Isolation of the Pharmacologically Active Saponin Ginsenoside Rb1 from Ginseng by Immunoaffinity Column Chromatography

Noriko Fukuda, Hiroyuki Tanaka, and Yukihiro Shoyama*

Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Received July 16, 1999

Immunoaffinity column chromatography using an anti-ginsenoside Rb1 monoclonal antibody has made possible a single-step separation of ginsenoside Rb1 from a crude extract of ginseng roots (*Panax ginseng*). The combination of immunoaffinity column chromatography and an enzyme-linked immunosorbent assay (ELISA) was also investigated.

Ginseng root [*Panax ginseng* C. A. Meyer (Araliaceae)] is one of the most important oriental medicines and is used worldwide to combat stress and disturbances of the central nervous system,[1,2] for hypothermia,[3] for its antioxidant and organ-protective actions,[4] and for radio-protection.[5,6] Ginseng root contains dammarane and oleanane saponins,[7,8] polyacetylene derivatives,[9] and polysaccharides,[10] of which the biological activities have been studied widely. A major ginsenoside, ginsenoside Rb1, has been investigated for its effects on the central nervous system.[11−14] More recently, Chang et al.[15] reported the effect of ginsenoside Rb1 on drug-induced memory impairment.

An immunological approach for assaying quantities of ginsenosides using a polyclonal antibody has been investigated by Sankawa et al.[16] However, no monoclonal antibodies related to ginsenoside Rb1 have been established yet. In our current investigation on the formation of monoclonal antibodies against naturally occurring bioactive compounds, we have reported previously the preparation of a monoclonal antibody against ginsenoside Rb1 and its characterization,[17] and a western blotting procedure using anti-ginsenoside Rb1 monoclonal antibody.[18] Herein we describe an immunoaffinity column chromatography procedure for ginsenoside Rb1 and its application in a single-step isolation of this component from a crude extract of ginseng root.

Because ginseng root contains a number of dammarane-type ginsenosides together with oleanane-type saponins, the isolation of individual saponins is quite tedious, requiring repeated Si gel column chromatography or preparative HPLC. To avoid this, we have established a simple and reproducible purification method for ginsenoside Rb1 using an immunoaffinity column conjugated with an anti-ginsenoside Rb1 monoclonal antibody.

The recovery of 400 μg of ginsenoside Rb1 was determined by an ELISA using various buffer solutions. The ginsenoside Rb1 concentration increased somewhat by eluting with a 20 mM phosphate buffer containing 0.5 M KSCN and 10% CH_3OH. When the 20 mM phosphate buffer was changed to 100 mM HOAc buffer (pH 4), the elution ability reached the optimal level. Although 20% CH_3OH enhanced the elution of ginsenoside Rb1, higher concentrations were ineffective in this regard. From these results, 100 mM HOAc buffer containing 0.5 M KSCN and 20% CH_3OH could be used routinely as an elution buffer solution.

* To whom correspondence should be addressed at the Graduate School of Pharmaceutical Sciences. Tel. and Fax: 81-92-642-6580. E-mail: shoyama@shoyaku.phar.kyushu-u.ac.jp.

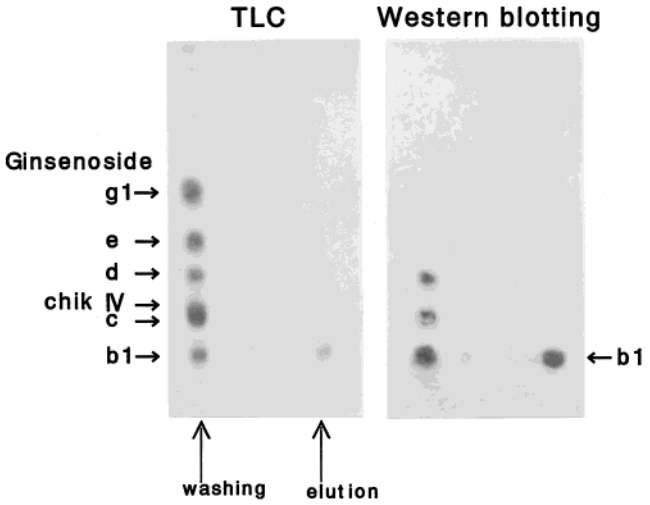

Figure 1. TLC and western blotting profiles for a standardized ginsenosides mixture (chik IV = chikusetsusaponin IV). The TLC plate and the western blotting membrane were stained by H_2SO_4 and incubated with anti-ginsenoside Rb1 monoclonal antibody, respectively, as indicated in the Experimental Section.

In a preliminary trial, a ginsenoside mixture containing ginsenosides Rg1, Re, Rd, Rc, and Rb1 and chikusetsusaponin IV (chik IV; an oleanane-type saponin), were separated by the newly prepared immunoaffinity column using the separation solvent system. Figure 1 shows the TLC and western blotting profiles of the ginsenosides and chik IV separated using this method. When the column was washed with 20 mM phosphate buffer containing 0.5 M NaCl (40 mL), ginsenosides Rg1, Re, Rd, Rc; chik IV; and overcharged ginsenoside Rb1 appeared. After these compounds were washed out, the combined ginsenoside Rb1 was eluted using 100 mM HOAc buffer containing 0.5 M KSCN and 20% CH_3OH.

A crude extract of *P. ginseng* roots was loaded onto the immunoaffinity column and washed with the washing solvent. Figure 2 shows the fractions 1−8 containing overcharged ginsenoside Rb1, which was determined by ELISA. Ginsenosides Rc, Rd, Re and, Rg1 were also detected in these fractions by the western blotting procedure (data not shown). A sharp peak appeared around fractions 20−24, which contained ginsenoside Rb1. However, ginsenoside Rb1 purified by the immunoaffinity column was still contaminated by a small amount of malonyl ginsenoside Rb1,[19] as detected by western blotting. This compound has almost the same cross-reactivity with ginsenoside Rb1 as indicated previously.[17] Therefore, the

10.1021/np990356s CCC: $19.00
Published on Web 01/28/2000

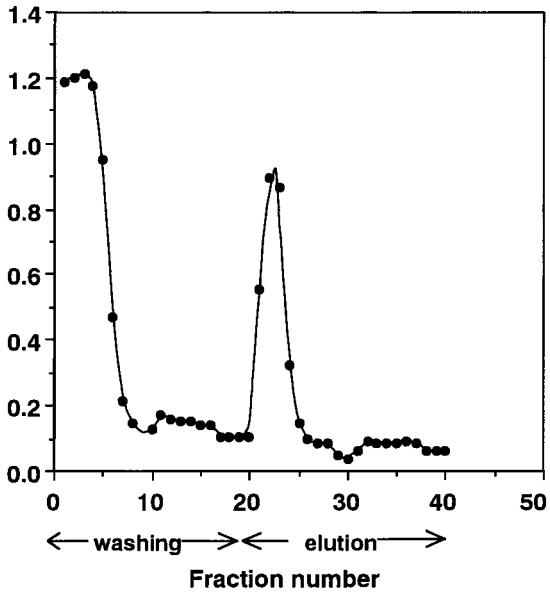

Figure 2. Elution profile of a crude extract of *Panax ginseng* on an immunoaffinity column. The column was washed by phosphate buffer and then eluted by 100 mM HOAc buffer containing 0.5 M KSCN and 20% MeOH (pH 4.0). Individual fractions (2 mL) were assayed by ELISA.

mixture was treated with a mild alkaline solution at room temperature for 1 h, as previously reported,[19] to give pure ginsenoside Rb1. Overcharged ginsenoside Rb1, eluted with washing solution, was repeatedly loaded and finally isolated in pure form. The antibody was stable when exposed to the eluent, and the immunoaffinity column showed almost no decrease in capacity (20 μg/mL gel) after repeated use more than 10 times under the same conditions, as was reported for a single-step separation of forskolin from a crude extract of *Coleus forskohlii* root.[20]

This methodology is effective for the rapid and simple purification of ginsenoside Rb1 and may open up a wide field of comparable studies with other families of saponins for which an acceptable method for single-step separation has not been developed. Therefore, to separate the total ginseng saponins based on panaxatriol or panaxadiol as the aglycon, a wide cross-reactive monoclonal antibody against ginsenosides could be designed, as was done for the total solasodine glycosides by an immunoaffinity column using an anti-solamargine monoclonal antibody.[21] A combination of immunoaffinity column chromatography, western blotting, and ELISA could be used to survey low concentrations of ginsenoside Rb1 of plant origin and/or in experimental animals and humans. We have succeeded in the isolation of ginsenoside Rb1 from a different plant, *Kalopanax pictus* Nakai, which was not known previously to contain ginsenosides, using this combination of methods (data not shown). This combination procedure has also been used in a breeding program of *P. ginseng* using tissue culture techniques,[22,23] resulting in a plant yielding higher ginsenoside Rb1 concentrations.

Experimental Section

Chemicals and Immunochemicals. Ginsenosides Rb1, Rc, Rd, Re, and Rg1 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and human serum albumin (HSA) were obtained from Pierce Chemical Co. (Rockford, IL). Peroxidase-labeled anti-mouse IgG was provided by Organon Teknika Cappel Products (West Chester, PA). Polyvinylidene difluoride (PVDF) membranes (Immobilon-N) were purchased from Millipore Corporation (Bedford, MA). Glass microfiber filter sheets (GF/A) were purchased from Whatman International Ltd. (Maidstone, U.K.). Ginsenoside Rb1-carrier protein conjugates were synthesized by a procedure used previously for solamargine.[24] The roots of *Panax ginseng* were obtained from Tochimototenn-kaido Corporation (Osaka, Japan). A voucher specimen of the root is deposited (no. 990225) at the herbarium of Graduate School of Pharmaceutical Sciences, Kyushu University. All other chemicals were standard commercial products of analytical grade.

Extraction of Ginseng Roots. Dried samples (50 mg) of ginseng roots were powdered and extracted five times with CH_3OH (2.5 mL) under sonication, then filtered, and the combined extract (3.8 mg) diluted with 20% CH_3OH.

Purification of Monoclonal Antibody. A monoclonal antibody prevent as previously reported[18] was purified using a Protein G FF column (0.46 × 11 cm, Pharmacia Biotech, Uppsala, Sweden). The cultured medium (500 mL) containing the IgG was filtered by a MILLEX-HV filter (0.45 μm filter unit, Millipore), and the adsorbed IgG was eluted with 100 mM citrate buffer (pH 3). The eluted IgG solution was neutralized with 1 M Tris solution, then dialyzed against H_2O, and finally lyophilized to give IgG.

Confirmation of the Purity of the Monoclonal Antibody by Matrix-Assisted Laser Desorption Ionization-Time-Of-Flight (MALDI-TOF) Mass Spectrometry. A small amount of the purified monoclonal antibody and BSA, used as an internal standard, was mixed with a 10^3-fold molar excess of sinapic acid in an aqueous solution containing 10% trifluoroacetic acid. The mixture was subjected to a JMS time-of-flight (TOF) mass monitor and irradiated with an N_2 laser (337 nm, 150 ns pulse). The ions formed by each pulse were accelerated by a 20-kV potential into a 2.0-m evacuated tube and were detected using a compatible computer.

ELISA Procedure. Ginsenoside Rb_1−HSA (5 molecules of ginsenoside Rb1 per molecule of HSA) (100 μL 1 μg/mL) was adsorbed to the wells of a 96-well immunoplate (NUNC, Roskilde, Denmark), then it was treated with 300 μL of phosphate buffered saline (PBS) 5% skim milk for 1 h to reduce nonspecific adsorption. Fifty milliliters of various concentrations of ginsenoside Rb1 dissolved in 20% of CH_3OH was incubated with the same volume of IgG solution (IgG: 0.418 μg/mL) for 1 h. The plate was washed three times with PBS containing 0.05% Tween 20 (TPBS), and then the monoclonal antibody was mixed with 100 μL of a 1:1000 dilution of peroxidase-labeled anti-mouse IgG for 1 h. After washing the plate three times with TPBS, 100 μL of substrate solution were added to each well and incubated for 15 min. Absorbance was measured at 405 nm.

Western Blotting of Ginsenosides. Ginsenosides were applied to a TLC plate and developed with *n*-BuOH−EtOAc−H_2O (15:1:4). The developed TLC plate was dried and then sprayed with a blotting solution mixture of *i*-PrOH−MeOH−H_2O (1:4:16). This TLC plate was placed on a stainless steel plate, then covered with a PVDF membrane sheet. After covering with a glass microfiber filter sheet, the whole was pressed for 50 s with a 120 °C hot plate as previously described.[18] The PVDF membrane was separated from the plate and dried. The blotted PVDF membrane was dipped in water containing $NaIO_4$ (10 mg/mL) under stirring at room temperature for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing gelatin (1%) was added and stirred at room temperature for 3 h. The PVDF membrane was washed with TPBS for 5 min twice. Then, the PVDF membrane was immersed in anti-ginsenoside Rb1 monoclonal antibody and stirred at room temperature for 1 h. After washing the PVDF membrane twice with TPBS and water, a 1000-times dilution of peroxidase-labeled goat anti-mouse IgG in PBS containing gelatin (0.2%) was added and stirred at room temperature for 1 h. The PVDF membrane was washed twice with TPBS and water, then exposed to 1 mg/mL of 4-chloro-1-naphthol−0.03% H_2O_2 in PBS solution, which was freshly prepared 10 min before use at room temperature.

The reaction was stopped by washing with water. The immunostained PVDF membrane was left to dry.

Preparation of an Immunoaffinity Column for Ginsenoside Rb1 Using an Anti-ginsenoside Rb1 Monoclonal Antibody. Purified IgG (10 mg) in Bio-Rad Affi-Gel Hz coupling buffer diluted was dialyzed against the coupling buffer two times. One hundred microliters of $NaIO_4$ solution (25 mg/1.2 mL H_2O) was added to the IgG solution (1 mL) and stirred gently at room temperature in the dark for 1 h. After the reaction, glycerol was added to the reaction mixture for 20 mM and stirred for 10 min for the inactivation of $NaIO_4$, then dialyzed against the coupling buffer. After the Affi-Gel Hz Hydrazide gel (3 mL) (Bio-Rad) was washed with the coupling buffer, the buffer was removed. IgG, dissolved in the coupling buffer (5 mL), was added to these gels and stirred gently at room temperature for 24 h. The immunoaffinity gel was packed into a plastic mini-column in volumes of 3 mL. Columns were washed with 20 mM of phosphate buffer containing 0.5 M NaCl (pH 7.0). The washing solvent was removed to measure unbound protein by direct ELISA, as previously reported[17] for determination of coupling efficiency. The immunoaffinity gel was washed with PBS until the ELISA value was equal to the background. The column was stored at 4 °C in PBS containing 0.02% sodium azide.

Determination of Adsorption and Elution Conditions and Capacity for the Immunoaffinity Column. Ginsenoside Rb1 (400 μg) was dissolved in PBS (3.5 mL) and loaded. After the column was incubated at 4 °C overnight, it was washed with PBS until the ginsenoside Rb1 disappeared by analyzing by ELISA. The immunoaffinity column loaded with ginsenoside Rb1 was eluted with various solvent systems, and ginsenoside Rb1 contents in individual fractions (10 mL each) were determined by ELISA and western blotting.

The ginsenoside Rb1-loaded immunoaffinity column was eluted with 100 mM HOAc buffer containing 0.5 M KSCN and 20% MeOH (pH 4.0), and the total ginsenoside Rb1 content was analyzed by ELISA to determine its capacity.

Purification of Ginsenoside Rb1 from a Crude Extract of the Roots of *P. ginseng* by Immunoaffinity Column Chromatography. An extract (3.8 mg) of ginseng roots was redissolved in PBS and then filtered using a MILLEX-HV filter (0.45 μm, Millipore) to remove insoluble portions. The filtrate was loaded on the immunoaffinity column and allowed to stand overnight at 4 °C. The column was washed with the washing buffer solution (40 mL), and then eluted with 100 mM HOAc buffer containing 0.5 M KSCN and 20% MeOH (pH 4.0, 20 mL each). The ginsenoside Rb1-containing fraction was concentrated and surveyed by TLC, developed with *n*-BuOH−EtOAc−H_2O (15:1:4), followed by western blotting.

Ginsenoside Rb1 contaminated with malonyl ginsenoside Rb1 was treated with 0.1% of KOH in MeOH at room temperature for 1 h to give pure ginsenoside Rb1.

Acknowledgment. This research was supported by a Grant-in-Aid for Scientific Research, Ministry of Education, Sciences and Culture (11470470, Japan), a the Sasakawa Scientific Research Grant from The Japan Science Society, and the Shorai Foundation for Science and Technology.

References and Notes

(1) Abe, K.; Cho, S. I.; Kitagawa, I.; Nishiyama, N.; Saito, H. *Brain Res.* **1994**, *649*, 7−11.
(2) Jie, Y. H.; Cammisuli, S.; Baggiolini, M. *Agents Actions* **1984**, *15*, 386−391.
(3) Fulder, S. J. *Am. J. Chin. Med.* **1981**, *9*, 112−118.
(4) Gillis, C. N. *Biochem. Pharmacol.* **1997**, *54*, 1−8.
(5) Tanaka, Y.; Akagi, K. *Metab. Dis.* **1992**, 29, 423−429.
(6) Shibata, S. *J. Trad. Sin.-Jpn. Med.* **1982**, *3*, 62−69.
(7) Besso, H.; Kasai, R.; Saruwatari, Y.; Fuwa, T.; Tanaka, O. *Chem. Pharm. Bull.* **1982**, *30*, 2380−2385.
(8) Koizumi, H.; Sanada, S.; Ida, Y.; Shoji, J. *Chem. Pharm. Bull.* **1982**, *30*, 2393−2398.
(9) Hansen, L.; Boll, P. M. *Phytochemistry* **1986**, *25*, 285−293.
(10) Tomoda, M.; Hirabayashi, K.; Shimizu, N.; Gonda, R.; Ohara, N.; Takada, K. *Chem. Pharm. Bull.* **1993**, *16*, 1087−1090.
(11) Saito, H.; Suda, M.; Schwab, M.; Thoenen, H. *Jpn. J. Pharmacol.* **1977**, *27*, 445−451.
(12) Takemoto, Y.; Ueyama, T.; Saito, H.; Horio, S.; Sanada, S.; Shoji, J.; Yahara, S.; Tanaka, O.; Shibata, S. *Chem. Pharm. Bull.* **1984**, *32*, 3128−3133.
(13) Saito, H. In *Recent Advances in Ginseng Studies*; Shibata, S., Otsuka, Y., Saito, H., Eds.; Hirokawa Publishing Company: Tokyo, 1989; pp 99−111.
(14) Himi, T.; Saito, H.; Nishizawa, N. *Chem. Pharm. Bull.* **1989**, *37*, 481−484.
(15) Chang, Y. S.; Wu, C. R.; Ho, Y. L.; Hsieh, M. T. In *Advances in Ginseng Research*; Huh, H., Ed.; The Korean Society of Ginseng: Seoul, 1998; pp 289−299.
(16) Sankawa, U.; Sung, C. K.; Han, B. H.; Akiyama, T.; Kawashima, K. *Chem. Pharm. Bull.* **1982**, *30*, 1907−1910.
(17) Tanaka, H.; Fukuda, N.; Shoyama, Y. *Cytotechnology* **1999**, *29*, 115−120.
(18) Fukuda, N.; Tanaka, H.; Shoyama, Y. *Biol. Pharm. Bull.* **1999**, *22*, 219−220.
(19) Kitagawa, I.; Taniyama, T.; Yoshikawa, M.; Ikenishi, Y.; Nakagawa, Y. *Chem. Pharm. Bull.* **1989**, *37*, 2961−2970.
(20) Yanagihara, H.; Sakata, R.; Minami, H., Tanaka, H.; Shoyama, Y.; Murakami, H. *Anal. Chim. Acta* **1996**, *335*, 63−70.
(21) Putalun, W.; Tanaka, H.; Shoyama, Y. *Cytotechnology* **1999**, 31, 151−156.
(22) Shoyama, Y.; Matsushita, H.; Zhu, X. X.; Kishira, H. In *Biotechnology in Agriculture and Forestry*; Bajaj, Y. P. S., Ed.; Springer-Verlag: Berlin, 1995; Vol. 31, pp 343−356.
(23) Shoyama, Y.; Zhu, X. X.; Nakai, R.; Shiraishi, S. *Plant Cell Rep.* **1997**, *16*, 450−453.

NP990356S