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COMPARISON OF THE KINETIC PARAMETERS OF *PSEUDOMONAS* ISOAMYLASE AND *AEROBACTER* PULLULANASE

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SUMMARY

The specific activities of *Pseudomonas* isoamylase (amylopectin 6-glucanohydrolase, EC 3.2.1.9) and *Aerobacter* pullulanase were measured as cleavage of glycosidic bonds per min per mg of protein at substrate concentrations of 2%. Those of *Pseudomonas* isoamylase were 110–280 μmoles for amylopectins and glycogens and 1.1 μmoles for pullulan. Those of *Aerobacter* pullulanase were 3–5 μmoles for amylopectins, 0.5–1.2 μmoles for glycogens and 53 μmoles for pullulan.

The K_m values (g/ml) of the isoamylase were about $1 \cdot 10^{-4}$–$2 \cdot 10^{-4}$ for amylopectins and glycogens and $2 \cdot 10^{-3}$ for pullulan while those of the pullulanase were about $8 \cdot 10^{-3}$–$1 \cdot 10^{-2}$ for amylopectins, about $2 \cdot 10^{-2}$–$5 \cdot 10^{-2}$ for glycogens and $1.7 \cdot 10^{-5}$ for pullulan. The molecular activities of the isoamylase were about 9500 to 26 700 for amylopectins and glycogens but only about 110 for pullulan.

INTRODUCTION

Isoamylase (amylopectin 6-glucanohydrolase, EC 3.2.1.9) catalyzes the hydrolysis of α-1,6-glucosidic linkages in amylopectin and glycogen. Previously[1–3], we reported the discovery, purification and properties of *Pseudomonas* isoamylase. Subsequently, we found a significant difference between the actions of *Pseudomonas* isoamylase and *Aerobacter* pullulanase on amylopectin and glycogen[4]. In this work, the debranching actions of the two enzymes on various substrates are compared.

MATERIALS AND METHODS

Enzymes

Crystalline *Pseudomonas* isoamylase was prepared by dropwise addition of $(NH_4)_2SO_4$ solution in the cold to a solution of the enzyme purified from the culture filtrate of *Pseudomonas* SB-15 (ref. 4). Crystalline *Aerobacter* pullulanase was prepared

from the culture filtrate of *Aerobacter aerogenes* ATCC 9621 by a modification of the method of Wallenfels and Rached[5].

Substrates

Amylopectins were obtained from commercial starch samples by the method of Lansky *et al.*[6]. Rabbit liver and oyster glycogens were obtained from Nutritional Biochemical Co. and Sigma Chemical Co., respectively. Phytoglycogen from sweet corn was kindly supplied by Dr Doi of Osaka University. Pullulan was a product from Hayashibara Co. Ltd. A 10% solution of the substrate in 1 M NaOH was neutralized with 1 M HCl and the resulting solution was diluted appropriately before use.

Analytical methods

Total carbohydrate was estimated by the phenol–H_2SO_4 method[7]. Reducing power of enzyme digests was measured by the method of Nelson[8]. Protein was determined by the method of Lowry *et al.*[9].

RESULTS AND DISCUSSION

Debranching activity was routinely assayed by measuring the linear rate of production of reducing groups. The initial rate was a linear function of the incubation

TABLE I

SPECIFIC ACTIVITIES OF *Pseudomonas* ISOAMYLASE AND *Aerobacter* PULLULANASE ON VARIOUS SUBSTRATES

Reaction mixtures contained 1.6 ml of substrate solution, 0.2 ml of 0.5 M acetate buffer (pH 3.5 for the isoamylase and pH 5.5 for the pullulanase) and 0.2 ml of crystalline enzyme solution containing 0.0416 μg isoamylase or 21.8 μg pullulanase for amylopectins and glycogens and 51 μg isoamylase or 0.106 μg pullulanase for pullulan. The final concentration of substrates was 2%. After incubation for 15 min at 40 °C, 1 ml of the mixture was taken out for assay of reducing power.

Substrate	*Specific activity (μmoles aldehyde groups/min per mg protein)*	
	Pseudomonas isoamylase	*Aerobacter pullulanase*
Amylopectins		
Potato	221	4.8
Sweet potato	235	3.2
Wheat	246	3.8
Maize	275	2.9
Waxy maize	213	3.2
Glutinous rice	163	3.4
Acid-treated amylopectins*		
Waxy maize	118	7.2
Glutinous rice	97	5.0
Glycogens		
Oyster	162	0.9
Rabbit liver	111	0.6
Phytoglycogen		
Sweet corn	233	1.2
Pullulan	1.1	53.0

* Amylopectins were treated with 4% HCl at 35 °C for 20 h.

time under the conditions used for the assay. Previously, we could not obtain a good linear relationship between the concentration of substrates and activities of the enzymes[4]. Drummond *et al.*[10] also observed a significant dilution effect caused in the action of pullulanase on amylopectin but not on pullulan, although they used a much lower enzyme concentration than we did. Thus, the enzyme concentration seems to be important in measuring the specific activity of an enzyme such as this. The specific activities on amylopectins, glycogens and pullulan are shown in Table I. The data were obtained under conditions considered from our previous results[4] to be suitable for the actions of the enzymes. One unit of specific activity is defined as the amount of enzyme which liberates reducing power equivalent to 1 μmole of glucose in 1 min at 40 °C. One mg of isoamylase had about 160–280 specific activity units when acting on various amylopectins while 1 mg of the pullulanase had only about 3–5 units. The isoamylase had lower activities on acid-treated amylopectins (treatment with 4% HCl at 35 °C for 20 h) than on their original amylopectins while the pullulanase had higher activities on these than on their original amylopectins. The iso-

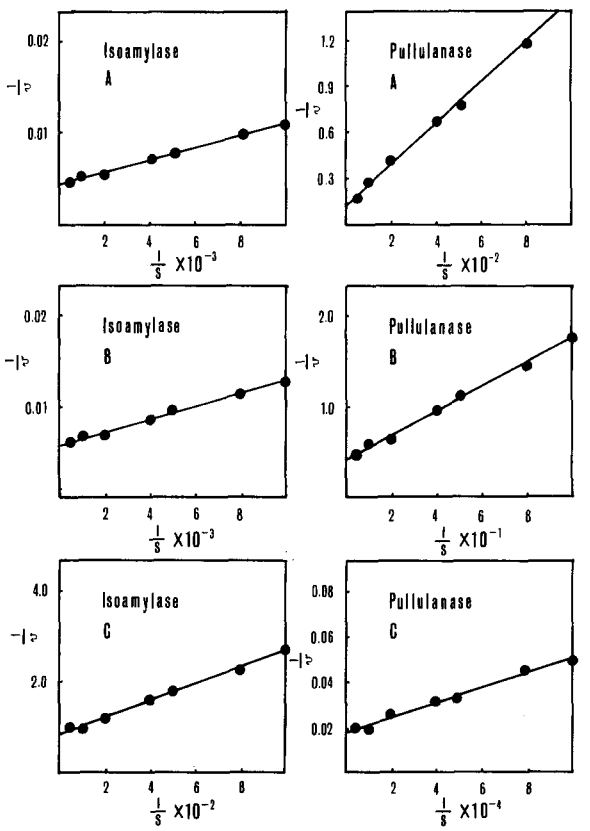

Fig. 1. Double-reciprocal plots of the kinetic data for potato amylopectin (A), oyster glycogen (B) and pullulan (C) with the isoamylase and pullulanase. The amounts of isoamylase and pullulanase used were 0.0134 μg and 31 μg, respectively, per 5 ml of incubation mixture with amylopectin and glycogen and 51 μg and 0.0077 μg, respectively, with pullulan. Substrate concentrations are expressed as g/ml and velocities as μmoles of aldehyde groups released per min per mg of enzyme protein.

amylase had slightly lower activities (110–160 units) on glycogens than on amylopectin while the pullulanase had much lower activities (0.5–1.2 units) The isoamylase had about 190- to 200-fold more activity on glycogen and 50- to 100-fold more activity on amylopectin than the pullulanase. On the other hand, values of 1.1 and 53 units for pullulan were obtained per mg of the isoamylase and pullulanase, respectively. Previously we concluded that the isoamylase could not hydrolyze pullulan[1]. This was probably because previously we used a lower concentration of enzyme. Thus, *Pseudomonas* isoamylase efficiently hydrolyzes α-1,6-glucosidic linkages in both amylopectin and glycogen, but scarcely hydrolyzes pullulan while *Aerobacter* pullulanase splits α-1,6-glucosidic linkages in pullulan, but scarcely hydrolyzes those in amylopectin or glycogen. Previously[4], we showed that pullulanase has much less activity on glycogen than on amylopectin from the time course of the actions of pullulanase on amylopectin and glycogen.

1 mg of *Pseudomonas* isoamylase showed 213 units of specific activity for maize amylopectin. In previous work 1 unit was expressed as the amount of enzyme causing an increase in $A_{610\ nm}$ of 0.1 in 1 h when coloration of the reaction mixture was developed by treatment with iodine solution. We reported previously[3] that the enzyme showed 59 100 units/mg of protein by the iodine coloration method. Here, we suggest adopting a new unit corresponding to the liberation of reducing power equivalent to 1 μmole of glucose in 1 min, because production of reducing power has been adopted as a method of expressing units of activity of many glucosidases[12]. One unit measured by this new method corresponds to 278 units by the old method and one unit by the old method corresponds to 0.0036 unit by the new method. When enzyme activity is low, units may be expressed as production of reducing power in 1 h rather than 1 min.

Fig. 1 shows Lineweaver–Burk plots of the effect of substrate concentration on the debranching activities of the two enzymes with potato amylopectin and oyster glycogen as representative substrates and with pullulan. The double-reciprocal plots were linear with all the substrates tested. The K_m values (g per ml) and V values (μmoles per min per mg protein) obtained from these results and results using other substrates are shown in Table II. With the isoamylase the K_m values were about $1 \cdot 10^{-4}$–$2 \cdot 10^{-4}$ for amylopectin, acid-treated amylopectin, glycogen and phytoglycogen. With the pullulanase the K_m values were about $8 \cdot 10^{-3}$–$1 \cdot 10^{-2}$ for amylopectin and acid-treated amylopectin and about $2 \cdot 10^{-2}$–$5 \cdot 10^{-2}$ for glycogen and phytoglycogen. The K_m values for pullulan were $2 \cdot 10^{-3}$ and $1.7 \cdot 10^{-5}$ with isoamylase and pullulanase, respectively. The difference between these two values was significant.

V values of the isoamylase for amylopectin and glycogen were similar to the values of specific activities obtained with 2% concentration of substrates, as shown in Table I. With pullulanase the V value with pullulan was also quite similar to the specific activity, shown in Table I, while the V values for amylopectins were a little larger, and those for glycogens were about 2- to 4-fold larger than the specific activities shown in Table I. This is probably because the K_m values of pullulanase for all these substrates except pullulan were quite high.

Molecular activity is defined as the number of equivalents of a certain group transformed per min per molecule of enzyme at the optimal substrate concentration. It can be calculated for the isoamylase since the molecular weight of *Pseudomonas* isoamylase is known to be 95 000 (ref. 3). The values obtained with amylopectin and

TABLE II

MICHAELIS CONSTANTS AND V VALUES OF *Pseudomonas* ISOAMYLASE AND *Aerobacter* PULLULANASE ON VARIOUS SUBSTRATES

Reaction mixtures contained 4 ml of various concentrations of substrate, 0.5 ml of 0.1 M acetate buffer (pH 3.5 for isoamylase, and pH 5.5 for pullulanase) and 0.5 ml of crystalline enzyme solution containing 0.077 μg pullulanase or 51 μg isoamylase with pullulan and 0.0134 μg isoamylase or 31 μg pullulanase with other substrates. After incubation for 30 min at 40 °C, the reducing power in the digest was assayed using 1 ml aliquots. In the cases of incubation of pullulanase with amylopectin or glycogen and of isoamylase with pullulan, whole incubation mixtures were used for assay.

Substrate	*Pseudomonas isoamylase*		*Aerobacter pullulanase*	
	$K_m \times 10^4$ *(g/ml)*	V *(μmoles/min per mg protein)*	$K_m \times 10^3$ *(g/ml)*	V *(μmoles/min per mg protein)*
Amylopectins				
Potato	1.4	225	10.1	7.2
Sweet potato	1.3	240	8.3	4.5
Wheat	1.4	250	7.6	5.3
Maize	1.3	280	9.9	4.4
Waxy maize	1.7	215	9.8	4.6
Glutinous rice	1.3	170	9.1	4.8
Acid-treated amylopectins*				
Waxy maize	1.5	120	8.9	10.2
Glutinous rice	1.0	100	7.7	7.0
Glycogens				
Oyster	1.1	165	33	2.3
Rabbit liver	1.2	115	20	0.9
Phytoglycogen				
Sweet corn	1.0	240	50	4.2
Pullulan	20	1.1	0.017	52.9

* See footnote to Table I.

glycogen were about 9500–26 700 while that for pullulan was only about 110. The molecular weight of the pullulanase of *A. aerogenes* ATCC 9621 has not been determined although crystalline pullulanase from another strain of the species was reported to have a molecular weight of 108 000–145 000 (ref. 12). Assuming that the molecular weight of our enzyme is 145 000, the molecular activity of this pullulanase with amylopectin and glycogen would be only 130–1400 and that with pullulan would be 7700. From these results the isoamylase was found to have higher affinity and activity than pullulanase for glycogen and amylopectin, especially for glycogen. On the other hand, unlike the isoamylase, the pullulanase has higher affinity and activity for pullulan than for amylopectin and glycogen.

The enzyme hydrolyzing α-1,6-glucosidic linkages in amylopectin was named isoamylase by Maruo and Kobayashi[13]. Manners[14] characterized isoamylase as an enzyme acting on both glycogen and amylopectin but not on pullulan. *Pseudomonas* isoamylase has slight activity towards pullulan. Manners[14] characterization of isoamylase as an enzyme which does not cleave pullulan may have to be changed to an enzyme that causes little or no cleavage of pullulan. Experiments using large amounts of highly purified enzyme are necessary before concluding that an enzyme does not act on a certain substrate. We regard our enzyme in *Pseudomonas* as an isoamylase although it has slight activity towards pullulan. We have analyzed the structures of glycogen and amylopectin using this enzyme[15,16]. This enzyme has strong activity

to cleave α-1,6-glucosidic linkages in glycogen and amylopectin and causes complete hydrolysis of these linkages. Thus, it is a useful tool in elucidating the structures of such polymers.

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