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Article

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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 measuring 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 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 U/mL, 0.067-0.53 U/mL and 0.15-1.13 U/mL, respectively. The β-D-glucosidase had a much higher V_{max} than both the naringinase and the 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, i.e., *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, i.e., α-L-rhamnosidase and β-D-glucosidase. Another commonly used method for determination of the naringinase activity is the Davis's method, which is based on the spectrophotometric determination of the reaction product between flavonones (e.g., naringin) and diethyleneglycol under an alkaline condition. [16] Likewise, this method can not 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 owed to 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 report 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) in concern of their solubilities in the enzymatic reaction solutions; (2) developing a reliable measurement of 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; (4) validating the reliability of the aforementioned method based on 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 Company Inc. (Ohio, 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 Corporation, Milford, MA, USA). [22] Briefly, twenty microliters of the sample solution which was pre-filtered through a 0.22 μm PTFE filter were injected into the column, and immediately isocratically eluted by a mobile phase in composition of 11.4% methanol, 26.6% acetonitrile and 62% purified deionized water at a flow rate of 0.4 mL/min at 35ºC 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, two milliliters of the stock solutions of the substrates were 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 50ºC for 5 min, heated at 100ºC for 5 min, and then cooled down to room temperature.

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

116 prunin; one unit of β-D-glucosidase was the enzyme that hydrolyzes 1 μM of prunin to
117 naringenin.

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

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

137 **Effect of Temperature on Solubility of Naringin, Prunin and Naringenin in the**
138 **Reaction Solutions.** Solutions of naringin (50, 100, 150, 200, 300, 400 and 500 μg/mL),
139 prunin (25, 50, 100, 150 and 200 μg/mL) and naringenin (25, 50, 75, 100 and 150 μg/mL)
140 were prepared in the reaction buffer. After incubation at 50ºC 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 (store temperature) for 12 h followed by filtration through 0.22 μm filters prior to the 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 *A. niger* fermentation broth and purified to have an activity 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 were mixed with 2 mL of a substrate (i.e., naringin or prunin) stock solution in 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 down to room temperature. After filtration through 0.22 μm filters, the solutions were subject to the 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 5 times, from which the RSD of each sample was calculated. Two samples, a broth of *A. niger* which was pre-diluted by 8 times and a purified naringinase which was pre-diluted by 32 times, were used to conduct the reaction.

Determination of the Enzymatic Kinetic Parameters. The naringinase, which was purified from *Aspergillus 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 50ºC for 5 min. After the activities were measured, their V_{max} and K_m were estimated based on the Lineweaver–Burk plot.

166 **Investigation of the Enzyme Production in Submerged Cultivation of *A. niger*.** The
167 cultivation of *A. niger* DB056 for the production of naringinase was conducted according to
168 the previous studies [22, 23] with minor modifications. Briefly, 50 mL of spore suspension
169 (OD_{600} at 0.2) of *A. niger* was inoculated into a NBS Bioflo-110 7 L fermentor (New
170 Brunswick Scientific, Connecticut, USA) which contained 5 L of fermental medium with
171 composition (g/L) of: naringin 10, $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 1.5, $(NH_4)_2SO_4$ 4.0,
172 $ZnSO_4 \cdot 7H_2O$ 0.09, $CaCl_2$ 0.1, yeast extract 1.0, soybean powder 2.0, and peptone 2.0,
173 followed by cultivation at 28ºC, pH 6.0 and 300 rpm for 8 days, during which samples were
174 fetched in every 24 h to measure the activities of naringinase, α-L-rhamnosidase and
175 β-D-glucosidase.
176 **Statistical Analysis.** Every experiment was done in triplicate except of the repeatability
177 that was conducted for 5 times, and means were calculated to represent the results. The SPSS
178 17.0 software (SPSS Inc. Chicago, IL) was used to analyze the significant difference ($p<0.05$)
179 of different samples through the Duncan's multiple range method.
180

■ RESULTS AND DISCUSSION

Qualification and Quantitation of Naringin, Prunin and Naringenin. Naringin, prunin and naringenin had their 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, Figure 1C). 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), accompanying with 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 unit of 10^7 mAu*s)=0.0103x+0.0035 (x refers to the analyzed chemical concentration in unit of μg/mL), y= 0.0148x+0.0024 and y=0.017x+0.007 (Figure 2), respectively. The R^2 were 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 RSD of 1.4-2.8%; prunin had a recovery of 98.9-102.8 and RSD of 1.8-3.3%; naringenin displayed a recovery of 98.8-103.6% and 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] while 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°C and 4°C, it cannot be sufficiently dissolved (e.g., 300 μg/mL) at 20°C and 4°C (Figure3A). By contrast, all the prunin solutions were detected to have their concentrations identical to the theoretical (or the actually prepared) concentrations in the range of 25-200 μg/mL at 50°C, 20°C 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 the Approach for Its Determination. Theoretically, there are three kinds of enzyme activities resulted from the naringinase complex, i.e., 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 a difficulty to directly determine the production of naringenin.

All the naringenin solutions were measured to have their detected concentrations remarkably lower than their theoretical (or actually prepared) concentrations at any tested temperatures, i.e., 50°C, 20°C and 4°C (Figure 3C). This result was consistent with previous

results that naringenin had a very poor solubility in the aqueous solution, [18, 28] and implied the naringenin generated in the enzymatic reaction was not be 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 that 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 be directly determined due to its poor solubility. Therefore, in order 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 following equations (1) and (2), and listed in Table 2, which allow 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 equation (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 the generated naringenin (z, equal to the concentration in micromole of the hydrolyzed prunin) was indirectly obtained 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 equation (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 ($a+b$).

$$(x + y + z)\ \text{nar} \xrightarrow{\text{Rha}} x\ \text{nar} + (y + z)\ \text{pru} \xrightarrow{\text{Glu}} x\ \text{nar} + y\ \ \text{pru} + z\ \text{narg} \quad (1)$$

$$(a + b)\ \text{pru} \xrightarrow{\text{Glu}} \text{a pru} + \text{b narg} \quad (2)$$

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

Desirable Detective Range and RSD of the Measured Enzyme Activities. The enzymatic activities were observed to have varying values depending on the dilution times (Table 3). 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 more than 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 more than 16 times, which implied the desirable applied ranges of the naringinase, α-L-rhamnosidase and β-D-glucosidase were 0.06-0.43 U/mL, 0.07-0.53 U/mL and 0.15-1.13 U/mL (Table 3), respectively. It suggests a proper dilution is needed to get the accurate result when the enzyme activities go beyond the up-limits of these concentrations.

Regarding the repeatability of the analyzed enzymatic activities, RSDs of the fermented broth and the purified enzymes were 8.25% and 7.33% for the naringinase; 4.29% and 4.54%

for the α-L-rhamnosidase, and 3.33% and 4.64% for the 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.

Validation of the Determinations of the Enzyme Activities. Although some naringinases, α-L-rhamnosidases and β-D-glucosidases have been characterized by the Davis and *p*-nitro phenol methods, [12-15, 24, 25, 30, 31] their enzymatic kinetic parameters towards the naringin hydrolysis were hardly studied based on the previous old methods. Our HPLC method has facilitated to reveal those kinetics. Based on the Lineweaver–Burk plots, the naringinase from *A. niger* had V_{max} of 0.60 U/mL and K_m of 0.13 mM (Figure 5). The α-L-rhamnosidase had V_{max} of 1.51 U/mL and K_m of 0.28 mM, while the β-D-glucosidase had V_{max} of 9.63 U/mL and K_m of 0.39 mM (Figure 5). The 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 to analyze the kinetic parameters of α-L-rhamnosidase. The 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 based on 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 the K_m value (0.13 mM) much lower than that of a previous counterpart from *A. niger* (1.9 mM) determined by the Davis method, [24] indicating the 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

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

311 Recently, naringinase has been confirmed to be an enzyme complex consisting of two
312 subunits rather than a solo protein with two catalytic sites. [33] Although a few naringinase
313 fermentations were studied according to the Davis method, [24, 25] the production of
314 α-L-rhamnosidase and β-D-glucosidase and their quantitative relationship during this process
315 are still not clear. The present HPLC method could help to monitor the production of
316 naringinase as well as its α-L-rhamnosidase and β-D-glucosidase (Figure 6). This study
317 revealed that *A. niger* excreted the α-L-rhamnosidase a little ahead of β-D-glucosidase, which
318 explained why the activity of naringinase was hardly observed in the initial 48 h of
319 fermentation (Figure 6). The α-L-rhamnosidase activity stopped its increase in the middle of
320 the fermentation (after 60 h). However, the β-D-glucosidase kept on rapid increase all over the
321 fermentation period, and its activity (3.45 U/mL) greatly outnumbered that of the
322 α-rhamnosidase (1.75 U/mL) at the end of fermentation (Figure 6). Since the naringinase was
323 affected by both the α-L-rhamnosidase and the β-D-glucosidase, it was reasonable to suggest
324 that increasing the production of the α-L-rhamnosidase and the β-D-glucosidase could
325 increase the production and/or activity of the naringinase. Nevertheless, the activity tendency
326 of α-L-rhamnosidase was not consistent to that of β-D-glucosidase, indicating these two
327 enzymes might be synthesized by two independent biochemical processes. Although the
328 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.

In summary, an improved procedure was developed and evaluated to accurately analyze the enzymatic activities of naringinase, α-L-rhamnosidase and β-D-glucosidase based on the HPLC determination of naringin, prunin and naringenin. 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 the 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, 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, 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.

■ ASSOCIATED CONTENT

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445

446 **Figure captions**

Figure number	Caption
Figure 1.	Chromatograms of the reaction mixture: (A) before and (B) after the naringinase hydrolysis ,and (C) standards of naringin, prunin and naringenin. Enzymatic hydrolysis of naringin is composed of two reactions: (A) At first α-L-rhamnosidase breaks naringin down into prunin and rhamnose, and (B) subsequently β-D-glucosidase splits prunin to release naringenin and glucose.
Figure 2.	Calibration curves of naringin, prunin and naringenin
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 ℃. Results are means of three independent determinations.
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.
Figure 5	Lineweaver–Burk plots of the naringinase, α-L-rhamnosidase and β-D-glucosidase from *A. niger*. The 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), the 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 the 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).
Figure 6	The 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 two times of those of the α-rhamnosidase (1.75 U/mL) and the naringinase (1.58 U/mL).

447

448

449
450 **Table 1.**

451 Recoveries and RSDs of the concentrations of naringin, prunin and naringenin after the
452 treatment under the enzymatic reaction condition

Naringin			Prunin			Naringenin		
Con. (μg/mL)	Rec. (%)	RSD (%)	Con. (μg/mL)	Rec. (%)	RSD (%)	Con. (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

453 Results were means of three independent determinations.
454

Table 2.

Calculation of the theoretical concentrations of naringin, prunin and naringenin before and after the enzymatic reactions

	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*

(*x*+*y*+*z)* was the number of mM of naringin before reaction, *x* mM of naringin and *y* mM of prunin could be measured after the reaction, and *z* mM of naringenin could be obtained by subtracting *y* and *z* from (*x*+*y*+*z*). (*a*+*b*) was the number of mM of prunin, *a* mM of prunin could be determined after the reaction, thus the b mM of naringenin could be calculated by taking *a* from (*a*+*b*).

Table 3.

Effect of diluted times of the enzyme solutions on the activity changes of naringinase, α-L-rhamnosidase and β-D-glucosidase

Diluted times	Naringinase		α-L-rhamnosidase		β-D-glucosidase	
	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±0.17^{e}	2.26±0.07	2.26±0.07^{e}	3.24±0.13	3.24±0.13^{e}
2	1.98±0.12	3.95±0.23^{d}	2.06±0.11	4.12±0.22^{d}	3.10±0.13	6.19±0.26^{d}
4	1.39±0.11	5.55±0.43^{c}	1.43±0.03	5.71±0.10^{c}	2.20±0.17	8.80±0.43^{c}
8	0.80±0.02	6.44±0.15^{b}	0.84±0.04	6.74±0.29^{b}	1.85±0.07	14.76±0.54^{b}
16	0.43±0.01	6.91±0.18^{a}	0.53±0.02	8.43±0.37^{a}	1.13±0.05	18.01±0.77^{a}
32	0.21±0.01	6.81±0.24^{a}	0.26±0.01	8.29±0.37^{a}	0.59±0.02	18.79±0.59^{a}
64	0.11±0.01	7.20±0.40^{a}	0.14±0.00	8.71±0.29^{a}	0.29±0.01	18.72±0.87^{a}
128	0.06±0.00	7.35±0.61^{a}	0.07±0.00	8.33±0.31^{a}	0.15±0.01	18.81±0.85^{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. $^{a, b, c}$ in superscript within the same column mean significant difference (P<0.05).

475 **Table 4.**

476 Test of repeatability of the enzyme acitivities

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

477

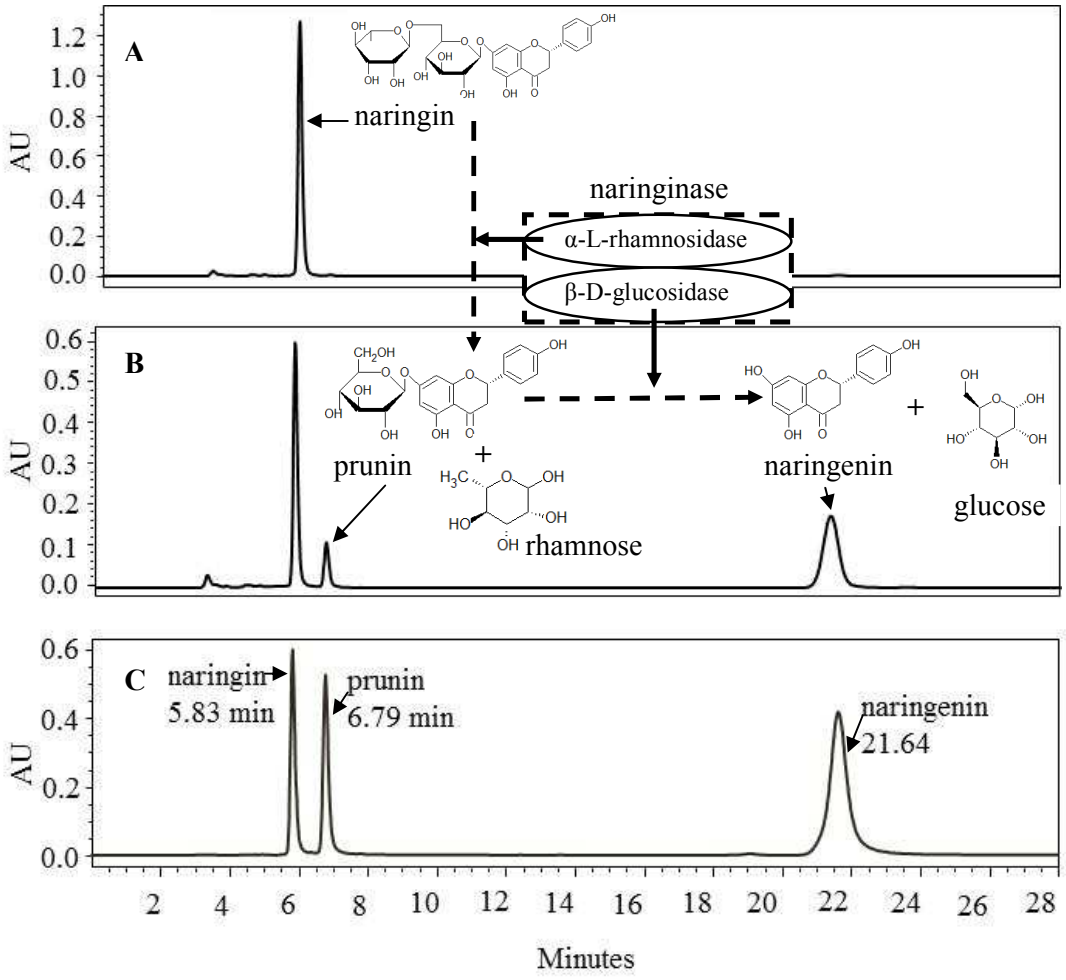
A
naringin
naringinase
α-L-rhamnosidase
β-D-glucosidase
B
prunin
rhamnose
naringenin
glucose
C
naringin
5.83 min
prunin
6.79 min
naringenin
21.64
AU
Minutes

Figure 1.

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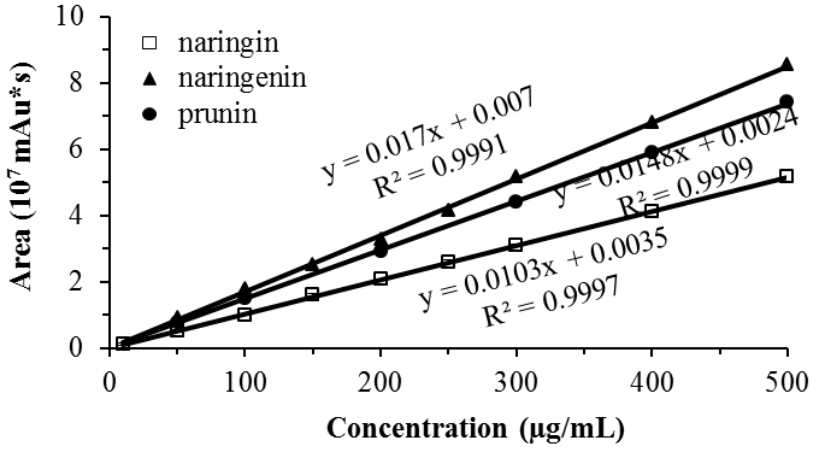

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486 **Figure 2.**

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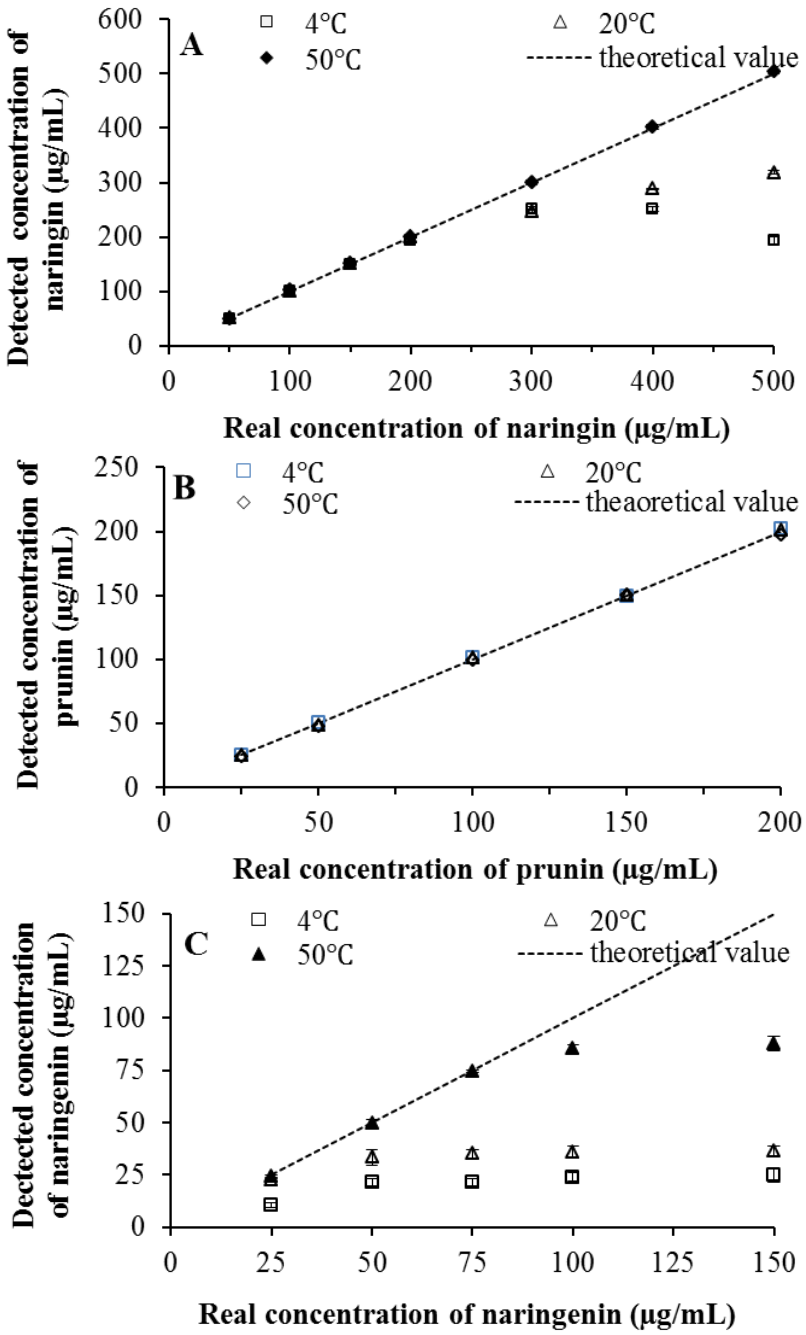
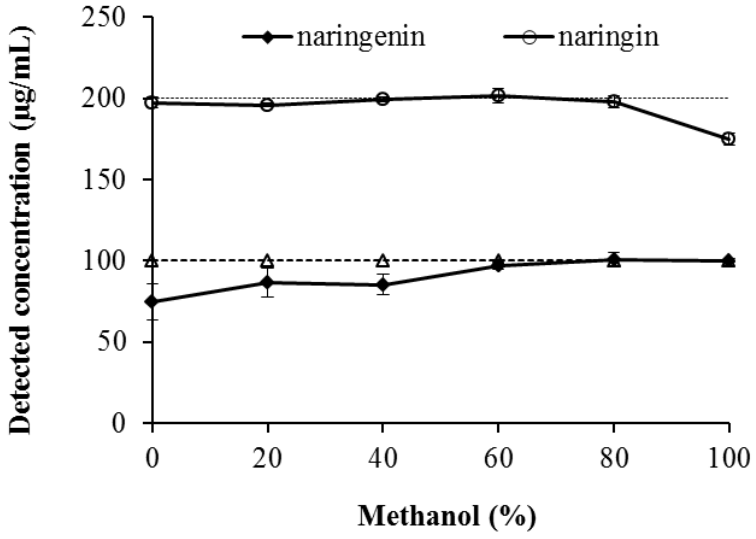

A
4°C
50°C
20°C
theoretical value
Detected concentration of naringin (μg/mL)
Real concentration of naringin (μg/mL)
B
4°C
50°C
20°C
theaoretical value
Detected concentration of prunin (μg/mL)
Real concentration of prunin (μg/mL)
C
4°C
50°C
20°C
theoretical value
Dectected concentration of naringenin (μg/mL)
Real concentration of naringenin (μg/mL)

Figure 3.

496



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498 **Figure 4.**

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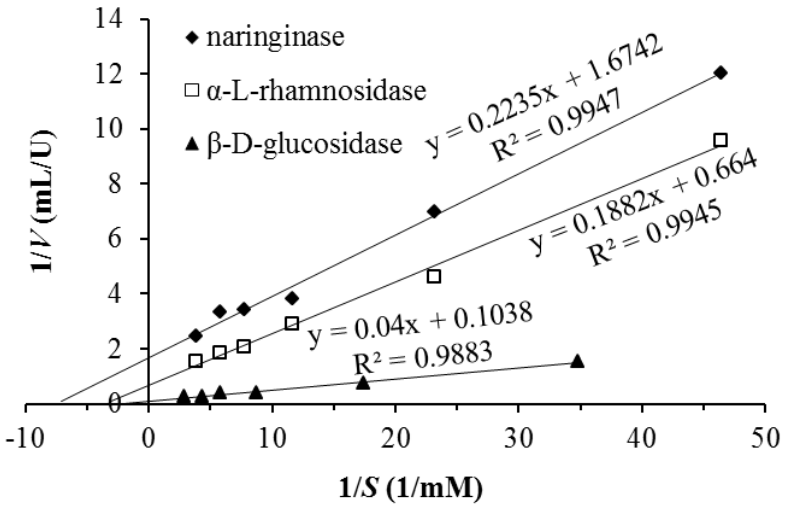

503

504 **Figure 5.**

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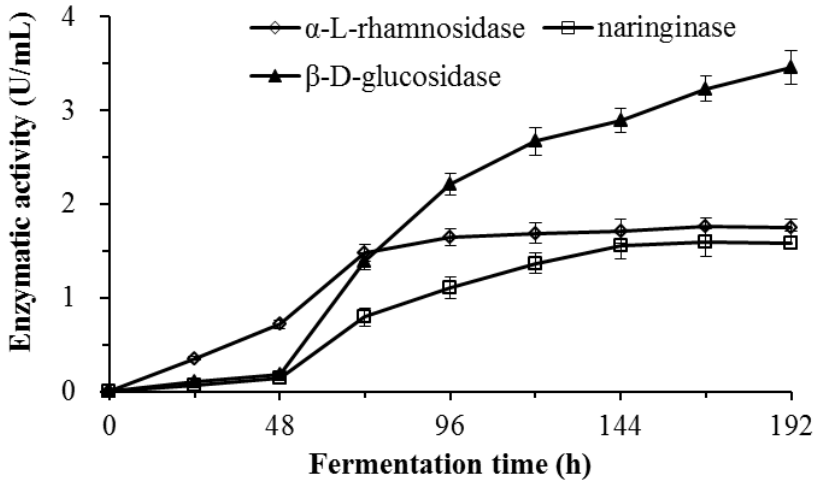

507

508 **Figure 6.**

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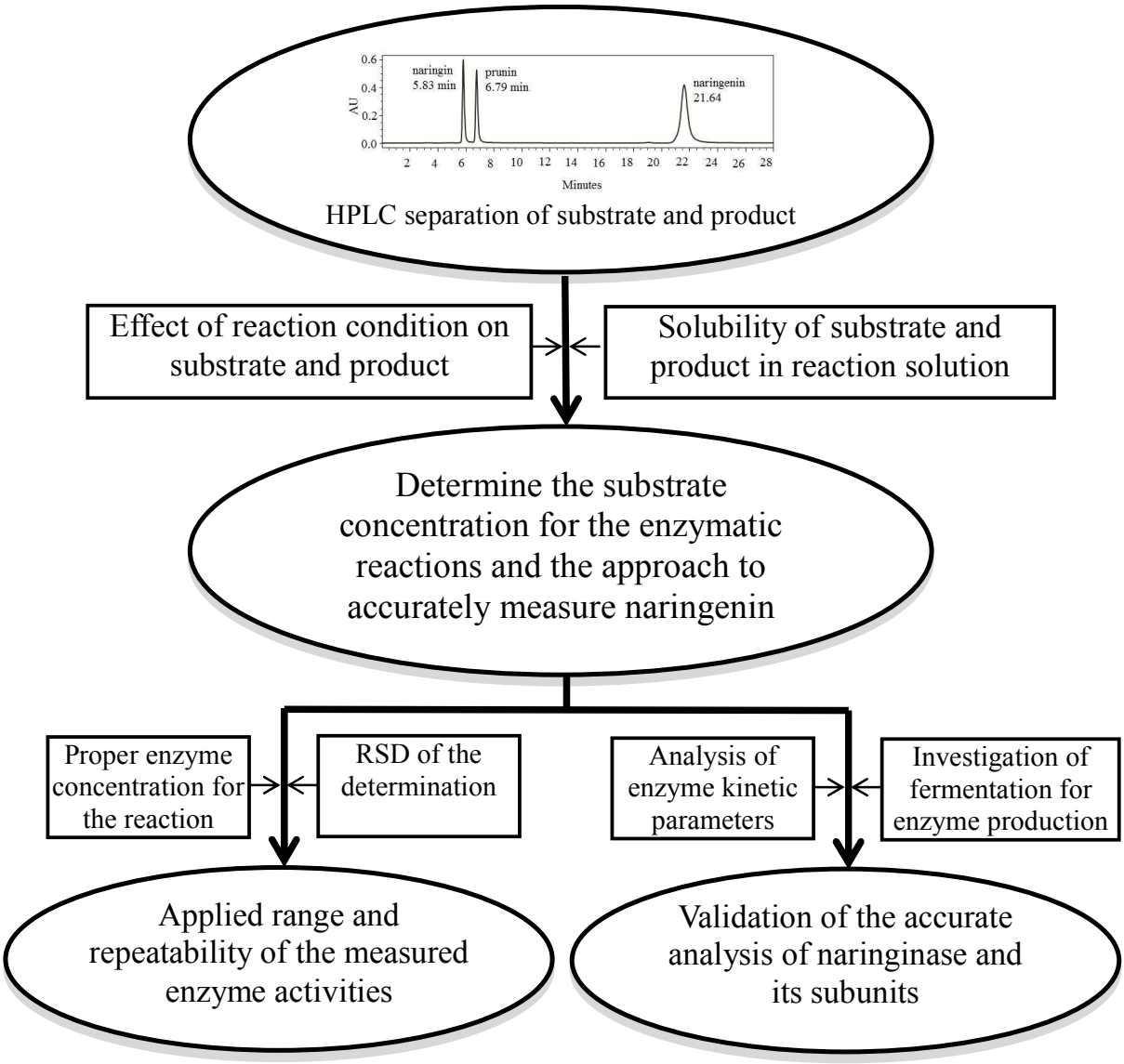

Graphic for the table of content