

Inhibitory Effects of Green Asparagus Extract, Especially Phospholipids, on Allergic Responses *in Vitro* and *in Vivo*

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ABSTRACT: Asparagus (*Asparagus officinalis* L.) is one of the widely consumed vegetables. To investigate the mechanism underlying the anti-allergic responses of asparagus, we extracted different fractions from asparagus and measured their inhibitory effects on β -hexosaminidase release in RBL-2H3 cells *in vitro* and an atopic dermatitis NC/Nga mouse model *in vivo*. The lipid fractions from asparagus were extracted with 50% ethanol, separated using chloroform by liquid–liquid phase separation, and fractionated by solid-phase extraction. Among them, acetone fraction (rich in glycolipid) and MeOH fraction (rich in phospholipid) markedly inhibited β -hexosaminidase release from RBL-2H3 cells. In NC/Nga mice treated with picryl chloride, atopic dermatitis was alleviated following exposure to the 50% EtOH extract, acetone fraction, and methanol fraction. The inhibitory effects of asparagus fractions *in vivo* were supported by the significant decrease in serum immunoglobulin E (IgE) levels. The phospholipid fractions showed significantly better inhibitory effects, and phosphatidic acid from this fraction showed the best inhibitory effect on β -hexosaminidase release. In mice challenged with ovalbumin (OVA), oral administration of asparagus extract and its fractions decreased the OVA-specific IgE level and total IgE, indicating that these effects may be partly mediated through the downregulation of antigen-specific IgE production. Taken together, the present study shows for the first time that asparagus extract and its lipid fractions could potentially mitigate allergic reactions by decreasing degranulation in granulocytes. Our study provides useful information to develop nutraceuticals and functional foods fortified with asparagus.

KEYWORDS: asparagus, phospholipids, degranulation, β -hexosaminidase, anti-allergy

INTRODUCTION

Allergy is one of the major health concerns in the modern world. While several therapeutic regimens have been developed to treat allergy, their undesirable side-effects are well-known.¹ Hence, plant-based therapeutics have gained popularity as relatively safe options. For instance, food components such as methylated catechin, strictonin, pectin, and metabolic enzymes are known to exhibit anti-allergic properties.^{2–5} Allergic diseases such as allergic asthma, rhinitis, and atopic dermatitis are categorized as type I hypersensitivity reactions that are provoked by the degranulation of granulocytes, including mast cells and basophils.^{6–8} Immunoglobulin (IgE) binds to high-affinity IgE receptor (Fc ϵ RI) expressed on granulocytes, and the cross-linking between an allergen and IgE to Fc ϵ RI results in the release of inflammatory mediators such as histamine, leukotriene, cytokines, and chemokines through degranulation. The binding of a specific allergen to a receptor-bound IgE may increase the concentration of intracellular Ca²⁺, leading to the granulocyte-mediated release of preformed mediators and arachidonic acid metabolites. The consequences include initiation of an allergic reaction, including vasodilation, smooth muscle contraction, and mucosal edema. Thus, the regulation of the degranulation process may serve as an important strategy to treat, relieve, or prevent allergic reaction.

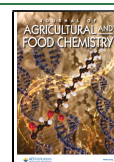
Asparagus (*Asparagus officinalis* L.) is one of the most widely consumed vegetables worldwide that is rich in several nutrients such as folic acid and potassium as well as non-nutrient ingredients such as rutin and saponin.⁹ Some beneficial effects of asparagus include its antioxidative, anti-dementia, anti-inflammatory, and anti-hypertensive activities.^{10–13} For instance, consumption of 6 g of powdered asparagus per day for 10 weeks was shown to improve hypertension, hyperglycemia, and dyslipidemia in generally healthy subjects.¹² Although not common, some individuals are allergic to asparagus. The lipid transfer proteins derived from asparagus are identified as relevant allergens in such individuals.¹⁴ How the non-protein components in asparagus, such as lipids, modulate allergic reactions is, however, unclear. In this direction, here we investigated the allergic response of different fractions extracted from asparagus using organic solvents. We extracted asparagus in 50% ethanol (EtOH) and fractionated the extract into lipid subclasses such as phospholipids. We measured the effects of these fractions on allergic reaction *in vitro*.

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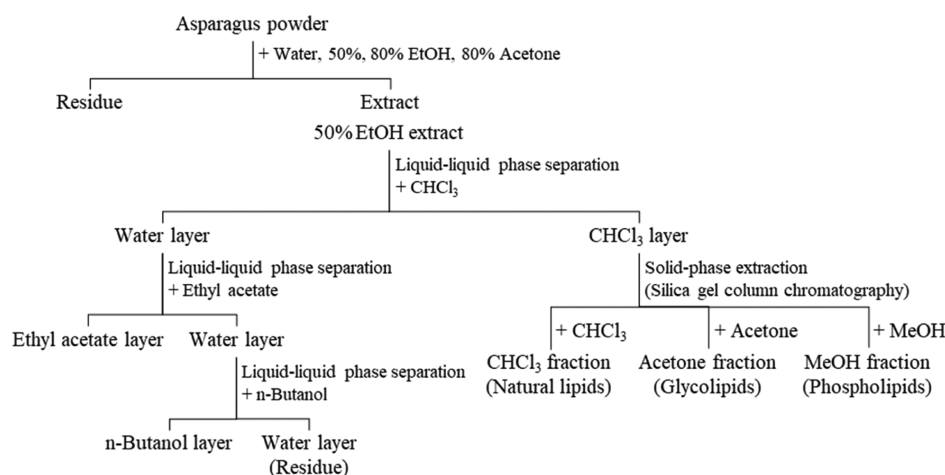


Figure 1. Scheme of the extraction and fractionation of lipids from asparagus.

MATERIALS AND METHODS

Chemicals. A23187 was obtained from BioVision Inc (Milpitas, CA, USA), and picryl chloride (2,4,6-trinitrochlorobenzene) was obtained from Tokyo Kasei (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo (Waltham, MA, USA), and glucosylceramide from rice, β -sitosterol-3-O-glucoside, and Wakogel C-300HG were procured from Wako Pure Chemical Industries (Osaka, Japan). Phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and thin-layer chromatography (TLC) silica gel 60 F254 and preparative liquid chromatography silica gel 60 F254 were supplied by Millipore (Burlington, MA, USA). Cell Counting Kit-8 (CCK-8) and *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide were acquired from Dojindo (Kumamoto, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively. To measure the serum IgE level, anti-mouse IgE and horseradish peroxidase (HRP)-conjugated anti-mouse IgE were obtained from Novus Biologicals (Centennial, CO, USA). Other reagents were of analytical grade and used without further purification.

Green Asparagus Extracts. Green asparagus shoot was obtained from JA Saga (Saga, Japan) in July 2016 and lyophilized after blanching at 90 °C for 10 min. The lyophilized asparagus was pulverized, stored at −20 °C, and extracted in 100 volumes of water, 50 or 80% EtOH, or 80% acetone for 4 h at room temperature, as shown in Figure 1. After centrifugation at 13,000g for 30 min, the supernatant was collected by filtration through no. 2 filter paper.

The 50% EtOH extract was recovered into water and chloroform (CHCl_3) layers by adding CHCl_3 . The extract was concentrated 10 times and mixed with the same volume of EtOH and two volumes of CHCl_3 and vigorously shaken. Each layer was collected after incubating overnight at 4 °C. The water layer was further separated by liquid–liquid phase separation using the same volume of ethyl acetate and *n*-butanol (Figure 1).

Extraction of Lipid Fractions from Asparagus. The CHCl_3 layer was fractionated into lipid classes by solid-phase extraction using silica gel column chromatography according to King *et al.* (Figure 1).¹⁵ Fifty volumes of activated silica gel were added to the sample and packed in an open column. The column was washed with CHCl_3 , and the dried CHCl_3 layer was re-dissolved in CHCl_3 at a concentration of 200–500 $\mu\text{g}/\text{mL}$. The CHCl_3 layer was applied onto the column, and each fraction was eluted with 5–10 volumes of CHCl_3 , acetone, and methanol (MeOH) against 1 volume of silica gel. Neutral lipids were eluted in the CHCl_3 fraction, and glycolipids were eluted in the acetone fraction. Phospholipids were finally collected in the MeOH fraction.

Phospholipids in the MeOH fraction were further isolated by TLC using $\text{CHCl}_3/\text{MeOH}/\text{acetic acid}/\text{water}$ (20/3.5/2.3/0.7, v/v/v/v) or

$\text{CHCl}_3/\text{EtOH}/\text{water}/\text{triethylamine}$ (30/35/7/35, v/v/v/v) as a developing solvent. Two solvents were used to verify that each phospholipid was not superimposed by another one. Phospholipid spots were detected by UV lamp (UV254 nm), and then individual phospholipids were scraped to purify by the method of Bligh & Dyer.¹⁶ Characterization of purified phospholipids was carried out using phospholipid standards and detected by 50% sulfuric acid.

Determination of Total Phosphorus. The content of phosphorus in phospholipid fractions was quantitatively determined. Dried phospholipid fractions (5–200 μg) were hydrolyzed using 70% perchloric acid by heating at 165 °C for 1.5 h and then 190 °C for 5 h. The hydrolyzed fractions were mixed with ammonium molybdate (2.5%) and then ascorbic acid (10%) and heated at 60 °C for 10 min. The mixture was cooled on ice, and its absorbance was measured at 450 nm wavelength using a microplate reader (Molecular Devices, LLC, San Jose, CA, USA).

Cells and Cell Culture. To determine the inhibitory effect of asparagus fractions on degranulation, rat basophilic leukemia RBL-2H3 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C under humidified 5% CO_2 -95% air.

Cell Viability. Cell viability was measured using the CCK-8 assay, according to manufacturer's instruction. RBL-2H3 cells (2.0×10^5 cells/mL) were cultured in DMEM supplemented with 10% FBS in the presence or absence of the prepared samples under humidified 5% CO_2 -95% air. After incubation, the culture medium was replaced with that containing 10% CCK-8 solution. The supernatant was collected, and its absorbance was measured at 450 nm (reference wavelength, 650 nm) using a microplate reader.

β -Hexosaminidase Release. As β -hexosaminidase is released in parallel with histamine from activated granulocytes, β -hexosaminidase released was measured as a degranulation marker in the presence of A23187. In brief, RBL-2H3 cells (5.0×10^5 cells) were washed and resuspended in Tyrode's buffer and treated with A23187 at a final concentration of 10 μM . The mixture was incubated at 37 °C for 30 min in the presence or absence of the asparagus fractions, and the reaction was terminated by cooling the system on ice for 5 min. The medium was centrifuged, and the release of β -hexosaminidase in the supernatant was measured. In total, 50 μL of the supernatant was transferred to a microtube and incubated with 125 μL of 2.0 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in 0.1 M citrate buffer (pH 4.5) for 30 min at 37 °C. The enzyme reaction was terminated by adding 100 μL of 0.1 M glycine buffer (pH 10). The absorbance was determined at 405 nm using a microplate reader. The β -hexosaminidase release was calculated as follows: $100 \times (\text{Abs}_{405}^{\text{sample}} / \text{Abs}_{405}^{\text{control}})$.

Experimental Animal. Male 6-week-old NC/Nga mice and female 6-week-old BALB/c mice were purchased from Japan SLC

(Shizuoka, Japan) and Kyudo (Fukuoka, Japan), respectively. Mice were housed under a controlled atmosphere (temperature, 22 ± 1 °C, humidity, $55 \pm 5\%$; 12 h light/dark cycle) and fed with a commercial chow and tap water *ad libitum* during acclimation. All experiments were undertaken in strict accordance with appropriate guidelines for handling laboratory animals and law (no. 105) and Notification (no. 6) of Japanese Government for the welfare of experimental animals. All procedures were approved by the Animal Care and Use Committee of Nishikyushu University (H28-3).

Evaluation of Severity in Skin Lesions. To investigate the effect of 50% EtOH extract, acetone fraction, and MeOH fraction on skin manifestation in atopic dermatitis mice, the animals were divided into four groups (9 mice per group) after a week's acclimation.

Atopic dermatitis was induced according to the pathogenic protocol described elsewhere.¹⁷ In brief, 5% picryl chloride dissolved in EtOH/acetone (3:1, v/v) was topically applied to the shaved chest and abdomen of each mouse from all groups on day 1. In addition, 1% picryl chloride dissolved in olive oil was applied to the shaved back and ear once a week from day 4. Mice were administered with the following samples each day *via* oral gavage: control [phosphate buffered saline (PBS)], 50% EtOH extract (500 mg/kg/day), acetone fraction (10 mg/kg/day), and MeOH fraction (10 mg/kg/day).

The severity of dermatitis was evaluated once a week. The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe).¹⁷ The sum of individual scores was taken as the dermatitis score. At week 12 after immunization, the level of mouse IgE antibody was measured using anti-mouse IgE and HRP-conjugated anti-mouse IgE.

Evaluation of Ovalbumin-Specific Allergic Reaction. To investigate the effect of the test agents on ovalbumin (OVA)-immunized mice, BALB/c mice were orally administrated with PBS or PBS containing 50% EtOH extract (500 mg/kg/day), acetone fraction (10 mg/kg/day), or MeOH fraction (10 mg/kg/day) once a day. All mice were primed with OVA (Sigma, St. Luis, MO, USA) to induce specific allergic response as follows: 20 μ g of OVA mixed with aluminum hydroxide (20 μ g OVA/1 mg Al(OH)₃ in 1 mL) was intraperitoneally injected on the first day of the experiment. This immunization was repeated four times at an interval of 1 week. At day 28 after the first intraperitoneal injection, total IgE and OVA-specific IgE levels in sera were measured by an enzyme-linked immunosorbent assay (ELISA).

Statistics. Data obtained are expressed as means $\pm$ SEM. One-way analysis of variance (ANOVA) was used to assess statistical significance of the difference against control groups. Values of * p < 0.05, ** p < 0.01, and *** p < 0.001 were considered statistically significant. Different small letters on bars represent statically significant difference at 0.05.

RESULTS

Effect of Asparagus Extracts on β -Hexosaminidase Release in RBL-2H3 Cells. We examined the effects of different asparagus extracts on the release of β -hexosaminidase from RBL-2H3 cells, which were incubated with the test agents. As shown in Figure 2, 50% EtOH extract and 80% EtOH extract significantly reduced β -hexosaminidase release as compared with the vehicle treatment without inducing any cytotoxicity. Thus, the asparagus extracts tested herein exerted the inhibitory effects on degranulation. In particular, the 50% EtOH extract at 10 μ g/mL concentration could significantly lower the A23187-induced β -hexosaminidase release by 45% as compared to the control, indicating that 50% EtOH extract was more potent than the others. Therefore, we used 50% EtOH extract in the subsequent experiment.

We separated 50% EtOH extract into four layers using CHCl₃, ethyl acetate, and *n*-butanol by liquid–liquid phase separation (Figure 1). The release of β -hexosaminidase from

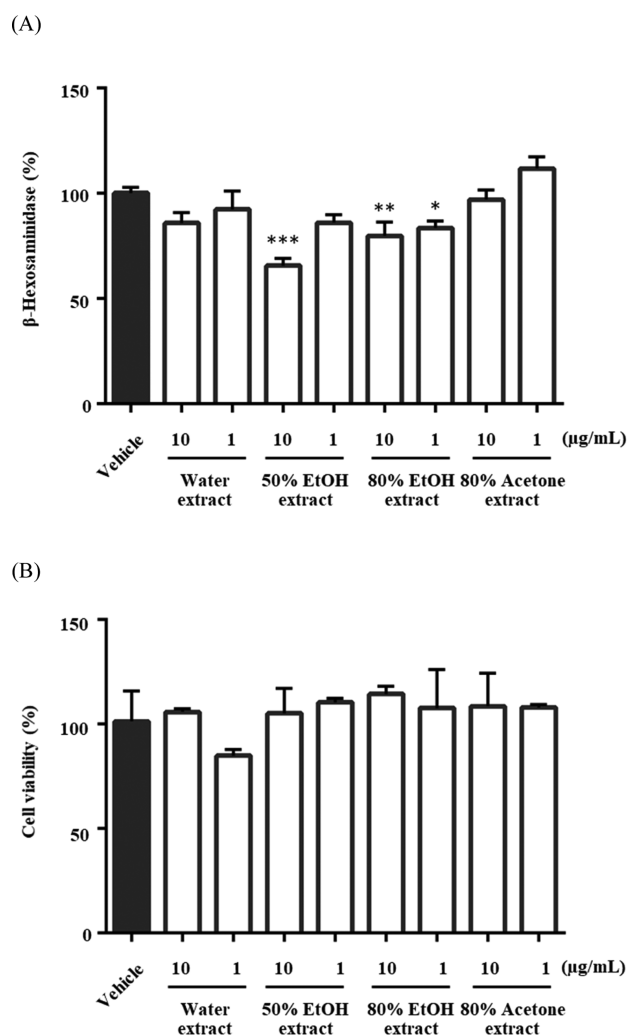


Figure 2. Effects of asparagus extracts on the degranulation of RBL-2H3 cells. RBL-2H3 cells were stimulated with A23187 (10 μ M) in the presence of asparagus extracts to induce the release of β -hexosaminidase. (A) Release of β -hexosaminidase. (B) Cell viability. Data are expressed as means $\pm$ SEM (n = 3). Statistically significant differences from the control are shown as * p < 0.05, ** p < 0.01, and *** p < 0.001.

RBL-2H3 cells was measured in the presence of 4 μ g/mL CHCl₃ layer, 20 μ g/mL ethyl acetate layer, *n*-butanol layer, or water layer (Figure 3). We found that the most effective suppression of β -hexosaminidase release was achieved using the CHCl₃ layer, although 50% EtOH extract and ethyl acetate layers also significantly repressed the release of the enzyme as compared with the control treatment.

Further Purification of the Bioactive Fractions from Asparagus. To identify the inhibitory molecules in the 50% EtOH extract against A23187-induced degranulation, the CHCl₃ layer derived from the 50% EtOH extract was fractionated into different lipid classes by solid-phase extraction using CHCl₃, acetone, and MeOH (Figure 1). These fractions were assessed for their effects on the degranulation of RBL-2H3 cells (Figure 4). Aside from the significantly enhanced effect of 50% EtOH extract on the release of β -hexosaminidase, the acetone and MeOH fractions also greatly inhibited the degranulation process as compared with the control. In particular, the strongest inhibitory effect was observed with 10 μ g/mL MeOH fraction. According to

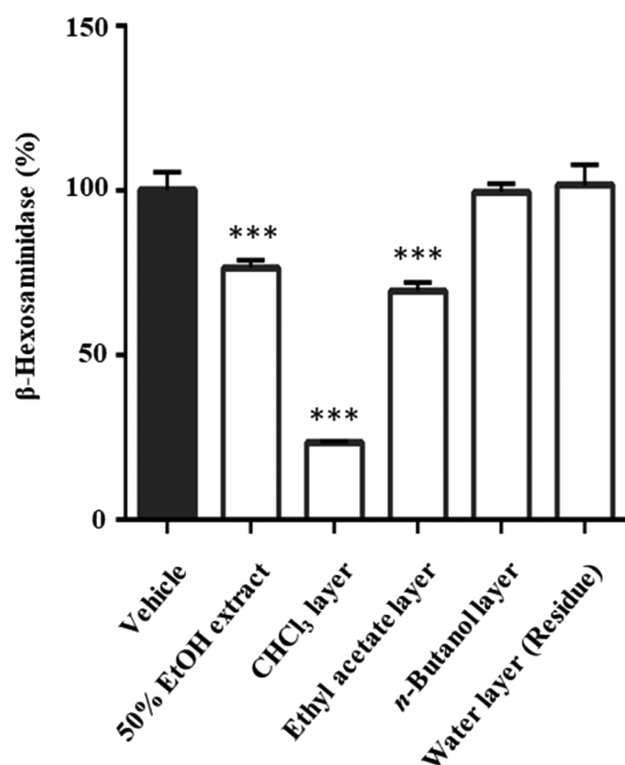


Figure 3. Inhibitory effects of asparagus extracts prepared by liquid–liquid phase separation on the A23187-induced release of β -hexosaminidase. RBL-2H3 cells were treated with the extracted fractions (50% EtOH extract at 20 μ g/mL, CHCl₃ layer at 4 μ g/mL, ethyl acetate layer, *n*-butanol layer, and water layer at 20 μ g/mL) in the presence of A23187. Data are expressed as means $\pm$ SEM ($n = 3$). Statistically significant differences from the control are represented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

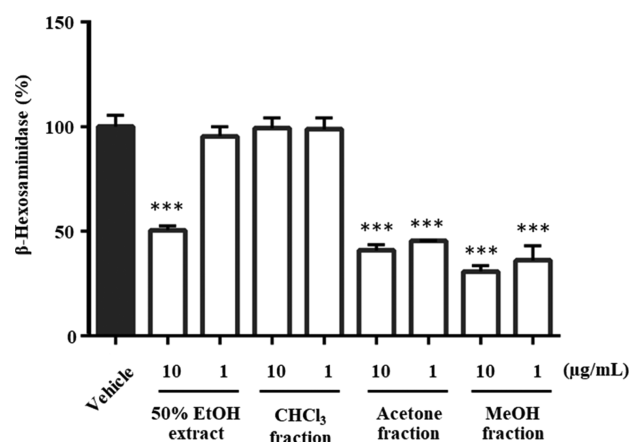


Figure 4. Effects of the 50% EtOH extract and lipid fractions prepared by solid-phase extraction on the A23187-induced release of β -hexosaminidase. RBL-2H3 cells were treated with the extracted fractions (1 and 10 μ g/mL) in the presence of A23187. Data are expressed as means $\pm$ SEM ($n = 3$). Statistically significant differences from the control are represented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

King *et al.*,¹⁵ the acetone fraction was defined as glycolipids and the MeOH fraction as phospholipids.

Oral Administration of the Lipid Fractions Resulted in the Suppression of Skin Manifestation in NC/Nga Mice. As our *in vitro* study showed that the effectiveness of the

acetone and MeOH fractions on the inhibition of the degranulation of RBL-2H3 cells, the effects of the 50% EtOH extract, acetone fraction, and MeOH fraction were compared in an atopic dermatitis model *in vivo* (Figure 5). NC/Nga mice developed skin inflammation-like atopic dermatitis following exposure to picryl chloride. After 12 weeks of feeding, no significant difference was observed in the body weight of mice from the four groups (data not shown). The severity of dermatitis was evaluated every week. The treatment with 50% EtOH extract (500 mg/kg/day), acetone fraction (10 mg/kg/day), and the MeOH fraction (10 mg/kg/day) remarkably improved the appearance of skin symptoms at the back and in the ear and inhibited the severity of dermatitis symptoms (Figure 5A). As early as at week 7, mice treated with acetone fraction or MeOH fraction showed significant amelioration of symptoms. Representative clinical features of NC/Nga mouse skin after treatment for 12 weeks are shown in Figure 5B. The mean score of skin severity following treatment with 50% EtOH extract, acetone fraction, and MeOH fraction decreased to 3.3 ± 0.9 , 4.9 ± 1.5 , and 5.2 ± 1.9 at the back, and 2.3 ± 1.3 , 3.2 ± 1.3 , and 3.1 ± 0.9 in the ear, respectively, at week 12. The control group, on the other hand, showed a gradual increase in skin severity score that remained high (6.4 ± 0.7 and 4.9 ± 0.9 for the back and ears, respectively). Furthermore, the levels of serum IgE also diminished in the mice administered with 50% EtOH extract, acetone fraction, or MeOH fraction (Figure 6). Matsumoto *et al.* showed that skin dermatitis symptoms correlate with total IgE levels in the plasma.¹⁸ These findings suggest that 50% EtOH extract, acetone fraction, and MeOH fraction may repress the dermatitis score at least in part by decreasing serum IgE levels and that asparagus may exert anti-allergic functions *in vivo*.

Composition of Lipid Components in Asparagus. The extraction of the constituents from the 50% EtOH extract yielded 97.6% water layer and 2.4% CHCl₃ layer. The acetone and MeOH fractions comprised 31.7% and 53.0% of CHCl₃ layer, respectively (Table 1A). Phospholipids were further isolated into subclass by TLC, as shown in the results using two development solvents (Figure 7). We measured the molar ratios based on phosphorous content and found that the composition of PA, PE, PG, PC, and PI was 7.3, 10.5, 22.1, 42.0, and 18.0%, respectively (Table 1B).

Effect of Phospholipids Derived from Asparagus on Degranulation. To investigate the exact class of phospholipids that exhibited anti-allergic properties, we examined the effect of phospholipids separated by TLC on the degranulation of RBL-2H3 cells. As shown in Figure 8, individual phospholipids significantly decreased the release of β -hexosaminidase, and the inhibitory activity was in the order of PA > PG = PE > PI > PC. While PC was the primary component, it exerted least inhibitory effects on the degranulation of β -hexosaminidase. Among the phospholipids derived from soybean, PE and PG were found to exhibit relatively strong inhibitory effects on the release of β -hexosaminidase (data not shown). Together with *in vivo* study results, we suggested that phospholipids mediate inhibitory effects on degranulation at least in RBL-2H3 cells.

Effect of the Asparagus Fractions on IgE Production. Considering the type 1 allergy, the binding of an allergen to Fc ϵ RI results in a series of biochemical reactions such as T cell activation and IgE production in B cells, consequently leading to IgE-mediated degranulation.⁸ As asparagus exerted inhibitory effects on β -hexosaminidase release, we investigated

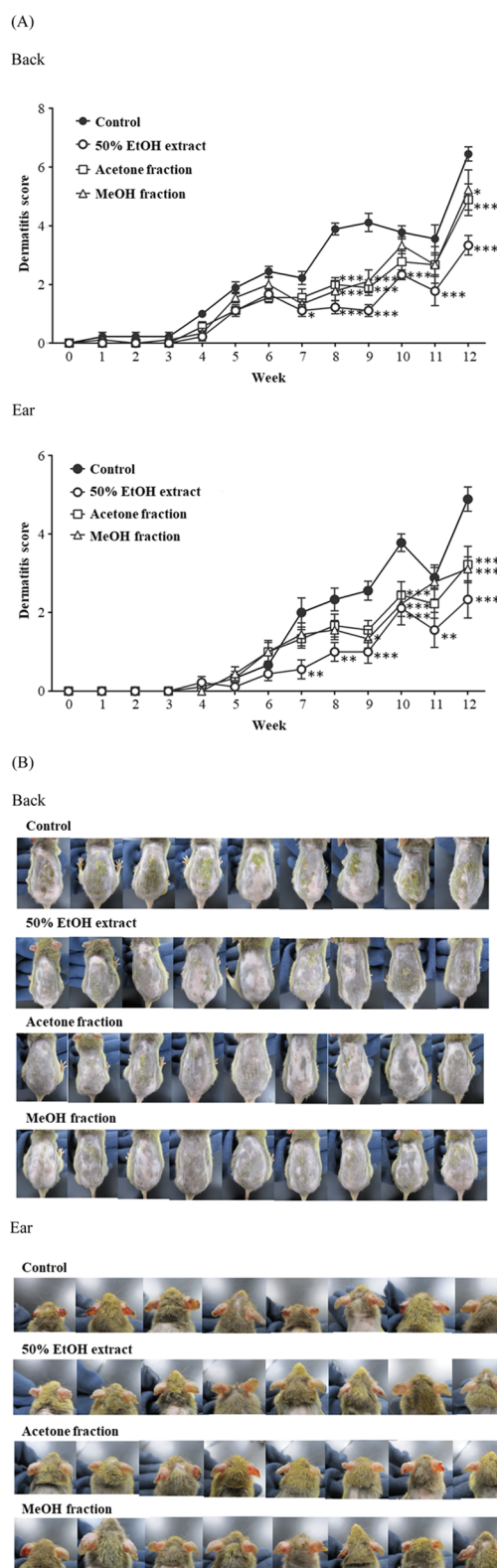


Figure 5. Effects of the 50% EtOH extract and lipid fractions on the clinical scores of skin symptoms in NC/Nga mice. The 50% EtOH extract (500 mg/kg) and the acetone and MeOH fractions (10 mg/kg) were orally administrated to NC/Nga mice daily for 12 weeks. (A) Dermatitis score at the back and in the ear. (B) Appearance of atopic dermatitis at the back and in the ear. Data are expressed as means $\pm$ SEM ($n = 9$). Statistically significant differences from the control are represented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

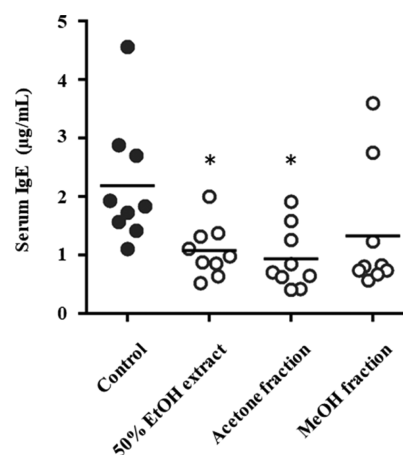


Figure 6. Effects of the 50% EtOH extract and lipid fractions on serum IgE levels in NC/Nga mice treated with 5% picryl chloride. The 50% EtOH extract (500 mg/kg/day) and acetone and MeOH fractions (10 mg/kg/day) were orally administrated to NC/Nga mice daily for 12 weeks. The concentrations of serum IgE were measured by ELISA. Data are expressed as means $\pm$ SEM ($n = 9$). Statistically significant differences from the control group are represented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

whether these asparagus fractions produced any effect on the allergy cascade other than degranulation by measuring the levels of total and antigen-specific IgE. Female 6-week-old mice were challenged with OVA/aluminum hydroxide on the first day of the experiment and then intraperitoneally injected with OVA once a week. Mice were orally administered PBS or PBS containing 50% EtOH extract (500 mg/kg/day), the acetone fraction (10 mg/kg/day), or the MeOH fraction (10 mg/kg/day) once every day for 28 days. No difference was observed in the body weight of mice from the four groups during the experimental period (data not shown). IgE levels in sera were detected on day 29 and found to be significantly decreased following oral administration with 50% EtOH extract, acetone fraction, and MeOH fraction (Figure 9A). Further, these test agents significantly lowered the OVA-specific IgE levels in sera as compared with the control treatment (Figure 9B). Thus, the oral administration of 50% EtOH extract, acetone fraction, and MeOH fraction could inhibit degranulation and production of allergen-specific IgE level in sera.

DISCUSSION

Asparagus exhibits various biological functions such as anti-inflammatory, anti-dementia, and anti-hypertensive effects.^{10–13} However, no reported study has evaluated the anti-allergic effects of asparagus fractions. Contrary to the fact that asparagus induces allergic reaction in some individuals,¹⁴ the current study shows that asparagus fractions could inhibit A23187-induced degranulation of RBL-2H3 cells and that its administration palliates skin manifestation in an atopic dermatitis mouse model. Further, we provide evidence that asparagus fractions, especially phospholipid fraction, potentially alleviate allergic response.

To gain insight into the constituents of asparagus that mediate anti-allergic responses, we first extracted lipids, and further fractionated the extracted asparagus (Figure 1). As the CHCl_3 layer exerted the most suppressive effect on degranulation *in vitro* among the four layers separated by liquid–liquid phase separation (Figure 3), the bioactive

Table 1. Composition Ratio of the Lipid Fractions and Phospholipids^a

(A)				
50% EtOH residue		50% EtOH extract		
46.5% (± 0.8)		53.5% (± 0.8)		
		Water layer	CHCl ₃ layer	
		97.6% (± 0.2)	2.4% (± 0.2)	
		CHCl ₃ fraction	Acetone fraction	MeOH fraction
		15.3% (± 1.7)	31.7% (± 0.7)	53.0% (± 1.7)
(B)				
Phospholipid fraction				
PA	PE	PG	PC	PI
7.3% (± 2.9)	10.5% (± 3.3)	22.1% (± 3.1)	42.0% (± 6.2)	18.0% (± 4.3)

^aRatio was calculated based on (A) weight of lipid fractions and (B) phosphorus content in phospholipids.

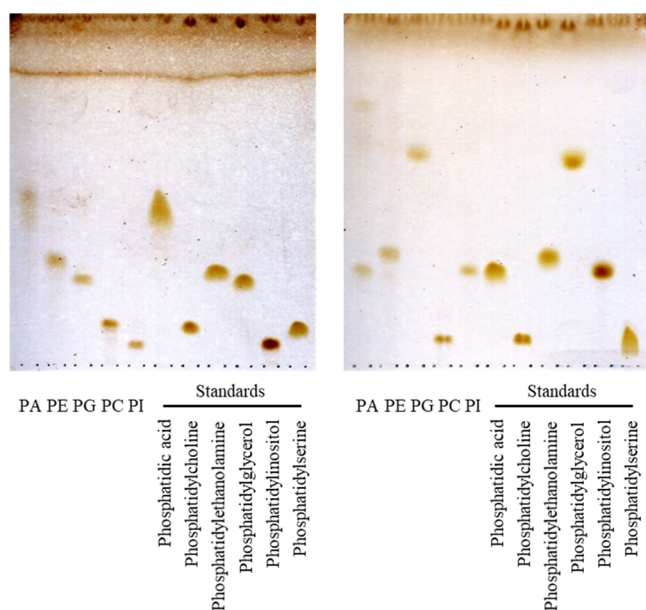


Figure 7. Thin layer chromatogram of phospholipid fractions from the MeOH fraction. TLC was developed by CHCl₃/MeOH/acetic acid/water (20/3.5/2.3/0.7, v/v/v/v, left panel) or CHCl₃/EtOH/water/triethylamine (30/35/7/35, v/v/v/v, right panel) and detected by 50% sulfuric acid.

constituent(s) was thought to be hydrophobic. Analysis of fractions prepared with solid-phase extraction showed that the acetone fraction rich in glycolipids and the MeOH fraction rich in phospholipids decreased the release of β -hexosaminidase from RBL-2H3 cells (Figure 4). Further, the administration of these fractions resulted in the amelioration of dermatitis skin manifestation in NC/Nga mice, accompanied with a decrease in serum IgE levels. Thus, the fractions of glycolipid and phospholipid in the extract were in part responsible for mediating inhibitory effects on degranulation. Moreover, these fractions could lower the levels of serum allergen-specific IgE in the OVA-immunized mice, indicative of the involvement of the asparagus fractions in the production of IgE. It is well known that the degranulation is induced by binding of IgE to Fc ϵ RI and then cross-linking to the allergen.⁶ From our *in vitro*

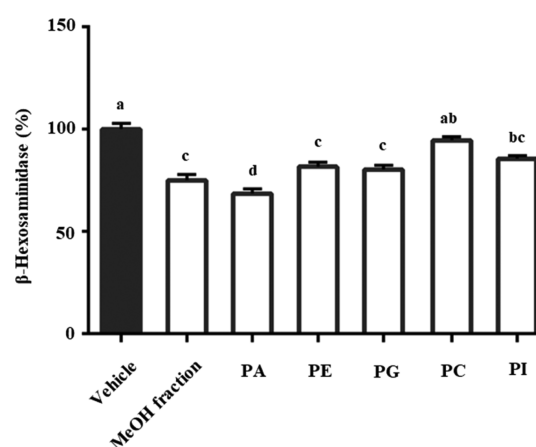


Figure 8. Effects of the MeOH fraction and isolated phospholipid fractions on the A23187-induced release of β -hexosaminidase. RBL-2H3 cells were treated with the fractions (10 nmol/mL) in the presence of A23187. Data are expressed as means $\pm$ SEM ($n = 3$). Different small letters on bars represent statistically significant differences at 0.05 significant level.

and *in vivo* studies, it is suggested that asparagus extract and its fractions alleviate allergic symptoms by affecting both the serum IgE level and the degranulation. Previous reports have shown that polyphenols from different plants are immunomodulators. Negi *et al.*⁹ found that asparagus is rich in rutin and saponin. Rutin, 3-rutinoside glycoside of quercetin, and quercetin were shown to inhibit IgE- or A23187-mediated histamine release from RBL-2H3 cells.^{19–22} Oral administration of rutin and quercetin (7.5 mg/kg) ameliorated allergic responses in an OVA-sensitized asthma model (guinea pig).²³ Furthermore, saponin derived from *Gleditsia* and *Quillaja* was found to exhibit inhibitory effects on histamine release from mast cells and RBL-2H3 cells,^{24,25} while the oral administration of saponin from tea leaf mitigated OVA-induced allergic reaction in guinea pigs and rats.²⁶ Up to now, no reports, however, have revealed the anti-allergic responses mediated by lipid classes such as phospholipids. Thus, to the best of our knowledge, this is the first study highlighting glycolipids and phospholipids in asparagus as the potential anti-allergic candidates.

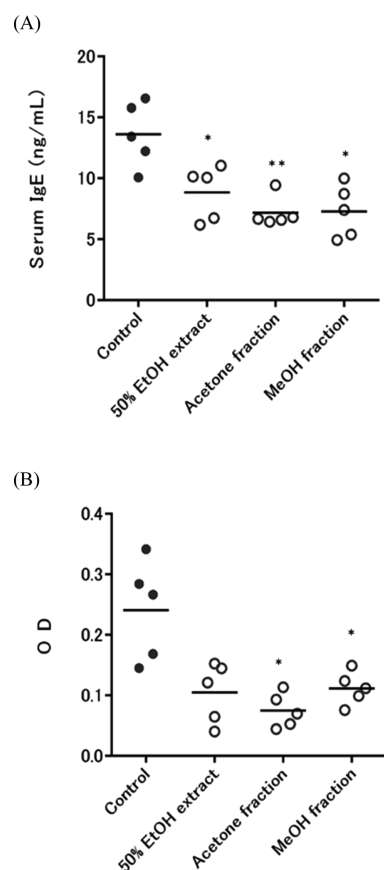


Figure 9. Effect of the oral administration of the 50% EtOH extract and lipid fractions on IgE levels in OVA-immunized mouse sera. Female 6-week-old BALB/c mice were orally administrated with the 50% EtOH extract (500 mg/kg/day) and acetone or MeOH fractions (10 mg/kg/day). Mice were challenged by OVA-mixed aluminum hydroxide (20 μ g OVA/1 mg Al(OH)₃ in 1 mL volume), and immunization was repeated for four times at an interval of 1 week intervals. At day 28 after first sensitization, sera Ig levels were measured by ELISA. (A) Total IgE levels in sera. (B) OVA-specific IgE levels in sera. Bar showing the average value of five independent measurements. Statistically significant differences from the control group are represented as * p < 0.05 and ** p < 0.01.

Besides phospholipids, glycolipids also accounted for the amelioration of degranulation. The role of glycolipids in modulating degranulation is not clear. However, our previous studies have shown changes in the intestinal microbial flora of C57BL/6N mice and cholesterol metabolism of obese mice fed with glycolipids derived from a fungus.^{27,28} Further, fungal 9-methyl-spingsadiene, a metabolite of glycolipids, is known to be a ligand for peroxisome proliferator-activated receptor and G-protein coupled receptor 120, both of which are known to be related to the development of obesity.²⁹ Although glycolipids modulate glucose and lipid metabolism, the lack of clarity about the relation between glycolipids and allergic response may encourage further studies in this field.

To elucidate the bioactive constituents, we focused on the MeOH fraction. As shown in Figure 7, the MeOH fraction was isolated by TLC and comprised PA, PG, PE, PC, and PI. The effects of these phospholipids on degranulation *in vitro* were determined. In comparison with MeOH fraction (unfractionated phospholipids), PA exerted significant inhibitory activity. The inhibitory activity of all the phospholipid components was in the order of PA > PG = PE > PI > PC. Phospholipids are

composed of hydrophobic (e.g., fatty acid) and hydrophilic (e.g., choline, ethanolamine, serine or inositol) constituents that exhibit different physiological functions.³⁰ Some of them play an important role in regulating signal transduction, membrane integrity, and neuronal development.^{31–33} Phospholipids have been known to be involved in degranulation.³⁴ Upon binding to the Fc ϵ RI in permeabilized RBL-2H3 cells,³⁵ antigens trigger a signaling cascade of degranulation, comprising phospholipids, phosphatidylinositol (3,4,5)-trisphosphate (PIP3), and proteins such as spleen tyrosine kinase and phospholipase C γ 1.³⁶ During degranulation, secretory granules are fused with the plasma membrane, wherein phospholipids form a major component. Thus, phospholipids may be one of the immunomodulators in cells. The present study clearly shows the strongest inhibitory activity of PA among all phospholipids. PA is important for cell signaling and is converted to diacylglycerol, a signaling molecule.³⁷ Taken together, phospholipids play a pivotal role in manipulating allergic reactions. However, no reports have thus far shown that the direct or indirect participation of dietary phospholipids in the inhibition of degranulation.

Considering the regulation of allergy by lipids, fatty acids, particularly polyunsaturated fatty acids, have been well studied. Lipid metabolites are involved in the induction and/or suppression of allergies.^{38–40} Prostaglandins and leukotrienes, the metabolites derived from n-6 fatty acids, trigger allergic reactions.^{41–45} In contrast, the metabolites derived from n-3 fatty acids, such as resolvins and protectins, have been reported to suppress allergy.^{46–49} Dietary fatty acids are incorporated into phospholipids in the plasma membrane *via* phospholipid biosynthesis and are known to regulate inflammation through the biosynthesis of lipid mediators.^{33,35} The MeOH fraction (phospholipids) was analyzed for fatty acid composition by gas chromatography–mass spectrometry, and the molar ratio of palmitic acid, linoleic acid, α -linolenic acid, and other fatty acids was found to be 33.5, 57.8, 7.4, and 1.2%, respectively (data not shown). As the amount of α -linolenic acid, anti-allergic fatty acid was found to be low in asparagus, the roles of individual fatty acids in regulating allergic response are currently unknown.

In conclusion, asparagus mitigated allergic response *in vitro* and *in vivo*, and phospholipids were at least in part responsible for the decreased release of β -hexosaminidase and relieved symptoms of atopic dermatitis. Further studies are warranted to understand the mechanism underlying the asparagus-mediated amelioration of allergic response to develop nutraceuticals and dietary supplement fortified with asparagus.

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Author Contributions

A.I. conceived and designed the experiment. A.I. and H.H. equally performed the experiment and wrote the paper. K.T. and Y.T. partially analyzed data. Y.N., H.Y., and T.Y. supervised and helped with the editing of the manuscript.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

IgE, immunoglobulin E; FcεRI, high-affinity IgE receptor; DMEM, Dulbecco's modified Eagles' medium; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; CCK-8, Cell Count Kit-8; HRP, horseradish peroxidase; TLC, thin layer chromatography; FBS, fetal bovine serum; PBS, phosphate buffered saline; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; PIP3, phosphatidylinositol (3,4,5)-trisphosphate

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