# Bat salivary proteins segregate according to diet

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*Summary.* – Whole saliva samples were gathered from four insectivorous (*Myotis tricolor, Pipistrellus africanus, Mops condylurus*, and *Chaerephon pumilla*) and two frugivorous (*Epomophorus labiatus, E. minor*) bat species following a 6-10 hour fasting period and immediately stored in liquid nitrogen. Proteins within the samples were visualized by SDS-PAGE techniques modified for saliva. With the exception of *E. minor*, each species is represented by four to five individuals. Only minor fluctuations in protein banding patterns were encountered among conspecifics. However, frugivores exhibit a major band at 60 Kd that is absent in insectivores, and insectivores exhibit a major 40 Kd band that is lacking in frugivores. Peptides from three sets of bands were extracted directly from the gels after *in-situ* proteolysis and isolation using HPLC. Comparison of amino acid sequences to the PIR, EC, and SwisProt databanks did not yield any compelling similarities, though some interesting potential matches were generated. Based on their molecular weights, potential identities of the proteins are discussed with respect to their roles in digestion and the maintenance of the oral cavity. These data constitute the first comparative analysis of bat salivary proteins and suggest that this novel source of data will prove useful in answering both functional and evolutionary questions.

*Résumé.* – Des échantillons de salive ont été recueillis de quatre espèces de chauves-souris insectivores (*Myotis tricolor, Pipistrellus africanus. Mops condylurus* et *Chaerephon pumilla*) et de deux espèces frugivores (*Epomorphorus labiatus, E. minor*) après une période de jeûne de 6 à 10 heures, et placés immédiatement dans l'azote liquide. Les protéines contenues dans les échantillons ont été rendues visibles par les techniques SDS-PAGE modifiées pour la salive. A l'exception de *E. minor*, chaque espèce est représentée par quatre à cinq individus. Parmi les individus conspécifiques, seules des fluctuations mineures ont été rencontrées dans les « banding patterns » des protéines. Cependant, les frugivores manifestent une bande majeure à 60 Kd, bande absente chez les insectivores. D'autre part, les insectivores montrent une bande majeure à 40 Kd, bande qui n'existe pas chez les frugivores. Des peptides ont été relevés directement des gels, après protéolyse *in situ* et isolation utilisant HPLC. La comparaison de séquences des acides aminés avec les banques d'information (Databanks) du PIR, EC et SuisProt, n'a abouti à

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aucune vraie similarité, quoique certaines similaritées potentielles ont été réalisées. Se basant sur leur poids moléculaire, les identités potentielles des protéines sont discutées, notamment à propos de leur rôle dans la digestion et dans l'entretien de la cavité buccale. Ces résultats constituent la première analyse comparative des protéines salivaires des chauves-souris et suggèrent que cette nouvelle source d'informations pourrait aider à résoudre les questions fonctionnelles aussi bien qu'évolutives.

## INTRODUCTION

Bats exhibit perhaps the broadest range of dietary adaptations of any order of mammals with specializations ranging from insectivory to frugivory, sanguivory, piscivory, and nectivory (Nowak 1994). Gross morphological variation in the cranial and postcranial skeleton associated with variation in dietary habits has been documented extensively (e.g., Dumont 1997a ; Freeman 1988 ; Schlosser-Sturm and Schliemann 1995). At the microscopic level, the dietary adaptations of bats are often reflected in the histochemistry and ultrastructure of the digestive tract and salivary glands (e.g., Forman 1972 ; Makanya *et al.* 1995 ; Phillips *et al.* 1984, 1993 ; Tandler *et al.* 1990).

Much of the variation in the salivary glands of bats is found within secretory granules of acinar cells, suggesting that acinar secretory products (i.e. saliva) vary among bat species (Phillips *et al.* 1987, 1993). Saliva is a complicated fluid that contains digestive enzymes, antibacterial proteins, growth factors, proteins that bind secondary compounds, a unique buffering system, as well as a host of compounds whose functions are not clearly understood (Lamkin and Oppenheim 1993 ; Turner *et al.* 1993 ; Zelles *et al.* 1995). As a whole, saliva plays a role in nutrition, protecting the teeth and oral tissues, lubricating food, and even interspecifc communication (Gray *et al.* 1995). Given the wide range of functions of saliva and the presence of variation in secretory granule morphology among bats, it is likely that salivary chemistry varies among bats in response to the requirements of mechanically processing and deriving nutrition from different types of foods.

Despite strong circumstantial evidence that salivary composition varies among bats, only one study has documented differences among species in the chemical composition of salivary glands (Junquierra *et al.* 1973). No study has produced a comparative survey of salivary proteins in a broad range of bats, although some salivary constituents have been described for vampires (Apitz-Castro *et al.* 1995). The primary goal of this study is to investigate variation in saliva composition between insectivorous and frugivorous bats. We hypothesize that salivary proteins differ between frugivores and insectivores, and that these differences are largely associated with the dissimilar demands of digesting high carbohydrate (fruit) versus high protein (insect) diets.

## MATERIAL AND METHODS

We sampled stimulated, whole saliva from the insectivorous families Molossidae (*Chaerephon pumilla* and *Mops condylurus*) and Vespertilionidae (*Myotis tricolor* and *Pipistrellus africanus*) and two frugivorous species (*Epomophorus labiatus* and *E. minor*) from the family Pteropodidae. All data were gathered from wild-caught bats under field conditions in southern Ethiopia. Animals were captured in mist nets, offered water or sugared-water, then placed in cloth bags and held overnight. Following a

six to ten hour fasting period, animals were anesthetized with Ketamine$_{TM}$ and salivation induced by an intraperitoneal injection of pilocarpine and isoproteranol (10 mg/kg) (Etzel *et al.* 1988). These two drugs together stimulate both adrenergic and cholinergic receptors in the salivary glands and induce protein secretion and saliva flow. Pooled, whole saliva was collected in micropipets from the floor and vestibule of the mouth, placed in cryotubes and immediately frozen in liquid nitrogen. Samples were transferred to a -80° freezer upon return from the field. Voucher specimens were euthanized with an overdose of anesthetic and accessioned into the collections of the Carnegie Museum of Natural History (Pittsburgh, USA) and the University of Addis Ababa (Addis Ababa, Ethiopia).

Saliva from 22 individuals was analyzed using SDS-PAGE (Laemmli and Favre 1973) modified for proteins of saliva (Iversen *et al.* 1982). Prior to analysis, all samples were centrifuged to separate cell fragments and any exogenous materials. Nine $\mu$l of saliva were drawn from the top of each sample and combined with 3 ci loading buffer (625 $\mu$l 0.5 TRIS (pH 6.8), 625 $\mu$l 10 % SDS, 62.5 $\mu$l Betamercaptoethanol, 625 $\mu$l glycerol, 7.5 mg Bromophenol Blue). Total protein content of the samples was not determined. Samples were boiled for five minutes and 10 $\mu$l of sample/buffer was loaded into 4 % - 20 % polyacrylamide gel (Diachi Separation Systems, Tokyo, Japan) and run at 35 $\mu$A (constant current) for 55-80 minutes. A molecular weight standard (Sigma Chemicals, St. Louis, USA) was included on each gel. Gels were fixed and stained overnight in a solution of 0.25 % Coomassie blue R-250 (Sigma Chemicals, St. Louis, MO) in 45 % methanol, 45 % distilled water and 10 % glacial acetic acid. Gels were destained with a solution of 45 % methanol and 7 % glacial acetic acid until bands were clearly visible.

To investigate the functional implications of differences in banding patterns, we derived amino acid sequences from the proteins within three different sets of bands (Fig. 1). The bands were extracted directly from the gels using a modification of the *in-situ* digestion methods described by Hellman *et al.* (1995). Briefly, gel pieces were washed and partialiy dehydrated with 50 % acetonitrile, then completely desiccated and reswollen for digestion using endopeptidase Lys-C (Wako Chemicals, Richmond, VA). Foliowing extraction with 60 % acetonitrile and 0.1 % TFA, and removal of solvent under a gentle stream of nitrogen, the eluate was applied to HPLC. Sequences were determined from seven individual peptides by direct Edman degradation using a Porton/Beckman PI2090 sequencer. PTH derivatives were identified by on-line HPLC. These techniques were successfully applied to a trypsin inhibitor band in the protein standard lane as a test of their effectiveness.

## RESULTS AND DISCUSSION

Figure 1 illustrates gel lanes representing individual saliva sample. Variation in banding patterns between species is greater than variation within individual species. The level of intraspecific variation is similar to that seen in the whole saliva of other mammals (Beeley *et al.* 1991 ; Marshall *et al.* 1993 ; Ekström *et al.* 1994 ; Schwartz *et al.* 1995). Despite evidence that saliva composition can be sexually dimorphic and is known to be a vehicle for intraspecific communication (Gray *et al.* 1984 ; Ikemoto and Matsushima 1984), banding patterns for male and female conspecifics within the present sample did not differ. In combination with the clarity of the banding patterns, the stability of banding patterns within species indicate that the techniques of collecting

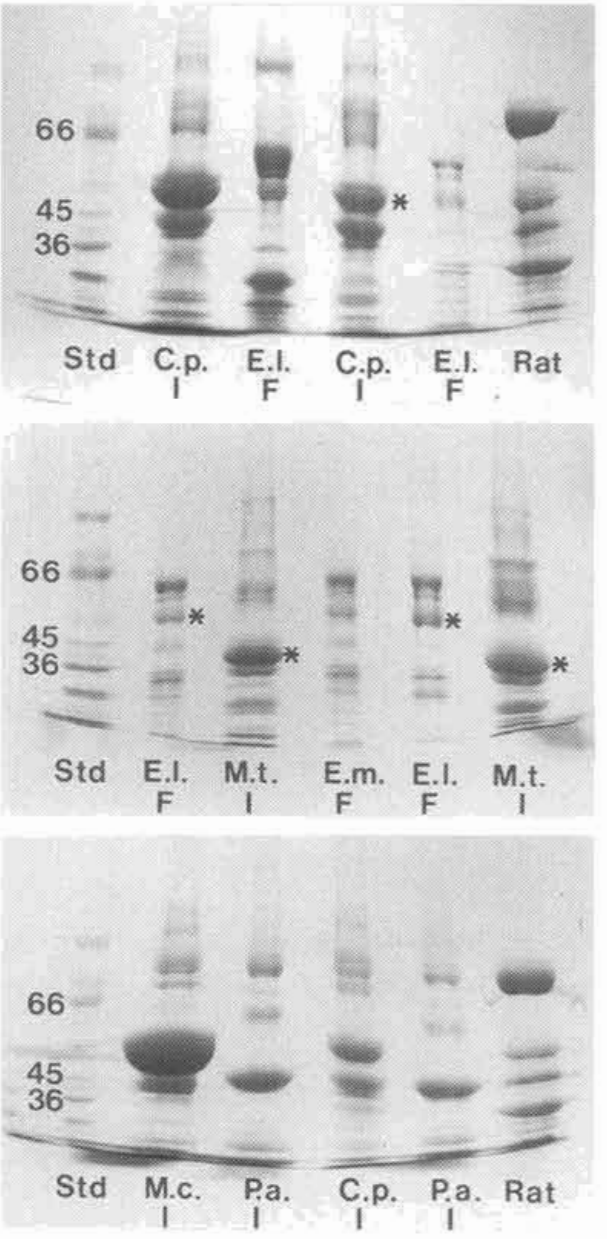


Fig. 1. – 4-20 % SDS-PAGE gels illustrating salivary protein banding patterns. Asterisks indicate bands that were extracted and sequenced. Bands from conspecific individuals were combined to increase sample volume. Abbreviations are : C.p. (*Chaerephon pumilla*), E.I. (*Epomophorus labiatus*), E.m. (*Epomophorus minor*), F (frugivore), I (insectivore), M.c. (*Mops condylurus*), M.t. (*Myotis tricolor*), P.a. (*Pipistrellus africanus*) Rat (whole rat saliva), and Std (Molecular Weight Standard).

saliva have largely circumvented the potential problems of protein degradation and sample contamination.

The data presented in Figure 1 support the the hypothesis that the saliva of frugivorous and insectivorous bats differs in protein composition. The frugivores exhibit a major 60 kD band that is lacking in insectivores, while insectivores exhibit a major band at 30 kD that is lacking in frugivores. An unexpected result of this study is the striking pattern of variation between species from the two insectivorous families. Saliva from the two molossids (*Chaerephon pumilla* and *Mops condylurus*) exhibit a major 50 kD band that is not present in any of the vespertilionid samples (*Myotis tricolor* and *Pipistrellus africanus*).

There are several potential explanations for the differences in banding pattern among the insect feeders in this sample. One alternative is that the insects consumed by vespertilionids and molossids differ enough in their nutritional quality that the two types of bats require different arrays of enzymes to break them down effectively. A second explanation is that molossids may require an additional salivary component to counteract noxious protective compounds found in some insects. In herbivorous mammals, proline-rich proteins in saliva serve a protective function by binding dietary tannins which otherwise inhibit nutrient transport in the gut (McArthur *et al.* 1995). A third explanation for the variation in the electrophoretic banding pattern of saliva among insectivorous bats is that differences in saliva composition reflect phylogenetic rather than functional divergence of vespertilionids and molossids.

Table 1 presents the amino acid sequences identified for peptides generated from the five gel bands identified in Figure 1. These sequences were tested against the PIR and EC protein data bases using the FASTA program (Pearson and Lipman 1988) to identify potential protein matches among the peptides from each species. In addition, the FASTA-SWAP program (Ladunga *et al.* 1996 ; Pearson and Lipman 1988) was used to compare the sequences in the EC database, and BLASTP (Altschul *et al.*, 1990) was used in conjunction with SwissProt and PIR databases.

None of the database searches yielded definitive identifications for any of the peptide sequences. Searches of the individual peptide sequences generated from bands of identical sizes in *Chaerephon pumilla* and *Myotis tricolor* failed to indicate common sequence similarities. However, a comparison of the 60 kD band in *Epomophorus labiatus* saliva to the EC database using FASTA-SWAP identified the salivary enzyme alpha amylase ( 55.6 % identity in 9 amino acid overlap, 22.2 % class identity) and a yeast serine/threonine protein kinase (55.6 % identity in 9 amino acid overlap, 0 % class identity) as possible matches. Of these two proteins, we suggest that the amylase is a more likely match.

TABLE 1. – Peptide sequences from gel bands identified (by asterisks) in Fig. 1.

| *Taxon* | *Mol. wt.* | *Peptide Sequence* |
|---|---|---|
| *Epomophorus labiatus* | 60 kD | (K)LLDDFTTELT |
| *Chaerephon pumilla* | 50 kD | (K)DVVSLTELFXPL<br>(K)LFEDXLEY<br>(K)IMGAWNDFYQQL |
| *Myotis tricolor* | 40 kD | (K)EVVXPLQDTAV<br>(K)LRVXTGADVTL |

Amylase functions in carbohydrate breakdown and is common in saliva of animals with a largely vegetarian diet (Beal 1991). It is dramatically reduced in the saliva of carnivores (Junquierra *et al.* 1973 ; McGeachin and Akin 1979) and marsupials with low-carbohydrate diets (Beal 1990 ; Scott and Beal 1994). Because *Epomophorus* consumes primarily high-carbohydrate, low-protein fruits, we would expect to find alpha-amylase in its saliva. That the 60 kD falls within the range of molecular weights of known mammalian salivary amylases (Beeley *et al.* 1991 ; Sanders and Rutter 1972) further supports the possibility that it is an amylase.

Although we have not identified the proteins in insectivorous bat saliva, we can speculate about their functions by looking to salivary proteins of similar molecular weights in other species. Within rat and human saliva, bands ranging between 30 and 50 kD in molecular weight have been identified as either carbonic anhydrase (Etzel *et al.* 1997 ; Feldstein and Silverman 1984 ; Henderson *et al.* 1976) or one of many different proline-rich proteins (Beeley *et al.* 1991 ; Iversen *et al.* 1982 ; Schwartz *et al.* 1995). In saliva, carbonic anhydrase is secreted to maintain a greater buffering capacity and optimal pH for enzymes present within the oral cavity. Elevated levels of carbonic anhydrase among insectivorous bats would fit well with the recent finding that buffering capacity of insectivorous bat saliva is significantly higher than that known for any other mammals (Dumont 1997b). Proline-rich proteins serve protective functions by keeping saliva supersaturated with calclum phosphate salts (thereby retaining high buffering capacities), contributing to the acquired enamel pellicle (a protective organic film that covers tooth surfaces), and promoting agglutination which inhibits bacterial attachment and hastens their removal from the oral cavity (Bennick *et al.* 1983 ; Douglass 1994 ; Hay *et al.* 1987 ; Gillece-Castro *et al.* 1991).

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