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AN ISOMALTOTRIOSE-PRODUCING DEXTRANASE FROM *Flavobacterium* SP. M-73: ACTION PATTERN OF THE ENZYME

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ABSTRACT

An isomaltotriose-producing dextranase from *Flavobacterium* sp. M-73 (dextranase II) effectively hydrolyzed virtually linear dextrans, such as clinical dextran, dextrans T-10, T-110, or B-512F native dextran, to degrees ranging from 25 to 32%, expressed as apparent conversion into D-glucose. Highly branched dextrans, such as those from strains B-1298, B-1299, B-1307, or B-1416, were not good substrates for this enzyme. Studies on the action pattern of dextranase II on linear dextrans showed that this dextranase hydrolyzed high-molecular-weight substrates in an endo-type fashion. The major hydrolysis product was isomaltotriose. When isomalto-oligosaccharides were used as substrates, however, dextranase II hydrolyzed the low-molecular-weight substrates less effectively. Kinetic studies of the enzyme, using substrates of various chain lengths, showed that the affinity of the enzyme increased with increase of the molecular size of the substrate. Simultaneous use of the dextran α-(1→2)-debranching enzyme (dextranase I) with dextranase II greatly increased the extent of hydrolysis of branched dextrans from strains B-1298 and B-1299.

INTRODUCTION

Two types of dextran-degrading enzymes, dextranase I and dextranase II, are produced by a strain (M-73) of a *Flavobacterium* species[1]. Whereas the former (dextranase I) has a strict specificity for the (1→2)-α-D-glucosidic linkage at the branch point[2,3], the latter (dextranase II) hydrolyzes virtually linear dextrans to give isomaltotriose as the major product. In our previous paper, we reported[4] the purification and properties of dextranase II. The present paper describes the detailed action-pattern of dextranase II on various dextrans and isomalto-oligosaccharides.

MATERIALS AND METHODS

α-D-Glucans. — Dextrans from *Leuconostoc mesenteroides* strains NRRL B-512F, B-1298, B-1299, B-1307, and B-1416 were prepared by essentially the same

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procedure as reported previously[5]. Clinical dextran was supplied by Meito Sangyo Co., Ltd., Japan. Dextrans T-10 and T-110 were the products of Pharmacia Fine Chemicals Co., Ltd.

Oligosaccharides of the isomaltose series. — Oligosaccharides having α-(1→6)-linkages were prepared from clinical dextran by partial acid hydrolysis (0.05M H_2SO_4, 7 h, 100°). The hydrolyzate was fractionated by charcoal–Celite column chromatography. Further purification of each oligosaccharide was performed by gel filtration on a column of Bio-Gel P-2. Reduction of isomaltopentaose was performed by the procedure of Wolfrom and Thompson[6].

Enzymes. — Dextranase II from *Flavobacterium* sp. M-73 was prepared by the procedure described in a previous paper[4]. Preparation of the dextran α-(1→2)-debranching enzyme from *Flavobacterium* sp. M-73 has been described[2].

Enzyme assay. — The standard assay-system[4] for dextranase II consisted of 0.5 mL of 0.5% clinical dextran solution in 50mM phosphate buffer (pH 7.0) and 0.5 mL of suitably diluted enzyme solution. The mixture was incubated for 10 min at 30°.

The assay system for dextran α-(1→2)-debranching enzyme[2] consisted of 0.5 mL of a 0.5% solution of B-1299 soluble dextran in 50mM acetate buffer (pH 5.6) and 0.5 mL of enzyme solution. The mixture was incubated for 10 min at 40°. In both cases, the reaction was stopped by the addition of 1.0 mL of alkaline copper reagent, and the mixture was assayed for reducing sugar by the Nelson–Somogyi method[7,8].

One unit of the debranching enzyme and dextranase II is defined as the amount of enzyme that catalyses the liberation of reducing sugar equivalent to 1.0 μmol of D-glucose per min under the foregoing conditions.

Analytical methods. — Throughout this study, reducing sugar was determined by the Nelson–Somogyi method[7,8], and total sugar by the phenol–sulfuric acid method[9] with anhydrous D-glucose as the reference standard. Multiple ascending-paper chromatography was performed on Toyo No. 50 filter paper at room temperature with 70% aqueous 1-propanol (v/v). Spots were detected by the alkaline silver nitrate dip-procedure[10] and by aniline hydrogenphthalate[11].

RESULTS

Action of dextranase II on various dextrans. — α-D-Glucans containing various linkages were digested with dextranase II, and the degree of hydrolysis (d.h.) of each substrate was determined. The results are summarized in Table I. Dextrans having almost linear structure were good substrates for dextranase II, and the native dextran from *L. mesenteroides* NRRL B-512F showed the highest d.h. values of 32.1%. With the virtually linear dextrans, the limit of hydrolysis became smaller with decrease of the molecular weight of the substrate (Table I). As shown in Fig. 1, the initial velocity of hydrolysis was much higher with the linear dextrans than with highly branched dextrans, and the hydrolysis of the former dextrans reached

TABLE I

CORRELATION BETWEEN THE STRUCTURE OF SUBSTRATE DEXTRANS AND DEGREE OF HYDROLYSIS[a]

Dextran	*Linkage*[b]	*D.h. (%)*
Clinical dextran	α-(1→6), (1→3)	25.7
Dextran T-10	α-(1→6), (1→3)	24.7
Dextran T-110	α-(1→6), (1→3)	26.8
B-512F	α-(1→6), (1→3)	32.1
Sephadex G-15	α-(1→6), (1→3)	3.1
Sephadex G-75	α-(1→6), (1→3)	16.0
Sephadex G-200	α-(1→6), (1→3)	21.1
B-1298	α-(1→6), (1→2), (1→3)	1.9
B-1299	α-(1→6), (1→2), (1→3)	1.2
B-1307	α-(1→6), (1→3), (1→4)	8.2
B-1416	α-(1→6), (1→3), (1→4)	8.7

[a]Hydrolysis was performed for 24 h at 30°. For details see the legend to Fig. 1. [b]Reviewed by Sidebotham[12].

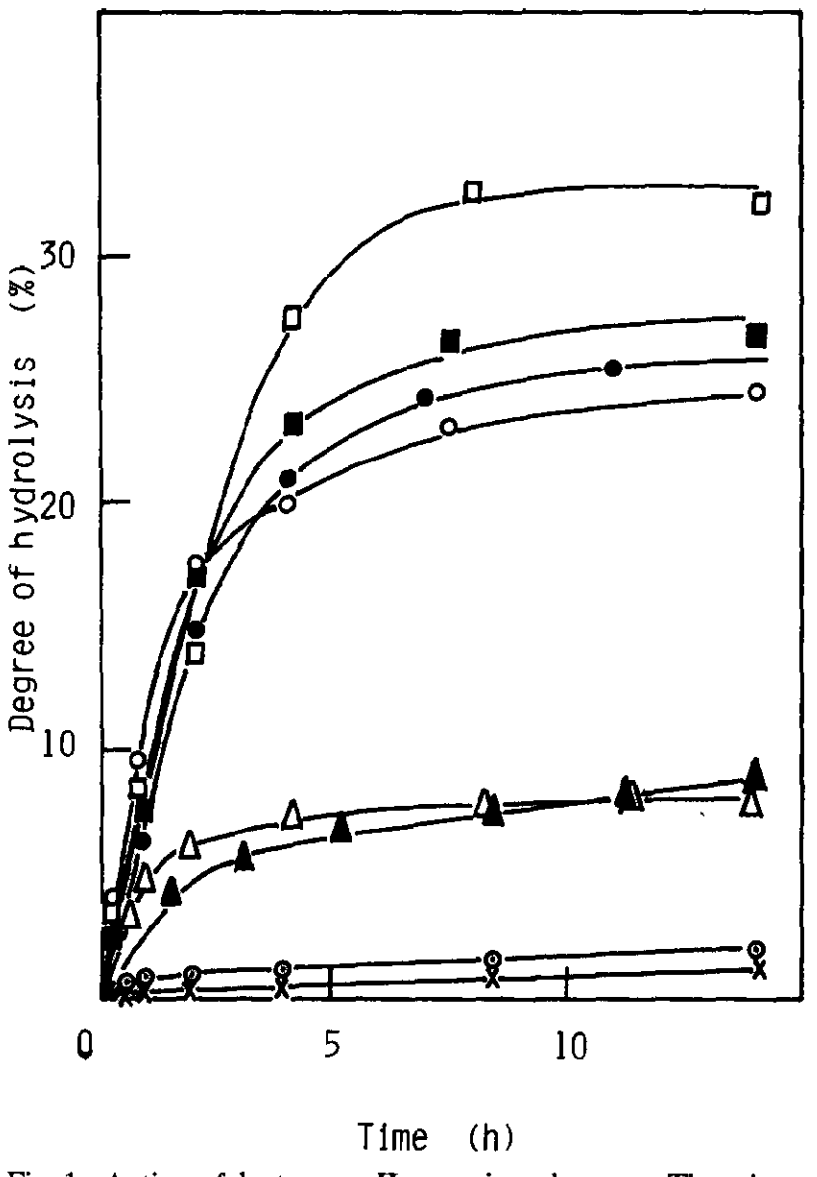

Fig. 1. Action of dextranase II on various dextrans. The mixture contained 0.5% of each dextran in 10mM phosphate buffer (pH 7.0) and 0.02 unit of enzyme. Incubation was conducted at 30° for the indicated intervals. The mixture was then assayed for increase in reducing sugar to determine the degree of hydrolysis (d.h.). (□), B-512F dextran; (■), Dextran T-110; (●), clinical dextran; (○), Dextran T-10; (△), B-1307 dextran; (▲), B-1416 dextran; (⊙), B-1298 dextran, and (x), B-1299 dextran.

Fig. 2. Paper chromatogram of the hydrolyzates of various dextrans with dextranase II. The hydrolyzate of each dextran by action of dextranase II (24 h, 30°) was prepared as described in the legend to Fig. 1. STD Glc, standard D-glucose; IM_2, isomaltose; IM_3, isomaltotriose, and IM_4, isomaltotetraose. Other abbreviations are the same as in Table I.

a maximum (d.h.; 25–32%) after 10 h. Paper chromatography of the hydrolyzates of linear dextrans showed that isomaltotriose was the main product (Fig. 2). Although the highly branched dextrans were hydrolyzed to small extents (Table I), the dextrans from strains B-1307 and B-1416 gave higher d.h. values than those from strains B-1298 and B-1299. The major hydrolysis product from the branched dextrans was also isomaltotriose (Fig. 2). Among the cross-linked dextran gels examined, Sephadex G-200 showed the highest d.h. value (Table I). The susceptibility of these gels to the enzyme seems to be related to the pore-size (that is, degree of cross-linking) of the gels.

Action of dextranase II on oligosaccharides of the isomaltose series. — The action of dextranase II on the series of α-(1→6)-linked oligosaccharides was examined by paper chromatography (Fig. 3). The final product from isomaltohexaose was isomaltotriose, whereas isomaltopentaose was degraded to isomaltose and isomaltotriose. In the latter case, part of the substrate (isomaltopentaose) remained unhydrolyzed, whereas isomaltohexaose was completely degraded to isomaltotriose. Isomaltotetraose and isomaltotriose were not hydrolysed at all. These results indicate that linear α-(1→6)-linked oligosaccharides consisting of five or more D-glucosyl residues are hydrolyzed by dextranase II. When isomaltopentaose was reduced by $NaBH_4$ and then digested with this enzyme, isomaltotriose and isomaltitol were detected on the paper chromatogram (Fig. 4). This result suggests that dextranase II produced an isomaltotriose molecule by splitting the D-glucosidic linkage between the third and fourth D-glucosyl residues from the nonreducing end of isomalto-oligosaccharides.

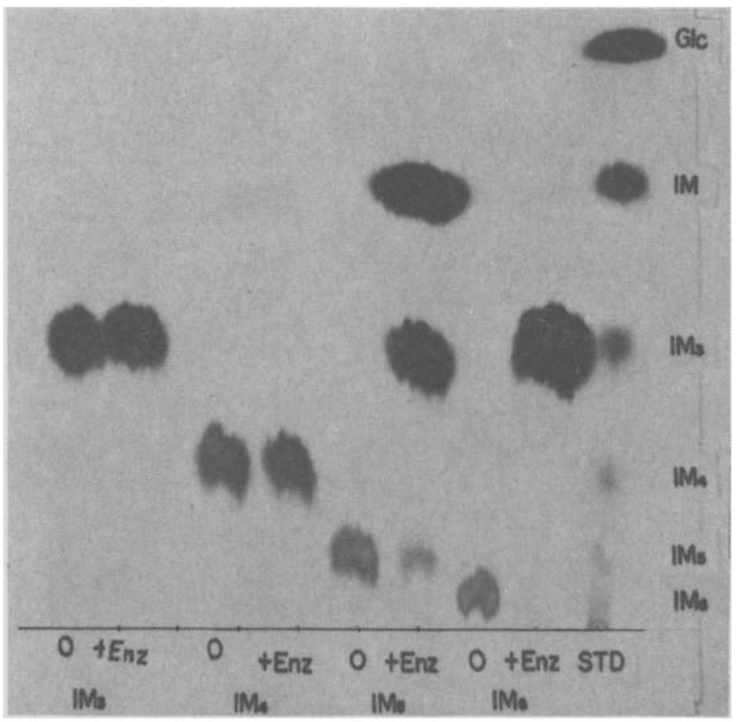

Fig. 3. Paper chromatogram of the hydrolyzates of various isomalto-saccharides with dextranase II. The mixture contained 2mM of each oligosaccharide in 10mM phosphate buffer (pH 7.0) and 0.02 unit of enzyme. Incubation was conducted for 24 h at 30°. Abbreviations are the same as in Fig. 2. +Enz; digested with dextranase II.

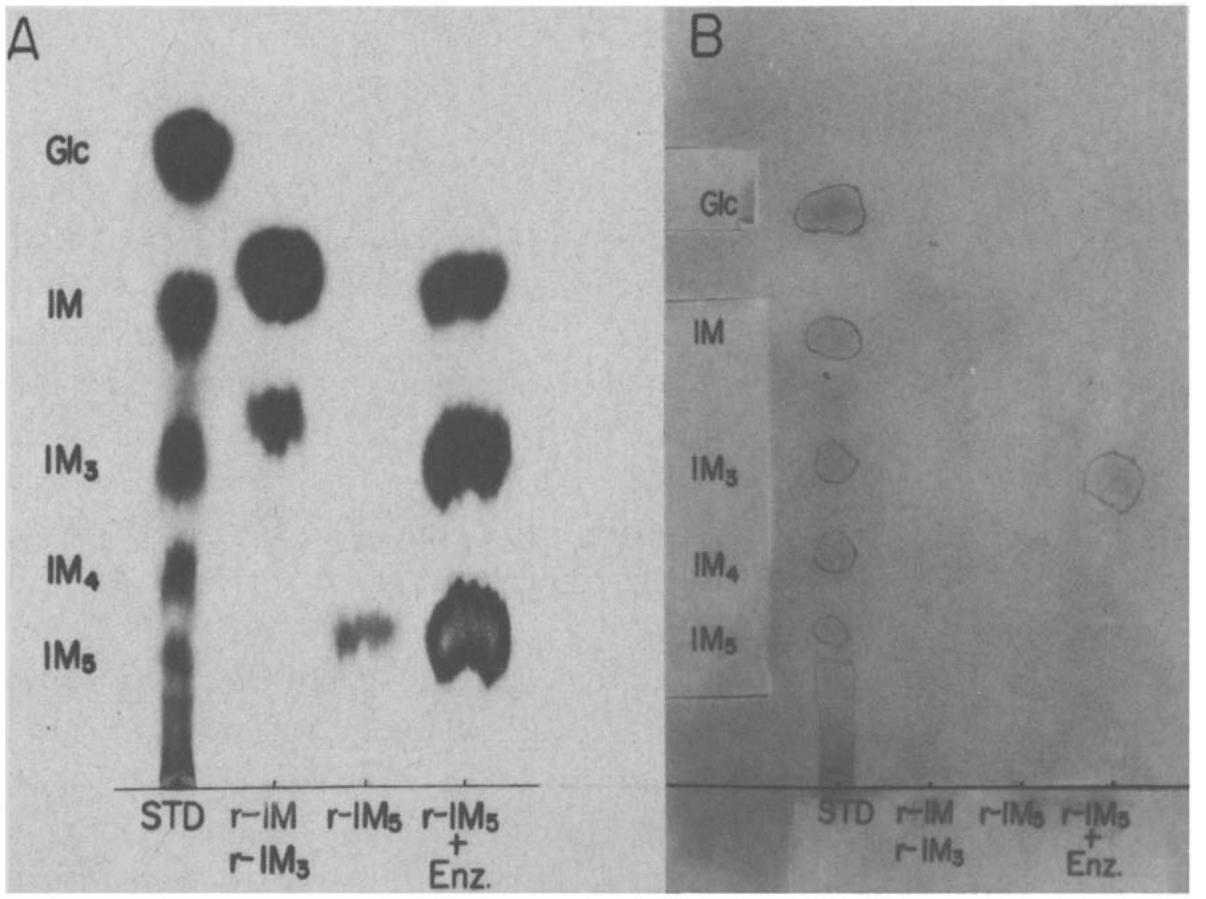

Fig. 4. Paper chromatogram of the hydrolyzate of reduced isomaltopentaose with dextranase II. The reaction conditions are the same as Fig. 3. r-; reduced form of oligosaccharides. (*a*) Detected by alkaline silver nitrate. (*b*) Detected by aniline hydrogenphthalate.

TABLE II

KINETIC PARAMETERS OF DEXTRANASE II FOR DEXTRANS AND ISOMALTOHEXAOSE[a]

Substrate	K_m (μM)	k_o[b]	k_o/K_m ($sec^{-1} \cdot mM^{-1}$)
Clinical dextran	4.9	22.8	4653
Dextran T-10	46	28.5	620
Dextran T-110	3.1	20.9	6742
Isomaltohexaose	530	11.6	22

[a]The mixture contained 0.1–1.0 mg/mL dextrans or 0.05–1.0mM isomaltohexaose in 10mM phosphate buffer (pH 7.0) and 0.01 unit of enzyme. Incubation was effected for 20 min at 30°, and the mixture was then assayed for increase in reducing sugar to determine the reaction velocity. [b]Calculated as V_{max}/e_o (mol.wt. of the dextranase II: 114,000, see ref. 4).

Kinetic measurements. — The effect of substrate concentration was examined by using three, virtually linear dextrans and isomaltohexaose. The Michaelis constants for dextrans T-10, T-110, and clinical dextran were 46, 3.1, and 4.9 μM, respectively (Table II). These values were significantly lower than that for isomaltohexaose (530 μM). Comparison of the k_o and k_o/K_m values also suggested that the substrates having higher molecular weights were more susceptible to this enzyme. Dextran T-110 and isomaltohexaose gave K_m values of 3.1 and 530 μM, and k_o/K_m values of 6742 and 22 $sec^{-1} \cdot mM^{-1}$, respectively.

Action pattern of dextranase II. — The molecular-weight distribution of the hydrolyzates of the native dextran from strain B-512F at various periods of hydrolysis was examined by gel filtration on a column of Sepharose 6B (Fig. 5). After 1 h, the d.h. value of the hydrolyzate was 4.6% and more than half of the product was eluted at the region between the void volume (V_o) and the internal volume (V_i). The d.h. values were 15.6% after 12 h and 18.7% after 38 h, and the molecular size of the product became smaller with the progress of hydrolysis. These results suggested that degradation of the high-molecular-weight substrate with dextranase II proceeded by an endo-type action. The action pattern of the enzyme was also characterized by the method of Tung and Nordin[13]. The relationship between the amount of solubilized total sugar (t.s.) and reducing sugar (r.s.) during the hydrolysis was examined with B-512F native dextran. The ratio of t.s. to r.s. was plotted against r.s. (Fig. 6), and the results indicated that dextranase II cleaved the substrate dextran by an endo action-pattern.

The endo action-pattern of this enzyme was also supported by the results from the hydrolysis of cross-linked dextran gels (Sephadex), as shown in Table I. The Sephadex gels having larger pore-sizes were better hydrolyzed than those with smaller ones.

Combined action of dextranase II and debranching enzyme. — Hydrolysis of highly branched dextrans with dextranase II was significantly restricted by the occurrence of branching (Fig. 1 and Table I). As the α-(1→2)-debranching enzyme (dextranase I) from *Flavobacterium* sp. M-73 is known to split the (1→2)-α-D-

glucosidic linkage constituting the branch points of B-1298 and B-1299 dextrans[3], this enzyme was expected to render these dextrans more susceptible to dextranase II. Table III shows the results of the combined use of dextranase II and α-(1→2)-debranching enzyme for the hydrolysis of various dextrans. Addition of the debranching enzyme to the mixture resulted in marked increase of the degree of hydrolysis. Thus, the B-1298 dextran was most effectively hydrolyzed (d.h. 33.8%); this value is comparable to that of the B-512F native dextran (Fig. 7). The d.h. value of B-1299 dextran was also increased by addition of the second enzyme, more than 10-fold over that with dextranase II only. The effect of the debranching enzyme on the B-1416 dextran was not remarkable and caused less than a doubling of the d.h. value. Paper chromatograms of the hydrolyzates of various dextrans are shown in Fig. 8. The major hydrolysis products from B-1298, B-1299, and B-1416 dextrans were D-glucose and isomaltotriose. In each instance, the amount of D-glucose was much larger than in the hydrolyzate of B-512F dextran, indicating that D-glucose originated from the branch points.

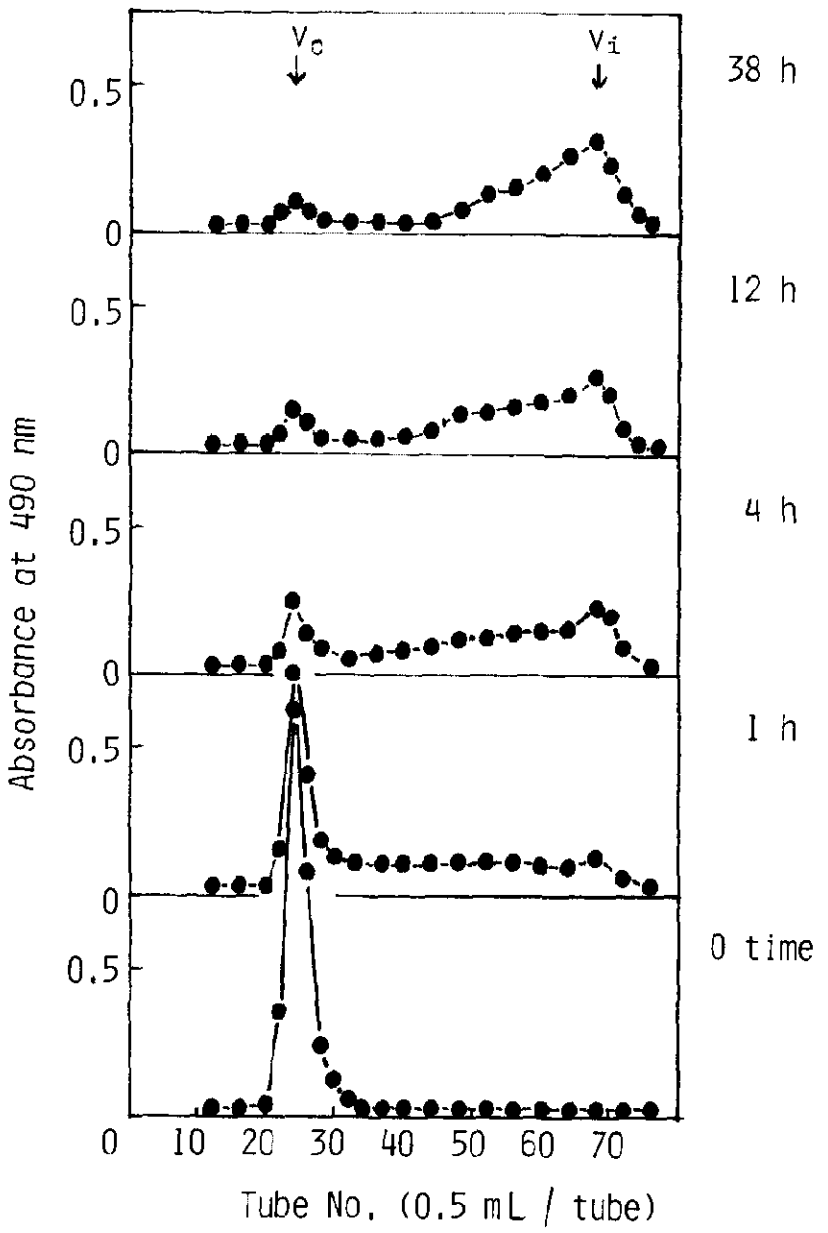

Fig. 5. Elution pattern of the hydrolyzates of dextran with dextranase II from a column of Sepharose 6B. The B-512F native dextran (0.2% solution in 0.1M phosphate buffer, pH 7.0) was incubated with dextranase II (0.004 unit) at 30° for the indicated periods. The digests were concentrated and applied to a column (1.2 × 29.5 cm) of Sepharose 6B and eluted with water.

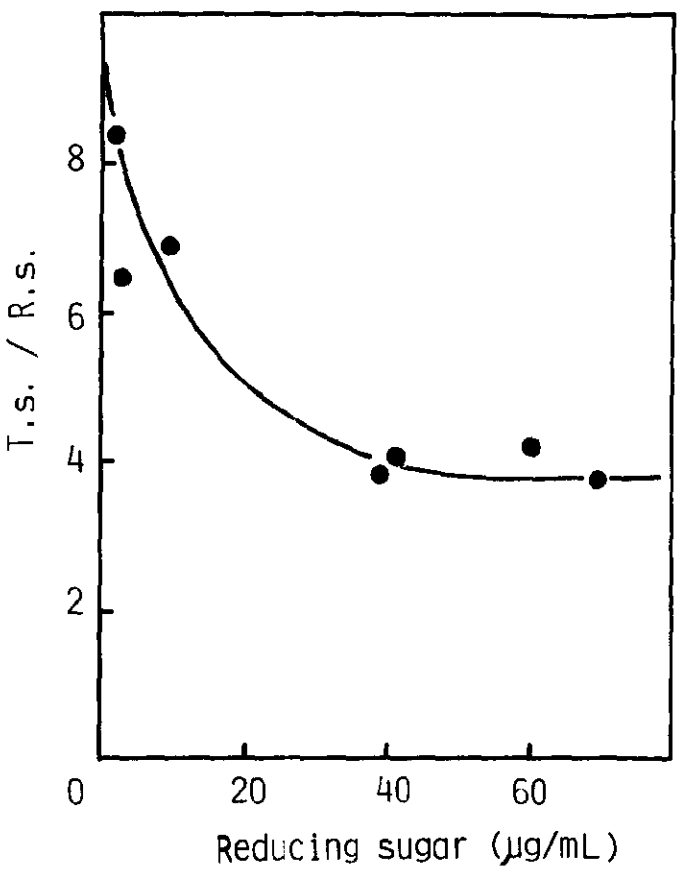

Fig. 6. Action pattern of dextranase II. The mixture, consisting of 1% of B-512F native dextran and various amounts of enzyme, was incubated for 10 min at 30°. The reaction was stopped by heating for 5 min in a boiling-water bath. After gel filtration on a column (1 × 20 cm) of Sephadex G-50 to remove the undigested substrate, the effluent was concentrated and assayed for total sugar (t.s.) and reducing sugar (r.s.).

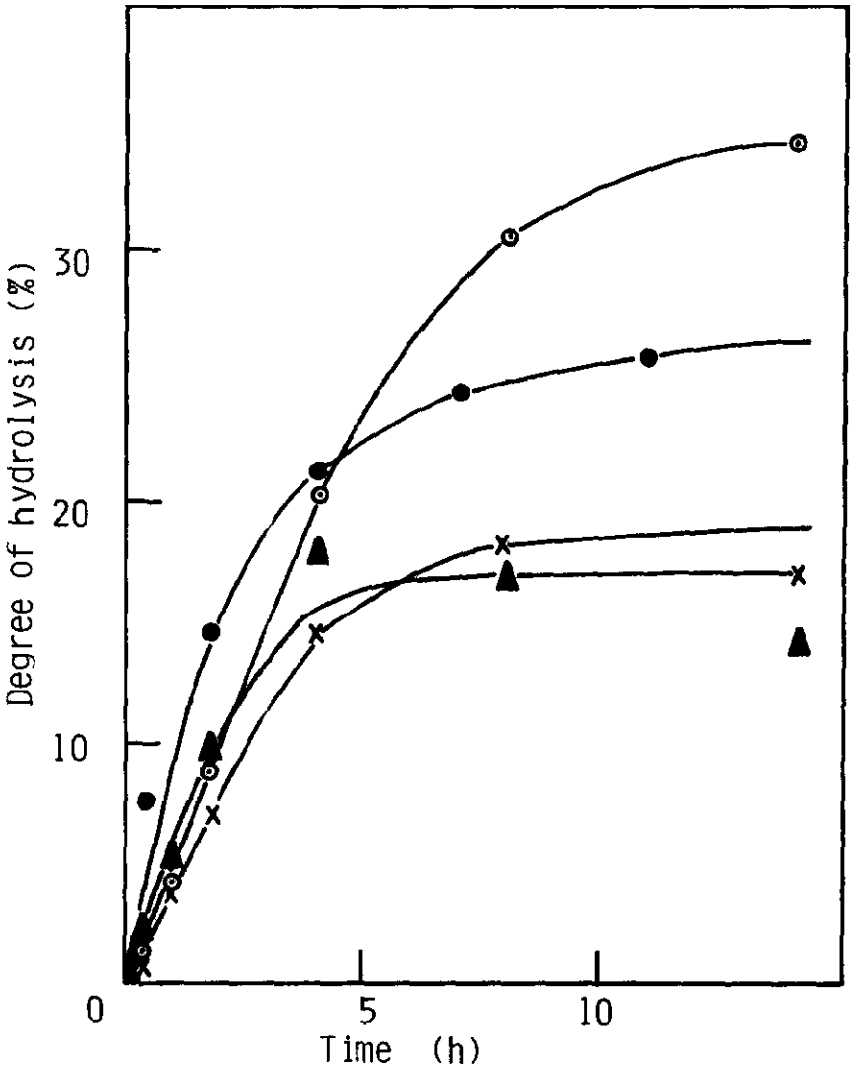

Fig. 7. Combined action of dextranase II and α-(1→2)-debranching enzyme on various dextrans. The reaction conditions and abbreviations are the same as in Fig. 1. Dextranase II (0.02 unit) and debranching enzyme (0.02 unit) were used.

TABLE III

EFFECTS OF α-(1→2)-DEBRANCHING ENZYME ON THE ACTION OF DEXTRANASE II[a]

Dextran	*B-512F*	*B-1298*	*B-1299*	*B-1416*
D.h. (%)	32.1	33.8	17.0	14.6

[a]Hydrolysis was performed for 24 h at 30°. For details see the legend to Fig. 7.

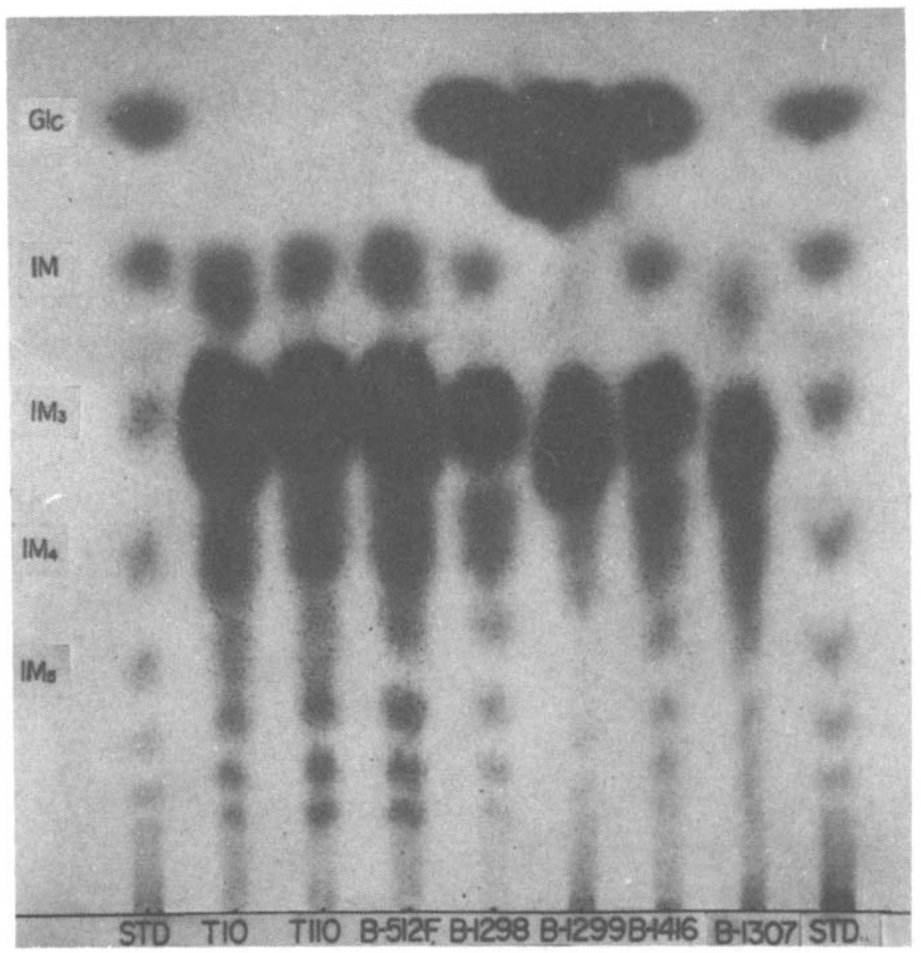

Fig. 8. Paper chromatogram of the hydrolyzates of various dextrans with dextranase II and α-(1→2)-debranching enzyme. The hydrolyzate of each dextran with the action of dextranase II and debranching enzyme (24 h, 30°) was prepared as described in the legend to Fig. 7. For abbreviations see Fig. 2.

DISCUSSION

Dextranase II from *Flavobacterium* sp. M-73 showed some unique characteristics in its action pattern. This enzyme hydrolyzed (1→6)-α-D-glucans in an endo-type fashion (Fig. 5 and Fig. 6). Further evidence is provided by the ease of hydrolysis of Sephadex gels, as shown in Table I and by the higher affinity for the high-molecular-weight substrates (Table II). The action of an exo-dextranase should be greatly restricted by the epoxide cross-linking present in substrate Sephadex gels. Unlike most of the endo-dextranases hitherto reported[14–17], dextranase II barely hydrolyzed isomaltotriose and isomaltotetraose (Fig. 3). The smallest substrate susceptible to this enzyme is isomaltopentaose, which is cleaved to isomaltotriose and isomaltose. Hydrolysis of isomaltohexaose with dextranase II yielded isomaltotriose as the sole product (Fig. 3), and isomaltopentaitol was

cleaved to isomaltotriose and isomaltitol (Fig. 4). These results indicated that dextranase II could remove a trisaccharide unit from the nonreducing terminal of the oligosaccharides. A similar endo-dextranase from *Penicillium lilacinum* has been reported by Walker and Dewar[15]. More-detailed information on the action pattern of dextranase II might be obtained by subsite-mapping studies such as those reported by Walker's group[18,19].

The kinetic study of dextranase II showed that substrates of higher molecular-weight were more susceptible to this enzyme (Table II). This result is in contrast to that obtained with the α-D-glucosidase of *Bacillus thermoglucosidius*. Although the latter enzyme hydrolyzed isomalto-oligosaccharides from the nonreducing end, it showed higher K_m values and lower k_c/K_m values for high-molecular-weight substrates[20].

The dextranase II of *Flavobacterium* hydrolyzed clinical dextran to isomaltotriose (63% of the total sugar) and, relatively, a very small amount of D-glucose and other oligosaccharides, as described in our previous paper[4]. Isomaltotriose was the major product throughout the incubation. Although the endo-dextranase from *Streptococcus mutans* K1-R was reported to hydrolyze linear dextrans to isomaltotriose[21], the end-products in the hydrolyzate of B-512F type dextran with most of the endo-dextranases was a mixture of D-glucose and isomalto-oligosaccharides[14–17]. In contrast, the exo-dextranase (exo-isomaltotriohydrolase: EC 3.2.1.95) from *Brevibacterium fuscum* has been reported to produce isomaltotriose in 42% yield from the B-512F type dextran[22].

Because the combined action of dextranase II and α-(1→2)-debranching enzyme led to increase of the d.h. values for the highly branched dextrans (Table III), the two enzymes from *Flavobacterium* sp. M-73 may provide useful information on the fine structure of highly branched dextrans. Structural studies on the B-1298 and B-1299 dextrans by the enzymic method are in progress.

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