



The clinical and immunomodulatory effects of green soybean extracts

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ARTICLE INFO

Article history:

Received 13 July 2012

Received in revised form 4 December 2012

Accepted 17 December 2012

Available online 22 December 2012

Keywords:

Glycine max

WEGS

WEYS

Th2 cytokines

BAFF

APRIL

IgE

Cedar pollinosis

Allergic rhinitis

Withdrawal parallel-group study

ABSTRACT

The present study was performed to investigate the immune-modulating activities of extracts from green soybean (*Glycine max*) in a 2,4-toluene diisocyanate (TDI)-inducing guinea pig rhinitis model and a human trial study for allergic rhinitis. Hot water extracts of green soybean were chosen for animal experimentation on the basis of their ability to regulate the production of B cell-activating factor of the TNF family and a proliferation-inducing ligand in mouse spleen cells. Green soybean extracts significantly decreased the levels of ovalbumin (OVA)-specific IgE in mice and significantly suppressed the TDI-induced nasal mucosa secretion. An open-label human pilot study was performed on 16 subjects, using Japanese cedar pollinosis. The symptom scores for Japanese cedar pollinosis were better in the long-term green soybean extracts intake group than in the withdrawal short-term intake group. Green soybean extracts had great potential as an orally active immune modulator for the treatment of various allergic diseases.

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1. Introduction

Type-1 allergic responses involve the degranulation of IgE-dependent mast cells and the accumulation of eosinophils at the sites of inflammation, which are considered to result from the unbalanced overactivation of type-2 helper T cell (Th2) cells and, consequently, the Th2-mediated overproduction of IgE (Abbas, Murphy, & Sher, 1996; Comstock, Gershwin, & Teuber, 2010; Ver-celli, 2001). The representative cytokines of Th2 cells, such as interleukin (IL)-4, IL-5, IL-10, and IL-13, are known to play important roles in these reactions. On the other hand, the survival of B cell subsets by BAFF (B cell-activating factor of the TNF family) and APRIL (a proliferation-inducing ligand) participates in immuno-

globulin-producing maintenance (Parry et al., 2001). In fact, the BAFF levels in asthma and atopic dermatitis patient serum were elevated relative to normal serum (Jee, Kim, Hong, Sohn, & Kim, 2010; Kang et al., 2006). Therefore, the modulation of type-1 helper T cell (Th1) and Th2 responses and the suppression of BAFF are a rational strategy for the treatment of allergic diseases.

Diisocyanates are the leading cause of occupational asthma, and epidemiological evidence suggests that occupational rhinitis is a comorbid and preceding condition in patients who develop asthma (Redlich & Karol, 2002). Some studies have developed and characterised the animal model of TDI-induced rhinitis. This model is appropriate for pollinosis evaluation (Tamura & Kimoto, 2009).

There is a need to develop an orally active and safe immune modulator for the treatment of allergic diseases. In an effort to find such immune modulators, we have been screening for natural re-agents from edible plants, and have identified green soybean as a possible candidate. Soybean research has indicated that there are various elements in foods made with soybean that moderate or lessen the development potential for chronic diseases (Mateos-Aparicio, Redondo, Villanueva-Suárez, & Zapata-Revilla, 2008). Examples include the hypocholesterolemic effects of soy phytoestrogen (Deng, 2009; Hall et al., 2006; Lund et al., 2007), the cancer risk-reducing properties of the isoflavonoids (Andres, Abraham, Appel,

Abbreviations: BAFF, B cell activating factor of the TNF family; APRIL, a proliferation-inducing ligand; IgE, immunoglobulin E; WEGS, water extracts from green soybean; WEYS, water extracts from yellow soybean; TDI, 2,4-toluene diisocyanate; DMSO, dimethyl sulfoxide; OVA, ovalbumin; TPA, 12-*o*-tetradecanoylphorbol-13-acetate; ELISA, enzyme-linked immune sorbent assay; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ADL, activities of daily living.

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& Lampen, 2011; Song et al., 2007) and the saponins (Tsai, Chen, Chien, Huang, & Lin, 2010), and the xenobiotic effect of genistein (Moon, Wang, & Morris, 2006). It appears that there are health benefits when including soybeans in one's diet. Soybeans are commercialised in a wide variety of colours and tones: from yellow to black through green, brown or red. The yellow pigmented soybean is the most commercial and abundant soybean. The health benefits have been reported to differ according to differences in colour. For example, the extracts from black soybean had a longer low-density lipoprotein oxidation lag time than those from yellow soybean because of the high total polyphenol contents in the seed coat (Takahashi et al., 2005). The brown soybean seed coat was shown to have a high radical-scavenging activity by a highly polymerised proanthocyanidins component (Takahata, Ohnishi-Kameyama, Furuta, Takahashi, & Suda, 2001). Another study showed that a diet enriched in soybean isoflavones and green tea enhanced the immune function of ovariectomised mice in natural killer activity and lymphoproliferation compared with control mice (Baeza et al., 1992). We hypothesised that there may not only be health benefits to consuming yellow soybeans, but also black, brown and green soybeans. A soybean seed is usually green before it is harvested, and becomes yellow during maturation. In this study, yellow, black, brown or green soybean refer to the seed coat colour of each mature soybean (*Glycine max*) at post-harvest. We evaluated and compared the effect of the green soybean and yellow soybean on immune-modification.

2. Methods

2.1. Materials

Soybean samples were purchased from local markets in Shizuoka (Japan). Briefly, hot water extracts were obtained by maceration of dried soybean powder (10 g) in a flask with 100 ml of distilled water, followed by stirring for 2 h at 90 °C and then filtration on a glass filter. Hot water extracts were concentrated by a freeze-drier. The extract was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Inc., Tokyo, Japan).

2.2. Cell culture

Six-week-old male Balb/cAnNCrIrlj mice were purchased from Charles River Japan (Kanagawa, Japan). The spleen cells were cultured in 15% FBS in RPMI1620 medium, and stimulated with 5 ng/ml of 12-*o*-tetradecanoylphorbol-13-acetate (TPA; Wako Pure Chemical Inc., Osaka, Japan) and 0.5 µM A23187 (Wako Pure Chemical Inc.). Three to 300 µg/ml of soybean extract samples and 25 µM concentrations of apigenin (Wako Pure Chemical Inc.), for positive control, were added to the culture medium for IL-4. The same doses of soybean extract samples and 100 ng/ml of concanavalin A (Wako Pure Chemical Inc.), for positive control, were added to the culture medium without TPA and A23187 for IFN-γ. Twenty-four hours later, the culture supernatants were harvested. IL-4 and IFN-γ concentrations were measured by an ELISA kit (Thermo Fisher Scientific K.K., Yokohama, Japan), following the manufacturer's instructions. Nc/Nga mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were given a dermal challenge by application of Biostir AD (100 mg of ointment/mouse; Biostir Inc., Kobe, Japan) to the dorsum once a week for 5 weeks. The spleen cells were cultured in 10% FBS in RPMI1620 medium, and 300 µg/ml of soybean extract samples were added to the culture medium. Six hours later, the culture cells were harvested. BAFF and APRIL mRNA levels were measured by quantitative RT-PCR.

2.3. Quantitative RT-PCR

Quantitative RT-PCR was performed on a Thermal Cycler Dice Real time PCR System (Takara Inc.) according to the manufacturer's instructions. Total RNA was extracted with a guanidinium isothiocyanate-based RNA isolation kit (RNeasy mini kit; Qiagen, CA), according to the manufacturer's instructions. Two µg of RNA were converted into single-stranded DNA by a standard 50 µl RT reaction with the PrimeScript RT reagent Kit (Takara Inc.). The cDNA generated from the reverse transcription reactions was amplified by PCR with the SYBR Premix Ex Taq™ II (Takara Inc.), in a total volume of 25 µl, according to the manufacturer's instructions. The primers used were as follows: β-Actin: 5'-TGACCA-CACCTTCTACAATGA-3'/5'-CAGCTGGATAGCAACGTACAT-3'; BAFF: 5'-GCTTTCAGGGACCAGAGGAAA-3'/5'-TTACAGCAGTTTAGGG-CACCAA-3'; APRIL: 5'-GAGTCTGGGACACTGGAATTT-3'/5'-AGAT-ACCACCTGACCATTTGTGA-3'. The level of messenger RNA of BAFF and APRIL was expressed as the relative fold change vs. the β-actin messenger RNA.

2.4. Mouse model of OVA sensitization

All experiments were approved by our institutional Animal Care and Use Committee and were performed in accordance with institutional guidelines. Five-week-old male Balb/cAnNCrIrlj mice were maintained on a purified diet and under controlled humidity conditions (55% ± 5%), light (12 h/12 h light/dark cycle) and temperature (23 ± 1 °C). The animals were carefully examined for 1 week prior to use in the experiments, and only healthy animals were chosen. The animals were immunised two times by intraperitoneal injections with 1 µg of OVA absorbed onto 2 mg of alum adjuvant every 2 weeks. One week after the sensitization, the mice were divided according to their serum OVA-specific IgE into four groups and then fed the AIN76 (Barnard, Lewis, Teter, & Thigpen, 2009) based experimental soybean extracts or control diets starting on Day 0. The experimental period was four weeks long, and a blood sample was collected from the tail vein every two weeks. OVA-specific IgE and total IgG levels were measured using an ELISA kit (DS Pharma Biomedical Corporate Profile). All animals were treated in accordance with the guidelines for the care and use of laboratory animals at the University of Shizuoka.

2.5. TDI induced-guinea pig rhinitis model

Hartley male guinea pigs were purchased from Charles River Japan. 2,4-Toluene diisocyanate (TDI) was purchased from Tokyo Chemical Industry (Tokyo, Japan). TDI ethyl acetate solutions (10%) were prepared immediately before use. To induce pulmonary lesions, the animals were exposed to TDI by the following method: the TDI solution (30 µl) was painted onto the nasal vestibule of the animals for 10 s with a thin cotton applicator once a day for 5 days. Control guinea-pigs received only ethyl acetate with the same technique. The animals were fed the AIN76-based diets, that include 2.5–10% of WEGS or 10% of β-glucan (for positive control), starting on Day 0. TDI-sensitised guinea pigs were challenged with 5% of TDI by the same method of sensitization four weeks after the first sensitization. Nasal secretion from the nostrils was measured as an index of the exocrine activity of the nasal mucosa. The secretion was wiped off with a piece of pre-weighed paper at 10 min after the last sensitization. The paper was then weighed again.

2.6. Clinical trials

The study consisted of two parts with open-label treatment (Fig. 1). In Part 1, a single dose (3 g/day) of WEGS was administered every day for 6 weeks. Part 2 was a withdrawal period, in which

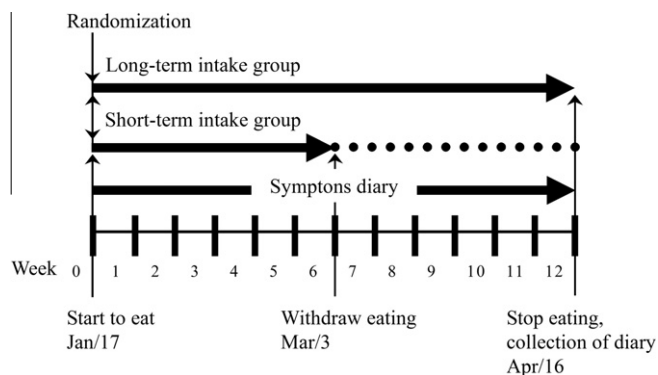


Fig. 1. Human study design.

the patients were randomly assigned by their total symptom score for the past year to receive either WEGS every day for 6 weeks or not receive it at all. Patients who had seasonal allergic rhinitis, and who required treatment, were eligible for enrolment if they were between the ages of 30 and 60 years. Patients who had not received treatment with any anti-allergic drugs, for six months before this study started, participated. Administration of other agents was permitted during the trial. As shown in Fig. 1, the study was carried out from January 17 to April 16, 2011. The subjects were instructed to keep a diary of allergic symptoms and medication throughout the experimental period. The studies were conducted in accordance with the guidelines of the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of Nisshin Pharma Inc. Written informed consent was obtained from all subjects.

Pollen counts in the atmosphere were obtained from the data acquisition system of the Ministry of the Environment of Japan (<http://kafun.taiki.go.jp/>). Levels were measured during the experimental period, near to the experimental area at the Hanno City Office, Saitama, Japan. Values are presented as particle number per cubic metre per day. Nasal and ocular symptoms described by the patients were classified as follows: sneezing, runny nose, and stuffy nose, itchy eyes, and watery eyes. Symptoms were evaluated using a 5-point scale: 0, no symptoms; 1, mild; 2, moderate; 3, se-

vere; 4, very severe symptoms present all day. The highest score for sneezing, runny nose, and stuffy nose was used as the nasal symptom score. Although no medication to prevent the allergy itself was administered before the pollen season, the patients in the study were concomitantly treated with commercial and/or prescribed medicine to relieve their symptoms. The use of medication was scored from 0 to 3. The sum of the symptom and medication score was used as the symptom-medication score (SMS), as defined by the Japanese Society for Allergology, to assess the severity of symptoms of Japanese cedar pollinosis (Baba, Konno, & Takenaka, 2002).

2.7. Statistical analysis

Data are presented as the mean $\pm$ standard error (SE). Statistical analyses were performed using Dunnett's multiple comparison tests for one-way analysis of variance, the Mann–Whitney *U*-test or the Student's *t*-test. Differences were considered significant when *P*-values were less than 0.05.

3. Results

3.1. WEGS-elicited Th2 cytokine, B cell stimulation cytokine suppression and Th1 cytokine expression in vitro

The effect of WEGS, at the indicated concentrations of 3–300 $\mu\text{g/ml}$, was evaluated on the cytokine production with TPA- and A23187-treated splenocytes for IL-4 and without stimulation for IFN- γ . The expression of Th1/Th2 cytokines was observed in the supernatant of the treated splenocytes, after 48 h, by ELISA. WEGS suppressed the expression of Th2 cytokine IL-4. The suppression level of IL-4, at the concentration of 150 $\mu\text{g/ml}$, was almost 0.34-fold that of the control level (Fig. 2A). WEGS slightly up-regulated the expression of Th1 cytokine IFN- γ (Fig. 2B). WEGS directly suppressed the expression of BAFF and APRIL (a proliferation-inducing ligand) in splenocytes from Nc/Nga mice, which induced atopic dermatitis by mite allergen. The suppression level of BAFF, at the concentration of 150 $\mu\text{g/ml}$, was almost 0.2-fold that of the control level (Fig. 2C). However, water extracts from yellow soybean (WEYS) did not suppress the expression of BAFF. Furthermore, WEGS suppressed the population of IgE positive B-

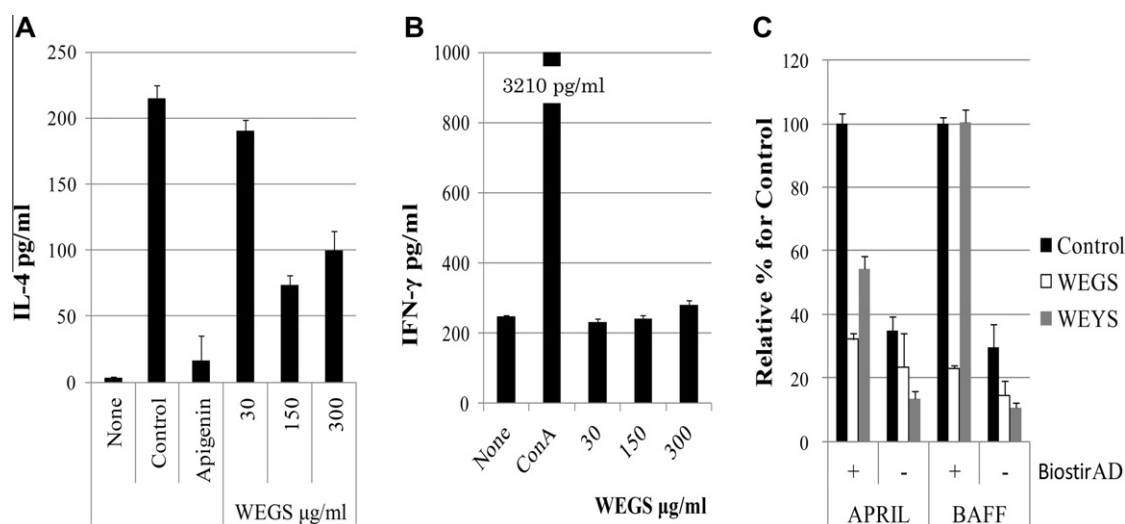


Fig. 2. WEGS down-regulates IL-4, BAFF and APRIL production. Whole splenic cells ($5 \times 10^6/\text{ml}$) from naive mice (A and B) and Nc/Nga mice, immunised with mite allergen (C), were cultured with WEGS. IL-4 and IFN- γ levels in the culture supernatants were quantified by ELISA. BAFF and APRIL mRNA levels were quantified by qRT-PCR. The mRNA levels were normalised using the level of β -actin, and are expressed as the mean $\pm$ SE relative to that for control with Biostir AD. The data represent the mean values and SE from five experiments. **P* < 0.01, compared with medium alone.

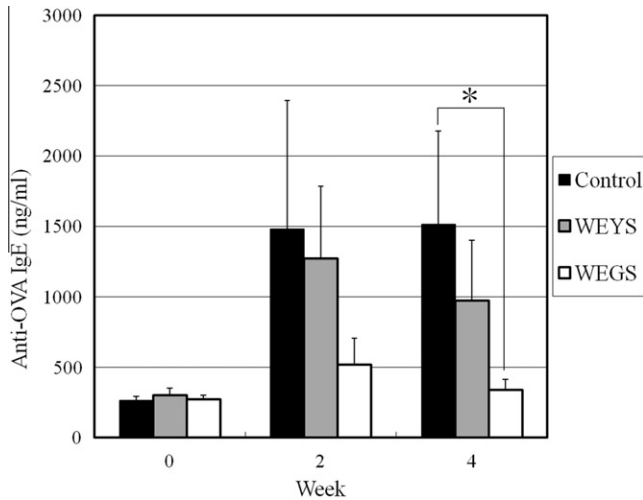


Fig. 3. Effect of WEGS and WEYS on ovalbumin (OVA)-specific IgE serum levels, in OVA-sensitized/challenged mice pre-sensitized for 21 days. Represented weeks are indicated as the period from the day administration was begun. IgE levels were determined by ELISA. Data are represented as means $\pm$ SE from 10 mice in each group. * $P < 0.05$, compared with OVA-sensitized/challenged group (OVA group).

Table 1
Effects of WEGS on nasal mucosa secretion levels of TDI induced guinea pig.

	Control	WEGS			β -Gulcan
		2.5%	5%	10	
Nasal secretion (mg)	121.8 $\pm$ 16.5	97.6 $\pm$ 16.9	74.1 $\pm$ 11.3*	67.1 $\pm$ 11.7*	61.2 $\pm$ 12.8**

Values are shown as mean $\pm$ SEM for 5 animals.

* $P < 0.5$ versus control animals (Mann–Whitney U -test).

** $P < 0.1$ versus control animals (Mann–Whitney U -test).

cell in splenocytes from Nc/Nga mice. The cell counts of IgE positive B-cell, at the concentration of 200 μ g/ml of WEGS, was almost 0.5-fold that of the control level (data not shown).

3.2. Effects of soybean extracts on production of IgE antibodies from OVA-sensitized mice

WEGS and WEYS did not cause significant changes in weight loss or drinking amount (data not shown). The level of OVA-specific IgE at the fourth week was significantly lower in the mice fed the 5% WEGS-supplemented diets than in those fed the control diet (Fig. 3). To confirm the significance of the green soybean in comparison with the yellow one, each hot water extract was determined in a range of IgE levels in the mice fed diet. The level of OVA-specific IgE was lower in the mice fed the 5% WEGS-supplemented diets than in those fed the 5% WEYS supplemented diets (Fig. 3). The levels of total IgG did not significantly differ between the mice groups (data not shown).

3.3. Effect of WEGS on TDI-induced nasal response in allergic rhinitis

Repeated application of TDI to the guinea pig nostril sensitises the animal, inducing allergic symptoms. In TDI-sensitized guinea

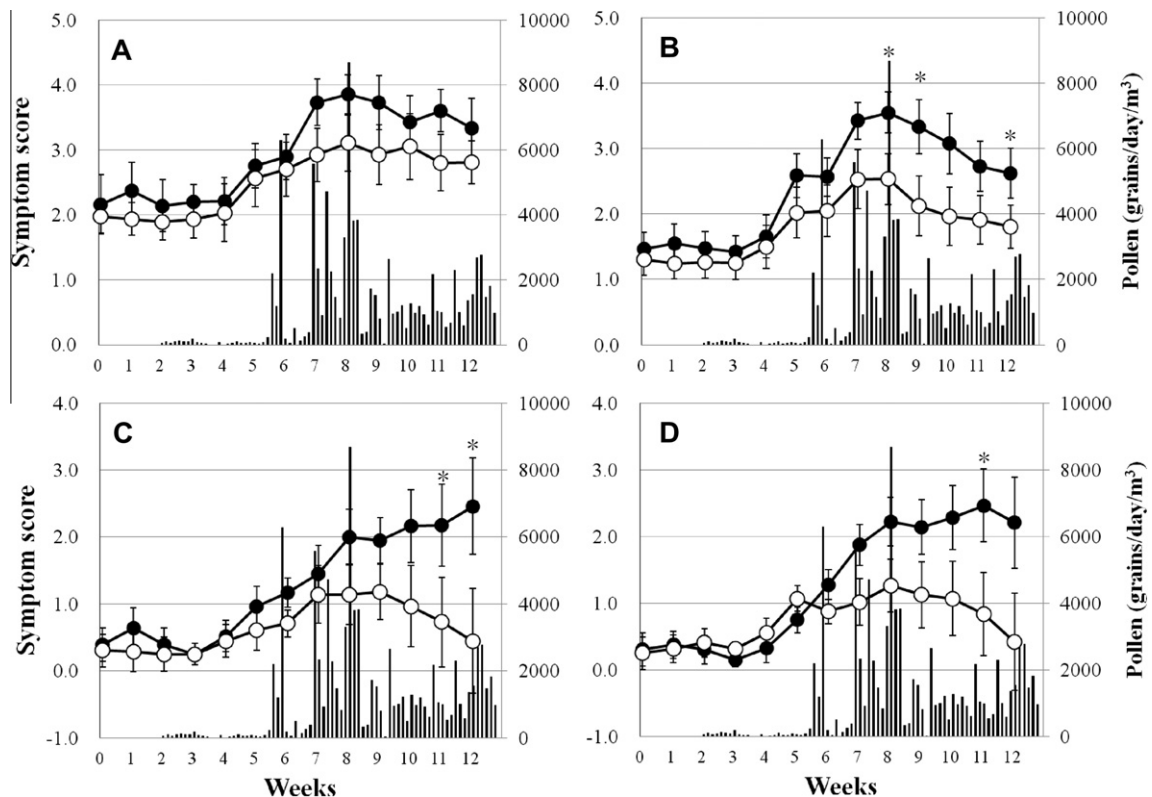


Fig. 4. The symptom scores of human study. The mean symptoms scores of subjects given WEGS in the long-term (open circles) and the short-term intake group (closed circle). Vertical bars represent the scattering number of cedar pollen. (A) nasal, (B) eye, (C) throat pain, (D) hindrance to ADL. A comparison between the two groups was performed using the Mann–Whitney U test. * $P < 0.05$ vs. another group.

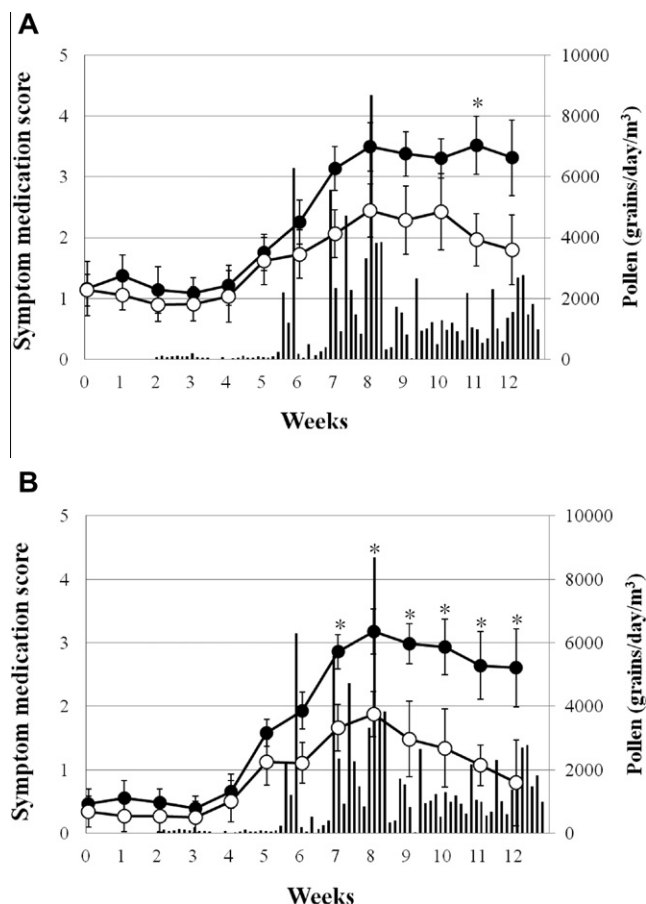


Fig. 5. The symptom medication scores of human study. The mean SMS of subjects given WEGS in the long-term (open circles) and the short-term intake group (closed circle). Vertical bars represent the scattering number of cedar pollen. The mean nasal (A) and eye (B) SMS of changes found in subjects given WEGS. A comparison between two groups was performed using the Mann–Whitney *U*-test. **P* < 0.05.

pigs, the application of TDI on day 28 induced vigorous nasal mucosa secretion. WEGS significantly suppressed the TDI-induced nasal mucosa secretion (Table 1). The levels of the nasal mucosa secretion in the guinea pigs fed 10% of the WEGS-supplemented diets were the same as those in the positive control (fed 1000 mg/kg/days of beta-glucan).

3.4. An open-label, single-dose, randomised, withdrawal parallel-group human study

Sixteen subjects were enrolled in the study (8 in the long-term group and 8 in the short-term group). An overall high compliance rate was obtained. No significant differences were found between the long-term and short-term intake groups with respect to patient characteristics (data not shown). The effects of WEGS intake, beginning before pollen dispersion, on allergic symptoms were examined by comparing the time-course changes in 3 nasal or 4 non-nasal symptom scores in the long-term intake group with those in the short-term intake group, in which WEGS intake was stopped when pollen scattering began. All symptom scores increased as the pollen count increased. The long-term intake group had a lower incidence of each symptom score (Fig. 4) and SMS (Fig. 5) than had the withdrawal short-term intake group throughout the pollen scattering period. The long-term intake group had a significantly (*P* < 0.05) lower incidence of itchy eyes at weeks 8 and 9 (Fig. 4B), tears at weeks 8 and 12 (Fig. 4B), throat pain at weeks 11 and 12 (Fig. 4C) and hindrance to activities of daily living (ADL)

at week 11 (Fig. 4D) compared with the withdrawal short-term intake group. From weeks 7 to 12, the mean eye SMS of the long-term intake group was significantly (*P* < 0.05) lower than that of the short-term intake group (Fig. 5B).

4. Discussion

To date, no one has investigated whether soybeans might possess positive effects for the large group of allergic diseases. We report here that green soybean extracts acts in an anti-allergic manner and simultaneously improve allergen-induced rhinitis. The results of these studies show that green soybean extracts appear to have such biological activity by modulating the TH1 and TH2 balance through a decrease in the level of selective TH2 cytokines and an increase in that of TH1 cytokines. The production of IgE is promoted by Th2 cells and their cytokines, such as IL-4 and IL-5, but is inhibited by IFN- γ secreted from Th1 cells (Mosmann & Coffman, 1989). The suppression levels of IL-4 by WEGS were mild. However, OVA-specific IgE was significantly suppressed by WEGS. We (surprisingly) found that WEGS was able to reduce IgE levels, even in the therapeutic protocol. Given these results, we believe that localised Th2 immune reactions are modified by treatment with WEGS after OVA sensitization. Because the mice were fed WEGS after OVA sensitization, immunoglobulin class switching had already occurred. However, WEGS suppressed OVA-specific IgE-production. In the human study, also, the short-term withdrawal WEGS intake group had a high incidence of each symptom score throughout the pollen scattering period. If WEGS modified systemic Th2 immune reactions, then the short-term withdrawal intake group should have experienced some symptom alleviation effects. On the other hand, WEGS directly suppressed the expression of BAFF and APRIL in splenocytes from Nc/Nga mice in which atopic dermatitis was induced by mite allergen. BAFF and APRIL may be related to the suppression of IgE-production in the OVA sensitization mouse by WEGS. It has been reported that BAFF is a key B cell survival factor associated with systemic autoimmune diseases, such as systemic lupus erythematosus (Vincent, Morand, & Mackay, 2012). The BAFF levels in asthma and atopic dermatitis patient serum were elevated relative to normal serum (Jee et al., 2010; Kang et al., 2006). However, there is no evidence that the suppression of BAFF alleviates allergic symptoms. Our results suggest the possibility that BAFF suppression controls allergic symptoms. The actual molecular mechanism by which WEGS suppresses IgE biosynthesis is still under investigation.

WEGS suppressed the TDI-induced nasal mucosa secretion and reduced the symptom scores for Japanese cedar pollinosis. In our clinical trial study, all the chosen subjects are office workers, and less likely to be exposed to a chemical substance. TDI is known to cause occupational asthma in workplaces (Kupczewska-Doback, Czerzak, & Brzezinski, 2012; Redlich & Karol, 2002). The relationship of chemical substance-induced asthma and pollinosis has been reviewed (D'Amato et al., 2005). The authors described that concentrations of airborne allergens and air pollutants are frequently increased contemporaneously, and that an enhanced IgE-mediated response to aeroallergens and enhanced airway inflammation could account for the increasing frequency of allergic respiratory allergy and bronchial asthma. Therefore, WEGS may be applicable for the chemical substance-induced asthma.

Many studies have used various strains of probiotics in murine models of type 1 allergy. In almost all of these studies, the total IgE levels were significantly lower in probiotics administration compared with the control. However, OVA-specific IgE levels were not significantly decreased by probiotics (Yu et al., 2010). This is consistent with the result reported that probiotics administration

prevents atopic dermatitis but does not decrease sensitization rates in humans (Kalliomaki, Salminen, Poussa, Arvilommi, & Isolauri, 2003). On the other hand, OVA-specific IgE levels were significantly decreased by the green soybean extracts. Therefore, green soybean extracts may be more effective than a lactic acid bacterium for human allergy patients. Some compounds that can be identified in soybean (e.g., genistein, daidzein, and saponins) have been independently reported to exhibit anti-inflammatory and immune modulatory properties, which might partially explain the bioactivities of WEGS. However, there is a slightly higher level of these compounds in green than in yellow soybeans (refer to [Supplementary data](#) for the components of the extracts). It is possible that there might be additional unknown compounds contributing to the full bioactivities of WEGS. We are investigating the identification of active compounds. As a result of the investigation, we found another isoflavone which suppresses production of the BAFF from green soybean. However, we cannot explain the full bioactivities of WEGS by this isoflavone. It is probable that this isoflavone and other compounds compositely affect the bioactivity of WEGS. Considering the biological activity of WEGS, further investigations into the identification of active compounds and other *in vivo* disease models are warranted. Because green soybean extracts has an allergic rhinitis symptom improvement effect, we plan to conduct a double blind trial for our next study.

5. Conclusion

Water extracts from green soybean had inhibitory activity with regard to the production of IgE, as well as the ability to regulate B cell apoptosis, probably by suppressing the BAFF and APRIL expression. The present results from the rhinitis model and human trial study indicate the great potential of WEGS as a natural suppressor of activated B cells through BAFF pathways and ultimately as anti-allergic reagents.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.12.014>.

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Component	WEYS	WEGS
Moisture	1.9	4.2
Protein	49.4	41.7
Lipid	9.4	5.1
Ash	8.3	7.6
Sugars	29.2	39
Fiber	1.8	2.4
Total saponin	0.35	0.41
Total isoflavon	0.45	0.65

All values are expressed on a grams per 100 g of powder, and data are reported as mean (n = 3).

Supplementary data

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Food Chemistry Volume 138, Issue 4 2013 2300 - 2305

<http://dx.doi.org/10.1016/j.foodchem.2012.12.014>