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Studies on the Anti-inflammatory Glycosides of Panax ginseng

Byung Hoon Han, Yong Nam Han, and Lin Keun Woo*

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Abstract—Two anti-inflammatory glycosides, Panax saponin A, $C_{47}H_{72}O_{14}\cdot 2H_2O$, m.p.208-10° and C, m.p. 196-202°, were isolated from the methanol extract of *Panax ginseng*. The anti-inflammatory activity of Panax saponin A was found to have delayed and prolonged characteristics. The partial structure of Panax saponin A was established to be β, β'-20S-protopanaxatriol-diglucoside. One of glucose residues was bound to the 20S-hydroxyl group of aglycone.

In our previous communication,[1] 60 species of crude drugs were screened for anti-inflammatory (or anti-rheumatic) activity, based on the stabilizing activity on serum albumin (Cohn Fr. V).[2,3] Methanol extract of Panax ginseng was observed to stabilize the albumin from heat denaturation even at 200-fold dilution. This result strongly suggested the presence of non-steroid anti-inflammatory substance in the extract.[2,3] Adopting both the albumin stabilizing activity and carrageenin edema test as the tracing index for the anti-inflammatory activity, the purification of effective principles were attempted and two triterpene glycosides, designated Panax saponin A and C, were isolated in a chromatographically pure state as the result.

Present paper describes the studies on the isolation of anti-inflammatory components, partial structure of the isolated substance and a part of anti-inflammatory characteristics of the substance.

The procedure for solvent fractionation of the extract and the result of the activity traced by albumin stabilizing test are outlined concomitantly in Scheme la. As shown in Scheme la, the albumin stabilizing activity was shown in both neutral and acidic glycoside fractions. The neutral glycoside fraction was adopted for further column chromatographic purification, since this fraction was dominant in the original glycoside mixture. From the step of column chromatographic purification the anti-inflammatory activity was traced by carrageenin edema

* Natural Products Research Institute, Seoul National University, Seoul.

test and the result is outlined in Scheme Ib. Nuetral glycoside fraction suppressed the carrageenin edema volume by 55% at the dose of 100 mg/kg.p.o., in one experiment. Furthermore, the edema was suppressed significantly even one week after the medication of this fraction in both single and multiple dose groups, as shown in Table I. The majour spots of this fraction, Panax saponin A and C, were obtained in a chromatographically single state by repeated column chromatographic purification and the anti-inflammatory activity was reconfirmed from the isolated substance. As shown in Table I, the specific anti-inflammatory activity was not significantly increased parallel with the progress of the purification of Panax Saponin A and C, therefore the possibility of the presence of other anti-inflammatory glycosides is not excluded. In order to reconfirm the prolonged anti-inflammatory activity from Panax Saponin A, time interval effect on carrageenin edema suppression was examined by varying the length of time intervals between the medication and carrageenin edma induction and the results are outlined in Table II. As shown in Table II, the statistical significances and the rates of edema suppression of Panax Saponin A were increased parallel with the length of time intervals and also with the increased doses, suggesting the delayed anti-inflammatory characteristics. The delayed anti-inflammatory activity of Panax Saponin A enable to guess delayed absorption of this substance in the gastro-intestinal tract of animals or some indirectly acting mechanisms as the mode of action.

Partial structure of Panax Saponin A was determined as following; ir-spectrum of PS-A displayed a strong absorption of ν c-o-c in the region of 1000-1150cm^{-1}, suggesting the presence of glycoside bond. On acid hydrolysis with d-H_2SO_4, PS-A afforded panaxatriol and glucose. The identity of panaxatriol was confirmed by direct comparison with the authentic spectrums*. The number of glucose moiety in the molecule was found to be 2 mole by the chemical analysis of glucose content in the hydrolysate and by the number of protons in the $\delta^{TMS}_{CD_3OD}$ 2.5-5.3(32-36 H) region in the nmr-spectrum of PS-A. The genuine aglycone of PS-A, obtained by periodate-alkali treatment,[5] was identified as 20S-protopanaxatriol by direct comparison with the reference compound which was prepared by the method of Shibata et al.[6].

Upon acetylation, deca-acetate of PS-A was obtained in a fine needle, mp. 250-2°, and the nmr spectrum of which showed deca-acetyl protons at $\delta^{TMS}_{CD_3OD}$ 1.85-2.06 (s. 10×3H) with the disappearance of hydroxyl protons. Peracetylation was also verified by the disappearance of hydroxyl absorption in the ir-spectrum of the acetate. Considering the strong resistance to acetylation of C-20 hydroxyl group in the 20 S-protopanaxatriol, due to steric hindrance[7], it was concluded that at least one mole of glucose should be attached to the 20S-hydroxyl group. Anomeric configuration of glycoside bond of PS-A was analyzed to be β, β'-diglucoside configuration by both Hudson rule[8] and by pyranose ring breathing absorption[9] in ir-spectrum of PS-A. Studies on PS-C is now proceeding.

* Ir and nmr spectrum of panaxatriol was kindly supplied from Dr. S. Shibata.

EEXPERIMENTAL

All melting points were determined on the heat block and uncorrected. The following chromatographic solvents were used; Solvent A; $CHCl_3$: MeOH:H_2O (75 : 25 : 2.5), B; BuOH satd. with H_2O, C;Et_2O, D; Phenol: H_2O(5 : 1), E; $CHCl_3$: MeOH(40 : 1).

Protein Stabilizing Activity—A simple screening method for determining anti-inflammatory activity, developed by Mizushima et al.[2,3], was modified appropriately in order to eliminate the salt effect in the extract. One ml of 1%-bovine serum albumin in 0.1 M-Phosphate buffer (pH 5.3) was mixed in a tube(10mm i.d.) with 1 ml of appropriately diluted test solution. Twenty minutes after incubation at room temperature, the reaction mixture was heated, being shaken for 120 sec., in a 70° water bath. The degree of heat denaturation of albumin was estimated by the turbidity of the precipitate (absorbancy at 570 mμ). The reduced absorbancy in the test solution is positive sign for the presence of anti-inflammatory components in the sample. A sodium chloride solution, of equi-conductivity with the test solution, was added to the control solution, in order to eliminate the false positive reaction which might be arisen by the inorganic components in the extract. Satisfactory inhibition of heat denaturation was adopted for the positive sign of the anti-inflammatory components.

Carrageenin Edema Test[4]—Male albino rats weighing 110-130(Wistar strain) were accomodated for one week in our institute and divided into experimental and control groups, each consisting of 5-6 animals. In the regular experiment, 1%-saline solution of glycosides was administered orally one hour before edema induction in the dose described in Table 1. Edema was induced by the subcutaneous injection of 0.05m*l* of 1%-saline solution of carrageenin in the hind paw of all rats. In order to observe the prolonged activity of the glycosides, edema was induced one week after the medication of the glycosides. Parallel experiments were conducted with aspirin as the references for both experiments. Edema volume was measured by the method of Harris and Spencer'[10] using a plethysmometer, and the results were tabulated as the mean value of the edema suppression in Table I.

In Table II, the time interval effects of PS-A on edema suppression are outlined. The length of time intervals between the medication of PS-A and the carrageenin edema induction was varied from zero to 240min. The edema suppression rates and the statistical significances of them are highly increased parallel with the length of time intervals. Similar tendency was observed in the group No. V in which the doses of PS-A was doubled. The apparent inconsistency of the edema suppression rate of group No. V of Table I with that of group No.II in Table II might be arisen by seasonal effect[11]. Edema suppression (E.S) was calculated by following equation;

$$(E.S)\% = \frac{Ec - Ee}{Ec} \times 100$$

in which Ec and Ee are the edema volume of control and experimental animals respectively.

Table I—Anti-inflammatory action of panax saponins.

Group No.	Samples	Daily doses mg/kg.p.o.	Medication period days	Edema suppression rate at the time of 2**	3	4	5	Remarks*
I	Neutral glycoside fraction	100	6	59.2	55	27.6	29	R
II	Neutral glycoside fraction	100	6	70	67.7	27.7	—	P
III	Neutral glycoside fraction	100	1	79.6	43	13	—	R
IV	Neutral glycoside fraction	100	1	55	43	27	19	P
V	Partially purified PS-A (B.C.D. impurity)	50	1	18	27	30	25	R
VI	PS-A	50	1	18.6	37.3	31	21	R
VII	PS-C	50	1	—	—	33	43	R

* R and P denote the experimental design of regular and prolonged activity.

** Edema suppression rate at the time of 2 hours are considered to be unsignificant.

Reference: Aspirin 300mg/kg.p.o.; 53%, 100mg/kg.p.o.; 39% edema suppression at the time of 3 hour, and no prolonged activity

Table II—Time interval effect of PS-A on edema suppression.

Group No.	Doses mg/kg	Time [a] Interval min.	Animals Weight(gm.)		Edema at the time after carrageenin administration 1	2	3	4	5 (hrs.)
Control	CMC-solution.		133 s.e.±12	e	2.58	4.67	6.15	5.33	5.15
				r	—	—	—	—	—
				S.E.	0.555	1.03	0.600	0.795	0.633
I	100	0	137 s.e.±8.8	e	1.7	5.1	5.43	4.45	4.0
				r	34.3	—	11	16.5	22.5
				S.E.	0.26	1.09	1.17	1.05	0.9
II	100	60	124 s.e.±10.8	e	2.11	3.47	4.23	4.45	4.28
				r	18.1	25.7	31.3	16.5	16.8
				S.E.	0.83	0.545	0.90	1.06	0.835
III	100	120	129 s.e.±10.2	e	1.45	3.35	3.05	3.95	3.67
				r	43.7	28.3	50.5*	26.0	28.7
				S.E.	0.51	0.76	0.76	0.92	0.575
IV	100	240	136 s.e.±9.85	e	2.41	3.50	3.43	2.66	3.93
				r	6.6	25.1	44.5*	50.1*	23.7
				S.E.	0.64	0.286	0.71	0.59	0.62
V	200	30	127 s.e.±8.75	e	1.37	2.55	2.58	3.23	2.53
				r	47.0	45.3	58.0*	39.5*	50.7*
				S.E.	0.328	0.59	0.525	0.38	0.515

abbreviation: CMC; carboxymethyl cellulose, e; Edema volume×10, r; Percent of edema reduction, S.E.; Standard error of edema volume, s.e.; Standard error of body weight of animals.

* Edema suppression is highly significant statistically. ($P<0.01$)

a) The length of time intevals between the medication of PS-A and carrageenin edema induction.

Ginseng roots(5kg)
homogenation with 20 litre cold MeOH and percolation
Filtrate(++) Residue
evapd.
Ext.
H_2O and Et_2O
H_2O layer(+++) Ether(−)
$CHCl_3$ and NH_4OH
Aqueous layer(+++) $CHCl_3$(−)
d-HCl and $CHCl_3$
Aq. layer(+++) $CHCl_3$(−)
n-BuOH
BuOH layer(+++) Aq. layer(+)
4%-NaOH
BuOH layer(++) NaOH layer(+)
Amberite IR-120 and IR-400
Filtrate
concentration
BuOH solution Sucrose cryst.
Et_2O
Amorphous powder(neutral glycoside).

Scheme 1a—Separation procedure of neutral glycoside fraction.

Neutral glycoside fraction(20gm)**
Silica-gel column(5×75cm)
n-BuOH-H_2O

Fr. I	Fr. II**	Fr. III
(Ve. 400-800)	(Ve. 800-1200)	(Ve. 1200)
A: (−)	(+)	(+)
L: (+)	(+)	(+)

Al_2O_3
EtOAc-MeOH gradient
(5%-MeOH. exhaustive)
(40%-MeOH)
PS-A
m.p. 208°
$[\alpha]_D^{20}$ 35
Ac_2O Pyridine
PS-C
m.p. 198-202°
Acetate
m.p. 252°
5%-NaOH
5 hours reflux
PS-A**
m.p. 204°

** Carrageenin edema test
A: Anthrone test
L: Liebermann Buchard test

Scheme 1b—Isolation procedure of PS-A and PS-C. Positive and negative signs are the approximate stabilizing activity of the fractions upon the heat denaturation of Cohn Fr. V(Serum Albumin).

Separation of Crude Saponins—Fresh roots of Korean ginseng(5kg) were homogenized with 20 L portion of MeOH and extracted by percolation three times at room temperature. Percolate was concentrated to dryness under reduced pressure and the resulting residue was fractionated by the procedure illustrated in Scheme la. In tracing the anti-inflammatory activity, the method of Y. Mizushima et al. was used. As shown in diagram, the protein stabilizing activity was found mainly in the BuOH layer. Hence the effective components were expected to have a glycosidic nature. Alkali-washing of BuOH layer resulted in the partial loss of activity, however the majour part of glycoside was remained in BuOH layer.

After being demineralized of this fraction by the treatment of Amberite IR-120 and IR-400, the BuOH layer was concentrated to a small volume by vaccum distillation. After being filtered off sucrose, which was crystalized during the concentration, an excessive amount of Et_2O was added to the BuOH layer to separate neutral glycosidic components as a white amorphous precipitate. This precipitate was positive in Liebermnn-Buchard reaction and Anthrone reaction and showed a significantly potent, suppressive activity on the carrageenin edema test. Prolonged activity of this substance was also noticed in one experiment in both single and multiple doses groups (Table I). Due to this data, primary interest was focused on the pure isolation of the main components of this glycosidic substance, which was subjected to further column chromatographic purification(Scheme Ib).

Isolation of PS-A and PS-C—T.L.C.-spots of neutral glycoside fraction, were designated tentatively as Panax Saponin A.B.C. etc. by order of their increasing polarity, upper majour spot being corresponded to Panax Saponin A(Solvent; A). The neutral glycoside mixture(20gm) was chromatographed over silica gel column(500gm, 5×75cm, particle size<0.08 mm) with solvent B. Eluate was divided into three fractions; Fraction-I(Ve. 400-800), Fraction-II(Ve. 800-1200), Fraction-III(Ve. 1200→). Fraction-I showed positive Liebermann reaction and negative Anthrone reaction, and consisted minor part, therefore discarded without further examination. Fraction-II, showing the both reaction positive, contained the majour components, Panax saponin A and C with some B and D impurity. Fraction-III was also positive to both reaction, however it was discarded without further examination on account of its minor quantity.

Fraction-II was rechromatographed over active alumina(Grade-1, 2.5×75c m) with EtOAc containing 5%-MeOH. Exhaustive elution with the solvent produced PS-A in a chromatographically single state, mp. 208-10°, $[\alpha]_D^{20}$ 35(0.2% in MeOH). Gradient elution by increasing the MeOH content up to 40% produced PS-C in a pure state, mp. 196-202° and was set aside for future study. Nmr. spectrum of PS-A showed eight methyl protons, $\delta_{CD_3OD}^{TMS}$ 0.9-1.0(4×3H), 1.07(3H), 1.30(3×3H), of which later four methyl protons were considered to be characteristic to those of protopanaxatriol.[6] Molecular weight of PS-A was estimated to be roughly 800 by the proton number in the n.m.r.-spectrum of PS-A, assuming the number of protons in the region of $\delta_{CD_3OD}^{TMS}$ 0-2.5 to be 44, based on the number of non-polar protons in aglycone.

Deca-acetate of PS-A—PS-A was peracetylated by the ordinary method and given a fine needle from MeOH, mp. 250-2°C; ir (KBr): strong OAc absoption and no OH band, 910 (Type-1 band of pyranose ring), 890(Type-Ⅱb); nmr $\delta_{CDCl_3}^{TMS}$: 0-2.3(72H), 1.85-2.06 (10×3H), 4.0-4.2(m, 4H), 3.3-5.3(18H); Anal. Calcd. for $C_{42}H_{72}O_{14}(10\times C_2H_2O)$: C, 60.98, H, 7.59. found: C, 60.95, H, 7.51.

Deca-acetate was also ascertained through the saponification of this acetate. In one of the experiments, 2.2706 gm of acetate was refluxed for 5 hours in 50ml of 0.5N-alcoholic NaOH. The excess NaOH was titrated with 0.5N-HCl (Ind.; Ph.pt.). The observed

value of alkali consumption was matched with the theoretical value of the deca-acetate within a 0.5% margin of error.

Hydrolysis of PS-A—PS-A(10mg) was hydrolysed with N-H_2SO_4 (50%-alcoholic) by boiling for 5 hours in a sealed capillary tube. Glucose was detected by thin layer chromatography(solvent D), and by gas chromatography, column; 3% SE-30 on Chromosorb W(80-100 mesh) 3×1500 mm, column temp; programmed 6°C/min from 120°C, carrier gas; N_2(1.2kg/cm²), tr; 8.0(β), 10.0(α). The main spot of aglycone from the Et_2O soluble part of hydrolysate, was identified to be panaxatriol by comparing with the reference compound, which was prepared in our laboratory by the method of Shibata et al.[6] In order to assay the glucose content in PS-A, 50mg of PS-A was refluxed with 2N-HCl in 75%-dioxane for 3 hours. The reducing sugar content was assayed by Somogyi's method.[12] From the calibration curve of glucose, prepared from a parallel experiment, the glucose content in PS-A was found to be 41%, approaching the theoretical value for 2 mol glucose in the molecule.

Genuine Aglycone of PS-A(Formation of 20S-Protopanaxatriol)[6]—A solution of Na-Periodate(1 gm) in 50 ml of water was added with stirring to a cooled solution of PS-A (500mg) in 50 ml of 10%-MeOH and kept at 4°C for one week, being shaken occasionally. To this reaction mixture 1 gm of Na-borohydride was added and kept at room temperature overnight. The reaction mixture was acidified to pH 2 through the carefull addition of d-H_2SO_4 and kept at room temperature for 2 days with occasional shaking. The reaction product was extracted with an excessive amount of Et_2O, and concentrated to a colourless syrupy residue. Triturating this residue with a small volume of benzene gave an amorphous powder, mp. 145°, which showed two spots on T.L.C., the lower one being majour component(Solvent; C). By compareing the majour spot with the reference compound, which was prepared by the method of Shibata et al,[6] this spot was identified as 20S-protopanaxatriol. The amorphous powder was converted to panaxatriol by refluxing with 5%-H_2SO_4(50%-MeOH) for one hour.

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