

Expression, cloning, and IgE-binding of the full-length dust mite allergen Der f 8

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Abstract *Dermatophagoides farinae*, a domestic mite species, produces some of the most potent allergens that contribute to allergy in China and worldwide. We sought to clone and express the group 8 allergen of *D. farinae* (Der f 8) to investigate its IgE-binding reactivity. The full-length cDNA encoding Der f 8 was generated by using RT-PCR and 5' RACE, cloned into pCold-TF expression vector, confirmed by nucleotide sequencing, sub-cloned into pET-28b (+), and transfected into *E. coli* BL21 cells for expression. After purification by nickel affinity chromatography and identified by SDS-PAGE, the recombinant Der f 8 bound with sera from 40.9 % (9/22) of mite-allergic patients according to ELISA testing. Analysis of the recombinant DNA sequence revealed a 231 amino acid open reading frame encoding a protein with a derived molecular mass of 26.4 kDa and an isoelectric point of 6.84. The deduced amino acid sequence has nine phosphorylation sites, displaying strong homology with glutathione S-transferase, and its secondary structure comprises alpha helix (45.5 %), extended strand (11.3 %), and random coils (43.3 %). BLAST through the National Center for Biotechnology Information database and alignment identified similarity with group 8 allergens or glutathione S-transferases of *Dermatophagoides pteronyssinus*, *Suidasia medanensis*, *Lepidoglyphus destructor*, *Glycyphagus domesticus*, and *Aleuroglyphus ovatus* (64, 65, 53, 53, and 50 %, respectively). The first recombinant Der f 8 protein produced in full length successfully bound with patient

IgE, demonstrating the importance of Der f 8 in mite allergy.

Keywords *Dermatophagoides farinae* · Recombinant allergen · Molecular cloning · Domestic mite

Introduction

Domestic mite allergy, which is found in populations around the world, produces asthma, atopic dermatitis, and perennial rhinitis, among other symptoms. Allergen avoidance and pharmacotherapy are currently the mainstay treatments of these diseases, but they are not easily able to change the course of disease [1]. Allergen-specific immunotherapy (ASIT) has been used with success in allergy treatment, including of mite allergy, and may offer more opportunity for changing the disease course. However, ASIT with mite extracts, a mixture of allergens, non-allergens, and other proteins cannot be standardized fully and can cause severe side effects like anaphylaxis.

Of the 24 groups of dust mite allergens listed in the International Union of Immunological Societies (IUIS) nomenclature dataset (<http://www.allergen.org/>), 22 have been identified from *Dermatophagoides* spp. The groups 1 and 2 allergens are considered the major potency allergens, reacting with sera from 80 % of mite-sensitive patients. Recombinant groups 1 and 2 allergens from *Dermatophagoides pteronyssinus* have varying IgE reactivity; the recombinant Der p 1 exhibits weak reactivity, while the recombinant Der p 2 exhibits comparable IgE recognition to the native allergen and shows promise for ASIT [2, 3]. Increasing efforts are devoted to developing recombinant allergens that effectively recapitulate their natural counterparts but that can be administered with defined concentrations and consistent quality. Further,

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recombinant allergens can be modified to reduce allergenicity and retain immunogenicity, which may increase safety and efficacy of allergy treatments [4–6].

In China, a cross-sectional survey in 6,304 patients suffering from asthma and/or rhinitis across 17 cities revealed that 59.5 % were hypersensitive to *Dermatophagoides farinae*, 57.6 % to *D. pteronyssinus*, and 40.7 % to *Blomia tropicalis* [7]. *D. farinae* therefore makes the most significant contribution to allergy for individuals in China with domestic mite hypersensitivity. Our laboratory has cloned and expressed several allergens from *D. farinae* [8–15] toward a better understanding of the allergens and how they induce hypersensitivity. Here, we sought to clone the full-length group 8 allergen from *D. farinae* (Der f 8) for the first time. The results reported here will provide a foundation for both component-resolved diagnosis and ASIT production for domestic mite allergy in China and, likely, other parts of the world.

Materials and methods

Patient sera

Peripheral blood samples were collected from patients with allergic asthma in the Respiratory Department, Affiliated Nanjing Children's Hospital, Nanjing Medical University, Jiangsu, P. R. China. Patients had not received any treatment. Asthma diagnosis followed WHO criteria: all children had cough, asthma, obvious wheeze, rhonchus and moist rale in the lung, and other clinical manifestations; and X-ray examination showed increased lung markings or lung hyperinflation and infection, etc. [16]. The diagnosis of allergy to dust mite allergens was established on the basis of a suggestive clinical history and positive serum IgE (sIgE) to *D. farinae* extracts as determined by the Allergy Screening test panel for atopy (Mediawiss Analytic, Moers, Germany). The inclusion criteria were (1) allergy to *D. farinae* extracts with more than 1 year of follow-up and (2) levels of sIgE to *D. farinae* extract higher than 3 kU/L at the time of inclusion into the study. A total of 22 pediatric patients, mean age 4.833(±2.041) years, were enrolled as serum positive for *D. farinae*, and then used for detection of specific IgE-binding to recombinant fusion allergen. Additionally, the sera of five non-atopic healthy children, mean age 5.1,667 (±1.169) years, were treated as negative controls. The study protocol was approved by the Ethics Committee for Clinical Investigation of Nanjing Children's Hospital.

Obtaining the full sequence of the group 8 allergen of *Dermatophagoides farinae* (Der f 8)

Dermatophagoides farinae were cultured and isolated as we previously reported (8–15), and total RNA was isolated

using RNA isolator (TaKaRa Biotech, Dalian, China. No. D312) and stored at −80 °C. DNA primers were designed and synthesized based on the published partial sequence of Der f 8 (GenBank Accession No. AY283295). The sequence of the forward primer F₁ was 5'-GGCTA TTGGGATGCACGTGGTCTT-3' and of the reverse primer R₁ was 5'-TATTCCGAAACTTTGGGCAATGCT-3'. cDNA was generated by RT-PCR as follows. Reaction mix [total RNA (1 µL), 10 mM dNTP mixture (1 µL), 20 µM random primer (1 µL), and RNase-free H₂O (7 µL)] was incubated at 65 °C for 5 mins then placed on ice for 2 mins. We then added 5 × PrimeScript RT Buffer (TaKaRa Biotech, No. DR027S) (4 µL), 40 U/µL of RNase Inhibitor (0.5 µL), PrimeScript RTase (TaKaRa Biotech No. 2680A, for the two-step method) (0.5 µL), and RNase-free dH₂O (5 µL) to the mixture. A fraction of the final reaction mixture (10 µL) was then incubated sequentially at 30 °C (10 min), 42 °C (60 min), and 70 °C (15 min). PCR was performed using TaKaRa LA Taq[®] with GC Buffer (TaKaRa Code No. DRR02AG). The total PCR reaction mixture (25 µL) contained the RT product (1 µL), 2.5 mM of dNTP mixture (4 µL), 20 µM of the forward primer F₁ (0.5 µL), 20 µM of the reverse primer (0.5 µL), 2 × GC Buffer (12.5 µL), 5 U/µL of TaKaRa LA Taq (0.25 µL), dH₂O (6.25 µL). Thermal cycling was performed as follows: 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; and a final step at 72 °C for 10 min. In total, 5 µL of the PCR product was analyzed by agarose gel electrophoresis (1.0 %) and visualized with ImageMaster[®] VDS. The PCR product was recovered by Agarose Gel DNA Purification Kit Ver 2.0 (TaKaRa Code No. DV805A) and sequenced using an ABI PRISM[™] 377XL DNA Sequencer (TaKaRa Biotech) by the above primers F₁ and R₁.

A second primer was designed and synthesized according to the above-sequencing results (R₂: 5'-TTCTGGACGATCGCTGCCCTATAC-3'). After the total RNA was treated with alkaline phosphatase (CIAP) and tobacco acid pyrophosphatase (TAP), the 5' RACE Adaptor (5'-Full RACE Kit, TaKaRa Code No. D315) was then used to join segments. The reaction mixture for reverse transcription (RT) contained the ligated RNA (6 µL), 50 random 9-mers (0.5 µL), and 10 mM dNTP Mixture (1 µL); mix was incubated at 70 °C for 10 min, then placed on ice for 2 min. 5 × M-MLV Buffer (2 µL), 40U/µL of RNase Inhibitor (0.25 µL), and 200 U/µL of reverse transcriptase M-MLV (0.25 µL) were added. The final reaction mixture (10 µL) was incubated at 42 °C for 60 min and 70 °C for 15 min. The first PCR was performed using LA Taq[®] with GC Buffer (TaKaRa Code No. DRR02AG) and with the primers R₁ and 5' RACE Outer Primer (CATGGCTACATGCTGACAGCCTA). The second PCR was performed with the primer R₂ and 5' RACE

Inner Primer (CGCGGATCCACAGCCTACTGATGATCAGTCGATG). After the second PCR, amplified DNA was recovered using the Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa Code No. DV805A), and the cDNA was cloned into pMD19-T simple vector (TaKaRa Code No. D102A). *E. coli* JM109 (TaKaRa Code No. D9052) was then transformed by the recombinant plasmid, positive clones were selected by blue/white screening on Luria-Bertani (LB) plates containing 100 µg/mL ampicillin, and samples were submitted for automatic DNA sequencing. Merging the sequencing results revealed the full sequence for Der f 8.

Cloning and DNA sequencing

Primers were designed to amplify full-length Der f 8: forward, 5'-TACCCTCGAGGGATCCATGAGCGAAACAAAACCAATTC-3'; reverse, 5'-TAGACTGCAGGTCGACTCATGCATATGTACCATTCCAT-3' with a *Bam* H I and *Sal* I site at their 5' end as underlined. First, reverse transcription was performed with PrimeScript™ RT-PCR Kit (Code No. DR014A) using the PCR Thermal Cycler Dice (TaKaRa Biotech Co. Ltd). The reverse transcription reaction mixture contained total RNA (1 µL), 10 mM dNTP Mixture (1 µL), 20 µM Random 6-mers (1 µL), and

RNase-free H₂O (7 µL), and was incubated at 65 °C for 5 min then placed on ice for 2 min. Next, 5 × PrimeScript RT Buffer (4 µL), 4 U/µL of RNase Inhibitor (0.5 µL), PrimeScript RTase (0.5 µL), and RNase-free dH₂O (5 µL) were added in. The final reaction mixture (10 µL) was incubated at 30 °C for 10 min, 42 °C for 30 min, and 95 °C for 5 min. The RT product was used as the template for PCR in the Thermal Cycler Dice with PrimeSTAR® HS DNA Polymerase (TaKaRa, DR044A). The total reaction mixture contained RT products (1 µL), 2 × PrimeSTAR GC Buffer (25 µL), 2.5 mM each of dNTP Mixture (4 µL), 20 µM of the forward primer F (1 µL), 20 µM of the reverse primer R (1 µL), 2.5 U/µL of PrimeSTAR® HS DNA Polymerase (0.5 µL), dH₂O (17.5 µL). PCR conditions were as follows: an initial incubation for 3 min at 94 °C; 30 cycles of 10 s at 98 °C, 15 s at 55 °C, and 1 min at 72 °C; and a final incubation for 10 min at 72 °C. In total, 5 µL of the PCR product was analyzed by agarose electrophoresis (1.0 %) and visualized with ImageMaster® VDS.

After the PCR-amplified DNA was recovered with a MiniBEST Agarose Gel DNA Purification Kit Ver 2.0 (TaKaRa Code No. D823A), it was then cloned into pCold-TF with solution of the DNA Ligation Kit (TaKaRa Code No. D6020A). *E. coli* JM109 (TaKaRa Biotech Co. Ltd,

Fig. 1 Nucleotide sequence and deduced amino acid sequence of Der f 8

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1  atggagctcggtaccctcgaggatccatgagcgaaacaaaccaattcttggctattgg
   M E L G T L E G S M S E T K P I L G Y W
61  gatgcacgtggtcttggccaagccatacgtttattattaacatatgccggtgttgatttc
   D A R G L G Q A I R L L L T Y A G V D F
121 atcgataaacggttatactgttgggtccaccacaaattatgatcggttcacaatgggtgaat
   I D K R Y T V G P P P N Y D R S Q W L N
181 gataaatataatcttggattagattttccaaattgtccatattatattgatggtaattga
   D K Y N L G L D F P N C P Y Y I D G N V
241 aaattatcacaatcattggccattattcgatatattgcacgtaaacaaaaattaattgga
   K L S Q S L A I I R Y I A R K Q K L I G
301 caaaatgaacatgaagaaatacgtgcatcattagctgaacaacagattatcgatatgaat
   Q N E H E E I R A S L A E Q Q I I D M N
361 atggctattgcacgtattgcatataatccaaattgtgaaaaattgaaaccagaatttctt
   M A I A R I A Y N P N C E K L K P E F L
421 aaatcattacctgaacaagtagaattattgtcaaaatttcttggcgatcaaccattcatt
   K S L P E Q V E L L S K F L G D Q P F I
481 gccggtgcaaacatttcttatgctgattttttgctatatgaatatctaactaaacttaag
   A G A N I S Y A D F L L Y E Y L T K L K
541 atactagtccagaagtttatgataaatttgaaaattcttaaaaaattccatggaaacgta
   I L V P E V Y D K F E N L K K F H G N V
601 ttggaagcattgccaaagagtttcagaatatattaaaaacaacaacaaaagcttttcat
   L E A L P R V S E Y I K K Q Q P K A F H
661 ggtccaacatcattatggaatggtacatatgcatga
   G P T S L W N G T Y A -

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Code No. D9052) was then transformed with the recombinant plasmid pCold-TF-Der f 8, positive clones were selected by blue/white screening on Luria–Bertani (LB) plates containing 100 µg/mL ampicillin, and samples were submitted for automatic DNA sequencing on ABI PRISM™ 377XL DNA Sequencer (Perkin Elmer) with primers for pCold-TF: P₁, 5'-GCGGGTCTGGAAGTTCT GTT-3' and P₂, (5'-CCAAATGGCAGGGATCTTAG -3').

Construction of expression plasmid pET-28b (+)-Der f 8

The full-length sequence of Der f 8 was used to design the following primers for amplification: forward, 5'-CTCC ATGGAGCTCGGTACCCTAGAGGGAT-3'; reverse, 5'-TCCTCGAGTGCATATGTACCATTCATAATGATGT-3' with an *Nco* I and *Xho* I site at their 5' end as underlined. The pCold-TF-Der f 8 was used as the PCR template. Total reaction mixture contained 10 × Pyrobest PCR Buffer (5 µL), 10 mM dNTP Mixture (1 µL), TaKaRa Pyrobest® DNA Polymerase (0.5 µL), 10 mM dNTP Mixture (8 µL), 20 µM of the forward primer F' (1 µL), 20 µM of the reverse primer R' (1 µL), the plasmid pCold-TF-Der f 8 (0.5 µL), and ddH₂O (34 µL). PCR conditions were as follows: initial incubation for 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 45 s at 72 °C; and final incubation for 10 min at 72 °C. The PCR product was analyzed by agarose electrophoresis (1.0 %) and visualized with ImageMaster® VDS. The recovered PCR product was digested with *Nco* I and *Xho* I and then sub-cloned into the expression vector pET-28b (+) (Novagen, Kit Lot No. 69865) to create pET-28b (+)-Der f 8 using the DNA Ligation Kit (TaKaRa Biotech, D6023). *E. coli* JM109 competent cells (TaKaRa Biotech, D9052) were transformed with pET-28b (+)-Der f 8 plasmids, positive clones were selected by blue/white screening and verified by restriction enzyme analysis with *Nco* I and *Xho* I.

Production and purification of recombinant fusion protein

pET-28b (+)-Der f 8 plasmid (0.5 µL) that was purified using the MiniBEST Plasmid Purification Kit Ver. 2.0 (TaKaRa Biotech, DV801A) was used to transform 100 µL of *E. coli* BL21 (DE3, Stratagene) and was identified by PCR with the primers F' and R'. Overnight culture of *E. coli* BL21 carrying pET-28b (+)-Der f 8 grown at 37 °C in L-broth containing 50 mg/L of Kanamycin was diluted to 100-fold with the same medium and further cultured for 2 h at 37 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 µM to induce the *tac* promoter controlling expression of the cloned Der f 8. After culturing at 37 °C, *E. coli* colonies were harvested by

Table 1 Motifs of functional site prediction of the mature Der f 8 allergen

Tools	Motifs of functional site	AA position
ScanProsite	<i>Soluble glutathione S-transferase N-terminal domain profile</i>	13–100
	<i>Soluble glutathione S-transferase C-terminal domain profile</i>	102–219
InterProScan	<i>Glutathione S-transferase, Mu Class</i>	43–55
		56–68
		99–110
		151–164
	<i>Glutathione S-transferase, N-terminal</i>	13–100
	<i>Glutathione S-transferase, C-terminal</i>	118–201
	<i>Glutathione S-transferase, C-terminal-like</i>	98–215
	<i>Thioredoxin-like fold</i>	10–93
	<i>Glutathione S-transferase/chloride channel, C-terminal</i>	102–219
MotifScan	<i>ASN_Glycosylation N-glycosylation site</i>	164–167
		227–230
	<i>cAMP- and cGMP-dependent protein kinase phosphorylation site</i>	43–46
	<i>Casein kinase II phosphorylation site</i>	9–12
		110–113
		142–145
		166–169
	<i>N-myristoylation site</i>	4–9
		24–29
		162–167
	<i>Soluble glutathione S-transferase C-terminal domain profile</i>	102–219
	<i>Soluble glutathione S-transferase N-terminal domain profile</i>	13–100
	<i>NAC domain profile</i>	1–95
	<i>Glutathione S-transferase profile (C-terminal sub-domain)</i>	146–203
	<i>Glutathione S-transferase profile (N-terminal sub-domain)</i>	15–107
	<i>CobN/Magnesium Chelatase</i>	16–50
	<i>Glutathione S-transferase, C-terminal domain</i>	116–204
	<i>Glutathione S-transferase, N-terminal domain</i>	15–94
	<i>Pyruvate ferredoxin/flavodoxin oxidoreductase</i>	105–129
	<i>Glutathione S-transferase, C-terminal domain</i>	116–204

H Alpha helix, *E* extended strand, *C* random coil

centrifugation, resuspended in buffer containing 100 mM Tris–HCl and 10 mM EDTA (pH 9.0), and disrupted by sonication. Then, 10 µL of the protein samples were subjected to sodium dodecyl sulphate-polyacrylamide gel

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MELGTLEGSMSSETKPILGYWDARGLGQAIRLLLTAGVDFIDKRYTVGPPPNYDRSQWLNDKYNLGLDFPNCPPYYIDGNV
ccccccccccccccccccccccccchhhhhhhhhhhccccccccccccccccccccccccchhhhhcccccccccccccccccccc
KLSQSLAIRYIARKQKLGQNEHEEIRASLAEQQIIDMNMAIARIAYNPNCCKLKPEFLKSLPEQVELLSKFLGDQPFII
chhhhhhhhhhhhhhhhhhhhhccccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh
AGANISYADFLLYEYLTCLKILVPEVYDKFENLKKFHNVLALPRVSEYIKKQQPKAFHGPTSLWNGTYA
ccccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh

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Fig. 2 The secondary structure of Der f 8 allergen. *H* Alpha helix, *E* extended strand, *C* random coil

electrophoresis (SDS-PAGE, 12.5 %) and CBB-R250 staining.

After the induced bacteria were collected and centrifuged, the cell pellet was treated with 40 mL of 1 × binding buffer (His•Bind® Purification Kit, Novagen company, Cat. No. 70665), placed on ice, and then sonicated for 10 min. The cell lysate was then centrifuged at 5,000×*g* for 15 min. The precipitate was a crude inclusion body, which was treated with 1 × binding buffer containing 6 M urea. Upon filtration, the supernatant was subject to SDS-PAGE. Before purification, 5 mL His•Bind® Resin (Novagen company, Cat. No. 70666) was used to balance the His•Bind® column (Novagen company, Cat. No. 70971) to get a resin column of about 3 mL. Super pure water (3 × volume) was added through the column, and then 1 × Charge Buffer (Novagen 69759 Lot N31043) and 1 × binding buffer containing 6 M urea were added. The *E. coli* BL21 (DE3) cells containing recombinant rDer f 8 protein were filtered through the column, and the filtrate was collected. The column was washed with 1 × binding buffer containing 6 M urea (10 × volume), 1 × washing buffer containing 6 M urea (5 × volume), and 1 × eluting buffer containing 6 M urea (2 × volume).

Specific IgE-binding to recombinant allergen

The IgE-binding ability of rDer f 8 protein was detected by enzyme-linked immunosorbent assay (ELISA). For antigen coating, 100 µL of rDer f 8 (1 µg/mL) diluted in coating buffer (0.1 M phosphate-buffer saline) was added to a 96-well microtiter plate, and incubated at 8 °C overnight. Protein samples were blocked with 300 µL of PBST buffer (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄, 2 mL of Tween-20, dissolved in 800 mL of distilled H₂O); then, 100 µL of diluted patient serum individually (diluted 1:4 with blocking buffer) were added for overnight incubation at 4 °C. Next, 50 µL of secondary antibody (HRP-mouse anti-human IgE, 1:5,000 dilution, Sigma Immunochemicals, St. Louis, MO, USA) were added and samples were incubated at 37 °C for 1 h. In total, 100 µL tetramethylbenzidine (TMB) substrate solution was added for 15-min incubation at room temperature. In total, 50 µL of NH₂SO₄ was added to stop the reaction. Optical density was measured at 405 nm wavelength (OD₄₀₅) for three wells using an iMark Microplate

Absorbance Reader (Bio-Rad). The reactivity was considered to be positive if the optical density (OD) values of the detected sera were higher than the cutoff ELISA value (mean ELISA value of healthy donors ±3 SD).

Characterization of the cloned Der f 8

Bioinformatics approaches were used to characterize rDer f 8. The open reading frame (ORF) was identified using ORF finder National Center for Biotechnology Information (NCBI). rDer f 8 amino acid sequence was predicted using Translate Tools in ExPaSy, its physicochemical properties using ProtParam, its hydrophilicity was determined using ProtScale, and its phosphorylation sites using NetPhos2.0 Server. Secondary structure was predicted using GOR4.0 and its functional sites were predicted by InterProScan, ScanProsite, and MotifScan. Alignment of Der f 8 amino acid sequence with sequences of ticks and other mites was performed with ClustalW 2.0 (EBI) and VECTOR NTI 11.0 software (IBI, New Haven, Conn., USA).

Results

Characterization of full-length Der f 8

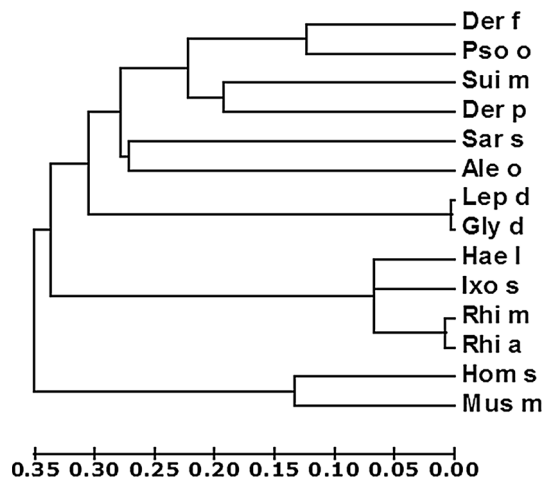
Full-length Der f 8 was cloned using the published partial sequence from *D. farinae* in GenBank. Following sequencing of the full-length clone, bioinformatics analyses were applied to characterize the protein. One ORF was identified, stretching 696 bp from the start codon ATG to the stop codon TGA and encoding 231 amino acids (Fig. 1). ProtParam prediction indicated a relative molecular mass of 26.4 kDa, a theoretical isoelectric point (PI) of 6.84, and an instability index (II) of 34.65, suggesting it is stable. The grand average of hydropathicity (GRAVY) was postulated as −0.33, suggesting it is a hydrophilic protein, which was confirmed by ProtScale. NetPhos2.0 predicted nine phosphorylation sites, including five Serines at amino acid positions 9, 83, 110, 142, and 208, one Threonine at amino acid 46, and four Tyrosines at amino acids 53, 75, 167, and 230. When this deduced amino acid sequence was input to ScanProsite, InterProScan, and MotifScan, homology with glutathione S-transferase was detected (Table 1).

Fig. 3 Alignment between Der f 8 and its homologous amino acid sequence by ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/>). Notes: ‘Asterisks’ means that the residues or nucleotides in that column are identical in all sequences in the alignment; ‘Colon’ indicates conserved substitutions; ‘Dot’ indicates semi-conserved substitutions. Group 8 allergens of *L. destructor*, *G. domesticus*, *Dermatophagoides farinae*, *P. ovis*, *S. medianensis*, *Dermatophagoides pteronyssinus*, *Sarcoptes scabiei*, *A. ovatus*, *Homo sapiens*, *Mus musculus*, *Rhipicephalus microplus*, *Rhipicephalus annulatus*, *Haemaphysalis longicornis*, and *Ixodes scapularis* are abbreviated as Lep d, Gly d, Der f, Pso o, Sui m, Der p, Sar s, Ale o, Hom s, Mus m, Rhi m, Rhi a, Hae l, and Ixo s, respectively

Lep_d	-----MASKPVLGYWDARGLGEDIRTMVYLNVDPEKRYKPS-----DRSKWLN	45
Gly_d	-----MASKPVLGYWDARGLGEDIRTMVYLNVDPEKRYKPS-----DRSKWLN	45
Der_f	MELGTLEGSMSETHPILGVWDARGLGQAIRLLLTVAGVDFIDKRYTVGPPFNDRSQWLN	60
Pso_o	-----MDTKPVLGYWDARGLGQAIRLLLTVADVDPIKRYKVPAPNDRSEWLN	50
Sui_m	-----MSSKPVLGYWDIRGLAQPIRLLLAYLDVDYEDKRYVLG--ANFDRSAWLT	48
Der_p	-----MSQPIGLGVWDIRGVAGPIRLLLTYSQVDFVDKRYVQIGPAPFDRSQWLN	49
Sar_s	-----MSSKPTLGVWNIIRGLAQPIRMLSYAGVDFVDKRYVNGPAPFDRSEWLN	50
Ale_o	-----MSTKPIGVWDIRGLGQPVRSLLAYAGVDFIDKRYRADG--KQGEWLN	48
Hom_s	-----MPMTLGVWDIRGLAHAIRLLLEYTDSYVEKKYTLGDAPFNDRSQWLN	48
Mus_m	-----EDHSTSTMPMILGVWNVRLTHPIRMLEVTDSYDEKRYTMGDAPFDRSQWLN	55
Rhi_m	-----MAPVLGYWDIRGLAQPIRLLLAHVDKRYVDCGPPFDRSQWLN	48
Rhi_a	-----MAPVLGYWDIRGLAQPIRLLLAHVDKRYVDCGPPFDRSQWLN	48
Hae_l	-----MAPILGVWDIRGLAQPIRLLLAHADKRYVDCGPPFDRSAWLK	48
Ixo_s	-----MAPVLGYWDIRGLAQPIRLLLAHMDKRYVDCGPPFDRSAWLK	48
	***: * : * : * : * : * : * : *	
Lep_d	EKFTLGLNFPNLPYYLDGVKLSQSCAILRYLGRKHKLDGETEKEHNNIAVVELQVMDKF	105
Gly_d	EKFTLGLNFPNLPYYLDGVKLSQSCAILRYLGRKHKLDGETEKEHNNIAVVELQVMDKF	105
Der_f	DRYNLGLDFPNCPPYIDGNVLSQSLAIRIYIARKQKLGQNEHEIRASLAEQQIIDM	120
Pso_o	DRYNLGLDFPNCPPYIDGNVLTQSLAIRIYIARKYNNLNGNEMEKIRISLAEQQIVDLN	110
Sui_m	EKPNLGLDFPNCPPYIDGNVLSQSLAIRIYIARKYKLTGANEPEELRVSLVEQQVVDGV	108
Der_p	EKPNLGLDFPNCPPYIDGMEDMTQTFAIRYLGRKRYKLSGNDHEIRISMAEQQTQDM	109
Sar_s	EKPNLGLDFPNCPPYIDGVKLTQSLAIRIYIARKHKLDGNEQELRIALCEQQIVDLV	110
Ale_o	EKPNLGLDFPNCPPYIDGNVLTQSLAIRIYIARKYKLTGANEAEHNAIVLEQQVVDLV	108
Hom_s	EKPNLGLDFPNCPPYIDGTHKITQSNAILRYIARKHNLGGETESEEKIRVDILNQMMDN	108
Mus_m	EKPNLGLDFPNCPPYIDGSHKITQSNAILRYIARKHNLGGETESEEIRADIVNQVMDTR	115
Rhi_m	EKPNLGLDFPNCPPYIDGVKLTQSMAILRYIARKHNLGGETEAEHQRVDVSEQQFADFR	108
Rhi_a	EKPNLGLDFPNCPPYIDGVKLTQSMAILRYIARKHNLGGETEAEHQRVDVSEQQFADFR	108
Hae_l	EKPNLGLDFPNCPPYIDGVKLTQSMAILRYIARKHNLGGETEAEHQRVDVTEQQFADFR	108
Ixo_s	EKPNLGLDFPNCPPYLDGVKITQSMAILRYIARKHNLGGETEAEHQRVDVMAEQQFADFR	108
	: * : * : * : * : * : * : * : * : * : * : * : * : *	
Lep_d	MANALVCYSPDCEKLRVDVLTLPDEIKLFANFLKNSVYVAGNKISYVDFLLHEFLTRIE	165
Gly_d	MANALVCYSPDCEKLRVDVLTLPDEIKLFANFLKNSVYVAGNKISYVDFLLHEFLTRIE	165
Der_f	MAIARIAYNPCEKLRPEFLKSLPEQVELLSKFLNQGPFISGTNISEYADFLLYEVLTKR	180
Pso_o	QALSRWAYNPCEKLRPEVLEKLPQELLESHFLNQGPFISGTNISEYADFLLYEVLTKR	170
Sui_m	QSLSRWAYNPADKLRPFDLKTLDPGSRVQLSHFLGNSPFFVAGTSITTYVDFWLYEVLTKR	168
Der_p	AAMIRVCYDANCCLKPDLVLSLDPCLKLSHFLVGEHFFVAGANISYVDFVLYEVLTKR	169
Sar_s	MAMRISYVDRNFEKLRPDVLEKLPDLNKLSEFLGSHFFVAGTNLYVDFVWLYEVLTKR	170
Ale_o	VALFKTIFDPNFTVKEVLEKLPDLKLSQISNFIGSKGFSVGPVNTTYVDFWLYEVLTKR	168
Hom_s	MELVRLCYDPDFEKLKPKYLEELPEKRLKLYSEFLGKRPWFAGDKITFVDFLAYDVLDMR	168
Mus_m	MQLIMLCYNPDFEKGKPEFLKTIPEKMKLYSEFLGKRPWFAGDKITYVDFLAYDVLDMR	175
Rhi_m	MNVVRLCYNPDFEKLKGVLEKLPASLKAFSDVLGTHFFAGDNLTYYVDFIAYEMLAQH	168
Rhi_a	MNVVRLCYNPDFEKLKGVLEKLPASLKAFSDVLGTHFFAGDNLTYYVDFIAYEMLAQH	168
Hae_l	MNVVRLCYNPDFDKLKVLEKLPDALKSFSEVLGKHFFAGDNLTYYVDFIAYEMLAQH	168
Ixo_s	MNVVRLCYNPDFDKLKVLEKLPASLKAFSDVLGSHYFAGDNLTYYVDFIAYEMLSQH	168
	: : : : * : * : * : : : : : * : * : * : * : *	
Lep_d	TFVPGTLAPHENLTKEV-ERINSLPRVSEYIYNRK--PSIFNGPMKWNATF----	214
Gly_d	TFVPGTLAPHENLTKEV-ERINSLPRVSEYIYNRK--PSIFNGPMKWNATF----	214
Der_f	ILVPEVYDRFENLKKFHNVLALPRVSEYIKQQ--PKAFHGPTLWNGTYA----	231
Pso_o	VLMPFLVYNENLKKFH-ERIEALPRINEYIKQQ--PKSFHGPTLWNGTYA----	220
Sui_m	VLVPEVFGQFENLKKFV-ERIESLPRVSEYIKQQ--PKLFGPMKWNNGQYA----	218
Der_p	VMPPEVFGQFENLKKYV-ERMESLPRVSDYIKQQ--PKTFNAPTENWNSYA----	219
Sar_s	AMTPEVFAKFNGLQNYV-NRFESMPKISAVLKQQ--PQFFNGLMKWNNGYV----	219
Ale_o	LFAPFAFNGFNLGAFV-TELETGPKLAWLKQQ--PQKFGV-FGPFQAEY----	216
Hom_s	IFPEKCLDAFLNKDFI-SRFEGKKISAYMKSSQFLRGLLFGKSATWNSK----	218
Mus_m	MFPEKCLDAFNLDFL-ARFEGKKISAYMKSSRYIATPFSKMAHWNSK----	225
Rhi_m	IFAPDCLKDFANLKAFV-DRIEALPHVAAYLKSDKCIHWPLNGDMASFQSRGKXP	223
Rhi_a	IFAPDCLKDFANLKAFV-DRIEALPHVAAYLKSDKCIHWPLNGDMASFQSRGKXP	223
Hae_l	LLAPDCLKDFANLKAFV-DRIEALPHVAAYLKSDKCIHWPLNGDMASFQSRGKXP	223
Ixo_s	LFAPDCLKEHANLKAFV-DRIEALPHVAAYLKSDKFIHWPLNGDMASFQSRGKXP	223
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Table 2 Identity of amino acid sequence Der f 8 and its homologous amino acid sequences. See Fig. 3 for explanation of the acronyms

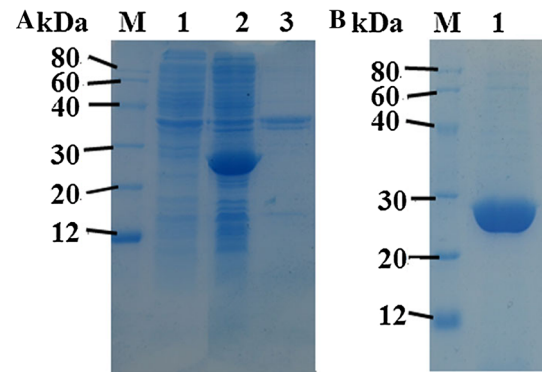
	Ale_o	Gly_d	Lep_d	Hae_l	Rhi_a	Rhi_m	Ixo_s	Hom_s	Mus_m	Sar_s	Der_p	Der_f	Pso_o	Sui_m
Ale_o		47	47	51	49	49	46	42	43	57	52	50	51	55
Gly_d			100	51	50	51	50	46	46	56	54	53	55	59
Lep_d				51	50	51	50	46	46	56	54	53	55	59
Hae_l					89	89	85	54	54	56	51	48	47	55
Rhi_a						99	87	56	54	55	50	48	48	53
Rhi_m							86	57	56	56	50	48	48	53
Ixo_s								53	50	53	51	46	48	53
Hom_s									76	53	48	48	49	50
Mus_m										50	45	45	43	46
Sar_s											66	59	60	64
Der_p												64	61	68
Der_f													78	65
Pso_o														66
Sui_m														

**Fig. 4** Phylogenetic tree for Der f 8 and its homologous amino acid sequences. See Fig. 3 for explanation of the acronyms

The secondary structure of Der f 8 was predicted using GOR4.0, which identified 45.5 % (105 amino acids) of the protein was in alpha helix, 11.3 % (26 amino acids) in extended strand, and 43.3 % (100 amino acids) in random coils (Fig. 2).

Homology with other protein sequences

BLASTp protein sequence alignment (NCBI) identified similar sequences in several other species: *Psoroptes ovis* (GenBank CAQ51257), *D. pteronyssinus* (GenBank AAX37326), *Suidasia medanensis* (GenBank AAX34055), *Sarcoptes scabiei* (AAX37321), *L. destructor* (GenBank AAQ55488), *Glycyphagus domesticus* (GenBank AAQ54608), *Aleuroglyphus ovatus* (GenBank ABL09318),

**Fig. 5** SDS-PAGE analysis of demonstrating successful expression of rDer f 8 in *E. coli* BL21 cells. **a** Expression of rDer f 8 in *E. coli* BL21 cells. Lane M, protein Marker (TransGen Biotech); Lane 1, whole *E. coli* BL21 containing pET-28b (+)-Der f 8 before IPTG induction; Lane 2, supernatant from cells containing pET-28b (+)-Der f 8 at 3 h after induction; Lane 3, the pellet of cells containing pET-28b (+)-Der f 8 at 3 h after induction. **b** rDer f 8 purified from *E. coli* BL21 cells. Lane M, Protein Marker (TransGen Biotech); Lane 1, purified protein

Rhipicephalus microplus (GenBank ADQ01061), *Haemaphysalis longicornis* (GenBank AAQ74441), *Rhipicephalus annulatus* (GenBank ABR24785), *Ixodes scapularis* (GenBank XP_002401749), *Homo sapiens* (GenBank AAH58881), and *Mus musculus* (GenBank EDL01938). ClustalW2 alignment detected 45 identical residues, 44 conserved substitutions, and 17 semi-conserved substitutions in the alignment (Fig. 3). VECTOR NTI identified similarity of the group 8 allergen or glutathione S-transferase of *D. farinae* with *P. ovis*, *D. pteronyssinus*, *S. medanensis*, *Sarcoptes scabiei*, *L. destructor*, *G. domesticus*, and *A. ovatus* as 78, 64, 65, 59, 53, 53, and 50 % (Table 2). In the phylogenetic tree for Der f 8 and its similar

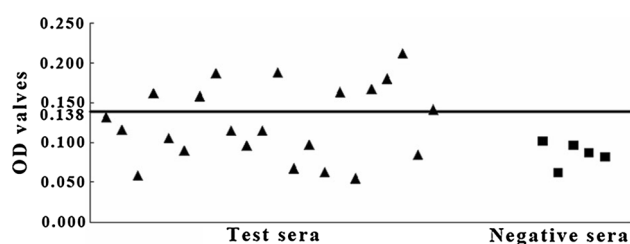


Fig. 6 IgE reactivity to rDer f 8. Each dot on the graph indicates an individual serum. The cutoff values (*dashed line*) were determined by the mean plus three standard deviations from rDer f 8-specific IgE of control sera

amino sequences, all the mites were clustered, all the ticks were clustered, and human and mouse were clustered (Fig. 4).

Expression and purification of Der f 8 as recombinant allergen

The cloned full-length Der f 8 was inserted into an expression plasmid [pET-28b (+)-Der f 8] to transform *E. coli* BL21(DE3) cells. Expression was induced in the presence of IPTG (Fig. 5a). The recombinant protein was successfully purified by nickel affinity chromatography (GE) successfully from 20 mL of sonication products (Fig. 5b).

IgE reactivity to rDer f 8

To assess the allergenicity of rDer f 8, IgE ELISA was performed using sera from 22 mite-allergic pediatric patients with asthma and five non-allergic control individuals. Positive IgE reactions to rDer f 8 were detected in sera of 40.9 % (9/22) of asthma patients using a cutoff of 3 standard deviations above the mean value from healthy controls (Fig. 6).

Discussion

We found that, after cloning, expression, and purification of full-length recombinant Der f 8, IgE reactivity to the recombinant protein was exhibited in 40.9 % of mite-allergic pediatric patients with asthma. This indicates Der f 8 is an important allergen for mite hypersensitivity.

Der f 8 is encoded by a 696-bp open reading frame, producing a predicted amino acid sequence of 231 amino acids. The predicted molecular mass of 26.4 kDa was confirmed by SDS-PAGE from the expression construct. Since the expression vector pET28b (+) contains a nickel-binding six-histidine tag, the recombinant product should contain the extra 6 histidines, and the exact molecular

weight of rDer f 8 would be lower than detected here by SDS-PAGE.

The group 8 allergen of *Dermatophagoides pteronyssinus* has been reported to exhibit strong homology with the active glutathione-binding site of glutathione transferases [17]. We also found homology between the predicted sequence of Der f 8 and glutathione S-transferase. Further, the amino acid sequence of Der f 8 is conserved across tick and other mite species. These identical and conserved sequences have functional value. For example, a *Glycosylation* N-glycosylation site was positioned at 164–167 residues, where was conserved substitutions. Additionally, secondary structure prediction identified alpha helix and random coils as the primary structural components of Der f 8. This represents the first report of predicted secondary structure of this allergen and is an important step toward understanding the allergen's ability to bind IgE epitopes.

Sequence alignment also highlighted the similarities among domestic mites, including *D. pteronyssinus* (Pyroglyphidae), *L. destructor* and *G. domesticus* (Glycyphagidae), *S. medianensis* and *A. ovatus* (Acaridae), all of which contained group 8 allergens with at least 50 % similarity, which indicates potential allergenicity for each of these mite species.

In conclusion, we demonstrate that the cloning, expression, and characterization of recombinant Der f 8 has enabled an initial evaluation of its potency as an allergen in mite-allergic individuals with asthma. These findings provide a foundation from which to explore the structural biology and biochemistry of Der f 8, thereby enabling future work with ASIT.

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References

1. Eifan AO, Calderon MA, Durham SR. Allergen immunotherapy for house dust mite: clinical efficacy and immunological mechanisms in allergic rhinitis and asthma. *Expert Opin Biol Ther.* 2013;13:1543–56.
2. Walgraffe D, Matteotti C, Bakkoury M, Garcia L, Marchand C, Bullens D, Vandenbranden M, Jacquet A. A hypoallergenic variant of Der p 1 as a candidate for mite allergy vaccines. *J Allergy Clin Immunol.* 2009;123:1150–6.
3. Bordas-Le Floch V, Bussi eres L, Airouche S, Lautrette A, Bouley J, Berjont N, Horiot N, Horiot S, Huet A, Jain K, Lemoine P, Chabre H, Batard T, Mascarell L, Baron-bodo V, Tourdot S, Nony E, Moingeon P. Expression and characterization of natural-like recombinant Der p 2 for sublingual immunotherapy. *Int Arch Allergy Immunol.* 2012;158:157–67.
4. Vrtala S, Huber H, Thomas WR. Recombinant house dust mite allergens. *Methods.* 2014;66:67–74.

5. Valenta R, Niespodziana K, Focke-Tejkl M, Marth K, Huber H, Neubauer A, Niederberger V. Recombinant allergens: what does the future hold? *J Allergy Clin Immunol*. 2011;127:860–4.
6. Valenta R, Linhart B, Swoboda I, Niederberger V. Recombinant allergen for allergen-specific immunotherapy: 10 years anniversary of immunotherapy with recombinant allergens. *Allergy*. 2011;66:775–83.
7. Li J, Sun B, Huang Y, Lin X, Zhao D, Tan G, Wu J, Zhao H, Cao L, Zhong N. China Alliance of Research on Respiratory Allergic Disease. A multicentre study assessing the prevalence of sensitization in patients with asthma and/or rhinitis in China. *Allergy*. 2009;64:1083–92.
8. Cui Y, Zhou P, Peng J, Peng M, Zhou Y, Lin Y, 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*. 2008;41:380–8.
9. Cui YB, Zhou Y, Shi W, Ma G, Yang L, Wang Y. Cloning, expression, and analysis of the group 2 allergen from *Dermatophagoides farinae* from China. *An Acad Bras Ciênc*. 2010;82:1–11.
10. Cui YB, Cai HX, Li L, Zhou Y, Gao CX, Shi WH, Yu M. Cloning, sequence analysis and expression in *E. Coli* of the group 3 allergen of *Dermatophgoides farinae*. *Chin Med J (Engl)*. 2009;122:2657–61.
11. Cui YB, Zhou Y, Ma G, Li Yang, Wang Y. Molecular cloning, bioinformatic analysis, and expression of a house dust mite allergen Der f 5 of *Dermatophagoides farinae* from China. *Braz J Med Biol Res*. 2012;45:746–52.
12. Cui YB, Zhou Y, Shi WH, Ma GF, Yang L, Wang YG, Li L. Molecular cloning, expression, sequence analyses of dust mite allergen Der f 6 and its ige-binding reactivity with mite allergic asthma patients in southeast China. *Mol Biol Rep*. 2012;39:961–8.
13. Cui YB, Cai HX, Zhou Y, Gao CX, Shi WH, Yu M, Li L. Cloning, expression and characterization of Der f 7, an allergen of *Dermatophagoides farinae* from China. *J Med Entomol*. 2010;47:868–76.
14. Cui Y, Gao C, Zhou Y, Peng M, Lin Y, Peng J. Phylogenetic analysis of house dust mites. *Cent Eur J Med*. 2010;5:69–74.
15. Cui Y, Zhou Y, Wang Y, Ma G, Yang L. The Group 10 Allergen of *Dermatophagoides farinae* (Acari: Pyroglyphidae): cDNA Cloning, Sequence Analysis, and Expression in *E. coli* BL21. *J Med Entomol*. 2013;50:205–8.
16. Nicklas RA. National and international guidelines for the diagnosis and treatment of asthma. *Curr Opin Pulm Med*. 1997;3:51–5.
17. O'Neill GM, Donovan GR, Baldo BA. The cloning and characterization of a major allergen of the house dust mite, *Dermatophagoides pteronyssinus*, homologous with glutathione S-transferase. *Biochim Biophys Acta*. 1994;1219:521–8.