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The Role of Conventional Two-Dimensional Electrophoresis (2DE) and Its Newer Applications in the Study of Snake Venoms

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1. Introduction

The objective of this chapter is to provide an overview of the different approaches that have been undertaken in our laboratory and by other researchers to investigate the different aspects of snake venoms using two-dimensional electrophoresis (2DE). It will also highlight the few novel modifications that we have employed to improve the protocol of 2DE, in order to further increase its versatility as a research tool in the study of snake venoms.

2. The utilization of proteomics to characterize snake venoms

The biological and pathological activities of snake venoms are associated with proteins and peptides in the venoms. These venom constituents are often conveniently classified as either neurotoxic or hemotoxic (Calvete *et al.*, 2009). The venoms of the Elapidae and Viperidae families are among the most thoroughly investigated. The main constituents of the Elapidae venoms are the neurotoxic proteins with lower molecular weights. On the other hand, the main constituents of the Viperidae venoms are the hemotoxic proteins with higher molecular weights. Nevertheless, this classification is not mutually exclusive, since in certain venoms, such as the Elapidae *Ophiophagus hannah*, the main constituents are the higher molecular weight enzymes, which are typically more characteristic of Viperidae venoms (Tan & Saifuddin, 1989). Apart from this widely accepted classification of neurotoxins and hemotoxins, the other aspect in the diversity of venom proteins includes the relative abundances of each protein family. High abundance proteins are important in generic killing and are generally the primary targets of immunotherapy while low abundance proteins are considered to be more important in evolutionary studies (Calvete *et al.*, 2009). Understanding the differences in venom proteins abundances is important as it also has an influence on the method that is required to study these proteins with different abundances in different venoms.

In the early studies of snake venoms, in order to dissect and to analyze the complexity of snake venom constituents, the typical workflow employed has been to isolate and subsequently characterize the biochemical characteristics of individual venom proteins

(Bougis *et al.*, 1986; Graham *et al.*, 2008; Ownby & Colberg, 1987; Tan & Saifuddin, 1989). For example, the crude venom of *O. hannah* was fractionated by Sephadex G75 gel filtration chromatography and DEAE-Sephacel ion-exchange chromatography and the biological properties of the individual chromatography fractions were subsequently determined by utilizing various biochemical assays (Tan & Saifuddin, 1989). The objectives of the study were to investigate the presence of toxic components in the *O. hannah* venom and to provide information for further investigations of the biochemistry and toxicology of *O. hannah* venom. Graham *et al.* (2008) analyzed 30 venoms from the Elapidae and Viperidae families by G50 gel filtration chromatography and following comparison of the chromatography profiles, definitive patterns that could be used for preliminary analyses of venom components were established.

However, the comparison of elution profiles was limited by the less-than-optimum resolution of peaks, especially those containing venom components that were present at higher amount in the venom, resulting in broader peaks within the chromatography profiles, masking the presence of other components (Chippaux *et al.*, 1991). Biochemical analysis and characterization also did not allow for the differentiation and comparison of venom constituents in terms of protein structure (Chippaux *et al.*, 1991). Nevertheless, with the development and refinement of chromatographic techniques that allow for further detailed analyses of fraction components, such strategy of isolation and characterization of venom constituents remains the mainstay of toxinology (Graham *et al.*, 2008).

Notwithstanding the few limitations of 2DE, its recent revitalization and its utilization as part of the workflow to analyze venom complexity has encouraged a new direction in venom studies that uses a more global approach in visualizing venom complexity (Fox & Serrano, 2008). Separating proteins based on two independent parameters – pI value by isoelectric focusing (IEF) in the first dimension and molecular weight by SDS-PAGE in the second dimension – 2DE is able to resolve venom proteins into a few thousand individual spots, producing a specific profile for each venom analyzed via 2DE (Carrette *et al.*, 2006). The different 2DE profiles of venoms will then be used for comparison and this concept of between-gel comparison, or comparative proteomics, has largely been put into a few different practical applications of snake venom study.

2.1 Venom variation

Venom variation is one of the very important aspects in the study of snake venom. Snake venom variation is essential to both basic venom research and the management of snake envenomation (Fox & Serrano, 2008). During the selection of a snake donor for crude venom that is to be used for research purposes, it is essential that the chosen venoms are rich in the components of study interests (Chippaux *et al.*, 1991). Therapeutically, the knowledge of venom variations at all levels, including inter-species and intra-species variations, would aid in the decision of an appropriate antivenom and allow for more effective treatment of envenomation victims (Chippaux *et al.*, 1991). Subsequently, the production of antivenom is also reliant on the knowledge of venom variations.

Within our laboratory, we have attempted to develop a 2DE-based approach to investigate the variations among the venoms of eight Malaysian snakes (Vejayan *et al.*, 2010). Even though there were venom proteins distributed throughout the entire 2DE profiles, as expected with such a complex sample, a closer examination revealed that each venom profile had its own distinguishing features. For instance, each of the three Crotalinae venoms (*Trimeresurus sumatranus*, *Tropidolaemus wagleri*, *Calloselasma rhodostoma*) had profiles

with heavy spotting of proteins at the molecular weight range 15-37kDa. The Elapidae venoms, on the other hand, had profiles with protein spots at the molecular weight range of 15-20kDa (Fig.1). These results clearly elicited the differences of the patterns between the Elapidae and Viperidae venoms that have been so well documented in other literatures discussed above, thus proving the feasibility of 2DE as an ancillary taxonomic tool (Calvete *et al.*, 2009; Nawarak *et al.*, 2003). Apart from inter-family difference in venom compositions, the 2DE analysis of the eight Malaysian snake venoms also demonstrated the obvious pattern of train of spots due to post-translational modifications in venom proteins (Fig. 1). Similarly, Guercio *et al* (2006) performed 2DE analysis on crude *Bothrops atrox* venoms obtained from three different stages of maturation - juveniles, sub-adults and adults . The 2DE profiles obtained demonstrated the alteration that occurred in the proteome composition of snake venoms following progression in developmental stages. Subsequently, the group identified new groups of ontogenetic molecular markers - for instance, P-III class metalloproteinases and serine proteinases were more abundant in juveniles while P-I class metalloproteinases were more abundant in adults.

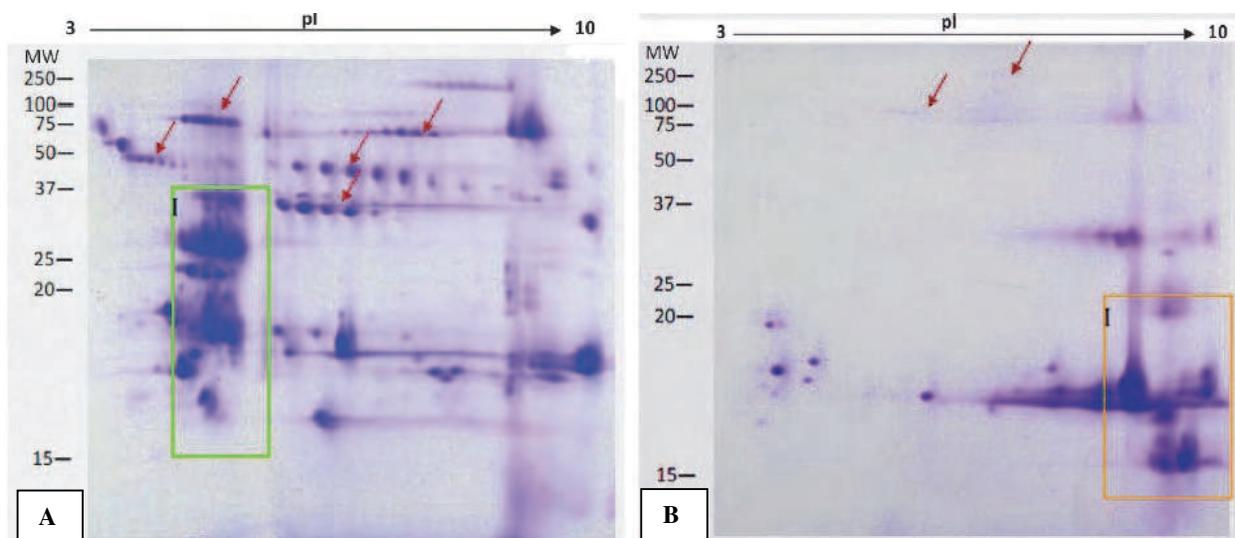


Fig. 1. 2DE images of venoms, (A) *Trimeresurus sumatranus* (Crotalinae) with scattered spots in region of 15 -37kDa while (B) *Bungarus fasciatus* (Elapidae) with spots predominantly at range of 15-20kDa. The boxed I zone show heavy spotting of acidic proteins for (A) compared to protein clustering in the lower right region, that is, the basic and lower molecular mass proteins (<20kDa) as shown for (B). Arrows indicate trains of spots due to post-translational modifications. Only two out of the eight 2DE venom profiles are shown here (Vejayan *et al.*, 2010).

2.2 Envenomation pathology

2DE also plays a role in the study of envenomation pathology and antivenom mechanisms. 2DE profiles can clearly demonstrate the venom components that are most immunogenic. In a study conducted by Correa-Netto *et al* (2010), crude *Bothrops jararacussu* venom fractionated by non-reducing 2DE was submitted to immunoblot analysis using anti-jararaca, anti-jararacussu and anti-crotalid sera. The results showed that the anti-jararaca and anti-jararacussu sera showed immune reactivity for venom proteins between 30 and

97kDa. The study also showed cross-reactivity of *B. jararacussu* venom with anti-crotalid serum. The importance of these results was that they allowed for the identification of the groups of proteins responsible for the horses' immune response in the process of antivenom production.

To investigate the effects of *Echis carinatus* envenomation on the human plasma proteome, an *in vitro* model utilizing 2DE as one of its core techniques has been established in two studies (Cortelazzo *et al.*, 2010; Guerranti *et al.*, 2010). The results from these two studies showed that 2DE was capable of demonstrating global proteomic changes when the human plasma was incubated with the *E. carinatus* venom. Upon comparison with the 2DE profile of the untreated control plasma, the 2DE profile of the human plasma treated with *E. carinatus* venom showed that some of the protein spots entirely disappeared or had a decreased level. Some of the protein spots in the 2DE profile of the venom-treated plasma also showed that the appearance of some new venom-dependent fragments. These proteins that were affected by the *E. carinatus* venom were identified by mass spectrometry analysis and have important functions in the blood coagulation process, which explained venom-induced thrombophilia in *E. carinatus* envenomation. The researchers, therefore, concluded that the 2DE proteomic approach was a valid method to study the molecular mechanism of envenomation on human blood proteins.

We tried to investigate the possibility of using the *Mimosa pudica* tannin (MPT) isolate as an antivenom of plant origin against the *Naja naja kaouthia* venom and have utilized 2DE as one of our core techniques in these studies. In an initial study, 2DE analysis was done on the crude *N. n. kaouthia* venom and MPT-treated *N. n. kaouthia* venom. In comparison, the 2DE results of the MPT-treated gel (Fig. 2A) showed a number of protein spots missing and within them we were able to identify 6 spots using mass spectrometry analysis to be isomers of the phospholipase A2 family of enzymes (Fig. 2) (Vejayan *et al.*, 2007). The disappearance of spots as detected via 2DE analysis indicated the binding mechanism of tannin from MPT that could potentially function as an antidote to the *N. n. kaouthia* venom. The results from this study, therefore, served as the preliminary findings before we progressed further into designing an *in vivo* study that looked into the efficacy of MPT to neutralize *N. n. kaouthia*.

In a following *in vivo* study, the crude *N. n. kaouthia* venom and *N. n. kaouthia* venom pre-incubated with MPT were injected into two different groups of mice (Ambikabothy *et al.*, 2011). There was a third group of mice in the study that served as the control group. The blood from all three groups of mice were collected with the cardiac puncture technique and centrifuged at 3000rpm for 10 min at 4°C to separate sera from cells. The sera from all three groups were then subjected to 2DE analysis in order to look into the different protein expression between the different groups of mice that had been given three different treatments respectively. Comparative analysis of the three 2DE profiles showed that a total of 5 protein spots were differentially expressed (>2 folds) (Fig. 3). It could be seen from the results that the serum of the venom-treated mice showed substantial proteomic changes that were absent from the serum of the mice treated with venom that was pre-incubated with MPT. Four of the five protein spots (Spots 2505, 4606, 3513, 3303) were identified as serine protease inhibitors, gelsolin, hemopexin and α 2-macroglobulin respectively. These four proteins were found to be upregulated in the serum of the venom-treated mice and could be the most possible candidates that were causative of mortality in *N. n. kaouthia* envenomation. The fifth spot, Spot 4407, could not be identified.

Based on the few examples elaborated above, it can be seen that 2DE is a versatile research tool that can be used in various aspects of snake venom study. This is underlined by its

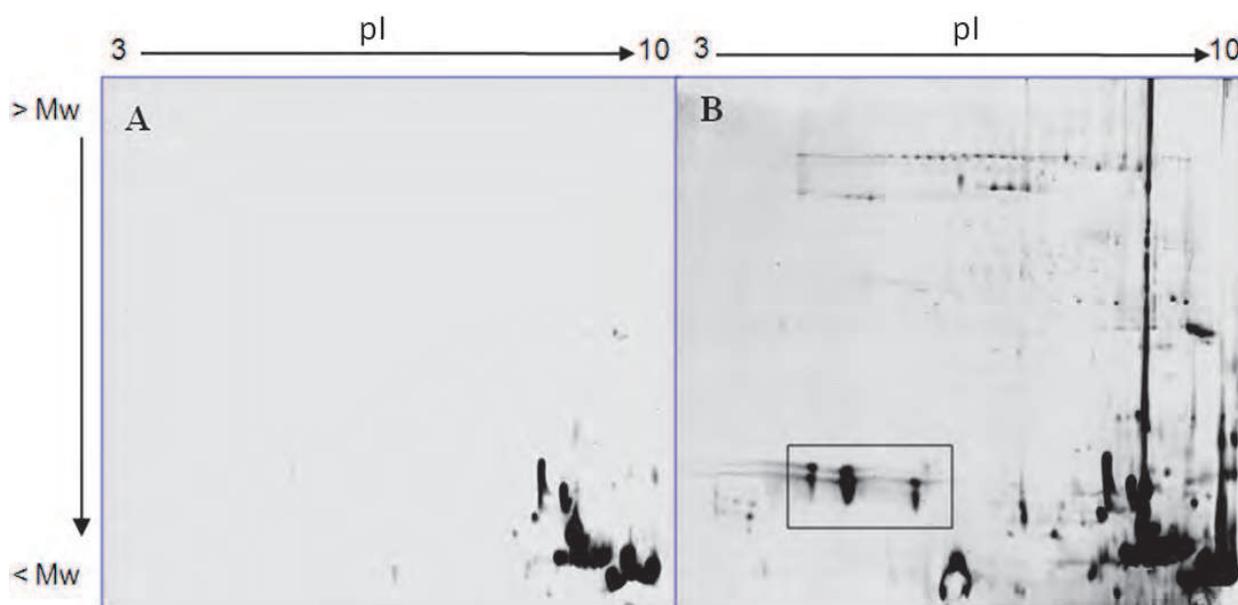


Fig. 2. The 2DE profiles of (A) MPT-treated *N. n. kaouthia* venom (B) crude *N. n. kaouthia* venom alone. Both treatments, upon incubation, were centrifuged and the supernatants were collected for 2DE analysis. The rectangular box outlined the six protein spots that had disappeared after the venom was treated with MPT (Vejayan *et al.*, 2007).

powerful resolving capability of separating venom proteins into hundreds of spots to provide a realistic overview of venom complexity. Such 2DE profiles can then be further subjected to comparative proteomic analysis for venom variation investigations. 2DE technique can also be easily and efficiently interfaced with other biochemical techniques (Rabilloud *et al.*, 2010), such as immunoblot analysis, to elucidate the mechanism of antivenom and pathology of envenomation.

In the next section, we will discuss how the resolution power of 2DE enables it to be used as a useful sample fractionation tool before mass spectrometry analysis, thus establishing the concept of integrative proteomics in venom study that is especially essential in novel protein identification.

3. 2DE-MS venom proteome mapping: Its importance and challenges

While 2DE has high resolving capability, detailed analysis of venom constituents and identification of novel proteins would not have been made possible without advances in mass spectrometry techniques. Subsequent to these combined efforts of 2DE mapping and protein identification by mass spectrometry, the concept of integrated proteomics has also been established, allowing for the systematic characterization of venom proteins.

The principles of integrated proteomics can largely be described in two stages - the first stage involves optimum sample fractionation by either electrophoretic methods (1D-PAGE or 2DE) or trypsin digest followed by liquid chromatography, while the second stage involves mass spectrometry analysis and database searches for protein identification (Brewis & Brennan, 2010). The concept of integrated proteomics in snake venom study was first pioneered with the study of venom glands from the sea snake *Laticauda colubrina* and the terrestrial *Vipera russelli* (Rioux *et al.*, 1998). The workflow employed was sample fractionation by 2DE followed by a combination of Edman sequencing and amino acid

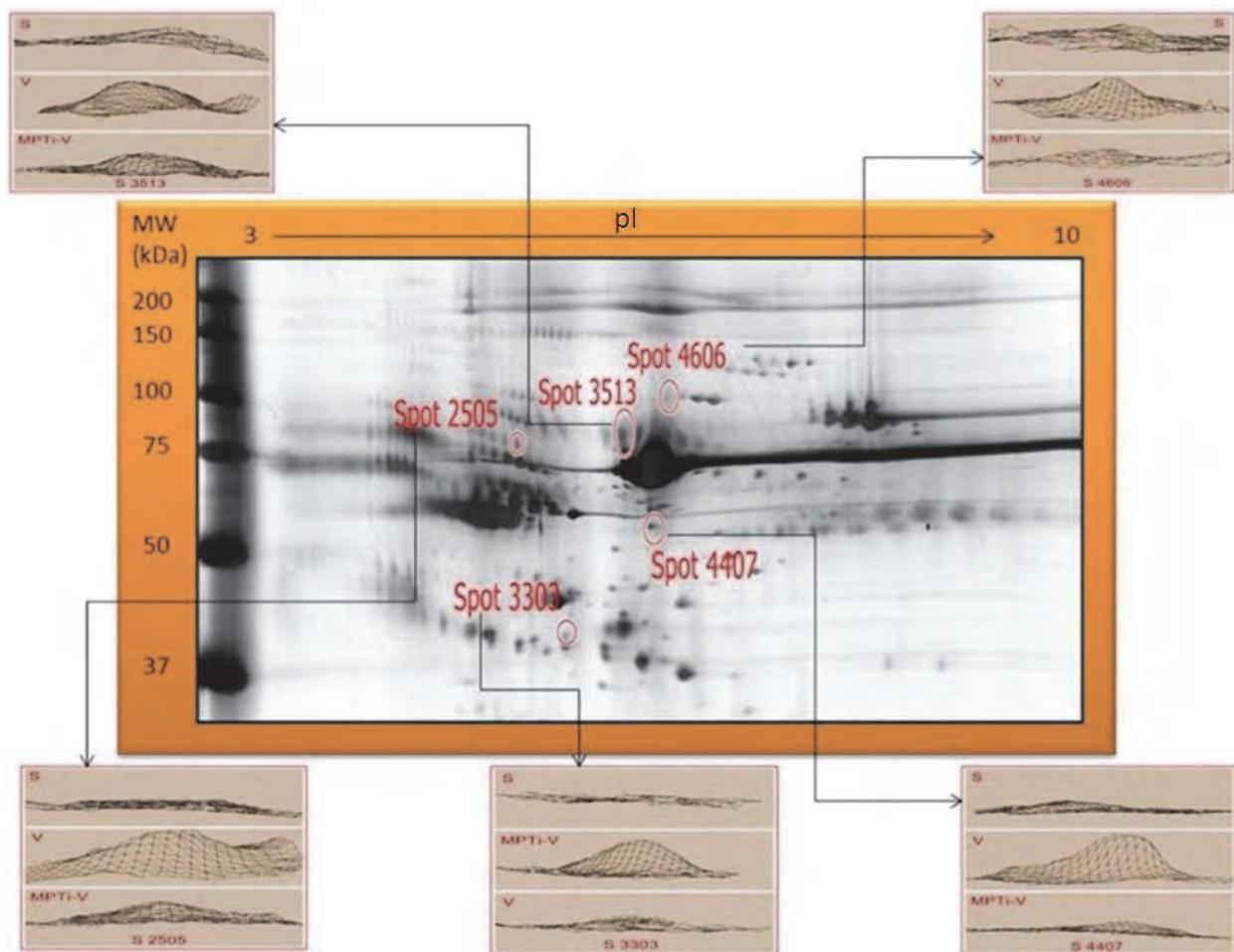


Fig. 3. Proteomic pattern of venom injected mice serum. 20 μ g mice serum protein after 2DE analysis at non-linear pH 3-10, stained by silver nitrate and 3D view of Spots 3513, 4606, 2505, 3303 and 4407 respectively in three different groups – control group (S), venom group (V) and MPT-treated group (MPT-V) (Ambikabothly *et al.*, 2011).

analysis. Subsequent to that, beginning in 2003, there was a rapid increase in literatures reporting on proteomic analysis of snake venoms. Using a variety of approaches, venom proteomes of 55 snake venoms have been analyzed (Fox & Serrano, 2008).

3.1 2DE-MS mapping of four venom proteomes

Following our initial 2DE analysis of the eight Malaysian snake venoms described earlier, we selected four of the venoms for further study on their constituents via mass spectrometry techniques. The four venoms selected were *N. n. kaouthia* (NK), *O. hannah* (KC), *Bungarus fasciatus* (BF) and *C. rhodostoma* (CR) (Fig. 4). We successfully identified a total of 64 proteins from the four venoms – 16 from *N. n. kaouthia*, 15 from *O. hannah*, 6 from *B. fasciatus* and 27 from *C. rhodostoma* respectively. All these proteins have biochemically and pharmacologically important properties. Evidently, based on the results from this study, the typical proteomic routine of 2DE and mass spectrometry, which was also the prototype workflow introduced when the concept of integrated proteomics was first pioneered, has been useful in investigating important venom constituents. Some of the major groups of proteins are discussed in the following.

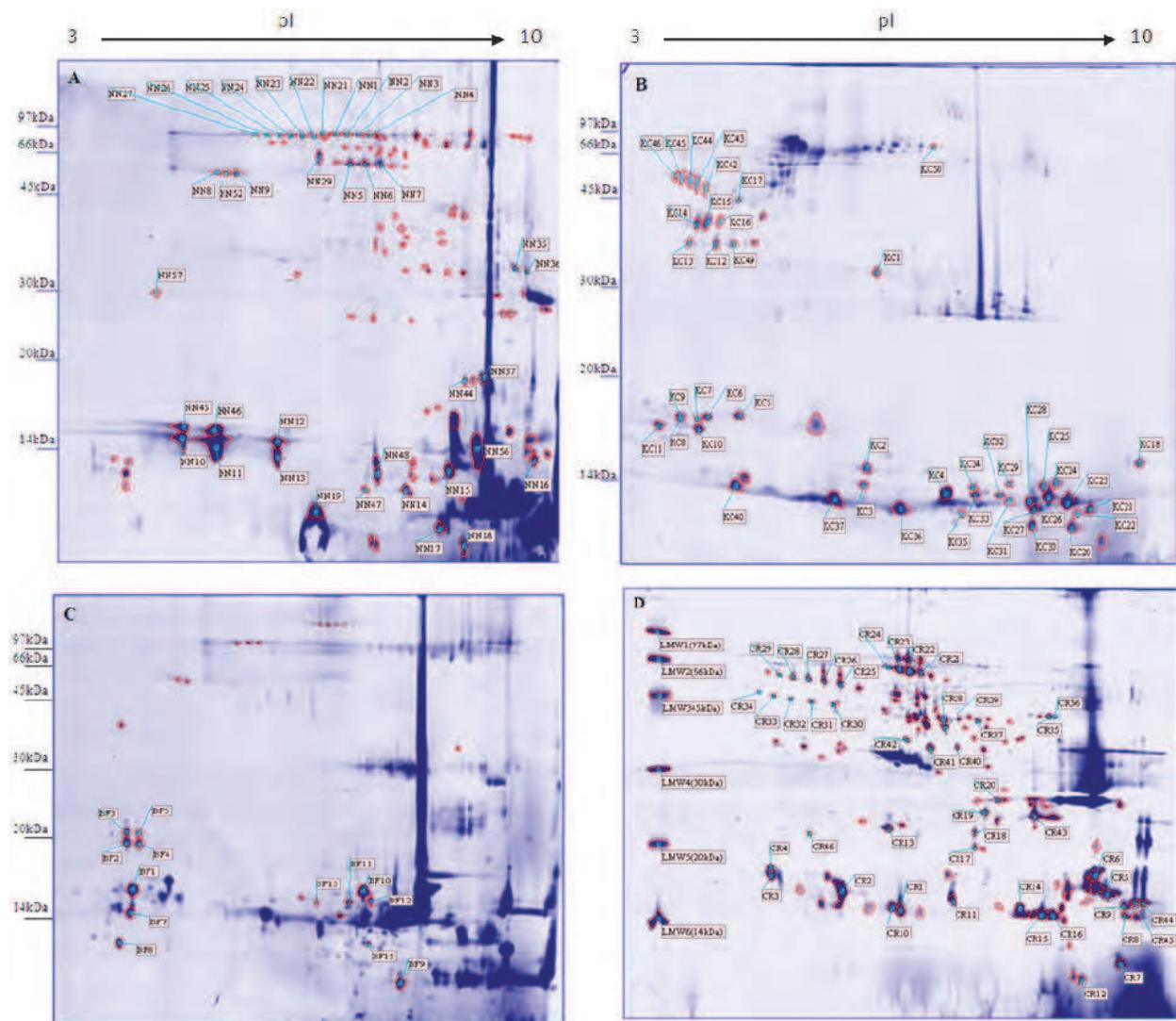


Fig. 4. Proteome maps of the snake venoms of *N. kaouthia* (A), *O. hannah* (B), *B. fasciatus* (C), and *C. rhodostoma* (D) with IPG 3-10 (300µg protein, 18cm), 15%T, Coomassie blue staining showing the annotated spots of the 4 venoms. The spots were detected and annotated by using the Image Master 2D Platinum software. These protein spots picked from the 2-DE gel stained by Coomassie blue, tryptic-digested, extracted and analyzed in duplicates per spot with MALDI-TOF MS in reflectron mode. The figure displays in (D) 6 annotated spots of the low molecular weight markers (Amersham Biosciences - Uppsala, Sweden).

The phospholipase A₂ (PLA₂) group of enzymes was identified in all 4 species (annotated as NN10, NN11, NN12, NN13, NN45, NN46, KC5, KC6, KC9, KC10, BF1, BF2, BF4, BF7, BF9, BF15, CR1, CR5 and CR6). At least one type of these enzymes was identified in the 4 species studied. It is not surprising as extensive studies on venom have demonstrated the accelerated natural selection force that drives the evolution of a multitude of extremely potent snake toxins from an ancestral PLA₂ with digestive function (Montecucco *et al.*, 2008; Ogawa *et al.*, 1996). Hundreds of species of venomous snakes of the families Elapidae and Viperidae were shown to have evolved a wide variety of venoms which contain varying proportions of toxins endowed with PLA₂ activity, characterized by their neurotoxicity, myotoxicity, as well as anticoagulant and edema-inducing properties (Boffa *et al.*, 1976;

Montecucco *et al.*, 2008; Vishwanath *et al.*, 1987). Hence, in snake venom, PLA₂ enzymes, in addition to their possible role in the digestion of the prey, exhibit a wide variety of pharmacological effects through interfering with normal physiological processes (Kini, 2003). It is well known that some of the most toxic and potent pharmacologically active components of snake venoms are either PLA₂ enzymes or their protein complexes. For example, all known presynaptic neurotoxins from snake venom are PLA₂ enzymes or contain PLA₂ as an integral part (Bon, 1997; Fletcher & Rosenberg, 1997). PLA₂ myotoxins are more potent and fast-acting than their non-enzymatic counterparts (Gubenek *et al.*, 1997).

PMF using MS also allowed the identification of long neurotoxins (NN14, KC23, KC25, KC26, KC27 and KC33) in both the cobra species studied. Unlike presynaptic beta-neurotoxins which exhibit varying PLA₂ activities, these long neurotoxins are classified as alpha-neurotoxins which affect the post-synaptic membrane (Lewis & Gutmann, 2004). Post-synaptic neurotoxins have only been identified in venoms from the families Elapidae and Hydrophiidae (sea snakes). They are antagonists of the nicotinic receptor on the skeletal muscle and display different binding kinetics and affinity for subtypes of nicotinic receptors (Hodgson & Wickramaratna, 2002). Other significant neurotoxins, such as the muscarinic toxin-like proteins (MTLP) (NN15, NN17 and KC32), ohanin (KC18) and thaicobrin (NN37), were also identified in the cobra venoms.

In *C. rhodostoma* venom another important protein was identified - the rhodocetin (CR2), a Ca²⁺-dependent lectin-related protein (CLPs), which is a potent platelet aggregation inhibitor induced by collagen. It is a prime example of a CLP dimer, in which the two subunits are held together by interactions and act synergistically to elicit the biological activity, affecting platelet aggregation and blood coagulation, important in cellular thrombosis and non-cellular processes in homeostasis (Kornalik, 1991; R Wang *et al.*, 1999). This protein also demonstrated the ability to antagonize stromal tumor invasion *in vitro* and other $\alpha 2\beta 1$ integrin-mediated cell functions (Eble *et al.*, 2002). According to this worker, its ability to inhibit tumor cell invasion through a collagen matrix, combined with its lack of cytotoxicity, high solubility, diffusibility and biochemical stability, may qualify rhodocetin to be one of the first snake venom disintegrins of potential practical importance in tumor therapy, *e.g.* in attempts to interfere with stromal invasion and metastasis.

The proteomic approach applied in this study also successfully identified a significant number of enzymes, namely zinc metalloproteinase or disintegrin (CR17, CR18 and CR43), Ancrod or venombin A (CR25, CR27, CR30, CR31, CR32, CR33, CR34, CR40 and CR41), and L-amino-acid oxidases (CR21, CR22, CR23, CR39), that display the well-documented hemotoxic properties of the viper's venom. Disintegrin exhibits hemorrhagic activities by binding to the glycoprotein IIb-IIIa receptor on the platelet surface, thus inhibiting fibrinogen interaction with platelet receptors while L-amino-acid oxidases exhibit hemorrhagic activities by catalyzing oxidative deamination of hydrophobic and aromatic L-amino acids (Au, 1993; Dennis *et al.*, 1990; Gould *et al.*; 1990, Macheroux *et al.*, 2001). L-amino-acid oxidases have also recently been shown to display antibacterial properties (Tonismagi *et al.*, 2006). On the other hand, ancrod is a thrombin-like serine protease that selectively cleaves the fibrinopeptides, resulting in aberrant fibrinogen that is unable to form dispersible blood clots (Au *et al.*, 1993).

As discussed earlier, venom proteins have different abundances and, thus, the identification of each of these proteins may require different methods. The high abundance proteins, for example, causes incomplete resolubilization during equilibration, resulting in vertical

streaking and tailing of the most intense protein spots (Berkelman *et al.*, 2004). During the mapping of the four selected venoms, we noticed that these vertical streaks so commonly observed on 2DE profiles of snake venoms were particularly prominent in the 2DE profile of *B. fasciatus*. The presence of these vertical streaks led to the lack of complete visualization of the separated protein spots on the 2DE profiles, limiting the identification of these proteins by mass spectrometry analysis.

Therefore, in order to eliminate these streaks for protein identification, various measures were taken, for instance, prolonging the equilibration time to facilitate sufficient equilibration, scavenging any excess or residual thiol reducing agent with iodoacetamide before loading the IPG strips onto the 2nd dimension gel (as this reducing agent known to exacerbate this effect) or by loading lesser content of protein. However, apart from loading far lesser amount of venom, none of the other measures produced the desired results. As shown in Fig. 5a and Fig. 5b, loading of only 0.8 μ g and 1.06 μ g protein, respectively, resulted in elimination of vertical streaks. As the load of protein was increased to 1.6 μ g (Fig. 5c), an apparent vertical streak begins to show up on the 2DE gel. Apart from highlighting the presence of certain highly abundant proteins in snake venoms, these results also demonstrated another important aspect of venom proteome mapping by 2DE-MS – the complete visualization of a venom proteome, additional steps must be taken, including loading different amounts of venom, use of different staining techniques and the use of a variety of pI ranges (Fox *et al.*, 2002). The end result will probably show that venom constituents are much more complex than originally shown.

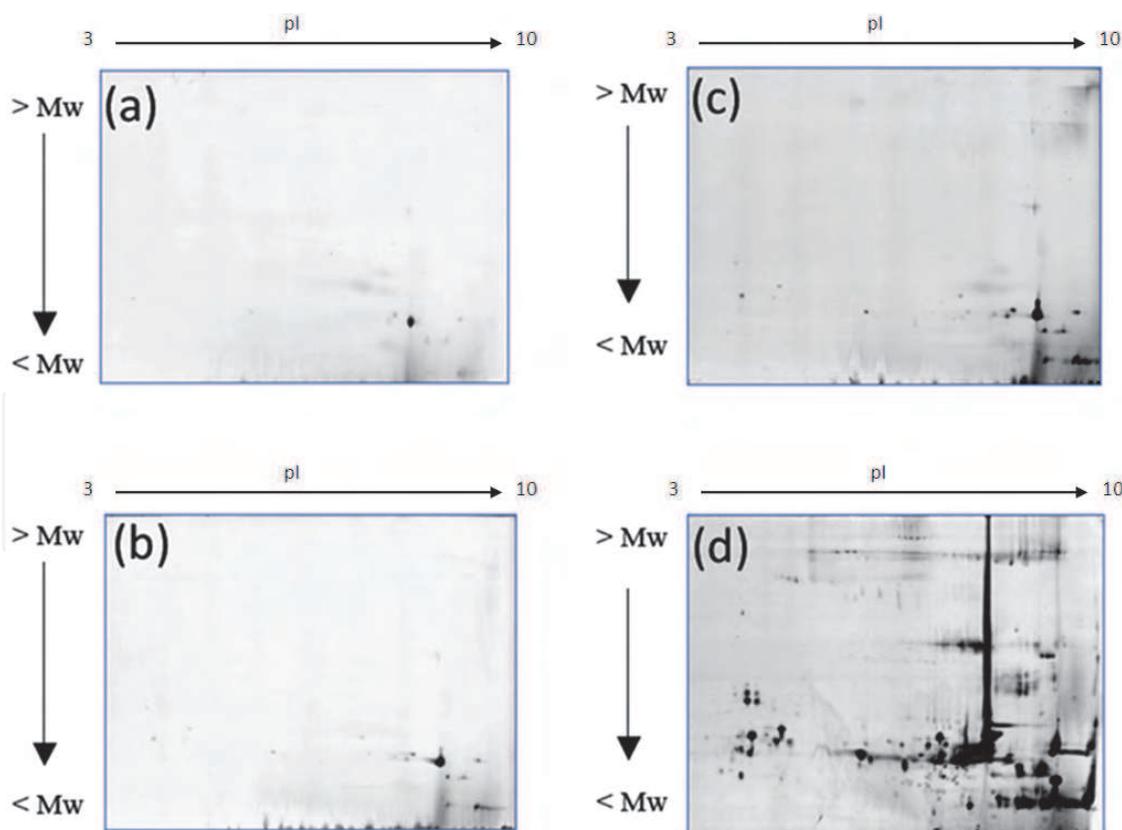


Fig. 5. 2DE profiles of *B. fasciatus* venom at different loading protein content during first dimension IEF separation, IPG 3-10, 15%T, Coomassie blue staining. (a) 0.8 μ g protein (b) 1.06 μ g protein (c) 1.6 μ g protein (d) 35 μ g protein.

3.2 Challenges in 2DE-MS venom proteome mapping

The main objective of integrated proteomics in venom study is to achieve full proteome coverage of snake venoms. Nevertheless, despite our successful identification of major proteins in the four venoms, a large number of other spots could not be identified, despite displaying high-quality mass spectra. As such, researchers are constantly establishing new, additional workflows to meet this objective and it is no longer limited to only 2DE followed by mass spectrometry analysis in the process of proteome coverage. For example, Li *et al* (2004) has described the novel identification of 124 and 74 proteins from the *Naja naja atra* and *Agkistrodon halys* venoms respectively through the utilization of four combined proteomic approaches, namely - (1) shotgun digestion plus HPLC with ion-trap tandem mass spectrometry, (2) 1D-PAGE plus HPLC with MS/MS, (3) gel filtration plus HPLC with MS/MS, (4) 2DE plus MALDI-TOF-MS. By using four different workflows to characterize the constituents of the same venoms, each approach could compensate the detection coverage of the venomous proteins, since it was found that a few proteins could only be identified by one specific approach (Li *et al.*, 2004).

While conducting database searches for protein identification during the profiling of the four venoms, we found that the limited database for snake venom proteins could pose as a major limitation to this approach of 2DE and mass spectrometry for protein identification. The Taxonomy Browser contained in the Entrez database (URL: <http://www.ncbi.nlm.nih.gov>) provided the total number of proteins available for matching for each of the 4 snake venoms. The number of known proteins available, as of 16 June 2011, was 122 for *N. n. kaouthia*, 176 for *O. hannah*, 119 for *B. fasciatus* and 76 for *C. rhodostoma*. The protein database was constructed based on the sequence data from the translated coding regions from DNA sequences in GenBank, EMBL, and DDBJ as well as protein sequences submitted to Protein Information Resource (PIR), SWISS-PROT, Protein Research Foundation (PRF), and Protein Data Bank (PDB) (sequences from solved structures). It was obvious that the database was incomplete for snake venom matching, compared with 534,370 available for *Homo sapiens* (human) proteins, or 88,882 for *Bos Taurus* (cattle) proteins. Nevertheless, the snake venom protein database is still of value in comparison to some other venomous species such as *Chironexyama guchii* (sea wasp) with only 2 proteins, *Hadrurus aztecus* (scorpion) with 1, and *Dolomedes plantarius* (spider) with none, to name a few, for matching.

A closer examination of the snake venom protein database revealed the following information. Some of the proteins were not found in the venom but were evident elsewhere in the snake's body, for example, the nerve growth factor (located in the nervous system), NADH dehydrogenase and cytochrome b (located in mitochondria) and oocyte maturation factor (located in the reproductive system). Also, most of the venom proteins were precursor forms or polypeptide chains of the same protein, example: phospholipase PLA₂ precursor and Chain A, crystal structure of L-Amino Acid Oxidase. Based on these factors, therefore, in reality, the snake venom proteins available for matching in the database were, in fact, much more limited. This limitation may be overcome in the future as the database for snake venoms is developing. This is evident by comparing the number of known proteins dated back to 3rd October 2005 (67 for *N. kaouthia*; 78 for *O. hannah*; 34 for *B. fasciatus*; and 60 for *C. rhodostoma*) to the most recent Entrez protein database. The protein database of *B. fasciatus* has shown its most promising development, displaying increment of up 250% while that of *O. hannah* increased by 126%, *N. kaouthia* by 82%, and *C. rhodostoma* by 27%. If not for the reason of incomplete database another common possibility is due to post-translational

modification (PTM) undergone by some of the proteins. PTM of venomous protein are common phenomenon in snake venoms. Of a number of mechanisms inducing chemical modification of protein, glycosylation is one of the most frequently found (Li *et al.*, 2004). It is expected the advances in mass spectrometry combined with posttranslational modification database specific for venoms may solve this shortcoming. A PTM database comprising of phosphorylation, N-glycosylation or acetylation sites is already existing and growing steadily for nine different species (none yet for snake)(Gnad *et al.*, 2011).

3.3 Relevance of 2DE-MS venom proteome mapping in the present proteomic landscape

With the advances in mass spectrometry techniques, non gel-based proteomic techniques such as LC/MS are now generally being considered as state-of-the-art, thus leaving the gel-based 2DE technique in a questionable state as to whether it still has any relevance as a proteomic method. Nevertheless, while the issue at hand is one that will be of continuous debate, we should keep in mind that the limitations of 2DE are well known and are probably most thoroughly investigated than any other proteomic techniques. Hence, researchers who choose to utilize the technique are generally aware of the limitations and are usually able to adapt the complexity of the sample to the resolution power of the 2DE method by narrowing the study subject to one focus in order to reduce the sample complexity (Rabilloud *et al.*, 2010). For instance, in a study done by Nawarak *et al* (Nawarak *et al.*, 2003) to investigate the proteomes of a number of selected Elapidae and Viperidae venoms, the group first fractionated the crude venoms on RP-HPLC before subjecting only the major eluted peaks to 2DE analysis.

In addition, the robustness of the 2DE technique has been tested thoroughly and the influence of the various parameters on the intra-laboratory reproducibility has been investigated (Choe & Lee, 2003), thus making the 2DE process a strong technique for proteome profile building and for subsequent deposition in databases to be accessed by researchers worldwide to be used for reference.

Specifically in the field of venom proteome study, the 2DE technique remains important when it comes to the study of (1) subpopulation of venom proteins and (2) post-translational modifications (PTM) in venom proteins. In two studies that were done in similar manner, Serrano *et al* (2005) and Birrell *et al* (2006) investigated the diversity of venom proteins in the viperid venoms (*Bothrops jararaca* and *Crotalid atrox*) and the Australian Brown Snake venom (*Pseudonaja textilis*) respectively. Taking advantage of the efficient interface of 2DE with other biochemical techniques, the two groups of researchers subjected the obtained 2DE profiles to immunoblot analysis with antisera raised against specific venom protein groups that were of study interests. Serrano *et al* (2005) did immunoblot analysis with antisera raised against metalloproteinase's, serine protease and phospholipase A2. Birrell *et al* (2006), on the other hand, did immunoblot analysis with antisera raised against prothrombinase complex, heavy chain of the Factor Xa-like protease, GIa residues, textilins and textilotoxins. The results were specific 2DE profiles of these protein groups, giving rise to a more thorough understanding of venom complexity and providing insights for investigators who want to focus on these subpopulations of proteins in future studies.

Large portions of venom proteins undergo PTM (Nawarak *et al.*, 2004). Protein spots that have undergone PTM appeared as train of spots on the 2DE profiles, owing to their differences in pI values that gave rise to their non-identical migration profiles (Birrell *et al.*, 2006). Both groups employed specific fluorescent dyes, Pro-Q Emerald and Pro-Q Diamond,

for the study of post-translational modifications (PTM) in venom proteins (Birrell *et al.*, 2006; Serrano *et al.*, 2005). Apart from specific staining methods, other modifications can also be applied on the conventional 2DE protocol for the study of PTM. Nawarak *et al.* (2004) performed lectin-affinity purification using Sepharose-bound Con A to fractionate venom glycoproteins. The bound glycoproteins were then eluted and studied using 2DE followed by mass spectrometry.

Furthermore, through efforts of venom proteome mapping by 2DE-MS, major protein groups have been identified, as evident from our results elaborated in Section 3.1. These proteins have potential values when it comes to pharmaceutical and diagnostic uses. The purification of these proteins is thus slowly emerging as another new aspect of investigation when it comes to snake venom research. Therefore, the profiling of these proteins on 2DE maps that can be stored in a reference library for the use of researchers worldwide will be very useful when a researcher wants to use these profiled protein spots for the purpose of 2DE-guided purification, a technique that will be described in greater details in a following section.

4. Modifications to the 2DE protocol

4.1 Spiking

In performing high-performance liquid chromatography (HPLC), a concept known as co-injection can be used to help identify unknown compounds. To perform co-injection, a process known as spiking has to be done. Spiking is done by first mixing a synthesized or isolated standard (some are available commercially) with the sample containing the compound to be identified and subsequently, if the co-injected standard and unknown compounds co-elute, then the relative peak intensity of the unknown compound on the chromatogram of the spiked sample will be higher than that for the unspiked sample (Peters *et al.*, 2005). Co-elution supports, but does not prove, the idea that the compounds are identical.

The spiking concept from HPLC was then adapted to proteomics and 2DE. It can be demonstrated by using alpha-bungarotoxin from the *Bungarus multicinctus* (Many banded krait) venom (Vejayan *et al.*, 2008) using the following steps:

1. Crude *B. multicinctus* venom was first subjected to 2DE analysis on an 18cm format gel, using the conventional 2DE protocol without any modification (Fig.6). Crude venom, containing 0.8µg protein, was loaded onto the IPG strip via a sample-loading cup on the anodic end.
2. A second 2DE analysis is done on the same venom, but with one modification – instead of using a single sample loading cup in IEF, two sample loading cups were used. One of the cups was placed at the anodic end (designated as Cup A) and loaded with crude *B. multicinctus* venom (0.8µg protein) alone while the other cup was placed at the cathodic end (designated as Cup B) and loaded with commercially purchased purified *B. multicinctus* alpha-bungarotoxin (0.1µg protein) (Fig. 7). The remaining of the first and second dimension separations was done as per the usual conventional 2DE protocol.
3. Both the 2DE profiles obtained from Steps 1 and 2 were then subjected to comparative analysis using the Image Master 2D Platinum software. A spot of increased intensity was identified on the 2DE profile obtained from Step 2. The increase of intensity was quantified as a 2.5 fold increase in the % volume (Fig. 8).
4. The spot with increased intensity was cleaved and subjected to MALDI-TOF-MS peptide mass fingerprinting. The protein was identified and confirmed as alpha-bungarotoxin.

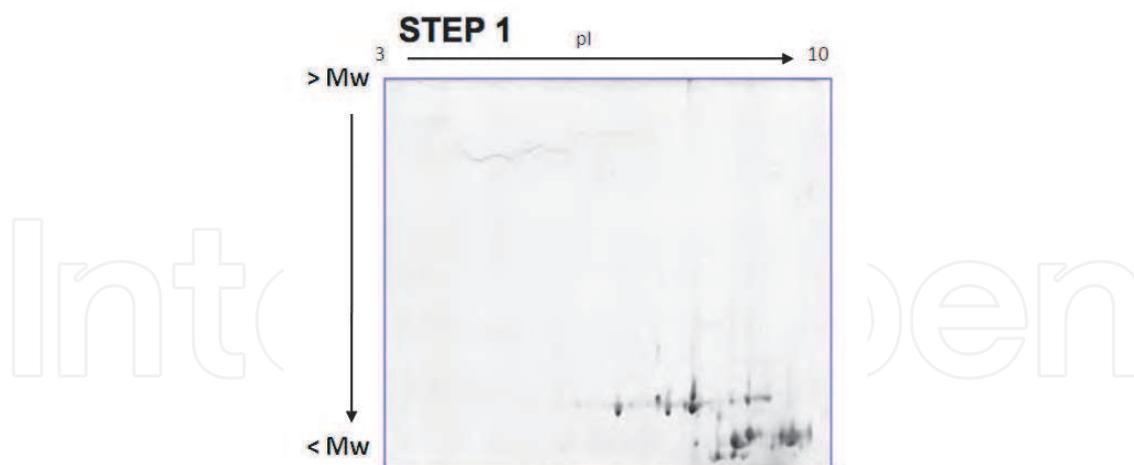


Fig. 6. Step 1 of spiking technique: 2DE analysis of the crude *B. multicinctus* alone (0.8 μ g protein) was done on an 18cm format gel using the conventional 2DE protocol.

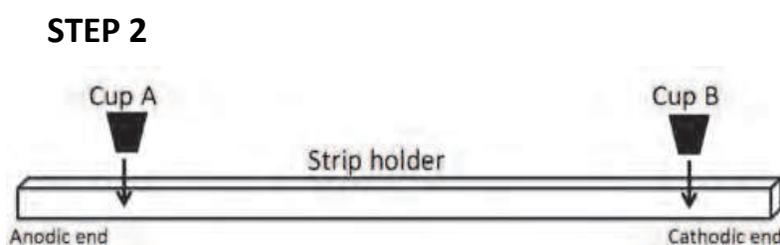


Fig. 7. Step 2 in spiking technique: A second 2DE analysis was done using two sample loading cups – Cup A at the anodic end is loaded with crude *B. multicinctus* venom while Cup B at the cathodic end is loaded with pure alpha-bungarotoxin.

This method was initially conceptualized with the intention to locate the spot of alpha-bungarotoxin on the 2DE profile of *B. multicinctus* by simply identifying the “spiked spot”, which was the spot of increased intensity, without using any mass spectrometry technique. However, we have also found the technique useful when we needed to affirm the identity of a compound purified from snake venom. After we have successfully purified rhodocetin from the venom of *C. rhodostoma*, we spiked the purified protein on the crude *C. rhodostoma* venom and found a spot of increased intensity that matches to the rhodocetin spot on the 2DE profile of *C. rhodostoma*. The usefulness of spiking for this purpose can be better demonstrated in the following section.

4.2 2DE-guided purification

In recent years, natural products drug discoveries have received a renewed interest and snake venoms are also being investigated for pharmacologically important components. Once a crude venom has been identified as an active source, bioassay-guided purification is typically used to isolate the active component. While the general paradigm of this process can be relatively straightforward in the academic laboratory setting, the design of a suitable bioassay can present as a practical challenge to the whole purification process. Notwithstanding the fact that the design of a bioassay has to take into consideration some

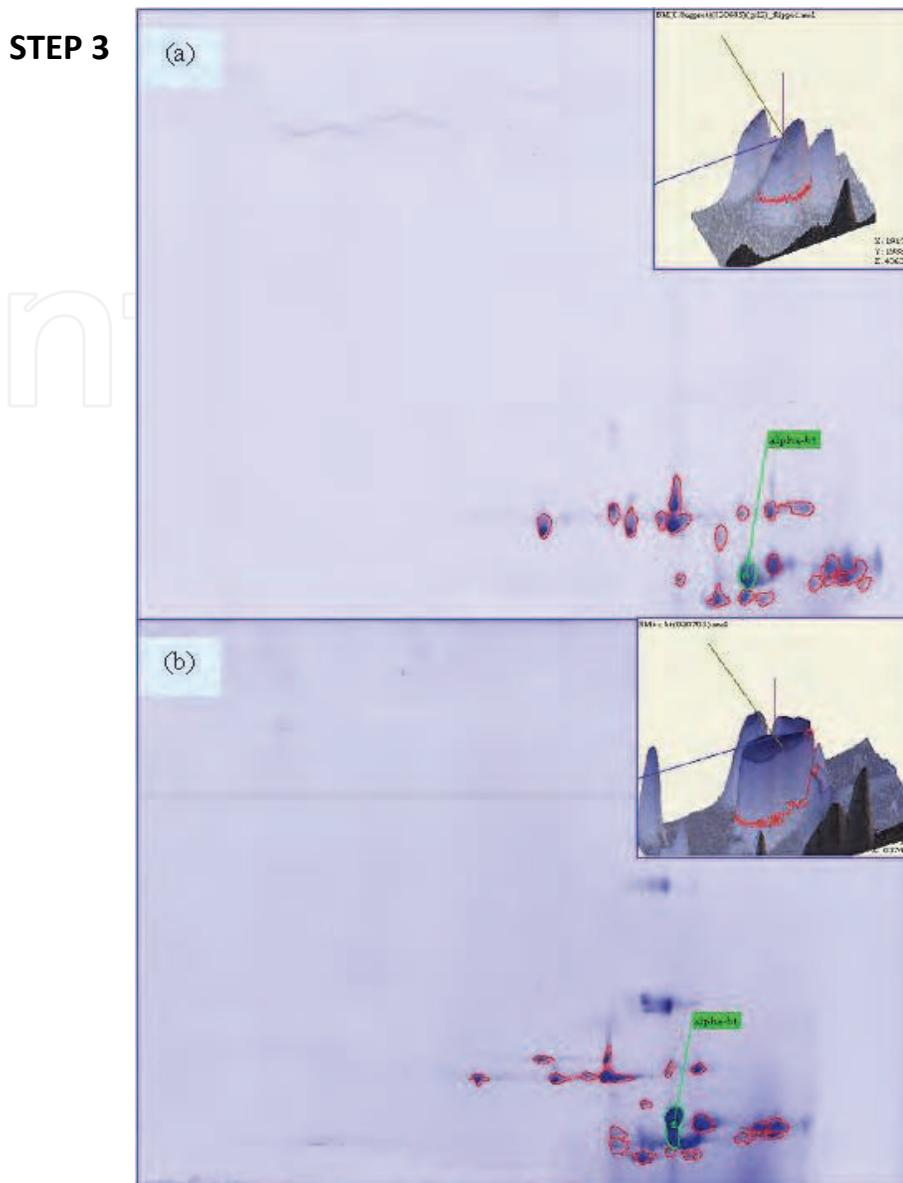


Fig. 8. Step 3 in spiking technique: Comparative analysis between the unspiked (a) and alpha bungarotoxin-spiked (b) 2DE profiles showed a spot with increased intensity (the spiked spot). The inset 3D figures showed a % volume increase quantified at 2.5 fold. The spiked spot was later confirmed as alpha-bungarotoxin by mass spectrometry.

important criteria – sensitivity, specificity, lack of ambiguity, accuracy, reproducibility and a reasonable cost – some bioassays also have lengthy turnaround time and require high amount of the active component, thus posing as challenges to the progression of the fractionation process.

All the above elaboration on the challenges of purifying therapeutically important proteins from snake venoms can perhaps be best illustrated using rhodocetin as an example. Rhodocetin is a CLP from the venom of *C. rhodostoma* and has been investigated for important therapeutic properties including platelet aggregation inhibition and stromal invasion inhibition. Two previous groups of researchers have employed bioassay-guided purification to purify this important protein. Wang *et al* (R Wang *et al.*, 1999) has employed the use of a platelet aggregation bioassay that required the use of human and rabbit blood

collection and the inhibition of platelet aggregation would prove the presence of rhodocetin. Eble *et al* (2002) on the other hand, utilized the ELISA-like procedure to detect the inhibition of the binding between $\alpha 1\beta 2$ integrin with type I collagen by rhodocetin. Given the importance of rhodocetin as a potentially therapeutic component, we would like to have purified rhodocetin in our laboratory for the purpose of further investigations. However, we were confronted by a few challenges in wanting to do so. Firstly, rhodocetin has yet to be made available as a commercially sold purified protein. Secondly, the purification of rhodocetin, as described above, required the design of complicated bioassays.

In view of all these mentioned challenges in rhodocetin purification, we attempted to find an alternative to bypass the requirement of a bioassay to purify rhodocetin. As such, we hypothesized that a concept known as “2DE-guided purification” would be able to achieve this purpose (Tang *et al.*, 2011). The concept of 2DE-guided purification is essentially using the presence of a protein spot on the 2DE gel as an indication of the presence of a protein within a particular sample. As discussed in Section 3, following our efforts of venom proteome profiling, rhodocetin has been successfully identified on the 2DE profile of *C. rhodostoma*. We could, therefore, take advantage of this result and use it for the 2DE-guided purification of rhodocetin, of which will be described in a detailed step-by-step description as follows:

1. The crude venom of *C. rhodostoma* was subjected to 2DE analysis on a 7cm format minigel. The 2DE profile obtained was then compared with our previous work of profiling the crude *C. rhodostoma* venom on a larger 18cm format 2DE gel. The rhodocetin spot was identified on the minigel, cleaved and sent for MALDI-TOF-MS peptide mass fingerprinting (Fig. 9). It was successfully identified as the alpha subunit of rhodocetin.
2. The crude *C. rhodostoma* venom was then subjected to fractionation by liquid chromatography. Out of the various chromatography techniques for protein purification, we have selected anion-exchange using the column Mono Q 5/50 GL (1ml). The chromatography profile showed six eluted peaks and we designated the peaks as U, P1, P2, P3, P4 and P5 (Fig. 10). The fractions of these peaks were collected for the subsequent desalting process.
3. The desalting process involves size exclusion chromatography using G25 HiTrap Desalting column. Interestingly, while each of the peaks typically gave a single protein peak upon desalting, P2 was an exception as it produced two distinct peaks on its desalting profile. As such, we assumed that the G25 gel filtration has further fractionated the contents of P2 and we designated the two peaks as DP1 and DP2 (Fig. 11).
4. After the two-step protein fractionation process, we collected seven peaks based on the chromatography profiles. All the seven peaks were then subjected to 2DE analysis respectively on minigels. The resulting profiles were then compared to the *C. rhodostoma* profile done in Step 1. Out of the seven profiles, only the profile of DP2 showed the presence of the rhodocetin spot. Therefore, we could conclude that the peak DP2 contained rhodocetin (Fig. 12).
5. To conclude the purification process, it was essential for us to determine the homogeneity of DP2, in order to decide if any further cycle of fractionation was necessary. The SDS-PAGE of DP2 showed two distinct bands at the low molecular weight region (Fig. 13) that was characteristic of purified rhodocetin, as shown by previous groups of researchers (Eble *et al.*, 2002; R Wang *et al.*, 1999).

6. Finally, to further confirm the identity of the purified compound as rhodocetin, we employed the spiking technique, as described in Section 4.1, by loading the crude *C. rhodostoma* venom into a sample loading cup located at the anodic end and the purified compound into another sample loading cup at the cathodic end. A spiked spot was identified and upon comparison with the 2DE profile of the unspiked crude *C. rhodostoma* venom, the location of the spiked spot correspond to the location of the rhodocetin (alpha subunit) and the increase in intensity was quantified at 1.6 fold (Fig. 14). Together, these confirmed the identity of the purified compound as rhodocetin.

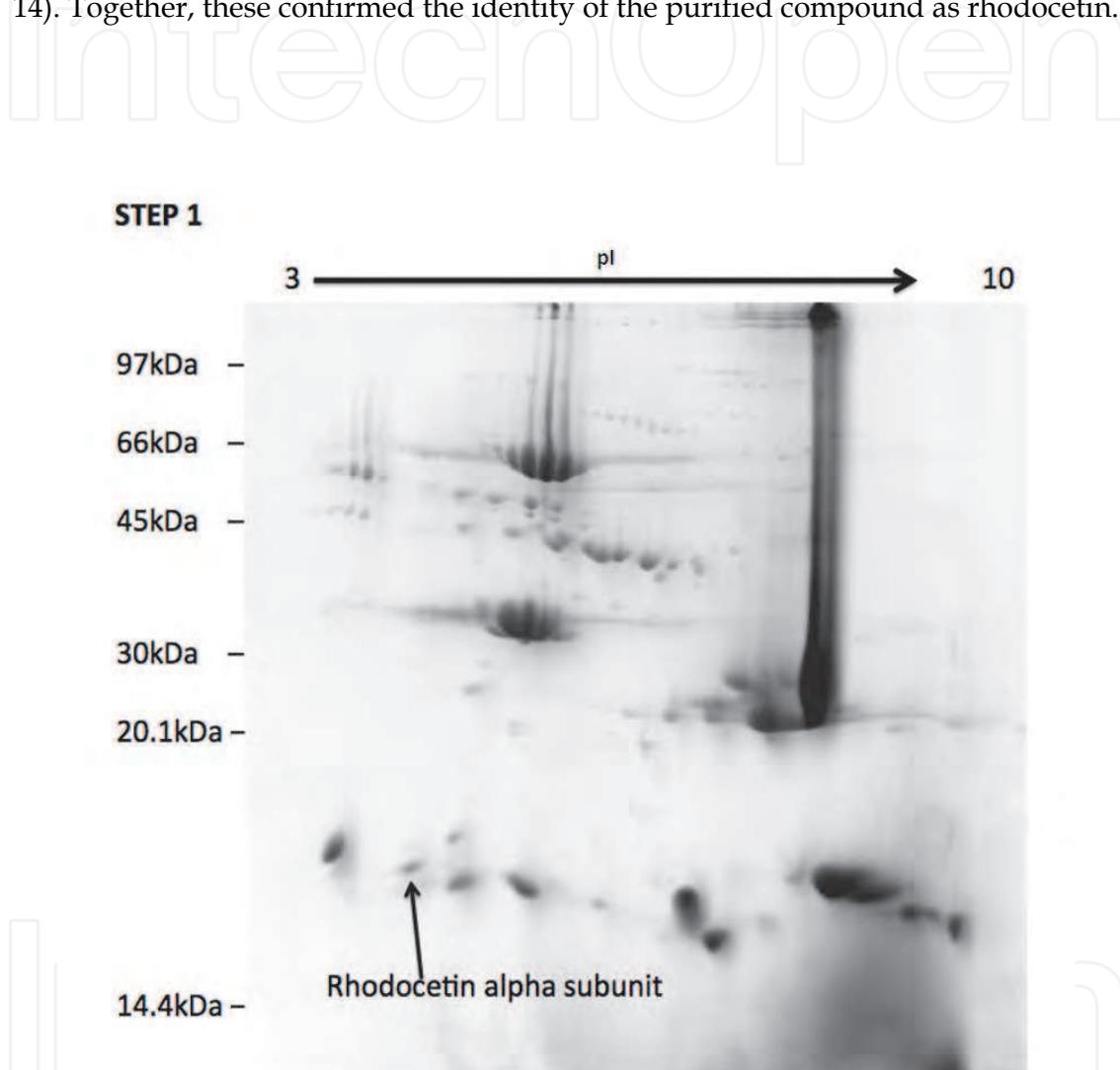


Fig. 9. Step 1 of 2DE-guided purification: 2DE profile of *C. rhodostoma* (60 μ g protein) with the rhodocetin (alpha subunit) spot annotated. The profile was obtained by IEF on a 7cm IPG strip (pH 3-10) and the proteins subsequently separated in the second dimension by 15% SDS-PAGE. The separated proteins were visualized by Coomassie Brilliant Blue staining.

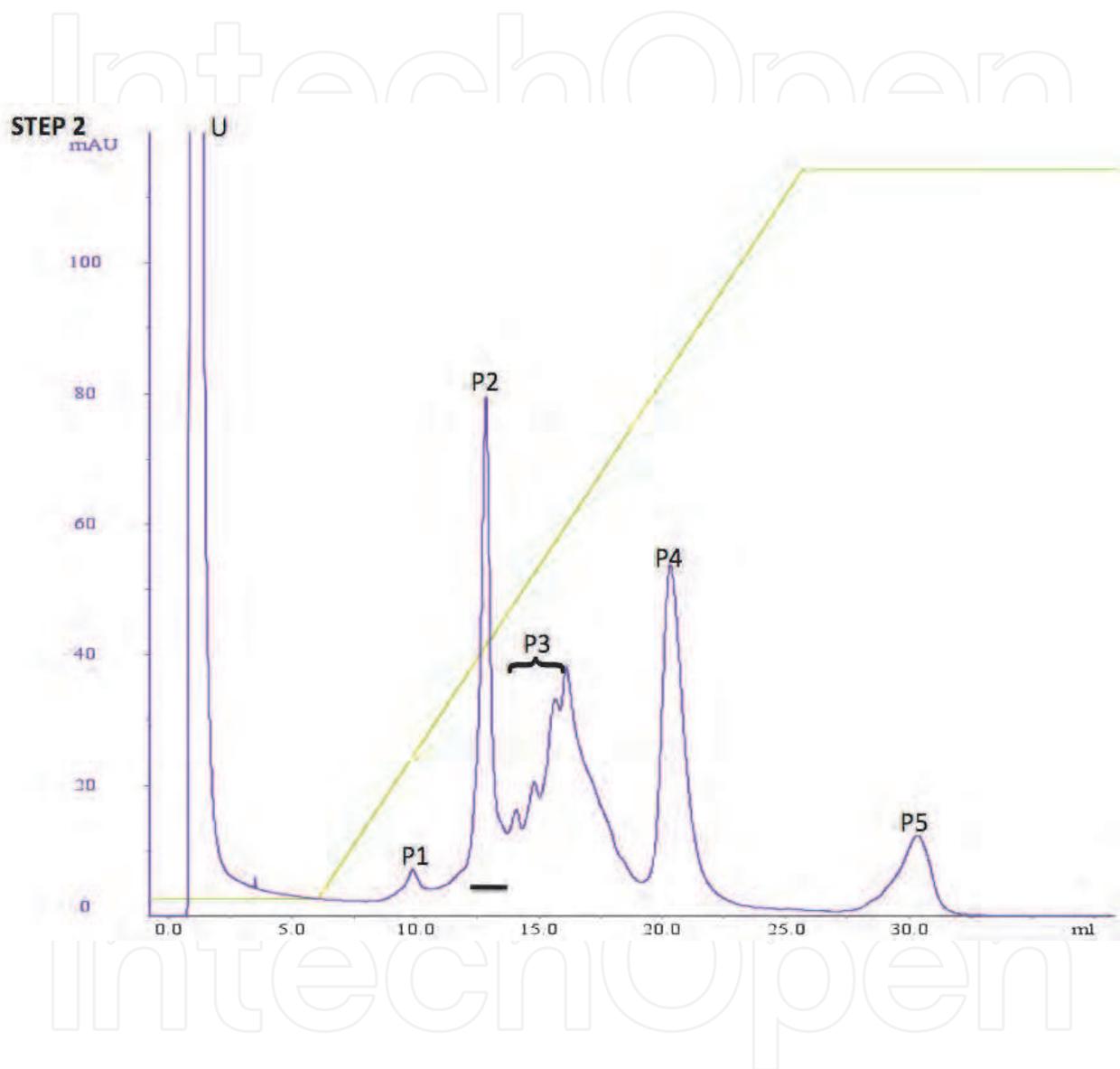


Fig. 10. Step 2 of 2DE-guided purification: Anion-exchange to fractionate crude C. *rhodostoma* venom – 5mg of crude *C. rhodostoma* venom dissolved in 250 μ l of 20mM Tris-HCl, pH 8.5 and loaded into a Mono Q 5/50 GL (1ml) column, equilibrated with 20mM Tris-HCl, pH 8.5. Six peaks (U, P1, P2, P3, P4 and P5) were obtained.

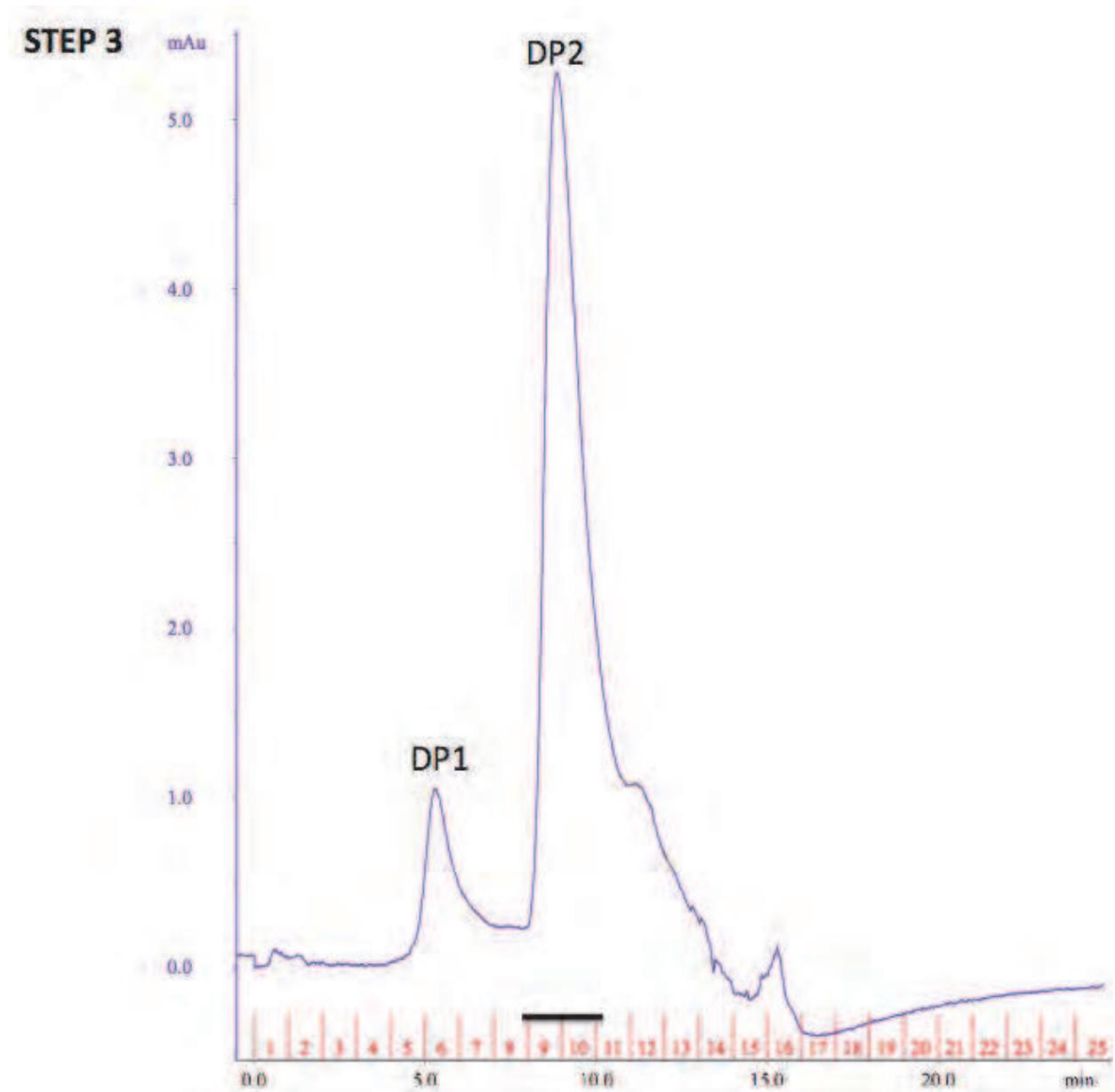


Fig. 11. Step 3 of 2DE-guided purification: The chromatography profile obtained when P2 fraction collected from the Mono Q column was directly injected into a G25 HiTrap Desalting column, equilibrated with distilled water. Two peaks of DP1 and DP2 were obtained.

STEP 4

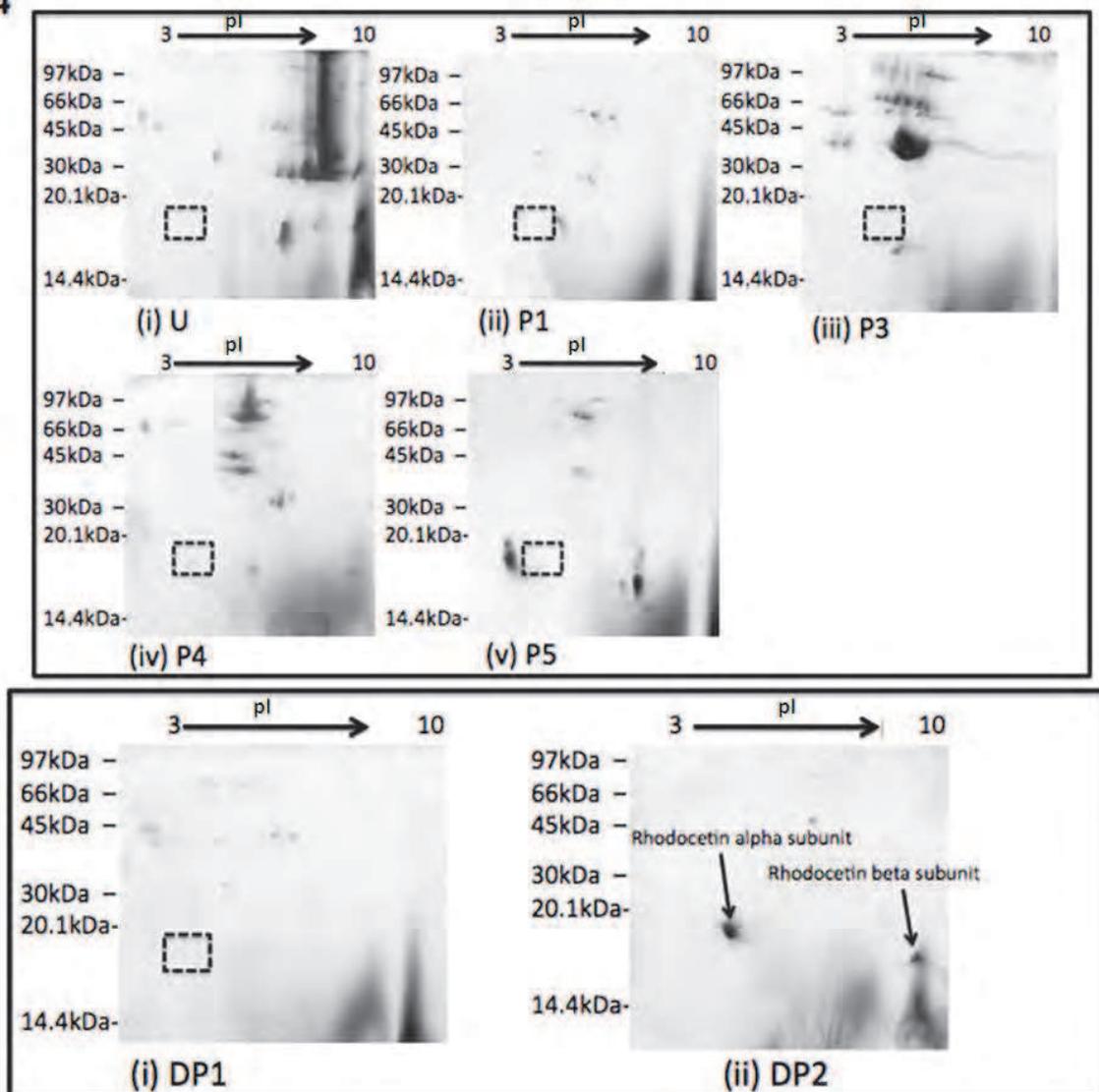


Fig. 12. Step 4 of 2DE-guided purification: 2DE assay was done on the seven collected peaks after the fractions were desalted and lyophilized. The 2DE profile of each peak is shown here and the small area outlined by the black grids on each 2DE gel represents our area of interest in which rhodocetin (alpha subunit) spot should have been present. The 2DE profile of DP2 clearly showed the presence of the alpha and beta subunits of rhodocetin.

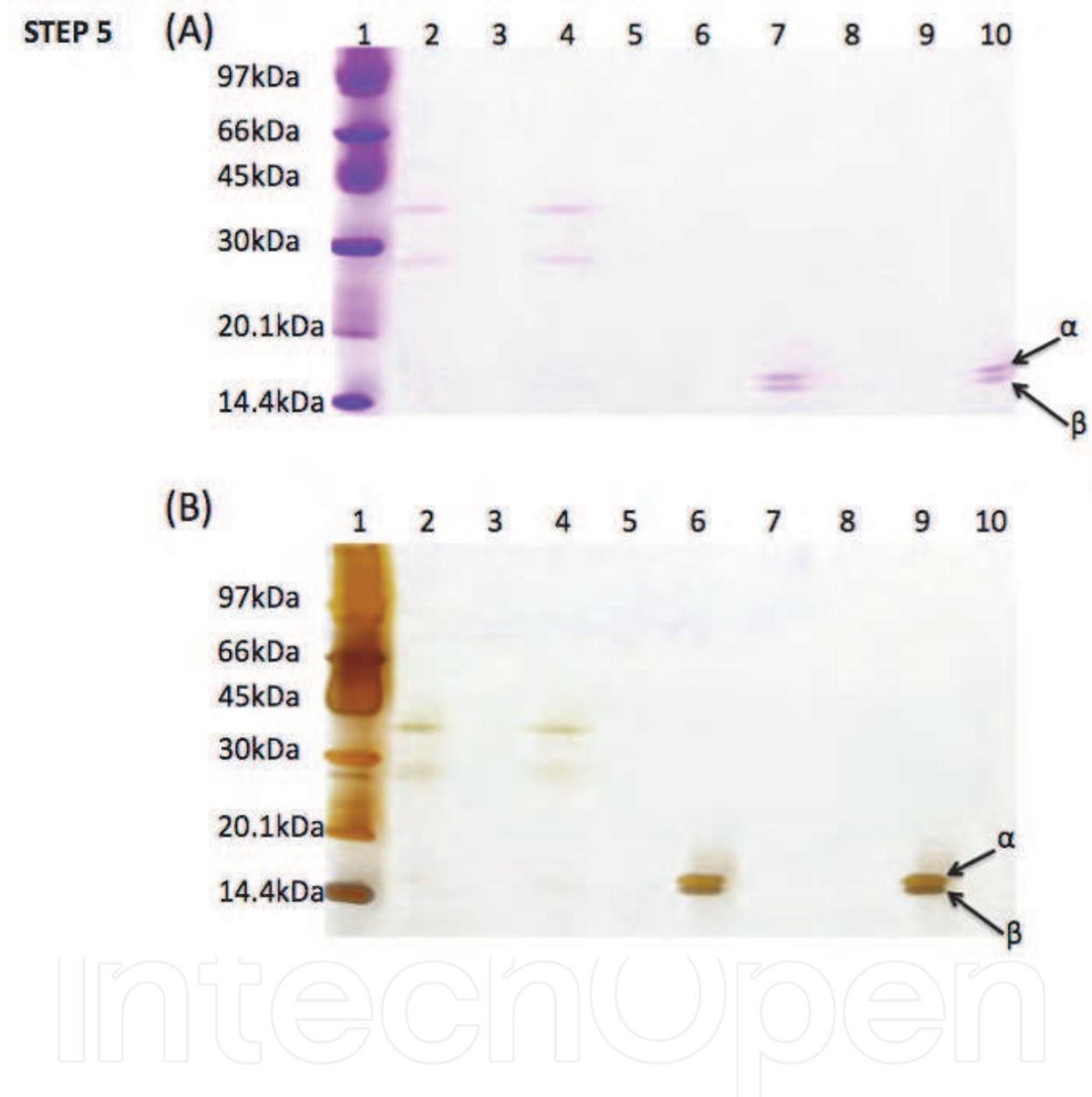


Fig. 13. Step 5 of 2DE-guided purification (SDS-PAGE): Homogeneity of purified rhodocetin from DP2 assessed using 15% SDS-PAGE. The purified rhodocetin showed two distinct bands due to the separation of the heterodimer into its alpha and beta subunits by SDS denaturation. The separated bands were visualized with both (A) Coomassie Brilliant Blue and (B) silver staining. (A) Lane 1: GE Healthcare Low Molecular Weight (LMW) markers; Lane 2: DP1; Lane 3: *blank*; Lane 4: DP1; Lane 5 and 6: *blank*; Lane 7: DP2; Lane 8 and 9: *blank*; Lane 10: DP2. (B) Lane 1: GE Healthcare LMW markers; Lane 2: DP1; Lane 3: *blank*; Lane 4: DP1; Lane 5: *blank*; Lane 6: DP2; Lane 7 and 8: *blank*; Lane 9: DP2; Lane 10: *blank*. The blank wells were intentionally skipped to prevent any effect of inter-well spillage.

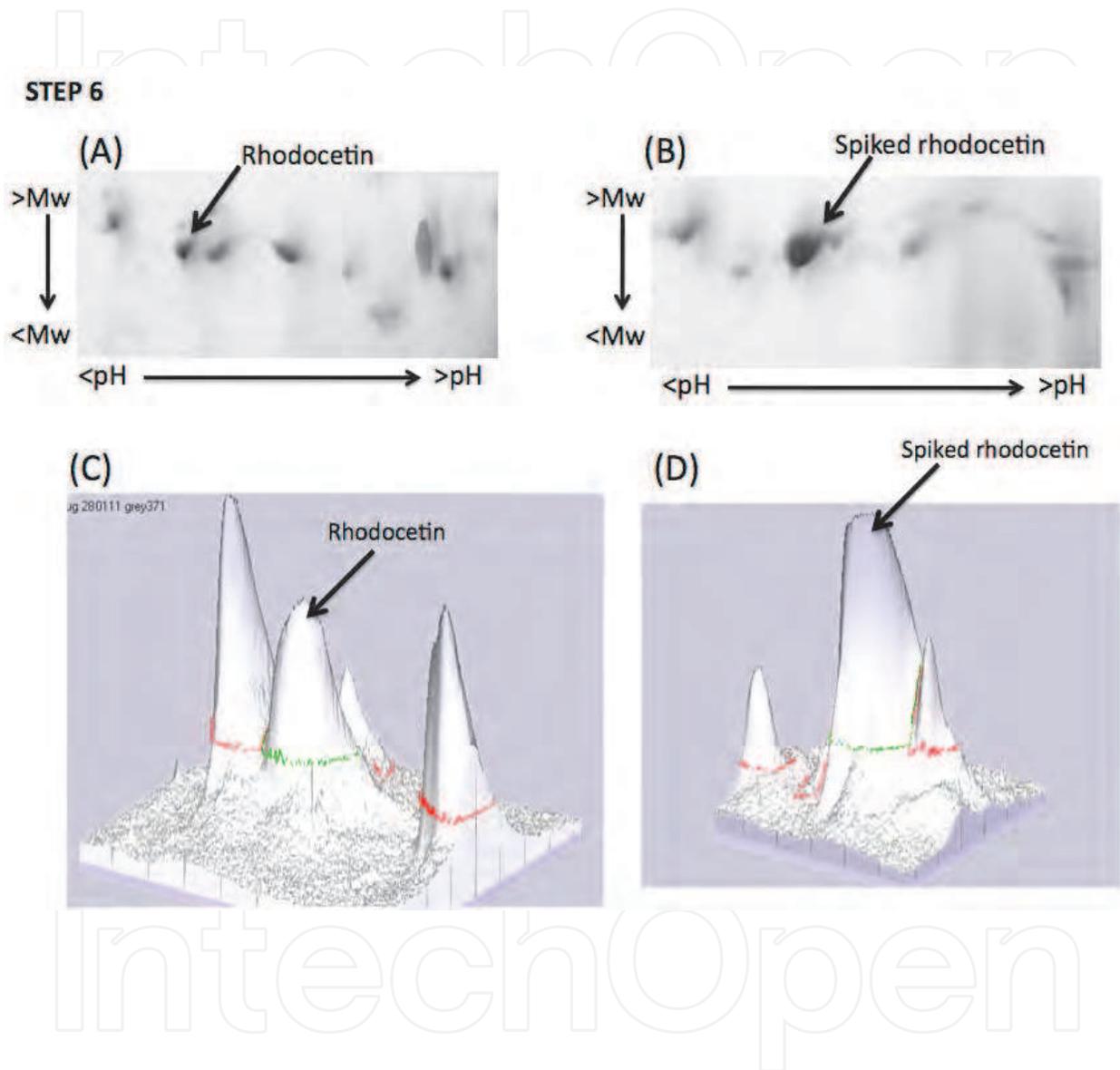


Fig. 14. Step 6 of 2DE-guided purification (spiking): (A) Area of interest on the 2DE profile of crude *C. rhodostoma* venom with the rhodocetin (alpha subunit) spot labelled. (B) The same area showing the spot of spiked rhodocetin with an observed increased intensity. (C) 3D representation views of the rhodocetin (alpha subunit) spot on the crude venom alone and (D) the spiked rhodocetin (alpha subunit) spot, with the latter spot having a quantified 1.6 fold increase in intensity.

Based on our results, we have successfully proved that rhodocetin could be purified using 2DE-guided purification. 2DE profile, in place of an assay, is sufficiently selective and specific to determine which peak contained rhodocetin, therefore allowing us to decide which peak should be selected for further fractionation. While we have only described the use of this method using rhodocetin and *C. rhodostoma*, 2DE is a versatile technique that can be applied to any sample, as long as it is protein containing (Carrette *et al.*, 2006; O' Farrell, 1975). Therefore, we see that this concept is probably one of the most important innovations that we have developed for our laboratory; especially given the fact that 2DE has undergone much development and effort of standardization since its initiation. These efforts have helped to improve 2DE to become a method with a standardized protocol that requires little optimization and is often reproducible. Hence, the following few paragraphs will discuss a few aspects of 2DE-guided purification that may be of concerns to researchers who are interested to utilize this concept in their own laboratories to purify therapeutically important proteins from snake venoms.

We have intentionally selected 2DE over the one-dimensional electrophoretic method SDS-PAGE as the assay to guide our progression in the purification process of rhodocetin, despite the fact that SDS-PAGE could be done much more easily. Given its one-dimensional separation capability, SDS-PAGE has only limited differentiation efficiency of crude venom proteins, owing to the overlapping of protein bands with similar molecular weights (Soares *et al.*, 1998). The protein spots on the 2DE profile, on the other hand, are more specific and are more definite indications of the presence of the proteins in a particular sample.

One of the major limitations of 2DE has always been the time required to perform a single run. The time needed to complete a general large format 2DE gel is often estimated to be 3-5 days (Carrette *et al.*, 2006; Felley-Bosco *et al.*, 1999). Nevertheless, we have selected minigels to be used as our assays in 2DE-guided purification. This has decreased the overall time required, making it possible to complete several simultaneous runs in a single day (Felley-Bosco *et al.*, 1999). In our context of study, the utilization of minigels was also adequate in identifying the rhodocetin spot by comparing the crude *C. rhodostoma* profile on the minigel with that previously done on a larger 18cm format 2DE gel. This is in line with the findings of a study that has also shown that data transfer between large format gel and minigel was compatible (Felley-Bosco *et al.*, 1999). Besides, with the recent advent of 2DE innovations such as the bench top proteomics system ZOOM® IPGRunner™ System (Invitrogen) that allows for rapid first and second dimension protein separation in 2DE, any laboratory can achieve high-resolution 2DE faster, simpler and easier (Pisano *et al.*, 2002).

The detection of spots in 2DE relies critically on the staining method and our utilization of Coomassie Brilliant Blue has been sufficiently sensitive for our progression. The two common staining methods, silver staining and Coomassie Brilliant Blue, stain between 0.04-2ng/mm² and 10-200ng/mm² respectively (Wittman-Liebold *et al.*, 2006). Several recent modifications to the Coomassie Brilliant Blue staining protocol has also greatly increased its sensitivity (Pink *et al.*, 2010; X Wang *et al.*, 2007). As such, the 2DE assay is a sensitive one requiring relatively low amount of sample, as compared to certain bioassays. In addition, the sensitivity of this technique is expected to improve with the development of fluorescent staining (Yan *et al.*, 2000). This is especially important, since progression into further cycle of fractionation only results in reduction of the available sample while bioassay-guided purification of venom's neurotoxins utilizing animal assays require fairly large amount of the sample material (Escoubas *et al.*, 1995). Although a microinjection technique has been

described to address this issue, this technique can be labour intensive and time consuming (Escoubas *et al.*, 1995).

Since liquid chromatography frequently employs salt gradient and utilizes non-volatile buffer (such as Tris-HCl), salt can still be present even after desalting and lyophilisation of the peaks. This was evident by our inability to increase the voltage during IEF resulting in underfocusing of the protein spots. Subsequently, whenever this problem appeared, we prolonged the IEF protocol to an overnight running by introducing an additional first step of 50V at step and hold for 12h. This was found to improve IEF and voltage could be increased up to 5000V. This is in line with the concept of electrophoretic desalting described by Gorg *et al* (1995) in which samples with high salt concentration were directly desalted in the IPG strip using a low voltage during the first few hours of IEF. Davidsson *et al* (2002) also previously reported that such prolonging of IEF run could improve the problem of incomplete focusing due to the presence of ampholytes in cerebrospinal fluid samples.

The biggest limitation of 2DE-guided purification is its dependence on protein profiling efforts and publications of 2DE reference maps. In our study, without prior profiling of rhodocetin into the 2DE reference map of CR, the rhodocetin spot will not be located and consequently, it will be impossible to determine the presence of rhodocetin in the chromatography peaks by 2DE testing. However, this challenge show prospects of improvisation as protein profiling efforts continue to be on the rise in recent years.

5. Conclusion

We hope that the role of 2DE in snake venom study has been effectively underlined in this chapter. While the present setting in the field of proteomic methods is one that tends to incline towards the rapidly advancing non-gel based proteomic methods, it is obvious that 2DE still has the advantages of being a robust technique with high resolution power. In terms of investigating the complexity of snake venoms, it is evident that the application of 2DE is not limited to only whole proteome analysis for taxonomic and envenomation pathology investigations, but is also feasible as an assay in the multistep protein purification process for pharmacologically important venom proteins. There is no standardized workflow as to how 2DE should be used in the investigation of snake venoms. Depending on the objective of the study, 2DE should be innovatively used along with other proteomic methods and its protocol should be appropriately modified in order to meet the study objectives.

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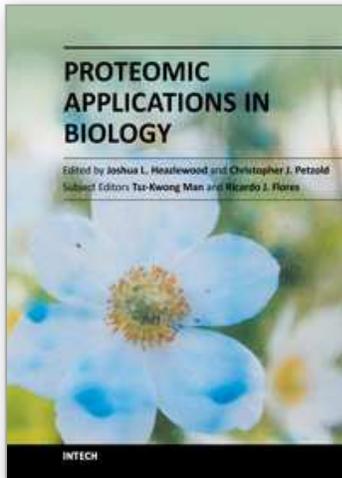
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The past decade has seen the field of proteomics expand from a highly technical endeavor to a widely utilized technique. The objective of this book is to highlight the ways in which proteomics is currently being employed to address issues in the biological sciences. Although there have been significant advances in techniques involving the utilization of proteomics in biology, fundamental approaches involving basic sample visualization and protein identification still represent the principle techniques used by the vast majority of researchers to solve problems in biology. The work presented in this book extends from overviews of proteomics in specific biological subject areas to novel studies that have employed a proteomics-based approach. Collectively they demonstrate the power of established and developing proteomic techniques to characterize complex biological systems.

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