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Aqueous Fraction from Korean Red Ginseng Inhibits the Protein Phosphorylation Induced by Tumor Promoter

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Abstract□Aqueous fractions from Korean red ginseng inhibited the phosphorylations of 40 KD and 20 KD polypeptides which were induced by phorbol-12-myristate-13-acetate (100 nM) in human platelets. Much more carbohydrates were contained in the aqueous fractions than proteins. An aqueous fraction extracted with methanol, mainly, consists of glycoproteins, molecular weights of which were below 18 KD. We may infer that the aqueous fraction from Korean red ginseng do antitumorous and antiplatelet functions.

Key words□Aqeous fraction from Korean red ginseng, antitumorous activity, inhibition on phosphorylation of 40 KD and 20 KD.

Introduction

An extracellular signalling molecule (agonist) phosphorylates the functional protein within a cell to make a physiological funtions.[1] Phorbol-12-myristate-13-acetate, a well known tumor promoter, stimulates the phosphorylations of 40 KD and 20 KD polypeptides in platelets, then, platelets aggregation follows.[1] It is known that 40 KD and 20 KD polypeptides are phosphorylated by diacylglycerol-dependent kinase C and by Ca^{2+}/calmodulin-dependent kinase respectively, and that the phosphorylations of these proteins are directly related to the release of platelet aggregating factors such as serotonin and ADP.[1] We investigated how the aqueous fraction from Korean red ginseng affected the phosphorylations of 40 KD and 20 KD polypeptides, and discussed the possibilities acting as antiplatelet or antitumorous drugs.

Materials and Methods

Carrier free-^{32}Pi(1 mC*i*) was obtained from Amersham Life Science Co. Phorbol-12-myristate-13-acetate and the other chemical reagents were obtained from Sigma Chemical Co.

1. Preparation of aqueous fractions from Korean red ginseng

Korean red ginseng was cut into 0.5 cm length, powdered, and extracted with methanol, chloroform, and acetone, successively. Pre'iminary extractions were concentrated under reduced pressure (25~30 ℃), and then separated into aqueous fractions and lipophilic fractions within a separatory funnel by adding chloroform and water successively to the funnel. The aqueous fractions were collected, freeze-dried, and stored as stock at −85℃.

2. Labelling of platelets with ^{32}Pi

Platelet-rich plasma (PRP) obtained from the antecubital vein of normal human volunteers, was purchased from Taejon Red Cross Blood Center, Korea. The blood was anticoagulated with CPD sol (sodium citrate, NaH_2PO_4, glucose, adenine mixture; Korean Green Cross Pharm. Co.) when PRP was prepared. PRP was centrifuged at 125 x *g* for 10 min to remove red blood cells, and PRP was incubated with 1 mC*i* of ^{32}Pi at 37℃ for 60 min. Labelled platelets were washed twice with Tris-citrate-bicarbonate buffer (pH 6.5,[4] containing 2 mM EDTA) by centrifugation at 1,100 x *g* for 10 min. The washed platelets were recentrifuged twice with suspending buffer (pH 6.9,[4] without EDTA) to remove EDTA.

Finally, platelet number was adjusted to 5×10^8 cells/ml with the suspending buffer. All of the above procedures were carried out at 25℃ to avoid platelet aggregations by cold condition.

3. Phosphorylation reaction of proteins

Protein-phosphorylations were carried out according to the method of Laemmli.[5] Platelet suspensions (10^9 cells/ml) containing 1.7 mg proteins were preincubated at 37℃ for 2 min in the presence of 1 mM $CaCl_2$ and aqueous fractions from Korean red ginseng (200 μg/ml), and then stimulated by phorbol-12-myristate-13-acetate (100 nM) for 3 min. The stop solution (0.125 M Tris, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) was added to stop the phosphorylation reaction with the same volume of the reaction volume. To denature proteins, samples were boiled for 5 min and 50 μg proteins of each sample were separated by SDS-PAGE (1.5 mm, 10% polyacrylamide). Separated proteins were stained, destained, dried, and autoradiographed onto X-ray film (Fuji Medical X-ray Film) at −70℃ for 15 days. Phosphorylated proteins are assayed in CAMAG TLC scanner II at 554 nm.

4. Other methods

Molecular weight of proteins in aqueous fractions was determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 1.5 mm, 10 %).[5] The slab gel was detached from the electrophoresis instrument, stained with the staining solution (0.04% Coomassie Brilliant Blue R-120/acetic acid/methanol [5 : 1 : 5, v/v]), and destained with 7% acetic acid solution. The molecular weights of separated proteins were calculated by comparing the moving distances with those of protein standards (albumin, bovine; 66,000, albumin, egg; 45,000, glyceraldehyde-3-p-dehydrogenase; 36,000, carbonic anhydrase, bovine; 29,000, trypsinogen, bovine pancreas; 24,000, trypsin inhibitor, soybean; 20,000, α-lactalbumin, bovine milk; 14,200, Sigma). Hexose and protein were assayed by anthrone colorimetric reaction[6] and by Lowry method, respectively. According to the method of Glossmann,[7] glycoprotein was identified by Schiff's reagent as red bands on the gel.

Results and Discussions

Phorbol-12-myristate-13-acetate (PMA) does not mediate the production of diacylglycerol from membrane inositolphospholipids that is usually seen in the signal transduction pathways of growth factor or neurotransmitter. PMA passes through the membrane and thus activates protein kinase C directly in the cytosol. Physiological phenomena such as cell growth, differentiation, transformation, etc. are known to be intimately associated with the activity of protein kinase C.[1] Especially in platelets, 40 KD polypeptide is phosphorylated, and serotonin is released when protein kinase C is activated.[2] Furthermore when 20 KD polypeptide is phosphorylated by Ca^{2+}/calmodulin-dependent kinase, it makes a synergistic effect with phosphorylated 40 KD poly-

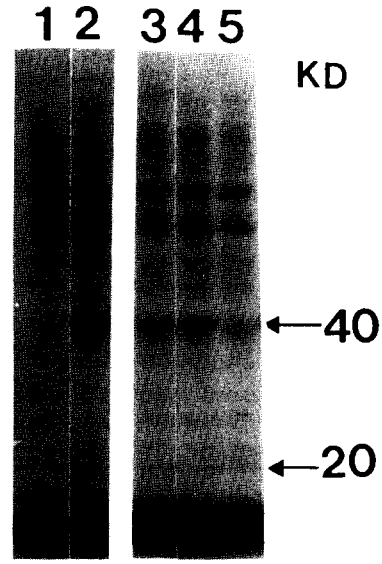

Fig. 1. Protein phosphorylation pattern caused by PMA and by aqueous fractions in human platelets. The phosphorylation reaction of proteins was performed as described in the "Methods". Phorbol-12-myristate-13-acetate (PMA) was dissolved in ethanol. Lane 1 : Ethanol 4 μl as control of PMA; lane 2 : PMA 100 nM; lane 3 : PMA 100 nM + aqueous fraction by methanol 200 μg/ml; lane 4 : PMA 100 nM + aqueous fraction by chloroform 200 μg/ml; lane 5 : PMA 100 nM + aqueous fraction by acetone 200 μg/ml.

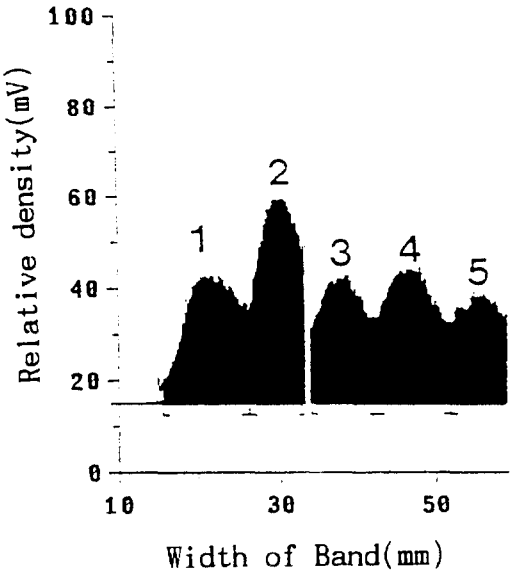

Fig. 2. Scanning pattern of the phosphorylation of 40 KD polypeptide which is shown in Fig. 1. The resulting peaks are from CAMAG TLC scanner II at 554 nm. The legend numbers of lanes are same as those used in Fig. 1.

Table 1. Inhibition of aqueous fraction on the phosphorylation of 40 KD polypeptide

Peak no.	Sample	Height (mm)	Area (mm^2)	Inhibition (%)
1	Control	27.6	2122.3	–
2	PMA 100 nM	42.9	2842.1	–
3	Methanol Ext.	26.8	1852.2	34.8
4	Chloroform Ext.	28.9	2293.3	19.3
5	Acetone Ext.	23.6	1550.5	45.4

The phosphorylation of 40 KD polypeptide which is represented in Fig. 2 was calculated as the area of peaks by CAMAG TLC scanner II.
The peak numbers from 1 to 5 are same as those of Fig. 1.

peptide in the release of serotonin which stimulate platelet aggregation.[2]

In our experiment, 40 KD polypeptide was phosphorylated by PMA (Fig. 1, lane 2), but the aqueous fractions extracted with methanol (Fig. 3, lane 2) and with acetone (Fig. 3, lane 4) inhibited the phosphorylation of 40 KD polypeptide by 34% and 45%, respectively (Fig. 1 & 2, lanes 3 & 5, Table 1). Glycoproteins whose molecular weight is below 18 KD

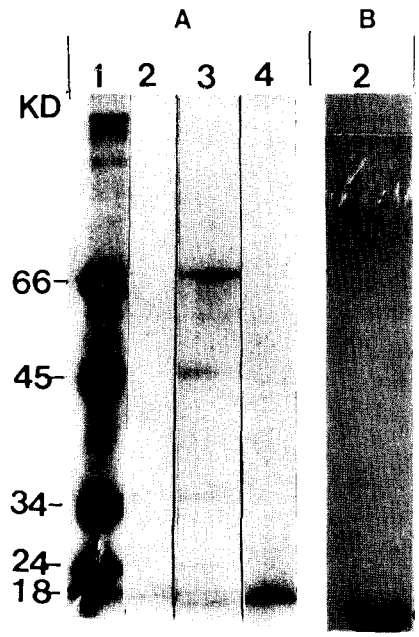

Fig. 3. Molecular weights of proteins from each aqueous fraction. A side : Stained with coomassie blue; B side : Stained with schiff's reagent; lane 1 : Protein standard (MW 66~18 KD); lane 2 : Protein from the aqueous fraction by methanol; lane 3 : Protein from the aqueous fraction by chloroform; lane 4 : Protein from the aqueous fraction by acetone.

Table 2. Contents of protein and hexose in aqueous fraction

	Protein (μg/mg of ext.)	Hexose (μg/mg of ext.)	Hexose/ Protin
Aqueous fraction by CH_3OH	121± 4.0	610	5.0
Aqueous fraction by $CHCl_3$	250± 14.0	430	1.7
Aqueous fraction by acetone	214± 11.0	530	2.5

The assay of protein and hexose were performed as describe in "Materials and Methods".

polypeptide were discovered in the aqueous fraction extracted with methanol fraction (Fig. 3, lane 2: B) and 18 KD polypeptide was contained in the aqueous fraction extracted with acetone (Fig. 3, lane 4). The phosphorylation of 20 KD polypeptide indu-

ced by PMA is slightly inhibited by all fractions as shown in Fig. 1 & 2. When the composition of the aqueous fractions, was analyzed carbohydrates were much more contained in the aqueous fractions than proteins (Table 2). Maltose, sucrose, glucose, and mannose were also found in the aqueous fractions (data not shown).

Proteins present in the aqueous fraction were analyzed by SDS-PAGE and subsequently stained with Coomassie Brillant Blue R-120. 66 KD and 45 KD polypeptides were major proteins observed in the aqueous fraction extracted with chloroform, and 18 KD polypeptide was observed in the aqueous fraction extracted with acetone. But any protein band was not detected in the aqueous fraction extracted with methanol by this detection method (Fig. 3A). The glycoprotein of low molecular weight, below 18 KD polypeptide, was detected only in the aqueous fraction extracted with methanol when stained with Schiff's reagent (Fig. 3B).

It is remarkable that the aqueous fractions extracted with organic solvent first and then successively washed with water inhibit the phosphorylation of 40 KD polypeptide. To elucidate possible role of contaminant saponin in the inhibitory effect of the aqueous fraction on the activity of protein kinase C, further study is required. These data suggest that the aqueous fractions from Korean red ginseng modulate the differentiation and growth of cells.

요 약

홍삼으로부터 추출한 수용성 분획은 tumor promoter인 phorbol-12-myristate-13-acetate(100 nM)가 유인한 사람 혈소판의 40 KD 및 20 KD 단백질의 인산화를 억제했다. 이 결과는 홍삼의 수용성 분획이 antitumor 또는 antiplatelet 작용을 하고 있음을 시사하는 것이다. 이와 같은 작용을 하는 성분은 확실히 모르지만, 이들 수용성 분획에는 단백질보다 탄수화물이 더 많이 함유되어 있었고, 특히 methanol로 추출한 수용성 분획에는 18 KD 이하의 glycoprotein이 함유되어 있었다.

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