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Purification and Characterization of Extracellular *Pseudomonas aeruginosa* Urate Oxidase Enzyme

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Abstract

Urate oxidase (uricase) was isolated and purified from *Pseudomonas aeruginosa* to apparent homogeneity using ammonium sulphate precipitation followed by ion exchange and gel filtration chromatography. The specific activity of the purified uricase enzyme was found to be 636.36 with the use of uric acid as a substrate. The purified uricase enzyme is a monomeric protein with molecular weight of 64 kilodaltons. The optimal pH and temperature of the purified enzyme is 9.0 and 30°C, respectively. The effect of some metal ions was studied. Sulphate forms of Fe^{+2}, Zn^{+2} and Co^{+2} inhibit the uricolytic activity whereas; NaCl and $CaCl_2$ enhance the enzyme activity. Moreover, the purified enzyme is inhibited by EDTA and KCN.

Key words: Uricase, *P. aeruginosa*, PCR, 16S rRNA, uricozyme

Introduction

Urate oxidase or uricase (urate:oxygen oxidoreductase, EC 1.7.3.3), an enzyme that catalyzes the oxidation of uric acid to allantoin, occupies a pivotal position in the chain of enzymes responsible for the metabolism of purines (Wu *et al*., 1994). The end product of purine metabolism varies from species to species; in bacteria and some marine invertebrates, purines are degraded to uric acid, allantoin, allantoic acid, and urea and then to ammonia and carbon dioxide (Wu *et al*., 1994). In human and other higher primates, uricase is absent as a result of two mutations in the gene coding sequence, which cause premature termination of the translation process. As a result, uric acid is the end product of purines metabolism, which is present largely as the relatively insoluble monosodium salt, elevate in the human blood serum causing gout symptoms (Lee *et al*., 1988). Gout is a painful disorder, characterized by uricemia, recurrent attacks of acute arthritis, deposition of sodium urate in and around joints, and in many cases, formation of uric acid calculi (Lee *et al*., 1988). Gout treatments generally include allopurinol, a potent competitive inhibitor of xanthine oxidase, the enzyme that catalyzes the conversion of hydroxyxanthine to xanthine and xanthine to uric acid (Massey *et al*., 1970). In the case of gout associated with renal complications, direct injection of urate oxidase allows a much more rapid resorption of urate nephrolithiases. Although several microbial sources of uricase have been proposed for this clinical indication, only one has actually been used commercially under the trademark of Uricozyme and is isolated and purified from *Aspergillus flavus*. In various microorganisms uricase synthesis is regulated by components of the growth medium and the ability to degrade uric acid and to use it for growth is an inducible property of these microorganisms (Van der Drift and Vogels, 1975). Moreover, it was suggested that uricase formation might be controlled by a repression in which a metabolite derived from both the nitrogen and carbon sources may participate (Bongaerts *et al*., 1977). In this work we reported for the first time the isolation, purification and characterization of *P. aeruginosa* uricase.

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Experimental

Materials and Methods

Bacterial isolate identification. The bacterial isolate used in this study was a gift from Dr. Yousry Gohar, department of Microbiology, Faculty of Science, Alexandria University, Egypt. This isolate was tested for uricase production using nutrient agar medium contained per liter: peptone, 5.0 g; Beef extract, 3.0 g; NaCl, 2.0 g; agar, 23.0 g and uric acid 3.0 g uric acid. The identification of the bacterial isolate was based on cell morphology, colony morphology, growth on nutrient broth and nutrient agar as well as several biochemical tests. The identification process was performed at the Fermentation and Biotechnology Center, El-Azhar University, Cairo. To confirm the biochemical tests results for isolate identification, 16S rRNA (rDNA) technique was carried out. DNA was isolated and purified according to Sambrook *et al*., 1989. Amplification of the 16S rDNA gene from the genome was carried out by polymerase chain reaction (PCR) using primers designed to amplify 16S rRNA gene. The forward primer was 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse primer was 5'-TACGGYTACCTTGTTACGACTT-3'. The polymerase chain reaction analysis was performed with 100 ng of genomic DNA in a final volume of 50 µl, including a reaction buffer 1x, 30 pmole of each primer, and 2 units of *Taq* polymerase. Thermocycling consisted of an initial denaturation of 5 minutes at 94°C and of 30 cycles of 1 minute at 94°C (denaturation), 1 minute at 55°C (primers annealing), and 1.5 minutes at 72°C (extension). Polymerase chain reaction products were analyzed for purity check on 1% agarose gel by electrophoresis, stained with ethidium bromide (0.5 µg/ml), and visualized using ultraviolet transillumination.

DNA sequencing. DNA was sequenced by the dideoxy chain termination method according to Sanger *et al*., 1977 using ABI Prism Ready Reaction Dye Terminator Sequencing Kit and analyzed on an ABI 377 automated sequencer. The nucleotide sequence obtained about 326 base pairs were then analyzed using nucleotide Blast search data base and have been deposited in the GenBank sequence data base and have the accession number AF419219.

Uricase assay. Uricase was assayed by following the disappearance of uric acid, detected by decrease in absorbance at 292 nm in the presence of cell free extract or purified uricase. The assay mixture contained about 50–100 µl of the enzyme preparation in 0.1 M borate buffer pH 9.0 and 0.12 mM of uric acid in a final volume of 3.0 ml. One unit is defined as the amount of enzyme necessary to transform 1 µmole of uric acid into allantoin in 1 minute at 30°C and pH 9.0 (Mahler, 1970).

Protein analysis. Total protein concentrations of cell free supernatant and purified samples were assayed by the method of Bradford (Bradford, 1976) using a calibration curve established with bovine serum albumin as a standard. Proteins in these preparations were analyzed by SDS-PAGE on 10% gels according to the method of (Laemmli, 1970).

Monitoring bacterial growth, uric acid consumption and uricase activity. Bacterial growth was monitored by measuring the absorbance of the *P. aeruginosa* culture at 550 nm, which highly correlated with the viable cell count on production medium pH 7.0 contained per liter: K_3PO_4, 10 g; glucose, 3.5 g; 1.0 g $MgCl_2$ and uric acid, 3.0 g. The activity of the extracellular uricase was monitored throughout the growth of the bacterial strain. Bacterial strain was activated by growing overnight on 100 ml of the production medium at 37°C with shaking at 200 rpm. In second day, 3 ml was transferred to 300 ml of production medium and allowed to grow at 37°C with shaking to the indicated time at which 1.5 ml of the growing culture was centrifuged in a microcentrifuge at 3500 g for 2 minutes. The supernatant was used to determine the uricase activity as described before and uric acid consumption by measuring the absorbance at 293 nm.

Enzyme production and purification. Cells were cultured aerobically in 1 liter flask at 37°C with rotatory shaking 200 rpm for 18 hours in a production medium contained per liter: K_3PO_4, 10 g; glucose, 3.5 g; 1.0 g $MgCl_2$ and uric acid, 3.0 g. The culture was then centrifuged at 8,000 rpm for 20 minutes at 4°C. Solid ammonium sulphate was then added slowly to the culture filtrate at 70% saturation with gentle stirring on ice bath after which the mixture was allowed to stand at 4°C for overnight. The mixtu re was then centrifuged at 12,000 rpm for 30 minutes at 4°C. Pellet was dissolved in 5 ml 0.02 M Tris-HCl pH 8.5 and dialyzed overnight against 2 liters of the same buffer. The concentrated and dialyzed cell free supernatant was applied to a column (2.5×7 cm) containing Quaternary amino methyl Sepharose fast flow (Q-Sepharose), which previously was equilibrated with 20 mM Tris-HCl, pH 8.5. The column was washed with 3 bed volumes of 20 mM Tris-HCl, pH 8.5 at a flow rate of 60 ml/hour and the bound proteins were eluted with a linear NaCl gradient (0–0.5 M) in the same buffer. Fractions containing uricase enzyme were pooled and concentrated using ammonium sulphate precipitation method as described above. The concentrated enzymes were applied to a Sephadex G-50 column (1.5×45 cm), which had been equilibrated with 20 mM Tris-HCl, pH 8.5. Slow flow rates (0.6 ml/min) were used to allow optimal separation of the enzyme from other proteins. Fractions with uricase enzymatic activity were pooled and concentrated as described earlier.

pH and temperature optima, thermostability and kinetic studies. Uricase optimum pH was determined over a pH values range from 6.0 to 10.0. Sodium phosphate buffer 0.1 M was used from pH 6.0 to 7.0, borate buffer 0.1 M for pH 8.0 to 9.0 and Tris-HCl 0.1 M for pH 10.0. The temperature optimum of the purified uricase was determined over a temperature range of 25°C–60°C in 0.1 M borate buffer pH 8.0. Thermostability of the purified uricase enzyme was examined at a temperatures range 40–70°C for different time intervals (10–60 min) in 0.1 M borate buffer pH 8.0. The effect of substrate concentration, enzyme concentration and that of incubation time was studied at pH 8.0 with 0.1 M borate buffer. In all cases uricolytic activity was determined as described earlier.

Effect of EDTA and KCN on the activity of uricase enzyme. To examine the effect of EDTA and KCN on the activity of the purified uricase, different concentrations of EDTA and KCN were used. Reaction mixtures and measurement were identical to those described earlier.

Effect of some metal ions on the activity of uricase enzyme. Different metal ions were tested for their effect on the activity of uricase, including; zinc sulphate, iron sulphate, cobalt sulphate, copper sulphate, NaCl and $CaCl_2$.

Results and Discussion

Identification of bacterial isolate. Based on cell morphology, colony morphology, growth on nutrient broth and nutrient agar as well as several biochemical tests, the bacterial isolate was identified as *Pseudo-*

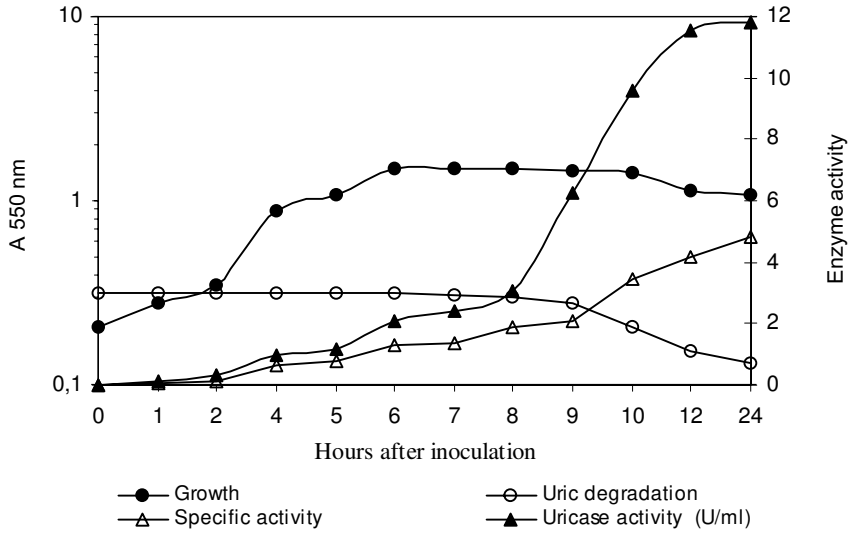

Fig. 1. Growth and uricase production by *Pseudomonas aeruginosa* upon growing on production medium supplemented with uric acid as a sole carbon and nitrogen source.

monas aeruginosa. To confirm the biochemical tests results for bacterial isolate identification, 16S rRNA methodology was carried out. The sequencing data obtained using this strategy indicated that the isolate under study was 98% *P. aeruginosa* and the nucleotide sequence was deposited in the GenBank sequence database, and given the accession number AF419219.

Monitoring the bacterial growth and uricase production. The growth and uricase production level of *P. aeruginosa* was monitored on production medium supplemented with uric acid as described earlier. Figure 1 illustrate the growth, uricase production and uric acid consumption by *P. aeruginosa*. The level of uricase production increased gradually with growth and reached its maximum level after 12 hours of inoculation (11.79 Units/ml and specific activity of 4.8). Moreover, it was noticed that, the increase in uricase activity was parallel to the consumption of uric acid in the fermentation medium, as indicated by the reduction in the absorbance at 293 nm. No significant change in uricase activity was noticed after 24 hours of inoculation. To investigate the effect of different concentration of uric acid on uricase production, different concentrations of uric acid (0.0–0.4% w/v) was added to the production medium. Table I shows a parallel correlation between uric acid concentration and uricase production. It was noticed that maximum induction level was 24 hours after inoculation in a medium supplemented with 0.3% uric acid (24.21 units/ml). It was reported that maximum uricase induction level for *Candida utilis* was obtained in a fermentation medium containing uric acid inducer at a concentration of 0.6% as the sole nitrogen source (Liu *et al*., 1994). Moreover, 0.25–0.6% w/v uric acid concentration range were used for uricase production by *C. utilis* and *C. tropicalis* (Liu *et al*., 1994 and Atsuo *et al*., 1977).

Purification and characterization of *P. aeruginosa* uricase. *P. aeruginosa* uricase enzyme was purified using $(NH_4)_2SO_4$ selective precipitation (70% saturation) followed by ion exchange chromatography

Table I
Effect of different concentrations of uric acid on the production of uricase

Uric acid concentrations (%)	Uricase activity (Units/ml) [a]		
	6 h	12 h	24 h
0	0	0	0
0.05	0	1.92	6.71
0.15	0	4.79	6.71
0.20	8.63	10.54	18.63
0.30	6.71	25.88	24.21
0.40	5.75	7.67	12.09

[a] One unit of uricase is the amount of enzyme that oxidizes one μM of uric acid per minute at 37°C

Table II
Purification table of uricase enzyme produced by *P. aeruginosa* using Q-Sepharose column followed by Sephadex G-50 column

Purification steps	Volume (ml)	Enzyme activity (Units/ml)[a]	Total units	Protein content (mg/ml)	Total protein	Specific ctivity (Units/mg protein)	Purification folds[b]	Recovery[c] (%)
Cell free supernatant (CFS)	350	5.28	1848	0.265	92.75	19.924	1	100
Pellet after 70% $(NH_4)_2SO_4$ ppt. and dialysis	20	92.30	1846	2.600	52.0	35.512	1.82	99
After Q-Sephadex column	6.0	94.73	568.4	0.160	0.96	592.062	29.71	31
After Sephadex G-50 column	3.0	70.0	210.0	0.110	0.33	636.360	31.93	11.5

[a] One unit of uricase enzyme is the amount of enzyme that consumed one micromole of uric acid per minute at 37°C.
[b] Purification folds was derived by dividing specific activity at any step by the specific activity of the initial step.
[c] Recovery (%) was derived by dividing total units of the enzyme entering the column by the total units comes purified from the column at the same step.

on Q-Sepharose Fast Flow anion exchanger and Sephadex G-50 column. The data in Table II illustrate that uricase was selectively purified using $(NH_4)_2SO_4$ precipitation to 1.8 fold with 35.5 specific activity. Fractionation pattern of uricase enzyme upon using Q-Sepharose anion exchanger is shown in Figure 2. *P. aeruginosa* uricase was bound to the anion exchange column at pH 8.5 and eluted with linear NaCl gradient (0–0.5 M). Most of the uricolytic activity was found in the third protein peak (absorbance at 280 nm) in the 0.5 M NaCl gradient (Figure 2). The enzyme was purified almost 29.71-fold with a specific activity and

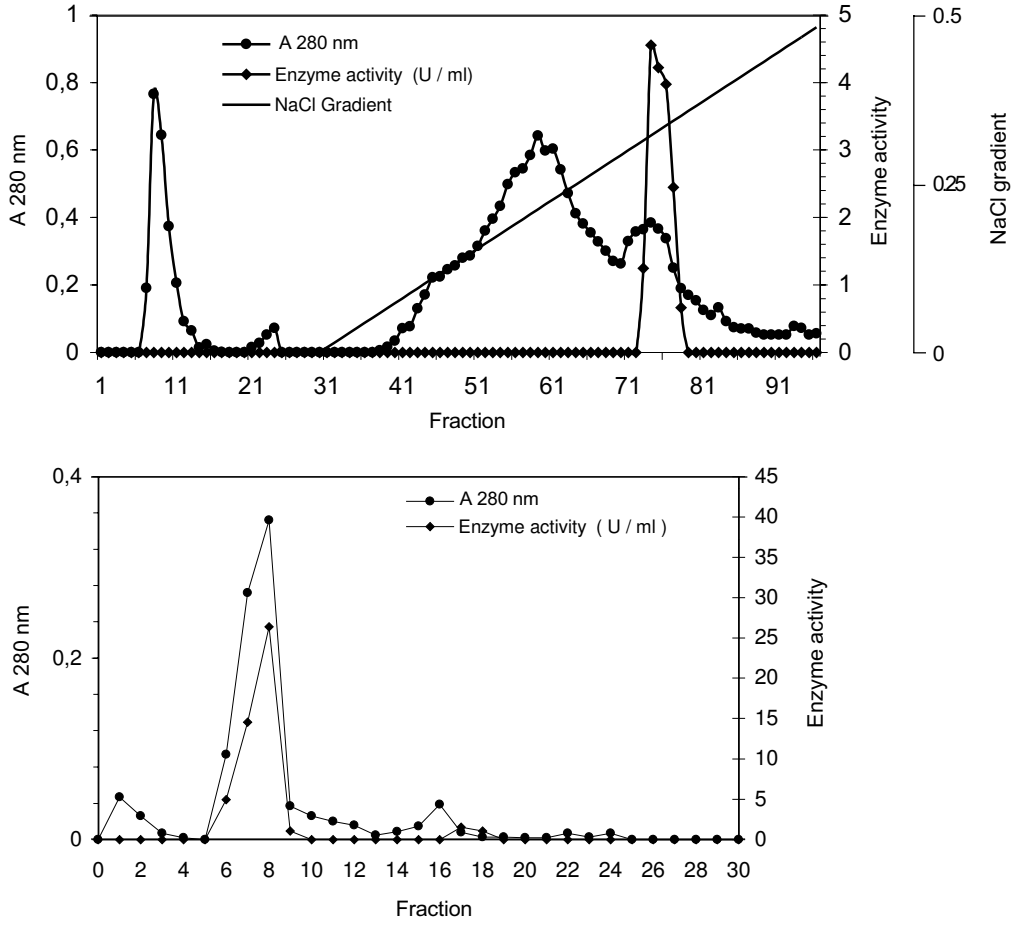

Fig. 2. Fractionation pattern of *P. aeruginosa* uricase enzyme on: (A) Q-Sepharose Fast Flow column, (B) Sephadex G-50 column.

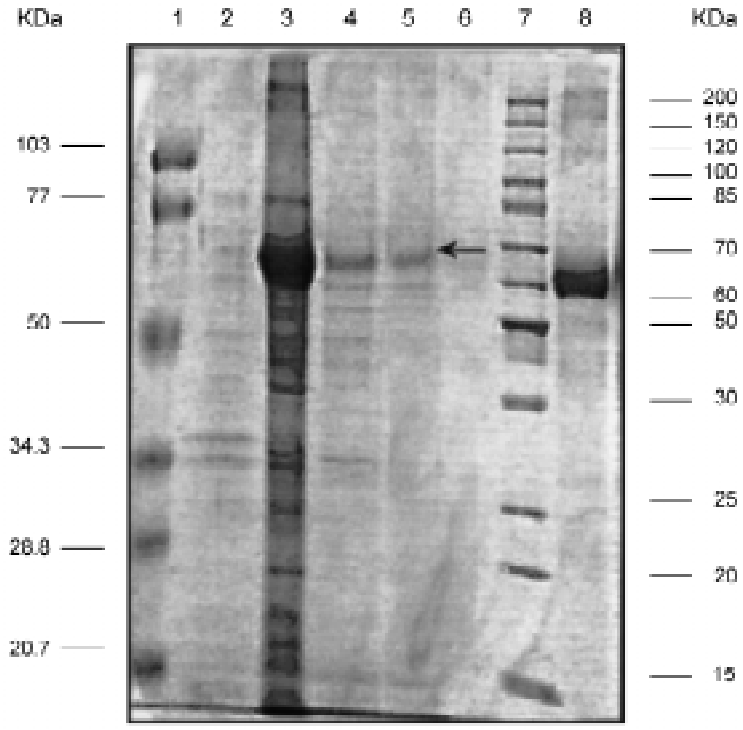

Fig. 3. Sodium dodecyl sulphate polyacrylamide gel (10%) electrophoresis of *P. aeruginosa* cell-free supernatant (lane 2); $(NH_4)_2SO_4$ concentrated and dialyzed enzyme (lane 3); enzyme after Q-Sepharose column (lane 4); and after Sephadex G-50 column (lane 5).
Lanes 1 and 7 represent BioRad prestained protein molecular weight markers and Fermentas ladder molecular weight markers respectively. Lane 8 represents commercial uricase enzyme and lane 6 is an empty lane.

recovery of 592 and 31% respectively (Table II). Q-Sepharose Fast Flow column active uricase fractions were pooled and applied to Sephadex G-50 column. Data presented on Figure 3 demonstrated that, only the second protein peak overlapped with the uricase activity. Upon using Sephadex G-50 column the enzyme was purified to 31.9-fold and with a specific activity of 636.360 (Table II). The purity was confirmed by the presence of a single major protein band on a denaturing SDS-PAGE (Figure 3). The molecular weight of the purified uricase was determined by SDS-PAGE to be 68.0 KDa for one subunit. It has been reported that the native molecular weight of uricase enzyme produced and purified from *Bacillus fastidiosus* is 145–150 KDa (Bongaerts and Vogels, 1976) and that of *C. utilis* 34 KDa and composed of one subunit. Moreover, pig, mouse and baboon uricase was found to be localized in the liver associated with the peroxisome and composed of tetramer with a subunit molecular mass of 32–33 KDa (Bernard *et al*., 1989).

Purified *P. aeruginosa* exhibited maximum uricolytic activity at pH 9.0 and a temperature 30°C (Figure 4a and b). Thermostability of the purified uricase enzyme was also investigated for one hour. Figure 4c illustrated that, *P. aeruginosa* uricase enzyme retained 12.4% of its original activity after exposure to 40°C for 50 minutes. Moreover, the purified enzyme lost all of its original activity after 10 minutes of exposure to 70°C. Thus, the purified *Pseudomonas* uricase enzyme has proved to be thermolabile at higher temperatures. This result agreed with those of other researchers (Itaya *et al*., 1971 and Zhu *et al*., 2001), they found that purified *C. utilis* uricase enzyme was sensitive to heat and upon cloning and expression in *E. coli* BL21 (DE3) of *C. utilis* uricase gene, thermostability of the expressed protein was enhance compared to the native *C. utilis* uricase enzyme (Zhu *et al*., 2001). The effect of substrate concentration, enzyme concentration and incubation time was also investigated. The results demonstrated that, 20 µg of uric acid substrate and 10 µl (2.0 µg) of the purified enzyme per reaction was enough to be used in the reaction mixture for incubation period of 30 minutes.

Table III displayed the effects of some various compounds on the uricase activity. EDTA, KCN, Fe^{+2}, Zn^{+2} and Co^{+2} inhibited the uricase activity by different degrees. This result also agreed with those of other researcher (Bongaerts and Vogels, 1976 and Kaltwasser, 1971). However, metal ions such as Na^{+2}, Ca^{+2} and Cu^{+2} strongly enhance the uricase activity by 182.2, 236.0 and 275.0% respectively. One possible

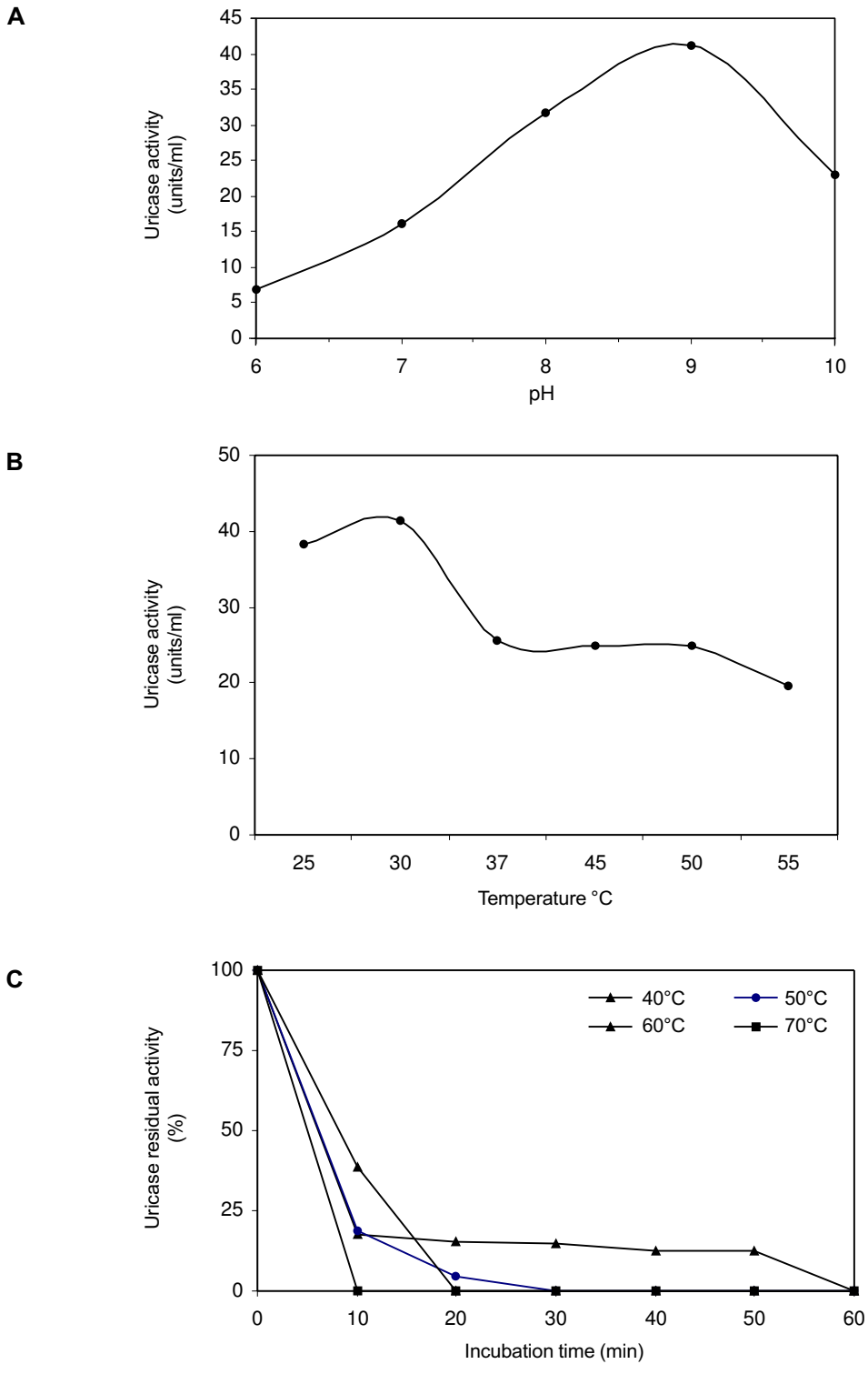

Fig. 4. pH optimum (a), temperature optimum (b) and thermostability (c) of the purified *P. aeruginosa* uricase enzyme.

explanation for the results of the above investigation is that some metal ions bind to the uricase enzyme, and alter the enzymatic activity by stabilization or destabilization of the enzyme's conformation. However, we have not yet demonstrated this experimentally.

The demonstration that *P. aeruginosa* produce extracellular uricase enzyme in fermentation medium supplemented with uric acid inducer and the possibility of obtaining this enzyme in purified form is highly encouraging to use such enzyme in uric acid determination in biological fluids. A comparative study using *P. aeruginosa* uricase and commercially available uricozyme was carried out to compare the urico-

Table III
Effect of some compounds on uricase activity

Compounds or Metal ions	Concentration (mM)	Uricase residual activity (%)
None	0.0	100.0%
EDTA	10.0	80.0%
KCN	1.0	0.0%
Fe^{2+}	1.0	5.2%
Zn^{2+}	1.0	20.4%
Cu^{2+}	1.0	275.0%
Co^{2+}	1.0	20.0%
Na^{2+}	1.0	182.2%
Ca^{+2}	1.0	236.0%

lytic activity of both preparations using the same amount of protein. Figure 5 shows that both enzyme preparations were able to utilize urate as a substrate as indicated by measuring the remained uric acid in the reaction mixtures. Surprisingly, the rate of uric acid consumption was higher in the case of *P. aeruginosa* uricase than that of uricozyme.

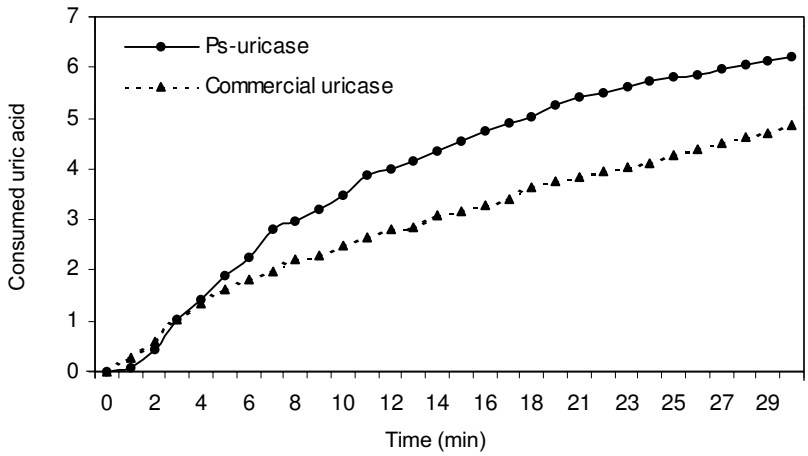

Fig. 5. Uric acid consumption by purified *P. aeruginosa* uricase and commercial uricase enzyme.

In the present work we demonstrated for the first time that *P. aeruginosa* produce extracellular inducible active uricase enzyme. This enzyme was purified and characterized and the data produced in this study demonstrated that the potency of the produced enzyme in uric acid degradation and hence its possible use in uric acid determination in biological fluids. Genomic library was constructed from purified *P. aeruginosa* DNA and screened for urate gene. A number of potentials positive uricase producing clones were obtained. Our future work will focus on the sequencing, sub-cloning and overexpression of *Pseudomonas* uricase gene in *E. coli* which will be necessary for elucidating the biochemical and biophysical characters of wild-type urate oxidase and the recombinant enzyme and its role in *Pseudomonas* purines metabolism.

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