
Purification of Metalloprotease Inhibitors That Neutralize Snake Venom Toxins in California Ground Squirrel Blood

Cheuk Yee L. Ho

Introduction

In snake venoms that cause extensive hemorrhage, such as those of rattlesnakes and other vipers and pitvipers, the toxins responsible for hemorrhage have been identified as snake venom metalloproteases (SVMPs). These enzymatic toxins cause localized hemorrhage, either through damage to endothelial cells (Ownby et al. 1978; Ownby and Geren, 1987) or through gaps produced between endothelial cells as a result of damage to the basement membrane of blood vessels (Ohsaka, 1979; Markland, 1998). Hemorrhage produced by SVMPs can subsequently lead to edema, shock, tissue necrosis, and reduced ability to regenerate muscle tissue (Gutiérrez and Rucavado, 2000). Additionally, leakage of blood from affected vessels also helps spread other venom toxins to their target tissues. Because of these effects, SVMPs have been proposed as an important target for new therapeutic approaches aimed at reducing human morbidity caused by snakebite.

Snake venom metalloprotease inhibitors (SVMPs) are being investigated as a potential complement to current antibody-based treatments (Pérez and Sánchez, 1999; Gutiérrez and Rucavado, 2000). Natural SVMPs have been isolated from several vipers and pitvipers, including *Crotalus atrox* (Weissenberg et al., 1991, 1992), as well as a selection of non-venomous snakes and other animals. Over a dozen antihemorrhagic factors have been isolated from the blood sera, plasma, or muscle tissue of mammals. Several reviews of this work have been published (Domont et al., 1991; Thwin and Gopalakrishnakone, 1998; Pérez and Sánchez, 1999; Perales and Domont, 2002), with an increasing emphasis on the biochemical and pharmacological properties of resistance factors. All of the proteins described to date protect mammals through their activity as SVMPs, inhibiting hemorrhage induced by both whole venoms and purified venom hemorrhagic toxins. Almost all of these proteins are small and slightly acidic, and present as circulating factors in the bloodstream (Pérez and Sanchez, 1999). In addition, proteins for which sufficient sequence information is available appear to belong to the *Ig Supergene* family of the innate immune system. Presumably because venom toxins, if they are to be effective, must be neutralized almost immediately after injection by a predator, these proteins are innately expressed in resistant mammals and do not seem to be inducible with previous exposure to venom (Perales and Domont, 2002).

However, SVMPIs are not homogeneous in structure and show a variety of structural themes and mechanisms of inhibition. For example, oprin, isolated from the Virginia opossum (*Didelphis virginiana*), is homologous to mammalian α_1 -antitrypsin (Catanesi and Kress, 1993) and is thought to work by irreversibly binding to the active site of target SVMP. In contrast, the antihemorrhagic factor from the European hedgehog (*E. europaeus*) is homologous to β_2 -macroglobulin (de Wit and Weström, 1987a, b), and is thought to act as a molecular cage for SVMP, preventing venom toxins from accessing target tissues. Erinacin, a 1090 kD protein isolated from *E. europaeus* muscle tissue, appears to be a member of the ficolin/opsonin P35 family of lectins (Omori-Satoh et al., 2000). The sugar-binding lectins within this protein family bind specifically to carbohydrate structures on the surface of a pathogen and recruit other cells and molecules to destroy the pathogen (Holmskov, 2000).

One important conclusion emerging from this growing literature on the molecular processes involved in venom neutralization in mammals is that the precursors to resistance factors are likely to be widespread. Because most mammal species are preyed upon by pitvipers, vipers, or other venomous species with hemorrhagic toxins that have not yet been identified, it is likely that there are many other SVMPIs that are as yet undiscovered. A second important conclusion is that prey species have capitalized on precursors in different ways: although inhibition of SVMPs appears to be a critical part of resistance, it has been achieved through a variety of molecular mechanisms. Given this variety, it is likely that there has been a kind of natural experimentation by rattlesnakes and their prey across generations, resulting in a wealth of unexplored data on the molecular basis of venom toxicity and resistance.

One species of mammal that is likely to contain SVMPIs is the California ground squirrel (*Spermophilus beecheyi*). These squirrels are an important food resource for rattlesnakes throughout California (Fitch, 1948; Linsdale, 1946). Fitch (1949) estimated that *S. beecheyi* account for up to 69% (by weight) of the diet of adult northern Pacific rattlesnakes. Accordingly, rattlesnakes are an important threat to ground squirrels. Field observations reveal that northern Pacific rattlesnakes are common near burrow systems during the squirrels' reproductive season and periods of pup emergence, and Fitch (1949) estimated that rattlesnakes are responsible for approximately 40% of pup and juvenile mortality in ground squirrel colonies.

It is not surprising, therefore, that California ground squirrels employ a variety of defenses against rattlesnake predators. In addition to vigilance, these squirrels will aggressively confront snake predators detected near their burrows. Prolonged staring, conspicuous tail flagging, substrate throwing, and biting are used to discourage hunting snakes and to provoke defensive displays such as hissing or rattling that help squirrels assess the danger posed by snake predators (Owings and Coss, 1977; Coss and Owings, 1989; Rowe and Owings, 1990, 1996; Swaisgood et al., 1999). This kind of active harassment, which requires a close approach to the rattlesnake, exposes squirrels to the risk of envenomation.

Some California ground squirrels possess an innate ability to neutralize venom hemorrhagic activity as a buffer against this risk. However, the ability to withstand envenomation varies among populations (Poran et al., 1987; Coss et al., 1993). Recent studies of the functional basis of venom resistance show that blood serum from California ground squirrels can reduce the hemolytic activity of rattlesnake venom, as well as the activity of venom metalloproteases and other enzymatic toxins (Biardi, 2000; Biardi et al., 2000).

The goal of this research project is to purify and characterize the plasma proteins responsible for ground squirrel venom resistance. Because ground squirrel blood plasma is effective at reducing metalloprotease activity, it seeks to identify the presence and identity of SVMPIs in this species, with the goal of determining whether they belong to the same *Ig Supergene* family of proteins as other SVMPIs already identified in mammals.

Materials and Methods

BLOOD SAMPLING AND STORAGE

Squirrels from locations in central California where rattlesnakes are common were live-trapped and anesthetized with Ketamine-HCl (50 mg/kg). Approximately 3 ml of whole blood was collected via cardiac puncture and stored in additive-free Vacutainer blood collection vials (Becton Dickinson, Irvine, CA). Samples were kept at 4°C overnight, and then clots were removed and discarded. The supernatant was centrifuged at 3000 g for 10 minutes at 4°C to separate out any remaining erythrocytes. Sera were stored at -70°C until used.

VENOM SOURCE AND PREPARATION

Lyophilized venom from the northern Pacific rattlesnake (*C. v. oreganus*) was purchased from Sigma Chemical Co., St. Louis, MO. Venom was then rehydrated in a 0.05 M Tris-HCl, 0.005 M calcium chloride, 0.05% Brij, pH 7.6 buffer and stored at -20°C until used.

AFFINITY CHROMATOGRAPHY

HiTrap Blue Affinity column contains a dye, Cibacron Blue F3G-A, which is capable of binding to enzymes that require adenylyl-containing cofactors, albumin, coagulation factors and interferon. Electrostatic interactions, hydrophobic interactions and structural similarity helped to separate and bind the different types of proteins, serum albumin and interferon within the column, which uses the 50mM KH_2PO_4 pH=7 buffer system.

ANION EXCHANGE CHROMATOGRAPHY

As the HiTrap Blue Affinity column does not sufficiently purify individual proteins, the HiTrap Blue fractions were further separated using the Mono-Q HR 5/5 ion exchange column. Ion exchange chromatography separates a sample according to the ions' charge. Mono Q stands for anion exchangers, indicating that the ion exchange column is positively charged and binds to anions. The Mono-Q HR 5/5 ion exchange column uses a 20mM Tris-HCl pH=8 buffer system, with a gradient of 0-40% NaCl. Different proteins precipitate under different salt concentrations: as NaCl concentration increases, proteins bind onto and leave the column at different times, thereby separating them.

ASSAY OF SVMP AND SVMPI ACTIVITY

SVMP activity in *C. o. oreganus* was determined using BODIPY-labelled gelatin (DQ™ gelatin, Molecular Probes, Inc.) as a substrate. Venom (1 µg) was combined with substrate (1 µg) in 200 µl of buffer (0.05 M Tris-HCl + 0.15 M NaCl + 0.5 mM CaCl_2 + 0.2 mM sodium azide, pH 7.6). Using a Perkin Elmer LS-50B Fluorescence Spectrophotometer, activity was measured over a 2-hr. period as fluorescence increased to 535 nm.

Ground squirrel chromatography fractions were screened for SVMPI activity using the same protocol, except that 5 µl of plasma or chromatography fractions were added to the reaction.

DE NOVO SEQUENCING AND IDENTIFICATION OF SDS-PAGE BANDS

Fractions that reduced the activity of *C. o. oreganus* SVMP were run on SDS-PAGE using Novex pre-cast gels (Invitrogen). Bands unique to fractions showing metalloprotease activity were cut from the gel and taken to the UCD Molecular Structure Facility. Bands were digested using a CNBr/trypsin double digest. Fragments were then identified and analyzed by de novo MS sequencing using a Perkin Elmer Sciex QSTAR Hybrid Quadrupole-TOF Mass Spectrometer. Fragment sequences were compared with the 'nr-prot' database using the MS BLAST algorithm (Schevchenko et al., 2001).

Results

Figure 1 shows the chromatogram from the HiTrap Blue column separation step. The HiTrap Blue column separated the protein into two peaks, which were then treated with fluorescence assay and checked for inhibitor activities. Peak 2 was found to contain the inhibitor of interest. Due to the fact that peak 2 is not purified enough to determine the specific inhibitor, it was subjected to another chromatographic purification using the Mono-Q HR 5/5 Ion Exchange column.

Figure 2 shows the chromatogram of the ion exchange step. Through the Mono-Q HR 5/5 ion exchange column, peak 2 from HiTrap Blue fractions are further separated into smaller but more purified fractions. Once again, the fractions were treated with fluorescence assay. Two ion exchange fractions were found to contain the inhibitor of interest, and one of these became the focus. The peak indicated by an asterisk (*) was found to contain the inhibitor of interest.

Table 1 shows the process by which each step further purifies the sample. In this table, it can be observed that as the sample was subjected to the two purification steps, the total amount of protein decreased as proteins that were not responsible for snake venom resistance were removed. However, the specific activities and the purification factors increased through the two-

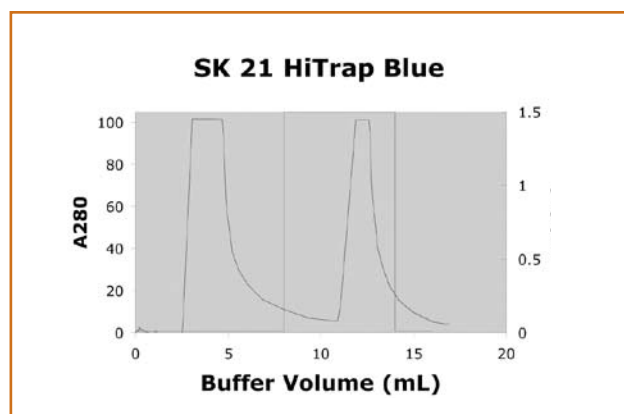


Figure 1. HiTrap Blue Affinity chromatogram for sample

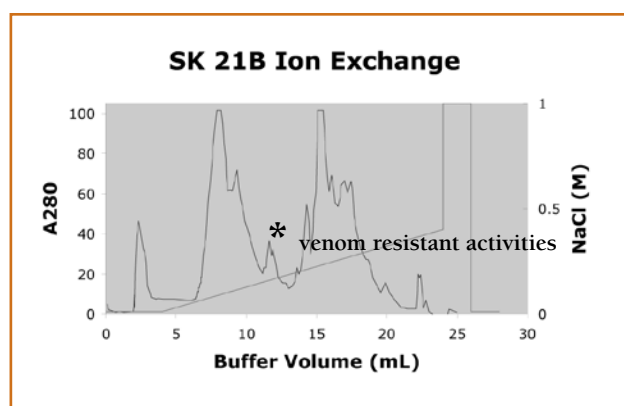


Figure 2. Mono-Q HR 5/5 ion exchange chromatogram for sample SK 21B. (Peak 2 from HiTrap Blue fractions)

Table 1. Summary of purification steps.

	Total protein (mg)	Total inhibitor (units)	Specific Activity (units/mg)	Purification factor	Yield
SK 21 plasma	824.00	2.59	0.27	1.01	100.0%
SK 21 HiTrap pea	20.03	0.92	0.60	2.24	2.4%
SK 21 Mono-Q peak 4	1.33	0.35	2.52	9.34	0.2%
SK 21 Mono-Q peak 5	1.28	0.86	5.34	19.78	0.2%

step purification process, proving that, as the sample went through the HiTrap Blue Affinity column and then the Mono-Q HR 5/5 ion exchange column, the sample became more purified and the inhibitor of interest was easier to isolate.

Figure 3 shows the results of denaturing SDS-PAGE of chromatography fractions from ground squirrel sera that show SVMPI activity. Lane 3 shows the fraction containing the inhibitor, and lane 4 shows an adjacent fraction that does not have activity. Bands unique to lane 3 are possible SVMPI proteins, and were cut out for MS analysis and identification.

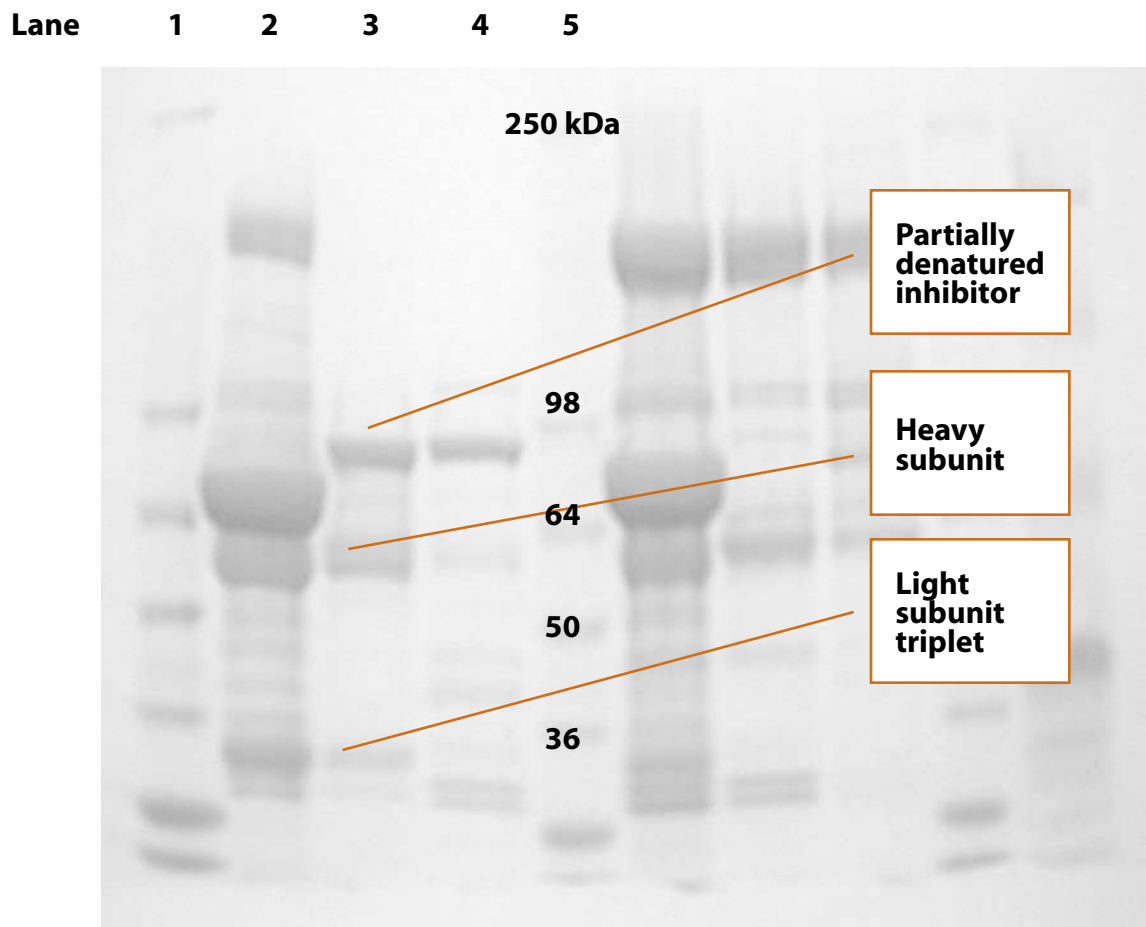


Figure 3. SDS-PAGE gel showing anion exchange chromatography fraction of ground squirrel blood serum containing SVMPI activity. Lanes 1 and 5: MW marker; Lane 2: HiTrap Blue fraction; Lane 3: Mono-Q HR 5/5 fraction containing SVMPI activity. Lane 4: anion exchange fraction missing SVMPI activity. The subunit bands indicated in Lane 4 were cut out and analyzed by de novo MS sequencing.

Table 2 shows the results of the MS BLAST homology searches of the gel bands of fractions containing inhibitors. The subunit bands have sequences that are similar to a known protease inhibitor, inter- α trypsin inhibitor. Mammalian inter- α trypsin inhibitor is a known protease inhibitor but has not been proven to be effective against metalloproteases and/or xenologous proteases (Bost et al., 1997). The plasma proteins HP25 and HP27 from the Siberian chipmunk (*Tamias sibericus*) have collagen-like domains that may make them potential ligands for the active site of venom metalloproteases (Takamatsu et

Table 2. Sequence information on ground squirrel blood proteins identified from SDS- PAGE bands in fractions showing SVMPI activity.

SDS-PAGE Band	Peptide sequence(s)	Database matches	Sequence ID†
High mass	LDYQEGPPG	mammalian	Q1462
	SPEHVVVTR	trypsin inhibitor	inter- α Q9GLY4 P79263
Low mass <i>top</i>	GDKVWLESK	HP27	Q60577
	LSERPPEPFQPIVFK		
<i>middle</i>	PPEPFQPLVFK FSGFLIHEN	HP25	Q60576
<i>bottom</i>	DPTVSLPR GELVDLVPHK NVSQFLFPLR	none	

al., 1993). The failure to match the smallest of the subunits may indicate a lack of protein information for squirrels in the sequence database, or it may reflect a novel subunit tuned to the challenges of venom toxins.

Discussion

When the sample was subjected to the HiTrap Blue Affinity column purification, unwanted materials were washed out at 0% salt gradient. The rest of the proteins—including the one represented by the peak in Fig. 2 (which contained the inhibitor of interest)—were separated out with a 100% salt gradient. However, the protein was not purified enough to identify the specific inhibitor of interest. The HiTrap Blue peak was then subjected to the Mono-Q HR 5/5 ion exchange purification. In this process, the proteins contained in the HiTrap Blue peak 2 were further separated as different proteins bound to the column at different times while the NaCl gradient rose gradually from 0% to 40%. Through the ion exchange column, the sample was separated into 10-plus fractions. After treating all the peaks with fluorescence assay, two peaks were found to contain snake venom resistance activity. Fractions that contained the inhibitor of interest were to be run through a SDS-PAGE gel along with the original sample. The SDS-PAGE gel is usually set up so that the original sample is on the left of the gel and proceeds to the right, following the order of the successive steps of chromatographic purification. The purity of the inhibitor of interest increases through the series of chromatographic purification steps, and thus, its corresponding band on the SDS-PAGE gel should appear darker through the series of fractions. The gel was then imaged and sent over to the Mass Spectrometry Facility to determine the protein gel band that corresponds to the inhibitor of interest.

The result was that California ground squirrels were found to possess blood proteins that work as SVMPIs. Those proteins are also present in the *Ig Supergene* family, but are not the same as SVMPIs from other species of mammals that are resistant to rattlesnake venom. This is the first demonstration that proteins homologous to inter- α trypsin inhibitor can neutralize snake venom metalloproteases. This finding means that prey may respond to rattlesnake predation in different ways. If this is the case, then there may be many other new forms of SVMPI in other species of mammals that are prey to rattlesnakes.

Snake venom metalloprotease inhibitors (SVMPIs) are being investigated as a potential complement to current antibody-based treatments of envenomation (Pérez and Sánchez, 1999; Gutiérrez and

Rucavado, 2000). The purification steps were significant as they helped in isolating the inhibitor of interest from the original blood plasma sample, which contained many proteins with complex structures. Future research into the structure and sequence of the SVMPI might prove important not only for understanding snake venoms but for developing effective therapies for rattlesnake bites as well.

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