Studies on Selective Modulators and Anti-anorexigenic Agents in Korean Red Ginseng.

Hiromichi Okuda, Keizo Sekiya, Hiroshi Masuno, Takeshi Takaku* and Kenji Kameda*

2nd Department of Medical Biochemistry, School of Medicine, Ehime University, Shigenobu, Onsen-gun, Ehime, Japan 791-02

**Central Research Laboratory, School of Medicine, Ehime University, Shigenobu, Onsen-gun, Ehime, Japan 791-02*

Summary

Isolated rat adipocytes are well known to possess opposite pathways of lipid metabolism: lipolysis and lipogenesis. Both of the metabolism respond to various biologically active substances such as epinephrine, ACTH and insulin. Epinephrine and ACTH stimulate lipolysis and insulin accelerates lipogenesis.

Recently, Korean red ginseng powder was found to contain adenosine and an acidic poptide which inhibited epinephrine-induced lipolysis and stimulated insulin-mediated lipogenesis from added glucose. The acidic peptide is consisted mainly of glutamic acid and glucose. Ginsenosides Rb_1 and Re inhibited ACTH-induced lipolysis in isolated rat adipocytes, while they did not affect insulin-stimulated lipogenesis. Thus, all these substances extracted from Korean red ginseng exhibited selective modulations toward the opposite metabolic pathways in rat adipocyte; They inhibited the lipolysis but not the lipogenesis. We call these substances"selective modulators". Recently, we isolated a toxic substance named "toxohormone-L " from ascites fluid of patients with various malignant tumors. The toxohormone-L stimulated lipolysis in rat adipocytes and induced anorexia in rats. Both the lipolytic and the anorexigenic actions of toxohormone-L were found to be inhibited by ginsenoside Rb_2 in Korean red ginseng.

Based on these results, physiological significances of these substances in Korean red ginseng were discussed.

Panax ginseng is a medicinal plant long used in treatment of various pathological states including general complaints such as head ache, shoulder ache, chilly constitution and anorexia in cancer patients. There have been many pharmacological studies on *Panax ginseng* roots. Petkov[1]reported that oral administration of an aqueous alcoholic extract of ginseng roots decreased the blood sugar level of rabbits. Saito[2]reported that *Panax ginseng* suppressed hyperglycemia induced by epinephrine and high carbohydrate diets. These findings suggest that *Panax ginseng* roots contain insulin-like substances. Previously, we[3]demonstrated that ginseng roots contain an insulin-like peptide which inhibits epinephrine-induced lipolysis and stimulated insulin-mediated lipogenesis.

In 1984, we suggested that such an insulin-like substance should be called a selective modulator[4]. Present investigation describes the details of the selective modulators in ginseng roots.

During progressive weight loss in patients with various neoplastic disease, depletion of fat stores have been observed. The depletion of body fat during growth of neoplasms is associated with increase in plasma free fatty acids.

Recently, we[5]found that the ascites fluid

from patients with hepatoma or ovarian tumor and the pleural fluid from patients with malignant lymphoma elicited fatty acid release in slices of rat adipose tissue *in vitro*. The lipolytic factor, named"toxohormone-L". was purifed from the ascites fluid of patients with hepatoma. The isolated preparation gave a single band on both disc gel electrophoresis and sodium dodecyl sulfate(SDS)-acrylamide gel electrophoresis in the presence of β-mercaptoethanol. Its molecular weight was determined to be 70,000-75,000 and 65,000 by SDS-acrylamide gel electrophoresis and analytical ultracentrifugation, respectively. Injection of toxohormone-L into the lateral ventricle of rats significantly suppressed food and water intakes. There was at least 5 hr delay between its injection and appearance of its suppressive effect.

In the present study, we also tried to find a inhibitory substance toward toxohormone-L from root powder of ginseng.

Materials and Methods

Animals

Young male Wistar King rats, weighing 160 to 200g, were given standard laboratory diet and water *ad lib.* They were sacrificed by a blow on the head, and their epididymal adipose tissues were quickly removed.

Panax ginseng

Panax ginseng powder(*Panax ginseng* C.M. Meyer) was kindly provided by Nikkan Korai Ninjin Co. Ltd., Kobe, Japan.

Measurement of anti-lipolytic activity

Isolated fat cells were prepared from rat epididymal adipose tissue by the method of Rodbell[6]. Fat cells equivalent of 100 mg of adipose tissue were incubated for 2 hr at 37℃ in Krebs-Ringer-phosphate buffer containing 2.5 % bovine albumin, 0.25 μg of adrenaline and test samples in a final volume of 1.0 m*l*. After incubation, 5 m*l* of Dole's extraction mixture was added and free acids released were estimated by the method of Dole[7]. One unit of anti-lipolytic activity was defined as the amount causing 10% inhibition of adrenaline -induced lipolytic activity.

Protein determination

Protein was estimated by the method of Lowry *et al*[8].

Column chromatography

Gel filtration was carried out on a Bio Gel P-2 column. Material was eluted with water or 0.01 N HCl. Dowex 50 W×4(200-400 mesh, in H^+ form) was used. Elution was performed with a linear gradient of 0 to 2 N HCl. An avicel column was washed with methanol and material was eluted with methanol: water(9: 1, pH 2.0 with HCl).

Thin layer chromatography

Thin layer chromatography was carried out on Silicagel 60 with n-butanol: acetic acid:

Table I. *Purification of Anti-lipolytic Substance.*

Procedure		Total activity (units×10^3)	Recovery (%)
Crude extract		1399	100.0
Inner dialysate		279	19.9
Outer dialysate		1184	84.6
Bio Gel P-2 column chromatography			
Fraction	I	0	0
	II	359	25.7
	III	100	7.2
	IV	256	18.9
	V	0	0
	VI	0	0
	VII	0	0
2nd Bio Gel P-2 column chromatography			
Fraction	I	0	0
	II	0	0
	III	0	0
	IV	0	0
	V	189	13.5
	IV	0	0
Dowex 50w×4 column chromatography			
Main peak		192	13.7

water(3:1:1, by vol.) or ethanol: water(50:50) as developing solvent.

High-speed liquid chromatography

High-speed liquid chromatography was done using a μ Bondapac C_{18} Waters column with MeOH:H_2O(15:85) as solvent.

Preparation of trimethylsilyl derivatives

Lyophilized material(50-100 μg) was heated with 100 μl of N, O-bis(trimethylsilyl) trifluoroacetamide and 1.5 μl of trimethylchlorosilane at 80℃ for 1 hr in a sealed ampoule on a glycerol bath. Adenosine was also trimethylsilylated by the same procedure.

Instrumentation

Gas chromatographic-mass spectrometric analysis was carried out with helium as carrier gas in a JMS-D 300 Mass Spectrometer. The column was packed with 80-100 mesh AW-D-MCS chromosorb W coated with 2% OV-17 silicone.

Results and Discussion

Panax ginseng powder was extracted with 9 volumes of water at 4℃ for 24 hr. The extract was centrifuged and the supernatant was concentrated and dialyzed against water at 4℃ for 24 hr. This procedure was repeated once more. The combined outer dialysate showed about 85% of the anti-lipolytic activity of the extract(Table 1). The outer dialysate was concentrated and applied to a Bio Gel P-2 column. As shown in Fig.1, anti-lipolytic activity was eluted mainly in fractions II and IV. Fraction IV had high absorption at 260 nm. Fraction IV was rechromatographed on a Bio Gel P-2 column as shown in Fig.2. The active fraction was collected and applied to a Dowex 50 W×4 column and material was eluted with a linear gradient of 0 to 2 N HCl.

As shown in Fig.3, all the anti-lipolytic activity was eluted in the main peak. This fraction also had high absorption at 260 nm(Fig.4). The absorption ratio(A 280 nm/A 260 nm) of the fraction was found to be 0.186, which is the same as that of adenosine. The active fraction was subjected to Silicagel 60 thin layer chromatography. The Rf value of the fraction

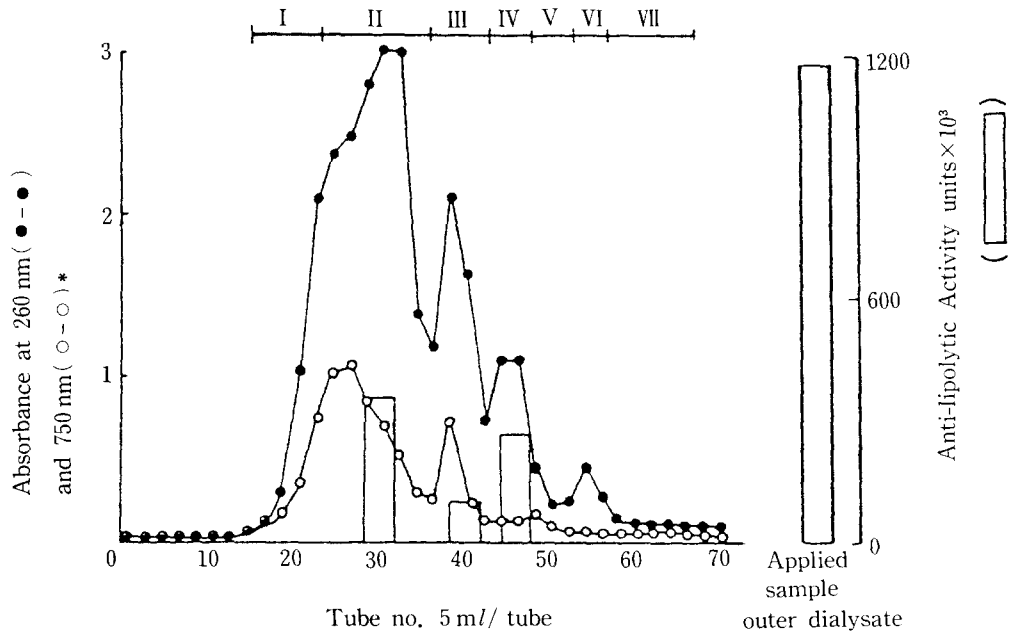

Fig.1. *Gel filtration of the outer dialysate on a Bio Gel P-2 column. Column size, 2.2×43 cm. Elution was carried out with water. *; Absorption of protein in the method of Lowry et al.*

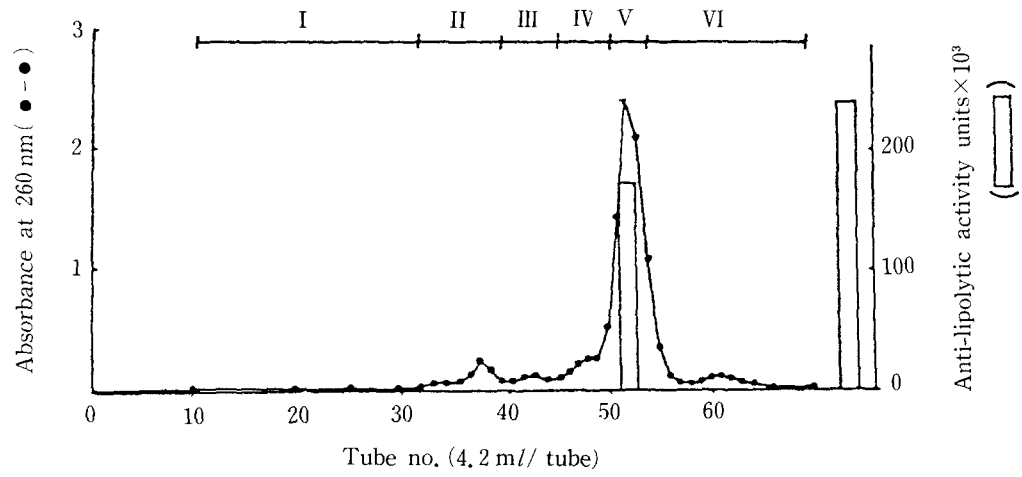

Fig.2. *Rechromatography of fraction IV from Bio Gel P-2. Column size, 2.2×43 cm. Elution was carried out with water(4.2 ml/tube)*

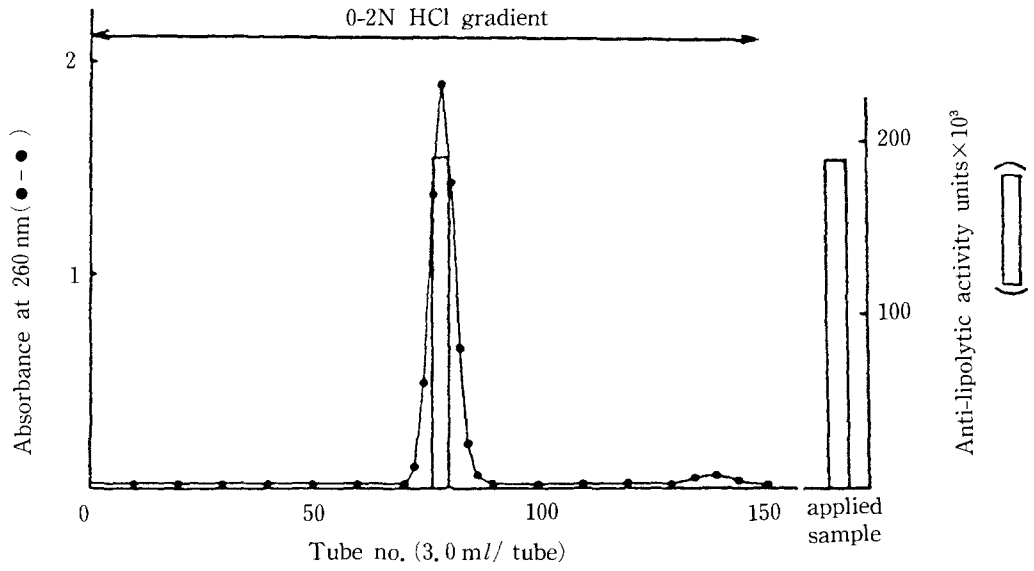

Fig.3. *Ion exchange chromatography on a Dowex 50 W×4 column. Column size, 1.0×15 cm(3.0 ml/ tube)*

was found to be the same as that of adenosine. Then the fraction was subjected to high-speed liquid chromatography. As shown in Fig.5, the peak material showed the same retention time as adenosine. Next, the fraction was analyzed by mass spectrometry. The mass spectra of the TMS-derivatives of the fraction and adenosine were compared. The TMS-derivative of the fraction show the same intensity of each fragment ion as that of the TMS-derivative of adenosine(Fig.6). From these findings, the active principle was identified as adenosine. The presence of adenosine in *Panax ginseng* has already been reported by Hiyama *et al*[9].

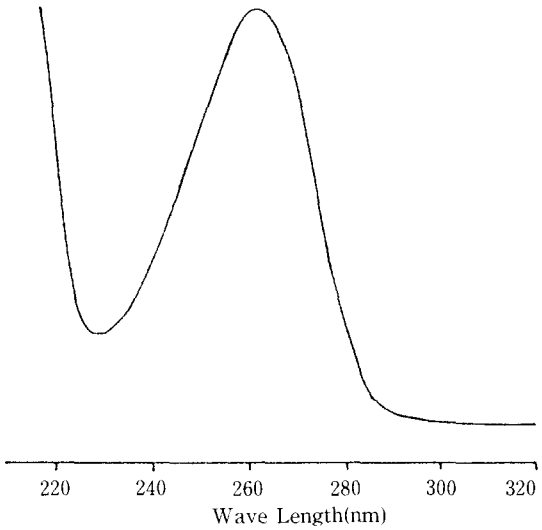

Fig.4. *Absorption spectrum of the main peak from the Dowex 50 W×4 column.*

Adenosine is widely distributed in living material, but its content in *Panax ginseng* is especially high. In 1961, Dole[10] observed that nucleic acids, uncleotides and uncleosides inhibit the lipolytic action of epinephrine on adipose tissue. Other lipolytic hormones, such as adrenoacor-

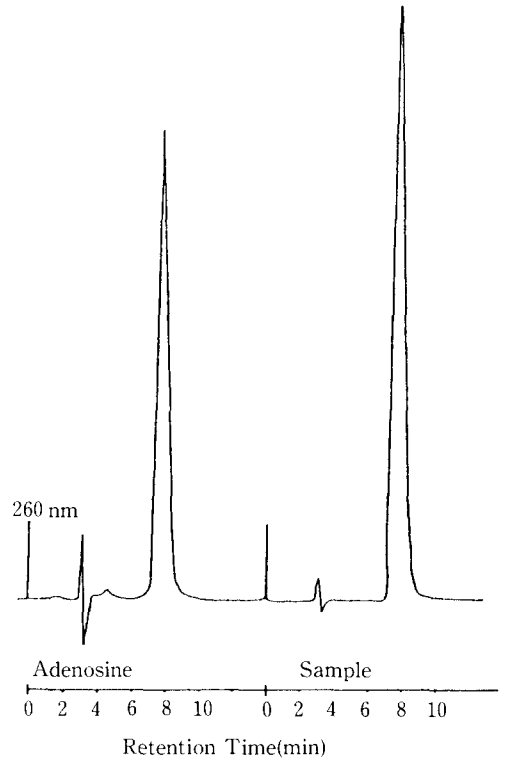

Fig.5. *High-speed liquid chromatogram of the main peak from the Dowex 50 W×4 column.*

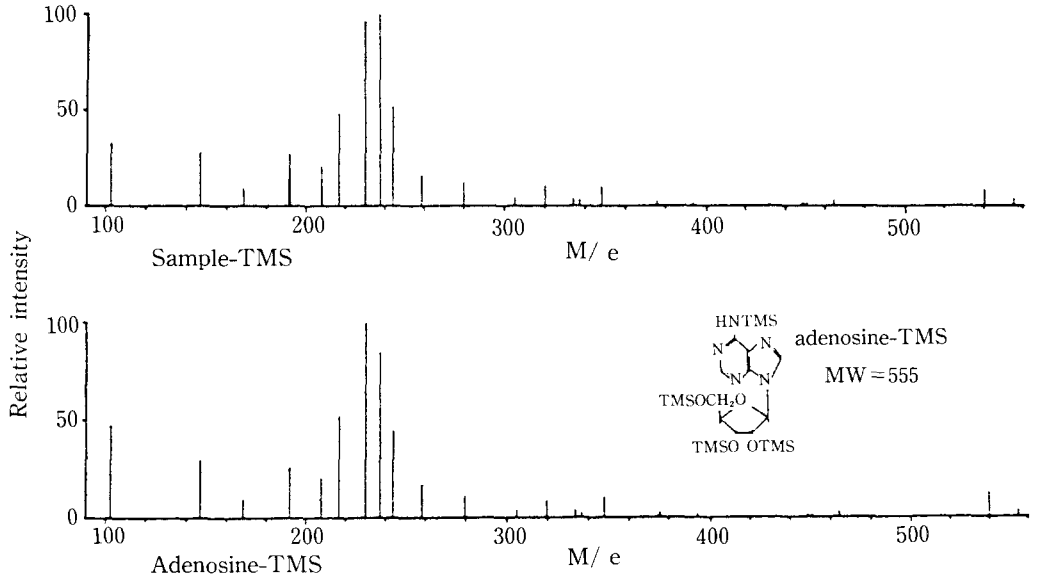

Fig.6. *Mass spectra of TMS derivatives of adenosine and the main fraction from the Dowex 50 W×4 column*

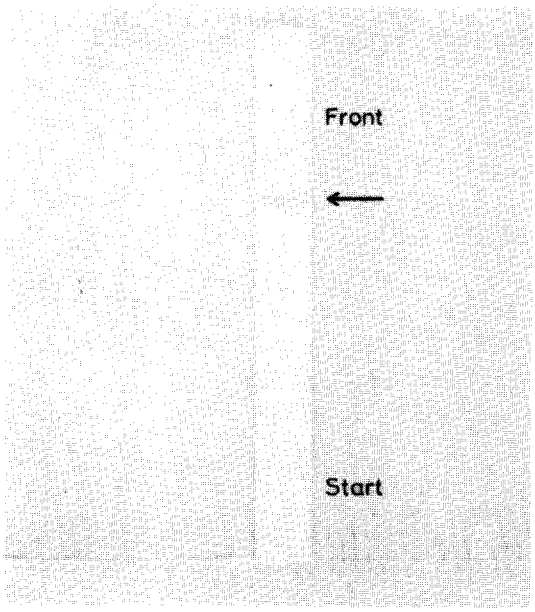

Fig.7. *Thin layer chromatography of ginseng peptide. Arrow indicates a ninhydrin positive band.*

ticotropic hormone, thyroid-stimulating hormone and growth hormone were also inhibited by nucleic acid[10]. He also reported that adenosine stimulated lipogenesis from glucose and from acetate in the presence of glucose, and inhibited synthesis of glycerides from lactate or pyruvate[11].

In this laboratory, it was found that adenosine has an anti-lipolytic effect on lipolysis induced by adrenocorticotropic hormone and glucagon in fat cells and that it stimulates lipogenesis from glucose in the cells(unpublished data). Namely, adenosine has selective modulating activities for inhibition of lipolysis and stimulation of lipogenesis.

Fraction II from the first Bio Gel column was then applied to Dowex-2 column(Cl^- form), washed with water and eluted with 0.05 N HCl. The eluate was subjected to dialysis with dialysis membrane to remove larger molecules than 1,000 dalton and the outer dialysate was concentrated. The concentrated material was applied to Sephadex G-25 column and eluted with water. The fractions which contained the active substance, were collected and concentrated. The concentrated solution was then subjected to thin layer chromatography with ethanol and water(50:50, v/v) as developing

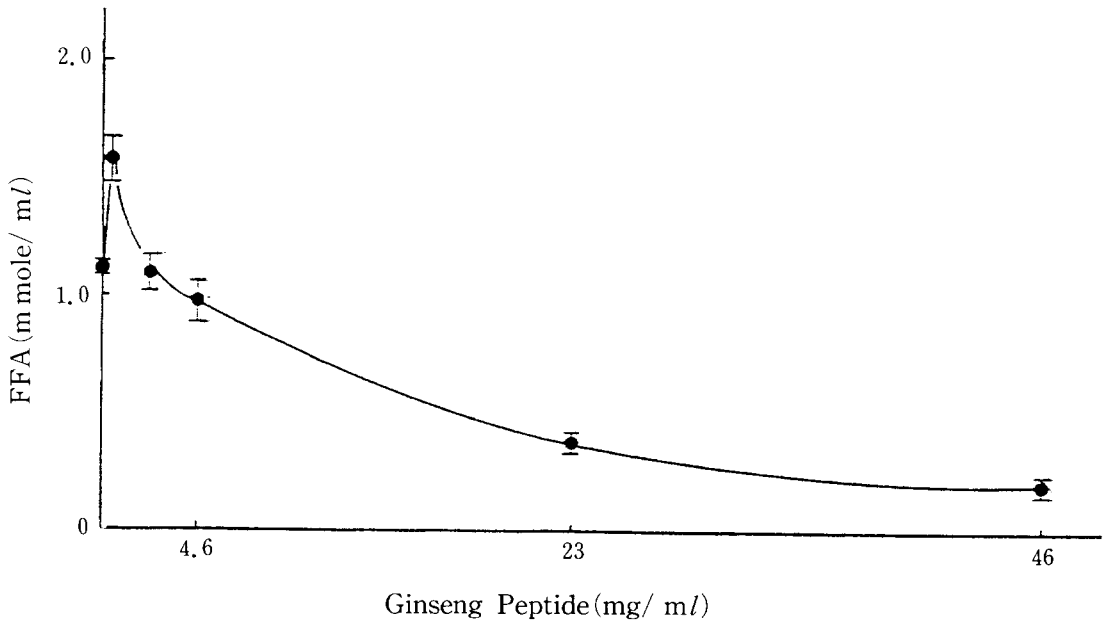

Fig.8. *Effect of ginseng peptide on epinephrine-induced lipolysis in fat cells.*

solution. Only one ninhydrin-positive band was found in the thin layer chromatography as shown in Fig.7. The ninhydrin-positive band was scraped off and extracted with water. Epinephrine-induced lipolysis in fat cells was clearly inhibited by the ninhydrin positive fraction, while insulin-stimulated lipogenesis was enhanced by the fraction as shown in Fig.8 and 9. There is no free amino acid in the ninhydrin-positive fraction. The ninhydrin-positive fraction was found to contain glutamic acid and glucose after its acid hydrolysis. There is one mole of glucose per 6 moles of glutamic acid in this fraction. Peptide sequence analysis was carried out by dansyl technique described by Hartley[12]. N-Terminal amino acid and suc-

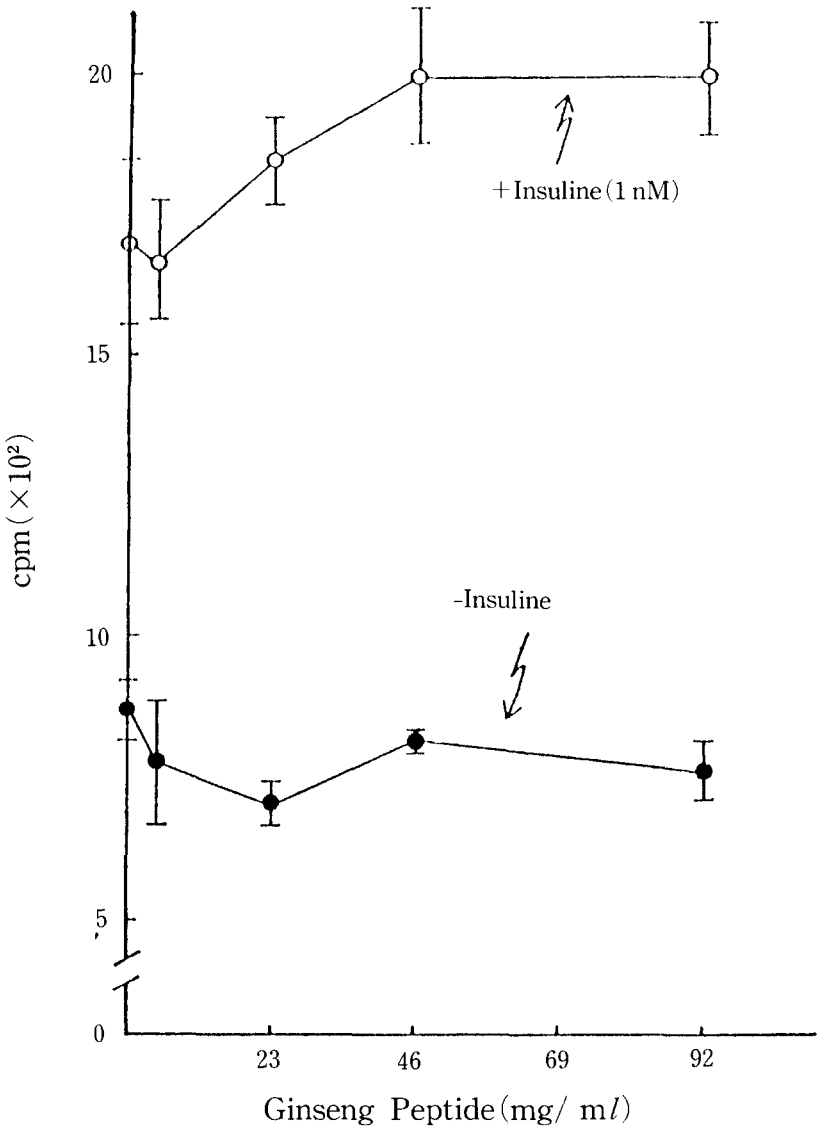

Fig.9. *Effect of ginseng peptide on lipogenesis from glucose in fat cells.*

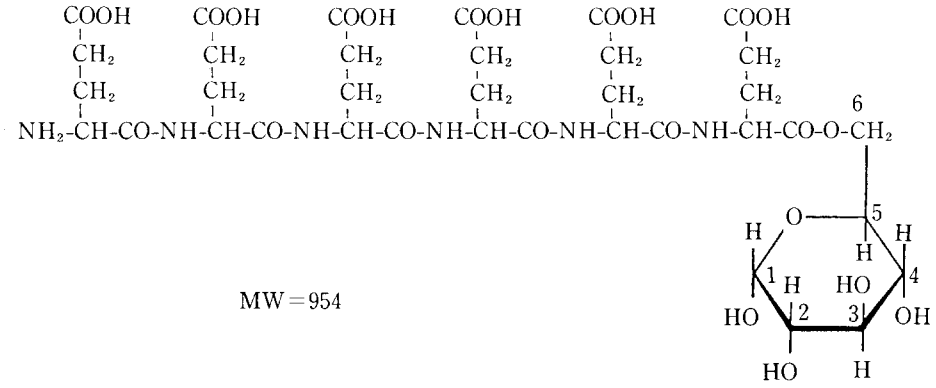

Fig.10. *Hypothetical structure of ginseng peptide.*

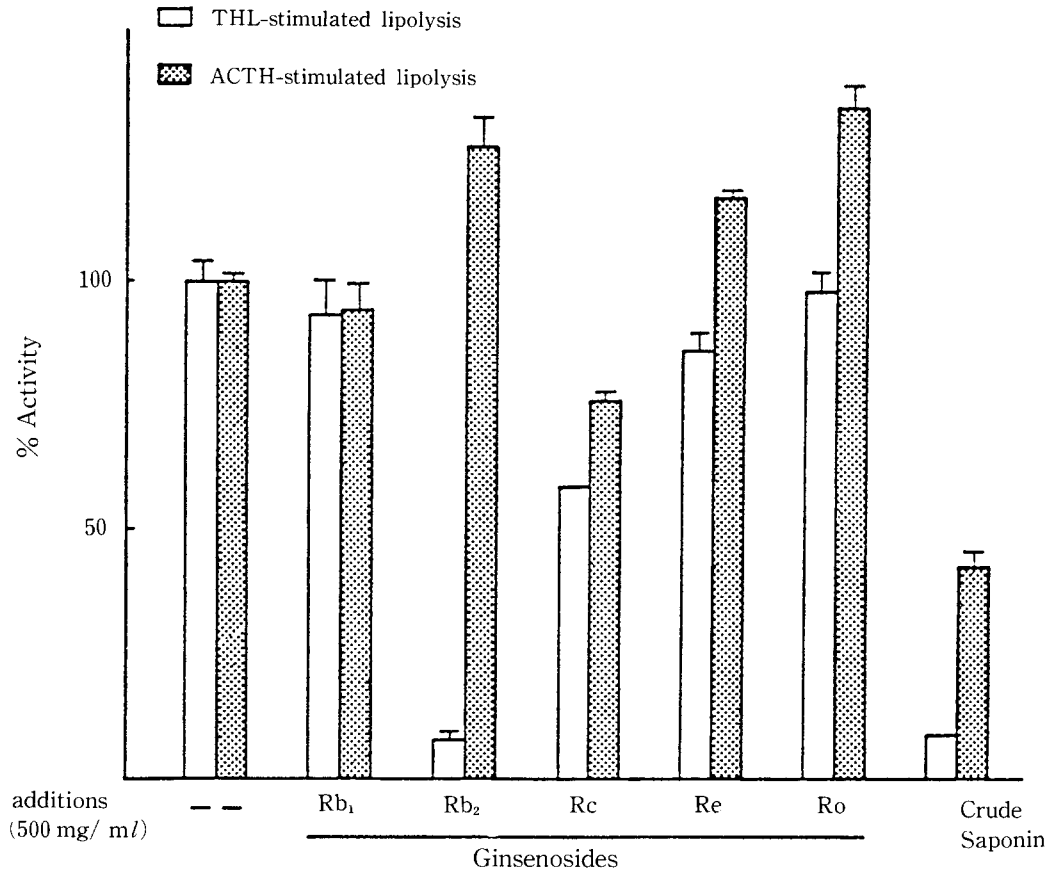

Fig.11. *Effects of ginsenosides of toxohormone(THL)-and ACTH-atimulated lipolysis in fat cells.*

cessive 5 amino acid residues were found to be glutamic acids. Further examination is needed to elucidate the whole structure of this gluco -peptide. Fig.10 shows the hypothetical structure of ginseng peptide.

Isolated rat adipocytes are well known to possess opposite pathways of lipid metabolism: lipolysis and lipogenesis. Both of the metabolism respond to various biologically active substances such as epinephrine, ACTH and insulin. Epinephrine and ACTH stimulate lipolysis and insulin accelerates lipogenesis.

As mentioned above, *Panax ginseng* was found to contain selective modulators such as adenosine and acidic gluco-peptide which inhibited lipolysis and stimulated lipogenesis from glucose. In addition to these substances, the ginseng possesses another selective modulators. These are ginsenosides Rb_1 and Re which inhibit ACTH-indiced lipolysis and do not affect insulin-mediated lipogenesis.

Tumor-bearing animals and patients with various neoplasms frequently show a striking depletion of body lipid. This depletion could be related to growth of the tumor and could contribute to debilitation of the host.

Recently, we found that the ascites fluids from patients with hepatoma or ovarian tumor and the pleural fluid from patients with malignant lymphoma elicited fatty acid release in rat fat cells *in vitro*.

The lipolytic factor, named toxohormone-L, was isolated from the ascites fluid of patients with hepatoma. The isolated preparation gave a single band on disc gel electrophoresis and its molecular weight was determined to be 70,000. Injection of toxohormone-L into the lateral ventricle of rats significantly suppressed food and water intakes. Therfore, it follows that both lipolytic and anorexigenic actions of toxohormone-L secreted from tumor cells contribute to weight loss in patients with cancer. It is of interest that *Panax ginseng* contains some inhibitory substances toward the actions of toxohormone-L. One of these is ginsenoside Rb_2 as shwon in Fig.11. The ginsenoside Rb_2 inhibits toxohormone-L-induced lipolysis in fat cells, while it stimulates ACTH-stimulated lipolysis. An inhibitory action of ginsenoside Rb_2 on the anorexigenic action of toxohormone-L will be reported by Dr. Sakata in this symposium. Other inhibitory substance was found to be a polysaccharide as reported previously[4].

These results suggest that *Panax ginseng* may protect debilitation of cancer patients through inhibiting the lipolytic actions of toxohormone-L.

References

1. Petkov, W., *Arzneim. Forsch.* (Drug Res.) **11,** 288(1961).
2. Saito, T., *Keio Igaku,* **2,** 149(1922).
3. Ando, T., Muraoka, T., Yamasaki, N. and Okuda, H., *Planta Medica,* **38,** 18(1980).
4. Okuda, H., Masuno, H. and Lee, S.J., *Proc. 4th Int. Ginseng Symp.,* p.145 (1984).
5. Masuno, H., Yoshimura, H., Ogawa, N. and Okuda, H., *Eur. J. Cancer Clin. Oncol.,* **20,** 1177(1984).
6. Rodbell, M., *J. Biol. Chem.,* **239,** 375(1964).
7. Dole, V.P., *J. Clin. Invest.,* **35,** 150(1965).
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., *J. Biol. Chem.,* **193,** 265(1951).
9. Hiyama, C., Miyai, S., Yoshida, H., Yamasaki, K. and Tanaka, O., *Yakugaku Zasshi,* **98,** 1132(1978).
10. Dole, V.P., *J. Biol. Chem.,* **236,** 3125(1961).
11. Dole, V.P., *J. Biol. Chem.,* **237,** 2758(1962).
12. Hartley, B.S., *Biochem. J.,* **119,** 805(1970).