

Major allergens in soybean and clinical significance of IgG4 antibodies investigated by IgE- and IgG4-immunoblotting with sera from soybean-sensitive patients

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Summary

Background Soybean occasionally causes food allergy but its major allergens have not been sufficiently confirmed. The relationship between food allergy and food-specific IgG4 has also not been defined.

Objective We investigated the allergenicity of soybean proteins and the clinical significance of soybean-specific IgG4.

Methods We detected IgE- and IgG4-binding proteins in soybean by immunoblotting with sera from 30 soybean-sensitive patients (including seven patients with positive soybean challenge tests). The extract from soybeans was fractionated into the whey fraction and the globulin fraction.

Results Ten and eight proteins were detected as IgE- and IgG4-binding proteins, respectively, with a significant difference between the patient and control groups. Among the IgE-binding proteins, the proteins with molecular weights of 20 000 and 58 000 in the whey fraction, and 26 000 and 31 000 in the globulin fraction, had a particularly high IgE detection rate and high specificity. Two patients with positive challenge tests showed a quite different pattern in which only a protein with a molecular weight of 78 000 in the globulin fraction was detectable with serum IgE in both patients. The majority of IgG4-binding proteins were not consistent with the IgE-binding proteins. The strong reactivity of IgG4 was observed in all five infants among seven patients with positive challenge tests, and three of them had a very weak IgE reactivity.

Conclusions There were various antigenic proteins in soybean. Five proteins with molecular weights of 20 000 and 58 000 in the whey fraction, and 26 000, 31 000 and 78 000 in the globulin fraction, were considered major allergens in the IgE-mediated reaction. Results of IgE- and IgG4-immunoblotting suggested that soybean-specific IgG4 may act anaphylactically in patients with soybean allergy.

Keywords: soybean, protein, allergen, specific IgE, specific IgG4, immunoblot

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Introduction

Soybean is an important food component of the Japanese diet and is often used in processed forms, such as soybean

paste (miso), bean curd (tofu), and fermented soybeans (natto). Soybean is also well known as one of the major allergenic foods. Although there have been several reports on the allergenicity of soybean proteins [1–6], there has been no consistency in the major soybean allergens.

The proteins specifically bound to serum IgE have been investigated as the allergenic components in food. Recently,

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Table 1. Study subjects

Subjects	No.	Sex M/F	Age (yr)	No. of subjects with AD or BA		
				AD	BA	AD + BA
Patient group*						
Group 1	7	4/3	7.7 (1 to 22)	4	0	3
Group 2	23	15/8	8.0 (0 to 17)	10	3	10
Control group						
Group 3	11	6/5	7.8 (0 to 13)	7	3	1
Group 4	11	7/4	8.0 (0 to 15)	—	—	—

AD = atopic dermatitis; BA = bronchial asthma.

* Soybean-sensitive patients.

Group 1: patients with positive challenge test to soybean (characteristics of each patient are shown in Table 3). Group 2: allergic patients with positive IgE to soybean (RAST score ≥ 2) (they had not undergone challenge test). Group 3: allergic patients with negative IgE to soybean (RAST score 0). Group 4: non-atopic individuals.

however, much attention has been focused on the role of IgG4 in allergic diseases. Some researchers have reported that IgG4 acts as an anaphylactic antibody [7, 8], while others have described its action as a blocking antibody [9, 10]. Several recent reports indicated that the measurement of food specific IgG4 was helpful in detecting the causative foods in allergic patients [11, 12]. An interesting approach to clarifying the action of IgG4 might be to investigate whether or not both IgG4 and IgE are produced in response to the same proteins.

We investigated to detect the major allergens in soybean and evaluate the clinical significance of soybean-specific IgG4 by IgE- and IgG4-immunoblotting, using the sera from 30 soybean-sensitive patients and 22 control subjects.

Materials and methods

Subjects

The sera were obtained from 30 soybean-sensitive patients with atopic dermatitis (AD) and/or bronchial asthma (BA) who were treated at Shinshu University Hospital (Matsuyama, Japan), Fraternity Memorial Hospital (Tokyo, Japan) or the Metropolitan Toshima Hospital (Tokyo, Japan). All patients were positive to soybean with a prick puncture test performed and read in the standard manner [13].

Among them, 27 patients had a score of 2 or higher in the IgE RAST to soybean. The remaining three patients had a score of 1, but their diet records strongly suggested the

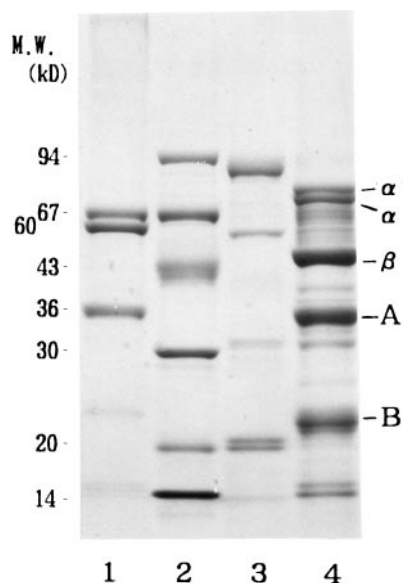


Fig. 1. SDS-PAGE patterns of soybean proteins stained with Coomassie brilliant blue. Lanes 1, 2 = molecular weight markers; lane 3 = whey fraction; lane 4 = globulin fraction. α' , α , β : β -conglycinin subunits, A, B: glycinin subunits. These bands of globulin fraction were tentatively identified by comparison with the known protein components [15] based on their mobilities.

existence of soybean allergy. Furthermore, elimination of soybean and soybean products from the diet alleviated their symptoms, but when they ingested soybean again their allergic symptoms worsened. Seven patients, including these three, yielded positive results on a soybean challenge test (group 1). The other patients did not undergo the challenge test (group 2). The control group consisted of 11 allergic patients who were not sensitive to soybean and 11 non-atopic individuals (groups 3 and 4). The characteristics of these subjects are listed in Table 1.

Soybean protein fractions

Soybean proteins were fractionated by the following procedure. Soybeans (Tsuruno-ko, a Japanese species) were crushed and defatted with n-hexane. The defatted meal was milled and passed through a 48-mesh sieve. The flour was extracted with water (1:10 ratio of flour to water) for 1 h with stirring at room temperature, then centrifuged at 10 000 rpm for 30 min at 20°C. The supernatant was acidified to pH 4.6 with 0.5 N HCl, and centrifuged at 10 000 rpm for 30 min at 20°C. The supernatant (whey fraction: WF) was separated from the precipitate (globulin fraction: GF), and these two fractions were dialysed against distilled water for 3 days.

Immunoblotting

The sera obtained were stored at -80°C until use. IgE- and

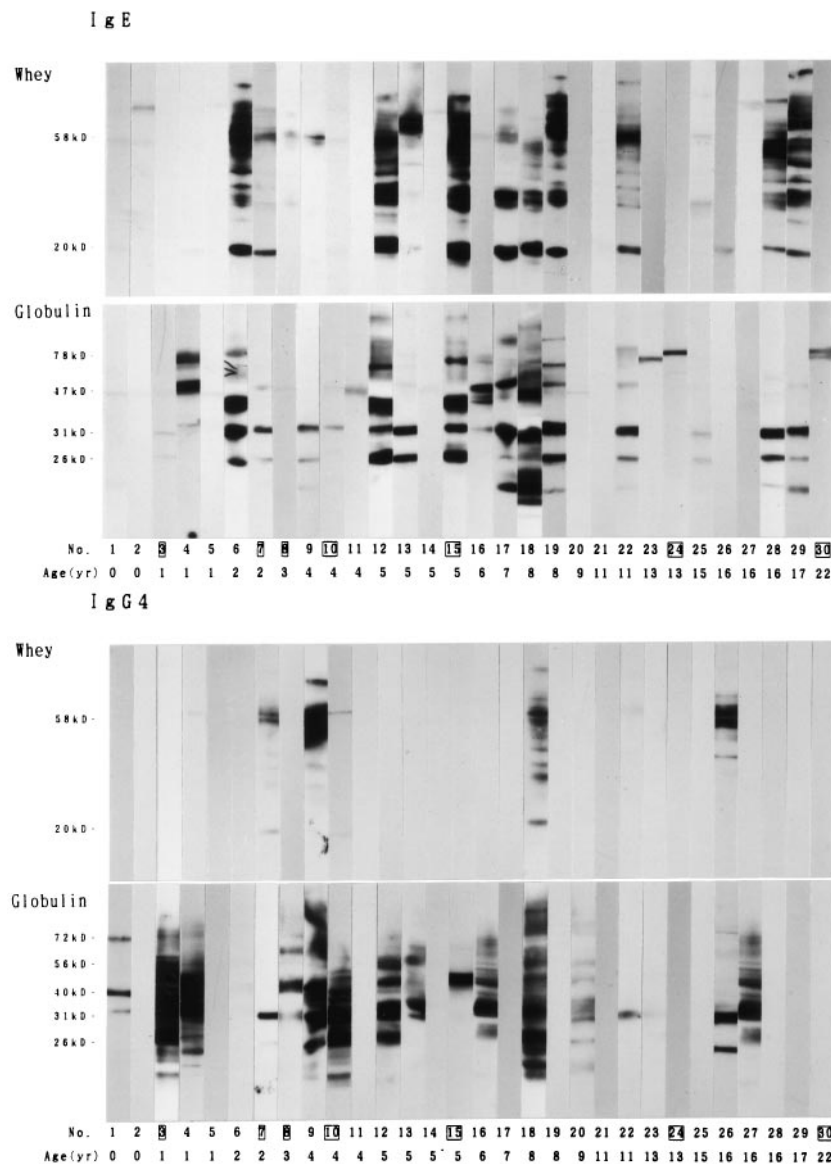


Fig. 2. IgE- and IgG4-immunoblots of 30 soybean-sensitive patients. Lanes 3, 7, 8, 10, 15, 24, 30 = patients in group 1 (with positive challenge test to soybean); the others = patients in group 2 (who had not undergone challenge test, RAST ≥ 2). Molecular weights were estimated by SDS-PAGE.

IgG4-immunoblotting of individual subjects were performed with the same serum using the same antigen preparation.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the WF and GF was performed on 5–20% gradient gel according to the method of Laemmli [14]. Samples were reduced with 2-mercaptoethanol prior to SDS-PAGE. The proteins separated on the gels were transferred onto the PVDF-membrane (ATTO Co., Tokyo, Japan) using the transblot apparatus (ATTO Co.). The membrane was blocked with 1% (wt/vol) polyvinylpyrrolidone dissolved in TBS (50 mM Tris-HCl, pH 7.6, 0.8% NaCl) containing 0.1% (vol/vol) Tween-20 for 2 h at room temperature,

followed by washing three times with TBS containing 0.02% Tween-20. Then the membrane was incubated with 2 ml of the patient serum (1:4 and 1:5 vol/vol dilution for detection of IgE and IgG4, respectively, with TBS containing 0.02% Tween-20) for 3 h at room temperature with rocking.

After washing three times with the washing buffer, the membrane was incubated with 2 ml of peroxidase-conjugated antihuman IgE (Tago Inc., Calif., USA) or peroxidase-conjugated antihuman IgG4 (Yamasa Fermenting Co., Tokyo, Japan) (1:20 000 vol/vol dilution in both antibodies) for 2 h at room temperature with rocking.

Table 2. Major IgE- and IgG4-binding proteins in soybean and the frequency detected with serum antibodies in study subjects

Fraction	Molecular weight (kD)	Frequency (%)			
		IgE		IgG4	
		Patient	Control	Patient	Control
Whey	91	33.3*	9.1	0.0	4.5
	75	33.3*	4.5	10.0	0.0
	58	60.0***	9.1	23.3	13.6
	41	36.7***	0.0	13.3	0.0
	28	40.0***	0.0	13.3	0.0
	20	53.3***	9.1	10.0	4.5
Globulin	91	16.7	0.0	33.3*	4.5
	78	16.7	0.0	36.7*	4.5
	72	23.3	9.1	40.0*	9.1
	64	33.3*	4.5	16.7	4.5
	56	13.3	0.0	40.0**	4.5
	47	66.7***	18.2	33.3*	4.5
	40	10.0	0.0	43.3**	4.5
	31	50.0***	0.0	56.7***	9.1
	26	46.7***	4.5	33.3*	4.5

Number of study subjects: patients 30, controls 22.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared to control with χ^2 test.

After washing five times with the washing buffer, peroxidase on the membrane was detected with the enhanced chemiluminescence (ECL) western blotting detection system (Amersham Japan Co., Tokyo). The hyperfilm-ECL (X-ray film for this system) was exposed to chemiluminescence caused by the peroxidase and subsequently developed. The membrane was then stained with Coomassie brilliant blue and used to determine the location of the bands appearing on the film.

Statistical analysis

Statistical analyses were done by the χ^2 test.

Results

Soybean proteins

SDS-PAGE patterns of the soybean WF and GF are shown in Fig. 1. The main bands of the GF were tentatively identified by comparison with the known protein components described in the literature [15], based on their electrophoretic mobilities.

Immunoblotting

Fig. 2 shows the immunoblots with sera of all patients

examined. Their characteristics are shown in Table 1. Results obtained with sera from group 1 are shown in lanes 3, 7, 8, 10, 15, 24 and 30, whereas the remaining lanes show results obtained from group 2.

Ten of the 22 control sera had IgE antibodies, though most of their blotting bands were faint. Furthermore their blotting patterns varied according to individual, so any IgE-binding protein was not detected with high frequency. IgG4 antibodies were detected from four control sera, among which one reacted weakly to the protein with molecular weight of 58 000 in the WF, the other one reacted to the proteins with molecular weights 64 000 and 72 000 in the GF, and the remaining two reacted to several proteins in both fractions (photograph is not shown. Detected frequency of each protein is shown in Table 2).

Many patients exhibited IgE-reactivity to several bands on their blotting patterns (Nos. 6, 12, 15, 17, 18, 19, 22, 28, 29). The bands with molecular weights of 20 000 and 58 000 in the WF and 26 000, 31 000 and 47 000 in the GF, were frequently detected. Two of these bands, with molecular weights of 20 000 in the WF and 31 000 in the GF, often showed a strong reactivity for IgE in the patient sera. In contrast, a band with a molecular weight of 47 000 in the GF showed a weak reactivity.

Table 3. Characteristics of patients in group 1

Subject No.	3	7	8	10	15	24	30
Sex	Male	Male	Male	Female	Male	Female	Female
Age (yr)	1.5	2.6	3.8	4.6	5.5	13.4	22.4
IgE (IU/ml)	350	55	6000	310	3800	2428	685
Atopic disease	AD	AD, BA	AD	AD, BA	AD, BA	AD	AD
IgE RAST positive foods except soybean	Egg, milk, wheat buckwheat	Egg, milk	Egg, milk, wheat rice, onion, salmon	Egg, milk, wheat, rice, sweet-corn, melon	Egg, rice, buckwheat	Wheat	Kidney bean
To soybean							
IgE RAST	2	1	1	1	2	2	2
Symptoms	AD, U	AD, U	AD	AD	AD	AD	AD

AD = atopic dermatitis; BA = bronchial asthma; U = urticaria.
Subject Nos. correspond to those in Fig. 2

Two patients in group 1, Nos. 24 and 30, showed a different pattern from those of the other patients. Only a band with a molecular weight of 78 000 in the GF was detected by IgE in both sera.

Subject No.	IgE							IgG4						
	3	7	8	10	15	24	30	3	7	8	10	15	24	30
Proteins														
Whey fraction														
91 kD		○	○											
75		○			○									
58		○	○	○	○				○		○			
41					○				○					
28		○			○				○		○			
20		○		○	○				○					
Globulin fraction														
91 kD					○				○		○	○		
78						○	○		○	○				
72						○	○		○	○	○	○		
64					○									
56									○		○	○		
47	○	○	○	○	○				○		○	○	○	
40					○				○		○	○		
31	○	○		○	○				○	○	○	○	○	
26	○	○		○	○				○	○	○			

Subject Nos. correspond to those in Table 3.

Fig. 3. IgE- and IgG4-antibodies specific to soybean proteins in sera of patients in group 1

IgG4 reacted rarely to the proteins in the WF, but often showed a strong reactivity to the proteins in the GF. A band with a molecular weight of 31 000 in the GF was most frequently detected by IgG4 in the patient sera. Also on the IgG4-blotting patterns many patients exhibited IgG4-reactivity to several bands (Nos. 3, 4, 9, 10, 12, 16, 18, 26, 27).

With almost all of the patient sera some IgE-binding protein was detected in the GF and/or WF, whereas there was a large number of patients with whose sera no IgG4-binding protein was detected in either fraction (Nos. 2, 5, 11, 14, 17, 19, 21, 24, 25, 28, 29, 30). The majority of the patients whose sera did not show IgG4 binding with any soybean protein were patients aged over 6 yr.

IgE- and IgG4-binding proteins

The major IgE- and IgG4-binding proteins showing a significant difference in the detection rate between the patient and control groups are summarized in Table 2. As major IgE-binding proteins, six proteins in the WF and four proteins in the GF were recognized. Eight major IgG4-binding proteins were found only in the GF. Among these proteins, only three proteins reacted with both IgE and IgG4. Of the major IgE-binding proteins, five proteins with molecular weights of 20 000 and 58 000 in the WF, and 26 000, 31 000 and 47 000 in the GF, showed a particularly high rate of IgE detection. Of the IgG4-binding proteins, the protein with a molecular weight of 31 000 in the GF showed an especially high rate of IgG4 detection.

Specific antibodies in the patients of group 1

Table 3 lists the characteristics of the seven patients in

group 1. Patient numbers correspond to those in Fig. 2. There were four boys and three girls, ranging in ages from 1 to 22 yr. Evaluation by the RAST showed that patients 3, 7, 8, 10 and 15, who were of a younger age, were sensitized to various antigens including egg and milk (although patient 15 was negative to milk), whereas patients 24 and 30, who were older, gave a negative response to egg and milk, and furthermore did not have many sensitizing antigens. The major allergic symptoms included AD alone in four patients and AD plus BA in three; the symptoms provoked by soybean products were mostly AD, although patients 3 and 7 developed urticaria also.

Fig. 3 shows the IgE- and IgG4-antibodies specific to soybean proteins in these seven patients which were detected on their immunoblots in Fig. 2. In the five younger patients (Nos. 3, 7, 8, 10, 15), the IgE detection rates for each protein showed a similarity to those in the all-patient group. Serum IgG4 specific to soybean was detected in all five patients. In patients 3, 8 and 10, the reaction of IgE was weak, and that of IgG4 was strong (Fig. 2). In both patients 24 and 30, serum IgE was detected in response to the protein with a molecular weight of 78 000 in the GF. This protein, however, resulted in a low IgE detection rate in the other patients. Furthermore, in both patients neither IgE nor IgG4 was detected in response to any of the proteins that had resulted in a high detection rate in the other patients.

Discussion

In the present study, proteins with molecular weights of 20 000 and 58 000 in the WF, and 26 000, 31 000 and 47 000 in the GF, were recognized by serum IgE of more than half of the patients. Among these components, the protein with a molecular weight of 47 000 in the GF showed a weak reactivity to serum IgE on the whole. Furthermore, the detection rate of this protein was comparatively high even in the control group. Therefore, this protein was assessed as having a low specificity. The other four had a high specificity, and so were presumed to be proteins with markedly high allergenicity.

In the seven patients with positive challenge tests, five younger patients and two older patients showed clearly different patterns of IgE detection, which suggested that there are at least two different types of soybean allergen pattern. In the five younger patients it was speculated that the four proteins mentioned above acted as the major allergens. It was, however, impossible to determine which of these four proteins served as the most significant allergen in each patient. It is probable that the major allergen differs from patient to patient, or several proteins act simultaneously in one patient. On the other hand, in the older two patients it was speculated that a protein with molecular weight of 78 000 in the GF acted as an allergen.

Herian *et al.* [5] identified three different patterns of immunoblots in adult patients (23–67 yrs) with soybean allergy. They classified their patients into three patterns whose serum IgE bound the proteins with molecular weights of 50 000–60 000, 20 000 or 14 000, respectively. Neither of these three patterns was in agreement with the two patterns identified in the present study. This discrepancy may have been caused by differences in the age and dietary behaviour of the subjects, or the method of preparing the sample proteins. In particular the diversity of soybean products and the high frequency of their consumption are characteristics of the Japanese diet, which may have an influence on the species of allergenic proteins. Only the protein with a molecular weight of 20 000 in the WF which had a high detection rate in our study may be identical to one of the allergens which Herian and his colleagues had reported.

Ogawa *et al.* [6] indicated that the allergenicity of the whey fraction was low, but the results of the present study demonstrated the high allergenicity of the two proteins in the WF. This disparity may have derived from differences in the method of preparing the whey fraction. Since whey proteins are not the main components of soybean proteins and are easy to separate, it should not be difficult to make soybean products free of whey proteins. It is thought that the protein with a molecular weight of 31 000 in the GF will probably be the same component as the protein called Gly m Bd 30K, which was recently named by Ogawa *et al.* [6] as the most important soybean allergen.

The protein with a molecular weight of 78 000 is speculated to be α' -subunit of 7S-globulin from its electrophoretic behaviour. In recent studies on breeding, a soybean strain lacking the α' -subunit has been found [16]. It may be possible to utilize this soybean strain for some soybean allergic patients.

In the present study, the majority of proteins resulting in a high IgG4 detection rate differed from proteins showing a high IgE detection rate. It is of interest that proteins in the WF could scarcely be recognized by serum IgG4 of the patients, while several proteins in the WF bound strongly to serum IgE of many patients. Approximately 90% of the water-extracted soybean proteins are globulin proteins, and the amount of whey proteins is small. This suggests that the amount of antigen may have to exceed a certain level to induce the production of IgG4 specific to it.

The frequency of IgG4 detection showed a tendency to be higher in the infant group than in the older patient group. Moroi [17] revealed that the peak of IgG4 titre to soybean protein was seen at an age of 4 to 5 yr. Thus, the production of IgG4 seems to be suppressed from around the latter stages of infancy. This may be related to the fact that the amount of antigen entering from the intestinal tract is reduced with the maturation of digestive tract function. It has been reported that IgG4 positivity to soybean increases after

7 months of age [18]. This may be because the opportunity to eat soybean products, such as bean curd and fermented soybean, increases around this age. It has been demonstrated that IgG4 increases as a result of desensitization therapy [19, 20] and decreases through elimination of allergen-food from the diet [21]. These phenomena also suggest a close relationship between IgG4 production and the amount of antigen entering the body.

From the results that IgG4 and IgE were not necessarily produced in response to the same proteins, it is difficult to consider that IgG4 acts as a blocking antibody at least in the IgE-mediated allergic reaction. Moreover, the five infants with positive challenge tests showed a strong IgG4 reaction. Of them, three patients showed a very weak IgE reaction, suggesting minor involvement of IgE in their allergic symptoms. These findings may indicate the possibility that soybean-specific IgG4 acts as an anaphylactic antibody. The higher frequency of IgG4 detection in the infant group suggests that IgG4 may play an important role especially in younger children. If these assumptions are true, a decrease in the production of IgG4 related to food allergy may be attributed in part to the spontaneous alleviation of allergic symptoms with growth. We may have to pay attention to IgG4-mediated allergenic components of soybean, too.

The present study showed the diversity of the allergenic proteins in soybean. It is desirable to determine which protein provokes the soybean-allergic disease of each patient, and desirable that various allergen-free soybean foods should be produced. The role of IgG4 in food allergy is a significant issue on the definitive diagnosis of allergens and establishment of dietary therapy. Although in this study IgG4 seemed to act as anaphylactic antibody, convincing evidence remains to be established.

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