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Lactic Acid Bacterial Fermentation Increases the Antiallergic Effects of *Ixeris dentata*

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***Ixeris dentata* (ID, family Asteraceae), called Seumbakuy in Korea, was fermented with lactic acid bacteria (LAB) and their antiallergic activities were investigated. Fermentation of ID with *Bifidobacterium breve* or *Lactobacillus acidophilus* increased its inhibition of degranulation in RBL-2H3 cells induced by the IgE-antigen complex. Oral administration of these extracts to mice inhibited the passive cutaneous anaphylaxis (PCA) reaction induced by the IgE-antigen complex and scratching behaviors induced by compound 48/80. The fermented ID more potently inhibited the PCA reaction and scratching behaviors than the non-fermented one. These extracts also inhibited mRNA expression of TNF-α and IL-4, as well as NF-κB activation in RBL-2H3 cells induced by the IgE-antigen complex. These findings suggest that LAB fermentation improves ID-mediated inhibition of IgE-induced allergic diseases such as rhinitis and asthma, and that ID works by inhibiting degranulation and NF-κB activation in mast cells and basophils.**

Keywords: *Ixeris dentata*, antiallergic effect, passive cutaneous anaphylaxis reaction, itching, lactic acid bacteria

Fermentation produces beneficial products for humans, often *via* manufacturing on an industrial scale. These processes are performed by lactic acid bacteria (LAB), such as *Bifidobacterium* sp. and *Lactobacillus* sp., and some molds, such as *Saccharomyces* sp. [17, 23, 27]. These microbes transform foods and convert sugars to alcohol and lactic acid. For example, LAB fermentation of ginseng produces lactic acid and compound K, a potent cytotoxic antitumor agent that is transformed from the ginsenosides Rb1, Rb2, and Rc [1, 11, 13].

Ixeris dentata (ID, family Asteraceae) is a herbal medicine used in Korea, China, and Japan for indigestion, pneumonia, hepatitis contusion, and tumors [25]. Fermented ID, as in *kimchi*, is frequently eaten in Korea. ID is neuroprotective against oxidative stress induced by kainic acid in mouse brain [18]. ID also inhibits the anaphylactic reaction induced by IgE or compound 48/80 [30], and ID increased the production of NO and TNF-α by rIFN-γ-primed macrophages [5]. The pharmacological effects, such as antiallergic activity, of fermented ID (FID) have not been thoroughly studied.

Mast cells and basophils are critical participants in allergic diseases [26]. These cells express surface membrane receptors with high affinity and specificity for IgE, which is induced by IL-4 [16]. The interaction of antigen-bound IgE with surface membrane receptors causes the release of histamine, prostaglandins, leukotrienes, and cytokines [16]. These cytokine-induced reactions cause tissue inflammation, anaphylaxis, and scratching behaviors. These allergic diseases are increasing chronic health problems in most countries [29].

Here, we investigated the inhibitory effects of ID fermented by lactic acid bacteria against allergic models, such as passive cutaneous anaphylaxis and scratching behaviors.

Materials and Methods

Materials

Betamethasone, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), LPS, 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 Griess reagent was purchased from Promega Co. (U.S.A.).

Extraction of ID and FID

ID (1 kg), artificially cultured in Yang-Yang, Kangwondo, Korea, was extracted with 5 l of boiling water for 2 h, and then concentrated under vacuum (Yields: water extract, 5.2%). These water extracts (20 g) were suspended in 1 l of water and incubated

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for 24 h at 37°C with *Bifidobacterium breve* K-110 (BB) isolated from human intestinal microflora [19] or *Lactobacillus acidophilus* KCTC3168 (LA) (2 g as dry weight), extracted with ethyl acetate, and concentrated under vacuum. These extracts were used as the fermented ID (FID) agent [yield: nontreated, 4.3%; BB-treated (BB-FID), 37%; LA-treated (LA-FID), 29%].

Inhibition of β-Hexosaminidase Release of RBL-2H3 Cells

The inhibitory activity of test agents against the release of β-hexosaminidase from RBL-2H3 cells was evaluated according to Choo *et al.* [4]. RBL-2H3 cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum and L-glutamine. Cells were dispensed into 24-well plates at 5×10^5 cells per well in medium containing 0.5 μg/ml of mouse monoclonal IgE, and 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. The cells were exposed to 40 μl of test agents for 20 min, and then treated with 20 μl of antigen (DNP-HSA, 1 μg/ml) for 10 min at 37°C to activate 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 2,000 ×*g* for 10 min, and then 25-μl aliquots of the supernatant were transferred to 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 was measured by using an ELISA reader at 405 nm.

Assay of NO Production in LPS-induced RAW264.7 Cells

RAW 264.7 cells were seeded at 5×10^4 cells per well in flat-bottomed 96-well plates. LPS (1 μg/ml) and test agents were added to the culture medium and incubated at 37°C for 16 h, briefly centrifuged, and then 150 ml of cell culture supernatant was mixed with 150 ml of Griess reagent and incubated for 10 min at room temperature (light protected). The absorbance was measured using an ELISA reader at 540 nm and compared with a standard calibration curve prepared from sodium nitrite [4].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For isolation of mRNA from RBL-2H3 cells, cultured cells were immediately placed in liquid nitrogen and pulverized in a mortar. mRNA was extract from the pulverized tissue by using TRI reagent (Cincinnati, OH, U.S.A.) according to the manufacturer's instructions. The respective primer sets were prepared according to the method of Shin *et al.* [22]: TNF-α, forward primer 5'-GATTTTATTTGT-TTAAAAGCAGATATC-3' and reverse primer 5'-CATCCTAA-GTCTACACAGGATCT-3' (size 206 bp); IL-4, forward primer 5'-CCGATTATGGTGTAATTTCCTATGCTG-3' and reverse primer 5'-GGCCAAGTCTACACAGGATCT-3' (size 11 bp); GAPDH, forward primer ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCCTGTTGCTGTA-3' (size 452 bp). RT-PCR was performed with AccPower RT/PCR Premix (Bioneer, Seoul, Korea). Optimization of cycle number was performed to ensure that production accumulation was in the linear range. Amplified products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide. The gels were photographed under UV light. The GAPDH gene was used as an internal control.

Immunoblot

RBL-2H3 cells (5×10^5 cells), previously cultured in DMEM, were treated with 0.5 μg/ml mouse monoclonal IgE for cell sensitization. The cells (1.8 ml) were exposed to 0.2 ml of the test agents (dissolved in 0.5% dimethyl sulfoxide) for 4 h, followed by treatment with 0.2 ml of DNP-HSA (1 μg/ml) for 40 min at 37°C. The supernatant (50 μl) was transferred into 96-well ELISA plates, and the IL-4 and TNF-α concentrations were then determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, U.S.A.) [14].

Collected cells (3×10^6 cells) for immunoblot assay were lysed on ice for 15 min in a hypotonic buffer containing 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin, and centrifuged at 12,000 ×*g* at 4°C for 15 min. The supernatant was used as the cytosolic fraction for the IκBα immunoblot assay. The pelleted nuclei fraction for the NF-κB immunblot assay was resuspended in extraction buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 300 mM NaCl, 1 mM DTT, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin, and then lysed on ice for 30 min. The lysed nuclei fraction was centrifuged at 12,000 ×*g* and 4°C for 30 min. Cell lysates (40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Hertfordshire, U.K.) at 30 V for 2 h. The membranes were blocked with 2% skim milk in PBS, containing 0.05% tween 20, and incubated for 2 h at room temperature. The cytosolic IκBα and nucleic p65 NF-κB were assayed using their corresponding antibodies, according to a previously reported method [7, 31]. Immunodetection was carried out using an enhanced chemiluminescence detection kit.

Animals

Male and female ICR mice (20–22 g, 5 weeks old) and male BALB/c mice (18–22 g, 5 weeks old) were supplied from the Charles River Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20–22°C, under a relative humidity of 50%±10%, a frequency of air ventilation of 15–20 times/h, and 12 h illumination (07:00–19:00; intensity, 150–300 Lux), 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).

PCA Reaction

An IgE-dependent cutaneous reaction was measured according to the method of Choo *et al.* [4]. Each group consisted of six male ICR mice. The 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 test compound (either orally or intraperitoneally), followed 1 h later by injection of 200 μl of 3% Evans blue in phosphate-buffered saline, containing 200 μg of DNP-HSA, *via* the tail vein. Thirty min after DNP-HSA injection, the mice were sacrificed and their dorsal skin was 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 (absorbance at 620 nm).

Scratching Behavior Experiments

Before the experiment, male BALB/c mice were put into acrylic cages (22×22×24 cm) for about 10 min for acclimation. Each group consisted of six male ICR mice. The behavioral experiments were performed according to the method of Sugimoto *et al.* [28]. The rostral part of the skin on the back was clipped, and 50 μg/50 μl of compound 48/80 was intradermally injected with a 29-gauge needle. Compound 48/80 was dissolved in saline. Control mice received a saline injection instead of the scratching agent. Immediately after intradermal injection, the mice (one animal/cage) were put back into the same cage and their behaviors recorded for the observation of scratching using an 8-mm video camera (SV-K80; Samsung, Seoul, Korea) under automated 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.

Statistics

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

Results

ID and FID Inhibit Degranulation of RBL-2H3 Cells

We investigated the ability of ID, BB-FID, and LA-FID to inhibit the degranulation of mast cells and basophils induced by IgE in RBL-2H3 cells (Fig. 1). ID, BB-FID, and LA-FID all inhibited β-hexosaminidase release (degranulation), with IC_{50} values of 113.9 μg/ml, 18.5 μg/ml, and 25.2 μg/ml, respectively. Even at 200 μg/ml, these agents did not show cytotoxicity under the same conditions.

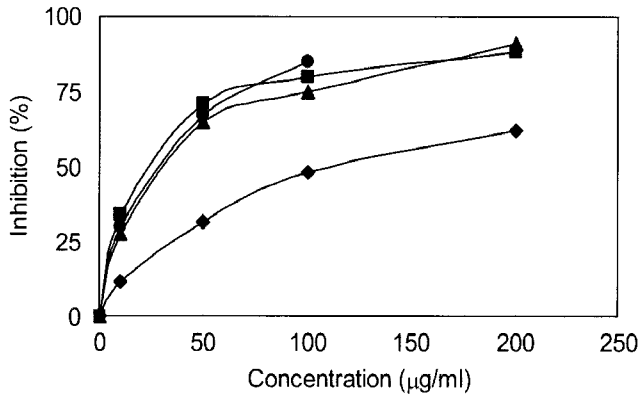

Fig. 1. ID, FID, and azelastine inhibit degranulation of RBL-2H3 cells induced by the IgE-antigen complex.

RBL-2H3 cells, which were grown in DMEM supplemented with 10% fetal bovine serum and L-glutamine, were dispensed into 24-well plates, at 5×10^5 cells per well, and sensitized using 0.5 μg/ml of mouse monoclonal IgE. They were then washed with 500 μl of Siraganian buffer, exposed to 40 μl of various concentrations of ID (◆), BB-FID (■), LA-FID (▲), and azelastine (●) for 20 min, and treated with 20 μl of antigen (DNP-HSA, 1 μg/ml) for 10 min at 37°C. The release of β-hexosaminidase from RBL-2H3 cells was measured. Inhibition values indicate the mean±SD (n=3).

Table 1. ID and BB-FID inhibit the mouse PCA reaction induced by the IgE-antigen complex and scratching behaviors induced by compound 48/80.

Agent	Dose (mg/kg)	Inhibition (%)	
		PCA reaction[a]	Scratching behavior[b]
ID	50	20±3[c]	18±8[c]
	100	28±5[c]	20±5[c]
FID	50	52±7[d]	51±6[d]
	100	61±8[d]	63±7[d,e]
Azelastine	10	81±3[e]	73±4[e]

[a]The amounts of Evans blue extravasated from the dorsal skin (1×1 cm) of the control stimulated with the IgE-antigen complex and vehicle-treated groups were 24±4 μg and 11±3 μg, respectively.

[b]Scratching behaviors of the normal control, which was treated with saline alone, and the control group, which was treated with compound 48/80 and saline instead of test agents, was counted for 1 h. The numbers of scratching behaviors in the normal and control groups were 3±2 and 245±23, respectively.

[c,d,e]Items with the same letter in each column were not significantly different.

Inhibition values indicate the mean±SD (n=6).

ID and BB-FID Inhibit PCA and Scratching Behavior Reactions in Mice

We next measured the ability of ID and BB-FID (the most potent inhibitor of degranulation) to inhibit the mouse PCA reaction induced by the IgE-antigen complex as a measure of *in vivo* antiallergic activity (Table 1). ID and BB-FID (100 mg/kg) inhibited the PCA reaction by 28% and 61%, respectively. ID and BB-FID (100 mg/kg) also significantly inhibited the scratching frequency induced by compound 48/80 by 20% and 63%, respectively. These extracts also decreased the skin vascular permeability induced by compound 48/80 (data not shown), in proportion to their inhibition of scratching behavior.

ID and BB-FID Inhibit Cytokine Production in RBL-2H3 Cells

We next measured whether ID and BB-FID inhibited the expression of TNF-α and IL-4 protein in RBL-2H3 cells stimulated with the IgE-antigen complex in an ELISA assay (Fig. 2). ID and BB-FID (50 μg/ml) inhibited TNF-α by 37% and 49% and IL-4 expression by 13% and 25%, respectively. ID and BB-FID also inhibited mRNA expression of these cytokines in RBL-2H3 cells stimulated with the IgE-antigen complex (Fig. 2). BB-FID inhibited protein expression more potently, but only barely inhibited IL-4 mRNA expression.

Effect of ID and FID on Activation of Transcription Factor NF-κB

We next determined the effects of ID and BB-FID on the activation of NF-κB in RBL-2H3 cells (Fig. 3). Treatment

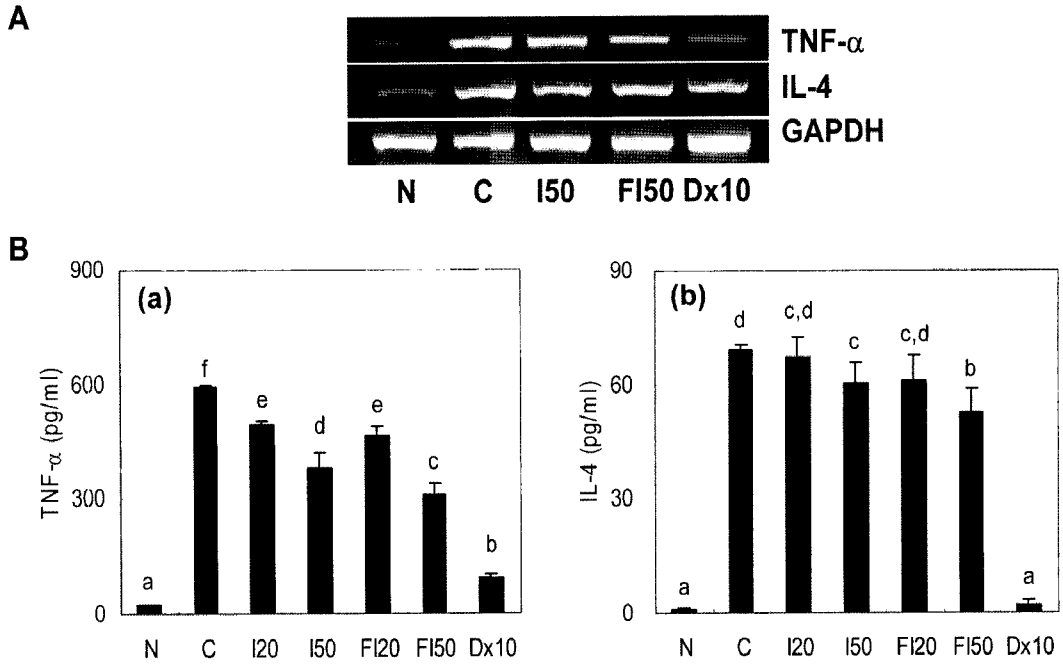

Fig. 2. The effects of ID and BB-FID on the mRNA and protein expression of TNF-α and IL-4 in RBL-2H3 cells induced by the IgE-antigen complex.

RBL-2H3 cells (5×10^5 cells) were treated with 0.5 μg/ml of mouse monoclonal IgE for 1 h, and then exposed to 0.2 ml of the test agents (N, normal; C, vehicle alone; I20, 20 μg/ml ID; I50, 50 μg/ml ID; FI20, 20 μg/ml FID; FI50, 50 μg/ml FID; Dx10, 10 μM dexamethasone) for 20 min, followed by treatment with 0.2 ml dinitrophenol-human serum albumin (DNP-HSA, 1 μg/ml) for 1 h (RT-PCR) or 4 h (for ELISA) at 37°C, and then RT-PCR (**A**) and ELISA (**B**) for TNF-α (a) and IL-4 (b) were performed. Normal (N) was treated with vehicle alone, control group (C) was treated with the vehicle and IgE-antigen complex. Values represent the mean±SD for duplicate experiments. Inhibition values indicate the mean±SD (n=3). ^{a,b,c,d,e,f}Items with the same letter in each figure were not significantly different ($P<0.05$).

with the IgE-antigen complex activated NF-κB in the nuclei fraction, and decreased IκB. Treatment with ID or BB-FID attenuated the NF-κB activation induced by the IgE-antigen complex. The inhibitory effect of BB-FID was slightly more potent than that of ID.

ID and BB-FID Inhibit LPS-stimulated NO Production in RAW264.7 Cells

Chronic allergic diseases cause macrophage activation to produce NO and proinflammatory cytokines [5]. Therefore, we tested whether ID or BB-FID inhibited LPS-induced NO production in RAW264.7 cells (Fig. 4). ID and BB-FID potently inhibited NO production, with IC_{50} values of 30.9 μg/ml and 7.1 μg/ml, respectively.

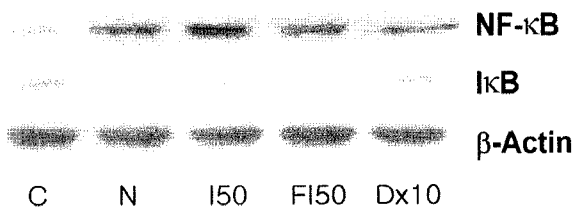

Fig. 3. ID and BB-FID inhibit NF-κB activation.

RBL-2H3 cells were stimulated by IgE for 1 h and then incubated overnight, treated with 50 μg/ml of ID (I50) and FID (FI50), 10 μM dexamethasone (Dx) or vehicle alone (C) for 30 min, and then stimulated with DNP-HSA. The normal control (N) was treated with vehicle alone, which did not contain IgE or test agents. The cells were collected 1 h after antigen treatment. The cell extract was analyzed for IκBα and β-actin in the cytosolic fraction and NF-κB in the nuclei fraction using immunoblotting.

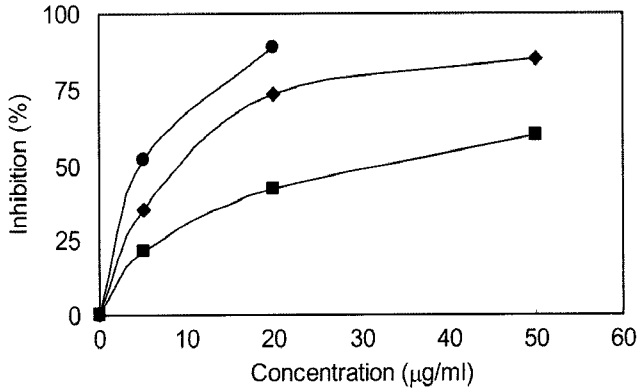

Fig. 4. Effects of ID and BB-FID on LPS-stimulated NO synthesis in RAW 264.7 cells.

Nitric oxide levels were determined by measuring the amount of nitrite in cell culture supernatant using Griess reagent, according to the manufacturer's protocol. RAW 264.7 (mouse macrophage leukemia) cells were stimulated with lipopolysaccharide (LPS, 1 μg/ml) and the test agents (■, ID; ◆, FID; ●, Dx) for 24 h. The cells were briefly centrifuged, and 150 μl of cell culture supernatant was then mixed with 150 μl of Griess reagent. Incubation was conducted for 10 min at room temperature (light protected), and absorbance was measured using an ELISA reader at 540 nm using a sodium nitrate determined calibration curve as a standard. Inhibition values indicate the mean±SD (n=3).

Discussion

Allergic reactions, including rhinitis, asthma, and anaphylaxis, produce inflammatory mediators and cause scratching, inflammation, pain, and increased vascular permeability [26, 29]. Antihistamines, NSAIDs, steroids, and immunosuppressants can treat these allergic diseases [6, 20, 21, 24], but their repeated application causes side effects [21]. Herbal medicines have received increasing attention as treatments for allergic diseases [2].

ID inhibits the systemic anaphylaxis reaction in mice [30]. In the present study, we found that ID inhibited PCA and scratching behavior reactions, as well as the degranulation of RBL-2H3 cells stimulated by the IgE-antigen complex. BB-FID more potently inhibited degranulation of RBL-2H3 cells than non-fermented ID. BB-FID also was more potent in inhibiting the PCA reaction induced by the IgE-antigen complex. BB alone showed a weak antiallergic effect [3, 12]. These results suggest that ID and BB-FID may inhibit the PCA reaction by inhibiting mast cell degranulation. ID and BB-FID inhibited the scratching behaviors stimulated by compound 48/80 in proportion to their ability to inhibit changes in vascular permeability.

Mast cells produce histamine, as well as proinflammatory cytokines, particularly TNF-α, IL-4, and IL-6 [10], which are important in allergic reactions. Mast cells are a principal source of TNF-α in human dermis, and degradation of mast cells in the dermal endothelium is abrogated by inhibition of TNF-α [27]. The IL-6 produced from mast cells, when locally accumulated, is associated with a PCA reaction [15]. IL-4 induces IgE production in B lymphocytes [26]. Inhibiting the cytokine expression in mast cells would reduce allergic symptoms. ID and BB-FID inhibited both degranulation and gene expression of TNF-α and IL-4 in IgE-stimulated RBL-2H3 cells, as well as NO production in RAW264.7 macrophage cells, as do other antiallergic natural agents, such as quercetin [8, 9]. However, ID and BB-FID did not inhibit degranulation and IL-4/TNF-α expression with the same potency, suggesting that they may proceed *via* different mechanisms. Thus, the antiallergic effects of ID and FID may be due to the inhibition of degranulation and cytokine biosynthesis.

The NF-κB activation is an important signaling pathway in immune responses [7, 31], and NF-κB activation is essential for the expression of the proinflammatory cytokines, such as TNF-α and IL-6. ID and BB-FID inhibited IgE-induced activation of NF-κB in RBL-2H3 cells.

Based on these findings, ID and FID may protect against PCA and itching reactions, which are representative the IgE-mediated skin allergic diseases, with increased potency after LAB fermentation.

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