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Glucoamylase I of Black *Aspergillus*

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Glucoamylase I of black *Aspergillus* was purified by $(NH_4)_2SO_4$ precipitation, ethanol fractionation, ion exchange chromatography on DEAE-cellulose, preparative isoelectric focusing and Sephadex G-100 gel filtration. The enzyme thus purified was found to contain no α-amylase and appeared to be homogeneous in polyacrylamide gel electrophoresis. The isoelectric point of glucoamylase I was at pH 3.4. The optimum conditions for its action on boiled soluble starch were at 60°C and pH 4.5. The enzyme was quite stable at pH 4.0-5.0 and temperature up to 40°C. It had a strong debranching activity (0.56) and it hydrolyzed about 75 % of soluble starch but glycogen and p-limit dextrin from glycogen almost completely. It could be almost completely adsorbed onto raw starch and was active in raw starch digestion. Maximum digestion of raw starch by this enzyme occurred at pH 3.4. Glucoamylase I contained about 7.6 % carbohydrate.

INTRODUCTION

In recent years, there has been considerable attention in mold glucoamylase (1, 4-α-D-glucan glucohydrolase, EC 3.2.1.3) because of its industrial usefulness. This enzyme is an exo-acting carbohydrase which cleaves glucose units from the non-reducing end of starch. Glucoamylase can digest raw starch and is used successfully for the alcoholic fermentation of raw starch without cooking (Ueda and Saha, 1981; Ueda, 1981). Ueda (1957) found that the action of black koji amylase on raw starch was much stronger than that of yellow koji or malt amylase. Ueda et *al.* (1974) separated glucoamylase I and glucoamylase II of black koji mold by raw starch adsorption and showed that glucoamylase I, the debranching active amylase, was very strong in raw starch adsorption and digestion whereas glucoamylase II, the debranching deficient amylase, could not be adsorbed onto raw starch and was weak in raw starch digestion. Hayashida (1975) also reported that glucoamylase I of *Aspergillus awamori* var. *kawachi* was active in raw starch adsorption and digestion. Glucoamylase I of *Rhizopus sp.* (Ueda and Kano, 1975) and *Aspergillus oryzae* (Miah and Ueda, 1977; Saha et *al., 1979)* had a high debranching activity and was active in raw starch digestion. Although glucoamylase I of black *Aspergillus* has already been studied by many workers yet many of the properties of this enzyme are still in dispute (Pazur et al., 1971; Lineback et al., 1969; Freedberg *et al.*, 1975; Yoshino and Hayashida, 1978). It was of our interest

to establish a more refined purification procedure of this debranching active glucoamylase I of black *Aspergillus* and to study extensively the physico-chemical characteristics of this enzyme.

MATERIALS AND METHODS

Enzyme preparation

A commercial glucoamylase preparation of black *Aspergillus* (Sumizyme AN, Lot No. 781114) was kindly supplied by Shin-Nihon Chemical Co., Japan. The supplied enzyme solution was dialyzed at first against running tap water and then deionized water with two changes for about 24 hr to remove reducing sugars present before use as crude enzyme solution.

Enzyme assay

Glucoamylase activity was assayed by measuring the release of glucose from boiled soluble starch. A mixture of 5.0 ml of 1% boiled soluble starch solution, 1. Oml of 0.2M acetate buffer, pH 4.5 and 1.0 ml of deionized water was preincubated for 5 min at 40°C. Then 1.0 ml of enzyme solution was added and mixed. After 10 min reaction, 1 ml of the reaction mixture was withdrawn and the amount of reducing sugar liberated was measured by micro-Bertrand method. One unit of glucoamylase activity is defined as the amount of enzyme which produces 1 mg glucose in 1 ml of the reaction mixture under the above conditions.

Protein determination

Protein was determined by measuring absorbance at 280nm using bovine serum albumin as standard.

Isoelectric focusing

Isoelectric focusing of the enzyme was performed by the method of Vesterberg and Svensson (1966).

Debranching activity

Debranching activity of glucoamylase was measured according to the method previously reported (Mitsue *et al.*, 1979).

Disc electrophoresis

Disc electrophoresis in 7.5 % polyacrylamide gel at pH 8.4 in Tris-glycine buffer was carried out according to the method of Davis (1964).

Substrate specificity

Initial reaction velocity was determined by adding a suitable quantity of enzyme to various substrates (1% for macromolecular polysaccharides and 0.25 % for oligosaccharides) to produce a linear increase of glucose during the first 15min of reaction and the glucose liberated was determined by glucose oxidase method (Dahlqvist, 1961).

Raw starch adsorption and digestion

The procedure followed for raw starch adsorption was a modification of the procedure described previously (Saha and Ueda, 1981). 0.5 g raw starch was washed with 5 ml of 0.1 M acetate-HCl buffer, pH 3.4. Five ml of purified glucoamylase I acidified to pH 3.4 with 1 N HCI was added to the washed raw starch at 4°C and stirred for 20 min. After centrifugation, amylase activity of the supernatant was assayed and the adsorption percent was calculated.

Raw starch digestion was determined according to the method reported previously (Ueda and Saha, 1980).

Estimation of carbohydrate

Total carbohydrate content was measured by the phenol-sulfuric acid method (Dobuis et *al.*, 1956) with glucose as standard.

RESULTS AND DISCUSSION

Purification of glucoamylase I

All the purification steps were carried out at 4℃, unless otherwise mentioned.

Step 1. *Ammonium sulfate fractionation*

To the dialyzed crude enzyme solution (880 ml), solid ammonium sulfate was added with constant stirring to give 0.8 saturation (25°C). After standing overnight, the precipitate formed was collected by centrifugation at 12,000 x g for 20min and dissolved in a small volume of 0.1 M phosphate-citrate buffer, pH 5.0. The solution was dialyzed at first against running deionized water and then 0.1 M phosphate-citrate buffer, pH 5.0 for 48 hr.

Step 2. *Ethanol fractionation*

To the dialyzed solution (220ml) from step 2, chilled ethanol (-20°C) was added drop by drop under continuous stirring to give a concentration of 75 % (v/v) and kept overnight at -20°C. The resultant precipitate was collected by centrifugation at 17,000 x g for 20 min, dissolved in a small volume of 0.1 M phosphate-citrate buffer, pH 5.0 and dialyzed against the same buffer for 24 hr.

Step 3. *DEAE-cellulose column chromatography*

The dialyzed enzyme solution from step 2 was concentrated to about 20 ml by ultrafiltration in an Amicon cell (Model 202, Amicon Corp., Lexington, Mass.) equipped with UM 20 membrane under a nitrogen pressure of 3 to 4 kg/cm². The concentrated solution was then applied to a DEAE-cellulose column (5 x 77 cm) equilibrated with 0.1 M phosphate-citrate buffer, pH 8.2. The column was eluted stepwise with the same buffer at two different pH of 8.2 and 4.2. Twenty ml fractions were collected at a flow rate of 30 ml/hr. A typical elution pattern of the absorbance at 280 nm and of enzyme activity is shown in Fig. 1. A small non-active protein fraction (Peak I) and the amylase fraction (Peak II) which could not be adsorbed on DEAI-cellulose at pH

8.2 were eluted at pH 8.2 whereas only one protein fraction (Peak III) was eluted at pH 4.2. Peak III and Peak II were referred to as glucoamylase I and glucoamylase II, respectively. The two active fractions were pooled separately, concentrated by ultrafiltration and dialyzed for 20 hr. Studies with these enzyme fractions revealed that glucoamylase I had a high dehranching activity and was active in raw starch adsorption and digestion whereas glucoamylase II had a weak debranching activity, could not be adsorbed onto raw starch and was weak in raw starch digestion. No further purification of glucoamylase II was carried out.

Step 4. *Preparative isoelectric focusing*

The dialyzed glucoamylase I solution obtained at step 3 was subjected to isoelectric focusing in a sucrose density gradient on carrier ampholyte (Pharmalyte, Pharmacia Fine Chemicals, Sweden) of pH 2.5—5.0 and was charged for 48 hr. 250 to 300 mg protein was applied at a time and 2 ml fractions were collected. pH and absorbance at 280nm of each fraction were measured. Fractions corresponding to each peak were pooled separately, dialyzed at first against deionized water and then with **0.** 1 M phosphate-citrate buffer, pH 4.2 and the activity was measured. The result is shown in Fig. 2. One major and two small peaks of protein were obtained. The major peak was found to be enzyme protein. The enzyme had an isoelectric point at pH 3.4 which indicates that the enzyme was an acidic protein.

Step 5. *First Sephadex G-100 gel filtration*

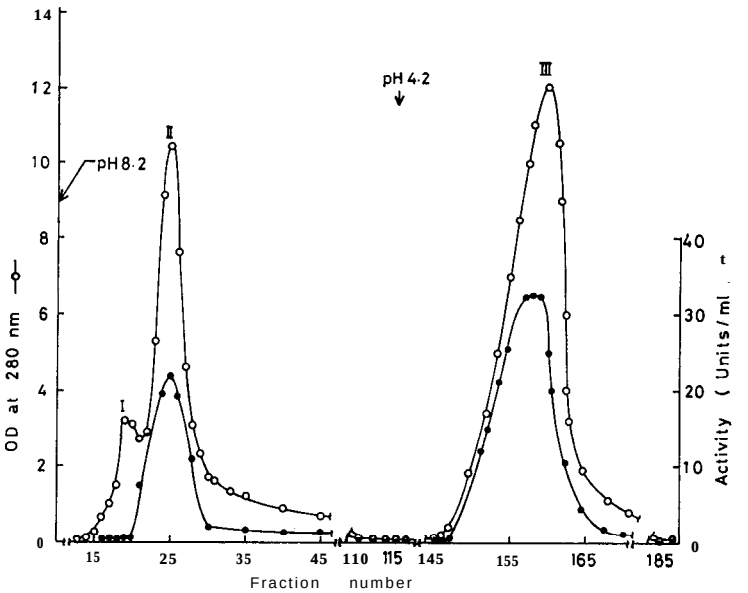

Fig. 1. DEAE-cellulose column chromatography. The dialyzed enzyme solution obtained from ethanol fractionation was applied to a column (5X 77 cm) equilibrated with 0.1 M phosphate-citrate buffer, pH 8.2 and eluted stepwise with the same buffer at pH 8.2 and 4.2. Fractions (20 ml) were collected at a flow rate of 30 ml/hr.

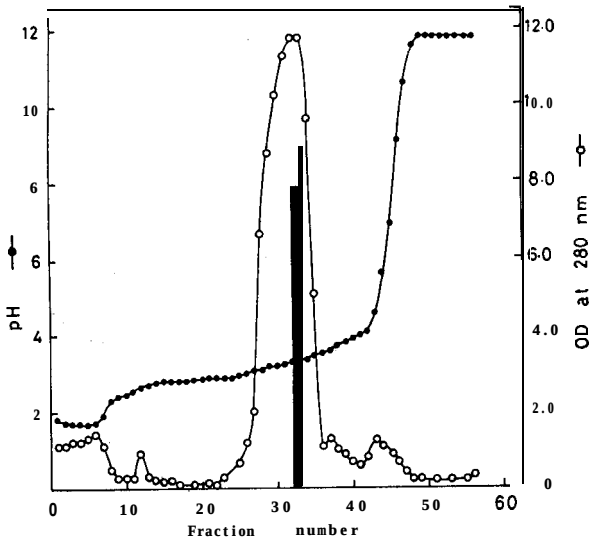

Fig. 2. Preparative isoelectric focusing of glucoamylase I. The dialyzed enzyme solution obtained after DEAE-cellulose column chromatography was subjected to isoelectric focusing on carrier ampholyte (pH 2.5-5.0) in a sucrose density gradient in a LKB 110 ml column at 15°C for 48 hr at 1.0 to 0.8 W. Two ml fractions were collected.

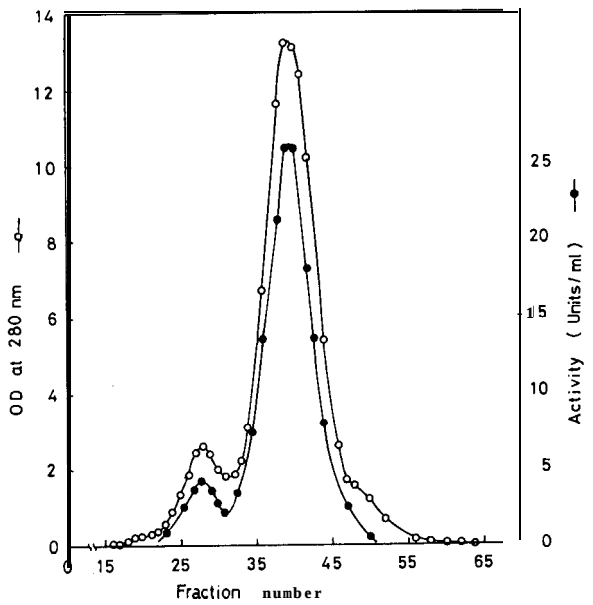

Fig. 3. First Sephadex G-100 gel filtration of glucoamylase I. The dialyzed enzyme solution obtained after isoelectric focusing was applied to a column (1.5×132 cm) equilibrated with 0.1 M phosphate-citrate buffer, pH 4.2. Fractions were collected at a flow rate of 9 ml/hr.

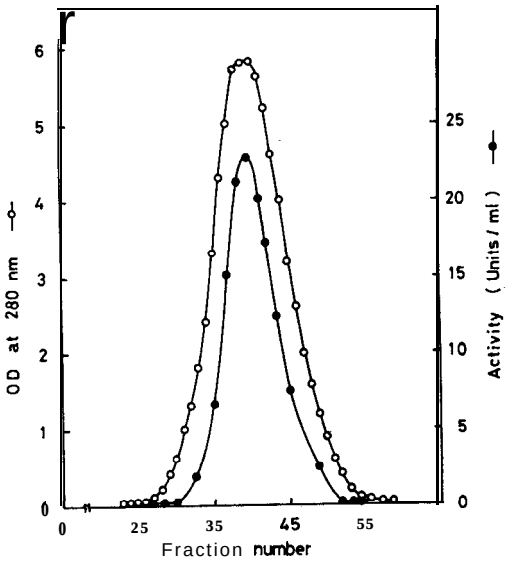

Fig. 4. Second Sephadex G-100 gel filtration of glucoamylase I. The dialyzed enzyme solution after Sephadex G-100 gel filtration was rechromatographed on Sephadex G-100 under the same conditions as in Fig. 3.

The glucoamylase I solution obtained from step 4 was concentrated by ultrafiltration and applied to a Sephadex G-100 column (1.5 × 132 cm) previously equilibrated with 0.1 M phosphate-citrate buffer, pH 4.2. Each 5 ml fraction was collected at a flow rate of 9 ml/hr. The elution profile is shown in Fig. 3. Active fractions of the two enzyme fractions were pooled separately and dialyzed against the eluting buffer for 12 hr. The enzyme solution of the first peak (Fraction No. 23-31) was found to contain mostly α-amylase and was discarded.

Step 6. *Second* ***Sephadex*** *G-100 gel* ***filtration***

The enzyme solution of the second peak obtained at step 5 was rechromatographed on Sephadex G-100 column (1.5 x 132 cm) under the same conditions as in step 5. Elution pattern is shown in Fig. 4. Active fractions were pooled and dialyzed against 0.1 M phosphate-citrate buffer, pH 4.2. The glucoamylase I preparation was found to show only one peak and contain no α-amylase as was evident from the retention of colour of starch in the reaction mixture with iodine even after 10 days of its reaction with boiled soluble starch.

The glucoamylase I thus purified was 4 fold pure over the crude enzyme and was used for subsequent studies.

A summary of the purification procedures is given in Table 1.

Criteria of purity

Polyacrylamide ***gel*** *electrophoresis*

The purified glucoamylase I displayed homogeneity in polyacrylamide gel

Table 1. Purification of glucoamylase I of black *Aspergillus*.

	Purification step	Total protein (mg)	Total activity (units)	Specific activity (units /mg protein)	Recovery (%)
1.	Crude enzyme	43,568	34,854	0. 80	100. 0
2.	$(NH_4)_2SO_4$ fractionation	14,813	23,701	1. 60	***68.0***
3.	Ethanol fractionation	8,313	14,298	1. 72	***41.0***
4.	DEAE-cellulose				
	Glucoamylase I	1,874	4,180	2.23	***12.0***
	Glucoamylase II	914	1,033	1. 13	***3.0***
5.	Isoelectric focusing				
	Glucoamylase I	1.410	3,427	2. 43	***9.8***
6.	First Sephadex G-100				
	Glucoamylase I	836	2,676	3.20	***7.7***
7.	Second Sephadex G–100				
	Glucoamylase I	805	2,578	3. 20	***7. 4***

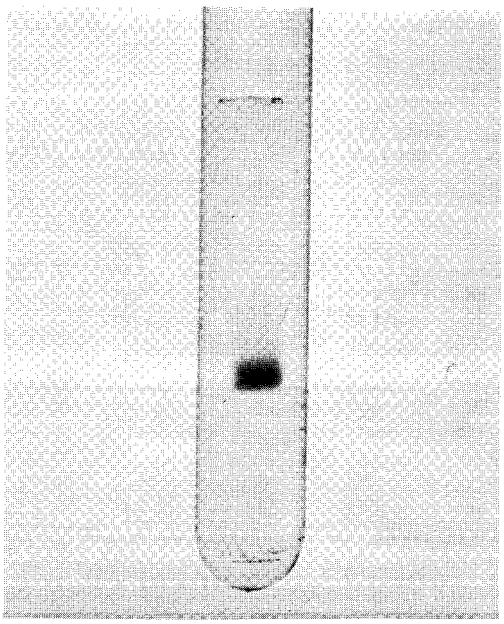

Fig. *5.* Disc gel electrophoresis of purified glucoamylase I. Electrophoresis in 7.5 % polyacrylamide gel at pH 8.4 in Tris-glycine buffer was performed with a current flow of 3 to 4 mA per tube. About 150 μg protein was applied in each tube. Gels were stained with 1 % Amido black 10-B.

electrophoresis indicating the presence of a single protein in it. Fig. 5 reveals that glucoamylase I had migrated as a single protein band towards anode.

Properties of glucoamylase I

Effect of pH on enzyme activity

Glycine–HCl buffer (pH 2.0), phosphate-citrate buffer (pH 3.0-7.0) and glycine–NaOH buffer (pH 8.0-10.0) -each of 0.2 M were used. The enzyme activity was measured according to standard assay method. The optimum pH for the action of glucoamylase I was found to be 4.5.

Effect of temperature on enzyme activity

The reaction mixture containing enzyme was incubated at various temperatures and the activity of the enzyme was measured according to standard method. The optimum temperature for the action of glucoamylase I was 60°C.

pH stability

Enzyme solutions in buffers of various pH (0.2 M) were incubated at 40°C for 30min. Then the pH of the solutions were adjusted at 4.5 (optimum pH) and the residual activities were measured. Glucoamylase I was found to be quite stable at pH 4.0-5.0.

Thermal stability

Enzyme in 0.1 M acetate buffer at pH 4.5 was incubated at the desired temperature for 30min. It was then cooled at 4°C and the residual activity of the enzyme was measured according to standard assay system. The enzyme was quite stable up to 40°C with essentially no loss of activity in 30min.

Substrate specijcity

The substrate specificity of the enzyme using various substrates was tested and the result is presented in Table 2. Glucoamylase I could hydrolyze soluble starch, β-limit dextrin from glycogen, amylose, amylopectin, glycogen, dextrin, maltodextrin, maltotriose and maltose, the initial rate of attack on

Table 2. Relative rates of initial reaction velocity of glucoamylase I on various substrates.

Substrate	Relative rate of hydrolysis (%)[1]
Soluble starch	100
Amylose A (MW 4,000)	95
Amylose B (MW 21,000)	75
Amylopectin	84
Glycogen	100
Dextrin	90
p-limit dextrin from glycogen	56
Maltodextrin	75
Pullulan	0.34
Maltotriose	20
Maltose[2]	10
α-Methyl-D-glucoside	0.00

1) Rates of initial reaction velocity expressed relative to soluble starch as 100.
2) Value for glucose released divided by 2.

different substrates being different.

Time course of hydrolysis of boiled soluble starch, glycogen and p-limit dextrin from glycogen

The hydrolysis pattern of boiled soluble starch, glycogen and β-limit dextrin from glycogen by glucoamylase I is shown in Fig. 6. The enzyme was found to hydrolyze about 75 % of soluble starch but glycogen and β-limit dextrin almost completely.

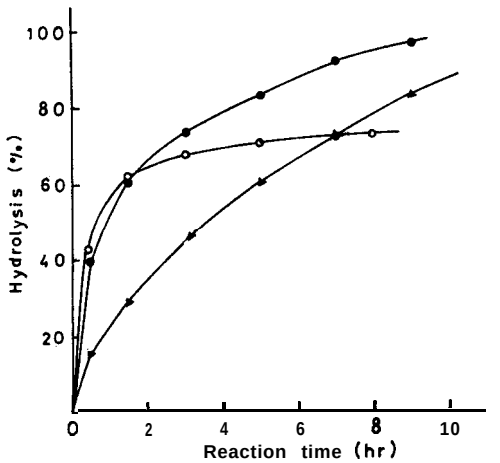

Fig. 6. Hydrolysis of boiled soluble starch (○), glycogen (●) and β-limit dextrin from glycogen (A) by glucoamylase I (0.85 unit). Reaction mixture (8 ml) contained substrate (50 mg) and acetate buffer (pH 4.5. final concentration 0.025 M). Samples were removed at various times for measurement of reducing sugar by the micro-Bertrand method.

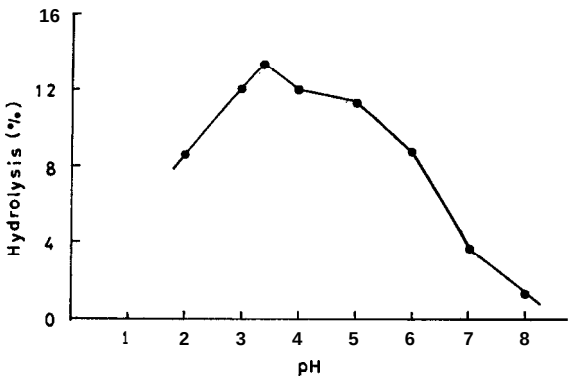

Fig. 7. Effect of pH on raw starch digestion by glucoamylase I (1.15 units). The reaction mixture containing 50mg raw wheat starch, 0.5 ml 0.5 M sodium citrate-HCl buffer of the desired pH, 1.0 ml deionized water, 1.0 ml enzyme and a few grains of thymol was incubated at 30°C for 24 hr. Reducing sugar liberated in 1 ml of the reaction mixture was determined by the micro-Bertrand method.

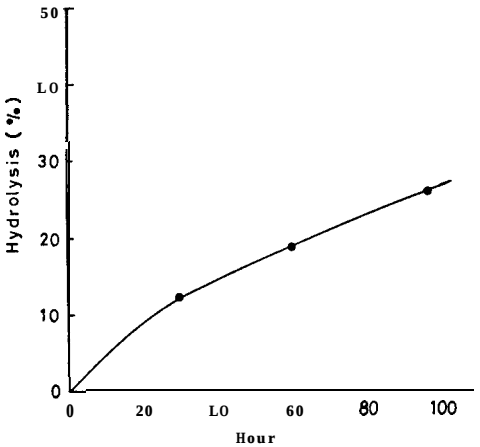

Fig. 8. Time course of raw starch digestion by glucoamylase I (0.85 unit). Conditions same as in Fig. 7.

Debranching activity

The debranching activity of glucoamylase I was found to be 0.56 which indicates that the enzyme had a strong debranching activity.

Raw starch adsorption and digestion

Glucoamylase I could be almost completely adsorbed onto raw wheat starch at pH 3.4 which was the isoelectric pH of the enzyme.

Fig. 7 shows the effect of pH on the digestion of raw wheat starch by glucoamylase I. It was found that glucoamylase I could digest raw wheat starch maximally at pH 3.4. From this result, it may be concluded that the optimum pH for raw starch hydrolysis is different from that of soluble starch hydrolysis in case of glucoamylase I of black *Aspergillus.*

A time course of raw starch digestion by glucoamylase I is presented in Fig. 8 which indicates that the enzyme was strong in raw starch digestion.

Total carbohydrate content

Analysis by phenol-sulfuric acid method revealed that glucoamylase I contained about 7.6 % carbohydrate relative to a standard of D-glucose. This amount of carbohydrate is similar to that of glucoamylase I of *Aspergillus awamori* var. *kawachi* (Yoshino and Hayashida, 1978) but different from that of glucoamylase I of *Aspergillus niger* (Lineback and Aira, 1972; Pazur *et al.*, 1980).

Raw starch adsorption and elution behaviour of this enzyme, especially the effect of amylase inhibitor on its adsorption and borate on elution will be reported in the next paper.

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