Proceedings of the 5th International Ginseng Symposium, Seoul, 1988

Studies on Selective Modulators and Antianorexigenic Agents in Korean Red Ginseng

Takeshi Takaku, Kenji Kameda, Yukinaga Matsuura*, Keizo Sekiya*,
Hideo Okai** and Hiromichi Okuda*

Central Research Laboratory, School of Medicine, Ehime University
* 2nd Department of Medical Biochemistry, School of Medicine, Ehime University
** Department of Fermentation Technology, Faculty of Engineering, Hiroshima University

Abstract

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. 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 substance which inhibited epinephrine-induced lipolysis and stimulated insulin-mediated lipogenesis from added glucose. The chemical structure of this acidic substance is determined to be pyro-glutamic acid (Pyro-Glu). Pyro-Glu exhibits selective modulations toward the opposite metabolic pathways in rat adipocyte ; It inhibits the lipolysis but stimulates the lipogenesis. We call these substances (adenosine, Pyro-Glu) "selective modulators" or "insulin-like substances". Based on these results, physiological significances of these substances in Korean red ginseng were discussed.

Introduction

Panax ginseng is a medicinal plant long used in treatment of various pathological states including general complaints such as head ache, shoudler ache, chilly constitution and anorexia in cancer patients.

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[1] 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 purified 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 65,200 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 tried to find a inhibitory substance toward toxohormone-L from red ginseng power. There have been many pharmacological stuidies on *Panax ginseng* roots. Petkov[2] reproted that oral administration of an aqueous alcoholic extract of ginseng roots decreased the blood sugar level of rabbits. Saito[3] reproted 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[4,5] demonstrated that ginseng roots contain insulin-like substance including adenosine which inhibited epinephrine-induced lipolysis and stimulated insulin-mediated lipogenesis.

In 1984, we suggested that such insulin-like substances should be called a selective modulators[5]. Present investigation describes the details of the selective modulators in red ginseng powder.

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* power (*Panax ginseng* C.A. 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°C in Krebs-Ringer-phosphate buffer containing 2.5% bovine albumin, and test samples in a final volume of 1.0 ml. After incubation, 5ml of Doles's extraction mixture was added and free fatty acids released were estimated by the method of Dole[7]. One unit of antilipolytic activity was defined as the amount causing 10% inhibition of adrenaline-induced lipolytic activity.

Measurement of lipogenic activity : Fat cells equivalent to 100 mg of adipose tissue were added to 325μl of Hank's buffer containing 4% BSA and 3 mM glucose at final volume of 0.5 ml in a polyprophylene tube and incubated for 20 min at 37°C. Insulin (25μl) and a test drug (25μl) were added to the incubation buffer. After 10 min incubation, ^{14}C-glucose (0.25μCi) was added and incubation was continued for 30 min. The reaction was stopped by adding 2.5 ml of Dole's extraction mixture[7]. The tube was shaken and 1.5 ml heptane and 1 ml water were added, and mixture was shaken and centrifuged. The heptane extracts were washed with same volume of alkaline ethanol (0.05 N NaOH in 50% ethanol) to remove free fatty acids. The radioactivity of upper heptane layer was counted.

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[9]. Material was eluted with water.

Results and discussion

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. Therefore, 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 shown in Fig. 1. 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 was clarified by Dr. Sakata (personal communication). Other inhibitory substance was found to be a polysaccharide as reported previously[5].

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

Then, we tried to isolate selective modulators or insulin-like substances from red ginseng. Isolated fat cells are well known to possess opposite pathways of lipid metabolism : Lipolysis and lipogenesis. Lipolysis is stimulated by epinephrine and ACTH, and lipogenesis is activated by insulin. In various pathological conditions, balance between lipolysis and lipogenesis is often broken. For example, lipolysis is accelerated in diabetes and lipogenesis is enhanced in obesity.

From ancient time, *Panax ginseng* is believed to improve pathological conditions of diabetes mellitus. If so, *Panax ginseng* should contain inhibitors toward lipolysis and stimulators toward lipogenesis, because lipolysis is accerelated and lipogenesis is inhibited in diabetes. We already reported that red ginseng contained such modulators, which were called selective modulators or insulin-like substances. One of the insulin-like substances is determined to be adenosine[9].

Now, we tried to isolate another insulin-like substance than adenosine. One hundred grams of red ginseng powder were mixed with water. The water extract of red ginseng was subjected to dialysis against water. The outer dialysate was then subjected to gel-filtration on Bio-Gel P-2 column as shown in Fig. 2. Anti-lipolytic activity was eluted mainly in fraction 2 and 4. Fraction 4 was determined to be adenosine[9]. Fraction 2 was then applied to Dowex-2 column (Cl^- form), washed with water and eluted with 0.5 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 then

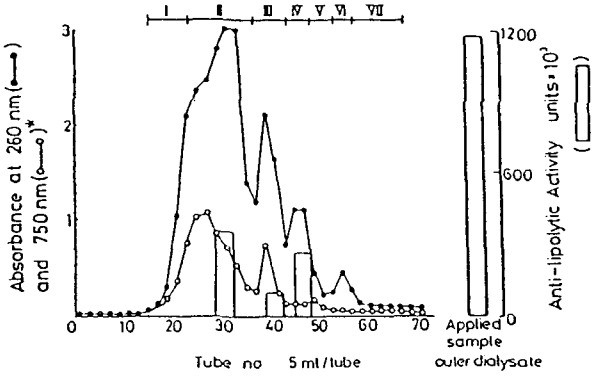

Fig. 2. Gel filtration of the outer dialysate on a Bio-Gel P-2 column. Column size, 2.2 x 43 cm. Elution was carried out with water. * ; Absorption of protein in the method of Lowry et al.

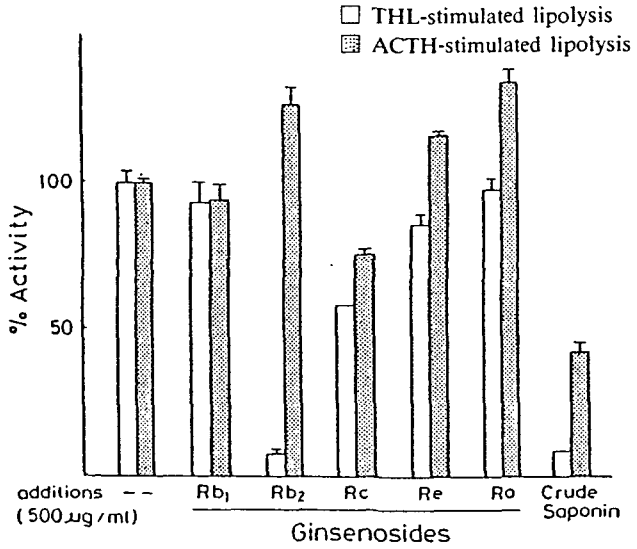

Fig. 1. Effects of ginsenosides on toxohormone (THL) -and ACTH-stimulated lipoysis in fat cells.

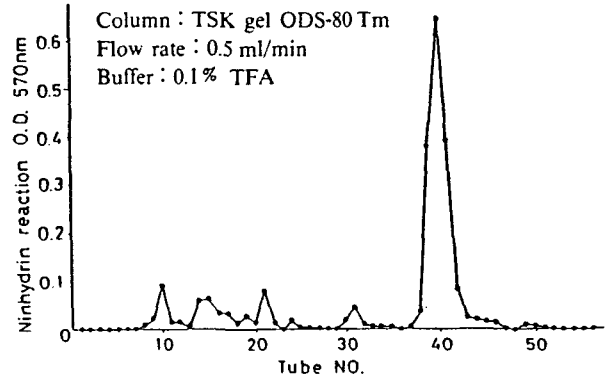

Fig. 3. Separation of insulin-like substance with reverse-phase chromatography.

applied to reverse phase chromatography as shown in Fig. 3. Each fraction was hydrolyzed (6N HCl, 100°C, 24h) and subjected to ninhydrin reaction. Peaks at around tube No. 40 showed high ninhydrin reaction. The peaks at around tube No. 40. did not contain any amino acids. On the other hand, only glutamic acid was demonstrated after acid hydroysis, suggesting that the active principle may be a derivative of glutamic acid. The fraction at peak No. 40 was found to inhibit epinephrine-induced lipolysis in fat cells as shown in Fig. 4. In addition to anti-lipolytic activity, the fraction at peak No. 40 stimulated lipogenesis from glucose in the presence of insulin (Fig.5).

Therefore, we concluded that this fraction is insulin-like substance or selective modulator. Table 1 shows the summary of the purification of insulin-like substance from red ginseng. The yield of the insulin-like substance is 0.1% from red ginseng powder. The fraction at tube No. 40 was found to contain D-glucose in addition to a derivative of glutamic acid. Therefore, we assume that the active principle might be glucosyl-glutamate. However, it is not the case as shown in Fig. 6. A derivative of glutamic acid was separated from D-glucose by ion-exchange chromatography.

The first peak contained D-glucose and the second a derivative of glutamic acid. A question arises what is a chemical structure of this derivative.

Fig. 7 shows mass spectra of insulin-like substance and pyro-glutamic acid.

In EI mass spectrum of insulin-like substance, a peak at M/Z 129 was found. In the CI mass spectrum, peaks at M/Z 130 and 259 were found and the peak at M/Z 130 was much stronger than that at M/Z 259. These

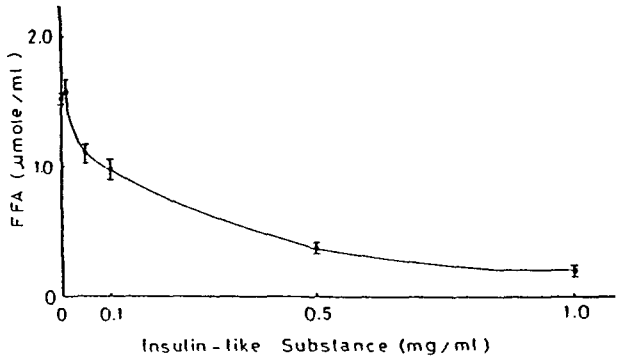

Fig. 4. Effect of insulin-like substance on epinephrine-induced lipolysis in fat cells.

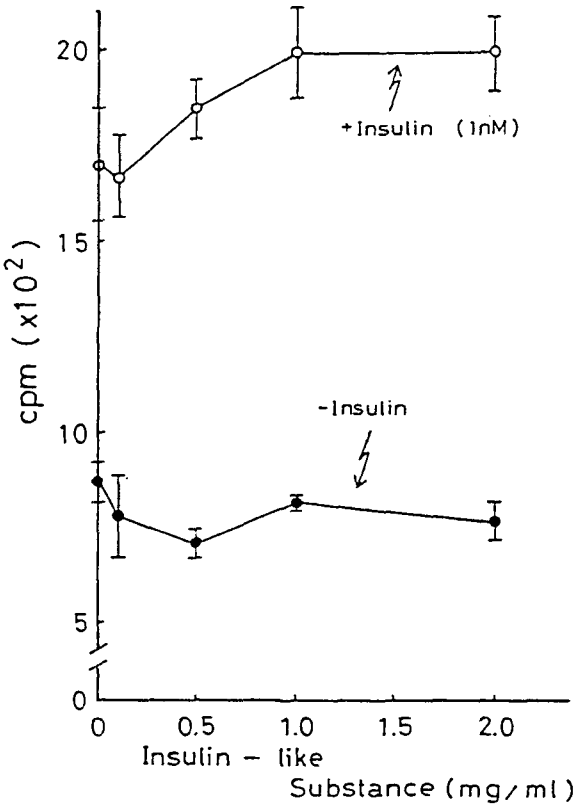

Fig. 5. Effect of insulin-like substance on lipogenesis from glucose in fat cells.

Table 1. Purification of insulin-like substance from red ginseng

	Weight	Specific activity	Total activity	
	(g)	(U/mg)	U×10³	%
Water extract from 100g red ginseng	33	2.8	92.4	100
Outer dialysate	26	2.0	51.8	56
Bio-gel column	21	1.4	29.5	32
Dowex -2×8 column	1.2	5.4	6.5	7
HPLC (Reverse phase)	0.1	23.0	2.3	2

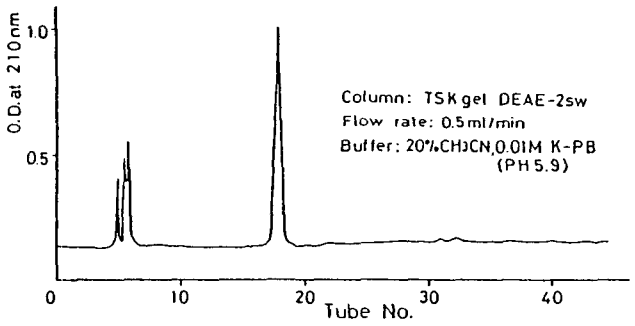

Fig. 6. Separation of insulin-like substance with ion-exchange chromatography.

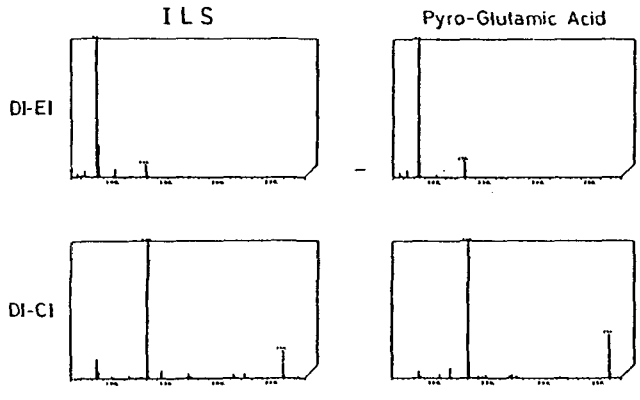

Fig. 7. Mass spectra of insulin-like substance (ILS) and pyro-glutamic acid.

results suggests that molecular weight of insulin-like substance may be 129.

From the facts that molecular weight of insulin-like substance is 129 and its amino group is covered, we assume that the chemical structure of insulin-like substance may be pyro-glutamic acid. Mass spectra of pyro-glutamic acid is essentially the same as thoses of insulin-like substance (Fig.7).

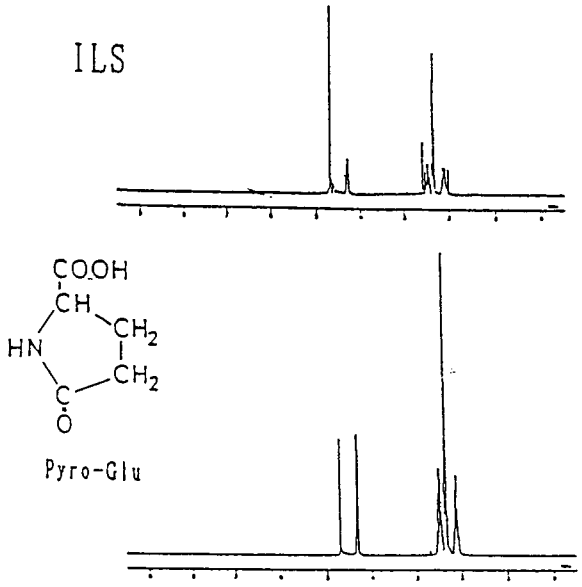

Fig. 8. NMR spectra (D_2O) of insulin-like substance (ILS) and pyro-glutamic acid.

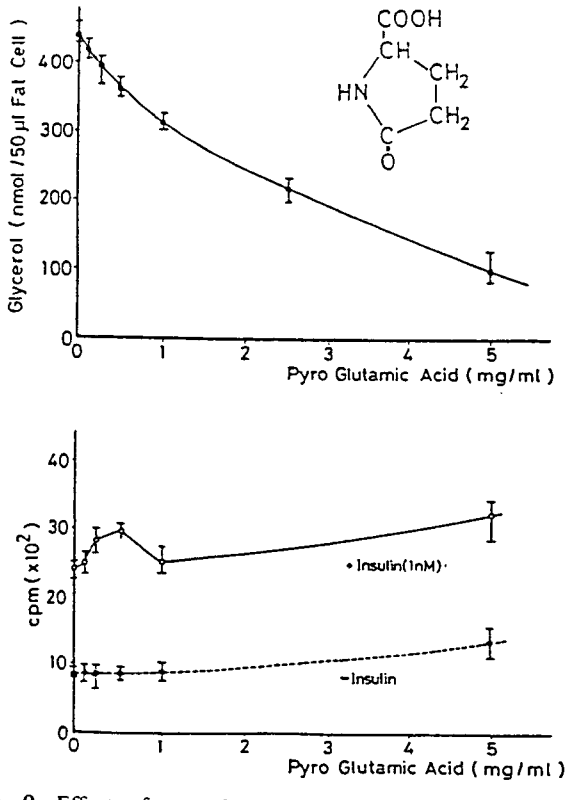

Fig. 9. Effect of pyro-glutamic acid on epinephrine-induced lipolysis and lipogenesis from glucose in fat cells.

Proton-NMR spectra of insulin-like substance and pyro-glutamic acid are shown in Fig.8. The same spectra were found in both substance. From these results, we concluded that the insulin-like substance is pyro-glutamic acid.

Pyro-glutamic acid clearly inhibits epinephrine-induced lipolysis and slightly elevated lipogensis from glucose both in the presence and absence of insulin, indicating that pyro-glutamic acid is insulin-like substance (Fig.9). However, the anti-lipolytic activity of pyro-glutamic acid is lower than that of the fraction at tube No. 40 in reverse chromatography, suggesting that contaminated material containing D-glucose might enhance the anti-lipolytic activity of pyro-glutmic acid.

Experiments are now in progress to elucidate another insulin-like substances in red ginseng powder.

References

1. Masuno, H., Yoshimura, H., Ogawa, N. and Okuda, H., Eur. J. Cancer Clin. Oncol., 20, 1177 (1984)
2. Petkov, W., Arzneim. Forsch. (Drug Res.) 11, 288 (1961)
3. Saito, T., Keio Igaku, 2, 149 (1922)
4. Ando, T., Muraoka, T., Yamasaki, N. and Okuda, H., Planta Medica, 38. 18 (1980)
5. Okuda, H., Masuno, H. and Lee, S, J., Proc. 4th Int. Ginseng Symp., p.145 (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. Okuda, H. and Yoshida, R., Proc. 3rd Int. Ginseng Symp., p. 53 (1980)

H. Oura : Is blood glucose decreased by cyclo-Glu?

H. Okuda : I haven't carried out such experiment. However I assume that cycloglutamate or pyro-glutamate might fail to reduce blood glucose.

B. Z. Ahn : Have you carried out any *in vivo* test in animal with panaxytriol?

M. Katano : No. We are planning to test *in vivo* in the near future.

고려홍삼에 함유된 선택조절제 및 항식욕감퇴 인자에 관한 연구

Takeshi Takaku, Kenji Kameda, Yukinaga Matsuura*, Keizo Sekiya*, Hideo Okai**, and Hiromichi Okuda*.

Central Research Laboratory, School of Medicine, Ehime University

* 2nd Department of Medical Biochemistry, School of Medicine, Ehime University

** Department of Fermentation Technology, Faculty of Engineering, Hiroshima University

에피네프린은 지방분해를 촉진하고 인슈린은 지방생성을 촉진한다는 것은 잘 알려져 있다. 최근에 한국홍삼분말 중에는 에피네프린으로 유도된 지방분해를 억제하며 인슐린이 매개하는 당으로부터의 지방합성을 촉진하는 아데노신과 산성펩티드 성분이 존재함이 밝혀졌다. 산성펩티드의 화학구조는 Glu-Glu-Glu-Glu-Glu-

Glu-Glucose인 것으로 밝혀졌다. 한국홍삼으로 부터 추출된 이러한 물질들은 흰쥐 지방세포에서 반대의 대사경로를 통하여 지방분해를 억제하는 반면 지방합성을 촉진하기도 하는 선택적 조절작용을 나타낸다. 최근에 우리는 여러가지 악성종양을 가진 환자의 복수액으로부터 toxohormone-L로 명명된 독성물질을 분리하였다. Toxohormone-L은 흰쥐 지방세포에서 지방분해를 촉진하고 흰쥐에서 식욕감퇴를 유발했다. Toxohormone-L의 이러한 작용은 한국홍삼에서 분리된 진세노사이드 Rb_2에 의해서 억제됨이 발견되었다. 한국홍삼에서 발견된 이러한 물질의 생리학적 유의성을 언급하고자 한다.