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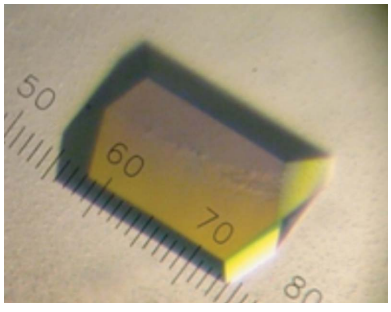
Cloning, expression, purification, crystallization and preliminary X-ray diffraction studies of a major group 7 allergen, Der f 7, from the dust mite *Dermatophagoides farinae*

Der f 7 is a major group 7 allergen from the dust mite *Dermatophagoides farinae* that shows 86% sequence identity to the homologous allergen Der p 7 from *D. pteronyssinus*. Der f 7 was successfully overexpressed in an *Escherichia coli* expression system and purified to homogeneity using Ni–NTA affinity and size-exclusion column chromatography. SeMet-labelled Der f 7 was crystallized by the hanging-drop vapour-diffusion method using a reservoir solution consisting of 0.1 *M* bis-tris pH 7.4 and 28% polyethylene glycol monomethyl ether 2000 at 293 K. X-ray diffraction data were collected to 2.24 Å resolution using synchrotron radiation. The crystals belonged to the orthorhombic system, space group $P2_12_12_1$, with unit-cell parameters $a = 50.19$, $b = 58.67$, $c = 123.81$ Å. Based on the estimated Matthews coefficient (2.16 Å^3 Da^{-1}), two molecules of Der f 7 could be present in the asymmetric unit of the crystal lattice.

1. Introduction

The house dust mite (HDM) has been known for decades to be a major causative agent of various allergic diseases, including asthma, atopic dermatitis (AD) and allergic rhinitis. 23 groups of allergens have been isolated from different species of dust mites (http://www.allergome.org), with a handful of them having been characterized using a broad range of immunoassays. The group 7 allergen of dust mite was first isolated from a *Dermatophagoides pteronyssinus* cDNA library and named Der p 7 (Shen *et al.*, 1993). Sera from 14 out of 38 allergic children (37%) reacted strongly with Der p 7. Skin-prick tests showed reactivity in 16 out of 30 allergic patients (53%; Shen *et al.*, 1993). The homologous allergen Der f 7 isolated from *D. farinae* is a 196-residue protein which has 86% identity to Der p 7. A Der f 7 fusion protein was shown to react with IgE antibodies in 19 out of 41 sera from asthmatic children (46%; Shen *et al.*, 1995). Monoclonal antibodies produced against Der p 7 and Der f 7 cross-reacted with the group 7 allergens of both species and blocked IgE binding to these allergens (Shen *et al.*, 1996, 1997), suggesting that Der f 7 and Der p 7 may share similar IgE epitopes. Recently, the crystal structure of Der p 7 at 2.35 Å resolution has been determined by the addition of maltose-binding protein (MBP) to the N-terminus of the protein. Der p 7 alone crystallized readily at room temperature, but did not show good diffraction (Mueller *et al.*, 2010). An isoform of Der f 7 has been cloned and expressed and its secondary structure has been characterized (Cui *et al.*, 2010), but a detailed three-dimensional structure of Der f 7 is not available. A three-dimensional model of Der f 7 was generated using homology modelling based on the crystal structure of Der p 7 and was used for monoclonal antibody (MAb) binding studies (Shen *et al.*, 2011). Immunodot-blot experiments using overlapping peptides derived from Der f 7 identified Leu48 and Phe50 as important residues for interaction with the Der f 7-specific MAb HD12. The corresponding residues on Der p 7, which does not react with MAb HD12, are Ile48 and Leu50, suggesting that Leu48



and Phe50 are unique epitopes in Der f 7 (Shen *et al.*, 2011). Subsequently, Chou and coworkers identified Asp159, which is located in the loop region based on the model of Der f 7, as an important residue that is responsible for IgE-mediated cross-reactivity between Der f 7 and Der p 7 (Chou *et al.*, 2011). However, this phenomenon was observed in only two out of 30 sera tested. A high-resolution crystal structure of Der f 7 is required to provide the detailed location of IgE/IgG-binding residues. Here, we describe the cloning, expression, crystallization and preliminary X-ray diffraction data of Der f 7 without an affinity tag. The detailed crystal structure will improve the mapping of IgE epitopes and the determination of the cross-reactivity properties between Der f 7 and Der p 7.

2. Cloning, expression and purification of Der f 7

Using the specific primers 5′-CGGAATTCGATCCAATTCACTAT-3′ (Derf7_forward) and 5′-ATTTTTTTCCAATTCAAGCTTCG-3′ (Derf7_reverse), DNA coding for Der f 7 (GenBank accession No. AAP35077.1) from residues Asp1 to Asn196 was amplified by PCR. The N-terminal 17 residues of Der f 7 were predicted to be a signal peptide by the *SignalP* 3.0 server (http://www.cbs.dtu.uk/services/SignalP/; Emanuelsson *et al.*, 2007). The DNA fragment was subcloned between the *Eco*RI and *Hin*dIII restriction-endonuclease sites of a modified pET-32a plasmid vector with the S-tag and thioredoxin tag removed and the last two residues of the thrombin cleavage site modified to merge with the *Bam*HI site. The correct sequence of the DNA insert was confirmed by sequencing before the plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells for protein expression. 10 ml overnight seed culture was inoculated into 1 l Luria–Bertani broth (with 0.1 mg ml^{-1} ampicillin) and grown at 310 K until the OD_{600} reached 0.6. Protein expression was induced with 0.3 m*M* IPTG and growth continued at 298 K overnight. The cell suspension was then harvested by centrifugation (4000 rev min^{-1}, 30 min, 277 K) and resuspended in 30 ml Ni-binding buffer (20 m*M* Tris–HCl pH 8, 5 m*M* imidazole, 0.5 *M* NaCl). The cells were lysed by sonication at an amplitude of 38% for 3 min (1 s pulse on and 0.1 s pulse off) followed by centrifugation at 18 000 rev min^{-1} at 277 K for 30 min. The supernatant was then loaded onto an Ni–NTA affinity column and left to bind for 1 h at 277 K. After vigorous washing with 500 ml washing buffer (20 m*M* Tris–HCl pH 8, 30 m*M* imidazole and 0.5 *M* NaCl), the protein was eluted slowly using 20 ml elution buffer (20 m*M* Tris–HCl pH 8, 0.5 *M* imidazole and 0.5 *M* NaCl). Subsequently, the protein was dialyzed against buffer consisting of 50 m*M* Tris–HCl pH 8, 0.5 *M* NaCl at 277 K for at least 16 h. Overnight thrombin cleavage was carried out by adding 2 U protease per milligram of protein at room temperature after dialysis. The recombinant Der f 7 without a His tag was then passed through a Superdex 200 gel-filtration column using the same buffer as used for overnight dialysis. L-Selenomethionine-labelled Der f 7 was expressed using

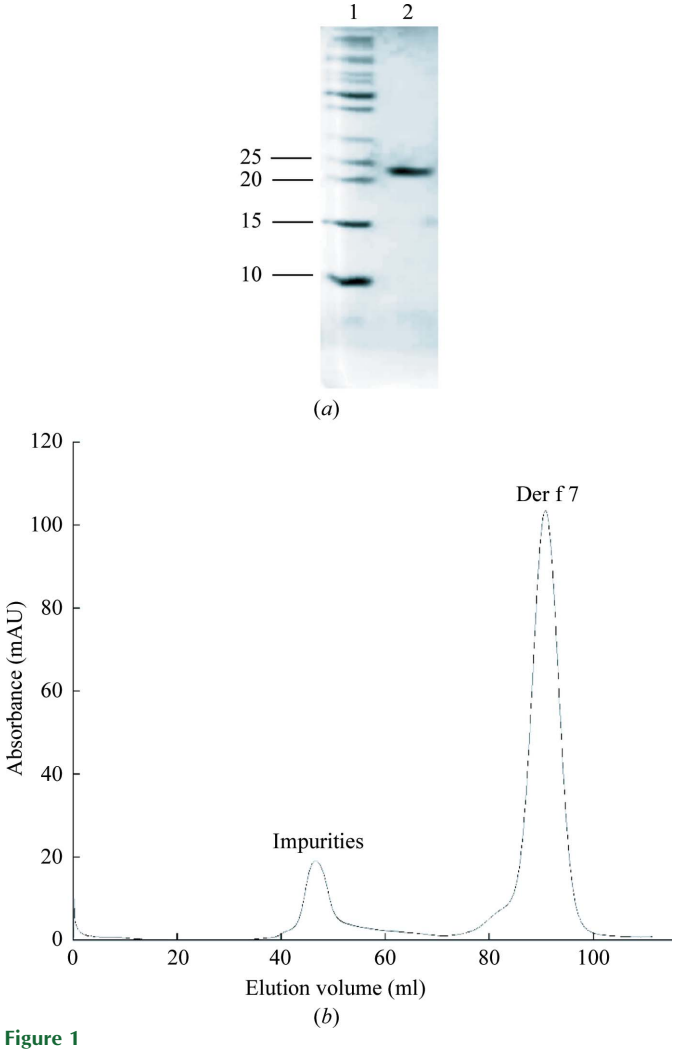


Figure 1
The recombinant Der f 7 was subjected to gel electrophoresis using 15% SDS–PAGE followed by Coomassie Blue staining. (*a*) Lane 1, protein markers (labelled in kDa). Lane 2, purified recombinant Der f 7. (*b*) The elution profile of Der f 7 shows the monomeric state of the protein on a Superdex-200 gel-filtration column.

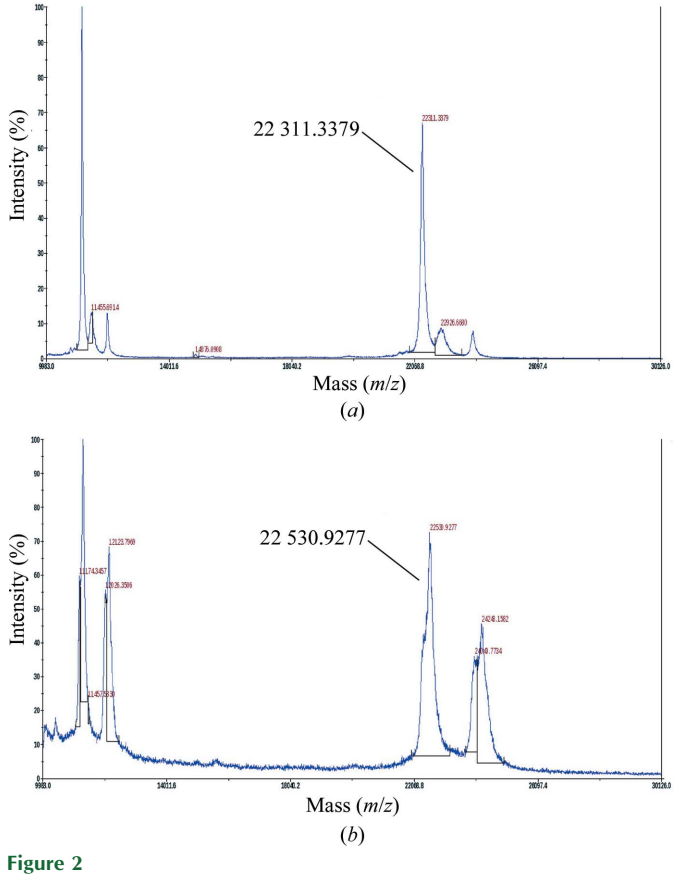


Figure 2
Substitution by SeMet in Der f 7 was confirmed using MALDI–TOF MS experiments. The mass shift of approximately 219.59 Da between native Der f 7 (*a*) and SeMet-labelled Der f 7 (*b*) indicates substitution by five Se atoms.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Peak	Inflection	High resolution
Beamline	BL-13B	BL-13B	BL-13B
Wavelength (Å)	0.97920	0.97939	0.96360
Oscillation angle (°)	0.5	0.5	0.5
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)			
a	50.26	50.19	50.19
b	58.81	58.66	58.67
c	123.94	123.81	123.81
Resolution limits (Å)	30.0–2.27 (2.35–2.27)	30.0–2.27 (2.35–2.27)	30.0–2.24 (2.32–2.24)
Observed reflections	163589	96729	103664
Unique reflections	17333	17373	18187
Multiplicity	9.4 (6.5)	5.6 (5.1)	5.7 (5.2)
Completeness (%)	97.8 (87.7)	98.1 (90.9)	97.9 (90.9)
$\langle I/\sigma(I)\rangle$	33.42 (3.66)	19.98 (4.78)	22.38 (3.08)
$R_{\rm merge}$† (%)	4.6 (39.4)	5.0 (31.7)	3.1 (27.6)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th measurement and $\langle I(hkl)\rangle$ is the mean intensity for that reflection.

the same bacterial strain grown in M9 minimal medium to which 25 mg l^{-1} L-selenomethionine (SeMet) was added when the OD_{600} reached 0.6 (Doublié & Carter, 1992). A purification protocol similar to that used for native recombinant Der f 7 was implemented for the SeMet-labelled Der f 7.

3. Characterization of Der f 7

After removal of the N-terminal His tag, the recombinant Der f 7 protein was comprised of residues Asp1–Asn196 (196 residues) and four additional residues (glycine, serine, glutamic acid and phenylalanine) at the N-terminus from the thrombin cleavage site and the *Eco*RI cloning site. The recombinant Der f 7 appeared as a single band on the SDS–PAGE gel with a molecular weight of approximately 24 kDa. As shown in Fig. 1(*a*), the purity of the recombinant Der f 7 was very high following gel filtration and it was suitable for crystallization. The gel-filtration experiment indicated that the recombinant Der f 7 formed a monomer in solution (Fig. 1*b*). The monomeric state of Der f 7 was also confirmed using NMR based on the overall correlation time of the protein (data not shown). The SeMet-labelled Der f 7 was subjected to MALDI–TOF mass spectrometry to confirm substitution by SeMet. The expected molecular weight of native recombinant Der f 7 is 22 307.4 Da, which agrees closely with the measured molecular weight of 22 311.4 Da (Fig. 2). A difference in molecular weight of 219.59 Da was observed when the mass spectrum of the native recombinant Der f 7 was compared with that of the SeMet-labelled Der f 7, confirming that five SeMet residues had been successfully substituted (Fig. 2). CD spectra obtained at room temperature showed that both the native and the SeMet-labelled forms of Der f 7 contained highly similar secondary-structure contents comprised of both α-helices and β-strands (data not shown).

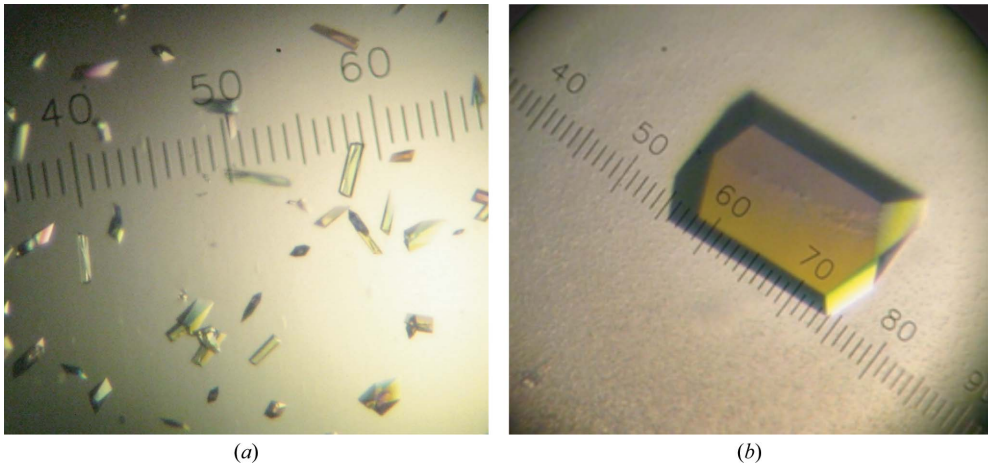
4. Crystallization of Der f 7

Prior to crystallization, the purified protein was dialyzed into 10 m*M* HEPES pH 7 and concentrated to 8 mg ml^{-1} using an Amicon filter (Millipore, 10 kDa molecular-weight cutoff). Crystallization screening for native Der f 7 was carried out using the Index screen (Hampton Research) employing the hanging-drop vapour-diffusion technique at 298 K with 1 µl protein solution (8 mg ml^{-1}) and 1 µl reservoir solution. Initial crystals were obtained after ∼24 h using a reservoir solution consisting of 0.1 *M* bis-tris pH 7 and 28% polyethylene glycol monomethyl ether (PEG MME) 2000. These crystals were too small for diffraction studies despite observable polarization (Fig. 3*a*). The crystallization was further optimized by varying the pH of the reservoir buffer and the concentration of the protein, as well as the temperature. Preliminary diffraction data were collected from native Der f 7 crystals using an in-house X-ray source (Bruker MICROSTAR X-ray generator and PLATINUM135 CCD detector). The best crystal of native Der f 7 diffracted to 2.8 Å resolution with a mosaicity in the range 0.79–0.87°.

Crystallization of the SeMet-labelled protein was optimized based on the conditions used for crystallization of native Der f 7. The best diffracting crystals of recombinant Der f 7 were grown using a reservoir solution consisting of 0.1 *M* bis-tris pH 7.4 and 28% PEG MME 2000 at 293 K. The crystals of SeMet-labelled Der f 7 grew to approximately 0.34 × 0.25 × 0.2 mm within one week (Fig. 3*b*). The best crystal of SeMet Der f 7 diffracted to 2.24 Å resolution with a mosaicity in the range 0.42–0.76°.

5. Data collection of SeMet-labelled Der f 7

The best crystal of SeMet-labelled Der f 7 was picked up in a clean nylon loop and flash-cooled at 100 K in a nitrogen-gas cryostream without additional cryoprotectant. Synchrotron data were collected



(*a*) (*b*)

Figure 3
Crystals of recombinant Der f 7. (*a*) Initial crystals of native Der f 7. (*b*) Optimized crystal of SeMet-labelled Der f 7.

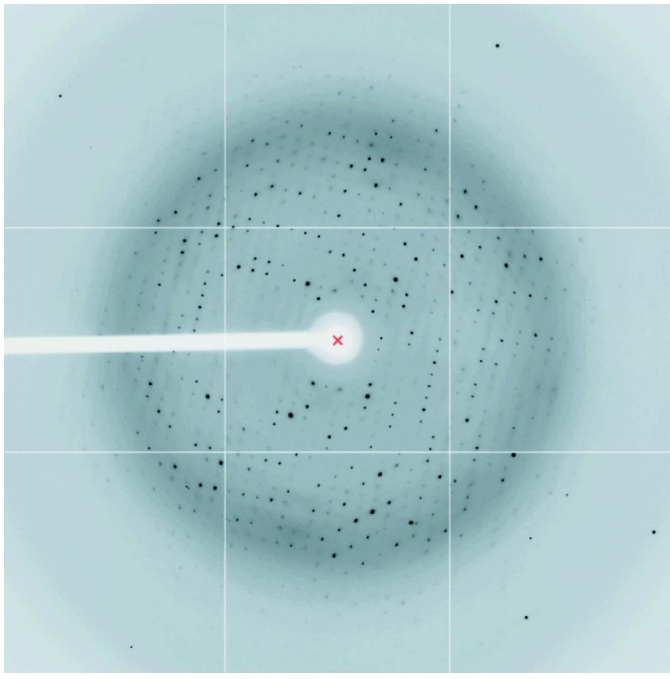


Figure 4
The SeMet-labelled Der f 7 crystal diffracted to a resolution of 2.24 Å. Data collection was performed on beamline BL-13B at the National Synchrotron Radiation Research Center (NSRRC), Taiwan.

on beamline BL-13B at the National Synchrotron Radiation Research Center (NSRRC), Taiwan. X-ray fluorescence spectra recorded from crystals of SeMet Der f 7 near the Se absorption edge were analyzed using the program *CHOOCH* (Evans & Pettifer, 2001). Complete MAD data were collected at three wavelengths using an ADSC Quantum 315 CCD detector with an oscillation angle of 0.5° and a crystal-to-detector distance of 340 mm (Table 1). The data processing was performed using the *HKL*-2000 suite (Otwinowski & Minor, 1997). The high-resolution data set was collected to a resolution of 2.24 Å (Fig. 4). The crystals of recombinant Der f 7 were orthorhombic and belonged to space group $P2_12_12_1$. The unit-cell parameters were $a = 50.19$, $b = 58.67$, $c = 123.81$ Å and the solvent content was ∼43.7%, with an estimated Matthews coefficient of 2.16 Å^3 Da^{-1} (Matthews, 1968; Kantardjieff & Rupp, 2003), suggesting that there could be two Der f 7 molecules in the asymmetric unit.

6. Discussion

We cloned, overexpressed and purified Der f 7 to homogeneity. Unlike the previously reported crystal of MBP-Der p 7, the purified Der f 7 was SeMet-labelled and crystallized without any affinity tag. Diffraction data from the SeMet-labelled crystal showed that it has an improved mosaicity range (0.42–0.76°) and resolution range (30.0–2.24 Å) compared with the MBP-Der p 7 crystal (mosaicity range of 1.1–1.4° and resolution of 50.0–2.35 Å; Mueller *et al.*, 2010). The above diffraction data were obtained without additional cryoprotectant. In fact, addition of cryoprotectant resulted in a deterioration in the resolution and increased mosaicity. Currently, we are determining the structure of Der f 7 using both molecular-replacement and MAD approaches. We are hopeful that the structure of Der f 7, when combined with site-directed mutagenesis and IgE-binding experiments, will provide detailed information on the IgE epitopes of Der f 7 and its cross-reactivity with Der p 7.

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