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Production of monoclonal antibodies against a major purgative component, sennoside B, their characterization and use in ELISA

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For immunization, sennoside B was conjugated with bovine serum albumin. The hapten density in the antigen conjugate was determined to be 3 mol mol^{-1} protein by matrix-assisted laser desorption–ionization TOF mass spectrometry. A hybridoma secreting monoclonal antibody against sennoside B was produced by fusing splenocytes from mouse immunized with the sennoside B conjugate and mouse myeloma cells. Weak cross-reactivities occurred with sennoside A which is a stereochemical isomer, and a monomer of sennoside B, rhein, but no cross-reactivity was observed with other related anthraquinones and phenolics. The range of the assay extended from 0.5 ng ml^{-1} to 15 ng ml^{-1} of sennoside B, and good correlation between ELISA and HPLC methods was obtained when crude extracts of rhubarb were analyzed.

Introduction

Rhubarb, the rhizome and root of *Rheum* spp. (Polygonaceae), has been an important drug in traditional Chinese herbal medicine as well as in Western medicine since ancient times. It was recorded in *Chinese Materia Medica* 2000 years ago. It is used in many traditional Chinese herbal medicines prescribed with other herbal medicines for the syndrome of stasis of blood, as an anti-inflammatory and sedative agent. Furthermore, it is widely used in 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,[2] together with various kinds of phenolics, like tannins,[3] stilbenes,[4] naphthalenes[5] and lindleyin.[6] The content of sennoside in rhubarb has been shown to fluctuate with the genetic heterogeneity of species, differences in soil condition and climate influence. Furthermore, variations in quality have occurred depending on the harvest or collection season and the method of processing. Therefore, standardization of quality is necessary for constant pharmacological activity. Sennosides are metabolized by intestinal bacteria to rheinanthrone which acts in the intestines as a direct purgative.[7,8] Despite the availability of a number of synthetic purgatives, sennoside-containing prescriptions are still among the most widely used today, and their importance is increasing.

In our ongoing study of the formation of monoclonal antibodies (MAbs) against naturally occurring bioactive compounds, we have produced MAbs against forskolin,[9,10] solamargine,[11] opium alkaloids,[12] marihuana compounds,[13] glycyrrhizin,[14] crocin[15] and ginsenoside Rb_1,[16] and applied them in affinity chromatography,[10,17,18] new Western blotting approaches and in immunocytolocalization.[19–21] Immunological approaches for assaying quantities of sennosides and sennoside A (SA) using polyclonal antibodies and MAb have been investigated by Atzorn *et al.*[22] and by us,[23] respectively. However, since no success with MAb against sennoside B (SB) has been reported, we herein communicate the preparation of MAb against the major purgative component, SB, its characterization and applications.

Experimental

Chemicals and immunochemicals

SB 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). Bovine serum albumin (BSA) and human serum albumin (HSA) were provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was provided by Organon Teknika Cappel Products (West Chester, PA, USA). All other chemicals were standard commercial products of analytical grade.

Plant materials

Samples of various rhubarb roots were purchased from the Tochimototenkaido Corporation (Osaka, Japan).

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 $NaHCO_3$, a solution suitable for the ELISA.

Synthesis 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 BSA was added. Then, 0.3 ml of 20 mM phosphate buffer (pH 5.5) containing 6 mg of EDC was added, with stirring at room temperature for 14 h. The reaction mixture was dialyzed five times against H_2O, and then lyophilized to give 5.5 mg of SB conjugate (SB–BSA). SB–HSA conjugate was also synthesized in the same manner.

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Determination of hapten density in SB–carrier protein conjugate by matrix-assisted laser desorption–ionization (MALDI)-TOF mass spectrometry

The hapten density in the SB–carrier protein conjugate was determined by MALDI-TOF mass spectrometry, as previously described.[24–26] 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. The mixture was subjected to a JEOL Mass Spectrometer (JMS) time-of-flight (TOF) mass monitor 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.[24,25]

Immunization and hybridization

SB–BSA was dissolved in 8 M urea (20 mg ml^{-1}), and then diluted one hundred times with phosphate buffered saline (PBS). BALB/c female mice were injected intraperitoneally with SB–BSA solution three times. The first immunization was injected as a 1 : 1 emulsion in Freund's complete adjuvant (0.5 ml, 100 μg ml^{-1} SB–BSA). The second immunization was injected the same way twelve days after the first injection. On the third day after the final immunization, injected as a PBS solution (0.5 ml, 200 μg ml^{-1} SB–BSA), splenocytes were isolated and fused with a hypoxanthine–aminopterin–thymidine (HAT)-sensitive mouse myeloma cell line, P3-X63-Ag8-653, by the polyethylene glycol (PEG) method.[27] Hybridomas producing MAb reactive to SB were cloned by the limiting dilution method.[28] Established hybridomas were cultured in enriched RPMI 1640-Dulbecco's-Ham's F 12 (eRDF) medium supplemented with 10 μg ml^{-1} insulin, 35 μg ml^{-1} transferrin, 20 μM ethanolamine and 25 nM selenium (ITES).[29]

Purification of MAb

The MAb was purified using a Protein G FF column (0.46 × 11 cm; Amersham Pharmacia Biotech, Uppsala, Sweden). The cultured medium (120 ml) containing the IgG was adjusted to pH 7 with 1 M TRIS solution (pH 9) and applied to the column. The column was washed with 20 mM phosphate buffer (pH 7). Bound IgG was eluted with 100 mM citrate buffer (pH 2.7) at a flow of 1 ml min^{-1}. Fractions of 30 ml were collected. Elution was monitored photometrically at 280 nm. The eluted IgG was neutralized with 1 M TRIS–HCl buffer (pH 9), then dialyzed against H_2O four times, and finally lyophilized.

Direct ELISA using SB–HSA

The reactivity of MAb to SB–HSA was determined by a direct ELISA. SB–HSA (100 μl, 1 μg ml^{-1}) dissolved in 50 mM carbonate buffer (pH 9.6) was adsorbed to the wells of a 96 well-immunoplate (Nalge Nunc, Roskilde, Denmark) then treated with 300 μl PBS containing 5% (w/v) skimmed milk (SPBS) for 1 h to reduce non-specific adsorption. The plate was washed three times with PBS containing 0.05% (v/v) Tween 20 (TPBS) and reacted with 100 μl of testing MAb for 1 h. The plate was washed three times with TPBS, and then incubated with 100 μl of a 1 : 1000 dilution peroxidase-labeled anti-mouse IgG for l h. After washing the plate three times with TPBS, 100 μl of substrate solution, [0.1 M citrate buffer (pH 4.0) containing 0.003% (v/v) H_2O_2 and 0.3 mg ml^{-1} ABTS (2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt; Wako Pure Chemical Ind., Ltd.)], was added to each well and incubated for 20 min. Absorbance was measured by a microplate reader (Model 450 Microplate Reader Bio-Rad Laboratories, Richmond, CA, USA) at 405 nm and 490 nm. All reactions were carried out at 37 °C.

Competitive ELISA

SB–HSA (four molecules of SB per molecule of HSA) (100 μl, 1 μg ml^{-1}) dissolved in 50 mM carbonate buffer (pH 9.6) was adsorbed to the wells of a 96 well immunoplate then treated with 300 μl SPBS for 1 h 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^{-1}) for 1 h. The plate was washed three times with TPBS, and then incubated with 100 μl of a 1 : 1000 dilution of peroxidase-labeled anti-mouse IgG for 1 h. After washing the plate three times with TPBS, 100 μl of substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H_2O_2 and 0.3 mg ml^{-1} of ABTS] was added to each well and incubated for 15 min. The absorbance was measured by a microplate reader at 405 nm and 490 nm.

The cross-reactivities (CR) of sennosides and related compounds were determined as follows:

$$\mathrm{CR}(\%) = \frac{\mu\mathrm{g\ ml^{-1}\ of\ SB\ yielding}\ A/A_0 = 50\%}{\mu\mathrm{g\ ml^{-1}\ of\ compound\ under\ investigation\ yielding}\ A/A_0 = 50\%} \times 100$$

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.

Recovery experiments

Various amounts of SB were added to dried powdered rhubarb root (30 mg). The amount of SB in the unspiked sample was determined to be 178.2 μg. For each level three samples were analyzed. The root powders were extracted five times with MeOH containing 0.1% NH_4OH (0.5 ml) with sonication and filtered using a Cosmonice Filter W. The combined extract was diluted with 10 mM $NaHCO_3$. The recovery was calculated from the added SB in the same concentration ranges.

$$\mathrm{Recovery}\ (\%) = \frac{\mathrm{measured\ amount} - 178.2}{\mathrm{added\ amount}} \times 100$$

Results and discussion

Direct determination of hapten–carrier protein conjugate by MALDI-TOF mass spectrometry

Fig. 1 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 the SB component (MW 862) were 2500 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.[14] The number of SB contained in the SB–HSA conjugate was also determined to be around four molecules by its spectrum.

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.[9] Hybridoma producing MAbs reactive to SB were obtained, and classified as IgG1 (5G6, 7H12) and IgG2b (5C7) which had κ light chains.

The reactivity of IgG type MAb, 7H12, was tested by varying the antibody concentration and by performing a dilution curve as indicated in Fig. 2. The antibody concentration (0.121 μg ml^{-1}) at which the absorbance was about 1.0 in Fig. 2 was selected for competitive ELISA.

Assay sensitivity and assay specificity

The free MAb 7H12, following incubation with competing antigens, was bound to the polystyrene microtitre plates precoated with SB–HSA. Under these conditions, the full measuring range of the assay extended from 0.5 ng ml^{-1} to 15 ng ml^{-1} as indicated in Fig. 3 and the ELISA using MAb 7H12 was more sensitive than when using MAb 5C7 or 5G6.

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 (Fig. 4). Moreover, SB possesses an *erythro*-configuration between C-10 and C-10′ as indicated in Fig. 4. Therefore, MAbs should distinguish all these functional groups, and also recognize the stereochemistry of this complicated compound.

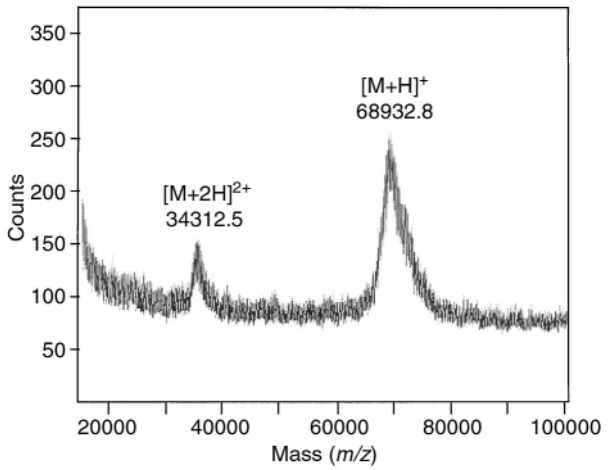


Fig. 1 Direct determination of sennoside B–BSA conjugate by matrix-assisted laser desorption–ionization TOF mass spectrometry. [M + H]$^+$, [M + 2H]$^{2+}$ are single and double protonated molecules of sennoside B–BSA, respectively.

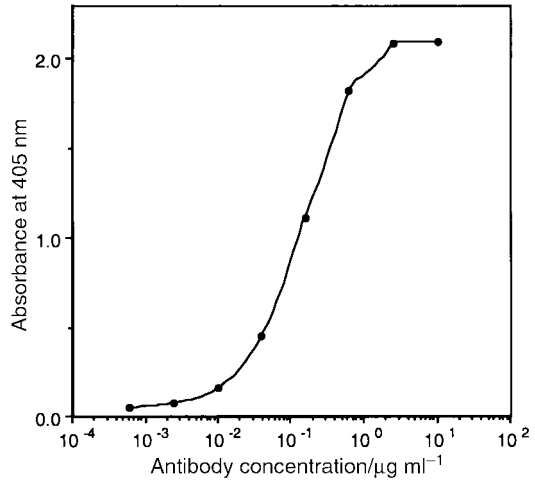


Fig. 2 Reactivities of IgG type MAb (7H12) against sennoside B. To examine the reactivity of the antibody, varying concentrations of antibody were added to each well of a 96 well-immunoplate coated with SB–HSA (1 μg ml^{-1}).

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 the MAbs were examined by the competitive ELISA and calculated by the method reported by Weiler and Zenk.[30] Table 1 indicates the cross-reactivities of anti-SB MAbs against related anthraquinones and phenolics. MAb 7H12 has weak cross-reactivity with sennoside A (SA) (2.45%) and rhein (0.012%). However, the other related anthraquinones did not cross-react. From these results it is suggested that the epitope consists of a basal structure of rhein and a 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. Moreover, MAb 7H12 does not react with other anthraquinone and phenolic compounds as indicated in Table 1. 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%),[23] these two MAbs make it possible to investigate stereochemical recognition precisely.

Recovery of SB by ELISA using MAb 7H12

The recovery was calculated from samples containing added SB in the same concentration range as endogenous SB together with variations in replicates (Table 2). Table 2 indicates excellent recovery (96–104%) between approximately 180 to

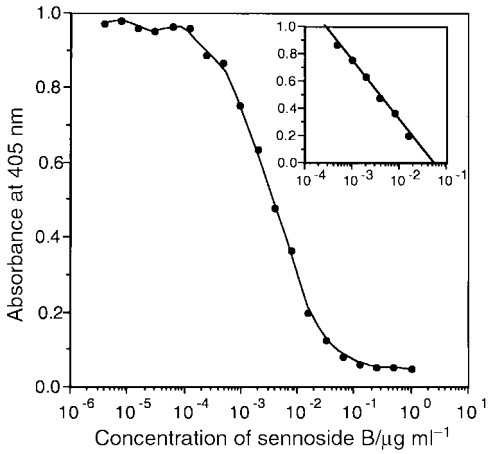
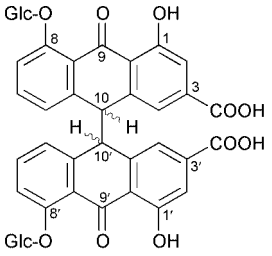


Fig. 3 Calibration curve of sennoside B. Various concentrations of sennoside B were incubated with MAb precoated with sennoside B–HSA (1 μg ml^{-1}). After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm.



Sennoside	10-10′	Formula	M.W.
B	erythro	$C_{42}H_{38}O_{20}$	862
A	threo	$C_{42}H_{38}O_{20}$	862

Fig. 4 Structures of sennoside B and A. Sennoside B possessed an *erythro*-configuration between C-10 and C-10′.

270 μg and lower variations (3.7–4.9 %RSD). From these results, it is evident that the ELISA using anti-SB MAb can be routinely used for the phytochemical investigations involving crude plant extracts.

Table 1 Cross-reactivities of anti-sennoside B MAbs against various compounds[a]

	MAb		
Compound	7H12	5G6	5C7
Anthraquinone			
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	< 0.004	< 0.023	< 0.006
Barbaloin	< 0.004	< 0.023	< 0.006
1,4-Dihydroxyanthraquinone	< 0.004	< 0.023	< 0.006
Stilbene			
Rhaponticin	< 0.004	< 0.023	< 0.006
Phenolic acids			
Gallic acid	< 0.006	< 0.022	< 0.009
Vanillic acid	< 0.006	< 0.022	< 0.009
Caffeic acid	< 0.006	< 0.022	< 0.009
Homogentisic acid	< 0.006	< 0.022	< 0.009
Coumarin			
Esculin	< 0.006	< 0.022	< 0.009
Tannins			
Cinnamtannin B_1	< 0.006	< 0.022	< 0.009
Flavonoids			
Baicalin	< 0.006	< 0.022	< 0.009
Naringin hydrate	< 0.006	< 0.022	< 0.009
Wogonine	< 0.006	< 0.022	< 0.009
Wogonine 7-*o*-β-glucuronide	< 0.006	< 0.022	< 0.009
Curcuminoid			
Curcumin	< 0.006	< 0.022	< 0.009
Cannabinoid			
Δ^1-Tetrahydrocannabinolic acid	< 0.006	< 0.022	< 0.009
Δ^1-Tetrahydrocannabinol	< 0.006	< 0.022	< 0.009

[a] The cross-reactivities of various compounds were determined according to Weiler's equation.[30]

Table 2 Recovery experiment for sennoside B

Added amount of SB/μg	Measured amount of SB/μg[a]	%RSD[b]	Recovery (%)[c]
0	178.2 ± 7.1	[4.0]	—
25	204.2 ± 7.6	[3.7]	104.1
50	228.7 ± 10.5	[4.6]	101.0
100	274.1 ± 13.3	[4.9]	95.9

[a] Dried powder of rhubarb (30 mg) was used. [b] Relative Standard Deviation. [c] Recovery (%) = (measured amount −178.2)/(added amount) × 100.

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

The calibration curve for SB was obtained over the concentration ranges 0.5–15 ng ml^{-1} by the ELISA. This assay was approximately 10^4 times sensitive than that of the HPLC method as reported previously.[31] In order to determine the correlation between ELISA and HPLC results, we performed SB determinations in various rhubarb roots using HPLC. Fig. 5 shows the correlation of SB concentration in the rhubarb root determined by HPLC and ELISA for which $R^2 = 0.987$. We conclude that the ELISA can be used for the determination of SB concentrations without any pre-treatment of samples.

Determination of concentration of SA and SB in various rhubarb samples

The concentrations of SA and SB in various rhubarb samples were determined by ELISA (Table 3). Shinshu Daio, bred by crossing *R. palmatum* with *R. coreanum* in order to increase the concentration of SB and SA in Japan, contained the highest SB level of 6.01 ± 0.18 μg mg^{-1} dry wt. Ga-wo, estimated to be high grade in traditional Chinese medicine, contained SB at 3.14 ± 0.27 μg mg^{-1} dry wt. These results are in good agreement with previous reports.[32] Interestingly, the concentration of SA in rhubarb is approximately 2.1–2.4 times higher than that of SB (except for one sample: Kinmon Daio), in good agreement with a previous report.[32] The correlation between results from ELISA and HPLC is good.

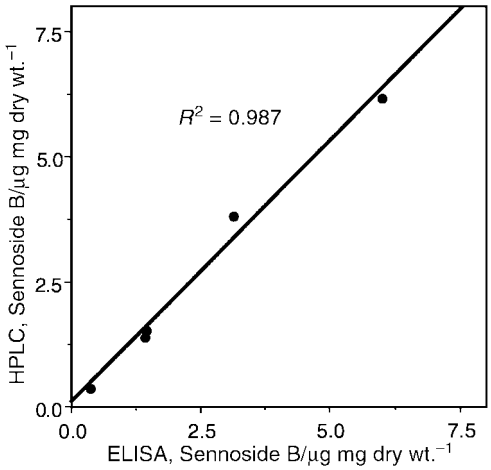


Fig. 5 Correlation curve of results from ELISA and HPLC. The correlation coefficient was calculated from fitting a straight line to results from ELISA and HPLC methods.

Table 3 Total sennosides contents in various rhubarb samples

	Content/μg mg^{-1} dry wt. powder									
	Sennoside B				Sennoside A					
Sample	ELISA	%RSD[a]	HPLC	%RSD	ELISA	%RSD	HPLC	%RSD	Total sennosides	%RSD
Itto-Ga-wo (refuse)	1.42 ± 0.07	[4.93]	1.40 ± 0.11	[7.86]	3.43 ± 0.16	[4.66]	3.69 ± 0.28	[7.59]	4.85 ± 0.23	[4.74]
Itto-Ga-wo (powder)	1.44 ± 0.12	[8.33]	1.52 ± 0.18	[11.84]	3.27 ± 0.20	[6.12]	3.69 ± 0.32	[8.67]	4.71 ± 0.32	[6.79]
Kinmon Daio	0.35 ± 0.01	[2.86]	0.38 ± 0.02	[5.26]	3.34 ± 0.02	[0.60]	0.85 ± 0.04	[4.71]	3.69 ± 0.03	[0.81]
Ga-wo	3.14 ± 0.27	[8.60]	3.80 ± 0.16	[4.21]	6.62 ± 0.42	[6.34]	6.93 ± 0.02	[0.29]	9.76 ± 0.69	[7.07]
Shinshu Daio	6.01 ± 0.18	[3.00]	6.15 ± 0.59	[9.60]	13.69 ± 0.69	[5.04]	12.28 ± 0.41	[3.34]	19.70 ± 0.87	[4.42]

[a] Coefficient of variation Relative Standard Deviation.

Determination of total sennosides in *Rheum* and *Senna* species

The total sennoside content is important for the purgative effect.[33] Therefore we determined the total sennosides (SA + SB) in *Rheum* root and *Senna* leaf (*Cassia angustifolia*, listed in Japanese Pharmacopeia) by ELISA. Shinshu Daio contained the highest total sennosides of 19.70 ± 0.87 μg mg^{-1} dry wt. (Table 3). Ga-wo (see Table 3) and *C. angustifolia* contained around 9.7 μg mg^{-1} dry wt. of total sennosides and these two species were estimated to be higher grade than the other species.

Analysis of interconversion of SA and SB

Interconversion of SA to SB treated under a mild alkaline condition was reported previously.[34] We observed that SA rapidly changes into SB by UV irradiation. In order to clarify this point we irradiated SA with UV light (254 nm) for 2 h resulting in 1.24% SB production. This phenomenon was also observed when SA in MeOH solution was stored at room temperature for several weeks. SB also changed to SA by UV irradiation, but at a lower conversion of 0.39%. These results are understandable because the *threo* configuration changes to the *erythro* (which is of a higher energy state) following UV irradiation. This application of ELISA is possible because of its specificity and high sensitivity.

In conclusion, the limit of detection of the ELISA for SB was determined to be 100 pg ml^{-1}. The ELISA was more sensitive than TLC[2] or HPLC[32] methods. This ELISA methodology can be utilized to study large numbers of plant samples, and to analyze small samples obtained in breeding studies of *Rheum* and *Senna* species. In addition, it appears likely that the ELISA could be used in pharmacokinetic studies. The method developed is straightforward and convenient and requires no expensive equipment.

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