

# Structural property of soybean protein P34 and specific IgE response to recombinant P34 in patients with soybean allergy

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**Abstract.** Soybean allergy is one of the important food allergies because soybean is widely used in processed foods. P34 has been identified as the main allergen in soybeans. The main objective was to analyze the structural property of recombinant P34 and the P34 antigen-specific IgE response in soybean allergy using recombinant P34. Recombinant P34 was expressed by the BL21 (DE3) strain of *Escherichia coli*. Purified recombinant P34 showed oligomerization and binding to endotoxin. The binding of recombinant P34 to endotoxin was confirmed by LPS pull-down assay. High-density SDS treatment dissociated oligomeric recombinant P34 and removed endotoxin. Both native P34 and purified recombinant P34 showed almost identical structural properties as determined by circular dichroism analysis. We analyzed recombinant-P34-specific IgE antibodies by the ImmunoCAP System. In ImmunoCAP using recombinant P34, all sera from healthy controls were classified as negative. A correlation was found between the specific IgE antibodies to whole soybean and recombinant P34 ( $r=0.526$ ,  $P<0.05$ ). The sera from 3 of 9 (33%) patients with outgrown soybean allergy and 6 of 9 (66%) patients with soybean allergy were classified as positive. SDS-treated recombinant P34 retained its structure and biological activity. Recombinant P34 is a useful tool for the analysis of antigen-specific response in soybean allergy. It may be possible to develop a modified form of recombinant P34 for the diagnosis or treatment of soybean allergy using specific immunotherapy techniques.

## Introduction

Soybean is one of the main sources of protein in human nutrition (1,2), and soybean is used in an increasing number of products because of its health benefits (3,4). However, in the United States, approximately 0.4% of children are allergic to soybean and many infants must not be fed soybean-based formula and baby foods (5). Soybean-induced allergic symptoms may range from skin, gastrointestinal, or respiratory reactions to anaphylaxis (5,6). Although the primary treatment for food allergies is to avoid the causative agent, it is difficult to avoid soy protein because of its extensive use in prepared and processed foods.

There are more than 20 soybean proteins that cause allergies (7). Recently, it has been reported that Gly m 5 ( $\beta$ -conglycinin) and Gly m 6 cause severe allergic reactions in Europe (8). In other reports, three proteins, Gly m Bd 60k, Gly m Bd 30k (P34), and Gly m Bd 28k represent the main seed allergens in soybean-sensitive patients (9,10). P34 is the allergen most strongly and frequently recognized by the IgE antibodies in the sera of soybean-sensitive patients with atopic dermatitis (3,11). In several IgE binding studies, more than 65% of soybean allergic patients with atopic dermatitis exhibited an allergic response to P34 (11-14). P34 is regarded as the major, or immunodominant, soybean allergen and is a target allergen for producing low-allergen-content hypoallergenic soybean products (15-17). Moreover, P34 shares high sequence homologies with the main peanut allergen Ara h 1, the dust mite allergen Der p 1, cow's milk caseins, and papain (18-20). Thus, peanuts and soybeans contain common allergenic components and, for this reason, IgE antibodies to peanut proteins may cross-react with soybean proteins.

First, we tried to express recombinant P34 (rP34) in *Escherichia coli* using a previously reported method (21). However, recombinant proteins expressed in *E. coli* carry the risk of endotoxin contamination. Endotoxin causes false-positive results in biological-cell-based assays, such as the cell proliferation assay and must be removed particularly when evaluating allergen-stimulated T cell proliferation (22). The methods for removing endotoxin vary greatly and depend on the structure and characteristics of particular allergens (13,22,23).

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**Abbreviations:** CD, circular dichroism; DLS, dynamic light scattering; LST, lymphocyte stimulation test; rP34, recombinant P34

**Key words:** endotoxin, ImmunoCAP, recombinant P34, SDS, soybean allergy, specific IgE

On the other hand, for serological diagnosis of soybean allergy, specific IgE antibodies to soybean proteins can be detected using commercially available tests. However, these tests are unsatisfactory for the identification of patients with soybean allergy. Specific IgE antibodies to major allergens in other foods, such as omega-5 gliadin, are highly useful for the diagnosis of food allergies (24). The measurement of specific IgE antibodies to rP34 may be useful. Furthermore, for a precise serological diagnosis, a tertiary folded soluble recombinant allergen protein is necessary.

In this study, we expressed rP34 using an *E. coli* expression system for the evaluation of clinical manifestation. Our purified rP34 had almost identical structural properties to native P34. Furthermore, ImmunoCAP System using rP34 was carried out to analyze the specific IgE antibodies to P34 in patients with and without soybean allergy.

## Materials and methods

**Soybean extracts.** Soybean (Fukuyutaka) flakes from commercial sources were ground to a fine powder using pestle and mortar and extracted by incubation with 20 mmol sodium phosphate and 1 mmol sodium chloride (1:20 wt/vol, pH 7.2) overnight at 4°C. Soybean mRNA was obtained using an RNeasy Plant Mini kit (Qiagen). Reverse transcription was carried out using an RNasin ribonuclease inhibitor (Promega) and M-MLV reverse transcriptase (Invitrogen). The sense and antisense primers used for PCR were designed on the basis of the cDNA sequences: 5'-GATTCGATCGAAGGTCGTAAGAAAATGAAGAAGGAAC-3' for the sense primer and 5'-GCGGCCGCAAGAGGAGAGTGATCAACTCTTC-3' for the antisense primer. The resulting DNA fragment was purified using a Gene Clean kit, ligated to a pUC118 plasmid, and transformed into the *E. coli* strain JM109. The resulting plasmid, pUC118/P34, was digested with *Eco*RI and *Not*I, and the fragment containing the P34 gene was inserted into pET24b and expressed in *E. coli* BL21 (DE3). The initial expression, purification and refolding of rP34 were performed in accordance with the method of Babiker *et al* (21).

**Purification of histidine-tagged recombinant P34 using Ni Sepharose column.** rP34 was purified using a Ni Sepharose column (Ni Sepharose 6 Fast Flow, 5 ml, GE Healthcare). The bound protein was eluted with 20 ml of 100 mM phosphate buffer containing 500 mM imidazole, pH 7.4.

**Conformational change of recombinant P34.** To produce the monomeric form of P34, we used a previously reported method with modification (14). The homogeneity of rP34 was determined by dynamic light scattering (Dyna Pro-99, Proterion). The solution containing the oligomeric form of rP34 was treated with an equivalent volume of 4% SDS solution containing 10% 2-ME and heated at 98°C for 10 min. The solution was then loaded onto a Sephacryl S-200 column pre-equilibrated with 0.1 M sodium phosphate buffer (pH 7.6) containing 1% SDS and 10 mM 2-ME. The column was run at 20°C using the same buffer and the fractions containing P34 were collected.

**Endotoxin removal from recombinant P34.** Detoxi-Gel (Pierce) was used to remove contaminating endotoxin in

accordance with the manufacturer's instructions. The collected samples were dialyzed against 50 mM sodium phosphate buffer (pH 7.6) overnight at 4°C. The level of endotoxin was measured by a chromogenic limulus amoebocyte lysate assay (endotoxin single test; Wako Pure Chemical Industries). The collected sample was dialyzed against 50 mM sodium phosphate buffer (pH 7.6) overnight at 4°C to remove any remaining SDS and stored at -20°C.

Endotoxin was also removed by Triton X-114 phase separation, as described by Liu *et al* (25). Triton X-114 was added to rP34 to a final concentration of 1% and incubated for 30 min at 4°C with constant stirring, followed by a 10-min incubation at 37°C and centrifugation at 20,000 x g at 25°C for 10 min.

**N-terminal sequencing of recombinant P34.** After separation by SDS-PAGE for 1 h at 200 mA in 25 mM Tris, 192 mM glycine, and 10% methanol, the proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was briefly stained with CBB R250 (Wako, Japan) and destained extensively in 45 and 90% methanol solutions containing 7% acetic acid. Amino acid sequence analysis of rP34 was carried out using an Edman degradation technique using a pulse liquid automatic sequencer (Model 491HT, Applied Biosystems).

**Circular dichroism (CD).** For SDS-treated proteins, SDS concentrations of 0, 1 and 4% (0,  $3.5 \times 10^{-2}$  and  $1.4 \times 10^{-1}$  M) were used. Optically clear solutions in phosphate buffer were used to record CD spectra (195–250 nm) in a 1 mm rectangular quartz cell (JASCO) using a CD spectrometer (JASCO). The secondary structure was determined by visual assessment of the spectra and using the computer program CDPPro. Native P34 was provided by Professor S. Nagaoka of Gifu University.

**Dynamic light scattering (DLS).** DLS was carried out using a DynaPro-99 molecular-sizing instrument equipped with a micro-sampler (Protein Solutions). rP34 samples containing 0, 1 and 4% SDS were used in the experiment. The DynaPro-99 instrument was operated in accordance with the DLS machine protocol to estimate the molecular weight of rP34. Data were analyzed using the Dynamics 5.0 software (Protein Solutions).

**LPS pull-down assay.** A pull-down assay of the recombinant protein using labeled LPS was carried out as previously described (26,27). Briefly, 10 µg of biotinylated LPS (Alexis Biochemicals) was absorbed onto 20 µl of streptavidin agarose (Vector Laboratories) and incubated with 10 µg of LPS-free rP34 at room temperature for 1 h. After washing with PBS containing 0.1% Triton X-100, the precipitated protein was detected by silver staining.

**Homology modeling of P34.** The 31 kDa cysteine protease SPE31 (PDB code, 2bl m) was selected from a protein database (www.rcsb.org/pdb) as the most homologous template for P34. The structural modeling of P34 was performed using the MOE software (Chemical Computing Group, Inc.).

**Subjects.** Patients with a history of soybean allergy were selected as subjects of this study (Table I), and grouped on the basis of the results of the open challenge test or the accidental

episodes of ingestion as follows: the outgrown group (n=9) had a history of soybean allergy and produced soybean-specific IgE, but was currently tolerant to soybean; the allergic group (n=9) was reactive to soybean; and the healthy control group (n=13) was negative for all allergens in ImmunoCAP System (Phadia AB, Uppsala, Sweden) and had no history of food allergies. Five patients (Patients 11, 13, 15, 16 and 18) had allergic reactions (urticaria, cough and wheeze) within 2 h after soybean ingestion, and six patients (Patients 10, 12-15 and 17) had skin symptoms after more than two hours. Informed consent was obtained from the families of all the subjects.

**Western blot analysis.** Western blotting using an anti-P34 monoclonal antibody was carried out in accordance with the method of Tsuji *et al* (28). Samples were electrophoresed on 15-25% gradient gels (XV PANTERA GEL). Separated proteins were then transferred onto membranes using an iBlot system (Invitrogen) at 30 mA for 14 min. The membranes were blocked with blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 3% BSA) for 2 h at room temperature. After two 5-min washes in TBS-T, they were reacted with the patients' sera for 2 h. After washing four times, the blots were incubated with goat anti-human IgE conjugated to HRP (1:5,000) for 2 h. Blots were washed four times as described above. The P34 monoclonal antibody was a kind gift from Professor T. Ogawa of Kyoto University.

**Specific IgE antibody.** The level of specific IgE antibody to our rP34 in these patients was measured by ImmunoCAP System. In the case of using an undiluted reagent, ImmunoCAP enables the quantification of specific serum IgE antibodies in the range of 0.35 to 100 kUA/l. However, for comparative purposes, the above-mentioned range is often converted into 7 scores, in accordance with the internal calibrator system, as follows: class 0, negative; class 1, 0.35-0.7 kUA/l; class 2, 0.7-3.5 kUA/l; class 3, 3.5-17.5 kUA/l; class 4, 17.5-50 kUA/l; class 5, 50-100 kUA/l; and class 6, >100 kUA/l. IgE levels >0.35 kUA/l in serum are considered to indicate positivity for IgE by the manufacturer.

**Statistical analysis.** Pearson's correlation coefficient was used to estimate correlations between two numerical variables. P-values <0.05 are considered statistically significant.

## Results

**High-density SDS-treatment was useful for removal of endotoxin from rP34.** The purified rP34 was detected using an anti-P34 monoclonal antibody (Fig. 1A). The N-terminal amino acid sequence of the recombinant protein (KKMKKEQTS) was identical to that of soybean P34 (amino acid number, 123-131). As mentioned above, recombinant proteins expressed in *E. coli* carry the risk of endotoxin contamination. To remove endotoxin from this protein, rP34 was applied to the endotoxin removal column. However, all of the proteins absorbed onto the column. To further investigate the binding ability of P34 to LPS, we performed pull-down assay. LPS was clearly pulled down with rP34 (Fig. 2). We found that high-density SDS treatment, but not Triton X-114 treatment, dissociated oligomeric recombinant P34 and

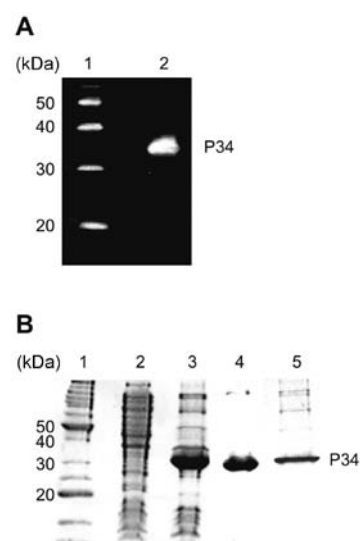


Figure 1. Purification of recombinant P34. (A) Immunoblot of recombinant P34 with an anti-P34 monoclonal antibody (1:3,000). Lane 1, molecular weight marker; lane 2, recombinant P34. (B) SDS-PAGE using a 15% gel. Polypeptides were visualized by Coomassie blue staining. Lane 1, molecular weight marker; lane 2, soluble fraction; lane 3, insoluble fraction; lane 4, refolded protein; lane 5, recombinant P34 without endotoxin.

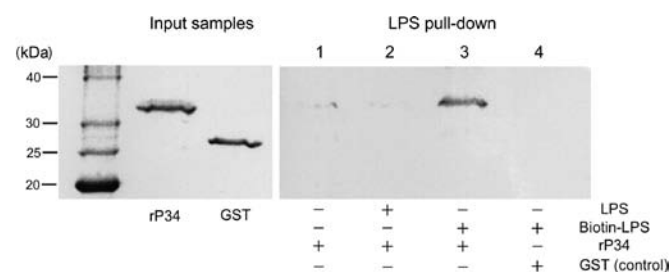


Figure 2. LPS pull-down assay. Lane 1, 10 µg of recombinant P34 was incubated with 30 µl of packed avidin beads. Lanes 2-3, 10 µg of recombinant P34 was incubated with 10 µg of non-biotinylated (lane 2) or biotinylated LPS (lane 3) and 30 µl of packed avidin beads. Lane 4, GST protein was added as a control. After washing, recombinant P34 bound to LPS was detected by silver staining.

removed endotoxin. The final yield of purified rP34 decreased to ~40% of that in the sample prior to endotoxin removal (Fig. 1B). The final yield after purification was about 11.6 mg/l of LB medium culture.

**Both native P34 and SDS-treated endotoxin-free rP34 showed almost identical structural properties.** SDS-induced structural conformation changes were determined from the appearance of a minimum peak at 205 nm and a weak shoulder at 222 nm in the CD spectra. No significant differences were observed between native P34 and SDS-treated rP34 in terms of the shape of the CD spectra and molar ellipticity values at 222 nm, which reflect the  $\alpha$  helix content of the folded protein (Fig. 3). It should be noted that there was actually a minor difference between native and rP34, but this may be attributable to the C-terminal hexa-histidine tag.

Structurally, P34 belongs to the papain family, whose individual members share a common mechanism of catalysis. The model of the P34 structure was constructed on the basis of the structure of the papain-like protein family protein SPE31

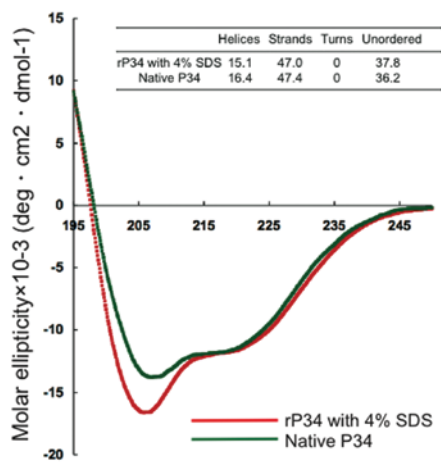


Figure 3. Circular dichroism spectra of native P34 and recombinant P34 exposed to SDS. The secondary structures of both native and recombinant P34 after SDS treatment were estimated using a computer program (CDPro).

(Fig. 4). Analysis of the sequence alignments for P34 identified SPE31 as sharing 78% sequence identity (29). The overall structure of P34 contains two independent domains, namely, the L domain and R domain, which form a cleft. The major secondary structural part of the L domain forms the  $\beta$ -sheet and that of the R domain forms the  $\alpha$ -helix.

**Reactivity of IgE in sera from soybean allergic patients to rP34 examined by ImmunoCAP System.** A total of 31 sera (13 controls, 9 outgrown, and 9 patients with soybean allergy) were available for the present study. The levels of rP34-specific IgE antibodies in the sera from outgrown and allergic patients measured by ImmunoCAP System are shown in Table I. Among the 31 sera investigated, 9 were positive for the specific IgE antibodies to rP34 and 22 were negative. A correlation was found between specific IgE antibodies to soybean and rP34. The correlation value was 0.526 and  $P < 0.05$ . All sera from healthy controls were negative for the antibodies. Six of the 9 (66%) outgrown and 3 of the 9 (33%) allergic patients were also negative for the antibodies. The positive sera belonged to class 1 (4 sera), class 2 (3 sera) and class 3 (2 sera). No serum belonged to classes 4

to 6. Four of 5 patients with sera belonging to class 2 or 3 were not tolerant to soybean. The median concentrations of specific IgE antibodies to rP34 were 1.63 kUA/l (range, 0.35–5.00) in the allergic group and 0.50 kUA/l (range, 0.35–1.26) in the outgrown group.

Discussion

Recently, recombinant allergens have become available for diagnostic and therapeutic purposes (23,30,31). It is particularly important to evaluate T cell activation for diagnosis and specific immunotherapy using allergens. However, the use of recombinant antigens contaminated with endotoxin leads to nonspecific cytokine production and affects T cell proliferation, thereby masking any antigen-specific responses. In our previous study, we showed that endotoxin-free rP34 might be a useful tool for evaluation of soybean allergy (32). It is essential to remove endotoxin from recombinant proteins before using them in the analysis of antigen-specific responses.

During the processes of protein purification, we noted a strong affinity of rP34 to endotoxin derived from the host strain. In this study, we clearly showed the LPS binding ability of P34 by the LPS pull-down assay (Fig. 2). It is reported that P34 contains many hydrophobic residues and is, thus, insoluble in aqueous solutions (16). The structural model of rP34 (Fig. 4C) indicated that many of the hydrophobic residues (shown in yellow) are located superficially, and thus may contribute to the strong affinity of rP34 to endotoxin. On the other hand, DLS analysis showed that rP34 treated with 4% SDS had a molecular weight of 34.9 kDa, whereas non-SDS-treated rP34 had a molecular weight of 790 kDa, indicating that SDS treatment disrupted the rP34 oligomers. This finding was consistent with the finding of a previous study, in which Ogawa *et al* (14) isolated native P34 in its oligomeric form (>300 kDa). In our recent study and in this study, it was also possible that these SDS-induced conformational changes enabled the separation of endotoxin from rP34 (32). From this findings, we speculated that the oligomerization property of P34 may influence its affinity to endotoxin.

It was reported that P34 is a receptor of the glycolipid elicitor syringolide produced by *Pseudomonas syringae*

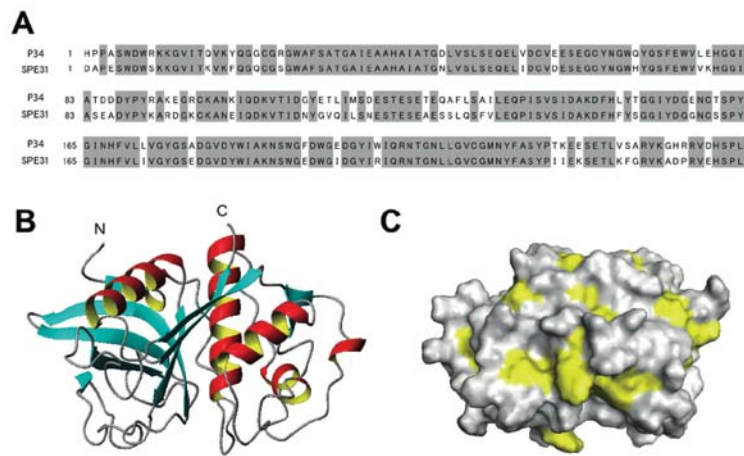


Figure 4. Structural model of recombinant P34. (A) Alignment of P34 and SPE31 amino acid sequences. P34 shares 78% amino acid sequence identity with SPE31. (B) Structural model of mature P34. (C) Distribution of hydrophobic residues on P34. The hydrophobic residues are shown in yellow.



Table I. Characteristics of outgrown subjects and soybean allergic subjects.

Patient	Gender	Age (years)	Symptoms caused by soybean intake	Total IgE (IU/ml)	ImmunoCAP values			
					Soybean		rP34	
					Class	kUA/l	Class	kUA/l
1	M	6	Outgrown	3,540	2	4.14	2	1.26
2	F	1	Outgrown	133	1	1.40	1	0.56
3	M	4	Outgrown	156	0	<0.35	0	<0.35
4	M	5	Outgrown	233	2	0.84	0	<0.35
5	M	4	Outgrown	108	2	1.07	0	<0.35
6	M	3	Outgrown	377	2	1.94	0	<0.35
7	F	2	Outgrown	426	2	2.14	0	<0.35
8	F	3	Outgrown	374	2	3.60	1	0.61
9	M	9	Outgrown	977	2	1.02	0	<0.35
10	M	1	Urticaria	>16,000	5	88.2	3	5.00
11	M	4	Wheeze, urticaria	1,960	4	20.9	2	1.13
12	M	2	Erythema	31	1	0.59	0	<0.35
13	F	1	Cough, urticaria	626	2	1.63	0	<0.35
14	F	1	Urticaria	23.2	0	<0.35	0	<0.35
15	M	6	Cough, urticaria	1,490	5	76.4	1	0.41
16	M	1	Wheeze, urticaria	2,460	3	9.31	3	3.61
17	M	2	Urticaria	1,850	6	>100	1	0.45
18	F	6	Wheeze, urticaria	>16,000	5	57.1	2	2.93

(29,33). This suggests that P34 plays an important role in plant defenses against bacteria. Endotoxin is also recognized as a glycolipid elicitor and can induce defense responses in rice cells, including reactive oxygen generation and defense-gene expression. Both endotoxin and syringolide have considerable similarities in their immunological roles as pathogen-associated molecular patterns (PAMPs) (34). This suggests that P34 also recognizes the endotoxin in soybean in a manner similar to that of the Toll-like receptor 4 (TLR-4), MD-2, and CD14 in the mammalian innate immune systems (34-36). It is of particular interest that a recent report showed that the LPS-binding protein Der p 2 which is one of the major allergens of house dust mites, modulates mouse TLR-4 signaling and is associated with atopic dermatitis (37). Trompette *et al* (37) suggested that lipid-binding proteins possess the intrinsic adjuvant activity that underlies the phenomenon of allergenicity. The endotoxin binding ability of P34 may also be associated with allergic reactions in humans through a similar mechanism.

A previous report has described the linear B cell epitope on P34 (13). However, the conformational B cell epitopes on P34 remain to be fully elucidated. It is desirable to maintain the three-dimensional structure of P34, both for accurate evaluation of T cell responses and for the elucidation of conformational epitopes. High-density SDS may cause conformational changes in proteins. In a previous report, CD analysis of soybean glycinin (11S) and  $\beta$ -conglycinin showed that SDS treatment increased both the  $\alpha$ -helical and unordered structures of both proteins at the expense of the  $\beta$ -sheet structure (38). In this case, the relatively low-density SDS treatment ( $2 \times 10^{-3}$  M) may have changed the conformation of these proteins. However, in the

present study, no significant changes in the secondary structure of P34 occurred after high-density SDS treatment. Estimation of the secondary structure from the recorded CD spectra indicated no significant differences between non-SDS-treated native P34 and SDS-treated rP34 (Fig. 3). This suggests that both of the proteins form a similar folding pattern. Both CD and DLS analyses indicated that rP34 retained its secondary structure even after high-density SDS treatment. Our purified rP34 is useful for the analysis of linear or tertiary epitopes because it retains the tertiary structure of its protein.

We established the method using the ImmunoCAP system with purified rP34 for the first time. Measurement of the concentration of specific IgE antibodies to rP34 is more useful than IgE Western blotting (data not shown). A correlation was found between specific IgE antibodies to soybean and rP34. Patient 16, whose serum with specific IgE antibody to soybean had a relatively low score of 3, showed severe allergic reactions within two hours after soybean ingestion. It is assumed that the allergic reaction in this patient was caused mainly by P34 in soybean. On the other hand, the finding that patients 15 and 17 showed a high score for their serum with specific IgE antibodies to soybean but a low score of 1 for specific IgE antibodies to rP34 may indicate an allergic reaction to soy protein other than P34. These results showed that the rP34 ImmunoCAP system we established could play a role as a soy allergic diagnostic tool or could indicate outgrown allergy.

In conclusion, our proposed new purification method for rP34 did not affect the tertiary protein structure. The strong affinity of P34 for endotoxin/LPS suggests that P34 may modulate the immunological responses underlying allergenicity.

Furthermore, the ImmunoCAP system using our purified rP34 that retains its tertiary structure may be useful for the identification of patients with soybean allergy or for the indication of outgrown allergy.

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