

CASE REPORT

Soybean β -conglycinin as the main allergen in a patient with food-dependent exercise-induced anaphylaxis by *tofu*: food processing alters pepsin resistance

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Summary

Background Food-dependent exercise-induced anaphylaxis (FDEIA) due to soybeans is a rare disorder. The allergen responsible for FDEIA due to soybeans has not yet been determined.

Objective We characterized the clinical features of a patient with FDEIA due to *tofu*, who was well tolerant to drinking soy milk. We then sought to identify the responsible soybean allergen(s) in that patient. We further studied whether different stabilities of the allergen(s) to pepsin digestion between two soybean products are related to their clinical allergenicity.

Methods Skin prick tests and provocation tests using soybean products were performed to detect the responsible food and other factors that induced the allergic symptoms. Specific IgE to various soybean allergens were examined by ImmunoCAP®, ELISA and protein microarray assays. Immunoblotting for soybeans and soybean products using the patient's serum was also performed. Soybean products were serially digested by pepsin to disclose the stability of the allergens.

Results Provocation with ingestion of *tofu* and exercise induced the allergic symptoms, while ingestion of soy milk and exercise did not. Immunoblot analysis, ELISA and protein microarray assay revealed that β -conglycinin mainly reacts with IgE antibodies in the patient's serum. By immunoblot analysis, β -conglycinin in soy milk completely disappeared after pepsin digestion within 20 min, whereas β -conglycinin in *tofu* was almost intact after more than 120 min of pepsin digestion.

Conclusion We identified β -conglycinin as the causative allergen in a patient with FDEIA induced by *tofu*. The difference in resistance to pepsin digestion between *tofu* and soy milk suggests that the presence of undigested allergens in the digestive tract is a prerequisite for the development of FDEIA.

Keywords β -conglycinin, food-dependent exercise-induced anaphylaxis, pepsin-digestion, soy milk, soybean, *tofu*

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Introduction

Food-dependent exercise-induced anaphylaxis (FDEIA) is a distinct form of food allergy, because its symptoms are characteristically induced by physical exercise after ingestion of the causative food [1]. Patients with FDEIA usually have IgE antibodies to the causative food and exhibit symptoms such as urticaria, erythema, pruritus

and dyspnea, which is typical for IgE-mediated allergic reactions [2]. The symptoms of FDEIA are induced only by the combination of food and exercise or the combination of aspirin and exercise [3, 4]. Matsuo et al. [5] determined that ω -5 gliadin and high molecular weight (MW) glutenin are causative allergens of wheat-dependent exercise-induced anaphylaxis (WDEIA), the most frequent type of FDEIA. They also demonstrated that exercise and aspirin

increase the levels of circulating gliadin peptides after wheat intake in patients with WDEIA, suggesting that the increased absorption of the allergen from the intestine due to exercise and aspirin is one of the reasons why this unique allergic disorder develops by the combination of food intake and exercise [6].

As for soybean allergies, a variety of allergens have already been determined [7]. The major allergens of soybean are Kunitz soybean trypsin inhibitor (KSTI) for asthma in laboratory worker [8], Gly m 1, Gly m 2 for Barcelona asthma which is a symptom of inhalation of soybean dust [9], Gly m Bd 30k [10, 11], Gly m Bd 28k [12] and Gly m Bd 60k [13] which was identified as the α subunit of β -conglycinin. [13] The latter three were detected using the patient with food allergy to soybean. Additionally, Helm *et al.* [14] reported 22 kDa allergen as soybean G2 glycinin allergen as an important allergen of soybean food allergy. Recently, oral allergy syndrome (OAS) and/or food allergy including anaphylaxis caused by soybean protein-containing foods in patients with birch pollen allergy have been reported. Starvation-associated message 22 (SAM 22: Gly m 4), with a MW of 17 kDa [15, 16], and soybean profilin (Gly m 3), with a MW of 14 kDa [17], have been reported as allergens due to soybeans. So far, FDEIA induced by soybean products has never been reported and the major allergen responsible for soybean-induced FDEIA has not yet been determined.

We recently examined a patient with FDEIA induced by a soybean product. The most interesting feature was the fact that she experienced FDEIA only after ingesting *tofu*, which is a gelatinized material made from soy milk using *nigari* (mainly magnesium chloride), but she was not similarly affected by soy milk. By immunoblotting analysis, ELISA and protein microarray assay, we determined that β -conglycinin is the causative allergen of her FDEIA induced by *tofu*. Whereas β -conglycinin of soy milk completely disappeared after pepsin digestion within 20 min, β -conglycinin of *tofu* was almost completely intact after pepsin digestion for more than 120 min. The markedly higher stability of β -conglycinin in *tofu* against pepsin digestion may explain why our patient developed FDEIA symptoms only after ingesting *tofu* but not soy milk. This is the first report showing that resistance to pepsin digestion during food processing is important to the cause of FDEIA.

Patient and methods

A 16-year-old girl with atopic dermatitis developed generalized urticaria, abdominal pain and dyspnea and became semiconscious on her 20 min ride to school on a bicycle. She was brought by ambulance to the Emergency Clinic of the Hyogo Prefectural Kakogawa Hospital on 6 August 2004. Upon her arrival at the hospital, dyspnea, flushing and swelling of her entire body was observed and

she had a systolic blood pressure of 40 Torr. She was treated with subcutaneous epinephrine. Her symptoms subsided 2 days later, and she was referred to our Dermatology Clinic to determine the cause of her symptoms. She had previously experienced a similar anaphylaxis on her way to school on a bicycle twice. She often eats rice and *miso* soup with *tofu* at breakfast before attending school. She never experienced food allergy to soy, peanut and others so far.

Laboratory tests

Routine blood tests were carried out. Total IgE levels were determined by means of an IgE test kit (Phadia, Uppsala, Sweden). Specific IgE levels for soybean, Gly m 4 and other respiratory and food allergens were measured by ImmunoCAP[®] from Phadia, the range of which was 0.35–100 kU/L.

Skin prick tests

Standard procedure skin prick tests were performed with various soybean products: *tofu*, soy milk, boiled green soybean, soybean flour, *miso*, soy sauce and *okara* (soybean fibres) and *nigari* as is on the patient's volar forearm with a Prick-Lancetter (Ewo Care AB, Gislaved, Sweden). Physiological saline and histamine (10 mg/mL, Sigma-Aldrich, Tokyo, Japan) served as controls. The prick test reaction was regarded as weak positive (2+) when it was one-half the size of the histamine control, and was clear positive (3+) when the reaction was the same size as that resulting from histamine at 20 min.

Provocation test

A series of challenge procedures using combinations of foods and exercise were carried out to identify the causative factors for the development of her FDEIA symptoms. For oral provocation of soybean products, the patient took 200 g of boiled green soybeans, 200 mL of soy milk and 200 g of *tofu*. Written informed consent was obtained following a full explanation of the tests to be given to the patient and her parents. Open challenge tests were performed with the suspected foods followed by exercise at 30 min after the meal containing the suspected food or foods. [18] The exercise part of the challenge consisted of a treadmill ergonometric stress test with progressively increasing speeds (up to Bruce grade V) for 15 min. [19] Aspirin (500 mg) was administered 30 min before the food ingestion. During the tests, the patient's heart rate, blood pressure and electrocardiogram were monitored.

Protein microarray assay

IgE reactivity with 14 different allergens and allergen extracts were tested in a multiplexed immunoassay [20].

Table 1. Result of protein microarray assay to various allergens of soybean, peanut and panallergen of plants*

	Array components							Peanut components				PR-10	Profilin	CCD
	Soybean components						Peanut extract					rBet v 1	rPhl p 12	MuXF ₃
	Soy extract	Glycinin	β-Conglycinin	rGly m 4	Gly m T1	SBA		Ara h 1	Ara h 2	Ara h 3	rAra h 8			
Patient's serum	383	37	4501	4	22	9	10	3	1	3	1	0	13	2
Background†	68	26	12	13	32	17	48	31	36	31	25	482	26	29

*Results for the different array components were converted from fluorescence units into response units and corrected for background binding.

†Background around all individual array components are reported as average response units for four IgE negative sera plus 3 standard deviations.

SBA: soybean agglutinin, PR-10: pathogenesis-related protein-10, CCD: cross-reactive carbohydrate determinant.

Soybean and peanut extracts, were ground, freeze-dried and extracted in phosphate buffer at neutral pH. Allergenic components were either purchased or purified essentially according to previously published methods and included Gly m T1 (Sigma-Aldrich, St. Louis, MO, USA), SBA (soybean agglutinin; Vector Laboratories, Peterborough, UK) β-conglycinin [21], glycinin [21], Ara h 2 [22], Ara h 1 [22], Ara h 3 [23] and cross-reactive carbohydrate determinants (CCD [24]). Recombinant allergenic components were produced with a histidine tag and purified using IMAC [25] and included rGly m 4 [15, 16], rAra h 8 [26], rBet v 1 [27] and rPhl p 12 [28]. Allergens and extracts were covalently immobilized to polystyrene particles (0.11 µm diameter, Bangs Laboratories Inc., Carmel, IN, USA) and the IgE reactivities were evaluated using human reference sera with known IgE levels (determined using ImmunoCAP[®]) for the corresponding allergens. Particles were spotted in duplicate in an array format on a nitrocellulose-based capillary flow membrane using a nano-plotter (Type 2.0 system by Gsellgesellschaft für Silizium-Mikrosysteme mbH, GeSim, Grosserkmannsdorf, Germany). Diameter of arrayed spots was approximately 200 µm and the density of the array was around 400 spots/cm². The assay procedure comprised sequential additions of 30 µL washing buffer (phosphate buffer at neutral pH with blocking agents and detergent), serum (30 µL), washing buffer (30 µL), fluorophore-conjugated monoclonal anti-human IgE antibody (20 µL) and washing buffer (2 × 30 µL), before reading the fluorescence intensity of each spot using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA, USA). IgE reactivity was reported as response units corrected for background binding adjacent to each spot, as well as the average response of four IgE negative sera plus three standard deviations (Table 1).

Immunoblotting analysis

The immunoblotting analysis was performed according to previous reports. [29] Briefly, the soybean extracts were treated with sodium dodecylsulphate (SDS) sample buffer and were boiled for 5 min, followed by SDS-polyacryla-

mid gel-electrophoresis (SDS-PAGE). Twenty µg proteins were loaded per lane. After SDS-PAGE, the separated protein samples were transferred to polyvinylidene fluoride (PVDF) membranes by semi-dry blotting methods. The PVDF membranes were incubated with the patient's serum [20 × dilution in the presence of 5% skin milk in phosphate buffered saline with Tween 20 (PBST)]. After incubation with the patient's serum, the membranes were washed with PBST and were again incubated with horseradish peroxidase (HRP)-labelled anti-human IgE. Finally, signals were detected by enhanced chemiluminescence (ECL) western blotting reagents (GE Healthcare, Tokyo, Japan). Chemiluminescent signals obtained were recorded on X-ray film.

For the immunoblotting inhibition, the purified soybean β-conglycinin was prepared as previously described. [21] The competitor protein (purified soybean β-conglycinin; 0.25 mg/mL) was pre-incubated with the patient's sera for 10 min at room temperature, and then immunoblotting of soybean extracts were performed.

Enzyme-linked immunosorbent assay

Purified soybean β-conglycinin [21] (100 µL : 20 µg/mL) or soybean protein extract (100 µL : 20 µg/mL) in PBS was added to wells of ELISA plates (IWAKI, Asahi Technoglass, Tokyo, Japan) and were coated on the plates overnight at 4.0 °C (39.2 °F). After coating, the wells were blocked with Zepto Block (Zepto Metrix, Buffalo, NY, USA) for 2 h at room temperature. After blocking, the wells were washed with PBST and the patient's sera (200 × dilution) were added. After 1 h incubation, the sera were removed and the wells were washed with PBST five times. Then, biotin-labelled anti-human IgE (5000 × dilution) was added and incubated for 1 h. After incubation, the solution was removed and the wells were washed five times. After washing, avidin-HRP (10 000 × dilution) was added and incubated for 1 h at room temperature. Finally, antigen-antibody complexes were detected by tetramethylbenzidine (TMB) reagent (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The TMB reaction was stopped after 5 min by adding 1 M phosphoric acid. The absorbance of yellowish

colour was measured by Wallac ARVO sx 1420 multilabel counter (Wallac, Turku, Finland) at 450 nm.

For the inhibition ELISA, the competitor protein (purified soybean β -conglycinin [21]; 0.25 mg/mL) was pre-incubated with the patient's sera for 10 min at room temperature.

Pepsin digestion

Soybean curd, that is, *tofu*, was made from 100 mL soy milk by adding 1.2 mL *nigari* and, following microwave treatment for 1 min, 250 mg of the soybean curd and soy milk were homogenized in 1 mL 0.1 M HCl (pH 1.8–2.0) and then were incubated with 10 μ L pepsin (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for the indicated times. The protein contents at the start of the procedure of the digestion were adjusted. After incubation, the samples were subjected to SDS-PAGE. After SDS-PAGE, the gels were stained with coomassie brilliant blue (CBB) for total protein staining. For the immunoblotting analysis, the digested samples were treated as described above.

Results

Skin prick tests

Skin prick testings showed 2+ positive reactions to *tofu*, soy milk, boiled green soybean and soybean flour, but were negative to *miso*, soy sauce, *nigari* and *okara* (soybean fibres).

Provocation tests

For oral provocation of soybean products, we choose 200 g of boiled green soybeans, 200 mL of soy milk and 200 g of *tofu*. The patient exhibited no symptoms in response to ingestion of boiled green soybeans or soy milk, any combination of exercise after ingestion, aspirin intake before ingestion or aspirin intake before and exercise after ingestion. In contrast, when she exercised 30 min after ingestion of 200 g of *tofu*, she developed severe urticaria and swelling of her face. No symptoms were evident when she took a similar amount of *tofu* at rest. We therefore diagnosed her FDEIA as provoked only by *tofu*.

Immunoglobulin E assay

Laboratory tests revealed a high level of serum total IgE (542 IU/mL). IgE tests showed that her serum contained specific IgE against soybean (34.1 kU/L), peanut (1.25 kU/L) and candida (1.94 kU/L), but not against wheat, gluten, shrimp, orchard grass, timothy, Japanese cedar, mugwort, ragweed, birch, latex or Gly m 4 (Phadia). As for the microarray assay to various soybean proteins, she reacted highly to β -conglycinin (4501 units), but did not react to

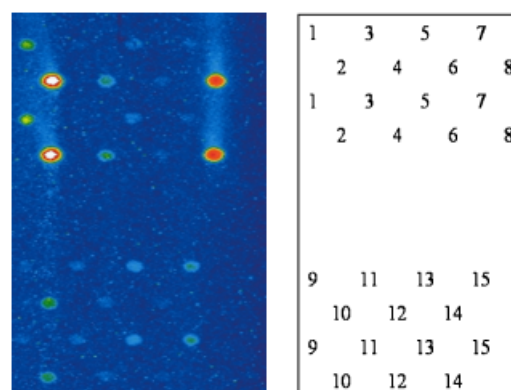


Fig. 1. Protein microarray assay. The spot IDs of allergens are as follows – 1: Glycinin, 2: β -conglycinin, 3: rGly m 4, 4: Gly m T1, 5: SBA, 6: no allergen, 7: cross-reactive carbohydrate determinants (CCD), 8: soybean extract, 9: rBet v 1, 10: rPhl p 12, 11: Ara h 1, 12: Ara h 2, 13: Ara h 3, 14: rAra h 8, 15: peanut extract.

glycinin (37 units), Gly m T1 (22 units), thimosy profilin (13 units), Gly m 4 (four units), CCD (two units) or SBA (nine units) (Table 1, Fig. 1).

Immunoblotting of soybean extracts with the patient's serum

Immunoblotting of soybean extracts demonstrated that this patient's serum IgE mainly bound to the proteins with MWs identical to β -conglycinin and had weak binding to other protein bands, including soybean trypsin inhibitor as well as others (Fig. 2a). Those bands were identical for α , α' and β subunits of β -conglycinin which are glycoproteins with MWs of 50–70 kDa [13]. Pre-incubation of patient's serum with purified β -conglycinin resulted in the diminished binding of patient's serum with 50–70 kDa molecules (Fig. 2b).

We further assessed the reactivity of the patient's IgE to soybean β -conglycinin using an ELISA. When β -conglycinin was coated on the plate, the absorbance values obtained with the patient's serum were 1.607 ± 0.016 (mean SE), while those values with healthy serum were 0.159 ± 0.009 . In addition, the absorbance values obtained with the patient's serum pre-treated with purified β -conglycinin were 0.160 ± 0.094 . Thus, the IgE binding to soybean proteins was almost inhibited with purified β -conglycinin. These results indicate that the IgE in this patient's serum binds tightly to soybean β -conglycinin in the soybean protein extract.

Coomassie brilliant blue staining of pepsin-digested soy milk and tofu

In order to test the resistance of *tofu* and soy milk to pepsin digestion, we generated soy curd, that is, *tofu*, by the addition of *nigari* to soy milk and subsequent microwave treatment. Extracts of soy milk and *tofu* materials were subjected to pepsin digestion under acidic conditions

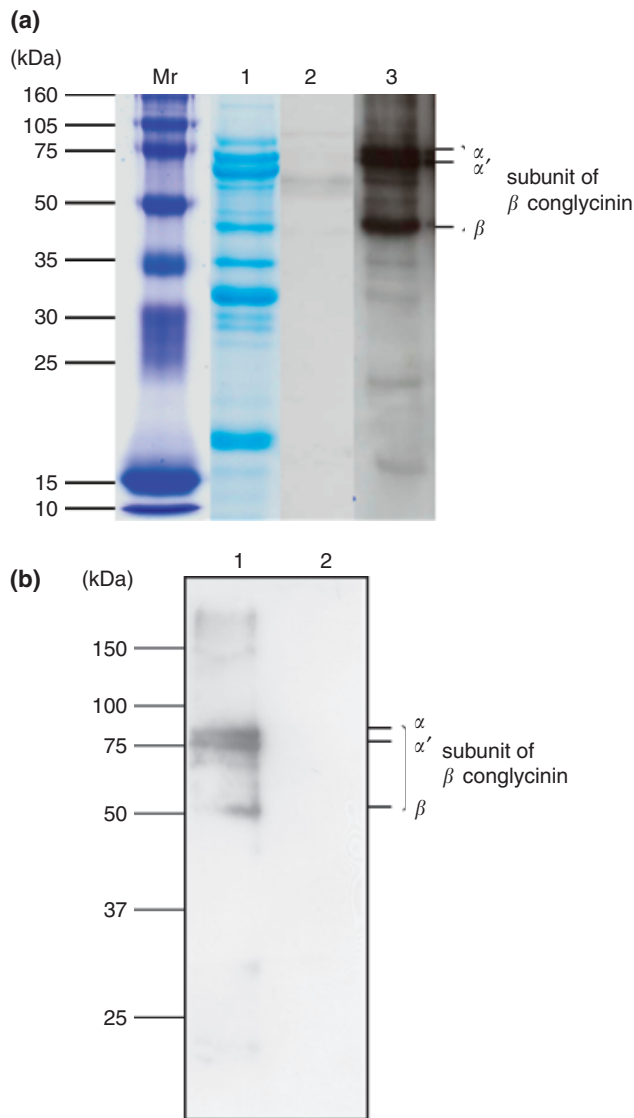


Fig. 2. (a) Coomassie brilliant blue (CBB) staining (lane 1) of the soybean extracts and immunoblotting using the normal (lane 2) and patient's sera (lane 3), respectively. The patient's serum reacted with α , α' and β subunits of β -conglycinin from the soybean proteins. (b) In contrast to the immunoblotting of soybean extracts (lane 1), pre-incubation of patient's serum with purified β -conglycinin resulted in the diminished binding of patient's serum with 50–70 kDa molecules (lane 2).

(pH 2.0) (Fig. 3a, left). Proteins of *tofu* were almost intact even after pepsin digestion for more than 120 min. In contrast, 50–70 kDa proteins of soy milk disappeared within 20 min following pepsin digestion (Fig. 3a, right). During pepsin digestion, no pH changes were observed (data not shown).

Immunoblotting analysis with the patient's serum of pepsin-digested soy milk and tofu

To confirm the time-dependent degradation of soy milk and *tofu* components reactive with the patient's serum,

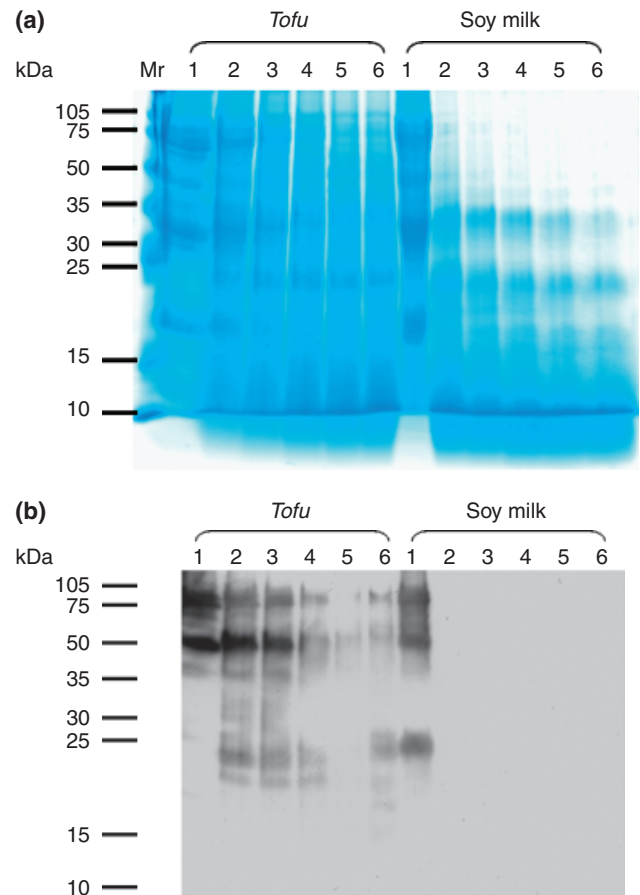


Fig. 3. Difference of resistance to pepsin digestion between *tofu* and soy milk. Coomassie brilliant blue (CBB) staining (a) and immunoblotting (b). Lanes are as follows – lane 1: pepsin digestion 0 min, lane 2: pepsin digestion 20 min, lane 3: pepsin digestion 60 min, lane 4: pepsin digestion 120 min, lane 5: pepsin digestion 180 min, lane 6: pepsin digestion 240 min.

immunoblotting analysis was performed. For *tofu*, her serum IgE reacted with antigens of 50–70 kDa more than 120 min after pepsin digestion (Fig. 3b, left). In contrast, her serum IgE detected no antigens in soy milk after pepsin digestion for 20 min (Fig. 3b, right). Purified β -conglycinin in solution was similarly digested to soy-milk with pepsin (data not shown).

Discussion

Here we present a rare condition of FDEIA induced by the combination of *tofu* and exercise. A survey of literature reveals that this is the first case of FDEIA induced by soybean products and exercise. We found that the patient's serum reacted strongly with the 50–70 kDa molecules of soybean products by IgE immunoblotting. The molecular sizes of those proteins are compatible with α , α' or β subunits of β -conglycinin [13]. This was confirmed by the results of immunoblotting inhibition experiments using purified β -conglycinin. We confirmed

that IgE antibodies in the patient's serum react with purified β -conglycinin by ELISA and by a protein microarray assay. These results indicate that the major allergens for this patient are α , α' or β subunits of β -conglycinin.

All soybean products except fermented foods contain β -conglycinin [13], which is compatible with the finding that various soybean products induced positive responses in her skin prick tests. Nevertheless, the patient did not develop any symptoms following the ingestion of soybean products (except for *tofu*) even though she exercised following their intake. Because *tofu* is made using *nigari* that gelatinizes proteins in soy milk, it is possible that *nigari*-gelatinized proteins in *tofu* may be difficult to digest by peptic enzymes. We therefore examined whether pepsin digestion destroys the allergenicity of β -conglycinin in soy milk and/or in *tofu*. The 50–70 kDa protein bands that represent β -conglycinin in soy milk disappeared within 20 min after the addition of pepsin, and the patient's serum no longer reacted with the allergens thereafter. However, digestion with pepsin did not reduce levels of those protein bands in *tofu* and the binding ability to IgE remained even 120 min after addition of the enzyme. In other words, β -conglycinin peptides in soy milk are susceptible to degradation by pepsin digestion, while those in *tofu* are not. It is thus likely that β -conglycinin peptides in soy milk are easily digested by pepsin in the stomach and do not reach the intestine as peptides with intact epitopes recognized by IgE antibodies. On the other hand, β -conglycinin peptides in *tofu* cause FDEIA because they enter the intestine intact in a state preserving IgE reactivity. Recently, it has been described that pepsin treatment at lower pH decreases the allergenicity of codfish [30]. Thus, digestion of appropriate allergens by gastric fluid in the stomach is a critical part of the development of this food allergy. In the previous report [31], β -conglycinin was stable by pepsin digestion. The difference of these results might be caused by the difference of experimental conditions. We focussed on the different digestive modes between *tofu* and soymilk in the present experiment.

The 50–70 kDa proteins seem to be too large to be absorbed by normal intestinal or oral mucosal membranes. In fact, the patient did not develop an OAS when she drank soy milk, which contains a considerable amount of intact β -conglycinin. Thus, additional factors must be necessary for these large molecules to be absorbed, which results in the development of anaphylaxis in the patient. It has been shown that prolonged or intense exercise increases the permeability of macromolecules in the small intestine through a paracellular route in humans assessed by the lactulose and rhamnose differential urinary test [32]. This phenomenon may be related to the small intestinal tract ischaemia because exercise causes epithelial barrier dysfunction following the reduction of splanchnic blood flow levels [33]. In addition, acute

exercise induces the gastrointestinal leakage of allergen in lysozyme-sensitized mice [34]. In accordance with such evidence, Matsuo et al. [6] showed that the development of FDEIA symptoms depends on the increased absorption of allergens from the intestinal tract following exercise or aspirin intake. It is thus possible that large 50–70 kDa molecules may get into the circulation only when acute exercise is performed.

Conclusion

We confirmed that α , α' and β subunits of soybean β -conglycinin are the causative major allergens in a patient with FDEIA induced by *tofu*. The difference in resistance to pepsin digestion among various soybean products may explain why only *tofu* and not soy milk causes FDEIA in this patient who has a specific IgE against β -conglycinin of soybeans. Development of certain food allergies thus involves resistance to peptic fluid digestion, food processing and probably absorption from the intestine, which may be influenced by exercise.

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