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Stabilization of β-D-galactosidase from Heat and Chemical Inactivation with the Extract of *Panax ginseng* C.A. Meyer

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Abstract □ Staiblization effect of *Panax ginseng* C. A. Meyer on β-D-galactosidase inactivation was pro ved by kinetic studies of thermal inactivation of the enzyme. The water extract *Panax ginseng* C.A. Meyer showed stabilization activity at minimal concentration of 10ppm. The methanolic extract was purified to obtain ginseng saponins, and two groups of the ginsenosides, *i.e.*, protopanaxadiol and protopanaxatriol were isolated. They also showed a protective effect against the thermal and chemical inactivation of the enzyme; *p*-chloromercuribenzoic acid and hydroxylamine known as protein modifier greatly inactivated the enzyme but inactivation was significantly blocked by the ginseng component.Mg^{2+}. known as a cofactor, stabilized the enzyme and the poor stabilization effect by it was potentiated by ginseng components.

Keywords □ Ginseng saponin, β-D- galactosidase, Stabilization, Thermal inactivation, Kinetic studies, Protopanaxadiol, Protopanaxatriol, *p*-Chloromercuribenzoic acid, Hydroxylamine.

It is well known that ginseng exerts numerous pharmacologic effects, and is widely used to improve the ill state of human body or to cure diseases[1]. Compared with other drugs ginseng shows differences in its pharmacology, in which the biological activity is very diverse and mild.[2] The characteristics of ginseng pharmacology was described as adaptogenic effect by Brekhmann[3] which means nomalization of physiological condition to maintain the individual homeostasis.

Bioactive materials among numerous natural products, especially drugs,interact with proteins in body fluids to give some pharmacologic activities. The molecular interaction between the drug and protein is dependant on the structural specificity of a protein which is the binding material. The conformational structure of a protein acts an important role in its function[4], because the stability of macromolecules is very sensitive to its conformation[5~9]. The various physiological activities of ginseng may be appeared through proteins,and thus researches on the interaction of protein-ginseng components and clarification of the reaction mechanism may be very important.

Kim *et al.*[10] suggested the possibility of stabilization of an enzyme, cabonic anhydrase, by ginseng extracts. Han *et al.*[11] found that bovine serum albumin was stabilized by a ginseng saponin, ginsenoside Rg_1 against thermal denaturation. Back *et al.*[12] proved the red blood cell membrane was protected against physical or mechanical stress by ginseng extract.

The purpose of the present work is to elucidate the effect of ginseng components on the confo rmational stability of proteins by employing β-D-galactosidase and heat as a model enzyme and an inactivation method, respectively.

EXPERIMENTAL METHODS

Preparation of the Ginseng Saponin

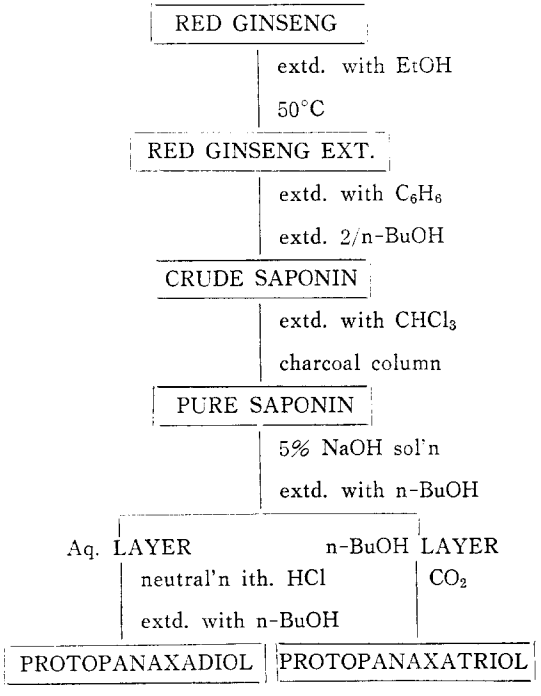
Side roots of Korean red ginseng, manufactured by the office of Monopoly, the Republic of Korea, were extracted with 70% ethanol for six hours at 50°C and this extraction was repeated twice.

The combined ethanolic extract was concentrated to about 40% moisture content with a rotary evaporator. The above processes were operated on a pilot plant scale at Ilhwa Company in Seoul, Korea. The amount of 430kg of ginseng extract was yielded from 865kg of the raw material. In order to remove lipophilic components, benzene was added to the ethanolic extract, agitated for 1 hour and decanted. The volume ratio of the ethanolic extract to benzene was 1 to 5. After two times of benzene treatment the residue was dissolved in water at volume ratio of 1 to 5 and the pH of aqueous phase was adjusted to about 7.3 with 5% NaOH solution. The saponin fractions in the aqueous solution was extracted 5 times with the same volume of butanol. The amount of 80kg of crude saponins was obtained from the combined butanol layer after evaporation at 50°C.

The processes of benzene and butanol treatm ents of the ethanol extract were done on a pilot plant scale at the Pacific Chemical Co. in Suwon, Korea. The water extract of Red Ginseng was purchased from the office of Monopoly, the Republic of Korea and used without further treatment.

Isolation of Protopanaxadiol and Protopanaxatriol Saponins

The amount of 100g of crude saponins was treated with 500ml of chloroform 3 times to remove the residual lipophilic impurities. The residue was dissolved in 200ml of 90% methanol and it was loaded on a 7×50cm charcoal column which was prewashed with methanol. The residue was then eluted using 90% methanol, and the eluted methanol was evaporated with a vacuum evaporator at 65°C to obtain 80g of white saponin powder. The amount of 50g of saponins was dissolved in 5% NaOH solution, followed by extraction 5 times using butanol, and the combined butanol layer was treated by employing CO_2 gas to remove Na^+ as a Na_2CO_3 precipitate. The butanol layer was evaporated to obtain mainly protopanaxatriol saponins[13]. The aqueous layer was neutralized with conc. HCl and extracted by butanol. The combined butanol layer was evaporated under vacuum at 70°C. The dryed powder was dissolved in absolute methanol to precipitate the remained NaCl. The supernatant was evaporated to dryness to give protopanaxadiol saponins. (Scheme I)



SchemeI: Isolation of ginseng saponin, protopanaxadiol and protopanaxatriol saponins.

Deteramination of the Rate Constant of the Enzyme Inactivation

Lyophilized powder of β-D-galactosidase(EC. 3.2.1.23) from *E. coli* (Sigma Chemical Co.) was used and the specific activity was 250 ONPG units at pH 7.3, 37°C. The rate constant and half-life of the enzyme inactivation was determined from the asymptotic curve of enzyme activity under thermal inactivation using the Chemistry Auto-Ananlyser, Gilford System 3500 and spectrofluorophotometer Farrand Mark I equipped with a circulation water bath, as described previously.[14] Artificial substrates of the enzyme, *o*-nitrophenyl-β-D-galactopyranoside(ONPG) and 4-methylumbelliferyl-β-D-galactopyranoside were used for the enzyme assay.

The enzyme, 1.0μg in 0.05ml Tris buffer of pH 7.3 was added to the solution of 0.068 M *o*-nitrophenyl-β-D-galactopyranoside in 0.1M Tris buffer, pH 7.3, containing 0.03M $MgCl_2$ and NaCl and the reaction mixture was pre-incubated for 3 seconds at room temperature and suctioned into the thermocuvette after 12 seconds of the temperature equilibration. The absorbance of the reaction mixture in the thermocuvette was recorded continuously. All the procedures were operated automatically using the program card of General Kinetics I or II furnished by Gilford System.

For the spectrofluorometric measurement a solution of 0.48mM methylumbelliferyl-β-D-galactopyranoside was used as the enzyme substrate and other reagents were as the same as those for the spectrophotometric measurement.

RESULTS

Preparation and Isolation of the Ginseng Saponin

Ginsenosides Ro, Ra, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 and Rg_2 could be identified from the charcoal treated saponin mixture on an HPLC chromatogram(Fig. 1-A). Impurities were almost disappeared on the HPLC chromatogram but some UV absorbing peaks were observed at 254nm. The HPLC pattern of each protopana-

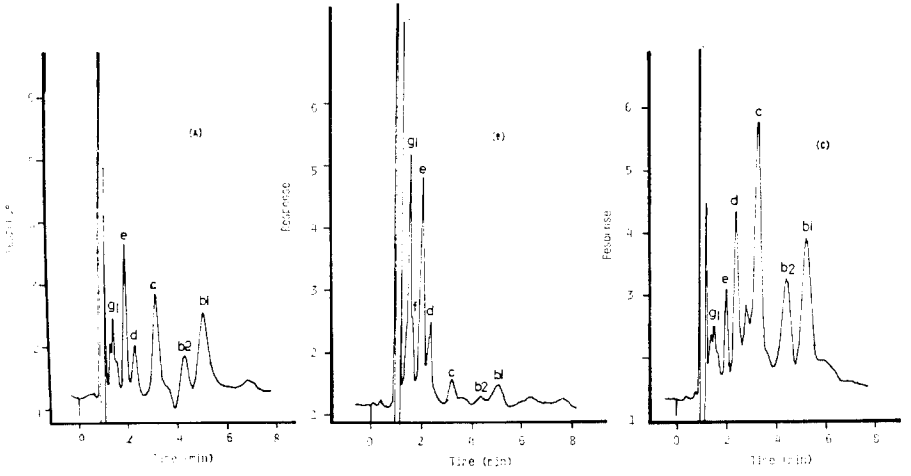


Fig. 1: HPLC pattern of total (A), triol (B) ad diol (C) saponin mixture.
Column: Carbohydrate Analysis (3.9mm×30cm)
Solvent: Acetonitrile/H_2O/Butanol (85/20/15)
Sample: 20μl (15% aquaous solution)
Flowrate: 3.0ml/min.
Detector: Reflective index

xatriol and protopanaxadiol saponins showed a good separation, showing Re, Rf and Rg_1 as the main components of the protopanaxatriol saponins (Fig. 1-B) and Rb_1, Rb_2, Rc and Rd as the major components of the protopanaxadiol saponins (Fig. 1-C).

Enzyme Stabilization against Heat Heat Inactivation

The half-life of β-D-galactosidase activity was determined in low concentration of protopanax-

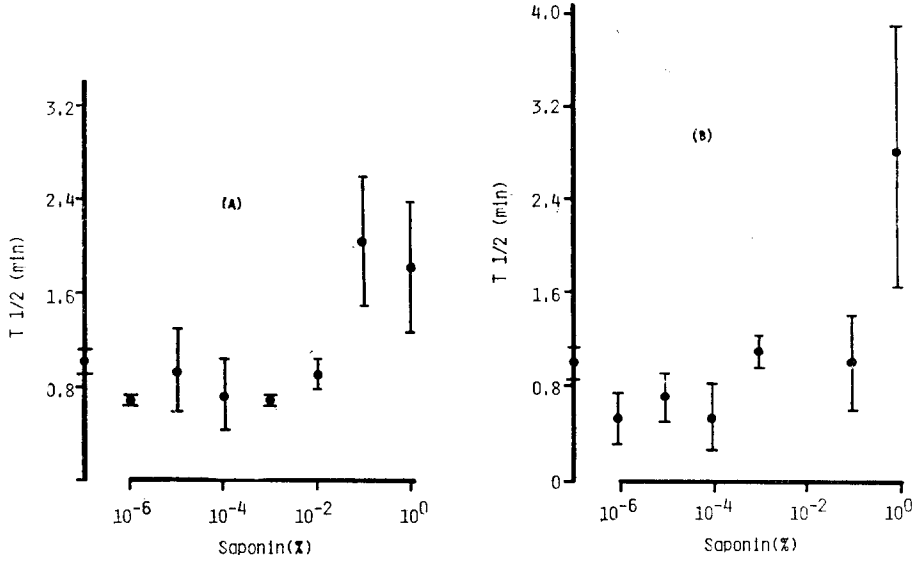


Fig. 2: Stabilization of β-D-galactosidase by diol (A) and triol(B) saponin mixture. Half-life of the enzyme under inactivation condition was measured in the reacion mixture of each saponin concentration using 4-methylumbellifery β-D-galactoside as enzyme substrate. Reaction mixture was 2.55ml.

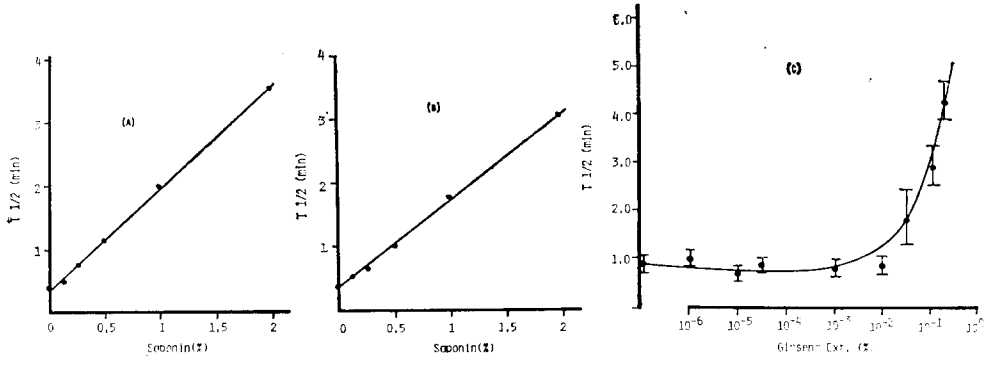


Fig. 3: Stabilizing activity of protopanaxadiol (A), protopanaxatriol (B) sapon mixture and ginseng water extract (C) for β-D-galactosidase. Half-life of the enzyme activity was measured under inactivation condition with increasing ginseng concentration in the reaction mixture. Temperature, 53°C, pH7.3: 0.1M Tris buffer, enzyme 10μg. The absorbance of *o*-nitrophenol hydrolyzed from *o*-nitrophenyl-β-D-galactoside (ONPG) was recorded continuously by Chemistry Autoanalyzer, Gilford System 3500. Asymptotic curve of the absorbance was analyzed by the asymptotic regression method and half-life of the enzyme was calculated. ONPG concentration was 6.3mM and the total reaction volume was 1.0ml.

atriol and protopanaxatriol saponins using 4-methylumbelliferyl β-D-galactopyronoside as the enzyme substrate. The halflife was significantly increased at the concentra ion of 100 ppm and 10 ppm of protopanaxatdiol and protopanaxatriol saponins, respectively(Fig. 2-A, 2-B).

The half-life of the enzyme activity was about 0.8 minutes at 45°C in lower concentration than the critical concentration of saponin. But it increased to about 2.0 minutes at 1000 ppm of protopanaxatriol saponins.

The stabilizing effect of the water extract of Korea Red Ginseng was studied and it showed a great effect against thermal inactivation of β-D-galactosidase as shown in Fig. 3-C. The half-life of this enzyme began to increase above the concentration of 10ppm of the extract. The stabilizing effect of ginseng saponins was determined at higher concentrations of saponins by using ONPG as the enzyme substrate and Chemistry Autoanalyzer at 53°C. The half-life of enzyme activity increased linearly with increasing the saponin concentration up to 20,000 ppm. 0.3 minutes of the half-life at control increased to about 3.5min. (protopanaxadiol) and 3.2min. (protopanaxatriol) as shown in Fig. 3-A and 3-B.

Effect of $MgCl_2$ on the Protective Effect of Ginseng Saponin

Magnesium ion is required as a cofactor in the enzyme catalysis although not indispensable for the coenzyme activity. To elucidate the stabilization mechanism of ginseng saponins, magnesium free enzyme, magnesium complexed enzyme and both form of the enzyme were thermally inactivated and the half-life was measured in the presence and absence of ginseng saponins.

The half-life of the metal free enzyme was not significantly changed by the ginseng saponins. But it increased significantly in the presence of magnesium ion and the ginseng saponins (Table I).

Table 1: Effect of $MgCl_2$ on the protective activity of triol saponin.

Material	$MgCl_2$(mM)	t 1/2(min)
Tris buffer	0.00	0.56
Tris buffer	0.36	1.55
Tris buffer	1.43	1.60
Tris buffer	2.85	1.62
Triol 1%	0.00	0.66
Triol 1%	0.89	4.68
Triol 2%	0.00	1.30
Triol 2%	0.89	9.02

The metal ion content of the saponin sample was measured with an atomic absorption spectrophotometer. The concentrations of Na^+,K^+ and Mg^{2+}, were determined but they were negligble to effect the enzyme stability. From this it could be concluded that the stabilizing effect of the ginseng saponin was not contributed by the metal ions contaminated in saponin sample.

Enzyme Stabilization against Chemical Reagents

p-chloromercuribenzoic acid(PCMB) and Hydroxylamioe (HA) are known very strong enzyme inhibitors by attacking sulhydrl groups or carbonyl groups which may play an important role in the catalytic site of an enzyme or may participate in the intra or intermolecular hydrogen boning to influence the conformational stabilty of a protein molecule.

Although the sulfhydryl groups of β-D-galactosidase were proved not to be participated in the catalytic site[24], and thus the enzyme activity was known not to be decreased by blocking the sulfhydryl groups to some extents. But the stability of the enzyme activity was significantly

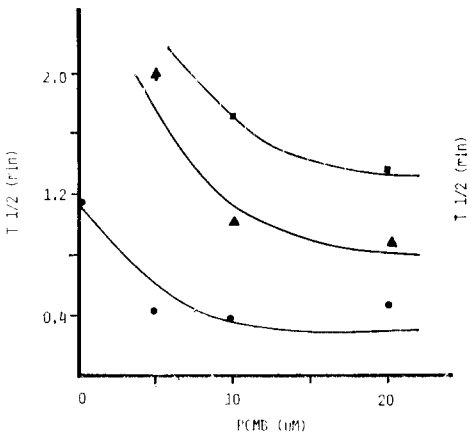


Fig.4. : Stablization of β-D-galactosidase from *p*-chloromercuribenzoic acid inactivation.
The enzyme was inactivated by the PCMB and the half-life of the enzyme in each PCMB concentration was measured under triol saponin mixture.
Control —●— saponin 0.3% —▲— saponin 1.0% —■—
Reaction temperature 48°C, pH7.3 0.1 M Tris buffer.

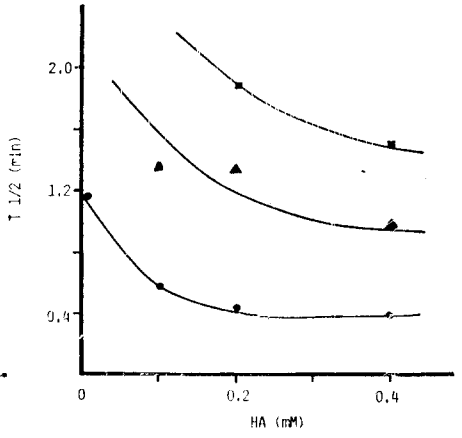


Fig.5. : Stabilization of β-D-galactosidase from hydroxylamine.
The half-life of the enzyme was measured in each reaction mixture of hydroxylamine and triol saponin.
Control —●— saponin0.1% —▲— saponin 0.2% —■—
Temperature 48°C, pH 7.3 0.1M Tris buffer, total reaction mixture 1.0ml, substrate 6.8mM ONPG.

decreased by PCMB in the present investigation and the half-life of the enzyme increased in the presence of the ginseng saponin (Fig.4).

The protective effect of the ginseng saponin against the inactivation by hydroxylamine was similar to that against PCMB inactivation as shown in Fig.5. But the ginseng saponin was more effective for the hydroxylamine inactivation than for the PCMB inactivation.

DISCUSSION

The HPLC chromatogram of the total saponin mixture in Fig. 1 showed all the ginsenosides known. Sugars, the most abundant impurities in the saponin fraction, was not detected by HPLC equipped with RI detector except only trace amount of sucrose,of which retention time lies between those of the ginsenoside Rc and Rd in the given HPLC condition.

Some impurities absorbing UV at 254nm was also observed on the HPLC chromatogram under the same instrumental condition as above. To obtain the absorbing spectrum of the sample, 0.1% methanolic solution was scanned from 700nm to 350nm. No characteristic spectrum was obtained, but the absorbance was simply increased at the wavelength shorter than 470nm.

The conformational structure of a protein is largely contributed by hydrogen bonding, and van der Waals interaction in the macromolecule [15]. The structure can be changed by the change of these binding forces, and this conformational change is defined as denatruation in general[16]. If these macromolecules are enzymes, the catalytic activity will be influenced by the conformation of the enzyme. The inactivation of an enzyme is caused by denaturation of the

enzyme by denaturing factors including heat, ultraviolet light, organic solvents, urea, and salts[16].

In this work, it was proved that some components in ginseng, whether it is saponin or not stabilized β-D-galactosidase, but the stabilizing mechanism is still unknown and some possibilities should be discussed. The inactivation of β-D-galactosidase is known to be caused by the dissociation of the tetramer enzyme molecule[17]. Stabilization of the quaternary structure of this enzyme may be established by blocking the dissociation of the tetramer resulting from the stabilizing the conformation of each subunit. But it should not be excluded the possibility that the stablization is caused by inhibiting the dissociation of the tetramer molecule without the conformational change of each subunit.

It is well known that the catalytic activity of an enzyme is decreased by modification of some functional groups in the enzyme. β-D-galactosidase was studied in this respect. From these studies, it may be expected that this enzyme can be catalytically stabilzed by preventing the chemical modification of functional groups. The protein, β-D-galactosidase, may be protected from the modification of functional groups by heat as well as chemicals.

The possibilities of stabilization of this enzyme may be supported by numerous works in this fields[18-22]. Recent advances in this fields show that much of the allosteric regulation in biological system is caused by the chemical modification of some enzymes as well as metabolic intermediates. The famous typical example is the α-glucanphosphorylase in glucose metabolism[23].

The catalytic activities of enzymes is known be to mainly controlled by the conformational change of the enzymes. From these fact it is evident that the activity change of enzymes can be prevented by inhibiting the conformational change. And consequently the catalytic activity can be stabilized.

Sulfhydryl groups in β-D-galastosidase are known to contribute largely to the catalytic activity, although they do not directly participate in the catalytic reaction[24]. Nineteen sulfhydryl groups in each subunit is known to be easily modified by *p*-chloromercuribenzoic acid, resulting in inactivation of this enzyme.

In this work, the protecting effect of ginseng components against the chemical modification of -SH groups was studied and it was confirmed that the catalytic activity of β-D-galactosidase was stabilized by ginseng components. But at the present time it is uncertain whether some unknown compounds in ginseng blocked-SH groups or they protected β-D-galactosidease from subunit dissociation.

Hydroxylamine is known to modify the carbonyl group of a protein and thus inactivate the enzyme. β-D-galactosidase was largely inactivated by this reagent and the ginseng component protected it from the modification. Further knowledge in stablization of this enzyme from those chemical modification should be clarified.

Many enzymes need metal ions as a cofactor and they are activated in the presence of them [25, 26]. Metal ions are known to activate enzymes by binding with the enzyme itself or with its substrate[27-29]. The stabilizing effect of ginseng on this enzyme, as shown in this work, was enhanced by Mg^{2+} ion. But it should be clarified whether a certain ginseng component binds directly to the enzyme, consequently increasing the affinity of the enzyme to metal ions or it exerts its activity directly by effecting the electrostatic interaction between the enzyme and metal ions.

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