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PURIFICATION AND SOME PROPERTIES OF A NOVEL MALTOHEXAOSE-PRODUCING EXO-AMYLASE FROM *AEROBACTER AEROGENES*

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Summary

Maltohexaose producing amylase (EC 3.2.1.-) is the fourth known exo-amylase, the three previously known being glucoamylase, β-amylase and *Pseudomonas stutzeri* maltotetraose producing amylase. The enzyme after release from *Aerobacter aerogenes* cells by 0.1% sodium lauryl sulfate extraction was purified by ammonium sulfate precipitation, DEAE-Sephadex column chromatography and Sephadex G-100 gel filtration to 80-fold of the original sodium lauryl sulfate extract activity. It gave a single band on disc electrophoresis, and the molecular weight by gel filtration was 54 000. This amylase showed maximal activity at 50°C and pH 6.80. The pH stability range was relatively wide, the enzyme retaining more than 90% of its initial activity in the range of 6.50—9.0. 80% of the activity was retained after 15 min at 50°C. This enzyme produced maltohexaose from starch, amylose and amylopectin by exo-attack, but did not act on α- or β-cyclodextrin, pullulan or maltohexaitol. Also the enzyme acted on β-limit dextrins of amylopectin and glycogen to form branched oligosaccharides. The unusual reaction of this enzyme on β-limit dextrin is discussed from the standpoint of the stereochemistry of 1,4-α- and 1,6-α-glucosidic bonds. This is the anomalous amylase for which it is recognized that 1,6-α-glucosidic linkages in the substrates can mimic the effect of 1,4-α-bonds, as previously observed in pseudo-priming reactions of *E. coli* phosphorylase.

Introduction

In the previous papers [1,2], we reported the discovery of a novel maltohexaose-producing amylase (EC 3.2.1.-). The new amylase was obtained by the

Symbols and abbreviations: G_1, G_2, G_3... etc. are glucose, maltose and maltotriose ... etc. B_6, B_7, B_8 are "branched" maltooligosaccharides containing a single 1,6-α-link. In symbolic formulae 0 represents an α-glucopyranose unit; ϕ, represents a reducing end D-glucose unit; — is a 1,4-α-link; and an arrow (↓ or →) between glucose units is a 1,6-α-link.

extraction of *Aerobacter aerogenes* cells with 0.1% sodium lauryl sulfate solution, and produced more than 30% maltohexaose from starch. The action pattern of the partially purified amylase was exo, and it cleaved the sixth glucosidic bond from the non-reducing end of reducing end-labeled malto-oligosaccharides prepared by the *Bacillus macerans* amylase coupling reaction [3]. The action pattern of the enzyme was analogous to glucoamylase, β-amylase and *Pseudomonas stutzeri* maltotetraose producing amylase, the last as reported by Robyt and Ackerman [4]. The *Aerobacter* amylase is therefore the fourth known exo-amylase.

This paper summarizes our studies of the purification, characterization and action of this new amylase on various substrates.

Materials and Methods

(1) Materials

Soluble starch (Merck Co. pro analysis), pullulan (Hayashibara Biochem. Research Lab.) and glycogen (Fluka AG, Bucks SG) were commercial materials. Short chain amylose, prepared by debranching of amylopectin by *Pseudomonas* isoamylase, then fractionated by precipitation and determined an average degree of polymerization ($\overline{DP}$) as 23 by reducing end determination, was donated by Hayashibara Biochemical Research Laboratories. A commercial waxy-maize starch was defatted as described by Schoch [5] and used as amylopectin.

Crystalline α- and β-cyclodextrin were prepared by the action of *Bacillus macerans* enzyme [6]. To prepare the β-amylase limit dextrin of amylopectin, defatted waxy-maize starch was extensively treated with soybean β-amylase. The degree of β-amylolysis was 58%. Maltohexaose was separated from an *Aerobacter aerogenes* amylase digest by carbon column chromatography [7], then the homogeneity was confirmed by paper chromatography. Reduced short chain amylose and maltohexaitol were prepared by the reaction of short chain amylose and maltohexaose with sodium borohydride at 100° C for 20 min, then deionized by Amberlite MB-3 [8]. In order to prepare reduced substrate quickly, we employed conditions of 100° C for 20 min, instead of the ordinary mild conditions at room temperature. In our preliminary experiments, we did not observe any difference in the initial velocity of the enzyme action on the reduced short chain amylose prepared at high temperature and at room temperature. A series of maltosaccharides was obtained as by Kainuma et al. [2].

Porcine pancreatic alpha-amylase was a twice crystallized preparation purchased from Worthington Biochemical Corp. Twice crystallized pullulanase and crystalline *Pseudomonas* isoamylase prepared as by Yokobayashi [9] were purchased from Hayashibara Biochemicals Research Laboratory (Okayama, Japan). *Bacillus macerans* cyclodextrin glycocyl transferase was prepared as by Tilden and Hudson [1]. Crude soybean β-amylase was a gift from Nagase Co. This preparation was absolutely free from maltose-splitting activity, compared with a crystalline sweet potato β-amylase which frequently contains the maltose splitting activity [11].

(2) Purification of the enzyme

Aerobacter aerogenes IFO-3321 (Type Culture Collection of Institute for

Fermentation, Osaka, 4-54 Juso-Nishinomachi, Higashi-Yodogawa, Osaka, Japan) was cultured in a modified Wallenfels medium [12] as for pullulanase production. The collected Aerobacter cells were stored in a deep freezer at −10°C. The enzyme was extracted by suspending 55 g of the frozen cells in 275 ml of 0.1% sodium lauryl sulfate solution with reciprocal shaking at 30°C for 24 h. After the cells were removed, the extract was fractionated by adding a fine powder of ammonium sulfate. The fraction precipitating between 0.2 and 0.7 saturated ammonium sulfate was collected by centrifugation, then dissolved in 100 ml of 5 mM Tris · HCl buffer solution at pH 7.0. The solution was dialyzed against the same buffer solution at 4°C for 18 h with several changes of the outer solution. Following dialysis, approximately 110 ml of the enzyme solution was chromatographed on a DEAE-Sephadex A-50 column (2.64 × 45 cm), which was eluted by a gradient of 0 to 0.5 M sodium chloride solution. The enzyme fraction was rechromatographed on a smaller DEAE-Sephadex column (1.9 × 25 cm) using a lower gradient system (0 to 0.3 M sodium chloride). The rechromatographed enzyme fraction was further purified using a Sephadex G-100 column (1.9 × 100 cm).

At each step of the purification, the enzyme activity and the protein concentration were assayed by the Technicon Auto-Analyzer [8,13]. One unit of the enzyme activity was expressed as 1 μmol of glucosidic bond hydrolyzed/min at 40°C and pH 7.0, using reduced short chain amylose as substrate.

(3) Analytical methods and paper chromatography

Total carbohydrate of the digest was determined by the phenol-sulfuric acid method [14]. For the determination of the reducing value, both the Somogyi-Nelson method [15] and the automated ferricyanide method [16] were employed. The enzyme activities of column fractions, the determination of the optimum reaction conditions and the effects of metal ions were determined by the Auto Analyzer procedure using a reduced short chain amylose as substrate [8]. Total protein was determined by the automated Folin-Lowry procedure [8,17], using bovine serum albumin as a standard.

Ascending paper chromatography was carried out using Toyo filter paper No. 50 and 51 with the solvent system *n*-butanol/pyridine/water (6 : 4 : 4, by vol.) at 60°C. This solvent system was used for the discrimination between linear and branched oligosaccharides. In order to survey the action of the enzyme on oligosaccharides, the two dimensional paper chromatographic method was employed [18]. After irrigation, all the chromatograms were treated by the glucoamylase dip method [19] to convert the weakly reducing oligosaccharides into glucose, the glucose then being revealed by the silver nitrate dip method. This technique is effective to detect the spots of maltosaccharides, except maltose which is hydrolyzed extremely slowly during the glucoamylase treatment.

(4) Polyacrylamide disc gel electrophoresis

Disc gel electrophoresis was employed to test the homogeneity of the purified enzyme as described by Davis [20]. Samples were run on 5 × 70 mm gels by using 3 mA/gel. Electrophoresis was stopped when the dye marker (bromophenol blue) reached the bottom of the gels. After being removed from the glass tubes, the gels were stained by Amido Black for 1 h.

(5) Determination of the optimum reaction conditions and enzyme stability

For most of the experiments, a 200 mM Tris maleate buffer (pH 7.0) and 0.4% reduced short chain amylose solution were used as buffer solution and substrate, respectively. The reducing value formed by enzyme action was determined by the automated ferricyanide method.

To determine the optimum pH of the reaction, the increase in reducing value was determined after incubation for 30 min at 40° C. The reaction mixture consisted of 1 ml of enzyme (15 munits/ml), 0.4 ml of the buffer solution of different pH value and 1 ml of the substrate.

For the determination of the pH stability curve, 1 ml of the enzyme solution was incubated with 0.1 ml of 200 mM buffer solution at various pH values (acetate buffer for pH 3—5.2, Tris maleate buffer for pH 5.6—8.6, or sodium carbonate/bicarbonate buffer for pH 9.0—10.0) for 60 min at 40° C. At the end of the period, 1 ml of substrate solution and 1 ml of 200 mM Tris maleate buffer (pH 7.0) were added. The mixture was incubated at 40° C for 40 min, then the remaining enzyme activity was determined. For determining the thermal stability of the enzyme, 1 ml of the enzyme solution was incubated for 15 min with 0.2 ml of 200 mM Tris maleate buffer (pH 7.0) at elevated temperatures using the gradient temperature incubator (Toyo Kagaku, Tokyo, Japan). Then 1 ml of 0.4% substrate solution was added and the reducing sugar formed was determined after the incubation at 40° C for 30 min. The effect of calcium ion on the stability was determined in the same way using a dialyzed enzyme. The optimum temperature of the reaction was determined by incubating 1 ml of the enzyme solution with 0.3 ml of 200 mM Tris maleate buffer solution and 1 ml of 0.4% substrate solution at various temperatures for 30 min.

Results and Discussion

(1) Purification of the enzyme

The sodium lauryl sulfate extract of *Aerobacter aerogenes* cells contained a highly active pullulanase (EC 3.2.1.9) and weak α-amylase. A part of the α-amylase and inactive proteins were removed by the ammonium sulfate precipitation.

The new amylase was eluted from a DEAE-Sephadex A-50 column at a sodium chloride concentration of 0.1—0.15 M compared with a large peak of pullulanase eluted at 0.3—0.4 M. The α-amylase activity was eluted at a still higher sodium chloride concentration. Although the maltohexaose producing amylase fraction obtained by the DEAE-Sephadex chromatography was already free from pullulanase activity, we removed contaminating proteins by rechromatography on the same column by using a lower gradient of sodium chloride. Subsequent gel filtration on Sephadex G-100 separated a small protein peak from the active enzyme as shown in Fig. 1. At this stage of the purification, the enzyme showed a single band on disc gel electrophoresis. A summary of the purification is given in Table I.

At the stage where the preparation showed a single band on disc gel electrophoresis, the enzyme had been purified about 80-fold and had a specific activity of 8.20 units/mg of protein. This specific activity was extremely low

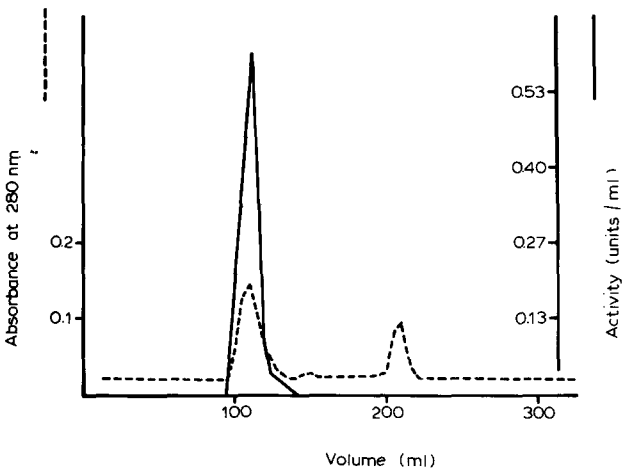


Fig. 1. Sephadex G-100 gel filtration of *A. aerogenes* maltohexaose producing amylase after the second DEAE-Sephadex A-50 purification. Small protein peak was removed by gel filtration. Void volume (V_0) of the column (1.9 X 100 cm) was 55 ml and fractions were collected each 5 ml.

compared with 2500 units/mg of *Pseudomonas stutzeri* amylase [4], 1100 units/mg of sweet potato β-amylase [21] and 500 units/mg porcine pancreatic α-amylase [21]. The specific activity was comparable with that of *Rhizopus niveus* glucoamylase (40 units/mg) [22] or *Aerobacter aerogenes* pullulanase (30 units/mg) [23]. The molecular weight obtained by gel filtration on Sephadex G-100 was about 54 000 and the isoelectric point of the purified amylase by Ampholine electrofocusing was 6.40.

(2) Properties of the enzyme

Fig. 2 shows pH activity and pH-stability of the enzyme. The pH optimum of the enzyme was 6.80, although the enzyme had more than 80% of the maximum activity between pH 5.5 and 8.0. After being kept at various pH values for 60 min at 40°C in the absence of substrate, the enzyme retained

TABLE I

PURIFICATION SCHEME FOR *A. AEROGENES* MALTOHEXAOSE-PRODUCING AMYLASE

Purification steps	Total activity* (units)	Protein** (mg)	Spec. act. (units/mg)	Purification factor	Enzyme recovery (%)
Sodium lauryl sulfate extract	360	3488	0.10	1	100
$(NH_4)_2SO_4$ ppt 0.2—0.7 S	194	1537	0.12	1	54
DEAE-Sephadex-1	83	36.3	2.27	22	23
DEAE-Sephadex-2	49	8.5	5.75	56	14
Sephadex G-100	28	3.4	8.20	80	8

a One unit of activity is expressed as 1 μmol of glucosidic bond hydrolyzed/min at 40°C and pH 7.0.
b Protein was determined by the Folin-Lowry procedure using bovine serum albumin as a standard.

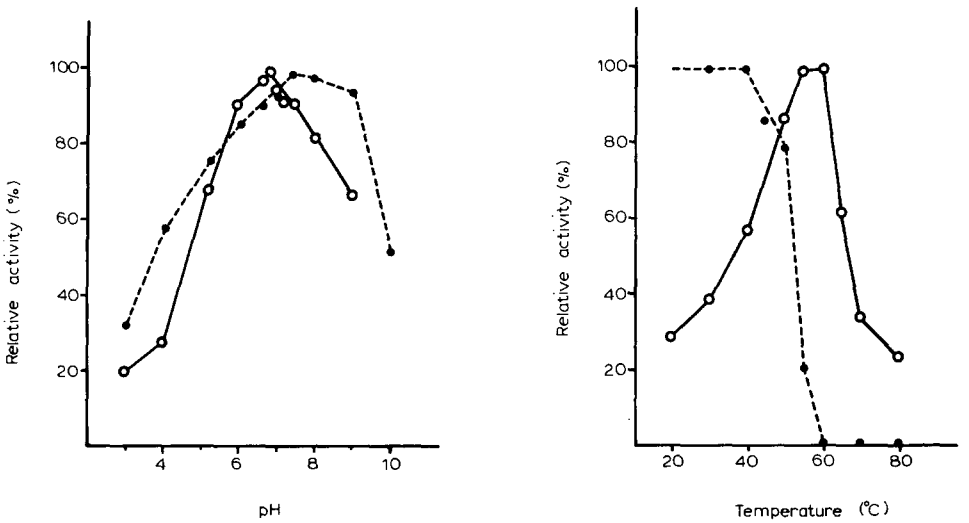


Fig. 2. pH-activity and stability curves for maltohexaose producing amylase. In order to obtain pH-activity curve (o———o), the enzyme solution (15 munits/ml) was incubated with borohydride-reduced short chain amylose ($\overline{DP}$. 23) for 30 min at 40°C with 200 mM buffer solution of various pH. The increase of reducing value was determined by the automated ferricyanide method. To obtain pH stability curve (•- - - - -•), the enzyme solution was kept for 60 min at 40°C at various pH values, then the activity remaining was determined.

Fig. 3. Optimum reaction temperature and thermostability for the maltohexaose producing amylase. To obtain optimum reaction temperature curve, (o———o), 1 ml of enzyme solution was incubated with 0.3 ml of 200 mM tris-maleate buffer (pH 7.0), 1 ml of 0.4% substrate for 30 min at various temperatures. Thermostability (•- - - - -•) was determined by following: 1 ml of the enzyme solution was incubated at various temperatures with buffer (pH 7.0) without substrate for 15 min, then the remaining activity was determined.

about 80% of its activity between pH 6 and 9. Seventy percent of the activity was lost at pH 3.0 and 50% was lost at pH 10.0. The optimum temperature of activity for a 30 min incubation and the thermostability at various temperatures for 15 min without substrate are shown in Fig. 3. The enzyme was extremely unstable above 40°C, and all activity was lost after 15 min at 60°C. The discrepancy between the optimum temperature and the thermostability of the enzyme apparently results from the protective effect of substrate on enzyme stability.

To examine the effect of calcium ion on the thermostability of the enzyme, the enzyme was dialyzed against deionized water for 8 h and incubated for 60 min at various temperatures in the presence and absence of 4.2 mM $CaCl_2$. The activity remaining was assayed by incubating with substrate at 40°C for 60 min. The results are shown in Fig. 4.

Table II shows the effects of metal ions on the activity of the enzyme. Only calcium and strontium ions increased the activity of the enzyme in 1 mM concentration. Mercuric ion completely inactivated the enzyme, and cupric and zinc ions strongly inhibited it.

The effects of several inhibitors on the dialyzed enzyme are shown in Table III. The maltohexaose producing amylase was slightly inhibited by 1 mM iodoacetamide or *p*-chloromercuribenzoate, whereas by comparison β-amy-

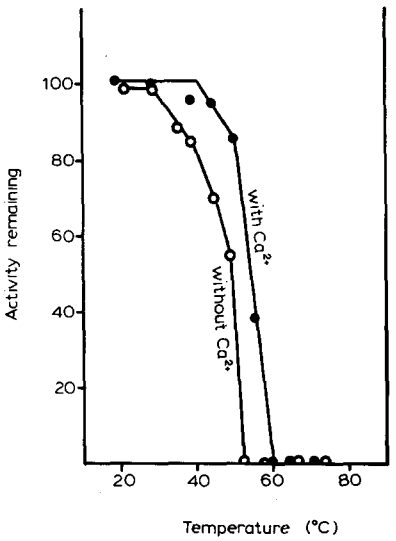


Fig. 4. Effect of Ca^{2+} on the enzyme stability at various temperatures. Dialyzed enzyme was incubated for 60 min at various temperatures in the presence and absence of 4.2 mM $CaCl_2$, then the remaining activity was determined.

TABLE II

EFFECTS OF METAL IONS ON THE ACTIVITY OF *A. AEROGENES* MALTOHEXAOSE-PRODUCING AMYLASE

The activity of the dialyzed maltohexaose-producing amylase was determined by the automated ferricyanide method [7] after incubating with substrate in the presence of divalent metal ion for 9 min.

No ion	100	Hg^{2+}	0
Ca^{2+}	108	Zn^{2+}	20
Ba^{2+}	98	Cu^{2+}	11
Sr^{2+}	111	Sn^{2+}	89
Co^{2+}	99	Mg^{2+}	89

TABLE III

EFFECTS OF VARIOUS INHIBITORS ON THE ACTIVITY OF *A. AEROGENES* MALTOHEXAOSE-PRODUCING AMYLASE

The activity of the dialyzed maltohexaose-producing amylase was determined by the automated ferricynide method after incubation of the enzyme solution (15 munits/ml) with 0.4% reduced short chain amylose, dissolved in 200 mM Tris/maleate buffer (pH 7.0), in 1 mM inhibitor solution for 9 min.

	Maltohexaose producing amylase	β-amylase*
No inhibitor	100	100
Iodoacetamide	86	77
p-Chloromercuribenzoate	96	1
$HgCl_2$	0	—
$AgNO_3$	14	—
Cystein	105	—

* Crystalline sweet potato β-amylase purchased from Worthington Inc.

TABLE IV

RELATIVE REACTION RATES OF MALTOHEXAOSE-PRODUCING AMYLASE ON VARIOUS SUBSTRATES

Details of the substrates are described in Materials. The substrate solutions were prepared as follows: 50 mg of each substrate was dissolved in 1 ml dimethyl sulfoxide, then added 1 ml of 200 mM Tris/maleate buffer (pH 7.0) and 8 ml of distilled water. Five ml of the substrate solution was incubated with 1 ml of the enzyme (33 munits/ml) at 40° C, then each 1 ml was sampled at 30, 60 and 90 min to determine the increase of the reducing value by Somogyi-Nelson method.

Soluble starch	100
Amylose	58
Short chain amylose ($\overline{DP}$ = 23)	115
Reduced short chain amylose	109
Waxy maize starch	65
β-limit dextrin of waxy maize starch	49
Glycogen	46
β-limit dextrin of glycogen	11
α-cyclodextrin	0
β-cyclodextrin	0
Pullulan	0
Maltohexaose	<2
Maltohexaitol	0

lase was almost totally inhibited by *p*-chloromercuribenzoate. On the other hand, $HgCl_2$ and $AgNO_3$ inhibited the enzyme strongly. Cysteine (1 mM) did not increase the activity of the enzyme.

(3) Action of maltohexaose producing amylase on various substrates

To study the substrate specificity, the initial reaction velocity of the enzyme acting on various substrates was determined by reducing value measurements. The results are presented in Table IV. No reaction was observed on α- and β-cyclodextrin, pullulan or maltohexaitol. Maltohexaose was cleaved mainly into maltose and maltotetraose at a rate less than 2% of the rate on soluble starch. Glycogen and high molecular weight amylose were cleaved at 46% and 58%, respectively. β-Amylase limit dextrin of amylopectin was cleaved faster than glycogen. β-Amylase limit dextrin of glycogen was also cleaved at a rate of 11% of the rate on soluble starch. These reactions, which were surprising for an exo-amylase, will be discussed later in this paper. The only substrate which gave a higher relative reaction rate than soluble starch was short chain amylose ($\overline{DP}$ = 23). The action of the enzyme on soluble starch, glycogen, amylopectin β-amylase limit dextrin and glycogen β-amylase limit dextrin etc. are shown in Fig. 5. Under the same reaction conditions, maltohexaose and small amounts of maltotetraose and maltose were formed from soluble starch and glycogen, while B_6, B_7 and B_8, the branched oligosaccharides, were main products from β-amylase limit dextrins. This chromatogram evidently proved that the branched oligosaccharides from β-limit dextrins were not formed by the action of contaminated α-amylase.

(4) Reaction of the amylase on β-amylase limit dextrin and the structures of the resulting branched oligosaccharides

As mentioned previously, we observed an anomalous reaction of the en-

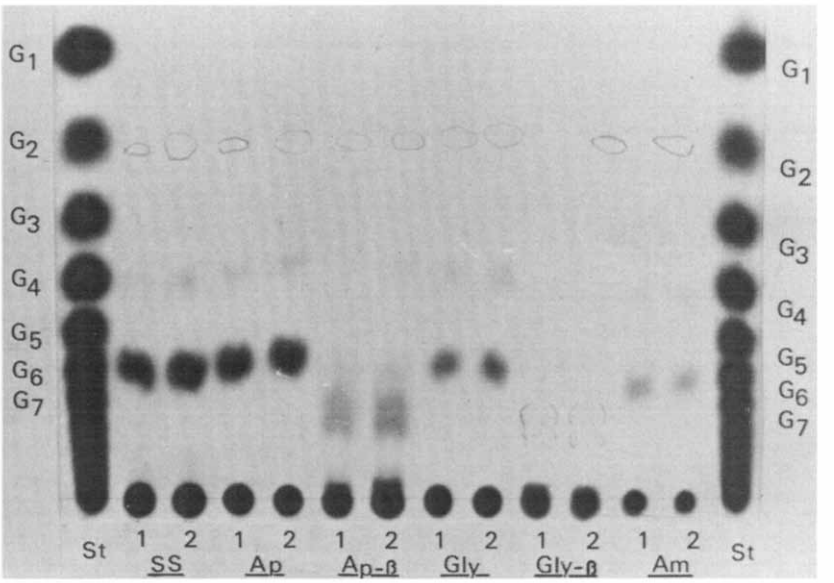


Fig. 5. Paper chromatogram of the reaction products of the enzyme on soluble starch, amylopectin, amylose, glycogen, β-limit dextrin of amylopectin and glycogen. Samples were taken from the experiments described in Table IV. SS, soluble starch; Ap, amylopectin; Ap-β, amylopectin β-amylase limit dextrin; Gly, glycogen; Gly-β, glycogen β-amylase limit dextrin; Am, amylose; St, standard maltooligosaccharides. 1 and 2 are samples reacted for 30 min and 60 min, respectively.

zyme on β-amylase limit dextrin of amylopectin and glycogen to form branched oligosaccharides. To identify the branched saccharides, the digest was fractionated on a Biogel P-2 column [24]. Fig. 6 shows that B_6, B_7 and B_8 are the main branched oligosaccharides which formed presumably from the non-reducing ends of the substrate. The smaller peaks eluted between 400 to 500 ml are

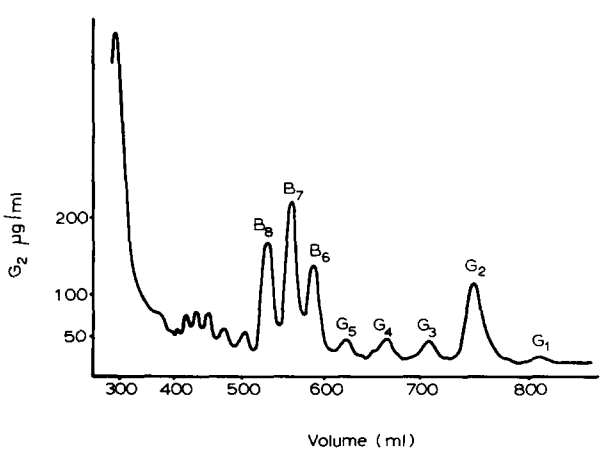


Fig. 6. Biogel P-2 chromatogram (2.64 × 200 cm) of the maltohexaose-producing amylase digest of β-amylase limit dextrin for 24 h. B_6, B_7 and B_8 are the predominant branched oligosaccharides.

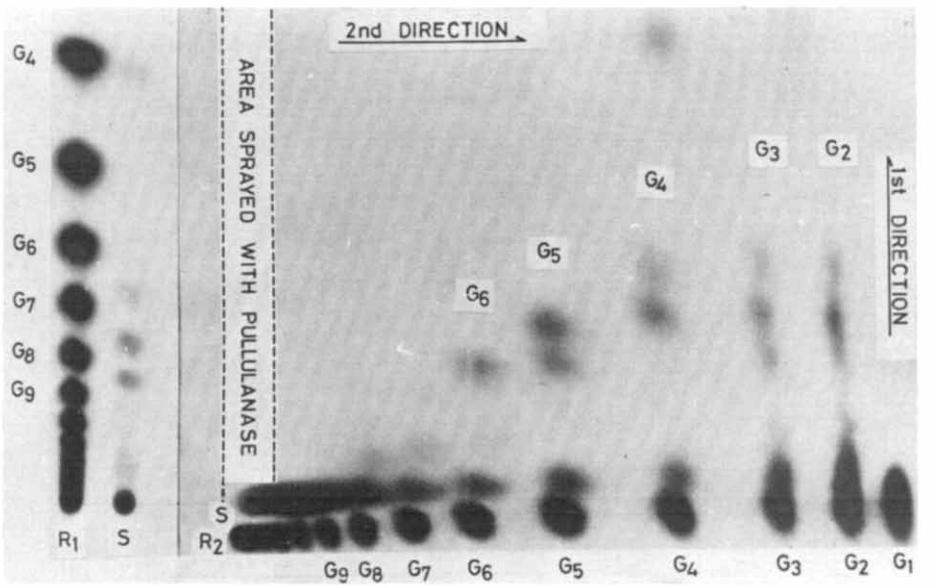


Fig. 7. Two-dimensional chromatogram, showing action of pullulanase on the maltohexaose producing amylase digest of β-amylase limit dextrin of amylopectin. R_1 and R_2 are reference series for the first and second direction of chromatogram. S is the point of application of the sample. After irrigation in the first direction, the left side of the chromatogram containing R_1 and one of the S channels, was cut off for reference. The remaining S channel was sprayed with pullulanase. After allowing enzyme action on the paper, the chromatogram was dried, reference R_2 was applied and the chromatogram was redeveloped in the second direction (perpendicular to the first development).

considered to be multiply branched oligosaccharides which have also been formed by the action of the enzyme on β-amylase limit dextrin. At an early stage of the reaction, we observed a small amount of maltohexaose which was produced from the inner linear part of β-limit dextrin after removal of the branched outer part. Then the maltohexaose was degraded slowly into smaller fragments by the same enzyme.

Two dimensional chromatography of the digest was carried out by spraying with pullulanase shown in Fig. 7. In the chromatogram, we observed faint spots of maltohexaose and maltotetraose and also products of pullulanase debranching as follows:

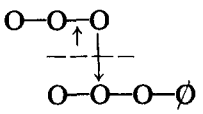
$$B_6 \rightarrow G_3 + G_3$$
$$\rightarrow G_4 + G_2$$

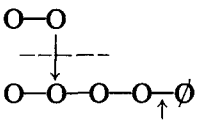
$$B_7 \rightarrow G_4 + G_3$$
$$\rightarrow G_5 + G_2$$

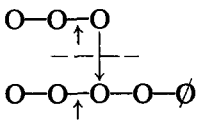
$$B_8 \rightarrow G_5 + G_3$$
$$\rightarrow G_6 + G_2$$

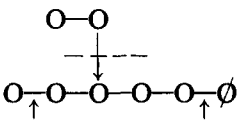
The branched saccharides were also examined by the action of porcine pancreatic α-amylase by the two dimensional paper chromatographic method

[19,21]. From the results of the two dimensional paper chromatography using the known specificity of both pullulanase and porcine pancreatic α-amylase, the structures of B_7 and B_8 were determined to be as follows:

B_{7-1} 

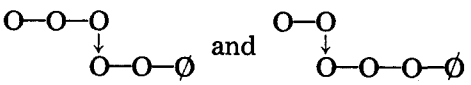
B_{7-2} 

B_{8-1} 

B_{8-2} 

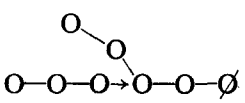
where — —↓— — is the glucosidic bond cleaved by pullulanase and ↑ is the bond cleaved by porcine pancreatic α-amylase.

Similar analysis indicated two possible structures,

 and for B_6.

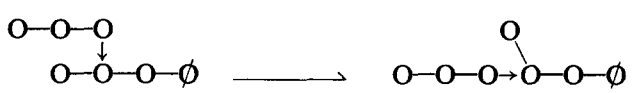
However these are uncertain at this moment because they do not correspond to generally accepted structures at the non-reducing end of amylopectin β-amylase limit dextrin [25].

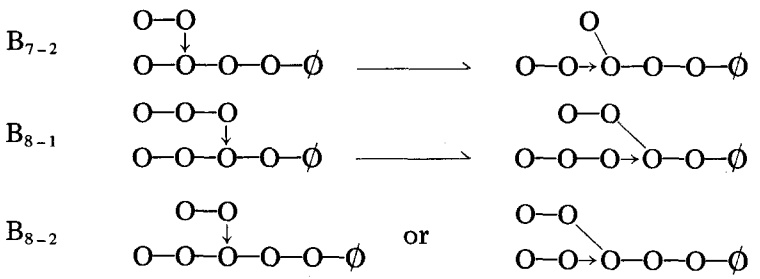
As we have already shown in our previous papers, this enzyme has the strict product specificity of maltohexaose when acting on linear 1,4-α-glucan. To see whether there is any stereochemical similarity between B_7 or B_8 and maltohexaose, molecular models of the branched and linear maltosaccharide were compared. Fig. 8 shows a model of B_{8-1} (3 on 5 B_8), where A and B correspond to the A- and B-chains of an amylopectin molecule. When this model is viewed in the following way,



the six glucose chain was almost the same size as maltohexaose. The binding site of the enzyme, which normally requires a chain of 1,4-α-linked glucose residues, may be able to accept a substrate chain in which one of the 1,4-α-links is replaced by a 1,6-α-link.

If we compare the models, the glucose units of maltohexaose can be exactly superimposed on B_8, except for the distortion of the third glucose from the reducing end. Thus B_7 and B_8 structures apparently mimic the linear maltohexaose structure at the *A. aerogenes* maltohexaose producing amylase binding site, in a manner analogous to that reported by Giri and French for the binding site of *E. coli* phosphorylase [26]. The formation of B_7 and B_8 from β-limit dextrins can be explained by rewriting the symbolic formulas as follows:

B_{7-1} 



By this means, all the B_7 and B_8 structures may be seen to possess a common hexaose structure including one 1,6-α-linkage at the third or fourth glucose unit from the reducing end.

In agreement with Giri and French, we observed that the O_1-O_6 distance in α-D-glucopyranose can be exactly the same as the O_1-O_4 distance. We also observed that maltohexaose and maltohexaose with one 1,6-α-bond are exactly superimposable except for the third glucose unit for B_{7-1} and B_{8-1}, and the fourth glucose unit for B_{7-2} and B_{8-2}. Fig. 9 is a schematic interpretation of substrate binding on maltohexaose producing amylase.

Though we do not know the size of the binding site of this enzyme, from the product specificity of the enzyme, we assume that the size of the site on the left hand side in the schematic representation of Fig. 9 of the catalytic site is six glucose units. Fig. 9 (I) shows binding of a linear substrate. Maltohexaose is mainly degraded into maltotetraose and maltose. (II) and (III) indicate pro-

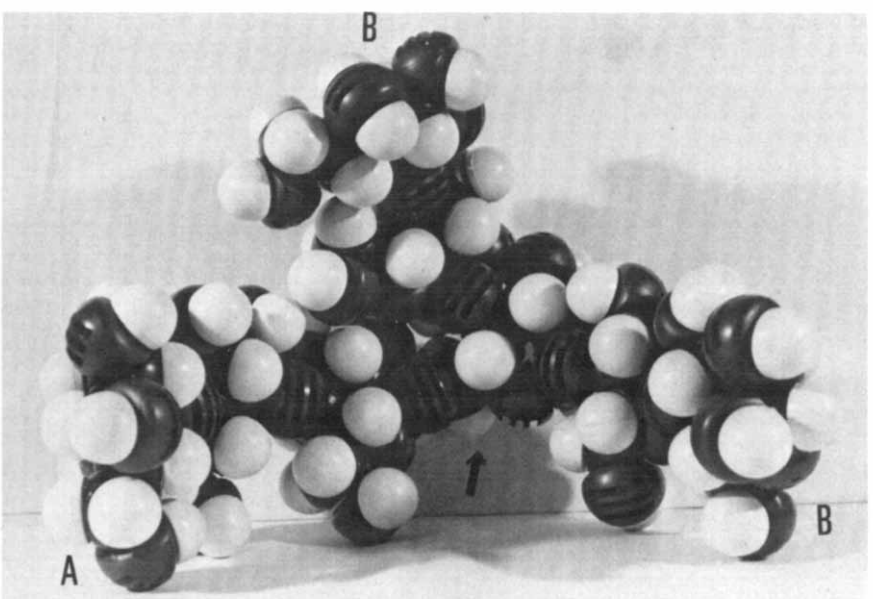


Fig. 8. Molecular model of B_{8-1} (3 on 5 B_8) A and B indicate chains of B_8 coming from the A- and B-chains of amylopectin. (↑) arrow indicates the glucose unit of the branch point which is the only distorted unit compared with maltohexaose.

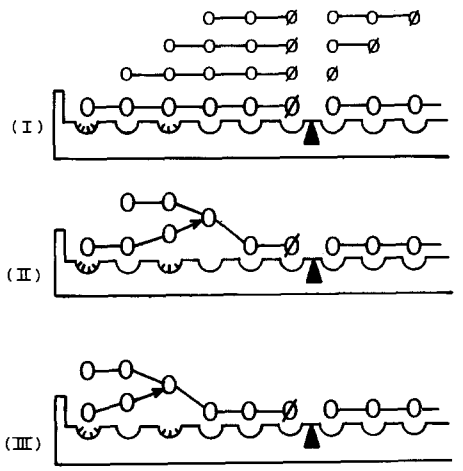


Fig. 9. Schematic representation of the substrate binding site of *A. aerogenes* maltohexaose-producing amylase. The wedge indicates the catalytic site. (I) indicates the mode of binding of linear substrates including maltohexaose. Maltohexaose is mainly degraded into maltotetraose and maltose but also gives a very small amount of maltopentaose, glucose, two molecules of maltotriose. (II) and (III) indicate productive binding of the outer branches of β-limit dextrin. The third glucose unit from the reducing end in (II), and the fourth in (III), are distorted compared with (I).

ductive binding of branched substrates. Although in these diagrams the branched substrate appears somewhat distorted, the enzyme still cleaves the indicated bond to produce B_6, B_7 and B_8 from β-limit dextrin. Bindings and reaction as in (II) and (III) would be possible if the geometry around the 1,6-α-bond was not critically important at the designated sites. The sixth and fourth sites from the wedge are supposed to be more important sites of the enzyme, due to the fact that the product specificity produces only maltohexaose from 1,4-α-glucan. Maltohexaose is then mainly degraded into maltotetraose and maltose.

Although the maltohexaose-producing amylase degrades β-amylase-limit dextrin of amylopectin and glycogen to form branched oligosaccharides, the enzyme is not able to degrade amylopectin completely into small molecular weight oligosaccharides. Further studies on the comparison of the structures between β-amylase limit dextrin and the maltohexaose producing amylase limit dextrin will be reported elsewhere.

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