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Research article

Purification and characterization of a non-S-RNase and S-RNases from styles of Japanese pear (*Pyrus pyrifolia*)

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Abstract

In this study we biochemically characterized stylar ribonucleases (RNases) of Japanese pear (*Pyrus pyrifolia*), which exhibits S-RNase-based gametophytic self-incompatibility. We separated the RNase fractions NS-1, NS-2, and NS-3 from stylar extracts of the cultivar Nijisseiki (S_2S_4). The RNase in each fraction was purified to homogeneity through a series of chromatographic steps. Chemical analysis of the proteins revealed that the basic RNases in the NS-2 and NS-3 fractions were the S_4- and S_2-RNases, respectively. Five additional S-RNases were purified from other cultivars. An acidic RNase in the NS-1 fraction was also purified from other cultivars, and identified as a non-S-allele-associated RNase (non-S-RNase). The non-S-RNase is composed of 203 amino acids, is non-glycosylated and is a N-terminal-pyroglutamylated enzyme of the RNase T_2 family. The substrate specificities and optimum pH levels of the non-S-RNase and S-RNases were similar. Interestingly, the specific activity of the non-S-RNase was 7.5–221-fold higher than those of the S-RNases when tolura yeast RNA was used as the substrate. The specific activity of the S_2-RNase was 8.8–28.6-fold lower than those of the other S-RNases. These differences in specific activities among the stylar RNases are discussed.

Keywords: Japanese pear; Primary structure; Purification; Ribonuclease; Self-incompatibility

1. Introduction

The stylar S-ribonucleases (S-RNases) in the families Rosaceae, Solanaceae, and Scrophulariaceae are involved in gametophytic self-incompatibility (for a recent review, see Ref. [22]). They are encoded by a single S-locus with multiple alleles. Their ribonuclease activity is essential for rejection of self-pollen [8]. It was reported that the ribonuclease activity in the styles of the self-incompatible *Nicotiana alata* (Solanaceae) was 130–1000-fold higher than that of the related self-compatible *Nicotiana tabacum* [23]. However, Clark et al. reported that comparable levels of stylar RNase activity were detected in self-incompatible and self-compatible *Petunia hybrida* [3], indicating that the levels of RNase activity in styles are not always correlated with self-incompatibility phenotypes. In fact, RNases other than S-RNases were detected in the styles of *Petunia inflata* [18,30]. These petunia RNases are not encoded by S-locus genes and are called S-like RNases because of their sequence similarities to those of S-RNases [18]. Some cDNAs encoding S-like RNases or non-S-RNases have also been cloned in the Rosaceae [20,24,34,35]. Based on their amino acid sequences, they

Abbreviations: API, *Achromobactor* protease I; CM, carboxymethyl; EDTA, ethylene diamine tetraacetic acid; ESI-MS, electrospray ionization-mass spectrometry; HCl, hydrochloric acid; HPLC, high performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; NaOH, sodium hydroxide; RCM, reduced and carboxymethylated; RNase, ribonuclease; TFA, trifluoroacetic acid.

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can be divided into two subfamilies [9]: the basic S-like RNases [20,35] and the acidic RNases [24,34], which are induced by phosphate starvation. However, their biochemical properties, including specific activities and chemical structures, have not been extensively studied. More work is needed to clarify the differences in biochemical characteristics between the non-S-RNases and the S-RNases in the family Rosaceae.

In Japanese pear, a member of the Rosaceae family, several cDNAs encoding S-RNases have already been cloned [2,15,24,28,31]. In addition, a self-compatible stylar part mutant of the cultivar Nijisseiki (S_2S_4), Osa-Nijisseiki, was identified. The S_4-RNase is not expressed in the styles of this self-compatible mutant [14,24,26,27]. However, we observed that the levels of RNase activity in the styles of Osa-Nijisseiki were comparable to those of Nijisseiki. Therefore we set out to clarify the abundance levels and specific activities of the S-RNases and non-S-RNase(s) expressed in the styles of Japanese pear.

Here, we report on the purification of RNases from the styles of Japanese pear and the identification of these enzymes as S-RNases and a non-S-RNase. The biochemical characterization of these stylar RNases is also reported here. Our studies revealed that the specific activity of the non-S-RNase is much higher than those of the S-RNases.

2. Materials and methods

2.1. Materials

Flowers of Nijisseiki (S_2S_4), Chojuro (S_2S_3), Kosui (S_4S_5), Imamuraaki (S_1S_6), and Okusankichi (S_5S_7) were collected at the balloon and mature flower stages at the Tottori Horticultural Experiment Station (Yura, Tottori). Pistils were excised from the flowers, rapidly frozen in liquid nitrogen, and stored at −70 °C until used. A lysylendopeptidase, *Achromobactor* protease I (API) was purchased from Wako Pure Chemical Industries. Pre-packed MonoS, Fast Desalting and Phenyl Superose columns were from GE Healthcare Biosciences. The C18 reversed-phase and hydroxyapatite columns were purchased from YMC Co. Ltd. All of the other chemicals used were of the highest grades available commercially.

2.2. Assay of ribonuclease activity

Ribonuclease activity was measured with torula yeast RNA as the substrate. The reaction buffer (490 μL) of 0.1 M imidazole-hydrochloric acid (HCl) (pH 7.0) containing 0.1 M KCl and 2 mg RNA, was incubated at 37 °C for 10 min. The enzyme solution (10 μL) was then added, and the reaction incubated at 37 °C. After 20 min, 100 μL of stop solution (25% perchloric acid, 0.75% (w/v) lanthanum acetate) were added. After 30 min of incubation on ice, the solution was centrifuged at 14,000 × *g* for 5 min. The absorbance of the supernatant at 260 nm was measured with a Hitachi U-2000 spectrophotometer. One unit of RNase activity was designated as the amount of RNase which gave an increase in absorbance of one at 260 nm, in 1 min of reaction time.

Specific activity was calculated as the number of RNase activity units per nmol of enzyme. RNase specific activity was measured by the method described above, but 0.1 M Tris–HCl (pH 7.0) was used as the reaction buffer instead of 0.1 M imidazole-HCl (pH 7.0) containing 0.1 M KCl. The amount of enzyme was estimated by amino acid analysis.

2.3. Purification of RNases from the styles of Japanese pear

All the chromatographic procedures were carried out at 4 °C. Styles from 500 flowers of Nijisseiki (S_2S_4) were ground in liquid nitrogen with a mortar and pestle. After evaporation of the liquid nitrogen, the extraction buffer (10 mL, 0.1 M Tris–HCl (pH 7.8) containing 25 mM ethylene diamine tetraacetic acid (EDTA), 15 mg/mL sodium ascorbate and 30 mg/mL polyclar AT) was added immediately, and the whole stirred for 30 min at 0 °C then centrifuged at 9500 × *g* for 10 min at 4 °C. The supernatant was used for further purification. The supernatant was applied to a Sephadex G-50 column (2.6 × 45 cm) equilibrated with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-sodium hydroxide (NaOH) (pH 6.5) containing 5 mM EDTA and was eluted with the same buffer. RNase-positive fractions (87 mL) were pooled and applied to a carboxymethyl (CM)-cellulose column (1.4 × 20 cm) equilibrated with 50 mM MES-NaOH (pH 6.5) containing 5 mM EDTA. The column was washed with the same buffer, and NS-1 was eluted under these conditions. Two other fractions bearing RNase activity (NS-2 and NS-3) were eluted with a linear gradient of sodium chloride from 0 to 0.5 M.

For purification of NS-1 (the non-S-RNase), RNase-positive NS-1 fractions were pooled and applied to another CM-cellulose column (2.4 × 52 cm) equilibrated with 50 mM sodium acetate (pH 5.0). The RNase fractions were pooled, dialyzed against water, and lyophilized. The sample was then dissolved in 50 mM sodium acetate buffer (pH 5.0) containing 2 M ammonium sulfate, and applied onto a Phenyl Superose column (1.6 × 50 mm) equilibrated with the same buffer, at a flow rate of 50 μL/min. The column was washed with the same buffer for 80 min, and the NS-1 enzyme was eluted with a linear gradient of ammonium sulfate, from 2 to 0 M, over 40 min. The fractions containing RNase were pooled on ice.

For purification of NS-2 (the S_4-RNase) and NS-3 (the S_2-RNase), fractions containing NS-2 (45 mL) and NS-3 (45 mL) were each dialyzed against water and concentrated to 3.7 mL. The concentrated fractions were applied (separately) to a hydroxyapatite column (6 × 330 mm) equilibrated with 10 mM sodium phosphate (pH 6.8) at flow rates of 0.5 mL/min. The enzymes were eluted with linear gradients of sodium phosphate from 10 mM to 480 mM. The pooled NS-2 fraction was further purified using a Mono S column (1.6 × 50 mm) equilibrated with 50 mM MES-NaOH (pH 6.0) at a flow rate of 100 μL/min. The column was washed with the same buffer for 20 min, and the enzyme was eluted with a linear gradient of sodium chloride from 0 M to