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Purification and Characterization of a Naringinase from *Aspergillus aculeatus* JMUdb058

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Supporting Information

ABSTRACT: A naringinase from *Aspergillus aculeatus* JMUdb058 was purified, identified, and characterized. This naringinase had a molecular mass (MW) of 348 kDa and contained four subunits with MWs of 100, 95, 84, and 69 kDa. Mass spectrometric analysis revealed that the three larger subunits were β-D-glucosidases and that the smallest subunit was an α-L-rhamnosidase. The naringinase and its α-L-rhamnosidase and β-D-glucosidase subunits all had optimal activities at approximately pH 4 and 50 °C, and they were stable between pH 3 and 6 and below 50 °C. This naringinase was able to hydrolyze naringin, aesculin, and some other glycosides. The enzyme complex had a K_m value of 0.11 mM and a k_{cat}/K_m ratio of 14 034 s^{-1} mM^{-1} for total naringinase. Its α-L-rhamnosidase and β-D-glucosidase subunits had K_m values of 0.23 and 0.53 mM, respectively, and k_{cat}/K_m ratios of 14 146 and 7733 s^{-1} mM^{-1}, respectively. These results provide in-depth insight into the structure of the naringinase complex and the hydrolyses of naringin and other glycosides.

KEYWORDS: *naringinase, Aspergillus aculeatus, identification, hydrolysis, kinetics*

INTRODUCTION

Naringinase is an enzyme complex that possesses the activities of both α-L-rhamnosidase (EC 3.2.1.40) and β-D-glucosidase (EC 3.2.1.21). This enzyme has many important applications in the food and pharmaceutical industries because of its ability to hydrolyze many glycosides, e.g., 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides, naringin, hesperidin, and rutin.[1,2] Naringinase specifically hydrolyzes naringin to naringenin,[1,3,4] resulting in an improvement in the taste of citrus juice.[5] In addition, it efficiently removes the hesperidin haze from orange products[1] and enhances the aroma of wine and grape juices.[6,7] Moreover, it also plays an important role in modification of flavonoids to yield compounds of high bioactivity.[2,8]

Naringinase has been found in some fungi, yeasts, and bacteria, e.g., *Aspergillus niger*,[9] *Penicillium decumbens*,[10] *Williopsis californica*,[11] and *Staphylococcus xylosus*.[12] Among these, the fungi *Aspergillus* has been regarded as the best source because many strains of *Aspergillus* have been approved by the FDA to produce enzymes for the food industry, and their naringinases are active and stable at acidic pH values.[1] Several research groups have purified naringinase, α-L-rhamnosidase, and β-D-glucosidase from *Aspergillus* and other organisms[8,13−19] and cloned some of these genes.[14,20−25] However, most naringinases exhibited a single band upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis,[1,16−19] which made it difficult to separate the subunits and study their structures. Thus far, the structure (how do the α-L-rhamnosidase and β-D-glucosidase subunits form the naringinase complex) has yet to be elucidated.

Although some studies have focused on determining the kinetic parameters of naringinase, α-L-rhamnosidase, or β-D-glucosidase using the Davis and *p*-nitrophenyl methods,[4,10] the hydrolysis kinetic study of naringin, which represents its most important application, has been difficult because of the intrinsic limitations of these methods. Naringinase is commonly assayed by the Davis method, which is based on the colonization reaction between flavonones and alkaline diethylene glycol.[26] However, naringin (substrate), prunin (intermediate), and naringenin (product) all generate colonization complexes with similar absorption spectra, which makes it difficult to accurately determine the kinetic parameters of naringinase.[27] The artificial substrates *p*-nitrophenyl-α-L-rhamnopyranoside (pNPR) and *p*-nitrophenyl-α-L-glucopyranoside (pNPG) allow rapid analysis of α-L-rhamnosidase and β-D-glucosidase,[28,29] but they could not monitor the reaction velocity in the hydrolysis of special glycosides such as naringin. In comparison, a high-performance liquid chromatography (HPLC) method based on distinguishing naringin, prunin, and naringenin could determine the activities of naringinase, α-L-rhamnosidase, and β-D-glucosidase using naringin and prunin as substrates.[19] This HPLC method would overcome the limitations of the Davis and *p*-nitrophenyl methods and enable the kinetic study of the hydrolyses of naringin and other glycosides.

Recently, the naringinase synthesizing *Aspergillus aculeatus* strain JMUdb058 has been isolated in our lab. In this study, its naringinase (an enzyme complex consisting of α-L-rhamnosidase and β-D-glucosidase) was purified to determine its primary

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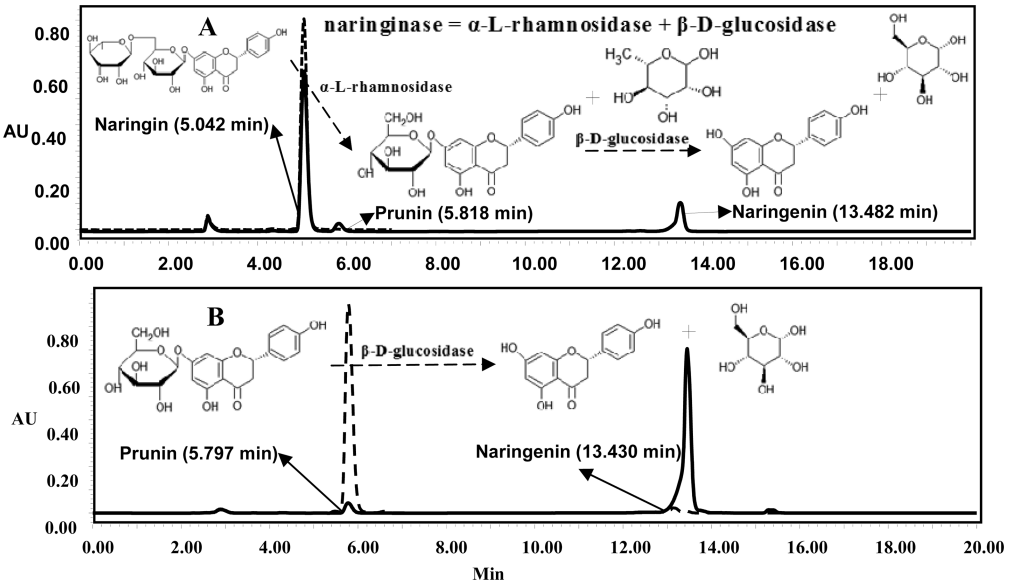

Figure 1. (A) Chromatographs of the reaction mixtures before (solid line) and after (dashed line) enzymatic hydrolysis of naringin (4,5,7-trihydroxy-flavanone-7-rhamnoglucoside). Activity of naringinase complex that releases naringenin (4,5,7-trihydroxyflavonone) from naringin is determined by quantification of the liberated naringenin using naringin as the substrate, and the α-L-rhamnosidase activity that hydrolyzes naringin to release prunin (4,5,7-trihydroxy-flavanone-7-glucoside) and rhamnose is determined by quantification of the consumed naringin. (B) Chromatographs of reaction mixtures before (solid line) and after (dashed line) the enzymatic hydrolysis of prunin, from which either the prunin consumed or the naringenin generated is quantified to determine the β-D-glucosidase activity.

structure, which would reveal the basic structure of the enzyme. In addition, kinetic study of the hydrolysis of naringin was performed using an HPLC method to monitor enzyme activity, which provides insight into the kinetic characteristic of the hydrolysis of this glycoside.

MATERIALS AND METHODS

Chemicals and Reagents. DEAE-Sepharose Fast Flow, Sephacryl S 300HR, acrylamide, and sodium dodecyl sulfate (SDS) were purchased from Amersham Biosciences Inc. (Uppsala, Sweden). Protein markers for SDS-PAGE were products from Fermentas VAB (Vilnius, Lithuania). Prunin was purchased from Extrasysthese (Genay Cedex, France). Naringin, naringenin, dithiothreitol (DTT), bovine serum albumin (BSA), Coomassie Brilliant Blue-R250, and ammonium persulfate were purchased from Sigma-Aldrich (St. Louis, MO). Hesperidin, arbutin, myricitrin, salicin, and aesculin were purchased from Xian Xiaocao Botanical Development Co. Ltd. (Xian, China). HPLC-grade methanol and acetonitrile were products from Tedia Co. Inc. (Fairfield, OH). All other reagents were of analytical grade.

Strain and Cultivation. JMUdb058 was identified as a strain of *A. aculeatus*. In brief, its mycelium and spores were identical to characteristics of a typical *A. aculeatus*. In the NCBI database, the *A. aculeatus* JMUdb058 26S rDNA sequence (accession number JQ301899) was 100% homologous with the 26S rRNA gene (accession number EU381181.1) of the *A. aculeatus* strain NRRL 360.

A. aculeatus JMUdb058 was cultured by a modified method of our previous study.[19] This strain was activated on media containing (g/L) $MgSO_4 \cdot 7H_2O$ (1.0), KH_2PO_4 (1.0), $(NH_4)_2SO_4$ (1.5), KCl (0.5), KNO_3 (1.5), $CaCl_2$ (0.1), yeast extract (2.0), naringin (2.5), and agar (20) at 28 °C for 96 h. Spores were then collected in water. Subsequently, 50 mL of the spore suspension (OD_{600} = 0.2) were inoculated into a NBS Bioflo-110 7-l fermenter with 5 L of medium containing (g/L) $MgSO_4 \cdot H_2O$ (0.5), KH_2PO_4 (1.5), $(NH_4)_2SO_4$ (4.0), $ZnSO_4 \cdot 7H_2O$ (0.09), $CaCl_2$ (0.1), yeast extract (1.0), naringin (5.0), soybean powder (2.0), and peptone (2.0), pH 6.0. After 7 days of cultivation at 28 °C (300 rpm and air flow of 0.8 L/min), a broth with a naringinase activity of 1.16 U/mL was obtained.

Purification of Naringinase. Naringinase was purified by a modified method based on previous studies.[16,19] The broth (460 mL) was centrifuged (Avanti J-25, Beckman Coulter, USA) at 4 °C and 15 000*g* for 20 min. Then the supernatant (450 mL, 1.10 U/mL) was fractionally precipitated by ammonium sulfate from 40% to 80% saturation. The resultant pellet was dissolved in the minimum necessary quantity of 20 mM citrate buffer (pH 6.0), followed by dialysis against the same buffer for 24 h at 4 °C. The dialysate was applied to a DEAE-Sepharose Fast Flow column (1.6 × 20 cm, Amersham Biosciences Inc., Uppsala, Sweden) that was previously equilibrated with five times the column volume of 20 mM citrate buffer (pH 6.0). With a fraction size of 3 mL, elution (after washing with 300 mL of 20 mM citrate buffer) was performed at a flow rate of 1 mL/min with a linear NaCl gradient from 0 to 0.8 M in a final volume of 200 mL and an isocratic elution of 0.8 M NaCl. The naringinase fractions were pooled and concentrated using an ultrafilter membrane (cutoff of 10 kDa) and an Amicon 8050 Ultra filtration Cell (Millipore Corp., MA). Subsequently, the concentrated fraction was loaded onto a Sephacyl S 300 HR column (1.6 × 60 cm, Amersham Biosciences Inc., Uppsala, Sweden) that had been equilibrated with 20 mM citrate buffer containing 0.15 M sodium chloride. This process was followed by elution with a fraction size of 1 mL at a flow rate of 0.4 mL/min with 20 mM citrate buffer. The fractions were collected to analyze the activity of naringinase and the concentration of protein.

Analysis of Enzyme Activities. Enzyme activities were determined according to the reaction scheme proposed by Puri and Banerjee[1] and Yadav et al.[4] As shown in Figure 1, naringin was used as the substrate to determine the activities of α-L-rhamnosidase and naringinase, and prunin was applied to determine the activity of β-D-glucosidase. Units of naringinase and β-D-glucosidase were defined as the amounts of the enzymes that hydrolyzed naringin and prunin, respectively, to release 1 μM of naringenin per minute at 50 °C and pH 4.0. Additionally, one unit of α-L-rhamnosidase was defined as the amount of enzyme that consumed 1 μM of naringin per minute at 50 °C and pH 4.0.

The analysis procedure, which included performing the enzymatic reactions and quantifying the substrates and products, was conducted according to Ni et al.[11] with some modifications. An assay mixture composed of 1.9 mL of 50 mM citrate buffer (pH 4.0), 2 mL of 200 μg/mL substrate solution (naringin for the analysis of naringinase and α-L-rhamnosidase activities or prunin for the analysis of β-D-glucosidase activity), and 0.1 mL of enzyme solution was incubated

at 50 °C for 5 min followed by heating at 100 °C for 30 min to inactivate the enzyme. After filtration through a 0.22 μm filter (Generay Biotech Co., Ltd., Shanghai, China), the reaction mixture was subjected to HPLC for analysis. This process was repeated for the control experiment using inactivated enzyme instead of fresh enzyme solution. A Waters 2695 HPLC coupled with a Symmetry C_{18} reverse phase column (bonded C_{18} ligands on a high-purity base-deactivated silica) (4.6 × 150 mm, 3.5 μm) and a 2487 UV detector (Waters Corp., Milford, MA) was used to determine the concentrations of the substrates and products. Following an injection of 20 μL of reaction mixture, the column was eluted using a gradient elution at 35 °C and 0.4 mL/min. The mobile phase was composed of water (A), methanol (B), and acetonitrile (C). The gradient procedure began with A:B:C = 62:12:26 within 0−7 min. This procedure was followed by a linear change to A:B:C = 15:35:50 within 7−9 min, another linear change to A:B:C = 15:35:50 within 9−15 min, a linear return to A:B:C = 62:12:26 within 15−17 min, and maintaining at A:B:C = 62:12:26 within 17−20 min. The target compounds were captured in the 2487 UV detector at 280 nm.

Analysis of the Concentration of Protein. The protein content in the purification was monitored by ultraviolet spectrophotometry (A_{280}), whereas the concentration of protein for the kinetic studies was determined using the Bradford assay with bovine serum albumin (BSA) standards.[30]

Determination of the Molecular Mass (MW) of Naringinase and Its Subunits. The MW of naringinase was analyzed by comparing the elution volume of the enzyme with the protein standard (HMW, GE Health, USA) using a standard curve. The MWs of the subunits were measured by SDS-PAGE using a Mini-protean III dual-slab cell electrophoresis[31] and a 10.0% gel, which was stained with Coomassie Brilliant Blue R-250.

Identification of Subunits of Naringinase. Naringinase was separated on a 10.0% native-PAGE gel, which was run at 8 mA and 4 °C for approximately 120 min. The resulting protein bands were extracted to analyze the activities of α-L-rhamnosidase and β-D-glucosidase. Furthermore, the protein bands on the SDS-PAGE gel from the above section were extracted followed by washing with water twice, destaining with 10% ethanol and 10% acetic acid, dehydrating twice with acetonitrile for 20 min, and in-gel digesting overnight with a sequencing grade modified porcine trypsin. Peptides were extracted with a 50% acetonitrile and 0.1% formic acid solution, dried thoroughly using a Savant SpeedVac concentrator (Thermo Electron Corp., Pittsburgh, PA), and dissolved in a 5% acetonitrile and 0.1% formic acid solution. Identification of the peptides was performed on a Nano Aquity UPLC system (Waters Corp., Milford, USA) connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with an online nanoelectrospray ion source (Michrom Bioresources, Auburn, USA). Peptides were separated by a Cap trap column (0.5 × 2 mm, Michrom Bioresources Inc.) and a 15 cm reverse-phase column (100 μm i.d., Michrom Bioresources Inc.).

The peptide mixtures were injected onto the trap column at a flow of 20 μL/min for 5 min and subsequently eluted with a 0.1% formic acid solution (mobile phase A) and acetonitrile containing 0.1% formic acid (mobile phase B) by a three-step linear gradient (0−35 min, B increased from 5% to 45%; 35−40 min, B increased to 80%; 40−45 min, B held at 80%; 45−60 min, B reduced from 80% to 5%). Flow rate was maintained at 500 nL/min, and column temperature was kept at 35 °C. An electrospray voltage of 1.9 kV versus the inlet of the mass spectrometer was used.

An LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to allow an automatic switch between MS and MS/MS acquisition. Full-scan MS survey spectra with two microscans (m/z 400−2000) were acquired using the Orbitrap, with a mass resolution of 60 000 at m/z 400, followed by eight sequential LTQ-MS/MS scans. Dynamic exclusion was used with two repeat counts: a 10 s repeat duration and a 60 s exclusion duration. For MS/MS, the precursor ions were activated using the 25% normalized collision energy at the default activation q of 0.25.

The MS/MS spectrum was identified by searching against the Swissprot database using the Mascot algorithm. Search parameters were set up as follows: taxonomy of fungi; partial trypsin (KR) cleavage; two missed cleavage; fragment mass tolerance of ±1 Da; instrument ESI-TRAP. Peptide identification was accepted with significance threshold $P < 0.05$.

Investigation of the Effects of pH and Temperature on Naringinase. The optimal pH was investigated by analysis of the enzymatic activities at different pH values using 50 mM of citrate buffer (pH 3.0−6.0), phosphate buffer (pH 6.0−7.0), and Tris-HCl buffer (pH 7.0−8.0) at 50 °C. The pH stability was assessed by measuring the residual activities after preincubation of the enzyme by the method described in Analysis of Enzyme Activities.

To study the optimum temperature, enzymatic activities were determined at pH 4.0 in 50 mM of citrate buffer and between 30 and 80 °C. The thermal stability was investigated by incubating the enzyme in the citrate buffer (50 mM, pH 6.0) at 30−80 °C for 60 min prior to analysis of the residual enzyme activities by the method mentioned in Analysis of Enzyme Activities.

Investigation of Substrate Specificity. Incubation of 0.1 mL of naringinase (1.08 U/mL) with 1.9 mL of citrate buffer (50 mM, pH 4.0) and 2 mL of 200 μg/mL substrate (naringin, hesperidin, myricitrin, prunin, arbutin, salicin, or aesculin) at 50 °C for 5 min was followed by measurement of the concentration changes of substrates and products using the HPLC method described in Analysis of Enzyme Activities.

Determination of Kinetic Parameters for Hydrolysis of Naringin. The enzyme activities of naringinase, α-L-rhamnosidase, and β-D-glucosidase were determined at various concentrations of substrate (6.25, 12.5, 25, 50, 75, 100, 150, and 200 μg/mL) at 50 °C and pH 4.0. From the data, kinetic constants such as the Michaelis constant (K_m) and maximum velocity (V_{max}) were computed from Lineweaver−Burk plots, and the turnover constant (K_{cat}) and the catalytic coefficient (ratio of K_{cat} to K_m) were then estimated.

Statistical Analysis. Every experiment was performed in triplicate, and the results represent the mean values that were calculated. SPSS 17.0 (SPSS Inc. H, Chicago, IL) was used to analyze the significant difference ($p < 0.05$) of the various samples through Duncan's multiple range test.

RESULTS AND DISCUSSION

Purification of Naringinase. Many studies referred to purification of naringinase, α-L-rhamnosidases, and β-D-glucosidases.[8] In general, a combined procedure is always needed to purify naringinase,[1,8,32] whereas affinity chromatography is effective for purification of α-L-rhamnosidases and β-D-glucosidases using one-step chromatography.[15] A combined procedure consisting of ammonium sulfate precipitation and chromatography on a DEAE-Sepharose Fast Flow and a Sephacyl S 300 HR column was used to purify naringinase in this study. As shown in Figure 2A, naringinase activity was eluted from the DEAE-Sepharose Fast flow column in 0.45−0.55 M NaCl. The naringinase fraction was then separated from other proteins on a Sephacyl S 300HR column (Figure 2B). Table 1 showed that after the final purification step (size exclusion chromatography with the Sephacyl S 300HR column), the naringinase was purified by 17.2-fold with a yield of 7.1%.

Molecular Mass and Structure of the Naringinase Complex. Previous studies showed that the MW of naringinase varied depending on its origin and fermentation conditions.[1,8,32] When analyzed by SDS-PAGE, most naringinases behaved homogenously, appearing as a solo band.[16−19] However, some microorganisms have the ability to synthesize several α-L-rhamnosidases and β-D-glucosidases that result in different MWs on an SDS-PAGE.[14,15] Ni et al.[19] purified a naringinase that exhibited a MW of 132 kDa by gel filtration

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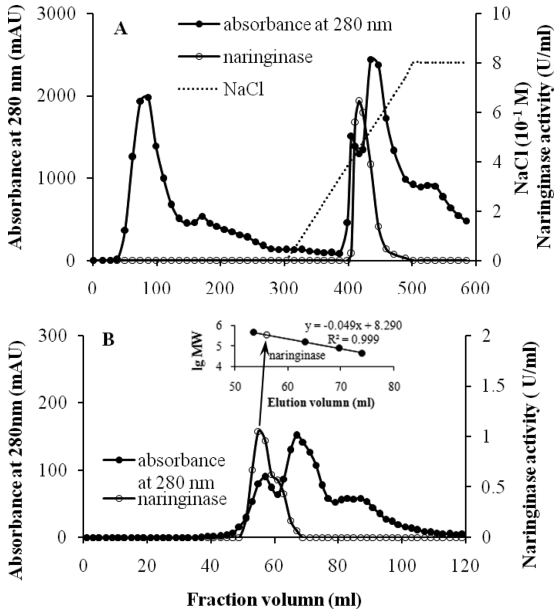

Figure 2. In all, 90 mL (399–489 mL) of the fractions from the DEAE-Sepharose FF column (A) was pooled and subsequently concentrated to 2.5 mL prior to separation on a Sephacyl S 300HR column (B). Naringinase exhibited the highest activity at an elution volume of 56 mL. (B, insert) Linear relationship of the log [MW] versus the elution volume (V_e). Ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (44 kDa) were used as standards and had V_es values of 53.66, 63.25, 69.74, and 73.94 mL, respectively.

Table 1. Purification Summary of Naringinase from *A. aculeatus* JMUdb058

purification step	total protein (mg)	total activity (U)	specific activity (U/mg)	purification fold	yield (%)
crude extract	385.0	532.2	1.38	1	100
$(NH_4)_2SO_4$ precipitation	268.0	411.3	1.53	1.1	77.3
DEAE-Sepharose FF	23.5	146.6	6.24	4.5	27.5
Sephacyl S 300HR	1.6	38.0	23.75	17.2	7.1

chromatography and 66 kDa by SDS-PAGE. Another naringinase from *A. niger* was estimated to have a MW of approximately 168 kDa by gel filtration chromatography and 85 kDa by SDS-PAGE analysis.[16] A naringinase from *A. sojae* was purified and determined to have a MW of 70 kDa by SDS-PAGE.[18] Two α-L-rhamnosidases with different protein sequences, MWs, and properties were purified from *A. aculeatus*.[14] Two β-D-glucosidases and one α-L-rhamnosidases were purified from the naringinase broth of *A. terreus* CECT 2663.[15] Recently, Puri[32] reviewed the latest research advancement on the structure of naringinase, in which he assumed that naringinase was a complex with different subunits. These studies raise questions regarding the makeup of the naringinase complex and whether all of the different α-L-rhamnosidases and β-D-glucosidases are components of naringinase.

Naringinase from *A. aculeatus* JMUdb058 was estimated to have a MW of 348 kDa based on the elution volume and standard curve (Figure 2B). Native-PAGE analysis showed three protein bands. Among these, two had β-D-glucosidase activity and the remaining one was an α-L-rhamnosidase (Figure 3A), which indicates that the naringinase contains at

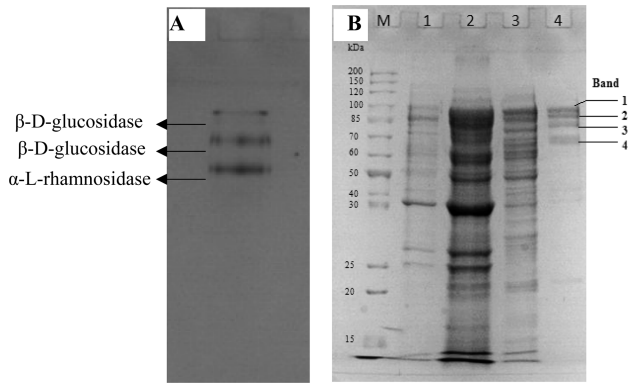

Figure 3. (A) Native-PAGE analysis of the naringinase from Sephacyl TM S 300 HR chromatography (at an elution volume of 56 mL). (B) SDS-PAGE analysis of the purifications. Lane M was the protein marker (#SM0661, Fermentas); lanes 1, 2, 3, and 4 (from left to right) were crude broth, fractions pooled in $(NH_4)_2SO_4$ precipitation, DEAE-Sepharose Fast Flow chromatography, and Sephacyl S 300HR chromatography (at 56 mL of elution volume), respectively.

least of one α-L-rhamnosidase and two β-D-glucosidase subunits. Furthermore, SDS-PAGE, which was performed for 150 min with a 10% gel, showed four protein components with MWs of approximately 100, 95, 84, and 69 kDa (Figure 3B). The sum of the MWs of the four bands was equal to the counterpart estimated by gel filtration chromatography (100 + 95 + 84 + 69 = 348 kDa), which indicate that naringinase contains four subunits. Identification based on mass spectra analysis and Mascot searching against the Swissport database revealed that three of the subunits (bands 1 (100 kDa), 2 (95 kDa), and 3 (84 kDa) in Figure 3B) were β-D-glucosidase 1 precursors[24] and the other (band 4 (69 kDa) in Figure 3B) was an α-L-rhamnosidase A precursor[14] from *A. aculeatus* (Table 2, Supporting Information Tables 1–4). Although the native-PAGE analysis produced different results from SDS-PAGE analysis because more uncertain factors affect the native-PAGE results, the SDS-PAGE and mass spectra analyses indicated that this naringinase was a tetramer consisting of one α-L-rhamnosidase subunit and three β-D-glucosidase subunits. These results confirmed the assumption that naringinase is composed of α-L-rhamnosidase and β-D-glucosidase subunits.[32,33]

A. aculeatus synthesizes two α-L-rhamnosidases (RhaA and RhaB) that are encoded by different genes (*rhaA* and *rhaB*);[14] however, only α-L-rhamnosidase A (RhaA) was present in the naringinase of this study. Thus far, α-L-rhamnosidase has been associated with the glycoside hydrolysis family (GH) of 28, 78, and 106,[33] but only the α-L-rhamnosidase of GH 78 is found in naringinase,[14,35,36] which further indicates the sequence selectivity of naringinase. A crystal structure of α-L-rhamnosidase of GH 78 was resolved by Cui et al. (2007).[33] This structure showed that the catalytic domain was an (α/α) 6-barrel fold structure with several highly conserved residues, such as Asp 567, Glu 572, Asp 579, and Glu 841, thus providing a potential interpretation for sequence selectivity.

Most α-L-rhamnosidases and β-D-glucosidases from the *Aspergillus* family are glycoproteins with variable MWs due to post-translational processing.[14,24,25,29] The α-L-rhamnosidase subunit had a MW (69 kDa) similar to that of the

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Table 2. Identification of the Subunits by MS Analysis and Mascot Searching[a]

band no.	MW (kDa)	matched peptide	score	accession no.	protein name	pI
1	100	50(36)	3796	BGL1_ASPAC	β-D-glucosidase	5.0
2	95	44(31)	2188	BGL1_ASPAC	β-D-glucosidase	5.0
3	84	36(24)	1685	BGL1_ASPAC	β-D-glucosidase	5.0
4	69	5(3)	100	gil13241312	α-L-rhamnosidase A	5.8

[a]Peptide identification was accepted with a significance threshold of $P < 0.05$. Band number corresponds to the number of bands mentioned in Figure 3B. p*I* (isoelectric point) represents the theoretical results predicted based on the protein sequence. Matched peptide was the number of parings between the experimental fragmentation spectra and the theoretical segments of the protein. Accession no. is the unique number given when a protein sequence is entered into a primary or secondary database (i.e., GenBank).

deglycosylated α-L-rhamnosidase A (Rha A) from *A. aculeatus*,[14] indicating that the post-translational glycosylation that is found in almost all liberated α-L-rhamnosidases did not occur in formation of naringinase. On the other hand, the three β-D-glucosidase subunits had different MWs, which is consistent with an earlier study in which three different β-D-glucosidases were purified from *A. aculeatus*.[34] Because only one gene encoding β-D-glucosidase was found in this fungus,[24] it is reasonable to believe that the three β-D-glucosidases occur from different post-translational processing. These results indicate that the post-translational processing of β-D-glucosidase plays an important role in forming the structure of the naringinase complex.

Effects of pH and Temperature on Naringinase. Most of the fungal naringinases, α-L-rhamnosidases, and β-D-glucosidases have an optimal activity at an acidic pH value and are stable in a broad pH range.[1,8,32] Naringinases from *A. niger* 1344,[16] *P. decumbens* PTCC 5248,[10] and *A. sojae*[18] have optimum pH values of 4.0, 4.5, and 6.0, respectively.

Both α-L-rhamnosidase and β-D-glucosidase described in this study exhibited optimal activity at pH 4.0, which is similar to the optimal pH of some previously characterized α-L-rhamnosidases[8,14,33,34] and β-D-glucosidases[1,8,29] and explains why the naringinase had an optimal pH of 4.0 (Figure 4A). α-L-Rhamnosidase was very stable between pH 3 and 8 (Figure 4B). However, the stability of the β-D-glucosidase ranged from pH 3 to 6, which suggests that naringinase is stable from approximately pH 3 to 6 (Figure 4B). These characteristics allow the naringinase to hydrolyze naringin and hesperidin in acidic pH conditions, which is found in wine and citrus juices.

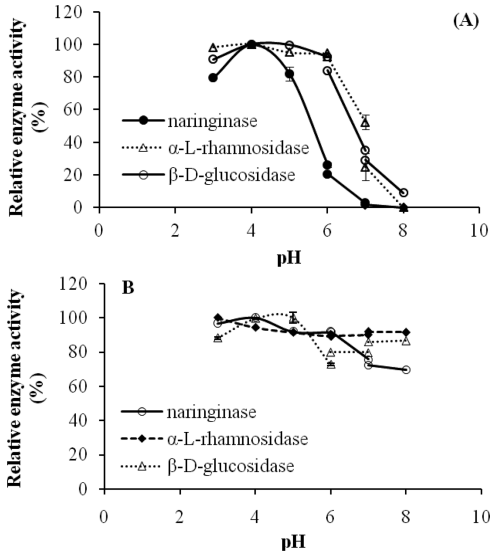

Figure 4. Optimal pH (Figure 4A) was estimated by analysis of the enzymatic activities in different buffers (pH 3−8). pH stability (Figure 4B) was measured by analysis of the activity that remained after the enzyme was incubated at 4 °C for 24 h in buffers with different pH values from 3 to 8. Relative activity was the percentage of a detected activity compared with the activity at the peaks.

The optimal temperature of the α-L-rhamnosidase and β-D-glucosidase subunits was 50 °C and within the range from 50 to 60 °C, which is consistent with some other α-L-rhamnosidases[32,35,36] and β-D-glucosidases[29,37] and thus presents a reasonable explanation for the occurrence of the optimal temperature of naringinase at 50 °C (Figure 5A). In addition,

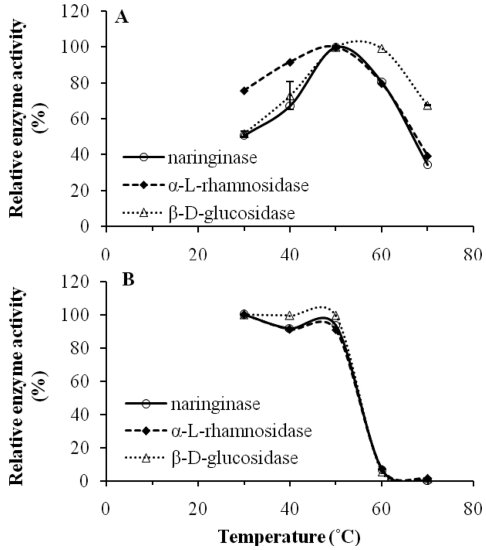

Figure 5. Optimal temperature (A) was estimated by analysis of the enzymatic activities at different temperatures (30−70 °C). pH stability (B) was measured by analysis of the activity that remained after the enzyme was incubated at different temperatures for 24 h in citrate buffer.

the naringinase complex and its α-L-rhamnosidase and β-D-glucosidase subunits exhibited similar temperature stability ranges below 50 °C (Figure 5B), which is consistent with most naringinases from fungi.[8,10,16,18,32]

Substrate Specificity. Naringinase hydrolyzed hesperidin, myricitrin, arbutin, salicin, and aesculin (Figure 6) in addition to naringin and prunin (Figure 1), which was supported by the ability of α-L-rhamnosidase to cleave both α-1,2 and α-1,6 linkages to release the terminal rhamnose[4] and of β-D-glucosidase to cleave several types of beta glycosidic bonds.[35] Among the substrates tested, naringin was the best substrate for the α-L-rhamnosidase and aesculin and prunin were the best

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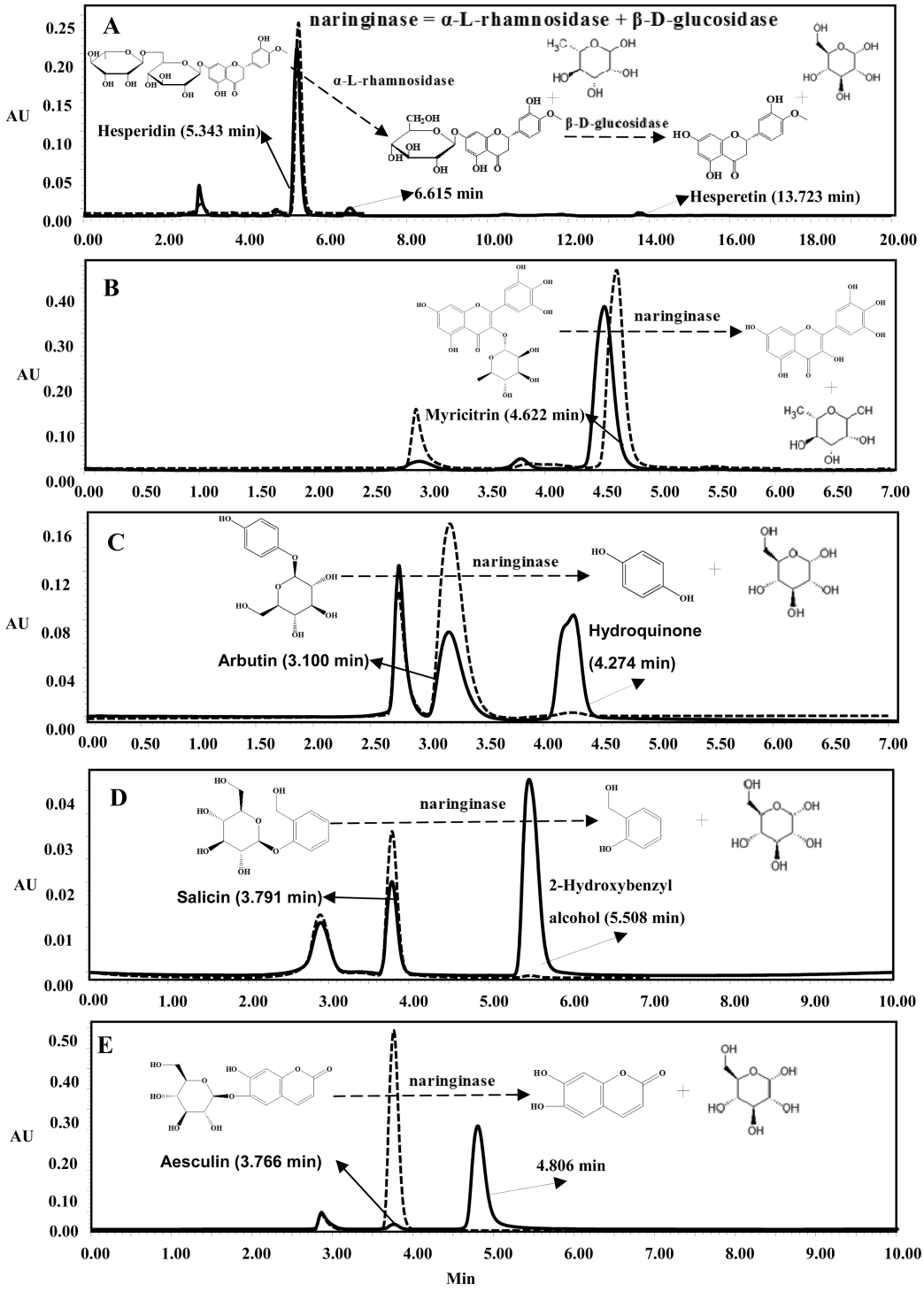

Figure 6. Chromatographs A–E show the reaction mixtures before (solid line) and after (dashed line) enzymatic hydrolysis of hesperidin, myricitrin, arbutin, salicin, and aesculin, respectively. Hesperidin was hydrolyzed by α-L-rhamnosidase first, releasing rhamnose and hesperetin-7-glucoside, which was further catalyzed to hesperetin and glucose by the β-D-glucosidase activity of naringinase. Myricitrin (myricetin-3-rhamnoside) was hydrolyzed to myricetin and rhamnose by α-L-rhamnosidase. β-D-Glucosidase split arbutin (C), salicin (D), and aesculin (E) to generate hydroquinone, salicyl alcohol, and aesculetin, respectively.

substrates for β-D-glucosidase (Table 3). These results suggest the effectiveness of naringinase in modification of glycosides.

Kinetic Parameter for Hydrolysis of Naringin. Although some studies have characterized naringinase using *p*-nitrophenol methods,[8,14,29,32] the kinetic parameters of the hydrolysis of naringin were difficult to accurately determine. The HPLC method that distinguished naringin, prunin, and naringenin was used to determine the reaction velocities of the α-L-rhamnosidase (transforming naringin to prunin), β-D-glucosidase (cleaving prunin to naringenin), and naringinase complex (transforming naringin to naringenin via prunin; Figure 1).[19] This HPLC method can determine the kinetics of the hydrolysis of naringin by the Lineweaver–Burk plot method. As shown in Table 4, the α-L-rhamnosidase was

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Table 3. Substrate Specificity of the Naringinase[a]

active components	substrate	hydrolysis rate (%)
α-L-rhamnosidase	naringin	22.68 ± 0.22[a]
	myricitrin	5.54 ± 0.76[b]
	hesperidin	4.04 ± 0.33[c]
β-D-glucosidase	aesculin	97.28 ± 0.04[a]
	prunin	95.33 ± 0.16[b]
	arbutin	55.06 ± 0.71[c]
	salicin	33.88 ± 0.31[d]

[a]The substrate specificity of naringinase was studied by measuring the hydrolysis rates of α-L-rhamnosidase and β-D-glucosidase. Naringin, myricitrin, and hesperidin, three glycosides containing a terminal rhamnose, were used to investigate the substrate specificity of the α-L-rhamnosidase. Aesculin, prunin, arbutin, and salicin, four substrates containing a terminal glucose, were used to analyze the substrate specificity of β-D-glucosidase. The hydrolysis rate (%) was calculated by the formula: $(C_I - C_R) \times 100/C_I$, where C_I was the initial concentration of the substrates before the enzymatic reaction and C_R was the residual concentration of the substrates after the enzymatic reaction. The numbers followed by different lowercase superscript letters indicate a statistical difference ($P < 0.5$).

Table 4. Kinetic Parameters of the Purified Naringinase[a]

enzyme	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
naringinase	0.11 ± 0.003	258.6 ± 3.5	1500 ± 20	14 034
α-L-rhamnosidase	0.23 ± 0.005	565.1 ± 11.2	3278 ± 65	14 146
β-D-glucosidase	0.53 ± 0.002	707.6 ± 2.9	4104 ± 17	7733

[a]The kinetic parameters were calculated at pH 4.0 and 50 °C.

estimated to have a K_m value of 0.23 mM, which is less than that of the pNPR (2.8 mM)[14] and indicates that α-L-rhamnosidase A has a higher affinity for naringin than pNPR. Furthermore, the K_m value of the α-L-rhamnosidase A is less than that of the β-D-glucosidase (0.53 mM), which indicates α-L-rhamnosidase has a stronger affinity for naringin than the β-D-glucosidase has for prunin. However, the α-L-rhamnosidase showed lower values of both V_{max} (565.1 vs 707.6 U/mg) and k_{cat} (3278 vs 4104 s^{-1}) than β-D-glucosidase, which suggests that α-L-rhamnosidase is the main factor affecting the velocity of naringin hydrolysis. Although the β-D-glucosidase had lower k_{cat}/K_m (7733 s^{-1} mM^{-1}) than α-L-rhamnosidase (14 146 s^{-1} × mM^{-1}), the k_{cat}/K_m (14 034 s^{-1} × mM^{-1}) of the naringinase was similar to that of the α-L-rhamnosidase because the β-D-glucosidase had a higher V_{max}. This result further suggests that α-L-rhamnosidase is the rate-limiting step of the enzymatic hydrolysis of naringin.

■ ASSOCIATED CONTENT

Supporting Information

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Notes

The authors declare no competing financial interest.

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