# ELISA for the Determination of Saikosaponin a, an Active Component of Bupleuri Radix

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**In order to quantify saikosaponin a (SSA), one of the major active components of Bupleuri Radix, a competitive and indirect ELISA method was developed.**

**High titer rabbit polyclonal antibodies (pAbs) were raised against a conjugate of SSA and bovine serum albumin, coupled with a periodate oxidation method. SSA competitively inhibited the binding of rabbit anti-SSA pAbs to SSA–ovalbumin on the solid phase, a coated antigen on the well. The quantity of pAbs bound to the well was monitored using a peroxidase-conjugated anti-rabbit IgG as a secondary antibody, and tetramethylbenzidine solution as a substrate. The measuring range extended from 50 pg/ml to 20 ng/ml of SSA, with a detection limit of 40 pg/ml (5.13 pM). Antibodies showed some cross-reactivity with saikosaponin c (12.74%). However, the antibodies showed only slight cross-reactivities with saikosaponin d (0.3%), which differs from SSA only in the stereochemistry of the 16-hydroxyl group, and the artificial saikosaponins, saikosaponin $b_1$ (2.1%) and saikosaponin g (0.53%). The specific and sensitive ELISA is especially suited for determination of SSA in samples when only small quantities of materials can be extracted for analysis.**

**Key words** saikosaponin a; polyclonal antibody; periodate oxidation; Bupleuri Radix; ELISA

Saikosaponin a (SSA) is one of the major active components of Bupleuri Radix (root of *Bupleurum falcatum* L., Umbelliferae), and has been widely prescribed in Oriental medicine. SSA has been shown to have a number of biological actions, which include anti-inflammation,[1] corticosterone secretion,[2] lowering plasma-cholesterol,[3] protection of liver damage caused by D-galactosamine[4] and anti-tumor effects.[5] To assure safety, it is important to monitor the disposition of the active component in the body, however, pharmacokinetic studies of SSA have been hampered by the lack of specific and sensitive methods for its quantification.

Recently, several laboratories have been engaged in developing cell and tissue cultures of *Bupleurum falcatum* L.[6] The manipulation of culture conditions, and the screening of SSA-producing clones from a large number of clones require a simple and sensitive assay technique to measure trace amounts of the compound.

HPLC methods have often been used for the quantitative analysis of SSA.[7] However, they are time consuming and not sensitive enough to detect the SSA in biological fluids and in extremely small samples from cell or tissue cultures. Furthermore, HPLC requires purification of crude extracts and/or derivatization prior to analysis. These factors taken together make HPLC unsuitable for large screening programs.

ELISA is one of the most useful techniques for quantification of plant secondary metabolites in plant materials,[8] as well as in biological fluids.[9] It has many advantages over conventional methods, such as sensitivity, specificity, speed and relative simplicity in handling of the assay. Herein, we report a highly sensitive and specific ELISA, suitable for picogram quantities of SSA. The ELISA method developed in this study should be a helpful tool for pharmacokinetic studies, intact plant and cell culture screening, as well as for routine analysis of SSA.

## Results and Discussion

**Production of Antibodies against SSA** The presence of a $C_{13\beta,28}$-allyl oxide ring and $C_{16}$-$\beta$-hydroxyl function in SSA is a structural characteristic of this compound. These groups should be distal to the point of conjugation, ensuring that they are exposed to the immune system. Therefore, we used the glycone, and not the aglycone moiety of the hapten for cross-linking with a carrier protein.

The conjugate of SSA and bovine serum albumin (BSA) was prepared as described (Chart 1) in Materials and Methods. The possible reaction mechanism shown in Chart 1 is based on the studies of Butler and Chen[10] and Smith.[11] Rabbits that were inoculated with the SSA–BSA conjugate obtained by the periodate oxidation method, provided antibodies suitable for detection of SSA. The antibody titer reached a plateau after the fourth booster injection.

**Assay Sensitivity** After the assay procedure was optimized, the standard curve of SSA was established. When the absorbance for each standard was plotted *versus* its SSA concentration on a linear-log scale, a sigmoidal curve was obtained and plotting logit ($B/Bo$) *versus* log standard SSA concentration yielded a linear response (Fig. 1). The practical measuring range of the competitive ELISA extends from 50 pg/ml—20 ng/ml samples. The sensitivity of the assay, defined as two standard deviations from zero, was 5.13 pM, corresponding to 40 pg/ml.

**Assay Specificity** Antibody specificity was evaluated by cross-reactivity assays using various natural and synthetic SSA-like structures, including ginsenosides. The results are summarized in Table 1. The reactivity towards SSA was taken as 100%. All ginsenosides tested showed no cross reactivities. The antibodies showed a high specificity for the functionality of the $C_{13\beta,28}$-allyl oxide ring and $C_{16}$-$\beta$-hydroxyl of the SSA structure. 6″-

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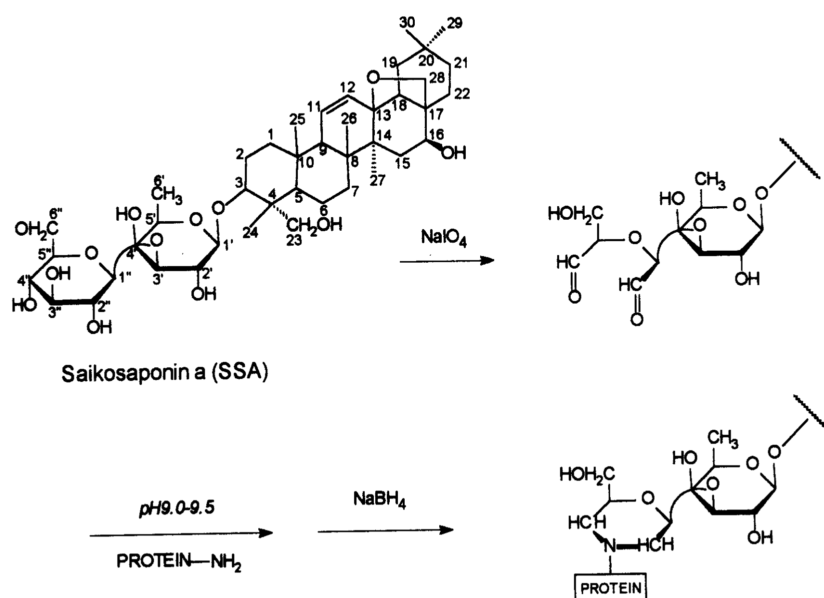


Chart 1. Proposed Mechanism for Conjugation of SSA to Carrier Protein by the Periodate Oxidation Method

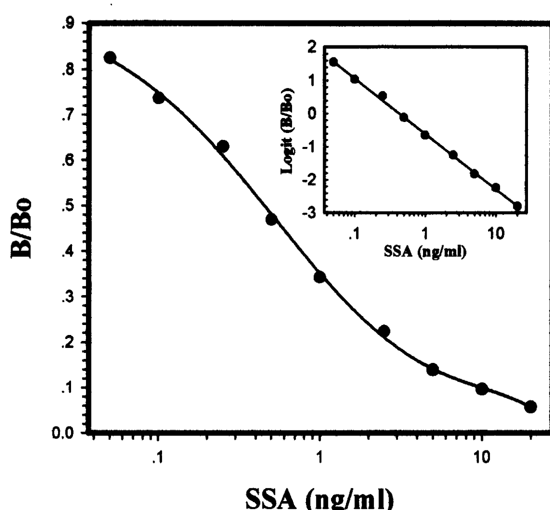


Fig. 1. ELISA Standard Curve of SSA

*B* and *Bo* are the percentages of binding in the presence and absence of SSA respectively. The logit-log plot is obtained from $\ln[(B/Bo)/(1-B/Bo)]$. Coating antigen concentration: 200 ng/ml, rabbit anti-SSA antibody dilution: 1/120000, peroxidase-labeled anti-rabbit IgG dilution: 1/2000

Table 1. Cross-Reactivities of Anti-SSA Antibodies

| Compound | Cross reaction (%)[a] |
|---|---|
| SSA | 100 |
| SSAS-1 | 19.85 |
| Saikosaponin c | 12.74 |
| Saikosaponin $b_1$ | 2.1 |
| 16-keto SSA | 1.3 |
| Prosaikogenin G | 1.3 |
| Saikosaponin g | 0.53 |
| Saikosaponin d | 0.3 |
| Ginsenoside $Rg_1$ | $<0.1$ |
| Ginsenoside Rc | $<0.1$ |
| Ginsenoside $Rb_1$ | $<0.1$ |
| Ginsenoside $Rb_2$ | $<0.1$ |

*a*) Determined as the amount of compound required for 50% inhibition of the binding of the antibodies to the solid-phase antigen, as compared to SSA itself (100%).

Hemisuccinyl SSA (SSAS-1), synthesized from SSA, is not present in methanol (MeOH) extracts of Bupleuri Radix. Therefore, the cross-reacted compound was not likely to cause interference in the assay. The cross-reactivity of the anti-SSA antibody to saikosaponin c (12.74%) was expected because the compound contains both the $C_{16}$-$\beta$-hydroxyl and allyl ether linkage.

However, the polyclonal antibodies showed only slight cross-reactivity with saikosaponin d (0.3%), which differs from SSA only in the stereochemistry of the 16-hydroxyl group, and the synthetic saikosaponin 16-keto SSA. The breakage of the $C_{13\beta,28}$-allyl oxide ring resulted in a loss of immunoreactivity with the SSA antibodies. Thus, saikosaponin $b_1$ (2.1%) showed only minor cross reactivity.

**Assay Variability and Recovery** The accuracy of an assay method and the presence of matrix interferences can be assessed by spiking samples with known concentrations of analyte and comparing recovered amounts with input amounts.[12] Studies with an MeOH extract of the sample, to which known quantities of SSA (5, 1.3, 0.3 ng/ml) was added, showed a mean analytical recovery of 104.9% for SSA. The resulting estimates of SSA concentration suggest that the matrix did not interfere with the ELISA. In comparison with HPLC methods, a preliminary treatment of crude extracts such as solvent partition or derivatization was not required. Therefore, the immunoassay was found to be suitable for the detection of SSA in crude methanolic extracts.

Within-assay coefficients of variation ($n=3$) were 7.0%, 6.0%, and 9.1% at SSA concentrations of 5, 1.3, 0.3 ng/ml, respectively. Between-assay coefficients of variation ($n=3$) were 13.2%, 7.3%, and 6.0% for the low, medium, and high concentrations, respectively.

**Comparison of ELISA and HPLC** The values obtained with the HPLC procedure were compared with those determined for the same samples using the ELISA method. The crude saponin fraction of Bupleuri Radix was

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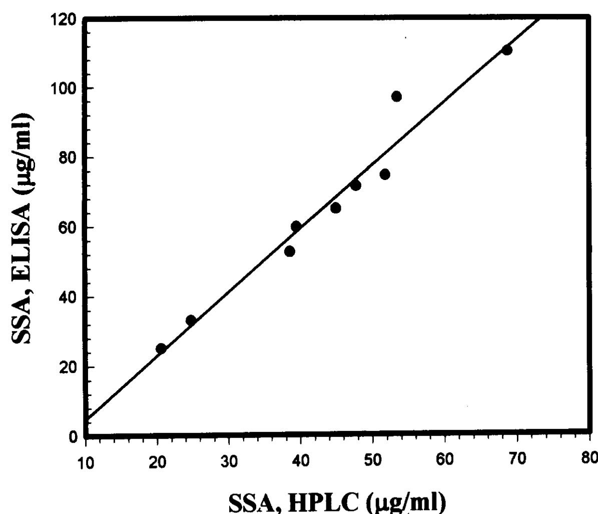


Fig. 2. Correlation of SSA Contents between ELISA and HPLC

analyzed by the two methods. The correlation coefficient ($\gamma$) was calculated from fitting a straight line by least squares. There was good correlation ($\gamma=0.958$) between the assay values obtained by these two methods (Fig. 2).

## Experimental

**Media** Phosphate-buffered saline (PBS, 0.01 M phosphate buffer, pH 7.2, containing 0.154 M NaCl); coating buffer (0.05 M carbonate–hydrogen carbonate, pH 9.6); blocking buffer (C-PBS, PBS containing 0.1% casein); washing buffer (T-PBS, PBS containing 0.05% Tween 20).

**Purification of Saikosaponins** Bupleuri Radix, the root of *Bupleurum falcatum* L. (Umbelliferae), are both commercially available and cultivated in fields and pots, which were used for the present studies. Saikosaponins a, c and d were isolated from the crude drug by the modified method of Ishii *et al.*[13] The isolated saikosaponins were identified by comparison with authentic samples using $^1$H- and $^{13}$C-NMR spectra.

**Chemicals and Immunochemicals** BSA, ovalbumin (OVA), $NaIO_4$, $NaBH_4$, and complete and incomplete forms of Freund's adjuvant were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The tetramethylbenzidine (TMB) substrate solution was from Kirkegaard and Perry Laboratories (Maryland, U.S.A.). Casein was purchased from Merck (Darmstadt, Germany). Peroxidase-labeled anti-rabbit IgG was obtained from Vector Laboratories, Inc. (Burlingham, U.S.A.)

All other chemicals used were of analytical grade from commercial sources.

**Preparation of Antigenic SSA–BSA Conjugate** SSA was coupled to BSA by a modification of the procedure previously used for colchicoside.[14]

To a solution of SSA (390.0 mg, 0.5 mmol) in 80% EtOH (18.75 ml), a solution of $NaIO_4$ (856.0 mg, 4 mmol) in $H_2O$ (11.25 ml) was added dropwise over 40 min at room temperature. Aqueous ethylene glycol (50%, 3 drops) was then added to neutralize excess $NaIO_4$. After 30 min of further stirring, the precipitate was removed by centrifugation. The supernatant was added to a solution of BSA (280.0 mg, 4.2 $\mu$mol) in $H_2O$ (30 ml) dropwise, with stirring at room temperature and the pH was kept at 9.0—9.5 by adding 5% aqueous $K_2CO_3$. After an additional 100 min, Thirty milliliters of a fresh solution of $NaBH_4$ (295 mg, 15.5 eq) in $H_2O$ were added dropwise and the mixture was stirred for 3 h. Subsequently, 1 N HCOOH, pH 6.5, was added to neutralize excess $NaBH_4$ followed by 1 h of stirring. Thereafter, the pH of the reaction mixture was adjusted to 8.5 with 1 N $NH_4OH$. The reaction mixture was dialyzed against $H_2O$ for 8 d at 4 °C and lyophilized. The reaction product was dissolved in phosphate buffered saline and insoluble material was separated by centrifugation. The soluble fraction was dialyzed against $H_2O$ for 4 d at 4 °C. After dialysis, the conjugate solution was lyophilized and stored at −70 °C.

**Determination of SSA Molecules Bound to BSA Molecule** UV spectral analysis was performed after acid treatment. Treatment of SSA–BSA conjugate with 2% HCl–50% MeOH for 22 h converted SSA bound to BSA to saikosaponin $b_1$[15] which shows absorbance maxima at 244, 252, and 260 nm. A comparison of the absorbance at 252 nm of the SSA–BSA conjugate to standard SSA treated with the acidic solution determined that the molar ratio of SSA and BSA was 7.8 : 1.

**Preparation of SSA–OVA Conjugate for the Solid Phase Coating** To a solution of SSA (195.0 mg, 0.25 mmol) in 80% EtOH (9.375 ml), a solution of $NaIO_4$ (374.0 mg, 1.75 mmol) in $H_2O$ (5.625 ml) was added dropwise over 45 min, stirring at room temperature. Excess $NaIO_4$ was neutralized by the addition of 3 drops of 50% aqueous ethylene glycol followed by further stirring for 30 min. Subsequently, the precipitate was removed by centrifugation. The supernatant was added to a solution of OVA (112.5 mg, 2.5 $\mu$mol) in $H_2O$ (15 ml) dropwise at room temperature and the pH was kept at 9.0—9.5 by adding 5% aqueous $K_2CO_3$. After an additional 110 min, fifteen milliliters of a fresh solution of $NaBH_4$ (147 mg, 15.5 eq) in $H_2O$ were added dropwise and the mixture was stirred for 3 h. Subsequently, 1 N HCOOH, pH 6.5, was added to neutralize excess $NaBH_4$ followed by 1 h of stirring. Thereafter, the pH of the reaction mixture was adjusted to 8.5 with 1 N $NH_4OH$. The reaction mixture was dialyzed against $H_2O$ for 8 d and lyophilized.

**Immunization and Antiserum Production** Two rabbits and two guinea pigs were used for immunization with the SSA–BSA conjugate. However, guinea pigs did not produce specific antibodies and studies were discontinued.

Preimmune sera were collected from the rabbits used in this study before immunization. One milligram of conjugate was dissolved in 1 ml of saline, mixed and emulsified with complete Freund's adjuvant. Rabbits were given the emulsion (700—800 $\mu$l) by injection into their backs and leg muscles biweekly. Four weeks later, intramuscular booster injections were made at biweekly intervals with 400—500 $\mu$g of conjugate mixed with Freund's incomplete adjuvant in a 1 : 1 ratio. Bleedings were performed one week after the booster injections. 5—10 ml of blood per rabbit were collected from the marginal vein of the ear with 23G needles. The blood was coagulated at room temperature. Clots were cut in squares, and stored at 4 °C overnight for better serum separation. After centrifugation at 13000 × *g* for 20 min, sera were separated. Aliquots of 20 $\mu$l serum were separated for analysis, and the remaining bulk of the serum was stored at −70 °C.

**ELISA Procedure** Each well of a microtiter plate (flat-bottomed, Nunc) was coated overnight at 4 °C, or for 2 h at 37 °C, with 50 $\mu$l of SSA–OVA conjugate (0.2 $\mu$g/ml) dissolved in coating buffer. The plates were then washed four times with T-PBS. The unbound sites of wells were blocked by 200 $\mu$l of C-PBS and incubated for 2 h at 37 °C. The plates were washed four times. Fifty microliters of anti-SSA antiserum diluted 1/120000 in C-PBS and 50 $\mu$l of sample solution diluted in C-PBS were added to each well. After incubation for 2 h at 37 °C, the plates were washed 4 times with T-PBS. 50 $\mu$l of peroxidase-labeled goat anti-rabbit IgG diluted 1/2000 in C-PBS was added to each well. After incubation for 1 h at 37 °C, the plates were again washed 5 times. 50 $\mu$l of TMB peroxidase substrate solution was added to each well. After incubation for 30 min in the dark at 37 °C, the reaction was stopped by adding 50 $\mu$l of 1 M $H_2SO_4$. The activity of enzyme bound to the solid phase was measured photometrically at 450 nm using a microplate reader (Labsystems, Finland).

**Quantitative Analysis of SSA by HPLC** The crude saponin fraction was prepared by solvent fractionation from the methanolic extract of Bupleuri Radix[13] and subjected to HPLC analysis. HPLC was performed using a JASCO HPLC-system (Japan), equipped with a pump (PU-980) and UV-VIS detector (UV-975). The following conditions were applied: column, Crestpak C-18 (150 mm × 4.6 mm i.d.); flow rate, 1.0 ml/min; eluent, acetonitrile–water (40:60); detection, UV at 203 nm.

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## References

1) Yamamoto M., Kumagai A., Yamamura Y., *Arzneim.-Forsch.*, **25**, 1021—1023 (1975).
2) Hiai S., Yokoyama H., Nagasawa T., Oura H., *Chem. Pharm. Bull.*, **29**, 495—499 (1981).
3) Yamamoto M., Kumagai A., Yamamura Y., *Arzneim.-Forsch.*, **25**, 1240—1243 (1975).
4) Abe H., Sakaguchi M., Yamada M., Arichi S., Odashima S., *Planta Med.*, **40**, 366—372 (1980).

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5) Motoo Y., Sawabu N., *Cancer-Letters*, **86**, 91—95 (1994).
6) Uomori A., Seo S., Tomita Y., *Shiyakugaku Zasshi*, **28**, 152—160 (1974); Hiraoka N., Kodama T., Oyanagi M., Nakano S., Tomita Y., Yamada N., Iida O., Satake M., *Plant Cell Reports*, **5**, 319—321 (1986); Fujioka N., Tanaka A., Koyama A., Hamamoto Y., Namera A., Yamasaki K., Kohda H., *Shoyakugaku Zasshi*, **41**, 308—312 (1987).
7) Terauchi M., Kanamori H., Sakamoto I., Mochiike A., Katoh M., Kohda H., *Shoyakugaku Zasshi*, **47**, 213—217 (1993); Kimata H., Hiyama C., Yahara S., Tanaka O., Ishikawa O., Aiura M., *Chem. Pharm. Bull.*, **27**, 1836—1841 (1979).
8) Ferreira J. F. S., Janick J., *Phytochemistry*, **41**, 97—104 (1996); Yoshimatsu K., Satake M., Shimomura K., *J. Nat. Prod.*, **53**, 1498—1502 (1990).
9) Saita T., Matsunaga H., Yamamoto H., Nagumo F., Fujito H., Mori M., Katano M., *Biol. Pharm. Bull.*, **17**, 798—802 (1994).
10) Butler V. P., Chen J. P., *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 71—78 (1967).
11) Smith T. W., *J. Clin. Invest.*, **51**, 1583—1593 (1972).
12) Rodbard D., "Ligand Assay," ed. by Langan J., Clapp J. J., Masson, New York, 1981, p. 45.
13) Ishii H., Nakamura M., Seo Sh., Tori K., Tozuo T., Yoshimura Y., *Chem. Pharm. Bull.*, **28**, 2367—2383 (1980).
14) Poulev A., Deus-Neumann B., Zenk M. H., *Planta Med.*, **60**, 77—83 (1993).
15) Kimata H., Hiyama C., Yahara S., Tanaka O, Ishikawa O., Aiura M., *Chem. Pharm. Bull.*, **27**, 1836—1841 (1979).

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