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Production, crystallization and preliminary crystallographic study of the major cat allergen Fel d 1

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The domestic cat (*Felis domesticus*) is an important cause of allergic disease worldwide. The major cat allergen 1 (Fel d 1) has been expressed in *Escherichia coli*, purified and refolded in a soluble form. Crystals of Fel d 1 were obtained in 13% 2-methyl-2,4-pentanediol, 0.1 *M* sodium acetate pH 4.8. The Fel d 1 crystals belong to space group $P2_1$, with unit-cell parameters $a = 43.3$, $b = 51.5$, $c = 67.7$ Å, and diffract to 1.9 Å resolution.

1. Introduction

Exposures to small airborne particles derived from the domestic cat are responsible for allergic disease in a significant part of the population in the western world. Of all known cat allergens, *Felis domesticus* allergen 1 (Fel d 1) is the most potent and well studied. IgE antibodies to Fel d 1 are present in the sera of more than 90–95% of cat-allergic patients (van Ree *et al.*, 1999). The protein was first described three decades ago (Ohman *et al.*, 1974) and numerous studies have characterized its biochemical and immunological nature. Fel d 1 is a 38 kDa acidic glycoprotein with N-linked carbohydrate content (Kristensen *et al.*, 1997) present in the pelt, saliva and lacrimal glands of feline animals (Anderson *et al.*, 1985; van Milligen *et al.*, 1990). The allergen consists of two 18 kDa non-covalently linked heterodimers, each composed of two chains, chain 1 (8 kDa) and chain 2 (10 kDa) (Morgenstern *et al.*, 1991), that are encoded by separate genes (Griffith *et al.*, 1992). Three disulfide bridges connect the two chains, *i.e.* Cys3 (1)–Cys73 (2), Cys44 (1)–Cys48 (2), Cys70 (1)–Cys7 (2) (Kristensen *et al.*, 1997), suggesting an antiparallel orientation of chain 1 and chain 2. A homologue of Fel d 1, uteroglobin (Klug *et al.*, 2000), has been described as an anti-inflammatory cytokine-like molecule present, for example, in the human lung (Mukherjee *et al.*, 1999). The human orthologue, Clara cell 10 kDa protein, shares 22% sequence identity with chain 1 of Fel d 1 and displays similar properties to uteroglobin (Mantile *et al.*, 1993).

Attempts to refold separate recombinant Fel d 1 chains into a native-like tetramer composed of two identical heterodimers have been limited to date (Bond *et al.*, 1993; Keating *et al.*, 1995; Slunt *et al.*, 1995; van Ree *et al.*, 1999). Using a direct fusion of chain 2 and chain 1 (Fig. 1), we have succeeded in creating *in vitro* conditions for the appropriate folding of the two chains. The stable recombinant Fel d 1 acts in a very similar way to the native allergen. It displays an identical disulfide-bonding pattern to the native protein and also forms a homodimer with a comparable secondary structure as revealed by circular dichroism. Most importantly, it acts as the native allergen with respect to immunoreactivity *in vitro* (unpublished results).

The purpose of the present study was to establish a system for structural studies of the major cat allergen Fel d 1. We report the conditions for successful refolding, purification and crystallization of Fel d 1. The structure will provide insights into the function of Fel d 1 and facilitate the improvement of candidates for cat-allergy immunotherapy. Furthermore, a soluble and folded recombinant Fel d 1 molecule with correct disulfide formation will be useful for improved *in vitro* and *in vivo* diagnosis.

2. Materials and methods

2.1. Protein cloning, expression and purification

The Fel d 1 construct was created by directly linking the C-terminal residue of chain 2 (arginine residue Arg92) with the N-terminal residue of chain 1 (glutamate residue Glu1) 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). Briefly, Fel d 1 was expressed following induction with IPTG as a six-histidine fusion protein in *Escherichia coli* strain BL21 (DE3) pLysS and purified

Figure 1
Expression construct used for Fel d 1 production. Chains 2, 1 and the six-histidine tag are coloured grey, white and black, respectively.

from inclusion bodies. The protein was solubilized in 20 m*M* Tris–HCl containing 6 *M* guanidine–HCl, 0.5 *M* NaCl and 5 m*M* imidazole pH 8.0 and bound to a Hi-Trap Ni^{2+}-chelate affinity column (Amersham Biosciences). The column buffer was changed to 6 *M* urea, 20 m*M* Tris–HCl, 0.5 *M* NaCl, 20 m*M* imidazole pH 8.0 and a linear 12 column-volume gradient was used to 20 m*M* Tris–HCl, 0.5 *M* NaCl, 20 m*M* imidazole pH 8. The protein was eluted with 20 m*M* Tris–HCl, 0.5 *M* NaCl, 0.5 *M* imidazole pH 8.0. Fel d 1 was further purified to homogeneity by size-exclusion and 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

Crystals were obtained by hanging-drop vapour diffusion. Crystal Screens (Hampton Research) were used to establish initial crystallization conditions for Fel d 1 which were then refined in a finer grid. Typically, 2 µl of 7.5 mg ml^{-1} protein in 20 m*M* Tris–HCl pH 7.5 was mixed in a 1:1 ratio with the crystallization reservoir solution and allowed to equilibrate at both room temperature and at 277 K.

2.3. Data collection and processing

Crystals were soaked in cryoprotectant solution (20% MPD) before flash-freezing in a cold nitrogen stream. A data set for Fel d 1 was collected at beamline I711 at MAX-II, Lund. A total of 250 images were collected with 0.7° oscillations per image. Data were processed using *MOSFLM* (Leslie, 1999) and scaling and reduction of the data was performed using programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The space group and unit-cell parameters were determined using the autoindexing option of *MOSFLM* and by the analysis of pseudo-precession images (Lu, 1999).

3. Results and discussion

The Fel d 1 construct is composed of 170 amino acids with a calculated molecular weight of 19 177 Da and a theoretical pI of 5.0. We succeeded in the production and proper folding of the Fel d 1 allergen by fusing the two chains that form Fel d 1 (Fig. 1). Recombinant Fel d 1 displays an identical disulfide-bonding pattern to the native protein and also forms a homodimer with a comparable secondary structure as revealed by circular dichroism (unpublished results). Most importantly, the stable recombinant Fel d 1 displays immunoreactivity *in vitro* similar to the native allergen.

Table 1
Statistics of data collection.

Values in parentheses are for the highest resolution shell.

X-ray source	I711
Wavelength (Å)	1.134
Resolution range (Å)	25–1.9
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 43.3, b = 51.5, c = 67.7, β = 95.3
Total No. of unique reflections	23462
No. of observed reflections	68839
Completeness (%)	99.4 (99.9)
R_{sym}	0.087 (0.35)
$I/\sigma(I)$	11.1 (3.1)
Mosaicity (°)	0.7

The recombinant protein was produced in *E. coli*, refolded *in vitro* and isolated using a Ni^{2+}-chelate column, gel-filtration and ion-exchange chromatography. A minimum of 10 mg of highly purified refolded protein was obtained from 1 l of culture (Fig. 2). The initial Fel d 1 crystals appeared after one week and continued to grow for two more weeks in a 2-methyl-2,4-pentanediol (MPD) grid screen (10–65% MPD pH 4–9) at 277 K; the size of the crystals was further optimized in a finer grid (14–18% MPD pH 4.4–5.4). The quality of the crystals was improved by seeding from crystals grown in 16% MPD, 0.1 *M* sodium acetate pH 4.8 using a cat whisker. The size of the crystals was further increased by decreasing the protein concentration to 2.5 mg ml^{-1}. The final crystals were obtained in 13% MPD, 0.1 *M* sodium acetate pH 4.8 at 277 K (Fig. 3).

The native crystals diffracted to 1.9 Å resolution using synchrotron radiation. The statistics of the data set are listed in Table 1. The crystals belong to space group $P2_1$, with unit-cell parameters a = 43.3, b = 51.5, c = 67.7 Å, β = 95.3°. The average B factor as derived from a Wilson plot was 18 $Å^2$. The

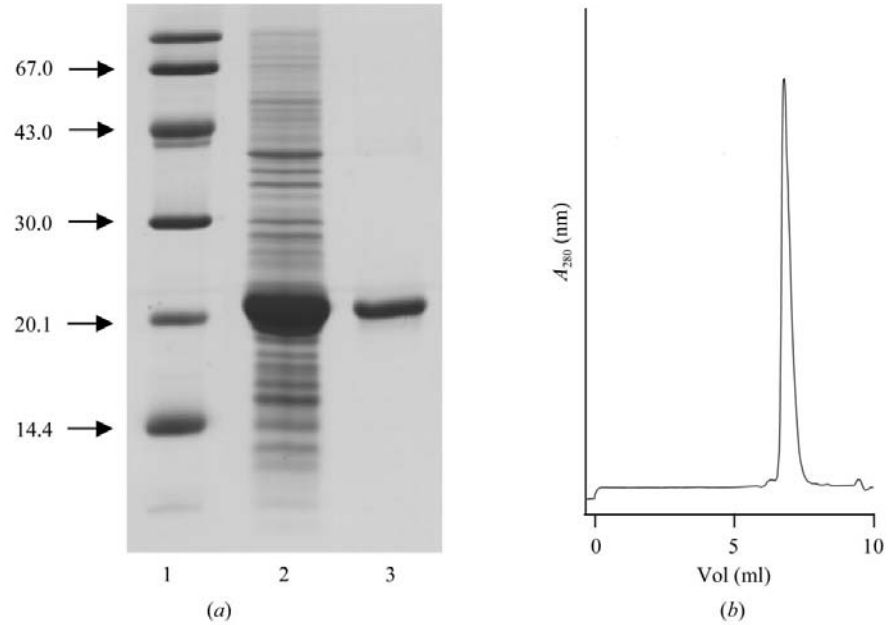

Figure 2
(*a*) Proteins separated by SDS–PAGE (15% gel) under denaturing and reducing conditions, stained with Coomassie brilliant blue. Lane 1, molecular-weight markers in kDa; lane 2, *E. coli* lysate; lane 3, purified Fel d 1. (*b*) Fel d 1 elution profile from gel filtration, FPLC Superose 12 column (Amersham Biosciences) in 0.5 ml min^{-1} PBS, 50 µl sample volume. The elution was monitored at 280 nm.

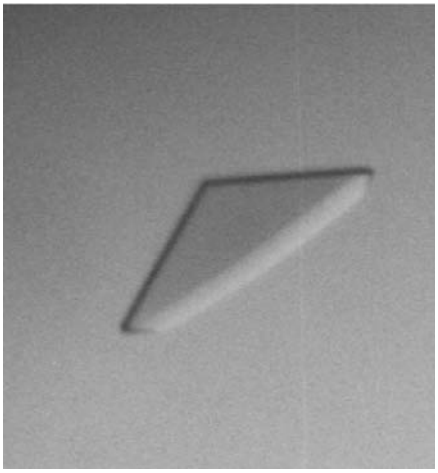

Figure 3
Photograph of a typical crystal of Fel d 1. The longest dimension is ∼0.2 mm.

crystals are very likely to contain two Fel d 1 molecules per asymmetric unit, which would give a Matthews coefficient V_M = 2.0 Å^3 Da^{-1}, corresponding to 40% solvent.

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