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[illegible]g Alpha-bungarotoxin in 2-DE Gel of *Bungarus multicinctus* [illegible] Banded Krait) Venom

[illegible] J.[1]*, Ibrahim, H.[2] and Othman, I.[1]

[illegible] Medicine and Health Sciences, Monash University Malaysia, Jalan Lagoon Selatan, 46150 [illegible]a, Selangor Darul Ehsan, Malaysia
[illegible]an@med.monash.edu.my Facsimile: 6 03 55146323
[illegible] Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia
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[illegible]T Snake venom being one of the most prominent subsets of natural toxins, has been [illegible] studied as it causes many crippling injuries and death to mankind. It is a well-known fact that [illegible]m is the most complex of all known toxins. In this study identification of a lethal protein in [illegible]m was carried out using the integrated approach of proteomics. Initially, 2D gel [illegible]sis, a component of proteomics, was used to separate low molecular weight proteins from [illegible]lticinctus venom. Next, the location of alpha-bungarotoxin was determined by spiking this [illegible]er with *B. multicinctus* venom using the sample cup loading technique. An image based 2D [illegible]*B. multicinctus* venom only and that spiked with alpha-bungarotoxin showed a 2.5 fold [illegible] volume of one spot. Consequently, identification of this protein spot using 'matrix-assisted [illegible]on ionization-time of flight (MALDI-TOF) mass spectrometry', matched it to alpha-[illegible] This study demonstrated the usefulness of proteomics in locating the position of important [illegible] map of a complex sample such as that of snake venoms.

[illegible] Bisa ular yang merupakan salah satu subset paling menonjol antara toksin semulajadi [illegible]ara terperinci memandangkan keupayaannya menyebabkan kecederaan melumpuhkan dan [illegible]da manusia sejagat. Adalah sedia maklum, bisa ular merupakan toksin paling kompleks di [illegible]ksin yang diketahui. Dalam kajian ini, pengenalan bagi suatu protein berbahaya di dalam [illegible] dilakukan melalui pendekatan bidang proteomik. Bermula dengan 2D gel elektroforeis, [illegible]u komponen kaedah-kaedah proteomik, protein-protein yang mempunyai berat molekul [illegible] dipisahkan dari bisa *Bungarus multicinctus*. Ini diikuti oleh penentuan lokasi alpha-[illegible] dengan cara memperkenalkan protein berkenaan bersama dengan bisa *B. multicinctus* [illegible]h muatan sampel bercawan. Analisis berdasarkan imej 2D bagi bisa *B. multicinctus* sahaja [illegible]i yang ditambah dengan alpha-bungarotoxin telah menunjukkan peningkatan peratusan [illegible]anyak 2.5 ganda bagi salah satu tompok. Seterusnya pengenalan bagi tompok protein ini [illegible]ggunakan 'matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass [illegible]y' telah memadankannya kepada alpha-bungarotoxin. Dengan demikian kajian ini telah [illegible]an kegunaan bidang proteomik dalam menentukan kedudukan bagi protein-protein yang [illegible]m pemetaan 2D bagi sampel yang kompleks seperti bisa ular.

[illegible]n, 2-D electrophoresis, snake venom, krait)

INTRODUCTION

[illegible]ation of proteomics with the use of 2-[illegible]ass spectrometry in studying venoms [illegible] recently received much attention by [illegible]. Probable reason for this could be due [illegible] resurgence of two-dimensional gel electrophoresis (2-DE) and the recent advances in mass spectrometry [1 and 2]. Based on previous records most work done used this application to de-convolve the extreme complexity of natural venoms. For instance, Li *et al.* [3] assessed the venom proteomics profiles from *Naja naja atra* and *Agkistrodon halys* by various different

approaches: (i) shotgun digestion plus HPLC with ion-trap tandem MS, (ii) one-dimensional SDS/PAGE plus HPLC with tandem MS, (iii) gel filtration plus HPLC with tandem MS and (iv) gel filtration and 2-DE plus MALDI-TOF MS [3]. They reported the novel identification of 124 and 74 proteins and peptides in cobra and viper venom respectively. Nawarak *et al.* [4] used two-dimensional electrophoresis and MALDI-TOF MS to identify moderate to high molecular weight glycoproteins in *N. n. kaouthia* venom which were previously fractionated by binding with Con A [4]. These approaches have also been utilized for characterizations of those novel proteins as yet inserted into protein databases. For instance, the molecular mass of purified proteins were determined by MALDI-TOF MS [5 and 6] while 2-DE has been used to determine both molecular weight as well as pI values of the isolated proteins [7]. Other venoms have also been characterized with the use of proteomics applications. Venom of the spider *Selenocosmia huwena* was separated by 2-DE gel to over 300 protein spots and they were analyzed and identified by employing both N-terminal sequencing as well as MALDI-TOF MS [8]. Considerable work has also been performed on scorpion and bee venoms. In one study, molecular mass fingerprint analysis of *Orthochirus innesi* scorpion venom, and identification of components from bumblebee *Bombus lapidaries* venom have been attempted [9].

Certainly, the potential of proteomics technology in unravelling the complexity of natural venoms have been highlighted. In addition this tool also provides enormous versatility to be used in diverse applications ranging from uncovering information of novel proteins to potentially identifying the minute differences of a very closely related organism. All this, together with the fact that this approach is relatively new in the study of snake venom, an investigation utilizing proteomics approach to locate alpha-bungarotoxin from *Bungarus multicinctus* has been attempted in this study

B. multicinctus (many banded krait) is morphologically very much similar to *B. candidus*, the Malayan krait which is common in Malaysia [10]. Members of this genus belonging to the Elapid family are commonly known as kraits and their venom is very toxic, frequently causing death of people who are bitten. Generally, the non-enzymatic protein or polypeptide toxins in the form of neurotoxins dominate the lethal actions of these elapids. Those venoms affecting the release of acetylcholine from the pre-synaptic membrane are called beta-neurotoxins and those affecting the post-synaptic membrane are called alpha-neurotoxins.

MATERIALS AND METHODS

Venoms

B. multicinctus venom and its purified constituent, alpha-bungarotoxin was purchased from Sigma Chemical Co. (U.S.A). Both were in lyophilized form and stored at -20°C.

Two Dimensional Electrophoresis (2-DE)

IPG strips (18 cm) with a linear range of pH 3 – 10 were rehydrated overnight with 340 µl of rehydration solution. After rehydration, the IPG strip was introduced with the venomous proteins via a sample loading cup. Prior to this the venomous proteins were dissolved in 100 µl of rehydration solution containing 8M urea, 2% (w/v) CHAPS, 20 mM DTT (dithiothreitol), 0.5% (v/v) IPG buffer, 0.002% (w/v) Bromophenol Blue. Electrofocusing was carried out at 30 kV.h using IPGphor at 20°C according to the manufacturer's instruction. Before the second dimension, the IPG strips were equilibrated by two equilibration steps: reduction buffer with 50 mM Tris/HCL, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, a trace of Bromophenol Blue and 1 % (w/v) DTT on a rocking table for 10 min; alkylation buffer with 50 mM Tris/HCL, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, a trace of Bromophenol Blue and 2.5% (w/v) iodoacetamide for an additional 10 min. The equilibrated strip was loaded and run on 15% polyacrylamide Laemmli gels (26cm x 20 cm) using the Ettan Dalt II system with a programmable power control, initially 0.5W per gel for 40 min, followed by 15 W per gel till the dye front reached the bottom of gel. The separated gel proteins were visualized by Coomassie Brilliant Blue staining.

MALDI-TOF MS

In-gel digestion of selected spots obtained from 2-DE gels after the final destaining process was performed according to Schevchenko *et al.* (11). The coomasie stained spots were excised and 50µl of acetonitrile. To rehydrate, the supernatant was then replaced with 25µl of 25mM NH_4HCO_3

for 10 minutes. These steps of rehydration followed by dehydration were repeated to give a total of 3 washes. Finally the spots were dried for 5min using a Speedvac.

In-Gel Tryptic Digestion

The dried spots were then resuspended in 10μl of 10ng/μl trypsin in 25mM NH_4HCO_3 and incubated for complete digestion at 37^{0}C overnight. On completion, the gel was once again dehydrated with addition of acetonitrile. Finally the gel piece was removed and the supernatant was dried down in a Speedvac for 20min.

Sample Preparation

An equal amount of tryptic digested samples were mixed together with a matrix solution consisting of α-cyano-4-hydroxy cinnamic acid in acetonitrile acidified with trifluroacetic acid (1mg/ml). Thereafter 0.4μl of this mixture was spotted on the slide and air-dried.

Mass Spectrometry

MALDI-TOF mass spectra of peptide mixture were obtained on an Ettan MALDI-TOF-Pro mass spectrometer with a delayed ion source with nitrogen laser of 337nm. The acceleration voltage was set to 20kV with positive ion reflectron mode. The low mass rejection was activated. Instrument was calibrated externally with peptide samples of adrenocorticotropic hormone and (Ile7) angiotensin III.

RESULTS

Figure 1 shows the 2D map of 0.8μg protein of *B. multicinctus* venom. Next, on another 2-DE run, 2 sample loading cups were inserted at separate location of a IPG strip. One sample loading cup close to the anodic end while the other close to the cathodic end. The anodic located sample loading cup, similar to the earlier experiment, was loaded with 0.8μg protein of *B. multicinctus* venom while the other cup was loaded with 0.1μg protein of the comercially available alpha-bungarotoxin. The results of this experiment showed a significant increase in intensity of one spot found in the 2D map of *B. multicinctus* venom. Spot detection and matching of the 2D gels containing *B. multicinctus* venom only and that of the venom spiked with alpha-bungarotoxin protein was carried out with the aid of Image Master 2D Platinum software. Figure 2 shows the analysis using the software which quantified a 2.5 fold increase in % volume of alpha-bt spot in 2D gel of venom spiked with alpha-bungarotoxin protein. Also inserted in the figure at the top right hand corner of each gel was a clear 3D view representation of the increase in % volume.

Protein in gel plug, corresponding to the spot alpha-bt was cut from the two-dimensional gel stained by CBB and was subjected to MALDI-TOF MS peptide mass fingerprinting. The mass spectra of the peptide from this spot showed multiple peaks ranging from 800 to 4,000 Da (Figure 3). The prominent peaks within this range were selected for comparison with two established databases. The protein with the highest correlation with this spot identified by the internet search database, ExPASy [12], was alpha-bungarotoxin, isoform A31 (Swiss-Prot accession number: P60615). The sequence coverage was 86% with the total number of peptide hits amounting to eight (1319.549, 1335.526, 1390.641, 1447.613, 1463.641, 1576.819, 1616.714, 2804.056). The theoretical mass and pI of this protein was calculated to be 7994 Da and 8.38, respectively. In contrast, MASCOT [13] database search done produced no match to any protein related to the species studied.

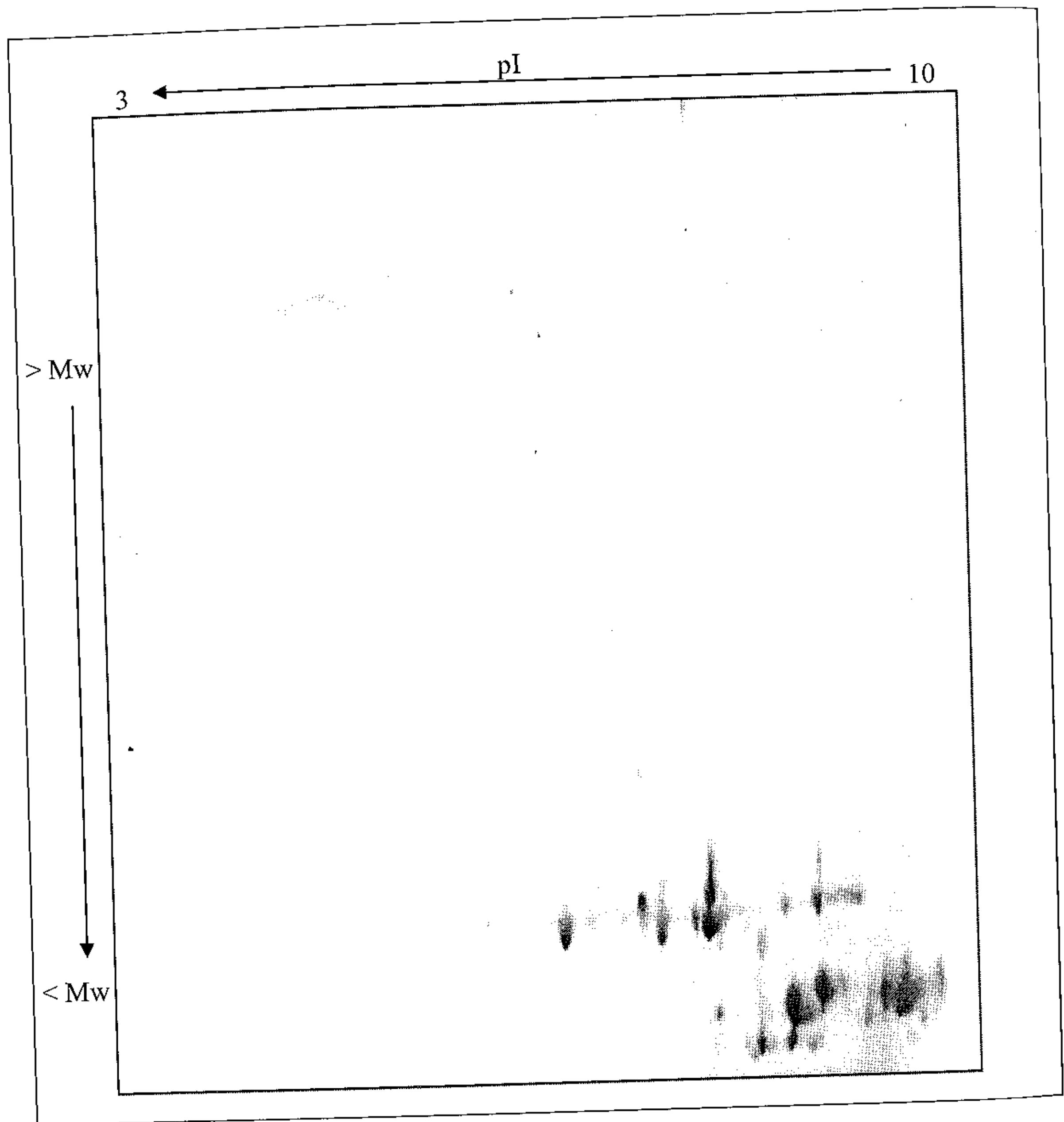

Figure 1. 2-DE of *B. multicinctus* as detected by Coomassie Blue staining (0.8μg protein). The denatured venom was separated by isoelectric focusing in 18cm Immobiline dry strips (pH 3-10; Amersham Biosciences, Sweden). Then, proteins were separated in the second dimension by 15% SDS-PAGE and visualized after coomassie blue staining

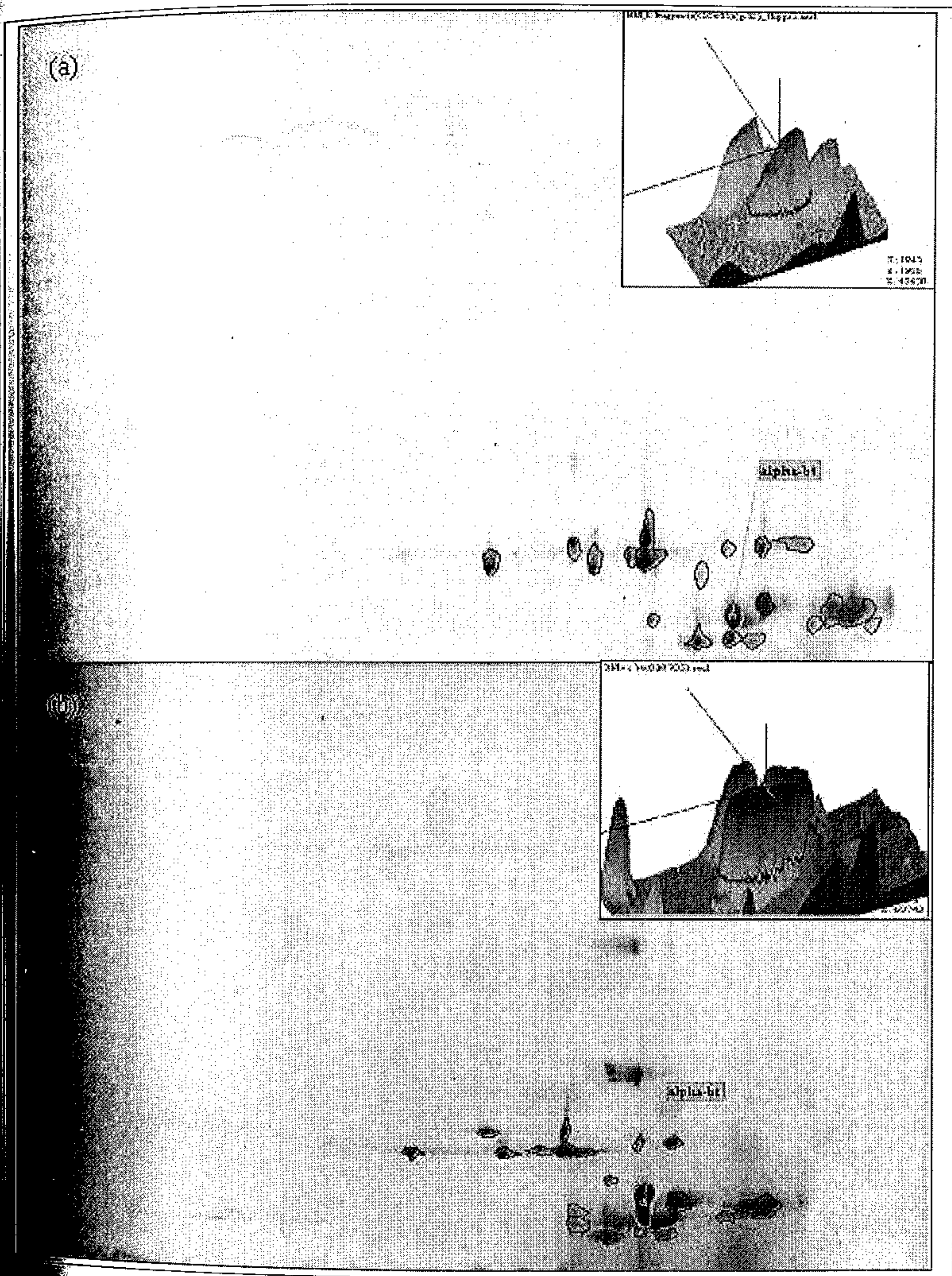

Figure 2. 2D gels containing (a) *B. multicinctus* venom (0.8μg protein) only and (b) that of the venom (0.8μg protein) spiked with alpha-bungarotoxin (0.1μg protein) analysed using Image Master 2D Platinum software. Inserted in the figure at the top right hand corner of each gel was a clear 3D view representation of the 2.5 fold increase in % volume

Figure 3. MALDI-TOF MS profile of a tryptic digest of protein in the gel plug of alpha-bt spot obtained from an Ettan MALDI-TOF Pro mass spectrometer. The prominent mass peaks were chosen for database searches

DISCUSSION

These experiments were done to demonstrate the potential of identifying a known protein in a 2D map consisting of several unidentified protein spots. The protein chosen for the purpose was alpha-bungarotoxin. This protein is the first alpha-neurotoxin purified from *B. multicinctus* wayback in 1963 [14]. Alpha-bungarotoxin belongs to the type II alpha-neurotoxin subfamily (formerly long-chain). Alpha-neurotoxins can bind much more specifically and irreversibly to nicotinic acetylcholine receptor at skeletal muscle neuromuscular junction, producing blockade of neuromuscular transmission at the postsynaptic membrane, leading to paralysis and quick death of victims [15]. Most alpha-neurotoxins are derived from the Elapid (e.g. cobra and krait) and Hydrophid (seasnake) snake venoms [16].

Briefly the method involved spiking the alpha-bungarotoxin protein during the 1st dimension IPGphor run of the 2-DE of *B. multicinctus* venom. Prior to that, the 2-DE of *B. multicinctus* venom was optimized by using the cup loading technique in the 1st dimension isoelectric focusing and consequently, the proteins were separated in the 2nd dimension by 15% SDS-PAGE homogenous gel. Sample application using cup loading was found to produce better results instead of in-gel rehydration. It was also ideal for separating the more basic proteins of snake venoms by applying the loading cup at the anodic site. The selection of 15% homogenous gel was found to be suitable choice to display very low molecular weight proteins as alpha-bungarotoxin was known to have a molecular weight of 8,000 Da with 74 amino acid peptide. A image based analysis between 2-DE gel of *B. multicinctus* venom and that of another spiked with alpha-bungarotoxin revealed the whereabout of the spot belonging to this protein. MALDI-TOF MS peptide mass fingerprinting of this spot identified it to alpha-bungarotoxin protein. Hence, the results of the 2-DE and MALDI-TOF MS both confirmed the location of this protein to the particular spot.

CONCLUSION

Clearly, this technique of utilizing two loading cups with one used for spiking the alpha-bungarotoxin protein can be useful in locating the position of many more important proteins of snake venoms in their respective 2-DE gel.

REFERENCES

1. Serrano, S. M., Shannon, J. D., Wang, D., Camargo, A. C. and Fox, J. W. (2005). A multifaceted analysis of viperid snake venoms by two-dimensional gel electrophoresis: an approach to understanding venom proteomics. *Proteomics* **5**: 501 - 510.
2. Fox, J. W., Ma, L., Nelson, K., Sherman, N. E. and Serrano, S. M. (2006). Comparison of indirect and direct approaches using ion-trap and Fourier transform ion cyclotron resonance mass spectrometry for exploring viperid venom proteomes. *Toxicon*, In Press.
3. Li, S., Wang, J., Zhang, X., Ren, Y., Wang, N., Zhao, K., Chen, X., Zhao, C., Li, X., Shao, J., Yin, J., West, M. B, Xu, N. and Liu, S. (2004). Proteomic characterization of two snake venoms: *Naja naja atra* and *Agristrodon halys*, *Biochem J.* **15**: 119 - 127.
4. Nawarak, J., Phutrakul, S. and Chen, S. T. (2004). Analysis of lectin-bound glycoproteins in snake venom from the Elapidae and Viperidae families. *J. Proteome Res.* **3**: 383 - 392.
5. Juarez, P., Sanz, L. and Calvete, J. J. (2004). Snake venomics: Characterization of protein families in *Sistrurus barbouri* venom by cysteine mapping, N-terminal sequencing, and tandem mass spectrometry analysis. *Proteomics* **4**: 327 - 338.
6. Toyama, O. D., Boschero, C. A., Martins, A. M., Fonteles, C. M., Monteiro, S. H. and Toyama, H. M. (2005). Structure-function relationship of new crotamine isoform from the *Crotalus durissus cascavella*. *Protein J.* **24**: 9 - 19.
7. Oliveira, D. G., Toyam, M. H., Novello, J. C., Beriam, L. O. and Marangoni, S. (2002). Structural and functional characterization of basic PLA2 isolated from *Crotalus durissus terrificus* venom. *Protein Chem.* **21** (3): 161 - 168
8. Liang, S., Li, X., Cao, M., Xie, J., Chen, P. and Huang, R. (2000). Indentification of venom proteins of spider *S. huwena* on two-dimensional electrophoresis gel by N-terminal microsequencing and mass spectrometric peptide mapping. *Protein Chem.* **19**: 225 - 229.
9. Favreau, P., Menin, L., Michalet, S., Perret, F., Cheneval, O., Stocklin, M., Bulet, P. and Stocklin, R. (2006). Mass spectrometry strategies for venom mapping and peptide

sequencing from crude venoms: Case applications with single arthropod specimen. *Toxicon*. 47: 676 - 687.

Soderberg, P. (1973). On eleven Asian elapid snakes with specific references to their occurance in Thailand. *Nat. Hist. Bull. Siam. Soc.* **24**: 203 - 317.

Schevchenko, A., Wilm, M., Vorm. O., and Mann, M. (1996). Mass spectrometric sequencing of proteins from silver-stained polyacrylamaide gels. *Electrophoresis* **68**: 850 - 858.

ExPASy (URL's: http://www.expasy.org/tools/aldente/)

MASCOT (URL's: http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF)

Chang, C. C. and Lee, C. Y. (1963). Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes neuromuscular blocking action. *Arch. Int. Pharmacodyn.* **144**: 241 - 257.

Lewis R. L. and Gutmann L. (2004). Snake venoms and the neuromuscular junction. *Semin Neurol.* **24**: 175 - 179.

Hall, Z. W. (1999). Alpha neurotoxins and their relatives: Ffoes and friends? *Neuron* **23**: 4 - 5.

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N. Eriyusni*, T. K. M

Institute of Biological Sc
Malaysia
* eriyusni@hotmail.com
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