Pharmacologically Active Saikosaponin in *Bupleurum falcatum* Detected by Competitive ELISA and Eastern Blotting Using Monoclonal Antibodies

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Abstract: This study showed the effective microanalytic methods for detection of saikosaponin by a competitive enzyme-linked immunosorbent assay (ELISA) using anti-saikosaponin a (SSa) monoclonal antibody (MAb). The results showed that the competitive ELISA had higher sensitivity than high performance liquid chromatography (HPLC) for the detection of saikosaponin. ELISA showed a highly significant difference in SSa concentrations between the roots of *Bupleurum falcatum* with different origins. In addition, a difference in SSa concentrations was observed between cytologically different cultivars originating from Japan (2n = 26) and Korea (2n = 20). It also showed excellent performance in quantitative analysis of SSa in the small samples from regenerated plantlets and calli. In addition, Eastern blotting using anti-SSa MAb 3G10 multispecific to saikosaponins was applied to analyze the distribution of saikosaponins in *Bupleurum falcatum* roots and leaves. The localized distribution of saikosaponins in whole leaves and cork layer of the roots in *B. falcatum* was successfully demonstrated by this novel immunoassay.

Key words: *Bupleurum falcatum*, Eastern blotting, ELISA, Monoclonal antibody, Saikosaponin, Umbelliferae.

*Bupleurum falcatum* L. (*Umbelliferae*) is a perennial herb indigenous to China, Korea and Japan. Its roots are considered one of the most important traditional Chinese medicines, and are used in pain-killer, anti-allergy, anti-inflammatory, anti-ulcer, and anti-pyretic medications (Yamamoto et al., 1975a,b; Kimata et al., 1982). The quality of *B. falcatum* is evaluated by analysis of ash, extract, essential oil, saikosaponin content, etc. (Korea Food and Drug Administration, 2007). Saikosaponin is believed to be the major component of *B. falcatum* (Park et al., 1992), and its pharmacologically active ingredients are saikosaponin a, c and d (Shibata et al., 1973; Yamada et al., 1991; Sakurai et al., 1998).

Saikosaponin production and growth characteristics of *B. falcatum* vary with the environmental and genetic conditions and are important factors in breeding (Shon et al., 1997a,b; Shon and Yoshida, 1997). The traditional method for selecting *B. falcatum* yielding a higher amount of secondary metabolites is time-consuming, laborious and cost-inefficient. A large amount of sample is needed for the quantitative analysis of saikosaponin content using HPLC (Shon et al., 1997a).

Enzyme assays are gaining popularity in plant breeding studies. It is highly sensitive, simple and reproducible assay systems for quantitative analysis using small amount of samples (Shoyama et al., 1992, 1995, 1998; Zhu et al., 2006). Competitive ELISA using two kinds of monoclonal antibodies (MAbs), i.e. monospecific to saikosaponin a (SSa) and multispecific to saikosaponins, have also been developed and their reliability and accuracy evaluated for the quality control of *Bupleuri radix* (Zhu et al., 2004, 2006). Recently, competitive ELISA and eastern blotting using MAbs have been developed and used in breeding of *Panax ginseng* (Morinaga et al., 2006), *Glycyrrhiza uralensis* (Shan et al., 2001), *Artemisia annua* (Putalun et al., 2007) and *Bacopa monnieri* (Phrompittayarata et al., 2007) at a very young growth stage of these plants. These studies showed that the use of MAbs can save time, labor and cost as compared with the traditional methods.

The objective of this work was to assess and optimize competitive ELISA using anti-SSa MAb in quantitative analysis of SSa in *B. falcatum* from different origins, regenerated plantlets, and plant tissues.
Materials and Methods

1. Chemicals
Saikosaponins a, b1, c and d were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Polyvinylidene difluoride (PVDF) membrane (Immobilon-N) was purchased from Millipore Corporation (Bedford, MA, USA). Glass microfiber filter sheet (GF/A) was purchased from Whatman International Ltd. ( Maidstone, England). All other chemicals were standard commercial products of analytical grade.

2. Preparation of plant material
*B. falcatum*, originating from Japan (2n=26) and Korea (2n=20), were grown in the experimental field. Seeds were obtained from the National Institute of Crop Science, Suwon, Korea, and Research Center for Medical Plant Resources, National Institute of Health Sciences, Japan. The plants were cultivated following the cultivation method of the medicinal plant as described by Shon et al. (1998). Plant roots were sampled in late November and were dried for one week under natural conditions. After drying, it was preserved in a tight container for SSA analysis by ELISA and HPLC. Dried samples (20 mg) of various *B. falcatum* roots were powdered, extracted with methanol (MeOH) (0.5 mL) under sonication for 5 times, filtered, and then evaporated. For the elimination of acyl group from acylated saikosaponins, the extracts were treated with 5% of KOH in MeOH at room temperature for 2 hr as previously reported (Kitagawa et al., 1989), then neutralized with 1 M HCl in MeOH, and assayed by ELISA. For the immunohistochemical analysis of fresh leaves, leaves on the third node from the youngest leaf bud were used. Fresh and dried root samples were also subjected to immunohistochemical analysis. To conduct SSA analysis on regenerated plantlets in Tsukuba strain, we prepared regenerated plantlets and calli from anther cultures following the method of Shon et al. (1998). Plant roots in Tsukuba strain, we prepared regenerated plantlets in Tsukuba strain. For the determination of SSA in plantlets and calli from anther cultures following the cultivation method of the medicinal plant as described by Shon et al. (1998). Plant roots were sampled in late November and were dried for one week under natural conditions. After drying, it was preserved in a tight container for SSA analysis by ELISA and HPLC. Dried samples (20 mg) of various *B. falcatum* roots were powdered, extracted with methanol (MeOH) (0.5 mL) under sonication for 5 times, filtered, and then evaporated. For the elimination of acyl group from acylated saikosaponins, the extracts were treated with 5% of KOH in MeOH at room temperature for 2 hr as previously reported (Kitagawa et al., 1989), then neutralized with 1 M HCl in MeOH, and assayed by ELISA. For the immunohistochemical analysis of fresh leaves, leaves on the third node from the youngest leaf bud were used. Fresh and dried root samples were also subjected to immunohistochemical analysis. To conduct SSA analysis on regenerated plantlets in Tsukuba strain, we prepared regenerated plantlets and calli from anther cultures following the method of Shon et al. (2004) and Shon and Yoshida (1997). Roots were used for the SSA analysis in the regenerated plantlets. For the determination of SSA in the callus, we prepared successively subcultured calli from a single anther.

3. Direct ELISA using SSA-HSA
The reactivity of the MAbs to SSA-HSA was determined by direct ELISA method. SSA-HSA dissolved in 50 mM sodium carbonate buffer (pH 9.6) (1 μg mL−1; 100 μL) was adsorbed to the wells of a 96-well immunoplate (MaxiSorp™ Surface, Nalgene NUNC Roskilde, Denmark) and then treated with 300 μL of phosphate buffered saline (PBS) containing 5% skim milk (SPBS) for 1 hr to reduce non-specific adsorption. The plate was washed three times with PBS containing 0.05% Tween 20 (TPBS), and then incubated with 100 μL of various concentrations of anti-SSA MAb. After washing the plate three times with TPBS, 1000-time-diluted peroxidase (POD)-labeled anti-mouse IgG (ICN Biomedicals, Inc., Aurora, OH, USA) was added to each well and allowed to react for 1 hr. After washing the plate three times with TPBS, 100 μL of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.003% H2O2 and 0.3 mg mL−1 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Wako Pure Chemical Ind., Ltd., Osaka, Japan) was added to each well and incubated for 10 min. Absorbance at 405 nm was measured with a micro plate reader (MODEL 450 Microplate Reader BIO-RAD Laboratories, CA, USA). The plates were kept at 37˚C for the reactions.

4. Competitive ELISA
A 96-well immunoplate after adsorption of 100 μL of 1 μg mL−1 SSA-HSA was treated with 300 μL of SPBS for 1 hr to reduce non-specific adsorption. Fifty microliter of SSA at various concentrations or samples dissolved in 10% MeOH was separately incubated with 50 μL of anti-SSA MAb solution for 1 hr. The plate was washed three times with TPBS, and then the anti-SSA MAb was combined with 100 μL of a 1000-time diluted POD-labeled anti-mouse IgG for 1 hr. After washing the plate three times with TPBS, 100 μL of ABTS solution was added to each well and incubated for 10 min. The absorbance was measured with the micro plate reader at 405 nm. All reactions were carried out at 37˚C. The cross-reactivities of anti-SSA MAb against SSA and related compounds were determined according to Weiler and Zenk’s equation (Weiler and Zenk, 1976).

5. Quantitative analysis of saikosaponins by HPLC
Standard solutions of SSA (400, 200, 100, 50 and 25 μg mL−1) in MeOH were freshly prepared and assayed by HPLC. After alkaline treatment of the extract of *B. falcatum*, the SSA concentration in each sample was determined by HPLC following the modified method of Kimata et al. (1979). HPLC was performed using a Model LC-10AD (Shimadzu Co., Ltd., Kyoto, Japan) pump connected with a TSK-gel ODS-120A (4.6 I.D.×250 mm. Tosoh Co., Ltd., Tokyo, Japan), and equipped with a UV-8 Model II Spectrophotometer (Tosoh Co., Ltd.) set at 203 nm. The mobile phase was 50 mM phosphate buffer containing 44% of CH3CN.

6. Eastern blotting using anti-SSA MAb 3G10
Fresh roots and leaves of *B. falcatum* were placed on the PVDF membrane, and were pressed together evenly for 6 hr. After drying, the PVDF membrane was treated with NaOH solution (10 mg mL−1) for 1 hr. Then, the PVDF membrane was dipped in 1% BSA-50 mM carbonate buffer (pH 9.6) and incubated overnight. After washing with PBS twice for 5 min, the PVDF membrane was treated with SPBS for 3 hr to
reduce non-specific adsorption. The PVDF membrane was then immersed in anti-SSa MAb 3G10 dissolved in PBS containing 0.2% gelatin (GPBS) (IgG : 0.46 μg mL⁻¹) and stirred for 2 hr at room temperature. After washing with TPBS twice for 5 min, the membrane was dipped into anti-SSa MAb 3G10 dissolved in GPBS, and stirred at room temperature for 1 hr. The PVDF membrane was again washed twice with TPBS. Finally, the membrane was exposed to a freshly prepared 4-chloro-1-naphthol (1 mg mL⁻¹)-0.03% H₂O₂ in PBS for 10 min at room temperature for color development.

Results and Discussion

1. Quantitative analysis of SSa in root tissues of B. falcatum with different origins by competitive ELISA using anti-SSa MAb 1G6

We prepared anti-SSa MAb 1G6 having higher specificity against SSa, and established competitive ELISA for the determination of SSa concentration in Bupleuri radix and traditional Chinese medicines (Zhu et al., 2004). Fig. 1 shows the correlation of the SSa concentrations in B. falcatum determined by ELISA and HPLC. The coefficient of determination between the SSa concentration determined by the two methods in 30 individual samples was 0.991. In addition, their deviations from the standard curve were within the limits of experimental error. Furthermore, the calibration curve of SSa using anti-SSa MAb 1G6 showed that the measurable range by the assay covers from 26 ng mL⁻¹ to 1.5 μg mL⁻¹. Since the range measurable by HPLC in our laboratory was 25 to 400 μg mL⁻¹, the ELISA is about 1000 times more sensitive than HPLC. In the present study, SSa concentrations in the cultivated B. falcatum with different origins determined by the competitive ELISA were significantly correlated with those determined by HPLC (Table 1). Therefore, ELISA can be used for quantitative analysis of SSa.

The SSa concentration in two types of B. falcatum samples, Korean (2n =20) and Japanese species (2n =26) having cytological differences, was also analyzed by HPLC and competitive ELISA. The concentration of SSa in the Japanese and Korean species obtained by HPLC was 6.32–7.85 and 10.47 mg g⁻¹, respectively, and that determined by competitive ELISA was 4.58–4.80 and 6.66 mg g⁻¹ respectively, suggesting that the SSa concentration varies with the origin of B. falcatum (Table 1). The result confirmed that the ELISA test is more sensitive in quantitative analysis of saikosaponin content in the roots of B. falcatum than HPLC. Furthermore, both analyses showed a higher concentration of SSa in the Jeongsun strain.

Shon et al. (1998) and Minami et al. (1995) found that the clear polymorphism of B. falcatum in both morphological property and concentration of SSa depended on different habitats, but the concentrations of SSc and d were within the standard deviation. Genetic variation was also investigated in this species. Mizukami et al. (1993) found several types of cultivars in B. falcatum species in Japan by DNA fingerprints. However, the relation between genetic variation and saikosaponin concentration is still unclear. The major problem for the breeding of B. falcatum yielding high concentration of saikosaponins is the requirement of

![Graph](image_url)

**Table 1.** The concentration of saikosaponin α in B. falcatum with different origins determined by ELISA using anti-SSa MAb 1G6 and HPLC methods.

| Origin          | Concentration | ELISA (mg g⁻¹ dwt.) | HPLC (mg g⁻¹ dwt.) |
|-----------------|---------------|---------------------|--------------------|
| Japan (Kumamoto) |               | 4.58 ± 0.42         | 6.32 ± 0.78        |
| Japan (Mishima) |               | 4.70 ± 0.19         | 6.58 ± 0.96        |
| Japan (Nara)    |               | 4.78 ± 0.14         | 7.85 ± 0.84        |
| Japan (Yasato)  |               | 4.80 ± 0.17         | 7.10 ± 1.02        |
| Korea (Jeongsun)|               | 6.67 ± 0.72         | 10.47 ± 2.3        |
| Correlation*    |               |                     | 0.997              |

*: Correlation between the content of saikosaponin α obtained by ELISA and HPLC. Data are means ± standard deviations of 10 replications.
195

Shon et al. —— Determination of Saikosaponin by Competitive ELISA and Eastern Blotting in *B. falcatum*

large-scale root samples. However, the newly developed competitive ELISA method can be used as a simple and fast method for breeding of plants containing high concentration of saikosaponin using a small amounts of the sample.

2. Determination of saikosaponin concentration in the regenerated plantlets and the calli of *B. falcatum* L. by ELISA method using anti-SSa MAb 1G6 and MAb 3G10

Table 2 shows SSa and saikosaponin concentrations in plants regenerated through anther culture determined by ELISA using anti-SSa MAb 1G6 and 3G10. The SSa and saikosaponin concentrations in the different lines of Tsukuba strain determined by ELISA using anti-SSa MAb 1G6 and 3G10 ranged from 1.3 to 2.7 and 23.8 to 30.9 mg g⁻¹, respectively. The mean values of SSa and saikosaponin concentrations obtained by the ELISA using anti-SSa MAb 1G6 and 3G10 were 2.0 and 28.1 mg g⁻¹, respectively. Considering the specificity of each anti-SSa MAb, the values obtained by ELISA using anti-SSa MAb 1G6 and anti-SSa MAb 3G10 showed concentrations of SSa and total saikosaponins in the samples, respectively. Even in calli, the competitive ELISA method using the two monoclonal antibodies (data not shown) showed clear differences in SSa among individual calli.

These results showed that the ELISA method using the two monoclonal antibodies can enable microanalysis of saikosaponin in various samples using callus culture and the plants regenerated from anther cultures. ELISA offers higher sensitivity than HPLC and gas chromatography (GC) and is important and useful for plant breeding research of *B. falcatum* species.

3. Immunohistochemical analysis of *B. falcatum* by Eastern blotting method

Eastern blotting, which is a novel immunostaining method for detection of low molecular weight compounds, was applied to analysis of saikosaponins in *B. falcatum* using anti-SSa MAb 3G10 multispecific for saikosaponins. Dried and fresh cross sections of roots and vertical sections of leaves of *B. falcatum* L. were stamped onto PVDF membrane directly. After saikosaponins blotted onto the membranes were treated with NaIO₄ and conjugated with BSA, saikosaponins were specifically stained with the general protocol of Eastern blotting (Fig. 2).

Fig. 2 shows strong color development in the tissue and cork of the root suggesting higher concentration of saikosaponins in these parts. On the other hand, no conspicuous color development was observed in phloem and xylem inside the cork. This was similar to the results obtained by Shon et al. (1997a) and Minami et al. (1995), wherein total saikosaponin content was higher in outer tissues than in the inner tissues of the roots. Saikosaponin was also detected in the leaf tissues by immunohistochemical analysis (Fig. 2C). A higher saikosaponin concentration in the fresh leaf of *B. falcatum* was also detected using anti-SSa MAb 3G10.

Analysis by ELISA showed that saikosaponin concentrations in the roots were 3.37 mg g⁻¹ in the cork layer, 0.28 mg g⁻¹ in phloem, and 1.02 mg g⁻¹ in xylem. It has been reported that secondary metabolites accumulated in cork layer or xylem of *Bupleurum falcatum* (Minami et al., 1995). The cork tissue was found to contain higher concentration of SSa than phloem and xylem by using ELISA, indicating a good agreement with the above immunohistochemical analysis. With regard to the distribution of saikosaponins in the main root, saikosaponins accumulated most in the outer tissues of the phloem layer, especially in the pericycle and its neighboring tissues (Minami et al., 1995; Shon et al., 1997a).

**Conclusion**

This study showed that the ELISA method using anti-monoclonal antibodies could quantitatively detect SSa in two *B. falcatum* plants with different

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Table 2. Determination of saikosaponins in the roots of regenerated plantlets of *B. falcatum* by ELISA using anti-SSa MAb 1G6 and anti-SSa MAb 3G10.

| Origin | ELISA using anti-SSa MAb 1G6 | ELISA using anti-SSa MAb 3G10 |
|--------|-----------------------------|-----------------------------|
|        | SSa content (μg g⁻¹ dwt.)   | Total saikosaponin content (μg g⁻¹ dwt.) |
| Tsukuba B* | 1.4 ± 0.13                  | 23.8 ± 0.82                  |
| Tsukuba D  | 1.3 ± 0.09                  | 28.8 ± 0.43                  |
| Tsukuba E  | 1.8 ± 0.13                  | 28.0 ± 1.09                  |
| Tsukuba E  | 2.6 ± 0.15                  | 29.0 ± 0.65                  |
| Tsukuba F  | 2.7 ± 0.14                  | 30.9 ± 0.95                  |
| Mean     | 2.0 ± 0.66                  | 28.1 ± 2.63                  |

*B, D, E, and F are *B. falcatum* Tsukuba varietal lines. Values are means ± S.D. of 3-replicated analysis.
origins having different genotypes (2n = 20, 2n = 26) even SSa and saikosaponins in the calli or young plants regenerated from anther cultures. The results showed that the ELISA test was more sensitive than HPLC, making it possible to conduct screening even at a very young growth stage of the plant. The results further demonstrated that ELISA gave precise results, and its sensitivity was 1000-fold higher than HPLC. Moreover, our study showed the usefulness of Eastern blotting using anti-SSa MAb in quantitative and immunohistochemical analyses for saikosaponins in B. falcatum. Thus, this newly developed ELISA method could be effective in accelerating breeding of B. falcatum saving time, labor and cost.

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