Biochimica et Biophysica Acta, 391 (1975) 96—108

BBA 67489

A *PSEUDOMONAS* INTRACELLULAR AMYLASE WITH HIGH ACTIVITY ON MALTODEXTRINS AND CYCLODEXTRINS

KANJI KATO, TOSHIYUKI SUGIMOTO, AKINORI AMEMURA and TOKUYA HARADA*

The Institue of Scientific and Industrial Research, Osaka University, Yamada-kami, Suita-shi, Osaka, 565 (Japan)

(Received December 9th, 1974)

Summary

An intracellular amylase from *Pseudomonas* MSl was purified 95-fold in a 14% yield by fractionation with $(NH_4)_2SO_4$, followed by chromatographies on CM-cellulose, hydroxylapatite and Sephadex G-200. On polyacrylamide gel electrophoresis at pH 8.3 it gave a single band and its molecular weight was determined to be 96 000 from its mobility on sodium dodecylsulfate gel electrophoresis. Its activity was maximal at pH 5.5 and 50° C. This enzyme hydrolyzed maltotriose and maltotetraose faster than amylose, but did not hydrolyze maltose. It also rapidly hydrolyzed a series of cyclomaltodextrins. Amylopectin and glycogen, which have branched structures, were attacked much slower than amylose.

The enzyme cleaved the substrates in an endo-wise fashion and produced almost equimolar amounts of glucose and maltose as the final products from various substrates. The enzyme mechanism is discussed on the basis of quantitative autoradiographic data. The K_m values for maltotriose, maltotetraose, cyclomaltohexaose, corn amylose and waxycorn amylopectin were 1.0, 1.4, 3.5, 3.3 and 13 · 10^{-3} g/ml, respectively. The products of the enzyme had an α-configuration. Thus, this enzyme is a specific α-amylase.

Introduction

Pseudomonas SB15, produces an extracellular isoamylase [1] (EC 3.2.1.9) and also intracellular amylases [2], when grown on maltose. A mutant strain, MSl, produces these enzymes constitutively in much larger amounts than those

* To whom reprint requests should be sent.
Abbreviations: G_1, G_2, G_3, etc: glucose, maltose, maltotriose, etc; G_1^*, G_2^*, G_3^*, etc: glucose, maltose, maltotriose, etc., labeled at the reducing end.

induced in the parent strain [2]. These intracellular amylases were separated into three active fractions by CM-cellulose column chromatography. One of them was identified as an α-glucosidase [3] and another as a new, specific amylase. The present paper describes purification and some of the physical and chemical properties of the intracellular amylase.

Methods and Materials

Cultures

A mutant strain *Pseudomonas* MSI which produces extracellular isoamylase and intracellular α-glucosidase and amylase constitutively, was used [2]. The medium contained 2% maltose, 0.4% sodium glutamate, 0.15% $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.001% $MnCl_2 \cdot 4H_2O$ and 0.001% NaCl [2]. The pH was adjusted to 7.0 with 0.01 M HCl.

Assay of amylase activity

The standard reaction mixture (0.9 ml) contained 0.2% or 0.002 M substrate in 0.1 M acetate buffer (pH 5.5). The reaction was initiated by adding 0.1 ml of enzyme solution, and the mixture was incubated at 40° C for an appropriate time. Then the reaction was stopped by adding 1 ml of Somogyi alkaline copper reagent. Increase of reducing power was determined by the method of Nelson [4]. Maltose, maltotriose and maltotetraose have high reducing power, so glucose release during the reactions with these compounds was assayed directly with glucose oxidase [5]. One unit of enzyme activity is defined as the amount liberating 1 μmol of aldehyde group as glucose per min.

Determination of protein concentration

Protein concentration was estimated by the method of Lowry et al. [6] using bovine serum albumin as a standard. The amounts of protein in effluents from columns were determined from their absorbance at 280 nm, using a Hitach 124 UV-VIS spectrophotometer.

Gel electrophoresis

Polyacrylamide gel electrophoresis was carried out at pH 8.3 by the method of Davis [7] with a current of 2 mA/tube for 3.5 h. Then gels were stained with Coomassie brilliant blue [8]. To relate the position of the protein band to that of amylase activity, two identical gels were subjected to electrophoresis at the same time. One gel was stained immediately after electrophoresis while the other was cut into 0.2-cm sections. Each section was extracted with 0.5 ml of 0.1 M acetate buffer (pH 5.5) and the enzyme activity of the extract was measured. Sodium dodecylsulfate gel electrophoresis was run at pH 7.2 at 8 mA/tube for 5 h [9].

Paper chromatography

The reaction products were examined as follows. At zero time and after appropriate incubation times, samples (1 ml) of reaction mixture were heated in a boiling water bath for 5 min to inactivate the enzyme, cooled and treated

with Amberlite IR-120 to remove cations. The resulting supernatant was evaporated to dryness in vacuo and the residue was dissolved in 50 μl of water. 10 μl of the solution was subjected to paper chromatography on Toyo-Roshi No. 50 paper. The chromatogram was developed by the descending method with *n*-butanol/pyridine/water (6 : 4 : 3, by vol.) as solvent. Reducing sugars on the chromatogram were detected with alkaline $AgNO_3$ reagent. Two-dimensional paper chromatography was used to examine the action of the enzyme on maltodextrins labeled at the reducing end. After chromatography in the first direction and removal of the reference channels, the area containing oligosaccharides was treated with enzyme on the paper. The paper was then dried and a new reference spot was applied. Then chromatography was carried out in the second direction using the same solvent system. Radioautograms of the chromatograms were prepared using X-ray film (Film, Kx., for Medical use, Fuji Film Co.) and an exposure time of 14 days.

Substrates

Maltotriose and maltotetraose were prepared by partial acid hydrolysis of amylose, followed by chromatography on a charcoal column [10]. Waxycorn amylopectin, obtained from Nihon Shokuhin Co., was defatted with methanol. β-Limit dextrin was prepared by prolonged incubation of waxycorn amylopectin with sweet β-amylase, removal of maltose by dialysis, and freeze-drying. A series of cyclodextrins and pullulan were products of Hayashibara Biochemical Laboratory, Okayama. ^{14}C-labeled maltodextrins labeled at the reducing end were prepared by the reaction of ^{14}C-labeled glucose and cyclomaltohexaose in the presence of amylase from *Bacillus macerans* [11]. Labeled C_1—C_5 compounds were separated and isolated by multiple ascending paper chromatography [12]. Other substrates were commercial products of the highest purity available.

Preparation of crude enzyme

Cells of *Pseudomonas* MS1 were harvested from 20 l of culture by continuous centrifugation at 20 000 × *g* and washed twice with 2 l of 0.01 M acetate buffer (pH 5.5). Then they were suspended in 2 l of the same buffer. This cell suspension in batches of 50 ml was disrupted by sonication for 30 min at 20 kcycles using a Kaijo Denki type 4280 ultrasonic oscillator in an ice-water bath. The broken cell debris was removed by centrifugation at 50 000 × *g* for 60 min and the supernatant was used as crude enzyme.

Symbols and abbreviations

G_1, G_2, G_3, etc. represent glucose, maltose, maltotriose, etc. and G_1^*, G_2^*, G_3^*, etc. represent glucose, maltose, maltotriose, etc. labeled at the reducing end.

Results

Purification of intracellular amylase

All operations were carried out at 4° C, unless otherwise stated. The sonicated supernatant (1.8 l) obtained from the cells in 20 l of culture was brought

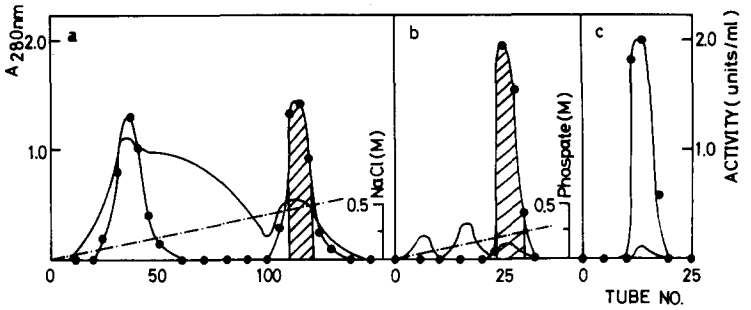


Fig. 1. Elution pattern of intracellular amylase from columns of CM-cellulose (Fig. 1a), hydroxylapatite (Fig. 1b) and Sephadex G-200 (Fig. 1c). Each fraction contained 10 ml of effuluent. ———, absorbance at 280 nm; •——•, amylase activity; — · —, concentration of NaCl or phosphate, Shaded areas indicate fractions pooled for the subsequent step. Corn amylose (Sigma co.) was used as the substrate.

to 50% saturation of $(NH_4)_2SO_4$ and the resulting precipitate was removed by centrifugation. Further solid $(NH_4)_2SO_4$ was added to the supernatant to give 70% saturation. The precipitate was collected by centrifugation, dissolved in 250 ml of 0.01 M acetate buffer (pH 5.5) and dialyzed against running water for 16 h at room temperature and then against 5 l of 0.01 M acetate buffer (pH 5.5) for 24 h. The precipitate formed during dialysis was removed by centrifugation and the supernatant obtained was applied to a CM-cellulose column (5 cm × 15 cm) equilibrated with 0.01 M acetate buffer, pH 5.5. The column was eluted with a linear gradient obtained by placing 500 ml of 0.01 M acetate buffer (pH 5.5) in the mixing chamber and 500 ml of the same buffer containing 0.5 M NaCl in the reservoir. Amylases were eluted as two peaks at about 0.2 and 0.4 M NaCl (Fig. 1a). The first peak corresponded to α-glucosidase [3]. The second peak in fractions Nos 110—120 was collected. Next most of the NaCl was removed by concentrating the solution to 15 ml by vacuum ultrafiltration through a collodion membrane. The dialysed amylase was then applied to a hydroxylapatite column (2 cm × 15 cm), equilibrated with 0.01 M of phosphate buffer (pH 6.0). The column was eluted with a linear gradient of 0.01 M to 0.5 M phosphate buffer (pH 6.0). The active fractions, Nos 24—30, were collected (Fig. 1b). Then the solution was concentrated and desalted in a collodion membrane against the same 0.01 M buffer. The amylase solution (10 ml) was further purified by gel filtration on a Sephadex G-200 column (3 cm × 45 cm) equilibrated with 0.1 M acetate buffer (pH 5.5). Amylase activity

TABLE I

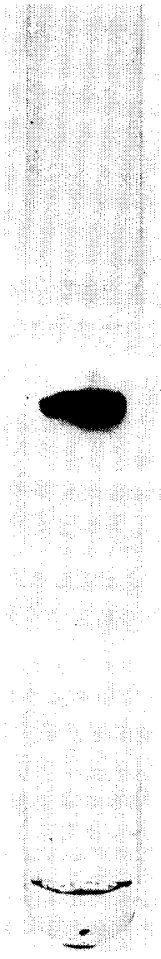
ENZYME PURIFICATION

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)
1. Crude enzyme	1800	2900	650	0.22
2. $(NH_4)_2SO_4$, 50—70% satn	250	2200	560	0.25
3. CM-cellulose	110	71	230	3.2
4. Hydroxylapatite	64	6.2	110	19
5. Sephadex G-200	74	4.3	92	21

emerged as a single symmetrical peak (Fig. 1c) coinciding with the protein. Fractions Nos 13—20 were collected and concentrated to 10 ml. The purified amylase retained more than 90% of its activity after storage for 1 month at 4° C in 0.1 M acetate buffer (pH 5.5). The purification procedure is summarized in Table I. The amylase was purified about 95-fold with a yield of about 4.3 mg of enzyme from 20 l of culture.

Purity of the enzyme preparation

On disc-gel electrophoresis at pH 8.3, the purified amylase behaved as a single protein component (Fig. 2) and extraction of slices of unstained gel showed that amylase activity coincided with the protein band.



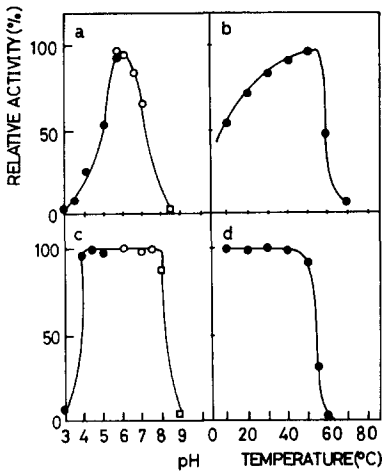


Fig. 2. Polyacrylamide gel electrophoresis of the purified amylase at pH 8.3.

Fig. 3. Effect of pH (a,c) and temperature (b,d) on the activity and stability, respectively, of amylase. 0.1 M acetate buffer (pH 3.0—5.5) (○———○), 0.1 M phosphate buffer (pH 5.5—7.5) (●———●) and 0.1 M ammonium buffer (pH 8.0—9.0) (□———□) were used. For examination of pH stability, enzyme solutions of various pH values were incubated for 30 min at 40° C, and then adjusted to pH 5.5 and residual activity was measured by the standard method. For examination of temperature stability, in 0.1 M acetate buffer solution (pH 5.5) was incubated for 30 min at various temperatures. Then solutions were cooled rapidly to 4° C and remaining activity was assayed.

Properties of the amylase

The molecular weight of the enzyme was estimated by comparing its mobility on sodium dodecylsulfate disc gel electrophoresis [9] with those of a series of compounds of known molecular weight (Mann Res. Lab.). The molecular weight of the enzyme determined by sodium dodecylsulfate gel electrophoresis along with standard graphical interpolation gave a molecular weight of 96 000.

Activity was maximal at pH 5.5 (Fig. 3a). The activity in a 30-min reaction period increased with increase in temperature up to 50° C (Fig. 3b). The amylase was stable on incubation for 30 min at 40° C at about pH 4.5—7.5 (Fig. 3c). Its thermal stability at 10—80° C was measured in 0.1 M acetate buffer (pH 5.5). As shown in Fig. 3d, the enzyme was stable at 50° C for 30 min but incubation at 60° C for 30 min resulted in complete loss of activity.

The effects of 17 kinds of metal ions (2 mmol) on the activity of this amylase were also examined by preincubating the enzyme with the metals for 30 min at 40° C and then assaying residual activity. The amylase activity was completely inhibited by Hg, Cu and Ag ions. Fe, Pb and Cd ions reduced the activity to 49%, 53% and 65%, respectively. The enzyme was also inhibited significantly by the metal-ion chelating reagent, EDTA.

Substrate specificity

The rates of the reactions with various substrates were measured quantitatively from the initial velocities (Table II). The amylase hydrolyzed amylose much faster than amylopectin and glycogen and had a high specificity for maltodextrins of small molecular weight. Maltotriose and maltotetraose were hydrolyzed better than amylose, while maltose was not hydrolyzed. Activity was similar with maltodextrins of two different lengths* of $\overline{DP}$ 13 and $\overline{DP}$ 100 and corn amylose (Sigma Co., $\overline{DP}$ 300). The amylase also hydrolyzed three cyclomaltodextrins as rapidly, or more rapidly than amylose.

Kinetic parameters of the amylase on various substrates

The K_m and V values of the enzyme for various substrates were determined (Table III). The K_m value for maltotriose was lowest, being about $1.0 \cdot 10^{-3}$ g/ml. However, this value did not differ significantly from those for maltotetraose, cyclomaltohexaose and amylose. Thus, this amylase had a broad specificity for various maltodextrins, regardless of their size or cyclization. Maltose competitively inhibited the hydrolysis of amylose and its K_i value was $5 \cdot 10^{-3}$ g/ml. The K_m value for amylopectin ($13.0 \cdot 10^{-3}$ g/ml) was high compared with those for the above substrates. Thus, this amylase has a high affinity for straight-chain or cyclic dextrins but a low affinity for branched dextrins.

Product of hydrolysis of amylase

Samples (1%) of corn amylose, cyclomaltohexaose, maltotetraose and amylopectin were incubated with the purified enzyme (0.005 unit) in 50 mM buffer in a total volume of 10 ml at 40° C, and the products were examined by

* Dextrins of low molecular weight were prepared by complete debranching of defatted corn starch (1% v/v) by *Pseudomonas* isoamylase [1] followed by acetone fractionation; the average degrees of polymerization ($\overline{DP}$) determined by periodate oxidation were 100 and 13.

TABLE II

INITIAL VELOCITIES IN HYDROLYSIS OF VARIOUS SUBSTRATES BY THE AMYLASE

Substrates were prepared in 0.1 M acetate bugger (pH 5.5) at a final concentration of 0.2% for macromolecular polysaccharides and 0.002 M for oligosaccharides. A suitable quantity of enzyme was added to produce a linear increase in reducing value and in glucose release during the first 15 min of reaction.

Substrate	Initial velocity* (units/mg)	
	Reducing power	Glucose oxidase
0.002 M		
Maltose		0.0
Isomaltose		0.0
Maltotriose		30
Isomaltotriose		0.0
Maltotetraose		25
Cyclomaltohexaose	44	26
Cyclomaltoheptaose	20	14
Cyclomaltooctaose	28	18
0.1%		
Maltodextrin ($\overline{DP}$ = 13)**	22	9.3
Maltodextrin ($\overline{DP}$ = 100)	21	
Corn amylose (Sigma Co.)	21	
Waxycorn amyltopectin	1.2	
Waxycorn β-limit dextrin	1.1	
Glutinous rice amylopectin	1.6	
Oyster glycogen (Wako Chem)	0.37	
Pullulan	0.0	
Dextran	0.0	

* The initial velocity is expressed as the specific activity.

** Prepared as described in the text.

paper chromatography after appropriate incubation times. The products from amylose were G_1, G_2, G_3, G_4 and G_5 (Fig. 4a). At the final stage of digestion, G_3, G_4 and G_5 disappeared and only G_1 and G_2 were detected. This can be explained by the facts that the amylase has relatively high activity for smaller maltodextrins such as G_3 and G_4, but does not attack G_2 (Table II). The products from cyclomaltohexaose (Fig. 4b) were similar to those from amylose. However, at an early stage of the reaction, maltohexaose appeared predominantly. The ring of cyclomaltohexaose was initially opened, giving the

TABLE III

KINETIC PARAMETERS OF THE AMYLASE ON VARIOUS SUBSTRATES

The reaction mixture contained various concentrations of substrate, 0.1 ml of 1.0 M acetate buffer (pH 5.5) and 10 μl of amylase solution containing 2.1 units of amylase activity in a total volume of 1 ml. After incubation for 20 min at 40° C, the reducing power in the digest was assayed.

Substrate	K_m		V (μmol/ml)
	(10^{-3} M)	(10^{-3} g/ml)	
Maltotriose	2.0	1.0	3.8
Maltotetraose	2.1	1.4	4.0
Cyclomaltohexaose	3.6	3.5	4.0
Corn amylose		3.3	3.4
Waxycorn amylopectin		13	0.63

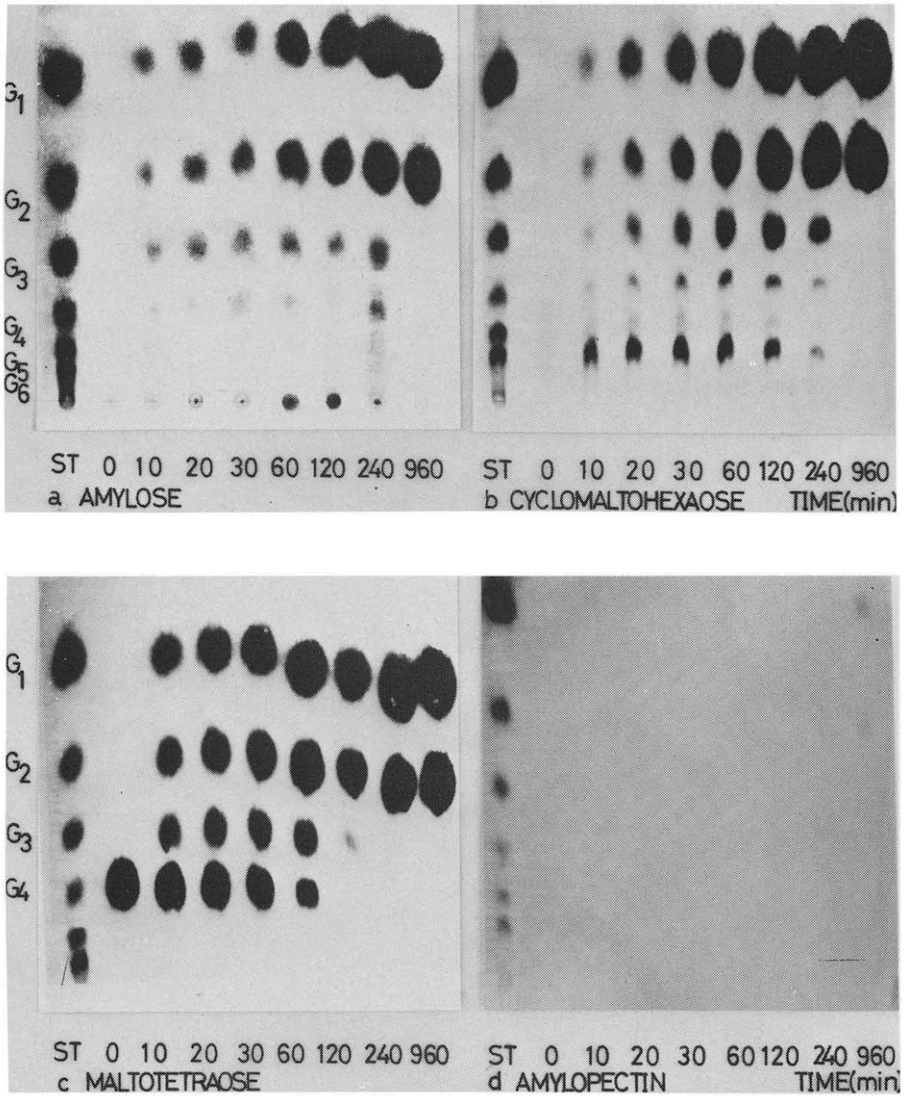


Fig. 4. Chromatography analysis of hydrolyzates by the amylase. Samples taken at intervals were applied to the bottom of the paper. The first spot on the left was a mixture of G_1 through G_6, for reference.

acyclic form, G_6, and then G_6 was further degraded to give G_1 and G_2. Other cyclodextrins (cyclomaltoheptaose and cyclomaltooctaose) were hydrolyzed in the same manner as cyclomaltohexaose. Maltotetraose was rapidly hydrolyzed to G_1, G_2 and G_3 and the G_3 was then converted to G_1 and G_2 (Fig. 4c). Amylopectin was degraded slowly and very small amounts of G_1 and G_2 were detected on paper (Fig. 4d).

The final hydrolysis products were extracted from the paper with water and estimated quantatively from their reducing values. Almost equimolar amounts of G_1 and G_2 were formed on complete hydrolyses of various substrates (Table IV).

TABLE IV

RATIO OF MALTOSE TO GLUCOSE PRODUCED AS FINAL PRODUCTS IN THE ENZYMATIC REACTION

Complete hydrolysis was performed by incubation at 40° C for 24 h using 2 units of amylase and 0.1% of each substrate. The areas of glucose and maltose were cut out from the paper chromatograms for quantitative analysis. Glucose and maltose were extracted into 1 ml of water (50° C, 1 h) and their concentrations were measured as reducing power.

Substrate	Molar ratio (maltose/glucose)
Maltotriose	0.94
Maltotetraose	0.98
Cyclomaltohexaose	0.92
Corn amylose	0.90
Soluble starch	0.96

Reaction with ^{14}C-labeled maltodextrins

Fig. 5 shows the radioactive products formed on reaction with substrates labeled at the reducing end (G_2^* to G_5^*). The results indicate the points which were hydrolyzed. The enzyme concentration and the reaction time were carefully chosen to give products at the early stage of the reaction. The areas of the

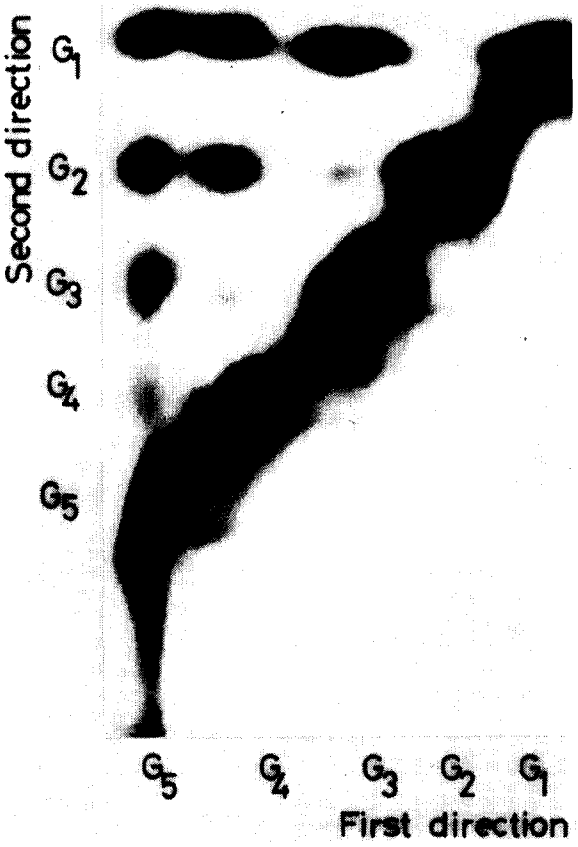


Fig. 5. Radiograph showing action of amylase on maltodextrins labeled at the reducing end.

TABLE V

SUSCEPTIBILITY OF α-1,4-GLUCOSIDIC LINKAGES IN MALTODEXTRINS

G_1^*, G_2^*, G_3^*, etc. are glucose, maltose, amltotriose, etc. labeled at the reducing end.

Substrate	Hydrolysis products (cpm)		
	G_1^*	G_2^*	G_3^*
G_3^*	22 000 (0.95)	1000 (0.05)	
G_4^*	14 100 (0.56)	9200 (0.36)	2000 (0.08)
G_5^*	7 200 (0.52)	4900 (0.32)	2000 (0.13)
G_6^*	6 200 (0.43)	4200 (0.29)	4000 (0.28)

paper with the products were cut out and counted in toluene in a liquid scintillation spectrometer (Table V). Hydrolysis of G_3^* gave mainly G_1^* with a little G_2^*. Hydrolysis of G_4^* yielded G_1^*, G_2^* and G_3^* and their ratio calculated from the results in Table V was 0.56, 0.36 and 0.36 and 0.08, respectively. Degradation of G_5^* gave G_1^* (0.52), G_2^* (0.32), G_3^* (0.13) and a slight amount of G_4^*. These data are discussed in detail later.

Optical-rotation study

The reaction of the amylase with amylose was studied by observing the changes in the optical rotation with time. Sweet potato β-amylase was also studied for comparison. The purpose of the study was the determination of the configuration of the anomeric carbon atom of the products. The products that are initially formed from amylase action are usually one of two forms: α or β. Mutarotation occurs slowly with the establishment of an equilibrium between these forms. It is known that alkali will catalyze the mutarotation reaction [13]. Hence, the addition of alkali to a digest producing α-products should result in a decrease in the optical rotation, and the addition of alkali to a digest producing β-products should result in an increase in the optical rotation. 20 mg of corn amylose were dissolved in 10 ml of water and 5 units of enzyme were added. Optical-rotation measurements were made at various intervals using a 1-dm tube. The temperature of the reaction was 20°C. At appropriate time 4 mg of anhydrous sodium carbonate was added per 10 ml of digest. This brought the pH to 10 and accelerated the mutarotation reaction. Fig. 6 shows

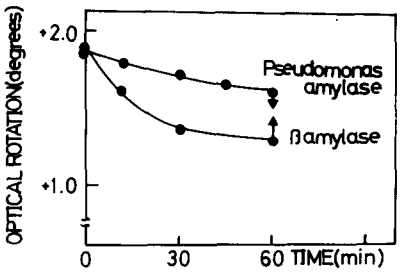


Fig. 6. Optical-rotation study of the action of *Pseudomonas* intracellular amylase and sweat potato β-amylase with amylose. The triangular symbol indicates the optical rotation after the addition of alkali to the digests.

the change in optical rotation with time for the two enzymes. Our enzyme was found to be of the α-type.

Discussion

The intracellular amylase from *Pseudomonas* MSl studied in this work is an endo-type amylase. In general, this amylase hydrolyzes maltotriose and other higher maltodextrins with a wide range of molecular sizes. Kinetic studies showed that this enzyme has nearly equal K_m values for various maltodextrins. Endo-type amylases from bacteria [14], fungi [14], saliva [14], pancreas [15] and other sources [16,17] are known to accumulate maltotriose at an achroic stage of reaction, because of their low activity on maltotriose, and the resultant maltotriose is slowly hydrolyzed to glucose and maltose.

Pseudomonas amylase has much lower activities on amylopectin and glycogen, its K_m value for amylopectin being approximately ten times that for amylose. This intracellular amylase which has low activity on α-1,6-branched dextrins may degrade starch molecules well in combination with extracellular isoamylase. Furthermore, in cooperation with the latter and intracellular α-glucosidase it can hydrolyze starch completely.

The striking characteristic of this amylase is that it attacks a series of cyclomaltodextrins as rapidly as linear maltodextrins. Cyclomaltodextrins appear to be immune to the action of most amylases [16,17], but microorganisms such as *Bacillus polymyxa* [18] and *Aspergillus oryzae* [19,20] have been reported to produce an enzyme which can attack cyclomaltodextrins. However, extensive studies showed that *B. polymyxa* amylase [21] did not attack cyclomaltodextrins and possessed all the characteristics of a β-amylase. *Asp. oryzae* amylase [19,20] could hydrolyze a series of cyclodextrins but the initial rates of degradation of cyclomaltodextrins were quite low in comparison with those of linear dextrins. The present amylase rapidly hydrolyzed a series of cyclodextrins and its K_m value for cyclomaltohexaose was about $3.5 \cdot 10^{-3}$ g/ml, which was nearly the same as that for linear amylose. Paper chromatography showed that this amylase degrades cyclomaltohexaose in two steps (Fig. 7): first cyclomaltohexaose is cleaved to G_6 with very rapid accumulation of G_6 and second, the linear G_6 is degraded to smaller molecules. This second step appears to be rate-limiting in the overall enzyme reaction, so that the rate of degradation of cyclodextrins probably becomes almost the same as that of linear dextrins. Hydrolysis of cyclodextrins by this amylase is in contrast with the reactions of sweet-potato β-amylase [13], potato phosphorylase [22] and glucoamylase in which cyclodextrins act as competitive inhibitors. The position specificity of this amylase on lower maltodextrins was examined using maltodextrins labeled at the reducing end as substrates. Analysis of the labeled

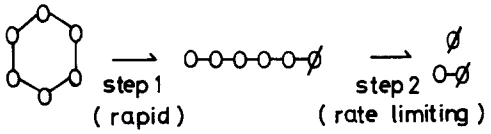


Fig. 7. Schematic representation of the cleavage and degradation of cyclomaltohexaose. The symbols used in this figure are explained in Fig. 8.

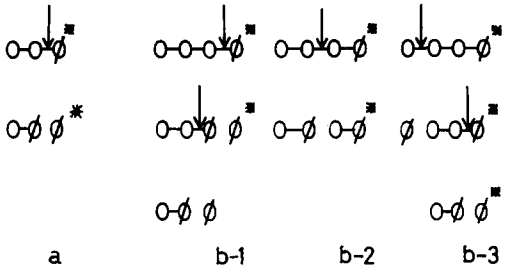


Fig. 8. Possible mechanisms for cleavage of the α-1,4 glucosidic bonds where ○ represents a glucosyl unit, ϕ represents a reducing-end glucosy moiety, ϕ* is a glucosyl moiety labeled at the reducing-end, and — is an α-1,4 glucosidic bond.

products from these substrates showed the frequency distribution of hydrolysis of glucosidic bonds in each maltodextrin. The mechanism of degradations of G_3 and G_4 are discussed here in detail only to simplify the explanation of the mechanism. In the case of G_3^*, this amylase attacks the first bond from the reducing end to produces G_1^* exclusively (Fig. 8a). The degradation of G_4^* leads mainly to formation of G_1^* and G_2^*, as shown in Fig. 5. The products are more similar to those of *Bacillus subtilis* amylase [23] and pancreatic amylase [23] than to those of the amylases of *Asp. oryzae* [23] and *Endomycopsis* [23] The formation of those products by the enzymatic reaction may occur as follows. If the first bond from the reducing end is attacked, G_1^* is produced from the beginning of reaction (Fig. 8b1). If the second bond is hydrolyzed, only G_2^* is produced, and this is resistant to further amylolysis (Fig. 8b2). If the third bond is cleaved, hydrolysis of G_4^* must proceed via G_3^* to G_1^* (Fig. 8b3). The data in Table V may indicate that the former two types of cleavage contribute mainly to the degradation of G_4. The contents of the final products (G_1 and G_2) can be calculated as follows:

$$\frac{G_2}{G_1} = \frac{0.56 \cdot 1 \text{ mol } G_2 \text{ (Fig. 8b1)} + 0.36 \cdot 2 \text{ mol } G_2 \text{ (Fig. 8b2)}}{0.56 \cdot 2 \text{ mol } G_1 \text{ (Fig. 8b1)}} = \frac{1.28}{1.12} = 1.1$$

The quantitative data in Table IV give a value of 0.98 for the hydrolysis of G_4, which is rather close to that calculated from the results of experiments using maltodextrin labeled at the reducing end as substrate. The calculated value obtained by using labeled substances becomes much closer to that (0.98) directly determined since it is considered that the third bond is also cleaved to a small extent. This result supports our explanation for the mechanism of G_4 degradation to produce almost equimolar amounts of G_1 and G_2.

A saccharifying enzyme from *B. subtilis* is known to produce small molecules of maltodextrins from the beginning of the reaction, but this enzyme hardly hydrolyzes maltose [24]. This saccharifying amylase has a much lower activity toward maltotriose than maltotetraose and produces much more glucose than maltose as final product from amylose. Our enzyme has higher activities toward maltodextrins of small molecular weight than toward amylose, unlike the enzyme from *B. subtilis*. As shown in Fig. 8, our enzyme is an α-amylase.

Thus, it is concluded that the intracellular amylase from *Pseudomonas* B15 is an α-amylase which can attack a variety of maltodextrins, including maltotriose, and cyclomaltodextrins to form almost equimolar amounts of maltose and glucose.

Acknowledgement

The authors would like to express their thanks to members in Hayashibara Co, Okayama-shi for their aid in the preparation of maltodextrins.

References

1 Yokobayashi, K., Misaki, A. and Harada, T. (1970) Biochim. Biophys. Acta 212, 458—469
2 Sugimoto, T., Amemura, A. and Harada, T. (1974) Appl. Microbiol. 28, 336—339
3 Amemura, A., Sugimoto, T. and Harada, T. (1974) J. Ferment. Technol. 52, 778—780
4 Nelson, N. (1944) J. Biol. Chem. 153, 375—380
5 Saifer, A. and Gerstenfield, S. (1958) J. Lab. Clin. Med. 51, 448—449
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
7 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
8 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
9 Shapiro, A.L., Vinuela, E. and Maisel, J.V. (1967) Biochem. Biophys. Res. Commun. 28, 815—820
10 Whelan, W.J., Bailey, J.M. and Roberts, D.J.P. (1953) J. Chem. Soc. 1293—1298
11 French, D., Levine, M.L., Norberg, E., Nordin, P., Puzur, J.H. and Wild, G.M. (1954) J. Am. Chem. Soc. 76, 2387—2390
12 Pazur, J.H. and Okada, S. (1966) J. Biol. Chem. 241, 4146—4151
13 Thoma, J.A. and Koshland, Jr, D.E. (1960) J. Am. Chem. Soc. 82, 3329—3333
14 Bird, R. and Hopkins, R.H. (1954) Biochem. J. 56, 86—99
15 Robyt, J.F. and French, D. (1970) J. Biol. Chem. 15, 3917—3927
16 Radley, J.A. (1968) Starch and its Derivatives, 4th edn, pp. 300—301
17 Radley, J.A. (1968) Starch and its Derivatives, 4th edn, pp. 430—476
18 Robyt, J. and French, D. (1964) Arch. Biochem. Biophys. 104, 338—344
19 Hanrahan, V.M. and Caldwell, M.L. (1953) J. Am. Chem. Soc. 75, 2129—2197
20 Suetugu, N., Koyama, S., Takeo, K. and Kuge, T. (1974) J. Biochem. Tokyo 76, 57—63
21 Marshall, J.J. (1974) FEBS Lett. 46, 1—4
22 Husemann, E. and Lindemann, E. (1954) Stärke 6, 141—148
23 Okada, S., Kitahama, S., Higashihara, M. and Fukumoto, J. (1969) Agr. Biol. Chem. 33, 900—906
24 Mantani, S., Yamamoto, T. and Fukumoto, J. (1967) Proceedings of the Symposium on Amylase, Japan, pp. 95—104