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A method has been devised for the chromatographic resolution of glucosidic compounds, ginsenosides, on polyethersulphone (PES) membrane. The method results in good resolution and quantitative immunoassay for ginsenoside Rb1 (G-Rb1), G-Rc, and G-Rd in crude extracts of various ginsengs. The newly established method is simpler and applies for quantitative analysis. Ginsenosides developed by acetonitrile–water–acetic acid solvent system on a PES membrane were directly treated with a NaIO₄ solution followed by bovine serum albumin (BSA), resulting in a ginsenoside–BSA conjugate on a PES membrane. Anti-G-Rb1 monoclonal antibody (MAb) was bound, and then a second antibody labeled with peroxidase directed against the first antibody. Finally a substrate reacted to the enzyme and gave staining. The stained membrane was scanned, and spots were analyzed quantitatively using NIH Image software. At least 62.5 ng of G-Rb1, G-Rc, and G-Rd were clearly detectable individually. Three ginsenosides can be analyzed quantitatively between 0.125 and 2.0 µg.

Key words: chromatographic resolution/eastern blotting/ginsenosides monoclonal antibody/NIH Image software

Introduction

In the continuation of immunostaining of several compounds, Granger et al. (1988) and Reig and Klein (1988) found a polyvinyliden difluoride (PVDF) membrane which is most widely used in the analysis of proteins in western blotting experiments (Towbin et al., 1979). Regarding small molecule compounds, Towbin et al. (1984) reported immunostaining of glycosphingolipids transferred from a thin-layer chromatography (TLC) plate to a nitrocellulose membrane, but the transfer efficiency and the reproducibility were low. Uemura et al. (1983), Kundra et al. (1983), and recently Suetake and Yu (2003) and Meisen et al. (2004) succeeded in the direct immunostaining on a TLC plate only limited to glycolipids and glycosphingolipids because the other small molecule compounds are easily washed out by buffer solution without fixing.

We have investigated the immunostaining of naturally occurring bioactive glucosides in our ongoing studies of preparation of monoclonal antibodies (MAbs) and their applications. We have developed improved western blotting techniques with new immunostaining methodology for the identification of glucoalkaloids (Tanaka et al., 1997), ginsenoside Rb1 (G-Rb1), G-Rc, and G-Rd (Fukuda et al., 1999, 2000, 2001), eastern blotting technique (Shan et al., 2001) which make it possible to visualize small molecule compounds on a PVDF membrane. In this method, we blotted glucosides from a TLC plate developed by solvent system to a PVDF membrane and separated the glucoside molecule into two functional parts, the epitope and sugar parts. The sugar parts in glucosides were oxidatively cleaved to give aldehyde groups which were conjugated with carrier protein to fix on a PVDF membrane. However, because the transfer efficiency was not efficient, the method could not be applicable for the quantitative immunoassay.

In this article, we describe a new approach for the chromatographic resolution of glucosidic compounds, ginsenosides in crude extracts of various ginsengs on a PES membrane, and apply to the quantitative immunoassay arranged by the newly developed eastern blotting technique and NIH Image software system for G-Rb1, G-Rc, and G-Rd by using a single MAb, anti-G-Rb1 MAb.

Results

Eastern blotting for G-Rb1, G-Rc, and G-Rd

Previously we succeeded in the eastern blotting of G-Rb1 on a PVDF membrane using anti-G-Rb1 MAb which resulted in staining together with G-Rc and G-Rd (Fukuda et al., 2001). The structures of the above ginsenosides are shown in Figure 1.

Seven membranes were obtained in the market and their suitabilities were tested for the eastern blotting as indicated in Table I. Although we previously reported that positive-charged PVDF membrane was suitable for the eastern blotting (Tanaka et al., 1997), the transfer efficiency was variable indicating it impossible for quantitative analysis. Therefore, the direct development of glucosides by solvent system without transfer from a TLC plate was needed. Because positive-charged PES membrane is also good for eastern blotting as indicated in Table I and no success of immunoblotting has been reported, we investigated the eastern blotting using PES membrane without transfer from a TLC plate. Figure 2 shows the eastern blotting of...
ginsenoside standards, G-Rb1, G-Rc, G-Rd, and the crude extract of Panax quinquefolium (American ginseng) root on the positive-charged PES membrane using anti-G-Rb1 MAb. The result suggests that the positive-charged PES membrane is suitable for the eastern blotting, and its hydrophilic property may be applied to the chromatographic resolution of ginsenosides on this membrane.

**Chromatographic resolution of ginsenosides on PES membrane**

G-Rb1, G-Rc, G-Rd, and the crude extract of American ginseng root were applied to a PES membrane and developed by various ratios of methanol–water or acetonitrile–water containing various ratios of acetic acid. As shown in Figure 3, all ginsenoside standards and that of American ginseng could be developed by acetonitrile–water–acetic acid (25:75:1, by volume). G-Rb1, G-Rc, and G-Rd can be detected in at least in 62.5 ng level (Figure 3A–C).

**Assay sensitivity and assay variation**

We applied the chromatographic resolution in eastern blotting technique to the quantitative immunoassay for G-Rb1, G-Rc, and G-Rd using graphic analysis of NIH Image software because newly established method reflected direct sample amounts without transfer efficiency. Figure 4 shows the standard curves of ginsenosides by plotting the area against the logarithm of ginsenoside concentrations. Under these conditions, the full linear range of the assay was extended from 0.125 to 2.0 µg as indicated in Figure 4.

Reproducibility and precision are important criteria for an immunoassay. Standard curves for the chromatographic resolution of G-Rb1, G-Rc, and G-Rd in eastern blotting technique from four consecutive days were compared, and the variations were calculated (Figure 4). The variations between replicates from membrane to membrane...
Chromatographic resolution of glucosidic compounds (interassay) and spot to spot (intra-assay) were measured (Table II). It is typical that intra-assay variations are generally lower than interassay. Although many factors such as applying spot, multichannel pipette, edge effects due to chromatographic resolution, 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.

Quantitative and qualitative analysis of ginsenosides by chromatographic resolution in eastern blotting technique, high-performance liquid chromatography, and NIH Image analyses in crude extractions of various ginsengs

Various ginsengs were analyzed, and G-Rb1, G-Rc, and G-Rd were detected by the chromatographic resolution of ginsenosides in eastern blotting technique (Figure 5). The areas of coloring spots on this membrane were calculated using graphic analysis of NIH Image software as described above. Fibrous ginseng, Panax notoginseng (Sanchi ginseng), and American ginseng contained high levels of ginsenosides (Table III). Panax japonicus (Japanese ginseng) contained only G-Rb1. These results were in a good agreement with those from the high-performance liquid chromatography (HPLC) analysis (Table III).

Discussion

Ginsenosides (ginseng saponins) are known to be the bioactive components of various ginsengs. According to the difference in the aglycone in these saponins, ginsenosides are classified into three types: the 20-(S)-protopanaxadiol type (e.g., G-Rb1, G-Rc, G-Rb2, and G-Rd), the 20-(S)-protopanaxatriol type (e.g., G-Rg1, G-Rf, and G-Re), and the oleanolic acid type (e.g., G-R0). We reported here the application of the eastern blotting technique for the quantitative and qualitative analysis of G-Rb1, G-Rc, and G-Rd as marker components of various ginsengs.

Previously we succeeded the eastern blotting of G-Rb1 on a PVDF membrane using anti-G-Rb1 MAb resulting in staining together with G-Rc and G-Rd (Fukuda et al., 2001). 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. Because it was evident that a part of sugar moiety in G-Rb1 was immunized, the cleavage of sugar moiety by NaIO4 expanded its cross-reactivity against other ginsenosides resulting in possibility of staining for G-Rc and G-Rd, though anti-G-Rb1 MAb had weak cross-reactivity with G-Rc and G-Rd (0.024% and 0.020%, respectively).

In a new approach, we demonstrated the immunoblotting of ginsenosides on the positive-charged PES membrane instead of a PVDF membrane. PES membranes are widely used for the ultrafiltration system (Duarte et al., 2003) and enzyme immobilization unit (Gomes et al., 2004). However, no success with immunoblotting using PES membrane has been reported yet. Fortunately, we found that the positive-charged PES membrane was suitable for the immunoblotting of ginsenosides. This membrane is a highly cross-linked quaternized amine charge polymer coating based on polyethyleneimine on a 0.2-µm pore size membrane. This gives very high dynamic capacities under selected conditions for the removal of endotoxin from process feedstreams, buffer and water. We noticed its intrinsic hydrophilicity and strong physical property against organic solvents may be applied to the chromatographic resolution of ginsenosides.
on this membrane. Although we tested various ratios of methanol–water containing various ratios of acetic acid for the solvent system, acceptable resolution data was not obtained (data not shown). However, from this we found its reverse phase property and tested various ratios of acetonitrile–water containing various ratios of acetic acid just following HPLC mobile phase of ginsenosides. All ginsenoside standards and American ginseng root extract were separated well with acetonitrile–water–acetic acid (25:75:1, by volume) and when directly applied their individual detection limit were 62.5 ng. From these results, we applied the chromatographic resolution in eastern blotting technique to the quantitative immunoassay using graphic analysis of NIH Image software calculating the areas of coloring spots on membrane. Assay sensitivity and assay variation of this new method were estimated to be useful for the quantitative analysis of ginsenosides.

The other important merit is the same as PVDF membrane depending on the increase of cross-reactivity as reported in the previous paper (Fukuda et al., 1999). Almost all 20-(S)-protopanaxadiol type ginsenosides can be stained by using anti-G-Rb1 MAb. Therefore, we can distinguish what kind of aglycon it has and how many sugar it contains in a molecule depending on their Rf value. Such information make it possible to suggest the structure of ginsenoside easily. However, the reactivity of anti-G-Rb1 MAb does not cross over to 20-(S)-protopanaxatriol and oleanolic acid type saponins indicating that the specificity still remains though it expanded.

In addition, ginsenosides in crude extracts of various ginsengs were determined using the combination assay

### Table II. Variations among NIH Image runs for the analysis of ginsenosides

<table>
<thead>
<tr>
<th>Amount (µg)</th>
<th>CV (%)</th>
<th>Ginsenoside Rb1</th>
<th>Ginsenoside Rc</th>
<th>Ginsenoside Rd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inter-assay</td>
<td>Intra-assay</td>
<td>Inter-assay</td>
<td>Intra-assay</td>
</tr>
<tr>
<td>0.25</td>
<td>11.78</td>
<td>5.36</td>
<td>8.56</td>
<td>6.39</td>
</tr>
<tr>
<td>0.5</td>
<td>9.82</td>
<td>2.60</td>
<td>7.34</td>
<td>0.73</td>
</tr>
<tr>
<td>1.0</td>
<td>8.91</td>
<td>0.51</td>
<td>4.60</td>
<td>0.66</td>
</tr>
<tr>
<td>2.0</td>
<td>9.14</td>
<td>2.05</td>
<td>10.13</td>
<td>3.84</td>
</tr>
</tbody>
</table>

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

### Table III. Ginsenosides concentration of various ginsengs determined by NIH Image and high-performance liquid chromatography (HPLC)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/mg dry weight powder)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ginsenoside Rb1</td>
</tr>
<tr>
<td></td>
<td>NIH Image</td>
</tr>
<tr>
<td>White ginseng</td>
<td>2.28 ± 0.08</td>
</tr>
<tr>
<td>Red ginseng</td>
<td>3.51 ± 0.15</td>
</tr>
<tr>
<td>Fibrous ginseng</td>
<td>16.94 ± 0.32</td>
</tr>
<tr>
<td><em>Panax notoginseng</em></td>
<td>14.35 ± 0.64</td>
</tr>
<tr>
<td><em>Panax quinquefolium</em></td>
<td>12.92 ± 0.15</td>
</tr>
<tr>
<td><em>Panax japonicus</em></td>
<td>2.62 ± 0.33</td>
</tr>
</tbody>
</table>

HPLC, high-performance liquid chromatography; ND, not detectable. Data are the means of triplicate assays.
system of chromatographic resolution in eastern blotting technique and NIH Image analysis. The contents determined by newly established method agreed well with those determined by HPLC. Many analytical approaches have been used to identify ginsenosides in ginseng extracts. Among these methods, the use of HPLC in conjunction with mass spectrometry (MS) or tandem MS (MS/MS) is most frequently and widely used (Chan et al., 2000; Harkey et al., 2001). The advantages of the new approach over the HPLC method are mainly its saving cost-performance (e.g., organic solvents and analytical equipments), speed, and ease of use, which are useful if large numbers of smaller samples are to be analyzed. Moreover, the assay system described here can analyze quantitatively and/or qualitatively at least three ginsenosides, G-Rb1, G-Rc, and G-Rd by using a single MAb, anti-G-Rb1 MAb.

This is apparently the first report of the chromatographic resolution of ginsenosides in eastern blotting technique on positive-charged PES membrane and its application. Although it has been believed difficult to determine the concentrations of small molecular compounds by western and/or eastern blotting, and 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.

Materials and methods

Chemicals and immunochemicals

G-Rb1, G-Rc and G-Rd were purchased from Wako Pure Chemical (Osaka, Japan). Bovine serum albumin (BSA) was provided by Pierce (Rockford, IL). Peroxidase-labeled anti-mouse IgG was obtained from Organon Teknika Cappel Products (West Chester, PA). PVDF membranes (Immobilon-N and Immobilon-P) were purchased from Millipore Corporation (Bedford, MA). Nitrocellulose membrane (Bio Trace), nylon membranes (N6, 6 Posidyne and Biodyne), and PES membranes (Mustang E and Mustang Q) were purchased from Pall Corporation (East Hills, NY). Glass microfiber filter sheets (GF/A) were purchased from Whatman International ( Maidstone, UK). All other chemicals were standard commercial products of analytical reagent grade.

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 Costomonic filter W (0.45-µm Filter unit, Nacalai Tesque, Kyoto, Japan). The combined extracts were diluted with methanol for the eastern blotting, HPLC, and NIH Image analyses.

MAb against G-Rb1

Immunization and hybridization of MAb against G-Rb1 (9G7) have been described previously (Tanaka et al., 1999). Anti-G-Rb1 MAb was purified using a Protein G FF column (Amersham Biosciences, Piscataway, NY). The culture medium (125 ml) containing IgG was 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.

Eastern blotting for G-Rb1, G-Rc, and G-Rd on PES membrane

Eastern blotting was performed as reported previously (Tanaka et al., 1997), as follows. G-Rb1, G-Rc, G-Rd, and the extracts of ginseng were applied to a silica gel TLC plate and developed by n-butanol–ethanol–water (15:1:4, by volume). The developed TLC plate was dried and then sprayed with a blotting solution mixture of 2-propanol–methanol–water (1:4:16, by volume). It was placed on a stainless steel plate and covered with a piece of PES membrane. After covering with a glass microfiber filter sheet, the whole assembly was pressed evenly for 50s with a 120°C hot plate as previously described (Taki et al., 1994) with some modifications. The PES membrane was separated from the TLC plate and dried.

The blotted PES membrane was dipped into water containing NaI04 (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 phosphate buffer, the membrane was treated with phosphate buffer containing 5% skim milk for 2 h to reduce nonspecific adsorption. The PES membrane was immersed in anti-G-Rb1 MAb and stirred at room temperature for 3 h. After washing the PES membrane twice with phosphate buffer containing 0.05% Tween 20 (T-PBS) and water, a 1:1000 dilution of peroxidase-labeled goat anti-mouse IgG in phosphate buffer 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% H2O2 in phosphate buffer for 15 min at room temperature. The reaction was stopped by washing with water, and the immunostained PES membrane was allowed to dry.

Chromatographic resolution of G-Rb1, G-Rc, and G-Rd on PES membrane

G-Rb1, G-Rc, G-Rd, and the extract of American ginseng root were applied to a PES membrane and developed by various ratios of methanol–water or acetone–water containing various ratios of acetic acid as follows. Methanol–water (100:0, 75:25, 50:50, and 25:75, by volume, respectively) containing acetic acid (2.0%, 1.0%, 0.5%, and 0%, respectively). Acetone–water (100:0, 75:25, 50:50, and 25:75, by volume, respectively) containing acetic acid (2.0%, 1.0%, 0.5%, and 0%, respectively).

HPLC instruments and conditions for G-Rb1, G-Rc, and G-Rd

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 × 150 mm internal diameter, Naclalai Tesque, Kyoto, Japan) maintained at room temperature. The mobile phase was acetonitrile–water (30:70, by volume) containing 50 mM KH2PO4, and the flow rate was 1.0 ml/min. UV absorbance detection at 202 nm was used for monitoring the effluent. In this condition, retention times of G-Rb1, G-Rc, and G-Rd were 13, 17, and 37 min, respectively.

Image analysis system and image acquisition
A graphic analysis system, which consisted of a personal computer (Macintosh Power Book G4, Apple Computer, Irvine, CA), a public domain program NIH Image 1.62 (developed at the US 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, San Jose, CA) was used.

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

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 (Masters et al., 1992; Root and Wang, 1993).

Acknowledgments
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Abbreviations
BSA, bovine serum albumin; G-Rb1, ginsenoside Rb1; HPLC, high-performance liquid chromatography; MAb, monoclonal antibody; PES, polyethersulphone; PVDF, polyvinylidene difluoride; TLC, thin-layer chromatography.

References