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Purification and crystallization of Japanese pear S-RNase associated with gametophytic self-incompatibility

Takanori Matsuura,[a] Masaki Unno,[b] Hiroaki Sakai,[b] Tomitake Tsukihara[b] and Shigemi Norioka[a]*

[a]Division of Protein Chemistry, Institute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan, and [b]Division of Protein Crystallography, Institute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan

Correspondence e-mail: norioka@protein.osaka-u.ac.jp

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S-RNase is a 25 kDa pistil-specific protein associated with gametophytic self-incompatibility. The S-RNase is secreted into the transmitting tissue of the pistil and is responsible for the discrimination of pollen *S*-alleles. Crystals of Japanese pear S_3-RNase were obtained by the hanging-drop vapour-diffusion method. Preliminary X-ray data showed that the crystals diffract to a 1.5 Å resolution and belong to the space group $P2_1$, with unit-cell parameters a = 45.4, b = 52.4, c = 47.4 Å, $\alpha = \gamma = 90$, $\beta = 106.5^\circ$.

1. Introduction

Self-incompatibility is a mechanism that prevents self-fertilization in flowering plants (de Nettancourt, 1977). The self-incompatibility in solanaceous, rosaceous and scrophulariaceous plants is gametophytically controlled by a single multi-allelic locus (*S*-locus; Newbigin *et al.*, 1993). When a pollen grain lands on a stigma, a discriminating process as to whether an *S*-allele of the pollen matches one of two *S*-alleles of the pistil takes place. The pollen grain germinates on the stigma and grows toward the embryo, but if its *S*-allele matches one of the two *S*-alleles of the pistil, the pollen-tube growth is arrested in the style and no fertilization takes place. A pistil-specific ribonuclease (S-RNase) encoded by the *S*-locus has been shown to be responsible for the self-incompatibility reaction, in particular the discrimination of the pollen *S*-alleles, by experiments using transgenic plants (Huang *et al.*, 1994; Lee *et al.*, 1994; Murfett *et al.*, 1994). However, the mechanism of the self-incompatibility is still ambiguous because the pollen *S*-gene product(s), which interact(s) with S-RNase and cause(s) self and non-self recognition between the pollen and pistil, has not yet been found.

The primary structural features of the solanaceous, rosaceous and scrophulariaceous S-RNases suggested that a hypervariable (HV) region, in which many amino-acid substitutions occur, may be responsible for the discrimination of the *S*-alleles (Kao & McCubbin, 1996). The analysis of synonymous (*dS*) and non-synonymous (*dN*) substitutions in rosaceous S-RNase genes detected four regions with an excess of *dN* over *dS* in which positive selection may operate (PS regions; Ishimizu, Endo *et al.*, 1998). As well as the HV region, the PS regions may also be important for the self-incompatibility reaction.

To elucidate the mechanism of the self-incompatibility on a molecular level, we have initiated a three-dimensional structure analysis of Japanese pear S-RNase. Although seven S-RNases (S_1-RNase to S_7-RNase) have been identified in and purified from the styles of the Japanese pear (Ishimizu, Shinkawa *et al.*, 1998), we chose the S_3- and S_5-RNases for X-ray crystallographic analysis because they have only two N-glycosylation sites (Ishimizu *et al.*, 1999) and their amino-acid sequences are highly homologous to each other (95.5% identity). If the three-dimensional structures of the S_3- and S_5-RNases are determined, the conformational difference between the two S-RNases will provide very important information on the recognition site of the S-RNase toward the pollen *S*-gene product(s). As the first step in the structure analysis of the Japanese pear S-RNases, we now report the preliminary X-ray crystallographic data as well as the crystallization conditions of the S_3-RNase.

2. Materials and methods

2.1. Protein purification

As we have not yet succeeded in the construction of expression system of recombinant S-RNase, we directly purified the S-RNase from Japanese pear styles. The styles from about 5000 flowers of 'Chojuro' (S_2S_3), a cultivar of the Japanese pear, were ground in liquid nitrogen with a mortar and pestle and then extracted with 100 ml 40 m*M* MES/NaOH pH 6.5 containing 5 m*M* Na_2EDTA, 1.5%(*v*/*v*) sodium ascorbate and 3%(*v*/*v*) Polyclar-AT (polyvinylpyrrolidone, GAF Chemicals Co.) for 30 min at 277 K. After centrifugation at 18 800*g* for 10 min at 277 K, the supernatant was collected and ammonium sulfate was added to the supernatant to the final concentration of 90%(*w*/*v*). After keeping the solu-

tion for 2 h at 277 K, it was centrifuged at 18 800*g* for 30 min and the precipitate was dissolved in 15 ml of 40 m*M* MES/NaOH pH 6.5 containing 5 m*M* Na_2EDTA and 1.5%(*v*/*v*) sodium ascorbate and chromatographed on a Sephadex G-75 column (3.6 × 58 cm) equilibrated with 40 m*M* MES/NaOH pH 6.5 containing 5 m*M* Na_2EDTA. Fractions having RNase activity were collected and applied to a CM-cellulose column (2.2 × 30 cm) equilibrated with the same buffer. Proteins bound to the column were eluted with a linear gradient of NaCl from 0 to 0.4 *M*. The S-RNase fractions were dialyzed against 10 m*M* sodium phosphate pH 6.5 and applied to a hydroxyapatite column (1.3 × 18 cm) equilibrated with 10 m*M* sodium phosphate pH 6.5. S-RNase was eluted with a linear gradient of sodium phosphate from 10 to 250 m*M*. The yield of S_3-RNase was 5 mg per 5000 'Chojuro' flowers.

2.2. Crystallization and X-ray data collection

Crystals of the Japanese pear S_3-RNase were grown using the hanging-drop vapour-diffusion method at 277 K by mixing 2 µl of protein solution (4 mg ml^{-1} in 50 m*M* MES/NaOH pH 7.0) and an equal volume of reservoir solution [100 m*M* MES/NaOH pH 7.0, 200 m*M* sodium acetate and 30%(*w*/*v*) polyethylene glycol 6000]. Crystals appeared within 5 d and grew to average dimensions of approximately 0.1 × 0.1 × 0.03 mm (Fig. 1).

The crystals were picked up with a nylon loop and flash-cooled at 100 K in a stream of gaseous nitrogen. The X-ray diffraction data were collected at a wavelength of 0.9 Å at SPring-8 (Hyogo, Japan) BL40B2 beamline using the R-AXIS IV (Rigaku) detector system. The crystal-to-detector distance was 200.0 mm. A total of 83 rotation images were collected with an oscillation angle of 2.0° with an exposure time of 40 s for each image. The data processing was carried out using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The diffraction intensities were indexed using the program *DENZO* and scaled and merged to 1.5 Å resolution using the program *SCALEPACK*.

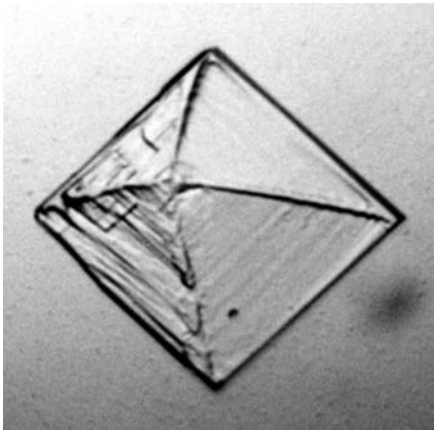

Figure 1
Crystal of Japanese pear S_3-RNase. The size of this crystal is approximately 0.1 × 0.1 × 0.03 mm.

3. Results and discussion

A complete data set was collected to a resolution of 1.5 Å using a single crystal. Detailed data statistics are shown in Table 1. Using the results from the autoindexing by *DENZO* and pseudo-precession photographs created by *HKLVIEW* (Collaborative Computational Project, Number 4, 1994), the space group was determined to be $P2_1$, with unit-cell parameters a = 45.4, b = 52.4, c = 47.4 Å, $\alpha = \gamma = 90$, $\beta = 106.5°$. Assuming that one S_3-RNase molecule (MW = 25 000 Da) is contained in the asymmetric unit, the Matthews coefficient (V_M; Matthews, 1968) was calculated to be 2.16 Å^3 Da^{-1}; the estimated solvent content is thus 43%, which is in the range typically found in protein crystals.

The Japanese pear S_3-RNase has a sequence homology of 26% with RNase MC1, whose crystal structure has already been published (Nakagawa *et al.*, 1999). Therefore, the molecular-replacement method (MR) using the coordinates of RNase MC1 was tried extensively. However, all promising MR solutions did not result in interpretable electron-density maps. This is probably because of the substantial conformational differences between the Japanese pear S_3-RNase and RNase MC1. Therefore, the crystal structure of the Japanese pear S_3-RNase will be solved by the multiple isomorphous replacement method. A search for heavy-atom derivatives is now in progress.

It is important for understanding the molecular recognition of the self-incompatibility reaction to clarify the tertiary structures of the S-RNases. The Japanese pear S_3-RNase is a particularly exciting molecule because of the existence of the highly homologous (95.5%) isomer S_5-RNase (Ishimizu, Shinkawa *et al.*, 1998). In this pair, there are only two and four amino-acid substitutions within the HV and PS regions, respectively, but the pollen S-gene product(s) can recognize them as different materials. Determination of the tertiary structures of the S_3- and S_5-RNases at high resolution and careful comparison between the two structures will give us very important information on the recognition site of the S-RNase toward pollen factor(s) and on the discrimination mechanism of pollen *S*-alleles by the S-RNase.

Table 1
X-ray data-collection statistics.

Space group	$P2_1$
Unit-cell parameters†	
a (Å)	45.39 (6)
b (Å)	52.40 (5)
c (Å)	47.41 (6)
$\alpha = \gamma$ (°)	90.00
β (°)	106.60 (6)
Resolution‡ (Å)	1.5 (1.53–1.5)
Observed reflections‡	105257 (5999)
Independent reflections‡	33441 (2095)
R_{merge}‡§	5.7 (14.6)
Completeness‡ (%)	96.3 (90.6)
$I/\sigma(I)$‡	14.7 (11.3)
Averaged redundancy‡	3.2 (2.6)

† Values in parentheses are estimated standard deviations. ‡ Values in parentheses refer to the highest resolution shell, 1.53–1.5 Å. § $R_{merge} = (\sum|I_i - \langle I\rangle|/\sum I_i)/100$, where I_i is an individual intensity observation, $\langle I\rangle$ is the mean intensity for that reflection and the summation is over all reflections.

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