

Anti-Allergic Action of Aged Black Garlic Extract in RBL-2H3 Cells and Passive Cutaneous Anaphylaxis Reaction in Mice

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ABSTRACT Garlic (*Allium sativum*) has been used as a food as well as a component of traditional medicine. Aged black garlic (ABG) is known to have various bioactivities. However, the effect of ABG on allergic response is almost unknown. In the present study, we investigated whether ABG can inhibit immunoglobulin E-mediated allergic response in RBL-2H3 cells as well as *in vivo* passive cutaneous anaphylaxis (PCA). In *in vitro* tests, ethyl acetate extract (EBG) of ABG significantly inhibited the release of β -hexosaminidase (IC₅₀, 1.53 mg/mL) and TNF- α (IC₅₀, 0.98 mg/mL). Moreover, BG10, an active fraction of EBG, dramatically suppressed the release of β -hexosaminidase (IC₅₀, 53.60 μ g/mL) and TNF- α (IC₅₀, 27.80 μ g/mL). In addition, BG10 completely blocked the formation of prostaglandin E₂ and leukotriene B₄ at ≥ 25 μ g/mL. When the effect of BG10 on Fc ϵ RI receptor cascade was investigated, BG10 significantly inhibited the phosphorylation of Syk, but not Lyn. Furthermore, BG10 dose dependently decreased the phosphorylation of cytosolic phospholipase A₂ (cPLA₂) and 5-lipoxygenase (5-LO) as well as the expression of cyclooxygenase-2 (COX-2). Consistent with what has been mentioned earlier, BG10 also significantly inhibited the PCA reaction in mice. In conclusion, these results indicate that ABG suppresses the allergic response, and the mechanism for its anti-allergic action may involve suppressions of Syk, cPLA₂, 5-LO, and COX-2. The anti-allergic actions of ABG, EBG, or BG10 suggest that they may be useful as functional foods for allergic diseases.

KEY WORDS: • allergic inflammation • degranulation • LTB₄ • mast cells • PGE₂ • TNF- α

INTRODUCTION

ALLERGIC RESPONSES are classified into various types, including type 1 (anaphylactic shock).¹ In type 1 allergic responses, the activation of Fc ϵ RI receptor (the high-affinity immunoglobulin E [IgE] receptor) on the plasma membrane of mast and basophilic cells is known to liberate β -hexosaminidase, a general marker of degranulation, as well as allergic mediators such as histamine, cytokines, prostaglandins, and leukotrienes.² Especially, arachidonic acid metabolites, including prostaglandins and leukotrienes, mediate numerous acute and chronic allergic inflammatory reactions.^{3,4} Thus, mast cells and basophils are implicated in the development of allergic diseases such as asthma, allergic rhinitis, and inflammatory arthritis.⁵

RBL-2H3 cells originated from rat basophilic leukemia⁶ have been widely used to study IgE–Fc ϵ RI interactions, related to intracellular signaling pathways in degranulation, and to screen anti-allergic agents.^{7–9} Thus, RBL-2H3 cells have been regarded as a useful model for *in vitro* screening.

Separately, passive cutaneous anaphylaxis (PCA) is used as an animal model of IgE-mediated allergic response.^{10–13}

Garlic (*Allium sativum*), a member of the Lily family, has been used as a culinary spice and as a traditional medicine, and has some beneficial effects on various pathologies such as bacterial infection, hypoglycemia, oxidative stress, carcinogenesis, inflammation, hyperlipidemia, and immune system dysfunction.^{14,15} Raw garlic is known to possess several polyphenol compounds such as apigenin, quercetin, and myricetin.¹⁶ Although pungent organosulfur compounds in raw garlic possess potent biological activities,¹⁷ they cause undesirable effects such as gastric side effects, allergic reactions, and contact dermatitis.¹⁸ Therefore, the ingestion of raw garlic may limit its usefulness as a health-enhancing functional food. Aged garlic, prepared by treating raw garlic with several aging processes,^{19–27} possesses the advantage that it has lesser side effects than raw garlic.^{19,20} In addition, aged garlic expresses greater anti-oxidative capacity than raw garlic due to the increased levels of *s*-allyl cysteine and polyphenols.^{15,19} Especially, aged black garlic (ABG), prepared by employing high temperature and humidity,^{28,29} is characterized by increased viscosity, and the decreases in its unique flavor and odor.³⁰ Although ABG is known to have some biological activities,^{22,23,31} the effect of ABG on allergic responses has not been previously examined.

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In this study, we examined the anti-allergic effects of ethyl acetate extract (EBG) of ABG and BG10 (a fraction from C₁₈ chromatography of EBG) on IgE-mediated allergic responses in RBL-2H3 cells and the PCA reaction in mice. In addition, the mechanism for the anti-allergic action of BG10, one of the most active fractions of EBG, was elucidated.

MATERIALS AND METHODS

Reagents

Minimum essential medium (MEM) medium, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA). 4-[3-[4-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) was obtained from Dojindo (Kumamoto, Japan). Specific antibodies against phospho-Syk, phospho-Lyn, phospho-cPLA₂, Syk, Lyn, cytosolic phospholipase A₂ (cPLA₂), cyclooxygenase-2 (COX-2), and β -actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kit for TNF- α was obtained from e-Bioscience, Inc. (San Diego, CA, USA). Specific antibodies against 5-lipoxygenase (5-LO), phospho-5-LO, and enzyme immunoassay (EIA) kits for prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) were purchased from Cayman Chemical (Ann Arbor, MI, USA). 4-Nitrophenyl *N*-acetyl- β -D-glucosaminide (p-NAG), dinitrophenyl-IgE (DNP-IgE), and DNP-human serum albumin (DNP-HSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Plant material and fractionation

ABG was prepared according to a modification of a process previously reported;³¹ fresh garlic or ABG (2.6 kg), after homogenization, were extracted with 80% methanol (MeOH; 5 L) in a bath sonicator for 36 h, and then, the mixtures were filtered by Whatman No. 3 filter paper. The total filtrate was evaporated, and then, the residue of (892 g dry weight) ABG was dissolved in deionized water. The solution was mixed with three volumes of ethyl acetate, and then, the layer of ethyl acetate was separated and evaporated. The dried residue of ethyl acetate extract (11 g dry weight) was dissolved in MeOH, and then suspended in deionized water. The mixture was loaded onto an open column (C₁₈, 40–63 μ m), and then, the column was eluted by the mixture of MeOH and water (500 mL of each) to yield 15 fractions (Fig. 2A); 20% MeOH for BG1–3, 40% MeOH for BG4–7, 60% MeOH for BG8–11, 80% MeOH for BG12–13, and 100% MeOH for BG14–15. The dried residue of BG10 was estimated to be ~1 g with a yield of 9.09% from EBG. Raw garlic extract (RGE), EBG, or BG10 was dissolved in ethanol for *in vitro* tests, and in phosphate-buffered saline (PBS) buffer for *in vivo* experiments.

Determination of total phenolic and flavonoid contents

Total phenolic content in a sample was determined with Folin–Ciocalteu reagent according to the method.³² BG10

was dissolved using 20 mM PBS buffer (pH 7.4), to a final concentration of 100 mg/mL. The solution (0.33 mL) was transferred into a test tube containing 2.5 mL of distilled water, and then mixed with 0.16 mL of Folin–Ciocalteu reagent. After 5 min, 0.3 mL of 10% sodium bicarbonate solution was added. The mixture was incubated for 30 min in darkness, and the absorbance at 760 nm was measured using a spectrophotometer (DU650; Beckman Coulter, Brea, CA, USA). A standard curve was prepared to express the results as tannic acid equivalents. Separately, the content of total flavonoid in a sample was determined according to the method previously reported.³³ Briefly describing, 0.4 mL of BG10 was added to 4 mL of 90% diethylene glycol containing 0.4 mL of 1 N NaOH. The mixture was incubated for 1 h. The absorbance of the solution at 420 nm was measured using a spectrophotometer. A standard curve was prepared to express the results as naringin equivalents.

Animals

ICR mice, known as Swiss CD-1 mice³⁴ (5–6 weeks, 25–30 g), were procured from Nara Biotech Co. (Pyeongteak, Korea), and housed in cages (10 mice per cage) under specific pathogen-free conditions (21–24°C and 40–60% relative humidity) with a 12-h light/dark cycle, and were given free access to standard rodent food (Sangyang Co., Osen, Korea) and water. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publications No. 85-23, 1985, revised 1996), and approved by the Committee of Animal Care and Experiment of the Chungnam National University (CNU-00137).

Passive cutaneous anaphylaxis

IgE-mediated PCA reaction was evaluated following the previous method.³⁵ ICR mice were subcutaneously via ears that were injected with anti-DNP-IgE (1 μ g) diluted in 1 \times PBS using an insulin syringe. After 24 h, mice were orally administrated with BG10 (16.7–66.7 mg/kg), and 1 h later, they were intravenously administered by 100 μ g of DNP-HSA in 1 \times PBS containing 0.5% Evans blue. Thirty minutes later, the mice were euthanized by inhalation anesthesia, and the ear was harvested and incubated with 1 mL formamide for 2 h at 80°C. The mixture was homogenized and centrifuged (17,000 g, 10 min) at 4°C. The absorbance at 620 nm was measured using a micro-plate reader (Emax; Molecular Devices, Inc., Sunnyvale, CA, USA).

Cell culture

RBL-2H3 cells were cultured in MEM medium containing 5% (v/v) FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂ as previously described.³⁶ All *in vitro* tests contain a vehicle control group (0.1% ethanol).

Cytotoxicity assay

Cell respiration, an indicator of cell viability, was determined by measuring the mitochondrial-dependent reduction

of WST-1 to water-soluble tetrazolium salt.³⁷ Briefly, RBL-2H3 cells were seeded on a 96-well plate (2.5×10^4 cells/well) in MEM with 5% FBS at 37°C overnight. The cells were washed with $1 \times$ PBS, and then incubated with $1 \mu\text{g}/\text{mL}$ DNP-IgE for 24 h. The cells mentioned earlier, preincubated with EBG (0–2 mg/mL) or BG10 (0–100 $\mu\text{g}/\text{mL}$) for 1 h, were simultaneously mixed with 100 ng/mL DNP-HSA and 10 μL WST-1 reagent, and then incubated for another 4 h. The cell viability was determined by measuring the difference of absorbance at wavelength 450 nm.

β -Hexosaminidase release activity

RBL-2H3 cells were incubated in a 24-well plate (1×10^5 cells/well) at 37°C overnight. IgE-sensitized cells were preincubated with EBG or BG10 for 1 h, and then stimulated with DNP-HSA for 4 h. To measure the activity of β -hexosaminidase released from the cells, cultured media were centrifuged (17,000 g, 10 min) at 4°C. The supernatant (25 μL) was mixed with 50 μL of 0.1 M sodium citrate buffer (pH 4.5) containing 10 mM p-NAG in a 96-well plate, and then incubated for 1 h at 37°C. The reaction was terminated by 0.1 M bicarbonate buffer (pH 10.0). The absorbance was measured at 405 nm using a micro-plate reader.

ELISA of TNF- α

To measure TNF- α level in cultured media, all cultured media were centrifuged at 4°C, and the samples were stored at -80°C until assay. TNF- α was quantified using an ELISA kit according to the manufacturer's instructions.

EIA analysis of PGE₂ and LTB₄

To determine the levels of PGE₂ and LTB₄ in cultured media, all cultured media were centrifuged at 4°C, and the samples were stored at -80°C until they were analyzed. PGE₂ and LTB₄ were determined using EIA kits according to the manufacturer's instructions.

Immunoblotting analysis

Immunoblotting analysis was evaluated following the method previously reported.³⁸ The membranes were incubated with a 1:1000 dilution of specific antibodies against phospho-Syk, Syk, phospho-Lyn, Lyn, phospho-cPLA₂, cPLA₂, phospho-5-LO, 5-LO, COX-2, or β -actin. The blots were washed with TBS-T, and then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated IgG secondary antibody (Cell Signaling Technology, Inc.). The proteins on membranes were detected using a chemiluminescent reaction (ECL plus kit; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), followed by exposure of the membranes to Hyperfilm ECL (Amersham Pharmacia Biotech). The levels of target proteins were compared with those of loading controls (β -actin or nonphosphorylated protein), and the results were expressed as a ratio of density of each protein, identified by protein standard size marker. The density of each band was determined using ImageJ software (version 1.46r for Windows; NIH).

Statistical analyses

The experimental results were listed as means \pm SD or SEM. One-way analysis of variance (ANOVA) was used for multiple comparisons (GraphPad Prism version 4.03 for Windows, San Diego, CA, USA). If there was a significant variation between treated groups, the Dunnett test was applied. Differences at the $*P < .05$ and $**P < .01$ levels were considered statistically significant.

RESULTS

Inhibitory effects of RGE, EBG, or BG10 on IgE-induced allergic response

To evaluate the effect of RGE or EBG on IgE-induced allergic response, IgE-sensitized RBL-2H3 cells were preincubated with RGE or EBG (0.1–2 mg/mL) for 1 h before antigen challenge. RGE significantly inhibited the release of β -hexosaminidase at 0.1 or 0.5 mg/mL, but not at ≥ 1 mg/mL (Fig. 1A). Although RGE strongly suppressed the release of TNF- α (Fig. 1C), it showed remarkable cytotoxicity at ≥ 0.05 mg/mL (Fig. 1E). However, EBG dramatically decreased IgE-induced release of β -hexosaminidase (IC₅₀, 1.53 mg/mL) and TNF- α (IC₅₀, 0.98 mg/mL) as shown in Figure 1B and D. Moreover, EBG, at concentrations showing a remarkable anti-degranulation effect, did not show any significant cytotoxicity (Fig. 1F). Taken together, these findings suggest that EBG preparation can exhibit anti-allergic action at noncytotoxic concentrations.

Effects of fractions, derived from EBG, on IgE-induced allergic response

To obtain the fractions containing anti-allergic components from EBG, EBG was fractionated by C₁₈ open column (40–63 μm) chromatography to give rise to 15 fractions (Fig. 2A). To evaluate the inhibitory effect of each fraction on allergic response, IgE-sensitized RBL-2H3 cells were exposed to each fraction (100 $\mu\text{g}/\text{mL}$) for 1 h before stimulation with DNP-HSA, and then, the release of β -hexosaminidase and TNF- α was determined. As shown in Figure 2B and C, some fractions (BG3–13) significantly attenuated the release of β -hexosaminidase and TNF- α . Especially, BG10 and BG11 at 100 $\mu\text{g}/\text{mL}$ had a potent inhibitory effect on the release of β -hexosaminidase with 81.3–82.5% inhibition and TNF- α with 94.1–95.5% inhibition, in comparison with the positive control (HSA). These results indicate that two fractions, BG10 and BG11, exhibit a greater suppression of degranulation than EBG.

Inhibitory effect of BG10 on IgE-induced allergic response and formation of pro-inflammatory lipid mediators

Next, to determine the inhibitory effect of BG10 on IgE-induced allergic response, IgE-sensitized RBL-2H3 cells were preincubated with BG10 (25–100 $\mu\text{g}/\text{mL}$) before antigen challenge. As shown in Figure 3, BG10 markedly inhibited the release of β -hexosaminidase (IC₅₀, 53.60 $\mu\text{g}/\text{mL}$)

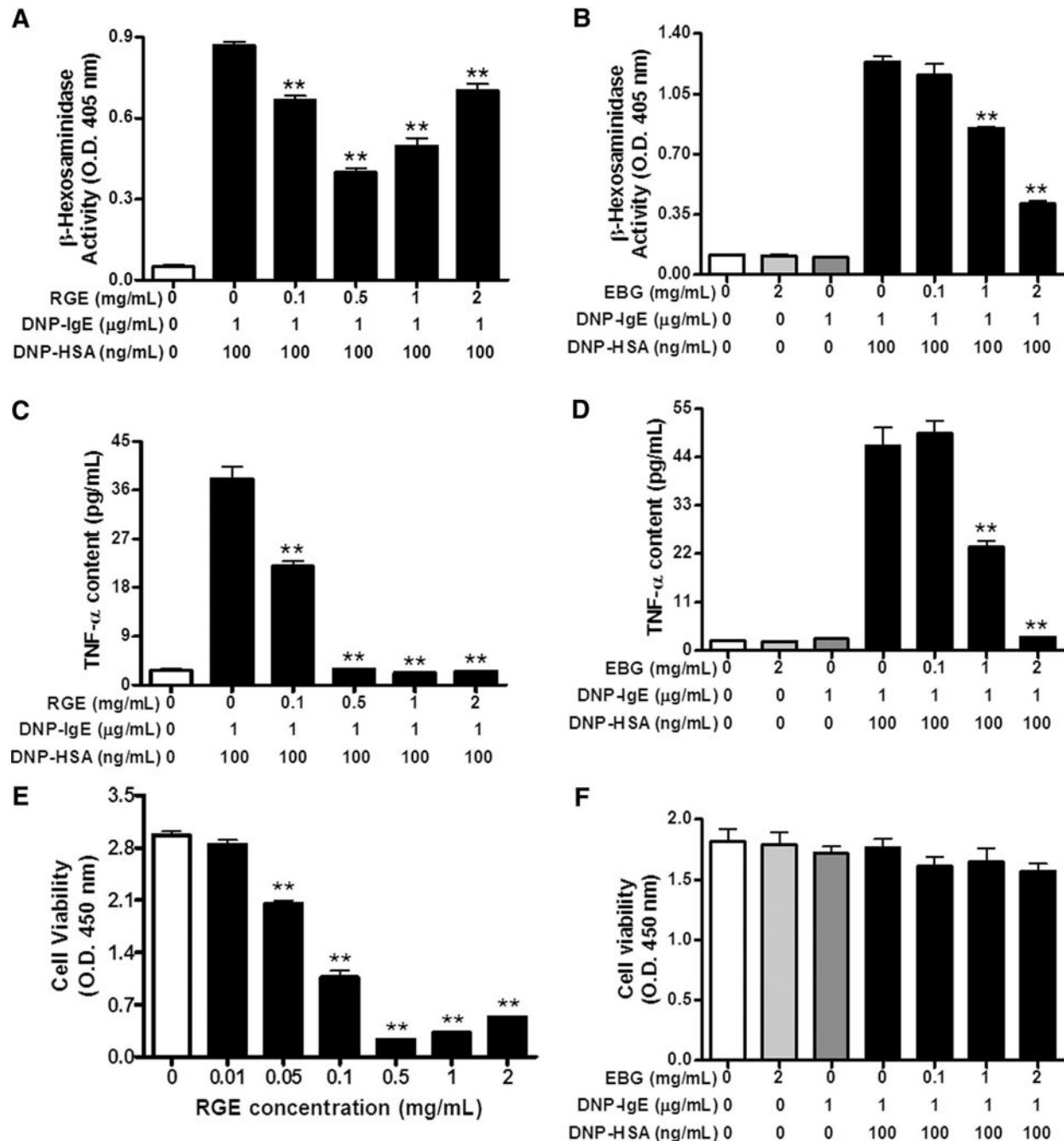


FIG. 1. Effect of raw garlic extract (RGE) or ethyl acetate extract (EBG) on the release of β -hexosaminidase (A, B) or TNF- α (C, D) and the cell viability (E, F) in immunoglobulin E (IgE)-activated RBL-2H3 cells. RBL-2H3 cells were seeded on a 24-well plate (1×10^5 cells/well) in MEM with 5% FBS at 37°C overnight, and further incubated with dinitrophenyl-IgE (DNP-IgE) for 24 h. IgE-sensitized cells were exposed to RGE or EBG (0–2 mg/mL) for 1 h, and then stimulated with DNP-human serum albumin (HSA; 100 ng/mL) for 4 h. Separately, IgE-sensitized cells were exposed to RGE or EBG (0–2 mg/mL) for 1 h, and then simultaneously treated with DNP-HSA (100 ng/mL) and WST-1 reagent (10 μ L) for 4 h. The β -hexosaminidase activity, TNF- α level, and cell viability were determined as described in **Materials and Methods**. Data are the mean \pm SD values of triplicate determinations. ** $P < .01$ versus DNP-HSA-treated group.

and TNF- α (IC_{50} , 27.80 μ g/mL). Moreover, BG10, at concentrations showing anti-degranulation effects, had no cytotoxicity (Fig. 3C). Subsequently, we investigated the effect of BG10 on the production of PGE₂ and LTB₄, representative pro-inflammatory lipid mediators that are im-

plicated in acute and chronic allergic reactions.^{2–4} As shown in Figure 3D and E, the BG10 at ≥ 25 μ g/mL remarkably inhibited the formations of PGE₂ ($\geq 125\%$) and LTB₄ ($\geq 84\%$). These results suggest that BG10, rich in phenolic (10.6%) and flavonoid (3.5%) components (Table 1), may

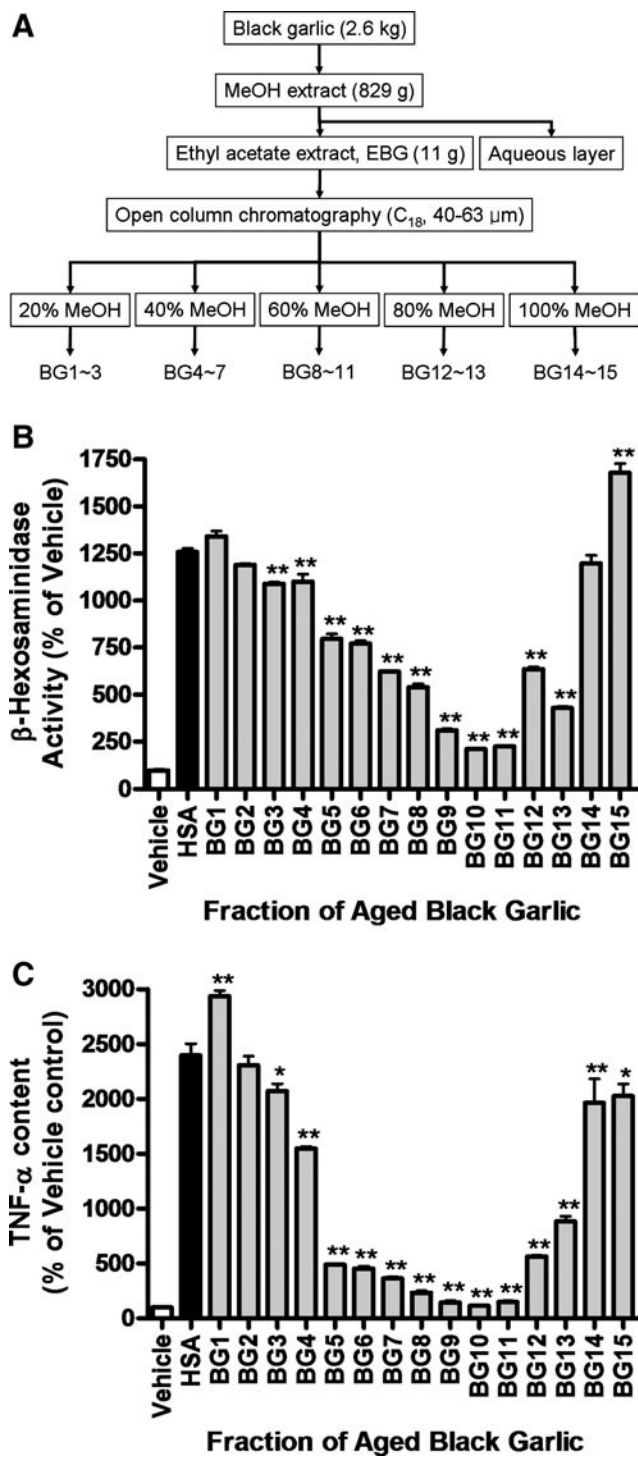


FIG. 2. (A) The fractionation scheme of aged black garlic. Effect of fractions, derived from EBG, on the release of β -hexosaminidase (B) or TNF- α (C) in IgE-activated RBL-2H3 cells. The release of β -hexosaminidase activity or TNF- α was determined as described in Figure 1. Data are the mean \pm SD values of triplicate determinations. * P < .05 and ** P < .01 versus DNP-HSA-treated group.

be effective for alleviating acute and chronic allergic response.

Effect of diallyl sulfide, diallyl disulfide, diallyl trisulfide, or ajoene on IgE-mediated degranulation of RBL-2H3 cells

Aged garlic is known to contain various organosulfur compounds such as ajoene, diallyl sulfide (DAS), diallyl disulfide (DADS), or diallyl trisulfide (DATS).³⁹ Therefore, we investigated whether an anti-allergic action of BG10 was associated with these organosulfur compounds. When IgE-sensitized RBL-2H3 cells were preincubated with DAS, DADS, DATS, or ajoene (1–100 μ M) before antigen challenge, there was no significant suppression of IgE-induced degranulation by these organosulfur compounds. Although ajoene inhibited the degranulation in a concentration manner (Fig. 4D), it showed a remarkable cytotoxicity at ≥ 50 μ M (Fig. 4E). From these results, it is suggested that anti-allergic action of BG10 may not be associated with organosulfur compounds.

Regulatory effect of BG10 on Fc ϵ RI or arachidonate cascade

We further examined the mechanism for anti-allergic action of BG10. The activation of Fc ϵ RI receptor is known to be associated with phosphorylation of Lyn and Syk, followed by degranulation of mast cells.⁴⁰ In this regard, BG10, showing a strong anti-allergic action, was supposed to potentially affect phosphorylation of Lyn or Syk. When IgE-sensitized RBL-2H3 cells were preincubated with BG10 (100 μ g/mL) for 1 h before antigen challenge, and the incubation was extended for another 10 min, BG10 was found to partially inhibit phosphorylation of Syk, but not phosphorylation of Lyn (Fig. 5A). In a separate experiment to determine the effect of BG10 on arachidonate cascade, IgE-sensitized RBL-2H3 cells were preincubated with BG10 (100 μ g/mL) for 1 h before antigen challenge, and the incubation was extended for another 4 h. As shown in Figure 5B, BG10 was observed to inhibit phosphorylation of cPLA₂, an enzyme of a limiting step for arachidonate cascade, and 5-LO, an enzyme for leukotriene biosynthesis, as well as expression of COX-2, an enzyme for prostaglandin biosynthesis. All these findings indicate that BG10 inhibits not only activation of Syk, but also activation of cPLA₂ and 5-LO in IgE-activated mast cells. In addition, it also inhibits the expression of COX-2. Thus, it is suggested that the anti-allergic action of BG10 may be mediated by the suppression of the arachidonate cascade.

Suppressive effect of BG10 on IgE-mediated PCA

Finally, we evaluated the anti-allergic action of BG10 in an *in vivo* system. The PCA model is a general animal model for evaluation of anti-allergic drugs.¹² Therefore, we investigated the anti-allergic action of BG10 in PCA model using mice. When IgE-sensitized ICR mice were orally administrated with BG10 at various doses (16.7–66.7 mg/kg)

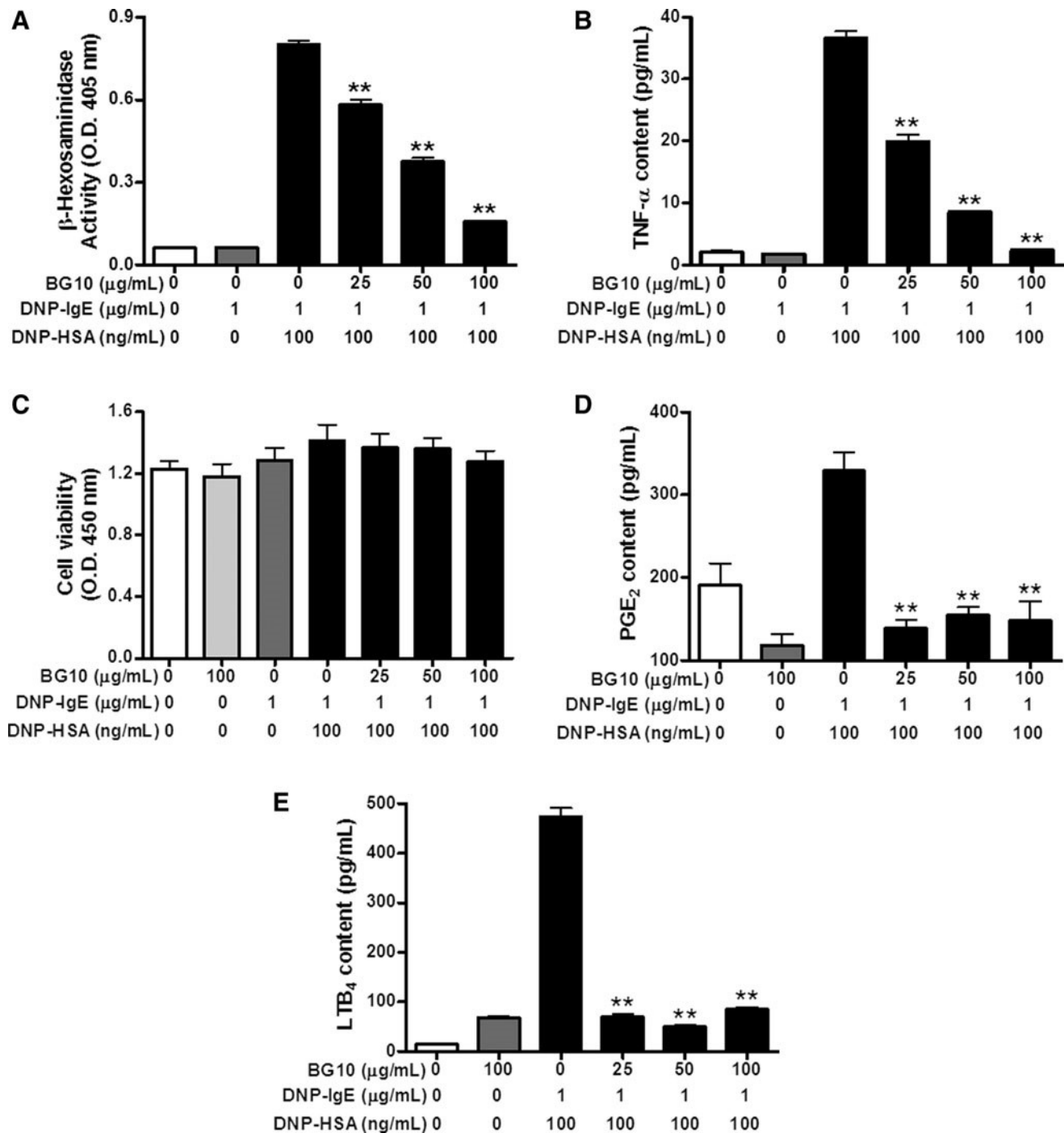


FIG. 3. Effect of BG10 on the release of β -hexosaminidase (A) or TNF- α (B) the formation of prostaglandin E₂ (D; PGE₂) or leukotriene B₄ (E; LTB₄), and the cell viability (C) in IgE-activated RBL-2H3 cells. The release of β -hexosaminidase activity or TNF- α , and cell viability were determined as described in Figure 1, respectively. Separately, IgE-sensitized cells were exposed to BG10 (0–100 $\mu\text{g/mL}$) for 1 h, and then stimulated with DNP-HSA (100 ng/mL) for 4 h. The amount of PGE₂ or LTB₄ was determined as described in **Materials and Methods**. Data are the mean \pm SD values of eight determinations. ** $P < .01$ versus DNP-HSA-treated group.

for 1 h before antigen challenge, it was found that BG10 at 66.7 mg/kg significantly decreased the PCA reaction (Fig. 6). This result provides evidence that BG10 can effectively suppress IgE-induced allergic response *in vivo* system.

DISCUSSION

Although garlic has a long history of use as a spice in foods and as a component of traditional medicine, it can induce allergic responses and cytotoxicity due to some

TABLE 1. COMPONENTS OF TOTAL PHENOLICS AND TOTAL FLAVONOIDS IN BG10

	Total phenols	Total flavonoids
BG10 (mg/g dry weight)	106.20 ± 5.00	35.35 ± 2.10

Data are the mean ± SD values of triplicate determinations.

allergen proteins or organosulfur compounds such as alliin.^{18,39} Therefore, aged garlic, devoid of cytotoxic components such as alliin or allergenic proteins, has been preferred by some as a functional food and an element of traditional medicine.

Previously, aged garlic was reported to possess various biological actions such as anti-oxidant, anti-cancer, anti-inflammation, antibiotic, and anti-arteriosclerosis actions.^{41,42}

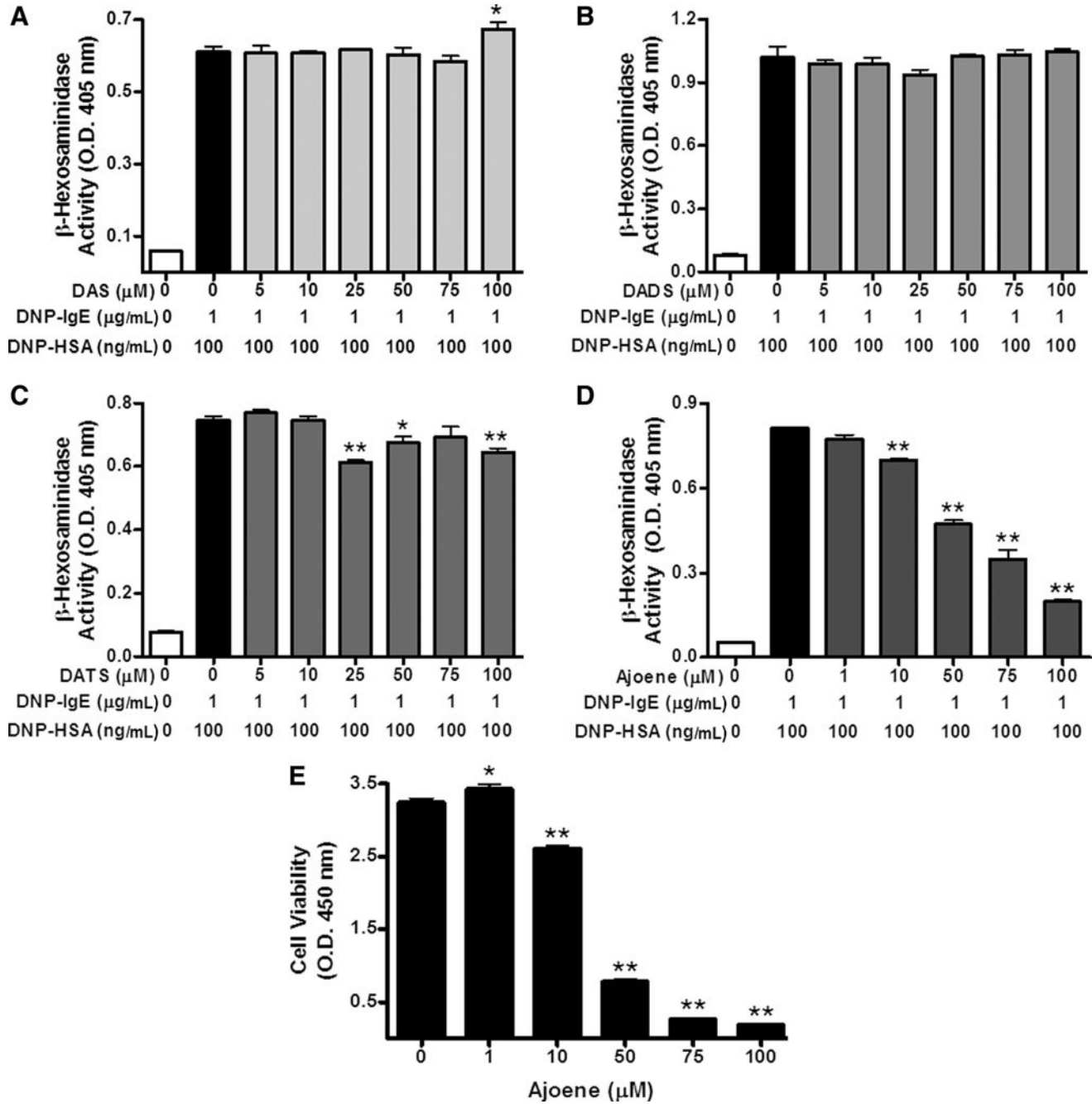


FIG. 4. Effect of diallyl sulfide (A; DAS), diallyl disulfide (B; DADS), diallyl trisulfide (C; DATS), or ajoene (D) on degranulation, and the cell viability (E) in IgE-activated RBL-2H3 cells. The release of β-hexosaminidase activity and cell viability were determined as described in Figure 2, respectively. Data are the mean ± SD values of eight determinations. *P < .05 and **P < .01 versus DNP-HSA-treated group.

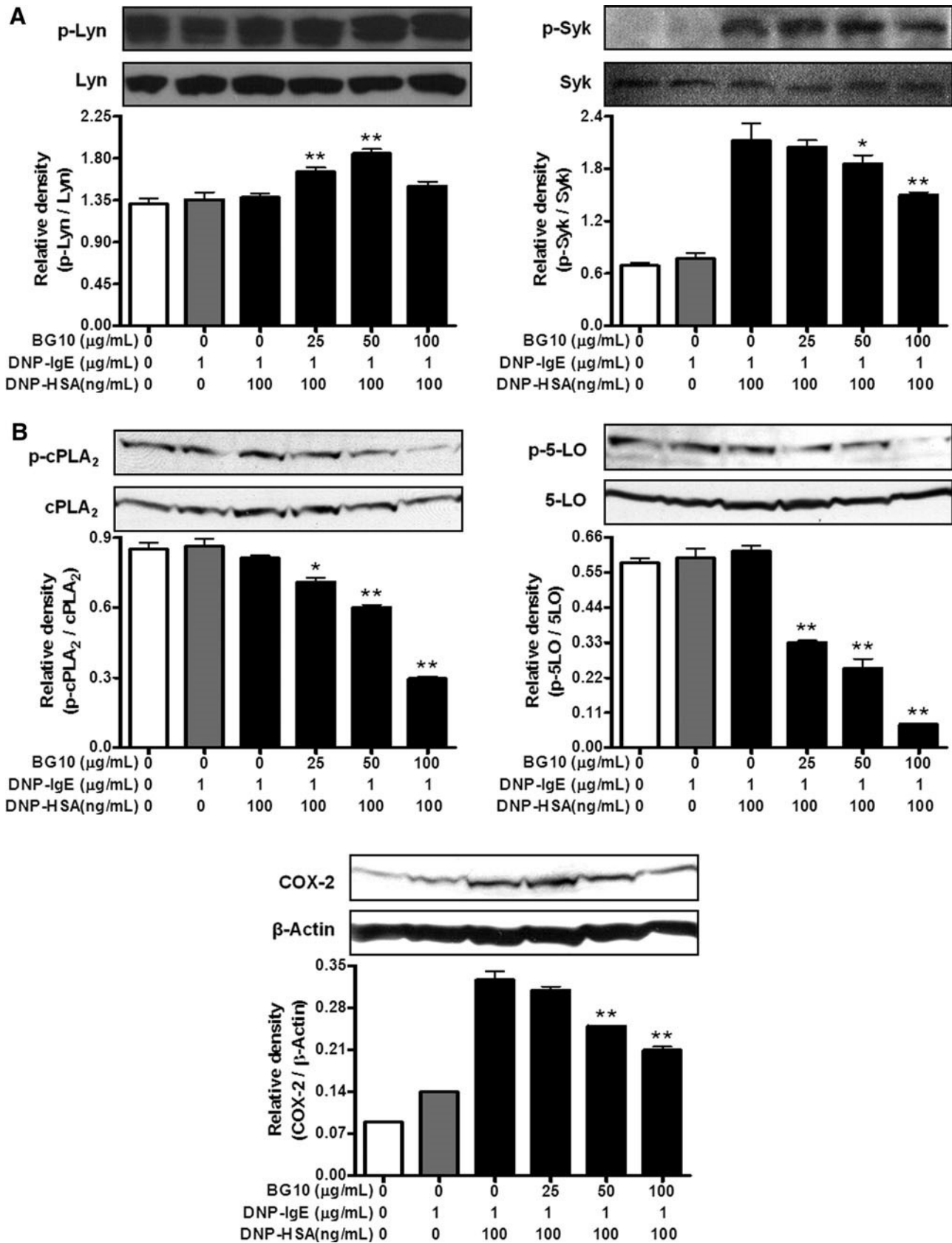


FIG. 5. Effect of BG10 on Fc ϵ RI cascade or arachidonate cascade in IgE-activated RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were exposed to BG10 (0–100 μ g/mL) for 1 h, and then stimulated by DNP-HSA (100 ng/mL) for 10 min or 4 h. The cells were rinsed with 1 \times phosphate-buffered saline, and lysed with cell lysis buffer. The expression of Lyn and p-Lyn, (A), Syk and p-Syk (B), cytosolic phospholipase A₂ (cPLA₂) and p-cPLA₂ (C), 5-lipoxygenase (5-LO) and p-5LO (D), or cyclooxygenase-2 (COX-2) and β -actin (E) was determined as described in **Materials and Methods**. Similar results were obtained in three independent experiments. * P < .05 and ** P < .01 versus DNP-HSA-treated group. (A) p-Lyn & p-Syk; (B) p-cPLA₂, p-5-LO & COX-2.

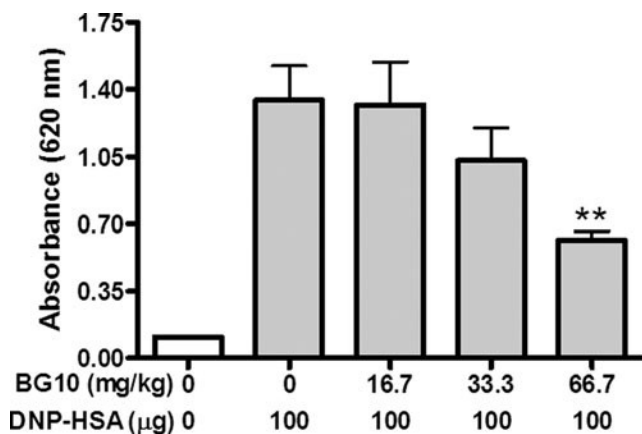


FIG. 6. Inhibitory effect of BG10 on IgE-mediated passive cutaneous anaphylaxis reaction in mice. IgE-sensitized mice were orally administrated with BG10 (16.7–66.7 mg/kg) for 1 h, and then intravenously injected with 100 μ g DNP-HSA containing 2% Evans blue. After 30 min, the mice described earlier were euthanized, and their ears were excised. Extravasated dye in the ear was analyzed as described in **Materials and Methods**. Data are listed as mean \pm SEM values of eight determinations. ** $P < .01$ compared with DNP-HSA-treated group.

However, there are limited reports with regard to anti-allergic actions of ABG except immunomodulatory actions of ethanol-treated garlic⁴³ and anti-bronchitis effect of glycoprotein derived from aged garlic.⁴⁴

Our present data demonstrate that EBG exerts anti-allergic actions, based on *in vitro* and *in vivo* tests. Especially, EBG had an advantage compared with RGE, as the former had a much lower cytotoxicity than the latter. Moreover, BG10, derived from EBG, was much more potent than EBG in suppressing the release of β -hexosaminidase (~ 29 times) and TNF- α (~ 35 times). Although the anti-allergic action of BG11 seems to be somewhat higher than that of BG10, the yield of BG11 (0.91%) is lower than that of BG10 (9.09%).

Generally, aged garlic is known to contain various sulfides such as ajoene, DAS, DADS, or DATS, in addition to allyl cysteine and alliin corresponding to major organosulfur compounds in fresh garlic.³⁹ Although ajoene showed anti-degranulation activity in our study, it showed a remarkable cytotoxicity at concentrations exerting anti-degranulation action. Furthermore, DAS, DADS, or DATS failed to inhibit IgE-mediated degranulation. Thus, the sulfide compounds present in ABG are not likely to be responsible for the anti-allergic action of EBG or BG10. Alternatively, polyphenols such as flavonoids may be responsible for anti-allergic action, as some flavonoids such as apigenin or quercetin⁴⁵ and some phenolic acids such as cinnamic acid⁴⁶ present in garlic have been observed to inhibit phosphorylation of Syk in IgE-activated RBL-2H3 cells.⁴⁷ Probably consistent with this, the level of polyphenols was observed to be increased in ABG.^{28,30} Likewise, BG10 seemed to be rich in phenolic (10.6%) and flavonoid contents (3.5%).

One possible mechanism for the anti-allergic activity of BG10 may be related to a direct suppression of the Fc ϵ RI

receptor activation by BG10. The degranulation of IgE-activated mast cells is associated with the activation of Fc ϵ RI receptor, a high-affinity IgE receptor, and activation of the receptor induces liberation of various inflammatory mediators such as TNF- α , histamine, leukotrienes, and prostaglandins via phosphorylation of some tyrosine kinases⁴⁸; some of the immunoreceptor tyrosine-based activation motifs are phosphorylated by Lyn after antigen-mediated activation of the receptor.⁴⁹ In turn, the activation of Syk leads to an increase in intracellular Ca²⁺ and the activation of MAP kinases.^{50,51} Thus, Lyn and Syk are important intracellular mediators of the early signaling pathway of Fc ϵ RI receptor activation. In the present study, a remarkable, although partial, inhibition of Syk activation by BG10 may support the notion that one of the targets for BG10 may be Syk, an important upstream protein in Fc ϵ RI receptor activation. In support of this, BG10 dramatically decreased the activation of cPLA₂, 5-LO, and COX-2, belonging to downstream proteins in Fc ϵ RI receptor activation. In further support, BG10 decreased the levels of PGE₂ and LTB₄, corresponding to COX-2 product and 5-LO product, respectively, which are known to be enhanced in diverse immune cells, including mast cells.^{52,53} Nevertheless, we cannot exclude the possibility that the inhibition of 5-LO or COX-2 may be caused through other routes, as the effect of BG10 on Syk activation was lower than that of BG10 on activation of 5-LO or COX-2. Such a suppressive effect of BG10 on levels of PGE₂ and LTB₄ may contribute to its anti-allergic action, as LTB₄ is a potent chemoattractant and activator for neutrophils and other immune cells in severe asthma,^{52,54} and PGE₂ may contribute to asthma development and inflammation associated with type 2 cytokines such as IL-4 and IL-5.⁵³ Taken together, it is suggested that BG-10 can suppress allergic reactions by decreasing the activation of Syk as well as activities of cPLA₂, 5-LO, and COX-2. Further, such an effect of BG10 on arachidonate cascade enzymes may be extended to anti-inflammatory action in other cells or tissues. Several cytokines may play critical roles in allergic inflammation.⁵⁵ For example, TNF- α , secreted from IgE-activated mast cells,⁵⁶ is known to play an important role in allergic response.⁵⁵ Therefore, the inhibitory effect of EBG or BG10 on TNF- α formation may provide an additional advantage of EBG or BG10 as an anti-allergic agent.

Finally, BG10, at a moderate dose, was devoid of acute toxicity and significantly suppressed IgE-induced anaphylaxis in PCA model. Furthermore, ABG had no chronic toxicity in rats.³¹ Thus, it is affirmed that ABG may be a safe and effective agent for the alleviation of allergic response.

In conclusion, this study demonstrated that EBG or BG10 possesses anti-allergic functions *in vitro* and *in vivo* systems. Moreover, BG10 may be concentrated with anti-allergic components that are extracted from ABG, because BG10 has more potent anti-allergic action than EBG. These findings revealed a beneficial feature of EBG in IgE-induced allergic responses. The mechanisms for the anti-allergic action of EBG or BG10 may include multiple targets such as Syk, cPLA₂, 5-LO, and COX-2. Such anti-allergic actions of

EBG or BG10 may provide further information for the application of ABG preparation as functional food or a preventive agent.

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AUTHOR DISCLOSURE STATEMENT

No potential conflicts of interest were disclosed.

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