

Full Length Research Paper

Purification and characterization of *Aspergillus niger* α -L-rhamnosidase for the biotransformation of naringin to prunin

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An α -L-rhamnosidase, which was extracted from the fermented broth of *Aspergillus niger* was purified, characterized and confirmed via biotransformation of naringin to prunin. After being purified to homogeneity by ammonium sulphate fractionation and chromatography on diethylaminoethanol (DEAE) Sepharose and Sephacryl S-200 HR columns, the enzyme was determined by the exclusive gel chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to have a molecular weight of approximately 87 kDa. Its optimal pH and stable pH values were within the range of 4.5 to 5 and 3.5 to 7.5, respectively while its optimal temperature was in 50 to 60°C. In addition, the enzyme was strongly inhibited by Fe²⁺, Fe³⁺, Zn²⁺, Al³⁺, Mn²⁺, Cu²⁺, Ag⁺, Hg²⁺ ions and sodium dodecyl sulfate (SDS) and slightly activated by K⁺ and Ba²⁺ ions. Its K_m towards naringin was 0.27 mM and the V_{max} was 9805.15 U/mg. The enzyme could efficiently convert naringin to prunin with a transforming rate above 97%. These results indicate that the α -L-rhamnosidase separated from *A. niger* could be a promising candidate for its commercial application in food and pharmaceutical industries.

Key words: *Aspergillus niger*, α -L-rhamnosidase, naringinase, naringin, prunin, transformation.

INTRODUCTION

Naringin (4',5,7'-trihydroxyflavanone-7-rhamnoglucoside) and naringenin (4',5,7'-trihydroxyflavanone) are two flavonoids, which possess strong anti-inflammatory, antiulcer, anticancer and antioxidative activities (Parmar, 1983; Chen et al., 1990; Martin et al., 1994; Gordon et al., 1995; So et al., 1996; Heim et al., 2002; Calgarotto et al., 2007; Ekambaram et al., 2008). However, they also have some undesirable properties. For example, naringin has an intense bitter taste with a low bitter taste threshold of 20 μ g/ml (Ribeiro and Ribeiro, 2008). Naringenin hardly dissolve in water (Tommasini et al., 2004).

Prunin (4',5,7'-trihydroxyflavanone-7- β -D-glucoside) is a

structure analogue of naringin and naringenin (Kaul et al., 1985; Choi et al., 1991a, b; Chang et al., 2011). It exhibits combined advantages of both naringin and naringenin, that is, strong bioactivity, good solubility and little bitter taste (Puri et al., 1996). However, prunin naturally exists in a low quantity. As a result, much effort has been tried to develop an efficient process to transform naringin to prunin, taking advantage of the naringin that is commercially available in a large quantity as a citrus byproduct. Fox et al. (1953) reported a procedure by acidic transformation of naringin to prunin, but it needed strict reaction conditions and complicated purification steps. In contrast, enzymatic method is more desirable due to its simpler process and lower production cost because the enzymatic reaction can be controlled under a milder and more environmentally friendly condition with high efficiency and high specificity.

Although some studies (Roitner et al., 1984; Soria and

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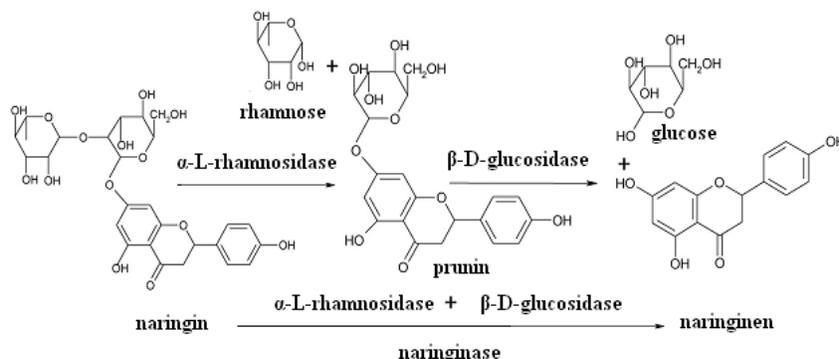


Figure 1. Mechanism of enzymatic conversion of naringin to prunin.

Ellenrieder, 2002; Kaur et al., 2010) have demonstrated the possibility of preparing prunin by the enzymatic method, so far prunin has not been commercially produced due to the lack of industrial biocatalyst. The enzyme, α -L-rhamnosidase (E. C. 3.2.1.40) which cleaves the α -1, 2-glycosidic bond is responsible for the biotransformation of naringin to prunin (Figure 1) (Yadav et al., 2010). It has been reported that α -L-rhamnosidase usually joins together with β -D-glucosidase to form naringinase (Figure 1) (Yadav et al., 2010). Naringinase and α -L-rhamnosidase has been reported from some microorganisms (Yoshikazu et al., 1973; Manzanares et al., 1997; Spagna et al., 2000; Yadav et al., 2010; Puri, 2011), among which *Aspergillus niger* is considered the most potential and promising resource for industrial practice because this fungus not only has been listed in the Food and Drug Administration (FDA'S) approved microbial category and proven safe for food and medicinal use, also is able to be induced to efficiently produce some food-grade enzymes (Pel et al., 2007), including the naringinase and the α -L-rhamnosidase. Moreover, the fermentation process is easy to be scaled up because its technology is pretty mature and has been widely used in industry (Pel et al., 2007).

Studies have concerned in the purification of α -L-rhamnosidase from *Aspergillus niger* and other microorganisms (Yadav et al., 2010; Chang et al., 2011; Puri, 2011; Ribeiro, 2011), but α -L-rhamnosidase has been seldom investigated in the preparation of prunin. Recently, a new strain DB056 of *A. niger* that could produce a high yield of naringinase (complex of α -L-rhamnosidase and β -D-glucosidase) has been screened, along with a successful optimized scale-up process for naringinase production in a 200 L fermentor (data not shown) in our laboratory in Jimei University, China. Therefore, it presented a commercially available source of α -L-rhamnosidase. In this context, it is valuable to purify and characterize the α -L-rhamnosidase for the enzymatic preparation of prunin. The p-nitrophenyl- α -L-rhamnopyranoside (pNPR) method (Romero et al., 1985) is commonly used to monitor the

activity of α -L-rhamnosidase. However, it could not monitor the biotransformation of prunin. In the present study, the α -L-rhamnosidase was purified and characterized to transform the prunin by using a high performance liquid chromatography (HPLC) method enabled to simultaneously differentiate naringin, prunin and naringenin instead of the pNPR method.

MATERIALS AND METHODS

Reagent and chemical

Diethylaminoethanol (DEAE)-Sephacryl S-200 HR, acrylamide and sodium dodecyl sulfate (SDS) were purchased from Amersham Biosciences (Uppsala, Sweden). Protein markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Fermentas VAB (Vilnius, Lithuania). Naringin, prunin, naringenin, ethylene diamine tetraacetic acid (EDTA), dithiothreitol (DTT), pNPR, bovine serum albumin, Coomassie brilliant blue, ammonium persulfate and substrates were purchased from Sigma (St. Louis, MO). Methanol, acetonitrile were HPLC grade and purchased from Tedia Company Inc. (Fairfield, Ohio, USA). All other reagents were of analytical grade.

Cultivation of *A. niger* for producing naringinase

The strain *A. niger* DB056 was inoculated onto slant media in composition (g/L) of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0, KH_2PO_4 1.0, $(\text{NH}_4)_2\text{SO}_4$ 1.5, KCl 0.5, KNO_3 1.5, CaCl_2 0.1, yeast extract 2.0, naringin 2.5 and agar 20. The spores were grown at pH 6.0 and 28°C for 3 days before they were washed and adjusted to make spore suspension to OD_{600} 0.2. Then they were inoculated into a NBS Bioflo-110 7 L fermentor which contained 5 L of fermental media according to Puri and Kalra (2005) with minor modification. The medium composition was (g/L): naringin 10, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, KH_2PO_4 1.5, $(\text{NH}_4)_2\text{SO}_4$ 4.0, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.09, CaCl_2 0.1, yeast extract 1.0, soybean powder 2.0 and peptone 2.0. The strain was cultured at 28°C, pH 6.0, in 300 rpm for 7 days for yielding enough amount of naringinase. The broth was centrifuged at 4500 rpm for 15 min to remove the cells and collect the supernatant.

Determination of enzymatic activity of α -L-rhamnosidase

A HPLC method which was developed based on the principle of Yadav et al. (2010) was applied to simultaneously determine naringin

prunin and naringenin so that the α -L-rhamnosidase activity could be determined. Detailed procedure is described as follows; Two milliliters of 300 μ g/mL naringin solution was mixed with 1.9 ml of 10 mM citric acid buffer (pH 5.0) and warmed up in a 40°C water bath for 5 min. Then, the reaction was immediately initiated by adding 100 μ l of properly diluted enzyme solution and incubated at 40°C for 15 min. The reaction solution was heated to 100°C to denature the enzyme. The mixture was then filtered through 0.22 μ m membranes prior to the HPLC analysis. A Waters 1525 HPLC instrument equipped with a 2487 UV detector and a Symmetry C18 reversed-phase column (4.6 \times 150 mm, 3.5 μ m) was controlled by the Breeze Chromatographic software. The mobile phase was composed of 11.4% methanol, 26.6% acetonitrile and 62% purified deionized water, with an isocratic elution flow at 0.4 ml/min. Sample injection was in a volume of 20 μ l. The column temperature was at 35°C, the detective wavelength was at 280 nm and the running time was 28 min under an isocratic elution procedure. One unit of α -L-rhamnosidase was defined as the enzyme degraded 1 μ g naringin within 1 min.

Determination of protein concentration

For the purification, the protein was estimated by measuring the absorbance at 280 nm. While for the characterization, the protein was analyzed by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Purification of α -L-rhamnosidase

The broth was centrifuged at 4°C, 10,000 g for 30 min in a centrifuge (Avanti J-25, Beckman Coulter, USA). The supernatant was fractionated with ammonium sulphate from 50 to 80% concentration. The resulting pellet was dissolved in a minimum volume of 10 mM sodium citrate buffer (pH 5.5) containing 5 mM DTT and dialyzed against the same buffer extensively. The dialysate was subsequently applied to a DEAE-Sepharose Fast Flow column (2.5 \times 16 cm), which was previously equilibrated with the dialysis buffer. Unabsorbed fractions were removed by washing the column with the dialysis buffer 5 times of volume of the column. Then, binding proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M in a total volume of 420 ml.

Fractions were collected in 3 ml/tube for analyses of the α -L-rhamnosidase activity and content of the protein. Those fractions showing the α -L-rhamnosidase activity (that is, displaying the ability to degrade naringin to prunin) were pooled together and dialyzed against 10 mM sodium citrate buffer (pH 5.5) containing 5 mM DTT. The pooled fractions from the DEAE-Sepharose Fast Flow column were concentrated by ultra filtration using a YM-30 membrane (Millipore, MA, USA) and applied to a Sephacryl S-200 HR gel-filtration column (1.5 \times 98 cm) equilibrated and washed with 20 mM citric acid buffer (pH 5.5) containing 0.15 M NaCl and 5 mM DTT at 0.5 ml/min. The fractions were collected in 2 ml/tube for analyses of the α -L-rhamnosidase activity and the concentration of protein. The eluted active fractions were pooled for enzyme characterization.

SDS-PAGE electrophoresis and molecular weight measuring

Molecular weight (MW) of the α -L-rhamnosidase was determined both by gel filtration chromatography (GFC) and SDS-PAGE. GFC was performed on the same Sephacryl S-200 HR column as aforementioned, while the SDS-PAGE was carried out in a Mini-protean III dual-slab cell electrophoresis according to the method of Laemmli (1970), using a 10% gel. The proteins were stained with the Coomassie brilliant blue R-250.

Effects of pH and temperature

Enzymatic activities of the purified α -L-rhamnosidase over the pH range of 3.0 to 8.5 were determined at the temperature of 40°C for 15 min using 50 mM of the following buffers: sodium citrate buffer (pH 3.0 to 5.5), sodium phosphate buffer (pH 6.0 to 7.0) and Tris-HCl buffer (pH 7.5 to 8.5). For temperature profile study, the activities were assayed at a temperature range between 20 and 65°C for 15 min at pH 5.0 using the 50 mM sodium citrate buffer.

pH and thermal stability

Effect of pH on stability of the purified α -L-rhamnosidase was evaluated by measuring the residual enzymatic activity after incubation under various pH values at 4°C for 24 h. The pH values (3.0 to 8.5) were kept by 50 mM sodium citrate buffer of pH 3.0 to 5.5, sodium phosphate buffer of pH 6.0 to 7.0 and Tris-HCl buffer of pH 7.5 to 8.5. To investigate the thermal stability, the purified α -L-rhamnosidase dissolved in 50 mM sodium citrate buffer (pH 5.0) was incubated at different temperatures from 4 to 60°C and sampled in different intervals depending on the temperature. Then the samples were immediately cooled in ice water before the residual activity was determined.

Effects of metal ions and reagents

The purified α -L-rhamnosidase were incubated with Fe^{2+} , Fe^{3+} , Zn^{2+} , Ca^{2+} , K^{+} , Al^{3+} , Mg^{2+} , Ba^{2+} , Mn^{2+} , Cu^{2+} , Na^{+} , Ag^{+} , Hg^{2+} , EDTA- Na_2 , DTT, SDS, urea, dimethylsulfoxide (DMSO), mercaptoethanol, glycerol and citric acid at final concentrations of 1 and 5 mM in the 50 mM sodium citrate buffer (pH 5.0) at room temperature ($25 \pm 1^\circ\text{C}$) for 24 h. Then, the residual enzymatic activity was measured as aforementioned. The experimental control was performed without any addition of metal ions or chemical reagents.

Determination of Michaelis constant

The reaction solutions were prepared to contain 0.06 mg/mL of the enzyme and 300, 200, 100, 50, 25, 12.5 and 6.25 μ g/ml of naringin, respectively. The reaction was performed at 40°C for 3 min. Thereafter, the amount of consumed naringin was determined to calculate the enzymatic activities. The V_{max} and K_m were determined based on the Lineweaver-Burk plot.

Enzymatic transformation of naringin to prunin

The purified α -L-rhamnosidase at final concentration of 18.23 U/ml was applied to hydrolyze naringin under the condition described. The reaction mixture was sampled every 8 min to measure the content change of naringin, prunin and naringenin by the same HPLC method described. Meanwhile, the transforming rate was calculated according to the remaining amount of naringin.

RESULTS

Purification of α -L-rhamnosidase

The α -L-rhamnosidase was purified from the fermented broth of *A. niger* by ammonium sulfate precipitation and column chromatographies of DEAE-Sepharose Fast Flow column and Sephacryl S-200 HR. As shown in Figure 2A,

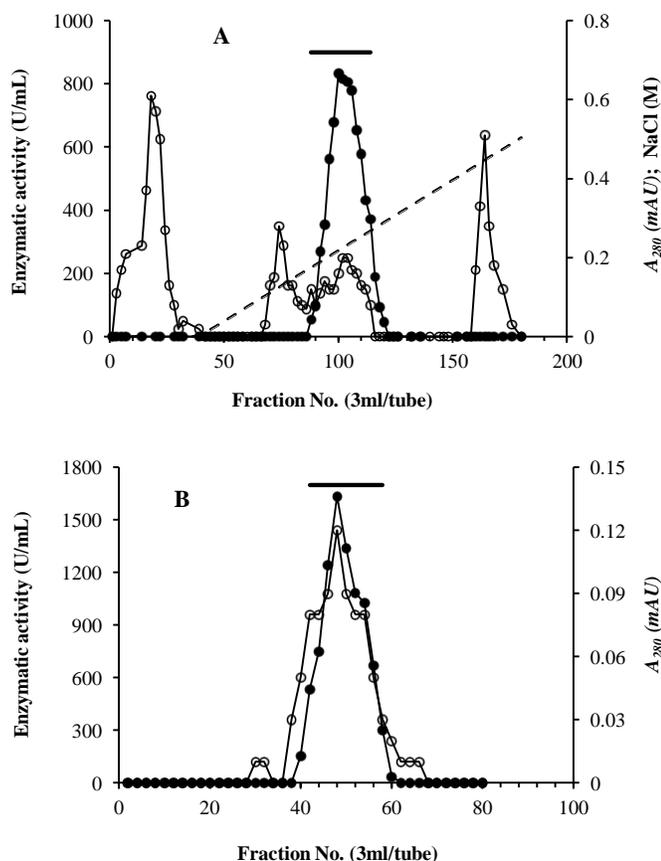


Figure 2. Chromatographic purification and analysis of α -L-rhamnosidase. (A) DEAE-sephrose column chromatography. (B) Sephacryl-S200 chromatography. Absorbance is at 280 nm. (\circ); α -L-Rhamnosidase activity (\bullet); concentration of NaCl (---); collected fractions (—).

a total number of 4 protein peaks were eluted from the DEAE-sepharose separation. The expected α -L-rhamnosidase was eluted out with NaCl concentration of 0.18 to 0.28 M and detected mainly in the second protein peak. All the peaks were separated well without overlaying, indicating that the DEAE-Sephacryl chromatography was efficient to be able to separate the α -L-rhamnosidase from other proteins.

The active fractions collected from the ion exchange chromatography were further applied to the Sephacryl S-200 column. The result (shown in Figure 2B) demonstrated that a single protein peak concurrently exhibited the enzyme activity. The molecular weight of this peak (that is, α -L-rhamnosidase) was measured around 87 kDa both by the SDS-PAGE (Figure 3) and Sephacryl S-200 (data not shown) after comparison with the standard protein markers. After the purification steps aforementioned, the α -L-rhamnosidase was purified by 77.68 fold with yield (recovery) of 36.53% and had a specific activity at 7678.8 U/mg (Table 1). Only one protein band shown in the SDS-PAGE analysis (Figure 3) indicated that the protein had been sufficiently purified to

homogeneity.

pH optimum and stability

The purified α -L-rhamnosidase (Figure 4) exhibited its maximal activity in pH range of 4.5 to 5.0. Also, it showed stabilities over a broad pH range of 3.5 to 7.5 (Figure 4), especially within pH 5 to 6, where the relative activity was maintained around 90% after 24 h. However, the enzyme became unstable when pH approached below 3.0 and above 8.0, where the relative activity showed a significant loss.

Temperature optimum and stability

The maximal activity of the α -L-rhamnosidase was at 50 to 60°C (Figure 5A). For its thermal stability, slight changes in activity were observed at 4°C after 10 days (Figure 5B). However, activities of the α -L-rhamnosidase tended to decrease at 25 and 37°C after 4 days (Figure 5B), at 50 and 55°C within 1 h (Figure 5C), at 60°C within 10 min (Figure 5D), respectively.

Effects of metal ions and reagents

As shown in Tables 2 and 3, the α -L-rhamnosidase was strongly inhibited by Fe^{2+} , Fe^{3+} , Al^{3+} , Cu^{2+} , Ag^{+} , Hg^{2+} ions and SDS and 5 mM citric acid; slightly restricted by Zn^{2+} , Ca^{2+} , Mg^{2+} and Mn^{2+} . However, it was slightly activated by K^{+} , Ba^{2+} ions at 1 mM and 5 mM, as well as DTT, urea, DMSO, glycerol and citric acid at 1 mM. Na^{+} , EDTA- Na_2 and mercaptoethanol seemed having no significant effect on α -L-rhamnosidase.

Validity of the α -L-rhamnosidase for transformation of naringin to prunin

The specific activity of the α -L-rhamnosidase increased with the increment of substrate concentration within 0 to 100 $\mu\text{g}/\text{ml}$ (Figure 6). The Michaelis constants, K_m and V_{max} of the α -L-rhamnosidase for hydrolysis of naringin were determined (Figure 6) at 156.74 $\mu\text{g}/\text{ml}$ (0.27 mM) and 9805.15 U/mg, respectively. Almost all the naringin was hydrolyzed (Figures 7B and 7C). No naringenin was observed during this process (Figure 7C). After the hydrolysis, 97% of the naringin was transformed to prunin (Figure 7D).

DISCUSSION

As described in the introduction, the α -L-rhamnosidase from *A.niger* naringinase was possibly the best feasible and practical option for industrial catalyses of naringin to

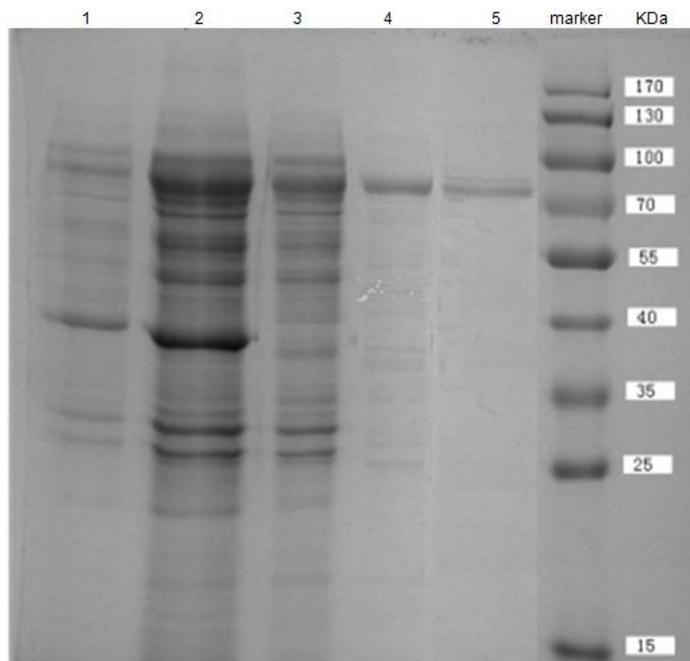


Figure 3. SDS-PAGE of the purified α -L-rhamnosidase. Lane 1 is the broth supernatant, lane 2 is the ammonium sulphate precipitation fraction, lane 3 is the dialysate of the ammonium sulphate precipitation, lane 4 is the fraction from DEAE-sephrose column, and lane 5 is the fraction from Sephacryl-S200 column.

Table 1. Purification of α -L-rhamnosidase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purified fold
Broth supernatant	922.96	91243.8	97.2	100	1
Ammonium sulphate precipitation	161.5	54442.8	334.8	59.67	3.41
Dialysis	32.7	44857.8	1371.6	49.17	13.88
DEAE-Sepharose chromatography	5.64	40294.8	7144.2	44.16	72.27
Sephacryl S-200 chromatography	4.34	33328.8	7678.8	36.53	77.68

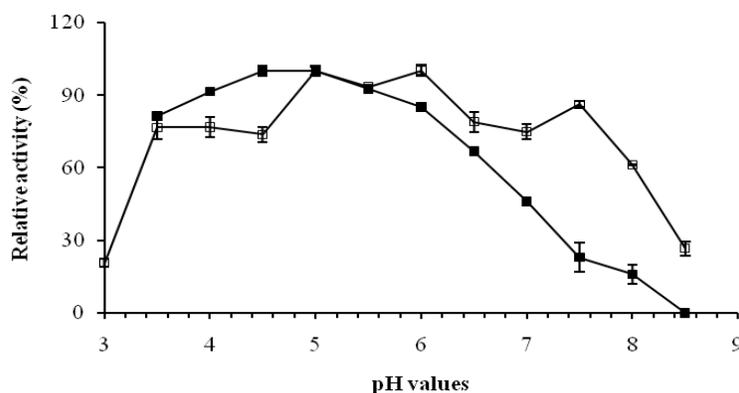


Figure 4. Effect of pH on α -L-rhamnosidase activity: pH optimum (■); pH stability(□).

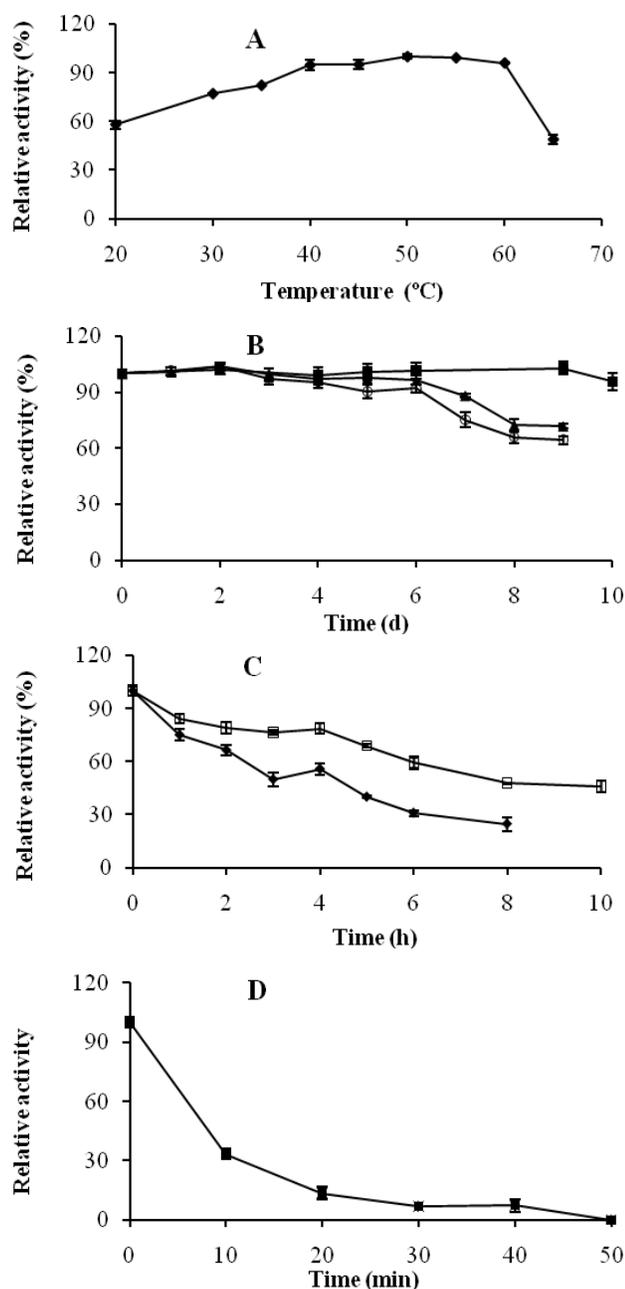


Figure 5. Effect of temperature on α -L-rhamnosidase activity. (A) Temperature profiles; (B) temperature stability at 4(■), 25(▲), 37°C(○); (C) temperature stability at 50(□), 55°C(◆); (D) thermal stability at 60°C.

prunin so far. By using the HPLC method to facilitate detection of the enzymatic activity (Figure 7A), the α -L-rhamnosidase from *A. niger* has been purified to homogeneity (Figures 2 and 3) by ammonium sulphate precipitation and chromatographies on DEAE-sepharose and Sephacryl S-200 HR columns. The MW of the purified α -L-rhamnosidase was measured to be around 87 kDa, similar to a previously reported α -L-rhamnosidase from

A. niger (Manzanares et al., 1997). Although our purified α -L-rhamnosidase had similar pH optimal value in range of pH 4.0 to 5.0 as the previously reported α -L-rhamnosidases determined by the pNPR method (Manzanares et al., 1997; Spagna et al., 2000; Yoshikazu et al., 1973), their stable pH values are significantly different. In addition, our α -L-rhamnosidase showed an optimal temperature within 50 to 60°C, which was also different from other reported α -L-rhamnosidases from *A. niger* (Yoshikazu et al., 1973; Manzanares et al., 1997). Yet, its optimal pH, stable pH, optimal temperature and stable temperature were all in agreement with those of the naringinase from *A. niger* (Puri and Kalra, 2005).

Moreover, the molecular weight of our α -L-rhamnosidase which was similar to the SDS-PAGE analysis of the naringinase from *A. niger*, but less than the result of gel filtration analysis of the same naringinase (168 kDa) (Puri and Kalra, 2005), suggesting it was a subunit from naringinase. This enzyme was easy to be purified. Based on the chromatogram, remaining enzymatic activity and calculated purified fold (Figure 2B, Table 1), it is obvious that the ion exchange chromatography was so efficient to purify the α -L-rhamnosidases that further purification procedures, for example, gel filtration chromatography and affinity chromatography, were not necessary. Its K_m value (0.27 mM) indicating that our purified α -L-rhamnosidase has a high affiliation to naringin. Furthermore, the transforming rate and the residual naringin (Figure 7) were above 97% and less than 4%, respectively revealing the high efficiency and validity of the new purified α -L-rhamnosidase. These properties suggest our α -L-rhamnosidase was in high purity that can be used in food and pharmaceutical industries.

The purified enzyme and its process possessed the following three advantages: the enzyme that displayed a strong enzymatic activity and good stability in a wide pH and temperature range (Figures 3 and 4) would facilitate the bioconversion of naringin to prunin. It also facilitated the purification of prunin because the transforming rate was very high and the remaining naringin was left in small amount (Figure 7) and the enzymatic reaction can be promoted by K^+ ion and some other chemicals (Tables 2 and 3). Moreover, this α -L-rhamnosidase can be applied to eliminate the bitter taste of citrus juice, which is expected to having the following four advantages: the process is safe; it will not cause other nutritional loss, for the enzyme only specifically hydrolyzes naringin; it can hydrolyze naringin at natural and weak acidic pH value of the juice and unlike naringinase, it would not convert naringin to non-soluble naringenin which has to be removed by centrifuge and filtration during the juice processing.

Conclusion

The α -L-rhamnosidase from a fermented broth of *A. niger* was purified and characterized in light of its MW, optimal

Table 2. Effect of metal ions on the α -L-rhamnosidase activity.

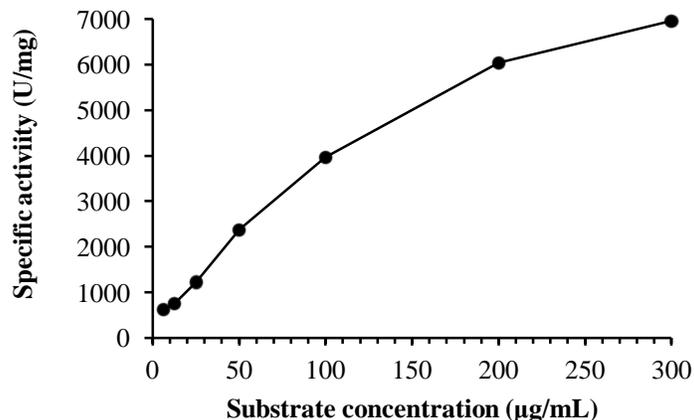
Metal ion	Relative activity of α -L-rhamnosidase (%)	
	1 mmol/L	5.0 mmol/L
Fe ²⁺	41.81 \pm 0.15 ^h	32.75 \pm 2.68 ^f
Fe ³⁺	10.51 \pm 0.28 ⁱ	5.79 \pm 2.14 ^{gh}
Zn ²⁺	79.47 \pm 0.86 ^f	72.14 \pm 0.98 ^e
Ca ²⁺	87.67 \pm 0.66 ^e	94.65 \pm 1.27 ^c
K ⁺	119.49 \pm 2.19 ^a	107.58 \pm 1.04 ^a
Al ³⁺	8.93 \pm 2.78 ⁱ	5.26 \pm 2.69 ^{gh}
Mg ²⁺	80.39 \pm 3.74 ^f	85.71 \pm 3.23 ^d
Ba ²⁺	113.58 \pm 2.64 ^b	106.01 \pm 0.46 ^a
Mn ²⁺	96.87 \pm 2.35 ^d	83.64 \pm 1.98 ^d
Cu ²⁺	58.79 \pm 2.30 ^g	7.26 \pm 0.14 ^g
Na ⁺	102.39 \pm 2.61 ^c	104.06 \pm 4.30 ^{ab}
Ag ⁺	9.40 \pm 1.83 ⁱ	4.00 \pm 0.57 ^h
Hg ²⁺	8.33 \pm 1.21 ⁱ	5.74 \pm 1.46 ^{hg}
Control	100 \pm 2.20 ^c	100 \pm 1.40 ^b

Means with different superscript letters within the same column are significantly different ($P < 0.05$).

Table 3. Effects of potential activator and inhibitor on activity of α -L-rhamnosidase.

Chemical	Relative activity of α -L-rhamnosidase (%)	
	1 mM	5 mM
EDTA-Na ₂	102.22 \pm 2.95 ^c	100.80 \pm 0.92 ^b
DTT	121.51 \pm 2.76 ^a	98.99 \pm 2.96 ^b
SDS	15.25 \pm 3.14 ^d	3.33 \pm 1.56 ^d
Urea	109.35 \pm 4.74 ^b	105.45 \pm 2.15 ^a
DMSO	110.74 \pm 1.35 ^b	101.00 \pm 0.75 ^b
Mercaptoethanol	100.12 \pm 1.06 ^c	100.56 \pm 1.45 ^b
Glycerol	109.58 \pm 0.18 ^b	100.33 \pm 1.14 ^b
Citric acid	111.68 \pm 1.89 ^b	32.65 \pm 3.26 ^c
Control	100 \pm 2.59 ^c	100 \pm 1.05 ^b

Means with different superscript letters within the same column are significantly different ($P < 0.05$).

**Figure 6.** Effect of substrate concentration on specific activity and the Lineweaver-Burk of α -L-rhamnosidase.

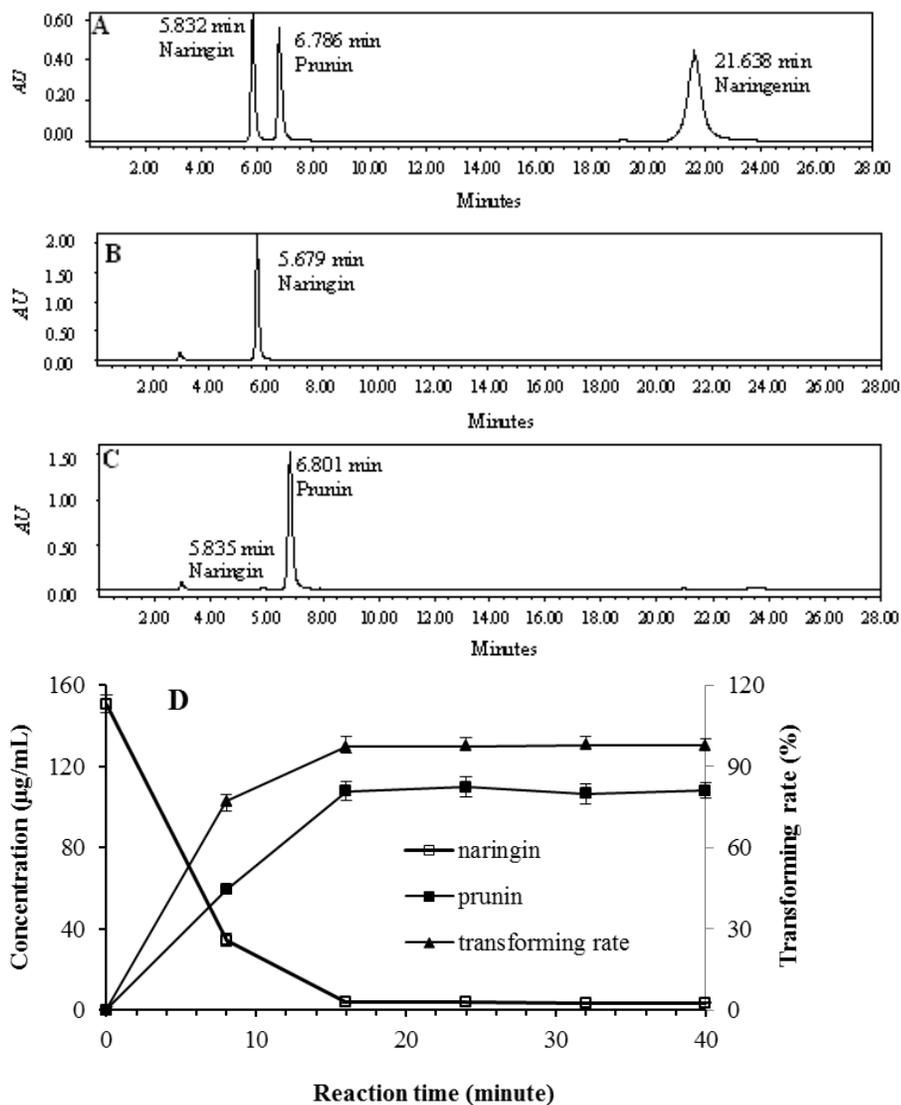


Figure 7. Enzymatic conversion of naringin to prunin by the α -L-rhamnosidase. A. The chromatogram of naringin, prunin and naringenin. B. The chromatogram of the control of the conversion experiment. C. The chromatogram of the sample of the conversion experiment. D. The concentration change of naringin and prunin.

pH value and its stable range, optimal temperature and its thermal stability, activators, inhibitors, substrate specificity, kinetic parameters and its ability to hydrolyze naringin to prunin. The present α -L-rhamnosidase showed a MW of about 87 kDa, with optimal pH 4.5 to 5.0, stable pH range of 3.5 to 8.0 and optimal temperature of 55 to 60°C. This enzyme could be inhibited by Fe^{2+} , Fe^{3+} , Zn^{2+} , Al^{3+} , Mn^{2+} , Cu^{2+} , Ag^+ , Hg^{2+} ions and SDS. It had a higher specificity to naringin than pNPR. Its K_m was 0.27 mM; the V_{max} was 9805.15 U/mg. This enzyme was highly efficient to convert the naringin to prunin with a transforming rate greater than 97%. These results suggest that this α -L-rhamnosidase from *A. niger* naringinase can have meaningful applications in food and pharmaceutical industries.

REFERENCES

- Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248-254.
- Calgarotto AK, Miotto S, Honorio KM, da Silva ABF, Marangoni S, Silva JL, Comar JM, Oliveira KMT, da Silva SL (2007). A multivariate study on flavonoid compounds scavenging the peroxynitrite free radical. *J Mol. Struct. Theochem.*, 808: 25-33.
- Chang H, Lee Y, Bae H, Huh J, Nam S, Sohn H, Lee HJ, Lee S (2011). Purification and characterisation of *Aspergillus sojae* naringinase: The production of prunin exhibiting markedly enhanced solubility with in vitro inhibition of HMG-CoA reductase. *Food Chem.*, 124: 234-241.
- Chen YT, Zheng RL, Jia ZJ (1990). Flavonoids as superoxide scavengers and antioxidants. *Free Radical Biol. Med.*, 9: 19-21.
- Choi JS, Yokozawa T, Oura H (1991a). Improvement of hyperglycemia and hyperlipemia in streptozotocin-diabetic rats by a methanolic

- extract of *Prunus davidiana* stems and its main component, Prunin. *Planta Med.*, 57: 208-211.
- Choi JS, Yokozawa T, Oura H (1991b). Antihyperlipidemic effect of flavonoids from *Prunus Davidiana*. *J. Nat. Prod.*, 54: 218-224.
- Ekambaram G, Rajendran P, Magesh V, Sakthisekaran D (2008). Naringenin reduces tumor size and weight lost in n-methyl-N'-nitro-N-nitrosoguanidine-induced gastric carcinogenesis in rats. *Nutr. Res.*, 28: 106-112.
- Fox DW, Savage WL, Wender SH (1953). Hydrolysis of some flavonoid rhamnoglycosides to flavonoid glucosides. *J. Am. Chem. Soc.*, 75: 2504-2505.
- Gordon PB, Holen I, Seglen PO (1995). Protection by naringin and some other flavonoids of hepatocytic autophagy and endocytosis against inhibition by okadaic acid. *J. Biol. Chem.*, 270: 5830-5838.
- Heim KE, Tagliaferro AR, Bobilya DJ (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.*, 13: 572-584.
- Kaul TN, Middleton EJrMD, Ogra PL (1985). Antiviral effects of flavonoids on human viruses. *J. Med. Virol.*, 15: 71-79.
- Kaur A, Singh S, Singh RS, Schwarz WH, Puri M (2010). Hydrolysis of citrus peel naringin by recombinant α -L-rhamnosidase from *Clostridium stercorarium*. *J. Chem. Technol. Biot.*, 85: 1419-1422.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- Manzanares P, de Graff LH, Visser J (1997). Purification and characterization of an α -L-rhamnosidase from *Aspergillus niger*. *FEMS Microbiol. Lett.*, 157: 279-283.
- Martin MJ, Marhuenda E, Perez GC, France JM (1994). Antiulcer effect of naringin on gastric lesions induced by ethanol in rats. *Pharmacology*, 49: 144-150.
- Parmar NS (1983). The gastric anti-ulcer activity of naringenin, a specific histidine decarboxylase inhibitor. *Intl. J. Tissue React.*, 5: 415-420.
- Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.*, 25: 221-231.
- Puri M (2012). Updates on naringinase: structural and biotechnological aspects. *Appl. Microbiol. Biotechnol.*, 93: 49-60.
- Puri M, Kalra S (2005). Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for transformation of flavonoids. *W. J. Microbiol. Biotechnol.*, 21: 753-758.
- Puri M, Marwaha SS, Kothari RM (1996). Studies on the applicability of alginate-entrapped naringinase for the debittering of kinnow juice. *Enzyme Microb. Tech.*, 18: 281-285.
- Ribeiro IA, Ribeiro MHL (2008). Naringin and naringenin determination and control in grapefruit juice by a validated HPLC method. *Food Control*, 19: 432-438.
- Ribeiro MH (2011). Naringinases: occurrence, characteristics, and applications. *Appl. Microbiol. Biotechnol.*, 90: 1883-1895.
- Roitner M, Schalkhammer TH, Pittner F (1984). Preparation of prunin with the help of immobilized naringinase pretreated with alkaline buffer. *Appl. Biochem. Biotechnol.*, 9: 483-488.
- Romero C, Manjon A, Bastida J, Iborra JL (1985). A method for assaying rhamnosidase activity of naringinase. *Anal. Biochem.*, 149: 566-571.
- So FV, Guthrie N, Chambers AF, Moussa M, Carroll KK (1996). Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr. Cancer*, 26: 167-181.
- Soria F, Ellenrieder G (2002). Thermal inactivation and product inhibition of *Aspergillus terreus* CECT 2663 α -L-rhamnosidase and their role on hydrolysis of naringin solutions. *Biosci. Biotechnol. Biochem.*, 66: 1442-1449.
- Spagna G, Barbagallo RN, Martino A, Pifferi PG (2000). A Simple method of purifying glycosidases: α -L-rhamnopyranosidase from *Aspergillus niger* to increase the aroma of Moscato wine. *Enzyme Microb. Tech.*, 27: 522-530.
- Tommasini S, Calabrò ML, Raneri D, Ficarra P, Ficarra R (2004). Combined effect of pH and polysorbates with cyclodextrins on solubilization of naringenin. *J. Pharm. Biomed. Anal.*, 3: 327-333.
- Yadav V, Yadav PK, Yadav S, Yadav KDS (2010). α -L-Rhamnosidase: a review. *Process Biochem.*, 45: 1226-1235.
- Yoshikazu K, Kayoko I, Fujio E (1973). α -L-rhamnosidase of the live of *Turbo cornutus* and *Aspergillus niger*. *J. Biochem.*, 73: 31-37.