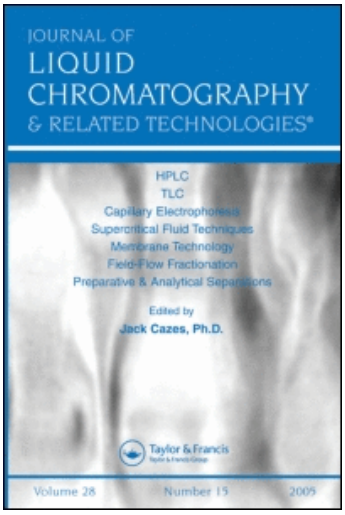

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WESTERN BLOTTING OF STEROIDAL ALKALOID GLYCOSIDES USING MONOCLONAL ANTIBODY AGAINST SOLAMARGINE

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ABSTRACT

A method for the determination of solasodine glycosides using western blotting was investigated. Solasodine glycosides, separated by silica gel thin-layer chromatography, were transferred to a polyvinylidene difluoride membrane. The membrane was treated with sodium periodate solution followed by bovine serum albumin, resulting in a solasodine glycoside-bovine serum albumin conjugate. Individual spots were stained with monoclonal antibody against solamargine. Immunostaining of solasodine glycosides was more sensitive compared with other staining methods. The newly established western blotting method is extended to the distribution of solasodine glycosides in plants.

INTRODUCTION

The immunoassay system using MAb is indispensable to biological investigations.[1] It is, however, rare for naturally occurring bioactive compounds to have small molecular weights. In the process of MAb formation, the confirmation of antigen conjugate is necessary for immunization. The hapten number conjugated with carrier protein has been determined by MALDI mass analysis, resulting in a more accurate molecular ratio of hapten and carrier protein especially for those compounds having no specific UV absorbance.[2-4] In our studies on the production of MAb against naturally occurring bioactive compounds, we have established MAbs against forskolin,[5,6] opium alkaloids,[7] marihuana compounds,[8] solamargine,[9] crocin,[10] and ginsenoside Rb1.[11]

In our continuing studies on the breeding of medicinal plants, we have reported the higher yielding strain of cannabidiolic acid which is considered a non-toxic marihuana strain,[12] and clonal and homogeneous medicinal strains,[13-17] and virus free plants[18] using plant biotechnology.

The natural sources of adrenocortical hormones and sex hormones, which have been supplied mainly by diosgenin, are becoming rare. The most important feature of solasodine is that it can be converted to dehydropregnenolone. Solasodine is found with a series of sugar residues attached to the oxygen at C-3 position. The most common forms are the triglycosides with solamargine being predominant.[19] Therefore, the steroidal alkaloid glycosides of solasodine type, such as solamargine are important as starting material for the production of steroidal hormones in the pharmaceutical and medicinal areas. Rapid, simple, highly sensitive and reproducible assay systems are required for a large number of plants, and limited amount of samples, in order to select the strain of higher yielding steroidal alkaloid glycosides.

We present, here, a simple determination method for solasodine glycosides using western blotting in this capture.

EXPERIMENTAL

Chemicals and Immunochemicals

BSA and HSA were provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was provided by Organon Teknika Cappel Products

(West Chester, PA, USA). PVDF membranes (Immobilon-N) were purchased from Millipore Corporation (Bedford, MA, USA). Glass microfiber filter sheets (GF/A) were purchased from Whatman International Ltd. (Maidstone, England). All other chemicals were standard commercial products of analytical grade.

Fruits of *Solanum khasianum* were obtained from the herbal garden of the Faculty of Pharmaceutical Sciences, Kyushu University in Japan. Solamargine and solasonine were isolated from fresh fruits of *S. khasianum* as previously described.[19] Solasodine was obtained from solamargine by acid hydrolysis as previously described.[19] Solamargine (1 mg) was dissolved in MeOH containing 1 M HCl (1 mL). The mixture was heated at 70°C for 10, 20, 30, 60, and 90 min. Individual hydrolysates were evaporated, *in vacuo*, and applied to TLC. Spots developed by TLC were determined by H_2SO_4 and Dragendorff reagent.

An immunogen, solamargine-BSA conjugate was synthesized from solamargine by treatment with $NaIO_4$ followed by conjugation with BSA as previously reported.[20] Immunization and hybridization were carried out as previously reported.[5] A culture medium (50 mL) containing IgG (SMG-BD9)[9] was purified by using a CHROMATOP Protein A column[21] (0.46 x 25 cm, NGK INSULATORS, LTD, Nagoya, Japan), and dialyzed against H_2O 5 times, and finally lyophilized to give IgG (0.53 mg).

TLC

Solasodine glycosides were applied to a TLC plate and developed with $CHCl_3$: MeOH : NH_4OH (7 : 2.5 : 1). The developed TLC plate was dried and then sprayed with a blotting solution mixture of isopropanol : methanol : water (5 : 20 : 40, by volume). It was placed on a stainless steel plate, then covered with a PVDF membrane sheet. After covering with a glass microfiber filter sheet, the plate was pressed evenly for 45 s with a 130°C iron as previously described.[22] The PVDF membrane was separated from the plate and dried.

Western Blotting of Solasodine Glycosides on PVDF Membrane

The PVDF membrane blotted was dipped in water containing $NaIO_4$ (10 mg/mL), under stirring, at room temperature for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA (1%) was added, and stirred at room temperature for 3 h. The PVDF membrane was washed with TPBS for 5 min twice, and then washed with water. The PVDF membrane

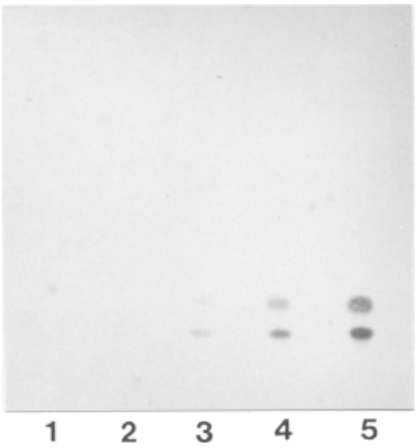

Figure 1. Western blotting of solamargine and solasonine. Solamargine (upper spot) and solasonine (bottom spot) were developed by $CHCl_3$-MeOH-NH_4OH solvent system on a silica gel TLC plate. Lanes 1 to 5 show the concentrations of both alkaloids, 0.8, 1.6, 8, 40, and 200 ng, respectively. After transfer to PVDF membrane, the membrane was treated with $NaIO_4$ and stainned by MAb.

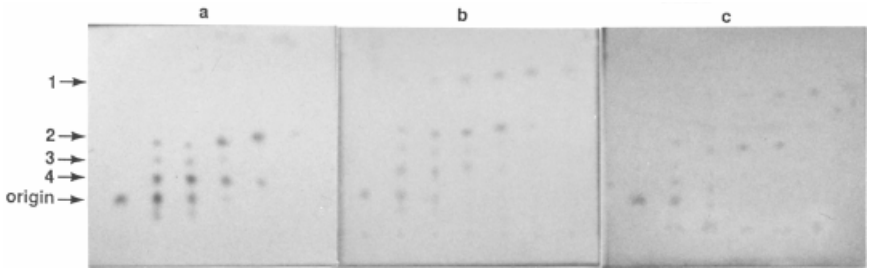

Figure 2. Hydrolyzed products of solamargine by HCl. a, b, and c show western blotting, the staining with sulfuric acid and with Dragendorff reagent, respectively. Solamargine was hydrolized by 1M HCl for 10, 20, 30, 60, and 90 min, respectively. Spots 1-4 were identified with solasodine, 3-O-β-D-glucopyranosyl solasodine, L-rhamnosyl-(1→4)-O-3-β-D-gucopyranosyl solasodine, L-rhamnosyl-(1→2)-3-β-O-D-glucopyranosyl solasodine, respectively.

was immersed in anti-solamargine MAb, and stirred at room temperature for 1 h. After washing the PVDF membrane twice with TPBS and water, 1000 times dilution of peroxidase-labeled goat anti-mouse IgG in GPBS 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, and the reaction was stopped by washing with water. The PVDF membrane was allowed to dry.

RESULTS AND DISCUSSION

After solasodine glycosides were transferred to a PVDF membrane sheet from the TLC plate by heating (see materials and methods section), as previously reported,[22] the PVDF membrane was treated with $NaIO_4$ solution followed to conjugate with BSA since free solasodine glycosides on PVDF membrane are washed out by buffer solution or water without the formation of conjugate with carrier protein. The PVDF membrane was immersed in anti-solamargine MAb, and then peroxidase-labeled secondary MAb. When added substrate and H_2O_2, clear blue spots appeared as indicated in Fig. 1.

Fig. 1 shows western blotting of solamargine and solasonine. Lanes 1 to 5 show the concentrations of both alkaloids at 0.8, 1.6, 8, 40, and 200 ng, respectively. The different sensitivities between solamargine and solasonine were observed in individual concentrations, and the sensitivity of solasonine was somewhat higher than that of solamargine. Detectable limits were 1.6 ng of solasonine.

To expand this newly established western blotting method, solamargine was hydrolyzed by 1M HCl for 10, 20, 30, and 60 min. Individual hydrolysates were applied to three TLC plates and then developed with $CHCl_3$: MeOH : NH_4OH solvent system. Two plates were sprayed and visualized with H_2SO_4 and Dragendorff reagents, respectively. One plate was transferred to PVDF membrane. Fig. 2 shows the western blotting (a), and staining by Dragendorff reagent (b) and H_2SO_4 (c). When compared the staining sensitivities of three methods, western blotting was the highest, followed by the H_2SO_4, and then the Dragendorff reagent. Moreover, compared with two stainings between western blotting (a) and H_2SO_4 (c), solasodine was not detected by immunostaining despite 44% cross-reactivity,[9] since solasodine can not conjugate on PVDF membrane due to the absence of a sugar moiety. It is easily suggested that product 1 may be an aglycone of solamargine, solasodine and products 2, 3, and 4 might be solasodine mono and diglycosides. Therefore, products 1, 2, 3, and 4 were identified as solasodine, 3-*O*-α-D-glucopyranosyl-solasodine, *O*-α-L-rhamnosyl-(1→4)-3-*O*-β-D-glucopyranosyl-solasodine and *O*-α-L-rhamnosyl-(1→2)-3-*O*-β-D-glucopyranosyl-solasodine, respectively, by direct comparison with authentic samples.

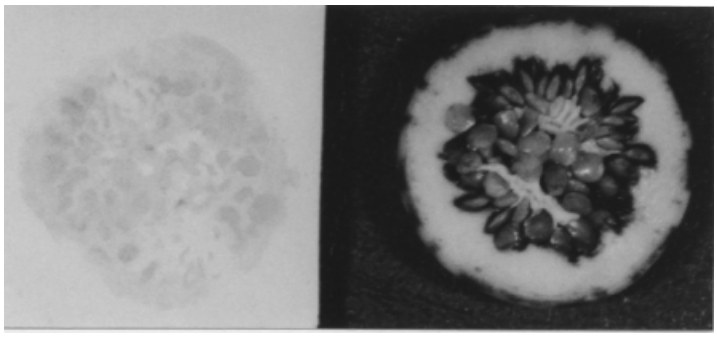

Figure 3. The fruit slice of *Solanum khasianum* and its immunostaining. The PVDF membrane covered the surface of the sliced fruit, and stained with the same manner with western blotting as indicated in Fig. 1.

Table 1

Cross-Reactivities of Monoclonal Antibody (SMG-BD9) Against Steroidal Compounds

Compound	Cross-Reactivities (%)
Solmargine	100
Solasonine	92.1
3-*O*-β-D-glucopyranosyl-solasodine	112.9
O-α-L-rhamnosyl-(1→4)-3-*O*-β-D-glucopyranosyl-solasodine	80.7
O-α-L-rhamnosyl-(1→2)-3-*O*-β-D-glucopyranosyl-solasodine	98.5
Tomatine	2.1
Tomatigine	0.3

Table 1 shows the cross-reactions of individual hydrolyzed products. The cross-reactivities of three hydrolyzed products were 112.9, 80.7, and 98.5%, respectively, together with solamargine (100%) and solasonine (92.1%). The cross-reactions of tomatidine and tomatine, which are structurally related to solasodine and its glycoside, were insignificant in tomatine (2.1%) and undetectable in tomatidine (0.3%). Other steroidal compounds did not cross-react with anti-solamargine MAbs (data not shown).

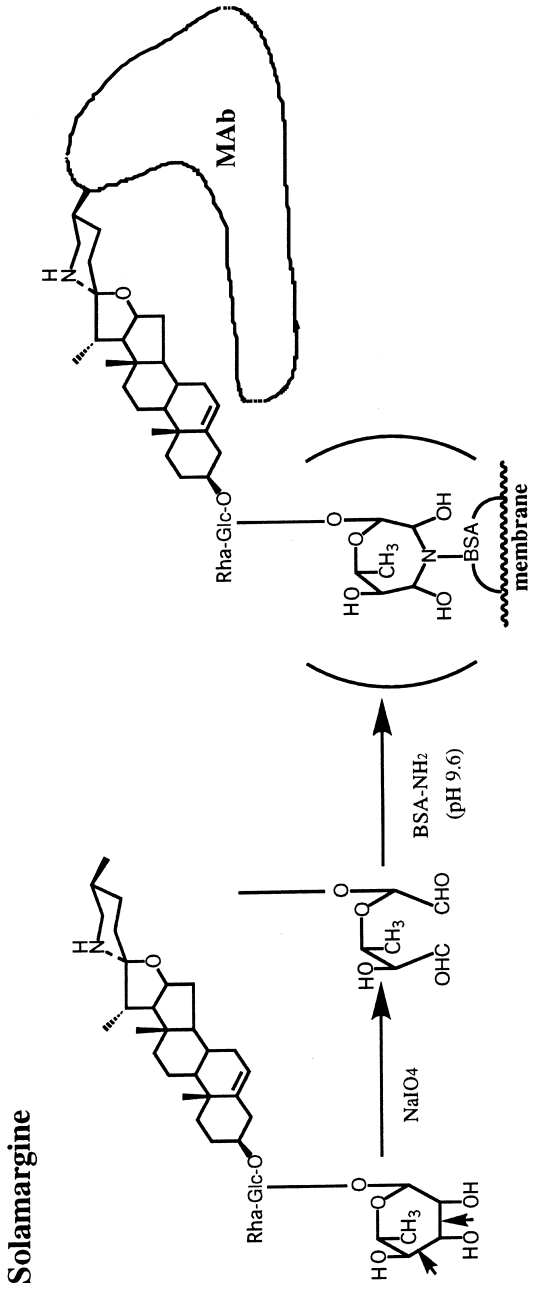

Figure 4. Mechanism of the newly established western blotting.

Fig.3 shows a western blot and the sliced fruit of *S. khasianum*. It became evident that seeds and pericarp contained a higher concentration of solasodine glycosides compared to other tissues. To confirm this result, we analyzed these tissues individually by ELISA. Seeds contained 18 ± 2 μg/mg of solasodine glycosides, inner and outer pericarp had 0.43 and 0.12 μg/mg, respectively, resulting in good agreement with that of the western blot.

This is the first report on the use of western blots for solasodine glycosides and its application. Fig. 4 summarizes the newly established western blotting. This method divides two functions of solasodine glycosides, namely sugar moiety and aglycone. 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. On the other hand, the MAb binds to the steroidal part of the molecule. This assay method can be routinely used to survey natural resources of solasodine glycosides as a simple and rapid analysis. Moreover, this methodology may be available for the assay *in vitro* of plants of *S. khasianum*, therefore making it possible to study a large number of cultured plants, and a limited small amount of sample *in vitro* for the breeding of *Solanum* species containing a higher amount of steroidal alkaloids in the continuation of our medicinal plant breedings. Furthermore, this system may be available for the analysis of animal plasma samples of glycoside or glucuronide not limited to solasodine glycosides and/or distributions in organs or tissues since very low concentrations are expected.

Although it is difficult to detect a small molecular compound by the western blotting method, the approach described here will be particularly attractive in a wide variety of comparable situations as indicated in the distribution of solasodine glycosides in the fruit of *S. khasianum* (Fig. 3). In continuing studies of this result, naturally occurring pharmacologically active glycosides for the central nervous system, such as ginsenosides Rb_1[23] and crocin[24] have been investigated. These results will be reported elsewhere.

ABBREVIATIONS

TLC, thin-layer chrom.; PVDF, polyvinylidene difluoride; FABMS, fast atom bombardment mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; BSA, bovine serum albumin; HSA, human serum albumin; TPBS, phosphate buffered solution containing 0.05% of Tween 20; GPBS, phosphate buffered solution containing 0.2% of gelatin; ELISA, enzyme linked immunosorbent assay; MAb, monoclonal antibody; ABTS, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammon. salt.

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