

Identification of an IgE-Binding Epitope of a Major Buckwheat Allergen, BWp16, by SPOTs Assay and Mimotope Screening

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Key Words

Allergen · Buckwheat · Immediate hypersensitivity · Anaphylaxis · Recombinant proteins · 2S albumin · SPOTs membrane · Mimotope · ELISA · Alanine scanning

Abstract

Background: The buckwheat 16-kDa protein (BWp16), as reported in our previous study, is a major allergen in buckwheat; however, the IgE-binding epitopes of BWp16 have not as yet been identified. **Methods:** We screened candidates for IgE-binding epitopes on BWp16 by using arrays of overlapping peptides synthesized on activated cellulose membranes (SPOTs membrane). The mimotope method was also used to analyze IgE-binding epitopes of BWp16. Nine single alanine (Ala) mutants of BWp16 expressed in *Escherichia coli* were used to confirm the epitopes of BWp16. The IgE-binding activity of single Ala mutants of BWp16 was determined by ELISA with mouse anti-BWp16 polyclonal antiserum or ELISA inhibition with sera from buckwheat allergic patients. **Results:** The SPOTs assay identified amino acid residues 99–110, i.e. EGVRDLKELPSK, as a candidate for the linear IgE-binding epitope of BWp16. The mimotope method indicated that peptides similar to EGVRDLKE were candidate sequences for epitopes of BWp16. Ala scanning of rBWp16 revealed that all EGVRDLKE peptides containing a single amino acid mutation had weaker IgE-binding activity than

rBWp16 WT. An ELISA inhibition assay for rBWp16 WT revealed the inhibitory effect of rBWp16 D103A to be less than that of rBWp16 WT. **Conclusions:** We identified the peptide EGVRDLKE as a very likely candidate for the IgE-binding epitope of BWp16, and Asp103 as the critical amino acid in BWp16. This is the first report on the identification of IgE-binding epitopes of BWp16. Our findings will contribute to the production of BWp16 hypoallergens, and to allergen-specific immunotherapy for buckwheat allergy.

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Introduction

Buckwheat (*Fagopyrum esculentum*) is a common pseudocereal consumed in noodles, pancakes, soup, and gruels across the world. Since the first buckwheat-allergic patient was reported in 1909, it has become well known that buckwheat allergy is one of the most important food allergies, manifesting as severe and critical symptoms induced by ingestion or inhalation of even a small amount of the flour or food products, not only in Japan but also in other countries [1–4]. Buckwheat allergy presents with a type I immune response, i.e. an immediate hypersensitivity reaction that includes anaphylaxis mediated by a specific IgE antibody. It is therefore important to identify buckwheat allergen molecules and clarify the mechanism

Table 1. Characterization of sera from patients with positive IgE binding to buckwheat

Serum No.	Immediate hypersensitivity reaction	Age years	Sex	Major diagnosis/symptoms	Total IgE IU/ml	Buckwheat-specific IgE levels, IU/ml	Buckwheat-specific IgE score
001	no data	6	M	food allergy (buckwheat, egg white, milk), atopic dermatitis, bronchial asthma, attention deficit/hyperactivity disorder	936	12.6	3
002	positive	34	M	food allergy (buckwheat anaphylaxis), hay fever (cedar pollen)	278	5.31	3
003	no data	3	M	food allergy, atopic dermatitis	288	17.5–49.9	4
004	positive	13	M	food allergy (buckwheat, bonito, yams), severe atopic dermatitis	22,600	6.8	3
005	positive	1	F	food allergy (buckwheat)	53	4.79	3
006	positive	7	M	food allergy (buckwheat)	448.6	45.3	4

Serum No. 001, 002, and 003 were previously labelled 3 [11], 001 [12], and 7 [11], respectively.

of buckwheat allergy to develop an accurate diagnostic procedure and safer immunotherapy.

Urisu et al. [5], from an immunoblotting analysis of sera from buckwheat-allergic patients, reported that 11 proteins in buckwheat have IgE-binding activities, and among them a 24-kDa protein is one of the major allergens in buckwheat (based on the results of IgE-RAST, prick tests, and RAST inhibition assays). The 24-kDa allergen, Fag e 1, is the β -subunit of the 13S globulin seed storage protein. Fag e 1 has been identified as having 8 linear IgE-binding epitopes by means of a SPOTs assay [6]. In addition, 14- and 18-kDa proteins have been identified as major allergens in buckwheat by an immunoblotting analysis [7]. Furthermore, recent studies have shown that a 19-kDa protein is also a major buckwheat allergen that has weak homology with vicilin-like allergen and 7S globulin [8, 9]. On the other hand, a 16-kDa protein has been reported to be resistant to pepsin digestion and to have IgE-binding activities with sera from patients with immediate hypersensitivity reaction to buckwheat [10]. Our previous studies have shown that the 16-kDa buckwheat seed storage protein (BWp16) is a strong candidate for the major buckwheat allergen involved in the 2S albumin family based on the character of the reaction between BWp16 and the sera of patients positive for IgE-binding activity to buckwheat [11, 12].

To better understand the mechanisms of the allergic immune response, it is important to isolate allergen molecules, characterize their 3-dimensional structures, and

identify the IgE-binding epitopes. Since IgE antibodies recognize the surfaces of allergen molecules, there are 2 types of IgE-binding epitopes: linear and conformational. SPOTs assay is a useful tool for rapid identification of the linear epitopes in allergens with covalently attached synthesized overlapping peptides on the cellulose membrane (SPOTs membrane) [13, 14]. On the other hand, screening of a random peptide phage display library is an effective method of analysis for mimotopes, small peptides that mimic the structure of epitopes [15–17]. Mimotope analysis reveals the conformational as well as the linear epitopes of allergen molecules.

We previously reported that recombinant BWp16 (rBWp16) generated in *Escherichia coli* (*E. coli*) is immunologically equivalent to purified native BWp16 from buckwheat extracts [11]. We also demonstrated that ELISA using rBWp16 could be employed for in vitro diagnosis of buckwheat allergy [12]. In addition, we showed the involvement of cysteine residues in the allergenicity of BWp16 [12]. In the present study, we have identified a peptide as the linear IgE-binding epitope of BWp16, as well as the amino acid residues critical for IgE binding in BWp16.

Materials and Methods

Patients' Sera

Sera from Japanese patients whose buckwheat-specific IgE CAP RAST score was positive (table 1) and control sera from

healthy volunteers were used in this study. Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethics Review Committee of the National Institute of Health Sciences.

Peptide Synthesis and SPOTs Assay

The 58 overlapping peptides consisting of 12 amino acids, offset by 2 amino acids derived from BWp16, were synthesized and bound to membranes (SPOTs membranes, custom-ordered from Sigma-Genosys; The Woodlands, Tex., USA). The SPOTs membranes were stored at -20°C until use in the IgE-binding assay. The membranes were washed with Tris-buffered saline and blocked overnight as described previously [14].

Affinity Purification of Anti-BWp16 IgE Antibody in Patient Serum

Anti-BWp16 IgE antibody was purified by the method described previously [17] with minor modifications. Two-hundred fifty milligrams of CNBr-activated Sepharose 4B (GE Healthcare UK, Little Chalfont, UK) was suspended in 50 ml of 1 M HCl, and the solution was allowed to stand at room temperature for 15 min. The CNBr-activated Sepharose 4B was washed with 5 ml coupling buffer (0.1 M NaHCO_3 , 0.5 M NaCl, pH 8.3) twice, and the buffer was removed by centrifugation. Seven milligrams of rBWp16 WT protein prepared as previously described [12] was dissolved in coupling buffer, and the solution was mixed with the CNBr-activated Sepharose 4B. After allowing it to react for 1 h at room temperature, the rBWp16 protein-coupled Sepharose 4B gel was washed with coupling buffer, then mixed with 5 ml of 0.1 M Tris-HCl (pH 8.0) and allowed to stand for 2 h at room temperature. The protein-coupled gel was then washed once with 5 ml of 100 mM acetate buffer (pH 4.0) containing 0.5 M NaCl, once with 5 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.5 M NaCl, and twice with phosphate-buffered saline (PBS).

The serum No. 003 was diluted 1:1 with saturated ammonium sulfate and allowed to stand for 30 min at 4°C . After centrifugation, the 50% ammonium sulfate fraction of serum was collected, the suspension buffer was then replaced with PBS 3 times with Microcon YM-30 Centrifugal Filter Unit (Millipore Corporation, Bedford, Mass., USA), and finally redissolved in PBS as partially purified IgE. The partially purified IgE was mixed with the rBWp16 protein-coupled gel and incubated overnight at 4°C with gentle shaking. After washing with PBS 3 times, bound antibodies were eluted with a half volume of 3 M KSCN/PBS by incubation for 15 min at 4°C . The eluted fractions were immediately neutralized by mixing with an equal volume of 1 M Tris-HCl (pH 8.0), and then in PBS (pH 7.4).

To remove IgG antibody, the anti-BWp16 IgE antibody solution was mixed with Protein G Sepharose 4B (GE Healthcare UK) and allowed to stand for 30 min at 4°C with gentle shaking. The solution was then centrifuged, and the supernatant was collected as almost purified anti-BWp16 IgE antibody.

Screening for Mimotopes with a Phage Display Peptide Library

A 96-well plate (Nunc LockWell™ Module Plate, C8, M type, cat No. CN-446469; Nunc A/S, Roskilde, Denmark) was coated with the purified anti-BWp16 IgE antibody diluted 1:4 with 0.1 M NaHCO_3 (pH 8.6; 50 $\mu\text{g}/\text{well}$), and then used to screen for mimotopes with a phage display peptide library. The screening was per-

formed using the Ph.D.™-12 Phage Display Peptide Library Kit (New England BioLabs, Ipswich, Massachusetts, USA) according to the manufacturer's protocol.

Expression of the Wild-Type and Alanine Mutants of rBWp16 in E. coli

BWp16 WT cDNA encoding amino acid residues 23–149 was cloned into the pGEX 6P-1 vector (GE Healthcare UK) as described previously [12]. Nine single alanine (Ala) mutants of BWp16 cloned into the pGEX 6P-1 vector were constructed (Takara Bio, Shiga, Japan). The BWp16 WT and 9 single Ala mutant recombinant plasmids were transfected into *E. coli* BL21 (DE3) pLysS cells, as described previously [12]. All recombinant mutated proteins and rBWp16 WT were expressed and purified as described previously [12].

ELISA

The coating of 96-well plates (Nunc LockWell) each with rBWp16 protein (1.0 $\mu\text{g}/50\ \mu\text{l}$ of 50 mM sodium carbonate buffer per well; pH 9.6) was followed by incubation, washing, and finally a blocking step as described previously [12]. The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated overnight at 4°C with mouse anti-BWp16 polyclonal antiserum (1:500 dilution) [11] in 0.1% casein-PBS. After the wells were washed with PBS-T, and then they were incubated with rabbit anti-mouse IgE (1:1,000 dilution; Nordic Immunological Laboratories, Tilburg, The Netherlands) for 1 h at room temperature. For colorimetric detection, the wells were exposed to horseradish-peroxidase-conjugated goat anti-rabbit Ig antibodies (1:1,000 dilution; Amersham Biosciences, Piscataway, N.J., USA) in 0.1% casein-PBS for 1 h at room temperature and then reacted with substrate solution (TMB reagent, cat No. 555214; BD Biosciences, San Diego, Calif., USA). Colorimetric intensity (OD450) was measured according to the manufacturer's protocol.

ELISA Inhibition

Diluted patients' and control sera (1:50 dilution) were pre-incubated overnight at 4°C with an equal volume of serially diluted rBWp16 WT or rBWp16 mutants in 0.2% casein-PBS or PBS. Then, 96-well plates were coated with rBWp16 WT protein (1.0 $\mu\text{g}/50\ \mu\text{l}$ of 50 mM sodium carbonate buffer per well; pH 9.6) and incubated as described previously [12]. After washing, blocking, and washing again, the plates were incubated with pre-incubated serum samples overnight at 4°C . The wells were then washed, and detection was performed as described previously [12].

Results

Mapping of IgE-Binding Epitopes in BWp16 through SPOTs Assay

In order to identify the regions of the primary sequence of BWp16 that were recognized by the sera from allergic patients, 58 overlapping peptides of BWp16 were attached to cellulose membrane (SPOTs membrane; fig. 1a). Serum No. 001 showed IgE-specific binding with peptide numbers 50, EGVRDLKELPSK, and 51,

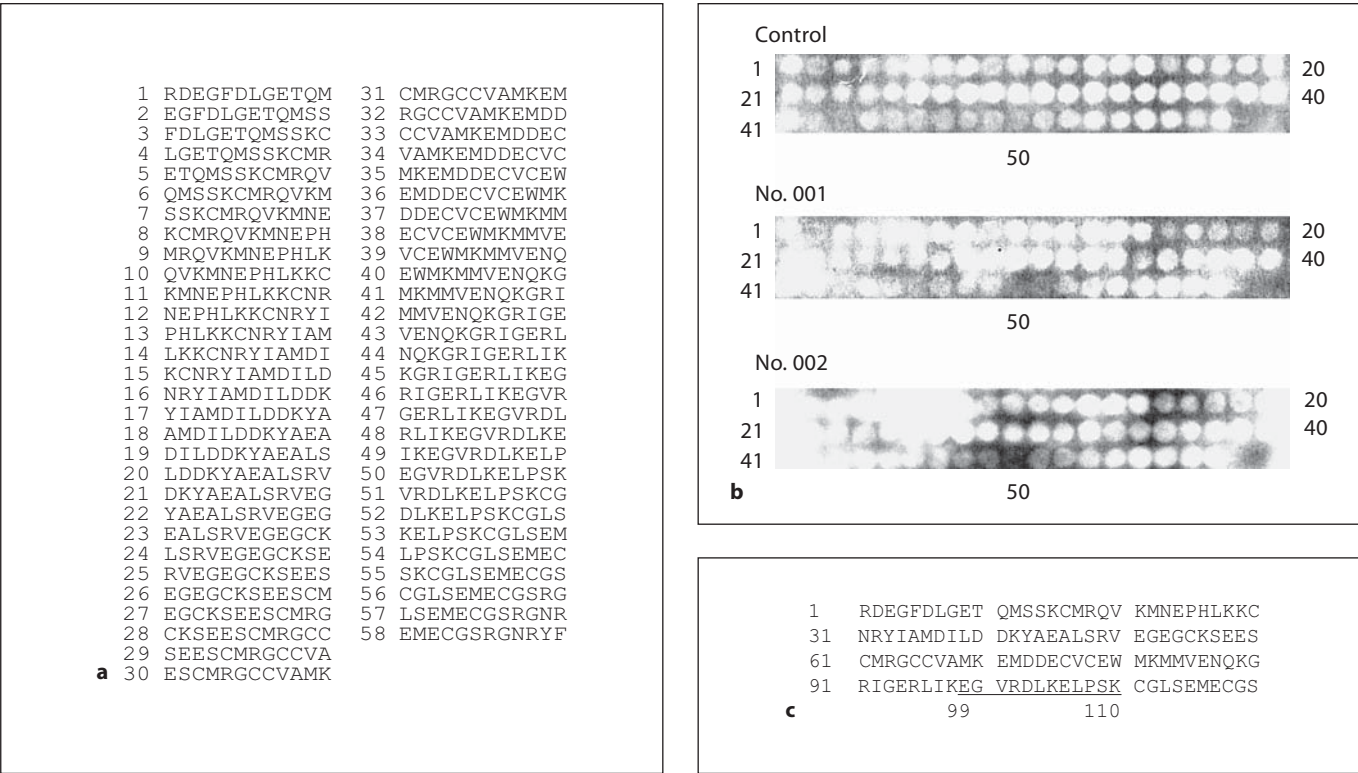


Fig. 1. Epitope mapping of BWp16 by SPOTs assay. **a** Overlapping peptide synthesis of BWp16. Fifty-eight 12-amino acid peptides, offset by 2 amino acids, were synthesized. **b** Membranes containing overlapping peptides were tested with sera from Japanese patients whose buckwheat-specific IgE CAP RAST scores were positive (No. 001 and 002) and with control serum from a healthy volunteer (control). **c** Deduced amino acid sequence of BWp16 protein. Peptide number 50, corresponding to residues Glu99 to Lys110, is underlined.

VRDLKELPSKCG, on the SPOTs membrane, but the control serum showed few signals with these peptides (control and No. 001; fig. 1b). Furthermore, serum No. 002 showed strong IgE-specific binding, especially with peptide number 50 (No. 002; fig. 1b). These results indicated amino acid residues 99–110, EGVRDLKELPSK, represented by peptide number 50, to be the most probable candidate for the linear IgE epitope of BWp16 (fig. 1c).

Screening for BWp16 Mimotopes with a Phage Display Library

Phage display peptide libraries are extensively utilized to map the antigenic epitopes of proteins. Several biopannings of IgE-antibody-binding-phage clones in vitro and subsequent amplification in vivo allowed identification of mimotopes. After being coated with the refined anti-BWp16 IgE antibody, 96-well plates were used to screen for mimotopes of BWp16 with the Ph.D.TM-12 Phage Display Peptide Library Kit. The third panning yielded

several clones containing sequences with high homology to EGVRDLKE (fig. 2). Significantly, the EGVRDLKE sequence was contained within the candidate sequence for the linear IgE epitope of BWp16 revealed by the SPOTs assay (fig. 1), suggesting even more strongly that EGVRDLKE is the most likely candidate for the linear IgE epitope of BWp16.

Alanine Scanning Mutagenesis of the Residues in EGVRDLKE

The SPOTs assay and mimotope screening yielded similar results indicating that one of the IgE epitopes on BWp16 is a sequence containing EGVRDLKE, amino acid residues 99–106. To identify the amino acids in this sequence that are critical for IgE binding, we generated 9 different rBWp16 mutants, each with a different amino acid residue replaced by an Ala residue, and referred to them as rBWp16 K98A, E99A, G100A, V101A, R102A, D103A, L104A, K105A, and E106A (fig. 3a). We then ana-

Fig. 2. Epitope mapping of BWp16 with the phage display library. Selected amino acid sequences from the third panning are shown aligned with BWp16. The conserved amino acid residues are shown in white against a black background. Gray boxes indicate residues conserved among less than 5 clones.

3rd2-1	-----	1
3rd2-10	-----	1
3rd2-11	-----	1
3rd2-12	-----	1
3rd2-13	-----	1
3rd2-14	-----	1
3rd2-15	-----	1
3rd2-2	-----	1
3rd2-3	-----	1
3rd2-4	-----	1
3rd2-5	-----	1
3rd2-6	-----	1
3rd2-7	-----	1
3rd2-8	-----	1
3rd2-9	-----	1
mature BWp16	RDEGFDLGETQMSSKCMRQVKMNEPHLKKCNRYIAMDILDDKYAEALSRVEGEGCKSEES	60
3rd2-1	-----	1
3rd2-10	-----EHYDRN-----T-----	7
3rd2-11	-----EHYDRN-----T-----	7
3rd2-12	-----EHFCR-----D-----	6
3rd2-13	-----EHFQRD-----LLLPA-----	11
3rd2-14	-----EHYDRN-----T-----	7
3rd2-15	-----	1
3rd2-2	-----	1
3rd2-3	-----EHYDRT--SPRQT-----	11
3rd2-4	-----EHYDRS-----S-----	7
3rd2-5	-----EHYIRD--TSPAY-----	11
3rd2-6	-----EHYDRS-----S-----	7
3rd2-7	-----EHYDRN-----T-----	7
3rd2-8	-----	1
3rd2-9	-----EHYDRS-----S-----	7
mature BWp16	CMRGCCVAMKEMDDECVCCEWMKMMVENQKGRIGERLIKEGV-RDLKE-LPSKCGLSELEC	118
3rd2-1	-----WHWPW-----YQ-----GQLWP	12
3rd2-10	-----FKSWE-----	12
3rd2-11	-----FKSWE-----	12
3rd2-12	-----CQTPML-----	12
3rd2-13	-----P-----	12
3rd2-14	-----FKSWE-----	12
3rd2-15	-----AMNAPPS-L--TFPR---	12
3rd2-2	-----WHWNYMLQPPPP-----	12
3rd2-3	-----L-----	12
3rd2-4	-----NYLLA-----	12
3rd2-5	-----T-----	12
3rd2-6	-----NYLLA-----	12
3rd2-7	-----FKSWE-----	12
3rd2-8	-----TGPSQSLGTTFD-----	12
3rd2-9	-----NYLLA-----	12
mature BWp16	GSRGNRYFV-----	127

lyzed the reactivity of IgE with the wild-type and mutated rBWp16 by ELISA with mouse anti-BWp16 polyclonal antiserum. IgE-binding activity to all of the rBWp16 mutants was lower than that to rBWp16 WT (fig. 3b), suggesting that the EGVRDLKE sequence functions as the BWp16 epitope, and that all of its amino acid residues play a significant role in the allergenicity of rBWp16.

ELISA Inhibition Assay of rBWp16 by E99A and D103A Mutants with Patients' Sera

As shown in figure 3, substitution of each of the amino acid residues in EGVRDLKE decreased the binding activity of IgE for BWp16, and substitution of Glu99 and Asp103 by Ala resulted in greater reductions in IgE-bind-

ing activity. These amino acid residues are conserved in some clones isolated as BWp16 mimotopes (fig. 2). We then focused on mutants rBWp16 E99A and D103A and assessed the competitive inhibitory effect of these mutants on IgE binding by the ELISA inhibition method with sera from buckwheat-allergic patients. In all patients' sera used for the assay, the inhibitory effects by rBWp16 D103A were reduced as compared with those of rBWp16 WT (fig. 4). In serum No. 004, the 50% inhibition dosages of rBWp16 D103A and WT were 0.9 μ M and 0.07 μ M, respectively (fig. 4a). In sera No. 005 and 006, more than 1 μ M rBWp16 D103A were required to inhibit IgE binding to rBWp16 by 50% as compared with rBWp16 WT (fig. 4b, c). On the other hand, the inhibitory effi-

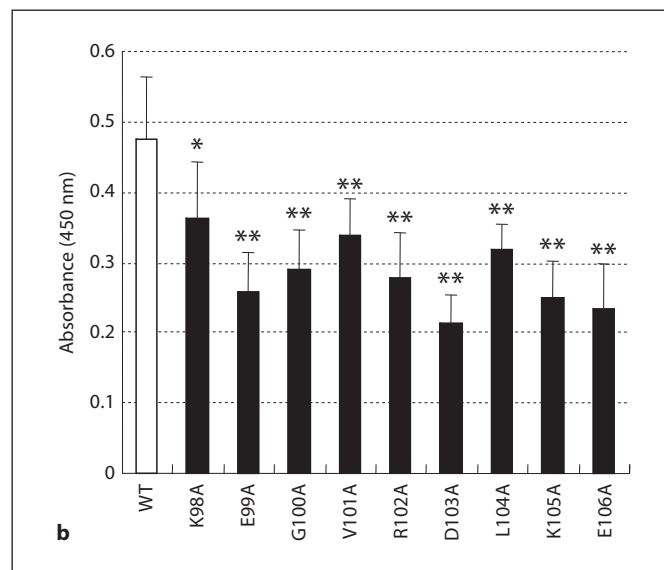
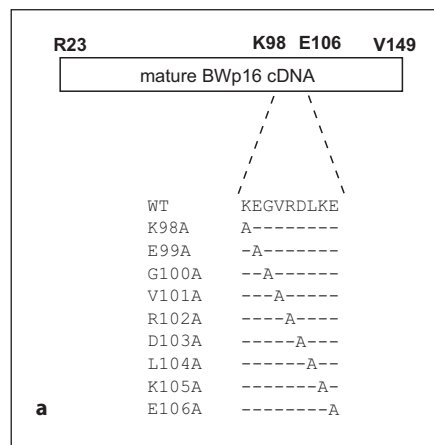


Fig. 3. Ala scanning mutagenesis of the residues in EGVRDLKE. **a** Construct used in Ala scanning analysis. The mutated rBWp16 contained EGVRDLKE sequences with a substitution of each amino acid residue by an Ala residue. **b** ELISA of rBWp16 with or without an Ala substitution in the EGVRDLKE sequence, with mouse anti-BWp16 polyclonal antiserum. Values are means $\pm$ SD of 3 independent experiments, each performed in duplicate. * $p < 0.05$; ** $p < 0.01$.

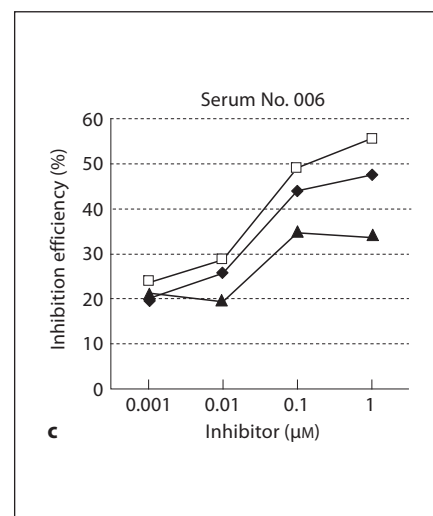
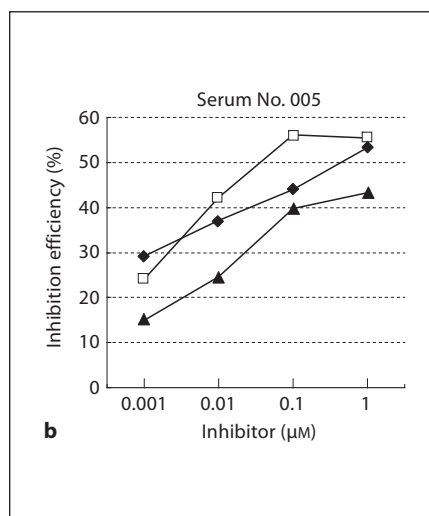
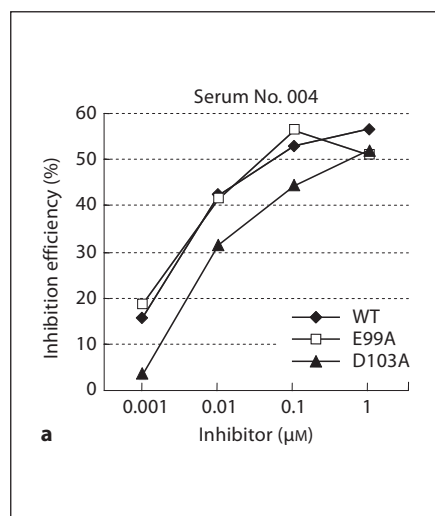


Fig. 4. ELISA inhibition of rBWp16 WT, E99A, and D103A with sera from buckwheat-allergic patients. Sera from buckwheat-allergic patients No. 004, 005, and 006 (1:50 dilution) were pre-incubated with different concentrations of rBWp16 with or without an Ala substitution. The pre-incubated serum samples were transferred to rBWp16 WT-coated wells, and bound IgE was analyzed by ELISA. A representative result of 2 similar experiments is

shown. The percentage of binding inhibition after pre-incubation with the different inhibitors was calculated as: $[(OD - OD_{inhibitor}) / OD] \times 100$, where OD (optical density) and OD inhibitor indicate the absorbance values obtained without or with inhibitors before incubation, respectively. $\blacklozenge$ = rBWp16 WT; $\square$ = rBWp16 E99A; $\blacktriangle$ = rBWp16 D103A.

ciency of rBWp16 E99A differed minimally from that of rBWp16 WT (fig. 4). These results indicate that Asp103 is more important than Glu99 for IgE-binding activity to BWp16.

Discussion

In this study, we focused on the identification of IgE-binding epitopes of BWp16. The results of both mimotope screening and the SPOTs assay suggested that EGVRDLKE is one of the linear IgE-binding epitopes of BWp16 (fig. 1, 2). Due to recent advances in molecular biology, several allergen molecules are now known to belong to the 2S albumin family [18], and IgE-binding epitope mapping studies have been conducted on several 2S albumin family allergens, including Ana o 3 [19], Ara h 2 [20], and Jug r 1 [21]. We previously reported that BWp16 contains a conserved motif of 8 cysteine residues, which is one of the characteristic features of members of the 2S albumin family, and that it exhibits homology with several 2S albumin family allergens, Ara h 6, Ric c 1, and Ara h 2 [12]. Alignment of the 2S albumins suggested EGVRDLKE to be located in helix IV [12, 18]. Unfortunately, the EGVRDLKE sequence in BWp16 had low sequence homology with the helix IV sequences in all other 2S albumin family allergens, as reported previously, even though linear IgE-binding epitopes of several 2S albumin proteins have been indicated to be located in helix IV. Moreover, a search for known epitopes in the Allergen Database for Food Safety (<http://allergen.nih.go.jp/ADFS/>) yielded no sequences homologous with EGVRDLKE among known allergen epitopes except Fag e 1, a major buckwheat allergen that belongs to the 13S globulin seed storage protein family. These results support those few reports of cross-reactivity between buckwheat and other foods [22, 23]. A case report revealed buckwheat to have a cross-reactive allergen to one poppy seed-allergic patient's IgE [23]. On the other hand, it has been reported that sesame proteins, including Ses i 1 and Ses i 2, may cross-react with a 2S albumin protein from poppy seeds [24, 25]. Identification of the poppy seed allergens that are the cause of the cross-reactions with buckwheat and epitope analysis of those allergens might clarify the mechanism underlying the cross-reactions between poppy seeds and buckwheat.

Our findings suggest that all of the amino acid residues in the EGVRDLKE sequence of BWp16, especially Glu99 and Asp103, are involved in the IgE-binding activity of BWp16 (fig. 3). Furthermore, the results of ELISA

inhibition suggested that Asp103 plays the role of a critical amino acid in EGVRDLKE, although the substitution of Glu99 had little impact on the IgE-binding activity of BWp16 with sera from buckwheat allergic patients (fig. 4). The differences in these results, as reflected by the role of Glu99, may be attributable to differences in the individual variations of buckwheat allergic patients. To determine the position of EGVRDLKE in the 3-dimensional structure of BWp16, we predicted the 3-dimensional structure model of BWp16 by using ROKKY [12]. The results indicated that the region containing EGVRDLKE may form an α -helix and is located on the surface of BWp16 (data not shown). Crystallization and X-ray-diffraction analyses of rBWp16 also suggested that the amino acid residues in EGVRDLKE may be located on the surface of the protein (our unpublished data). Our results are consistent with the predicted 3-dimensional model of BWp16. These results corroborated those of ELISA and ELISA inhibition in this study, and indicated more strongly that EGVRDLKE is an epitope of BWp16.

This is the first report identifying an IgE-binding epitope peptide and its critical amino acid residues in the major buckwheat allergen BWp16. Analysis of sera from other buckwheat allergic patients might reveal other epitopes. The results of this study can be used to develop allergen-specific immunotherapy for patients with the buckwheat allergy.

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