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Immunological Studies on the Natural Products I.

Production of Antibody Specific to Saikosaponin a

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Abstract—In the course of the immunological studies on the natural products, the antibody specific to saikosaponin a was produced. Saikosaponin a was treated with succinic anhydride to give 6″-O-hemisuccinyl saikosaponin a, which was successively converted to saikosaponin a—BSA conjugate (4.5 mole saikosaponin a/mole of BSA) by carbodiimide method. The antibody obtained from rabbits immunized with saikosaponin a—BSA conjugate as usual manner reacted with both the conjugate and BSA, while after the absorption with BSA, the antibody reacted with the conjugate alone.

Keywords—Immunological technology • saikosaponin • *Bupleurum falcatum* • saponin • antibody • 6″-O-hemisuccinyl saikosaponin a

Recently immunological techniques have been applied in the field of plant natural product sciences.[1]

Saponin, one of the plant constituents which is widely distributed in plants, whose function in plant is not fully understood, is steroidal or triterpenoidal glycoside that has several distinctive properties such as forming froth in water, poisoning fish, hemolytic activity and formation of insoluble complex with steroid. Saponin hemolysis has been considered as to be caused by the formation of ligid complex with cholesterol existing in biomembrane.

The saponin crude drugs, such as Ginseng Radix, Glycyrrhizae Radix, Platycodi Radix, Senegae Radix, Bupleuri Radix and so on, play important roles in oriental medicine. Their representative activities are elixir, expectorant, detoxification, antiinflammation and so on. However, the mode of action of saponin has not been clarified yet. It was just postulated that saponin could react with cholesterol in biomembrane and extensive studies have been done.[2] Bupleuri Radix(roots of *Bupleurum falcatum* L.: Umbelliferae), one of the most famous crude drugs in oriental medicine, has an antiinflammatory activity,[3] protective action against hepatic injury,[4] plasma cholesterol-lowering activity,[5] corticosterone secretion-inducing acti-vities[6] and so on. From this crude drug, saponins such as saikosaponin a, c, d and many other saponins are isolated and considered as active components.[7]

For the purpose to elucidate the mode of action of saponin by immunological methods and to prepare the immunoassay method, the author produced an antibody specific to saikosaponin a which was used as a model saponin.

Experimental

All melting points were measured with Gallenkamp MFB-600-030W and were uncorrected. IR spectra were determined on a Perkin-Elmer

Infrared spectrophotometer Model 783. UV spectra were determined on a Perkin-Elmer UV-VIS spectrophotometer Lambda 5. ^{1}H-NMR spectra and ^{13}C-NMR spectra were measured on a Bruker Spectrospin 80 with TMS as an internal standard. TLC was performed on Kieselgel G precoated TLC plate(Merck). For column chromatography, Kieselgel 60(above 230 mesh, Merck) and $CHCl_3$-MeOH-H_2O(30:10:1) and EtOAc-EtOH-H_2O(8:2:1) as solvent systems were used. All solvents were redistilled and other reagents are all experimental grade.

Preparation of saikosation a

Saikosaponin a(Sa) was prepared by the method of H. Ishii *et al.*.[8] Crude saikosaponin a was isolated by the silica gel column chromatography with $CHCl_3$-MeOH-H_2O(30:10:1) as solvent system and successive silica gel column chromatography with EtOAc-EtOH-H_2O(8:2:1) as solvent system was per-acetylated and purified with the silica gel column chromatography [benzene-EtOAc (3:2) as solvent system]. Saikosaponin a octaacetate was hydrolyzed with 2% KOH-MeOH under reflux for 3 hr to give saikosaponin a. It was identified by direct comparison with authentic sample of mixed mp, IR, ^{1}H-NMR and ^{13}C-NMR.

Synthesis of 6″-O-hemisuccinyl saikosaponin a

Sa(780 mg, 1 mmol) and succinic anhydride (120 mg, 1.2 mmol) were dissolved in pyridine (2.5 ml) and the mixture was heated at 70~80° under N_2 stream for 6 hrs. After reaction, the solution was poured into cooled 10% H_2SO_4 (50 ml) and extracted with BuOH(20 ml, 3 times). The BuOH layer was washed with water (20 ml, 3 times), dried over anhydrous sodium sulfate and evaporated to dryness. TLC of the reaction mixture using EtOAc-EtOH-H_2O(8:2:1) as solvent system showed four spots at Rf values of 0.50, 0.34, 0.18 and 0.08, including starting material. The compound which showed Rf value of 0.34 was separated by silica gel column chromatography using EtOAc-EtOH-H_2O(8:2:1) as solvent system. The eluate was precipitated from MeOH-ether to yield 6″-O-succinyl saikosaponin a(SaS-1, 126 mg, 14%), mp 205~210°, Anal. Cald. for $C_{46}H_{72}O_{16}$: C, 62.71; H, 8.28. Found: C, 63.14; H, 8.38. UV(end absorption); $IR\nu_{max}^{KBr}$: 3400 (broad, hydroxyl), 3000~2,500(acid), 1730 (carbonyl), 1380, 1065(broad); ^{1}H-NMR δ: 0.73(3H), 0.94(3H), 0.97(3H), 1.00(3H), 1.05(3H), 1.11(3H)(all singlet, angular methyl), 1.30(3H, doublet, 6′-Me). 2.60(4H, multiplet, —$COCH_2CH_2CO$—); ^{13}C-NMR(see Table 1).

Synthesis of saikosaponin a—BSA conjugate

SaS-1(30 mg) and BSA(38 mg) were dissolved in mixture of water and DMF(pH 5.5, 5 ml). 1-Ethyl-3-(3-dimethylaminopropyl) carboiimide (EDC, 33 mg) was poured into this solution and the mixture was stirred at room temperature over night. After reaction, the mixture was dialysed over water at 4° for 5 days. After dialysis, the solution was centrifuged and the supernatant was lyophilized.

Determination of Sa molecule bound to BSA molecule

UV spectrometric analysis after acid treatment and phenol-H_2SO_4 method for determination of sugar contents were performed. Treatment of conjugate with N-HCl(pH 1, for 16 hrs.) converted Sa bound to BSA to saikosaponin b_1 which shows absorbances at 242.5 nm (ε 23700), 251 nm(ε 27600) and 260.5 nm(ε 17400).[9] Comparison of the absorbance at 251 nm of the conjugate to standard Sa treated same way determined that 4.5 molecules of Sa coupled to one BSA molecule.

Preparation of antiserum to Sa

The Sa—BSA conjugate(3 mg) was dissolved in sterile isotonic saline(1.5 ml) and emulsified

with the same amount of complete Freund's adjuvant(Difco, Detroit, Mich., USA). One ml of the emulsion was injected into each of 3 house male rabbits subcutaneously and intramuscularly at multiple sites on the back and foot pads. Booster injections with the same amount of immunogen were administered once every two weeks for two months and monthly thereafter. The blood was collected from the ear vein 2 weeks after the last booster injection. The serum was separated by centrifugation for 30 min at 3,000 rpm and was stored at −20° until use.

Absorption of antiserum with BSA

The antiserum was mixed with an equal volume of BSA solution(1.0 mg/ml), and the mixture was allowed to stand at 37° for 1 hr and at 4° overnight, then the precipitates were removed by centrifugation. The presence and specificity of untreated and treated antibody to the conjugate was examined by Ouchterlony's method.[11]

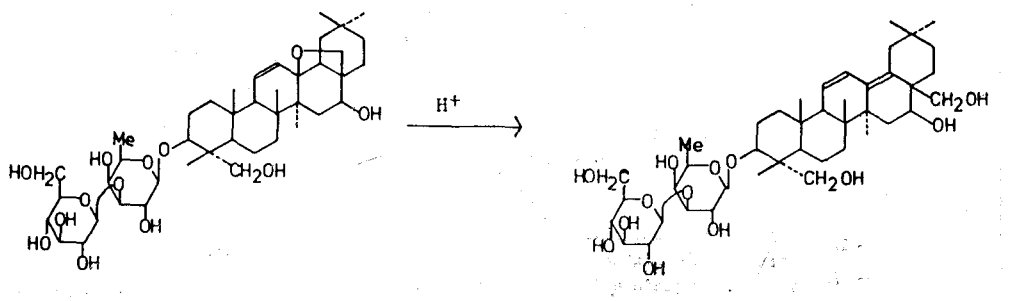
Results and Discussion

Separation of Sa

Saikosaponin a(Sa) is a major genuine saponin obtained from *Bupleurum falcatum*. It was reported that this genuine saponin contains very unstable allyl oxide linkage, and are easily converted into artificial diene-saponins by mild acid treatment (Fig. 1). This artificial diene-saponin, saikosaponin b_1(Sb_1), is also formed during the process of extraction. H. Kimata *et al.*[12] reported that formation of the diene-saponin could be minimized by extraction of the sample at room temperature or by refluxing in methanol containing a base. In this experiment, the extraction was carried out at room temperature for more than three days. Sb_1 runs concurrently with Sa on silica gel chromatography system, so that it is impossible to separate Sa from Sb_1. As mentioned on experimental section, crude Sa was peracetylated with acetic anhydride and pyridine and Sa octaacetate was purified by silica gel column chromatography. Sa was obtained by hydrolysis of Sa octaacetate. Purified Sa showed no absorption due to a heteroannular diene system.

Synthesis of ligand for immunogen

For immunological studies on natural products, various types of antibody derived from varieties of immunogen, BSA and hapten conjugate, where different position of hapten molecule is attached to BSA molecule. Hydroxyl group is used as attaching position in formation

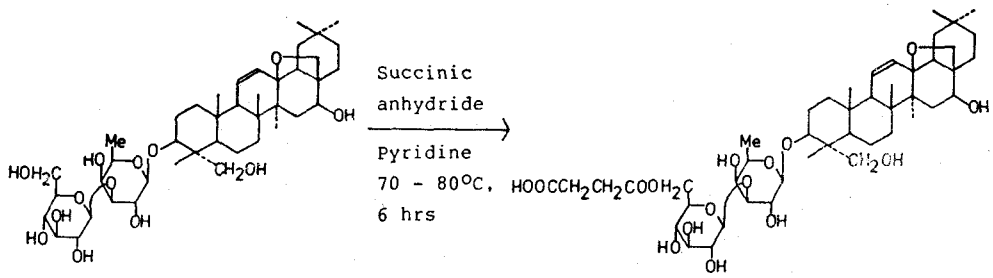


Saikosaponin a

Saikosaponin b_1

λ max EtOH 242.5 (ε 23700)
251 (27600)
260.5nm (17400)

Fig. 1. Acid catalized transformation of saikosaponin a.



Saikosaponin a(Sa) 6''-O-Hemisuccinyl-saikosaponin a(SaS-I)

Scheme 1. Synthesis of 6''-O-hemisuccinyl saikosaponin a(SaS-I).

of immunogen of several natural products.[13] Among several hydroxyl functional group contained in Sa, there are two primary hydroxyl groups at C23 and C6''. When we use these primary hydroxyl groups, we can synthesize two different types of ligand for immunogen. Succinyl group is usually used as spacing group for hydroxyl group. Succinic anhydride could react selectively with primary hydroxyl group under mild condition as mentioned in experimental section, while secondary hydroxyl group could not react under this mild condition. The reaction product of Sa and succinic anhydride showed 4 spots at Rf values of 0.50, 0.34. 0.18 and 0.08, which corresponds to Sa, 6''-O-hemisuccinyl Sa(SaS-I), 23-O-hemisuccinyl Sa(SaS-II, not identified) and 6'', 23-O-dihemisuccinyl Sa(SaS-III, not identified) on thin layer chromatogram(Scheme 1)(Fig. 2). SaS-I was separated as one of the ligand that could be obtained from the reaction mentioned above. In IR spectra, SaS-I showed absorption of carbonyl group of carboxylic acid at 1730 cm^{-1} and typical absorption of carboxylic acid at 3000~2500 cm^{-1}. In ^{1}H-NMR spectra of SaS-I, the peak of succinyl group was observed at δ 2.60(4H, multiplet). In ^{13}C-NMR spectra (Table 1), the signal of C6''-carbon carrying a hemisuccinylated OH group shifted downfield by +1.8 ppm and the signal of C5''-carbon moved upfield by −2.5 ppm. Therefore hemisuccinyl group was readily pointed out to be positioned at 6'' carbon of glucosyl moiety. From these spectral data it was indicated that SaS-I was synthesized.

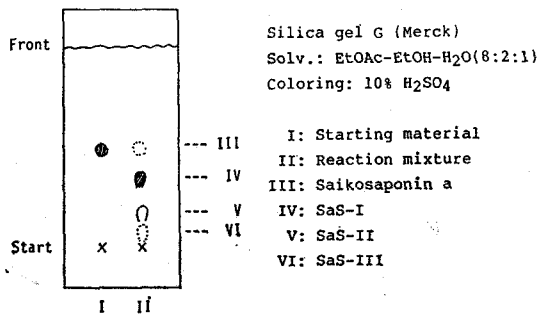


Fig. 2. Chromatogram of reaction mixture of saikosaponin a and succinic anhydride.

Synthesis of immunogen

Sa—BSA conjugate was synthesized by the methods of P. Westekemper *et al.*.[14] SaS-I and BSA was coupled via CDI method(Scheme 2). Based on the determination of UV absorption after acid treatment it was calculated that 4.5 molecules of SaS-I were bound to one molecule of BSA.

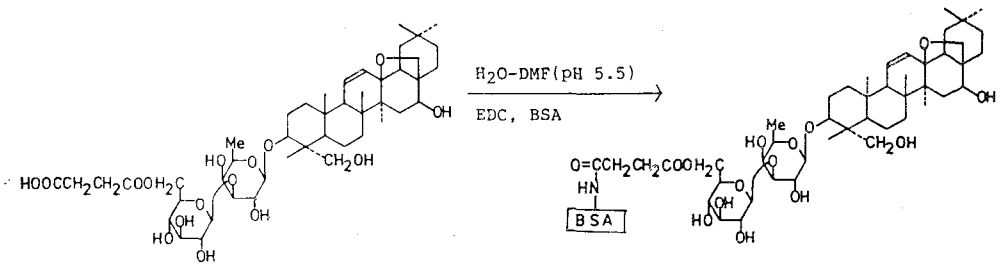
Production of antibody

House male rabbits were immunized by the method of K. Kawashima.[15] Antiserum that could bind Sa-BSA conjugate was obtained after 3 monthes of first immunization. The antiserum obtained was tested for the antibody production by Ouchterlony method. Since the

Table 1. Carbon-13 chemical shifts of Sa and Sa hemisuccinate(δ in ppm)

Carbon No.	Sa	Sa hemi-succinate	$\Delta\delta$*	Carbon No.	Sa	Sa hemi-succinate	$\Delta\delta$	Carbon No.		Sa	Sa hemi-succinate	$\Delta\delta$
1	38.9	39.0	+0.1	16	64.4	64.5	+0.1		1′	105.2	105.6	+0.4
2	25.6	25.8	+0.2	17	46.9	47.1	+0.2		2′	71.7	71.5	−0.2
3	82.5	82.4	−0.1	18	52.4	52.4	0.0	Fuc	3′	85.0	85.4	+0.4
4	43.5	43.7	+0.2	19	38.2	38.2	0.0		4′	71.7	71.7	0.0
5	48.0	48.1	+0.1	20	31.6	31.7	+0.1		5′	70.8	71.0	+0.2
6	17.8	17.9	+0.1	21	35.0	35.0	0.0		6′	16.9	17.1	+0.2
7	31.9	32.0	+0.1	22	25.6	25.8	+0.2		1″	105.5	105.6	+0.1
8	42.4	42.5	+0.1	23	65.1	65.0	−0.1		2″	75.3	75.4	+0.1
9	53.2	53.3	+0.1	24	12.7	12.8	+0.1	Glu	3″	77.9	78.1	+0.2
10	36.6	36.7	+0.1	25	18.6	18.7	+0.1		4″	71.7	72.0	+0.3
11	132.0	132.0	0.0	26	19.8	19.8	0.0		5″	77.9	75.4	−2.5
12	131.0	131.1	+0.1	27	20.8	20.8	0.0		6″	62.8	64.6	+1.8
13	84.0	84.1	+0.1	28	72.9	72.9	0.0	$-\underline{C}OCH_2CH_2COOH$			173.1	
14	46.0	46.0	0.0	29	33.7	33.7	0.0	$-CO\underline{C}H_2CH_2COOH$			29.0	
15	36.2	36.4	+0.2	30	23.9	23.9	0.0	$-CCOCH_2\underline{C}H_2COOH$			31.0	
								$-COCH_2CH_2\underline{C}OOH$			177.9	

* Succinylation shift($\Delta\delta$): $\delta_{Sa\ hemisuccinate}-\delta_{Sa}$



6′′-O-Hemisuccinyl-saikosaponin a(SaS-I)

Saikosaponin a - BSA (3.8 - 4.5 : 1)

Scheme 2. Synthesis of saikosaponin a—BSA conjugate.

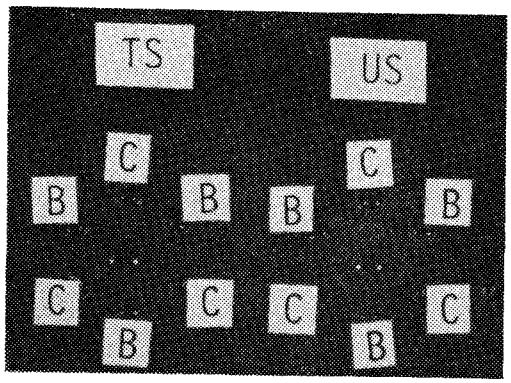


Fig. 3. Ouchterlony test for treated and untreated serum.
TS: treated serum US: untreated serum
B: BSA C: Sa—BSA conjugate

serum shows precipitation with carrier molecule (BSA), it is necessary to remove antibody that could bind with BSA in order to test antibody production to hapten. Antiserum was incubated with enough amount of BSA solution and precipitate was centrifuged to get treated antiserum. This treated antiserum showed precipitin reaction with Sa-BSA conjugate alone (Fig. 3). This conjugate was proved to be immunogenic in rabbits and antiserum could be obtained.

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