

The 18-kDa form of cat allergen *Felis domesticus* 1 (Fel d 1) is associated with gelatin- and fibronectin-degrading activity

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Summary

Background Fel d 1, an important allergen from domestic cats, is a significant cause of asthma. In addition to directly promoting IgE synthesis, other biological activities of allergens may contribute to either allergic sensitization or the magnitude of allergic effector responses. For example, allergens that degrade proteins have been suggested to facilitate allergen presentation by increasing paracellular permeability of airways epithelium. However, little information exists to indicate whether Fel d 1 has other activities relevant to allergic responses.

Objective To study whether Fel d 1 is associated with enzyme activity.

Methods Fel d 1 was obtained by a rigorous purification strategy and its identity confirmed by laser desorption mass spectrometry, cleavage and sequencing. The ability of Fel d 1 to degrade gelatin, fibronectin and the artificial substrate N-benzoyl-FVR-p-nitroanilide was studied. The effect of Fel d 1 on the morphology of tight junctions in epithelial cell monolayers was also investigated.

Results The 18-kDa form of Fel d 1 caused degradation of denatured collagens (gelatin) and cleaved a 20-kDa fragment from the A chain of plasma fibronectin. Catalytic activity was not altered by inhibitors of cysteine peptidases, matrix metallopeptidases or by removal of divalent cations. In contrast, aprotinin and TLCK were inhibitors of Fel d 1. The absence of a serine peptidase catalytic triad in Fel d 1, together with the stoichiometry of the inhibition of TLCK and aprotinin, suggest that their inhibitory action may be due to noncatalytic site interactions. Alternatively, highly purified Fel d 1 may be associated with an active contaminant, although none were found.

Conclusion These results suggest that Fel d 1 is another example of a domestic allergen which is associated with enzyme activity. It remains to be established whether the activity resides in Fel d 1 itself or in an unresolved, and possibly related, protein.

Keywords: Fel d 1 allergen, peptidase activity, serine peptidase inhibitors

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Introduction

Allergy to domestic cats is found in 10–30% of patients with allergic asthma [1,2]. Feline airborne allergens are a significant risk in asthma and rhinoconjunctivitis [3,4–6] as

demonstrated by the diminution in the symptoms of patients when cats are washed regularly or removed from households [6,7]. A greater understanding of the biology of allergens derived from the cat is therefore of importance in understanding an important cause of allergic reactions.

Accumulating evidence indicates that some denominated or presumptive allergens possess significant biological activity in addition to their ability to trigger IgE-mediated

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responses [8,9]. For example, several house dust mite allergens possess proteolytic activity that is capable of disrupting intercellular adhesion in the airway epithelium [10], promoting epithelial cell apoptosis, cleaving the low affinity IgE receptor (CD23) of B lymphocytes [11,12], cleaving the α subunit of the T-cell interleukin-2 receptor [13] and inactivating α_1 -proteinase inhibitor [14]. Evidence exists to suggest that some of these allergens also augment cytokine production in airway epithelial cells [15]. Collectively, these studies have led to the suggestion that it would be more appropriate to consider allergens as polypeptides with a diverse range of biological effects, rather than proteins which simply trigger the synthesis of specific IgE [9–17].

Fel d 1 is an important allergen of the domestic cat (*Felis domesticus*) and is derived from lachrymal, skin and anal sebaceous glands and saliva [18–21]. It is an acidic glycoprotein with a multimeric structure [22–25], the latter feature probably explaining the wide range of estimates of its molecular mass reported in early purification studies [26–29]. Biochemical and immunoaffinity purification [23], partial protein sequencing, cDNA cloning [25] and genomic DNA cloning [30], suggest that Fel d 1 exists as a non-covalently-linked multimer of approximately 38 kDa. Under dissociating conditions, the multimer reveals an 18-kDa form [23] which can be separated by reductive alkylation into two subunits with apparent molecular weights of approximately 4 kDa and 14 kDa as determined by SDS-PAGE [23]. These subunits are referred to as chain 1 and chain 2, respectively, and genomic cloning has established that they are encoded by discrete genes [30]. However, the apparent molecular weights of the native chains observed on SDS-PAGE differ from those encoded in the gene sequences.

Difficulty was initially encountered in establishing the amino acid sequence of chain 2 by conventional protein sequencing, and several segments were not unambiguously identified by Edman degradation [25]. Genomic cloning studies have suggested the existence of sequence polymorphisms in the chain 2 DNA sequence [30] and the existence of these may explain the earlier difficulties in amino acid sequencing.

Insights into the structural and functional biology of some allergens, most notably the house dust mite allergen Der p 1, have been revealed by their similarity to well-characterized peptidases [9–17]. However, computational approaches to Fel d 1 are less revealing. Chain 1 of Fel d 1 is ~50% identical to the α -subunit of the mouse salivary androgen-binding protein [31], and shares weak identity with uteroglobin [25]. However, no significant conservation of sequence identity has been noted for chain 2. Reduction and alkylation of Fel d 1 results in loss of its allergenic activity, suggesting that the IgE-binding sites are likely to

be conformation-dependent [23–32]. This is consistent with difficulties encountered in the epitope mapping of this protein with oligopeptides [33,34], but which are not apparently encountered when using recombinant Fel d 1 chains [35]. The extensive presence of asparagine-linked oligosaccharides in chain 2 (~20%) has previously been suggested to indicate that glycosylation may maintain the active conformation of the epitopes that trigger IgE synthesis [23]. However, this seems less likely because nonglycosylated recombinant Fel d 1 chains bind IgE effectively [35].

In order fully to understand the characteristics that may make certain proteins highly allergenic it is necessary to complement the recent advances in their structure elucidation with functional tests of their biological activities. Given the recent interest in allergens with proteolytic activity, the purpose of the present study was therefore to investigate whether highly purified preparations of Fel d 1 exhibited enzymatic activity.

Materials and methods

Purification of Fel d 1

Fel d 1 was purified by two-step immunoaffinity chromatography. In the first step, murine monoclonal antibody (Cat 1, ALK-Albelló, Hørsholm, Denmark) (32.5 mg) with specificity for Fel d 1 was coupled to 2.5 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) overnight at 5 °C according to the manufacturer's instructions. Subsequently, 50 mg of freeze-dried cat hair and dander extract (ALK-Albelló, Hørsholm, Denmark) was redissolved at 20 mg/mL in 0.05 M carbonate buffer (pH 9.0) and applied to the immunoaffinity matrix at 30 mL/h and washed with 50 mL of application buffer. Fractions (5 mL) were collected throughout. The bound material was eluted with 50 mL of 0.05 M glycine/NaOH, pH 11.0 and the fractions were immediately neutralized by addition of 2 M HCl. Purifications were conducted at 5 °C and all of the fractions collected were analysed by crossed immunoelectrophoresis (CIE) [36] against a rabbit antibody raised to cat hair and dander extract (Ra @Fel d, ALK-Albelló, Hørsholm, Denmark). To remove albumin that coeluted in the first step, a second stage of purification was used. Fractions containing Fel d 1 were pooled and dialysed overnight at 5 °C against 1000 volumes of pH 7.4 phosphate-buffered saline (PBS) before application at 30 mL/h to an immunoaffinity matrix of rabbit antihuman albumin (A001, Dako, Glostrup, Denmark). The matrix was washed with 16 mL PBS and bound material eluted with 16 mL 0.05 M glycine/NaOH, pH 11.0. All operations were conducted at 5 °C and the fractions recovered were analysed as in purification step 1. The effluent and the first wash fraction contained Fel d 1

without any detectable contamination by albumin. These fractions were pooled, dialysed against 1000 volumes of 0.05 M ammonia-bicarbonate buffer and lyophilized. As analysed by silver staining SDS-PAGE [37], the Fel d 1 preparation did not contain any obvious contaminants.

For some experiments, Fel d 1 prepared by two stages of immunoaffinity chromatography was subjected to further affinity purification on a column containing monospecific rabbit polyclonal anti-Fel d 1 IgG (ALK-Albelló, Hørsholm, Denmark). This procedure yielded three fractions. Fraction A was the Fel d 1 preparation before percolation through the additional affinity column (and thus identical to the material prepared by the standard procedure above). Fraction B was the material eluted from the additional affinity matrix, whereas fraction C contained material that was not captured by this column during sample loading.

Instrumental analyses

Reversed phase high performance liquid chromatographic analysis (HPLC) of Fel d 1 was performed at 1 mL/min using two LKB 2150 pumps and an LKB 2141 variable wavelength detector monitoring $A_{214\text{ nm}}$. The stationary phase was a Vydac C₄ column (25 × 0.4 cm, 10 µm particle size) maintained at 45 °C. Initially, the column was eluted isocratically with 100% of 0.1% v/v trifluoroacetic acid (solvent A) for 5 min. Solvent B (90% v/v acetonitrile in 0.08% v/v trifluoroacetic acid) was then introduced over 30 min as a binary linear gradient to produce a 40:60% mixture of A and B. The proportion of A and B was then changed linearly to produce a 20:80% mixture after a further 5 min. Samples of eluate were collected corresponding to the appearance of UV-absorbing peaks in the developing chromatogram.

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-MS) was performed using either a Bio-Ion (Applied Biosystems Inc), or Bruker Reflex (Bruker-Franzen, Analytik, Bremen, Germany) mass spectrometer. Samples were dissolved in 0.1% v/v trifluoroacetic acid (TFA) to a concentration of approximately 0.05 µg/µL and applied to a stainless steel probe tip precoated with 1 µL matrix solution (α -cyano-4-hydroxy cinnamic acid in acetone, 15 µg/µL) and washed with 3 × 10 µL 0.1% v/v TFA before introduction into the mass spectrometer. Mass spectra were acquired for at least 50 shots and analysed using BioMac or LaserOne software running on an Apple Macintosh computer. For reduction on target, 3 mL of dithiothreitol solution (155 mM dithiothreitol in 100 mM ammonium bicarbonate solution, pH 7.8) was applied to the sample on the probe tip. After 10 min, a new layer of matrix was applied and, after washing the sample, it was analysed as described above.

For reduction and alkylation, lyophilized protein (2 nmol)

was dissolved in 200 µL 0.5 M Tris-HCl, 6 M guanidine-HCl, pH 7.6, 5 mM EDTA and 5 µL 1.4 M DTT was added. After 10 min at room temperature, 1 µL 4-vinyl-pyridine was added and, after a further 10 min the reaction was stopped by the addition of 5 µL 1.4 M DTT. Excess reagents were removed by dialysis against ultrapure water for 2–3 days, after which the protein was lyophilized.

Automated Edman degradation was performed on a Knauer 910 pulsed-liquid gas phase sequencer. Peptides were dissolved in 0.1% v/v TFA or 70% v/v formic acid and spotted onto polybrene-coated PVDF membranes. The PTH derivatives were identified by gradient elution on narrow bore HPLC using a 0.2 × 25 cm column packed with 5 µm SuperSpher C18 (Merck, Darmstadt, Germany) eluted with a mixture of 7% v/v acetonitrile in pH 4.6 sodium acetate (buffer A) and 100% v/v acetonitrile (buffer B).

SDS-PAGE

SDS-PAGE was done in 2 mm thick 7.5% w/v acrylamide resolving gels containing 0.125% SDS w/v at neutral pH overlaid with 2.5% w/v acrylamide stacking gels. Samples were boiled for 2 min in loading buffer (composition: 2% w/v SDS, 2% w/v DTT, 13.4% w/v sucrose, 0.05% w/v Tris and 0.007% w/v bromophenol blue) before being applied to the gels. Electrophoresis was performed at 15 mA for the first 90 min followed by 45 mA for 7 h using a Multiphor II system with a Multidrive XL power supply (Pharmacia). Gels were stained with 0.025% w/v Coomassie blue R, 50% v/v methanol and 5% v/v acetic acid for 18 h and destained in 7.6% v/v acetic acid and 5% v/v methanol for 2 h.

Linear 4–18% gradient SDS-PAGE was used to study the effects of Fel d 1 on the integrity of fibronectin and collagens. Electrophoresis was performed at 15 mA for 90 min followed by 25 mA for 6 h. A reduction in apparent molecular weight or complete loss of staining of the matrix proteins was taken as evidence of their proteolytic cleavage.

Western blotting

Immunoblotting of Fel d 1 was carried out by conventional procedures using the immunoabsorbed rabbit polyclonal antibody @Fel d (ALK-Albelló).

Effect of Fel d 1 on fibronectin and collagens

Fibronectin was freshly prepared in HEPES-buffered MEM (minimum essential medium) at pH 7.6. Aliquots of this solution (30 µL) were incubated with 15 µL of HEPES-buffered MEM containing either Fel d 1 or plasmin at 37 °C for 16 h. The final protein concentrations were fibronectin 167 µg/mL; plasmin 4.4 µg/mL and Fel d 1 8.7 µg/mL. Type IV collagen as a putative substrate was studied as

described above. Type I collagen was stored in 0.2 M acetic acid and a 30- μ L aliquot mixed with 15 μ L of either HEPES-buffered MEM containing Fel d 1 as above, or 50 mM Tris at pH 7.4 containing 222 ng/mL collagenase. At the end of the incubation period, an aliquot of each reaction mixture was sampled and subjected to gradient SDS-PAGE. Controls consisted of structural proteins that had not been treated with putative degradative enzymes.

Substrate gel zymography

Zymography was performed using a 2-mm thick SDS-polyacrylamide resolving gel (8%) containing either gelatin or casein as substrate. Electrophoresis was performed at 400 V for 3 h after an initial period of running samples through the stacking gel (1.5 h, 100 V). To prevent proteolysis during electrophoresis, gels were maintained at 4 °C using a Pharmacia MultiTemp II thermostatically controlled cooling system connected to the gel support plate of the Multiphor II electrophoresis unit.

Following electrophoresis, gels were washed once with water and permeabilized at room temperature in 10% v/v Triton X-100 for 1 h, the solution being renewed after 15 min. After three washes with water, followed by two washes in pH 8.0 50 mM Tris/5 mM CaCl₂/0.02% w/v azide incubation buffer, the gels were gently shaken in this buffer for 16 h at 37 °C. Zymograms were then washed with water, stained for 5–20 min in aqueous 0.5% w/v Coomassie blue R: 10% v/v acetic acid: 30% v/v methanol, and then regressed in 30% v/v methanol: 10% v/v acetic acid and then in water. For characterization of the enzymatic activity, electrophoresis conditions were modified to include an inhibitor in the electrophoresis sample and incubation buffers.

Radiochemical quantification of gelatin degrading activity

Labelled gelatin was prepared by heat inactivation of [¹⁴C]-labelled collagen at 60 °C for 20 min in buffer comprising 50 mM Tris/10 mM CaCl₂ and 0.05% Brij-35. Aliquots of this solution (100 μ L, 2500 dpm) were added to each tube containing assay samples (100 μ L) or an excess of trypsin (the latter to give an estimate of maximal degradation) and incubation performed at 37 °C for 16 h. In some experiments, gelatinolytic activity was also quantified after pretreating samples for 2 h at 37 °C with 4-aminophenylmercuric acetate (APMA). After incubation, unlabelled gelatin carrier was added to each tube (50 μ L of a 6-mg/mL solution) and the degraded and undegraded gelatin separated by precipitation with 500 μ L 32% w/v polyethylene glycol, during which time the tubes were kept on ice for 30 min. Following centrifugation for 15 min at 2000 g and 4 °C, aliquots (300 μ L) of the supernatant were transferred to scintillation vials and,

after addition of 1.8 mL water and 10 mL Optifluor scintillant, radioactivity determined by liquid scintillation counting (Beckman LS60000IC). Inter-assay and intra-assay coefficients of variation determined using APMA-activated recombinant human matrix metalloproteinase-2 (MMP-2, gelatinase A) were 12.1% and 6.6%, respectively.

N-benzoyl-Phe-Val-Arg-p-nitroanilide degradation

Peptidase activity was also assessed using a colourimetric assay with the substrate N-benzoyl-Phe-Val-Arg-p-nitroanilide (N-benzoyl-FVR-p-nitroanilide, Sigma). Purified Fel d 1 fraction (15 μ g/mL) was incubated with 0.625 mM substrate at 37 °C. Enzymatic activity was studied spectrophotometrically at A₄₀₅.

Protein assay

Fel d 1 protein in the various batches was measured using the Coomassie blue technique with serum albumin standards.

Cell culture and assay of the effects of Fel d 1 on epithelial tight junctions

MDCK epithelial cells were cultured as described [38,39]. Fel d 1 was applied to the confluent cell monolayer grown on 13 mm diameter coverslips and incubated for 6 h. Cells were then washed twice in PBS and fixed in ice-cold methanol for 10 min. The tight junction protein ZO-1 and desmoplakin of desmosomes were visualized by indirect immunocytochemistry. Nonspecific binding sites were blocked with 10% v/v goat serum in PBS containing 0.2% v/v Tween 20 for 30 min before incubation with either ZO-1 mAb R40.76 or desmoplakin mAb 11-5F for 1 h. After three rinses in PBS containing 0.2% v/v Tween 20, cells were further incubated for 1 h with affinity purified rat or mouse secondary antibody conjugated with FITC or TRITC, respectively. After the final rinse, the coverslip was mounted on a slide for microscopy (Zeiss Axiovert 10 with $\times 40$ Fluor oil immersion objective). Photographic documentation was made using Kodak TMAX 400 film.

Data presentation

Data are presented as the mean value $\pm$ SE mean of *n* separate experiments. Statistical comparisons were made by ANOVA and probability values assigned by the least significant difference test. Probability values ≤ 0.05 were considered statistically significant.

Materials

Aprotinin, APMA, tosyl lysyl chloromethyl ketone (TLCK),

N-ethylmaleimide (NEM), *L-trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), gelatin (type A from porcine skin), prestained molecular weight markers, were purchased from Sigma (Poole, Dorset, UK). Fibronectin purified from human plasma was obtained from Boehringer Mannheim (Lewes, East Sussex, UK). The matrix metalloproteinase inhibitors BB-94 ([4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(thienylthiomethyl)succinyl]-*L*-phenylalanine-*N*-methylamide) and BB-250 ([4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-thiophen-2-ylsulphonylmethyl)succinyl]-*L*-phenylalanine-*N*-methylamide) were kindly provided by British Biotech Ltd (Cowley, Oxfordshire, UK). 4-(2-Aminoethyl)-benzenesulphonylfluoride hydrochloride (AEBSF) was purchased from Pentapharm, Basel, Switzerland. Protein assay reagents were purchased from Pierce and Warriner (Cheshire, UK). Endopeptidase Asp-N (EC 3.4.24.33) was from Boehringer-Mannheim. HPLC-grade acetonitrile was obtained from Rathburn Chemicals (Walkerburn, UK), Nucleosil C₁₈ column packing was obtained from Machery Nagel (Duren, Germany) and Vydac C4 from Vydac (Hesperia, CA, USA). Sequencing agents were purchased from Knauer (Berlin, Germany). Reagents for cell culture and fluorescent antibody labelling were sourced as described [38,39]. All other laboratory reagents were purchased from BDH (Poole, Dorset, UK) and were of the highest grade obtainable.

Results

Characterization of purified Fel d 1

After elution from the Fel d 1 immunoaffinity column, the allergen-enriched preparations were found to contain albumin (Fig. 1a). This was removed using an albumin immunoaffinity column (Fig. 1a). The purified protein appeared as a broad band of apparent mass ~18–20 kDa on nonreducing SDS-PAGE and immunoblotting (Fig. 1b). Under reducing conditions, the light chain of Fel d 1 was also detectable. The MALDI mass spectrum of the immunoaffinity-purified protein consisted of a broad peak with a maximum at *m/z* 19 161 (Fig. 2, panel A). The doubly-charged molecular ion was also observed (maximum at *m/z* 9525) as was the triply-charged molecular ion in both monomeric (*m/z* 6288) and dimeric (*m/z* 12 800) forms (Fig. 2, panel A). After reduction on target (Fig. 2, panel B), the molecular ion of chain 1 appeared at *m/z* 7 998, although reduction was incomplete since the molecular ion of the intact molecule at *m/z* 19 100 was also detected. Chain 2 was not detected after reduction, possibly because the small amount of material produced by the incomplete reduction underwent poor laser desorption.

In reversed phase HPLC, Fel d 1 eluted as a doublet, suggesting the existence of a series of isoforms. MALDI mass spectra obtained from five individual fractions collected through the elution of the doublet were similar to each other

and to the native material that had not undergone HPLC (data not shown). Identification of the immunoaffinity protein at Fel d 1 was further confirmed by digestion of the protein using endoproteinase Asp-N after treatment with DTT and 4-vinyl pyridine. The peptides produced by endoproteinase Asp-N treatment were isolated by reversed phase HPLC using a Nucleosil C₁₈ stationary phase (Fig. 3), analysed by MALDI MS (Fig. 3) and sequenced. The derived sequences were in good agreement with those from cDNA and genomic cloning. MALDI MS and Edman degradation confirmed the existence of isoforms, e.g. K29 substitution by R in chain 1 (Fig. 3). In addition, chain 1 isoforms were found with V33 to S and K29 to N variants (not shown). Chain 2 isoforms contained N19 to S, G20 to L and F15 to T variants and both NH₂- and COOH-terminal truncation was evident (not shown).

Fel d 1 is associated with enzymatic activity

Fel d 1 produced significant degradation in gelatin zymograms. Activity was principally concentrated at apparent molecular weights between 17 and 19.6 kDa, but there was a characteristic 'comet tail' of degrading activity from approximately 20–100 kDa (Fig. 4). Because activity was detected at a relatively low mass in an 8% w/v acrylamide gel, further experiments were undertaken using 12% w/v gels. The extra resolving power failed to reveal any further bands of activity with apparent molecular weights below 17–19 kDa (data not shown). Gelatin-degrading activity was not inhibited when zymography was performed in the presence of 10 mM EDTA, 2 mM NEM or 5 μ M BB-94 (data not shown). However, the presence of aprotinin (10 μ M) produced marked inhibition of activity (Fig. 4). A modest degree of inhibition was also observed with 4-(2-aminoethyl)-benzenesulphonylfluoride hydrochloride (AEBSF), but not with the thiol peptidase inhibitor E-64 (Fig. 4).

Very weak substrate-degrading ability was exhibited by a band of apparent mass 19.4 kDa in casein zymograms (data not shown). This weak activity was not inhibited by development of the zymogram at pH 8.0 in the presence of 10-mM EDTA, 5- μ M BB-94 or 2-mM NEM.

Fel d 1 also degraded the artificial substrate *N*-benzoyl-FVR-*p*-nitroanilide, although the rate at which this occurred was slow (Fig. 5).

Degradation of ¹⁴C-labelled gelatin by Fel d 1 and its inhibition

Fel d 1 catalysed the degradation of radiolabelled gelatin and this was significantly inhibited by aprotinin and TLCK (Fig. 6). To determine whether this aprotinin-sensitive gelatin degrading activity was a coincidental contaminant

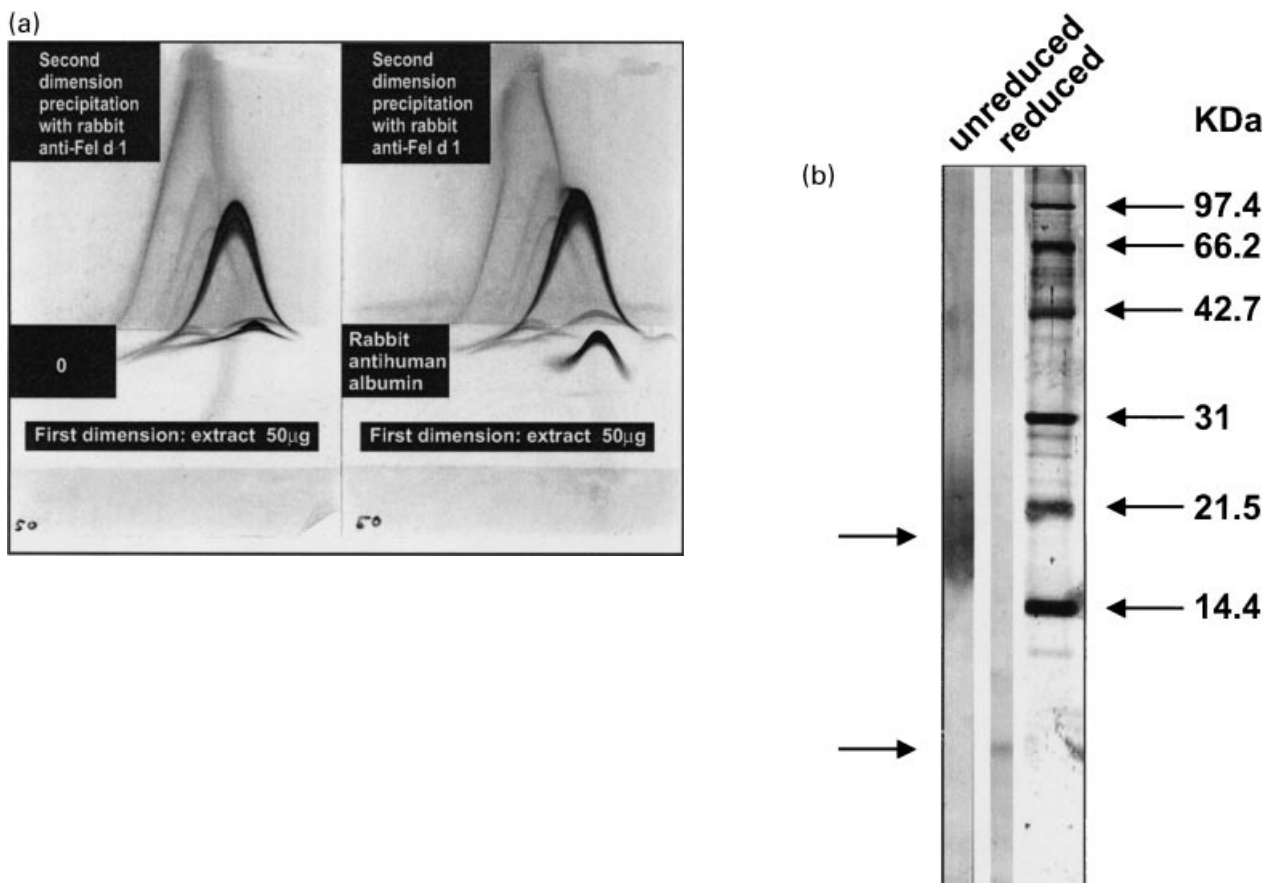


Fig. 1. (a) Crossed immunoelectrophoresis (CIE) of cat hair and dander extract. Fifty micrograms of extract was separated in the first dimension gel and precipitated in the second dimension gel using rabbit antibody to cat hair and dander extract. In the CIE on the left, no antibody was added to the intermediary gel. In the CIE on the right, the intermediary gel contained rabbit antihuman serum albumin. The antibody to the albumin precipitated the cat serum albumin completely, thus demonstrating a strong cross-reactivity. The antibody to serum albumin was thus used in affinity chromatography to remove albumin from the Fel d 1 extract. (b) Western blotting of Fel d 1 under nonreducing and reducing (DTT) conditions. Fel d 1 preparation (20 µg) was applied to each lane and immunoblotting performed according to conventional procedures using immunoabsorbed anti-Fel d 1 polyclonal antibody. Arrows on the left of the blots indicate the major bands of protein detected. A major band of 18–20 kDa was found in the case of unreduced protein and the light chain only in the case of the reduced fraction. Molecular mass standards are shown in the right hand lane.

in Fel d 1 purified by monoclonal antibody immunoabsorption, we conducted further tests using Fel d 1 fractionated by a further step of immunoabsorption using a monospecific rabbit polyclonal antibody. The results of this fractionation are displayed in Fig. 7. Fraction A in these experiments is equivalent to the Fel d 1 used in the other analytical procedures described in this paper. Figure 7 (panel A) shows that the second Fel d 1 immunoabsorption procedure quantitatively removed all of the gelatin-degrading activity from the sample. Gelatin-degrading activity was recovered by elution bound material from the column (fraction B). No protein was detected in fraction C (nonbinding material). Treatment of the fractions with APMA resulted in a small decrease in the enzymatic activity assayed in fractions A

and B (Fig. 7, panel A). Fraction B obtained from this purification strategy was inhibited by 10 µM aprotinin and 100 µM TLCK. The combination of both aprotinin and TLCK together failed to produce significantly greater inhibition of gelatin-degrading activity than that produced by aprotinin alone (Fig. 7, panel B).

Effect of Fel d 1 on fibronectin, type I collagen and type IV collagen

Fel d 1 had no effect on type I or type IV collagen. However, it had modest activity in cleaving fibronectin, reducing the apparent molecular weight of the chain A form from 220 to 200 kDa. This action is in contrast to the degradation of

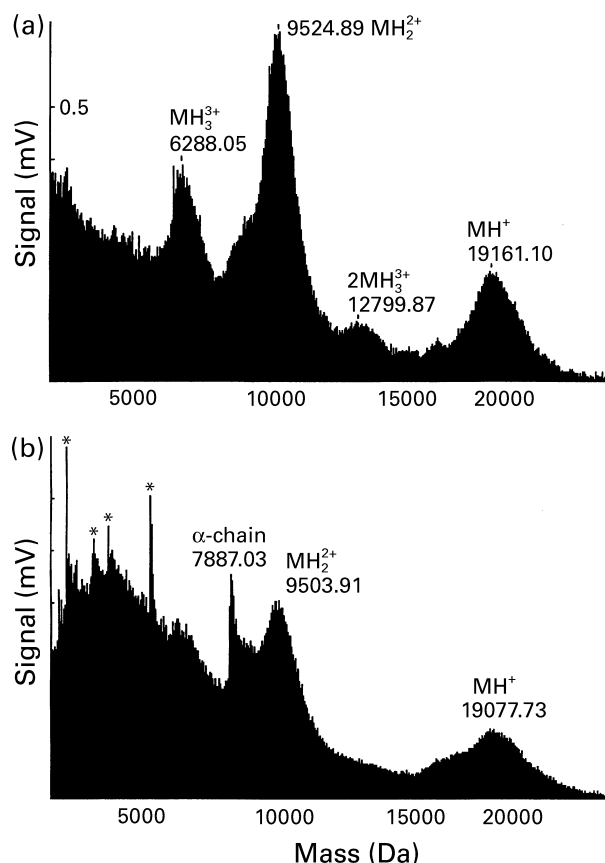


Fig. 2. (a) shows MALDI spectra of the immunoaffinity purified protein showing the singly, doubly and triply charged molecular ion of the protein, as well as the triply charged ion in dimeric form. (b) shows that following reduction directly on the mass spectro-metric target the singly charged molecular ion of Fel d 1 chain 1 is seen, but chain 2 is not observed. Peaks marked with asterisks are artefacts from the reduction.

fibronectin catalysed by plasmin, where cleavage products of approximately 185, 85, 35 and 30 kDa were observed (Fig. 8).

Effects of Fel d 1 on epithelial tight junctions

MDCK canine kidney epithelial cells were used in an experimental model to investigate whether Fel d 1 exhibited

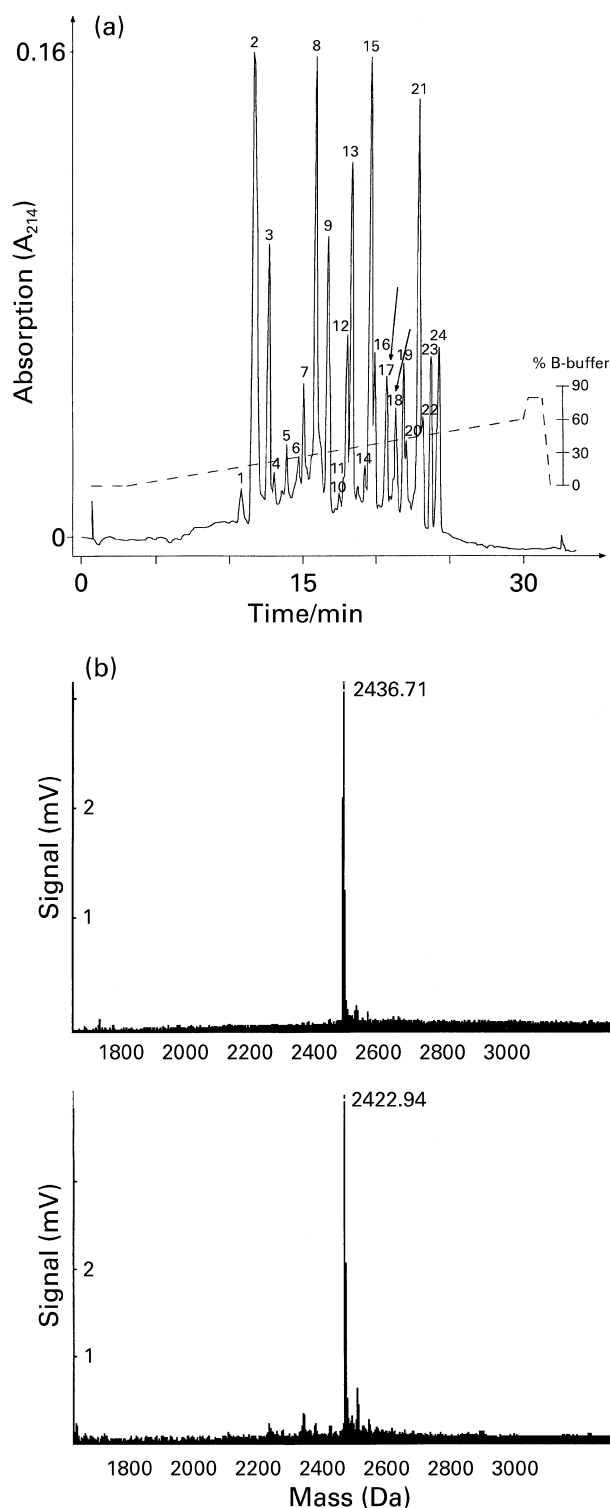


Fig. 3. Shown in (a) is the reversed phase-HPLC of 1 nmol reduced and alkylated Fel d 1 digested with endoproteinase Asp-N. The elution gradient is shown by the dotted line. The fractions containing the K/N isoforms of chain 1 are indicated with arrows. MALDI spectra of fractions 17 and 18 are shown in (b) (upper and lower traces, respectively); m/z 2436.7 corresponds to residues 19–39 of chain 1 (calculated molecular mass 2434.7), when residue 29 is a Lys and m/z 2422.9 correspond to K substituted by N (calculated molecular mass 2420.7).

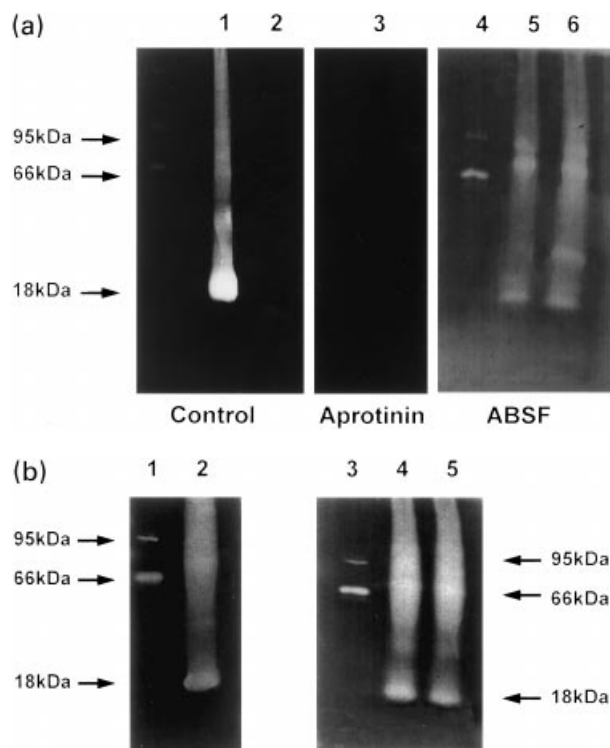


Fig. 4. Analysis of Fel d 1 by gelatin zymography. (a), lane 1 shows gelatin degradation caused by 2 nmol Fel d 1 in the absence of inhibitors. The stored diluent without Fel d 1 is shown in lane 2. The remaining lanes show samples incubated with the respective inhibitor for 1 h prior to electrophoresis and throughout zymogram development. Lane 3 shows the zymographic profile of 2 nmol Fel d 1 in the presence of 10 μ M aprotinin. Lanes 4–6 were all incubated with 10 equivalents of 4-(2-aminoethyl)-benzenesulphonyl fluoride and show the zymographic profiles of gelatinase A (lane 4) and Fel d 1 (lanes 5 and 6). The lack of gelatinase degradation by the diluent (lane 2) which was stored and handled in exactly the same way as the Fel d 1 samples excludes the development of degradative activity due to contamination during reagent storage. Shown in (b) are standards of gelatinase A (66 kDa) and gelatinase B (95 kDa) (lane 1), 1.3 nmol Fel d 1 alone (lane 2), the gelatinase positive controls incubated with E-64 prior to and during zymogram development (lane 3), and the activity of 1.3 nmol Fel d 1 incubated with 10 equivalents of E-64 for 1 h prior to electrophoresis and throughout zymogram development (lanes 4 & 5).

effects on tight junction structure. Figure 9 shows that Fel d 1 failed to alter tight junction morphology. Consistent with this, we found that permeability of MDCK cell monolayers to mannitol was unaltered by incubation for up to 28 h with Fel d 1 (data not shown).

Discussion

These experiments present novel evidence that highly purified preparations of cat allergen Fel d 1 can degrade

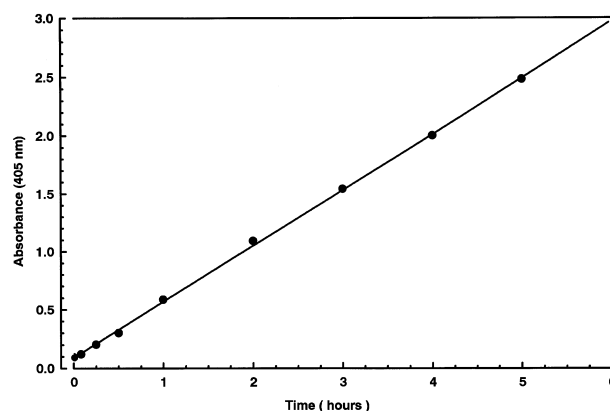


Fig. 5. Time-dependent degradation of N-benzoyl-FVR-*p*-nitro-analide by Fel d 1.

gelatin, fibronectin and the artificial substrate N-benzoyl-FVR. The degradation of gelatin was inhibited by aprotinin and TLCK, but not by inhibitors of thiol- or metallo-peptidases. The finding of enzymatic activity in highly purified preparations of Fel d 1 was unexpected and raises two possibilities, both of which could be of significance in understanding the interactions between allergens and the human immune system.

The first possibility is that Fel d 1 exhibits peptidase activity *per se*, suggesting that this biological property may be relevant to allergic sensitization in a similar way to that postulated for house dust mite allergens [10–15]. The second possibility that must be considered is that the enzyme activity could have resulted from the presence of

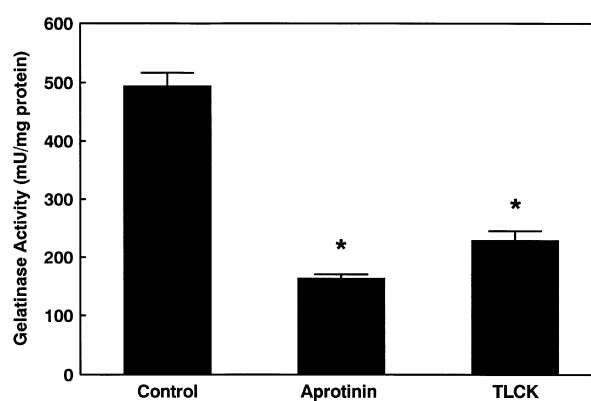


Fig. 6. Degradation of ^{14} C-labelled gelatin and its inhibition by 10 μ M aprotinin or 100 μ M TLCK. Results are displayed as milliunits of gelatinase activity per milligram of protein, where one gelatinase milliunit is defined as the degradation of one nanogram of gelatin per minute at 37 °C. Data are the mean $\pm$ SE mean of five experiments. *Differences that are significantly different ($P < 0.05$) compared to the control activity.

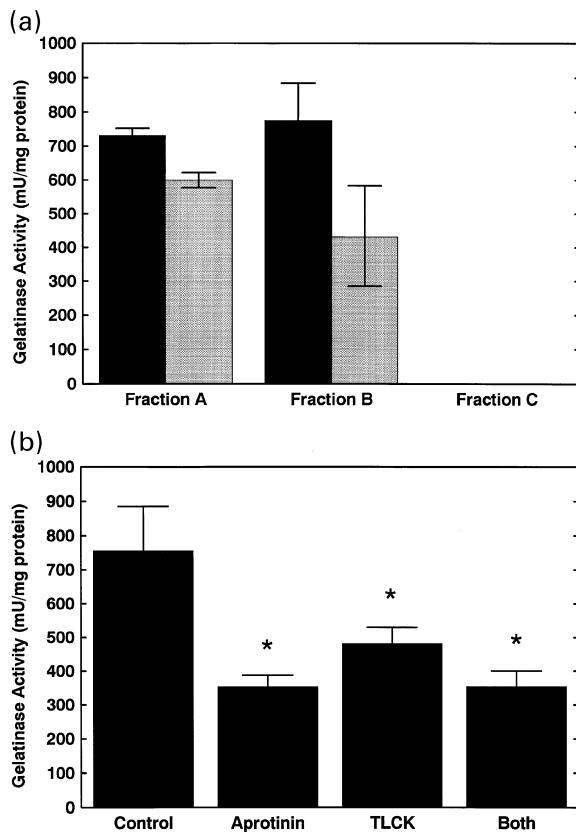


Fig. 7. Degradation of ^{14}C -labelled gelatin by Fel d 1 fraction A (equivalent to Fel d 1 used in the other experiments in this manuscript), by fraction B (Fel d 1 subjected to a second immunoaffinity step using a polyclonal antibody), and by fraction C (the material that was not captured by the immunoaffinity column during the preparation of Fel d 1B). Panel A shows the results obtained with these fractions with (grey columns) or without (black columns) pretreatment of the samples with APMA. Panel B shows the results for Fel d 1 fraction B and its inhibition by $10\ \mu\text{M}$ aprotinin alone or $10\ \text{mM}$ aprotinin and $100\ \mu\text{M}$ TLCK combined. Results are displayed as the milliunits of gelatinase activity per milligram of protein (see Fig. 6 for definition). Data are mean $\pm$ SE mean of 3–7 experiments. *Differences that are significantly different ($P < 0.05$) compared to the control activity.

peptidases copurifying with Fel d 1. However, the contaminant or 'accessory' peptidase(s) must: (i) be recognized by both of the Fel d 1 antisera used in these experiments; (ii) coincidentally have a mass no lower than 17–19 kDa like the (chain1 + chain2)₁ form Fel d 1; (iii) be present in vanishingly small quantities to escape detection by SDS-PAGE and MALDI MS and; (iv) be found in every batch of Fel d 1 that we have examined. If a contaminant peptidase were to meet all of these criteria then it would be virtually impossible to distinguish unambiguously between the two broad interpretations of our data. No positive evidence was obtained which supported the view that the immunoaffinity

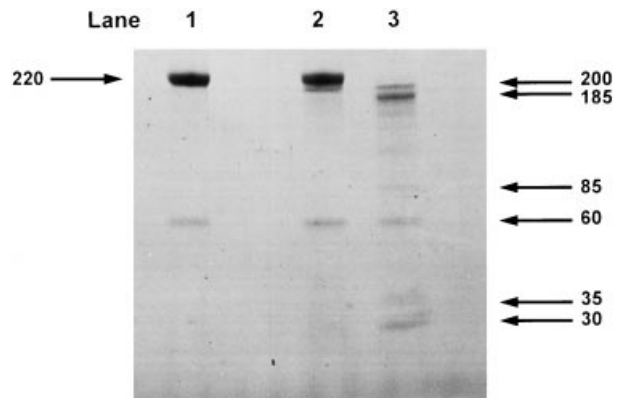


Fig. 8. Degradation of fibronectin by Fel d 1. Reactions were carried out as described in Materials and Methods and the products analysed by SDS-PAGE under reducing conditions using 4–18% linear gradient gels. Lane 1, fibronectin incubated without other additions. Lane 2, Fel d 1 incubated with fibronectin. Lane 3, fibronectin incubated with plasmin.

purified Fel d 1 used in these experiments was contaminated by detectable amounts of other proteins. However, because even techniques such as MALDI MS are subject to performance limitations, this absence of evidence is not proof of absolute purity. Nevertheless, it supports the view that the Fel d 1 preparations were extremely pure. Rigorous precautions and controls were instituted to prevent stored materials from acquiring contamination from exogenous sources after purification. However, Fel d 1 was itself heterogeneous. Full details of its identified isoforms are reported elsewhere [40]. Whilst some of the identified variants are consistent with those found by other workers [23–30], the NH_2 - and COOH -termination truncations of chain 2 have not been widely established [40]. Collectively, our data lead to the conclusion that Fel d 1 is either an enzyme *per se* or so closely associated with such activity that the 'accessory' proteins may be relevant to cat allergy and of potential significance to allergen standardization. Whether any such 'accessory' protein is an unknown contaminant (possibly allergen) or one of the isoforms of Fel d 1 cannot be deduced with certainty, although the sequences of the isoforms suggest that they are unlikely to be classical serine peptidases (see later).

On gelatin zymograms, Fel d 1 exhibited a band of substrate-degrading activity that had an apparent molecular mass of approximately 17–19 kDa consistent with the (chain1 + chain2)₁ form of the protein. However, substrate degradation was not confined to this band and an extensive 'tail' of activity was visible in the track of the electrophoresed protein. This type of tailing can sometimes occur when zymograms are overloaded with enzyme activity. This is unlikely to be the explanation here, because it was not

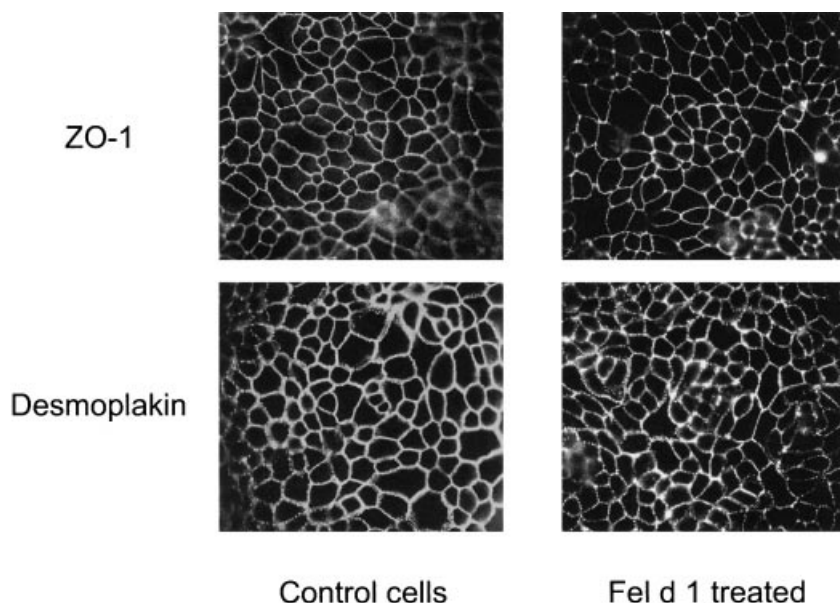


Fig. 9. Fluorescent antibody labelling of tight junction protein ZO-1 and the desmosomal plaque protein desmoplakin under control conditions (sham-treated cells) or following exposure of MDCK epithelial cells to 7.5 μ g Fel d 1 for 6 h at 37 °C. Specific enzymatic activity of Fel d 1 was 0.1 nmol N-benzoyl-FVR degraded/mg protein/min.

possible to dilute out the 'tails' without losing all activity. In zymography, 'tailing' can also be seen when the temperature of gels is not kept low during electrophoresis. Temperature was maintained at 4 °C in these experiments, the effectiveness of this being illustrated by the fact that positive control samples containing gelatinases A and B did not exhibit any tailing in the same gels (Fig. 4). Multiple batches of Fel d 1 were studied using zymography and all exhibited similar behaviour. We therefore consider it highly unlikely that the unusual behaviour of Fel d 1 on zymograms is due to gel overloading or temperature effects.

The gelatin-degrading activity of Fel d 1 was inhibited by aprotinin, TLCK and, to a lesser extent AEBSF, whereas inhibitors of cysteine or metallo peptidases were inactive. Superficially, these findings suggest that Fel d 1 might be a serine peptidase. However, the absence of a histidine residue in Fel d 1 and its isoforms means that it would not have the H, D and S-containing catalytic triad found in the majority of enzymes within this class. Whilst serine peptidases lacking a catalytic H residue are known, such as clan SF that have a catalytic dyad of S and K, this deficiency suggests that if Fel d 1 is an enzyme it may not be a serine peptidase and that inhibition of TLCK and AEBSF must arise nonspecifically. Alternatively, the serine peptidase activity may arise from an undiscovered contaminant. Clarification of these issues will require further investigation using kinetic analyses when suitable physiological substrates have been identified. Other attempts to resolve whether Fel d 1 is an enzyme, using serine peptidase inhibitors to capture Fel d 1 by affinity chromatography, have, to date, failed definitely to establish whether or not the enzyme activity is Fel d 1 itself.

Gelatin-degradation activity was only slightly affected by prior treatment of Fel d 1 with APMA, indicating that sulphhydryl residues contribute little to the mechanisms involved. Fel d 1 also cleaved a 20-kDa C-terminal fragment from the A chain of plasma fibronectin. However, the intact 20 kDa fragment from fibronectin was not itself detected in these experiments suggesting that Fel d 1 might exert some endopeptidase activity.

Fel d 1 shares only weak sequence identity with other proteins of well-described function. Similarity (50%) is seen between chain 1 of Fel d 1 and the α -subunit of mouse salivary androgen binding protein [31], a protein putatively involved in sex/subspecies recognition [41]. cDNA cloning has also identified a related lachrymal gland protein that shares identity with Fel d 1 (EMBL accession code AF008595). A 25% match also exists between chain 1 of Fel d 1 and uteroglobin and with the related Clara cell phospholipid binding protein CC10. Uteroglobin and CC10 contain two covalently linked chains [42–44], and by inference from the sequence data chains 1 and 2 of Fel d 1 should similarly be arranged in antiparallel fashion. Tryptic cleavage and sequence analysis has been used to confirm the general validity of this concept [32,40]. If one interchain disulphide were to be absent in a proportion of Fel d 1 molecules, then the allergen might be able to exist in a variety of oligomeric forms, perhaps accounting for the unusual mass range of activity seen on gelatin zymograms. However, we consider this unlikely since no supportive evidence was accrued in the other analytical procedures we performed.

Whatever the nature of the enzymatic activity associated with Fel d 1 it appears weak in comparison with the

peptidase allergens of house dust mites. Cysteine (Der p 1) and serine (Der p 3, Der p 6 and Der p 9) peptidase allergens from dust mites have a miscellany of potent effects on epithelia [16,17,38,39], most notably cleaving the occludin and claudin family proteins of tight junctions to increase paracellular delivery of allergens [10]. Although Fel d 1 does not seem able to emulate the disruptive effects of dust mite peptidase allergens on epithelial cell adhesion, there may nevertheless be other peptidase-dependent effects associated with the cat allergen Fel d 1 which are of potential relevance to allergy.

In summary, highly purified Fel d 1 was unexpectedly found to behave as an enzyme. Although no contaminating proteins were identified, the inhibition of enzyme activity by serine peptidase inhibitors is not consistent with expectations from the amino acid sequence of Fel d 1 or its known isoform variants, suggesting that the catalytic activity may reside in a copurified substance. These findings have implications for understanding the role of Fel d 1 as a cause of human disease and the standardization of this allergen.

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References

- Bryant DH, Burns MW. Skin test reactions to inhalant allergens in asthmatic patients. *Med J Aust* 1996; 1:918–26.
- Starsfield JK, Boyle AG, Rowell AG, Rowell FC, Moriarty SC. Pet sensitivities in asthmatic children. *Arch Dis Child* 1976; 51:186–92.
- Munir AK, Kjellman NI, Bjorksten B. Exposure to indoor allergens in early infancy and sensitization. *J Allergy Clin Immunol* 1997; 100:177–81.
- Custovic A, Taggart SC, Woodcock A. House dust mite and cat allergen in different indoor environments. *Clin Exp Allergy* 1994; 24:1164–8.
- Kleine-Tebbe J, Kleine-Tebbe A, Jeep S, Schou C, Lowenstein H, Kunkel G. Role of the major allergen (Fel d 1) in patients sensitized to cat allergens. *Int Arch Allergy Immunol* 1993; 100:256–62.
- de Blay F, Chapman MD, Platts-Mills TA. Airborne cat allergen (Fel d 1). *Environmental control with the cat in situ. Am Rev Respir Dis* 1991; 143:1334–9.
- Wood RA, Chapman MD, Adkinson NF, Eggleston PA. The effect of cat removal on allergen content in household dust samples. *J Allergy Clin Immunol* 1989; 83:730–4.
- Stewart GA. Molecular Biology of Allergens. In: *Asthma, Rhinitis*. Holgate ST, Busse WW eds. Blackwell Science, in press.
- Robinson C, Kelsheker NA, Srinivasan N, King CM, Garrod DR, Thompson PJ, Stewart GA. On the potential significance of the enzymatic activity of mite allergens: clues to structure and function revealed by molecular characterization. *Clin Exp Allergy* 1997; 27:10–21.
- Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, Robinson C. *J Clin Invest* 1999; 104:123–33.
- Hewitt CRA, Brown AP, Hart BJ, Pritchard DI. A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: Innate protection by antiproteases. *J Exp Med* 1995; 182:1537–44.
- Schulz O, Laing P, Sewell H, Shakib F. Der p 1, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23). *Eur J Immunol* 1995; 25:3191–4.
- Schulz O, Sewell H, Shakib F. Proteolytic cleavage of CD25, the α subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J Exp Med* 1998; 187:271–5.
- Kalsheker NA, Deam S, Chambers L, Sreedharan S, Brocklehurst K, Lomas DA. The house dust mite allergen Der, p. 1 catalytically inactivates α 1-antitrypsin by specific reactive centre loop cleavage: a mechanism that promotes airway inflammation and asthma. *Biochem Biophys Res Commun* 1996; 221:59–61.
- King CM, Brennan S, Thompson PJ, Stewart GA. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 1998; 161:3645–51.
- Robinson C, Winston HL, Wan H. Epithelial repair in asthma. Do the benefits of house dust mite avoidance result from proteinase avoidance? *Clin Exp Allergy* 1998; 28:530–3.
- Robinson C, Wan H, Winton Herbert CA, Ring PC, Garrod DR. House Dust Mite Proteinase Allergens and Their Interactions with the Bronchial Epithelium. In: *Asthma, Rhinitis*. Holgate ST, Busse WW eds. Blackwell Science, in press.
- Brown PR, Leitermann K, Ohman JL Jr. Distribution of cat allergen 1 in cat tissues and fluids. *Int Arch Allergy Appl Immunol* 1984; 74:67–70.
- Charpin C, Mata P, Charpin D, Lavaut MN, Allasia C, Vervloet D. Fel dI allergen distribution in cat fur and skin. *J Allergy Clin Immunol* 1991; 88:77–82.
- van Milligen FJ, Vroom TM, Aalberse RC. Presence of *Felis domesticus* allergen-1 in the cat's salivary and lacrimal glands. *Int Archs Allergy Appl Immunol* 1990; 92:375–8.
- De Andrade AD, Birnbaum J, Magalon C, Magnol JP, Lanteaume A, Charpin D, Vervloet D. Fel d 1 levels in cat anal glands. *Clin Exp Allergy* 1996; 26: 178–80.
- Leitermann K, Ohman JL Jr. Cat allergen I. Biochemical, antigenic and allergenic properties. *J Allergy Clin Immunol* 1984; 74:147–53.
- Duffort O, Carreira I, Nitti G, Polo F, Lombardero M. Studies on the biochemical structure of the major cat allergen *Felis domesticus*. *Mol Immunol* 1991; 28:301–9.
- Duffort O, Carreira J, Lombardero M. Monoclonal antibodies against Fel d 1 and other relevant cat allergens. *Immunol Lett* 1988; 17:71–7.

- 25 Morgenstern JP, Griffith IJ, Brauer AW, Rogers BL, Bond JF, Chapman MD, Juo M-C. Determination of the amino acid sequence of Fel dI, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. *Proc Natl Acad Sci USA* 1991; 88:9690-4.
- 26 Stokes CR, Turner MW. Isolation and characterization of cat allergens. *Clin Allergy* 1975; 5:251-54.
- 27 Didierlaurent A, Foglietti MJ, Guerin B, Hewitt BE, Percheron F. Comparative study on cat allergens from cat fur and saliva. *Int Arch Allergy Appl Immunol* 1984; 73:271-31.
- 28 Chapman MD, Aalberse RC, Brown MJ, Platts-Mills TAE. Monoclonal antibodies to the major feline allergen Fel d I. II. Single step affinity purification of Fel d I, N-terminal sequence analysis, and development of a sensitive two-site immunoassay to assess Fel d I exposure. *J Immunol* 1988; 140:812-8.
- 29 Duffort O, Carreira J, Lombardero M. Monoclonal antibodies against Fel d I and other relevant cat allergens. *Immunol Lett* 1988; 17:71-7.
- 30 Griffith IJ, Craig S, Pollock J, Xu X-B, Morgenstern JP, Rogers BL. Expression and genomic structure of the genes encoding FdI, the major allergen of the domestic cat. *Gene* 1992; 113:263-8.
- 31 Karn RC. The mouse salivary androgen-binding protein (ABP) alpha subunit closely resembles chain 1 of the cat allergen Fel dI. *Biochem Genet* 1994; 32:271-7.
- 32 Bond JF, Brauer AW, Segal DB, Nault AK, Rogers BL, Kuo M-C. Native and recombinant Fel dI as probes of the relationship of allergen structure to human IgE immunoreactivity. *Mol Immunol* 1993; 30:1529-51.
- 33 van Milligen FJ, van't Hof W, Aalberse RC. IgE and IgG4 binding to synthetic peptides of the cat (*Felis domesticus*) major allergen Fel dI. *Int Arch Allergy Immunol* 1994; 103:274-9.
- 34 van Milligen FJ, van't Hof W, van den Berg M, Aalberse RC. IgE epitopes on the cat (*Felis domesticus*) major allergen Fel d I, a study with overlapping synthetic peptides. *J Allergy Clin Immunol* 1994; 93:34-43.
- 35 Rogers BL, Morgenstern JP, Garman RD, Bond JF, Kuo M-C. Recombinant Fel dI: expression, purification, IgE binding and reaction with cat allergic human T cells. *Mol Immunol* 1993; 30:559-68.
- 36 Svendsen PJ. Fused rocket immunoelectrophoresis. *Scand J Immunol* 1993; 1:69-70.
- 37 Schafer-Nielsen C, Rose C. Separation of nucleic acids and chromatin proteins by hydrophobic interaction chromatography. *Biochim Biophys Acta* 1982; 696:323-31.
- 38 Winton HL, Wan H, Cannell MB, Thompson PJ, Garrod DR, Stewart GA, Robinson C. Class specification inhibition of house dust mite proteinases which cleave cell adhesion, induce cell death and which increase the permeability of lung epithelium. *Br J Pharmacol* 1998; 124:1048-59.
- 39 Winton HL, Wan H, Cannell MB, Gruenert DC, Thompson PJ, Garrod DR, Stewart GA, Robinson C. Cell lines of pulmonary and non-pulmonary origin as tools to study the effects of house dust mite proteinases on the regulation of epithelial permeability. *Clin Exp Allergy* 1998; 28:1273-85.
- 40 Kristensen AK, Schou C, Roepstorff P. Determination of isoforms, N-linked glycan structure and disulphide bond linkages of the major cat allergen Fel d I by mass spectrometric approach. *Biol Chem* 1997; 378:899-908.
- 41 Karn RC, Dlouhy SR. Salivary androgen-binding protein in Mus and other rodents. *J Hered* 1991; 82:453-8.
- 42 Mornon JP, Fridlansky F, Bally R, Milgrom E. X-ray crystallographic analysis of a progesterone-binding protein. The C221 crystallized form of oxidized uteroglobin at 2.2 Å resolution. *J Mol Biol* 1980; 137:415-29.
- 43 Singh G, Katyal SL, Brown WE, Phillips S, Kennedy AL, Anthony J, Squeglia N. Amino acid and cDNA nucleotide sequence of human Clara cells 10 kDa protein. *Biochim Biophys Acta* 1988; 950:329-37. Published erratum appears in 1007:243.
- 44 Miele L, Cordella-Miele E, Mukherjee AB. High-level bacterial expression of uteroglobin, a dimeric eukaryotic protein with two interchain disulfide bridges, in its natural quaternary structure. *J Biol Chem* 1990; 265:6427-35.