

Cloning of the panallergen profilin from lychee fruit and its cross-reactivity with birch pollen profilin Bet v 2

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Abstract

Lychee fruits can cause severe anaphylactic reactions and has cross-reactivity with other allergens. Panallergen profilin is partly responsible for the cross-reactivity between allergens. In the present study, the cDNA of lychee profilin was performed by RT-PCR and 3'RACE from total RNA. The lychee profilin cDNA includes an open reading frame coding for 131 amino acids. The deduced amino acid sequence of the corresponding protein show high identity with other allergenic profilins. Expression of the recombinant lychee profilin was carried out in *Escherichia coli* BL21 (DE3) using vector PET-28a, and the purification of the recombinant protein was performed via affinity chromatography with Ni⁺ coupled to sepharose. IgE reactivity of recombinant lychee profilin was investigated by immunoblot, and five of 15 lychee-allergic patients tested presented specific IgE antibodies to recombinant lychee profilin. IgE inhibition and ELISA inhibition experiments were performed to analyze lychee profilin cross-reactivity with profilins from birch pollen, and a high cross-reactivity between lychee profilin and birch profilin has been found in sera from lychee allergic patients.

Keywords: *Lychee, profilin, recombinant allergen, cross-reactivity.*

Introduction

The lychee (*Litchi chinensis* Sonn.) is the most renowned of a group of edible fruits of the family Sapindaceae, which also contains the two related species rainbutan (*Nephelium lappaceum*) and longan (*Euphoria longana*). Lychee is native to south-east Asia, but cultivation is now worldwide. Although a delicious fruit, lychee can also cause severe allergic reactions. The clinical symptoms include urticaria, pruritus and dyspnoea (Fäh et al. 1995; Giannatasio et al. 1995; Montag et al. 1996; Scarpa et al. 1996; Niggemann et al. 2002; Saraswat & Kumar 2005). Moreover, allergy to lychee fruit shows cross-reactivity with other fruits (Saraswat & Kumar 2005), vegetables (Lüttkopf et al. 2000), pollen (Wellhausen et al. 1996; Vieths et al. 1998; Eriksson et al. 2003), and latex (Niggemann et al. 2002).

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Several plant allergens have been identified as responsible for the cross-reactivity. These allergens are proteins that share common epitopes with Bet v 1 (cross-reactive carbohydrate determinants; CCD), Bet v6-related proteins and profilins (Vieths et al. 2002). Profilins are proteins found in all eukaryotic cells with molecular mass about 14–17 kDa. They were first described as actin-binding proteins in calf spleen by Carlsson et al. (1977). In 1991, allergologists' attention was drawn to profilin when a highly cross-reactive minor birch pollen allergen Bet v 2 was identified as a profilin (Valenta et al. 1991). Since then, profilins were identified as the allergen common to many fruits, vegetables, foods, as well as pollen. Further studies have shown that profilins are highly conserved with homologies in the range of 70–80% with known allergenic plant profilins (Radauer & Hoffmann-Sommergruber 2004). Its ubiquity and high sequence conservation have led to the use of the term 'pan-allergen' for this protein (Valenta et al. 1992).

In a patient study, Füh et al. (1995) found that there is a significant amount of profilin in lychee fruit, and that this profilin was the protein responsible for the patient's complex allergy syndrome. A lychee profilin gene has been submitted to GenBank (Accession No. AY049013), but the character of recombinant lychee profilin and its cross-reactivity with other profilin has not been studied. In the present study, the amino acid sequence of lychee fruit profilin was obtained from its corresponding cDNA and was expressed in *Escherichia coli*. The recombinant protein was purified by Ni²⁺ affinity chromatography and its IgE-binding capability was studied by means of immunoblot experiment. Moreover, the cross reactivity between recombinant lychee profilin and Bet v 2 was investigated by immunoblot inhibition and enzyme-linked immunosorbent assay (ELISA) inhibition experiments.

Materials and methods

Recombinant allergens and protein extracts

Recombinant birch pollen profilin Bet v 2 was obtained from Biomay (Linz, Austria). Protein extracts from fresh lychee fruit was prepared by a low temperature method as described by Westphal et al. (2004) with minor modifications. In brief, pieces of lychee fruit were ground in a mortar filled with liquid nitrogen. The powder obtained was homogenized in pre-chilled acetone and stored overnight at -20°C . The precipitates were washed twice with acetone, filtered and lyophilized. Protein extracts were obtained by incubating the powder with phosphate buffer (0.15 M NaCl/0.01 M NaH_2PO_4) at 4°C . After centrifugation, the supernatant was collected, filtered and freeze-dried. The lyophilized extracts were stored at -80°C .

Cloning of lychee fruit profilin cDNA and sequence analysis

Total RNA was isolated from lychee fruits using the 'RNeasy Plant RNA Mini Kit' (Qiagen) according to the manufacturer's instructions. The reverse transcription was carried out with 2 μg total RNA using AMV First-strand cDNA synthesis Kit (BBi), the reaction was performed at 42°C for 60 min followed by heat denaturation at 95°C for 5 min.

The cDNA obtained was amplified by polymerase chain reaction (PCR) using the degenerated oligonucleotide primers (5'-primer: 5'-ATGTCGTGGCAGGCGTACGT-3', 3'-primer: 5'-CAA CAA(C/T)CATGTTGCA(T/C)TTGTCC-3') which

were designed from the known sequence of different plant profilins. The amplification was performed with Ex-Taq DNA polymerase (Takara), as recommended by the manufacturer. After purification, the PCR products were ligated into pMD18-T vector (Takara), and the constructed plasmids were transformed into *E. coli* Top10. The positive clones were sequenced by the Sangon Biotechnology Co. (Shanghai, China) using the ABI 337 sequencer.

The clones obtained from the degenerated primers did not cover the full coding region. For amplification of the unknown 3'-end of the lychee profilin, the 3'RACE method was used according to the manufacturer's instructions (3'-full RACE Core set, Takara). First-strand cDNA synthesis was primed with an Oligo dT-3-sites adaptor primer enclosed in the 3'RACE system. The first strand cDNA was amplified by PCR using a gene-specific primer and the 3-sites adaptor primer supplied by the 3'RACE system. PCR products were also cloned into the pMD18-T vector and transformed into *E. coli* Top10, the positive clones were sequenced. The full-length cDNAs were obtained by the overlapping of the truncated gene and its 3'-end. The identities of the lychee profilin cDNA and its deduced amino acid sequences with profilin from other plant sources were analyzed using the Blast program on the NCBI website (<http://www.ncbi.nlm.nih.gov>) and the alignment of amino acid sequences were generated by CLUSTAL W (1.83) multiple sequence alignment program.

Expression and purification of the recombinant lychee profilin

In order to ligate into the PET-28a expression vector, *Nde*I and *Xho*I restriction sites were introduced at the 5'-end and 3'-end of the lychee profilin cDNA respectively, by PCR using the primers MpNde (5'-CATATGTCGTGGCAGGCGTAC-3') and MpXho (5'-ATCCTCGAGTTA CAGGCCCTGTTCAAG-3'). Initial cloning was carried out in *E. coli* strain Top10. Positive clones were selected first by colony PCR with the MpNde and MpXho primers, and then digested with the *Nde*I and *Xho*I restriction enzymes and sequencing. The target plasmids were transformed into *E. coli* BL21(DE3) cells for expression.

The cells were grown in LB-medium supplemented with 50 µg/ml kanamycin to an absorbance of 0.6 at 600 nm. Expression of lychee profilin was induced by adding isopropyl-d-thiogalactopyranoside (IPTG) to LB medium at a final concentration of 0.1 mM and then incubating overnight at 37°C. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5. After 3 cycles of freeze-thawing, cells were sonicated to obtain the lysate, insoluble material was removed by centrifugation. Finally, the recombinant protein was purified by affinity chromatography using the Ni⁺ as solid phase. The supernatant of the lysate was applied to the column followed by washing with 40 mM imidazole and elution with 200 mM imidazole. The elution products were dialyzed three times against 0.02 M Tris-HCl buffer and then freeze-dried.

Patients' sera

Serum samples used in this study were taken from birch pollen-allergic patients with a positive case history of immediate-type reaction to lychee fruits, and only patients' sera with a positive skin prick testing result to lychee (>7 mm²) and with a positive CAP result to birch pollen were used in the study. Sera from non-allergic subjects served as

negative control. All sera were kindly supplied by Shenzhen People's Hospital, and all samples were stored at -80°C .

IgE-immunoblotting and immunoblot inhibition

Protein from *E. coli* lysate and purified recombinant profilin were analyzed by SDS-PAGE. Proteins were visualized by Coomassie Brilliant Blue R250 staining or immunoblotting. For immunoblot analysis, recombinant profilin samples ($0.5\text{ }\mu\text{g/cm}$ slot) were electrically transferred onto the nitrocellulose membranes, and the membrane were blocked overnight with 2% bovine serum albumin solution (BSA) at 4°C , then allergic patients' sera (diluted 1:5) were added to each membrane strip for 2 h at 37°C . The biotin-labeled goat anti-human IgE antibody (KPL) was used as the secondary antibody, followed by incubation with the streptavidin-conjugated-HRP at 37°C for 2 h. The blotting bands were finally visualized with diaminobenzidine (DAB) chromogenic substrate solution.

For inhibition of IgE-binding to recombinant lychee profilin, a pooled patient serum was pre-incubated with $100\text{ }\mu\text{g}$ protein from lychee fruit extracts, or $15\text{ }\mu\text{g}$ of recombinant birch profilin Bet v 2 or $15\text{ }\mu\text{g}$ BSA (negative control). The pre-absorbed serum pool was then used for immunoblot experiments.

ELISA and ELISA inhibition

For ELISA, 96-well Maxisorb plates were coated with $0.5\text{ }\mu\text{g}$ recombinant lychee profilin in PBS overnight at 4°C . Plates were blocked in PBS/2% BSA for 2 h at 37°C , and washed three times with PBS/0.05% Tween-20, and then incubated with patients' sera (diluted 1:5) overnight at 4°C . The biotin-labeled goat anti-human IgE antibody was used as the secondary antibody, followed by incubation with the streptavidin-conjugated-HRP at 37°C for 1 h. Each incubation step was followed by washing three times with PBS/0.05% Tween-20. After visualization of the enzymatic activity with the AB liquid as substrate, the reaction was stopped by the addition of $100\text{ }\mu\text{l}$ 2 M H_2SO_4 , and absorption was measured at a wavelength of 450 nm.

For ELISA inhibition, sera were pre-incubated for 2 h at 37°C with 0.1, 1, 10 and $100\text{ }\mu\text{g/ml}$ of the inhibition protein (lychee extracts, rBet v 2) before overnight incubation on the allergen-coated plates. Then the inhibition experiment was performed as ELISA experiments described above.

Results

Cloning of the lychee profilin cDNA and sequence analysis

By amplification of cDNA from first strand synthesis products with degenerated oligonucleotide primers based on the known profilin sequences from other plants, a dominant fragment of approximately 360 bp were obtained from lychee fruit. The unknown 3'-end of cDNAs were obtained by using 3'-RACE method. The PCR resulted in a fragment about 500 bp length with an overlap of 110 bp with the coding region of lychee profilin. Full-length cDNA sequences were obtained by bridging the 3'-end and the corresponding gene fragments. The cDNA sequence was submitted to the NCBI GenBank under the Accession No. DQ309464, and the deduced protein was named Lit c 1.

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1  ATGTCGTGGCAGGCGTACGTCGACGATCACCTCATGTGCGAGACTGACGGCCAGCACCTC
   M S W Q A Y V D D H L M C E T D G Q H L
61  ACTGCCGCCGCCATCATCGGCCACGACGGCAGCGTCTGGGCCAGAGCGCCAACTTCCCT
   T A A A I I G H D G S V W A Q S A N F P
121 CAGTTCAAGCCTGTGAGATTACAGCGATCATGAAAGACTTTGATGAACCTGGTTCCCTT
   Q F K P V E I T A I M K D F D E P G S L
181 GCCCCCACTGGGTTGCACCTCGGTGGCACAAGTACATGGTTATCCAGGGTGAGCCTGGA
   A P T G L H L G G T K Y M V I Q G E P G
241 GCTGTTCATCCGTGGCAAAAAGGGTCCTGGTGGCATCACCGTGAAGAAAACAACTCAGGCT
   A V I R G K K G P G G I T V K K T T Q A
301 CTGATTATCGGAATATATGATGAACCAATGACCCCGGGACAGTGTAAACATGGTTGTGGAG
   L I I G I Y D E P M T P G Q C N M V V E
361 AGGTTGGGGATTACCTCGTCGATCAGGGCCTGTAAatgcacctcatctcagcatgatat
   R L G D Y L V D Q G L *
421 ggtatttccttatactggttttcggttttgggattgcctcttcttgaagatttggttaat
481 tgttgctgaagaaataaatgtgtgagggcagagtgatttttcggtttttttagatttgat
541 ttatgttttgccttaaggaaatcaattgtattgcagttttagaagaatttgaaatgtttt
601 ttggatattatgatattggtgagaattaagctttgagtttttaaaaaaaaaaaaaaaaaa
661 aaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

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Figure 1. Nucleotide sequence (upper) and deduced amino acid sequence (lower) of lychee profilin. Nucleotide numbers on the left.

The entire lychee profilin cDNA length was 660 bp containing a complete 396 bp open reading frame, a 3'-untranslated region and a poly(A) tail (Figure 1). The length of the deduced amino acid sequence was 131 amino acid residues, the calculated molecular mass was 14.06 kDa and its isoelectric point was 4.89. The deduced amino acid sequence of lychee profilin had a high identity with the existing lychee profilin sequence. Moreover, it also had high identities with profilin from other plants, such as fruits (Pru av 4 from cherry, Pru p 4 from peach and Mal d 4 from apple), and pollen (Bet v 2 from birch) (Figure 2).

Expression and purification of recombinant lychee profilin protein

For expression of the recombinant lychee fruit profilin, the complete coding cDNA sequence, including the start and the stop codon, was cloned into the PET-28 expression vector and then transformed into *E. coli* BL21(DE3). After inducing with IPTG overnight at 37°C, the recombinant lychee profilin protein was achieved. The soluble material, which includes most of the target protein, was chromatographed over a Ni²⁺ Sepharose affinity column. After elution with 200 mM imidazole, recombinant lychee profilin with an apparent molecular mass of 14 kDa was isolated with a high degree of purity (Figure 3). The yield of purified recombinant protein from 1 L bacterial culture was approximately 5 mg.

Sensitization to lychee profilin

Serum samples taken from 15 patients who had adverse symptoms after ingestion of lychee fruits and were suffering from pollinosis were investigated for IgE reactivity to recombinant lychee profilin by immunoblot experiment. A pooled serum from

Lit c 1	MSWQAYVDDHLMCETDQHLT--AAAIIGHDGSVWQSANFPQFKPVEITAIMKDFDEPG	58
AAL07320.1	***T*****--*****A**A*****	58
Pru av 4	*****DI**NR**--***L*Q*****S***T**A***E**A**L**L*Q**	58
Pru p 4	*****IE**N**S--*****T**L**E**V**C**L**N**N**	58
Mal d 4	*****DI**NR**--***L*Q*****S***S**A***E**A**L**L*Q**	58
Bet v 2	***T**E***DI***ASNSL*S**V*****S*****Q***C*****E***	60
Lit c 1	SLAPTGLHLGGTKYMNVIQGEPGA VIRGKKPGGITVKKTTQAL IIGIYDEPMTPGQCNMV	118
AAL07320.1	*****	118
Pru av 4	T*****F*****A*****S*****N*****L*****I	118
Pru p 4	*****Y*****V***S*L*****I	118
Mal d 4	T*****F*****S***I***S**L*****V*****I*	118
Bet v 2	H*****I*****A*****S***I***G***V**F**E**V*****	120
Lit c 1	VERLGDYLV DQGL	131 identity
AAL07320.1	*****	131 97%
Pru av 4	*****IE***	131 82%
Pru p 4	*****E***	131 85%
Mal d 4	*****IE***	131 82%
Bet v 2	*****I***	133 80%

Figure 2. Alignment of amino acid sequences of lychee profilin (Lit c 1) with those of allergenic profilins from the existing lychee profilin (AAL0732.1), sweet cherry (Pru av 4), peach (Pru p 4), apple (Mal d 4) and birch pollen (Bet v 2). Asterisk indicates identical amino acids, dashed lines are gaps. The sequence identities between Lit c 1 and other profilins are included at the bottom right of figure.

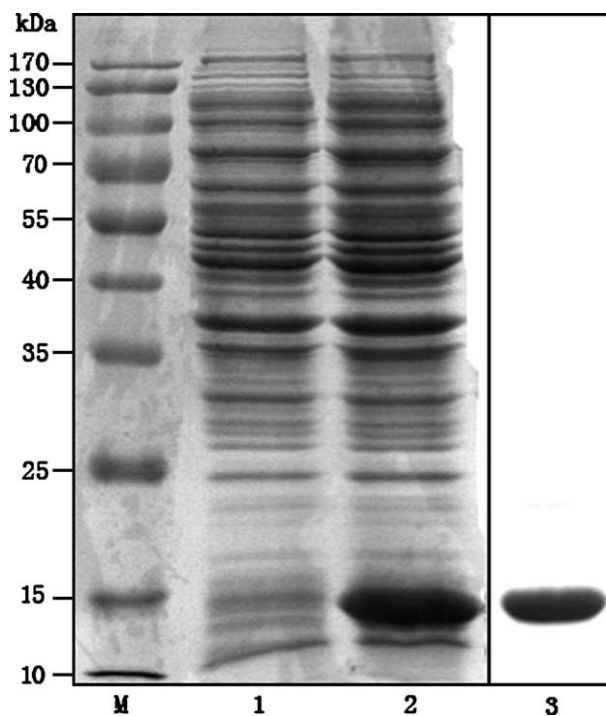


Figure 3. Coomassie-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the supernatant fraction from transformed *E. coli*-lysates with PET-28a vector carrying the lychee profilin cDNA. M, protein molecular weight marker; lane 1, before induced; lane 2, after induced; lane 3, purified recombinant lychee profilin.

10 people with no allergic reaction was used as a control. In the immunoblot experiments, IgE antibodies from 5 of 15 serum samples (33%) reacted with the purified recombinant lychee profilin. No reaction was found with sera from control subjects (Figure 4). All sera with positive reactions to the lychee fruit profilin had specific IgE to recombinant birch pollen profilin rBet v 2 in the ELISA experiment.

Inhibition experiments

To investigate the IgE cross-reactivity to profilin from Birch pollen, immunoblot inhibition and ELISA inhibition experiments were carried out with the pool of lychee allergic patients' sera. These patient sera showed positive reactions in the immunoblot experiments. The results of immunoblot inhibition experiment are shown in Figure 5. The recombinant lychee profilin band was almost completely inhibited by lychee fruit extracts, and the recombinant birch pollen profilin rBet v 2 inhibited a large part of the recombinant lychee profilin. A similar inhibition pattern was obtained in the ELISA inhibition experiments (Figure 6). The lychee fruits extracts inhibited >90% of the IgE binding to the recombinant lychee profilin. A high degree of inhibition was also obtained with birch pollen profilin (about 71%). No inhibition occurred with the control inhibitor BSA.

Discussion

Lychee can cause severe anaphylactic reactions. Sensitization to lychee fruit has been described. Thus far, little information about lychee allergen is available. Recently, only one 28-kDa lychee allergen was identified as a triose-phosphate isomerase (Hoppe et al. 2006). However, cross-reactivities between lychee fruit allergens and other fruits, vegetable, pollen, latex have been described (Wellhausen et al. 1996; Vieths et al. 1998; Lüttkopf et al. 2000; Niggemann et al. 2002; Eriksson et al. 2003; Saraswat et al. 2005). As to the allergens result in the cross-reactivity between lychee fruits and other food or pollen, there were some discussions. Saraswat et al. (2005) reported a case of a 33-year-old woman with severe reaction to apple, banana and lychee fruit,

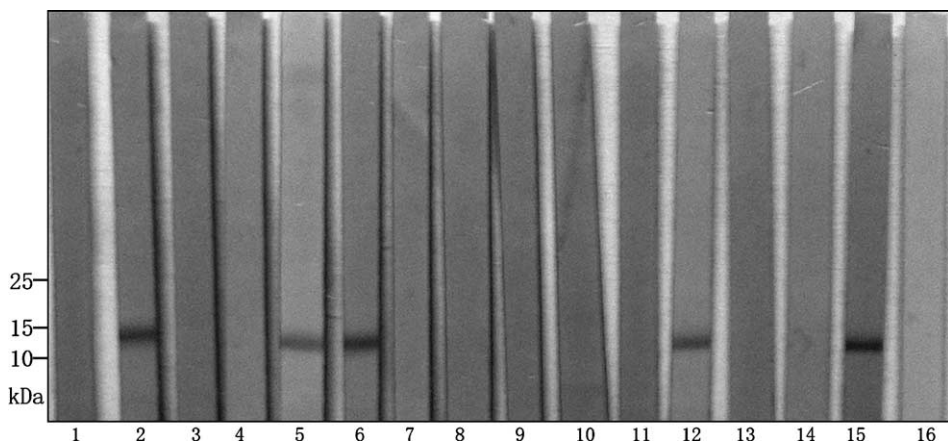


Figure 4. IgE-immunoblot of purified recombinant lychee profilin with individual sera (1–15) from patients with allergy to lychee fruits and with the control serum (16).

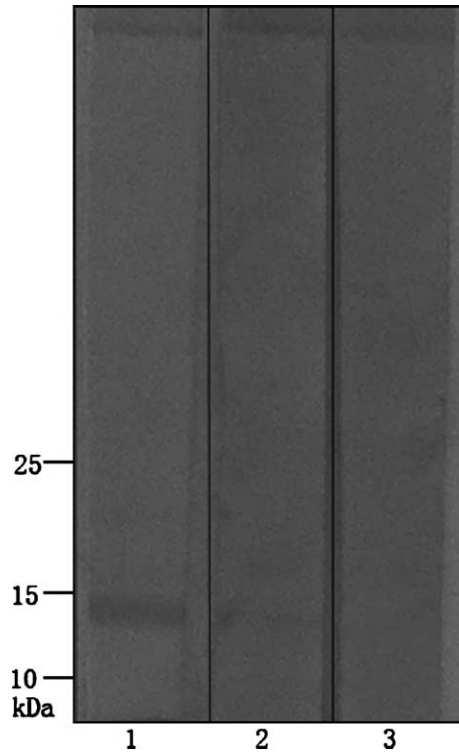


Figure 5. Inhibition of IgE-binding to recombinant lychee profilin after pre-incubation of a pooled sera from lychee allergic patients with BSA control (1), Bet v 2 (2), and lychee extract (3).

and discussed the possible responsible allergen for the cross-reactivity between those fruits; Wellhausen et al. (1996) and Vieths et al. (1998) identified an IgE-binding 35-kDa protein (Bet v5) from birch pollen with cross-reacting homologues in various plant foods, including lychee fruit. The immunoblot inhibition results of Föh's

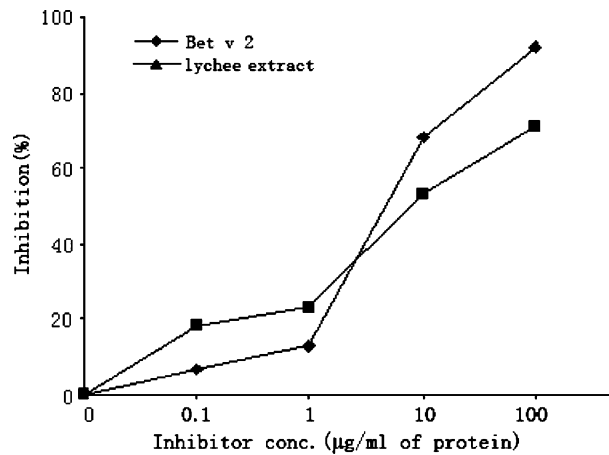


Figure 6. ELISA inhibition experiments. Sera pool from lychee allergic patients were pre-incubated with lychee extract and Bet v 2.

indicated a 16-kDa allergen as the cross-reactive protein between lychee, birch pollen and celery, and this allergen was identified as profilin (Fäh et al. 1995), but, until now, the recombinant lychee profilin has not been studied.

Profilin is a protein present in almost all eukaryotic cells. It was first identified as a minor allergen in birch pollen, however profilins are now described as panallergens because of their presence in botanically closely and distantly related plants or unrelated plants (Vallier et al. 1992; Van et al. 1992; Van et al. 1995; Scheurer et al. 2000; Asturias et al. 2005). The aim of this study was to investigate the role of the panallergen, profilin, as a cross-reaction allergen in lychee fruit. The cDNAs coding for lychee profilin isoforms has been isolated and sequenced. The complete deduced amino acid sequences consist of 131 amino acid residues with a calculated molecular mass of 14.06, according to sequence data correlating with the molecular characteristics to the known plant profilins. Moreover, high degrees of amino acid sequence homologies with existing lychee profilin sequence (97%) and other previously described allergenic profilins from fruits, pollen and vegetables (80–85%), were found. The high homology between the sequence presented here and the existing lychee profilin sequence may indicate that the profilin sequence is highly conserved in different lychee varieties cultivated in different areas.

In order to express the recombinant lychee fruit profilin, the coding region of lychee profilin was induced into PET-28(a) vector and expressed in *E. coli*, and a high level of recombinant protein was obtained when protein synthesis was induced by IPTG at 37°C overnight. Highly purified lychee profilin was chromatographically isolated from the soluble fraction by Ni⁺ Sepharose affinity chromatography.

The IgE reactivity of purified lychee profilin was studied by immunoblot experiment, and five of 15 sera (33%) from patients with suspected allergy to lychee fruits had IgE specific to the recombinant lychee profilin. Profilins have been identified as minor allergens in several plant species of tree, grass, and weed pollen, and the profilin specific IgE was detected in 10–30% of pollen- and food-allergic patients' sera (Radauer et al. 2004). In the present study, the prevalence of IgE binding to lychee profilin is 33%, a little higher than 10–30%, but lower than the prevalence reported by Vieths et al. (2002). In their report, the prevalence of IgE binding was 70% in seven lychee allergic subjects (Vieths et al. 2002). This difference may be caused by the different patient numbers used in the experiment.

The cross-reactivity of purified recombinant lychee profilin with lychee extract and rBet v 2 was determined by IgE-inhibition and ELISA inhibition experiments. The results revealed that the IgE reactivity of recombinant lychee profilin was almost fully inhibited by lychee extracts. This may indicate the similar IgE-binding capabilities of the natural profilin and the recombinant profilin. Immunoblot and ELISA inhibition experiments also revealed high cross-reactivity of the lychee profilin with the profilin from birch pollen Bet v 2 (71%). The high inhibition levels in the cross-inhibition experiments may suggest that lychee profilin shares epitope with profilin from different botanical origins, such as birch.

In conclusion, the present study cloned and expressed the lychee profilin, investigated the immunological characteristics of the recombinant lychee profilin, and discussed the cross-reactivity between lychee profilin and recombinant birch pollen allergen Bet v 2. The results showed that the panallergen profilin is assuredly partly responsible for the cross-reactivity between lychee fruits and other pollen allergen.

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