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Development of Eastern Blotting Technique for Sennoside A and Sennoside B using Anti-Sennoside A and Anti-Sennoside B Monoclonal Antibodies

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ABSTRACT:

Introduction – Rhubarb, senna and sennoside-containing preparations are currently widely employed as purgatives. The major active components of these medications are sennoside A (SA) and sennoside B (SB).

Objective – To develop an eastern blotting technique for the specific visualisation and easy determination of SA and SB in plant extracts for application in the standardisation and authentication of rhubarb and senna.

Methodology – SA and SB were separated by TLC, transferred to a PVDF membrane, treated with 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride solution and finally treated with bovine serum albumin (BSA). The resulting membrane-bound SA–BSA and SB–BSA conjugates were linked to anti-SA and anti-SB monoclonal antibodies (MAbs) and then to secondary antibodies labelled with peroxidase. SA and SB were detected by visualisation of the peroxidase reaction products.

Results – The limit of detection of the eastern blotting was 62.5 ng for both sennosides. The method was applied to the immunohistochemical localisation of SA in fresh rhubarb root. Phloem and radiate wood were found to contain higher concentrations of SA compared with other tissues (pith and bud) in agreement with results obtained by ELISA. The concentrations of SA in the phloem, radiate wood, pith and bud were 64.4, 48.1, 15.0 and 1.8 ng/mg fresh weight, respectively.

Conclusion – The technique described permitted the visualisation of small molecular weight compounds that had been bound to a membrane, using immunostaining. Owing to the specificity of the MAbs, the eastern blotting may prove to be a useful method for the identification of SA and SB in a background containing large amount of impurities.

Keywords: Eastern blotting; immunohistochemistry; sennoside A; sennoside B

Introduction

Sennoside A (SA) and sennoside B (SB) are the major purgative constituents of rhubarb [the rhizome and root of *Rheum* spp. (Polygonaceae)], and of senna [the leaf and pod of *Cassia* spp. (Leguminosae)] (Oshio *et al*., 1978; Yamagishi *et al*., 1987). Sennosides are metabolised by intestinal bacteria into rheinanthrone, which acts directly on the intestines as a purgative (Yang *et al*., 1996), functioning in the same way as natural prodrugs. Despite the numerous synthetic purgatives available, sennoside-containing medications are widely used worldwide and are among the most important pharmaceutical products of plant origin.

Members of the Leguminosae family, especially *Cassia* spp., are primarily used as natural laxatives (Elujoba *et al*., 1989) and also as natural dietary supplements for enhancement of blood flow and metabolism in the USA, Europe and Australia. Therefore, phytochemical studies of such plants require simple assays for the analysis of SA and SB, such as immunoassay systems that use monoclonal antibodies (MAbs) against bioactive compounds of low molecular weight. Indeed, these methods have become important tools for the qualitative and quantitative analyses of active constituents from natural medicines and plants (Weiler *et al*., 1980). The production and characterisation of anti-SA and anti-SB MAbs have already been described (Morinaga *et al*., 2000, 2001). Furthermore, assays employing these antibodies (ELISA and immunochromatography) have been applied in the investigation of members of the Leguminosae family (Putalun *et al*., 2004). The immunoblotting method, which is based on western blotting techniques that utilise antigen–antibody binding properties (Towbin *et al*., 1979), provides a specific and sensitive detection of high molecular weight analytes such as peptides and proteins. As an extension of this approach, the present paper describes the development of an

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eastern blotting technique for the identification of SA and SB, and immunohistochemical localisation of SA in fresh rhubarb roots.

Experimental

Chemicals and immunochemicals. SA and SB were purchased from Wako Pure Chemical (Osaka, Japan). 1-Ethyl-3-(3′-dimethylaminopropyl)-carbodiimide HCl (EDC) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Bovine serum albumin (BSA) was provided by Pierce (Rockford, IL, USA). Peroxidase-labelled anti-mouse IgG was provided by Organon Teknika Cappel Products (West Chester, PA, USA). Polyvinylidene difluoride (PVDF) membranes (Immobilon-N) were purchased from Millipore Corporation (Bedford, MA, USA). Glass microfibre filter sheets (GF/A) were purchased from Whatman International (Maidstone, England). All other chemicals were standard commercial products of analytical reagent grade.

Plant materials and extraction. Leaves of various *Cassia* species (*Cassia angustifolia*, *C. alata*, *C. bakeriana*, *C. fistula*, *C. mimosoides*, *C. floribunda*, *C. surattensis*, *C. tora* and *C. siamea*) were collected by our group in Thailand. Root samples of a *Rheum* plant (Hokkai Daio) were kindly donated by the Graduate School of Pharmaceutical Sciences, Hokkaido University (Sapporo, Japan). Dried leaf samples of *Cassia* species (60 mg) were powdered and extracted five times with methanol containing 0.1% (w/v) ammonium hydroxide (0.8 mL) under constant sonication and the extract was filtered using a Cosmonice Filter W (0.45 μm Filter Unit, Nacalai Tesque, Kyoto, Japan). The combined extracts were evaporated, and the respective residues were diluted with 10 mM sodium bicarbonate (0.5 mL) and used for eastern blotting (3 μL each) and ELISA (1 μL each).

Preparation of MAbs against SA and SB. Immunisation and hybridisation of MAbs against SA and SB have been described previously (Morinaga *et al*., 2000, 2001). Antibodies were purified using a Protein G FF column (Amersham Biosciences, Piscataway, NY, USA). The culture medium (125 mL) containing the IgG was adjusted to pH 7.0 with 1 M Tris–HCl buffer (pH 9.0) and applied to the column, which was then washed with phosphate-buffered saline (0.15 M sodium chloride in 10 mM potassium phosphate, pH 7.4; PBS). Adsorbed IgG was eluted with 100 mM citrate buffer (pH 2.7), neutralised with 1 M Tris–HCl buffer (pH 9.0), dialysed four times against water and finally lyophilised.

Thin-layer chromatography and sulfuric acid staining. SA and SB standards (3 μg each) and plant extracts were applied to a thin-layer chromatography (TLC) plate (Silica gel 60; Merck, Darmstadt, Germany) and eluted with 1-propanol:ethyl acetate:water:acetic acid (40:40:30:1, by volume). After drying, the developed TLC plate was sprayed with a 10% (v/v) sulfuric acid solution and then heated.

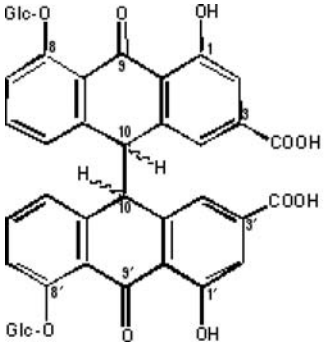
Eastern blotting and double staining. SA and SB were applied to a TLC plate and eluted with 1-propanol:ethyl acetate:water:acetic acid (40:40:30:1, by volume). The developed TLC plate was dried and then sprayed with a blotting solution mixture of isopropanol:methanol:water (1:4:16, by volume). The plate was placed onto a stainless steel base and covered with a PVDF membrane sheet followed by a glass microfibre filter sheet. The whole assembly was pressed evenly for 70 s with a 120°C hot plate. The PVDF membrane was separated from the TLC plate and dried. The blotted PVDF membrane was dipped into a 20 mM carbonate buffer solution (pH 9.6) containing BSA (1%, w/v) and EDC (20 mg/mL), and maintained at room temperature for 6 h under constant agitation. Afterwards, the PVDF membrane was washed twice for 5 min with PBS containing 0.05% (v/v) Tween 20 (T-PBS) and then treated with PBS containing 5% (w/v) skimmed milk for 2 h to reduce non-specific adsorption. The PVDF membrane was washed with T-PBS twice for 5 min, immersed into a reconstituted anti-SA MAb solution, and maintained at room temperature for 2 h under constant agitation. After washing the PVDF membrane twice with T-PBS and water, a 1:1000 dilution of peroxidase-labelled goat anti-mouse IgG in PBS containing 0.2% (w/v) of gelatine was added, maintaining the system at room temperature for 1 h under agitation. Finally, the PVDF membrane was washed twice with T-PBS and water, and then treated with freshly prepared 1 mg/mL 4-chloro-1-naphthol-0.03% (v/v) water in PBS for 10 min at room temperature.

The consecutive staining of the anti-SA MAb-stained membrane with anti-SB MAb was performed as described for anti-SA MAb, except that the membrane was treated with 2 mg/10 mL 3-amino-9-ethylcarbazole-0.03% (v/v) water in acetate buffer (0.05 M, pH 5.0) containing 0.5 mL of *N*,*N*-dimethyl formamide.

Results and Discussion

Some years ago our group established a new immunostaining method for the analysis of glycosides, such as solasodine glycosides, ginsenosides and glycyrrhizin, in which specific MAbs were employed (Tanaka *et al*., 1997; Shan *et al*., 1999; Fukuda *et al*., 2000, 2001). This technique, which was later named eastern blotting (Shan *et al*., 2001; Tanaka *et al*., 2006, 2007; Zhu *et al*., 2007), was used for the separation of the glycoside molecules into two portions, the aglycone (epitope) and the sugar moiety (fixation ability). After separation by TLC, the small molecules were blotted onto PVDF membranes. Although the preparation of the SA–BSA conjugate from the SA sugar moiety was performed according to the original methodology, the staining procedure was unsuccessful. Thus, another fixation method was developed, which resulted in the successful immunostaining of SA onto the PVDF membrane.

SA was transferred to the PVDF membrane according to the method described in the present paper. The treatment of the membrane with EDC (and BSA) solution was necessary because the SA molecule contains a carboxylic acid group (Fig. 1) and the reaction with EDC enhanced the fixation of the SA portion of the SA–BSA conjugate onto the PVDF membrane. In the absence of EDC or BSA, the blotted PVDF membrane could not be immunostained (data not shown). Figure 2 shows the eastern blotting of SA, SB and other structurally related compounds using anti-SA MAb [Fig. 2(a)] and the sulfuric acid staining [Fig. 2(b)]. The eastern blotting indicated only limited staining of SA as shown in Fig. 2(a), lane 7. Since the anti-SA MAb cross-reacts against SB and rhein (0.28 and 0.35%, respectively; Table 1), these molecules can be stained very weakly with anti-SA MAb. Previously, our group succeeded in performing the eastern blotting of ginsenoside Rb1 (G-Rb1) using anti-G-Rb1 MAb, which resulted in the staining of G-Rc, -Rd, -Re and -Rg1 (Fukuda *et al*., 2001). The difference between the eastern blotting technique presented in this paper and the previous procedure is the sugar moiety. In the ginsenosides the sugar moiety was oxidatively cleaved to release



Compound	10-10'	formula	M.W.
Sennoside A	*threo*	$C_{42}H_{38}O_{20}$	862
Sennoside B	*erythro*	$C_{42}H_{38}O_{20}$	862

Figure 1. Chemical structures of sennoside A and sennoside B.

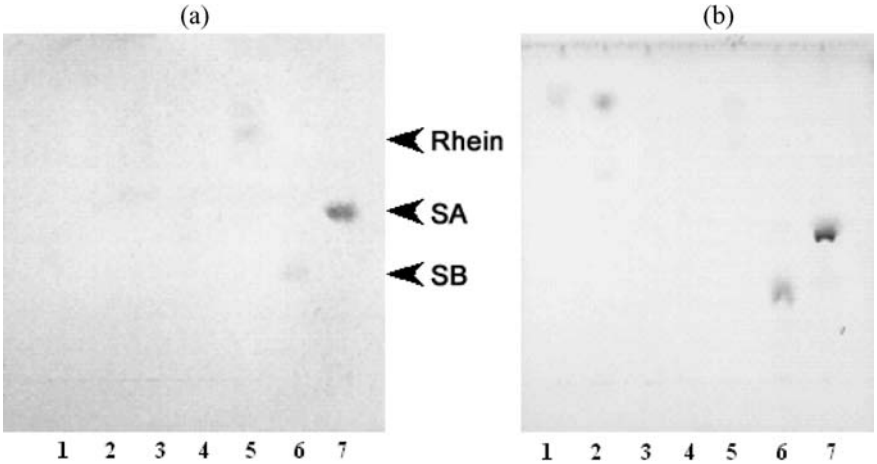


Figure 2. Eastern blotting of sennoside A, sennoside B and related compounds stained by anti-sennoside A monoclonal antibody (a) and TLC plate stained by 10% H_2SO_4 (b). Lanes 1, 2, 3, 4, 5, 6 and 7 indicate rhaponticin, barbaloin, aloe-emodin, emodin, rhein, sennoside B and sennoside A (3 μg each), respectively.

the aldehyde groups, which were conjugated with protein, in order to permit the fixation onto the PVDF membrane. Since part of the G-Rb1 sugar moiety was immunised, the cleavage of the sugar moiety by sodium periodate expanded its cross-reactivity against other ginsenosides resulting in the staining of G-Rc, -Rd, -Re and -Rg1, despite their weak cross-reactivities (0.024, 0.020, 0.005 and 0.005%, respectively). In contrast, the newly developed eastern blotting did not obstruct the SA sugar moiety and, therefore, the intensity of the staining of SA, SB and rhein was proportional to their cross-reactivities, as indicated in Table 1.

Since 4-chloro-1-naphthol and 3-amino-9-ethylcarbazole are peroxidase substrates, both substances are suitable for the binding of the secondary antibody (peroxidase-labelled anti-mouse IgG) used in the detection of the anti-SA and anti-SB MAbs. Since the sensitivity of the 3-amino-9-ethylcarbazole substrate is higher than that of 4-chloro-1-naphthol, it is justifiable to use first 4-chloro-1-naphthol (for detection of SA) and then 3-amino-9-ethylcarbazole (for detection of SB), which could be clearly identified by the purple and red colour, respectively, as indicated in Fig. 3. Such result demonstrated that both MAbs can distinguish the stereochemical configurations, *threo* and *erythro* between C-10 and C-10′ positions, of the sennoside molecule bound to a double-stained PVDF membrane. Furthermore, the results presented confirm that the epitope consists of a rhein basal structure and a sugar moiety as reported previously (Morinaga *et al*., 2000, 2001). Under the conditions described, the detection limit for both sennosides was 62.5 ng (data not shown).

Table 1. Cross-reactivities (%) of anti-sennoside A and anti-sennoside B monoclonal antibodies against various compounds

Compound	Anti-SA Mab	Anti-SB MAb
Anthraquinone		
Sennoside A	100	2.45
Sennoside B	0.28	100
Rhein	0.35	0.012
Emodin	<0.04	<0.004
Aloe-emodin	<0.04	<0.004
Barbaloin	<0.04	<0.004
1,4-dihydroxy-anthraquinone	<0.04	<0.004
Stilbene		
Rhaponticin	<0.04	<0.004
Phenolic acid		
Gallic acid	<0.04	<0.006
Vanillic acid	<0.04	<0.006
Caffeic acid	<0.04	<0.006
Homogentisic acid	<0.04	<0.006

Leaf samples from *Cassia* species were analysed using the double staining system and the results are shown in Fig. 4. It is clear that *C. angustifolia*, *C. alata*, *C. bakeriana* and *C. fistula* contained high levels of SA and SB [Fig. 4(a), lanes 1–6]. The results were confirmed using a quantitative competitive ELISA method employing

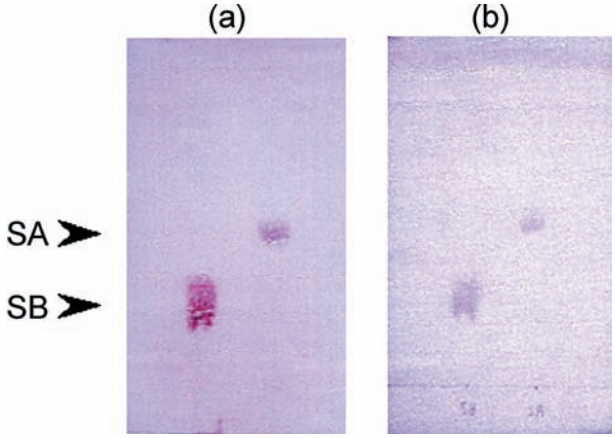


Figure 3. Double staining of sennoside A and sennoside B using eastern blotting technique (a) and TLC plate stained by 10% H_2SO_4 (b). Purple and red colours were stained by anti-sennoside A monoclonal antibody and anti-sennoside B monoclonal antibody, respectively.

anti-SA and anti-SB MAbs (Table 2). Although sulfuric acid staining [Fig. 4(b)] detected many spots in the various *Cassia* species, including probably sugars and different types of anthraquinone glycosides, the double staining technique [Fig. 4(a)] unmistakably detected SA and SB [Fig. 4(a); bands A and B], whilst the detection of others sennosides was weaker. Chlorophylls appear at the top of the membrane. Band A presents a purple colour corresponding to a *threo*-configuration between C-10 and C-10′ position typical of SA. The higher R_f value, compared with the SA standard, suggests that a COOH group in the SA molecule was replaced by a non-polar group such as -CH_2OH. Based on evidence reported in the literature, we suggest that band A represents sennoside C, which has a *threo*-configuration (Oshio *et al*., 1978). Band B presented a red colour and was identified as a compound with an *erythro*-configuration. The lower R_f value, compared with the SA standard, suggests that band B was conjugated with a polar group (Ex: oxalic acid) of a molecule. Following treatment of the crude extracts in lane 2 (Fig. 4) with a mild alkaline solution, band B disappeared, although band A still remained (data not shown). Based on these results we suggest that band B represents sennoside F (Oshio *et al*., 1978), which has an *erythro*-configuration and contains an oxalic acid group. The advantages of the eastern blotting technique over the sulfuric acid staining are the specificity, clear visualisation and easy identification of SA and SB. Such attributes are useful when SA or SB are analysed in the presence of compounds with similar R_f values of [e.g. samples of lanes 7–10/Fig. 4(b)].

Table 2. Sennoside A and sennoside B contents in leaves of various *Cassia* species

	Content (μg/mg dry wt powder)	
Sample	Sennoside A	Sennoside B
(1) *Cassia angustifolia*	4.56 ± 0.25	5.10 ± 0.15
(2) *C. alata*	1.19 ± 0.12	1.16 ± 0.15
(3) *C. bakeriana*	0.40 ± 0.03	0.44 ± 0.02
(4) *C. fistula* (A)	1.14 ± 0.08	0.75 ± 0.08
(5) *C. fistula* (B)	2.04 ± 0.32	1.52 ± 0.12
(6) *C. fistula* (C)	1.90 ± 0.16	2.05 ± 0.24
(7) *C. fistula* (D)	0.10 ± 0.01	0.13 ± 0.00
(8) *C. mimosoides*	$(1.30 \pm 0.24) \times 10^{-2}$	$(1.88 \pm 0.29) \times 10^{-4}$
(9) *C. floribunda*	$(2.78 \pm 0.11) \times 10^{-3}$	$(1.04 \pm 0.03) \times 10^{-4}$
(10) *C. surattensis*	$(1.15 \pm 0.18) \times 10^{-2}$	$(2.44 \pm 0.17) \times 10^{-4}$
(11) *C. tora*	$(2.13 \pm 0.21) \times 10^{-3}$	$(3.64 \pm 0.21) \times 10^{-5}$
(12) *C. siamea*	$(4.45 \pm 0.14) \times 10^{-3}$	$(1.87 \pm 0.13) \times 10^{-3}$

Data are the means of triplicate assays.

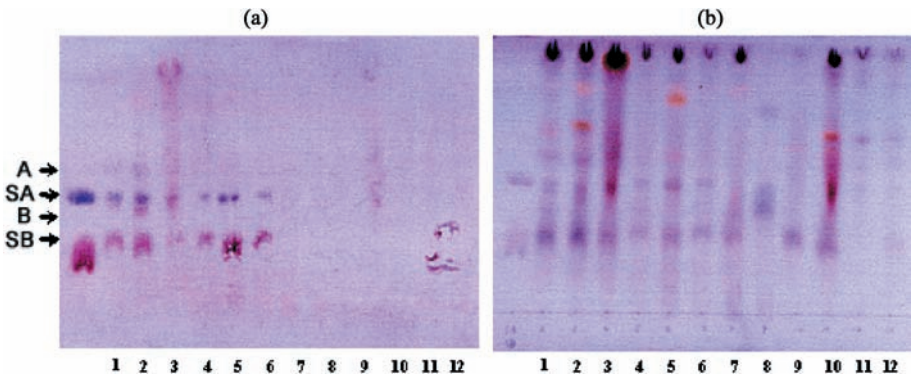


Figure 4. Double staining of sennoside A and sennoside B in various *Cassia* species (a) and TLC plate stained by 10% sulfuric acid (b). Left-hand lane indicates sennoside A (4 μg) and sennoside B (3 μg). Lanes 1–12 indicate various *Cassia* species, *Cassia angustifolia* (lane 1), *C. alata* (lane 2), *C. bakeriana* (lane 3), *C. fistula* (A) (lane 4), *C. fistula* (B) (lane 5), *C. fistula* (C) (lane 6), *C. fistula* (D) (lane 7), *C. mimosoides* (lane 8), *C. floribunda* (lane 9), *C. surattensis* (lane 10), *C. tora* (lane 11) and *C. siamea* (lane 12) (3 μL each), respectively.

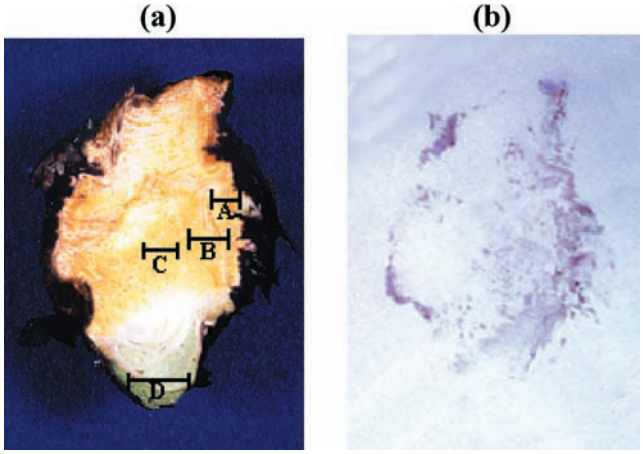


Figure 5. Immunohistochemical localisation of sennoside A using anti-sennoside A monoclonal antibody in rhubarb root. (a) Cross section of rhubarb root; (b) direct eastern blotting on PVDF membrane of a cross section of rhubarb root. (A) Phloem; (B) radiate wood; (C) pith; (D) bud, respectively.

Another application of the eastern blotting technique is the immunohistochemical localisation of SA in rhubarb root (Fig. 5). A fresh rhubarb root was sliced, placed onto the PVDF membrane, and pressed evenly for 1 h. The blotted PVDF membrane was stained using a procedure similar to that described for the extracts. The phloem and radiate wood contained a higher concentration of SA compared with other tissues (pith and bud). In order to confirm this result, these tissues were analysed individually using ELISA. The concentrations of SA, as determined by ELISA, were 64.4 ± 4.5, 48.1 ± 8.2, 15.0 ± 1.6 and 1.8 ± 0.3 ng/mg fresh weight for phloem, radiate wood, pith and bud, respectively. These values agreed substantially with those obtained by HPLC, i.e. the concentrations were 58.4 ± 2.6, 49.0 ± 3.9 and 13.3 ± 0.5 ng/mg fresh weight for phloem, radiate wood and pith respectively.

In conclusion, the eastern blotting method permitted the visualisation of low molecular weight compounds on a membrane using immunostaining. Owing to the specificity of the MAbs, the eastern blotting technique may prove to be a useful for the identification of SA and SB in a background containing a large amount of impurities. Moreover, this technique can be used to investigate the distribution of SA in fresh rhubarb root using immunohistochemical localisation.

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