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## PROTEINS OF PAROTOID GLAND SECRETIONS FROM TOADS OF THE GENUS *BUFO*

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*Abstract* - Freeze-dried parotoid gland secretions from toads of the genus *Bufo* contained large proportions of protein (25-35% by weight). SDS-PAGE suggested that secretions from several species of *Bufo* contained mixtures of proteins in the relative molecular mass range of approximately 12 - 200 kDa, which exhibited markedly different banding patterns from species to species. These proteins were presumably not discovered before because the previous extraction procedures used with these secretions were designed to examine low molecular mass compounds and would denature the proteins. SDS-PAGE of secretions from *B. mauritanicus* and *B. calamita* are shown here. The N-terminal amino acid sequence of one of the bands (approx. 58 kDa) of *B. mauritanicus* was found to be LPIPAFPGLDHGF and of a *B. calamita* band (30.5 kDa) was VQVFGLQKEA. No significant similarities to these two sequences and to three separate but partial N-terminal sequences obtained from these species were found in genetic databases.

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The granular ('venom') gland secretions from amphibians are known to be rich in low molecular weight constituents of varied molecular types such as steroids, amines and peptides. Much of the interest in these secretions is a consequence of their bioactivities, which may have partly evolved as one mode of anti-predator defense. However, as pointed out by Barthalmus (1994) in reviewing biological roles of amphibian skin secretions (using the well-studied *Xenopus laevis* as a model), there are few pointers to any role other than defense for this complex biochemical cocktail; more careful study may therefore prove biologically enlightening.

Toads of the genus *Bufo* have compact parotoid glands situated on the neck, sides of the head or shoulder regions which accumulate a milky secretion. A variety of compounds in such secretions from different *Bufo* species have been separated by two-dimensional paper chromatography with multiple staining techniques and the patterns used in evolutionary studies (Low, 1972). No antimicrobial peptides such as discovered in *X. laevis* skin (reviewed by Bevins and Zasloff, 1990; relative molecular mass up to some 3 kDa) have been reported yet in *Bufo*. While examining secretions from two *Bufo* species for the presence of such low relative molecular mass peptides by SDS-PAGE, it was observed in this laboratory that there were a number of proteins present of much higher relative molecular mass than might have been expected (reported by Clarke, 1997). This report reveals that a complex array of proteins is present in such secretions and provides the results of some preliminary N-terminal amino acid analysis of proteins from *B. mauritanicus* and *B. calamita*.

## MATERIALS AND METHODS

Buffer components and SDS were obtained from Sigma (Poole, Dorset, UK). Acrylamide and PVDF membrane were obtained from Bio-Rad (Hemel Hempstead, Herts, UK). *Bufo mauritanicus* (originally from Morocco) and *B. calamita* (originally from the United Kingdom) were kindly loaned from the collection of M. Linley. Using gentle pressure, the parotoid secretions were expressed into ammonium bicarbonate solution (1.0 or 2.0 mL, 0.05 M) and agitated gently to produce a milky solution. This method of obtaining the secretions is like that described by Bolliger and Meyer (1957), except that they expressed the secretions onto a glass plate and allowed them to harden before solvent extraction. Subsequent freeze-drying yielded white-to-cream flocculent solids. The freeze-dried secretions were dissolved in 0.1% NaCl and assayed by the Bradford (1976) or biuret methods using gamma-globulin as standard.

SDS Polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) using mini gels (12% T; 3.3 % C). Sample loadings were typically 5 microL of a 20 mg/mL solution in sample buffer. When required, protein bands were visualized by Coomassie Blue R-250 staining. Molecular weight standards were from Bio-Rad.

Proteins were blotted onto PVDF membrane using a semi-dry method after preparation according to Matsudaira (1987). Gels were soaked in blotting buffer (10 mM CAPS in 10% methanol) for 5 minutes. The membrane was

wetted with methanol and then transferred into blotting buffer. The gel/PVDF sandwich was assembled between filter paper (2 pieces each side; Whatman no. 3) soaked in blotting buffer and transfer carried out for 1.5 h (3 mA/cm<sup>2</sup>). The blot was rinsed with distilled water, followed by methanol, and then stained with Coomassie Blue R-250 (0.1% in 10% acetic acid/50% methanol) until the bands appeared. Destaining was with 10% acetic acid/50% methanol followed by rinsing with distilled water.

N-Terminal sequencing of selected bands was carried out commercially. Database searching for sequence similarities was performed using TBLASTN (Altschul *et al.*, 1990) and FASTA (Pearson and Lipman, 1988).

## RESULTS AND DISCUSSION

The parotoid gland secretions harden if left to stand and in the past have often been used in that state for extraction of low molecular mass components. By expressing the extracts immediately into a volatile buffer as described here in Methods, the proteins are rendered amenable for study. The extraction procedures used in the past were aimed at low molecular mass components and would often have denatured the protein components. However, determination of protein concentration in solutions of freeze-dried secretions by both the Bradford (1976) and biuret methods showed that the gland secretions contain some 25-35% protein by dry weight.

Initial experiments with 6 species of *Bufo* (up to 3 individuals from each species) suggested that the protein patterns obtained after SDS-PAGE and Coomassie Blue staining were markedly different from species to species. [Figure 1](#) shows the results of SDS-PAGE after Coomassie Blue staining of secretions from individual specimens of *B. mauritanicus* and *B. calamita*. Despite the crude nature of these secretions, the gel resolution was satisfactory, although improvements might be expected by prior molecular weight fractionation using membrane filters. The protein patterns from these two species are clearly different and cover a wide range of molecular size. Such gel patterns may prove useful in taxonomic or evolutionary studies. Parotoid secretions could be obtained easily in the field and prove useful in species identification, particularly where anatomical criteria are ambiguous.

Unstained gels similar to those shown were blotted onto PVDF membrane, the protein bands visualized with Coomassie Blue and submitted for N-terminal sequencing. Due to the complexity of the protein patterns the choice of bands was made on their ease of excision as a single band and their

preliminary sequencing behaviour. Of three bands from a *B. mauritanicus* blot (marked as A, B and C in [Figure 1](#)), two gave only partial or ambiguous sequences and one gave a complete run of twelve residues as shown in [Table 1](#). The marked similarities in these 3 sequences suggests post-translational processing or a family of related proteins. Proteolytic processing has been deduced to occur in the production of frog skin peptides (see Kreil, 1996). Two different N-terminal sequences were obtained from the somewhat diffuse major band of the *B. calamita* gel and are also given in [Table 1](#). It is not uncommon for such broad bands to be found to consist of more than one protein. One component gave a complete run of 9 residues from the N-terminus. The other component is probably N-blocked since the first residue could not be identified and there was also ambiguity at the third residue. No significant similarities to known sequences were found for any of these small *Bufo* sequences by searching protein and nucleic acid databases.

The presence of these arrays of proteins in *Bufo* parotoid gland secretions suggests a more complex role for these secretions than simply anti-predator defense. The peptides found in various species of toads and frogs which possess antimicrobial activities are of a much smaller molecular size range than encompassed by SDS-PAGE as used here. For instance, the magainins found in skin secretions of *Xenopus* are typically of 21-26 amino acid residues in length (reviewed by Bevins and Zasloff, 1990), resulting in molecular masses of 2.300-2.8 kDa. A peptide of a somewhat larger size than this (6.7 kDa) found in *Bombina variegata pachypus* was suggested to possess antimicrobial activity (Barberio *et al.*, 1987). Peptides of these sizes are not retained by the sieving effects of polyacrylamide gels as used here and run to the bottom (or run off the bottom) of the gel. The experiments of Barberio *et al.* (1987) with *Bombina* also used lyophilized skin secretions, but obtained from the skin of sacrificed animals. Interestingly, proteins in the larger size range described in this paper for *Bufo* were observed also in SDS-PAGE of the *Bombina* secretions, but they appear to be less abundant than the low molecular weight species - the reverse of what is found here in *Bufo* parotoid gland secretions.

It seems unlikely that so many large proteins as seen here in the *Bufo* would be directly involved in antimicrobial defense (as are the magainins for example). There are possibilities for indirect means of involvement, however, since enzymes have been suggested to be involved in the conversion of bufotoxins to free bufogenins (cardioactive steroids) by ester hydrolysis in *Bufo* parotoid gland secretions (Meyer and Linde, 1971) and have been deduced to be involved in the 'processing' of high molecular mass protein

precursors into the smaller active magainin peptides as mentioned earlier. A peptidase activity was detected in the skin secretion of *Bombina variegata* (Molzer and Michl, 1967).

Further work is in progress to assess the similarities and differences in banding patterns from different individuals of the same species and from a greater range of *Bufo* species. As pointed out by Clarke (Clarke, 1997), the use of *Bufo* for this sort of biochemical investigation is convenient compared with sampling from other types of amphibians. The secretion 'milking' can be repeated frequently without any harm to the animal.

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