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PURIFICATION AND SOME PROPERTIES OF THE ISOMALTODEXTRANASE OF *Actinomadura* STRAIN R10 AND COMPARISON WITH THAT OF *Arthrobacter globiformis* T6

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ABSTRACT

A newly isolated soil-actinomycete, *Actinomadura* strain R10 (NRRL B-11411), produces an extracellular isomaltodextranase (optimal pH, 5.0) that was purified to homogeneity. It exolytically releases isomaltose and a minor trisaccharide product, α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc, from dextran B-512 and, in addition, forms transient transisomaltosylation products. This pattern of products is qualitatively similar to that previously reported for the isomaltodextranase (EC 3.2.1.94, optimal pH, 4.0) of *Arthrobacter globiformis* T6 (NRRL B-4425). The *Arthrobacter* isomaltodextranase is most active on the (1→6)-α-D-glucopyranosidic linkage, but the relative activity increases with the degrees of polymerization of isomalto-oligosaccharide substrates. In contrast, the relative activity of *Actinomadura* isomaltodextranase is almost constant throughout the same series of substrates, and is much higher on 3-*O*- and 4-*O*-α-isomaltosyl-oligosaccharides than that exhibited by the *Arthrobacter* enzyme; the activity of *Actinomadura* isomaltodextranase on the α-(1→4) linkage is 3–4 times greater than on the α-(1→6). These results indicate that, generically, the bacterial isomaltodextranase is a glycanase, whereas the actinomycetal enzyme is a glycosidase. This difference is reflected in the hydrolysis of dextrans, especially of dextran B-1355 (fraction S), which has a high content of unbranched α-(1→3)-linked residues. In the digest of this dextran with *Arthrobacter* isomaltodextranase, short-chain fragments accumulated that were absent when the *Actinomadura* enzyme was employed.

INTRODUCTION

In 1974, we first reported isomaltodextranase (EC 3.2.1.94), an exo-dextranase produced extracellularly by a bacterium isolated from soil[1]: the bacterium was later re-identified as *Arthrobacter globiformis* T6 (IAM 12103, NRRL B-4425)[2].

Studies employing α-D-gluco-oligosaccharides of known structure have demonstrated that isomaltodextranase recognizes the α-isomaltosyl residue and catalyzes

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the cleavage of whatever α-linkage attaches that disaccharide to a glucose residue[3,4]. Because of this broad specificity, isomaltodextranase can bring about the exolytic release of isomaltose from both α-D-gluco-oligosaccharides and from dextrans by the hydrolysis of (1→2)-, (1→3)-, and (1→4)-*O*-α-isomaltosyl linkages in addition to the (1→6) linkages. The presence of such non-(1→6)-linked residues in all biologically and enzymically synthesized dextrans is well established[5-10]. It has been shown that *Arthrobacter* isomaltodextranase also liberates three kinds of trisaccharide [isomaltose residues linked by (1→2)-, (1→3)-, or (1→4)-*O*-α-D-glucopyranosyl groups on the non-reducing D-glucopyranosyl residues] from certain oligosaccharides and dextrans that contain these linkages[4,11]. The amounts of trisaccharides released from dextrans may reflect the extents to which the non-(1→6)-linked residues occur in these polysaccharides[11].

In order to obtain a similar dextranase from an Actinomycete, we screened a number of strains of this group obtained from many institutes and also isolated from soil. As a result, we obtained one (strain R10) from soil that was identified as *Actinomadura* sp. (NRRL B-11411). The new dextranase produced extracellularly by this microorganism showed the same mode of action on dextrans as did *Arthrobacter* isomaltodextranase: release of both isomaltose and trisaccharides containing non-(1→6)-linkages, together with transient transisomaltosylation. Some interesting differences were also found between *Actinomadura* and *Arthrobacter* isomaltodextranases. This report deals with purification of the new isomaltodextranase and comparison of its catalytic properties with those of the bacterial one.

MATERIALS AND METHODS

Carbohydrates. — Dextran T2000 ($\overline{M}_w$, 2,000,000) derived from dextran B-512 was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and dextran 40 ($\overline{M}_w$, 40,000) derived from dextran N-4 (ref. 11) was supplied by Meito Sangyo Co., Nagoya, Japan. A series of isomalto-oligosaccharides (degree of polymerization, 4–8) was prepared from a partial acid-hydrolyzate of dextran T2000 by paper chromatography on Toyo filter paper No. 131. Dextrans B-512(F) and B-1355 (fraction S)[5] were gifts from Dr. A. Jeanes of the Northern Regional Research Center, Peoria, IL, U.S.A., as were isomaltose, isomaltotriose, and panose [α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc]. Isomaltotriitol was prepared by reduction of isomaltotriose with sodium borohydride.

α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Glc [3-*O*-α-isomaltosyl D-glucose) was a gift from Dr. M. Torii of The Research Institute For Microbial Diseases, Osaka University, Suita, Japan, and α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc (4^2-α-isomaltosylmaltose) was donated by Dr. T. Yamamoto, Osaka City University, Osaka, Japan. α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc was prepared as before from a digest of dextran B-1355 fraction S and *Arthrobacter globiformis* T6 isomaltodextranase[11].

Phenyl α-isomaltoside was synthesized by the method used by Helferich and

Schmitz-Hillebrecht to synthesize phenyl α-D-glucopyranoside[12]. The isomaltose for this synthesis was prepared by extensively digesting dextran T2000 with the bacterial isomaltodextranase and then removing the residual dextran with 80% methanol. Because this starting material was impure, phenyl isomaltoside was isolated from the deacetylated product by paper chromatography with Toyo filter paper No. 131.

The concentrations of oligosaccharides and glycosides in solution were obtained by converting the saccharides in aliquots completely into D-glucose with the aid of *Candida* α-D-glucosidase (transglucosylamylase)[13,14] and estimating the D-glucose with D-glucose oxidase reagent[15]. As the synthetic phenyl isomaltoside was found by analysis with *Candida* α-D-glucosidase to be a mixture of α and β anomers in the ratio of 7:3, the concentration of this substrate was determined with respect to the α anomer.

Cultures. — *Actinomadura* sp. (designated as strain R10, NRRL B-11411) was a new isomaltodextranase producer isolated from a dry road-soil in Nagoya, Japan. This organism was characterized by Dr. T. G. Pridham of Northern Regional Research Center, Peoria, IL, U.S.A. Analyses of a whole-cell hydrolyzate revealed that the organism contains both *meso* and LL isomers of diaminopimelic acid, in addition to glucose, galactose, mannose, madurose, and an unidentified sugar. Morphological studies place the organism in the Section Rectiflexibiles. It is non-chromogenic and forms gray aerial mycelia. It could not be identified with any known species of *Actinomadura* and, pending further work, is best designated as *Actinomadura* sp.[16].

The bacterial isomaltodextranase-producer was *Arthrobacter globiformis* T6 (IAM 12103, NRRL B-4425)[1-3].

Enzymes. — For production of isomaltodextranase, *Actinomadura* R10 was grown in a medium composed of 0.2% ammonium nitrate, 0.1% potassium dihydrogenphosphate, 0.05% magnesium sulfate heptahydrate, 1.5% Meito dextran 40, and 1.5% Daigo Polypeptone (Wako Pure Chemical Industries, Osaka, Japan) at pH 6.0. The inoculated medium was either shaken (180 r.p.m.) in 500-mL flasks (100 mL of liquid in each) or aerated (one liquid volume of air per min) in 30-L jars (20 L of liquid each) for 2 days at 20–22°. Isomaltodextranase was excreted into the medium. Production of the enzyme was low (0.1 unit per mL of culture filtrate at best), and the culture filtrate contained other carbohydrases that released D-glucose from dextran. The isomaltodextranase was purified as described in Results and Discussion.

Isomaltodextranase of *Arthrobacter globiformis* T6 was prepared and assayed as reported before[1]. *Actinomadura* isomaltodextranase was assayed basically in the same way as was *Arthrobacter* isomaltodextranase[1], except that the mixtures were maintained at pH 5.0 with acetate buffer. The unit amount of each isomaltodextranase was calculated by using for calibration a straight-line tangent to curves of the amount of enzyme *vs.* liberation of isomaltose from dextran T2000 (see Fig. 3).

Enzymic digestion of dextrans or oligosaccharides. — Miscellaneous digests, incubated at 37°, were composed of either 0.5 or 1.0% of dextran or 1.0mM oligo-

saccharide (3-*O*-α-isomaltosyl-D-glucose, 0.4mM) in 0.025M acetate buffer at pH 5.0 (*Actinomadura* isomaltodextranase) or at pH 4.0 (*Arthrobacter* isomaltodextranase), together with an appropriate amount of enzyme. When incubation extended longer than overnight, the pH was raised by 1.0 unit. The reaction was stopped by heating for 5 min at 100°, or by mixing with copper reagent or with 2% aqueous sodium carbonate.

The increase of reducing power was determined by either the Somogyi[17] or Somogyi–Nelson[17] procedure, with D-glucose as the standard. The extent of substrate consumption was calculated on the assumption that the reducing powers of isomaltose and other reducing oligosaccharides used or produced in the digests were equal to that of D-glucose, on a molar basis. When D-glucose was released from substrates, it was estimated by the D-glucose oxidase method[15].

Paper chromatography. — Paper chromatograms were developed by descending or ascending irrigation of Toyo filter paper No. 131 with solvents described in the legends. Sugars were detected by the silver nitrate–sodium hydroxide dipping-technique[18].

RESULTS AND DISCUSSION

Purification of Actinomadura *isomaltodextranase.* — Isomaltodextranase was first separated from a 36-L jar-culture filtrate by precipitation with 70% saturated ammonium sulfate. The precipitate, taken up to 800 mL of deionized water, was dialyzed against deionized water to remove the sulfate and mixed with one-tenth of its volume of citrate–phosphate buffer at pH 7.5. About half of the crude enzyme-solution thus obtained was placed on a column (2.4 × 18 cm) of DEAE-cellulose (DE 52, Whatman Ltd., England) and washed with ten-fold-diluted citrate–phosphate buffer at pH 7.5 (column chromatography was conducted at 5°). An alpha amylase contaminant (starch–iodine color reaction) passed through the column. The column was washed with ~400 mL of buffer, and adsorbed enzymes were eluted with 1200 mL of the same buffer (0–0.3M gradient in sodium chloride). Elution of isomaltodextranase as a single peak began toward the end of the washing, and was virtually complete within one sixth of the salt gradient. Enzymes liberating D-glucose from starch or dextran, or splitting maltose or isomaltose were eluted later from the column. The isomaltodextranase fractions were combined and concentrated by ultrafiltration through Dia-filter G-05T (Bio-Engineering Co., Japan); the yield from the culture filtrate was ~300 units (~30% recovery), with specific activity of 5.0 units per mg of protein (absorbance at 280 nm with egg-albumin standard).

The purified, concentrated, enzyme solution did not release D-glucose from dextran or from starch, split neither maltose nor isomaltose, and possessed no β-D-glucosidase activity (cellobiose). Electrofocusing (a 110-mL apparatus of LKB Produkter AB, Sweden) of this enzyme solution showed a single isomaltodextranase peak, having an isoelectric point of pH 4.9, after a two-day run on a double gradient of glycerol (0–50%) and pH (4–6) under a potential of 900 V.

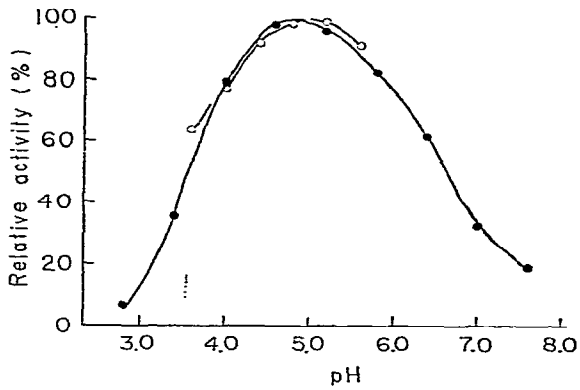

Fig. 1. pH-Dependency of *Actinomadura* isomaltodextranase. Each mixture (5.0 mL) contained 1.0% of dextran T2000, 0.18 unit of enzyme and either 0.04M acetate (○) or 5-fold diluted citrate–phosphate (●) buffer, and was incubated for 45 min at 37°.

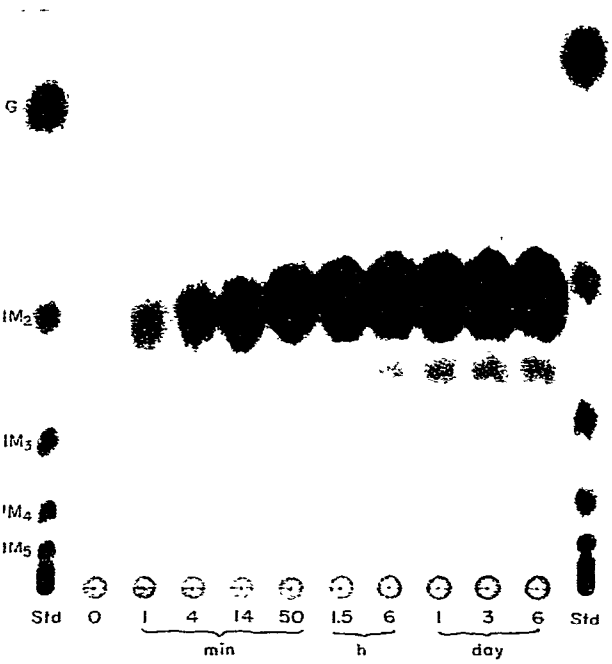

Fig. 2. Paper chromatogram of an *Actinomadura* isomaltodextranase (1.0 unit/mL) digest of dextran T2000 (1.0%). Spot samples, 50-μL each. G, D-glucose; IM_2, isomaltose; IM_3, isomaltotriose; *etc.* The paper chromatogram was developed by descending irrigation with 6:4:3 (v/v) 1-butanol–pyridine–water for 2 days.

pH–Activity relationship of Actinomadura *isomaltodextranase.* — By using dextran T2000 as substrate, the activity of *Actinomadura* isomaltodextranase was measured at different pH values in acetate and citrate–phosphate buffers. The result illustrated in Fig. 1 shows the optimal activity of this dextranase to be at pH 5.0. The activity of *Arthrobacter* isomaltodextranase was optimal[1] at pH 4.0.

Action on dextran T2000. — Fig. 2 is a paper chromatogram of an *Actinomadura* isomaltodextranase digest of 1.0% dextran T2000. Besides isomaltose (the main hydrolytic product), transient formation of isomalto-oligosaccharides as the result of transisomaltosylation occurs at an early stage, and then a trisaccharide [α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc] gradually accumulates. This pattern of product is qualitatively identical to that observed for digestion of dextran by *Arthrobacter* isomaltodextranase[11], and demonstrates a similar mode of hydrolysis of dextran. In a separate experiment, it was observed that 33% consumption of 1.0% dextran T2000 by *Actinomadura* isomaltodextranase brought about only a 17% decrease in relative viscosity of the digest, showing the reaction to be exolytic. The absence of D-glucose, even after extensive digestion of the dextran (Fig. 2), shows that the enzyme solution used was completely free from other, interfering carbohydrases. The transisomaltosylation activity of the *Actinomadura* enzyme seems less pronounced than that of the *Arthrobacter* enzyme[3].

Fig. 3 illustrates calibration curves for hydrolysis of dextran T2000 by both isomaltodextranases in terms of amounts of enzyme *vs.* liberation of isomaltose.

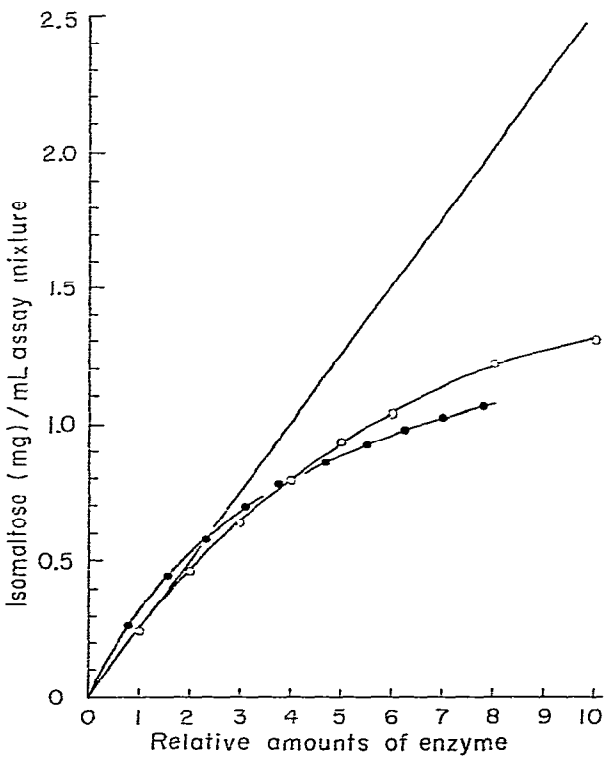

Fig. 3. Calibration curves (amount of enzyme *vs.* liberation of isomaltose from dextran T2000) of *Arthrobacter* (○) and *Actinomadura* (●) isomaltodextranases, and a tangent drawn to the curve for the former isomaltodextranase to obtain the amount of isomaltose liberated proportional to the amount of either enzyme.

TABLE I

RELATIVE ACTIVITIES OF *Arthrobacter* AND *Actinomadura* ISOMALTODEXTRANASES ON VARIOUS OLIGOSACCHARIDES

Structure[a]	*Activities*		*Structure*[a]	*Activities*		*Structure*[a]	*Activities*	
	Arthrobacter	*Actinomadura*		*Arthrobacter*	*Actinomadura*		*Arthrobacter*	*Actinomadura*
IM_3	10[b]	10	$IM_2 \rightarrow S$	0.08	0.8	$IM_2 \rightarrow Ph$	15	18
IM_4	17	10	O			O		
			↓			↓		
IM_5	20	10	$O_1 \rightarrow {}_3O$	0.5	5	$O_1 \rightarrow {}_4O$	7.8	28
IM_6	31	11	O			O		
			↓			↓		
IM_7	30	10	$O_1 \rightarrow {}_3O$	0.2	6	$O_1 \rightarrow {}_4O_1 \rightarrow {}_4O$	6.9	40
			↓					
IM_8	30	10	O					

[a]IM_3–IM_8 is a series of isomalto-oligosaccharides from isomaltotriose to isomalto-octaose; $IM_2 \rightarrow S$, isomaltotriitol; $IM_2 \rightarrow Ph$, phenyl α-isomaltoside. O represents a D-glucose residue. ↓ indicates an α-(1→6) linkage. Other α-linkages are indicated by numerals. Each of the test digests contained 1.0mM oligosaccharide (3-*O*-α-isomaltosyl-D-glucose, 0.4mM) and an appropriate amount of enzyme, and was incubated at 37° for 10–50 min. Experiments were conducted with each of two substrates, isomaltotriose and one of the other oligosaccharides, and the activity ratios were calculated from the initial rates of reaction (within 30% substrate consumption) taking the activity on isomaltotriose as 10. (The series of isomalto-oligosaccharides was examined at the same time). [b]Relative activities of *Arthrobacter* isomaltodextranase on a series of isomalto-oligosaccharides were reported[3] in 1975.

Because the curves are not identical, amounts of enzyme were added such that the relative extents of dextran hydrolysis were as close as possible. The tangent to the curve for *Arthrobacter* isomaltodextranase helped in assaying this enzyme because the amount of isomaltose liberated may be obtained, even when it is not strictly proportional to the amount of enzyme. The straight line in Fig. 3 was used as such for calculating the unit amount of *Actinomadura* isomaltodextranase, even though it is not a correct tangent to the curve. In this way, the units for both isomaltodextranases with respect to hydrolysis of dextran T2000 were made as similar as possible.

The dissimilarity between the calibration curves in Fig. 3 does not seem to arise from dissimilarity in product inhibition of the enzymes. Separate experiments showed that consumption of 1.0% dextran T2000 by both isomaltodextranases was equally inhibited by added isomaltose; that is, ~20% inhibition by 2mM isomaltose and ~60% inhibition by 10mM isomaltose.

Relative activities of isomaltodextranases on various oligosaccharides. — Table I lists the relative activities of *Arthrobacter* and *Actinomadura* isomaltodextranases on various oligosaccharides. Isomaltose is released by both isomaltodextranases from all of the oligosaccharides listed in the table. As explained in the legend, the relative activities were calculated independently for each enzyme by taking as 10 the activity on isomaltotriose. From the table, it is evident that the isomaltodextranases differ

from each other in relative activities on all substrates tested, except for phenyl α-isomaltoside. The activity on this glycoside is similar for both enzymes, relative to isomaltotriose. The initial rate of hydrolysis of isomaltotriose by *Actinomadura* isomaltodextranase was only ~20% higher than that by the *Arthrobacter* enzymes, when the enzymes were compared at the same unit-levels calculated from the calibration curves of Fig. 3. Because the activities on isomaltotriose are similar, all values in Table I may be taken as comparable.

Some features of Table I may be pointed out. The activity of bacterial isomaltodextranase on isomalto-oligosaccharides increases with degree of polymerization up to isomaltohexaose, and then remains constant as far as tests were made, whereas that of the actinomycetal enzyme is the same throughout the series. With the other oligosaccharides, however, the activity of the latter isomaltodextranase is generally higher, sometimes markedly higher, than that of the bacterial enzyme, if activities on isomaltotriose are equalized as shown in the table. It is conspicuous that, apart from the relatively high activities of both enzymes on phenyl α-isomaltoside, the (1→4)-linkages of panose and 4^2-*O*-α-isomaltosylmaltose were hydrolyzed faster by the actinomycetal isomaltodextranase than was the (1→6)-linkage of isomalto-oligosaccharides. This fact, when taken together with the constant rate of hydrolysis of isomalto-oligosaccharides, implies that *Actinomadura* isomaltodextranase may be considered, in accord with Reese's concept of carbohydrases[19], as an α-isomaltosidase rather than an exo-dextranase.

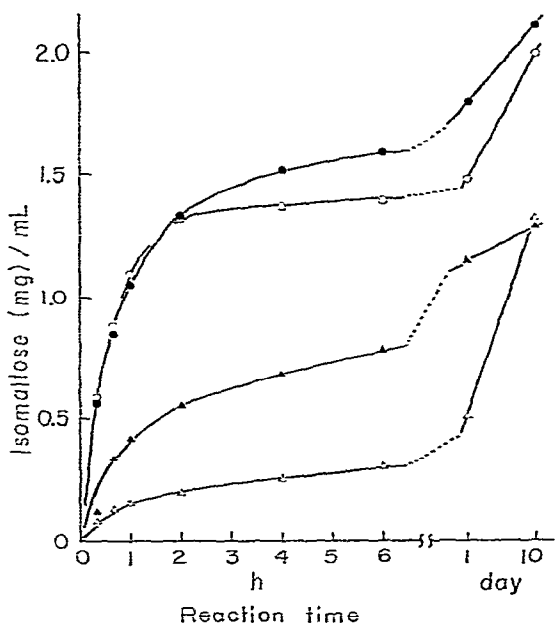

Fig. 4. Comparison of hydrolysis of dextran by both isomaltodextranases. Hydrolysis at 37° of two kinds of dextrans, B-512(F) (●○) and B-1355 (fraction S) (▲△), by *Actinomadura* (●▲) and *Arthrobacter* (○△) isomaltodextranases was monitored by reductometry. The amount of each enzyme was 0.1 unit/mL for dextran B-512(F) and 0.3 unit/mL for dextran B-1355 (fraction S).

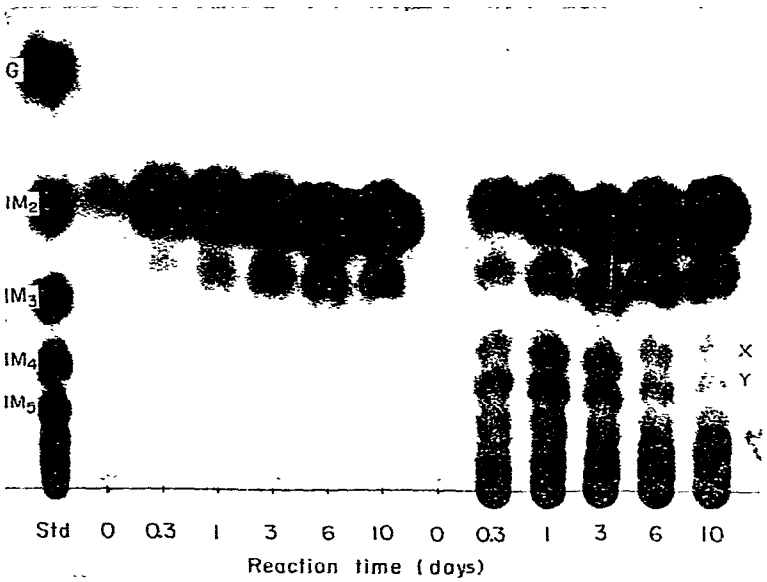

Fig. 5. Paper chromatogram showing the hydrolysis of dextran B-1355 (fraction S) by *Actinomadura* (left) and by *Arthrobacter* (right) isomaltodextranases. Each digest contained 1.0% of dextran and 2 units/mL enzyme. Spot samples were 85-μL each. The paper chromatogram was developed by ascending irrigation with aqueous 65% 1-propanol for two days. Symbols are as in Fig. 2.

The much higher activities displayed by actinomycetal isomaltodextranase on (1→3)- and (1→4)-linkages than by the bacterial enzyme is reflected in the enzymic hydrolysis of dextrans, especially of those dextrans having a high content of non-(1→6)-linkages, as observed in the experiments described next.

Comparison of dextran hydrolysis by both isomaltodextranases. — The isomaltodextranases were compared with regard to dextran hydrolysis (Fig. 4). In hydrolysis of dextran B-512(F), both enzymes initially display almost the same activity but, from the time the reaction slows down, the rate of action of *Actinomadura* isomaltodextranase exceeds that of the *Arthrobacter* enzyme. This divergence may arise from more-frequent encountering of (1→3)-linkages, which are much less susceptible to hydrolysis by the *Arthrobacter* enzyme. In hydrolysis of dextran B-1355 (fraction S), the rates of hydrolysis differ from the outset (Fig. 4). This result is expected, because (1→3)-linked residues alternate with (1→6)-linked residues in this dextran[7,20].

Fig. 5 is a paper chromatogram showing the progress of hydrolysis of dextran B-1355 (fraction S) by both isomaltodextranases. The chromatographic patterns are quite different from each other, as are the progressive increases in reducing power shown in Fig. 4. We earlier reported accumulation and gradual disappearance of two higher oligosaccharides in the *Arthrobacter* isomaltodextranase digest of this dextran, and presumed that they were the last chain-fragments at the reducing ends of the dextran molecules, which are more readily consumable to this stage by this exo-dextranase[11]. Because of heavier sample-spotting, the accumulation of still higher saccharides is now evident in the chromatogram (Fig. 5).

In our previous paper[11], the saccharide migrating between isomalto-triose and -tetraose standards was designated X and identified as α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc. Transient accumulation of saccharide X in the *Arthrobacter* isomaltodextranase digest is understandable in view of the much lower activity of this enzyme on the (1→3)-linkage in comparison to the (1→6)-linkage (Table I). The succeeding oligosaccharide (designated[11] Y) has now been identified as α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc because, in addition to the reason just given, this saccharide was resistant to *Arthrobacter* glucodextranase [which has negligible activity on the (1→3)-linkage[11]]. Furthermore, saccharide Y was split by isomaltodextranase into 6-*O*-α-nigerosyl-D-glucose and isomaltose[11].

The structure of dextran B-1355 (fraction S) as consisting primarily of alternating (1→3)- and (1→6)-linked D-glucose residues has been established by acetolysis and methylation analyses[7,20]. Recent enzymic-degradation studies that employed *Cladosporium* exo-(1→3)-α-D-glucanase in conjunction with *Arthrobacter* isomaltodextranase[21] indicated that a significant proportion of the alternating sequences begin with (1→3)-linkages at the non-reducing ends[21]. If oligosaccharides X and Y constitute fragments remaining after enzymic digestion, and are not transisomaltosylation products, their characterization provides, for the first time, an indication of the sugar sequences at the reducing end of a dextran.

Accumulation of still higher saccharides in the digest of *Arthrobacter* isomaltodextranase must similarly be due to the (1→3)-linkages being much less susceptible to this enzyme. The structures of the saccharides accumulating in the digest of *Arthrobacter* isomaltodextranase, but not in that of the *Actinomadura*, accord with evidence that the linear portions of dextran B-1355 (fraction S) consist of alternating (1→3)- and (1→6)-linked α-D-glucopyranosyl residues[7,20]. It is evident that *Actinomadura* isomaltodextranase readily consumes the aforementioned saccharides (X and Y, and higher ones in the right chromatogram of Fig. 5), without allowing their accumulation, because the enzyme not only readily splits (1→3)-linkages but also has relatively higher activity on smaller saccharides than does the *Arthrobacter* enzyme. These properties of *Actinomadura* isomaltodextranase reflect its oligosaccharidase character in contrast to the *Arthrobacter* isomaltodextranase, which is more like an exo-polysaccharidase, even though there is retention of the anomeric configuration of the product at the time of reaction[3].

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