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Purification and Properties of Uricase from *Candida* sp. and Its Application in Uric Acid Analysis in Serum

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ABSTRACT

The purification of uricase from *Candida* sp. was carried out by precipitation with ammonium sulfate then further proceeded with Sephadex G200, and DEAE-cellulose DE52 chromatographies. The specific activity of the enzyme was enhanced from 0.05–12 (U/mg protein). The purity of the enzyme was judged to be homogeneous by SDS-PAGE. Some of the general properties of enzyme were investigated. The optimum reaction pH and temperature were 8.5 and 30°C, respectively. The enzyme was stable at a pH range from 8.5–9.5 and at temperatures lower than 35°C. The apparent K_m value of the enzyme was calculated to be about 5.26×10^{-6} mol/L. The molecular weight was determined to be 70,000–76,000 by the gel filtration and SDS-PAGE techniques. The isoelectric point was determined to be pH 5.6. The effects of some metallic ions on enzyme activity and stability were discussed. The partial purified uricase was used in serum uric acid determination. The within-batch imprecision percentage ranged from 2.16–2.63 and the between-batch imprecision percentage ranged from 2.4–3.6. The recovery ratio were from 96–101%. The correlation among this method and Boehringer, Roche, or Biotrol enzymatic kits were $Y = 1.086x - 0.50$ ($r = 0.981$), $Ya = 0.959x - 0.29$ ($r = 0.97$), and

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$Yb = 1.110x - 0.45$ ($r = 0.956$), respectively. A linear calibration curve was obtained at 2.5–15 mg/dL uric acid. The stability of reagents and the effects of some substances in serum were also surveyed.

Index Entries: Uricase; *Candida* sp.; uric acid analysis.

INTRODUCTION

Uricase (EC 1.7.3.3), which catalyzes the oxidation of uric acid to allantoin, has been widely used as clinical diagnosis enzyme to determine the uric acid concentration in blood serum (*1,2*). The enzyme from different sources has been investigated (*3–5*).

Recently, we succeeded in screening of an uricase-producing rich strain from serveal cultures of *Candida*. The optimum culture conditions of enzyme production were investigated and the results were reported in the previous paper (*6*). In this study, the purification and some of the general properties of this uricase as well as its application to uric acid determination in the serum are reported.

MATERIALS AND METHODS

Candida sp. was obtained from the Institute of Microbiology, Academia Sinica.

Chemicals: Uric acid (Aldrich Chemical Co., Milwaukee, WI); mol wt marker (MW-SDS-70, Sigma Cat No 104540, Boehringer (Mannheim GmbH, Germany); carrier ampholytes for isoelectric focusing-pharmalyte (Pharmacia Chemicals, Uppsala, Sweden). Uric acid kits (Boehringer, Cat. No. 556718, Roche Cat. No. 0714453, Biotrol Cat. No. A02455); Peroxidase (Boehringer); 3,5-dichlorophenosulfonic acid (Boehringer). Culture medium and procedure for uricase production were as previously described (*6*).

Determination of uricase activity: The uricase activity was determined as previously described (*6*). One unit was defined as the amount of enzyme which catalyzed the oxidation of 1 μmol of uric acid/min at pH 8.5 and 25°C.

Serum uric acid determination: The principle of assay is as follows:

$$\text{uric acid} + H_2O + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2 + CO_2$$

$$H_2O_2 + \text{3,5-dichlorophenolsulfonic acid} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{red compound} + H_2O$$

Table 1
Purification of Uricase from *Candida* sp.

Step	Total protein, mg*[a]	Total activity, U	Specific activity, U/mg	Recovery, %	Purification factor
Crude enzyme	2080	107.4	0.05	100.00	1
$(NH_4)_2SO_4$ precipitation	576.35	94.85	0.17	88.31	3.4
Sephadex G200	137.34	70.68	0.52	65.81	10.4
DEAE-cellulose DE52	29.40	45.75	1.56	42.60	31.2
DEAE-cellulose DE52	3.91	21.40	5.47	19.93	109.4
Sephadex G200	1.30	15.86	12.20	14.77	244.0

[a]The concentration of protein was determined by the method of Lowry (*9*).

The red compound has a absorbance peak around 510 nm and its absorbance is proportional to the amount of uric acid in sample assayed.

Calculation (6 mg/dL uric acid as standard solution):

$$\text{uric acid (mg/dL)} = [(\text{absorbance of assayed sample}) / (\text{absorbance of standard sample})] \times 6$$

RESULTS AND DISCUSSION

Purification of Uricase

After cell cultivation, the yeast cells were collected by centrifugation (5,000 rpm for 20 min), resuspended in 0.1 mol/L pH 8.5 Tris-HCl-glycine buffer and lysed by sonic oscillation (20 Kc/s 300W for 5 min). The supernatant of the lysed cell suspension served as the crude enzyme preparation. The ammonium sulfate fraction between 40–70% saturation was collected by centrifugation, the precipitate was dissolved in 0.01 mol/L pH 8.5 borate buffer, and dialyzed against the same buffer. The supernatant was placed onto a Sephadex G200 column and the enzyme was eluted with 0.01 mol/L, pH 8.5 borate buffer. The active fractions were pooled and placed onto a DEAE-cellulose DE52 column. After washing the column with the equilibrium buffer the enzyme was eluted with a linear gradient of ammonium sulfate ranging up to the concentration of 0.25 mol/L. The active fractions were rechromatographed on a DEAE-cellulose DE52 column and stepwisely eluted with 0.1 mol/L sodium chloride. The active fractions were pooled and placed onto a Sephadex G200 column. The enzyme was eluted with 0.01 mol/L, pH 8.5 borate buffer. Table 1 is the summary of purification procedure described above. The specific activity of uricase was enhanced from 0.05 U/mg to 12 U/mg, and about 14% enzyme activity was recovered.

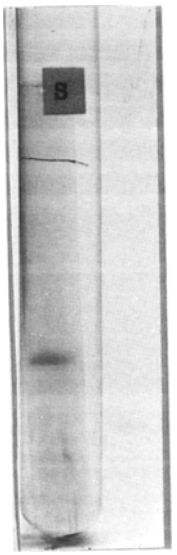

Fig. 1. SDS polyacrylamide gel electrophoresis of purified uricase.

Table 2
Properties of Uricase Purified from *Candida* sp.

Items	Value
Optimum pH	8.5
Optimum temp.	30°C
pH stability	8.5–9.5
Thermal stability ($t_{1/2}$)	45°C; 5 min
K_m (uric acid)	5.26×10^{-6} mol/L
Molecular weight	70,000–76,000
Isoelectric point	pH 5.6
Inhibitory ions	Hg^{2+}, Cu^{2+}, Ba^{2+}, Fe^{3+};
Ions affected stability	Fe^{3+}, Hg^{2+}, Zn^{2+}

Polyacrylamide Disc Gel Electrophoresis

The purified enzyme was analyzed by SDS polyacrylamide disc gel electrophoresis (7). Coomassie brilliant blue was used for protein staining and a single band was obtained (Fig. 1).

Effect of pH on Uricase Activity

Measuring the enzyme activity at different pH buffer system (borate buffer pH 7.0–9.0, borax-Na_2CO_3 buffer pH 9.0–11.0), the optimum pH for the enzyme reaction was 8.5. After incubation at 40°C for 8 min, uricase was stable in 0.1 mol/L borate buffer of pH between 8.5–9.5 (Table 2).

Effect of Temperature on Uricase Activity

Measuring the enzyme activity at different temperature, the optimum temperature for the enzyme reaction was 30°C. After incubation at 45°C for 5 min, the uricase remained at about 50% of its initial activity (Table 2).

Molecular Weight of Uricase

The mol wt of purified uricase was determined to be 70,000–76,000 by the use of SDS-PAGE (7) and gel filtration methods (Table 2). The mol wt of uricase reported by Kimikazu Itaya (4) from *Candida utilis* was 120,000. By the gel filtration method, the commercial uricase from *Candida utilis* was determined as 120,000 but with no difference in enzymatic activity between the purified and commercial enzyme product.

K_m Value of Uricase

The apparent K_m value of purified uricase was determined in 0.1 mol/L, pH 8.5 borate buffer (uric acid: 1.25–10 μmol/L) and calculated to be approx 5.26×10^{-6} mol/L from the Lineweaver-Burk plot (Table 2).

Isoelectric Point of Purified Uricase

Using a sucrose density gradient electrofocusing method (*8*) with carrier ampholyte of pH 3–10, it was shown that the isoelectric point of purified uricase is about pH 5.6 (Table 2).

Effect of Metallic Ions and Compounds on Uricase Activity

The different compounds were added to substrate separately and their effect on the enzyme reaction was assayed. The results showed that the enzyme reaction was inhibited by Hg^{2+}, Cu^{2+}, Ba^{2+}, and Fe^{3+}. After incubation with each compound at 30°C for 60 min and at 4°C for 16 h, the remaining activity was then determined. The enzyme activity was greatly reduced by incubation of uricase with Fe^{3+}, Hg^{2+}, and Zn^{2+} (Table 2).

Application of the Purified Uricase

In this study, the partial purified enzyme was used for the serum uric acid determination. The results of this experiment are as follows:

Precision

The within-batch standard deviation and coefficient of variation ranged from 0.13–0.18 and 2.16–2.63%, respectively, the between-batch standard deviation and coefficient of variation for 20 assays in a 5-d period ranged from 0.18–0.20 and 2.40–3.60%, respectively (Table 3).

Table 3
Evaluation Data of Kit Prepared by This Method

Precision	Within batch		Between batch	
	SD	0.13–0.18	SD	0.18–0.20
	CV%	2.16–2.63	CV%	2.40–3.60
Recovery	96–101%			
Linear range	2.5–15 mg/dL			
Correlation and regression	Boehringer kit	$r = 0.981$	$Y = 1.086X - 0.50$	
	Roche kit	$r = 0.97$	$Y_a = 0.959X - 0.29$	
	Biotrol kit	$r = 0.956$	$Y_b = 1.110X - 0.45$	

Accuracy

The percentage of recovery of the method ranged from 96–101%. It is good enough for routine determination of serum uric acid in clinical chemistry (Table 3). A comparison experiment of 50 serum samples was carried out by this method with Boehringer, Roche, and Biotrol enzymatic kits. The results can be summarized as follows:

$$Y = 1.086x - 0.50\ (r = 0.981),\ Ya = 0.959x - 0.29\ (r = 0.97),$$

and $Yb = 1.110x - 0.45$ ($r = 0.956$) (Y, Ya, and Yb expressing this method, x, Boehringer, Roche, and Biotrol enzymatic kits, respectively) (Table 3).

Calibration Curve of Uric Acid

A good linear calibration curve was obtained for this method at 2.5–15 mg/dL uric acid. This effectively covers the normal range of serum uric acid (2.5–7 mg/dL) (Table 3).

Effect of Serum Compounds on this Method

Hemoglobin (< 200 mg/dL) and bilirubin (< 2 mg/dL) had no effect on the serum uric acid determination, but vitamine C (> 2 mg/dL) had a negative effect on it.

Stability

The stock reagent at room temperature was stable for at least 3 mo. The working reagent was stable for 24 h in use.

It is obvious that the prepared enzymatic kit using partial purified uricase by this method can replace the imported enzymatic kit for serum uric acid assay in clinical diagnosis in China.

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