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Formation of monoclonal antibody against a major ginseng component, ginsenoside Rg_1 and its characterization

Monoclonal antibody for a ginseng saponin

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Abstract

The ratio of hapten and bovine serum albumin in an antigen conjugate was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. A hybridoma secreting monoclonal antibody against ginsenoside Rg_1 was produced by fusing sprenocytes immunized with a ginsenoside Rg_1-bovine serum albumin conjugate with HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-653. A very small cross-reaction appeared with ginsenoside Re. The full measuring range of the assay extends from 0.3 μg ml^{-1} to 10 μg ml^{-1} of ginsenoside Rg_1.

Abbreviations: MAb, monoclonal antibody; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; HSA, human serum albumin; PBS, phosphate buffered saline; TPBS, PBS containing 0.05% of Tween 20; SPBS, PBS containing 5% skim milk; ABTS, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt

Introduction

Almost all *Panax* spp. (Araliaceae family) have been used in folk medicine. The most famous variety is *P. ginseng*, which was recorded in Chinese Materia Medica 2000 years ago. Ginseng is one of the most important Chinese medicines used in the world in tonics to combat stress and cancer, disturbances of the central nervous system, and hypothermia, and for radio-protection (Tanaka and Akagi, 1992; Shibata, 1982). It contains many dammarane and oleanane saponins (Besso et al., 1982; Koizumi et al., 1982), polyacetylene derivertives (Hansen and Boll, 1986) and polysaccharides (Tomoda et al., 1993) of which the biological activities have been studied widely.

In our ongoing study of the formation of monoclonal antibody (MAb) against naturally occurring bioactive compound, we have set up the MAbs against forskolin (Sakata et al., 1994; Yanagihara et al., 1996), solamargine (Ishiyama et al., 1996), codeine and thebaine (Shoyama et al., 1996), marihuana compounds (Tanaka et al., 1996) and their applications used for an affinity chromatography (Yanagihara et al., 1996) and an immunostaining (Tanaka et al., 1997). An immunological approach for assaying quantities of ginsenosides using a polyclonal antibodies has been investigated by Sankawa et al. (1982) and Yoon et al. (1998). However, since no result of MAb except against ginsenoside Rb_1 reported by us (1999), we herein communicate the formation of MAb against a major ginseng component, ginsenoside Rg_1 having pharmacological activities like excitation of central nervous system and improvement of memory and learning capability, and its characterizations.

Materials and methods

Chemicals and immunochemicals

Ginsenoside Rg_1, Rb_1, Rc, and Re was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and human serum albumin (HSA) were provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was provided from Organon Teknika Cappel Pruducts (West Chester, PA, USA). Complete Freund's adjuvant was provided by Difco (Detroit, USA). All other chemicals were standard commercial products of analytical grade.

Extraction of various ginseng sample

Dried samples (50 mg) of various ginseng were powdered, and then extracted with methanol (5 ml) under sonication 5 times, filtered and the combined extracts was diluted with 20% methanol suitably for enzyme-linked immunosorbent assay (ELISA).

Synthesis of antigen conjugates

Ginsenoside Rg_1-carrier protein conjugates were synthesized by a modification of the procedure already used for solamargine (Ishiyama et al., 1996) which is based on the method of Erlanger and Beiser (1964). To the H_2O solution (0.5 ml) containing $NaIO_4$ (4 mg), 80% MeOH solution (0.7 ml) of ginsenoside Rg_1 (10 mg) was added dropwise, and stirred at room temperature for 1 h. To the above reaction mixture, the carbonate buffer solution (pH 9.6, 1 ml) containing BSA (10 mg) was added, and stirred at room temperature for 5 h. The reaction mixture was dialyzed against H_2O 5 times, and then lyophilized to give ginsenoside Rg_1 BSA conjugate (17 mg). Ginsenoside Rg_1-HSA conjugate was also synthesized in the same manner to that of ginsenoside Rg_1-BSA conjugate.

Determination of hapten number in ginsenoside Rg_1-carrier protein conjugate by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry

The hapten number in the ginsenoside Rg_1-BSA conjugate was determined by MALDI TOF mass spectrometry as previously described (Shoyama et al., 1993a,b; Goto et al., 1994). A small amount (1–10 pmol) of antigen conjugate was mixed with a 10^3-fold molar excess of sinapinic acid in an aqueous solution containing 0.15% trifluoroacetic acid. The mixture was subjected to a JMS TOF mass monitor and irradiated with a N_2 lazer (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 detected using compatible computer as previously reported.

Immunization and hybridization

BALB/c female mice were injected intraperioneally with ginsenoside Rg_1-BSA dissolved in phosphate buffered saline (PBS) four times. The first immunization (50 μg protein) was injected as a 1:1 emulsion in Freund's complete adjuvant. The second and third immunization (50 μg protein in each injection) were injected as a 1:1 emulsion in Freund's incomplete adjuvant. On the third day after the final immunaization (100 μg protein, i.p.), splenocytes were isolated and fused with a HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-653, by the polyethylene glycol method (Galfre and Milstein, 1981). Hybridomas producing MAb reactive to ginsenoside Rg_1 were cloned by the limited dilution method (Goding, 1980). Established hybridomas were cultured in eRDF medium supplemented with 10 μg ml^{-1} insulin, 35 μg ml^{-1} of transferrin, 20 μM ethanolamine and 25 nM selenium (ITES) (Murakami et al., 1982).

Purification of MAb

A MAb 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 adjusted to pH 7 with 1 M Tris solution and subjected to the column, and washed the column with 10 mM phosphate buffer (pH 7). Absorbed IgG was eluted with 100 mM citrate buffer (pH 3). The eluted IgG was neutralized with 1 M Tris solution, then dialyzed against PBS (pH 7.4) 3 times, and finally lyophilized.

Direct ELISA using ginsenoside Rg_1-HSA

The reactivity of MAbs to ginsenoside Rg_1-HSA was determined by a direct ELISA. Ginsenoside Rg_1-HSA conjugate (100 μl, 1 μg ml^{-1}) was adsorbed to the wells of a 96 well-immunoplate (NUNC. Roskilde, Denmark), then it was treated with 300 μl PBS containing 5% skim milk (SPBS) for 1 h to reduce non-specific adsorption. The plate was washed three times with PBS containing 0.05% of Tween 20 (TPBS) and reacted with 100 μl of testing MAb for 1 h. The plate was washed three times with TPBS, and

then the MAb combined with 100 μl of a 1:1000 dilution of peroxidase-labeled anti-mouse IgG (Organon Teknika Cappel Products, West Chester, USA) for l h. After washing the plate three times with TPBS, 100 μl of substrate solution, [0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2, and 0.3 mg ml^{-1} 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, Wako Pure Chemical Ind., Ltd., Osaka, Japan)] was added to each well and incubated for 20 min. Absorbance was measured by a micro plate reader (Model 450 Microplate Reader Bio-Rad Laboratories) at 405 nm. All reactions were carried out at 37 °C.

Competitive ELISA

Ginsenoside Rg_1-HSA (15 molecules of ginsenoside Rg_1 per molecule of HSA) (100 μl, 1 μg ml^{-1}) was adsorbed to the wells of a 96 well-immunoplate (NUNC. Roskilde, Denmark), then it was treated with 300 μl SPBS for 1 h to reduce non-specific adsorption. Fifty μl of various concentrations of ginsenoside Rg_1 dissolved in 20% of MeOH solution was incubated with 50 μl (IgG: 1.018 μg ml^{-1}) of IgG solution for 1 h. The plate was washed three times with TPBS, and then the MAb was combined 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 [0.1 M citrate buffer (pH 4) containing 0.003% H_2O_2, 0.3 mg/ml of ABTS] was added to each well and incubated for 15 min. The absorbance was measured by micro plate reader at 405 nm. Concentration of the standard solutions were 1×10^5, 2.5×10^4, 6.25×10^3, 1.56×10^3, 3.91×10^2, 97.7, 24.4, and 6.10 (ng/ml) and the corresponding absorbances were 0.09, 0.20, 0.47, 0.89, 1.11, 1.24, 1.25, and 1.29, respectively.

The cross-reactivities (CR%) of ginsenoides and related compounds were determined according to Weiler's equation (Weiler and Zenk, 1976):

$$\text{CR\%} = \frac{\mu\text{g ml}-1 \text{ of GRg}_1 \text{ yielding A/A}_0=50\%}{\mu\text{g ml}-1 \text{ of compound under investigation}} \times 100$$
$$\text{yielding A/A}_0 = 50\%$$

A is the absorbance in the presence of the test compound and A_0 is the absorbance in the absence of the test compound (20% MeOH soln.).

Sample preparation

Dried samples (50 mg) of various ginseng were powdered, extracted with MeOH (0.5 ml) under sonication 5 times, filtered, and then evaporated. The residue was redissolved in 1 ml of MeOH. Sample solutions were diluted 2∼200 times in terms of individual samples with 20% MeOH when analyzed by the competitive ELISA.

Quantitative analysis of ginsenoside-Rg1 by HPLC

HPLC system (Tosoh, Tokyo, Japan) composed of a LC-10AD pump (Shimazu, Japan) and UV-8 model Spectrometer equipped with a Cosmosil 5 C18 (4.5 × 150 mm, Nacalai Tesque) was used. The column was eluted with H_2O-CH_3CN containing 0.5% H_3PO_4 (80:20 v/v) at a flow rate of 1.0 ml min^{-1}. The effluent was monitored by absorption at UV 202 nm (Yamaguti et al., 1988).

Table 1. Hapten number of conjugate

Conjugate	Hapten number
Ginsenoside Rg1-BSA	13
Ginsenoside Rg1-HSA	15

Results and discussion

Direct determination of hapten-carrier protein conjugate by MALDI TOF mass spectrometry

Figure 1 shows the MALDI TOF mass spectra of the antigen, ginsenoside Rg_1-BSA conjugate. A broad peak coinciding with the conjugate of ginsenoside Rg_1 and BSA appeared from *m/z* 70,000 to 90,000 centering at around *m/z* 76,470. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of ginsenoside Rg_1 component (MW800) are 10,037 resulting in the 13 molecules of ginsenoside Rg_1 conjugated with BSA. The hapten number was estimated to be enough for immunization. The number of ginsenoside Rg_1 contained in the ginsenoside Rg_1-HSA conjugate was also determined to be around 15 molecules by its spectrum (Table 1).

Production and characteristics of MAb against ginsenoside Rg_1

The hyperimmunized BALB/c mice used to derive the cell clone described in this study yielded splenocytes which were fused with P3-X63-Ag8-653 myeloma cells by the routinely established procedure in this laboratory (Sakata et al., 1994). Hybridoma producing

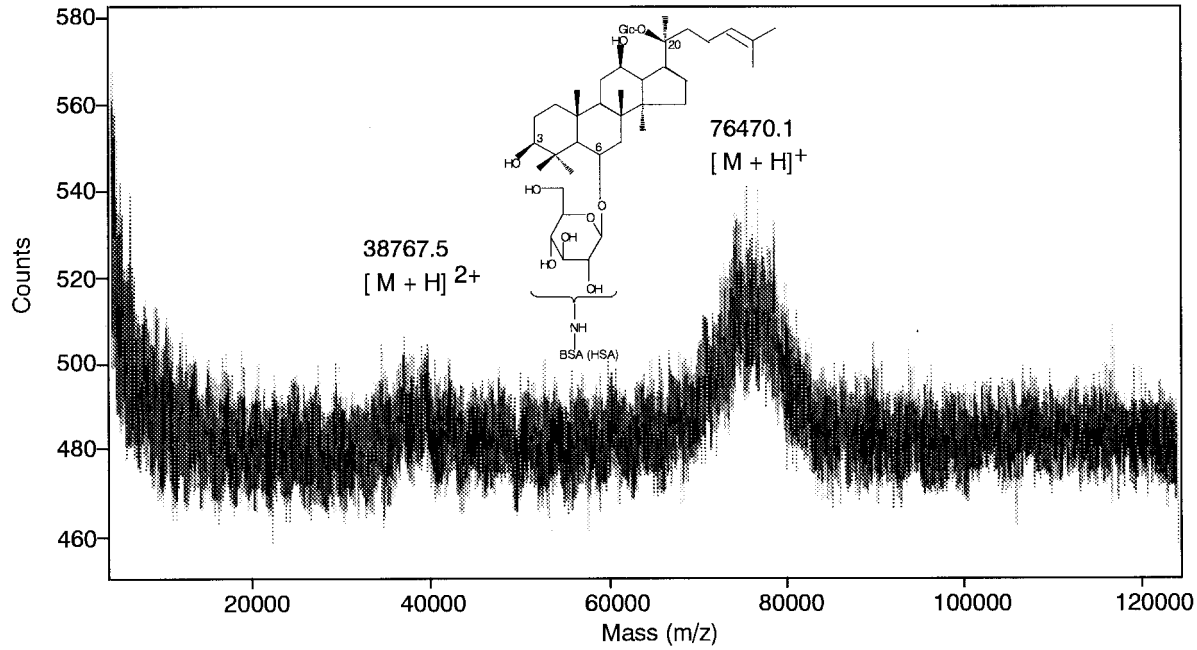


Figure 1. Direct determination of ginsenoside-Rg_1 BSA conjugate by matrix-assisted laser desorption/ionization mass spectrometry. $[M+H]^+$, $[M+2H]^{2+}$ are single and double protonated molecules of ginsenoside Rg_1-BSA, respectively.

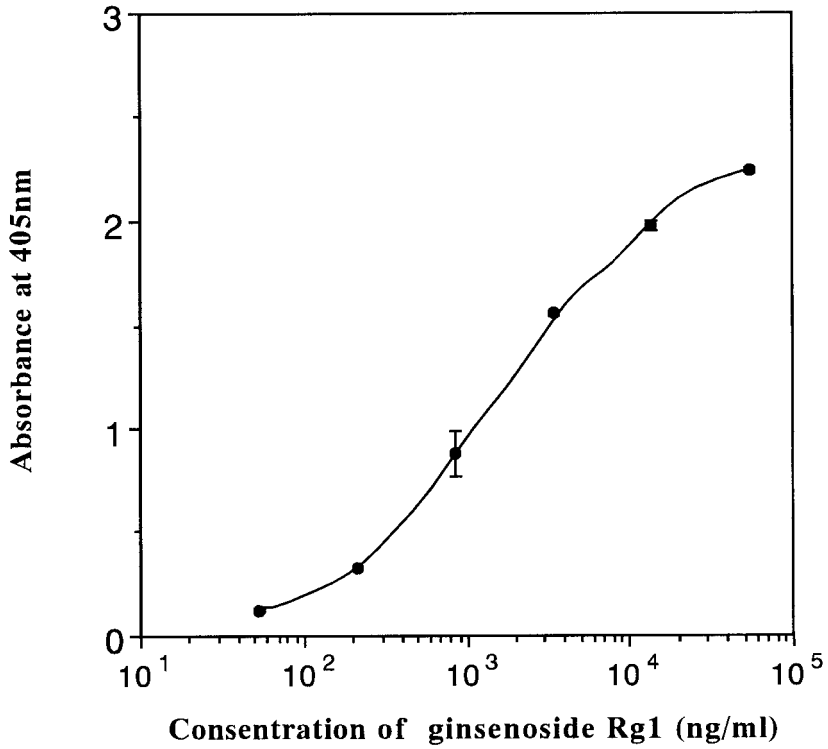


Figure 2. Reactivities of IgG type MAb (1F4) against ginsenoside Rg_1. To examine reactivity of antibody, varying concentration of antibody was added to each well of a 96 well-immunoplate coated with ginsenoside Rg_1-HSA (1 μg ml^{-1}).

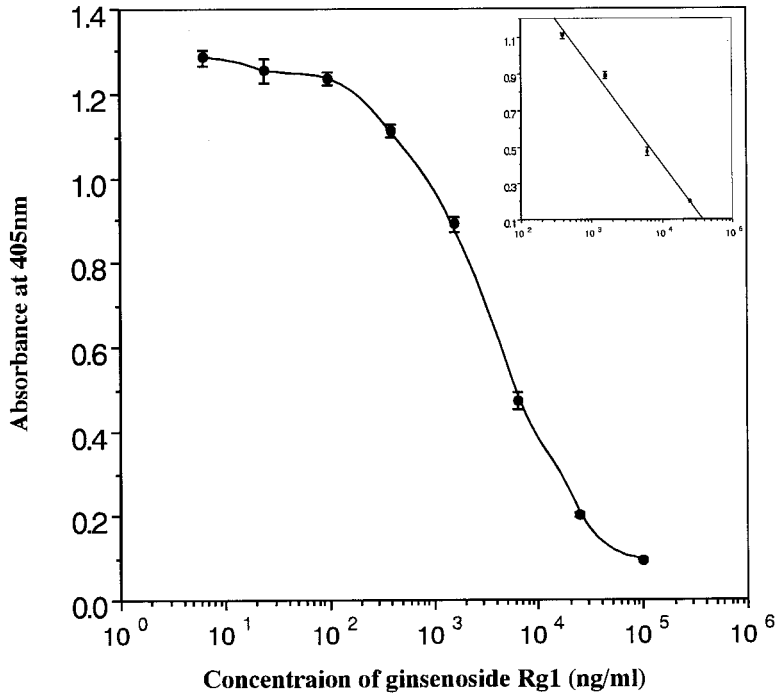


Figure 3. Calibration curve of ginsenoside Rg_1. Various concentrations of ginsenoside Rg_1 were incubated with MAb in the wells precoated with ginsenoside Rg_1-HSA (1 μg ml^{-1}). After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm.

MAb reactive to ginsenoside Rg_1 was obtained, and classfied into IgG2b which had *k* light chains.

The reactivity of IgG type MAb, 1F4 was tested by varying antibody concentration and by performing a dilution curve as indicated in Figure 2. The antibody concentration (1.081 μg ml^{-1}) at which the OD was about 0.8 in Figure 2 was selected for competitive ELISA.

Assay sensitivity and assay specificity

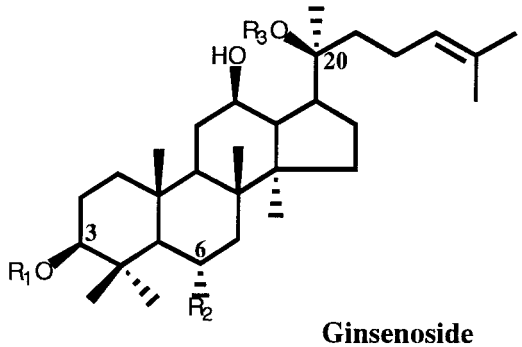
The free MAb following competition is bound to polystyrene microtitre plates precoated with ginsenoside Rg_1-HSA. Under these conditions, the full measuring range of the assay extends from 0.3 to 10 μg ml^{-1} as indicated in Figure 3.

Cross-reactivity is the most important factor in determining the value of antibody. Since the ELISA for ginsenoside Rg_1 was established for phytochemical investigations involving crude plant extracts, the assay specificity was checked by determining the cross reactivities of the MAb with various related compounds. The cross-reactivities of MAb obtained was examined by competitive ELISA and calculated using

Table 2. Cross-reactivities (%) of MAb (1F4) against Ginsenosides and another steroidal compounds

Compound	Cross-reactivities (%)
Ginsenoside Rg_1	100
Ginsenoside Re	3.3
Ginsenoside Rb_1	<0.93
Ginsenoside Rc	<0.93
Ginsenoside Rd	<0.93
Saikosaponin a	<0.93
Digitonin	<0.93
Solasonine	<0.93
Deoxycholic acid	<0.93
Glycyrnhizin	<0.93
Ergosterol	<0.93
Solamargine	<0.93
Cholesterol	<0.93
β-Sitosterol	<0.93
Chikusetsusaponi IV	<0.93
Tomatine	<0.93

The cross-reactivities of ginsenosides and other steroidal compounds were determined according to Weiler's equation (Weiler et al., 1976; see Materials and methods section



Ginsenoside

	R_1	R_2	R_3
Rb1	Glc-(2)-Glc-	H	Glc-(6)-Glc-
Rc	Glc-(2)-Glc-	H	Ara(f)-(6)-Glc-
Rd	Glc-(2)-Glc-	H	Glc-
Re	H	Rha-(2)-Glc-*O*-	Glc-
Rg1	H	Glc-*O*-	Glc-

Figure 4. Structure of ginsenosides.

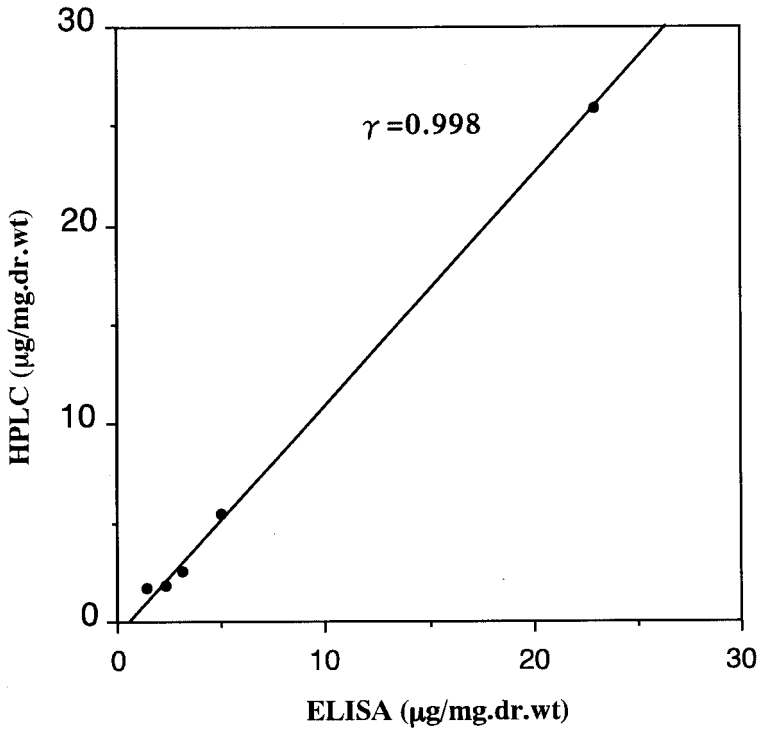


Figure 5. Correlation between ELISA and HPLC determination of ginsenoside Rg_1 in various ginseng extraction. γ = correlation coefficient.

Table 3. Ginsenoside Rg_1 contents of various ginseng by competitive ELISA

Sample	Content (μg/mg dr.wt.)			
	ELISA	C.V.(%)[a]	HPLC	C.V.(%)
Ginseng (*P. ginseng*)	2.28±0.02	0.0	1.781±0.07	0.0
Red Ginseng	1.34±0.08	0.0	1.645±0.05	0.0
Fibrous Ginseng	4.98±0.04	0.0	5.392±0.25	0.1
San-chi Ginseng (*P. notoginseng*)	22.9±3.20	1.1	25.926±0.32	0.1
American Ginseng (*P. quinquefolium*)	3.15±0.23	0.1	2.489±0.04	0.0
Japanese Ginseng (*P. japonicus*)	0.12±0.01	0.0	–	–

[a] C.V. (%) were means of trhee replicate assays.

p mole of ginsenoside Rg_1 yielding midrange and p mole of derivatives of ginsenoside Rg_1 under investigation yielding midrange by the method reported by Weiler and Zenk (1976). The cross-reactivity of ginsenoside Re which possess a sugar moeity attached to C-6 hydroxygroup are weak comparing with ginsenoside Rg_1 (Figure 4). Moreover, ginsenosides Rb_1, Rc and Rd show no cross-reactivity. From these results a sugar moiety at C-6 position was necessary. On the other hand a sugar moiety at C-20 position is not related for the reactivity. It becomes evident that the MAb reacted only with small number of structurally related ginsenoside Rg_1 very weakly, and did not react with other steroidal compounds as shown in Table 2 resulting in that the MAb against ginsenoside Rg_1 exhibited high specificity. Therefore, the newly established MAb against ginsenoside Rg1 can be routinely used for the phytochemical investigations involving crude plant extracts without any pretreatment.

Analyses of ginsenoside Rg_1 in various ginseng by using competitive ELISA

The content of ginsenoside Rg_1 in various ginsengs were analyzed by the competitive ELISA method described above (Table 3). Kitagawa et al. (1987) analyzed the ginsenoside contents in various crude ginsengs by HPLC. Recently Yamaguchi et al. (1988), Samukawa et al. (1995) and Xuan et al. (1998) reported the comparative contents of gingenosides in the various commercial ginseng radices analyzed by HPLC. In the present study, the Sanchi ginseng showed the highest ginsenoside Rg_1 content. Also Fibrous ginseng showed the higher content. These results were a good agreement with their previous reports as discribed above. The newly established ELISA was more sensitive than the TLC (Tani et al. 1981) or HPLC methods (Sticher and Soldati 1979; Soldati and Sticher 1980). The correlation coefficient was calculated from fitting a straight line analyzed by ELISA and HPLC methods. There was a good correlation (γ=0.998) between both assay values by the two methods (Figure 5). This methodology can be utilized for the assay of ginsenoside Rg_1, therefore it is possible to study a large number of plantlets cultured in this laboratory (Shoyama et al., 1995, 1997), and a small sample size *in vitro* for the breeding of *Panax* species to yield high concentration of ginsenoside Rg_1 related compounds.

In our knowledge this is the first time the immunoassay system of ginsenoside Rg_1 has been achieved.

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