Journal of Chromatography, 497 (1989) 268–275
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5005

Note

Purification of microbial uricase

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(First received April 12th, 1989; revised manuscript received August 22nd, 1989)

Uricase (urate:oxygen oxidoreductase, EC 1.7.3.3), the enzyme that plays an important part in nitrogen metabolism, catalyses specifically the oxidation of uric acid:

$$\text{uric acid} + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2 \quad (1)$$

Uricase is used in medicine and clinical biochemistry as a diagnostic reagent for the determination of uric acid.

Uricase was originally isolated from mammalian organism [2–4]. Recently, interest was concentrated on microbial preparations from various fungi, yeasts [5] and bacteria [6]. The microbial enzyme is inducible [7] and therefore the presence of uric acid or some other inducer in the medium is necessary for enzyme production.

This paper describes the purification and characterization of microbial uricase from *Candida utilis*.

EXPERIMENTAL

Candida utilis DBM 1120 (Collection of Microorganism of the Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, Czechoslovakia) was cultivated in a liquid medium with uric acid for 66 h as inducer (0.1%) on a rotary shaker. Uricase was extracted into borate buffer (pH 8.5) after X-press (Biox, Sweden) disintegration of the biomass.

0378-4347/89/$03.50

Fig. 1. Elution profile of ion-exchange chromatography on a DEAE-cellulose column. Glass column (100 mm × 15 mm I.D.); 5 ml of sample after salting out with ammonium sulphate; flow-rate, 40 ml/h; binding buffer, 0.01 *M* borate (pH 8.5); elution gradient, 0–1 *M* NaCl. Solid line, proteins; dashed line, activity of enzyme.

Uricase activity assay

The enzyme activity was calculated from the decrease in uric acid in the presence of uricase. To 2 ml of a solution containing uric acid (10 μg per 1 ml of borate buffer, pH 8.5), 0.8 ml of water and 0.1 ml of enzyme at 25 °C were added. After 5 min, 0.2 ml of 0.1 *M* potassium cyanide solution was added to the mixture to stop the enzyme reaction. In the reference sample, the solution

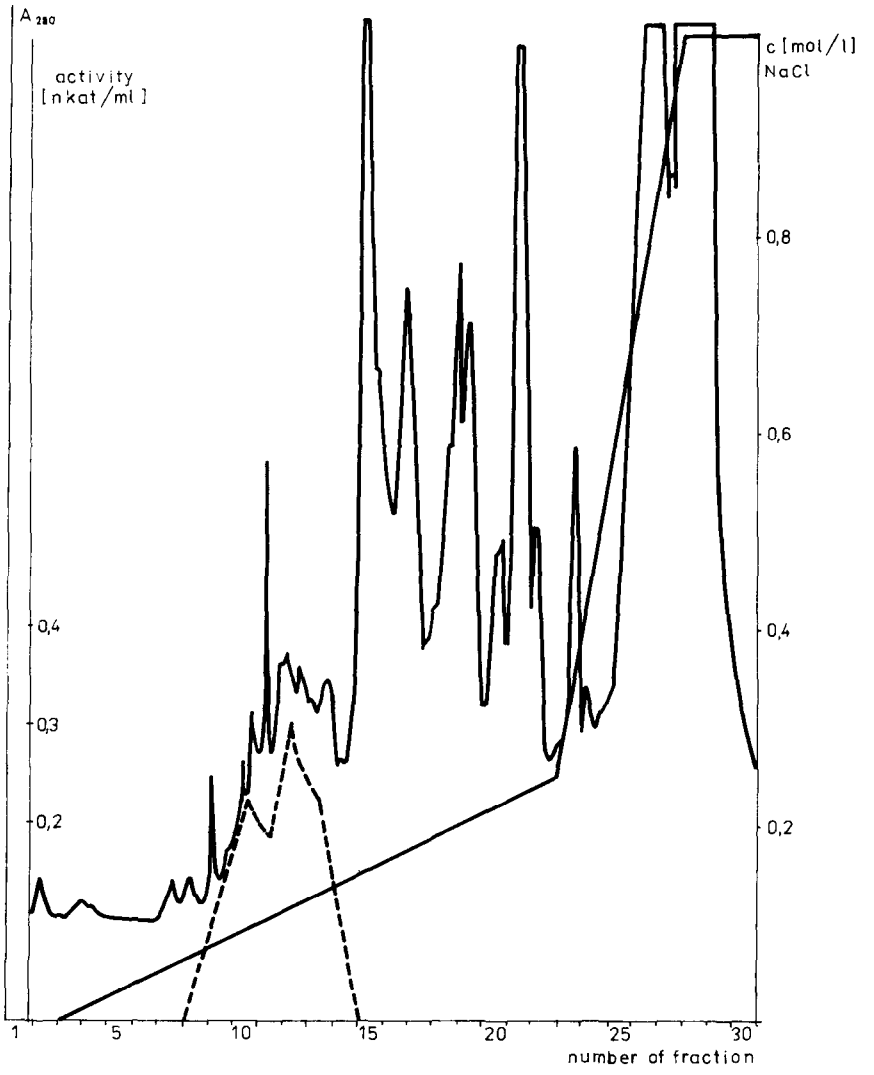

Fig. 2. Elution profile of FPLC ion-exchange chromatography on a Mono Q HR 5/5 column; 2 ml of sample after salting out with ammonium sulphate; flow-rate, 30 ml/h; binding buffer, 0.01 *M* borate (pH 8.5); elution gradient, 0–0.25 *M* NaCl. Curves as in Fig. 1.

of potassium cyanide was added to the mixture before the addition of the enzyme. The absorbance of both samples was measured at 293 nm. The difference between the absorbance of the sample and reference is equivalent to the decrease in uric acid during the enzyme reaction [8].

Determination of proteins

The content of proteins was determined after every purification step using the method of Lowry et al. [9].

Ion-exchange chromatography on DEAE-cellulose

A glass column (15 mm I.D.) was filled with DEAE-cellulose to a height of 100 mm. The binding of sample proteins was performed in 0.01 *M* borate buffer (pH 8.5); for elution an ionic strength gradient of sodium chloride (0–1 *M*) was applied at a flow-rate of 40 ml/h using a Microperpex peristaltic pump (LKB). For detection a UV detector at 280 nm was used.

Ion-exchange chromatography by means of a fast protein liquid chromatographic (FPLC) system

A commercial column of Mono Q HR5/5 anion exchanger (Pharmacia–LKB, Bromma, Sweden) was equilibrated with 0.1 *M* borate buffer (pH 8.5). After application of 2 ml of sample the active fractions were eluted by an ionic strength gradient of sodium chloride (0–0.25 *M*) at a flow-rate of 30 ml/h. For detection a UV detector at 280 nm was used.

TABLE I

PURIFICATION OF URICASE BY ONE-STEP ION-EXCHANGE CHROMATOGRAPHY ON DEAE-CELLULOSE OR MONO Q

Starting buffer: 10 m*M* borate buffer (pH 8.5). Elution: gradient of 0–1 *M* NaCl in starting buffer.

Purification step	Total protein (mg)	Activity		Yield (%)
		Total (nkat)	Specific (nkat/mg)	
Extraction	103.4	30.0	0.29	–
Salting out	29.6	29.3	0.99	98
Desalting	29.6	29.3	0.99	98
Chromatography on:				
DEAE-cellulose	4.9	20.2	4.12	67
Mono Q	5.0	24.5	4.90	82

RESULTS AND DISCUSSION

Purification by ion-exchange chromatography

Crude enzyme extract was treated with ammonium sulphate (60% saturation) and the precipitate that formed was resolved and desalted on a PD-10 column (Pharmacia–LKB) and then applied either to a DEAE-cellulose or a Mono Q column. The results are shown in Figs. 1 and 2, respectively.

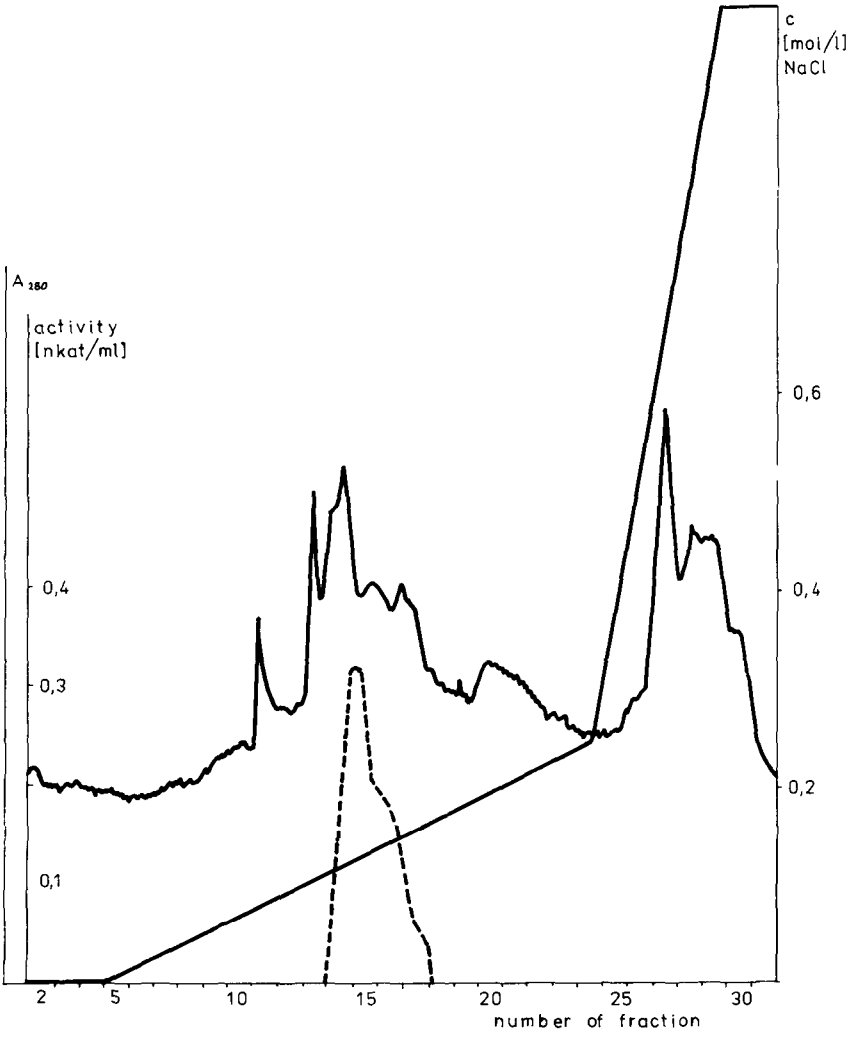

Fig. 3. Elution profile of FPLC ion-exchange chromatography on a Mono Q HR 5/5 column; 2 ml of sample after salting out and ion-exchange chromatography on the DEAE-cellulose column; flow-rate, 30 ml/h; binding buffer, 0.01 *M* borate (pH 8.5); elution gradient, 0–0.25 *M* NaCl. Curves as in Fig. 1.

The preliminary experiment on DEAE-cellulose showed that at the chosen pH the active fraction of uricase was released from the column at a low ionic strength (0.1–0.2 *M* sodium chloride). The main portion of the unwanted contaminating proteins was eluted at higher concentrations of sodium chloride. With regard to the chromatographic profile in Fig. 1, in the FPLC experiment the elution gradient was adjusted so as to obtain a better separation of proteins (0–0.25 *M* within 20 ml; 0.25–1.00 *M* within 5 ml). According to the course of the uricase activity in both experiments, it seems that the isolated enzyme is not a single protein. This corresponds with the finding of Bongaerts et al. [10], who reported the occurrence of two subunits with uricase activity produced by the microorganism *Bacillus fastidiosus*.

The balance of the two processes (gravity chromatography and FPLC) and their comparison are shown in Table I. As might be expected, better results were achieved with the FPLC system, not only as far as purification was concerned but also in terms of recovery of activity.

The sample after salting-out contained a large number of non-active proteins (see Fig. 2) and therefore its application directly to a Mono Q column is not advantageous. The most effective procedure for the purification of uricase from *C. utilis* was found to be a combination of gravity chromatography on DEAE-cellulose followed by FPLC on Mono Q (Fig. 3). As the main task was to obtain uricase with a high specific activity, fractions with low enzyme activity after preliminary chromatography on DEAE-cellulose were not applied to FPLC. This may be one of the reasons why only one peak of uricase activity appeared in Fig. 3.

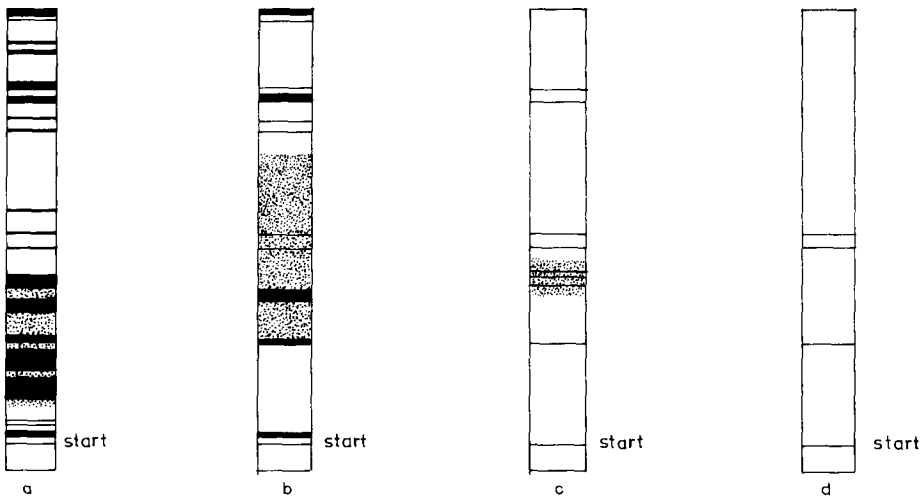

Fig. 4. Scheme of polyacrylamide disc electrophoresis in rod-shaped gel system. The process was performed in Tris-glycine buffer pH 8.6. Proteins were detected using amido black 10B. (a) Crude enzyme; (b) after salting out; (c) after one-step chromatography; (d) after two-step chromatography.

TABLE II

PURIFICATION OF URICASE BY TWO-STEP ION-EXCHANGE CHROMATOGRAPHY ON DEAE-CELLULOSE AND MONO Q

Conditions as in Table I.

Purification step	Total protein (mg)	Activity		Yield (%)
		Total (nkat)	Specific (nkat/mg)	
Extraction	33.3	10.0	0.30	–
Salting out	10.3	9.6	0.93	96
DEAE-cellulose	1.4	6.4	4.59	64
Mono Q	0.2	3.6	18.00	36

The efficiency of the entire purification process is summarized in Table II. Using the above procedure, a 60-fold purification of the uricase afforded a recovery of 34% of the activity. The uricase preparation obtained from the two-step chromatography provided almost a three-fold higher specific activity compared with that obtained by the one-step process.

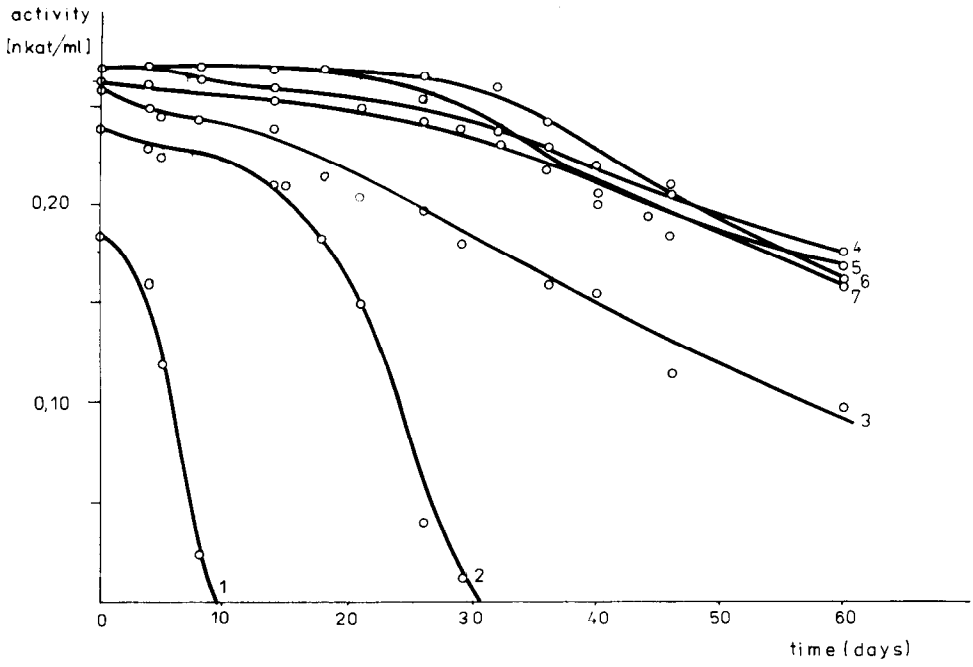

Fig. 5. Storage stability of the uricase preparation in a refrigerator (4°C) at different pH values of the medium. (1) pH 5; (2) pH 6; (3) pH 7; (4) pH 8; (5) pH 9; (6) pH 10; (7) pH 11.

Main characteristics of the uricase preparation obtained

Several parameters of the uricase preparation obtained by two-step chromatography were determined and compared with the data published for preparations isolated from *C. utilis* [11–13].

The optimum temperature of the uricase was found to be 30 °C and the optimum pH was 8.5. The homogeneity was checked by gel electrophoresis (Fig. 4); three zones of proteins appeared. The relative molecular mass of the zone referring to uricase was estimated to be 102 000; those of contaminants were significantly lower.

The stability during storage of the enzyme preparation in a refrigerator (4 °C) at different pH values of the medium is shown in Fig. 5. The best conditions for storage are in the pH range 8–11, where 65% of the original activity was preserved after 60 days.

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