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Purification, crystallization and preliminary X-ray analysis of urease from jack bean (*Canavalia ensiformis*)

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Plant urease is a seed protein that is common in most legumes. It is also common in many bacteria and fungi and several species of yeast. Urease allows organisms to use exogenous and internally generated urea as a nitrogen source by catalyzing the hydrolysis of urea to ammonia and carbon dioxide. Urease from jack bean meal was purified to electrophoretic homogeneity using a series of steps involving acetone precipitation and size-exclusion and ion-exchange chromatography. The jack bean urease was crystallized and the resulting crystals diffracted to 2.05 Å resolution using synchrotron radiation. The crystals belonged to the hexagonal space group $P6_322$, with unit-cell parameters $a = b = 138.57$, $c = 198.36$ 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 uncatalyzed reaction (Andrews *et al.*, 1984; Mobley *et al.*, 1995). Urease is produced by bacteria, fungi, yeast and plants, where it catalyses urea degradation to supply these organisms with a source of nitrogen for growth (Mobley & Hausinger, 1989). While fungal and plant ureases are homohexameric proteins consisting of 90 kDa subunits, bacterial ureases are multimers of two-subunit or three-subunit complexes. Plant, fungal and bacterial ureases exhibit high sequence similarity, suggesting that they have conserved secondary structures and similar catalytic mechanisms (Mobley *et al.*, 1995; Sirko & Brodzik, 2000). Despite their closely related amino-acid sequences, the biological activities of ureases, such as insecticidal activity, lethal activity towards mice and platelet-aggregating activity, differ significantly among plant and bacterial ureases (Follmer, Real-Guerra *et al.*, 2004).

Jack bean (*Canavalia ensiformis*) urease (JBU) was the first enzyme to be crystallized, a feat that was accomplished by James B. Sumner in 1926 and for which he was awarded the Nobel Prize in chemistry in 1946. Although JBU was the first protein to be crystallized, its structure has yet to be determined. JBU was also the first enzyme that was shown to contain nickel at its active site (Dixon *et al.*, 1975). Bacterial ureases serve as a virulence factor in human and animal infections of the urinary and gastrointestinal tracts, being involved in kidney-stone formation, catheter encrustation, pyelonephritis, ammonia encephalopathy and hepatic coma (Mobley & Hausinger, 1989; Mobley *et al.*, 1995).

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 and in seedling nitrogen metabolism (Zonia *et al.*, 1995). Plant ureases have also been found to participate in defence against insect predation (Polacco & Holland, 1994). The insecticidal properties of plant ureases were first described for canatoxin, an isoform of JBU (Carlini *et al.*, 1997), and subsequently for JBU and soybean seed-specific urease (Follmer, Real-Guerra *et al.*, 2004).

Table 1
Data-collection statistics for the JBU crystals.

Values in parentheses are for the highest resolution shell (2.12–2.05 Å).

Wavelength (Å)	1.0
Space group	$P6_322$
Unit-cell parameters (Å, °)	$a = b = 138.57$, $c = 198.36$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution range (Å)	30–2.05
R_{merge}† (%)	11.19 (32.73)
Total No. of reflections	1193744
No. of unique reflections	72810
Mean $I/\sigma(I)$	4.3 (1.2)
Completeness (%)	99.9 (99.9)
Multiplicity	16.8 (13.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of a reflection with Miller indices hkl and $\langle I(hkl)\rangle$ is the average intensity of that reflection and all of its symmetry-related reflections.

To date, knowledge of the structural and functional aspects of bacterial ureases has surpassed that of those of plants. X-ray crystal structures of the enzymes from the microbes *Klebsiella aerogenes* (PDB code 1fwj), *Bacillus pasteurii* (PDB code 4ubp) and *Helicobacter pylori* (PDB code 1e9z) have been determined and analyzed (Jabri *et al.*, 1995; Benini *et al.*, 1999; Ha *et al.*, 2001). Among the plant ureases, only the crystallization and preliminary X-ray analysis of JBU at resolutions of 3.5 and 3.3 Å have been reported using synchrotron radiation (Jabri *et al.*, 1992; Sheridan *et al.*, 2002). In order to better understand the biological properties of plant ureases, we initiated structural studies on some of them. We have previously reported the purification, crystallization and preliminary X-ray analysis of pigeon pea urease (Balasubramanian & Ponnuraj, 2008) at a resolution of 2.5 Å using an in-house X-ray source. Here, we report the preliminary structural analysis of JBU at 2.05 Å resolution using synchrotron radiation.

2. Experimental

2.1. Protein purification

The protein was purified from jack bean meal (Sigma) using a method based on that described by Blakeley *et al.* (1969). 175 ml deionized water was heated to 313 K and 75 ml acetone was added; the mixture was then immediately removed from the heat. 50 g jack bean meal was then added while stirring and the mixture was stirred for 5 min. The solution was centrifuged at 277 K for 10 min at 10 000 rev min^{-1}. The supernatant was left at 277 K for 60 h and the solution was centrifuged at 277 K for 15 min at 10 000 rev min^{-1}. The supernatant was discarded and the pellet was resuspended in a buffer containing 50 m*M* Tris, 5 m*M* EDTA and 5 m*M* β-mercaptoethanol pH 7.5. The solution was centrifuged at 277 K for 30 min at 10 000 rev min^{-1}. The supernatant was concentrated and loaded onto a Sephacryl S-300 gel-filtration column (GE Healthcare Lifesciences) which was pre-equilibrated in the same buffer and the peak containing active urease fractions was collected and pooled. The sample was loaded onto a DEAE column equilibrated with the same buffer and gradient elution with sodium chloride (0–1 *M*) was performed. The fractions from the ion-exchange column that had urease activity were dialysed overnight against the same buffer and concentrated to a final concentration of 20 mg ml^{-1} using a Centriprep concentrator. The purified urease showed a single band at 90 kDa on a 10% SDS–PAGE (Fig. 1).

2.2. Crystallization

Crystallization experiments were carried out using the hanging-drop vapour-diffusion method at 294 K. Hampton Research screens were employed to screen the protein. The condition that yielded crystals was further optimized in order to obtain crystals that were suitable for X-ray diffraction studies. A drop containing 2 µl protein solution was mixed with 2 µl precipitant and equilibrated against 1 ml reservoir solution consisting of 1.6 *M* ammonium phosphate dibasic, 100 m*M* Tris pH 8.8 and 10% ethylene glycol. Diffraction-quality crystals of dimensions 0.25 × 0.05 × 0.04 mm were obtained in 2 d (Fig. 2).

2.3. X-ray analysis

Crystals suitable for X-ray diffraction were transferred into a cryoprotectant solution, which had the same composition as the reservoir solution with the addition of 30% ethylene glycol, for a few seconds. Crystals were mounted on cryoloops (Hampton Research) and flash-cooled in a nitrogen-gas stream at 100 K. Initial diffraction experiments were carried out using an in-house MAR345 image-plate detector and a Bruker Microstar copper rotating-anode generator operated at 60 mA and 45 kV. The crystals diffracted to 3.0 Å resolution with an exposure time of 30 min per frame and the diffraction decayed gradually. Subsequent diffraction experiments were carried out at the Elettra synchrotron-radiation laboratory in Italy. A native data set was collected from one crystal on the XRD1 beamline. A total of 140 frames were collected with an oscillation step of 1° and an exposure of 20 s per frame. Diffraction images were recorded on a

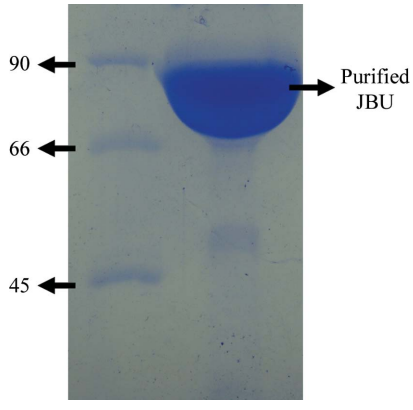

Figure 1
SDS–PAGE of purified jack bean urease. Lane 1 contains the standard proteins jack bean urease (90 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa); lane 2 contains purified jack bean urease.

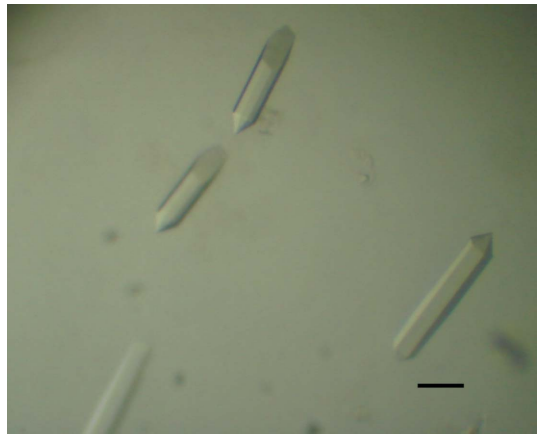

Figure 2
Crystals of jack bean urease. The scale bar represents 0.1 mm.

MAR165 CCD detector to a resolution of 2.05 Å and 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

The first crystals of jack bean urease were obtained by Sumner (1926) from water/acetone extraction of jack bean meal allowed to stand at 277 K overnight. In 1992, Jabri and coworkers obtained diffraction-quality crystals of JBU; these crystals diffracted to 3.5 Å resolution and belonged to the complex space group $F4_132$ with a very large unit-cell length of $a = 364$ Å (Jabri *et al.*, 1992). In 2002, crystallization of JBU was again reported (Sheridan *et al.*, 2002); in this case, antibodies were used to aid the crystallization of JBU and to obtain better diffraction. However, the crystals obtained only diffracted to 3.3 Å resolution and the diffraction decayed rapidly. The final data set was derived from the five best crystals and had a completeness of only 77.5%.

Follmer (2008) reported that the great heterogeneity, insolubility and high polydispersity of JBU (Fishbein *et al.*, 1969; Fishbein & Nagarajan, 1971; Follmer, Pereira *et al.*, 2004) in solution were possible reasons for the failure to determine its structure by X-ray crystallography. We employed the same purification protocol as previously used by Sheridan *et al.* (2002) with modifications of the buffer conditions. In addition, one extra purification step using a DEAE ion-exchange column was introduced into the purification protocol. The final purified protein that we obtained showed a single band on 7% native PAGE that corresponds to a hexamer, which is in contrast to the previously reported purification (Sheridan *et al.*, 2002) in which it existed as monomers, trimers and hexamers; this may be the reason for our success in crystallization. The crystallization condition that we have used also differs from the previously reported condition and results in good diffraction-quality crystals.

In this crystallization communication, we report the crystallization of JBU in a hexagonal ($P6_322$) form, with unit-cell parameters $a = b = 138.57$, $c = 198.36$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. These data were collected using one crystal at 100 K with a completeness of 99.9% to a resolution of 2.05 Å. Given the molecular weight of the protein (90 kDa) and assuming the presence of one molecule in the asymmetric unit, the resultant Matthews coefficient (Matthews, 1968) is 3.05 $Å^3\ Da^{-1}$, corresponding to a solvent content of 59.67%. If two molecules were present in the asymmetric unit the solvent content would be only 18%.

A preliminary solution of the structure was obtained by molecular replacement with a homology model of JBU derived from *H. pylori* urease (PDB code 1e9z) using the *AMoRe* program (Navaza, 1994). Rotation and translation searches were carried out in the resolution range 20–4 Å. This resulted in a single clear solution with a correlation coefficient of 48.2% and an R value of 45.6%. Further refinement of the structure is in progress.

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