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Detection and quantification of ginsenoside Re in ginseng samples by a chromatographic immunostaining method using monoclonal antibody against ginsenoside Re

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Abstract

A chromatographic immunostaining method has been developed for the determination of ginsenoside Re (G-Re) in ginseng samples on a polyethersulphone (PES) membrane. G-Re standard and the extracts of ginseng roots were applied to a PES membrane and developed by methanol–water–acetic acid (45:55:1, by volume). G-Re was clearly detected by an immunostaining method using a monoclonal antibody against G-Re. The coloring spots of G-Re were analyzed quantitatively using NIH Image software indicating at least 0.125 μg of G-Re was detectable. G-Re can be analyzed quantitatively between 0.25 and 4.0 μg.

Keywords: Ginseng; Ginsenoside Re; Chromatographic immunostaining; NIH Image software

1. Introduction

Ginseng, the crude drug of *Panax ginseng*, is one of the most important Chinese medicines. It has been used to enhance stamina and 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 [1–4]. Major active components are the ginsenosides, which consist of protopanaxatriol or protopanaxadiol possessing a dammarane skeleton in their molecules (Fig. 1). Ginsenoside Re (G-Re) especially has an anti-diabetic effect [5] and is a major constituent of *Panax quinquefolium* (American Ginseng). In the United States, diabetes is the sixth leading cause of death [6] and *P. quinquefolium* is one of the top 10 selling natural health products [7]. It is well known that the percentages of individual ginsenosides vary in the ginseng root or the root extract depending on the method of extraction, subsequent treatment, or even the season of its collection [8,9]. Therefore, the use of standardized, authentic ginseng root both in research and by the public is to be advocated.

In our on-going study to prepare monoclonal antibodies (MAbs) for naturally occurring bioactive compounds, we prepared MAbs against ginsenoside Rb1 (G-Rb1) [10] and G-Rg1 [11] and applied to an enzyme-linked immunosorbent assay (ELISA) for G-Rb1 and G-Rg1 and developed eastern blotting technique for ginsenosides [12–14] which make it possible to visualize small molecule compounds on a PVDF membrane. However, because the transfer efficiency was not efficient, the eastern blotting method could not be applied for the quantitative immunoassay. In this paper, we report on the preparation of the anti-G-Re MAb, and its application in the development of a chromatographic immunostaining method for the quantitative analysis of G-Re arranged by the newly developed eastern blotting technique on a positive charged polyethersulphone (PES) membrane and NIH Image analysis software.

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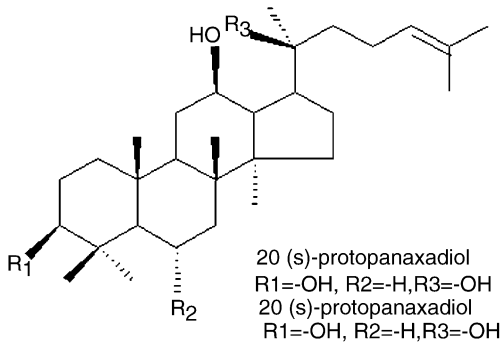

	Aglycone	R1	R2	R3
Ginsenoside Rb1	protopanaxadiol	-O-Glc-^{2}Glc	-H	-O-Glc-^{6}Glc
Ginsenoside Rc		-O-Glc-^{2}Glc	-H	-O-Glc-^{6}Glc
Ginsenoside Rd		-O-Glc-^{2}Glc	-H	-O-Glc
Ginsenoside Re	protopanaxatriol	-OH	-O-Glc-^{2}Rha	-O-Glc
Ginsenoside Rg1		-OH	-O-Glc	-O-Glc

Fig. 1. Structures of ginsenosides.

2. Experimental

2.1. Chemicals and immunochemicals

G-Rb1, G-Rc, G-Rd, G-Re and G-Rg1 were purchased from Wako Pure Chemical (Osaka, Japan). Bovine serum albumin (BSA) was provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was obtained from Organon Teknika Cappel Products (West Chester, PA, USA). Positive charged polyethersulphone (PES) membranes (Mustang E) was purchased from Pall Corporation (East Hills, NY, USA). All other chemicals were standard commercial products of analytical-reagent grade.

2.2. Plant material and extraction

The crude materials of various ginsengs were purchased from Nakai Koshindo (Kobe, Japan). Dried samples (50 mg) of various ginsengs were powdered, extracted five times with 0.5 ml methanol with sonication, and filtered using a Cosmonice filter W (0.45 μm Filter unit, Nacalai Tesque, Kyoto, Japan). The combined extracts were diluted with methanol for the chromatographic immunostaining and HPLC analyses.

2.3. Synthesis of antigen conjugate

G-Re-BSA conjugate was synthesized by a modification of the procedure of Erlanger and Beiser [15]. An 80% methanol solution (1.0 ml) of G-Re (10 mg) was added drop wise to a solution (0.5 ml) containing $NaIO_4$ (4 mg) and stirred at room temperature for 1 h. Carbonate buffer (50 mM, pH 9.6, 1.0 ml) containing BSA (10 mg) was added to the above reaction mixture and stirred at room temperature for 5 h. The reaction mixture was dialyzed against four changed of water for 2 days, and then lyophilized to produce G-Re-BSA conjugate (17 mg).

The hapten number in the G-Re-BSA conjugate was determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) as previously described [16].

2.4. Immunization, hybridization and purification of MAb

Six-week-old BALB/c female mice were immunized with the G-Re-BSA conjugate by an intraperitoneal injection of 50 μg of conjugate dissolved in phosphate-buffered solution (0.15 M NaCl in 10 mM potassium phosphate, pH 7.4, PBS) and emulsified in an equal volume of Freund's complete adjuvant. Two weeks later, the injection was repeated with the same amount of immunogen, but emulsified in Freund's incomplete adjuvant. Two and four weeks later, a booster dose of 100 μg conjugate dissolved in PBS without adjuvant was administered intraperitoneally. On the third day after the final immunization, the splenocytes were isolated and fused with a logarithmically growing hypoxanthine–aminopterin–thymidine (HAT)-sensitive mouse myeloma cell line, SP2/0-Ag14, by the polyethylene glycol (PEG) method [17]. Hybridomas producing MAbs reactive to G-Re were cloned by the limited dilution method [18]. Established hybridomas were cultured in enriched RPMI 1640-Dulbecco's-Ham's F12 (eRDF) medium supplemented with 9 μg/ml of insulin, 20 μg/ml of transferrin, 20 μM of ethanolamine and 25 nM of sodium selenite (ITES) [19].

MAb was purified using a Protein G FF column (Amersham Biosciences, Piscataway, NY, USA). The culture medium (200 ml) containing IgG was filtered and adjusted to pH 7.0 with 1 M Tris–HCl buffer solution (pH 9.0), applied to the column, and the column was washed with 20 mM phosphate buffer (pH 7.0). Adsorbed IgG was eluted with 100 mM citrate buffer (pH 2.7). The eluted IgG was neutralized with 1 M Tris–HCl buffer solution (pH 9.0), and dialyzed four times against water and lyophilized.

2.5. Chromatographic immunostaining method for G-Re in eastern blotting technique on PES membrane

Eastern blotting was performed as reported previously [20] except separation by thin-layer chromatography (TLC) and transfer to PVDF membrane, as follows.

G-Re and the extracts of ginseng were applied to a positive charged PES membrane. After dry, this membrane was hung in the tank and immersed and developed by methanol–water–acetic acid (45:55:1, by volume). The developed PES membrane was dried and dipped into water containing $NaIO_4$ (10 mg/ml) and stirred at room temperature for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA (1%) was added, and stirred at room temperature for 3 h. After washing the PES membrane with PBS, the membrane was treated with S-PBS for 2 h to reduce non-specific adsorption. The PES membrane was immersed in anti-G-Re MAb and stirred at room temperature for 3 h. After washing the PES membrane twice with T-PBS and water, a 1:1000 dilution of peroxidase-

labeled goat anti-mouse IgG in PBS containing 0.2% gelatin was added, and the mixture was stirred at room temperature for 1 h. The PES membrane was washed twice with T-PBS and water and then exposed to freshly prepared 1 mg/ml 4-chloro-1-naphthol–0.03% H_2O_2 in PBS for 40 min at room temperature. The reaction was stopped by washing with water, and the immunostained PES membrane was allowed to dry.

2.6. Image analysis system and image acquisition

A graphic analysis system, which consisted of a personal computer (Macintosh Power Book G4, Apple Computer Inc., Irvine, CA, USA), a public domain program NIH Image 1.62 (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/), a desktop scanner (GT-9700F, Seiko Epson Corporation, Nagano, Japan) and a scanning software Photoshop CS (Adobe Systems Inc., San Jose, CA, USA) was used.

Images were captured as 256 levels grayscale. The file size was 300 kb (200 dots/in. resolution). Digital output was transferred from the scanner to the computer and stored as a PICT file.

2.7. Image analysis using NIH Image software

The immunostained PES membrane grayscale image was loaded and thresholded to make a binary image for the separation of objects and background. Then, we chose the analyze menu options and selected the area, ellipse major axis and ellipse minor axis commands to calculate the objects area as previously described [21,22].

2.8. HPLC analysis for G-Re

The chromatograph used in this study consisted of a LC-10AD equipped with a SPD-10A vp UV detector (Shimadzu, Kyoto, Japan) and a Chromatopac C-R8A data analyzer system (Shimadzu, Kyoto, Japan). The analytical column was a Cosmosil 5C18-AR column (4.6 mm × 150 mm i.d., Nacalai Tesque, Kyoto, Japan) maintained at room temperature. The mobile phase was acetonitrile–water (20:80, by volume) containing 0.5% H_3PO_4, and the flow rate was 1.0 ml/min. UV absorbance detection at 202 nm was used and in this case retention times of G-Re was 19 min.

3. Results and discussions

In this study, we prepared the hybridomas showing reactivity with G-Re, a clone secreting IgG1 MAb having κ chain was successfully established after repeated subcloning by limited dilution method, and termed MAb-4G10. MAb-4G10 has cross-reactivities to ginsenosides with different affinities, as shown in Table 1. From this, we suggested that a part of sugar, especially C-20-glucose may be immunized and may function as an epitope for the structure of ginsenosides.Previously we succeeded the eastern blotting of G-Rb1 on a PVDF membrane using anti-G-Rb1 MAb [12]. In this methodology we separated the G-Rb1 molecule into two functional parts, the epitope part (mainly aglycone) and the sugar parts. The sugar parts in ginsenosides were oxidatively cleaved to release aldehyde groups which were conjugated with protein to fix on a PVDF membrane. The aglycone part of G-Rb1 was bound by the anti-G-Rb1 MAb as shown diagrammatically in Fig. 2. However, since separation of G-Rb1 and staining were impossible at the same time, we first separated G-Rb1 by TLC developing and transferred to PVDF membrane by blotting. The membrane was treated by $NaIO_4$ and then protein, and finally stained by anti-G-Rb1 MAb.

Table 1
Cross-reactivities (%) of MAb-4G10

Compound	Cross-reactivities
Ginsenoside Re	100
Ginsenoside Rg1	70.94
Ginsenoside Rd	76.23
Ginsenoside Rc	0.046
Ginsenoside Rb1	0.045
Saikosaponin a	<0.009
Digitonin	<0.009
Solasonine	<0.009
Deoxycholic acid	<0.009
Glycyrrhizin	<0.009
Ergosterol	<0.009
Solamargine	<0.009
Cholesterol	<0.009
β-Sitosterol	<0.009
Chikusetsusaponin IV	<0.009

The cross-reactivities of G-Re and related compounds were determined according to Weiler and Zenk's equation [23].

Here anti-G-Re MAb was applied for the new approach, a chromatographic immunostaining of G-Re utilizing a positive charged PES membrane instead of a PVDF membrane. PES membrane is widely used for the ultrafiltration system [24] and enzyme immobilization unit [25]. However, no success with immunostaining using PES membrane has been reported yet. Fortunately, we found that the positive charged PES membrane was suitable for the immunoblotting of G-Re (data not shown). We noticed that its intrinsic hydrophilicity and strong physical property against organic solvents may it possible to separate G-Re chromatographically on this membrane. G-Re and the crude extracts of ginseng roots were applied to a PES membrane and developed by various ratios of methanol–water or acetonitrile–water containing various ratio of acetic acid or formic acid. After dry, the membrane was treated by $NaIO_4$ solution to cleave the sugar moiety in G-Re and visualized using the eastern blotting technique. As shown in Fig. 3, G-Re and that of ginseng roots could be developed by methanol–water–acetic acid (45:55:1, by volume). On the other hand, a blank extract and extract containing structurally related compounds (i.e., G-Rg1, G-Rd, G-Rc and G-Rb1) indicated no band in this method (data not shown). The diffuse bands may be due to the presence of two co-migrating bands (G-Re and G-Rg1) which are inseparable after solvent separation on PES membrane.

From these results we applied the chromatographic immunostaining technique to the quantitative immunoassay using graphic

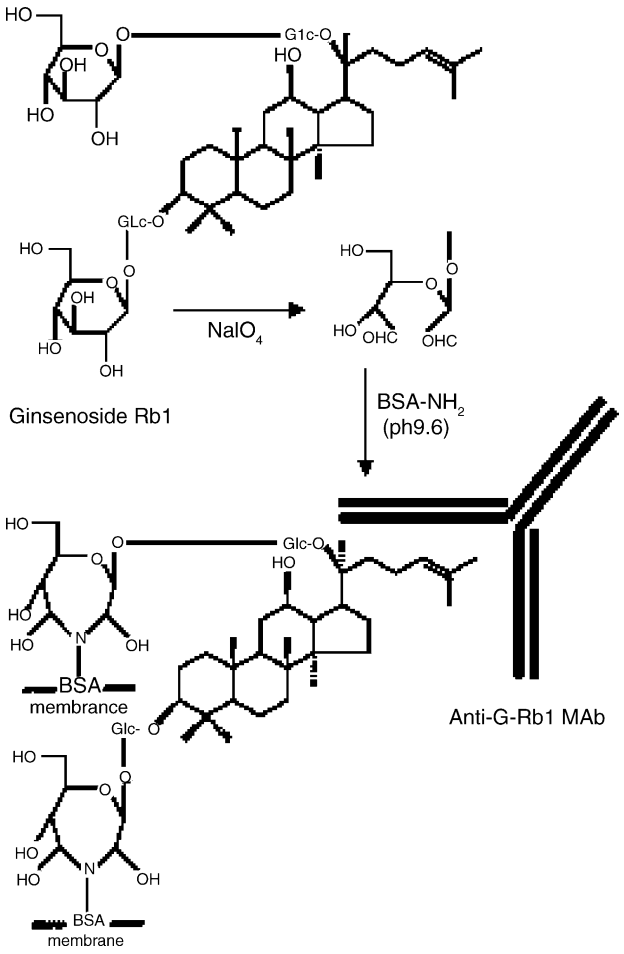

Fig. 2. Schematic diagram illustrating the eastern blotting of ginsenoside Rb1 onto membrane.

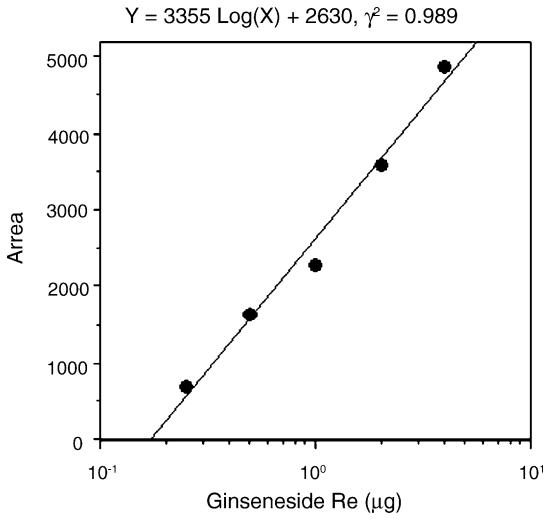

Fig. 4. Standard curve of ginsenoside Re using NIH Image.

analysis of NIH Image software because the newly established method reflected direct sample amounts without transfer efficiency. Fig. 4 shows the standard curve of G-Re by plotting the area against the logarithm of G-Re concentrations. Under these conditions, the full linear range of the assay was extended from 0.25 to 4.0 μg as indicated in Fig. 4. The detection limit of G-Re was 0.125 μg of direct applied amount.

Reproducibility and precision are important criteria for an immunoassay. Standard curve for the chromatographic separation of G-Re using the eastern blotting technique from 4 consecutive days were compared, and the variations were calculated. The variations between replicates from membrane to membrane (inter-assay) and from spot to spot (intra-assay) were measured (Table 2). It is typical that intra-assay variations are generally lower than inter-assay ones. Although many factors such as applying the spot, the multi-channel pipette, edge effects due to chromatographic separation, uneven temperature during incubation, and day-to-day variation in the preparation of reagents affected variations. However, they might be reduced when a new standard curve is prepared each time.

Various ginsengs were analyzed, and G-Re was detected by the chromatographic immunostaining method using MAb against G-Re on positive charged PES membrane (Fig. 3, lanes I–VI). The areas of coloring spots on this membrane were calculated using graphic analysis of NIH Image software. The

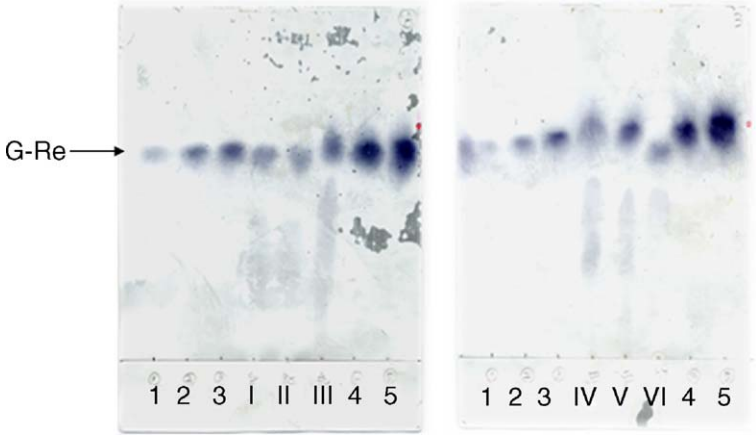

Fig. 3. Chromatographic immunostaining of ginsenoside Re in eastern blotting technique on polyethersulphone membrane. Lanes 1–5 indicate ginsenoside Re standard with the loaded amount being 0.25, 0.5, 1.0, 2.0 and 4.0 μg, respectively. Lanes I–VI indicate White ginseng [*P. ginseng*], Red ginseng [*P. ginseng*], Fibrous ginseng [*P. ginseng*], *P. notoginseng* [Sanchi ginseng], *P. quinquefolium* [American ginseng] and *P. japonicus* [Japanese ginseng], respectively.

Table 2
Variations among NIH Image runs for the analysis of ginsenoside Re

G-Re amount (μg)	CV (%)	
	Inter-assay	Intra-assay
0.25	8.57	5.37
0.5	9.83	4.61
1.0	8.92	0.67
2.0	9.15	3.85
4.0	10.12	6.40

The measured values are the mean ± standard deviation (SD) for four membranes and three replicate spots for each concentration within one membrane from 4 consecutive days. The variations in replicates from membrane to membrane and spot to spot are defined as inter- and intra-assay variation, respectively.

Table 3
Ginsenoside Re concentration in ginseng roots determined by NIH Image and HPLC analyses

Sample	Concentration (μg/mg dry weight powder)	
	NIH Image	HPLC
White ginseng	1.84 ± 0.58	1.23 ± 0.10
Red ginseng	1.82 ± 0.57	1.16 ± 0.09
Fibrous ginseng	8.68 ± 0.70	8.28 ± 0.11
P. notoginseng	4.23 ± 1.22	3.58 ± 0.06
P. quinquefolium	12.94 ± 2.26	16.63 ± 0.75
P. japonicus	0.66 ± 0.35	0.25 ± 0.02

Data are the mean of triplicate assays ± S.D. ND, not detectable.

concentrations of G-Re in ginseng samples determined by NIH Image agreed well with that determined by HPLC (Table 3) and the least-squares fit had a coefficient of determination (γ^2) of 0.987.

4. Conclusion

Many analytical approaches have been used to identify G-Re in ginseng extracts. Among these methods, the use of HPLC in conjunction with mass spectrometry (MS or MS/MS) is most frequently and widely used [9,26,27]. The advantages of the new approach over the HPLC method are mainly its better cost-performance ratio (e.g., organic solvents and analytical equipment), speed and ease of use, which are useful if large numbers of small samples are to be analyzed. In our conditions, the determination of G-Re concentration in six varieties of ginseng roots were required 1000 min and 300 ml of organic solvents using by HPLC method; in contrast NIH Image analysis did not need as much as those of HPLC analysis.

This is apparently the first report of MAb against G-Re and its application to the chromatographic immunostaining method for G-Re utilizing positive charged PES membrane. Although it has been believed difficult to determine the concentrations of small molecular compounds by western blotting technique, the methodology described here may open a wide field of comparable studies with other families of carbohydrates containing compounds of low molecular weight, such as saponins, glycosides, glucuronides, aminosugar conjugates and/or glycolipids and glycosphingolipids.

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