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PURIFICATION AND BIOCHEMICAL CHARACTERISTICS OF UREASE FROM *PROTEUS RETTGERI*

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SUMMARY

1. Urease (urea amidohydrolase, EC 3.5.1.5) from *Proteus rettgeri* was purified by a procedure that included heat shock, precipitation with ethanol and chromatography on Sephadex G-200, DEAE-Sephadex and hydroxyapatite, and crystallyzation as a phosphate complex. This preparation gave a single band when analyzed by either polyacrylamide electrophoresis or Ouchterchlony's agar diffusion technique.

2. The reaction rate was linearly dependent on the amount of enzyme assayed. The optimal pH of the enzyme was 7.5 and optimal temperature 55°. Heavy metals and oxygen were deleterious.

3. K_m and v_{max} were strictly dependent on pH and temperature. When the temperature was increased, the kinetics of enzymic activity changed from Michaelian to a sigmoidal type. The same effect was obtained at lower temperatures by addition of ammonium ions.

INTRODUCTION

Bacterial ureases have been studied for a long time, mainly from a taxonomic viewpoint[1-6]. However, the physiology and biochemistry of bacterial ureases have been studied to a lesser extent (PASSMORE AND YUDKIN[7]). Purification of bacterial urease has been carried out only by LARSON AND KALLIO[8] from *Bacillus pasteurii*, and GUO AND LIU[9] who partially purified urease from several Proteus species and studied their serological relationships. In a previous paper from our laboratory[10] we showed that urease from a strain of *Proteus rettgeri* is an inducible enzyme, synthesized only in the presence of the specific substrate and under control by catabolite repression, end-product repression and inhibition of activity by NH_4^+.

The present paper deals with the purification of urease (urea amidohydrolase, EC 3.5.1.5) from *P. rettgeri* accomplished in order to study its biochemical characteristics and the mechanism of inhibition by NH_3. A preliminary report has appeared elsewhere[11].

MATERIAL AND METHODS

Microorganism

A strain of *P. rettgeri* ENCB from our culture collection was used as source of the enzyme. This strain was mantained on agar slants of the medium previously described[10].

Preparation of cell-free extracts

P. rettgeri was grown in a 10 l fermentor, containing the basal medium[10] *plus* 5 g/l NaCl, 25 ml/l glycerol, 5 g/l neopeptone (Difco) and 5 g/l peptone (Difco), at 28° with shaking and forced aeration. After 4 h, urea was added under sterile conditions to a final concentration of 2% (w/v), and incubation continued for 4 more hours. Cells were harvested by centrifugation and washed with deionized water until $[NH_4^+]$ reached a minimal value. The batch of cells were resuspended in 0.05 M phosphate buffer (pH 7.7) with 10^{-4} M EDTA[1] and GSH, and disrupted with a Ribi cell press. The crude extract was centrifuged at 14 000 × *g* for 60 min, and the supernatant was used as crude starting material.

Analytical polyacrylamide gel electrophoresis

Disk electrophoresis was used to study the purity of the enzyme. Routine runs and staining were performed by the methods described by DAVIS[12] and ORNSTEIN[13]. Enzyme was detected in unstained gels as follows: the gel was immersed in 30 mM urea dissolved in phosphate buffer containing EDTA and GSH, and incubated for 20 min. The gel was washed with buffer and immersed in Nessler's reagent. When orange-yellow bands appeared, the gel was washed with deionized water.

Enzyme assays

The enzymic activity was assayed by two methods: Nessler's conventional technique and the method described by RUIZ-HERRERA AND GONZALEZ[14]. The following protocols were used unless otherwise indicated. For Nessler's method, 15 μmoles urea, 100 μmoles potassium phosphate (pH 7.7), EDTA and GSH 0.2 μmole each, and enzyme were mixed in a final volume of 2.0 ml. After 5 min of incubation at 37°, 5 ml of Nessler's reagent were added and the absorbance was immediately measured at 415 nm. Appropriate controls were run simultaneously.

Measurements of activity by the RUIZ-HERRERA AND GONZALEZ technique were carried out as follows: in a 3 ml cuvette, 30 μmoles triethanolamine (pH 6.9), 40 μmoles phenol red, 100 μmoles urea, 0.3 μmole EDTA, 0.3 μmole GSH and enzyme in a final volume of 3 ml were mixed. Activity was followed by increase of absorbance at 555 nm.

In both methods, activity was expressed in units, one unit being the amount of enzyme that hydrolyzed 1 μmole of urea per min. As units calculated by the two methods are not equivalent, the method used is indicated for each experiment.

The protein content of the samples was calculated from the ratio $A_{280\text{ nm}}/A_{260\text{ nm}}$ (ref. 15).

Immunological methods

Rabbits were injected with crude enzyme, and when antibodies reached a high

titer, the animals were bled. Antigen–antibody reactions using enzyme at different steps of purification were measured by means of Ouchterchlony's agar diffusion technique.

Chemicals

Sephadex G-200 and DEAE-Sephadex were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydroxyapatite was from Calbiochem, Los Angeles, Calif., U.S.A. All other reagents used were of analytical grade.

RESULTS

Purification of urease

The following method was adopted. The crude extract obtained from cells recovered from 30 l of medium was treated with deoxyribonuclease (2.5 μg/ml) for 10 min at room temperature and centrifuged in the cold at 14 000 $\times$ *g* for 20 min. The supernatant was recovered and heated at 55° for 10 min. The insoluble material was eliminated by centrifugation. To the supernatant, protamine sulfate (108 mg/g of protein) was added and the mixture shaken gently for 15 min. The precipitate was eliminated by centrifugation. The supernatant was added very slowly and with continuous stirring to 3 volumes of 95% ethanol maintained at −15°. Stirring was continued for 15 min and the precipitate was recovered by centrifugation, resuspended in about 100 ml of phosphate buffer (pH 7.7) with EDTA and GSH (see MATERIAL AND METHODS) and subjected to chromatography on a column (40 cm $\times$ 3 cm) of Sephadex G-200 previously equilibrated with the above-mentioned buffer. Aliquots of 10 ml were collected, urease appearing after the void volume, which for this column was 120 ml. Active material was pooled and concentrated by ultradialysis under argon. This fraction was further resolved in a hydroxyapatite column (15 cm $\times$ 2 cm) with the same type of buffer solution. Aliquots of 10 ml were collected. Urease was not adsorbed under these conditions and came out in Fractions 4–8. Active fractions were pooled, concentrated by ultradialysis and subjected to chromatography on a column of DEAE-Sephadex (15 cm $\times$ 2 cm). Fourteen 10-ml fractions were collected, the column being washed with the above-mentioned buffer. A linear gradient, of NaCl (500 ml) from 0.1 to 0.5 M dissolved in the same buffer, was next applied. Activity was recovered in Fractions 27–33. This material was concentrated by ultradialysis.

Crystallization of urease

Material obtained in the last step crystallized when less than 0.5 vol. of 70% ethanol was slowly added. Crystals are shown in Fig. 1. All activity (with low yield) was recovered in the crystalline fraction. The crystals contained about 33.7% of phosphate, expressed as K_2HPO_4. They gave no reaction with anthrone.

In Table I, the results obtained in the purification of one batch of crude extract are summarized. Purification was about 43-fold, but the yield was low. Determination of protein of the most purified fractions may not be strictly correct since purified urease had a very low absorbance at 280 nm, maybe because it has a low content of aromatic amino acids.

Fig. 1. Crystals of urease from *P. rettgeri*. Photographs were taken with a Nachet Model 300 Photomicroscope by phase interference and with an Ektachrome (Kodak) film. Magnification: 75 ×.

Characterization of the purified enzyme

The purity of urease was assessed by polyacrylamide gel electrophoresis and immunodiffusion techniques. Only one band was observed when the crystalline fraction was analyzed by either method.

The reaction rate was linearly dependent on the amount of enzyme added down to 0.006 unit as measured by either of the methods described.

The purified fraction had an optimal pH of 7.5. The activity decreased sharply at lower and higher pH values. When the enzyme was maintained at pH lower than 7.0, activity was irreversibly lost.

Enzyme activity was maximal at 55°. At 80° the enzyme was more active than at room temperature. When the enzyme was incubated without urea at 80° it was rapidly inactivated, indicating that the substrate protected urease.

Arrhenius plots of the effect of temperature on urease showed a biphasic curve

TABLE I

PURIFICATION OF UREASE FROM *Proteus rettgeri*

Stage of purification	*Vol. (ml)*	*Protein*		*Activity*		*Purification*	*Yield (%)*
		mg/ml	*Total (mg)*	*Specific**	*Total*		
Crude extract	506	19.3	9800	0.71	7007	1.0	100
Protamine sulfate	240	26.4	6340	0.30	1902	0.0	27
Ethanol (95%)	100	19.0	1905	0.31	590	0.0	8.4
Sephadex G-200	71	2.96	210.1	1.60	336	2.2	4.7
Hydroxyapatite	46.5	0.67	31.2	5.2	163	7.2	2.6
DEAE-Sephadex	75	0.06	4.5	30.6	137	42.8	2.2

* Specific activity is expressed in units measured by Nessler's technique.

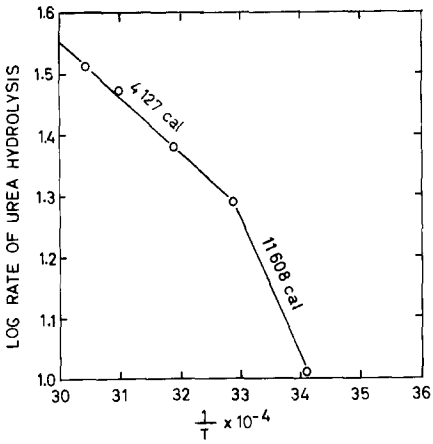


Fig. 2. Arrhenius plot of the effect of temperature on urease activity. The values on the line represent the calculated energies of activation.

(Fig. 2) with an inflexion point at about 30°. The energy of activation above this temperature was 4127 kcal/mole, and below it nearly three times as much, 11 608 kcal/mole. Values of Q_{10} from *P. rettgeri* urease are compared with those from Jack bean and *B. pasteurii* in Table II. Above 30°, values for urease from *P. rettgeri* were somewhat lower than those from the above-mentioned enzymes.

Substrate affinity

The variation of the rate of reaction with the purified enzyme, using various amounts of urea was measured by incubation at 37° and at pH of 7.7. The results

TABLE II

TEMPERATURE COEFFICIENTS OF THREE UREASES

Temperature	*Jack bean urease** Q_{10}	*B. pasteurii urease*** Q_{10}	*P. rettgeri urease* Q_{10}
10–20°	1.95	1.80	—
20–30°	1.63	1.44	1.90
30–40°	1.49	1.30	1.22
40–50°	1.34	1.24	1.22

* Data of SUMNER[28].
** Data of LARSON AND KALLIO[8].

obtained (Fig. 3) showed Michaelis–Menten kinetics. Using a lower pH and temperature, lower K_m and v_{max} were obtained than at higher temperature and pH.

V_{max} and K_m were dependent on temperature when measured at pH 6.9. At 22° the lowest K_m and v_{max} were obtained; when the temperature was increased to 27° both K_m and v_{max} increased. At 40° only v_{max} increased and K_m remained constant; and at 51° the enzyme largely lost the affinity for urea. At these two last temperatures, the enzyme kinetics changed from Michaelian to sigmoidal.

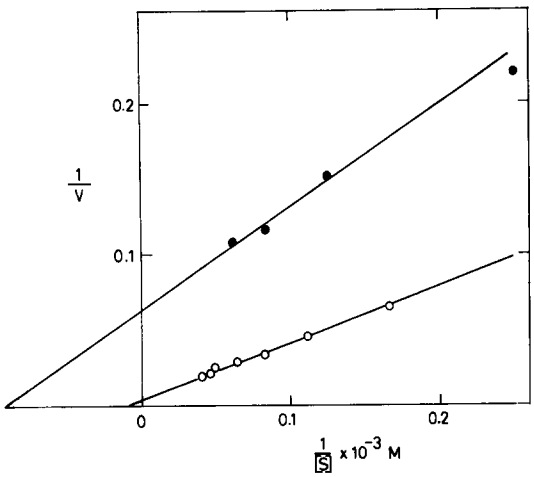
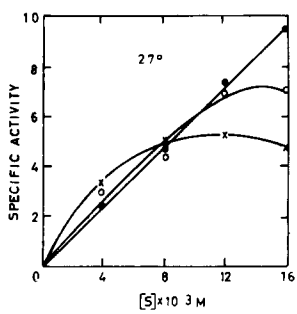


Fig. 3. Effect of concentration of substrate on urease activity. The affiinity of the enzyme for its substrate was measured by Nessler's method at 37° and pH 7.7. The K_m was calculated to be $7.1 \cdot 10^{-2}$ M (○). By the method of RUIZ-HERRERA AND GONZALEZ[14] at 22° and pH 6.9, the K_m was calculated to be $1.05 \cdot 10^{-2}$ M (●).



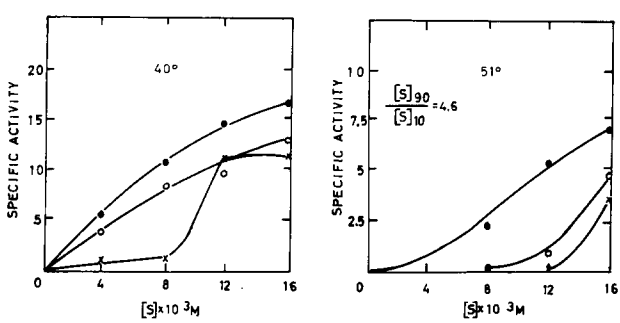


Fig. 4. Effect of NH_4^+ on enzyme kinetics at several temperatures. The activity was measured by the method of RUIZ-HERRERA AND GONZALEZ[14] at three different temperatures, and in the presence of various amounts of NH_4Cl .The specific activity is expressed as usual. ●, no NH_4Cl; ○, *plus* $5 \cdot 10^{-3}$ M NH_4Cl; ×, *plus* 10^{-2} M NH_4Cl.

Effect of NH_4^+ on urease

Results previously reported[10] showed that NH_4^+ ions inhibited partially purified urease by a competitive mechanism. In the present study, the effect of NH_3 on the purified enzyme was tested at three different temperatures and using various amounts of substrate. At 22° there was little effect by NH_3, but as temperature increased, the inhibitory effect also increased (Fig. 4). More interesting is the change of kinetics by NH_4^+ from a Michaelian to a sigmoidal type. Considering Hill's equation[16] it was observed that the calculated values $[S]_{90}/[S]_{10}$ and the slopes of the curves were far from normal. These results suggest a cooperative effect for enzymic activity.

Effect of some divalent cations on urease activity

Urease was very sensitive to divalent cations, the most toxic being Cu^{2+} followed by Co^{2+}, Mg^{2+}, Ca^{2+} and Mn^{2+}. Higher concentrations of Mn^{2+} not only did not inhibit urease, but were stimulatory.

DISCUSSION

Urease from *P. rettgeri* was very sensitive to dialysis. Similarly, ONODERA[17] reported that Jack bean urease was inactivated by dialysis. It was also inactivated when subjected to chromatography on ion exchange celluloses. The enzyme was strongly affected by pH, being more active at alkaline values. VAN SLYKE AND CULLEN[18] and LARSON AND KALLIO[8] reported that ureases from Jack bean and *B. pasteurii*, respectively, were also more active at an alkaline pH.

The most deleterious substances were heavy metals and oxygen. To avoid their effects, urease was always maintained in a buffer solution containing EDTA and GSH, and under a positive pressure of argon.

The effect of heavy metals on ureases from several sources is well-documented[19–21]. In regard to the stimulatory effect of manganese that we found, we may mention that JONES[22] described the stimulation of ureolytic activity of a crude extract obtained from the rumen by Ba^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+}.

P. rettgeri urease was heat-stable in the presence of substrate. When protected from O_2 and heavy metals it retained its activity after prolonged storage at 4°.

The crystals of urease we obtained appear to be a product of cocrystallization between urease and inorganic phosphate. In the absence of phosphate, urease precipitated when treated with ethanol, but not in a crystalline form.

The kinetic analysis of urease gave interesting results. K_m and v_{max} were strongly dependent on pH and temperature. DIXON[23] has suggested the possibility of studying the nature of the ionizing groups in the active site of the enzyme by observation of the changes of K_m as a function of pH, and such an analysis has been carried out for arginase by GREENBERG AND MOHAMED[24].

By observation of the effect of temperature and NH_4^+ ions on the kinetic behavior of urease, it may be concluded that urease has several active groups and that NH_4^+ inhibits the enzymic activity by an allosteric mechanism, such as occurs in aspartate transcarbamylase[25] and threonine deaminase[26].

We do not have evidence that urease from *P. rettgeri* is a polymeric enzyme, but we have observed some changes in the mobility of the enzyme through Sephadex G-200 after subjection to various treatments. BLATTER AND GORIN[27] have reported

that Jack bean urease may exist in different stages of aggregation of a monomer whose molecular weight was calculated to be 40 000 to 60 000. Further experiments are needed to determine whether urease from *P. rettgeri* is indeed a polymeric enzyme.

Finally, the ureases isolated from three different sources—Jack bean, *B. pasteurii* and *P. rettgeri*—have some similarities. A more thorough study of this enzyme, we think, would be valuable to establish a phylogenetic and evolutionary pattern for ureolytic organisms.

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