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Note

Removal of contaminating proteinases from crude preparations of microbial enzymes

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Proteinases are common contaminants of crude enzyme preparations of microbial origin. Their presence usually causes considerable losses of enzyme during its purification and storage. Their removal in the early stages of purification is, thus, essential.

For removal of proteinases, we have employed column chromatographic methods based principally on bioaffinity binding of proteinases, the purified enzyme remaining unbound. In the case of thermally stable enzymes, the proteinases were denatured by heating.

MATERIALS AND METHODS

Crude preparations

For glucose isomerase and uricase, respectively, cell extracts were prepared from *Streptomyces nigrificans*[1] and *Candida utilis*[2].

Chromatographic sorbents

Uric acid, analytical grade, and casein, thermally modified[3,4], were employed.

Analytical methods

Glucose isomerase activity This was determined with fructose as a substrate. The reaction mixture contained 0.5 ml of Britton Robinson buffer (pH 8.5), 0.1 ml of 0.1 *M* magnesium sulphate, 0.2 ml of enzyme. After 30 min at 60°C, the amount of glucose formed from fructose was estimated by the glucose oxidase/peroxidase method (Lachema kit, Czechoslovakia).

Uricase activity. This was measured in a mixture containing 2 ml of reagent (10 parts of 0.6 *M* ammonium phosphate pH 7.0, 1.7 *M* methanol, 700 U/ml catalase, together with 0.5 parts of 58 g/l acetylacetone and 85 g/l methanol, 0.5 ml of uric acid (6 mg/100 ml) and 0.1 ml of sample [uricase activity between 0 and 1.4 nkat/ml (1 nkat = $6 \cdot 10^{-2}$ U)]. The reaction was stopped after 5 min at 37°C by addition of 0.2 ml of 0.1 *M* potassium cyanide and the colour measured spectrophotometrically at 410 nm against a blank. The uricase activity was read from a calibration curve[5].

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Proteinase activity. This was determined with azocasein as a substrate. The reaction mixture contained 1 ml of 1% azocasein solution in 0.2 *M* phosphate buffer (pH 7.2) and 0.5 ml of the sample solution. After 30 min at 37°C the reaction was stopped by addition of 1.5 ml of 5% trichloroacetic acid. After centrifugation (3000 *g*, 15 min), the absorbance at 366 nm (against a blank) was read, which is proportional to the proteinase activity.

Protein content. This was determined by the method of Lowry *et al.*[6].

Removal of proteinases

Heat inactivation. The cell extract of *S. nigrificans* (crude preparation of glucose isomerase) with 1.0 *M* Mg^{2+} and 1.0 m*M* Co^{2+} was heated for 20 min at 60°C. After cooling any precipitate was centrifuged off.

Column with thermally modified casein. The cell extract of *Candida utilis* (crude preparation of uricase) was applied to a column of thermally modified casein (bed dimensions 200 mm × 10 mm), equilibrated with 0.01 *M* borate buffer pH 8.5. The procedure was carried out at 2°C.

Column with uric acid. The cell extract of *C. utilis* was applied to a column of uric acid (bed dimensions 100 mm × 10 mm) equilibrated with distilled water. The procedure was carried out at 2°C.

RESULTS

The crude preparation of glucose isomerase, obtained after disintegration and extraction of cells of *S. nigrificans* 82/20 contained proteins with proteinase activity of 86 nkat/ml, which caused considerable losses of glucose isomerase activiity. Considering the thermal stability of glucose isomerase, heat denaturation was tried as a method for proteinase inactivation. The effects of heating at 60°C for different times on the stability of proteinases, and of Mg^{2+} and Co^{2+} which stabilize glucose oxidase, are shown in Fig. 1. It is seen that heating is very effective for proteinase inactivation, which was practically complete in 10 min if the stabilizing ions Mg^{2+} and Co^{2+} were absent. However, the activity of glucose isomerase was decreased,

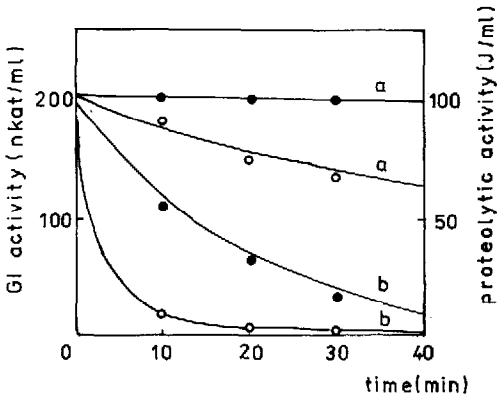

Fig. 1. Effect of heat (60°C) on the activity of glucose isomerase (a) and proteinase (b) in the presence (●—●) and absence (○—○) of stabilizing ions, Mg^{2+} and Co^{2+}.

TABLE I

CHROMATOGRAPHY OF CRUDE URICASE PREPARATION ON THERMALLY MODIFIED CASEIN

Sample	*Total protein (mg)*	*Total activity (nkat)*		*Specific uricase activity (nkat/mg)*
		Proteinase	*Uricase*	
Before column	171.0	582	150.0	0.88
After column	129.5	570	146.0	1.13
24 h after column	127.5	–	75.0	0.59
48 h after column	120.1	–	53.0	0.44
72 h after column	109.0	–	36.0	0.35

too, and so in this case the presence of stabilizing ions is necessary. Even in the presence of stabilizing ions, proteinases were significantly inactivated after 20 min. This method may be used only in the case when a thermally stable enzyme is purified.

In further experiments a less thermally stable enzyme preparation was treated (uricase from *Candida utilis*), which contained contaminating proteinases activity (23.3 nkat/ml). A 25-ml volume of enzyme solution was used for chromatography on thermally modified casein, previously described[4] as an effective sorbent for trypsin. The results are shown in Table I. It can be concluded that practically no proteinase activity was removed from the crude extract on the column of thermally modified casein. However, some other balast proteins were probably retained on the column, so that the specific activities of both uricase and proteinase increased after the chromatography. During storage at 2°C the amount of protein and the uricase activity gradually decreased, probably due to the presence of proteinase activity. After 72 h only 24% of uricase activity remained.

Another 25 ml of crude uricase preparation was applied to a column of uric acid. Table II shows the results. During the chromatography, 92% of proteinase activity was removed. This gave a stabilizing effect on the uricase activity, which after 72 h was 68% of the original value.

The removal of proteinase activity from a crude enzyme preparation at the

TABLE II

CHROMATOGRAPHY OF CRUDE URICASE PREPARATION ON THE COLUMN OF URIC ACID

Sample	*Total protein (mg)*	*Total activity (nkat)*		*Specific uricase activity (nkat/mg)*
		Proteinase	*Uricase*	
Before column	171.0	582	150.0	0.88
After column	109.5	42.5	146.0	1.33
24 h after column	104.2	–	132.0	1.26
48 h after column	102.0	–	110.4	1.08
72 h after column	100.1	–	99.5	0.99

beginning of the purification can improve the total yield of enzymatic activity. For thermally stable glucose isomerase from *S. nigrificans*, the inactivation of proteinase by heat is recommended. This cannot be used in the case of uricase from *C. utilis* having low thermal stability. Instead, the majority of the proteinase activity (92%) can be removed chromatographically on a column of uric acid, on which practically no uricase is bound.

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