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HYDROLYSIS OF FOURTEEN NATIVE DEXTRANS BY *Arthrobacter* ISOMALTODEXTRANASE AND CORRELATION WITH DEXTRAN STRUCTURE*

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ABSTRACT

The isomaltodextranase (EC 3.2.1.94) from *Arthrobacter globiformis* T6 hydrolyzed thirteen dextrans to various extents (11–64% after 13 days) at initially large but gradually decreasing rates. Dextran B-1355 fraction S was, unlike the other dextrans, hydrolyzed by the dextranase initially at the lowest rate among the dextrans used, but the rate was maintained for a long period with little decrease, so that the hydrolysis reached as high as 85% after 13 days. Paper chromatography of these dextran digests revealed that this dextranase produces in addition to isomaltose, one or two trisaccharides [isomaltose residues substituted by (1→2)-, (1→3)-, or (1→4)-α-D-glucopyranosyl groups at the non-reducing D-glucopyranosyl residues] from every dextran used. It is evident that the non-(1→6)-linkages of these trisaccharide products constitute the "anomalous" linkages of the corresponding dextrans. The relative amounts of these trisaccharide products appear to indicate the approximate relative amounts of a particular linkage among the dextrans, or the relative amounts of two kinds of linkages of each dextran. The kinds and the relative amounts of "anomalous" linkages of some dextrans were established on the basis of the trisaccharides produced by isomaltodextranase.

INTRODUCTION

Recently, we found and studied two bacterial exo-dextranases; the glucodextranase[1,2] [exo-(1→6)-α-D-glucosidase, EC 3.2.1.70] that is produced extracellularly by *Arthrobacter globiformis* I42, and isomaltodextranase[3–5] (exo-isomaltohydrolase, EC 3.2.1.94) that is also produced extracellularly by strain T6 of the same bacterial species. (The latter enzyme was new and was registered for entry in the Enzyme Nomenclature[6] in 1975.) The major action of these purified enzymes is exo-lytic release of D-glucose or isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose), respectively, from dextrans and isomalto-oligosaccharides. However, both enzymes also show minor, specific activities on certain non-(1→6)-α-D-glucopyranosidic linkages of gluco-oligosaccharides or of dextrans[2,5]. Formation of transient

*Dedicated to Dr. Allene Jeanes on the occasion of her retirement.

trans(isomaltosyl)ation products from dextrans by isomaltodextranase also occurs[4]. The known identities and proportions of "anomalous" linkages in many dextrans have permitted further observation of the catalytic peculiarities and specificities of this isomaltodextranase. Understanding of the molecular structure of the dextrans used is extended by the results of the unique activity of this enzyme and by comparison of these results with those from the *Arthrobacter* glucodextranase, which are reported in detail elsewhere[2].

MATERIALS AND METHODS

Dextrans. — Dextran N-4 (native)[7] was supplied by Meito Sangyo Co., Nagoya, Japan. Thirteen native NRRL dextrans[8] (enumerated in Table I) and structural information on them were provided by Dr. Allene Jeanes of the Northern Regional Research Center, Peoria, Ill., U.S.A. Three of the dextrans (dextrans B-742, fractions L and S, and B-1355 fraction S) are essentially homogeneous fractions separated from the corresponding polydisperse, native dextrans by graded precipitation with aqueous alcohol[9].

Enzymes. — Isomaltodextranase was prepared with *Arthrobacter globiformis* T6 (IAM 12103, NRRL 4425) and assayed as described previously[3,4]. The enzyme solution used had neither amylase (reductometry and starch–iodine color-reaction) nor endo-dextranase (viscometry) activities. The activity of the isomaltodextranase decreased only 5–10% during 2 weeks at 37° in 20mM acetate buffer at pH 5.0. *Arthrobacter globiformis* I42 glucodextranase[1,2] and *Candida tropicalis* var. *japonica* α-D-glucosidase (transglucosyl-amylase) (EC 3.2.1.20)[10,11] were those used in the previous work. *Rhizopus niveus* glucoamylase (twice crystallized, EC 3.2.1.3) was purchased from Seikagaku Kogyo Co., Tokyo, Japan.

Hydrolysis of dextrans by isomaltodextranase. — The dextran digest was composed of 0.16% or 1.0% of dextran, an indicated amount of isomaltodextranase, and 20mM acetate buffer at either pH 4.0 (incubation for less than 60 min) or at pH 5.0 (incubation for more than a day); toluene was used to inhibit microbial growth. At intervals during incubation at 37°, samples were withdrawn for analysis and heated for 3 min at 100° to arrest the reaction.

The increase of reducing power (with D-glucose as standard) in the digests was measured by either Nelson's[12] or Somogyi's[13] method. The percentage of enzymic hydrolysis of the dextran was calculated from substrate concentrations obtained by the phenol–sulfuric acid method[14] (D-glucose as standard), and was based on the assumption that the reducing power of isomaltose is equal to that of D-glucose.

Paper chromatography. — Paper chromatograms were developed by descending irrigation of Toyo filter paper No. 131 with 6:4:3 (v/v) 1-butanol–pyridine–water for 2 days. Sugars were made visible by the silver nitrate–sodium hydroxide dipping technique[15].

Analytical methods. — Some of the products in dextran digests were isolated

by preparative paper-chromatography. The amounts of the oligosaccharides in the dextran digests were measured with Glucostat (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) after completely conversion into D-glucose by *Candida* α-D-glucosidase. Some of the oligosaccharide products were enzymatically treated with dilute carbohydrases overnight at 37° and rechromatographed on paper. Both gluco- and isomalto-dextranase used for this purpose contained ~0.3 unit per 1 ml digest. The activity of *Rhizopus* glucoamylase used was such that it converted 5–10% of 1.0% starch in digests into D-glucose during 30 min at 37°.

RESULTS AND DISCUSSION

Processes and extents of hydrolysis. — Fig. 1 illustrates the initial and advanced phases of digestion of the fourteen dextrans by isomatodextranase. The degree of hydrolysis generally differs among the dextrans used, but, initially, all of the dextrans, except for the fraction of S of dextran B-1355, are rapidly hydrolyzed to some extent, even by a small amount of enzyme. The rates of hydrolysis then decrease gradually. After one day of incubation in this experiment, thirteen of the dextrans seem to have reached the limit-dextrin stage, but hydrolysis still proceeds at very low

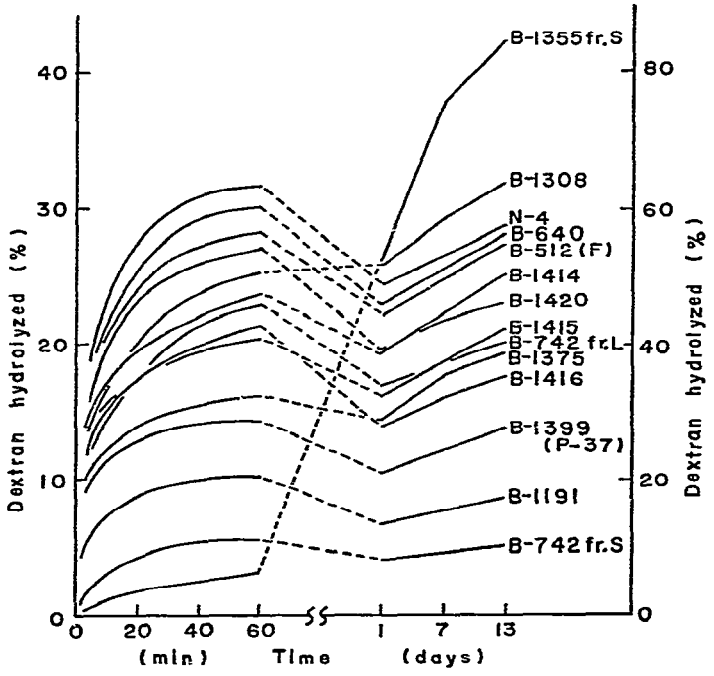

Fig. 1. Hydrolysis of fourteen dextrans (0.16%) by isomatodextranase. Digests for the initial stage (min scale) each contained 0.1 unit/ml enzyme, whereas those for the advanced stage (time scale) each contained 2 units of enzyme/ml.

rates. The lowest initial rate of hydrolysis for dextran B-1355 fraction S is maintained for a long time with little decrease, so that its ultimate extent of hydrolysis eventually exceeds those of all of the other dextrans. As the activity of isomaltodextranase is largely inhibited by the reaction products (unpublished experiments), it is very difficult to attain such high extents of dextran hydrolysis as depicted in Fig. 1 with higher substrate concentrations, under otherwise similar conditions.

The foregoing observations, and the extent of digestion of fourteen dextrans, by isomaltodextranase, differed notably from that in the digestion of the same dextrans by *Arthrobacter* glucodextranase, which we have reported elsewhere[2]. The highest percentages of conversion of dextrans by two dextranases thus far attained

TABLE I

CORRELATION WITH DEXTRAN STRUCTURE OF THE ACTIVITY OF ISOMALTODEXTRANASE AND GLUCODEXTRANASE ON DEXTRANS

Dextran identification[a]	*Linkages in dextran* (%)[b]			*Conversion of dextran* (%)	
	1→ and 1→6	*1→2 and/or 1→4*	*1→3*	*Isomalto-dextranase, 13 days*[c]	*Gluco-dextranase, 1 day*[d]
N-4	93–96	0	4–7	58	30
B-512(F)[e]	95	0	5	55	29
B-640	95	(5)	0	57	29
B-1414	96	(4)	0	50	30
B-1308[f]	95	(5)	0	64	32
B-1420	81	19[g]	0	46	82
B-1415	89	11[g,h]	0	42	47
B-1416	84	16[i]	0	36	43
B-742, fraction L	81	19[j]	0	40	61
B-1375	81	(6)	13	39	17
B-1191	77	(9)	14	18	19
B-1399(P-37)[k]	65	35[l]	0	28	14
B-742, fraction S	57	17[m]	26[m]	11	11
B-1355, fraction S[n]	57	(8)	35	85	3

[a]Dextrans (except for N-4) from the Northern Regional Research Center[8] are identified by the NRRL number of the corresponding bacterial strains in the Culture Collection at that Center. [b]Values of α-D-glucopyranosidic linkages are from periodate-oxidation analyses[8], unless otherwise stated. The accuracy of the periodate values may be influenced by the structurally related tendency of some dextrans to over-oxidation and of others to under-oxidation. [c]From Fig. 1. [d]From Fig. 3 of ref. 2. [e]Methylation structure-analysis established the linkages shown[16]. [f]Linkages by methylation structure-analysis are 96% (1→ and 1→6) and 4% (1→3) at branch points[17]. [g]These linkages presumably are (1→4), as (1→2) linkages were not indicated by serological analysis[18]. [h]Combined enzymic and chemical analyses showed 14% of (1→4) linkages at branch points[19]. [i]Branch-point linkages were shown by combined enzymic and chemical analyses to be 7% of (1→4) and 10% of (1→3)[19]. [j]Methylation structure-analysis has shown[20] these linkages to be (1→4). [k]This strain, from which the dextran initially reported was obtained[8], is now designated in this manner to differentiate it from the variant strain whose dextran has been reported more recently[17]. [l]These linkages have been shown to be mainly (1→2)[18,21]. [m]By methylation structure-analysis[20], branch-point linkages in this dextran fraction are almost exclusively (1→3). [n]Linkages by methylation structure-analysis are 54% of (1→ and 1→6), 35% of (1→3) in linear positions, and 11% of (1→3) at branch points[17].

are shown in Table I, which also summarizes the structural information thus far known for the dextrans used. The difference in the action of the two exo-dextranases may be ascribed partly to their differences in relative rates of splitting four types of α-D-glucopyranosidic linkages. The ratio of the rates of splitting four α-linked glucobioses, isomaltose, maltose, nigerose, and kojibiose, by glucodextranase was[2] 100:3–4:0.03–0.05:0.01–0.02[2]. As these ratios demonstrated very low rates for (1→3) and (1→2) linkages in dextrans, it was presumed that the extents of hydrolysis of dextrans by *Arthrobacter* glucodextranase depend on the proportions of these two kinds of linkages in dextrans[2]. Thus, the very low value (3%) of conversion of dextran B-1355 fraction S by glucodextranase would be reasonable, because the (1→3) linkage content of this dextran is reported to be as high as 46% (Table I). The relatively high conversion-values of dextran B-1420, B-1415, and B-742 fraction L by glucodextranase agree with the finding[18–20] by other methods that their main "anomalous" linkage is (1→4). The (1→4) linkage is much more susceptible to glucodextranase than other "anomalous" linkages.

As for isomaltodextranase, comparison of the rates of splitting of various α-D-glucopyranosidic linkages is complicated, in contrast to results for glucodextranase, because the former dextranase removes not only isomaltose but also "modified isomaltoses", namely, isomaltose residues substituted by (1→2)-, (1→3)-, or (1→4)-*O*-α-D-glucopyranosyl groups at their non-reducing D-glucopyranosyl groups, from dextrans and gluco-oligosaccharides[5]. Although even the relative rates of splitting of various α-isomaltosidic linkages are not available [it is evident that the rate of splitting of the (1→6) linkages is highest; see the last section], it is presumed from the results depicted in Fig. 1 and from the structural information for dextrans in Table I that the rates of splitting of (1→3) and perhaps also (1→2) linkages are not extremely low, but rather are comparable to that of splitting of the (1→4) linkage. The low but steady consumption of dextran B-1355 fraction S by isomaltodextranase (Fig. 1 and Table I), in spite of the very high content of (1→3) linkages, and the limited extents of hydrolysis of the other dextrans having less "anomalous" linkage-contents, imply that distribution of the "anomalous" linkages in dextran molecules largely affects the mode of isomaltodextranase action on dextrans. Thus, two dextrans, the B-742 fraction S and B-1355 fraction S, both of which contain the highest amounts of (1→3) linkage among the dextrans (Table I), show hydrolysis processes widely different from each other, indicating a large dissimilarity between the two dextrans as regards the distribution of this linkage.

Trisaccharide products. — Fig. 2 shows a paper chromatogram of the fourteen dextran–isomaltodextranase digests listed in Fig. 1 after extensive enzymolysis. D-glucose is produced from all dextrans. It is presumed that the D-glucose was not formed by any contaminant enzyme but originated from a readily consumable fraction of each dextran, because it is almost negligible in some digests in spite of extensive digestion of dextran and heavy sample-loading. Only dextran B-1420 contained D-glucose from the outset. Production of one or two higher saccharides, moving between isomaltose and isomaltotriose on paper is noteworthy. These

saccharides constitute one of the three kinds of modified isomaltoses mentioned earlier and now identified.

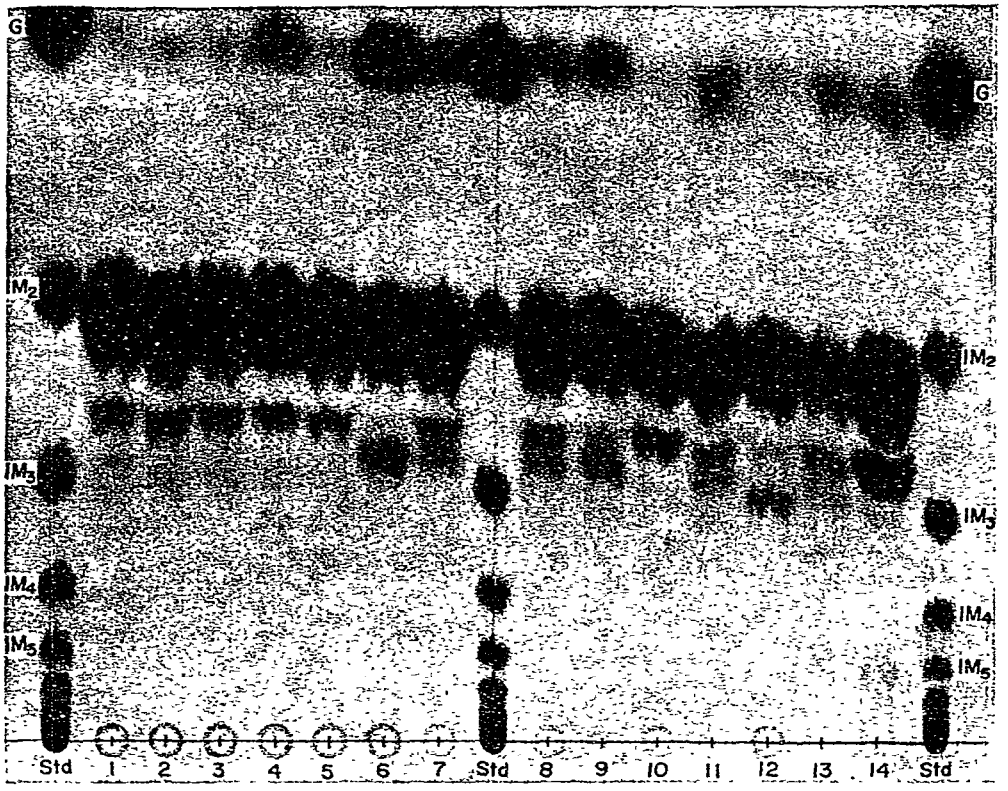

Fig. 2. Paper chromatogram showing trisaccharide products appearing in the dextran–isomaltodextranase digests. The residual portions of the fourteen digests in Fig. 1 continued to be incubated at 37°. After 50 days from the onset, they were heated to stop the reaction and chromatographed. (About one fourth of the enzyme originally given remained active.) Spot samples, 220 μl each. G, D-glucose; IM_2, isomaltose; IM_3, isomaltotriose: and so on. Dextrans of the digests; 1, N-4; 2, B-512(F); 3, B-640; 4, B-1414; 5, B-1308; 6, B-1420; 7, B-1415; 8, B-1416; 9, B-742 fraction L; 10, B-1375; 11. B-1191; 12, B-1399(P-37); 13, B-742 fraction S; and 14, B-1355 fraction S.

The trisaccharide product from dextran N-4 and B-1355 fraction S was already identified by us[5] by liquid chromatography as *O*-α-D-Glc*p*-(1→3)-*O*-α-D-Glc*p*-(1→6)-D-Glc (6-*O*-α-nigerosyl-D-glucose). This saccharide is produced to some extent from every dextran used (Fig. 2). It is evident that the second saccharide produced from several dextrans (B-1420, B-1415, B-1416, B-742 fraction L and S, and B-1191) is *O*-α-D-Glc*p*-(1→4)-*O*-α-D-Glc*p*-(1→6)-D-Glc and the third one, from dextran B-1399(P-37), is *O*-α-D-Glc*p*-(1→2)-*O*-α-D-Glc*p*-(1→6)-D-Glc; this is evident from the relative order of movement on paper of α-linked glucobioses obtained by Aso and Yamauchi[22] with the same solvent as used in the present work. The trisaccharide products from dextrans B-1420, B-1415, B-1416, and B-742 fraction

L were eluted from the preparative paper-chromatograms and treated with *Rhizopus* glucoamylase. In all instances, only the second trisaccharide was degraded to isomaltose and D-glucose, whereas 6-*O*-α-nigerosyl-D-glucose remained intact, indicating that the second trisaccharide has a (1→4)-α-D-glucopyranosyl residue. The third trisaccharide produced from dextran B-1399(P-37), and also 6-*O*-α-nigerosyl-D-glucose isolated from dextran B-1355 fraction S, were resistant to both glucoamylase and glucodextranase.

Fig. 2 shows that production of not only isomaltose but also of 6-*O*-α-nigerosyl-D-glucose from five dextrans, N-4, B-512(F), B-640, B-1414, and B-1308, is quite similar. It is already known[7,17] that the "anomalous" linkage of dextrans N-4, B-512(F) and B-1308 is (1→3). The "anomalous" linkage of dextrans B-640 and B-1414 is now proved to be (1→3), although periodate-oxidation analysis failed to reveal this linkage[8] (see Table I). These five dextrans were hydrolyzed by glucodextranase to a similar extent[2]. The reaction processes and extents of degradation of these five dextrans in isomaltodextranase digestion are also close to each other (Fig. 1), suggesting that not only the amounts but also the distribution of their (1→3)-linkages are similar among the five dextrans. The apparently larger spot of 6-*O*-α-nigerosyl-D-glucose relative to that of isomaltose from dextran B-1375 than those from the foregoing five dextrans would indicate a larger content of (1→3) linkages in this dextran, as also suggested by periodate-oxidation analysis. The result is consistent with the lower extent of digestion by glucodextranase than the foregoing five dextrans (Table I). Production of this trisaccharide from dextran B-1355 fraction S was the largest among the dextrans used. This is as expected, because this dextran contains the largest amount of (1→3) linkages and was the most extensively hydrolyzed by isomaltodextranase.

It is certain that the dextrans giving two trisaccharide products by isomaltodextranase digestion contain two "anomalous" linkages, of which the (1→3) is the more common (Fig. 2). Not only periodate-oxidation analysis, but also many other careful methods employed to determine the kinds and amounts of "anomalous" linkages still failed to reveal the minor linkages. For instance, the (1→4) linkage and its proportion in dextrans B-1420 and B-1415 could be determined by periodate oxidation, serologically, enzymically and chemically, but the other linkage, (1→3), was still not observed. This linkage is clearly demonstrated by production of a corresponding trisaccharide following digestion of these dextrans by isomaltodextranase. It appears that the relative intensities of the two trisaccharide spots on paper from each dextran indicate the approximate relative amounts of the two "anomalous" linkages contained in the corresponding dextran. For example, the reported values [10% of (1→3) and 7% of (1→4) linkages] for dextran B-1416, as obtained by enzymic and chemical analyses[19], appear to agree (Fig. 2) with relative intensities of the two trisaccharide spots from the dextran. The approximate content of (1→3) linkages in dextran B-1399(P-37) could be calculated from the relative amounts of the two trisaccharide products obtained by isomaltodextranase digestion, based on the value (35%) for the content of (1→2) linkages, assuming this value to be

true. The much higher content of (1→4) linkages than (1→3) in dextrans B-1420 and B-742 fraction L coincides with the very high consumption of these dextrans by glucodextranase, to which the (1→4) linkage is much more susceptible than the (1→3) linkage.

Paper-chromatographic monitoring of dextran hydrolysis. — In order to observe the process of dextran hydrolysis by isomaltodextranase in greater detail, two digests of 1.0% dextrans B-512(F) and B-1355 fraction S, respectively, were examined by paper chromatography.

In the case of dextran B-512(F) (Fig. 3), isomaltotetraose (a transglycosylation product) appears temporarily in the early stages, but the trisaccharide product, 6-*O*-α-nigerosyl-D-glucose, appears somewhat later and gradually increases. This fact would indicate that the transfer product tended to accumulate during rapid consump-

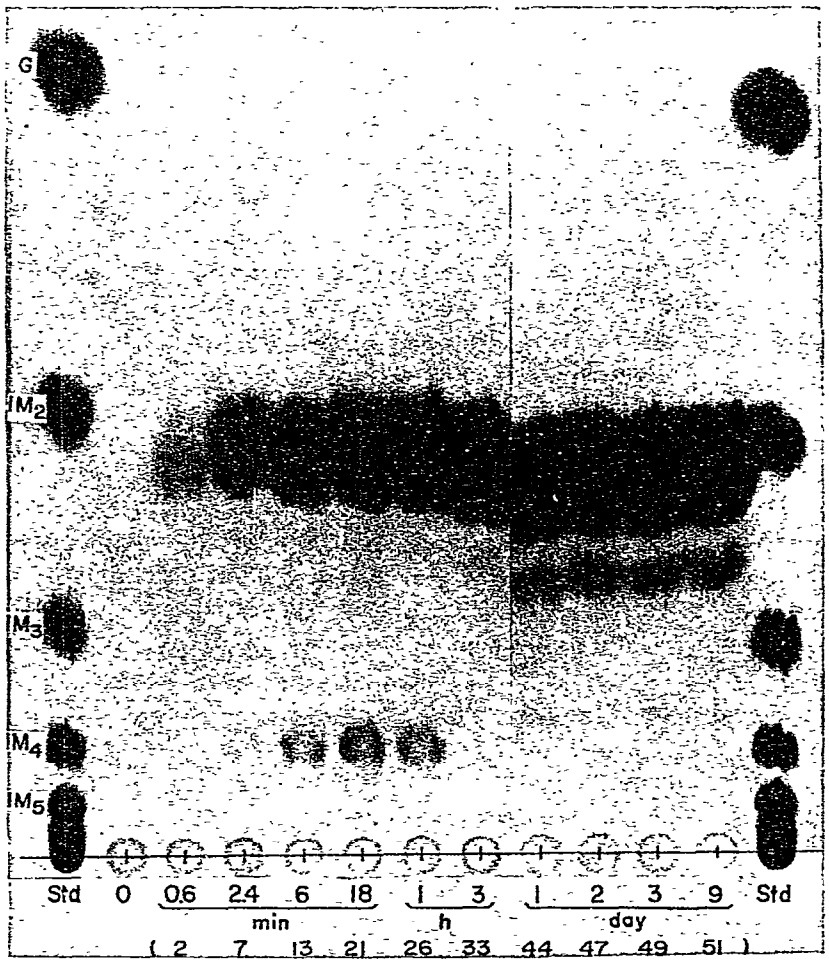

Fig. 3. Paper chromatogram of a dextran B-512(F) (1.0%)–isomaltodextranase (1.0 unit/ml) digest. Spot samples, 100 μl each. Symbols are as in Fig. 2.

tion of successively (1→6)-linked side chains of the dextran, and that, as the enzyme encountered branching points more frequently with the rate of hydrolysis decreasing, the transfer product was consumed and the trisaccharide product gradually accumulated. From this process, it is presumed that the extents of rapid hydrolysis of all dextrans, except B-1355 fraction S in the initial stage illustrated in Fig. 1, indicate approximately the relative amounts of successively (1→6)-linked α-D-glucopyranosyl residues in the side chains. It is also presumed that the rates of release of the three types of trisaccharides mentioned here ("modified isomaltoses") are very low compared with that of isomaltose release in digestion of dextran by isomaltodextranase.

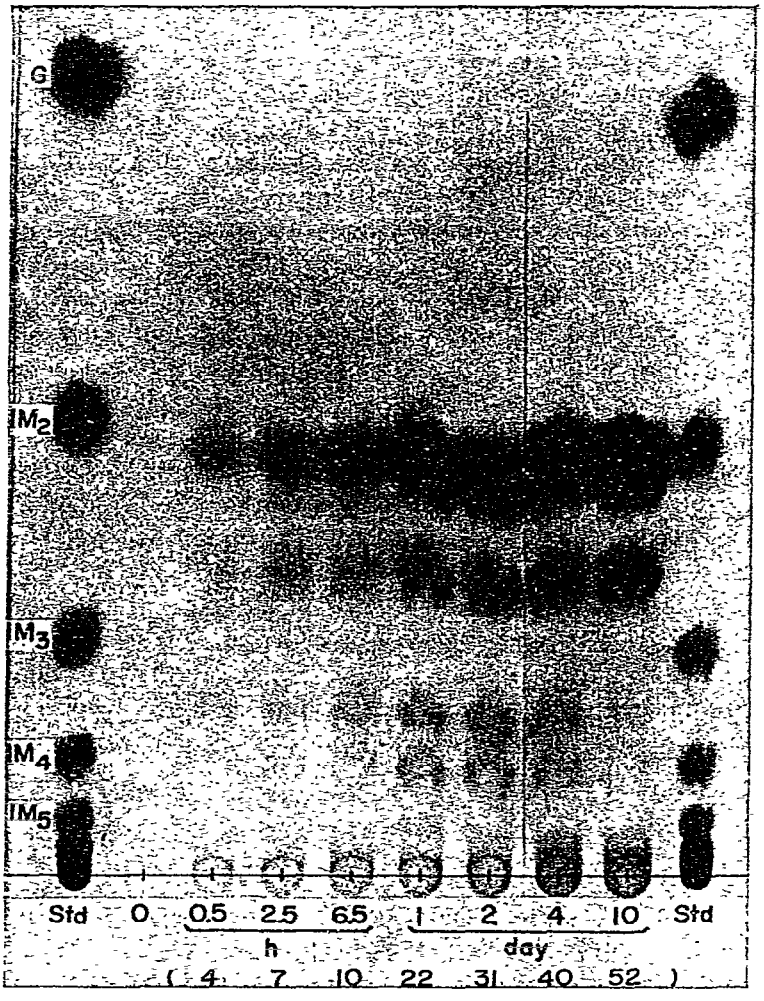

Fig. 4. Paper chromatogram of a digest of dextran B-1355 fraction S (1.0%)–isomaltodextranase (2.0 units/ml). Spot samples, 37 μl each. Symbols are as in Fig. 2.

In the case of dextran B-1355 fraction S (Fig. 4), the trisaccharide product, 6-*O*-α-nigerosyl-D-glucose, appears from the outset and increases together with isomaltose. This fact, and the hydrolysis process of this dextran as depicted in Fig. 1, indicate that the (1→3) linkage unusually abundant in this dextran is uniformly distributed in the molecules without the presence of successive, (1→6)-linked, side chains. The structure of the linear portions of the dextran as being composed of alternating (1→3)- and (1→6)-linked α-D-glucopyranosyl residues has been suggested by Goldstein and Whelan[24] and by Misaki *et al.*[25]. The molar ratio of the trisaccharide product to isomaltose in the later stage of this digest of 1.0% dextran B-1355 fraction S was 1:6.6–6.9. Misaki *et al.*[25] obtained 1:6 as the corresponding ratio for a similar digest.

The paper chromatogram (Fig. 4) shows two still higher saccharides that appear and then later disappear during digestion by isomaltodextranase of dextran B-1355 fraction S ;one migrates between isomalto-triose and -tetraose, and the other coincides with isomaltotetraose. These products, designated as X and Y, respectively, are not trans(isomaltosyl)ation products, but are the last chain fragments that tend to accumulate because of difficult digestibility by the present glycanase[4] of a relatively readily consumable fraction of this dextran.

The products were separately eluted from preparative paper and analyzed by enzymic digestion and by paper chromatography. Saccharide X was completely converted into isomaltose by isomaltodextranase, whereas glucodextranase (whose activity on either (1→3) or (1→2) linkages is very low compared with that on (1→6) linkages[2]) completely split it into glucose and a trisaccharide resistant to isomaltodextranase. As this trisaccharide must be 6-*O*-α-nigerosyl-D-glucose, saccharide X was identified as *O*-α-D-Glc*p*-(1→6)-*O*-α-D-Glc*p*-(1→3)-*O*-α-D-Glc*p*-(1→6)-D-Glc.

Complete splitting of saccharide Y by isomaltodextranase to give isomaltose and 6-*O*-α-nigerosyl-D-glucose indicates that it is a pentasaccharide. As glucodextranase did not liberate glucose from saccharide Y under conditions similar to those used for digestion of saccharide X by the same enzyme, it is highly probable that saccharide Y has a 6-*O*-α-nigerosyl group as its non-reducing end.

Even after exhaustive digestion, either by isomaltodextranase or by glucodextranase, every dextran used still gave a limit dextrin. Analyses of these limit-dextrins from dextran could provide valuable structural information on the corresponding dextrans, such as the distribution of "anomalous" linkages and density of branching.

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