Chapter 1

Quality Control of *Rheum* and *Cassia* Species by Immunological Methods Using Monoclonal Antibodies Against Sennosides

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Additional information is available at the end of the chapter

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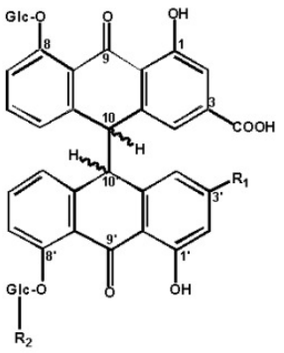
1. Introduction

Recently, medical usage of Japanese traditional medicine has been expanded by reaching aging society and increasing various chronic diseases. Therefore, the demand of crude drugs prescribed for Japanese traditional medicine has been increased. However, over 90% of crude drugs are imported in our country, and those over 70% are supplied by the collection of wild species. It is well known that the natural resources bring the difficulty of quality control depending on collection season, cultivation place, a variety of species and so on. The other problem, shortage of crude drug comes up. For these general environment, micropropagation and clonal propagation systems using tissue and cell culture were investigated in this laboratry.

Sennoside A (SA) and B (SB) have the strong catharsis activity and contained in rhubarb and senna (Figure 1) [1]. The concentration of sennosides in rhubarb and senna is variously dependent on the genetic heterogeneity of species, differences in soil condition and climate influence. Sennosides are metabolized by intestinal bacteria to rheinanthrone which acts in the intestines as a direct purgatives [2, 3] and functions as similar to a natural prodrug (Figure 2). Despite the rising availability of a number of synthetic cathartics, sennoside- containing prescriptions are still among the most widely used today, and their importance is increasing.

Rhubarb, the rhizome and root of *Rheum* spp. (Polygonaceae), is an important drug in traditional Japanese herbal medicine as well as in western medicine since ancient times. It was already recorded in *Chinese Materia Medica* 2000 years ago. It is used in many traditional Japanese herbal medicines prescribed with other herbal medicines for the syndrome of stasis of blood, as an anti-inflammatory, sedative agent and as a stomachic. Furthermore, it is widely

used as cathartics in Japan. The main purgative principles of rhubarb have proved to be sennosides [1], identical with those isolated from senna leaves, and rheinosides, which were also isolated as purgatives of rhubarb, together with various kinds of phenolics, like tannins, stilbenes, naphthalenes and lindleyin. The quality of rhubarb is severely regulated by Japanese Pharmacopeia as rhubarb contains SA of over 0.25% dry weight in root [4].



Compound	R_1	R_2	10-10'
Sennoside A	COOH	H	*threo*
Sennoside B	COOH	H	*erythro*
Sennoside C	CH_2OH	H	*threo*
Sennoside D	CH_2OH	H	*erythro*
Sennoside E	COOH	HOOC-CO	*threo*
Sennoside F	COOH	HOOC-CO	*erythro*

Figure 1. Structures of sennosides

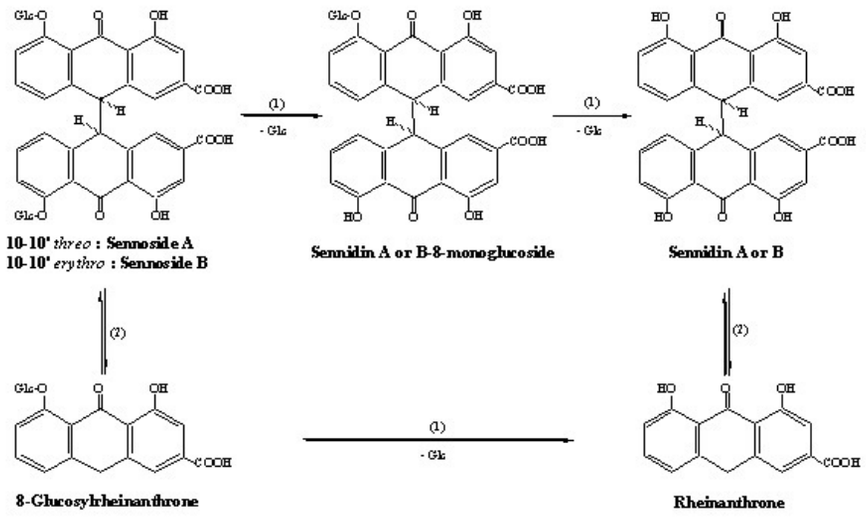


Figure 2. Metabolic pathways of sennosides by intestinal bacteria.

Senna, the leaf and pod of *Cassia* spp. (Leguminosae), is one of the most popular in herbal remedies and in health food industry. It has been widely used in cathartics for the relief of constipation prescribed with other health teas and dieter's teas in Japan, and often used as natural dietary supplements for enhancement of bloodflow and metabolism in USA, Europe and Australia. These pharmaceutical properties are due to sennosides, which are contained

in *Cassia acutifolia* Delile and *Cassia angustifolia* Vahl. *C. angustifolia* listed in Japanese Pharmacopeia, and the quality is severely regulated as senna contains total sennosides (SA and SB) of over 1.0% dry weight in leaf [4].

In the breeding research on the plant, a lot of stages are required as follows : dedifferentiation, extension of mutation by the mutagen, redifferentiation, analysis of the redifferentiated plant, mass propagation of the higher yielding plant and transplanting to soil. Therefore, it is very important to study a large number of plant samples in the phytochemical field and a small sample size *in vitro* for the breeding of *Rheum* and *Cassia* species yielding high concentration of sennosides. Many analytical approaches have been investigated for the determination of sennosides in plant extracts. Among these methods, the use of high-performance liquid chromatography (HPLC) appears most frequently and widely today. However, when the assay of very low concentration of sennosides in the regenerated plantlets is needed, the HPLC method is not appropriate and efficient.

Recently, the immunological assay method is widely developed for the purpose of analysis for a small amount of constituent. In general immunological methodologies in particular enzyme-linked immunosorbent assay (ELISA) have promoted the development of higher sensitive assay system.

On the one hand, monoclonal antibodies (MAbs) have many potential uses in addition to immunological methods in plant sciences. MAbs are superior to polyclonal antibodies (PAbs) in the antigenic specificity and stability. Therefore, immunoassay using MAbs against pharmacologically active compound having small molecular weight has become an important tool for the studies on receptor binding analysis, enzyme assay and quantitative and/or qualitative analytical techniques in plants owing to its specific affinity, and possesses an extremely high possibility in the phytochemical analysis. Up to now, immunological approach for assaying quantities of sennosides in *C. angustifolia* using PAb against SB has been investigated by Atzorn *et al* [5]. However, since no success with MAbs against SA and SB has been reported, objectives of this work are shown as following.

1. Production of MAb against SA, its characterization and use for ELISA.
2. Production of MAbs against SB, their characterization and use for ELISA.
3. Establishments of a new eastern blotting, double staining and immunohistochemical staining using anti-SA and SB MAbs.

2. Production of MAb against SA, its characterization and use for ELISA

2.1. Preface

In the immunologically analytical methodology, there are two measuring methods using the antiserum (polyclonal antibody ; PAb) and MAb in general. PAb is a heterogeneous mixture of antibody molecules arising from a variety of constantly evolving B lymphocytes. Therefore, PAb can often show high affinity because different antibody populations react with the

variety of epitopes that characterize the antigen. On the other hand, there are some problems of PAb that the extensive cross-reactivity occurs between the antibody and the multiple antigens which have the same antigenic determinant, and it is impossible to supply for identical antibody permanently. In the meantime, MAb is produced from a single B lymphocyte and can react with one antigenic determinant of the specific antigen. Besides MAb has identical specificity and affinity. There are some advantages that the complete purity of the immunized antigen is not required and the hybridoma cells can be preserved as freeze stock, and it is possible to get MAb depending on necessary respond.

There are several formats for ELISA like direct ELISA, competitive ELISA, sandwich ELISA and competitive ELISA according to the immune complexes formed during manipulation. Analysis of low molecular weight compound by immunoassay is still limited to competitive format.

Quality control of the Japanese herbal medicine is necessary because it is believed that approximately 70% of these crude drugs prescribed are collected from natural resource. Furthermore, since MAbs become necessary for the assay of concentrations of active constituents in our on-going plant biotechnological projects, we have already produced MAbs against natural compounds such as forskolin [6], solamargine [7], opium alkaloids [8], marihuana compounds [9], glycyrrhizin [10], crocin [11], ginsenoside Rb1 [12] and Rg1 [13], and developed individual competitive ELISAs. An immunological approach for assaying quantities of sennosides using a PAbs has been investigated by Atzorn *et al*.[5]. However, since no result of MAb related to sennosides has been reported yet, anti-SA MAb was produced as described [14].

2.2. Experimental

2.2.1. Chemicals and immunochemicals

SA was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). 1-Ethyl-3-(3'-dimethylaminopropyl)-carbodiimide HCl (EDC) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). 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). Enriched RPMI1640-Dulbecco's-Ham's F12 (eRDF) medium and RD-1 additives (containing 9 μg/mL insulin, 20 μg/mL transferrin, 20 μM ethanolamine, 25 μM sodium selenite) were purchased from Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo, Japan). Hypoxanthine-aminopterin-thymidine (HAT) additives were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fetal calf serum (FCS) was purchased from Cambrex Corporation (Walkersville, MA, USA). All other chemicals were standard commercial products of analytical grade. Samples of various rhubarb roots were purchased from the Tochimototenkaido Corporation (Osaka, Japan).

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2.2.2. Extraction of various rhubarb samples

Dried samples (30 mg) of various rhubarb roots were powdered, and then extracted five times with MeOH containing 0.1% (w/v) NH_4OH (0.5 mL) with sonication, filtered using a Cosmonice Filter W (0.45 μm Filter Unit, Nacalai Tesque Inc., Kyoto, Japan), and the combined extracts were diluted with 10 mM $NaHCO_3$ to prepare a solution suitable for the ELISA.

2.2.3. Synthesis of antigen conjugates

To SA (6 mg) dissolved in 1 mL of tetrahydrofuran-20 mM phosphate buffer of pH 5.5 (7:3), 0.3 mL of 20 mM phosphate buffer (pH 5.5) containing 6 mg of EDC was added. Then, 0.3 mL of 20 mM phosphate buffer (pH 5.5) containing 6 mg of BSA was added, with stirring at room temperature for 14 hr. The reaction mixture was dialyzed five times against H_2O, and then lyophilized to give 5.8 mg of SA conjugate (SA-BSA). SA-HSA conjugate was also synthesized in the same manner.

2.2.4. Determination of hapten density in SA-carrier protein conjugate by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry

The hapten number in the SA-carrier protein conjugate was determined by MALDI-TOF mass spectrometry as previously described [15]. A small amount (1-10 pmol) of antigen conjugate was mixed with a 10^3-fold molar excess of sinapinic acid in an aqueous solution containing 0.15% trifluoroacetic acid (TFA). The mixture was subjected to a JEOL Mass Spectrometers (JMS) time-of-flight (TOF) mass monitor (model Voyager Elite, PerSeptive Biosystems Inc., Framingham, MA, USA) and irradiated with a N_2 laser (337 nm, 150 ns pulse). The ions formed by each pulse were accelerated by a 20 kV potential into a 2.0 m evacuated tube and detected using a compatible computer as previously reported [15].

2.2.5. Competitive ELISA for SA

SA-HSA (five molecules of SA per molecule of HSA) (100 μL, 1 μg/mL) dissolved in 50 mM carbonate buffer (pH 9.6) was adsorbed to the wells of a 96-well immunoplate then treated with 300 μL S-PBS for 1 hr to reduce non-specific adsorption. Fifty μL of various concentrations of SA or samples dissolved in 10 mM $NaHCO_3$ solution were incubated with 50 μL of MAb solution (0.218 μg/mL) for 1 hr. The plate was washed three times with T-PBS, and then incubated with 100 μL of a 1:1000 dilution of POD-labeled anti-mouse IgG for 1 hr. After washing the plate three times with T-PBS, 100 μL of substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H_2O_2 and 0.3 mg/mL of ABTS] was added to each well and incubated for 15 min. The absorbance was measured by a micro plate reader at 405 nm and 490 nm.

The cross-reactivities (CR) of sennosides and related compounds were determined as following.

$$\mathrm{CR}(\%) = \frac{\mu g/\mathrm{mL} \ \text{ of SA yielding } A/A_0 = 50\%}{\mu g/\mathrm{mL} \ \text{ of compound under investigation yielding } A/A_0 = 50\%} \times 100$$

where A is the absorbance in the presence of the test compound and A_0 is the absorbance in the absence of the test compound.

2.3. Results and discussion

2.3.1. Direct determination of SA-carrier protein conjugate by MALDI-TOF mass spectrometry

In general, the low molecular weight compounds (hapten) like plant secondary metabolite have no immunogenicity. Therefore, it should be conjugated with some high molecular compound like protein resulting in immunogenic. The specificity of immunoassay method is dependent on the site of linkage between hapten and carrier protein moiety, and enumeration of hapten in immunogen conjugate. SA-BSA and SA-HSA conjugates were synthesized as immunogen and the immobilization antigen for ELISA, respectively. Figure 3 shows the typical synthetic pathway of SA-BSA conjugate. The commonly used methods to link carboxyl group and amino group in a hapten or carrier involve activation by carbodiimides, isobutylchloroformate or carbonyldiimidazole. Carbodiimides react with carboxyl groups to form an unstable *O*-acetylisourea intermediate, which reacts with amines to form amide bonds. EDC can be used commonly as a carbodiimide. In this case, carrier protein combined directly to antigen as indicated in Figure 3.

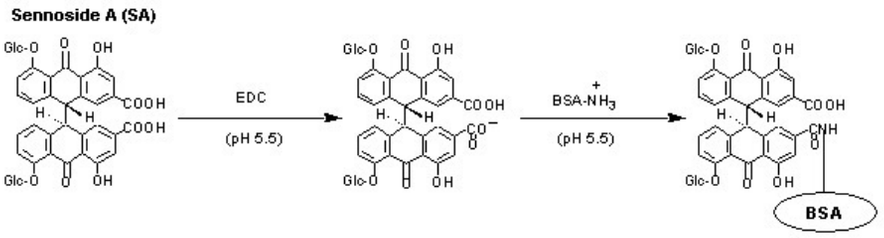

Figure 3. Typical synthetic pathway of SA-BSA. Carboxyl group of SA was activated by EDC and subsequently combined to amino residues of lysine and/or arginine on the protein to form amide bond.

Figure 4 shows the MALDI-TOF mass spectrum of the antigen, SA-BSA conjugate. A broad peak coinciding with the conjugate of SA and BSA appeared from *m*/*z* 68,500 to 73,500 centering at around *m*/*z* 70,600. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of SA component (MW 862) are 4,218 resulting in the range of two to eight molecules of SA (five on average) conjugated with BSA. In general eight to twenty five molecules of hapten conjugated with carrier protein in the conjugate were sufficient for immunization. Therefore, the hapten number was estimated to be sufficient for immunization because an antigen conjugate having a similar hapten number was sufficient for immunization in a previous study [10]. The number of SA contained in the SA-HSA conjugate was also determined to be around five molecules by its spectrum.

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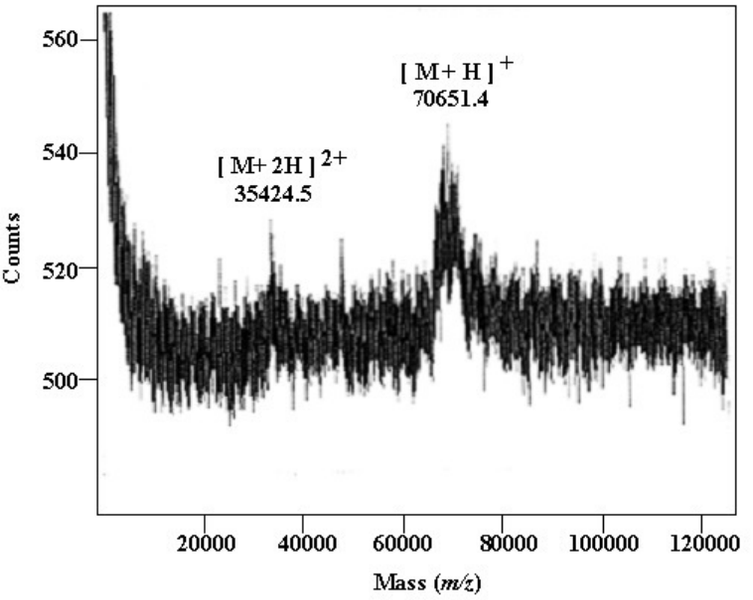

Figure 4. Direct determination of SA-BSA by MALDI-TOF MS. [M+H]+, $[M+2H]^{2}+$ are single and double protonated molecules of SA-BSA, respectively.

2.3.2. Production and characteristic of MAb against SA

After the cell fusion and HAT selection, hybridoma producing MAb reactive to SA was obtained, and classfied into IgG_1 which had *k* light chains. Refined MAb was confirmed to be IgG compared to the MALDI-TOF MS measurement. The molecular weight of MAb was 151,396 calculated [16]. The reactivity of IgG type MAb 6G8 was tested for varying the antibody concentration and for performing a dilution curve in direct ELISA. The antibody concentration of 0.218 μg/mL showed the absorbance at 0.8 in direct ELISA, therefore it was selected for the competitive ELISA.

2.3.3. Assay sensitivity and assay specificity

The free MAb 6G8, following incubation with competing antigen, was bound to the polystyrene microtitre plates precoated with SA-HSA. Under these conditions, the full measuring range of the assay extended from 20 to 200 ng/mL as indicated in Figure 5.

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. Moreover, SA possessed a *threo*- configuration between C-10 and C-10' positions as indicated in Figure 6. Therefore, a MAb should detect all these functions, and also the stereochemical recognition is needed for this complicated compound. Since the newly established ELISA against SA is expected to be applied for phytochemical investigations involving crude plant extracts, the assay specificity was checked by determining the cross-reactivities of MAb with various related compounds. The cross-reactivities of the MAb was examined by competitive ELISA.

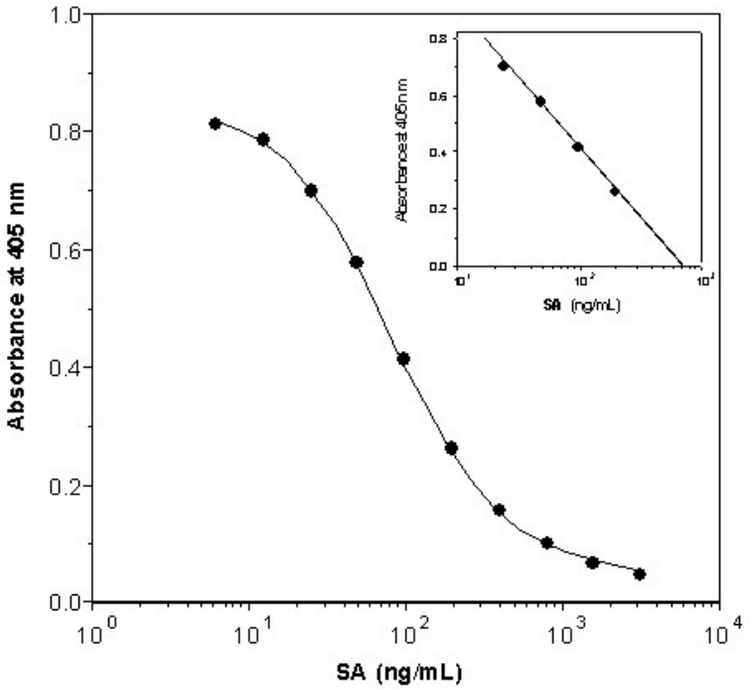

Figure 5. Calibration curve for SA.

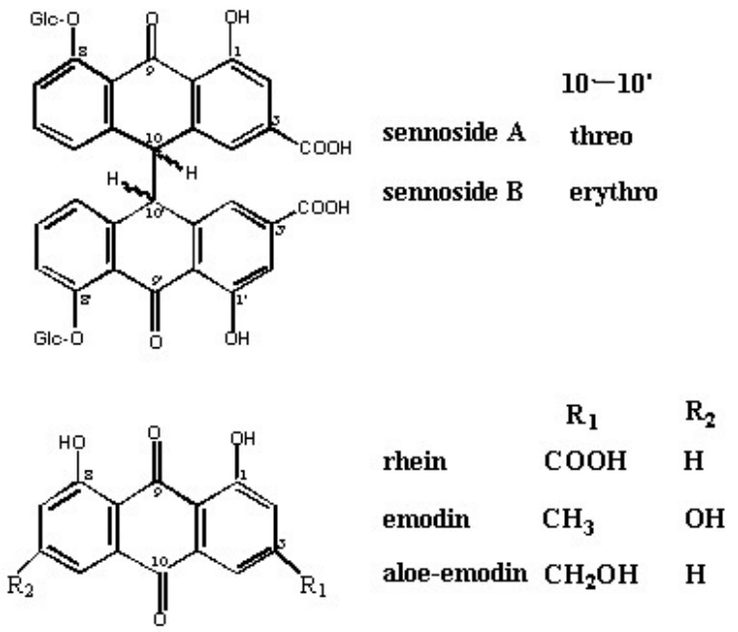

Figure 6. Chemical structures of SA, SB and its structurally related compounds.

Table 1 indicates the cross-reactivities of anti-SA MAb against related anthraquinone, anthrone and phenol carboxylic acid. MAb 6G8 cross-reacted with rhein and SB weakly; 0.28 and 0.35%, respectively. However, the other related anthraquinone and anthrone did not

have appreciable cross-reactivities. From these results it is suggested that a basal structure of rhein and sugar moiety caused immunization. In addition the most important property of MAb 6G8 is an ability of stereochemical recognition because the differences of structure between SA and SB are only the stereochemical configuration at the C-10 and C-10' positions. Therefore, it is suggested that *threo*-configurational structure of bisanthrone is indispensable as an immunodominant molecule for reactivity of MAb 6G8.

Compound	Cross-reactivities (%)
Anthraquinone and anthrone	
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
Phenol carboxylic acid	
gallic acid	< 0.04
vanillic acid	< 0.04
caffeic acid	< 0.04
homogentisic acid	< 0.04

Table 1. Cross-reactivities (%) of MAb-6G8 against sennosides and other compounds.

2.3.4. Correlation of results of SA determination in crude extracts of rhubarb roots between HPLC and ELISA using MAb 6G8

The ELISA was utilized to measure the concentrations of SA in various rhubarb (Table 2). Oshio and Kawamura determined sennoside concentrations in various crude rhubarbs by HPLC [17]. More recently Seto *et al.* reported the comparative concentrations of sennosides determined by HPLC in various commercial rhubarbs [18]. They required a lager sample size compared to the newly established ELISA due to some pretreatments because the crude materials contained several kinds of phenolics such as tannins, stilbens, naphthalen derivatives and lindleyin as previously indicated.

Table 2 shows the SA concentrations in various rhubarbs. Shinshu Daio bred by crossing *R. palmatum* and *R. coreanum* in order to increase the concentration of SA in Japan, contained the highest SA; 13.69±0.69 μg/mg dry wt. Ga-wo which was estimated to be high grade, con-

tained 6.62±0.42 μg/mg dry wt. The other three species showed almost the same concentrations of SA, around 3.3 μg/mg dry wt. These results are in good agreement with the previous reports [18]. The correlation between results from ELISA and HPLC is reasonable except for Kinmon Daio. The concentration analyzed by HPLC was very low compared to the others. The reason is still obscure although individual peaks separated by HPLC were analyzed by ELISA.

Sample	Concentration (μg/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

Table 2. SA concentrations in various rhubarb samples. Data are the means of triplicate assays.

3. Production of MAbs against SB, their characterization and use for ELISA

3.1. Preface

SB is a very important natural bioactive component of rhubarb and senna as well as SA. Total sennoside (SA and SB) concentrations are important, when rhubarb and senna are used as a raw material of medical supply and traditional Japanese herbal medicine for the purgative effect.

A number of methods for the quantification of SB have been published, most of which have been performed by HPLC [17]. Immunological approaches for assaying quantities of sennosides and SA using PAb and MAb have been investigated by Atzorn *et al*. [5] and by us [14], respectively. However, no success with MAb against SB has been reported. In here, production of anti-SB MAb and the competitive ELISA using anti-SA and SB MAbs for the direct determination of SA and SB in various samples are described [19].

3.2. Experimental

3.2.1. Plant materials

Samples of various rhubarb roots were purchased from the Tochimototenkaido Corporation (Osaka, Japan). Samples of leaves of *Cassia* plants were collected in Thailand. Traditional Japanese prescriptions were procured from Tsumura & Co. (Tokyo, Japan). Dietary supplements (health teas and dieter's teas) were purchased from drug and department stores.

3.2.2. Sample preparation

Dried samples (30 mg) of various rhubarb roots, *Cassia* plant leaves, traditional Japanese prescriptions and dietary supplements were powdered, and then extracted five times with MeOH containing 0.1% (w/v) NH_4OH (0.5 mL) with sonication, filtered using a Cosmonice Filter W (0.45 μm Filter Unit, Nacalai Tesque Inc., Kyoto, Japan), and the combined extracts were diluted with 10 mM $NaHCO_3$ to prepare a solution suitable for the ELISA.

3.2.3. Synthesisi of antigen conjugates

To SB (6 mg) dissolved in 1 mL of tetrahydrofuran-20 mM phosphate buffer of pH 5.5 (7:3), 0.3 mL of 20 mM phosphate buffer (pH 5.5) containing 6 mg of EDC was added. Then, 0.3 mL of 20 mM phosphate buffer (pH 5.5) containing 6 mg of BSA was added, with stirring at room temperature for 14 hr. The reaction mixture was dialyzed five times against H_2O, and then lyophilized to give 5.5 mg of SB-BSA conjugate. SB-HSA conjugate was also synthesized in the same manner.

3.2.4. Determination of hapten density in SB-carrier protein conjugate by MALDI-TOF mass spectrometry

The hapten number in the SB-carrier protein conjugate was determined by MALDI-TOF mass spectrometry as previously described [15].

3.2.5. Competitive ELISA for SB

SB-HSA (four molecules of SB per molecule of HSA) (100 μL, 1 μg/mL) dissolved in 50 mM carbonate buffer (pH 9.6) was adsorbed to the wells of a 96-well immunoplate then treated with 300 μL S-PBS for 1 hr to reduce non-specific adsorption. Fifty μL of various concentrations of SB or samples dissolved in 10 mM $NaHCO_3$ solution were incubated with 50 μL of MAb solution (0.121 μg/mL) for 1 hr. The plate was washed three times with T-PBS, and then incubated with 100 μL of a 1:1000 dilution of POD-labeled anti-mouse IgG for 1 hr. After washing the plate three times with T-PBS, 100 μL of substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H_2O_2 and 0.3 mg/mL of ABTS] was added to each well and incubated for 15 min. The absorbance was measured by a micro plate reader at 405 nm and 490 nm.

3.3. Results and discussion

3.3.1. Direct determination of SB-carrier protein conjugate by MALDI-TOF mass spectrometry

It is well known that hapten number in an antigen conjugate is important for immunization against low molecular weight compounds. Figure 7 shows the MALDI-TOF mass spectrum of the antigen, SB-BSA conjugate. A broad peak coinciding with the conjugate of SB and BSA appeared from *m*/*z* 67,300 to 70,700 centering at around *m*/*z* 68,900. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of SB component

(MW 862) are 2,500 resulting in the range of one to five molecules of SB (three on average) conjugated with BSA. This conjugate, although having a relatively low hapten number, proved sufficiently immunogenic in agreement with our previous results [10]. The number of SB contained in the SB-HSA conjugate was also determined to be around four molecules by its spectrum.

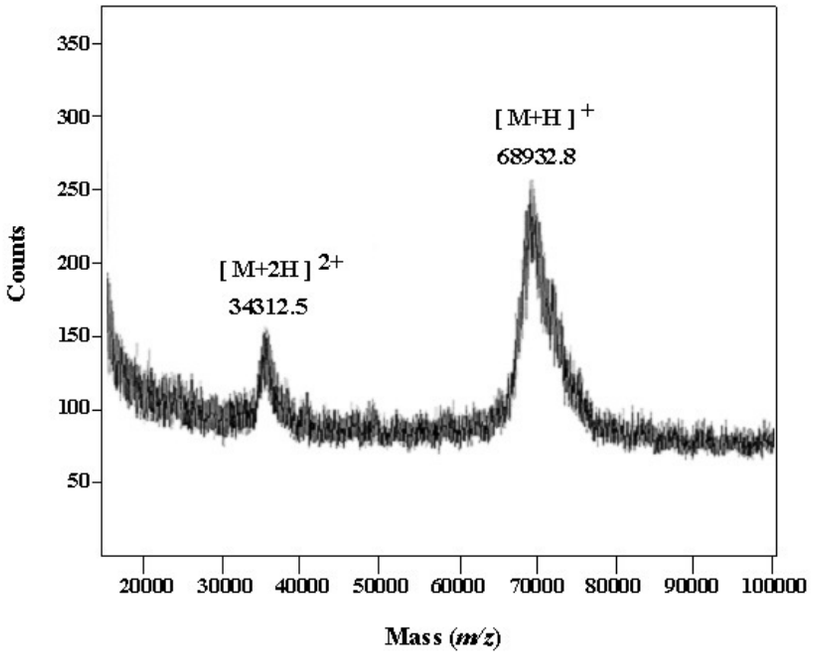

Figure 7. Direct determination of SB-BSA by MALDI-TOF MS.

3.3.2. Production and characteristics of Mabs against SB

The immunized BALB/c mice yielded splenocytes which were fused with P3-X63-Ag8-653 myeloma cells by the routinely established procedure in this laboratory [6]. Hybridoma producing MAbs reactive to SB were obtained, and classified as IgG1 (5G6, 7H12) and IgG2b (5C7) which had *k* light chains. The reactivity of IgG type MAb 7H12 was tested by varying the antibody concentration and by performing a dilution curve in direct ELISA. The antibody concentration (0.121 μg/mL) at which the absorbance was about 1.0 in direct ELISA was selected for competitive ELISA.

3.3.3. Assay sensitivity and assay specificity

The free MAb 7H12 following competition was bound to the polystyrene microtitre plates precoated with SB-HSA. Under these conditions, the full measuring range of the assay extends from 0.5 ng/mL to 15 ng/mL as indicated in Figure 8 and the ELISA using a MAb 7H12 is more sensitive than those using MAb 5C7 and 5G6.

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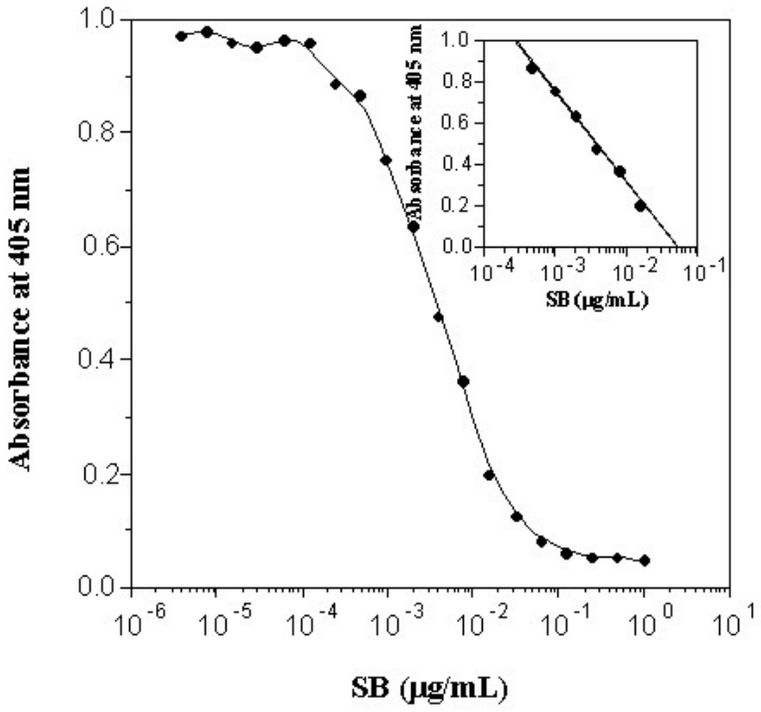

Figure 8. Calibration curve for SB.

SB is a unique anthraquinone having individual double-carboxylic acid-, hydroxyl-, carbonyl- and *O*-glucosyl-groups at C-3, C-1, C-9 and C-8 positions in the molecule, respectively. Moreover, SB possesses an *erythro*-configuration between C-10 and C-10' positions. Therefore, MAbs should distinguish all these functional groups, and also recognize the stereochemistry of this complicated compound. Since the newly established ELISA against SB is expected to be used for phytochemical investigations involving crude plant extracts, the assay specificity was checked by determining the cross-reactivities of the MAbs with various related compounds. The cross-reactivities of MAbs were examined by the competitive ELISA. Table 3 indicates the cross-reactivities of anti-SB MAbs against related anthraquinone, anthrone and phenol carboxylic acid. MAb 7H12 has weak cross-reactivities with SA (2.45%) and rhein (0.012%). However, the other related anthraquinone and anthrone did not have appreciable cross-reactivities. From these results it is suggested that the epitope consists of a basal structure of rhein and sugar moiety. In addition the most important property of MAb 7H12 is its ability to distinguish between SB and SA, which differ only in the stereochemical configuration at the C-10 and C-10' positions. Therefore, it is suggested that *erythro*-configurational structure of bisanthrone is indispensable as an immunodominant molecule for the reactivity of MAb 7H12. So the ELISA using a MAb 7H12 possesses apparently high sensitivity and specificity for SB. Because we have also prepared an anti-SA MAb having a weak cross-reactivity with SB (0.28%) as already discussed, these two MAbs make it possible to investigate stereochemical recognition precisely.

Compound	Cross-reactivities (%)		
	7H12	5G6	5C7
Anthraquinone and anthrone			
sennoside B	100	100	100
sennoside A	2.45	2.30	8.53
rhein	0.012	0.030	0.007
emodin	< 0.004	< 0.023	< 0.006
aloe-emodin	< 0.040	< 0.023	< 0.006
barbaloin	< 0.004	< 0.023	< 0.006
1,4-dihydroxy-anthraquinone	< 0.004	< 0.023	< 0.006
Stilbene			
rhaponticin	< 0.004	< 0.023	< 0.006
Phenol carboxylic acid			
gallic acid	< 0.004	< 0.023	< 0.006
vanillic acid	< 0.004	< 0.023	< 0.006
caffeic acid	< 0.004	< 0.023	< 0.006
homogentisic acid	< 0.004	< 0.023	< 0.006

Table 3. Cross-reactivities of anti-SB MAbs against various compounds.

3.3.4. Correlation of results of SB determination in crude extracts of rhubarb roots between HPLC and ELISA using MAb 7H12

The concentrations of SB in various rhubarb samples were determined by ELISA (Table 4). Shinshu Daio, bred by crossing *R. palmatum* and *R. coreanum* in order to increase the level of SB concentration in Japan, contained the highest SB level of 6.01±0.18 μg/mg dry wt. Ga-wo, estimated to be high grade in the traditional Japanese medicine, contained SB level of 3.14±0.27 μg/mg dry wt. These results are in good agreement with previous reports [18]. The correlation between results from ELISA and HPLC is also good.

Sample	Concentration (μg/mg dry wt. powder)	
	ELISA	HPLC
Shinshu Daio	6.01±0.18	6.15±0.59
Ga-wo	3.14±0.27	3.80±0.16
Kinmon Daio	0.35±0.01	0.38±0.02
Itto-Ga-wo (powder)	1.44±0.12	1.52±0.18
Itto-Ga-wo (refuse)	1.42±0.07	1.40±0.11

Table 4. SB concentrations in various rhubarb samples. Data are the means of triplicate assays.

3.3.5. Determination of concentrations of SA and SB in various Cassia species

The concentrations of SA and SB in leaves of various *Cassia* species were determined by ELISA using anti-SA and SB MAbs (Table 5). The results indicate that *C. angustifolia* contains 4.56±0.25 μg/mg dry wt. powder of SA and 5.10±0.15 μg/mg dry wt. powder of SB indicating higher amounts of SA and SB compared to the other species. *C. alata* contains 1.19±0.12 μg/mg dry wt. powder of SA and 1.16±0.15 μg/mg dry wt. powder of SB. *C. fistula* (A)~(D) contain 0.10-2.04 μg/mg dry wt. powder of SA and 0.13-2.05 μg/mg dry wt. powder of SB, respectively.

Sample	Concentration (μg/mg dry wt. powder)		
	Sennoside A	**Sennoside B**	**Total sennosides**
Cassia angustifolia	4.56±0.25	5.10±0.15	9.66±0.40
C. alata	1.19±0.12	1.16±0.15	2.35±0.27
C. bakeriana	0.40±0.03	0.44±0.02	0.84±0.05
C. fistula (A)	1.14±0.08	0.75±0.08	1.89±0.16
C. fistula (B)	2.04±0.32	1.52±0.12	3.56±0.44
C. fistula (C)	1.90±0.16	2.05±0.24	3.95±0.40
C. fistula (D)	0.10±0.01	0.13±0.00	0.23±0.01
C. mimosoides	$(1.30\pm0.24)\times10^{-2}$	$(1.88\pm0.29)\times10^{-4}$	$(1.32\pm0.24)\times10^{-2}$
C. floribunda	$(2.78\pm0.11)\times10^{-3}$	$(1.04\pm0.03)\times10^{-4}$	$(2.88\pm0.11)\times10^{-3}$
C. surattensis	$(1.15\pm0.18)\times10^{-2}$	$(2.44\pm0.17)\times10^{-4}$	$(1.17\pm0.18)\times10^{-2}$
C. tora	$(2.13\pm0.21)\times10^{-3}$	$(3.64\pm0.21)\times10^{-5}$	$(2.17\pm0.23)\times10^{-3}$
C. siamea	$(4.45\pm0.14)\times10^{-3}$	$(1.87\pm0.13)\times10^{-3}$	$(6.32\pm0.27)\times10^{-3}$

Table 5. Total sennoside concentrations in leaves of various *Cassia* species. Data are the means of triplicate assays.

4. Establishments of a new eastern blotting, double staining and immunohistochemical staining using anti-SA and SB MAbs

4.1. Preface

Thin-layer chromatography (TLC) is most widely used for detection, separation and monitoring of small molecular compounds like sennosides. If the direct TLC immunostaining with MAb can be done, this procedure must be contributive to the development of structural analysis of small molecular compounds. However, this procedure cannot be used for the direct detection of small molecular compounds on a TLC plate because the silica gel is sloughed off from the plate and the compounds on the plate are easily washed out without fixing during treatment. If the compounds are transferred from the TLC plate to a plastic

membrane with hydrophobic properties and immobilized on the membrane, these difficulties can be solved. Therefore, I examined the transfer of sennosides from a TLC plate to a plastic membrane. Towbin *et al*. first reported the transfer of glycosphingolipids using nitrocellulose membranes [20]. However, since its transfer efficiency was poor and reproducible results were not obtained, I tested various plastic membranes and transfer conditions resulting in a polyvinylidene difluoride (PVDF) membrane to be the best [21]. The membrane is very stable against heating and various organic solvents in addition to retaining sennosides with high efficiency. I named this new method as eastern blotting (EB), because theoretically same methodology compared to previous EB except the way of sennoside-BSA conjugation for fixing sennosides on the membrane [22]. I communicate here the EB procedure for sennosides and its application for analytical survey of sennosides [23].

4.2. Experimental

4.2.1. Chemicals and immunochemicals

Polyvinylidene difluoride (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.

4.2.2. EB and Double staining

Sennosides were applied to a TLC plate and developed 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). It was placed on a stainless steel plate and then covered with a PVDF membrane sheet. After covering with a glass microfiber filter sheet, the whole assembly was pressed evenly for 70 s with a 120 ˚C hot plate as previously described with some modifications [24, 25]. The PVDF membrane was separated from the TLC plate and dried.

The blotted PVDF membrane was dipped in 20 mM carbonate buffer solution (pH 9.6) containing BSA (1%) and EDC (20 mg/mL), and stirred at room temperature for 14 hr. After washing the PVDF membrane twice with T-PBS for 5 min and then treated with S-PBS for 3 hr to reduce non-specific adsorption. The PVDF membrane was washed with T-PBS twice for 5 min, and then immersed in anti-SA MAb (6G8) and stirred at room temperature for 3 hr. After washing the PVDF membrane twice with T-PBS for 5 min, a 1:1000 dilution of POD-labeled goat anti-mouse IgG in PBS cotaining 0.2% of gelatin (G-PBS) was added and stirred at room temperature for 1 hr. The PVDF membrane was washed twice with T-PBS and water, then exposed to 1 mg/mL 4-chloro-1-naphtol-0.03% H_2O_2 in PBS solution which was freshly prepared before use for 10 min at room temperature. The protocol of the EB technique is shown in Figure 9.

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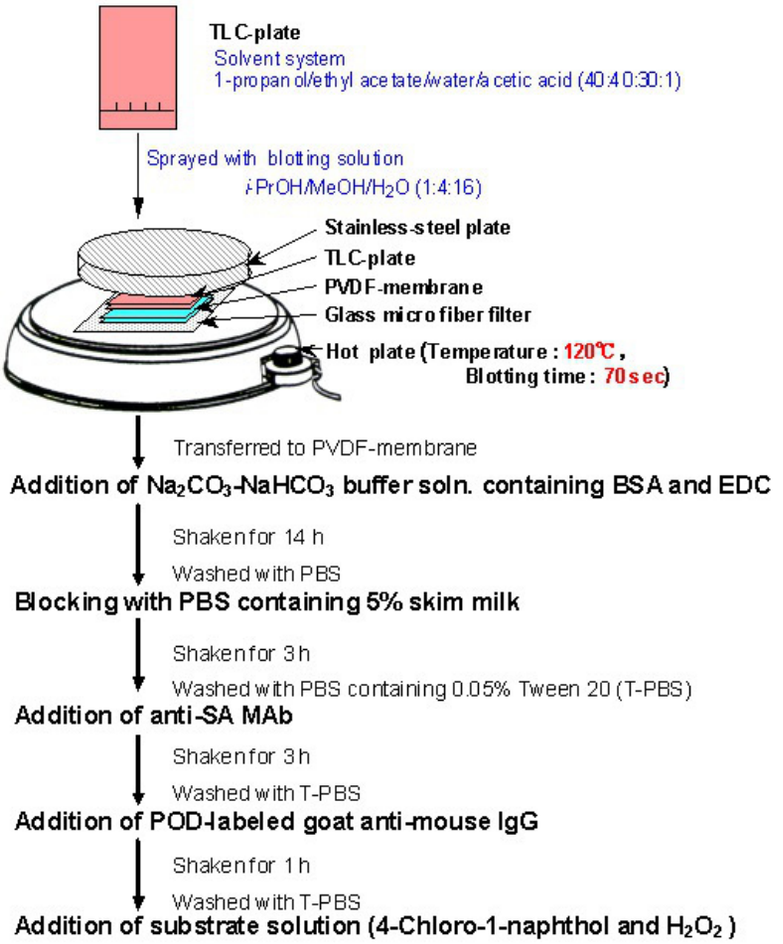

Figure 9. Eastern blotting protocol.

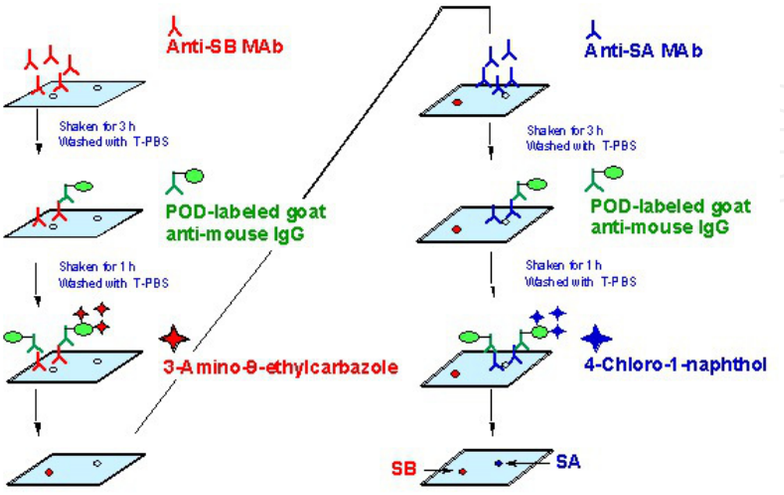

Figure 10. Double staining protocol.

For successive staining by anti-SB MAb (7H12), the PVDF membrane stained by anti-SA MAb was treated in the same way as anti-SA MAb (6G8) except that it was exposed to 2 mg/10 mL 3-amino-9-ethylcarbazole-0.03% H_2O_2 in acetate buffer (0.05 M, pH 5.0) containing 0.5 mL of *N,N*-dimethyl formamide. The protocol of double staining is shown in Figure 10.

4.2.3. EB for immunohistochemical staining of SA

A piece of PVDF membrane was placed on a glass microfiber filter sheet. A sliced fresh rhubarb root was placed on the PVDF membrane, and they were pressed together evenly for 1 hr. The blotted PVDF membrane was stained using the same procedure described for the EB method.

4.3. Results and discussion

4.3.1. EB of SA using anti-SA MAb

Previously we established a new immunostaining method named as eastern blotting for several glycosides like solasodine glycosides [21], ginsenosides [26, 27] and glycyrrhizin [22, 28] by using individual MAbs. In this methodology we separated the sugar moiety in a molecule into two functions, the epitope part and fixation ability part on a membrane after blotted to a PVDF membrane from a TLC plate, since small molecular compounds can not be fixed on the membrane. Although I followed the previous methodology for SA, unfortunately staining was not succeeded. Therefore, a new blotting method onto a PVDF membrane from the developed TLC plate is required. SA was transferred to the PVDF membrane by the same way as previously described, and treated with EDC solution followed by the addition of BSA as indicated in Figure 9. This reaction enhanced the fixation of SA via SA-BSA conjugate on the PVDF membrane and the pathway was indicated diagrammatically in Figure 11. When the blotted PVDF membrane was incubated in the absence of EDC, it was essentially free of immunostaining (data not shown).

Figure 12 shows the EB of sennosides and other structurally related compounds using anti-SA MAb (A) and the H_2SO_4 staining (B). The EB indicated only limited staining of SA as shown in Figure 12A, lane 7. Moreover, the EB method was considerably more sensitive than that of H_2SO_4 staining. Since anti-SA MAb cross-reacts against SB and rhein as 0.28 and 0.35%, respectively, they can be stained very weakly by anti-SA MAb, as described in the previous section. Previously Fukuda *et al.* succeeded the EB of ginsenoside Rb1 by using anti-ginsenoside Rb1 MAb resulting in staining together with ginsenoside Rc, Rd, Re and Rg1 [26, 27]. The difference between the newly established EB and the previous methodology is combine system of sugar moiety to PVDF membrane. The sugar moiety in ginsenosides was oxidatively cleavaged to release aldehyde groups which were conjugated with a protein to fix on a PVDF membrane. Since it was evident that a part of sugar moiety in ginsenoside Rb1 was immunized, the cleavage of sugar moiety by $NaIO_4$ expanded its cross-reactivity against other ginsenosides resulting in possibility of staining for ginsenoside Rc, Rd, Re and Rg1, though their cross reactivities are weak. On the other hand, the newly established EB in here does not hinder around sugar moiety in SA. Therefore, strength of staining for SA, SB and rhein was proportional to their cross-reactivities as described in ELISA.

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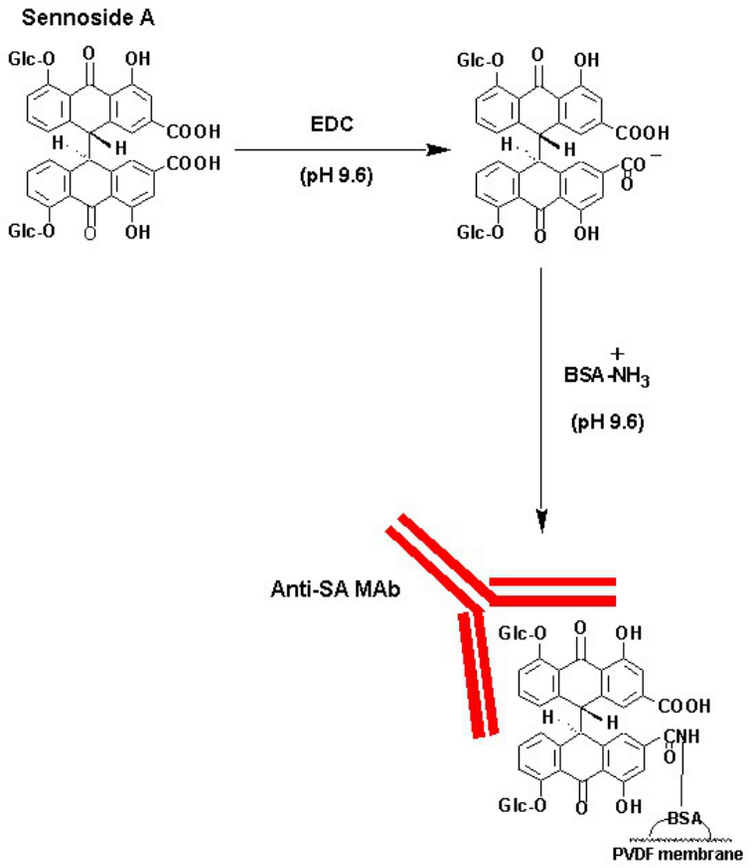

Figure 11. Schematic diagram illustrating the eastern blotting of SA onto the PVDF membrane and the detection using anti-SA MAb.

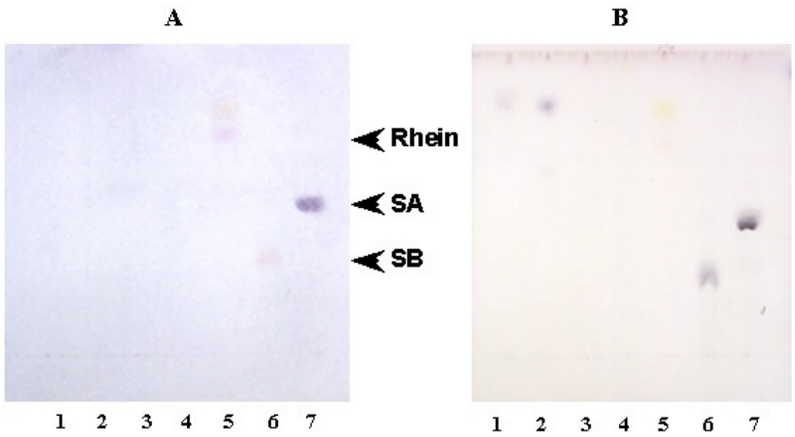

Figure 12. Eastern blotting of sennosides and related compounds stained by anti-SA MAb (A). B shows a TLC plate stained by 10% H_2SO_4. Lanes 1, 2, 3, 4, 5, 6 and 7 indicate rhaponticin, barbaloin, aloe-emodin, emodin, rhein, SB and SA (3 μg), respectively.

4.3.2. Double staining of sennosides using anti-SA and SB MAbs

Previously, I used 4-chloro-1-naphthol for staining of SB. However, since it could not function well for SB, the combination of 4-chloro-1-naphthol and 3-amino-9-ethylcarbazole was selected to improve double staining of sennosides as indicated in Figure 10. SA and SB were stained clearly by the purple and red color, respectively (Figure 13). From this result both antibodies can distinguish stereochemical configurations, *threo* and *erythro* between C-10 and C-10′ positions in a molecule on PVDF membrane stained as double coloring, respectively.

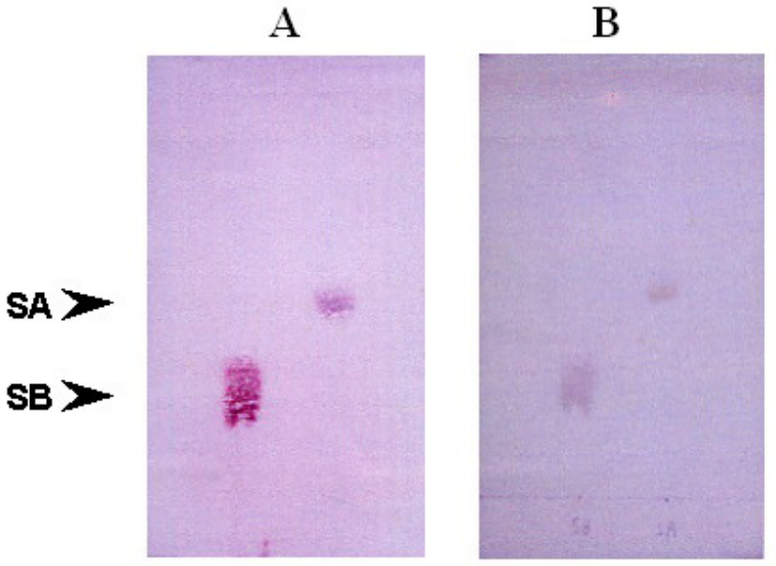

Figure 13. Double staining of sennosides using eastern blotting technique (A). B shows a result of H_2SO_4 staining. Red and purple colors were stained by anti-SB and SA MAb, respectively.

4.3.3. Detection of SA and SB in various Cassia species using double staining with a new EB technique

The crude extracts of various *Cassia* species were analyzed by the newly developed double staining system and TLC stained with H_2SO_4 as shown in Figure 14. Although H_2SO_4 staining (Figure 14B) detected many spots including probably sugars and different types of anthraquinone glycosides in various *Cassia* species, double staining (Figure 14A) detected clearly SA and SB, and very weakly other sennosides except appearance of chlorophylls around top. Band 1 indicated a purple color that means a *threo*-configuration between C-10 and C-10′ positions detected by EB using anti-SA MAb as shown in Figure 14A. Moreover, its *R*f value indicated that band 1 has one sugar moiety and a CH_2OH group instead of COOH group in a molecule. I surveyed the previous papers regarding sennosides in senna [1]. Judging from these evidences, I suggested that band 1 is sennoside C (SC) having *threo*-configuration as indicated previously [1]. Band 2 was easily suggested to be *erythro*-configu-

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ration from its red color. The *R*f value clearly showed that band 2 includes one sugar moiety having a HOOC-CO group. From these results I supposed that band 2 is sennoside F (SF) that has *erythro*-configuration as indicated previously [1]. The double staining by EB indicates that *C. angustifolia*, *C. alata*, *C. bakeriana* and *C. fistula* contain a higher concentration of sennosides compared to the other species. This result has a good agreement with that of ELISA. The limit of detection by the double staining method was confirmed to be 48 μg/mL of both SA and SB.

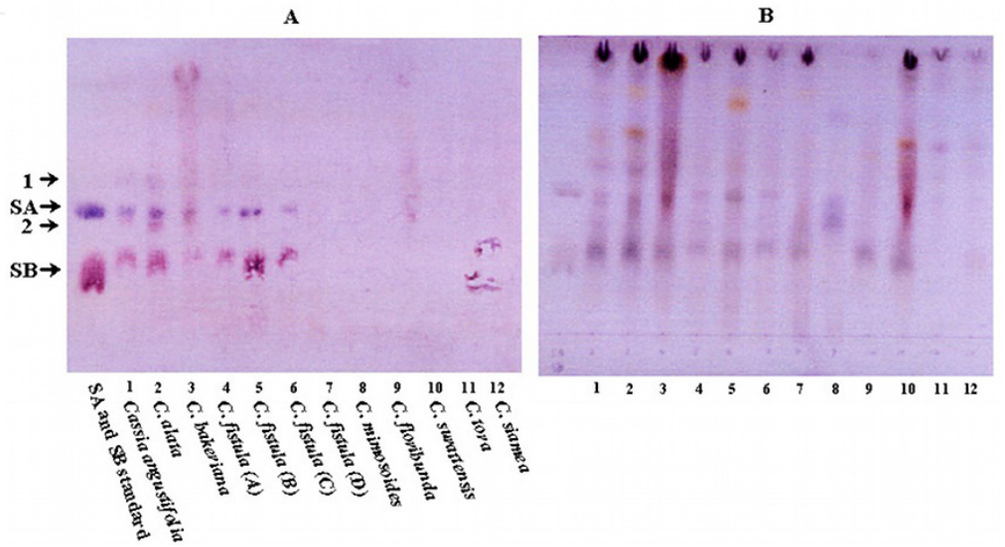

Figure 14. Double staining of SA and SB in various *Cassia* species (A). B shows a result of H_2SO_4 staining. Lefthand lane indicates SA (4 μg)and SB (3 μg). Lanes 1~12 indicate various *Cassia* species (3 μL).

4.3.4. Validation of EB for immunohistochemical staining of SA

As an other application of the EB method, the immunohistochemical staining of SA in rhubarb root, was investigated. A sliced fresh rhubarb root was placed on the PVDF membrane, and they were pressed together evenly for 1 hr. The blotted PVDF membrane was stained using the same procedure described for the EB method. Figure 15II illustrates the immunohistochemical staining of SA in fresh Hokkai Daio root. The phloem and cambium contained a higher concentration of SA compared to other tissues, pith and bud. To confirm this result, I analyzed these tissues individually by ELISA and HPLC. The concentrations of SA were determined by ELISA to determine 64.4±4.5, 48.1±8.2, 15.0±1.6 and 1.8±0.3 ng/mg fresh wt. in phloem, cambium, pith and bud, respectively. This result was a good agreement with those of HPLC resulting in 58.4±2.6, 49.0±3.9 and 13.3±0.5 ng/mg fresh wt. in phloem, cambium and pith, respectively.

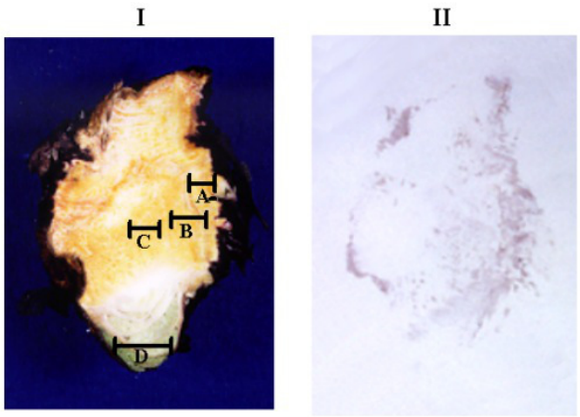

Figure 15. Immunohistochemical staining of SA using anti-SA MAb in rhubarb root. I, cross section of Hokkai Daio root; II, direct eastern blotting on PVDF membrane of a cross section of Hokkai Daio root. A, Phloem; B, Cambium; C, Pith; D, Bud, respectively.

5. Conclusion

The recent developments of molecular biosciences and their biotechnological applications have opened up many new avenues of pharmaceutical areas. MAbs have many potential uses in addition to immunological methods to plant sciences. Therefore, immunoassay system using MAbs against pharmacologically active natural products having low molecular weight have become an important tool for the studies on receptor binding analysis, enzyme assay, and quantitative and/or qualitative analytical techniques in plants owing to their specific affinity.

In order to analyze the stereochemical isomers, SA and SB in plants, medicaments, prescriptions, health foods and patients'sera, I have produced MAbs against them. These MAbs have the most important ability to distinguish between SA and SB, which differ only in the stereochemical configuration at the C-10 and C-10′ positions, respectively. Moreover, they have no detectable cross-reaction with the other related anthraquinone and anthrone.

Analytical systems of SA and SB by competitive ELISA using anti-SA and SB MAbs were established. These ELISA systems are capable of measuring SA and SB in complex matrics without any pretreatments. Furthermore, these ELISA methods are approximately 2,000 times for SA and 10,000 times for SB more sensitive than that of HPLC method.

The newly developed EB methodology can be theoretically expanded for all compounds having carboxylic acid such as phenol carboxylic acids, glucuronides, furthermore compounds having only a carboxylic group in a molecule. A new double staining with EB method for sennosides using anti-SA and SB MAbs was established. SA and SB were stained purple and red color, respectively. This system visualized sennosides on a PVDF membrane. In fact, SA and SB in the crude extracts of various *Cassia* species were distinguished by their coloring and *R*f values. Moreover, it could make it possible to survey the natural resour-

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ces of sennosides and quickly determine their structures. Furthermore, EB also can be used for the survey of distribution of SA and/or SB in the *Rheum* specimen by immunohistochemical staining.

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