Biochimie 93 (2011) 659–668

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Cloning and characterisation of novel cystatins from elapid snake venom glands

Renée Richards [a,b], Liam St Pierre [a], Manuela Trabi [a,c], Lambro A. Johnson [c], John de Jersey [d], Paul P. Masci [c], Martin F. Lavin [a,b,*]

[a] The Queensland Institute of Medical Research, 300 Herston Rd., Herston, Brisbane, QLD 4029, Australia
[b] University of Queensland, Centre for Clinical Research, Brisbane 4029, Australia
[c] University of Queensland, School of Medicine, South Brisbane Region, Centre for Integrative Clinical and Molecular Medicine, Princess Alexandra Hospital, Brisbane 4102, Australia
[d] University of Queensland, School of Chemistry and Molecular Biosciences, Brisbane 4072, Australia

ARTICLE INFO

Article history:
Received 17 August 2010
Accepted 9 December 2010
Available online 21 December 2010

Keywords:
Elapid venom
Cysteine-protease inhibitor
Cystatin

ABSTRACT

Snake venoms contain a complex mixture of polypeptides that modulate prey homeostatic mechanisms through highly specific and targeted interactions. In this study we have identified and characterised cystatin-like cysteine-protease inhibitors from elapid snake venoms for the first time. Novel cystatin sequences were cloned from 12 of 13 elapid snake venom glands and the protein was detected, albeit at very low levels, in a total of 22 venoms. One highly conserved isoform, which displayed close sequence identity with family 2 cystatins, was detected in each elapid snake. Crude *Austrelaps superbus* (Australian lowland copperhead) snake venom inhibited papain, and a recombinant form of *A. superbus* cystatin inhibited cathepsin L ≅ papain > cathepsin B, with no inhibition observed for calpain or legumain. While snake venom cystatins have truncated N-termini, sequence alignment and structural modelling suggested that the evolutionarily conserved Gly-11 of family 2 cystatins, essential for cysteine protease inhibition, is conserved in snake venom cystatins as Gly-3. This was confirmed by mutagenesis at the Gly-3 site, which increased the dissociation constant for papain by 10^4-fold. These data demonstrate that elapid snake venom cystatins are novel members of the type 2 family. The widespread, low level expression of type 2 cystatins in snake venom, as well as the presence of only one highly conserved isoform in each species, imply essential housekeeping or regulatory roles for these proteins.

1. Introduction

Snake venoms contain a myriad of polypeptide components, including protease inhibitors, which are designed to interfere with prey homeostasis. Potent inhibition of papain-like cysteine proteases has previously been observed for several snake venoms and attributed to a cystatin-like protein in *Bitis arietans* (African puff adder) [1] and *Naja naja atra* (Taiwan cobra) [2]. The only Australian elapid snake venom previously examined for cysteine protease inhibition is that of *Notechis scutatus* (tiger snake), which demonstrated potent inhibition of cathepsin L and papain [3], although the specific inhibitor was not identified. More recently, the cDNA for *Bitis gabonica* (Gaboon viper) "bitiscystatin" has been identified (GenBank® accession # AY430403) [4].

The cystatin superfamily of cysteine-protease inhibitors is subdivided into three families: Family 1 stefins are single chain polypeptides of approximately 100 amino acids; family 2 cystatins are 120 amino acid single chain polypeptides with two intra-molecular disulfide bonds; and family 3 kininogens are glycosylated proteins which consist of three cystatin-like subunits. Recent data-mining analyses indicate that family 1 and family 2 cystatins are the intracellular and secreted ancestors, respectively, for a whole range of multi-cystatin domain proteins in addition to the kininogens, which do not necessarily function as cysteine-protease inhibitors [5].

The few snake venom cystatins previously identified show high sequence identity with the family 2 cystatins. The best characterised family 2 cystatins are chicken cystatin/ovocystatin and human cystatin C. These proteins form 1:1 stoichiometric complexes with C1 family cysteine proteases, in competition with the substrate [6]. Crystal structure analyses and docking studies indicate that the N-terminal region, loop 1 (Gln-55-Gly-59) and loop 2 (Pro-105-Trp-106) in cystatin C form a wedge-shaped enzyme binding region essential

Abbreviations: BSL, back-side loop; G3S, glycine-3-serine; ECM, extra-cellular matrix; PLA_2, phospholipase A_2.

* Corresponding author. The Queensland Institute of Medical Research, 300 Herston Rd., Herston, Brisbane, QLD 4029, Australia. Tel.: +617 3362 0341; fax: +617 3362 0106.
E-mail address: martin.lavin@qimr.edu.au (M.F. Lavin).

0300-9084/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2010.12.008

for inhibition of C1 family cysteine proteases [7,8]. The topology of this binding area is shared by cystatins, stefins and also the evolutionarily distinct chagasins [9,10]. The evolutionarily conserved Gly-11 of cystatin C is essential for inhibition as it allows the N-terminal segment of the inhibitor to adopt a conformation appropriate for interaction with the substrate binding pocket of the C1 protease [11]. This part of the molecule constitutes the structurally most highly conserved region across these three inhibitor families. A number of cystatins also exhibit inhibition of legumain, a C13 family cysteine protease, which is mediated by a separate region known as the backside loop (BSL), located between the main α-helix and the second strand of the β-sheet [12].

Family 2 cystatins are conserved throughout evolution, are present in both prokaryotic and eukaryotic organisms and the proteins are expressed in a wide array of tissues and fluids in higher eukaryotic organisms [5]. Tight-binding inhibition of C1 family cysteine proteases led to the early assumption that cystatins primarily function to protect multi-cellular organisms from tissue injury caused by unregulated proteolytic activity. However, it is evident that the specific and elegant regulation of C1 family cysteine protease activity influences many aspects of vertebrate biology. Lysosomal cysteine proteases (cathepsins) effect tissue remodelling and tumour cell invasion through degradation of extra-cellular matrix (ECM) proteins [13]. Site-specific cleavage by various cathepsins leads to activation of immune, inflammatory and apoptotic cascades, which may be relevant to cancer progression and other diseases [13–15]. Cystatin interaction with exogenous proteases and non-protease targets has also been observed. For instance, cystatins and cystatin-derived peptides inhibit microbial growth through both cysteine protease inhibition-dependent and -independent mechanisms [16].

To date, very few snake venom cystatins have been isolated and only one cDNA sequence has been cloned. Here we describe the identification of 12 new cystatin cDNA sequences from elapid snake venom glands. These sequences show high conservation between snake species and demonstrate high identity with family 2 cystatins. Moreover, cDNA and/or protein expression was observed, albeit at very low levels, in all of the 22 snake species examined. We further demonstrate by recombinant expression and mutagenesis that the functional characteristics of *Austrelaps superbus* "AsCystatin" are consistent with family 2 cystatins. This study represents the first description of cystatin cDNA sequences for elapid snakes and demonstrates widespread expression within elapid snake venoms as well as conservation of sequence and functional characteristics of family 2 cystatins. We propose that these proteins are not toxins *per se*, but instead play essential housekeeping or regulatory roles.

2. Materials and methods

2.1. RNA isolation and cDNA synthesis

Venom glands were excised from a number of Australian, Papuan and Asian elapid snakes including *A. superbus* (Australian lowland copperhead snake), *N. scutatus* (tiger snake), *Oxyuranus scutellatus* (coastal taipan), *Oxyuranus microlepidotus* (inland taipan), *Pseudonaja textilis* (common brown snake), *Tropidechis carinatus* (rough-scaled snake), *Hoplocephalus stephensii* (Stephen's banded snake), *Pseudechis porphyriacus* (red-bellied black snake), *Pseudechis australis* (mulga), *Rhinoplocephalus nigrescens* (Australian small-eyed snake) (collected under National Parks and Wildlife's permit number W4\00261\01\SAA), *Micropechis ikaheka* (Papua New Guinean small-eyed snake) (supplied by David Williams and Owen Paiva, The University of Papua New Guinea and the Australian Venom Research Unit, The University of Melbourne) and *Naja kaouthia* (Thai cobra) (Venom Supplies Pty Ltd). Snap frozen glands were homogenised with a polytron and RNA isolated using the Tri Reagent method (Sigma, St Louis, USA). First strand cDNA was synthesised from 1 μg of total RNA with an oligo$(dT)_{12-18}$ primer via reverse transcription with 200 units of Superscript III RNAse H^- Reverse Transcriptase (Invitrogen, Mt Waverly, Australia). The final reaction was ethanol precipitated and cDNA samples resuspended in sterile water and stored at −20 °C.

2.2. Identification of cystatin cDNA sequences

A partial cDNA clone demonstrating similarity to other known venom cystatin sequences via BLAST homology searches was identified from a cDNA library from the venom gland of *A. superbus*. Subsequently, a primer was designed to the 3′UTR of this sequence (5′-GCT GGA CAC ACT CAG GAT CAT TTG CC-3), and 5′RACE performed to identify the full-length sequence from the venom gland of this snake using a SMART RACE cDNA Amplification kit according to the manufacturer's instructions (Clontech, Palo Alto, CA). Briefly, upon amplification of the full-length transcript, a band was excised from a 1% TAE agarose gel containing ethidium bromide and purified using an Eppendorf (North Ryde, Australia) Perfectprep Gel Cleanup Kit. This transcript was subsequently cloned via the pGEM-T vector system (Promega, Madison, WI) and the ligation mix transformed into competent dH5α *Escherichia coli*, with clones selected on LB-Ampicillin (50 μg/mL) plates supplemented with IPTG and X-Gal. Multiple clones were isolated and sequenced with an ABI Big Dye Terminator cycle sequence ready reaction kit (Perkin–Elmer, Norwalk, CT) and alignments performed with BioEdit Software (Isis Pharmaceuticals Inc., Carlsbad, CA).

From the identified 5′RACE *A. superbus* cystatin transcript, a forward (5′-CGC TCT GCC TTG TCT GCC AGC-3′) primer was designed within the 5′UTR and used along with the above reverse primer to amplify full-length homologues from the other elapid species. A 518 bp PCR product was amplified from 12 elapid snake cDNAs with 1 unit of AmpliTaq gold (Applied Biosystems, Foster City, USA) buffered in 1× buffer, 2.25 mM $MgCl_2$ and 200 μM dNTPs with 25 pmol of each of the primers. The reaction was thermocycled at 95 °C for 8 min followed by 30 cycles of 95 °C for 25 s, 52 °C for 25 s and 72 °C for 1 min with a final extension of 72 °C for 3 min. All PCR products were visualised on a 1% TAE agarose gel, purified, cloned, sequenced and aligned as described above.

2.3. Recombinant expression of AsCystatin

Recombinant expression was performed using two different systems. Initially, a clone was generated for mature AsCystatin in *Xho*I and *Xba*I restriction sites of pPICZα for expression as a secreted yeast 6xHis-tag fusion protein (Invitrogen, Mt Waverly, Australia). Constructs were then transformed into KM71H *Pichia pastoris*, positive clones selected and protein expression induced by addition of 0.5% methanol. Recombinant protein (rAsCystatin) was then purified by immobilised metal affinity chromatography with Ni-NTA nickel agarose beads according to manufacturer's instructions (Qiagen, Hilden, Germany).

Subsequently, AsCystatin was cloned and expressed in the pGex6P *E. coli* expression system (GE Healthcare, Rydalmere, Australia), which generates an N-terminal GST-tagged protein with a specific protease cleavage site to enable tag removal. The mature cystatin sequence was cloned into the vector via *BamH*I and *EcoR*I restriction sites. A mutant Gly-3-Ser (G3S) AsCystatin pGex6P1 construct was generated using an alternate 5′ (*BamH*I) cloning primer that incorporated the mutant sequence. Constructs were confirmed by sequencing and transformed into BL21 (DE3) strain *E. coli* (Invitrogen), and GST-fusion proteins were expressed and purified according to the protocols of Frangioni and Neel [17]. Briefly, bacteria

were grown to OD_{600} 1.0 and expression was induced at 16 °C for 16 h with 0.5 mM IPTG. Cells were lysed and the soluble GST-fusion protein was purified by the glutathione sepharose 4B batch method, as per the manufacturer's instructions (GE healthcare, Rydalmere, Australia). The GST tag was removed and the recombinant protein eluted by cleavage of the bead-bound material with PreScission protease (a fusion of GST and human rhinovirus type 14 3C protease, GE Healthcare). Residual GST and GST-fusion proteins were removed from the final recombinant protein preparation by binding to fresh glutathione sepharose 4B beads. Recombinant protein (wild-type and mutant) integrity was confirmed by N-terminal sequencing (Australian Proteome Analysis Facility).

2.4. Antibody production and immunoblot detection of cystatins in venom

Polyclonal antibodies were raised in rabbits injected with rAs-Cystatin expressed in the *P. pastoris* yeast expression system, with the immunisation protocol performed by the Institute of Medical Veterinary Sciences (Adelaide, Australia). Subsequently, these antibodies were used to determine the presence and relative quantity of cystatins within a number Australian and non-Australian venoms via immunoblotting. Briefly, a total of 12.5 μg (immunoblot) or 25 μg (coomassie stain) of lyophilised crude venom resuspended in 50% glycerol and 50% saline was separated under non-reducing conditions on a 12% SDS-polyacrylamide gel. Venom samples were run from a total of 22 snake species including *Austrelaps superbus, Austrelaps ramsayi, N. scutatus, Notechis ater serventi, Notechis ater niger, O. microlepidotus, O. scutellatus, Oxyuranus scutellatus canni, P. porphyriacus, P. australis, Pseudechis gutatus, Pseudechis colletti, P. textilis, Pseudonaja affinis, Pseudonaja nuchalis, H. stephensii, Demansia vestigiata, R. nigrescens, T. carinatus, Acanthophis antarcticus, M. ikaheka* and *Naja naja* along with 8 ng recombinant AsCystatin expressed via the pGex6P system. Gels were either subsequently stained with Coomassie R-250 or venom proteins transferred to PVDF membrane at 100 V for 1 h at 4 °C and probed with the rabbit antibody raised against yeast rAsCystatin. Immunoblots were then probed with anti-rabbit secondary antibody conjugated to either horseradish peroxidase or IRDye 800 CW (Li-Cor) and the signal detected with either enhanced chemiluminescence reagent (Perkin–Elmer) or by infrared detection using a Li-Cor Odyssey scanner, as indicated in figure legends.

2.5. Enzyme inhibition studies

To determine the inhibitory properties of rAsCystatin, spectrofluorometric and colorimetric assays were performed. The concentration of papain was calculated by active site titration with E64 and the concentration of rAsCystatin subsequently calculated by titration with the known concentration of papain, based on the assumption of 1:1 stoichiometry, using benzyloxycarbonyl-Phe-Arg-p-nitroanilide (Z-FR-pNA) substrate (Enzo LifeSciences) [18]. Inhibition of human calpain 1 (44 nM by protein content) (Sigma–Aldrich) was assessed using azocasein substrate as previously described [19]. At least two independent spectrofluorometric assays were performed for each enzyme target. The assays were performed in 96-well format with triplicates of each inhibitor concentration and 2.0 μM Z-FR-7-amino-4-methylcoumarin (Z-FR-AMC) substrate (Enzo LifeSciences) for papain and cathepsins and 100 μM Z-Ala-Ala-Asn-7-amido-4-methylcoumarin (Z-AAN-AMC) substrate (Bachem) for legumain. Enzymes (*Carica papaya* papain 0.086 nM by E64 titration (Sigma–Aldrich); *Homo sapiens* cathepsin B 0.157 nM (Sigma–Aldrich), cathepsin L 0.33 nM (Merck), legumain 10.0 nM (R&D systems), by protein estimation) were incubated with various concentrations of inhibitor or venom (Venom Supplies Pty Ltd, Tanunda, Australia) for 60 min before addition of substrate. The following assay buffers were used for the different enzymes: papain, 100 mM potassium phosphate pH 6.5, 300 mM KCl, 1 mM EDTA, 0.01% Tween 20, 5 mM DTT; cathepsins, 50 mM NaAcetate pH 5.5, 1 mM EDTA, 5 mM DTT, 0.01% Tween 20; legumain, 50 mM NaAcetate pH 4, 100 mM NaCl, 5 mM DTT. Fluorescence was measured using a FLUOstar OPTIMA microplate reader (BMG Labtech) with excitation and emission wavelengths of 380 and 460 nm, respectively.

The apparent dissociation constants were calculated from the equation for simple reversible inhibition, $v_i = v_o/(1 + I_o/K_{iapp})$, or in the case of titrating inhibitors by the method of Morrison & Walsh [20] using the equation for tight-binding inhibition. The experimentally measured reaction rates (v_i and v_o) were fitted to the appropriate equation over a range of inhibitor concentrations (I_o), by non-linear regression analysis performed with SigmaPlot v11 (Systat Software Inc. 225 W Washington St., Suite 425, Chicago, IL 60606, USA).

For time-dependent inhibitors, residual activity (v_i) was measured after pre-incubating enzyme and inhibitor for 60 min. Dissociation constants were generally determined at substrate concentrations below the Michaelis constant (K_m) to minimise substrate effects and any difference between the apparent and the substrate-independent values. These estimates were corrected for substrate concentration assuming competitive inhibition, using the formula $K_i = K_{iapp}/(1 + [S]/K_m)$ and K_m values of 80 μM for papain [21], 6 μM for cathepsin L [21] and 118 μM for cathepsin B [22]. Standard deviations were determined from independent replicate estimates of K_i.

2.6. Molecular modelling of the interaction of AsCystatin variants with cysteine proteases

Homology models of AsCystatin and the Gly-3-Ser (G3S) mutant were created with SwissModel [23] using the structure of ovocystatin (PDB ID 1yvb:I; in complex with falcipain 2) as template. Models of the papain/inhibitor complexes were assembled based on PDB ID 3ima (papain in complex with tarocystatin), while the model of the cathepsin B/AsCystatin complex was based on PDB ID 3k9m (cathepsin B/stefin A). Finally, we used the structure of apo-cathepsin L C25A (PDB ID 3k24) to create the model of the cathepsin L/AsCystatin complex. All models were subjected to energy minimisation in Swiss Pdb Viewer [24] (3 cycles of 100 steps of steepest descent in the GROMOS force field) and Insight II (Accelrys Inc.; 1000 steps of steepest descent *in vacuo* in the Consistent Valence Force Field CVFF). Protein–protein interactions were analysed with ProtorP [25] and hydrogen bonds using the optimal hydrogen bonding network feature on the WhatIf web server (http://swift.cmbi.ru.nl/servers/html/hnet.html) [26].

2.7. In vitro toxicity assay

The toxicity of rAsCystatin to the PC3 human prostate cancer cell line was assessed using Cell Proliferation Reagent Wst-1 (Roche), according to the manufacturer's instructions. Briefly, cells were grown to 50% confluency on a 96-well plate then treated with rAs-Cystatin (final concentration 6.0 μM) or control buffer, each diluted in phenol-red free RPMI (Invitrogen) containing 10% foetal calf serum. Following a continuous 24 h treatment, Wst-1 reagent was added to wells and the formation of Wst-1 cleavage products produced by metabolically active cell enzymes was monitored at A_{440}.

3. Results

3.1. Identification of cystatin-like cDNA sequences in elapid snakes

As part of a comprehensive screen of Australian elapid snake venom gland cDNA libraries [27], a partial cDNA transcript

demonstrating similarity to known venom cystatin sequences via BLAST homology searches was identified from the Australian lowland copperhead snake, *A. superbus*. Subsequently, 5′RACE was used to obtain the full-length sequence and primers based on this sequence were designed to clone the cystatin-like sequences from related elapid snake species.

The full-length cDNA and deduced protein sequence obtained from the venom gland of *A. superbus* is depicted in Fig. 1. A 26 amino acid propeptide is predicted based on N-terminal sequence data previously obtained for cystatins from the venom of *B. arietans* (African puff adder) [1] and *N. naja atra* (Taiwan cobra) [2]. This propeptide designation also conforms to bioinformatic signal peptide predictions (www.cbs.dut.dk/services/SignalP), with the predicted M_r of the mature protein, "AsCystatin" being 13,087 Da.

An alignment of the deduced protein sequences of cystatin-like transcripts from all 12 elapid snake species identified in this study, compared to the five other known venom cystatins, is displayed in Fig. 2. These 12 cystatins are all 141 amino acids in length and display 75% identity across the deduced pre-protein sequences. The 26 amino acid propeptide is highly conserved across all elapid snake species, a feature common to all elapid venom-derived proteins [28], although it varies considerably from the one known viper sequence (*B. gabonica*) and from tarantula (*Chilobrachys jingzhao*) and eastern bearded dragon (*Pogona barbata*) sequences.

It is notable that attempts to amplify a cystatin-like transcript from the related Australian elapid *D. vestigiata* (black whip snake) were unsuccessful, although the protein was detected by immunoblot (see Section 3.3). This suggests sequence differences may exist in the cystatin transcript from *D. vestigiata* as compared with other elapid species, which would be consistent with the evolutionarily distinct nature of this particular elapid snake [29].

3.2. Conservation of family 2 cystatin structural elements in snake venom cystatin

The sequence alignment in Fig. 3A illustrates the high identity between mature AsCystatin and prototypical family 2 cystatin proteins. The highest identity is observed for cystatin M (approximately 40%) which, like AsCystatin, contains a multiple amino acid insertion between the two cysteine residues that form the first

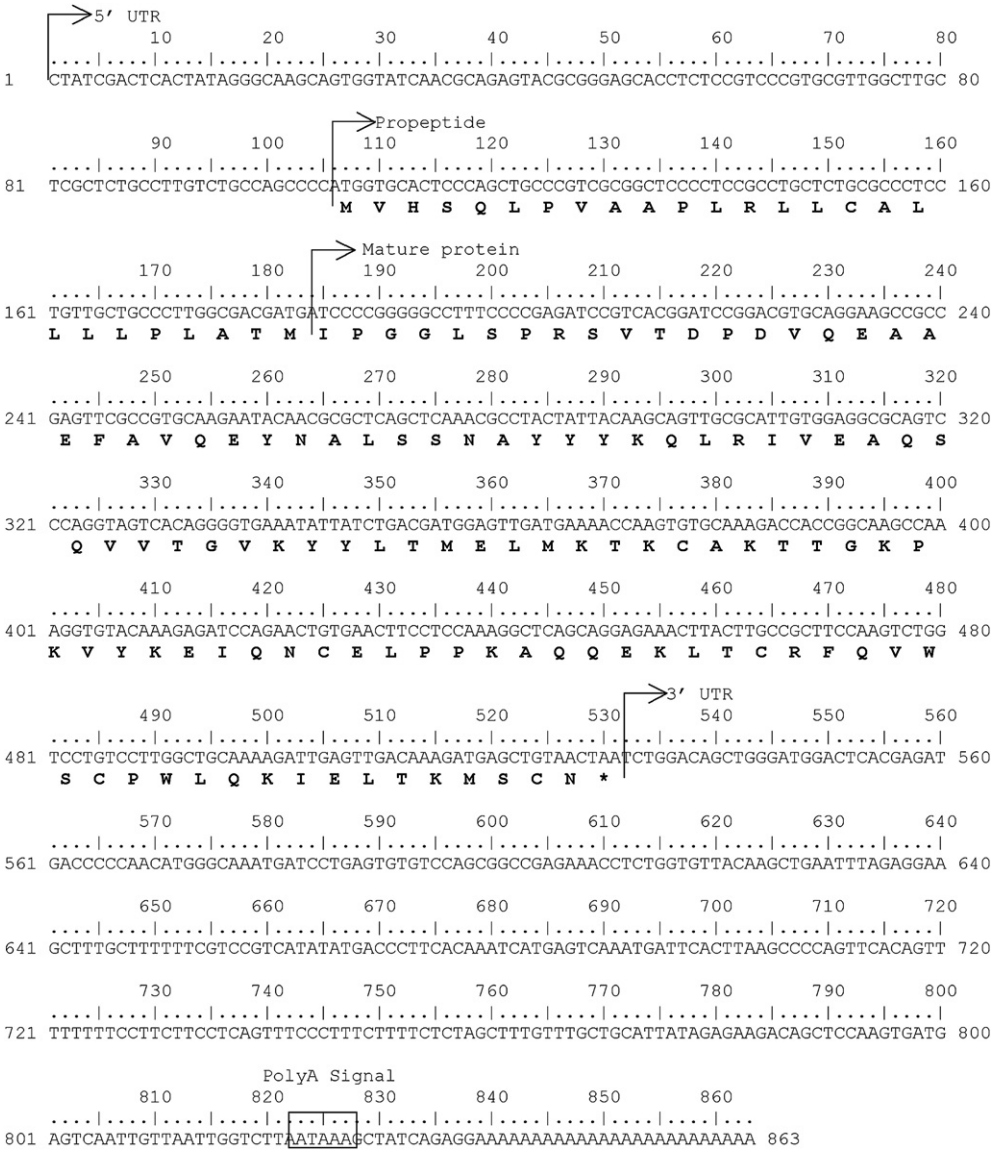

Fig. 1. Full-length cDNA sequence and corresponding translated protein sequence of AsCystatin, a cystatin-like molecule identified from the venom gland of the Australian copperhead snake, *A. superbus* (GenBank accession number FJ411278). mRNA features are indicated where appropriate.

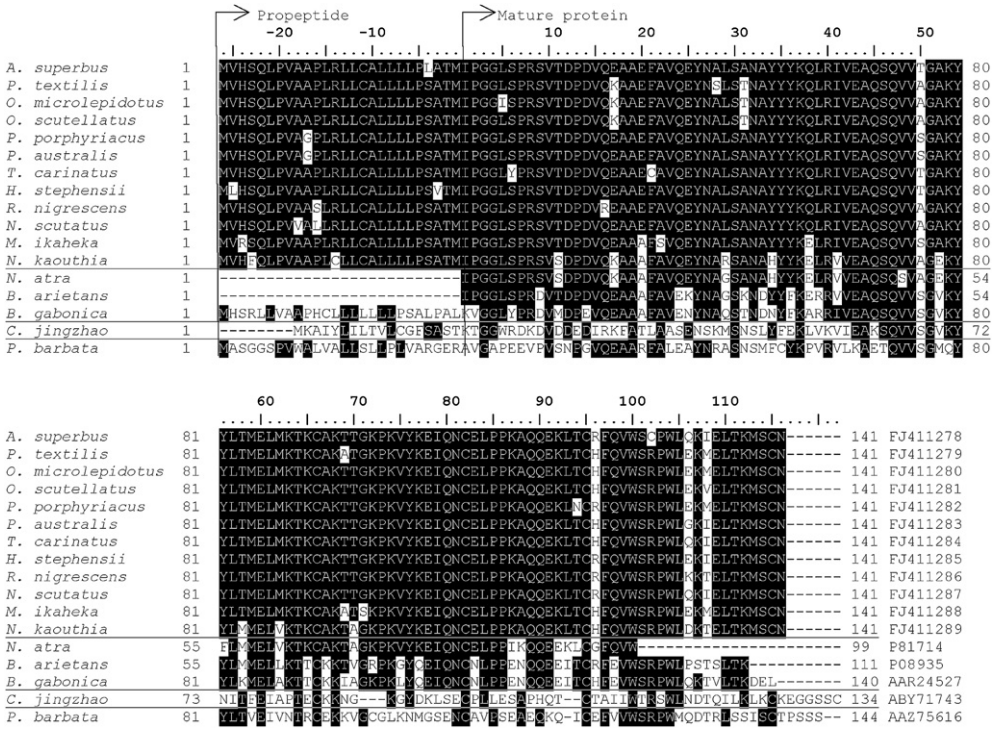

Fig. 2. Alignment of the deduced protein sequences of cystatin-like transcripts from a number of Australian, Papuan and Asian elapids determined in this study compared to all other known venom cystatin sequences from the snakes *Naja atra* (Chinese cobra), *Bitis arietans* (African puff adder), *Bitis gabonica* (Gaboon viper), the spider *Chilobrachys jingzhao* (Chinese tarantula) and the lizard *Pogona barbata* (Eastern bearded dragon). GenBank or UniProtKB accession numbers are provided at the end of each sequence. Cleavage sites between the propeptide and mature protein are indicated by arrows.

disulfide bridge. The disulfide bridge cysteine residues, as well as loop 1 and loop 2 required for C1 cysteine protease inhibition [7], are highly conserved across all family 2 members. Interestingly, Asn-39, an essential element of the BSL required for legumain inhibition [12], is absent in AsCystatin.

The area of highest divergence between AsCystatin and the other type 2 cystatins is the N-terminus, which is truncated in the snake venom cystatins. As previously stated, N-terminal residues are essential for inhibitor activity. The N-terminus is structurally highly conserved in cysteine-protease/cystatin inhibitor complexes, while inhibitor loops 1 and 2, the other two contact areas between enzyme and inhibitor, demonstrate considerable structural variation. Despite the variation from prototypical family 2 cystatins seen in this area of snake venom cystatins, the sequence alignment (Fig. 3A) indicates conservation of the essential Gly-11 (cystatin C numbering) in AsCystatin (as Gly-3).

Structural modelling was used to verify the conservation of cystatin family 2 structural elements in AsCystatin and to assess its potential interactions with selected cysteine proteases. We modelled the three dimensional structure of AsCystatin and its G3S mutant based on the structure of ovocystatin (PDB ID 1yvb; in complex with falcipain 2), which showed the highest sequence similarity in the PDB to AsCystatin (approximately 25% identity). The papain/AsCystatin complex was assembled based on PDB ID 3ima, the crystal structure of papain in complex with the N-terminal domain of tarocystatin (Fig. 3B–D). The backbone atoms of the enzyme contact regions (N-terminal segment, L1, L2) of the AsCystatin model and tarocystatin superimpose with an RMSD of 0.52 Å, indicating a high structural conservation in these regions. The models for cathepsin L and cathepsin B complexes were based on PDB ID 1yvb (ovocystatin/falcipain 2), as outlined in Section 2.6, since no complexes between these two enzymes and a family 2 cystatin are currently available in the PDB. Analysis of protein–protein interactions in the three modelled AsCystatin complexes revealed interface areas between 770 and 1120 Å^2, comparable in size to those of known cysteine protease/cystatin complexes. It should be noted that the size of the interface area as calculated with ProtorP [25], the method used here, was generally smaller than published values, *e.g.* 763 Å^2 *vs.* 922 Å^2 for the papain/chagasin complex (PDB ID 3eiz).

It has been noted repeatedly that the conserved Gly residue near the N-terminus is crucial for the ability of this segment to form a tight turn. Energy minimisation of the G3S AsCystatin mutant resulted in a local change in conformation in this N-terminal segment, rotating the three terminal residues approximately 55° towards the body of the inhibitor (Fig. 3E). This altered conformation is stabilised by a backbone hydrogen bond between HN of Ser-3 and O of Val-48, a contact seen in neither the model of AsCystatin nor the crystal structure of ovocystatin (PDB ID 1yvb). It would be impossible for the G3S mutant to enter the substrate binding cleft of most cysteine proteases without conformational rearrangement, which would also involve breaking this hydrogen bond that ties the N-terminus to loop 1, since the N-terminal region is now too wide for the catalytic groove of the enzyme. It is conceivable that the structural changes seen in the N-terminus of our G3S mutant model are an artefact of the energy minimisation *in vacuo*. However, preserving the backbone angles for residue 3 in the G3S mutant would result in strained conformation of the peptide backbone, while the rotation mentioned above puts Ser-3 into the favourable regions of the Ramachandran plot.

3.3. Detection of cystatin-like cysteine-protease inhibitors in venom

To biochemically analyse the structural and functional characteristics of snake venom cystatins, recombinant proteins were

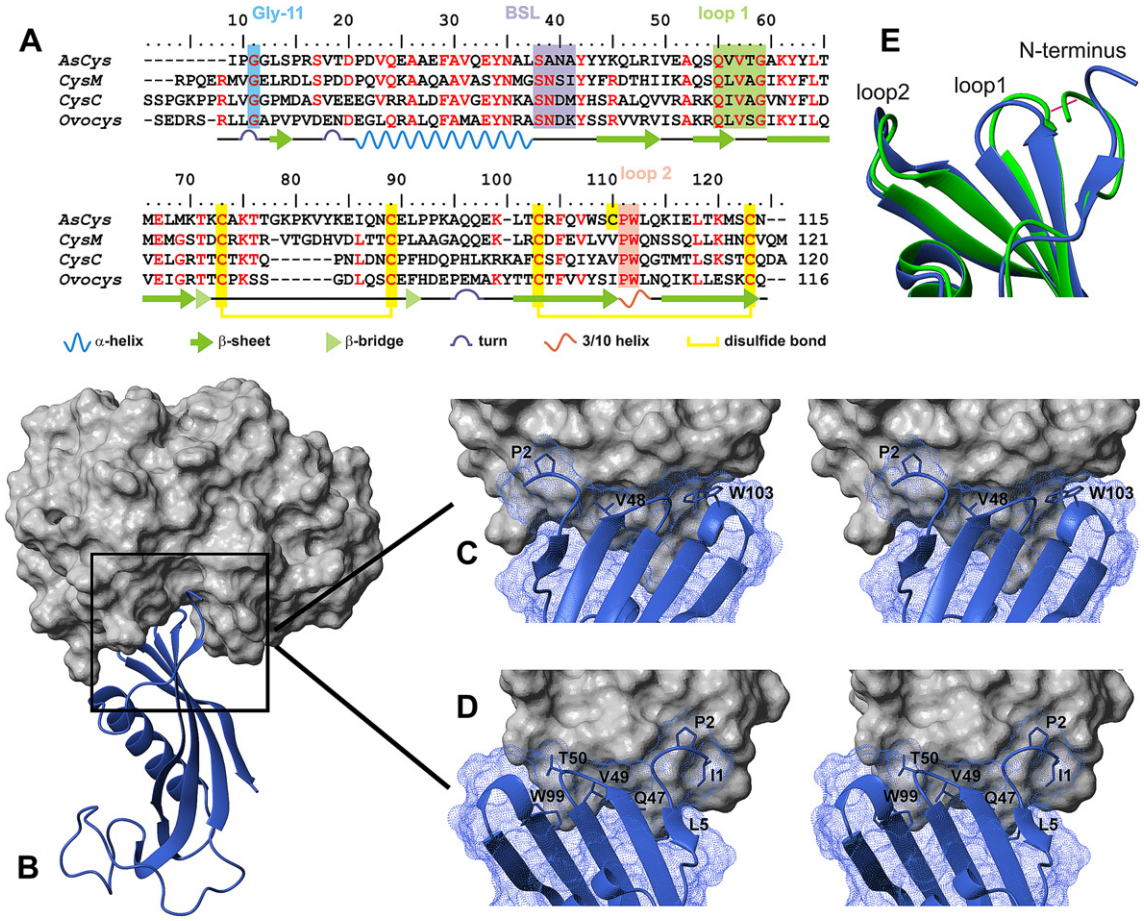

Fig. 3. Conservation of cystatin family 2 structural elements in snake venom cystatin. (A) Alignment of mature protein sequences from *Austrelaps superbus* cystatin (AsCys), *Homo sapiens* cystatin M (CysM, NP_001314), *H. sapiens* cystatin C (CysC, CAA36497) and *Gallus gallus* ovocystatin (Ovocys, NP_990831). Conserved functionally significant regions (Gly-11, back-side loop (BSL), loop 1, loop 2) are highlighted by coloured blocks and disulfide bridges are indicated. DSSP assigned secondary structure elements for PDB ID 1yvb (ovocystatin) are shown below the sequences. (B) Molecular model of the AsCystatin/papain complex. The N-terminal region, loop 1 and loop 2 of the inhibitor (shown as blue ribbon) are inserted into the substrate binding cleft of papain (shown as grey surface). (C) and (D): Stereo close-up views of each face of the substrate binding cleft, highlighting the inhibitor residues in contact with papain. The inhibitor is again depicted as blue ribbon, with the side chains of the contacting residues given as sticks and labelled (AsCystatin numbering). Views C and D are rotated 90 and −90° about the y-axis with respect to A. (E) The three enzyme contact areas of the G3S mutant (in green) in comparison with those of AsCystatin (in light blue). Note that the N-terminal region of the mutant is twisted towards loop 1 to allow for more favourable backbone dihedral angles around the mutated residue Ser-3. This conformation is stabilised by a hydrogen bond between Ser-3 and Val-48 (AsCystatin numbering), shown in magenta.

produced. AsCystatin was initially produced as a C-terminal His-tagged recombinant protein using the pPICZα system (Invitrogen), which utilises a *P. pastoris* signal sequence to mediate secretion. The signal sequence is cleaved upon secretion to produce a recombinant protein with a native N-terminus. Unfortunately, expression trials indicated that recombinant *P. pastoris* AsCystatin consisted of several slightly modified forms, as indicated by the presence of more than one band by SDS-PAGE (Supp. Fig. 1A). However, this recombinant protein was successfully used to induce production of a polyclonal anti-cystatin antibody, which was shown by dot blot to be sensitive and specific for the bacterially expressed AsCystatin (Supp. Fig. 1B).

The antibody was subsequently used to probe an array of snake venom samples by immunoblot analysis. The majority of elapid snake venom proteins are low molecular weight, resolving below 17 kDa by reducing SDS-PAGE. A very strong Coomassie-stained band was present at approximately 13 kDa for most snakes, and we have previously demonstrated that this is composed primarily of phospholipase A_2 (PLA_2) isozymes [30]. In initial immunoblot work, cystatin-like proteins appeared to be masked by the intense 13 kDa phospholipase A_2 band present on reducing gels (data not shown). However, in native venom, some PLA_2s form disulfide linked complexes [31,32] which exhibit reduced electrophoretic mobility. Concordantly, using non-reducing SDS-PAGE, a broad M_r range of Coomassie-stained bands was apparent (Fig. 4A), which enabled the specific detection of cystatin proteins in a panel of venoms. By immunoblot analysis it was shown that cystatin is expressed in 22 of 22 snake venoms, as indicated by the presence of immunoreactive bands at approximately 13 kDa (Fig. 4B), consistent with the predicted size of snake venom cystatins. Slight differences in electrophoretic mobility were observed for cystatin protein across genera, despite high sequence identity, and this is possibly a consequence of the non-reducing environment and interactions with other snake venom components. Certainly, the similarly shifted electrophoretic mobility of *T. carinatus* (rough-scaled snake) and the two copperhead snake venom cystatins compared with rAsCystatin is intriguing because the predicted protein sequences of *A. superbus* cystatin and *T. carinatus* cystatin each contain one unique unpaired cysteine residue (at position 102 and position 21, respectively, see Fig. 2), which may mediate formation of disulfide linked complexes with other venom components.

It would appear that cystatin expression is subject to gene regulation. Variation in expression levels within individual genera

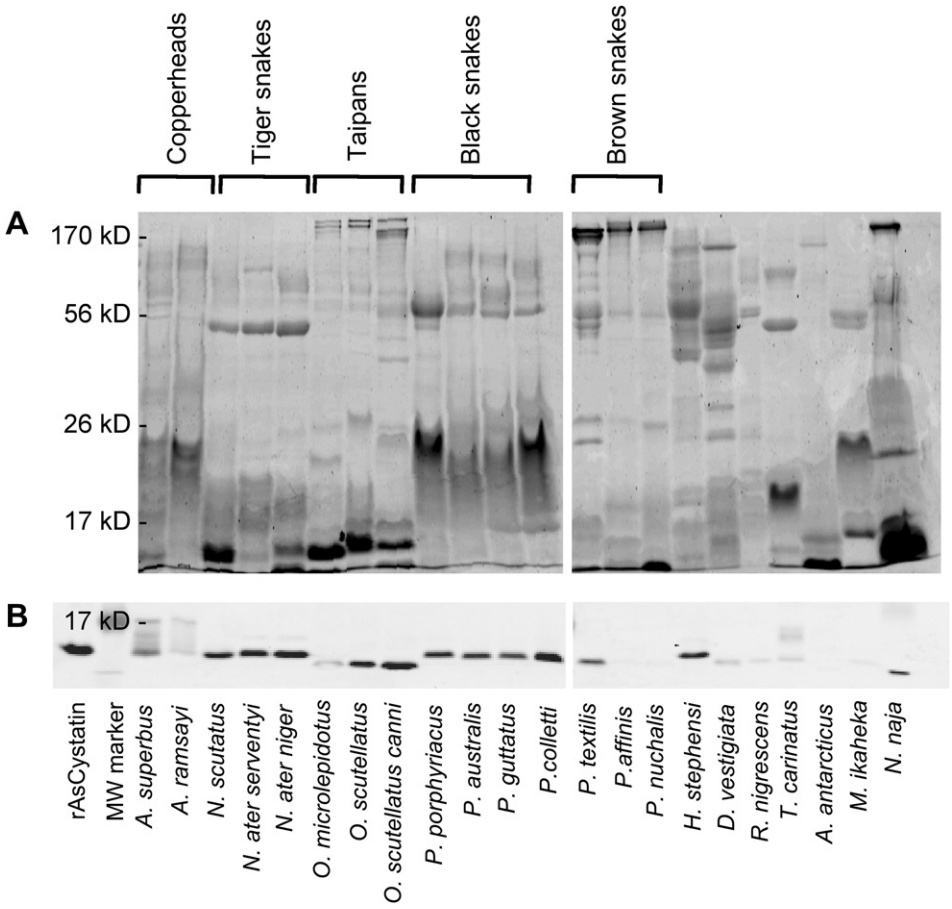

Fig. 4. Detection of cystatin protein in elapid snake venoms. (A) Composite of two Coomassie-stained gels (separated by a white line) loaded with 25 μg each of non-reduced whole venom from a number of Australian, Papuan and Asian elapid snakes. (B) Immunoblot detection of AsCystatin within the venom (12.5 μg) of these snakes alongside 8 ng of bacterially expressed recombinant AsCystatin (rAsCystatin). The protein was detected using an IRDye 800 CW (Li-Cor) secondary antibody and an infrared scanner.

was observed; in particular, *O. microlepidotus* venom exhibited lower expression levels than the other taipans, while *P. textilis* venom appeared to contain higher levels of cystatin than the other brown snake species. Weaker cystatin immunoblot reactivity was also observed in *R. nigrescens, M. ikaheka* and *N. naja* venoms, with extremely low levels of protein detected in *A. antarcticus* venom. Snake venom proteomic variation is documented to occur at the inter-family, inter-species and intra-species levels and is influenced by a number of factors including habitat, diet, age and sex [33–35]. Our immunoblot data indicate inter-species variations in the quantity of cystatin protein in the venom, but cystatin expression quite likely displays intra-species and individual variation also.

By comparison of immunoblot band intensities with the 8 ng of bacterial recombinant protein in lane 1 (Fig. 4B), it is apparent that the relative quantity of cystatin protein in venom is low (less than 0.6 μg/mg crude venom), even for the snakes with the largest amounts. Such low expression levels may explain why previous proteomic analysis of Australian elapid venoms failed to detect the cystatins, particularly given that these proteins may be masked by the significantly more abundant PLA_2 isozymes [30]. Concordant with the presence of active cystatin protein, crude *A. superbus* venom was shown to inhibit papain activity in a dose-dependent and tight-binding fashion. The cysteine-protease inhibitor content of one preparation of crude *A. superbus* venom was estimated at 3.9 pmol/mg by titration with papain, or 0.05 μg active inhibitor per mg of crude venom (Supp. Fig. 2). This is consistent with the protein estimation obtained for cystatin by immunoblotting, and confirms the presence of an active cysteine-protease inhibitor in *A. superbus* venom.

3.4. Functional characterisation of AsCystatin

For functional analysis of AsCystatin, a recombinant protein with minimal modifications was produced using the pGex6P1 *E. coli* expression system. In this system, the recombinant protein was expressed as an N-terminal GST-fusion which retained just five extra N-terminal amino acids, GPLGS, following PreScission protease cleavage (confirmed by N-terminal sequencing, Australian Proteome Analysis Facility). Fig. 5 demonstrates the expression and purification of recombinant AsCystatin (rAsCystatin). IPTG induction of GST-fusion protein expression (lane 2) and efficient removal of the GST tag by PreScission protease cleavage (lane 3) was observed. The final purified recombinant protein, with a predicted M_r of 13,498 Da is evident in lane 4. The cleaved protein was produced at a yield of 5 mg/L of induced culture.

Inhibition of various C1 cysteine proteases by rAsCystatin was tested (see Table 1). Consistent with ovocystatin and cystatin C, rAsCystatin displayed tight-binding inhibition of C1 family cysteine proteases. Cathepsin L and papain were inhibited with K_i values of 0.9 pM and 1.7 pM, respectively. rAsCystatin also inhibited cathepsin B, albeit with a weaker K_i of 0.69 nM. The active site groove of cathepsin B is partially blocked by the so-called occluding loop, requiring a two-step mechanism of inhibition [36]. While the model of the cathepsin B/rAsCystatin complex showed the largest

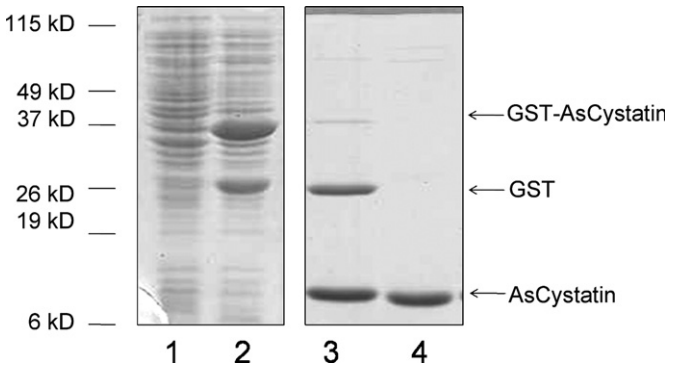

Fig. 5. Recombinant expression and purification of AsCystatin in bacteria. Representative Coomassie-stained gels demonstrating the purification of the IPTG-inducible GST-fusion protease inhibitor. (1) Uninduced whole cell lysate, (2) IPTG-induced whole cell lysate, (3) elution of cleaved recombinant cystatin, (4) final purified recombinant cystatin.

interface area (1124 Å^2) of the complexes modelled in this study, it can be assumed that the energy expenditure necessary to displace the occluding loop contributed to the higher overall K_i.

The ability of rAsCystatin to inhibit other cysteine protease families was also examined. At concentrations up to 140 nM, and at least 3-fold in molar excess of the enzyme, rAsCystatin did not inhibit calpain 1, a C2 family cysteine protease not generally inhibited by family 2 cystatins [37]. The deduced AsCystatin protein sequence diverged from prototypical family 2 cystatins by the apparent absence of Asn-39 (cystatin C numbering, see Fig. 3A) required for legumain (C13 family) inhibition [12]. As expected, rAsCystatin did not inhibit legumain at inhibitor concentrations up to 660 nM and at least 6-fold in molar excess of enzyme (Table 1).

Despite the truncated N-terminus of native AsCystatin relative to other family 2 cystatins, sequence alignment and structural modelling suggested Gly-3 of AsCystatin is homologous to the cystatin C Gly-11 essential for C1 cysteine protease inhibition (see Fig. 3). To further assess the homology between Gly-3 of AsCystatin and Gly-11 of cystatin C and to confirm our modelling results, a recombinant Gly-3-Ser mutant, rAsCystatin G3S, was produced in the pGex6P expression system. This protein was expressed and purified in the same manner as the wild-type protein and the mutation was confirmed by N-terminal sequencing. Mutagenesis of rAsCystatin Gly-3 to Ser increased the dissociation constant (K_i) for papain from 1.7 pM to approximately 18 nM (see Table 1), which equates to a 10^4-fold reduction in inhibitory activity. This provides biochemical evidence that the inhibitory mechanism of snake venom cystatins is consistent with family 2 cystatins.

These data do not indicate elapid snake venom cystatins are toxins, but their widespread presence in strongly toxic venoms nevertheless begs the question. Snake venom components from many different species have been shown to be toxic to multiple cell types *in vitro* [38–40]. Consistent with the view that snake venom cystatins are not toxins *per se*, we saw no evidence of cell death (PC3 prostate cancer cells) following continuous exposure to 6 μM of rAsCystatin for 24 h (data not shown).

4. Discussion

Family 2 cystatin-like proteins have been detected in various tissues and fluids from diverse organisms including sugarcane (*Saccharum officinarum*) [41], horseshoe crab (*Tachypleus tridentatus*) [42], tick (*Ixodes scapularis*) [43], jellyfish (*Cyanea capillata*) [44], and several mammalian and reptilian species [5]. A cystatin-like protein from snake venom (*B. arietans*) was first purified and its amino acid sequence determined in 1987 [1,45]. Subsequently, a cystatin-like cysteine-protease inhibitor was isolated from *N. atra* venom and phylogenetic analysis based on amino acid composition identified it as a member of a new sub-family of type 2 cystatins which included human cystatin M [2]. In 2002, a screen of nine elapid snake venoms demonstrated varying levels of cathepsin L and papain inhibitory activity which isolated to a 12–13 kDa gel filtration fraction [3]. Prior to the present study, only one snake venom cDNA (bitiscystatin from the viper *B. gabonica*) had been cloned [4]. In the current study, cystatin-like cysteine-protease inhibitor cDNAs were cloned from elapid snake venom glands for the first time. Close sequence identity was observed across all cystatins from elapid snake species and the protein was shown to be widely expressed in snake venoms. We also demonstrated that the structural and functional characteristics of family 2 cystatins have been conserved in snake venoms.

Given the high sequence identity of cystatin-like cDNAs in elapid snakes, a single species was chosen as a model for further characterisation. *A. superbus* cystatin (AsCystatin) cDNA codes for a pre-protein with a novel 26 amino acid signal sequence. The predicted mature secreted protein, AsCystatin, has an apparent M_r of 13,087 Da by amino acid composition. The presence of an active cysteine-protease inhibitor in *A. superbus* venom was demonstrated through inhibition of papain, and immunoblotting confirmed cystatin protein is secreted in the venom.

Sequence elements essential for inhibition of C1 family cysteine proteases are conserved in snake venom cystatins and the inhibition profile of rAsCystatin is in line with classical family 2 cystatins. Unlike canonical serine protease inhibitors, which mimic the conformation of a substrate, cysteine-protease inhibitors are thought to elicit their inhibitory activity by blocking the catalytic groove of the enzyme, and thereby preventing access for potential substrates. This type of enzyme/inhibitor interaction would allow for a greater variability in both conformation and peptide sequence of the inhibitor. Elapid snake venom cystatins contain the three structural elements (N-terminus; loop 1 Gln-55-Gln59; loop 2 Pro-105-Trp-106) required to form the wedge-shaped binding motif for C1 protease active site binding and inhibition [7,11]. In accordance with prototypical family 2 cystatins, mutagenesis of Gly-3 (Gly-11 in cystatin C) strongly reduced the potency of papain inhibition. Molecular modelling of the G3S mutant indicated that a conformational change in the N-terminal segment centred around Ser-3

Table 1
Inhibition of cysteine proteases by recombinant *Austrelaps superbus* cystatin (rAsCystatin) variants.

Enzyme	rAsCystatin K_i(pM) ± SD	rAsCystatin G3S K_i(pM) ± SD	Ovocystatin [48,58] K_i(pM)	Cystatin C [12,48] K_i(pM)
Papain	1.74 ± 0.04 (3)	1.81 ± 0.05 × 10^4 (2)	5	5
Cathepsin L	0.89 ± 0.38 (2)	n.d.	19	5
Cathepsin B	690 ± 7 (2)	n.d.	1700	250
Calpain	>1.4 × 10^5	n.d.	>1 × 10^7	>1 × 10^7
Legumain	>6.6 × 10^5	n.d.	<5.0 × 10^3	200

Dissociation constants and error estimates, K_i(pM) ± SD, were determined in continuous rate assays in replicate experiments (n). Dissociation constants for prototypical family 2 cystatins, ovocystatin and human cystatin C were obtained from the literature [12,48,58]. (n.d. not determined).

would be required for the G3S mutant to interact with the substrate binding groove of papain.

The importance of amino acids preceding the crucial Gly-11 residue has been reported for other cystatins. Hall et al. (1995) showed that replacement of Leu-9 or Val-10 of cystatin C with Gly affected enzyme affinity and selectivity, in particular for cathepsins B and H [36]. In this study, rAsCystatin, with an N-terminal sequence of Ser-8-Ile-9-Pro-10-Gly-11 (cystatin C numbering) displayed similar selectivity as cystatin C [36,46]. It would thus appear that Leu-9 and Val-10 are interchangeable with some other hydrophobic residues, as present in rAsCystatin (Ile-9-Pro-10). Cathepsin B has a somewhat unique status amongst cysteine proteases in that it contains an 18-residue insertion, termed the occluding loop, that controls access to the enzymatic groove in a pH dependent manner [47]. While the association rate constant for the interaction of cystatins with papain and similar cysteine proteases is generally similar to that of a diffusion controlled reaction, inhibition of cathepsin B follows a two-step mechanism that includes displacement of the occluding loop to allow the inhibitor access to the substrate binding groove [36]. The initial step of cathepsin B inhibition involves interaction of the N-terminal section of the inhibitor with the enzyme. In cystatin C, one of the most potent inhibitors for cathepsin B with a K_i of 0.22–0.25 nM [11,48], the side chains of Val-10 and to a lesser extent Leu-9 were found to be crucial for efficient inhibition, with an approximately 2000-fold higher K_i for the V10G mutant and a 180-fold higher K_i for the L9G mutant [36]. As mentioned above, the corresponding residues in native AsCystatin are Ile-1 and Pro-2, identical to those found in cystatin A. Indeed, the K_i determined here for rAsCystatin is very similar to that reported for cystatin A (0.7 nM *vs.* 0.9 nM for the latter) [49], suggesting a similar initial interaction of the two inhibitors with cathepsin B.

The AsCystatin sequence differs from cystatin C in three significant ways: an altered N-terminus; the absence of Asn-39 for legumain inhibition; and the presence of an additional stretch of amino acids between the cysteine residues that form the first disulfide bridge. Our results suggest that the altered N-terminus has no significant impact on the inhibitory profile and the critical Gly-11 residue is conserved. It is not clear why some family 2 cystatins harbour the Asn-39 and the capacity to inhibit legumain, while others do not [12]; however, the absence of Asn-39 proscribes legumain inhibition for AsCystatin. As observed for cystatin M, the amino acid insertion in AsCystatin is unlikely to affect overall conformation as it is in a largely unstructured region which lies on the opposite side to the conserved hair pin loops that form the inhibitory wedge [50].

There are varying reports regarding the relative quantity of cystatin protein in snake venoms. In two separate studies, the cystatin (bitiscystatin) content of viper *B. gabonica* venom is reported to be very high. One group demonstrated that bitiscystatin accounts for 9.8% of the total venom protein [51] and another report suggested that Kunitz-type inhibitors and cystatin combined represent 10% of the total venom protein [4]. Another viper species, *B. arietans*, has also been reported to contain a significant level of cystatin protein in the venom, 1.7% of the total protein content [52]. In the current study, the papain inhibitory activity of *A. superbus* venom was presented simply as an illustration of the presence of active cysteine-protease inhibitor. Nevertheless, it is interesting that we observed a much lower level of active protein in the venom from an *A. superbus* sample compared with that seen for the vipers. Our immunoblot results also suggest that cystatins are a minor component of elapid snake venoms (less than 0.6 μg/mg crude venom), but that protein concentration varies considerably across and within genera. It is likely that the quantity of cystatin protein in elapid snake venoms is generally very low since this protein was not detected in a comprehensive proteomic screen of 18 elapid snake species from nine different genera which identified proteins from 17 different families [30].

The presence of cystatin-like proteins in the venom of elapid snakes would seem to implicate them as toxins. However, their close structural and functional identity with family 2 cystatins, which are important regulatory proteins, is not entirely consistent with this. Venom toxins are often present as multiple isoforms varying within and across species and it is hypothesised that this has occurred via adaptive evolution induced by differing prey availability and susceptibility [53,54]. This is not the case with cystatins, for which only one highly conserved isoform was detected in each elapid snake species. Some toxic venom components are highly similar to commonly expressed regulatory proteins. However, in the case of the snake venom prothrombin activators for example, the venom proteins have been altered in specific ways to improve stability and activity, are controlled by specialised promoters which mediate high level expression, and are expressed in only a few species [55–57]. In contrast, widespread, low level expression and high level conservation are observed for snake venom cystatins. Add to this the absence of *in vitro* toxicity, and a strong case for snake venom cystatins as essential housekeeping or regulatory proteins, rather than specific prey-targeted toxins emerges.

It remains unclear what specific purpose snake venom cystatins serve. As previously suggested, cystatins may indirectly promote venom toxicity by protecting snake venom proteins from prey ECM proteases [2]. On the other hand, given that snake venom glands are modified salivary glands it is pertinent that some mammals secrete cystatins into the saliva. The specific role of mammalian salivary cystatins is not clear but these proteins appear to function in various ways to maintain oral health [16]. It is thus possible that snake venom cystatins play roles independent from envenomation, comparable to mammalian salivary cystatins.

Acknowledgments

We wish to thank Dr Masego Johnstone, Ms Simone Reynolds and Dr Katja Fischer for assistance with spectrofluorometric assays, Dr Amanda Wraith Kijas and Dr Stephen Earl for helpful discussions, Ms Jennifer McCarron for PC3 cells, Dr Janelle Hancock for the Wst-1 reagent, and Mr Joe Sambono for provision of venom glands. Financial assistance was provided by an ARC linkage grant between Venomics Pty Ltd and the Australian Research Council. MT was funded by an Australian National Health and Medical Research Council industry fellowship. N-terminal sequencing was facilitated by access to the Australian Proteome Analysis Facility supported under the Australian Government's National Collaborative Research Infrastructure Strategy (NCRIS).

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biochi.2010.12.008.

References

[1] H.J. Evans, A.J. Barrett, A cystatin-like cysteine proteinase inhibitor from venom of the African puff adder (*Bitis arietans*), Biochem. J. 246 (1987) 795–797.
[2] M. Brillard-Bourdet, V. Nguyen, M. Ferrer-di Martino, F. Gauthier, T. Moreau, Purification and characterization of a new cystatin inhibitor from Taiwan cobra (*Naja naja atra*) venom, Biochem. J. 331 (Pt. 1) (1998) 239–244.
[3] H. Mashiko, H. Takahashi, Cysteine proteinase inhibitors in elapid and hydrophiid snake venoms, Toxicon 40 (2002) 1275–1281.

[4] I.M. Francischetti, V. My-Pham, J. Harrison, M.K. Garfield, J.M. Ribeiro, *Bitis gabonica* (Gaboon viper) snake venom gland: toward a catalog for the full-length transcripts (cDNA) and proteins, Gene 337 (2004) 55–69.
[5] D. Kordis, V. Turk, Phylogenomic analysis of the cystatin superfamily in eukaryotes and prokaryotes, BMC Evol. Biol. 9 (2009) 266–287.
[6] M. Abrahamson, A. Ritonja, M.A. Brown, A. Grubb, W. Machleidt, A.J. Barrett, Identification of the probable inhibitory reactive sites of the cysteine proteinase inhibitors human cystatin C and chicken cystatin, J. Biol. Chem. 262 (1987) 9688–9694.
[7] W. Bode, R. Engh, D. Musil, U. Thiele, R. Huber, A. Karshikov, J. Brzin, J. Kos, V. Turk, The 2.0 A x-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases, EMBO J. 7 (1988) 2593–2599.
[8] S.X. Wang, K.C. Pandey, J.R. Somoza, P.S. Sijwali, T. Kortemme, L.S. Brinen, R.J. Fletterick, P.J. Rosenthal, J.H. McKerrow, Structural basis for unique mechanisms of folding and hemoglobin binding by a malarial protease, Proc. Natl. Acad. Sci. U S A 103 (2006) 11503–11508.
[9] A. Ljunggren, I. Redzynia, M. Alvarez-Fernandez, M. Abrahamson, J.S. Mort, J.C. Krupa, M. Jaskolski, G. Bujacz, Crystal structure of the parasite protease inhibitor chagasin in complex with a host target cysteine protease, J. Mol. Biol. 371 (2007) 137–153.
[10] I. Redzynia, A. Ljunggren, A. Bujacz, M. Abrahamson, M. Jaskolski, G. Bujacz, Crystal structure of the parasite inhibitor chagasin in complex with papain allows identification of structural requirements for broad reactivity and specificity determinants for target proteases, FEBS J. 276 (2009) 793–806.
[11] A. Hall, H. Dalboge, A. Grubb, M. Abrahamson, Importance of the evolutionarily conserved glycine residue in the N-terminal region of human cystatin C (Gly-11) for cysteine endopeptidase inhibition, Biochem. J. 291 (Pt. 1) (1993) 123–129.
[12] M. Alvarez-Fernandez, A.J. Barrett, B. Gerhartz, P.M. Dando, J. Ni, M. Abrahamson, Inhibition of mammalian legumain by some cystatins is due to a novel second reactive site, J. Biol. Chem. 274 (1999) 19195–19203.
[13] N. Obermajer, Z. Jevnikar, B. Doljak, J. Kos, Role of cysteine cathepsins in matrix degradation and cell signalling, Connect. Tissue Res. 49 (2008) 193–196.
[14] V. Hook, T. Toneff, M. Bogyo, D. Greenbaum, K.F. Medzihradszky, J. Neveu, W. Lane, G. Hook, T. Reisine, Inhibition of cathepsin B reduces beta-amyloid production in regulated secretory vesicles of neuronal chromaffin cells: evidence for cathepsin B as a candidate beta-secretase of Alzheimer's disease, Biol. Chem. 386 (2005) 931–940.
[15] U. Meyer-Hoffert, Reddish, scaly, and itchy: how proteases and their inhibitors contribute to inflammatory skin diseases, Arch. Immunol. Ther. Exp. (Warsz) 57 (2009) 345–354.
[16] D.P. Dickinson, Salivary (SD-type) cystatins: over one billion years in the making—but to what purpose? Crit. Rev. Oral Biol. Med. 13 (2002) 485–508.
[17] J.V. Frangioni, B.G. Neel, Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins, Anal. Biochem. 210 (1993) 179–187.
[18] A.J. Barrett, H. Kirschke, Cathepsin B, cathepsin H, and cathepsin L, Meth. Enzymol. 80 (Pt. C) (1981) 535–561.
[19] E. Dransfield, Calpains from thaw rigor muscle, Meat Sci. 43 (1996) 311–320.
[20] J.F. Morrison, C.T. Walsh, The behavior and significance of slow-binding enzyme inhibitors, Adv. Enzymol. Relat. Areas Mol. Biol. 61 (1988) 201–301.
[21] J.R. Tchoupe, T. Moreau, F. Gauthier, J.G. Bieth, Photometric or fluorometric assay of cathepsin B, L and H and papain using substrates with an amino-trifluoromethylcoumarin leaving group, Biochim. Biophys. Acta 1076 (1991) 149–151.
[22] T. Fox, P. Mason, A.C. Storer, J.S. Mort, Modification of S1 subsite specificity in the cysteine protease cathepsin B, Protein Eng. 8 (1995) 53–57.
[23] K. Arnold, L. Bordoli, J. Kopp, T. Schwede, The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling, Bioinformatics 22 (2006) 195–201.
[24] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling, Electrophoresis 18 (1997) 2714–2723.
[25] C. Reynolds, D. Damerell, S. Jones, ProtorP: a protein-protein interaction analysis server, Bioinformatics 25 (2009) 413–414.
[26] R.W. Hooft, C. Sander, G. Vriend, Positioning hydrogen atoms by optimizing hydrogen-bond networks in protein structures, Proteins 26 (1996) 363–376.
[27] L. St Pierre, R. Woods, S. Earl, P.P. Masci, M.F. Lavin, Identification and analysis of venom gland-specific genes from the coastal taipan (*Oxyuranus scutellatus*) and related species, Cell Mol. Life Sci. 62 (2005) 2679–2693.
[28] L. St Pierre, S.T. Earl, I. Filippovich, N. Sorokina, P.P. Masci, J. De Jersey, M.F. Lavin, Common evolution of waprin and kunitz-like toxin families in Australian venomous snakes, Cell Mol. Life Sci. 65 (2008) 4039–4054.
[29] L. St Pierre, G.W. Birrell, S.T. Earl, T.P. Wallis, J.J. Gorman, J. de Jersey, P.P. Masci, M.F. Lavin, Diversity of toxic components from the venom of the evolutionarily distinct black whip snake, *Demansia vestigiata*, J. Proteome Res. 6 (2007) 3093–3107.
[30] G.W. Birrell, S.T. Earl, T.P. Wallis, P.P. Masci, J. de Jersey, J.J. Gorman, M.F. Lavin, The diversity of bioactive proteins in Australian snake venoms, Mol. Cell Proteomics 6 (2007) 973–986.
[31] R. Doley, R.M. Kini, Protein complexes in snake venom, Cell Mol. Life Sci. 66 (2009) 2851–2871.
[32] L.D. Possani, B.M. Martin, A. Yatani, J. Mochca-Morales, F.Z. Zamudio, G.B. Gurrola, A.M. Brown, Isolation and physiological characterization of taicatoxin, a complex toxin with specific effects on calcium channels, Toxicon 30 (1992) 1343–1364.
[33] J.P. Chippaux, V. Williams, J. White, Snake venom variability: methods of study, results and interpretation, Toxicon 29 (1991) 1279–1303.
[34] S. Flight, P. Mirtschin, P.P. Masci, Comparison of active venom components between Eastern brown snakes collected from South Australia and Queensland, Ecotoxicology 15 (2006) 133–141.
[35] H.L. Gibbs, L. Sanz, J.J. Calvete, Snake population venomics: proteomics-based analyses of individual variation reveals significant gene regulation effects on venom protein expression in *Sistrurus* rattlesnakes, J. Mol. Evol. 68 (2009) 113–125.
[36] A. Hall, K. Hakansson, R.W. Mason, A. Grubb, M. Abrahamson, Structural basis for the biological specificity of cystatin C. Identification of leucine 9 in the N-terminal binding region as a selectivity-conferring residue in the inhibition of mammalian cysteine peptidases, J. Biol. Chem. 270 (1995) 5115–5121.
[37] C. Crawford, Inhibition of chicken calpain II by proteins of the cystatin superfamily and alpha 2-macroglobulin, Biochem. J. 248 (1987) 589–594.
[38] C.A. Cotte, E. Essenfeld-Yahr, A.C. Lairet, Effects of *Crotalus* and *Bothrops* venom on normal and malignant cells cultivated *in vitro*, Toxicon 10 (1972) 157–161.
[39] C. Gaertner, N. Goldblum, S. Gitter, A. De Vries, The action of various snake venoms and their chromatographic fractions on animal cells in culture, J. Immunol. 8 (1962) 526–534.
[40] J.C. Oliveira, H.M. de Oca, M.M. Duarte, C.R. Diniz, C.L. Fortes-Dias, Toxicity of South American snake venoms measured by an *in vitro* cell culture assay, Toxicon 40 (2002) 321–325.
[41] A. Gianotti, W.M. Rios, A. Soares-Costa, V. Nogaroto, A.K. Carmona, M.L. Oliva, S.S. Andrade, F. Henrique-Silva, Recombinant expression, purification, and functional analysis of two novel cystatins from sugarcane (*Saccharum officinarum*), Protein Expr. Purif. 47 (2006) 483–489.
[42] K.L. Agarwala, S. Kawabata, M. Hirata, M. Miyagi, S. Tsunasawa, S. Iwanaga, A cysteine protease inhibitor stored in the large granules of horseshoe crab hemocytes: purification, characterization, cDNA cloning and tissue localization, J. Biochem. 119 (1996) 85–94.
[43] M. Kotsyfakis, A. Sa-Nunes, I.M. Francischetti, T.N. Mather, J.F. Andersen, J.M. Ribeiro, Antiinflammatory and immunosuppressive activity of sialostatin L, a salivary cystatin from the tick *Ixodes scapularis*, J. Biol. Chem. 281 (2006) 26298–26307.
[44] Y. Yang, S. Cun, L. Peng, X. Xie, J. Wei, W. Yang, A. Xu, cDNA cloning, identification and characterization of a novel cystatin from the tentacle of *Cyanea capillata*, Biochimie 85 (2003) 1033–1039.
[45] A. Ritonja, H.J. Evans, W. Machleidt, A.J. Barrett, Amino acid sequence of a cystatin from venom of the African puff adder (*Bitis arietans*), Biochem. J. 246 (1987) 799–802.
[46] A.J. Barrett, The cystatins: a diverse superfamily of cysteine peptidase inhibitors, Biomed. Biochim. Acta 45 (1986) 1363–1374.
[47] R. Frlan, S. Gobec, Inhibitors of cathepsin B, Curr. Med. Chem. 13 (2006) 2309–2327.
[48] A.J. Barrett, The cystatins: a new class of peptidase inhibitors, TIBS (1987) 193–196 12-May 1987.
[49] E. Pol, S.L. Olsson, S. Estrada, T.W. Prasthofer, I. Bjork, Characterization by spectroscopic, kinetic and equilibrium methods of the interaction between recombinant human cystatin A (stefin A) and cysteine proteinases, Biochem. J. 311 (Pt. 1) (1995) 275–282.
[50] G. Sotiropoulou, A. Anisowicz, R. Sager, Identification, cloning, and characterization of cystatin M, a novel cysteine proteinase inhibitor, down-regulated in breast cancer, J. Biol. Chem. 272 (1997) 903–910.
[51] J.J. Calvete, C. Marcinkiewicz, L. Sanz, Snake venomics of *Bitis gabonica gabonica*. Protein family composition, subunit organization of venom toxins, and characterization of dimeric disintegrins bitisgabonin-1 and bitisgabonin-2, J. Proteome Res. 6 (2007) 326–336.
[52] P. Juarez, S.C. Wagstaff, J. Oliver, L. Sanz, R.A. Harrison, J.J. Calvete, Molecular cloning of disintegrin-like transcript BA-5A from a *Bitis arietans* venom gland cDNA library: a putative intermediate in the evolution of the long-chain disintegrin bitistatin, J. Mol. Evol. 63 (2006) 142–152.
[53] J.C. Daltry, W. Wuster, R.S. Thorpe, Diet and snake venom evolution, Nature 379 (1996) 537–540.
[54] D. Kordis, F. Gubensek, Adaptive evolution of animal toxin multigene families, Gene 261 (2000) 43–52.
[55] M.H. Bos, M. Boltz, L. St Pierre, P.P. Masci, J. de Jersey, M.F. Lavin, R.M. Camire, Venom factor V from the common brown snake escapes hemostatic regulation through procoagulant adaptations, Blood 114 (2009) 686–692.
[56] S. Kwong, A.E. Woods, P.J. Mirtschin, R. Ge, R.M. Kini, The recruitment of blood coagulation factor X into snake venom gland as a toxin: the role of promoter cis-elements in its expression, Thromb. Haemost. 102 (2009) 469–478.
[57] V.S. Rao, R.M. Kini, Pseutarin C, a prothrombin activator from *Pseudonaja textilis* venom: its structural and functional similarity to mammalian coagulation factor Xa-Va complex, Thromb. Haemost. 88 (2002) 611–619.
[58] J.M. Chen, P.M. Dando, N.D. Rawlings, M.A. Brown, N.E. Young, R.A. Stevens, E. Hewitt, C. Watts, A.J. Barrett, Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase, J. Biol. Chem. 272 (1997) 8090–8098.
[59] P.P. Masci, Studies of coagulation and fibrinolysis using Australian snake venoms: from molecular toxinology to novel therapeutic agents, PhD thesis (2000).