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SHORT COMMUNICATION

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"Venom" of the slow loris: sequence similarity of prosimian skin gland protein and Fel d 1 cat allergen

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Abstract Bites inflicted on humans by the slow loris (*Nycticebus coucang*), a prosimian from Indonesia, are painful and elicit anaphylaxis. Toxins from *N. coucang* are thought to originate in the brachial organ, a naked, gland-laden area of skin situated on the flexor surface of the arm that is licked during grooming. We isolated a major component of the brachial organ secretions from *N. coucang*, an approximately 18 kDa protein composed of two 70–90 amino-acid chains linked by one or more disulfide bonds. The N-termini of these peptide chains exhibit nearly 70% sequence similarity (37% identity, chain 1; 54% identity, chain 2) with the two chains of Fel d 1, the major allergen from the domestic cat (*Felis catus*). The extensive sequence similarity between the brachial organ component of *N. coucang* and the cat allergen suggests that they exhibit immunogenic cross-reactivity. This work clarifies the chemical nature of the brachial organ exudate and suggests a possible mode of action underlying the noxious effects of slow loris bites.

Introduction

The slow loris, *Nycticebus coucang* (Lorisidae), an arboreal denizen of the forests of southeast Asia and western Indonesia, is one of few mammals and the only primate regarded as venomous. The noxiousness of *N. coucang*, which is recounted in the folklore of Thailand (Wilde 1972) and recent zookeepers' manuals (Fitch-Snyder and Schulze 2001), is substantiated by reports of painful and occasionally lethal bites on humans. Wilde (1972) reported that a bite by a pet slow loris on an adult man resulted in anaphylaxis characterized by hypotension, cyanosis of the extremities, and microhematuria. No evidence of a salivary toxin was found, and it was concluded that the victim had been sensitized to allergens in loris saliva by prior minor bites and scratches. Alterman (1990) proposed that noxious bites by *N. coucang* are caused by toxins originating in the brachial organ, a glandular area of naked skin on the flexor surface of the arm that is licked and used in grooming (Montagna et al. 1961; Ehrlich and Musicant 1977). *N. coucang* toxins have not been characterized in detail, but polypeptides generated from brachial organ secretions when mixed with saliva (Alterman and Hale 1991; Alterman 1995), and an unidentified steroid (Alterman 1989), have been suggested as the active components. We report a protein in the brachial organ secretions of *N. coucang* that exhibits extensive amino-acid sequence similarity to Fel d 1, the major allergen from the saliva and integument of the domestic cat, *Felis catus*. Our results suggest that the chief protein in the brachial organ exudate is an allergen.

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Materials and methods

Brachial organ secretions were obtained from an 11-year old male *N. coucang* (663 g) born to captive adults at the Wildlife Conservation Society, Bronx, New York, and euthanized due to health problems. Secretions were collected by first scraping away a tan exudate encrusting the surface of the glandular area. Pressure was then applied to the brachial organ, and a tan fluid expressed from the skin surface was taken up into capillary tubes, which were broken off into glass vials and placed on dry ice. Samples were stored at −80°C until analysis. Approximately 5 μl of secretion were extracted with acetonitrile (2×25 μl). Deionized water was then added to the brachial organ exudate until a cloudy solution resulted (about 60 μl).

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis was performed using the Voyager DE PRO (AB Biosystems, Framingham, Mass.) with an acceleration voltage of 20 kV and an N_2 laser at 337 nm. Sinapinic acid was used as the matrix for protein analysis, while α-cyano-4-hydroxycinnamic acid was used for peptides. Samples were measured both with and without insulin as an internal standard. The electrospray ionization (ESI) instrument was a Q-

TOF mass spectrometer (Micromass, Manchester, UK) fitted with a Z-spray electrospray ion source. The atmospheric pressure chemical ionization (APCI) instrument was a LCmate (JEOL, Tokyo, Japan).

Ten percent acrylamide gels and a tris-tricine buffer system (Schagger and von Jagow 1987) were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis under reducing conditions. Gels were visualized following a standard Coomassie stain–destain protocol. A polyvinylidine difluoride (PVDF) membrane blot was made using a Hoeffer semi-dry electroblotter.

High performance liquid chromatography (HPLC) separation was performed with a Cosmosil C18 (4.6×150 mm) column and using a Waters 600 controller pump with manual injection. A Waters 996 photo diode array detector and Millennium 2010 (version 2.15.01) were used. All injections were monitored at 214 nm and fraction collection was done manually. Solvent system: solvent A = water, 0.1% trifluoroacetic acid (TFA); solvent B = acetonitrile, 0.1% TFA. Gradient: 75% A, ramp to 5% A over 20 min, and continue for 10 min.

The protein was dissolved in 6 M guanidine–Tris (pH 8.5) and then reduced using 0.1 M dithiothreitol for 3 h at 50°C in the dark under argon. Iodoacetamide was added to a final concentration of 0.15 M and the alkylation was allowed to proceed for 3 h at room temperature. The method for HPLC purification of the reduction–alkylation reaction mixture was the same as described above but included 4 min at the initial conditions to remove salts.

Edman sequencing was done at the Columbia University protein core facility on an Applied Biosystems 494 sequencer. The BLAST program was used to search a protein sequence database and in sequence alignment.

Results

ESI and APCI mass spectrometry (MS) revealed no small, organic components in the acetonitrile extract. MALDI-MS of the aqueous extract indicated a peak at 17,656 Da (Fig. 1A). An 80-kDa band (band *a*), a band at 18 kDa (*b*), and a broad band at lower molecular weight (*c*), were visualized by Coomassie staining of an SDS-PAGE gel run under reducing conditions (Fig. 1B). HPLC of the crude aqueous extract showed a single peak; however, when this peak was collected and run on SDS-PAGE, the distribution of bands *b* and *c* on the gel was the same as that prior to HPLC purification, while the 80-kDa band was no longer observed on the gel. Edman sequencing from PVDF blots of both bands *b* and *c* produced indistinguishable, binary mixtures of amino acids. Band *a* yielded no product upon Edman degradation, indicating that it may be N-terminally modified while the other protein chains are not. Further investigation is required to determine whether the 80-kDa component is unrelated to the other bands or is a multimeric form of another protein in the exudate.

The 18-kDa protein was isolated by HPLC (Fig. 1C), reduced, and then alkylated. Reversed phase HPLC of this mixture afforded numerous fractions (Fig. 1C). Edman sequencing of the two major peaks, 14 and 17 (Fig. 1C), showed that they are the deconvolution of the mixture obtained in the previous sequencing experiment. This suggests that slow loris brachial organ protein contains two peptide chains linked by one or more disulfide bonds, and that band *c* on the gel is a mixture of these two components of the intact protein. MALDI-MS of peaks 14 (8 kDa) and 17 (10 kDa) clarified the extent of sequence determination: 50% of chain 1 (35 of ~70 amino acids) and 35% of chain 2 (31 of ~90 amino acids). A protein

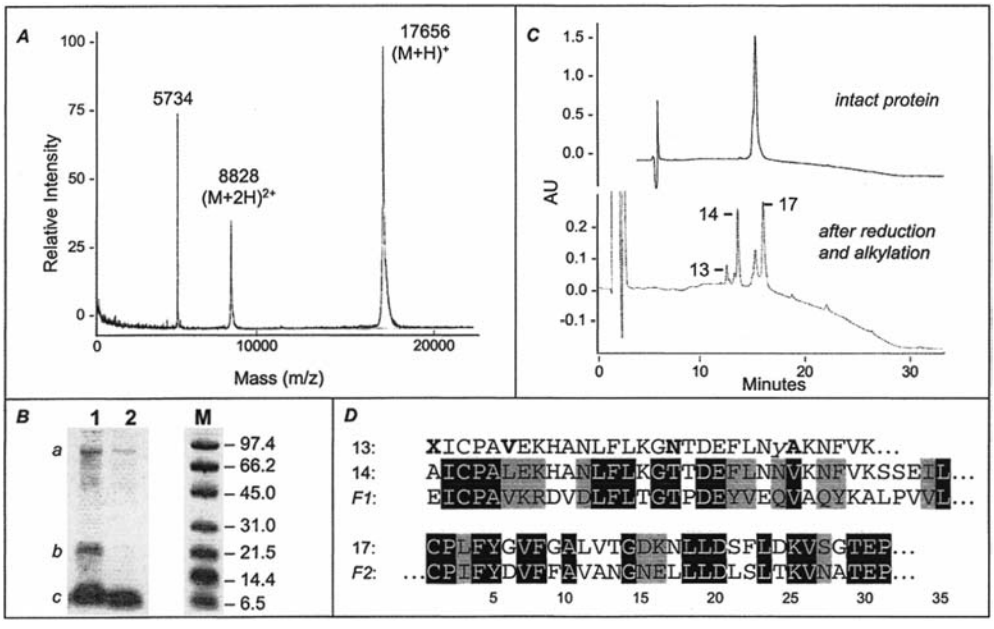

Fig. 1 A–D Analysis of *N. coucang* brachial organ exudate. **A** Matrix-assisted laser desorption/ionization mass spectrometry (*MALDI-MS*) of crude aqueous extract, internal standard = insulin (MW 5,733). **B** SDS-PAGE. *Lane 1*, brachial organ exudate, *Lane 2*, brachial organ exudate, boiled for 5 min under reducing conditions, *Lane M*, MW marker. **C** HPLC chromatograms of intact and reduced and alkylated brachial organ exudate. The numbered fractions were collected. **D** Sequence of N-terminus of the HPLC-purified, reduced–alkylated peptides, and alignment with Fel d 1 chain 1 (*F1*) and chain 2 (*F2*). X=S, G, D, A, or T; *y*=Y or N

database search indicates that the N-termini of the two main chains of the brachial organ exudate exhibit nearly 70% sequence similarity (37% identity, chain 1; 54% identity, chain 2) with the two chains of Fel d 1, the major allergen from the domestic cat, *Felis catus* (Leitermann and Ohman 1984; Morgenstern et al. 1991) (Fig. 1D). Additionally, Fel d 1 chain 2 is expressed in two forms, resulting from alternative splicing, alternative initiation, or the expression of different alleles (Griffith et al. 1992). Our experiments have not identified a second form of loris chain 2; however, an alternative form of chain 1, in which 10% of the N-terminal amino acids vary, has been discovered (Fig. 1D).

Discussion

The brachial organ protein of *N. coucang* shares a high degree of sequence similarity, as well as the unusual disulfide-bridged heterodimeric structure, with Fel d 1. Fel d 1 is the only allergen with this type of structure heretofore elucidated. The nearest match to a known protein, indicated by a BLAST search of chain 1 of both Fel d 1 and the protein from *N. coucang*, is an androgen-binding protein from the mouse, *Mus musculus* (Karn 1994). No matches were indicated for loris chain 2, other than Fel d 1, with E-values less than 79. Recent work indicates that Fel d 1 may possess serine protease activity with gelatin and fibronectin, suggesting a potential endogenous biological activity for this protein (Ring et al. 2000). Additionally, T-cell reactive regions of Fel d 1 have been mapped, with the N-terminal portion of chain 1 containing most of the allergenic epitopes (Counsell et al. 1996). The activity and epitope specificity of the slow loris allergen, and comparison with Fel d 1, remain to be examined pending full characterization of the protein. However, the N-terminal sequence similarity suggests the existence of immunogenic cross-reactivity between the two proteins (Valenta et al. 1996). The exhibition of anaphylaxis following loris bites, and the reported variability in sensitivity to them (Wilde 1972), are consistent with the hypothesis that *N. coucang* brachial organ protein acts as an allergen.

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