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Eastern Blotting and Immunoaffinity Concentration Using Monoclonal Antibody for Ginseng Saponins in the Field of Traditional Chinese Medicines

HIROYUKI TANAKA, NORIKO FUKUDA, AND YUKIHIRO SHOYAMA*

Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Ginsenosides separated by silica gel TLC blotted to a PVDF membrane that was treated with a $NaIO_4$ solution followed by bovine serum albumin (BSA) resulted in a ginsenoside−BSA conjugate on a PVDF membrane. The blotted spots were stained by anti-ginsenoside Rb1 (G-Rb1) and -Rg1 (G-Rg1) monoclonal antibodies (MAbs). The newly established immunostaining method, Eastern blotting, was applied for the determination of ginsenosides possessing protopanaxadiol and/or protopanaxatriol in the traditional Chinese medicine (TCM). This method developed a new way to separate the ginsenoside molecule into two functional parts using a simple and well-known chemical reaction. The sugar parts were oxidized by $NaIO_4$ to give dialdehydes, which reacted with amino groups of the protein and covalently bound to the adsorbent PVDF membrane. The MAb bound to the aglycon part of the ginsenoside molecule for immunostaining. Double staining of Eastern blotting for ginsenosides using anti-G-Rb1 and -Rg1 MAbs promoted complete identification of ginsenosides in *Panax* species. The immunoaffinity concentration of G-Rb1 was deteremined by immunoaffinity column conjugated with anti-G-Rb1 MAb leading to the knock-out extract, which will be useful for the pharmacological investigation. To concentrate and determine G-Rb1 in *P. japonicus*, the crude extract of *P. japonicus* was fractionated by immunoaffinity column conjugated with anti-G-Rb1 MAb. Two ginsenosides, chikusetsusaponins III and IV having protopanaxadiol as an aglycon, were identified by Eastern blotting, although it was expected that G-Rb1 might be a component of *P. japonicus* by enzyme-linked immunosorbent assay (ELISA) analysis.

KEYWORDS: Ginseng saponins; Chinese medicine; immunoaffinity

INTRODUCTION

With the rapid development of the molecular biosciences and their biotechnological applications, immunoassays using monoclonal antibodies (MAbs) against drugs and low molecular weight bioactive compounds have become an important tool, due to their specificity, for receptor binding analyses, enzyme assays, and quantitative and qualitative analytical techniques in both animals and plants. On the other hand, the immunoblotting method is based on a Western blotting technique that utilizes antigen−antibody binding properties and has provided a specific and sensitive detection of higher molecule analyte such as peptides and proteins. Previously we have prepared many MAbs against naturally occurring bioactive compounds such as forskolin (*1*), crocin (*2*), solamargine (*3*), opium alkaloids (*4*), marijuana compounds (*5*), ginsenosides (*6, 7*), saikosaponin a (*8*), paeoniflorin (*9*), sennosides (*10, 11*), ginkgolic acid (*12*), glycyrrhizin (*13*), and berberine (*14*) and established individual competitive enzyme-linked immunosorbent assays (ELISAs) as highly sensitive, specific, and simple methodologies. As an extension of this approach, an immunostaining method using anti-solamargine MAb was established by us (*15*).

Ginseng, the crude drug of *Panax ginseng* root, is one of the most important components of traditional Chinese medicine (TCM). 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, hypothermia, carbohydrate and lipid metabolism, immune function, the cardiovascular system, and radioprotections (*16*). Its major active components are the ginsenosides, which consist of protopanaxatriol and/or protopanaxadiol possessing a dammarane skeleton in their molecules. It is well-known that the concentrations of ginsenosides vary in the ginseng root or the root extracts depending on the method of extraction, subsequent treatment (*17*), or even the season of its collection (*18*). Therefore, standardization of quality is required in the field of TCM. On the other hand, *Panax japonicus* is morphologically different from *P. ginseng*. It became evident that major components were

* Author to whom correspondence should be addressed (e-mail shoyama@phar.kyushu-u.ac.jp). Present address: Faculty of Pharmaceutical Science, Nagasaki International University, 2825-7 Hausutenbosu, Sasebo-city, Nagasaki 859-3298, Japan (e-mail Shoyama@niu.ac.jp).

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oleanane-type saponins such as chikusetsusaponins; therefore, the content of ginsenosides might be low.

To control the quality of ginseng prescribed in TCM, we previously prepared anti-G-Rb1, -G-Rg1, and -G-Re MAbs, respectively (*6*, *7*), and set up the ELISA, a new staining method, Eastern blotting for ginsenosides, and an immunoaffinity concentration method for quantitative analysis for the samples containing lower concentrations of G-Rb1 (*19*).

In our ongoing studies on MAbs against naturally occurring bioactive compounds, we review here a new Eastern blotting method for ginsenosides in TCM-prescribed ginseng and the double staining for ginsenosides in the crude drug of *Panax* species using anti-G-Rb1 and -Rg1 MAbs. Furthermore, the immunoaffinity concentration of G-Rb1 by immunoaffinity column is also discussed.

EXPERIMENTAL METHODS

Eastern Blotting (*15*). Ginsenosides were applied to a silica gel TLC plate and developed with *n*-BuOH/EtOAc/H_2O (15:1:4). The developed TLC plate was dried and then sprayed with a blotting solution mixture of *i*-PrOH/MeOH/H_2O (1:4:20 by volume). It was placed on a stainless steel plate and covered with a piece of PVDF membrane. After glass microfiber filter sheet had been placed over the plate, the whole assembly was pressed evenly for 50 s with a 120 °C hot plate as previously described (*15*) with some modifications. The PVDF membrane was separated from the TLC plate and dried.

The blotted PVDF membrane was dipped in water containing $NaIO_4$ and stirred at room temperature for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA was added and stirred at room temperature for 3 h. After the PVDF membrane had been washed with PBS, the membrane was treated with PBS containing 5% skim milk for 3 h to reduce nonspecific adsorption. The PVDF membrane was immersed in anti-G-Rb1 MAb and stirred at room temperature for 1 h. After the PVDF membrane had been washed twice with PBS containing 0.05% Tween 20 and water, a 1:1000 dilution of peroxidase-labeled goat anti-mouse IgG in PBS containing 0.2% gelatin was added and stirred at room temperature for 1 h. The PVDF membrane was washed twice with TPBS and water and then exposed to freshly prepared 1 mg/mL 4-chloro-1-naphthol/0.03% H_2O_2 in PBS for 10 min at room temperature. The reaction was stopped by washing with water, and the immunostained PVDF membrane was allowed to dry.

For staining by anti-G-Rg1 MAb, the blotted PVDF membrane was treated in the same way as anti-G-Rb1 MAb except that it was exposed to 0.2 mg/mL of 3-amino-9-ethylcarbazole/0.03% H_2O_2 in acetate buffer (0.05 M, pH 5.0) containing 5% *N*,*N*-dimethylformamide.

Preparation of Immunoaffinity Column Using Anti-G-Rb1 MAb and Immunoaffinity Concentration of G-Rb1 (*19*). Purified anti-G-Rb1 MAb in diluted Bio-Rad Affi-Gel Hz coupling buffer was dialyzed against the coupling buffer two times. $NaIO_4$ solution was added to the MAb solution and stirred gently at room temperature. After the reaction, glycerol was added to the reaction mixture and stirred for the inactivation of $NaIO_4$ and then dialyzed. Affi-Gel Hz Hydrazide gel was added to the above reaction mixture and reacted, resulting in hydrazone gel, and immunoaffinity gel, which was packed into a plastic minicolumn.

The extracts of ginseng roots were redissolved in PBS and then filtered with a 0.45 μm MILEX-HV filter (Millipore) to remove insoluble portions. The filtrate was loaded on the immunoaffinity column and allowed to stand overnight at 4 °C. The column was washed with the washing buffer solution (40 mL) and then eluted with 100 mM AcOH buffer containing 0.5 M KSCN and 20% MeOH (pH 4.0). The G-Rb1 containing fraction was used for ELISA to determine its concentration and then subjected to TLC with $CHCl_3$/MeOH/H_2O (7:4:1) and *n*-BuOH/AcOH/H_2O (15:1:4) as developing solvents, followed by Eastern blotting.

Concentration of ginsenoside from the crude extracts of *P. japonicus* root by immunoaffinity column was carried out in the same way as for *P. ginseng* as described above. Individual fractions containing ginsenosides were performed for Eastern blotting as indicated above.

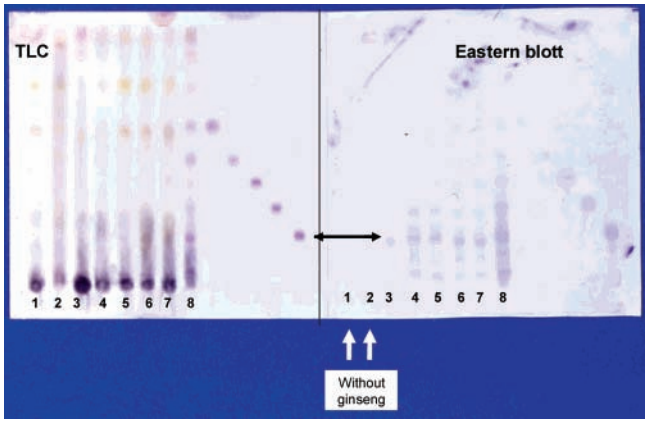

Figure 1. Eastern blotting of ginsenosides in traditional Chinese medicine (TCM) prescriptions by anti-ginsenoside Rb1 MAb. Samples: 1, Kikyoto; 2, Daiokanzoto; 3, Ninjin'yoeito; 4, Shikunshito; 5, Ninjinto; 6, Hange-shashinto; 7, Shosaikoto; 8, crude extract of ginseng. Samples 1 and 2 do not contain ginseng. Standard of ginsenosides indicated G-Rg1, -Re, -Rd, -Rc and -Rb1 from upper.

RESULTS AND DISCUSSION

Eastern Blotting of Ginsenosides. Although Western blotting is a common assay methodology for substances of high molecular weight, this method has not been applied for small molecules, as direct immunostaining of such compounds on a TLC plate is yet unknown. Therefore, a new method for such small molecular compounds is needed. Moreover, if small molecules can be blotted to a membrane, the fixing on it also requires a new methodology. Previously we succeeded in separating the function of small molecule compounds such as solasodine glycosides into a part of epitope and the fixing on membrane as follows (*15*). The PVDF membrane blotted was treated with $NaIO_4$ solution. This reaction enhanced the fixing of solasodine glycoside via solasodine glycoside−BSA conjugates on PVDF membrane. The PVDF membrane incubated in the absence of $NaIO_4$ was essentially free of staining for solasodine glycoside. From this finding we have applied this new methodology to various glycosides such as glycyrrhizin (*13*) and saikosaponins (*20*). In this paper we investigate the Eastern blotting of ginsenosides.

Figure 1 shows the H_2SO_4 staining and Eastern blotting of ginsenoside standards and the TCM using anti-G-Rb1 MAb. It is impossible to determine the ginsenosides by TLC stained by H_2SO_4 because of the complicated profile as indicated in **Figure 1**. On the other hand, clear staining of G-Rb1 occurred by Eastern blotting. Although H_2SO_4 staining detected all standard compounds, Eastern blotting indicated only limited staining of G-Rb1 and two other ginsenosides, G-Rc and -Rd, of which cross-reactivities were 0.02% as shown in **Figure 1**. The Eastern blotting method was considerably more sensitive than H_2SO_4 staining. Furthermore, it became evident that Kikyoto and Daiokanzoto prescriptions, which did not contain ginseng, indicated no spot of G-Rb1. We suggested that an aglycon, protopanaxadiol, and a part of the sugars may be of importance to the immunization and may function as an epitope in the structure of ginsenosides. In addition, it is suggested that the specific reactivity of the sugar moiety in the ginsenoside molecule against anti-G-Rb1 MAb may be modified by $NaIO_4$ treatment of ginsenosides on the PVDF membrane, causing G-Rc and -Rd to become detectable by Eastern blotting.

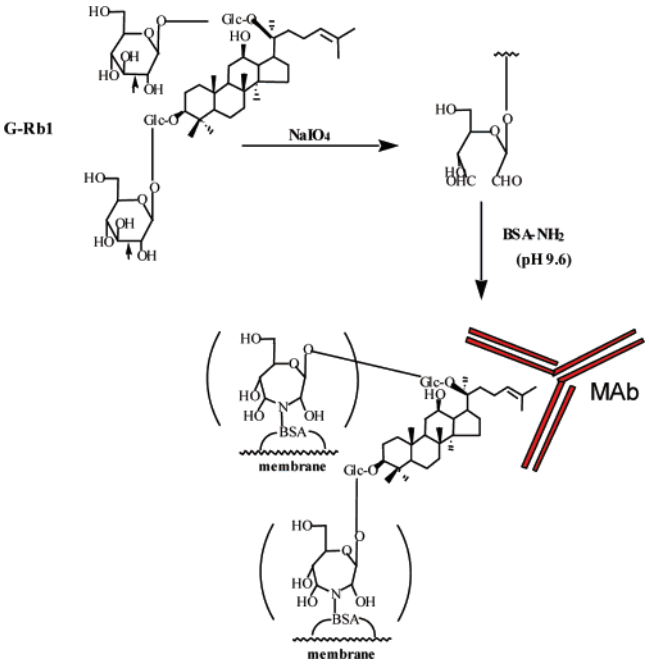

Figure 2. Mechanism of Eastern blotting.

We report here a new methodology to separate the G-Rb1 molecule into two functional parts. The sugar parts are oxidized to give dialdehydes, which react with amino groups of lysine and/or arginine of the protein that can bind strongly to the adsorbent membrane, PVDF. The aglycon part of the G-Rb1 molecule is bound by the anti-G-Rb1 MAb for visualization of G-Rb1 by the enzyme-labeled specific antibody. The method is shown diagrammatically in **Figure 2**.

When the mixture of anti-G-Rg1 and -Rb1 MAbs and the pair of substrates were tested for staining of ginsenosides, all ginsenosides, G-Rg1, -Re, -Rd, -Rc, and -Rb1, were stained blue (data not shown), although the purple staining for G-Rg1 was expected because 3-amino-9-ethylcarbazole was used as a substrate. It is easily suggested that the sensitivities of substrate between 3-amino-9-ethylcarbazole and 4-chloro-1-naphtol might be different. Therefore, we performed successive staining of the membrane using anti-G-Rg1 and then anti-G-Rb1. Finally, we succeeded in the double staining of ginsenosides, indicating that G-Rg1 and -Re were stained purple and the others blue as indicated in **Figure 3**. From this result both antibodies can distinguish the individual aglycons, protopanaxatriol and protopanaxadiol. For this application the crude extracts of various *Panax* species were analyzed by the double-staining system (*21*), allowing all ginsenosides to be determined clearly as indicated in **Figure 3**.

Interestingly, this makes it possible to suggest that the staining color shows the pharmacological activity; for example, the purple spots indicate ginsenosides having the stimulation activity for the central nervous system. On the other hand, the blue color indicated ginsenosides possessing the depression effect for the central nervous system. Moreover, the R_f value of ginsenoside roughly suggests the number of sugars attached to the aglycon. Therefore, both analyses make it possible to suggest that the aglycon attached and the number of sugars combined elucidate the structure of ginsenosides.

As a further application we surveyed Araliaceae species by Eastern blotting using anti-G-Rb1 MAb as indicated in **Figure 4**. ELISA analysis and the Eastern blotting profile of *Kalopanax pictus* Nakai (**Figure 4B**, line 13, as indicated by an arrow) suggest that this species may contain G-Rb1. Depending on this

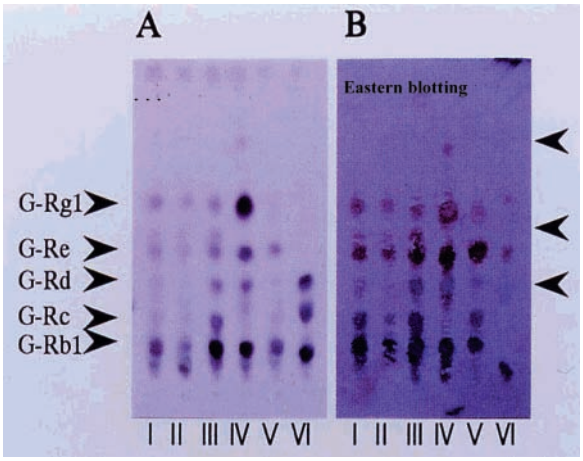

Figure 3. Double staining of Eastern blotting for ginsenosides contained in various ginseng samples using anti-G-Rb1 and anti-G-Rg1 monoclonal antibodies: (**A**) TLC profile stained by sulfuric acid; (**B**) Eastern blotting by anti-G-Rb1 and anti-G-Rg1 monoclonal antibodies I, II, III, IV, V, and VI indicated white ginseng, red ginseng, fibrous ginseng (*Panax ginseng*), *Panax notoginseng*, *Panax quinquefolius*, and *Panax japonicus*, respectively. Upper purple color spots and lower blue color spots were stained by anti-G-Rg1 and anti-G-Rb1 monoclonal antibodies, respectively.

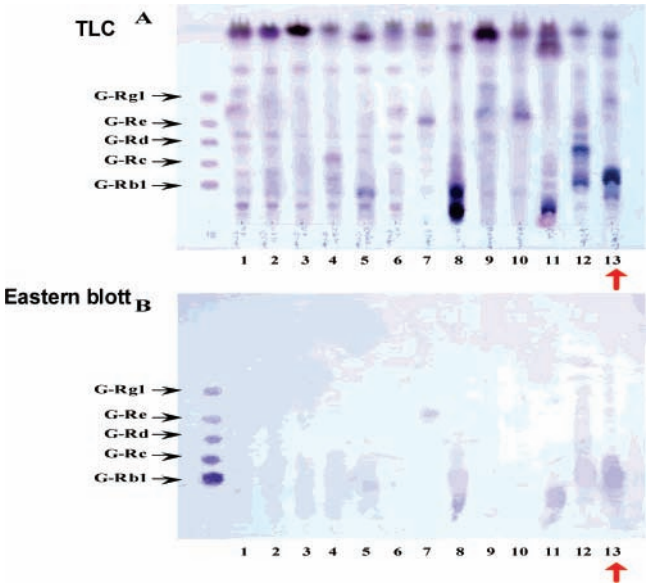

Figure 4. Ginsenosides in Alariacea plants analyzed by Eastern blotting. Arrow indicates *Kalopanax pictus*.

information, we succeeded in isolating G-Rb1 from the barks of *K. pictus* even though the concentration is 0.0009% dry weight as reported previously (*22*).

Immunoaffinity Concentration by Immunoaffinity Column Conjugated with MAb for the Determination of Ginsenosides (*19*). To confirm the concentration for G-Rb1 by immunoaffinity column conjugated with anti-G-Rb1 MAb, a crude extract of *P. ginseng* roots was loaded onto the immunoaffinity column and washed with the washing solvent. **Figure 5** shows fractions 1−8 containing overcharged G-Rb1, which was determined by ELISA. G-Rc, -Rd, -Re, and -Rg1 were also detected in these fractions by the Eastern blotting procedure. A sharp peak appeared around fractions 40−44, which contained G-Rb1. However, G-Rb1 purified by the immunoaffinity column was still contaminated by a small amount of malonyl G-Rb1 as detected by Eastern blotting. This compound has almost the same cross-reactivity with G-Rb1 (data not shown). Therefore,

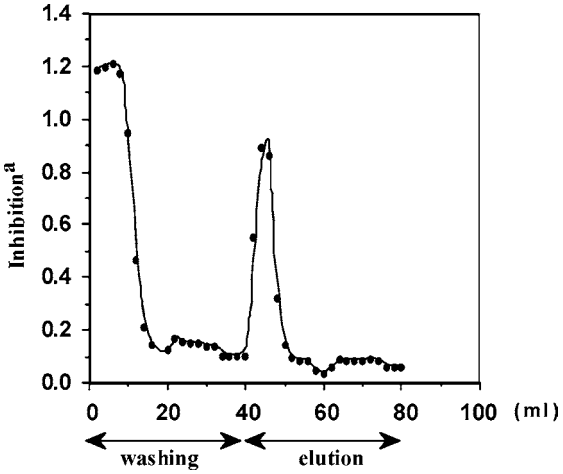

Figure 5. Elution profile of *Panax ginseng* crude extract used immunoaffinity column monitoring by ELISA using anti-G-Rb1 MAb.

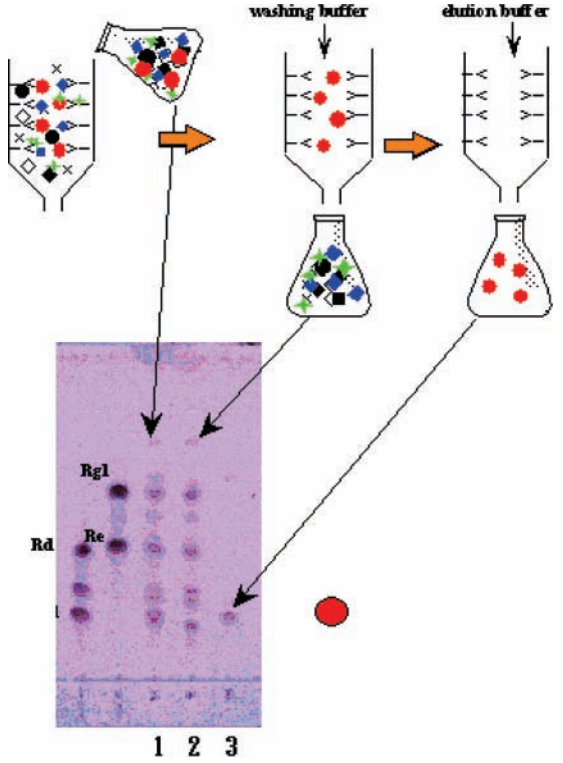

Figure 6. Preparation of knock-out extract eliminated G-Rb1 from *Panax ginseng* crude extract using immunoaffinity column conjugated with anti-G-Rb1 MAb. Lines 1, 2, and 3 indicate crude extract, knock-out extract, and purified G-Rb1, respectively. Red spot shows G-Rb1.

the mixture was treated with a mild alkaline solution at room temperature for 1 h to give pure G-Rb1. Overcharged G-Rb1, eluted with washing solution, was repeatedly loaded and finally isolated in pure form. From this result we confirmed that the immunoaffinity column can concentrate G-Rb1 from the ginsenoside mixture.

After washing, fractions were deionized and the solvent was lyophilized. **Figure 6** indicates the TLC profile of the purification step. Lines 1, 2, and 3 were the crude extract, the washing fraction, and the eluted fraction, respectively. Interestingly, the washing fraction contained all of the compounds in the ginseng crude extract except G-Rb1. It became evident that the G-Rb1 molecule can be eliminated by an immunoaffinity column conjugated with anti-G-Rb1 MAb, and the washing fraction was knocked out only by the antigen molecule, G-Rb1. Therefore,

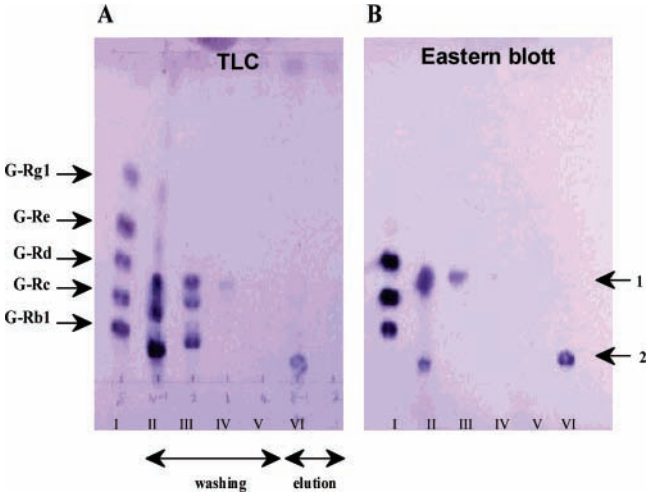

Figure 7. Purification and determination of ginsenosides of *P. japonicus* by immunoaffinity column and Eastern blotting.

we named this washing fraction a knock-out extract. This knock-out extract may be useful for the determination of real pharmacologically active principle in the TCMs. The antibody was stable when exposed to the eluent and the immunoaffinity column, indicating almost no decrease in capacity (20 μg/mL gel) after repeated use more than 10 times under the same conditions, as reported for a single-step separation of forskolin from a crude extract of *Coleus forskohlii* root (*23*). Furthermore, because we succeeded in the preparation of MAbs having a wide cross-reactivity such as anti-solamargine MAb (*3*), anti-saikosaponin a (*24*), and G-Re (*25*), the related total saponins can be concentrated by an immunoaffinity column conjugated with MAb as reported previously in the case of solasodine glycosides (*26*).

P. japonicus is distributed in Japan and China and is morphologically different from the other *Panax* species. Yahara et al. reported that no G-Rbl was found in *P. japonicus* (*27*) and isolated oleanane-type saponins called chikusetsusaponins and elucidated their structures. Morita et al. examined the varieties of *P. japonicus* by chemical analysis of saponins (*28*). From these results, the concentration of G-Rb1 might be trace levels. However, we determined it by ELISA and found higher concentrations compared with previous reports (*29*), although approximately half the concentration of G-Rb1 was found by HPLC analysis as compared with ELISA. To make clear these differences, we used an immunoaffinity column for immunoaffinity concentration of G-Rb1. The crude root extract of *P. japonicus* was loaded on the immunoaffinity column and washed with the washing solvent and then with elution solvent, as already indicated. **Figure 7** shows the H_2SO_4 staining (**A**) and the Eastern blotting (**B**) profiles of the two fractions separated by the immunoaffinity column. Fraction 1 eluted with the washing solvent showed many spots, including chikusetsusaponins, similar to the original extract of *P. japonicus*. However, fraction 2 contained a higher concentration of compound **1**, although two other bands were still detected on Eastern blotting. Compound **1** clearly indicated a dammarane saponin having protopanaxadiol as a framework and three sugars in a molecule compared to the R_f value of G-Rd, suggesting that compound **1** is chikusetsusaponin III. Finally, we identified compound **1** as chikusetsusaponin III in a direct comparison with authentic sample.

A clear unknown band of compound **2** appeared in fraction 5 eluted with the elution solvent. G-Rb1 was, however, not detected by Eastern blotting, although it was detected by TLC

as indicated in **Figure 7A**. It can be suggested that compound **2** has molecular structure and cross-reactivity similar to those of G-Rb1 and seems to be related to the ginseng saponin having protopanaxadiol as an aglycone. Moreover, compound **2** might have the same sugar fragments but possess five sugar moieties in the molecule compared with G-Rb1, as indicated by their R_f values. From these pieces of evidence compound **2** might be chikusetsusaponin III-20-*O*-gentiobiose, chikusetsusaponin IV, which was identified by direct comparison with authentic sample (*29*). Therefore, we concluded that *P. japonicus* did not contain G-Rb1, but did contain chikusetsusaponin IV, having the same aglycon and the same sugar component instead of G-Rb1.

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