-diffusion pattern of the degraded mannans. The lines formed by the three mannan fraction, 0.5, 1.0 and 2.0 hr-hydrolysate, fused completely with a line formed by the intact mannan, whereas the mannan
digested for 5.0 hr and the core mannan moiety did not form lines at all. Fig. 7 shows the result of the quantitative precipitation inhibition test of the core mannan. No significant inhibitory activity was observed in the range employed up to 1500 μg.

**DISCUSSION**

It has been known that the major component of polysaccharides in the yeast cell wall are a mannan and a glucan, and that the former plays an important role in antigenic activities, especially the immunological specificity of the yeasts, while the latter does not seem to participate in antigenic activities [16, 17, 23].

Several investigations into the chemical structure of mannans from baker’s yeast have been carried out by Haworth et al. [5], Cifonelli et al. [1] and Peat et al. [14, 15]. Recently, Lee et al. [12] have isolated a tetrasaccharide, Man$_{1}$$\alpha$$\rightarrow$3Man$_{1}$$\alpha$$\rightarrow$2Man$_{1}$$\alpha$$\rightarrow$2Man, in the acetolysate from yeast mannan. Stewart et al. have proposed an alternative structure of this mannan as shown in Fig. 8. This is regarded as the most adequate structure of this polysaccharide at present [20, 21].

Acetolysis of the yeast mannan preferentially splits $\alpha$$1$$\rightarrow$6 linkages in the polysaccharide, giving mannose plus $\alpha$$1$$\rightarrow$2 and $\alpha$$1$$\rightarrow$3 linked oligosaccharides, and provides an excellent procedure for the analysis of the chemical structure. The acetolysis products for the intact mannan obtained in the present study, were mannose, di-, tri- and tetrasaccharide in the ratios 1.0:2.0:2.3:1.1 respectively, which resembled that reported by Lee et al. [12] and Stewart et al. [21].

Various methods of chemical treatment for the structural modification of polysaccharide antigens have been reported by many workers in order to elucidate the relationships between structure and immunological specificity of the antigenic substances [10]. The use of a splitting enzyme on the antigens has also contributed to the immunochemical studies on the antigenic determinants, e.g., the blood group substances [6].

*Arthrobacter* $\alpha$-mannosidase is an exoglycosidase, which cleaves one by one, from non-reducing end, groups of the branching parts of the mannan, producing free mannose [8, 9]. It is apparent that the $\alpha$-mannosidase digestion procedure is an excellent tool for elucidating the antigenic activities of mannan.

The quantitative acetolysis study of each degraded mannan in the present study showed that the digestion process seems to proceed at first from the tetrasaccharide moieties composed of the longest branching parts of mannan, Man$_{1}$$\alpha$$\rightarrow$3Man$_{1}$$\alpha$$\rightarrow$2Man$_{1}$$\alpha$$\rightarrow$2Man, and then the tri- to disaccharide moieties in order of the size of the branching parts, finally leaving a resistant core mannan moiety containing mainly $\alpha$$1$$\rightarrow$6 linkages. The results of the quantitative precipitation reaction showed that the precipitating ability of the mannan was destroyed partially or completely by the action of the enzyme. From the above facts, it is clear that the tetrasaccharide moieties affect remarkably the antigenic

![Fig. 8. The chemical structure of mannan proposed by Stewart and Ballou [21]. M= α-D-mannopyranosyl residue.](image-url)
activities of the mannan; mannan which digested for 5.0 hr (59%) and 23 hr (62%) no longer contained tetrasaccharide moieties, and showed no significant antigenic activities. These findings were also confirmed by results of agar gel double diffusion reactions.

As seen in Fig. 5, the decreasing ratios of either the maximum amount of antibody N precipitable or per cent degradation of the intact mannan are proportional and gave a nearly straight line through 0 hr to 2.0 hr digestion. Furthermore, the molar ratios of the tetrasaccharide relative to the trisaccharide also decreased in an essentially straight line. Therefore, it is evident that the presence of \( \alpha 1 \rightarrow 3 \) linkages in the non-reducing residue are very important to fit the combining site of the antibody. These observations provide further evidence that the tetrasaccharide moiety plays an important role for the antigenic determinants of the mannan of \( S. cerevisiae \), as reported previously [24]. A recent investigation in this laboratory of the antigenic determinants of this mannan have made clear that the trisaccharide moieties are another important factor to fit the combining sites of the antibody. The trisaccharide moieties contain Man1→3Man1→2Man in addition to Manα1→2Manα1→2Man, and the former constitutes about 20% of the trisaccharide fractions. The result of the quantitative precipitation-inhibition test showed that the former is apparently a better inhibitor than the latter; as 0.06 \( \mu \)mole of the former was required for 50%-inhibition as compared with 0.11 \( \mu \)mole of the latter. This observation also confirmed definitely that the \( \alpha 1 \rightarrow 3 \) linked mannose residue is important for antigenic activities.

In the first paper of this series, we observed the effects of the antigenic activities of the mannan by chemical modification; partial acid hydrolysis and periodate oxidation, and indicated that the presence of 3-O-substituted mannose residues adjacent to the several mannose residues joined with \( \alpha 1 \rightarrow 2 \) linkage in the branching chains are essential to fit the combining site of the antibody [24]. All the results in this study, thus far obtained by the enzymatic modification of mannan, support strongly these findings. Furthermore, the core mannan containing only \( \alpha 1 \rightarrow 6 \) linkages recovered from culture filtrate completely lost either its precipitating ability or its inhibitory activity when tested in amounts from 60 to 1500 \( \mu \)g. This observation gave a direct proof that the core moiety of mannan is not responsible directly for any antigenic activity, but rather merely functions as a "carrier" of the antigenic determinants dominating the immunological specificity.

The molecular weight of the mannan of \( S. cerevisiae \) has been estimated to be 22 000 [9]. Basing on the above value for the parent mannan, molecular weights for three fractions, 9, 21 and 35%-digested products obtained in the present study, were assessed to be about 20 000, 17 000 and 14 000 respectively. Studies on the effect of molecular weight on polysaccharide antigens and their quantitative precipitation reactions have been reported for the dextran–anti-dextran system by Kabat and Berg [11], and recently on the dextrans, glycogens and levans–concanavalin A systems by So and Goldstein [18]. According to the descriptions of these workers, the lower molecular weight antigens reached an equivalence point more rapidly in the region of excess antibody or concanavalin A than the higher weight antigens, but both of them gave nearly equal maximum amounts of precipitated N. While in the region of excess anti-
gen, the former were much more effective in causing inhibition than the latter. Based on the above facts, the interpretation that the chemical structures of antigenic determinants of all the polysaccharides employed by the above workers are inalterable by any modification procedure to cleave the glycosidic linkages, since the arrangement of linkages was relatively uniform in these molecules, and therefore, the partially degraded products still possessed strong precipitation activity. However, the results obtained in the present study clearly differed from those of the above workers; when the molecular weight of mannan was decreased by the action of the enzyme, the equivalence point shifted from left to right to the region of excess antibody and the maximum amount of antibody N decreased inversely proportional to the per cent degradation (0 to 35%). Therefore, the decreasing precipitation activity of this mannan by α-mannosidase digestion is attributable to the elimination of the branching chains composed of the antigenic determinants.

In conclusion, we found, in the present study, our results to substantiate the chemical structure of this mannan as proposed by Lee et al. [12] and Stewart et al. [21].

ACKNOWLEDGEMENT

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