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Effects of Ginseng Saponin on the Lysosomal Enzyme Activities in Streptozotocin-induced Diabetic Mice

C. K. Moon, H. S. Kim* and K. S. Kim
College of Pharmacy, Seoul National University

인삼 Saponin이 Lysosome 효소 활성에 미치는 영향

문창규 · 김희수* · 김강석
서울대학교 약학대학

ABSTRACT—Lysosomal enzymes might play a most important role in the pathogenesis of diabetic microangiopathy. Some glycosidases, which participate in the catabolism of glycoprotein, are significantly decreased in diabetic mice. In search of new potential lysosomal enzyme inducers, we examined the effects of crude red-ginseng saponin fraction on N-acetyl-β-D-glucosaminidase, β-D-galactosidase and α-D-mannosidase activities in the liver and kidney of normal and streptozotocin induced diabetic mice. It was found that i.p. administration of ginseng saponin produced the induction of lysosomal enzymes in the kidney more intensively than in the liver. The obtained results suggest the possibility that ginseng saponin might prevent the diabetic microangiopathy.

INTRODUCTION

Microangiopathy has become one of the most important problems of diabetes mellitus. Although the pathogenesis of diabetic microangiopathy is not completely known, evidence to date indicates that altered metabolism of glycoproteins is involved in the occurrence of the diabetic vascular lesions, characterized by a thickening of the basement membrane and the deposition of PAS-positive materials,[1,2] chronic hyperglycemia stimulates glycosylation. Glycosylation produces capillary basement membrane thickening (CBMT) and glomerular deposits of glycoproteins primarily in retina and kidney.

Glycosidases in lysosomes have been suggested to participate in the catabolism of glycoprotein[3], so decrease in their activities in the kidney could be an important factor in development of microangiopathy in diabetes. CBMT is caused by retarded catabolism of glycoprotein which follows decrease of lysosomal enzyme activities and/or increased membrane biosynthesis. Actually it was reported that a hydroxylysine-linked α-glucosyl-β-galactose disaccharide unit was significantly increased in renal glomerular basement membrane isolated from diabetic human patients[4]. Such an increase could arise from enhanced synthesis and/or decreased degradation of the disaccharide unit. A diabetes-dependent decrease in renal lysosomal enzyme activities has been reported in various animal models with chemically induced[5-8] and spontaneous[8-10] diabetes. Since diabetic human patients[11-15] and animals[5,8,16] also contain excessive plasma levels of lysosomal enzymes, it appears that the reciprocal changes in the levels of these enzymes in the plasma and kidney of diabetic subjects may arise from an abnormal release of these enzymes into the extracellular milieu. However, the mechanism of hyperglycemia-induced down-regulation of these renal enzymes remains unknown.

Under the consideration that the normalization

* Present address is College of Pharmacy, Chung-Nam University

of altered lysosomal enzyme activities in diabetic state might have an important role to prevent the diabetic microangiopathy, we started to search the substances affecting lysosomal enzyme activities.

Panax ginseng has been reported to have the improving effects on the defected glucose metabolism in diabetes. But any report has not yet been published on the effects of ginseng components on lysosomal enzymes which might have an important role in diabetic microangiopathogenesis. This fact motivates us to investigate the effects of ginseng saponin on lysosomal enzymes.

MATERIALS AND METHODS

Crude ginseng saponin (saponin 1)

Crude ginseng saponin is obtained from 6 year-old red-ginseng root according to the procedure as shown in Fig. 1.

Purified ginseng saponin (saponin 2)

Neutral Al_2O_3 (ca. 10 g) was deactivated with distilled water and packed in 2×30 cm column. After elution with 20 times column volume of methanol, about 12 g of saponin dissolved in methanol was loaded. After the elution with 50 column volume methanol, the eluate was concentrated by rotary evaporator in vacua. To remove the still remained fluorescent materials, charcoal was added to the concentrated solution and the mixture was refluxed for 3 hrs. And the solution was filtered and concentrated.

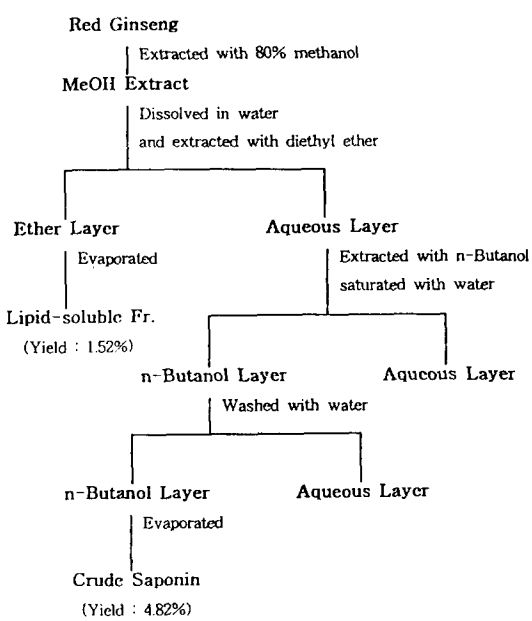

Fig. 1. Extraction and fractionation of crude saponin from Korean red ginseng.

Reagents

p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-α-D-mannopyranoside, and p-nitrophenyl-β-D-galactopyranoside were purchased from Sigma Chemical Company. Other chemicals used in this experiment were guaranteed grade.

Experimental animals

Male ICR mice (20~25 g) supplied from the Experimental Animal Breeding Center of Seoul National University were used as experimental animals. Laboratory chow of Sam-Yang Industries, LTD was used.

Induction of experimental diabetes mellitus

Male ICR mice (20~25 g) bred in same condition were adapted in neighboring environment for about 1 week. 24 hrs fasted mice were injected intraperitoneally with 200 mg/kg body weight of streptozotocin in approximately 0.2 m*l* of sodium citrate buffer (10 mM, pH 4.5). 48 hrs after the treatment of streptozotocin the blood was obtained from orbital sinus and blood glucose levels were determined using Reflotest-Glucose Boehringer. Animals with fasting blood glucose levels of about 350 mg/d*l* were used as diabetic mice. Summerized experimental conditions are shown in Table 1.

Preparation of enzyme source

The livers and kidneys were quickly excised, washed with ice-cold normal saline solution and weighed after the animals had been killed by cervical dislocation. All subsequent procedures were done

Table 1. Experimental condition

Group (animal No.)	Gr. 1(5)	Gr. 2(5)	Gr. 3(5)	Gr. 4(5)
FBGL*	90~110	350~360	340~350	340~360
FBGL**	90~110	350~360	340~350	340~360
Agent	saline	saline	saponin 1 in saline	saponin 2 in saline
Dose	10 ml/kg	10 ml/kg	150 mg/10ml/kg	
Route	intraperitoneally			
Treatment	10 days			

FBGL*: Fasting blood glucose level before treatment (mg/dl), FBGL**: Fasting blood glucose level after treatment (mg/dl). Gr. 1: Control group, Gr. 2: Diabetic control group, Gr. 3: Diabetic saponin 1 treated group, Gr. 4: Diabetic saponin 2 treated group.

at 4℃ unless otherwise mentioned. Two kidneys of mice or about 1 g of liver were finely cut off and homogenized in 9 volumes of 0.01 M sodium phosphate buffer, pH 6.0 a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,000 ×g for 10 min and the supernatant was frozen at −70℃ and assayed within one week.

Assay of enzyme activities

Activities of N-acetyl-β-D-glucosaminidase, α-D-mannosidase and β-D-galactosidase were assayed using the following p-nitrophenyl derivatives as substrates; p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-α-D-mannopyranoside, p-nitrophenyl-β-D-galactopyranoside.

p-Nitrophenyl glycosides were dissolved in 0.125 M sodium acetate buffer (pH 5.0) to make 6 mM.

Substrate (0.25 ml) was added to 0.25 ml of appropriately diluted enzyme solution. The mixture was incubated for 30 min at 37℃ in stoppered glass tubes and reactions were stopped by adding 0.5 ml of 0.5 M glycine-sodium hydroxide buffer (pH 10.45). After centrifugation of reaction mixture at 4,000 rpm for 15 min, the produced p-nitrophenol was measured spectrophotometrically at 410 nm and the activity was calculated from a standard curve prepared simultaneously in each assay.

Preparation of standard curve

p-Nitrophenol solutions of various concentrations were prepared using sodium acetate buffer. Glycine-sodium hydroxide buffer (pH 10.45, 0.5 M, 0.5 ml) was added to 0.5 ml p-nitrophenol solution of each concentration and the absorbance of phenolate was measured at 410 nm spectrophotometrically. From the obtained data the standard curve was prepared. ($y=0.007553x-0.02586$).

Protein assay

Directly before use, alkaline sodium carbonate solution and potassium sodium tartrate-cupric sulfate solution were mixed at the volume ratio of 50 to 1. This mixture (5 ml) was added to each glass tubes contained 1 ml of the diluted enzyme solution. After leaving them as it is for 24 min, 0.5 ml of Foline reagent was added to 0.5 ml p-nitrophenol solutions of each concentrations and it was allowed to react for 30 min.

The protein content was determined by measuring the absorbance at 750 nm. Blank was prepared by the same experimental procedure described as above except distilled water instead of the diluted enzyme solution. Bovine serum albumin solutions of various concentrations were used to draw up standard curve. From the standard curve, the protein content in the enzyme solution was determined. ($y=0.00339x-0.0104$)

Statistical analysis

Values of each group were expressed as the means and its standard error. T-value were calculated by Student's t-test. p-value were determined in an exchange table. It was judged that p-value of less than 0.05 is significant.

RESULTS AND DISCUSSION

Changes of lysosomal enzymes activities in diabetic state were investigated by Fushimi and Tasu,

Table 2. Effect of saponin from Red-Ginseng on some lysosomal enzyme activities in the liver and kidney of mice

Enzyme \ Group / Organ		Group 1 (Control)	Group 2 (Diabetic control)	Group 3 (Diabetic saponin 1 treated)	Group 4 (Diabetic saponin 2 treated)
		Specific activity nmol/min/mg protein (Percentage of control)			
N-acetyl-β-D-glucosaminidase	Liver	26.870± 1.322(100.00)	23.100± 0.996(85.97)*	31.628± 0.950(117.71)**	26.868± 0.645(99.99)
	Kidney	39.876± 1.568(100.00)	30.131± 0.990(75.56)*	76.965± 8.887(193.01)**	61.879± 3.083(155.18)**
β-D-galactosidase	Liver	1.052± 0.203(100.00)	1.007± 0.176(95.70)	1.268± 0.099(120.59)**	1.056± 0.064(100.44)
	Kidney	3.920± 0.161(100.00)	3.330± 0.416(84.95)*	8.033± 1.657(204.94)**	5.755± 0.844(146.81)**
α-D-mannosidase	Liver	3.113± 0.178(100.00)	2.935± 0.055(94.27)*	3.132± 0.152(100.59)	2.993± 0.106(96.14)
	Kidney	4.454± 0.576(100.00)	3.012± 0.627(67.62)*	8.002± 1.682(179.66)**	6.426± 0.429(144.27)**

* $P<0.05$, ** $P<0.01$.

in which the markedly decreased enzyme activities were observed in the cases of N-acetyl-glucosaminidase, α-mannosidase and β-D-glactosidase.

Lysosomal enzymes catalyze the metabolism of glycoproteins and these enzymes might be most important in the pathogenesis of diabetic microangiopathy, a condition in which glycoproteins accumulate. The deposition of glycoproteins in the vasular system and the kidney of diabetic subject could conceivably be a consequence of glycosidases-deficiency.

Therefore, it is meaningful to search the agents to stimulate the activities of those enzymes. In this study, we started to investigate the effects of ginseng saponin on the lysosomal enzyme activities. There are some reports on the effects of ginseng on the experimental diabetes.

White and red ginseng-extract showed the hypoglycemic effect in alloxan or streptozotocin induced diabetic animals. But any experimental trial has not yet been done to investigate the effects of ginseng saponin on the lysosomal enzymes which might have important role in diabetic microangiopathogenesis.

From this reason we examined the effects of ginseng saponin on the lysosomal enzyme activities. Streptozotocin induced-diabetic mice were treated with ginseng saponin (i.p.) for 10 days. Assayed results on the effects of ginseng saponin on lysosomal enzyme activities are summarized in Table 2.

In the liver; In streptozotocin-induced diabetic mice, N-acetyl-β-D-glucosaminidase and α-D-mannosidase activities were significantly decreased by 14% and 6% respectively. But β-D-galactosidase activity was not changed compared to that of normal control group. This result accorded with those of Fushimi and Tarui[17]. In the diabetic animals treated with saponin 1(group 3) N-acetyl-β-D-glucosaminidase and β-D-Galactosidase activities were increased by 18% and 21% respcetively. But α-D-mannosidase activity was not affected by the treatment of saponin 1. In the case of saponin 2 treatment (group 4) no significant changes were observed in the activities of lysosomal enzymes examined in this experiment.

In the kidney: Lysosomal enzyme activities were changed in diabetic animals as found in already reported papers[8-13]; In diabetic control mice (group 2) the activities of α-D-mannosidase and β-D-galactosidase were significantly decreased as shown in Table 2 by 24%, 15% and 32%. But these three enzymes were markedly stimulated by the i.p. injection of saponin 1(group 3) in the ratio of 93% (N-acetyl-β-D-glucosaminidase), 105% (β-D-galactosidase) and 80% (α-D-mannosidase). Most activating effect was observed in the case of β-D-galactosidase.

In saponin 2 treated diabetic group (group 4) sig-

nificant changes of lysosmal enzyme activities were also observed. Activities of N-acetyl-β-glucosaminidase, β-D-glucosaminidase, β-D-galactosidase and α-D-mannosidase were found to be increased by 55%, 47% and 44% compared to those of control group. Crude ginseng saponin posesses more potent stimulating effect on the lysosomal enzyme activities than the purified ginseng saponin. This fact suggests that the active principle for this effect is not only the saponin but also some other substances in the lipid soluble fraction of ginseng extract. The obtained results also indicate that ginseng extract might have a possibility to prevent the diabetic microangiopathy even in the worsely controlled diabetic condition.

국문요약

인삼 saponin이 diabetic microangiopathy를 예방할 수 있는 활성이 있는가의 여부에 관한 연구의 일환으로 특히 고혈당 상태에서 그 활성이 저하되어 기저막에서 glycoprotein의 이화작용을 억제함으로써 microangiopathy를 촉진시키는 주요 lysosomal 효소인 N-acetyl-β-D-glucosaminidase, α-D-mannosidase와 β-D-galactosidase 활성에 미치는 인삼 saponin의 영향을 streptozotocin 유도 당뇨 동물을 대상으로 검토한 결과 매우 강한 효소 유도 작용을 나타냄을 확인하였다. 이는 당뇨 동물의 혈당 수준에는 무관한 작용으로 microangiopathy의 예방 가능성을 시사한다.

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