Antiplatelet Activity of Soy Sauce as Functional Seasoning

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In seeking the functionality of foodstuffs applicable to medicine, soy sauce was found to show antiplatelet activity. Therefore, the active components in soy sauce were purified, structurally identified, and studied for their inhibitory effects on the aggregation of human platelets. Aqueous 2-fold diluents of soy sauce inhibited platelet aggregation induced by collagen and epinephrine depending on the dilution factor. Since a basic extract with diethyl ether completely inhibited collagen-induced aggregation, it was subjected to serial extractions and multistep HPLC fractionations for purifying antiplatelet components. The finally obtained isolates were identified as 1-methyl-1,2,3,4-tetrahydro-β-carboline and 1-methyl-β-carboline on the basis of EI-MS, ^{1}H NMR, diode array, and fluorescence spectra. Their spectral data and chromatographic behaviors were the same as those of synthetic ones. 1-Methyl-1,2,3,4-tetrahydro-β-carboline showed mean concentrations (n = 5−6) of 4.6, 4.2, 28.6, 11.6, and 65.8 μg/mL to produce 50% inhibition of the maximal aggregation response induced by epinephrine, platelet-activating factor, collagen, adenosine 5′-diphosphate, and thrombin, respectively. Its inhibitory effect was much greater than that of 1-methyl-β-carboline on platelet aggregation by all the tested inducers. The quantitative HPLC analysis revealed that the significant amounts of both antiplatelet compounds were uniformly contained in commercially available soy sauce. From these results, soy sauce may be referred to as functional seasoning containing alkaloidal components with the potent preventive effect on thrombus formation.

Keywords: *Soy sauce; antiplatelet; functional seasoning; platelet aggregation*

INTRODUCTION

While the belief that foodstuffs are closely linked to optimal health may not be a worldwide concept, the concept of "medicine and eating are the same origin" has been traditionally accepted in Japan. Increasing attention has been recently directed toward health benefits of functional foods and beverages beyond their nutritional significance (Malaspina, 1996; Johns and Romeo, 1997). Ingestion of plant foods and beverages such as onions, citrus fruits, green tea, and their products relates to reduction of the risk and mortality of various diseases (Johns and Romeo, 1997; Yamamoto et al., 1997). Functional foods and beverages, unlike drugs, are presumed to be more safe, and they also have the potent preventive and/or therapeutic effects especially on chronic diseases such as cardiovascular disorder, possibly treating an entire population (Clydesdale, 1997). A study seeking the functionality of foods and beverages would be an important strategy to develop a new medicinal tool.

Although previous studies have dealt with functional foodstuffs, specific foods and beverages are not ingested daily even if their pharmacological effects were found (Johns and Romeo, 1997; Yamamoto et al., 1997). In contrast, seasoning is consumed almost everyday. Common soy consumption in Japan has been speculated to relate to reduction in the rate of cardiovascular disorder (Anthony et al., 1998; Barnes, 1998). Recent studies suggest that phytochemicals in soyfoods are responsible for their beneficial effects on health (Messina, 1995; Barnes, 1998). If soy sauce contains certain bioactive components in addition to taste and aroma compounds, it would be considered as functional seasoning.

On the basis of this consideration, we screened the pharmacological activity of soy sauce and consequently found that soy sauce had the ability to inhibit the aggregation of human platelets. In this paper, we describe the purification, isolation, and identification of antiplatelet components in soy sauce and their inhibitory effects on platelet aggregation induced by different agents.

MATERIALS AND METHODS

Materials. Soy sauce was obtained from commercial outlets. Collagen (MC Medical, Tokyo, Japan), epinephrine (Daiichi-Seiyaku, Tokyo, Japan), platelet-activating factor (PAF; Funakoshi, Tokyo, Japan), adenosine 5′-diphosphate Na (ADP; MC Medical), and thrombin (Sigma, St. Louis, MO) were used for induction of platelet aggregation. 1-Methyl-1,2,3,4-tetrahydro-β-carboline (MTBC) and 2-ethyl-1,2,3,4-tetrahydro-β-carboline (ETBC) were synthesized as reported previously (Peura and Nousiainen, 1981; Tsuchiya et al., 1994). 1-Methyl-β-carboline (MBC), 3-hydroxymethyl-β-carboline (HMBC), genistein, and daidzein were purchased from Funakoshi. Dimethyl sulfoxide (DMSO) of spectroscopic grade and acetonitrile of HPLC grade were purchased from Kishida (Osaka, Japan). All other reagents were of the highest quality available. Used H_2O was redistilled in an all-glass apparatus after purifying by a Milli-RO water purification system (Nihon Milli-Pore, Tokyo, Japan).

Platelet Aggregation. The experiments were designed and performed according to the guidelines of the Japanese Pharmacological Society. Informed consent was obtained from all

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subjects after the nature and consequences of their participation were explained. Human male subjects aged 43−46 years, without any medication in the preceding 20 days, followed a diet not containing soy sauce and soyfoods for 2 days prior to the study and were not allowed to eat for 15 h before blood drawing. They were subjected to blood collection at 10:00 am of the study day. Blood was collected from an antecubital vein using a syringe with 20 gauge needle and a tourniquet. The collected blood was anticoagulated with 3.8% (w/v) trisodium citrate (1 volume to 11 volume of blood) and centrifuged at 160*g* for 8 min to obtain platelet-rich plasma (PRP). After isolation of PRP, the remaining plasma was recentrifuged at 1500*g* for 20 min to obtain platelet-poor plasma (PPP). The platelet count of PRP was adjusted to 300 000/μL by diluting with PPP. PRP was stabilized by standing at room temperature for 30 min. Both PRP and PPP were used within 3 h after preparation.

Platelet aggregation was analyzed by a 604 HEMA TRACER aggregometer (Niko Bioscience, Tokyo, Japan) connected to a PC-9801US personal computer (NEC, Tokyo, Japan) and a PL 500 printer (Yokogawa, Tokyo, Japan). Aggregation responses were monitored by an increase of percent light transmission (% T) as a function of time, where PPP was 100% T and unstimulated PRP was 0% T. Throughout an analytical period, plasma samples were stirred at 1000 rpm and at 37 °C. PRP (170 μL) was prestirred to adjust the temperature. Sample solutions were prepared by dissolving and diluting the extracts, fractions, isolates, and synthetic compounds with H_2O, 1% (v/v) aqueous DMSO solution, or 0.1 M sodium phosphate buffer (pH 7.0). Genistein and daidzein as reference antiplatelet compounds were dissolved in 1% (v/v) aqueous DMSO solution. A 20 μL aliquot of the solutions was added to PRP after adjustment of % T to 0. Solvent alone was added for control samples. The volume of DMSO was adjusted to be less than 0.1% (v/v) of the final volume, which did not influence platelet aggregation by all the tested inducers. After 1 min, the aggregation was induced by adding 10 μL of each aqueous solution of collagen (50 μg/mL), epinephrine (40 μg/mL), PAF (5 μM), ADP (60 μM), and thrombin (5 U/mL). The time of inducer addition was defined as 0 min. Except epinephrine, maximal % T of aggregation response (T_{max}), area under curve of aggregation response (AUC, from 0 to 5 min), and a single slope of aggregation response were determined. For epinephrine producing the biphasic aggregation, first T_{max} (at 1 min) and second T_{max} (at 5 min), first AUC (from 0 to 1.5 min) and second AUC (from 1.5 to 5 min), and slopes of the first (% T at 45 s) and second (% T at 4 min) phases were determined.

For MTBC and MBC, the concentration to produce 50% inhibition (IC_{50}) was determined using T_{max}, AUC, and slope of aggregation responses induced by epinephrine, PAF, collagen, ADP, and thrombin. Sample solutions of MTBC and MBC were prepared by dissolving them in 1% (v/v) aqueous DMSO solution and serially diluting 2-fold with the same solvent. All aggregation experiments were replicated five to six times, and each result was expressed by mean ± SE.

Sample Dilution and Extraction. (a) Dilution. To confirm the antiplatelet activity of intact soy sauce, soy sauce was diluted 2- to 10-fold with H_2O. The diluents were subjected to platelet aggregation analysis.

(b) Preliminary Extraction. As a pilot extraction study, 1.0 mL of soy sauce was extracted with 10 mL of diethyl ether after the addition of 0.5 mL each of 2.0 M NaOH and of 2.0 M HCl to obtain basic and acidic extract, respectively. Each extract solution was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in 0.1−0.5 mL of 0.1 M sodium phosphate buffer (pH 7.0), and then the solution was subjected to platelet aggregation analysis.

Sample Preparation. Soy sauce was prepared to isolate antiplatelet components according to the procedure schematically shown in Scheme 1.

(a) Extraction. A basic extract found to be active in the preliminary extraction was prepared by extracting 200 mL of soy sauce with 500 mL of diethyl ether after the addition of 30 mL of 2.5 M NaOH. The extract solution was evaporated to dryness after drying over anhydrous Na_2SO_4. The residue was dissolved in 3.0 mL of 50% (v/v) aqueous acetonitrile solution, followed by HPLC fractionation.

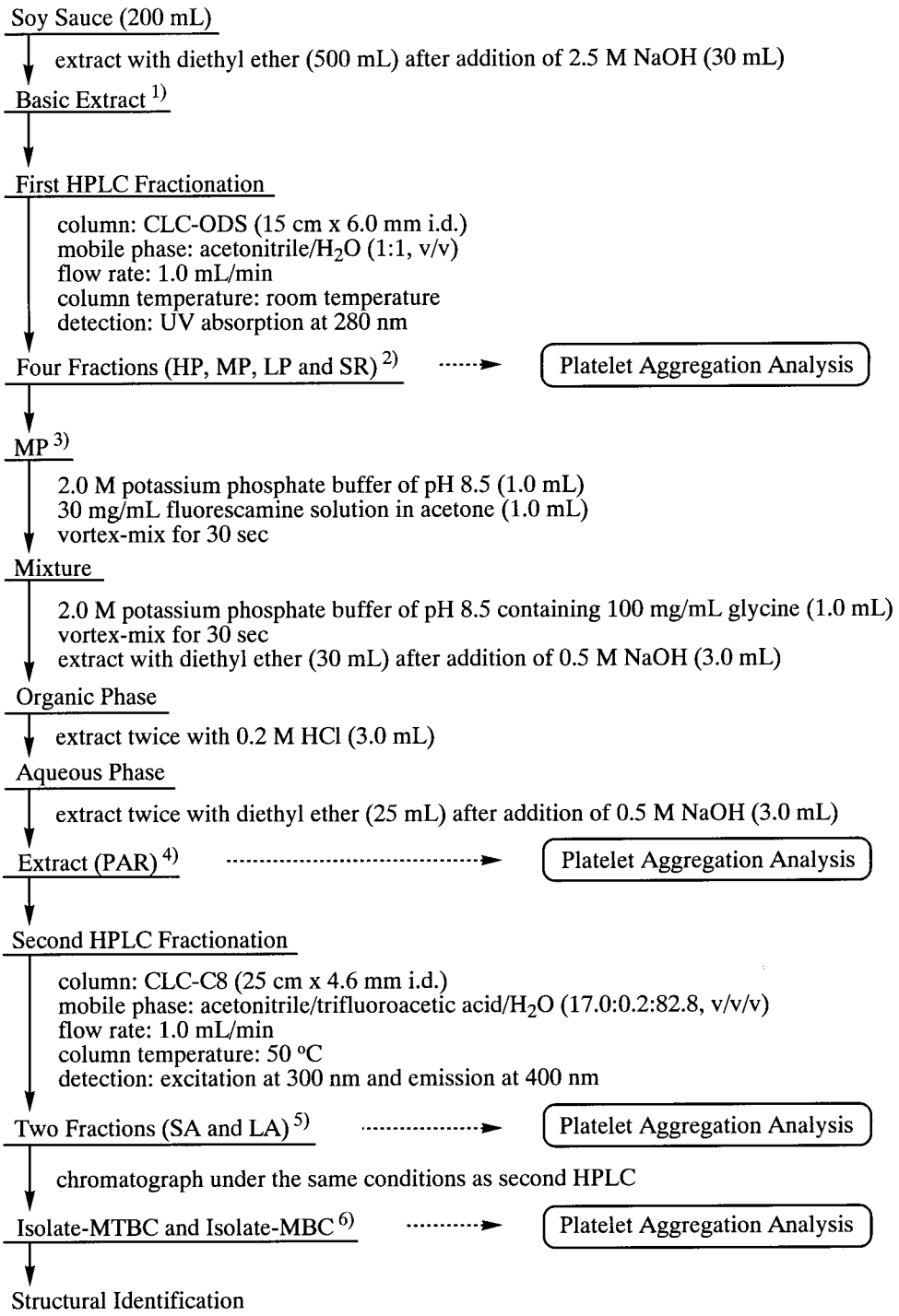
(b) First Fractionation. The extract prepared in (a) was divided into four fractions based on their polarity by reversed-phase partition chromatography. The HPLC system used consisted of a 655A-11 liquid chromatograph (Hitachi, Tokyo, Japan), a 7125 sample injector (sample volume 200 μL; Rheodyne, Cotati, CA), a Shim-pack CLC-ODS column (particle size 5 μm, 15 cm × 6.0 mm i.d.; Shimadzu, Kyoto, Japan), a UVILOG-5IIIA UV-detector (Õyõ-Bunkõ, Tokyo, Japan), and a CR-6A Chromatopac data processor (Shimadzu). Fractionation was performed by delivering a mixture of acetonitrile and H_2O (1:1, v/v) at a flow rate of 1.0 mL/min and at room temperature. By detecting absorption at 280 nm, eluates were divided into high polar (HP), moderate polar (MP), low polar (LP), and strongly retained fraction (SR) in their elution order. Each fraction was evaporated to remove organic solvent. The residual solution was extracted with 120 mL of diethyl ether after adding 8 mL of 0.5 M NaOH. The extract solution was evaporated to dryness after drying over anhydrous Na_2SO_4. The residue was dissolved with 1% (v/v) aqueous DMSO solution to be the concentration corresponding to the original soy sauce 20 mL per mL. The solution was subjected to platelet aggregation analysis.

(c) Derivatization. Derivatization with fluorescamine prior to extraction was performed in order to remove primary amine components from MP, which was found to be the most active in (b). The MP residue prepared from 200 mL of soy sauce was dissolved in 0.5 mL of 0.1 M HCl. The solution was vortex-mixed for 30 s with 1.0 mL of 2.0 M potassium phosphate buffer (pH 8.5) and 1.0 mL of a fluorescamine solution in acetone (30 mg/mL) and then vortex-mixed for 30 s with 1.0 mL of 2.0 M potassium phosphate buffer (pH 8.5) containing L-glycine (100 mg/mL). The mixture was extracted with 30 mL of diethyl ether after the addition of 3.0 mL of 0.5 M NaOH. The organic phase was back-extracted twice with 3.0 mL of 0.2 M HCl. After the addition of 3.0 mL of 0.5 M NaOH, the combined back-extracts were reextracted twice with 25 mL of diethyl ether to obtain primary amine removed extract (PAR). A 1.0 mL aliquot of the combined PAR solutions was evaporated to dryness. The residue was dissolved in 100 μL of 1% (v/v) aqueous DMSO solution, and then the solution was subjected to platelet aggregation analysis.

(d) Second Fractionation. The remaining PAR solution was fractionated by HPLC with fluorometric detection in order to subdivide PAR, which was found to be active in (c). After evaporation to dryness, the PAR residue was dissolved in 0.5 mL of the mobile phase solution: a mixture of acetonitrile, trifluoroacetic acid, and H_2O (17.0:0.2:82.8, v/v/v). The solution was chromatographed by the HPLC system consisting of an LC-10AD liquid chromatograph (Shimadzu) connected to an SCL-10A system controller (Shimadzu), a 7125 sample injector (sample volume 100 μL; Rheodyne), a Shim-pack CLC-C8 column (particle size 5 μm, 25 cm × 4.6 mm i.d.; Shimadzu) placed in a CTO-6A column oven (Shimadzu), and an RF-550 spectrofluorometric detector (Shimadzu) connected to a CR-6A Chromatopac data processor (Shimadzu). The mobile phase was delivered at a flow rate of 1.0 mL/min and at a column temperature of 50 °C. Eluates were detected at 300 nm for excitation and at 400 nm for emission. Two major peak fractions, defined as small area (SA) and large area fraction (LA) by their peak areas, were collected. Each fraction was evaporated to remove organic solvent, and the residual solution was extracted with 35 mL of diethyl ether after the addition of 1.0 mL of 0.5 M NaOH. The extract solution was evaporated to dryness after drying over anhydrous Na_2SO_4. The residue was dissolved with 1% (v/v) aqueous DMSO solution to be the concentration corresponding to the original soy sauce 20 mL per mL. The solution was subjected to platelet aggregation analysis.

(e) Isolation. The SA and LA residues prepared from 200 mL of soy sauce as in (a), (b), (c), and (d) were dissolved in 0.5 mL of 0.2% (v/v) aqueous trifluoroacetic acid solution. Each solution was chromatographed under the same conditions as second HPLC to obtain isolate-MTBC from SA and isolate-

Scheme 1. Schematic Presentation of Soy Sauce Preparation[a]



[a] (1) Evaporated to dryness and dissolved in 3.0 mL of 50% (v/v) aqueous acetonitrile solution for first HPLC fractionation. (2) Evaporated, extracted with 120 mL of diethyl ether after adding 8 mL of 0.5 M NaOH, evaporated to dryness, and dissolved with 1% (v/v) aqueous DMSO solution for platelet aggregation analysis. (3) Evaporated, extracted with 120 mL of diethyl ether after addition of 8 mL of 0.5 M NaOH, evaporated to dryness, and dissolved in 0.5 mL of 0.1 M HCl for derivatization. (4) Evaporated to dryness and dissolved with 1% (v/v) aqueous DMSO solution for platelet aggregation analysis or with the mobile phase solution for second HPLC fractionation. (5) Evaporated, extracted with 35 mL of diethyl ether after addition of 1.0 mL of 0.5 M NaOH, evaporated to dryness, and dissolved with 1% (v/v) aqueous DMSO solution for platelet aggregation analysis or with 0.2% (v/v) aqueous trifluoroacetic acid solution for isolation. (6) Evaporated to dryness and dissolved with 1% (v/v) aqueous DMSO solution to be 5−500 μg/mL for platelet aggregation analysis.

MBC from LA. After evaporation to dryness, each isolate was dissolved with 1% (v/v) aqueous DMSO solution to be 5−500 μg/mL. The solution was subjected to platelet aggregation analysis.

Isolate-MTBC and isolate-MBC were rechromatographed to confirm their purity together with measurement of their fluorescence spectra.

Structural Identification. EI-MS spectra of isolate-MTBC and isolate-MBC were measured by a JMS-D300 EI-MS instrument (JEOL, Tokyo, Japan). ^{1}H NMR spectra were also obtained using a JNM EX-400 instrument (JEOL) at 400 MHz in $CDCl_3$ or CD_3OD. For chromatographic and spectral comparison, isolated and synthesized compounds were chromatographed under the same conditions as second HPLC using an

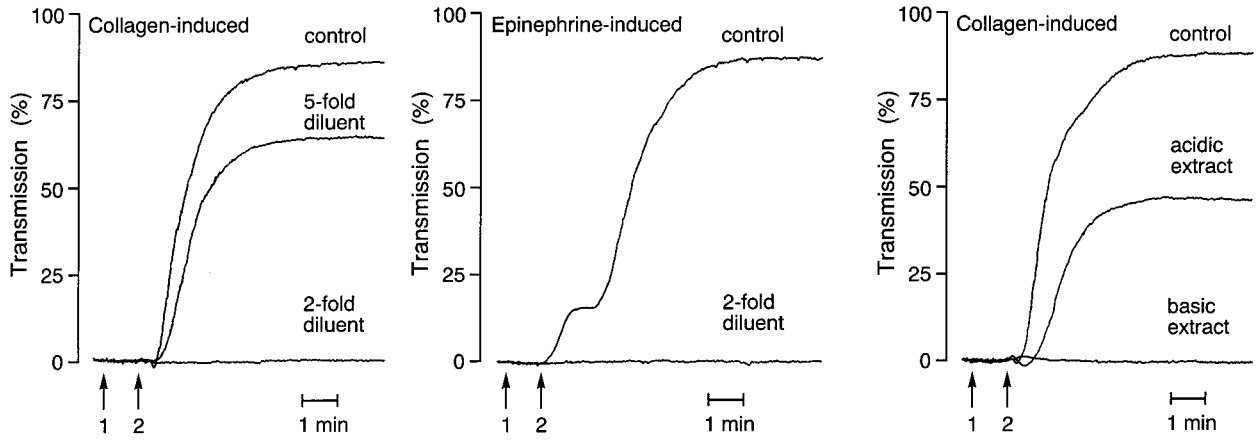


Figure 1. Platelet aggregation inhibited by diluent and extract samples of soy sauce. To PRP, an aqueous diluent, a basic extract, an acidic extract, or solvent alone as control was added at the indicated time (1). After 1 min, collagen or epinephrine was added to induce platelet aggregation at the indicated time (2).

SPD-M10AVP diode array detector (Shimadzu) controlled by an FMV-5133D5 personal computer (Fujitsu, Tokyo, Japan) in addition to a spectrofluorometric detector.

Quantitative Analysis of Soy Sauce. Soy sauce of different brands ($n = 5$) was diluted 1000- to 2000-fold with H_2O. To 0.25 mL of each diluent was added 50 μL of an aqueous solution of ETBC (100.0 ng/mL) and HMBC (25.0 ng/mL) as an internal standard for MTBC and MBC quantitation, respectively. The solution was vortex-mixed for 30 s with 0.5 mL each of 2.0 M potassium phosphate buffer (pH 8.5) and of a fluorescamine solution in acetone (5 mg/mL). Immediately after that, 0.5 mL of 2.0 M potassium phosphate buffer (pH 8.5) containing L-glycine (100 mg/mL) was added with vortex-mixing for 30 s. The mixture was extracted with 7.0 mL of diethyl ether after addition of 2.0 mL of 0.5 M NaOH. The organic phase was extracted with 1.0 mL of 0.2 M HCl. The aqueous phase was reextracted with 7.0 mL of diethyl ether after addition of 2.0 mL of 0.5 M NaOH. The organic phase was evaporated to dryness, and then the residue was dissolved in 200 μL of 0.2% (v/v) aqueous trifluoroacetic acid solution. An aliquot (50−100 μL) of the resulting solution was subjected to HPLC analysis using the same system as the second fractionation. An RF-535 fluorometric detector (Shimadzu) for detecting MBC and HMBC at excitation 300 nm and at emission 430 nm was connected in series to an RF-550 spectrofluorometric detector for detecting MTBC and ETBC at excitation 275 nm and at emission 350 nm. The mobile phase, a mixture of acetonitrile, trifluoroacetic acid, and H_2O (20.0:0.2:79.8, v/v/v), was delivered at a flow rate of 1.0 mL/min and at a column temperature of 50 °C. MTBC and MBC were quantified on the basis of their peak area ratios to ETBC and HMBC, respectively, by reference to the calibration graphs.

RESULTS

Figure 1 shows the typical results of platelet aggregation in which monophasic and biphasic responses of PRP were induced by collagen and epinephrine, respectively. Aqueous 2-fold diluents of soy sauce completely inhibited both collagen- and epinephrine-induced aggregation. Their inhibition potency depended on the dilution factor for soy sauce.

In a pilot extraction study, the antiplatelet activity was found in both basic and acidic extracts. A basic extract completely inhibited the aggregation induced by collagen, whereas an acidic extract showed much less inhibitory effect (Figure 1).

Isoflavones were tested for the inhibitory effect as reference antiplatelet components contained in soy and its products. Although genistein and daidzein (100 μg/mL of each) showed the antiplatelet activity, neither isoflavone inhibited platelet aggregation induced by collagen and epinephrine as intensively as the basic extract.

The active basic extract was divided into four fractions (HP, MP, LP, and SR) by reversed-phase HPLC with UV detection. The greatest antiplatelet activity was specified in MP, which almost completely inhibited collagen- and epinephrine-induced aggregation (Figure 2). However, HP, LP, and SR did not show the inhibitory effect, while very slight inhibition by HP was observed in the second phase aggregation induced by epinephrine.

The most active MP was extracted after a reaction with fluorescamine to separate amine components. The obtained PAR showed the intensive inhibitory effect on platelet aggregation induced by collagen and epinephrine.

The active PAR was further fractionated by HPLC with fluorometric detection to obtain two fractions (SA and LA). Both SA and LA significantly inhibited the aggregation induced by collagen (Figure 2). In epinephrine-induced aggregation, the second phase aggregation was completely inhibited by SA, and less intensively by LA. SA also inhibited the first phase aggregation, but not LA.

Isolate-MTBC from SA and isolate-MBC from LA showed a single peak in HPLC analysis with fluorometric detection (Figure 3). Both isolates were confirmed to have the purity over 98.0% based on their peak areas. Maximal excitation and emission wavelengths were 275 and 350 nm for isolate-MTBC and 300 and 430 nm for isolate-MBC. EI-MS spectra at *m/z* (relative intensity at 70 eV) were 186 (M^+, 58), 171 (100), 157 (42), 144 (11), 130 (10), and 115 (8) for isolate-MTBC and 182 (M^+, 100), 154 (14), 140 (4), and 127 (5) for isolate-MBC. The results of ^1H NMR spectral measurements of isolate-MTBC in $CDCl_3$ and isolate-MBC in CD_3OD are shown in Figure 4. Isolate-MTBC and isolate-MBC were structurally identified as 1-methyl-1,2,3,4-tetrahydro-β-carboline and 1-methyl-β-carboline, respectively (see Figure 4), on the basis of these spectral data, which agreed with those of synthesized compounds and previous reports (Peura and Nousiainen, 1981). In rechromatography, their retention time, diode array spectra, and fluorescence spectra were the same as those of synthetic MTBC and MBC.

Table 1 shows the IC_{50}s of MTBC and MBC for epinephrine-, PAF-, collagen-, ADP-, and thrombin-induced aggregation. MTBC was more active than MBC

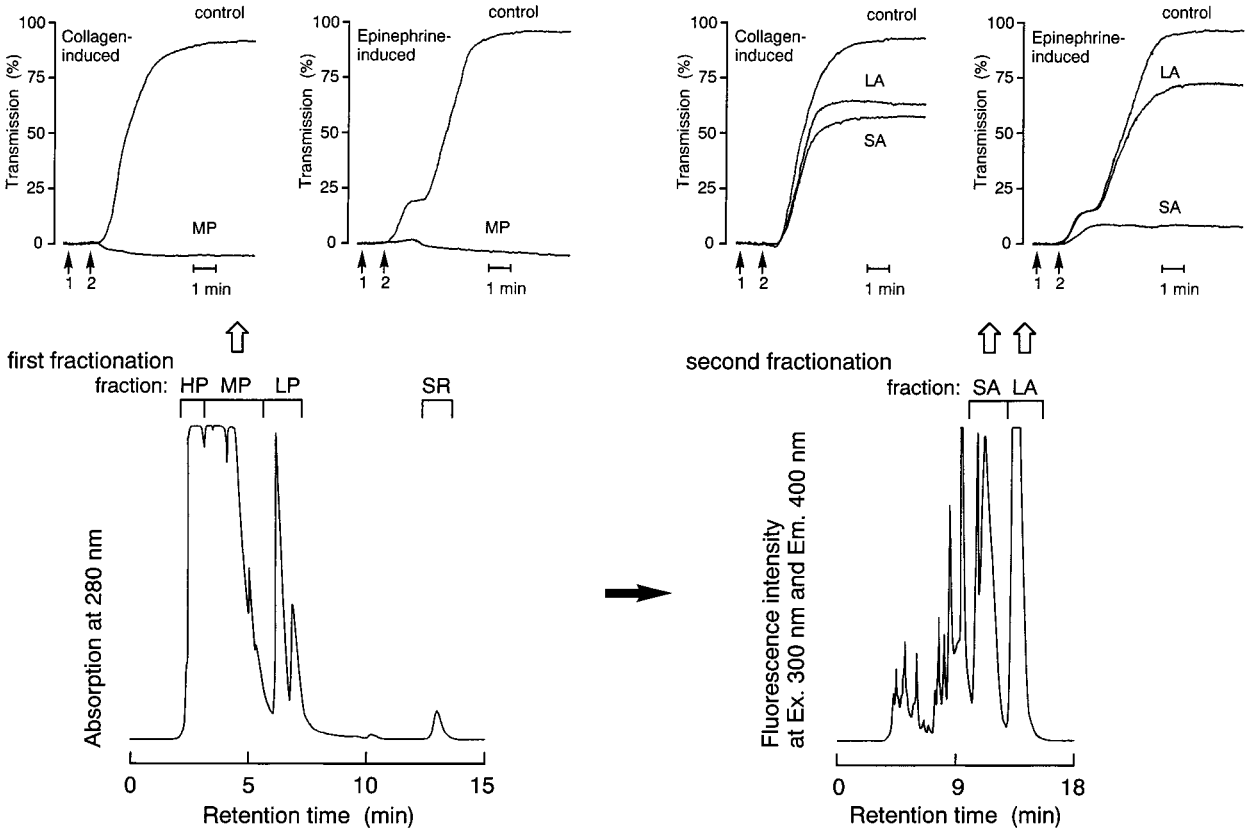


Figure 2. First and second HPLC fractionation profiles and platelet aggregation inhibited by fractions. MP, SA, and LA dissolved with 1% (v/v) aqueous DMSO solution or solvent alone as control was added to PRP at the indicated time (1). After 1 min, collagen or epinephrine was added to induce platelet aggregation at the indicated time (2).

Table 1. Inhibitory Effects of 1-Methyl-1,2,3,4-tetrahydro-β-carboline and 1-Methyl-β-carboline on Human Platelet Aggregation

		IC_{50}[a] (μg/mL)	
aggregation inducer[b]		1-methyl-1,2,3,4-tetrahydro-β-carboline	1-methyl-β-carboline
epinephrine (2.0 μg/mL) first phase:	T_{max}[c]	12.4 ± 1.7	81.9 ± 1.6
	AUC[d]	18.5 ± 3.0	88.8 ± 5.6
	slope[e]	38.4 ± 1.4	106.6 ± 7.9
epinephrine (2.0 μg/mL) second phase:	T_{max}	4.6 ± 1.2	22.4 ± 3.4
	AUC	5.0 ± 0.8	30.1 ± 2.9
	slope	13.3 ± 1.1	23.5 ± 6.2
PAF (0.25 μM)	T_{max}	4.2 ± 0.2	44.4 ± 0.7
	AUC	2.3 ± 0.7	30.3 ± 1.3
	slope	>20*	>50*
collagen (2.5 μg/mL)	T_{max}	28.6 ± 4.0	184.5 ± 18.8
	AUC	23.4 ± 3.2	170.0 ± 23.6
	slope	65.3 ± 7.5	278.0 ± 30.3
ADP (3 μM)	T_{max}	11.6 ± 1.8	47.4 ± 4.3
	AUC	12.5 ± 2.0	49.7 ± 4.2
	slope	>50*	>100*
thrombin (0.25 U/mL)	T_{max}	65.8 ± 4.5	548.5 ± 19.2
	AUC	60.1 ± 6.9	550.9 ± 30.7
	slope	>100*	>500*

[a] Mean ± SE of concentration to produce 50% inhibition (n = 5−6). [b] Platelet aggregation was induced by each agent of the indicated concentration 1 min after adding each alkaloid to platelet-rich plasma. [c] Maximal percent light transmission of aggregation response. [d] Area under curve of aggregation response. [e] Slope of aggregation response. *No significant inhibition at the indicated concentration.

on inhibition of platelet aggregation by all the tested inducers. Especially, MTBC intensively inhibited the epinephrine-induced second phase aggregation and PAF-induced aggregation to show IC_{50}s ranging from 2.3 to 5.0 μg/mL for T_{max} and AUC. The inhibitory effect of MTBC was greater on PAF-induced, epinephrine-induced second phase, ADP-induced, epinephrine-induced first phase, collagen-induced, and thrombin-induced aggregation in this order.

Table 2. 1-Methyl-1,2,3,4-tetrahydro-β-carboline and 1-Methyl-β-carboline in Soy Sauce

	concentration[a] (μg/mL)	
soy sauce	1-methyl-1,2,3,4-tetrahydro-β-carboline	1-methyl-β-carboline
brand A	85.23	1.22
brand B	43.92	0.55
brand C	49.53	4.18
brand D	28.35	0.29
brand E	52.67	1.46
mean ± SD	51.94 ± 18.63	1.54 ± 1.39

[a] Values represent the means in duplicate HPLC quantitation for each brand.

In quantitative analysis, all the tested soy sauce samples were found to contain both MTBC and MBC (Table 2). The concentrations in soy sauce were 28.35−85.23 μg/mL for MTBC and 0.29−4.18 μg/mL for MBC.

DISCUSSION

In crude extraction of soy sauce, an acidic extract inhibited collagen-induced aggregation as well as a basic extract, although the former was inferior in inhibition potency to the latter. Salicylates and their related compounds, which show the inhibitory effect on platelet aggregation (Weiss et al., 1968; Dupin et al., 1986), have

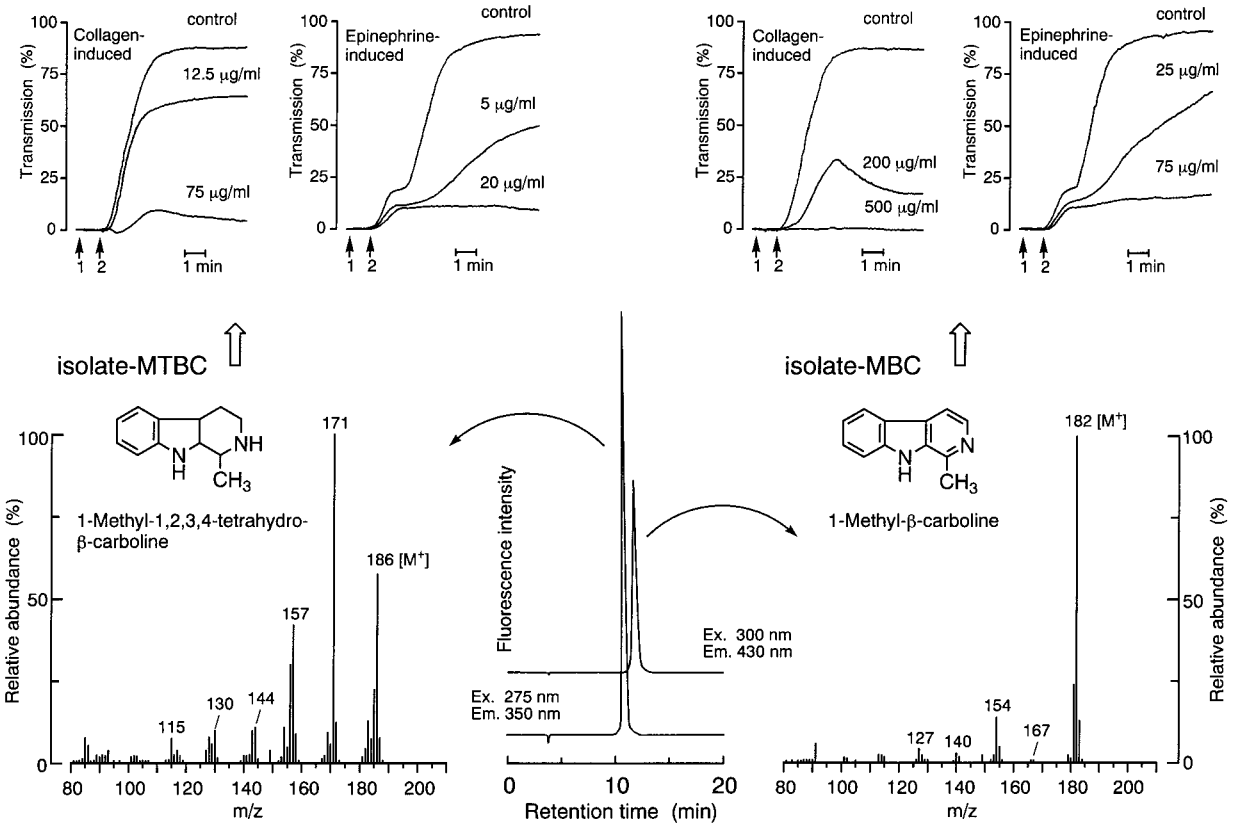
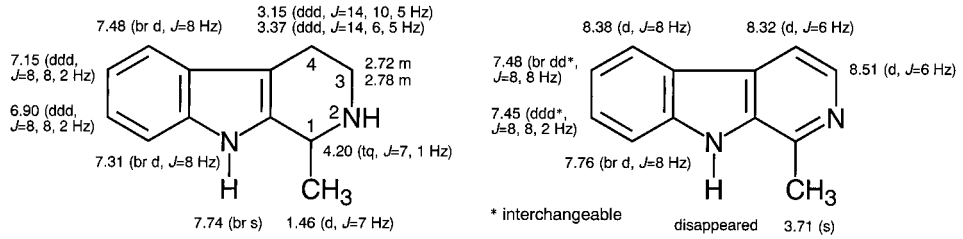


Figure 3. HPLC profiles and EI-MS spectra of the finally obtained isolates and platelet aggregation inhibited by them. Isolate-MTBC and isolate-MBC dissolved with 1% (v/v) aqueous DMSO solution or solvent alone as control was added to PRP at the indicated time (1). After 1 min, collagen or epinephrine was added to induce platelet aggregation at the indicated time (2).



1-Methyl-1,2,3,4-tetrahydro-β-carboline (isolate-MTBC)

1-Methyl-β-carboline (isolate-MBC)

Figure 4. Chemical structures of antiplatelet components isolated from soy sauce with ^{1}H NMR data measured in $CDCl_3$ for isolate-MTBC and in CD_3OD for isolate-MBC.

been widely added to soy sauce as a preservative. Aspirin-like antiplatelet activity is known in a number of food additives (Williams et al., 1989). Since an antiplatelet preservative possibly transfers into the acidic extract, the following purification was focused only on the basic extract.

Soy sauce is made by fermentation of soybeans, and it contains a number of compounds including soy components and fermentation products (Yokotsuka, 1986). Among them, isoflavones are also known to inhibit platelet aggregation (Nakashima et al., 1991; Wilcox and Blumenthal, 1995). However, genistein and daidzein were not as active as the basic extract. Considering the isoflavone concentration in soy sauce (Fukutake et al., 1996), certain basic components, possibly amines, are more responsible for the inhibitory effect of the active extract than genistein and daidzein.

MP, specified as the active fraction by reversed-phase HPLC of the basic extract, was reacted with fluorescamine prior to extraction with diethyl ether under alkaline conditions. Fluorescamine is a derivatization reagent to react predominantly with a primary amino group (Udenfriend et al., 1972). Since the compounds highly reactive to fluorescamine remain in the alkaline aqueous phase as their acidic derivatives, the following extraction enables to obtain PAR without containing primary amine components. The obtained PAR inhibited collagen- and epinephrine-induced platelet aggregation, indicating that the antiplatelet activity of soy sauce derives from basic components other than primary amines.

The active PAR was incidentally found to show fluorescence when being irradiated with UV light.

Therefore, it was further fractionated by HPLC with fluorometric detection, providing active SA and LA. As presumed from differences in chromatographic behavior and inhibition potency, two kinds of components (MTBC and MBC) with high purity were finally isolated and structurally identified. The reason MTBC was fractionated into SA despite its overwhelmingly high concentration in soy sauce is accounted for by the used detection conditions that are almost comparable to maximal excitation and emission wavelengths for MBC but not for MTBC.

MTBC shows IC_{50}s ranging from 2.3 to 65.8 μg/mL against T_{max} and AUC of aggregation responses induced by epinephrine, PAF, collagen, ADP and thrombin. MBC also inhibits platelet aggregation, although its IC_{50} is larger. The structurally analogous β-carbolines were recently found to inhibit platelet aggregation (Given and Longenecker, 1987; Begum et al., 1996). Compared with the IC_{50}s of 7-methoxy-1-methyl-3,4-dihydro-β-carboline, 1,2,3,4-tetrahydro-β-carboline and β-carboline (Given and Longenecker, 1987), MTBC is more active on inhibition of platelet aggregation by all the tested inducers.

The inhibitory effects of β-carboline alkaloids were reported to occur through antagonism of adrenoceptors in platelets (Given and Longenecker, 1983, 1987), suggesting the pharmacological mechanism underlying inhibition of epinephrine-induced aggregation by MTBC and MBC. However, MTBC and MBC also inhibit platelet aggregation induced by PAF, collagen, ADP, and thrombin. Their antiplatelet activity is not interpreted only by the effect on adrenoceptors. Membrane fluidity regulates the platelet function and various membrane-fluidizing agents are known to inhibit platelet aggregation (Kitagawa et al., 1993; Vlasic et al., 1993). Certain β-carbolines influence the fluidity of model membranes (Peura et al., 1982). The alteration of membrane fluidity may be involved in the antiplatelet effects of MTBC and MBC.

The daily consumption of soy sauce in Japan is estimated as 23−30 mL per person according to data from the Ministry of Health and Welfare of Japan (1980) and the Japan Soy Sauce Brewers Association (1998). Therefore, it is presumed that MTBC of 1.2−1.6 mg and MBC of 35−46 μg are daily supplied through soy sauce consumption by calculation using their mean concentrations obtained from quantitation of several soy sauce samples. The in vivo levels of β-carbolines increasingly change by their exogenous supply, suggesting that MTBC and MBC are readily absorbed (Tsuchiya et al., 1996a,b). To speculate the beneficial effect of soy sauce, other active components must be addressed because genistein and daidzein inhibit platelet aggregation as well as MTBC and MBC (Nakashima et al., 1991; Wilcox and Blumenthal, 1995). The possibility that soy sauce consumption leads to the antiplatelet action in the human body has remained to be investigated, while it would be expected by the coexistence of alkaloidal and flavonoidal active components in soy sauce.

Since β-carboline alkaloids are the natural components in plants belonging to Solanaceae and they are produced during fermentation, tomato, ketchup, tabasco, vinegar, miso (soybean paste), cheese, yogurt, etc. contain MTBC and MBC (Adachi et al., 1991; Tsuchiya et al., 1996b). These seasonings and foods are also the dietary sources for supplying MTBC and MBC in addition to soy sauce.

MTBC, MBC, and their relating β-carbolines are present in platelets of humans and animals (Bidder et al., 1979; Honecker et al., 1980; Schouten and Bruinvels, 1985). Their contents are elevated by alcohol intake, especially in chronic intake like alcoholics (Peura et al., 1980; Rommelspacher et al., 1984; Collins, 1988). Although the platelet count is kept within the normal range, alcohol intake decreases platelet aggregability (Cowan, 1980; Fink and Hutton, 1983), which was observed in platelet aggregation induced by epinephrine, ADP, thrombin, and collagen (Haut and Cowan, 1974). The link between alcohol-induced increase of β-carbolines and alcohol-induced depression of platelet aggregability may support the in vivo inhibition of platelet aggregation by MTBC and MBC.

Concerning the functionality of soyfoods, their consumption has been recognized to be associated with reduction of various cancers (Messina et al., 1994), blood cholesterol (Carroll and Kurowska, 1995), and cardiovascular diseases (Barnes, 1998). These associations have been attributed to bioactive components such as soy proteins, amino acids/peptides, fibers, and isoflavones (Barnes, 1998; Potter, 1998). The present study has revealed that soy sauce additionally contains two alkaloidal antiplatelet components. In conclusion, soy sauce is considered to be not only a mixture of taste and aroma compounds but also functional seasoning with the potent preventive effect on thrombus formation.

ABBREVIATIONS USED

MTBC, 1-methyl-1,2,3,4-tetrahydro-β-carboline; MBC, 1-methyl-β-carboline; PRP, platelet-rich plasma; PPP, platelet-poor plasma; PAF, platelet-activating factor; ADP, adenosine 5′-diphosphate; ETBC, 2-ethyl-1,2,3,4-tetrahydro-β-carboline; HMBC, 3-hydroxymethyl-β-carboline; DMSO, dimethyl sulfoxide; % T, percent light transmission; T_{max}, maximal % T of aggregation response; AUC, area under curve of aggregation response; IC_{50}, concentration to produce 50% inhibition; HP, high polar; MP, moderate polar; LP, low polar; SR, strongly retained; PAR, primary amine removed; SA, small area; LA, large area.

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