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Development and Evaluation of an HPLC Method for Accurate Determinations of Enzyme Activities of Naringinase Complex

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ABSTRACT: An HPLC method that can separate naringin, prunin, and naringenin was used to help accurately measure the activities of naringinase and its subunits (α-L-rhamnosidase and β-D-glucosidase). The activities of the naringinase and β-D-glucosidase were determined through an indirect calculation of the naringenin concentration to avoid interference from its poor solubility. The measured enzymatic activities of the naringinase complex, α-L-rhamnosidase, and β-D-glucosidase were the as same as their theoretical activities when the substrates' (i.e., naringin or prunin) concentrations were 200 μg/mL, and the enzyme concentrations were within the range of 0.06−0.43, 0.067−0.53, and 0.15−1.13 U/mL, respectively. The β-D-glucosidase had a much higher V_{max} than either naringinase or α-L-rhamnosidase, implying the hydrolysis of naringin to prunin was the limiting step of the enzyme reaction. The reliability of the method was finally validated through the repeatability test, indicating its feasibility for the determinations of the naringinase complex.

KEYWORDS: *HPLC, naringinase, α-L-rhamnosidase, β-D-glucosidase, kinetics, Aspergillus niger*

INTRODUCTION

Naringinase is an enzyme complex that contains both an α-L-rhamnosidase (EC 3.2.1.40) that hydrolyzes naringin to prunin and a β-D-glucosidase (EC 3.2.1.21) that further degrades prunin to naringenin.[1,2] The naringinase activity refers to its capability to convert naringin to naringenin and glucose (Figure1). The naringinase has many valuable applications, such as debittering citrus fruit juices,[3−5] producing rhamnose,[6] enhancing wine aromas,[7] removing hesperidin crystals from canned orange products,[8] yielding clinical important products by (or from) modification of steroids, etc.[9−11]

The traditional method to measure the enzymatic activities of the α-L-rhamnosidase and β-D-glucosidase often uses the following two artificial substrates, *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR) and *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG),[12−15] respectively. However, lack of specificity of the substrates has cast some doubts on the method for the accurate determinations of the enzymatic activities of the naringinase and its subunits, that is, α-L-rhamnosidase and β-D-glucosidase. Another commonly used method for determination of the naringinase activity is the Davis method, which is based on the spectrophotometric determination of the reaction product between flavonones (e.g., naringin) and diethylene glycol under an alkaline condition.[16] Likewise, this method cannot accurately determine the activities of naringinase and the α-L-rhamnosidase because naringin (the substrate), prunin (an enzymatic reaction intermediate), and naringenin (the product) have similar absorptive spectra.[16,17] Later, a combined procedure consisting of an aldohexose analysis and naringenin analysis was proposed to measure the activities of the α-L-rhamnosidase and naringinase, [17] but its application was limited because of its operation complexity.

It has been reported that HPLC is able to conduct accurate and precise determination of naringin, prunin, and naringenin.[18−21] Recently, an HPLC method has been used to analyze the concentration changes of naringin, prunin, and naringenin after the hydrolysis of naringin, resulting in measurements of the activities of naringinase, α-L-rhamnosidase, and β-D-glucosidase.[22] However, there was a problem that was neglected in the previous paper that naringin is subject to precipitation or crystallization in aqueous solution at room temperature, which might lead to an inhomogeneous reaction solution and affect the accuracy and repeatability of the determinations. Moreover, the poor solubility of naringenin in water might cause an inaccurate HPLC determination of the chemical, leading to inaccurate determinations of the activities of naringinase and/or β-D-glucosidase.

Therefore, the general objective of the present study was to develop a reliable HPLC analysis for simultaneous determination of the activities of naringinase, α-L-rhamnosidase, and β-D-glucosidase regardless of the limited solubility of naringin, prunin, and naringenin in the enzymatic solution. It included the following four specific aspects: (1) determining appropriate ranges of concentrations of the substrates (i.e., naringin or prunin) with regard to their solubilities in the enzymatic reaction solutions; (2) developing a reliable measurement of

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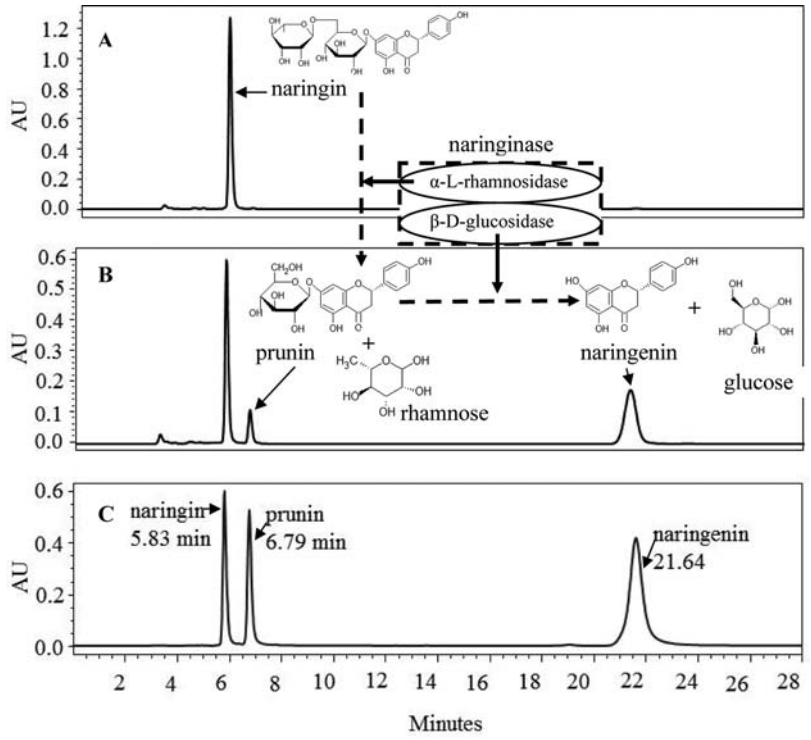

Figure 1. Chromatograms of the reaction mixture (A) before and (B) after the naringinase hydrolysis and of (C) standards of naringin, prunin, and naringenin. Enzymatic hydrolysis of naringin is composed of two reactions: (A) First, α-L-rhamnosidase breaks naringin down into prunin and rhamnose, and (B) subsequently β-D-glucosidase splits prunin to release naringenin and glucose.

naringenin to ensure accurate determinations of the enzymatic activities of naringinase and β-D-glucosidase; (3) investigating the proper ranges of the enzyme concentrations and enzymatic kinetics for the accurate determinations of these enzymes activities; and (4) validating the reliability of the aforementioned method on the basis of the repeatability of the enzymatic activities.

MATERIALS AND METHODS

Materials and Reagents. Naringin and naringenin were bought from Sigma-Aldrich Inc. (St. Louis, MO, USA). Prunin was bought from Extrasysthese (Genay Cedex, France). HPLC grade methanol and acetonitrile were purchased from Tedia Co. Inc. (Fairfield, OH, USA). All other chemicals in analytical grade were obtained from various chemical companies in China.

HPLC Condition. Qualitative and quantitative analyses of naringin, prunin, and naringenin were conducted on a Waters 1525 HPLC instrument, which was equipped with a Symmetry C18 reversed phase column (4.6 × 150 mm, 3.5 μm) (Waters Corp., Milford, MA, USA).[22] Briefly, 20 μL of the sample solution, which was prefiltered through a 0.22 μm PTFE filter, was injected into the column and immediately isocratically eluted by a mobile phase with a composition of 11.4% methanol, 26.6% acetonitrile, and 62% purified deionized water at a flow rate of 0.4 mL/min at 35 °C for 28 min. Signals were detected at 280 nm by a 2487 UV detector.

Enzymatic Reaction Condition. The substrate naringin was used for analyses of the naringinase and α-L-rhamnosidase, and the substrate prunin was used for analysis of the β-D-glucosidase. The enzymatic reaction solutions and reaction conditions were set up according to the previous studies.[1,12,15,20−25] In brief, 2 mL of the stock solutions of the substrates was mixed with 1.9 mL of 20 mM citric acid buffer (pH 5.0) and 0.1 mL of the enzyme solution followed by incubation at 50 °C for 5 min, heating at 100 °C for 5 min, and then cooling to room temperature.

Definition of Enzymatic Activity Unit. One unit of naringinase was defined as the amount of enzyme that releases 1 μM naringenin from naringin; 1 unit of α-L-rhamnosidase was defined as the amount of enzyme that hydrolyzes 1 μM naringin to prunin; 1 unit of β-D-glucosidase was the enzyme that hydrolyzes 1 μM prunin to naringenin.

Construction of Calibration Curves of Naringin, Prunin, and Naringenin. The standard solutions of naringin and prunin were prepared at 10, 50, 100, 150, 200, 300, 400, and 500 μg/mL in 10 mM citric acid buffer (pH 5.0) followed by heating at 100 °C for 5 min (imitating inactivation of the enzyme) in a water bath prior to HPLC analysis. The naringenin solutions were prepared in methanol because of its poor aqueous solubility. After filtration through 0.22 μm syringe filters (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA), all of the standard solutions were subjected to HPLC analysis as mentioned under HPLC Condition. The calibration curves were plotted by the peak areas against concentrations in a linear regression.

Effect of the Enzymatic Reaction Condition on the Enzyme Substrates and Products. To investigate the reaction condition on the substrates and products (i.e., naringin, prunin, and naringenin), the standard solutions were prepared at 50, 150, 250, 350, and 450 μg/mL in the reaction solution, which were incubated at 50 °C for 5 min, then heated at 100 °C for 5 min, and kept in a water bath at 50 °C. Thereafter, the naringin and prunin solutions were filtered through 0.22 μm filters prior to HPLC analysis; the naringenin solutions were dried in a rotary evaporator and subsequently dissolved in the same volume of methanol prior to HPLC analysis. Recovery (the percentage of a detected concentration relative to the actually prepared concentration) and relative standard deviation (RSD) of the detected concentration of the three chemicals were calculated.

Effect of Temperature on Solubility of Naringin, Prunin, and Naringenin in the Reaction Solutions. Solutions of naringin (50, 100, 150, 200, 300, 400, and 500 μg/mL), prunin (25, 50, 100, 150, and 200 μg/mL), and naringenin (25, 50, 75, 100, and 150 μg/mL) were prepared in the reaction buffer. After incubation at 50 °C for 5 min, the solutions were subsequently heated at 100 °C for 5 min and respectively kept at 50 °C, 20 °C (room temperature), and 4 °C (storage temperature) for 12 h followed by filtration through 0.22 μm filters prior to HPLC analysis.

Investigation of Desirable Ranges of Enzyme Concentrations for Enzymatic Reactions. To investigate the desirable ranges of the enzyme concentrations for accurate determinations of the enzymatic activities, the naringinase that was extracted from the *Aspergillus niger* fermentation broth and purified to have an activity of about 7.24 U/mL[22] was serially diluted by 2, 4, 8, 16, 32, 64, and 128 times. One hundred microliters of the respectively prepared enzyme solutions was mixed with 2 mL of a substrate (i.e., naringin or prunin) stock solution at a concentration of 400 μg/mL and 1.9 mL of 20 mM citric acid buffer (pH 5.0) at 50 °C for 5 min and subsequently heated at 100 °C for 5 min and cooled to room temperature. After filtration through 0.22 μm filters, the solutions were subject to HPLC analysis. Thereafter, the enzymatic activities were obtained by multiplying the activity of each dilutee with its corresponding diluted factor.

Repeatability of Enzymatic Activities. To test the repeatability of the measured enzymatic activities, the enzymatic reactions were repeated five times, from which the RSD of each sample was calculated. Two samples, a broth of *A. niger* that was prediluted by 8 times and a purified naringinase that was prediluted by 32 times, were used to conduct the reaction.

Determination of the Enzymatic Kinetic Parameters. The naringinase, which was purified from *A. niger* according to our previous study[22] and diluted to a final concentration at about 0.43 U/mL, was applied to reactions with 200, 150, 100, 75, 50, 25, 12.5, and 6.25 μg/mL of the substrate at 50 °C for 5 min. After the activities were measured, their V_{max} and K_m were estimated on the basis of the Lineweaver−Burk plot.

Investigation of the Enzyme Production in Submerged Cultivation of *A. niger*. The cultivation of *A. niger* DB056 for the production of naringinase was conducted according to previous studies[22,23] with minor modifications. Briefly, 50 mL of spore suspension (OD_{600} at 0.2) of *A. niger* was inoculated into a NBS Bioflo-110 7 L fermentor (New Brunswick Scientific, Enfield, CT, USA), which contained 5 L of fermental medium with a composition (g/L) of naringin, 10; $MgSO_4 \cdot 7H_2O$, 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; soybean powder, 2.0; and peptone, 2.0; followed by cultivation at 28 °C, pH 6.0, and 300 rpm for 8 days, during which samples were fetched in every 24 h to measure the activities of naringinase, α-L-rhamnosidase, and β-D-glucosidase.

Statistical Analysis. Every experiment was done in triplicate except of the repeatability, which was conducted five times, and means were calculated to represent the results. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the significant difference ($p < 0.05$) of different samples through Duncan's multiple-range method.

RESULTS AND DISCUSSION

Qualification and Quantitation of Naringin, Prunin, and Naringenin. Naringin, prunin, and naringenin had HPLC chromatographic retention times at 5.83, 6.79, and 21.64 min (Figure 1), respectively. Obviously they were completely separated from each other under the current HPLC condition. The chromatographic resolutions between the chemicals were more than 2.0 (Figure1B,C). The enzymatic hydrolysis of naringin resulted in a decrease of concentration of naringin from a peak height of 1.2 AU before the hydrolysis reaction (Figure 1A) to 0.6 AU after the reaction (Figure 1B), accompanied by the appearance of prunin and naringenin, which was in accordance with the results of the previous studies that the naringinase hydrolyzes naringin to naringenin through intermediate prunin.[1,2,18−21] This result indicates the HPLC method is able to distinguish the substrate (naringin), intermediate (prunin), and product (naringenin) after the naringinase hydrolysis and able to be used for the determination of the enzyme activity.

The established calibration curves of naringin, prunin, and naringenin were expressed as y (peak area in units of 10^7 mAu·s) = $0.0103x + 0.0035$ (x refers to the analyzed chemical concentration in units of μg/mL), $y = 0.0148x + 0.0024$, and $y = 0.017x + 0.007$ (Figure 2), respectively. The R^2 values were

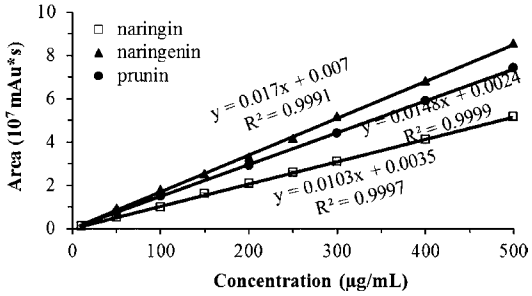

Figure 2. Calibration curves of naringin, prunin, and naringenin.

0.9997 for naringin, 0.9999 for prunin, and 0.9991 for naringenin, which indicated a good linearity of the three calibration curves within the concentration range of 10−500 μg/mL (Figure 2). After incubation and heating within the enzymatic reactions, naringin showed a recovery of 98.0−100.2% and an RSD of 1.4−2.8%; prunin had a recovery of 98.9−102.8 and an RSD of 1.8−3.3%; and naringenin displayed a recovery of 98.8−103.6% and an RSD of 1.7−2.9% (Table 1). This result suggests the naringin, prunin, and naringenin are stable and not significantly affected by the reaction condition.

Solubility of Naringin and Prunin in the Reaction Solution and Desirable Substrate Concentration. The solubility of naringin in water was reported to be dependent on the temperature and acidity. A higher temperature favors a higher solubility,[26] whereas a higher acidity (lower pH) results in a lower solubility.[27] Although naringin can be dissolved up to 500 μg/mL at 50 °C and showed an acceptable solubility (e.g., 200 μg/mL) at 20 and 4 °C, it cannot be sufficiently dissolved (e.g., 300 μg/mL) at 20 and 4 °C (Figure3A). In contrast, all of the prunin solutions were detected as having their concentrations identical to the theoretical (or the actually prepared) concentrations in the range of 25−200 μg/mL at 50, 20, and 4 °C (Figure 3B). Therefore, it was evident that choosing 200 μg/mL of the substrates (naringin or prunin) was desirable for this experiment in an effort to ensure the complete solubility of the substrates (i.e., naringin and prunin) in the processes of the enzymatic reaction (50 °C), sample keeping (4 °C), and room temperature (20 °C).

Solubility of Naringenin in the Reaction Solution and Approach for Its Determination. Theoretically, there are three kinds of enzyme activities resulting from the naringinase complex; those are naringinase, α-L-rhamnosidase, and β-D-glucosidase. The activity of naringinase (the combination of the activities of both α-L-rhamnosidase and β-D-glucosidase) can be measured by determining the amount of the appearance of naringenin released from naringin. By comparison, the α-L-rhamnosidase can be measured by determining either the degraded naringin or the released prunin, and the β-D-glucosidase can be analyzed by measuring either the hydrolyzed prunin or the production of naringenin (Figure 1). However, naringenin had a very poor solubility in the aqueous solution,[18,30] which has caused difficulty in directly determining the production of naringenin.

Table 1. Recoveries and RSDs of the Concentrations of Naringin, Prunin, and Naringenin after Treatment under the Enzymatic Reaction Condition[a]

naringin			prunin			naringenin		
concn (μg/mL)	rec (%)	RSD (%)	concn (μg/mL)	rec (%)	RSD (%)	concn (μg/mL)	rec (%)	RSD (%)
450.2	99.5	1.4	450.5	101.3	2.6	450.1	98.8	1.9
350.1	100.2	0.6	350.0	98.9	1.8	350.2	102.8	2.6
250.2	100.0	1.7	250.3	99.1	2.2	250.0	103.6	2.9
150.3	99.7	2.8	150.8	102.8	1.5	150.3	99.4	1.7
50.6	98.0	2.4	50.1	101.1	3.3	49.8	100.6	2.2

[a]Results are means of three independent determinations.

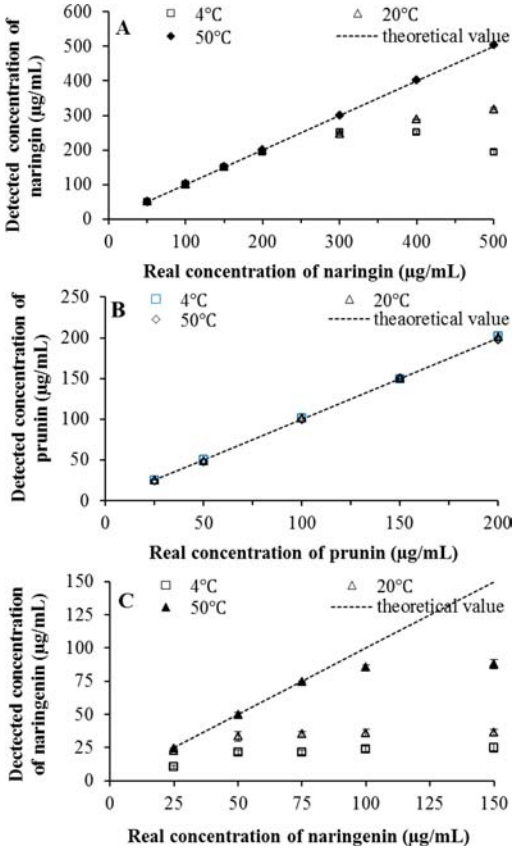

Figure 3. Solublities of (A) naringin, (B) prunin, and (C) naringenin in the reaction solution (10 mM citric acid buffer) at 4, 20, and 50 °C. Results are means of three independent determinations.

All of the naringenin solutions were measured to have their detected concentrations remarkably lower than their theoretical (or actually prepared) concentrations at any tested temperatures, that is, 50, 20, and 4 °C (Figure 3C). This result was consistent with previous results that naringenin had very poor solubility in the aqueous solution[18,28] and implied the naringenin generated in the enzymatic reaction was not sufficiently dissolved in water. It was reported that addition of methanol to a final concentration of 50% increased the solubility of naringenin in grapefruit juice.[18] A similar result was also found in our study: an addition of 60−80% methanol achieved a simultaneous dissolution of 200 μg/mL of naringin and 100 μg/mL of naringenin (Figure 4). However, the addition of methanol to dilute the sample solutions by 4−5 times may cause difficulty in analyses of low concentrations of the analytes.[29] These results indicate the released naringenin is hard to directly determine due to its poor solubility. Therefore,

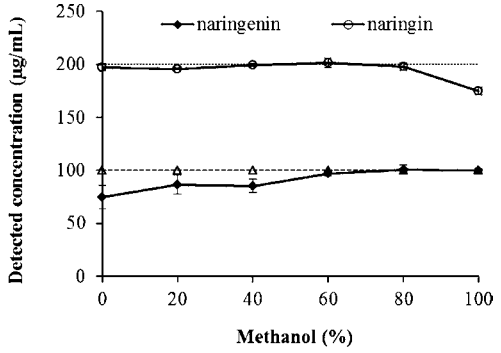

Figure 4. Effect of methanol concentration on the determinations of naringin and naringenin. The detected values were linked by a solid line, compared with the theoretical values in a dotted line.

to ensure the accuracy of the determination of the activities of naringinase and β-D-glucosidase, the production of naringenin was measured through an indirect approach according to the consumption of naringin and prunin.

Given the case above, the concentration changes of naringin, prunin, and naringenin before and after the reactions are expressed in the eqs 1 and 2 and listed in Table 2, which allow

Table 2. Calculation of the Theoretical Concentrations of Naringin, Prunin, and Naringenin before and after the Enzymatic Reactions[a]

	reaction with naringin for determination of naringinase and α-L-rhamnosidase (mM)			reaction with prunin for determination of β-D-glucosidase (mM)	
	naringin	prunin	naringenin	prunin	naringenin
before reaction	$x + y + z$	0	0	$a + b$	0
after reaction	x	y	z*	a	b

[a]$(x+y+z)$ was the mM naringin before reaction, x the mM naringin and y the mM prunin that could be measured after the reaction, and z the mM naringenin that could be obtained by subtracting y and z from $(x + y + z)$. $(a + b)$ was the mM prunin and a the mM prunin that could be determined after the reaction; thus, the b mM naringenin could be calculated by taking a from $(a+b)$.

the calculation of the enzymatic activities. The concentration change of substrate naringin could be used to determine the activity of α-L-rhamnosidase as shown in eq 1, where the amount of consumed naringin $(y + z)$ was obtained by taking its remaining concentration (x) from its original concentration before the hydrolysis reaction $(x + y + z)$ to calculate the activity of α-L-rhamnosidase. Meanwhile, the amount of generated naringenin (z, equal to the concentration in micromoles of the hydrolyzed prunin) was indirectly obtained

Table 3. Effect of Diluted Times of the Enzyme Solutions on the Activity Changes of Naringinase, α-L-Rhamnosidase, and β-D-Glucosidase[a]

	naringinase		α-L-rhamnosidase		β-D-glucosidase	
dilution times	diluted solution (U/mL)	sample (U/mL)	diluted solution (U/mL)	sample (U/mL)	diluted solution (U/mL)	sample (U/mL)
control	2.15 ± 0.17	2.15 ± 0.17e	2.26 ± 0.07	2.26 ± 0.07e	3.24 ± 0.13	3.24 ± 0.13e
2	1.98 ± 0.12	3.95 ± 0.23d	2.06 ± 0.11	4.12 ± 0.22d	3.10 ± 0.13	6.19 ± 0.26d
4	1.39 ± 0.11	5.55 ± 0.43c	1.43 ± 0.03	5.71 ± 0.10c	2.20 ± 0.17	8.80 ± 0.43c
8	0.80 ± 0.02	6.44 ± 0.15b	0.84 ± 0.04	6.74 ± 0.29b	1.85 ± 0.07	14.76 ± 0.54b
16	0.43 ± 0.01	6.91 ± 0.18a	0.53 ± 0.02	8.43 ± 0.37a	1.13 ± 0.05	18.01 ± 0.77a
32	0.21 ± 0.01	6.81 ± 0.24a	0.26 ± 0.01	8.29 ± 0.37a	0.59 ± 0.02	18.79 ± 0.59a
64	0.11 ± 0.01	7.20 ± 0.40a	0.14 ± 0.00	8.71 ± 0.29a	0.29 ± 0.01	18.72 ± 0.87a
128	0.06 ± 0.00	7.35 ± 0.61a	0.07 ± 0.00	8.33 ± 0.31a	0.15 ± 0.01	18.81 ± 0.85a

[a]Results are means of three independent determinations. The listed activity of the sample was the product of the measured activity of a diluted enzyme solution multiplied by its corresponding dilution time. Letters (a−e) within the same column mean significant difference ($P < 0.05$).

by subtracting the remaining naringin (x) and prunin (y) from the original concentration of naringin ($x + y + z$) to measure the activity of naringinase. Substrate prunin was used to determine the activity of β-D-glucosidase by measuring the consumed prunin (equal to the production of naringenin) as shown in eq 2, where the amount of consumed prunin (b, theoretically equal to the amount of the generated naringenin) was obtained by taking the remaining concentration (a) from the original concentration ($a + b$).

$$(x + y + z)_{nar} \xrightarrow{Rha} x_{nar} + (y + z)_{pru} \xrightarrow{Glu} x_{nar} + y_{pru} + z_{narg} \quad (1)$$

$$(a + b)_{pru} \xrightarrow{Glu} a_{pru} + b_{narg} \quad (2)$$

In eqs 1 and 2, nar, pru, narg, Rha, and Glu are the abbreviations of naringin, prunin, naringenin, α-L-rhamnosidase, and β-D-glucosidase, respectively; x, y, z, a, and b are variables as defined in Table 2. $(x + y + z)$ is the number of mM of the reaction naringin before the enzymatic reaction for analyzing the activity of α-L-rhamnosidase, $(y + z)$ is the total amount (mM) of prunin that was released by α-L-rhamnosidase after the reaction, and z is the amount (mM) of naringenin that was released from prunin by β-D-glucosidase after the reaction. $(a + b)$ is total amount (mM) of prunin before a reaction for analyzing the activity of β-D-glucosidase; a is the amount (mM) of remaining prunin after the reaction, b is the amount (mM) of naringenin generated after the reaction.

Desirable Detection Range and RSD of the Measured Enzyme Activities. The enzymatic activities were observed to have varying values depending on the dilution times (Table 3). The more times the enzyme solution was diluted, higher activity (product of the activity of a diluted enzyme multiplied by the dilution time) of the solution was obtained. When the enzyme was diluted >16 times, its activity had no significant changes along with the increasing dilution times. This result indicated the dilutees had enzymatic activities linearly related to the diluted times when diluted >16 times, which implied the desirable applied ranges of the naringinase, α-L-rhamnosidase, and β-D-glucosidase were 0.06−0.43, 0.07−0.53, and 0.15−1.13 U/mL (Table 3), respectively. This suggests a proper dilution is needed to get the accurate result when the enzyme activities go beyond the upper limits of these concentrations.

With regard to the repeatability of the analyzed enzymatic activities, the RSDs of the fermented broth and the purified enzymes were 8.25 and 7.33% for naringinase, 4.29 and 4.54% for α-L-rhamnosidase, and 3.33 and 4.64% for β-D-glucosidase, respectively (Table 4). These results demonstrated good repeatability for determinations of activities of the enzymes in both the fermented broth and their purified forms.

Table 4. Test of Repeatability of the Enzyme Activities

sample	enzyme	enzyme activity (U/mL)	RSD (%)
fermented broth	naringinase	0.20	8.25
	α-L-rhamnosidase	0.22	4.29
	β-D-glucosidase	0.43	3.33
purified enzyme	naringinase	0.21	7.33
	α-L-rhamnosidase	0.24	4.54
	β-D-glucosidase	0.59	4.64

Validation of the Determinations of the Enzyme Activities. Although some naringinases, α-L-rhamnosidases, and β-D-glucosidases have been characterized by using the Davis and p-nitrophenol methods,[12−15,24,25,30,31] their enzymatic kinetic parameters toward naringin hydrolysis were hardly studied via the previous old methods. Our HPLC method has facilitated the revelation of those kinetics. On the basis of Lineweaver−Burk plots, naringinase from *A. niger* had V_{max} of 0.60 U/mL and K_m of 0.13 mM (Figure 5). α-L-Rhamnosidase had V_{max} of 1.51 U/mL and K_m of 0.28 mM,

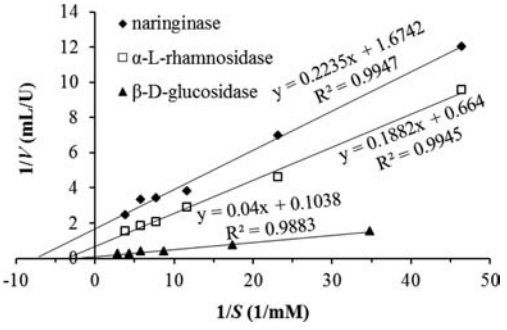

Figure 5. Lineweaver−Burk plots of the naringinase, α-L-rhamnosidase, and β-D-glucosidase from *A. niger*. Naringinase has V_{max} of 0.60 U/mL (the reciprocal of 1.6742) and K_m of 0.13 mM (the quotient of 0.2235 divided by 1.6742); α-L-rhamnosidase has V_{max} of 1.51 U/mL (the reciprocal of 0.664) and K_m of 0.28 mM (the quotient of 0.1882 divided by 0.664); and β-D-glucosidase has V_{max} of 9.63 U/mL (the reciprocal of 0.1038) and K_m of 0.39 mM (the quotient of 0.04 divided by 0.1038).

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whereas β-D-glucosidase had V_{max} of 9.63 U/mL and K_m of 0.39 mM (Figure 5). α-L-Rhamnosidase was determined to have a K_m similar to that of a previous study obtained by a HPLC method,[33] indicating both HPLC methods were accurate in analyzing the kinetic parameters of α-L-rhamnosidase. β-D-Glucosidase from *A. niger* was determined to have a much lower K_m (0.39 mM) than that of a previous study (K_m = 2.59 mM) analyzed on the basis of the direct detection of naringenin, verifying that direct analysis of the production of naringenin did cause inaccuracy to the determination of the activity of β-D-glucosidase, whereas the indirect calculation of naringenin production is necessary and more desirable to ensure the accuracy of the determination of naringinase and β-D-glucosidase. It is evident that the present naringinase had a K_m value (0.13 mM) much lower than that of a previous counterpart from *A. niger* (1.9 mM) determined by using the Davis method,[24] indicating that our improved method has overcome the inaccuracy of the Davis method and provided an accurate and sensitive determination of naringinase.

The K_m of this α-L-rhamnosidase (0.28 mM) was much smaller than those (about 2.8 mM) of previous α-L-rhamnosidases from *A. niger* using the substrate *p*NPR, which indicates the enzyme has a higher affinity to naringin than *p*NPR.[7,8,14,32] In addition, the K_m of our β-D-glucosidases (0.39 mM) is much lower than that of a β-D-glucosidase extracted from *A. niger*, which had K_m values of 1.03 mM for *p*NPG and 5.36 mM for cellobiose,[30] indicating the β-D-glucosidases had a higher affinity to naringin than to pNPG and cellobiose. In addition, the α-L-rhamnosidase has a lower V_{max} than the β-D-glucosidase, implying the hydrolysis of naringin to prunin was the velocity-limiting step of the naringin degradation, which is similar to our previous study of the naringinase from *A. aculeatus*.[33]

Recently, naringinase has been confirmed to be an enzyme complex consisting of two subunits rather than a solo protein with two catalytic sites.[33] Although a few naringinase fermentations were studied according to the Davis method,[24,25] the production of α-L-rhamnosidase and β-D-glucosidase and their quantitative relationship during this process are still not clear. The present HPLC method could help to monitor the production of naringinase as well as its α-L-rhamnosidase and β-D-glucosidase (Figure 6). This study revealed that *A. niger* excreted the α-L-rhamnosidase a little ahead of β-D-glucosidase, which explained why the activity of naringinase was hardly observed in the initial 48 h of fermentation (Figure 6). The α-L-rhamnosidase activity stopped its increase in the middle of the fermentation (after 60 h). However, the β-D-glucosidase maintained a rapid increase throughout the fermentation period, and its activity (3.45 U/mL) greatly outpaced that of the α-rhamnosidase (1.75 U/mL) at the end of fermentation (Figure 6). Because the naringinase was affected by both α-L-rhamnosidase and β-D-glucosidase, it was reasonable to suggest that increasing the production of α-L-rhamnosidase and β-D-glucosidase could increase the production and/or activity of the naringinase. Nevertheless, the activity tendency of α-L-rhamnosidase was not consistent with that of β-D-glucosidase, indicating these two enzymes might be synthesized by two independent biochemical processes. Although the α-L-rhamnosidase and a β-D-glucosidase were purified from a fermented broth of *A. niger*,[34,35] their synthetic pathways for the formation of the naringinase complex is not elucidated yet.

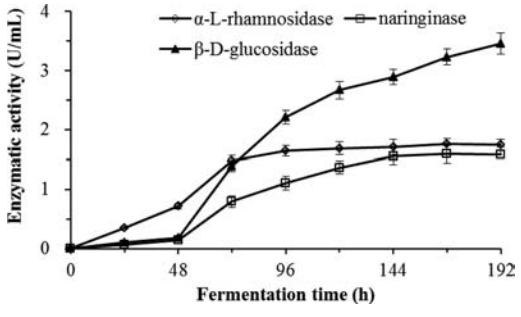

Figure 6. Activities of naringinase, α-L-rhamnosidase, and β-D-glucosidase produced in a submerged fermentation of *A. niger* during a fermentation period of 192 h. The final activity of β-D-glucosidase (3.45 U/mL) was about 2 times those of α-L-rhamnosidase (1.75 U/mL) and naringinase (1.58 U/mL).

In summary, an improved procedure based on the HPLC determination of naringin, prunin, and naringenin was developed and evaluated to accurately analyze the enzymatic activities of naringinase, α-L-rhamnosidase, and β-D-glucosidase. It is critical to contain about 200 μg/mL of substrate (naringin and prunin) in the reaction solution and measure the product naringenin through the indirect calculation approach instead of direct HPLC analysis. The desirable ranges of the enzymes for the enzymatic reactions were 0.06−0.43 U/mL for naringinase, 0.07−0.53 U/mL for α-L-rhamnosidase, and 0.15−1.13 U/mL for β-D-glucosidase. The analyses of repeatability of RSDs of the enzymatic activities were below 9% for naringinase and below 5% for both α-L-rhamnosidase and β-D-glucosidase. In addition, this new method could help to study the fermented production and characteristics of naringinase. This HPLC method provides accurate determinations of the activities of naringinase, α-L-rhamnosidase, and β-D-glucosidase, which overcomes the shortcomings of previous methods and facilitates further studies on the naringinase complex.

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Notes

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