

# A cystatin-like cysteine proteinase inhibitor from venom of the African puff adder (*Bitis arietans*)

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Venoms from eight snakes have been screened for inhibitory activity against papain, strong activity being found in that of the African puff adder, *Bitis arietans*. The inhibitor from *B. arietans* venom has been purified by affinity chromatography on carboxymethyl-papain-Sepharose and ion-exchange chromatography. The inhibitor had an apparent  $M_r$  of 13000 in SDS/polyacrylamide gel electrophoresis, and pI value of 6.5 (major component) or 6.3 (minor component). Values of  $K_i$  for the inhibition of papain, cathepsin B and dipeptidyl peptidase I were 0.10, 2.7 and 0.23 nM, respectively; chicken calpain was not inhibited.

## INTRODUCTION

Protein inhibitors of serine proteinases are extremely numerous, and have been studied in great detail, but much less is known about inhibitors of cysteine, aspartic and metalloproteinases. All of the tight-binding protein inhibitors of the papain-like cysteine proteinases that have been examined in detail have been from avian or mammalian species. These have all proved to be members of the cystatin superfamily, and each falls into one of the three distinct but homologous protein types or families (Barrett *et al.*, 1986a,b).

Proteinase inhibitors of reptiles have received far less attention than those of mammals, but are of considerable evolutionary interest. An inhibitor of the cysteine proteinases, papain and cathepsins B and H, was isolated from the venom of *Vipera ammodytes*, but not further characterized (Kregar *et al.*, 1981). We have now examined other snake venoms for cysteine proteinase inhibitors, and, having found one, have isolated and characterized it.

## EXPERIMENTAL

### Materials

The venoms of *Agkistrodon piscivorus piscivorus*, *Bitis arietans*, *Bitis nasicornus*, *Crotalus atrox*, *Naja nivea*, *Naja naja atra*, *Vipera ammodytes* and *Vipera russelli*, as well as papain and bovine dipeptidyl peptidase I, were obtained from Sigma Chemical Co. The substrates Z-Phe-Arg-NHMec, Gly-Phe-NHMec and Suc-Leu-Tyr-NHMec were obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland.

Human cathepsin B (Rich *et al.*, 1986) and chicken gizzard calpain (Parkes *et al.*, 1985) were available in the laboratory. Cm-papain-Sepharose and egg-white cystatin were prepared as described by Anastasi *et al.* (1983).

## Methods

**Purification of inhibitor from venom of puff adder.** *B. arietans* venom (1.0 g) was suspended in 100 ml of 0.05 M-sodium phosphate buffer, pH 6.5, containing 1 mM-EDTA, 1 mM-iodoacetic acid and 1 mM-phenylmethanesulphonyl fluoride, and insoluble material was removed by two passes through a Whatman glass microfibre filter (type GF/D). The resulting solution was applied to a Cm-papain-Sepharose column (14 cm × 5 cm, 275 ml bed volume) that had been equilibrated with 0.05 M-sodium phosphate buffer, pH 6.5. The column was washed with the same buffer until the  $A_{280}$  approached zero, and bound protein was eluted with 0.05 M-potassium phosphate/NaOH (pH 11.5). Fractions (4 ml) were collected into tubes containing 1 ml of 0.25 M- $\text{KH}_2\text{PO}_4$  to bring the pH to 7.0.

Fractions from the Cm-papain-Sepharose column that showed significant inhibitory activity for papain were combined, and the pool was centrifuged at 8000 g for 30 min. The supernatant solution was concentrated by ultrafiltration over an Amicon YM-5 membrane under pressure of  $\text{N}_2$ , dialysed against 20 mM-Tris/HCl (pH 8.0), centrifuged at 8000 g for 30 min, and applied to an f.p.l.c. (Pharmacia system) Mono Q anion exchange column (10 cm × 1 cm) that had been equilibrated with the same buffer. A linear gradient from 20 mM to 220 mM-Tris/HCl (pH 8.0) was applied (2 ml/min) to elute the inhibitor. Again, the most active fractions were combined.

**Electrophoresis and isoelectric focusing.** SDS/polyacrylamide gel electrophoresis was as described (Bury, 1981), with slab gels containing 12.5% total acrylamides. Isoelectric focusing was with ampholines covering the range pH 5.0–8.0 (Barrett, 1973).

**Inhibition studies.** Fluorimetric enzyme assays were as follows: papain and cathepsin B with Z-Phe-Arg-

Abbreviations used: Cm, S-carboxymethyl; NHMec, 7-(4-methyl)coumarylamide; Suc, succinoyl; Z, benzyloxycarbonyl; f.p.l.c., fast protein liquid chromatography.

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**Table 1. Screening of snake venoms for ability to inhibit papain**

Data are values of relative fluorescence in assays with Z-Phe-Arg-NHMec as substrate, as described in the text. The amount of papain used was 5 ng, with 3.6  $\mu\text{g}$  of each venom (or 0.36  $\mu\text{g}$  in one experiment with *B. arietans* venom, as indicated). Assays of venom without added papain showed the presence of an enzyme active against Z-Phe-Arg-NHMec in some venoms (probably a trypsin-like serine proteinase). These control activities were subtracted from those obtained with added papain, and the difference was taken as the true activity of papain in the presence of the venom reflecting the activity of inhibitors.

Venom	Relative fluorescence			Inhibition (%)
	With added papain	Without papain	Difference	
None	0.822	0.014	0.808	(0)
Family Crotalidae				
<i>A. piscivorus piscivorus</i>	1.136	0.431	0.705	13
<i>C. atrox</i>	1.368	0.690	0.678	16
Family Viperidae				
<i>B. arietans</i> (3.6 $\mu\text{g}$ )	0.136	0.135	0.001	100
(0.36 $\mu\text{g}$ )	0.057	0.031	0.026	97
<i>V. russelli</i>	0.840	0.034	0.806	0
Family Elapidae				
<i>N. nivea</i>	0.818	0.025	0.793	2
<i>N. naja atra</i>	0.737	0.020	0.717	11

NHMec as substrate at pH 6.0, dipeptidyl peptidase I with Gly-Phe-NHMec at pH 6.5, and calpain with Suc-Leu-Tyr-NHMec at pH 7.5 (Green *et al.*, 1984; Parkes *et al.*, 1985). In screening assays to detect inhibitory activity for papain in the snake venoms, papain (5 ng) was used at pH 6.0, with Z-Phe-Arg-NHMec as substrate (5  $\mu\text{M}$ ) in stopped assays as described by Barrett (1980). Initially, 3.6  $\mu\text{g}$  of each venom was used, and the assay with *B. arietans* venom was repeated with 0.36  $\mu\text{g}$ .

Kinetic values for the inhibition of papain, cathepsin B and dipeptidyl peptidase I were determined as described by Nicklin & Barrett (1984). The concentrations of solutions of the venom inhibitor were determined by  $A_{280}$  on the assumption that  $A_{280}^{1\%} = 8.5$ , as for chicken cystatin (Anastasi *et al.*, 1983).

## RESULTS AND DISCUSSION

### Screening of venoms for ability to inhibit papain

The initial screening of venoms for inhibition of papain gave the results shown in Table 1. The venoms of eastern cottonmouth moccasin (*A. piscivorus piscivorus*), puff adder (*B. arietans*) and western diamondback rattlesnake (*C. atrox*) contained significant endogenous activity against the papain substrate, Z-Phe-Arg-NHMec, in the absence of papain. When the papain activity in the presence of each venom was corrected for the activity of the venom alone, it was obvious that only puff adder venom showed strong inhibition of papain. A 10-fold dilution of the puff adder venom also inhibited papain completely. In a second experiment (not shown), venoms from two other viper species were examined in exactly the same way. *Bitis nasicornus* showed slightly weaker activity than *B. arietans*, and *Vipera ammodytes* much weaker (although significant) inhibitory activity.

The species tested represent three families of snakes, the Elapidae, Viperidae and Crotalidae, but only three of the four species from the Viperidae showed significant activity. The only previous report of a cysteine proteinase

inhibitor from a snake venom was for *Vipera ammodytes* (Kregar *et al.*, 1981).

### Purification of the cysteine proteinase inhibitor from puff adder venom

The inhibitor was purified by an affinity chromatography procedure used previously for chicken cystatin (Anastasi *et al.*, 1983) and f.p.l.c. anion exchange chromatography. Affinity chromatography of puff adder venom on Cm-papain-Sepharose gave a single protein peak comprising about 4% of the starting material. Much inactive protein was precipitated following the neutralization of the fractions, as was reported previously for the purification of human liver cystatins (Green *et al.*, 1984).

All of the protein recovered in the supernatant solution after concentration, dialysis and centrifugation was loaded on to the f.p.l.c. Mono Q column. One large peak containing most of the inhibitory activity was resolved from several smaller peaks which also inhibited papain. The final column wash with 1.0 M-Tris/HCl, pH 8.0, eluted only inactive protein. Fractions comprising the major peak were combined, and this material (5.7  $A_{280}$  units) was the purified inhibitor used in further work.

The purified inhibitor ran as a single component in SDS/polyacrylamide gel electrophoresis, with mobility similar to those of cytochrome *c* and chicken cystatin, indicating an  $M_r$  of about 13000. Further evidence of homogeneity was obtained by isoelectric focusing, which gave a major band with a pI of 6.5 estimated to comprise about 90% of the preparation; the other 10% migrated with a pI of 6.3.

### Inhibitory activities

The inhibition constants for the interaction of puff adder inhibitor with papain, cathepsin B and dipeptidyl peptidase I are summarized and compared with those of various cystatins in Table 2. Values of  $k_{+1}$  and  $k_{-1}$  were

**Table 2. Comparison of inhibition constants for puff adder inhibitor with other cystatins**

Values of apparent  $K_i$  were obtained as described in the text, but depend on the assumption that  $A_{280}^{1\%} = 8.5$ . Data for other cystatins are from Barrett *et al.* (1986b).

Enzyme	Inhibition constant (apparent $K_i$ , nM)				
	Chicken cystatin	Human cystatin A	Human cystatin B	Human cystatin C	Puff adder cystatin
Papain	< 0.005	0.019	0.12	< 0.005	0.10
Cathepsin B	1.7	8.2	73	0.25	2.7
Dipeptidyl peptidase I	0.35	33	0.23	3.5	0.23

found to be  $8.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$  and  $2.3 \times 10^{-4} \text{ s}^{-1}$  for cathepsin B, and  $5.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$  and  $1.4 \times 10^{-3} \text{ s}^{-1}$  for dipeptidyl peptidase I.

The venom inhibitor (92 nM) showed no inhibition of chicken calpain.

The inhibitory spectrum of the puff adder inhibitor against cysteine proteinases is similar to those of Type 1 and Type 2 cystatins; Type 3 cystatins (kininogens) inhibit calpain with  $K_i$  about 1 nM (Salvesen *et al.*, 1986), which the venom inhibitor did not.

The molecular size and inhibitory properties of the *B. arietans* venom cysteine proteinase inhibitor are consistent with its being a member of the cystatin superfamily (Barrett *et al.*, 1986a,b), and the results of amino acid sequence analysis (Ritonja *et al.*, 1987) confirm this classification.

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