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Note

Rapid procedure for purification of β-amylase from *Ipomea batata**

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Methods for the preparation of β-amylase[2–6] are generally lengthy and give low yields. We have earlier reported an affinity method for the purification of β-amylase[7] that gave a high yield of the enzyme. Here we report a quicker and simpler chromatographic procedure, which gives β-amylase of high purity. β-Amylase is a commercial enzyme and an alternative quicker procedure is of interest.

EXPERIMENTAL

Extraction of the enzyme

A 100-g amount of peeled and sliced red sweet potato (*Ipomea batata*) was blended in a Waring blender in 0.1 *M* acetate buffer (pH 4.8) containing 0.002 *M* 2-mercaptoethanol and filtered through cheese-cloth. This extract was kept at 4°C for 30 min. After removing the starch, the supernatant was centrifuged in the cold for 10 min at 5000 *g*. The supernatant at this stage was taken as a crude extract.

Determination of enzyme activity and protein

Enzyme activity was assayed by the 3,5-dinitrosalicylic acid method[8]. The assay mixture was prepared by mixing 0.5 ml of 1% soluble starch in 0.1 *M* acetate buffer (pH 4.8) and 0.5 ml of suitably diluted enzyme. This mixture was incubated at 30°C for 3 min, then the reaction was terminated by addition of 1 ml of dinitrosalicylic acid reagent. After heating for 5 min on a boiling water-bath, the mixture was cooled to room temperature and 10 ml of distilled water were added. The colour that developed was measured at 550 nm on a Klett–Summerson colorimeter.

Protein was determined by using the method of Lowry *et al.*[9]. The phenol–sulphuric acid method of Dubois *et al.*[10] was used for the determination of the carbohydrate content in each step of the purification.

Enzyme unit, specific activity and enrichment ratio

One unit (U) of β-amylase activity is defined as the amount of enzyme that liberates 1 μmole/min of maltose at pH 4.8 and 30°C.

The specific activity of the enzyme is defined as the number of enzyme units per milligram of protein.

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The enrichment ratio is expressed as the ratio of the specific activity after a given step after the initial step.

Polyacrylamide gel electrophoresis

This was carried out according to Gabriel[11]. In order to check the β-amylase activity in the gel, 1% of starch was incorporated during polymerization and, after completion of electrophoresis, one gel was incubated at 30°C for 20 min in acetate buffer. Then the gel was immersed in 0.01 *M* potassium iodide–iodine solution. The zone that did not undergo iodine staining corresponded to β-amylase protein.

Ammonium sulphate precipitation

To the crude extract ammonium sulphate was slowly added with constant stirring at 4°C until 40% saturation and then kept at 4°C for 30 min. This was followed by centrifugation in the cold at 5000 *g* for 10 min. After discarding the precipitate, the ammonium sulphate concentration was increased to 60% very carefully as described above. After centrifugation in the cold at 5000 *g* for 10 min a clear precipitate was collected in acetate buffer (pH 4.8), which was dialysed for 2 h against 0.025 *M* phosphate buffer (pH 8) containing 0.002 *M* 2-mercaptoethanol. This sample was subjected to chromatography as describerd below.

Chromatographic studies

A 2.0-g amount of dry DEAE-Sephadex A-50 (Pharmacia) was soaked in water for 4 h at room temperature and packed in a Pyrex column (50 × 2 cm I.D.). Then 500 ml of distilled water, 400 ml of 1.0 *M* sodium chloride solution and 500 ml of distilled water were successively run through the column, which was subsequently equilibrated with 500 ml of 0.025 *M* phosphate buffer (pH 8) containing 0.002 *M* 2-mercaptoethanol.

A 5-ml volume of β-amylase solution prepared by dialysis against phosphate buffer was applied to the above column at a flow-rate 9 ml/h. Five bed volumes of phosphate buffer were passed through the column, then elution was effected by using a gradient of sodium chloride (0.1 to 0.5 *M*) at a flow-rate 30 ml/h. Fractions of 10 ml were collected and assayed for activity, protein and carbohydrate.

The most active fractions were pooled and precipitated by ammonium sulphate saturation to 60%. The precipitate obtained after centrifugation was dissolved in phosphate buffer (pH 8) and chromatographed again on the same column after regeneration.

RESULTS AND DISCUSSION

Sweet potato β-amylase was first crystallized by Balls *et al.*[2]. This method, with some modifications, is still in use for commercial preparations. Englard and Singer[3] also followed this method and crystallized β-amylase eight times to obtain highly purified enzyme. Nakayama and Amagase[4,5] further improved this method by introducing acetone precipitation, which resulted in a 3-fold increase in recovery and purity. Takeda and Hizukuri[6] later developed a faster method that took 6 h to give a crystalline preparation with a yield of only 19%.

Removal of some contaminating activity such as that due to α-amylase, α-

glucosidase (maltase), glycoamylase and phosphatase was a problem. To overcome this difficulty, several procedures have been reported, *e.g.*, affinity chromatography[12], glycogen precipitation[13,14], thymol–amylose complexation[7] and the use of DEAE-Sephadex A-50[15]. The method described here not only rapidly gives a purified preparation of β-amylase but is also devoid of the above contaminating activity (data not shown).

It can be seen from the elution profile in Fig. 1 that a major amount of carbohydrate in the extract passes into the effluent and a very small portion, perhaps associated with the pure enzyme, is eluted with the active fractions. Apart from the active protein fractions eluted with 0.2 *M* sodium chloride, two more protein peaks with higher carbohydrate association appear at higher sodium chloride concentrations. On staining the gels with iodine solution, amylolytic activity (data not shown) was seen only in peak 1 (Fig. 1).

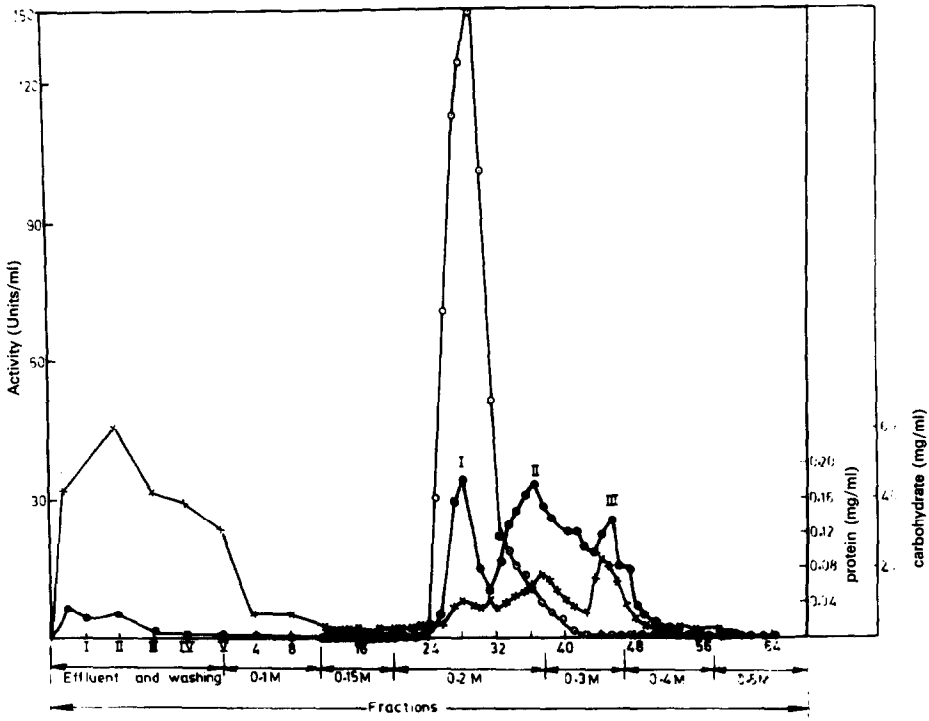

Fig. 1. Typical chromatographic profile of fraction 2 on DEAE-Sephadex A-50: ○, activity; ●, protein; ×, carbohydrate.

It can be seen from Table I that β-amylase is completely retained on the column and also recovered from it. Differential retention and elution of carbohydrate and other proteins eventually result in a 4-fold purification in this operation.

The validity of this procedure was ascertained by rechromatography of the fraction represented by peak 1 in Fig. 1. On the same column after regeneration, the active fractions were eluted at the same position (Fig. 2). However, this step increased the specific activity (Table II), and is a desirable step in this purification procedure.

TABLE I

BEHAVIOUR OF β-AMYLASE, PROTEINS AND CARBOHYDRATES OF FRACTION 2 ON DEAE-SEPHADEX A-50

Component tested	*Amount loaded*	*Amount retained*	*Retention (%)*	*Total eluted*	*Recovery (%)*
β-Amylase	10200 U	10200 U	100	9656 U	94.6
Protein	30.0 mg	28.6 mg	95.3	26.8 mg	93.7
Carbohydrate	5.0 mg	2.41 mg	48.2	2.383 mg	98.9

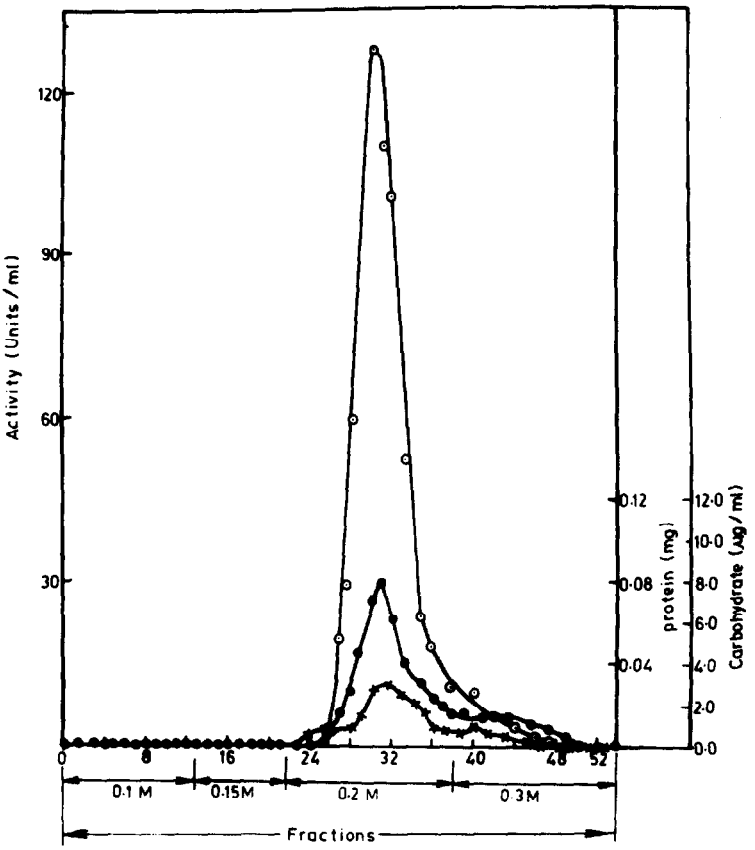

Fig. 2. Rechromatographic profile of peak 1 after ammonium sulphate precipitation on a DEAE-Sephadex A-50 column. ○, Activity; ●, protein; ×, carbohydrate.

To assess the extent of purification, gel electrophoretic analysis of crude and other fractions was carried out, and the results are shown in Fig. 3.

By comparison of lanes A and F in Fig. 3, it can be seen that band I corresponds to β-amylase, the three bands in the ammonium sulphate fraction become segregated after passage through Sephadex A-50 and peak 1 gives one homogeneous band corresponding to β-amylase.

TABLE II

SUMMARY OF β-AMYLASE PURIFICATION PROCEDURE

Fraction No.	*Stage of purification*	*Volume (ml)*	*Total activity (U)*	*Total protein (mg)*	*Total carbohydrate (mg)*	*Specific activity (U/mg)*	*Carbohydrate protein ratio*	*Recovery (%)*	*Enrichment ratio*
1	Crude extract	80	14000	380	530	36.84	1.390	100	–
2	40–60% ammonium sulphate fraction dissolved in buffer	5	10200	30	5	340	0.166	72.9	9.2
3	Pooled active fractions 26–30	50	8065	6.13	0.41	1318	0.066	57.6	35.8
4	Active fraction from rechromatography	50	7040	4.0	0.16	1760	0.04	50.3	47.8

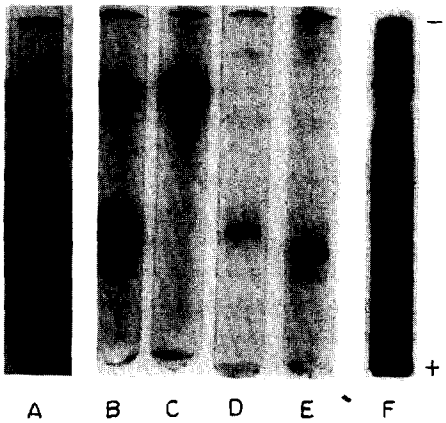

Fig. 3. Polyacrylamide gel electrophoresis (7.5% acrylamide) at pH 8.3 at various stages of purification. A, Crude sample; B, 40–60% ammonium sulphate fraction; C, D, E, peaks 1, 2 and 3 of the fractions from Fig. 1, respectively; F, activity staining.

Association of carbohydrate in the pure fractions was noted by Uhera *et al.*[16], suggesting the possibility that β-amylase is a glycoprotein. We observed that whenever the carbohydrate content in the pure fraction of β-amylase is less than 2%, the enzyme is very unstable. Although carbohydrate may not be essential for the β-amylase molecule to exhibit biological activity, it may be necessary to impart stability to the molecule.

This procedure for the isolation and purification of β-amylase has the following advantages: (1) purification can be completed within 1 day; (2) the same column can be reused after regeneration; (3) the procedure does not include any drastic treatment and gives significantly higher recoveries; (4) the usual contamination of yellow impurities[15] found in commercial preparations is eliminated (data not shown).

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