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Enzymatic Synthesis of Novel Phenol Acid Rutinosides Using Rutinase and Their Antiviral Activity in Vitro

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Supporting Information

ABSTRACT: Novel rutinosides of vanillic acid, sinapic acid, ferulic acid, and caffeic acid were prepared via a rutinase-catalyzed transglycosylation reaction. Reaction mixtures containing rutin, phenolic acid, and rutinase derived from tartary buckwheat (*Fagopyrum tataricum*) seeds were incubated in 20 mM acetate buffer (pH 5.0) at 40 °C. The resulting rutinoside of each phenolic acid was purified by HPLC, and the structure was determined by NMR and FAB-MS analysis. Antiviral activity was determined using feline calicivirus (FCV) strain F9, which is a typical norovirus surrogate. It was found that rutinosylation of the phenolic acids increased their antiviral activity against FCV, with the sinapic acid rutinoside being the most effective. These results will contribute to the development of antiviral agents against noroviruses.

KEYWORDS: *antiviral activity, norovirus, phenolic acid, rutinoside, transglycosyaltion reaction*

INTRODUCTION

Noroviruses are recognized as a major cause of nonbacterial gastroenteritis and viral foodborne infection in both children and adults throughout the world.[1] Noroviruses are highly communicable, with transmission occurring through the ingestion of contaminated food and water and by person-to-person contact.[2] Currently there is no vaccine to prevent human norovirus infection and no specific therapy available to treat it. Feline calicivirus (FCV) has thus been chosen as a surrogate virus to study noroviruses, because noroviruses could not be grown in cell culture systems.[3] FCV has a similar primary sequence and genome organization and shares biochemical properties with noroviruses.[4]

Plant-derived natural products are abundant sources of useful compounds for antiviral chemotherapy.[5] Many studies have confirmed the antiviral activity of phenolic compounds (phenolic acids, flavonoids, lignans, and tannins) against several viruses such as rotavirus,[6] herpes virus,[7] and influenza virus.[8] Flavonoid glycoside also had potent antiviral activity. For example, quercetin 7-rhamnoside has stronger antiviral activity against porcine epidemic diarrhea virus compared to flavonoid aglycones.[9] Additionally, flavonoid rhamnoglycosides exhibit an in vitro inhibitory effect on rotavirus infection,[10] whereas their aglycones exhibit no inhibitory activity. Thus, the sugar moiety likely plays an important role in the antiviral activity of plant-derived natural products.

The molecular modification of phenolic acids is currently an important field of research. Many studies have confirmed that the biological activities of phenolic acids can be improved by lipophilization[11] and glycosylation.[12] In particular, glycosylation allows phenolic acids to reinforce their hydrophilic character by conjugating sugars. Because the use of phenolic acids is often limited by their weak solubility either in hydrophilic or hydrophobic formulations, glycosylation of a hydrophilic compound can be a potent strategy to alter its solubility and cell penetration ability, as well as its biological activity.

Recent studies have revealed that plant-derived polyphenols, including tannins, have potent antivirus effects against FCV.[13] We thus hypothesized that phenolic acids are good candidates as antiviral agents against noroviruses and that the glycosylation of phenolic compounds could aid in the inactivation of noroviruses. In this study, we prepared rutinosides of phenolic acids (vanillic acid, sinapic acid, ferulic acid, and caffeic acid) using the reverse reaction of hydrolysis by rutinase, derived from tartary buckwheat (*Fagopyrum tataricum*) seeds (Figure 1) and investigated their antiviral activity against FCV strain F9 in vitro.

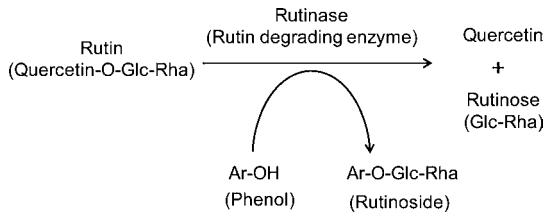

Figure 1. Scheme showing rutin hydrolysis and transglycosylation of phenol acceptors by rutinase.

MATERIALS AND METHODS

Materials. Tartary buckwheat (*F. tataricum*) seeds were supplied by Nikkoku Seihun (Nagano, Japan). Rutin, vanillic acid, and ferulic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sinapic acid and caffeic acid were obtained from Sigma-Aldrich Japan Co. Ltd. (Tokyo, Japan). MEMα was purchased from Nacalai Tesque (Kyoto, Japan). Crandell–Reese feline kidney

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(CRFK) cells were obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan). FCV strain F9 was purchased from the American Type Culture Collection (Manassas, VA, USA). All other reagents were of biochemical and HPLC grade.

Rutinase. Rutinase was isolated from tartary buckwheat seeds according to the method of Yasuda et al.[14] with some modifications. Briefly, tartary buckwheat flour (5 g) was extracted with 150 mL of 20 mM acetate buffer (pH 5.0) by stirring for 1 h at room temperature. After centrifugation at 15000*g* for 30 min at 4 °C, the supernatant was filtered through filter paper, and then the filtrate was applied to a DEAE-Sepharose column (GE Healthcare, Tokyo, Japan) pre-equilibrated with 20 mM acetate buffer (pH 5.0) and eluted with a linear gradient of 0−1 M NaCl in 20 mM acetate buffer (pH 5.0) at a flow rate of 1.0 mL/min. The fractions with rutin-degrading activity were combined and dialyzed overnight against 20 mM acetate buffer (pH 5.0) containing 0.1 M NaCl. The dialysate was freeze-dried, dissolved in 5 mL of 20 mM acetate buffer (pH 5.0), then applied to a Sephacryl S-100 column (GE Healthcare, Tokyo, Japan) pre-equilibrated with 20 mM acetate buffer (pH 5.0) containing 0.1 M NaCl and fractionated at a flow rate of 0.5 mL/min. The fractions with rutin-degrading activity were combined. Finally, the solution was dialyzed using 10−12 kDa cutoff dialysis tubing against distilled water, freeze-dried, and stored at −30 °C. Eight and a half milligrams of rutinase was obtained from 5 g of tartary buckwheat seed powder.

Measurement of Rutinase Activity. The reaction mixture, consisting of 0.3 mL of 0.1% (w/v) rutin in 20% (v/v) methanol and 0.1 mL of enzyme solution during the purification of rutinase, was incubated at 40 °C for 3 min, then the reaction was stopped by the addition of 1.2 mL of methanol. The quenched reaction mixture was passed through a 0.45 μm syringe filter and then analyzed by HPLC. The HPLC system was composed of an HPLC pump (PU-2089 Plus, Jasco, Tokyo, Japan), column oven (CTO-10A, Shimadzu, Kyoto, Japan), UV detector (UV-2075 Plus, Jasco), and RI detector (RI-1530, Jasco). HPLC analysis was performed on an Inertsil ODS-3 column (4.6 × 250 mm, GL Sciences, Tokyo, Japan) using 2.5% acetic acid/methanol/acetonitrile (55:35:15, v/v/v) at a flow rate of 1 mL/min. One unit (kat) of rutinase was defined as the amount of enzyme activity that produces 1 mol of quercetin per second under the reaction conditions. The activity of rutinase prepared as described above was 1092 μkat/mg of protein.

Enzymatic Synthesis of Phenolic Acid Rutinosides. The enzymatic reaction was conducted in closed tubes. The reaction mixture was composed of 10 mM rutin, 3.75% (w/w of rutin substrate) rutinase, and 10 mM phenolic acid in 1 mL of 20 mM acetate buffer (pH 5.0). The tubes were incubated at 40 °C for 0−48 h on a thermomixer (Eppendorf, Hamburg, Germany) with shaking at 1300 rpm. Control experiments were conducted in the absence of enzyme. To examine the effect of temperature, pH, and rutinase concentration on enzymatic synthesis of rutinosides, the reaction was conducted at different temperatures ranging from 30 to 50 °C in the pH range of 4.0−7.0 (20 mM acetate buffer for pHs 4.0 and 5.0; and 20 mM phosphate buffer for pH 6.0 and 7.0) using different enzyme concentrations (0−15.00%, w/w of rutin substrate), respectively. Fifteen percent (w/w of rutin substrate) of enzyme concentration was equivalent to 1500 μkat/mL of enzyme activity.

Purification and Structural Analysis of Phenolic Acid Rutinosides. Products were monitored by HPLC analysis on an Inertsil NH_2 column (4.6 × 250 mm) (GL Sciences, Tokyo, Japan). The resultant mixtures were centrifuged at 13000 rpm for 30 min at 4 °C, then the supernatant was passed through a 0.45 μm syringe filter and applied to the HPLC column. Elution was carried out with 75% acetonitrile at a flow rate of 1 mL/min, and the fractionated compounds were detected using an RI detector and a UV detector set at 280 nm. The column temperature was set at 30 °C. The synthesized rutinosides was collected, the acetonitrile was evaporated, and the residue was freeze-dried. The HPLC retention times for the substrates and products are as follows: rutin, 11.9 min; vanillic acid, 11.5 min; sinapic acid, 14.7 min; ferulic acid, 11.8 min; caffeic acid, 15.1 min; vanillic acid rutinoside, 6.3 min; sinapic acid rutinoside, 5.6 min; ferulic acid rutinoside, 5.5 min; caffeic acid rutinoside, 6.4 min. Calibration curves for phenolic acid rutinosides were obtained using purified samples, and the yield was calculated from the calibration curves.

The structures of the rutinosylated phenolic acids were determined by ^{1}H NMR and ^{13} C NMR (Bruker Avance DRX-500 spectrometer, Rheinstetten, Germany) at 500 and 125 MHz, respectively. Acetone-d_6 and DMSO-d_6 were used as solvents. Chemical shifts are given in parts per million relative to TMS, used as an internal standard. Fast atom bombardment mass spectrometry (FAB-MS) spectra were measured with a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan).

Identification of Phenolic Acid Rutinosides. *Vanillic acid rutinoside:* ^{1}H NMR [$(CD_3)_2SO$] δ 7.47 (dd, J = 8.5, 1.5 Hz, 1H), 7.38 (d, J = 1.5 Hz, 1H), 6.74 (d, J = 8.5 Hz, 1H), 5.49 (d, J = 7.5 Hz, 1H), 4.53 (br, 1H), 3.80 (d, J = 10.0 Hz, 1H), 3.74 (s, 3H), 3.75 (s, 1H), 3.59 (d, J = 2.0 Hz, 1H), 3.45−3.38 (m, 4H), 3.31−3.24 (m, 4H), 3.17−3.10 (m, 3H), 1.67 (s, 2H), 1.10 (d, J = 6.5 Hz, 3H); ^{13}C NMR [$(CD_3)_2SO$] δ 164.77, 148.32, 146.44, 125.02, 122.47, 115.86, 112.84, 100.86, 94.53, 76.56, 72.70, 72.13, 70.82, 70.53, 69.88, 68.60, 66.75, 55.64, 18.08. High-resolution FAB-MS (negative ion mode), m/z 475.1522 $[M - H]^-$ (calcd for $C_{20}H_{27}O_{13}$, 475.1530).

Sinapic acid rutinoside: ^{1}H NMR [$(CD_3)_2CO$] δ 7.66 (d, J = 16.0 Hz, 1H), 7.06 (s, 2H), 6.42 (d, J = 16.0 Hz, 1H), 5.61 (d, J = 8.0 Hz, 1H), 4.72 (s, 1H), 4.65−4.00 (br, 3H), 3.97−3.30 (m, 10H), 3.85 (s, 6H), 1.18 (d, J = 6.5 Hz, 3H); ^{13}C NMR [$(CD_3)_2CO$] δ 166.07, 148.96, 147.33, 139.72, 126.01, 115.51, 107.01, 101.73, 95.24, 77.99, 77.40, 73.96, 73.79, 72.45, 71.80, 70.98, 69.10, 67.53, 56.70, 18.13. FAB-MS negative ion mode, m/z 531.1795 $[M - H]^-$ (calcd for $C_{23}H_{31}O_{14}$, 531.1792).

Ferulic acid rutinoside: ^{1}H NMR [$(CD_3)_2SO$] δ 7.62 (d, J = 15.8 Hz, 1H), 7.32 (d, J = 1.8 Hz, 1H), 7.13 (dd, J = 8.0, 1.8 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.47 (d, J = 15.8 Hz, 1H), 5.44 (d, J = 8.0 Hz, 1H), 5.35 (br s, 1H), 5.21 (br s, 1H), 4.70 (br s, 2H), 4.52 (s, 1H), 3.81 (m, 2H), 3.80 (s, 3H), 3.58 (s, 2H), 3.46−3.05 (m, 6H), 1.10 (d, J = 6.0 Hz, 3H); ^{13}C NMR [$(CD_3)_2SO$] δ 165.49, 148.22, 146.54, 125.28, 123.62, 115.77, 113.77, 111.51, 100.82, 94.28, 76.53, 76.49, 72.68, 72.10, 70.81, 70.52, 69.79, 68.53, 66.64, 55.84, 18.03. FAB-MS negative ion mode, m/z 501.1611 $[M - H]^-$ (calcd for $C_{22}H_{29}O_{13}$, 501.1686).

Caffeic acid rutinoside: ^{1}H NMR [$(CD_3)_2CO$] δ 7.62 (d, J = 16.0 Hz, 1H), 7.17 (d, J = 2.0 Hz, 1H), 7.06 (dd, J = 8.0, 2.0 Hz, 1H), 6.86 (d, J = 8.0 Hz, 1H), 6.27 (d, J = 16.0 Hz, 1H), 5.59 (d, J = 8.0 Hz, 1H), 4.71 (s, 1H), 4.00−3.30 (m, 10H), 1.18 (d, J = 6.0 Hz, 3H); ^{13}C NMR [$(CD_3)_2CO$] δ 166.09, 149.32, 147.08, 146.49, 127.37, 122.81, 116.43, 115.28, 114.91, 101.75, 95.30, 77.99, 77.45, 73.95, 73.82, 72.48, 71.81, 71.05, 69.12, 67.59, 18.13. FAB-MS negative ion mode, m/z 487.1528 $[M - H]^-$ (calcd for $C_{21}H_{27}O_{13}$ 487.1530).

Solubility Measurements. Native and rutinosylated phenolic acids were suspended in 1 mL of 20 mM acetic buffer (pH 5.0) at a concentration of 0.01 M. After centrifugation at 16000*g* for 10 min, the pellet was dried in a vacuum desiccator and weighed.

Cell Culture. CRFK cells were maintained in MEMα medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Wako, Osaka, Japan) at 37 °C in a humidified atmosphere of 5% CO_2 in air. CRFK cells were seeded at 2 × 10^5 cells/well in a 96-well plate (Iwaki, Tokyo, Japan) and incubated in 100 μL/well of MEMα supplemented with 10% FBS at 37 °C, 5% CO_2, for 24 h to form a cell monolayer. Native and rutinosylated phenolic compounds (100 or 200 μM) dissolved in DMSO (0.1% DMSO in final solution) diluted with buffer were tested for their cytotoxicity on the tissue culture prior to viral infection assays.

Determination of Antiviral Activity. FCV strain F9 was propagated and titrated on CRFK cells. Virus stocks were prepared as previously described by Matemu et al.,[15] and the resulting fluids were harvested, titrated, and stored at −80 °C until use. The assay of antiviral activity was based on the inhibition of norovirus-induced cell death, which was performed using the methyl thiazolyl tetrazolium (MTT) method[16] and 50% tissue culture infectious dose ($TCID_{50}$) method.[15]

Antiviral assay for vanillic, sinapic, ferulic, and caffeic acid and their rutinosides was performed by a MTT method. Briefly, the native and rutinosylated phenolic acids were suspended with FCV in equal ratio

at 100 or 200 μM final concentration and preincubated for 1 h before inoculation. Aliquots (25 μL) of different concentrations of samples−FCV were added to the confluent cell monolayers in a 96-well plate. The cells were incubated at 37 °C, 5% CO_2, for 1 h. The culture medium was removed, and adherent cells were rinsed with PBS. Fresh MEMα containing 2% FBS was added, and the cells were incubated at 37 °C, 5% CO_2, for 72 h. As a positive control, cells were infected with the same concentration of virus without sample, and as a negative control only 0.1% DMSO was added to the cells. The culture medium was removed, and 50 μL of 1.0 mg/mL MTT solution was added. After the cells were further incubated for 4 h at 37 °C, 5% CO_2, the medium was removed and 250 μL of DMSO was added to solubilize the MTT−formazan products. The absorbance was read at 570 nm using a microplate reader (Bio-Rad model 680, Tokyo, Japan). The antiviral activity was taken as the percentage cell viability by comparing the absorbance of the treated cells with the controls for 72 h.

Antiviral assay for sinapic acid and its rutinoside was performed by a $TCID_{50}$ method as described previously.[15] Briefly, serial 2-fold dilutions of the virus was mixed with an equal volume of sample (final concentration, 200 μM) or control and preincubated for 0, 10, 30, and 60 min before inoculation. Aliquots (80 μL) of samples−FCV were added to each of eight wells in the confluent CRFK cells monolayer of a 96-well plate. Following 1 h of incubation at 37 °C, 5% CO_2, for viral adsorption, the culture medium was removed and adherent cells were rinsed with PBS. Fresh MEMα containing 2% FBS was added, and the cells were incubated at 37 °C, 5% CO_2, for 72 h. $TCID_{50}$ was calculated using the Reed−Muench method.[17]

Statistical Analyses. The results were expressed as the mean ± SD. Statistical evaluation was carried out using an unpaired Student's *t* test and ANOVA, followed by Fisher's test. All statistical analyses were performed using Statcel software ver. 2.0 (OMS-Publishing, Saitama, Japan).

RESULTS

Synthesis of Rutinosides. The enzymatic glycosylation of various phenolic acids with rutinose was carried out using the reverse reaction of rutinase. Rutinase was used to transfer the rutinose moiety from rutin (sugar donor) to phenolic acids. Vanillic acid, sinapic acid, ferulic acid, and caffeic acid were chosen as acceptors. The reaction products were analyzed by HPLC, and a new peak corresponding to the transglycosylation product of each substrate was detected using the RI detector. The molecular formulas of newly synthesized rutinosides of vanillic acid, sinapic acid, ferulic acid, and caffeic acid were found to be $C_{20}H_{28}O_{13}$, $C_{23}H_{32}O_{14}$, $C_{22}H_{30}O_{13}$, and $C_{21}H_{28}O_{13}$, respectively, by FAB-MS. The structure of each newly synthesized rutinoside was determined by NMR analysis. Figure 2 shows the chemical structures of the rutinosides of vanillic acid, sinapic acid, ferulic acid, and caffeic acid.

The progress of the enzymatic rutinosylation of vanillic, sinapic, ferulic, and caffeic acid was examined in a time course experiment (Figure 3). A reaction mixture containing 10 mM rutin, 3.75% (% of ruin substrate), and 10 mM phenolic acid in 1 mL of 20 mM acetate buffer (pH 5.0) was incubated at 40 °C for 0−48 h with shaking at 1300 rpm. In all cases, rutinase catalyzed the transrutinosylation of the various phenolic acids, although the reactivity of sinapic acid was very low. Product yield decreased in the order vanillic acid, ferulic acid, caffeic acid, and sinapic acid.

The effect of reaction temperature on the synthesis of vanillic acid rutinoside was studied in a time course experiment (Figure 4A). The results showed that the yield increased rapidly during the first 12 h of incubation and then remained constant. In the temperature range 30−40 °C, the yield increased with increasing temperature; however, high temperatures such as 50 °C caused a significant drop in the yield. In the pH range

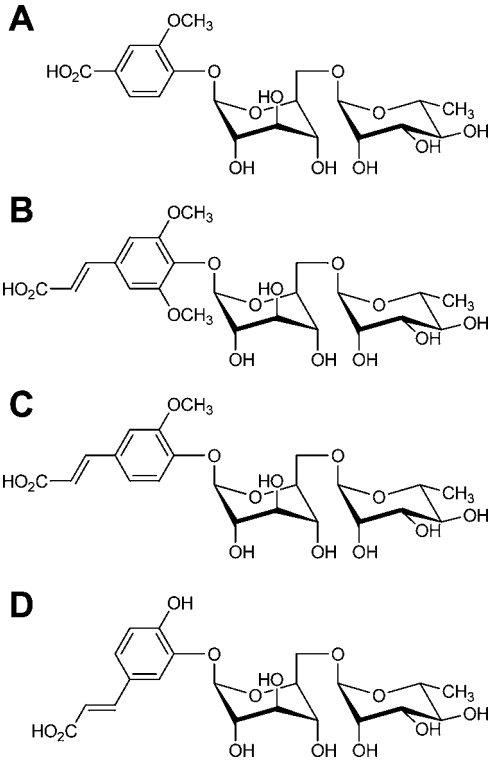

Figure 2. Structures of enzymatically synthesized vanillic acid rutinoside (A), sinapic acid rutinoside (B), ferulic acid (C), and caffeic acid rutinoside (D).

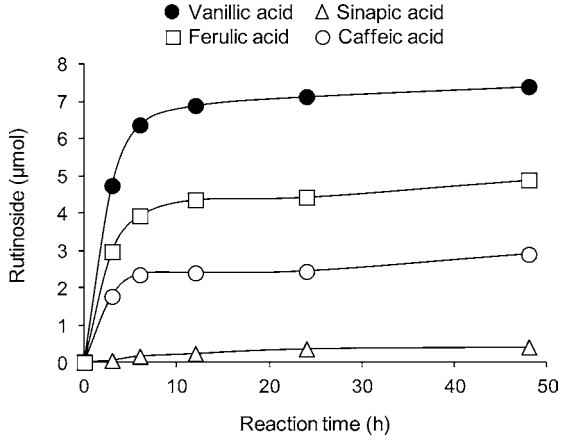

Figure 3. Time course of the synthesis of vanillic acid, sinapic acid, ferulic acid and caffeic acid rutinoside catalyzed by rutinase. Reaction temperature, 40 °C; buffer, 20 mM acetate buffer (pH 5.0); rutinase, 3.75% (w/w of rutin substrate).

4.0−7.0, higher yield was observed between pH 4.0 and 5.0, and the yield reached a maximum in 20 mM acetate buffer (pH 5.0) (Figure 4B). The effect of enzyme concentration on transglycosylation yield was examined next (Figure 4C). The yield increased as the enzyme concentration increased, reaching a maximum at 3.75% (w/w of rutin substrate) of rutinase, then decreased rapidly at higher enzyme concentration. The other three phenolic acid rutinosides also showed similar results (data not shown). These results indicated that the optimum conditions for the synthesis of rutinosides are a reaction temperature of 40 °C, 20 mM acetate buffer (pH 5.0), and 3.75% (w/w of rutin substrate) rutinase.

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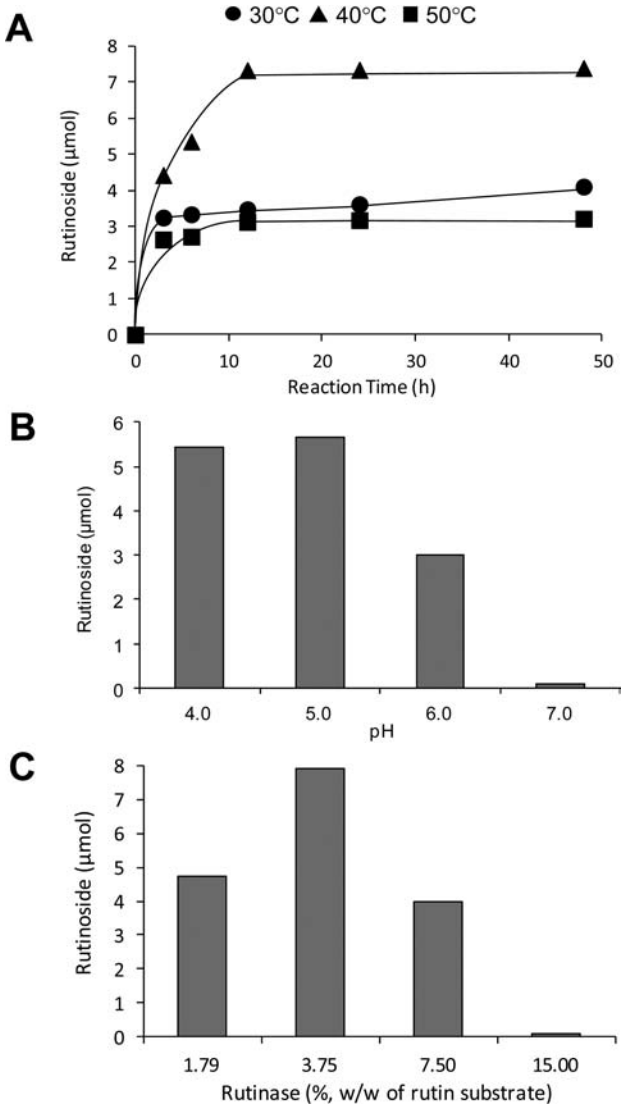

Figure 4. Optimization of the reaction conditions for synthesis of vanillic acid rutinoside using rutinase: (A) effect of reaction temperature [buffer, 20 mM acetate buffer (pH 5.0); rutinase, 3.75% (w/w of rutin substrate)]; (B) effect of buffer pH [reaction temperature, 40 °C; rutinase, 3.75% (w/w of rutin substrate); reaction period, 24 h]; (C) effect of different concentrations of enzyme [reaction was carried out in 20 mM acetate buffer (pH 5.0) and at 40 °C for 24 h].

Effect of Solubility on Rutinosylation. The solubility of phenolic acids significantly improved by glycosylation with rutinose (Figure 5). The solubility of ferulic acid, sinapic acid, caffeic acid, and vanillic acid increased from 0.35 to 1.31 mg/mL, 0.85 to 1.56 mg/mL, from 0.87 to 1.58 mg/mL, and from 1.40 to 2.17 mg/mL, respectively.

Antiviral Activity. The phenolic acids and their synthesized rutinosides were tested for antiviral activity using the MTT assay by measuring CRFK cell viability after infection with FCV F9. As shown in Figure 6, cell viability decreased to 27.5% after viral infection. The results showed that treatment with phenolic acids inhibited the decrease in cell viability mediated by FCV infection and that the rutinosides of phenolic acids had stronger inhibitory activity compared with the corresponding aglycone. Of the rutinosides tested, sinapic acid rutinoside had the highest inhibitory activity, followed by caffeic acid rutinoside.

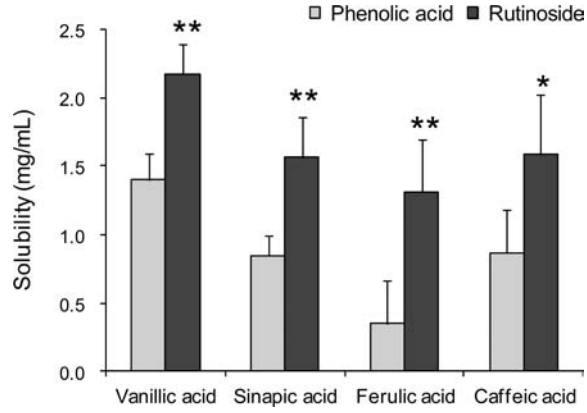

Figure 5. Effect of rutinosylation on the solubility of phenolic acids. Data are presented as means ± SD of three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$, when compared with the control.

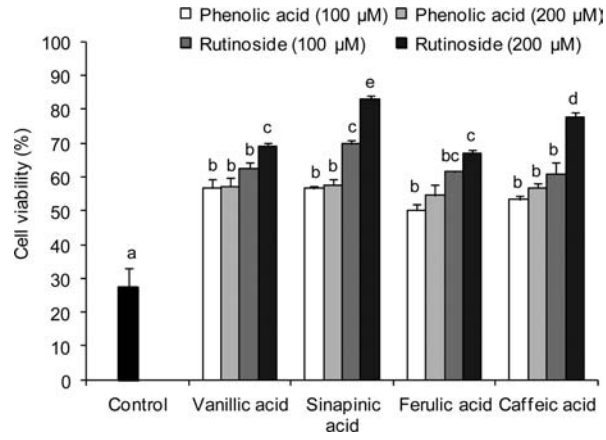

Figure 6. Inhibitory activity of phenolic acids and their rutinosides against FCV infectivity. Data are presented as means ± SD of three independent experiments. Different letters above bars indicate statistically different data ($p < 0.05$).

We further examined the inhibitory effect of sinapic acid and its rutinoside on FCV infection using the $TCID_{50}$ assay (Figure 7). FCV was treated with sinapic acid or its rutinosides for different

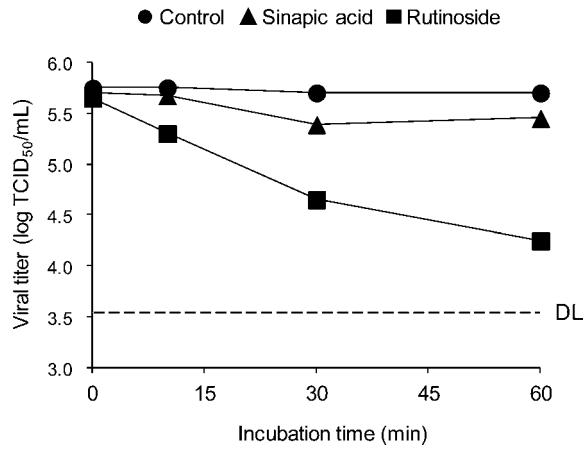

Figure 7. Effects on sinapic acid and its rutinoside on FCV infectivity at different incubation times. Virus infectivity was measured with the standard $TCID_{50}$ assay. DL, detection limit of virus infectivity with the method.

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incubation times, and the virus infectivity was measured with the $TCID_{50}$ assay. A significant decrease in viral titer was observed when FCV was treated with sinapic acid rutinosides for 60 min.

DISCUSSION

The present study demonstrates that phenolic acid rutinosides synthesized by the transglycosylation reaction of rutinase exhibit potent antiviral activity against FCV strain F9 in vitro. The rutinosides of vanillic acid, sinapic acid, ferulic acid, and caffeic acid are reported for the first time. The antiviral activity of the rutinosides was more potent than that of the corresponding aglycone.

Some glycosidases catalyze the formation of glycoside as well as the hydrolysis of glycosidic linkages.[18,19] For example, Tramice et al.[20] demonstrated the transglycosylation reactions of glycosyl hydrolases from *Thermotoga neapolitana*, and Minig et al.[21] reported that α-rhamnosyl-β-glucosidase from *Acremonium* sp. efficiently transglycosylated the rutinose moiety from hesperidin to 2-phenylethanol. Rutinase not only hydrolyzes the glycosidic bonds between quercetin and rutinose but also catalyzes the reconstitution of rutin. We thus attempted to transglycosylate the rutinose moiety hydrolyzed from rutin to phenolic acids. The NMR data indicate that rutinosylation proceeded at the primary hydroxyl group of rutinose. In the rutinosylation of vanillic acid, sinapic acid, and ferulic acid, a hydroxyl group at the 4-position of the aromatic ring was rutinosylated. When caffeic acid, which contains two hydroxyl groups in the ortho position, was used as the acceptor, the hydroxyl group at the 3-position of the aromatic ring was conjugated to rutinose. On the other hand, phenolic compounds containing two terminal aromatic groups such as curcumin, NDGA, and resveratrol did not form rutinosides (data not shown). Therefore, these results indicate that the transglycosylation reaction of rutinase proceeds in a highly regioselective manner. The yield of synthesized rutinoside depended on the phenolic acid used as the acceptor. Further studies using many kinds of phenolic acids are necessary to reveal the relationship between the structure of the acceptor and the yield of rutinoside following transglycosylation.

The degree of rutinosylation depended on the enzyme concentration. Higher enzyme concentration reduced the yield, perhaps due to accelerated hydrolysis of the rutinosides. The optimum reaction temperature and pH for rutinoside synthesis were 40 °C and pH 5.0; yield decreased above 50 °C and pH 6.0, respectively. Yasuda et al. reported that the optimum enzyme activity of rutinase was observed at 50 °C and between pH 4.0 and 6.0 and that this enzyme was stable up to 60 °C and between pH 4.0 and 7.0.[22] According to our results, the optimum conditions for the transglycosylation reaction are 40 °C and pH 5.0, so the optimum conditions for rutinosylation and hydrolysis differ. Rutinase has high substrate specificity for rutin (quercetin-3-rutinoside) and does not degrade structural homologues such as naringin (naringenin-7-rhamnoglucoside) and quercitrin (quercetin 3-rhamnoside).[14] When naringin was used in our experiments instead of rutin, no synthesis of phenolic acid rhamnoglucoside was observed. It is possible that rutin is essential for the synthesis of new rutinosides using the transglycosylation reaction of rutinase.

On the basis of our in vitro model of FCV infection, phenolic acid rutinosides exhibited antiviral activity when FCV was pretreated with phenolic acid rutinosides before incubation with CRFK cells. No significant activity was observed when the CRFK cells were pretreated with rutinosides, followed by FCV inoculation into CRFK cells (data not shown). The results strongly suggest that phenolic acid rutinosides inhibit FCV activity by directly binding to the virus and inactivate it and, consequently, block viral penetration into the cells. FCV strain F9 binds to the cellular receptor feline junctional adhesion molecule 1 (fJAM-1),[23] and FCV has been shown to bind α2,6-sialic acid.[24] According to previous studies,[15] acylation substantially enhances anti-FCV activity of 7S peptides, and higher surface hydrophobicity may contribute to the enhanced anti-FCV activity. With these findings taken into consideration, the carbohydrate moiety in rutinosides could bind to FCV; subsequent diffusion or trafficking of bound virus across the cell surface may then lead to virus entry after interactions with specific protein receptors such as fJAM-1. Among the rutinosides tested, sinapic acid rutinoside had the strongest antiviral effect against FCV. Further detailed studies are necessary to investigate the relationship between rutinoside structure and antiviral activity.

In conclusion, phenolic acid rutinosides were synthesized enzymatically using rutinase from tartary buckwheat and exhibited potent antiviral activity against FCV. These results should contribute to the development of antiviral agents against noroviruses.

ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

CRFK, Crandell−Reese feline kidney; FBS, fetal bovine serum; FCV, feline calicivirus; fJAM-1, feline junctional adhesion molecule 1; MTT, methyl thiazolyl tetrazolium

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