

A simple determination of steroidal alkaloid glycosides by thin-layer chromatography immunostaining using monoclonal antibody against solamargine

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Abstract A method of determination for solasodine glycosides by using thin-layer chromatography (TLC) immunostaining was investigated. Solasodine glycosides separated by silica gel TLC were transferred to a polyvinylidene difluoride membrane. The membrane was treated with sodium periodate solution followed by bovine serum albumin (BSA), resulting in a solasodine glycoside-BSA conjugate. Conjugation was confirmed by matrix-assisted laser desorption/ionization mass spectrometry. Individual spots were stained by monoclonal antibody against solamargine. Immunostaining of solasodine glycosides was more sensitive compared to other stainings. The newly established immunostaining method can be extended to analysis of the distribution of solasodine glycoside in the plant body.

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Key words: Solasodine glycoside; Solamargine-BSA conjugate; Monoclonal antibody; FAB-MS; MALDI-MS; TLC immunostaining

1. Introduction

In the recent rapid development of the molecular biosciences and their biotechnological applications, immunoassay systems using MAbs against drugs and biologically active compounds of low molecular weight have become important tools [1] for studies on receptor binding analysis, enzyme assays and quantitative and/or qualitative analytical techniques in animals or plants, owing to their specific affinity. In previous papers, we reported methods for the direct determination for antigen conjugates by MALDI mass spectrometry [2–4], and the production of MAbs and enzyme immunoassay for solamargine [5], forskolin [6,7], opium alkaloids [8] and marihuana compounds (cannabinoids) [9].

In continuation of studies on the breeding of medicinal plants, we have already reported the higher yielding strain of cannabidiolic acid which is designated a non-toxic marihuana strain [10], and clonal and homogeneous medicinal strains [11–13], and virus-free plants [14] using plant biotechnology.

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Abbreviations: TLC, thin-layer chromatography; PVDF, polyvinylidene difluoride; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; BSA, bovine serum albumin; HSA, human serum albumin; TPBS, phosphate buffer solution containing 0.05% Tween 20; GPBS, phosphate buffer solution containing 0.2% gelatin; ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.

The natural resources of adrenocortical hormones and sex hormones, which are mainly obtained from 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 the C-3 position. By far the most common forms are the triglycosides, solamargine being predominant [15]. Therefore, the steroidal alkaloid glycosides of the solasodine type like solamargine have become important as a 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 a limited small amount of samples, in order to select the strain of higher yielding steroidal alkaloid glycosides.

We here wish to present a simple determination method for solasodine glycosides by using TLC immunostaining.

2. Materials and methods

2.1. 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 Corp. (Bedford, MA, USA). Glass microfiber filter sheets (GF/A) were purchased from Whatman International Ltd. (Maidstone, UK). All other chemicals were standard commercial products of analytical grade.

Fruit of *S. khasianum* was obtained from the herbal garden of the Faculty of Pharmaceutical Sciences, Kyushu University, Japan. Solamargine and solasonine were isolated from fresh fruits of *S. khasianum* as previously described [15]. Solasodine was obtained from solamargine by acid hydrolysis as described in [15]. 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, respectively. Individual hydrolysates were evaporated in vacuo and applied to TLC. Spots developed on TLC were determined using H₂SO₄ and Dragendorff reagent.

An immunogen, solamargine-BSA conjugate, was synthesized from solamargine by NaIO₄ treatment followed by conjugation with BSA as reported previously [16] with modifications. Immunization and hybridization were carried out as reported [5] from this laboratory. A cultured medium (50 ml) containing the IgG (SMG-BD9) [5] was purified by using a Chromatop Protein A column [17] (0.46×25 cm, NGK Insulators, Ltd., Nagoya, Japan), dialyzed 5 times against H₂O, and finally lyophilized to give IgG (0.53 mg).

2.2. TLC

Solasodine glycosides were applied to a TLC plate and developed with chloroform/methanol/ammonia solution (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 vol.). It was placed on a stainless-steel plate, then covered with a PVDF membrane sheet. After covering with a glass microfiber filter sheet, the whole was pressed evenly for 45 s with a 130°C iron as previously described

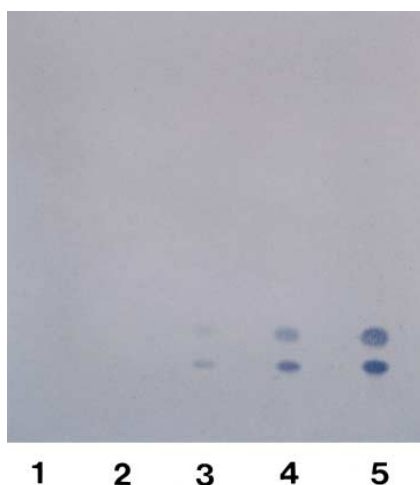


Fig. 1. TLC immunostainings of solamargine and solasonine. Solamargine (upper spot) and solasonine (bottom spot) were developed by CHCl_3 -MeOH- NH_4OH solvent system on silica gel TLC plate. Lanes 1–5 show the concentrations of both alkaloids, 0.8, 1.6, 8, 40 and 200 ng, respectively. After transfer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

[18] with modifications. The PVDF membrane was separated from the plate and dried.

2.3. Immunostaining of solasodine glycosides on PVDF membrane

The blotted PVDF membrane 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) con-

taining BSA (1%) was added, and stirred at room temperature for 3 h. The PVDF membrane was washed twice with TPBS for 5 min, and then washed with water. The PVDF membrane was immersed in anti-solamargine MAb, 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 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 immunostained PVDF membrane was allowed to dry.

2.4. Extraction of solasodine glycoside-BSA conjugate from PVDF membrane

The band of solasodine glycoside-BSA conjugate on PVDF was extracted with 0.1 M acetate buffer (pH 4) under stirring at 4°C overnight. The solution was dialyzed against H_2O .

2.5. Analysis by MALDI mass spectrometry

A small amount (1–10 pM) of the solasodine glycoside-BSA conjugate extracted from the PVDF membrane was mixed with a 10^3 -fold molar excess of sinapinic acid in an aqueous solution containing 10% trifluoroacetic acid. The mixture was placed inside a JMS-LDI 1700 TOF mass monitor and irradiated with a N_2 laser (337 nm, 3 ns pulse). The ions formed by each pulse were accelerated by a 30 kV potential into a 1.7 m evacuated tube and detected using a Micro-channel Plate Detector with an IBM PC compatible computer as reported in [2–4].

3. Results and discussion

After the solasodine glycosides had been transferred to the PVDF membrane sheet from the TLC plate by heating (see

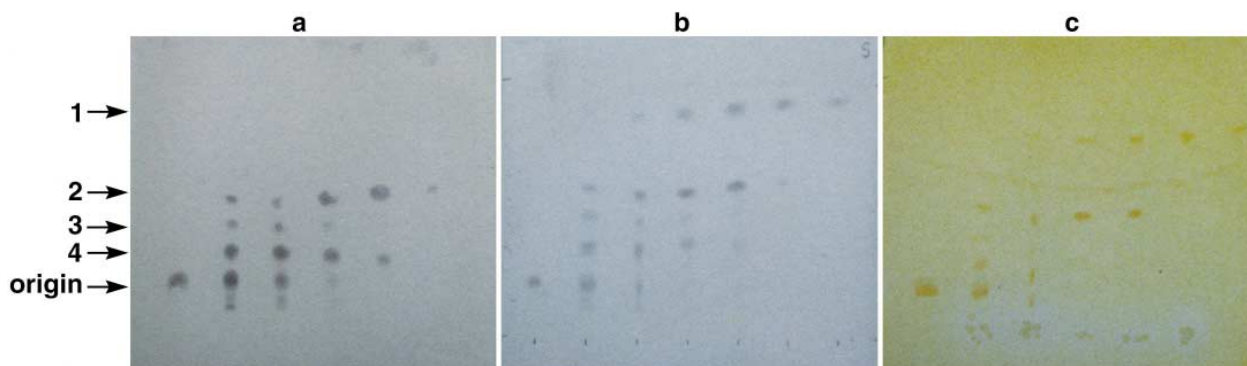


Fig. 3. Products of HCl hydrolysis of solamargine. a–c depict TLC immunostaining, staining with sulfuric acid and staining with Dragendorff reagent, respectively. Solamargine was hydrolyzed using 1 M HCl for 10, 20, 30, 60 and 90 min, respectively. Spots 1–4 were identified with solasodine, 3- O - β -D-glucopyranosyl solasodine, L-rhamnosyl-(1 \rightarrow 4)- O -3- β -D-glucopyranosyl solasodine and L-rhamnosyl-(1 \rightarrow 2)-3- β - O -D-glucopyranosyl solasodine, respectively.

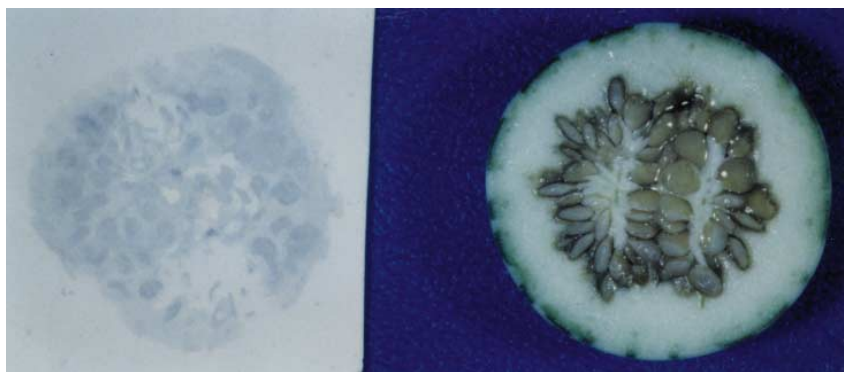


Fig. 4. The sliced fruit of *Solanum khasianum* and its immunostaining. The PVDF membrane covered the surface of the sliced fruit, and was stained in the same manner via TLC immunostaining as indicated in Fig. 1.

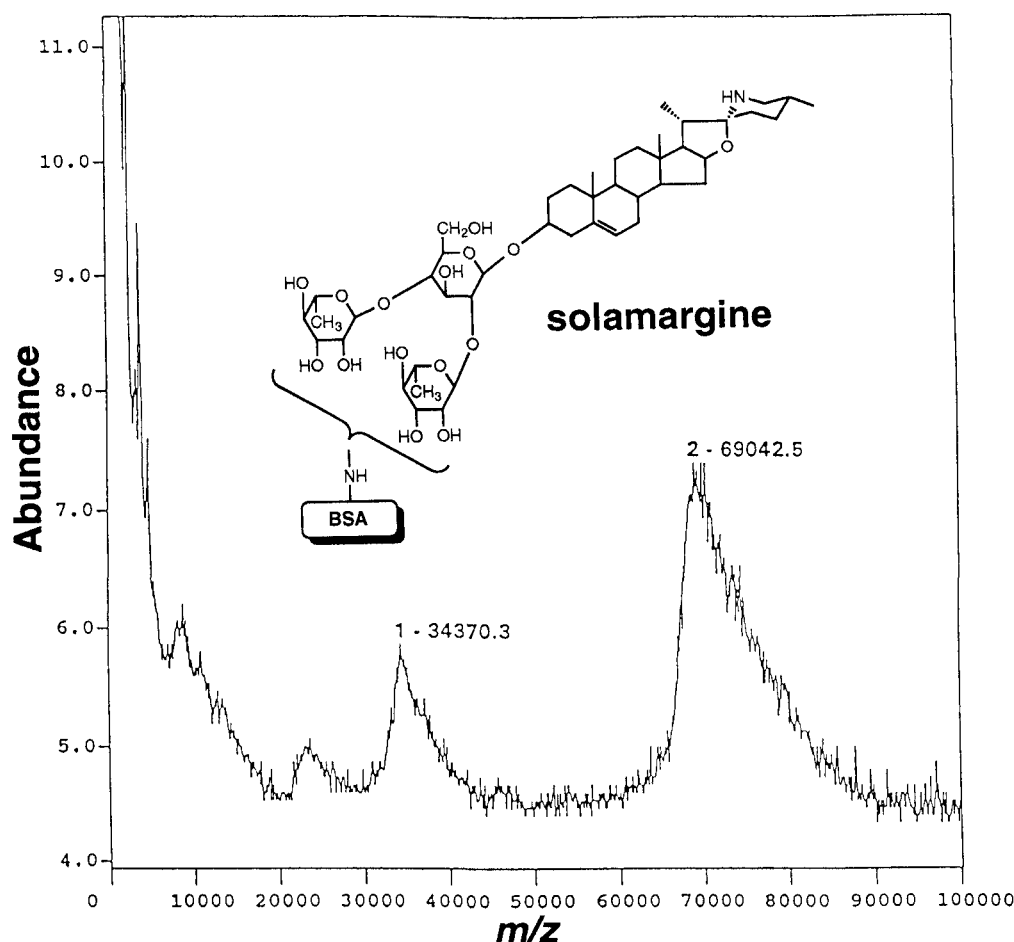


Fig. 2. MALDI mass spectrometry of solamargine-BSA conjugate.

Section 2) as previously reported [18] with modifications, the PVDF membrane was treated with NaIO_4 solution followed by conjugation with BSA since solasodine glycosides on PVDF membranes are washed out by buffer solution or water without the formation of a conjugate with the carrier protein. The PVDF membrane was immersed in anti-solamargine MAb, and then peroxidase-labeled secondary MAb. on addition of substrate and H_2O_2 , clear blue spots appeared as shown in Fig. 1. Fig. 1 shows the immunostaining of solamargine and solasonine. Lanes 1–5 demonstrate the concentrations of both alkaloids, 0.8, 1.6, 8, 40 and 200 ng, respectively. Different sensitivities between solamargine and solasonine were observed in individual concentrations, and the sensitivity of solasonine was somewhat higher than that of solamargine. The detection limit was 1.6 ng solasonine.

In order to confirm the preparation of solamargine-BSA conjugate on the PVDF membrane, the band corresponding to the solamargine-BSA conjugate was assessed by MALDI mass spectrometry (Fig. 2). A broad peak 2 $[\text{M}+\text{H}]^+$ of solamargine-BSA conjugate appeared at around m/z 69043 in MALDI mass spectrometry demonstrating that at least 1–2 molecules and up to 15 molecules of solamargine had combined. Therefore, it becomes clear that the sugar moiety which was conjugated with BSA is necessary in this staining system.

To expand this newly established immunostaining method, solamargine was hydrolyzed by 1 M HCl for 10, 20, 30 and 60 min, respectively. Individual hydrolysates were applied to

three TLC plates and then developed with a $\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$ solvent system. Two plates were sprayed and colored with H_2SO_4 and Dragendorff reagents, respectively. One plate was transferred to a PVDF membrane. Fig. 3 shows the immunostaining (a), and stainings by H_2SO_4 (b) and Dragendorff reagent (c). On comparing the staining sensitivities of the three methods, the immunostaining was found to be the most extensive followed by H_2SO_4 and then Dragendorff reagent. It is easily suggested that product 1 may be an aglycone of solamargine, solasodine and that products 2–4 might be solasodine mono- and diglycosides. Therefore, products 1–4 were identified as solasodine, 3-*O*- β -D-glucopyranosyl-solasodine, *O*- α -L-rhamnosyl-(1 \rightarrow 4)-3-*O*- β -D-glucopyranosyl-solasodine and *O*- α -L-rhamnosyl-(1 \rightarrow 2)-3-*O*- β -D-glucopyranosyl-solaso-

Table 1
Cross-reactivities of monoclonal antibody (SMG-BD9) against steroidal compounds

| Compound | Cross-reactivities (%) |
|---|------------------------|
| Solamargine | 100 |
| Solasonine | 92.1 |
| 3- <i>O</i> - β -D-Glucopyranosyl-solasodine | 11 |
| <i>O</i> - α -L-Rhamnosyl-(1 \rightarrow 4)-3- <i>O</i> - β -D-glucopyranosyl-solasodine | 17 |
| <i>O</i> - α -L-Rhamnosyl-(1 \rightarrow 2)-3- <i>O</i> - β -D-glucopyranosyl-solasodine | 36 |
| Tomatine | 2.1 |
| Tomatigine | 0.3 |

dine, respectively, by direct comparison with authentic samples. As compared with the staining between immunostaining (a) and sulfuric acid (b), solasodine was not detected by immunostaining despite having 44% cross-reactivity [5] resulting in good agreement with previous evidence. Table 1 lists the cross-reactions of individual hydrolyzed products. The cross-reactivities of three hydrolyzed products were 11, 17 and 36%, 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 antibodies (data not shown).

Fig. 4 depicts immunostaining and the sliced fruit of *S. khasianum*. It became evident that seeds and pericarp contained a higher concentration of solasodine glycosides as compared to other tissues. To confirm this result, we analyzed these tissues individually by ELISA. The seeds contained 18 ± 2 µg/ml of solasodine glycosides, the inner and outer pericarp having 0.43 and 0.12 µg/ml, respectively, thus showing good agreement with immunostaining.

This is the first report in which immunostaining for solasodine glycosides and its application have been described. This assay method can be routinely used for surveying natural resources of solasodine glycosides as a simple and rapid analysis. Moreover, this methodology may be available for the assay of in vitro plantlets of *S. khasianum*, therefore making it possible to study a large number of cultured plantlets, and a limited small amount of sample in vitro for the breeding of *Solanum* species containing higher amounts of steroidal alkaloids for the continuation of our medicinal plant breeding. Furthermore, this system may be available for the analysis of animal plasma samples of glycoside or glucuronide, and not be limited to solasodine glycosides and/or distributions in organs or tissues since very low concentrations are expected. Although it is difficult to detect a low molecular weight compound by western blotting, 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. 4). In extending studies of this result, naturally occurring pharmacologically active glycosides for the central nervous system like ginsenosides Rb₁ [19] and crocin [20] have been investigated. These results will be communicated elsewhere.

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