Spinach (Spinacia oleracea) is an outstanding food source of folic acid (Prinz-Langenohl et al., 1999), vitamin A (Edwards et al., 2001), and so on. It is known that aqueous extracts from spinach have the effects of anti-oxidation (Bergman et al., 2001; Lomnitski et al., 2003) and anti-carcinogenesis (Sani et al., 2004). However, it is unknown whether spinach has an inhibitory effect on platelet aggregation-induced thrombosis.

Platelet aggregation is essential to the formation of a hemostatic plug when normal blood vessels are injured. However, the interactions between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction (Schwartz et al., 1990). Inhibition of the platelet-collagen interaction might be a promising approach to the prevention of thrombosis. An important role in the mechanism by which collagen induces platelet aggregation is played by thromboxane A2 (TXA2) production, an intracellular Ca2+-agonist as an aggregation-inducing autacoidal molecule. In addition, SSEF significantly increased the formation of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), intracellular Ca2+-antagonists as aggregation-inhibiting molecules, in collagen-stimulated platelets. These results suggest that SSEF might inhibit Ca2+-elevation and TXA2 formation by increasing the production of Ca2+-antagonistic molecules cAMP and cGMP. These mean that SSEF is the potent inhibitor of collagen-stimulated platelet aggregation. On the other hand, prothrombin time (PT) and activated partial thromboplastin time (APTT) were potently prolonged by SSEF. These findings suggest that SSEF prolongs the internal time between the conversion of fibrinogen to fibrin. Accordingly, our data demonstrate that SSEF may be a crucial tool for a negative regulator during platelet activation and blood coagulation on thrombotic diseases.

Key Words: Spinach saponin-enriched fraction (SSEF), Platelet aggregation, Blood coagulation, Thrombotic diseases

INTRODUCTION

Spinach (Spinacia oleracea) is an outstanding food source of folic acid (Prinz-Langenohl et al., 1999), vitamin A (Edwards et al., 2001), and so on. It is known that aqueous extracts from spinach have the effects of anti-oxidation (Bergman et al., 2001; Lomnitski et al., 2003) and anti-carcinogenesis (Sani et al., 2004). However, it is unknown whether spinach has an inhibitory effect on platelet aggregation-induced thrombosis.

Platelet aggregation is essential to the formation of a hemostatic plug when normal blood vessels are injured. However, the interactions between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction (Schwartz et al., 1990). Inhibition of the platelet-collagen interaction might be a promising approach to the prevention of thrombosis. An important role in the mechanism by which collagen induces platelet aggregation is played by thromboxane A2 (TXA2) formation (Cattaneo et al., 1991), which also contributes to an increase in cytosolic free Ca2+ level ([Ca2+]i) in collagen-activated platelets. An increase in [Ca2+]i activates both the Ca2+/calmodulin-dependent phosphorylation of myosin light chain (MLC) and the diacylglycerol (DG)-dependent phosphorylation of cytosolic pleckstrin to induce platelet aggregation (Nishikawa et al., 1980). On the other hand, both cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), as
anti-platelet regulators, decrease the [Ca\(^{2+}\)], an essential factor for platelet aggregation.

Blood coagulation systems, another common region in generation of thrombosis, have intrinsic blood coagulation system (activated partial thromboplastin time, APTT) from the injured blood vessel, and extrinsic blood coagulation system (prothrombin time, PT) derived from tissue. The prolongation of blood coagulation time such as PT and APTT means the prolonged time of forming fibrin clot and is the mark of inhibited blood coagulation. Extended APTT and PT hint that thrombin is produced with delay (Davie and Ratnoff, 1964; Furie and Furie, 1988; Akoum et al., 1990; Sano et al., 2003).

As described above, there is no report showing its anti-thrombotic activity with spinach saponin. In order to clarify the mode of anti-thrombotic action of SSEF, we investigated the effect of SSEF on various parameters associated to collagen-induced platelet aggregation and blood coagulation.

**MATERIALS AND METHODS**

**Materials**

Collagen was obtained from the Chrono-Log Corporation (Havertown, PA, U.S.A.), and other reagents were obtained Sigma Chemical Corporation (St. Louis, U.S.A.). cAMP-, cGMP-, and TXB2-enzymeimmunoassay (EIA) kits were purchased from Amersham Bioscience (Buckinghamshire, U.K.). Ginseng total saponin was donated from Central Institutes of KT&G, Daejon, Korea.

**Preparation of SSEF**

We used fresh spinach from a local market. Spinach roots were broken and dried. A total 1 kg of dry spinach roots was denatured by heating at 95°C for 6 h with 5 L distilled water, and then 0.6 L of resultants was extracted by a dianion HP-20 column (Supelco, Bellefonte, PA, USA) with 40% ethanol. SSEF in 40% ethanol was evaporated, frozen, and dried. SSEF was dissolved with distilled water to investigate the effects on platelet aggregation and blood coagulation.

**Determination of saponin concentration in SSEF**

The SSEF 0.1 ml was added in 8% vanillin-ethanol 0.3 ml and 72% sulfuric acid 4 ml, and then heated at 60°C for 10 min. The concentration of saponin in SSEF was measured at 545 nm of UV/visible spectrophotometer (Optizen 2120 UV, daejon, Korea), and was calculated by using the calibration curve of ginsenoside-Rc (G-Rc). The total saponin content was expressed as %.

**Preparation of washed rat platelets**

Blood was collected from Sprague-Dawley rats (6-7 weeks, male), and anti-coagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose). Platelet-rich plasma was centrifuged at 125×g for 10 min to remove the red blood cells, and the platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO\(_3\), 36 mM NaH\(_2\)PO\(_4\), 5.5 mM glucose, and 1 mM EDTA, pH 7.4). The washed platelets were then resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO\(_3\), 36 mM NaH\(_2\)PO\(_4\), 0.49 mM MgCl\(_2\), 5.5 mM glucose, 0.25% gelatin, pH 7.4) to a final concentration of 5×10\(^{9}\)/ml. All of the above procedures were carried out at 25°C to avoid platelet aggregation on cooling. The Ethical Committees for Animal Experiments of Inje University approved this study.

**Measurement of platelet aggregation**

Washed platelets (10\(^9\)/ml) were preincubated for 3 min at 37°C in the presence of 2 mM exogenous CaCl\(_2\), and activated for 5 min with collagen (10 μg/ml) for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log, Corp., Haverton, PA, USA) at a constant stirring speed of 1,000 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspension buffer was used as the reference. SSEF was reconstituted with distilled water.

**Measurement of TXB2**

Washed platelets (10\(^9\)/ml) were preincubated with or without SSEF for 3 min in the presence of 2 mM CaCl\(_2\), and activated for 5 min with collagen (10 μg/ml). The reactions were terminated by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB\(_2\), a stable metabolite of TXA\(_2\), was determined with Synergy HT Multi-Model Microplate Reader (Bio Teck Instrument, Winoosku, U.S.A.) using a TXB2 EIA kit. SSEF was reconstituted with DW.

**Measurement of cAMP and cGMP**

Washed platelets (10\(^9\)/ml) were preincubated for 3 min at 37°C with various concentrations of SSEF in the presence of 2 mM CaCl\(_2\), and then stimulated with collagen (10 μg/ml) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP and cGMP were measured with Synergy HT Multi-Model Microplate Reader (Bio-Teck Instrument, Winoosku, U.S.A.) using cAMP and cGMP EIA kits. SSEF was reconstituted with DW.

**Measurement of PT and APTT**

Citrated platelet-poor plasma (PPP) was prepared by centrifuging the blood remaining after the removal of PRP at 1,300 ×g for 10 min. The PPP (0.1 ml) was preincubated in a two-channel coagulator (KG Behnik Elektronik GMBH & Co., Germany) cup (BioMérieux, 95-662) with gentle stirring for 1 min at 37°C. PT was determined as the time interval between the addition of PT reagent (0.1 ml) to the PPP and the formation of a fibrin clot. After preincubation for APTT measurement, 0.1 ml of APTT reagent was added to the PPP and incubated for 3 min at 37°C. Following incubation, 0.1 ml of 25 mM CaCl\(_2\) was rapidly added to the PPP solution containing APTT reagent. APTT was determined as the time required to form a fibrin clot. SSEF was reconstituted with distilled water.

**Statistical analysis**

The experimental results are expressed as the means ± S.E.M. and are accompanied by the number of observations. Data were with analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. A p-value less than 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Saponin concentration of SSEF**

The saponin level in SSEF, as shown in Fig. 1, was 10% when was calculated by calibration curve of G-Rc. It is known