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Purification, crystallization and preliminary X-ray crystallographic analysis of rice bifunctional α-amylase/subtilisin inhibitor from *Oryza sativa*

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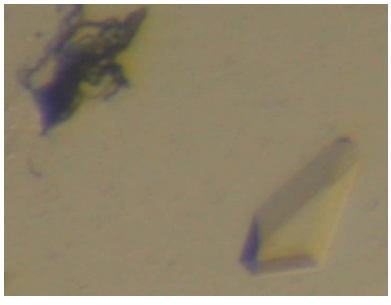

Rice bifunctional α-amylase/subtilisin inhibitor (RASI) can inhibit both α-amylase from larvae of the red flour beetle (*Tribolium castaneum*) and subtilisin from *Bacillus subtilis*. The synthesis of RASI is up-regulated during the late milky stage in developing seeds. The 8.9 kDa molecular-weight RASI from rice has been crystallized using the hanging-drop vapour-diffusion method. According to 1.81 Å resolution X-ray diffraction data from rice RASI crystals, the crystal belongs to space group $P2_12_12$, with unit-cell parameters a = 79.99, b = 62.95, c = 66.70 Å. Preliminary analysis indicates two RASI molecules in an asymmetric unit with a solvent content of 44%.

1. Introduction

It has been suggested that proteins from plants and microorganisms that inhibit α-amylases exert their natural roles in the control of endogenous α-amylase activity or are involved in plant defence mechanisms against pathogens and pests (Garcia-Olmedo *et al.*, 1987; Whitaker *et al.*, 1988; Franco *et al.*, 2002; Svensson *et al.*, 2004). Inhibitors of certain types have been reported to be anti-nutritional factors for humans (Garcia-Olmedo *et al.*, 1987; Ho *et al.*, 1994; Yoshikawa *et al.*, 1999) and hence have received much attention for potential applications in therapies against obesity and certain diseases (Garcia-Olmedo *et al.*, 1987; Layer *et al.*, 1985). However, no definitive functions for these proteins has yet been identified in plants. According to sequence and three-dimensional structure similarities, these inhibitors can be classified into seven types: microbial, knottin-like, γ-thionin-like, CM proteins, Kunitz-type, thaumatin-like and legume lectin-like inhibitors (for a review, see Svensson *et al.*, 2004). An inhibitor of one of these types, called α-amylase/subtilisin inhibitor, was discovered in barley (Yoshikawa *et al.*, 1976). It possesses the ability to inhibit subtilisin (*Bacillus subtilis* serine protease) and has sequence similarity to the Kunitz soybean trypsin inhibitor family (Onesti *et al.*, 1991); it was also identified in a complex with an endogenous α-amylase (Weselake *et al.*, 1983; Mundy *et al.*, 1983). Rice, like most other cereals, contains several types of enzyme inhibitors (Tashiro *et al.*, 1987; Abe *et al.*, 1987; Feng *et al.*, 1991; Yu *et al.*, 1988), including a 20 kDa molecular-weight rice bifunctional α-amylase/subtilisin inhibitor (RASI) that can inhibit both α-amylase from the larvae of the red flour beetle (*Tribolium castaneum*) and subtilisin from *B. subtilis* (Ohtsubo & Richardson, 1992). Yamagata and coworkers performed a detailed characterization of RASI and suggested that the expression of RASI occurred during the late milky stage in developing seeds (Yamagata *et al.*, 1998). RASI was shown to be located/accumulated in the outermost part of the rice grain and the subcellular site of aleurone cells in the form of aleurone particles. This is the opposite of the distribution of BASI (barley α-amylase/subtilisin inhibitor from barley seed), which accumulates in the starchy endosperm (Leah & Mundy, 1989).

BASI is the best characterized inhibitor of the α-amylase/subtilisin family (Mundy *et al.*, 1983; Weselake *et al.*, 1983). It contains a single chain of 181 amino acids with two disulfide bridges (Svendsen *et al.*, 1986) and shows 58% sequence identity to RASI (Ohtsubo & Richardson, 1992). BASI is highly specific for an endogenous high-pI

α-amylase isoenzyme 2 (AMY2), but shows no effect on isoenzyme 1 (AMY1) that shares 80% sequence identity to AMY2 (Mundy *et al.*, 1983). The K_i for BASI acting on barley AMY2 was determined to be ∼0.1 n*M* (Abe *et al.*, 1993; Bonsager *et al.*, 2003), whereas WASI (wheat α-amylase/subtilisin inhibitor, 92% sequence identity to BASI; Mundy *et al.*, 1984) was less potent. RASI showed even less activity against endogenous plant α-amylase (Yamagata *et al.*, 1998). A rapid and tight simple two-step mechanism was proposed for the binding of BASI to AMY2 (Sidenius *et al.*, 1995), which differs from the mode of action of α-AI1, AAI and RBI (lectin-type, knottin-type and cereal-type α-amylase inhibitors, respectively) as the inhibition process of these proteins involves the insertion of inhibition loops into the α-amylase active site (Franco *et al.*, 2002), while BASI does not interact directly with any catalytic acidic residues of the enzyme (Valle *et al.*, 1998). Further properties of BASI have been reviewed by Nielsen *et al.* (2004).

Details of the function of RASI and the related BASI and WASI in plant seeds remain unclear. RASI shows a sequence, enzymatic and physicochemical properties and physiological expression patterns that differ from homologous proteins from other cereals. Here, we describe the successful purification, crystallization and crystallographic characterization of 176-amino-acid RASI, which is the first reported α-amylase/subtilisin inhibitor from rice seed. The objective of our present work is to provide an alternative structural basis for and ultimately a functional illustration of α-amylase–inhibitor interactions in rice plants.

2. Materials and methods

2.1. Protein purification and identification

RASI was purified from rice bran (*Oryza sativa japonica*) as described by Ohtsubo & Richardson (1992) with some modifications. Briefly, rice bran (200 g) was defatted with hexane and extracted with 50 m*M* phosphate buffer pH 7.5 containing 0.68 *M* 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 continually 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 using HCl. The resulting soluble proteins were filtered with a 0.45 µm filter and applied onto a column (CM-Sepharose, 26 × 40 cm) equilibrated with 50 m*M* sodium acetate pH 4.5 containing 0.15 *M* NaCl. After elution with a linear gradient (0.15–1.0 *M*) of NaCl, the fractions containing α-amylase-inhibiting activity were collected and applied onto a size-exclusion column (Superdex 75, 26 × 60 cm). The rice bran RASI 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 0.1% aqueous TFA. The yield of protein was approximately 30 mg. The purity of RASI, as examined by 12.5% SDS–PAGE (Fig. 1) and analytical reverse-phase HPLC (Vydac, Protein and Peptide C18, 4.6 × 260 mm), was found to exceed 99%; the protein identity was determined by protein N-terminal sequencing. The protein sample was then vacuumed to remove acetonitrile and dried by lyophilization.

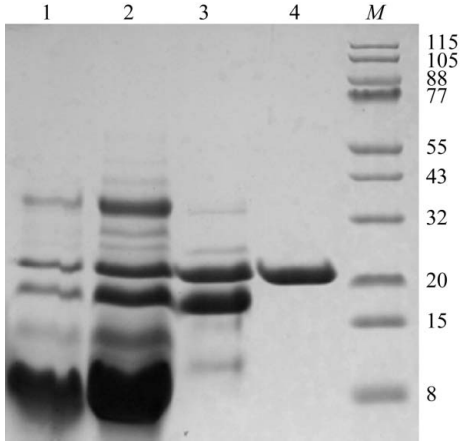

Figure 1
Coomassie-blue stained 12.5% tricine SDS–PAGE of pools from RASI purification. Purification fractions containing RASI activity were collected at each step. All samples were boiled at 373 K for 10 min in 4× sample buffer with 10 m*M* DTT. Lane 1, pool from soluble crude extracts of rice bran in 50 m*M* sodium acetate pH 4.5; lane 2, pool from CM-Sepharose column; lane 3, pool from Superdex 75 column; lane 4, pool from RP-HPLC; lane *M*, molecular-weight markers in kDa.

2.2. Crystallization

Prior to crystallization trials, the purified protein sample was redissolved at a concentration of 10 mg ml^{-1} in double-deionized water. Crystallization was performed using VDX48 plates (Hampton Research) by the hanging-drop vapour-diffusion method at 291 K. Small crystals were obtained from a condition consisting of 30%(*w*/*v*) PEG 8000 and 0.2 *M* ammonium sulfate (Crystal Screen kit condition No. 30; Hampton Research) within 5 d of setup. This condition was refined further without ammonium sulfate to produce larger RASI crystals using 2 µl hanging drops containing 1 µl protein solution and 1 µl reservoir solution equilibrated against 200 µl reservoir resolution containing 20%(*w*/*v*) PEG 8000 and 0.2 *M* Na_2HPO_4 buffer pH 8.5. Crystals of good diffraction quality were used for data collection (Fig. 2).

2.3. X-ray data collection and processing

The protein crystals were initially tested and characterized with the use of 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 5 µl cryoprotectant solution containing 20%(*w*/*v*) PEG 8000, 20%(*w*/*v*) glycerol and 0.2 *M* Na_2HPO_4 buffer pH 8.5 for a few seconds, mounted on a glass loop (0.1–0.2 mm, Hampton Research) and flash-cooled in liquid nitrogen at 100 K. For

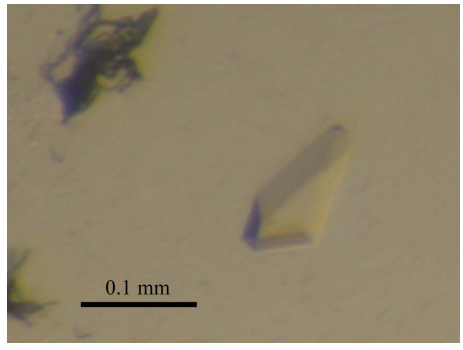

Figure 2
A single crystal of rice RASI grown by the hanging-drop method.

Table 1
Crystal diffraction statistics of RASI.

Values in parentheses are for the highest resolution shell (1.87–1.81 Å).

Wavelength (Å)	1.00
Temperature (K)	100
Resolution range (Å)	30.0–1.81
Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 79.99, b = 62.95, c = 66.70
Unique reflections	31303
Completeness (%)	99.5 (99.9)
$\langle I/\sigma(I)\rangle$	12.8 (5.7)
Average redundancy	4.7
R_{sym}† (%)	6.0 (34.1)
Mosaicity (°)	0.534
No. of molecules in ASU	2
Matthews coefficient (Å^3 Da^{-1})	2.24
Solvent content (%)	44.05

† $R_{sym} = \sum_{\mathbf{h}}\sum_l |I_{\mathbf{h}l} - \langle I_{\mathbf{h}}\rangle| / \sum_{\mathbf{h}}\sum_l \langle I_{\mathbf{h}}\rangle$, where $I_{\mathbf{h}l}$ is the lth observation of reflection **h** and $\langle I_{\mathbf{h}}\rangle$ is the weighted average intensity for all observations l of reflection **h**.

complete data collection, a 125° rotation was measured with 0.25° oscillations using an X-ray wavelength of 1.00 Å, an exposure duration of 15 s and a crystal-to-detector distance of 200 mm at 110 K in a nitrogen stream using a cryo-system (X-Stream, Rigaku/MSC). All data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Under SDS denaturating and reducing conditions, SDS–PAGE showed a single band corresponding to a molecular weight of about 20 000 Da (Fig. 1), in agreement with the molecular weight of RASI determined by ESI–tandem MS of 18 953 Da. Protein crystals of rectangular shape appeared after 5 d and continued to grow to final dimensions of 0.07 × 0.05 × 0.03 mm after two weeks in an incubator at 291 K (Fig. 2). The protein crystals were sensitive to change in precipitant concentration, which increased the mosaicity during transfer to the cryoprotectant solution consisting of crystallization liquid containing 20%(*w*/*v*) glycerol. Crystals of satisfactory quality were identified by careful screening and selection for data collection, as they typically 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}. Thus, although data were collected for a total rotation of 180°, only the first 125° range was selected for high-resolution data processing after an inspection of data statistics with regard to crystal decay. Analysis of the diffraction pattern indicated that these crystals exhibit orthorhombic symmetry and systematic absences indicated the space group to be $P2_12_12$. Assuming the presence of two RASI molecules per asymmetric unit, the Matthews coefficient is estimated to be 2.24 Å^3 Da^{-1}, corresponding to a solvent content of 44.05% (Matthews, 1968), which is within the general range for protein crystals. Details of data statistics are provided in Table 1.

Initial attempts to solve the crystal structure of RASI were performed by molecular replacement using the structure of BASI from barley (*Hordeum vulgare*; 58% sequence identity; PDB code 1ava; Vallee *et al.*, 1998) with non-conserved residues altered to alanines as a search model. A molecular-replacement solution was obtained using the *AMoRe* program (Navaza, 1994) which confirmed the presence of two protein molecules per asymmetric unit. After rigid-body refinement using *CNS* v.1.1 (Brünger *et al.*, 1998) in the resolution range 25–3.5 Å, R and R_{free} were 47.5 and 46.8%, respectively. Complete model building and refinement of the structure to 1.81 Å resolution is in progress; structural details will be described in a separate paper.

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