

In vitro and *in vivo* anti-allergic activity of soy sauce

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Abstract. Soy sauce (*Shoyu*) is a traditional fermented seasoning of Japan and available throughout the world. Polysaccharides were obtained from dialysate of *Shoyu*, and these *Shoyu* polysaccharides (SPS) were examined for anti-allergic activity *in vitro* and *in vivo*. The SPS originated from partially-degraded polysaccharides of soybeans by mold enzymatic hydrolyses, and *Shoyu* contained about 1% (w/v) SPS. First, the inhibitory effects of SPS on hyaluronidase, which is known to be related to inflammation and allergic responses, were as potent as those of an anti-allergic medicine, disodium cromoglycate. Second, SPS significantly inhibited the release of histamine from rat basophilic leukemia (RBL-2H3) cells, which had been induced by the antigen. Third, orally administered SPS had a significant suppressive effect on passive cutaneous anaphylaxis induced in the ears of mice. These results suggest that SPS may have anti-allergic activities, and soy sauce is a potentially promising seasoning for the treatment of allergic diseases through food.

Introduction

In Japanese soy sauce, soybeans and wheat are the two main raw materials, used in almost the same quantity (1). We previously reported that proteins of the raw materials were completely degraded into peptides and amino acids by microbial proteolytic enzymes after fermentation (2), and no allergen of the raw materials was contained in soy sauce (2-4). In contrast, polysaccharides that originated from the cell wall of soybeans were resistant to the enzymatic hydrolyses, and these polysaccharides were contained in soy sauce even after fermentation (5,6). Although soy sauce has

been traditionally used in Japan and several other oriental countries, many of its ingredients are unknown and the biological activities of soy sauce itself remain to be elucidated. Recently, soy sauce has been reported to have anti-carcinogenic (7,8), anti-microbial (9), anti-oxidative (10-12) and anti-platelet (13) activities, and to inhibit angiotensin I-converting enzyme (14). Further study will be necessary to elucidate the biological functions of soy sauce ingredients.

Allergic responses can be divided into four general categories, based on the mechanism of immunological involvement (15-17). A type I allergy, which causes various symptoms of asthma, rhinitis, atopic dermatitis and anaphylactic shock, is the most common (15-17). The pathological mechanism of the type I allergy has been explained as the degranulation of mast cells and the release of chemical mediators such as histamine, leucotrienes and prostaglandins from these cells (15-17). Mast cell degranulation occurs in response to immunological stimuli in which the antigen-IgE antibody reaction predominates on the cell membrane (15-17).

Hyaluronidase, which is one of the mucopolysaccharide-splitting enzymes, has been implicated in allergic reactions, the migration of cancer cells, inflammation and an increase in the permeability of the vascular system (18-24). Kakegawa *et al* (25-27) have reported a good correlation between the inhibitory effect of hyaluronidase and that of histamine release from mast cells in response to acidic anti-allergic agents. Rat basophilic leukemia (RBL-2H3) cells release histamine when exposed to various stimuli *in vitro* (28,29). RBL-2H3 cells have the same function as mast cells and basophils, which are involved in allergic type I reactions (19-22,28,30-35).

Therefore, in the present study, we examined the effect of soy sauce ingredients on the inhibition of hyaluronidase activity, as well as histamine release in RBL-2H3 cells. Furthermore, we evaluated the anti-allergic effect of orally-administered soy sauce ingredients using a passive cutaneous anaphylaxis (PCA)-reaction induced in the ears of mice, which is one of the animal models for type I allergy (36-40).

Materials and methods

Shoyu polysaccharides (SPS) preparation. Soy sauce (*Shoyu*) was fermented by Higashimaru Shoyu Co. Ltd. (Tatsuno, Hyogo, Japan) as described previously (1,2). *Shoyu*

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Table I. Polysaccharides from soy sauce brewing and their inhibitory effects on the activation of hyaluronidase.

Samples from soy sauce brewing ^a	Polysaccharide content %(w/w) or %(w/v)	Hyaluronidase inhibition IC ₅₀ (mg/ml) ^b
Positive compound ^c		
Disodium cromoglycate		0.20
<i>Koji</i>		
Soybeans + wheat (ethanol precipitation)	2.47% (w/w)	1.26
Soybeans + wheat (dialysate after ethanol precipitation)	0.99% (w/w)	0.39
Soybean hulls (ethanol precipitation)	1.86% (w/w)	0.17
Treated materials		
Wheat (dialysate after ethanol precipitation)	0.29% (w/w)	ND ^d
Soybeans (dialysate after ethanol precipitation)	1.18% (w/w)	ND ^d
Soybean hulls (dialysate after ethanol precipitation)	2.20% (w/w)	ND ^d
Soy sauce (raw <i>Shoyu</i>)		
Soybeans + wheat (dialysate)	1.05% (w/v)	0.30

^aFor details of sample preparation and assay of hyaluronidase inhibition, see Materials and methods. ^bInhibitory concentration of 50% (IC₅₀) was calculated using the mean of 3 observations at each of the 5 concentrations. ^cDisodium cromoglycate was used as an inhibitor of hyaluronidase. ^dND, not detected.

polysaccharides (SPS) were prepared according to the method of Kikuchi and Sugimoto (6). The solid sample was prepared as follows. Heat-treated material (10 g) and the *koji* were extracted with 100 ml of water at 25°C for 30 min. Each extract was centrifuged at 1,300 x g for 10 min to obtain the supernatant, and then 200 ml of ethanol was added to 100 ml of the supernatant. The supernatant was immediately fractionated into ethanol-soluble and ethanol-insoluble fractions after the addition of ethanol. The precipitate of the ethanol-insoluble fraction was recovered by centrifugation at 1,300 x g for 10 min, and dissolved in 10 ml of water. Dissolution (10 ml) in the seamless cellulose tubing (small size 18, pore size: 25Å, molecular weight cut-off: 12,000, Wako Pure Chemical Industries Ltd., Osaka, Japan) was dialyzed overnight in water at 4°C, and then freeze-dried. In contrast, liquid sample (*Shoyu*) was prepared as follows: 10 ml of raw soy sauce in the seamless cellulose tubing (small size 18, Wako Pure Chemical Industries Ltd.) was dialyzed overnight with water at 4°C, and then freeze-dried.

Assay of inhibitory effects on hyaluronidase. The effects of samples on hyaluronidase were determined by a previously-reported procedure (18-24). The test sample, hyaluronic acid potassium salt from cockscomb (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan), hyaluronidase from bovine testes (type IV-S, Sigma Chemical Co., St. Louis, MO, USA), Compound 48/80 (Sigma Chemical Co.), CaCl₂ and NaCl were dissolved in 0.1 M acetate buffer (pH 4.0), respectively.

Hyaluronidase (400 unit/ml), which had been preincubated with the test sample at 37°C for 20 min in advance, was incubated with compound 48/80 (0.1 mg/ml), CaCl₂ (2.5 mM) and NaCl (0.15 M) at 37°C for 20 min (24). After the incubation, hyaluronic acid potassium salt (0.4 mg/ml) was added and the reaction mixture was incubated at 37°C for

40 min (24). Then the reaction was stopped by adding 0.4 M NaOH and the mixture was allowed to stand for 10 min in ice water (24). The inhibitory effect was determined by the modified Morgan-Elson method (24). Test samples were replaced with the buffer solution for the control, while the enzyme solution was replaced with the buffer solution for the blank. Percent inhibition was calculated as follows: Inhibition (%) = [(A-B)-(C-D)]/(A-B) x 100. A, control OD at 585 nm; B, control blank OD at 585 nm; C, sample OD at 585 nm; D, sample blank OD at 585 nm.

The 50% inhibitory concentration (IC₅₀) was calculated using the mean of 3 observations at each of the 5 concentrations. Disodium cromoglycate (DSCG, Biomol Research Laboratories Inc. Plymouth Meeting, PA, USA) was used as an inhibitor of hyaluronidase (18,20-24).

Assay of histamine release from stimulated rat basophilic leukemia cells. The rat basophilic leukemia cell line RBL-2H3 (JCRB0023) was obtained from Health Science Research Resources Bank (Osaka, Japan). Histamine release from RBL-2H3 was assayed according to a previous method (19-22,28,30-35). Briefly, RBL-2H3 cells were cultured at 37°C in an atmosphere containing 5% CO₂, in a medium prepared by supplementing RPMI-1640 medium (Sigma Chemical Co.) with 10% fetal bovine serum (Sigma Chemical Co.). Cells were plated at 5x10⁵ cells/well in 24-well culture plates (0.4 ml/well, Multiwell, 24 well, Becton-Dickinson, Franklin Lakes, NJ, USA) and cultured overnight with anti-dinitrophenyl (DNP) IgE (0.5 µg/ml, Sigma Chemical Co.) at 37°C. The sensitized cells were washed 2 times with PBS, and added to 260 µl of releasing medium containing 117 mM NaCl, 5.4 mM KCl, 2.0 mM CaCl₂, 0.8 mM MgSO₄, 5.6 mM D-glucose, 25 mM HEPES and 1 mg/ml of BSA (pH 7.7). The test sample dissolved in the releasing medium was also

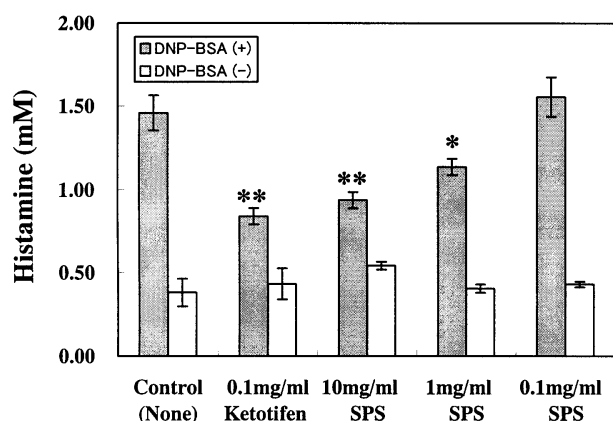


Figure 1. Inhibitory activity of SPS on histamine release from RBL-2H3 cells. For details of the sample preparation and assay of histamine release, see Materials and Methods. SPS were prepared as a dialysate from raw soy sauce, as shown in Table I. Ketotifen fumarate was used as an inhibitor of histamine release. RBL-2H3 cells in 24-well culture plates were cultured overnight with anti-DNP IgE (0.5 µg/ml) at 37°C. The sensitized cells were washed, and added to the releasing medium. The test sample dissolved in the releasing medium was also added to the cell suspension, and then incubated for 10 min at 37°C. After the addition of 20 µl of DNP-BSA (4.0 µg/ml), the stimulated cells were further incubated for 30 min at 37°C. After incubation, the histamine concentration in the supernatant was measured with an ELISA kit. Each value is the average of triplicate cultures, and each bar indicates the mean ± SE (n=3). Significantly different from control at *p<0.05, **p<0.005, respectively (Student's t-test).

added to the cell suspension, and then incubated for 10 min at 37°C. After the addition of 20 µl of DNP-BSA (4.0 µg/ml, Cosmo Bio Co. Ltd., Tokyo, Japan), the stimulated cells were further incubated for 30 min at 37°C. After the incubation, the histamine concentration in the supernatant was measured with an ELISA kit (A05890, SPI-Bio, Bonde, France). Ketotifen fumarate salt (Sigma Chemical Co.) was used as an inhibitor of histamine release from RBL-2H3 cells (22,32,34).

Passive cutaneous anaphylaxis (PCA) reaction. The PCA reaction was evaluated according to a previous method (36-40). Briefly, 7-week-old male BALB/c mice were obtained from CLEA Japan Inc. (Tokyo, Japan) and housed at 25°C in 55% humidity with a 12 h/12 h light-dark cycle (lights on, 7 a.m. to 7 p.m.). After preliminary breeding for 4 days, the mice were divided into 2 groups of 7. The test sample (0.3 mg) was dissolved in 400 µl of water, and filtrated through a sterile membrane (0.45 µm filter unit, Millex-HV, Millipore Co., Bedford, MA, USA). The filtrated sample solution (400 µl) was orally administered using a metal stomach tube for 3 days (test group, 0.3 mg of sample x 3 days). To the control group, distilled water was orally administered in the same manner. Three days after the oral administration, 20 µl of anti-DNP IgE (10 µg/ml, Sigma Chemical Co.) in PBS was injected intradermally into the ears of mice. At 24 h after the sensitization, the mice were tail-intravenously challenged with 100 µl of DNP-BSA (1 mg/ml, Cosmo Bio Co. Ltd.) containing 5 mg/ml of Evans blue dye (Wako Pure Chemical Industries Ltd.). Then, 30 min after the antigen challenge, mice were sacrificed and the ears were removed. Each ear was extracted with 1 ml of 0.5% Na₂SO₄/acetone (3:7) for

24 h, and the OD at 620 nm was measured (36). The amount of extravasated dye was calculated from the standard curve of dye and OD at 620 nm (36).

Results

Inhibitory effect of polysaccharides from soy sauce on activation of hyaluronidase. In Japanese soy sauce (*Shoyu*), soybeans and wheat are the two main raw materials present in almost the same quantities. Briefly, the brewing of soy sauce (*Shoyu*) from raw materials was conducted in the following order: heat treatment of the raw materials, the *kaji* (mold cultivation: enzyme production and enzymatic degradation of materials), the *moromi* (the mixing of the *kaji* with salt water: further enzymatic degradation of materials, lactic acid bacteria and yeast fermentation), and filtration (pressing of the aged *moromi*). Finally, the liquid is raw soy sauce.

First, ethanol-insoluble fractions from the *kaji* were prepared, and examined for inhibitory effects on the activation of hyaluronidase (Table I). The IC₅₀ of DSCG, used as an anti-allergic medicine, was 0.20 mg/ml in this study. The inhibitory effect of the ethanol-insoluble fraction from the *kaji* of soybeans and wheat was 1.26 mg/ml. The ethanol-insoluble fraction of the *kaji* was dialyzed through a membrane having a molecular exclusion limit of 12 kDa, and the inhibitory effect of the residue dialysate (over 12 kDa) was stronger (0.39 mg/ml) than that of the ethanol-insoluble fraction of the *kaji*. These results suggested that the ethanol-insoluble fraction of the *kaji* might include an anti-allergic substance and the substance would be purified by dialysis to enhance its inhibitory effect.

Second, the inhibitory effect of the ethanol-insoluble fraction from the *kaji* of soybean hulls was stronger (0.17 mg/ml) than that of the dialysate from the *kaji* of soybeans and wheat. Generally, soybean hulls are a byproduct of the soybean oil industry, and high levels of pectinaceous polysaccharides are also contained in the ethanol-insoluble fraction of soybean hulls (41). The amount of the anti-allergic substance in the ethanol-insoluble fraction from the *kaji* of soybean hulls was also 2-fold that in the dialysate from the *kaji* of soybeans and wheat. These results suggested that the anti-allergic substance of the *kaji* might be abundant in soybean hulls. However, none of the heat-treated materials, wheat, soybeans or even soybean hulls, had inhibitory effects. Therefore, the formation of an anti-allergic substance while making the *kaji* was required for mold-enzymatic degradation of soybeans.

Third, it was examined whether the anti-allergic substance was present in raw soy sauce after the pressing of the aged *moromi*. The inhibitory effect of the dialysate from raw soy sauce was 0.30 mg/ml, and the content was about 1% (w/v). Kikuchi and Yokotsuka (5) confirmed that the dialysate mainly contained soybean polysaccharides from soy sauce. In this study, the dialysate from raw soy sauce was termed *Shoyu* polysaccharides (SPS). In conclusion, the anti-allergic substances, SPS, were biosynthesized by the *kaji* molds from soybean polysaccharides, and were even stable during the *moromi* stage. Soy sauce contained about 1% (w/v) SPS, the novel anti-allergic substances that had almost the same inhibitory effects on activation of hyaluronidase as DSCG.

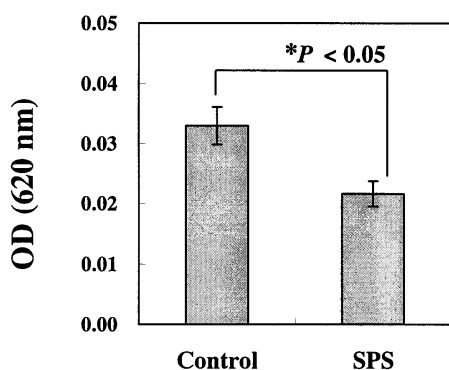


Figure 2. Suppressive effect of SPS on the PCA-reaction induced in the ears of mice. For details of the sample preparation and assay of the PCA reaction, see Materials and methods. SPS were prepared as a dialysate from raw soy sauce, as shown in Table I. SPS were orally administered to BALB/c mice for 3 days (0.3 mg of SPS $\times$ 3 days). The mice were injected intradermally into the ears with 20 μ l of anti-DNP IgE (10 μ g/ml). At 24 h after sensitization, the mice were tail-intravenously challenged with 100 μ l of DNP-BSA (1 mg/ml) containing 5 mg/ml of Evans blue dye. Then, 30 min after the antigen challenge, mice were sacrificed and the ears were removed. The dye was extracted from each ear, and the OD at 620 nm was measured. The amount of extravasated dye was calculated from the standard curve of dye and OD at 620 nm. Each value is the average of triplicate measures, and each bar indicates the mean $\pm$ SE ($n=7$). Significantly different from control at $*p<0.05$ (Student's *t*-test).

Inhibitory activity of SPS on histamine release from sensitized RBL-2H3 cells induced by antigen. The inhibitory effect of SPS on the release of histamine from RBL-2H3 cells was examined further (Fig. 1). A test sample is normally added to releasing medium containing cells challenged with the antigen (DNP-BSA). In this case, it is thought that the test sample has an effect downstream from the antigen-stimulated stage in the cascade of an immediate type of allergic reaction. The inhibitory effect of SPS was concentration-dependent, and the histamine release at 1.0 mg/ml of SPS was significantly decreased compared to the control ($p<0.05$), but no inhibitory effect at 0.1 mg/ml of SPS was observed. In contrast, 0.1 mg/ml of ketotifen, which is an anti-allergic drug and was used as a positive control, significantly inhibited the histamine release ($p<0.005$), but 1.0 mg/ml of ketotifen was severely cytotoxic (data not shown), as described previously (34). No cytotoxicity of SPS was observed between 0.1 mg/ml and 10 mg/ml. Although the inhibitory effect of SPS was estimated to be about one tenth that of ketotifen, the cytotoxicity of SPS was at least 10-fold lower than that of ketotifen. In this study, SPS were only a crude sample from the dialysate from soy sauce, and thus more purified samples will be needed. Furthermore, DSCG, which is a major anti-allergic drug and an effective inhibitor of hyaluronidase, did not show an inhibitory effect on histamine release in the present experimental system (data not shown), as described previously (20,22).

Effect of oral administration of SPS on passive cutaneous anaphylaxis (PCA) in mice. The effect of SPS on the PCA-reaction induced in the ears of mice was examined further (Fig. 2). SPS was orally administered to mice for 3 days (0.3 mg of SPS $\times$ 3 days), and the amount given per day corresponded to about 12 mg/kg because the average weight

of the mice was about 25 g. Compared to the control, SPS had a significantly suppressive effect on PCA activated by anti-DNP IgE when administered orally ($p<0.05$). Since raw soy sauce contained about 1% (w/v) SPS (Table I), the effective dose of SPS for anti-allergic activity was estimated as 1.2 ml/kg per day for soy sauce.

Discussion

Hyaluronidase is a mucopolysaccharide-splitting enzyme, and contributes to the permeability of the vascular system and inflammation (18-24). Also, hyaluronidase may be one of the target enzymes of influent calcium ion in mast cells and may directly control mast cell degranulation (18-24). As a first screening *in vitro*, the inhibitory effects on hyaluronidase can be usually measured to evaluate anti-allergic action (18-24). In previous studies (18-24), extracts of many vegetables, fruits and herbs were evaluated for inhibitory effects, and several anti-allergic constituents were determined. Notably, in 1992, Sawabe *et al* (19) showed that pectic substances such as apple, citrus, etc. inhibited both activated hyaluronidase and histamine release from mast cells. This was the first report on the anti-allergic activity of polysaccharides. In 1997, it was also reported that alginic acids from brown algae had inhibitory effects (21).

In 1972, Kikuchi and Yokotsuka (5) purified polysaccharides from soy sauce, and investigated their properties in detail. Cell wall polysaccharides of soybean, one of the main raw materials of soy sauce, contained a large amount of galacturonic acid and were little hydrolyzed by mold enzymes during the *koji* and *moromi* stages (5,6). Although it is about 30 years since it was reported that polysaccharides like pectic substances are present in soy sauce (5,6), the biological activities of polysaccharides have not been investigated as yet. In this study, we indicated that *Shoyu* polysaccharides, SPS, had a potent inhibitory effect on activation of hyaluronidase the same as an anti-allergic medicine, DSCG (Table I). In contrast to the previously-mentioned pectic substances, interestingly, polysaccharides prepared from treated materials such as soybeans, wheat, and soybean hulls had no inhibitory effect (Table I). Therefore, the anti-allergic SPS originated from partially-degraded polysaccharides of soybeans in the *koji*, and were stable even during the *moromi* stage. Soy sauce contained about 1% (w/v) SPS, the novel anti-allergic substances similar to DSCG (Table I).

Furthermore, we examined the anti-allergic activities of SPS by assaying histamine release from rat basophilic leukemia (RBL-2H3) cells, which had been induced by the antigen (Fig. 1). Kakegawa *et al* (25-27) reported that there was a good correlation between the inhibitory effect of hyaluronidase and that of histamine release from mast cells. Similarly, SPS that inhibited hyaluronidase significantly inhibited histamine release from RBL-2H3 cells. The inhibitory effect of SPS on histamine release was concentration-dependent, and estimated at about one tenth that of ketotifen, an anti-allergic drug (34). However, the cytotoxicity of SPS was at least 10-fold lower than that of ketotifen. Therefore, SPS from soy sauce would be safe and is expected to act as an anti-allergic seasoning for foods.

Finally, we evaluated the anti-allergic effect of orally administered SPS using a PCA-reaction induced in the ears of mice, one of the animal models for a type I allergy (Fig. 2). Despite the fact that many anti-allergic substances showed both inhibition of hyaluronidase and histamine release from basophils or mast cells *in vitro* (19-22), no study has been reported on the effects on the PCA reaction *in vivo*. SPS had a significant suppressive effect on the PCA reaction when 12 mg/kg per day was administered orally for 3 days. The effective concentration of SPS was lower than that of anti-allergic substances described previously (37-40). For example, the minimum effective oral doses to suppress the PCA reaction were reported as follows: 100 mg/kg of a persimmon leaf extract (37), 125 mg/kg of a herb *Perilla frutescens* extract (38,39), and 100 mg/kg of a buckwheat grain extract (40), respectively. In contrast, 12 mg/kg of SPS per day corresponded to only about 1 ml/kg per day of raw soy sauce. The daily consumption of soy sauce in Japan is estimated at about 30 ml per person according to data from the Japan Soy Sauce Brewers Association (1988). Therefore, soy sauce is useful as an anti-allergic therapy in normal daily life.

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