Formation of monoclonal antibody against a major ginseng component, ginsenoside Rb₁ and its characterization

Hiroyuki Tanaka, Noriko Fukuda & Yukihiro Shoyama*

Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

E-mail: shoyama@shoyaku.phar.kyushu-u.pc.jp

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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 mass spectrometry. A hybridoma secreting monoclonal antibody against ginsenoside Rb₁ was produced by fusing splenocytes immunized with a ginsenoside Rb₁-bovine serum albumin conjugate with HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-653. A very small cross-reaction appeared with ginsenoside Rc and ginsenoside Rd. The full measuring range of the assay extends from 20 ngml⁻¹ to 400 ngml⁻¹ of ginsenoside Rb₁.

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 buffer saline; GPBS, PBS containing 0.2% of gelatin; 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, 1973; Shibata, 1982). It contains many dammarane and oleanane saponins (Besso et al., 1982; Koizumi et al., 1982), polyacetylene derivatives (Hansen and Boll, 1986) and polysaccharides (Tomoda et al., 1993) of which the biological activity has been studied widely.

In our ongoing study of the formation of monoclonal antibody (MAb) against naturally occurring bioactive compound, we have set up the MAb 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). However, since no result of MAb related to ginsenosides has been reported yet, we herein communicate the formation of MAb against a major ginseng component, ginsenoside Rb₁ having a sedative activity, and its characterizations.

Materials and methods

Chemicals and immunochemicals

Ginsenoside Rb₁ was purchased from Wako Pure

* To whom correspondence should be addressed.

Chemical Ind., Ltd. (Osaka, Japan). BSA and HSA were provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was provided from Organon Teknika Cappel Products (West Chester, PA, USA). PVDF membranes (Immobilon-N) were purchased from Millipore Corporation (Bedford, MA, USA). Glass microfiber filter sheet (GF/A) were purchased from Whatman International Ltd. (Maidstone, England). All other chemicals were standard commercial products of analytical grade.

Extraction of various ginseng sample

Dried samples (10 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 ELISA.

Synthesis of antigen conjugates

Ginsenoside Rb₁-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 H₂O solution (0.5 ml) containing NaIO₄ (4 mg), 80% MeOH solution (0.7 ml) of GRb₁ (10 mg) was added dropwise, and stirred at room temperature for 1 hr. 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 hr. The reaction mixture was dialyzed against H₂O 5 times, and then lyophilized to give ginsenoside Rb₁ conjugate (GRb₁-BSA) (17 mg). Ginsenoside Rb₁-HSA (GRb₁-HSA) conjugate was also synthesized in the same manner to that of GRb₁-BSA conjugate.

Determination of hapten number in GRb₁-carrier protein conjugate by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry

The hapten number in the GRb₁-BSA conjugate was determined by MALDI 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 10³-fold molar excess of sinapinic acid in an aqueous solution containing 0.15% trifluoroacetic acid. The mixture was subjected to a JMS time-of-flight (TOF) mass monitor and irradiated with a N₂ 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 detected using compatible computer as previously reported.

Immunization and hybridization

BALB/c female mice were injected intraperitoneally with GRb₁-BSA dissolved in phosphate buffer 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 immunization (100 µg protein), splenocytes were isolated and fused with a HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-653, by the polyethylene glycol (PEG) method (Galfre and Milstein, 1981). Hybridomas producing MAb reactive to ginsenoside Rb₁ were cloned by the limited dilution method (Goding, 1980). Established hybridomas were cultured in eRDF medium supplemented with 10 µg/ml insulin, 35 µg/ml 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 GRb₁-HSA

The reactivity of MAbs to Rb₁-HSA was determined by an ELISA. GRb₁-HSA conjugate (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 GPBS for 1 hr to reduce non-specific adsorption. The plate was washed three times with TPBS and reacted with 100 µl of testing MAb for 1 hr. The plate was washed three times with TPBS, and then MAb combined with 100 µl of a 1:1000 dilution peroxidase-labeled anti-mouse IgG (Organon Teknika Cappel Products, West Chester, USA) for 1 hr. After washing the plate three times with TPBS, 100 µl of substrate solution, [0.1 M citrate buffer (pH 4.0) containing 0.003% H₂O₂ and 0.3 mg/ml ABTS (Wako Pure Chemical Ind., Ltd., Osaka, Japan)] was added to each well and incubated for 20 min. Absorbance

Figure 1. Direct determination of ginsenoside-Rb₁-BSA (GRb₁-BSA) conjugate by matrix-assisted laser desorption/ionization mass spectrometry. [M+H]⁺, [M+2H]²⁺ are single and double protonated molecules of GRb₁-BSA, respectively.

was measured by a micro plate reader (Model 450 Microplate Reader Bio-Rad Laboratories) at 405 nm and 490 nm. All reactions were carried out at 37 °C.

Competitive ELISA

GRb₁-HSA (5 molecules of GRb₁ per molecule of HSA) (100 μl, 1 μg/ml) was absorbed to the wells of a 96 well-immunoplate (NUNC, Roskilde, Denmark) then it was treated with 300 μl SPBS for 1 hr to reduce non-specific adsorption. Fifty μl of various concentrations of ginsenoside dissolved in 20% of MeOH solution was incubated with 50 μl (IgG:0.418 μg/ml) of IgG solution for 1 hr. 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 hr. After washing the plate three times with TPBS, 100 μl of substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H₂O₂, 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 and 490 nm.

The cross-reactivities (CR%) of ginsenosides and related compounds were determined according to Weiler's equation (Weiler et al., 1976):

$$\text{CR\%} = \frac{\mu\text{g/ml of GRb}_1 \text{ yielding } A/A_0 = 50\%}{\mu\text{g/ml of compound under investigation}} \times 100$$

yielding $A/A_0 = 50\%$

A is the absorbance in the presence of the test compound and A₀ is the absorbance in the absence of the test compound (20% MeOH soln.).

Results and discussion

Direct determination of hapten-carrier protein conjugate by matrix-assisted laser desorption tof mass spectrometry

Figure 1 shows the MALDI mass spectra of the antigen, GRb₁-BSA conjugate. A broad peak coinciding with the conjugate of GRb₁ and BSA appeared from m/z 70,000 to 90,000 centering at around m/z 79,469. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of GRb₁ component (MW1,109) are from 3,327 to 23,289 resulting in the range of 3 to 21 (12 in average) molecules of GRb₁ conjugated with BSA. The hapten number was estimated to be enough for immunization. The number of GRb₁ contained in the GRb₁-HSA conjugate

Table 1. Hapten numbers of conjugates

Conjugate	Hapten number
Ginsenoside Rb ₁ -BSA	12
Ginsenoside Rb ₁ -HSA	5

Figure 2. Reactivities of IgG type MAb (9G7) against GRb₁. To examine reactivity of antibody, varying concentration of antibody was added to each well of a 96 well-immunoplate coated with GRb₁-HSA (1 μ g/ml).

was also determined to be around 5 molecules by its spectrum (Table 1).

Production and characteristics of MAb against GRb₁

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 MAb reactive to GRb₁ was obtained, and classified into IgG2b which had *k* light chains.

The reactivity of IgG type MAb, 9G7 was tested by varying antibody concentration and by performing a dilution curve as indicated in Figure 2. The antibody concentration (0.418 μ g/ml) 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 GRb₁-

Figure 3. Calibration curve of GRb₁. Various concentrations of GRb₁ were incubated with MAb in the precoated with GRb₁-HSA (1 μ g/ml). After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm.

HSA. Under these conditions, the full measuring range of the assay extends from 20 to 400 ngml⁻¹ as indicated in Figure 3.

Cross-reactivity is the most important factor in determining the value of antibody. Since the ELISA for GRb₁ 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 p mole of GRb₁ yielding midrange and p mole of derivatives of GRb₁ under investigation yielding midrange by the method reported by Weiler and Zenk (1976). The cross-reactivity of GRc and GRd which possess a diglucose moiety attached to C-3 hydroxygroup are weak comparing with GRb₁. Moreover, GRE and GRg₁ show no cross-reactivity. From these results a diglucose moiety at C-3 position was necessary. On the other hand a sugar moiety at C-20 position is not related for the reactivity. The effect of the sugar moiety conjugated at C-6 is not clear because such derivatives are not available from natural resources. It becomes evident that the MAb reacted only with small number of structurally related GRb₁ very weakly, and did not react with other steroidal compounds as shown in Table 2 resulting in that the MAb against GRb₁ exhibited high specificity. Therefore, the newly established MAb against GRb₁ can be routinely used for

Table 2. Cross-reactivities of MAb (9G7) against Ginsenosides

Compound	Cross-reactivities (%)
Ginsenoside Rb ₁	100
Ginsenoside Rc	0.024
Ginsenoside Rd	0.020
Ginsenoside Re	<0.005
Ginsenoside Rg ₁	<0.005
Glycyrrhizin	<0.005
Gigitonin	<0.005
Tigogenin	<0.005
Tigogenin	<0.005
Gitogenin	<0.005
Digitonin	<0.005
Solamargine	<0.005
Solasoinine	<0.005
Cholesterol	<0.005
Ergosterol	<0.005
Ursolic acid	<0.005
β -Sitosterol	<0.005
Cholic acid	<0.005
Deoxycholic acid	<0.005

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

Table 3. Ginsenoside Rb₁ contents of various ginseng

Sample	Content (μ g/mg dry wt.)
Ginseng (<i>P. ginseng</i>)	6.20 $\pm$ 0.39
Red Ginseng	3.75 $\pm$ 0.36
Fibrous Ginseng	48.44 $\pm$ 4.70
San-chi Ginseng (<i>P. notoginseng</i>)	35.46 $\pm$ 1.38
American Ginseng (<i>P. quinquefolium</i>)	36.09 $\pm$ 1.97
Japanese Ginseng (<i>P. japonicus</i>)	2.01 $\pm$ 0.26

the phytochemical investigations involving crude plant extracts without any pretreatment.

Analyses of GRb₁ in various ginseng

The ELISA was utilized to measure the contents of GRb₁ in various ginseng (Table 3). Kitagawa et al. (1987) analyzed the ginsenoside contents in various crude ginsengs by HPLC. Recently Yamaguchi et al. (1988) and Samukawa et al. (1995) reported the comparative contents of ginsenosides in the various commercial ginseng radices analyzed by HPLC. In the present study, the fibrous ginseng that is made of an

active growing part in *P. ginseng* showed the highest GRb₁ content. Also San-chi ginseng and American ginseng showed the higher content. These results were a good agreement with their reports. The newly established ELISA was more sensitive than the TLC (Tani et al., 1981) or HPLC methods (Sticher et al., 1979; Soldati et al., 1980). The correlation coefficient was calculated from fitting a straight line analyzed by ELISA and HPLC methods. There was a good correlation ($\gamma = 0.997$) between both assay values by the two methods except the range of lower concentration (0.1 μ g/ml) of GRb₁ (data not shown). This methodology can be utilized for the assay of GRb₁ related compounds, therefore it is possible to study a large number of plantlets cultured in this laboratory (Shoyama et al., 1995; Shoyama et al., 1997), and a small sample size *in vitro* for the breeding of *Panax* species to yield high concentration of GRb₁ related compounds.

In our knowledge this is the first time the immunoassay system of GRb₁ has been achieved.

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