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Identification of immunoglobulin E (IgE)-binding epitopes and recombinant IgE reactivities of a latex cross-reacting Indian jujube Ziz m 1 allergen

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Summary

Ziz m 1 is a major Indian jujube (*Zizyphus mauritiana*) allergen involved in latex-fruit syndrome, and cDNA of the allergen has been cloned, sequenced and expressed in yeast by our laboratory previously. In this study, we performed an immunoglobulin E (IgE)-binding epitope analysis of Ziz m 1 using overlapping recombinant fragments. Eight overlapping recombinant fragments were generated from the recombinant Ziz m 1 allergen. The fragments were expressed in *Escherichia coli* and IgE-binding activities were evaluated by sera of latex–Indian jujube-allergic subjects and normal subjects using immunoblotting. Human allergic sera are not able to recognize fragments consisting of amino acid sequences 26–71, 119–280 and 119–291. However, residues at positions 26–199, 26–105, 26–86, 119–320 and 238–330 were found relevant in the IgE-binding. Our results indicate that ^{72}NISGHCSD CTFLGEE^{86} and ^{292}VWNRYYDLKTNYSSSIILEYVNSGTKYLP^{320} of Ziz m 1 are the sequences required for human IgE binding. Four corresponding peptides, ^{72}NISGHCSDCTE^{86}, ^{292}VWNRYYDLKT^{301}, ^{300}KTNYSSSIILEY^{311} and ^{309}LEYVNSGTKYLP^{320}, were synthesized, and these peptides reacted with 70%, 100%, 70% and 70% of 10 allergic sera tested, as revealed by enzyme-linked immunosorbent assay. Sensitization to ^{292}VWNRYYDLKT^{301} correlated significantly with the presence of allergic symptoms ($P < 0{\cdot}001$). These findings will be useful in designing diagnostic and therapeutic approaches, thereby contributing to the development of specific immunotherapy for subjects with latex–fruit syndrome.

Keywords: IgE-binding regions, Indian jujube, latex–fruit syndrome, recombinant Ziz m 1 allergen, synthetic peptides

Introduction

Latex allergy has been the most important occupational allergy among medical workers worldwide [1–4], including Taiwan [5,6]. So far, 13 allergens from natural rubber latex are characterized [7], and some of them have been documented to play a dominant role in food cross-reactivity to certain fruit [8]. Previously, our study showed that 8·6% (26 of 302) of medical workers in Taichung had immediate allergic reactions to latex gloves, and 26·2% (seven of 26) of the latex-allergic subjects had latex–fruit syndrome. Among those subjects, 28·6% (two of seven) were allergic to Indian jujube (*Zizyphus mauritiana*) [6]. Indian jujube, a native species of Yunnan province of southern China, is a popular sweet fruit growing in warm climates and is cultivated abundantly in southern Taiwan. Patients suffering from Indian jujube allergy may present with oral allergy syndrome, urticaria, angioedema, asthmatic attack or worsening of pre-existing atopic dermatitis [9].

Recently, we have cloned a 30-kDa major Indian jujube allergen, Ziz m 1 [10]. The recombinant Ziz m 1 (rZiz m 1), with chitinase activity, exhibits immunoglobulin E (IgE) cross-reactivity with the latex allergen hevamine. Moreover, a computer-assisted homology search found that Ziz m 1 had 39–45% sequence identity to many class III chitinases of plants, including hevamine, of natural rubber latex and belonged to a family of 18 glycosyl hydrolases. Hevamine is a 30 kDa protein with lysozyme and chitinase activity and has been classified into the PR-8 family of pathogenesis-related proteins [11]. Although it has been regarded as a minor latex allergen in western countries [12], our study showed that hevamine was recognized by 55% of the sera from

Table 1. Sequences of primers used for the cloning of Ziz m 1 fragment in pET30.

Overlapping fragment	Nucleotide sequence	MW[§] (kDa)
N1 (26–199)[†]		
Sense	GAATTC[140‡]GGTGGCATAGCAACCTAC[157]	24·8
Anti-sense	GCGGCCGC[661]TGTCCAAAGATAAGCGTC[644]	
N2 (26–105)		
Anti-sense	GCGGCCGC[379]ATAAGGTCCTCCAAGAGA[362]	14·4
N3 (26–86)		
Anti-sense	GCGGCCGC[322]CTCTTCGCCGAGAAAGGT[305]	12·4
N4 (26–71)		
Anti-sense	GCGGCCGC[277]TAAACTCAGGTACCGGCC[260]	10·8
C1 (119–280)		
Sense	GAATTC[419]GTTGCAGAGCAACTGTGG[436]	23·7
Anti-sense	GCGGCCGC[904]CACCGAAACAATTTGATC[887]	
C2 (119–291)		
Anti-sense	GCGGCCGC[937]GTTTACACCTCCAAACTT[920]	24·8
C3 (119–320)		
Anti-sense	GCGGCCGC[1024]TGGTAAATACTTGGTTCC[1007]	28·2
C4 (238–330)		
Sense	GAATTC[776]GATTGGACTGTGTCGCTT[793]	16·1
Anti-sense	GCGGCCGC[1075]GTAATGGTGCGTTATGGA[1058]	

[†]Numbers in parenthesis represent the position of the amino acid sequences of cDNA of Ziz m 1 (NCBI Accession no. AY839230). [‡]The numbers represent nucleotides position of the Ziz m 1. [§]Indicates molecular weight deduced from amino acid sequences of recombinant fragments expressed in BL21 (DE3). Restriction sites for *Eco*RI and *Not*I are underlined.

latex-allergic patients [13]. It is one of the major latex allergens among Taiwanese medical workers and plays an important role in the latex–fruit syndrome.

The structural characteristics of proteins are major determinants of cross-reactivity [14]. Knowledge of IgE-binding epitopes on the Ziz m 1 molecule will advance understanding of the molecular basis of latex–fruit syndrome. In the present study, we describe the high-level expression of Ziz m 1 cDNA in *Escherichia coli* (rZiz m 1–*E. coli*), and compare the biochemical and immunological properties with *Pichia*-expressed recombinant Ziz m 1 allergen (rZiz m 1–*P*) [10]. Eight overlapping recombinants were generated, and recombinant proteins were expressed in *E. coli* BL21 (DE3). Subsequently, IgE binding regions of Ziz m 1 were identified. Heterogeneous IgE-binding patterns exist among latex–Indian jujube-allergic subjects as revealed by enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Subjects

The project was reviewed and approved by the Institutional Review Board of Taichung Veterans General Hospital. A total of 10 latex–Indian jujube-allergic subjects (P1–P10) and five healthy non-allergic individuals (NA1–NA5) were included in this study. Informed consent was obtained from all the subjects. Patients with asthma or angioedema involving the airway to Indian jujube are classified as the severe allergic group, and those with only allergic rhinoconjunctivitis, dermatitis or oral allergy syndrome were designated the mild allergic group.

Serum samples were obtained from all subjects and assayed for specific IgE antibodies to latex glove and Indian jujube extracts and recombinant Ziz m 1 (rZiz m 1) by ELISA [10]. Equal volumes of sera from seven patients were pooled to constitute an allergic serum pool for immunoblotting studies.

Polymerase chain reaction cloning of Ziz m 1 fragments

The previously cloned cDNA coding for Ziz m 1 (GeneBank database, Accession no. AY839230) was used as a template for polymerase chain reaction (PCR) amplification of Ziz m 1 fragments. For PCR, gene-specific primers were designed with restriction sites for cloning into the pET30 expression vector (Novagen, Madison, WI, USA) (Table 1). PCR was performed with a hot start at 94°C for 3 min, and subsequently 30 cycles of amplification were performed under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The PCR products were purified by BandPrep kit (Genepure, Taichung, Taiwan) and ligated into pCR2·1 vector (Invitrogen, Carlsbad, CA, USA).

Expression of recombinant Ziz m 1 in *E. coli* and *P. pastoris*

After double digestion with *Eco*RI and *Not*I, the cDNAs were then ligated into vector pET30a. Sequences of cDNA inserts

were performed using the ABI PRISM Dye Terminator Kit and ABI 377 sequencer (Perkin-Elmer Biosystems, Warrington, UK). The resulting plasmids were then transformed into *E. coli* BL21 (DE3) for expression [15]. Expression of *P. pastoris* was performed as described previously [10].

Purification of recombinant proteins

Plasmid pET30 contains the His-tag sequence, a stretch of six histidine residues that was expressed at both N and C-terminal ends of the target protein. The sequence of His-tag binds to divalent cations (Ni^{2+}) immobilizing the histidine-binding metal chelation resin (Novagen), and the resulting fusion protein is recovered by elution with 1 M imidazole. Briefly, an overnight culture was diluted 1:100 into 200 ml Luria–Bertani broth containing 25 μg/ml kanamycin. Protein expression was induced with isopropyl thio-β-D-galactoside at a final concentration of 0·4 mM, while the culture reached an optical density (OD) of 0·5 at 600 nm. The cells were harvested and suspended in 20 ml sonication buffer [50 mM Tris-HCl, pH 8·0, 200 mM NaCl, 0·1 mM ethylenediamine tetraacetic acid and 0·1% Nonidet P-40] after 16 h incubation at 37°C. Cells were subjected to 20 min sonication in an ice-water bath with a Branson sonifier-250 (Branson Ultrasonics, Danbury, CT, USA). The inclusion bodies were obtained after 30 min, 10 000 ***g*** centrifugation at 4°C. The inclusion bodies were then dissolved in 1× binding buffer containing 6 M urea, and recombinant proteins were purified using the rapid affinity column chromatography with pET-His-Tag system, as described by the manufacturers (Novagen). The rZiz m 1 was refolded using dialysis with a gradual removal of urea in 0·02 M phosphate-buffered saline (PBS), pH 7·2 and concentrated by Amicon Ultra PL-10 (Millipore, Billerica, MA, USA). Protein concentration was determined using the Bio-Rad Bradford assay (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (BSA) (Sigma Biochemical Co., St Louis, MO, USA) was used as protein standard.

Skin prick test

Subjects were skin prick-tested with laboratory-prepared glove extract [13], crude Indian jujube extract [9], rZiz m 1 from yeast expression system (rZiz m 1–*P*) and rZiz m 1 from *E. coli* system (rZiz m 1–*E. coli*) at a concentration of 100 μg/ml in PBS containing 50% glycerol (PBSG), as described previously [9]. Briefly, the test was performed with a sterile disposable SharpTest applicator (Greer Laboratories, Lenoir, NC, USA). PBSG and 1·0 mg/ml of histamine were used as negative and positive controls respectively. The diameters of erythema and wheals were measured. The mean diameter of the wheal and erythema was graded according to controls. A reaction comparable to the negative control was considered negative. A response with a wheal or an erythema greater than those produced by the negative control but less than half the diameter of positive control was graded as 1+ and a reaction range between half to the size of the positive control was graded as 2+. A reaction comparable with the positive control was graded as 3+. A reaction larger than the positive control was graded as 4+.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting

Protein samples were loaded onto a 4% polyacrylamide stacking gel above a 12% separating gel, and the gel was run with discontinuous buffer by Laemmli's method [16]. After electrophoresis, gels were fixed and stained with 0·2% Coomassie Brilliant Blue R250 (Sigma). For immunoblotting, gels were transferred electrophoretically under semidry conditions (Bio-Rad) for 30 min at 0·8 mA/cm^2 [17] to nitrocellulose membranes (Millipore). After transfer, the membranes were blocked in Tris-HCl buffered saline containing 0.1% Tween 20 (TBST) (50 mM Tris-HCl, pH 7·5; 150 mM NaCl; 0·05% Tween 20) containing 5% skimmed milk for 2 h at room temperature. Blots were incubated with a 1:10 dilution of patient serum pool or negative control serum pool overnight at 4°C. The blots were washed for three 10-min periods in TBST and then incubated with a 1:500 dilution of an anti-human IgE alkaline phosphatase conjugate (PharMingen, San Diego, CA, USA) for 2 h at room temperature. The reaction was detected using a chemiluminescent substrate solution (Applied Biosystems, Bedford, MA, USA) and signals were recorded by exposure to X-ray film.

Synthetic peptides

Four biotinylated overlapping peptides, ^{72}NISGHCSDCTE^{86}, ^{292}VWNRYYDLKT^{301}, ^{300}KTNYSSSIILEY^{311} and ^{309}LEYVNS GTKYLP^{320}, corresponding to IgE-binding amino acid sequences of recombinant fragments, were purchased from United Biochemical Research, Inc. (Seattle, WA, USA). Peptides were purified by high-performance liquid chromatography (greater than 80% purity) and characterized using matrix-assisted laser desorption mass spectrometry.

Immunodetection of synthetic peptides by ELISA

The specificity of IgE binding regions was evaluated further using four synthetic peptides and tested for IgE-binding reactivity with 10 allergic and five non-allergic sera. IgE-binding reactivity of peptides was determined by ELISA, as described previously [15,18]. Optimal concentration of antigen and conjugate was determined by checkerboard titration. Biotin-labelled synthesized peptides (10–50 ng/well) in coating buffer (0·1 M sodium carbonate, pH 9·6) were used to coat the streptavidin immobilizer plate (Nunc, Taichung, Denmark) in duplicate and incubated at 37°C for 2 h. The plate was blocked with 2% BSA and incubated over-

Table 2. Clinical history and specific IgE values in patients with latex–Indian jujube allergy and control subjects.

Subjects	Clinical manifestation	Glove extract† ELISA (OD)/SPT	Indian jujube extract† ELISA (OD)/SPT	rZiz m 1–*E. coli* ELISA (OD)/SPT	rZiz m 1–*P* ELISA (OD)/SPT
P1	AE, OAS	0·72/3+	1·25/4+	0·34/+	0·58/2+
P2	AS, OAS	2·45/4+	2·78/4+	0·37/2+	1·26/4+
P3	AR, AD	0·38/2+	0·73/2+	0·38/+	0·29/+
P4	AS, OAS	0·62/4+	1·22/3+	0·52/2+	0·45/4+
P5	AR, OAS	2·60/2+	2·99/4+	3·85/2+	3·86/2+
P6	AC, OAS	1·45/4+	0·42/2+	1·82/+	2·10/4+
P7	AR, OAS	0·69/3+	1·76/4+	0·53/+	0·81/2+
P8	AR, AD	3·39/2+	2·93/3+	0·42/+	3·13/3+
P9	AS, AR	0·95/2+	0·34/2+	1·06/2+	0·82/2+
P10	AN, AS	2·95/4+	2·84/4+	0·85/2+	0·46/4+
NA1	None	0·068/–	0·07/–	0·079/–	0·064/–
NA2	None	0·077/–	0·05/–	0·074/–	0·081/–
NA3	None	0·069/–	0·06/–	0·065/–	0·075/–
NA4	None	0·071/–	0·08/–	0·069/–	0·064/–
NA5	None	0·072/–	0·11/–	0·076/–	0·073/–

†Laboratory-prepared crude extract. AE, angioedema; AN, anaphylaxis; OAS, oral allergy syndrome; AS, asthma; AR, allergic rhinitis; AD, atopic dermatitis; AC, allergic conjunctivitis; ELISA, enzyme-linked immunosorbent assay; OD, optical density; SPT, skin prick test.

night at 4°C with 1:10 dilution of patients or non-allergic sera. After washing, antibody binding was detected using alkaline phosphatase-labelled mouse anti-human IgE monoclonal antibody along with p-nitrophenyl phosphate (Sigma) as a substrate. The OD was determined at 405 nm on a Sunrise Absorbance Reader (TECAN, Austria). The cut-off value was defined as 2 × [mean OD values of 20 non-allergic samples + 2 standard deviations (s.d.)].

Statistical analysis

Statistical analysis was performed with SPSS version 10·0 software (SPSS Inc., Chicago, IL, USA) using appropriate methods. *P*-values less than 0·05 were considered to be significant.

Results

Comparison of rZiz m 1–*P* and rZiz m 1–*E. coli*

The clinical manifestations, the OD of specific IgE by ELISA and the results of skin prick test (SPT) to latex glove extract, Indian jujube extract, rZiz m 1–*E. coli* and rZiz m 1–*P* are summarized in Table 2. The *in vitro* IgE reactivity determined by ELISA to latex glove and Indian jujube extracts; rZiz m 1–*E. coli* and rZiz m 1–*P* were consistent in all subjects. There was no difference regarding *in vitro* IgE-binding activity to rZiz m 1–*E. coli* and rZiz m 1–*P* by ELISA ($P = 0{\cdot}51$). However, the *in vivo* IgE reactivity determined by SPT showed significant reaction (2+~4+) to the rZiz m 1–*P* but gave only marginal readings (1+~2+) to the rZiz m 1–*E. coli* from allergic subjects ($P = 0{\cdot}01$). All the non-allergic subjects showed negative SPT results and no significant specific IgE binding activities to latex and Indian jujube extracts, rZiz m 1–*E. coli* and rZiz m 1–*P*.

Identification of IgE-binding epitopes using PCR-derived fragments

First, according to the prediction of domain linkers by amino acid composition (http://armadillo.blueprint.org), three gene segments (N1, C1 and C4) of Ziz m 1 were generated by PCR, but not to destroy the structure of protein domains. The preliminary results showed that two potential IgE-reactive regions were located within amino-acid residues 26–119 and 280–330 (data not shown). Following this, we began the process of systematically narrowing down the epitope sites. Finally, eight overlapping fragments of Ziz m 1 covering the whole allergen molecule, except the 25 residues of signal peptide, were generated by PCR (Table 1). The complete Ziz m 1 and the fragments were expressed as fusion proteins in *E. coli*. Affinity-purified recombinant proteins were subjected to sodium dodecy sulphate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane and assayed for IgE reactivity with allergic serum pool. Results are shown in Fig. 1. The mature Ziz m 1 (Ziz m 1: 26–330, 39·5 kDa), the N-terminus fragments N1 (Ziz m 1: 26–199), N2 (Ziz m 1: 26–105), N3 (Ziz m 1: 26–86), the C-terminus fragments C3 (Ziz m 1: 119–320) and C4 (Ziz m 1: 238–330) reacted with IgE antibodies. On the other hand, no detectable IgE-binding activity was found for fragments N4 (Ziz m 1: 26–71), C1 (Ziz m 1: 119–280) and C2 (Ziz m 1: 119–291). These results indicate that human linear IgE-binding epitopes of Ziz m 1 are located at amino acid sequences ^{72}NISGHCSDCTFLGEE^{86} and ^{292}VWNRYYDLKTNYSSSIILEYVNSGTKYLP^{320}. Results of immunoblotting are summarized, and the schematic map of the location of recombinant fragments on the Ziz m 1 molecule is presented in Fig. 2. Oligonucleotide primers and molecular weight deduced from amino acid sequences of recombinant fragments expressed in BL21 (DE3) are listed in Table 1.

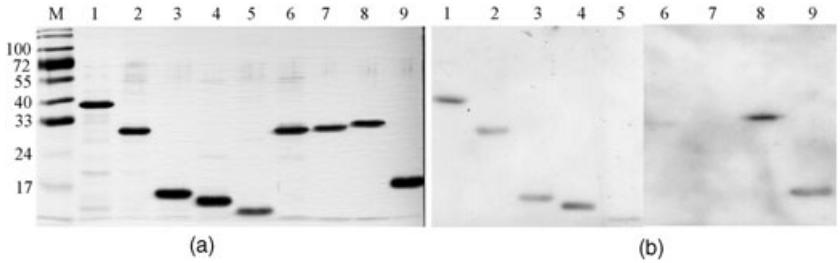

Fig. 1. Silver-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (a) and immunoblotting (b) of purified proteins expressed from deleted Ziz m 1 cDNA. Mature Ziz m 1: 26–330 (lane 1), N1 (Ziz m 1: 26–199, lane 2), N2 (Ziz m 1: 26–105, lane 3), N3 (Ziz m 1: 26–86, lane 4), N4 (Ziz m 1: 26–71, lane 5), C1 (Ziz m 1: 119–280, lane 6), C2 (Ziz m 1: 119–291, lane 7), C3 (Ziz m 1: 119–320, lane 8) and C4 (Ziz m 1: 238–330, lane 9). Numbers on the left indicate sizes (kDa) of protein marker (lane M). The non-specific weak band in lanes 5 and 6 of (b) may be due to the high sensitivity of chemiluminescent exposure.

Individual IgE-binding of the synthesized peptides by ELISA

We further synthesized four biotinylated peptides corresponding to two IgE-binding amino acid sequences of Ziz m 1. Sera from 10 latex–Indian jujube-allergic patients (P1-P10) and five non-allergic (NA1-NA5) subjects were used for analysing the IgE-binding activities to synthetic peptides by ELISA (Fig. 3). Subjects P1, P2, P4, P9 and P10 were designated as the severe allergic group and P3, P5, P6, P7 and P8 were designated as the mild allergic group. An OD level higher than 0·2 was considered positive. The sera from patients P1, P4, P5, P8 and P10 were able to bind all peptides tested, while IgE from subjects P3 and P7, P2 and P9 and P6 recognized 75%, 50% and 25% of peptides tested respectively. Results revealed heterogeneous IgE-binding

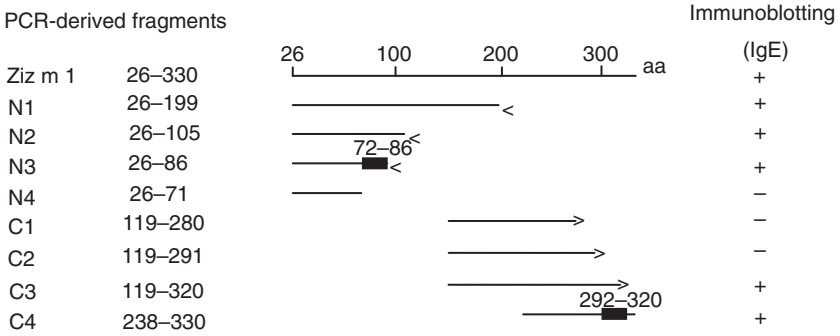

Fig. 2. B cell epitope mapping on Ziz m 1. Schematic map location of deletion mutants on the Ziz m 1. ■ represents the region recognized by human IgE. Arrows (< or >) represent the direction of deletion construction.

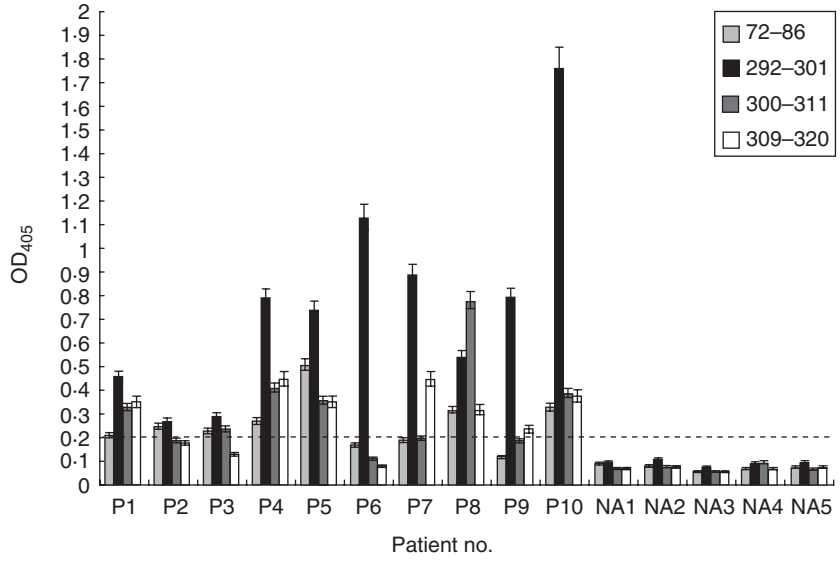

Fig. 3. Binding profiles of immunoglobulin E antibodies to synthetic peptides by enzyme-linked immunosorbent assay. Sera from 10 latex–Indian jujube-allergic subjects (P1–P10) and five from non-allergic subjects (NA1–NA5) were analysed. The cut-off value was 0·2, as shown by the dotted line.

Table 3. Immunoglobulin E (IgE)-binding capacity of Ziz m 1 synthetic peptides to latex- and Indian jujube-sensitive atopic sera.

	Serum number										
Peptide no.	1	2	3	4	5	6	7	8	9	10	% of IgE reactivity
^{72}NISGHCSDCTFIGEE^{86}	+	+	+	+	2+	−	−	+	−	+	70%
^{292}VWNRYYDLKT^{301}	+	+	+	2+	2+	3+	2+	2+	2+	4+	100%
^{300}KTNYSSSIILEY^{311}	+	−	+	+	+	−	+	2+	−	+	70%
^{309}LEYVNSGTKYLP^{320}	+	−	−	+	+	−	+	+	+	+	70%

An optical density (OD) level higher than 0.2 was considered positive. Responses were classified with respect to the OD values of peptides according to the following criteria: < 0.2 = −; 0.21–0.50 = +; 0.51–1.0 = 2+; 1.0–1.5 = 3+; > 1.5 = 4+.

patterns to synthetic peptides. No IgE-binding activity was detected in all non-allergic controls.

In addition, peptide ^{292}VWNRYYDLKT^{301} showed strong IgE reactivity to 100% of patient sera tested. Peptides ^{72}NISGHCSDCTE^{86}, ^{300}KTNYSSSIILEY^{311} and ^{309}LEYVNS GTKYLP^{320} bound 70% of the allergic sera tested. These results are summarized in Table 3. In addition, it was found that the presence of anti-peptide ^{292}VWNRYYDLKT^{301} IgE antibody in allergic subjects correlated significantly with the presence of allergic symptoms ($P < 0{\cdot}001$). The reactivities of the peptide-specific IgE antibodies are in concordance with the intergroup severity of allergic symptoms, as revealed by the analysis of variance (ANOVA) test ($P = 0{\cdot}024$). The number of IgE-binding peptides presented in the subjects had no significant correlation with clinical severity ($P = 0{\cdot}736$).

Discussion

Well-defined recombinant products have become valuable tools for diagnostic as well as therapeutic purposes in allergic diseases. Previously, we reported the cloning and expression of an important Indian jujube Ziz m 1 allergen in yeast *P. pastoris* [10]. In this report, rZiz m 1 was expressed in both *Pichia* and *E. coli*. *Pichia* and *E. coli* are currently the most commonly used expression systems for the production of recombinant allergens and have their own advantages and disadvantages [19]. For rZiz m 1, to reach the highest expression level (15 mg/l) requires 6 days in *Pichia*. However, only 16 h are required to produce as much as 28 mg/l recombinants in *E. coli*. We have demonstrated previously that the rZiz m 1–*P* is glycosylated and possesses chitinase activity [10], but the rZiz m 1–*E. coli* showed no chitinase activity (data not shown). In the present study, both rZiz m 1–*P* and rZiz m 1–*E. coli* bound comparable levels of serum IgE antibodies *in vitro* ($P = 0{\cdot}51$). Although long known to be potentially antigenic and be able to bind IgE, the clinical significance and ability to elicit an allergenic response of glycans is still a controversial subject [20,21]. It has been reported that the presence of the minimal core of N-linked glycans is required for the cross-linking of IgE antibodies on the surface of mast cells and basophils [22,23]. It is possible that the rZiz m 1–*E. coli* gave significantly poorer results ($P = 0{\cdot}01$) in *in vivo* IgE-binding (SPT) than rZiz m 1–*P* was due to the differences of protein glycosylation among the *E. coli* and yeast expression systems. However, further experiments are required to prove the hypothesis. Nevertheless, our data suggested that the rapid and excellent yields and lower cost compared with the yeast expression system gave the *E. coli* expression system great advantages in the development of *in vitro* diagnostic allergens.

In this study, we performed an IgE-binding epitope analysis of the major Indian jujube allergen Ziz m 1 using overlapping recombinant fragments. Although further study is needed to determine whether additional human linear IgE-binding epitopes of Ziz m 1 exist between positions 86–119 and 320–330, our results indicate that ^{72}NISGHCSDCTFL GEE^{86} and ^{292}VWNRYYDLKTNYSSSIILEYVNSGTKYLP^{320} of Ziz m 1 are the sequences required for human IgE binding. In addition, a short peptide, ^{292}VWNRYYDLKT^{301}, that bound 100% of the sera tested from latex–Indian jujube-allergic patients, seems sufficient to predict the presence of latex–Indian jujube-allergic reactions. However, we need more cases to validate the clinical significance.

Investigation of IgE-binding epitopes on allergen molecules is important for a clearer understanding of the allergen–antibody interaction and may contribute to the development of more effective strategies for immunotherapy. There is evidence that both linear and conformational epitopes are important in allergic reactions [24,25]. Different sensitization pathways may contribute to the different biochemical processing of the molecules at their first encounter with the immune system [26]. Inhalational allergens such as latex allergens may be able to penetrate the epithelial layer at full size, and these molecules are found to possess mainly conformational epitopes. In contrast, food allergens are degraded to small fragments during their passage through the digestive tract. Peptides as short as 6–10 amino acids in length were sufficient to bind IgE, as those being identified on major peanut allergen, Ara h 1 [27]. It has been reported that some peanut IgE-binding peptides are 'predictive peptides' that correlate with persistent symptomatic allergies, asymptomatic sensitization and outgrowth in the toleration of peanuts [28]. The number and diversity of binding epitopes is reported to correlate with the severity of clinical allergic symptoms to peanuts [29]. However, although binding to peptide ^{292}VWNRYYDLKT^{301} is present in all the latex–Indian jujube-allergic patients, the diversity and number of IgE-binding peptides to Ziz m 1 showed no

significant correlation with the severity of disease in our latex–Indian jujube-allergic patients.

Ziz m 1 was found to have significant sequence identify to latex hevamine (45·2%), which is an allergen, and many plant chitinases including class III chitinases of *Vigna angularis* (45·3%), *Capsicum annuum* (44·7%) and *Oryza sativa* (41·2%). Recently, a hevein-like domain of class I chitinases has been reported to be responsible for IgE-mediated *in vitro* and *in vivo* reactions in latex–fruit syndrome [30,31]. However, further sequence homology searches reveal no significant sequence homology between Ziz m 1 and Hev b 6·02 and Prs a 1 class I endochitinase from avocado (Z78202).

Many chitinases have been well documented, as found in a large variety of organisms, including bacteria, plants, insects and fungi [32]. More recently, it has become clear that mammals also contain such enzymes [33,34]. Acidic mammalian chitinase has been proved to be an important mediator of interleukin (IL)-13-induced responses in T helper 2 (Th2)-dominated asthma [35]. IL-13 is an immunoregulatory cytokine secreted predominantly by activated Th2 cells, and has been implicated in the pathogenesis of asthma in human and animal studies [36]. Ziz m 1 and the cross-reactivity among other chitinase families may represent important therapeutic targets for Th2 cytokine-mediated diseases. The cross-reactivity among allergens from different botanical families should be determined by investigating further the roles of these defined epitopes in the immune mechanism of the disease process.

In conclusion, our study identified two IgE binding regions of Ziz m 1 and found that peptide ^{292}VWNRYY DLKT^{301} was able to bind all sera of our latex–Indian jujube-allergic patients. Our findings are important in elucidating that the antigenic structures responsible for allergenicity to Indian jujube could thus contribute to the strategic development of more specific and potentially more efficacious immunotherapy.

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References

1 Turjanmaa K, Alenius H, Makinen-Kiljunen S, Reunala T, Palosuo T. Natural rubber latex allergy. Allergy 1996; **51**:593–602.

2 Charous BL, Blanco C, Tarlo S *et al.* Natural rubber latex allergy after 12 years: recommendations and perspectives. J Allergy Clin Immunol 2002; **109**:31–4.

3 Ahmed DD, Sobczak SC, Yunginger JW. Occupational allergies caused by latex. Immunol Allergy Clin North Am 2003; **23**:205–19.

4 Bousquet J, Flahault A, Vandenplas O *et al.* Natural rubber latex allergy among health care workers: a systematic review of the evidence. J Allergy Clin Immunol 2006; **118**:447–54.

5 Lai CC, Yan DC, Yu J, Chou CC, Chiang BL, Hsieh KH. Latex allergy in hospital employees. J Formos Med 1997; **96**:266–71.

6 Chen YH, Lan JL. Latex allergy and latex–fruit syndrome among medical workers in Taiwan. J Formos Med 2002; **101**:622–6.

7 Wagner S, Breiteneder H. *Hevea brasiliensis* latex allergens: current panel and clinical relevance. Int Arch Allergy Immunol 2005; **136**:90–7.

8 Brehler R, Theissen U, Mohr C, Luger T. 'Latex–fruit syndrome': frequency of cross-reacting IgE antibodies. Allergy 1997; **52**:404–10.

9 Lee MF, Chen YH, Lan JL, Tseng CY, Wu CH. Allergenic components of Indian jujube (*Zizyphus mauritiana*) show IgE cross-reactivity with latex allergen. Int Arch Allergy Immunol 2004; **133**:211–6.

10 Lee MF, Hwang GY, Chen YH, Lin HC, Wu CH. Molecular cloning of Indian jujube (*Zizyphus mauritiana*) allergen Ziz m 1 with sequence similarity to plant class III chitinases. Mol Immunol 2006; **43**:1144–51.

11 Jekel PA, Hartmann BH, Beintema JJ. The primary structure of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex. Eur J Biochem 1991; **200**:123–30.

12 Alenius H, Kalkkinen N, Lukka M *et al.* Prohevein from the rubber tree (*Hevea brasiliensis*) is a major latex allergen. Clin Exp Allergy 1995; **25**:659–65.

13 Lee MF, Chen YH, Lin HC, Wang HL, Hwang GY, Wu CH. Identification of hevamine and Hev b 1 as major latex allergens in Taiwan. Int Arch Allergy Immunol 2006; **139**:38–44.

14 Ferreira F, Hawranek T, Gruber P, Wopfner N, Mari A. Allergic cross-reactivity: from gene to the clinic. Allergy 2004; **59**:243–67.

15 Wu CH, Lee MF, Tseng CY. IgE-binding epitopes of the American cockroach Per a 3 allergen. Allergy 2003; **58**:986–92.

16 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; **227**:680–5.

17 Towbin H, Staehelin T, Gordon J. Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; **76**:4350–4.

18 Wu CH, Lee MF, Yang JS, Tseng CY. IgE-binding epitopes of the American cockroach Per a 1 allergen. Mol Immunol 2002; **39**:459–64.

19 Schmidt M, Hoffman DR. Expression systems for production of recombinant allergens. Int Arch Allergy Immunol 2002; **128**:264–70.

20 Van der Veen MJ, van Ree R, Aalberse RC *et al.* Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. J Allergy Clin Immunol 1997; **103**:327–34.

21 Mari A, Iacovacci P, Afferni C *et al.* Specific IgE to cross-reactive carbohydrate determinants strongly affects the *in vitro* diagnosis of allergic diseases. J Allergy Clin Immunol 1999; **103**:1005–11.

22 Batanero E, Villalba E, Rodriguez R. Glycosylation site of the major allergen from olive tree pollen: allergenic implications of the carbohydrate moiety. Mol Immunol 1994; **31**:31–7.

23 Batanero E, Crespo JF, Monsalve RI, Martin-Esteban M, Villalba M, Rodriguez R. IgE-binding and histamine-release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen, Ole e 1. J Allergy Clin Immunol 1999; **103**:147–53.

24 Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 2006; **6**:761–71.
25 Valenta R, Niederberger V. Recombinant allergens for immunotherapy. J Allergy Clin Immunol 2007; **119**:826–30.
26 Chardin H, Raulf-Heimsoth M, Chen Z *et al.* Interest of two-dimensional electrophoretic analysis for the characterization of the individual sensitization to latex allergens. Int Arch Allergy Immunol 2002; **128**:195–203.
27 Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon GA. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. Eur J Biochem 1997; **245**:334–9.
28 Beyer K, Ellman-Grunther L, Järvinen KM, Wood RA, Hourihane J, Sampson HA. Measurement of peptide-specific IgE as an additional tool in identifying patients with clinical reactivity to peanuts. J Allergy Clin Immunol 2003; **112**:202–7.
29 Shreffler WG, Beyer K, Chu TT, Burks AW, Sampson HA. Microarray immunoassay: association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. J Allergy Clin Immunol 2004; **113**:776–82.
30 Karisola P, Kotovuori A, Poikonen S *et al.* Isolated hevein-like domains, not 31-kd endochitinases, are responsible for IgE-mediated *in vitro* and *in vivo* reactions in latex–fruit syndrome. J Allergy Clin Immunol 2005; **115**:598–605.
31 Diaz-Perales A, Blanco C, Sanchez-Monge R, Varela J, Carrillo T, Salcedo G. Analysis of avocado allergen (Prs a 1) IgE-binding peptides generated by stimulated gastric fluid digestion. J Allergy Clin Immunol 2003; **112**:1002–7.
32 Cohen-Kupiec R, Chet I. The molecular biology of chitin digestion. Curr Opin Biotechnol 1998; **9**:270–7.
33 Reknema GH, Boot RG, Muijsers AO, Donker-Koopman WE, Aerts JM. Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. J Biol Chem 1995; **270**:2198–202.
34 Boot RG, Blommaart EF, Swart E *et al.* Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. J Biol Chem 2001; **276**:6770–8.
35 Zhu Z, Zheng T, Homer RJ *et al.* Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 2004; **304**:1678–82.
36 Daines MO, Chen W, Tabata Y *et al.* Allergen-dependent solubilization of IL-13 receptor α2 reveals a novel mechanism to regulate allergy. J Allergy Clin Immunol 2007; **119**:375–83.

