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Isoflavonoids and the Other Constituents in Callus Tissues of *Pueraria lobata*

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Puerarin, daidzin, daidzein, genistein, coumesterol, methyl 2,4-dihydroxybenzoate, a mixture of methyl *p*-hydroxybenzoate and methyl *trans*-*p*-hydroxycinnamate, phytosterols and aliphatic acid esters were isolated from the methanol extract of callus tissues, which were induced from the stem of *Pueraria lobata* Ohwi. The main products at the final stage of callus culture were daidzin and puerarin in the suspension culture and daidzin in the static culture. The quantitative changes of daidzin, puerarin and daidzein during the growth of callus tissues were investigated.

Keywords——*Pueraria lobata*; Leguminosae; tissue culture; isoflavonoid; quantitative change

Pueraria root prepared from *Pueraria lobata* Ohwi or *P. lobata* Ohwi var. *chinensis* Ohwi (Leguminosae) is one of the most important crude drugs in Chinese medicine. The previous studies on the constituents of Pueraria root[1,2] and flower[3-6] showed the presence of various isoflavonoids. Hypothermic, spasmolytic and hypotensive activities of Pueraria root[7-13] have been reported. The present investigation was undertaken to determine the constituents of callus tissues induced from the stem of *P. lobata* and to elucidate the quantitative changes of isoflavonoids during the growth of callus tissues.

The callus tissues are pale yellow in the logarithmic phase and gradually become brown after reaching the stationary phase. They were subcultured at about 4-week intervals. When the methanol extract prepared by homogenizing the callus tissues in methanol was dissolved in water and extracted with chloroform, a large amount of precipitation occurred. A similar precipitate also developed during concentration of the chloroform-extracted solution. Repeated recrystallizations of the precipitates gave a pure compound (V) and a mixture of III, IV and V. The chloroform extract was chromatographed on silica gel to give aliphatic acid esters, phytosterols, compounds I, II, III, IV and V. Also, column chromatography of the water-soluble fraction obtained after the chloroform extraction on Amberlite XAD-2 gave compounds VI and VII. The aliphatic acid esters were established as a mixture of methyl palmitate, methyl linolenate, methyl linoleate and methyl stearate (3:1:1:2), and the phytosterols as a mixture of stigmasterol and β-sitosterol (7:4) by comparing their t_R values on gas chromatography (GC) with those of authentic samples. Compound I was identified as methyl 2,4-dihydroxybenzoate by direct comparison with an authentic sample. Compound II was confirmed to be a mixture of methyl *p*-hydroxybenzoate and methyl *trans*-*p*-hydroxycinnamate by GC–mass spectrum (MS) comparison with authentic samples. Compounds III, V, VI and VII were assumed to be isoflavonoids on the basis of their ultraviolet (UV) spectral pattern and characteristic singlet-signal of the C-2 proton at δ 8.2—8.3 in the ^{1}H-nuclear magnetic resonarce (NMR) spectra,[14,15] and were respectively identified as genistein, daidzein, daidzin and puerarin by direct comparison with authentic samples. The UV spectrum of compound IV was similar to that of flavone or flavonol, but its IR spectrum differed from that of the γ-chromone in having a band at 1700 cm^{-1} (CO) due to the lactone carbonyl group.[16] Compound IV was methylated with diazomethane[17] to give a dimethyl compound. Compound IV was confirmed to be coumesterol by comparing the various spectral and physical data with those in the literature.[18] 3′-Hydroxypuerarin and 3′-methoxypuerarin[19] contained in the original plant were not detectable in the methanol extract of callus tissues on thin layer chromatography (TLC).

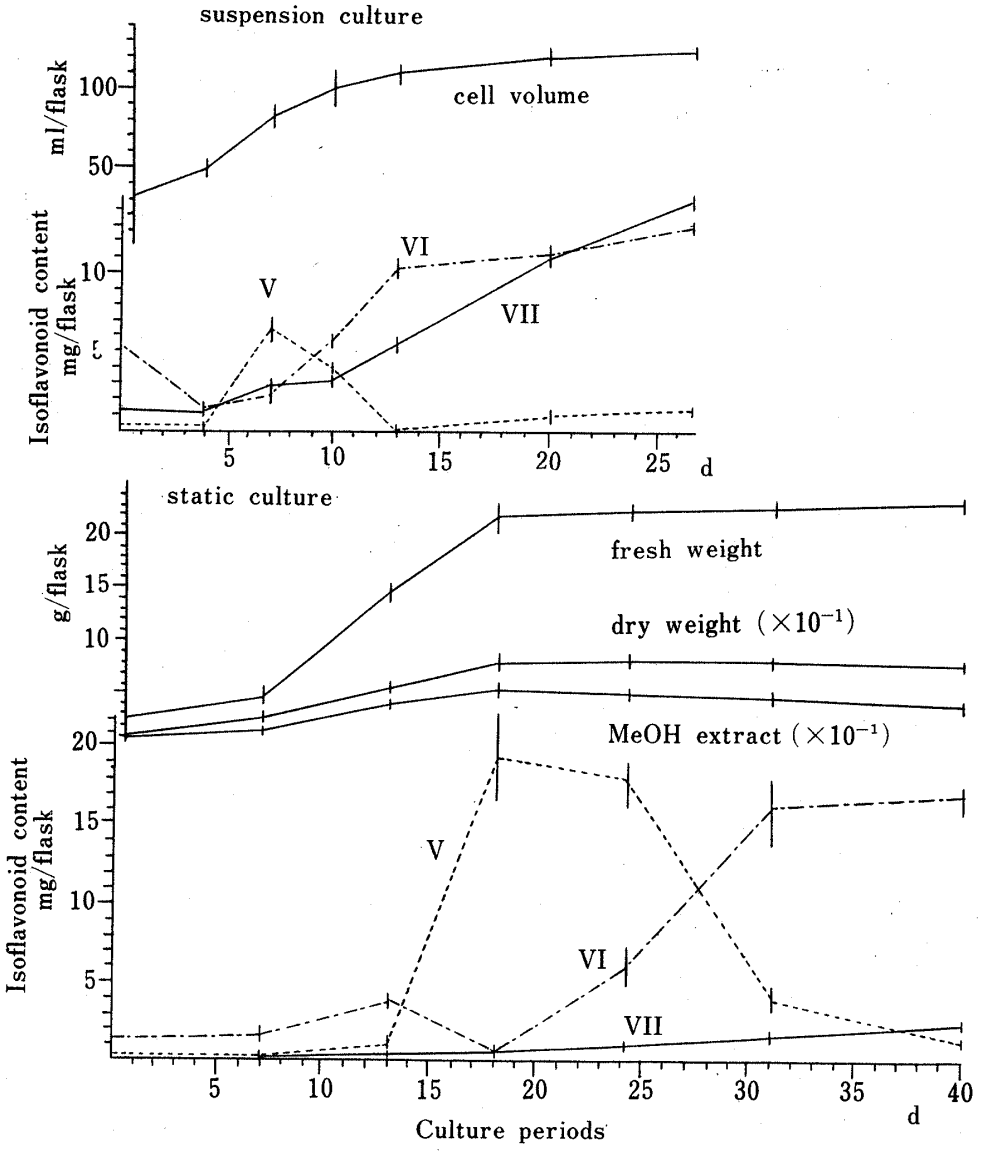

Fig. 1. Quantitative Changes of Daidzein, Daidzin and Puerarin during the Growth of Callus Tissues induced from *Pueraria lobata*

Volume of medium per flask; suspension culture 250 ml.
static culture 40 ml.
The cultured solution was allowed to stand in a measuring cylinder for 6 h, then the volume of precipitated cells was measured as the cell volume.

Next, the quantitative changes of daidzein, daidzin and puerarin during the growth of callus tissues in static and suspension cultures were investigated and the results are shown in Fig. 1. The quantitative analysis[19,20] of isoflavonoids in the callus tissues was carried out using a dual-wavelength TLC scanner at λ_S 250 nm and λ_R 400 nm. The total amount of genistein and coumesterol based on the daidzein was always 6 to 8% at each stage of the callus culture. As can be seen from Fig. 1, the amount of daidzein based on the dry weight at the 18th day in the static culture was 2 to 3%, and the main products at the final stage of callus culture were daidzin and puerarin in the suspension culture and daidzein in the static culture. The amount of daidzein in the callus tissues increased during the logarithmic growth phase and decreased after the stationary growth phase had been reached. The amount of daidzin (daidzein 7-*O*-glucoside) increased in proportion to the decrease of daidzein, but the amount of puerarin (daidzein 8-*C*-glucoside) increased gradually from the initial stage. This finding indicated that *O*- and *C*-glucosidations probably proceed at different stages.[21]

Experimental

Melting points were recorded on a Yanagimoto micro melting point apparatus and are uncorrected. Spectral data were obtained on the following machines; UV on a Shimadzu UV-210, NMR on a JEOL JNM-PS-100 or FX-100, MS on a Hitachi RMU-7L and IR on a Hitachi 260-10. A Yanaco G-80 gas chromatograph equipped with a flame ionization detector and a 1.5% SE-30 glass column (1.5 m×4 mm), and a Shimadzu CS-900 dual-wavelength TLC scanner were employed for analysis.

Tissue Culture——Callus tissues were induced from the stem of *Pueraria lobata* on Murashige and Skoog (MS) medium[23] supplemented with 0.1 ppm of kinetin, 1.0 ppm of 2,4-D, 3% sucrose and 0.8% agar. The callus tissues were subcultured on the same medium as above, changing the medium at about 4-week intervals, at 26°C in the dark for 2 years. The suspension culture was carried out in MS medium supplemented with 0.05 ppm of kinetin, 0.5 ppm of 2,4-D and 3% sucrose, on a rotary shaker (84 rpm) at 27°C in the dark.

Materials——Authentic methyl 2,4-dihydroxybenzoate, methyl *p*-hydroxybenzoate, methyl *trans-p*-hydroxycinnamate, aliphatic acid esters and phytosterols were purchased from Tokyo Kasei Kogyo Co., Ltd., Japan. Authentic daidzein, daidzin and puerarin were isolated from the root of *P. lobata*. Authentic genistein was prepared according to the method of Baker and Ollis.[22]

Extraction and Isolation——The callus tissues (fresh weight 12 kg) were homogenized with methanol and filtered through a Buchner funnel. The filtrate was concentrated to 1 l under reduced pressure. The extracted solution was diluted with 1 l of water and extracted with chloroform in a separatory funnel. This procedure yielded 9.2 g of precipitate. Repeated recrystallizations of the precipitate from methanol-water solution gave 4.9 g of daidzein (V) and 2.7 g of a mixture consisting of genistein (III), coumesterol (IV) and daidzein (V). Upon concentration of the chloroform-extracted solution, 5.6 g of precipitate and 3.0 g of chloroform extract were obtained. The extract was subjected to column chromatography on silica gel to give 439 mg of aliphatic acid esters, 62 mg of phytosterols, 3 mg of methyl 2,4-dihydroxybenzoate (I), 2 mg of a mixture consisting of methyl *p*-hydroxybenzoate (IIa) and methyl *trans-p*-hydroxycinnamate (IIb), 12 mg of genistein (III), 9 mg of coumesterol (IV) and 321 mg of daidzein (V). Water solution (2 l), after extraction with chloroform, was subjected to column chromatography on Amberlite XAD-2. Elution with the solvent system of water-methanol gave 65 mg of daidzin (VI) and 2.5 g of puerarin (VII). The GC conditions for analysis of aliphatic acid esters were as follows; column temperature 170°C, velocity of N_2 carrier gas 32 ml/min. Retention times were as follows: methyl palmitate (t_R 4.6 min), methyl linolenate (8.7), methyl linoleate (9.1) and methyl stearate (10.3). For phytosterols the conditions were as follows: column temp. 260°C, N_2 32 ml/min; stigmasterol (t_R 5.6) and β-sitosterol (6.2). The GC–MS data for II were as follows; *m/z* (%): IIa (t_R 1.3 min), 152 (68, M^+), 121 (100), 92 (29), 65 (25), IIb (8.8 min), 178 (53, M^+), 147 (64), 137 (100), 119 (21), 90 (18), 65 (12). The spectral and physical data for I and III—VII were as follows; (I) mp 120—122°C, UV λ_{max}^{MeOH} nm: 222, 259, 295, NMR ($(CD_3)_2CO$, δ, ppm): 3.90 (3H, s, CH_3), 6.36 (1H, dd, J=2 and 1 Hz, 3-H), 6.43 (1H, dd, J=8 and 2 Hz, 5-H), 7.69 (1H, dd, J=8 and 1 Hz, 6-H), 9.31 (1H, s, 4-OH), 10.89 (1H, s, 2-OH), MS *m/z* (%): 168 (54, M^+), 136 (100), 108 (49), (III) mp 296—298°C, UV λ_{max}^{MeOH} nm: 261, 328 (sh), NMR (DMSO-d_6, δ, ppm): 6.23 (1H, d, J=2 Hz, 6-H), 6.38 (1H, d, J=8 Hz, 8-H), 6.78 (2H, d, J=9 Hz, 3′ and 5′-H), 7.32 (2H, d, J=9 Hz, 2′ and 6′-H), 8.32 (1H, s, 2-H), MS *m/z* (%): 270 (100, M^+), 153 (41), 152 (24), 118 (20), (IV) mp >300°C, UV λ_{max}^{MeOH} nm: 244, 304, 343, $\lambda_{max}^{MeOH+NaOH}$ nm: 245, 272, 311, 377, $\lambda_{max}^{MeOH+NaOAc}$ nm: 244, 264, 311, 364, $\lambda_{max}^{MeOH+AlCl_3}$ nm: 244, 304, 343, IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 1700 (CO), NMR (DMSO-d_6, δ, ppm): 6.86 (1H, d, J=2 Hz, 8-H), 6.90 (2H, dd, J=9 and 2 Hz, 3′ and 6-H), 7.12 (1H, d, J=2 Hz, 5′-H), 7.63 (1H, d, J=9 Hz, 2′-H), 7.80 (1H, d, J=9 Hz, 5-H), MS *m/z* (%): 268 (100, M^+), 240 (15), 134 (27), 120 (17), dimethyl ether of IV: mp 197—198°C, UV λ_{max}^{MeOH} nm: 244, 304, 343, $\lambda_{max}^{MeOH+NaOAc}$ nm: 244, 304, 343, MS *m/z* (%): 296 (100, M^+), 281 (90), 148 (20), (V) mp 325—330°C, UV λ_{max}^{MeOH} nm (log ε): 248 (4.42), 260 (4.03, sh), 304 (1.73, sh), NMR (DMSO-d_6, δ, ppm): 6.79 (2H, d, J=9 Hz, 3′ and 5′-H), 6.83 (1H, d, J=2 Hz, 8-H), 6.90 (1H, dd, J=9 and 2 Hz, 6-H), 7.36 (2H, d, J=9 Hz, 2′ and 6′-H), 7.94 (1H, d, J=9 Hz, 5-H), 8.22 (1H, s, 2-H), MS *m/z* (%): 254 (80, M^+), 137 (100), 118 (54), (VI) mp 240—242°C, UV λ_{max}^{MeOH} nm (log ε): 249 (4.48, sh), 260 (4.93), 304 (1.41, sh), NMR (DMSO-d_6, δ, ppm): 6.78 (2H, d, J=9 Hz, 3′ and 5′-H), 7.12 (1H, dd, J=9 and 2 Hz, 6-H), 7.17 (1H, d, J=2 Hz, 8-H), 7.38 (2H, d, J=9 Hz, 2′ and 6′-H), 8.02 (1H, d, J=9 Hz, 5-H), 8.34 (1H, s, 2-H), 5.12 (1H, d, J=7 Hz, anomeric proton), MS *m/z* (%): 416 (1, M^+), 254 (92), 137 (100), 118 (66), (VII) mp 186—187°C (dec.), UV λ_{max}^{MeOH} nm (log ε): 249 (4.44), 260 (3.78, sh), 304 (1.62, sh), NMR (DMSO-d_6, δ, ppm): 6.78 (2H, d, J=9 Hz, 3′ and 5′-H), 6.97 (1H, d, J=9 Hz, 6-H), 7.36 (2H, d, J=9 Hz, 2′ and 6′-H), 7.90 (1H, d, J=9 Hz, 5-H), 8.29 (1H, s, 2-H), 4.83 (1H, d, J=10 Hz, anomeric proton).

Quantitative Analysis of Isoflavonoids——Plates pre-coated with Silica gel $60F_{254}$ (Merck) were used and developed with the lower-layer of $CHCl_3$–MeOH–H_2O (35 : 65 : 40). After development, the estimation of isoflavonoids in the methanol extract of callus tissues was carried out by using a dual-wavelength TLC scanner with wavelengths of λ_S 250 nm and λ_R 400. The calibration curves of authentic samples were prepared in a similar manner.

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References and Notes

1) S. Shibata, T. Murakami, and Y. Nishikawa, *Yakugaku Zasshi*, **79**, 757 (1959).
2) T. Murakami, Y. Nishikawa, and T. Ando, *Chem. Pharm. Bull.*, **8**, 688 (1960).
3) T. Kurihara and M. Kikuchi, *Yakugaku Zasshi*, **93**, 1201 (1973).
4) T. Kurihara and M. Kikuchi, *Yakugaku Zasshi*, **95**, 1283 (1975).
5) T. Kurihara and M. Kikuchi, *Yakugaku Zasshi*, **96**, 1486 (1976).
6) M. Kubo and K. Fugita, *Phytochemistry*, **12**, 2547 (1973).
7) Y. Tanno, *Nippon Yakurigaku Zasshi*, **33**, 263 (1941).
8) S. Shibata, M. Harada, and T. Murakami, *Yakugaku Zasshi*, **79**, 863 (1959).
9) K. Miura, T. Takeda, Y. Nakamoto, and H. Saito, *Oyo Yakuri*, **5**, 247 (1971).
10) M. Harada and K. Ueno, *Chem. Pharm. Bull.*, **23**, 1798 (1975).
11) H. Nakamoto, Y. Iwasaki, and H. Kizu, *Yakugaku Zasshi*, **97**, 103 (1977).
12) G. Ceng, Y. Zhou, L. Zhang, and L. Fan, *Chinese Medical Journal*, **54**, 265 (1974).
13) X. Lu, S. Chen and T. Sun, *Acta Pharmaceutica Sinica*, **15**, 218 (1980).
14) T.J. Mabry, K.R. Markham, and M.B. Thomas, "The Systematic Identification of Flavonoids," Springer, New York, 1970.
15) J.B. Harborne, T.J. Mabry, "The Flavonoids," Chapman and Hall, London, 1975.
16) E. Wong, *Fortschr. Chem. Organ. Naturstoffe*, **28**, 41 (1971).
17) Edited by A.H. Blatt, "Org. Syn." Coll. Vol. II, 1943, p. 165 and p. 461.
18) E.M. Bickoff, R.L. Lyman, A.L. Livingston, and A.N. Booth, *J. Am. Chem. Soc.*, **80**, 3969 (1958); L. Jurd, *J. Org. Chem.*, **24**, 1786 (1959); H.N. Khastgir, P.C. Duttagupta, and P. Sengupta, *Tetrahedron*, **14**, 275 (1961); A.L. Livingston, E.M. Bickoff, R.E. Lundin, and L. Jurd, *Tetrahedron*, **20**, 1963 (1964).
19) "Experimental Methods of Natural Organic Products," edited by S. Natori, N. Ikegawa, and M. Suzuki, Kodansha, Japan, 1977, pp. 467—472.
20) Y. Akada, S. Kawano, and M. Yamagishi, *Yakugaku Zasshi*, **100**, 1057 (1980).
21) J.W. Wallace, J.T. Mabry, and R.E. Alston, *Phytochemistry*, **8**, 93 (1969); J.W. Wallace and H. Grisebach, *Biochim. Biophys. Acta*, **304**, 837 (1973).
22) W. Baker, J. Chadderton, J.B. Harborne, and W.D. Ollis, *J. Chem. Soc.*, **1953**, 1852.
23) T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).