EXTRACTION AND PURIFICATION OF SAXITOXIN-BINDING PROTEIN FROM MARINE RESOURCE

MUHD FAIZUL HELMI AHAMAD HASMI

THIS DISSERTATION IS SUBMITTED IN PARTIAL FULLFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
BACHELOR OF SCIENCE WITH HONOURS

BIOTECHNOLOGY PROGRAMME
SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SABAH

2007

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS@

JUDUL: EXTRACTION AND PURIFICATION OF SAXITOXIN-BINDING PROTEIN FROM MARINE RESOURCE

Ijazah: SARJANA KEPUJIAN (BIOTEKNOLOGI)

SESI PENGAJIAN: _____

Saya MUHD FAIZUL HELMI AHAMAD HASMI

(HURUF BESAR)

mengaku membenarkan tesis (LPS/Sarjana/Doktor Falsafah)* ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. **Sila tandakan (/)

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)
☑	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
☐	TIDAK TERHAD	

Disahkan oleh

(TANDATANGAN PENULIS)

(TANDATANGAN PUSTAKAWAN)

Alamat Tetap: 2326, JLN 3/8
BANDAR BARU SG. BULOH,
47000 SG BULOH, SELANGOR.

PROF DATUK DR. KAMARUZAMAN AMPON
Nama Penyelia

Tarikh: 30 NOV 2007

Tarikh: _____

CATATAN: * Potong yang tidak berkenaan.

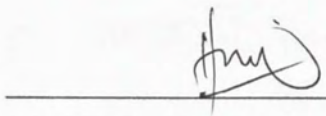
** Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.

@ Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau disertasi bagi pengajian secara kerja kursus dan penyelidikan, atau Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I admit that this report is my own effort except for all the footnotes and references which I have mentioned their sources in detailed.

9 NOVEMBER 2007



MUHD FAIZUL HELMI AHAMAD HASMI

HS 2004-2612

AUTHENTICATION

Signature

1. **SUPERVISOR**

   (PROF. DATUK DR. KAMARUZZAMAN AMPON)

2. **EXAMINER 1**

   (DR. ROZIAH BTE HJ. KAMBOL)

3. **EXAMINER 2**

   (DR. IVY WONG NYET KUI)

4. **DEAN**

   (PROF. MADYA DR. SHARIFF A. K. S. OMANG)

ACKNOWLEDGEMENT

In the first place, I am thankful to Allah Almighty on the success and completion of this project.

I am grateful to both of my parents Ahamad Hasmi Bin Sulaiman and Jalilah Bte Saleh for being very supportive throughout the time I spent in Universiti Malaysia Sabah. Thanks also to all of my sisters; Hasliza, Shahidah and Khadijah for their help during the hard time.

Moreover, I would also like to record my sincere appreciation to my supervisor Prof. Datuk Dr. Kamaruzzaman Ampon, who had kindly facilitated and advised me in conducting this project. Without him, this research will lose its dignity. Furthermore, a very special thanks to all of the biotechnology lecturers in University Malaysia Sabah, who have guided and taught me with the fundamental concept of biotechnology, where it is the basic of everything result in this dissertation.

Here, I also would like to convey my gratitude to Mr Chong Tong Seng, for his willingness to teach me and provided valuable thoughts and ideas in completing this project.

Finally, I would like to say thank you to all of my friends Faiz Khodri, Azli Ariffin, Asis Hannase, Fakhrul Amin, Mohd Haizal, Azfar Karim, Nik Adham, Hamdi Mat Isa, Azlan Ismail, Mohd Saufi, Mohd Shahin, Mohd Kamal, Mohd Hazwan, Amrul Amin and Hafiz Halim for being supportive and helpful for all of the time. Special thanks to all of you and may Allah s.w.t bless you.

ABSTRAK

Kajian ini dijalankan adalah untuk mengekstrak dan menulenkan protein yang mengikat kepada saxitoxin daripada plasma darah ikan buntal. Kesemua ikan adalah dari sepsis yang sama iaitu *Arothron reticularis*. Kesemua sampel ikan di tangkap di sekitar Pantai Tanjung Lipat dan Sungai Likas. Sebanyak 20 ml protein plasma telah dikumpulkan menerusi pengemparan 39.6 ml darah yang dikeluarkan daripada kesemua 12 ekor ikan buntal yang telah ditangkap. Plasma darah ini mengandungi protein sebanyak 811.2 mg. Kemudian, jumlah jumlah protein di dalam plasma darah telah menurun dengan drastik kepada 184.79 mg setelah ditambahkan dengan pepejal ammonium sulfat kepada 50 dan 70 peratus kepekatan. Setelah itu, penulenan protein dengan menggunakan kolum kromatografi penukaran anion Uno Q1 telah memisahkan protein tersebut kepada lima pecahan. Di dalam proses ini, 50 mM Tris-HCl (pH 7.4) telah digunakan sebagai larutan penimbal bergerak dan 0.25 M NaCl sebagai larutan penimbal pemisah. Pecahan yang menunjukkan penyerapan pada 280 nm tertinggi adalah di pecahan yang kelima iaitu F5, dengan penyerapan sebanyak 0.2382 dan pecahan yang ketiga, F3 telah menunjukkan penyerapan terendah iaitu pada 0.0093. Kemudiannya, tiga lagi pecahan iaitu F1 dengan serapan sebanyak 0.0317, F2 dengan serapan 0.0174 dan akhirnya F4 dengan serapan sebanyak 0.0699. Proses pemisahan ini diulang sehingga kesemua sampel telah habis dipisahkan. Kemudian, kesemua pecahan protein yang sama dari setiap pemisahan telah dikumpulkan dan jumlah protein didalam setiap pecahan tersebut ditentukan. Akhirnya, F1 menunjukkan kehadiran protein sebanyak 4.423 mg, F2 dengan 3.907 mg, F3 dengan 2.268 mg, F4 dengan 9.421mg and F5 dengan kehadiran sebanyak 27.568 mg protein.

ABSTRACT

This study aims to extract and partially purify the saxitoxin-binding protein from the plasma protein of *Arothron reticularis*, puffer fish. The fish samples were collected along Pantai Tanjung Lipat and Sungai Likas. A total of 20 ml of plasma was collected after centrifugation of 39.6 ml of blood withdrawn from 12 fresh fish samples. This plasma contained 811.2 mg of protein. The amount of protein decreased drastically to 184.79 mg after the addition of solid ammonium sulfate to 50 and 70 percent of saturations. Next, the purification using Uno Q1 anion exchange column chromatography with 50 mM Tris-HCl (pH 7.4) as the running buffer and 0.25 M NaCl as the elution buffer separated the proteins into five fractions. The highest fraction peak collected is in the F5 fraction where the absorbance was 0.2382 and the lowest peak is in the F3 fraction, with absorbance of 0.0093. The other three fractions are F1 with 0.0317, F2 with 0.0174 and finally the F4 with absorbance of 0.0699. After further separation of the total protein sample left, the same fractions from each separation were pooled together and the concentrations of each fraction were determined. Finally, F1 shows the presence of 4.423 mg of protein, F2 with 3.907 mg, F3 with 2.268 mg, F4 with 9.421mg and F5 with 27.568 mg.

CONTENT

LIST OF TABLE

LIST OF FIGURE

LIST OF PHOTO

LIST OF SYMBOL

cm	centimeter
Da	Dalton
V	volt
°C	degree Celsius
%	percent
g	gram
kg	kilogram
mg	milligram
mm	millimeter
nm	nanometer
M	molar
mM	millimolar
µM	micromolar
L	liter
ml	milliliter
µl	microliter
nl	nanoliter
Fe^{3+}	Ferum ion
NaCl	Sodium chloride
Na^{+}	Sodium ion
PEG	Polyethylineglycol
cDNA	deoxyribonuclease acid
HCl	Hydrochloride acid
UV	ultra violet
PSP	Paralytic Shellfish Poisoning
HPLC	High Performance Liquid Chromatography

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

Over the last few decades, many coastal regions throughout the world have experienced incidences of algal blooms, which are harmful because of their potential threat to humans as well as marine organisms. In Malaysia, harmful algal blooms or HABs occurs in the South China Sea in the coastal waters of west Sabah, Sebatu in Malacca and also Tumpat in Kelantan (Lim *et al.*, 2002). The causative organism is the dinoflagellate, *Pyrodinium bahamense* var. *compressum*. During algal blooms, the microorganisms which usually are not very numerous in the seas undergo a population explosion. The numbers become so large and dense that sometimes they impart a brownish-red color to the sea. These blooms result in massive fish kills and mortalities to marine mammals, sea birds and also human due to the production of the natural neurotoxins which are saxitoxin and tetrodotoxin (Kirkpatrick *et al.*, 2005).

Once the toxin is synthesized, it is accumulated and metabolized by the shellfish. This will become a public health problem when people eat these toxic shellfish and suffer the Paralytic Shellfish Poisoning, or simply known as PSP. PSP toxins are potent, reversible blockers of the voltage-activated sodium channels (Okumura *et al.*, 2005). As

we all know, the conductance and amplification of the electrical impulse in nerve and muscle membrane results from the action of the voltage-sensitive ion conductance channels. So, when the channel is blocked, the patient suffers neurological distress, which typically appears within 15 to 30 minutes after consumption of the contaminated food and can result in death (Aversano *et al.*, 2005).

Besides the shellfish, saxitoxin also has been isolated from the puffer fish. Puffer fish is well known as a very poisonous fish and may cause a characteristic clinical poisoning with a high mortality rate. It is known that, the poison of puffers are composed of saxitoxin and also another cationic toxin which is known as tetrodotoxin, the predominant toxin is depending on the species (Oliveira *et al.*, 2006). In countries like Japan and Australia, the flesh of the puffer fish is well considered as a delicacy (Isbister *et al.*, 2002). It is prepared by specially trained chefs who are certified by the government to prepare the flesh free of the toxic liver, gonads, and skin (El-Sayed *et al.*, 2003). Despite of these precautions, there are still many cases of tetrodotoxin and saxitoxin poisoning reported each year in patients ingesting puffer fish.

In particular, there is an increasing need to monitor the occurrences of these toxins in the sea. At present, it is impossible to predict with any degree of accuracy either the precise timing and location of red tide blooms or the duration of the contamination of shellfish with saxitoxin during and after the blooms. There are also at present, no effective strategies to destroy or control red tide blooms. Consequently, both extended monitoring, in terms of the number of monitored locations and the frequency of

monitoring and the ability to respond rapidly to red tide blooms are of critical importance to prevent the loss of human life.

Through the research done by Krishnan *et al.* (2001), the saxitoxin actually binds to one type of protein named saxiphilin. This protein is contained in the plasma and tissue of certain vertebrates and invertebrates that specifically binds the neurotoxin saxitoxin. Thus, from a better understanding about the binding of saxitoxin to saxiphilin, we can develop a biosensor which can detect, monitor and forecast the development the algal blooms in the sea water.

Thus, the objective of this research is to extract and partially purify the saxitoxin-binding protein from the pufferfish.

CHAPTER 2

LITERATURE REVIEW

2.1 Algal blooms

Algal bloom also known as red tide is the result of a massive multiplication or blooming of the tiny, single celled algae which usually found in warm saltwater. It is a natural phenomenon, apparently unrelated to man made pollution. In high concentrations, the algae may create a brownish red color on the surface of the water; in other instances, it may look yellow green, or may not be visible at all (El-Sayed *et al.*, 2003). Some red tides have covered up to several hundred square miles of water. Most importantly, until now, no one can predict when or where the red tides will appear or how long they will last since they are affected by many variables such as weather and currents.

Algae, like the other microscopic single celled organisms, grow by asexual reproduction. Then, each of the resulting cells can go on to divide again, and again, and so on. Starting with only one cell, if the cell population from each generation increases by a factor of 2^n (where n is the number of generations), it is clear that after a relatively small number of generations, the number of cells will be very large. In the oceans a generation

can range from hours to a few days. Most noticeable algal blooms in the aquatic environment range from 100,000 to 1,000,000 cells per liter (Plumley, 1997).

It is generally accepted that bloom initiation is caused by the right set of environmental conditions, which are the combination of nutrients, sunlight and temperature. These conditions can be provided on a local basis by natural basis from land or by human inputs, like the treated or untreated sewage, farming or urban gardening practices. Those algae give significant impact on ecosystem processes as they synthesized potent neurotoxins such as tetrodotoxin and saxitoxin. These toxins affect the viability, growth and recruitment of a wide range of organisms. Many researchers view these toxins as secondary metabolites of algae (Plumley, 1997).

In Malaysia, the blooms occur periodically every year in the coastal waters of west Sabah, Sebatu in Malacca and also Tumpat, Kelantan (Lim *et al.*, 2002). Since 1976, these blooms were reported to contribute to too many paralyses and deaths of humans. This is because the dinoflagellate actually produces potent neurotoxins that will cause Paralytic Shellfish Poisoning (Lim *et al.*, 2005). Besides that, the red tide toxins are also deadly to fishes, marine mammals and birds. Filter feeding shellfish, such as oysters, clams, mussels and other bivalve mollusks, are unaffected because they were able to consume the algae and concentrate the toxin in various organs. Furthermore, it was also reported that the puffer fish was tolerant to the neurotoxins (Naguchi *et al.*, 2005).

2.2 Puffer fish

Puffer fish belongs to the family Tetradontidae, and are also called blowfish. There are many different species of puffer fish which live in fresh water, some live best in brackish water and some puffers live best in marine or sea water. Because they live in this kind of water, they have various colors and sizes. They are called puffer fish because of their ability to inflate themselves with water or air when they feel threatened. It is a defensive mechanism (Mohsin & Ambak, 1996).

Puffer fish are well known as poisonous and may cause a characteristic clinical poisoning with a high mortality rate. The poison of puffer fish may be compose of both tetrodotoxin and saxitoxin (Oleveira *et al.*, 2006). Both toxins are cationic neurotoxins. These toxins in puffer fish come from external sources like the dinoflagellates and accumulate in tissues such as liver and eggs (Tanner *et al.*, 1996). Although that, puffer fish is not poisoned by the toxin that accumulates insides its bodies. This is because the puffer fish has a mutation in the protein sequence of the sodium channel pump. This point mutation in the amino acid sequence compared to the sequence in human shows that these fish is highly resistant to those toxin poisoning (Naguchi *et al.*, 2005). As a result, the toxin does not recognize the channel in puffer fish and therefore does not bind to it and block it. The puffer fish store high concentrations of tetrotodoxin and saxitoxin in various organs.

2.2.1 Type of puffer fish

In this research, the saxiphilin protein is isolated from *Arothron reticularis* puffer fish found in the coastal region around Kota Kinabalu. Photo 2.1 shows the picture of the fish.

Photo 2.1 The picture of *Arothron reticularis.*

This type of puffer fish is nearly cylindrical in a cross-section of it body, with a broad head and back. The entire head and body is covered with small prickles, except for the lips, posterior end of caudal peduncle and fins. It has moderate eyes that are situated below the head dorsal profile. More over, this *Arothron reticularis* does not breathe like a normal fish, because it lacks of the gills. This means that this type of fish breathe air using the lungs. Besides that, it has a moderate terminal mouth, where the teeth fused into a beak with a median suture. The lateral line of the fish is inconspicuous but single and strongly arched in its anterior part.

The habitat of this fish covers the region from India to Indo-west Pacific including Japan. Thus, this means that this type of fish also can be found in the Malaysian waters. It feeds mainly on planktonic items such as copepods, amphipods, cumaceans and fish eggs. The fish can grow up to 11 to 38 cm and feed mainly on crabs. The maximum size of this fish can achieved is up to 42 cm (Mohsin & Ambak, 1996).

2.3 Neurotoxin

A neurotoxin is a toxin that acts specifically on the nerve cells, neurons, usually by interacting with membrane proteins and ion channels. The toxin actually affects the nervous system by causing depolarization of nerve and muscle fibers due to increased sodium ion permeability of the excitable cell membrane. Ion channels catalyze the diffusion of inorganic ions down their electrochemical gradients across cell membranes. Because the ionic movements are passive, ion channels would seem to be extraordinarily simple physical systems, and yet they are responsible for electrical signaling in living cells. Among their many functions, ion channels control the pace of the heart, regulate the secretion of hormones into the bloodstream, and generate the electrical impulses underlying information transfer in the nervous system. So, once the neurotoxin interferes with the ions channels, it will cause paralyzing and death. In this research, there are two types of neurotoxin that is important in the study of saxiphilin, tetrodotoxin and saxitoxin. Both of these neurotoxin shares the same binding site at the saxiphilin protein and affect the sodium ion channels by causing depolarization (Freitas *et al.*,1996).

2.3.1 Saxitoxin

Saxitoxin is a cationic neurotoxin that possess guanidinium group in it chemical structure. It is a product of marine dinoflagellates belonging to *Alexandrium*, *Pyrodinium* and *Gymnodinium* genera and freshwater cyanobacteria such as *Aphanizomenonflos-aquae*, *Anabaena circinalis* and *Lyngbya wollei* (Arvesano *et al.*, 2005). This toxin has a molecular mass of 299.29 $gmol^{-1}$. The chemical structure of saxitoxin is shown in the Figure 2.1.

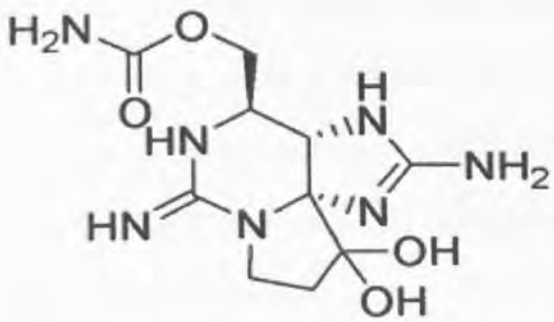


Figure 2.1 The chemical structure of saxitoxin (Yamashita *et al.*, 2001).

Although it is a natural product of the dinoflagellates, but once synthesized, the toxin is accumulated and metabolized by the shellfish. Besides that, it is found that puffer fish also accumulated the toxin at an extremely high concentration in their tissues. This will contribute to the public health problem as the peoples eat the contaminated shellfish and puffer fish and suffer the Paralytic Shellfish Poisoning as the toxin blocks the voltage-activated sodium channels.

However, the toxin accumulation and metabolism systems in shellfish and puffer fish have not been well characterized. Although that, it is known that there is a soluble proteins that have the ability to binds with the toxin. This protein is found in body fluid of many mammals and vertebrates and known as saxiphilin.

a. Saxitoxin modes of action

As stated before, saxitoxin is a type of cationic neurotoxin. Therefore, it affects the neurological system of the victims. More specifically, saxitoxin inhibits the conductance of action potentials within excitable membranes by binding to it and blocking the sodium ion channels (Yamashita *et al.*, 2001). Saxitoxin binds to what is known as site 1 of the sodium channel. Site 1 is located at the extracellular pore opening of the ion channel. The binding of any molecules to this site will temporarily disable the function of the ion channel.

The sodium ion channel is an important transmembrane glycoprotein composed of three subunits: alpha, beta-1, and beta-2. An alpha subunit forms the core of the channel. When the alpha subunit protein is expressed by a cell, it is able to form channels which conduct Na^+ in a voltage-gated way, even if beta subunits are not expressed. When beta subunits assemble with alpha subunits, the resulting complex can display altered voltage dependence and cellular localization. The alpha and beta-2 subunits are connected by one or several disulfide bonds (Cooper, 2000). In vitro removal of the beta-2 subunit

REFERENCE

Abdul Latif, R. and Mokhtar, N. M. 2001. *Asas Biokimia*. Dewan Bahasa dan Pustaka, Kuala Lumpur.

Agnew, W. S., Levinson, S. R., Brabson, J. S. and Raftery, M. A. 1978. Purification of the tetrodotoxin-binding component associated with the voltage-sensitive sodium channel from *Electrophorus electricus electroplax* membranes. *Proc. Nati. Acad. Sci. USA* **75(6)**: 2606–2610.

Ahn, Y. H. and Shanmugam, P. 2006. Detecting the red tide algal blooms from satellite ocean color observations in optically complex Northeast-Asia Coastal waters. *Remote Sensing of Environment* **103(4)**: 419-437.

Aversano, C. D., Hess, P. and Quilliam, M. A. 2005. Hydrophilic interaction liquid chromatography–mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins. *Journal of Chromatography A* **1081**: 190–20.

Campbell, M. K. and Farrell, O. S. 2003. *Biochemistry*. Fourth Edition. Thomas Learning, Inc., United States of America.

Cooper, G. M. and Hausman, R. E. 2000. *The Cell: A Molecular Approach.*Third Edition. ASM Press, Washington.

Dixon, M. and Webb, E. C. 1979. *Enzymes*. Third Edition. Longman Group Limited, London.

El-Sayed, M., Yacout, G. A., El-Samra, M., Ali, A. and Kotbb, S. M. 2003. Toxicity of the Red Sea pufferfish *Pleuranacanthus sceleratus* "El-Karad". *Ecotoxicology and Environmental Safety* **56**:367–372.

Freitas, J. C., Ogata, T., Veit, C. H. and Kodama, M. 1996. Occurrence of tetrodotoxin and paralytic shellfish toxins in *Phallusia nigra* (Tunicata ascidicea) from the Brazillian coast. *Journal Venom Animal Toxins* **2**: 104-793.

Isbister, G. K., Son, J., Wang, F., Maclean, C. J., Lin, C., Ujma, J., Balit, C. R., Smith, B., Milder, D. G. and Kiernan, M. C. 2002. Puffer fish poisoning: a potentially life-threatening condition. *The Medical Journal of* Australia **177 (11-12)**: 650-653.

Janson, J. C. and Ryden, L. 1996. Protein purification, principles, high resolution methods and applications. Wiley VCH, New York.

Karlson, P. 1975. *Introduction to Modern Biochemistry*. Fourth Edition. Academic Press, New York.

Karp, G. 2003. *Cell and Molecular Biology Concept and Experiment*. Third Edition. John Willey and sons, Inc. United States of America.

Kirkpatrick, B., Fleming, L. E., Backer, L. C., Bean, J. A., Tamer, R., Kirkpatrick, G., Kane, T., Wanner, A., Dalpra, D., Reich, A. and Baden, D. G. 2005. Environmental exposures to Florida red tides: Effects on emergency room respiratory diagnoses admissions. *Harmful Algae* **5(5)**: 526-533.

Krishnan, G., Morabito, M. A. and Moczydlowski, E. 2001. Expression and characterizations of Flag-epitope and hexahistidine-tagged derivatives of saxiphilin for use in detection and assay of saxitoxin. *Toxicon* **39**: 291-301.

Lehninger, A. L. 1975. *Biochemistry*. Second Edition. Worth Publisher, USA.

Lim, P. T., Leaw, C. P. and Usup, G. 2002. Status of HAB and potential remote sensing application in detection of HAB events in Malaysia water. Universiti Kebangsaan Malaysia, Bangi.

Lim, P. T., Usup, G., Leaw, C. P. and Ogata, T. 2005. First report of *Alexandrium taylori* and *Alexandrium peruvianum* (Dinophyceae) in Malaysia waters. *Harmful Algae* **4**: 391-400.

Llewellyn, L. E. and Moczydlowski, E. G. 1994. Characterization of saxitoxin binding to saxiphilin; a relative of the transferrin family that displays pH-dependent ligand binding. *Biochemistry* **33(40)**:12312-12322.

Llewellyn, L. E. and Doyle, J. 2000. Microtitre plate assay for paralytic shellfish toxins using saxiphilin: gauging the effects of shellfish extract matrices, salts and pH upon assay performance. *Toxicon* **39(2-3)**: 217-224.

Mahar, J., Lukács, G. L., Li, L., Hall, S. and Moczydlowski, E. 1990. Pharmacological and biochemical properties of saxiphilin, a soluble saxitoxin-binding protein from the bullfrog (*Rana catesbeiana*). *Toxicon* **29(1)**: 53-71.

Mohammad, C. J. and Pihie, A. H. L. 1998. *Asas Biologi dan Biokimia 1*. Universiti Putra Malaysia, Serdang.

Mohsin, M. A. K. and Ambak, M. A. 1996. *Marine Fishes and Fisheries of Malaysia and Neighbouring Countries*, Serdang.

Naguchi, T., Arakawa, O. and Takatani, T. 2005. TTX accumulation in pufferfish. *Comparative Biochemistry and Physiology* **Part D1**: 145-152.

Oliveira, J. S., Fernandes, C. R., Schwartz, C. A., Bloch, C., Meloc, J.A.A., Pires, O. R. and Freitas, J. C. 2006. Toxicity and toxin identification in *Colomesus asellus*, an Amazonian (Brazil) freshwater puffer fish. *Toxicon* **48**: 55–63.

Okumura, M., Tsuzuki, H. and Tomita, B. 2005. A rapid detection method for paralytic shellfish poisoning toxins by cell bioassay. *Toxicon* **46**: 93-98.

Plumley, G. F. 1997. Marine algal toxin: Biochemistry, genetics and molecular biology. *Limnol. Oceanogr* **42**: 1252 – 1264.

Price, N. C. 1996. *Proteins LabFax*. BIOS Scientific Publishers Limited, United Kingdom.

Scrimgeour, K. G. 1977. *Chemistry and control of enzyme reactions*. Academic Press Inc. London.

Scopes, R. K. 1994. *Protein purification, principles and practices*. Springer Verlag. New York.

Smith, C. 2005. Striving for purity: advances in protein purification. *Nature Methods* **2**: 71 – 77.

Tanner, P., Prezekwas, G., Clark, R., Ginsberg, M. and Waterman, S. 1996. TTX Poisoning Associated with Eating Puffer Fish Transported from Japan–California. *Assoc. J. Am. Med.* **275**:1631.

Voet, D., Voet, G. J. and Pratt, C. W. 1996. *Fundamental of Biochemistry*, John Wiley and Sons, USA.

Wua, Z., Yang, Y., Xie, L., Xia, G., Hud, J., Wang, S. and Zhang, R. 2005. Toxicity and distribution of tetrodotoxin-producing bacteria in puffer fish *Fugu rubripes* collected from the Bohai Sea of China. *Toxicon* **46**: 471–476.

Yamashita, M. Y., Sugimoto, A., Terakawa, T., Shoji, Y., Miyazawa, T. and Yasumoto, T. 2001. Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish, *Fugu pardalis*. *Biochemistry* **268**: 5937–5946.