Korean J. Ginseng Sci.
Vol. 14, No. 1, 1–5 (1990)

Effect of Acidic Polysaccharide of Red Ginseng on Lipolytic Action of Toxohormone-L from Cancerous Ascites Fluid

Sung Dong Lee, Kenji Kameda*, Takeshi Takaku*, Keizo Sekiya, Kumi Hirose**, Kazuhiro Ohtani**, Osamu Tanaka** and Hiromichi Okuda§

2nd Department of Medical Biochemistry, School of Medicine, Ehime University, Ehime 791-02
**Central Research Laboratory, School of Medicine, Ehime University and*
***Institute of Pharmaceutical Sciences, School of Medicine, Hiroshima University, Hiroshima 734, Japan*
(Received August 16, 1989)

Abstract □ Toxohorome-L is a lipolytic factor, found in ascites fluid of sarcoma 180-bearing mice and of patients with hepatoma. A substance that inhibited the lipolytic action of Toxohormone-L was isolated from red ginseng powder. This substance had a pectin-like α-1,4-polygalacturonan backbone with some acetoxyl groups, and so was an acidic polysaccharide. It inhibited Toxohormone-L-induced lipolysis in a dose dependent manner at concentrations higher than 10 ug/ml.

Keywords □ *Panax ginseng* C.A. Meyer, red ginseng, toxohormone-L.

Introduction

Panax ginseng is a medicinal plant long used in treatment of various pathological states including general complaints such as headache, shoulder ache and chill and especially debilitation in cancer patients.

Depletion of fat stores has been observed during progressive weight loss in patients with various neoplastic diseases. This depletion of body fat during growth of neoplasms is associated with increase in the plasma level of free fatty acids.

We found that the ascites fluid from sarcoma 180-bearing mice and patients with hepatoma or ovarian tumor, and the pleural fluid from patients with malignant lymphoma elicited fatty acid release from slices of rat adipose tissue *in vitro*[1]. A lipolytic factor, named "Toxohormone-L", was purified from the ascites fluid of sarcoma 180-bearing mice and of patients with hepatoma. Injection of Toxohormone-L into the lateral ventricle of rats significantly suppressed their food and water intakes. Therefore, Toxohormone-L has two actions, lipolytic and anorexigenic actions, which may cause reduction of body fat in cancer patients.

In the present investigation, we tried to find a substance in red ginseng powder that inhibited the lipolytic action of Toxohormone-L in ascites fluid of sarcoma 180-bearing mice.

§Address for correspondence: Hiromichi Okuda, 2nd Department of Biochemistry, School of Medicine, Ehime University, Shigenobucho, Onsen-gun, Ehime 791-02, Japan
Abbreviation: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid.

Materials and Methods

Animals

Young male Wistar King tats, weighing 160 to 200 g, were allowed free access to standard laboratory diet and water. They were sacrificed by a blow on the head and their epididymal adipose tissues were quickly removed. Male DDK mice, weighing 17 to 20 g, were also given standard laboratory diet and water ad libitum.

Red ginseng

Red ginseng powder (*Panax ginseng* C.A. Meyer) was kindly provided by Nikkan Korai Ninjin Co. Ltd., Kobe, Japan and Korea Ginseng and Tobacco Research Institute, Deajeon, Korea.

Preparation of Toxohormone-L fraction

Male DDK mice were inoculated i.p. with 0.5 m*l* of sarcoma 180 suspension (4 to 5×10^9 cell/mouse), and 10 to 14 days later, the ascites fluid was harvested. The ascites fluid was centrifuged at $1,0000 \times g$ for 10 min at 4 °C and the resultant supernatant was used as the Toxohormone-L fraction.

Measurement of anti-lipolytic activity

Isolated fat cells were prepared from rat epididymal adipose tissue by the method of Rodbell[2]. Fat cells (50 μl packed volume) were incubated for 30 min at 37 °C in 175 μl of Hanks buffer (pH 7.4) buffered with 25 mM HEPES contaning 4% bovine serum albumin, 25 μl of test sample and 50 μl of Toxohormone-L fraction in a final volume of 0.30 m*l*. After incubation, the free fatty acids released were extracted with 3 m*l* of a 1:1 (v/v) mixture of chloroform and heptane containing 2% (v/v) methanol and measured with copper reagent and bathocuproine by the method on Zapf *et al.*[3].

Determination of carbohydrate

Carbohydrate was measured by the phenol-sulfuric acid method[4].

NMR measurement

NMR spectrum was determined on JEOL GX-400 in C_5H_5N solution using TMSi ether as a internal standard: ^{1}H-NMR at 400 MHz, ^{13}C-NMR at 100 MHz.

Results

Red ginseng powder was extracted with 10 volumes of deionized water at 4 °C for 24 h. The extract was centrifuged, and the supernatant was concentrated and dialyzed against deionized water at 4 °C for 24 h in a dialyzed membrane to remove molecules smaller than 10,000 daltons. The inner dialysate was then concentrated and freeze-dried. The resulting powder was treated with methanol at room temperature and then with hot methanol to remove ginsenosides. The residual material was extracted with deionized water at room temperature and then with hot water, and the water extracts were combined, concentrated and mixed with 4 volumes of ethanol. The resulting precipitate fraction (ginsenoside-free ethanol precipitate) was dialyzed against deionized water and the inner dialysate was applied to a DEAE-TOYOPEARL 650 M column (28 mm × 50 cm) equilibrated with 0.02 M NH_4HCO_3. Elution was carried out with 0 M, 0.05 M, 0.10 M, 0.15 M, 0.20 M, 0.25 M and 0.30 M NaCl in 0.02 M NH_4HCO_3, successively, and the resulting fractions of eluate were named PG_1, PG_2, PG_3, PG_4, PG_5, PG_6 and PG_7, respectively. The yields of these fractions from 500 g of red ginseng powder were 32.3 g, 777 mg, 311 mg, 197 mg, 25 mg and 25 mg, respectively.

Table 1. Inhibitory effects of PG fractions on lipolysis induced by Toxohormone-L. The rate of Toxohormone-L-induced lipolysis was 2.23 free fatty acid μEq/g cells/2h in the absence of PG fractions.

Concentration (μg/m*l*)	Fraction						
	PG1	PG2	PG3	PG4	PG5	PG6	PG7
	Percent inhibition						
10	12.2	-1.1	-2.5	-1.7	-1.5	-4.9	-6.6
50	35.8	3.3	9.0	22.0	4.2	13.6	18.4
100	44.4	10.9	24.5	42.7	27.1	25.1	25.0
200	47.3	11.6	25.9	53.2	32.0	28.1	25.9
500	62.3	12.5	35.1	72.2	52.9	42.0	27.4
1000	80.0	19.9	45.0	87.9	77.9	61.6	31.6

The inhibitory effects of these fraction on lipolysis induced of Toxohormone-L were examined. A shown in Table 1, PG_1, the unabsorbed fraction, and PG_4 were strongly inhibitory.

For further purification the PG_4 fraction (100 mg) was dissolved in 0.02 M NH_4HCO_3 and subjected to gradient elution on a DEAE-TOYOPEARL 650 M column (14 mm × 20 cm). The elution profile is shown in Fig. 1. The effluent fractions

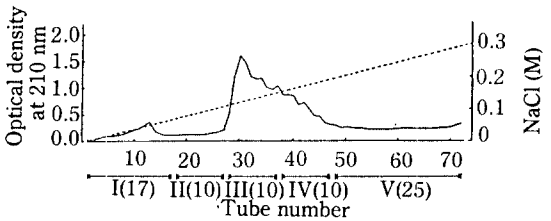

Fig. 1. DEAE-TOYOPEARL column chromatography of PG_4 fraction from red ginseng.
Gradient elution was carried out with 0 to 0.3 M NaCl in 0.02 M NH_4HCO_3. Fractions of 15 m*l* of effluent were collected.

Table 2. Inhibitory effects of various fractions obtained by gradient elution on lipolysis induced by Toxohormone-L. The rate of Toxohormone-L-induced lipolysis was 2.46 free fatty acid μEq/g cells/2h in the absence of the fractions.

Concentration (μg/m*l*)	Fraction				
	PG4-I	PG4-II	PG4-III	PG4-IV	PG4-V
	Percent inhibition				
10	13.1	20.3	11.3	26.7	-1.5
50	14.8	31.6	66.7	44.8	7.6
100	40.2	47.6	80.2	65.1	19.6
200	40.6	48.4	82.5	70.5	20.0
500	76.6	52.2	97.7	88.4	76.1
1000	79.1	59.9	98.9	91.3	—

were combine as indicated in the figure, and their inhibitory effects on Toxohormone-L-induced lipolysis were examined. As shown in Table 2, PG_4-III and PG_4-IV were strongly inhibitory.

The yields of PG_4-III and PG_4-IV were 57 mg and 36 mg, respectively. Fractions PG_4-III and PG_4-IV were combined and subjected to further purification.

A sample of 50 mg of the mixture of the PG_4-III and PG_4-IV fractions was subjected to high performance liquid chromatography on a TSK gel ODS-120T column (4.6 mm × 250 mm). Elution was carried out with CH_3CN in 0.1% trifluoroacetic acid at a flow rate 0.5 m*l*/min. In the first chromatography, two separate peaks were eluted as shown in Fig. 2.

As inhibitory activity toward Toxohormone-L-induced lipolysis was found in the second peak, this fraction was collected and again subjected to reverse phase HPLC. A single sharp peak was obtained, as shown in Fig. 3. The homogeneity of material in this peak was confirmed by analytical gel permeation high performance liquid chromatography in the following: Pump, TOSO CCPM; RI detector, TOSO RI-8000; UV detector, TOSO-8000 at 203 nm; Column, Joint column of TSK gel G-3000 PW (7.5 mmi.d. × 30 cm) and TSK gel G-5000PW (7.5 mmi.d. × 30 cm); Column temperature, 80°C; Mobile phase, 0.5 M NaCl; Flow rate, 0.7 m*l*/min. The ^{13}C-NMR spectrum of the purified material mainly showed signals of the methyl ester of 4-linked α-galacturonide: 171.6 (C-6,$\underline{C}OOCH_3$), 99.3 (C1), 77.7 (C-5), 69.8 (C-4), 67.6 (C-3), 67.4 (C-2)

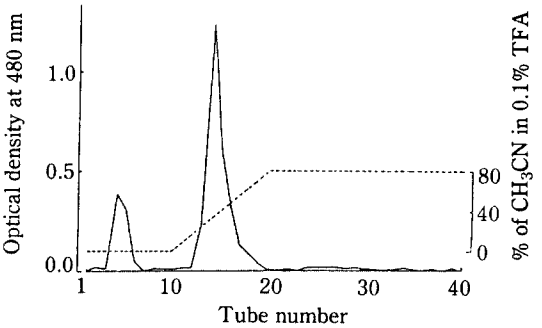

Fig. 2. 1st reverse phase HPLC of PG_4-III and PG_4-IV fractions.
The effluent was collected in fractions of 1 m*l*.
——: Carbohydrate determined with phenol-sulfuric acid. (4)
------: Percent of CH_3CN in 0.1% trifluoroacetic acid (TFA)

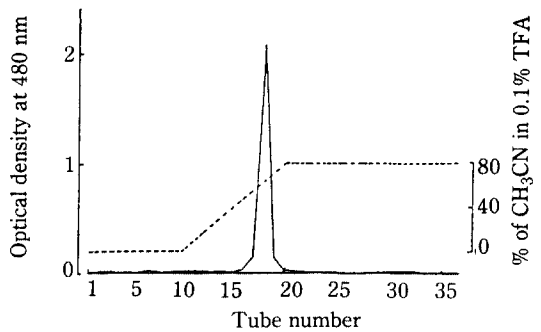

Fig. 3. 2nd reverse phase HPLC of the PG_4-III and PG_4-IV fractions.
The explanation is as for Fig. 2.

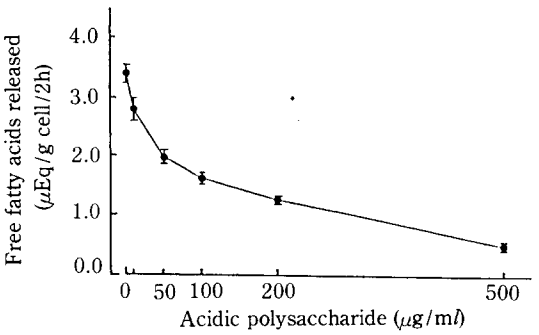

Fig. 4. Inhibitory effect of the acidic polysaccharide purified from red ginseng on Toxohormone-L-induced lipolysis.

and 25.4 ($COO\underline{C}H_3$). The proton signal at δ 3.56 (singlet) was assignable to a carbomethoxyl group. From these date, the purified materials seems to have a pectin-like α-1,4-polygal-acturonan backbone. The presence of some acetoxyl groups were demonstrated by weak carbon signals at δ 170.1 ($\underline{C}OCH_3$) and 19.6 ($CO\underline{C}H_3$) as well as a proton signal at δ 1.98 (singlet, $COCH_3$). We are now determining the molecular weight and sugar composition of this acidic polysaccharide.

Eight mg of the finally purified acidic polysaccharide was obtained from 50 mg of the mixture of PG_4-III and PG_4-IV by repeated reverse phase HPLC. Free fatty acids released by Toxohormone-L decreased in response to increasing concentrations of the purified acidic polysaccharide (Fig. 4). The minimum effective concentration of acidic polysaccharide was 10 μg/m*l*. The acidic polysaccharide at concentrations of 100 and 500 μg/m*l* decreased Toxohormone-L-induced lipolysis 50 and 83%, respectively. On the other hand, the acidic polysaccharide did not affect both epinephrine- and ACTH-induced lipolysis at its concentration of 500 μg/m*l*: Epinephrine-induced lipolysis in the absence and presence of the acidic polysaccharide was 6.8 ± 0.3 μEq/g and 6.4 ± 0.2 μEq/g, respectively. ACTH-induced lipolysis in the absence and presence of the acidic polysaccharide was 6.8 ± 0.5 μEq/g and 6.9 ± 0.3 μEq/g, respectively.

Discussion

Our previous studies suggested that a polysaccharide fraction of red ginseng might inhibit Toxohormone-L-induced lipolysis in adipocytes[5]. In the present investigation, we purified an inhibitory component of the polysaccharide fraction, and found by ^{13}C-NMR spectral analysis that this component had a pectin-like α-1,4-polygalacturonan backbone with some acetoxyl groups. Therefore, it is an acidic polysaccharide. This acidic polysaccharide inhibits adipocyte lipolysis induced by Toxohormone-L, but dose not affect lipolysis induced by epinephrine or ACTH. In previous studies, we found that ginsenoside Rb_2 inhibited Toxohormone-L-induced lipolysis in adipocytes but not ACTH-induced lipolysis[6].

Most pharmacological effects of red ginseng are though to be due to Ginsenosides[7-9]. Kubo *et al.* reported that ginsenosides are located in the surface region of the red ginseng root[10]. Therefore, the ginsenoside content is more in small ginseng roots than in big ones. However, from ancient times big ginseng roots have been thought to be far more effective than ones. Thus there is a contradiction between the ginsenoside content of ginseng roots and their appreciated value. On the other hand, the acidic polysaccharide described here may be located in the inner part of the red ginseng root, and if so, big ginseng roots should contain more of this acidic polysaccharide than small ones.

Experiments are now in progress to determine the exact structure of this acidic polysaccharide.

Acknowledgement

This work supported by Medical Society for Red Ginseng Research.

Literature Cited

1. Masuno, H., Yoshimura, H., Ogawa, N. and Okuda, H.: *Eur. J. Cancer Clin. Oncol.* **20,** 1177 (1984).
2. Rodbell, M.: *J. Biol. Chem.* **239,** 375 (1964).
3. Zapf, J., Schoenle, E., Waldvogel, M., Sand, M. and Froesch, E.R.: *Eur. J. Biochem.* **113,** 605 (1981).
4. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers,

P.A. and Smith, F.: *Anal. Chem.* **28**, 350 (1956).

5. Okuda, H., Masuno, H., and Lee, S.J.: Proc. 4th Internat. Ginseng Symp. 145-152 (1984).
6. Okuda, H., Sekiya, K., Masuno, H., Takaku, T. and Kameda, K.: *Proc. Korea-Japan Panax Ginseng Symp.* 1-9 (1989).
7. Shibata, S.: New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity. (H. Wagner and P. Wolff, Springer Verlag, Berlin) 185-190 (1977).
8. Yokoyama, T., Kanai, K., Takefuji, M. and Oura, H.: *Chem. Pharm. Bull.* **24**, 3202 (1976).
9. Shibata, Y., Nozaki, T., Higashi, T., Sanada, S. and Shoji, J.: *Chem. Pharm. Bull.* **24**, 2818 (1976).
10. Kubo, M., Samukawa, K., Tani, T., Katuki, T. and Arichi, S.: *Ginseng Review* **2**(1), 33 (1984).