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Purification and characterization of urease from dehusked pigeonpea (*Cajanus cajan* L.) seeds

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Abstract

Urease has been purified from the dehusked seeds of pigeonpea (*Cajanus cajan* L.) to apparent electrophoretic homogeneity with approximately 200 fold purification, with a specific activity of 6.24×10^3 U mg^{-1} protein. The enzyme was purified by the sequence of steps, namely, first acetone fractionation, acid step, a second acetone fractionation followed by gel filtration and anion-exchange chromatographies. Single band was observed in both native- and SDS–PAGE. The molecular mass estimated for the native enzyme was 540 kDa whereas subunit values of 90 kDa were determined. Hence, urease is a hexamer of identical subunits. Nickel was observed in the purified enzyme from atomic absorption spectroscopy with approximately 2 nickel ions per enzyme subunit. Both jack bean and soybean ureases are serologically related to pigeonpea urease. The amino acid composition of pigeonpea urease shows high acidic amino acid content. The N-terminal sequence of pigeonpea urease, determined up to the 20th residue, was homologous to that of jack bean and soybean seed ureases. The optimum pH was 7.3 in the pH range 5.0–8.5. Pigeonpea urease shows K_m for urea of 3.0 ± 0.2 mM in 0.05 M Tris–acetate buffer, pH 7.3, at 37 °C. The turnover number, k_{cat}, was observed to be 6.2×10^4 s^{-1} and k_{cat}/K_m was 2.1×10^7 M^{-1} s^{-1}. Pigeonpea urease shows high specificity for its primary substrate urea.

Keywords: Urease; Pigeonpea (*Cajanus cajan*); Enzyme purification; Urea; Characterization

1. Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) catalyses the hydrolysis of urea at a rate 10^{14} times faster than the uncatalysed reaction, eventually yielding ammonia and carbon dioxide (Andrews et al., 1984; Mobley et al., 1995). Urease has been reported to be ubiquitous in virtually all plants and has been reported to be present in all tissues of soybean (Polacco and Havir, 1979). Most extensively studied plant urease is from jack bean (*Canavalia ensiformis*). The complete jack bean embryo-specific urease gene has been cloned in *E. coli* (Riddles et al., 1991). Recently, soybean genes involved in nickel insertion into urease (Polacco et al., 1999) and the one encoding Ni-binding protein necessary for urease activity have been identified (Freyermuth et al., 2000).

However, knowledge of bacterial urease has surpassed that of plants. Recently, X-ray crystal structure of the enzyme from microbes, *Klebsiella aerogenes*, *Bacillus pasteurii* and *Helicobacter pylori* has been determined and characterised (Jabri et al., 1995; Benini et al., 1999; Ha et al., 2001). Furthermore, many pathologies are associated with the activity of ureolytic bacteria, and the efficiency of soil nitrogen fertilisation with urea is severely decreased by microbial urease activity (Bremner and Krogmeier, 1988; Collins and D'Orazio, 1993).

It has been recently shown that urease from pigeonpea can be used for analytical purposes (Das et al., 1997, 1998; Kayastha and Srivastava, 2001; Srivastava et al., 2001). Jack bean does not grow in India and importing the purified jack bean urease for various analytical applications turns out to be quite expensive. Therefore, an attempt has been made to purify the enzyme from a

Abbreviations: BSA; bovine serum albumin; DTT; dithiothreitol; HEPES; N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulphonic acid]; PBS; phosphate buffered saline; TPBS; PBS containing Tween-20.

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locally available rich source, pigeonpea (*Cajanus cajan* L.) with a view to determining its molecular properties and exploring various avenues of its commercial exploitation. Our further aim is also to carry out high-resolution crystallographic studies from pigeonpea. Among plant ureases, the evaluation of the crystal fine structure of jack bean urease has been attempted but was only successful up to 3Å resolution (Jabri et al., 1992, 1995).

2. Results and discussion

Urease is especially abundant in legume seeds but it is possibly widespread in all plants albeit at lower levels (Hogan et al., 1983). Pigeonpea (*Cajanus cajan* L.), a locally available legume, is a rich source of urease. Urease from dehusked pigeonpea seeds has been purified approximately 200 fold to an apparent electrophoretic homogeneity. A series of purification steps were used, namely, acid step, two acetone fractionations, chromatographies on Sephadex G-200 and DEAE-cellulose (Fig. 1). The sample protocol of a typical purification batch is given in Table 1. The specific activity of the final preparation varied generally from 4.5–5.5×10^3 U mg^{-1} protein depending on the batch of the seeds used. Earlier workers have also reported that variation in yield and specific activity of the purified enzyme from jack bean (Blakeley et al., 1969a). Besides the jack bean enzyme, urease has also been purified from soybean seeds and cell suspension (Polacco and Havir, 1979), watermelon seeds (Prakash and Bhushan, 1997) and recently from mulberry leaves (Hirayama et al., 2000). The specific activity of pigeonpea urease is comparable with highly purified jack bean urease.

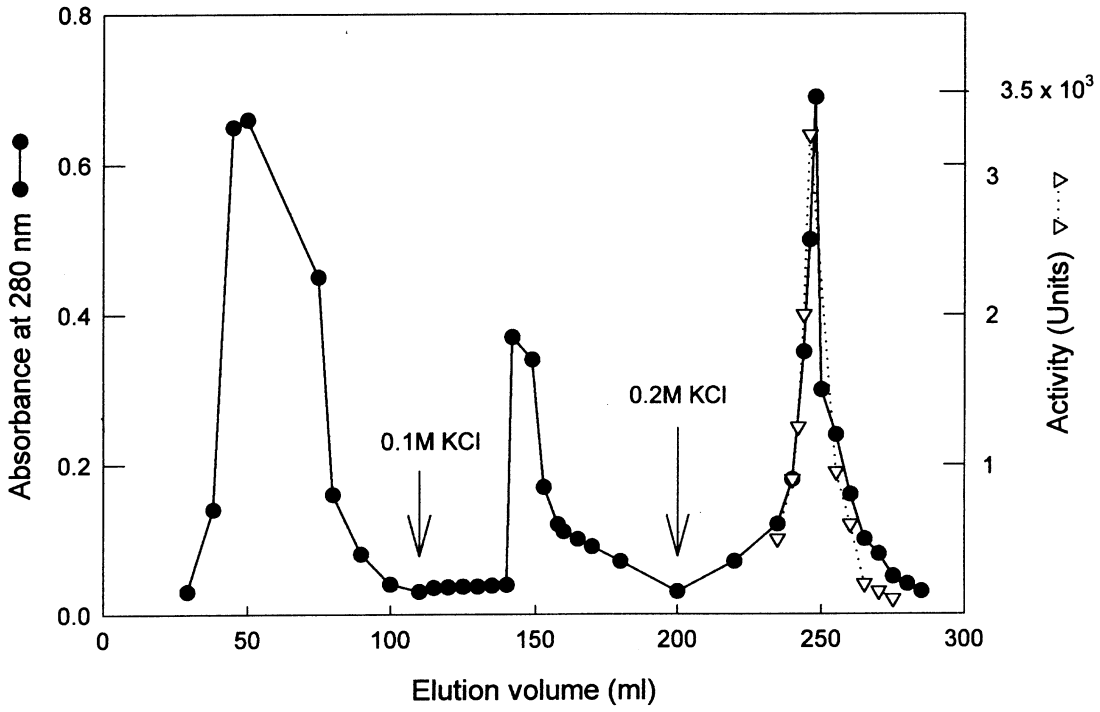

Fig. 1. Elution profile of pigeonpea urease on DEAE-Cellulose chromatography; (▽) enzyme activity, (●) protein in mg. The enzyme was eluted from the column with buffer containing 0.2 M KCl as shown.

Table 1
Purification of urease from 100 g of pigeonpea seeds

Step	Total activity (units ×10^3)	Total protein (mg)	Specific activity (units mg^{-1})	Purification[a] (−fold)	Yield (%)
Crude extract	200	6344	32	–	–
1st Acetone fractionation	122	302	404	13	61
Acid step	106	145	730	23	53
2nd Acetone fractionation	70	34	2042	65	35
Sephadex G-200	53	10	5300	170	27
DEAE-Cellulose	24	3.8	6240	198	12

[a] Fold purification calculated with respect to the specific activity of the crude extract.

The purified enzyme when stored frozen at −20 °C in 0.1 M Tris–acetate buffer, pH 6.8 showed a half-life of about 50 days; on storage at 4 °C, the $t_{1/2}$ reduced to 31 days. However, addition of 5 mM DTT increased the half-life to 180 days at −20 °C.

A 7% native-PAGE of the purified sample showed a single band on staining with Coomassie Brilliant Blue (Fig. 2, lane A) corresponding to the jack bean protein band (Fig. 2, lane B). Activity staining of the native gel with Cresol Red in the presence of 0.25 M urea also showed a single band corresponding to the protein bands (result not shown).

A 10% SDS–PAGE of the enzyme showed a single band on staining with Coomassie Brilliant Blue [Fig. 3(i)]. Densitoscan analysis of the Coomassie Blue stained gel showed a single peak corresponding to the protein band (result not shown).

The native molecular mass estimated by gel filtration on a Sepharose 6B column was approximately 540 kDa (Fig. 4). The migrations of jack bean and pigeonpea urease, under identical conditions, were very similar, both in gel-filtration and native-PAGE.

The subunit molecular mass of urease has been determined by comparing its electrophoretic mobility in the presence of SDS with mobilities of known proteins under identical conditions [data of Fig. 3(i)]. The subunit molecular mass of pigeonpea urease was found to be 90 kDa, which is similar to that reported from jack bean and soybean ureases. Presence of a single protein-staining band following SDS/PAGE suggests that the pigeonpea urease is made up of subunits of identical molecular mass and is, therefore, a homohexameric protein. The protein sequence of jack bean urease was

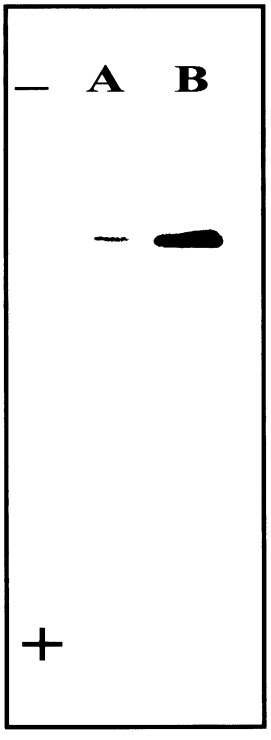

Fig. 2. 7% Native-PAGE Lane A: Purified pigeonpea urease (5 μg). Lane B: Commercially available jack bean urease (15 μg) (Sigma Chemicals, Type VII). Protein bands were visualised by staining with Coomassie Brilliant R250 Blue.

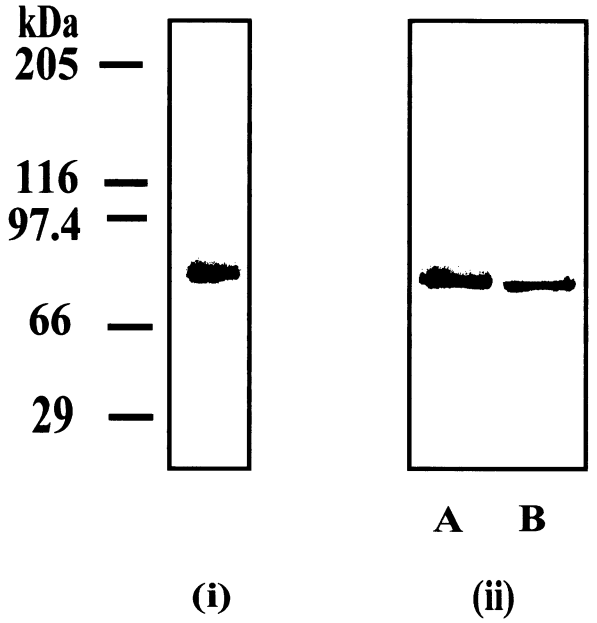

Fig. 3. SDS/PAGE of purified pigeonpea urease. (i) 10 μg of pigeonpea urease in a 10% SDS/PAGE gel. Standard proteins were myosin (205 kDa), β-galactosidase (*E. coli*, 116 kDa), phosphorylase-b (rabbit muscle, 97.4 kDa), BSA (66 kDa) and carbonic anhydrase (bovine erythrocytes, 29 kDa). (ii) Western blotting of urease adsorbed to nitrocellulose membrane: 2 μg of each antigen (jack bean urease in Lane A and pigeonpea urease in Lane B) reacted with anti-urease (soybean) antibody.

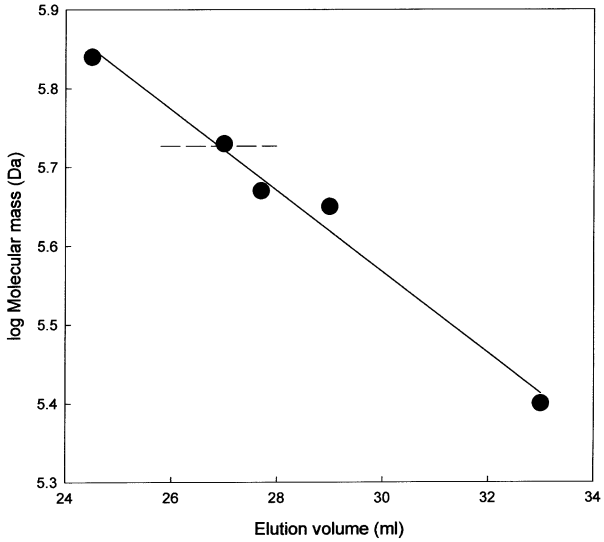

Fig. 4. Estimation of native molecular mass by gel filtration on a Sepharose 6B chromatography column. The standard proteins used (●) were thyroglobulin (bovine thyroid, 669 kDa); urease (jack bean, 540 kDa); urease (watermelon, 470 kDa); ferritin (horse spleen, 440 kDa); pyruvate kinase (rabbit muscle, 240 kDa). A broken line shows the elution volume of pigeonpea urease.

determined (Mamiya et al., 1985) and the cDNA is cloned (Riddles et al., 1991); the subunit molecular mass is therefore known to be 90.7 kDa. Several studies show that the enzyme exists as a trimer or hexamer in its native form (Wang et al., 1994). The molecular mass of urease from soybean has been reported to be 480 kDa (Polacco and Havir, 1979) and the molecular mass of urease from watermelon has been reported to be slightly lower, at 470 kDa (Prakash and Bhusan, 1997). However, a recent report from mulberry leaf urease shows the molecular mass to be 175 kDa and was found to be a homodimer (Hirayama et al., 2000).

Atomic absorption spectroscopy of pigeonpea urease (freshly prepared) showed the presence of 1.96 nickel ions per enzyme subunit or approximately 12 nickel ions per mole of enzyme. Nickel has been recognised as a universal component of ureases known so far (Benini et al., 1999). In both, jack bean and soybean ureases, 2-nickel ions per enzyme subunit have been reported (Dixon et al., 1975, 1980a; Polacco and Havir, 1979).

Serological relationship was determined against anti-urease antibodies. Significant cross-reactivity was obtained with pigeonpea urease against both jack bean monoclonal antibody and affinity purified soybean polyclonal antibodies for both ELISA (result not shown) and Western blotting studies. The ELISA results showed an increased binding between pigeonpea urease and soybean antibody to be approximately 30% higher than the corresponding response between jack bean urease and soybean antibody at similar dilutions of antigen and antibody. Pre-immune serum from both rabbit and mouse did not show any reaction with either of the antigens. From Western blotting, an immunoreactive protein migrating with a mass of 90 kDa was observed when both jack bean and pigeonpea urease were treated with anti-soybean [Fig. 3(ii)] and anti-jack bean urease antibodies (result not shown). Furthermore, no band was observed when pre-immune serum was used instead of the anti-soybean and anti-jack bean urease antibodies (results not shown). SDS treatment was not found to destroy the epitopes against which antibodies had been raised, as both the antigens (pigeonpea and jack bean ureases) after SDS treatment showed significant reaction with antibodies. On the basis of the aforesaid observations it can be concluded that the three ureases share common epitopes in native and denatured conformations. The presence of some common and unique determinant(s) between soybean urease antiserum and jack bean urease has been reported earlier (Polacco and Havir, 1979).

The overall amino acid profile of pigeonpea urease is found to be very similar to those of jack bean and soybean ureases (Table 2). This is expected in light of their identical subunit masses and serological cross-reactivity. Trp is destroyed during acid hydrolysis and cysteine is greatly reduced. Thr and Ser values were corrected for hydrolytic losses. High content of acidic amino acids (Glu and Asp) is observed in all the three ureases. Jack bean and soybean are reported to contain high Met content although this amino acid is generally present in limiting amount in comparison to other legume seed proteins indicating urease to be a seed reserve protein (Milton and Taylor, 1969; Staples and Reithel, 1976; Polacco and Havir, 1979). Met level of pigeonpea urease was slightly higher than either of the two plant sources mentioned above.

The first 20 amino acids of the 90 kDa subunit of the pigeonpea seeds were determined and the results were compared with the relevant amino acid sequences of ureases from mulberry leaf, soybean seeds and seedling axis, and jack bean seeds (Fig. 5). The data of Fig. 5 shows the first 20 amino acids of the pigeonpea seed urease has a high degree of conservation with those of mulberry leaf urease (80% identity), jack bean urease (100% identity), soybean ureases from seeds and seedling axes (100 and 90% identity), respectively. However, compared with the subunit of *K. aerogenes* urease, a benchmark representative of bacterial urease, N-terminal sequence of pigeonpea urease showed a relative lower structural similarity (50%). High similarity of amino acid sequences between plant ureases suggests that these enzymes are closely related.

The rate of hydrolysis of urea by urease at 37 °C showed an optimum pH at 7.3 in the pH range 5.0–8.5 in the buffering conditions of 0.05 M of citrate-phosphate (pH 5.0–7.0), HEPES-KOH (pH 6.8–8.2) and Tris–acetate (pH 7.3–8.5) buffers (data not shown). Pigeonpea urease is only 50% as efficient at pH 5.0 as compared to its activity at pH 7.3 whereas, jack bean urease has 68% activity at pH 5.2 compared to its activity at pH 7.0 (Blakeley and Zerner, 1984). For soybean ubiquitous urease, pH optima at 7.0 (in vitro studies) and at 5.25 and 8.75 (in vivo studies) have been observed (Kerr et al., 1983). The differences in pH optima between the in vitro and in vivo studies was suggested by the authors to be due to the interaction of some other factor(s) as urea uptake across plasma membrane could be pH-dependent. The pH optimum of soybean seed urease (in vitro studies) was reported to be very broad and ranged from 5.0 to 9.7 (Kerr et al., 1983). Watermelon urease showed a sharp pH optimum at 8.0 (Prakash and Bhusan, 1997), while the optimum pH for mulberry leaf urease is 9.0, the highest value among the ureases purified so far (Hirayama et al., 2000).

Variation in rate of hydrolysis of urea by pigeonpea urease with different urea concentration (10 points measured between 0.5 and 500 mM viz., 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 mM) was found to follow simple Michaelis–Menten relationship (data not shown). The K_m of pigeonpea urease for its substrate, urea, determined from the double reciprocal plot in 0.05 M Tris–acetate buffer, pH 7.3, at 37 °C was 3.0 ± 0.2 mM

Table 2
The amino acid composition of plant ureases[a]

Amino acid	Soybean[b]	Jack bean[b]	Jack bean[c]	Pigeonpea[d]
Cys (C)	0.47±0.34	0.23±0.08	2.14	0.38±0.05
Trp (W)	ND	ND	0.7	ND
Asp (D) and Asn (N)	12.76±0.33	10.9±0.06	11.61	11.37±0.09
Thr (T)	5.28±0.28	6.72±0.02	6.79	5.98±0.01
Ser (S)	5.79±0.21	5.96±0.04	5.95	6.05±0.03
Gln (Q) and Glu (D)	10.09±0.42	10.42±0.14	8.86	10.94±0.03
Pro (P)	6.09±0.63	5.09±0.06	5.36	4.69±0.05
Gly (G)	10.35±0.23	9.72±0	10.11	10.11±0.09
Ala (A)	7.44±0.84	9.76±0.15	9.54	9.11±0.04
Val (V)	6.34±0.24	6.11±0.22	4.6	6.85±0.02
Met (M)	2.04±0.1	1.99±0.04	2.83	2.32±0.06
Ile (I)	6.16±0.21	5.53±0.03	5.28	5.54±0.09
Leu (L)	8.17±0.12	7.77±0.07	8.15	8.26±0.04
Tyr (Y)	2.43±0.08	2.72±0.22	2.47	3.04±0.18
Phe (F)	4.23±0.18	2.89±0.03	2.81	3.51±0.05
His (H)	2.09±0.16	3.04±0.03	2.80	2.00±0.02
Lys (K)	5.64±0.09	6.54±0.07	5.69	5.76±0.05
Arg (R)	4.62±0.08	4.64±0.03	4.2	4.13±0.05

[a] Data expressed as residues/100 amino acids.
[b] Data of Polacco and Havir (1979).
[c] Data of Staples and Reithel (1976).
[d] Mean of 0.3 and 0.6 nmol enzyme analysed.

	1				5					10					15					20	
Pigeonpea (seed)	M	K	L	S	P	R	E	V	E	K	L	G	L	H	N	A	G	Y	L	A
Mulberry (leaf)	*	*	*	T	*	*	*	I	*	*	*	D	*	*	*	*	*	F	*	*
Soybean (seed)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Soybean (seedling)	*	*	*	*	*	*	*	I	*	*	*	D	*	*	*	*	*	*	*	*
Jack bean (seed)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
K. aerogenes	*	E	*	T	*	*	*	K	D	*	*	L	*	F	T	*	A	L	V	*

Fig. 5. Sequence alignment of N-terminal amino acid sequence of pigeonpea seed urease compared with those of other plant ureases and *K. aerogenes*, a bench mark representative of bacterial ureases. Shown are the N-terminal 20 amino acid residues of pigeonpea urease (the present study); Mulberry leaf urease (Hirayama et al., 2000); Soybean ureases from seeds and seedling axes (Torisky et al., 1994); Jack bean seed urease (Takishima et al., 1988); *Klebsiella aerogenes* (Mulrooney, S.B. & Hausinger, R.P., 1990). Asterisks represent amino acid residues identical to those of pigeonpea seed urease.

(Table 3). The V_{max} was 7.7×10^3 μmol NH_3/min/mg protein. Kinetic studies of jack bean urease show a K_m of 2.9 to 3.3 mM (Blakeley et al., 1969a; Blakeley and Zerner, 1984). An apparent K_m of 0.85 mM for soybean leaf urease (Kerr et al., 1983) and a K_m of 19–476 mM for the soybean seed urease depending on the buffer systems chosen has been reported (Talsky and Klunker, 1967). Most recently from mulberry leaf urease K_m of urea was reported to be 0.16 mM using Lineweaver–Burk plot; this is the lowest value among the plant ureases purified so far (Hirayama et al., 2000).

Urease is one of the most catalytically efficient enzymes with k_{cat} hundred times higher than the k_{cat} for the action of any peptidase in the hydrolysis of carboxamides and approximately 10^{14} increase in the rate of breakdown of urea as compared to the uncatalysed one (Andrews et al., 1984). k_{cat} for pigeonpea urease is 6.2×10^4 s^{-1} and the specificity constant, k_{cat}/K_m was

Table 3
Substrate specificity of pigeonpea urease with urea and various substrate analogues and further inhibition effect of these analogues on urease activity

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Nature of inhibition	K_i (mM)
Urea	3.0±0.2	6.2×10^4	2.1×10^7	–	–
Thiourea	40±3	3.4×10^1	85	Competitive	23±2
Hydroxy urea	125±5	1.7×10^1	1.4×10^2	Competitive	100±5
N-methyl urea	1000±10	2×10^2	2.0×10^2	Competitive	980±10

2.1×10^7 M^{-1} s^{-1} (Table 3). The k_{cat} reported for jack bean urease is $2.15–2.34\times10^4$ s^{-1} (Blakeley et al., 1969a; Norris and Brocklehurst, 1976).

Besides urea, different analogues of urea were also examined as a substrate, namely thiourea, hydroxy urea and *N*-methyl urea, for their specificity and inhibitory studies on pigeonpea urease. A comparison data for the values of K_m, K_i and k_{cat}/K_m of these analogues with urea was shown in Table 3. It is clear from the table that k_{cat}/K_m value for urea is highest and that for thiourea is least. All these analogues appeared to be reversible competitive inhibitor for pigeonpea urease. Thiourea, hydroxy urea, and *N*-methyl urea were shown to be substrates for pigeonpea urease, although the rate of thiourea hydrolysis was very slow (data not shown) but a significant amount of ammonia released was detected over a longer period of incubation. Recently, Lopreore and Byers (1998) have studied the kinetics of hydrolysis of thiourea with jack bean urease and reported the K_i value in 702 mM range. Blakeley et al. (1969b) found hydroxy urea, a substrate for jack bean urease and also examined its inhibition pattern and found it a completely reversible inhibitor. Dixon et al. (1980b) have also determined the K_m and K_i values for different substrate analogues, namely urea, *N*-methyl urea, acetamide etc., but they did not find thiourea as a substrate for jack bean urease.

3. Experimental

3.1. Plant materials and chemicals

Dehusked pigeonpea seeds were purchased locally. Nunc immunoplate 96F was from BRL (Gibco). Nitrocellulose paper (pore size 0.2 μm) was from Schleicher & Schuell. Standard proteins for molecular mass determination (thyroglobulin, ferritin and catalase) were from Pharmacia, Sweden. Acetone (HPLC grade) and acrylamide (4×recrystallised) were purchased from Spectrochem, India. Urea (enzyme grade) was from Sisco Research Laboratories, India. Affinity purified polyclonal antibodies against soybean urease were a kind gift from Professor J.C. Polacco, University of Missouri, Columbia, MO, USA. Monoclonal jack bean urease antibody, second antibodies (anti-mouse and anti-rabbit IgG) conjugate with horseradish peroxidase, molecular weight markers and jack bean urease (Type VII) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

All solutions were prepared in triple distilled water from an all-quartz distillation assembly.

3.2. Urease activity assay

Urease activity was routinely assayed by measuring ammonia liberated at a fixed time interval from urea according to Nessler's method (Das et al., 1998) unless stated otherwise. An enzyme unit is defined as the amount of enzyme required to liberate one μmol of ammonia per min under our test conditions (0.1 M urea, 0.05 M Tris–acetate buffer, pH 7.3, 37 °C).

Protein was estimated by the method of Lowry et al. (1951). Specific activity is expressed as enzyme units/mg protein.

3.3. Enzyme purification

Urease has been purified from dehusked pigeonpea (*Cajanus cajan* L.) seeds by a succession of steps, which were carried out at 0–4 °C, unless stated otherwise.

3.3.1. Extraction

Dehusked pigeonpea seeds (100 g) were soaked in extraction buffer (0.025 M Tris–acetate, pH 6.8) containing 50 μg ml^{-1} leupeptin and homogenised in a kitchen blender. The homogenate was centrifuged at 26,890 g for 15 min. The clear yellow supernatant was collected.

3.3.2. First acetone fractionation

The above extract was fractionated with acetone in the range 25–40% (v/v) at −15° C. After removing the traces of acetone, the precipitate obtained in this fraction was dissolved in minimum volume of buffer (0.05 M Tris–acetate, pH 6.8).

3.3.3. Acid step

The pH of the above enzyme fraction was brought down to 5.1 by adding chilled 0.1 M acetic acid. The suspension was immediately centrifuged at 26,890 *g* for 1 min to remove precipitate and the pH of the clear supernatant brought back to 6.8 with Tris solution.

3.3.4. Second acetone fractionation

To the acid fractionated enzyme acetone was added to a concentration of 67%; (v/v) acetone at −15 °C. Precipitate was suspended in 0.1 M Tris–acetate, pH 6.8.

3.3.5. Sephadex G-200 chromatography

The second acetone fractionated enzyme was loaded on to a gel filtration column (Sephadex G-200) of dimensions 1.6×20 cm. The column was equilibrated with 4 bed volumes of running buffer (0.1 M Tris–acetate buffer, pH 6.8) at flow rate was 18 ml/h. The A_{280} was monitored of all fractions and activity and protein of the peak fractions assayed. High specific activity fractions were pooled (21.3 ml).

3.3.6. DEAE-cellulose chromatography

The enzyme from the gel filtration column was loaded on a DEAE-cellulose column of dimensions 1.6×20 cm equilibrated with 4 bed volumes of running buffer (0.1 M Tris–acetate buffer, pH 6.8). The flow rate was 30 ml/h. Urease was adsorbed on the column material and unadsorbed proteins were washed out with several volumes of running buffer till the washings were free of protein ($A_{280} \leqslant 0.04$). A discontinuous KCl gradient was applied for enzyme elution. Fractions with high A_{280} were assayed for urease activity and protein. Fractions with high specific activity were pooled.

Pooled enzyme was concentrated to 4.2 ml by a Centriplus-10 (Amicon) and 5 mM DTT was added to stabilise the enzyme. Enzyme was stored in small batches at −20 °C.

3.4. Polyacrylamide gel electrophoresis

PAGE was performed by the method of Laemmli (1970). Native-PAGE was run at 7% and SDS-PAGE at 10% acrylamide concentration of the resolving gel. Gels were run in electrode buffer Tris–glycine, pH 8.3 and were stained with Coomassie Brilliant Blue R-250.

For activity staining, the gels were equilibrated with Cresol Red (0.5 g/l) and Na_2EDTA (1 g/l) followed by incubation with the same solution containing urea (15 g/l) (Blattler et al., 1967).

Densitoscanning analysis of the Coomassie Blue stained SDS–PAGE gel was done in a Beckman DU 8B Spectrophotometer. Scanning from separating gel up to the dye front was carried out with 0.1×4 mm slit and 600 nm filter at a speed of 5 cm/min with slab gel scanning mode.

3.5. Molecular mass determination and immunoblotting

The molecular mass of the purified protein was estimated in a gel filtration column of Sepharose 6B (exclusion limit 10^4–3×10^6 for proteins). The dimensions of the column was 1.1×51 cm. Column was washed extensively with 0.1 M Tris–acetate buffer, pH 6.8 for equilibration. Flow rate of the column was 10 ml/h. The column was calibrated with standard proteins. 1 ml fractions were collected and A_{280} monitored for all fractions. The molecular mass of urease subunits was determined in 10% SDS-PAGE gel calibrated with standard proteins The molecular mass was determined by plotting a graph of the logarithm of molecular masses of the standard proteins and their relative migration in the gel.

Proteins were separated by SDS–PAGE (10% gel) and transferred to nitrocellulose membrane. The resultant membrane was incubated in PBS containing 0.2% (w/v) BSA for 1 h at room temperature. The membrane was then sequentially incubated with primary antibody (dilution 1:16000) in TPBS (PBS containing 0.1% Tween-20 v/v) at 4 °C overnight and then with second antibodies (anti-mouse and anti-rabbit IgG) conjugate with horseradish peroxidase. Second antibodies, diluted 1:2000 in TPBS, were incubated for 1 h. Reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/ml prepared in PBS with 1 μl H_2O_2/ml). Reaction was terminated by rinsing with water.

3.6. ELISA

ELISA was performed with 1–2 μg antigen (pigeonpea or jack bean urease) in 100 μl PBS in a 96-well microtitre plate at 37 °C. Non-specific sites were blocked with 0.1% (w/v) BSA in PBS. Anti-urease (Jack bean/soybean) antibodies were serially diluted from 1:500 to 1:16000 and from 1:100 to 1:3200, respectively. Pre-immune serum from mouse and rabbit, respectively, at similar dilutions were also incubated with the antigens. Anti-mouse and anti-rabbit horseradish peroxidase-labelled second antibodies were diluted 1:10,000 in PBS containing 0.1% (v/v) Tween-20 (TPBS). Reaction was developed with *o*-phenylenediamine dihydrochloride and read at 490 nm in an ELISA plate reader, E_{max} precision microplate reader (Molecular devices).

3.7. Amino acid composition

Desired amount of enzyme solution was centrifuged at 180,000 *g* for 2 h at 4 °C in an analytical ultracentrifuge. During centrifugation the enzyme protein moved to the bottom of the centrifuge tube which was taken up with a pipette. Acid hydrolysis was carried out in 6 N HCl for 48 h at 110 °C and sample for analysis was prepared after complete elimination of HCl in vacuo and making the original volume with HPLC grade water. The amino acid analysis was performed in an Amino Acid Analyser (Model 835, Hitachi, Japan).

3.8. N-terminal amino acid sequencing

Purified enzyme was subjected to SDS–PAGE (7.5%), electroblotted onto PVDF membrane according to the

procedure of Towbin et al. (1979) and visualized by Ponceau S dye. The 90 kDa protein band was cut out and 500 pmol protein was subjected to N-terminal sequence analysis by automated Edman degradation on a gas phase sequencer (Model PSQ-2, Shimadzu, Japan). Phenylthiohydantoin (PTH) derivatives of individual amino acids were identified by reverse phase HPLC.

3.9. Specificity studies

The activity of pigeonpea urease was determined in varying concentrations of substrate analogues like, thiourea, hydroxy urea and methyl urea with hypochlorite method (Weatherburn, 1967) where ammonia released was monitored by reacting with phenol-hypochlorite to form indophenol, due to their interference with Nesslers's reagent. K_i were determined by the Dixon method. K_m was calculated by Lineweaver–Burk plot method.

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