Inhibitory effect of fermented *Arctium lappa* fruit extract on the IgE-mediated allergic response in RBL-2H3 cells

JAE-MYUNG YOO, JU HYE YANG, HYE JIN YANG, WON-KYUNG CHO and JIN YEUL MA

Korean Medicine (KM)-Application Center, Korea Institute of Oriental Medicine (KIOM), Daegu 41062, Republic of Korea

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Abstract. *Arctium lappa* fruit has been used in traditional medicine, and it is known to exert beneficial effects, such as antioxidant, anti-inflammatory and anticancer effects. However, the effects of the *Arctium lappa* fruit on the allergic response remain unknown. In this study, we evaluated the anti-allergic effects of *Arctium lappa* fruit extract (AFE) and its fermented form (F-AFE) using immunoglobulin E (IgE)-activated RBL-2H3 cells. To investigate the anti-allergic effects of AFE or F-AFE, we examined the release of β-hexosaminidase, a key biomarker of degranulation during an allergic reaction, and the production of pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) in the cells treated with or without the above-mentioned extracts. AFE weakly inhibited the release of β-hexosaminidase, whereas F-AFE significantly suppressed the release of β-hexosaminidase in a dose-dependent manner. Consistently, F-AFE suppressed the production of TNF-α and PGE_2 in a dose-dependent manner. F-AFE exerted an inhibitory effect on the production of β-hexosaminidase, TNF-α and PGE_2 with an IC_{50} value of 30.73, 46.96 and 36.27 μg/ml, respectively. Furthermore, F-AFE inhibited the phosphorylation of Lyn, Fyn and Syk, which are involved in the FcεRI signaling pathway, that of phosphoinositide phospholipase C (PLC)γ1/2 and protein kinase C (PKC)δ, which are associated with the degranulation process, as well as that of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun *N*-terminal kinase 1/2 (JNK), p38 and Akt, which are associated with cytokine expression. In the late phase, F-AFE partially suppressed the phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$), but not the expression of cyclooxygenase (COX)-2. To compare and identify the major components of the two extracts, we used high-performance liquid chromatography. The levels of arctigenin, one of the major compounds, were elevated 6-fold in F-AFE compared with AFE, whereas the levels of arctiin, an arctigenin glycoside, were decreased in F-AFE by approximately 57.40%. These results suggest that arctigenin plays an important role in the anti-allergic effects of F-AFE. Taken together, F-AFE containing anti-allergic phytochemicals, including arctigenin, inhibited the activation of the FcεRI receptor induced by the antigen-IgE complex. Such effects may provide further information for the development of a phytomedicine for allergic diseases.

Correspondence to: Dr Won-Kyung Cho or Dr Jin Yeul Ma, Korean Medicine (KM)-Application Center, Korea Institute of Oriental Medicine (KIOM), 70 Cheomdan-ro, Dong-gu, Daegu 41062, Republic of Korea
E-mail: wkcho@kiom.re.kr
E-mail: jyma@kiom.re.kr

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Introduction

Arctium fruit (*Arctium lappa* L.) has been used in traditional herbal medicine in East Asia, and is known to exert various beneficial, such as antioxidant (1), anticancer (2), anti-aging (3), anti-diabetes (4), neuroprotective (5), hepatoprotective (6) and anti-colitis (7) effects. Such effects are associated with polyphenol compounds, such as arctigenin, arctiin and other lignan compounds in *Arctium lappa* fruit (1,2,4,6,8). However, to the best of our knowledge, the effects of *Arctium lappa* fruit or its fermentation product on allergic reactions have not been reported to date.

The fermentation process for medicinal herbs has been frequently used for improving the biological effects of the extracts (9,10). Fermentation using microorganisms, such as yeast (11), fungi (12) and bacteria (9,10) is known to yield metabolites, or to convert glycosylated compounds into aglycones, one of the active forms.

Anaphylactic shock is known as the type I of allergic response classes (13), and is the most important clinically, as anaphylactic shock is closely associated with the degranulation of mast cells (14). When mast cells are activated by the antigen-immunoglobulin E (IgE) complex, they are then able to release a number of allergic inflammatory mediators, such as histamine, cytokines, prostaglandins and leukotrienes from numerous granules in a matter of minutes (15). Consequently, tissues or organs stimulated by allergic inflammatory mediators induce acute inflammatory responses simultaneously. In signaling cascades associated with allergic reactions, the FcεRI receptor is known as the high affinity IgE receptor, and is located on the plasma membrane of mast cells and basophilic cells (15). Therefore, the activation of the receptor on mast cells is intimately associated with degranulation or biosynthesis of the allergic inflammatory mediators in mast cells.

In present study, we found that *Arctium lappa* fruit extract (AFE) exerted mild anti-allergic effects. Based on this

finding, we hypothesized that the fermentation process may improve the anti-allergic effects of AFE on IgE-activated mast cells. To investigate anti-allergic effects of fermented AFE (F-AFE), degranulation was determined by measuring β-hexosaminidase activity in IgE-activated RBL-2H3 cells. To examine the anti-allergic and anti-inflammatory effects of F-AFE, the levels of inflammatory biomarkers, including tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) were measured. Finally, to elucidate the mechanisms and active compounds responsible for the anti-allergic effects of F-AFE, the FcεRI signaling cascade was analyzed by immumoblotting analysis, and the active phytochemical composition of F-AFE was analyzed by high-performance liquid chromatography (HPLC). Herein, we demonstrate that F-AFE suppresses IgE-mediated allergic events in mast cells. Furthermore, our data may aid in the development of a phytomedicine for allergic diseases.

Materials and methods

Reagents. MEM-α medium, 1X Dulbecco's phosphate-buffered saline (DPBS), penicillin, streptomycin and fetal bovine serum (FBS) were purchased from GE Healthcare Life Sciences (HyClone, Logan, UT, USA). The EZ-Cytox cell viability assay kit was purchased from DAEIL Lab Service Co. Ltd. (Seoul, Korea). Specific antibodies against phosphorylated (p-)Lyn (#2731), p-Syk (#2710), p-phosphoinositide phospholipase C (PLC)γ1/2 (#2821, #3871, respectively), p-protein kinase C (PKC)δ (#2055), p-extracellular signal-regulated kinase 1/2 (ERK1/2; #9101), p-c-Jun *N*-terminal kinase 1/2 (p-JNK; #9251), p-p38 (#9211), p-Akt (#9271), p-cytosolic phospholipase A_2 ($cPLA_2$; #2831) and cyclooxygenase (COX)-2 (#4842) were obtained from Cell Signaling Technology (Beverly, MA, USA). A specific antibody against p-Fyn (orb128087) was purchased from Biorbyt Ltd. (Cambridge, UK). A specific antibody against β-actin (sc-47778) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The enzyme immunoassay (EIA) kit for PGE_2 was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). The enzyme-linked immunosorbent assay (ELISA) kit for TNF-α was purchased from e-Bioscience, Inc. (Science Center Drive, San Diego, CA, USA). 4-Nitrophenyl *N*-acetyl-β-*D*-glucosaminide (p-NAG), dinitrophenyl (DNP)-immunoglobulin E (DNP-IgE) and DNP-human serum albumin (DNP-HSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The standards of arctiin and matairesinol were purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, China). A standard of arctigenin was obtained from Must Bio-Technology Co., Ltd. (Chengdu, China). All other chemicals were of analytical grade.

Preparation of extracts of Arctium lappa fruit or fermented Arctium lappa fruit. AFE or F-AFE were prepared according to a modification of a process reported previously (16); *Arctium lappa* fruits were purchased from the Yeongcheon Oriental Herbal Market (Yeongcheon, Korea), and were then verified by Dr Ki-Hwan Bae, who holds the position of Professor Emeritus at the College of Pharmacy, Chungnam National University (Daejeon, Korea). *Arctium lappa* fruits (1 kg) were boiled in distilled water (10 liter) for approximately 3 h at 115˚C. The aqueous extract was filtered through a testing sieve (aperture 500 and 150 μm). AFE was fermented using *Lactobacillus rhamnosus* (*L. rhamnosus)* (KFRI 128, KCTC 2182) provided from the Korea Food Research Institute. Briefly, the water extract adjusted to pH 7.0 with 1 N NaOH was autoclaved for 5 min, inoculated with *L. rhamnosus* ($1x10^5$-$1x10^7$ CFU/ml), and then incubated for 48 h at 37˚C for 48 h. F-AFE was filtered through a 60-μm nylon net filter (Millipore, Billerica, MA, USA), deposited overnight. The supernatant was lyophilized, and the dried pellet was then stored at -20˚C until use. AFE and F-AFE were dissolved in 10% DMSO solution for use in all the experiments.

HPLC analysis. HPLC analysis was evaluated following a modification of a previously described method (17). To analyze the lignan compounds, HPLC analysis was performed using a Chromeleon 7 system equipped with a Dionex UltiMate 3000 Pump, an autosampler, a column compartment and a diode array detector (Thermo Fisher Scientific Inc., Foster City, CA, USA) and a Phenomenex Luca C_{18} column (4.6x250 mm, 5 μm; Phenomenex Inc., Torrance, CA, USA). The lignan chemicals were eluted in a gradient system composed of solvent A (deionized water) and solvent B (acetonitrile). The gradient was 20-30-36-45-90-20% of solvent B at gradient time, *t*G = 0-20-35-50-53-75 min, column oven temperature was 30˚C and the flow rate was 1.0 ml/min; an injection volume of 10 μl was applied. The UV/Vis detector was set at a wavelength range from 190 to 400 nm. The standards of arctiin, arctigenin or matairesinol and sample solutions (1.0 mg/ml) were dissolved and diluted in 60% methanol. Calibration curves constructed using linear least-squares regression were linear over the concentration range of the standards used (Table I). The relative standard deviation of the measured concentrations was used to assess the precision. A comparison of the mean measured concentration versus the corresponding nominal concentration was used to assess the accuracy.

Cell culture. RBL-2H3 cells, originating from rat basophilic leukemia and known as one of the mast cell lines, were obtained from the Korean Cell Line Bank (Seoul, Korea), and were cultured in MEM-α medium containing 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2 as previously described (18). All the experiments included a control group, which was a vehicle control group (0.1% DMSO).

Cell viability assay. Cell viability was determined by measuring the mitochondrial-dependent reduction of WST-1 to water-soluble tetrazolium salt (19). Briefly, the RBL-2H3 cells were seeded on a 96-well plate ($1x10^4$ cells/well) in MEM-α medium with 10% FBS at 37˚C overnight. The cells were washed by 1X DPBS, and then incubated with 50 ng/ml DNP-IgE for 24 h. The above-mentioned cells, pre-incubated with AFE (0-2,500 μg/ml) or F-AFE (0-500 μg/ml) for 1 h in Hank's balanced salt solution (HBSS) containing 0.1% BSA, were simultaneously mixed with 0.1 μg/ml DNP-HSA and 10 μl EZ-Cytox reagent, and then incubated for a further 4 h. Cell viability was determined at 450 nm using a microplate reader (SpectraMax i3; Molecular Devices, Sunnyvale, CA, USA).

β-hexosaminidase activity assay. β-hexosaminidase activity was evaluated according to a previously described method (20). The

Table I. Calibration curves of three lignan standards.

Lignan compound	Regression equation[a]	Correlation coefficient (r^2)[b]	Linear range (μg)
Arctiin	y=0.0916x+0.0737	10.000	37.5-600.0
Arctigenin	y=63597x-2277.6	10.000	12.5-200.0
Methylarctigenin	y=119193x-18355	10.000	5.0-80.0

[a]y, peak area; x, concentration (μg/ml); [b]regression coefficient (n=5).

supernatant (25 μl) was mixed with 50 μl p-NAG (10 mM) in 0.1 M sodium citrate buffer (pH 4.5) in a 96-well plate, followed by incubation for 1 h at 37˚C. The reaction was terminated by stop buffer (0.1 M sodium carbonate buffer, pH 10.0). The absorbance was measured at 405 nm using a microplate reader.

ELISA for determining the levels of TNF-α. To determine the levels of TNF-α in the culture media, the IgE-sensitized cells were pre-incubated with F-AFE (0-500 μg/ml) for 1 h in MEM-α medium with 0.1% FBS for 1 h, spiked with DNP-HSA, and then incubated for a further 4 h. All culture media were centrifuged (17,000 x g for 10 min) at 4˚C, and the samples were stored at -80˚C until use. TNF-α was detected using an ELISA kit according to the manufacturer's instructions (e-Bioscience, Inc.).

EIA for determining the levels of PGE_2. To measure the levels of PGE_2 in the culture media, all culture media were centrifuged (17,000 x g for 10 min) at 4˚C, and the samples were stored at -80˚C until use. PGE_2 was measured using an EIA kit according to the manufacturer's instructions (Cayman Chemical Co.).

Immunoblot analysis. Immunoblot analysis was carried out according to a previously described method (20). PVDF membranes containing blotted proteins were visualized using a chemiluminescent reaction (ECL plus kit) and an Imaging system (ChemiDoc Touch Imaging System) (both from Bio-Rad, Hercules, CA, USA). The levels of target proteins were compared to those of a loading control (β-actin), and the results were expressed as a ratio of the density of each protein identified by a protein standard size marker (BIOFACT Co., Ltd., Daejeon, Korea). The density of each band was measured using ImageJ software (version 1.49v for Windows; NIH, Bethesda, MD, USA).

Statistical analyses. The experimental results are presented as the means ± SD. One-way analysis of variance (ANOVA) was used for multiple comparisons (GraphPad Prism version 5.01 for Windows; GraphPad Software, San Diego, CA, USA). If there was a significant variation between the treatment groups, the Dunnett's test was applied. Values of $P<0.05$ and $P<0.01$ were considered to indicate statistically significant differences.

Results

Inhibitory effects of AFE or F-AFE on IgE-mediated degranulation in RBL-2H3 cells: comparison between AFE and F-AFE. First, to investigate effects of AFE and F-AFE on IgE-mediated degranulation in mast cells, the IgE-sensitized RBL-2H3 cells were incubated with various concentrations of AFE or F-AFE prior to exposure to the antigen (0.1 μg/ml DNP-HSA). Although AFE weakly inhibited the release of β-hexosaminidase, a marker of degranulation, from the IgE-activated RBL-2H3 cells until 2,500 μg/ml, the inhibitory effect of AFE was not enhanced after 250 μg/ml (Fig. 1A). On the other hand, F-AFE markedly inhibited degranulation (IC_{50} value, 30.73 μg/ml) in the IgE-activated RBL-2H3 cells in a dose-dependent manner (Fig. 2A). Notably, neither AFE nor F-AFE exerted any severe cytotoxic effects at concentrations of up to 1,000 μg/ml and 250 μg/ml, respectively (Figs. 1B and 2B). These results indicate that fermentation of the *Arctium lappa* fruit potently enhances the anti-allergic effects of AFE within non-cytotoxic concentrations.

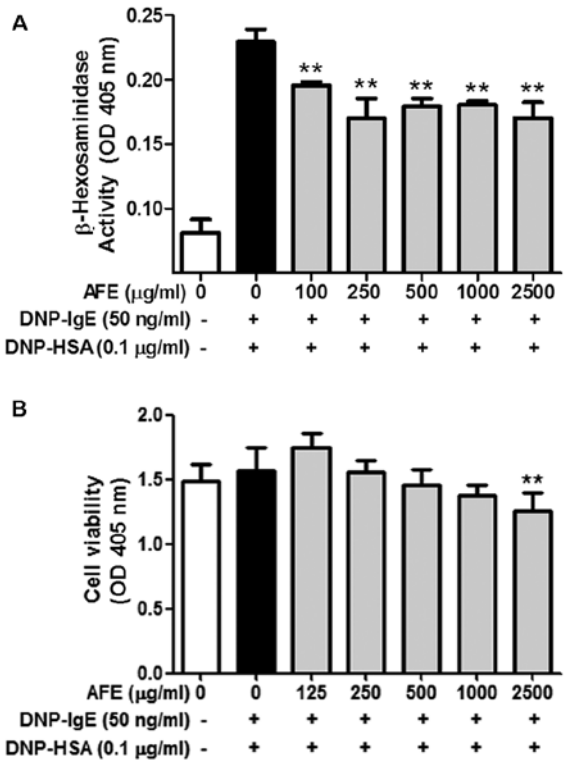

Figure 1. Effects of *Arctium lappa* fruit extract (AFE) on degranulation and cell viability in immunoglobulin E (IgE)-activated RBL-2H3 cells. RBL-2H3 cells were seeded on a 24-well plate ($1x10^5$ cells/well) or a 96-well plate ($1x10^4$ cells/well) in MEM-α with 10% FBS at 37˚C overnight, and further incubated with dinitrophenyl (DNP)-IgE for 24 h. IgE-sensitized cells were exposed to AFE (0-2,500 μg/ml) for 1 h, and then stimulated with DNP-HSA (0.1 μg/ml) for 4 h. β-hexosaminidase activity and cell viability were determined as described in the Materials and methods. Data are the mean ± SD values of triple or octuplex determinations. **P<0.01 vs. DNP-HSA-treated group. (A) β-hexosaminidase; (B) cell viability.

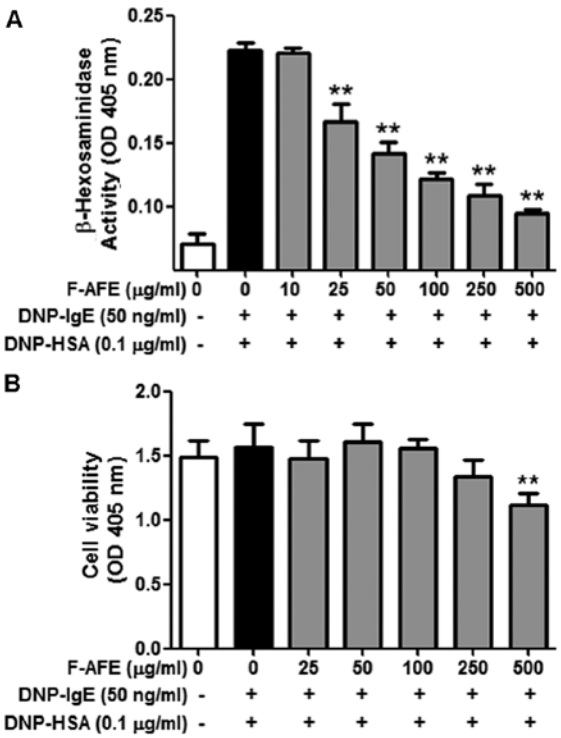

Figure 2. Effect of fermented *Arctium lappa* fruit extract (F-AFE) on degranulation and cell viability in immunoglobulin E (IgE)-activated RBL-2H3 cells. β-hexosaminidase activity and cell viability were determined as described in Fig. 1. Data are the mean ± SD values of triple or octuplex determinations. **P<0.01 vs. DNP-HSA-treated group. (A) β-hexosaminidase; (B) cell viability.

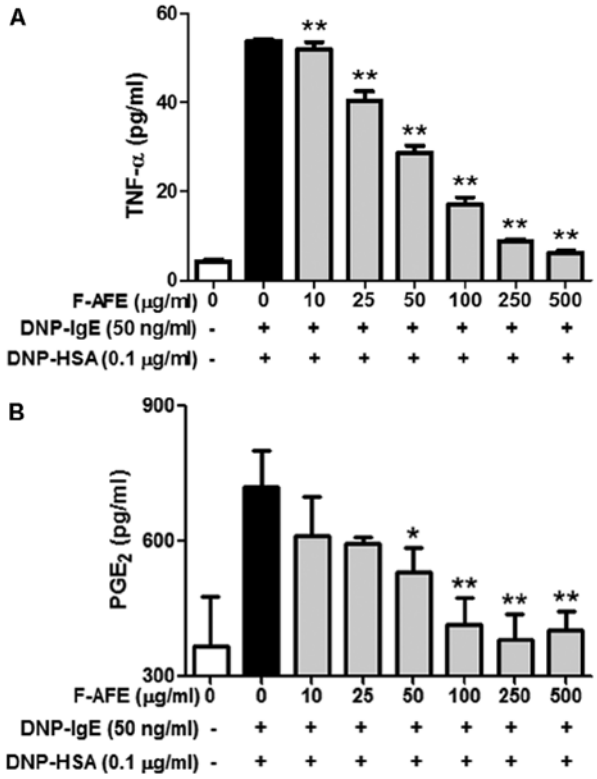

Figure 3. Inhibitory effects of fermented *Arctium lappa* fruit extract (F-AFE) on pro-inflammatory mediators. Immunoglobulin E (IgE)-sensitized RBL-2H3 cells were exposed to F-AFE (0-500 μg/ml) for 1 h, and then stimulated with dinitrophenyl-human serum albumin (DNP-HSA) (0.1 μg/ml) for 4 h. The levels of tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) were determined as described in the Materials and methods. Data are the mean ± SD values of triple determinations. *P<0.05 and **P<0.01 vs. DNP-HSA-treated group. (A) TNF-α; (B) PGE_2.

Inhibitory effects of F-AFE on the release of pro-inflammatory mediators. It is known that IgE-activated mast cells are able to release pro-inflammatory mediators, such as TNF-α, associated with the initiation of airway inflammation and the generation of airway hyper-responsiveness in asthma (21), and PGE_2, associated with asthma development and inflammation (22). Therefore, we focused on the effects of F-AFE on the release of TNF-α and PGE_2 from IgE-activated RBL-2H3 cells. When the IgE-sensitized RBL-2H3 cells were pre-incubated with F-AFE and stimulated with the antigen, F-AFE significantly decreased the release of TNF-α with an IC_{50} value of 46.96 μg/ml (Fig. 3A) and that of PGE_2 with an IC_{50} value of 36.27 μg/ml (Fig. 3B). These results suggest that F-AFE suppresses acute or chronic allergic inflammatory responses by inhibiting the release of various inflammatory mediators, including cytokines and lipid mediators from IgE-activated mast cells.

Regulatory effects of F-AFE on the activation of the arachidonate cascade. We wished to examine the effects of F-AFE on the formation of pro-inflammatory lipid mediators, as pro-inflammatory lipid mediators, including PGE_2, prostaglandin D_2 (PGD_2), leukotriene B_4 (LTB_4) and leukotriene C_4 LTC_4) are known to aggravate allergic diseases (23-26). Thus, we investigated the effects of F-AFE on the phosphorylation of $cPLA_2$, a rate-limiting enzyme of eicosanoid synthesis, and the expression of COX-2, a rate-limiting enzyme of prostaglandins. When the IgE-sensitized RBL-2H3 cells were incubated with F-AFE prior to antigen challenge for 4 h, F-AFE partially suppressed the phosphorylation of $cPLA_2$, but not the expression of COX-2 (Fig. 4). These findings

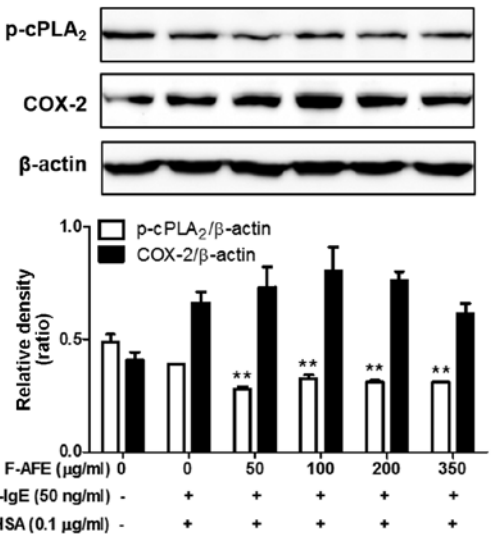

Figure 4. Inhibitory effects of fermented *Arctium lappa* fruit extract (F-AFE) on the activation of rate-limiting enzymes in the arachidonate cascade. RBL-2H3 cells were seeded on a 6-well plate ($5x10^5$ cells/well) in MEM-α with 10% FBS at 37˚C overnight, and further incubated with dinitrophenyl-immunoglobulin E (DNP-IgE) for 24 h. IgE-sensitized RBL-2H3 cells were exposed to F-AFE (0-350 μg/ml) for 1 h, and then stimulated with dinitrophenyl-human serum albumin (DNP-HSA) (0.1 μg/ml) for 4 h. The above cells were rinsed with 1X DPBS, and lysed with cell lysis buffer. The expression of p-$cPLA_2$, COX-2 or β-actin was determined as described in the Materials and methods. Similar results were obtained from 3 independent experiments. **P<0.01 vs. DNP-HSA-treated group.

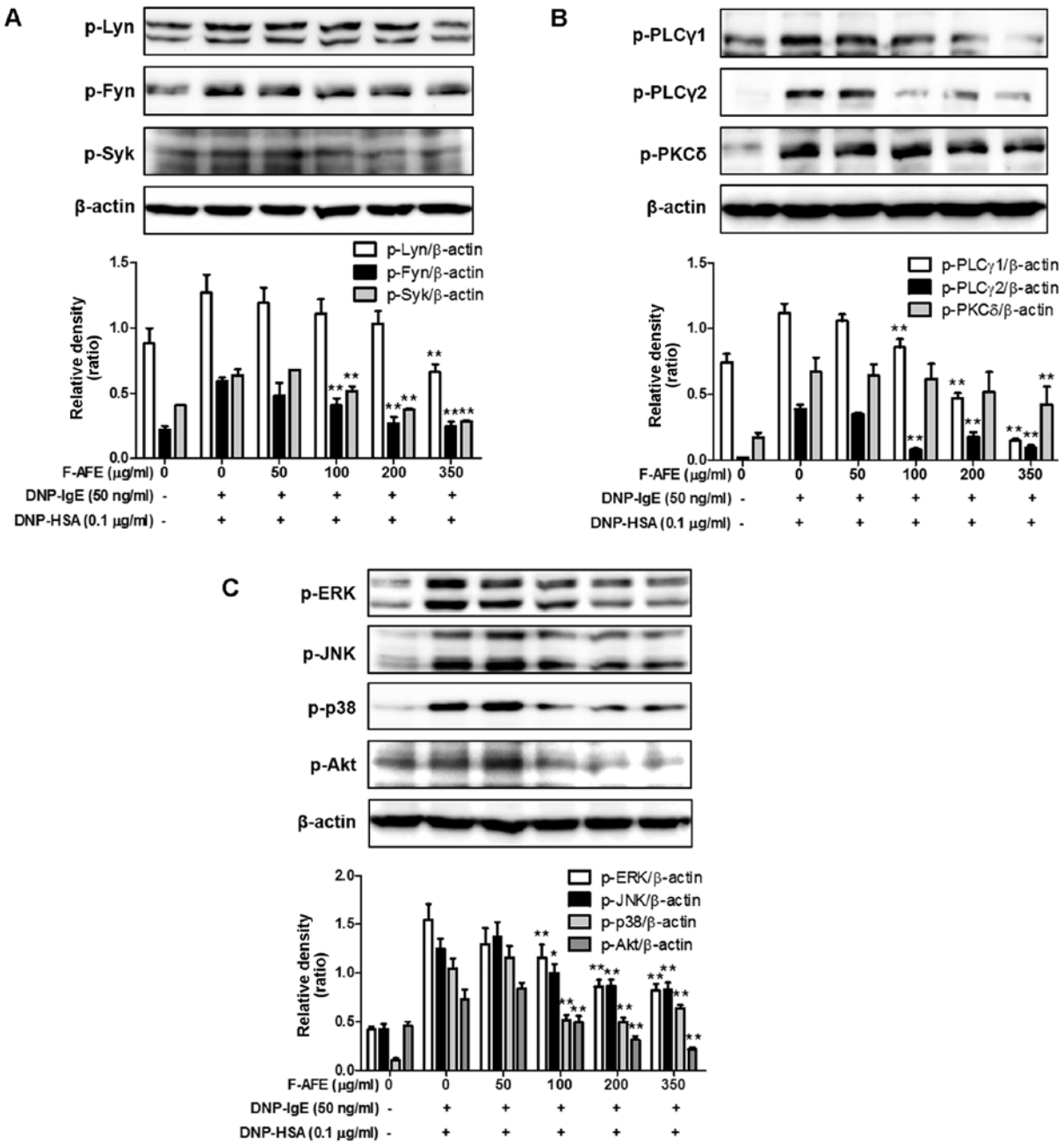

Figure 5. Effects of fermented *Arctium lappa* fruit extract (F-AFE) on the phosphorylation of intermediate proteins in the FcεRI cascade. immunoglobulin E (IgE)-sensitized RBL-2H3 cells were exposed to F-AFE (0-350 μg/ml) for 1 h, and then stimulated with dinitrophenyl-human serum albumin (DNP-HSA) (0.1 μg/ml) for 10 min. The above-mentioned cells were rinsed with 1X DPBS, and lysed with cell lysis buffer. The expression of p-Lyn, p-Fyn, p-Syk, p-PLCγ1, p-PLCγ2, p-PKCδ, p-ERK, p-JNK, p-p38, p-Akt, or β-actin was determined as described in the Materials and methods. Similar results were obtained from 3 independent experiments. *P<0.05 and **P<0.01 vs. DNP-HSA-treated group. (A) p-Lyn, p-Fyn and p-Syk; (B) p-PLCγ1, p-PLCγ2 and p-PKCδ; (C) p-ERK, p-JNK, p-p38 and p-Akt.

indicate that F-AFE regulates the formation of eicosanoids, including prostaglandins by regulating the activation of $cPLA_2$ in the arachidonate cascade.

Regulatory effects of F-AFE on the activation of the FcεRI cascade. Since F-AFE reduced the rate-limiting enzymes of the arachidonate cascade in the late phase (4 h), we further investigated the rate-limiting proteins and intermediate proteins in the FcεRI cascade in the early phase (10 min). The activation of the arachidonate cascade is involved in the IgE-mediated activation of the FcεRI cascade in mast cells (20,27). Thus, we wished to determine whether F-AFE regulates the activation of rate-limiting proteins and/or intermediate proteins in the IgE-mediated FcεRI cascade. When the IgE-sensitized RBL-2H3 cells were stimulated with the antigen (DNP-HSA) for 10 min after the cells were incubated with F-AFE for 1 h, F-AFE inhibited the phosphorylation of Lyn and Fyn, as well as that of Syk (Fig. 5A). In addition, F-AFE significantly reduced the phosphorylation of intermediate proteins, the downstream targets of Syk, such as ERK, JNK, p38 and Akt (Fig. 5C). Additionally, F-AFE significantly suppressed the phosphorylation of PLCγ1/2 and PKCδ, which are involved

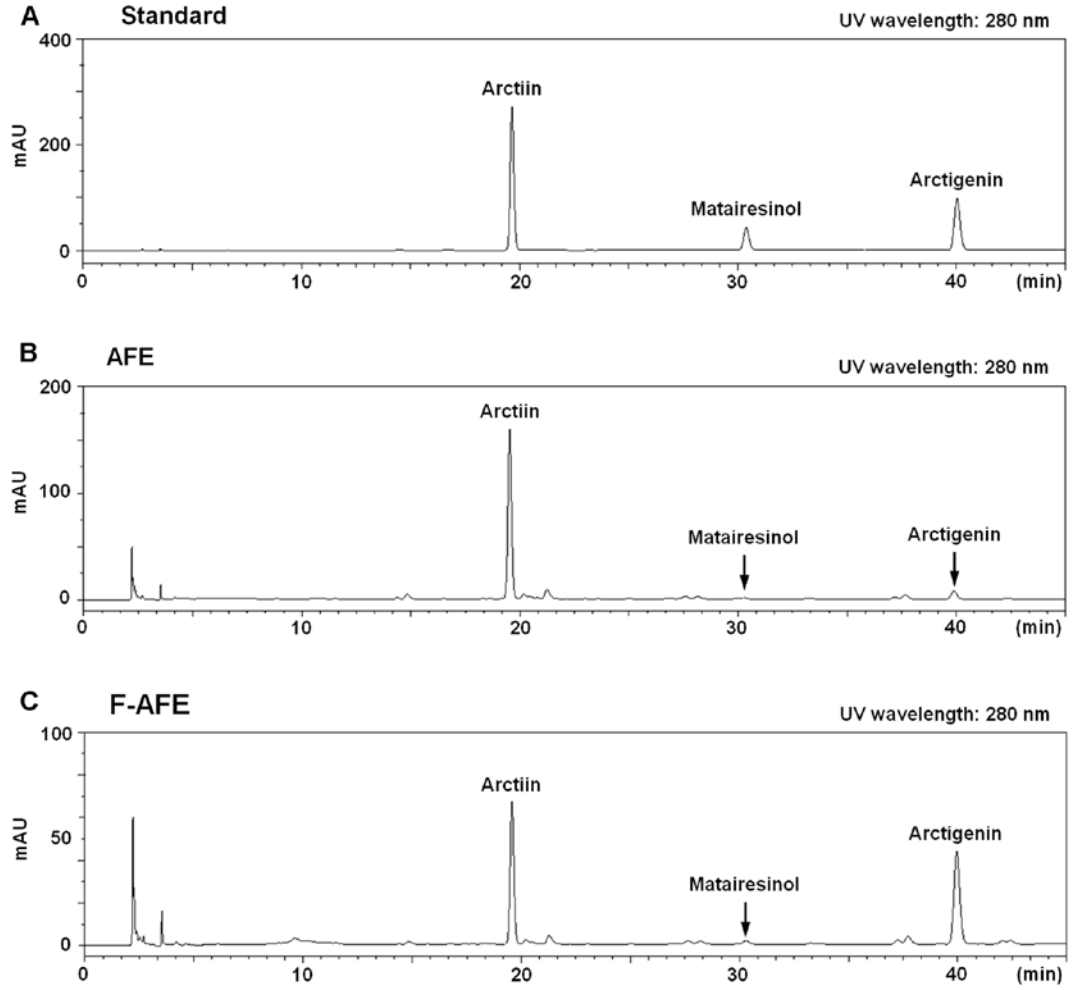

Figure 6. HPLC chromatograms of *Arctium lappa* fruit extract (AFE) or fermented AFE (F-AFE). These are HPLC chromatograms of a standard mixture of three standard chemicals in AFE or F-AFE at 280 nm. Acrtiin (1), matairesinol (2) and acrtigenin (3) were identified. HPLC anlaysis was carried out as described in the Materials and methods. (A) Standard chromatogram; (B) AFE chromatogram; (C) F-AFE chromatogram.

Table II. Composition of AFE or F-AFE: lignan compounds.

	Lignan compounds		
	Arctiin	Arctigenin	Methylarctigenin
AFE	338.79±0.01	14.69±0.01	1.80±0.37
F-AFE	144.31±0.05	88.35±0.07	2.99±0.22

Data are the means ± SD values of triple determinations. Unit: mg/g dry weight.

in the degranulation process of mast cells (Fig. 5B). These findings indicate that the anti-allergic effects of F-AFE are associated with the regulation of the activation of the FcεRI cascade through the inhibition of the activation of Lyn, Fyn and Syk in mast cells.

Identification of lignan compounds, one of the major components, in F-AFE: comparison between AFE and F-AFE. Finally, to substantiate what is behind the anti-allergic effects of F-AFE, we analyzed and compared the chemical composition of F-AFE and AFE using an HPLC system. It is known as that *Arctium lappa* fruit contains a number of lignan compounds, such as arctiin, arctigenin and other lignan compounds (1,2,4,6,8). Based on the findings of these previous studies, we analyzed the amounts of the 3 major compounds: arctiin, matairesinol and arctigenin. The retention times for the peaks of the arctiin, matairesinol or arctigenin standard were 19.64, 30.38 or 40.06 min, respectively, as shown on an HPLC chromatogram (Fig. 6A). The peaks of matairesinol and arctigenin in F-AFE were increased, and the peak of arctiin in F-AFE was decreased on the HPLC chromatogram (Fig. 6B and C). Table II summarizes the amounts of arctiin, matairesinol and arctigenin in AFE and F-AFE. These results suggest that fermentation using *L. rhamnosus* elevates the levels of aglycones, such as arctigenin in AFE through conversion from lignan glycosides, such as arctiin. Taken together, our findings indicate that F-AFE has the potential for use as an anti-allergic remedy, and that aglycones including arctigenin in AFE are responsible for the anti-allergic effects.

Discussion

Arctium lappa fruit has long been used in traditional herbal medicine in East Asia, and has been shown to exert various

beneficial effects, such as antioxidant (1), anticancer (2), anti-aging (3), anti-diabetic (4), neuroprotective (5), hepatoprotective (6) and anti-colitis (7) effects. Such effects of the *Arctium lappa* fruit are caused by lignan compounds (1-7). However, to the best of our knowledge, the effects of the *Arctium lappa* fruit on allergic reaction have not been reported to date.

Various fermented foods have been consumed for centuries worldwide. In addition, several fermented foods have also been used in traditional medicine. Currently, the application of the fermentation process using microorganisms, such as fungi, yeast and bacteria has been often used to improve the beneficial effects of medicinal herbs (9-12). Fermentation is known for yielding metabolites, or converting glycosylated compounds to aglycones, one of the active forms. The fermentation process is known to yield metabolites of microorganisms, or convert glycosides to aglycones (11,12,28). Nevertheless, the application of the fermentation process for the *Arctium lappa* fruit has been not reported to date, to the best of our knowledge.

Generally, *Arctium lappa* fruit is known to contain various lignan compounds, such as arctiin, arctigenin and methylarctigenin (1,2,4,6,8,29). In particular, arctigenin is known as one of the major lignan compounds (29), and is a major effective component (5,7,30) in the *Arctium lappa* fruit. These data demonstrate that AFE possesses weak anti-allergic properties. By contrast, as shown in this study, F-AFE, fermented using *L. rhamnosus*, exerted potent anti-allergic effects, as shown by cultured cell-based assays. F-AFE also contains elevated levels of aglycones, such as arctigenin, as shown by HPLC analysis. Therefore, F-AFE possesses an advantage in comparison with AFE, as F-AFE is much more potent than AFE in suppressing the biomarkers for degranulation (β-hexosaminidase) and inflammation (TNF-α and PGE_2) at several dozen microgram per milliliter levels. Actually, F-AFE contains 6-fold higher levels of arctigenin than AFE, whereas the acrtiin level was decreased in F-AFE by approximately 57.40% compared to AFE. This indicates that the anti-allergic effects of F-AFE may be caused by the elevated levels of arctigenin. Although F-AFE at 500 μg/ml significantly had a low cytotoxicity, F-AFE at the same concentration suppressed the release of β-hexosaminidase, TNF-α and PGE_2. These results suggest that F-AFE at only 500 μg/ml may inhibit the growth of mast cells or induces the apoptosis of mast cells. To conclude, F-AFE (at 500 μg/ml) may not only directly inhibit the allergic response in IgE-activated mast cells, but may also induce a decrease in the number of mast cells. Such effects of F-AFE may contribute to the enhancement of its anti-allergic effects.

As regards the intracellular mechanisms responsible for the anti-allergic effects of F-AFE, one possible mechanism is associated with a direct suppression of a signaling cascade of FcεRI receptor, a high affinity IgE receptor (31). The degranulation in IgE-activated mast cells correlates with the activation of the FcεRI receptor, located on the extracellular plasma membrane of mast cells or basophils (31). When IgE-activated mast cells liberates granules to the extracellular space, a number of pro-inflammatory mediators, such as TNF-α, histamine, prostaglandins and leukotrienes are released from the cells (15), and these mediators then induce acute and chronic inflammation. Therefore, the key function of an anti-allergic action is to regulate the activation of the FcεRI cascade. In this regard, Fyn, Lyn and Syk are known as rate-limiting factors of the FcεRI signaling cascade (32,33). Their activation induces the elevation of intracellular Ca^{2+} levels and the activation of the mitogen-activated protein kinase family, such as ERK, JNK and p38 (32). Therefore, Fyn, Lyn and Syk are the key signaling proteins of FcεRI cascade in the early phase. In the present study, the inhibitory effects of F-AFE on the phosphorylation of Lyn, Fyn or Syk may support the notion that one of the anti-allergic targets for F-AFE may be Lyn, Fyn or Syk. In support of this hypothesis, in our study, F-AFE significantly inhibited the phosphorylation of ERK, JNK, p38 and Akt. Additionally, F-AFE also reduced the phosphorylation of PLCγ1/2 and PKCδ in a concentration-dependent manner. It is known that the degranulation of IgE-activated mast cells is involved in the activation of the Syk/PLCγ/PKCδ pathway in the FcεRI cascade (20).

Separately, as previously demonsrated, the production of eicosanoids, including prostaglandins and leukotrienes in IgE-activated mast cells is intimately related with the activation of the FcεRI cascade (20,22). Eicosanoids, such as PGE_2, PGD_2, LTB_4 and LTC_4 are able to induce or aggravate acute and chronic inflammation in allergic diseases, such as asthma and allergic rhinitis (34). Therefore, the formation of eicosanoids in IgE-activated mast cells is another target of the anti-allergic actions of F-AFE. In this study, F-AFE not only reduced the levels of PGE_2, which is involved in asthma development and inflammation related with interleukin (IL)-4 and IL-5 (23), but also suppressed the phosphorylation of $cPLA_2$ in IgE-activated RBL-2H3 cells. This suggests that F-AFE inhibits the biosynthesis of prostaglandins through the inhibition of $cPLA_2$, a rate-limiting enzyme of the arachidonate cascade. Besides, the inhibitory effects of F-AFE on the activation of $cPLA_2$ may support the reduction of leukotriene biosynthesis in IgE-activated mast cells. Such effects of F-AFE may contribute to the improvement of its anti-allergic effects on allergic diseases.

In conclusion, this study demonstrates that AFE or F-AFE exert anti-allergic effects, and that the fermentation process enhances the anti-allergic effects of AFE by converting arctiin, a lignan glycoside, to arctigenin, an aglycone. These findings reveal a novel feature of F-AFE in allergic reactions. The mechanisms responsible for its anti-allergic effects may involve various targets, such as Lyn, Fyn, Syk and $cPLA_2$, as well as pro-inflammatory mediators. Such effects may be due to several compounds in the *Arctium lappa* fruit, and may aid in the development of a functional food or a preventive agent for allergic diseases.

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