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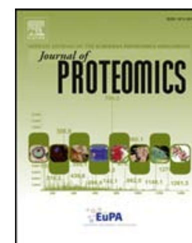

Proteomic comparison of *Hypnale hypnale* (Hump-Nosed Pit-Viper) and *Calloselasma rhodostoma* (Malayan Pit-Viper) venoms

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ABSTRACT

Treatment of *Hypnale hypnale* bites with commercial antivenoms, even those raised against its sister taxon *Calloselasma rhodostoma*, has never been clinically successful. As these two genera have been separated for 20 million years, we tested to see whether significant variations in venom had accumulated during this long period of evolutionary divergence, and thus could be responsible for the failure of antivenom. Proteomic analyses of *C. rhodostoma* and *H. hypnale* venom were performed using 1D and 2D PAGE as well as 2D-DIGE. *C. rhodostoma* venom was diverse containing large amounts of Disintegrin, Kallikrein, L-amino acid oxidase, Lectin, phospholipase A_2 (acidic, basic and neutral) and Snake Venom Metalloprotease. In contrast, while *H. hypnale* also contained a wide range of toxin types, the venom was overwhelmingly dominated by two molecular weight forms of basic PLA_2. 2D-DIGE (2-D Fluorescence Difference Gel Electrophoresis analysis) showed that even when a particular toxin class was shared between the two venoms, there were significant molecular weights or isoelectric point differences. This proteomic difference explains the past treatment failures with *C. rhodostoma* antivenom and highlights the need for a *H. hypnale* specific antivenom.

Biological significance
These results have direct implications for the treatment of envenomed patients in Sri Lanka. The unusual venom profile of *Hypnale hypnale* underscores the biodiscovery potential of novel snake venoms.

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1. Introduction

The Hump-Nosed Pit-Viper (*Hypnale hypnale*) inhabits Sri Lanka and southern India and is classified by the World Health Organisation (WHO) as a snake of the highest medical importance in Southern Asia [1]. Contact between humans and *H. hypnale* is common due to its abundance and tolerance of disturbed habitat in both wet and dry deciduous zones. Its small

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size (rarely exceeding 0.5 m) and effective camouflage make the snake difficult to detect, and bites most often result from victims accidentally stepping on coming into contact with an unseen snake [2]. *H. hypnale* has been estimated to be responsible for 35% of venomous snake bites in Sri Lanka and envenomation causes local necrosis, pain and haemorrhagic blisters in most patients [3]. While *H. hypnale* bites are rarely lethal (fatality rate 1.8%), up to 40% of patients experience systemic effects including haemostatic dysfunction, thrombocytopenia, spontaneous haemorrhage and acute kidney injury [2,3]. These effects have been replicated in mouse models, in which it has been demonstrated that *H. hypnale* venom has procoagulant, fibrinolytic, oedema-triggering and platelet aggregation activities [4,5]. Recently, thrombotic microangiopathy has also been described in a number of *H. hypnale* bites, which may contribute to tissue damage at the bite site and the development of renal complications [6].

Despite the medical importance of this snake, effective venom neutralisation has not been achieved in humans. Treatment with Bharat polyvalent antivenom (ASVS) and Haffkine polyvalent antivenom, both raised against the "big four" Asian venomous snakes (*Naja naja, Bungarus caeruleus, Daboia russelii* and *Echis carinatus*), has been found to be completely ineffective against *H. hypnale* envenomation both in rodent assays and clinical envenomations [7]. Mitochondrial DNA analysis suggests that *H. hypnale* and the Malayan Pit-Viper *Calloselasma rhodostoma* form a phylogenetic clade, although they have been diverging from one another for approximately 20 million years [8]. As their envenomations show similar clinical features, the efficacy of Thai Red Cross Malayan Pit Viper (MPV) monovalent antivenom in neutralising *H. hypnale* venom has also been investigated. There is controversy in the literature regarding the effectiveness of MPV in supressing the haemorrhagic, procoagulant and necrotic activies of *H. hypnale* venom in rodent models [9], and it has never been successful in a clinical setting [2]. The Hemato polyvalent antivenom (HPA) produced against *C. rhodostoma* and two other haemotoxic Thai snakes, *Cryptelytrops albolabris* and *Dixonius siamensis*, has been demonstrated to abrogate the lethality of *H. hypnale* venom in rats, but has not yet been clinically trialled [9].

Both MPV and HPA are substantially more effective in neutralising *C. rhodostoma* venom than that of *H. hypnale*, suggesting that some toxins present in the latter venom may not be neutralised [9]. However, immunological profiling of *H. hypnale* venom using indirect ELISA with antisera revealed 90% cross-reactivity with *C. rhodostoma* venom [10]. *C. rhodostoma* venom toxins have been comprehensively described and include the metalloproteases kistomin and rhodostoxin [11]; multiple phospholipase A_2 isoforms (PLA_2) [12]; L-amino oxidases [13]; C-type lectins [14] and serine proteases such as Ancrod, which is being trialled as a clinical anticoagulant (under the brand name Viprinex) [15]. These toxins act in concert to produce haemostatic dysfunction: metalloproteases and serine proteases cleave or inhibit fibrinogen, while enzymatic PLA_2 isoforms and C-type lectins inhibit platelet aggregation. Non-enzymatic PLA_2 isoforms have been shown to be myotoxic and to cause oedema in vivo [5]. L-amino oxidases trigger deamination cascades that produce hydrogen peroxide, leading to a variety of clinical sequelae including haemorrhage, haemolysis, oedema and apoptosis of vascular endothelial cells [13]. An enzymatic comparison with *C. rhodostoma* venom found that *H. hypnale* venom had similar protease, L-amino acid oxidase, thrombin-like enzyme and hemorrhagin activities but much higher levels of PLA_2 activity [9], consistent with the fact that PLA_2 isoforms are readily purified from the venom [5].

Gaining a better understanding of the venom composition of *H. hypnale* may be invaluable in the development of more effective treatments for envenomation in addition to providing insight into divergent venom evolution subsequent to geographical separation and speciation of snakes as well as highlighting potential regional variations in *H. hypnale* venom. Additionally, as snake venoms are a rich source of pharmacologically active proteins and peptides, the comprehensive characterisation of venom proteomes is an important avenue of biodiscovery [16]. We have previously shown that comparative 2D-PAGE gel with in-gel protein digestion followed by MS/MS sequencing is an effective way to compare snake venom profiles [17]. In this paper we compare the proteomic profiles of *C. rhodostoma* and *H. hypnale*, providing insight into compositional differences predicted by previous paraspecific antivenom cross-neutralisation studies.

2. Materials and methods

2.1. Venom samples

Venom samples were obtained from a single captive bred specimen each of *H. hypnale* from Sri Lanka and *C. rhodostoma* from Malaysia. After milking by BGF, samples were immediately placed in liquid nitrogen until being lyophilised and stored at −70 °C.

1D gel, 2D gel, Shotgun sequencing, LC-MS/MS conditions were as previously described by us [17].

2.2. Differential In-Gel Analysis (2D DIGE)

For DIGE Cy-Dye labelling, venom samples were first cleaned and quantified by 2-D Clean-up and 2-D-Quant Kits (GE Healthcare) respectively. 50 μg of the two venoms were labelled with 1 μl of 400 pmoles/μl of Cy3 and Cy5 dyes at pH8.5 for 30 min on ice, while 25 μg of each pooled venom were labelled with Cy2 dye as an internal standard according to the manufacturer's instructions (GE Healthcare). Labelling reactions were quenched by the addition of 1 μl of 10 mM lysine for 10 min (Sigma, USA). All reaction mixtures were combined and loaded onto 24 cm, pH 3–11 non-linear IPG strips and run as described under "2D Gels" above. After electrophoresis, gels were scanned on a Typhoon™ laser scanner (GE Healthcare) for three dyes and the resultant images were overlaid and analysed using the Progenesis SameSpots software (Nonlinear Dynamics).

3. Results and discussion

In this study we undertook the first detailed proteomic characterisation of *H. hypnale* venom and provided a comparison with the sister-taxon *C. rhodostoma* venom using 1D and

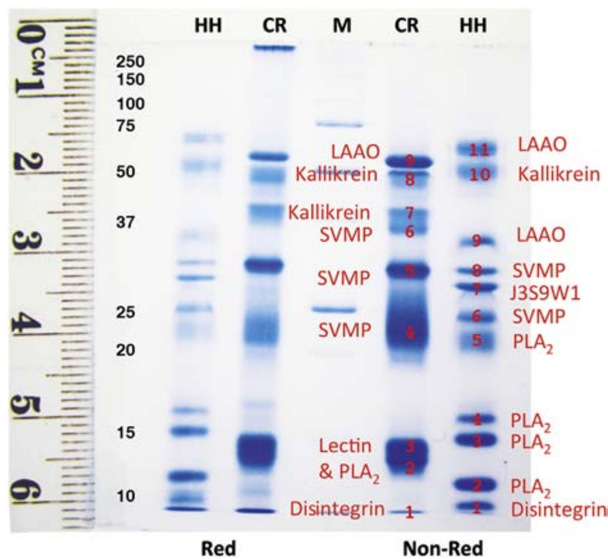

Fig. 1 – 12% SDS-PAGE analysis of *Calloselasma rhodostoma* (CR) and *Hypnale hypnale* (HH) venoms carried out under dissociating (reducing, R) and dissociating and denaturing (non-reducing, NR) conditions. Gel was stain in 0.2% colloidal coomassie brilliant blue G250. M = molecular mass standard markers.

2D PAGE, as well as DIGE. The combined proteomics approaches revealed a diverse composition of venom components (Figs. 1–4, summarised in Table 1, full-details in Supplementary Tables 1–6). Horizontal trains of spots were common on the 2D gels from these species, indicating extensive isoform and glycoform variation. All three techniques revealed considerable differences between the two venoms in terms of protein composition and expression levels.

C. rhodostoma venom was particularly diverse containing disintegrin, kallikrein, L-amino acid oxidase, Lectin, phospholipase A_2 (acidic, basic and neutral) and Snake Venom Metalloprotease. The comparison of our *C. rhodostoma* sample with previously annotated gels of *C. rhodostoma* [18–21], *Crotalus durissus terrificus* [22] and *Daboia. russelii siamensis* [20,23] venoms showed a generally similar patterns of basic PLA_2, C-type lectins, serine proteases and metalloproteases but with significant differences in the specific relative composition. This is congruent with previous work demonstrating that *C. rhodostoma* venom shows significant differences between populations [24].

In contrast, *H. hypnale* venom was dominated by PLA_2, with two molecular weight isoforms of basic PLA_2 being particularly abundant. The low expression levels of high molecular weight proteins in the *H. hypnale* sample were unexpected as previous studies on the enzymatic activity of *H. hypnale* venom had reported comparable protease, thrombin-like enzyme, hemorrhagin and hyaluronidase activities to *C. rhodostoma* venom [25]. While there is no evidence yet of individual or

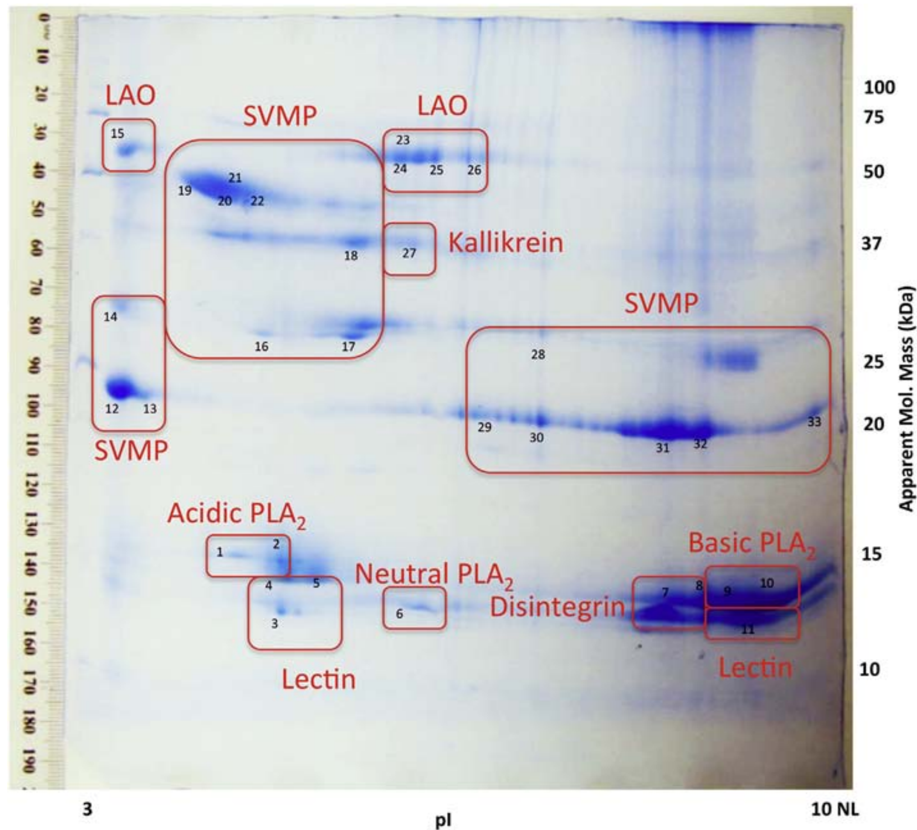

Fig 2 – 2-dimension gel of the *Calloselasma rhodostoma* crude venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown.

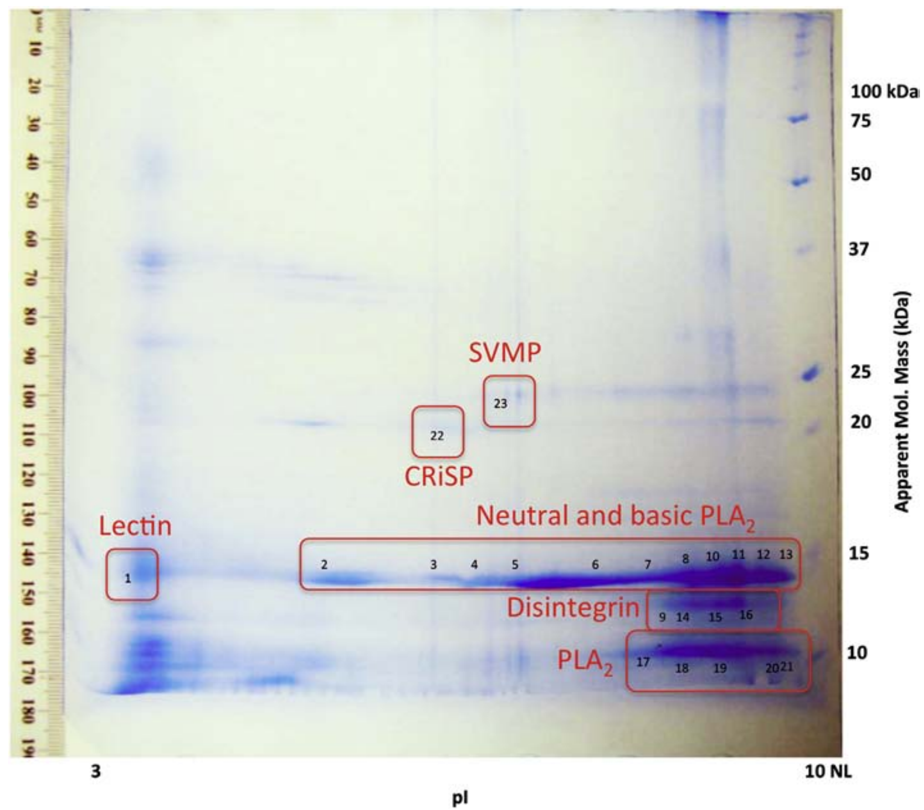

Fig. 3 – 2-dimension gel of the *Hypnale hypnale* crude venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown. The marked spots were analysed by MS2 and the positive results are summarised in Supplementary Table 4.

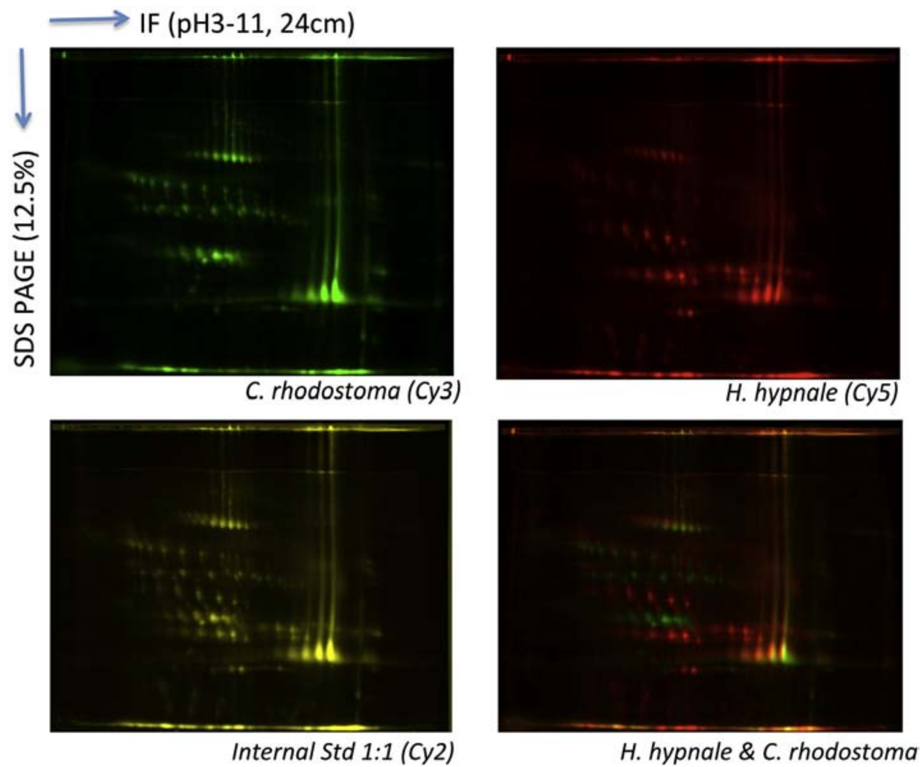

Fig. 4 – 2D-DIGE comparison of *Calloselasma rhodostoma* and *Hypnale hypnale* venoms.

Table 1 – Homologues of toxin types identified in *Calloselasma* and *Hypnale* venoms.

Toxin type	UniProt accession of representative	Characterised bioactivities
Disintegrin-domain of SVMP	P0cb14	Anticoagulant
Type II phospholipase A2	Q9PVF4; Q9PVF0	Anticoagulant, oedema-inducing and myotoxic
Lectin	Q9PSM4 (homomeric); P81398 (heteromeric)	Anticoagulant
Kallikrein type S1 protease	Q91053	Fibrinolytic and also release of kinin from kininogen
SVMP	O57413	Nectrotic and also anticoagulant
LAAO	P81382	Apoptotic and anticoagulant

SVMP = snake venom metalloprotease; LAAO = L-amino acid oxidase.

geographic variation in *H. hypnale* venom, as noted above significant sexual and regional differences have been described in *C. rhodostoma* venom. As intraspecific variation in venom toxins is clinically significant, future studies exploring this aspect of *H. hypnale* venom are needed prior to any production of a *H. hypnale* specific antivenom.

DIGE revealed that even where protein types were shared between the two venoms, they existed in subtly different isoforms (with variations in both molecular weight and charge) between the two venoms, which could have considerable effects on antigenicity. This latter result demonstrates the value of DIGE analysis, the sensitivity of which uncovers variations in venom profiles which may be too subtle to be detected by standard SDS-PAGE techniques. The dominance of *H. hypnale* venom by PLA_2 isoforms obscured the presence of other toxins. 1D gel showed that these toxins are indeed present, but at very low quantities. The sensitivity of DIGE allowed for more accurate relative qualitation. Qualitatively higher expressions of serine proteases, metalloproteases with disintegrin domains and PLA_2, along with L-amino oxidases were observed in the *C. rhodostoma* sample, while *H. hypnale* venom was clearly dominated by PLA_2.

The regional variation of *C. rhodostoma* venom not only has significant implications for the relative neutralisation of *C. rhodostoma* antivenom for treatment of envenomation of different populations of this species, but also for any potential cross-reactivity with *H. hypnale* venom. Such extreme variation of *C. rhodostoma* venom is likely responsible for the conflicting reports regarding the relative usefulness of different *C. rhodostoma* antivenoms in neutralising *H. hypnale* envenomations [2,9]. Compounding this are suggestions by our results that the venom of *H. hypnale* may also vary significantly across its range.

In addition to regional variation within *C. rhodostoma* venoms leading to different antivenom stoichiometry, whereby antivenoms raised against different populations will have differential composition in regards to antibodies targeting specific toxin types, the differential toxin expression between *C. rhodostoma* and *H. hypnale* also contributes to described failures of *C. rhodostoma* antivenom in neutralising *H. hypnale* venom [2]. As antibodies are more readily generated against large antigens [26], *C. rhodostoma* antivenom is expected to contain disproportionately more antibodies against medium to large proteins in *C. rhodostoma* venom. Conspicuously such large enzymatic toxins are only in trace expression levels in *H. hypnale* venom. Instead, *H. hypnale* venom is dominated by PLA_2 isoforms that differ significantly from those found in *C. rhodostoma* venom. It is perhaps unsurprising, therefore, that this antivenom is less successful in neutralising the toxic effects of *H. hypnale* venom, particularly those resulting from phospholipase activity such as platelet aggregation inhibition and possibly myotoxicity. This finding has real medical significance and provides a compelling argument for the development of a *H. hypnale* specific antivenom.

Our proteomics results show that *H. hypnale* differs significantly in toxin composition and expression levels from that of its sister taxon, *C. rhodostoma*. Our studies showed that while both venoms contained PLA_2, and that this toxin type dominates *H. hypnale* venom, the *H. hypnale* forms were either higher or lower molecular weight than the *C. rhodostoma* type, with these size variations having implications for antigenic recognition and also potentially indicating differential bioactivity. Such differences may account for the previous failures of Thai Red Cross MPV monovalent antivenom to neutralise *H. hypnale* venom in a clinical setting. While the presence of many high molecular mass enzymes in the *C. rhodostoma* venom sample supports previous findings, our results bore little visual resemblance to previously published 2D studies and our results also suggest that *H. hypnale* venom may also vary considerably across its range. As the distribution of *H. hypnale* ranges from Sri Lanka to southern India and across a wide range of habitats, intraspecific variation of *H. hypnale* venom may be clinically relevant and is an important avenue of future research.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.07.020.

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