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Purification, crystallization and preliminary X-ray analysis of urease from pigeon pea (*Cajanus cajan*)

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Urease is a seed protein that is common to most Leguminosae. It also occurs in many bacteria, fungi and several species of yeast. Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide, thus allowing organisms to use exogenous and internally generated urea as a nitrogen source. Urease from pigeon pea seeds has been purified to electrophoretic homogeneity using a series of steps involving ammonium sulfate fractionation, acid precipitation, ion-exchange and size-exclusion chromatography techniques. The pigeon pea urease was crystallized and the resulting crystals diffracted to 2.5 Å resolution. The crystals belong to the rhombohedral space group *R*32, with unit-cell parameters $a = b = 176.29$, $c = 346.44$ Å.

1. Introduction

Ureases (urea amidohydrolases; EC 3.3.1.5) are nickel-dependent enzymes (Dixon *et al.*, 1975) that catalyze the hydrolysis of urea to form ammonia and carbon dioxide at a rate that is 10^{14} times faster than the uncatalysed reaction (Andrews *et al.*, 1984; Mobley *et al.*, 1995). Ureases have been isolated from a wide variety of organisms, including plants, fungi and bacteria (Mobley & Hausinger, 1989).

Bacterial urease is one of the key enzymes used by pathogenic bacteria that cause gastric ulcers, urinary stone formation and oral biofilm formation (Mobley *et al.*, 1995). In agriculture, ureases in soil bacteria can decompose soil-based fertilizers, resulting in both nitrogen deficiency and ammonia toxicity, leading to plant damage (Mobley & Hausinger, 1989).

In plants, urease is abundant in the seeds (embryo-specific urease; Polacco & Holland, 1994) and is also found at lower levels in the vegetative tissues of most species (tissue-ubiquitous urease; Hogan *et al.*, 1983). The best biochemically characterized plant urease is that from jack bean (*Canavalis ensiformis*; Follmer *et al.*, 2004). Soy bean has been found to possess both a ubiquitous urease that is synthesized in all tissues and an embryo-specific urease that is confined to the developing embryo and is retained in the mature seed; its activity is 1000-fold greater than that of the ubiquitous urease in many tissues (Polacco & Sparks, 1982; Polacco & Winkler, 1984). Pigeon pea urease has been characterized as being serologically related to jack bean and soy bean ureases (Das *et al.*, 2002). In addition, ureases from sources such as watermelon seeds (Prakash & Bhusan, 2003) and *Chenopodium album* leaves (Shora, 2000) have also been characterized.

Despite the abundance of urease in some plant tissues and its ubiquity in virtually all plants, little has been revealed about its physiological roles. Both embryo-specific and tissue-ubiquitous ureases have been suggested to play an important role in seed germination, in which urease-negative mutants tend to germinate more slowly and with a lower frequency than urease-positive ones, and in seedling nitrogen metabolism (Zonia *et al.*, 1995). Ureases are also found to participate in plant defence against insect predation (Polacco & Holland, 1994). To date, knowledge of the structural and functional aspects of bacterial ureases has surpassed that of plant ureases. X-ray crystal structures of the enzymes from the microbes *Klebsiella aerogenes* (PDB code 1fwj), *Bacillus pasteurii* (PDB code

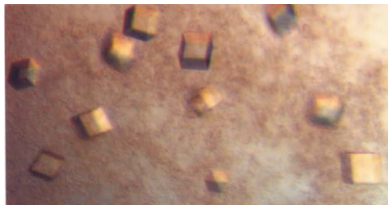

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (2.59–2.50 Å).

Wavelength (Å)	1.5418
Space group	*R*32
Unit-cell parameters (Å, °)	$a = b = 176.29$, $c = 346.44$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution range (Å)	30–2.5
Total No. of reflections	558518
No. of unique reflections	133788
Completeness (%)	96.3 (91.9)
$R_{\rm merge}$	9.7 (40.5)
Mean $I/\sigma(I)$	5.4 (0.8)

4ubp) and *Helicobacter pylori* (PDB code 1e9z) have been determined and analysed (Jabri *et al.*, 1995; Benini *et al.*, 1999; Ha *et al.*, 2001). Of the plant ureases, only the crystallization and preliminary X-ray analysis of jack bean urease at resolutions of 3.5 and 3.3 Å has been reported using synchrotron radiation (Jabri *et al.*, 1992; Sheridan *et al.*, 2002), although this was the first enzyme to be crystallized (Sumner, 1926). Here, we report the purification, crystallization conditions and preliminary data on crystals of pigeon pea urease, which diffract to 2.5 Å resolution using an in-house X-ray source.

2. Experimental

2.1. Protein purification

Dry pigeon pea seeds were ground into a fine powder and extracted overnight with hexane [1:2(*w*:*v*)]. The solvent was removed and the meal was dried at room temperature. 100 g of defatted meal was extracted with buffer *A* (20 m*M* Tris–HCl, 10 m*M* EDTA and 10 m*M* β-mercaptoethanol pH 7.5) at 277 K for 1 h. The meal was removed by centrifugation at 10 000 rev min^{-1} for 30 min. Ammonium sulfate was added to the supernatant to 30% saturation and stirred at 277 K for 1 h. The precipitated proteins were removed by centrifugation at 10 000 rev min^{-1} for 30 min at 277 K. Ammonium sulfate was raised to 55% saturation in the supernatant and stirred at 277 K for 1 h. This was followed by centrifugation at 10 000 rev min^{-1} for 30 min at 277 K. The resultant pellet was redissolved in 20 ml buffer *B* (50 m*M* Tris–HCl, 5 m*M* EDTA and 5 m*M* β-mercaptoethanol pH 7.5) and dialyzed overnight against buffer *B*.

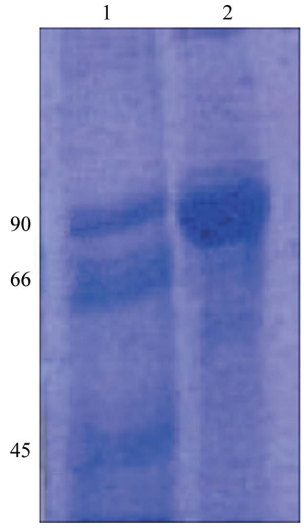

Figure 1
SDS–PAGE of purified pigeon pea urease. Lane 1 contains the standard proteins jack bean urease (90 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa) and lane 2 contains purified pigeon pea urease.

For acid precipitation, the pH of the dialyzed sample was adjusted to 5.1 using 0.1 *M* acetic acid and centrifuged at 10 000 rev min^{-1} for 5 min. The pellet was discarded and the pH of the supernatant was brought back to 7.5. The sample was loaded onto a Q-Sepharose column (GE Healthcare Life Sciences) equilibrated with buffer *B* followed by a gradient elution using sodium chloride. The urease eluted at around 400 m*M* NaCl. The sample was concentrated using Centriprep concentrators and loaded onto a Sephacryl S-300 gel-filtration column (GE Healthcare Life Sciences) which was pre-equilibrated with buffer *B*. The peak fractions containing urease were pooled and loaded onto a DEAE cellulose column equilibrated with buffer *B* and eluted using a potassium chloride gradient. This sample was concentrated and loaded onto a Superdex 75 column equilibrated with buffer *C* (50 m*M* Tris–HCl, 5 m*M* EDTA, 5 m*M* β-mercaptoethanol and 100 m*M* NaCl pH 7.5). The fractions containing urease were pooled and concentrated. The final concentration was estimated to be 12 mg ml^{-1} using the Bradford absorption method (Bradford, 1976). The purified urease showed a single band at 90 kDa on a 10% SDS–PAGE gel (Fig. 1). An assay for urease activity using Nessler's method (Das *et al.*, 1998) was carried out at all purification steps. The typical yield of the protein was 6 mg from 100 g of dry seeds.

2.2. Crystallization

Crystallization experiments were carried out using the hanging-drop vapour-diffusion method at 294 K. Home-made screens such as ammonium sulfate *versus* pH and polyethylene glycol *versus* pH were employed to screen the protein. The condition that yielded crystals was further optimized to obtain crystals that were suitable for X-ray diffraction studies. Final diffraction-quality crystals of dimensions 0.3 × 0.2 × 0.15 mm (Fig. 2) were obtained from drops containing 2 µl protein sample mixed with 2 µl reservoir solution and equilibrated against 1 ml reservoir solution (23% PEG 2000 monomethyl ether, 100 m*M* Tris–HCl pH 9.0 and 1 *M* $MgCl_2$).

2.3. X-ray analysis

Crystals suitable for X-ray diffraction were transferred into cryoprotectant solution, which had the same composition as the mother liquor but with the addition of 20% ethylene glycol. Crystals were mounted on cryoloops (Hampton Research) and flash-cooled in liquid nitrogen at 100 K. Diffraction experiments were carried out using an in-house MAR345 image-plate detector at a crystal-to-detector distance of 120 mm and a Bruker Microstar rotating copper-anode generator operating at 60 mA and 45 kV. A native data set was collected from a single crystal. A total of 120 frames were collected

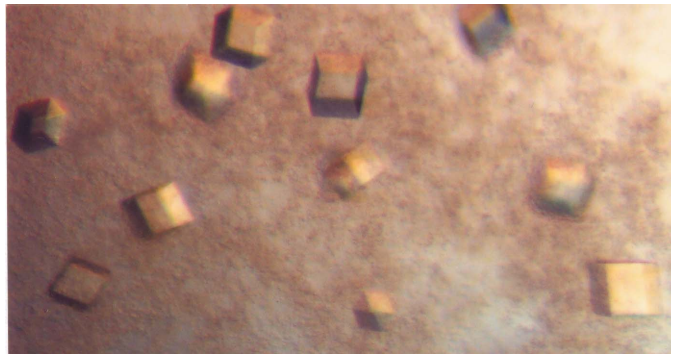

Figure 2
Crystals of pigeon pea urease.

with an oscillation step of 1° and an exposure of 120 s per frame. Diffraction images to a resolution of 2.5 Å were indexed, integrated, merged and scaled using the *AUTOMAR* software package (Bartels & Klein, 2003). Data-collection statistics are reported in Table 1.

3. Results and discussion

Pigeon pea urease crystallized in a rhombohedral (*R*32) form, with unit-cell parameters $a = b = 176.29$, $c = 346.44$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Given the molecular weight of 90 kDa of the protein and assuming the presence of two molecules in the asymmetric unit, the resultant Matthews coefficient is 2.86 Å^3 Da^{-1}, corresponding to a solvent content of 57.06%. Alternatively, the Matthews coefficient may be 1.91 Å^3 Da^{-1} and the solvent content 35.60% assuming the presence of three molecules in the asymmetric unit. To determine the location of noncrystallographic symmetry, a self-rotation function and native Patterson analysis were performed with the programs *POLARRFN* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) and *CNS* (Brünger *et al.*, 1998), respectively. The results of these studies were not conclusive in determining noncrystallographic twofold or threefold symmetry. Hence, the actual noncrystallographic symmetry will need to be determined during subsequent structure-solution steps.

Previous biochemical studies indicated that pigeon pea urease exists as a hexamer of identical 90 kDa subunits, like jack-bean urease (Das *et al.*, 2002). In the present crystal, since the asymmetric unit contains a dimer or a trimer, the assumption is that the biologically active hexamer will be generated by a combination of crystallographic and noncrystallographic symmetry.

The complete amino-acid sequence of pigeon pea urease has yet to be determined. However, previous studies indicated that the amino-acid compositions of jack bean and pigeon pea ureases are highly similar and that the N-terminal 20 amino acids are identical in these two ureases (Das *et al.*, 2002). Currently, we are working on a molecular-replacement solution using a homology model of jack bean urease derived from the crystal structure of *Helicobacter pylori* urease (PDB code 1e9z). The sequence of jack bean urease is 54.6% identical to that of *H. pylori* urease.

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