Preparation of Monoclonal Antibodies against Ginsenosides and its Application with the Aim of Breeding Ginseng

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Introduction

Ginseng is one of the most important medicinal plants used for traditional medicines. It is used to enhance stamina and the capacity to cope with fatigue and physical stress and as a tonic against cancers, disturbances of the central nervous system (memory, learning and behavior), hypothermia, carbohydrate and lipid metabolism, immune function, the cardiovascular system and radioprotection. Major active components of ginseng are the ginsenosides, which are mainly triterpenoid dammarane derivatives. In the breeding for superior ginseng with high-yield ginsenosides, it is critical that the analytical methods for ginsenosides could be highly sensitive and rapid.

In this study, we developed immunological methods that are convenient, highly sensitive, and reproducible, as well as being able to simultaneously analyze multiple samples in order to screen a superior sample with high-yield ginsenosides in the above-mentioned research. Specifically, we have prepared monoclonal antibodies (MAbs) against ginsenoside Rb1 and ginsenoside Rg1, and developed enzyme-linked immunosorbent assay (ELISA) for the quantitative detection of these compounds with high sensitivity, reliability and simplicity. Also, we established the new immunostaining method for the determination of ginsenosides in ginseng and traditional herbal medicines.

Experimental

Chemicals and immunochemicals

Ginsenosides (G) -Rb1, -Rc, -Rd, -Re, -Rf and -Rg1 were purchased from Wako (Osaka, Japan). Bovine serum albumin (BSA) and human serum albumin (HSA) were provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was obtained from Organon Teknika Cappel Products (West Chester, PA, USA). PVDF membranes (Immobilon-N) were purchased from Millipore (Bedford, MA, USA). Glass microfiber filter sheets (GF/A) were purchased from Whatman International (Maidstone, UK). All other chemicals were standard commercial products of analytical-reagent grade. Anti-G-Rb1 and anti-G-Rg1 MAbs were purified using a Protein G FF column (11 × 0.46 cm i.d.) (Pharmacia Biotech, Uppsala, Sweden). The cultured medium (500ml) containing the IgG was adjusted to pH 7 with 1 M Tris solution (pH 9.0) and loaded onto the column, which was washed with 10 mM phosphate buffer (pH 7). Adsorbed IgG was eluted with 100 mM citrate buffer (pH 2.7). The eluted IgG was neutralized with 1 M Tris solution, then dialyzed against phosphate buffered saline (PBS) three times, and finally lyophilized.

Plant and drug materials

The crude drug materials of ginsengs were purchased from Nakai Koshindo (Kobe, Japan). Traditional medicine extracts were kindly provided by Mr T. Somehara, Saga Medical School.

Sample preparation

Dried samples (50mg) of various ginsengs were powdered, extracted with MeOH (5ml) under sonication five times, filtered and then evaporated. For the elimination of the malonyl group from malonylated ginsenosides (MGs), the extracts were treated with 0.1% KOH in MeOH at room temperature for 1 h, and assayed by ELISA.

Competitive ELISA

GRb1-HSA (100 μℓ, 1 μg/ml) was adsorbed in the wells of a 96-well immunoplate (Nalge NUNC, Roskilde, Denmark), then treated with 300 μℓ of 5% skim milk (S) in PBS for 1 h to reduce non-specific adsorption. A 50 μℓ of ginsenoside and samples solution (20% MeOH solution) was incubated with 50 μℓ of 0.4 μg/ml IgG solution for 1 h. The plate was washed three times with 0.05% Tween 20 (T) in PBS, 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 and 0.3 mg/ml 2,2 ' -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS)] was added to each well and incubated for 15 min. The absorbance was measured with a micro plate reader at 405 nm.

Competitive ELISA for G-Rg1 was performed with the same manner as that for G-Rb1.

Eastern blotting

Ginsenosides, the extracts of ginsengs and the traditional herbal medicines were applied to TLC plates and developed with BuOH–EtOAc–H_2O (15 : 1 : 4). One TLC plate was sprayed with H_2SO_4 and another TLC plate was blotted on the PVDF membrane with the blotting solution with heating. The blotted PVDF membrane was immersed in water containing $NaIO_4$ for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA was added and stirred for 3 h. The PVDF membrane was stained by standard protocols of Eastern blotting using MAb.

Results and discussion

Cross-reactivities of anti-G-Rb1 MAb and anti-G-Rg1 MAb

Cross-reactivity is the most important factor in determining the value of an antibody. Since ELISA for G-Rb1 was established for phytochemical investigations involving crude plant extracts, the assay specificity was checked by determining the cross reactivities of anti-G-Rb1 MAb with related various compounds. It became evident that the MAb reacted only very weakly with a small number of structurally related ginsenosides, G-Rc (0.024%) and G-Rd (0.020%), and did not react with other steroidal compounds resulting in that the MAb against G-Rb1 exhibited high specificity. Anti-G-Rg1 MAb had a weak cross-reactivity against G-Re, 3.3% and was very weak for G-Rd, G-Rc and G-Rb1 (0.93% respectively, or lower) indicating high specificity. From these results it became clear that two of the MAbs against G-Rb1 and G-Rg1 could be used for phytochemical investigations

Assay sensitivity and accuracy

The sensitivity of the newly established ELISAs was higher than that of the TLC or HPLC methods. The free MAb following competition is bound to polystyrene microtiter plates precoated with G-Rb1-HSA. Under these conditions, the full measuring range of the assay extends from 20 to 400 ng/ml. On the other hand, the concentration of G-Rg1 can be analyzed between 0.3 to 10 μg/ml.

The contents of G-Rb1 and G-Rg1 in various ginsengs were determined by competitive ELISA and

HPLC. As a result of calculating the correlation coefficient from fitting a straight line analyzed by ELISA and the HPLC method, there were good correlations for G-Rb1 (γ=0.997) and G-Rg1 (γ=0.998) between assay values from both methods. Therefore, it is possible to analyze a large number of in vitro ginseng samples precisely with these systems in the field of breeding.

Table 1. Quantitation of G-Rb1 and G-Rg1 in various ginsengs by competitive ELISA[a]

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

[a] Data were mean ± SD from triplicate analyses for each sample.

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 designed the system that ginsenosides was blotted onto a PVDF membrane from the developed TLC plate for immunostaining. After ginsenosides were 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 ginsenosides-BSA conjugate on the PVDF membrane. Figure 1-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*, respectively. Figure 1-C shows the Eastern blotting of double staining using anti-G-Rb1 and -Rg1 MAbs for ginsenoside standards. Moreover, we performed successive staining of the PVDF membrane using anti-G-Rg1 and then anti-G-Rb1. Finally we succeeded the double staining of ginsenosides indicating that G-Rg1 and -Re were stained as purple color and the other did blue color separately as indicated in Fig.1-C. From these result, both antibodies can distinguish individual aglycons, 20(*S*)-protopanaxatriol and 20(*S*)-protopanaxadiol. For this application the crude extracts of three *Panax* spp. were analyzed by the newly developed double staining system. Major ginsenosides can be determined clearly by the double staining method. G-Rb1, -Rc and -Rd possessing 20(*S*)-protopanaxadiol as an aglycon were stained as blue bands, and G-Re and -Rg1 did purple depending on the substrate (4-chloro-1-naphthol). Figure 1-D shows double staining of Eastern blotting for three *Panax* spp. When compared to single staining of Eastern blotting by anti-G-Rb1 MAb (Fig.1-B), it became evident that the quality of *Panax* spp. regarding ginsenosides was varying. *P. notoginseng* contained higher amount of G-Rg1, but G-Rc disappeared (Fig.1-D lane 4). Higher amount of G-Re and trace amounts of G-Rd and -Rg1 were observed in *P. quinquefolius* as indicated in Fig.1-D lane 5. Three bands stained as red color by anti-G-Rg1 MAb were found as indicated by arrows although they could not be detected by the staining with anti-G-Rb1 MAb. It is easily suggested that these bands might be related to G-Rg1 and G-Re having 20(*S*)-protopanaxatriol as an aglycon in a molecule. The survey of previous papers induced a conclusion that the three unknown bands could be determined to be G-Rh1, G-Rf

and 20-gluco G-Rf that are mono-, di- and triglycoside of 20(*S*)-protopanaxatriol respectively depending upon individual Rf values. This finding makes possible to suggest that the structures of aglycon can be recognized from band color, and Rf value is reflected from the sugar number. Depending upon this result, we determined two bands under G-Rb1 as MG-Rb1 and 20-*O*-gentiobiosyl-chikusetsusaponin III which has five sugars in a molecule in *P. ginseng* in good agreement with the Rf value previously reported. Finally these two compounds were identified by comparison with the authentic samples.

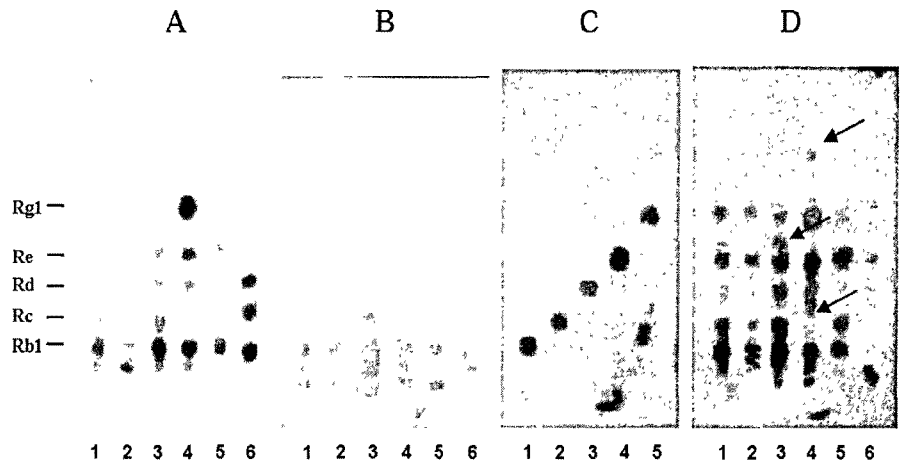

Fig.1. Eastern blotting of various *Panax* samples.

A: H_2SO_4 staining; B: Eastern blotting using anti-G-Rb1 MAb; D: Double stained Eastern blotting using anti-G-Rb1 and anti-G-Rg1 MAbs. Lane 1, 2, 3, 4, 5, and 6 indicate white ginseng, red ginseng, fibrous ginseng, *P notoginseng, P. quinquefolius*, and *P. japonicus* (60 □), respectively.

C: Double stained Eastern blotting of ginsenoside standards using anti-G-Rb1 and anti-G-Rg1 MAbs. Lane 1, 2, 3, 4, and 5 indicate G-Rb1, -Rc, -Rd, -Re, -Rg1 (3 □), respectively.

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