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Note

Action of alkaline sodium borohydride on fractions of D-mannans from bakers' yeast (*Saccharomyces cerevisiae*)*

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D-Mannans elaborated by many species of yeasts can be considered as glycoproteins consisting of a phosphate-containing polysaccharide and relatively small proportions of peptide components[1]. These D-mannans were shown to be composed of heterogeneous molecular species with respect to phosphate and protein content, and could be resolved into several subfractions containing various phosphate groups and peptide moieties[2]. The D-mannan subfractions free of phosphate and peptide, prepared from the bulk D-mannan of bakers' yeast (*Saccharomyces cerevisiae*) by DEAE-Sephadex chromatography, were shown to display a marked growth-inhibitory activity against mouse-implanted sarcoma 180 and Ehrlich carcinoma solid-tumor[3–5]. The yield of the neutral D-mannan fraction was relatively low (up to 10%, based on the amount of the parent bulk D-mannan) and that of the acidic D-mannan fraction, which contains phosphate and peptide moieties, was high (70%, accounting for the major part of the bulk D-mannan). As the latter fraction is toxic to the host animal, an attempt was made to prepare a neutral D-mannan by eliminating both prosthetic groups.

In the present study, four fractions of bakers' yeast D-mannan (Fractions A, B, C, and D) were treated with hot alkaline sodium borohydride solution, which has been shown by Nakajima and Ballou[6] to cleave the linkage between the 2-acetamido-2-deoxy-D-glucopyranosyl and L-asparaginyl residues of the D-mannan of the *S. cerevisiae* X-2180 strain. The peptide component had been eliminated by this treatment, although no definite reaction conditions were reported.

The results of the chemical analysis of borohydride-treated D-mannan fractions from bakers' yeast, designated Fractions A′, B′, C′, and D′ (see Table I), indicate

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TABLE I

CHEMICAL COMPOSITION OF FRACTIONS OF D-MANNAN TREATED AND UNTREATED WITH ALKALINE SODIUM BOROHYDRIDE

Fraction	*Sugar (%)*	*P (%)*	*Protein (%)*	*HexN*[a] *(%)*	$[\alpha]_D^{20}$[b]	S_{20}[b]
A	96 0	0.0	0.0	0.0	+80.5°	3.2
A′	95.5	[c]	[c]	[c]	+81.0°	[c]
B	90.0	0 1	2 5	6 5	+71.8°	3.2
B′	94.5	0.1	0 4	0.9	+81.5°	3.0
C	86.0	0.4	3.3	6.0	+74.0°	3.4
C′	92 7	0.3	0.6	1.1	+80 5°	3.0
D	80.1	0.4	5.7	6.5	+59.0°	3.5
D′	90 5	0.4	0.5	0.8	+72 5°	2.8

[a]Abbreviation: HexN, hexosamine. [b]For solutions in water (*c* 1). [c]Not determined

TABLE II

AMINO ACID ANALYSIS FRACTIONS OF D-MANNAN TREATED, AND UNTREATED WITH ALKALINE SODIUM BOROHYDRIDE

Amino acid (%)	*Fraction*					
	B	*B′*	*C*	*C′*	*D*	*D′*
Arg	0 00	0.00	0.00	0.00	0.15	0.00
Asp	0 46	0.18	0.44	0.15	0.54	0.21
Thr	0.17	0.02	0 02	0.00	1.13	0.01
Ser	0 34	0.03	0 23	0.00	0.29	0.03
Glu	0.37	0.04	0.47	0.00	1.04	0.06
Pro	0.37	0.00	0 37	0.00	0.49	0.00
Gly	0.15	0.02	0.11	0.00	0.20	0.00
Ala	0.46	0.06	0 39	0.00	0.59	0.00
Cys	0 00	0.00	0.00	0.00	0.00	0.00
Val	0.30	0.03	0 34	0.00	0.56	0.06
Met	0.00	0 00	0 00	0.00	0.00	0.00
Ile	0.12	0.00	0.14	0.00	0 25	0.00
Leu	0.10	0.00	0 18	0.00	0 26	0.00
Tyr	0.00	0.00	0.42	0.00	0.05	0.10
Phe	0.04	0.00	0.06	0.00	0.10	0.08
Total amino acid content (%)	2.88	0.38	3.17	0.15	5.65	0.45

that removal of the peptide component of these fractions was successfully achieved, *i.e.*, the total nitrogen content of Fractions B′, C′, and D′ was quite small, suggesting that the only amino acid residue remaining in the modified D-mannans is asparatic acid (Table II). It is also evident that the proportion of total hexosamine also decreased in Fractions B′, C′, and D′. On the other hand, however, the phosphate content of these fractions was not significantly changed (Table I).

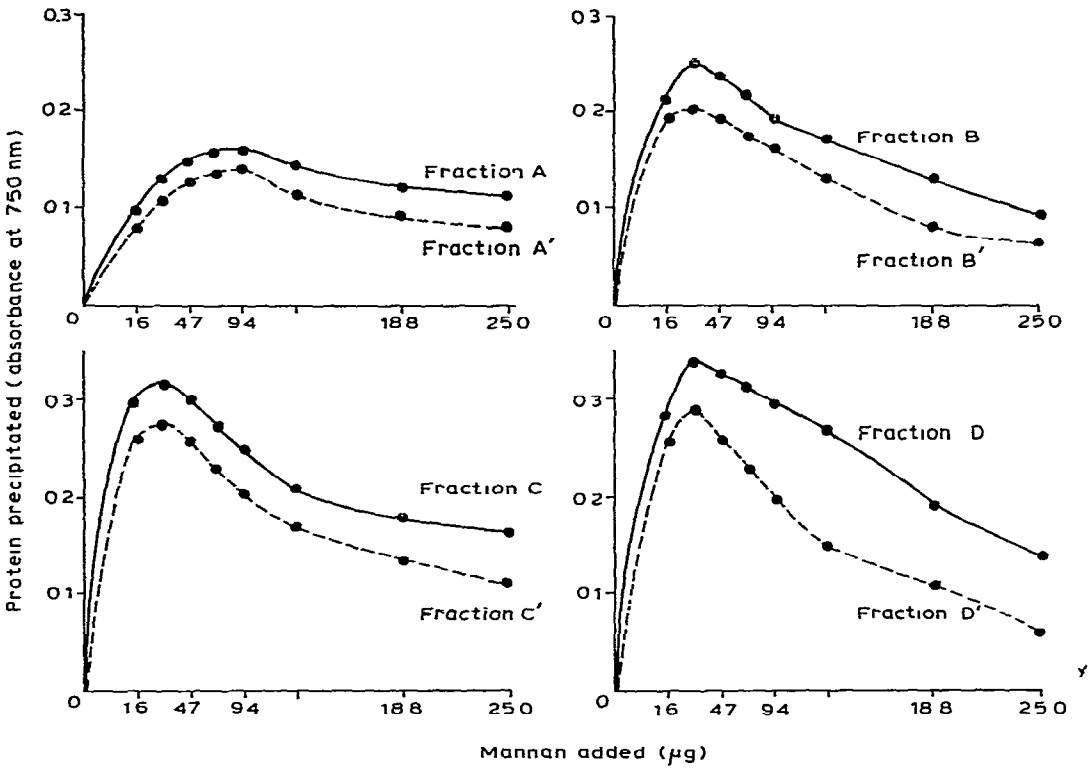

Fig 1. Quantitative precipitation curves of treated and untreated fractions of D-mannan against homologous anti-*S. cerevisiae* serum.

The four borohydride-treated D-mannan fractions were assayed for antibody-precipitating activity against the homologous anti-*S. cerevisiae* serum. As depicted in Fig. 1, each modified D-mannan fraction showed an antibody-precipitating activity slightly lower than that of the corresponding parent D-mannan fractions, indicating that degradation of the polysaccharide compounds occurred simultaneously with the modification reaction.

In order to assess the change in the chemical structure caused by the borohydride reaction, Fractions A', B', C', and D' were analyzed for difference of average length of branches by controlled acetolysis[7] (Table III). This showed a decrease in the average length of branches, especially in the number of the longest branches (Man_4)*, in all modified D-mannans. Decrease in molecular weight was also indicated by the change of S_{20} values (Table I).

These findings suggest that the alkaline borohydride reduction eliminates most

*Abbreviations: Man_4, *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranose; Man_3, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranose; Man_2, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranose; and Man, D-mannose.

TABLE III

MOLECULAR RATIOS OF ACETOLYSIS PRODUCTS OF TREATED AND UNTREATED FRACTIONS OF D-MANNAN PRODUCTS

Fraction	*Molecular ratios of acetolysis products*			
	Man	Man_2	Man_3	Man_4[a]
A	2.6	3.9	3.7	1.0
A′	9.2	5.8	3.3	1.0
B	4.6	6.2	4.7	1.0
B′	7.7	9.5	5.8	1.0
C	3.1	4.0	2.0	1.0
C′	5.4	7.6	3.0	1.0
D	2.4	2.7	1.7	1.0
D′	3.2	3.9	2.1	1.0

[a]The molecular ratios are expressed relative to Man_4 taken as unity

of the peptide component in yeast D-mannan, but also partially degrades the α-D-mannopyranosyl units from the branch chains to yield D-mannans containing shorter branch-chains. Also, the phosphate groups, which are assumed to exist as a monoester type in the parent D-mannan[6], are unexpectedly resistant to the borohydride reaction.

EXPERIMENTAL

General methods. — Specific rotations were determined for solutions in a 1-dm semimicrotube with an Applied Electric Automatic Polarimeter. Ultracentrifugal analyses were performed with a Hungarian Optical MOM 3170B ultracentrifuge. The content of carbohydrate and phosphate was determined with the phenol–sulfuric acid reagent[8], and by the Ames and Dubin method[9], respectively. Total protein content was determined by the Folin method of Lowry *et al.*[10]. Total hexosamine content was determined by the method of Elson and Morgan[11]. Amino acid residues were determined by the following method: Each D-mannan or modified D-mannan fraction (50 mg) was kept in 6M constant-boiling-point hydrochloric acid (2 mL) for 12 h at 110°. The solution was evaporated to dryness, and the residue dissolved in distilled water (2 mL). Some insoluble material was filtered off (Toyo filter paper No. 2), and the amino acid residues were analyzed with a Hitachi KLA-3B amino acid analyser.

Materials. — The fresh, whole cells of bakers' yeast (*Saccharomyces cerevisiae*) were supplied by Oriental Yeast Co., Ltd., Tokyo, Japan.

Preparation of the bulk D-mannan from bakers' yeast. — The method used to prepare the bulk D-mannan was similar to that followed in the preceding study[7].

Chromatographic fractionation of the bulk D-mannan. — A solution of the bulk

D-mannan (3 g) in water (50 mL) was applied to a column (8 × 100 cm) of DEAE-Sephadex A-50 (AcO^-), and the elution was performed stepwise, successively with water, 0.05, 0.1, and 0.25M sodium chloride solution to give fractions A, B, C, and D. The flow rate was 0.5 mL/min, and a 0.1-mL sample of each fraction was analyzed for carbohydrate content with the phenol–sulfuric acid reagent[8]. Each eluate was evaporated *in vacuo* and the residue dissolved in a minimum amount of water. The solution was dialyzed against running tap-water for 48 h, concentrated to 50 mL, and then poured into absolute ethanol containing 0.1% of sodium acetate (200 mL). The precipitate was centrifuged off at 3000 r.p.m. for 10 min, washed with absolute ethanol, and dried *in vacuo* in the presence of phosphorus pentaoxide (yield: 0.23, 0.98, 1.41, and 0.61 g, respectively).

Alkaline sodium borohydride reduction of D-*mannan fractions.* — Alkaline sodium borohydride reduction of D-mannan fractions was performed as follows[6]. A solution of each fraction (1.2 g) in M sodium hydroxide (100 mL) containing sodium borohydride (3.8 g) was heated for 8 h at 98°. The mixture was made neutral with acetic acid, and evaporated *in vacuo*. A solution of the residue in water (50 mL) was applied to a column of Amberlite IR-120 (H^+) cation-exchange resin, and the eluate was evaporated *in vacuo*. Methanol (50 mL) was added and evaporated. A solution of the residue in water (20 mL) was poured into ethanol containing 0.1% of sodium acetate (100 mL) and the precipitate was centrifuged off at 3000 r.p m. for 10 min, washed with absolute ethanol, and dried *in vacuo* in the presence of phosphorus pentaoxide, to give the modified D-mannan fractions A′, B′, C′, and D′ (yield: 1.05, 0.99, 0.82, and 0.78 g, or 88, 82, 68, and 65%, respectively).

Acetolysis of D-*mannan fractions.* — The acetolysis of the D-mannan fractions was performed as described in the preceding study[1]. The resulting *O*-deacetylated manno-oligosaccharide mixture was fractionated on a column (3 × 100 cm) of Bio-Gel P-2. The four peaks of neutral carbohydrate in the elution profile of each of the four acetolyzates were identified by t.l.c. with authentic specimens as Man_4, Man_3, Man_2 and Man, respectively[7]. The proportions of each component for each D-mannan fraction was calculated from the dimensions of the peak in the elution profile, and then converted into a molecular ratio.

Calculation of average length of branches of D-*mannan.* — The average length of branches of D-mannan X, was calculated using the formula[12] (*1*), where *A*, *B*, *C*, and *D* represent the molecular proportion of Man, Man_2, Man_3, and Man_4, respectively.

$$X = (A \times 1) + (B \times 2) + (C \times 3) + (D \times 4)/A + B + C + D \qquad (1)$$

Immunological assay. — An antiserum was prepared by immunizing a rabbit with heat-killed, whole cells of bakers' yeast. The antiserum had an agglutinin titer of 1:2560 against the immunizing cell suspension, and showed a high precipitin-activity against the homologous yeast D-mannan. The quantitative precipitin reaction was performed according to the method of Okubo *et al*[1]. The amount of protein precipitated was determined by the Folin method, according to Lowry *et al.*[10].

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