

ELISA, western blotting, immunocytochemical localization and immunoaffinity column for naturally occurring bioactive compounds using monoclonal antibodies

Follow this and additional works at: <https://www.jfda-online.com/journal>

 Part of the [Food Science Commons](#), [Medicinal Chemistry and Pharmaceutics Commons](#), [Pharmacology Commons](#), and the [Toxicology Commons](#)



This work is licensed under a [Creative Commons Attribution-Noncommercial-No Derivative Works 4.0 License](#).

Recommended Citation

Shan, S.-J.; Putalun, W.; Fukuda, N.; Morinaga, O.; Tanaka, H.; and Shoyama, Y. (2000) "ELISA, western blotting, immunocytochemical localization and immunoaffinity column for naturally occurring bioactive compounds using monoclonal antibodies," *Journal of Food and Drug Analysis*: Vol. 8 : Iss. 4 , Article 14.
Available at: <https://doi.org/10.38212/2224-6614.2827>

ELISA, Western Blotting, Immunocytochemicalization and Immunoaffinity Column for Naturally Occuring Bioactive Compounds Using Monoclonal Antibodies

SHAO-JIC SHAN, WARAPORN PUTALUN, NORIKO FUKUDA, OSAMU MORINAGA,
HIROYUKI TANAKA AND YUKIHIRO SHOYAMA*

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

(Received: June 29, 2000; Accepted: December 14, 2000)

ABSTRACT

Anti-sennoside A (SA) and anti-glycyrrhizin (GC) MAb were used for screening and cloning. And they were also used for ELISA. New western blotting method of determination for GC and ginsenoside was established that compounds separated by silica gel TLC were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was treated with NaIO₄ solution followed by BSA, resulting in a ginsenoside-BSA conjugate on the PVDF membrane which was stained by the general western blotting. Immunocytochemicalization of GC in the fresh root of *Glycyrrhiza* species was conducted using anti-GC MAb after blotting to PVDF membrane. Immunoaffinity column chromatography using anti-ginsenoside Rb1 (GRb1) MAb performs better than previously published separation methods resulting in pure GRb1 from the crude extracts of ginseng. On the other hand, total solasodine glycosides were separated by an immunoaffinity column using a wide-cross reactive anti-solamargine (SM) MAb.

Key words: monoclonal antibody, bioactive compounds, ELISA, western blotting, immunoaffinity column

INTRODUCTION

The immunoassay system using monoclonal antibodies (MAbs) is not so often used to detect naturally occurring bioactive compounds having smaller molecular weight except for drugs like morphine. However, it is now indispensable to various investigations. Quality control of the Chinese herbal medicine is necessary because it is believed that approximately 70% of these crude drugs prescribed are collected from natural sources. Furthermore, since MAbs are essential for the assay of concentrations of active constituents in our on-going plant biotechnological projects, we prepared MAbs against forskolin⁽¹⁻³⁾, marihuana compounds^(4,5), opium alkaloids⁽⁶⁾, glycoalkaloid⁽⁷⁾, glycyrrhizin (GC)⁽⁸⁾, crocin⁽⁹⁾, ginsenoside Rb1 (GRb1)⁽¹⁰⁾, sennoside A (SA)⁽¹¹⁾. We applied these MAbs to conduct immunoaffinity concentration⁽¹²⁻¹⁴⁾ and western blotting⁽¹⁵⁻¹⁷⁾. In the process of MAb production the confirmation of antigen conjugate is necessary for the immunization. The strategy for the determination of molecular weight of hapten-carrier protein conjugate by MALDI tof mass analysis provides UV more accurate molecular ratio of hapten and carrier protein especially for those compounds having no specific UV absorbance where difficulties and ambiguities remain⁽¹⁸⁻²⁰⁾.

Three applications of MAb are discussed in this review article; a high sensitive competitive ELISA, a new western blotting and an immunoaffinity column chromatography regarding four naturally occurring bioactive compounds, SA, GC, GRb1 and a glycoalkaloid, solamargine (SM).

PREPARATION OF MAB AND ESTABLISHMENT OF COMPETITIVE ELISA

I. Anti-GC MAb and ELISA

Licorice (*Glycyrrhiza* spp.) is one of the most important Chinese medicines used in the Chinese traditional medicine prescribed with other herbal medicines for anti-tussive, expectorant and corrigent⁽²¹⁾. These pharmaceutical properties are mainly due to GC which has, anti-ulcer⁽²²⁾ and anti-viral⁽²³⁾ activities, and is now a drug in Japan for anti-allergic⁽²⁴⁾ and protection of liver⁽²⁵⁾. Immunological approaches for assaying quantities of GC in body fluid and crude extracts of licorice root in Chinese traditional medicine using the anti-glycyrrhetic acid MAb has been investigated by Mizugaki et al.⁽²⁶⁾. We now review the formation of MAb against GC using GC-carrier protein conjugate synthesized by NaIO₄.

(I) Direct Determination of Hapten-carrier Protein Conjugate by MALDI-tof Mass Spectrometry

Figure 1 shows the MALDI tof mass spectrum of the antigen, GC-BSA conjugate. A broad peak coinciding with the conjugate of GC appeared at around *m/z* 70,021. Using a molecular weight of 66,433 for BSA, the calculated values of GC component (MW 823) is 3,588 resulting in at least 4 molecules of GC conjugated with BSA. The hapten number was estimated to be enough for immunization as compared to the previous results of SM⁽⁷⁾. The number of GC moiety contained in the GC-HSA conjugate was also determined by its

* Author for correspondence. Tel: +81-926426580; Fax: +81-926426545; E-mail:shoyama@shoyaku.phar.kyushu-u.ac.jp

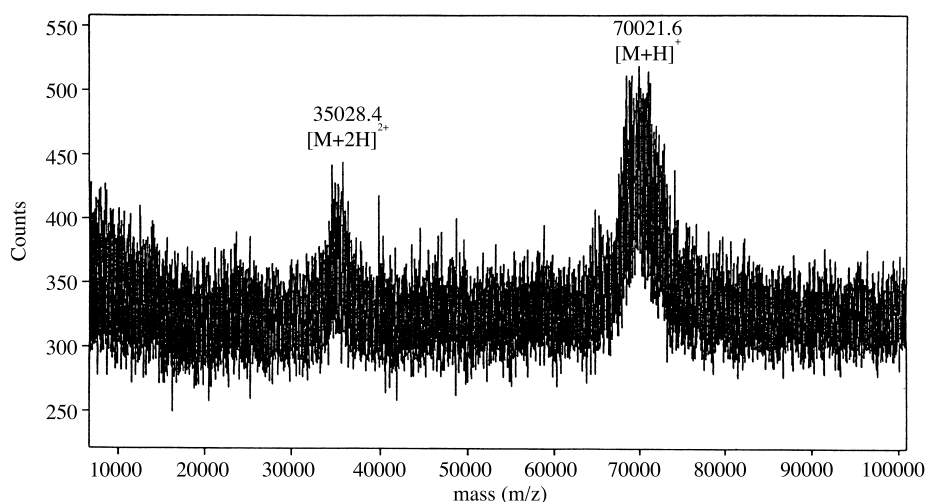


Figure 1. MALDI-tof mass of glycyrrhizin-BSA conjugate.

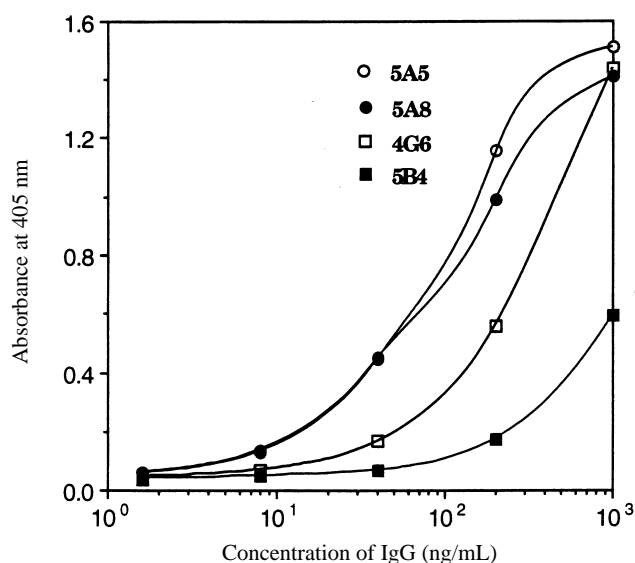


Figure 2. Reactivities of IgG type MAbs against glycyrrhizin.

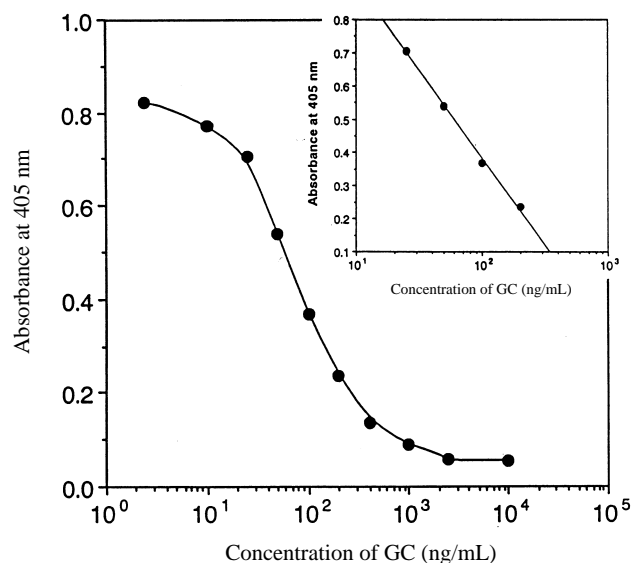


Figure 3. Calibration curve of glycyrrhizin.

spectrum to be around 5 molecules.

(II) Production and Characteristics of MAbs against GC

The hyperimmunized BALB/c mice, used to derive the cell clone described in this study yielded splenocytes which were fused with myeloma cells by the routinely established procedure in this laboratory⁽¹⁾. Four hybridomas producing MAb reactive to GC were obtained, and classified into IgG1 which had λ light chains (Table 1).

The reactivities of IgG type MAbs against to GC were tested by direct ELISA using varying antigen (GC-HSA) concentrations. MAbs 5A5 and 5A8 had higher reactivities than those of other MAbs 4G6 and 5B4 as described in Figure 2.

The free MAb 5A8 following competition is bound to polystyrene microtitre plates precoated with GC-HSA. Under these conditions, the full measuring range of assay extends from 20 to 200 ng/ml as indicated in Figure 3.

Table 1. List of monoclonal antibodies against GC

MAb	IgG subclass	Light chain
4G6	1	λ
5A5	1	λ
5A8	1	λ
5B4	1	λ

The ELISA for GC was established for phytochemical investigations involving crude plant extracts, and the assay specificity was checked by determining the cross reactivities of the MAb with various related compounds. The cross-reactivities of MAb obtained was examined by competitive ELISA and calculated using p mole of GC. The cross-reaction of anti-GC MAbs against various related compounds is indicated in Table 2. MAb 5A8 had cross-reactivities of 4.6% against the monoglucuronide of glycyrrhetic acid and 2.1% against glycyrrhetic acid, respectively. MAbs, 4G6, 5A5 and 5B4 had an extensive cross-reaction against the monoglucuronide of glycyrrhetic acid. Moreover, aglycone of GC,

Table 2. Cross-reactivities (%) of anti-GC MABs

Compound	5A8	5A5	4G6	5B4
Glycyrrhizin	100	100	100	100
Glycyrrhetic acid 3/ <i>O</i> -glucuronide	4.36	46.30	73.50	81.03
Glycyrrhetic acid	2.13	18.72	18.35	20.04
11-Deoxo-18 β -glycyrrhetic acid	2.32	5.45	11.37	1.17
18 α -Liquiritic acid	<0.02	<0.02	<0.02	<0.02
18 β -Liquiritic acid	<0.02	<0.02	<0.02	<0.02
11-Deoxo-18 β -liquiritic acid	<0.02	<0.02	<0.02	<0.02
Deoxycholic acid	0.34	0.09	0.16	0.16
Ursolic acid	<0.02	<0.02	0.04	<0.02
Oleanolic acid	<0.02	<0.02	<0.02	<0.02
Hederagenin	<0.02	<0.02	<0.02	<0.02
Betulin	<0.02	<0.02	<0.02	<0.02
Lupeol	<0.02	<0.02	<0.02	<0.02
Cholic acid	<0.02	<0.02	<0.02	<0.02
Cholesterol	<0.02	<0.02	<0.02	<0.02
Ginsenoside Rb1	<0.02	<0.02	<0.02	<0.02
Saikosaponin a	<0.02	<0.02	<0.02	<0.02
Solamargine	<0.02	<0.02	<0.02	<0.02
Solasonine	<0.02	<0.02	<0.02	<0.02
Digitonin	<0.02	<0.02	<0.02	<0.02
Ergosterol	<0.02	<0.02	<0.02	<0.02
β -Sitosterol	<0.02	<0.02	<0.02	<0.02

The cross-reactivities were determined according to Weiler's equation (Weiler *et al.* 1976).

Table 3. Glycyrrhizin contents in traditional Chinese prescription samples

sample	content (mg/g dry drug)
Shakuyaku-kanzo-to	15.77 \pm 2.89
Sho-saiko-to	1.42 \pm 0.34
Dai-saiko-to	0

Data were mean \pm SD from three replicate wells for each concentration.

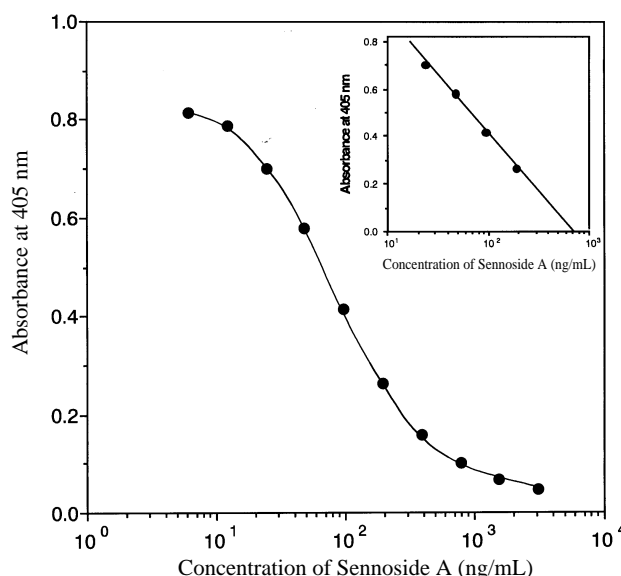
glycyrrhetic acid, cross-reacted with anti-GC MAb, 5B4 resulting in a 20 % cross-reaction. There was an undetectable cross-reaction with other steroidal compounds. Judging from these findings, it is evident that an aglycone and part of the glucuronic acid are immunogenic.

(III) Assay for Traditional Chinese Medicines Prescribed with Licorice

Table 3 shows the concentration of GC in two Traditional Chinese medicines prescribed with licorice, Shakuyaku-kanzo-to and Shosaiko-to, and in Dai-saiko-to which dose not contain licorice. Two former prescriptions contained GC and the concentrations depended on the components of licorice because Shakuyaku-kanzo-to contained two times of licorice than that of Shosaiko-to.

II. Preparation of anti-SA MAb and ELISA

Rhubarb (Da-Huang in Chinese), the rhizome and root of *Rheum* spp. (Polygonaceae), is an important crude drug in the traditional Chinese herbal medicine as well as in western medicine. It is used in many traditional Chinese herbal medicines prescribed for the syndrome of stasis of blood, anti-inflammatory and sedative agent, and as stomachache and for chronic constipation in western civilization. The main purga-

**Figure 4.** Calibration curve of sennoside A.

tive principle of rhubarb was proved to be sennosides⁽²⁷⁾, identical with those isolated from *Senna* leaves, and rheinonides which were isolated also as purgative principles of rhubarb, together with various kinds of phenolics, like tannins, stilbenes, naphthalene derivatives and lindleyin. Sennosides are metabolized by the intestinal bacteria to rheinanthrone which is a direct purgative⁽²⁸⁾. Despite the availability of a number of synthetic purgatives, sennoside-containing prescriptions are still among the most widely used today, and their importance is increasing. Therefore sennosides are listed as the most important pharmaceuticals of plant origin.

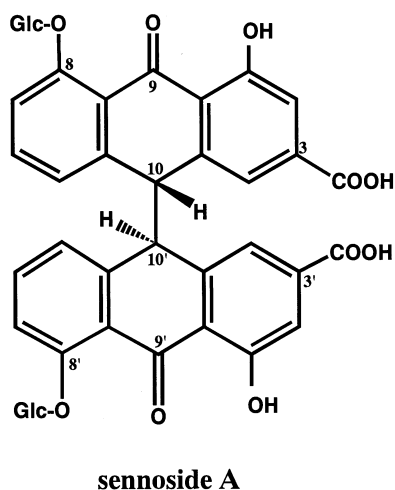


Figure 5. Structure of sennoside A.

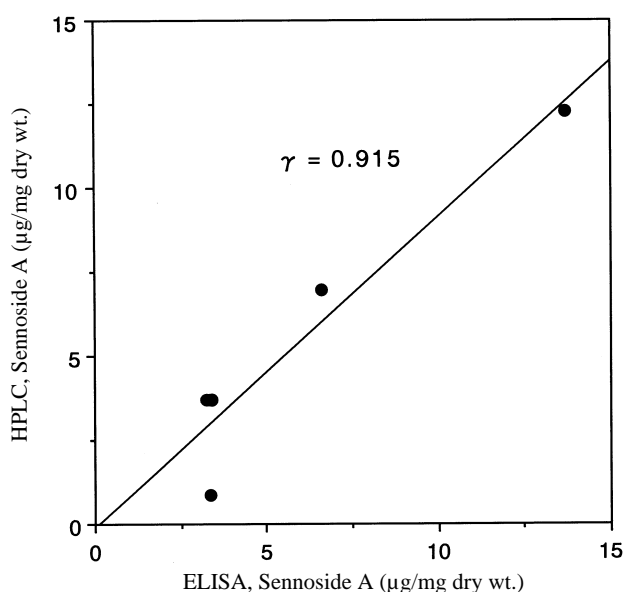


Figure 6. Correlation of sennoside A concentration in rhubarb root between HPLC and ELISA.

(I) Production and Characteristics of MAb against SA

Hybridoma producing MAb reactive to SA was obtained, and classified into IgG1 which had *k* light chains. The reactivity of IgG type MAb, 6G8 was tested by varying antibody concentration and by performing a dilution curve. The antibody concentration (0.218 µg/mL) at which the OD was about 0.8 was selected for competitive ELISA.

The full measuring range of the assay extends from 20 to 200 ngmL⁻¹ as indicated in Figure 4.

SA is a unique anthraquinone having individual double of carboxylic acid-, hydroxyl-, carbonyl- and *O*-glucosyl-groups at C-3, C-1, C-9 and C-8 positions in a molecule, respectively (Figure 5). Moreover, SA possessed a *threo* configuration between at C-10 and C-10' as indicated in Figure 5. Therefore, a MAb should detect all these functions, and also the stereochemical recognition is needed for a complicated compound, SA. Since the newly established ELISA

Table 4. Cross-reactivities of anti-sennoside A MAb (6G8) against various compounds

Compound	Cross-reactivities (%)
Anthraquinone	
sennoside A	100
sennoside B	0.28
rhein	0.35
emodin	< 0.04
aloe-emodin	< 0.04
barbaloin	< 0.04
1,4-dihydroxy-anthraquinone	< 0.04
Stilbene	
rhaponticin	< 0.04
Phenolic acid	
gallic acid	< 0.04
vanillic acid	< 0.04
caffeic acid	< 0.04
homogentisic acid	< 0.04
Coumarin	
esculin	< 0.04
Tannin	
cinnamtannin B1	< 0.04
penta galloyl glucose	< 0.04
Flavonoid	
baicalin	< 0.04
naringin hydrate	< 0.04
wogonine	< 0.04
wogonine 7- <i>O</i> - β -glucuronide	< 0.04
Curcuminoid	
curcumin	< 0.04
Cannabinoid	
Δ^1 -Tetrahydrocannabinolic acid	< 0.04
Δ^1 -Tetrahydrocannabinol	< 0.04

The cross-reactivities of various compounds were determined according to Weiler's equation.

Table 5. Recovery experiment for sennoside A

Added amount of SA (µg)	Measured amount of SA (µg) ^a	Recovery (%) ^b
0	201.7 ± 6.4	
25	228.2 ± 5.7	105.8
50	251.7 ± 2.1	99.9
100	298.7 ± 6.4	97.0

^a Dried powder of rhubarb (30 mg) was used.

^b recovery (%) = (measured amount - 201.7)/(added amount) × 100.

against SA is expected to be applied for phytochemical investigations involving crude plant extracts, the assay specificity is checked by determining the cross-reactivities of the MAb with various related compounds. The cross-reactivities of MAb obtained was examined by competitive ELISA and calculated using *p* mole of SA yielding mid-range and *p* mole of compounds related to SA under investigation yielding mid-range by the method reported by Weiler and Zenk⁽²⁹⁾. Table 4 indicates the cross-reactivities of anti-SA MAb against related anthraquinones and phenolics. MAb 6G8 cross-reacted with rhein and sennoside B (SB) weakly, 0.28 and 0.35%, respectively. However, the other related

anthraquinones did not. From these results it is suggested that a basal structure of rhein and sugar moiety were immunized. In addition most important property of MAb 6G8 is an ability of stereochemical recognition because the differences of structure between SA and SB are only stereochemical configuration at C-10 and C-10' positions. Moreover, MAb 6G8 does not react with other anthraquinone and phenolic compounds as indicated in Table 4 resulting in the high specificity of MAb against SA.

(II) Competitive ELISA and Correlation of SA in Crude Extracts of Rhubarb Root between HPLC and ELISA

The recovery was calculated from the added SA in the same concentration ranges by the following equation. A measured amount of SA in the absence of the added SA was considered as 201.7 (μg).

$$\text{Recovery \%} = \frac{\text{measured amount} - 201.7}{\text{added amount}} \times 100$$

Table 5 indicates good recover ratios between approximately 200 to 300 μg levels. From these results, it is evident that the ELISA using anti-SA MAb can be routinely used for the phytochemical investigations involving crude plant extracts.

The ELISA was approximately 2×10^3 times sensitive than the HPLC method as reported previously⁽³⁰⁾. In order to confirm the correlation of SA between ELISA and HPLC, we also performed the SA contents in various rhubarb roots using HPLC, and calculated the correlation coefficient from fitting a straight line analyzed by ELISA and HPLC methods. Figure 6 shows the correlation of SA concentration in the rhubarb root between HPLC and ELISA indicating that a higher concentration than 3 $\mu\text{g}/\text{mg}$ dry wt. was good. We decided the newly established ELISA can be used for the analysis of SA concentration without any pretreatment.

Oshio *et al.*⁽³¹⁾ analyzed the sennoside contents in various crude rhubarbs by HPLC. Recently Seto *et al.*⁽³²⁾ reported the comparative contents of sennosides analyzed by HPLC in the various commercial rhubarbs. They needed more sample size compared to the established ELISA due to some pretreatments because the crude materials contained several kinds of phenolics like tannins, stilbenes, naphthalen derivatives and lindleyin as previously indicated. Table 6 shows the SA concentrations in various rhubarb. Shinshu-Daio bred by the crossing between *R. palmatum* and *R. coreanum* in order to increase the concentration of SA in Japan, contained the highest SA, 13.69 $\mu\text{g}/\text{mg}$ dry wt.. Ga-wo which was estimated to be a high grade contained 6.62 $\mu\text{g}/\text{mg}$ dry wt.. Other three species showed almost same concentrations of SA, around 3.3 $\mu\text{g}/\text{mg}$ dry wt.. These results are a good agreement with the previous reports⁽³²⁾.

The newly established ELISA was more sensitive than that of TLC or HPLC method. This methodology can be utilized for the assay of SA, therefore it is possible to study a large number of plant samples in the phytochemical field and a small sample size *in vitro* for the breeding of *Rheum* species yielding high concentration of SA. As documented already,

since SA is an important pharmacologically active compound, pharmacokinetics are needed for the body fluids. For these purposes the ELISA can be available because lower concentrations of SA in a large number of samples will be expected.

NEW WESTERN BLOTTING

I. Western Blotting of Ginsenosides Using anti-Ginsenoside Rb1 MAb

Ginseng is one of the most important Chinese medicines used in the world in tonics to combat stresses and cancer, disturbances of the central nervous system⁽³³⁾ and hypothermia⁽³⁴⁾, for antioxidant and organ-protective actions⁽³⁵⁾, for radio-protection⁽³⁶⁾. It contains many dammarane and oleanane saponins⁽³⁷⁾, polyacetylene derivatives⁽³⁸⁾ and polysaccharides⁽³⁹⁾ of which the biological activities have been studied widely. A major ginsenoside, G-Rb1 has been investigated in the nervous system⁽⁴⁰⁻⁴³⁾. More recently, Chang *et al.*⁽⁴⁴⁾ reported the effect of G-Rb1 on drug-induced memory impairment. We previously reported the preparation of MAb against G-Rb1 and its characterizations, using anti-G-Rb1 MAb⁽¹⁰⁾.

Although the western blotting is one of the most common methodology in the fields of higher molecular substances, small molecular compounds have been ruled out. It

Table 6. Sennoside A contents in various rhubarb samples

Sample	Content ($\mu\text{g}/\text{mg}$ dry wt. powder)	
	ELISA	HPLC
Shinshu Daio	13.69 ± 0.69	12.28 ± 0.41
Ga-wo	6.62 ± 0.42	6.93 ± 0.02
Kinmon Daio	3.34 ± 0.02	0.85 ± 0.04
Itto-Ga-wo (powder)	3.27 ± 0.20	3.69 ± 0.32
Itto-Ga-wo (refuse)	3.43 ± 0.16	3.69 ± 0.28

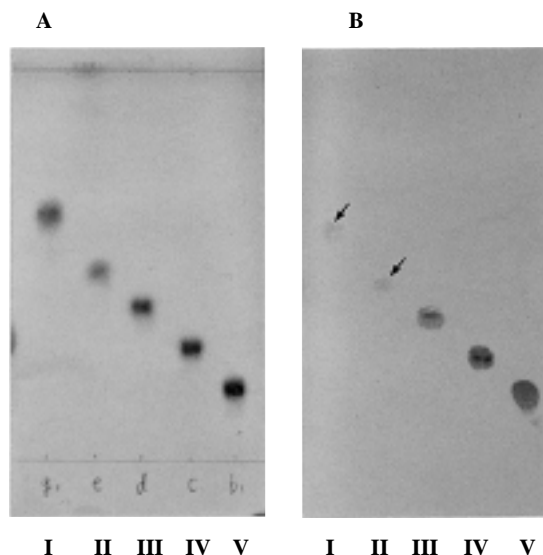


Figure 7. TLC profile stained by H_2SO_4 (A) and western blotting (B) for ginsenosides I: ginsenoside Rg1, II: ginsenoside Rf, III: ginsenoside Rd, IV: ginsenoside Rc, V: ginsenoside Rb1.

is well known that the direct immunostaining of small molecular compounds on TLC plate is impossible. Therefore, a new surveying method bearing an ability to react with small molecular compounds like GRb1 is required for the finding of naturally occurring bioactive compounds. Moreover, if they can be blotted to a membrane, the fixing on membrane needs some new idea. Since we synthesized the hapten-BSA conjugate as indicated above, it seems to be that this reaction can be induced for the fixing on membrane.

Ginsenosides including GRb1 were applied to TLC plate and developed with *n*-BuOH-EtOAc-H₂O (15:1:4). One TLC plate developed was sprayed and colored with H₂SO₄. Other TLC plate developed was blotted on the PVDF membrane by heating at around 120°C. The PVDF membrane

blotted was dipped in water containing NaIO₄ for 1 hr. After washing with water, 50 mM carbonate buffer solution containing BSA was added, and stirred for 3 hr. The PVDF membrane was stained by standard protocols of western blotting using MAb 9G7.

Figure 7 shows the H₂SO₄ staining (A) and western blotting (B) of ginsenosides standards. Lanes I to V show the bands of ginsenosides, GRg1, GRe, GRd, GRc and GRb1, respectively. Different sensitivities between ginsenosides were observed, and that of GRb1 was higher than that of the other ginsenosides. The detectable limit was 360 *p* mole of GRb1. Relations between the binding site of sugar moiety and the staining phenomenon were ruled out that the sugar moiety at C-3 position was necessary for staining, but that at

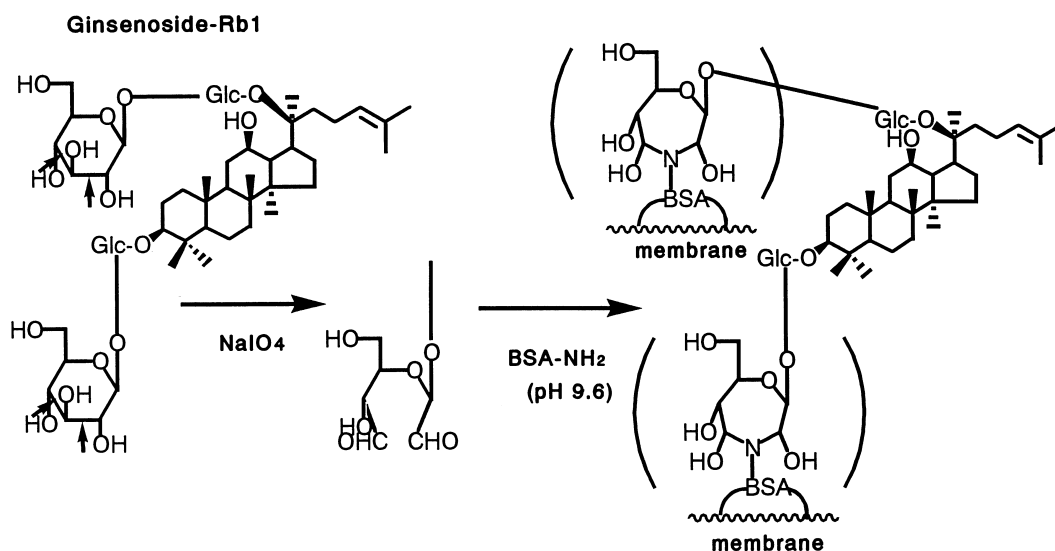


Figure 8. Hypothetic pathway of new western blotting.

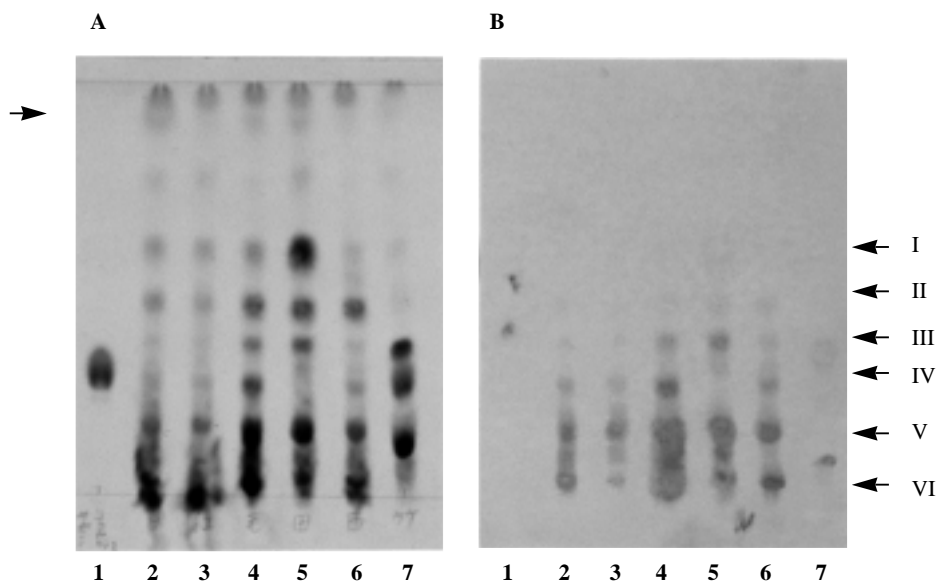


Figure 9. TLC stained by H₂SO₄ (A) and western blotting (B) of crude ginseng extract I to V are same as in Fig.8. VI: malonyl ginsenoside Rb1. Lanes 1 to 7 indicate chikusetsaponin IV, white ginseng, red ginseng, fibrous ginseng, *P. notoginseng*, *P. quinquefolium* and *P. japonicus*, respectively.

C-6 position inhibited staining. Differences of sensitivity on ginsenosides may depend upon their structures and cross-reactivities. The bands of GRe and GRg1 were stained instead of almost no cross-reactivity against MAb 9G7, but disappeared after few hours. This phenomenon suggested that the frame of aglycone, panaxatriol and a part of sugars might be immunized, and functioned as epitopes in the structure of ginsenosides. In addition it is suggested that the specific reactivity of sugar moiety in the ginsenoside molecule against MAb may be modified by the NaIO_4 treatment of ginsenosides on the membrane resulting that GRe and GRg1 become detectable.

Namely this reaction enhanced the fixing of ginsenosides via ginsenoside-BSA conjugates on PVDF membrane. From the evidence that the PVDF membrane incubated in the absence of NaIO_4 were essentially free of staining for ginsenosides (data not shown) the above hypothesis became clear as indicated in Figure 8. Moreover, the antigen synthesized by NaIO_4 cleavage via the conjugation with carrier protein is necessary. Because when the different type of hapten-carrier protein conjugate is used, the western blotting can not be detectable.

The H_2SO_4 staining (Figure 9-A) detected many spots, the western blotting (Figure 9-B) did only ginsenosides in various *Panax* samples bearing panaxatriol resulting that an oleanane-type saponin, chikusetsusaponin III contained in *P. japonicus* can not be detected by the western blotting (Figure 9 lane 1). Interestingly, an aglycone of ginsenosides, panaxatriol (arrow shows) was not detected by the western blotting evidently indicating that the newly established western blotting needed sugar moieties as discussed previously. Compound VI appeared together with ginsenosides on the western blotting suggesting that compound VI might be malonyl GRb1. Therefore, compound VI was directly identified to malonyl GRb1 by the comparison with authentic sample.

II. Western Blotting of GC and Related Compounds Using anti-GC MAb

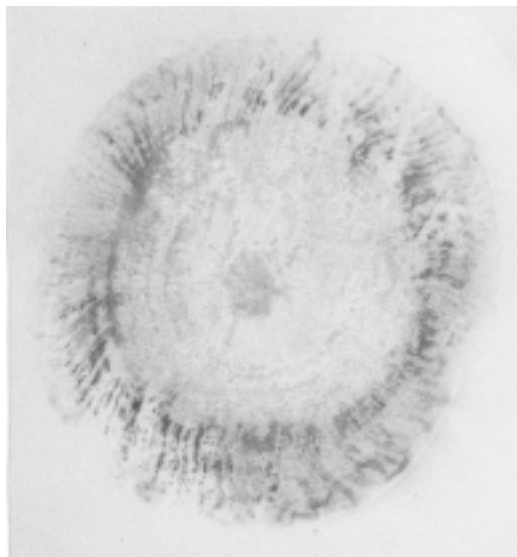


Figure 11. Immunocytochemical localization of glycyrrhizin in fresh *Glycyrrhiza* root.

Figure 10 shows the H_2SO_4 staining (A) and western blotting (B) of GC and various samples. Lanes 1 to 3 show the bands of GC, glycyrrhetic acid monoglucuronide and glycyrrhetic acid, respectively. The western blotting method was more sensitive than the H_2SO_4 staining. A GC aglycone, glycyrrhetic acid was not detected by the western blotting, indicating that the western blotting requires sugar moieties as the same with that of GRb1. In addition it is suggested that the specific reactivity of sugar moiety in GC against MAb may be modified by the NaIO_4 treatment of GC on the membrane, causing glycyrrhetic acid monoglucuronide which has a small cross-reactivity (4.4%) to become detectable by the western blotting as indicated in Figure 10-B, lane 2. The phenomenon that glycyrrhetic acid monoglucuronide is more sensitive than that of GC may depend on their blotting efficiencies because GC is strongly bounded to TLC plate due to two carboxylic acid groups in a molecule.

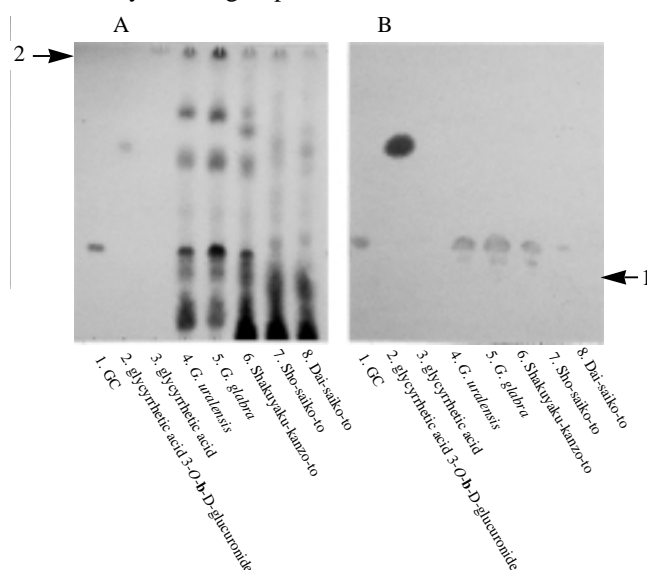
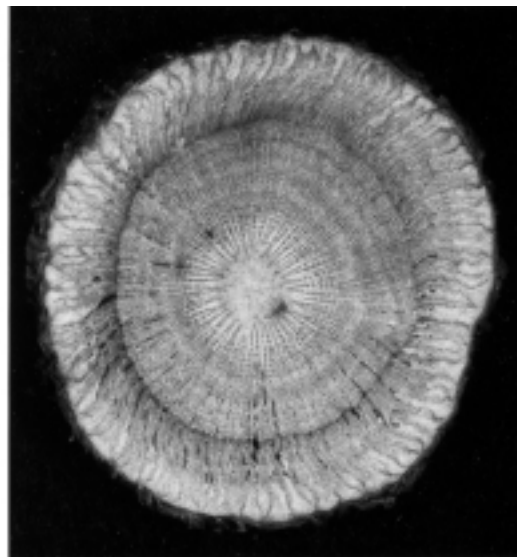


Figure 10. TLC (A) and Western blotting (B) of glycyrrhizin and related compounds. Arrows 1 and 2 indicated an unknown compound and glycyrrhetic acid respectively.



Although the H_2SO_4 staining (Figure 10-A) detected more spots including probably sugars and different types of saponins (Figure 10-A lanes 4 and 5) in various *Glycyrrhiza* samples, the western blotting (Figure 10-B) did only GC, together with a small amount of unknown compound (arrow-1) which may be structurally related to GC, although it has not been identified yet. Two prescriptions of the Chinese traditional medicine containing licorice (Figure 10-B, lanes 6 and 7) were analyzed to detect the typical profile of licorice, and one prescription without licorice, Dai-saiko-to indicated to be free of staining by the western blotting as indicated in Figure 10-B, lane 8. There forth this assay method can be routinely used for survey of natural resources of GC, and of

concentrations of GC in drugs and/or in animal plasma samples as a simple and rapid analysis.

III. Immunocytolocalization of GC in the Fresh *Glycyrrhiza* root

As an application of western blotting methodology the immunocytolocalization of GC in the fresh *Glycyrrhiza* root has been investigated. A sliced *Glycyrrhiza* root was placed on a PVDF membrane, and they were pressed evenly for 6 hr. The blotted PVDF membrane was dipped in water containing NaIO_4 for 1 hr. After washing with water, carbonate buffer solution containing BSA was added, and stirred for 3

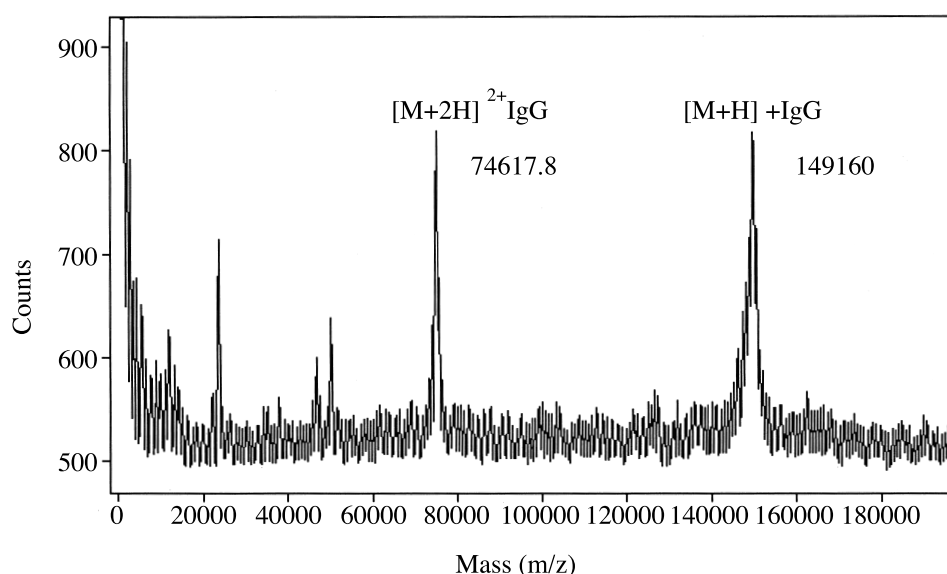


Figure 12. MALDI-tof mass spectra of purified anti-ginsenoside RB1 MAb.

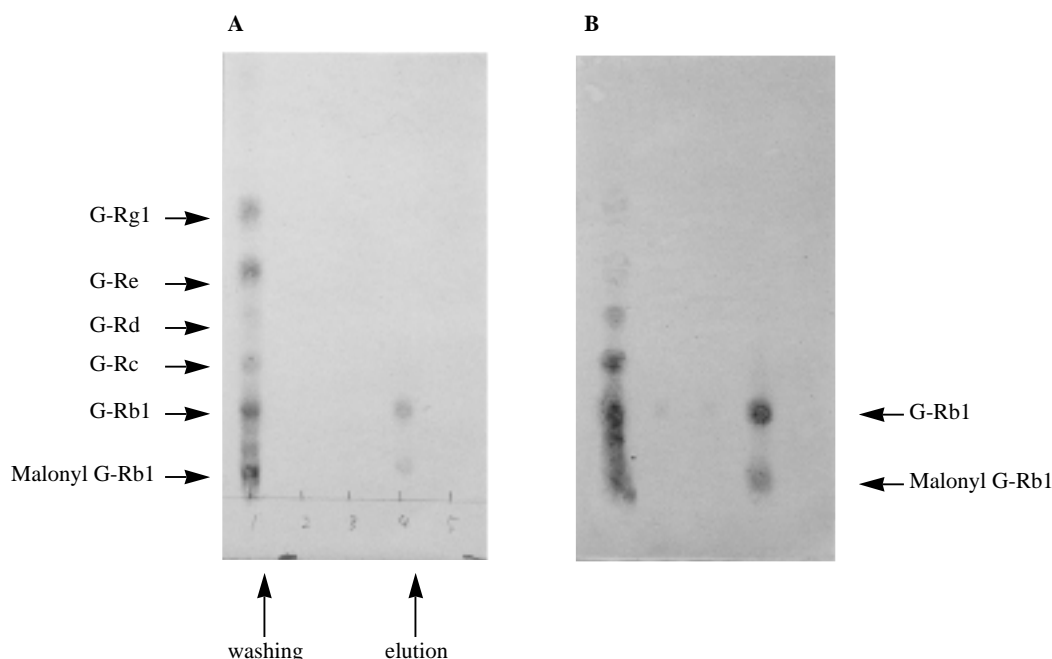


Figure 13. TLC stained by H_2SO_4 (A) and western blotting (B) for elution profile of ginsenosides.

hr. The PVDF membrane was stained by the same method as indicated above using MAb.

Figure 11 indicates the distribution of GC in the fresh root. Endodermis cells (or the pericycle) contained the highest contents of GC, and decreasing to that of exodermis tissues and then that of radial vascular bundle. The immunocytochemical localization of GC may be available for the analysis of animal tissue samples of GC not limited to GC distributions in plant organs or tissues since very low concentrations are expected.

IMMUNOAFFINITY COLUMN CHROMATOGRAPHY

I. Single Step Isolation of GRb1 from Crude Extracts of Ginseng by Immunoaffinity Column Using an anti-GRb1 MAb

After MAb from the original clone was purified by Protein G Sepharose 4FF column, the MALDI mass spectrometry was measured to confirm the purity of the MAb as previously reported⁽¹⁾. The molecular weight was 149,160, which is in good agreement with that of human IgG1 being determined as 146,000 (Langone, 1982) (Figure 12). It became evident that the purified MAb using protein G column can be used for the immunoaffinity column.

Since the ginseng root contains a number of dammarane-type saponins, ginsenosides together with oleanane-type saponins, the isolation of a saponin are very troublesome requiring the repeated silica gel column chromatography. For this evidence we established a simple and reproducible purification method for GRb1 using an immunoaffinity column conjugated with a purified anti-GRb1 MAb.

The GRb1 concentration increased little by eluting with the 20 mM phosphate buffer containing 0.5 M KSCN and 10% CH₃OH. If the 20 mM phosphate buffer was changed to the 100 mM AcOH buffer (pH 4), the elution ability reaches to the optimum. Although 20 % CH₃OH enhanced the elution of GRb1, higher concentrations of CH₃OH than 20 % did not affect the sample. From these results 100 mM AcOH buffer containing 0.5 M KSCN and 20 % CH₃OH can be routinely used as an elution buffer solution. The antibody was stable when exposed to the eluent, and the immunoaffinity column showed almost no decrease in capacity (20 µg/mL gels) after repeated use more than 10 times under the same conditions.

The crude extracts of root of *P. ginseng* were loaded on the immunoaffinity column, and washed with 20 mM phosphate buffer containing 0.5 M NaCl (PBS, 40 ml). Figure 13 shows the fractions 1 to 14 contained over-charged GRb1 and which was determined by ELISA. GRc, GRd, GRE and GRg1 were detected by western blotting. A sharp peak appeared around fractions 20 to 30 which contained GRb1 determined by ELISA. However, GRb1 purified by the immunoaffinity column was still contaminated by a small amount of malonyl GRb1⁽⁴⁵⁾ detected by the western blotting as shown in Figure 13. This compound has almost the same

cross-reactivity with GRb1. Therefore, the mixture was treated with alkaline solution at room temperature for 1 hr, as previously reported⁽⁴⁵⁾, to give pure GRb1. Over charged GRb1, eluted using washing solution, was repeatedly loaded and finally isolated.

This methodology is useful for the rapid and simple supplement of pure GRb1, and may open up a wide field of comparable studies with other families of saponins that an acceptable method for a quick separation is not known yet, although we reported a single step separation of forskolin using anti-forskolin MAb⁽¹²⁾.

II. Immunoaffinity Purification of Solasodine Glycosides by Using Wide-cross Reactive anti-Solamargine MAb

The purified MAb was coupled to Affi-Gel Hz hydrazide gel to give an immunoaffinity gel.

To assess the capacities and the recoveries of solamargine, solasonine and solasodine from the immunoaffinity column, each substance was added separately and run through the column. The content of individual fractions were determined by ELISA after washing with PBS, and then completely eluting with PBS containing 40% methanol. The established elution buffer system resulted in 95.31%, 97.20% and 95.80% recovery of solamargine, solasonine and solasodine, respectively. The capacity of immunoaffinity column was determined to be 6.19, 12.92, 3.92 µg of solamargine, solasonine and solasodine, respectively per ml of immunoaffinity gel.

The crude extract of *S. khasianum* fruit was loaded on the immunoaffinity column, washed with PBS and eluted with 40% methanol in PBS. Figure 14 shows a chromatogram detected by ELISA. Fractions 1-8 contained over loaded solasodine glycosides like solamargine, solasonine, L-rhamnosyl-(1->4)-O-3-β-D-glucopyranosyl solasodine and the other non-related unknown compounds which were detected by TLC stained with H₂SO₄ as indicated in Figure

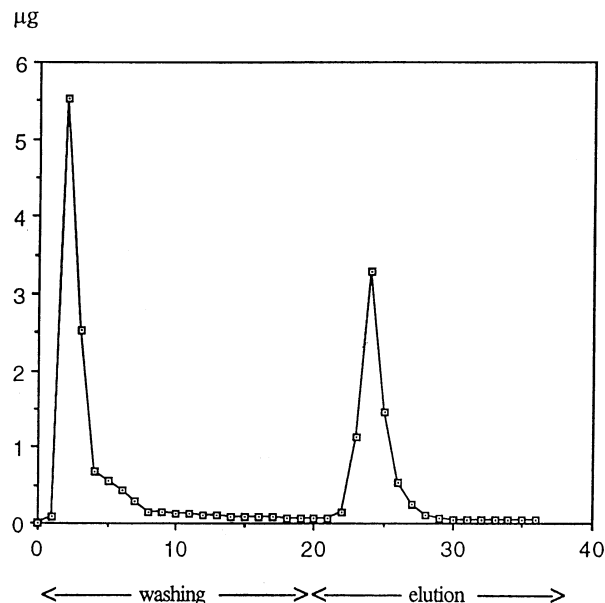


Figure 14. Elution profile of solasodine glycosides.

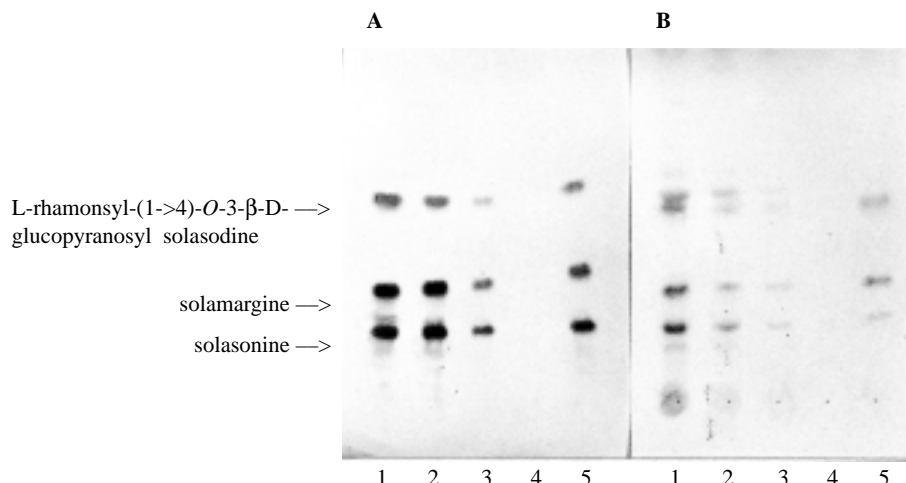


Figure 15. Elution profile of solasodine glycosides detecting by western blotting (A) and TLC stained by H_2SO_4 (B).

15(B). The peak of fractions 22-29 shows the elution of total solasodine glycosides eluted with 40% methanol in PBS. The fractions contained only solamargine, solasonine and L-rhamnosyl-(1->4)-O-3- β -D-glucopyranosyl solasodine which were determined by TLC stained with H_2SO_4 and the western blotting (Figure 15-A,B). The above over loaded solasodine glycosides can be separated completely from the non-related unknown compounds by repeated column chromatography (data not shown). This result indicates that solasodine-type steroidal alkaloids can be separated by the newly established immunoaffinity column.

CONCLUSION

Western blotting methodology reviewed in this article can be theoretically applied to all sugar conjugates such as saponins and glycosides. Moreover, glucuronides and aminosugar conjugates, with at least two vicinal hydroxyl groups in a molecule may apply also. Our result indicates that the new western blotting method can detect the related compounds possessing very small cross-reactivity because the reactivity may be modified when the sugar moiety is opened by $NaIO_4$ as indicated that ginsenosides can be detected by anti-GRb1 MAb instead of very small cross-reactivity against GRc, GRd, GRf and GRg1.

In general, competitive ELISA is a more sensitive method compared to TLC and/or HPLC as described for GC⁽⁸⁾, SA⁽¹¹⁾ and GRb1⁽¹⁰⁾. When detecting very low concentration of these compounds is required, the combination of ELISA and immunoaffinity concentration may be useful techniques for the samples from natural resources and animal plasma.

All solasodine glycosides can be cross-reacted with anti-SM MAb⁽⁷⁾. This wide-cross reaction is the major advantage of this MAb reagent because it is pointed out that the mixture of total solasodine glycosides can be separated by a single immunoaffinity column. Therefore, this method can be applied to the rapid and simple separation of total solasodine glycosides. This methodology is also useful for

the detection of higher yielding solasodine glycosides plantlets *in vitro* of *S. khasianum* by the combination with ELISA because the regenerated plantlets contain a small amount of solasodine glycosides. Therefore, if there is need to separate the total saponins, such as panaxatriol or panaxadiol as an aglycone, the wide cross-reactive MAb against ginsenosides can be used for this purpose.

ACKNOWLEDGEMENTS

This research project was supported by the Grant-in-Aid for Scientific Research, Ministry of Education, Sciences and Culture (# 11470470, Japan), Sasakawa Scientific Research Grant, The Shorai Foundation for Science and Technology and The Uehara Memorial Foundation. The authors are grateful to these financial supports.

REFERENCES

1. Sakata, R., Shoyama, Y. and Murakami, H. 1994. Production of monoclonal antibodies and enzyme immunoassay for typical adenylate cyclase activator, forskolin. *Cytotechnology* 16: 101-108.
2. Yanagihara, H., Sakata, R., Shoyama, Y. and Murakami, H. 1995. Relationship between the content of forskolin and growth environments in clonally propagated *Coleus forskohlii* Brig. *Biotronics* 24: 1-6.
3. Yanagihara, H., Sakata, R., Shoyama, Y. and Murakami, H. 1996. Rapid analysis of small samples containing forskolin using monoclonal antibodies. *Planta Medica*. 62: 169-172.
4. Tanaka, H., Goto, Y. and Shoyama, Y. 1996. Monoclonal antibody based enzyme immunoassay for marihuana (cannabinoid) compounds. *J.Immunoassay* 17: 321-342.
5. Tanaka, H. and Shoyama, Y. 1999. Monoclonal antibody against tetrahydrocannabinolic acid distinguishes *Cannabis sativa* samples from different plant species. *For.Sci.Int.* 106: 135-146.
6. Shoyama, Y., Fukada, T. and Murakami, H. 1996.

- Production of monoclonal antibodies and ELISA for thebaine and codeine. *Cytotechnology* 19: 55-61.
7. Ishiyama, M., Shoyama, Y., Murakami, H. and Shinohara, H. 1996. Production of monoclonal antibodies and development of an ELISA for solamargine. *Cytotechnology* 18: 153-158.
 8. Tanaka, H. and Shoyama, Y. 1998. Formation of a monoclonal antibody against glycyrrhizin and development of an ELISA. *Biol.Pharm.Bull.* 21: 1391-1393.
 9. Xuan, L., Tanana, H., Xu, Y. and Shoyama, Y. 1999. Preparation of monoclonal antibody against crocin and its characterization. *Cytotechnology* 29:65-70.
 10. Tanaka, H., Fukuda, N. and Shoyama, Y. 1999. Formation of monoclonal antibody against a major ginseng component, ginsenoside Rb1 and its characterization. *Cytotechnology* 29: 115-120.
 11. Morinaga, O., Tanaka, H. and Shoyama, Y. 2000. Production of monoclonal antibody against a major purgative component, sennoside A, its characterization and ELISA. *Analyst* 125: 1109-1113.
 12. Yanagihara, H., Sakata, R., Minami, H., Tanaka, H., Shoyama, Y. and Murakami, H. 1996. Immunoaffinity column chromatography against forskolin using an anti-forskolin monoclonal antibody and its application. 335:63-70.
 13. Putalun, W., Tanaka, H. and Shoyama, Y. 1999. Rapid separation of solasodine glycosides by an immunoaffinity column using anti-solamargine monoclonal antibody. *Cytotechnology* 31: 151-156.
 14. Fukuda, N., Tanaka, H. and Shoyama, Y. 2000. Isolation of the pharmacologically active saponin ginsenoside Rb1 from ginseng by immunoaffinity column chromatography. *J.Nat.Prod.* 63: 283-285.
 15. Tanaka, H., Putalun, W., Tsuzaki, C. and Shoyama, Y. 1997. A simple determination of steroidal alkaloid glycosides by thin-layer chromatography immunostaining using monoclonal antibody against solamargine. *FEBS Lett.* 404: 279-282.
 16. Fukuda, N., Tanaka, H. and Shoyama, Y. 1999. Western blotting for ginseng saponins, ginsenosides using anti-ginsenoside Rb1 monoclonal antibody. *Biol.Pharm.Bull.* 22: 219-220.]
 17. Shan, S., Tanaka, H. and Shoyama, Y. 1999. Western blotting method for the immunostaining detection of glucuronides of glycyrrhetic acid using anti-glycyrrhizin monoclonal antibody. *Biol.Pharm.Bull.* 22: 221-223.
 18. Shoyama, Y., Sakata, R., Isobe, R. and Murakami, H. 1993. Direct determination of forskolin-bovine serum albumin conjugate by matrix-assisted laser desorption ionization mass spectrometry. *Org.Mass.Spect.* 28: 987-988.
 19. Shoyama, Y., Fukada, T., Tanaka, T., Kusai, A. and Nojima, K. 1993. Direct determination of opium alkaloid-bovine serum albumin conjugate by matrix-assisted laser desorption/ionization mass spectrometry. *Biol.Pharm.Bull.* 16: 1951-1053.
 20. Goto, Y., Shima, Y., Morimoto, S., Shoyama, Y., Murakami, H., Kusai, A. and Nojima, K. 1994. Determination of tetrahydrocannabinolic acid-carrier protein conjugate by matrix-assisted laser desorption/ionization mass spectrometry. and antibody formation. *Org.Mass Spectrometry* 29: 668-671.
 21. Japanese Pharmacopoeia. 1996. 13th ed. pp. 1140-1141.
 22. Doll R., Hill I. D., Hutton C. and Underwood D. L. 1962. Clinical trial of a triterpenoid liquorice compound in gastric and duodenal ulcer. *Lancet*, ii, 793-796.
 23. Pompei, R., Flore, O., Marccialis, M. A., Pani, A. and Loddo, B. 1979. Glycyrrhizic acid inhibits virus growth and inactivates virus particles. *Nature* 281(5733): 689-690.
 24. Kuroyanagi, T. and Saito, M. 1966. Effect of prednisolone and glycyrrhizin on passive transfer in experimental allergic encephalomyelitis. *Jpn. J. Allerg.* 15: 67-74.
 25. van Rossum T.G., Vulto A.G., de Man R.A., Brouwer J.T., Schalm S.W., 1998. Review article: glycyrrhizin as a potential treatment for chronic hepatitis C. *Alimentary Pharmacology & Therapeutics.* 12:199-205.
 26. Mizugaki, M., Itoh, K., Hayasaka, M., Ishiwata, S., Nozaki, S., Nagata, N., Hanadate, K. and Ishida, N. 1994. Monoclonal antibody-based enzyme-linked immunosorbent assay for glycyrrhizin and its aglycon, glycyrrhetic acid. *Journal of Immunoassay* 15:21-34.
 27. Oshio, H., Naruse, Y. and Tsukui, M. 1978. Quantitative analysis of the purgative components of rhubarb and senna. *Chem. Pharm. Bull.* 26: 2458-2464.
 28. Yang, L., Akao, T., Kobashi, K. and Hattori, M. 1996. Purification and characterization of a novel sennoside hydrolyzing β -glucosidase from *Bifidobacterium* Sp. Strain SEN, a human intestinal anaerobe. *Biol. Pharm. Bull.* 19: 705-709.
 29. Weiler, E. W. and Zenk, M. H. 1976. Radioimmunoassay for the determination of digoxin and related compounds in *Digitalis lanata*. *Phytochemistry* 15: 1537-1545.
 30. Sagara, K., Oshima, T. and Yoshida, T. 1987. Rapid and simple determination of sennoside A and B in rhei rhizoma by ion-pair high-performance liquid chromatography. *J. Chromatogr.* 403: 253-261.
 31. Oshio, H. and Kawamura, N. 1985. Quantitative analysis of the laxative components in rhubarb by high performance liquid chromatography. *Shoyakugaku Zasshi* 39: 131-138.
 32. Seto, T., Yasuda, I., Hamano, T., Takano, I., Kiyono, S., Nishijima, M. and Akiyama, K. 1996. Determination method of sennoside A, sennoside B, rhein 8-glucoside in kampo or crude drug preparations and the comparison of these components in processed rhubarb. *Natural Medicines* 50: 138-144.
 33. Abe, K., Cho, SI., Kitagawa, I., Nishiyama, N. and Saito, H. 1994. Differential effects of ginsenoside Rb1 and malonylginsenoside Rb1 on long-term potentiation in the dentate gyrus of rats. *Brain Res.* 649:7-11.
 34. Ben-Hur E. and Fulder, S. 1981. Effect of *Panax ginseng* saponins and *Eleutherococcus senticosus* on survival of

- cultured mammalian cells after ionizing radiation. Am. J. Chin. Med. 9: 48-56.
35. Gillis, C.N. 1997. Panax ginseng pharmacology: a nitric oxide link?. Biochem. Pharmacol. 54: 1-8.
 36. Tanaka, Y. and Akagi, K. 1992, Radiation damage. In: Okuda, T, Kimura, M. Miyamoto, A. Wada, H. (ed.) Metabolism and disease, supplementary issue, Vol. 29. pp. 423-429. Nakayama Shoten, Tokyo.
 37. Besso, H., Kasai, R., Saruwatari, Y., Fuwa, T. and Tanaka, O. 1982. Ginsenoside-Ra1 and ginsenoside-Ra2, new dammarane-saponins of ginseng roots. Chem. Pharm. Bull. 30: 2380-2385.
 38. Hanasen, L. and Boll, PM. 1986. Polyacetylenes in Araliaceae: Their chemistry, biosynthesis and biological significance. Phytochemistry 25: 285-293.
 39. Tomoda, M., Hirabayashi, K., Shimizu, N., Gonda, R., Ohara, N. and Takada, K. 1993. Characterization of two novel polysaccharides having immunological activities from the root of Panax Ginseng. Chem. Pharm. Bull. 16: 1087-1090.
 40. Saito, H., Suda, K., Schwab, M. and Thoenen, H. 1977. Potentiation of the NGF-mediated fiber outgrowth by ginsenoside Rb1 in organ cultures of chicken dorsal root ganglia. Jpn. J. Pharmacol. 27: 445-451.
 41. Takemoto, Y., Ueyama, T., Saito, H., Horio, S., Sanada, S., Shoji, J., Yahara, S., Tanaka, O. and Shibata, S. 1984. Potentiation of nerve growth factor-mediated nerve fiber production in organ cultures of chicken embryonic ganglia by ginseng saponins: structure-activity relationship. Chem. Pharm. Bull. 32: 3128-3133.
 42. Saito, H., Tsuchiya, M., Naka, S. and Takagi, K. 1977. Effect of Panax Ginseng root on conditioned avoidance response in rats. Jpn. J. Pharmacol. 27: 509-516.
 43. Himi, T., Saito, H. and Nishizawa, N. 1989. Effect of ginseng saponins on the survival of cerebral cortex neurons in cell cultures. Chem. Pharm. Bull. 37: 481-484.
 44. Chang, Y. S., Wu, C. R., Ho, Y. L. and Hsieh, M. T. 1989. In "Advances in Ginseng Research". Huh, H. ed. The Korean Society of Ginseng, Seoul, 15: 289-299.
 45. Kitagawa, I., Taniyama, T., Yoshikawa, M., Ikenishi, Y. and Nakagawa, Y. 1989. Chemical studies on crude drug processing. IV. Chemical structures of malonyl-ginsenosides Rb1, Rb2, Rc, and Rd isolated from the Root of Panax ginseng C. A. MEYER. Chem. Pharm. Bull. 37: 2961-2970.

利用單株抗體於酵素連接免疫吸附分析、免疫細胞分佈和免疫親合性管柱以檢測及分離天然物活性成分

單小傑 Waraporn Putalun 福田憲子 森永紀 田中宏幸 正山征洋*

九州大學 藥學院
日本 福岡市 東區 馬出3-1-1

(收稿: June 29, 2000; 接受: December 14, 2000)

摘 要

抗番瀉甙甲 (sennoside A; SA) 及抗甘草甜素 (glycyrrhizin; GC) 之單株抗體已經過選篩 (screening) 及選殖 (cloning), 並且應用於酵素連接免疫吸附分析 (enzyme link immuno sorbent assay; ELISA)。檢測抗甘草甜素及人參皂甙 (ginsenoside) 之新西方墨點法 (new western blotting method) 已建立。首先將甘草甜素和人參皂甙以矽膠薄層層析板分離, 再轉移到聚偏二氟乙烯 (polyvinylidene difluoride; PVDF) 膜, 隨後以碘酸鈉 (NaIO_4) 溶液處理, 再以牛血清蛋白 (bovine serum albumin; BSA) 浸潤, 結果 PVDF 膜就附上人參皂甙-血清蛋白複合物之抗原, 接下來的染色步驟與傳統西方墨點法相同。檢驗甘草屬 (*Glycyrrhiza* species) 植物新鮮根部中甘草甜素在細胞內之分配情形, 可先將甘草甜素以上述方法轉移到聚偏二氟乙烯膜, 再以抗甘草甜素之單株抗體偵測。應用免疫親合性管柱 (immunoaffinity column) 接上人參皂甙 Rb1 (GRb1) 之單株抗體來分離人參皂甙 Rb1, 遠優於先前所發表由人參粗萃取物分離 Rb1 的方法。另一方面, solasodine glycosides 的總含量測定, 可藉由免疫親合性管柱接上 anti-solamargine (SM) 之單株抗體, 經廣泛性交反應來分離。

關鍵詞: 單株抗體, 生物活性成分, 酵素連接免疫吸附分析法, 西方墨點法, 免疫親合性管柱