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Protein Fraction from *Panax ginseng* C.A. Meyer Regulates the Glycogen Contents by Modulating the Protein Phosphorylation in Rat Liver

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Abstract□When rat liver homogenates were incubated with 1 mM CCl_4 for five min, glycogen level was decreased, while treatment with protein fraction G_4 increased the glycogen level. In addition, G_4 inhibited the phosphorylation of 34 KD and 118 KD polypeptides induced by CCl_4. These proteins were more strongly phosphorylated by Ca^{2+}/calmodulin-dependent kinase than by C-kinase. Since 34 KD polypeptide was solely phosphorylated by NaF (50 mM), an inhibitor of both glycogen synthase and phosphoprotein phosphatase, it is inferred that 34 KD polypeptide is glycogen synthase-like protein. Because glycogen synthesis is inhibited by phosphorylation of Ca^{2+}-dependent glycogen synthase, it is suggested that G_4 increased liver glycogen level by inhibiting phosphorylation of 118 KD polypeptide (Ca^{2+}-pump) which modulates intracellular Ca^{2+} level, and by inhibiting phosphorylation of 34 KD polypeptide which is thought to glycogen synthase-like protein.

Key words□Rat liver, CCl_4, protein fraction, glycogen, inhibition of protein phosphorylation.

Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), is a well known Oriental folk medicine. It improves or invigorates a physiological condition that is weakened by stress or disease. It had been reported that ginseng polypeptide decreased the level of liver glycogen which was increased by alloxan.[1] But the mechanism was not known.

The hepatotoxin carbon tetrachloride (CCl_4) has been extensively studied as a model compound producing hepatocellular necrosis. It is known that hepatotoxicity by CCl_4 is due to an excessive accumulation of Ca^{2+} in hepatocytes.[2,3]

The intracellular Ca^{2+} is an important regulator of cellular metabolism. Increase of cytosolic free Ca^{2+} above the resting level>0.1 μM is associated with stimulation or inhibition of cellular functions.[4] Endoplasmic reticulum (or sarcoplasmic reticulum), mitochondria and plasma membranes have been considered as the three important sites for the regulation of intracellular calcium levels. Suggestion that plasma membrane,[5] endoplasmic reticulum[4] in liver contain ATP-dependent Ca^{2+}-pump has been allowed to study for cellular functions in relation to intracellular calcium levels.

Glycogen synthase is the key enzyme in the control of glycogen synthesis. Its activity is regulated by phosphorylation/dephosphorylation reactions affecting multiple sites. Dephosphorylated forms are physiologically active, whereas the more phosphorylated forms are inactive.[6,7] The phosphorylation of glycogen synthase is triggered by the Ca^{2+} accumulation into liver cells.[6] Ca^{2+}-pump in both plasma membrane and endoplasmic reticulum is 118 KD polypeptide.[4,8] It has also been reported that 63 KD polypeptide is a calcium binding protein in rat liver endoplasmic reticulum and has a Ca^{2+}-ATPase activity.[9] In several reports, hepatic glycogen synthase is reported to be 90 KD, 73 KD and 85 KD

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polypeptides.[10] [12] In this report, we suggested that G_4 regulates the protein phosphorylation (34, 63 and 118 KD polypeptides) related to the modulation of the levels of hepatic glycogen and hepatic Ca^{2+}.

Materials and Methods

1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), and N-(6-aminohexyl)-5-chloro-1-naphtalene sulfonamide (W-7) were obtained from Sigma Chemical Co. [γ-^{32}P]ATP (250 μCi) was purchased from Amersham Life Science Co.

1. Preparation of partially purified protein fraction from *Panax ginseng* C.A. Meyer (Korean red ginseng)

Korean red ginseng was cut into 0.5 cm length, powdered, and extracted with chloroform. Preliminary extracts were concentrated under a reduced pressure (25~30℃), and then separated into aqueous fractions and lipophilic fractions in a separatory funnel by adding chloroform and water successively to the funnel. The aqueous fractions were collected, freeze-dried, and fractionated with Bio-Rad Econo System. Freeze-dried sample (5 g) was dissolved in 0.01 M Tris buffer (pH 7.0) and loaded on a Sephadex G-75 column (2.5×75.0 cm). 2 m*l* of each fraction were collected and absorbance was measured at 254 and 280 nm. The flow rate was maintained at 0.1 m*l*/min. Fractions which had absorption-peak at 254 and 280 nm were lyophilized and termed G_4 fraction. Molecular weight of protein in G_4 fraction was determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 1.5 mm, 10%).[13] 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 pancrease; 24,000, trypsin inhibitor, soybean; 20,000, α-lactalbumin, bovine milk; 14,200, Sigma).

2. Preparation of liver homogenates

Five m*l* of cold homogenate buffer (0.25 M sucrose, 5 mM Tris, 5 mM $MgCl_2$, pH 7.4) containing 1 mM mercaptoethanol were added per gram of liver immediately after removing the liver from rats fed for 24 hr. Liver tissues were cut into small piecies and homogenated in a glass potter homogenizer with three passage of fitting teflon pestle (600~1,000 rpm, 5 times). The homogeneates was filtered by a cotton cheese cloth to remove tissue debris and centrifuged for 10 min at 700 x g. The upper layer of pellet was carefully collected and a part of it was used as a homogenate fraction.

3. Protein phosphorylation of homogenates

After the liver homogenate containing 100 μg of protein was preincubated for 3 min at 37℃ with or without 1 mM $CaCl_2$, H-7, W-7, NaF and protein fraction G_4, 2 μCi of [γ-^{32}P]ATP was added and incubated for 5 min. The reaction was stopped by adding an equal volume of SDS buffer (0.125 M Tris, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01 % bromophenol blue, pH 6.8). The reaction tubes were boiled for 5 min to denature the proteins completely. Aliquotes containing 20 μg of protein from reaction tubes were loaded onto SDS-PAGE (10%, 1.5 mm gel) according to Laemmli.[13] Separated proteins were stained, destained, dried and autoradiographed onto X-ray film (Fuji Medical X-ray Film) at −70℃ for 15 days. Phosphorylated protein bands are scanned by a transmittance/reflectance scanning densitometer (Hoefer Sci., GS 3000).

4. Determination of glycogen level.

After the liver homogenate containing 1 mg of protein was preincubated for 3 min at 37℃ for 5 min. The reaction was stopped by adding 3 m*l* of 1 N NaOH and glycogen was precipitated by the addition of 2 m*l* ethanol. Liver glycogen was determined according to the method of Carroll *et al.*[14]

5. Other methods

Hexose and protein were assayed by anthrone colorimetric reaction[15] and by Lowry method,[16] respectively.

Results and Discussion

An protein fraction extracted with chloroform

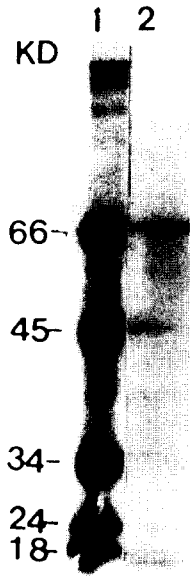

Fig. 1. Molecular weights of protein fraction. Lane 1 : protein standard (MW 66~18 KD); lane 2 : protein from the aqueous fraction stained with Coomassie blue.

Table 1. Contents of protein and hexose in a partially purified protein (G_4)

Protein (μg/mg)	Hexose (μg/mg)	Hexose/ protein
29.7± 3.4	83.5± 5.0	2.8

The assay of protein and hexose were performed as described in "Material and Methods".

mainly consisted of 66 KD and 45 KD polypeptides (Fig. 1), and when was more purified with Sephadex G-75 column, 45 KD polypeptide band in the protein fraction was not detected (Fig. 1).[17] In a partially purified protein (G_4) the protein content was less than that of hexose (Table 1). This protein seems to be the same as hydrophobic proteins which were extracted using organic solvents, such as chloroform, methanol, and acetone.[18] It is inferred that hexose is not bound to the protein but free one. It had reported that carbon tetrachloride (CCl_4) dr-creased the level of hepatic glycogen,[19,20] which is due to the abnormal glycogenesis and decreased glucose uptake by CCl_4.[19,20] This means that glucose is increased by CCl_4 in blood. When the homo-

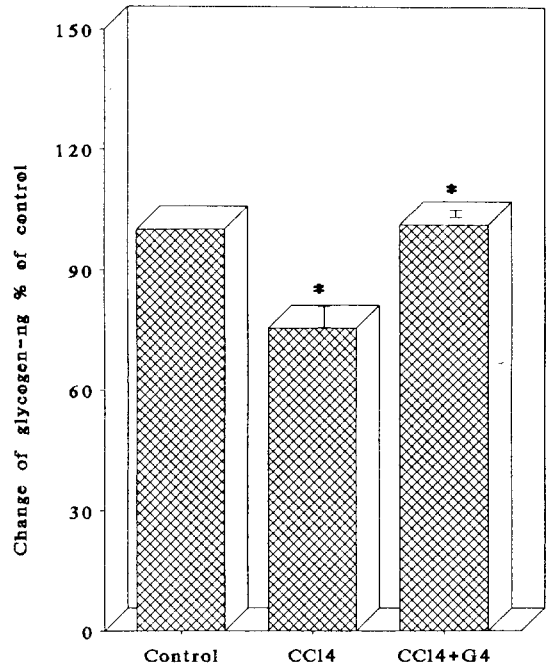

Fig. 2. Effect of protein fraction (G_4) on glycogen production by exogenous CCl_4 in rat liver. The assay of glycogen level was performed as described in "Materials and Methods". The data are given as the Mean± S.E. (n=3) and change of ng % of control. 22.2± 0.3 (Mean± S.E.) ng % was contained in control. Cont : control, G_4 : protein fraction purified with Sephadex G-75 column chromatography.
*Significant at $p<0.01$.

genates containing 1 mM CCl_4 were incubated for 5 min at 37℃, the level of glycogen was decreased (Fig. 2), G_4, however, increased the level of glycogen reduced by CCl_4 (Fig. 2). This suggests that G_4 regulates the glycogen synthase. Glycogen synthase, an enzyme involved in the rate-limiting step in glycogen synthesis, is under metabolic and endocrine controls. Regulation of the activity of glycogen synthase is believed to occur via the phosphorylation/dephosphorylation reaction catalyzed by specific kinase and phosphatase.[6,7]

As mentioned in the introduction, the phosphorylation of glycogen synthase is triggered by the Ca^{2+} accumulation into liver cells.[6] This Ca^{2+} is regulated by Ca^{2+}-pump (Ca^{2+}-ATPase) in plasma membrane and endoplasmic reticulum.

As shown in Fig. 3, when homogenates containing

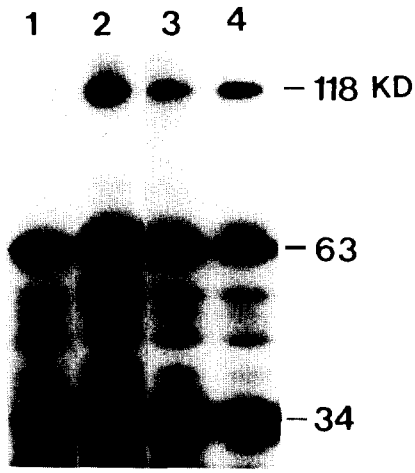

Fig. 3. Effect of protein fraction (G_4) on protein phospholyation induced by CCl_4. Lane 1 : control; lane 2 : 1 mM CCl_4; lane 3 : CCl_4 + protein fraction 200 μg/m*l*; lane 4 : CCl_4 + protein fraction 400 μg/m*l*.

Table 2. Inhibition of protein fractin (G_4) on protein phosphorylation

	118 KD	63 KD	34 KD
Control	100%	100	100
G_4 fraction			
200 μg/m*l*	37	59	95
400 μg/m*l*	60	54	58

Phosphorylation of polypeptides which is represented in Fig. 3 was calculated as the area of peaks by transmittance/reflectance scanning densitometer (Hoefer Sci., GS 3,000).

1 mM $CaCl_2$ were incubated for 5 min at 37℃, 118 KD, 63 KD and 34 KD polypeptides were phosphorylated by CCl_4 (Fig. 3, lane 2, Table 2). However, protein fraction G_4 of 200 μg/m*l* and 400 μg/m*l* potently inhibited the phosphorylation of 118 KD, 63 KD and 34 KD polypeptides (Fig. 3, lane 3, 4, Table 2). Because 118 KD and 63 KD polypeptides functioning as Ca^{2+}-pump are present at plasma membrane and endoplasmic reticulum, it is reasoned that CCl_4-induced these protein phosphorylation is related to Ca^{2+} accumulation into liver cell, and because this Ca^{2+} is associated with the phosphorylation of glycogen stnthase,[6] it is also suggested that protein fraction (G_4) regulates the glycogen synthesis by inhibiting the phosphorylation of 118 KD, 63 KD and 34 KD polypeptides.

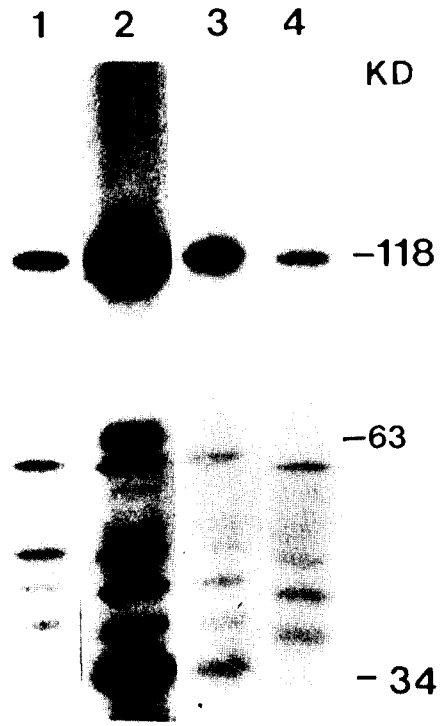

Fig. 4. Inhibition of protein phosphorylation. Lane 1 : control; lane 2 : 1 mM CCl_4; lane 3 : CCl_4 + H-7 (6 μM); lane 4 : CCl_4 + W-7 (25 μM).
H-7 : 1-(5-isoquinolinylsulfonyl)-2-methyl piperazine
W-7 : N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide

On the other hand, Ca^{2+}/phospholipid-dependent protein kinase C (C-kinase) and Ca^{2+}/calmodulin dependent kinase are stimulated by Ca^{2+}. CCl_4-induced the phosphorylation of 118 KD, 63 KD and 34 KD polypeptides (Fig. 4, lane 2) were inhibited by 1-(5-isoquinolinylsulfonyl)-2-methylpiperzine (H-7), an inhibitor of C-kinase, and N-(6-aminohexyl)-5-chloro-1-naphthalalene sulfonamide (W-7), an inhibitor of Ca^{2+}/calmodulin-dependent kinase (Fig. 4, lane 3, 4). Inhibitory degree of these was stronger by W-7 than by H-7 (Fig. 4, lane 3, 4). These results suggest that these protein phosphorylation is more potently occurred by Ca^{2+}/calmodulin dependent kinase than by C-kinase. It is thought that 118 KD polypeptide is not Ca^{2+}-pump in plasma

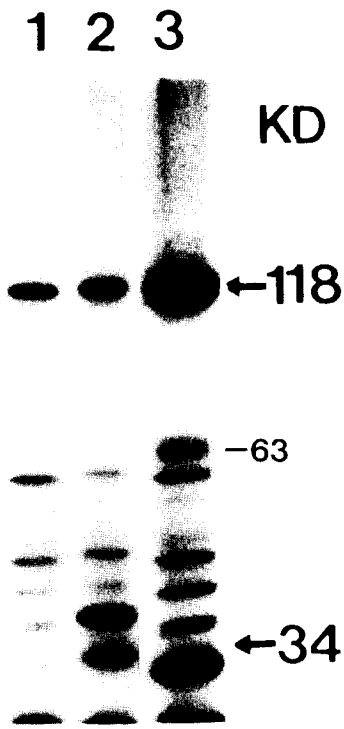

Fig. 5. Inhibiton of protein phosphorylation by NaF. Lane 1 : control; lane 2 : 50 mM NaF; lane 3 : 1 mM CCl_4.

membrane but Ca^{2+}-pump in endoplasmic recticulum. The reasons are as follows: first, Ca^{2+} was accumulated in liver microsome by CCl_4 (data not shown); second, when liver homogenates were seperated by centrifugating at 9,200 x g, the phosphorylation of 118 KD polypeptide was identified in the supernatant which has endoplasmic reticulum-rish fractions, and its phosphorylation also was identified in liver microsome fraction (data not shown).

118 KD phosphorylation was not occurred, but 34 KD phosphorylation was solely occurred by 50 mM NaF, an inhibitor of both glycogen synthase and phosphoprotein phosphatase (Fig. 5, lane 2). From these results, it is thought that 34 KD polypeptide phosphorylated by CCl_4 may be a protein related to glycogen synthase. Accordingly, it is suggested that the increase of glycogen level by protein fraction G_4 is likely due to the inhibition of the phosphorylation of 34 KD polypeptide by protein fraction (Fig. 3, lane 3, 4). The protein fraction (G_4) contained both free-hexose and protein (Table 1, Fig. 1). In order to understand the role of the protein-hexose complex in inhibiting the protein phosphorylation and increasing the synthesis of glycogen, further study is required.

요 약

쥐간 homogenate에 1 mM CCl_4를 첨가하여 5분간 incubation시켰을 때, glycogen level이 감소하였으나, 단백질 분획 G_4의 첨가에 의하여 증가되었다. 또한 CCl_4에 의해 일어나는 34 KD 및 118 KD polypeptides (Ca^{2+}-pump)의 인산화는 G_4에 의해 저해되었고, 이들 단백질의 인산화는 C-kinase보다 Ca^{2+}/calmodulin-dependent kinase에 의해 더 강하게 일어났다. 그리고 glycogen synthase의 저해제 및 phosphoprotein phosphatase의 저해제인 NaF(50 mM)에 의해 34 KD polypeptide가 유일하게 인산화되었기 때문에 이 34 KD polypeptide가 glycogen synthase-like protein인 것 같이 생각되어진다.

Glycogen의 합성은 Ca^{2+} 의존성 glycogen synthase의 인산화에 의해 억제되므로, G_4는 칼슘 조절 작용을 하는 118 KD polypeptide(Ca^{2+}-pump)의 인산화와 glycogen 합성효소와 유사한 단백질인 34 KD polypeptide의 인산화를 저해시킴으로써 쥐간에서 CCl_4에 의해 저하된 glycogen의 생성을 촉진하는 것으로 추론된다.

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