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Presence of peptide inhibitors in rattlesnake venoms and their effects on endogenous metalloproteases

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Abstract

Long-term storage of proteins with retention of biological activity is a concern for many actual and potential protein drugs. A model for stabilization of proteins for long periods could exist in natural systems, particularly among viperid snakes whose venoms are rich in lytic enzymes, because when secreted into the lumen, they are stored in an inactive and competent state for many months. One mechanism inhibiting autolysis is the production of (relatively) low affinity peptide enzyme inhibitors. We investigated the distribution of two of these peptides (pEQW and pENW) in venoms from nine species of rattlesnakes and evaluated the role of these peptides in inhibiting and stabilizing isolated major venom metalloproteases (Cvo Pr V and cromipyrrhin) from *Crotalus oreganus oreganus* and *C. mitchelli pyrrhus* venom. We show that two endogenous peptides, pEQW and pENW, are present in venoms from ten taxa of *Crotalus* and *Sistrurus* and that pEQW inhibits Cvo PrV and cromipyrrhin. The peptide inhibitor pEQW also stabilizes cromipyrrhin against autoproteolysis under extreme conditions (heat). Using these peptides as models, it may be possible to design similar low affinity peptide inhibitors of protein drugs which will increase their stability and/or allow for storage under less stringently controlled conditions.

Keywords: Autolytic degradation; Coelution; Enzymes; Protein stabilization; Venom gland; Venom storage

1. Introduction

Modeling of the effects of local environmental factors in proteins has provided a better understanding of the balance between protein stability and function (Bai et al., 1994; Muñoz et al., 1996). Factors affecting protein stability also have an effect on the protein's retention of biological activity on long-term storage, a concern for many actual and potential protein drugs. Many proteins could be useful for pharmacological or industrial applications, but they are often not stable enough for these purposes (Costantino et al., 1995; Dong et al., 1995; Cowan, 1995; Ó Fágáin, 1995; Villegas et al., 1996; Van den Burg and Eijsink, 2002). Consequently, proteins from many different organisms in a variety of environments have been examined and evaluated for their potential as models of protein stability (Arnold, 1993; Cowan, 1995; Ó Fágáin, 1995; Pikal-Cleland et al., 2002; Lin-Lee et al., 2002). This broad search for potential models has included protein sources ranging from bacteria to snake venoms (Jakob et al., 1992; Shoichet et al., 1995; Burgess and Grant, 1997), and protein stability has been evaluated under extreme conditions of temperature, pH, salinity, dilution, or various combinations of these factors (Hampe et al., 1978; Izutsu et al., 1994; Ownby et al., 1994; Cowan, 1995; Butler and Falke, 1996; Munekiyo and Mackessy, 1998).

In the main venom gland of rattlesnakes, venom is secreted into a lumen and may be stored in an inactive but competent state for many months (Mackessy, 1991). Because these venoms contain numerous proteases and other lytic enzymes (e.g. Bjarnason and Fox, 1988–1989,

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1994; Tu, 1996; Stocker, 1998; Bailey, 1998), endogenous mechanisms must be present within the gland and stored venom that inhibit autolysis and promote protein stability during long-term storage. Stability of *Crotalus m. molossus* venom under numerous storage conditions was evaluated by Munekiyo and Mackessy (1998). The results of this study found that electrophoretic, toxic and enzymatic activities of *C. m. molossus* venom, which contained at least 24 distinct protein components, were largely unaffected by extremes of temperature and storage conditions. This study suggested that most venom activities should remain stable even if collected or stored under potentially adverse conditions, and endogenous inhibitors likely promote stable long-term storage.

There appear to be redundant mechanisms by which venomous snakes store potent hydrolytic enzymes (Robeva et al., 1991; Francis et al., 1992; Francis and Kaiser, 1993) and protect themselves from venom toxins (Straight et al., 1976). These mechanisms include the presence of high citrate levels (Francis et al., 1992; Freitas et al., 1992; Odell et al., 1998) and the production of low affinity peptide enzyme inhibitors (Robeva et al., 1991; Francis and Kaiser, 1993; Huang et al., 1998). Citrate is an organic component of venom that has been studied extensively (Francis et al., 1992; Freitas et al., 1992; Fenton et al., 1995; Odell et al., 1998) and has been demonstrated to inhibit metalloproteases, phospholipase A_2 (PLA_2) and phosphodiesterase (PDE) from venoms of several different species of venomous snakes. Citrate can form strong complexes with divalent metal ions (Ca^{+2}, Mg^{+2}, and Zn^{+2}) and thus may inhibit Ca^{+2}-dependent PLA_2, Mg^{+2}-dependent PDE, and Zn^{+2}-dependent proteases, all of which require these cations for activity, by chelation of metals (Francis et al., 1992; Odell et al., 1998). Francis et al. (1992) suggested that high concentrations of citrate may represent an important mechanism used by the snakes to protect themselves against the toxic effects of their own venom. However, Mackessy (1996) reported that citrate did not significantly inhibit activity of a purified metalloprotease (Cvo PrV), even at high (100 mM) concentrations. Based on these data, there must also be other protective mechanisms that inhibit enzymes in stored venoms, particularly proteases.

Peptide enzyme inhibitors are present in some venoms at millimolar concentrations (Francis and Kaiser, 1993), and these endogenous peptides have been examined for their role in the inhibition of human serine proteases and snake venom metalloprotease activity (Robeva et al., 1991; Francis and Kaiser, 1993; Zhung et al., 1994; Bjarnason and Fox, 1994; Portaro et al., 1997; Gomis-Rüth et al., 1998; Huang et al., 1998). Grams et al. (1993) suggested that the venom enzymes, especially the metalloproteases, are stored as inactive zymogens in order to prevent autodigestion, and there is evidence that protease activation of some of the matrixin-zymogens takes place in two steps (Woessner, 1991). The study of Grams et al. (1993) confirmed one of these steps: a cysteine switch-like mechanism is involved in the activation of some snake venom metalloproteases. Therefore, it seems likely that some venom components may serve a multifunctional role, both individually and in concert, as a stabilizer and/or inhibitor controlling metalloprotease activation.

In the current study, we examined the distribution of two peptides, pyroglutamyl-glutamyl-tryptophan (pEQW) and pyroglutamyl-asparagyl-trytophan (pENW), in venoms from nine species of rattlesnakes and evaluated the role of (synthetic) pEQW in stabilizing isolated venom metalloproteases from *Crotalus mitchelli pyrrhus* (termed cromipyrrhin) and *Crotalus oreganus oreganus* (Cvo Pr V) venoms. We predicted that all venoms would contain at least one of these peptide inhibitors and that pEQW would stabilize metalloproteases by inhibiting autolysis, as previous investigators have noted inhibitory effects on other metalloproteases (e.g. Francis and Kaiser, 1993).

2. Materials and methods

2.1. Reagents

All reagents (analytical grade or better) were obtained from Sigma Chemical Co., USA. Novex tris-glycine 14% acrylamide gels and Mark 12 molecular weight standards were obtained from Invitrogen, Inc., USA. Acetonitrile (HPLC grade) was obtained from Fisher Scientific, USA. Synthetic peptides were synthesized and HPLC-purified by MacroMolecular Facilities, Colorado State University, Ft Collins, CO, USA.

2.2. Venom extractions

Venom was extracted from captive snakes using standard methods (Mackessy, 1988), lyophilized and stored frozen with desiccant. Venoms used were from *Crotalus atrox* (SE Arizona), *C. horridus atricaudatus* (Arkansas), *C. mitchelli pyrrhus* (SE California), *C. molossus molossus* (SE Arizona), *C. ruber* (S California), *C. scutulatus* (type A venom; S Mojave Desert, CA), *C. oreganus concolor* (Sweetwater Co., WY), *C. oreganus oreganus* (Santa Barbara Co., CA), *C. viridis viridis* (Weld Co., NE Colorado), and *Sistrurus catenatus edwardsii* (Lincoln Co., SE Colorado); taxonomy for rattlesnakes of the *C. viridis* complex follows Ashton and de Queiroz (2001).

2.3. Isolation and identification of pEQW and pENW from rattlesnake venoms

Four hundred microliters of reconstituted venom (4.0 mg/ml ddH_2O) was centrifuge-fractionated using Millipore Micron YM-3 3.0 kD cutoff centrifugal filters (Bedford, MA, USA). Fifty microliters of filtrate obtained was applied to a Vydac C_{18} reverse phase column (4.6×300 mm) on a Waters HPLC (Milford, MA, USA)

operating under Empower Pro software, and peptides were eluted with an increasing acetonitrile (ACN) gradient (20–25% B over 15 min; solvent $A = 0.1\%$ trifluoroacetic acid (TFA) in water; $B = 80\%$ ACN in 0.1% TFA; 30 min run time). For all venoms, at least two samples from different individual snakes were analyzed. Venom peptides were identified by comparing elution times to those of synthetic purified peptides pEQW and pENW (50 μL injections of 0.2 mM peptide, individually and combined), a well-established method for compound identification (e.g. Krull and Swartz, 2001; Lee and MacKinnon, 2004). Synthetic peptide standards were run between every other venom sample to correct for potential temperature and replicate effects on elution times. Relative percent abundance of pENW and pEQW in each venom were calculated by integrating chromatograms, eliminating extraneous peaks (if any) and calculating % area for each peptide. Approximate molar concentrations of these two peptides in venoms were calculated based on peak areas of venom samples and peak areas of known concentrations of synthetic pENW and pEQW. This measure makes the assumption that filtrate obtained from spin columns samples venoms equally and that the filtrate is representative of peptide concentration in the whole venoms. Coelution experiments also were used to confirm peptide identity. Fifteen microliters spin column filtrate from *C. m. molossus* venom was combined with either 5 μL of ddH_2O, 0.2 mM synthetic pENW or pEQW and injected under the same conditions as above. Chromatograms of 'spiked' samples were then compared to venom filtrate alone.

For peptides obtained from *C. m. molossus* venom, mass spectroscopic analysis was carried out at MacroMolecular Resources, Colorado State University (Fort Collins, CO). Dried peptide fractions were dissolved in 0.2% formic acid in 50/50 acetonitrile/water at an approximate concentration of 0.5 mg/ml, and masses were determined by MALDI MS spectroscopy (Kratos, MALDI I equipment).

2.4. Inhibition of Cvo Pr V and cromipyrrhin metalloproteases by metal chelators and pEQW

The dominant venom metalloproteases Cvo Pr V (from *Crotalus oreganus oreganus* venom) and cromipyrrhin (a homolog of Cvo PrV from *Crotalus mitchelli pyrrhus* venom), both approximately 53 kD, were purified from crude venoms using anion exchange chromatography on Sephacel columns at pH 8.25 (Mackessy, 1996). Metalloproteases (10 μg; approx. 190 pmol) were incubated with metal chelators (EDTA, EGTA, or 1,10-phenanthroline) or synthetic pEQW for 30 min at room temperature and then assayed for residual protease activity (Mackessy, 1996).

2.5. Stabilization of cromipyrrhin metalloprotease

Stability of cromipyrrhin was evaluated over a 96-h period of incubation at 37 °C with or without 5.0 mM synthetic pEQW. Aliquots were withdrawn at 0, 1, 2, 4, 8, 12, 24, 48, 72 and 96 h, frozen immediately and then assayed for azocasein-hydrolyzing activity (Aird and da Silva, 1991); aliquots were also evaluated electrophoretically using SDS-PAGE (Novex 14% tris–glycine acrylamide gels) after reduction with 2-mercaptoethanol; seven micrograms of protein were loaded in each lane.

3. Results

Chromatograms of synthetic peptides (Fig. 1A–B) established mean elution times for pENW of 7.55 ± 0.05 min and for pEQW of 7.95 ± 0.05 min; elution times were preserved when an equimolar mixture of both

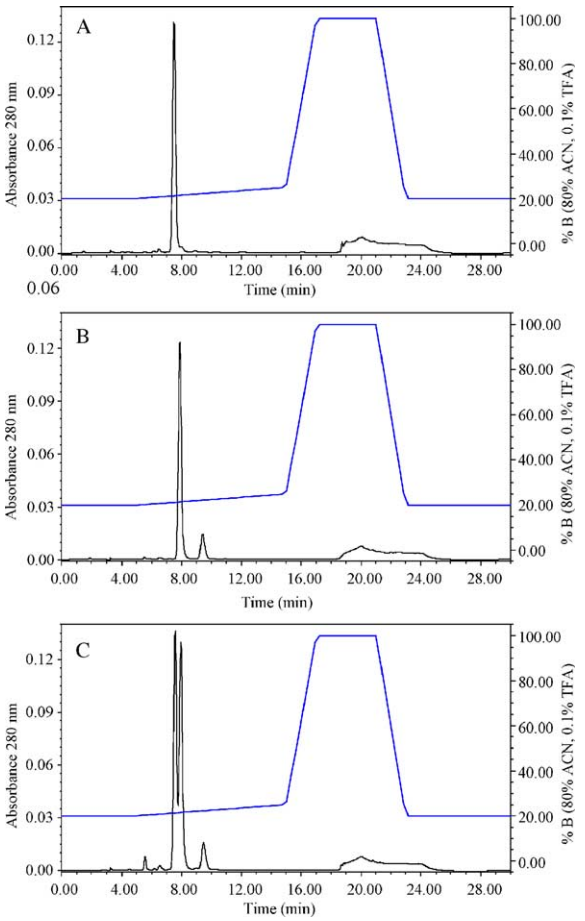

Fig. 1. RP-HPLC chromatograms for synthetic tripeptides. (A) Elution profile for 50 μL 0.2 mM pyroglutamyl-asparagyl-tryptophan (pENW); the peptide elutes at 7.55 min. (B) Elution profile for 50 μL 0.2 mM pyroglutamyl-glutamyl-tryptophan (pEQW); the peptide elutes at 7.95 min. (C) Elution profile for 50 μL equimolar mixture (0.2 mM each) of pENW and pEQW; note that the elution times of the individual peptides are preserved. The small peak at ~9.4 min in B and C is a synthesis artifact.

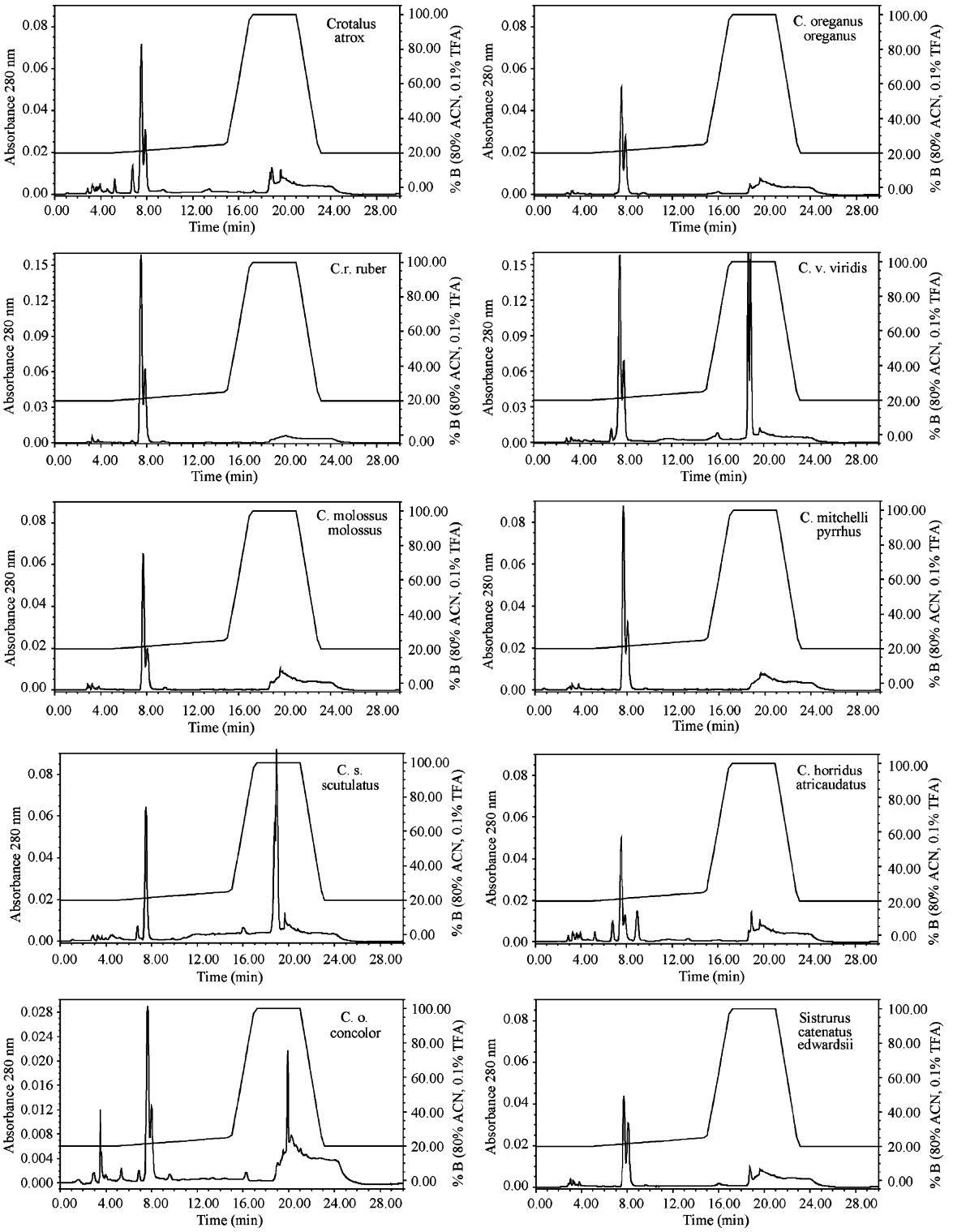

Fig. 2. RP-HPLC chromatograms for crude venoms (50 μL) following spin-fractionation using 3 kD cutoff cartridges. Peptides were eluted with a gradient of 20–25% B over 10 min. All venoms contained peptides which eluted between 7.5–8.0 min; peptide identities and relative amounts are given in Table 1. Note the difference in *Y* axis scale for *C. ruber*, *C. v. viridis* and *C. o. concolor* venoms.

peptides was subjected to RP-HPLC (Fig. 1C). Mass spectrometry of *C. m. molossus* venom peptides confirmed the identity of fractionated venom peptides (pENW: predicted monoisotopic mass $[M+H^+]=430.17$, found = 430.2; pEQW: predicted mass = 444.17, found = 444.1). Coelution experiments demonstrated that the synthetic peptides each coeluted with the venom peptide at the predicted elution times (as indicated above), further confirming identification of venom peptides as pENW and pEQW; no peak broadening or extraneous peaks were observed in these 'spiked' samples. Elution times for synthetic peptides were used to identify peptides in the following venom chromatograms. Evaluation of venoms from nine species and two genera of rattlesnakes demonstrated the presence of at least one of these tripeptides in all 10 venoms investigated (Fig. 2). Venoms of all 10 taxa sampled contained pENW, and pEQW was present in all venoms except *C. s. scutulatus* (Fig. 2; Table 1); in all cases, pENW was the more abundant tripeptide detected. Venoms of *C. o. concolor* contained the lowest concentrations of both peptides; this highly toxic venom also contains very low metalloprotease activity (Mackessy et al., 2003). Most venoms contained very little material (peptides/A280 nm-absorbing compounds) besides these two peptides, but venoms from *C. v. viridis* and *C. s. scutulatus* contained significant amounts of an unidentified peptide which eluted during column wash; minor amounts of unidentified peptides were observed in three other venoms. Approximate concentrations of peptides in venom samples (pENW = 228–1741 μM; pEQW = 0–691 μM) are listed in Table 1.

Table 1
Distribution and relative abundance of two peptide inhibitors in rattlesnake venoms

Venom	% pENW	μM pENW	% pEQW	μM pEQW	Unidentified peak(s)
Crotalus atrox	69.3	704	30.7	293	(+)
C. horridus atricaudatus	81.3	511	18.7	110	(+)
C. mitchelli pyrrhus	71.3	914	28.7	345	−
C. molossus molossus	75.6	665	24.4	201	−
C. ruber ruber	81.4	790	18.6	169	−
C. s. scutulatus type A	100	635	0	0	(+)
C. oreganus concolor	70.4	228	29.6	90	(+)
C. oreganus oreganus	62.1	507	37.9	291	−
C. viridis viridis	70.3	1741	29.7	691	+
Sistrurus catenatus edwardsii	55.5	452	44.5	340	−

pENW, pyroglutamyl-asparagyl-trytophan; pEQW, pyroglutamyl-glutamyl-tryptophan; % peptide based on integrated peak area; μM peptide based on standards (see Section 2); '+' indicates presence of unidentified peptides/peaks; '−' indicates absent from venom; (+), minor component.

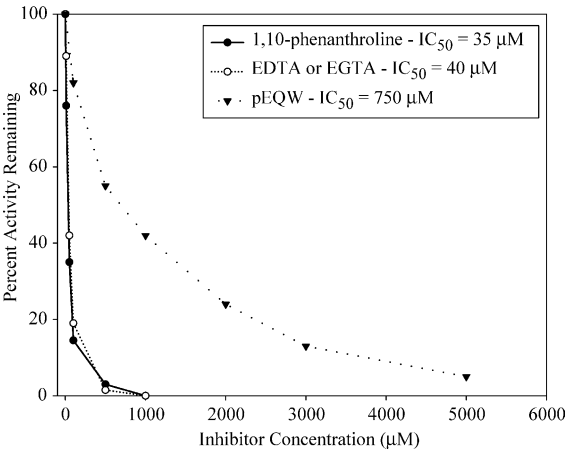

Fig. 3. Effects of metal chelators or synthetic pEQW on Cvo Pr V, the major metalloprotease from *C. o. oreganus* venom. pEQW shows relatively low affinity for this protease.

When compared with known inhibitors of metalloproteases (metal chelators), synthetic pEQW showed approximately 20-fold lower inhibition efficacy ($IC_{50}=750$ μM) for purified Cvo Pr V than did either EDTA or EGTA ($IC_{50}=40$ μM) or 1,10-phenanthroline ($IC_{50}=35$ μM) (Fig. 3). At inhibitor concentrations above 1.0 mM, EDTA or EGTA produced complete inhibition, and pEQW gradually inhibited activity; at the highest concentration used (5.0 mM), protease activity approached zero. When cromipyrrhin was subjected to the same metal chelators and pEQW (Fig. 4), inhibition of metalloprotease activity followed a pattern similar to that of Cvo Pr V with EDTA ($IC_{50}=50$ μM), but

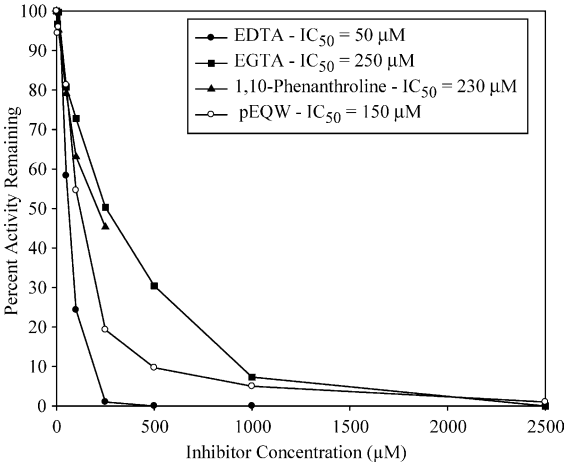

Fig. 4. Effects of metal chelators or synthetic pEQW on cromipyrrhin, the major metalloprotease from *C. m. pyrrhus* venom.

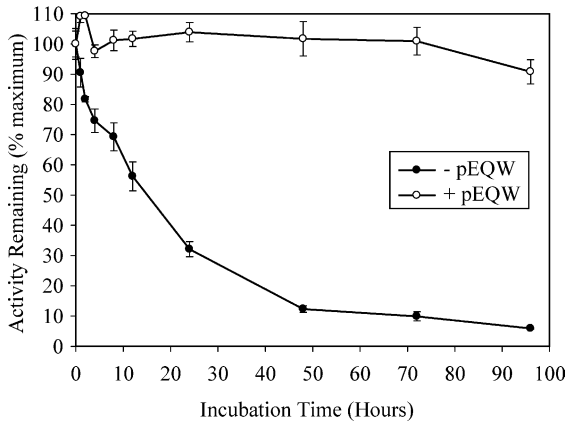

Fig. 5. Synthetic peptide pEQW at 5.0 mM protects cromipyrrhin from autolytic degradation at 37 °C. Points show means of three replicates ±1 standard deviation.

not with synthetic pEQW (150 μM), EGTA (250 μM) or 1,10-phenanthroline (250 μM).

When cromipyrrhin was incubated at 37 °C without 5.0 mM synthetic pEQW, protease activity showed a rapid and exponential decline; protease incubated with synthetic pEQW did not show any decline in activity until 96 h of incubation (Fig. 5). When time course aliquots were run on 14% acrylamide gels, partial loss of the 53 kD metalloprotease was apparent by ~8 h incubation (no synthetic pEQW present), and by 48 h, the protein was completely degraded (Fig. 6). Major degradation products appeared at approximately 34, 29, 7 and 4.5 kD; these were apparently stable. Minor degradation products appeared at ~48 and 45 kD, and they were completely degraded by 72 h. When incubated at 37 °C with synthetic pEQW, no loss of the 53 kD protease was noted for up to 72 h. It should be noted that purified cromipyrrhin and Cvo Pr V are unstable when stored without inhibitor (prior to stabilization experiments), hence the minor degradation bands in lanes containing synthetic pEQW.

4. Discussion

It is likely that a model for stabilization of proteins for long periods could exist in natural systems, particularly among viperid snakes whose venoms are rich in lytic enzymes. Previously, we showed that undefined venom inhibitors/stabilizers protected *Crotalus m. molossus* venom components against autolytic and heat/freezing-induced degradation (from −80 to +37 °C) for up to one week in vitro (Munekiyo and Mackessy, 1998). Other studies have demonstrated that endogenous peptide inhibitors occur at low millimolar concentrations in the venoms of *Crotalus atrox* (Robeva et al., 1991), *Bothrops asper* (Francis and Kaiser, 1993: 1.0 mM pENW and 4.5 mM pEQW) and *Trimeresurus mucrosquamatus* venom (Huang et al., 1998; 7.3 mM pENW and 5.4 mM pEQW), and it appeared likely that these peptides could act as stabilizers of metalloproteases.

In the present study, pENW was found in venoms of 10 taxa of two genera of rattlesnakes and pEQW was found in 9 taxa, suggesting that these endogenous peptides are widespread among rattlesnake venoms and likely viperid venoms generally. Additional peptides/280 nm-absorbing material in some venoms were not identified in this study, but they may be additional small peptide inhibitors such as pEKW (c.f. Huang et al., 1998). Approximate concentrations of pENW (0.23–1.74 mM) and pEQW (0.089–0.69 mM) were not as high as observed for *B. asper* and *T. mucrosquamatus* venoms. As commonly observed among viperids

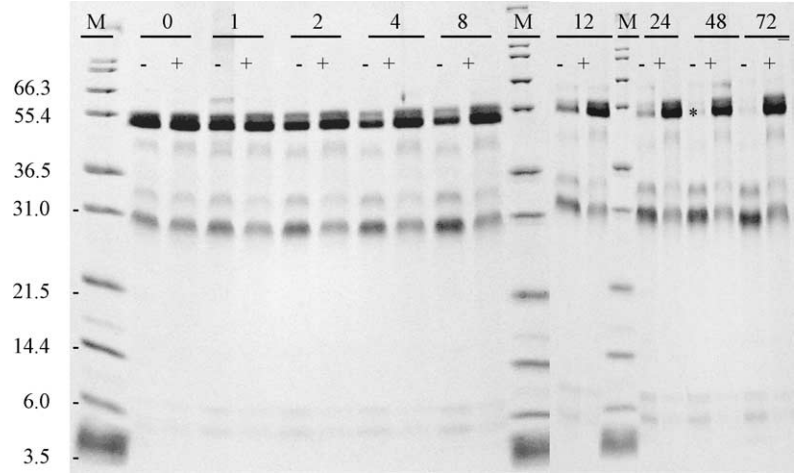

Fig. 6. Electrophoretic evidence that synthetic peptide pEQW protects cromipyrrhin from autolysis at 37 °C. Note that by 48 h incubation, in the absence of pEQW the native 53 kD protease band is completely lost. *M*, molecular mass standards (in kD); 0–72 h, incubation; –, pEQW not present during incubation; +, 5.0 mM pEQW present during incubation.

(e.g. Bjarnason and Fox, 1994), venoms from *C. ruber* (Mackessy, 1985), *C. o. oreganus* (Mackessy, 1996) and *C. molossus* (Munekiyo and Mackessy, 1998) also contain at least four different metalloproteases. It is likely that small peptide inhibitors of metalloproteases are a common means by which these characteristic and abundant enzymes are stored inactive within the venom glands of viperid snakes. It may be of functional significance that the venom lacking pEQW (*C. scutulatus*) and the venom with very low tripeptide concentrations (*C. o. concolor*) are much more toxic (LD_{50} <0.75 μg/g; Bieber et al., 1975; Mackessy et al., 2003) than most of the other venoms, and metalloprotease activity of both *C. o. concolor* and C. *scutulatus* (type A) venoms is extremely low (Mackessy, 1988; Mackessy et al., 2003). High metalloprotease activity and high toxicity of venoms appear to be incompatible qualities of many venoms generally (Mackessy et al., 2003), a quality perhaps also consistent with distribution of particular peptide inhibitors in venoms.

When compared with known inhibitors of metalloproteases (metal chelators), pEQW inhibited the metalloprotease activities of cromipyrrhin and Cvo Pr V with generally lower efficacy. In both cases, results showed that the peptides inhibited most metalloprotease activity at low millimolar concentrations but did not completely inhibit activity at the highest concentrations used, whereas the metal chelators completely inhibited activity at lower concentrations. However, pEQW appeared to be a more potent inhibitor of cromipyrrhin ($IC_{50}=150$ μM) than of Cvo Pr V ($IC_{50}=750$ μM). The metalloprotease inhibitors EGTA and 1,10-phenathroline appeared to be less effective inhibitors of cromipyrrhin, while EDTA inhibition was similar for both enzymes. Results from cromipyrrhin and Cvo Pr V inhibition suggested that pEQW has a low affinity for and is a relatively weak inhibitor of these metalloproteases. This combination of low affinity and high concentration provides an efficient mechanism for inactivation within the gland and rapid activation in tissues of prey upon envenomation (c.f. Gomis-Rüth et al., 1998).

Following incubation with synthetic pEQW, cromipyrrhin retained significant activity ($>90\%$) even after 96 h of incubation at 37 °C. However, cromipyrrhin without pEQW showed an exponential loss in activity within the first few of hours of incubation, with less than 10% of original activity remaining by 50 h of incubation. This indicates that pEQW provided some protective mechanism that preserved biological activity even under conditions of extended heating. These results are consistent with and help explain earlier observations of stability of heated crude venoms (Ownby et al., 1994; Munekiyo and Mackessy, 1998).

Electrophoretic analysis showed similar results: in the absence of pEQW, cromipyrrhin degraded over time. The 53 kD protease band showed evidence of degradation after 1 h of incubation, and by $\sim$48 h, the band is completely lost. A 33 kD fragment appeared as a minor degradation product, while a 31.0 kD fragment intensified with incubation time. Two minor bands at 5 and 8 kD also tended to increase in intensity as incubation proceeded. The enzymatic and electrophoretic data show that pEQW provided protection against autolytic degradation of cromipyrrhin when incubated at 37 °C for up to 96 h, and protein stability is enhanced as well.

Results presented here indicate that natural products are a fruitful source of protein-stabilizing compounds. The tripeptides pEQW and pENW appear to be widespread among venoms as low affinity/high concentration inhibitors of venom metalloproteases and perhaps other enzymes. Together with other mechanisms which inhibit venom enzymes (low pH, high citrate levels, cysteine switch activation), the peptide inhibitors stabilize and inhibit autolytic proteins, thereby protecting venom integrity during long-term storage and protecting the snake against potential damage by its own venom components. Recently, the crystal structure of the metalloprotease TM-3 (isolated from *Trimeresurus mucrosquamatus* venom) complexed with these tripeptides was determined (Huang et al., 2002). Crystallographic analysis demonstrated that the tripeptides bind in the active site of the protease, and the tryptophan residue in the P^{-1} site confers specificity of these inhibitors. It appears likely that inhibition and stabilization of Cvo Pr V and cromipyrrhin occurs via a similar mechanism of active site occupation by the peptides. A thorough physical investigation of these peptides and their interaction with venom proteins may assist design of low affinity (and nontoxic) inhibitors/stabilizers for protein drugs which will extend their shelf life and/or allow for storage under less stringently controlled conditions.

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