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FRACTIONAL PRECIPITATION OF D-MANNAN FROM BAKERS' YEAST WITH CONCANAVALIN A*

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ABSTRACT

The neutral component of D-mannan of bakers' yeast *(Saccharomyces cerevisiae)*, consisting solely of D-mannose residues, was precipitated with concanavalin A to give four fractions. The first three displayed similar reactivities in quantitative precipitin reaction against concanavalin A and homologous anti-*S. cerevisiae* serum, but the fourth showed different precipitin curves. Analysis of the fractions by acetolysis indicated structural differences. The different behavior of the last fraction in precipitin reactions could be due to a lower content of branching points, or to shorter chain-lengths.

INTRODUCTION

In recent years, lectins have been increasingly used in fractionating complex carbohydrates. Lloyd[1] reported the separation of yeast D-mannan and bacterial dextran on a concanavalin A–Sepharose 4B column, whereas Torii and Misaki[2] employed concanavalin A in solution for the separation. Therefore, we attempted the fractionation of the neutral component of D-mannan of bakers' yeast *(Saccharomyces cerevisiae)*, by precipitation with concanavalin A.

RESULTS AND DISCUSSION

In order to establish the maximum precipitation of D-mannan with concanavalin A, the quantitative precipitin reaction was investigated by the previously described procedure[3] (see Fig. 1).

The equivalent points for the precipitation of protein and carbohydrate correspond to 150 and 500 μg of D-mannan, respectively. The maximum recovery of precipitated D-mannan (84.0%) corresponds to that of the curve of protein recovery. The ratio of D-mannan to protein in the precipitate obtained at this point was 1 : 5.76 on a weight basis, or a recovery of 78.0 and 99.8%, respectively. Therefore,

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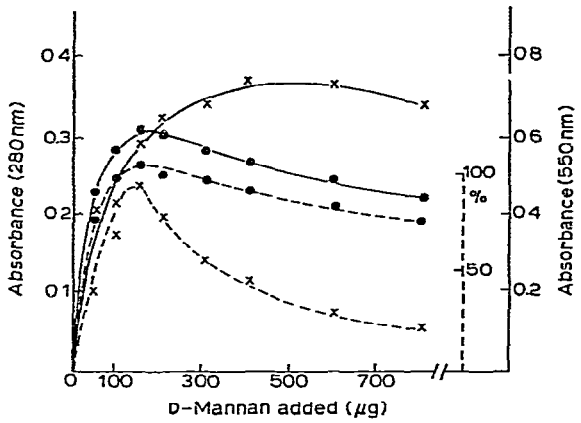

Fig. 1. Preliminary, quantitative precipitin-reaction between concanavalin A and D-mannan of *S. cerevisiae*: —●—, protein, —×— carbohydrate, —●— protein yield, and —×— carbohydrate yield.

concanavalin A is a specific precipitating agent for D-mannan, giving a good recovery.

Based on the results of a preliminary precipitin study, in which the D-mannan: concanavalin A ratio at equivalent point was 1.5:9.0, a 1:4.5 ratio of D-mannan to concanavalin A was used for large-scale fractionation. At this antigen excess concentration, one-fifth of D-mannan possessing lesser reactivity was expected to remain in the supernatant of the reaction mixture.

Thus, a fractional precipitation of D-mannan with concanavalin A gave four subfractions (Fraction I, II, III, and IV). The yields of these fractions ranged from 17 to 23%, and the total recovery was 80%. Dissociation of Fractions I, II, and III from the corresponding specific precipitates with either D-mannan or methyl α-D-mannopyranoside was unsuccessful, due to the strong affinity between the mannans and concanavalin A. All four mannan preparations were found to be pure polysaccharides with nearly identical specific optical rotations (see Table I). The intact

TABLE I

CHEMICAL COMPOSITION OF ORIGINAL D-MANNAN AND OF FRACTIONS OBTAINED WITH CONCANAVALIN A

Fractions	*Total carbohydrate* (%)[a]	*Total protein* (%)[b]	$[\alpha]_D^{20}$ (°)[c]
Original D-mannan	99.0	0.0	+82
Fraction I	94.1	0 0	+80
Fraction II	97.3	0.0	+82
Fraction III	98 3	0.0	+83
Fraction IV	99.6	0.0	+81

[a]Determined by the modified Molisch method[10]. [b]Determined by the modified Lowry–Folin method[11]. [c]In water, *c* 1.0.

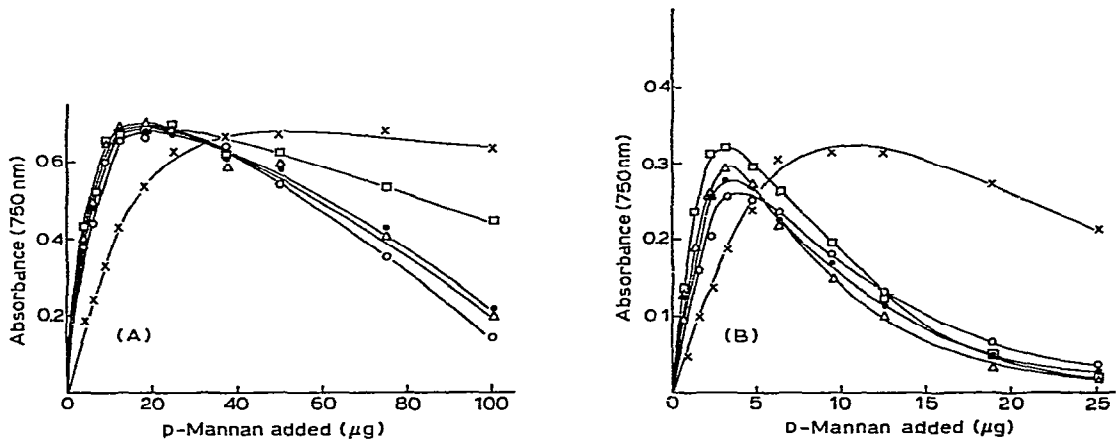

Fig. 2. Quantitative precipitation curves of intact D-mannans and derived subfractions with concanavalin A (A), and anti-*S. cerevisiae* serum (B): —●— original D-mannan, —○— Fr. I, —△— Fr. II, —□— Fr. III, and —×— Fr. IV.

D-mannan, and Fractions I, II, and III gave similar curves in both quantitative precipitin reactions with concanavalin A and anti-*S. cerevisiae* serum (see Fig. 2). On the other hand, Fraction IV gave precipitin curves different from those of all other D-mannans, *i.e.*, the equivalent points shifted largely to the antigen-excess regions in both reaction systems, and the overall shapes of the curves were more gently sloped than those of the other fractions. The differences in branching of the mannan fractions were analyzed by controlled acetolysis[4]. The oligosaccharide mixtures obtained after deacetylation were separated on a column of Bio-Gel P-2 to give the corresponding acetolysis fingerprints (see Fig. 3-A, -B, -C, -D, and -E). The molecular ratio of oligosaccharides and mannose in the acetolysis product from Fraction I is $Man_4 < Man_3 < Man_2 \fallingdotseq Man$*, and the ratios of Fractions II and III are identical ($Man_4 < Man_3 < Man_2 > Man$). Fraction IV, however, was found to possess a significantly different molecular ratio ($Man_4 < Man_3 \ll Man_2 < Man$), the acetolyzate containing a considerably larger amount of Man, whereas the content of Man_3 was quite low (see Table II).

Recently, Torii and Misaki[2] succeeded in fractionating a dextran B-1397 preparation with a concanavalin A solution and reported eight subfractions possessing different precipitin activities against both concanavalin A and the corresponding antiserum. Sakakibara *et al.*[5] further described a fractional precipitation procedure of dextran B-1397 and B-512 with an anti-dextran antibody partially purified by

*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; Man, D-mannose.

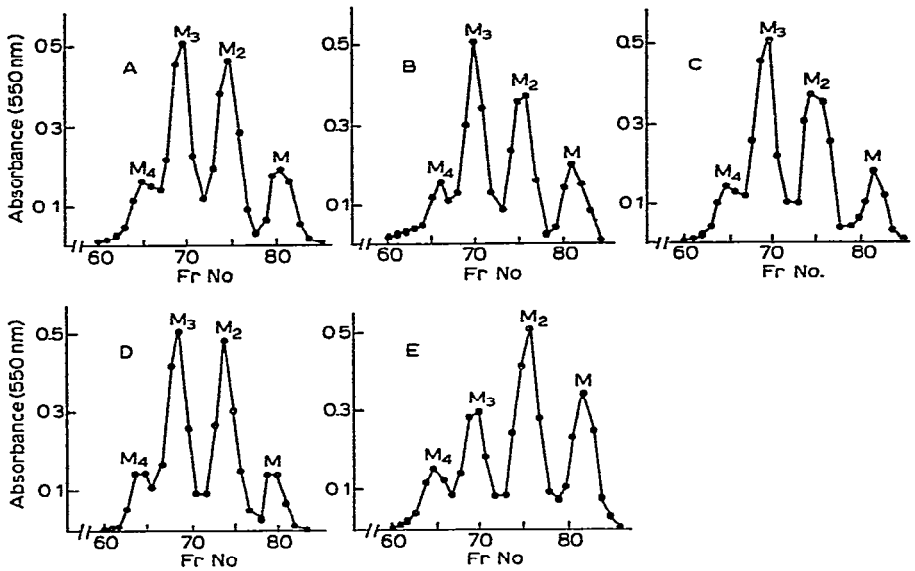

Fig. 3. Acetolysis fingerprints of D-mannan and derived subfractions on a Bio-Gel P-2 column: A, D-mannan; B, Fr. I; C, Fr. II; D, Fr. III; and E, Fr. IV.

TABLE II

MOLECULAR RATIOS OF D-MANNO-OLIGOSACCHARIDES AND D-MANNOSE PRODUCED BY ACETOLYSIS OF ORIGINAL D-MANNAN AND DERIVED SUBFRACTIONS

Fractions	*Man*$_4$[a]	*Man*$_3$	*Man*$_2$	*Man*
Original D-mannan	1	4.2	5.9	5.0
Fraction I	1	4.4	5.8	5.9
Fraction II	1	4 9	5.2	4.6
Fraction III	1	5.3	7.7	4.3
Fraction IV	1	3.0	7.2	9.1

[a]The molecular ratios are expressed with Man_4 as unity.

specific absorption on a column of Sephadex G-75. The D-mannan of *S. cerevisiae* has been found[6] to be strongly reactive against concanavalin A and is capable of precipitating up to 5.76-fold of concanavalin A by weight basis (Fig. 1). This could be due to the existence of a large amount of branching moieties corresponding to Man_4 and Man_3 in this polysaccharide. Therefore, the different behavior of Fraction IV in quantitative precipitin reactions may be due to its shorter average-length and to a lower density of the branches. In order to obtain additional information on the structure of the four D-mannan fractions, a series of precipitin-inhibition assays of the D-mannan–anti-*S. cerevisiae* serum systems with Man_4, Man_3, Man_2, and Man was also performed. However, all precipitin inhibition patterns were similar.

From the results obtained in the earlier[2,3,5] and present study reported by us, it may be concluded that many polysaccharides can be resolved into corresponding fractions having varying precipitin activities by fractional precipitation with a specifically precipitating protein, which indicates structural differences of the active sites or antigenic determinants.

EXPERIMENTAL

General method. — Specific rotations were determined in 1-dm, semimicro tubes with an Applied Electric automatic polarimeter.

Materials. — The neutral D-mannan from bakers' yeast was prepared according to the method of Sakaguchi *et al.*[7]. An aqueous solution of the bulk D-mannan obtained from whole cells of bakers' yeast (Oriental Yeast Co. Ltd., Tokyo) was repeatedly passed through a column of DEAE-Sephadex (A-50, AcO^-) to remove the acidic D-mannan(s) containing phosphorus and nitrogen. The D-mannan preparation, $[\alpha]_D^{20}$ +82.0° (*c* 1.0, water), was completely free from glucose, nitrogen, or phosphorus, and was homogeneous in ultracentrifugal analysis showing an S_{20} value of 3.0. D-Mannotetraose (Man_4) $[\alpha]_D^{20}$ +65.7° (*c* 1.0, water), D-mannotriose (Man_3) $[\alpha]_D^{20}$ +62.0° (*c* 1.0, water), and D-mannobiose (Man_2) $[\alpha]_D^{20}$ +60.1° (*c* 1.0, water), were prepared by acetolysis of the parent D-mannan[8].

Anti-S. cerevisiae whole-cell serum. — This serum was prepared by immunizing a rabbit with heat-killed, whole-cells of bakers' yeast[8]. It had an agglutinin titer of 1:2560 against the immunizing cell-suspension, and showed a high precipitin-activity against the homologous yeast D-mannan.

Concanavalin A. — Concanavalin A (2 × crystallized, carbohydrate-free) was a commercial product of Miles-Yeda Ltd., Israel, and was used as a solution in phosphate-buffered saline (140mM NaCl, 3mM KH_2PO_4, and 7mM Na_2HPO_4, pH 7.2) (PBS). The determination of concanavalin A concentration was based on the value of $A_{1cm}^{1\%}$ 13.0 at 280 nm in M NaOH according to the manufacturer's manual, although the $A_{1cm}^{1\%}$ value for this lectin at the same wave length in 0.1M NaOH has been reported[9] to be 12.7 ± 0.1.

Fractional precipitation of yeast D-mannan with concanavalin A. — To a solution of the D-mannan (120 mg) in PBS (600 ml) was added a 0.1% solution of concanavalin A in PBS (180 ml), and the mixture was kept for 48 h at 4°. The precipitate was collected by centrifugation at 14,000 r.p.m. for 30 min, washed carefully with chilled PBS (100 ml), and dissolved in a minimum amount of 2M NaOH. To this clear solution was added a 20% aqueous solution of trichloroacetic acid (~15 ml) until no more precipitate occurred. The cloudy suspension was centrifuged at 3000 r.p.m. for 15 min, and the clear supernatant solution was neutralized by addition of 40% NaOH. The solution was dialyzed against running tap water, and then against distilled water. After concentration of the solution *in vacuo*, the mannan was obtained by the addition of a large amount of abs. ethanol. It was washed thoroughly with abs. ethanol, and then dried over P_2O_5 *in vacuo* (Fraction I, 20.5 mg, 17.1%). The

supernatant solution of the centrifugation at 14,000 r.p.m. was treated with an equal volume of concanavalin A solution and processed, as described for the isolation of Fraction I, to give Fraction II (yield, 27.8 mg, 23.1%). The third fractional precipitation with the same procedure gave Fraction III (yield 21.0 mg, 17.5%). Finally, the remaining supernatant solution was concentrated to 20 ml, deproteinized with 20% trichloroacetic acid (15 ml) and 40% NaOH, and dialyzed to recover unprecipitated D-mannan (Fraction IV, yield 24.2 mg, 20.2%). The total recovery of D-mannan fractions was 77.9%.

Acetolysis of D-*mannan on a microscale.* — This was performed according to the procedure of Kocourek and Ballou[4] modified as follows: D-mannan (10 mg) was suspended in anhydrous formamide (500 μl) and the mixture was heated on a steam-bath until the polysaccharide was completely dissolved. Pyridine (500 μl) and acetic anhydride (500 μl) were added to the cooled solution which was kept for 12 h at 40°. The light-brown solution was concentrated *in vacuo* below 40°, and the last traces of the solvent were removed in a high vacuum. The light-brown residue was treated with acetic anhydride (500 μl), and then with 50:1 (v/v) acetic acid–conc. sulfuric acid (0.51 ml). The solution was kept for 12 h at 40°, and then neutralized with pyridine (250 μl), and evaporated on a rotary evaporator at 50°. The residue was dissolved in 1:1 (v/v) water–chloroform (50 ml).

The aqueous layer, after separation from the chloroform, was extracted several times with chloroform (25 ml) until the carbohydrate reaction was no longer positive with the 1-naphthol–sulfuric acid reagent. The combined chloroform extracts were washed with water (20 ml), dried (sodium sulfate), and evaporated to dryness. The residue was dissolved in methanol (2 ml), and to this solution was added, dropwise, a 0.5M methanolic sodium methoxide solution until the deacetylated product precipitated. After ~20 min at room temperature, the mixture was neutralized by careful addition of 50% acetic acid. The solution was evaporated to dryness *in vacuo*, and the residue was dissolved in water (2 ml). The solution was passed through a column of Amberlite IR-120 (H^+) and IR-410 (OH^-). The eluate was concentrated to a small volume *in vacuo*, and applied to a column (0.8 × 190 cm) of Bio-Gel P-2. Fractions of 1.0 ml each were collected, and the carbohydrate content was determined by the modified Molisch method[10]. Oligosaccharides in the eluate were identified with authentic Man_4, Man_3, Man_2, and Man by t.l.c. (Kiesel Gel G plate, 5:3:2, v/v, butanol–ethanol–water).

Quantitative precipitin reaction. — In order to assess the reactivity between the D-mannan and concanavalin A, both the precipitated protein and D-mannan were determined by the following procedure: To each tube (1.6 × 10.4 cm) containing a known quantity of D-mannan in PBS (1.0 ml), was added a 0.1% PBS solution of concanavalin A (1.0 ml). The mixture was kept for 12 h at room temperature and then centrifuged at 2500 r.p.m. for 12 min. The precipitate was washed carefully twice with ice-cold PBS (1.0 ml each), and then dissolved in M NaOH (4.0 ml). The concentration of the protein was determined at 280 nm. For the determination of the amount of D-mannan in each precipitate, the alkaline solution (1.0 ml) was mixed

with the 1-naphthol–sulfuric acid reagent (2.5 ml). The color developed was determined at 550 nm and D-mannose was used as the standard. The quantitative precipitin assay using anti-*S. cerevisiae* serum on a relatively smaller scale was performed according to the procedure of Sunayama[11].

Chemical analysis. — Total carbohydrate and protein contents were determined by the methods of Devor[10], and Lowry and Folin, as modified by Eggstein and Kreutz[12], respectively.

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REFERENCES

1 K. O. Lloyd, *Arch. Biochem. Biophys.*, 137 (1970) 460–468.
2 M. Torii, K. Sakakibara, B. P. Alberto, and A. Misaki, *Biochem. Biophys. Res. Commun.*, 72 (1976) 236–242.
3 S. Suzuki, H. Hatsukaiwa, H. Sunayama, and N. Honda, *Jpn. J. Microbiol.*, 15 (1971) 437–442.
4 J. Kocourek and C. E. Ballou, *J. Bacteriol.*, 100 (1969) 1175–1181.
5 K. Sakakibara, M. Torii, B. P. Alberto, and A. Misaki, *Biochem. Biophys. Res. Commun.*, 73 (1976) 741–750.
6 L. L. So and I. J. Goldstein, *J. Biol. Chem.*, 243 (1968) 2003–2007.
7 O. Sakaguchi, S. Suzuki, M. Suzuki, and H. Sunayama, *Jpn. J. Microbiol.*, 11 (1967) 119–128.
8 S. Suzuki, H. Sunayama, and T. Saito, *Jpn. J. Microbiol.*, 12 (1968) 19–24.
9 B. B. L. Agrawal and I. J. Goldstein, *Arch. Biochem. Biophys.*, 124 (1968) 218–229.
10 A. W. Devor, *Anal. Chem.*, 24 (1948) 1626.
11 H. Sunayama, *Jpn. J. Microbiol.*, 14 (1970) 27–39.
12 M. Eggstein and F. H. Kreutz, *Klin. Wochenschr.*, 33 (1955) 879–884.