International Journal of Biomedical and Pharmaceutical Sciences ©2012 Global Science Books

A New Strategy of Immunostaining for Identification of Ginsenosides

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ABSTRACT

In the newly established immunostaining method, Eastern blotting, we 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. Eastern blotting indicated the specific staining of ginsenoside (G)-Rb1 together with other ginsenosides, G-Rc and -Rd of which cross-reactivities were 0.02% compared with H_2SO_4. 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 as a blue and a purple color, respectively. As an application we analyzed several Alariacean plants by Eastern blotting using anti-G-Rb1 MAb resulting in the isolation of G-Rb1 from *Kalopanax pictus*. We succeeded one-step isolation of G-Rb1 from the crude ginseng extract using immunoaffinity column combined with anti-G-Rb1 MAb. It became evident that the washing fraction contained all of components except antigen, G-Rb1 giving a knockout extract named by us. As other application we purified and identified two known ginsenosides from the crude extract of *P. japonicus* using Eastern blotting and immunoaffinity column combined with anti-G-Rb1 MAb.

Keywords: Eastern blotting, ginseng, ginsenoside, immunostaining, knockout extract, one-step purification
Abbreviations: **ELISA**, enzyme-linked immunosorbent assay; **G-**, ginsenoside-; **HPLC**, high-performance liquid chromatography; **MAb**, monoclonal antibody; **PVDF**, polyvinylidene fluoride; **TCM**, traditional Chinese medicine

INTRODUCTION

With the rapid development of the molecular biosciences and their biotechnological applications, the immunoassays using monoclonal antibodies (MAbs) against drugs and bioactive compounds possessing higher molecular weight have become an important tool, due to their specificity, for receptor binding analyses, enzyme assays, quantitative and qualitative analytical techniques in the wide research field. The immunoblotting method is based on western blotting technique that utilizes antigen-antibody binding properties and has been provided a specific and sensitive detection of higher molecule analyte like peptides and proteins. In our ongoing phytochemical studies we prepared many MAbs against naturally occurring bioactive compounds like forskolin (Sakata *et al.* 1994), crocin (Xuan *et al.* 1999), solamargine (Ishiyama *et al.* 1996), opium alkaloids (Shoyama *et al.* 1996), marihuana compounds (Tanaka *et al.* 1996), ginsenosides (Tanaka *et al.* 1999; Fukuda *et al.* 2000b), saikosaponin a (Zhu *et al.* 2004), paeoniflorin (Lu *et al.* 2003), sennosides (Morinaga *et al.* 2000, 2001), ginkgolic acid (Loungratana *et al.* 2004), glycyrrhizin (Shan *et al.* 2001) and berberine (Kim *et al.* 2004) and so on, and then established individual competitive enzyme-linked immunosorbent assay (ELISA) as a high sensitive, specific and simple methodology. As an extension of this approach, an immunostaining method using anti-solamargine MAb was established by us (Tanaka *et al.* 1997).

Ginseng, the crude drug of *Panax ginseng* root is one of the most important components of traditional Chinese medicines (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 (Cho *et al.* 1995). Major active components are the ginsenosides, which consist of protopanaxatriol and/ or protopanaxadiol possessing a dammarane skeleton in their molecules. More than 60 ginsenosides have been isolated from *Panax* species like ginsenoside (G)-Rb1, -Rc and -Rd belonged to protopanaxadiol group, and G-Rg1, -Re and -Rf possessing protopanaxatriol as an aglycone. Recently wide pharmacological activities of ginsenosides have been reported like cancer chemoprevention (Wang *et al*. 2009), cardiovascular activities (Wang *et al*. 2007a, 2007b), antidiabetic effects (Sievenpiper *et al*. 2004; Xie *et al*. 2005; Shang *et al*. 2007) and central nervous system (Xu *et al*. 2009; Liu *et al*. 2010). It is well known that the concentration of ginsenosides in *P. ginseng* are varying in the ginseng root or the root extracts depending on the method of extraction, subsequent treatment (Kitagawa *et al.* 1989), or even the season of its collection (Tanaka 1989). Moreover, two other major *Panax* species, *P. quinquefolium* and *P. notoginseng* are marketing now. Furthermore, *P. japonicus* that is morphologically different from *P. ginseng* and contains oleanane-type saponins like chikusetsusaponins is also important as a folkmedicine. From these evidences standardization of quality for *Panax* species is required in the field of traditional Chinese medicine (TCM).

High-performance liquid chromatography (HPLC) has been widely used for the analysis of ginsenosides in general. The identification of ginsenosides in *Panax* species has been carried out by HPLC-mass spectrometry (MS) (Breemen *et al*. 1995; Chan *et al.* 2000; Harkey *et al.* 2001; Gu *et*

Received: 3 October, 2010. Accepted: 3 August, 2011.

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al. 2006; Mo *et al*. 2006). However, since HPLC-MS needs the pretreatment of sample, much longer time and the cost-performance for analysis, we previously prepared anti-G-Rb1, -G-Rg1 and -G-Re MAbs, respectively (Tanaka *et al.* 1999; Fukuda *et al.* 2000b) and set up the ELISA, a new staining method, Eastern blotting, for ginsenosides. an immunoaffinity concentration method for quantitative analysis for the samples containing lower concentration of G-Rb1 (Fukuda *et al.* 2000c) in order to control the quality of *Panax* species prescribed in TCM.

In our continuing 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 G-Rg1 MAbs. Furthermore, the immunoaffinity concentration of G-Rb1 by immunoaffinity column is also discussed in this review.

Eastern blotting of ginsenosides

Although the 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 is necessary for the new methodology. Previously we succeeded in identifying the function group of small molecule compounds like solasodine glycosides into a part of epitopes and demonstrating the fixing ability on a membrane (Tanaka *et al.* 1997). The PVDF membrane blotted was treated with $NaIO_4$ solution. This reaction enhanced the fixing of solasodine glycoside via solasodine glycoside-albumin conjugates on the PVDF membrane. The PVDF membrane incubated in the absence of $NaIO_4$ was free of staining for solasodine glycoside. From this finding we have applied this new methodology to various glycosides like glycyrrhizin (Shan *et al.* 2001) and saikosaponins (Morinaga *et al.* 2006) together with ginsenosides (Fukuda *et al.* 2001). In this review we will focus on the application of the Eastern blotting for ginsenosides.

Fig. 1 shows the H_2SO_4 staining (A) and the Eastern blotting of ginsenoside standards and the TCM using anti-G-Rb1 MAb (B). It is impossible to determine the ginsenosides by TLC stained by H_2SO_4 because of complicated profile as indicated in **Fig. 1A**. On the other hand the clear staining of G-Rb1 occurred by Eastern blotting (B). Compared with H_2SO_4 staining, the Eastern blotting indicated the specific staining of G-Rb1 together with other ginsenosides, G-Rc and -Rd of which cross-reactivities were 0.02% as shown in **Fig. 1B**. The Eastern blotting method was considerably more sensitive than that of H_2SO_4 staining. Furthermore, it became evident that Kikyoto and Daiokanzoto formulas which did not contain ginseng indicated no spot of G-Rb1. We suggested that an aglycon, protopanaxadiol and a part of 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 sugar moiety in the ginsenoside molecule against anti-G-Rb1 MAb may be modified by the $NaIO_4$ treatment of ginsenosides on the PVDF membrane causing G-Rc and -Rd become detectable by the Eastern blotting even though very small cross-reactivities like 0.02% as described above.

The new method is to separate the G-Rb1 molecule into two functional parts. The sugar parts are oxidized to release 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 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 in **Fig**. **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

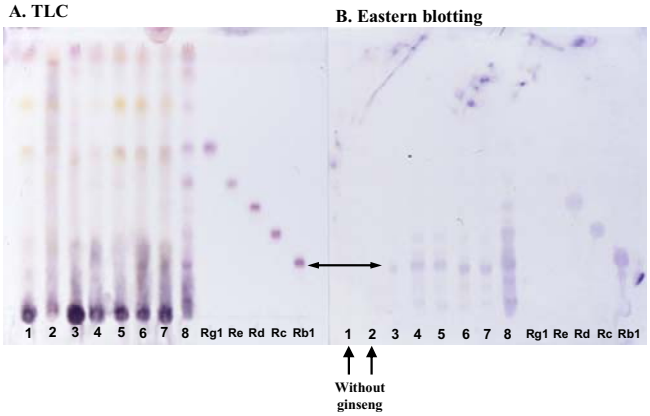


Fig. 1 Ginsenoside profiles on TLC (A) stained by sulfuric acid and Eastern blotting (B) using anti-G- Rb1 MAb in traditional Chinese medicine (TCM) prescriptions. Samples 1: Kikyoto, 2: Daiokanzoto, 3: Ninjin'yoeito, 4: Shikunshito, 5: Ninjinto, 6: Hangeshashinto, 7: Shosaikoto, 8: Crude extract of ginseng. Samples 1 and 2 do not contain ginseng. Standard of ginsenosides indicated G-Rg1, -Re, -Rd, -Rc and -Rb1 from upper.

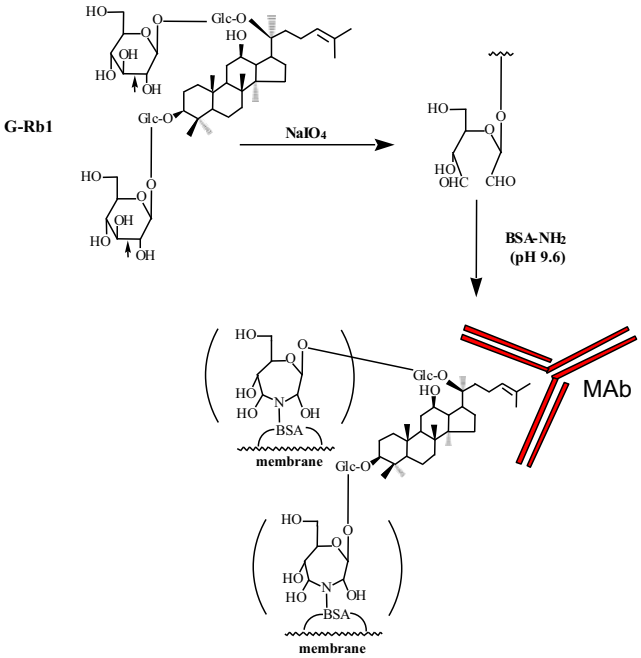


Fig. 2 Mechanism of Eastern blotting.

stained as a blue color (data not shown), although the purple color 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 established the double staining of ginsenosides indicating that G-Rg1 and -Re were stained as purple color and the others as blue color separately as indicated in **Fig. 3**. These results showed that using double antibodies can distinguish individual aglycons, protopanaxatriol and protopanaxadiol. For this application the crude extracts of various *Panax* species were analyzed by the double staining system (Fukuda *et al.* 2001) resulted that all ginsenosides can be determined clearly as indicated in **Fig**. **3**.

Furthermore, the results suggest that the staining color shows the different pharmacological activity like the purple spots indicate ginsenosides having the stimulation activity for the central nervous system (CNS), and the blue color indicated ginsenosides possessing the depression effect for the CNS. The Rf value of ginsenoside roughly suggests the

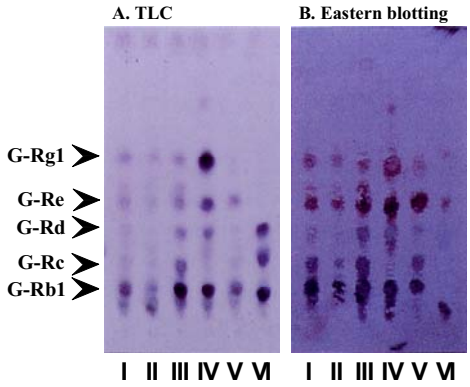


Fig. 3 Double staining of Eastern blotting for ginsenosides contained in various ginseng using anti-G-Rb1 and anti-G-Rg1 MAbs. TLC profile stained by sulfuric acid (**A**). Eastern blotting by anti-G-Rb1 and anti-G-Rg1 MAbs (**B**). I, II, III, IV, V and VI indicated white ginseng, red ginseng, fibrous ginseng (*Panax ginseng*), *Panax notoginseng*, *Panax quinquefolius* and *Panax japonicus*, respectively. Purple color spots and blue color spots were stained by anti-G-Rg1 and anti-G-Rb1MAbs, respectively.

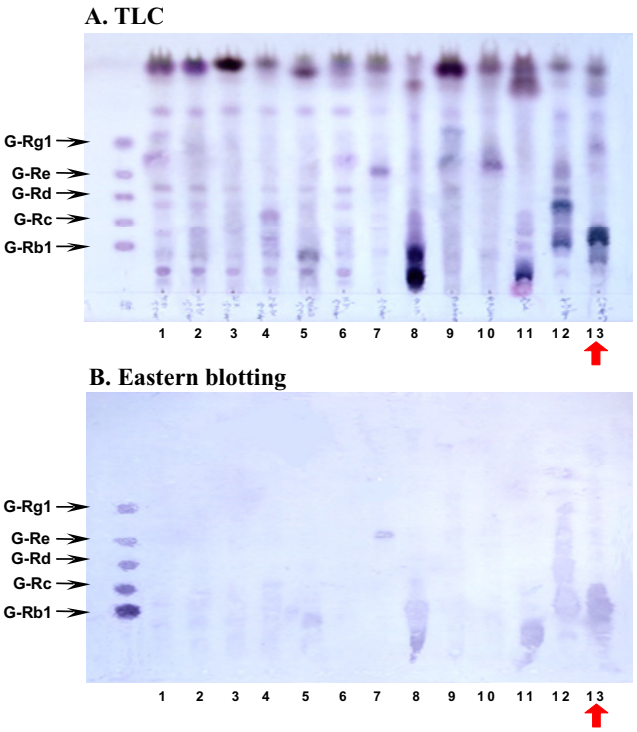


Fig. 4 Ginsenosides in Alariacea plants analyzed by TLC (A) and Eastern blotting (B). Red arrow indicates *Kalopanax pictus*.

number of sugar attached to the aglycon. Therefore, the double staining analysis could be used to elucidate the structure of the aglycon attached and the number of sugars in ginsenosides.

As one of further application we surveyed Araliaceae species by Eastern blotting using anti-G-Rb1 MAb as indicated in **Fig**. **4**. ELISA analysis and Eastern blotting profile of *Kalopanax pictus* Nakai (**Fig**. **4B**, line 13 as indicated by an arrow) suggest that this species may contain G-Rb1. Depending on this information we succeeded to isolate G-Rb1 from the barks of *K. pictus* even thought the concentration is 0.0009% dry wt (Tanaka *et al.* 2005).

Immunoaffinity concentration by immunoaffinity column conjugated with MAb for determination of ginsenosides

In order to confirm the ability of concentration for G-Rb1

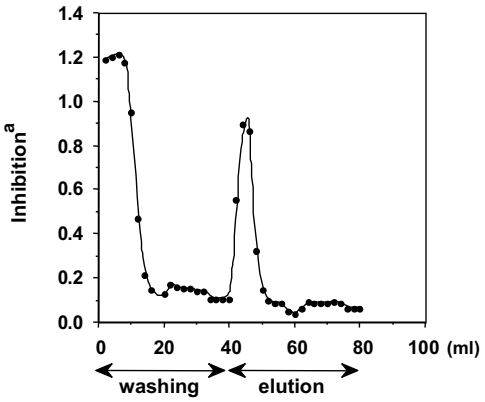


Fig. 5 Elution profile of *Panax ginseng* crude extract used immunoaffinity column monitoring by ELISA using anti-G-Rb1 MAb.

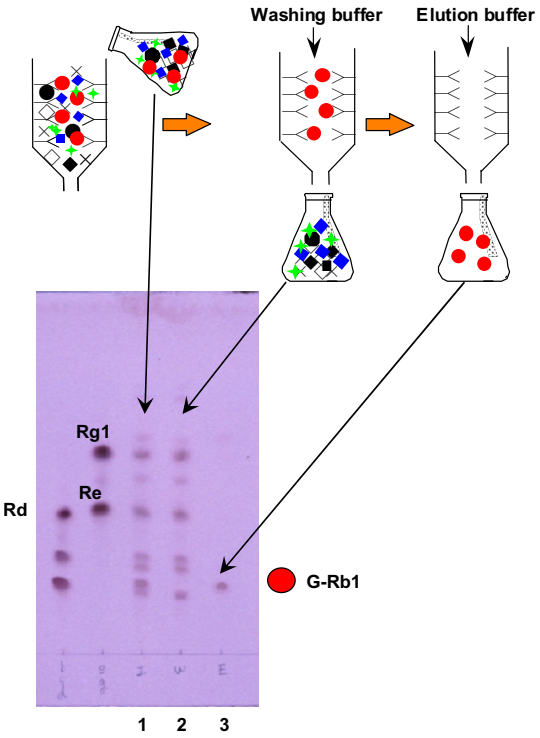


Fig. 6 Preparation of knock-out extract eliminated G-Rb1 from *Panax ginseng* crude extract using immunoaffinity column conjugated with anti-G-Rb1 MAb. Lines 1, 2 and 3 indicate crude extract, knock-out extract and purified G-Rb1, respectively. Red spot shows G-Rb1.

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 (Fukuda *et al.* 2000c). **Fig. 5** shows the 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, 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. **Fig. 6** indicates the TLC profile of purification step. Lanes 1, 2 and 3 are the crude extract, the

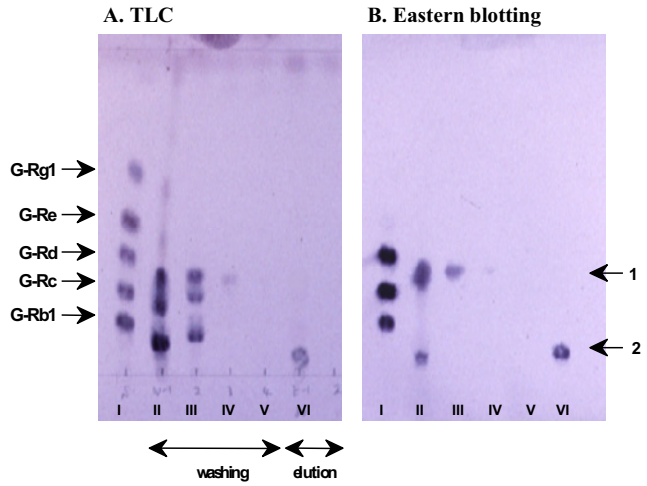
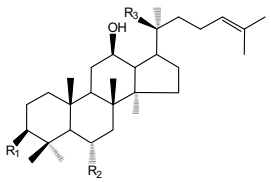


Fig. 7 Purification and determination of ginsenosides of *P. japonicus* by immunoaffinity column and Eastern blotting. TLC profile stained by sulfuric acid (**A**). Eastern blotting by anti-G-Rb1(B). I: Standard of ginsenosides, II-V: washing fractions, VI: elution fractions. 1 and 2 indicated isolated ginsenoside, respectively.



		R1	R2	R3
Compound 1	Chikusetsusaponin III	-O-Glc(6-Xyl, 2-Glc)	-H	-OH
Compound 2	Chikusetsusaponin VI	-O-Glc(6-Xyl, 2-Glc)	-H	-O-Glc^{6}Glc
	Ginsenoside Rb1	-O-Glc^{2}Glc	-H	-O-Glc^{6}Glc
	Ginsenoside Rc	-O-Glc^{2}Glc	-H	-O-Glc^{6}Ara
	Ginsenoside Rd	-O-Glc^{2}Glc	-H	-O-Glc

Fig. 8 Isolated and identified ginsenosides from *Panax japonicus.*

washing fraction and the eluted fraction, respectively. Interestingly the washing fraction contained all of compounds in the ginseng crude extract except G-Rb1. It becomes clear that G-Rb1 molecule can be eliminated by an immunoaffinity column conjugated with anti-G-Rb1 MAb and the washing fraction knocked out only the antigen molecule, G-Rb1. Therefore, we named this washing fraction a knockout extract. This knockout 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 was reported for a single-step separation of forskolin from a crude extract of *Coleus forskohlii* root (Yanagihara *et al.* 1996). Furthermore, since we succeeded the preparation of MAbs having a wide cross-reactivity like anti-solamargine MAb (Ishiyama *et al.* 1996), anti-saikosaponin a (Zhu *et al.* 2006) and G-Re (Morinaga *et al.* 2006), the related total saponins can be concentrated by an immunoaffinity column conjugated with MAb as reported previously in the case of solasodine glycosides (Putarun *et al.* 1999).

P. japonicus distributed in Japan and China is morphologically different from the other *Panax* species. Yahara *et al.* (1977) reported that no G-Rbl was found in *P. Japonicus*, and isolated oleanane-type saponins named as chikusetsu saponins and elucidated their structures. Morita *et al.* (1985) examined the varieties of *P. japonicus* by chemical analysis of saponins. 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 (Fukuda *et al.* 2000a), although approximately half the concentration of G-Rb1 was found by high-performance liquid chromatography (HPLC) analysis compared with ELISA. To make clear these differences, we used 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. **Fig**. **7** shows the H2SO4 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 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 Rf value of G-Rd suggesting that compound 1 might be chikusetsusaponin III. Finally we identified compound 1 to be chikusetsusaponin III in a direct comparison with authentic sample as indicated **Fig. 8**.

A clear unknown band 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 **Fig**.**7A**. It can be suggested that compound 2 has similar molecular structure and cross-reactivity with G-Rb1, and seems to be related ginseng saponin having protopanaxadiol as an aglycone. Moreover, compound 2 might have the same sugar fragments possessing 5 sugar moieties in the molecule compared with G-Rb1, as indicated by their Rf value. From these evidences compound 2 might be chikusetsusaponin III-20-*O*-gentiobiose, chikusetsusaponin IV which was identified by the direct comparison with authentic sample (Fukuda *et al.* 2000a) as indicated in **Fig**. **8**. Therefore, we concluded that *P. japonicus* did not contain G-Rb1, but did chikusetsusaponin IV having the same aglycon and same sugar component instead of G-Rb1.

ACKNOWLEDGEMENTS

The authors thank for the financial support for Special Coordination Funds for Promoting Science and Technology in Japan Society for the Promotion of Science.

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