



Partial purification and characterization of urease from germinating chickpea (*Cicer arietinum* L.) seed

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ABSTRACT

Urease, the urea-hydrolyzing enzyme, was identified abundantly in germinating chickpea (*Cicer arietinum* L.) seed. The enzyme was purified to homogeneity by the sequential steps of 20% acetone extraction, followed by 50% acetone fractionation, gel filtration on Sephadex G-200, and DEAE-cellulose chromatography. The purification fold was 44.99 with a final specific activity of 489.57 mMmin⁻¹mg⁻¹. The purified urease was a hexamer of identical subunits. The native enzyme had a molecular mass of 510 kDa (Gel filtration, Sephadex G-200) whereas subunit values of 85 kDa were determined on PAGE with sodium dodecyl sulfate. The optimum pH and temperature of the purified urease were 7.2 and 48°C, respectively, using urea as substrate. The half-life of urease was 30 days in 50 mM phosphate buffer (pH 7.2) at 40°C. Km value of the purified urease for urea was 3.1 mM. Urease activity was decreased by 50% within 5 minutes at 70°C. The optimum substrate (urea) concentration for urease was 25 mM. The enzyme showed the highest activity when incubated for 30 minutes at 48°C. Ca²⁺ enhanced urease activity by 120.47%, while Pb²⁺, Cu²⁺, Zn²⁺ and Hg²⁺ almost completely inhibited the urease activity.

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KEYWORDS

Chickpea (*Cicer arietinum* L.) seed;
Urease;
Urea;
Purification;
Characterization.

INTRODUCTION

There are at least three key enzymes involved in urea metabolism in plants: arginase, urease and glutamine synthetase. The primary role of urease is to allow the organism to use external or internally generated urea as a nitrogen source^[1-2]. Significant amounts of plant nitrogen flow through urea. This compound derives from arginine and possibly from degradation of purines and ureides^[3]. The nitrogen present in urea is unavailable to the plant unless hydrolyzed by urease. Ammonia, the

product of urease activity, is incorporated into organic compounds mainly by glutamine synthetase. It may function coordinately with arginase in the utilization of seed protein reserves during germination^[4]. Germination of Arabidopsis seeds in water containing urease inhibitor was delayed by 36 hours and completely blocked in case of aged seeds. Immobilization of urease has been carried out in several matrices for clinical/analytical applications^[5-6] and has also been used for the treatment of ureacontaining effluents^[7-8]. So far ureases have been purified from several different sources, including a fun-

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gal respiratory pathogen of human *Coccidioides immitis*^[9-10] and *Schizosaccharomyces pombe*^[11], from jack bean (*Canavalia ensiformis*)^[12-15], mulberry (*Morus alba*) leaves^[16], silkworm (*Bombyx mori*)^[17], pigeon pea (*Cajanus Cajan L.*)^[18-21], water melon (*Citrullus vulgaris*)^[22], soybean (*Glycine max*)^[3,23], *Staphylococcus leei*^[24] and also from *Helicobacter pylori*^[25].

In this study, we gave attention to the purification and characterization of urease from germinating chickpea (*Cicer arietinum*) seed for the first time.

MATERIALS AND METHODS

Materials

Chickpea (*Cicer arietinum L.*) Seeds were collected from Bangladesh Agriculture research Institute (BARI), substation of Ishwardi, pabna, Bangladesh. The seeds were soaked in distilled water for 6 hrs, germinated in the dark at 22°C for 48, 72, 96, 120, 144, 168, 192 and 216 hrs including soaking time. The germinated seeds at different intervals were stored separately in the deep freeze (-10°C) for further experimental purpose.

BSA, SDS/PAGE-chemicals and Sephadex G-75 were purchased from Sigma Chemicals Ltd., USA. Standard proteins, DEAE-cellulose were purchased from Pharmacia Fine Chemicals Ltd., Sweden. All other chemicals used for this research were of analytical grade.

Enzyme extraction

Unless mentioned otherwise all the operations were done at 4°C. Ten grams of germinated seeds were pisted in a mortar and pestle and then suspended in 40 ml of 20% chilled (-20°C) acetone. After occasional gentle stirring for 3 hours the suspension was filtered through double layer of cheese cloth. The filtrate was then collected and centrifuged for 15 minutes. The supernatant was used as "crude extract".

Purification of urease

Acetone precipitation

The "crude extract" was adjusted to 50% saturation by the addition of acetone (chilled to -20°C) under constant and gentle stirring. The resulting precipitate was collected by centrifugation, dissolved in minimum volume of pre-cold 50 mM phosphate buffer pH 7.0 and dialyzed against the same buffer for 24 hours. The dia-

lyzed solution was then centrifuged for 10 minutes and the clear supernatant thus obtained was designate as "crude enzyme solution".

Gel filtration chromatography

Gel filtration was carried out on Sephadex G-200 column (150×3.0 cm). The "crude enzyme solution" after dialysis against 50 mM phosphate buffer, pH 7.0, was loaded onto the Sephadex G-200 column pre-equilibrated with the same buffer and the protein was eluted with the buffer. The enzymatically active fractions were pooled and dialyzed against 50 mM phosphate buffer, pH 7.8 for 24 hrs.

DEAE-cellulose chromatography

The dialyzed enzyme solution was loaded on the DEAE-cellulose column (20×3.0 cm), preequilibrated with 50 mM phosphate buffer, pH 7.8. The bound proteins were eluted with a linear gradient of NaCl (100-500 mM) in the same buffer at a flow rate of 0.5 ml min⁻¹. Absorbance at 280 nm, protein concentration and urease activity was determined. The active fractions were collected.

Enzyme assay

Urease activity was assayed following the method as described^[26]. Urea solution (3% in 0.2 M phosphate buffer, pH 7.0) was used as substrate. One unit of urease activity was defined as the amount required for liberating 1 μmol of ammonia per min at 55°C. Protein concentration was determined by the method of Lowry et al.^[27], using BSA as the standard.

Determination of molecular weight by gel filtration

The molecular weight of the purified urease was determined by the method of Andrews^[28] by gel filtration on Sephadex G-200 column (150×3.0 cm) equilibrated with 50 mM phosphate buffer, pH 7.0. Phosphorylase-b (97.4 kDa), β-galactosidase (116 kDa), β-amylase (200,000 kDa) and Catalase (*Aspergillus niger* 385 kDa) were used as marker proteins^[29]. The molecular weight of the enzyme was calculated from a standard curve, constructed by plotting the elution volume against log of molecular weight of standard proteins.

SDS-PAGE pattern of subunit

SDS-PAGE was performed according to the method of Laemmli^[30] on a Bio-rad mini electrophoresis system. The standard proteins used were β-lacto-

globulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase-b (97.4 kDa) and β -galactosidase (116 kDa). PAGE was performed with 7% gels and the electrophoresis was run at 2000 V and 50 A.

Optimum pH of the enzyme

To study the effect of pH on enzyme activity, the enzyme solutions (0.6%) were dialyzed against 50 mM buffer of different pH (AcONa - HCl, pH 2.0-3.0; AcONa - CH_3COOH , pH 4.0-5.0; NaH_2PO_4 - Na_2HPO_4 , pH 5.5-8.0; $\text{Na}_2\text{B}_4\text{O}_7$ -HCl, pH 8.5-9.0; $\text{Na}_2\text{B}_4\text{O}_7$ - Na_2CO_3 , pH 9.5.) for 24 h with frequent changes of buffers. After necessary adjustment of pH by adding 0.1 N HCl or 0.1 N NaOH, the enzyme activity was assayed using urea as substrate.

Optimum temperature and thermal inactivation

In order to determine the optimum temperature, the enzyme solutions (0.5%) in 50 mM phosphate buffer, pH 7.2, were incubated at various temperatures ranging from 10°C - 90°C for 15 min in a temperature controlled water bath and the activity was assayed.

Approximately 25-30 different enzyme solutions were incubated in assay buffer (50 mM phosphate buffer pH 7.2) at the desired temperature (70°C). At definite time intervals two solutions were withdrawn, cooled and transferred immediately to the assay solution (50 mM phosphate buffer pH 7.2 and 1 ml of 0.2% urea). Residual activity was determined by the usual enzyme activity assay method at 48°C.

Substrate specificity

Substrate specificity of chickpea urease was determined using urea, hydroxyurea, thiourea, and urea-phosphate as substrate. In the procedure, 1 ml of 50 mM phosphate buffer pH 7.2, 1 ml of substrate (0.2%) of different types and 1 ml of enzyme solution were taken in different test tubes and incubated at 48°C for 15 min and the urease reaction was stopped by adding 1 ml of 0.6N H_2SO_4 . Then 1 ml of sodium tungstate solution (0.1%) was added to each test tube to precipitate out the protein and centrifuged. The amount of ammonia released during incubation was estimated by the reaction with Nessler's reagent^[31].

Storage stability

For storage stability studies, the enzyme solution

was kept at 4°C. The activity of urease was determined on different days by the usual method described earlier. Effect of incubation time The effect of incubation time on the activity of enzyme was examined. For the study the enzyme was incubated at various time (10, 20, 30, 40, 50, 60, 70 min.) using urea as substrate, keeping other operational conditions (pH and temperature) constant and the enzyme activity was subsequently monitored.

Effect of various chemicals and metal ions on the activity of urease

Effect of various compounds and metal ions on the activity of urease was examined by incubating the enzyme solution at room temperature in the presence of different ion or compound for 5 minutes and aliquots were withdrawn and assayed under standard reaction conditions (pH 7.2, Tem. 48°C).

Measurement of Km of urease

Michaelis constant (K_m) was determined by the assay of urease activity for various concentration of the substrate (urea 0.1 - 2.0 mM) at definite interval. Initial velocity of respective substrate conc. were calculated. K_m was calculated from Lineweaver-Burk double reciprocal plot^[32].

RESULTS AND DISCUSSION

Urease plays an important role in germination and in seedlings' nitrogen metabolism. After 120 h of ger-

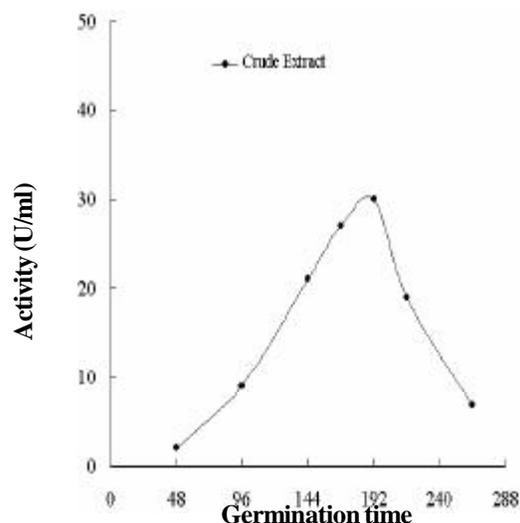


Figure 1: Time course study of crude urease extract from germinating *C. arietinum* seed

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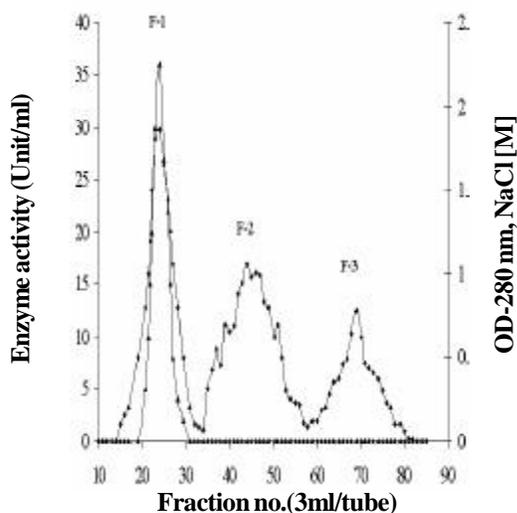


Figure 2: Gel filtration of 50% acetone fractionated crude extract on Sephadex G-200. The crude enzyme solution was applied to the column (2.0 × 50.0 cm) pre-equilibrated with 50 mM phosphate buffer, pH 7.0, at 4°C and developed with the same buffer with a flow rate of 25ml/hr. (◆) OD at 280 nm and (▲) enzyme activity

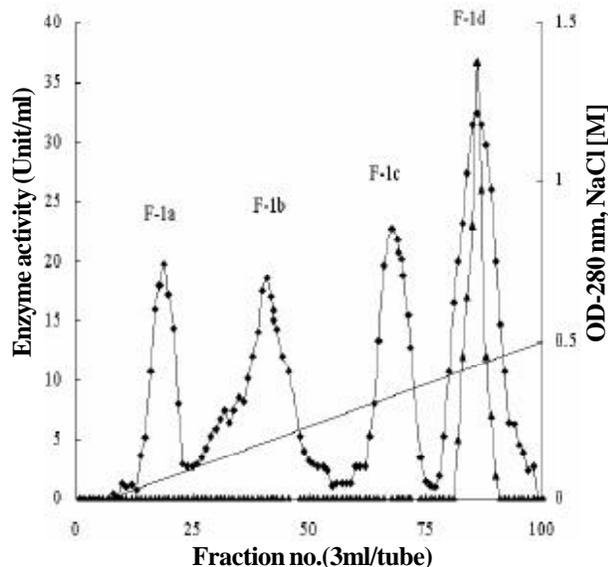


Figure 3: Ion exchange chromatography of F-1 fraction on DEAE cellulose, obtained by gel filtration, was applied to the column (1.5×3.0 cm) pre washed with 50 mM phosphate buffer, pH 7.5 and eluted with a gradient of NaCl (0.1- 0.5 M) in the same buffer with a flow rate of 25 ml/hour. (◆) OD at 280 nm and (▲) enzyme activity.

mination the activity increased gradually and showed maximum activity at 192 h after germination and then declined rapidly (figure 1). Therefore, we used 192 h germination for further experimental purpose.

The purification results of germinated chickpea seed

TABLE 1 : Urease activities in the course of purification of chickpea seed urease

Step of purification	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification fold.
Crude extract	1676	18236	10.88	100	1
Acetone precipitation and dialysis	430.42	12513	29.07	68.61	2.67
Gel filtration	87.39	9532	109.07	52.27	10.02
DEAE-cellulose	18.03	8827	489.57	48.40	44.99

urease is summarized in TABLE 1. Specific activity of the extracted enzyme increased throughout the purification steps and the final purification fold achieved was nearly 45. The specific activity of the final preparation was 489.57 mU mg⁻¹.

Purification of enzyme

The dialyzed enzyme solution obtained from 50% acetone fractionation was applied to Sephadex G-200 column pre-equilibrated with 50 mM phosphate buffer, pH 7.0 and was eluted with the same buffer at a flow rate of 25 ml/h. The components of the crude enzyme solution separated as two major peaks, F-1 and F-2 and a minor peak F-3 (figure 2). The active fraction (F-1) indicated by solid bar was pooled and concentrated by freeze dryer. The concentrated enzyme was dialyzed against 50 mM phosphate buffer, (pH 7.5) for 24 h and applied to a DEAE-cellulose column, pre-equilibrated with the same buffer and eluted by gradient of sodium chloride (0.1 - 0.5 M) in the same buffer. As shown in figure 3, the components of F-1 fraction were separated into three minor peaks (F-1a, F-1b and F-1c) without activity and a major peak (F-1d) having urease activity. F-1d fraction as indicated by solid bar was pooled and used for further experimental purposes.

The molecular weight of the purified urease (F-1d fraction) as determined by gel filtration on Sephadex G-200 was 510,000. The molecular weight of the chickpea seeds urease reported in this investigation is in relative agreement with molecular weights estimated for urease from other sources. Fishbein et al.^[33] have purified urease from seeds of jack bean has a molecular mass of 480,000; while Das et al.^[18] have isolated urease from dehusked pigeonpea (*Cajanus cajan* L.) seeds with molecular weight of 540,000. The plant and fungal ureases are homo-oligomeric proteins consisting

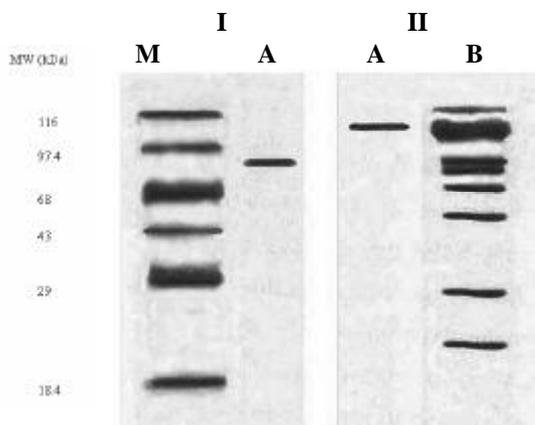


Figure 4: Native (II) and SDS-PAGE (I) patterns of chickpea seed urease. (II) lane A and B, pure enzyme and crude; (I) lane A and M, pure enzyme and molecular wt. marker proteins

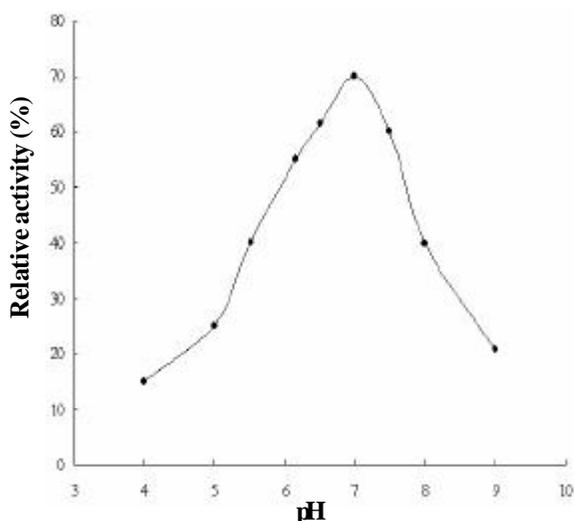


Figure 5: Effect of pH on the activity of chickpea seed urease

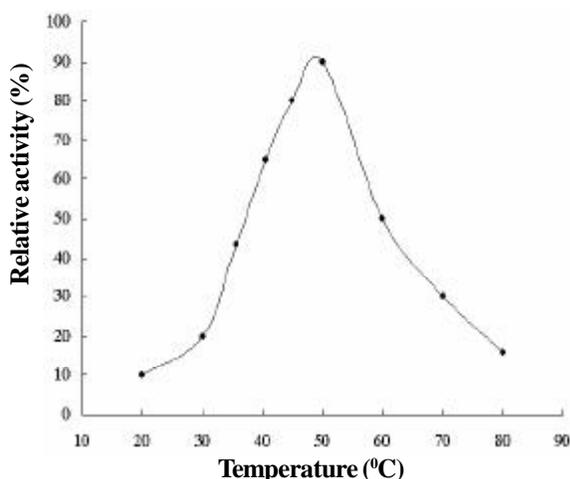


Figure 6 : Effect of temperature on the activity of chickpea seed urease. The activity was expressed as 100%.

of identical subunits, while the bacterial ureases are multimers formed from a complex of two or three subunits of different size^[34].

The purified chickpea seeds urease also had six subunits. Molecular mass of each subunit was 85,000 (figure 4). Sung et al.^[35] found that purified urease from seeds of jack bean contain six subunits, each of 80,000. Das et al.^[18] investigated that urease from dehusked pigeonpea seeds was a hexamer of identical subunits (90,000). Our result on subunit molecular mass relatively coincided with those results.

The optimum pH calculated for the urease activity of chickpea seeds was 7.2 (figure 5). From the result it might be concluded that the urease isolated from chickpea seeds belongs to the category of basic urease. This result is similar to those reported for urease from jack bean^[35] and pigeonpea^[18] but different from that isolated from mulberry leaves^[16] and the pathogenic fungus *Coccidioides immitis*^[9].

Temperature of incubation was optimized by controlling the reaction mixture at 20-80°C (figure 6) at optimum pH 7.2. The activity increased with increasing temperature 45 - 55°C, followed by a sharp decline at 80°C. This result is closely related to those reported by Das et al.^[18] and Srivastava et al.^[20] but differs from that stated by El-Shora^[36].

The half-life of urease was determined to be 30 days in 50 mM phosphate buffer (pH 7.2) at 4°C. This value for urease from seeds of pigeonpea was reported to be 31 days^[18]. Half of the initial activity of urease was destroyed within 5 min when incubated in 50 mM phosphate buffer (pH 7.2) at 70°C while that of urease from seeds of pigeonpea was reported to be destroyed within 7 min^[18]. The optimum substrate concentration of urease was 25 mM. The enzyme showed the highest activity when incubated for 30 min under standard conditions (48°C, pH 7.2).

Urease purified from chickpea seeds catalyzed the hydrolysis of urea and hydroxyurea (TABLE 3) similar to the urease isolated from jack bean^[37-38].

Enzyme activation or inhibition

Effect of various metal ion and chemicals on the activity of chickpea urease was studied (TABLE 2). Calcium ions exhibited distinct role in the urease action. The urease activity increased in presence of calcium ion at low concentrations (3 mM or less) but decreased at higher calcium concentrations, which is consistent with

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TABLE 2: Effect of various metallic salts and chemicals on the activity of urease purified from chickpea seed

Reagent	Relative activity (%)		
	1mM	3mM	5mM
None	100	100	100
BaCl ₂	105	113	103
MgCl ₂	103	110	104
NaCl	98	97	95
KCl	99	98	96
CuCl ₂	32	30	31
ZnCl ₂	50	49	45
HgCl ₂	12	12	14
PbSO ₄	24	23	22
CaCl ₂	105	115	92
*EDTA	61	50	42

*Ethylene diamine tetraacetic acid, EDTA

TABLE 3: Substrate specificity of chickpea seed urease (Action of urease on different substrates)

Substrate	Specific activity (mU/mg/min)
Urea	192
Hydroxyurea	185
Thiourea	0
Ureaphosphate	0

the results reported elsewhere^[39].

EDTA, a metal chelator, decreased the enzyme activity significantly. This may be due to the removal of metal ions located on or near the active site. Divalent cations like Ba⁺⁺ and Mg⁺⁺ slightly stimulated the enzyme at a concentration of 1-3 mM whereas Na⁺ and K⁺ produced little or no effect on the activity. Heavy metals such as Cu⁺⁺, Zn⁺⁺, Pb⁺⁺ and Hg⁺⁺ almost completely inhibited enzyme activity, indicating the presence of thiol (-SH) group in the enzyme active site. These results are in good agreement with those from pigeon pea urease^[40] and jack bean ureases^[13-14].

Kinetic constant

As determined from the Lineweaver-Burk double reciprocal plot, Km value for urease was 3.1 mM. Reddy et al.,^[19] and Mirbod et al.,^[9] observed the Km values of 4.75 and 4.1 mM for jackbean and pathogenic fungus urease, respectively whereas Das et al.,^[18] determined the Km of 3.0 mM for pigeonpea urease, that agrees well with our result.

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