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GROWTH-INHIBITORY ACTIVITY OF THE D-MANNAN OF *Saccharomyces cerevisiae* X2180-1A-5 MUTANT STRAIN AGAINST MOUSE-IMPLANTED SARCOMA 180 AND EHRLICH-CARCINOMA SOLID TUMOR

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ABSTRACT

The D-mannan of *Saccharomyces cerevisiae* X2180-1A-5 mutant strain, which possesses a main chain composed of α-(1→6) linked D-mannopyranosyl residues and a small proportion of branches composed of α-(1→2)- and α-(1→3)-linked D-mannopyranosyl residues, showed strong growth-inhibitory activity against mouse-implanted Sarcoma 180 and Ehrlich-carcinoma solid tumor The observation that the level of this activity was nearly identical with that of the D-mannan of a wild-type strain of bakers' yeast, which possesses a high proportion of branches composed of α-(1→2)- and α-(1→3)-linked D-mannopyranosyl residues, suggests that the branches are not essential for antitumor activity The partial acid-degradation products of both D-mannans, the molecular weight of which was one-third of that of each parent D-mannan, had only one half of the antitumor activity of the parent D-mannans This suggests that molecular size is the most important factor for the differences in activity of the polysaccharides of wild and mutant strains

INTRODUCTION

Various polysaccharides have been shown to be growth-inhibitory against mouse-implanted, allogeneic tumors[1,2] The D-mannans of several species of yeasts have also been shown to manifest similar antitumor activity[3] For understanding of the mechanism of antitumor activity of these polysaccharides, which was designated by Nakahara *et al* [4] as the "host-mediated defence", we have studied the immunological properties of the D-mannan of bakers' yeast (wild-type strain of *Saccharomyces cerevisiae*). The results clearly indicate that this polysaccharide is able to exert adjuvant action enhancing both humoral and cellular antibody responses to the host animal[5]. However, the relationship between chemical structure and antitumor activity of this D-mannan was not investigated, although the structure consists of a main chain of α-(1→6)-linked D-mannopyranosyl residues and of a large proportion of branches consisting of α-(1→2)- and α-(1→3)-linked D-mannopyranosyl residues[6,7]

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In order to analyze the active site(s) of the antitumor activity in bakers' yeast D-mannan, a comparative study of the D-mannan of *S cerevisiae* X2180-1A-5 mutant strain (*M*-D-mannan), developed by Raschke *et al.*[6], was of interest, because this D-mannan possesses an extremely small proportion of branches, as compared with the D-mannan of *S. cerevisiae* wild-type strain (*W*-D-mannan)

RESULTS AND DISCUSSION

Many polysaccharides of plant and microbial origins show growth-inhibitory activity against mouse-implanted tumors, *e g*, Sarcoma 180, Sarcoma 37, adenocarcinoma, etc [8-10]. However, the chemical structure of the active site of antitumor activity in these polysaccharides has not been elucidated

The *W*-D-mannan of bakers' yeast is an antitumor polysaccharide, the chemical structure of which has been extensively investigated[7]; it is highly branched Therefore, it may be assumed that the nonreducing terminal groups of the branches occupying the most external part of the molecule correspond to the active site(s) of various biological activities, in a manner similar to that observed for the serological activity of the *W*-D-mannan[7]

The *W*-D-mannan used in the present study is the same as that used earlier[11]. The *M*-D-mannan was prepared from fresh, wet, whole cells by the same method as that used for the *W*-D-mannan Neither phosphorus nor nitrogen was detected in either D-mannan (Table I). Their chemical structures were determined by partial acetolysis, and the molecular ratios of the resultant D-manno-oligosaccharides and D-mannose were calculated from the peak dimensions of each elution profile From these results, it is evident that the *W*-D-mannan possesses a large proportion of branches (average d p 2 3), whereas the proportion of branches (average d p 1 1) of the *M*-D-mannan is relatively small

TABLE I

CHEMICAL COMPOSITIONS AND PROPERTIES OF THE D-MANNANS OF *S cerevisiae* WILD-TYPE[a] AND MUTANT[b] STRAINS, AND OF THEIR PARTIAL HYDROLYZATES[c]

Composition and properties	W-D-*Mannan*			M-D-*Mannan*	
	Orig	*Fr* W-*I*	*Fr* W-*II*	*Orig*	*Fr* M-*I*
Components					
Sugar (%)	96 0	95 2	94 0	93 0	96 5
P (%)	0 0	0 0	0 0	0 0	0 0
N (%)	0 0	0 0	0 0	0 0	0 0
$[\alpha]_D^{20}$ (degrees)	+80 5	+69 5	+57 5	+62 5	+60 5
D p	201	135	66	149	50
S_{20}	3 2	2 1	1 7	2 2	1 5

[a] *W*-D-Mannan [b] X2180-1A-5 strain, *M*-D-mannan [c] Fractions *W*-I, *W*-II, and *M*-I

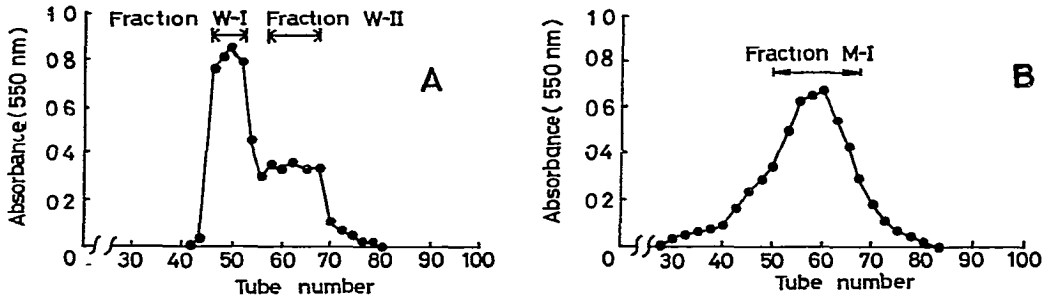

Fig 1 Gel-filtration patterns of partially acid-hydrolyzed *W*- and *M*-D-mannans on a column (4 × 90 cm) of Sephadex G-75, with elution at a rate of 2 mL/15 min and 3 mL/tube A, *W*-D-mannan, B, *M*-D-mannan

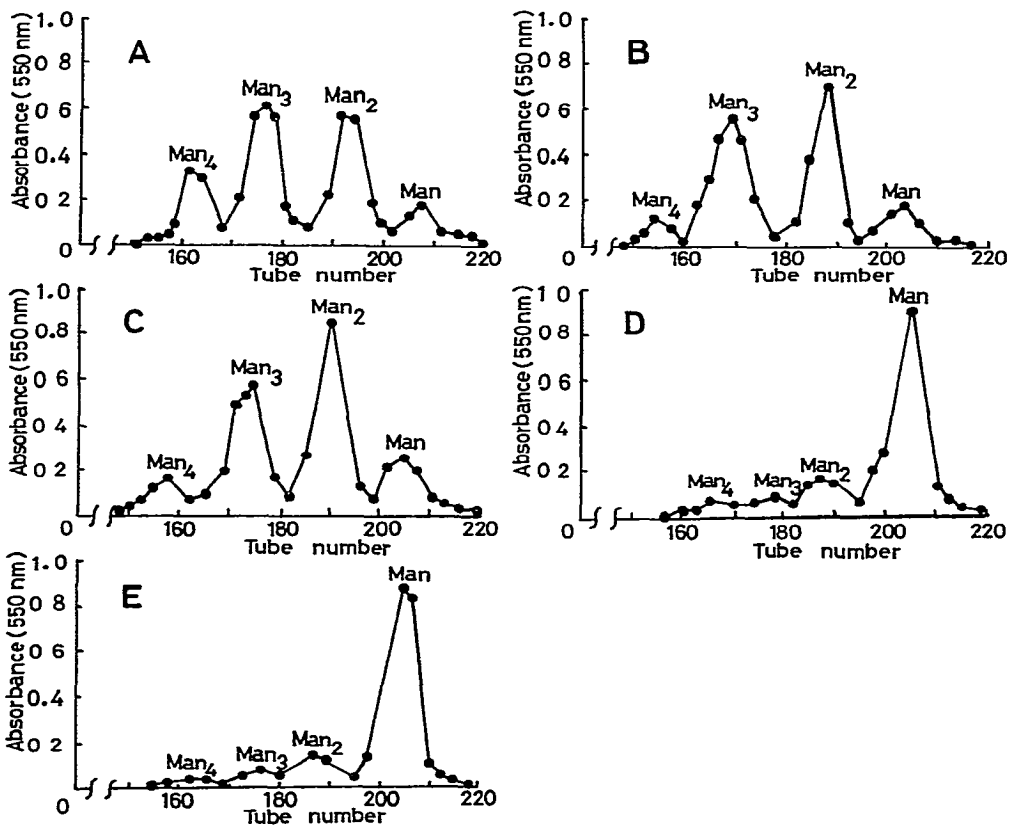

Fig 2 Acetolysis fingerprints of the D-mannan of *S cerevisiae* and of partial acid-hydrolyzate of the fractions obtained by chromatography on a column of Bio-Gel P-2 A, *W*-D-mannan, B, Fraction *W*-I, C, Fraction *W*-II, D, *M*-D-mannan, and E, Fraction *M*-I

TABLE II

MOLECULAR RATIOS AND AVERAGE LENGTH OF BRANCHES OF ACETOLYSIS PRODUCTS OF D-MANNANS OF *S cerevisiae* WILD-TYPE AND MUTANT STRAINS, AND OF THEIR PARTIAL HYDROLYZATES

Property	W-D-*Mannan*			M-D-*Mannan*	
	Orig	*Fr* W-*I*	*Fr* W-*II*	*Orig*	*Fr* M-*I*
Molecular ratios of acetolysis products					
Man Man_4	2 6 1	5 6 1	7 4 1	655 1	1400 1
Man_2 Man_4	3 9 1	14 2 1	9 2 1	41 0 1	109 1
Man_3 Man_4	3 7 1	11 3 1	6 3 1	11 5 1	14 5 1
Average length of branches	2 3	2 2	2 2	1 1	1 1

Both D-mannans were subjected to partial degradation with dilute sulfuric acid, and the resultant hydrolyzates were fractionated by gel chromatography on a column of Sephadex G-75 The hydrolyzate of *W*-D-mannan gave two fractions (Fractions *W*-I and *W*-II), whereas *M*-D-mannan gave one product (Fraction *M*-I) (see Fig 1). The molecular weight of the D-mannans and of their degradation products was obtained by determination of the degree of polymerization according to the method of Hirst *et al* [12], and the sedimentation coefficient of both polysaccharides and of the degradation products was recorded (Table I) The mol wts of Fractions *W*-I and *W*-II were, respectively, about two-thirds and one-third of that of *W*-D-mannan, whereas the mol wt of Fraction *M*-I was about one-third of that of *M*-D-mannan

The differences between the structures of the branches of the two D-mannans and of their partial acid-degradation products were analyzed by partial acetolysis[13] The oligosaccharide mixtures obtained after *O*-deacetylation were separated on a column of Bio-Gel P-2 to give the corresponding acetolysis fingerprints (Fig 2) The D-manno-oligosaccharides contained in each peak were identified by *t l c* using authentic specimens of Man_4*, Man_3, Man_2, and Man It is evident that the molar ratio of D-manno-oligosaccharides (Man_4, Man_3, and Man_2) and Man detected in each acetolyzate of Fractions *W*-I, *W*-II, and *M*-I resemble that of the corresponding D-mannan (Table II)

The results of antitumor assay indicate that both D-mannans show growth-inhibitory activity against mouse-implanted Sarcoma 180 and Ehrlich-carcinoma

*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, and Man_2, *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranose

TABLE III

GROWTH-INHIBITORY ACTIVITY OF *W*- AND *M*-D-MANNAN AND THEIR PARTIAL HYDROLYZATES AGAINST MOUSE-IMPLANTED SARCOMA 180 SOLID TUMOR

Sample	*Dose (mg/kg/day × 10)*	*Average tumor weight (g)*	*Inhibition rat o (%)*	*Complete regression*[a]
Control		12 8		0/9
W-D-Mannan	300	2 0	84	6/10
	200	1 7	87	6/9
	150	0 6	95	8/10
	100	1 9	85	6/10
	50	3 9	69	3/10
Fraction *W*-I	300	3 0	77	5/10
	200	2 8	78	6/10
	150	3 1	76	5/9
	100	3 4	73	4/9
	50	7 2	43	1/10
Fraction *W*-II	300	9 6	30	0/9
	200	8 7	32	1/10
	150	10 7	16	0/10
	100	12 4	3	0/9
	50	13 5	−5	0/8
M-D-Mannan	300	3 1	76	6/9
	200	2 7	79	8/10
	150	2 4	81	8/9
	100	1 8	86	9/10
	50	3 7	71	5/10
Fraction *M*-I	300	9 6	25	0/10
	200	11 4	11	0/8
	150	12 0	6	0/8
	50	10 1	21	0/10

[a]Ratio of number of ddY-strain mice showing complete regression to number of mice tested

solid tumors In terms of growth-inhibition ratio, *W*-D-mannan showed 95 and 79% regression against Sarcoma 180 and Ehrlich carcinoma, respectively, at a dose of 150 mg/kg/day for ten successive days On the other hand, *M*-D-mannan showed 81% regression against Sarcoma 180 at the same dose, and this mannan was also able to display a strong activity, 91% regression, against Ehrlich carcinoma at a dose of 50 mg/kg/day (see Tables III and IV) These results indicate that the antitumor activity of both D-mannans is not dependent on the complexity of the branches, and, consequently, that this activity is far less specific than the antigen–antibody reaction, which distinguishes[7] clearly between the structures of *W*- and *M*-D-mannans Fraction *W*-I (the degradation product of *W*-D-mannan of higher mol. wt) showed lower growth-inhibition ratios than did the parent D-mannan (76 and 67% against

TABLE IV

GROWTH-INHIBITORY ACTIVITY OF *W*- AND *M*-D-MANNAN AND THEIR PARTIAL HYDROLYZATES AGAINST MOUSE-IMPLANTED EHRLICH-CARCINOMA SOLID TUMOR

Sample	*Dose (mg/kg/day × 10)*	*Average tumor weight (g)*	*Inhibition ratio (%)*	*Complete regression*[a]
Control		13 0		0/10
W-D-Mannan	300	3 1	76	5/10
	150	2 7	79	5/9
	100	6 1	53	4/10
	50	13 0	0	0/10
	20	14 8	−14	0/10
	10	18 6	−43	0/9
Fraction *W*-I	300	3 6	72	0/10
	150	4 3	67	3/9
	100	9 5	27	2/9
	50	17 9	−38	0/9
	20	16 3	−25	0/10
	10	17 5	−35	0/9
Fraction *W*-II	300	16 6	−28	0/10
	150	16 4	−26	0/9
	100	17 8	−37	0/9
	50	14 8	−14	0/10
	20	13 2	−2	0/10
	10	15 9	−22	0/10
M-D-Mannan	150	4 5	65	3/9
	100	4 5	65	4/10
	50	1 1	91	8/10
	20	9 5	27	0/10
	10	9 1	30	0/10
Fraction *M*-I	150	7 7	41	0/10
	100	9 1	30	0/10
	50	13 3	−2	0/10

[a]Ratio of number of ddY-strain mice showing complete regression to number of mice tested

Sarcoma 180 and Ehrlich carcinoma, respectively, at a dose of 150 mg/kg/day for ten successive days) On the other hand, Fraction *W*-II (the smaller degradation-product of the same parent D-mannan) showed only borderline antitumor activity Similarly, the degradation product of *M*-D-mannan (Fraction *M*-I) also exhibited inhibition ratios lower than those of the parent D-mannan (6 and 41% against Sarcoma 180 and Ehrlich carcinoma at the same dose, respectively)

Thus, the present study indicates that the presence of a dense network of branches in the parent D-mannan is not essential for antitumor activity, and that the size of the D-mannan molecule is a major factor for this activity

EXPERIMENTAL

General methods — The total carbohydrate content was determined by the modified Molisch method[14] Total phosphorus and nitrogen contents were determined by the method of Allen as modified by Nakamura[15], and by microelementary analysis, respectively The degrees of polymerization of D-mannans and the acid-hydrolyzates were determined by the alkaline iodine method according to Hirst *et al*[12], using 50 mg of each specimen Ultracentrifuge analyses were performed with Hitachi UCA 2-A and MOM 3170B ultracentrifuges at 60 000 r p m All samples were dissolved in water to give 1% (w/v) solutions The specific rotations were measured, with an Applied Electric automatic polarimeter, on solutions (*c* 1 0, water) in 1-dm semimicrotubes Thin-layer chromatography was carried out according to the method described previously[13]

Materials. — The *Saccharomyces cerevisiae* wild-type strain was a commercial product (Oriental Yeast Industries Co Ltd , Tokyo), and the same as that used previously[13] The *S cerevisiae* X2180-1A-5 mutant strain (provided by Dr C E Ballou) was maintained on an agar-slant consisting of 2% of D-glucose, 1% of peptone, 0 1% of yearst extract, and 1 5% of agar D-Manno-oligosaccharides, Man_4, Man_3, and Man_2 were the same specimens as those used previously[13]

S cerevisiae *X2180-1A-5 strain culture* — The mutant strain was inoculated into Sabouraud liquid medium in a 500-mL shaking-flask, and grown for 72 h at 37° under shaking in a reciprocal shaker The cells were harvested by centrifugation, and washed thoroughly with a saline solution by centrifugation The yield of the wet-packed cells was ~12 g/L of medium

Preparation of D-*mannans* — The D-mannans of *S cerevisiae* wild-type and mutant strains were prepared by the Fehling-solution method described previously[11]

Partial-acetolysis study of D-*mannans* — Acetolysis of D-mannans on a micro-scale (samples of 10 mg) was carried out according to the description of Okubo *et al*[11] The *O*-deacetylated acetolysis products were passed through a column (2 × 100 cm) of Bio-Gel P-2, and aliquots of eluates were examined for carbohydrate content with the modified Molisch reagent[14] The oligosaccharides in the eluate were identical with authentic Man_4, Man_3, Man_2, and Man by t l c (Kieselgel G plate, 5 3 2, v/v, 1-butanol–ethanol–water)

Calculation of the average length of branches of D-*mannans* — The average length (X) was calculated by the following formula $X = [(A \times 1) + (B \times 2) + (C \times 3) + (D \times 4)]/[A + B + C + D]$, where A, B, C, and D represent the values of the molecular ratios of Man, Man_2, Man_3, and Man_4, respectively

Preparation of partially acid-hydrolyzed D-*mannan fractions* — To a solution of 165mM sulphuric acid (300 mL) maintained at 97° was added the D-mannan (3 g) of each strain The mixture was heated for 90 min and then neutralized with barium carbonate After centrifugation, the supernatant was concentrated *in vacuo* to a thin syrup, which was chromatographed on a column (4 × 90 cm) of Sephadex G-75, and eluted with water (2 mL during 15 min) Aliquots of the eluates were

examined for carbohydrate content, and the eluates corresponding to each fraction were combined and lyophilized The results of reducing-power assays indicated that the degree of hydrolysis of Fraction *W*-I, *W*-II, and *M*-I was 67 2, 32 8, and 33 6%, respectively

Assay of antitumor activity — This assay was performed according to the method described previously[3] as follows male mice of ddY strain initially weighing $\sim 18 \pm 2$ g were implanted subcutaneously in the left groin with Sarcoma 180 and Ehrlich carcinoma ascites tumors (10^7 cells per mouse), and, 24 h later, each D-mannan fraction in 0 9% sodium chloride solution was injected intraperitoneally at a dose of 10–300 mg/kg/day after tumor inoculation for 10 days The growth-inhibitory ratio was calculated by the following formula inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the average tumor-weight of the control group 30 days after tumor implantation, and B is the average tumor-weight of the D-mannan-administered group

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