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Production of Antibody against Saikosaponin a, an Active Component of Bupleuri Radix

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High titer rabbit polyclonal antibodies (pAbs) which show a specificity for saikosaponin a (SSA), have been generated. The immunogen used was a conjugate of SSA linked through its glucose moiety to bovine serum albumin by periodate oxidation method. The antibody titers obtained from two rabbits, innoculated with the immunogen, reached a plateau after the fourth and third booster injection, respectively. The specificity of the pAbs was determined by hapten inhibition assays using several SSA-like structures. SSA competitively inhibited the binding of the rabbit anti-SSA pAbs to SSA-ovalbumin on solid phase, a coated antigen on the well. The antibodies showed high specificity to SSA, exhibiting no significant cross-reactivity with any of SSA analogues tested.

Key words : Saikosaponin a, Polyclonal antibodies, Periodate oxidation, Hapten inhibition assay

INTRODUCTION

Saikosaponin a (SSA) is one of the major and active saponins of Bupleuri Radix. SSA has been known to have several biological actions such as anti-inflammatory actions (Yamamoto *et al.*, 1975a), stimulatory action on the pituitary-adrenocortical axis (Hiai *et al.*, 1981), plasma-cholesterol lowering action (Yamamoto *et al.*, 1975b), protective action against hepatic damage by D-galactosamine (Abe *et al.*, 1980), and antitumor effects (Motoo and Sawabu, 1994).

Recently, several laboratories have been engaged in developing cell and tissue cultures of *Bupleurum falcatum* L. (Fujioka *et al.*, 1987; Hiraoka *et al.*, 1986; Uomori *et al.*, 1974). The manipulation of culture conditions and the screening of high SSA-producing clones from a large number of clones require a simple and sensitive assay technique to measure trace amounts of the compound.

SSA has been determined by a number of analytical methods, which include colorimetry (Akahori and Kagawa, 1974), TLC-densitometry (Kimata *et al.*, 1980), droplet counter current chromatography (DCC, Otsuka *et al.*, 1978) and HPLC with UV detection (Terauchi *et al.*, 1993). However, complete separation of saikosaponins could not be achieved by TLC, and the separation of SSA by DCC or HPLC required a lot of time and effort. Furthermore, the conventional methods for SSA determination are not sensitive enough to detect SSA contained in extremely small scale of samples from cell or tissue cultures.

Enzyme-linked immunosorbent assay (ELISA) would be one of the most suitable techniques for the screening of plant materials from small scale of samples because of the high sensitivity and simplicity of the assay (Ferreira and Janick, 1996; Yoshimatsu *et al.*, 1990).

The purpose of this study was to raise pAbs against SSA to establish an ELISA, which is able to detect and quantify SSA in crude extracts with minimal sample preparation. ELISA can be a faster, simpler and more sensitive for the quantification of SSA than the conventional methods.

This report describes the immunization of rabbits with a conjugate of SSA covalently linked to bovine serum albumin (BSA) by periodate oxidation method. SSA-specific antisera obtained after this challenge were investigated in terms of specificity and time course of rise in titer.

MATERIALS AND METHODS

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).

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Purification of saikosaponins

Bupleuri Radix, the root of *Bupleurum falcatum* L. (Umbelliferae), is commercially available, which was used for the present studies. Saikosaponins a, c and d were isolated from the crude drug by the modified method of Ishii *et al.* (1980). The isolated saikosaponins were identified by comparison with authentic samples using ^{1}H- and ^{13}C-NMR spectra (Tori *et al.*, 1976)

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, USA). The tetramethylbenzidine (TMB) substrate solution was obtained from Kirkegaard & Perry Laboratories (Maryland, USA). Casein was purchased from Merck (Darmstadt, Germany). Peroxidase-labeled anti-rabbit IgG was obtained from Vector Laboratories, Inc. (Burlingham, USA).

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 already used for colchicoside (Poulev *et al.*, 1993).

To a solution of SSA (390.0 mg, 0.5 mmole) in 80% EtOH (18.75 ml), a solution of $NaIO_4$ (856.0 mg, 4 mmole) in H_2O (11.25 ml) was added dropwise within 40 minutes under stirring at room temperature. Subsequently, 3 drops of 50% aqueous ethylene glycol were added to neutralize excess $NaIO_4$. After 30 minutes of further stirring, the precipitate was removed by centrifugation. The supernatant was added to a solution of BSA (280.0 mg, 4.2 μmol) in H_2O (30 ml) dropwise under 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, 30 ml of a fresh solution of $NaBH_4$ (295 mg, 15.5 equiv.) in H_2O were added dropwise and the whole mixture was further stirred for 3 hours. Subsequently, 1 N HCOOH, pH 6.5, was added to neutralize excess $NaBH_4$ followed by 1 hour 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 days 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 days. After dialysis, the conjugate solution was lyophilized and stored at -70°C.

Determination of SSA molecule bound to BSA molecule

UV spectral analysis was performed after acid treatment. Treatment of conjugate with 2% HCl-50% MeOH for 22 hours converted SSA bound to BSA to saikosaponin b_1 which shows absorbance maximum at 244 nm, 252 nm, and 260 nm. 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 coating conjugate for the solid phase coating

To a solution of SSA (195.0 mg, 0.25 mmole) in 80% EtOH (9.375 ml), a solution of $NaIO_4$ (374.0 mg, 1.75 mmole) in H_2O (5.625 ml) was added dropwise within 45 minutes under stirring at room temperature. Excess $NaIO_4$ was neutralized by the addition of 3 drops of 50% aqueous ethylene glycol followed by a 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 μmol) in H_2O (15 ml) dropwise under stirring 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, 15 ml of a fresh $NaBH_4$ (147 mg, 15.5 equiv.) solution were added dropwise and the whole mixture was further stirred for 3 hours. Subsequently, 1 N HCOOH, pH 6.5, was added to neutralize excess $NaBH_4$ followed by 1 hour 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 days and lyophilized.

Immunization and antiserum production

Two outbred female rabbits (2.2~2.3 kg) and two guinea pigs (Hartly, female) were used for immunization with the SSA-BSA conjugate. However, guinea pigs did not produce specific antibodies.

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 emusified with complete Freund's adjuvant. Rabbits were given the emulsion (700~800 μl) by injection into the backs and leg muscles biweekly. Four weeks later, intramuscular booster injections were made in biweekly intervals with 400~500 μg of conjugate mixed 1:1 with Freund's incomplete adjuvant. Bleedings were performed 10 days 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 13,000× g for 20 min, sera were separated. Aliquots of 20 μl serum were separated for analysis, and the remaining bulk of the serum was stored at -70°C.

Determination of titers

Microtiter plates (flat-bottomed, Nunc) were coated overnight at 4°C with 50 μl/well of 0.2 μg coating conjugate dissolved in 1 ml coating buffer. The plates were then washed four times with T-PBS. The unbound sites of wells were blocked by 200 μl of C-PBS and incubated for 2 hours at 37°C. The plates were washed four times. Fifty microliters of anti-SSA antiserum serially diluted in C-PBS and 50 μl of C-PBS were added to each well. After incubation for 2 hours at 37°C, the plates were washed four times with T-PBS. 50 μl of peroxidase-labeled goat anti-rabbit IgG diluted 1/2,000 in C-PBS was added to each well. After incubation for 1 hour at 37°C, the plates were again washed five times. 50 μl of TMB peroxidase substrate solution was added to each well. After incubation for 30 minutes in the dark at 37°C, the reaction was stopped by adding 50 μl of 1 M H_2SO_4. The activity of the enzyme bound to the solid phase was measured photometrically at 450 nm using a microplate reader (Labsystems, Finland).

Hapten inhibition studies

Antibody specificity was assessed by addition to detection system of varying concentrations of several SSA-like structures before addition of antibody. Compounds tested included the genuine saikosaponins SSA, saikosaponin d and saikosaponin c as well as the artificial saikosaponins saikosaponin b_1 and saikosaponin g.

RESULTS AND DISCUSSION

SSA (M.W. 780) itself with low molecular weight is not immunogenic. Thus, the hapten must be conjugated covalently to a macromolecule (*i.e.* BSA, OVA, *etc.*) in order to build up specific antibodies from animal. The hapten-protein conjugate will induce specific antibodies against the hapten moiety, as well as, the carrier protein *in vivo*. The coupling of small molecule with a carrier protein, is therefore, a crucial factor in producing the specificity of the antibody. In addition, the linkage between the hapten and the macromolecule should be stable and non-hydrolyzable.

The presence of 13β, 28-oxide ring and 16-hydroxyl function of 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 glycone, not aglycone, moiety of the hapten for the cross-linking with a carrier protein.

The conjugate of SSA and BSA was prepared as described (Fig. 1) in Materials and Methods and injected into animals as immunogen. Rabbits, inoculated with

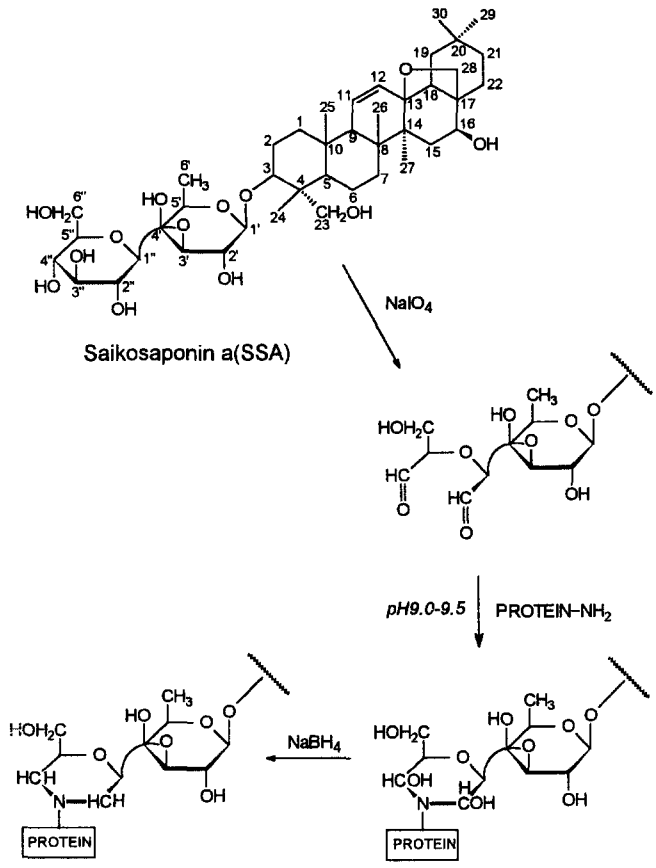

Fig. 1. Proposed mechanism for conjugation of SSA to carrier protein by periodate oxidation method.

the SSA-BSA conjugate obtained by periodate oxidation method, provided antibodies suitable for detection of SSA. The specific binding of the serum was detected by reacting variously diluted anti-SSA serum with a solid phase antigen, the immobilized SSA-OVA conjugate on the surface of wells of a microtiter plate, using the peroxidase-labeled anti-rabbit goat IgG as a tracer. In order to avoid a nonspecific reaction between the antibody and the protein, which is used for a coating conjugate, the protein carrier used for immunization must be different from the protein used for the coating conjugate. In this study, BSA and OVA were used for the synthesis of an immunogen and a coating conjugate, respectively.

Typical binding curves of anti-SSA sera from bleeding of the two rabbits are shown in Fig. 2. The antibody titers obtained from rabbits C and T reached a plateau after the fourth and third booster injection, respectively. Use of preimmunization serum showed little non-specific binding to the solid phase antigen.

The specificity of the pAbs produced was evaluated by hapten inhibition assays using several SSA-like structures. SSA competitively inhibited the binding of the rabbit anti-SSA pAbs to an SSA-OVA on solid phase, a coated antigen on the well. The amounts of

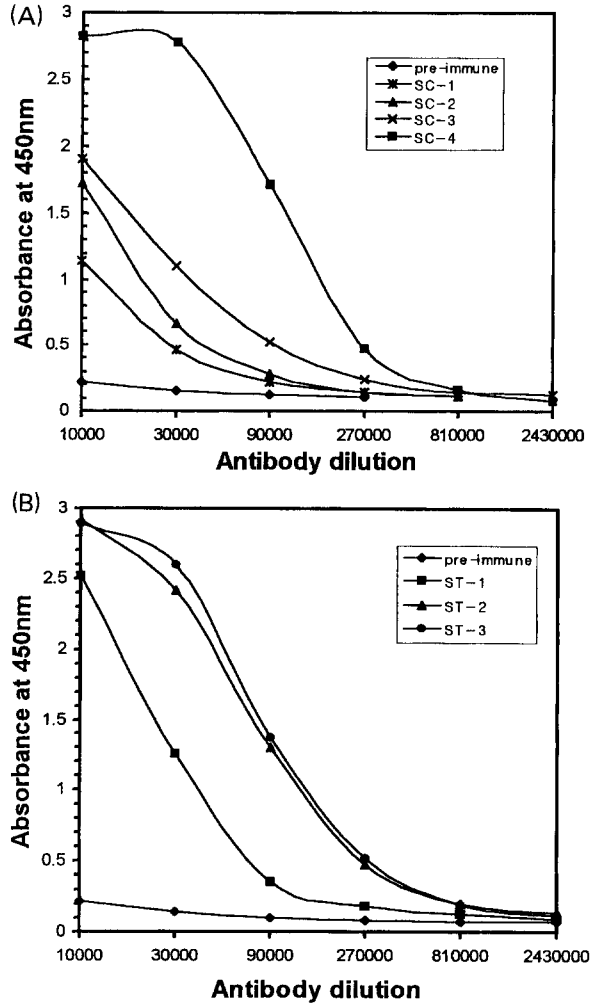

Fig. 2. Titers of anti-SSA antiserum samples detected by indirect ELISA.; A) antisera obtained from rabbit C: samples SC-1, SC-2, SC-3 and SC-4 were collected 10 days after first, second, third, and fourth boosting, respectively; B) antisera obtained from rabbit T: samples ST-1, ST-2, and ST-3 were collected 10 days after first, second, and third boosting, respectively. Preimmune sera were collected from the rabbits before immunization.

inhibitors required to give 50% inhibition of the binding of the antibodies to the solid-phase antigen are shown in Table I. Percent cross-reactivities determined as the amount of compound required for 50% inhibition, as compared to SSA itself (100%). The antibodies showed a very high specificity for the functionality of $C_{13\beta,28}$-allyl oxide ring and C_{16}-β-hydroxyl of SSA structure.

The cross-reactivity of the anti-SSA antibody to saikosaponin c (12.74%) was expected because the compound contains both C_{16}-β-hydroxyl and the allyl ether linkage. The polyclonal antibodies showed only slight cross-reactivity with saikosaponin d (0.3%), which differs from SSA only on the stereochemistry of the 16-hydroxyl group. The breakage of $C_{13\beta,28}$-allyl oxide ring

Table I. Hapten inhibition assays using several SSA-like structures

Inhibitor	Amount of inhibitor required to give 50 % inhibition (ng/ml)
SSA	0.28
Saikosaponin c	2.17
Saikosaponin b_1	13.19
Saikosaponin g	52.26
Saikosaponin d	92.33

resulted in loss of immunoreactivity with the SSA antibodies. Thus artificial saikosaponins b_1 (2.1%) and g (0.53%) showed minor cross reactivity.

In this work, we have shown that high titer and specific antibody capable of recognizing the SSA molecule can be obtained by immunizing rabbits with an antigen in which SSA is coupled to BSA through the glucose moiety using periodate oxidation method. Thus, the glycone moiety of SSA appears to be suitable for attachment of a carrier protein in the preparation of immunogen for SSA-specific ELISA.

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REFERENCES CITED

Abe, H., Sakaguchi, M., Yamada, M., Arichi, S. and Odashima, S., Pharmacological actions of saikosaponin isolated from *Bupleurum falcatum* L. Effects of saikosaponins on liver. *Planta Med.*, 40, 366-372 (1980).

Akahori, A. and Kagawa, K., Quantitative determination of saikosaponins in *Bupleurum* extract. II. Thin layer chromatographic separation of saikosaponins. *Shoyakugaku Zasshi*, 28, 122-126 (1974).

Ferreira, J. F. S. and Janick, J., Immunoquantitative analysis of artemisinin from *Artemisia annua* using polyclonal antibodies. *Phytochemistry*, 41, 97-104 (1996).

Fujioka, N., Tanaka, A., Koyama, A., Hamamoto, Y., Namera, A., Yamasaki, K. and Kohda, H., Propagation of *Bupleurum falcatum* by shoot tip culture. *Shoyakugaku Zasshi*, 41, 308-312 (1987).

Hiai, S., Yokoyama, H., Nagasawa, T., and Oura, H., Stimulation of the pituitary-adrenocortical axis by saikosaponin of *Bupleuri Radix. Chem. Pharm. Bull.*, 29, 495-499 (1981).

Hiraoka, N., Kodama, T., Oyanagi, M., Nakano, S., Tomita, Y., Yamada, N., Iida, O. and Satake, M., Characteristics of *Bupleurum falcatum* plants propagated through somatic embryogenesis of callus cultures. *Plant Cell Reports*, 5, 319-321 (1986).

Ishii, H., Nakamura, M., Seo, S., Tori, K., Tozuo, T., Yoshimura, Y., Isolation, characterization, and nuclear magnetic resonance spectra of new saponins from the roots of *Bupleurum falcatum* L. *Chem. Pharm. Bull.*, 28, 2367-2383 (1980).

Kimata, H., Fujimoto, N., Tanaka, O. and Miyazaki, Y., Separatory determination of saponins of *Bupleuri Radix* by dual wavelength thin layer chromatograph-densitometry and its application to the evaluation of roots of cultivated *Bupleurum falcatum* L. *Shoyakugaku Zasshi*, 34, 311-315 (1980).

Motoo, Y. and Sawabu, N., Antitumor effects of saikosaponins, baicalin and baicalein on human hepatoma cell lines. *Cancer-Letters*, 86, 91-95 (1994).

Otsuka, H., Kobayashi, S. and Shibata, S., Separation and determination of saponins of *Bupleuri Radix* by droplet counter current chromatography (DCC). *Planta Med.*, 33, 152-159 (1978).

Poulev, A., Deus-Neumann, B. and Zenk, M. H., Immunoassays for the quantitative determination of colchicine. *Planta Med.*, 60, 77-83 (1993).

Terauchi, M., Kanamori, H., Sakamoto, I., Mochiike, A., Katoh, M. and Kohda, H., Simultaneous analysis of saikosaponin a, c, and d in *Bupleurum* root by high-performance liquid chromatography. *Shoyakugaku Zasshi*, 47, 213-217 (1993).

Tori, K., Seo, S., Yoshimura, Y., Nakamura, M., Tomita, Y. and Ishii, H., Carbon-13 spectra of saikosaponins a, c, d and f isolated from *Bupleurum falcatum* L. *Tetrahedron Letters*, 46, 4167-4170 (1976).

Uomori, A., Seo, S. and Tomita, Y., Studies on the constituents in tissue cultures of *Bupleurum falcatum* L. *Shiyakugaku Zasshi*, 28, 152-160 (1974).

Yamamoto, M., Kumagai, A. and Yamamura, Y., Structure and action of saikosaponins isolated from *Bupleurum falcatum* L. I. Anti-inflammatory action of saikosaponins. *Arzneim.-Forsch.*, 25, 1021-1023 (1975a).

Yamamoto, M., Kumagai, A. and Yamamura, Y., Structure and action of saikosaponins isolated from *Bupleurum falcatum* L. II. Metabolic actions of saikosaponins, especially a plasma cholesterol-lowering action. *Arzneim.-Forsch.*, 25, 1240-1243 (1975b).

Yoshimatsu, K., Satake, M. and Shimomura, K. Determination of cardenolides in hairy root cultures of *Digitalis lanata* by enzyme-linked immunosorbent assay. *J. Nat. Prod.*, 53, 1498-1502 (1990).