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Purification, crystallization and preliminary X-ray crystallographic analysis of rice Bowman–Birk inhibitor from *Oryza sativa*

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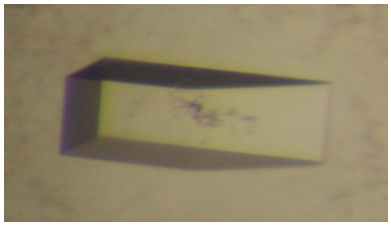

Bowman–Birk inhibitors (BBIs) are cysteine-rich proteins with inhibitory activity against proteases that are widely distributed in monocot and dicot species. The expression of rice BBI from *Oryza sativa* is up-regulated and induced by pathogens or insects during germination of rice seeds. The rice BBI (RBTI) of molecular weight 15 kDa has been crystallized using the hanging-drop vapour-diffusion method. According to the diffraction of rice BBI crystals at a resolution of 2.07 Å, the unit cell belongs to space group $P2_12_12_1$, with unit-cell parameters $a = 74.37$, $b = 96.69$, $c = 100.36$ Å. Preliminary analysis indicates four BBI molecules in an asymmetric unit, with a solvent content of 58.29%.

1. Introduction

In plants, two major serine protease inhibitors of Kunitz and Bowman–Birk (BBI) type (Ryan, 1990; Mosolov & Valueva, 2005) that inhibit exogenous trypsin and chymotrypsin are generally expressed in developing seeds and other organs and are thought to play an important role in the plant defence system (Laskowski & Kato, 1980; Ryan, 1990; Doares *et al.*, 1995; Sivasankar *et al.*, 2000; Kennedy, 1998). BBIs of certain types also display anticarcinogenic or anti-inflammatory activities *in vitro* (Correa, 1981; Troll & Kennedy, 1989; Kennedy *et al.*, 1993). BBIs are small cysteine-rich proteins with molecular weights ranging from 8 to 20 kDa which are widespread both in monocot and dicot species and have highly conserved cysteine motifs (Qi *et al.*, 2005). According to an extensive investigation of the inhibitory activity of BBI against proteases, the BBIs are able to inhibit two molecules of protease bound to separate reactive sites and the relative affinity becomes altered when one site is already occupied. Steric hindrance between two competing proteases has been suggested to play a role in the modulation of this inhibition potency (Tur Sinal *et al.*, 1972; Song *et al.*, 1999).

Of the numerous BBIs that have been found, only one protein has been purified to homogeneity from rice (*Oryza sativa*) bran and is referred to as RBTI (rice bran trypsin inhibitor; Tashiro & Maki, 1978), with a molecular weight of about 15 kDa. Members of the BBI gene family display varied expression patterns during the germination of rice seeds, with wounding also having an effect on the expression of rice BBI transcripts (Qu *et al.*, 2003). Moreover, the induction of rice BBI is regulated by light and is sensitive to phytohormones; such events have been suggested to be associated with a kinase-signalling cascade mechanism (Rakwal *et al.*, 2001). RBTI has long been recognized to differ characteristically from other BBIs in terms of amino-acid sequence: it comprises four domains and shows a duplicated structure of the Bowman–Birk type inhibitor. The extremely variable sequence in the P1′ domain and both the numerous cysteine residues and the location of disulfide bridges are the main structural distinctions compared with other BBIs (Tashiro *et al.*, 1987; Rakwal *et al.*, 2001). The novelty of the rice BBI results not only from its expression, which is induced by pathogens or insects, but also from its endogenous regulation by phytohormones, *e.g.* jasmonic acid and abscisic acid, and its activation mechanism that is coupled with kinase activity. Although the exact physiological role of the rice BBI is not fully understood, it seems likely to play a role in the plant's response to well defined stress factors (Rakwal *et al.*, 2001) and to infection with the pathogenic fungus blast disease *Pyricularia oryzae*

(Qu *et al.*, 2003). For these reasons, we have directly isolated the BBI from rice bran, consisting of 133 amino-acid residues, and established a successful large-scale protein-purification procedure for the purpose of structural studies. To further understand the biological significance of BBI in rice development, it is important and necessary to understand its detailed three-dimensional structure and functional relationships. Here, we report the protein purification, identification, crystallization and preliminary X-ray diffraction characterization of rice BBI.

2. Materials and methods

2.1. Protein purification and identification

RBTI was purified from rice bran (*O. sativa japonica*) as described by Tashiro & Maki (1978) using modified procedures. Briefly, rice bran (200 g) was defatted with hexane and extracted with 50 m*M* phosphate buffer pH 7.5 containing 4% NaCl and 1 m*M* PMSF at 296 K for 2 h. After centrifugation (10 000*g*, 30 min), solid ammonium sulfate was added to the supernatant to attain 85% saturation and the mixture was stirred at 277 K overnight. The precipitated proteins were collected by centrifugation (25 000*g*, 30 min) and then redissolved and dialyzed against 50 m*M* phosphate buffer pH 7.5 containing 0.15 *M* NaCl for 3 d at 277 K. After dialysis, the insoluble precipitants were removed by centrifugation (25 000*g*, 20 min) and the supernatant was collected and adjusted to pH 4.5 with HCl. The resulting soluble proteins were filtered using a 0.45 µm filter and applied onto a CM-Sepharose column (26 × 40 cm) equilibrated with 50 m*M* sodium acetate pH 4.5 containing 0.15 *M* NaCl. After elution with a linear gradient of NaCl (0.15–1.0 *M*), fractions containing trypsin-inhibiting activity were collected and applied onto a size-exclusion column (Superdex 75, 26 × 60 cm). RBTI was finally purified by semi-preparative reverse-phase HPLC (Cosmosil, 5C18-AR-300, 10 × 250 mm), eluted with a linear gradient of acetonitrile (10–60%) in aqueous TFA (0.1%). The yield of protein was approximately 100 mg; the purity of RBTI, as examined by 12% SDS–PAGE (Fig. 1) and analytical reverse-phase HPLC (Vydac, Protein and Peptide C18, 4.6 × 260 mm), was found to exceed 99% and the protein identity and molecular weight were determined by protein N-terminal sequencing and electrospray ionization–tandem mass spectrometry (ESI–tandem MS). The protein sample was then vacuumed to remove acetonitrile and dried by lyophilization.

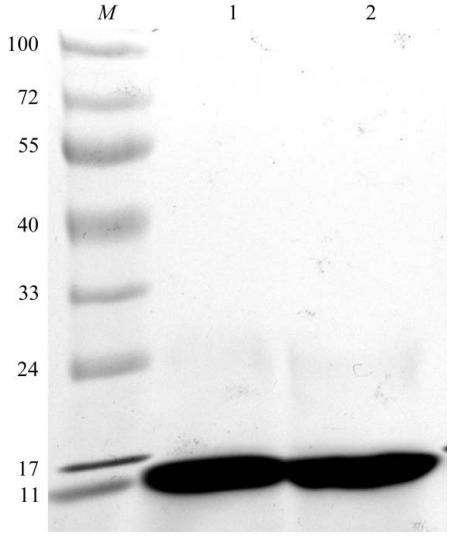

Figure 1
RBTI proteins collected from the final purification step (RP-HPLC) were analyzed by 12% SDS–PAGE. Lane *M*, molecular-weight markers in kDa; lane 1, 10 µg BBI, 4× sample buffer boiled at 373 K for 15 min; lane 2, 10 µg BBI, 4× sample buffer with 10 m*M* DTT boiled at 373 K for 15 min.

2.2. Crystallization

Prior to crystallization, the protein sample was redissolved to a concentration of 10 mg ml^{-1} in double-deionized water. Crystallization was performed by the hanging-drop vapour-diffusion method at 293 K. Small crystals were observed from a condition using PEG 6000 and Na_2HPO_4 within 1 d after initial screening using Crystal Sceeen kits (Hampton Research). This condition was further refined to produce larger RBTI crystals using 2 µl hanging drops containing equal volumes of protein solution (1 µl) and reservoir solution (1 µl) equilibrated against 200 µl reservoir resolution containing 21–25%(*w*/*v*) PEG 6000 and 0.2 *M* Na_2HPO_4 pH 9.1 (Fig. 2).

2.3. X-ray data collection and processing

The protein crystals were initially screened and characterized using synchrotron radiation at the Taiwan-contracted protein crystallographic beamline BL12B2 equipped with a CCD detector (Quantum-4R, ADSC) at SPring-8 in Japan. Data collection was completed at the SPXF beamline BL13B1 equipped with a CCD detector (Q315, ADSC) at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan. The crystal was transferred from a crystallization drop into 10 µl cryoprotectant solution containing 21%(*w*/*v*) PEG 6000, 15%(*v*/*v*) glycerol and 0.2 *M* Na_2HPO_4 pH 9.1 for a few seconds, mounted on a glass loop (0.2–0.3 mm; Hampton Research) and flash-cooled in liquid nitrogen at 100 K. For complete data collection, 200° rotations with 1.0° oscillation were measured using an X-ray wavelength of 1.00 Å with 15 s exposures and a crystal-to-detector distance of 200 mm at 110 K in a nitrogen stream using a cryosystem (X-Stream, Rigaku/MSC Inc.). All data were indexed, integrated and scaled using the *HKL*2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

SDS–PAGE of purified RBTI showed a single band under SDS-denaturating and reducing conditions corresponding to a molecular weight of about 15 kDa (Fig. 1). The molecular weight of RBTI determined by ESI–tandem MS is 15 057 Da. Protein crystals of rectangular shape appeared in 1 d and continued to grow to final dimensions of 0.05 × 0.05 × 0.2 mm within 3 d in an incubator at 293 K (Fig. 2). The protein crystals were sensitive to changes in

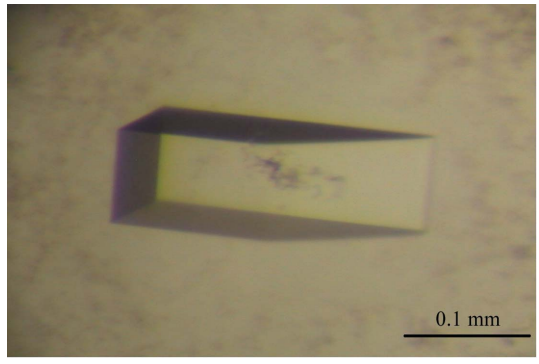

Figure 2
A single crystal of rice RBTI grown by the hanging-drop method.

Table 1
Crystal diffraction statistics of RBTI.

Values in parentheses are for the highest resolution shell (2.15–2.07 Å).

Wavelength (Å)	1.00
Temperature (K)	100
Resolution range (Å)	30.0–2.07
Space group	$P2_12_12_1$
Unique reflections	44886
Completeness (%)	99.8 (99.9)
$\langle I/\sigma(I)\rangle$	30.9 (7.6)
Average redundancy	8.0
R_{sym}† (%)	6.0 (32.2)
Mosaicity	0.285
Unit-cell parameters (Å)	a = 74.37, b = 96.69, c = 100.36
No. of molecules per ASU	4
V_M (Å^3 Da^{-1})	3.0
Solvent content (%)	58.29

† $R_{sym} = \sum_h \sum_i [|I_i(h) - \langle I(h)\rangle|/\sum_h \sum_i I_i(h)]$, where I_i is the ith measurement and $\langle I(h)\rangle$ is the weighted mean of all measurements of $I(h)$.

precipitant concentration, which increased crystal mosaicity, especially during transfers to the cryoprotectant solution containing 15%(*v*/*v*) glycerol. Good-quality crystals were identified through careful screening and selection as they frequently exhibited fairly high mosaicity (>1°). Radiation damage was observed after protracted exposure during data collection, which caused a decrease in $I/\sigma(I)$ and an increase in R_{sym}. Analysis of the diffraction pattern indicates that these crystals exhibit orthorhombic symmetry and systematic absences indicated the space group to be $P2_12_12_1$. The self-rotation function of the crystals using data in the 15–4 Å resolution range and the program *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) showed two non-crystallographic twofold-symmetry (NCS) axes at polar angles (θ, φ, χ) = (29.2, 90, 180°) and (60.8, 90, 180°). Assuming the presence of four RBTI molecules per asymmetric unit, the Matthews coefficient is estimated to be 3.0 Å^3 Da^{-1}, corresponding to a solvent content of 58.29% (Matthews, 1968), which is within the normal range for protein crystals. Details of the data statistics are given in Table 1.

Initial attempts to solve the crystal structure of RBTI were performed using the molecular-replacement method with the structures of BBI from barley (46% sequence identity; PDB code 1c2a; Song *et al.*, 1999), soybean (29% sequence identity; PDB code 1k9b; Voss *et al.*, 1996) and pea seeds (21% sequence identity; PDB code 1pbi; Li de la Sierra *et al.*, 1999) as search models. None of these attempts provided a clear solution. The preparation of heavy-atom derivatives for MAD and sulfur-SAD experiments is currently in progress for phase determination. The completion of the structure determination will be described in a separate paper.

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