

Anti-allergic effects of the brown alga *Eisenia arborea* on Brown Norway rats

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ABSTRACT: To investigate the anti-allergic effects of the brown alga *Eisenia arborea*. A strain of Brown Norway rats known to strongly respond to immunoglobulin E (IgE) were used as an allergy model animal. The rats were immunized with ovalbumin by oral administration. The levels of serum IgE and histamine were suppressed in the rats fed a diet supplemented with dried *E. arborea* powder. As for the cytokine pattern, the interferon- γ production in the spleens and mesenteric lymph nodes (MLN) was enhanced, and the interleukin-4 (IL-4) production in the spleens and/or IL-10 production in the spleens and MLN were suppressed. These results, together with the change in the Th1/Th2 balance, indicate that the rats fed with *E. arborea* became more anti-allergic, suggesting that *E. arborea* might possess anti-allergic effects.

KEY WORDS: anti-allergy, BN rat, cytokine, *E. arborea*, histamine, immunoglobulin.

INTRODUCTION

Some anti-allergic investigations for components of terrestrial products have already been reported using experimental model animals such as mice or rats. For example, polyphenols such as tea catechins,^{1,2} astragalin from persimmon leaf extracts,³ polycyanidine from apple extracts⁴ and naringenin chalcone from tomato skin extracts⁵ are reported to have anti-allergic effects. Other than polyphenols, oligosaccharides⁶ and lipids⁷ are also reported to have anti-allergic effects.

Fucoidan^{8,9} and alginate^{10,11} from brown algae have also been reported to have anti-allergic effects in experimental animals and *in vitro* experiments, respectively. Moreover, some unsaturated fatty acids from *Undaria pinnatifida* are also known to have anti-allergic effects due to the suppression of chemokine production in cultured mouse mast cells.¹² Previous studies showed that the 80% methanol and methanol/chloroform extracts from a brown alga *Eisenia arborea* (*E. arborea*) possess an anti-allergic effect based on an *in vitro* experiment.^{13–15} The active compounds

included in the methanol/chloroform extract were phlorotannins such as eckol, 6,6'-bieckol, 6,8'-bieckol, 8,8'-bieckol, phlorofucofuroeckol-A and phlorofucofuroeckol-B.^{14,15} However, since *E. arborea* is generally used as a food, it is necessary to study the anti-allergic effects of the alga by *in vivo* experiments. Therefore, in this present study, the authors attempted to demonstrate the anti-allergic effects of the *E. arborea* algal powder on a Brown Norway (BN) rat strain known as a high IgE responder,¹⁶ and found expression of interleukin-4 (IL-4) and interferon (IFN)- γ .¹⁷

MATERIALS AND METHODS

Preparation of dried *Eisenia arborea* powder

Eisenia arborea (Phaeophyta) was collected on the coast of Mugizaki in Mie prefecture. After washing off all debris with tap water, the alga was stored at -40°C until used. Just before its use, the alga was thawed and dried in the shade for 3 days, followed by an air oven at 35°C for 3 days. The dried alga was powdered using an ultracentrifugal mill (ZM200; Retsch, Haan, Germany) to a size of approximately 0.2 mm in diameter, and stored at 4°C in an airtight container with desiccant until used.

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Received 13 June 2007. Accepted 16 August 2007.

Table 1 Composition of the diets

Constituent	Amount (g)			
	Control or basal diet	Test diet 1	Test diet 2	Test diet 3
Casein	20	20	19.5	19
DL-Methionine	0.3	0.3	0.3	0.3
Corn starch	15	15	15	15
Sucrose	50	49	48	46
Corn oil	5	5	5	5
Mineral mixture AIN-76 [†]	3.5	3.5	2.5	1.5
Vitamin mixture AIN-76 [‡]	1	1	1	1
Choline bitartrate	0.2	0.2	0.2	0.2
Fiber	5	5	3.5	2
<i>E. arborea</i> powder	0	1	5	10
Total	100	100	100	100

Except for choline bitartrate (Wako Pure Chemical, Tokyo, Japan) and *Eisenia arborea* (*E. arborea*) powder, all components were purchased from CLEA Japan (Tokyo, Japan).

[†]Mineral mixture AIN-76;[‡]vitamin mixture AIN-76.¹⁸

Animals and diets

In order to acclimate to the laboratory conditions, 4-week-old female BN rats (Japan SLC, Hamamatsu, Shizuoka, Japan) were kept in an animal room under a 12 h light : 12 h dark cycle at $24 \pm 2^\circ\text{C}$, and a relative humidity of $50 \pm 10\%$ for 7 days on an AIN-76¹⁸ diet. Food and water were freely available. The compositions of the experimental diets are shown in Table 1.

Designs of experiments

After acclimating for 1 week, the rats were divided into five (1 basal diet, 1 control and 3 test) groups ($n = 6$). The three test groups were fed diets containing 1% (test diet 1), 5% (test diet 2) and 10% (test diet 3) algal powder, as shown in Table 1, until the end of the oral immunization. The basal diet group was not immunized, and the control and test groups were immunized with egg white ovalbumin ([OVA], Grade V: Sigma-Aldrich, St. Louis MO, USA; 0.1 mg/mL tap water). The oral immunizations with OVA of the control and experimental groups were performed daily at a volume of 1 mL/rat using a stainless steel animal feeding needle for 42 days. After 24 h from the last immunization, blood was collected from the heart under diethylether anesthesia. Serum was collected as usual and stored at -20°C until used.

All of the animal experiments were performed under the Mie University Charter for Animal Experiments and the Mie University Guideline for Animal Experiments.

Assay for immunoglobulins in sera

Immunoglobulin E

The detection of the OVA-specific immunoglobulin E (IgE) was performed according to the method of Knippels *et al.*¹⁹ with some modifications. A 50 μL OVA solution (50 $\mu\text{g}/\text{mL}$) in a 50 mM sodium carbonate buffer (pH 9.6) was added to each well of a 96-well microtiter plate (flat bottom; Maxisorp, Nunc, Roskilde, Denmark), and the plate was incubated overnight at 4°C . The supernatant was discarded and the wells were washed three times with 200 μL of phosphate buffered saline (PBS) containing 0.05% Tween 20. A total of 200 μL of 0.1% bovine serum albumin (BSA) in PBS was then added to the plate and incubated for 1 h at room temperature. After discarding the BSA and washing the wells with PBS, the serum sample (50 μL) adequately diluted with 0.1% BSA was added to each well, and incubated for 20 h at 4°C . After discarding the samples and washing the wells with PBS, 50 μL of mouse anti-rat IgE (Technopharm Biotechnology, Villejuif-Cedex, France) diluted with 0.1% BSA was added to each well and incubated for 1 h at room temperature. After discarding the antibody and washing the wells with PBS, biotinylated antimouse Ig (50 μL) (Amersham Bioscience, Chalfont, UK) diluted with 0.1% BSA was added to each well and incubated for 1 h at room temperature. After discarding the antibody and washing the wells with PBS, 50 μL of streptavidin-horseradish peroxidase (Amersham Bioscience) diluted with PBS was added to each well and incubated for 1 h at room temperature. After discarding and washing, 100 μL of 3, 3', 5, 5'-tetramethylbenzidine (Sigma-Aldrich) was added

to each well and incubated for 15 min at room temperature. To stop the reaction, 2 N sulfuric acid (100 μ L) was added to each well. The optical densities at 450 nm were measured on microplate-reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA).

Total Immunoglobulin E

For the detection of the total IgE, serum samples (50 μ L) diluted with sodium carbonate buffer were added to each well of a 96-well microtiter plate and the plate was incubated for 16 h at 4°C. The procedure from the incubation with 0.1% BSA for 1 h to the measurement of the optical density was the same as that for the OVA specific IgE.

Histamine measurement

For histamine release in the rats, the active systemic anaphylaxis reaction was induced according to the method of Akiyama *et al.*²⁰ The BN rats of all groups were immunized with OVA (1 mg) in an alum adjuvant (Imject alum; Pierce Chemical, Rockford, IL, USA) by ip administration after 24 h from the last oral administration of the OVA. After 12 min^{20,21} from the ip administration, blood was collected as described above. Histamine in the sera was immediately detected by a fluorescent detection method according to the procedure of Shore *et al.*²²

A preparation of lymphocyte and detections of cytokine

A lymphocyte preparation was performed according to the method of Lim *et al.*²³ with some modifications. The spleens and mesenteric lymph nodes (MLN) were extirpated from the rats into an RPMI-1640 culture medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Lot no. AND18640; HyClone, South Logan, UT, USA). The lymphocytes in the spleen and MLN were then squeezed into RPMI-1640 medium containing 10% fetal bovine serum and 1 mM sodium bicarbonate. To remove the connective tissues, the medium containing the lymphocytes was filtered through a stainless steel mesh (#100) and aggregated with incubation for 30 min at 37°C, followed by a second filtration through the stainless steel mesh. Lymphocyte separation reagent (Lympholyte-Rat; Cedarlane Laboratories, Burlington, ON, Canada) was layered onto the filtrates and centrifuged (200 $\times$ g) for 30 min at room tempera-

ture. The lymphocyte layer at the interface was collected and suspended in RPMI-1640 medium containing Con A (5 μ g/mL; LKT Laboratories, St. Paul, MN, USA) at a density of 2×10^6 cells/mL. The cell suspension was divided into a 24-well microplate and incubated for 24 h at 37°C. The culture media was collected and applied to the cytokine detection using ELISA kits (Amersham Bioscience). The detection of IFN- γ , IL-4 and -10 in the culture media were conducted according to manufacturer's protocol.

Statistical analysis

Data were expressed as the mean $\pm$ standard deviation, and the statistical analyses were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Growth of rats

Throughout the feeding periods, the rats maintained a good appetite. Among all the groups, no diarrhea or abnormal symptoms were observed. Although the mean amounts of diet fed were 9.1 ± 0.8 g/day per body in the basal diet, 8.8 ± 0.9 g/day per body in the control, 10.4 ± 1.4 g/day per body in the test diet 1, 9.9 ± 0.8 g/day per body in the test diet 2 and 10.7 ± 0.8 g/day per body in the test diet 3, the difference was not significant ($P > 0.05$). Although the final body weight and spleen weight were not the same in each group (Table 2), no statistical difference was observed ($P > 0.05$).

Immunoglobulins

Immunoglobulin E

The value of the OVA specific IgE in the control (0.84 ± 0.39 μ g/mL) significantly ($P < 0.01$) increased when compared to that of the basal diet group (not detected), and the rats were absolutely sensitized with OVA. However, the levels of the OVA specific IgE were significantly ($P < 0.01$) suppressed from control to 0.12 ± 0.03 μ g/mL (test group 1) or 0.16 ± 0.03 μ g/mL (test group 2), and slightly ($P > 0.05$) suppressed to 0.59 ± 0.08 μ g/mL in test group 3 (Table 3).

Table 2 Body and spleen weights of rats

Body weight (g)	Basal diet	Control	Test diet 1	Test diet 2	Test diet 3
Initial weight [†]	94.5 ± 5.3	100.2 ± 7.4	96.8 ± 4.8	103.5 ± 4.7	99.5 ± 3.3
Final weight [†]	156.7 ± 4.3	167.2 ± 6.5	168.2 ± 8.7	168.5 ± 6.0	169.5 ± 3.7
Weight gain (g) [†]	62.2 ± 7.7	67.0 ± 9.5	71.3 ± 9.6	65.0 ± 8.1	70.0 ± 5.1
Spleen weight (g) [‡]	0.385 ± 0.019	0.423 ± 0.044	0.481 ± 0.116	0.400 ± 0.032	–

Values are mean ± standard deviation.

[†]*n* = 6; [‡]*n* = 3.

(–), not determined.

Table 3 Immunoglobulin contents of Brown Norway rats fed on algal powder

Experimental group	OVA specific IgE (μg/mL)	Total IgE (μg/mL)
Basal diet	ND	270.33 ± 85.82
Control	0.84 ± 0.39	472.74 ± 124.26
1	0.12 ± 0.03**	260.40 ± 51.05*
2	0.16 ± 0.03**	244.67 ± 79.35*
3	0.59 ± 0.08	589.97 ± 42.49

Values are mean ± SD (*n* = 6).

Asterisks indicate significant differences from control.

P* < 0.05; *P* < 0.01.

1, Contained 1% *Eisenia arborea* (*E. arborea*) powder; 2, contained 5% *E. arborea* powder; 3, contained 10% *E. arborea* powder.

IgE, immunoglobulin E; ND, not detected; OVA, ovalbumin.

Total Immunoglobulin E

The IgE level of the basal diet group was 270.33 ± 85.82 μg/mL and the value of the total IgE in the control (472.74 ± 124.26 μg/mL) significantly (*P* < 0.05) increased when compared to that of the basal diet group. The levels of the test groups were significantly (*P* < 0.05) suppressed from the control to 260.40 ± 51.05 μg/mL (test group 1) or 244.67 ± 79.35 μg/mL (test group 2), respectively, although no effect was found in test group 3 (589.97 ± 42.49 μg/mL) (Table 3).

Histamine

Although the histamine level of the control significantly (*P* < 0.01) increased to 298.56 ± 49.63 nM from that of the basal diet group (124.86 ± 19.89 nM), that of all test groups (test group 1, 163.11 ± 55.90 nM; test group 2, 144.90 ± 62.98 nM; test group 3, 182.39 ± 33.94 nM) were significantly (*P* < 0.05) suppressed relative to that of the control (298.56 ± 49.63 nM) and slightly increased relative to that of the basal diet group (124.86 ± 19.89 nM) (*P* > 0.05) (Fig. 1).

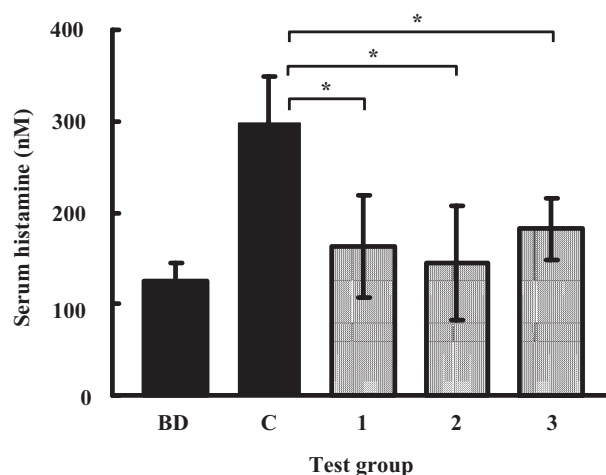


Fig. 1 Histamine levels of sera detected by a fluorescence method. The test groups are as follows: BD, basal diet; C, control; 1, contained 1% *Eisenia arborea* (*E. arborea*) powder; 2, contained 5% *E. arborea* powder; 3, contained 10% *E. arborea* powder. Values are given as means ± standard deviation (*n* = 6). Asterisks indicate significant differences from C. **P* < 0.05.

Cytokines

Interferon-γ

Table 4 shows the IFN-γ levels of the spleen and MLN-derived lymphocytes. Both IFN-γ levels of the spleens and MLN in the control were significantly increased when compared to that of basal diet groups (spleens, 2.77 ± 0.15 pg/mL; MLN, not detected) (*P* < 0.01). The IFN-γ level in the spleens of test group 1 was significantly (*P* < 0.01) elevated to 126.01 ± 3.16 pg/mL relative to the control (42.23 ± 2.16 pg/mL). However, that of test group 2 (48.71 ± 1.66 pg/mL) only slightly increased relative to the control. In the MLN, the IFN-γ level of test group 1 significantly (*P* < 0.05) increased to 33.18 ± 1.66 pg/mL relative to the control (12.32 ± 3.48 pg/mL). Test group 2 showed the greatest elevation of IFN-γ to 133.93 ± 4.25 pg/mL (*P* < 0.01).

Table 4 Cytokine contents of Brown Norway rats fed on algal powder

Experimental group	IFN- γ in spleens	IFN- γ in MLN	IL-4 in spleens	IL-10 in spleens	IL-10 in MLN
Basal diet	2.77 $\pm$ 0.15	ND	ND	46.25 $\pm$ 0.38	20.39 $\pm$ 1.29
Control	42.23 $\pm$ 2.16	12.32 $\pm$ 3.48	3.47 $\pm$ 0.48	73.49 $\pm$ 19.45	48.31 $\pm$ 19.07
1	126.01 $\pm$ 3.16**	33.18 $\pm$ 1.66*	2.46 $\pm$ 1.13	53.69 $\pm$ 24.88	20.65 $\pm$ 6.43*
2	48.71 $\pm$ 1.66	133.93 $\pm$ 4.25**	2.62 $\pm$ 0.77	30.99 $\pm$ 2.31*	24.05 $\pm$ 11.85*

Values are mean $\pm$ standard deviation ($n = 3$).

The orders of cytokine concentrations were pg/mL. Asterisks indicate significant differences from control. * $P < 0.05$; ** $P < 0.01$.

1, contained 1% *Eisenia arborea* (*E. arborea*) powder; 2, contained 5% *E. arborea* powder.

IFN- γ , interferon- γ ; IL-4, interleukin-4; MLN, mesenteric lymph nodes; ND, not detected.

Interleukin-4

Table 4 shows the IL-4 levels of the spleen-derived lymphocytes. Both IL-4 levels in the spleen of the test group 1 (2.46 $\pm$ 1.13 pg/mL) and 2 (2.62 $\pm$ 0.77 pg/mL) tended to be lower than that of the control (3.47 $\pm$ 0.48 pg/mL) ($P > 0.05$). The level of the control significantly increased when compared to the basal diet group (not detected) ($P < 0.01$).

Interleukin-10

Table 4 shows the IL-10 levels of lymphocytes derived from the spleen and MLN. In the spleens, although the IL-10 level of the control (73.49 $\pm$ 19.45 pg/mL) tended to increase to the basal diet group (46.25 $\pm$ 0.38 pg/mL) ($P > 0.05$), the level of test group 1 tended to be suppressed to 53.69 $\pm$ 24.88 pg/mL relative to the control ($P > 0.05$), showing a level similar to the basal diet group. However, that of test group 2 was significantly suppressed to 30.99 $\pm$ 2.31 pg/mL relative to the control ($P < 0.05$). In the MLN, the level of control (48.31 $\pm$ 19.07 pg/mL) significantly increased compared to the basal diet group (20.39 $\pm$ 1.29 pg/mL) ($P < 0.05$), and those of test groups 1 and 2 were significantly suppressed to 20.65 $\pm$ 6.43 pg/mL and 24.05 $\pm$ 11.85 pg/mL, respectively ($P < 0.05$). These levels are comparable to the basal diet group relative to the control.

DISCUSSION

In this study, both the OVA-specific and total serum IgE levels were suppressed. The histamine levels of all the test groups were suppressed compared to that of the control and were similar to that of the basal diet group. For the cytokine release of lymphocytes derived from the spleen and MLN, only some of the test groups released IFN- γ . The release of IL-4 from the splenocytes tended to be sup-

pressed ($P > 0.05$). The release of IL-10 from the lymphocytes derived from the spleen and MLN in the test diet 1 and 2 groups had no increase compared to the basal diet group.

There are two types of T helper cell clones, defined as Th1 and Th2 cells,²⁴ and it is well known that if Th2 is more dominant than Th1, an allergic disease occurs.²⁵ Th1 cells mainly produce IFN- γ ²⁵ as a cytokine that relates to a class switch to IgG2a producing B cell and cell-mediated immunity.²⁶ However, Th2 cells mainly produce IL-4, -5, -10 and -13;²⁵ in particular, IL-4 is related to a class switch to IgE and IgG1 producing B cell and humored-mediated immunity.^{26,27} Therefore, cytokines from Th1 and Th2 cells mutually crosslink and suppress each other. IL-10 suppresses Th1 cells to mediate inhibition of IL-12 production of macrophages or monocytes,²⁸ and IL-12 is known to enhance biological activity of Th1 cells.²⁹ Based on these facts, the results from the present study are likely to show that *E. arborea* might express an anti-allergic effect to change the Th1/Th2 balance. This is because, based on the results of the present study, it appears that the suppression of the OVA-specific and total IgE levels or histamine levels contribute to the suppressed release of IL-4 and IL-10 from the spleen and MLN-derived lymphocytes, and to the enhancement of the IFN- γ release from the spleen and MLN-derived lymphocytes. Therefore, *E. arborea* is likely to possess an anti-allergic effect. Further studies of the fluctuations of the IgG subclasses (IgG1 and IgG2a) and quantitative Th1/Th2 balance measured by flow cytometry are required.

It is well known that *E. arborea* contains phenolic compounds called phlorotannins³⁰ that are thought to be the best candidates as anti-allergic compounds. In fact, it was found that phlorotannins from the brown alga *Eisenia bicyclis* inhibited hyaluronidase,³¹ phospholipase A2s, lipoxygenases and cyclooxygenases³² that are related to allergic or inflammatory reactions, and those from brown alga *Ecklonia cava* suppressed the prostaglandin E₂ production in cultured murine macrophage cells.³³ In previous studies, six kinds of phlorotannins

from *E. arborea* were isolated and identified, and they also showed anti-allergic effects that inhibit degranulation of histamine from rat basophilic leukemia cells.^{14,15} Moreover, since it is known that strictinin, a tea polyphenol, inhibited IgE production from the cultured human B cell line DND39,³⁴ suppression of the IgE levels in the present study may be induced by phlorotannins with a mechanism similar to strictinin. Therefore, it is theorized that the previously identified phlorotannins^{14,15} are the best candidates as active compounds in *E. arborea*. Phlorotannins may show not only inhibition of inflammation-related enzymes and degranulation from some inflammatory lymphocytes downstream in the allergy response pathway, but may also inhibit IgE production upstream in a manner similar to strictinin.

However, as shown in Table 3, since the total IgE level of test group 3 fed with 10% algal powder is higher than the other test groups and similar to the control, the excessive intake of *E. arborea* may have an adverse effect. Because *E. arborea* contains some phlorotannins, the results of the present study could be supported by the finding in mice that phenolic compounds enhanced the IgE production due to enhancement of the IL-4 production.³⁵ Since overproduction of IgE by phenolic compounds leads to degradation of allergic symptoms via degranulation enhancement of chemical mediators such as histamine, leukotrienes and prostaglandins, the excessive intake of phlorotannins and algal powder as an anti-allergic food stuff could cause adverse effects from an anti-allergy standpoint. In fact, since tea catechins and cocoa polyphenols are known to modulate cytokine production from mice and mononuclear cells,^{36,37} changes in the cytokines related to the Th1/Th2 balance and overproduction of IgE of test group 3 in the present study might be due to the previously identified phlorotannins.^{14,15} If the appropriate intake of *E. arborea* is maintained, the phlorotannins may express beneficial immunomodulating effects such as those found in tea catechins and cocoa polyphenols. For the allergic response in the test group 3, further studies are required to measure the cytokine productions.

Because it is reported that BN rats immunized with OVA by ip administration showed a higher OVA specific IgE production than rats immunized by oral administration,¹⁹ we immunized BN rats with OVA by ip administration according to a method described in the report. When the rats were fed the test diet 2, the IgE and histamine levels in the sera tended to be suppressed compared to the basal diet group, and changes in the cytokine patterns similar to the oral administration groups were found in the spleens. Although the IL-4 sup-

pression in the oral administration test groups were not significant, the IL-4 level of the ip administration test group was significantly suppressed from 6.41 ± 0.60 pg/mL (control) to 3.34 ± 0.35 pg/mL (test diet 2) ($P < 0.05$, $n = 3$).

Other than phlorotannins, several algal polysaccharides such as fucoidan and alginate are known to be anti-allergic compounds.^{8–11} Because it is well known that *E. arborea* includes fucoidan (approx. 3%)³⁸ and alginate (approx. 30%),³⁹ further studies of the anti-allergic effects of polysaccharides from *E. arborea* are required.

Therefore, since *E. arborea* is likely to express an anti-allergic effect in BN rats by inhibition of IgE and anti-degranulation of chemical mediators via a change in the Th1/Th2 balance, this alga seems to be useful as a food stuff to relieve allergic symptoms such as atopic dermatitis, pollenosis, asthma and allergic rhinitis.

ACKNOWLEDGMENTS

The authors thank Dr Y. Atoji and Dr Y. Kanamaru, Faculty of Applied Biological Sciences, Gifu University, for discrimination of MLN. Thanks also to Dr D. A. Coury for reading this manuscript.

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