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Original Article

Molecular characterization of buckwheat major immunoglobulin E-reactive proteins in allergic patients

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Abstract

Buckwheat extract was analyzed by immunoblotting experiments using sera from nine allergic and three non-allergic individuals. Major IgE-reactive bands were 73, 70, 62, 58 and 54 kDa under non-reducing conditions and were detected in allergic subjects, but not in non-allergic ones. Under reducing conditions, the 73, 70, 62 and 58 kDa bands split to 56 and 24, 52 and 24, 45 and 24, and 43 and 24 kDa, respectively. The 24 kDa molecule was the most prominent band recognized with IgE as well as IgG or IgA. The FA02 cDNA clone, encoding the α and β subunits of the legumin-like storage protein, was isolated from a cDNA library made of immature buckwheat seeds. The deduced amino acid sequence of the cDNA clone is substantially identical to the N-terminal amino acid sequence of the 24 kDa molecule, which may be identical to that of BW24KD reported by Urisu *et al*. Consistent with these results, the translation product of the cDNA encoding the putative β subunit was strongly recognized with serum IgE, IgG and IgA from buckwheat-allergic patients. These results suggested that the 24 kDa molecule may be the β subunit of the legumin-like storage molecule of buckwheat.

Key words: allergen, buckwheat, cDNA, immunoblot analysis, legumin.

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Introduction

Inhalation or ingestion of a very small amount of buckwheat allergens can initiate severe symptoms in patients with hypersensitivity to buckwheat.[1] The immunologic properties of buckwheat allergens have been studied by several investigators,[2–11] but their molecular natures are still unclear. Yanagihara has described buckwheat allergen proteins with molecular masses of approximately 100, 50 and 17 kDa.[9] Yano *et al*. have reported proteins of 8–9 kDa, which have strong IgE-binding activity.[10] Recently, Urisu *et al*. have extensively characterized buckwheat allergens by immunoblotting analysis using sera from 15 patients and have reported the N-terminal 29 amino acid sequence of a protein with a molecular mass of 24 kDa as a major allergen (BW24KD) of buckwheat.[6]

In the present report, we prepared the sample by extracting finely ground powder of buckwheat by cold alkaline solution. Using this preparation, we analyzed the buckwheat extract by an immunoblotting assay.

We show here that: (i) Four major IgE-reactive bands, 73, 70, 62 and 58 kDa molecules, were detected by immunoblot analysis under non-reducing conditions; (ii) under reducing conditions, each band was resolved in two bands containing a 24 kDa molecule, of which the N-terminal amino acid sequence is substantially identical to the BW24KD named by Urisu *et al*;[6] (iii) the 24 kDa molecule was recognized with not only IgE but also IgG and IgA; (iv) the N-terminal amino acid sequence of the 24 kDa molecule was completely identical to the deduced amino acid sequence of the cDNA clone encoding the legumin-like storage protein; and (v) the translation product of the cDNA clone encoding the β subunit of the legumin-like storage protein (21.1 kDa

without sugar moiety) was strongly recognized with IgE, IgG, and IgA of the sera from buckwheat-allergic patients. Thus, these results suggested that, as a major buckwheat allergen, the 24 kDa molecule might be the β subunit of the legumin-like storage molecule of buckwheat.

METHODS

Patients

Nine buckwheat-allergic patients, five male (aged 9–68 years) and four female (aged 12–25 years), and five non-allergic individuals (male, aged 22–25 years) were selected for this study according to case histories and radioallergosorbent tests (RAST). None of the patients had received hyposensitization therapy before collection of sera. Laboratory data of non-allergic individuals showed that the percentage of eosinophils in white blood cells was between 2.6 and 3.5%. Total serum IgE levels were between 40 and 123 IU/mL. The RAST scores measured by Pharmacia CAP System (Pharmacia, Uppsala, Sweden), including house dust 1, house dust 2, Derf and Derp, were all negative among these individuals.

Buckwheat extracts

Buckwheat (15 g) was finely ground and extracted in 100 mL 0.125 mol/L $NaHCO_3$ at 4°C for 1 h. After extraction, the supernatant was separated by centrifugation at 10 000 *g* for 30 min. The precipitates were re-extracted in 100 mL 0.125 mol/L $NaHCO_3$ at 4°C for 1 h. The combined supernatants were used for the sample.[12,13] To prevent protein degradation by protease or freezing and thawing, extracted samples were aliquoted and snap-frozen for use.

Antibodies and other reagents

Alkaline phosphatase (AP)-conjugated antihuman IgE antibody was obtained from Biosource (Camarillo, CA, USA). The AP-conjugated antihuman IgG and antihuman IgA antibodies were obtained from TAGO (Burlingame, CA, USA). The AP-conjugated antihuman IgG1 (BS-MA003) and antihuman IgG4 (BS-MA007) were purchased from MBL Co. Ltd. (Nagoya, Japan). Trizma base was purchased from Sigma (St Louis, MO, USA). Disodium *p*-nitrophenylphosphate hexahydrate, diethanolamine and Coomasie Brilliant Blue G250 were obtained from Wako (Osaka, Japan).

Immunoblotting

Immunoblotting analysis was performed as described previously.[14] The samples were prepared by boiling for 5 min in a sample buffer (0.06 mol/L Tris, 1% sodium dodecyl sulfate (SDS), 5% glycerol, 0.01% bromophenol blue) with or without 5% 2-mercaptoethanol, electrophoresed in SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride microporous membrane (PVDF, Immobilon, Millipore, Bedford, MA, USA) using a semidry system (Sartorius, Tokyo, Japan). The membranes were visualized by incubation with patients' sera as a primary antibody in the presence or absence of buckwheat extracts or purified 24 kDa molecule, followed by the addition of AP-conjugated antihuman IgG or IgE, IgA (1:1000) as a second antibody, and developed by 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/Nitro blue tetrazolium (NBT) visualization solution (Promega, WI, USA).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed as described previously.[14] Briefly, microtiter plates (MS-8596F, Sumitomo Bakelite Co., Tokyo, Japan) were coated with the buckwheat extracts (100 mg/50 mL per well). The remainder of the binding site was blocked by the addition of 300 mL phosphate-buffered saline containing 2% fetal bovine serum (FBS-PBS) for 2 h at room temperature. The wells were incubated with 300 mL undiluted sera for 2 h at room temperature. Alkaline phosphatase-antihuman IgE antibody (65 mL) was added at a dilution of 1:250 for 2 h at room temperature. The wells were incubated with 100 mL substrate containing 10% disodium *p*-nitrophenylphosphate hexahydrate and 10% diethanolamine (Wako Co.). Absorbance was read at 405 nm by an automated microplate reader (InterMed NJ-2011, Tokyo, Japan).

Amino acid sequencing

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described and transferred onto a PVDF membrane using Sartorius's semidry system. The band of 24 kDa was cut out and the membrane washed in distilled water. The N-terminal amino acid sequence of the sample was determined with Model 473A Protein sequencer (PE Applied Biosystems, Chiba, Japan).

Plasmids

FA02 was cloned from a buckwheat seed cDNA library (Fujino, unpubl. data, 1997). The GenBank accession

number is D87980. The FA02 β-subunit gene (nucleotide numbers of the coding region: 1153–1717, plus 189 bp containing a non-coding region and polyA tail) was polymerase chain reaction (PCR) amplified using the FA02 gene as a template, the 5′ primer (5′-AATGGATCCGAGCAAGCGTTCTG-3′), and the oligo(dT)14-*Not*I primer. The PCR product was inserted into the *Bam*HI–*Not*I sites of the pGEX-6P-1 vector (Pharmacia) to construct the expression vector GF0403.

Gene expression of pGF0403 and preparation of *Escherichia coli* lysates

Escherichia coli strain XL1Blue was transformed with pGF0403. Isopropyl-β-D-thiogalactopyranoside (IPTG, Wako, Tokyo, Japan) was used at 10 mmol/L to induce gene expression. Cells were harvested by centrifugation and resuspended in 50 mmol/L Na_2HPO_4, pH 7.0, containing 0.1 mmol/L phenylmethylsulfonylfluoride (PB buffer). Cells were disrupted by sonication (Branson Co.) until the OD600 decreased to one-tenth of its original value. Supernatants were prepared by centrifugation at 5000 *g* for 10 min. Insoluble inclusion bodies were resuspended in the PB buffer and used for samples.

Results

Immunoblotting analysis of buckwheat extracts

Non-reduced samples were examined by immunoblotting analysis using sera from nine allergic patients (aged 9–68 years) and two non-allergic individuals as primary antibodies and AP-conjugated antihuman IgG, IgE, or IgA antibody as secondary antibody. Representative results are shown in Fig. 1. The sera from both allergic and non-allergic individuals reacted to molecules extracted from buckwheat by IgG and IgA isotypes. The marked difference between allergic patients and non-allergic subjects was that the IgE class was detected in the sera from allergic individuals only. In a separate experiment, three non-allergic individuals (cases 12, 13 and 14) were tested on reactivity to only IgE and showed no reactivities. To examine the sensitivity of the assay system, an ELISA was performed on the IgE-binding activity of sera. It was shown that the sera from non-allergic individuals showed no IgE-binding reactivity to buckwheat, while those of allergic subjects were strongly positive (data not shown). The results are summarized in Fig. 2. The blotting patterns were almost identical for IgG and

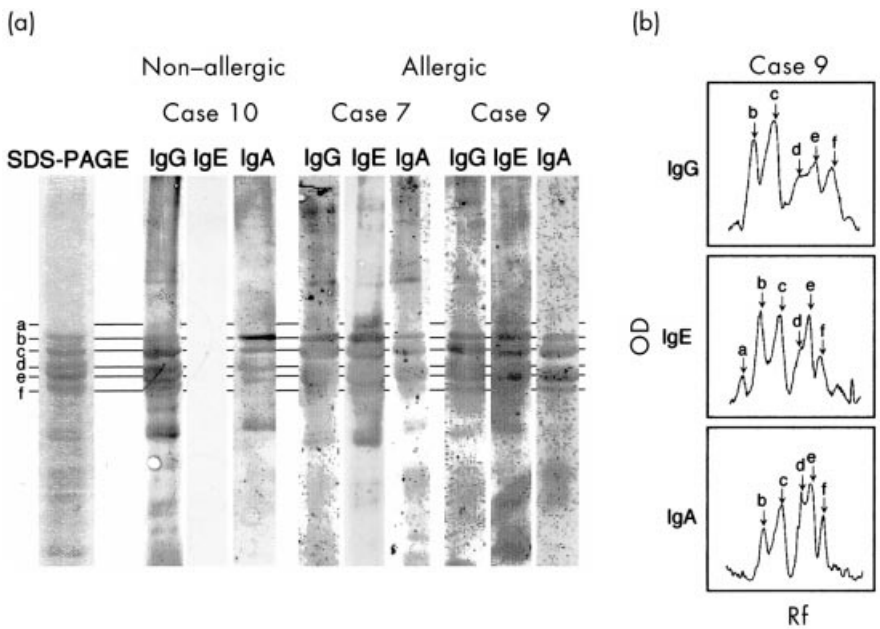

Fig. 1 Immunoblotting analysis using sera from allergic and non-allergic individuals. Representative results are shown in this figure. Data that are not included in this figure are summarized in Fig. 2. (a) Samples were electrophoresed in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The membrane was visualized by incubation with 500 μL sera (diluted at 1:3 for IgG and IgA and non-diluted for IgE) as a primary antibody. The sizes of marked bands are as follows (in kDa): a, 79; b, 73; c, 70; d, 62; e, 58; f, 54. (b) Intensity of the bands was determined by a densitometer (Image Master, Pharmacia Biotech AB, Uppsala, Sweden). Rf, rate of migration to the front marker.

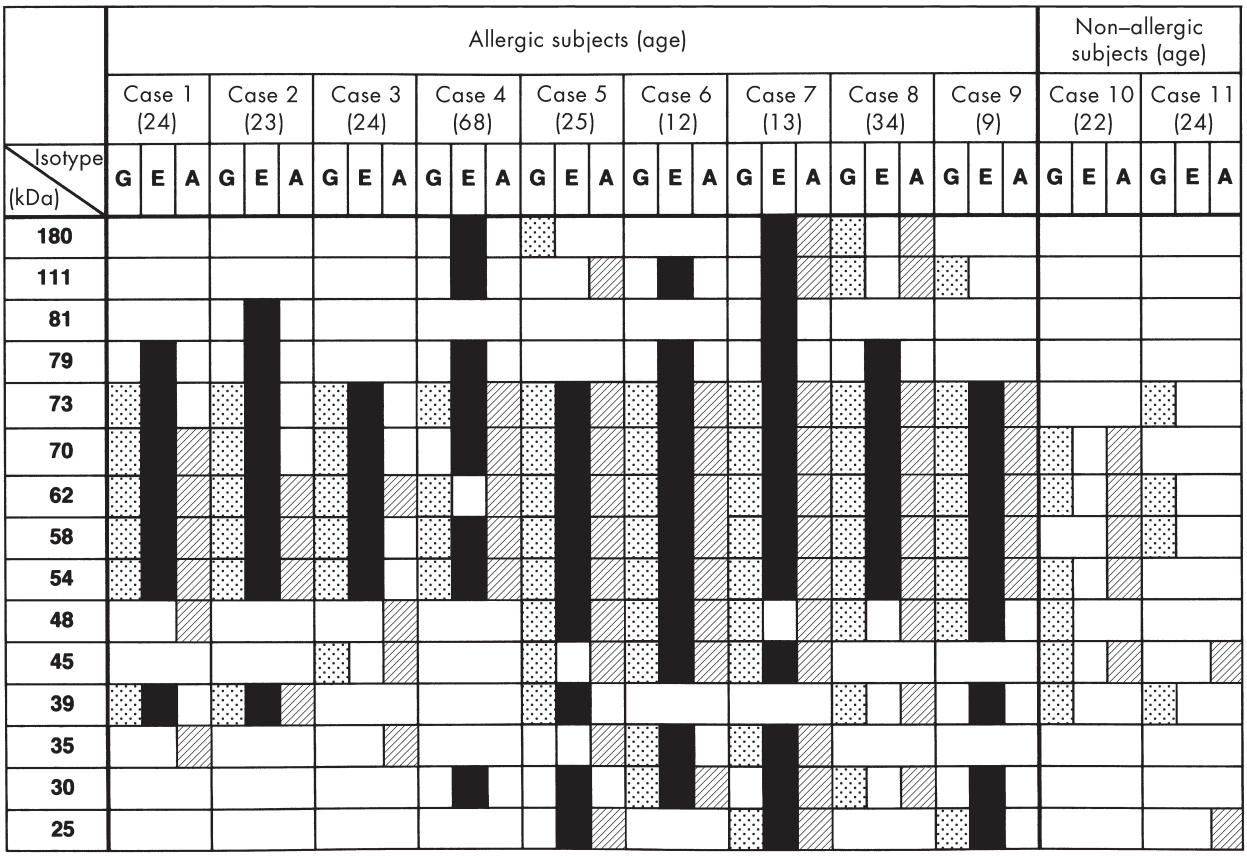

Fig. 2 Summary of immunoblotting analysis. Experiments were performed as described in Fig. 1. The results of nine allergic and two non-allergic individuals are represented. Each serum was tested for IgG (G) and IgA (A) at a dilution of 1:3 and for IgE (E) reactivities at 1:1. Blanks indicate non-reactivity. Three non-allergic individuals (cases 12, 13 and 14) were tested for reactivity to only IgE and showed no reactivities (not included in this figure).

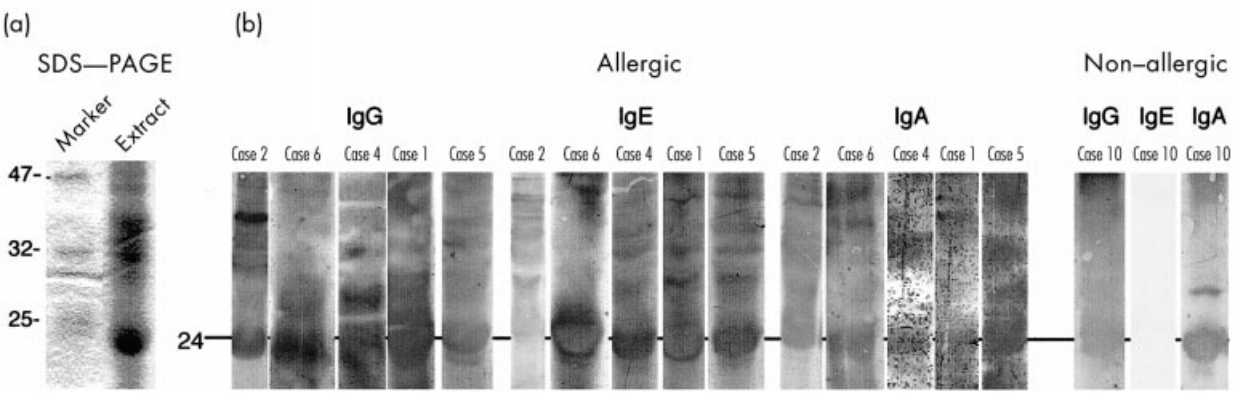

Fig. 3 Immunoblotting analysis of buckwheat extracts under reducing conditions. Samples (15 μg/lane) were electrophoresed in 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions. Representative results are shown in this figure, with sizes shown in kDa. (a) The gel was stained with Coomasie brilliant blue; molecular markers are shown on the left. (b) Immunoblotting using sera from allergic cases 1,2,4–6) or non-allergic individuals (case 10). Sera were used as dilutions of 1:3 to detect IgG or IgA and 1:1 to detect IgE. In this analysis, only IgE reactivities were analysed in the cases 3, 7, 8, 9, 11 and 12. Allergic cases (3, 7, 8 and 9) gave positive results, whereas non-allergic cases (11 and 12) resulted in negative patterns (these results are not included in this figure).

IgA, although not exactly the same in every single individual. Major reacting bands were detected at 73, 70, 62, 58 and 54 kDa. The 73 kDa band reacted to 100% of the sera from allergic individuals in the IgG class, 66% in the IgA class and 100% in the IgE class. The 70 kDa band reacted to 88% in IgG, 77% in IgA and 100% in IgE. The 62 kDa band reacted to 100% in IgG, 100% in IgA and 88% in IgE. The 58 kDa band reacted to 100% in IgG, 88% in IgA and 100% in IgE. The 54 kDa band reacted to 100% in IgG, 88% in IgA and 100% in IgE. The faint band of 79 kDa was recognized mainly with patients' IgE, but not IgG or IgA (Fig. 1). This result was obtained in 66% of allergic individuals.

Immunoglobulin E-reactive major bands under reduced conditions of SDS-PAGE

When the immunoblotting analysis was performed by 15% acrylamide gels under reducing conditions, these major bands molecules (73, 70, 62 and 58 kDa) disappeared, with the concomitant appearance of a 24 kDa molecule (Fig. 3a). In order to characterize their molecular nature, these bands were excised and eluted from gels under non-reducing conditions. When these bands were electrophoresed under reducing conditions, the 73, 70, 62 and 58 kDa bands split to 56 and 24, 52 and 24, 45 and 24, and 43 and 24 kDa, respectively (Fig. 4). The 24 kDa molecule was strongly recognized by sera from allergic patients in the IgE, IgG, and IgA classes, whereas it reacted to IgG and IgA but not IgE in the cases of non-allergic individuals (Fig. 3b). Thus, these molecules appeared to share the 24 kDa molecule.

Molecular relationship of the 24 kDa molecule and the β chain of the legumin-like storage molecule

The 24 kDa band was separated by 15% SDS-PAGE under reducing conditions and the amino acid sequence of the N-terminal 22 residues was determined. The sequence is GLEQAF(X)NLKFKQNVNRPSRAD and is substantially identical to that of BW24KD, which has been reported by Urisu *et al*. (Fig. 5).[6] In our study, we identified a cDNA clone, pGF0403 DNA, which encodes the β subunit of the legumin-like storage protein and has been registered in GENBANK. The N-terminal sequence of the 24 kDa molecule was completely identical to the deduced amino acid sequence of pGF0403 DNA, except for seventh cysteine residue that could not be detected in our analysis (Fig. 5).

In order to examine whether the β subunit of the legumin-like storage protein is recognized with the IgE of allergic patients, immunoblotting analysis was carried out using the translation product of pGF0403. The DNA was transfected into XL1Blue. Cells were harvested at the indicated time after the induction of gene expression using IPTG. Extracts of cells were separated into fractions of supernatants and precipitates by centrifugation and analyzed by 15% SDS-PAGE. The pattern of Coomassie staining on gels is shown in Fig. 6a. The GF0403 gene product is a fusion protein of glutathione *S*-transferase (GST; 26 kDa) at the N-terminal end with the β-subunit of legumin (17 kDa) at the C-terminal end. Thus, the 43 kDa protein was produced and visible in precipitates but not in supernatants of extracts 1 h after gene induction. These electrophoresed gels were blotted onto the PVDF membrane and tested using sera from allergic patients. The 43 kDa protein strongly showed positive development in every case of IgG, IgE, and IgA isotypes (Fig. 6b). The antisera used in these experiments showed no reactivity to GST alone (data not shown).

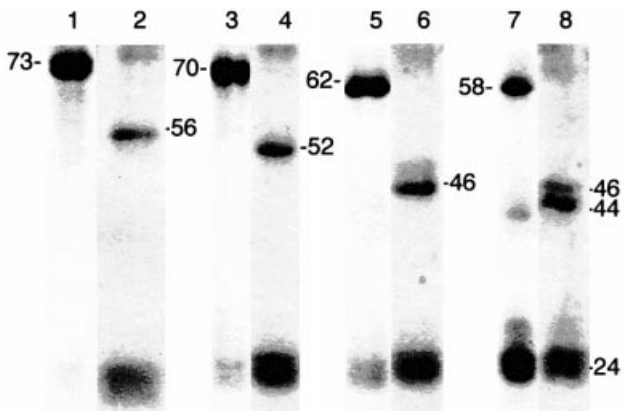

Fig. 4 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of 73, 70, 62 and 58 kDa molecules under reducing conditions on 15% SDS-PAGE gels. The 73, 70, 62 and 58 kDa protein bands were excised from 10% SDS-PAGE under non-reducing conditions. Crushed gels containing these bands were boiled in a digestion buffer with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) 5% 2-mercaptoethanol for 5 min and subjected to 10% SDS-PAGE. The 24 kDa band may have become contaminated in lanes 3, 6 or 7 during the elution of the 70, 62 or 58 kDa bands under non-reducing conditions. The 46 and 44 kDa bands were visible in lane 8 because of possible contamination of the 62 kDa band in the 58 kDa band under non-reducing conditions. All sizes shown are in kDa.

Fig. 5 Comparison of the amino acid sequence of the β subunit of legumin-like protein (FA02-β) with the N-terminal sequence of the 24 kDa molecule and BW24KD.[6] The 24 kDa protein purified by Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was directly sequenced using a Model 473A protein sequencer (PE Applied Biosystem). The amino acid sequence of FA02 was deduced from the nucleotide sequence accepted by GENBANK (asscession no. D87980). The asterisks indicate the positions of identical residues. X, unidentified amino acid; in the case of the 24 kDa molecule, phenylthiohydantoin amino acids (cysteine) were not detected. Underline, signal peptide; arrowheads predicted cleavage sites of post-translational modification; boxed region , α-subunit of legumin; dotted line, β-subunit of legumin.

Discussion

Both allergic and non-allergic individuals showed remarkable levels of serum IgA and IgG antibody responses and T cell proliferative responses to buckwheat allergens (data not shown). However, only allergic patients, but not non-allergic subjects, showed IgE antibody responses to buckwheat allergens (Figs 1–3). In the present study, the characterization of buckwheat allergens was performed by immunoblotting analysis using sera from allergic patients.

Under non-reducing conditions, 15 bands were visible, ranging from 25 to 180 kDa. Among them, 73, 70, 62, 58 and 54 kDa were major bands recognized with IgG, IgA and IgE of the sera from allergic patients. These bands were also reactive to IgG and IgA but not IgE of the sera from non-allergic individuals. No age-related shift on the blotting pattern was evident, suggesting that the allergenic epitopes rarely changed during the subjects' lifetimes due to repetitive exposure to allergens. Under reducing conditions, these major molecules each split into two bands; one band was characteristic to each molecule and the other one appeared to be a common major band of 24 kDa. Both of these were strongly reactive to IgG, IgA and IgE of the sera from allergic patients (Figs 1,3). When the antigen specificity of these bands was examined using the serum for IgE class from case 5, binding to the 24 kDa band was inhibited in the presence of buckwheat extracts, but not cedar pollen extracts (data not shown). An identical finding has been reported by Urisu *et al*.[6] N-terminal amino acid sequencing analysis suggested that the 24 kDa molecule might be the BW24KD.[6]

Recently, Fujino *et al*. (unpubl. data, 1997) have isolated a cDNA clone (FA02) from the buckwheat seed library, which encodes the legumin-like gene. The amino acid sequence of FA02 predicted two cleavage sites by

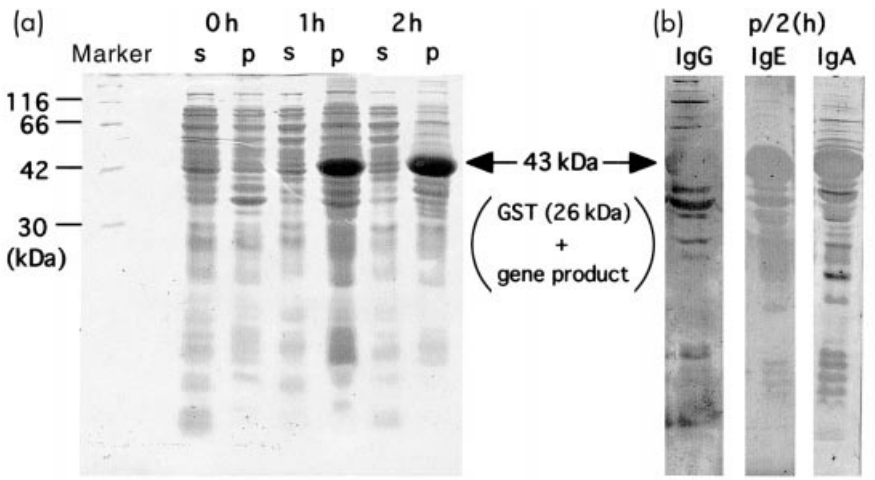

Fig. 6 Immunoblotting analysis of the GF0403 gene product. The plasmid, GF0403, bears the FA02 β-subunit gene at the *Bam*HI–*Not*I sites of the pGEX-6P-1 vector. The GF0403 gene product is a fusion protein of glutathione S-transferase (GST) (26 kDa) at the N-terminal half and the β-subunit of legumin of (17 kDa) at the C-terminal half. *Escherichia coli* extracts were prepared at 0, 1 and 2 h after inducing GF0403 gene expression in XL1Blue cells with isopropyl-1-thio-β-D-galactoside (IPTG). Aliquots (20 μL) of supernatants (s) or pellets (p) were electrophoresed in 15% sodium dodecyl sulfate–polyacrylamide gels under reducing conditions. (a) The gel was stained with Coomassie brilliant blue. (b) Immunoblotting analysis of the GF0403 gene product. The blotting was performed with an identical dilution of serum (from an allergic individual, case 2), as shown in Fig. 3. A representative result is shown in this figure.

post-translational modification, suggesting that FA02 would be cleaved into a signal peptide (20 amino acid residues), an acidic α subunit of 41.3 kDa and a basic β subunit of 21.1 kDa. The C-terminal fragment (amino acids 378–565) of FA02 bears an N-terminal amino acid sequence that is substantially identical to the 24 kDa molecule, as well as BW24KD described by Urisu *et al*.[6]

There is a high degree of similarity between the N-terminal amino acid sequence of BW24KD and those of the β subunit of 11 or 12S globulins of garden pea, soybean, upland cotton, mouse-ear cress, rape, pumpkin, cucurbit, common sunflower, rice and oat. These plants range from dicot to monocot species in angiosperm. Urisu *et al*. have pointed out that the majority of subjects with allergies to buckwheat do not develop immediate hypersensitivity to the ingestion of these plants.[6] It is conceivable that the epitope specificities of anti-24 kDa IgE antibodies generated in buckwheat-allergic individuals may be different from the N-terminal amino acid sequences of anti-β subunits of 11 or 12S globulins of other related plants. Thus, it is intriguing to examine the epitopes of anti-24 kDa IgE in relation to the molecular structure of the legumin-like storage protein in these plants.

The IgE-reactive major bands, as well as the 24 kDa molecule, showed strong reactivity to IgE as well as to IgG and IgA (Figs 1,3). The most notable difference between allergic patients and non-allergic subjects was that only allergic individuals, not non-allergic subjects, showed IgE antibody production against these major allergens. Regarding IgA production, it is known that transforming growth factor-β is a immunoglobulin class-switching factor for B cells to IgA^+ cells and plays an important role in oral tolerance as well.[15] However, the level of serum IgA reactive to these allergens was almost comparable in both allergic and non-allergic subjects (Figs 1,3). Therefore, serum IgA per se may not be involved in immuno-unresponsiveness. In our preliminary experiments, IgG4 was predominantly produced in allergic subjects relative to the level of IgG1. The reverse relationship was found in non-allergic subjects (data not shown). The study analysing a number of allergic and non-allergic individuals may reveal that T helper cell (Th)2-type responses are primarily provoked in allergic patients, while Th1-type responses are generated in non-allergic individuals.

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