



Identification, cloning, and characterization of a major cat flea salivary allergen (Cte f 1)

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Abstract

An 18 kDa protein isolated from saliva of the cat flea, *Ctenocephalides felis*, elicits a positive intradermal skin test (IDST) in 100 and 80% of experimental and clinical flea allergic dogs, respectively. Using solid-phase enzyme-linked immuno assay (ELISA), this protein detected IgE in 100 and 80% of experimental and clinical flea allergic dogs, respectively. A cDNA (pFSI) encoding a full-length Cte f 1 protein was isolated from a *C. felis* salivary gland cDNA library, using a combination of PCR and hybridization screening. This cDNA is 658 bp in length, and contains an open reading frame of 528 bp. The open reading frame encodes a protein of 176 amino acids, consisting of an 18 amino acid signal sequence and a 158 amino acid mature protein. The calculated molecular weight and pI of the mature protein are 18 106 Da and 9.3, respectively. The protein, named Cte f 1, is the first novel major allergen described for canine flea allergy. Recombinant Cte f 1 (rCte f 1) was expressed in *Escherichia coli*, *Pichia pastoris* and baculovirus infected *Trichoplusia ni* cells. Approximately, 90% of the rCte f 1 expressed in *E. coli* accumulated in insoluble inclusion bodies, which could be refolded to a soluble mixture of disulfide isomers with partial IgE binding activity. Small quantities of an apparently correctly refolded form of rCte f 1, which had IgE binding activity equal to the native antigen, was isolated from the soluble fraction of *E. coli* cells. However, *P. pastoris* and baculovirus infected insect cells expressed and secreted a fully processed, correctly refolded and fully active form of rCte f 1. Mass spectrometry analysis of the active forms of rCte f 1 confirmed that eight intact disulfide bonds were present, matching the number observed in the native allergen. The relative ability of rCte f 1 to bind IgE in the serum of flea allergic animals, produced in these three expression systems, matched that of the native allergen. Competition ELISA demonstrated that approximately 90% of the specific IgE binding to native Cte f 1 could be blocked by the different forms of rCte f 1. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Flea allergy dermatitis (FAD) is the most common dermatological disease of cats and dogs living in flea endemic areas. Susceptible animals develop an intensely

pruritic papular reaction to bites of the cat flea, *Ctenocephalides felis*. Subsequent self-trauma results in excoriations, alopecia, and secondary bacterial or fungal infections of the damaged skin. Animals with chronic FAD develop disfiguring hyperpigmentation and thick-

Abbreviations: bp, base pairs; Cte f 1, a major allergen of canine flea-bite allergy from the cat flea *Ctenocephalides felis* (DNA sequence deposited 10/27/98, accession # AF102502) named according to the WHO/IUIS Allergy Nomenclature Sub-Committee; CV, column volumes; ESI, electrospray ionization; FAD, flea allergy dermatitis; FcεRIα, extracellular domain of the human high affinity IgE receptor; FSA, flea saliva antigen; HFBA, heptafluorobutyric acid; HIC, hydrophobic interaction column; IDST, intradermal skin testing; kDa, kiloDaltons; M_r , relative molecular mass; MALDI, matrix assisted laser desorption; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PFU, plaque forming units; PVDF, polyvinyl difluorobenzene; rCte f 1, recombinant Cte f 1; R/D, reduced and denatured; RT, room temperature; TFA, trifluoroacetic acid; SPF, specific pathogen-free; SSC, 0.15 M NaCl, 0.15 M NaC₆H₅O₇ · 2H₂O, 5 × Denhardt's, 0.1% BSA, ficoll and polyvinylpyrrolidone; TMB, 3,3',5,5'-tetramethylbenzidine.

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ening of the skin. In dogs, the distribution of the skin lesions of FAD differs from that seen in atopic dermatitis induced by other allergens such as house dust mite and pollens. FAD lesions are concentrated over the lower back, rump and base of the tail, while other allergens induce lesions mainly in the thin-skinned areas around the face, belly and groin. This characteristic distribution of lesions, in conjunction with response to flea control, is used in the differential diagnosis of FAD. (For review of FAD, see Reedy et al., 1997).

Intradermal testing with commercially available extracts of whole fleas may also be used for FAD diagnosis but the sensitivity of these reagents is controversial (van Winkle, 1981; Slacek and Opdebeeck, 1993). In addition, double blind controlled whole flea extract immunotherapy trials have shown the extracts to be ineffective in alleviating FAD in both dogs and cats (Kunkle and Milcarsky, 1985; Halliwell et al., 1987). However, FAD is elicited by flea bites and allergens present in saliva may be present in only minute quantities in whole flea (McKeon and Opdebeeck, 1994; Cook et al., 1996).

In an effort to improve the sensitivity and specificity of in vivo and in vitro diagnostic methods for FAD, several groups have attempted to purify the major allergens responsible for FAD. Several putative allergens have been identified in whole flea extracts ranging in molecular weight from 14 to 45 kDa (Trudeau et al., 1993; Greene et al., 1993a,b). Lee and co-workers (Lee et al., 1997, 1999) recently reported the detection of flea salivary antigens ranging from 8 to 12 and 40 kDa. Using western blot analysis, the proteins that they isolated have been shown to bind IgE in the sera of flea allergic individuals. Dogs hypersensitive to flea bites reacted positively to intradermal skin testing (IDST) using either the putative 8–12 and 40 kDa allergens (Lee et al., 1999). We have reported the chromatographic separation of flea saliva proteins into several fractions which induce positive IDST reactions in FAD dogs (Frank et al., 1996). In this study, we extend the characterization of one of these fractions. We isolated an 18-kDa protein, which is recognized by IgE antibodies present in a high percentage of FAD dogs. The cDNA encoding the protein was cloned and subsequently expressed in three heterologous expression systems, (1) bacteria (*Escherichia coli*); (2) insect cells (*Trichoplusia ni* using a baculovirus vector); and (3) yeast (*Pichia pastoris*). This study describes the structural characterization of these recombinant proteins isolated from the different expression systems and their ability to bind serum IgE from flea allergic dogs.

2. Materials and methods

2.1. Flea saliva collection and allergen purification

Flea saliva was collected on protein binding membranes in a saliva collection apparatus as previously described (Frank et al., 1996). Extracted saliva (500 ml) was loaded onto a C4 reverse phase column (4.6 × 150 mm, Vydac, Hesperia, CA) using a Waters 626 HPLC system (Waters Corp., Milford, MA). Bound proteins were eluted with an increasing gradient from 94% solvent A (0.1% trifluoroacetic acid (TFA) in water), 6.0% solvent B (0.085% TFA in 90% CH₃CN) to 50% solvent B over 50 min, and finally to 100% B over 20 min at a flow rate of 0.8 ml/min. Fractions (1.0 ml) were collected and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and tested for their allergenicity using the IDST procedure previously described (Frank et al., 1996). Fractions containing a major allergen of canine flea-bite allergy from the cat flea *C. felis* (DNA sequence deposited 10/27/98, accession # AF102502) named according to the WHO/IUIS Allergy Nomenclature Sub-Committee (Cte f 1) were further purified on a C4 column (2.1 × 250 mm, Vydac) using 0.13% heptafluorobutyric acid (HFBA) in water as solvent A and 0.11% HFBA in 90% CH₃CN as solvent B. A gradient of 30–100% solvent B over 55 min was used (data not shown). Purified Cte f 1 was analyzed by Edman N-terminal sequencing as described below.

2.2. Identification of a full length cDNA encoding Cte f 1

A *C. felis* salivary gland cDNA library was prepared from approximately 6000 salivary glands dissected from fed and unfed fleas. RNA was prepared by direct extraction using the guanidinium thiocyanate procedure followed by centrifugation through cesium chloride. PolyA⁺ mRNA was prepared using the FAST-TRACK™ kit (Invitrogen Corp., San Diego, CA) according to the manufacturer's instructions. Isolated mRNA was used directly for cDNA synthesis and molecular cloning was performed using the Lambda-Zap-cDNA Synthesis Kit™ (Stratagene Cloning Systems, LaJolla, CA) according to the manufacturer's instructions.

N-terminal sequence analysis of purified Cte f 1 yielded a 26 residue sequence: E D I W K V N K K X T S G G K N Q D R K L D Q I I Q. This amino acid sequence was used to design a set of synthetic degenerate oligonucleotide primers for polymerase chain reaction (PCR) amplification. The first degenerate primer, constructed from amino acid residues 1 through 8, had the nucleic acid sequence 5'-GAR GAY ATH TGG AAR GTN AAY AA-3'. The second degenerate

primer, constructed from residues 11 through 18, had the nucleic acid sequence 5'-CAN TCN GGN AAR AAY CAR GA-3'. These primers were used in conjunction with two vector primers, in a nested PCR reaction, to screen the *C. felis* salivary gland cDNA library. PCR was carried out under the following conditions: a 4.0- μ l fraction of the flea cDNA library, containing approximately 4.0×10^6 plaque forming units (PFU), was used as template in a 100- μ l PCR reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 200 μ M dNTP's and 1.0 μ M PCR primers, as described above. AmpliTaq Gold DNA Polymerase (2.5 U, PE Applied Biosystems, Branchburg, NJ) was added, and the reaction was carried out in the following manner, one cycle of 10 min at 95°C; 35 cycles of 30 s at 94°C; 30 s at 50°C; 1 min at 72°C; and one cycle of 7 min at 72°C. Using this approach a 573 bp PCR product was generated. This 573 bp product codes for an open reading frame that matched the N-terminal amino acid sequence determined from the native 18 kDa protein.

A DNA probe labeled with ³²P, comprising nucleotides from the 573 bp PCR-generated clone, was used to screen a whole flea cDNA library using standard hybridization techniques. Approximately 1.0×10^6 plaques were screened under the following conditions: plaques were denatured in the presence of 0.5 M NaOH, 1.5 M NaCl, and neutralized in a solution containing 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0). Following neutralization, the filters were rinsed briefly in $2 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl, 0.15 M NaC₆H₅O₇ · 2H₂O) and subjected to UV crosslinking using a UV Stratalinker 1800™ (Stratagene Cloning Systems, La Jolla, CA). The filters were then blocked in hybridization buffer containing $5 \times$ SSC, $5 \times$ Denhardt's (0.1% BSA, ficoll and polyvinylpyrrolidone), 0.5% SDS, and 100 mg/ml ssDNA for 3 h at 52°C. Approximately, 4.0×10^6 cpm of probe was added for each filter, and hybridization was carried overnight at 52°C. Following hybridization, the filters were washed in $2 \times$ SSC, 0.5% SDS at room temperature (RT) for 15 min, followed by two washes, for 10 min, in the presence of $0.2 \times$ SSC, 0.1% SDS at 55°C. The filters were rinsed in $2 \times$ SSC, air dried and subjected to autoradiography. Positive plaques were picked and phagemid DNA was isolated. Using this approach, a nucleic acid molecule encoding a full-length cDNA for Cte f 1 (pFSI) was identified (accession number AF102502).

2.3. Expression and isolation of recombinant Cte f 1

2.3.1. Construction of Cte f 1 *E. coli* recombinant gene expression vector

A DNA fragment encoding the entire Cte f 1 protein was amplified from the cDNA designated pFSI using primers jmk21 (5'-GCGCGGATCCGCATATGGAA-GACATCTGGAAAGTTAATAAAAAATGTACAT-

CAGG-3') and jmk20 (5'-CCGGAATTCTTATTATT-TATTTTTTGGTCGACAATAACAAAAGTTTCC-3'). The PCR reaction was carried out in a 50- μ l reaction volume using 20-ng template DNA, 1.0 μ M of each primer, 0.2 mM of each of the four deoxyribonucleoside triphosphates, 20 mM Tris-HCl, pH 8.8, 2.0 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.1 mg/ml BSA and 5 U cloned *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, CA). Amplification was carried out under the following conditions: 25 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; followed by 7 min at 72°C.

The PCR product was digested with the restriction enzymes *Bam*HI and *Eco*RI and ligated into similarly digested p λ cro (plasmid p λ cro is a ColE1-derived plasmid developed by Heska Corporation for producing foreign proteins in *E. coli* using the thermally inducible λ pR promoter). The resulting plasmid was cleaved with *Nde*I, self-ligated to remove non-essential sequences, and transformed into *E. coli* BL21 cells. An ampicillin resistant transformant with the desired plasmid sequence was saved as strain JK115. The plasmid in JK115 fuses the entire Cte f 1 coding sequence, along with an initiating methionine residue, to the λ pR promoter.

2.4. Isolation of rCte f 1 expressed in *E. coli*

Overnight cultures (25 ml) of *E. coli* cells (Strain JK115) were grown from 5 to 10 ml inoculations of frozen stock cultures in Superbroth (3.2% Difco Bacto tryptone, 2% Difco Bacto yeast extract, 0.5% NaCl, 5.0 mM NaOH, 100 μ g/ml ampicillin). Scale-up cultures were inoculated with 2–5 ml of the overnight cultures and initially grown in shake flasks (300 ml/1 liter flask, total volume = 4–20 l) at 32°C to a cell density of 0.8–1.0 (absorbance 600 nm (A_{600})). The culture was induced by shifting the temperature to 42°C then incubating for an additional 2–3 h. Fermentation runs were performed using a 6-l (4.5-l working volume) fermenter (BioFlo III, New Brunswick Scientific, Edison, NJ). Overnight cultures (100 ml) were grown in superbroth containing 100 mg/ml ampicillin and inoculated into 4 l of ampicillin containing media in the fermenter. Cells were grown at 32°C to a density of $A_{600} \sim 10.0$ –20.0 and induced for 1–2 h at 42°C. Cells grown in either shake flasks or the fermenter were harvested after chilling by centrifugation at $2000 \times g$ for 30 min in a refrigerated large volume centrifuge (IEC PR-7000 M, International Equipment Company, Needham Heights, MA). Aliquots of the whole cells were analyzed by SDS-PAGE.

Harvested cells were suspended (10:1, v:w, buffer:cells) in phosphate buffered saline, pH 7.2 (PBS-breaking buffer) and passed twice through a mi-

crofluidizer at 120 PSI (Microfluidizer Model 1108, Microfluidics Corporation, Newton, MA). The burst cell suspension was centrifuged at $30\,000 \times g$ for 30 min. The supernatant (S1) was saved for further processing of soluble recombinant Cte f 1 (rCte f 1). The pellet (P1) was resuspended in breaking buffer (10:1, v:w), and re-centrifuged at $30\,000 \times g$ for 30 min. The supernatant (S2) was saved for analysis and the washed pellet (P2) was resuspended in breaking buffer (10:1, v:w) containing 1.0% Triton X buffer and re-centrifuged for a final time for 30 min. The supernatant (S3) and the pellet (P3, inclusion bodies) were saved for analysis. SDS-PAGE and western blot analysis of S1–S3 and P1–P3 showed that rCte f 1 was distributed between the soluble fractions (S1–S2, ~10–15%) and the insoluble (inclusion bodies) fractions (P3, ~85–90%, data not shown). Therefore, S1 and S2 were combined and processed as described below. The inclusion body fraction (P3), containing 85–90% of the rCte f 1 was also processed as described below.

2.5. Isolation of soluble rCte f 1 produced in *E. coli*

Soluble fractions S1 and S2 were combined, adjusted to pH 6.5 and loaded onto an SP-sepharose (Amersham-Pharmacia Biotech, Piscataway, NJ) cation exchange column previously equilibrated with 50 mM Tris, pH 6.5 (SP–Tris-buffer A). Bound protein was eluted with a linear gradient to 40% SP–Tris-buffer B (50 mM Tris, pH 6.5, 1.0 M NaCl) in 10–15 column volumes (CV). SDS-PAGE analysis showed that rCte f 1 bound the column and eluted at 0.4–0.5 M NaCl.

Fractions containing rCte f 1 were pooled and ammonium sulfate was added to 1.2 M. This pool was then loaded onto a butyl-sepharose (Pharmacia-Amersham Biotech) HIC column previously equilibrated with 50 mM Tris, pH 7.0, 1.2 M ammonium sulfate. SDS-PAGE and western blot analysis showed that rCte f 1 did not bind to the column and was present in the flow-through. The fractions containing rCte f 1 were analyzed by reverse phase chromatography.

2.6. Isolation of insoluble rCte f 1 from inclusion bodies

Initial experiments (see above) demonstrated that ~90% of expressed rCte f 1 was present in an insoluble form in inclusion bodies (P3 fraction). The rCte f 1 present in the inclusion bodies was solubilized in 8.0 M urea, 15 mM Na_2HPO_4 , pH 10.7, 0.1 M β -mercaptoethanol. Insoluble contaminants were removed by centrifugation at $30\,000 \times g$ for 30 min as described above, yielding S4 and P4 fractions. The S4, urea solubilized rCte f 1, was adjusted to pH 7.5 and loaded onto a SP-sepharose column previously equilibrated with 8 M urea, 15 mM Tris, pH 7.5, 100 mM β -mercap-

toethanol (SP–urea-buffer A). Bound protein was eluted with a linear salt gradient to 40% SP–urea-buffer B (8 M urea, 1 M NaCl, 50 mM Tris, pH 7.5, 100 mM β -mercaptoethanol) in 15–20 CV. SDS-PAGE analysis showed that rCte f 1 bound to the column and eluted at 0.4–0.5 M NaCl. Isolated Cte f 1 was renatured by dialysis against PBS, pH 7.2, centrifuged at $10\,000 \times g$ for 60 min then analyzed by SDS-PAGE and RP-C18 reverse phase chromatography.

2.7. Construction of Cte f 1 *P. pastoris* recombinant gene expression vector

The expression plasmid pBC102, used for secreting rCte f 1 from *P. pastoris*, was constructed by inserting a *Sna*BI–*Eco*RI PCR fragment containing the Cte f 1 coding sequence into similarly digested pPIC9K (Invitrogen, Carlsbad, CA). The PCR fragment was amplified from the plasmid DNA designated pFSI with primers EB3 (5'-GCTGTAGCTTACGTAGAAGACATCTGGAAAGTTAA) and EB5 (5'-GAGTCAGGTGAATTCTTATTTATTTTGGTTCGACA) using the following reagents, 10 ng template DNA; 1.0 mM of each primer; 20 mM Tris–HCl; pH 8.75; 10 mM KCl; 10 mM $(\text{NH}_4)_2\text{SO}_4$; 2.0 mM MgCl_2 ; 0.1% Triton X-100; 0.2 mM each of the four deoxyribonucleoside triphosphates, and 2.5 U of *Pfu* polymerase (Stratagene Cloning Systems, Dutova et al., 1998; Raymond et al., 1998). Amplification was performed using the following cycle conditions, 25 cycles of 1 min at 94°C; 30 s at 94°C; 30 s at 50°C; 1 min at 72°C; and one cycle of 7 min at 72°C. The resulting plasmid fuses the codon encoding the first residue of the mature Cte f 1, glutamic acid, to the codon encoding the 89-amino acid *S. cerevisiae* α -mating factor leader sequence. Following secretion and signal sequence cleavage at the KEX2 and STE13 sites, the *P. pastoris*-produced rCte f 1 is expected to contain two additional N-terminal amino acids — tyrosine and valine.

Electrocompetent SMD1168 cells were prepared essentially as described by Becker and Guarante (1991). Approximately, 5.0 μg of *Sac*I-linearized pBC102 DNA was pulsed with 80 ml electrocompetent SMD1168 cells in a sterile 0.2-cm sterile cuvette using a BTX electroporator set at 1.5 kV, 25 mF, 400 Ω . Immediately following pulsing, 1.0-ml ice-cold 1.0 M sorbitol was added to the cuvette, and cells were plated on MD plates (described below) at 30°C to select for His⁺ integrants. Integration of pBC102 into the chromosome of His⁺ integrants was confirmed by PCR. PCR (50 μl) reactions containing the following reagents were prepared: ~5.0 μg *P. pastoris* genomic DNA (isolated using the QIAamp™ Tissue Kit; Qiagen, Valencia, CA), according to the manufacturer's recommended protocol, 1.0 mM of each 5'AOX1 and 3'AOX1 primers, 20 mM Tris–HCl, pH 8.75, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$,

2.0 mM MgCl₂, 0.1% Triton X-100, 0.2 mM each of the four deoxyribonucleoside triphosphates, and 2.5 U of *Pfu* polymerase (Stratagene Cloning Systems). Amplification was performed using the following cycle conditions, one cycle of 1 min at 94°C; 25 cycles of 45 s at 94°C; 45 s at 54°C; 90 s at 72°C; and one cycle of 7 min at 72°C. Aliquots of 10 µl of the reactions were evaluated by agarose gel electrophoresis. Several strains were shown to have PCR products of the predicted sizes. One clone designated HCY148 was saved for future studies.

2.8. Isolation of rCte f 1 expressed in *P. pastoris*

P. pastoris strains were routinely cultured on YPD (1.0% yeast extract, 2.0% peptone, 2.0% dextrose). His⁺ transformants were selected on MD medium (1.34% yeast nitrogen base, 0.00004% biotin, 2.0% dextrose). Small-scale induction of *P. pastoris* strains HCY148 was performed using BMG or BMM which were composed of the following: 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 0.00005% biotin and either 1.0% glycerol (BMG) or 0.5% methanol (BMM). Single colonies of these strains were used to inoculate 25 ml of BMG in a 250 ml baffled flask. Cultures were grown at 28°C with shaking to $A_{600} \sim 1.0$. Cultures were pelleted for 10 min at 3000 $\times g$ and resuspended in 250 ml BMM ($A_{600} \sim 1.0$) in eight 2 l baffled flasks, incubated at 28°C for 4 days and induced by daily addition of 0.5% methanol (v/v). The cell cultures were centrifuged at 15 000 $\times g$ for 30 min (Avanti centrifuge, Beckman, Fullerton, CA) to remove the cells.

The medium (2 l total volume) containing secreted Cte f 1 was concentrated to 400 ml using a tangential flow filtration (3000 MW cutoff (MWCO), AG Technologies Needham, MA) and dialyzed against 20 mM CH₃COONa pH 6.0 (SP-buffer A). SDS-PAGE analysis showed that a band co-migrating with rCte f 1 isolated from *E. coli* was present in the concentrated media. The dialyzed media was loaded onto a 5 $\times$ 20 mm SP-sepharose column (Pharmacia Biotech, Piscataway, NJ) previously equilibrated with 20 mM sodium acetate, pH 6.0. Bound protein was eluted with a linear salt gradient to 60% SP-buffer B (20 mM CH₃COONa, 1.0 M NaCl, pH 6.0) in 20–25 CV. Fractions (0.5 ml) were collected and analyzed by SDS-PAGE and reverse phase RP-C18 chromatography. It was observed that rCte f 1 eluted at 0.4–0.46 M NaCl.

2.9. Construction of Cte f 1 baculovirus recombinant gene expression vector

A recombinant baculovirus expressing Cte f 1 under transcriptional control of the Autographa californica polyhedrin promoter was constructed using the Bac-to-

BacTM baculovirus expression system (Life Technologies, Gaithersburg, MD). The polymerase chain reaction was used to amplify the entire Cte f 1 coding sequence and the native 18 amino acid signal sequence as a 531 bp *Bam*HI–*Hind*III fragment for subcloning into *Bam*HI–*Hind*III-digested pFastBac1. The forward and reverse primers for PCR amplification were 5'-CCGGGATCCTATAAATATGAATTATTGTTTTT-TAGTATTTTTAG-3' and 5'-GCCAAGCTTTTAT-TTATTTTTTGGTCGACAATAAC-3', respectively. The amplification reaction included 5-ng template DNA, 0.25 mM each primer, 5.0 U cloned *Pfu* polymerase (Stratagene), 1 $\times$ cloned *Pfu* buffer (as supplied by the manufacturer), 0.25 mM each dNTP. Cycle conditions were, one cycle of 4 min at 95°C; 25 cycles of 30 s at 95°C, 30 s at 55°C, 1 min 15 s at 72°C; and 1 cycle of 5 min at 72°C. The DNA sequence of the resulting pFASTBac1 recombinant plasmid pFAST-Bac1-Cte f 1 was confirmed using gene specific primers. Plasmid DNA was transformed into competent DH10Bac cells (Life Technologies) to produce a recombinant bacmid. The DNA from this recombinant bacmid was used to transfect *Spodoptera frugiperda* (Sf9) cells, essentially as described in the manufacturer's instruction manual (Life Technologies Bac-to-BacTM Baculovirus Expression Systems).

The supernatant containing recombinant baculovirus particles was harvested 72-h post-transfection and further amplified in Sf9 cells for 1 week. The resulting viral stock was titered on Sf9 cells and used to infect *T. ni* cells. *T. ni* cells were grown according to the manufacturer's instructions and infected with recombinant baculovirus particles at a multiplicity of infection (MOI) of one. Cells were harvested 48 h later and the supernatant fraction, containing rCte f 1, was further purified as described below.

2.10. Isolation of rCte f 1 expressed in baculovirus infected *T. ni* insect cells

Culture supernatants were harvested by centrifugation of the cell cultures at 5000 $\times g$ for 30 min and sterile filtered (0.2 µm, Corning, NY). The pH of the Hi five cell (*T. ni*) supernatant (4 l) was adjusted to pH 6.0 and loaded onto a Q-SP-sepharose tandem column 2 $\times$ 5 ml, (Amersham-Pharmacia Biotech) previously equilibrated with 20 mM CH₃COONa, pH 6.0 (SP-buffer A). After loading the sample, the Q-sepharose column was disconnected. The S-sepharose column was washed with 3–4 CV of SP-buffer A. Bound protein was eluted with a linear gradient to 80% SP-buffer B (20 mM CH₃COONa, pH 6.0, 1.0 M NaCl) in 20 CV. SDS-PAGE analysis of protein present in the S-sepharose fractions showed that rCte f 1 eluted at 0.55 M NaCl. Fractions containing rCte f 1 were pooled and aliquots were saved for SDS-PAGE analysis and quan-

titation by reverse (data not shown) RP-C18 chromatography.

The SP-sepharose pool was diluted with four volumes of 10 mM Na₂HPO₄, 10 mM NaCl, pH 7.0 (HAP-buffer A). The diluted sample was loaded onto a hydroxyapatite column (Hydroxyapatite type II, Bio-Rad, Hercules, CA, 1.0 × 8.0 cm) previously equilibrated with HAP-buffer A. Bound protein was eluted with a linear gradient to 100% HAP-buffer B (500 mM Na₂HPO₄, 10 mM NaCl, pH 7.0) in 40 CV. The flow-through, wash and elution fractions (1.5 ml) were collected and analyzed by SDS-PAGE.

2.11. Structural characterization of Cte f 1

2.11.1. Reverse phase refold assay

To determine conformational forms of rCte f 1 produced using each of the expression systems (*E. coli*, *P. pastoris*, baculovirus infected *T. ni* cells) an analytical reverse phase protocol was developed. Briefly, 0.5–3.0 mg of rCte f 1 or native Cte f 1 was loaded onto a RP-C18 column (1.0 × 250 mm, Vydac) previously equilibrated with 5.0% acetonitrile, 0.1% TFA. Bound protein was eluted with a linear gradient (0.1 ml/min) to 50% buffer B (100% acetonitrile, 0.085% TFA) in 20 CV ($\Delta\% B = 1\% B$ per min), 50–100% B in 2 CV. The eluent was monitored at 200–300 nm. The elution time of the recombinant forms of Cte f 1 were compared with that of native Cte f 1.

2.12. N-terminal sequencing

All protein samples were sequenced using standard pulsed liquid Edman degradation chemistry on either a Model 473A or a Procise™ HT 494-01 Protein Sequencer (Applied Biosystems). All sequencer and chromatography reagents were from Applied Biosystems. Buffer B is composed of 88% CH₃CN and 12% isopropanol (EM Science), both HPLC grades.

2.13. Alkylation of rCte f 1

Reduction and alkylation of rCte f 1 was performed as previously described (Brune, 1992; Sechi and Chait, 1998). The relative activity of alkylated Cte f 1 to serum IgE was measured using solid phase and competition ELISA as described below.

2.14. Mass spectrometry analysis of FS-I

Samples containing isolated native Cte f 1 and rCte f 1 were subjected to RP-C18 chromatography prior to mass spectrometry analysis (see methods described above). Separate aliquots were denatured, reduced, and subjected to alkylation then isolated by RP-C18 chromatography. RP-C18 fractions were analyzed by both

matrix assisted laser desorption (MALDI) and nano-electrospray ionization quadrupole (ESI) mass spectrometry (Stults, 1995).

2.15. Characterization of Cte f 1 as a major allergen of FAD

2.15.1. Detection of canine IgE using ELISA

In order to achieve highly specific detection of IgE, an ELISA using the extracellular domain of the alpha chain of a high affinity human IgE receptor (FcεRIα) was developed (Jelsema and Kinet, 1994; Klubal et al., 1997). The recombinant receptor consists of the extracellular portion of the alpha chain (amino acids 1–172) expressed in *T. ni* insect cells using a baculovirus expression system (Ye et al., 1992; Klubal et al., 1997; McCall et al., 1997). FcεRIα detects IgE antibody in the serum of allergic dogs and cats. Solid phase ELISAs were performed by coating varying concentrations of antigens at 4°C overnight in 50 mM carbonate/bicarbonate buffer (pH 9.6) in 96-well plates (Immulon II, Dynatech Labs). Excess antigen was removed and the plates were blocked with assay buffer (PBS, 4% fetal calf serum, 0.05% Tween 20) for 1 h. Sera were diluted to 1:10 in assay buffer, added to the plates then incubated for 2 h at RT. Plates were then washed four cycles with PBS, 0.05% Tween 20 (PBS-T20) and incubated at RT for 1 h with 1.5 ng per well of biotinylated FcεRIα (McCall et al., 1997). After a further wash cycle, 10 ng per well of streptavidin-HRP (KPL Labs, Gaithersburg, MD) was added and incubated for 1 h at RT. Following a final wash, the plates were developed with 3,3', 5,5'-tetramethylbenzidine (TMB, KPL Labs) for 10–30 min and the reaction was stopped by the addition of 1 M H₃PO₄. The concentration of IgE was determined by measuring absorbance at 450 nm (SpectraMax 250 ELISA plate reader, Molecular Devices, Sunnyvale, CA). The relative concentration of IgE was expressed as absorbance at 450 × 1000 nm after background subtraction.

Competition ELISAs were performed by preincubating titrated concentrations of inhibitory antigens (up to 10 µg per well) with sera from FAD animals diluted 1:30 for 1–2 h at RT. Unbound IgE present in the sera was detected by incubating the sera for 1 h in FSA or native Cte f 1 coated plates. Percent inhibition was calculated by comparing the relative amount of IgE bound with either FSA or native Cte f 1 using inhibiting antigens against the amount of bound IgE observed using uninhibited sera.

2.16. Intradermal skin test (IDST)

IDST was carried out by administering separate 50-µl injections of antigens, (dissolved in PBS), whole flea extract (1:250 dilution, Greer Labs, Lenoir NC), PBS

negative control, and histamine phosphate positive control (1/100 000 w/v dilution). The reactions were evaluated objectively and subjectively at 15 min. Objective evaluation included two perpendicular measurements of the wheels with a caliper. Subjective evaluation included assessment of the wheal for size and erythema and palpation for turgidity. A score of 0 was assigned to the negative saline control site and 4+ assigned to the positive histamine control site.

2.17. Allergic dog populations

2.17.1. Experimental FAD dogs

Outbred laboratory dogs were sensitized to fleas by exposing them once a week to 25 unfed female fleas for 20 min. The fleas were contained in mesh-fronted chambers strapped to the shaved lateral thorax of the dog. After 40-week exposure, approximately 75% of the dogs were IDST positive to flea saliva antigen (FSA) prepared as described in Frank et al. (1996), but no FAD dermatological symptoms were observed. When these sensitized dogs were infested with fleas (100 unfed female fleas released into the dorsal hair once per week for 6 weeks), all dogs developed IgE antibodies to FSA, and all developed symptoms of FAD, although the extent of the symptoms varied widely. Dogs with severe symptoms were indistinguishable from clinical cases of FAD seen in client owned dogs after environmental flea exposure. The dogs were intradermally skin tested with FSA and with rCte f 1. A pool of sera from 14 experimental FAD dogs, designated pool A, used in ELISA experiments were noted.

2.18. Clinical FAD dogs

Sera from client owned dogs with clinical FAD were recruited in collaboration with Dr Ken Kwochka and Dr Andrew Hillier, Department of Clinical Veterinary Sciences, Ohio State University, Columbus, OH. The diagnosis of FAD was made on the basis of clinical signs and response to flea control. The dogs were also intradermally skin tested with whole body flea extract (Greer Labs., NC), FSA, native Cte f 1, and rCte f 1. Serum samples were obtained from all dogs and anti-FSA and anti-Cte f 1 IgE antibodies were measured by ELISA. Negative controls consisted of sera from unexposed laboratory dogs or 'specific pathogen-free' (SPF) sera purchased from Harlan Bioproducts, Indianapolis, IN.

3. Results

3.1. Isolation of native Cte f 1

Reverse phase chromatography resolved flea saliva

proteins into at least 15 major peaks (Frank et al., 1996). SDS-PAGE and IDST analysis of these peaks showed a protein with a relative molecular mass (M_r) of 18 kDa that eluted at approximately 32% acetonitrile and gave a positive IDST reaction in FAD dogs (Frank et al., 1996). This protein was later termed Cte f 1 according to the WHO/IUIS Allergen Nomenclature Sub-Committee conventions (King et al., 1994). In order to obtain N-terminal sequence, fractions containing Cte f 1 were pooled and re-injected onto the same reverse phase column using HFBA as the ion-pairing agent. Cte f 1 eluted at 63% acetonitrile and was approximately 95% homogeneous. The purified protein was subjected to N-terminal sequence analysis. The following sequence was obtained, EDIWKVNKKXTSGGKNQDRKLDQIIQ. This sequence was used to construct primers for the PCR-directed cloning of the cDNA encoding the 18 kDa allergen.

3.2. Cloning of cDNA encoding Cte f 1

A cDNA (pFSI) encoding a full-length Cte f 1 protein was isolated from a *C. felis* salivary gland cDNA library, using a combination of PCR and hybridization screening, as described in Section 2. This cDNA (accession # AF102502) is 658 bp in length, and contains an open reading frame of 528 bp. The open reading frame encodes a protein of 176 amino acids, consisting of an 18 amino acid signal sequence and a 158 amino acid mature protein. The mature protein has a calculated molecular weight of 18 106 Da and pI of 9.3 calculated as described in Ribeiro and Sillero (1990). The molecule contains 16 cysteines and is composed of 22% basic residues (arginine and lysine). Database searches, using basic local algorithm search tool (BLAST) (Altschul et al., 1997) revealed no significant homologies to any sequence deposited in GenBank™.

3.3. Structural characterization of Cte f 1

Fig. 1 shows that rCte f 1 was successfully expressed in *E. coli*, *P. pastoris*, and baculovirus infected insect cells. SDS-PAGE analysis confirmed that all three expression systems produced a protein with a M_r of 18 kDa, consistent with an unglycosylated product. The relative yields were as follows, *E. coli*, 200 mg/l; *P. pastoris*, 0.3 mg/l; and baculovirus infected insect cells, 4–5 mg/l.

3.4. Recombinant Cte f 1 expressed in *E. coli*

After expression in *E. coli*, rCte f 1 was detected in both soluble and insoluble cellular fractions. The recombinant protein in the soluble fraction was subsequently purified using a series of chromatographic steps

including cation exchange, hydrophobic interaction and reverse phase chromatography. Reverse phase chromatography showed that the soluble form of rCte f 1 eluted as a major peak at 25% acetonitrile (fractions 28.5–29.5 min, Fig. 2) adjacent to poorly resolved shoulder peaks (fractions 26.0–29.0, 30.0–31.5 min;

Fig. 2). The number of shoulder peaks observed varied with the preparation (Fig. 3A–D). SDS-PAGE analysis of the reverse phase peaks showed that a single, apparently homogeneous, protein (18–19 kDa) was present in all of the peaks. A lower MW protein (16–17 kDa) eluting before the bulk of the rCte f 1 product was also

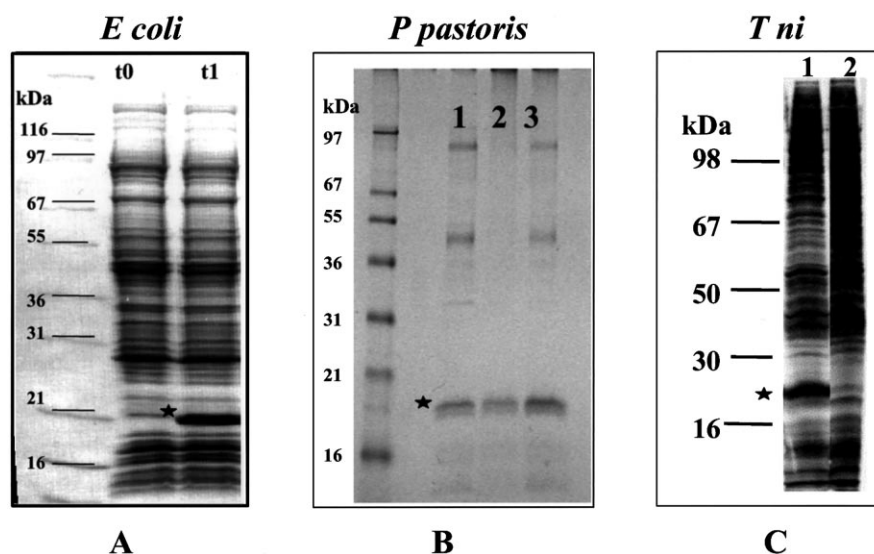


Fig. 1. Expression of rCte f 1. SDS-PAGE analysis of *E. coli* cells, and *P. pastoris* and *T. ni* media. (A) *E. coli* strain JK115 induced for 2–3 h at 42°C (lane t0, uninduced; lane t1, induced); (B) *P. pastoris* media of clone HCY148 containing pBC102 (lanes 1–3); (C) media of *T. ni* cells containing pFASTBac1-Cte f 1 (lane 1, baculovirus infected cells; lane 2, uninfected cells). ★ Indicates rCte f 1.

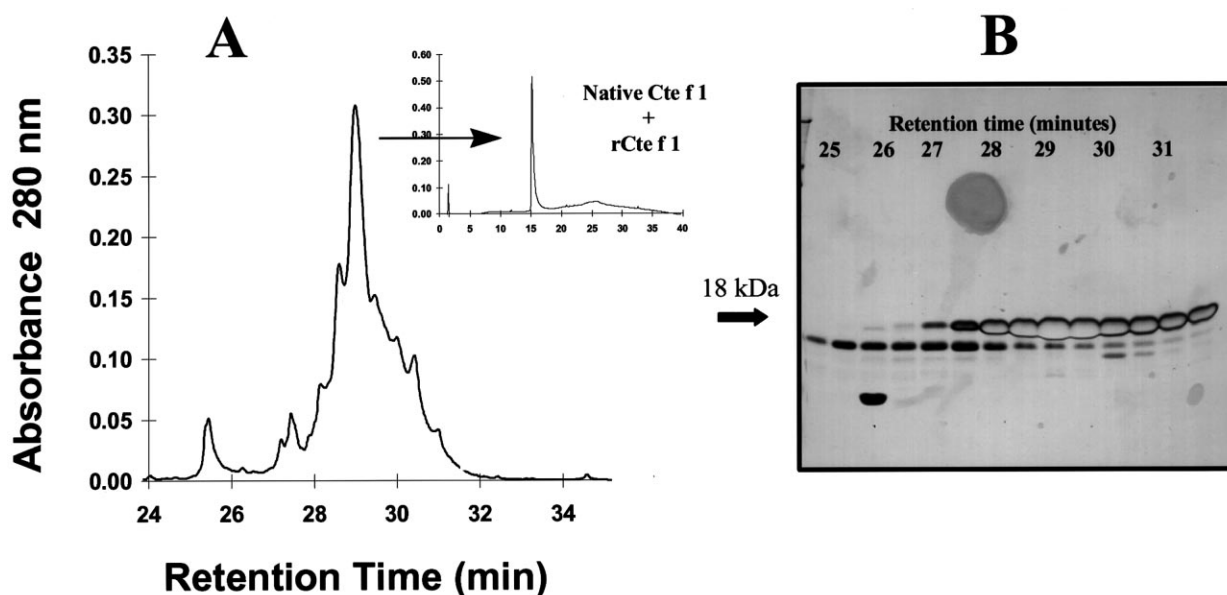


Fig. 2. Reverse phase (RP-C4) chromatography of *E. coli* produced rCte f 1. Butyl sepharose fractions containing *E. coli* produced rCte f 1 were pooled as described in text. The sample was loaded onto an RP-C4 column (2.1 × 250 mm), previously equilibrated with 0.05% TFA and bound protein was eluted as described (A). Fractions were collected and analyzed by SDS-PAGE (B). The major peak eluting at 28.5–29.5 min was pooled and re-injected onto the RP-C4 column with native Cte f 1 and eluted using a 20 column volume linear gradient with 100% acetonitrile, 0.045% TFA at 0.20 ml/min (A inset).

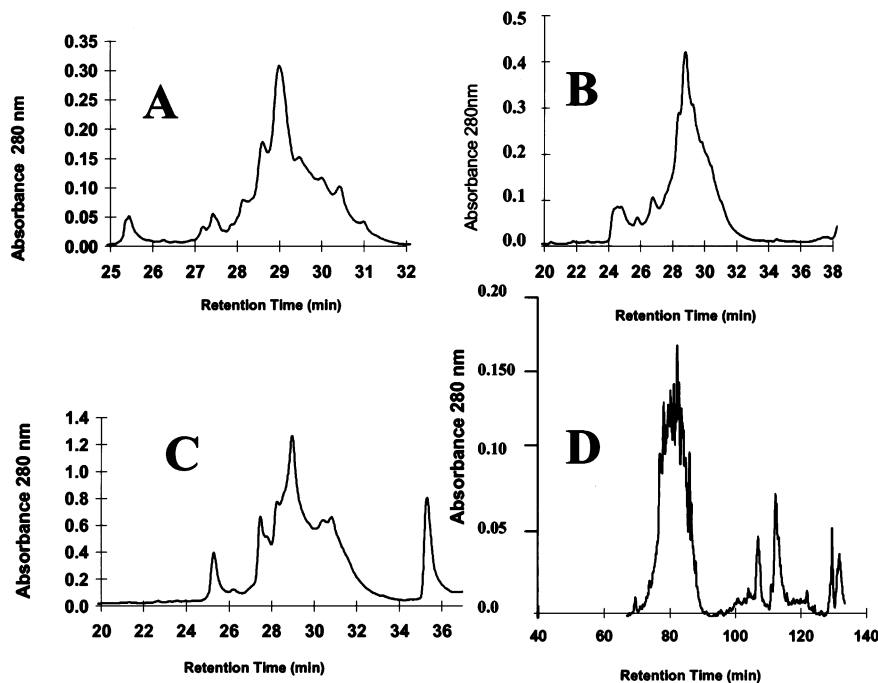


Fig. 3. Comparison of reverse phase (RP-C4) chromatography profiles of *E. coli* produced rCte f 1 isolated from different preparations: Butyl sepharose fractions containing rCte f 1 were pooled, concentrated 5–10 $\times$ and loaded onto a RP-C4 column (1.0 $\times$ 25.0 or 2.5 $\times$ 25.0 cm), previously equilibrated with 0.05% TFA. The sample was loaded in 5–50 ml aliquots. Bound protein was eluted with a linear gradient to 40% buffer B (CH₃CN + 0.045% TFA) in 20 CV and 40–100% B in 2 CV. The flow rate was 3.0–6.0 ml/min. The eluent was monitored from 200–350 nm using a photodiode array detector (PDA). Fractions were collected and analyzed by SDS-PAGE. (A–B) Four liter shake flask preparation; (C) 20-l shake flask preparation; (D) 4-l fed batch fermentation.

observed in all preparations (Fig. 2). N-terminal sequence analysis showed that the 18–19 kDa proteins had intact N-termini beginning with the uncleaved methionine residue (MEDI...). The lower molecular weight band had an identical N-terminal sequence, suggesting that it may be a C-terminal protease digestion product. SDS-PAGE analysis under non-reducing conditions showed that <10% of the rCte f 1 present in the RP-C4 fractions was a 38 kDa dimer containing intermolecular disulfide bonds (data not shown). Mass spectrometry analysis of fractions 29.0–31.5 min showed that these peaks contained mixtures of disulfide isomers of Cte f 1 (masses ranging from 18 220 to 18 238 amu). The major peak (28.5–29.5 min), which co-eluted with native Cte f 1 when co-injected onto a reverse phase C18 column (Fig. 2 inset), was collected and pooled *E. coli* produced soluble rCte f 1. Reverse phase (RP-C4) analysis of soluble rCte f 1 produced in fed batch fermentation runs showed that the product contained a mixture of Cte f 1 disulfide isomers (Fig. 3D).

Insoluble rCte f 1 was also isolated from inclusion bodies, solubilized and refolded by dialysis. Reverse phase chromatography showed that, prior to refolding, reduced and denatured rCte f 1 eluted later than the soluble disulfide isomers of rCte f 1 (data not shown). However, refolded rCte f 1 eluted as a series of poorly

resolved peaks between reduced and denatured rCte f 1, and the major disulfide isomer isolated from the soluble fraction of the *E. coli* cells and native Cte f 1 (data not shown). These results strongly suggest that solubilized and refolded rCte f 1 isolated from the inclusion bodies is composed of mis-folded disulfide isomers.

3.5. Recombinant Cte f 1 expressed in *P. pastoris*

P. pastoris cells were engineered to express and secrete rCte f 1. The medium containing the recombinant product was concentrated 5-fold, dialyzed against 20 mM NaCH₃COOH, pH 6.0. The recombinant product was isolated using cation exchange chromatography. Reverse phase chromatography of rCte f 1 present in SP-sepharose fractions showed that *P. pastoris* expressed rCte f 1 eluted as a major peak at 25% acetonitrile (data not shown). SDS-PAGE analysis showed that rCte f 1 represented the bulk of the material (>95%); however, lower molecular weight bands were visible. The major peak co-eluted with native Cte f 1 (data not shown). RP-C18 fractions containing rCte f 1 were either loaded onto SDS-PAGE, blotted to polyvinyl difluorobenzene (PVDF) membranes, or subjected to N-terminal sequencing. The sequence determined for the SDS-PAGE band co-migrating with native Cte f 1 was EAEAYVEDI.... This

sequence matched the predicted sequence of rCte f 1 cleaved at the predicted KEX2 protease site in the α -factor signal sequence encoded by the *P. pastoris* expression vector (data not shown). N-terminal sequence analysis of the lower MW bands (5–10 kDa) eluting at 23.5 min, yielded the sequence YVEDIW... (data not shown). These data indicate that a significant proportion (~10%) of the rCte f 1 secreted by *P. pastoris* was cleaved at the carboxy-terminus to yield a truncated molecular species.

3.6. Recombinant Cte f 1 expressed in baculovirus infected insect cells

T. ni cells were engineered to express and secrete rCte f 1. The medium containing the recombinant product was adjusted to pH 6.0 and rCte f 1 was isolated using sequential cation exchange and hydroxyapatite chromatography steps. SDS-PAGE analysis of protein, which eluted from the SP-sepharose cation exchange column, showed that rCte f 1 eluted at 0.55 M NaCl. The SP-sepharose fractions containing rCte f 1 were diluted with four volumes of 10 mM Na₂HPO₄, 10 mM NaCl, pH 7.0, and loaded onto the hydroxyapatite column. SDS-PAGE analysis of the final hydroxyapatite chromatographic step showed that rCte f 1 bound to the column and eluted at 0.35–0.40 M Na₂HPO₄ (data not shown). Fractions containing rCte f 1 were pooled, with aliquots saved for SDS-PAGE and reverse phase HPLC analysis. The final product was >95% homogeneous.

3.7. Mass spectrometry analysis

The masses of non-reduced and reduced-denatured native rCte f 1 are 18 090 and 18 107 amu, respectively (Table 1). The masses of non-reduced and reduced-denatured rCte f 1 expressed in *E. coli*, are 18 220 and 18 238 amu, respectively (Table 1). The masses of non-reduced rCte f 1 expressed in *P. pastoris* and baculovirus infected insect cells are 18 752.5 and 18 089 amu, respectively. The observed masses of reduced and denatured native Cte f 1 correspond to the calculated masses of the predicted amino acid sequences (Table 1).

Table 1
Mass spectrometry analysis of native Cte f 1 and rCte f 1

Protein	Calculated mass (no disulfide bonds)	Observed mass MH ⁺	Δ amu	Number of disulfide bonds
Native Cte f 1 non-reduced	18106	18089	–17	8
Native Cte f 1 reduced/denatured	18106	18105.6	–0.4	–
Fully active <i>E. coli</i> soluble rCte f 1 non-reduced	18238.4	18219.7	–18.7	8
Fully active <i>E. coli</i> soluble rCte f 1 reduced/denatured	18238.4	18236.9	–1.5	–
Baculovirus rCte f 1	18106	18089	–17	8
Pichia rCte f 1	18768.7	18752.5	–16.2	8

These results strongly suggest that native Cte f 1 is not glycosylated, contains eight disulfide bonds and has no free cysteine residues. The mass spectrometry data also show that the rCte f 1 isolated from the soluble fraction of *E. coli*, and the media of *P. pastoris* and baculovirus infected cells each also contains eight disulfide bonds.

3.8. Characterization of Cte f 1 as a major allergen of FAD

3.8.1. Relative affinity of Cte f 1 for IgE in sera from experimentally sensitized dogs

As discussed above, rCte f 1 isolated from the soluble fraction of *E. coli* was eluted as multiple peaks on reverse phase chromatography, and is composed of a mixture of disulfide isomers (Fig. 2A and B, Fig. 3A–D). Mass spectrometry analysis confirmed that the number of disulfide bonds in the isomers varied from two to eight (data not shown). The relative affinities of these disulfide isomers were measured in a competition ELISA using a serum pool from 14 experimental dogs sensitized to flea bite (pool A). This serum pool has high levels of FSA specific IgE as measured by IgE ELISA. The relative amounts of different native Cte f 1 and rCte f 1 isomers required to inhibit the binding of IgE to FSA by 50% (IC₅₀), are shown in Fig. 4B. The IC₅₀ is a measurement of relative affinity of the Cte f 1 isomers for IgE. Approximately, 25 ng/ml of native Cte f 1 is required for 50% inhibition of the binding of IgE to FSA in this ELISA. The rCte f 1 isomers show 100-fold variation in their IC₅₀'s. Fractions 2 and 4 bind IgE as well as the native molecule, while fraction 10 requires over 350 ng/ml of protein to reduce the level of IgE binding by 50%. Reduction and alkylation of the *E. coli*-produced rCte f 1 abolished its IgE binding activity in a standard ELISA (Fig. 5A). The rCte f 1 isolated from baculovirus infected cells and isolated from *P. pastoris* bound IgE as efficiently as native Cte f 1 in both the standard and competition ELISA formats (Fig. 5A and B). However, greater concentrations (20–100-fold) of refolded rCte f 1 isolated from *E. coli*, were needed to inhibit the binding of native Cte f 1 to IgE (Fig. 5B).

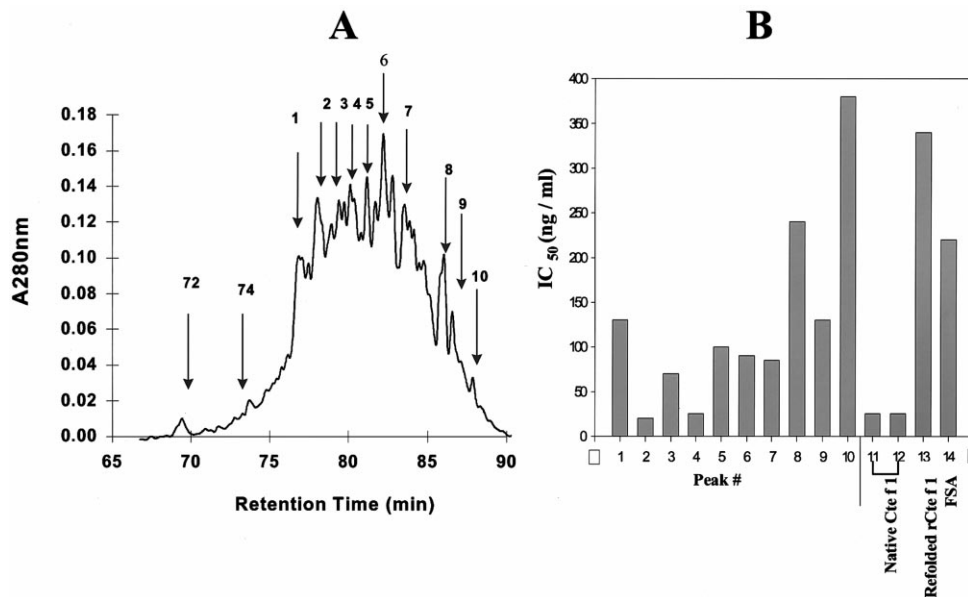


Fig. 4. Relative affinity of FAD dog serum IgE (pool A) for disulfide isomers of *E. coli* produced rCte f 1. Resolution of Cte f 1 disulfide isomers isolated from the soluble fraction of *E. coli* cells produced from a 4.5-l fermentation run (A). Relative amount of Cte f 1 disulfide isomers required to inhibit 50% (IC₅₀) of the binding of serum IgE to antigens present in FSA (B).

3.9. Prevalence of anti-Cte f 1 IgE in flea allergic dogs: experimental FAD dogs

A pilot study challenging a group of eight flea bite sensitized research dogs showed that the animals became symptomatic and developed strong IDST reactions to flea saliva. ELISA showed that the concentration of IgE against FSA and native Cte f 1 in these dogs also greatly increased (data not shown). The IDST and relative amounts of anti-FSA and anti-Cte f 1 IgE that were present in the sera of a larger group of 30 flea allergic research dogs are shown in Table 2. At the initiation of the flea sensitization study, the dogs were both IDST and ELISA negative to FSA (data not shown). All dogs were IDST and ELISA positive, using both FSA and *E. coli* produced soluble rCte f 1, following 2 weeks of exposure to chambered fleas, and 6 weeks exposure to free ranging fleas. During flea infestation, all dogs demonstrated allergic symptoms using the FAD symptom scale. The scale ranges from 1 to 25 with scores of 1–5 indicating mild erythema, papule and crust formation, and 25 indicating serious erythema, alopecia, hyperpigmentation and crusting (Kwochka et al., 1998). The relative importance of Cte f 1 in the IgE response of these experimentally sensitized dogs was determined using the competition ELISA discussed above (Fig. 4B and Fig. 5B). As discussed above, approximately 25 ng/ml of native Cte f 1 is required for 50% inhibition of the binding of IgE to the antigens present in FSA in this ELISA (Fig. 4B). Sera from 14 experimental FAD dogs were pooled (pool A) and preincubated with increasing concentra-

tions of soluble rCte f 1 produced in *E. coli*, *P. pastoris* and baculovirus infected *T. ni* cells (Fig. 5B). The relative amount of serum IgE that was able to bind to native Cte f 1 coated plates, after pre-incubation, was measured using ELISA. Approximately 90% of the specific IgE binding to native Cte f 1 could be blocked by the different recombinant forms of Cte f 1 (Fig. 5B). These data suggest that most of the major IgE epitopes were present on rCte f 1 produced in *E. coli*, *P. pastoris* and baculovirus infected *T. ni* cells.

3.10. Prevalence of anti-Cte f 1 IgE in flea allergic dogs: clinical FAD dogs

The IDST scores and relative amounts of anti-FSA and anti-Cte f 1 IgE for ten client-owned dogs allergic to fleas are shown in Table 2. These data were obtained at the end of the flea season in Ohio. In addition, all dogs had been subjected to a controlled flea challenge of 100 fleas for 48 h. All the animals had a very strong positive IDST (score = 4) to FSA, although two of the ten animals were negative using ELISA (dogs 3 and 4). Eight of the ten allergic dogs were IDST positive to both native and *E. coli* produced rCte f 1 (score = 3–4). Using FSA and fully active soluble rCte f 1 produced in *E. coli*, 70% (seven of ten) and 87.5% (seven of eight), respectively, of the animals that were IDST positive were also positive in the solid phase ELISA (Table 2). The relative importance of Cte f 1 in the IgE response of these clinical dogs was indicated by competition ELISA. Diluted serum from each of five flea allergic dogs was preincubated with saturating amounts (30

$\mu\text{g/ml}$) of *E. coli* produced soluble rCte f 1 or FSA. The remaining amount of unbound serum IgE able to bind to FSA coated plates was measured using ELISA. Eighty to one hundred percent of the FSA-specific IgE could be blocked by *E. coli* produced soluble Cte f 1 (Fig. 6) indicating that most of the IgE produced in response to exposure to flea bites was directed against Cte f 1.

4. Discussion

Canine hypersensitivity to flea bite has been the subject of many clinical and biochemical investigations, but the identity of the relevant allergens has remained largely unknown. Early studies on flea oral secretions, using guinea pigs as test animals, identified low molecu-

lar weight peptides and aromatic compounds in preparations obtained by allowing fleas to bite through a membrane into distilled water. The small size of these putative allergens led the authors to speculate that they functioned as haptens, which bound to host skin proteins to elicit an allergic response (Benjamini and Feingold, 1963). In more recent studies (Trudeau et al., 1993; Greene et al., 1993a,b), allergens in flea body extracts were separated using SDS-PAGE analysis followed by IgE immunoblot analysis with sera from clinically flea allergic dogs. Considerable heterogeneity of response was observed between dogs, and several putative IgE-reactive proteins ranging in molecular mass from 14 to 150 kDa were observed. The same group also carried out in vitro translation of mRNA isolated from *C. felis* and immunoprecipitated the allergens present with IgE in sera from flea allergic dogs.

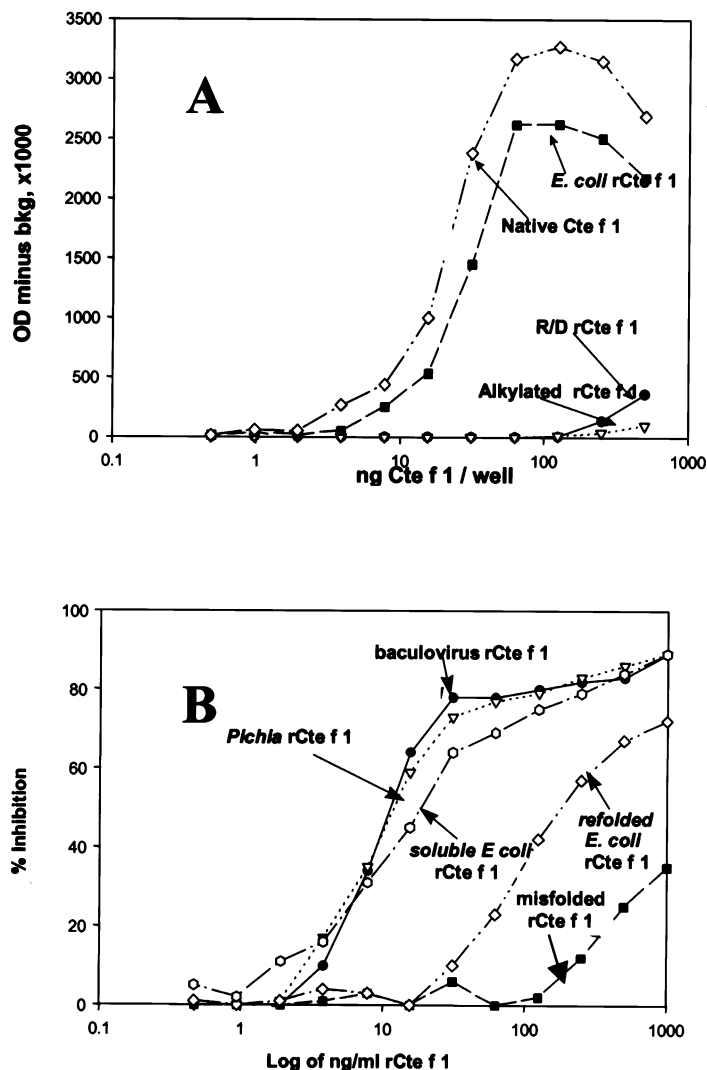


Fig. 5. (A) Relative affinity of FAD dog serum IgE (pool A) for reduced and denatured forms of *E. coli* produced rCte f 1. (A) Solid Phase ELISA — native Cte f 1 ($\diamond$); rCte f 1 ($\blacksquare$); (R/D) rCte f 1 ($\blacklozenge$); alkylated rCte f 1 (∇). (B) Competition ELISA measuring the relative affinity of FAD dog serum IgE (pool A) for native Cte f 1 vs. rCte f 1 expressed in different expression systems — *E. coli* rCte f 1, fully active isolated from soluble fraction ($\circ$); refolded ($\diamond$); misfolded ($\blacksquare$); baculovirus rCte f 1 ($\bullet$); *Pichia* rCte f 1 (∇). Competition ELISA performed as described in the text.

Table 2
Detection of serum IgE by solid phase ELISA and IDST in experimental and clinical FAD dogs using flea saliva antigen (FSA) or fully active rCte f 1 isolated from the soluble fraction of *E. coli*^a

Dog ID #	IDST FSA	ELISA FSA	IDST rCte f 1	ELISA rCte f 1	Symptom score
<i>(A) Experimental FAD dogs</i>					
0030	4–5	1159	3	1262	8
0161	3	1121	4	1250	4
0251	5	1222	4–5	1240	5.5
0820	4–5	1018	5	1088	6.5
1630	5	1331	5	1425	7.5
2563	4	516	4	1232	5
2938	4	851	4	786	8.5
3985	Nd	838	nd	853	9
3993	5	1232	4	1198	14.5
4132	5+	1106	5	1183	14.5
4205	5	1080	4	1238	8.5
4218	4–5	1177	4	1338	4
4957	4	1089	4	1179	8.5
5376	3–4	330	4	356	9.5
5532	5	979	4	1109	12.5
5546	4	834	4	605	5
6020	4	1119	4	1200	4
6041	5	992	5	832	10.5
5064	Nd	1311	nd	1238	10.5
6224	5	1126	3	1286	8
6628	4–5	1146	4	1234	19.5
6887	5+	578	5	396	6
6992	5+	419	4	472	10
7247	5	1116	3	1224	7.5
7492	4	889	3	862	11
7497	4	1179	3	1253	21.5
7969	5	1064	5	1254	2.5
8311	5	228	4	259	6.5
9287	4	1001	4	1117	5
9775	5	267	3–4	413	5.5
<i>(B) Clinical FAD dogs</i>					
1	4	343	4	241	
4	4	117	4	200	
3	4	1	3	27	
4	4	43	4	276	
5	4	1513	4	3188	
6	4	485	0	206	
7	4	794	4	613	
8	4	100	2	0	
9	4	423	4	588	
10	4	1228	4	1680	

^a ELISA readings for antigen specific IgE $< 100A_{450} \times 1000$ nm and IDST scoring < 2 were considered negative. IDST scoring and ELISA were performed as described in the text. For the intradermal skin test, 1000 ng of FSA and 200 ng of rCte f 1 were used.

Eight proteins ranging in molecular weight from 14 to 45 kDa were observed (Greene et al., 1993b). In a study looking at humans with reported hypersensitivity to fleas, Trudeau et al. (1993) identified several IgE reactive proteins in flea extracts by immunoblotting. A 34-kDa protein was particularly IgE reactive. Lee and co-workers have also recently reported the detection of flea salivary antigens ranging from 40 to 60 kDa (Lee et al., 1997). Our recent analysis of collected flea saliva suggests that the major flea allergens for companion animals are proteins in the molecular weight range of 14–40 kDa (Frank et al., 1996).

In this paper, we describe the cloning of the cDNA and characterization of a flea saliva protein, Cte f 1, a major allergen for flea allergic dogs and cats. Native Cte f 1 has a calculated molecular weight of 18 106 Da and *pI* of 9.3. Mass spectrometry analysis indicates that the native molecule has no post-translational modifications and that all of the 16 cysteines are involved in intramolecular disulfide bonds (Table 1). In research dogs experimentally sensitized to flea bite, Cte f 1 is a major allergen. Using a fully active *E. coli* produced rCte f 1 as the antigen in intradermal skin test and in solid phase ELISA, IgE can be detected in 100% of

these experimental FAD dogs (Table 2). In addition, competition ELISA performed using sera from 14 sensitized dogs demonstrated that rCte f 1 produced in three different expression systems (*E. coli*, *P. pastoris* and baculovirus infected insect cells) could inhibit approximately 95% of the binding of antigen specific IgE to native Cte f 1 (Fig. 5B). Despite the observation that rCte f 1 isolated from the *E. coli* soluble subcellular fraction consisted of numerous disulfide isomers with varying affinities for IgE (Fig. 4), sufficient quantities of *E. coli* produced rCte f 1, with IgE activity equal to native Cte f 1, could be isolated from this fraction (Fig. 5). In contrast, any rCte f 1 produced in either the *P. pastoris* or baculovirus infected *T. ni* cell expression systems bound serum IgE with affinity comparable to native Cte f 1 (Fig. 5B). Reduced, denatured and alkylated *E. coli* produced rCte f 1 had greatly reduced affinities for IgE (Fig. 5A). These data strongly suggest that the epitopes of Cte f 1 recognized by IgE from these dogs are conformational and are dependent upon intramolecular disulfide bonds.

In contrast to experimental FAD animals, approximately 80% of naturally flea-bite sensitized dogs had detectable levels of anti-Cte f 1 IgE (Table 2). Clinical animals appear to react to a broader range of allergens in flea saliva (Frank et al., 1996; Lee et al., 1997) possibly due to greater genetic heterogeneity of the pet dog population compared with a single laboratory breed (de Weck et al., 1997). Alternatively, the greater length of flea exposure experienced by the clinical animals may have increased the IgE response to multiple allergens present in flea saliva or in other parts or secretions of the flea.

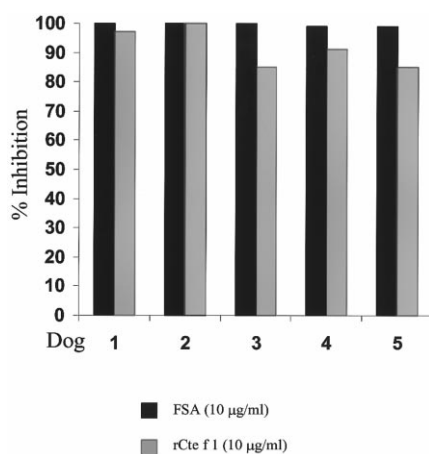


Fig. 6. Competition ELISA measuring the relative affinity of clinical dog sera IgE (dogs A–E) for FSA, and soluble rCte f 1 produced in *E. coli*: competition ELISA was performed by preincubating diluted serum from each of five flea allergic dogs with 10 µg per well of *E. coli* produced soluble rCte f 1, whole flea extract or FSA. The relative amount of unbound serum IgE able to bind to FSA coated plates was measured using ELISA as described.

The function of Cte f 1 is unknown and cannot be inferred from its sequence, since BLAST homology searches failed to reveal significant homology with any protein or DNA sequence in the major databases (Altschul et al., 1997). The presence of Cte f 1 in the saliva of fleas suggests that its function may be related to blood feeding. Recently, Cheeseman (1998) partially purified authentic apyrase activity from the salivary glands of the cat flea, *C. felis*. Two separate peaks of apyrase activity were present in 44 and 57 kDa peaks eluting from a gel filtration column (Cheeseman, 1998). The low sequence homologies observed among molecules performing identical functions required for blood feeding in insects strongly suggest that hematophagy evolved independently in different families of insects (Ribeiro et al., 1990; Valenzuela et al., 1998). This phenomenon, in which molecules with common functions evolve from molecular ancestors with unrelated structures, is termed convergent evolution (Bork et al., 1993; Doolittle, 1994). For example, mosquito *Aedes aegypti* and the bedbug *Cimex lectularis* anti-coagulant apyrases (ATP-diphosphohydrolase) perform essentially identical functions but share little sequence homology, either with each other or with apyrases from mammals, birds or nematodes (Bork et al., 1993; Doolittle, 1994; Smartt et al., 1995). It is, therefore, not unexpected that the function of flea salivary proteins cannot be readily inferred by sequence comparisons.

The lack of potent flea extracts has hampered both the accurate diagnosis and immunotherapy of flea bite allergy. Flea saliva collected as described by Frank et al. (1996) is a sensitive and specific diagnostic reagent for intradermal and in vitro tests, and forms the basis for a commercial in vitro test for anti-flea saliva IgE (McCall et al., 1997). Furthermore, a recent double blind clinical trial of flea saliva in pet dogs with FAD showed clinical benefit (Kwochka et al., 1998). Unfortunately, flea saliva is not readily prepared on a commercial scale. The availability of recombinant flea allergens such as rCte f 1 can be expected to increase the specificity while decreasing the cost of flea-bite allergy diagnosis and treatment.

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