

Quality Control of *Panax* Species by Monoclonal Antibody and DNA Analysis

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1. Introduction

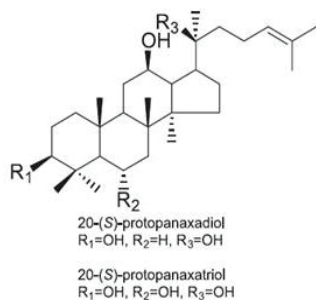
Almost all *Panax* spp. (family Araliaceae) have been used in folk medicine. The most famous variety is *P. ginseng*, which was recorded in Chinese Materia Medica 2000 years ago. It is used to enhance stamina and capacity to cope with fatigue and physical stress, and in tonics against cancers, disturbances of the central nervous system (memory, learning, and behavior), hypothermia, carbohydrate and lipid metabolism, immune function, the cardiovascular system and radioprotection. Meanwhile, *P. notoginseng*, cultivated extensively in Kuming province in China, is also an important crude drug used as an astringent and tonic. *P. japonicus* grows mainly in Japan and China and is used as a stomachic and a hair-growth tonic in Japan. In ginseng markets worldwide, *P. quinquefolius* usually commands a much higher price than *P. ginseng*. Since the roots of these ginseng products are similar in appearance and many commercial ginseng products are in the form of a powder or shredded slices, identification of the origins of ginseng products is not an easy task. Authentication of the sources of ginseng and ginseng products based on scientifically profiling has aroused much interest.

Ginsenosides (ginseng saponins) are known to be bioactive components of ginseng. According to the difference in aglycon in these saponins, ginsenosides are classified into three types: the 20(S)-protopanaxadiol type [e.g., ginsenosides Rb1 (G-Rb1), -Rc (G-Rc), -Rb2 (G-Rb2), -Rd (G-Rd); malonylginsenosides Rb1 (MG-Rb1), -Rb2 (MG-Rb2) and -Rc (MG-Rc)], the 20(S)-protopanaxatriol type [e.g., ginsenosides Rg1 (G-Rg1), -Rf (G-Rf), and -Re (G-Re)], and the oleanolic acid type [e.g., ginsenoside Ro (G-Ro)] (see Figure 1). Ginsenosides have been shown to affect various biological processes: e.g., tumor metastasis and anti-diabetes effects (Wu *et al.*, 1992; Newman *et al.*, 1992), the central nervous system (Kim *et al.*, 1990; Kim *et al.*, 1996; Tokuyama *et al.*, 1996), and retardation of the aging process (Metori *et al.*, 1997). It is well known that the content of ginsenosides varies in ginseng root or root extract depends on the method of extraction, subsequent treatment, or even the season of collection (Kim *et al.*, 1981); therefore, standardization of quality is essential.

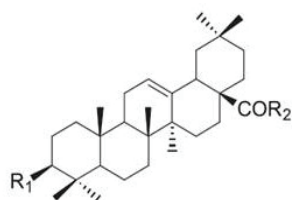
Many analytical approaches have been used to identify ginsenosides in ginseng extract (Sticher and Soldati, 1979; Soldati and Sticher, 1980; Tani *et al.*, 1981; Kitagawa *et al.*, 1987; Yamaguchi *et al.*, 1988; Samukawa *et al.*, 1995; Wang *et al.*, 1999; Chan *et al.*, 2000; Li *et al.*, 2000). We have prepared monoclonal antibodies (MAbs) against major active compounds of *P. ginseng*, G-Rb1 (Tanaka *et al.*, 1999) and G-Rg1 (Fukuda *et al.*, 2000a), and established an

ELISA and an immunoaffinity concentration for ginsenosides (Fukuda *et al.*, 1999; Fukuda *et al.*, 2000b; Fukuda *et al.*, 2001); however, no formation of MAb against the crude drug of *P. ginseng* has been reported, although immunological approaches for analyses of *P. ginseng* using a polyclonal antibody against *P. ginseng* have been investigated (Kitagawa *et al.*, 1996). The present study describes the establishment of two MAbs against *P. ginseng* protein component, and the application of an ELISA for the qualitative analysis and classification of *Panax* spp. using the anti-*P. ginseng* MAbs.

Previously, we used RAPD analysis for the homogeneity of regenerated *Panax* plants in vitro (Shoyama *et al.*, 1995); however, we have not yet succeeded in distinguishing individual *Panax* spp. using their crude drugs. Therefore, we planned to perform a combination of RAPD and new double staining for ginsenosides by Eastern blotting using anti-G-Rb1, -Rg1 MAbs and to standardize the quality of ginseng.



Aglycone	Ginsenoside	R1	R2	R3
Protopanaxadiol	G-Rb1	-O-Glc[2->1]Glc	H	-O-Glc[6->1]Glc
	G-Rb2	-O-Glc[2->1]Glc	H	-O-Glc[6->1]Ara(p)
	G-Rc	-O-Glc[2->1]Glc	H	-O-Glc[6->1]Ara(f)
	G-Rd	-O-Glc[2->1]Glc	H	-O-Glc
	MG-Rb1	-O-Glc[2->1]Glc[6->]Ma	H	-O-Glc[6->1]Glc
	MG-Rb2	-O-Glc[2->1]Glc[6->]Ma	H	-O-Glc[6->1]Ara(p)
	MG-Rc	-O-Glc[2->1]Glc[6->]Ma	H	-O-Glc[6->1]Ara(f)
Protopanaxatriol	G-Rg1	OH	-O-Glc	-O-Glc
	G-Rh1	OH	-O-Glc	OH
	G-Re	OH	-O-Glc[2->1]Rha	-O-Glc
	G-Rf	OH	-O-Glc[2->1]Glc	OH
	20-Gluco-G-Rf	OH	-O-Glc[2->1]Glc	-O-Glc



Aglycone	Ginsenoside	R1	R2
Oleanolic acid	G-Ro	-O-GlcA[2->1]Glc	-O-Glc

Glc, glucose; Ma, malonyl; Ara (p), arabinose in pyranose form; Ara (f), arabinose in furanose form; Rha, rhamnose.

Fig. 1. Structures of ginsenosides

2. Experimental

2.1 Instrumentation

Microtiter plates (Maxisorp) were purchased from Nalge Nunc (NY). The microplate reader was Model 450 (Bio-Rad Laboratories, CA). The PCR thermal cycler was a 1605 Air Thermo-Cycler (Idaho Technology, UT).

2.2 Chemicals and immunochemicals

Five kinds of commercial ginseng roots and other crude drugs were obtained from Nakai Koshindo (Kobe, Japan). Standards of ginsenosides were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and human serum albumin (HSA) were provided by Pierce (Rockford, IL). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corporation (Bedford, MA). A glass microfiber filter sheet (GF/A) was purchased from Whatman International Ltd. (Maidstone, UK). Peroxidase (POD)-labeled goat IgG fraction to mouse IgG was provided by ICN Pharmaceuticals Inc. (Aurora, OH). All other chemicals were standard commercial products of analytical grade.

2.3 Immunization and hybridization

Powdered white ginseng (*P. ginseng*) was suspended in phosphate-buffered saline (PBS), and extracted under sonication for 2 min. The white ginseng water-soluble fraction (WSF) obtained was immediately used as the immunogen. Mice were immunized by a protocol modified from Kitagawa (Kitagawa *et al.*, 1996). First, BALB/c male mice were injected intraperitoneally with an initial injection of 500 µg WSF in PBS emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, MI). Two boosts of 500 µg WSF in Freund's incomplete adjuvant (Difco Laboratories, MI) were performed intraperitoneally at intervals of 2 weeks. The final immunization (1 mg) was injected as a PBS solution. On the third day after the final immunization, splenocytes were isolated and fused with a hypoxanthine-thymidine-aminopterin (HAT)-sensitive mouse myeloma cell line, P3-X63-Ag8-U1, by the polyethylene glycol (PEG) method (Galfre and Milstein, 1981). Hybridoma-producing MAb reactive to *P. ginseng* WSF were selected in enriched RPMI 1640-Dulbecco's-Ham's F12 (eRDF; Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) medium containing HAT, and cloned twice by the limited dilution method (Goding, 1980).

Another hybridoma-producing MAb reactive to *P. ginseng* was prepared using red ginseng (*P. ginseng*) WSF as an immunogen by the above procedure.

2.4 ELISA using MAbs against ginseng

Dried samples (2.0 mg) of various ginseng and other crude drugs were powdered, suspended in 50 mM carbonate buffer at pH 9.6 (1.0 ml) under sonication for 10 min. The suspensions of ginseng WSF were diluted with 50 mM carbonate buffer at pH 9.6 and then assayed using ELISA.

Reactivities of MAbs to various kinds of ginseng WSFs were determined by ELISA. The wells of a 96 well-immunoplate were coated with a suspension of 100 µl/well of various concentrations of ginseng WSF for 1 hr. The plate was washed three times with PBS containing 0.05% Tween 20 (TPBS), and then treated with 300 µl PBS containing 5% skim milk (SPBS) for 1 hr to reduce non-specific adsorption. The plate was again washed three times with TPBS and reacted with 100 µl test MAb for 1 hr. Again, the plate was washed three times with TPBS, and then incubated with 100 µl of 1,000 times dilution of POD-labeled goat IgG fraction to mouse IgG for 1 hr. After washing a further three times with TPBS, 100 µl substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H₂O₂ and 0.3 mg/ml of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)] was added to each well and incubated for 20 min. Absorbance was measured by a microplate reader at 405 nm. All reactions were carried out at 37 °C.

The cross-reactivities (CR %) of various ginseng and other crude drugs were determined as follows:

$$\text{CR}\% = \frac{A - A_o}{A_{\text{PG}} - A_o} \times 100$$

where A is the absorbance of the test crude drug, A_{PG} is the absorbance of white ginseng, and A_o is the absorbance of the blank.

2.5 Western blot analyses of *P. ginseng* WSFs

Western blot analysis was carried out according to a reported method (essentially the method of Towbin) (Towbin *et al.*, 1979). Reduced protein WSFs contained in suspensions of various ginseng (20 μg) in electrophoresis sample buffer were separated by SDS-polyacrylamide electrophoresis (SDS-PAGE) (Laemmli, 1970) on 10% polyacrylamide gel, and electroblotted onto a PVDF membrane (Immobilon-P), which was then incubated with MAb to *P. ginseng* for 3 hr. The membrane was then incubated with 1,000 times dilution of POD-labeled goat IgG fraction to mouse IgG in PBS containing 0.2% gelatin (GPBS) for 1 hr. The POD-labeled secondary antibody bound to the membrane was then developed to blue bands with a substrate solution (4-chloro-1-naphthol soln.).

2.6 Competitive ELISA using anti-G-Rb1, -Rg1 MAbs

Dried samples (50 mg) of various ginseng were powdered and extracted with MeOH (5.0 ml) under sonication five times. The combined extract was filtered and then evaporated to dryness. To eliminate the malonyl group from malonylated ginsenosides, the extracts were treated with 0.1% KOH in MeOH at room temperature for 1 hr. The combined extracts were diluted with 20% methanol suitable for competitive ELISA.

G-Rb1-HSA (100 μl , 1 $\mu\text{g}/\text{ml}$) dissolved in 50 mM carbonate buffer (pH 9.6) was adsorbed to the wells of a 96 well-immunoplate, and then treated with 300 μl SPBS for 1 hr to reduce non-specific adsorption. Fifty microliters of various concentrations of G-Rb1 or samples dissolved with 20% methanol were incubated with 50 μl MAb solution (83 $\mu\text{g}/\text{ml}$) for 1 hr. The plate was washed three times with PBS containing TPBS, and then incubated with 100 μl of 1,000 times dilution of POD-labeled goat IgG fraction to mouse IgG for 1 hr. After washing the plate three times with TPBS, 100 μl substrate solution (ABTS soln.) was added to each well and incubated for 15 min. The absorbance was measured by a microplate reader at 405 nm. Quantitative analysis of G-Rg1 was performed by a similar competitive ELISA procedure as above.

2.7 Double-stained Eastern blotting

Ginsenosides and the extract of various ginseng were applied to TLC plates and developed with *n*-BuOH-EtOAc-H₂O (15:1:4). One developed TLC plate was dried and sprayed with H₂SO₄. Another TLC plate was dried and then sprayed with blotting solution mixture of isopropanol-methanol-H₂O (1:4:16, by volume). It was placed on a stainless steel plate and then covered with a PVDF membrane sheet. After covering with a glass microfiber filter sheet, it was pressed evenly for 50 s with a 120°C hot plate as previously described with modification (Fukuda *et al.*, 2000b). The PVDF membrane was separated from the plate and dried. The blotted PVDF membrane was dipped in water containing NaIO₄ (10 mg/ml) under stirring at room temperature for 1 hr. After washing with water, 50 mM carbonate buffer solution containing BSA was added and stirred for 3 hr. The PVDF membrane was washed with TPBS for 5 min twice and then washed with water. The PVDF membrane was immersed in anti-G-Rb1 MAb and stirred at room temperature for 1 hr. After washing the

PVDF membrane twice with TPBS and water, 1,000 times dilution of POD-labeled goat IgG fraction to mouse IgG in GPBS was added and stirred at room temperature for 1 hr. The POD-labeled secondary antibody bound to the membrane was then developed to blue bands with a substrate solution (4-chloro-1-naphthol soln.).

In the experiment of the immunocytolocalization of G-Rb1, a sliced fresh *P. ginseng* root was placed on the PVDF membrane and they were pressed together evenly for 6 hr. The blotted PVDF membrane was stained using the same procedure as described for the Eastern blotting method.

For staining with anti-G-Rg1 MAb, the blotted PVDF membrane was treated in the same way as anti-G-Rb1 MAb except that it was exposed to 2 mg of 3-amino-9-ethylcarbazole in 10 ml of 50 mM acetate buffer (pH 5.0) containing 0.03% H₂O₂ and 5% *N,N*-dimethylformamide.

2.8 RAPD analysis

DNA for RAPD analysis was extracted from the crude drug powder (100 mg) by the method of Murray and Thompson (1980) with some modification (Nakai *et al.*, 1996). The obtained crude DNA was further purified by the GeneClean II kit (Bio 101, Illkirch, France) as follows: to the DNA solution, 6 M NaI at three times the volume of the DNA solution, plus Glass milk (5 µl) were added and mixed using a vortex mixer. The mixture was incubated at 0 °C for 10 min, with mixing every 1 min. After 10 min incubation, the mixture was centrifuged at 6,000 rpm at 2 °C for 30 s. The supernatant was removed and then washed with New Wash 3 times. Finally, the DNA fraction was centrifuged at 14,000 rpm at 2 °C for 30 s. The supernatant was removed. After the addition of sterile pure water (50 µl), the DNA solution was incubated at 55 °C for 5 min. The mixture was again centrifuged at 15,000 rpm at room temperature for 20 min. A 45 µl supernatant sample was recovered and the DNA concentration was determined using a U-3210 Spectrophotometer (Hitachi, Tokyo, Japan). Amplifications were carried out in 10 µl reaction volumes composed of 1.5 ng/µl plant DNA, 50 mM Tris/HCl (pH 8.5), 5 mM MgCl₂, 500 µg/ml BSA, 2.0 % Ficol, 4 mM tartrazine, 10 µM EDTA, and 0.04 units/µl *Tth* DNA polymerase (Toyobo, Osaka, Japan) (Nei and Li, 1979).

The Air Thermo-Cycler was programmed for 60 s at 94 °C, followed by 60 cycles of 10 s at 94 °C, 30 s at 36 °C, and 60 s at 72 °C, followed by 120 s at 72 °C for amplification. Amplification products were separated in 1% agarose gels in 0.5 Tris/borate/EDTA (TBE) buffer. Gels containing ethidium bromide (5 µl/100 ml) were developed at 120 V for 3 hr. Gels were detected and photographed by a DNA transilluminator Model NTM-20 (UVP Inc., Upland, CA) at 302 nm. The sizes of the amplification products were estimated using a 100 bp (100 bp-2.0 kb) ladder (Amersham Pharmacia Biotech, Buckinghamshire, UK). The random primers consisted of ten base sequences from a kit purchased from QIAGEN Operon Technologies Inc. (Chatsworth, CA). The OPB and OPO series, having ten base sequences that had been synthesized previously (Nakai *et al.*, 1996), were as follows:

OPB-01 (5'-GTTTCGCTCC-3'), OPB-02 (5'-TGATCCCTGG-3'),
OPB-03 (5'-CATCCCCCTG-3'), OPB-04 (5'-GGACTGGAGT-3'),
OPB-05 (5'-TGCGCCCTTC-3'), OPB-06 (5'-TGCTCTGCCC-3'),
OPB-07 (5'-GGTGACGCAG-3'), OPB-08 (5'-GTCCACACGG-3'),
OPB-09 (5'-TGGGGGACTC-3'), OPB-12 (5'-CCTTGACGCA-3'),
OPB-13 (5'-TTCCCCCGCT-3'), OPB-14 (5'-TCCGCTCTGG-3'),
OPB-18 (5'-CCACAGCAGT-3'), OPB-19 (5'-ACCCCCGAAG-3'),

OPO-03 (5'-CTGTTGCTAC-3'), OPO-04 (5'-AAGTCCGCTC-3'),
 OPO-08 (5'-CCTCCAGTGT-3'), OPO-09 (5'-TCCCACGCAA-3'),
 OPO-10 (5'-TCAGAGCGCC-3'), OPO-12 (5'-CAGTGCTGTG-3'),
 OPC-18 (5'-CTCGCTATCC-3')

Genetically similar (F) values were determined by Nei and Li's method (1979).

3. Results and discussion

3.1 ELISA using MAbs against ginseng

This paper demonstrated ELISA for *Panax* spp. using two kinds of MAbs against *P. ginseng* protein components. Hyperimmunized BALB/c mice with ginseng WSF yielded splenocytes which were fused with P3-X63-Ag8-U1 myeloma cells by a routine procedure established in our laboratory (Sakata *et al.*, 1994). After additional testing with ELISA employing various ginseng, two hybridoma-producing MAbs were cloned and expanded. MAb 3H4 was produced from white ginseng WSF as an immunogen, whereas MAb 5H8 was produced from red ginseng WSF as an immunogen. Two MAbs reactive to *P. ginseng* were classified into IgG1 with k light chains.

MAbs that bound to the ginseng protein components on the surface of the wells were then reacted with POD-labeled goat anti-mouse IgG as the secondary antibody in ELISA using MAbs 3H4 and 5H8. First, the reactivity of IgG-type MAbs 3H4 and 5H8 to various concentrations of *P. ginseng* protein components was tested by varying antibody concentrations and by performing a dilution curve. In general, the color development intensity was regulated at around 1 for a highly reliable ELISA result. Optical antibody concentrations (MAb 3H4; 0.01 $\mu\text{g}/\text{mL}$, MAb 5H8; 5 $\mu\text{g}/\text{mL}$) of absorbance of approximately 1 were selected for the determination of cross-reactivities against crude drugs by ELISA.

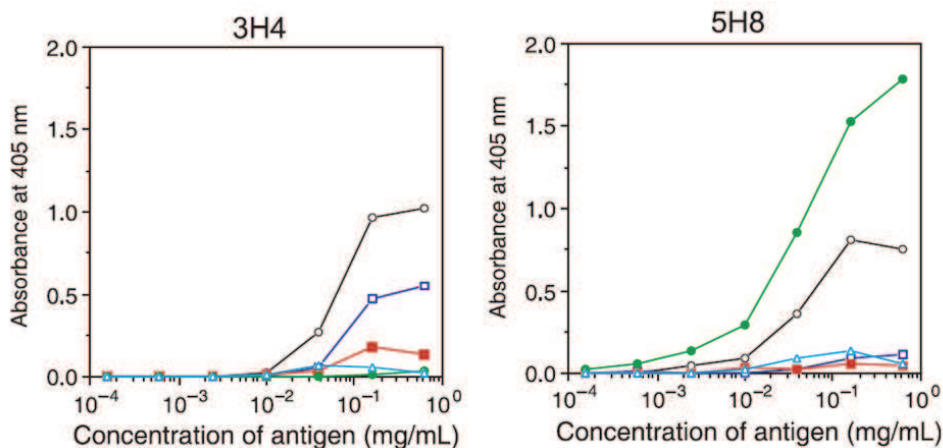


Fig. 2. Typical dose-response curves for five types of ginsengs measured by ELISA using MAbs 3H4 and 5H8. In order to examine the specificity of the ELISA, MAbs reactive to *P. ginseng* (MAb 3H4, 0.01 $\mu\text{g}/\text{mL}$; MAb 5H8, 5 $\mu\text{g}/\text{mL}$) were added to each well of a 96-well immunoplate coated with various concentrations of powdered drug material from white ginseng (\circ), red ginseng (\bullet), *Panax notoginseng* (\square), *P. quinquefolius* (\blacksquare) and *P. japonicus* (Δ). Values plotted are means ($n = 3$)

3.2 Cross-reactivity of MABs 3H4 and 5H8 against various crude drugs

Figure 2 shows typical dose response curves of five kinds of ginseng measured by ELISA using MABs 3H4 and 5H8. ELISA using MAB 3H4 was not specific to *P. ginseng* and showed cross-reactivity with *P. notoginseng* and *P. quinquefolius*, but did not react with other crude drugs. This wide cross-reactivity is the major advantage of the antibody reagent used in this ELISA. Although both MABs 3H4 and 5H8 cross-reacted with the *P. ginseng* protein components, the cross-reactivity against other *Panax* spp. varied, as shown in Table 1. The most important property of this MAB 3H4 is its ability to distinguish ginseng produced from *P. ginseng* by processing, since 3H4 did not react with red ginseng. MAB 5H8 has weak cross-reactivity with *P. notoginseng*, *P. quinquefolius* and *P. japonicus*; however, since MAB 5H8 cross-reacted with *Scopoliae rhizoma*, other assay methods such as our ELISA using anti-G-Rb1 and -Rg1 MABs might be needed.

Crude drug	Cross-reactivity (%) ^a	
	3H4	5H8
White ginseng (<i>Panax ginseng</i>)	100	100
Red ginseng (<i>P. ginseng</i>)	<1	204
San-chi ginseng (<i>P. notoginseng</i>)	53.4	4.4
American ginseng (<i>P. quinquefolius</i>)	43.1	2.4
Japanese ginseng (<i>P. japonicus</i>)	1.4	3.2
<i>Scopoliae Rhizoma</i>	<1	20.2
<i>Gardeniae Fructus</i>	<1	4.5
<i>Glycyrrhizae Radix</i>	<1	3.0
<i>Uvae Ursi Folium</i>	<1	2.7
<i>Phellodendri Cortex</i>	<1	2.7
<i>Sennae Folium</i>	<1	2.1
<i>Paeoniae Radix</i>	<1	1.5
<i>Bupleuri Radix</i>	<1	1.3
<i>Senegae Radix</i>	<1	1.3
<i>Rhei Rhizoma</i>	<1	1.2
<i>Aurantii Pericarpium</i>	<1	<1
<i>Coptidis Rhizoma</i>	<1	<1
<i>Cinnamomi Cortex</i>	<1	<1
<i>Scutellariae Radix</i>	<1	<1

^aValues are means \pm standard deviation ($n = 3$).

Table 1. Cross-reactivity (CR%) of MABs against various ginseng and other crude drugs assayed using the ELISA method

3.3 Detection of protein specific to MABs against ginseng

Blot analysis demonstrated that MAB 3H4 reacted with two major bands, M_r 24 kDa and 29 kDa, contained in 3 kinds of ginseng, as indicated in Figure 3. This observation suggests that the epitope is common to the two proteins. Judging from these results, MAB 3H4 might recognize common ginseng protein components contained in all *Panax* spp. On the other hand, MAB 5H8 selectively reacted with a broad band which roughly corresponds with bands at approximately M_r 24-29 kDa in *P. ginseng*, shown in Figure 3. This does not correspond to the result of the ELISA where MAB 5H8 reacted with *P. ginseng* and red

ginseng, suggesting that the immunogenic proteins were probably denatured during the manufacturing process and might not have been separated by SDS-PAGE.

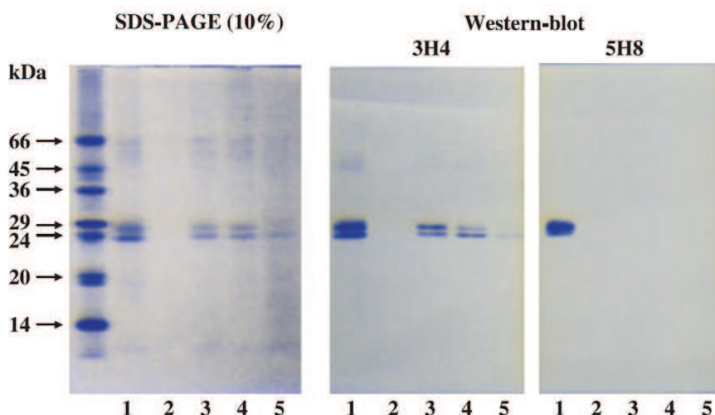


Fig. 3. SDS-PAGE and western blotting analyses of various ginsengs using MAbs 3H4 and 5H8. Samples were resolved by electrophoresis on a 10% acrylamide gel. Proteins were stained with Coomassie Brilliant Blue. Proteins separated by SDS-PAGE were transferred onto the PVDF membranes and incubated with MAbs 3H4 and 5H8. The bound MAb was reacted with PODlabelled secondary antibody, followed by reaction with substrate in order to develop blue protein bands. Lane 1, white ginseng (*Panax ginseng*); lane 2, red ginseng (*P. ginseng*); lane 3, sanchi-ginseng (*P. notoginseng*); lane 4, American ginseng (*P. quinquefolius*); lane 5, Japanese ginseng (*P. japonicus*). Molecular weights are as indicated by arrows

We established two MAbs that reacted predominantly with *P. ginseng* protein components to develop new immunological methods for analysis of *Panax* spp., resulting in two ELISAs using two MAbs. MAb 3H4, showing specific reactivity against ginseng, is predicted to be a probe for immunochemical discrimination between *Panax* spp. and other crude drugs. In addition, ELISA using MAb 5H8 can distinguish *P. ginseng* and red ginseng from other *Panax* spp. It became evident that the discrimination between white ginseng and red ginseng could be performed by Western blotting. This assay is also technically easy to perform and allows small amounts of sample to be analyzed, as demonstrated by this study. The ELISA developed in this paper can be utilized for a homogeneity assay of *Panax* spp., therefore making it possible to study in the phytochemical field.

3.4 G-Rb1 and -Rg1 concentrations in *Panax* spp. measured by ELISA

G-Rb1 and -Rg1 were quantitatively determined by the competitive ELISA that we developed previously (Tanaka *et al.*, 1999; Fukuda *et al.*, 2000a). Kitagawa *et al.* isolated malonyl ginsenosides from white ginseng, finding that non-processed ginseng contents are higher than in processed ginseng (Kitagawa *et al.*, 1983). It is thought that MG-Rb1 influences ELISA since it is suggested that MG-Rb1 has similar cross-reactivity with G-Rb1. Therefore, G-Rb1 concentrations in various *Panax* spp. were measured after MG-Rb1 had been converted into G-Rb1 by mild alkaline conditions. The concentrations of G-Rb1 and -Rg1 are shown in Table 2 and vary according to species. Fibrous ginseng, which is made from the actively growing part of *P. ginseng*, showed the highest G-Rb1 concentration, $64.4 \pm$

3.6 mg/g dry wt. *P. notoginseng* and *P. quinquefolius* also showed high concentrations, 47.1 ± 3.3 and 48.5 ± 1.8 mg/g dry wt., respectively; however, the G-Rb1 concentration in *P. japonicus* analyzed by ELISA must be inaccurate since a very high S.D. of the G-Rb1 concentration in *P. japonicus* is shown in Table 2. In the present study, *P. notoginseng* showed the highest G-Rg1 concentration, 22.9 ± 3.2 mg/g dry wt. Also fibrous ginseng showed a high concentration, 4.98 ± 0.04 mg/g dry wt. The concentrations determined by ELISA agreed well with those determined by HPLC (Kitagawa *et al.*, 1987). ELISA was more sensitive than TLC (Tani *et al.*, 1981) or HPLC (Sticher and Sticher, 1979; Soldati and Sticher, 1980). Moreover, Yamaguchi *et al.* (1988) and Samukawa *et al.* (1995) reported the comparative concentrations of ginsenosides in various commercial ginseng radices analyzed by HPLC. The results obtained in this investigation indicate that the determination of ginsenoside concentrations could be carried out semi-quantitatively by ELISA, which might become a promising tool for the standardization of ginseng samples.

Sample	G-Rb1 content (mg/g dry weight) ^a	G-Rg1 content (mg/g dry weight) ^a
Ginseng (<i>Panax ginseng</i>)	5.49 ± 0.75	2.28 ± 0.02
Red Ginseng (<i>P. ginseng</i>)	3.57 ± 0.62	1.34 ± 0.08
Fibrous Ginseng (<i>P. ginseng</i>)	64.4 ± 3.6	4.98 ± 0.04
San-chi Ginseng (<i>P. notoginseng</i>)	47.1 ± 3.3	22.9 ± 3.2
American Ginseng (<i>P. quinquefolius</i>)	48.5 ± 1.8	3.15 ± 0.23
Japanese Ginseng (<i>P. japonicus</i>)	1.37 ± 0.34	0.12 ± 0.01

^a Values are means \pm standard deviation ($n = 3$).

Table 2. Quantitative determination of ginsenosides Rb1 and Rg1 in various ginsengs using the ELISA method

3.5 Double-stained Eastern blotting of ginsenosides using anti-G-Rb1 and anti-G-Rg1 MAbs

Direct detection of small molecular compounds on a TLC plate by immunostaining is impossible because they are easily washed out by buffer solution without fixing. We therefore designed a system in which ginsenosides were blotted onto a PVDF membrane from the developed TLC plate for immunostaining. After ginsenosides had been transferred to the PVDF membrane, it was treated with NaIO_4 solution, followed by the addition of BSA. This reaction enhanced the fixation of ginsenosides via ginsenoside-BSA conjugate on the PVDF membrane. Figure 4-B indicates the Eastern blotting patterns of ginsenosides using anti-G-Rb1 MAb in three kinds of *Panax* spp., *P. ginseng*, *P. notoginseng* and *P. quinquefolius*.

Figure 4-C shows the Eastern blotting of double staining using anti-G-Rb1 and -Rg1 MAbs for ginsenoside standards. When the mixture of anti-G-Rg1 and -Rb1 MAbs and the pair of substrates were tested for staining of ginsenosides, all ginsenosides, G-Rg1, -Re, -Rd, -Rc and -Rb1, stained blue (data not shown), although purple staining for G-Rg1 was expected because 3-amino-9-ethylcarbazole was used as the substrate. It can therefore be suggested that the sensitivities of the substrate between 3-amino-9-ethylcarbazole and 4-chloro-1-naphthol might be different, so we performed successive staining of the PVDF membrane using anti-G-Rg1 and then anti-G-Rb1. Finally, we succeeded in the double staining of ginsenosides, indicating that G-Rg1 and -Re were stained purple and the other blue,

separately, as indicated in Fig. 4-C. From these results, both antibodies could distinguish individual aglycons, 20(S)-protopanaxatriol and 20(S)-protopanaxadiol. For this application, crude extracts of three *Panax* spp. were analyzed by the newly developed double-staining system. Major ginsenosides could be determined clearly by this double-staining method. G-Rb1, -Rc and -Rd possessing 20(S)-protopanaxadiol as an aglycon stained as blue bands, and G-Re and -Rg1 as purple, depending on the substrate (4-chloro-1-naphthol). Figure 4-D shows the double staining of Eastern blotting for three *Panax* spp. When compared to the single staining of Eastern blotting by anti-G-Rb1 MAb (Fig. 4-B), it became evident that the quality of *Panax* spp. regarding ginsenosides varied. *P. notoginseng* contained a higher amount of G-Rg1, but G-Rc disappeared (Fig. 4-D, lane 4). A higher amount of G-Re and trace amounts of G-Rd and -Rg1 were observed in *P. quinquefolius*, as indicated in Fig. 4-D, lane 5. Three bands stained red by anti-G-Rg1 MAb were found, as indicated by arrows, although they could not be detected by staining with anti-G-Rb1 MAb. It is suggested that these bands might be related to G-Rg1 and G-Re having 20(S)-protopanaxatriol as an aglycon in the molecule. A survey of previous papers (Shoji, 1990) suggested that the three unknown bands could be determined as G-Rh1, G-Rf and 20-gluco G-Rf, which are mono-, di- and triglycosides of 20(S)-protopanaxatriol, respectively, depending upon individual Rf values. This finding suggests that the structures of aglycon can be recognized from the band color, and the Rf value is reflected by the sugar number. Using this result, we determined two bands under G-Rb1 as MG-Rb1 (Kitagawa *et al.*, 1983) and 20-O-gentiobiosyl-chikusetsusaponin III (Shoji, 1990), which has five sugars in a molecule of *P. ginseng*, in good agreement with the Rf value previously reported (Kitagawa *et al.*, 1983; Shoji, 1990). Finally, these two compounds were identified by comparing with authentic samples (data not shown).

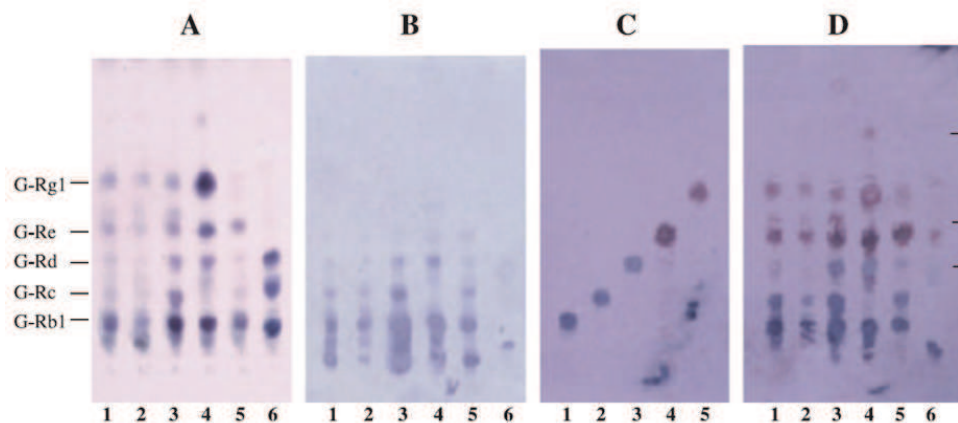


Fig. 4. Staining of various *Panax* samples. (A) Sulphuric acid staining; (B) eastern blotting using anti-G-Rb1 MAb; and (D) double-stain eastern blotting using anti-G-Rb1 and -Rg1 MAbs. Lanes 1–6 show white ginseng, red ginseng, fibrous ginseng, *P. notoginseng*, *P. quinquefolius* and *P. japonicus* (60 μ g), respectively. (C) Double-stain eastern blotting of ginsenoside standards using anti-G-Rb1 and -G-Rg1 MAbs. Lanes 1–5 show G-Rb1, -Rc, -Rd, -Re and -Rg1 (3 μ g), respectively

Figure 5 illustrates the immunocytolocalization of G-Rb1 in fresh *P. ginseng* roots. The phloem contained a higher concentration of G-Rb1 than the xylem. We conducted a

controlled study with a slice of fresh *Glycyrrhiza* root using the same procedure to evaluate the specific detection of ginsenosides in the *P. ginseng* root with this system. In consequence, the *Glycyrrhiza* root, although it contained saponins structurally related to G-Rb1, was not immunostained by the Eastern blotting method using anti-G-Rb1 MAb 9G7, indicating the high specificity for *Panax* spp. of this method (data not shown).

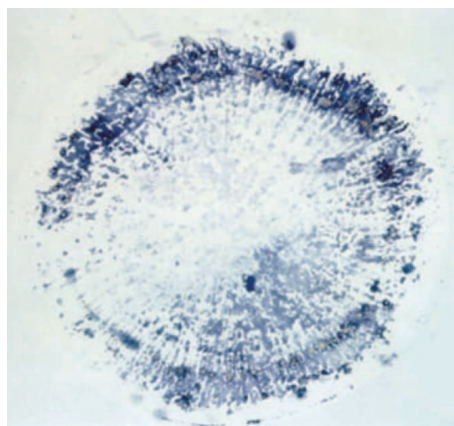


Fig. 5. Immunocytochemical localisation of G-Rb1 in *Panax ginseng* root using anti-G-Rb1 MAb

3.6 RAPD analysis

The total DNA extracted from the crude drugs of *Panax* spp. was analyzed by RAPD using 21 random primers, resulting in 113 total bands. Figure 6 shows a representative amplified band pattern identified by primers.

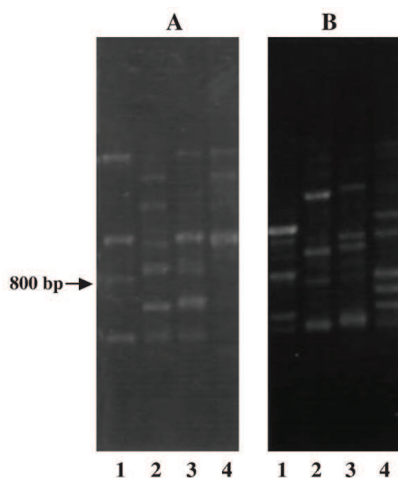


Fig. 6. Detection of polymorphic bands among *Panax* species using primer (A) OPO-08 (5'-CCTCCAGTGT-3') and (B) OPB-05 (5'-TGCGCCCTTC-3'). Lanes 1-4 show *P. notoginseng*, *P. japonicus*, *P. ginseng* and *P. quinquefolius*, respectively

The genetic distance matrix between individual species was calculated according to Nei and Li's method (1979). The degree of similarity among their fingerprints confirmed that *P. ginseng* is more closely related to *P. quinquefolius* than to *P. japonicus*. This result corresponds to the conclusions based on morphological taxonomic studies (Hara, 1970). It is suggested that RAPD analysis may be a useful method for determining the quality and homogeneity of *Panax* spp. as previously reported for regenerated plants of *Panax* spp. in vitro (Shoyama *et al.*, 1988).

4. Conclusion

The combination of RAPD and the developed double-stained Eastern blotting method can authenticate *P. notoginseng* and *P. japonicus*. Double-stained Eastern blotting indicated that *P. notoginseng* roots did not contain G-Rc, although the TLC profile was unclear. These results therefore suggest that a combination system of ELISA using MAbs, double-stained Eastern blotting and RAPD could identify four *Panax* spp. Moreover, a newly established immunocytochemical method clearly shows not only the distribution of ginsenosides in ginseng roots, but also differentiates ginseng from other crude drugs.

5. Acknowledgment

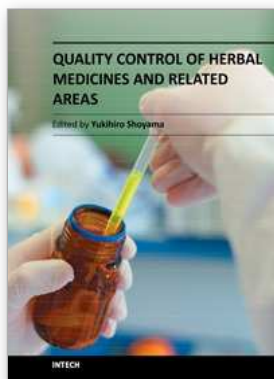
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The authors of this thematic issue provide a comprehensive summary of most recent knowledge and references on quality control in wide fields. Quality control is essential for natural products like natural medicine and related food products. In this issue fifteen chapters have been included, discussing in detail various aspects of quality control. It will certainly prove useful not only for phytochemical researchers, but also many scientists working in numerous fields. Much effort has been invested by the contributors to share current information. Without their efforts and input 'Quality Control of Herbal Medicine and Related Areas' could not exist.

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