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Research Article

POLYMORPHIC FORMS OF UREASE FROM THE DEHUSKED SEEDS OF PIGEONPEA (*CAJANUS CAJAN* L.)

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Abstract: Urease, purified from the dehusked seeds of pigeonpea (*Cajanus cajan* L.), was characterized for formation of polymorphic forms of the enzyme using polyacrylamide gel electrophoresis and activity staining techniques. The purified enzyme tended to form multimeric forms with three species of molecular mass of approximately 540, 600 and 700 kDa, respectively. Enzymatic activity was concentrated in the 540 kDa form and the polymorphic forms showed low (600 kDa) or no (700 kDa) catalytic activity. The formation of the polymorphic forms was a function of increasing salt concentration and presence of hydroxyl reagents. The multimeric forms had sulfhydryl bridges connecting them; addition of a reducing agent such as dithiothreitol (5 mM) resulted in formation of the basic unit of 540 kDa and loss of the higher molecular mass forms. Unlike urease from soybean or jack bean, no 240 kDa form was observed in any of the conditions studied.

Keywords: Urease; pigeonpea; *Cajanus cajan* L.; polymorphic forms.

Introduction

Urease (urea amidohydrolase; E.C. 3.5.1.5) catalyses the hydrolysis of urea to carbon dioxide and ammonia. Although it is known to be an abundant seed protein in legumes (Bailey and Boulter, 1969), urease is present in almost all plant tissues (Hogan *et al*., 1982). Till date, it has been purified to electrophoretic homogeneity from a number of sources including plant and microbial sources. Urease has a number of firsts to its credit. It was the first enzyme to be crystallized (Sumner, 1926) for which the Nobel Prize was awarded to J.B. Sumner in 1946. His discovery of the crystallized form of the protein struck the first major blow against the hypothesis that enzymes are non-protein catalysts. The second breakthrough in the history of urease was the discovery that it contains nickel at the active site (Dixon *et al*., 1975). Till date there is no report on another Ni containing enzyme in the plants. The third achievement in the study of urease was the X-ray crystal structure of the enzyme from *Klebsiella aerogenes* at 2.0 Å resolution revealing the intimate details of the molecular geometry at the active site (Jabri *et al*., 1995). Recently, the 3-D molecular structure of the jack bean urease (JBU) and pigeonpea urease (PPU) has also been determined (Balasubramanian and Ponnuraj, 2010; Balasubramanian *et al*., 2013).

Urease from plant sources has been purified from the seeds of jack bean (*Canavalia ensiformis*) (Sumner, 1926), soybean (*Glycine max*) (Polacco and Havir, 1979), pigeonpea (Das *et al*., 2002), watermelon (*Citrullus vulgaris*) (Prakash and Bhushan, 1997) and leaves of mulberry (*Morus alba*) (Hirayama *et al*., 2000) and characterized in terms of its physico-chemical properties to various degrees. Reports on the molecular mass of plant seed ureases have been controversial. Reports on the molecular mass of JBU and soybean urease

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(SBU) are generally reported to be from 480-590 kDa (Sumner *et al*., 1938; Reithel *et al*., 1964; Reithel and Robbins, 1967; Blakeley *et al*., 1969; Polacco and Havir, 1979; Dixon *et al*., 1980). Molecular mass of watermelon and PPU are reported to be 470 and 540 kDa, respectively (Tanis and Naylor, 1968; Prakash and Bhushan, 1997; Das *et al*., 2002). However, on the basis of the amino acid composition data, the currently accepted value of molecular mass for plant seed ureases is 540 kDa. Subunit molecular mass of plant seed ureases are approximately 90 kDa (Polacco and Havir, 1979; Dixon *et al*., 1980; Das *et al*., 2002). Hence, plant seed ureases are hexameric proteins.

Polyacrylamide gel electrophoresis (PAGE) of the purified PPU preparations (Das *et al*., 2002) sometimes showed some additional bands besides the 540 kDa band which, however, resulted in a single band of 90 kDa in sodium dodecyl sulfate-PAGE (SDS-PAGE). The purpose of this work was to investigate the molecular mechanism for formation of these additional protein bands.

Materials and methods

Plant materials and chemicals: Dehusked pigeonpea seeds were purchased locally. Standard proteins for molecular mass determination (thyroglobulin, ferritin and catalase) were from Pharmacia, Sweden. Acetone (HPLC grade) and acrylamide (4x recrystallised) were purchased from Spectrochem, India. Urea (enzyme grade) was from Sisco Research Laboratories, India.

Enzyme puriûcation: Urease was puriûed from dehusked seeds of pigeonpea (*Cajanus cajan* L.) following the protocol of Das *et al*. (2002). Briefly the seeds soaked overnight in the extraction buffer (0.025 M Tris–acetate, pH 6.8) were homogenized in a kitchen blender and the crude extract fractionated with acetone and acid steps followed by purification by chromatography on Sephadex G-200 and diethylaminoethyl-cellulose columns.

PAGE: Native-PAGE (7% gel) was performed by the method of Ornstein (1964) and Davis (1964). Protein loading was from 20-25 mg per lane of the native-PAGE gel. Gels were run in Tris–glycine, pH 8.3, electrode buûer and were stained with Coomassie brilliant blue R-250.

Native molecular mass determination: The molecular mass of the native protein was determined by the method of Zwaan (1967) as modified by Fishbein *et al*. (1970). Two native-PAGE gels with the resolving gel at 7.5% and another at 5%, were run. These gels were calibrated with standard proteins, catalase (bovine liver, 232 kDa), ferritin (horse spleen, 440 kDa), urease (jack bean, 540 kDa), thyroglobulin (bovine thyroid, 669 kDa) by running the standard proteins in both the gels and the relative mobility (R_f) or the ratio of distance migrated by the proteins versus the dye front in the two gels calculated, i.e., R_f in 7.5% gel/ R_f in 5% gel. A standard graph was drawn by plotting a graph between the R_f in the two gels, i.e., (R_f in 7.5% gel/ R_f in 5% gel) versus the logarithm of the molecular masses of the standard proteins. The R_f of pigeonpea urease in the two gels was plotted on the graph and its mass determined by extrapolation. The molecular mass of the purified protein was also estimated by our group in a gel filtration chromatography column of Sepharose 6B (separation range 10^4-$3x10^6$ for proteins) by the method of Andrews (1964) as described in Das *et al*. (2002).

Activity staining: For activity staining, the gels were equilibrated with an aqueous solution of cresol red (0.5 g/L) and Na_2EDTA (1 g/L) followed by incubation with urea (15 g/L) (Blattler *et al*., 1967). Urease activity was visualized as crimson bands in a golden-yellow background.

Estimation of protein: Total protein was estimated by the method of Lowry *et al*. (1951) using Folin-Ciocalteau reagent (1927) and bovine serum albumin as a standard protein.

Results and discussion

PAGE and activity staining: Activity staining of the purified and dialyzed preparation of PPU in a 7% native-PAGE gel using cresol red showed a single band corresponding to the protein band (Figure 1). Native-PAGE of the purified and dialyzed urease sample showed a single band on staining with Coomassie brilliant blue (Das *et al*., 2002). This band co-migrated with a commercial preparation of JBU (Das *et al*., 2002).

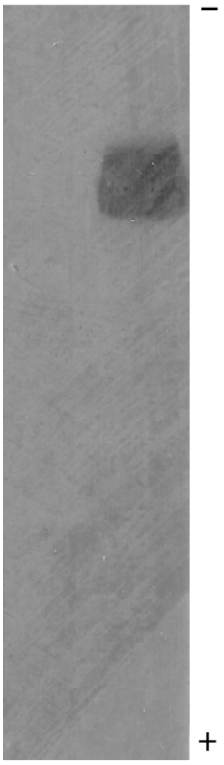

Figure 1: **Activity staining of purified dialyzed pigeonpea urease in native-PAGE gels. Native-PAGE (7%) of purified dialyzed enzyme sample (2 µg) stained with cresol red in the presence of substrate, urea.**

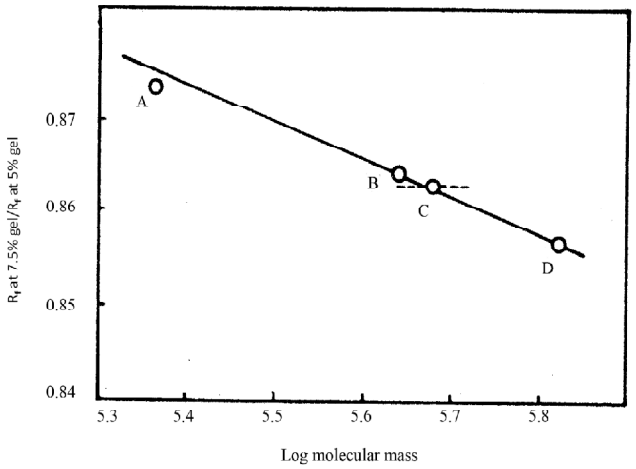

Figure 2: **Determination of molecular mass of pigeonpea urease by native-PAGE. Electrophoresis was performed in 7.5% and 5% acrylamide gels. The standard reference proteins were A, catalase (bovine liver, 232 kDa); B, ferritin (horse spleen, 440 kDa); C, urease (jack bean, 540 kDa); D, thyroglobulin (bovine thyroid, 669 kDa). The ratio of migration of pigeonpea urease in the two gels has been indicated by a broken line.**

Molecular mass: The molecular mass of the native protein was determined by PAGE using the method described earlier by Zwaan (1967). On plotting a graph of the ratio of R_f (relative mobility) in the gel, i.e., (R_f at 7.5%/ R_f at 5% gel) versus the logarithm of the molecular masses of the standard proteins, a straight line was obtained (Figure 2). From this graph, the molecular mass of PPU was determined as 540±10 kDa. Gel filtration chromatography of the protein also showed identical results as has been reported by our group earlier (Das *et al*., 2002).

Multimeric forms of urease: During the purification of PPU from dehusked seeds, it was noted that under certain conditions, the purified urease existed as more than a single protein species. The electrophoresis of aliquots of peak fractions from diethylaminoethyl-cellulose column, eluted with 0.2 M KCl and concentrated with dry powdered sucrose showed two and, sometimes, three bands on native-PAGE of the finally purified enzyme (Figure 3; Lane B). The protein band of lowest molecular mass (the basic

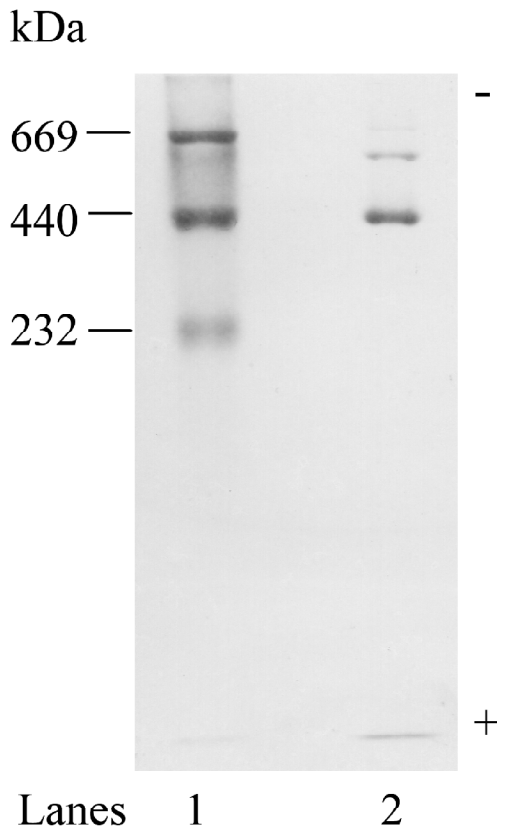

Figure 3: **Native-PAGE (7%) of pigeonpea urease enzyme eluted from anion-exchange chromatography column. Standard proteins (thyroglobulin, ferritin and catalase) were run in Lane 1. Enzyme sample (5 mg protein) without dialysis was run in Lane 2. The proteins in the gel were visualized by staining with Coomassie brilliant blue R-250.**

monomeric unit designated as species 1 of approximately 540 kDa) usually showed the highest enzymatic activity on staining with cresol red (Figure 1). The second, presumably a multimeric form, immediately above this (designated as species 2 of approximately 600 kDa), usually showed appearance of the activity band on prolonged period of incubation with the substrate, urea (Figure 4A; Lane 1). The enzymatic activity of the basic monomeric unit was higher than the mutimeric species 2 by many-folds. The polymeric protein band of approximately 700 kDa (species 3) did not show any enzymatic activity.

On native-PAGE of the finally purified enzyme after dialysis against 0.1 M Tris-acetate buffer, pH 6.5 (for approximately 10 hours), the higher molecular mass bands disappeared and only one band (species 1) remained (Figure 4B; Lane 1). SDS-PAGE of all these samples (with or without dialysis) showed only one band of 90 kDa as has been reported earlier (Das *et al*., 2002).

Addition of 5 mM dithiothreitol to a PPU enzyme sample showing species 1 and 2 was found to result in the dissociation of species 2 (Figure 4A; Lane 2). Hence, the loss of the multimeric forms in the sample suggests the involvement of sulfhydryl bridges in formation of the multimers.

Addition of a hydroxyl compound (50% glycerol, v/v), to a dialyzed preparation of urease resulted in the re-assembly of the multimeric forms (both species 2 and 3) (Figure 4B; Lane 2). Thus, hydroxyl compounds can be suggested to cause the association of urease molecules. However, contrary to our findings on PPU, Contaxis and Reithel (1971) have reported the dissociation of the 480 kDa species of JBU to a half-mer form of approximately 240 kDa in presence of 50% glycerol (v/v). Based on the present data, we are unable to offer an explanation for this discrepancy in the findings.

Addition of 10-200 mM KCl to the dialyzed urease preparation resulted in the formation of the multimeric forms at concentrations at and above 20 mM KCl (Figure 5, Lanes 2 to 6). Addition of 10 mM KCl did not cause the formation of the multimers and only the 540 kDas species was observed (Figure 5, Lane 1). Thus, the

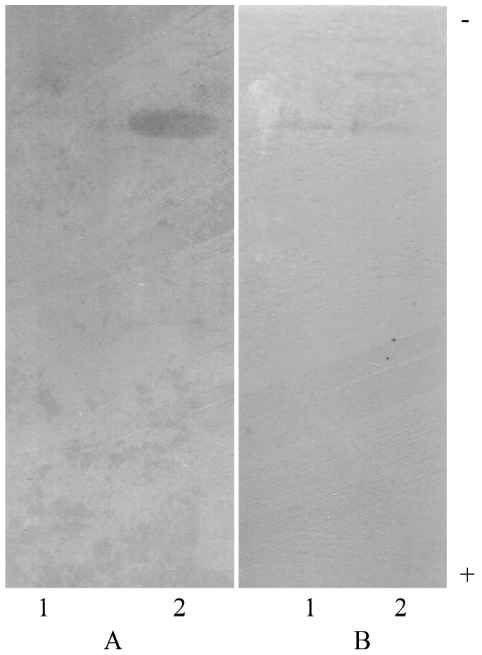

Figure 4: **(A) Effect of dithiothreitol on purified urease enzyme. Purified urease was treated with 5 mM dithiothreitol for 72 h and subjected to native-PAGE and activity staining. Lane 1: 0.5 mg protein without dithiothreitol treatment; Lane 2: 2 mg protein after treatment with 5 mM dithiothreitol. (B) Effect of 50% glycerol on dialyzed urease. Purified and dialyzed PPU (2 mg) was run in Lane 1. In Lane 2, dialyzed enzyme sample to which 50% glycerol was added and stored overnight at -10°C was run. Proteins were stained with Coomassie blue.**

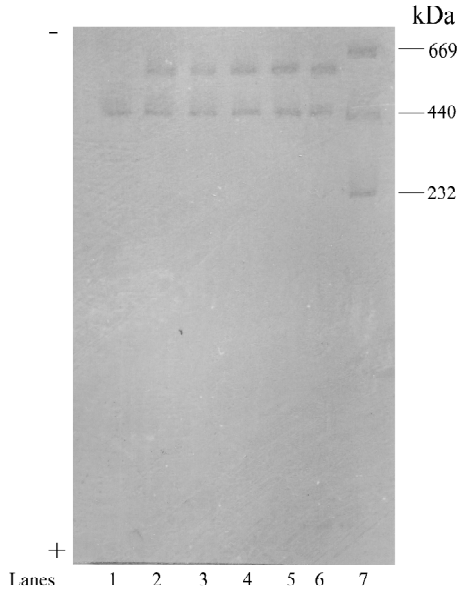

Figure 5: **Effect of ionic strength on purified urease. Purified and dialyzed enzyme with 10, 20, 50, 100 and 200 mM KCl were run in Lanes 1,2,3,4 and 5, respectively. Urease protein purified by the protocol outlined in Methods section (undialyzed) was run in Lane 6. Standard proteins were run in Lane 7. Proteins were stained with Coomassie blue.**

association-dissociation equilibrium seems to be a function of salt concentration/ionic strength (or salt-in effect). Furthermore, the inter-conversion of two urease species by merely altering salt concentration indicates that one species is not a proteolytic product of the other. It may be noted here that because the 600 kDa form showed minor catalytic activity and as the forms could be inter-converted to each other, they are not impurities in the enzyme preparation. Further studies using size exclusion chromatography and dynamic light scattering may broaden our understanding further.

There are probably a number of other factors involved in the inter-conversion of the various forms which have not been investigated. Similar findings are reported for urease from seeds of watermelon. Prakash and Bhushan (1998) observed two oligomeric forms of urease from watermelon, which disappeared on storage at -4°C. Similar to PPU, no 240 kDa species of watermelon urease was observed. Earlier reports on watermelon urease indicated the absence of a 12 S form in this species contrary to results from both JBU and SBU (Tanis and Naylor, 1968).

The presence of non-genetic isozyme variants has been reported by Polacco and Havir (1979) for SBU (the seed isozyme) and by Fishbein and co-workers (1970, 1975, 1976) for JBU. Polacco and Havir (1979) also reported salt concentration to be one of the controlling factors in the inter-conversion of the two species in soybean urease. However, Fishbein *et al*. (1975, 1976) have designated 12 different forms of JBU whose presence depended on salt concentration, pH, sulfhydryl reagents, hydroxyl compounds (glycerol), etc. They reported that an arithmetic polymer series of disulphide bonded α units (of approximately 500 kDa) of JBU formed with higher values of M_r and S in the absence of β-mercaptoethanol indicating involvement of sulfhyldryl bridges in formation of the multimers (Fishbein *et al*., 1970) similar to the present report. However, contrary to our report on PPU, all the polymeric isozymes of JBU were catalytically active. Glycerol at a concentration lower than (approximately one-third) that used by Contaxis and Reithel (1971) on JBU did not have any effect on the quaternary structure/activity of SBU. Again, contrary to the present report, glycerol resulted in dissociation of the 480 kDa JBU species to a 240 kDa one (Contaxis and Reithel, 1971). Polacco and Havir (1979) also stored commercial JBU (from Sigma Chemical Co.) in the presence of salt leading to the formation of two and sometimes three electrophoretically distinct species. It may be noted that unlike reports from jack bean or soybean, we did not observe the 240 kDa form of urease.

In conclusion, association-dissociation of the purified protein has been observed in the urease from the seeds of pigeonpea. The polymeric forms usually exhibited lower activity than the basic form. Salt concentration or ionic strength seems to be crucial determinants as dialysis resulted in a urease preparation without the polymeric forms. The multimeric forms are likely linked by sulfhydryl linkages.

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Abbreviations

PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PPU, pigeonpea urease; JBU, jack bean urease; SBU, soybean urease.

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