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Enzyme-linked immunosorbent assay for total sennosides using anti-sennside A and anti-sennoside B monoclonal antibodies

Osamu Morinaga [a], Takuhiro Uto [a], Seiichi Sakamoto [b], Hiroyuki Tanaka [b], Yukihiro Shoyama [a,*]

[a] Department of Pharmacognosy, Faculty of Pharmaceutical Science, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki 859-3298, Japan
[b] Department of Medicinal Plant Breeding, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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ABSTRACT

Total sennosides concentration is a very important factor when rhubarb and senna will be used as crude drugs. However, one-step analytical technique for total sennosides has not been reported except HPLC. An enzyme-linked immunosorbent assay (ELISA) for total sennosides concentration by using the combination of anti-sennoside A (SA) and anti-sennoside B (SB) monoclonal antibodies (MAbs) in a single assay has been investigated. Total sennosides concentration in rhubarb and senna samples determined by newly developed assay system showed good agreement with those analyzed by ELISA using anti-SA MAb and anti-SB MAb, respectively.

1. 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)] [1,2]. It is well known that the total concentrations of SA and SB reflect the purgative activity, related to the metabolic conversion of SA and SB into rheinanthrone by intestinal bacteria as shown in Fig. 1 [3]. Despite the availability of a number of synthetic purgatives, sennosides-containing medications are still widely used, and these compounds are considered as some of the most important pharmaceutical compounds of plant origin. Therefore, methods for the detection of the total sennosides concentration are discussed in most Pharmacopoeias [4].

Immunoassay systems using monoclonal antibodies (MAbs) against biologically active compounds of low molecular weight have become important tools for qualitative and quantitative analyses of active constituents from natural medicines and plants [5]. In our ongoing study to prepare MAbs for naturally occurring bioactive compounds, we prepared MAbs against SA [6] and SB [7] and applied to an enzyme-linked immunosorbent assay (ELISA) for SA and SB. However, one-step analytical system of total sennosides concentration has not been reported except HPLC. In this paper, we report a new approach for one-step analysis of total sennosides concentration in rhubarb and senna samples by using the combination of anti-SA and anti-SB MAbs in a single ELISA.

2. Experimental

2.1. Reagents and plant materials

SA and SB were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). 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. Rhubarb roots were purchased from Tochimototenkaido Corporation (Osaka, Japan). Senna leaves were collected from Thailand.

2.2. Sample preparation

Dried samples (30 mg) of various kinds of rhubarb and senna were powdered, extracted five times with 0.5 ml

* Corresponding author. Tel.: +81 956 20 5653; fax: +81 956 20 5622.
E-mail address: shoyama@niu.ac.jp (Y. Shoyama).

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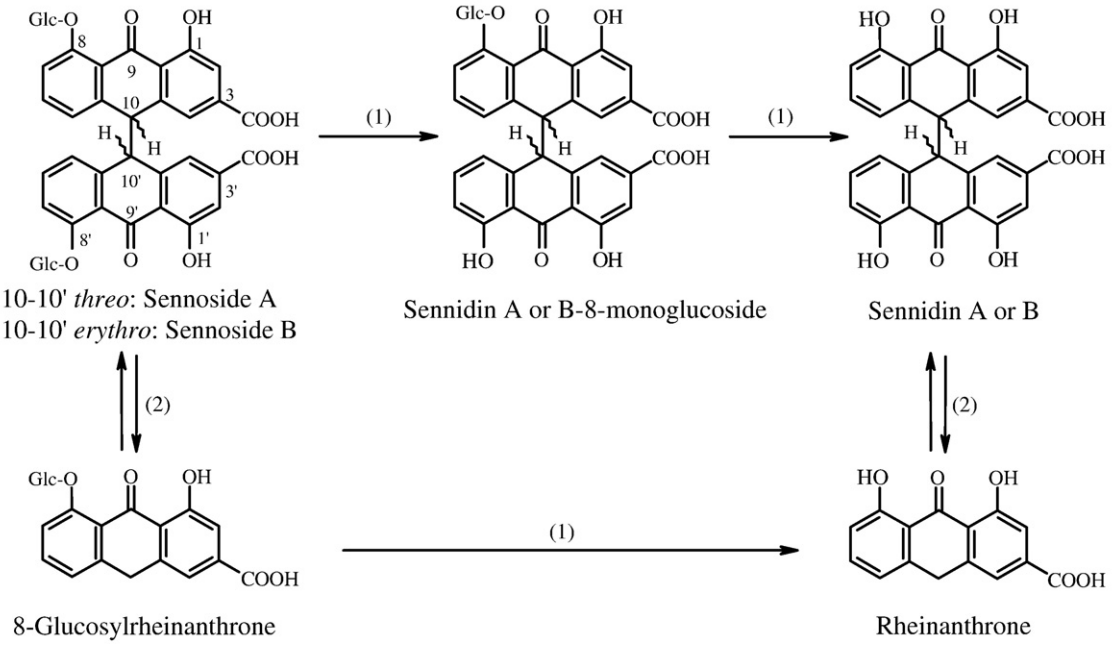

Fig. 1. Metabolic pathways of sennosides by enzymes of intestinal bacteria. (1): β-Glucosidase. (2): NADH-dependent flavin reductase.

methanol (MeOH) containing 0.1% NH_4OH with sonication, and filtered using a Cosmonice filter W (0.45 μm Filter unit, Nacalai Tesque, Kyoto, Japan). The combined extracts were diluted with 10 mM $NaHCO_3$ for the ELISA analysis.

2.3. Preparation of MAbs against SA and SB

Immunization and hybridization of MAbs against SA and SB have been described previously [6,7]. 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 washed with 20 mM phosphate buffer (pH 7.0). Adsorbed IgG was eluted with 100 mM citrate buffer (pH 2.7), neutralized with 1 M Tris–HCl buffer (pH 9.0), dialysed four times against water and lyophilized.

2.4. ELISA for total sennosides concentration by using the combination of anti-SA and anti-SB MAbs

Mixture of SA–HSA and SB–HSA (average 5 molecules of SA/molecule of HSA, average 4 molecules of SB/molecule of HSA) (100 μl. 1 μg/ml each in 50 mM carbonate buffer) were adsorbed in the wells of a 96-well immunoplate (NUNC, Roskilde, Denmark). The plate was treated with 300 μl of phosphate-buffered solution (0.15 M NaCl in 10 mM potassium phosphate, pH 7.4, PBS) containing 5% skimmed-milk (S-PBS) for 1 h to reduce nonspecific adsorption, washed three times with PBS containing 0.05% Tween 20 (T-PBS). A 50 μl volume of various concentrations of sennosides (ratio of sennosides; SA:SB=1:1) and samples dissolved in 10 mM $NaHCO_3$ solution was incubated with 50 μl of anti-SA and anti-SB MAbs mixture solution (1.51 μg/ml IgG solution) for 1 h. The plate was washed three times with T-PBS, and then the MAbs were combined with 100 μl of a 1:1000 dilution of peroxidase-labeled anti-mouse IgG for 1 h. After washing the plate three times with T-PBS, 100 μl of substrate solution, 100 mM citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg/ml of 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was added to each well and incubated for 15 min. The absorbance was measured with a micro-plate reader (Immuno Mini NJ-2300, Nalge Nunc, Roskilde, Denmark) at 405 nm. All reactions were carried out at 37 °C.

3. Results and discussion

SA and SB are unique bisanthraquinones having double carboxylic acid-, hydroxyl-, carbonyl- and *O*-glucosyl-groups at the C-3, C-1, C-9 and C-8 positions respectively and possessing *threo*- and *erythro*-configurations between C-10 and C-10′ positions (Fig. 1). Since cross-reactivity is the most important factor for determining the value of an antibody, a specific assay was performed in order to determine the cross-reactivity of MAbs with various related compounds. As indicated in Table 1, both MAbs had weak cross-reactivity with rhein (0.35% and 0.012%, respectively), but not cross-reacted with other related anthraquinones. These results suggest that the epitope consists of a basal structure of rhein dimer and sugar moieties. In addition, the antibodies could distinguish between SA and SB, which differ only in the stereochemical configuration at the C-10 and C-10′ positions. It means that hydrogen groups at the C-10 and C-10′ positions are immunized individually. Therefore, ELISA using anti-SA MAb and anti-SB MAb should possess the sensitivity and specificity necessary for the detection of SA and SB, respectively.

Table 1
Cross-reactivities (%) of anti-SA and anti-SB MAbs 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
Phenol carboxylic 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

In a new approach, we demonstrated the combination of both MAbs in a single ELISA for the determination of total sennosides concentration as a one-step analysis. Fig. 2A shows individual standard curves of sennosides (ratio of sennosides; SA:SB=1:1) using anti-SA MAb (0.523 μg/ml) and anti-SB MAb (0.985 μg/ml), respectively, preparing in a 96-well immunoplate precoated with mixture of SA–HSA and SB–HSA (1 μg/ml each). Although the assay sensitivities and specificities of both MAbs were different, both full measuring ranges were almost same from 0.0005 μg/ml to 0.2 μg/ml of sennosides. Therefore, we decided on both concentrations of MAbs depending on the activities of anti-SA MAb and anti-SB MAb, respectively.

Next we prepared the standard curve of sennosides (ratio of sennosides; SA:SB=1:1) using the mixture of both anti-SA and anti-SB MAbs (1.51 μg/ml) in a 96-well immunoplate precoated with the mixture of SA–HSA and SB–HSA (1 μg/ml each). Under these conditions, the full measuring range of the assay extended from 0.005 μg/ml to 0.15 μg/ml of sennosides as indicated in Fig. 2B. Moreover, the composition of the mixture of sennosides may have any influence on this assay system, because both antibodies obviously have a different affinity to their respective analyte. Previously we determined the total sennosides concentration in various rhubarb and senna plants by competitive ELISA using anti-SA MAb and anti-SB MAb individually and the average of total sennosides concentration in extract solution was approximately 300 μg/ml (5 μg/mg dry weight powder) [6,7]. Therefore we determined the sennosides concentration at the various ratios of sennosides (SA:SB) using by ELISA with combination of anti-SA MAb and anti-SB MAb in a single assay (Table 2). These results were reasonable between 7:3 and 3:7 of sennosides (see Table 2) and this methodology can be utilized to analyze rhubarb and senna samples because the concentration of SA in rhubarb is approximately 2 times higher than that of SB and the ratio of sennosides in senna is almost same as previously reported [6,7].

Total sennosides concentration is a very important when rhubarb and senna will be used as crude drug. Furthermore, variations in quality have occurred depending on the harvest or collection season and the method of processing. Therefore, the total concentration of sennosides which are major pharmacologically active compounds is appropriate for the

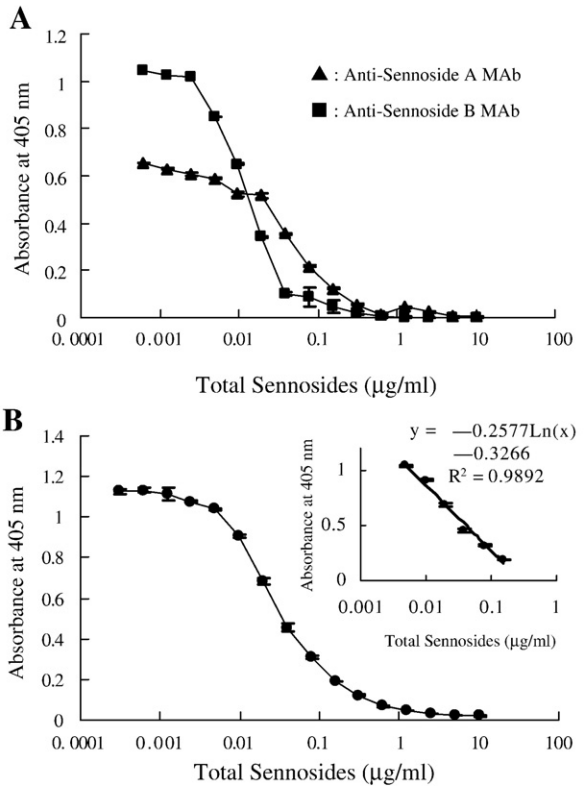

Fig. 2. Standard curves of inhibition by sennosides using anti-SA MAb and anti-SB MAb: (A) Various concentrations of sennosides (ratio of sennosides; SA:SB=1:1) were incubated with anti-SA MAb (0.523 μg/ml) and anti-SB MAb (0.985 μg/ml), respectively in a 96-well immunoplate precoated with mixture of SA–HSA and SB–HSA (1 μg/ml each). (B) Various concentrations of sennosides (ratio of sennosides; SA:SB=1:1) were incubated with mixture of anti-SA and anti-SB MAbs (1.51 μg/ml) in a 96-well immunoplate precoated with mixture of SA–HSA and SB–HSA (1 μg/ml each).

Table 2
Analysis of sennosides at various ratios using ELISA system employing the combination of anti-SA and anti-SB MAbs

Ratio of sennosides (SA:SB)	Concentration of sennosides (μg/ml)	%RSD [a]	Recovery (%) [b]
10:0	–	–	–
9:1	147.7±4.9	[3.3]	49.2
8:2	227.3±6.6	[2.9]	75.8
7:3	291.2±2.1	[0.7]	97.1
6:4	292.6±7.4	[2.5]	97.5
5:5	297.4±8.4	[2.8]	99.1
4:6	326.9±7.7	[2.4]	109.0
3:7	304.2±7.5	[2.5]	101.4
2:8	367.5±17.4	[4.7]	122.5
1:9	281.1±12.4	[4.4]	93.7
0:10	150.0±12.2	[8.1]	50.0

Data are the means of triplicate assay±SD.
[a] Coefficient of variation Relative Standard Deviation.
[b] Recovery (%)=actual measurement (μg/ml)/300 (μg/ml)×100.

Table 3
Total sennosides concentration in various rhubarb and senna samples determined by individual ELISA and combination ELISA using anti-SA and anti-SB MAbs

Sample	Concentration (μg/mg dry weight powder)			
	Individual ELISA	%RSD[a]	Combination ELISA	%RSD
Shinshu Daio	18.43±1.00	[5.43]	18.37±1.24	[6.75]
Ga-wo (A)	5.21±0.50	[9.60]	4.59±0.55	[12.0]
Ga-wo (B)	5.09±0.39	[7.66]	4.58±0.20	[4.37]
Cassia angustifolia	13.05±1.10	[8.43]	12.10±0.34	[2.81]
C. alata	2.35±0.27	[11.5]	2.33±0.13	[5.58]
C. bakeriana	0.84±0.05	[5.95]	0.88±0.05	[5.68]
C. fistula (A)	1.89±0.16	[8.47]	1.81±0.08	[4.42]
C. fistula (B)	3.56±0.44	[12.4]	3.45±0.16	[4.64]
C. fistula (C)	3.95±0.40	[10.1]	4.45±0.27	[6.07]
C. fistula (D)	0.23±0.01	[4.35]	0.27±0.02	[7.40]

Data are the means of triplicate assay±SD.
[a] Coefficient of variation Relative Standard Deviation.

quality control of rhubarb and senna and their extracts in the market. Two *Cassia* species, *C. angustifolia* and *C. acutifolia* which are listed in Japanese Pharmacopoeia satisfy that the total concentration of SA and SB is 1% dry weight or more as shown in Table 3. In the case of rhubarb, the concentration of SA is only ruled out as 0.25% dry weight or more. The total concentration of SA and SB in Shinshu Daio, bred by crossing *R. palmatum* with *R. coreanum* in order to increase the concentration of SA and SB in Japan, is 1.84% indicated in Table 3 suggesting that the SB concentration might be 0.61% since we determined SA is 1.23% by the usual ELISA method as indicated previously [6]. Furthermore, total sennosides concentration in rhubarb and senna samples determined by newly established method agreed well with those determined by ELISA using anti-SA MAb and anti-SB MAb respectively as indicated in Table 3 and these values also showed good agreement with those analyzed by HPLC method [6,7]. Moreover, coefficients of variation (CV) were determined by the ratios of standard deviations (SD) as indicated in Table 3 (shown as %RSD) and almost all of CVs were below 10%, suggesting that this assay system is useful for the analysis of total sennosides in rhubarb and senna plant extracts.

4. Conclusions

Many analytical techniques have been investigated for the determination of anthraquinones including SA and SB in plant materials and marketed formulations. Recently, HPLC and capillary electrophoresis (CE) are frequently and widely used [8–10]. However, these methods need pre-treatments (e.g., partition, purification and concentration) before the sample solution is injected into the analytical equipment. The advantages of the newly developed ELISA system employing the combination of anti-SA and anti-SB MAbs in a single assay over traditional analytical methods are speed, ease of use, sensitivity, analysis without pre-treatments and more environmentally soft-determination method (e.g., organic solvents as mobile phase and waste fluids) for screening of total sennosides concentration and for the quality control of drug production. Regarding sensitivity this ELISA was at least 2×10^3 times more sensitive than the HPLC method under our conditions. In our conclusions, the combination assay system of anti-SA and anti-SB MAbs by competitive ELISA represents a useful methodology for the qualitative and quantitative analysis of total sennosides concentration in biochemical and pharmaceutical sciences.

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