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ENZYMIC DEGRADATION OF WATER-SOLUBLE DEXTRAN FROM *Leuconostoc mesenteroides* NRRL B-1299

MIKIHIKO KOBAYASHI, YASUSHI MITSUISHI, SHIRO TAKAGI, AND KAZUO MATSUDA
Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Miyagi 980 (Japan)
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ABSTRACT

Structural studies on the water-soluble dextran elaborated by *Leuconostoc mesenteroides* NRRL B-1299 gave several important results that not only supported previous results but afforded an insight into the association of average repeating-units in the whole molecule. Sequential degradation of soluble dextran from its nonreducing terminals was achieved with two different enzymes, namely, α-D-(1→2)-debranching enzyme and D-glucodextranase. The debranching enzyme removed four separate residues of α-(1→2)-branched D-glucose from the average repeating unit consisting of 15 D-glucosyl residues. D-Glucodextranase continuously produced D-glucose from the nonreducing terminals by an exo type of action, but internal branches greatly restricted its action. Extensive digestion of soluble dextran B-1299 with the two enzymes released 74.3% of D-glucose and gave a limit dextrin of high molecular weight containing 8.4% of 2,6-di-*O*-substituted D-glucosyl residues. ^{13}C-N.m.r. studies indicated a characteristic pattern of the α-D-(1→2)-branched structure, which was significantly changed on treatment with the debranching enzyme. Moreover, an ~8-fold increase in the degree of linearity was observed after action of the debranching enzyme. The possible structure of water-soluble dextran B-1299 is discussed, based on a comparison of the limit dextrin with the native dextran, in regard to chemical structure, molecular-weight distribution, and degree of hydrolysis with two exo-enzymes. The native dextran might be constructed with at least ~8,200 "twigs" of repeating unit, and there are 14 steps of connected twigs between the reducing and nonreducing terminals. Upon consecutive hydrolysis with the two exo-enzymes, most of the twigs located at the 14th step (*i.e.*, nonreducing terminals) were hydrolyzed to D-glucose.

INTRODUCTION

We have recently reported[1] that two types of dextran-hydrolyzing enzyme, namely, an α-D-(1→2)-debranching enzyme and dextranase II, are produced by a Gram-negative strain of *Flavobacterium* sp. M-73. The former enzyme has a strict specificity for the α-D-(1→2)-glucosidic linkage at branch points of dextran, and

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produces D-glucose as the sole reducing sugar[2,3]. The latter enzyme belongs to the endo-dextranase type, but produces isomaltotriose units from the nonreducing end of oligosaccharide substrates[4,5]. Moreover, Sawai *et al.*[6] isolated glucodextranase [exo-α-D-(1→6)-glucosidase, EC 3.2.1.70] from *Arthrobacter globiformis* I42, and described its action on isomaltodextrins and 14 dextrans[7]. The effectiveness of enzymic fragmentation of the highly branched dextran from *Leuconostoc mesenteroides* NRRL B-1299 has been demonstrated[8]; this gave three branched oligosaccharides containing α-D-(1→2) linkages at the nonreducing terminals.

Among the 96 dextrans isolated, and characterized, by Jeanes *et al.*[9], the chemical structure of dextran B-1299 has been extensively studied. We showed[10] polymolecularity and polydispersity among five fractions from native dextran B-1299. Chemical analyses of the structure of dextran B-1299 were summarized in 1974 by Sidebotham[11]. Later, a series of studies on unusual dextrans, including B-1299 dextran, by ^{13}C-n.m.r. spectroscopy was reported by Seymour *et al.*[12]. Fragmentation analysis of both insoluble and soluble dextran B-1299 by acetolysis revealed the presence of consecutive sequences of α-D-(1→3) linkages[13] and α-D-(1→2) linkages[14], in addition to abundant single D-glucosyl groups joined to the back-bone through α-D-(1→2) linkages.

We now describe an approach involving enzymic analysis of water-soluble dextran B-1299. Differing from the usual enzymic analysis, sequential degradation of dextran B-1299 using α-D-(1→2)-debranching enzyme, or D-glucodextranase, or both, was applied, in order to compare the structure of native dextran with those of the limit dextrins* isolated from consecutive, hydrolysis steps. A typical endo-dextranase (EC 3.2.1.11) from *Chaetomium gracile*[15] and dextranase II from[4,5] *Flavobacterium* sp. M-73 were also used in some experiments. Structural features of soluble dextran B-1299 are discussed, based on the results of the enzymic hydrolysis and subsequent, chemical analyses.

RESULTS AND DISCUSSION

Action of α-D-(1→2)-debranching enzyme and D-glucodextranase on soluble dextran B-1299. — The progress of hydrolysis of the water-soluble dextran B-1299 with dextran α-(1→2)-debranching enzyme has already been shown (see ref. 3, Fig. 6). The degree of hydrolysis (d.h.) at 24 h was[3] 31.5%; this indicated that about one third of the D-glucosyl units in dextran B-1299 constitute α-D-(1→2)-branch points, and each consists of one D-glucosyl group. The d.h. value showed good correlation to the results from methylation analysis[16–18]. D-Glucodextranase from *Arthrobacter globiformis* I42 produces only D-glucose as a hydrolysis product from the

*The abbreviations used are: D-I, limit dextrin prepared by the digestion of the soluble dextran with the debranching enzyme; D,G-I, limit dextrin prepared from D-I by D-glucodextranase; D-II, limit dextrin prepared from D,G-I by the debranching enzyme; D,G-II, limit dextrin prepared from D,G-I by D-glucodextranase; D-III, D,G-III, ,,,,,,, defined in the same way. D,E, limit dextrin prepared by the digestion of soluble dextran with the debranching enzyme and dextranase II.

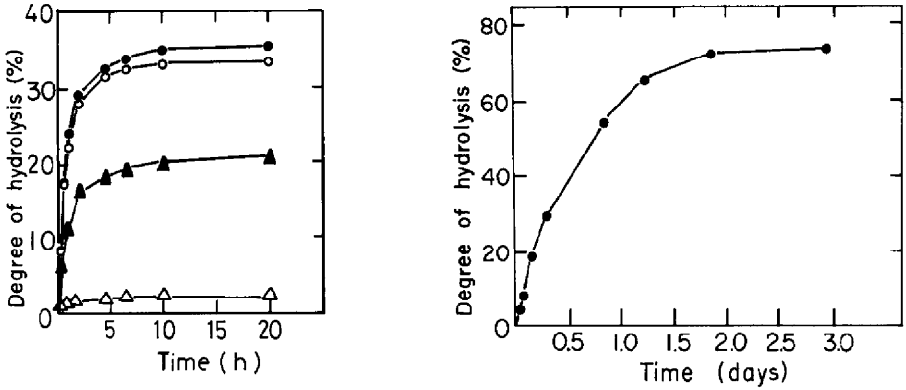

Fig. 1. Action of D-glucodextranase on the water-soluble dextran B-1299, its D-I limit dextrins, and clinical dextran. [Each dextran (50 mg/5 mL of 50mM acetate buffer (pH 6.0)–5mM $CaCl_2$) was hydrolyzed with D-glucodextranase (0.18 U) from *Arthrobacter globiformis*[6] at 40°, and an aliquot of the digest (5–30 μL) was withdrawn to determine the degree of hydrolysis (d.h., %) at suitable intervals. (●), D-I limit dextrin of dextran B-1299; (○), clinical dextran; (▲), D-I limit dextrin of dextran B-1299, CS fraction; and (△), dextran B-1299.]

Fig. 2. Synergistic action of α-D-(1→2)-debranching enzyme and D-glucodextranase on the water-soluble dextran B-1299. [The reaction mixture contained 50 mg of soluble dextran B-1299, 0.18 U of the debranching enzyme and 0.18 U of D-glucodextranase in 5 mL of 50mM acetate buffer (pH 6.0)–5mM $CaCl_2$. Incubation was performed at 40°, and an aliquot of the digest (5–30 μL) was withdrawn to determine the degree of hydrolysis (d.h., %) at suitable intervals.]

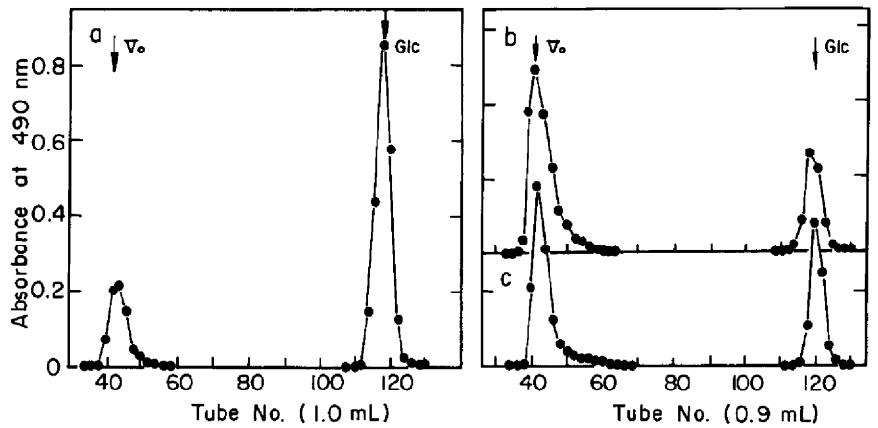

Fig. 3. Elution patterns of dextran hydrolyzates from a Sepharose CL-6B column. [(a) The soluble dextran B-1299 hydrolyzate by α-D-(1→2)-debranching enzyme and D-glucodextranase. The digest was prepared as described in the legend to Fig. 2. A portion of the reaction mixture (d.h. 73.6%) was treated for 3 min in a boiling-water bath, and applied to a Sepharose CL-6B column (1.5 × 74 cm). The column was eluted with water, and fractions (1.0 mL or 0.9 mL, as indicated) were assayed for the total sugar. (b) The digest with debranching enzyme. The soluble dextran B-1299 (100 mg/10 mL of 50mM acetate buffer (pH 6.0)–5mM $CaCl_2$) was hydrolyzed with debranching enzyme (1.0 U) for 24 h at 40°. The reaction was terminated by heating, and the digest was applied to the column. (c) The digest with D-glucodextranase following the debranching-enzyme action. The D-I limit dextrin, *i.e.*, V_o fraction of experiment (b), was hydrolyzed with D-glucodextranase (1.0 U) for 24 h at 40°, and subjected to the column. V_o, void volume; Glc, elution volume of standard D-glucose.]

nonreducing end of various dextrans, and the ratios of the initial velocity of hydrolysis of isomaltose, maltose, nigerose, and kojibiose are[7] 100:3–4:0.03–0.05:0.01–0.02. The d.h. values, after 24 h at 40°, of soluble dextran B-1299 and standard clinical dextran were 3.0 and 32.2%, respectively (see Fig. 1). Thus, the proportion of α-(1→6)-linked D-glucose at the terminal was estimated to be <3% of the total. When dextran B-1299 was digested with a mixture of the α-(1→2)-debranching enzyme and D-glucodextranase, release of D-glucose reached a maximum d.h. of 73.6% (see Fig. 2). The hydrolyzate gave two peaks, at V_0 and V_t, on a column of Sepharose CL-6B (see Fig. 3a). The ratio of peaks eluted at V_0 (limit dextrin) and V_i was ~1:3. Paper chromatography of the V_i fraction showed that glucose was the sole product. On the other hand, on rechromatography in a column of Sepharose CL-2B, ~70% of the limit dextrin was again eluted at the V_0 position, and ~30% of the total was eluted in the subsequent, high-molecular-weight region as a tailing shoulder (data not shown). Therefore, the molecular weight of the limit dextrin was estimated to be $>5 \times 10^6$.

Consecutive hydrolysis of soluble dextran B-1299. — Based on the substrate specificity and action pattern of α-D-(1→2)-debranching enzyme and D-glucodextranase, sequential degradation of the water-soluble dextran B-1299 (WS) from the nonreducing end was examined. Clinical dextran and the CS fraction[10] of native dextran B-1299 were used as references. As reported previously[10], the CS fraction was derived from the fraction of the native dextran B-1299 soluble in 0.1M sodium tetraborate (pH 9.3), and had no ability to form a quarternary ammonium complex with 4% Cetavlon (cetyltrimethylammonium bromide).

The first step in the sequential degradation was debranching of the WS and CS dextrans with the α-D-(1→2)-debranching enzyme. The d.h. values of WS and

TABLE I

CONSECUTIVE HYDROLYSIS OF SOLUBLE DEXTRAN B-1299 AND ITS CS FRACTION WITH THE DEBRANCHING ENZYME AND D-GLUCODEXTRANASE[a]

Hydrolysis step[b]	*WS dextran*		*CS dextran*	
	D.h. (%)[c]	*Total d.h. (%)*	*D.h. (%)*[c]	*Total d.h. (%)*
1st D	27.3	27.3	26.7	26.7
1st G	26.0	53.3	16.6	43.3
2nd D	5.4	58.7	5.0	48.3
2nd G	10.4	69.1	9.3	57.6
3rd D	1.0	70.1	2.1	59.7
3rd G	3.0	73.1	2.2	61.9
4th D	—	—	—	—
4th G	1.2	74.3	1.1	63.0

[a]D.h. (%), degree of hydrolysis (%). [b]D, α-D-(1→2)-debranching enzyme; G, D-glucodextranase. For details see the legend to Fig. 3b and 3c. [c]D.h. (%) values for 1st G to 4th G are represented as the corrected values against the amount of original dextrans.

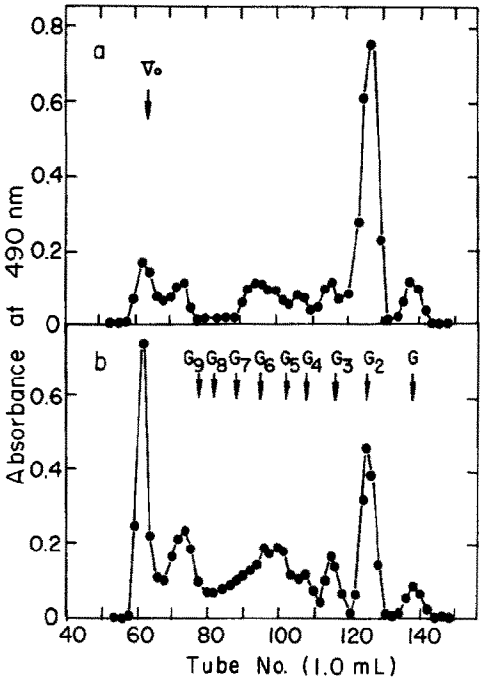

Fig. 4. Elution patterns of dextran hydrolyzates from a Bio-Gel P-2 column. [(a) Hydrolyzate of clinical dextran by endodextranase. Clinical dextran (2.0 mg/2 mL of 50mM acetate buffer, pH 6.0) was hydrolyzed with endo-dextranase (0.01 U) from *Chaetomium gracile*[15] for 24 h at 40°. After heat inactivation, the digest (d.h. 46.9%) was applied to a Bio-Gel P-2 column (1.5 × 93 cm). The column was eluted with water, and fractions (1.0 mL) were assayed for the total sugar. (b) Hydrolyzate of D,G-I limit dextrin by endo-dextranase. The digest (d.h. 34.6%) was subjected to the column. G–G_9, elution volumes of isomalto-oligosaccharides.]

CS were 27.3 and 26.7%, respectively (see Table I). The subsequent, D-I limit dextrins were then hydrolyzed with D-glucodextranase (see Fig. 1). The d.h. values of the D-I limit dextrins of WS and CS were 35.8 and 21.1%, respectively. The d.h. value of clinical dextran (33.6%) was comparable to that of the D-I limit dextrin of the WS fraction, which indicated that these limit dextrins might contain a long sequence of α-D-(1→6)-linked, linear structure, and are readily attacked by D-glucodextranase.

Fig. 4 shows the gel-filtration pattern of clinical and B-1299 D,G-I limit dextrin hydrolyzed with the endo-dextranase from *Chaetomium gracile*. The d.h. values of clinical dextran and D,G-I limit dextrin were 46.9 and 34.6%, respectively. Although the elution profiles lying between the higher-oligosaccharide and D-glucose regions were almost identical, 22.1% of the total sugar (corresponding to ~5% of the original, WS dextran) was eluted at the V_o position (tubes No. 58–65) of the column of Bio-Gel P-2. This fraction might contain a relatively higher amount of D-glucosidic linkages other than those of the linear, α-D-(1→6) type, because of the structure, highly resistant to the three hydrolyzing enzymes employed.

The resistant structure was substantiated by the following observations. As exemplified by the structure of the average repeating-units[19] of dextran B-1299, some of the branches might be terminated by α-D-(1→3)-linked D-glucosyl groups. We have also demonstrated the occurrence of consecutive, α-D-(1→2)-linked D-glucosyl residues by the isolation[14] of fragment oligosaccharides from the acetolyzate of soluble dextran B-1299.

Gel-filtration patterns of the hydrolyzates obtained by the first (debranching enzyme and D-glucodextranase) digestion are shown in Fig. 3b and c, respectively. In both cases, more than half the amount of total sugar was eluted at the V_o position of a column of Sepharose CL-6B. The V_o fraction was subjected to the next cycle of consecutive hydrolysis, and this was repeated until the release of D-glucose reached an apparently stationary state (fourth cycle). Table I is a summary of the sequential degradation of WS and CS dextrans. Although the first and second cycles of hydrolysis were very effective in removing D-glucosyl groups from the non-reducing terminal, the resulting limit dextrin (D,G-II limit dextrin) gave no significant amount of reducing sugar on enzymic digestion in the next two cycles. The d.h. value of 74.3% for WS dextran was comparable to that obtained from the combined use of the debranching enzyme and D-glucodextranase (see Fig. 2).

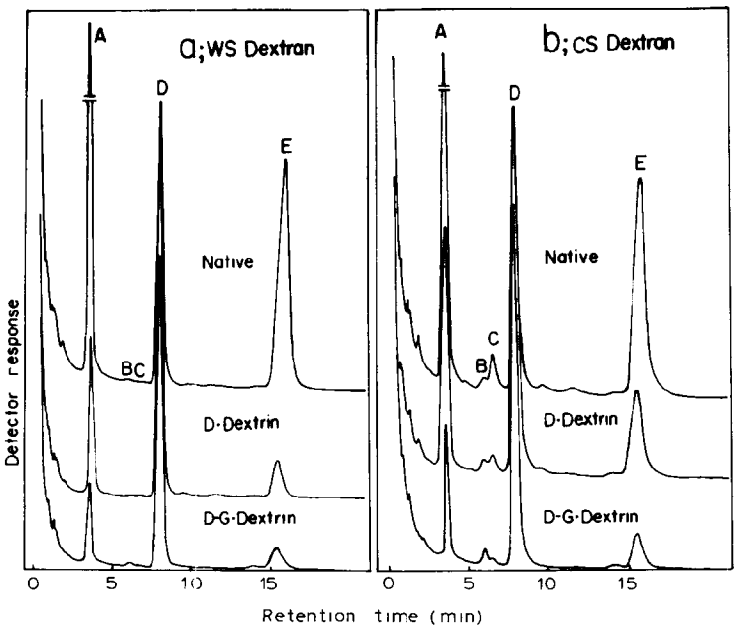

Fig. 5. Gas–liquid chromatogram of the partially methylated alditol acetates from methylated dextrans and their limit dextrins. [(a) WS Dextran, water-soluble dextran B-1299; D-Dextrin, D-I limit dextrin; D-G-Dextrin, D,G-I limit dextrin. (b) CS Dextran, CS fraction from WS dextran; D-Dextrin, D-I limit dextrin from CS dextran, D-G-Dextrin, D,G-I limit dextrin from the D-I limit dextrin. Peaks A to E correspond to those components described in Table II.]

TABLE II

PROPORTIONS OF THE METHYLATED D-GLUCOSIDES IN THE DEGRADATION PRODUCTS OF THE METHYLATED DEXTRANS AND LIMIT DEXTRINS[a]

Peak[b]	O-*Me*-D-*glucose*	*Relative mol (%)*					
		WS dextran			*CS dextran*		
		Native	*D-I*	*D,G-I*	*Native*	*D-I*	*D,G-I*
A	2,3,4,6-Tetra-	33.0	11.2	10.4	32.9	20.2	15.2
B	3,4,6-Tri-	—	—	—	3.7	1.2	1.2
C	2,4,6-Tri-	—	—	trace	1.1	1.0	2.4
D	2,3,4-Tri-	34.0	77.0	81.1	28.1	62.2	69.7
E	3,4-Di-	33.0	11.7	8.4	34.2	15.3	11.5

[a]The alditol acetate derivatives of *O*-methylated sugars were separated by g.l.c., as described in the text. [b]Corresponds to Fig. 5.

Therefore, most of the D-glucosyl linkages susceptible to these enzymes could be removed by the sequential degradations.

Methylation analysis of the limit dextrins. — The structure of the native dextrans and their limit dextrins was studied by methylation analysis. As shown in Fig. 5, the native dextrans WS and CS gave almost equal amounts of 2,3,4,6-tetra- (A), 2,3,4-tri- (D), and 3,4-di-*O*-methylglucose (E). The action of the α-D-(1→2)-debranching enzyme significantly lowered the ratio of 3,4-di-*O*-methylglucose, corresponding to the branch points, to 2,3,4-tri-*O*-methylglucose (0.97 to 0.15; see Table II). Although the limit dextrins of WS gave no tri-*O*-methylglucoses derived from the linear structure of α-D-(1→2) or α-D-(1→3) linkages, those of the CS fraction gave small amounts of 3,4,6-tri- and 2,4,6-tri-*O*-methylglucose. This structure seemed to restrict the action of both the α-D-(1→2)-debranching enzyme and the D-glucodextranase, as the debranching enzyme could not hydrolyze kojibiose[3] and kojihexaose[20], and D-glucodextranase could scarcely hydrolyze[7] nigerose and kojibiose, as already described.

Structural studies on the water-soluble dextran B-1299 by the Bourne group[17,19], the NRRL group[12,18], and this laboratory[10,13,14,16] indicated that the α-D-(1→2) linkage in this dextran may occur in three different forms. (*i*) Most of the α-D-(1→2) linkages constitute branch points of a single D-glucosyl group, as substantiated by the isolation[8,14,19] of oligosaccharide fragments from the partially degraded dextran B-1299. (*ii*) About one-fifth of the branched, α-D-(1→2) linkages intersect a continuous chain of α-D-(1→6)-linked D-glucosyl residues to the other α-D-(1→6)-linked backbones. Isolation of oligosaccharides having alternating, α-D-(1→2) and α-D-(1→6) linkages[14,19] supported this structure. (*iii*) A small amount of α-D-(1→2) linkages might occur in continuous, or alternating, or both, α-D-(1→2) and α-D-(1→3) linkages with more than two D-glucosyl residues. Identification of 3,4,6-tri-*O*-methylglucose from the methylated dextran[16], and of kojitriose

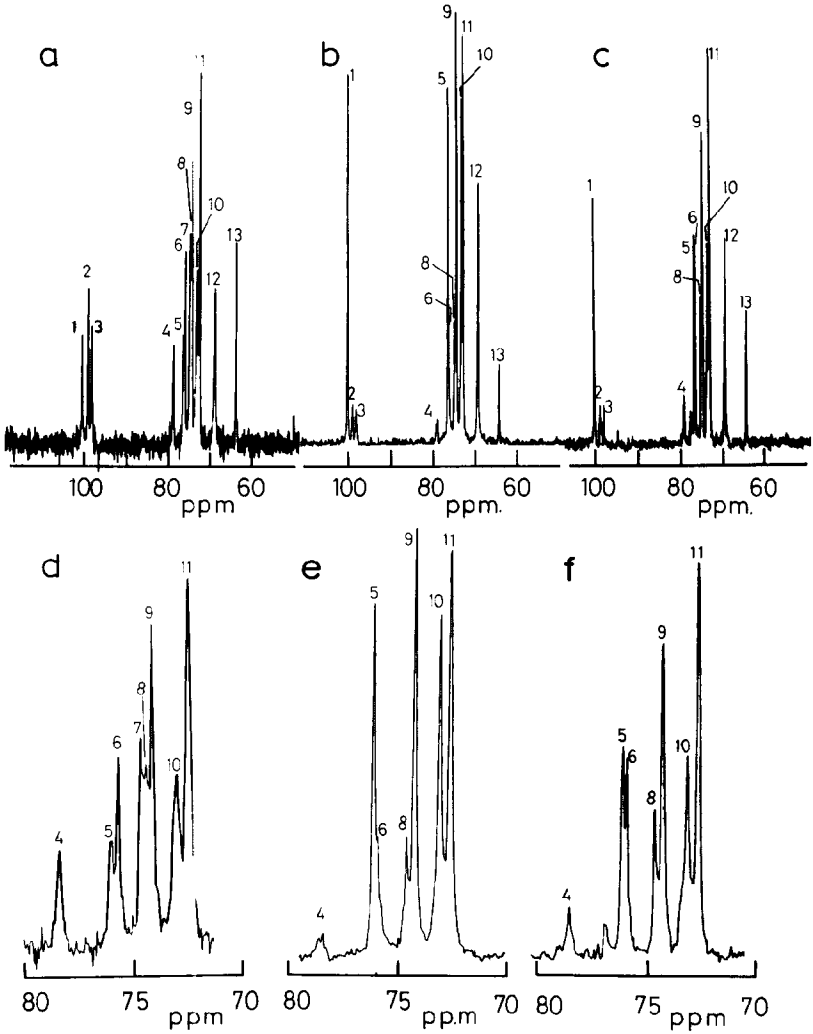

Fig. 6. ^{13}C-N.m.r. spectra, at 70°, of water-soluble dextran B-1299, D-I limit dextrin, and D,E limit dextrin. [The numerical assignments of resonances correspond to those resonances listed in Table III. (a) Soluble dextran B-1299. (b) D-I limit dextrin; for details, see the legend to Fig. 3b. (c) D,E limit dextrin. The soluble dextran B-1299 (250 mg/100 mL of 50mM phosphate buffer, pH 7.0) was hydrolyzed with the mixture of debranching enzyme (11.9 U) and dextranase II (6.3 U) for 40 h at 30°. The reaction was terminated by heating for 3 min in a boiling-water bath, and the digest was extensively dialyzed against water. (d), (e), and (f) are the spectra of (a), (b), and (c), expanded in the 70–80-p.p.m. region.]

or kojitriose-containing tetrasaccharides from the acetolyzate[14] may constitute evidence for the former structure. A mixture of trisaccharides containing 2-*O*-nigerosyl-D-glucose (identified from among the acetolyzed fragments[14]) gave a clue suggesting the latter structure.

^{13}C-N.m.r. spectroscopy of the soluble dextran B-1299 and limit dextrins. —

TABLE III

CHEMICAL SHIFTS[a] OF ^{13}C-N.M R. SPECTRA, AT 70°, OF SOLUBLE DEXTRAN B-1299 AND LIMIT DEXTRINS

Signal No.	*Dextran B-512F-type*[c]	*Dextran B-1299*			*Assignment of resonance*[b]	
		Native	*D-I*	*D,E*	*Carbon atom*	*Type of residue*
1	99.6	100.6 (99.6)[c]	100.5 ↑ ↑[d]	106.6 (↓)	C-1	chain-extending
2		99.2 (98.2)	99.2 ↓ ↓	99.3 n.c.	C-1	branch-terminating
3		98.4 (97.4)	98.4 ↓ ↓	98.4 n.c.	C-1	branch point
4		78.7 (77.8)	78.6 ↓ ↓	78.7 ↑	C-2	branch point
		—	—	77.1		
5	75.2	76.2 (75.2)	76.2 ↑ ↑	76.2 ↓		
6		75.8 (74.8)	76.0 (↓)	76.0 ↑		
7		74.8 (73.8)	75.0 n.d.	75.6 n.d.		
8		74.6 (73.6)	74.6 (↑)	74.7 (↑)		
9	73.2	74.2 (73.2)	74.2 (↓)	74.3 (↓)		
10	72.0	73.0 (72.0)	73.0 (↓)	73.0 (↓)		
11	71.6	72.4 (71.5)	72.5 n.c.	72.5 n.c.	C-4	(1→6)-linked pyranosidic, of linear-type chain
12	67.8	68.8 (67.8)	68.7 (↓)	68.7 (↓)	C-6	linked, both chain-extending and branch-point types
13		63.5 (62.5)	63.5 ↑	63.5 ↑	C-6	free, branch-terminating

[a]The chemical shifts are given in p.p.m. relative to tetramethylsilane. [b]These assignments were made by Seymour *et al.*[12]. [c]Data of Seymour *et al.*[12]. [d]Symbols designate the relative change of resonance intensity compared with that indicated in the previous column. ↑ ↑ or ↓ ↓, strong increase or decrease; ↑ or ↓, medium increase or decrease; (↓) or (↑), small increase or decrease; n.c., not clearly changed; n.d., not detected.

TABLE IV

CHARACTERISTICS OF THE SOLUBLE DEXTRANS B-1299 AND LIMIT DEXTRINS[a]

Dextran fraction	*D.h. (%)*	*Yield (%)*	n *(calculated from ^{13}C-n.m.r. data*[b]*)*	n *(calculated from methylation analysis*[c]*)*
Native	—	—	0.73	1.03 (0.67)[12]
D-I	28.5	60	7.71	6.88
D,E	41.3	40	5.19	(7.80)[d]

[a]The analytical procedure was the same as those described in the legend to Fig. 6. [b]Represents the degree of linearity of dextran[12]. The peak height of the resonance at 100.6 p.p.m. was divided by the peak height of the resonance at 99.2 p.p.m. [c]The amount of 2,3,4-tri-*O*-methyl derivative was divided by that of 2,3,4,6-tetra-*O*-methyl derivative; data taken from Table II. [d]Data from the methylation analysis of D,G-I limit dextrin (see Table II).

Fig. 6 shows the ^{13}C-n.m.r. spectra of the water-soluble dextran B-1299 and the limit dextrins. The spectrum of native dextran (see Fig. 6a) was exactly the same as that reported by Seymour *et al.*[12], and corresponded to the features of dextrans containing 2,6-di-*O*-substituted α-D-glucosyl residues at branch points. The 13 sig-

nals obtained for dextran B-1299 were compared with the 6 signals for the virtually linear, B-512F type of dextran (see Table III). The D-I limit dextrin of dextran B-1299 prepared by exhaustive digestion with the α-D-(1→2)-debranching enzyme showed a significant decrease of the resonances in the 78.7–99.2-p.p.m. region and at 63.5 p.p.m. (see Fig. 6b). Apparent changes in the intensity of 13 signals of dextran B-1299 caused by action of the debranching enzyme are summarized in Table III. Based on the assignment of the ^{13}C-n.m.r. resonances described by Seymour *et al.*[12], a drastic decrease in the intensity of the signals at 99.2, 98.4, 78.7, and 63.5 p.p.m. (see Fig. 6b) might be attributable to the removal of single D-glucosyl groups at O-2 branch points.

The D,E limit dextrin was prepared by the simultaneous action of the debranching enzyme and the dextranase II from[4] *Flavobacterium* sp. M-73. Although it had been demonstrated that dextranase II hydrolyzes α-D-(1→6)-linked, linear dextrans in an endo type of action[5], the action of this enzyme on dextran B-1299 might be restricted by the secondary linkages of the α-(1→2) type. Therefore, the d.h. value corresponding to the D,E limit dextrin (41.3%; see Table IV) was somewhat smaller than that of D,G-I limit dextrin (53.3%; see Table I). The ^{13}C-n.m.r.

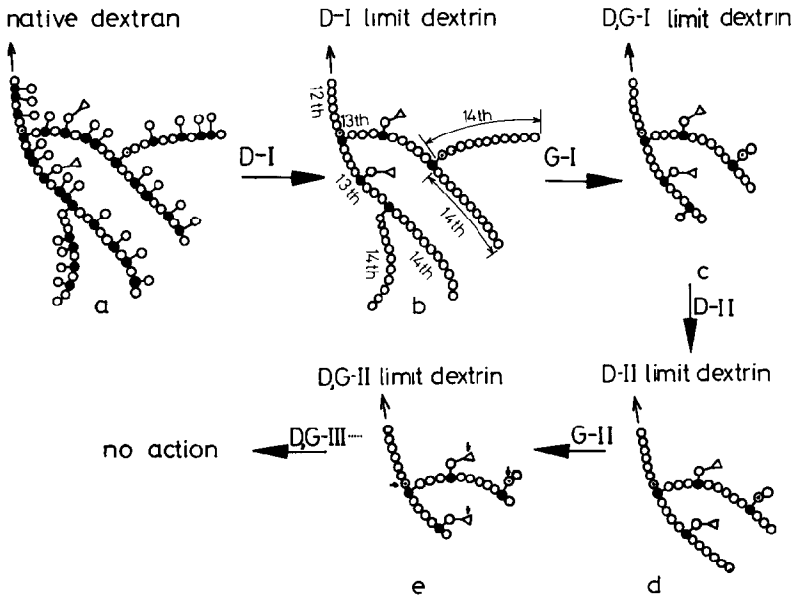

Fig. 7. Model for the sequential degradation of water-soluble dextran B-1299. [(a) Soluble dextran B-1299, (b) D-I limit dextrin, (c) D,G-I limit dextrin, (d) D-II limit dextrin, (e) D,G-II limit dextrin, D-I, first digestion of native dextran with debranching enzyme; G-I, first digestion of D-I limit dextrin with D-glucodextranase; D-II, second digestion of D,G-I limit dextrin with debranching enzyme, and so on. D-Glucosyl residues are joined through: (○), α-D-(1→6) linkage; (●), α-D-(1→2)-branched linkage; (⊙), α-D-(1→2)-linear linkage; (▽), α-D-(1→3)-linear linkage. (○→) shows direction to the reducing terminal. The cardinal number indicates the position of the individual repeating-unit arranged from the reducing terminal. The smallest arrows indicate the D-glucosyl residues not susceptible, or resistant, to both the debranching enzyme and D-glucodextranase.]

spectrum of D,E limit dextrin (see Fig. 6c) showed a pattern intermediate between that of the native dextran and that of D-I limit dextrin (see Fig. 6a, b). Although the D,E limit dextrin gave a new signal at 77.1 p.p.m., the new resonance could be assigned to neither α-(1→2) nor α-(1→6) linkages. Interestingly, the same signal was detected for the D,E limit dextrin of dextran B-1298 (data not shown). According to the diagnostic evaluation of the ^{13}C-n.m.r.-spectral data made by Seymour *et al.*[12], the degree of linearity (*n*, the ratio of chain-extending residues to branch-point residues) of native dextran and of limit dextrins was estimated (see Table IV). The native dextran B-1299 had a constant of $n = 0.73$, which closely agreed with the value ($n = 0.67$–1.03) obtained by methylation analysis. The average chain-length ($\overline{c.l.}$) of 3 was calculated from the methylation-analysis data[16,17]. The limit dextrins D-I and D,E had constants of $n = 7.71$ and $n = 5.19$, respectively, indicating that the degree of linearity of these limit dextrins had increased 7 to 10-fold, compared with the native dextran.

Structure of the water-soluble dextran B-1299. — The results of the sequential enzymic degradation of soluble dextran B-1299 are depicted in Fig. 7. The first cycle of enzymic digestion with the α-D-(1→2)-debranching enzyme and D-glucodextranase released 53% of D-glucose from the dextran B-1299 (see Table I). The former enzyme removed four single D-glucosyl groups at the α-D-(1→2) branch points of every repeating-unit (see Fig. 7b), and the latter enzyme successively hydrolyzed α-D-(1→6) linkages from the nonreducing end of dextran molecules (see Fig. 7c). In the second cycle of digestion, most of the outer chains were processed and shortened by about two to six D-glucosyl residues (see Fig. 7d, e). Further action of glucodextranase would be greatly restricted in the immediate neighborhood of the branch points, and it therefore leaves intact most of the internal, branched α-D-(1→2) linkages connecting the continuous, α-D-(1→6)-linked backbones. Moreover, the sequence of a kojitriosyl unit[14] and 2-*O*-α-nigerosyl-D-glucosyl units[17,19] also hinders the D-glucodextranase action. Thus, 8.4% of the 2,6-di-*O*-substituted D-glucosyl residues in the D,G-IV limit dextrin (see Table II) are approximately attributable to the type (*ii*) already described (*i.e.*, D-glucosyl residues connecting the A and B chains to the B and C chains).

Because more than 70% of the limit dextrin prepared by the combined action of the debranching enzyme and D-glucodextranase was eluted at the V_o of the Sepharose CL-2B column, the molecular weight of this limit dextrin would be $>5 \times 10^6$, even after the hydrolysis of 75% equivalent to D-glucose. Therefore, the native dextran B-1299 has a molecular weight of $>2 \times 10^7$. The average repeating-units (containing 5 branched D-glucosyl residues in the average structure having 15 residues) proposed by Bourne *et al.*[17] are consistent with the results of the debranching-enzyme action. If it is presumed that repeating units of 15 D-glucosyl residues mutually diverge from the B and C chains, the native dextran might be constructed with ~8,200 twigs of repeating units containing 12.3×10^4 D-glucosyl units (see Fig. 8). For convenience, every internal twig was considered to have one intersecting point. Thus, a simple calculation suggested that there are 14 steps of con-

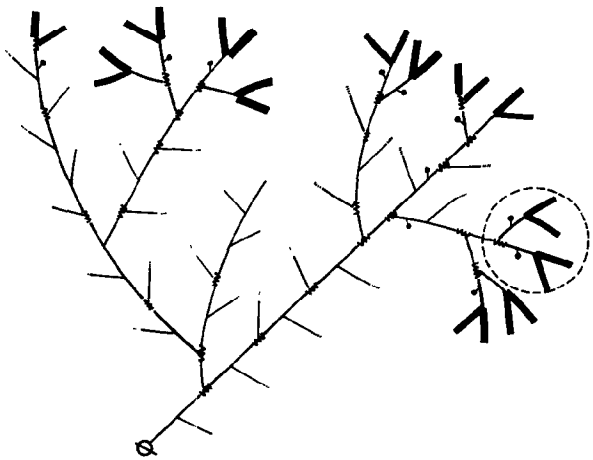

Fig. 8. Tentative model of the whole sequence of the water-soluble dextran B-1299. [(Ø), reducing terminal D-glucose; (——), average repeating-unit composed of 15 D-glucosyl residues; (——-), further sequence of repeating-units was omitted for convenience; (▬▬), 14th repeating-unit from the reducing terminal; (∿∿∿), branched D-glucosyl residues not susceptible to the consecutive enzymic hydrolysis; (—•—), branched structure terminated by α-D-(1→3)-linked D-glucose. The broken circle corresponds to structure (a) shown in Fig. 7.]

nected twigs between the reducing and nonreducing ends. Almost all of the 14th twigs (see Fig. 8, thick lines) could be removed by the concerted action of the debranching enzyme and D-glucodextranase, and the resulting D,G-I limit dextrin was presumably constructed with ~4,100 twigs of repeating units (5.7×10^4 D-glucose). The final D,G-IV limit dextrin may contain <3,000 twigs and ~3,100 residues of α-D-(1→2)-branched D-glucose, which corresponds to 12 to 13 steps of connected twigs. About 4.1×10^4 D-glucosyl residues are branched in the native dextran, and $>3.4 \times 10^4$ residues could be split by the debranching enzyme, indicating that almost four-fifths of the branches were removed. These estimates show good correspondence with the d.h. values obtained with the various enzymic hydrolyses.

The sequential, degradation method presented here was restricted by the occurrence of "anomalous" linkages, *i.e.*, α-D-(1→2)-linked linear chains and α-D-(1→3) linkages, in the dextran molecule. The three dextran-degrading enzymes mainly used in these experiments could not attack these "anomalous" linkages, and produced a limit dextrin having a high molecular weight. The enzymic degradation of polysaccharide from the nonreducing terminal, illustrated herein, would provide an effective approach to clarifying the average repeating-structure of the whole molecule. An accurate determination of the molecular weight of the polysaccharide, and the use of exo-enzymes having a high specificity for the substrate would be essential for depicting the structure.

EXPERIMENTAL

Dextrans. — The water-soluble dextran B-1299 and CS fraction were prepared by the procedure described previously[10]. Clinical dextran was provided by Meito Sangyo Co., Ltd., Japan.

Enzymes. — Dextran α-D-(1→2)-debranching enzyme[2] and dextranase II (ref. 4) from *Flavobacterium* sp. M-73 were prepared by procedures described previously. D-Glucodextranase[6] from *Arthrobacter globiformis* I42 was kindly provided by Prof. T. Sawai (Aichi Kyoiku University, Aichi, Japan). Endo-dextranase from *Chaetomium gracile*[15] was supplied by Sankyo Co., Japan.

Enzyme assay. — The standard methods for assay of the dextran α-D-(1→2)-debranching enzyme and other dextran-hydrolyzing enzymes have been described[1].

Analytical methods. — Assays of total sugar and reducing sugar, and paper chromatography, were conducted as described previously[3], and methylation was performed by the previous procedures[16]. The corresponding alditol acetates were separated in an OV-210 column[21] at 190°. ^{13}C-N.m.r. spectra were recorded with a JEOL FX-100 spectrometer, each dextran (20–50 mg) being dissolved in deuterium oxide (0.5 mL).

REFERENCES

1 M. KOBAYASHI, Y. MITSUISHI, AND K. MATSUDA, *Biochem. Biophys. Res. Commun.*, 80 (1978) 306–312.
2 Y. MITSUISHI, M. KOBAYASHI, AND K. MATSUDA, *Agric. Biol. Chem.*, 43 (1979) 2283–2290.
3 Y. MITSUISHI, M. KOBAYASHI, AND K. MATSUDA, *Carbohydr. Res.*, 83 (1980) 303–313.
4 M. KOBAYASHI, S. TAKAGI, M. SHIOTA, Y. MITSUISHI, AND K. MATSUDA, *Agric. Biol. Chem.*, 47 (1983) 2585–2593.
5 S. TAKAGI, M. SHIOTA, Y. MITSUISHI, M. KOBAYASHI, AND K. MATSUDA, *Carbohydr. Res.*, (1984) in press.
6 T. SAWAI, T. YAMAKI, AND T. OHYA, *Agric. Biol. Chem.*, 40 (1976) 1293–1299.
7 T. OHYA, T. SAWAI, S. UEMURA, AND K. ABE, *Agric. Biol. Chem.*, 42 (1978) 571–577.
8 Y. MITSUISHI, M. KOBAYASHI, AND K. MATSUDA, *Carbohydr. Res.*, 127 (1984) 331–337.
9 A. JEANES, W. C. HAYNES, C. A. WILHAM, J. C. RANKIN, E. H. MELVIN, M. J. AUSTIN, J. E. CLUSKEY, B. E. FISHER, H. M. TSUCHIYA, AND C. E. RIST, *J. Am. Chem. Soc.*, 76 (1954) 5041–5046.
10 M. KOBAYASHI, K. SHISHIDO, T. KIKUCHI, AND K. MATSUDA, *Agric. Biol. Chem.*, 37 (1973) 357–365.
11 R. L. SIDEBOTHAM, *Adv. Carbohydr. Chem. Biochem.*, 30 (1974) 371–444; see pp. 390–399.
12 F. R. SEYMOUR, R. D. KNAPP, E. C. M. CHEN, A. JEANES, AND S. H. BISHOP, *Carbohydr. Res.*, 71 (1979) 231–250.
13 T. WATANABE, K. SHISHIDO, M. KOBAYASHI, AND K. MATSUDA, *Carbohydr. Res.*, 61 (1978) 119–128.
14 T. WATANABE, M. CHIBA, Y. MATSUDA, F. SAKURAI, M. KOBAYASHI, AND K. MATSUDA, *Carbohydr. Res.*, 83 (1980) 119–127.
15 A. HATTORI, K. ISHIBASHI, AND S. MINATO, *Agric. Biol. Chem.*, 45 (1981) 2409–2416.
16 M. KOBAYASHI, K. SHISHIDO, T. KIKUCHI, AND K. MATSUDA, *Agric. Biol. Chem.*, 37 (1973) 2763–2769.
17 E. J. BOURNE, R. L. SIDEBOTHAM, AND H. WEIGEL, *Carbohydr. Res.*, 22 (1972) 13–22.
18 F. R. SEYMOUR, E. C. M. CHEN, AND S. H. BISHOP, *Carbohydr. Res.*, 68 (1979) 113–121.
19 E. J. BOURNE, R. L. SIDEBOTHAM, AND H. WEIGEL, *Carbohydr. Res.*, 34 (1974) 279–288.
20 T. WATANABE, Y. KAMO, K. MATSUDA, AND W. F. DUDMAN, *Carbohydr. Res.*, 110 (1982) 170-175.
21 B. LINDBERG, *Methods Enzymol.*, 28 (1972) 178–195.