

The *Dermatophagoides farinae* group 22 allergen: cloning and expression in *Escherichia coli*

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Background: *Dermatophagoides farinae* (Hughes) (Acari: Pyroglyphidae) and other domestic mites produce allergens that affect people worldwide. Here, the complementary DNA (cDNA) coding for group 22 allergen of *D. farinae* (Der f 22) from China was cloned, sequenced, and expressed successfully.

Methods: The cDNA encoding Der f 22 was synthesized by reverse transcription polymerase chain reaction (RT-PCR), then ligated to the pCold-TF for expression in *Escherichia coli* BL21 cells. The purified recombinant fusion protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western-blotting, and tandem matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF).

Results: The full-length cDNA comprised 468 nucleotides and was 99.57% (466/468) identical with the reference sequence (GenBank: DQ643992). After the plasmid pCold-TF-Der f 22 was transformed into *E. coli* BL21 and expressed with the induction of IPTG, SDS-PAGE showed a specific band for the recombinant fusion protein. The recombinant fusion protein, which was purified by chromatography, bound with a His-tagged antibody by Western blot-

ting. MALDI-TOF/TOF mass spectrometry revealed that the structure of the recombinant protein was identical to the predicted Der f 22 structure. The hydrophilic protein contains a signal peptide of 20 amino acids, and the mature Der f 22 consists of 135 amino acid residues with a molecular weight of 14.7 kDa and theoretical isoelectric points (pI) of 6.38. Its secondary structure comprises an alpha helix (38.5%), beta-sheet (45.9%), random coils (11.85%), and beta-turns (11.1%).

Conclusion: This work represents the first reported full-length sequence and successful cloning of Der f 22 from *D. farinae* in China; bioinformatics analysis can be used to further study the allergenicity and clinical utility of the recombinant Der f 22. © 2015 ARS-AAOA, LLC.

Key Words:

Dermatophagoides farinae; recombinant allergen; molecular cloning; domestic mite; Der f 22

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Allergic diseases such as asthma and rhinitis can be triggered by hypersensitivity to domestic mite species,

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which commonly exist in homes around the world. House dust mites, including the *Dermatophagoides* spp., produce a number of proteins that induce hypersensitivity.¹ Indeed, 33 groups of house dust mite allergens have been identified to date (World Health Organization and International Union of Immunological Societies [WHO/IUIS], Allergen Nomenclature Sub-committee; <http://www.allergen.org>). Although some of these allergens have been characterized in detail and are currently being used in allergen-specific immunotherapy, many of them remain to be studied.² Importantly, because individuals display diverse patterns of allergen recognition, the identification and characterization of each allergen is necessary to advance the diagnosis and treatment of immunoglobulin E (IgE)-mediated hypersensitivity to house dust.³⁻⁷

Dermatophagoides farinae hypersensitivity is common in China; therefore, characterizing the allergens produced

by this species is relevant to mitigating allergic disease.¹ To better understand house dust mite hypersensitivity, our laboratory has cloned and expressed multiple allergens from *D. farinae*.^{8–15} Here, we report the cloning and expression of the full-length sequence encoding the group 22 allergen from *D. farinae* (Der f 22). Combined with an initial bioinformatics analysis of the sequence, these results will enable future study of this allergen for its contribution to house dust mite hypersensitivity in China and worldwide.

Materials and methods

Preparation of Der f 22 complementary DNA and polymerase chain reaction

Polymerase chain reaction (PCR) primers were designed using the published sequence of Der f 22 (GenBank: DQ643992), as follows: forward primer, 5'-TACCCTC GAGGGATCCATGAACC GATTCCTCATTGTTTGC-3', and reverse primer, 5'-TAGACTGCAGGTCGACTTA GTTTTGAAGACTAATGTGCATTC-3', with a *Bam* HI and a *Sal* I site, respectively, at the 5' end (underlined). Total RNA was obtained using RNA isolator (TaKaRa Biotech Co. Ltd, Dalian, China; Code No.D312) and stored at -80°C. Reverse transcription (RT) was performed using the total RNA isolated from mites with PrimeScript™ RT-PCR Kit (TaKaRa; Code No. DR014A) in the PCR Thermal Cycler Dice (TaKaRa; Code No. TP 600). The reaction mixture for RT, containing total RNA (1 µL), 10 mM of dNTP Mixture (1 µL), 20 µM of Random 6-mers (1 µL), and RNase Free H₂O (7 µL), was incubated at 65°C for 5 minutes, and placed on ice for 2 minutes. Then, 5× PrimeScript RT Buffer (4 µL), 40 U/µL of RNase Inhibitor (0.5 µL), PrimeScript RTase (0.5 µL), and RNase Free dH₂O (5 µL) were added. The 20-µL reaction mixture was incubated at 30°C for 10 minutes, 42°C for 30 minutes, and, finally, 70°C for 15 minutes. The RT product was used as a template for PCR in the same thermal Cycler Dice with PrimeSTAR® HS DNA Polymerase with GC Buffer (TaKaRa; Code No. DR044A). The total reaction mixture contained RT products (1 µL), 2× PrimeSTAR GC Buffer (25 µL), 2.5 mM of dNTP Mixture (4 µL), 20 µM of the forward primer (1 µL), 20 µM of the reverse primer (1 µL), 2.5 U/µL of PrimeSTAR HS DNA Polymerase (0.5 µL), and dH₂O (16.5 µL). PCR conditions comprised an initial incubation for 3 minutes at 94°C, followed by 30 cycles of 10 seconds at 98°C, 15 seconds at 55°C, and 1 minute at 72°C. After a final incubation for 10 minutes at 72°C, the amplicons were analyzed by agarose gel electrophoresis (1.0%) and visualized with ImageMaster® VDS.

Cloning and DNA sequencing

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 the DNA Ligation Kit (TaKaRa; Code No.

D6020A). *Escherichia coli* JM109 (TaKaRa; Code No. D9052) cells were transformed with the recombinant plasmid pCold-TF-Der f 22, positive clones were selected by blue/white screening on lysogeny broth (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: VP1 (5'-GCGGGTCTGGAAGTTCTG TT- 3') and VP2 (5'-CCAAATGGCAGGGATCTTAG-3').

Construction of expression plasmids of pCold TF-Der f 22

After sequencing, the verified pCold-TF-Der f 22 plasmids were transformed into *E. coli* BL21 cells (DE3; Stratagene, La Jolla CA). The *E. coli* BL21 carrying pCold-TF-Der f 22 were grown on LB plates containing 100 µg/mL ampicillin at 37°C overnight. A single colony was inoculated into 2 mL LB and cultured at 37°C overnight; 10 µL of culture were added into a glass tube containing 2 mL LB with ampicillin (100 µg/mL) and then cultured at 37°C. Then 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG; 50 µL, final concentration of 1 mM) was added, and the sample was incubated for 23 hours at 15°C. *E. coli* were harvested by centrifugation. After resuspension in phosphate buffered saline (PBS) (160 µL/tube), cells were subjected to ultrasonic disruption until the suspension became transparent. A total of 8 µL of the suspension were collected, 2 µL of 5× sodium dodecyl sulfate (SDS) sample buffer were added, and the mixture was heated for 10 minutes at 95°C. In parallel, an empty pCold-TF plasmid was used as a control. The recombinant protein was expressed and isolated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) and visualized by Coomassie Brilliant Blue R-250 (CBB-R250) staining.

Production and purification of recombinant fusion protein

The *E. coli* BL21 carrying pCold-TF-Der f 22 was grown on 400-mL LB plates containing 100 µg/mL ampicillin, and IPTG was added to a final concentration of 100 µM. After culturing at 28°C, *E. coli* colonies were harvested by centrifugation (8000g). Cells were treated with lysis buffer (10 mM imidazole, 50 mM PBS, 0.3 M NaCl, 5% glycerol) and disrupted by sonication. After the induced bacteria were collected and centrifuged, the supernatant was collected for purification. Before purification, lysis buffer (10 mM imidazole, 50 mM PBS, 0.3 M NaCl, 5% glycerol) was used to balance the His•Bind® column (Novagen; Cat. No. 70971). The collected supernatant of the culture medium was added through the column at 1 mL/minute, and the eluent was collected and stored at 4°C. The column was washed with washing buffer 1 (20 mM imidazole, 50 mM PBS, 0.3 M NaCl, 5% glycerol) (5× volume), washing buffer 2 (50 mM imidazole, 50 mM PBS, 0.3 M NaCl, 5% glycerol) (5× volume), and eluting buffer (250 mM imidazole, 50 mM PBS, 0.3 M NaCl, 5% glycerol) (2× volume). Then 10 µL

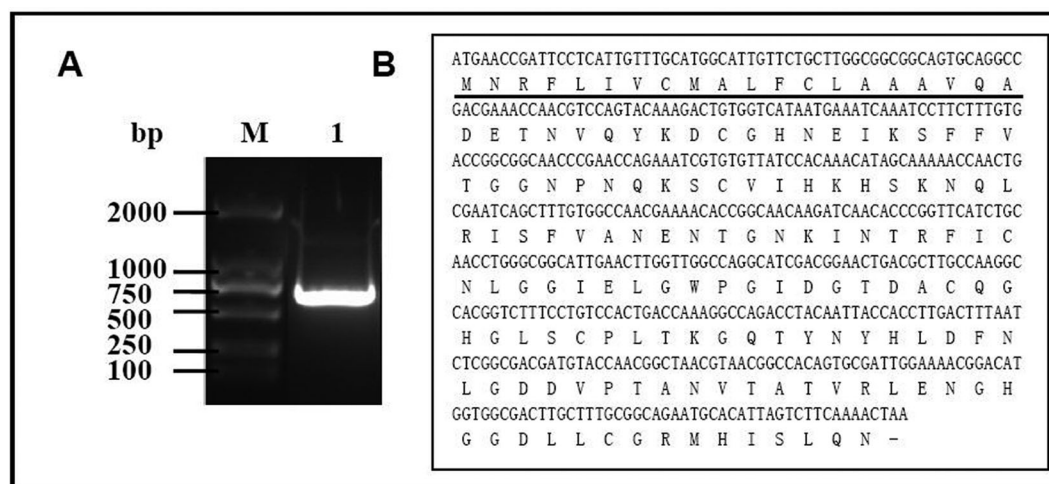


FIGURE 1. Cloning and sequencing of cDNA encoding Der f 22. (A) PCR amplification of Der f 22 identified by agarose electrophoresis (1.0%). Lane M, DNA Marker DL 2000; Lane 1, the PCR product. (B) Nucleotide sequencing results for Der f 22 and its deduced amino acid sequence (the signal peptide sequence is underlined). cDNA = complementary DNA; PCR = polymerase chain reaction.

of the purification product was subjected to separation by SDS-PAGE. Subsequent Western blotting analysis was conducted as described in our previous reports.⁸⁻¹⁵

Matrix-assisted laser desorption ionization mass spectrometry analysis of the recombinant fusion protein

The purified recombinant fusion protein was analyzed using a tandem matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrometer (Model 4800; Applied Biosystems). The spectra generated were mass-calibrated using known standards and the peaks were deisotoped. Databases were searched with the masses obtained using the MASCOT search engine (Swiss-Prot Database, http://web.expasy.org/docs/swiss-prot_guideline.html) and a 50 ppm mass tolerance window. Significant matches from the Peptide Mass Fingerprint data were confirmed by tandem mass spectroscopy (MS/MS) analysis using the search criteria described and an MS/MS-tolerance window of 0.5 Da.

Bioinformatics of the cloned Der f 22

The open reading fragment (ORF) was determined by using the ORF finder on the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) website. The amino acid sequence of Der f 22 was determined by Translate Tools on the ExPASy Web server (<http://www.expasy.org/>); its physicochemical properties were predicted by the ProtParam tool (<http://web.expasy.org/protparam/>), its signal peptide sequence by SignalP 4.1 software (<http://www.cbs.dtu.dk/services/SignalP/>), its hydrophilicity by ProtScale tools (<http://web.expasy.org/protscale/>), and the secondary structure by SOPMA on NPS@server (<http://nhjy.hzau.edu.cn/kech/swxxx/jakj/dianzi/Bioinf7/Expasy/Expasy8.htm>).

Results

Cloning and expression of Der f 22 in *E. coli* BL21 cells

Total RNA was isolated from adult mites and used for RT-PCR to generate the complementary DNA (cDNA) using primers based on the reference sequence of Der f 22; the amplified product was approximately 468 bp (Fig. 1A). The recovered PCR product was cloned into the plasmid vector pCold-TF, which was used to transform *E. coli*-competent cells (JM109). The positive clones were selected by blue/white screening, digested with restriction enzymes, and sequenced for validation (Fig. 1B; the vector sequence and the added restriction sites were removed from the sequencing results). The sequencing results showed that the amplified Der f 22 cDNA was 99.57% (466/468) identical with the previously published sequence (DQ643992), with variations at position 285 (C>T) and position 381 (A>G); however, these base changes would not affect the amino acid sequence. Using the ORF Finder in the NCBI Web server, a complete ORF was found within the Der f 22 cDNA; the full-length sequence comprises 468 bp from the start codon ATG to the stop codon TAA (Fig. 2).

Expression and purification of recombinant protein Der f 22

E. coli BL21 cells were then transformed with the pCold-TF-Der f 22 plasmid, and expression was induced by treatment with IPTG. The optical density values were 0.57 and 0.78 before and after induction, respectively, for the *E. coli* containing the pCold-TF-Der f 22 plasmid. By comparing the molecular weight for proteins expressed by the control plasmid (55 kDa) with that of pCold-TF-Der f 22 (69.7 kDa), the molecular weight for Der f 22 was predicted to be 14.7 kDa; this prediction was confirmed by SDS-PAGE (Fig. 2A, B). *E. coli* BL21 cells carrying

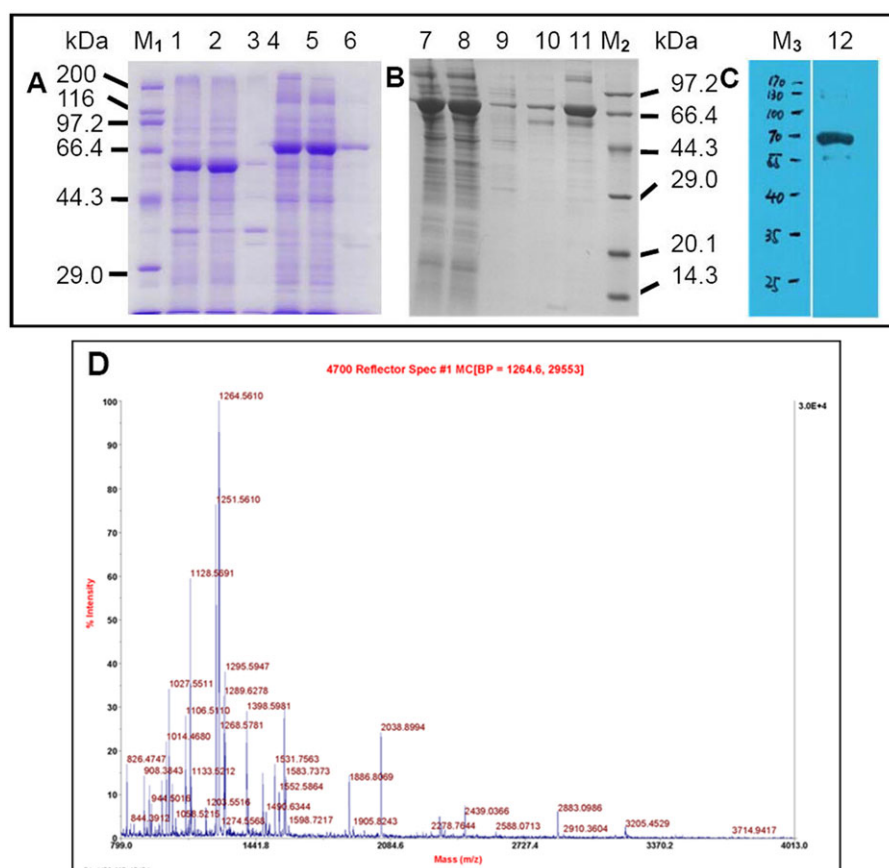


FIGURE 2. Expression of rDer f 22 in *E. coli* BL21 cells. *E. coli* BL21 cells were transformed with either pCold-TF-Der f 22 or empty vector pCold TF as control. (A) SDS-PAGE was used to detect rDer f 22 expressed from *E. coli* BL21 cells. Lane M₁, TaKaRa Protein Marker (Broad); Lane 1, whole cell lysate of *E. coli* BL21 cells containing pCold-TF; Lane 2, supernatant of cells containing pCold-TF; Lane 3, pellet of cells containing pCold-TF; Lane 4, whole-cell lysate of *E. coli* BL21 cells containing pCold-TF-Der f 22; Lane 5, supernatant of cells containing pCold-TF-Der f 22; Lane 6, pellet of cells containing pCold-TF-Der f 22. (B) SDS-PAGE was used to identify the purified rDer f 22. Lane 7, the supernatant of purified cells containing pCold-TF-Der f 22; Lane 8, the flow-through of the His-bind column for cells containing pCold-TF-Der f 22; Lane 9, 10, 11, the elution for the purification of the rDer f 22 from *E. coli* BL21 cells after applying imidazole solutions with the concentration of 20 mM, 50 mM, 250 mM; Lane M₂, protein weight marker; (C) Western blotting analysis of recombinant fusion protein; Lane M₃, Protein weight marker; Lane 12, the purified rDer f 22. (D) The first-order mass spectrum for the purified rDer f 22 by MALDI-TOF/TOF. (E) The second-order mass spectrum for the purified rDer f 22 by MALDI-TOF/TOF. MALDI-TOF/TOF = tandem matrix-assisted laser desorption/ionization time-of-flight; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

the pCold-TF-Der f 22 construct were harvested to purify the protein by nickel affinity chromatography and isolate it by SDS-PAGE (Fig. 2B). The purified recombinant fusion protein was obtained with a concentration of 1.5 mg/mL and with a purity of 90%. The recombinant protein also bound a His-tagged antibody by Western blotting (Fig. 2C). The peptide mass fingerprint (PMF), shown in Figure 2D and E, was consistent with the structure of Der f 22.

Inferred amino acid sequence and its structural and functional prediction

After removal of the vector sequence and added restriction sites, the length of the Der f 22 nucleic acid sequence was 468 bp from the start codon ATG to the stop codon TAA. The nucleotide sequencing result was 99.57% (466/468) identical to the reference sequence; bases differed at position 285 (C>T) and position 321 (A>G). The amino acid sequence was deduced from the nucleotide sequence using

ExPaSy Translate Tools. The gene encodes a pro-protein with 155 residues, relative molecular weight of 16.9 kDa, and theoretical isoelectric point of 6.63. A signal peptide sequence from amino acids 1 to 20 was predicted by SignalP 4.1 software (Fig. 3A). After removal of this sequence, the mature Der f 22 consists of 135 amino acid residues with a molecular weight of 14.7 kDa and theoretical pI of 6.38; its instability index (II) is 31.50. Using the glycosylation prediction program (GPP) Prediction Server (University of Nottingham; <http://comp.chem.nottingham.ac.uk/cgi-bin/glyco/bin/getparams.cgi>), the mature Der f 22 is glycosylated at amino acid positions 3, 4, 47, 52, 107, 109, 113, 118, 132, and 135. The grand average of hydropathicity (GRAVY) was postulated to be -0.527 , as demonstrated by the ProtScale tool (Fig. 3B). Further analysis indicated some of the predicted structural properties of the Der f 22 protein. Figure 3C shows the average flexibility indexes for each amino acid residue. The lowest score was 0.404, at position 97; the highest score was 0.491, at position 25.

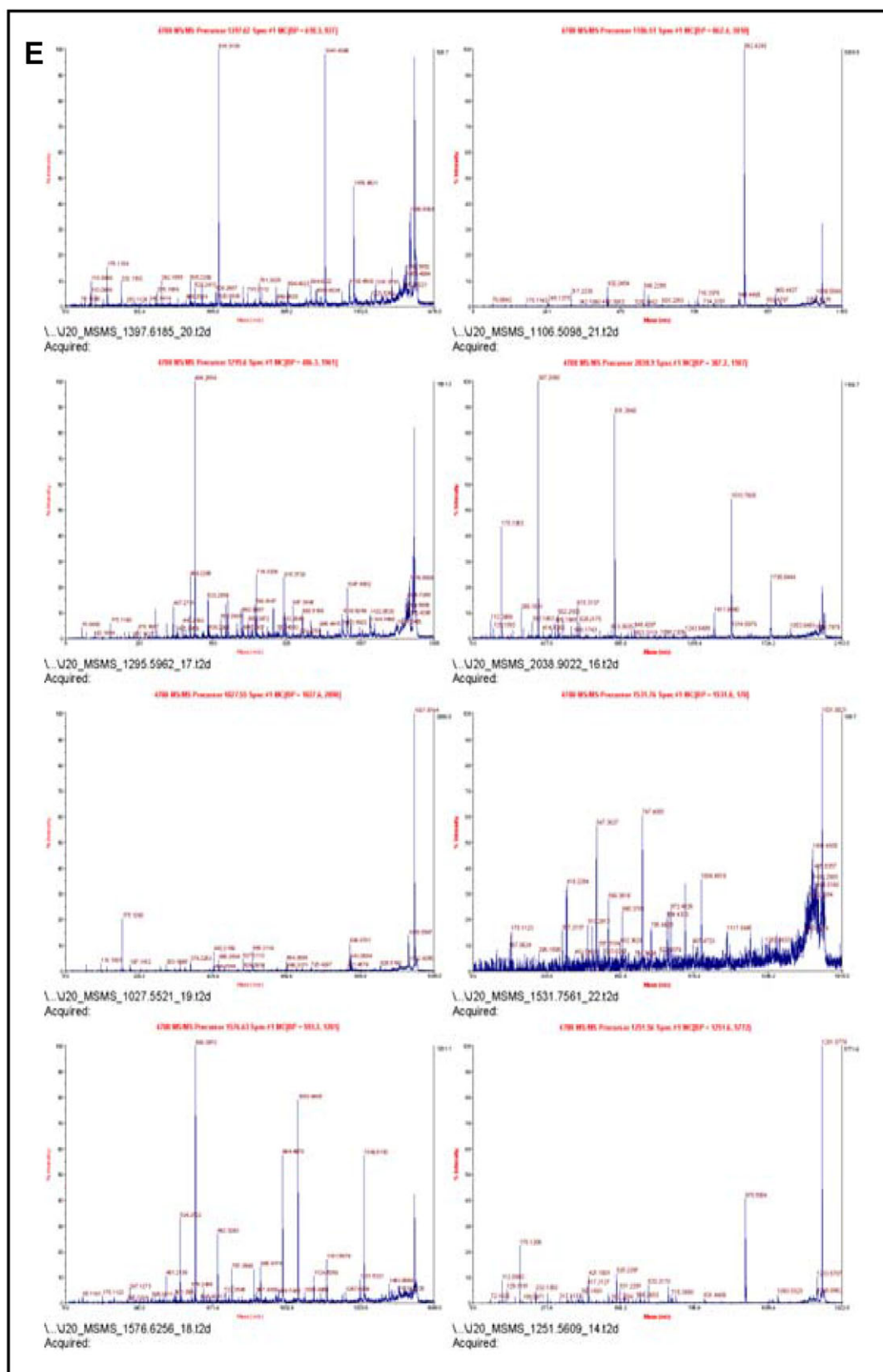


FIGURE 2. Continued.

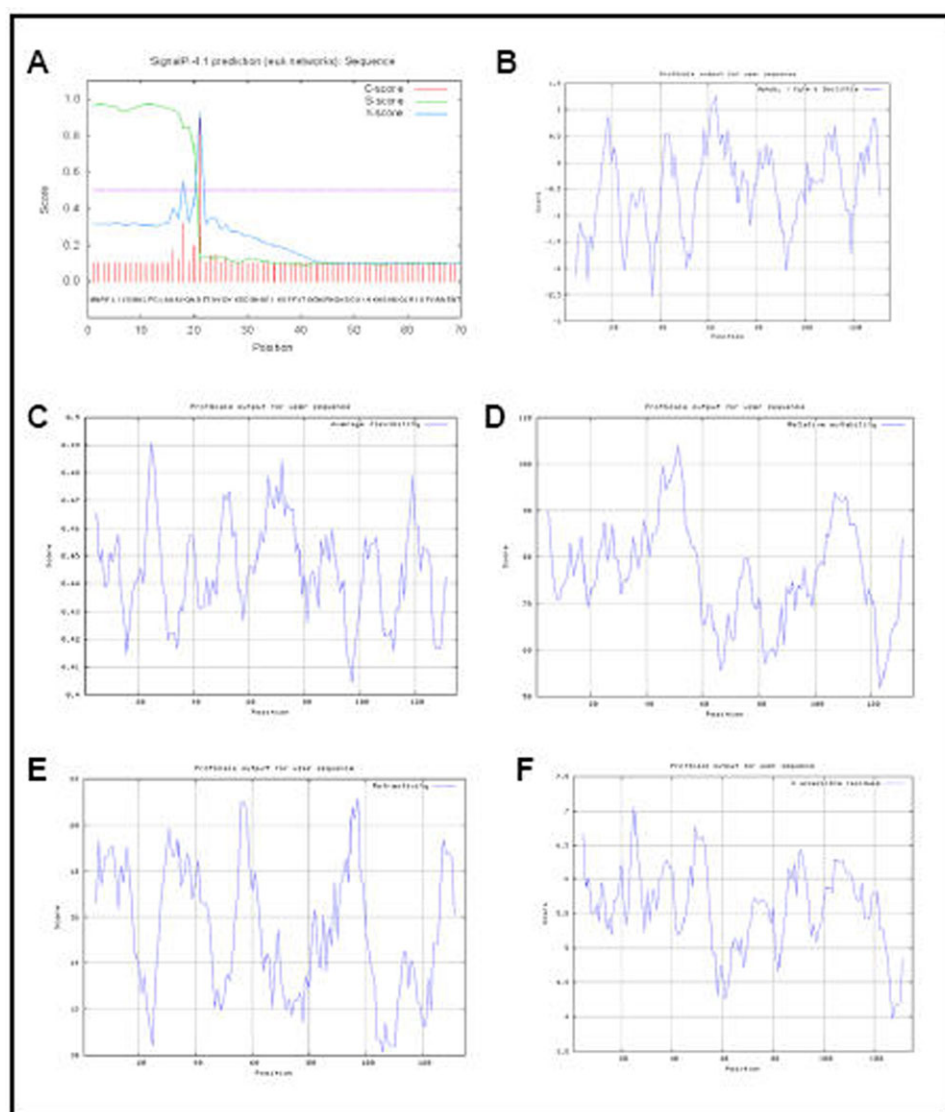


FIGURE 3. Bioinformatics analyses using the mature amino acid sequence of Der f 22. X-axis represents protein length from N- to C-terminus. Y-axis represents the score. The higher score, the higher probability. (A) The most likely cleavage site of Der f 22 pro-protein (SignalP 4.1). (B) Hydrophilicity analysis (ProtScale software, Kyte and Doolittle). (C) Average flexibility (ProtScale software). (D) Relative mutability (ProtScale software). (E) Refractivity (ProtScale software). (F) Accessible residues (ProtScale software).

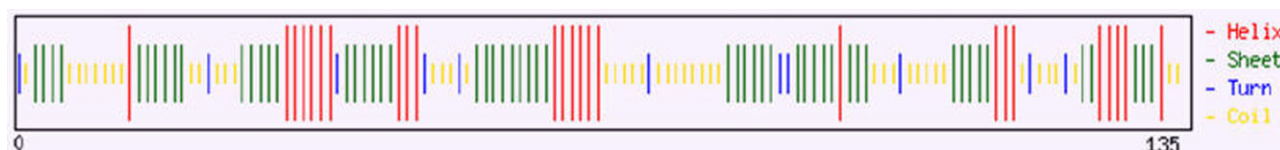


FIGURE 4. The secondary structure analysis of the mature allergen Der f 22 by CFSSP software (<http://www.biogem.org/cgi-bin/cho-fas.pl>). The secondary structure of the mature Der f 22 allergen comprises alpha-helix (52 aa, 38.5%), beta-sheet (62 aa, 45.9%), random coils (16 aa, 11.85%), and beta-turn (15 aa, 11.1%). aa = amino acid; CFSSP = Chou and Fasman Secondary Structure Prediction; coil = random coil; helix = alpha-helix; sheet = beta-sheet; turn = beta-turn.

Relative mutability for each amino acid residue is depicted in Figure 3D. The lowest score was 52, at position 123; the highest score was 104, at position 51. Figure 3E shows the refractivity for each amino acid residue. The lowest score was 10.156, at residue 106; the highest score was 21.154 at residue 97. Figure 3F shows the accessible residues for each

amino acid. The lowest score was 3.967 at residue 127; the highest score was 7.056 at residue 25. The secondary structure of Der f 22 was predicted to comprise alpha helix (52 amino acids, 38.5%), beta-sheet (62 amino acids, 45.9%), random coils (16 amino acids, 11.85%), and beta-turns (15 amino acids, 11.1%), as shown in Figure 4.

Discussion

This is the first report of the successful cloning and expression of the recombinant group 22 allergen from *D. farinae* in China. The gene fragment encoding Der f 22 was amplified by PCR to a full length of 468 base pairs. The deduced amino acid sequence encodes a pro-protein with 155 residues, relative molecular weight of 16.9 kDa, and theoretical isoelectric point of 6.63. After removing the signal peptide sequence, the mature Der f 22 consists of 135 amino acid residues with a molecular weight of 14.7 kDa and theoretical pI of 6.38. The analyses of the predicted sequence indicate that it is a stable, hydrophilic protein. Further analysis on the average flexibility indexes, relative mutability, refractivity, accessible residues enriched our knowledge of Der f 22. This information will help form a foundation for the future design of vaccines and therapies for Der f 22-related allergy.

The secondary structure is the general 3-dimensional form of local segments of proteins, which is defined by patterns of hydrogen bonds between backbone amide and carboxyl groups. The predicated secondary structure of Der f 22 comprises alpha helix (38.5%), beta-sheet (45.9%), random coils (11.85%), and beta-turns (11.1%). The beta sheet, a common recognition motif in peptides and proteins, is a peptide chain typically 3 to 10 amino acids long with a backbone in an almost fully extended conformation.^{16,17} The beta-sheet represents nearly 46% of the Der f 22 sequence, thus it is likely involved in the maintenance of the biological function for the allergen. The alpha-helix

is a common secondary structure in proteins involved in protein-protein interactions, such as enzyme-receptor and antibody-receptor interactions.^{18,19} Thus, the alpha-helix of Der f 22 may be involved in the protein's interactions with IgE.

The expression plasmid pCold-TF-Der f 22 was constructed successfully in the present study. *E. coli* expression systems are used extensively for the production of recombinant proteins due to a simple genetic background, easy handling, and low costs. However, some recombinant proteins do not fold correctly upon expression in *E. coli*. The pCold DNA vectors developed by Takara Bio provide increased in vivo protein yield, purity, and solubility for expressed recombinant using "cold shock" technology. To be more exact, the cold shock protein A (*cspA*) promoter and related elements have been incorporated into these vectors to upregulate target protein production at lowered incubation temperatures (37°C to -15°C). This temperature drop also suppresses expression of other cellular proteins and temporarily halts overall cell growth. Analysis by SDS-PAGE showed a clear band in the supernatant and whole *E. coli* BL21 cells containing pCold-TF-Der f 22 or pCold-TF, which demonstrates the advantages for this expression system. After nickel-affinity chromatography, the purified recombinant fusion protein was obtained and confirmed as consistent with the structure of Der f 22. This work provides a possibility for the production of a recombinant allergen that enables future studies of the structural biology, allergenicity, and clinical utility of this protein in the diagnosis and treatment of *D. farinae* hypersensitivity.

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