Western Blotting for Ginseng Saponins, Ginsenosides Using Anti-ginsenoside Rb1 Monoclonal Antibody

Noriko FUKUDA, Hiroyuki TANAKA, and
Yukihiro SHOYAMA*

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

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Ginsenosides separated by silica gel TLC were blotted to a polyvinylidene difluoride (PVDF) membrane which was treated with a NaIO_4 solution followed by bovine serum albumine (BSA), resulting in a ginsenoside-BSA conjugate on a PVDF membrane. The blotted bands were stained with monoclonal antibody (MAb). The newly established Western blotting method was used for the determination of ginsenosides and their distribution in various *Panax* species.

Key words monoclonal antibody; ginseng; ginsenoside Rb1; Western blotting

Ginseng is one of the most important Chinese medicines used in tonics to combat stress and cancer, disturbances of the central nervous system,¹⁾ and hypothermia,²⁾ for antioxidant and organ-protective actions,³⁾ and for radioprotection.⁴⁾ It contains many dammarane and oleanane saponins,^{5,6)} polyacetylene derivatives,⁷⁾ and polysaccharides⁸⁾ of which the biological activities have been studied thoroughly. A major ginsenoside (G)-Rb1 has been investigated in terms of effects on the nervous system.⁹⁾ More recently, Chang *et al.*¹⁰⁾ reported the effect of G-Rb1 on drug-induced memory impairment.

In our current study on the formation of MAb against naturally occurring bioactive compounds, we have established MABs against forskolin,¹¹⁾ solamargine,¹²⁾ codeine and thebaine,¹³⁾ marijuana compounds,¹⁴⁾ and crocin.¹⁵⁾ An immunological approach for assaying quantities of ginsenosides using a polyclonal antibody has been investigated by Sankawa *et al.*¹⁶⁾ However, since no results of MABs related to ginsenosides have yet been confirmed, we previously reported the preparation of a MAB against G-Rb1.¹⁷⁾ We herein describe Western blotting for ginsenosides in our ongoing investigations of applications of MABs.

Ginsenosides were applied to TLC plates and developed with *n*-BuOH-EtOAc- H_2O (15:1:4, by volume). One developed TLC plate was dried and sprayed with H_2SO_4 . Another TLC plate was dried and then sprayed with a blotting solution mixture of isopropanol-methanol- H_2O (1:4:16, by volume). It was placed on a stainless steel plate and then covered with a PVDF membrane sheet. After covering with a glass microfiber filter sheet, the whole was pressed evenly for 50 s with a 120 °C hot plate as previously described¹⁸⁾ with modification. The PVDF membrane was separated from the plate and dried. The blotted PVDF membrane was dipped in water containing NaIO_4 under stirring at room temperature for 1 h. After washing with water, 50 mM of carbonate buffer solution containing BSA was added and stirred for 3 h. The PVDF membrane was washed with TPBS (PBS containing 0.05% Tween 20) twice for 5 min and then washed with water. The PVDF membrane was immersed in anti-G-Rb1

MAb-9G7¹⁷⁾ and stirred at room temperature for 1 h. After washing the PVDF membrane twice with TPBS and water, a 1000-fold dilution of peroxidase-labeled goat anti-mouse IgG in GPBS (PBS containing 0.2% of gelatin) was added and stirred at room temperature for 1 h. The PVDF membrane was washed twice with TPBS and water, then exposed to 1 mg/ml 4-chloro-1-naphthol-0.03% H_2O_2 in PBS solution which was freshly prepared before use for 10 min at room temperature. The reaction was stopped by washing with water. The immunostained PVDF membrane was left to dry.

Although Western blotting is a common assay methodology for substances of high molecular weights, it has not been employed with small molecules, as direct immunostaining of such compounds on a TLC plate has not yet been confirmed. Therefore a new method for such compounds is required. Moreover, if small molecules can be blotted to a membrane, a new method for fixing is also required. Since we have synthesized a hapten-BSA conjugate by cleavage of the sugar moiety using NaIO_4 solution, as previously reported,¹²⁾ it seemed likely that this reaction could be used for fixation on a membrane.

The developed PVDF membrane was treated with NaIO_4 solution. This reaction enhanced the fixing of ginsenosides via ginsenoside-BSA conjugates on the PVDF membrane. The PVDF membranes incubated in the absence of NaIO_4 were essentially free of staining for ginsenosides (data not shown). The antigen synthesized by NaIO_4 cleavage via conjugation with carrier protein is necessary, since when a different type of hapten-carrier protein conjugate is used, Western blotting is not detectable. Figure 1 shows H_2SO_4 staining (A) and Western blotting (B) of ginsenosides. Lanes I to V show the bands of ginsenosides G-Rg1, -Re, -Rd, -Rc, and -Rb1, respectively. Different sensitivities between ginsenosides were observed, and the sensitivity of G-Rb1 was higher than that of the other ginsenosides. The detectable limit was 360 pmole of G-Rb1. The substitution pattern of the sugars is important: the sugar moiety at the C-3 position was necessary for staining, but that at the C-6 position inhibited staining. The sensitivity of the reaction depends upon their structures and cross-reactivities. Thus the bands of G-Re and -Rg1 were stained with almost no cross-reactivity (under 0.005%)¹⁷⁾ against MAB (Fig. 1), but disappeared after a few hours (arrows in Fig. 1-B and Fig. 1-B', lanes I and II). On the other hand, G-Rc and G-Rd had cross reactivities of 0.024 and 0.02%, respectively, and these remained (Fig. 1, lanes III and IV). In addition, it is suggested that the specific reactivity of the sugar moiety in the ginsenoside molecule against MAB may be modified by NaIO_4 treatment of ginsenosides on the membrane, causing G-Re and -Rg1 to become detectable by Western blotting.

Although H_2SO_4 staining (Fig. 2-A) detected numerous spots including probably sugars and different types of saponins like chikusetsusaponin IV (Fig. 2-A, lanes 1 to 7) in various *Panax* samples, Western blotting (Fig. 2-B) detected only ginsenoside spots I to VI. This indicates that chikusetsusaponin IV, which is an oleanane-type saponin contained in *P. japonicus*, cannot be detected by Western blotting as shown in Fig. 2-B, lane 1. A ginsenoside aglycone panaxatriol (arrow) was not detected by Western blotting, indicating that our new Western blotting method requires sugar moi-

* To whom correspondence should be addressed.

Fig. 1. Western Blotting of Ginsenosides

Ginsenosides (3 μ g) were chromatographed on silica gel TLC plates, and then developed with an *n*-BuOH-EtOAc-H₂O solvent system. After blotting to a PVDF membrane, the membrane was treated with NaIO₄ solution and BSA solution and then stained by the MAb. A: H₂SO₄ staining; B: Western blotting; B': after 5-h standing. Lanes I, II, III, IV, and V indicate G-Rg1, -Re, -Rd, -Rc, and -Rb1, respectively. Two arrows show G-Rg1 and -Re, respectively.

Fig. 3. Scheme of New Western Blotting Method for Ginsenoside Rb1

eties.

This is apparently the first report of Western blotting for ginsenosides and its application. We propose that in this process the sugars are oxidized to give dialdehydes, which then react with amino groups on the protein that sticks to the membrane. The MAb then binds to the steroid part of the molecule, as indicated in Fig. 3. Since it has previously been difficult to detect small molecular compounds by Western blotting and/or immunocytochemicalization, the methodology described here may open up a wide field of comparable studies with other families of carbohydrates containing compounds with low molecular weight, such as saponins, glycosides, glucuronides, and amino-sugar conjugates.

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Fig. 2. Western Blotting of Ginsenosides in Various *Panax* Samples

A: H₂SO₄ staining; B: Western blotting. Lanes 1, 2, 3, 4, 5, 6, and 7 indicate the standard of chikusetsusaponin IV, white ginseng, red ginseng, fibrous ginseng, *P. notoginseng*, *P. quinquefolium*, and *P. japonicus* (60 μ g), respectively. Compounds I, II, III, IV, V, and VI indicate G-Rg1, -Re, -Rd, -Rc, -Rb1, and malonyl G-Rb1, respectively. The arrow near the top of A shows aglycone of ginsenosides.