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Molecular cloning of Indian jujube (*Zizyphus mauritiana*) allergen Ziz m 1 with sequence similarity to plant class III chitinases

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Abstract

Indian jujube (*Zizyphus mauritiana*) is a sweet fruit that is abundantly cultivated in Taiwan. We have previously identified 42 and 30 kDa allergens that are cross-reactive with latex allergen from crude Indian jujube extract. This study aimed to clone the 30 kDa Ziz m 1 *Z. mauritiana* allergen. The Ziz m 1 encoding cDNA was isolated from a ZAPII cDNA library constructed from *Z. mauritiana* mRNA, sequenced and expressed in *Pichia pastoris*. The protein predicted from the cDNA sequence has 330 amino acids, the first 25 of which constituted a putative signal peptide. The deduced molecular mass of the mature protein is 33.86 kDa, while its isoelectric point is estimated at 4.36. The recombinant Ziz m 1 showed chitinase activity, possessed IgE binding capacity, and had IgE cross-reactivity with the latex allergen. Moreover, anti-recombinant Ziz m 1 antibody-based ELISA was able to detect commercial skin testing latex reagent, laboratory prepared latex and Indian jujube extracts. Recombinant Ziz m 1 showed 87.5% skin reactivities on eight latex- and Indian jujube-sensitive subjects. Although no sequence similarity was found to other known allergens, Ziz m 1 was found to have amino acid sequence identity (39–45.3%) to many plant chitinases including chitinase (45.2%) of *Hevea brasiliensis* (hevamine), class III chitinases of *Vigna angularis* (45.3%), *Capsicum annuum* (44.7%) and *Oryza sativa* (41.2%). A conserved domain search revealed that Ziz m 1 belongs to the family 18 glycosyl hydrolases. The recombinant allergen may therefore be of value for diagnosis and therapeutic purposes, and the further characterization of Indian jujube allergen may help to elucidate the mechanism underlying latex–fruit syndrome.

Keywords: Cloning; Recombinant Indian jujube Ziz m 1 allergen; Class III plant chitinases; Cross-reactivity; Latex–fruit syndrome

1. Introduction

Approximately 50% of patients who are allergic to latex (*Hevea brasiliensis*) have evidence of concomitant food allergies (Condemi, 2002; Wanger and Breiteneder, 2002), while fruit-allergic patients show 86% (49/57) sensitization to latex in one study (Garcia-Ortiz et al., 1998). This type of cross-reactivity is known as the latex–fruit syndrome (Blanco et al., 1994; Brehler et al., 1997). A prevalence of latex allergy among medical workers of 6.8–8.6% had been reported in Taiwan (Lai et al., 1997; Chen and Lan, 2002). Indian jujube (*Zizyphus mauritiana*), a native species of Yunnan province in southern China, is a sweet fruit growing in a warm climate and is abundantly cultivated in southern Taiwan. It is becoming an increasingly popular fruit during its season, late autumn. Recently, we identified 30 and 42 kDa allergens from Indian jujube and 42 kDa allergen from latex using patients' IgE by immunoblotting (Lee et al., 2004). The homologous and heterologous inhibition studies demonstrated marked inhibition using crude extracts as inhibitors as defined by immunoblot and enzyme-linked immunosorbent assay (ELISA) inhibitions. Moreover, anti-Indian jujube protein antibody-based ELISA was able to detect latex extracts.

Here, we report the cloning and purification of a 33.86 kDa Indian jujube glycoprotin that is immunoreactive with IgE antibody and has a sequence similarity to that of plant class III chitinases. This protein, according to World

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Health Organization (WHO) and the International Union of Immunological Societies (IUIS) nomenclature, is designated as Ziz m 1. Recombinant Ziz m 1 (rZiz m 1) was produced in methylotrophic yeast *Pichia pastoris* and displayed chitinase activity. Skin prick test (SPT) was performed using the natural and recombinant proteins, and IgE-binding capacity was tested by ELISA with sera from latex- and Indian jujube-sensitive subjects.

2. Materials and methods

2.1. Crude Indian jujube and ammoniated latex extracts, SPT and serum samples

Preparation of crude Indian jujube and ammoniated natural rubber latex (*H. brasiliensis*) extracts were performed as previously described (Lee et al., 2004). Commercial latex extract (100 IR/ml, Stallergenes SA, France), extracts of Indian jujube and latex prepared in the laboratory, and purified recombinant protein at a concentration of 100 μg/ml in phosphate buffered saline (PBS) containing 50% glycerol were used for skin testing. SPT was performed, and reaction was read and graded against controls as previously described (Lee et al., 2004). Eight sera were obtained from Indian jujube-sensitive patients, and an equal volume of serum from latex and Indian jujube allergic patients were pooled for immunoblotting studies.

2.2. Allergen specific IgE

Diagnostic measurements of allergen specific IgE were performed with the CAP-FEIA system (Pharmacia Diagnostic, Sweden). Specific IgE to laboratory prepared latex and Indian jujube extracts (3.0 μg/well) were measured by using a commercial ELISA kit according to the manufacturer's instructions (Allergopharma, Reinbek, Germany).

2.3. Anion-exchange chromatography

Crude Indian jujube extracts was purified by anion-exchange chromatography with RESOURCE Q column (Pharmacia) using a FPLC system (Pharmacia) according to the methods recommended by the manufacturer.

2.4. Two-dimensional gel electrophoresis (2D) and Western blot analysis

Partially purified Indian jujube and latex proteins were subjected to 2D under denatured conditions. The first dimension of 2D was performed with Bio-Rad protean II xi 2D cell system (Bio-Rad) according to the methods recommended by the manufacturer. The pH gradient (Bio-Rad) was 3–10, and the total volt-hour was 14,200. The second dimension standard SDS-PAGE under denatured conditions was performed with a discontinuous buffer, and proteins were transferred onto PVDF membranes and immunodetected according to the method already described (Lee et al., 2004).

2.5. Mass spectrometry

Proteins were visualized with silver stain after 2D, and the target protein spots were excised for in-gel digestion using a sequencing-grade modified trypsin (Promega, Madison, WI). The tryptic peptide mixtures were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS, Finnigan MAT, San Jose, CA), and search with a Mascot computer program (Matrix Science).

2.6. Extraction and isolation of mRNA from Indian jujube

Total RNA was extracted from fresh buds of Indian jujube tree with the concert plant RNA reagent kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Poly $(A)^+$ RNA was purified by oligo-(dT) cellulose chromatography (Ambion, Inc., Austin, TX).

2.7. Construction and screening of Indian jujube cDNA library

Indian jujube cDNA library was prepared from 5.0 μg of mRNA with the cDNA synthesis kit (Stratagene, La Jolla, CA), ligated into the λZAPII vector with *Eco*RI and *Xho*I linkers, and packaged in vitro by using the Gigapack III Gold packaging system (Stratagene). The cDNA library was plated on Luria-Bertani agar and screened with Indian jujube-sensitive patients' sera (1:10) that had been pre-absorbed with *E. coli* lysate. Plaques producing positive signals were identified and further screened against Indian jujube-sensitive sera or non-atopic control sera by plaque immunoassay. The sizes of insert of the positive clones were amplified by polymerase chain reaction (PCR) using λ forward (T3: AATTAACCCTCACTAAAGGG) and reverse primers (T7: GTAATACGACTCACTATAGGGC) and analyzed by agarose gel electrophoresis. PCR was performed with 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C for 35 cycles in a 50 μl volume. An initial 5 min incubation step at 99 °C was performed, and final extension was 6 min at 72 °C. The isolated PCR products were cloned into pCR2.1 (Invitrogen) and sequenced.

2.8. DNA sequencing

Sequence analysis was performed using the ABI PRISM Dye Terminator Kit and ABI 377 sequencer (Perkin-Elmer Biosystem, Warrington, UK). Both strands were analyzed to yield the final sequence of the amplified fragment.

2.9. Computer analysis and search for sequence homology

Analysis of the signal peptide and the potential *N*-glycosylation site were performed using the SignaIP 3.0

(Bendtsen et al., 2004) and NetNGlyc 1.0 of ExPASy Proteomics Server. Sequence comparisons and homology searches were performed using the NCBI BLASTP 2.2.10. ClustalW (1.82) alignments were performed using EMBI-EBI server.

2.10. Expression of the recombinant Ziz m 1 in the yeast P. pastoris

The lambda cDNA coding region of Ziz m 1 was amplified by PCR using the following primers: sense, 5′-AGA*GAATTC*ATGGTTCCACAAGCCAAAC-3′; antisense, 5′-*GCGGCCGC*TTATGCATTCTGATACATA (priming regions underlined, *Eco*RI and *Not*I sites in italics) and Taq polymerase (Invitrogen). The primers were designed to recreate the *Eco*RI/*Not*I restriction sites to ligate the DNA fragment into the pPIC9K vector (Invitrogen) and transformed into *E. coli* TOP10 F′ and sequenced to confirm the identity of the insert. After linearization of the pPIC9K/Ziz m 1 DNA with *Sal*I, the transformation of the *P. pastoris strain* KM71 (Invitrogen), screening of recombinant of Ziz m1-producing clones, and extracellular expression were performed according to the instruction manual.

2.11. Purification of rZiz m 1 allergen

The culture supernatants of *P. pastoris* clones producing rZiz m 1 were separated from the cells by centrifugation at 4000 × *g* for 10 min and filtered through a 0.2 μm filter. The secreted proteins were then concentrated and 20 mM Tris–Cl, pH 8.0 buffer added using Amicon Ultra centrifugal filter devices with an exclusion size of 30 kDa (Millipore, Bedford, MA) and purified by RESOURCE Q column as described above.

2.12. Chitinase activity assay

Colloidal chitin was prepared from crab shell chitin (Sigma, St. Louis, MO), and the chitinase activity was performed using colorimetric method according to the method of Sheng et al. (2002) with minor modification. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmole GlcNAc/min at pH 8.0 and 60 °C.

2.13. Inhibition studies

Three micrograms of partially purified Ziz m 1 or latex extract was subjected to SDS-PAGE. In immunoblot inhibition, sera were mixed with an equal volume of recombinant bovine serum albumin (rBSA) produced by *P. pastoris* (Invitrogen), or crude latex extract or rZiz m 1 (150 μg/ml) at 4 °C overnight, and immunoblotting was then performed as described above. ELISA inhibition assay was performed as previously described (Lee et al., 2004). Briefly, after coating with 100 μl of 30 μg/ml rZiz m 1, wells were incubated with a 1:10 dilution of serum which had been preincubated overnight at 4 °C with different concentration of Ziz m 1 or Indian jujube extract or latex extract (range, 1–20 μg/ml). Preincubation of inhibitor with rBSA was used as the negative control. Results were expressed as the percent inhibition of the reaction of antibody without any inhibitor.

2.14. Anti-rZiz m 1 antibody and anti-rZiz m 1 antibody-based ELISA

Polyclonal antibody against rZiz m 1 was raised in rabbits (Lee et al., 2004), and antisera were collected and purified by Protein A-agarose (Bio-Rad) affinity chromatography. The reactivities of proteins were evaluated in a direct binding ELISA as previously described (Lee et al., 2004). Briefly, microtiter plates were coated with 3.0 μg/well of rZiz m 1, a commercial skin testing latex (Stallergenes SA), latex and Indian jujube extracts or unimmunized rabbit IgG in triplicate. Anti-rZiz m 1 antibody (5.0 mg/ml, 1:5000) was added, and antibody binding and the optical density were determined at 415 nm.

3. Results

3.1. 2D and ESI-MS/MS

Molecular masses of the partially purified natural Indian jujube and ammoniated latex allergens are around 30 kDa with an isoelectric point (p*I*) value of 4.5 and 42 kDa with p*I* of 5.2, respectively, as revealed by 2D (data not shown). The stable signals at 1054.3 and 1054.4 *m*/*z* obtained from

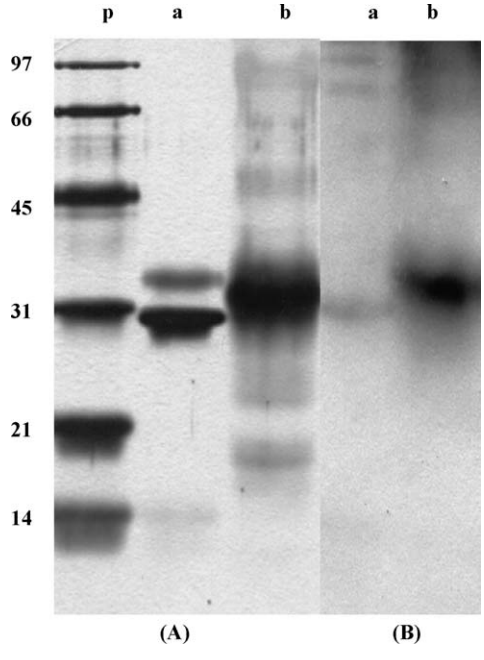

Fig. 1. Silver-stained SDS-PAGE (A) and immunoblotting (B) of partially purified natural Ziz m 1 (lane a) and rZiz m 1 (lane b). Numbers at left indicate size of standard proteins (lane p) in kDa.

tryptic peptide mixture of the 30 kDa allergen were analyzed by ESI-MS/MS and identified as the tryptic peptide ^{273}YGGIMLWSK^{282} from the class III chitinases (31.6 kDa, p*I* 4.80) of *Oryza sativa* (GenBank GI: 50936023). By using the same technique, nine tryptic peptides, ranging from 601.6 to 1727.5 *m*/*z*, obtained from the 42 kDa latex allergen exhibited sequence identity with latex Hev b 7 (42.8 kDa, p*I* 5.0; GenBank GI: 6707018) allergen, and the identified peptides overlap with 33% of the sequence of Hev b 7.

3.2. Isolation of Ziz m 1 cDNA and immunodetection of recombinant protein

A ZAPII cDNA library derived from Indian jujube mRNA was screened with human IgE antibodies. Ten immunoreactive clones containing inserts ranging from around 0.15 to 1.39 kilobases (kb) were identified (data not shown). Subsequently, the 1.39 kb cDNA clone (Ziz m 1) was expressed in *P. pastoris*. The highest expression level was observed at day 6 after induction, and a yield of approximately 15.0 mg of the recombinant allergen was obtained per liter of culture medium. Purified recombinant protein yielded a prominent band of 30 kDa (Fig. 1A, lane b), and migration was similar to that of natural Ziz m 1 (Fig. 1A, lane a). IgE-binding capacity of rZiz m 1 and natural Ziz m 1 was demonstrated in Fig. 1B.

3.3. Nucleotide and amino acid sequences of Ziz m 1

The cDNA and deduced amino acid sequences are presented in Fig. 2. The cDNA comprised 1223 base pairs with an open reading frame that encodes a protein of 330 amino acids including a leader sequence of 25 residues. The deduced

```
  ggcacgaggcctcgtgccggacgaggtaaaaaccc**gcagatag**tattaaaaaaggggggaaaaaaatggttcca   73
                                                                **M  V  P**     3
caagccaaacttgttgtggcttctctgatcctcacctcagccttaatccaaacctctgaggctgtaggtggcata  148
**Q  A  K  L  V  V  A  S  L  I  L  T  S  A  L  I  Q  T  S  E  A  V**  G  G  I    28

gcaacctactggggtcaatatactgagaccgaagaagggagtctagccgaagcctgtgctagtaatttgtattct  223
A  T  Y  W  G  Q  Y  T  E  T  E  E  G  S  L  A  E  A  C  A  S  N  L  Y  S    53

tacataaacatagcctatcttaacattttcggcgaaggccggtacctgagtttaaacatctctggtcactgcagt  298
Y  I  N  I  A  Y  L  N  I  F  G  E  G  R  Y  L  S  L  N  I  S  G  H  C  S    78

cattgcacctttctcggcgaagagataaaggcttgtcagagccagggcgttaagatcttcctctctcttggagga  373
D  C  T  F  L  G  E  E  I  K  A  C  Q  S  Q  G  V  K  I  F  L  S  L  G  G   103

ccttatggagattatcatctaacaaccgatggggatgccgatagggttgcagagcaactgtggagttcctttctc  448
P  Y  G  D  Y  H  L  T  T  D  G  D  A  D  R  V  A  E  Q  L  W  S  S  F  L   128

ggaggttctaaaagtactggagtttatcaaccacttctcggagacgttgaactcgatggcatagatttggacata  523
G  G  S  K  S  T  G  V  Y  Q  P  L  L  G  D  V  E  L  D  G  I  D  L  D  I   153

caaattggtcccccagaagagtatgacgttctggcacgtaacttgaaggacttaaccaaagatcgaacaagacct  598
Q  I  G  P  P  E  E  Y  D  V  L  A  R  N  L  K  D  L  T  K  D  R  T  R  P   178

ttctatttatctgcagctcccaagtgttctgcttataatgatagcgacgcttatctttggacagccgttgagacg  673
F  Y  L  S  A  A  P  K  C  S  A  Y  N  D  S  D  A  Y  L  W  T  A  V  E  T   203

gggcttttcgacttcgtttgggttaagttctataatgatacttcatgccagtacaataatgacacagctgctggt  748
G  L  F  D  F  V  W  V  K  F  Y  N  D  T  S  C  Q  Y  N  N  D  T  A  A  G   228

cttgacgctttttaccgttcgtggtatgattggactgtgtcgcttgcggaagggaataagttgctcatagggata  823
L  D  A  F  Y  R  S  W  Y  D  W  T  V  S  L  A  E  G  N  K  L  L  I  G  I   253

ccggcttccaatgaaactgataattccccactcggcggctacatcccgtctgatgtgctgaacgatcaaattgtt  898
P  A  S  N  E  T  D  N  S  P  L  G  G  Y  I  P  S  D  V  L  N  D  Q  I  V   278

tcggtgataatgacgtcgtccaagtttggaggtgtaaacgtatggaataggtattatgatcttaagacgaattac  973
S  V  I  M  T  S  S  K  F  G  G  V  N  V  W  N  R  Y  Y  D  L  K  T  N  Y   303

tcttcctccattattctcgagtatgttaattcgggaaccaagtatttaccattacggactaaatttatgtatcag 1048
S  S  S  I  I  L  E  Y  V  N  S  G  T  K  Y  L  P  L  R  T  K  F  M  Y  Q   328

aatgca**taa**tccataacgcaccattaccaactatgtatgagtgatttgtatccttgaatatatatatatatat 1123
N  A  *                                                                      330

atatatgtatccactgctacataaatggggtaccactatgtatcactcttgttta**aataaa**caatggtgtgaaat 1198
gtctaaaaaaaaaaaaaaaaaaaaa                                                   1223
```

Fig. 2. The nucleotide and deduced amino acid sequence of Ziz m 1. The putative TATA-box, the initiation methionine codon and the potential *N*-glycosylation sites are underlined. The putative cap signal, the putative signal peptide, the stop codon and polyadenylation site are indicated by bold print. The nucleotide sequence reported in this paper has been deposited in GenBank database with accession number AY839230.

```
Ziz m 1 -MVPQAKLVVASLILTSALIQTS--EAVGGIATYWGQYTETEEGSLAEACASNLYSYINI  57
Hev b   --MAKRTQAILLLLLAISLIMSSSHVDGGGIAIYWGQ--NGNEGTLTQTCSTRKYSYVNI  56
Cap a   ----MTINLLLPSILFLALIQTS--IARSGIAIYWGQ--NGNEATLNDTCASGNYAYVNL  52
Vig a   MACLKQVSALLLPLLFISFFKPS---HAGGISVYWGQ--NGNEGSLADACNTGNYKYVNI  55
Ory s   MANKSSLLQLLLIAAVASQFVSS---QAGSIAIYWGQ--NNGEGTLADTCATGNYKFVNI  55
                  :       : : .*     ..*: **** :   *.:* ::* :  * ::*:

Ziz m 1 AYLNIFGEGRYLSLNISGHCS----DCTFLGEEIKACQSQGVKIFLSLGGPYGDYHLTTD 113
Hev b   AFLNKFGNGQTPQINLAGHCNPAAGGCTIVSNGIRSCQIQGIKVMLSLGGGIGSYTLASQ 116
Cap a   SFLNKFGNGQTPEINLAGHCNPAVNGCTILGPQIKFCQKLGVKVMLSMGGGVGNYSLASK 112
Vig a   AFLFTFGGGQTPQLNLAGHCNPSINNCNVFSDQIKECQSKDIKVLLSLGGASGSYSLTSA 115
Ory s   AFLAAFGNGQPPVFNLAGHCDPTNGGCASQSSDIKSCQSRGVKIMLSIGGGAGSYYLSSS 115
        ::*  ** *:   :*::***.    .*    .  *: **  .:*::**:**   *.* *::

Ziz m 1 GDADRVAEQLWSSFLGGSKSTGVYQPLLGDVELDGIDLDIQIGPPEEYDVLARNLKDLTK 173
Hev b   ADAKNVADYLWNNFLGGKSSS----RPLGDAV[LDGIDFDIE]HGSTLYWDDLARYLSAYSK 172
Cap a   KDAKDVARYLYNNFLGGRSSF----RPLGNAR[LDGIDFDIE]LGSSLYYEDLAQYLKRYSK 168
Vig a   DDATQVANYIWNNFLGGQSSS----RPLGDAI[LDGVDFDIE]SGTGEHWDDLARALKGFNS 171
Ory s   EDAKNVATYLWNNFLGGQSSS----RPLGDAV[LDGIDFDIE]GGTNQHWDDLARYLKGYSN 171
         **  **   ::..**** .*       **:. ***:*:**: *.    :: **: *.  ..

Ziz m 1 DRTRPFYLSAAPKCSAYNDSDAYLWTAVETGLFDFVWVKFYNDTSCQYNNDTAAGLDAFY 233
Hev b   Q-GKKVYLTAAPQCPFP---DRYLGTALNTGLFDYVWVQFYNNPPCQYS---SGNINNII 225
Cap a   L-GRKMYLTAAPQCPFP---DRLLGTALNTGLFDNVWIQFYNNPSCQYT---TNNVDDLK 221
Vig a   ----QLLLTAAPQCPIP---DAHLDTAIKTGLFDIVWVQFYNNPPCQYS---SGNTNDLI 221
Ory s   S-GRRVYLTAAPQCPFP---DACIGDALNTGLFDYVWVQFYNNPPCQYS---SGSTSNLA 224
              . *:***:*.    *   :  *::***** **::***:..***.    : . . :

Ziz m 1 RSWYDWTVSLAEGNKLLIGIPASNETDNSPLGGYIPSDVLNDQIVSVIMTSSKFGGVNVW 293
Hev b   NSWNRWTTS-INAGKIFLGLPAAPEAAGS---GYVPPDVLISRILPEIKKSPKYGGVMLW 281
Cap a   NSWTRWTTS-VNARRIFLGLPAAPQAAGS---GFIPAEVLTGGILPVIKKSRKYGGVMLW 277
Vig a   SSWNQWTS--SQAKQLFLGVPASTAAAGS---GFIPADVLTSQVLPTIKGSSKYGGVMLW 276
Ory s   DAWKQWLS--VPAKQIFLGLPASPQAAGS---GFIPADDLKSQVLPVIKSSGKYGGIMLW 279
         :*  *      . ::::*:**:   : .*   *::*.: * . ::.  *  * *:**: :*

Ziz m 1 NRYYDLKTNYSSSIILEYVNSGTKYLPLRTKFMYQNA 330
Hev b   SKFYDDKNGYSSSILDSVLFLHSEECMTVL------- 311
Cap a   SKFWDEQTGYSASIVKSV------------------- 295
Vig a   DRFNDGQSGYSGAIIGSV------------------- 294
Ory s   SKYYDDQDDYSSSVKSDV------------------- 297
        ::: * : .**.::  .
```

Fig. 3. Comparison of Ziz m 1 with the sequences of chitinase from *Hevea brasiliensis* (Hev b), and chitinase III from *Capsicum annuum* (Cap a), *Vigna angularis* (Vig a) and *Oryza sativa* (Ory s). The motifs of family 18 glycosyl hydrolase are indicated by bold print. (*), (:) and (.) indicate that a position in alignment is perfectly, conserved and semi-conserved, respectively.

amino acid sequence of Ziz m 1 contains five potential *N*-glycosylation sites. The calculated molecular mass of mature protein (excluding leader sequences) is 33.86 kDa with a p*I* value of 4.36. The discrepancy between the predicted and observed molecular mass may have been due to glycosylation. A computer-assisted homology search revealed that Ziz m 1 sequence has significant degrees of identity with many plant chitinase proteins (39–45%) including chitinase (hevamine) from *H. brasiliensis* (rubber tree, 45.2%), class III chitinases from *Vigna angularis* (bean, 45.3%), *Capsicum annuum* (pepper, 44.7%) and *O. sativa* (rice, 41.2%), and all of these sequences contain a motif of the family 18 glycosyl hydrolases. These results are in concordance with our ESI-MS/MS finding. Alignments of these representative amino acid sequences are presented in Fig. 3. Moreover, a computer-assisted conserved domain search (NCBI, RPS-BLAST 2.2.9) revealed that Ziz m 1 belongs to the family 18 glycosyl hydrolases.

3.4. Chitinase activity of rZiz m 1

The specific chitinase activity of purified rZiz m 1 determined by colorimetric method was 1.185 ± 0.145 U/mg protein. This value was higher than specific activity of natural Ziz m 1 which displayed only 0.16 U/mg protein.

3.5. Inhibition studies

No inhibition of IgE binding to 42 kDa latex allergen was observed when purified rZiz m 1 was used as inhibitor as defined by immunoblot inhibition (data not shown). However, when using the crude latex extract or rZiz m 1 as an inhibitor, a weakening of IgE binding to the purified natural Ziz m 1 (Fig. 4, lanes b and c) was observed. To confirm the immunoblot inhibition, ELISA inhibition studies were performed using three selected patients. Preincubation of natural Ziz m 1 or latex extract with all of the atopic sera resulted in marked inhibition of IgE binding to rZiz m 1 (Fig. 5). No significant inhibition was observed when rBSA was used as inhibitor.

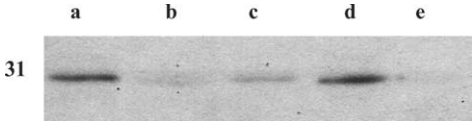

Fig. 4. Immunoblot inhibition. Binding of IgE from latex and Indian jujube-sensitive subjects to partially purified natural Ziz m 1, inhibited by 150 μg/ml proteins from rZiz m 1 (lane b) or latex extract (lane c) or rBSA (lane d). Lane a, no inhibitor and lane e, non-allergic serum. Number at left indicates size of standard proteins in kDa.

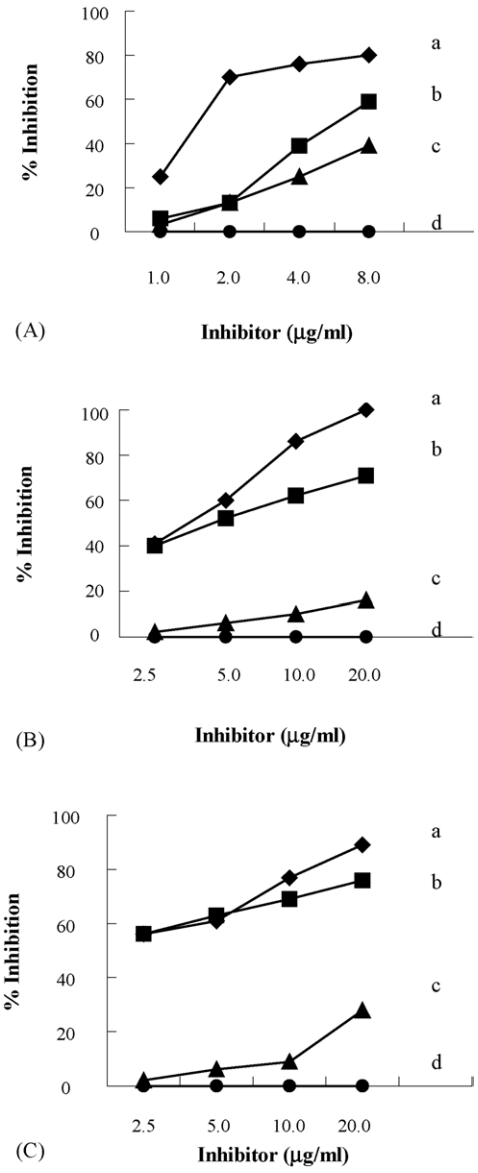

Fig. 5. Inhibition of binding of IgE from patients two (A), six (B) and seven (C) to rZiz m 1 by ELISA. Inhibitors: (a) partial purified natural Ziz m 1; (b) Indian jujube extract; (c) latex extract and (d) rBSA.

3.6. Detection of extracts by anti-rZiz m 1 antibody

A polyclonal antibody-based ELISA using anti-rZiz m 1 was performed for the laboratory prepared latex extract, commercial latex skin test reagent proteins and Indian jujube extract. The response of rZiz m 1 to anti-rZiz m 1 was dose dependent, and no rZiz m 1 protein was detected when preimmunized rabbit serum was substituted for anti-rZiz m 1 (data not shown). The optical densities at 415 nm for rZiz m 1, Indian jujube extract, latex extract, commercial skin testing latex reagent, and unimmunized rabbit IgG were 2.41, 1.14, 0.64, 0.93 and 0.032, respectively (the average of experiments performed in triplicate).

Table 1
Skin test results of the latex–Indian jujube allergic patients

Patient	Age/sex	Diagnosis[a]	SPT (kU/l)		
			Latex[b]	Indian jujube extract[c]	rZiz m 1[c]
1	42/F	AE, OAS	+++/1.48	++++/2.50	−/<0.35
2	31/F	A, OAS	++++/27.4	++++/16.6	+++/6.65
3	22/F	U	++/2.30	+++/25.0	+++/25.3
4	27/F	OAS	++/0.66	++/0.60	++/0.87
5	32/F	OAS	++++/20.2	++/5.62	++++/9.32
6	26/M	A, AD	+++/25.5	++/15.2	++++/10.3
7	76/M	AD	+/0.68	++/3.28	+++/14.3
8	42/M	OAS	+++/12.9	++++/10.3	++/3.61

[a] U, urticaria; AE, angioedema; A, asthma; OAS, oral allergy syndrome; AD, atopic dermatitis.
[b] CAP-FEIA system.
[c] ELISA.

3.7. Allergy skin test and IgE antibodies

Skin tests were performed on eight latex and Indian jujube-sensitive subjects with recombinant protein, and rZiz m 1 showed positive skin reactions in 87.5% (7 of 8). Ten of the non-allergic subjects showed negative skin reaction. Latex-, Indian jujube extract- and rZiz m 1-specific IgEs were also determined either by CAP-FEIA or ELISA in these eight atopic subjects. All patients were found to contain specific IgE to latex, Indian jujube extract and rZiz m 1 except one patient, ranging from 0.66 to 27.4, 0.60 to 25.0 and 0.87 to 25.3 kU/l, respectively. Patient 1 contains specific IgE to latex and Indian jujube extract, but contains no specific IgE (<0.35 kU/l) and negative skin reaction to rZiz m 1. The results are summarized in Table 1.

4. Discussion

In our previous study, only one prominent 42 kDa allergen was identified from ammoniated latex extract, and immunoblot and ELISA inhibition studies showed marked inhibition of IgE-binding to 30 kDa Indian jujube allergen when crude latex extract was used as an inhibitor (Lee et al., 2004). In the present study, no inhibition of IgE binding to 42 kDa latex allergen was observed when rZiz m 1 was used as inhibitor. It appears that Ziz m 1 cross-reacted with protein(s) of the crude latex extract that was not identified in our previous report.

Recombinant Ziz m 1 showed chitinase activity: a homology search revealed that Ziz m 1 showed 39–45% sequence identity to many plant class III chitinases including *H. brasiliensis* (hevamine) and belongs to the family 18 hydrolases. All family 18 class III chitinases share a 'chitinases-family-2' motif of [LIMFY]-[DN]-G-[LIVMF]-[DN]-x-E (Alvarado et al., 2002). The sequence of Ziz m 1 showed only one difference with respect to this motif present at amino acid position 154, substituting a glutamine for a glutamic acid (^{146}LDGIDLDIQ^{154}). These results are consistent with our

ESI-MS/MS finding. Hevamine is also a class III chitinase with molecular mass of 30 kDa and an essential rubber tree protein (Henrissat, 1991), and the purified hevamine bound IgE from only 1/29 latex-allergic sera tested in one study (Hoffmann-Sommergruber, 2000). Recently, a brief report on one case of anaphylactic reactions to azufaifa (Chinese jujube, *Ziziphus jujuba*) fruit was published, but the 30 kDa protein of azufaifa fruit has not yet been cloned. The binding of IgE to a 30 kDa allergen of azufaifa fruit can be inhibited by latex extract, and weakly inhibited by the recombinant chestnut class I chitinase (Alenis et al., 1995).

Chitinases are abundant proteins and have different functions in many organisms (Collinge et al., 1993; Hamel et al., 1997). In plants and mammals, they play a major role in the defense against pathogen attack (Kasprzewska, 2003). Plant chitinases can be subdivided into five classes (Collinge et al., 1993; Melcher et al., 1994; Brunner et al., 1998), and these classes can be grouped into two families of glycosyl hydrolases, family 18 and 19 (Hamel et al., 1997). In addition to the signal peptide found in all chitinases, class I with a hevein domain, class II without a hevein domain and class IV with a hevein domain share a homologous catalytic domain and make up the family 19 glycosyl hydrolases. Class III chitinases are mainly plant and fungal in origin, mostly acidic proteins without a hevein domain. Class III and V plant chitinases make up to family 18 glycosyl hydrolases with no sequence similarity to other classes of plant chitinases (Jekel et al., 1991; Hamel et al., 1997). Class V chitinases have two hevein domains and their catalytic domain is similar to that of class III chitinases.

Pathogenesis-related (PR) proteins are expressed by plants in response to stress conditions, such as infection, exposure to certain chemicals, wounding and environmental conditions, and can be regarded as a part of the defense system. However, PR proteins are constitutively expressed in some plant tissues, and remarkably many amino acid sequences and functions of characterized plant allergens can be classified as PR proteins (Alenis et al., 1995; Yagami, 2002; Breiteneder and Radauer, 2004). To date, 13 proteins of natural rubber latex (Hev b 1-13) have been described as allergens, and 12 of them have been cloned and sequenced (Sussman et al., 2002; Wanger and Breiteneder, 2002; Arif et al., 2004). Hev b 2 (β-1,3-glucanase, PR-2), Hev b 6.01, (prohevein, PR-3 and PR-4), Hev b 6.02 (hevein, PR-4), Hev b 7 (patatin-like protein) and Hev b 8 (profilin) have been reported to be associated with latex–fruit syndrome. Recently, class I chitinases have been identified in several fruits as the main cross-reacting allergen with hevein-latex (Hev b 6.01 and Hev b 6.02) (Diaz-Perales et al., 1998, 1999; Mikkola et al., 1998; Sanchez-Monge et al., 1999). At present, fruit class I chitinases and their relationship with hevein-latex seem to be the main clue to explain the latex–fruit syndrome (Salcedo et al., 2001). The other latex allergens, Hev b 2, Hev b 7 and Hev b 8 patterns of cross-reactivities with plant-derived foods have yet to be clearly confirmed. Class III chitinases and hevamine have been classified into PR-8 because of their inducible expression upon infection by pathogens (Lawton et al., 1992; Alenis et al., 1995), and their precise functions in the plant defense mechanism are not well understood. Hevamine has never been reported to be associated with latex–fruit syndrome. Our results open the possibility that plant class III chitinases and hevamine may play certain role in the latex–fruit syndrome. However, more studies are needed to elucidate the role of class III chitinases-related allergens in the association of latex and plant food allergens.

The present study revealed that both natural and rZiz m 1 were closely similar in their migration on SDS-PAGE (30 kDa), and inhibition studies and anti-rZiz m 1 antibody-based ELISA clearly indicated that cross-reactivity determinant(s) exists between Ziz m 1 and certain latex allergen(s). SPT revealed a rZiz m 1 allergy prevalence rate of 87.5% (7/8), thus Ziz m 1 is a candidate for a major *Z. mauritiana* allergen (at least 50% according to WHO–IUIS). To our knowledge, this report represents the first time a full-length cDNA from Indian jujube has been analyzed. Recombinant Ziz m 1 and sequence data could be useful in structural studies and may be valuable for diagnostic and therapeutic purposes. Furthermore, they may help shed some light on the mechanism underlying latex–fruit syndrome.

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