Aspergillus niger DLFCC-90 Rhamnoside Hydrolase, a New Type of Flavonoid Glycoside Hydrolase

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A novel rutin- α -L-rhamnosidase hydrolyzing α -L-rhamnoside of rutin, naringin, and hesperidin was purified and characterized from *Aspergillus niger* DLFCC-90, and the gene encoding this enzyme, which is highly homologous to the α -amylase gene, was cloned and expressed in *Pichia pastoris* GS115. The novel enzyme was classified in glycoside-hydrolase (GH) family 13.

α -L-Rhamnosidase (EC 3.2.1.40) cleaves terminal α -L-rhamnose from a large number of natural products, such as flavonoids, saponins, and many other natural glycosides, and this enzyme has been used in the food industry for eliminating the naringin bitterness of citrus juices (4, 20, 25, 26), removing hesperidin crystals from orange juices and releasing 7-O- β -D-glucoside-hesperetin, an important precursor in sweetener production (17, 24), and enhancing wine aromas (7, 6, 22). In addition, the deglycosylation of flavonoids *in vitro* by α -L-rhamnosidase has ushered in a new era of drug development (5, 8, 19, 27). Hitherto, α -L-rhamnosidases from fungi, bacteria, and animals have been purified and characterized (29), and several genes encoding α -L-rhamnosidases have also been cloned (1, 2, 3, 9, 16). Thus far, the α -L-rhamnosidases are categorized into four glycoside-hydrolase (GH) families, 28, 78, 106, and NC (nonclassified), in the CAZy (carbohydrate-active enzymes) database based on amino acid sequence similarities (10, 11).

In this paper, a novel rutin- α -L-rhamnosidase was purified and characterized from *Aspergillus niger* DLFCC-90, and the gene (*rhaR*) encoding this enzyme was also cloned and expressed in *Pichia pastoris* GS115.

The strain *A. niger* DLFCC-90, obtained from the Culture Collection of Biotechnology Engineering of Dalian Polytechnic University (Dalian, China), was cultured with shaking at 28 to 30°C for 72 to 96 h in a wort medium of 5.0 Baume degrees containing 2% extract from flowers of *Sophora japonica* (Huai Hua in Chinese). To obtain pure enzyme, the cell-free culture was treated by a 3-step method, i.e., ammonium sulfate precipitation (75% saturation), a Sephadex G-75 column (Amersham Pharmacia) eluted with 0.02 M acetate buffer (pH 5.0), and a DEAE 52-cellulose column (Amersham Pharmacia) eluted with a linear gradient of KCl (0.0 to 0.5 M) in 0.02 M acetate buffer (pH 5.0). The determination of the protein concentration was performed as described in reference 13. The enzymatic activity was measured using 2.0 mg/ml rutin (Sigma) in 0.02 M acetate buffer (pH 5.0) as a substrate after a reaction at 50°C for 18 h, and products of rhamnose and isoquercitrin from the enzyme reaction were detected to calculate the enzyme activity (18, 28). After the above-described 3 steps of purification, the enzyme's specific activity was increased 5.3-fold. The purified enzyme migrated as a single band on the 12% SDS-PAGE gel (12) with an apparent molecular mass of about 66 kDa (Fig. 1A), which was similar to those for the previ-

FIG 1 SDS-PAGE of the rutin- α -L-rhamnosidase. (A) SDS-PAGE of the enzyme from *A. niger* DLFCC-90. Lane 2, DEAE-purified enzyme; lane 3, Sephadex-purified enzyme; lane 4, ammonium sulfate precipitation of the culture supernatant proteins. (B) SDS-PAGE of rutin- α -L-rhamnosidase expressed in *P. pastoris* GS115. Lane 2, expression of the enzyme without methanol; lane 3, expression of the enzyme with methanol as an inducer. The arrow marks the predicted enzyme expressed in *P. pastoris* GS115. (A and B) Lane 1 in both panels, molecular mass markers (TaKaRa, Dalian, China).

ously reported α -L-rhamnosidase from *Curvularia lunata* (6) and naringinase from *Aspergillus sojae* (5). The 3-step-purified enzyme gave one major peak by high-performance liquid chromatography (HPLC) analysis (Tosoh TSKgel G2000SW; Φ , 7.8 mm by 300 mm), which indicates that it was almost pure protein. The purified enzyme can convert rutin to isoquercitrin from the qualitative analysis of the enzymatic product by the methods of HPLC

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Substrates	Hydrolyzed linkage
Rutin [3- <i>O</i> - α -L-Rha-(1 $\rightarrow$ 6)- β -D-Glc-quercetin]	3- <i>O</i> - α -L-(1 $\rightarrow$ 6)-Rha
Naringin [7- <i>O</i> - α -L-Rha-(1 $\rightarrow$ 2)- β -D-Glc-naringenin]	7- <i>O</i> - α -L-(1 $\rightarrow$ 2)-Rha
Hesperidin [7- <i>O</i> - α -L-Rha-(1 $\rightarrow$ 6)- β -D-Glc-hesperetin]	7- <i>O</i> - α -L-(1 $\rightarrow$ 6)-Rha
Hyperin (3- <i>O</i> - β -D-Gal-quercetin)	NA
Quercitrin (3- <i>O</i> - α -L-Rha-quercetin)	NA
Isoquercitrin (3- <i>O</i> - β -D-Glc-quercetin)	NA
Icariin (3- <i>O</i> - α -L-Rha, 7- <i>O</i> - β -D-Glc-icariin)	NA
Re [6- <i>O</i> - α -L-Rha-(1 $\rightarrow$ 2)- β -D-Glc, 20(<i>S</i>)- <i>O</i> - β -D-Glc-protopanaxatriol]	NA
Dioscin { 3- <i>O</i> - α -L-Rha-(1 $\rightarrow$ 4), [α -L-Rha-(1 $\rightarrow$ 2)]- β -D-Glc-diosgenin }	NA
<i>p</i> NPR (<i>p</i> -nitrophenyl- α -L-Rhamnoside)	NA
Soluble starch	NA

FIG 2 Substrate specificity of the rutin- α -L-rhamnosidase. Rha, rhamnose; Glc, glucose; Gal, galactose; NA, no activity.

(Knauer C-18; Φ , 3 mm by 300 mm) and nuclear magnetic resonance (NMR) (Bruker DR-400; Germany) (30).

The optimal pH and temperature of the purified enzyme (with rutin as a substrate) were 5.0 and 50°C, respectively. The enzyme had over 75% activity in the pH interval from 2.0 to 6.5, had over 80% activity below 60°C after 1 h of incubation, and still retained 40% activity at 70°C, but the activity was inactivated at 80°C after 1 h of incubation. The K_m and V_{max} of this enzyme for rutin were approximately 11.7 mM and 56.1 mM $\cdot$ h $^{-1}$, respectively.

The substrate specificity of rutin- α -L-rhamnosidase was analyzed with flavonoids (28), saponins (6), *p*-nitrophenyl- α -L-rhamnoside (*p*NPR) (6), and soluble starch (7) as described previously (all substrates from Sigma). The purified enzyme can hydrolyze 3-*O*- α -(1 $\rightarrow$ 6)-L-rhamnoside of rutin, 7-*O*- α -(1 $\rightarrow$ 6)-L-rhamnoside of hesperidin, and 7-*O*- α -(1 $\rightarrow$ 2)-L-rhamnoside of naringin (Fig. 2) but cannot hydrolyze *p*NPR, α -L-rhamnosides of saponins, such as ginsenoside Re and dioscin, or other glycosides of flavonoids, such as 3-*O*- β -D-galactoside of hyperin, 3-*O*- α -L-rhamnoside of quercitrin, 3-*O*- α -L-rhamnoside or 7-*O*- β -D-glucoside of icariin, or 3-*O*- β -D-glucoside of isoquercitrin (Fig. 2).

The N-terminal amino acid sequence of the new enzyme was identified as LSAAEWRTQSIYFLLT... using a liquid sequenator from Applied Biosystems (model 491) (23). Based on the homology blasted from NCBI, the upstream primer (5'-TGAAGTCCA

ACTTCGGCACT-3') and downstream primer (5'-ATGTAGCT GAGGACGTTTTTSG-3') were designed. The total RNA was isolated from *A. niger* DLFCC-90 with the Catrimox-14 RNA isolation kit (TaKaRa, Dalian, China). Using isolated total RNA as the template, first-strand cDNA was synthesized by a reverse transcription (RT)-PCR method with the TaKaRa 3'-Full rapid amplification of cDNA ends (RACE) core set, version 2.0, and then a partial cDNA of 640 bp was subsequently amplified using the two specific primers mentioned above. Using primers based on the known sequence described above, the 5' and 3' cDNA ends of *rhaR* were amplified as 612 bp and 745 bp long with the TaKaRa 5'-Full RACE kit and the TaKaRa 3'-Full RACE kit, respectively. The complete sequence of *rhaR* was identified to be 1,865 bp long from the three overlapping PCR amplification products mentioned above. The open reading frame (ORF) of *rhaR* encodes 505 amino acids, with a signal peptide of 21 amino acids. The molecular mass of this enzyme, calculated from the amino acid sequences, is 53 kDa, which is much smaller than the 66 kDa estimated from the SDS-PAGE gel (Fig. 1A), indicating that the enzyme secreted from *A. niger* DLFCC-90 is possibly glycosylated (14, 15, 16).

The new enzyme gene (*rhaR*) exhibits high similarity with the α -amylase gene (EC 3.2.1.1) from aspergilli. The ORF of *rhaR* is completely identical to those of two predicted α -amylase genes from aspergilli (GenBank accession numbers XM_001394298 and HQ186299).

The *rhaR* cDNA was subcloned into the vector pPIC9K (Invitrogen) and then transformed into *P. pastoris* GS115 (Invitrogen), and the recombinant *Pichia* strain was grown in BMGY medium using methanol as an inducer (21). There appeared to be a methanol-inducible (0.5%, vol/vol) protein with an apparent molecular mass of 66 kDa, which is in agreement with the molecular mass of the enzyme from *A. niger* DLFCC-90, but there was no specific band in noninduced cells (Fig. 1B). The crude cell extracts from *P. pastoris* GS115 which harbored expression vector showed derhamnosylation activity for rutin, naringin, and hesperidin (data not shown).

The amino acid sequence of rutin- α -L-rhamnosidase was compared with that of an α -amylase (Protein Data Bank [PDB] accession number 2AAA). Both enzymes consist of 484 amino acids, and similarity of above 95% was found between these two enzymes (Fig. 3). The new rutin- α -L-rhamnosidase contains amino acids (Asp206, Glu230, and Asp297) relating to α -amylase catalysis, but the new enzyme hydrolyzes α -L-rham-

FIG 3 Sequence alignment of rutin- α -L-rhamnosidase and 2AAA. The identical residues in the individual columns are indicated with a black background. The conserved amino acid residues (Asp206, Glu230, and Asp297) of 2AAA are indicated by black rectangles.

noside of flavonoid and cannot hydrolyze soluble starch (Fig. 2), unlike α -amylase. The new rutin- α -L-rhamnosidase is therefore obviously a novel type of flavonoid glycoside-hydrolase. Based on amino acid sequence similarities, the novel enzyme was classified as GH family 13.

Nucleotide sequence accession number. The nucleotide sequence for *rhaR* has been submitted to the GenBank database (accession number [EU200666](#)).

Protein structure accession number. The predicted amino acid sequence of the new rutin- α -L-rhamnosidase has been submitted to the NCBI Protein Database (accession number [ABW81206](#)).

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