

Anti-allergic action of buckwheat (*Fagopyrum esculentum* Moench) grain extract

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Abstract

The anti-allergic action of buckwheat grain extract (BGE) was investigated using rodent experimental models. The oral, intraperitoneal and intradermal administration of BGE significantly inhibited the compound 48/80-induced vascular permeability documented by Evans blue extravasation. In addition, BGE showed potent inhibitory effect on passive cutaneous anaphylaxis (PCA) activated by anti-dinitrophenyl (DNP) IgE when orally administered. In an in vitro study, BGE revealed to possess inhibitory potential on the compound 48/80-induced histamine release from rat peritoneal mast cells (RPMC). Moreover, BGE inhibited the IL-4 and TNF- α mRNA induction by PMA and A23187 in human leukemia mast cells, HMC-1. Taken together, these results suggest that anti-allergic action of BGE may be due to the inhibition of histamine release and cytokine gene expression in the mast cells.

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1. Introduction

It is well known that allergic inflammation is induced by IgE-dependent mast cell activation, which manifests as bronchial asthma, allergic rhinitis and atopic dermatitis. This includes not only rapidly provoked immediate phase reaction, but also protracted late phase reaction. Upon IgE-dependent activation, mast cells release immediately the preformed and de

novo synthesized mediators such as histamine, proteases, leukotrienes, prostaglandins and cytokines [1]. As a consequence, the acute reactions such as vasodilation, increased vascular permeability and bronchocontraction were induced. In addition, allergic responses also trigger the influx and activation of a variety of inflammatory cells including eosinophils and lymphocytes. Rapidly released mediators and numerous cytokines produced by mast cells are strongly believed to induce and sustain these responses, which may contribute to chronic inflammation [2]. In atopic dermatitis, the repeated acute responses to environmental factors exacerbate the condition of chronic disease. Therefore, the inhibition of immediate phase reaction is

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still regarded as the most important step in the treatment of allergic inflammation [3].

Recently, many investigators have concentrated their efforts on finding the effective therapeutics for allergic inflammation. Among those, complementary and alternative (CAM) medicine is one interesting area. Although it has not yet been incorporated into the mainstream of medical care because of limited scientific evidences and lack of mechanistic understanding, CAM is an increasingly attractive approach all over the world [4]. For instance, an extract of *Petasites hybridus* was reported as a possible agent for treatment of allergic rhinitis [5].

Buckwheat grain is one of the widely consumed foodstuffs. It has been previously documented that the intake of buckwheat gives beneficial effects on hypertension and hypercholesterolemia [6,7]. Besides, buckwheat has long been used as therapeutics for anti-inflammation, detoxification and lowering the fever in Korean folk medicine. The precise action mechanism of buckwheat, however, remains to be elucidated. In the present study, we have demonstrated the anti-allergic action of buckwheat grain extract (BGE) using rodent experimental models and in vitro cell culture systems.

2. Materials and methods

2.1. Materials

Buckwheat grain powder was purchased from Bongpyeong Nonghyup (Pyeongchang, Gangwon, Korea). Iscove's modified Dulbecco's medium (IMDM), α -minimal essential medium (α -MEM) and fetal calf serum (FCS) came from Gibco BRL (Gaithersburg, MD, USA). Compound 48/80, anti-DNP IgE, DNP-HSA, metrizamide and Evans blue were purchased from Sigma (St. Louis, MO, USA). Sprague–Dawley rats were supplied from Dae-Han Biolink (Eumsung, Chungbuk, Korea). M-MLV reverse transcriptase and Taq polymerase were purchased from Promega (Madison, WI, USA).

2.2. Preparation of buckwheat grain extract (BGE)

Buckwheat grain powder was extracted with 100% cold methanol. The methanol extract was concentrated

in a vacuum evaporator (Büchi, Switzerland) and the resulting residue was weighed and dissolved to 1% solution in appropriate vehicles. For polarity-based fractionation, methanol extract of buckwheat grain was suspended in 80% methanol then extracted with *n*-hexane. In the same way, additional fractionations were carried out. Fractionation scheme is depicted in Fig. 1.

2.3. Compound 48/80-induced cutaneous reaction

Male Sprague–Dawley rats, each weighing approximately 200 g, were used in compound 48/80-induced cutaneous reaction. Fifty microliters of 0.1% compound 48/80 solution freshly prepared in PBS was injected intradermally into the dorsal skin of rats using a microsyringe with a 28-gauge hypodermic needle. Immediately after injection of compound 48/80 solution, 0.5 ml of 1% Evans blue was administered intravenously. BGE was administered in three different ways. For oral treatment, BGE was suspended in DW at appropriate concentrations and administered once a day for 3 days. Intradermal injection of compound 48/80 was performed 2 h after oral administration of BGE at day 3. In the experiments of intraperitoneal and intradermal administration, BGE dissolved in 50% ethanol was diluted with PBS to appropriate concentrations. Intraperitoneal and intradermal injections of BGE were carried out 2 h and/or 10 min before treatment of compound 48/80. Control groups were treated with appropriate vehicles. The rats were sacrificed 60 min after intradermal injection of compound 48/80 and the dorsal skins were removed for measurement of extravasated dye. The amount of dye was

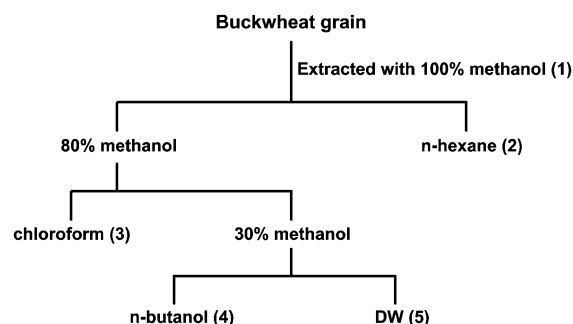


Fig. 1. Schematic representation of polarity-based fractionation of buckwheat grain extract (BGE).

measured as previously reported [8]. Briefly, skin tissue was solubilized with 1.0 N KOH at 37 °C, for overnight. Then, nine volumes of a mixture of 0.6 N KOH and acetone (5:13) was added and Evans blue dye was extracted. The absorbance of extracted dye was measured at 620 nm in a spectrophotometer (Beckman).

2.4. Passive cutaneous anaphylaxis (PCA) reaction

Sprague–Dawley rats were passively sensitized according to the method previously described [9]. The dorsal skins of rat were injected intradermally with 0.5 µg of anti-DNP IgE, followed 48 h later by injection of 1 µg of DNP-HSA in PBS containing 1% Evans blue via tail vein. After 60 min, the rats were sacrificed and reaction sites were excised for determination of extravasated dye. BGE was suspended in DW at appropriate concentrations and administered orally for 3 days, before challenging DNP-HSA.

2.5. Histamine release from rat peritoneal mast cells (RPMC)

Rat peritoneal mast cells (RPMC) from Sprague–Dawley rats were purified as previously described [10] with slight modifications. Briefly, rats were anesthetized by ether and Tyrode solution (150 mM NaCl, 0.1% glucose, 3 mM NaHCO₃, 3.7 mM KCl, 3.5 mM NaH₂PO₄) containing 0.1% gelatin was injected intraperitoneally. After gentle massage, the peritoneal cavity was opened and the fluid was collected. Mast cells were separated from major components of rat peritoneal cells using 22.5% metrizamide. Mast cells of 90–95% purity were routinely obtained, as estimated by toluidine blue staining. For testing histamine release, 2×10^5 /ml of RPMC was resuspended with α -MEM, FCS (1:1) and preincubated for 20 min at 37 °C. BGE and several solvent fractions were prepared at $100 \times$ concentrate in DMSO and added 30 min before treatment of compound 48/80 (10 µg/ml). Control group received 10 µl/ml of DMSO. After incubation with compound 48/80 for 20 min at 37 °C, the cells were separated from the released histamine by centrifugation at $400 \times g$ for 5 min. Residual histamine was released by disrupting the cells with perchloric acid and centrifugation. The amount of histamine was determined by *o*-phthalal-

dehyde spectrofluorometric method [9]. The fluorescence was measured at 440 nm (excitation at 360 nm).

2.6. Culture of HMC-1 cells and RT-PCR analysis

Human leukemia mast cells, HMC-1, were kept routinely in IMDM supplemented with 2 mM L-glutamine, 200 U penicillin/ml, 200 µg streptomycin/ml and 10% FCS. For RT-PCR analysis, 1×10^6 /ml of HMC-1 cells were preincubated with serum-free medium for overnight and activated with a combination of phorbol myristate acetate (PMA, 5 µM) and calcium ionophor A23187 (25 µM) for 4 h at 37 °C [11]. BGE was dissolved in DMSO and added to the incubation medium 30 min prior to the treatment of PMA and A23187. Control group was treated with appropriate amount of DMSO. Total RNAs were extracted by acid guanidinium thiocyanate–phenol–chloroform method. Two micrograms of total RNAs was reverse-transcribed with M-MLV reverse transcriptase in the presence of random hexamer. The resultant RT mixtures were then subjected to PCR cycles as follows: 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min for 30 cycles (IL-4, TNF- α), 35 cycles (IL-5) and 20 cycles (G3PDH). Primers for amplifying the respective fragments are listed in Table 1. After agarose gel electrophoresis, PCR products were

Table 1
Nucleotide sequence of the primers and expected size of PCR products

Primer	Sequence	Size (bp)
IL-4	Forward (5' → 3')	280
	TGAACAGCCTCACAGAGCAG	
IL-4	Reverse (5' → 3')	
	CTCTGGTTGGCTTCCTTCAC	
IL-5	Forward (5' → 3')	341
	GAGACCTTGGCACTGCTTTC	
IL-5	Reverse (5' → 3')	
	ATCTTTGGCTGCAACAAACC	
TNF- α	Forward (5' → 3')	449
	AGCCCATGTTGTAGCAAACC	
TNF- α	Reverse (5' → 3')	
	ATGTTCTGCTCCTCCTACAGG	
G3PDH	Forward (5' → 3')	655
	GAGTCAACGGATTGGTCGT	
G3PDH	Reverse (5' → 3')	
	TTCAGCTCAGGGATGACCTT	

quantified using a densitometer (Imagemaster, Pharmacia Biotech).

2.7. Statistical analysis

The results obtained were statistically evaluated using Student's *t*-test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect on compound 48/80-induced vascular permeability

It has been previously demonstrated that intradermal injection of compound 48/80 into the dorsal skin of rat provokes the mast cell-dependent vasodilation and increased vascular permeability documented by

Evans blue extravasation [12]. To determine the potential effect of BGE on the compound 48/80-induced vascular permeability, rats were orally administered with BGE for 3 days and skin extravasation was investigated. BGE produced potent inhibitory effect on the compound 48/80-induced vascular permeability, resulting in 82% and 40% inhibition at the dose of 1.0 and 0.1 g/kg bodyweight, respectively (Fig. 2A,B). BGE also showed significant inhibitory effect on compound 48/80-induced vascular permeability when intraperitoneally injected; however, its potency was somewhat weaker than that of oral administration (Fig. 2C). We then examined the effect of local application of BGE by intradermal injection at the sites of compound 48/80-induced skin extravasation. As shown in Fig. 2D, pretreatment of BGE (250 μ g/injection site) 10 min before compound 48/80 resulted in 96% inhibition of vascular permeability. Ketotifen was used as a positive control in all experiments. Although the

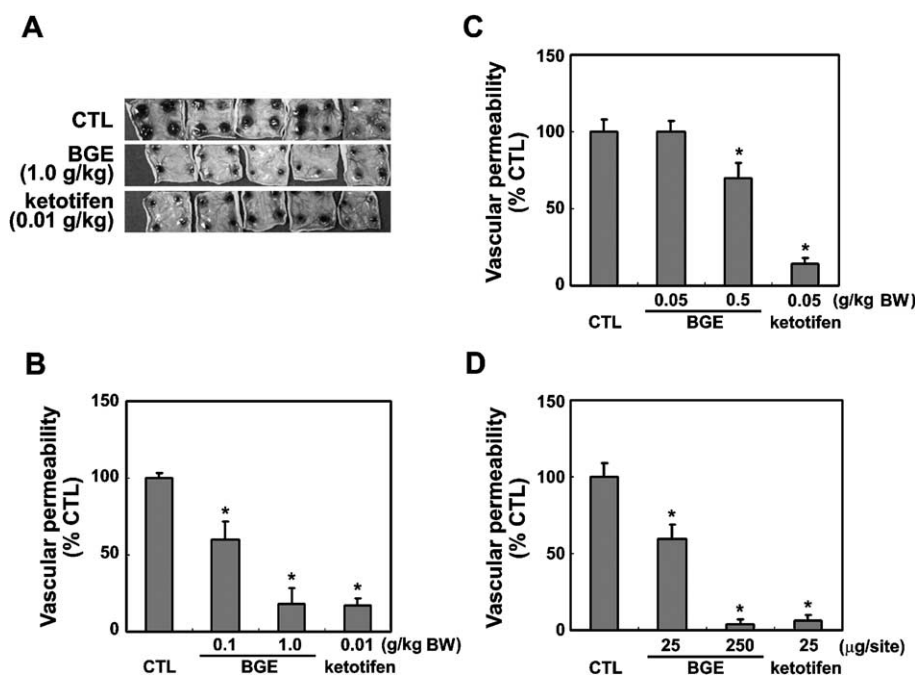


Fig. 2. (A) Representative data of vascular permeability documented by Evans blue extravasation. Sprague–Dawley rats were orally administered with buckwheat grain extract (BGE) for 3 days, followed by injection of compound 48/80 and Evans blue dye. Then, back skins were removed for determination of extravasated dye. Ketotifen was used as a positive control. (B) Quantification of effect of BGE on compound 48/80-induced vascular permeability when orally administered. (C) Effect of BGE on compound 48/80-induced vascular permeability when intraperitoneally injected, or (D) when intradermally injected. Data are expressed as percentage of control (CTL). The mean values $\pm$ S.E. are averages of four separate experiments (* $P < 0.05$ vs. CTL).

effects of BGE on allergic reaction were prominent as compared with control groups, it revealed that ketotifen was more potent in anti-allergic action.

3.2. Effect on passive cutaneous anaphylaxis (PCA) reaction

It has been well documented that PCA reaction is a typical model for mast cell-dependent immediate hypersensitivity and can be used to assess anti-allergic activities of potential drugs [13]. These previous findings led us to investigate the effect of BGE on PCA reaction. When orally administered for 3 days, BGE produced marked inhibitory effect (92% and 28% at the doses of 1.0 and 0.1 g/kg bodyweight) on PCA reaction induced by anti-DNP IgE and DNP-HSA (Fig. 3).

3.3. Effect on histamine release from rat peritoneal mast cells (RPMC)

In an attempt to examine the effect on histamine release, RPMC were preincubated with BGE, 30 min prior to the addition of compound 48/80. As shown in Fig. 4, BGE clearly inhibited compound 48/80-induced histamine release from RPMC, which showed 66% and 35% inhibition at the doses of 0.1 and 0.01 mg/ml, respectively. We next tested the effect of several solvent fractions to determine the active components in BGE. However, the effect of these fractions upon histamine release from RPMC was

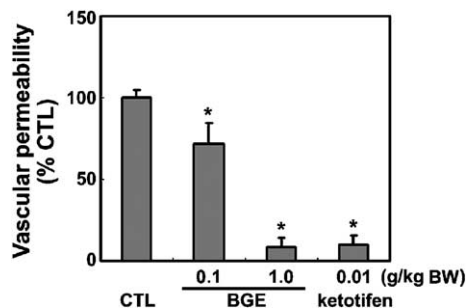


Fig. 3. Effect of buckwheat grain extract (BGE) on passive cutaneous anaphylaxis (PCA) reaction. BGE was orally administered for 3 days at indicated concentrations, and PCA reaction was induced by injection of anti-DNP IgE and DNP-HSA. Data are expressed as percentage of control (CTL). The mean values $\pm$ S.E. are averages of triplicate measurements (* $P < 0.05$ vs. CTL).

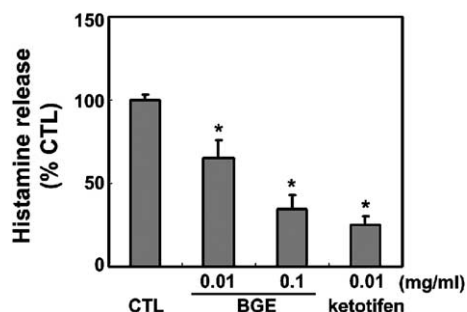


Fig. 4. Effect of buckwheat grain extract (BGE) on compound 48/80-induced histamine release from rat peritoneal mast cells (RPMC). RPMC were preincubated with BGE for 30 min prior to incubation with compound 48/80. The amounts of histamine release from RPMC were expressed as percentage of control (CTL). The mean values $\pm$ S.E. are averages of quadruplicate measurements (* $P < 0.05$ vs. CTL).

diminished significantly as compared with methanol extract (Table 2).

3.4. Effect on mRNA levels of cytokines in HMC-1 cells

In order to explore the effect of BGE on the mRNA levels of several cytokines implicated in pathogenesis of allergic inflammation, RT-PCR analysis was carried out using human leukemia mast cells, HMC-1 (Fig. 5A). When activated with PMA and ionophor A23187, the mRNA levels of IL-4, IL-5 and TNF- α were increased in HMC-1 cells. Treatment of BGE resulted in dose-dependent inhibition of IL-4 and TNF- α mRNA induction by PMA and A23187. However,

Table 2

Effect of several solvent fractions upon histamine release from rat peritoneal mast cells (RPMC)

Fraction	Concentration (mg/ml)	Percent inhibition
(1) Methanol	0.01	34.8
	0.1	66.3
(2) <i>n</i> -Hexane	0.01	15.1
	0.1	16.9
(3) Chloroform	0.01	16.4
	0.1	24.1
(4) <i>n</i> -Butanol	0.01	9.4
	0.1	16.7
(5) DW	0.01	0.9
	0.1	9.7
(6) Ketotifen (positive control)	0.01	75.0

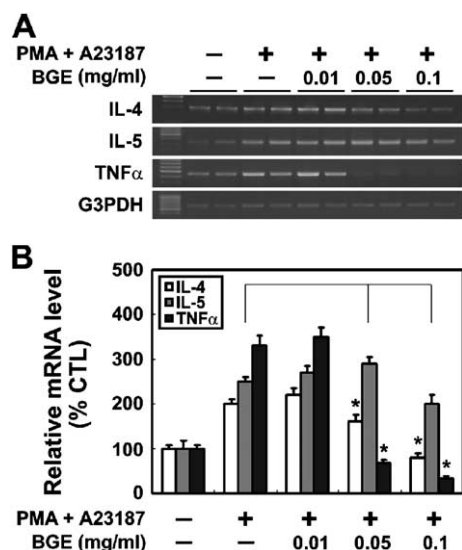


Fig. 5. (A) Effect of buckwheat grain extract (BGE) on the mRNA level of cytokines in human leukemia mast cells, HMC-1, by semi-quantitative RT-PCR analysis. HMC-1 cells were pretreated with BGE as indicated concentrations and then activated by PMA and calcium ionophor A23187. (B) Quantification of RT-PCR products for cytokines using a densitometer. The amount of RT-PCR products for each cytokines was corrected according to the quantity of G3PDH and expressed as a percentage of control ($n=4$, mean $\pm$ S.E., $*P<0.05$).

the mRNA level of IL-5 was not affected significantly by BGE treatment (Fig. 5B).

4. Discussion

Mast cells play a key role in immediate-type allergic reactions through the release of numerous mediators and cytokines. Among those, histamine is still regarded as a principal mediator of antigen-induced skin reactions [14]. As a histamine-related in vivo allergic model, the compound 48/80-induced skin extravasation has been well established [12,15]. In addition, the PCA reaction experimentally induced by injection of anti-IgE antibody has been also adopted successfully to test anti-allergic effects of certain drugs [16,17]. In the present study, we have demonstrated that BGE has potent inhibitory effects on compound 48/80-induced vascular permeability and anti-DNP IgE-induced PCA reaction. These effects could be simply inferred that the anaphylactic degranulation

of mast cells was inhibited by the treatment of BGE. Indeed, this notion was supported by the finding that BGE could inhibit significantly the compound 48/80-induced histamine release from RPMC. Furthermore, BGE showed prominent inhibitory potential on the gene expression of IL-4 and TNF- α induced by PMA and A23187 in human leukemia mast cells, HMC-1. It is now well established that human mast cell is a source of several multifunctional cytokines [18], and considerable evidences indicate that IL-4 from mast cells may be implicated in the pathogenesis of allergic inflammation such as asthma and rhinitis [19,20]. IL-4, a pleiotropic cytokine, is well known to be required for B cell isotype switching in favor of IgE production and to enhance selective eosinophil recruitment through induction of the endothelial expression of VCAM-1 [21,22]. Tissue infiltration by large numbers of eosinophils is one of consistent features in the perpetuation of the inflammatory response [23]. Therefore, it can be suggested that IL-4 plays an important role in establishing chronic allergic inflammation. This is supported by evidence that ear swelling in late phase reaction was significantly suppressed by the treatment with anti-IL-4 monoclonal antibody in ovalbumin-immunized mice model [24]. In addition, the important role of IL-4 in chronic nonallergic inflammatory reactions, such as scleroderma and liver fibrosis, was also reported [25]. TNF- α is a key mediator in many cytokine-dependent inflammatory events. TNF- α is released in allergic responses from both mast cells and macrophages via IgE-dependent mechanisms. It has been well known that TNF- α induces chemotaxis of neutrophils and T cells, enhances cytotoxicity of macrophages, and stimulates the expression of adhesion molecules from endothelial cells [26]. Previous data also indicated that TNF- α released from mast cells is responsible for eosinophil survival through partial inhibition of apoptosis, thereby contributes to chronic inflammation [27]. In this regard, our results suggest that BGE could affect the acute allergic reaction as well as chronic inflammation through the regulation of cytokines gene expression in the mast cells.

The active components of BGE in anti-allergic action are not clear. To gain more insight into the definite chemical identity of active components in BGE, we performed polarity-based fractionation. Unfortunately, we experienced that active ingredients were split into several solvent fractions (Table 2).

These results suggest that anti-allergic potential is synergistically increased by the combination of active components in BGE.

Recently, the interest of polyphenolic compounds as therapeutic agents against many diseases is growing. Buckwheat grain is used as a source of several polyphenolic compounds, such as rutin, orientin, vitexin and quercetin, and many evidences indicate that these compounds have anti-oxidant potential [28–30]. Interestingly, it has been reported that mast cells generate intracellular reactive oxygen species (ROS) in response to antigen challenge, and this may be involved in histamine release [31]. Moreover, it has been also reported that naturally occurring polyphenolic anti-oxidants reduced ROS levels in antigen-IgE activated mast cells and concomitantly inhibited histamine release by these activated mast cells [32]. Therefore, it could be suggested that the anti-allergic action of BGE is partly due to anti-oxidant potential of polyphenolic components in BGE. The precise action mechanism of BGE and its components on the allergic reactions, however, should be further investigated.

In summary, we have demonstrated that BGE has profound anti-allergic potential in mast cell-dependent test models, suggesting that BGE may be applicable to the treatment of allergic inflammation.

Acknowledgements

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