

Changes in Aurantio-Obtusin and Glucoaurantio-Obtusin Content in Cassiae Semen via Treatment with a Crude Enzyme Extract from *Aspergillus usamii*

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Received: March 28, 2007

Accepted: April 29, 2007

Abstract Cassiae Semen (seeds of *Cassia tora*) showed a remarkably different HPLC chromatogram after being treated with a crude enzyme extract from *Aspergillus usamii*. Increased and decreased compounds were identified as aurantio-obtusin and glucoaurantio-obtusin, respectively. The aurantio-obtusin content reached its maximum level (133.58±0.39 µg/mg extract) after being incubated for 50 min at 37°C, whereas the inactivated crude enzyme-treated control remained unchanged (54.13±1.33 µg/mg). On the other hand, the glucoaurantio-obtusin content decreased by less than one-third (51.09±1.63 µg/mg) of the untreated control (143.19±2.12 µg/mg), suggesting that an increase in aurantio-obtusin content originated from the enzymatic cleavage of its glucoside glucoaurantio-obtusin.

Keywords: Cassiae Semen, aurantio-obtusin, glucoaurantio-obtusin, *Aspergillus usamii*, processes, enzyme treatment

We have recently found that simple food processing techniques such as roasting, extrusion, and enzyme treatment (fermentation) could change the contents of active components in Oriental drugs. For example, the amount of liquiritigenin in fermented licorice increased up to 400 times over that of the control [10]. Moreover, the content of paeonol in Moutan Cortex and 5-hydroxymethyl furfural in Asparagi Tuber dramatically increased after the roasting process [7, 11].

Regarding chemical changes in Oriental drugs after processing, fifty popularly used medicinal plants were investigated. As a result, dramatic changes were found in the enzyme-treated Cassiae Semen (via HPLC chromatogram).

C. Semen purchased from an herbal medicine supplier in Daegu, Korea, was identified by Dr. Jong Hwan Kwak of Sung Kyun Kwan University, Suwon, Korea. Crude enzyme extract was prepared from *Aspergillus usamii* according to the previous report [10]. The inactivated enzyme was prepared by autoclaving crude enzyme solution. One g of lyophilized 95% EtOH extract of C. Semen was treated with about 260 U (1 U is defined as the enzyme activity needed to produce 1 mmol *p*-nitrobenzene from *p*-nitrophenyl-β-D-glucopyranoside per min) of a crude enzyme solution for 60 min at 37°C. Inactivated crude enzyme treated, crude enzyme treated, and untreated C. Semen extracts were analyzed by HPLC. In the crude enzyme treated extract, one peak was dramatically increased (Fig. 1).

To isolate an increased compound, the C. Semen (13 kg) was extracted with 95% EtOH (2×, 3 h) and the extract was evaporated to dryness. The residue (100 g) was then mixed with the 26,000 U of crude enzyme and incubated for 120 min at 37°C. The reaction mixture was suspended in 1 l of water and partitioned with an equivalent volume of CH₂Cl₂ (2×). The organic layer was dried with anhydrous Na₂SO₄ and evaporated so as to obtain a CH₂Cl₂ soluble fraction (32.7 g). This fraction was chromatographed onto a silica gel column (Merck Art. 7734, 5.5×40.0 cm, hexane-acetone=100:1-0:100). The eluates were combined into eleven fractions based on their TLC (Merck Art. 5715 and 1.15685) patterns. The fraction 7 (16.2 g), which contained the increased compound, was further purified by silica gel open column chromatography (Merck Art. 7734, 3.5×30.0 cm, hexane-acetone=20:1-8:1). Five fractions (Fr. 7-1 to 7-5) were obtained and the repeated silica gel chromatography (Merck Art. 7734; 1st, 2.0×30.0 cm, hexane-acetone=15:1-5:1; 2nd, 1.4×28.0 cm, hexane-acetone

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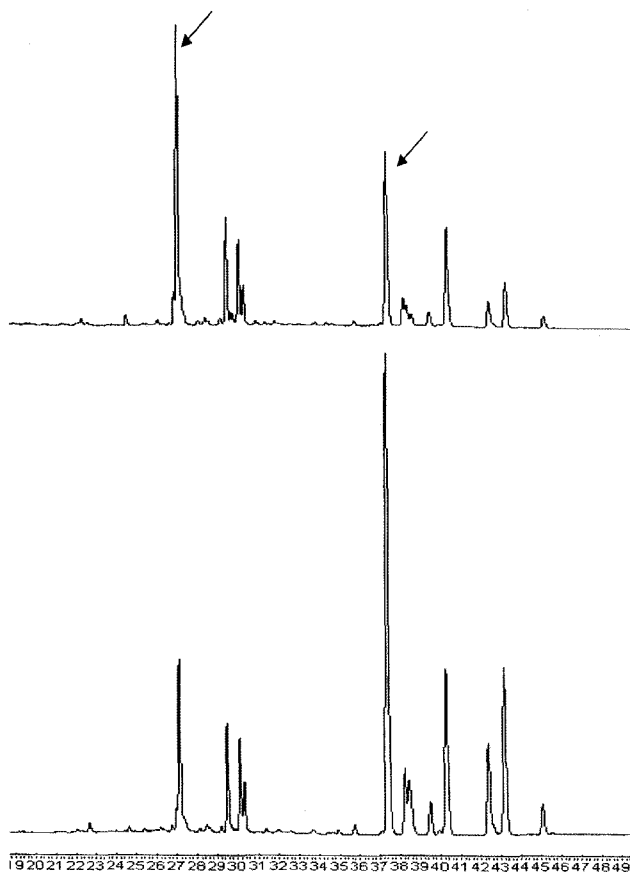


Fig. 1. HPLC profiles of Cassiae Semen EtOH extract after treatment with crude enzyme from *Aspergillus usamii*. Upper trace; EtOH extract+inactivated crude enzyme. Lower trace; EtOH extract+crude enzyme. Arrows indicate glucoaurantio-obtusin (left) and aurantio-obtusin (right), respectively.

=15:1-10:1) of Fr. 7-4 afforded compound **1** (381.0 mg). To isolate a decreased compound, untreated *C. Semen* (1 kg) was extracted with 95% EtOH (2 \times , 3 h) and the extract (19.2 g) was further purified with MPLC (ODS-S-50A, 26 \times 300 mm, 50%, 100% MeOH). The purification of Fr. 2 (155.6 mg) using MPLC (ODS-S-50A, 11 \times 300 mm, 20%, 25%, 35% MeOH) gave compound **2** (31.2 mg).

Compound **1**, isolated from the crude enzyme treated *C. Semen*, showed the same retention time as the increased peak in the HPLC. Compound **1** was obtained as a yellow crystalline powder and was visible under UV. Compound **1** gave a deprotonated molecular ion $[M-H]^-$ at m/z 329 in the ESI-MS. Characteristic fragmentation ions at m/z 314 (329-15), 299 (314-15), and 284 (299-15) were produced by the consequential loss of a methyl group. In the 1H -NMR analysis (400 MHz, $DMSO-d_6$), two characteristic signals at δ 3.80 and 3.84 (s, each 3H) were from two methoxyl groups. The signal at δ 2.29 (3H, s) indicated a methyl group that directly bonded to an aromatic ring. In addition, the signals at δ 7.17 (1H, s) and 7.78 (1H, s) confirmed that compound **1** had an aromatic ring. A total

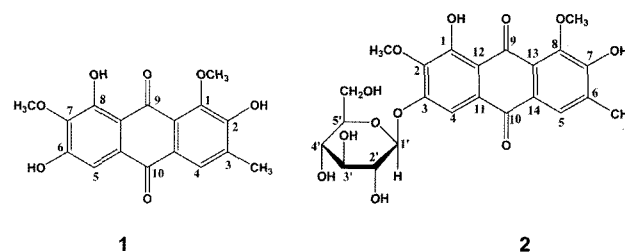


Fig. 2. Chemical structures of aurantio-obtusin and glucoaurantio-obtusin.

1, Aurantio-obtusin. **2**, Glucoaurantio-obtusin.

of 17 carbon signals including two methoxy carbon signals (δ 57.86 and 59.07) were detected in the ^{13}C -NMR spectrum (100 MHz, $DMSO-d_6$). By comparing these NMR data with previous reports [8], **1** was identified as aurantio-obtusin.

Compound **2**, whose retention time corresponded to the decreased peak in HPLC, was isolated from untreated *C. Semen*. Compound **2** was also obtained as a yellow crystalline powder and was visible under UV. In the ESI-MS, a protonated molecular ion $[M+H]^+$ at m/z 493 implied a molecular weight of 492. The fragmentation ion at m/z 331 $[M+H-Glc]^+$ suggested that **2** might contain one glucose unit in the structure. Other spectral data were very similar to those of **1** except for the additional signals of a sugar moiety. By comparing the NMR data with those of the references [20], **2** was identified as glucoaurantio-obtusin.

One g of ethanolic extract of *C. Semen* was treated with 260 U of crude enzyme and incubated for 10, 20, 30, 40, 50, 60, 90, and 120 min. The temperature was fixed at 37 $^\circ C$ since this is the optimal temperature of the main glucosidase in *Aspergillus usamii* [4]. An HPLC analysis was performed on a Gemini 5 μ C18 (4.6 \times 250 mm) column with a gradient solvent system, by varying the proportion of solvent A (water 99%-acetic acid 1%) to solvent B (acetonitrile 99%-acetic acid 1%). Solvent B the increased to 100% in 50 min and kept at 100% for 10 min at a flow rate of 0.8 ml/min. Detection was carried out under UV 280 nm. The aurantio-obtusin and glucoaurantio-obtusin isolated in this work (more than 99% under UV 280 nm in HPLC analysis) were used as standards. The calibration curve for aurantio-obtusin was $Y=0.297X-0.108$ ($r^2=0.9996$) and it was $Y=0.0037X-0.00006$ ($r^2=0.9996$) for glucoaurantio-obtusin, where Y represents the peak area and X is the weight in μg .

Aurantio-obtusin reached its maximum level at 50 min after crude enzyme treatment (Fig. 3). Under these conditions, the content of aurantio-obtusin increased about 2.4 times (133.58 ± 0.39 $\mu g/mg$ extract) that of the control (54.13 ± 1.33 $\mu g/mg$ extract). On the other hand, glucoaurantio-obtusin content decreased about 2.8 times (51.09 ± 1.63 $\mu g/mg$ extract) compared with that of the control (143.19 ± 2.12 $\mu g/mg$ extract).

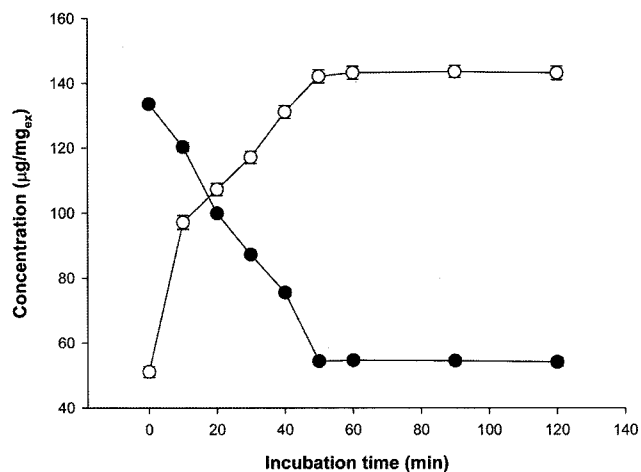


Fig. 3. Time-course change in aurantio-obtusin and glucoaurantio-obtusin contents of Cassiae Semen after treatment with crude enzyme.

Each data point is the mean value of duplicated experiments. -○-, aurantio-obtusin; -●-, glucoaurantio-obtusin.

mg extract). Recently, *A. usamii* has been documented as having strong glucosidase activity [4]; therefore, aurantio-obtusin could be produced *via* the enzymatic cleavage of its corresponding glucoside glucoaurantio-obtusin.

Food processing techniques have generally focused on promoting nutritional values and/or improving specific flavors or color in foodstuffs. Previous studies have mainly focused on the sensory evaluation of roasted *C. Semen* and identification of compounds produced by the roasting process [13, 19, 21]. Recently, as the tertiary functions (physiological characteristics) of food are being emphasized, biological as well as chemical changes after food processing

Table 1. Comparison of biological activities before and after enzyme treatment.

	Untreated	+Enzyme	+Inactivated enzyme
DPPH ^a	10.5±0.2	11.2±0.2	11.0±1.1
PEP ^b	0.8±0.1	1.0±0.0	1.5±0.2
BACE1 ^c	10.3±0.3	12.3±0.2	9.5±1.1
ACE ^d	15.3±1.0	18.3±1.3	16.3±1.2
APTT ^e	20.3±0.3	19.2±2.0	18.9±0.5
NIH-3T3 ^f	48.5±1.2	52.3±3.1	44.0±2.0
HUVECs ^g	65.0±2.3	65.0±3.0	63.0±0.9

^aAntioxidative effect (scavenging % at 100 ppm).

^{b,c}Antidementia (inhibition % at 40 ppm; PEP, prolyl endopeptidase; BACE1, β -secretase).

^dAntihypertensive effect (inhibition % at 20 ppm; ACE, angiotensin converting enzyme).

^eAnticoagulation effect (inhibition % at 167 ppm; APTT, activated partial thromboplastin times).

^fCytotoxicity to human cell line (survival % at 40 ppm).

^gCytotoxicity for normal cells (survival % at 200 ppm; HUVECs, human umbilical vein endothelial cells). All data are the mean values of duplicated experiments.

have become a hot topic. The enzyme-mediated synthesis or degradation of glycosides has been widely investigated to enhance the physicochemical properties of biologically important compounds such as ascorbic acid and isoflavone glycosides [5, 12]. In our experiments, no significant changes in biological activity of *C. Semen* were recognized (*i.e.*, antioxidative using DPPH [1], antidementia by PEP [14] and β -secretase inhibition [6], anticoagulating activity by APTT (activated partial thromboplastin times) [16], antihypertension by ACE inhibition [3], and cytotoxicity both in tumor (NIH-3T3) [9] and normal (HUVECs) [15] cells), between the before and after treatments of the crude enzyme solution (Table 1). Our results, however, are noteworthy in that aurantio-obtusin has been known to have antimutagenic effect [2], to possess inhibitory activity toward cAMP phosphodiesterase [17], and to direct inhibitory activity on aldose reductase [18]. Only one report was available regarding glucoaurantio-obtusin, describing its antiplatelet aggregation activity [20]. Simple processing techniques are expected to be useful in increasing the content of biologically active substances in Oriental drugs.

Acknowledgments

This work was supported by a grant from the "Traditional Technology Innovation Research Lab. Program," Korea Ministry of Science and Technology. We also extend our gratitude to the Korea Ministry of Commerce, Industry and Energy for their financial support.

REFERENCES

- Blois, M. S. 1985. Antioxidant determination by the use of a stable free radical. *Nature* **181**: 1199–1201.
- Choi, J. S., H. J. Lee, K. Y. Park, J. O. Ha, and S. S. Kang. 1997. *In vitro* antimutagenic effects of anthraquinone aglycones and naphthopyrones glycosides from *Cassia tora*. *Planta Med.* **63**: 11–14.
- Cushman, D. W., E. M. Gordon, F. L. Wang, H. S. Cheung, R. Tung, and N. G. Delaney. 1983. Purification and characterization of enkephalinase, angiotensin converting enzyme, and a third peptidyl dipeptidase from rat brain. *Life Sci.* **33**: 25–28.
- Do, H. J. 2003. Purification and characterization of β -glucosidase produced by *Aspergillus usamii* D5 isolated from traditional fermented food. MS thesis. Kyungpook National University, Daegu, Korea.
- Jeon, K. S., I. K. Hwang, and G. E. Ji. 2002. Assay of β -glucosidase activity of Bifidobacteria and the hydrolysis of isoflavone glycosides by *Bifidobacterium* sp. Int-57 in soymilk fermentation. *J. Microbiol. Biotechnol.* **12**: 8–13.
- Jeon, S. Y., K. Bae, Y. H. Seong, and K. S. Song. 2003. Green tea catechins as a BACE1 (β -secretase) inhibitor. *Bioorg. Med. Chem. Lett.* **13**: 3905–3908.

7. Jeon, S. Y., E. K. Kim, H. M. Kwak, J. Y. Kim, J. H. Lim, S. K. Chung, and K. S. Song. 2004. Changes in chemical composition and biological activities of Oriental crude drugs by food processing techniques (II) - Changes in paeonol contents in roasted Moutan Cortex. *Kor. J. Pharmacogn.* **35**: 388–392.
8. Ju, H. K., B. A. Hwang, S. J. Kang, S. Y. Chang, D. H. Won, J. S. Ro, and K. S. Lee. 2001. Isolation and quantitative analysis of aurantio-obtusin from Cassiae Semen. *Kor. J. Pharmacogn.* **32**: 157–162.
9. Kanamaru, H. and O. Yoshida. 1989. Assessment of *in vitro* lymphokine activated killer (LAK) cell activity against renal cancer cell lines and its suppression by serum factor using crystal violet assay. *Urolog. Res.* **17**: 259–264.
10. Kim, S. I., J. E. Kim, J. H. So, I. K. Rhee, S. K. Chung, K. B. Lee, Y. C. Yoo, and K. S. Song. 2004. Changes in chemical composition and biological activities of Oriental crude drugs by food processing techniques (I) - Changes in liquiritigenin contents in licorice extract treated by the crude enzyme extract from *Aspergillus kawachii*. *Kor. J. Pharmacogn.* **35**: 309–314.
11. Kwak, H. M., J. Y. Kim, J. H. Lim, S. K. Chung, S. H. Kwon, H. H. Jeong, J. M. Hur, and K. S. Song. 2005. Changes in chemical composition and biological activities of Oriental crude drugs by food processing techniques (III) Changes of 5-HMF contents from roasted Asparagi Tuber. *Kor. J. Pharmacogn.* **36**: 235–239.
12. Lee, C. Y., K. H. Kim, S. Y. Hur, J. H. Heo, M. H. Choi, S. K. Rhee, and C. H. Kim. 2006. Enzymatic synthesis of ascorbic acid fructoside by transfructosylation using levan fructotransferase. *J. Microbiol. Biotechnol.* **16**: 64–67.
13. Lee, H. J., J. H. Jung, S. S. Kang, and J. S. Choi. 1997. A rubrofusarin gentiobioside isomer from roasted *Cassia tora*. *Arch. Pharm. Res.* **20**: 513–515.
14. Lee, J. H., S. Y. Lee, K. S. Lee, H. J. Jang, K. H. Lee, T. R. Hahn, and Y. S. Paik. 2004. Prolyl endopeptidase inhibitors from the leaves of *Ginkgo biloba*. *Planta Med.* **70**: 1228–1230.
15. Lin, P. S., K. C. Ho, and S. J. Sung. 1993. Combined treatments of heat, radiation, or cytokines with flavone acetic acid on the growth of cultured endothelial cells. *Int. J. Hyperthermia* **9**: 517–528.
16. Morin, R. J. and D. Willoughby. 1975. Comparison of several activated partial thromboplastin time methods. *Am. J. Clin. Pathol.* **64**: 241–247.
17. Nikaido, T., T. Ohmoto, U. Sankawa, S. Kitanaka, and M. Takido. 1984. Inhibitors of cAMP phosphodiesterase in medicinal plants. VIII. Inhibitors of adenosine 3',5'-cyclic monophosphate phosphodiesterase in Cassia seed. *Chem. Pharm. Bull.* **32**: 3075–3078.
18. Yamaguchi, T., K. Hayashi, M. Takito, and H. Mihashi. 1990. Pharmaceuticals containing anthraquinones as aldose reductase inhibitors. *Jpn. Kokai Tokkyo Koho*, JP 02149515 A2 19900608 Heisei, p. 6.
19. Yen, G. C. and D. Y. Chung. 1999. Antioxidant effects of extracts from *Cassia tora* L. prepared under different degrees of roasting on the oxidative damage to biomolecules. *J. Agric. Food Chem.* **47**: 1326–1332.
20. Yun-Choi, H. S., J. H. Kim, and M. Takido. 1990. Potential inhibitors of platelet aggregation from plant sources, V. Anthraquinones from seeds of *Cassia obtusifolia* and related compounds. *J. Nat. Prod.* **53**: 630–633.
21. Zhang, Q., J. Yin, and J. Zhang. 1996. Comparison of contents of some active components between crude and processed seeds of sickle senna (*Cassia tora*) and their detections by HPLC. *Chinese Herb* **27**: 79–81.