Effect of acidic polysaccharide of Red Ginseng on lipolytic action of Toxohormone-L from cancerous ascites fluid

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Abstract

Toxohormene-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 liploysis in a dose dependent manner at concentrations higher than 10 μg/ml.

Key words Red Ginseng, Toxohormone-L.
Abbreviation HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

Panax ginseng is a medicinal plant long used in treatment of various pathological states including general complaints such as headache, shoulderache, chills 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, 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.

Materials and Methods

Animals : Young male Wistar King rats, weighing 160 to 200 g, were allowed free access to the 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 the 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

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Other materials : DEAE-TOYOPEARL 650 M, TSK gel ODS-120T column, TSK gel G-3000 PW column and TSK gel G-5000 PW column were purchased from TOSOH Co., Ltd. (Tokyo, Japan). HEPES and bovine serum albumin were obtained from Wako Pure Chemical Industries. Bovine serum albumin extracted to remove free fatty acid by the method of Chen.[2]

Preparation of Toxohormone - L fraction : Male DDK mice were inoculated *i.p.* with 0.5 ml 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 $1,000\times g$ for 10 min at 4℃ and the resultant supernatant was used as the Toxohormone-L fraction.

Measurement of anti-lipolytic activity : Isolated fat cells wera prepared from rat epididymal adipose tissue by the method of Rodbell.[3] Fat cells (50 μl packed volume) were incubated for 30 min at 37℃ in 175 μl of Hanks buffer (pH 7.4) buffered with 25 mM HEPES containing 4% bovine serum albumin, 25 μl of the test sample and 50 μl of Toxohormone-L fraction in a final volume of 0.30 ml. After incubation, the free fatty acids released were extracted with 3 ml 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*.[4]

Purification of acidic polysaccharide : DEAE-TOYOPEARL 650 M column (28 mm×50 cm) was 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. Then gradient elution was carried out with the same column from 0 to 0.3 M NaCl in 0.02 M NH_4HCO_3. TSK gel ODS-120T column (4.6 mm×250 mm) was equilibrated with 0.1% TFA. Elution was carried out with CH_3CN in 0.1% trifluoroacetic acid at a flow rate 0.5 ml/min.

Analytical gel permeation high performance liquid chromatography : Analytical gel permeation high performance liquid chromatography was carried out as follows : Pump, TOSO CCPM ; RI detector, TOSO RI-8000 ; UV detector, TOSO UV-8000 at 203 nm ; Column, joint column of TSK gel G-3000 PW (7.5 mm i.d. ×30 cm) and TSK gel G-5000PW (7.5 mm i.d. ×30 cm) ; Column temperature, 80 ℃ ; Mobile phase, 0.5 M NaCl ; Flow rate, 0.7 ml/min.

Nuclear magnetic resonance spectrum (NMR) : The NMR spectra were obtained with a JEOL GX-400 spectometer, at 400 MHz for ^1H at 100 MHz for ^{13}C in D_2O at 45° (internal dioxan, 67.4 ppm relative to the signal for tetramethylsilane).

Determination of carbohydrate : Carbohydrate was measured by the phenol - sulfuric acid method.[5]

Analysis of data : Student's t-test was used to determine the significance of difference.

Results

Purification of inhibitory substance toward Toxohormone-L-induced lipolysis from Red Ginseng powder

Red Ginseng powder was extracted with 10 volumes of deionized water at 4℃ for 24 hr. The extract was centrifuged, and the supernatant was concentrated and dialyzed against deionized water at 4℃ for 24 hr in a dialysis 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 (50℃) to remove ginsenosides. The residual material was extracted with deionized water at room temperature and then with hot water (50℃), and then 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. Step-wise elution was carried out as described in "Materials and Methods." 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

Table I Inhibitory effects of PG fractions on lipolysis induced by Toxohormone-L.

Concentration (μg/ml)	Fraction PG_1	PG_2	PG_3	PG_4	PG_5	PG_6	PG_7
	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 rate of Toxohormone-L-induced lipolysis was 2.23 free fatty acid μEq/g cells/2 hr in the absence of PG fractions.

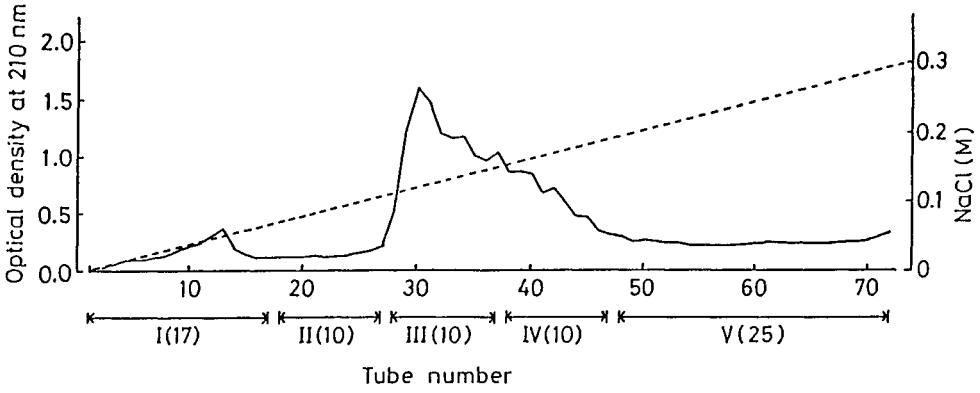

Fig. 1 DEAE-TOYOPEARL column chromatography of PG_4 fraction from Red Ginseng. Gradient elution was carried out with 0 to 0.3 M NaCl in 0.02 M NH_4HCO_3. Fractions of 15 ml of effluent, were collected.

Table II Inhibitory effects of various fractions obtained by gradient elution on lipolysis induced by Toxohormone-L.

Concentration (μg/ml)	Fraction PG_4-I	PG_4-II	PG_4-III	PG_4-IV	PG_4-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	-

The rate of Toxohormone-L-induced lipolysis was 2.46 free fatty acid μEq/g cells/2 hr in the absence of the fractions.

Ginseng powder were 32.3 g, 777 mg, 311 mg, 197 mg, 94 mg, 25 mg and 25 mg, respectively.

The inhibitory effects of these fractions on lipolysis induced by Toxohormone-L were examined. As shown in Table I, 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. The elution profile is shown in Fig. 1. The effluent fractions were combined as indicated in the figure, and their inhibitory effects on Toxohormon - L - induced lipolysis were examined. As shown in Table II, 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 as described in "Materials and Methods." In the first chromatography, two separate peaks were eluted as shown in Fig. 2.

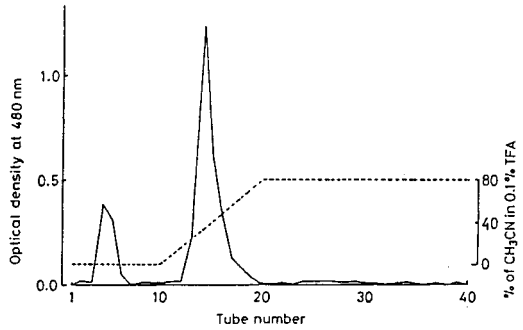

Fig. 2 First reverse phase HPLC of PG_4-III and PG_4-IV fractions.
The effluent was collected in fractions of 1 ml. —— : Carbohydrate determined with phenol-sulfuric acid. (4) ····· : Percent of CH_3CN in 0.1% trifluoroacetic acid (TFA).

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 as described in "Materials and Methods."

Eight mg of the finally purified acidic polysaccharide was obtained from 50 mg of the mixture of PG_4-III and PG-IV by repeated reverse phase

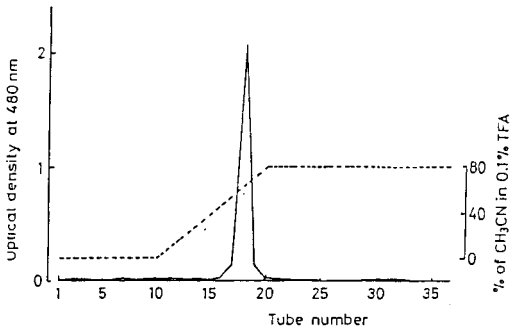

Fig. 3 Second reverse phase HPLC of PG_4-III and PG_4-IV fractions.
The explanation is as for Fig. 2.

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/ml. The acidic polysaccharide at concentrations of 100 and 500 μg/ml decreased Toxohormone-L-induced lipolysis 50% and 83%, respectively. On the other hand, the acidic polysaccharide did not affect either epinephrine- or ACTH-induced lipolysis at its concentration of 500 μg/ml : Epinephrine-induced lipolysis in the absence and presence of the acidic polysaccharide was 6.8±0.3 μEq/g and

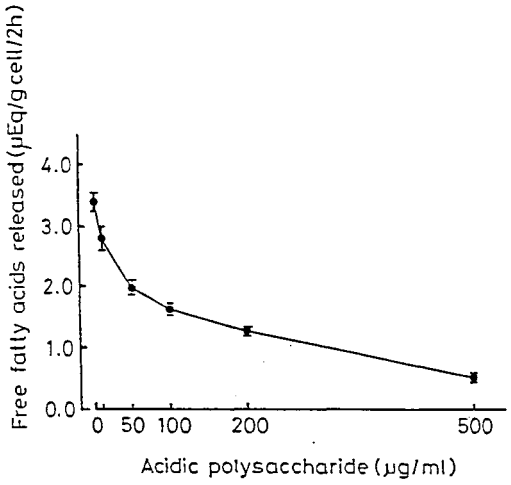

Fig. 4 Inhibitory effect of the acidic polysaccharide purified from Red Ginseng on Toxohormone-L-induced lipolysis.

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.

Analysis of chemical structure of the inhibitory substance

The ^{13}C-NMR spectrum of the purified material mainly showed signals of the methyl ester of 4-linked α-galacturonide : δ171.6 (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) and 25.4 ($COO\underline{C}H_3$). The proton signal at δ3.56 (singlet) was assignable to a carbomethoxyl group. From these data, the purified materials seem to have a pectin-like α-1, 4-polygalacturonan backbone. The presence of some acetoxyl groups was demonstrated by weak carbon signals at δ170.1 ($\underline{C}OCH_3$) and 19.6 ($CO\underline{C}H_3$) as well as a proton signal at δ1.98 (singlet, $COCH_3$). We are now determining the molecular weight and sugar composition of this acidic polysaccharide.

Discussion

Our previous studies suggested that a polysaccharide fraction of Red Ginseng might inhibit Toxohormone-L-induced lipolysis in adipocytes.[6] 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 does not affect lipolysis induced by epinephrine or ACTH. However, the mechanism of the inhibitory action of the acidic polysaccharide on Toxohormone-L-induced lipolysis remains to be solved. In previous studies, we found that ginsenoside Rb_2 inhibited Toxohormone-L-induced lipolysis in adipocytes but not ACTH-induced lipolysis.[7] We have already clarified that ginsenoside Rb_2 inhibited both lipolytic and anorexigenic actions of Toxohormone-L.[8] Currently, an experiment is underway to prove an inhibitory action of the acidic polysaccharide on anorexigenic activity of Toxohormone-L.

Most pharmacological effects of Red Ginseng are thought to be due to ginsenosides.[9-11] Kubo *et al.* reported that ginsenosides are located in the surface region of Red Ginseng.[12] Therefore, the ginsenoside content is greater in small ginseng roots than in large ones. However, from ancient times large ginseng roots have been thought to be far more effective than small 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 Red Ginseng, and if so, large ginseng roots should contain more of this acidic polysaccharide than small ones.

This possibility was confirmed as follows: Activity of the acidic polysaccharide was assayed by measuring the inhibitory effect of the ginsenoside-free ethanol precipitate fraction on Toxohormone-L-induced lipolysis, and defining 10% inhibition as 1 unit. The activity in large roots of Red Ginseng (average diameter ; 2 cm) was 3847 units/g Red Ginseng powder whereas in small roots (average diameter ; 0.5 cm>) it was 1387 units/g Red Ginseng powder. Thus large roots contain more of the acidic polysaccharide than small ones.

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

Acknowledgements

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和文抄録

トキソホルモン-Lは，sarcoma 180担癌マウスや肝癌患者の腹水に存在する脂肪動員因子である。我々は，トキソホルモン-Lの脂肪分解作用を阻害する物質を紅参末から単離した。この物質は，いくつかのアセトキシル基をもつα-1, 4-polygalacturonanを主成分とするペクチン様酸性多糖体であった。この酸性多糖は，10 μg/ml以上の濃度で濃度依存的にトキソホルモン-Lの脂肪分解作用を阻害した。

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