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Black soybean extract can attenuate thrombosis through inhibition of collagen-induced platelet activation☆

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Abstract

Many clinical trials have demonstrated the beneficial effects of soybean (*Glycine max*) on general cardiovascular health. Among a variety of soybeans, black soybean is known to display diverse biological activities superior to those of yellow and green soybeans, such as in antioxidant, anti-inflammatory and anticancer activities. However, few studies have been directed on the effect of black soybean on cardiovascular function. In this study, we aimed to investigate the effect of black soybean extract (BB) on platelet activation, a key contributor to thrombotic diseases. In freshly isolated human platelets, BB has shown potent inhibitory activity on collagen-induced platelet aggregation, while yellow soybean extract had marginal activity only. BB also attenuated serotonin secretion and P-selectin expression, which are important factors for the platelet–tissue interaction along with thromboxane A_2 formation. These *in vitro* results were further confirmed in an *ex vivo* platelet aggregation measurement and *in vivo* venous thrombosis model where oral administration of BB reduced collagen-induced platelet aggregation and $FeCl_3$-induced thrombus formation significantly. A potential active ingredient for antiplatelet effects of BB was isolated and identified to be adenosine through bioassay-directed fractionation and NMR and ESI-MS analyses. These results indicate that black soybean can be a novel dietary supplement for the prevention of cardiovascular risks and the improvement of blood circulation.
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Keywords: Black soybean; Antiplatelet activity; Antithrombotic activity; Collagen

1. Introduction

Soybean (*Glycine max*) has been one of the most important nutritional sources from ancient times. More than 20–80 g of soybean foods is consumed everyday in Asia [1], and soybean processed foods, such as tofu and fermented soybean sauce, are gathering popularity also in the western diet. Soybeans consist of protein (38%), soluble carbohydrate (15%), fiber (15%), oil (18%) and minor nutritional constituents [1,2]. Other than these nutritional components, a variety of health-beneficial ingredients, including isoflavones, saponins and unsaturated fatty acids, and plant-derived ingredients, such as glycinin and β-sitosterol, are contained in soybeans. Probably due to this enrichment of bioactive components, soybean is reported to have various therapeutic effects, such as anticancer [3–5], antiobesity [6,7], antidiabetes [8] and anti-osteoporosis [9] activities. In particular, benefits of soybeans on cardiovascular health have become an object of huge interest recently. Indeed, several clinical trials have been performed and demonstrated the beneficial effects of soybean consumption on cardiovascular diseases, such as hypertension [10,11] and dyslipidemia [12–14].

Cardiovascular diseases, including thrombosis, stroke, ischemic and coronary heart diseases, are a leading cause of mortality, accounting for nearly 30% of global deaths in 2005 [15]. Especially, thrombotic diseases constitute a major cardiovascular complication affecting a great number of patients. Consequently, a variety of antithrombotic drugs, including antiplatelet, anticoagulant and thrombolytic agents, have been developed to prevent thrombosis [16]. However, chemically synthesized or biologically modified drugs are frequently associated with serious adverse effects, such as internal bleeding and gastrointestinal adverse effects, casting doubt on their health benefit vs. the cost for lifetime management of cardiovascular

Abbreviations: Anti-CD62P-FITC Ab, FITC-labeled anti-CD62P antibody; anti-GPIb-PE Ab, phycoerythrin-labeled monoclonal antibody against human glycoprotein Ib; BB, black soybean extract; IC_{50}, half maximal inhibitory concentration; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2; YB, yellow soybean extract.

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risks [17,18]. In this background, much attention has been given to the development of dietary supplements or herbal medicines for preventive treatment against cardiovascular diseases for their merits in safety and nature-friendly image [19,20].

Soybean can be subdivided into yellow, green, brown and black, based on the color of their seed coats [21]. Among a variety of soybeans, the black soybean was reported to have discriminating components, such as phenolic acids, anthocyanins and isoflavones [22]. Owing to this, black soybean is known to display superior biological activities to yellow and green soybeans, such as free radical scavenging activities [22] and inhibition of LDL oxidation [23]. In addition, anti-inflammatory [24] and anticancer [25] activities of black soybean have been reported, arousing strong interest in its biological and nutritional merits. However, there are few studies focused on the antithrombotic effects of black soybeans to our best knowledge.

In this study, we discovered that black soybean extract (BB) has potent inhibitory activities on collagen-induced platelet aggregation and granule secretion, a major contributor to thrombus formation and the development of thrombotic diseases. Interestingly, yellow soybean extract (YB) had marginal activity only, suggesting the specificity and the novelty of the antiplatelet effects of BB. *In vivo* thrombosis experiment was conducted along with the identification of potential active ingredient through bioassay-directed fractionation in an effort to develop a novel nutritional supplement with antithrombotic activities.

2. Materials and methods

2.1. Materials

Trisodium citrate, ethanol, ethyl acetate, methanol, dimethyl sulfoxide, adenosine, NaCl, KCl, $MgCl_2$, HEPES, glucose, $NaHCO_3$, Na_2HPO_4, $CaCl_2$, glutaraldehyde, EDTA, indomethacin, urethane, ferric chloride and bovine serum albumin were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Collagen was from Chrono-log (Harvertown, PA, USA), and phycoerythrin-labeled monoclonal antibody against human glycoprotein Ib (anti-GPIb-PE Ab) and FITC-labeled anti-CD62P antibody (anti-CD62P-FITC Ab) were from BD Bioscience (San Diego, CA, USA). ^{14}C-labeled serotonin (55 mCi/mmol) and ACSII scintillation cocktail were obtained from GE Healthcare (Buckinghamshire, UK); thromboxane B_2 (TxB_2) ELISA kit was from Cayman Chemical (Ann Arbor, MI, USA). SCH 58261 and ZM 241385 were from Tocris Bioscience (Bristol, UK).

2.2. Soybean extraction and isolation

Black soybean (black seed coat and a green cotyledon) and yellow soybean (yellow seed coat and a yellow cotyledon) were from Boeun and Goesan, Korea. Dry matured soybeans were extracted for 3–5 h at 50°C–60°C with 20% ethanol. The extraction was repeated three times and left for 12 h at room temperature. After filtration and concentration under reduced pressure, the extract was lyophilized. The final yields of BB and YB were 7.6% and 8.7%, respectively, and the resultant powder was stored at −20°C. The BB consisted of protein (1.0%), lipid (1.2%), carbohydrate (86.8%), ash (10.2%) and water (0.8%), and the content of total phenolic compounds was 1.29%. For *in vitro* experiments, soybean extracts were dissolved in 50% ethanol; for *in vivo* experiments, soybean extract was dissolved in saline.

BB was suspended in water and partitioned to ethyl acetate or butanol. The ethyl acetate fraction (yield=1.2%) underwent solid-phase extraction using Sep-Pak cartridges (10 g, Waters, Milford, MA, USA) and was eluted with aqueous methanol step gradient (50%–100%) to seven fractions (A-1 to A-7). The A-1 (yield=28%) and A-2 (yield=14.5%) fractions were combined and applied on Sephadex LH 20 (GE Healthcare, Uppsala, Sweden) column chromatography (30 mm ID×800 mm) with 70% methanol, which yielded 10 fractions (B-1 to B-10). The B-1 (yield=18%) fraction was subjected to preparative HPLC [eluent: 10% methanol and 0.05% trifluoroacetic acid; column: ODS (20×150 mm); flow rate: 6 ml/min; detection: UV at 260 nm] and separated into seven fractions (C-1 to C-7). The final active fraction C-3 was obtained with a yield of 6.6% from the B-1 fraction. Fractions were dissolved in 50% ethanol or dimethyl sulfoxide and stored at −20°C.

2.3. Preparation of human platelets

Human blood was collected from healthy male volunteers (18–25 years old) who had not taken any drugs for at least 14 days with an approval from the ethics committee of the Seoul National University Health Service Center. Blood was anticoagulated with 3.8% trisodium citrate solution (1:9 citrate/blood, v/v). All procedures were conducted at room temperature, and the use of glass containers and pipettes was avoided. Platelet-rich plasma (PRP) was prepared by centrifugation at room temperature for 15 min at 150*g*. Platelet-poor plasma (PPP) was obtained from the precipitated fraction of PRP by centrifugation at room temperature for 20 min at 2000*g*. The platelet count in PRP was adjusted to 3×10^8 platelets/ml using PPP.

2.4. Platelet aggregation measurement

Platelet aggregation was determined by the turbidometric method using an aggregometer (Chrono-log). After incubation with soybean extracts or fractions for 10 min at 37°C, PRP was loaded on the aggregometer and stimulated with collagen (2–4 μg/ml) for 6 min. Platelet aggregation was measured by light transmission, with 100% calibrated as the absorbance of PPP and 0% calibrated as the absorbance of PRP.

For microscopic observation, PRP was fixed with 0.5% glutaraldehyde after incubation with BB and collagen. Fixed platelets were loaded on a slide glass and covered with a cover glass. Platelets were observed with an optical microscope (CX41, Olympus, Japan).

2.5. Measurement of P-selectin expression

After incubation with BB at 37°C for 10 min, PRP was stimulated with collagen (2–4 μg/ml) for 6 min. The resultant PRP was diluted with Tyrode's buffer (134 mM NaCl, 2.9 mM KCl, 1.0 mM $MgCl_2$, 10.0 mM HEPES, 5.0 mM glucose, 12.0 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 2 mM $CaCl_2$ and 0.3% bovine serum albumin, pH 7.4). Anti-CD62P-FITC Ab was used as a marker for P-selectin expression, while platelets were identified by anti-GPIb-PE Ab. Platelets were incubated with anti-CD62P-FITC Ab and anti-GPIb-PE Ab for 20 min in the dark and analyzed on a BD FACSCaliber flow cytometer (BD Bioscience) equipped with argon laser (excitation=488 nm). Data from 5000 events were collected and analyzed using CellQuest Pro software (BD Bioscience).

2.6. Measurement of serotonin secretion

Serotonin secretion was measured using ^{14}C-labeled serotonin as described by Bae *et al.* [26] with a minor modification. PRP was preincubated with 0.5 μCi/ml ^{14}C-labeled serotonin (55 mCi/mmol) for 45 min at 37°C. ^{14}C-labeled serotonin-loaded PRP was incubated with BB for 10 min at 37°C and stimulated with collagen (2 μg/ml) for 6 min. The reaction was terminated by the addition of EDTA (final concentration=5 mM). The resultant platelet suspensions were centrifuged at 12,000*g* for 1 min, and the supernatant was obtained for determination of ^{14}C-labeled serotonin secretion. Radioactivity in each sample was measured in a Wallac 1409 liquid scintillation counter (Perkin Elmer, Boston, MA, USA) after dilution with ACSII scintillation cocktail. Serotonin secretion was expressed as the percentage of total serotonin content as measured in the supernatant from the cell lysed with 0.3% Triton X-100.

2.7. Measurement of thromboxane A_2 formation

After incubation with BB for 10 min at 37°C, PRP was loaded in the aggregometer and stimulated with collagen (2–4 μg/ml) for 6 min. The reaction was terminated by adding EDTA (final concentration=2 mM) and indomethacin (final concentration=50 μM). The resultant platelet suspensions were centrifuged at 12,000*g* for 2 min, and the supernatant was obtained for determination of thromboxane A_2 (TxA_2) formation. The TxA_2 content in each sample was determined by TxB_2 level, the stable metabolite of TxA_2, measured using a commercial TxB_2 ELISA kit.

2.8. In vivo experiments

Male Sprague–Dawley rats (SamTako, Osan, Korea) weighing 200–300 g were used for animal studies. Before the experiments, animals were acclimated for 1 week, and food and water were provided *ad libitum*. All the protocols were approved by the ethics committee of the Seoul National University Animal Service Center. BB (50 and 100 mg/kg body weight) was administered through oral gavage to simulate the normal daily intake of soybean foods (20 to 80 g/day). For measurement of *ex vivo* platelet aggregation, 1 h after single oral administration of BB, whole blood was collected from abdominal aorta anticoagulated with 3.8% trisodium citrate solution (1:9 citrate/blood, v/v) under anesthesia. PRP preparation and platelet aggregation measurements were done as described above except for the concentration of collagen used (8–10 μg/ml).

For the estimation of thrombus formation, *in vivo* $FeCl_3$-induced rat venous thrombosis was used based on the method of Wang *et al.* [27] with modification. After single or 14-day multiple oral administration of BB once daily, rats were anesthetized with urethane (1.25 g/kg ip), and their abdomen was surgically opened. Loose cotton threads were placed 15 mm apart around the vena cava, and all side branches were ligated. Ferric chloride (5% in saline)-soaked filter paper (3 mm×10 mm) was applied to the exposed vena cava for 5 min and removed. Thirty minutes after application of $FeCl_3$, cotton threads were tightened and the ligated vena cava was isolated. The vena cava was opened and the thrombus was isolated, blotted and immediately weighed. Thrombus obtained surgically was oriented for cross-sectioning on disposable base molds in the optimal cutting temperature compound (Tissue-Tek Products, Torrance, CA, USA) and then frozen at −72°C. Frozen thrombus was cut into 10-μm thickness in a

cryostat (CM1850, Leica, Wetzlar, Germany) and dried for 30 min at room temperature before hematoxylin and eosin staining.

2.9. Analytical identification of active principle

The ^{1}H and ^{13}C NMR measurements were carried out in a JNM-ECA600 600-MHz FT-NMR spectrometer (JEOL, Tokyo, Japan) in D_2O with tetramethylsilane as internal standard. ^{1}H–^{1}H COSY, HMQC and HMBC NMR experiments were performed on the same spectrometer. ESI-MS spectrum was obtained on a Q-TRAP-3200 mass spectrometer (Applied Biosystems, Foster City, CA, USA). MS condition was ESI; positive mode with capillary voltage (kV), 3.2; cone voltage (kV), 30; source temperature, 120°C; desolvation temperature, 350°C; desolvation gas (L/h), 850; and collision energy, 17. The HPLC system (Hitachi, Tokyo, Japan) consisted of autosampler, pump and photodiode array detector. Mightysil C18 (4.6×250 mm, 5 μm) column was used with isocratic elution of methanol/water (90:10) at 1 ml/min. Adenosine was detected at 260 nm.

2.10. Statistical analysis

All data are shown as the mean±S.E.M., and the data were subjected to one-way analysis of variance followed by Duncan's multiple range tests to determine which means were significantly different from the control. Statistical analysis was performed using SPSS software (Chicago, IL, USA). In all cases, a *P* value <.05 was used to determine significance.

3. Results

3.1. In vitro antiplatelet effects of soybean extracts

To examine the effect of soybean extracts on platelet aggregation, we treated freshly isolated human platelets with BB (100 μg/ml) for 10 min and then initiated platelet aggregation with collagen (2–4 μg/ml). Notably, BB that was extracted with 20% ethanol showed the strongest inhibitory effect against collagen-induced platelet aggregation than other extraction conditions (Fig. 1A). Therefore, the following studies were done with BB extracted with 20% ethanol.

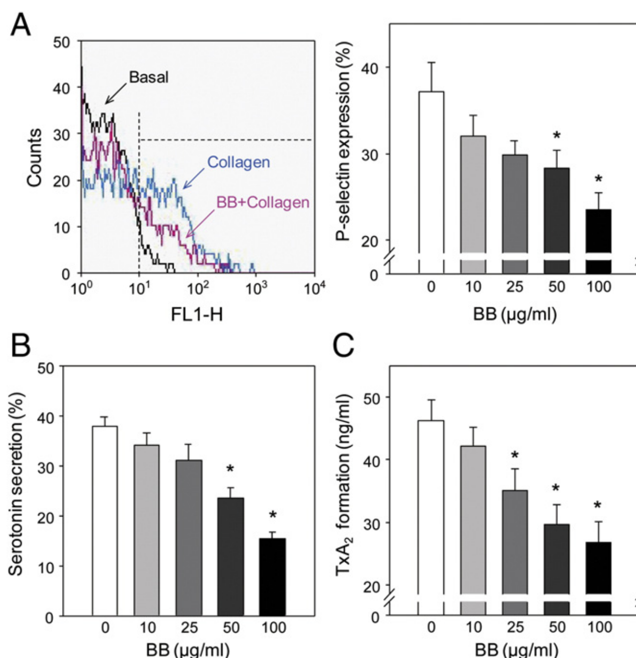

Fig. 2. Effects of BB on collagen-induced platelet activation. Various concentrations of BB were treated for 10 min, and human platelets were stimulated with collagen (2–4 μg/ml) for 6 min. (A) P-selectin exposure was measured using flow cytometric analysis. Representative histogram of nontreated platelets and platelets treated with vehicle or BB (100 μg/ml) and collagen is shown. (B) Serotonin secretion was determined using ^{14}C-labeled serotonin-loaded PRP. (C) TxA_2 formation was measured using a commercial TxB_2 ELISA kit. Values are the mean ±S.E.M. of three independent experiments. Representative histograms of three independent experiments are shown. The asterisk represents significant differences from the corresponding control, *P*<.05.

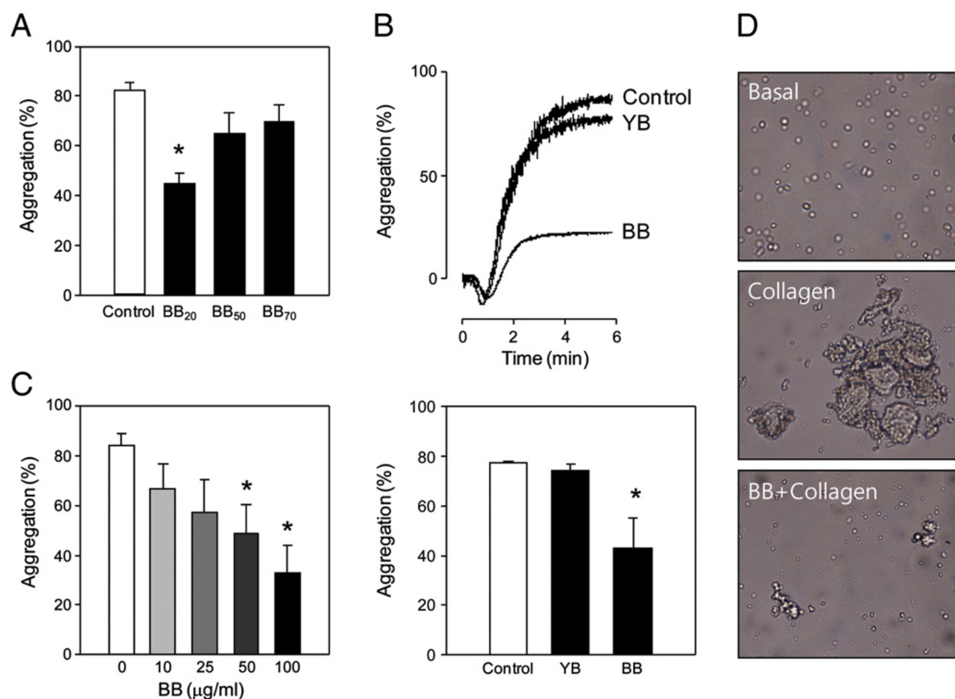

Fig. 1. Effects of soybean extracts on collagen-induced platelet aggregation. (A and B) (A) BB (100 μg/ml) extracted with various concentrations of ethanol and (B) YB or BB were treated for 10 min, and human platelets were stimulated with collagen (2–4 μg/ml) for 6 min. (C) Various concentrations of BB were treated for 10 min, and human platelets were stimulated with collagen (2–4 μg/ml) for 6 min. (D) Nontreated platelets or platelets treated with vehicle or 100 μg/ml of BB and collagen were fixed and observed. Values are the mean ±S.E.M. of three to six independent experiments. Representative tracing data of more than three independent experiments are shown. The asterisk represents significant differences from the corresponding control, *P*<.05.

The effects of BB and YB on platelet aggregation were compared in the same way. BB inhibited collagen-induced platelet aggregation significantly in a concentration-dependent manner (Fig. 1B and C). Surprisingly, YB did not show any effect on collagen-induced platelet aggregation at 100 μg/ml (Fig. 1B), although YB had marginal activity at 250 μg/ml (data not shown). The antiplatelet effect of BB could be also confirmed by microscopic observation where BB reduced the formation of platelet aggregate (Fig. 1D).

During agonist-induced activation, platelets can actively interact with vascular tissues and inflammatory cells, accelerating thrombus formation through P-selectin expression, serotonin secretion and TxA_2 formation. To investigate if BB can suppress the collagen-induced platelet activation along with aggregation, we measured P-selectin expression using anti-CD62P-FITC Ab with flow cytometry. As a result, BB suppressed collagen-induced P-selectin expression in a concentration-dependent manner (Fig. 2A). Collagen-induced serotonin secretion and TxA_2 formation were also inhibited by BB in a concentration-dependent manner as determined by reduced serotonin secretion from ^{14}C-labeled serotonin-loaded platelets (Fig. 2B) and decreased TxB_2 formation (Fig. 2C), indicating that BB can suppress platelet activation and further interactions with vascular tissues.

3.2. Ex vivo antiplatelet effect and in vivo antithrombotic effects of BB

Before the evaluation of the *in vivo* significance of these *in vitro* results, the effect of BB on rat platelet aggregation was examined using freshly isolated rat platelets. BB inhibited collagen-induced rat platelet aggregation in a similar pattern to that observed with human platelets (Fig. 3A). To confirm the antiplatelet effect of BB, we conducted an *ex vivo* platelet aggregation measurement and *in vivo* $FeCl_3$-induced rat venous thrombosis model after single or multiple oral administration of BB (50–100 mg/kg body weight once daily). As a result, collagen-induced platelet aggregation was significantly inhibited by BB (Fig. 3B). In addition, venous thrombus formation was attenuated by BB at 100 mg/kg as measured by decreased thrombus weight (Fig. 3C and D). As shown in Fig. 3C, thrombus formation was reduced even with lower dosage (50 mg/kg) of BB when BB was administered repeatedly for 14 days, indicating that the chronic intake of BB can manifest stronger antithrombotic effects.

3.3. Identification of active ingredients of BB

For the isolation and identification of active ingredients for antiplatelet effects of BB, BB was fractionated and antiplatelet effect was measured as described above to undertake the bioassay-directed fractionation. The ethyl acetate fraction showed the strongest inhibitory effect against collagen-induced platelet aggregation when compared with other fractions (Fig. 4A), and it was therefore subfractionated by solid-phase extraction and gradient elution. The A-1 and A-2 subfractions showed the most potent inhibitory effects on collagen-induced platelet aggregation (Fig. 4B), and the A-1 and A-2

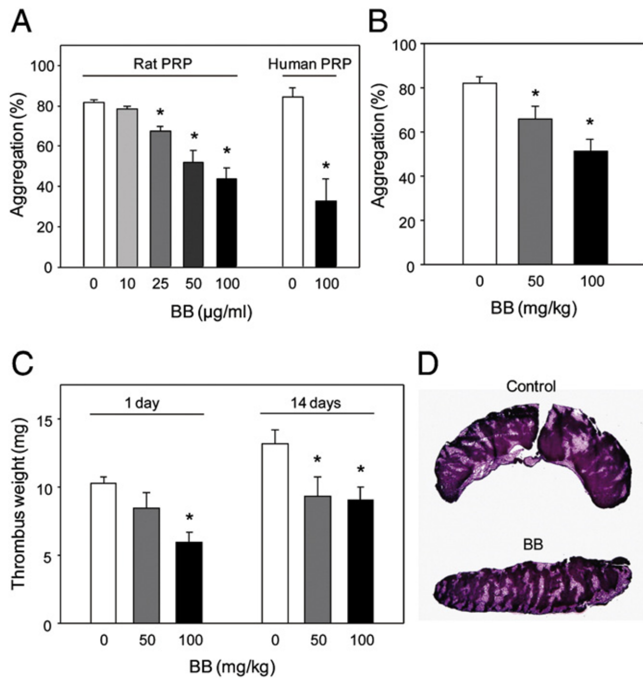

Fig. 3. Effects of BB on rat platelet aggregation and thrombus formation. (A) Various concentrations of BB were treated for 10 min, and rat platelets were stimulated with collagen (8–10 μg/ml) for 6 min. (B) One hour after oral administration of BB to rats, platelets were isolated and stimulated with collagen (10 μg/ml). (C) After oral administration once or once daily for 14 days, thrombus formation was determined in the $FeCl_3$-induced rat venous thrombosis model. (D) Isolated thrombus from vehicle- or BB-administrated (100 mg/kg) rats was fixed and stained. Values are the mean±S.E.M. of three to six animals. The asterisk represents significant differences from the corresponding control, $P<.05$.

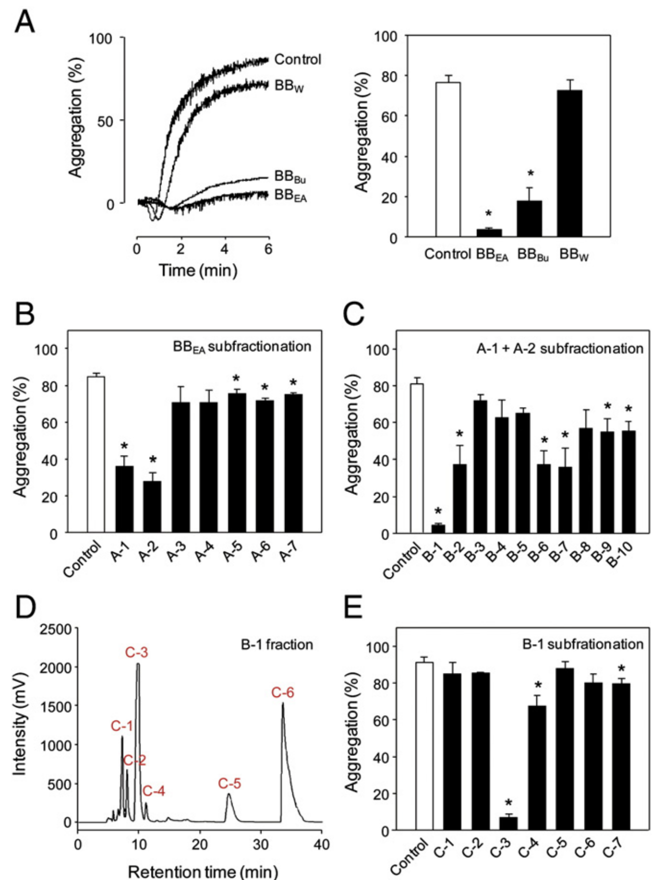

Fig. 4. Isolation of the active ingredient of BB. (A) Ethyl acetate (BB_{EA}), butanol (BB_{Bu}) and water (BB_w) fractions from BB (100 μg/ml) were treated for 10 min, and human platelets were stimulated with collagen (2–4 μg/ml) for 6 min. Subfractions (25 μg/ml) from the (B) ethyl acetate fraction, (C) A-1+A-2 fraction and (E) B-1 fraction were treated for 10 min, and human platelets were stimulated with collagen (2–4 μg/ml) for 6 min. (D) Preparative HPLC profile of the B-1 fraction is shown. The C-7 fraction is the remainder after isolation of the C-1 to C-6 fractions. Values are the mean±S.E.M. of three independent experiments. Representative tracing data of three independent experiments are shown. The asterisk represents significant differences from the corresponding control, $P<.05$.

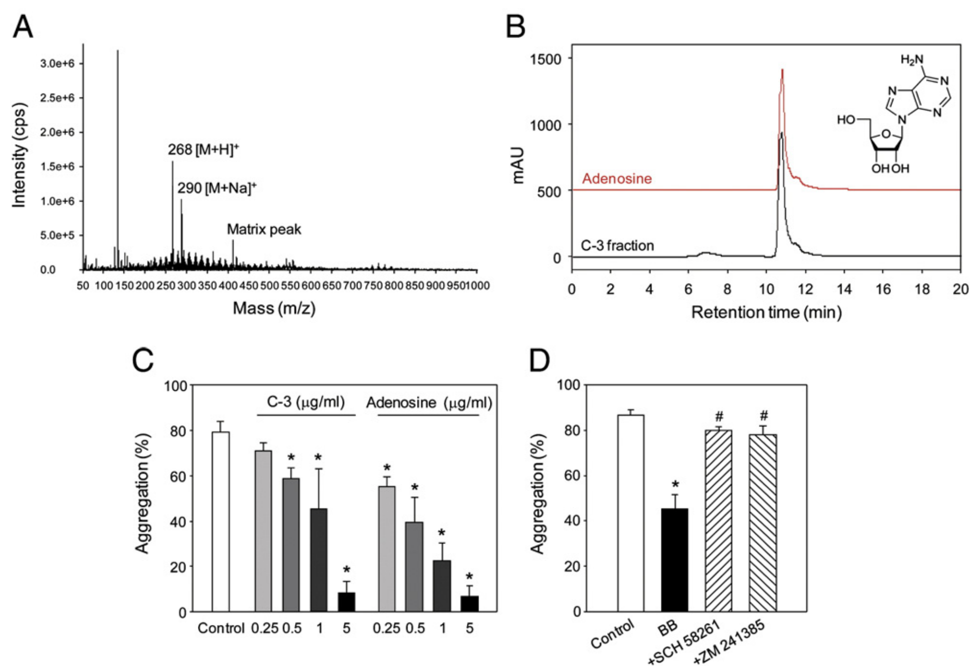

Fig. 5. Identification of the active ingredient of BB. (A) The ESI-MS spectrum of the C-3 fraction is shown. (B) HPLC profiles of the C-3 fraction and adenosine are compared. (C) Various concentrations of the C-3 fraction and adenosine were treated for 10 min, and human platelets were stimulated with collagen (2–4 μg/ml) for 6 min. Values are the mean±S.E.M. of three to four independent experiments. (D) BB (100 μg/ml) was treated for 10 min in the presence of adenosine receptor antagonist SCH 58261 (300 nM) or ZM 241385 (100 nM), and human platelets were stimulated with collagen (2–4 μg/ml) for 6 min. The asterisk represents significant differences from the corresponding control, *P*<.05. The pound sign represents significant differences from BB-only treatment, *P*<.05.

fractions were combined and subfractionated further by column chromatography. The B-1 subfraction showed the most potent inhibitory effects on collagen-induced platelet aggregation (Fig. 4C) and thus the B-1 fraction was subfractionated on peak-by-peak basis based on HPLC-UV profile through preparative HPLC chromatography (Fig. 4D). As a result, the C-3 subfraction with a single peak showed the most potent inhibitory effect on collagen-induced platelet aggregation (Fig. 4E).

The C-3 fraction was analyzed with NMR and MS for the identification of the active ingredient. Through ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC and HMBC spectra, the major ingredient of the C-3 fraction was identified as adenosine or adenosine derivative (data not shown). In addition, the mass spectrum of the C-3 fraction showed that the mass peaks of the active ingredient were identical to those of authentic adenosine standard (Fig. 5A). Confirming this, the HPLC profile of the C-3 fraction and adenosine standard matched well (Fig. 5B). To corroborate that adenosine might be the major active ingredient for the antiplatelet effect of BB, we compared the activities of the C-3 fraction and adenosine, of which results showed similar potencies (Fig. 5C). In addition, the adenosine receptor antagonists SCH 58261 and ZM 241385 blocked the inhibitory effects of BB (Fig. 5D), reflecting that adenosine might explain the antiplatelet effects of BB.

4. Discussion

In this study, we demonstrated that BB can inhibit collagen-induced platelet aggregation along with suppression of platelet activation as determined by reduced granule secretion (serotonin secretion and P-selectin expression) and TxA_2 formation. Conspicuously, YB showed only weak antiplatelet effects, suggesting that there is a discriminating difference in the composition of BB from that of YB, affecting platelet activation. These *in vitro* results were further confirmed in an *ex vivo* platelet aggregation and *in vivo* venous thrombosis model where single and repeated oral administration of BB reduced platelet aggregation and thrombus formation indeed. In addition, we isolated and identified adenosine as a potential active ingredient for antiplatelet effects of BB through bioassay-directed fractionation.

Commonly used antithrombotic drugs are frequently associated with adverse effects, and their benefit for prevention of cardiovascular risks is often being questioned [17,18]. In support of this concern, an analysis of 22 clinical trials using aspirin, a representative antiplatelet drug, as a preventive treatment for thrombosis has demonstrated that bleeding risk increased by aspirin intake from 0.07% to 0.1%, while the rate of serious thrombotic events decreased minutely from 0.57% to 0.51% [28]. For this reason, food materials have gathered huge attention as an alternative and preventive measure against cardiovascular risks, especially for thrombotic events, and many efforts have been made to develop functional foods with antithrombotic activities. In 7 clinical trials, garlic (*Allium sativum*), a common spice, has shown antiplatelet activity in both healthy people and patients with cardiovascular disease [29]. The antithrombotic effect of onions (*Allium cepa*) has been also demonstrated in *in vivo* thrombosis animal models [30,31]. However, the spicy taste and odor of garlic and onion are unacceptable for everyday use; moreover, excessive consumption of garlic or onion can cause diverse problems such as stomach disorder, anemia, allergic reaction and so on, which makes these food materials inadequate for lifetime management of cardiovascular risks [32]. In this study, we have demonstrated that black soybean has antithrombotic effect via antiplatelet activity. Different from the food materials described above, black soybean can be consumed everyday without any significant side effects, suggesting the utility of black soybean as the lifetime preventive management of cardiovascular risks.

The half maximal inhibitory concentration (IC_{50}) of BB against collagen-induced platelet aggregation in PRP was estimated to be 100±46.0 μg/ml, which might appear somewhat high. However, this

IC_{50} level is in a proximate range to those observed with antiplatelet drugs, aspirin (25±2.0 μg/ml) and ticlopidine (134±10.1 μg/ml), in PRP [33]. Moreover, C-3, the active fraction of BB, showed potent activities of which IC_{50} reached a level as low as 1 μg/ml (Fig. 5C), suggesting that antithrombotic effects of BB might be pharmacologically and nutritionally meaningful. Confirming this, an *in vivo* thrombosis experiment with BB indicated that single or multiple administration of BB indeed can manifest clinically meaningful antithrombotic effects. Especially, the doses employed in *in vivo* experiment were in a close range to daily consumption of soybean foods (20–80 g) [1]. Since the extraction yield was 7%–20%, 50–100 mg BB/kg body weight should amount to 500–1000 mg soybean/kg, which was calculated to be 30–60 g/60 kg adult. This indicates that daily intake of black soybean might be able to confer a certain extent of protection against thrombosis and thrombosis-associated cardiovascular diseases.

In previous studies, black soybean showed biological activities superior to other soybeans. Takahashi *et al.* [23] suggested that black soybean shows superior antioxidant effect against LDL oxidation due to the higher polyphenol content in the seed coat, especially anthocyanins. Xu and Chang [22] reported that black soybean has more potent free radical scavenging activities than other soybeans. According to this study, the contents of such phenolics as protocatechuic acid, protocatechualdehyde and caffeic acid are higher in black soybean than in yellow and other soybeans; furthermore, anthocyanins are only contained in black soybean. Several studies have demonstrated the inhibitory effect of these components on platelet activation or aggregation. However, protocatechuic acid (>100 μg/ml) and caffeic acid (>100 μg/ml) are known to inhibit collagen-induced platelet aggregation at relatively higher concentrations [34,35], which do not match the potent antiplatelet effects of BB (IC_{50}=100±46.02 μg/ml) in this study. In addition, anthocyanins have shown weak or no inhibitory effect on platelet activation or aggregation in previous studies [36,37] and the extract of the seed coat of black soybean, which contains most anthocyanins in black soybean, failed to show any effect against collagen-induced platelet activation (data not shown), suggesting that other antiplatelet ingredients might be contained in black soybean.

In this study, the major active ingredient for the antiplatelet effect of BB has been identified and characterized as adenosine through bioassay-directed fractionation and NMR and ESI-MS analyses. In previous studies, adenosine is known to be present in many other food sources, such as garlic, onion and mushrooms, and they suggested that adenosine might contribute at least in part to the *in vitro* antiplatelet activities of these food materials [38–40]. In this study, we could confirm that adenosine inhibited collagen-induced platelet aggregation potently (IC_{50}=0.59±0.13 μg/ml), which correlated well with the inhibitory potency of BB and the actual content of adenosine in BB (0.36%). In addition, in line with the stronger potencies of BB (Fig. 1A), the content of adenosine in BB was substantially higher than that in YB (0.16%).

Adenosine is known to inhibit platelet aggregation through a series of events, including the activation of adenylate cyclase, accumulation of cAMP and inhibition of intracellular calcium increase via interaction with platelet adenosine A_{2A} receptors [41]. These antiplatelet activities of adenosine correlate well with the inhibition of platelet aggregation and granule secretion manifested by BB [42–43]. Further corroborating this, antiplatelet potencies of the active fraction (C-3) matched those of the adenosine standard well (Fig. 5C). Of note, preliminary pharmacokinetic analysis for plasma adenosine following the oral administration of BB or pure adenosine revealed that the intake of BB resulted in a substantially higher plasma adenosine level than that of pure adenosine (data not shown). This suggests that some other compounding ingredients in BB have facilitated the absorption, or attenuate the clearance, of adenosine or possible contributions of other ingredients could not be excluded in the antiplatelet effects of BB, although further study is required to clarify these issues.

As shown in Fig. 2, BB inhibited collagen-induced P-selectin expression, serotonin secretion and TxA_2 formation that are indicators of platelet activation. P-selectin is known to play a major role in platelet interactions with other blood cells, leading to thrombosis [44,45]. Serotonin and TxA_2 can directly act on platelets as a secondary agonist causing further amplification of platelet responses [46,47]. In addition, serotonin and TxA_2 can cause vasoconstriction or proliferation of vascular smooth muscle cells, which could aggravate the development of cardiovascular risks [48,49]. In this context, the general inhibitory effect of BB on platelet degranulation and TxA_2 formation might be synergistic in the prevention of cardiovascular and thrombotic risks.

In summary, we have demonstrated that BB inhibited collagen-induced platelet aggregation and activation, leading to reduced thrombus formation. In addition, repeated oral administration of BB manifested more potent antithrombotic effects *in vivo*, indicating that long-term intake of BB could be more effective in the prevention of thrombotic events. More importantly, this study has provided, we believe, important evidence for the utility of the common food materials for the effective prevention of cardiovascular diseases.

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