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ARTICLE

Isolation and Characterization of Nicotiflorin Obtained by Enzymatic Hydrolysis of Two Precursors in Tea Seed Extract

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Two flavonol triglycosides, camelliaside A (CamA) and camelliaside B (CamB), of tea seed extract (TSE) were subjected to enzymatic hydrolysis. Among five kinds of glycosidases investigated, β-galactosidase (Gal) induced selective hydrolysis of CamA. On the other hand, pectinase (Pec) and cellulase (Cel) induced hydrolysis of CamB. For Gal and Pec, only kaempferol diglycoside (nicotiflorin, NF) was produced; on the other hand, significant amounts of kaempferol monoglycoside (astragalin, AS) and kaempferol (KR) were also detected for Cel. The combination of the use of Gal and Pec in the enzymatic hydrolysis of TSE afforded NF with high specificity. Crude NF with 22% purity was recovered from the enzymatic reaction mixture by extraction with organic solvent, and pure NF with >95% purity was obtained by crystallized in water. The chemical structure of NF was confirmed by ^{1}H and ^{13}C NMR analyses.

KEYWORDS: Tea seed extract; flavonol triglycosides; enzymatic hydrolysis; flavonol diglycoside; nicotiflorin

INTRODUCTION

Tea [*Camellia sinensis* (L.) O. Kuntze, Theaceae] is known to contain caffeine, vitamin C, tannins, saponins, and polyphenols. Tea is generally consumed as a beverage or in traditional medicines in Asia and classified into three forms: green, oolong, or black.

Since the cancer-protective effect of drinking green tea was reported (*1*, *2*), much research has been focused on the isolation and biological effect of each component contained in tea, especially polyphenols (*3*, *4*). The antiaging, wound-healing, and disease treatment effects of green tea have also been investigated (*4*). Anticancer effects (*5*,*6*) and potential health benefits (*7*) of tea were well reviewed in earlier literature. The major polyphenols present in tea are flavan-3-ols and flavonols. Flavan-3-ols account for about 77% of phenolic compounds in tea; however, they are structurally less stable than flavonols. The major flavonols in tea are glycosides of quercetin or kaempferol. As the popularity of tea increases, production of tea seed (TS) has also increased. TS also contains many biologically active compounds such as saponin, flavonoid, vitamins, and tannins (*3*, *8*).

Tea seed extract (TSE), a byproduct of oil processing of TS, is produced by hot-water extraction of tea seed. Because TSE has been found to inactivate human influenza viruses almost completely (*8*), many studies have been focused on the biological activities of TSE (*9*−*11*). Two kinds of kaempferol triglycosides were isolated from TSE and identified as camelliaside A (CamA) and camelliaside B (CamB) (*3*). Detailed study (*12*) of the constitution of TSE showed the presence of CamC (kaempferol-3-*O*-[2-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside), but the amount of CamC was <1% of the amount of CamA or CamB.

Recently, enzymatic hydrolysis of CamA and CamB in TSE was investigated to produce kaempferol (KR), and the combination of β-galactosidase and hesperidinase was found to afford KR with >95% purity (*13*, *14*). The antioxidant activity (*13*) and activity on 5α-reductase inhibition (*14*, *15*) of KR were also investigated. In the course of bioconversion, the appearance of two major intermediates such as kaempferol diglycoside (nicotiflorin, NF) and monoglycoside (astragalin, AS) were reported. However, these intermediates were not isolated. The chemical names and structures of KR and its glycosides are summarized in **Figure 1**.

A minute quantity of NF can be obtained by isolation from *Carthamus tinctorius* (*16*), *Acalypha indica* (*17*), *Staphylea bumalda* (*18*), the petals of *Clitoria ternatea* (*19*), and *Trigonotis peduncularis* Benth. (*20*). Even though antioxidant activity as well as protective effects on reducing memory dysfunction of NF has been reported (*16*, *21*), the rareness of NF in nature has prevented its widespread study and application. However, no study on the synthesis of NF by either chemical or biochemical methods has been performed.

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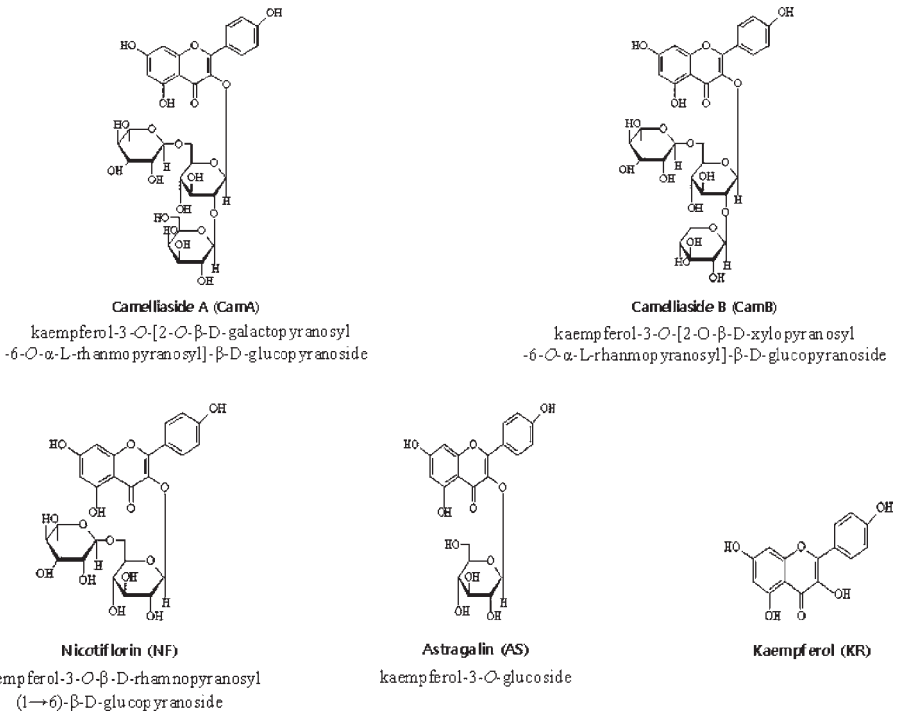

Figure 1. Chemical names and structures of KR and its glycosides.

In this paper, several enzymes were investigated for the selective hydrolysis of CamA and CamB in TSE. The combination of the use of β-galactosidase and pectinase was found to afford NF with high specificity. The isolation of NF from the enzymatic reaction mixture was carried out by extraction with organic solvent, and the pure NF obtained by crystallization was characterized by ^{1}H and ^{13}C NMR studies.

MATERIALS AND METHODS

Materials. TSE (product name "tea saponin") was purchased from Shanghai Youngsun Foods Co. Ltd. and used without further purification. Cellulase (Cel; EC 3.2.1.4, 1.2 U/mg), pectinase (Pec; EC 3.2.1.15, 1.32 U/mg), and β-galactosidase (Gal; EC 3.2.1.23, 10 U/mg) were purchased from Sigma. Reagent grade organic solvents were purchased from Aldrich and used without further purification. An authentic sample of NF was purchased from ChromaDex, Inc. (Irvine, CA).

Analysis of TSE Transformation. The analysis of extracts and enzyme reactants was carried out by the following HPLC and TLC method. The HPLC system consisted of Waters 600 and 486 detector. A Monitor C18 reverse phase column (5 μm, 250 × 4.6 mm) was employed. The detector wavelength was set at 254 nm. The mobile phases used for the analysis of samples were a mixture of distilled water (A) and acetonitrile (B). Isocratic elution (20% of B) was used as mobile phase for 14 min, and the gradient elution was 20→50% of B for the next 16 min at a flow rate of 1 mL/min. TLC analyses were performed on a silica gel plate (Merck, Kieselgel 60 F_{254}) using ethyl acetate/methanol/water (16:2:1, v/v/v). The separated spots on the TLC plate were visually observed by UV lamp, followed by dipping of the plates into 2% molybdo(VI)phosphoric acid hydrate in ethanol.

Isolation and Quantification of CamA and CamB from TSE. Preparative HPLC was performed using a SunFire Prep C18 OBD 5 μm (i.d. 19 × 150 mm, 5 μm particle diameter, Waters) column on a Waters HPLC system consisting of a Waters 600 controller, a Waters 2489 UV−visible detector, and a Waters Delta 600 pump with 70% 0.1 mM potassium phosphate buffer, 20% methanol, and 10% tetrahydrofuran (THF) as mobile phase. UV detection was carried out at 254 nm. The flow rate was 17 mL/min.

The analysis was carried out on a SunFire C18 (i.d. 4.6 × 150 mm, 3.5 μm particle diameter, Waters). The mobile phase was 70% 0.1 mM potassium phosphate buffer, 20% methanol, and 10% THF, and the flow rate was 0.5 mL/min.

Two compounds were analyzed by ESI-MS. Negative-ion ESI-MS spectra were obtained. The quantification of CamA and CamB from TSE was performed from a calibration curve for isolated CamA and CamB using Empire 2.0 software (Waters).

Enzymatic Hydrolysis of TSE. TSE (2.0 g) in 16 mL of phosphate buffer (pH 4.5) was dissolved at 40 °C, and 100 U of each enzyme predissolved in 4 mL of buffer was added. The reaction mixture was incubated with stirring at 40 °C for 48 h.

To monitor the progress of bioconversion, 0.1 mL of sample, which was taken every 2 h until 12 h and also taken at 24 and 48 h, was diluted with 0.5 mL of methanol. After centrifugation at 2000 rpm for 10 min, TLC and HPLC were measured.

Scale-up Reaction and Purification of NF. One hundred grams of TSE in 600 mL of phosphate buffer (pH 4.5) was dissolved at 40 °C. To this solution were added 4 kU of Gal and 3 kU of Pec predissolved in 100 mL of buffer. The reaction mixture was then incubated with stirring at 40 °C for 24 h.

After completion of the reaction had been confirmed by HPLC, 60 g of sodium chloride was added and the reaction mixture was extracted with 1200 mL of THF. The organic layer was washed with 1000 mL of water containing 50 g of sodium chloride and dried over $NaHCO_3$. After filtration and evaporation of the organic layer, 47.8 g of a crude powder was obtained.

The crude powder was dissolved with 240 mL of ethanol and precipitated with 2.4 L of diethyl ether. After filtration and drying under vacuum, 37.8 g of a yellowish solid was obtained. Crystallization in 380 mL of water afforded 4.3 of a yellowish crystal of NF.

The purity of NF at each step was measured by comparing the HPLC area with that of the authentic sample. To confirm the chemical structure of the purified NF, ^{1}H and ^{13}C NMR spectra were obtained with a Bruker spectrometer FT-NMR (500 MHz) using CH_3OH-d_4 as a solvent.

RESULTS AND DISCUSSION

Separation of CamA and CamB from TSE. Two main compounds, observed in the analytical HPLC, were separated by using preparative HPLC, followed by desalting as described under Materials and Methods.

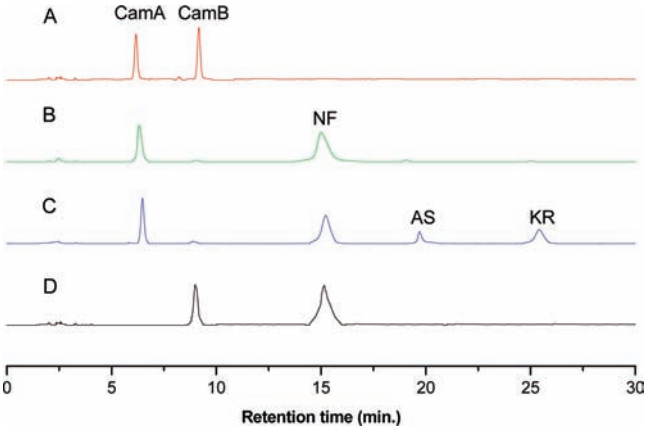

Figure 2. HPLC profiles of TSE (**A**) and samples taken at 24 h in the enzymatic reactions with Pec (**B**), Cel (**C**), and Gal (**D**).

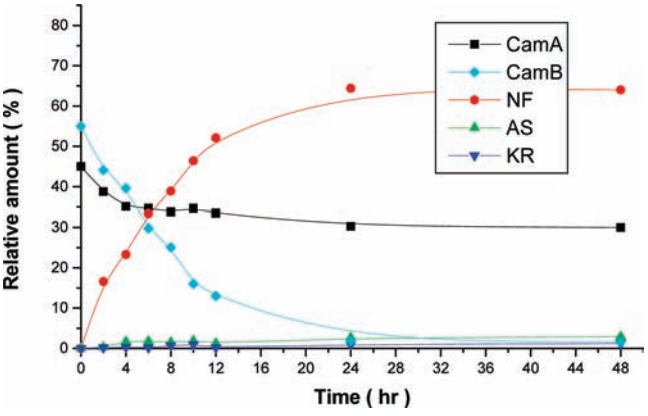

Figure 3. Relative amounts of each compound in the reaction using Pec (50 U/g of TSE).

To confirm the structure of the isolated compounds, mass spectra were acquired by ESI-MS and purity was assessed by HPLC. The ESI mass spectra of CamA and CamB exhibited molecular ion peaks at m/z 755.27 [$(MH)^-$] and 725.22 [$(MH)^-$] in negative ion mode. These ion peaks are clearly equivalent to the molecular weights of CamA (756.9 g/mol) and CamB (726.9 g/mol), respectively.

Amount of CamA and CamB in TSE. Calibration involves running a known concentration (2.500, 1.250, and 0.625 mg/mL) of CamA and CamB to determine the detector response. The quantification of CamA and CamB from TSE was applied into a calibration curve for isolated CamA and CamB using Empire 2.0 software (Waters). The result showed that the contents of CamA and CamB in TSE were 9.6707 (±0.1428) and 11.7278 (±0.0742) wt %, respectively.

Enzymatic Hydrolysis of TSE. The TSE, which contains CamA and CamB in the amounts of 9.67 and 11.73 wt %, respectively, was subjected to enzymatic hydrolysis at 40 °C, and the reaction progress was monitored by HPLC. As shown in **Figure 2A**, TSE itself showed only CamA and CamB. Among five kinds of glycosidases investigated in this work, two enzymes, hemicellulase and glucosidase, showed no reactivities. Therefore, the reactivity and specificity of Pec, Cel, and Gal were quantitatively investigated by measuring the relative amounts, which were calculated from the peak areas of each compound in HPLC. HPLC profiles of samples taken from the reaction mixture at 24 h are described in **Figure 2B** (reaction with Pec), **Figure 2C** (reaction with Cel), and **Figure 2D** (reaction with Gal).

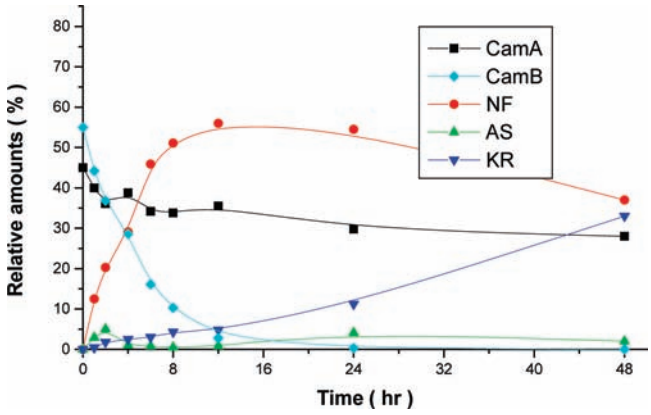

Figure 4. Relative amounts of each compound in the reaction using Cel (50 U/g of TSE).

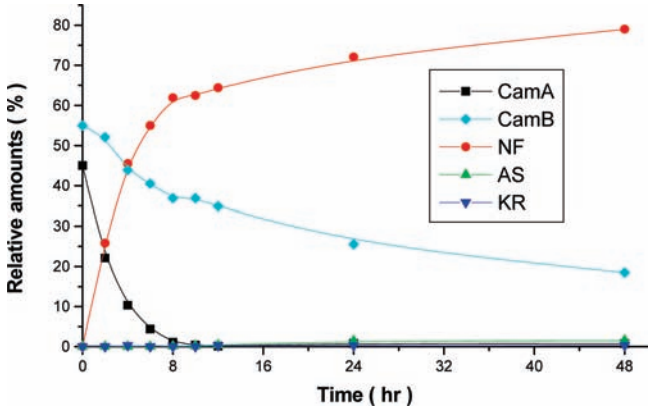

Figure 5. Relative amounts of each compound in the reaction using Gal (50 U/g of TSE).

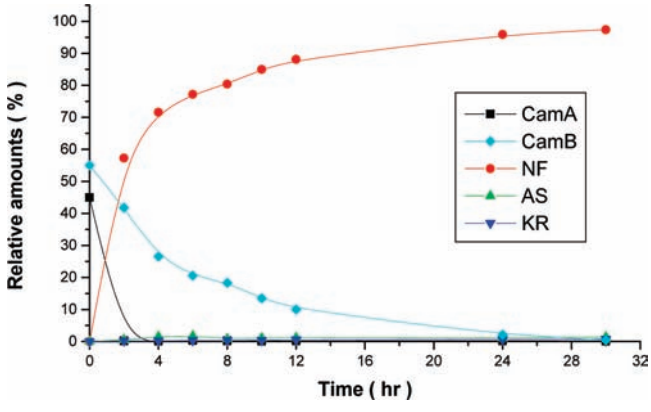

Figure 6. Relative amounts of each compound in the reaction using Gal (200 U/g of TSE).

Figure 3 shows the relative amounts of each compound when Pec in the amount of 50 U/g of TSE was applied for enzymatic hydrolysis of TSE. CamB was immediately deglycosylated, and the relative amount of NF increased. On the other hand, most of CamA remained unreacted even at 24 h, and no change in the compositional profile was observed at 48 h. It is also remarkable that, throughout the reaction, the amount of AS and KR was less than 2 and 1%, respectively. **Figure 3** indicates that Pec induces the formation of NF from CamB; however, no further reaction occurred once NF was formed. Pec is also called polygalacturonase and is able to break down or transform pectins, which are a

family of complex polysaccharides that contain 1,4-linked α-D-galacturonic acid residues. However, in this study, Pec was found to hydrolyze the xylosyl moiety of CamB,; on the other hand, no further deglycosylation of other sugars such as rhamnose and glucose was observed.

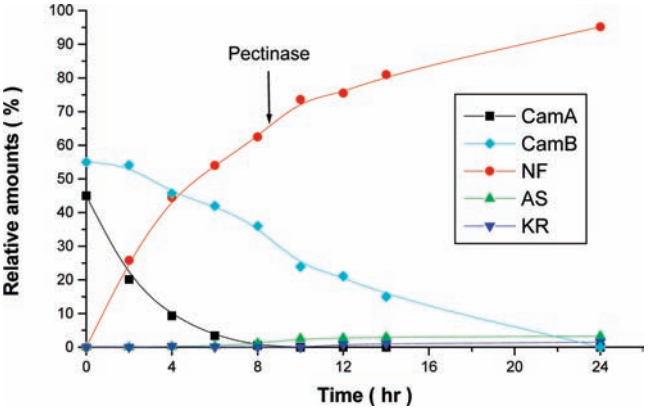

Figure 7. Relative amounts of each compound when the reaction was started in the presence of Gal (50 U/g of TSE) and Pec (50 U/g of TSE) was added at 8 h.

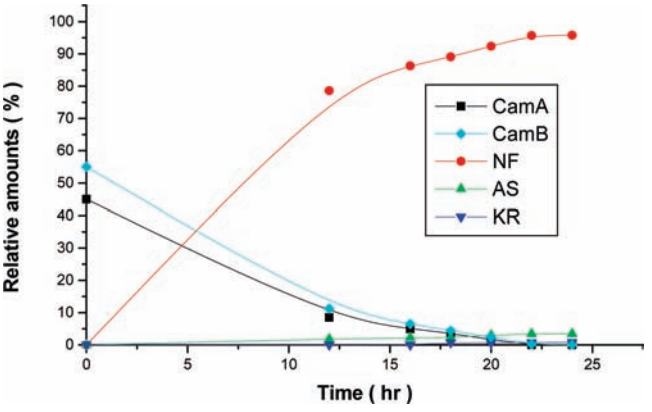

Figure 8. Relative amounts of each compound in the reaction using Gal (20 U/g of TSE) and Pec (35 U/g of TSE).

For Cel, CamB specificity was also observed, as described in **Figure 4**. However, significant amounts of AS and KR were detected and, for example, the relative amounts of AS and KR at 24 h were 11.2 and 4.1%, respectively. In general, Cel is known to cleave glycosidic bonds from some natural glycosides. However, these results suggest that Cel preferably hydrolyzes the xylosyl moiety of CamB in a manner similar to that of Pec; on the other hand, Cel also induces the deglycosylation of rhamnosyl and glucosyl moieties.

CamA was immediately deglycosylated for Gal and the relative amount of NF increases, as described in **Figure 5**. The decrease in the amount of CamB is very slow, and almost half of CamB remained unreacted. It is not surprising that Gal preferably hydrolyzes the galactosyl moiety of CamA; however, the xylosyl moiety of CamB was also hydrolyzed, even though the hydrolysis rate was very low. No appearance of AS or KR in a sample at 24 h indicates that Gal does not induce the deglycosylation of other sugars such as rhamnose and glucose.

In an earlier study (*13*) investigating the enzymatic hydrolysis of TSE with Gal, both CamA and CamB were reported to be transformed to NF in 24 h. However, it is not clear whether the transformation completely occurred. According to our results, when 50 U/g of Gal was used, CamA disappeared within 10 h; on the other hand, the hydrolysis of CamB was not complete in 24 h. However, 7 and 2% of CamB remained after 24 h when 100 and 150 U/g of Gal were applied, respectively. For 200 U/g, complete disappearance of CamB was observed in 30 h, as shown in **Figure 6**. It is noticeable that a meaningful amount of AS or KR was not formed even if the amount of Gal increased.

The complete hydrolysis of CamA and CamB to NF with a lesser amount of enzyme could be accomplished by the stepwise reaction of two enzymes such as Gal (CamA-specific) and Pec (CamB-specific). The reaction started with 50 U/g of Gal and, after confirming the disappearance of CamA by HPLC at 8 h, 50 U/g of Pec was added to the reaction mixture and the reaction continued. As shown in **Figure 7**, complete conversion to NF was

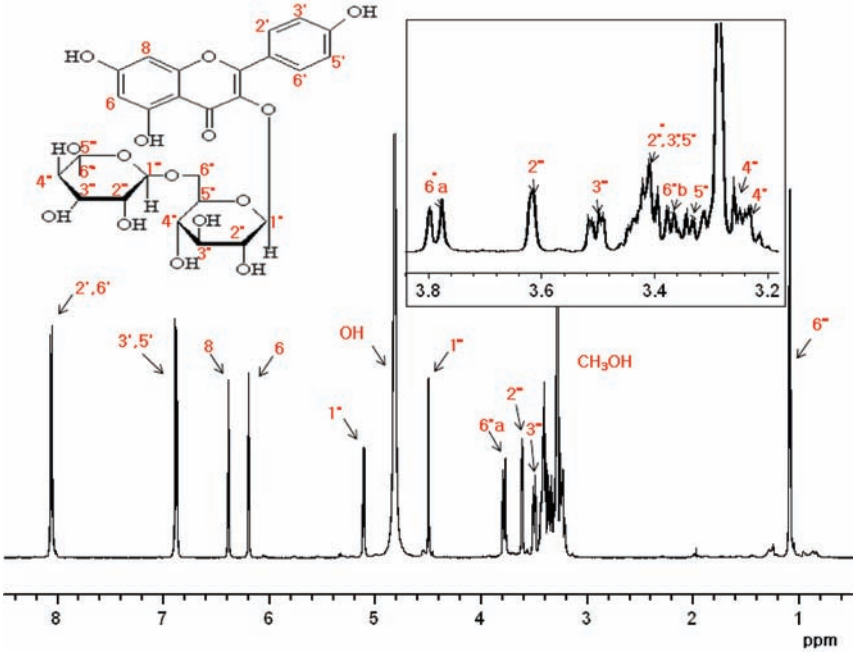

Figure 9. ^{1}H NMR of NF in CH_3OH-d_4.

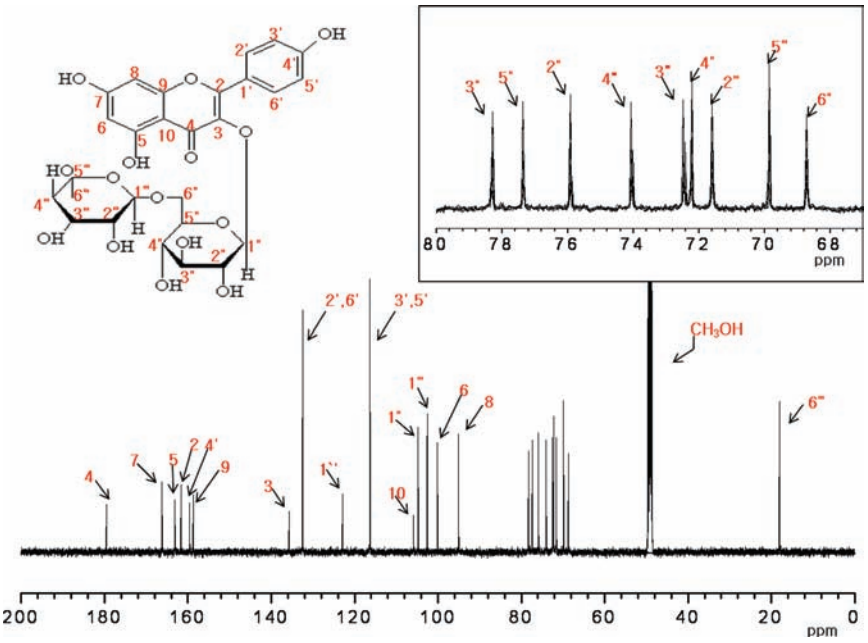

Figure 10. ^{13}C NMR of NF in CH_3OH-d_4.

observed at 24 h. A similar result was also obtained when the reaction started in the presence of 50 U/g of Gal and Pec.

Scale-up Reaction and Purification of NF. To prepare NF from TSE with a minimum amount of enzyme, the hydrolysis reaction of 100 g of TSE was carried out in the presence of two enzymes, Gal (CamA-specific) and Pec (CamB-specific).

As shown in **Figure 8**, both CamA and CamB completely disappeared at 24 h and only 3.4 and 0.8% of AS and NF, respectively, were observed in the presence of 40 U/g of Gal and 30 U/g of Pec. The recovery of NF by extraction with THF afforded 47.6 g of crude NF. The purity measured by comparing the HPLC area with that of authentic sample was 22%.

The mole-based yield of NF from TSE was 61.0% by calculation according to the equation

$$\text{yield}\ (\%) = \frac{\text{Pu} \times \text{Wt/MW (NF)}}{\{9.67\ (\text{g})/\text{MW (CamA)}\} + \{11.73\ (\text{g})/\text{MW (CamB)}\}} \times 100 \quad (1)$$

where Pu and Wt are the purity (%) and weight (g) of crude NF obtained at each step, respectively, and MW(X) indicates the molecular weight of X; 9.67 and 11.73 g are the weights of CamA and CamB contained in 100 g of TSE, respectively. According to the equation, the mole-based yield was calculated as

$$\text{yield}\ (\%) = \frac{0.22 \times 47.6\ (\text{g})/594\ (\text{g/mol})}{\{9.67\ (\text{g})/757\ (\text{g/mol})\} + \{11.73\ (\text{g})/727\ (\text{g/mol})\}} \times 100 = 61.0\ (\%)$$

It was reported that NF isolated from the seed of *C. sinensis* can be purified by recrystallization from water (*3*). In this study, crude NF was also recrystallized from water, and 3.2 g of pure NF was obtained. The final product was found to have a purity of 97%, which was measured by comparing the HPLC area with that of authentic sample. A yield of 18.1% from TSE on a molar base was obtained by calculation according to eq **1**.

The identification of final NF was carried out by ^1H and ^{13}C NMR studies. As shown in **Figures 9** and **10**, all signals in ^1H NMR and ^{13}C NMR spectra of NF are in good agreement with those of NF isolated from the petals of *C. ternatea* (*20*) and from *T. peduncularis* Benth (*21*). The integration ratios of each peak in ^1H NMR were also identical to the ideal ratios.

In conclusion, for the selective hydrolysis of CamA and CamB in TSE, combination of the use of β-galactosidase (Gal) and pectinase (Pec) was found to afford NF with high specificity. NF was isolated from the enzymatic reaction mixture by extraction with THF and purified by crystallization in water. The final product, which was characterized by ^1H and ^{13}C NMR studies, was found to have a purity of 97% from HPLC and 18.1% yield from CamA and CamB contained in TSE on a molar base. This paper is the first study to prepare pure NF from natural sources.

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