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Employing Bifunctional Enzymes for Enhanced Extraction of Bioactives from Plants: Flavonoids as an Example

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ABSTRACT: A cost-effective and environmentally friendly approach was developed to improve the extraction of active ingredients from plants, in which a bifunctional enzyme was employed for not only facilitating cell wall degradation but also increasing the bioactivity of target compounds in the extract. In the aqueous extraction of flavonoids from *Glycyrrhizae radix*, *Trichoderma viride* cellulase, a commercial cell-wall-degrading enzyme, was found to efficiently deglycosylate liquiritin and isoliquiritin, which are of high content but low bioactivity, into their aglycones that have much higher physiological activities for dietary and medicinal uses. Under optimized conditions, the extraction yield of liquiritigenin and isoliquiritigenin aglycones reached 4.23 and 0.39 mg/g of dry weight (dw) with 6.51- and 3.55-fold increases, respectively. The same approach was expanded to the extraction of flavonoids from *Scutellariae radix* using *Penicillium decumbens* naringinase, where enhanced production of more bioactive bacalein and wogonin was achieved via enzymatic deglycosylation of bacalin and wogonoside.

KEYWORDS: *Enzyme-assisted extraction, bifuncitonal, flavonoid, Glycyrrhizae radix, Scutellariae radix*

INTRODUCTION

Enzymes have been widely used in the production of natural products from plants in recent years for their advantages in easy operation, high efficiency, and environmental friendliness.[1−3] Most of the works in this field either use cell-wall-degrading enzymes, such as cellulase, pectinase, and β-glucosidase,[4−7] to hydrolyze cell wall constituents to enhance the extraction of intracellular contents or use enzymes that can modify compound chemical structures, such as glycosidases and lipases, to convert the extracts into their analogues with higher medical activities.[8−10] To obtain highly active extracts from plants, a two-step approach, including extraction followed by transformation, is usually adopted.[11] In this study, we propose an integrative enzyme-assisted approach, in which the facilitation of cell wall degradation and the transformation of target compounds are coupled in a single extraction process by taking advantage of a bifunctional enzyme. We show that this one-step, one-enzyme approach significantly improves the yield as well as the bioactivity of flavonoid extracts from *Glycyrrhizae radix* (*GR*) and *Scutellariae radix* (*SR*), showing high cost effectiveness and environmental friendliness.

GR (Chinese licorice) and *SR* (Chinese skullcap) are recognized as important medicinal herbs since ancient times in China, which have found wide applications in pharmaceutical, flavor, and food additive industries.[12,13] *GR* and *SR* both contain flavonoids as major active ingredients (Figure 1). Previous studies have reported that *GR* and *SR* extracts possess various physiological activities, such as anti-inflammatory, antitumor, antioxidant, and antiviral effects.[13−18] Flavonoids in plant cells generally occur as glycosylated derivatives.[19] In *GR*, the main flavonoid contents are liquiritin and isoliquiritin [6.9−9.6 and 1.3−2.1 mg/g of dry weight (dw), respectively] rather than their aglycones of liquiritigenin and isoliquiritigenin (0.4−0.6 and 0.2−0.3 mg/g of dw, respectively).[20] Likewise, the main flavonoids in *SR* are baicalin and wogonoside (101.1 and 35.5 mg/g of dw, respectively), instead of baicalein and wogonin (54.1 and 13 mg/g of dw, respectively).[17]

Flavonoid glycosides that make up the majority of natural flavonoids are, however, poorly absorbed by the human body until they have undergone hydrolysis by bacterial enzymes in the intestine, whereupon their aglycones can be absorbed.[19,21] For instance, balicalin itself is poorly absorbed from the rat gastrointestinal tract but is hydrolyzed to baicalein by intestinal bacteria and then restored to its original form in the body.[22] In addition, flavonoid aglycones often exhibit higher physiological activities than flavonoid glycosides when used as phytomedicines. Isoliquiritigenin from *GR*, for example, has been reported to be more effective than isoliquiritin in therapies for liver injury, menopausal symptom, and cancer.[23,24] Likewise, bacalein from *SR* shows higher free radical scavenging and antioxidant activities than bacalin,[18] while wogonin exhibits significant antitumor activity and wogonoside contrastively shows no activity at all.[17] Therefore, flavonoid extracts need to be converted from a glycoside-dominant composition to an aglycone-dominant composition for better absorption and higher bioactivity in the human body for dietary and medicinal uses. This transformation is conventionally achieved via

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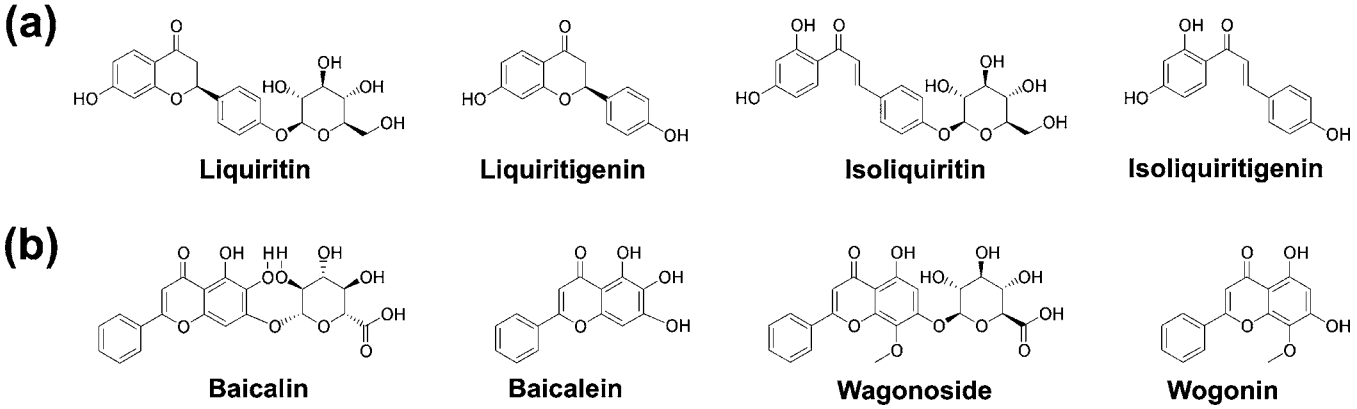

Figure 1. Chemical structures of predominant flavonoids in (a) *GR* and (b) *SR*.

chemical hydrolysis catalyzed by HCl or NaOH,[19] which may unfavorably lead to serious product loss and environmental issues.

Here, we came up with an idea to integrate the extraction and transformation of plant flavonoids in a single cost-effective and environmentally friendly process. A hydrolytic enzyme was used to deglycosylate flavonoid glycosides into aglycones during their extraction in the mild ethanol–water extractant, while the activity of the enzyme in cell wall degradation was employed simultaneously to increase the extraction yield. We first used *Trichoderma viride* cellulase (EC 3.2.1.4), a commercially available cell-wall-degrading enzyme, to assist the extraction of flavonoids from *GR*, leading to a remarkable increase of flavonoid aglycone contents in the extract. The same strategy was further expanded to the extraction of flavonoids from *SR* using *Penicillium decumbens* naringinase (EC 232.962.4). To our knowledge, this study presents the first example of using enzymatic bifunctionality, for cell wall degradation and product activity enhancement, in the extraction of natural compounds from plants. This is also the first report of enzyme-assisted extraction of flavonoids from either *GR* or *SR*.

MATERIALS AND METHODS

Plant Materials. *GR* and *SR* dry powders were made from cultivated Chinese licorice and *Scutellaria baicalensis* plants, respectively. Fresh *Glycyrrhizae* and *Scutellaria* roots were desiccated at 50 and 60 °C, respectively, and ground into fine powder. Powders were filtered through a 40-mesh sieve before use.

Enzymes and Chemicals. *T. viride* cellulase (3–10 units/mg) and *P. decumbens* naringinase (≥300 units/g) were purchased from Sigma-Aldrich (St. Louis, MO). Liquiritigenin, isoliquiritigenin, liquiritin, isoliquiritin, baicalin, wogonoside, baicalein, wogonin, and glycyrrhizic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other reagents were of analytical grade and commercially available.

Enzyme-Assisted Extraction of Total Flavonoids from *GR* and *SR*. *T. viride* cellulase or *P. decumbens* naringinase was added to ethanol–water solution and adjusted with acetate buffer to obtain a 100 mL extractant with the desired enzyme concentration, ethanol–water ratio, and pH. A total of 1 g of *GR* or *SR* dry powder was added to the extractant in an Erlenmeyer flask. The mixture was incubated under 200 rpm stirring for 4 h on a multichannel magnetic stirrer with a temperature controller (Guohua Electronics Co., Changzhou, China). After incubation, the mixture was filtered, and the filtrate was forwarded to analysis.

Sonication-Assisted Extraction of Total Flavonoids from *GR* and *SR*. In the optimized procedure of sonication-assisted extraction, 1 g of *GR* or *SR* dry powder was added to 100 mL of extractant (70% ethanol, pH 5) in an Erlenmeyer flask. Sonication was performed at 50 °C and 40 kHz on the power value of 250 W (KQ-250DE, Kunshan Ultrasonic Instrument Co., Kunshan, China). The mixture was then filtered, and the filtrate was forwarded to analysis.

Determination of Total Flavonoids. The aluminumchloride colorimetric method described by Chang et al.[25] was used to determine the total content of flavonoids. A total of 0.5 mL of extract or standard solution was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride (substituted with distilled water in a blank probe), 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After 30 min of incubation, absorbance at 415 nm was determined against a distilled water blank on a UV-1206 spectrophotometer (Shimadzu, Kyoto, Japan). All samples were made in triplicate, and mean values of total flavonoid content are expressed as milligrams of quercetin equivalents per gram of dw, calculated according to the standard calibration curve.

Scanning Electron Microscopy (SEM) Analysis. After extraction, the residual plant sample was dried, fixed on adhesive tape, sputtered with gold, and then examined under a high-vacuum condition at the voltage of 15.0 kV (2000× magnification) using the SEM system of FEI Quanta 200 (Hillsboro, OR).

Enzymatic Deglycosylation Reaction. The reaction system consisted of 100 mg of *T. viride* cellulase or *P. decumbens* naringinase and 2 mg of substrate compound (liquiritin, isoliquiritin, baicalin, or wogonoside) in 50 mL of 30% ethanol–water solvent. Prior to enzyme addition, the solution was adjusted by acetate buffer to pH 5. The enzymatic catalysis was carried out for 4 h under 200 rpm stirring at 50 °C.

Chromatographic Fingerprint Analysis. High-performance liquid chromatography (HPLC) analysis was carried out using a Diamonsil C18 column (5 μm, 250 × 4.6 mm, Dikma Technologies, Inc., Lake Forest, CA) attached to a HPLC LC-10A system (Shimadzu, Kyoto, Japan).

For *GR* extract analysis, the mobile phase of actonitrile (A)–0.05% phosphoric acid (B) was used. The flow rate was set at 1 mL/min, and the column temperature was maintained at 25 °C. Elution was performed in a precisely designed gradient elution procedure: 0–8 min, 20% A; 8–30 min, 20–38% A; 30–42 min, 38–50% A; 42–45 min, 50–95% A; and 45–50 min, 95–20% A. This method has proven to be reproducible and reliable, with relative standard deviation (RSD) < 3%. Two wavelengths were used for detection. Liquiritin, liquiritigenin, and glycyrrhizic acid were detected at 237 nm, where all three compounds have strong absorption, while isoliquiritin and isoliquiritigenin were detected at 365 nm.

For *SR* extract analysis,[26] the mobile phase of methanol (C)–0.2% phosphoric acid (D) was used. The flow rate was set at 1 mL/min, and the column temperature was maintained at 25 °C. Elution was performed in a precisely designed gradient elution procedure: 0–10 min, 45% C; 10–55 min, 45–70% C; and 55–60 min, 70–45% C. This method has proven to be reproducible and reliable with RSD < 3%. Baicalin, wogonoside, baicalein, and wogonin were detected at the wavelength of 274 nm.

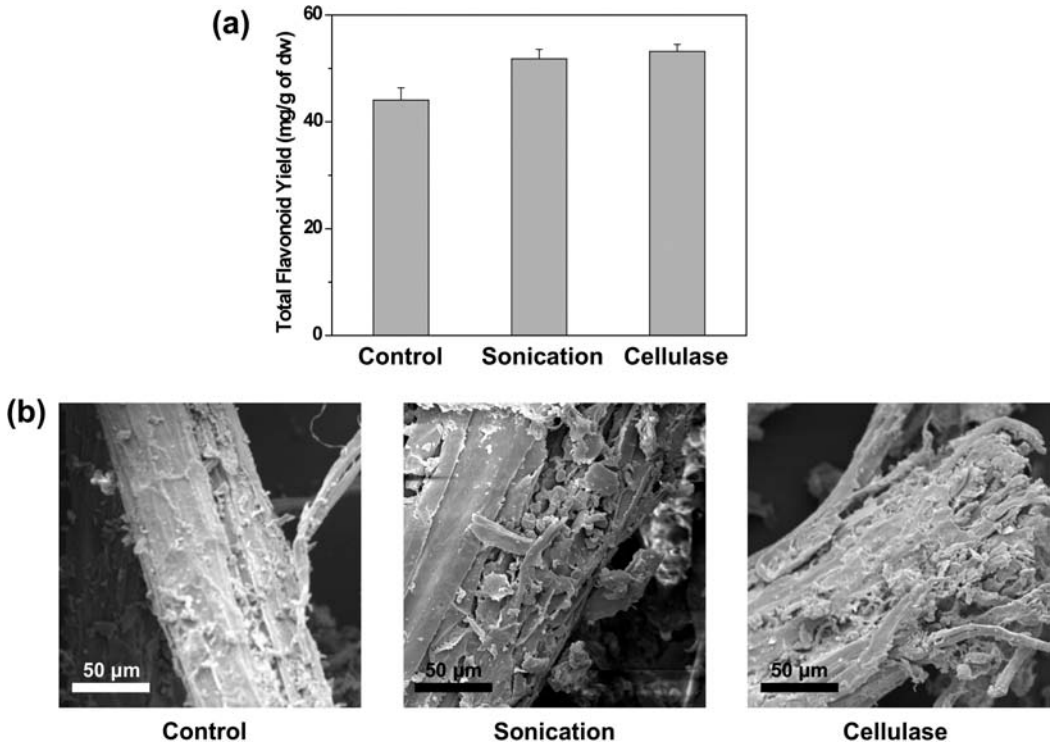

Figure 2. Extraction of total flavonoids from *GR* by different methods. (a) Extraction yields of total flavonoids by solvent-only, sonication-assisted, and cellulase-assisted methods. (b) Scanning electron micrographs of *GR* samples after extraction.

Table 1. Extraction of Flavonoids from *GR* by Different Methods

method	yield of total flavonoids (mg/g of dw)	yield of liquiritin (mg/g of dw)	yield of liquiritigenin (mg/g of dw)	yield of isoliquiritin (mg/g of dw)	yield of isoliquiritigenin (mg/g of dw)	ratio of liquiritigenin/liquiritin	ratio of isoliquiritigenin/isoliquiritin
solvent-only	44.12 ± 2.31	3.21 ± 0.15	0.65 ± 0.02	0.53 ± 0.03	0.11 ± 0.02	0.20	0.21
sonication-assisted	51.81 ± 1.83	4.33 ± 0.23	0.41 ± 0.03	0.49 ± 0.04	0.10 ± 0.01	0.09	0.20
cellulase-assisted	53.23 ± 1.32	0.57 ± 0.03	4.23 ± 0.19	0.13 ± 0.02	0.39 ± 0.02	7.42	3.00
naringinase-assisted	49.24 ± 2.53	0.56 ± 0.06	1.86 ± 0.13	0.15 ± 0.01	0.27 ± 0.03	3.32	1.80

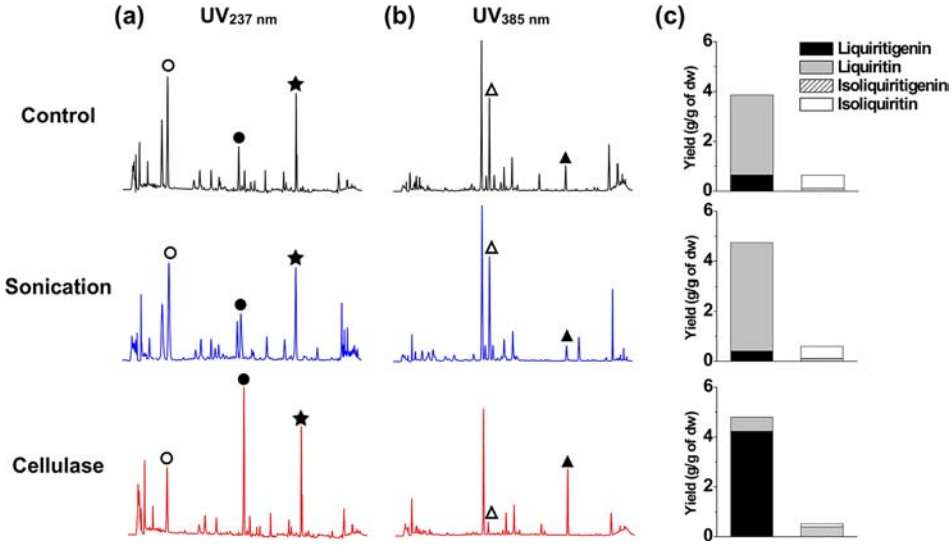

Figure 3. Chromatographic fingerprint analysis of *GR* extracts obtained by different methods. (a) Chromatograms of the extracts with marked peaks of liquiritin (○), liquiritigenin (●), and glycyrrhizic acid (★). Ultraviolet (UV) absorbance was detected at 237 nm. (b) Chromatograms of the extracts with marked peaks of isoliquiritin (△) and isoliquiritigenin (▲). UV absorbance was detected at 365 nm. (c) Calculated contents of liquiritigenin, liquiritin, isoliquiritigenin, and isoliquiritin in the extracts.

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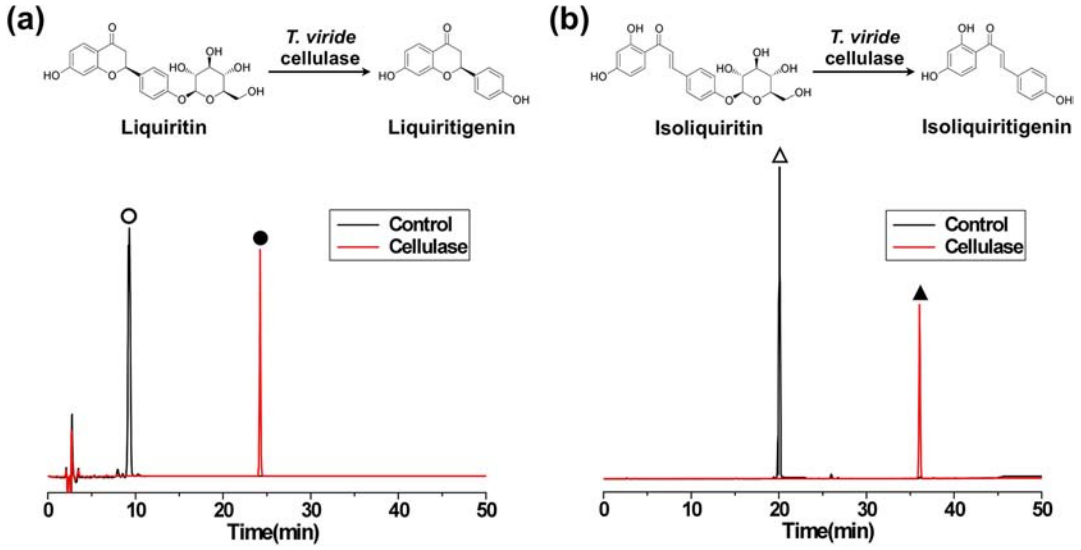

Figure 4. Deglycosylation of flavonoid glycosides by *T. viride* cellulase. (a) Transformation of liquiritin (○) into liquiritigenin (●). UV absorbance was detected at 237 nm. (b) Transformation of isoliquiritin (△) into isoliquiritigenin (▲). UV absorbance was detected at 365 nm.

Optimization of Enzyme-Assisted Extraction. Aqueous extractants with various ethanol ratios (20, 30, 40, 50, and 60%), enzyme concentrations (0.2, 0.5, 1, 2, and 5 mg/mL), and pH (4, 5, 6, 7, and 8) were incubated at various temperatures (30, 40, 50, 60, and 70 °C). The extraction yields under different conditions were assayed and compared.

RESULTS AND DISCUSSION

***T. viride* Cellulase Improves the Extraction Yield of Total Flavonoids from *GR*.** The major barrier to the release of flavonoids from plant materials is the cell wall rich in cellulose and pectins. Various methods have been used to facilitate cell wall destruction for enhanced extraction of plant flavonoids, including ultrasonication,[27] microwave,[28] supercritical fluids,[29] and enzymatic hydrolysis.[30] Here, we used a commercially available enzyme, *T. viride* cellulase, to assist the recovery of flavonoids from *GR* in the ethanol−water extractant. *T. viride* cellulase is known to have high cell-wall-degrading activity originated from its endoglucanase, exoglucanase, and β-glucosidase components.[31,32] The yield of total flavonoids reached 53.23 mg/g of dw with 2 mg/mL cellulase addition, 20.65% higher than that of the control (Figure 2a and Table 1). We also performed sonication-assisted extraction, which gave a total flavonoid yield of 51.81 mg/g of dw (Figure 2a and Table 1), on the same level as that of the enzyme-assisted extraction. The result indicates that cellulase addition had an identical enhancing effect on flavonoid extraction to sonication.

To validate the cell-wall-degrading role of *T. viride* cellulase, the morphology of *GR* samples after extraction was imaged by SEM. As shown in Figure 2b, the cell wall without cellulase treatment was intact and tight, while that after cellulase or sonication treatment was much looser. It can, therefore, be concluded that *T. viride* cellulase facilitated the destruction of the cell wall structure and, thereby, enhanced the release of intracellular flavonoids, in the same manner as sonication did in the extraction.

***T. viride* Cellulase Increases the Proportions of Liquiritigenin and Isoliquiritigenin in the *GR* Extract.** When we analyzed compositions of the GR extracts obtained from different methods, we surprisingly found that the contents of liquiritigenin and isoliquiritigenin in the sample with cellulase treatment were significantly higher than those without cellulase involvement (Figure 3 and Table 1). As shown on the chromatographic fingerprints, when *T. viride* cellulase was added, the peak of liquiritin decreased strikingly, while that of liquiritigenin became much larger (Figure 3a), implying that liquiritin was probably converted into liquiritigenin during the extraction. Likewise, growth of the isoliquiritigenin peak and reduction of isoliquiritin (Figure 3b) suggested that isoliquiritin was enzymatically transformed into isoliquiritigenin. Calculation based on the chromatographic profiles indicates that the ratio between liquiritigenin and liquiritin reached 7.42 and that the ratio between isoliquiritigenin and isoliquiritin reached 3.00 for the extract with cellulase addition, remarkably higher than those for the control (0.20 and 0.21, respectively) or the sonication-assisted extraction (0.09 and 0.20, respectively) (Figure 3c and Table 1). Meanwhile, the content of glycyrrhizic acid, another major flavonoid in the *GR* extract, remained on the same level after cellulase treatment (Figure 3a), indicating the absence of its enzymatic decomposition.

To validate the deglycosylation activity of *T. viride* cellulase, we used pure compounds of liquiritin and isoliquiritin as model substrates for enzymatic reactions. Chromatographic profiles of the reaction mixtures before and after enzymatic catalysis are shown in Figure 4. Both flavonoid glycosides could be completely converted to their aglycones after simply mixing with 2 mg/mL cellulase and stirring for 4 h at 50 °C, indicating high deglycosylation activity of the enzyme. Although *T. viride* cellulase has not yet been reported for its deglycosylating activity toward flavonoids, the β-glucosidase component in cellulases from other resources has proven efficient for the hydrolysis of glycosidic linkages in flavonoid glycosides.[33−35] For instance, the β-glucosidase component in *Aspergillus* sp. cellulase could readily deglycosylate the flavonoids from bergamot peel.[36] Thus, in our case, it is reasonable to suppose that the β-glucosidase constituent in *T. viride* cellulase[31,32] cleaved the glycosidic bonds within liquiritin and isoliquiritin and generated the corresponding aglycones.

With regard to the higher absorption and physiological activities of liquiritigenin and isoliquiritigenin in the human body, these results present an encouraging fact that *T. viride*

cellulase could significantly improve the bioactivity of the *GR* extract via deglycosylation of its main components of liquiritin and isoliquiritin into their aglycones.

Optimization of the Cellulase-Assisted *GR* Extraction. To maximize the yields of highly active liquiritigenin and isoliquiritigenin from *GR*, four factors, including ethanol/water ratio, cellulase concentration, pH, and temperature, in the enzyme-assisted extraction were selected for optimization (Figure 5).

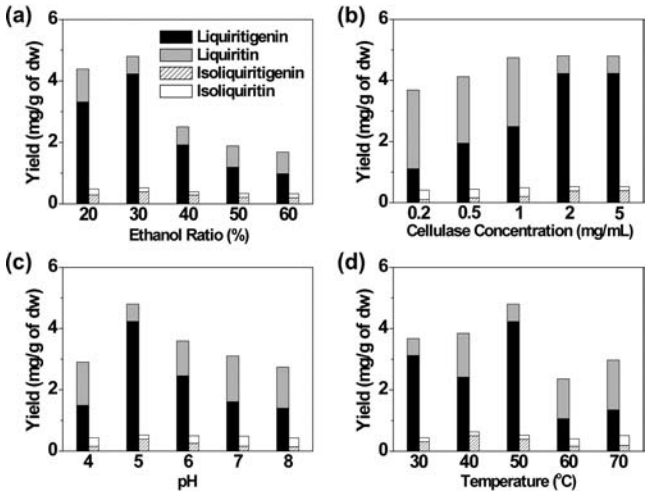

Figure 5. Effect of the (a) ethanol ratio, (b) cellulase concentration, (c) pH, and (d) temperature on the enzyme-assisted extraction of liquiritigenin, liquiritin, isoliquiritigenin, and isoliquiritin from *GR*.

Solubilities of the substrate and product generally have large effects on enzymatic reactions. In our case, although flavonoid glycosides are highly soluble in both water and ethanol, solubilities of aglycone products rise as the ratio of ethanol increases. When the requirement of water for enzymatic catalysis and that of ethanol for aglycone dissolution are taken into account, the ethanol−water ratio in the extractant needs to be controlled on a proper level to achieve the highest transformation efficiency of the flavonoid glycosides. As shown in Figure 5a, the highest yields of liquiritigenin and isoliquiritigenin were achieved at an ethanol ratio of 30%. When the ethanol ratio was increased slightly to 40%, the deglycosylation rate decreased dramatically, probably because of the enzyme inactivation caused by excess ethanol or the left shift of the reversible reaction toward glycosylation, owing to the increased solubility of the aglycone product.

We then optimized the cellulase concentration in the 30% ethanol extraction system (Figure 5b). The total yield of liquiritin and liquiritigenin increased with cellulase addition and reached the plateau at a cellulase dosage of 1 mg/mL, revealing enhancement of the extraction from facilitated cell wall degradation. When the cellulase concentration was further raised to 2 mg/mL, a remarkable increase of the ratio between liquiritigenin and liquiritin happened, indicating efficient enzymatic deglycosylation of the flavonoid glycoside. The yield of liquiritigenin and isoliquiritigenin reached the peak when 2 mg/mL cellulase was added, while a further increase of cellulase made no improvement.

The effect of different pH values and temperatures was also studied. Within a broad pH range, *T. viride* cellulase exhibited an improving effect on the extraction (Figure 5c). At pH 5, the yield of liquiritigenin and isoliquiritigenin reached the maximum. Moreover, 50 °C was identified as the optimal extraction temperature (Figure 5d). When the temperature was above 50 °C, the extraction yield decreased notably, probably because of the heat-induced inactivation of the enzyme.

Therefore, the optimum condition for the cellulase-assisted extraction was determined as 30% ethanol, 2 mg/mL cellulase, pH 5, and 50 °C. The highest yield of liquiritigenin was obtained as 4.23 mg/g of dw, 5.51 and 9.32 times higher than that of the control and the sonication-assisted extraction, respectively, while the yield of isoliquiritigenin reached 0.39 mg/g of dw, 2.55 and 2.90 times higher, respectively.

***P. decumbens* Naringinase Enhances the Extraction of Baicalein and Wogonin from *SR*.** To expand examples of employing bifunctional enzymes for improved ingredient extraction, we chose a different plant material, *SR*, for a second trial, whose main constituents are also flavonoids. Deglycosylation of the major contents of bacalin and wogonoside of *SR* will give their aglycones, baicalein and wogonin, which exhibit higher bioactivities, including antitumor effects.[17] A different

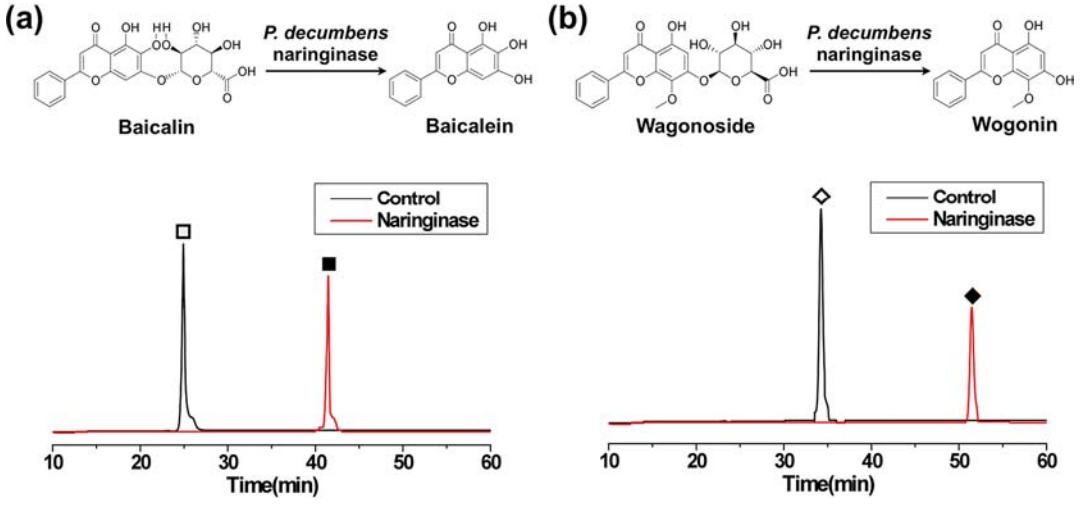

Figure 6. Deglycosylation of flavonoid glycosides by *P. decumbens* naringinase. (a) Transformation of baicalin (□) into baicalein (■). UV absorbance was detected at 274 nm. (b) Transformation of wogonoside (◇) into wogonin (◆). UV absorbance was detected at 274 nm.

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Table 2. Extraction of Flavonoids from *SR* by Different Methods

method	yield of total flavonoids (mg/g of dw)	yield of baicalin (mg/g of dw)	yield of baicalein (mg/g of dw)	yield of wogonoside (mg/g of dw)	yield of wogonin (mg/g of dw)	ratio of baicalein/baicalin	ratio of wogonin/wogonoside
solvent-only	542.33 ± 30.34	56.81 ± 5.63	7.01 ± 0.61	14.12 ± 0.82	1.23 ± 0.24	0.12	0.09
sonication-assisted	687.62 ± 25.13	61.83 ± 3.52	11.22 ± 0.53	19.24 ± 2.14	1.42 ± 0.12	0.18	0.07
naringinase-assisted	652.44 ± 29.82	10.14 ± 0.73	59.44 ± 1.92	18.83 ± 0.93	11.64 ± 0.73	5.86	0.62
cellulase-assisted	582.21 ± 18.73	9.82 ± 0.0.51	52.43 ± 3.23	17.62 ± 1.42	9.61 ± 0.82	5.34	0.55

commercially available enzyme, *P. decumbens* naringinase, was used, because its α-L-rhamnosidase (EC 3.2.1.40) and β-D-glucosidase (EC 3.2.1.21) activities have been reported to effectively hydrolyze the glycosidic bonds in flavonoid glycosides, such as those in naringin and rutin.[37−39]

Before extraction experiments, we first tested the deglycosylation activity of naringinase toward bacalin and wogonoside. Encouragingly, the enzyme could efficiently convert pure baicalin and wogonoside into their aglycones of baicalein and wogonin, respectively (Figure 6). This result promoted us to use naringinase for the extraction of *SR* flavonoids.

An extraction yield of 652.44 mg of total flavonoids/g of dw was obtained when naringinase was used in *SR* extraction, identical to that of the sonication-assisted extraction and 20.30% higher than that of the control (Table 2). Moreover, dramatic decreases in the peak areas of bacalin and wogonoside, along with increases in those of baicalein and wogonin, were found on the chromatographic fingerprints, indicating the deglycosylation of flavonoid glycosides during extraction (Figure 7). Under optimized conditions of the enzyme-assisted

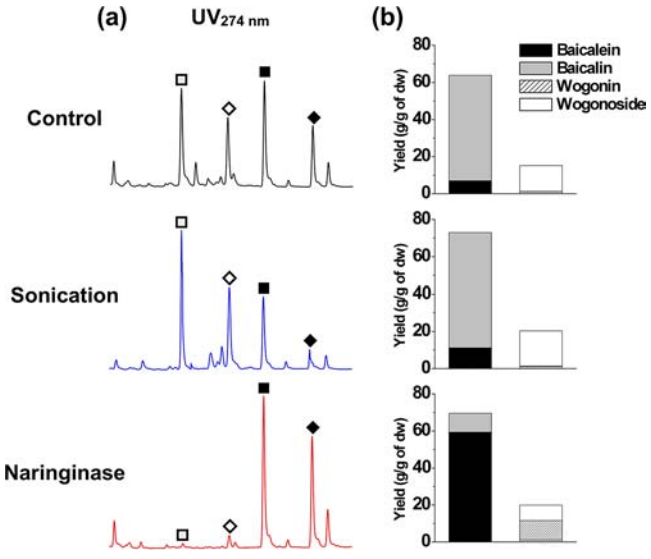

Figure 7. Chromatographic fingerprint analysis of *SR* extracts obtained by different methods. (a) Chromatograms of the extracts with marked peaks of baicalin (□), baicalein (■), wogonoside (◇), and wogonin (◆). UV absorbance was detected at 274 nm. (b) Calculated contents of baicalein, baicalin, wogonin, and wogonoside in the extracts.

extraction, the yield of baicalein achieved 59.44 mg/g of dw, 7.48 and 4.30 times higher than that of the control and the sonication-assisted extraction, respectively, while the yield of wogonin reached 11.64 mg/g of dw, 8.46 and 7.20 times higher, respectively (Table 2). Therefore, not only the yield of total flavonoids but also the proportions of baicalein and wogonin in the *SR* extract were largely enhanced upon naringinase addition, indicating dual roles of the enzyme in the extraction.

Effect of Plant−Enzyme Combinations on the Extraction Performance. In the final part of our research, we recombined the plant and enzyme options and examined their effects on the extraction performance. As presented in Figure 8,

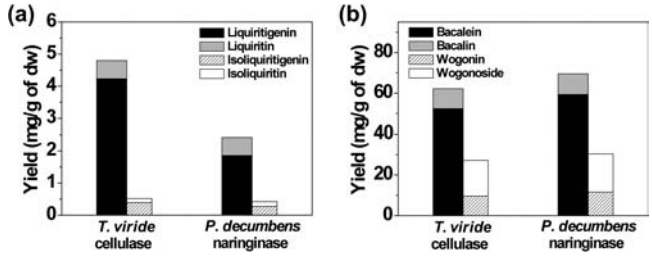

Figure 8. Effect of different plant−enzyme combinations on the extraction performance. (a) Extraction yields of major flavonoids from *GR* with the addition of *T. viride* cellulase and *P. decumbens* naringinase. (b) Extraction yields of major flavonoids from *SR* with the addition of *T. viride* cellulase and *P. decumbens* naringinase.

T. viride cellulase offered more effective extraction of flavonoids from *GR*, while *P. decumbens* naringinase worked slightly better for *SR* extraction. With regard to the more cellulosic property of *GR* compared to *SR*, it is reasonable to assume that the degradation of the cell wall played a more essential role in *GR* extraction compared to that in *SR* extraction. Thus, *T. viride* cellulase, which has much higher cell-wall-degrading activity than *P. decumbens* naringinase, delivered a better performance in flavonoid extraction from *GR*. Although rational considerations, such as those presented above, may be taken into account, determination of an effective enzyme for the ingredient extraction of a given plant is still quite an experiential practice in our opinion, which basically depends upon the maximum yield experimentally obtained via multiple trials.

In conclusion, the present study demonstrates a cost-effective approach employing bifunctional enzymes to enhance the extraction of highly active flavonoids from plants. Two enzymes, *T. viride* cellulase and *P. decumbens* naringinase, have shown significant improvement effects on flavonoid extraction from two plants, *GR* and *SR*, respectively. The advantages of this approach lie in not only the enzymatic enhancement of cell wall degradation but also the enzymatic transformation of target compounds into more bioactive analogues. One of our previous works has taken advantage of the bifunctionality of *P. decumbens* cellulase to facilitate the degradation of the cell wall and increase the solubility of extracts via transglycosylation, leading to an enhanced yield of total flavonoids from *Ginkgo biloba*.[40,41] Our study here, as an extension, uses the enzymatic bifunctionality to increase the bioactivity and, thus, quality of the extract, which would benefit its dietary and medicinal uses.

This novel strategy, in principle, can be applied to the extraction of, besides flavonoids, various plant ingredients that require biotransformation for enhanced bioactivities.[10,42] For

instance, ginsenoside Rb2, the major ginsenoside in ginseng, could be converted into more pharmacologically active minor ginsenosides, including compounds Y and K, if a hydrolytic enzyme is used in its extraction.[43] The major ingredient icariin in the commonly used *Epimedium* could also be enzymolyzed into icariin with low glycosyl or preicariin of higher activities during extraction.[42] The key in the new approach is to find an enzyme preparation that could accomplish dual functions of cell wall degradation and target compound transformation in the extraction process. In cases when it is hard to find a bifunctional enzyme commercially available, a mixed enzyme preparation may also be employed,[44] which is currently under investigation in our lab.

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Notes

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ABBREVIATIONS USED

GR, *Glycyrrhizae radix*; *SR*, *Scutellariae radix*; SEM, scanning electron microscopy

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