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Note

Characterization of an α-L-Rhamnosidase from *Streptomyces avermitilis*

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The putative α-L-rhamnosidase gene from *Streptomyces avermitilis* was cloned and expressed. The recombinant enzyme released L-rhamnose from *p*-nitrophenyl α-L-rhamnoside, *Citrus* flavonoids such as naringin, rutin, and hesperidin, and gum arabic which is an arabinogalactan-protein. Calcium ions increased L-rhamnose production by the enzyme from gum arabic, whereas enzyme activity was not affected by any metal ions.

Key words: α-L-rhamnosidase; glycoside hydrolase family 78; arabinogalactan-protein; *Streptomyces avermitilis*

Arabinogalactan proteins (AGPs) are a family of proteoglycans that are localized on the cell surfaces of higher plants. They play important roles in root formation, the promotion of somatic embryogenesis, and the attachment of pollen tubes.[1)] AGPs are characterized by the presence of large amounts of carbohydrates, such as D-galactose and L-arabinose, and the amino acids hydroxyproline, serine, threonine, alanine and glycine.[2)] Type II arabinogalactans and short oligosaccharides are two types of carbohydrate that are attached to the AGP backbone. Type II arabinogalactans have β-1,3-linked D-galactosyl backbones in mono- or oligo-β-1,6-D-galactosyl and/or L-arabinosyl side chains. L-Arabinose and lesser amounts of other auxiliary sugars, such as D-glucuronic acid, L-rhamnose, and L-fucose, are attached to the side chains primarily at the nonreducing termini.[3)] The complexity of AGPs complicates the structural and functional analysis of individual AGPs.

We are studying glycoside hydrolases that degrade the carbohydrate moieties of AGPs.[4–31)] We have first cloned two key enzymes, exo-β-1,3-galactanase and endo-β-1,6-galactanase, which hydrolyze the backbones of the AGP sugars.[11,12)] In the course of research on AGP-degrading enzymes, we found that *Streptomyces avermitilis* NBRC14893 possesses both an exo-β-1,3-galactanase and an endo-β-1,6-galactanase.[16,17)] Because these enzymes work more effectively when sugars such as L-arabinose and L-rhamnose are removed from the galactan backbone, we have focused on this bacterium as a source of enzymes that degrade the sugar linkages of AGPs. We cultivated this bacterium using gum arabic, an AGP, as carbon source, and found that a β-L-arabinopyranosidase (EC 3.2.1.88)[22)] and α-L-rhamnosidase (EC 3.2.1.40) are involved in AGP degrading enzyme system of *S. avermitilis* (Fig. 1A). In the present study, we focused on the characterization of a α-L-rhamnosidase from *S. avermitilis* NBRC14893.

Because the complete genome sequence of *S. avermitilis* has been determined,[32)] we knew how many putative α-L-rhamnosidase genes the strain has. Glycoside hydrolases (GHs) are categorized into various families in the Carbohydrate-Active enZymes (CAZy) database according to similarity of amino acid sequence.[33)] α-L-Rhamnosidases are classified into GH families 28, 78, and 106. *S. avermitilis* possesses only one putative α-L-rhamnosidase gene belonging to GH family 78 (GH78), and does not possess any genes belonging to GH families 28 or 106.

The full-length gene (3,090 bp) encoding a putative GH78 α-L-rhamnosidase (sav_828; GenBank accession no. BAC68538) was amplified from *S. avermitilis* genomic DNA by polymerase chain reaction using the following primers: forward, 5′-CAT ATG AGT GCC TTG CGA GTC ACC TCT-3′; and reverse, 5′-AAG CTT AAC GGT GAA TCG GTG GCT TCC-3′. The amplified DNA was cloned into pET30 vector (Novagen, Madison, WI) at the NdeI and HindIII restriction enzyme sites (underlined). A recombinant protein, SaRha78A, was successfully expressed using the T7 expression system in *Escherichia coli* Tuner (DE3) (Merck KGaA, Darmstadt, Germany). The protein was purified with a C-terminal histidine tag, and was dialyzed against 50 mM phosphate buffer, pH 7.0. The final preparation thus obtained was used as purified enzyme. Protein amounts were determined with the BCA protein assay reagent kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin (BSA) as standard. The molecular mass of SaRha78A on sodium dodecyl sulphate-polyacryamide gel electrophoresis was estimated to be 113 kDa (Fig. 1B, lane 3).

The recombinant protein showed the *p*-nitrophenyl-α-L-rhamnopyranoside (PNP-α-L-Rha*p*) hydrolyzing

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Abbreviations: AGPs, arabinogalactan-proteins; BSA, bovine serum albumin; CAZy, Carbohydrate-Active enZymes; GHs, glycoside hydrolases; GH78, glycoside hydrolase family 78; HPAEC-PAD, high-performance anion-exchange chromatography with a pulsed amperometric detection system; PNP, *p*-nitrophenol; PNP-α-L-Rha*p*, *p*-nitrophenyl-α-L-rhamnopyranoside; SaRha78A, *Streptomyces avermitilis* α-L-rhamnosidase

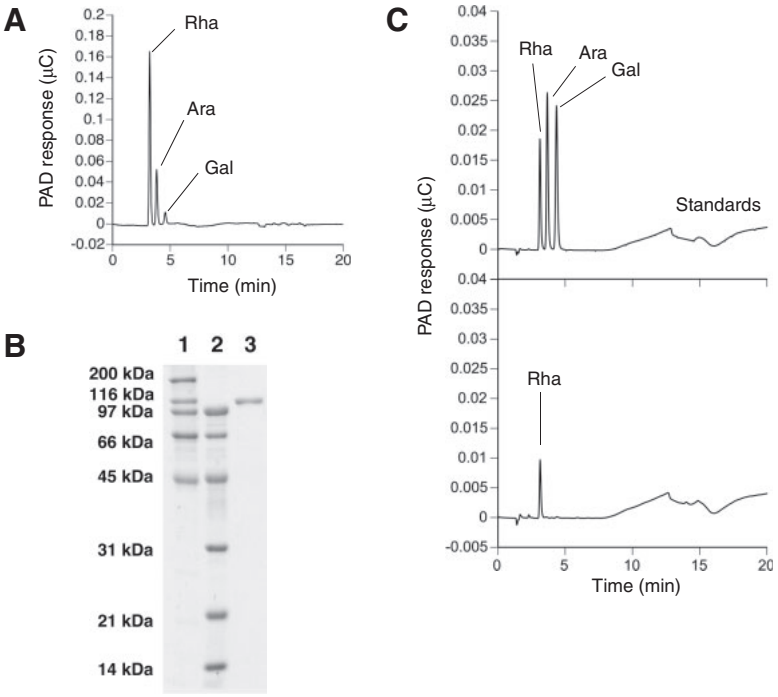

Fig. 1. Hydrolysis Product of Gum Arabic and Expression of the Recombinant Protein.

A, HPAEC-PAD analysis of the hydrolysis products of gum arabic by culture supernatant of *S. avermitilis* cultivated with gum arabic. Rha, rhamonose; Ara, arabinose; Gal, galactose. B, Sodium dodecyl sulphate-polyacryamide gel electrophoresis analysis of recombinant SaRha78A. Lanes 1 and 2, molecular mass marker; lane 3, recombinant SaRha78A. C, HPAEC-PAD analysis of the hydrolysis product of gum arabic generated by SaRha78A. Upper panel, standards (0.0002%); bottom panel, hydrolysis products of gum arabic by SaRha78A. Rha, rhamonose; Ara, arabinose; Gal, galactose.

activity, but did not hydrolyze PNP-α-L-arabinofuranoside, PNP-α-L-arabinopyranoside, PNP-β-L-arabinopyranoside, PNP-α-L-fucopyranoside, PNP-β-D-fucopyranoside, PNP-α-D-galactopyranoside, PNP-β-D-galactopyranoside, PNP-α-D-glucopyranoside, PNP-β-D-glucopyranoside, PNP-α-D-xylopyranoside, PNP-β-D-xylopyranoside, PNP-α-D-mannopyranoside, PNP-β-D-mannopyranoside, PNP-β-D-glucuronide, or PNP-β-D-galacturonide, suggesting that SaRha78A is an α-L-rhamnosidase. Maximal enzyme activity was detected at pH 6.0 at 50 °C (Fig. 2A and B), and enzyme was stable between pH 5.0 and 11.0 at 30 °C for 1 h, and also stable below 40 °C at pH 6.0 for 1 h (Fig. 2C and D). The specific activity of SaRha78A with PNP-α-L-Rha*p* as substrate was 7.6 ± 0.2 units/mg at pH 6.0 at 50 °C (Table 1). The kinetic parameters of SaRha78A for PNP-α-L-Rha*p* were determined by the following method: The reactions were performed in McIlvaine buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid), pH 6.0, containing 0.3–2 mM substrates, 0.1% (w/v) BSA, and 1.2 μM enzyme at 30 °C for up to 30 min. The K_m and k_{cat} values of SaRha78A for PNP-α-L-Rha*p* were 0.03 ± 0.00 mM and 1.93 ± 0.01 s^{-1} respectively (Table 1).

The effects of metal ions and EDTA on enzyme activity were examined by treatment with 2-mM solutions of metal ions, including Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Cs^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} and EDTA. The metal ions and EDTA did not affect enzyme activity when PNP-α-L-Rha*p* was used as substrate (data not shown).

The substrate specificities of SaRha78A were determined. The activities of SaRha78A toward flavonoids such as naringin, rutin, and quercitrin were analyzed by the method described by Koseki *et al.*[34] In contrast, the activity toward an arabinogalactan-protein was determined at 40 °C in McIlvaine buffer (pH 6.0) with 2% (w/v) gum arabic (Wako Pure Chemical Industries, Osaka, Japan) as substrate and 1.8 μM enzyme. After incubation for 24 h, the amount of L-rhamnose released

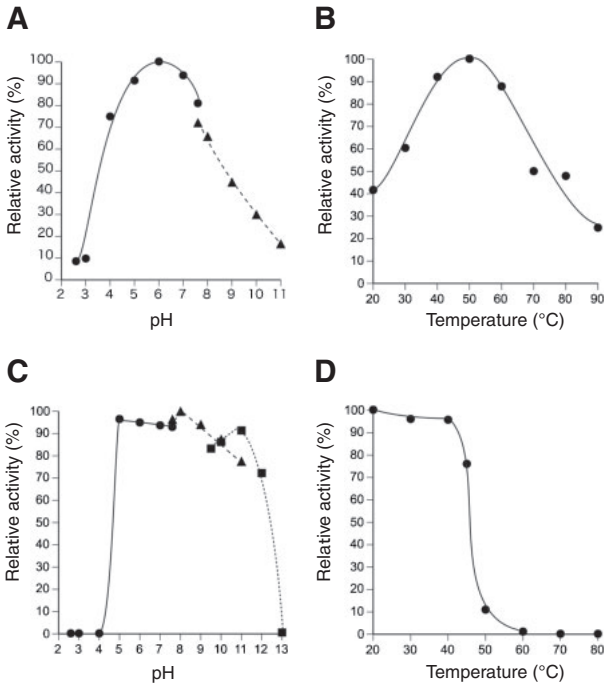

Fig. 2. Enzymatic Properties of Recombinant SaRha78A.

The effects of pH and temperature on enzyme activity and stability were investigated. A, Optimum pH; B, optimum temperature; C, stability for pH; D, stability for temperature. Symbols: ●, McIlvaine buffer; ▲, Atkins-Pantin buffer; ■, Glycine-NaOH buffer.

Table 1. Substrate Specificity of SaRha78A

Substrate	Linkage	Specific activity (units/mg)
PNP-α-L-Rha*p*	α-1	7.6 ± 0.2
Naringin	α-1,2	19.5 ± 1.1
Hesperidin	α-1,6	0.9 ± 0.07
Rutin	α-1,6	28.5 ± 2.9
Quercitrin	α-1	N.D.

was quantified by the HPAEC-PAD system. The sugar composition of the gum arabic was determined by a method described in a previous paper[22] and the molar ratio of L-rhamnose, L-arabinose, D-galactose, and D-glucuronic acid was 5:20:28:6.

As shown in Table 1, SaRha78A showed higher activity for rutin (28.5 ± 2.9 units/mg) and naringin (19.5 ± 1.1 units/mg). It also showed activity for hesperidin (0.9 ± 0.07 units/mg), but not for quericitrin. The activities for these flavonoids were much higher than that for PNP-α-L-Rha, which was caused by the difference between aglycon sides of the substrates. This suggests that SaRha78A possesses subsite +1, distinguishing the substrates. A similar tendency as to the activity for these substrates was reported for RhaB from *Aspergillus aculeatus*.[35] SaRha78A cleaved both the α-1,6 and the α-1,2-linked rhamnosyl residues. These activities have been reported for both bacterial and fungal enzymes belonging to GH78.[34–37] SaRha78A showed activity toward gum arabic, and the only L-rhamnose was detected as a hydrolysis product (Fig. 1C). The enzyme removed 0.7% L-rhamnose from the gum arabic. It removed 6.5% L-rhamnose from the substrate when 2 mM calcium chloride was added to the reaction mixture. These results suggest that SaRha78A has a region influenced by calcium ions, except for the catalytic domain, because enzyme activity was not affected by any of the metal ions (data not shown). Since the activity for polysaccharide increased, but not for glycosides, it is expected that the enzyme has a carbohydrate-binding module.[38,39] Two GH78 α-L-rhamnosidase structures have been solved. Both enzymes are composed of four or five distinct domains. However, the existence of a carbohydrate-binding module that binds to L-rhamnose has not been confirmed.

The present study indicates that *S. avermitilis* possesses a α-L-rhamnosidase acting on arabinogalactan-proteins. Calcium ions increased the amount of L-rhamnose produced from gum arabic by the enzyme. It remains to be determined which region of the enzyme is affected by calcium ions. A detailed structure-function study would be expected to answer the function of calcium ions in the arabinogalactan degradation by SaRha78A.

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