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Crystallization and preliminary crystallographic data of a Fel d 1 (1+2) construct corresponding to the major allergen from cat

The domestic cat (*Felis domesticus*) is one of the most important causes of allergic disease worldwide. A homologue of the major allergen Fel d 1 was created by linking the two chains that compose the protein. Fel d 1 (1+2) was expressed in *Escherichia coli* and subsequently purified using three chromatographic steps. Crystals of Fel d 1 (1+2) were obtained using the hanging-drop vapour-diffusion method in 22.5% PEG 3350, 0.5 M CaCl₂. The crystals belong to space group P1, with unit-cell parameters $a = 38.5$, $b = 42.9$, $c = 49.0$ Å, $\alpha = 70.7$, $\beta = 80.5$, $\gamma = 81.5^\circ$, and diffract to 1.6 Å resolution.

1. Introduction

Sensitization to the domestic cat (*Felis domesticus*) is a common cause of allergic disease, with a prevalence of approximately 10% in the westernized world (Freidhoff *et al.*, 1984; Roost *et al.*, 1999). The severity of symptoms ranges from mild rhinitis to life-threatening asthmatic responses. Cat allergens are abundant in society even in environments where cats are not present, such as schools and public transportation (Partti-Pellinen *et al.*, 2000; Almqvist *et al.*, 2001), and are therefore difficult to avoid. A careful structural and functional characterization of the major allergen is necessary to improve diagnostics and therapy of cat allergy.

The most prominent and potent allergen in cat dander, termed Fel d 1 (*Felis domesticus* allergen 1), was identified more than three decades ago (Ohman *et al.*, 1974). Fel d 1 elicits IgE responses in 95% of patients with cat allergy (van Ree *et al.*, 1999). The allergen is present in cat saliva and pelt, as well as in perianal and lachrymal glands (Anderson *et al.*, 1985; Bartholome *et al.*, 1985; van Milligen *et al.*, 1990). Fel d 1 is a 35 kDa tetrameric glycoprotein (Kristensen *et al.*, 1997) formed by two heterodimers (Duffort *et al.*, 1991). Each dimer is composed of two chains derived from independent genes, chain 1 (70 residues) and chain 2 (90 or 92 residues) (Morgenstern *et al.*, 1991; Griffith *et al.*, 1992). The two isoforms of the second chain are expressed in the skin and the saliva, respectively (Griffith *et al.*, 1992). The chains are linked by three disulfide bonds, formed between Cys3 of chain 1 and Cys73 of chain 2, between Cys44 of chain 1 and Cys48 of chain 2, and between Cys70 of chain 1 and Cys7 of chain 2 (Bond *et al.*, 1993; Kristensen *et al.*, 1997).

Using a direct linkage of chain 1 to chain 2 [construct Fel d 1 (1+2)] and chain 2 to chain 1 [construct Fel d 1 (2+1)], we have been able to establish *in vitro* conditions for the appropriate refolding of recombinant Fel d 1 fused homologues (Fig. 1). The refolded recombinant Fel d 1 proteins display an identical disulfide-bonding pattern and a comparable secondary structure to the native protein as revealed by

Figure 1
Expression constructs used for Fel d 1 production. (a) Fel d 1 (1+2). (b) Fel d 1 (2+1). Chains 1, 2 and the six-histidine tags are coloured white, grey and black, respectively.

circular dichroism (Grönlund *et al.*, 2003). Both proteins form dimers of the fused chains, corresponding to the native tetramer. Most importantly, the *in vitro* immunoreactivity of both stable recombinant Fel d 1 proteins is indistinguishable from the native allergen (Grönlund *et al.*, 2003). We have recently succeeded in solving the structure of the Fel d 1 (2+1) heterodimer to a resolution of 1.9 Å (Kaiser, Grönlund, Sandalova, Ljunggren, Schneider *et al.*, 2003; Kaiser, Grönlund, Sandalova, Ljunggren, van Hage-Hamsten *et al.*, 2003). However, while both native Fel d 1 and Fel d 1 (2+1) are purified as a tetramer/dimer, no dimer was formed in the crystallization conditions (16% MPD, 0.1 M sodium acetate pH 4.8) obtained for Fel d 1 (2+1). In addition, no electron density could be assessed for the Fel d 1 (2+1) region corresponding to the C-terminus of chain 2, indicating disorder in that part of the molecule. This disorder could also be attributed to the artificial connection of chain 2 to chain 1.

The purpose of the present study was to establish a system for structural studies of the dimeric form of the major cat allergen Fel d 1 as well as to attempt to determine its complete structure. We report here the conditions for successful refolding, purification and crystallization of Fel d 1 (1+2). The structure will provide deepened insights into the function of Fel d 1 and facilitate the improvement of candidates for cat-allergy immunotherapy.

2. Materials and methods

2.1. Protein cloning, expression and purification of Fel d 1 (1+2)

The Fel d 1 (1+2) construct was created by directly linking the C-terminal residue of chain 1 (Cys70) with the N-terminal residue of chain 2 (Val70) using overlapping oligonucleotides in PCR (Fig. 1). The sequenced PCR product was cloned into the pET 20b expression vector (Studier *et al.*, 1990) and transformed into BL21 (DE3) pLysS (Novagen). Fel d 1 (1+2) was expressed following induction with IPTG as a six-histidine fusion protein in *Escherichia coli* strain BL21 (DE3) pLysS and was purified from inclusion bodies. The protein was solubilized in 20 mM Tris-HCl containing 6 M guanidine-HCl, 0.5 M NaCl and 5 mM imidazole pH 8.0 and bound to a Hi-Trap Ni²⁺-chelate affinity column (Amersham Biosciences). The column buffer was changed to 6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole pH 8.0 and a linear 12-column gradient to 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole pH 8 was used. The protein was eluted with 20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole pH 8.0. Fel d 1 (1+2) was further purified to homogeneity by size-exclusion chromatography followed by MonoQ ion-exchange chromatography

(Amersham Biosciences). The concentration of the final product was determined spectrophotometrically and the purity of the product was assessed by SDS-PAGE under denaturing conditions (Laemmli, 1970) and gel filtration.

2.2. Crystallization of Fel d 1 (1+2)

Crystals were obtained by hanging-drop vapour diffusion. Crystal Screens (Hampton Research) were used to establish initial crystallization conditions for Fel d 1 (1+2), which were then refined in a finer grid. Typically, 2 µl 5 mg ml⁻¹ protein in 20 mM Tris-HCl pH 7.5 was mixed in a 1:1 ratio with the crystallization reservoir solution (22.5% PEG 3350, 0.5 M CaCl₂) and allowed to equilibrate both at room temperature and at 277 K.

2.3. Data collection and processing

A data set for Fel d 1 (1+2) was collected at beamline I711 at MAX-II, Lund. A total of 253 images were collected with 1.5° oscillation per image. The space group and unit-cell parameters were determined using the autoindexing option of *MOSFLM* (Leslie, 1999) and scaling and reduction of the data were performed using programs from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994). The space group and unit-cell parameters were determined using the autoindexing option of *MOSFLM*.

3. Results and discussion

The Fel d 1 (1+2) construct is composed of 170 amino acids with a calculated molecular weight of 19 177 Da and a theoretical pI of 5.0. The protein was produced in *E. coli*, refolded *in vitro* and isolated using an Ni²⁺-chelate column, gel-filtration and ion-exchange chromatography. Recombinant Fel d 1 (1+2) was purified as a dimer (corresponding to the native tetramer) and a minimum of 5 mg of highly purified refolded protein was obtained from 1 l bacterial culture (Fig. 2). As for Fel d 1 (2+1), the stable recombinant Fel d 1 (1+2) protein displays an identical disulfide-bonding pattern to the native protein and a comparable secondary structure as revealed by circular dichroism (Grönlund *et al.*, 2003). Most importantly, it also displays a similar immunoreactivity *in vitro* to the native allergen (Grönlund *et al.*, 2003). The initial Fel d 1 (1+2) crystals appeared after four weeks in 20% PEG 3350, 0.1 M CaCl₂ at 295 K. The size and quality of the crystals were improved by increasing the salt and precipitant concentration and the final crystals were obtained in 22.5% PEG 3350 and 0.5 M CaCl₂ at 295 K (Fig. 3).

Figure 2
(a) Proteins separated by SDS-PAGE (15% gel) under denaturing and reducing conditions, stained with Coomassie brilliant blue. Lane 1, molecular-weight markers in kDa; lane 2, purified Fel d 1 (1+2). (b) Fel d 1 (1+2) elution profile from gel-filtration FPLC Superdex 200 column (Amersham Biosciences) in 0.5 ml min⁻¹ PBS. The elution was monitored at 280 nm.

Figure 3
Photograph of typical crystals of Fel d 1 (1+2) in 22.5% PEG 3350 and 0.5 M CaCl₂. The longest dimension is ~0.2 mm.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	I711
Wavelength (Å)	1.087
Resolution (Å)	37.8–1.6
Space group	<i>P</i> 1
Unit-cell parameters	
<i>a</i> (Å)	38.5
<i>b</i> (Å)	42.9
<i>c</i> (Å)	49.0
α (°)	70.7
β (°)	80.5
γ (°)	81.5
Total No. unique reflections	33386
No. observed reflections	135810
Completeness (%)	92.1 (87.5)
<i>R</i> _{sym}	0.06 (0.26)
<i>I</i> / σ (<i>I</i>)	17.4 (4.2)
Mosaicity (°)	0.6

The native crystals diffracted to 1.6 Å resolution using synchrotron radiation. The statistics of the data set are listed in Table 1. The crystals belong to space group *P*1, with unit-cell parameters *a* = 38.5, *b* = 42.9, *c* = 49.0 Å, α = 70.7, β = 80.5, γ = 81.5°. The average *B* factor derived from a Wilson plot was 21 Å². The crystals are very likely to contain two Fel d 1 molecules per asymmetric unit, which would give a Matthews coefficient of *V*_M = 2.0 Å³ Da^{−1}, corresponding to 37% solvent content.

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