Affinity Chromatography of *Pseudomonas* Isoamylase on Cross-linked Amylose Gel

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The use of amylose gel cross-linked by epichlorohydrin for affinity chromatography of extracellular isoamylase of *Pseudomonas amyloclavata* was studied. The isoamylase was adsorbed well on the amylose gel and was eluted specifically with maltodextrin. The eluted enzyme was precipitated with ammonium sulfate to remove maltodextrin, and then the solution of the precipitate was dialyzed and concentrated by vacuum filtration. By this procedure 96 mg of the enzyme were purified to homogeneity from 20 liters of culture broth in about 70% yield.

Pseudomonas isoamylase¹⁾ (glycogen 6-glucanohydrolase, EC 3.2.1.68) splits the branching points of amylopectin and glycogen completely. The enzyme is useful for elucidating the structures of amylopectin and glycogen and for commercial production of amylose and maltose from starch, alone and in combination with β -amylase. In previous works in this laboratory, the isoamylase was purified about 720-fold in 23% yield from the culture fluid of the organism^{2,3)} and was also obtained in a crystalline form.⁴⁾ Recently, we found that this isoamylase was adsorbed to raw starch. α -Amylases from *Pseudomonas saccharophila*⁵⁾ and from *Bacillus licheniformis*⁶⁾ are known to be adsorbed to starch. Sandstedt and Ueda⁷⁾ reported the adsorption of four different α -amylases on raw starch and the relation of this to digestion of the starch. The affinity of α -amylase from *Streptomyces aureofaciens* for cross-linked starch was shown to be influenced by the presence of soluble substrates and proteins and the pH.⁸⁾ Because of increasing interest in the structure, function and practical uses of *Pseudomonas* isoamylase, we developed a simple new procedure using amylose gel for its isolation.

MATERIALS AND METHODS

Preparation of cross-linked amylose gel adsorbent. Amylose gel was prepared by a modification of the method of Matsumoto and Osawa⁹⁾ as follows. Amylose HyF-P was used as starting material, because branching points of amylopectin are split by the isoamylase but amylose is inert to it, although it is uncertain whether the branching points of cross-linked amylopectin gel are attacked by the isoamylase. A mixture of 30 ml of 5 N NaOH, 20 ml of epichlorohydrin and 20 ml of an aqueous suspension of 10 g potato amylose were stirred vigorously at 40°C. The suspension solidified in 10 min as a gel cake. This was stood for 30 min at 40°C, and then homogenized with a Universal Homogenizer from Nihon Seiki Co. The gel obtained was suspended in water and washed by decantation and the floating particles were discarded.

Preparation of crude isoamylase. *Pseudomonas amyloclavata* K1C was used. It is a revertant strain of K1, which is deficient in isoamylase and is derived from an isoamylase constitutive mutant strain MS1.¹⁰⁾ Strain K1C produces isoamylase constitutively as well as intracellular α -amylase and α -glucosidase. It was cultured aerobically at 30°C for 96 hr in the medium described by Harada *et al.*¹¹⁾: 2% maltose, 0.4% sodium glutamate, 0.15% $(\text{NH}_4)_2\text{HPO}_4$, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.01% each of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and 0.01% NaCl, at pH 5.5. After culture, cells were removed by centrifugation and the supernatant was used for purification of the extracellular isoamylase.

Assay of isoamylase activity. The reaction mixture contained 0.5 ml of 1% maize amylopectin and 0.4 ml of 0.1 M acetate buffer, pH 3.5. The mixture was incubated with 0.1 ml of the enzyme solution at 40°C for

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1 hr and then the reducing power of the digest was measured by the method of Somogyi and Nelson.¹²⁾ One unit of enzyme activity is expressed as the amount liberating 1 μ mole of aldehyde group as glucose per min.

Determination of protein concentration. Protein concentration was estimated by the method of Lowry *et al.*¹³⁾ using bovine serum albumin as a standard. The amount of protein in effluents from columns was determined from the absorbance at 280 nm, using a Hitachi 124 UV-VIS spectrophotometer.

Gel electrophoresis. Polyacrylamide gel electrophoresis was carried out at pH 8.3 by the method of Davis¹⁴⁾ with a current of 3 mA for 1.5 hr, and then the gel was stained with Amido Black 10B.

Reagents. Amylose HyF-P (Hayashibara Biochemical Laboratory), prepared from potato starch by treatment with *Pseudomonas* isoamylase,¹⁵⁾ was used to make cross-linked amylose gel. Maltose and maltotriose were the products of Hayashibara Biochemical Laboratory.

Maltodextrin (Ran) was also supplied from Hayashibara Biochemical Laboratory. This preparation was obtained by hydrolysis of corn starch with *Bacillus* α -amylase and about 60% of it was saccharides with a higher degree of polymerization than maltotetraose. It contained about 2.5% of saccharides that could not be removed by dialysis; these were removed by precipitation with two volumes of ethanol to prevent the purified enzyme from being contaminated. Maize amylopectin was purchased from Sigma Chemical Co. Isoamylase was purified in our laboratory as reported previously.¹⁾ Other chemicals were commercial products of the highest purity available.

RESULTS AND DISCUSSION

Adsorption of enzymes on amylose gel

We prepared cross-linked amylose gel and tested suitable conditions for its adsorption of *Pseudomonas* isoamylase. Results showed that isoamylase was adsorbed to the gel under acidic conditions (pH 3.5~5.5) where the enzyme is stable. To determine the optimal amount of amylose gel required for adsorption of the enzyme, we mixed various amounts of the gel in 2 ml of 0.01 M acetate buffer, pH 3.5, with 2 ml of buffer containing 170 μ g of isoamylase in a Micro Thermo-mixer (Thermomix Co.) for 1 min and then incubated the mixtures for 10 min at 4°C, centrifuged them at 3000 rpm for 10 min and measured the

FIG. 1. Influence of the Amount of Amylose Gel on Adsorption of Isoamylase Activity.

The amount of gel is shown as dry weight.

enzyme activity of the supernatant. As shown in Fig. 1, 50 mg of the gel adsorbed most of the enzyme, and one mg dry weight of the gel adsorbed about 3 μ g of isoamylase.

Elution of isoamylase from an amylose gel column

The abilities of various compounds to elute isoamylase from amylose gel were examined. For the tests isoamylase (50 μ g/10 ml) was applied to columns (7 $\times$ 50 mm) of amylose gel at 4°C. No activity could be eluted with 0.1 M acetate buffer (pH 3.0~5.5), 0.1 M phosphate buffer (pH 5.5~7.5) or 0.1 M ammonium buffer (pH 8.0~9.0). Elutions with solutions of 1 to 20% sodium chloride were

FIG. 2. Elution of Isoamylase from Amylose Gel.

Isoamylase (50 μ g) adsorbed to amylose gel was eluted with 20 ml of solutions of maltodextrin (○), maltotriose (●), maltose (△), glucose (▲) and sodium chloride (□). Recovery of the enzyme in the eluate is shown.

also tested under the same conditions, dialyzing the eluate against 0.01 M acetate buffer, pH 3.5, before measuring activity to avoid the effect of salt on the assay. However, less than 5% of the isoamylase was eluted from the column with any of the concentrations of sodium chloride tested (Fig. 2). Solutions of maltodextrin, prepared from corn starch by treatment with *Bacillus* α -amylase, maltotriose, a competitive inhibitor of isoamylase¹⁶⁾ and maltose were also examined (Fig. 2). Most of the adsorbed enzyme could be eluted with these compounds, but higher concentrations of maltose than of maltotriose and maltodextrin were required: 5 percent aqueous solutions of maltodextrin and maltotriose eluted almost all the isoamylase from the gel.

Purification of isoamylase using amylose gel

The isoamylase from *Pseudomonas amylo-deramosa* K1C was purified as follows. All operations were carried out at 4°C. Twenty liters of culture supernatant were divided into two portions. Each portion (10 liters) was directly applied to a column (65×210 mm) of the amylose gel equilibrated with 0.01 M acetate buffer, pH 3.5. The column was washed with 7 liters of the same buffer containing 0.5 M NaCl and then isoamylase was eluted with 1500 ml of 5% maltodextrin in the same buffer. The flow rate was 330 ml per hr and 100 ml fractions were collected. The elution pattern is shown in Fig. 3. The fractions of effluent containing the enzyme were pooled and treated with 60% saturation of ammonium sulfate. The resulting precipitate was collected by centrifugation, dissolved in 20 ml of 0.01 M acetate buffer, pH 3.5 and dialyzed and concentrated against 0.01 M

FIG. 3. Column Chromatogram of Isoamylase on Amylose Gel.

Arrows A and B indicate the points at which elution with 0.01 M acetate buffer (pH 3.5) containing 0.5 M sodium chloride and the same buffer containing 5% maltodextrin respectively were started. The solid bar indicates the fractions pooled for further purification. ○, absorbance at 280 nm; ●, isoamylase activity.

acetate buffer, pH 3.5, by vacuum filtration through a collodion membrane. In this way the isoamylase was purified 93-fold from the culture supernatant in a yield of 70%, as summarized in Table I. About 96 mg of the enzyme were obtained from 20 liters of culture supernatant. Electrophoresis on polyacrylamide gel gave a single protein band (Fig. 4). This affinity chromatography is much simpler and gives a better yield than the previous method involving 5 steps of purification.²⁾ The column of amylose gel could be used repeatedly: little or no loss of binding capacity of the column was detected in one month during repeated use.

Fortunately, the culture supernatant of *Pseudomonas amylo-deramosa* does not contain other amylases, such as α -amylase, glucoamylase and α -glucosidase. If they were present they would also be adsorbed to the amylose

TABLE I. ENZYME PURIFICATION

Step of purification	Vol. (ml)	Protein (mg)	Activity		
			Units	Yield (%)	Specific activity (units/mg)
Crude filtrate	20000	12800	26100	(100)	2.04
Amylose gel	1800	111	21100	81	190
(NH ₄) ₂ SO ₄ 0~60%	20	96.2	18300	70	190

FIG. 4. Polyacrylamide Disc Electrophoresis of Isoamylase at pH 8.3.

Approximately 0.1 mg of protein was applied to the gel. Electrophoresis was carried out for 2.5 hr at 2.0 mA at 4°C. The gel was stained with Amido Black dye.

gel and more strict conditions would be necessary for preparing the gel or adsorbing the isoamylase on it.

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