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Action patterns of amylolytic enzymes as determined by the [1-^{14}C]malto-oligosaccharide mapping method*

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ABSTRACT

A valuable technique for oligosaccharide mapping, utilizing radioactive malto-oligosaccharides, multiple-ascent p.c., and radioautography, has been developed for identifying the action patterns of the glucoamylase isozymes, alpha-amylases, beta-amylase, glucosyltransferase, and glucanosyltransferase. The glucoamylase isozymes act by multi-chain mechanisms on malto-oligosaccharides and most likely on starch and glycogen. The alpha-amylases act endo-wise and randomly hydrolyze α-(1→4)- but not α-(1→6)-glucosidic bonds. These amylases may act by single-chain and/or multi-chain mechanisms, depending on the number of hydrolytic attacks per single encounter of the enzyme and the substrate. The beta-amylases hydrolyze malto-oligosaccharides by a multi-chain mechanism. A fungal glucosyltransferase from *Aspergillus niger* transfers glucose units by a single-chain mechanism from maltose to glucosyl acceptors to yield new gluco-oligosaccharides with α-(1→4) and α-(1→6) linkages. A novel type of transferase isolated from *Bacillus subtilis* acts by a multi-chain mechanism and transfers segments of 2 to 5 glucose residues from malto-oligosaccharides to acceptor co-substrates. An alpha-amylase from the same organism removes maltotriose units from the non-reducing ends of oligosaccharides by a multi-chain mechanism.

INTRODUCTION

The action patterns of amylolytic enzymes on malto-oligosaccharides, starch, and glycogen have been investigated by many methods, including identification of the products of hydrolysis, measurement of the increases in reducing value, observation of decreases in iodine staining capacity, and measurement of decreases of viscosity. From the results, it has been proposed[1] that the hydrolyses proceed by single-chain and/or multi-chain mechanisms. Multi-chain mechanisms involve simultaneous and progressive shortening of all of the chains of the substrate from the non-reducing ends[2]. Single-chain mechanisms involve the complete hydrolysis of one chain before another chain is hydrolyzed[3,4]. A combination of the two mechanisms occurs during a multiple attack per single collision of enzyme and substrate[5,6]. The action patterns of several types of amylolytic enzymes, fungal glucoamylase[2], salivary alpha-amylase[7], bacterial alpha-amylase[8], sweet potato beta-amylase[9], fungal glucosyltransferase[10], and bacterial glucanosyltransferase[11] have been investigated by the unique method of oligosaccharide

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mapping[12]. Some of these enzymes are used industrially in the manufacture of crystalline glucose[13], high fructose syrup[14], citric acid[15], glutamic acid[15], fuel alcohol[16], corn syrup[13], maltose[17,18], and other gluco-oligosaccharides[19,20].

Malto-oligosaccharide mapping[12] involves separating [1-^{14}C]malto-oligosaccharides by 1D-p.c. with four ascents of a solvent system, then spraying the appropriate area of the dried chromatogram with a dilute solution of the enzyme under test, incubating at room temperature for 15 min, heating in an oven at 100° to inactivate the enzyme, developing the chromatogram in the second direction, preparing radioautograms, and identifying the products on the basis of R_F values.

RESULTS AND DISCUSSION

Radioactive malto-oligosaccharides, labeled at position 1 of the reducing units, were synthesized from [1-^{14}C]glucose and cyclomaltohexaose (α-cyclodextrin) utilizing the amylase from *Bacillus macerans*[21]. That the oligosaccharides were labeled at position 1 has been established in earlier studies[22]. Illustrative radioautograms are shown in Fig. 1. As expected, a plot of the R_F values versus d.p. for the malto-oligosaccharides yielded a linear relationship[23]. Fig. 1 shows that glucose and malto-oligosaccharides of d.p. 2–8 were separated readily.

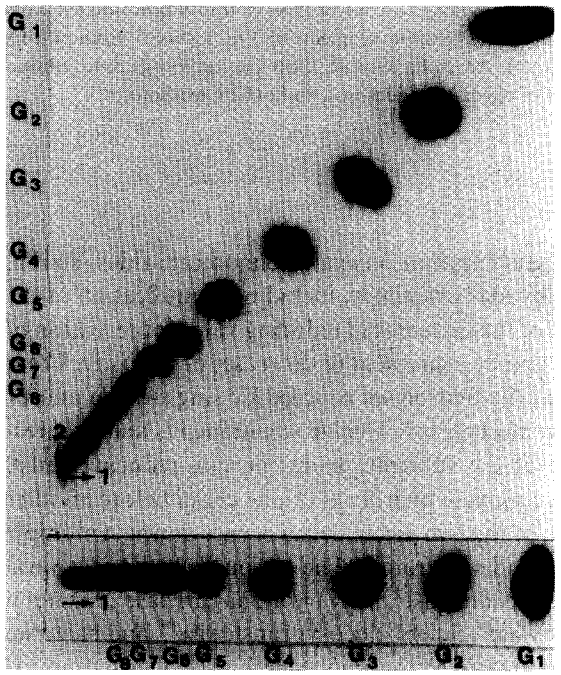

Fig. 1. Separation of malto-oligosaccharides by multiple ascent 1D- and 2D-p.c.: G_1, glucose; G_n malto-oligosaccharide of d.p. n. Arrows and numbers indicate the direction and sequence of chromatography, and the d.p. of the oligosaccharides.

The enzymes tested by the oligosaccharide mapping technique were two isozymes of glucoamylase from *Aspergillus niger*[24], glucoamylases from *Rhizopus niveus*[25] and *delemar*[26], a glucosyltransferase from *A. niger*[10,27], a glucanosyltransferase from *B. subtilis*[11], salivary alpha-amylase from human saliva[7], alpha-amylase from *B. subtilis*[8,28], and beta-amylase from sweet potato[9]. Each of the enzymes was subjected to paper electrophoresis[29] and ultracentrifugation[30] in order to check for purity. On electrophoresis, each enzyme preparation yielded a single protein band (Fig. 2) that contained the expected enzyme activity. The enzymes which migrated only slightly from the origin and reference glucoamylase were subjected to polyacrylamide gel electrophoresis[31]. Under the conditions employed, the protein bands in these preparations were located at the following distances from the origin: glucosyltransferase, 0.7 cm; *R. niveus* glucoamylase, 0.4 cm; salivary alpha-amylase, 1.3 cm; *A. niger* glucoamylase, 4.4 cm. The preparation of glucosyltransferase contained some minor protein components which did not possess amylolytic activity.

The enzyme preparations were subjected to ultracentrifugation in sucrose density gradients with analysis for u.v.-absorbing components by use of the ISCO density-gradient fractionator[30]. The results for fungal glucosyltransferase, fungal glucoamylase, salivary alpha-amylase, and plant beta-amylase are presented in Fig. 3. The molecular weights, calculated using an empirical formula[32], were 99 000 and 107 000 for the isozymes of the *A. niger* glucoamylase[24], 30 000 for glucosyltransferase[10], 95 500 for *R. niveus* glucoamylase[25], 101 000 for salivary alpha-amylase, 190 000 for beta-amylase[33], 70 000 for glucanosyltransferase[11], and 85 000 for bacterial alpha-amylase[12]. D-Glucose

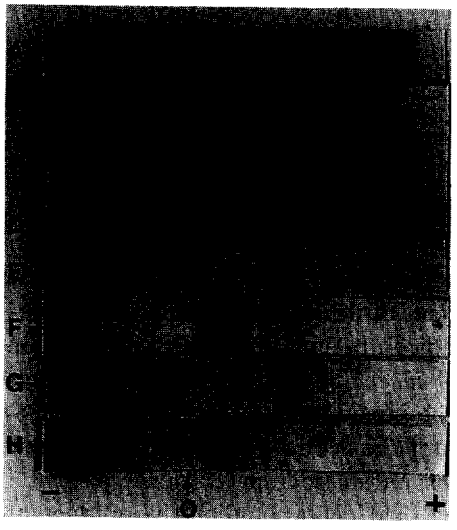

Fig. 2. Paper electrophoresis: A and B, isozymes of glucoamylase of *A. niger*; C, glucosyltransferase of *A. niger*; D, glucoamylase of *R. niveus*; E, alpha-amylase from human saliva; F, beta-amylase of sweet potato; G, glucanosyltransferase of *B. subtilis*; H, alpha-amylase of *B. subtilis*; O, origin.

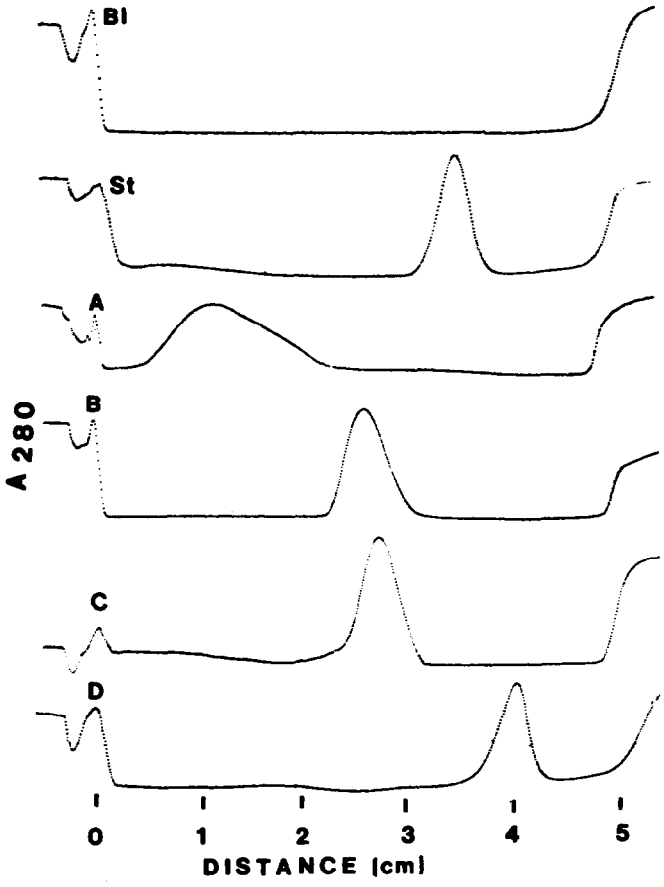

Fig. 3. Density-gradient centrifugation: Bl, blank; St, standard of D-glucose oxidase; A, glucosyltransferase; B, glucoamylase from *R. niveus*; C, salivary alpha-amylase; and D, beta-amylase.

oxidase with molecular weight of 150 000, as determined in the Model E analytical ultracentrifuge[34], was used as the standard.

The use of the mapping technique is illustrated in Figs. 4 and 5. The results in these figures were obtained by 2D-p.c. and four ascents of the solvent 1-butanol–pyridine–water (6:4:3 by vol.)[35]. Figs. 4A and 4B show radioautograms obtained after the action of the individual isozymes of glucoamylase of *A. niger*, and Fig. 4C shows the products after the action of glucosyltransferase from the same organism on the malto-oligosaccharides. Fig. 4D shows a chromatogram of the products of the action of one isozyme of glucoamylase stained with the silver nitrate–sodium hydroxide reagent[36]. The two isozymes of glucoamylase from *A. niger* yield identical radioactive products as well as non-radioactive glucose and evidently act by the same type of mechanism on the malto-oligosaccharides. Trace amounts of [1-^{14}C]glucose were produced from the radioactive maltose by the glucoamylase isozymes (Figs. 4A and 4B) but in low yields.

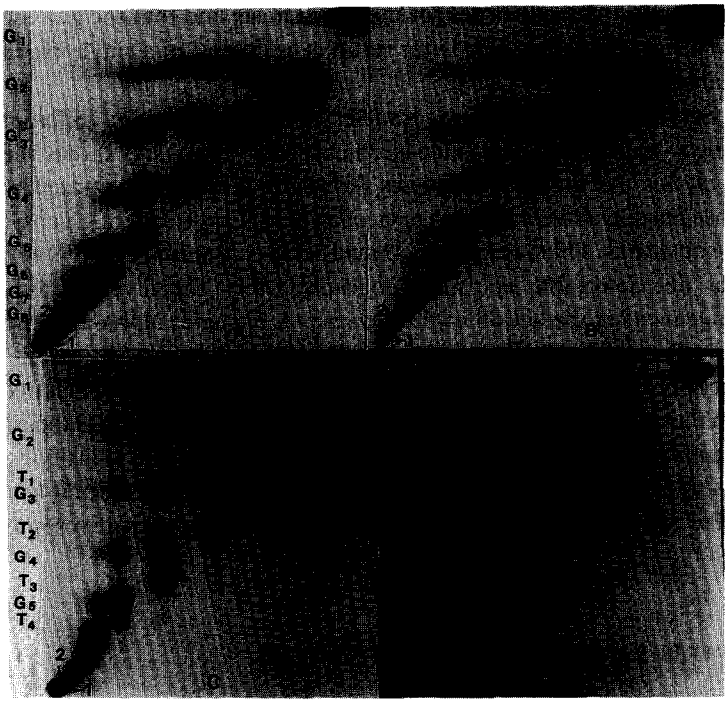

Fig. 4. Oligosaccharide maps: A and B, isozymes of glucoamylase of *A. niger*; C, glucosyltransferase; and D, stained chromatogram of the oligosaccharide map of A. Abbreviations as in Fig. 1 and in the text (Fig. 4D is reproduced with the permission of the publisher[26]).

Glucoamylase hydrolyzes maltose, but only at ~ 10% of the rate for the higher oligosaccharides[2]. The mapping results show that the hydrolysis of the α-(1→4) linked gluco-oligosaccharides by the glucoamylase proceeds by a multi-chain mechanism and not by a single-chain mechanism proposed earlier[38]. The α-(1→6) linkages in gluco-oligosaccharides, starch, and glycogen are hydrolyzed by glucoamylase[37] but at ~ 5% of the rate for maltose; consequently, starch and glycogen will be hydrolyzed completely to glucose by glucoamylase. The glucoamylases are used currently for the conversion of starch into glucose in the industrial production of glucose and high fructose syrup.

The oligosaccharide map in Fig. 4C shows that the fungal glucosyltransferase transferred glucose units from the malto-oligosaccharides to yield new oligosaccharides with α-(1→6) linkages. The simplest oligosaccharide (T_1) synthesized by this transferase has an R_F value lower than that of maltose. This compound has been identified[9] as isomaltose (6-α-D-glucosyl-D-glucose). The other oligosaccharides produced by the transferase are α-gluco-oligosaccharides with (1→4) and (1→6) linkages. The latter oligosaccharides are panose (4-α-isomaltosyl-D-glucose, T_2), dextrantriose (6-α-isomaltosyl-D-glucose, T_3), and dextrantriosyl-D-glucose (4-α-dextrantriosyl-D-glucose, T_4). Some of these oligosaccharides were synthesized for the first time with the transferase enzymes[19,20]. It will be noted that maltotriose, maltotetraose, maltopentaose, and higher

malto-oligosaccharides function as substrates for the transferase, and that the glucose liberated in the reactions is radioactive. The latter result is indicative of a single-chain mechanism for the transferase.

Bacterial glucanosyltransferase catalyzes transfer reactions which are different from those of the fungal transferase. The oligosaccharide map (Fig. 5A) shows the products synthesized by this enzyme. Earlier studies showed that the enzyme transfers segments of 2–5 glucose residues from malto-oligosaccharides to position 4 of glucosyl units of accepting co-substrates. Thus, the products synthesized contain only α-(1→4) linkages. Glucose was not detectable by radioautography or by the silver nitrate–sodium hydroxide reagents after the action of the enzyme. A bacterial alpha-amylase from the same organism, for which the oligosaccharide map is shown in Fig. 5B, exhibits a unique pattern of action on the malto-oligosaccharides by cleaving non-radioactive maltotriose units from the non-reducing ends and leaving radioactive glucose or labeled oligosaccharides. Results with the fungal and bacterial transferases may aid in clarifying the reaction mechanisms for the formation of branched glucose polymers from linear polymers[39,40] and for the conversion of 1-deoxy-1-fluoro carbohydrate derivatives into oligosaccharides[41].

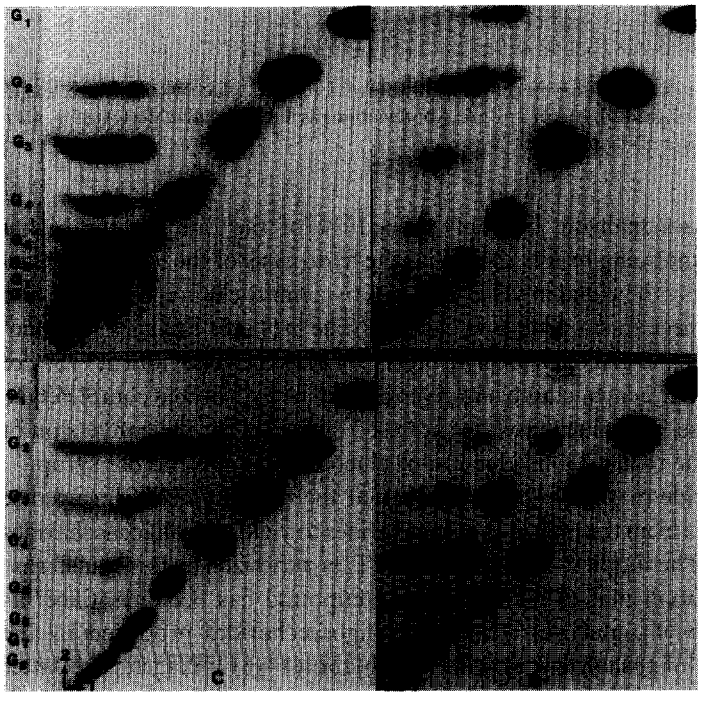

Fig. 5. Oligosaccharide maps: A, glucanosyltransferase; B, alpha-amylase of *B. subtilis*; C, salivary alpha-amylase; and D, sweet potato beta-amylase; T, transfer products (other abbreviations as in Fig. 1) (Fig. 5A is reproduced with the permission of the publisher[11] and Fig. 5C is reproduced with the permission of the publisher[12]).

Figs. 5C and 5D show the products of action of a mammalian salivary alpha-amylase and sweet potato beta-amylase on malto-oligosaccharides, and probably on starch and glycogen. The products of the alpha-amylase action are α anomers, whereas those of the beta-amylase action are β anomers. Fig. 5C shows that the salivary alpha-amylase action begins at the reducing ends of the malto-oligosaccharides, yielding several types of products. Maltotriose was hydrolyzed to yield labeled maltose and non-labeled glucose at a low rate in comparison to the hydrolysis of other oligosaccharides. Maltotetraose was hydrolyzed to give labeled and non-labeled maltose, and the higher malto-oligosaccharides were hydrolyzed to yield labeled maltose and non-labeled oligosaccharides. The nature of the products of hydrolysis indicates a multi-chain mechanism. The sweet potato beta-amylase acts on the non-reducing ends of the malto-oligosaccharides, to give non-radioactive maltose and radioactive oligosaccharides with two less glucose residues (Fig. 5D). Earlier experimental data of other investigators[3,4] have been interpreted to indicate a single-chain mechanism or a combination of single- and multi-chain mechanisms[42] for beta-amylase action, but the mapping results indicate a multi-chain mechanism as the major route for hydrolysis of malto-oligosaccharides by beta-amylase.

EXPERIMENTAL

Preparation of enzymes. — (*a*) *Glucoamylases.* The isozymes of glucoamylase were isolated from extracts of cultures of *A. niger* grown[24] on D-glucose and salt media at room temperature for 85 h. Solvent precipitation, ammonium sulfate fractionation, and chromatography on DEAE-cellulose[2] were used in the isolation. A filtered solution of the crude glucoamylase (5 g per 100 mL of distilled water) was mixed with 0.04M calcium acetate (10 mL), dialyzed against running water for 24 h, then diluted with an equal volume of 0.1M citric acid–disodium phosphate buffer (pH 8.0). The resulting solution was introduced onto a column of DEAE-cellulose (30 g), which had been thoroughly washed with the citrate–phosphate buffer. The protein components on the column were eluted with 0.05M citric acid–disodium phosphate buffer (pH 8.0, 500 mL) followed by 0.05M buffer (pH 6.0, 500 mL) and finally by 0.05M buffer (pH 4.0, 500 mL); 15-mL fractions were collected. On the basis of the products liberated from starch and maltose, the component which was eluted at pH 8.0 was a glucosyltransferase, that which was eluted at pH 7.0 was an alpha-amylase, and those which were eluted at pH 6.0 and 4.5 were glucoamylases. The two glucoamylases were isozymes and possessed different mobilities in paper electrophoresis, had the same pH optimum and the same temperature optimum, and yielded the same products on incubation with starch and malto-oligosaccharides. The isozymes were purified further by re-chromatography on DEAE-cellulose by the above procedure.

Glucoamylase preparations from *Rhizopus delemar*[25] and *niveus*[26] were obtained from Miles Laboratories Inc. (Elkhart, IN, U.S.A.) and from Seikagaku Kogyo Co. (Tokyo, Japan), respectively. A filtered solution of the *R. delemar* preparation (5 g in 100 mL of distilled water) was dialyzed for 24 h, then subjected successively to chroma-

tography on columns of Duolite A-2, Sephadex G-50, and Amberlite XE-64. The enzyme was desorbed from Amberlite XE-64 with 0.05M acetate buffer (pH 5.0). The u.v.-absorbing fractions were assayed for glucoamylase activity and those containing glucoamylase activity were combined, dialyzed, and concentrated ten-fold by lyophilization. This solution was used in the experiments described below. The glucoamylase from *R. niveus* was a highly purified sample[26] and was used as supplied.

(*b*) *Glucosyltransferase.* A preparation of amylases (DIAZYME) from *A. niger* is used for the industrial conversion of starch into D-glucose[13] and was obtained from Miles Laboratories. This sample contained glucosyltransferase, alpha-amylases, and glucoamylases. A sample (5 g) of the enzyme preparation was subjected to column chromatography on DEAE-cellulose (30 g) which had been washed with 0.1M citrate–phosphate buffer (pH 8.0) followed by elution of the transferase with the same buffer. Fractions of the eluate were assayed for transferase activity, using maltose as the substrate. P.c. was used to detect the transfer products. The appropriate fractions were combined, dialyzed, and concentrated by lyophilization. The chromatography on DEAE-cellulose was repeated in order to remove possible contaminants.

(*c*) *Glucanosyltransferase.* An enzyme preparation from *B. subtilis* and labeled HT concentrate was provided by Miles Laboratories. The glucanosyltransferase was assayed by incubating a mixture of the enzyme solution with aqueous 1% maltopentaose (Pierce Chemical Co., Rockford, IL, U.S.A.) in acetate buffer (pH 5.0) for 3 h at room temperature and determining the maltoheptaose synthesized by the enzyme by p.c. A solution of the enzyme preparation (5 g) in water (100 mL) was mixed with ammonium sulfate to 30% saturation, cooled to 4°, then passed through a column containing a mixture of corn starch granules (20 g) and Celite (15 g). Fractions (5 mL) were collected and assayed as described above. The transferase activity was located in the initial fractions. These fractions were combined and ammonium sulfate was added to 70% saturation. The solution was refrigerated for 24 h and the resulting white precipitate was collected by centrifugation. The precipitate was dissolved in water (20 mL) and the solution was dialyzed against distilled water for 24 h. The sample was then subjected to chromatography on DEAE-cullulose which had been washed with 0.1M sodium acetate buffer (pH 5). The column was washed with a gradient (0.1–0.5M) of sodium acetate, and fractions (5 mL) were collected and assayed for transferase activity. Fractions 19–27 containing enzymic activity were combined and dialyzed against distilled water. Ammonium sulfate was added to 50% saturation, and the precipitate which formed was collected by centrifugation and redissolved in water (5 mL). Enzyme activity measurements showed that this solution contained the transferase.

(*d*) *Salivary alpha-amylase.* Salivary alpha-amylase has been purified by solvent fractionation and crystallized from salt solution by multi-step procedures[7,8]. For the present experiments, salivary alpha-amylase was purified more easily by a density-gradient centrifugation method[30]. Human saliva (25 mL) was filtered, lyophilized to dryness, and dissolved in 5 mL of distilled water. Aliquots (0.2 mL) of this solution were subjected to a preparative density gradient centrifugation described in a later section. Fractions (0.2 mL) were collected and assayed for u.v.-absorbing components at 280 nm

and tested for salivary alpha-amylase activity by the starch–iodine method. The appropriate fractions were combined, dialyzed, and used for the experiments described.

(*e*) *Bacterial alpha-amylase.* A partially purified, bacterial alpha-amylase preparation, derived from strain K2 of *B. subtilis*, was provided by Dr. J. Fukumoto (Osaka City University, Osaka, Japan)[28]. The amylase was purified further by preparative density-gradient ultracentrifugation. Fractions from the density gradient containing the alpha-amylase were combined, dialyzed, concentrated, and used in the mapping experiments.

(*f*) *Sweet potato beta-amylase.* Purified beta-amylase from sweet potatoes was obtained from Sigma Chemical Co. Analysis of the sample by electrophoresis and by ultracentrifugation methods showed that the beta-amylase was free of contaminating proteins. Solutions of appropriate concentrations of the purified enzyme in acetate buffer of pH 5 were prepared and used in the experiments.

[1-^{14}C]Malto-oligosaccharides. — A solution of D-[1-^{14}C]glucose (2 mg, 5 μCi) and cyclomaltohexaose (12 mg) in water (0.1 mL) was mixed with a solution (0.1 mL) of *B. macerans* amylase[22]. The mixture was incubated at 37° for 18 h and the amylase was then inactivated by boiling for 10 min. P.c. of this solution revealed a series of gluco-oligosaccharides[21] whose R_F values were identical to those for the oligosaccharides produced by acid hydrolysis of amylose[23]. Radioactivity measurements by Geiger–Mueller counting showed that all of the oligosaccharides were radioactive, *e.g.*, 29 500 c.p.m. for glucose, 15 300 for maltose, 10 400 for maltotriose, and 6200 for maltotetraose. Densitometry of the spots detected after p.c. with the copper sulfate–molybdic acid reagents[2] gave concentration values for the individual oligosaccharides in the range of 0.14 to 0.19%. In this experiment, Whatman No. 1 paper[35] was used with four ascents of 1-butanol–pyridine–water (6:4:3). A radioautogram of the final dried chromatogram was prepared for locating the radioactive compounds by placing the chromatogram in contact with X-ray No Screen film for 3 days. The paper chromatogram was also stained with the silver nitrate–sodium hydroxide reagent in order to locate the reducing sugars[36].

Oligosaccharide mapping. — A 1D-chromatogram of 0.01 mL of the mixture of radioactive malto-oligosaccharides was prepared with the oligosaccharides located along one side of a square (30 cm) of Whatman No. 1 paper. Four ascents of 1-butanol–pyridine–water (6:4:3)[35] were used to separate the oligosaccharides. The area of the chromatogram containing the oligosaccharides was then sprayed with a 2×10^{-3} or 5×10^{-4}% solution of the enzyme prepared as described in the preceding sections[12]. The chromatograms were maintained at room temperature for ~ 15 min, then dried, developed in the second direction by four ascents of the above solvent, dried, and used to obtain a radioautogram. At these concentrations of the enzymes, approximately 10–20% of the glucosidic bonds of the substrates were hydrolyzed.

Paper electrophoresis. — A Spinco model R electrophoresis cell was used on solutions (0.01–0.03 mL) of the enzyme containing 30–100 μg of protein[29]. The buffer was 0.1 M phosphate buffer (pH 7.6) and electrophoresis was carried out for 18 h at 150 V and 10 mA. Starch-hydrolyzing enzymes on the paper electrophoretic strips were

located by spraying with aqueous 1% starch, incubating for 30 min, and spraying either with a solution of iodine (enzyme bands appeared as clear bands on a purple background), or a solution of D-glucose oxidase, followed by peroxidase and finally with *o*-tolidine (enzymes which liberated D-glucose appeared as blue bands).

Gel electrophoresis. — Samples (50 μg) of individual enzymes were subjected to gel electrophoresis in 7% polyacrylamide gels in Tris buffer (pH 8.3) for 6 h at 8 mA per gel[31]. The finished gels were stained with 0.2% Coomassie Blue G-250 in aqueous 10% trichloroacetic acid and then washed with aqueous 5% acetic acid.

Density-gradient ultracentrifugation. — Sucrose density-gradient ultracentrifugation was performed as described in an earlier publication[30]. Analysis for protein in the finished tubes was performed with an ISCO fractionator and analyzer. The gradients were prepared from solutions of 5, 10, 20, 30, and 40% concentrations of sucrose. Samples (0.2 mL) of the enzyme solutions with ~ 0.2% of protein were introduced on top of the gradient solution, the tubes were placed in an SW-65 swinging bucket rotor in a Beckman L2-65 centrifuge, centrifuged at 5.5×10^4 r.p.m. ($4.2 \times 10^5 g$) at 5° for 16 h, then subjected to a density-gradient fractionator[30]. By using standard proteins and an empirical formula[32], the molecular weights of the enzymes were calculated.

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