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Note

# Non-Saponin Fraction from *Panax ginseng* C.A. Meyer Inhibits Platelet Aggregation

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**Abstract**□Hexane, hexane/diethylether and chloroform fractions from *Panax ginseng* C.A. Meyer strongly inhibited human platelet aggregation induced by a high dose of thrombin (2 u/m*l*). Chloroform fraction more strongly inhibited the platelet aggregation than the other two fractions among them. There were fatty acid ester and phosphate ester instead of polyacethylene compounds in the chloroform fraction.

**Key words**□*Panax ginseng*, non-saponin fraction, inhibition of human platelet aggregation, fatty acid ester.

## Introduction

*Panax ginseng* C.A. Meyer (Araliaceae) is a traditionally, well-known oriental folk medicine reported to improve or invigorate the physiological condition that is weakened by various diseases. It was reported that panaxynol from *Panax ginseng* C.A. Meyer inhibited the platelet aggregation induced by a low dose of thrombin (0.1 u/m*l*).[1] Non-saponin fraction (NSF) devoid of panaxynol, nonetheless, inhibited both release of serotonin and human platelet aggregation induced by a low doses of thrombin (1~2 u/m*l*).[2] Platelet aggregation induced by a high dose of thrombin is irreversible and related to the release of serotonin.[3-6] Thrombosis is mainly resulted from irreversible aggregation which is intimately related with the release of serotonin.[3-6] The platelet aggregation induced by thrombin is primarily dependent upon the level of thromboxane $A_2$ ($TXA_2$) which is synthesized via cyclooxygenase pathway from arachidonic acids liberated from membrane phospholipids of platelets.[7-11]

This paper describes that NSF inhibits the aggregation of human platelets induced by a high dose of thrombin (2 u/m*l*).

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## Materials and Methods

### 1. Materials

Thrombin (from bovine plasma) and all the other chemical reagents were purchased from Sigma Chemical Co.

### 2. Preparation of Non-Saponin Fraction (NSF) from *Panax ginseng* C.A. Meyer

500 g of red *Panax ginseng* was fined to powder by a cut mill and deposited in 2500 m*l* of petroleum ether for 7 days in cold chamber (4℃). Then, it was extracted with petroleum ether 3 times at room temperature and concentrated at 25℃ with a rotary vacuum evaporator (EYELA, Tokyo RiKaKiKai, Co., LTD., Type:N-N). The yield was 0.2%. The concentrate was dissolved in petroleum ether and kept at −20℃ to be used for the following experiments.

### 3. Subfractions of NSF and their thin layer chromatography

To subfractionate NSF to fractions of polar and non-polar, NSF was successively eluted with 100 m*l* of hexane, hexane/diethylether (95 : 5, v/v) and chloroform on a silicic acid column (diameter; 1.5 cm, length; 25 cm). Thin layer chromatography was pe-

rformed in order to identify the substances in eluents as follows : The eluent was concentrated by $N_2$ gas, spotted onto the glass plate (silicic acid-coated, particle size; 1~17 μm, layer; 250 μm), and developed with petroleum ether/diethylether (7 : 3, v/v). Spots were identified by $I_2$ vapor and 10% $H_2SO_4$.

**4. Preparation of washed platelets**

Platelet-rich plasma (PRP) obtained from the antecubital vein of normal human volunteers was purchased from Taejon Red Cross Blood Center, Korea. The blood had been anticoagulated with CPD sol. (sodium citrate, $NaH_2PO_4$, glucose, adenine mixture; Korea Green Cross Pharm. Co.) during PRP preparation. PRP was centrifuged at 125 x g for 10 min to remove red blood cells. PRP was washed twice in Tris-citrate-bicarbonate buffer (pH 6.5[12], containing 2 mM EDTA) by centrifuging at 1,100 x g for 10 min. Because EDTA has an inhibitory action on platelet aggregations, the washed platelets were recentrifuged twice with suspending buffer (pH 6.9[12], without EDTA) to remove EDTA. Finally, the platelet number was adjusted to $5\times10^8$ cells/m*l* with suspending buffer. All the above procedures were carried out at 25℃ to avoid platelet aggregatins by cooling.

**5. Measurement of platelet aggregation**

The washed platelets were preincubated in a uv/visible spectrophotometer cuvette with gentle stirring for 3 min at 37℃ in the presence of 2 mM $CaCl_2$ with or without testing materials, and then stimulated with thrombin (2 u/m*l*) for 5 min with gentle stirring. Platelet aggregations were measured with a uv/visible spectrophotometer (Beckman DU-6) at 660 nm. Transmission (T) was calculated using the following formula.

$$T=\frac{1}{10^{\Delta A}}$$

$\Delta A = 5A - 3A$

5A = absorbance value after aggregation reaction occurred for 5 min

3A = absorbance value after preincubation had been carried out for 3 min

Suspending buffer was used as reference (absorbance 0). Because NSF was dissolved in dimethyl sulfoxide (DMSO), its pure activity was compensated by subtracting the absorbance of DMSO from that of NSF.

**6. Infrared absorption spectra of non-saponin fraction**

Non-saponin fraction (NSF) was scrapped from TLC plate and extracted with chloroform/methanol (1 : 2, v/v). The extract was concentrated with a vacuum evaporator (EYELA, Tokyo RiKaKiKai, Co., LTD., Type:N-N) and dried with a vacuum pump for 24 hr. The concentrate was pelleted with KBr and was scanned with an infrared spectrophotometer (Perkin Elmer, Model:1330).

## Results and Discussion

**1. Thin layer chromatography of subfraction of NSF and their effects on platelet aggregation**

NSF contains both non-polar substances ($R_f$; above 0.5) and polar substances ($R_f$; below 0.26) as well as polyacethylene compounds such as panaxynol ($R_f$; 0.35), panaxydol ($R_f$; 0.26), panaxytriol ($R_f$; 0.03) and so on (Fig. 1, lane 1). The hexane subfraction contains non-polar substances ($R_f$; above 0.5) without polyacethylene compounds (Fig. 1, lane 2) and inhibits the platelet aggregation induced by 2 units of thrombin/m*l* about to the extent of 55% (Table 1). The hexane/diethylether (95 : 5, v/v) subfraction includes the polyacethylene compounds and inhibits the platelet aggregations induced by thrombin (2 u/m*l*) about to 54% (Fig. 1, lane 3, Table 1). In the chloroform subfraction, polar compounds were detected on the origin, which inhibits platelet aggregations to 92% (Fig. 1, lane 4, Table 1). Above results imply that the inhibitory effect of NSF on thrombin-induced platelet aggregations is due to its subfraction of hexane, hexane/diethylether, and chloroform. Especially, it is interesting that the chloroform subfraction is devoid of polyacethylene compounds and inhibits platelet aggregations most strongly.

**2. Chemical characteristics of NSF**

Infrared spectrum of chloroform-subfraction showed typical absorption characteristics; C=O stretching of ester (1740 $cm^{-1}$), C-O stretching of ester (1120 $cm^{-1}$) and P=O stretching of phosphate ester (1250 $cm^{-1}$) (Fig. 2). Acethylene bonds in polyaceth-

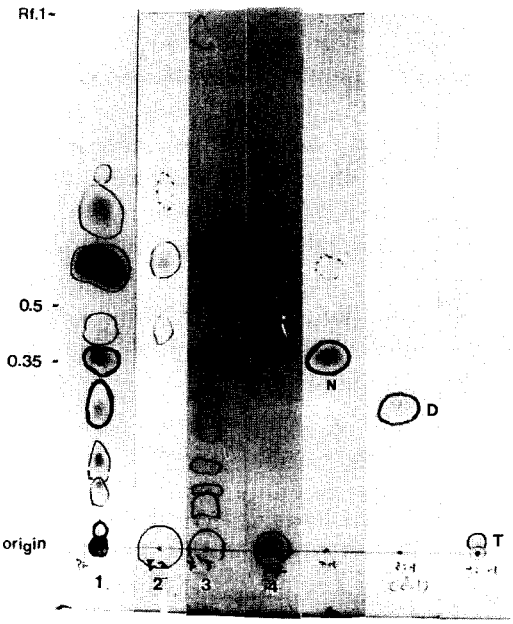


**Fig. 1.** Thin layer chromatography of subfractions eluted with non-polr solvent and polar solvent from NSF. NSF was loaded onto the silicic acid column (diameter : 1.5 cm, length : 25 cm) and continuously eluted with hexane, hexane/diethylether (95 : 5, v/v) and chloroform. 10 μl of each eluent was spotted onto the glass plate coated with silica gel (particle size : 1~17 μm, layer : 250 μm) and then developed with petroleum ether/diethylether (7 : 3, v/v). The spots were identified by $I_2$ vapor and 10% $H_2SO_4$.
1 : petroleum ether subfraction
2 : hexane subfraction
3 : hexane/diethylether (95 : 5, v/v) subfraction
4 : chloroform subfraction
N : panaxynol standard
D : panaxydol standard
T : panaxytriol standard

ylene compounds have absorption in $2260^{-1}$, $2250^{-1}$, $2240^{-1}$, $2220^{-1}$ and $2150^{-1}$ on infrared spectrum.[13] Especially in chloroform-subfraction were contained docosatrienoic acid more than 90% (data not shown). It is suggested that the chloroform-subfraction has antiplatelet activities and contains a kind of substance with fatty acid ester and phosphate ester.

Panaxynol is one of polyacethylene compounds and is reported to inhibit the platelet aggregation.[1]

**Table 1.** Effects of non-polar subfraction and polar subfraction of NSF on platelet aggregation induced by thrombin

| Subfraction | Inhibitory degree on platelet aggregation (%) |
|---|---|
| Hexane | 55.25± 7.42 |
| Hexane/diethylether (95 : 5, v/v) | 54.00± 1.60 |
| Chloroform | 92.50± 1.00 |

The inhibitory degree of subfractions of NSF on aggregation is indicated as percentage of what 80% aggregation induced by thrombin (2 u/ml) is allocated as control (100). The data are given as mean± S.D. (n=4~5).

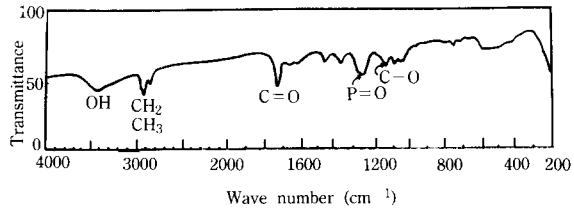


**Fig. 2.** Infrared absorption spectra of chloroform subfraction from *Panax ginseng* C.A. Meyer. The spots developed by chloroform from NSF on TLC were scrapped from the plate and extracted with chloroform/methanol (1 : 2, v/v). The purified chloroform subfraction was pelleted with KBr and was scanned with an infrared spectrophotometer (Perkin Elmer, Model:1330).

But polar subfraction (chloroform-subfraction) devoid of panaxynol inhibited platelet aggregation more strongly than both non-polar subfraction and polyacethylene compounds-containing subfraction which were eluted by hexane and diethylether (Fig. 1, Table 1). Although which subfraction of NSF has a major por ion in antiplatelet functions is not certain, it may be a clue to solve the problem that polar subfraction contains fatty acid ester and phosphate ester.

Generally the platelets incubated with thrombin are more susceptible to breakage in a gradient of glycerol-EDTA.[12] In our experiments, platelets were washed twice with buffer containing EDTA to remove a trace of $Ca^{2+}$.[9] Accordingly, we could not exclude the possibility that platelets were broken

during the washing process. If platelets were broken, the other intact platelets would be aggregated by stimulators of platelets such as ADP, serotonin, PAF and $Ca^{2+}$ released out pf granules. However, when platelets suspended with buffer without EDTA in the presence of NSF were preincubated, the aggregation (Table 1) did not occurr. EDTA also causes the partial proteolysis of actin-binding protein, fibronectin, a myosin and thrombin sensitive protein.[14] These mean that platelets can be inactivated by EDTA. If the inhibition of aggregation by NSF were due to the inactivation of platelets resulting from EDTA, our results may be false and platelet aggregation, $TXA_2$ productin[15] and serotonin release[2] would not occurr by thombin. However by thrombin, platelet aggregation occurred to 80% and platelet metabolites were produced (Table 1).[15] The above considerations mean that platelets were not broken and not inactivated by EDTA, and the inhibition on aggregation and production of platelet metabolites by NSF is not false.

## 요 약

높은 농도의 thrombin(2 u/ml)으로 유인된 사람 혈소판 응집반응에서, 한국홍삼에서 제조한 비사포닌 분획(지용성 분획) 중 hexane, hexane/diethylether 및 chloroform subfraction은 사람 혈소판 응집반응을 강하게 억제했다. 이들 중 chloroform subfraction이 다른 지용성 subfraction보다 강한 혈소판 응집억제 활성이 있었다. 이 분획에는 polyacethylene compound가 함유되지 않고, fatty acid ester 및 phosphate ester가 함유되어 있었다.

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