BBA 66147

PURIFICATION AND PROPERTIES OF PSEUDOMONAS ISOAMYLASE

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(Received April 13th, 1970)

SUMMARY

Isoamylase (EC 3.2.1.9) was purified about 720-fold in 23% yield to a final specific activity of 59 100 per mg protein from the culture fluid of *Pseudomonas* sp. strain SB-15. Ammonium sulfate fractionation followed by dialysis and DEAE- and CM-cellulose column chromatographies were used in purification. On ultracentrifugation the enzyme formed a single, sharp, symmetrical schlieren peak. Its $S_{20,w}$ was 5.7 S. Polyacrylamide gel electrophoresis at pH 8.3 gave a single band of protein. The molecular weight was determined as 95 000 by Archibald's method. The isoelectric point was 4.4.

The action of the enzyme on amylopectin and glycogen resulted in increases in the iodine coloration of 62 and 52%, respectively. The enzyme hydrolyzed all α-1,6-glucosidic inter-chain linkages in glycogen, amylopectin and their phosphorylase limit dextrins. The branching points of β-amylase limit dextrins were not hydrolyzed completely. 6-α-Maltotriosylmaltodextrins seemed to be hydrolyzed much faster than 6-α-maltosylmaltodextrins. The optimal pH of this enzyme was 3–4. Some other properties of the enzyme are also described.

INTRODUCTION

Enzymes (EC 3.2.1.9) hydrolyzing α-1,6-glucosidic interchain linkages are known to be present in yeast (isoamylase) [1,2] and higher plants[3] (R-enzyme). Pullulanase[4], produced by *Aerobacter aerogenes*, appears to be the first bacterial debranching enzyme found to hydrolyze the inter-chain linkages in both amylopectin and glycogen[5]. Similar enzymes were later found in certain strains of *Escherichia intermedia*[6] and *Streptococcus mitis*[7].

We found that a newly isolated soil bacterium, *Pseudomonas* sp. strain SB-15, produces an extracellular debranching enzyme, named *Pseudomonas* isoamylase.[8] This enzyme, unlike pullulanase, cannot hydrolyze the α-1,6-glucosidic linkages in pullulan. Moreover, it appears to differ from other known de-branching enzymes in several respects.

The present paper describes a procedure for purification of this isoamylase

from the culture fluid of *Pseudomonas* sp. strain SB-15, and some of the physical and chemical characteristics of the enzyme. A preliminary report of part of this work has been published[9].

MATERIALS AND METHODS

Assay of isoamylase activity

Isoamylase activity was determined by the method of KOBAYASHI[10] with a slight modification. Reaction mixture, containing 0.5 ml of 1.0% soluble glutinous rice starch, 0.1 ml of 0.5 M acetate buffer (pH 3.5) and 0.1 ml of enzyme solution, was incubated at 40° for 1 h, unless otherwise stated. A 0.5-ml aliquot of the reaction mixture was mixed with 0.5 ml of 0.01 M iodine–potassium iodide solution and diluted to 12.5 ml with water.

The solution was allowed to stand for 15 min at room temperature and the absorbance was then measured at 610 nm using a cuvette of 1-cm light-path with a substrate–iodine blank containing heat-inactivated enzyme. A unit of enzyme activity is expressed as the amount of enzyme causing an increase in $A_{610\ nm}$ of 0.1 in 1 h.

Determination of protein concentration

Protein concentration was estimated by the method of LOWRY *et al.*[11] 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 Hitachi 124 UV-VIS spectrophotometer.

Paper chromatography

Maltodextrins were separated on Toyo filter paper No. 50 with a solvent system of *n*-butanol–pyridine–water (9 : 5 : 7, by vol.) by the multiple ascending method. Sugars were detected with silver nitrate reagent[12]. The sugars separated by paper chromatography were eluted with water. Maltose and maltotriose in the eluates were determined with phenol–sulfuric acid reagent[13].

Substrates

Waxy maize amylopectin, obtained from Nihon Schokuhin Co., was defatted with methanol. Potato amylopectin and potato amylose, respectively, were kindly provided by Dr. K. Doi and Dr. T. Fukui of Osaka University. Oyster glycogen and rabbit liver glycogen were purchased from Wako Pure Chemical Ind. and Calbiochem Co., respectively. Maltose, maltotriose and pullulan were supplied by courtesy of Hayashibara Co.

Preparation of β-limit dextrin and phosphorylase limit dextrin

β-Limit dextrins were prepared by prolonged incubation of waxy-maize amylopectin and oyster glycogen with sweet-potato β-amylase, removal of maltose by dialysis, and isolation by freeze-drying.

Glycogen and amylopectin phosphorylase limit dextrins were prepared by the action of rabbit muscle phosphorylase A on rabbit liver glycogen and potato phosphorylase on waxy-maize amylopectin by the method of WALKER AND WHELAN[14].

Phosphorylase–β-amylase limit dextrins were prepared from the above phos-

phorylase limit dextrins by treatment with β-amylase. Low molecular weight products were removed from the limit dextrins by exhaustive dialysis. Oyster glycogen pullulanase limit-dextrin was obtained using pullulanase as follows. Reaction mixture containing 20 000 units of pullulanase, 400 mg of oyster glycogen and 0.01 M acetate buffer (80 ml, pH 5.5) was incubated for 48 h at 40°. The digest was heated, and denatured protein was removed by centrifugation. Then the solution was dialyzed for 72 h against distilled water at 5°. The non-diffusible material was concentrated to about 10 ml, precipitated by the addition of an equal volume of ethanol and dried *in vacuo*. The yield was about 300 mg.

Enzymes

Crystalline β-amylase and α-amylase were obtained from Sigma Chemical Co., and phosphorylase A from Worthington Biochemical Co. Crystalline potato phosphorylase was kindly supplied by Dr. A. Kamogawa of Osaka University. Crystalline pullulanase was prepared from the culture filtrate of *A. aerogenes* ATCC 9621 as described by WALLENFELS and coworkers[15,16].

Preparation of crude isoamylase

Pseudomonas sp. strain SB-15 was grown aerobically at 30° for 96 h in the medium of HARADA *et al.*[8] containing maltose (2%) and glutamate (0.4%) for production of isoamylase. The cells were removed by centrifugation at 10 000 × *g*, and the clear supernatant was used as the starting material for purification.

Electrophoretic analysis

Polyacrylamide gel electrophoresis was carried out as described by DAVIS[17]. Electrophoresis on cellulose polyacetate was carried out at 4°. Isoamylase located on the cellulose polyacetate strip was incubated on the surface of a thin layer of 2% agar containing 0.01 M sodium acetate and 0.5% glutinous rice starch (pH 3.5) at 35° for 30 min. The strip was then dipped in a solution of 0.001 M iodine-0.01 M potassium iodide. The position of isoamylase manifested as a purple zone on a red background.

Sedimentation experiments

Sedimentation studies were carried out in a Hitachi Model UCA-1 analytical ultracentrifuge equipped with both schlieren and interference optics. The temperature was maintained at 25°. The homogeneity of purified preparations was ascertained by schlieren photographs during high speed centrifugation. The $S_{20,w}$ was determined from the sedimentation data.

RESULTS AND DISCUSSION

Purification of enzyme

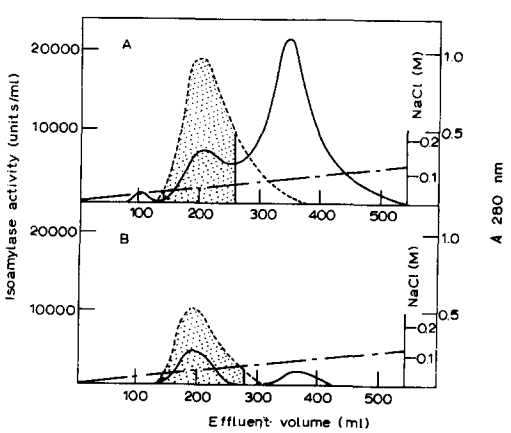
During purification all operations were carried out at 4°. Cultures of strain SB-15 were centrifuged and the supernatant (44 l) was brought to 70% saturation of ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 300 ml of 0.1 M acetate buffer (pH 6.0). The solution was dialyzed against tap-water for 16 h at 4°, centrifuged to remove the precipitate formed and made up to 400 ml.

Solid ammonium sulfate was added to the enzyme solution to give 15% saturation. The resulting precipitate was removed by centrifugation. Further solid ammonium sulfate was added to the supernatant to give 45% saturation. The precipitate formed was collected by centrifugation and dissolved in 40 ml of 0.01 M acetate buffer (pH 6.0). The enzyme solution was dialyzed against tap-water for 16 h and then against 5 l of 0.01 M acetate buffer (pH 6.0). The dialyzed material was passed through a column (2.5 cm × 30 cm) of DEAE-cellulose equilibrated with 0.01 M acetate buffer (pH 6.0). The column was eluted with a further 150 ml of 0.01 M acetate buffer (pH 6.0) and the eluate containing isoamylase was made up to 200 ml with distilled water. Pigmented matter and some protein were removed by this procedure.

The colorless active fraction was dialyzed against saturated ammonium sulfate solution overnight at 4°. The precipitate formed was collected by centrifugation and dissolved in 20 ml of 0.01 M acetate buffer (pH 3.5) and dialyzed against the same buffer for 16 h at 4°. The dialyzed enzyme solution was applied to a column (2.0 cm × 25 cm) of CM-cellulose equilibrated with 0.01 M acetate buffer (pH 3.5), and the column was washed with 150 ml of the same buffer. The enzyme was eluted with a linear gradient obtained by placing 500 ml of 0.01 M acetate buffer (pH 3.5) in the mixing chamber and 500 ml of the same buffer containing 0.3 M NaCl in the reservoir. The flow rate was about 1.5 ml per min. Fractions were collected in a fraction collector. The elution pattern is shown in Fig. 1A.

Fractions 14–26 were combined and rechromatographed under the same conditions as in the first chromatography. The isoamylase emerged at approximately the same NaCl concentration as in the first chromatography (Fig. 1B). The active fractions in Tubes 14–28 were pooled, dialyzed and concentrated against 0.01 M acetate buffer (pH 3.5) at 4° by vacuum ultrafiltration through a collodion membrane.

The purification procedure is summarized in Table 1. The overall yield was 23%, and the specific activity of the purified enzyme was 59 100 units/mg.



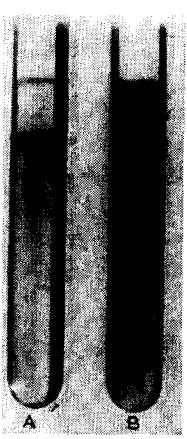


Fig. 1. Elution pattern of isoamylase from CM-cellulose columns. A, First CM-cellulose column. B, Second CM-cellulose column. ———, absorption at 280 nm; – – – –, isoamylase activity; — · —, concentration of NaCl. Shaded areas indicate fractions pooled for subsequent steps.

Fig. 2. Polyacrylamide disc electrophoresis of purified isoamylase at pH 8.3. Approx. 0.1 mg of protein was applied to each gel. Electrophoresis was carried out for 2.5 h at 2.0 mA per gel, at 4°. A, Stained with amido black dye. B, Assayed for isoamylase activity.

TABLE I

PURIFICATION OF ENZYME

Step of purification	*Vol. (ml)*	*Protein (mg)*	*Activity*		
			Units ($\times 10^3$)	*Yield (%)*	*Specific activity (units/mg)*
1. Crude filtrate	44 000	37 600	3080	(100)	82
2. $(NH_4)_2SO_4$, 0–70%	400	9 320	2328	76	250
3. $(NH_4)_2SO_4$, 15–45%	40	860	1988	65	2 310
4. DEAE-cellulose effluent	200	193	1692	55	8 790
5. 1st CM-cellulose, Fractions 14–26	20	27.4	1038	34	37 800
6. 2nd CM-cellulose, Fractions 14–28	150	11.7	693	23	59 100

Purity of the enzyme preparation

The enzyme preparation gave a single band associated with isoamylase activity on electrophoresis on acrylamide gel at pH 8.3 (Fig. 2). Ultracentrifugation of an identical preparation gave a single symmetrical schlieren peak (Fig. 3). The preparation was free of maltase, isomaltase, pullulanase, dextranase, α-amylase and D-enzyme. α-Amylase activity was determined by measuring the change in the iodine coloration intensity of the amylose used as substrate[18], and D-enzyme activity by measuring the amount of glucose formed from maltotriose[19]. Enzyme activity was not lost during storage of the solution for several months at 4°.

Absorption spectrum

The absorption spectrum of isoamylase was measured with a Hitachi 124 UV-VIS spectrophotometer. In 0.01 M acetate buffer (pH 4.5). the enzyme showed a typical spectrum of protein with an absorption maximum at 280 nm. The extinction coefficient $E_{280\ nm}$ was 22.6.

Isoelectric point

On electrophoresis on cellulose polyacetate strips the preparation gave a single band of isoamylase with an isoelectric point of 4.4.

Molecular weight

The sedimentation coefficient ($S_{20,w}$) was calculated as 5.75. The molecular weight was determined by the method of ARCHIBALD[20] as 94 000 assuming that the partial specific volume was 0.72.

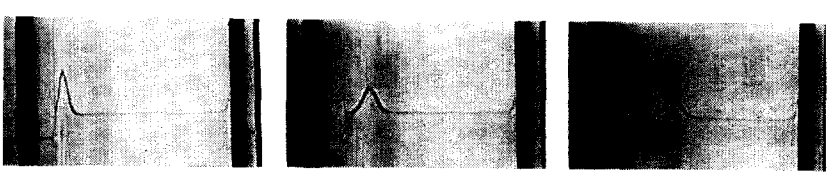
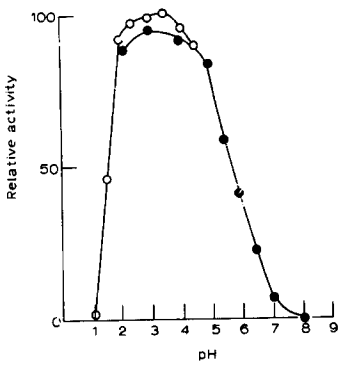


Fig. 3. Schlieren patterns of purified PSEUDOMONAS isoamylase. Sedimentation from left to right. Photographs were taken at 16-min intervals after a rotor speed of 54 500 rev./min had been attained using 0.05% protein in 0.01 M acetate buffer (pH 4.0). A double-sector cell was used, and the temperature was 25°. Bar angle, 70°.



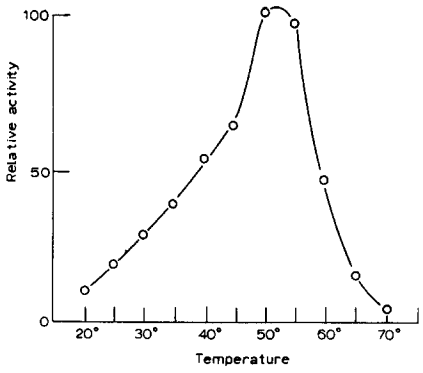


Fig. 4. Effect of pH on isomylase activity. The activity was determined under standard assay conditions except for change of pH as indicated. ○—○, 0.1 M acetate–HCl buffer (pH 1.0–5.0); ●—●, 0.1 M phosphate–citric acid buffer (pH 2.2–7.8).

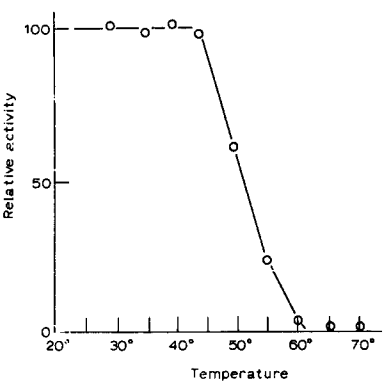
Fig. 5. Effect of temperature on isoamylase activity. Isomylase activity was determined under the standard assay conditions except for change of temperature as indicated.

Effect of pH on isoamylase activity

The effect of pH on isoamylase activity is shown in Fig. 4. The optimal pH was in the vicinity of 3–4.

Effect of temperature on isoamylase activity

The effect of temperature on isoamylase activity was measured at 20–70°. As



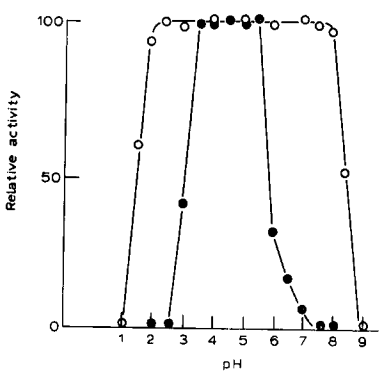


Fig. 6. Thermal stability of isoamylase. Enzyme dissolved in 0.25 M acetate buffer solution (pH 3.5) was incubated for 10 min. at the various temperatures indicated. The activity remaining was assayed under standard conditions.

Fig. 7. Effect of pH on stability of isoamylase. Enzyme solutions of various pH values were incubated for 24 h at 4° (○—○) and for 2 h at 40° (●—●). After incubation, each enzyme solution was adjusted to pH 3.5, and residual activity was measured by the standard method.

shown in Fig. 5, the optimal temperature of the isoamylase for a 10-min reaction was 52° at pH 3.5, and the activity at 40° was about 53% of that at 50°.

Thermal stability

Thermal stability was measured in 0.1 M acetate buffer (pH 3.5) by heating at 30–70° for 10 min. As shown in Fig. 6, the isoamylase was remarkably heat sensitive, *i.e.*, inactivation occurred at 45°, while at 50°, 55° and 60°, activity decreased by about 40, 77 and 95%, respectively. Heating for 10 min at 65° resulted in complete loss of activity.

pH stability

As shown in Fig. 7, the isoamylase was stable at 4° for 24 h at about pH 2.5–7.5, but inactivation occurred at lower and higher pH values. At 40°, the pH exerted a marked effect on the stability of the isoamylase within 2 h, almost complete activity remaining at pH 3.5–5.5, and being lost at below pH 2.5 and above pH 7.5.

TABLE II

EFFECTS OF VARIOUS REAGENTS ON ISOAMYLASE ACTIVITY

Reagent	*Final concn. (mM)*	*Inhibition (%)*
NaCl	1	0
$MgCl_2$	1	0
CuCl	1	30
$HgCl_2$	1	41
	0.1	34
NaF	1	19
Ammonium molybdate	1	2
Iodoacetate	10	13
	1	0
p-Chloromercuribenzoate	1	34
	0.1	0

Effect of various reagents on isoamylase activity

A wide range of reagents was tested, because they had been used in previous studies[2,7,21]. Acetate buffer at pH 3.5 was used. Results are expressed as percentage inhibition compared with the activity in control, reagent-free digests. The results of these experiments are given in Table II. Ammonium molybdate, which inhibits R-enzyme[21] and yeast isoamylase[2], did not inhibit isoamylase activity. Isoamylase was slightly inhibited by SH-group reagents, such as iodoacetate and *p*-chloromercuribenzoate, while R-enzyme and yeast isoamylase were only partially or not at all inhibited. Other experiments showed that the effects of ammonium molybdate, iodoacetate and *p*-chloromercuribenzoate on pullulanase were similar to their effects on isoamylase. The percentage inhibitions with ammonium molybdate (1 mM), iodoacetate (10 mM) and *p*-chloromercuribenzoate (1 mM) were 0, 24 and 16, respectively.

Increase in iodine coloration by the action of Pseudomonas isoamylase and pullulanase on amylopectin and glycogen

The actions of *Pseudomonas* isoamylase on amylopectin and glycogen were compared with those of *A. aerogenes* pullulanase (Fig. 8). The iodine coloration,

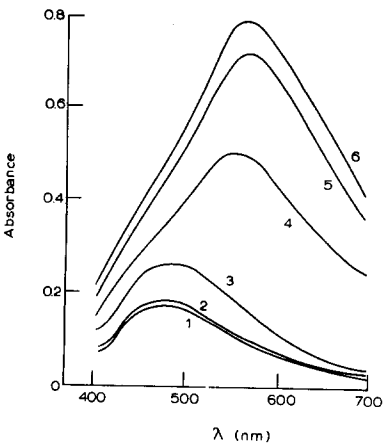


Fig. 8. Effect of Pseudomonas isoamylase and pullulanase on the iodine coloration of potato amylopectin and oyster glycogen. Potato amylopectin (2 mg) or oyster glycogen (2 mg) was incubated at 35° with either Pseudomonas isoamylase or pullulanase (44 units of either)and a final concentration of 0.01 M acetate buffer (pH 3.5 for isoamylase, pH 5.5 for pullulanase) in a total volume of 1 ml. After 20 h, 0.2-ml aliquots were mixed with 0.2 ml of 0.4% iodine–potassium iodide solution. The mixture was diluted with water to 10 ml and used for determination of the spectra of iodine-complexes. 1, oyster glycogen before treatment; 2, oyster glycogen after incubation with pullulanase; 3, oyster glycogen after incubation with Pseudomonas ioamylase; 4, potato amylopectin before treatment; 5, potato amylopectin after incubation with pullulanase; 6, potato amylopectin after incubation with Pseudomonas isoamylase; For experimental details see text.

measured at 570 nm using potato amylopectin as substrate, was increased by *Pseudomonas* isoamylase and pullulanase by 62 and 47%, respectively, and the maximum with amylopectin shifted from 550 to 570 nm.

When oyster glycogen was used as substrate there was a very small increase (11% at 470 nm) in the iodine coloration with pullulanase but a big increase with *Pseudomonas* isoamylase (52% at 470 nm).

Effects of Pseudomonas isoamylase and pullulanase on the β-amylolysis limits of amylopectin, glycogen and related dextrins

The simultaneous actions of a debranching enzyme and β-amylase were examined using mixtures of polysaccharide (2 mg), isoamylase or pullulanase (44 units) and β-amylase (80 units) in acetate buffer (pH 5.0) in a total volume of 2 ml. To allow the successive actions of a debranching enzyme and β-amylase, polysaccharide (2 mg) was first treated with either isoamylase (44 units) at pH 3.5 or pullulanase (44 units) at pH 5 for 2 h in 0.1 M acetate buffer in a total volume of 1.9 ml. After heat inactivation, the reaction mixture was incubated with 0.1 ml of β-amylase solution (80 units) in 0.1 M acetate buffer (pH 5) for 24 h. Samples (0.1 ml) were withdrawn and the maltose released was measured with SOMOGYI–NELSON[22] reagent. The concentrations of the polysaccharides added as substrates were determined by the phenol-sulfuric acid method[13]. The effects of Pseudomonas isoamylase on the β-amylolysis of amylopectin, glycogen and related dextrins were compared with those of the pullulanase of *A. aerogenes* (Table III).

Isoamylase, like pullulanase, caused complete degradation of amylopectin

both by simultaneous and successive action with β-amylase. Isoamylase hydrolyzed glycogen completely when acting either successively or simultaneously with β-amylase. However, although pullulanase caused complete degradation of glycogen on simultaneous action with β-amylase, it caused only a small increase in the β-amylolysis limit on successive action (38–46% for oyster glycogen, and 42–51% for rabbit liver glycogen), in agreement with the results of ABDULLAH *et al.*[5] The latter workers suggested that the incomplete degradation of glycogen by the successive action of pullulanase was due to its inability to penetrate into the interior of the glycogen molecule. Thus, these results indicate that Pseudomonas isoamylase, unlike pullulanase, can penetrate into the interior of the compact glycogen molecule and hydrolyze all α-1,6-glucosidic inter-chain linkages. This also seems likely from the results that Pseudomonas isoamylase caused complete degradation of oyster glycogen pullulanase-limit dextrin.

This difference between the two enzymes was also deduced from the iodine coloration of glycogen treated with the two enzymes, as shown in Fig. 8.

It has been reported that the R-enzyme has no appreciable action on glycogen,[3] and that the action of yeast isoamylase on glycogen is similar to that of pullulanase[5]. With glycogen β-limit dextrin, isoamylase caused extensive β-amylolysis (79%) by

TABLE III

EFFECTS OF PSEUDOMONAS ISOAMYLASE AND PULLULANASE ON β-AMYLOLYSIS OF AMYLOPECTIN, GLYCOGEN AND RELATED DEXTRINS

For experimental details see text.

Substrate	*Conversion to maltose (%)*				
	β-Amylase alone	*Successive action with β-amylase*		*Simultaneous action with β-amylase*	
		Pseudomonas isoamylase	*Pullulanase*	*Pseudomonas isoamylase*	*Pullulanase*
Waxy-maize amylopectin	50	99	95	95	103
Potato amylopectin	47	96	98	97	103
Oyster glycogen	38	102	46	100	99
Rabbit liver glycogen	42	100	51	99	97
Waxy-maize amylopectin β-limit dextrin	0	80	97	72	97
Oyster glycogen β-limit dextrin	0	79	31	76	99
Waxy-maize amylopectin phosphorylase limit dextrin	21	95	97	98	101
Rabbit liver glycogen phosphorylase limit dextrin	28	94	32	97	99
Waxy-maize amylopectin phosphorylase β-limit dextrin	0	48	96	56	100
Rabbit liver glycogen phosphorylase β-limit dextrin	0	44	29	50	97
Oyster glycogen pullulanase limit dextrin	34	97	35	98	98

successive action with β-amylase, whereas pullulanase caused only 31% degradation under the same conditions, showing that isoamylase can split the interior branch points in glycogen. Although the combined actions of Pseudomonas isoamylase with β-amylase caused degradation of β-limit dextrin, 20–30% of the amylopectin β-limit dextrin and 20–25% of the glycogen β-limit dextrin, respectively, were resistant to enzymic action. Pullulanase caused complete degradation of amylopectin β-limit dextrin by both simultaneous and successive action with β-amylase, and of glycogen β-limit dextrin by simultaneous action. However, Pseudomonas isoamylase, like pullulanase, caused almost complete degradation of amylopectin and glycogen phosphorylase-limit dextrin by both simultaneous and successive action with β-amylase, although pullulanase caused only 32% degradation of glycogen by successive action.

The difference in the degradation rates of β-limit, and phosphorylase limit dextrins by isoamylase may be due to the side chains in β-amylase limit dextrins being 2 or 3 glucose units long[23] whereas those in phosphorylase limit dextrins are 4 glucose units long[14]. On waxy-maize amylopectin and oyster glycogen phosphorylase β-amylase limit dextrins, which have 2 glucose unit stubs, the action of pullulanase is similar to that on amylopectin, glycogen and their phosphorylase or β-amylase limit dextrin. However, by the combined action of Pseudomonas isoamylase with β-amylase, 50% of amylopectin phosphorylase β-amylase limit dextrin and 56% of glycogen phosphorylase β-amylase limit dextrin were resistant to enzymic action.

Amylopectin, glycogen and their phosphorylase-limit dextrins were completely hydrolyzed by Pseudomonas isoamylase and β-amylase under standard conditions. The incomplete degradation of β-limit dextrins seems to be attributable to the specificity of the isoamylase toward the side-chain (A-chain) stubs which are shortened to two or three glucose residues[19] by the action of β-amylase.

To clarify this point the β-limit dextrins of waxy-maize amylopectin and oyster glycogen (10 mg each) were incubated with the debranching enzymes (220 units each) in acetate buffer (pH 5.0) in a total volume of 1 ml, at 35° for 2 h and the products were examined by quantitative paper chromatography as shown in Table IV. Pullulanase released maltose and maltotriose at almost equal rates together with higher maltosaccharides from both β-limit dextrins; during 20-min incubation the release of reducing sugars almost reached the maximum. Pseudomonas isoamylase also released similar sugars, but the amount of maltose released was very small.

TABLE IV

FORMATION OF MALTOSE AND MALTOTRIOSE FROM β-LIMIT DEXTRINS BY PSEUDOMONAS ISOAMYLASE AND PULLULANASE

For experimental details see text.

Substrate	*Debranching enzyme*	*Yield*		*Molar ratio of maltose to maltotriose*
		Maltose (wt.%)	*Maltotriose (wt.%)*	
Waxy-maize amylopectin β-limit dextrin	Pseudomonas isoamylase	0.59	9.43	1:13.4
	Pullulanase	5.84	12.30	1: 1.50
Oyster glycogen β-limit dextrin	Pseudomonas isoamylase	0.96	11.11	1: 9.7
	Pullulanase	8.62	8.79	1: 0.85

The molar ratios of maltose to maltotriose liberated by pullulanase were 1 : 1.5 with amylopectin β-limit dextrin, and 1 : 0.85 with glycogen β-limit dextrin. The molar ratios of maltose to maltotriose liberated by Psedomonas isoamylase were 1:13.4 with amylopectin β-limit dextrin, and 1:9.7 with glycogen β-limit dextrin. Under these conditions, most of the maltotriose residues attached to the main chain (B-chain) were liberated, but there was little liberation of maltose residues even on much longer incubation, owing to the low activity of the isoamylase on side-chains of two glucose units. This may explain why degradation of β-limit dextrins by the combined actions of isoamylase and β-amylase was incomplete.

Effect of Pseudomonas isoamylase and pullulanase on branched-chain oligosaccharides

Oligosaccharide mapping methods[24] were employed to compare the actions of debranching enzymes.

Malto-oligosaccharide containing branched-chain oligosaccharide as α-limit dextrin was prepared from waxy-maize amylopectin (1 g) by the action of bacterial α-amylase (50 mg) for 24 h. This material was developed by three ascents with the solvent *n*-butanol–pyridine–water (6:4:3, by vol.) by one-dimensional paper chromatography. The area of the chromatogram containing the oligosaccharides was sprayed uniformly with 4 ml of Pseudomonas isoamylase or pullulanase solution containing 400 units of activity per ml. The chromatogram was kept at 30° for approx. 30 min, and during this period it was dried. The chromatogram was developed in the second direction under similar conditions. The paper was dried in air and saccharides on the paper were detected with silver nitrate reagent[12], as shown in Fig. 9.

Pseudomonas isoamylase released maltotriose and higher maltosaccharides from the branched-chain oligosaccharides, but no detectable maltose. Pullulanase released maltose, maltotriose and much larger maltosaccharides from the branched-chain oligosaccharides. Especially large amounts of maltose and maltotriose were

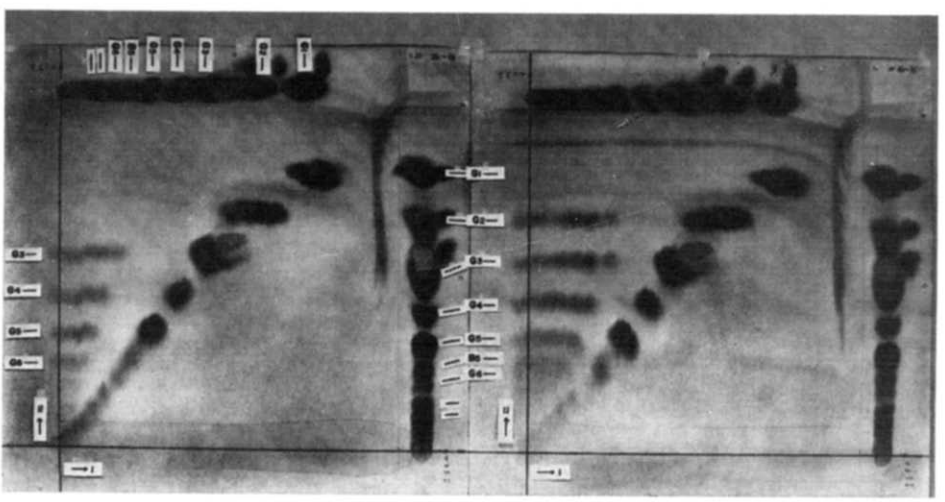


Fig. 9. Comparison of the action patterns of PSEUDOMONAS ISOAMYLASE AND AEROBACTER pullulanase on α-limit dextrin. Solvent, butanol–pyridine–water (6:4:3, by vol.). I, first direction of development; II, second direction of development after isoamylase action (left) or after pullulanase action (right).

released from the branched oligosaccharide having 5 glucose units which seemed to be 6^2- or 6^3-α-maltosylmaltotriose[25]. This is in agreement with the rsults shown in Table IV. These results indicate that the characteristic action of Pseudomonas isoamylase is hydrolysis of all α-1,6-glucosidic linkages in glycogen, but α-1,6-glucosidic linkages, attaching maltose residues to chains of α-1,4-linked glucose residues, are not readily hydrolyzed.

No enzyme is known to hydrolyze all the α-1,6-glucosidic inter-chain linkages in glycogen, although *S. mitis* pullulanase was found to hydrolyze these linkages well. An enzyme with such an ability seems very useful in the determination of the structures of glycogen and glycogen-like polysaccharides. 6-α-Maltotriosylmaltodextrins are known to be hydrolyzed over 10 times faster than 6-α-maltosylmaltodextrins by *S. mitis*, so Pseudomonas isoamylase is similar to *Streptococcus* pullulanase in this respect. However, the isoamylase clearly differs from the pullulanase in its action on pullulan, only the latter hydrolyzing this material.

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