Purification and characterization of a new cystatin inhibitor from Taiwan cobra (*Naja naja atra*) venom

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Cobra cystatin, a new cysteine-proteinase inhibitor of the cystatin superfamily, was isolated from the venom of the Taiwan cobra (*Naja naja atra*) by affinity chromatography on *S*-carboxymethylpapain–Sepharose and reverse-phase chromatography. The venom contained two forms of the inhibitor, one of 11870 Da and the other of 12095 Da, as determined by MS, and pI values of 6.2 and 6.1. Cobra cystatin strongly inhibits cysteine proteinases of the papain family, but not calpain. Papain, cathepsin L, cathepsin B and cathepsin S are inhibited with K_i values of 0.19, 0.1, 2.5 and 1.2 nM respectively. The amino acid sequence of cobra cystatin shows that it is a Type 2 cystatin. The amino acid sequence is 73% identical with that of the cystatin in African-puff-adder (*Bitis arietans*) venom, with which it shares a unique six-residue insertion in a region opposite the reactive inhibitory site. Cobra cystatin is 25–42% identical with other Type 2 cystatins, the most closely related being the recently described human cystatin M, which also has a similar five-residue insertion starting at position 76 (chicken cystatin numbering). A molecular phylogenetic tree of 16 representative members of Family 2 cystatins was constructed by parsimony analysis; it suggests that snake cystatins, together with *Tachypleus tridentatus* (Japanese horseshoe crab) cystatin and human cystatin M, form a new subfamily within cystatin Family 2.

INTRODUCTION

Snake venoms, particularly those of snakes of the Viperidae and Crotalidae families, contain neurotoxins and cytotoxins and a number of proteolytic enzymes that affect coagulation, haemorrhage and kinin release (see [1,2] for reviews). Snake venoms also contain Kunitz-type serine-protease inhibitors. Several such inhibitors, mostly from Elapidae and Viperidae snake venoms, have been isolated and characterized [3–7]. The contributions of these non-toxic basic polypeptides to the toxic properties of venoms is not clear, but they may inhibit the serine proteases of coagulation, fibrinolysis or inflammation [8,9]. The presence of inhibitors of cysteine, aspartic and metallo proteinases in snake venoms is much less well documented. Most cysteine-proteinase inhibitors belong to the cystatin superfamily and are present in a variety of mammalian and non-mammalian tissues and body fluids [10,11]. All cystatins are tight-binding inhibitors of papain-like cysteine proteinases, such as the cathepsins B, H, L and S. The known cystatins have been divided into three protein families [10]. Type 1 cystatins (stefins) have about 100 residues and no disulphide bonds or carbohydrates. Type 2 cystatins, including the well-characterized chicken cystatin, have about 120 residues and two characteristic disulphide loops. Type 3 cystatins, represented by the kininogens, are the most complex cystatins; they have three Type 2 cystatin-like domains, two of which are cysteine-proteinase inhibitors. Only two cysteine-proteinase inhibitors have been isolated from reptiles. One was isolated from African-puff-adder (*Bitis arietans*) venom and shown to be a Type 2 cystatin [12,13]; the other was isolated from the venom of the long-nosed viper (*Vipera ammodytes*) [14], but was not characterized. We have isolated a cystatin-like inhibitor from the venom of the Taiwan cobra (*Naja naja atra*). This inhibitor belongs to cystatin Family 2, based on its inhibition of cysteine proteinases and its primary sequence, which is 73% identical with that of African-puff-adder cystatin. Venom cystatins are also closely related to human cystatin M, a newly described cystatin that is down-regulated in breast cancer [15]. This, and the phylogenetic relationships between the known Type 2 cystatins, suggests the emergence of a new protein subfamily within cystatin Family 2.

EXPERIMENTAL

Materials

Starting material

Freeze-dried Taiwan-cobra venom was obtained from the snake breeding farm at the National Center of Natural Sciences and Technology, Hanoi, Vietnam.

Enzymes

Papain (EC 3.4.22.2) was from Boehringer-Mannheim, human recombinant cathepsin B (EC 3.4.22.1) and human recombinant cathepsin L (EC 3.4.22.15) were gifts from Dr. John S. Mort, (Joint Diseases Laboratory, Shriners Hospital for Children, Montreal, Quebec, Canada). Human cathepsin S was provided by Dr. Gregor Kopitar (Josef Stefan Institute, Ljubljana,

Abbreviations used: Cm, *S*-carboxymethyl; MCA, 7-(4-methyl)coumarylamide; Suc, succinoyl; Z, benzyloxycarbonyl; TFA, trifluoroacetic acid; E64, 1-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane; MALDI-TOF MS, matrix-assisted laser-desorption-ionization time-of-flight MS.

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Slovenia) and bovine micromolar calpain by Dr. Ahmed Ouali (Institut National de la Recherche Agronomique, Theix, France).

Miscellaneous

S-Carboxymethylpapain (Cm-papain)–Sepharose was prepared as described by Anastasi et al. [16]. Z-Phe-Arg-MCA and Suc-Leu-Tyr-MCA were from Bachem [Z is benzyloxycarbonyl, Suc is succinoyl and MCA is 7-(4-methyl)coumarylamide], CNBr-activated Sepharose 4B was from Pharmacia, and Aquapore C4 and C8 cartridges for reverse-phase chromatography were from Brownlee Labs. All other reagents were of analytical grade.

Methods

Purification of cobra cystatin

Freeze-dried Taiwan cobra venom (3.0 g) was dissolved in 10 ml of 0.05 M sodium phosphate buffer, pH 6.5, containing 1 mM EDTA, followed by the addition of water (5 ml). The resulting sample was mixed with 25 ml of Cm-papain–Sepharose gel equilibrated in 50 mM sodium phosphate buffer (pH 6.5)/0.5 M NaCl/1 mM EDTA and incubated for 2 h at room temperature. The gel was then poured into a column (1.6 cm × 15 cm) and washed with the equilibrium buffer until the A_{280} was close to zero. Bound material was eluted with 0.05 M K_2HPO_4/NaOH, pH 11.5, until A_{280} approached zero. Fractions (2 ml each) were collected in plastic tubes containing 1 ml of 0.25 M KH_2PO_4, pH 4.5, to bring the pH to neutral. Fractions containing bound material were pooled and concentrated by pressure ultrafiltration using a Diaflo YM-5 membrane (Amicon Corp.). The concentrated eluate (100 μl each run) was fractionated by reverse-phase chromatography using a C4 cartridge (2.1 mm × 30 mm; Brownlee), a Brownlee HPLC pump and a 1000S diode-array detector (Applied Biosystems) at a flow rate of 0.2 ml/min, with a linear (0–60 %) gradient of acetonitrile in 0.07 % trifluoroacetic acid (TFA) over 30 min.

Amino acid sequence analysis

Automated amino-acid sequence analysis was performed using an Applied Biosystems 477A pulsed liquid sequencer with the chemicals and program recommended by the manufacturer. Phenylthiohydantoin derivatives were identified with an on-line model 120A analyser.

The C-terminal part of the inhibitor was sequenced from a fragment obtained by CNBr cleavage of the native inhibitor. The fragments were reduced and alkylated with 4-vinylpyridine and separated by reverse-phase HPLC on a C4 column. Cysteine residues were identified as pyridylethylcysteinylphenylthiohydantoin [17].

Isoelectric focusing

The pI of cobra cystatin was determined using the Phastsystem® electrophoretic system of Pharmacia with isoelectric-focusing PhastGels 3–9 polyacrylamide gels containing Pharmalyte® carrier ampholytes [18] according to the manufacturer's instructions. The pI values were determined using the Pharmacia broad-pI calibration kit (pI range 3.65–9.30).

Matrix-assisted laser-desorption-ionization time-of-flight (MALDI-TOF) MS

The molecular mass of the inhibitor was determined by MALDI-TOF MS. The purified inhibitor (2 nmol) was mixed with an equal volume of 3,5-methoxy-4-hydroxycinnamic acid (sinapinic acid), deposited on the stainless-steel probe and allowed to evaporate. The system was calibrated with monoprotonated molecular ions from a standard mixture of angiotensin II, adrenocorticotrophic hormone-(18–39)-peptide and bovine insulin. The spectrometer was controlled, and data- processing done, with Bruker software (XMass) running on a Sun Sparc workstation.

Cystatin titration

Cobra cystatin was titrated with papain, previously titrated with the irreversible epoxide inhibitor 1-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E64) as described in [19]. A final papain concentration of 30 nM in 0.1 M phosphate buffer (pH 6.8)/1 mM EDTA/2 mM dithiothreitol/0.1 % Brij35, to which various volumes of cystatin were added, was used. The mixture was incubated for 10 min at 30 °C, and residual papain activity was measured using 5 μl of 0.25 mM Z-Phe-Arg-MCA and a Kontron spectrofluorimeter (excitation 350 nm, emission 460 nm).

Enzyme inhibition assays

Apparent inhibition constants ($K_{i,app}$) were determined as previously described [19,20] under experimental conditions that gave a non-linear dose–response curve. Z-Phe-Arg-MCA (3 μM final concentration) was used as the fluorigenic substrate for all four enzymes and the assay buffer (pH 6.8 for papain and pH 6.0 for cathepsins B, L and S) was 0.1 M phosphate buffer/1 mM EDTA/2 mM dithiothreitol/0.1 % Brij35.

The inhibition data were fitted to eqn. (1) by non-linear regression analysis using the Enzfitter software (1990 version, Biosoft).

$$a = 1 - \frac{[E]_0 + [I]_0 + K_{i,app} - \sqrt{[E]_0 + [I]_0 + K_{i,app}{}^2 - 4[E]_0[I]_0}}{2[E]_0} \quad (1)$$

where a is the residual enzyme activity (rate with inhibitor/rate without it).

True K_i values were calculated from the $K_{i,app}$ values according to eqn. (2), for simple competition [21]:

$$K_i = K_{i,app}/(1 + S_0/K_m) \quad (2)$$

These corrections for substrate competition were made using K_m values of 80 μM for papain [22], 105 μM for cathepsin B [23], 2.4 μM for cathepsin L [24] and 22 μM for cathepsin S [25]. The inhibition of calpain by cobra cystatin was assayed in 50 mM Tris/HCl (pH 7.5)/5 mM β-mercaptoethanol/1 mM $CaCl_2$ using the synthetic fluorogenic substrate Suc-Leu-Tyr-MCA (12 μM).

RESULTS AND DISCUSSION

Detection of cysteine-proteinase inhibitor activity in cobra venom

Several new members of the cystatin superfamily from insects, fish and plants have been identified recently (for a review, see [11]), but little work has been done on reptiles, although such studies could increase our knowledge of the way in which these proteins evolved. Venoms from snakes from the Viperidae, Elapidae and Crotalidae families have been assayed for their ability to inhibit papain [12], but only venoms from snakes of the Viperidae family were found to have significant inhibitory activity. We incubated crude venom of the Taiwan cobra (Elapidae) with papain and found that it contained a cysteine-

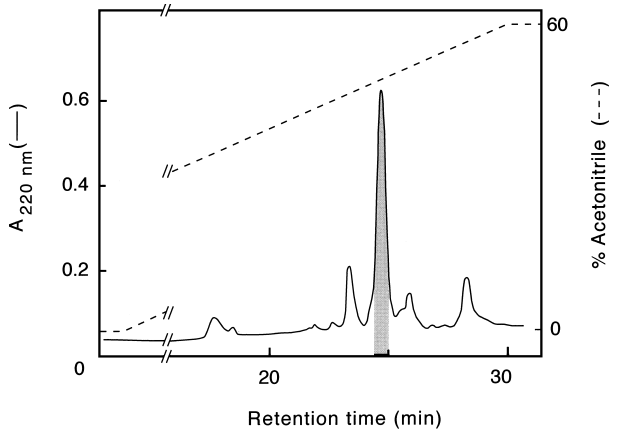

Figure 1 Reverse-phase HPLC of cobra cystatin eluted from Cm-papain–Sepharose

Aliquots (100 μl) of the pooled material eluted from Cm-papain–Sepharose were loaded on to a C4 reverse-phase column and eluted (at 0.2 ml/min) with a linear (0–60%) gradient of acetonitrile in 0.07% TFA developed over 30 min. The broken line shows the acetonitrile concentration. Acetonitrile was removed from eluted fractions by evaporation. Material under the shaded area containing cobra cystatin was pooled, dialysed and concentrated.

proteinase inhibitor. Papain (5 nM final concentration) was incubated with 5 μl of reconstituted venom (1:500 to 1:1 dilutions) in 0.1 M sodium phosphate buffer (pH 6.8)/1 mM EDTA/2 mM dithiothreitol/0.1% Brij35 for 10 min. Residual papain activity was measured using Z-Phe-Arg-MCA (5 μM final concentration). Inhibition was dose-dependent and was total with undiluted venom. Crude venom had no significant endogenous activity on Z-Phe-Arg-MCA in the absence of papain, in agreement with previous data reporting little proteolytic activity in Elapidae venoms [26,27].

Purification and characterization of the cystatin from Taiwan-cobra venom

Crude venom was fractionated by affinity chromatography on Cm-papain–Sepharose, and the fractions containing bound material were further purified by reverse-phase HPLC (Figure 1). Papain inhibition was associated with the major peak, which gave a single band on SDS/PAGE with a mobility similar to that of chicken cystatin (about 12 kDa). Isoelectric focusing of the purified product gave a major band with a pI of 6.2 and a minor band with a pI of 6.1. The minor peaks eluted from the C4 column (Figure 1) had no inhibitory activity. One of them was identified by N-terminal sequencing as cobrotoxin, a well-characterized component of cobra venom (results not shown). The cystatin-like nature of the inhibitor was emphasized by the stability of the molecule [10] under the acidic conditions of HPLC.

MALDI-TOF MS identified two components with slightly different molecular masses (average m/z of 11870 and 12095) (Figure 2). Two components were also identified by N-terminal sequencing of the native inhibitor, the minor one lacking the N-terminal Ile-Pro dipeptide found in the 57-residue sequence of the major component. The molecular mass of this dipeptide is consistent with the difference by MS and with the two isoforms detected by isoelectric focusing. The reason for this proteolytic 'clipping' at the N-terminus is unknown, but N-terminal heterogeneity has already been reported for several cystatins [10].

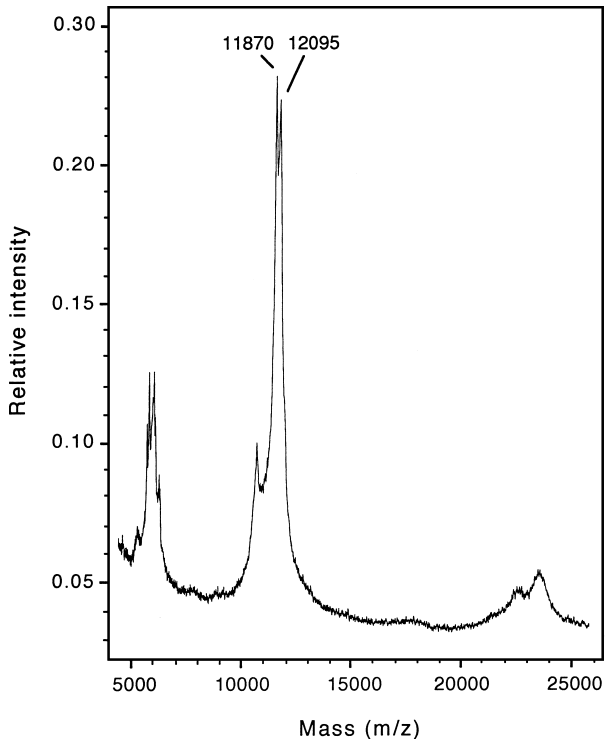

Figure 2 MALDI-TOF mass spectrum of cobra cystatin

The mass spectrum of purified cobra cystatin shows two peaks, one at m/z 11870 and the other at m/z 12095. (m/z is observed mass/charge.)

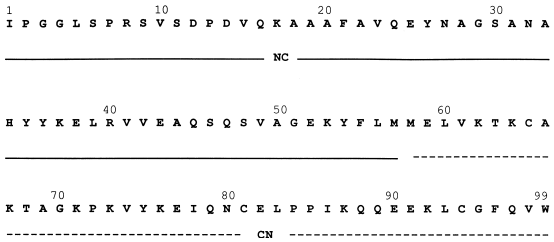

Figure 3 Primary structure of cobra cystatin

The continuous line indicates the sequence of the native cystatin (NC) and the broken line that of a CNBr fragment (CN). Cysteine residues were identified as pyridylethylcysteine after reduction–alkylation by 4-vinylpyridine of the CNBr lysate.

This could be due to the material being a mixture of venoms from several snakes or to the action of a dipeptidyl peptidase, an enzyme that releases N-terminal Xaa-Pro dipeptides [28]. The sequence of the C-terminal part of the cobra cystatin was tentatively determined after CNBr cleavage, since a Met doublet occurs at position 57-58 in the related puff-adder cystatin sequence. Three major fragments were obtained, one of which (fragment CN) contained two overlapping sequences starting at Met^{58} and Glu^{59} (Figure 3). The other two resulted from cleavage at the acid-labile Asp^{12}–Pro^{13} bond by the formic acid used in CNBr cleavage. The first 99 N-terminal residues of cobra cystatin were thus identified (Figure 3). This sequence probably covers

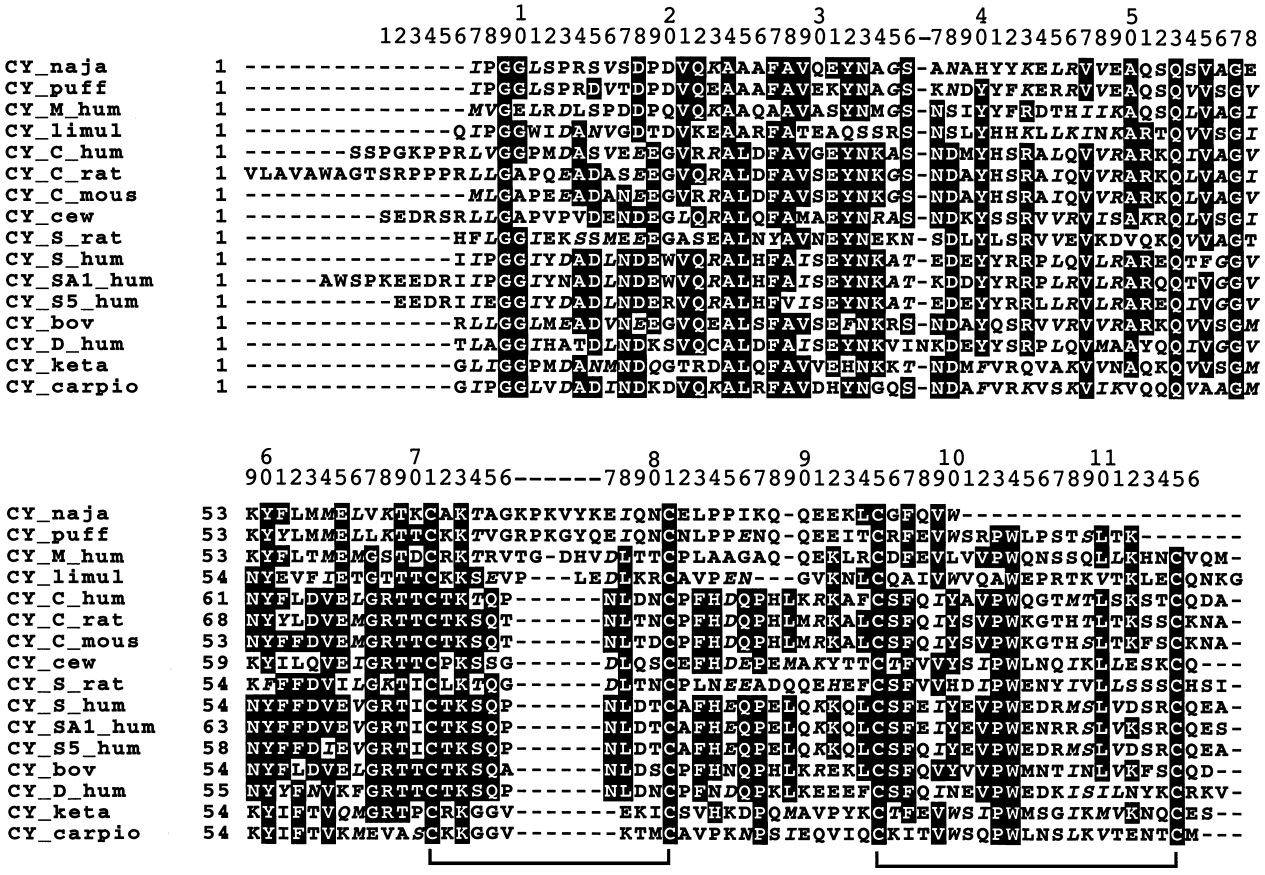

Figure 4 Alignment of the cobra cystatin sequence with those of other Family 2 cystatins

The cobra cystatin sequence was aligned with the most representative members of Family 2 cystatins using CLUSTAL W [41] and BOXSHADE (Version 3.2, University of Geneva) programs. The numbering above the sequences refers to chicken cystatin. Identical or similar residues in at least half the 16 aligned sequences are shown in black boxes or in italics respectively. The four cysteine residues that form two disulphide bridges in several Type 2 cystatins [10] are marked below the sequences. The abbreviations used and sequence databank accession numbers are as follows: cobra cystatin: CY_naja; puff-adder cystatin: CY_puff (PIR A28793); human cystatin M: CY_M_hum (Genbank HSU 62800); Japanese horseshoe-crab cystatin: CY_limul (PIR JC4536); human cystatin C: CY_C_hum (PIR UDHU); rat cystatin C: CY_C_rat (PIR S07085); mouse cystatin C: CY_C_mous (PIR A36163); chicken cystatin: CY_cew (PIR UDCH); rat cystatin S: CY_S_rat (PIR JQ1470); human cystatin S: CY_S_hum (PIR UDHUP1); human cystatin SAI: CY_SA1_hum (PIR A28110); human cystatin S5: CY_S5_hum (PIR B27015); bovine cystatin: CY_bov (PIR UDBO); human cystatin D: CY_D_hum (PIR S18212); chum salmon cystatin: CY_keta (PIR JC2040); carp cystatin: CY_carpio (SWISSPROT CYT_CYPCA)

Table 1 Inhibition constants (K_i) for the inhibition of cysteine proteinases by cobra cystatin and other representative Type 2 cystatins

Inhibition constants for cobra cystatin were determined by non-linear regression analysis using the method of Easson–Steadman with Enzfitter software (1990 version, Biosoft). Results are the means for at least two experiments carried out at 30 °C in 0.1 M phosphate buffer (pH 6.8 for papain and pH 6.0 for cathepsins B, L and S)/1 mM EDTA/2 mM dithiothreitol/0.1% Brij35 with Z-Phe-Arg-MCA (3 μM final concentration) as substrate. Inhibition constants for puff-adder cystatin are from [12], those for chicken cystatin and human cystatin C with papain and cathepsins B, L from [10], and from [40] for cathepsin S–cystatin C interaction. Abbreviation: n.d: not determined.

		K_i (nM)			
Enzyme	Source of cystatin ...	Cobra	Puff adder	Chicken	Human (C)
Papain		0.19	0.1	0.005	0.005
Cathepsin B		2.5	2.7	1.7	0.25
Cathepsin L		0.1	n.d	0.019	0.005
Cathepsin S		1.2	n.d	n.d	< 0.001

about 90% of the total sequence on the basis of a comparison with other cystatins (Figure 4) and the molecular mass of the inhibitor.

Inhibitory activities of cobra cystatin

The inhibition constants for the interaction of cobra cystatin with papain and human cathepsins B, L and S (Table 1) showed that, like other Family 1 and Family 2 cystatins, including puff-adder cystatin [10], cobra cystatin strongly inhibits cysteine proteinases and has a lower affinity for cathepsin B. This is a general feature of all cystatin inhibitors, owing to the occluding loop in the cathepsin B active site that limits the access of both substrates and inhibitors to the active site [29]. Though lacking the N-terminal extension which has been shown to be necessary for the tight binding of cysteine proteinases to chicken cystatin and cystatin C [30–32], cobra cystatin is, like several other cystatins [30], a potent inhibitor of cathepsin B. This could be due to the presence of a proline residue (Pro^8) ahead of the conserved Gly^9 residue, as suggested by Abrahamson et al. [30]. Like other low-molecular-mass cystatins, cobra cystatin (62 nM) does not inhibit bovine calpain.

Structural similarities with other Type 2 cystatins and phylogenetic relationships

The physico-chemical and inhibitory properties of cobra cystatin,

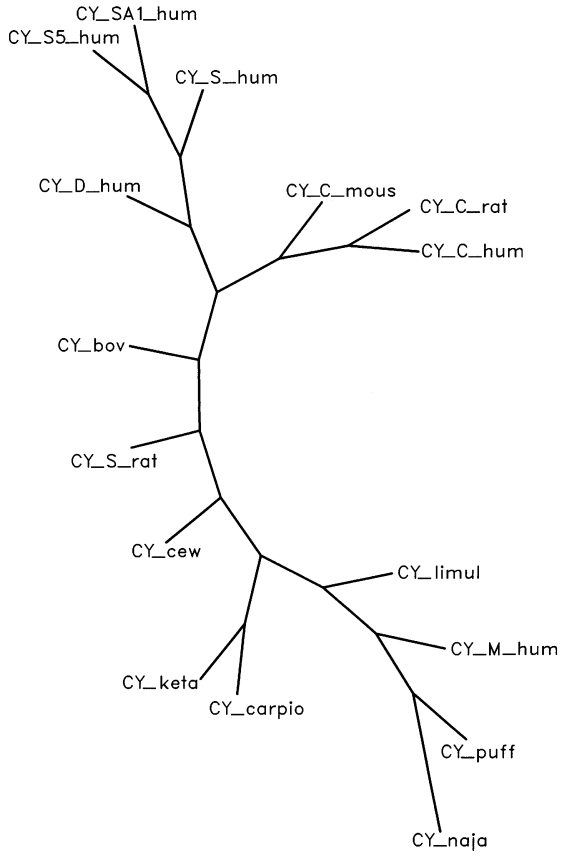

Figure 5 Phylogenetic relationships between the amino acid sequences of Type 2 cystatins of various origins

The 'unrooted' tree was generated using the maximum parsimony algorithm PROTPARS from the 16 aligned sequences and plotted with DRAWTREE from the PHYLIP program (Phylogeny Inference Package, version 3.5c, University of Washington). Abbreviations are the same as those used in Figure 4.

as well as its amino acid sequence, are all consistent with it being a Type 2 cystatin. Cobra cystatin is indeed closely related to Type 2 cystatins (Figure 4) and is most closely related to puff-adder cystatin (73% identity in the 100-residue overlap) and to the recently described human cystatin M (42% identity) [15]. It is less similar (25–35%) to the other Family 2 cystatins (Figure 4) or to Family 1 and Family 3 cystatins (15–20%). Cobra cystatin, like all other cystatins, contains the conserved Gly^{9} residue and the central Gln-Xaa-Val-Xaa-Gly motif, which participate in the cystatin inhibitory site [33,34]. But the third, Pro-Trp, conserved motif, was not identified because it lies in the C-terminal unsequenced part of the molecule. Similarly, Cys^{115}, which forms a disulphide bridge with Cys^{95} in Family 2 cystatins, has not been identified in cobra cystatin or in puff-adder cystatin [13]. But there is little doubt that this residue is present at that position, as cobra cystatin has a molecular mass consistent with that of a full-length Type 2 cystatin [10]. Unlike other Family 2 cystatins, cobra cystatin and puff-adder cystatin have a six-residue insertion between residues 76 and 77 (chicken cystatin numbering) and residue 89 is deleted. Human cystatin M and *Tachypleus* cystatin have similar features (Figure 4). The regions concerned lie within the first disulphide loop on the side opposite the inhibitory reactive site and probably do not affect inhibition. This agrees with reports that a chicken cystatin variant lacking the 71–89 segment is as effective as the wild- type protein [35]. Comparison of the X-ray and NMR structures of chicken cystatin [36] indicate that the conformation of this 18-residue segment changes according to its environment. The major difference is that the α-helix (residues 77–85) is not present in solution, but the integrity of the inhibitory site is maintained. Thus insertions or deletions in the 71–89 region of cystatins, or other post-translational modifications such as phosphorylation of Ser^{80} in chicken [36] or quail [37] cystatin, or glycosylation of Asn^{77} in rat cystatin C [38], may have occurred during evolution without affecting the inhibitory properties of these molecules.

The phylogenetic relationships between cobra cystatins and other Type 2 cystatins were determined by the protein sequence parsimony method [39], using the PROTPARS program (PHYLIP, Phylogeny Inference Package, version 3.5c, University of Washington) and multiple sequence alignment (Figure 4). The resulting 'unrooted' tree, plotted with DRAWTREE (Figure 5), shows that snake cystatins group with human cystatin M and *Tachypleus* cystatin, all of which have the unusual sequence characteristics mentioned above. These four cystatins may be the first members of a new subfamily within the cystatin Family 2. A similar tree was obtained using distance matrix methods, consistent with the proposed relationships between Type 2 cystatins.

Cobra cystatin is the second member of the cystatin superfamily to be identified in snake venoms. There is no evidence that cystatins are involved in venom toxicity, and their biological role in snake venom is therefore unclear. It could be, however, that the rather high concentration of cobra cystatin (about 0.5 μM), protects venom proteins from proteolytic inactivation by proteases of the snakebite victim.

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