

Antigenicity of the proteins in soy lecithin and soy oil in soybean allergy

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Summary

Background Soy lecithin and soy oil are usually produced from the hexane extract of soybean. Some of the soybean proteins are included in the extract and are therefore present in small amounts in both soy lecithin and soy oil. The antigenicity of the proteins present in defatted soybean has been studied with respect to soybean allergy, but the antigenicity of those found in the extract is yet to be investigated.

Objective The antigenicity of soy lecithin and soy oil proteins with regard to soybean allergy were investigated.

Methods The proteins present in soy lecithin and soy oil were determined according to already established method and analysed by SDS-PAGE. The IgE- and IgG4-binding abilities of the soy lecithin proteins were investigated by immunoblotting with sera from 30 soybean-sensitive patients, including seven with a positive challenge test. Immunoblotting of soy oil proteins was performed with the sera from some of these patients.

Results In 100 g of sample, the soy lecithin and soy oil contained 2.8 mg and 1.4–4.0 µg of proteins, respectively. The results of SDS-PAGE demonstrated the presence of only three proteins, with molecular weights of about 58–67 kDa in soy oil, and suggested that soy lecithin also contains these proteins. The soy lecithin also contained many proteins besides these. In the soy lecithin, the detection rate of only one protein, with a molecular weight of 31 kDa, by the serum IgE of patients was significantly different compared with controls (detection rate: 40%). The proteins with molecular weights of 58–67 kDa rarely bound to serum IgE. Only one of the patients who presented a positive challenge test had IgE antibodies to soy lecithin proteins. IgG4-binding proteins were found only rarely in soy lecithin. Neither the IgE nor the IgG4 present in the patients' sera reacted to any soy oil protein.

Conclusion Proteins present in soy lecithin and soy oil have little antigenicity with regard to soybean allergy.

Keywords: soy lecithin, soy oil, protein, antigenicity, specific IgE, specific IgG4, immunoblot

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Introduction

Soybean is known to be one of the major allergenic foods. It is well established that soy oil is also highly allergenic with

regard to soybean allergy. The major allergens in soybean have been identified by recent studies [1–7], and all of these have been investigated using defatted soybean or soybean products. Few studies into the allergenicity of soy oil have been carried out. Bush *et al.* [8] have reported that soybean oil is not allergenic to soybean-sensitive individuals. However, Doke *et al.* [9] have shown that proteins which have

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interacted with oxidized lipids are allergenic to soybean-sensitive patients, and that this effect is caused not only by soy oil, but also by other vegetable oils. On the other hand, the presence of soy proteins in soy lecithin and soy oil has been reported by Porras *et al.* [10], but the involvement of these proteins in soybean allergic diseases has not been established.

Soy oil is commonly obtained by removing the gummy matter from crude soy oil, which is extracted from soybean using hexane. Following dehydration, the gummy matter is known as soy lecithin and is used, for example, as a food additive. Most of the phospholipids and proteins present in crude soy oil are contained in soy lecithin, but it is considered that the proteins interacting with phospholipids are retained in soy oil. Thus, the proteins present in soy oil may also be found in soy lecithin.

In this study the quantity of proteins present in soy lecithin and soy oil were determined, their sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns compared, and their IgE- and IgG4-binding abilities investigated by immunoblotting to estimate their antigenicity in soybean allergy.

Materials and methods

Sera

Sera were obtained from 30 soybean-sensitive patients with atopic dermatitis (AD) and/or bronchial asthma (BA) who were treated at Shinshu University Hospital (Matsumoto, Japan), Doai Memorial Hospital (Tokyo, Japan), or the Metropolitan Toshima Hospital (Tokyo, Japan). The sensitivity of all patients to soybean was confirmed with a prick puncture test that was performed and read in the standard manner [11]. Of these patients, 27 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 existence of soybean allergy. Furthermore, the elimination of soybean and soybean products from the diet alleviated the symptoms of these three patients, but when they ingested soybean again their allergic symptoms worsened. Seven patients, including these three, yielded positive results in an open challenge test to soybean (group 1). Regarding three patients of group 1, their history suggested that soy oil also influenced their symptoms. The patients of group 2 had no history of a certain influence by ingesting soybean, and therefore were not subjected to the challenge test. Almost all of the patients in groups 1 and 2 were positive in the IgE-RAST to other foods in addition to soybean. 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. The sera from these subjects were stored at -80°C until use. For the immunoblotting of soy oil proteins the sera from four of the 15 patients who had IgE antibodies to soy lecithin proteins were used, including one patient who presented a positive challenge test.

Separation of the proteins from soy lecithin and soy oil

There is no common method to separate the proteins from soy lecithin and soy oil. A lipid extraction method was applied [12]. The food additive soy lecithin (Yoneyama Chemicals, Osaka, Japan) was used. Twenty millilitres of a mixture of *n*-hexane and iso-propylalcohol (3:2) and 4 mL of distilled water were added to 2 g of lecithin. This mixture was shaken, and then centrifuged at 800 *g* for 5 min at room temperature. The insoluble fraction including the proteins was thus obtained at the borderline between the two liquid layers. This fraction was washed three times with a mixed

Table 1. Study subjects

	Number	Sex (M/F)	Age (years)	Number of subjects with AD or BA		
				AD	BA	AD & BA
<i>Patient group*</i>						
Group 1	7	4/3	7.7 (1–22)	4	0	3
Group 2	23	15/8	8.0 (0–17)	10	3	10
<i>Control group</i>						
Group 3	11	6/5	7.8 (0–13)	7	3	1
Group 4	11	7/4	8.0 (0–15)	–	–	–

AD, atopic dermatitis. BA, bronchial asthma. *Soybean-sensitive patients. Group 1, patients with positive challenge to soybean. Group 2, allergic patients with positive IgE to soybean (RAST score ≥ 2)—had not undergone challenge test. Group 3, allergic patients with negative IgE to soybean (RAST score 0). Group 4, non-atopic individuals.

solvent of *n*-hexane, iso-propylalcohol and distilled water (6:4:1), and the solvent was removed under decompression (60 mm Hg, 40°C).

The protein content and SDS-PAGE patterns of soy oil samples from three different companies were examined. To collect the fraction that included the proteins in soy oil, a method similar to that used to remove the gummy matter from crude soy oil was used. One kilogram of soy oil and 250 mL of the saturated solution of ammonium sulphate were stirred for 1 h with a stirrer (LABO-STIRRER L-35, Yamato Kagaku, Tokyo, Japan). This mixture was then centrifuged at 15 600 *g* for 40 min at 20°C, and the creamy layer between the two layers of oil and ammonium sulphate solution was collected. This fraction included the proteins. The used oil produced the same creamy layer whenever this process was repeated, although the amount of product obtained was reduced with each cycle. Only the first and second product were collected as repeating the method more than three times has been shown to yield an insignificant amount of protein. Moreover, the products of three cycles of the method exhibited the same SDS-PAGE patterns. The collected creamy matter was shaken with three times as much of the mixed solvent (*n*-hexane, iso-propylalcohol and distilled water, 6:4:1). The mixture was then centrifuged at 800 *g* for 5 min at room temperature. By this means an insoluble fraction that included the proteins was obtained at the borderline between the two liquid layers. This fraction received the same treatment as that of the insoluble fraction obtained from soy lecithin.

Both the fractions from soy lecithin and soy oil seemed to contain not only protein, but also some kind of lipid.

Quantitative analysis of protein

The content of protein in the fraction separated from each sample as described above was determined by a modification of the method of Lowry *et al.* [13]. A definite quantity of distilled water was added to the dry fraction obtained from a definite quantity of lecithin or oil. This mixture was reacted directly with designated reagents. Some insoluble matter was observed floating in the final reacted solution; it was supposed that this was some kind of lipid. Prior to colourimetry, this insoluble matter was removed with a large pore filter paper.

SDS-PAGE

SDS-PAGE of the samples was performed on 5–20% gradient gels according to the method of Laemmli [14]. Samples were reduced with 2-mercaptoethanol prior to SDS-PAGE. About 15 and 10 µg of protein from soy lecithin and soy oil, respectively, were applied to the gels and run for about 70 min at 20 mA.

Immunoblotting

The proteins that separated on the gels were transferred onto the PVDF-membrane (ATTO, Tokyo, Japan) using a trans-blot apparatus (ATTO). The membrane was blocked with 1% (w/v) polyvinylpyrrolidone dissolved in TRIS-buffered saline (TBS 50 mmol/l TRIS-HCl, pH 7.6, 0.8% NaCl) containing 0.1% (v/v) Tween-20 for 2 h at room temperature, and then washed 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 v/v 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 anti-human IgE (Tago, CA, USA) or peroxidase-conjugated anti-human IgG4 (Yamasa Fermenting, Tokyo, Japan) (1:20 000 v/v dilution of both antibodies) for 2 h at room temperature with rocking. After washing five times with the washing buffer, peroxidase on the membrane was detected with an enhanced chemiluminescence (ECL) western blotting detection system (Amersham Japan, Tokyo, Japan). The hyperfilm-ECL (X-ray film for this system) was exposed to the chemiluminescence caused by the peroxidase, and was 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 analyses

Statistical analyses were performed using the chi-squared test.

Results

The proteins in soy lecithin and soy oil

The weight of protein in the soy lecithin and soy oil samples was 2.8 mg and 1.4–4.0 µg per 100 g of sample, respectively, as shown in Table 2. These samples, which were produced by three different companies, or at different times by the same company, contained slightly different protein contents. The proteins from these soy oil samples produced the same SDS-PAGE pattern, showing three bands with molecular weights of about 58–67 kDa (Fig. 1). The SDS-PAGE pattern of the soy lecithin proteins exhibited many bands, three of which were found at almost the same locations as those of the soy oil proteins.

Antigenicity of the proteins in soy lecithin and soy oil

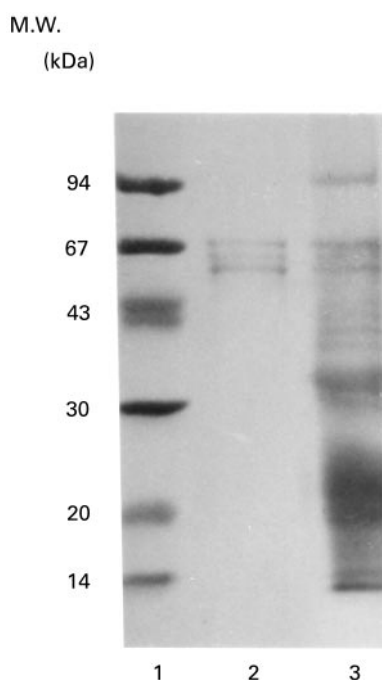
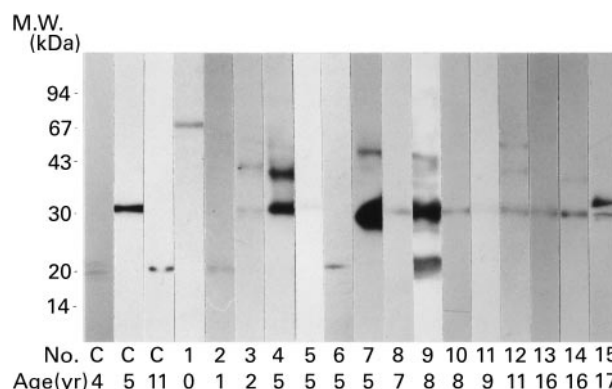
The IgE antibodies in the sera from 15 out of the 30 patients reacted to the proteins in the soy lecithin by

Table 2. Content of protein in soy lecithin and soy oil

Sample	Content ($\mu\text{g}/100\text{g}$)
Soy lecithin	2.8×10^3
Soy oil	
1	1.7
2	1.4
3	2.7
4	4.0
5	1.8
Average	2.3

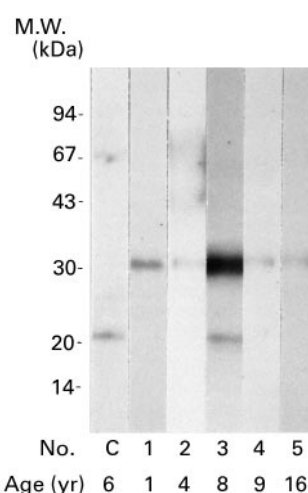
Soy oils 1–3 were produced by three different companies. Oils 3–5 were produced at different times by the same company.

immunoblotting, whereas the IgE antibodies in only three out of 22 control sera reacted to them. Only one of seven patients who presented a positive challenge test had IgE antibodies to the protein in soy lecithin (Number 7; Fig. 2). Figure 2 shows all of the immunoblot patterns of the IgEs detected in this study. The protein with a molecular weight of about 31 kDa was identified as a major IgE-binding proteins. Significantly different detection rates of 40% (12/30) and 4.5% (1/22) were obtained with patient and control sera, respectively. In addition to this, several proteins reacted to the IgE in patient sera, but their detection

**Fig. 1.** SDS-PAGE patterns of the proteins from soy lecithin and soy oil stained with Coomassie brilliant blue. Lane 1, molecular weight markers. Lane 2, proteins from soy oil. Lane 3, proteins from soy lecithin.**Fig. 2.** IgE-immunoblots of 15 soybean-sensitive patients (lanes 1–15) and three controls (lanes c). Lane 7, patient with positive challenge test to soybean.

rates were low and there was no significant difference between those of patients and controls. The proteins with molecular weights of about 58–67 kDa corresponding with those in soy oil did not react to the serum IgE of most of the patients. All of the proteins found in soy oil were considered to also be included in soy lecithin. Therefore immunoblotting to soy oil proteins was performed with sera from only four patients (Numbers 2, 5, 7 and 10; Fig. 2) with the intention of saving sera and oil. Their IgE-immunoblotting did not show any reactive band to soy oil proteins. The patient of number 7 belonged to group 1, and his history suggested that soy oil exacerbates his symptoms. Another two patients from group 1 also had a history suggesting that soy oil is responsible, at least in part, for their symptoms, but these patients did not have any IgE antibodies to soy lecithin proteins.

IgG4-antibodies to soy lecithin proteins were found in the sera of only five out of 30 patients and only one of the controls (Fig. 3). The IgG4 antibodies in all five of these

**Fig. 3.** IgG4-immunoblots of five soybean-sensitive patients (lanes 1–5) and one control (lane c).

sera reacted to the protein that had a molecular weight of about 31 kDa. None of these five patients presented a positive challenge test. Patient numbers 1, 3 and 5 in Fig. 3 correspond with numbers 2, 9 and 13 in Fig. 2, respectively. The remaining three patients represented in Fig. 3 did not show IgE-reactivity. None of the four patients who were tested for soy oil proteins had any IgG4 antibodies to those proteins.

Discussion

All samples of soy lecithin and soy oil tested in this study had a very small quantity of protein. Porras *et al.* [10] have detected soy protein in soy lecithin, margarine and soy oil, by an inhibition technique using ELISA. They found soy protein in six out of seven lecithin samples (1.03–27.2 mg/g) and three out of eight soy oil samples (0.11–3.3 mg/g). Soy protein was not detected in the remaining samples (one of lecithin and five of oil). The large quantitative difference between their samples and those investigated in the present study may be attributable to the different detection methods used. In addition, the samples used in this study may correspond with the samples that were found to be non-detectable in their report. It is thought that the manufacturing process, especially the degree of refinement, may influence the protein contents of these samples. A reliable method for separating the proteins from the lecithin and oil has not yet been established. Thus, it is not possible to prove that all of the proteins in these samples were detected and tested by immunoblotting.

During the manufacturing process of soy oil, gummy matter (lecithin containing water) is taken away from crude soy oil by mixing with hot water or steam. It is conceivable that the creamy matter, which is obtained by mixing soy oil with a solution of ammonium sulphate, is gummy matter that has been left in the soy oil. Lecithin is known to form complexes with proteins, therefore it is thought that a very small quantity of proteins is retained in soy oil in the form of a complex with lecithin. The results of SDS-PAGE analyses suggest that the soy oil proteins obtained from three different companies are all the same, and that the same proteins are also present in soy lecithin.

Half of the soybean-sensitive patients did not have IgE antibodies to any of the proteins extracted from the soy lecithin. The serum IgE of the remaining half of the patients was bound most frequently to the protein that had a molecular weight of about 31 kDa. The detection frequency of this protein was 40% of all soybean-sensitive patient sera. The result of immunoblotting of the soybean proteins in globulin and whey fractions using the same patient sera have previously been reported by the authors [7]. In this study it was shown that 10 of the proteins present in these two fractions were major IgE-binding proteins, and that five

of these proteins had bound with serum IgE in 50% or more of the patients. Almost all of the patients' sera that reacted to soy lecithin proteins reacted far stronger to the proteins from these two fractions. Regarding the patients who presented a positive challenge test, all seven patients reacted to some of the proteins from these two fractions, whereas only one reacted to soy lecithin proteins. These results suggest that the protein that had a molecular weight of about 31 kDa in soy lecithin is antigenic to the IgE antibody, but this antigenicity is far weaker than that of the proteins in globulin and whey fractions. Therefore, it is thought that this protein in soy lecithin is not a major allergen for soybean-allergic patients.

Serum IgG4 of the soybean-sensitive patients bound rarely with the proteins found in soy lecithin. The action of IgG4 in allergic disease has not been made clear. Whether it acts as an anaphylactic antibody or a blocking antibody, it seems that the proteins in soy lecithin are not concerned with the production of IgG4 antibodies. In a previous study [7] the authors showed that the proteins in whey fraction possess a very low IgG4-binding activity, whereas eight of the proteins from the globulin fraction bind significantly more frequently with the serum IgG4 of patients compared with controls. The major proteins present in soybean are globulin proteins, while whey proteins are minor components. Soy lecithin proteins are even smaller constituents. These results suggest that the production of IgG4 antibodies is affected by the quantity of the protein ingested as an antigen.

The proteins in soy oil are considered to be the same as some of the proteins in soy lecithin. Analyses of the immunoblotting patterns of the proteins from soy lecithin have demonstrated that equivalent bands to those of the proteins found in soy oil only rarely bind to both the IgE and IgG4 in 30 patient sera. Moreover, the proteins from soy oil did not bind with either the IgE or IgG4 of the four patient sera. One of these four patients who belonged to group 1 had a history of soy oil allergy. These results suggest that the proteins in soy oil have no antigenicity to IgE and IgG4 antibodies. Further investigations are required, including the development of a method to separate the proteins in lecithin and oil, if this problem is to be solved.

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