PHARMACOLOGICAL SEQUENTIAL TRIALS FOR THE FRACTIONATION OF COMPONENTS WITH HYPOGLYCEMIC ACTIVITY IN ALLOXAN DIABETIC MICE FROM GINSENG RADIX

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Three methods of fractionation of ginseng radix (*Panax ginseng* C.A. MEYER) components for a survey of hypoglycemic principle in alloxan diabetic mice were conducted and three groups of components tested; fat-soluble components, ginseng saponins and a third component with hypoglycemic activity. Pharmacological sequential trials of the fractionation yielded a most active fraction which was about 100-fold more effective than the original water-soluble extract of the ginseng radix. The ED₅₀ value was 0.4 mg/kg in lowering the blood level of glucose in alloxan diabetic mice. It was demonstrated that some ginseng fractions inhibited epinephrine-induced transient hyperglycemia in mice, increased glycogen content in rat liver, decreased the blood level of acetone bodies in alloxan diabetic mice, and inhibited the release of free fatty acid from rat epididymal fat pad. The results showed that hypoglycemic components existed in a new component of ginseng radix which is different from saponin.

Keywords—ginseng radix; hypoglycemic effect; alloxan diabetic mice; sequential fractionation; new hypoglycemic component

Panax ginseng C.A. MEYER is one of the crude drugs which is frequently used by Chinese, Korean and Japanese as a traditional medicine. It has long been used in various blended preparations (Kanpō Hōzai) with expectation of anti-diabetic effect, e.g. in Byakko-ka-Ninjin-tō, Chikuyō-Sekkō-tō and Bakumondō-tō. It is demonstrated¹⁾ that ginseng radix played a significant role in the hypoglycemic effect of Byakko-ka-Ninjin-tō on alloxan diabetic mice. As little attention has been drawn to the hypoglycemic effect²⁾ of ginseng radix, we attempted to obtain components effective in lowering the blood level of glucose in alloxan diabetic mice. Pharmacological^{3–5)} and chemical⁶⁾ properties of

various components of ginseng radix have been extensively investigated and determination of molecular structure of ginseng saponins^{7,8)} has led to the investigation of pharmacological activity of the saponins.⁹⁾ The purpose of this paper is to demonstrate that the saponins are not exclusive components responsible for the pharmacological action of ginseng radix, especially in respect to its hypoglycemic action.

METHODS AND MATERIALS

Animals — Male mice of dd-strain weighing 20–25 g and male rats of Wistar strain weighing 150–200 g were used. The animals were fasted for about 18 hr before each experiment.

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Alloxan Diabetic Mice — Alloxan monohydrate (75 mg/kg) dissolved in 0.9% NaCl solution was injected into a tail vein of mice. Seven days after the injection, blood glucose levels were determined at fasting, and the mice with a glucose level of 150 mg% or more were used as alloxan diabetics.

Assay for Blood Levels of Glucose and Acetone Bodies — Blood samples (20 μ l for glucose and 50 μ l for acetone bodies) were obtained from the orbital venous plexus using capillary glass tubes. The glucose level was determined by the method of Momose *et al.*¹⁰⁾ and the level of total acetone-bodies (acetone, acetoacetic acid and β -hydroxybutyric acid) by the method of Ahola *et al.*¹¹⁾

Evaluations of Hypoglycemic Activity — Alloxan diabetic mice were bled, injected *i.p.* with a ginseng fraction, and bled again 6 hr after the injection. Each blood glucose level in the first sample [B] and the second sample [A] was determined, and hypoglycemic activity of each ginseng fraction was expressed in terms of Fall% calculated by the following equation.

$$\text{Fall\%} = \frac{[B] - [A]}{[B] - 85} \times 100$$

This equation means that 100% is obtained when a blood glucose level in alloxan diabetic mice decreased to 85 mg% that is the mean blood glucose level in normal fasted mice of dd-strain. Median effective doses with its 95% confidence limits were calculated and used.

Assay for Glycogen Content — Four rat hemidiaphragms were incubated in an atmosphere of 95% O₂ : 5% CO₂ for 90 min at 37.5° in 4 ml Krebs-Hensleit bicarbonate buffer solution (KHBB) containing 300 mg% glucose in the absence or presence of a compound tested. In some experiments, the diaphragm and the liver were isolated 5 hr after the *i.p.* injection of a ginseng fraction. Glycogen was extracted by digestion of a 200 mg portion of the tissues in 0.4 ml of 30% KOH solution followed by precipitation of glycogen with 1.2 ml of 95% ethanol. Glycogen contents were determined according to the current method¹²⁾ using anthrone reagent containing

0.05% anthrone, 1% thiourea and 66% H₂SO₄ by volume.

Assay for Glucose Uptake — Two rat hemidiaphragms were incubated in 2 ml KHBB containing 200 mg% glucose under similar conditions to those of the assay for glycogen content. A decrease in glucose of the medium during the incubation period was expressed as the amount of glucose taken up by the diaphragms.

Assay for Release of Free Fatty Acid (FFA) — The epididymal fat pads were isolated from decapitated rats. Each fat pad weighing about 200 mg was placed in a test tube and cut into pieces. The adipose tissues were incubated for 2 hr at 37° in 2 ml KHBB, pH 7.2, containing 100 mg% glucose, 400 mg% bovine serum albumin and 0.02 μ g/ml isoproterenol in the presence or absence of a compound tested. Free fatty acids released to the incubation medium were deter-

FIG. 1. Fractionation I (for fat-soluble components) from Ginseng Radix (HAKUSAN)

mined colorimetrically according to the method of Itaya *et al.*¹³⁾

Compounds used — Ginseng radix was obtained from Takeda Chemical Industry Co., Ltd., tolbutamide and metformin from Toyama Chemical Industry Co., Ltd., carbutamide from Sumitomo Chemical Co., Ltd. and bovine crystalline insulin from Shimizu Seiyaku Co., Ltd. Others were obtained from commercial sources.

RESULTS

Isolation and Purification of Components with Hypoglycemic Activity from Ginseng Radix

Experiments for the survey of ginseng components with hypoglycemic activity were performed by application of three designs for fractionation and by using alloxan diabetic mice for a bioassay of the hypoglycemic activity.

The first design was made on the basis of an assumption that the hypoglycemic components were fat-soluble (Fig. 1). This classical assumption is widely accepted.

The second design was made to test hypoglycemic activity of the ginseng saponins which are not extracted from the fat-soluble fractions described. Ginsenoside R_g was isolated by the fractional precipitation with organic solvent followed by the chromatography on silica gel (Fig. 2).

The third design was made to perform pharmacologically sequential trials for fractionation. No assumption was made in respect to physical and chemical properties for the target components. A pharmacologically active, crude water-soluble extract was taken as an original material. Fig. 3 shows fraction procedures proposed from the results of trial and error for the pharmacological survey of the active components. Some portion of DPG-3 was subfractionated using a chromatography on silica gel eluted with chloroform and methanol. Hypoglycemic activity of each fraction expressed in terms of the ED₅₀ value and its yield are summarized in Table I.

The fat-soluble fractions (I in Table I) were

FIG. 2. Fractionation II (for isolation and purification of ginsenoside R_g) from Ginseng Radix Hair (HIGENINJIN)

FIG. 3. Fractionation III (Pharmacological sequential trials for components with hypoglycemic activity in alloxan diabetic mice) from Ginseng Radix (HAKUSAN)

TABLE I. Effect of Various Ginseng Fractions (in Fig. 1, 2, 3) on Blood Glucose Levels in Alloxan Diabetic Mice

Fractiona- tion	Fraction	Yield ^{a)} %	ED ₅₀ (95% confidence limits), mg/kg or Fall% ± S.E. at a given dose
I	D-8151		0.8 ± 15.0 at 40 mg/kg
	D-81513		1.5 ± 15.8 at 40 mg/kg
	D-81516		-35.9 ± 8.1 at 40 mg/kg
II	DC-1		52.7 (1.2-2290)
	DC-2		42.9 (10.8-169)
III	DPG-1	100	43.1 (13.5-139)
	DPG-2	Trace	
	DPG-3	3.9	8.8 (4.3-17.8)
	DPG-4	2.4	-26.4 ± 19.4 at 100 mg/kg
	DPG-5	93.7	56.3 (2.9-110)
	DPG-6		26.3 (13.5-51.3)
	DPG-3 IC1-3	0.2	1.5 (0.9-54.6)
	DPG-3 IC4-7	0.2	12.7 (1.6-104)
	DPG-3 IC8-9	0.2	10.6 (5.0-21.8)
	DPG-3 IC15-19	0.6	3.7 (0.05-262)
	DPG-3 IC20-22	0.3	0.41 (0.32-0.53)
	DPG-3 IC28	1.6	10.4 (5.9-18.6)

a) The yield was calculated as weight ratio to the original extract DPG-1.

TABLE II. *Yield of Ginsenosides and Hypoglycemic Activity in Ginseng Fractions*

	Yield, %				Hypoglycemic ED ₅₀ , mg/kg	
	Original extract		<i>n</i> -BuOH fraction		Original extract	3–2 ^{a)} fraction
	Whole saponin		Whole saponin			
DPG-series (H ₂ O extraction)	32	0.48	1.2	0.35	43.1	8.9
EPG-series (MeOH extraction)	6.7	1.34	1.9	1.28	70.5	17.9

The yield was calculated as weight ratio to the ginseng radix (HAKUSAN) used.

a) Each 3–2 fraction is a dialyzable portion of each *n*-BuOH soluble fraction, e.g., dialyzable DPG-3 is DPG-3-2.

ineffective in lowering the blood glucose level, and the ginsenoside R_g-rich fraction (II in Table I) were not more effective than the crude water-soluble extract (DPG-1 in Table I). Ginsenoside R_{g1} purified from DC-1 and -2 caused no hypoglycemic effect at 120 mg/kg. The hypoglycemic components drawn from DPG-1 were mainly collected in DPG-3, and subsequently in DPG-3 IC20-22. This fraction was about 100-fold more effective than the crude water-soluble extract DPG-1. DPG-3 IC20-22 contained unknown substances as major components and ginsenoside R_g and R_e as minor components, which were detectable by heating with 10% H₂SO₄ as a coloring reagent. On the contrary, DPG-4 containing ginsenoside R_b, R_c and D-81516 were shown to increase blood glucose level in alloxan diabetic mice.

Efficiency of the method for original extraction on the isolation of components with hypoglycemic activity from ginseng radix was determined in connection with that of saponins. For this purpose, two series of fractionation different only in the method for original extraction were carried out (Table II). Water-soluble extract (DPG-series) had smaller content of saponin and gave an *n*-BuOH fraction with smaller content of saponin than methanol-soluble extract (EPG-series). Each fraction derived from water-soluble extract had higher hypoglycemic potency than the corresponding fraction from methanol-soluble extract. Therefore, water-soluble extract was chosen as the material for a survey of hypoglycemic components from ginseng radix.

lycemic components from ginseng radix.

Effect on Carbohydrate Metabolism

Effect of EPG-3-2 fraction on the blood level of glucose in normal mice was studied. At a dose over the ED₅₀, value in lowering the level in alloxan diabetic mice, it did not influence the glucose level in normal mice at all (Table III). Transient hyperglycemia induced by epinephrine in normal mice was inhibited by DPG-1 at a dose about 9-fold higher than the ED₅₀ in alloxan diabetic mice (Fig. 4). The same dose of DPG-1 inhibited a glucose tolerance curve in normal mice in general (Fig. 5). Tolazoline, one of the α -adrenergic blockers, inhibited a humping pattern of the glucose tolerance curve. Whereas, DPG-1 at a high dose increased glycogen content in rat liver when injected into normal whole animals (Table IV). In isolated rat diaphragms, however, it showed no such effect at all. The ginseng component caused no stimulatory effect on glucose

TABLE III. *Effect on Blood Glucose Levels in Normal dd-Strain Mice*

Compound	Decreased blood glucose level, mg%
EPG-3-2	0.1 ± 3.4
Tolbutamide	11.3 ± 2.3 ^{a)}
Metformin	2.0 ± 2.2

Each value represents mean ± S.E. of 10–15 mice (25 mg/kg, 6-hr treatment).

a) Significantly different (one-tail *t*-test, *p* < 0.05).

FIG. 4. Effect of DPG-1 on Transient Hyperglycemia induced by Epinephrine in Normal Mice
DPG-1 was injected i.p. at 360 mg/kg 3 hr before the injection of epinephrine. Each point represents mean $\pm$ S.E. of 8 mice.

FIG. 5. Effect of DPG-1 and Tolazoline on Glucose Tolerance Curve in Normal Mice
DPG-1 was injected i.p. at 360 mg/kg 6 hr before the glucose injection, and tolazoline i.v. plus s.c. at 10 mg/kg 10 min before. Each point represents mean $\pm$ S.E. of 4 mice.

TABLE IV. Effect of Ginseng Fraction on Glycogen Content in Intact and Isolated Rat Preparations

Compound		Glycogen content, mg/kg ^{a)}		
		Isolated diaphragm	Intact Diaphragm	Liver
Control		2.2 $\pm$ 0.2	1.1 $\pm$ 0.1	2.6 $\pm$ 0.6
DPG-1	1.0 mg/ml	2.4 $\pm$ 0.3		
	200 mg/kg i.p.		1.4 $\pm$ 0.2	4.5 $\pm$ 0.8 ^{b)}
Insulin	100 mU/ml	4.0 $\pm$ 0.8 ^{b)}		

Each value represents mean $\pm$ S.E. of 4 experiments.

a) Glycogen content is expressed as the amount of equivalent glucose per wet weight of the tissues.

b) Significantly different from corresponding control (one-tail t-test, $p < 0.05$).

TABLE V. Effect of Ginseng Fractions on Blood Acetone-bodies Levels in Alloxan Diabetic Mice

Fraction	mg/kg	Acetone-bodies decreased ^{a)} μ mol/ml
DPG-1	120	0.265 $\pm$ 0.076 ^{b)}
DPG-3	12	0.231 $\pm$ 0.089 ^{b)}
DPG-5	173	-0.385 $\pm$ 0.184
DPG-6	38.7	0.202 $\pm$ 0.048 ^{b)}

Each value represents mean $\pm$ S.E. of 8–10 mice.

a) Total acetone-bodies concentration decreased below the control level during the 6-hr period after the i.p. injection of a ginseng fraction tested. The control value was 1.718 $\pm$ 0.189 μ mol/ml (mean $\pm$ S.E.)

b) Significantly different (one-tail t-test, $p < 0.05$).

uptake by isolated rat diaphragms, since an increase in the amount of glucose taken up by rat diaphragms incubated with 0.1 mg/ml DPG-3 was 0.06 $\pm$ 0.12 mg/g diaphragm (mean $\pm$ S.E., $n=10$, $p > 0.05$). Similar results were also obtained in the presence of 0.1 mg/ml DPG-3 IC20-22.

Effect on Lipid Metabolism

Effect of the ginseng fractions on the blood level of acetone bodies in alloxan diabetic mice was studied at each dose causing definite hypoglycemic effect on alloxan diabetic mice. Table V shows that the ginseng fractions except DPG-5 lowered the level of acetone bodies in parallel

TABLE VI. *Effect on FFA Release from Epididymal Fat Pad Stimulated by Isoproterenol*

Compound		FFA release, $\mu\text{eq/g/2 hr}$
Control		7.85 ± 1.60
EPG-3-2	0.5 mg/ml	$1.30 \pm 0.35^a)$
Insulin	1 mU/ml	$3.30 \pm 1.60^a)$

Each value represents mean $\pm$ S.E. of 9 experiments.

a) Significantly different from control (one-tail t-test, $p < 0.05$).

with the hypoglycemic potency. Carbutamide and metformin at doses over 150 mg/kg were ineffective in lowering the level of acetone bodies. Also, DPG-3-2 inhibited the isoproterenol-stimulated FFA release from isolated rat epididymal fat pad (Table VI).

DISCUSSION

Out of three experimental designs for fractionation, a pharmacological sequential trial alone yielded components with hypoglycemic activity in alloxan diabetic mice from ginseng radix. The most active fraction obtained consisted of unknown substances as major components and ginseng saponins (ginsenoside- $R_{g,e}$) as minor components. Ginsenoside- R_g rich fraction (DC-1, 2) lowered the blood glucose level to some extent but purified ginsenoside R_{g1} showed no such effect. DPG-4 containing ginsenoside- $R_{b,c}$ caused hyperglycemic effect. When a methanol-soluble extract was chosen as an original, each fraction derived from it was richer in saponins but not more effective in lowering the blood level of glucose than the corresponding fraction derived from a water-soluble extract. Hence, the hypoglycemic effect of ginseng fraction does not appear to be related to the saponins present as minor components. Ginseng radix contains fat soluble components, some of carbohydrate, amino acids, some of vitamins and choline beside saponins.⁶⁾ The hypoglycemic component may be a new principle different from saponin. It was evidenced that a substance proposed in a chemical but not in a pharmacological sense does not essentially mean

the main active component of an original material and, therefore, pharmacological sequential trial for fractionations is the most practical procedure for a survey of bioactive components. It is important that the total biological activity of the original material used should be recovered in an active component derived from it. In practice, both the ED_{50} value of each fraction and its yield are needed to determine whether or not it is the pharmacologically causative component. It is desirable to propose the term "component-activity causation" as a criterion. The component-activity causation is the basis for a pharmacological sequential trial for fractionation.

It is shown that experimentally pathologic animals were of great advantage for the assay for pharmacological activity of ginseng fractions, because the hypoglycemic fraction from ginseng radix was effective in lowering the blood level of glucose in alloxan diabetic mice but ineffective in normal mice. This observation seems to reflect such a characteristic in Chinese traditional medicines that the therapeutic effect is prominent in patients with disease conditions. At a dose far higher than that effective in alloxan diabetic mice, DPG-1 inhibited epinephrine-induced hyperglycemia. A high dose of DPG-1 lowered a glucose tolerance curve in both stages of glucose utilization and humping pattern.¹⁴⁾ These results suggest that the hypoglycemic fraction from ginseng radix did not antagonize epinephrine but stimulated the glucose utilization. The fraction caused also an increase of glycogen content in intact rat liver. In isolated rat diaphragm, it was not observed, however, that the ginseng fractions increased glycogen content or stimulated glucose uptake. These results suggest that the action of ginseng fractions on blood glucose level is indirect in large part and perhaps dependent on, e.g., insulin released from B-cell in pancreatic islets. In addition to the hypoglycemic effect, the main active fraction exhibited lowering effect on the blood level of acetone bodies in alloxan diabetic mice and on the isoproterenol-induced FFA release from isolated fat pad. Carbutamide and metformin showed no such effect. The action of

the ginseng extract on lipid metabolism seems to be, at least in part, direct on the adipose tissue. Hence ginseng extract seems to accelerate carbohydrate and/or lipid metabolism *via* direct and/or indirect mechanisms.

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