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DEXTRAN α-(1→2)-DEBRANCHING ENZYME FROM *Flavobacterium* Sp M-73 PROPERTIES AND MODE OF ACTION

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ABSTRACT

The general properties and specificity of a dextran α-(1→2)-debranching enzyme from *Flavobacterium* have been examined in order to apply this enzyme to the structural analysis of highly branched dextrans The optimum pH range and temperature were pH 5 5–6 5, and 45°, respectively The enzyme was stable up to 40° on heating for 10 min, and over a pH range of 6 5–9 0 on incubation at 4° for 24 h The effects of various metal ions and chemical reagents have also been examined The debranching enzyme has a strict specificity for the (1→2)-α-D-glucosidic linkage at branch points of dextrans and related branched oligosaccharides, and produces D-glucose as the only reducing sugar The degree of hydrolysis of the dextrans by this enzyme and the K_m value (mg/mL) were as follows B-1298 soluble, 25 2%, 0 21, B-1299 soluble, 31 5%, 0 27, and B-1397, 11 8%, 0 91 The debranching enzyme thus has a novel type of specificity as a dextranhydrolase We have termed this enzyme as dextran α-(1→2)-debranching enzyme, and its systematic name is also discussed

INTRODUCTION

A dextran α-(1→2)-debranching enzyme produced by soil bacterium strain M-73 completely hydrolyzes the highly branched dextran elaborated by *Leuconostoc mesenteroides* NRRL B-1299 Two types of enzyme, dextranase I and II, were detected from the strain M-73, and the former has a specificity for dextrans containing a (1→2)-α-D-glucosidic linkage at branch points[1] Our previous paper described the characterization of the strain (tentatively named *Flavobacterium* sp M-73) and purification of the α-(1→2)-debranching enzyme[2] The dextran-debranching enzyme was purified by chromatography on a column of DEAE-cellulose, by affinity chromatography on Sephadex G-150, and by preparative gel-electrophoresis The purified enzyme was electrophoretically homogeneous and had a molecular weight of 125,000

Enzymic analysis of polysaccharide structure is often more useful than non-enzymic analysis, as quantitative results may be obtained more rapidly by simple enzymic procedures. Although several types of dextran-degrading enzyme thus far

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isolated have been used in structural studies of dextrans[3,4], there is no enzyme that can specifically split (1→2)-α-D-glucosidic linkages in a linear or branched structure The present debranching enzyme should thus make a valuable contribution for the structural analysis of highly branched dextrans containing the (1→2)-α-D-glucosidic linkage

The present paper reports detailed examination of the general properties and substrate specificity of the dextran α-(1→2)-debranching enzyme.

MATERIALS AND METHODS

α-D-Glucans — The B-1299 soluble dextran was prepared by a procedure essentially the same as that reported previously[5] Dextran from *Leuconostoc mesenteroides* NRRL B-1307 (B-1307 dextran) was also prepared by the same procedure The B-1298 soluble dextran elaborated by *L mesenteroides* B-1298 was extracted with water from the methanol-precipitated, B-1298 dextran preparation The B-1397 dextran was kindly provided by Prof A Misaki (Osaka City University, Osaka, Japan) Clinical dextran was supplied by Meito Sangyo Co , Ltd , Japan Such other α-D-glucans as *Lentinus* α-(1→3)-glucan[6], pseudonigeran, and mutan[7] IG-1 were available in our laboratory collections

Enzymes — Dextran α-(1→2)-debranching enzyme was prepared by the procedure described in a previous paper[2] Endodextranase from *Chaetomium gracile* was supplied by Sankyo Co , Japan[1] Glucoamylase from *Rhizopus niveus* (2 × crystallized) was purchased from Seikagaku Kogyo Co , Japan

Preparation of α-(1→2)-branched oligosaccharides — α-(1→2)-Branched oligosaccharides were prepared from the B-1299 soluble dextran that had been previously treated with the dextran α-(1→2)-debranching enzyme and then hydrolyzed with endodextranase from *Chaetomium gracile* After fractionation by column chromatography on Bio-Gel P-2, three branched oligosaccharides A, B, and C were obtained The structures of these oligosaccharides were determined to be as follows[8], (A) 2^3-*O*-α-D-glucosyl-isomaltotriose, (B) 2^3-*O*-α-D-glucosyl-isomaltotetraose, (C) 2^3,2^4-di-*O*-α-D-glucosyl-isomaltotetraose

Enzyme assay — The standard method for assay of the dextran α-(1→2)-debranching enzyme has been described in a previous paper[1]

Analytical methods — Throughout this study, total sugar was determined by the phenol–sulfuric acid method[9], and reducing sugar by the Nelson–Somogyi method[10,11], with anhydrous D-glucose as reference standard.

Paper chromatography — Ascending paper chromatography was performed on Toyo No 50 filter paper at room temperature with *A*, 6 4 3 (v/v) 1-butanol–pyridine–water, and *B*, 65% aqueous 1-propanol (v/v) Spots were detected by the alkaline silver nitrate dip-procedure[12]

RESULTS

General properties of the dextran α-(1→2)-debranching enzyme — The proper-

ties of the purified dextran α-(1→2)-debranching enzyme were examined with an electrophoretically homogeneous preparation

The enzyme was most active at pH 6 0 (Fig 1a) and was stable in the range of pH 6 5–9.0 for 24 h at 4° (Fig 1b) Interestingly, the optimum pH for enzyme activity lay just outside the pH-stability region The temperature optimum for enzyme activity was ~45° (Fig 2a) The purified enzyme was stable at temperatures below 40° (Fig 2b)

The enzyme was strongly inhibited by Al^{3+}, Fe^{3+}, Hg^{2+}, and Cu^{2+} (1 33mM), whereas other metal ions had no significant effect on the activity (Table I) Among the chemicals tested, D-glucono-1,5-lactone and sodium dodecyl sulfate strongly inhibited (65%) the enzyme activity, and iodoacetic acid showed 40% inhibition

Substrate specificity and products formed by the debranching enzyme — α-D-Glucans containing various linkages were digested with the debranching enzyme, and the degree of hydrolysis of each substrate was determined The results are summarized

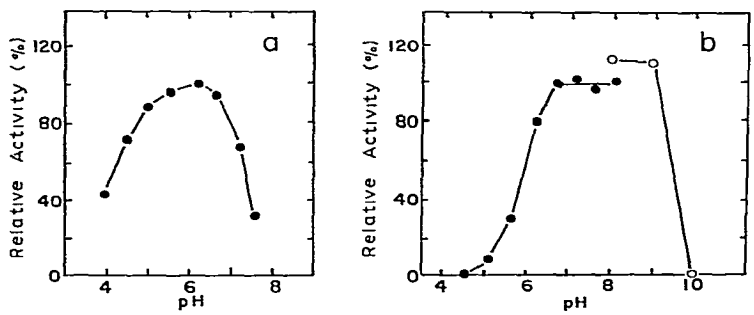

Fig 1 Effects of pH on the dextran α-(1→2)-debranching enzyme (a) pH activity The mixture consisted of the debranching enzyme (suitably diluted with water), 0 025M McIlvaine buffer (adjusted to various pH values as indicated), and 0 25% B-1299 soluble dextran (total volume, 1 0 mL) Incubation was conducted for 10 min at 40° (b) pH stability Aliquots (0 1 mL) of the enzyme solution were mixed with buffer solution (0 05M, 0 1 mL) adjusted to various pH values as indicated and incubated for 24 h at 4° The residual activity was assayed at pH 5 6 under the standard conditions —●—, 0 025M McIlvaine buffer, —○—, 0 025M borate buffer

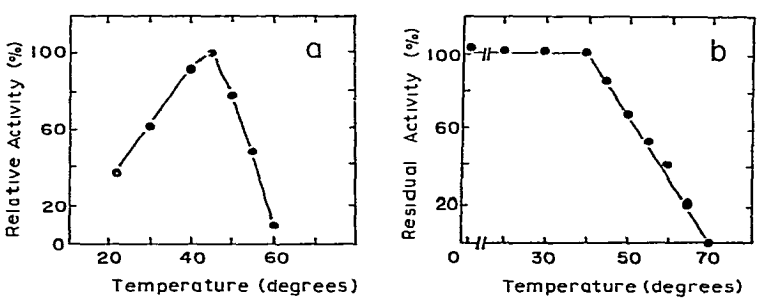

Fig 2 Effects of temperature on the dextran α-(1→2)-debranching enzyme (a) Optimal temperature The enzyme reaction was performed at pH 5 6 for 10 min at various temperatures (b) Thermal stability Aliquots (0 5 mL) of the suitably diluted enzyme solution (pH 5 6) were incubated for 10 min at various temperatures and the residual activity was assayed under the standard conditions

TABLE I

EFFECTS OF METAL IONS AND CHEMICALS ON THE ACTIVITY OF THE DEXTRAN α-(1→2)-DEBRANCHING ENZYME[a]

Compound[b]	*Relative activity (%)*	*Compound or ion*	*Relative activity (%)*
None	100 0	Ca^{2+}	101 4
Al^{3+}	0 9	Mn^{2+}	123 5
Fe^{3+}	2 6	EDTA	80 4
Hg^{2+}	1 2	NaF	99 2
Cu^{2+}	1 7	*p*-Chloromercuribenzoate	93 5
Ni^{2+}	82 0	Iodoacetic acid	60 1
Sr^{2+}	92 4	Phenylmethylsulfonyl fluoride	82 3
Co^{2+}	94 2	Glucono-1,5-lactone	34 2
Zn^{2+}	97 3	Sodium dodecyl sulfate	37 9
Ba^{2+}	98 9	L-Cysteine	102 7
Mg^{2+}	99 6	Guanidine HCl	97 4
Cd^{2+}	100 0	Urea	100 0

[a]The mixture consisted of the debranching enzyme (0 04 U), 0 25% of B-1299 soluble dextran and 0 025M acetate buffer (pH 5 6), and metal ion or chemical at the designated concentration (total volume, 1 0 mL) The enzyme was preincubated for 10 min at 40° with various metal ions or chemical reagents before the substrate was added After the incubation, the residual activity was measured by the standard method [b]Final concentration of metal ions (used in the chloride form) and chemical reagents was 1 33mM, except for *p*-chloromercuribenzoate (0 33mM)

TABLE II

SUBSTRATE SPECIFICITY OF THE DEXTRAN α-(1→2)-DEBRANCHING ENZYME

α-D-*Glucan*	*Major linkage*	*Degree of hydrolysis*[a] *(%)*
B-1299 dextran	α-(1→6),(1→2),(1→3)	31 5
B-1298 dextran	α-(1→6),(1→2),(1→3)	25 2
B-1397 dextran	α-(1→6),(1→3),(1→2)	11 8
B-1307 dextran	α-(1→6),(1→3),(1→4)	0 0
Clinical dextran	α-(1→6),(1→3)	0 0
Soluble starch	α-(1→4),(1→6)	0 0
Glycogen	α-(1→4),(1→6)	0 0
Pseudonigeran	α-(1→3)	0 0
(1→3)-α-D-Glucan (*Lentinus*)	α-(1→3) (1→4)	0 0
Mutan IG-1	α-(1→3),(1→6)	0 0

[a]The mixture (1 0 mL) consisted of the debranching enzyme (0 04 U/mL), 0 25% of each substrate, and 0 025M acetate buffer (pH 5 6) Incubation was performed for 24 h at 40° The percent of hydrolysis was calculated from the ratio of reducing sugar to total sugar in the mixture

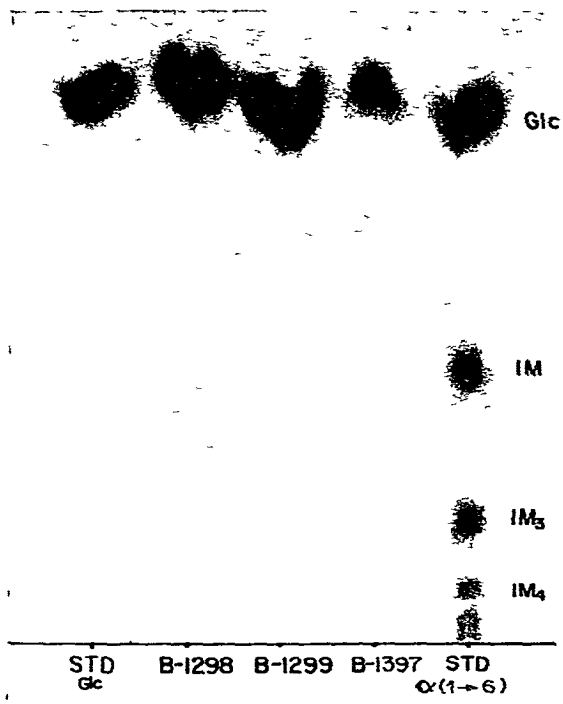

Fig 3 Paper chromatogram of the hydrolyzates of various dextrans, containing (1→2)-α-D-glucosidic linkages, by the dextran α-(1→2)-debranching enzyme The hydrolyzate of each dextran produced by the debranching enzyme was prepared as described in the footnote to Table II Chromatography was effected with solvent system *A* STD Glc, standard glucose, STD α-(1→6), standard α-(1→6)-oligosaccharides, Glc, glucose, IM, isomaltose, IM_3, isomaltotriose, IM_4, isomaltotetraose, B-1298, B-1298 soluble dextran, B-1299, B-1299 soluble dextran, and B-1397, B-1397 dextran

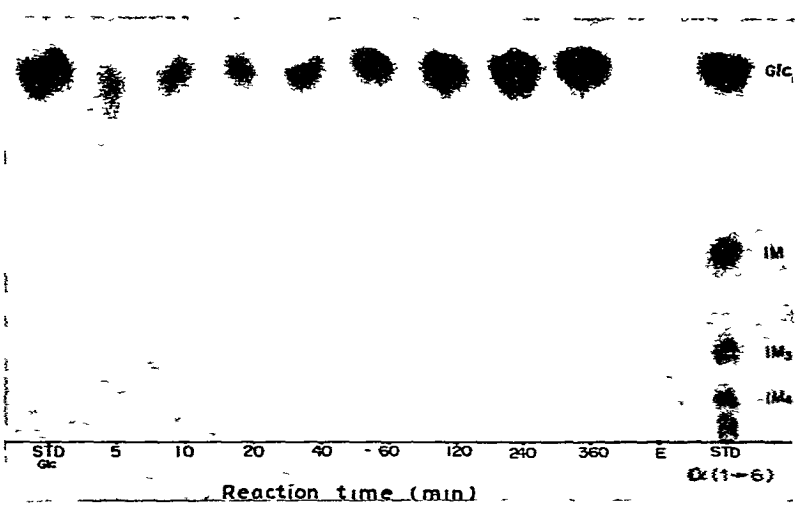

Fig 4 Paper chromatogram of the digest of B-1299 soluble dextran with the dextran α-(1→2)-debranching enzyme The analytical procedure and the abbreviations used are the same as those described in the legend to Fig 3

in Table II Dextrans having the (1→2)-α-D-glucosidic linkage were hydrolyzed by incubation at 40° for 24 h with this enzyme, and the degrees of hydrolysis of the B-1299, B-1298, and B-1397 dextrans were found to be 31 5, 25 2, and 11 8%, respectively Glucans and dextrans having no (1→2)-α-D-linkage in the structure were not, however, hydrolyzed by the enzyme (Table II)

Furthermore, paper chromatography of the hydrolyzates of the B-1298, B-1299, and B-1397 dextrans with the debranching enzyme showed glucose as the sole low-molecular-weight product (Fig 3) This fact was observed from the earliest time of incubation (Fig 4)

Action of the debranching enzyme on dextrans containing the (1→2)-α-D-glucosidic linkage — The effect of substrate concentration and the progress of hydrolysis were examined by using three kinds of dextrans These dextrans contained different proportions of (1→2)-α-D-glucosidic linkages, which mainly constituted branch-points of single D-glucose residues and were partly inter-chain linkages and/or double branch-points[13-17] The Michaelis constants for the B-1298, B-1299, and B-1397 dextrans were 0 21, 0 27, and 0 91 mg/mL, respectively (Fig 5) As shown in Fig 6, the progress of hydrolysis of the B-1298 and B-1397 dextrans reached a maximum at ~6 h after incubation, whereas that of the B-1299 dextran reached a maximum at ~12 h The final degree of hydrolysis was found to be proportional to the content of (1→2)-α-D-glucosidic linkages in each dextran (Table III).

Action of the dextran α-(1→2)-debranching enzyme on α-(1→2)-branched oligosaccharides — Three oligosaccharides used as model substrates had the following branched structures[8], 2^3-*O*-α-D-glucosyl-isomaltotriose (A), 2^3-*O*-α-D-glucosyl-isomaltotetraose (B), and 2^3,2^4-di-*O*-α-D-glucosyl-isomaltotetraose (C) The hydrolysis pattern of these α-(1→2)-branched oligosaccharides by the debranching enzyme is shown in Fig 7 In all instances, glucose was detected clearly as one of the fragments,

TABLE III

EFFECT OF THE CONTENT OF α-(1→2) LINKAGES AND AVERAGE CHAIN-LENGTH IN VARIOUS DEXTRANS ON THE DEXTRAN α-(1→2)-DEBRANCHING ENZYME ACTIVITY

Dextran	*Average chain-length*	*Content of α-(1→2)-linkage at branch point*	*Hydrolysis with debranching enzyme (%)*	*Initial velocity[a] (μg glucose/min)*	Km *(mg/mL)*
B-1298 soluble dextran	3 9[b]	19 2[b]	25 2	5 8	0 21
B-1299 soluble dextran	2 5[c]	34 9[c]	31 5	5 0	0 27
B-1397 dextran	5 2[d]	13 7[d]	11 8	4 0	0 91

[a]Initial velocity determined by the reaction system described in Fig 3 with a concentration of 1 0 mg/mL of dextran [b]Data of Lewis *et al*[16] [c]Data of Seymour *et al*[15] [d]Data of Miyaji *et al*[17]

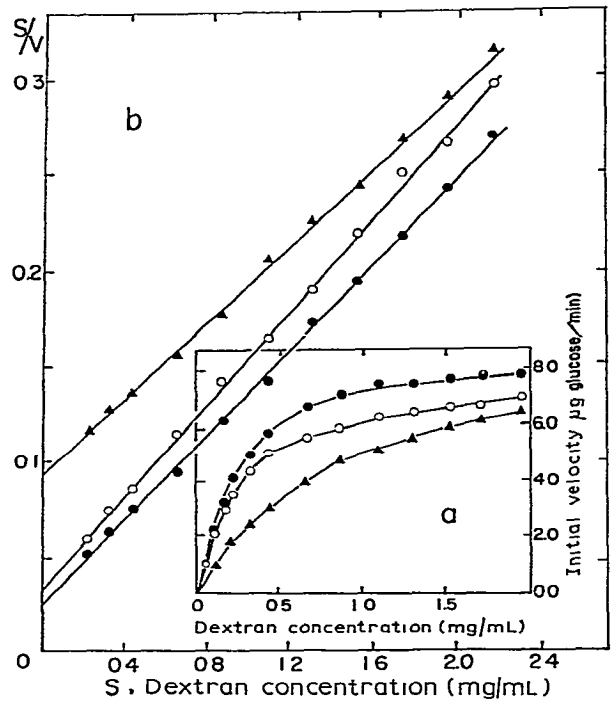

Fig 5 Effects of substrate concentration of various dextrans on the rate of reaction (a) Effect of substrate concentration Three kinds of dextran containing α-(1→2)-glucosidic linkages (various concentrations as indicated) were incubated with the debranching enzyme (0 04 U) for 10 min at 40° The mixture was then assayed for increase in reducing sugar, expressed as μg glucose per min (b) The results from (a) were plotted as S/V against S plots —●—, B-1298 dextran, —○—, B-1299 dextran, and —▲—, B-1397 dextran

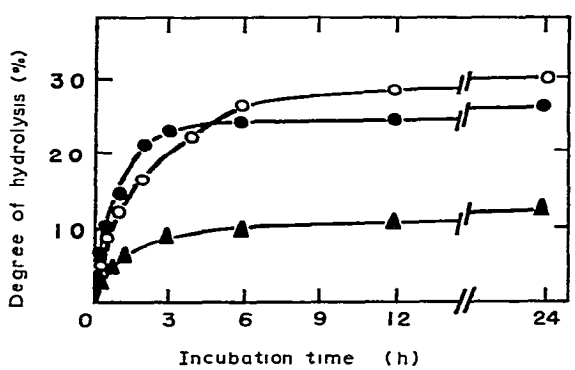

Fig 6. Action of the dextran α-(1→2)-debranching enzyme on various dextrans containing different proportions of α-(1→2)-glucosidic linkages Aliquots (2 mL) of the mixture contained 0 25% of substrate and 0 2 U of enzyme After incubation for 24 h at 40°, an aliquot of the digest was withdrawn to determine the degree (%) of hydrolysis at suitable intervals —●—, B-1298 dextran, —○—, B-1299 dextran, and —▲—, B-1397 dextran

being consistent with the preceding results on the digestion of α-(1→2)-branched dextrans Other fragments detected in the hydrolyzates of oligosaccharides A, B, and C were isomaltotriose (Fig 7, A-2) and isomaltotetraose (B-2 and C-3) Furthermore, a minor fragment (C-2), having almost the same R_F value as oligosaccharide B,

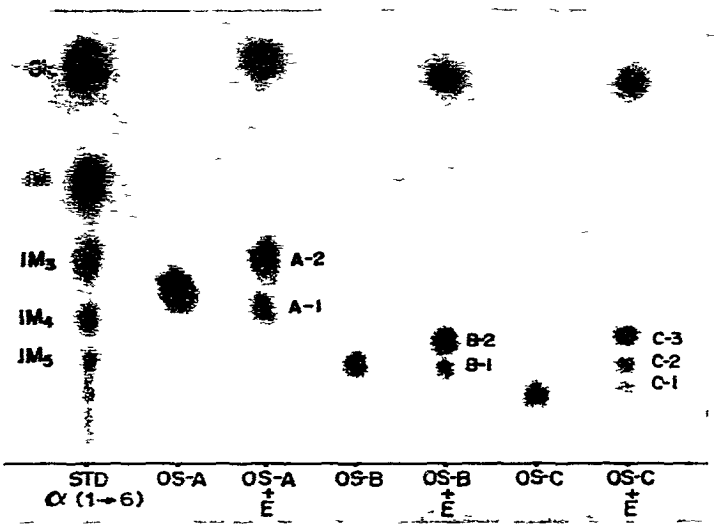

Fig 7 Paper chromatogram of hydrolyzates of α-(1→2)-branched oligosaccharides with the dextran α-(1→2)-debranching enzyme The system was the same as that described in Table II, except that the α-D-glucan was replaced by various branched oligosaccharides Chromatography was effected with solvent system *B* STD α-(1→6), standard α-(1→6) oligosaccharides, as described in the legend to Fig 3, OS-A, 2³-*O*-α-D-glucosyl-isomaltotriose, OS-B, 2³-*O*-α-D-glucosyl-isomaltotetraose, OS-C, 2³,2⁴-di-*O*-α-D-glucosyl-isomaltotetraose, A-1, B-1, and C-1, oligosaccharides remaining after action of the debranching enzyme, and E, debranching enzyme

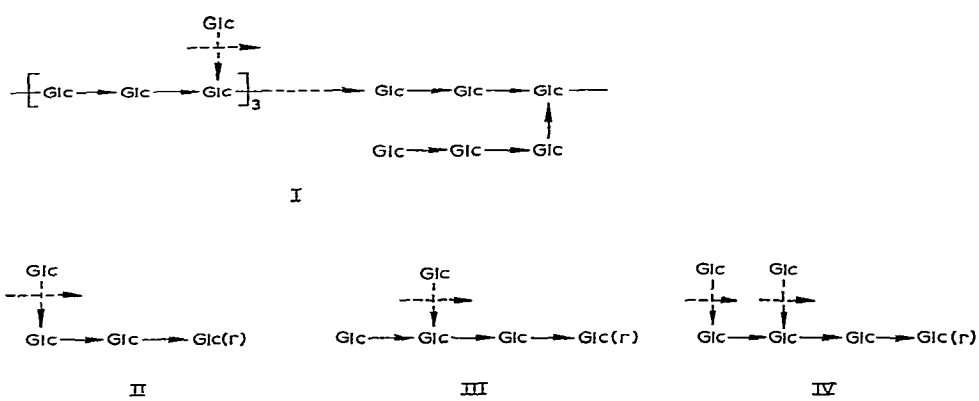

Fig 8 Action of the dextran α-(1→2)-debranching enzyme on B-1397 dextran and α-(1→2)-branched oligosaccharides I, average repeating-unit of B-1397 dextran[17], II, oligosaccharide A, III, oligosaccharide B, IV, oligosaccharide C, Glc, glucosyl residue, →, α-(1→6) linkage, ↓, α-(1→2) linkage, ↑, α-(1→3)-linkage, Glc(r), reducing glucose residue, and —↓—→, site of attack of the debranching enzyme.

was also detected in the digest from oligosaccharide C The fragment C-2 was identified as 2^3-*O*-α-D-glucosyl-isomaltotetraose (B), as its R_F value and hydrolysis pattern by the glucoamylase from *Rhizopus niveus*[18] were similar to those of an authentic sample (data not shown) The remaining spots, A-1, B-1, and C-1, were identified by two-dimensional paper chromatography with solvent system *B* as the original undigested oligosaccharides These results apparently indicate that the debranching enzyme is also specific for the (1→2)-α-D-glucosidic linkage of low-molecular-weight substrates It is noteworthy that a representative debranching activity could be demonstrated by the action of this enzyme on 2^3-α-D-glucosyl-isomaltotetraose (oligosaccharide B). However, this enzyme did not attack such disaccharides as maltose, isomaltose, nigerose, and kojibiose (data not shown) Therefore, it is concluded that the enzyme can split the single branch of a (1→2)-α-D-glucosidic linkage attached to the α-(1→6) backbone of the dextran molecule (Fig 8)

DISCUSSION

The present work has revealed the primary characteristics of the dextran α-(1→2)-debranching enzyme, and the applicability of this enzyme to the structural analysis of highly α-(1→2)-branched dextrans has been suggested The purified debranching enzyme was free from sugars detectable by the phenol–sulfuric acid method The debranching enzyme was stable at a temperature below 40° and was both active and stable over a range of pH about neutrality (Figs 1 and 2) These properties are similar to those of endodextranase from *Penicillium funiculosum*[19] It is noteworthy that almost half of the enzyme-activity was lost when the enzyme solution was kept at the optimum pH (pH 6 0) for 24 h in the absence of substrate

This enzyme could hydrolyze dextrans containing the (1→2)-α-D-glucosidic linkage, and the degree of hydrolysis of such dextrans (dextrans from strains B-1299 and B-1397) increased proportionally with the content of α-(1→2)-glucosidic linkages (Table III) The average repeating-unit of the B-1397 dextran, described by Miyaji and Misaki[17], consists of five glucosyl residues in which a single glucosyl residue occurs as a branch, linked through an α-(1→2) linkage (Fig 8, I) Hydrolysis of the B-1397 dextran with the debranching enzyme released 11 8% of D-glucose This value represents the proportion of α-(1→2) linkages susceptible to the enzyme, and is in close agreement with the value (13 7%) determined by methylation analysis[17], as shown in Table III Thus, the enzyme appeared specific to the α-(1→2)-glucosidic linkage at the branch point

Further studies on the action of the enzyme suggested that it could hydrolyze branched oligosaccharides by specific removal of a side-chain D-glucosyl group linked through an α-(1→2) linkage (Fig 7) The pattern of action of the debranching enzyme, summarized in Fig 8, II, III, and IV, clearly indicates that it has the ability to act on (1→2)-α-D-glucosidic linkages involved in the branch points A small amount of the original oligosaccharides that remained in the digests may arise from the low affinity of the enzyme for substrates of low molecular weight However,

further studies are required to clarify whether or not the enzyme hydrolyzes sequential chains and/or internal chains of α-(1→2) linkages

As shown in Fig 5a, the initial rate of hydrolysis of B-1299 soluble dextran was lower than that of B-1299 dextran The percent of hydrolysis of the former was, however, higher than that of the latter (Fig 6) The B-1397 dextran showed the lowest initial rate and percent of hydrolysis Moreover, K_m values for the three types of dextrans (Fig 5b) indicated that the affinity of the enzyme for the substrate corresponded to the initial rate of hydrolysis (Table III) For the B-1299 dextran, the initial rate of hydrolysis seemed to be lowered because of restriction of hydrolysis by the rather high proportion of branches, as indicated by the occurrence of adjacent branches

Whether or not the debranching enzyme requires the presence of α-(1→6)-linked D-glucosyl residues in the substrate still remains to be clarified, as substrates of high molecular weight containing solely of α-(1→2)-linkages are unavailable However, neither kojibiose nor any other α-linked glucobiose was susceptible to this enzyme Furthermore, we have previously suggested that this enzyme may have a particular subsite that interacts strongly with the α-(1→6)-linkage of the dextran[2] Thus, this enzyme seems to fall under the category of a debranching enzyme, and an α-(1→6)-linked skeletal chain appears to be necessary for recognition of the branch point in the substrate For the reasons already described, the systematic name dextran 2-glucohydrolase would be preferable for this enzyme

Various types of α-(1→2) linkage in the dextran molecule may be determined quantitatively by combined use of the dextran α-(1→2)-debranching enzyme and common dextranases Moreover, the debranching enzyme should provide useful information concerning the fine structure of dextrans

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