Hydrolysis of Flavanone Glycosides by β-Glucosidase from *Pyrococcus furiosus* and Its Application to the Production of Flavanone Aglycones from Citrus Extracts

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S Supporting Information

ABSTRACT: The hydrolytic activity of the recombinant β-glucosidase from *Pyrococcus furiosus* for the flavanone glycoside hesperidin was optimal at pH 5.5 and 95 °C in the presence of 0.5% (v/v) dimethyl sulfoxide (DMSO) and 0.1% (w/v) Tween 40 with a half-life of 88 h, a K_m of 1.6 mM, and a k_{cat} of 68.4 1/s. The specific activity of the enzyme for flavonoid glycosides followed the order hesperidin > neohesperidin > naringin > narirutin > poncirin > diosmin > neoponcirin > rutin. The specific activity for flavanone was higher than that for flavone or flavonol. DMSO at 10% (v/v) was used to increase the solubility of flavanone glycosides as substrates. The enzyme completely converted flavanone glycosides (1 g/L) to flavanone aglycones and disaccharides via one-step reaction. The major flavanone in grapefruit peel, grapefruit pulp, or orange peel extract was naringin (47.5 mg/g), naringin (16.6 mg/g), or hesperidin (18.2 mg/g), respectively. β-Glucosidase from *P. furiosus* completely converted naringin and narirutin in 100% (w/v) grapefruit peel extract to 22.5 g/L naringenin after 12 h, with a productivity of 1.88 g L^{-1} h^{-1}; naringin and narirutin in 100% (w/v) grapefruit pulp extract to 8.1 g/L naringenin after 9 h, with a productivity of 0.90 g L^{-1} h^{-1}; and hesperidin in 100% (w/v) orange peel extract to 9.0 g/L hesperetin after 9 h, with a productivity of 1.00 g L^{-1} h^{-1}. The conversion yields, concentrations, and productivities of flavanone aglycones in this study are the highest among those obtained from citrus extracts. Thus, this enzyme may be useful for the industrial hydrolysis of flavanone glycosides in citrus extracts.

KEYWORDS: *flavanone hydrolysis, Pyrococcus furiosus β-glucosidase, citrus extracts, naringenin, hesperidin*

■ INTRODUCTION

Flavonoids are a large group of polyphenolic compounds present in vegetables, especially in the genus *Citrus*, and have many health-related properties, including antioxidant, anticancer, antiviral, and anti-inflammatory activities.[1] Flavonoids are divided into four typical groups such as flavones, flavonols, flavanones, and flavanonols. Flavanones are colorless compounds derived from flavones, including hesperetin, naringenin, sakuranetin, isosakuranetin, eriodictyol, and homoeriodictyol. Hesperidin and hesperetin among the flavanone group are found abundantly in citrus fruits.[2] Hesperidin has a wide range of biological activities, including antiallergic,[3] anti-inflammatory,[4] analgesic,[4] antihypertensive,[5] diuretic, and antihypercholesterolemic activities.[6] However, the flavanone aglycone hesperetin has more profound pharmacological activity than the flavanone glycoside hesperidin.[7] As an example, the antioxidant activity of hesperidin is low or absent,[8] whereas that of hesperetin was high.[9] Thus, the enzymatic hydrolysis of flavanone glycosides in citrus extracts to flavanone aglycones has been performed.[10−12]

Naringin and naringenin have diverse biological activities, including antibacterial,[13] antioxidant,[14] anti-inflammatory, antifibrotic,[15] and antiulcer activities.[16] Naringin is a flavanone responsible for the bitter taste. In the citrus juice industry, to improve the quality and the commercial value of juice, naringin should be converted to naringenin.[17] The flavanone aglycones hesperetin and naringenin are produced by hydrolyzing sugar moieties in the flavanone glycosides hesperidin, naringin, neohesperidin, and narirutin. These flavanone aglycones have been obtained by hydrolysis reactions of microorganisms,

including *Aspergillus niger*, *Cryptococcus albidus*, *Cryptococcus diffluens*,[18] *Penicillium nigricans*,[19] *Penicillium brevicompactum*, *Penicillium funiculosum*, *Penicillium herquei*, *Penicillium rugulosum*,[20] and *Pullularia pullulans*.[18] Microbial hydrolysis exhibits low yield and poor productivity. Flavanone aglycones have also been achieved using naringinases from *A. niger*,[21] *Aspergillus sojae*,[22] *Aspergillus terreus*,[23] *Coniothyrium diplodiella*,[24] *Penicillium decumbens*,[25] *Pichia angusta*,[26] *Rhamnus dahuria*,[27] *Rhizopus nigricans*,[28] *Staphylococcus xylosus*,[29] and *Stilbella fimetaria*,[30] β-rutinosidases from *Acremonium* sp.,[31] *Fagopyrum tataricum*,[32] *P. rugluosome*,[33] and *Pseudomonas viridiflava*,[34] and β-glucosidase from *Sporobolomyces singularis*.[7] Although enzymatic hydrolysis shows higher conversion yield and productivity than microbial hydrolysis, the hydrolysis of flavanone glycosides by these enzymes is inefficient in terms of the conversion yield and productivity. Therefore, an enzyme with a high hydrolytic conversion yield and productivity is required for the effective hydrolysis of flavanone glycosides.

In this study, the hydrolytic activity and kinetic parameters for flavonoid glycosides were investigated using β-glucosidase from *Pyrococcus furiosus*. The reaction conditions such as pH, temperature, solvent, detergent, and enzyme and substrate concentrations were optimized for the production of the flavanone aglycone hesperetin. Under the optimized conditions,

P. furiosus β-glucosidase produced the flavanone aglycones hesperetin and naringenin from the flavanone glycosides hesperidin, neohesperidin, naringin, and narirutin from reagents and citrus extracts.

■ MATERIALS AND METHODS

Materials. The flavonoid aglycone standards, including hesperetin, naringenin, and quercetin, and the flavonoid glycoside standards, including hesperidin, neohesperidin, diosmin, rutin, and naringin, were purchased from Sigma (St. Louis, MO). The other flavonoid aglycones and flavonoid glycosides, including isosakuranetin, didymin, poncirin, and neoponcirin, were purchased from Apin Chemicals (Oxfordshire, U.K.). HPLC grade acetonitrile and acetic acid were purchased from Fisher Scientific (Hanover Park, IL). All of the restriction enzymes were purchased from New England Biolabs (Hertfordshire, U.K.). All other reagents were purchased from Sigma.

Preparation of Citrus Extracts. To extract flavonoids from citrus, 500 g of grapefruit peel, grapefruit pulp, or orange peel dry powder was added to 5 L of ethanol/water mixture (3:7, v/v) and incubated at 60 °C for 5 h. After cooling, the extract was filtered through a 0.45 μm filter, the ethanol was removed by evaporation, and then the residue was dissolved in 5 L of distilled water. The solution was adsorbed onto a Diaion HP-20 resin, which was rinsed with distilled water to remove the unbound sugars, leaving flavonoids attached to the resin. The adsorbed flavonoids were gradually eluted at a flow rate of 0.5 mL/min with ethanol. The ethanol was removed by evaporation, and then the residue was dissolved in 500 mL of distilled water. Sugar-free citrus extracts were used because Maillard reactions between unbound sugars and the enzyme occurred at temperatures above 70 °C.

Cloning and Gene Expression. The genomic DNA from *P. furiosus* DSMZ 3638 (Microbank, Daejeon, Korea), *E. coli* ER2566 (New Englands Biolabs, Herfordshire, U.K.), and pET24a(+) plasmid (Novagen, Madison, WI) were used as the source of the β-glucosidase gene, host cells for cloning and expression, and expression vector. The gene encoding the β-glucosidase was amplified by PCR using the genomic DNA from *P. furiosus* as a template. The sequence of the primers used for gene cloning was based on the DNA sequence of *P. furiosus* β-glucosidase (GenBank accession number AAC25555). Forward (5′-GGATCCATGAAGTTCTCCAAAAAAC-3′) and reverse primers (5′-GTCGACCTTTCTTGTAACAAATT-3′) were designed to introduce the *Bam*HI and *Sal*I restriction sites (underlined), respectively, and were synthesized by Bioneer (Daejon, Korea). The PCR-amplified DNA fragments were purified and inserted into the pET24a(+) vector digested with the same restriction enzymes. *E. coli* ER2566 strain was transformed with the ligation mixture using an electroporator (MicroPulser, Bio-Rad, Hercules, CA) and plated on Luria−Bertani (LB) agar containing 25 μg/mL kanamycin. A kanamycin-resistant colony was selected, and plasmid DNA from the transformant was isolated with a plasmid purification kit (Promega, Madison, WI). DNA sequencing was carried out using a DNA analyzer (ABI Prism 3730xl, Perkin-Elmer, Waltham, MA). Gene expression was evaluated by both SDS−PAGE and enzyme activity assay.

Culture Conditions. *E. coli* cells containing the β-glucosidase/pET24a(+) gene from *P. furiosus* were cultivated in a 2 L flask containing 500 mL of LB medium and 25 μg/L kanamycin at 37 °C with shaking at 200 rpm. When the optical density of bacteria reached 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce β-glucosidase expression, after which the culture was incubated with shaking at 150 rpm at 16 °C for 12 h as the conditions for obtaining the highest expression level.

Enzyme Preparation. The induced cells were harvested from the culture broth and disrupted by sonication on ice for 2 min in 50 mM phosphate buffer (pH 7.0) containing 300 mM NaCl with the addition of 1 mg/mL lysozyme. The unbroken cells and cell debris were removed by centrifugation at 13000*g* for 20 min at 4 °C, and the supernatant obtained was used as a crude extract. The enzyme solution of the supernatant was applied to a His-Trap affinity chromatography column (Amersham Biosciences, Uppsala, Sweden) equilibrated with

50 mM phosphate buffer (pH 7.0). The bound protein was subsequently eluted at 4 °C with the same buffer containing 250 mM imidazole at a flow rate of 1 mL/min. The active fractions were collected and dialyzed at 4 °C for 16 h against 50 mM citrate/phosphate buffer (pH 5.5). The resulting solution was used as a purified enzyme. The purification step using the column was conducted using a fast protein liquid chromatography (FPLC) system (Bio-Rad, Hercules, CA) in a cold room at 4 °C.

Enzyme Assay. Unless otherwise stated, the reaction was performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mM flavonoid glycoside, 0.017 U/mL enzyme, 0.5% (v/v) dimethyl sulfoxide (DMSO), and 0.1% (w/v) Tween 40 at 95 °C for 10 min. One unit (U) of enzyme activity for flavonoid glycoside was defined as the amount of enzyme required to liberate 1 μmol of flavonoid aglycone per minute at pH 5.5 and 95 °C. The specific activity (U/mg) was defined as the produced amount of flavonoid aglycone as a product per enzyme amount per unit reaction time. Various concentrations of hesperidin, neohesperidin, and naringin (0.1−5 mM) were used to determine the kinetics parameters of the enzyme. The amounts of flavonoid aglycones were detected by high-performance liquid chromatography (HPLC) assay. The K_m (mM) and k_{cat} (1/s) were determined by the Lineweaver−Burk plot from the Michaelis−Menten equation. To calculate the catalytic constant, k_{cat}, the amount of protein was divided by the subunit molecular mass of 55 488 Da.

Effects of pH and Temperature. To investigate the effects of temperature and pH on the hydrolytic activity of β-glucosidase for flavanone glycoside, temperature was varied from 75 to 95 °C at pH 5.5 and pH values were varied from 4.5 to 7.0 at 95 °C. The reactions were performed in 50 mM citrate/phosphate buffer containing 0.4 mM hesperidin, 0.017 U/mL enzyme, and 0.5% (v/v) DMSO for 10 min. The influence of temperature on enzyme stability was monitored as a function of incubation time by placing the enzyme solution at five different temperatures (75, 80, 85, 90, and 95 °C) in 50 mM citrate/phosphate buffer (pH 5.5). Samples were withdrawn at time intervals and were assayed. The half-life of the enzyme was calculated using Sigma Plot 9.0 software (Systat Software, San Jose, CA).

Effects of Solvent and Detergent. Several organic solvents at concentrations of 5% and 10% (v/v), including ethanol, methanol, 3-methyl-1-butanol, 2-propanol, isopropanol, formic acid, acetone, and DMSO, were tested for selecting the optimum solvent. The effect of solvent concentration on β-glucosidase activity was evaluated by varying the concentration of DMSO or methanol from 0.5% to 20% (v/v). The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mM hesperidin, 0.017 U/mL enzyme, and solvent at 95 °C for 10 min. Several detergents, including Tween 20, Tween 40, Tween 80, Span 20, Span 80, Triton-X 100, and Brij 58, were tested to find the optimum detergent for the hydrolytic activity. Tween 40 concentration was varied from 0.05% to 1% (w/v) to determine the optimum detergent concentration. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mM hesperidin, 0.017 U/mL enzyme, 0.5% (v/v) DMSO, and detergent at 95 °C for 10 min.

Hydrolysis of Flavanone Glycosides. The hydrolytic reactions of flavanone glycosides, including hesperidin, neohesperidin, naringin, and narirutin by β-glucosidase from *P. furiosus* was performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 1 g/L flavanone glycoside, 0.034 U/mL enzyme, 10% (v/v) DMSO, and 0.1% (w/v) Tween 40 at 95 °C for 90 or 150 min. DMSO at 10% (v/v) was used to increase the solubility of flavanone glycosides as substrates. In order to evaluate the effect of enzyme concentration on the production of hesperetin from hesperidin, the reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 12.2 g/L (20 mM) hesperidin, 10% (v/v) DMSO, and 0.1% (w/v) Tween 40 by varying the enzyme concentration from 0.017 to 0.85 U/mL at 95 °C for 10 min. The time course reactions for the production of hesperetin from hesperidin by *P. furiosus* β-glucosidase were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 12.2 g/L hesperidin, 0.85 U/mL enzyme, 10% (v/v) DMSO, and 0.1% (w/v) Tween 40 at 95 °C for 10 min. The production of hesperetin and naringenin from
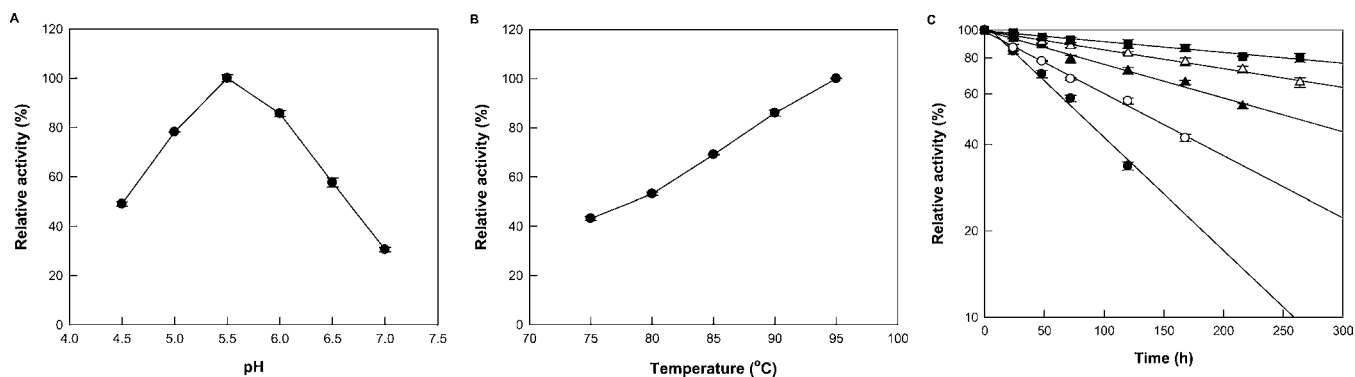
Figure 1. Effects of pH and temperature on the activity of *P. furiosus* β-glucosidase. (A) pH effect. The reactions were performed with 0.4 mM hesperidin, 0.017 U/mL enzyme, and 10% (v/v) DMSO at 95 °C for 10 min. (B) Temperature effect. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mM hesperidin, 0.017 U/mL enzyme, and 0.5% (v/v) DMSO for 10 min. (C) Thermal stability. The enzymes were incubated at 75 °C (■), 80 °C (△), 85 °C (▲), 90 °C (○), and 95 °C (●) for various incubation times. A sample was withdrawn at each time interval, and the relative activity was determined. Data are expressed as the mean of three experiments, and error bars represent standard deviation.
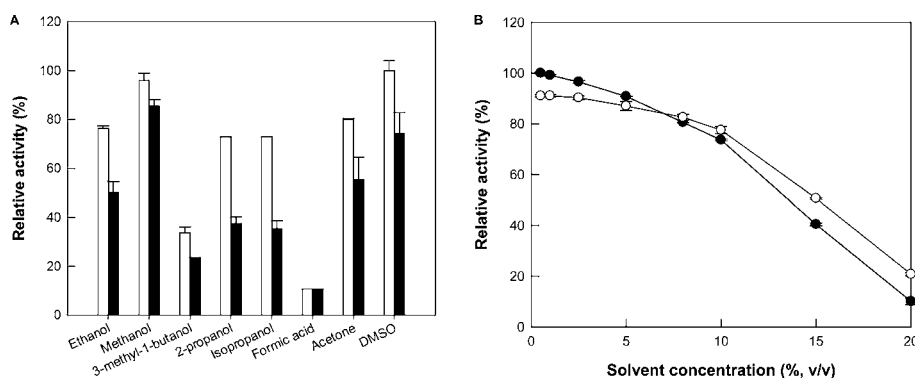

Figure 2. Effect of solvent on the activity of β-glucosidase from *P. furiosus*. (A) Effect of solvent type. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mM hesperidin, 0.017 U/mL enzyme, and 5% (v/v) (□) or 10% (v/v) (■) solvent at 95 °C for 10 min. (B) Effect of solvent concentration. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 0.4 mM hesperidin, 0.017 U/mL enzyme, and 1−20% (v/v) DMSO (●) or methanol (○) at 95 °C for 10 min. Data represent the mean of three experiments, and error bars represent standard deviation.

Table 1. Specific Activity and Kinetic Parameters of Recombinant *P. furiosus* β-Glucosidase for Flavonoid Glycosides

flavonoid	substrate	product	specific activity (U/mg)	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM s))
flavanone	hesperidin	hesperetin, rutinose	0.172 ± 0.001	1.6 ± 0.03	68.4 ± 2.23	42.1 ± 0.50
	neohesperidin	hesperetin, neohesperidose	0.103 ± 0.005	4.6 ± 0.04	84.8 ± 0.88	18.5 ± 0.34
	naringin	naringenin, rutinose	0.102 ± 0.003	1.7 ± 0.03	27.7 ± 1.28	16.5 ± 1.03
	narirutin	naringenin, neohesperidose	0.153 ± 0.003	3.7 ± 0.05	74.1 ± 0.29	19.8 ± 0.18
	poncirin	isosakuranetin, neohesperidose	0.091 ± 0.004			
	neoponcirin	isosakuranetin, rutinose	0.081 ± 0.004			
flavone	diosmin	diosmetin, rutinose	0.085 ± 0.002			
flavonol	rutin	quercetin, rutinose	0.011 ± 0.001			

citrus extracts was performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 100% (w/v) citrus extract, 0.85 U/mL enzyme, 10% (v/v) DMSO, and 0.1% (w/v) Tween 40 at 95 °C for 12 h.

Analytical Methods. Flavonoid aglycones and flavonoid glycosides were assayed using an HPLC system (Agilent 1100, Santa Clara, CA) equipped with a UV detector at 254 nm and a C18 column (50 mm × 4.6 mm, YMC, Kyoto, Japan). The column was eluted at 35 °C with a gradient of solvent A (3% acetic acid in water) and solvent B (3% acetic acid in mixture of water and acetonitrile of 50:50 (v/v)) from 75:25 to 40:60 for 12 min and then from 40:60 to 75:25 for 3 min. The flow rate was 1.5 mL/min. The substrates hesperidin, neohesperidin, naringin, narirutin, poncirin, neoponcirin, diosmin, and rutin were detected with retention times of 6.3, 6.9, 5.9, 6.1, 7.1, 2.8, 7.2, and 4.1 min, respectively. The products hesperetin, naringenin,

isosakuranetin, didymin, and quercetin were detected with retention times of 12.7, 11.7, 13.8, 9.1, and 9.3 min, respectively. The flavonoids in the reaction samples formed from different substrates were identified as the same retention times with the flavonoid standards. Rutinose was detected using a Bio-LC system (Dionex ICS-3000, Sunnyvale, CA) with an electrochemical detector and a CarboPac PA10 column. The column was eluted at 30 °C with 200 mM sodium hydroxide at a flow rate of 1 mL/min.

■ RESULTS AND DISCUSSION

Effects of pH and Temperature on the Activity of *P. furiosus* β-Glucosidase. A gene encoding *P. furiosus* β-glucosidase, with the same sequence as that reported in
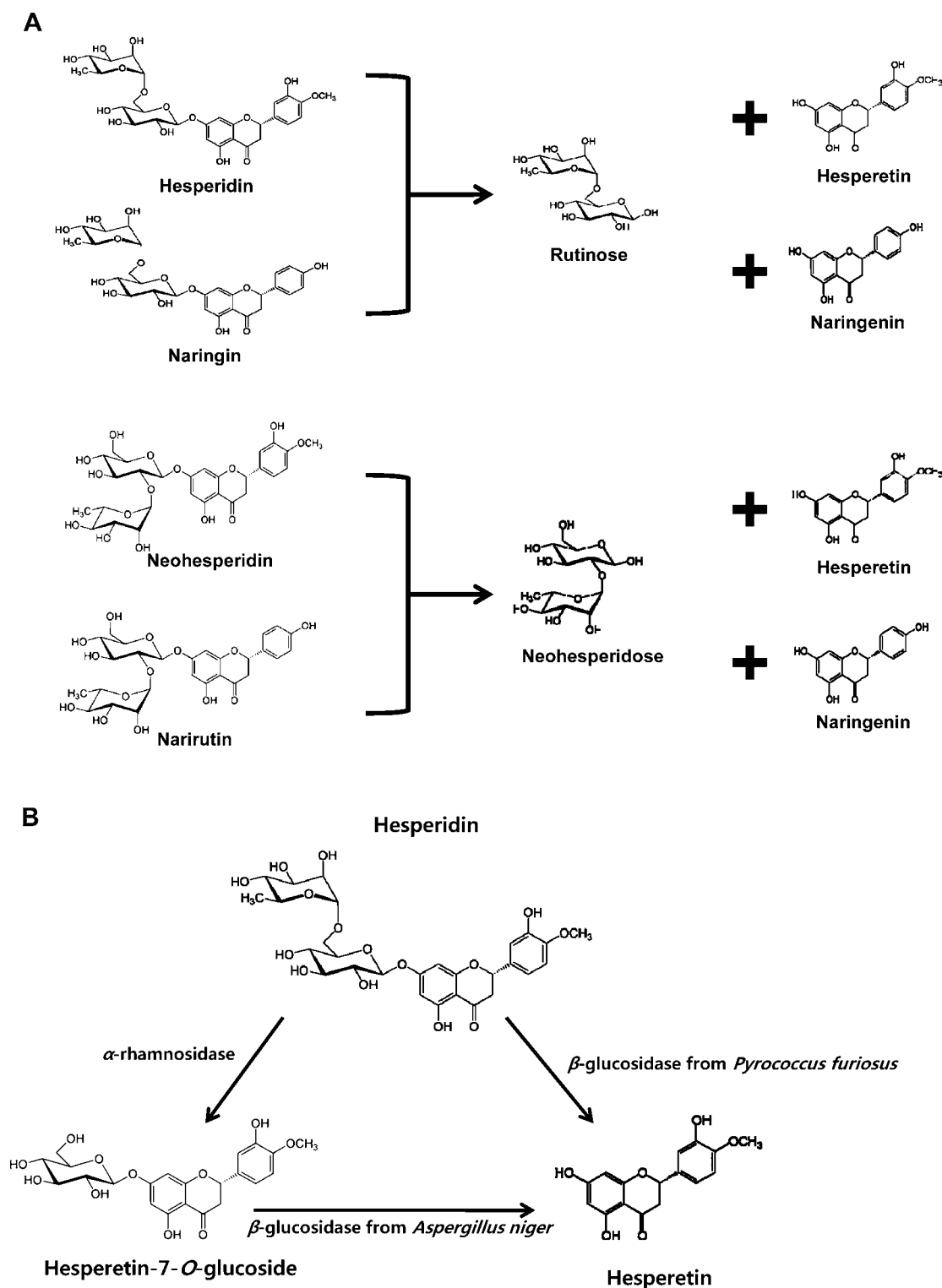
Figure 3. (A) Conversion of hesperidin, naringin, neohesperidin, and narirutin to hesperetin, naringenin, hesperetin, and naringenin by β-glucosidase from *P. furiosus*, respectively. (B) Hydrolytic pathways from hesperidin to hesperetin by two enzyme reactions with α-rhamnosidase plus β-glucosidase and one enzyme reaction with *P. furiosus* β-glucosidase. Two hydrolytic pathways are shown: hesperidin → hesperetin-7-O-glucoside → hesperetin; hesperidin → hesperetin.

GenBank (accession number AAC25555), was cloned and expressed in *E. coli*. The enzyme was purified from crude extract obtained from harvested cells as a soluble protein by His-trap affinity chromatography. β-Glucosidase from *P. furiosus* was purified with a purification of 7.4-fold and a yield of 59% compared to crude extract, and its specific activity was 172 U/mg for the flavanone hesperidin. The molecular mass of the expressed protein analyzed by SDS–PAGE was approximately 56 kDa, consistent with the calculated value of 55 488 Da based on the 472 amino acid residues plus six histidine residues (data not shown).
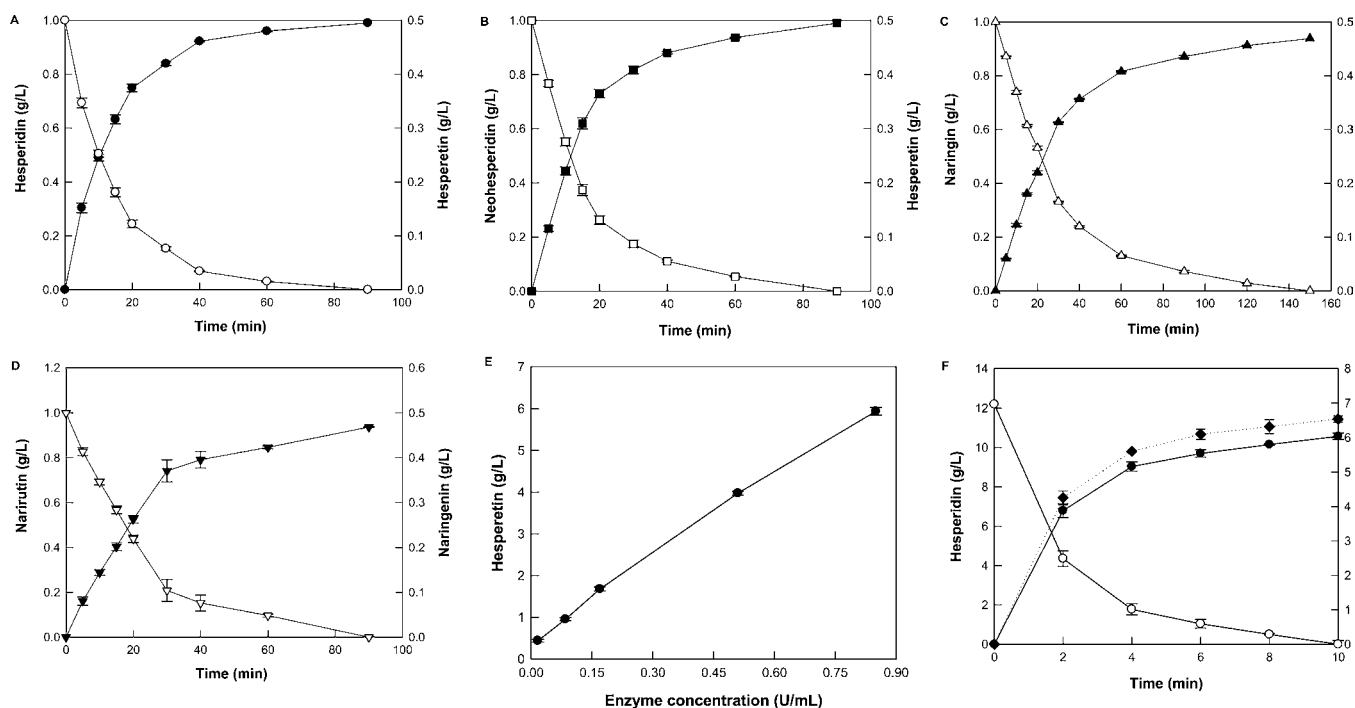
Figure 4. Conversion of flavanone glycoside to flavanone aglycone and production of hesperetin from hesperidin by β-glucosidase from *P. furiosus*. (A) Conversion of hesperidin (○) to hesperetin (●). (B) Conversion of neohesperidin (□) to hesperetin (■). (C) Conversion of naringin (△) to naringenin (▲). (D) Conversion of narirutin (▽) to naringenin (▼). The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 1 g/L flavonoid glycoside, 0.034 U/mL enzyme, 10% (v/v) DMSO, and 0.1% (w/v) Tween 40 at 95 °C. (E) Effect of enzyme concentration. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 12.2 g/L (20 mM) hesperidin, 0.017−0.85 U/mL enzyme, 10% (v/v) DMSO, and 0.1% (w/v) Tween 40 at 95 °C for 10 min. (F) Production of hesperetin (●) and rutinose (◆) from hesperidin (○). The reaction was performed in 50 mM citrate/phosphate buffer (pH 5.5) containing 12.2 g/L (20 mM) hesperidin, 0.85 U/mL enzyme, 10% (v/v) DMSO, and 0.1% (w/v) Tween 40 at 95 °C for 10 min. Data represent the mean of three experiments, and error bars represent standard deviation.

Table 2. Flavanone Contents in Citrus Extracts

	flavanone content[a] (mg/g)		
	grapefruit peel	grapefruit pulp	orange peel
hesperidin	2.3 ± 0.03	ND	18.2 ± 0.1
neohesperidin	1.1 ± 0.02	1.5 ± 0.02	ND
naringin	47.5 ± 0.5	16.6 ± 0.2	0.4 ± 0.01
narirutin	2.9 ± 0.03	3.3 ± 0.06	4.1 ± 0.02
hesperetin	ND	ND	ND
naringenin	ND	ND	ND

[a]Data represent the mean and standard deviation from three separate experiments. ND, not detected.

No flavonoid aglycones were formed when the reactions were conducted without the enzyme or with the grown cells of *E. coli* ER2566, which did not harbor the β-glucosidase gene from *P. furiosus*. The effect of pH on the hydrolytic activity of hesperidin to hesperetin by *P. furiosus* β-glucosidase was examined in a pH range from 4.5 to 7.0 (Figure 1A). Maximal activity was observed at pH 5.5. The activity at pH 4.5 and pH 6.5 was approximately 50% of the maximum. To prevent the evaporation of water at boiling point, the effect of temperature on the hydrolytic activity of hesperidin was investigated below 100 °C by varying the temperature from 75 to 95 °C at pH 5.5 (Figure 1B). As temperature was increased, the activity increased and was maximal at 95 °C in the used temperature range. The hydrolytic reactions of naringin and hesperidin by other flavanone glycoside hydrolytic enzymes have been performed at pH range of 4.0−6.0 and temperature range of

37−60 °C.[7,30,35] The thermal stability of *P. furiosus* β-glucosidase was examined at temperatures ranging from 75 to 95 °C (Figure 1C). Thermal inactivation of the enzyme followed first-order kinetics with half-lives of 637, 392, 235, 138, and 88 h at 75, 80, 85, 90, and 95 °C, respectively. β-Rutinosidase from *S. fimetaria* showed 45% relative activity at 70 °C after 10 min.[30] Naringinase from *A. niger* retained 60% of its initial activity when incubated at 50 °C for 90 min.[36] The half-life of *Penicillium* sp. naringinase was approximately 90 min at 50 °C.[35] Thus, the reaction temperature of *P. furiosus* β-glucosidase and its thermostability are the highest among flavanone glycoside hydrolytic enzymes.

Effects of Solvent and Detergent on the Activity of *P. furiosus* β-Glucosidase. The effect of organic solvent at the concentrations of 5% and 10% (v/v) on the hydrolytic activity for hesperidin by *P. furiosus* β-glucosidase was examined. Among the solvents tested, DMSO and methanol were effective solvents for hydrolysis activity (Figure 2A). The effect of the concentration of DMSO or methanol on the hydrolytic activity of hesperidin was evaluated by varying the concentration from 0.5% to 20% (v/v). The enzyme activity using DMSO was higher than that using methanol at low concentrations within 5% (v/v), whereas the enzyme activity using methanol was higher than that using DMSO at high concentrations above 5% (v/v) (Figure 2B). Therefore, maximum enzyme activity occurred at 0.5% (v/v) DMSO. The enzyme activity was determined with addition of solvent because some portion of hesperidin was insoluble under the reaction conditions without solvent. The effect of various detergents on the hydrolytic
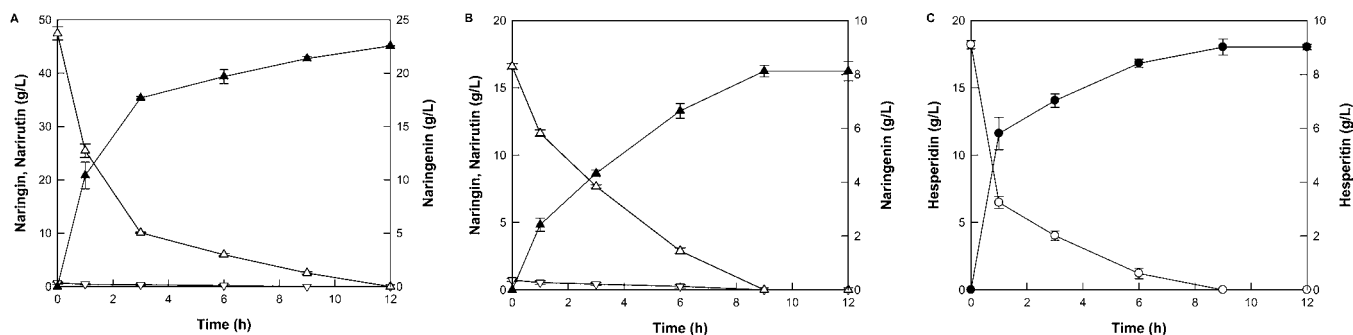
Figure 5. Production of hesperetin and naringenin from citrus extracts by *P. furiosus* β-glucosidase. (A) Production of naringenin (▲) from naringin (△) and narirutin (▽) in grapefruit peel extract. Hesperidin, neohesperidin, and hesperetin were not shown because their concentrations were low. (B) Production of naringenin (▲) from naringin (△) and narirutin (▽) in grapefruit pulp extract. Neohesperidin and hesperetin were not shown because their concentrations were low. (C) Production of hesperetin (●) from hesperidin (○) in orange peel extract. Naringin, narirutin, and naringenin were not shown because their concentrations were low. Data represent the mean of three experiments, and error bars represent standard deviation.

Table 3. Conversion of Flavanone Glycoside to Flavanone Aglycone by Flavanone Glycoside Hydrolytic Enzymes

main substrate	microorganism	enzyme	extract	main product	molar yield (%)	productivity (g L^{-1} h^{-1})	ref
naringin	*Aspergillus* sp.	pectinase	bergamot peel	naringenin			12
	Clostridium stercorarium	rhamnosidase	kinnow peel	prunin	72		22
	Aspergillus niger	naringinase	citrus juice	naringenin			41
	Penicillium decumbens	naringinase	white grapefruit	naringenin		0.18	42
	Pyrococcus furiosus	β-glucosidase	grapefruit peel	naringenin	100	1.88	this study
			grapefruit pulp	naringenin	100	0.90	
hesperidin	*Aspergillus sojae*	naringinase	orange juice	hesperetin-7-*O*-glucoside	71		11
			orange peel	hesperetin-7-*O*-glucoside	78		
	Penicillium sp.	hesperidinase	orange juice	hesperetin	45		43
			lime juice	hesperetin	57		
	Pyrococcus furiosus	β-glucosidase	orange peel	hesperetin	100	1.00	this study

activity for hesperidin was investigated. Of the detergents tested, Tween 40 showed the highest hydrolytic activity (Figure S1A in Supporting Information), with an optimal concentration of 0.1% (w/v) Tween 40 (Figure S1B in Supporting Information).

Substrate Specificity of *P. furiosus* β-Glucosidase for Flavonoid Glycosides. The hydrolytic activity was optimal under the conditions of 0.5% (v/v) DMSO and 0.1% (w/v) Tween 40 at pH 5.5 and 95 °C for 10 min. Under these conditions, the specific activity of *P. furiosus* β-glucosidase was investigated using flavonoid glycosides (Table 1). The specific activity of the enzyme followed the order hesperidin > narirutin > neohesperidin > naringin > poncirin > diosmin > neoponcirin > rutin. The specific activity for hesperidin was 1.7-fold higher than that for neohesperidin containing different sugar and 1.7-, 2.1-, 2.0-, and 15-fold higher than those for naringin, neoponcirin, diosmin, and rutin containing different aglycone flavonoids, respectively. Also, the specific activity for hesperidin was 1.9- and 1.1-fold higher than that for poncirin and narirutin containing both different sugar and different aglycone flavonoid. The specific activity for the flavanone glycosides hesperidin, neohesperidin, naringin, narirutin, and poncirin was higher than that for the flavone glycoside diosmin and the flavonol glycoside rutin. These results indicate that the β-glucosidase is an effective enzyme for hydrolyzing flavanone glycosides.

The Michaelis–Menten constants (K_m), turnover numbers (k_{cat}), and catalytic efficiencies (k_{cat}/K_m) for hesperidin, neohesperidin, naringin, and narirutin are presented in Table

1. The order of catalytic efficiency of *P. furiosus* β-glycosidase (hesperidin > narirutin > neohesperidin > naringin) was the same as that observed for specific activity. However, the turnover number and substrate affinity of the enzyme followed the order neohesperidin > narirutin > hesperidin > naringin and the order hesperidin > naringin > narirutin > neohesperidin, respectively.

The specific activities of β-glucosidase from *P. furiosus*[37] and *Stilbella singularis*[7] and of β-rutinosidase from *S. fimetaria*[30] for *p*-nitrophenyl-β-D-glucopyranoside are 914, 5.8, and 0.14 U/mg, respectively. The turnover number and catalytic efficiency of flavanone glycoside hydrolytic enzyme for flavanone glycoside have been reported only in *S. fimetaria* β-rutinosidase for hesperidin as 32.4 1/s and 18.3 1/(mM s), respectively.[30] The turnover number and catalytic efficiency of *P. furiosus* β-glucoosidase for hesperidin are 68.4 1/s and 42.1 1/(mM s), respectively (Table 1), which are 2.1- and 2.3-fold higher than those of *S. fimetaria* β-rutinosidase, respectively. These results indicate that *P. furiosus* β-glucosidase is the most efficient enzyme for hydrolyzing flavanone glycoside among flavanone glycoside hydrolytic enzymes.

One-Step Conversion to Flavanone Aglycone from Flavanone Glycoside by *P. furiosus* β-Glucosidase. The concentration of rutinose increased proportionally to hesperitin with a molar ratio of 1:1, and glucose and L-rhamnose were not detected in the Bio-LC during the reaction (Figure S2A). Moreover, rutinose was not hydrolyzed by the enzyme, and then the concentration of rutinose was not decreased (Figure S2B). This enzyme converted not only hesperidin and naringin

to hesperetin and naringenin containing rutinose but also neohesperidin and narirutin to hesperetin and naringenin containing neohesperidose (data not shown). Thus, *P. furiosus* *β*-glucosidase converts flavanone glycoside to flavanone aglycone and disaccharide via a one-step reaction (Figure 3A). *α*-Rhamnosidase plus *Aspergillus niger β*-glucosidase[38,39] or *P. furiosus β*-glucosidase can convert hesperidin to hesperetin (Figure 3B). Naringinase (or *α*-rhamnosidase) converts first hesperidin to hesperetin-7-*O*-glucoside and L-rhamnose, and *A. niger β*-glucosidase subsequently converts hesperetin-7-*O*-glucoside to hesperetin and glucose via a two-step reaction,[21] whereas *P. furiosus β*-glucosidase converts hesperidin to hesperetin and rutinose via a one-step reaction. *P. furiosus β*-glucosidase is more useful enzyme for obtaining hesperetin than naringinase (or *α*-rhamnosidase plus *β*-glucosidase) because the enzyme does not produce the intermediate hesperetin-7-*O*-glucoside.

Production of Flavanone Aglycones from Flavanone Glycosides by *P. furiosus β*-Glucosidase. *P. furiosus β*-glucosidase exhibited high hydrolytic activity for the flavanones. Thus, the production of flavanone aglycone by *P. furiosus* was performed with a high concentration of flavanone glycoside (1.0 g/L). For increasing solubility of flavanone glycosides, 10% (v/v) DMSO was used at pH 5.5, 95 °C and with 0.034 U/mL enzyme and 0.1% (w/v) Tween 40. To increase the solubility of flavanone glycosides as substrates, solvent concentration increased to 10% (v/v). Although the enzyme activity using 10% (v/v) methanol for 10 min was higher than that using 10% (v/v) DMSO, the boiling temperature of methanol (65 °C) was lower than that of DMSO (189 °C). Instead of 10% (v/v) methanol, DMSO at 10% (v/v) was used to prevent the evaporation of solvent because the production of flavanone was performed at 95 °C for more than 90 min. The enzyme completely hydrolyzed hesperidin, neohesperidin, naringin, and narirutin to hesperetin, hesperetin, naringenin, and naringenin after 90, 90, 150, and 90 min (parts A, B, C, and D of Figure 4), with productivities of 330, 330, 188, and 306 mg L^{-1} h^{-1}, respectively.

The effect of enzyme concentration on the production of hesperetin by *P. furiosus β*-glucosidase was investigated by varying the enzyme concentration from 0.017 to 0.85 U/mL with 12.2 g/L (20 mM) hesperidin as substrate for 10 min (Figure 4E). The hesperidin was completely hydrolyzed when 0.85 U/mL enzyme was used. Thus, the reaction conditions for the production of hesperetin from hesperidin by *P. furiosus β*-glucosidase were pH 5.5, 95 °C, 0.85 U/mL enzyme, 10% (v/v) DMSO, 0.1% (w/v) Tween 40, and 12.2 g/L (20 mM) hesperidin. Under these conditions, the enzyme produced not only 6.0 g/L (20 mM) hesperetin after 10 min, with a molar conversion yield of 100% and a productivity of 36 g L^{-1} h^{-1}, but also 6.5 g/L (20 mM) rutinose after 10 min, with a molar yield of 100% and productivity of 39 g L^{-1} h^{-1} (Figure 4F).

Naringinase from *A. niger* converted 200 mg/L naringin to 88 mg/L naringenin after 1 h, with a molar conversion yield of 90% and productivity of 88 mg L^{-1} h^{-1},[40] which are the previous highest yield and productivity in the production of flavanone aglycone. The molar conversion yield of hesperidin to hesperetin by endogalacturonase from *Aspergillus* sp. is 48%.[12] However, the concentration of hesperetin produced by flavanone glycoside hydrolytic enzymes has not been reported. The productivities of naringenin and hesperetin in the present study using *P. furiosus β*-glucosidase were 2.1- and 409-fold higher than that achieved using naringinase from *A. niger*,

respectively. *P. furiosus β*-glucosidase completely hydrolyzed hesperidin and naringin to hesperetin and naringenin, whereas the previously reported flavanone glycoside hydrolytic enzymes did not completely hydrolyze.

Production of Hesperetin and Naringenin from Citrus Extracts by *P. furiosus β*-Glucosidase. Citrus extracts from grapefruit peel, grapefruit pulp, and orange peel contained the flavanone glycosides hesperidin, neohesperidin, naringin, and narirutin but not their aglycone forms, hesperetin and naringenin (Table 2). The total flavanone contents in grapefruit peel, grapefruit pulp, and orange peel extracts were 53.8, 21.4, and 22.7 mg/g, respectively. The content of specific flavanone glycoside in grapefruit peel, grapefruit pulp, and orange peel extracts for the total flavanones followed the order naringin (88.3%) > narirutin (5.4%) > hesperidin (4.3%) > neohesperidin (2.0%); naringin (77.6%) > narirutin (15.4%) > neohesperidin (7.0%); and hesperidin (80.2%) > narirutin (18.1%) > naringin (1.8%), respectively. Under the reaction conditions for the production of hesperetin from hesperidin by *P. furiosus β*-glucosidase, time course reactions were performed from 100% (w/v) grapefruit peel, grapefruit pulp, and orange peel extracts for 12 h. The enzyme completely converted 50.4 g/L of naringin and narirutin in 100% (w/v) grapefruit peel extract to 22.5 g/L naringenin after 12 h, with a productivity of 1.88 g L^{-1} h^{-1} (Figure 5A); 19.9 g/L of naringin and narirutin in 100% (w/v) grapefruit pulp extract to 8.1 g/L naringenin after 9 h, with a productivity of 0.90 g L^{-1} h^{-1} (Figure 5B); and 18.2 g/L hesperidin in 100% (w/v) orange peel extract to 9.0 g/L hesperetin after 9 h with a productivity of 1.00 g L^{-1} h^{-1} (Figure 5C).

The conversion of flavanone glycoside to flavanone aglycone in citrus extracts by glycoside hydrolytic enzymes is summarized in Table 3. Pectinase from *Aspergillus* sp.,[12] rhamnosidase from *Clostriduim stercorarium*,[22] naringinase from *A. niger*,[41] and naringinase from *P. decumbens*[42] produced naringenin from naringin in citrus extracts, including bergamot peel, kinnow peel, citrus juice, and white grapefruit extracts. Naringinase from *A. sojae* converted hesperidin in orange juice and orange peel extracts to hesperetin-7-*O*-glucoside,[11] whereas hesperidinase from *Penicillium* sp. converted hesperedin in orange and lime juice extracts to hesperetin.[43] Naringinase from *P. decumbens* produced naringenin from naringin in white grapefruit extract with the highest previously reported productivity of 0.18 g L^{-1} h^{-1}.[42] However, the productivity of *P. furiosus β*-glucosidase for the production of naringenin from naringin in grapefruit peel extract is 10-fold higher than that of naringinase from *P. decumbens*. The molar conversion yield of *P. furiosus β*-glucosidase for hesperetin from hesperedin in orange peel extract is 43% higher than the highest previously reported molar conversion yield of 57% by hesperidinase from *Penicillium* sp.[43] *P. furiosus β*-glucosidase produced naringenin and hesperetin from naringin and hesperidin in citrus extracts with the highest productivity, concentration, and conversion yield reported to date, indicating that *P. furiosus β*-glucosidase is a potential producer of naringenin and hesperetin

In conclusion, *P. furiosus β*-glucosidase converted flavanone glycoside to flavanone aglycone via a one-step reaction. The enzyme was effective for hydrolyzing flavanone glycosides among flavonoid glycosides. The enzyme completely converted the flavanone glycosides from reagents and citrus extracts to flavanone aglycones with the highest productivity reported to date among enzymatic transformations. Thus, *P. furiosus β*-glucosidase may be useful in the production of the flavanone

aglycones naringenin and hesperetin from flavanone glycosides in citrus extracts.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Effect of detergent on activity, Bio-LC profiles for hydrolysis, and HPLC profiles for conversion of hesperidin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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