Expression, epitope prediction and IgE-binding of the *Tyrophagus putrescentiae* group 13 allergen

NAN WANG[1*], YING ZHOU[2*], MEILI WU[2], HANTING ZHU[3] and YUBAO CUI[3]

[1]Department of Basic Medicine, Jiangsu Vocational College of Medicine, Yancheng, Jiangsu 224005; [2]Department of Pediatrics Laboratory, Wuxi Children's Hospital; [3]Department of Clinical Laboratory, Wuxi People's Hospital Affiliated to Nanjing Medical University, Wuxi, Jiangsu 214023, P.R. China

Received April 1, 2020; Accepted July 22, 2020

DOI: 10.3892/etm.2020.9149

Abstract. Storage mites, such as *Tyrophagus putrescentiae*, are an important source of allergens that cause allergic diseases in humans. It has previously been indicated that *T. putrescentiae* has a high sensitization rate as an allergen in some Asian and European countries. Identifying and cloning the allergens in this species may enable improved diagnostic and therapeutic approaches. The aim of the present study was to clone and sequence the *T. putrescentiae* group 13 allergen (Tyr p 13) isolated from storage mites in China, to use bioinformatics tools to model its biophysical characteristics and to induce protein expression to test its IgE-binding activity. The full-length cDNA comprised 486 bp and was predicted to include a signal peptide of 22 amino acids. Its secondary structure was shown to comprise an α-helix (10.79%), extended strand (33.81%) and random coils (55.40%). Using homology modeling, the present study constructed a reasonable tertiary structure of Tyr p 13. Linear Bcell epitopes at amino acids 47-53, 70-76, 81-86, 101-105 and 112120 were predicted. Three discontinuous B-cell epitopes were also predicted: i) 47, 48, 49, 50, 51, 52, 53, 70, 71, 72 and 73; ii) 91, 92, 93, 94, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121 and 138; and iii) 74, 76, 79, 81, 82, 83, 84, 86, 101, 102, 103, 104 and 105. SDS-PAGE identified a specific band at the predicted molecular weight of the recombinant Tyr p 13 (rTyr p 13), demonstrating its successful expression. The rTyr p 13 bound to IgE in the serum of 13.2% (5/38) of patients allergic to *T. putrescentiae*, according to ELISA. The successful cloning of Tyr p 13 and basic bioinformatics analysis of the protein provided a foundation for the further study of this allergen with regards to the diagnosis and treatment of patients allergic to storage mites. These results provided a theoretical basis for the design of rTyr p 13 with modified B-cell epitopes.

Correspondence to: Professor Yubao Cui, Department of Clinical Laboratory, Wuxi People's Hospital Affiliated to Nanjing Medical University, 299 Qingyang Road, Wuxi, Jiangsu 214023, P.R. China
E-mail: ybcui1975@hotmail.com

*Contributed equally

Key words: *Tyrophagus putrescentiae*, recombinant allergen, *T. putrescentiae* group 13 allergen, homology modeling, Bcell epitopes

Introduction

The non-predatory storage mite *Tyrophagus putrescentiae* is distributed worldwide (1) and can be found in a variety of stored goods, particularly in high-fat and protein-rich stores (2). In addition to causing damage to food stores, *T. putrescentiae* can cause human allergic diseases (3,4). Allergic diseases caused by *T. putrescentiae* are more common in Asia (5) and Europe (6).

At present, the diagnosis and allergen immunotherapy (AIT) of mite allergic diseases depend on the use of natural allergen extracts. However, natural allergen extracts are associated with a number of issues, including unpredictable amounts of allergens and non-allergenic components in natural extracts, which can lead to serious immediate and late side effects (7,8). The majority of these issues in allergy diagnosis and AIT can be circumvented with the use of recombinant allergens and peptides (9). In order to improve the safety of AIT, researchers have turned their attention to recombinant allergens with modified B-cell epitopes, which can prevent IgE binding, but with intact T-cell epitopes to produce an immunotherapeutic benefit (10). With regards to allergy diagnosis, studies on food allergens have revealed the potential role of B-cell epitopes (IgE-binding epitopes) as biomarkers for characterizing various phenotypes (11). Thus, peptide-ELISA or epitope-ELISA has the potential to lead to advances in the clinical diagnosis of allergic diseases.

A better understanding of *T. putrescentiae*-produced allergens may aid the diagnosis and treatment of allergies to this mite. A series of studies on the *T. putrescentiae* allergens from China have thus been conducted. Using molecular cloning techniques, the sequencing and characterization of group 4, 28, 35 and 36 *T. putrescentiae* allergens from China were reported in our previous studies (12,13). The *T. putrescentiae* group 13 allergen (Tyr p 13) was another allergic component in our series of studies. The identification of B-cell epitopes of Tyr p 13 may be helpful in designing sequences for more accurate and safer peptide-based allergen diagnosis and immunotherapeutic agents. However, the tertiary structure and epitope of Tyr p 13 remain unclear.

The aim of the present study was to clone and express the full-length sequence encoding Tyr p 13 from China. Combined with the prediction analysis of Tyr p 13 structure and B-cell epitopes, these results were intended for use in the diagnosis and treatment of anaphylaxis caused by *T. putrescentiae* and may lay the foundation for the design of recombinant Tyr p 13 (rTyr p 13) with modified B-cell epitopes.

Materials and methods

Patient serum. Peripheral blood samples were collected from patients with allergic asthma at the Respiratory Department, Wuxi People's Hospital (Wuxi, China). The peripheral blood samples were placed at room temperature for 1 h, and then centrifuged at 1,000 x g for 10 min at 4˚C to obtain the serum. Patients had not received any treatment and asthma diagnosis was performed following the World Health Organization criteria (14). The diagnosis of allergy to *T. putrescentiae* allergens was established on the basis of a suggestive clinical history and positive serum IgE (sIgE) to *T. putrescentiae* extracts, as determined by the Allergy Screening test panel for atopy (Mediwiss Analytic GmbH). The inclusion criteria were as follows: i) Allergy to *T. putrescentiae* extracts with >1 year of follow-up, and ii) levels of sIgE to *T. putrescentiae* extract >3 kU/l at the time of inclusion in the study. A total of 38 patients (male:female ratio, 18:20), with a mean age of 35.263±8.374 years, were enrolled as serum-positive for *T. putrescentiae* and their serum was used to detect specific IgE-binding to recombinant allergens. In addition, serum from five non-atopic healthy people (malefemale ratio=2:3), with a mean age of 35.8±8.727 years, was used as the negative control. All participants provided written informed consent. The study protocol was approved by the Ethics Committee for Clinical Investigation of Wuxi People's Hospital Affiliated to Nanjing Medical University (Wuxi, China).

T. putrescentiae culture and isolation of adult mites. To isolate *T. putrescentiae*, flour and dust were obtained from the floors of a flour storage warehouse in Yancheng, China. The collected sample was placed in a 9-cm glass petri dish and the sample was observed under a stereomicroscope. The suspected pregnant *T. putrescentiae* were picked under a stereomicroscope and placed separately in different culture flasks containing culture medium for cultivation. Under the stereomicroscope, pregnant mites moved slowly, crept on the bottom of the petri dish, formed egg cells in the body and their body shape was larger. After ~2 months, the whole culture in the flask was examined under the stereomicroscope. According to the movement speed, body type and body surface of the *T. putrescentiae*, the species of mites in the culture medium could be simply judged (15). Subsequently, mites considered to be *T. putrescentiae* were cultivated in small culture flasks for pure culture. After a further 2 months, the mites previously isolated and cultured were identified again. The identification of *T. putrescentiae* was performed in strict accordance with the morphological characteristics of *T. putrescentiae* (15). If the mite was confirmed to be *T. putrescentiae*, all mites in that flask were considered to be *T. putrescentiae*. During the cultivation process, these *T. putrescentiae* were raised in an atmosphere of 25˚C and 85% relative humidity. In the culture process of *T. putrescentiae*, the culture medium consisted of rice bran medium, flour and yeast powder (11). The isolation method of pure *T. putrescentiae* mites from the culture medium was the same as that of pure *Dermatophagoides farinae* mites (16).

Preparation of Tyr p 13 cDNA. Total RNA was obtained from *T. putrescentiae* using the Takara MiniBEST Universal RNA Extraction Kit (cat. no. 9767; Takara Biotechnology Co., Ltd.). Based on the published sequence of Tyr p 13 (GenBank accession no. DQ983316; https://www.ncbi.nlm.nih.gov/nuccore/DQ983316.1/), a pair of primers were designed: Forward, 5'-GGAATTCCATATGTCGGTCGAAGAAC-3' and reverse, 5'-CCGCTCGAGTTAATCACCAGTCATCATCTCC-3', with an *Nde*I and an *Xho*I site at their 5' ends (underlined), respectively. Reverse transcription (RT)-PCR was performed using total mite RNA and the High-Fidelity PrimeScript RT Reagent Kit with gDNA Eraser (cat. no. DRR047A; Takara Biotechnology Co., Ltd.) on a PCR Thermal Cycler Dice (cat. no. TP600; Takara Biotechnology Co., Ltd.). gDNA was removed from the total RNA samples; briefly, the reaction mixture (10 μl) contained total RNA (1 μl), 5X gDNA Eraser Buffer (2 μl), gDNA Eraser (1 μl) and RNase-free H_2O (6 μl). The mixture was incubated at 42˚C for 2 min, and RT was performed according to the manufacturer's instructions. PrimeScript RT Enzyme Mix I (1 μl), RT Primer Mix (1 μl), 5X PrimeScript Buffer 2 (4 μl) and RNase-free dH_2O (4 μl) were added to the aforementioned reaction mixture. The reaction mixture (20 μl) was incubated at 37˚C for 15 min and 85˚C for 5 sec. The RT product was used as the template for PCR using PrimeSTAR® HS DNA Polymerase (cat. no. R010A; Takara Biotechnology Co., Ltd.).The PCR mix comprised RT products (1 μl), 5X PrimeSTAR PCR buffer (10 μl), 2.5 mmol/l dNTP mixture (4 μl), 20 μmol/l forward primer (1 μl), 20 μmol/l reverse primer (1 μl), 2.5 U/μl PrimeSTAR HS DNA polymerase (0.5 μl) and dH_2O (32.5 μl). For PCR, samples were incubated for 2 min at 94˚C, followed by 30 cycles at 98˚C for 10 sec, 65˚C for 30 sec and 72˚C for 40 sec. Finally, samples were incubated for 10 min at 72˚C and 5 min at 10˚C, and amplicons (5 μl) were analyzed by agarose gel electrophoresis (1.0%) containing ethidium bromide (cat. no. E7637; Sigma-Aldrich, Merck KGaA) and visualized with ImageMaster® VDS (Pharmacia Biotech).

Cloning and DNA sequencing. The PCR-amplified DNA was recovered from the gel using the Agarose Gel DNA Purification kit v2.0 (cat. no. DV805; Takara Biotechnology Co., Ltd.) and a poly-A tail was added using the DNA A-Tailing Kit (cat. no. 6109; Takara Biotechnology Co., Ltd.). The poly-A tailed product was cloned into the simple vector pMD20-T (cat. no. D107A; Takara Biotechnology Co., Ltd. according to the manufacturer's instructions. Competent *Escherichia coli (E. coli)* JM109 cells (cat. no. 9052; Takara Biotechnology Co., Ltd.) were transformed with the recombinant plasmid pMD20T-Tyr p 13 using the heat-shock method. Positive clones were selected by blue/white screening on Luria-Bertani (LB) plates containing 100 μg/ml ampicillin and samples were sequenced using *Bca* BEST sequencing primer RV-M/M13-47 (Takara Biotechnology Co., Ltd.) on an ABI PRISM 377XL DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

DNA sequence retrieval and analysis. The sequences were edited to remove the vector sequence and extra restriction sites using SeqMan on DNASTAR Lasergene software suite v7.1 (DNASTAR, Inc.). The open reading frame (ORF) was identified using the ORF finder (http://www.bioinformatics.org/sms2/orf_find.html) from the National Center for Biotechnology Information.

Physicochemical analysis and analysis of patterns. The sequence of Tyr p 13 was obtained by sequencing after pMD20T-Tyr p 13 was transformed into *E. coli* JM109 cells. The amino acid sequence of Tyr p 13 was predicted using Translate Tools in ExPaSy (https://web.expasy.org/protparam/). The family classification of rTyr p 13 was analyzed using InterPro v56.0 (www.ebi.ac.uk/interpro/) (17). The signal peptide sequence of rTyr p 13 was predicted by SignalP 4.1 software (http://www.cbs.dtu.dk/services/SignalP/), as previously described (17). Physiochemical analyses, including theoretical pI, aliphatic index, grand average of hydropathicity (GRAVY) and instability index of rTyr p 13, were predicted using ProtParam (web.expasy.org/protparam/) (17) and ProtScale tools (https://web.expasy.org/protscale/) (18). The phosphorylation sites of rTyr p 13 were determined using the NetPhos3.1 server (http://www.cbs.dtu.dk/services/NetPhos/), as previously described (17). The secondary structure was predicted using GOR4.0 (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html), as previously described (19). The TMHMM server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict transmembrane protein helices (20).

Homology modeling. Homology modeling was used to construct a tertiary structure of Tyr p 13. A BLASTP (blast.ncbi.nlm.nih.gov/Blast.cgi) search with default parameters was performed against the Protein Data Bank (PDB; www.rcsb.org/pdb/) to identify a suitable template for Tyr p 13. Based on this template, the tertiary structure of Tyr p 13 was predicted using SWISS-MODEL (https://www.swiss-model.expasy.org/). The generated model was evaluated using QMEAN, Global Model Quality Estimation (GMQE), ERRAT, VERIFY_3D, PROCHECK and ProSA-Web. QMEAN was used to to provide the global (for the entire structure) and local (per residue) error estimates on the basis of a single model, while GMQE was used to reflect the expected accuracy of a model built with that alignment and template and the coverage of the target, ERRAT (services.mbi.ucla.edu/SAVES) was used to analyze the statistics of non-bond interactions between different atom types, VERIFY_3D (services.mbi.ucla.edu/SAVES) was used to determine the compatibility of the atomic model (3D) with its amino acid sequence (1D) and compare the results to an advantageous structure, PROCHECK (services.mbi.ucla.edu/SAVES) was used to verify the stereochemical quality of the Tyr p 13 structure, and ProSA-Web (https://prosa.services.came.sbg.ac.at/prosa.php) was used to analyze whether the interaction between each residue of the model was within a reasonable range (21). Superimposition of the query and template structure and visualization of the generated models was performed using UCSF Chimera 1.10.2 (www.cgl.ucsf.edu/chimera/).

Structure-based prediction of Bcell epitopes. Although the reliable prediction of epitopes is challenging, the prediction of allergen epitopes is beneficial to the immunological diagnosis of allergic diseases. Linear and discontinuous B-cell epitopes were predicted using ElliPro: Antibody Epitope Prediction; IEDB analysis resource (http://tools.immuneepitope.org/tools/ElliPro/tutorial.jsp) (22). The rTyr p 13 protein sequence was analyzed using different analytical parameters, such as secondary structure, hydropathy, antigenicity, amphilicity, surface probability and flexibility in the Protean software of the DNASTAR Lasergene software suite v7.1 (23).

Construction of expression plasmids pET28a(+)-Tyr p 13. The recombinant pMD20-T-Tyr p 13 plasmid was digested with *Nde*I and *Xho*I to release the Tyr p 13 cDNA. The cDNA was separated by agarose gel electrophoresis and purified from the gel using the Agarose Gel DNA Purification kit v2.0. The DNA Ligation kit (cat. no. D6023; Takara Biotechnology Co., Ltd.) was used to sub-clone the cDNA into the expression vector pET28a(+) (cat. no. N72770; Novagen; Merck KGaA) to create pET28a(+)-Tyr p 13. *E. coli* DH5α cells (cat. no. D9057; Takara Biotechnology Co., Ltd.) were transformed with pET28a(+)-Tyr p 13 plasmids. Positive clones were selected by blue/white screening on LB plates containing 50 μg/ml kanamycin and verified by restriction enzyme analysis with *Nde*I and *Xho*I.

Production and purification of recombinant allergen. The pET-28a (+)-Tyr p 13 plasmid (0.5 μl) was purified using the MiniBEST Plasmid Purification Kit Ver. 4.0 (cat. no. 9760; Takara Biotechnology Co., Ltd.) and used to transform 100 μl *E. coli* BL21 (DE3) cells (cat. no. 200131; Agilent Technologies, Inc.). The pET28a(+)-Tyr p13-transformed *E. coli* BL21 cells were cultured in 3 ml LB liquid medium containing 50 μg/ml kanamycin for 8 h at 37˚C. After 8 h of cell culture, 2 ml cell culture was diluted into 100 ml LB + kanamycin in a glass tube and cultured at 37˚C until an approximate absorbance of 0.5 at 600 nm was reached. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mmol/l. The sample was incubated for 2 h at 30˚C. *E. coli* cells were then harvested by centrifugation (8,000 x g) at 4˚C for 10 min, resuspended in 15 ml Tris-HCl (pH 7.5) buffer and sonicated (20 kHz; ultrasound duration 3 sec, interval 10 sec) in an ice bath until the suspension became transparent. The cell debris and supernatant were separated by centrifugation (5,000 x g) at 4˚C for 40 min. The presence of the recombinant protein was verified by 10% SDS-PAGE and Coomassie Brilliant Blue R-250 (CBB-R250) staining.

The supernatant was applied to a Ni-NTA affinity chromatography column (cat. no. 70971; Novagen; Merck KGaA) pre-equilibrated with buffer [50 mmol/l Tris-HCl (pH 7.5), 0.3 mol/l NaCl]. The column was washed with 10 ml washing buffer 1 [50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l imidazole and 0.3 mol/l NaCl] to remove unbound proteins. Recombinant protein was eluted by a gradient of increasing concentrations of imidazole elution buffer. The elution flow rate was 1 ml/min. Eluents at different imidazole concentrations were collected and verified by 10% SDS-PAGE. The eluents containing high purity recombinant protein samples were mixed and desalinated by 12-h dialysis with several rounds

of buffer replacement. The purified product was separated by 10% SDS-PAGE and observed by Coomassie Brilliant Blue R-250 (CBB-R250) staining.

Specific IgE-binding to recombinant allergen. The ability of rTyr p 13 to bind to IgE was assessed by ELISA. The rTyr p 13 used for ELISA was purified by Ni NTA affinity chromatography. For antigen coating, 100 μl rTyr p 13 (1 μg/ml) diluted in coating buffer (0.1 mol/l PBS)was added to a 96-well microtiter plate and incubated at 8˚C overnight. Wells were washed with 300 μl PBS-0.05%Tween-20 (PBS-T) buffer and blocked with 3% bovine serum albumin (cat. no. A1933, Sigma-Aldrich; Merck KGaA) in PBS-T for 1 h at 37˚C. Subsequently, 100 μl patient serum and 100 μl healthy donor serum (diluted at 1:4 with PBS-T) were added, followed by overnight incubation at 4˚C. Secondary antibody (50 μl; HRP-mouse anti-human IgE; 1:5,000; Sigma-Aldrich; Merck KGaA) was added and samples were incubated at 37˚C for 1 h. To detect binding, 100 μl TMB substrate solution (cat. no. P0209; Beyotime Institute of Biotechnology) was added and samples were incubated for 15 min at room temperature. A total of 2 mol/l H_2SO_4 (50 μl) was added to stop the reactions. Optical density (OD) was measured for triplicate samples at a wavelength of 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.). The reactivity was considered positive if the OD values of the detected serum were higher than the cutoff ELISA value (mean ELISA value of healthy donors + 3 SD).

Results

Cloning Tyr p 13. Total RNA was isolated from adult mites, and Tyr p 13 cDNA was amplified by RT-PCR. A product of the expected size (486 bp) was produced (Fig. 1A). The product was cloned into the pMD20T vector and sequenced. Using the ORF Finder, a complete ORF was identified in the Tyr p 13 cDNA. The length from the start codon ATG to the stop codon TAA was 486 bp (Fig. 1B).

Inferred amino acid sequence, and its structural and functional prediction. Family classification revealed that Tyr p 13 belongs to the intracellular lipid-binding protein family. An amino acid sequence of 161 residues was predicted for Tyr p 13 using the Translate Tools on the ExPaSy web server. A signal peptide sequence from amino acids 1 to 22 was predicted using SignalP 4.1 software. The removal of the signal peptide sequence predicted by the SignalP 4.0 software (Fig. S1) yielded a predicted mature Tyr p 13 protein of 139 amino acid residues with a theoretical pI of 8.07. The instability index was 26.7, indicating a stable amino acid sequence. The grand average of GRAVY was -0.49, indicating that Tyr p 13 was hydrophilic. The NetPhos3.1 server was used to search the Tyr p 13 amino acid sequence for phosphorylation sites. Nineteen sites were predicted (Fig. S2), including seven serine sites positioned at residues 6, 17, 39, 63, 71, 87 and 103, nine threonine sites at residues 12, 53, 58, 61, 68, 84, 97, 107 and 133, and three tyrosine sites at residues 19, 43 and 120. The secondary structure of Tyr p 13 was predicted to comprise an α-helix (15 peptides, 10.79%), an extension chain (47 peptides, 33.81%) and a random coil (77 peptides,

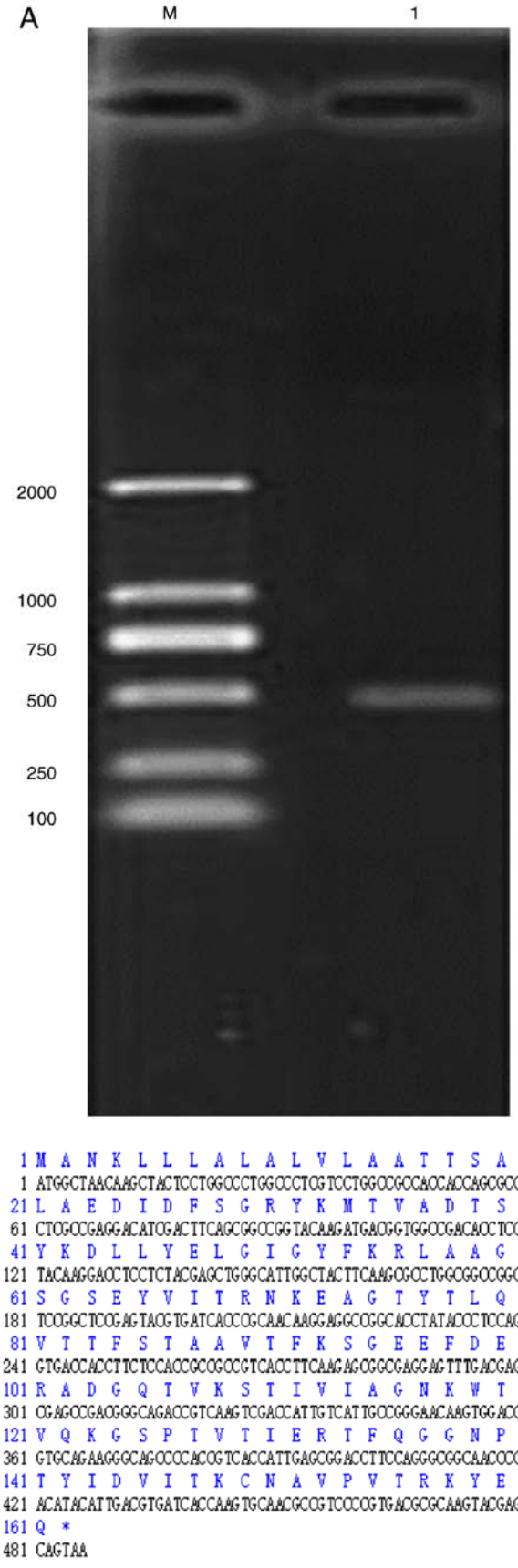

Figure 1. Cloning and sequencing of cDNA encoding Tyr p 13. (A) Amplification of Tyr p 13 cDNA by RT-PCR. Total RNA was isolated from mites and subjected to RT-PCR. The PCR products were separated on a 1% agarose gel containing ethidium bromide. Lane M, DNA Marker DL2, 000; Lane 1, PCR product (486 bp). (B) Nucleotide sequence and deduced amino acid sequence of Tyr p 13. Tyr p 13, *Tyrophagus putrescentiae* group 13 allergen; RT-PCR, reverse transcription-PCR.

```
EDIDFSGRYKMTVADTSNYKDLLYELGIGYFKRLAAGASGSEYVITRNKEAGTYTLQTVTTFSTAAVTFK
ccccccccceeeeccccchhhhhhhchhhhhhhhccccceeeeeecccccceeeeeeeecccceeecc
SGEEFDEPRADGQTVKSTIVIAGNKWTHVQKGSPTVTIERTFQGGNPPTYIDVITKCNAVPVTRKYERQ
ccccccccccccccceeeeeeeccccceeecccccceeeeeeccccccceeeeeeecccccccccccceec
```

Figure 2. Secondary structure analysis of the mature *Tyrophagus putrescentiae* group 13 allergen by GOR4.0 software. In the sequence, h, e, and c represent α-helix, extended strand and random coil, respectively.

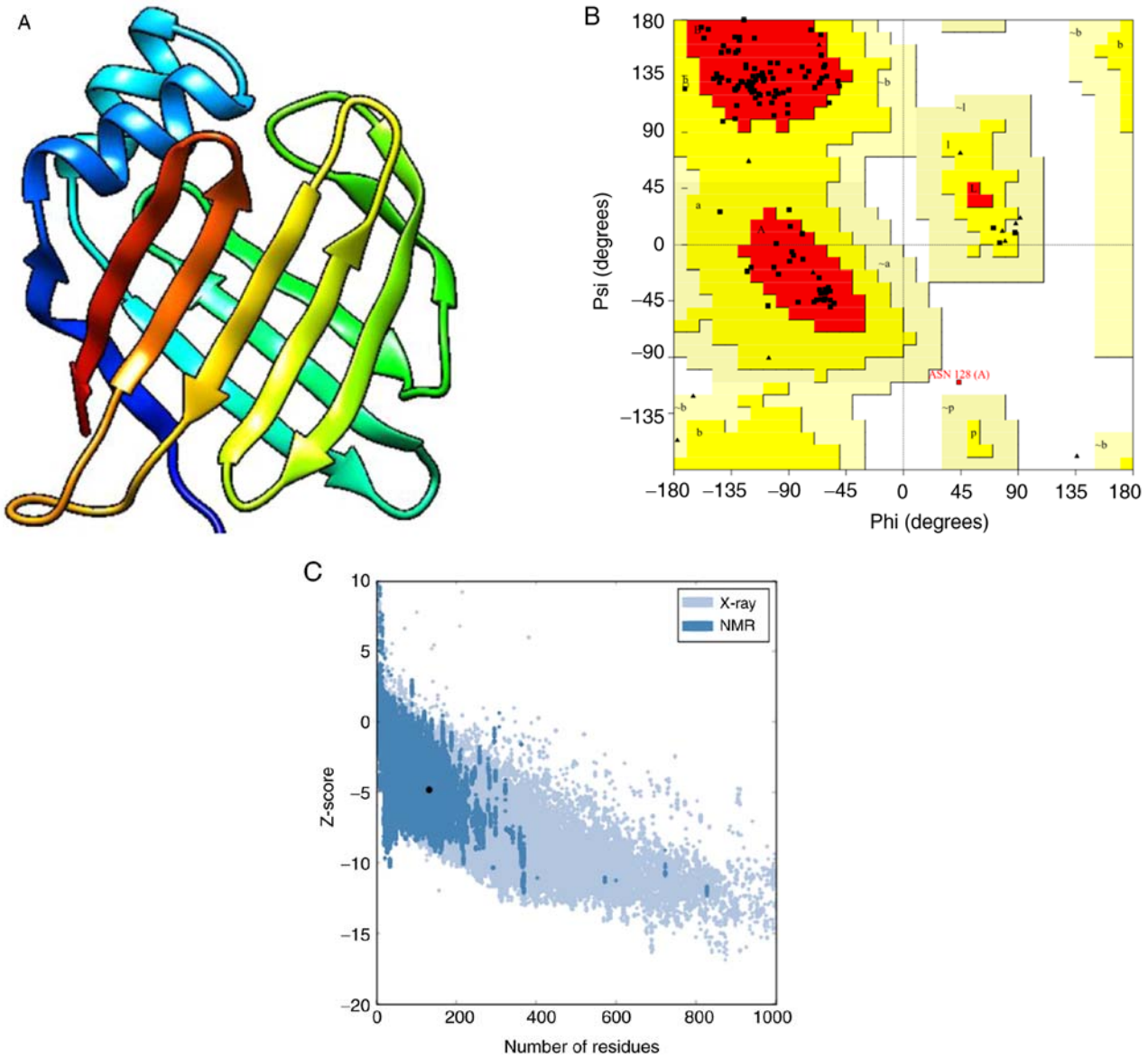

Figure 3. Three-dimensional structure and validation of Tyr p 13 homology model. (A) Structure of Tyr p 13 homology model. (B) Ramachandran plot of the Tyr p 13. (C) ProSA protein structure analysis results. Tyr p 13, *Tyrophagus putrescentiae* group 13 allergen.

55.40%; Fig. 2). The Tyr p 13 protein sequences were entered into the TMHMM Server 2.0, which predicted no transmembrane helices, and inferred that all protein sequences were located outside the membrane.

Homology modeling and model evaluation. The fatty acid-binding protein in brain tissue of *Drosophila melanogaster* (PDB accession no. 5GKB) belongs to the intracellular lipid-binding protein family (InterPro no. IPR031259), has a high sequence identity with Tyr p 13 (36.43%), and was used as the template for homology modeling (Fig. 3A). In the Ramachandran plot, 96.6% of the residues were in favored regions, 2.6% were in additional allowed parts, 0.8% were in generously allowed regions and 0% percent were in outlier regions (Fig. 3B). Based on the ERRAT results, the overall quality factor was 96.64%, indicating that the tertiary structure of Tyr p 13 had a high resolution. The Verify3D test requires an average 3D-1D score of ≥80% of the amino acids

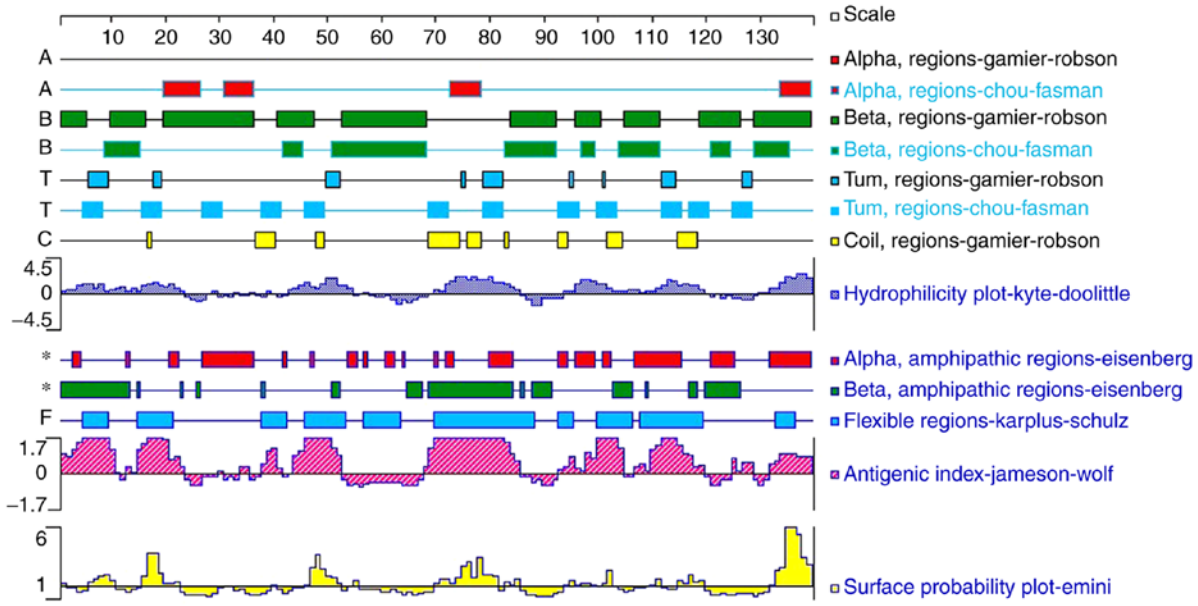

Figure 4. Analysis of *Tyrophagus putrescentiae* group 13 allergen secondary structure, hydropathy, antigenicity, surface probability and flexibility by Protean software.

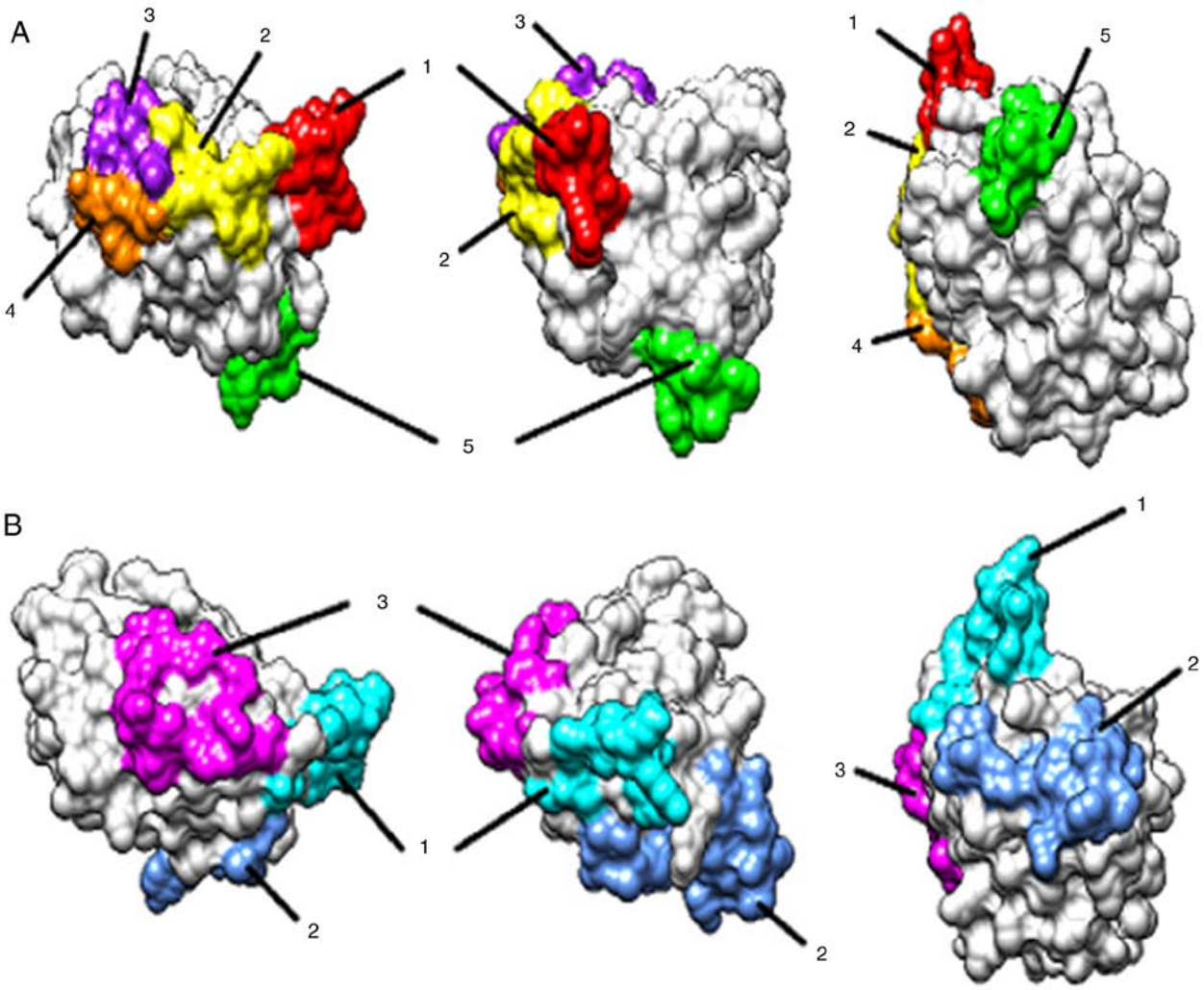

Figure 5. Possible B-cell epitopes within the 3D structure of Tyr p 13. (A) Linear Bcell epitopes within the 3D structure of Tyr p 13. (B) Discontinuous B cell epitopes within the 3D structure of Tyr p 13. Each color represents different linear/discontinuous epitopes, and the numbers correspond to the epitope numbers displayed in Table I. Tyr p 13, *Tyrophagus putrescentiae* group 13 allergen.

Table I. Predicted *Tyrophagus putrescentiae* group 13 allergen Bcell epitopes.

Epitopes	Peptide and position	Number of residues
Predicted linear epitopes		
1	RNKEAGT (47-53)	7
2	KSGEEFD (70-76)	8
3	DGQTVK (81-86)	7
4	KGSPT (101-105)	5
5	FQGGNPPTY (112-120)	9
Predicted discontinuous epitopes		
1	R 47, N 48, K 49, E 50, A 51, G 52, T 53, K 70, S 71, G 72, E 73	11
2	I 91, A 92, G 93, N 94, F 112, Q 113, G 114, G 115, N 116, P 117, P 118, T 119, Y 120, I 121, R 138	15
3	E 74, D 76, R 79, D 81, G 82, Q 83, T 84, K 86, K 101, G 102, S 103, P 104, T 105	13

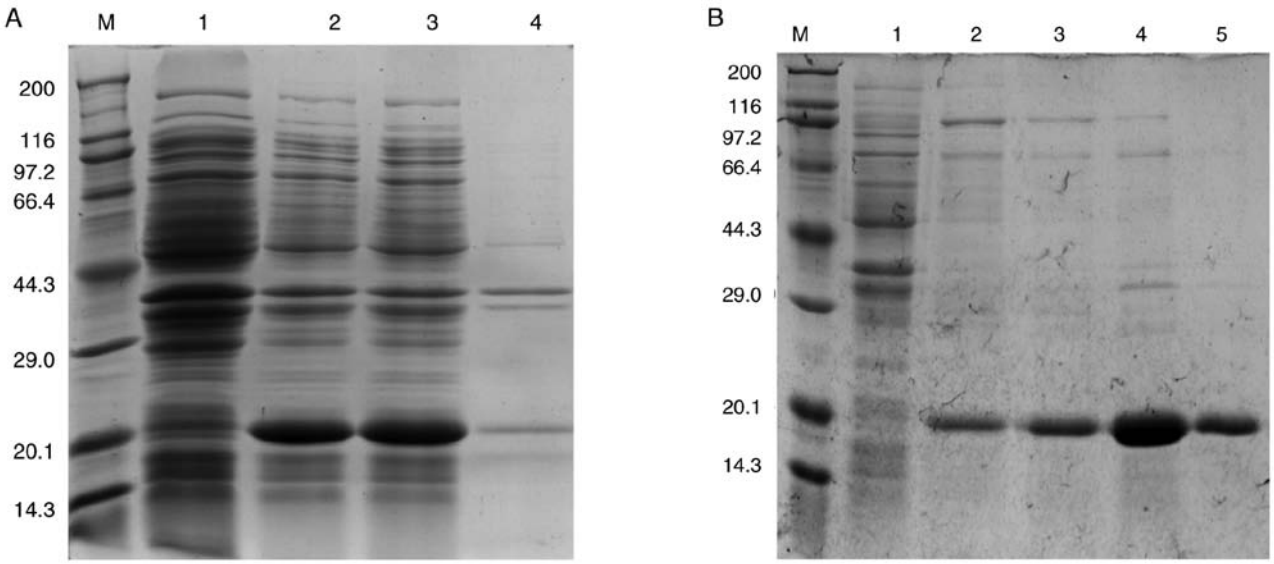

Figure 6. Expression of rTyr p 13 in *E. coli* BL21 cells. (A) SDS-PAGE of rTyr p 13 expressed in *E. coli* BL21 cells. Lane M, Takara Protein Marker (Broad); Lane 1, whole cell lysate of *E. coli* BL21 cells containing pET-28a as a negative control; Lane 2, whole-cell lysate of *E.coli* BL21 cells containing pET-28a (+)-Tyr p 13; Lane 3, supernatant of cells containing pET-28a (+)-Tyr p 13; Lane 4, pellet of cells containing pET-28a (+)-Tyr p 13. (B) SDS-PAGE of purified rTyr p 13. Lane M, Takara Protein Marker (Broad); Lane 1, protein flow-through from the column; Lanes 2, 3, 4 and 5, eluted fractions with 0, 10, 50 and 250 mmol/l imidazole elution buffer, respectively. rTyr p 13, recombinant *Tyrophagus putrescentiae* group 13 allergen.

to be ≥0.2. The VERIFY 3D results showed that 84.44% of residues had an average 3D1D score of ≥0.2, which suggested that the structures were favorable. ProSA analysis revealed that the predicted model was comparable to other acceptable proteins with a z score of -4.78 (Fig. 3C). The QMEAN server results revealed that the QMEAN Zscore was 1.61, thus suggesting that the protein model variation rate was low, overall folding and local structures had high accuracy rates, and stereochemistry was reasonable. Furthermore, the Q value was 0.7, which indicated that the predicted model of Tyr p 13 was reliable. These findings revealed that the tertiary structure model of Tyr p 13 was reliable and was suitable for use in the current study.

Prediction of B-cell epitopes by an integrated strategy. α-Helices and β-sheets have been reported to have high chemical bond energies and to be unlikely to form epitope sequences (24). By contrast, β-turns and random coils are located in surface-exposed regions of a protein, which often contain epitope sequences (21). The secondary structure, hydrophilicity, antigenicity, amphilicity, surface probability and flexibility of Tyr p 13 were analyzed using the Protean Software (for Protein Structure Analysis and Prediction) of the DNASTAR Lasergene software suite Ver. 7.1 (Fig. 4). Based on these results, linear and discontinuous B-cell epitopes were predicted by ElliPro software (Table I). Five amino acid peptide sequences were identified as promising linear epitopes (Fig. 5A) and three clusters were predicted to form discontinuous epitopes (Fig. 5B).

Expression and purification of recombinant allergen (rTyr p 13). Tyr p 13 cDNA was sub-cloned into the pET-28a (+) vector to produce a recombinant His-tagged protein. The recombinant

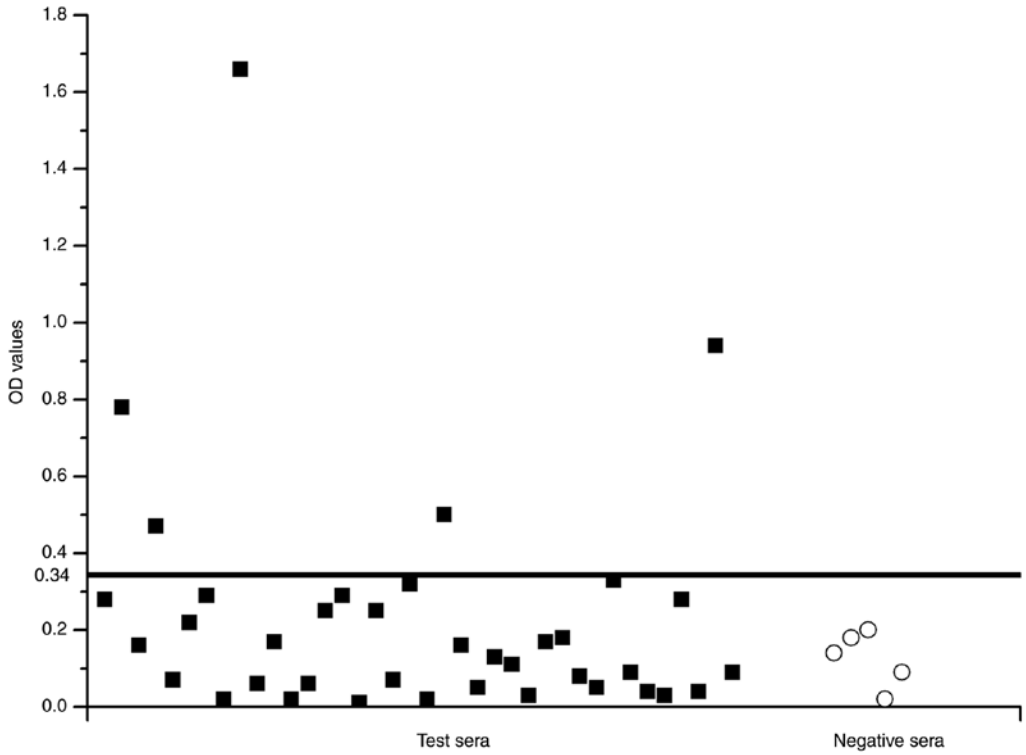

Figure 7. IgE reactivity of serum to recombinant *Tyrophagus putrescentiae* group 13 allergen. Each dot on the grapH indicates an individual serum sample. The cutoff value (straight line) is the mean OD value of healthy donors + three SD. OD, optical density.

His-tagged protein (rTyr p 13) was successfully purified by Ni-NTA affinity chromatography (Fig. 6).

IgE reactivity to rTyr p 13. ELISA was used to determine whether *E. coli*-produced rTyr p 13 had an IgE-binding ability with purified rTyr p 13 as a coat antigen. The mean OD value of the five healthy donors was 0.12 and the standard deviation (SD) was 0.0726. The postulated cutoff value was 0.34 (the mean OD value of healthy donors + three SD) (Fig. 7). Those with OD values >0.34 were judged to be IgE-positive. Positive IgE reactions to rTyr p 13 were detected in the serum of 13.2% (5/38) of the *T. putrescentiae*-allergic patients. The results indicated that rTyr p 13 had the ability to bind to IgE.

Discussion

Allergic diseases caused by *T. putrescentiae*, a type of storage mite, are common in several countries worldwide, including China. The positive skin prick test prevalence for *T. putrescentiae* in 2012 was 63% in Guangzhou, China (25). A better understanding of *T. putrescentiae* allergens may aid the diagnosis and treatment of *T. putrescentiae* allergies. For example, polymorphisms have been described for several storage mite allergens from different regions (25,26). Polymorphisms can have an important effect on the epitopes recognized by T lymphocytes, monoclonal antibodies and IgE of allergic patients (27). Therefore, in the present study, cDNA encoding Tyr p 13 was amplified from mites collected from a flour storage warehouse in China; subsequently, rTyr p 13 was expressed in an *E. coli* expression system and was purified by affinity chromatography.

Bioinformatics tools are crucial to allergy research, particularly the characterization of allergens by identification of structural motifs and epitopes and are complementary to experimental studies of allergens. Bioinformatics analysis of the sequence of Tyr p 13 revealed it to be a hydrophilic and stable protein, with no transmembrane helices and all protein sequences located outside the membrane. In addition, homology modeling based on a template was used to predict the tertiary structure of rTyr p 13. The key to homology modeling is to identify the right template; notably, quality of the homology model is dependent on high quality sequence alignment and template structure. The fatty acid-binding protein in brain tissue of *D. melanogaster* has a high sequence identity with Tyr p 13 (36.43%), and both proteins belong to the intracellular lipid-binding protein family (IPR031259). Therefore, the fatty acid-binding protein in brain tissue of *D. melanogaster* can be used as a modeling template. Following homology modeling using SWISS-MODEL, various additional parameters/programs were incorporated to establish a reliable model of Tyr p 13. Future work on these features can improve our understanding of Tyr p 13.

The specific interaction of allergens with IgE antibodies is a key event in allergic diseases. The present study assessed the allergenicity of rTyr p 13 and revealed that rTyr p 13 bound with serum from 13.2% (5/38) of patients allergic to *T. putrescentiae*, according to the results of ELISA. Furthermore, studies have determined a correlation between the severity or persistence of allergic diseases and the diversity of B-cell epitopes (IgE-binding epitopes) (27). The identification of B-cell epitopes of allergens is valuable for accurate and safe peptide-based allergen diagnosis, such as peptide-ELISA or epitope-ELISA,

and immunotherapy. The *in-silico* prediction of B-cell epitopes is considered a useful tool for selecting B-cell epitopes from immunologically relevant proteins and has been reported to be well correlated with the experimental approach (23). In most cases, B-cell epitopes are located on the surface of antigen molecules. Secondary and tertiary protein structures also contain important information regarding B-cell epitope prediction. For example, β-turns and random coils are detected in surface-exposed protein regions of, which often contain epitope sequences (21). Numerous algorithms have been generated to predict B-cell epitopes on protein sequences; these algorithms are based on the propensity values of the amino-acid properties of hydrophilicity, antigenicity, flexibility and accessibility. By integrating DNAStar Protean and ElliPro analysis results, and combining information from secondary and tertiary structures, the present study identified potential B-cell linear and discontinuous epitopes. However, further experimental verification is required for these predicted epitopes. Therefore, the findings of the present study may be useful not only for further work on Tyr p 13 but may also lay the foundation for the study of peptide-based allergen diagnosis and immunotherapy.

In conclusion, the present study demonstrated that the cloning, expression, characterization and B cell epitopes of recombinant Tyr p 13 protein provided initial evaluation of its potency as an allergen in mite-allergic individuals. These findings provided a foundation for which to explore the structural biology and biochemistry of Tyr p 13 protein, thereby enabling future work with ASIT.

Acknowledgements

Not applicable.

Funding

This work was supported by the National Sciences Foundation of China (grant nos. NSFC30060166, NSFC81001330 and NSFC31272369).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NW and YC conceived and designed the study. NW and YZ performed the experiments and wrote the manuscript. YC revised the manuscript critically. MW and HZ collected the patients' samples and analyzed the data. All authors contributed to the preparation of the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All participants provided written informed consent. The present study was approved by the Ethics Committee for Clinical Investigation of Wuxi People's Hospital Affiliated to Nanjing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Jeong KY, Lee H, Lee JS, Lee J, Lee IY, Ree HI, Hong CS and Yong TS: Molecular cloning and the allergenic characterization of tropomyosin from *Tyrophagus putrescentiae*. Protein Pept. Lett 14: 431-436, 2007.
2. Aygun O, Yaman M and Durmaz H: A survey on occurrence of *Tyrophagus putrescentiae* (Acari: Acaridae) in Surk, a traditional Turkish dairy product. J Food Eng 78: 878-881, 2007.
3. Uzunoğlu Karagöz E, Akdemir C, Direkel Ş and Cebeci Guler N: The investigation of the presence of mites in some served dry foodstuffs. Turkiye Parazitol Derg 41: 92-95, 2017.
4. Liao EC, Hsu EL, Tsai JJ and Ho CM: Immunologic characterization and allergenicity of recombinant Tyr p 3 allergen from the storage mite Tyrophagus putrescentiae. Int Arch Allergy Immunol 150: 15-24, 2009.
5. Mondal P, Dey D, Sarkar T, Laha A, Moitra S, Bhattacharyya S, Saha NC, Saha GK and Podder S: Evaluation of sensitivity toward storage mites and house dust mites among nasobronchial allergic patients of kolkata, india. J Med Entomol 56: 347-352, 2019.
6. Szilman E, Szilman P, Solarz K, Brewczyński P and Sieroń AL: Sensitization to the storage mite *Tyrophagus putrescentiae* in urban population of Upper Silesia (Poland). Wiad Parazytol 50: 471-476, 2004.
7. Tabesh S, Fanuel S, Fazlollahi MR, Yekaninejad MS, Kardar GA and Razavi SA: Design and evaluation of a hypoallergenic peptide-based vaccine for Salsola kali allergy. Int Immunopharmacol 66: 62-68, 2019.
8. Niederberger V and Valenta R: Molecular approaches for new vaccines against allergy. Expert Rev Vaccines 5: 103-110, 2006.
9. Ramos JD, Valmonte GR and de Guia RM: Recombinant proteins and peptides as diagnostic and therapeutic reagents for arthropod allergies. Protein Pept Lett 14: 992-1002, 2007.
10. Yang L and Kulis M: Hypoallergenic proteins for the treatment of food allergy. Curr Allergy Asthma Rep 19: 15, 2019.
11. Cui Y: Immunoglobulin E-binding epitopes of mite allergens: from characterization to immunotherapy. Clin Rev Allergy Immunol 47: 344-353, 2014.
12. Teng FX, Huang HF, Ge DZ, Yu LL, Xu C and Cui YB: *Tyrophagus putrescentiae* group 4 allergen allergenicity and epitope prediction. Allergol Immunopathol (Madr): 2020.
13. Cui Y, Yu L, Teng F, Zhang C, Wang N, Yang L and Zhou Y: Transcriptomic/proteomic identification of allergens in the mite *Tyrophagus putrescentiae*. Allergy 71: 1635-1639, 2016.
14. Nicklas RA: National and international guidelines for the diagnosis and treatment of asthma. Curr Opin Pulm Med 3: 51-55, 1997.
15. Li Y, Guifang M, Qisong L, Yubao C and Jinfang S: Use of a multimedia diagnostic microscope to morphologically identify adult *Tyrophagus putrescentiae*. J Pathogen Biology 13: 164-167, 2018.
16. Cui YB, Zhou P, Peng JL, Peng M, Zhou Y, Lin YZ and Liu L: Cloning, sequence analysis, and expression of cDNA coding for the major house dust mite allergen, Der f 1, in *Escherichia coli*. Braz J Med Biol Res 41: 380-388, 2008.
17. Mitchell A, Chang HY, Daugherty L, Fraser M, Hunter S, Lopez R, McAnulla C, McMenamin C, Nuka G, Pesseat S, *et al*: The InterPro protein families database: The classification resource after 15 years. Nucleic Acids Res 43: D213-D221, 2015.
18. de Castro E, Sigrist CJ, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A and Hulo N: ScanProsite: Detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Res 34: W362-W365, 2006.
19. Cui YB, Yu LL, Teng FX, Wang N, Zhou Y, Yang L and Zhang CB: Dust mite allergen Der f 4: Expression, characterization, and IgE binding in pediatric asthma. Pediatr Allergy Immunol 27: 391-397, 2016.

20. Krogh A, Larsson B, von Heijne G and Sonnhammer EL: Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J Mol Biol 305: 567-580, 2001.
21. Teng F, Yu L, Sun J, Wang N and Cui Y: Homology modeling and prediction of Bcell and Tcell epitopes of the house dust mite allergen Der f 20. Mol Med Rep 17: 1807-1812, 2018.
22. Shahsavani N, Sheikhha MH, Yousefi H and Sefid F: In silico homology modeling and epitope prediction of NadA as a potential vaccine candidate in neisseria meningitidis. Int J Mol Cell Med 7: 53-68, 2018.
23. Tong X, Guo M, Jin M, Chen H, Li Y and Wei JF: *In silico* epitope prediction, expression and functional analysis of Per a 10 allergen from the American cockroach. Int J Mol Med 38: 1806-1814, 2016.
24. Sikic K, Tomic S and Carugo O: Systematic comparison of crystal and NMR protein structures deposited in the protein data bank. Open Biochem J 4: 83-95, 2010.
25. Zhang C, Li J, Lai X, Zheng Y, Gjesing B, Spangfort MD and Zhong N: House dust mite and storage mite IgE reactivity in allergic patients from Guangzhou, China. Asian Pac J Allergy Immunol 30: 294-300, 2012.
26. Zakzuk J, Jiménez S, Cheong N, Puerta L, Lee BW, Chua KY and Caraballo L: Immunological characterization of a Blo t 12 isoallergen: identification of immunoglobulin E epitopes. Clin Exp Allergy 39: 608-616, 2009.
27. Cui Y, Zhou Y, Wang Y, Ma G and Yang L: The group 10 allergen of *Dermatophagoides farinae* (Acari: Pyroglyphidae): cDNA cloning, sequence analysis, and expression in *Escherichia coli* BL21. J Med Entomol 50: 205-208, 2013.