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Lactic Acid Bacteria Increase Antiallergic Effect of *Artemisia princeps* Pampanini SS-1

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Artemisia princeps Pampanini, which is called Ssajuarissuk in Korean (SS-1), was fermented with lactic acid bacteria (LAB) and their passive cutaneous anaphylaxis reaction-inhibitory activity was investigated. Of these fermented agents, SS-1 extract fermented with *Bifidobacterium infantis* K-525 (F-SS-1) most effectively inhibited the release of β-hexosamindase from RBL-2H3 cells induced IgE. In IgE-induced RBL-2H3 cells, F-SS-1 inhibited proinflammatory cytokines IL-6 and TNF-α mRNA expression. Oral administration of SS-1 and F-SS-1 to mice inhibited passive cutaneous anaphylaxis (PCA) reaction induced by IgE and scratching behaviors induced by compound 48/80. The inhibitory activity of F-SS-1 against scratching behaviors was more effective than that of SS-1. These findings suggest that the fermentation of SS-1 with LAB can increase its antiallergic activity.

Key words: *Artemisia princeps* SS-1, Ssajuarissuk, Antiallergic effect, Passive cutaneous anaphylaxis reaction, Fermentation

INTRODUCTION

Artemisia princeps Pampanini (Family Asteraceae), which contain eupatilin, acacetin, and eudesmane as main components (Ryu *et al.*, 2005), has long been used for the treatment of inflammation, diarrhea, gastric ulcer, and many circulatory disorders (Kim *et al.*, 1997). *Artemisia princeps*, which is called Ssajuarissuk in Korea (SS-1), cultivated in Ganghwado contains a high content of eupatilin, compared to plants from other places, such as China (Ryu *et al.*, 2005). Recently Shin *et al.* (2006) reported that, although SS-1 showed weak inhibitory effect against passive cutaneous anaphylaxis (PCA) reaction, these extracts did not inhibit the scratching behaviors stimulated by compound 48/80.

In addition, most herbal medicines contain bioactive secondary metabolites which are modified, such as sugars (Kobashi and Akao, 1997; Kim, 2002). To express their pharmacological activities, the secondary metabolites must be transformed. Therefore, we screened whether lactic acid bacteria (LAB) could transform these secondary metabolites, such as glycosides of ginsenoside Rb1 of ginseng, and increase the pharmacological activity of herbal medicines such as ginseng (Bae *et al.*, 2000).

In this study to investigate whether LAB could increase antiallerigic activity of SS-1, we fermented *Artemisia princes* SS-1 with some lactic acid bacteria isolated from human feces and investigated their PCA reaction and scratching behavior-inhibitory effects.

MATERIALS AND METHODS

Materials

Betamethasone compound 48/80, egg albumin, p-nitrophenyl-N-acetyl-β-D-glucosaminide, anti-dinitrophenol (DNP)-IgE, DNP-human serum albumin (HSA), and Evans blue were purchased from Sigma Co. (U.S.A). The RBL-2H3 cells were obtained from the Korean Cell line Bank (Korea). The intestinal LAB isolated in our previous study were used (Bae *et al.*, 2000).

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Extraction of Artemisia princeps SS-1

Artemisia princeps SS-1, which is cultivated in Ganghwado and brewed for 2 year, was extracted with 80% ethanol and then concentrated using a vaccum. The extract (0.5 g) was suspended in 100 mL of water and incubated for 24 h at 37°C with each lactic acid bacterium (0.2 g as dry weight). The same volume of ethanol was added in the fermented mixture, centrifuged, and then concentrated using a vaccum. These were used as fermented agents.

Assay of inhibitory activity against β-hexosaminidase release of RBL-2H3 cells

The inhibitory activity of test samples against the release of β-hexosaminidase from RBL-2H3 cells was evaluated according to Choo *et al.* (2003). RBL-2H3 cells were grown in Dulbecco's modified Eagle Medium supplemented with 10% fetal bovine serum and L-glutamine. Before the experiment, cells were dispensed into 24-well plates at a concentration of 5×10^5 cells per well. Using a medium containing 0.5 μg/mL of mouse monoclonal IgE, the cells were sensitized by incubation overnight at 37°C in 5% CO_2. They were then washed with 500 μL of Siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH) and incubated in 160 μL of Siraganian buffer containing 5.6 mM glucose, 1 mM $CaCl_2$ and 0.1% BSA for an additional 10 min at 37°C. Then cells were exposed to 40 μL of test agents for 20 min, treated with 20 μL of antigen (DNP-HSA, 1 μg/mL) for 10 min at 37°C to activate the cells and to evoke allergic reactions. The reaction was stopped by cooling in an ice bath for 10 min. The reaction mixture was centrifuged at 2000 rpm for 10 min and 25 μL aliquots of the supernatant were transferred into 96 well plates and incubated with 25 μL of substrate (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide) for 1 h at 37°C. The reaction was stopped by adding 200 μL of 0.1 M Na_2CO_3/$NaHCO_3$. Absorbance at 405 nm was measured by using an ELISA reader.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR analysis for the RBL-2H3 cells was performed using the modified method of Shin *et al.* (2005). Briefly, the total RNA from the collected RBL-2H3 cells was extracted by using RNeasy® Minikit (Qiagen, Germany) according to the manufacturer's instructions, and treated with RNase-free DNase. The concentration of RNA content was determined by measuring the absorbance at 260 and 280 nm and stored at -70°C until the RT-PCR analysis. The RT-PCR analysis was performed with AccPower® RT/PCR Premix (Bioneer, Seoul, Korea). The primers were designed as described in the following: IL-4, forward primer 5'-ACCTTGCTGTCACCCTGTTCTGC-3' and reverse primer 5'-GTTGTGAGCGTGGACTCATTCACCG-3' (product size 352 bp); IL-6, forward primer 5'-CTCCGCAAGAGACTTCCAGC-3 and reverse primer 5'-ACTCCAGGTAGAAACGGAACT-3' (product size 365 bp); tumor necrosis factor (TNF)-α, forward primer 5'-CGGAATTCGGCTCCCTCTCATCAGTTC-3' and reverse primer 5'-GCTCTAGACCCTTGAAGAGAACCTGGGA-3' (product size 238 bp); GAPDH, forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCCTGTTGCTGTA-3' (product size 452 bp). The amplification was performed at 94°C for 30-60 s, and 49-62°C for 30-40 s, and 72°C for 30-60 s with 30 cycles for IL-4, IL-6, TNF-α and GAPDH in a 20 μL reaction mixture. The RT-PCR products were electrophoresed on 2% agarose gel in a TBE buffer, stained with ethidium bromide and photographed under UV light. The GAPDH gene was used as an internal control. The signal intensity of each RT-PCR product was estimated using a Shimazu 9301-PC scanner (Shimazu Co., Tokyo, Japan).

Animals

Male and female ICR mice (20-22 g) and male BALB/c mice (18-22 g) were supplied from Charles River Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages, at a temperature of 20-22°C, with a relative humidity of 50 ± 10%, a frequency of air ventilation of 15-20 times/h, a 12 h illumination (07:00-19:00; intensity, 150-300 Lux), were fed standard laboratory chow (Charles River Orient Experimental Animal Breeding Center, Seoul Korea) and allowed water ad libitum. All procedures relating to the animals and their care conformed to the international guidelines 'Principles of Laboratory Animals Care' (NIH publication no. 85-23, revised 1985).

Passive cutaneous anaphylaxis (PCA) reaction

An IgE-dependent cutaneous reaction was measured according to the previous method of Choo *et al.* (2003). The male ICR mice were injected intradermally with 10 μg of anti-DNP IgE into each of two dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later each mouse received an injection of 200 μL of 3% Evans blue PBS containing 200 μg of DNP-HSA *via* the tail vein. The test agents were orally or intraperitoneally administered 1 h prior to DNP-HSA injection. Thirty minutes after the DNP-HSA injection, the mice were sacrificed and their dorsal skins were removed for measurement of the pigment area. After extraction with 1 mL of 1.0 N KOH and 4 mL of a mixture of acetone and 0.6 N phosphoric acid (13:5), the amount of dye was determined colorimetrically (the absorbance at 620 nm).

Scratching behavioral experiments

The male BALB/c mice were put into acrylic cages (22 × 22 × 24 cm) for about 10 min for acclimation. The behavioral experiments were performed according to the method of Sugimoto *et al.* (1998). The rostral part of the skin on the back of the mice was clipped, and 50 μg/50 μL of compound 48/80 for each mouse was intradermally injected with the use a 29 gauge needle. The scratching agent was dissolved in saline and then used. Control mice received a saline injection in the place of the scratching agent. Immediately after the intradermal injection, the mice (one animal/cage) were put back into the same cage. The observation of scratching behaviors was recorded using an 8-mm video camera (SV-K80, Samsung, Seoul, Korea) under unmanned conditions. Scratching of the injected site by the hind paws was counted and compared with that of other sites, such as the ears. Each mouse was used for only one experiment. The mice generally showed several scratches for 1 s, and a series of these behaviors was counted as one incident of scratching over 60 min. The samples were orally administered 1 h before the scratching agent.

Statistics

All the data were expressed as the mean ± the standard deviation, and statistical significance was analyzed using a one way ANOVA followed by a Student-Newman-Keuls test.

RESULTS

Inhibitory activity of SS-1 in β-hexosaminidase release of RBL-2H3 cells

To evaluate whether LAB can increase antiallergic activity of SS-1, we fermented SS-1 with some LAB isolated from human feces and investigated their inhibitory activity against β-hexosaminidase release of RBL-2H3 cells induced by IgE (Table I). SS-1 fermented with and without LAB all inhibited the degranulation of RBL-2H3 cells. Fermented SS-1 inhibited degranulation more strongly than SS-1.

Table I. Inhibitory activity of SS-1 extracts with and without lactic acid bacteria fermentation in β-hexosaminidase release of RBL-2H3 cells

Fermented lactic acid bacteria	Inhibition (%)
None	29±2
Bifidobacterium magnum K-321	44±1
Bifidobacterium minimum K-506	41±2
Bifidobacterium infantis K-525	46±3
Lactobacillus breve II-46	39±4
Streptococcus faecium	35±2

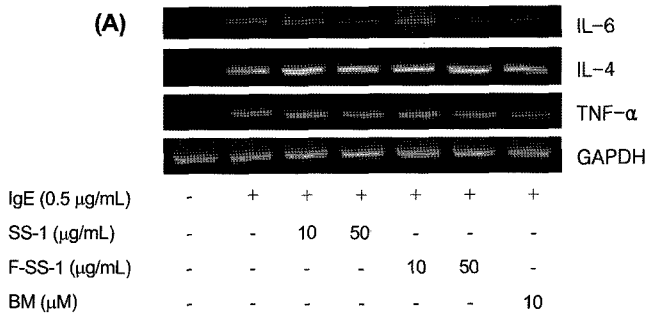
Final concentration of each extract of *Artemisia princeps* was 50 μg/mL. All values are means ± S.D. (n=3).

Among LAB, *B. infantis* K-525 most strongly increased the inhibitory effect.

We also investigated the inhibitory activity of SS-1 fermented with (F-SS-1) and without *B. infantis* K-525 (SS-1) against mRNA expression levels of cytokines IL-4, IL-6 and TNF-α in RBL 2H3 cells induced by IgE using an RT-PCR analysis (Fig. 1). IgE significantly induced mRNA levels of these cytokines. However, SS-1 and F-SS-1 inhibited the mRNA expression level of IL-6 and TNF-α induced by IgE. At a concentration of 50 μg/mL, F-SS-1 inhibited levels of IL-6 and TNF-α by 76% and 21%, respectively. However, it scarcely inhibit that of IL-4. F-SS-1 inhibited proinflammaotry cytokines IL-6 and TNF-α mRNA expression more strongly than SS-1.

Inhibitory activity of SS-1 on PCA reaction

To confirm *in vivo* anti-degranulation activity, we measured the inhibitory activity of SS-1 and F-SS-1 on the



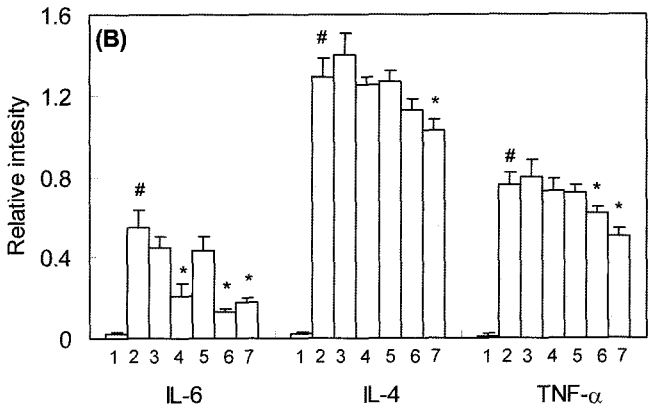


Fig. 1. Effect of SS-1 with and without fermentation on some cytokine mRNA expression levels of RBL-2H3 cells stimulated by IgE. RBL-2H3 cells (5×10^5 cells) were treated with 0.5 μg/mL of mouse monoclonal IgE, and exposed to 0.2 mL of agents for 20 min, followed by a treatment with 0.2 mL of dinitrophenol-human serum albumin (1 μg/mL) for 40 min at 37°C. Then RT-PCR for IL-4, IL-6, TNF-α and GAPDH were performed. SS-1, *Artemisia princeps* called Ssajuarissuk in Korea; F-SS-1, SS-1 fermented with *B. infantis* K-525; and BM, betamethasone. (A) RT-PCR of some cytokines; (B), Relative intensities of RT-PCR (IL-6, IL-4, TNF-α/GAPDH). All values are means ± S.D. (n=2). #Significantly different from normal group (#$P<0.05$). *Significantly different from control group (*$p<0.05$).

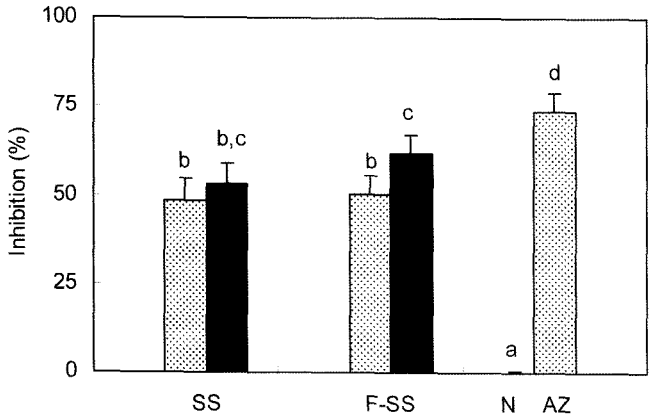


Fig. 2. Inhibitory effect of SS-1 with and without fermentation on IgE-induced passive cutaneous anaphylaxis in mice. The SS-1 was extracted with 80% ethanol and used as a SS-1 extract (SS). The extract was fermented with *B. infantis* K-525 for 24 h at 37°C, concentrated and used as fermented SS-1 extract (F-SS). The positive agent was orally administered 10 mg/kg of azelastine (AZ). Each extract (20 mg/kg-dotted bar and 50 mg/kg-black bar) was orally administered 1 h prior to DNP-HSA injection. The normal group (N) was treated with the vehicle alone. All values are means ± S.D. (n=5). ^{a,b,c,d} Items with the same letter are not significantly different.

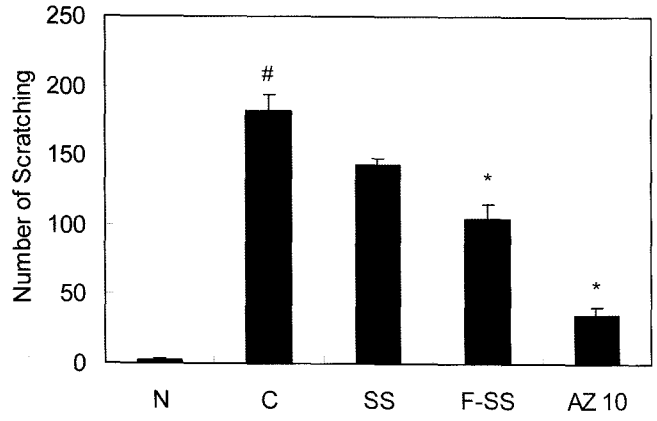


Fig. 3. Inhibitory effect of SS-1 with and without fermentation on compound 48/80-induced scratching behaviors in mice. The SS-1 was extracted with 80% ethanol and used as a SS-1 extract (SS). The extract was fermented with *B. infantis* K-525 for 24 h at 37°C, concentrated and used as a fermented SS-1 extract (F-SS). Each extract (50 mg/kg) was orally administered. The positive agent was orally administered 10 mg/kg of azelastine (AZ). The scratching agent compound 48/80 (50 g/50 l) for each mouse was intradermally injected 1 h after the administration of test agents. The normal group (N) was treated with the vehicle (saline) alone and the control group (C) was treated with compound 48/80 and the vehicle. All values are means ± the S.D. (n=5). #Significantly different from the normal control group ($^{\#}P<0.05$). *Significantly different from the compound 48/80 alone stimulated (control) group (*$p<0.05$).

mouse passive cutaneous anaphylaxis reaction induced by IgE (Fig. 2). Both SS-1 and F-SS-1 inhibited the PCA reaction induced by IgE. However, the PCA-inhibitory activity of SS-1 was not affected by LAB fermentation.

Inhibitory effect of SS-1 and F-SS-1 on scratching behaviors induced by compound 48/80

We investigated the inhibitory activity of SS-1 and F-SS-1 using a compound 48/80-induced scratching behavior animal model (Fig. 3). Both SS-1 and F-SS-1 extracts at a dose of 50 mg/kg inhibited the scratching frequency by 21% and 43%, respectively. These extracts at a dose of 50 mg/kg also decreased the vascular permeability of skin induced by the compound 48/80 (data not shown). The inhibitory activity of these extracts against vascular permeability was proportional to their inhibitions against scratching behaviors.

DISCUSSION

Allergic reactions including rhinitis, asthma and anaphylaxis produced many inflammatory mediators and caused scratching, inflammation, pain and increase of vascular permeability (Bielory, 2004; Plaut *et al.*, 1989; Stevens and Austen, 1989; Wuthrich, 1989;). Anti-histamines, steroids, and immunosuppressants not only have potent anti-inflammatory effects, but also cause intense side reactions (Schafer-Korting *et al.*, 1996; Sakuma *et al.*, 2001; Simons, 1992; Friedman *et al.*, 2002). Therefore, herbal medicines have been advanced methods for treating allergic diseases, and their effectiveness has received increasing attention. SS-1 cultivated in Ganghwado contains a high content of euphatilin compared to those produced in other places (Ryu *et al.*, 2005) and exhibited potent anti-inflammatory and antiallerigc activities. The pharmacological activity of many herbal medicines was increased by using intestinal bacteria (Kobashi and Akao, 1997; Kim, 2002). Therefore, we investigated whether LAB could increase antiallerigic activity of SS-1. First, we evaluate whether LAB can increase inhibitory activity of SS-1 against degranulation of RBL-2H3 cells and their cytokine biosynthesis. SS-1 inhibited the release of β-hexosamindase from RBL-2H3 cells induced by IgE. The SS-1 also inhibited the biosynthesis of cytokines IL-6 and TNF-α of RBL2H3 cells induced by IgE. The SS-1 also inhibited the PCA reaction as well as scratching behaviors induced by compound 48/80. These effects of SS-1, except for the inhibition of the PCA reaction, were increased by fermentation of LAB. F-SS-1, which is SS-1 fermented with *B. infantis* K-525, showed more potent antidegranulation and anti-scratching behavior activities than SS-1, although the extract of *B. infantis* K-525 alone did not show these inhibitions. Based on these findings, the antiallergic activity of SS-1 can be increased by LAB fermentation.

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