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Biochemical characterization of *Aspergillus oryzae* recombinant α-L-rhamnosidase expressed in *Pichia pastoris*

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An α-L-rhamnosidase-encoding gene from *Aspergillus oryzae*, which belongs to the glycoside hydrolase family 78, was cloned and expressed in *Pichia pastoris*. SDS-PAGE of the purified recombinant α-L-rhamnosidase protein revealed smeared bands with apparent molecular mass of 90–130 kDa. After *N*-deglycosylation, the recombinant enzyme showed a molecular mass of 70 kDa. The enzyme exhibited optimal activity at a pH of 5.0 and a temperature of 70 °C. Specific activity of the enzyme was higher toward hesperidin than toward naringin, which consist of α-1,6 and α-1,2 linkages, respectively. The activity was also higher toward hesperidin than toward rutin, which consist of 7-*O*- and 3-*O*-glycosyl linkages of flavonoids, respectively. Kinetic analysis of the enzyme showed that the Michaelis constant (K_m) was lowest toward rutin, moderate toward naringin, and higher toward *p*-nitrophenyl-α-L-rhamnopyranoside and hesperidin. Its high catalytic efficiency (k_{cat}/K_m) toward rutin was results of its low K_m value while its high catalytic efficiency toward hesperidin was results of a considerably high k_{cat} value.

[**Key words:** α-L-Rhamnosidase; Glycoside hydrolase family 78; *N*-glycosylation; *Aspergillus oryzae*; *Pichia pastoris*]

α-L-Rhamnosidase (EC 3.2.1.40) cleaves terminal α-L-rhamnose from natural glycosides, including flavonoids, such as naringin, hesperidin, and rutin. α-L-Rhamnosidases play a role in enhancing the aroma of grape juices and derived beverages (1–5). Additionally, they are used in citrus juices for removing the bitterness caused by naringin (6). Further, they have been used for the structural analysis of plant and bacterial polysaccharides, glycosides, and glycolipids (7).

Many microorganisms have been studied for their potential to produce glycosidases. α-L-Rhamnosidases have been classified as members of glycoside hydrolase (GH) families 13, 28, 78, and 106 in the continuously updated Carbohydrate-Active Enzymes (CAZy; http://www.cazy.org/index.html) database. The genus *Aspergillus* is important to the food industry owing to their ability to produce metabolites and extracellular glycosidases. It has been found that α-L-rhamnosidases from *Aspergillus terreus*, *A. nidulans*, *A. aculeatus*, *A. awamori* and *A. niger* have potential value in enology (3,8–11). The effect of carbon sources on α-L-rhamnosidase production and its gene expression in *A. nidulans* and *A. niger* has been investigated (12–14). Many α-L-rhamnosidase-encoding genes have been isolated and characterized from the genus *Aspergillus* including *A. aculeatus*, *A. brasiliensis*, *A. kawachii*, *A. nidulans*, *A. niger*, and *A. terreus* (12,15–19). *Aspergillus oryzae* has been safely used as a koji mould in the manufacture of *shoyu*, *miso*, and *sake* in Japan. *A. oryzae* has been recommended as one of the best microbial enzyme sources. To date, no α-L-rhamnosidase-encoding gene from *A. oryzae* has been reported. In this study, we cloned a gene encoding α-L-rhamnosidase, which is classified as a member of GH family 78, from *A. oryzae* and investigated biochemical characterization of recombinant enzyme expressed in *Pichia pastoris*.

MATERIALS AND METHODS

Chemicals *p*-Nitrophenyl-α-L-rhamnopyranoside (*p*NPαRha), *p*-nitrophenyl-α-D-glucopyranoside (*p*NPαGlu), *p*-nitrophenyl-β-D-glucopyranoside (*p*NPβGlu), *p*-nitrophenyl-β-D-galactopyranoside (*p*NPβGal), *p*-nitrophenyl-β-D-mannopyranoside (*p*NPβMan), *p*-nitrophenyl-β-D-xylopyranoside (*p*NPβXyl), *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPαAra), and naringin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hesperidin and rutin were obtained from Wako Pure Chemical Industries (Osaka, Japan). Naringenin-7-*O*-glucoside and quercetin-3-*O*-glucoside were obtained from Funakoshi Co., Ltd. (Tokyo, Japan).

Cloning and expression of *AorhaA* The *AorhaA* coding sequence was PCR amplified from genomic DNA of *A. oryzae* RIB40 using the following primers, which were designed based on the published genome sequence [GenBank: AOR_1_2262154 (AO090003001291), Gene ID: 83768215]: Forward, 5′-GCATCGATGGTGCCTTACAACGAATACAT-3′ (the *Cla*I site is underlined); Reverse, GCTCTAGACTATTGACTTGCAACTTCCAA-3′ (the *Xba*I site is underlined). The resulting PCR product was restriction digested with *Cla*I and *Xba*I and cloned into the *Pichia* expression vector pPICZαC (Invitrogen, Waltham, MA, USA). An intron deletion was performed by inverse PCR using the KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan), the forward primer 5′-AACATCGAAGCAAACGCTAT-3′, and the reverse primer 5′-GTGGCCGCCCATTCCGAACC-3′. The predicted sequences were verified by DNA sequencing analysis. This procedure yielded an expression plasmid vector containing the *AorhaA* gene under the control of the *AOX1* promoter and the gene was verified to be in-frame with the α-factor secretion signal sequence of *Saccharomyces cerevisiae*. *AorhaA* was expressed in *P. pastoris* GS115 according to the manufacturer's protocol (Invitrogen, Tokyo, Japan).

Enzyme assays The activity of α-L-rhamnosidase was routinely assayed as follows. A 1.4-ml volume of 50 mM sodium acetate buffer (pH 5.0) was mixed with 0.01 ml of 100 mM *p*NPαRha dissolved in dimethyl sulfoxide and 0.1 ml of the enzyme source was added to this mixture. The reaction mixture was incubated at

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50 °C for 20 min. The reaction was terminated by adding 1.5 ml of 0.5 M sodium carbonate, and the absorbance was then measured. The increase in the absorbance at 400 nm was proportional to the amount of *p*-nitrophenol released. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute under the assay conditions described above. The enzyme activity toward naringin, hesperidin, and rutin was assayed as follows. A 430-μl volume of 50 mM sodium acetate buffer (pH 5.0) was mixed with 50 μl of substrate (2 mg/ml) dissolved in methanol, and 20 μl of the enzyme source was added to this mixture. The reaction mixture was incubated at 50 °C for 5 min. The samples were analyzed to determine decreases in substrate or increases in hydrolyzate by using high-performance liquid chromatography with ultra violet detection (280 nm) on a TSKgel ODS-120T column (4.6 × 250 mm; Tosoh Co., Tokyo, Japan). 50 mM acetate buffer (pH 4.0), prepared in a 70:30 solution of water and acetonitrile was used for the mobile phase, and the column was operated at a flow rate of 0.5 ml/min at 45 °C. One unit of rhamnosidase activity was defined as the amount of enzyme required to release rhamnose at 50 °C and pH of 5.0 at the rate of 1 μmol min^{-1}.

Purification of recombinant AoRhaA *P. pastoris* was cultured in BMGY [1% yeast extract, 2% peptone, 1% glycerol, 1.34% yeast nitrogen base without amino acids, and 0.1 M potassium phosphate, (pH 6.0)] and BMMY (same composition as that of BMGY, except with 0.5% methanol instead of 1% glycerol), according to the manufacturer's instructions (Invitrogen). Microbial cultures were then centrifuged at 5000 ×*g* for 15 min and recombinant protein was purified from cell-free supernatants using a HiPrep DEAE FF 16/10 column (GE Healthcare, Little Chalfont, UK) equilibrated with 50 mM sodium acetate buffer (pH 5.0) using the ÄKTA fast protein liquid chromatography purification system (ÄKTA purifier UPC10; GE Healthcare). Samples were eluted using a linear gradient of NaCl concentrations (0–0.4 M) in 50 mM sodium acetate buffer (pH 5.0) at a flow rate of 3 ml/min. The active fractions were collected, pooled, and concentrated by ultrafiltration, and subsequently purified using an ENrich SEC650 gel filtration column (10 × 300 mm; Bio-Rad, Hercules, CA, USA). The column was eluted in 1 ml fractions with 50 mM sodium acetate buffer (pH 5.0) containing 0.2 M NaCl at a flow rate of 1.0 ml/min. The purity and concentration of the resulting samples were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; ATTO Corp., Tokyo, Japan) analysis and using the Micro BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), respectively.

Enzyme characterization The optimal pH and pH stability of the purified enzyme (4.6 μg) were determined by performing an activity assay in 50 mM HCl–KCl buffer (pH 2.0), 50 mM glycine–HCl buffer (pH 3.0), 50 mM sodium acetate buffer (pH 4.0–5.0), 50 mM sodium phosphate buffer (pH 6.0–8.0), 50 mM glycine–NaOH buffer (pH 9.0–11.0), and 50 mM Na_2HPO_4–NaOH buffer (pH 12.0). Protein stability was determined by analyzing the residual activity after incubating the enzyme at room temperature for 1 h at different pH levels. The optimal temperature for the purified enzyme was measured at pH 5.0 at temperature ranging from 45 °C to 75 °C. Aliquots of purified enzymes were incubated for 60 min at temperatures ranging from 45 °C to 75 °C and their residual activities were measured in 50 mM sodium acetate buffer (pH 5.0) at 50 °C.

Purified recombinant AoRhaA (rAoRhaA) (3.6 μg) was used for the determination of enzyme kinetics and substrate specificity. Specific activity and kinetic constants were measured using *p*NPαRha as an artificial substrate and naringin, hesperidin, and rutin as natural substrates (Fig. 1).

RESULTS AND DISCUSSION

Cloning and expression of *AorhaA* As shown in Table 1, the ClustalW alignment of the deduced amino acid sequence of AoRhaA revealed high identity values (64%–70%) with the α-L-rhamnosidases, which is classified as a member of GH family 78, from *A. aculeatus*, *A. kawachii*, and *A. niger* (15,16,18). However, this protein showed low identity values (6%–35%) with the α-L-rhamnosidases belonging to GH78 from *A. nidulans* (12), *A. terreus* (19), and *Xylaria polymorpha* (20). When fused with the *S. cerevisiae* α-factor secretion signal peptide and expressed in *P. pastoris*, rAoRhaA accumulated as an active enzyme in the culture supernatant (Supplementary Fig. S1). The culture supernatant of the *P. pastoris* transformants exhibited significant increases in hydrolytic activity (0.36 U/ml on day 4) toward *p*NPαRha. The enzyme produced by *P. pastoris* was purified in a two-step procedure using anion-exchange chromatography and gel filtration chromatography. Specific activity of purified enzyme was 5.4 U/mg toward *p*NPαRha. Purified enzyme showed a smeared band on SDS-PAGE with apparent molecular mass of 90–130 kDa. Following treatment with endoglycosidase H, the molecular mass of the resulting protein was 70 kDa (Fig. 2). The broad molecular mass (90–130 kDa) of rAoRhaA on SDS-PAGE was hypothesized to be a result of *N*-linked oligosaccharides. The hyperglycosylation of rAoRhaA is predicted to be due to a high degree of *N*-glycosylation. The NetNglyc server (http://www.cbs.dtu.dk/services/NetNGlyc/) predicted 14 *N*-glycosylation sites in AoRhaA. α-L-Rhamnosidase from fungi, including *A. aculeatus*, *A. kawachii*, *A. nidulans*, and *A. niger*, has been shown to be *N*-glycosylated (15,16,21,22). The molecular mass of the rAoRhaA that was examined in this study is greater than that of the native α-L-rhamnosidase from *A. aculeatus* (92 kDa and 85 kDa), *A. nidulans* (102 kDa), *A. niger* (85 kDa), and *A. kawachii* (90 kDa). Yeasts typically produce high-mannose-type *N*-glycans, suggesting that rAoRhaA possesses *N*-linked high mannose oligosaccharides. *P. pastoris* generates much longer glycosylation branches than fungi (23).

TABLE 1. Fungal GH78 α-L-rhamnosidases showing similarity with AoRhaA.

Strain	Accession no.	Identity (%)	Similarity (%)
A. kawachii	BAF98236.1	70	94
A. niger	XP_001389086.1	70	94
A. aculeatus (RhaA)	AF284761	68	94
A. aculeatus (RhaB)	AF284762	64	92
A. nidulans (RhaA)	C8VMJ6	35	78
A. nidulans (RhaE)	FR873475	6	46
A. terreus	AFH54529	7	44
X. polymorpha	AFA53085.1	7	48

General properties The optimal pH and temperature for rAoRhaA were observed to be 5.0 and 70 °C, respectively (Fig. 3). The enzyme exhibited more than 85% of its maximal activity in

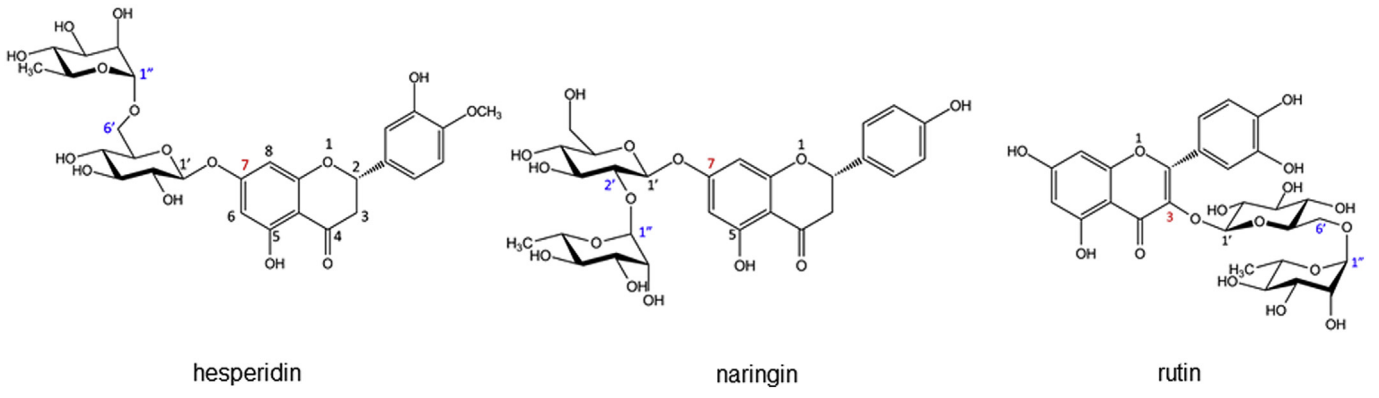

FIG. 1. Chemical structures of hesperidin, naringin, and rutin used for the α-L-rhamnosidase activity.

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