Natural Product Sciences
16(1) : 1-5 (2010)

Biotransformation of flavonoid-7-*O*-glucuronides by β-glucuronidases

Ran Joo Choi[1], In Jin Ha[1], Jae Sue Choi[2], Youmie Park[1]*, and Yeong Shik Kim[1,]*

[1]*Natural Products Research Institute, College of Pharmacy, Seoul National University, 599 Gwanangno, Gwanak-gu, Seoul 151-742, Republic of Korea*
[2]*Division of Food Science and Biotechnology, Pukyong National University, Busan 608-737, Republic of Korea*

Abstract – β-Glucuronidases (E.C. 3.2.1.31) from *Escherichia coli*, *Helix pomatia*, and bovine liver activity have been investigated on 7-*O*-glucuronides (baicalin, wogonoside, and luteolin-7-*O*-glucuronide) and 3-*O*-glucuronides (quercetin-3-*O*-glucuronide and kaempferol-3-*O*-glucuronide). Bovine liver enzyme was not active on any of these substrates. *E. coli* and *H. pomatia* enzymes were active on 7-*O*-glucuronides, however, 3-*O*-glucuronides were resistant to β-glucuronidase hydrolysis. These results suggest that glucuronic acid at 7-position is more susceptible to *E. coli* and *H. pomatia* β-glucuronidases than that at 3-position. In addition, the subtle difference of aglycone structure on 7-*O*-glucuronides affected the preference of enzyme. *E. coli* enzyme was favorable for the hydrolysis of baicalin, however, *H. pomatia* enzyme was found to be efficient for the hydrolysis of wogonoside. Both enzymes showed the similar hydrolytic activity towards luteolin-7-*O*-glucuronide. When the *Scutellaria baicalensis* crude extract was subjected to enzymatic hydrolysis, baicalin and wogonoside were successfully converted to their aglycone counterparts with *H. pomatia* at 50 mM sodium bicarbonate buffer pH 4.0. Accordingly, the enzymatic transformation of glycosides may be quite useful in preparing aglycones under mild conditions.

Keywords – β-Glucuronidase; *Scutellaria baicalensis* Georgi; Enzymatic hydrolysis; Flavonoid glucuronides

Introduction

Scutellaria baicalensis Georgi has been widely used in Asian traditional medicines and its root contains baicalin, baicalein, wogonoside and wogonin as major flavonoids. Flavonoids are found in a large number of plant families exhibiting various biological activities including antitumor (Li-Weber, 2008; Chao, *et al*., 2007), anti-inflammatory (Chi, *et al*., 2003), antioxidant (Gao, *et al*., 1999), anticardiovascular (Wang, *et al*., 2007), antithrombotic (Kimura and Okudu, 1997), and antiviral activities (Wu, *et al*., 2001; Guo, *et al*., 2007). A number of publications reported that the aglycones display more potent activities compared to those of glycoside counterparts. For example, baicalein (aglycone of baicalin) inhibited COX-2 gene expression in LPS-induced RAW 264.7 cells (Woo, *et al*., 2006). Therefore, a need has been arised for the purification of aglycone from crude extracts to test biological activities. However, it was reported that the contents of baicalein and wogonin in *S. baicalensis* dried roots of 42 samples were shown within the range of only 0.04 - 0.23% (Su, *et al*., 2008). Due to the low content of aglycones, it is not amenable to isolate aglycones from crude extracts. Instead, *S. baicalensis* contains flavonoid glycosides, baicalin and wogonoside, where glucuronic acid is β-linked to a 7-position of baicalein and wogonin. According to a recent publication, the contents of baicalin and wogonoside in dried roots ranged from 8.63 to 17.84%, and from 1.99 to 4.21%, respectively (Su, *et al*., 2008). This report suggests that the hydrolysis of glycoside linkages could be an excellent way to prepare aglycones in higher amounts from crude extracts. For a glycoside hydrolysis, two strategies have been applied: enzymatic hydrolysis and chemical hydrolysis (acid or base hydrolysis). Recently, Jianjun and Huiru reported the preparation of baicalein from baicalin in *S. baicalensis* extracts (Jianjun and Huiru, 2008). They used the acid hydrolysis of baicalin to generate baicalein and purified it by RP-HPLC. They found that hydrochloric acid shows better hydrolytic activity than other acids such as phosphoric acid and sulfuric acid.

For enzymatic hydrolysis, commercially available β-glucuronidases (E.C. 3.2.1.31) from three sources are of use: *Escherischia coli*, *Helix pomatia* and bovine liver. In order to develop an analytical method for psilocin in urine

*Author for correspondence
Tel: +82-2-880-2479; E-mail: kims@snu.ac.kr

sample, Kamata *et al*. investigated conditions including the acid, alkaline and enzymatic hydrolysis of psilocin glucuronide (Kamata, *et al*., 2003). The complete hydrolysis of psilocin was obtained by *E. coli* β-glucuronidase (5,000 units/mL urine, pH 6) on incubation at 37 °C for 2 hr. They also found that neither acid nor alkaline hydrolysis is applicable for psilocin sample. Ohkoshi mentioned about the simple enzymatic hydrolysis of baicalin with β-glucuronidase earlier, but no other flavone glucuronides were not compared (Ohkoshi, *et al*., 2008). Moreover, it has been reported that *Scutellaria* root itself is known to contain β-glucuronidase, which catalyzes the conversion of baicalin. They showed that baicalin present in the water extract can be hydrolyzed to its aglycone after 20 hr. However, prior to hydrolysis, the powderized water extract had to be kept in water (23 °C) for 75 hr, because higher temperature or 80% ethanol could not help the hydrolysis of baicalin (Ikegami, *et al*., 1995).

The present work describes β-glucuronidase hydrolysis on two types of flavonoid glucuronides as substrates: 7-*O*- and 3-*O*-glucuronides. In each case, β-glucuronidases from three sources (*E. coli*, *H. pomatia,* and bovine liver) were used to evaluate the activity. Furthermore, *S. baicalensis* extract was subjected to β-glucuronidase hydrolysis to find out optimum conditions and ensure the applicability of current methods to crude extracts.

Experimental

Materials and reagents – Propyl paraben and β-glucuronidases (E.C. 3.2.1.31) from *E. coli* (Type VII-A), *H. pomatia* (Type HP-2), and bovine liver (Type B-1) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. Quercetin-3-*O*-glucuronide and kaempferol-3-*O*-glucuronide were separated from *Nelumbo nucifera* and characterized as reported in the previous publications (Jung, *et al*., 2003; Jung, *et al*., 2008). The 70% ethanol extract of *S. bicalensis*, luteolin-7-*O*-glucuronide, and flavonoid aglycone standards (luteolin, quercetin, kaempferol) were provided through the National Research Center for Standardization of Herbal Medicines (Seoul National University, Seoul, Republic of Korea).

Sample preparation – Baicalin and wogonoside were purified by high-speed countercurrent chromatography (HSCCC) (Wu, *et al*., 2005). Baicalin and wogonoside were isolated by TBE-300A HSCCC (Shanghai Tauto Biotech Co. Ltd, Shanghai, China) [solvent system, ethyl acetate-methanol-1% acetic acid water (5 : 0.5 : 5, v/v)] and the purity was checked by RP-HPLC comparing with authentic standards to confirm their structures [A Zorbax SB-Aq C18 column (4.6 mm × 150 mm, 5 μm particle size); solvent, 0.5% phosphoric acid water-acetnonitrile; gradient, 0 - 40 min (5 - 35%, B), 40 - 41 min (35 - 85%, B), 41 - 48 min (85%, B), 48 - 49 min (85 - 5%, B); flow rate, 1.0 mL/min; detector, a Spectra 100 UV detector (Spectra Physics, USA); injector, a SIL-9A auto injector (Shimadzu, Japan); pump, a Hitachi L-6200 pump]. All ESI-MS^n experiments were performed on a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with ESI probe. MS conditions were selected as follows [solvent: methanol; sheath gas flow: 80 arbitrary units; capillary temperature: 275 °C; capillary voltage: 13 kV; spray voltage: 4.5 kV in positive mode]. For tandem mass spectrometry, collision energy with helium was set to 23% - 30%.

Biotransformation of flavonoid glucuronides – Each glucuronide standard (2 mM) was dissolved in the mixture of 250 μL sodium bicarbonate buffer (50 mM, pH 8.1) and 100 μL ethanol, and then mixed with 500 units of β-glucuronidase (from *E. coli*, *H. pomatia* and bovine liver). The amount of enzyme unit was set according to the previous publication (Kim, *et al*., 2003). The reaction mixture was incubated at 37 °C for 18 hours. Propyl paraben (2.5 mM, 10 μL) was added into this reaction mixture as an internal standard and it was extracted three times with ethyl acetate (350 μL/each time). The ethyl acetate fraction was combined and dried by N_2 for analysis by RP-HPLC and ESI-MS in the next step.

Biotransformation of 70% ethanol extract of *S. baicalensis* - For the hydrolysis of *S. baicalensis* 70% ethanol extract, 2 mg of the extract was dissolved in ethanol (200 μL) and 500 μL sodium bicarbonate buffer (50 mM). Buffer pH was varied at pH 4, 5, 6, and 7. Next, 1000 units of β-glucuronidase (*E. coli* and *H. pomatia*) were added to as the prepared solution. The reaction mixture was incubated at 37 °C for 18 hours. After the completion of digestion, the reaction mixture was filtered through 0.45 μm syringe filters before injecting onto RP-HPLC.

Results and Discussion

Five flavonoid glucuronides were tested for β-glucuronidase hydrolysis and their structures were shown in Fig. 1. Among them, baicalin and wogonoside were purified by preparative HSCCC, and the yield was 30 mg of baicalin and 6 mg of wogonoside from 120 mg crude

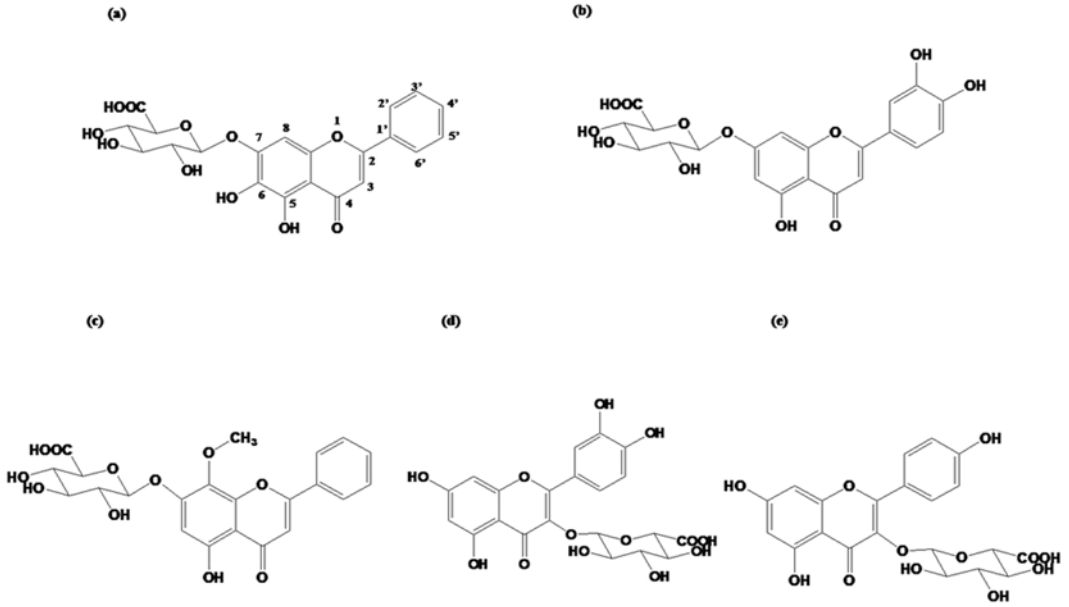

Fig. 1. Structures of flavonoid glucuronides used in this study (a) baicalin, (b) luteolin-7-*O*-glucuronide, (c) wogonoside, (d) quercetin-3-*O*-glucuronide, and (e) kaempferol-3-*O*-glucuronide.

extract. The purity of baicalin and wogonoside were 94.5 % and 92.8% in RP-HPLC. Structures of baicalin and wogonoside were confirmed by co-injection with authentic standards in RP-HPLC (data not shown) and ESI-MS analyses. Sodium adduct of *m/z* 468.9 $[M+Na]^+$ and *m/z* 483.1 $[M+Na]^+$ in ESI-MS analyses confirmed that the purified compounds were baicalin and wogonoside, respectively (data not shown). For baicalin, $[M+H]^+$ (*m/z* 447.1) and $[2M+Na]^+$ (*m/z* 914.8) adducts were also observed. In tandem MS fragmentations (MS^2) of baicalin, the characteristic loss of glucuronic acid (Δ176) was observed from *m/z* 468.9 generating a peak at *m/z* 292.9 shown in Fig. 2(a). Also, the loss of glucuronic acid (Δ176) was observed for wogonoside at *m/z* 307.0 from *m/z* 483.1 shown in Fig. 2(b).

For the enzymatic hydrolysis, each glucuronide standard was dissolved in 50 mM sodium bicarbonate buffer (pH 8.1) containing ethanol to fully dissolve the compound. Without the addition of ethanol in the buffer, the solubility of each compound was poor. Propyl paraben was chosen as an internal standard because of commercial availability, good solubility in ethyl acetate, and reasonable retention time in our RP-HPLC condition. The retention time of propyl paraben (38.9 min) did not overlap with our interested peaks. In our blank test (the same procedure without an enzyme and a substrate), the standard curve of propyl paraben in ranging from 0 mM to 10 mM displayed the linear relationship between concentration and peak area ($r^2 = 0.9949$). Ethyl acetate was chosen for liquid-liquid extraction of aglycones according to the previous report (Kim, *et al*., 2006). Generation of aglycone in ethyl acetate fraction was confirmed either by ESI-MS or by co-injection with an authentic standard in RP-HPLC (data not shown).

As shown in Table 1, *E. coli* and *H. pomatia* enzymes were active on 7-*O*-glucuronides, not on 3-*O*-glucuronides. Interestingly, it is likely that the position of glucuronic acid to aglycone moiety affects the activity. Glucuronic acid at 7-position was susceptible to *E. coli* and *H. pomatia* enzyms, however, glucuronic acid at 3-position was not susceptible at all. Among 7-*O*-glucuronides tested, luteolin-7-*O*-glucuronide showed almost the same susceptibility towards *E. coli* (0.15) and *H. pomatia* (0.16) enzymes. For the hydrolysis of baicalin, *E.coli* enzyme (0.14) was more useful than *H. pomatia* enzyme (0.09). On the other hand, *H. pomatia* enzyme (1.91) was found to be more efficient than *E. coli* enzyme (0.47) for the hydrolysis of wogonoside. These results suggest that the subtle difference in aglycone structures might affect the activity towards each enzyme. When the aglycone structure contains a 6-hydroxyl group (baicalin), it is likely that *E. coli* enzyme was more favorable. In case of luteolin-7-*O*-glucuronide, hydroxyl groups (5-, 3'- and 4'-) did not show any preference to either of *E. coli* or *H. pomatia* enzymes. When a methoxy group was substituted to an 8-position, *H. pomatia* enzyme was more favorable. However, β-glucuronidase from bovine liver was not active on any of glucuronides tested, and did not generate

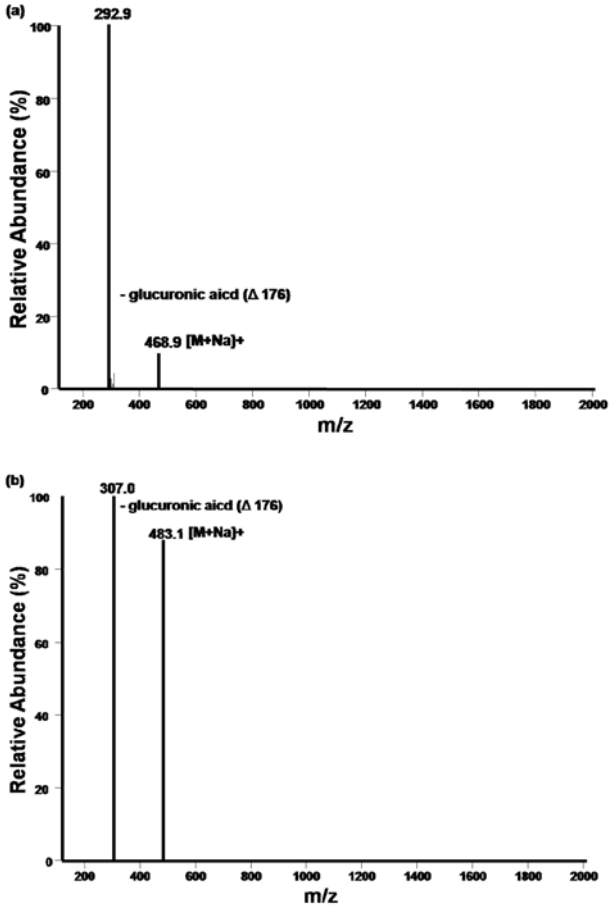

Fig. 2. Positive ion mode of ESI-MS^2 (a) baicalin, and (b) wogonoside purified by HSCCC.

Table 1. Hydrolysis of 7-*O*-glucuronides by glucuronidases

	E. coli.	*H. pomatia*	bovine liver
7-*O*-glucuronides			
baicalin	0.14[a]	0.09	–[b]
luteolin-7-*O*-glucuronide	0.15	0.16	–
wogonoside	0.47	1.91	–

[a]The peak area of aglycone was divided by the peak area of internal standard.
[b]No aglycone was observed in RP-HPLC after enzymatic digestion.

aglycones under the current experimental conditions. The similar results were reported on the hydrolysis of psilocin where the activity of β-glucuronidase was *E. coli* > *Ampullaria* > *H. pomatia* > bovine liver. Bovine liver enzyme showed the lowest activity on psilocin urine sample, and did not show any activities on flavonoid glucuronides tested in this study (Kamata, *et al*., 2003). This is possibly due to a fact that the hydrolysis of flavonoid glucuronides is dependent on the structure of

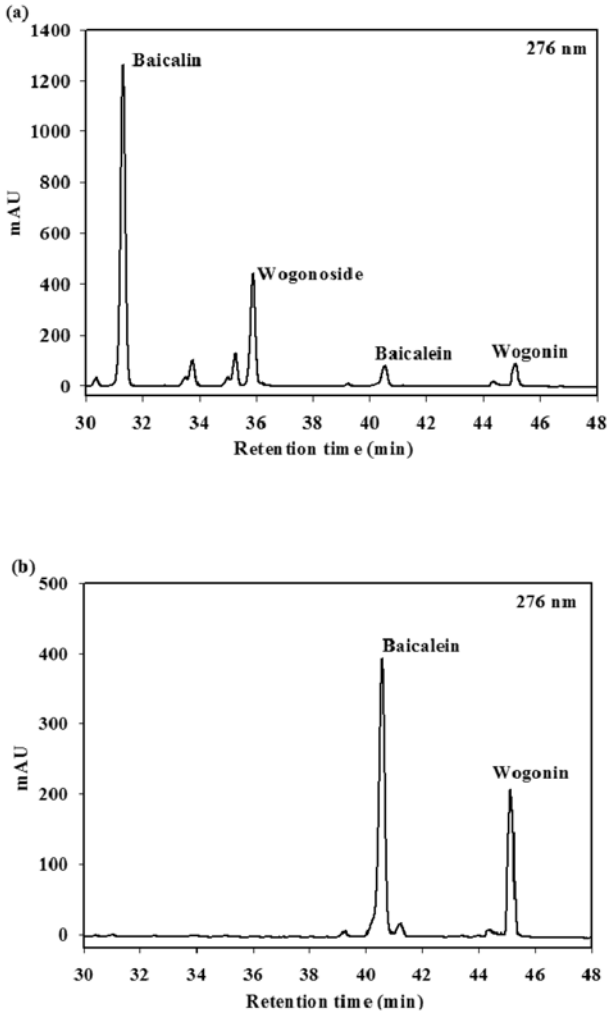

Fig. 3. RP-HPLC chromatograms. The 70% ethanol extract of *S. baicalensis* before the addition of β-glucuronidase from *H.pomatia* (a) and after the addition of β-glucuronidase from *H. pomatia* (b).

each substrate and sources of β-glucuronidase.

Next, we optimized the condition for conversion of baicalin and wogonoside in the 70% ethanol extract of *S. baicalensis*. *E. coli* and *H. pomatia* enzymes were tested under different pH (4, 5, 6, and 7, 50 mM sodium bicarbonate buffer). From RP-HPLC analysis, *H. pomatia* enzyme in buffer (pH 4.0) was optimum as shown in Fig. 3 (a-b). Baicalin and wogonoside in crude extracts were converted to their aglycones successfully.

Summary

It was demonstrated that β-glucuronidases (*E. coli* and *H. pomatia*) hydrolyze glucuronic acid at 7-position, not 3-position, of flavonoids. In terms of substrate specificity, enzymatic hydrolysis by β-glucuronidase is superior to

chemical hydrolysis. In addition, we extended the application of enzymatic hydrolysis to crude extracts, and it could be useful in the area of natural product research.

Acknowledgement

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 20090093533).

References

Chao, J.I., Su, W.C., and Liu, H.F., Baicalein induces cancer cell death and proliferation retardation by the inhibition of CDC2 kinase and surviving associated with opposite role of p38 mitogen-activated protein kinase and AKT. *Mol. Cancer Ther.* **6**, 3039-3048 (2007).

Chi, Y.S., Lim, H., Park, H., and Kim, H.P., Effects of wogonin, a plant flavones from *Scutellaria radix*, on skin inflammation: *in vivo* regulation of inflammation-associated gene expression. *Biochem. Pharmacol*. **66**, 1271-1278 (2003).

Gao, Z., Huang, K., Yang, X., and Xu, H., Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. *Biochim. Biophys. Acta* **1472**, 643-650 (1999).

Guo, Q., Zhao, L., You, Q., Yang, Y., Gu, H., Song, G., Lu, N., and Xin, J., Anti-hepatitis B virus activity of wogonin *in vitro* and *in vivo*. *Antivir. Res*. **74,** 16-24 (2007).

Jianjun, J., and Huiru, D., Preparation of high-purity baicalein from *Scutellaria baicalensis* Georgi. *Nat. Prod. Res.* **22**, 1410-1412 (2008).

Jung, H.A., Jung, Y.J., Yoon, N.Y., Jeong, D.M., Bae, H.J., Kim, D.W., Na, D.H., and Choi, J.S., Inhibitory effects of *Nelumbo nucifera* leaves on rat lens aldose reductase, advanced glycation endproducts formation, and oxidative stress. *Food Chem. Toxico.* **46**, 3818-3826 (2008).

Jung, H.A., Kim, J.E., Chung, H.Y., and Choi, J.S., Antioxidant principles of *Nelumbo nucifera* stamens. *Arch. Pharm. Res.* **26**, 279-285 (2003).

Kamata, T., Nishikawa, M., Katagi, M., and Tsuchihashi, H., Optimized glucuronide hydrolysis for the detection of psilocin in human urine samples. *J. Chromatogr. B* **796**, 421-427 (2003).

Kim, M.J., Kim, N.S., Kim Y.I., Kim, D.K., and Yang, J.H., Improvement of hydrolysis and bioavailability of coprecipitated products of *Coptidis rhizome* and *Scutellaria radix* by β-glucuronidase. *J. Kor. Pharm. Sci.* **33**, 91-97 (2003).

Kim, Y.H., Jeong, D.W., Paek, I.B., Ji, H.Y., Kim, Y.C., Sohn, D.H., and Lee, H.S., Liquid chromatography with tandem mass spectrometry for the simultaneous determination of baicalein, baicalin, oroxylin A and wogonin in rat plasma. *J. Chromatogr. B* **844**, 261-267 (2006).

Kimura, Y., and Okuda, H., Effects of flavonoids isolated from *Scutellaria Radix* on fibrinolytic system induced by trypsin in human umbilical vein endothelial cells. *J. Nat. Prod.* **60**, 598-601 (1997).

Li-Weber, M., New therapeutic aspects of flavones: The anticancer properties of *Scutellaria* and its main active constituents wogonin, baicalein and baicalin. *Cancer Treat. Rev.* **35**, 57-68 (2008).

Ohkoshi, E., Nagashima, T., Sato, H., Fujii, Y., Nozawa, K., and Nagai, M., Simple preparation of baicalin from Scutellariae Radix. *J. Chromatogr. A* **1216**, 2192-2194 (2008).

Su, S., He, C.M., Li, L.C., Chen, J.K., and Zhou, T.S., Genetic characterization and phytochemical analysis of wild and cultivated populations of *Scutellaria baicalensis*. *Chem. Biodivers.* **5**, 1353-1363 (2008).

Ikegami, F., Matsunae, K., Hisamitsu, M., Kurihara, T., Yamamoto, T., and Murakoshi, I., Purification and properties of a plant β-D-glucuronidase from *Scutellaria* root. *Biol. Pharm. Bull*. **18**, 1531-1534 (1995).

Wang, C.Z., Mehendale, S.R., and Yuan, C.S., Commonly used antioxidant botanicals: Active constituents and their potential role in cardiovascular illness. *Am. J. Chinese Med.* **35**, 543-558 (2007).

Woo, K.J., Lim, J.H., Suh, S.I., Kwon, Y.K., Shin, S.W., Kim, S.C., Choi, Y.H., Park, J.W., and Kwon, T.K., Differential inhibitory effects of baicalein and baicalin on LPS-induced cyclooxygenase-2 expression through inhibition of C/EBPβ DNA-binding activity. *Immunobiology* **211**, 359-368 (2006).

Wu, J.A., Attele, A.S., Zhang, L., Yuan, C.S., Anti-HIV activity of medicinal herbs: Usage and potential development. *Am. J. Chinese Med*. **29**, 69-81 (2001).

Wu, S., Sun, A., and Liu, R., Separation and purification of baicalin and wogonoside from the Chinese medicinal plant *Scutellaria baicalensis* Georgi by high-speed counter-current chromatography. *J. Chromatogr. A* **1066**, 243-247 (2005).

Received December 23, 2009
Revised March 3, 2010
Accepted March 3, 2010