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Crystallization and preliminary X-ray analysis of cryptolepain, a novel glycosylated serine protease from *Cryptolepis buchanani*

Monu Pande,[a] Vikash K. Dubey[b] and Medicherla V. Jagannadham[a]*

[a]Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India, and [b]Department of Biotechnology, Indian Institute of Technology, Guwahati 781039, Assam, India

Correspondence e-mail: vdubey@iitg.ernet.in

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Cryptolepain is a stable glycosylated novel serine protease purified from the latex of the medicinally important plant *Cryptolepis buchanani*. The molecular weight of the enzyme is 50.5 kDa, as determined by mass spectrometry. The sequence of the first 15 N-terminal resides of the protease showed little homology with those of other plant serine proteases, suggesting it to be structurally unique. Thus, it is of interest to solve the structure of the enzyme in order to better understand its structure–function relationship. X-ray diffraction data were collected from a crystal of cryptolepain and processed to 2.25 Å with acceptable statistics. The crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 81.78, b = 108.15, c = 119.86 Å. The Matthews coefficient was 2.62 Å^3 Da^{-1} with one molecule in the asymmetric unit. The solvent content was found to be 53%. Structure determination of the enzyme is under way.

1. Introduction

Serine endopeptidases and exopeptidases are of extremely widespread occurrence and diverse function. Many distinct families of serine proteases exist and they have been grouped into six clans (Rawlings & Barrett, 1994; Barrett & Rawlings, 1995), of which the two largest are the (chymo)trypsin-like and subtilisin-like clans. Although serine proteases from plant sources are rare, in recent years many serine proteases have been isolated and purified from various plant species, in which they occur in distinct parts, including the seeds, the latex and the fruits (Antão & Malcata, 2005). Very little information about the structural aspects of plant serine proteases is available. Most of the available data relating to plant serine proteases are focused on cucumisin-like proteases, a family of endopeptidases found in Cucurbitaceae (Kaneda & Tominaga, 1975, 1977; Kaneda *et al.*, 1986; Arima *et al.*, 2000).

The involvement of plant serine proteases in many physiological processes has been well documented (Palma *et al.*, 2002). These include microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation, senescence and protein degradation/processing. Plant proteases are well known for their industrial applications as a consequence of their broad substrate specificity and their activity over a wide range of pH and temperature. Quantitatively, more than half of commercially used industrial enzymes are proteases (Mantel *et al.*, 1985). In the food industry, proteases are indispensable for processes such as the tenderization of meat, brewing, cheese elaboration and bread manufacture (Caffini *et al.*, 1988).

Cryptolepain is a novel serine protease from the latex of the medicinally important plant *Cryptolepis buchanani*. The plant belongs to the family Apocynaceae (formerly Asclepidaceae) and is commonly distributed throughout India, especially in hot deciduous forests. It is well known in Ayurveda for its tremendous medicinal significance. Various parts of the plant are used as an antidiarrhoeal, antibacterial, anti-ulcerative and anti-inflammatory, as a blood purifier and in curing rickets in children (BhavPrakash, 1969). The ethanolic extract of the plant has potent immunostimulant activity

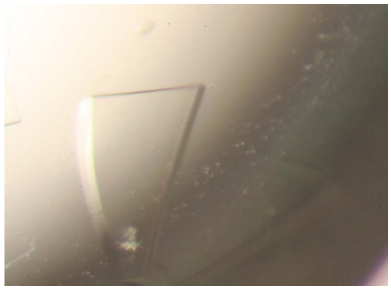


Table 1
Amino-terminal sequence of cryptolepain compared with those of other serine proteases.

Letters in italic represent similar amino-acid residues.

Enzyme	Amino-terminal sequence (first 15 residues)	Reference
Cryptolepain	*MEGASFGAFLSSTAR*	
Bamboo protease	TTRTPSFLR*L*SAVG*R*	Arima *et al.* (2000)
Pyrolysin	*MYN*STWVINALQFIQ	Shimamoto *et al.* (2001)
Cucumisin	TTRSWDFLGFPLTVP	Yagamata *et al.* (1994)
Tomato P69 *A*	TTHT*S*SFLG*L*QQNMG	Tornero *et al.* (1996)
Arabdopsis ara12	TTRTPLFLG*L*DEHTA	EMBL accession No. X85974
Alnus ag12	TTHTPRFLS*L*NPTGG	Ribeiro *et al.* (1995)

and can be used as therapeutic agent in immune-compromised patients (Kaul *et al.*, 2003). The role of the newly purified protease in the abovementioned activities cannot be ruled out.

The latex showed high caseinolytic activity, which is indicative of the presence of protease(s). A serine protease was purified to homogeneity by ammonium sulfate fractionation and cation-exchange chromatography. The N-terminal sequence of cryptolepain is unique and shows only little homology to other known serine proteases (Pande *et al.*, 2007). The amino-terminal sequence of cryptolepain has been compared with those of other known serine proteases (see Table 1). In the absence of significant sequence resemblance to other serine proteases, determination of the three-dimensional structure often provides an efficient approach to deriving strong hypotheses about protein structure. Here, we report the crystallization and preliminary X-ray crystallographic data of cryptolepain.

2. Materials and methods

2.1. Purification

The enzyme was purified following the protocol of Pande *et al.* (2007). Cryptolepain was purified from the latex of the medicinally important plant *C. buchanani*. The latex was collected in 10 m*M* phosphate buffer pH 7. After freezing at 253 K for 2–3 d, it was thawed and centrifuged at 10 000 rev min^{-1} for 10 min. The supernatant thus obtained was subjected to 50% ammonium sulfate precipitation. The supernatant from 50% ammonium sulfate fractionation was extensively dialyzed against 10 m*M* acetate buffer pH 4.5 and loaded onto CM-Sepharose cation-exchanger pre-equilibrated with 10 m*M* acetate buffer pH 4.5, followed by buffer wash with same buffer until no protein could be detected in the eluant. The bound proteins were eluted with a linear gradient of 0–0.6 *M* NaCl at a flow rate of 3 ml min^{-1}. The proteins were resolved into three peaks. The middle peak was found to be homogeneous and highly active. Prior to crystallization setup, the purity was further confirmed by the observation of a single band on MALDI–TOF (Fig. 1*a*) and SDS–PAGE (Fig. 1*b*).

2.2. MALDI–TOF of cryptolepain

The accurate molecular weight of cryptolepain was determined by MALDI–TOF using a Simadzu Axima CFR-Plus MALDI–TOF mass spectrometer in linear mode. The sample was prepared by mixing 7 m*M* protein with saturated sinapinic acid solution with 0.1% trifluoroacetic acid in 2:1 water:acetonitrile. 1 µl of the sample was spotted onto the sample plate and then dried at room temperature. MALDI–TOF-grade BSA was used for calibration of the instrument.

Table 2
Crystallographic data-collection and processing statistics of cryptolepain.

Values in parentheses are for the highest resolution shell.

Crystal data	
Space group	$C222_1$
Crystal system	Orthorhombic
Unit-cell parameters	a = 81.78, b = 108.153, c = 119.867
Maximum resolution (Å)	2.25
Mosaicity (°)	0.489
Molecules per ASU	1
Matthews coefficient (Å^3 Da^{-1})	2.62
Data collection and processing	
Total reflections	165072
Unique reflections	25533
Redundancy	6.5
$I/\sigma(I)$	17.33 (4.08)
R_{merge} (%)	10.7 (38.0)
Completeness (%)	100 (99.9)

2.3. Crystallization of cryptolepain

Purified protein was dialyzed against 10 m*M* sodium phosphate, 100 m*M* NaCl pH 7.0 and concentrated to 9 mg ml^{-1}. The protein solution (3 µl, 9 mg ml^{-1}) was mixed with an equal volume of reservoir solution for screening for crystallization conditions using the hanging-drop vapour-diffusion method. Initial crystallization conditions were obtained using Hampton Research Crystal Screen kits (Hampton Research, California, USA) and then further optimized to obtain diffraction-quality crystals. Cryptolepain crystals

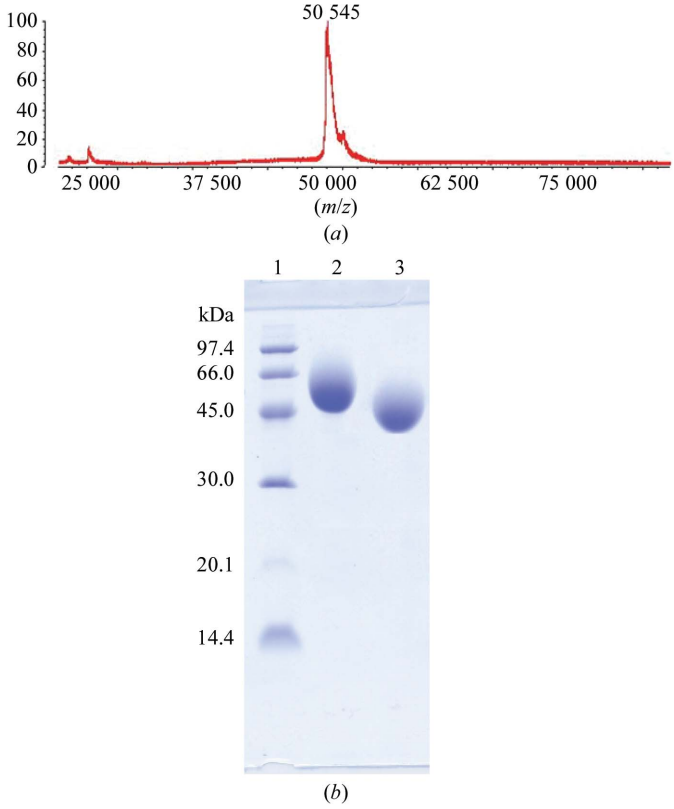


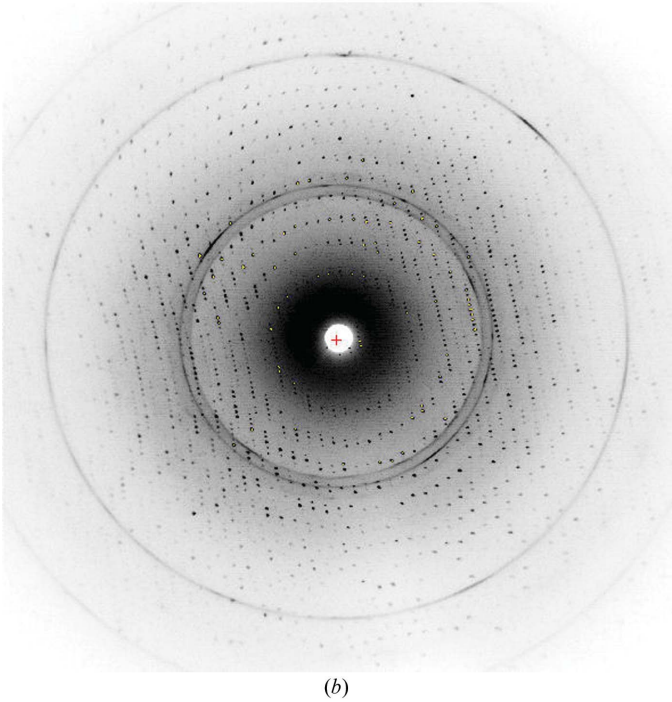
Figure 1
Prior to crystallization, the purity of the enzyme was checked by MALDI–TOF and SDS–PAGE. (*a*) Mass spectrometry of cryptolepain: MALDI–TOF-standard BSA was used for calibration of the instrument and data were collected in linear mode. (*b*) 12.5% SDS–PAGE of purified protease. Lanes 1, 2 and 3 contain molecular-weight markers and cryptolepain under nonreduced and reduced conditions, respectively. The molecular-weight markers used were phosphorylase b (97.40 kDa), BSA (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), soybean tyrosine inhibitor (20.1 kDa) and lactalbumin (14.4 kDa).

grew in two weeks at 293 K with 1 ml reservoir solution containing 1 *M* ammonium sulfate and 12% PEG 6000.

2.4. Data collection and data processing

Diffraction data were collected using a Rigaku RU-H2R copper rotating-anode (λ = 1.54 Å) X-ray generator (Rigaku MSC, The Woodlands, TX, USA) coupled to a Osmic Confocal Max-Flux Optics system (Osmic, Auburn Hills, MI, USA) and an R-AXIS IIc image plate (Rigaku MSC, The Woodlands, TX, USA). The rotating-anode generator was operated at 50 kV and 100 mA. The crystal was mounted using Hampton Research nylon mounted cryo-turns and frozen in a stream of gaseous nitrogen at 100 K. A crystal-to-detector distance of 120 mm and a 15 min exposure per frame were used to collect a total of 180 frames at 1° oscillation. Diffraction data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* (Otwinowski, 1993; Otwinowski & Minor, 1997).

(*a*)



(*b*)

Figure 2
(*a*) Crystal and (*b*) diffraction pattern of the serine protease cryptolepain from *C. buchanani*.

3. Results and discussions

3.1. Purification, assessment of the purity and crystallization

The enzyme cryptolepain was purified to high purity. To avoid any complications arising from autodigestion of the enzyme, the purity of the enzyme was checked prior to crystallization setup. The enzyme was found to be highly homogeneous and suitable for crystallization.

3.2. Preliminary crystallographic analysis

Cryptolepain crystals (Fig. 2*a*) belong to an orthorhombic space group, with unit-cell parameters a = 81.78, b = 108.153, c = 119.867 Å. The systematic absences (00l except for $l = 2n$) indicate that the space group is $C222_1$. The crystal diffracted to 2.25 Å resolution and the data statistics are summarized in Table 2. Assuming the presence of one molecule in the asymmetric unit, the Matthews coefficient (V_M value) is 2.62 Å^3 Da^{-1}, which falls into the normally observed range (Matthews, 1968) and corresponds to a solvent content of 53%. On the other hand, with two molecules per asymmetric unit the Matthews coefficient value would be 1.31 Å^3 Da^{-1}, which is outside the normal range.

Molecular-replacement calculations have been performed for cryptolepain and structure solution is in progress. Initially, serine proteases are being tested for a suitable search model for molecular replacement; concurrently, a search for heavy-atom derivatives is also in progress.

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