Immunological Characterization of Recombinant Soy Protein Allergen Produced by *Escherichia coli* Expression System

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To elucidate the molecular mechanism of the allergenicity of soybean P34 protein recognized as the most allergenic protein in soybean, the protein was expressed in *Escherichia coli* transformed with a plasmid carrying P34 cDNA. SDS−PAGE pattern showed that the molecular weight of the recombinant P34 was ∼2 kDa less than that of the native soybean P34. The difference in the molecular mass between these two proteins could be due to the native P34 in soybean being glycosylated at position Asn^{170}, whereas the recombinant protein generated in *E. coli* lacks this post-translational modification. Immunoblot analysis showed that both soybean and recombinant P34 proteins cross-reacted not only with polyclonal and monoclonal antibodies produced against P34 and crude soybean protein but also with patients' sera. The results suggest that the recombinant P34 is immunologically reactive, indicating that both proteins have similar epitope structures. Thus, the recombinant P34 produced by the *E. coli* expression system can be used as a standard allergen for molecular design to reduce the allergenic structure.

Keywords: *Soybean; recombinant allergen; immunoreactivity; P34; E. coli*

INTRODUCTION

Food allergy is a serious nutritional problem in both children and adults; a number of patients with atopic dermatitis are considered to be suffering from food hypersensitivity mediated by allergen-specific IgE antibodies, and the incidence of atopic dermatitis is increasing, mainly among children. Several causative allergenic proteins have been isolated on the basis of their specific binding with serum IgE antibody from patients allergic to certain foods (Sweeney et al., 1987). Soybean is one of the principal foodstuffs as a protein source, and soy protein isolate prepared from defatted soybean is also widely utilized as an ingredient in many processed foods. However, soybean is known as one of the typical allergenic foods with at least 15 allergenic proteins (Ogawa et al., 1991). Ogawa et al. (1993) have also reported that ∼65% of patients atopic to soybeans suffered from the 34 kDa protein. Thus, the 34 kDa protein has been recognized as a major allergen in soy protein. A 34000 Da protein (P34) is one of the four major soybean oil body proteins observed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis of organic solvent-extracted oil bodies from mature seeds (Kalinski et al., 1990) and is a moderately abundant protein in soybean seeds and cotyledons, but its level in leaves is low (Cheng et al., 1998). Kalinski et al. (1990) reported that the allergen of soy protein had an N-terminal amino acid sequence and amino acid composition identical with those of the soybean seed 34 kDa oil-body-associated protein or the soybean vacuolar protein with close homology to papain-like thiol proteases. The epitope analysis of P34 was done by using synthetic peptides and detected by two monoclonal antibodies, F5 of IgG and H6 of IgM. The epitopes were shown to be localized in peptides ^{31}QGGCGRGW AFSATGAIEA^{48}, containing the epitope for H6, and ^{115}DKVTIDGYETLIMSDEST^{132}, containing the epitope for F5 (Hosoyama et al., 1996).

To elucidate further the molecular mechanism of soybean P34 protein allergenicity, the protein was produced using *Escherichia coli* expression system because the recombinant protein is completely isolated at its gene level and can be easily prepared in large and pure amounts to enable the molecular design of the protein to reduce the allergenic structure by genetic engineering. Thus, a recombinant P34 is constructed and has been generated in *E. coli* as a standard allergen for further genetic modifications.

MATERIALS AND METHODS

Materials. *E. coli* BL21 (DE3), the expression vector pET-22b(+) (Ampicilin resistant), and pT7-BlueT vector were purchased from Takara, Co. Ltd. (Kyoto, Japan). A complementary DNA (cDNA) of P34 protein in a baculovirus transfer vector was supplied by Dr. Okamoto, Tokyo Metropolitan University. Male mice (C3H/He; 7 weeks old) and female mice (BALB/c; 8 weeks old) were purchased from Japan SLC, Co., Ltd. (Shizuoka, Japan). ^{125}I-labeled anti-human IgE (1.7×10^4 Bq/mL) was obtained from Pharmacia Co. (Uppsala, Sweden). Female New Zealand white rabbits were purchased from Kyudo Co. (Tosu, Japan). Gelatin, Tween 20, 3,3′-diaminobenzidine, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated goat anti-mouse IgG was from Organon Teknika (West Chester, PA); goat anti-rabbit IgG−peroxidase conjugate and Freund's complete and incomplete adjuvants were purchased from Sigma Chemical Co. Restriction enzymes and other modifying enzymes were purchased from Takara, Co. Ltd. The chemically synthesized oligonucleotides as sense primer and an antisense for PCR

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were purchased from Kurabo, Co. Ltd. (Osaka, Japan). Unless otherwise stated, all reagents used in this study were of reagent grade. *E. coli* cells were routinely cultured in LB broth or agar.

Bacteria harboring plasmids were cultured on medium supplemented with 60 μg/mL ampicillin.

Construction of pT7-Blue T or pET Vector Carrying P34. P34 cDNA in baculovirus transfer vector was digested by *Eco*RI, and it was used as a template DNA. Primers I and II used for PCR were designed on the basis of cDNA sequence: primer I, 5′ TTA CAT ATG AAG AAA ATG AAG AAG GAA CA 3′; primer II, 5′ TTAGTC GACTTCAAAGAGGAGAGTGATCA 3′. The reaction mixture consisted of template DNA (1 ng), primer I (10 pmol), primer II (10 pmol), 10xEx Taq buffer (8 μL), dNTPs mixture (5 μL), sterilized water (33.5 μL), and Ex Taq polymerase (0.5 μL, 5 units). Then the mixtures were subjected to Polymerase Chain Reaction using a Perkin-Elmer Gene Amp PCR System (Tokyo, Japan). PCR products were analyzed by electrophoresis on 1% agarose, and the target DNA band was purified and then ligated to the plasmid pT7Blue T vector using the TA system. The ligated DNA was transformed into *E. coli* XL1-Blue cells according to the Hanahan method (Hanahan, 1983), and the resultant plasmid was designated pT7/P34 and used for DNA sequencing. Plasmid pT7/P34 was digested with *Nde*I (CATATG) and *Sal*I (GTCGAC), and then the fragment including the P34 gene was inserted into the pET-22b plasmid directly behind the T7 promoter. The resultant plasmid was designated pET/P34

DNA Sequencing. DNA sequencing was performed by means of the dideoxy chain termination method (Sanger et al., 1977) using a thermo sequenase premixed cycle sequencing kit (Amersham) and a DNA Thermal Cycler model 2400 (Perkin-Elmer). A DNA sequencer SQ3000 (Hitachi) was used to determine the sequences.

Amino Acid Sequencing (N Terminal). After sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), soybean and recombinant P34 proteins were electroblotted onto PVDF membrane (Japan Genetics Co. Ltd.) for 1.5 h at 90 mA in blotting buffer (20 mM Tricine, 25 mM Tris-HCl, 20% methanol) by electroblotting in a semidry system (AE-6675, ATTO Co., Tokyo). The transferred protein was visualized by ponceau S staining, and the excised band was sequenced using a PSQ-2 (Shimadzu) equipped with recorder C-R4AX (Shimadzu).

Expression of P34 Gene in *E. coli*. pET/P34 was transformed into *E. coli* BL21 (DE3). *E. coli* carrying pET/P34 was cultured in 3 mL of LB medium containing 60 μg/mL ampicillin with shaking at 37 °C overnight. The preculture (0.5 mL) was inoculated into 50 mL of LB medium containing 60 μg/mL ampicillin in a 250 mL Erlenmeyer flask and grown with shaking at 37 °C. When the OD_{600} reached 0.6−1.0 (∼2.5 h), isopropyl β-D-(−)-thiogalactopyranoside (IPTG) was added (50 mM stock) to a final concentration of 0.5 mM. Then after 2 h of growth at 37 °C, the cells were harvested.

Purification of the Recombinant Protein Expressed in *E. coli*. Cultured cells were harvested by centrifugation at 10000 rpm for 5 min. The harvested cells were suspended in 25 mL of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Then the suspended cells were sonicated for 5 min with output of 5 (Tomy Ultrasonic Disruptor UD-201, Tokyo, Japan). The solution was centrifuged at 5000 rpm for 5 min. To separate the soluble and insoluble fractions, the supernatant was centrifuged at 18000 rpm for 30 min. The pellet was mixed (twice) with 5 mL of 1% Triton X-100 for 30 min and centrifuged at 18000 rpm for 30 min. The Triton X-100 insoluble fraction was mixed with 5 mL of 2 M urea and centrifuged at 18000 rpm for 30 min. The collected pellet solubilized in 8 M urea and the insoluble fraction were removed by centrifugation at 18000 rpm for 30 min. The solubilized protein was subjected to gradient dialysis with buffer containing 4, 2, 1, 0.5, and 0 M urea with 10 μL/L mercaptoethanol. After dialysis, the solution was centrifuged at 18000 rpm for 30 min to remove unfolded protein.

Preparation of Acid-Precipitated Soy Protein (APP). APP was prepared according to the method of Iwabuchi et al. (1987). A sample of defatted meal (100 g) was extracted once with 2 L of 0.03 M Tris-HCl buffer (pH 8) containing 10 mM 2-mercaptoethanol (2-ME) at 20 °C. After centrifugation (8000*g*), the supernatant was acidified to pH 4.8 with 2 N HCl and then recentrifuged. The precipitates were dissolved with water at 4 °C, and the pH was adjusted to 8. After centrifugation (8000*g*), the clear supernatant was dialyzed against distilled water for 24 h at 4 °C and then freeze-dried.

Preparation of Soybean P34 Protein. Soybean P34 protein was prepared as described by Ogawa et al. (1993) using the crude 7S globulin fraction prepared from defatted meal of soybean protein.

SDS−PAGE. SDS−PAGE was carried out using the method of Laemmli (1970) with a 15% acrylamide separating gel and a 3% acrylamide stacking gel containing 0.1% SDS; 0.2% sample (20 μL) was prepared in a Tris−glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was done at a current of 10 mA for 5 h in electrophoretic Tris−glycine buffer containing 0.1% SDS. After electrophoresis, the gel sheets were stained for proteins with 0.2% Coomassie brilliant blue R250 and then destained with 10% acetic acid containing 20% methanol.

Production of Polyclonal Antibody against Crude Soy Protein in Rabbits. The immunization schedule and methods of immunization via the multiple-injection technique were performed according to the guidelines of Chu et al. (1979). Two rabbits were each injected with crude soy protein (2.0 mg/mL) in 0.01 M phosphate buffer (PB; pH 7.5) containing 0.85% NaCl (PBS), emulsified with 2.0 mL of Freund's complete adjuvant. For booster injections, immunogen (1.0 mg/mL) in PBS and 2.0 mL of Freund's incomplete adjuvant were used for each rabbit. The collected antisera were precipitated with $(NH_4)_2SO_4$ to a final saturation of 35% by mixing 2 mL of antisera with 1 mL of saturated $(NH_4)_2SO_4$ solution. The precipitates were redissolved in water and reprecipitated twice. Finally, the precipitates were reconstituted to half of the original volume with distilled water, dialyzed against distilled water for 1 h followed by 0.01 M PB overnight (all at 4 °C), and then stored at −20 °C until they were used.

Production of Polyclonal Antibody against Soybean P34 Protein in Mouse. Three mice (C3H/He) were each injected intraperitoneally with P34 protein (10 μg/mL) adsorbed onto 2 μg of alum (P34−Alum; 0.5 mL/mouse) according to the method of Aramaki et al. (1995). One week later each mouse was injected with the same antigen (booster injection). As a control, three mice were injected intraperitoneally with a mixture of alum and phosphate-buffered saline (PBS). The collected antisera (heart bleeding) were precipitated with $(NH_4)_2SO_4$ to a final saturation of 35% by mixing 2 mL of antisera with 1 mL of saturated $(NH_4)_2SO_4$ solution. The precipitates were redissolved in water and reprecipitated twice. Finally, the precipitates were reconstituted to half of the original volume with distilled water, dialyzed against distilled water for 1 h followed by 0.01 M Tris-HCl buffer saline (TBS) overnight (all at 4 °C) to be ready for immunoblotting detection, and then stored at −20 °C until they were used.

Preparation of Monoclonal Antibody (mAb). The mAb against soybean P34 protein was prepared using female 8-week-old BALB/c mice injected interapertoneally with the allergenic protein (50 μg/mouse) according to the method of Tsuji et al. (1993). mAb in the ascites was fractionated with $(NH_4)_2SO_4$. An IgG mAb was further purified with a protein A column and termed the F5 monoclonal antibody.

Human Serum. Human serum samples were prepared from 361 patients with atopic dermatitis (mean age of 6.1 years, ranging from 3 months to 23 years) who were asked to donate blood samples for testing at Tokushima Kensei Hospital and National Kagawa Children's Hospital.

Immunoblotting with Polyclonal Antibodies. Antibodies produced in rabbits and mice raised against crude soy protein and soybean P34, respectively, were used to detect the immunoreactivity of the recombinant protein produced by *E. coli* expression system and soybean P34 protein. Immunoblotting was done according to the method of Ventling and Hurley (1988) with slight modifications. Samples were elec-

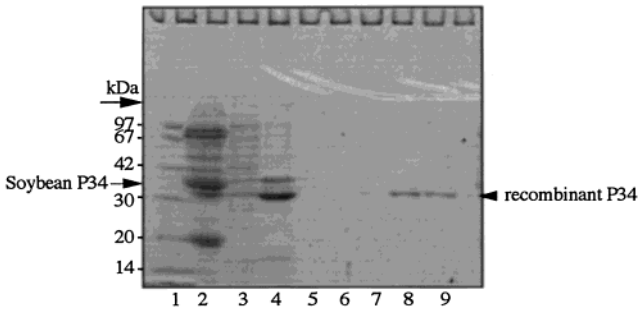

Figure 1. SDS−PAGE pattern of soy protein, soluble and insoluble fractions of the recombinant protein (P34), and urea-solubilized protein: (lane 1) molecular marker; (lane 2) soy protein; (lane 3) soluble protein; (lane 4) insoluble protein; (lanes 5−8) protein solubilized by 2, 4, 6, and 8 M urea, respectively; (lane 9) refolded protein by gradient dialysis.

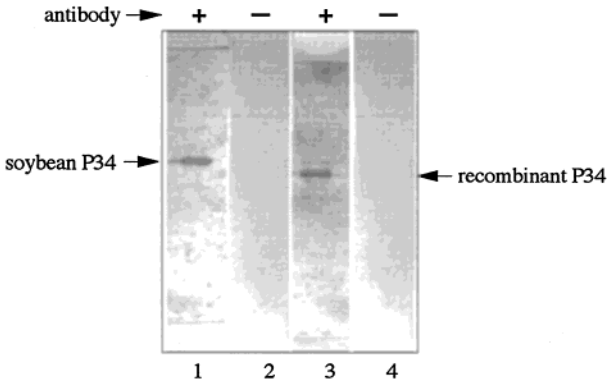

Figure 2. Immunoblotting of soybean and recombinant proteins (P34) with rabbit polyclonal antibody (1:10000) raised against crude soybean protein: (lanes 1 and 2) soy protein; (lanes 3 and 4) recombinant P34 protein (0.5 μg/lane); (+) with antibody; (−) without antibody.

trophoresed on 15% SDS−polyacrylamide slab gels (8 × 7 cm, 0.5 mm thick) as described by Laemmli (1970). Proteins were transferred to a nitrocellulose membrane by electroblotting in a semidry system (AE-6675, ATTO Co., Tokyo) at 40 mA for 2 h, in 24 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol (v/v). Protein blots were blocked with 1% gelatin in 0.1% Tween 20, 100 mM Tris-HCl (pH 7.5), 0.9% NaCl (TTBS) for 2 h at room temperature with agitation. The allergen on the membrane was reacted at room temperature for 1 h with mouse or rabbit polyclonal antibody (1/10000). After blots had been washed three times (10 min in each wash) in TTBS, the immunocomplex of the allergen was incubated with a purified goat anti-mouse IgG or goat anti-rabbit IgG (H & L) diluted (1:800) in 20 mL of blocking buffer for 1 h at room temperature with constant agitation. Blots were washed as described before. Immunodetection was carried out in 50 mL of reaction mixture containing 0.2% 3,3-diaminobenzidine, 50 mM Tris-HCl (pH 7.4), and 7.5 μL of 30% (v/v) H_2O_2 mixed just before use.

Immunoblotting with F5 Monoclonal Antibody (mAb). Antibody raised against soybean P34 was used to detect the immunoreactivity of the recombinant protein produced by the *E. coli* expression system and soybean P34 protein. Immunoblotting was done according to the method of Tsuji et al. (1993). Soybean P34 protein was transferred to a PVDF membrane. The membrane was blocked with 1% BSA in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20 (buffer A), and then the membrane was incubated at 37 °C for 1 h with an mAb (F5) against the allergen. As a control, nonimmune IgG was used. The bound mAb was reacted with peroxidase-conjugated sheep anti-mouse IgG, diluted 1:2000 with buffer A containing 1% BSA. The immunocomplexes on the membrane were detected by a 20 min incubation at room temperature with 4-chloro-1-naphthol (0.3 mg/mL) and 0.03% H_2O_2 as a substrate in 50 mM Tris-HCl buffer (pH 7.3).

Immunoblotting with Patients' Sera. Immunoblotting analysis of the IgE-binding proteins of soybean was carried out using the sera from 361 patients with atopic dermatitis. However, >65% of the patients had the specific IgE antibodies binding to soybean proteins. Therefore, immunoblotting with a representative sample serum was done according to the method of Ogawa et al. (1991).

RESULTS AND DISCUSSION

A P34 (34 kDa) protein of soybean was expressed in *E. coli* using a pET expression system. The P34 gene was inserted into pET-22b downstream of the T7 promoter and SD sequence as described under Materials and Methods. The target protein was expressed in *E. coli* after IPTG induction. The *E. coli* lysate was analyzed by SDS−PAGE (Figure 1). The SDS−PAGE pattern (Figure 1, lane 4) showed that the recombinant protein appeared as a major band (molecular mass of ~32 kDa) in the insoluble fraction of the cell lysate. The insoluble fraction (inclusion body) was solubilized in 2 M (lane 5), 4 M (lane 6), 6 M (lane 7), and 8 M (lane 8) urea. The results indicated that the recombinant P34 was found to be soluble in 8 M urea. The recombinant P34 was further purified and refolded by a gradient dialysis as described under Materials and Methods. The native P34 protein of soybean has a molecular mass of 34 kDa (Figure 1, lane 2). The difference in the molecular mass between these two proteins could be due to the native P34 in soybean being glycosylated at position Asn^{170}, whereas the recombinant protein generated in *E. coli* lacks this post-translational modification. The N-terminal amino acid sequence of the recombinant protein produced by the *E. coli* expression system was KKMKKEQY, similar to that of the native soybean P34 protein. Results indicated that the recombinant allergen produced by the *E. coli* expression system was highly pure, and no other bands were detected after purification and refolding (Figure 1, lanes 8 and 9). Furthermore, the size of the major protein is consistent with the molecular mass obtained by Kalinski et al. (1990). Thus, the results obtained suggest that the recombinant protein was properly expressed as a target protein in the *E. coli* expression system using the pET-22b (+) vector. Antibodies prepared from rabbits and mice immunized with crude soybean and purified soybean P34 protein, respectively, were used to detect the immunoreactivity of the recombinant protein produced by the *E. coli* expression system compared to the soy protein (P34). Figure 2 shows the immunoblotting of soy protein P34 and the recombinant protein using polyclonal antibody raised against rabbit immunized with crude soy protein. The purified soy protein (P34) was strongly cross-reacted with the polyclonal antibody, suggesting that the P34 protein predominantly induced the antibody production in rabbits (Babiker et al., 1998). The recombinant P34 protein was also cross-reacted with the antibody in a similar way. Figure 3 shows the immunoblotting of soy protein (P34) and recombinant P34 using polyclonal antibody raised against mouse immunized with soybean P34 protein. The mouse polyclonal antibody strongly cross-reacted with both soybean and recombinant P34 proteins, just like the rabbit antibody. This shows that P34 gives good immunological response in both of the experimental animals. Despite the difference in molecular size between the two proteins, the cross-reactivities of both proteins with the polyclonal antibodies are similar. To further confirm the

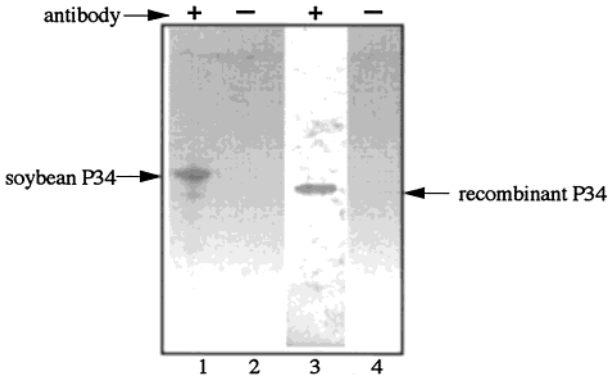

Figure 3. Immunoblotting of soybean and recombinant proteins (P34) with mouse polyclonal antibody (1:10000) raised against purified soybean P34: (lanes 1 and 2) soy protein; (lanes 3 and 4) recombinant P34 protein (0.5 μg/lane); (+) with antibody; (−) without antibody.

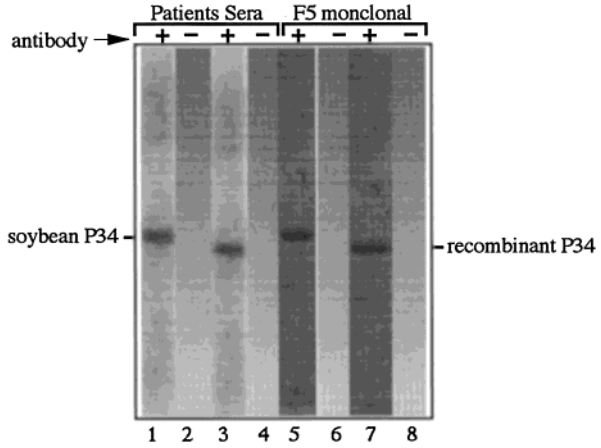

Figure 4. Immunoblotting of soybean and recombinant proteins (P34) with patients' sera and F5 monoclonal antibody raised against soybean P34 (1:10000) protein: (lanes 1, 2, 5, and 6) soybean P34; (lanes 3, 4, 7, and 8) recombinant P34 protein (0.5 μg/lane); (+) with antibody; (−) without antibody.

immunoreactivity of the recombinant protein produced by the *E. coli* expression system, patients' sera and F5 of IgG monoclonal antibody were used as shown in Figure 4. Each serum was tested separately, and >65% of the patients were found to have the specific IgE antibodies binding to the proteins. Therefore, a representative serum result is shown in Figure 4. The F5 mAb recognizes the epitope ^{115}DKVTIDG YETLIMSD-EST^{132} (Hosoyama et al., 1996). Patients' sera as well as the F5 monoclonal antibody (Figure 4) were strongly cross-reacted with the recombinant and soybean proteins. This provides evidence confirming the identities of the two proteins. The results indicate that the production of antibody is not dependent upon the presence of a carbohydrate chain in soybean P34 protein. Despite the presence of at least 15 allergenic proteins in soybean, P34 was found to be the most abundant and allergenic protein because it has been shown to enhance the IgE binding ability of soybean (Ogawa et al., 1991). In a previous study we (Babiker et al., 1998) have reported that the purified P34 protein is the most allergenic and abundant protein in soybean and its protease digests. Also, it has been reported that the SDS−PAGE epitope map of fragments of P34 protein using mAbs designated P3E1 and P4B5 raised against P34 were found to recognize both the native and denatured proteins (P34) even in the presence of 2 M urea without diminished label intensity, and some residual binding was still detected at 4 M urea (Herman et al., 1990), indicating that the P34 protein remained allergenic even under adverse chemical conditions. Therefore, the immunoreactivity of the recombinant protein produced by the *E. coli* expression system as well as the purified soybean P34 protein of soybean with the antibodies indicated that the immunoreactivity of the recombinant protein is similar to that of the purified P34 protein. Furthermore, the immunoreactivity of the recombinant protein implies also the fact that the protein has an antibody-recognizable conformation under Western blot conditions even after heat treatment in the presence of SDS and 2-ME. The similarity in amino acid sequence and cross-reactivity with the antibodies produced indicated that both the recombinant and soybean P34 proteins are identical and could have similar epitope structures. Although both proteins were observed to have a similar amino acid sequence, the molecular mass of the purified P34 protein of soybean was ∼2 kDa more than that of the recombinant protein. The difference in the molecular mass between these two proteins could be due to the native P34 in soybean being glycosylated at position Asn^{170}, whereas the recombinant protein generated in *E. coli* lacks this post-translational modification. This suggestion was supported by the fact that *E. coli* have no glycosylation system. In addition, carbohydrate staining of soy protein gave a stained band corresponding to the P34 protein (data not shown).

In conclusion, the recombinant protein, despite being 2 kDa less than the purified P34 protein of soybean, was immunologically reactive and can be used as a standard allergen for molecular design to reduce the allergenic structure by the introduction of genetic engineering.

LITERATURE CITED

Aramaki, Y.; Suda, H.; Tsuchiya, S. Interferon-Y Inductive Effect of Liposomes as an Immunoadjuvant. *Vaccine* **1995**, *13*, 1809−1814.

Babiker, E. E.; Azakami, H.; Matsudomi, M.; Iwata, H.; Ogawa, T.; Bando, N.; Kato, A. Effect of Polysaccharide Conjugation or Transglutaminase Treatment on the Allergenicity and Functional Properties of Soy Protein. *J. Agric. Food Chem.* **1998**, *46*, 866−871.

Cheng, J.; Boyd, C.; Slaymaker, D.; Okinaka, Y.; Takeuchi, Y.; Midland, S.; Sims, J. J.; Herman, E.; Keen, N. Characterization of a 34-kDa Soybean Binding Protein for the Syringolide Elicitors. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3306−3311.

Chu, F. S.; Grossman, S.; Wei, R. D.; Mirocha, C. J. Production of Antibody against T-2 toxin. *Appl. Environ. Microbiol.* **1979**, *37*, 104−108.

Hanahan, D. Studies on Transformation of *Escherichia coli* with Plasmid. *J. Mol. Biol.* **1983**, *166*, 557−580.

Herman, E. M.; Melroy, D. L.; Buckhout, T. J. Apparent Processing of a Soybean Oil Body Protein Accompanies the Onset of Oil Mobilization. *Plant Physiol.* **1990**, *94*, 341−349.

Hosoyama, H.; Obata, A.; Bando, N.; Tsuji, H.; Ogawa, T. Epitope Analysis of Soybean Major Allergen Gly m Bd 30K Recognized by the Mouse Monoclonal Antibody Using Overlapping Peptides. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 1181−1182.

Iwabuchi, S.; Yamauchi, F. Determination of Glycinin and β-Conglycinin in Soy Proteins by Immunological Methods. *J. Agric. Food Chem.* **1987**, *35*, 200−205.

Kalinski, A.; Weisemann, J.; Matthews, B.; Herman, E. M. Molecular Cloning of a Protein Associated with Soybean

Seed Oil Bodies That Is Similar to Thiol Proteases of the Papain Family. *J. Biol. Chem.* **1990**, *265*, 13843−13848.

Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Ogawa, T.; Tsuji, H.; Bando, N.; Okazima, H.; Nishikawa, H.; Sasaoka, K. Investigation of the IgE-binding proteins in soybeans by immunoblotting with the sera of the soy-bean-sensitive patients with atopic dermatitis. *J. Nutr. Sci. Vitaminol.* **1991**, *37*, 555−565.

Ogawa, T.; Tsuji, H.; Bando, N.; Kitamura, K.; Zhu, Y.; Hirano, H.; Nishikawa, H. Identification of the Soybean Allergenic Protein, Gly m Bd 30K, with the Soybean Seed 34-kDa Oil-body-associated Protein. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 1030−1033.

Sanger, F.; Nicklen, S.; Coulson, A. R. DNA Sequencing with Chain Terminating Inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463−5467.

Sweeney, M. J.; Klotz, S. D. In *Handbook of Food Allergies*; Breneman, J. C., Ed.; Dekker: New York, 1987; pp 17−18, 28.

Tsuji, H.; Bando, N.; Okazima, H.; Kimoto, M.; Okada, N.; Ogawa, T. Preparation and application of monoclonal antibodies for a sandwich enzyme-linked immunosorbent assay of the major soybean allergen, Gly mBd 30K. *J. Nutr. Sci. Vitaminol.* **1993**, *39*, 389−397.

Ventling, B. L.; Hurley, W. L. Soy Proteins in Milk Replacers Identified by Immunoblotting. *J. Food Sci.* **1988**, *54*, 766−767.

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