FUNDAMENTAL STUDY OF BIOACTIVE PEPTIDES ON SELECTED *Alexandrium spp.* AT DIFFERENT GROWTH PHASES

BY

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ABSTRACT

The naturally occurring phenomenon of harmful algae blooming (HAB) at the water column brought detrimental effects to the economy as well as the environmental health of the water ecosystem. Most cases of HABs reported in Malaysia waters are dominated by dinoflagellates *Alexandrium spp.* with some of the species were reported to cause shellfish poisoning. In this study, two *Alexandrium spp.*; *A. tamiyavanichii* and *A. leei* with different toxicity levels were analyzed using proteomic approaches involving two-dimensional PAGE and HPLC analysis. The growth pattern of both species were identified and compared by using protein profiles at each growth phases. Protein expression reduced throughout the growth phases of *A. tamiyavanichii* but elevated during stationary phase of *A. leei*. A short duration of stationary phase suggests the continuous expression of growth proteins in *A. leei*. GNAT family acetyltransferase and lipases were successfully identified enzyme protein in *A. tamiyavanichii* and *A. leei* respectively with growth regulatory functions. The toxin profiles of both species exhibited higher level of toxin content in *A. tamiyavanichii* with 88 mol % of total toxins recorded as compared to 12 mol% in *A. leei*. The highest toxin content was recorded during exponential phase of *A. tamiyavanichii* with dominance of GTX4 and STX congeners. Further investigation on the toxicity effects of *Alexandrium spp*. was studied by using oysters where the shellfish are fed with toxic *A. tamiyavanichii* and non-toxic *A. leei*. LC-MS results of the two up-regulated spots in oysters fed with *A. tamiyavanichii* were identified as arginine kinase and transgelin. The up-regulation of the proteins was induced as responsive mechanism towards accumulation of the toxic species *A. tamiyavanichii*. These responses reflect the oyster stability during HAB outbreak. Fundamental studies of dinoflagellates from its molecular as well as byproduct analysis are useful to understand the biochemistry of the HAB species. The findings from this study can provide the basis knowledge on the biochemical properties of HAB species and the behavioral of affected organisms.

خلاصة البحث

لقد سببت ظاهرة انتشار الطحالب المضرة في الماء آثارا مدمرة للاقتصاد وللصحة البيئية والنظام البيئي المائي. أن معظم حالات انتشار الطحالب المضرة الموثقة في المياه الماليزية يسيطر عليها رتبة الألكساندريوم من شعبة السوطيات الدوارة، حيث تسبب بعض الأنواع تسمم الصدفيات. تم في هذه الدراسة تحليل نوعي أليكساندريوم تاميافانيتشي (*Alexandrium tamiyavanichii*) وأليكساندريوم ليي (*Alexandrium leei*) بمستويات سمية مختلفة باستخدام الفصل الكهربائي لهلام كبريتات دوديكل الصوديوم متعدد الأكريلامايد (PAGE) والتحليل الكروماتوغرافي السائل العالي الأداء (HPLC). تم تحديد نمط النمو لكلا النوعين وتحليلهما بشكل أوسع عن طريق استخدام التنميط البروتيني في كل مراحل النمو. تم تخفيض التعبير البروتيني طوال مراحل النمو لنوع أليكساندريوم تاميافانيتشي، ولكنه ارتقى خلال المرحلة الثابتة لنوع أليكساندريوم ليي. تم التعرف بنجاح على أسيتيل ترانسفيراز من عائلة GNAT في نوع أليكساندريوم تاميافانيتشي، والليبازات في نوع أليكساندريوم ليي كبروتينات إنزيمية بوظائف تنظيم النمو. أظهرت بروفايلات السمية لكل من النوعين مستوى أعلى للسمية في نوع أليكساندريوم تاميافانيتشي بقيمة 88 مول نسبي من إجمالي السموم المسجلة، مقارنة بـ 12 مول نسبي للأليكساندريوم ليي. تم تسجيل أعلى محتوى للسموم خلال طور النمو المطرد لأليكساندريوم تاميافانيتشي مع هيمنة مجانسات GTX4 و STX. تم القيام بالمزيد من التحقيقات في آثار سمية رتبة الأليكساندريوم في المحار حيث تم تغذية المحار بالأليكساندريوم تاميافانيتشي السام وأليكساندريوم ليي غير السام. تم تحديد نتائج LC-MS من المناطق المرفوعة في المحارات التي تغذيها بأليكساندريوم تاميافانيتشي بكونها كينازات الأرجينين وترانسجيلين. تم استنتاج أن التنظيم الرفعي للبروتينات كان آلية استجابة لتراكم النوع السام أليكساندريوم تاميافانيتشي في المحار. أظهر هذه الاستجابات قدرة تحمل المحار في مواجهة انتشار الطحالب المضرة. تعتبر الدراسات الأساسية لشعبة السوطيات الدوارة كتعبير جزيئي وتحليل منتجاتها الثانوية مفيدة لفهم النشاط البيوكيميائي لأنواع الطحالب المضرة المنتشرة. بإمكان نتائج هذه الدراسة أن توفر معلومات أساسية حول الخصائص البيوكيميائية لأنواع الطحالب المضرة المنتشرة في المياه الماليزية وتأثيراتها المتسلسلة على النظام البيئي المائي.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology).

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D-PAGE	-	Two-Dimensional Polyacrylamide Gel Electrophoresis
2-DE	-	Two dimensional electrophoresis
AK	-	Arginine kinase
APS	-	Ammonium Persulphate
BSA	-	Bovine Serum Albumin
CHAPS	-	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DNA	-	Deoxyribonucleic acid
DTT	-	Dithiothreitol
GTX	-	Gonyautoxin
HAB	-	Harmful algal bloom
HCl	-	Hydrochloric Acid
IEF	-	Isoelectric Focusing
IPG	-	Immobilize pH Gradient
MALDI-TOF	-	Matrix Assisted Laser Desorption Ionization – Time of Flight
PMSF	-	Phenylmethylsulfonyl fluoride
PSP	-	Paralytic shellfish poisoning
RuBisCO	-	Ribulose-1,5-biphosphate carboxylase/oxygenase
SDS	-	Sodium dodecyl sulfate
STX	-	Saxitoxin
tRNA	-	Transfer ribonucleic acid
TEMED	-	Tetramethylethylenediamine

TCA	-	Trichloroacetic acid
Tris	-	Trisaminomethane
UV	-	Ultraviolet

LIST OF SYMBOLS

cm	-	Centimeter
°C	-	degree Celsius
p*I*	-	Isoelectric Point
g	-	gravitational acceleration
kDa	-	kilodalton
µg	-	microgram
µl	-	Microliter
M	-	Molar
ml	-	Milliliter
nm	-	Nanometer
nmol	-	nanomolar
ppt	-	parts per thousand
%	-	Percent
±	-	Plus-minus
V	-	Volts
v/v	-	volume in volume
w/v	-	weight in volume

CHAPTER ONE

BACKGROUND STUDY

1.1 HARMFUL ALGAL BLOOM (HAB)

Harmful algal bloom (HAB) is a world phenomenon of excessive growth of harmful algae within the water column. Diverse environmental conditions in different regions of the world explain on the tolerance of particular species of microalgae. Enormous fish killings and mortalities of marine mammals in west coast of Florida, severe shellfish poisoning cases within Gulf of Mexico and increasing public health incidents in Alaska are some of the significant impacts of HAB (Kirkpatrick et al., 2010).

Dinoflagellates are one of the primary producers of the aquatic ecosystem. It is also known as causative agent of harmful algal bloom events worldwide apart from diatoms and cyanobacteria. The size for this single-celled dinoflagellate ranges from 2 micrometers to 2 milimetres (Leaw et al., 2010). These microscopic planktonic organisms hold the key to the food chain in aquatic ecosystems. However, the major concern related to the massive growth of this primary producer is due to its capability of synthesizing toxins under different environmental conditions. Blooming of microalgae like dinoflagellates is considered harmful when it brought damaging effects to the biodiversity of the ecosystem. The long term effects of HAB event can only be seen after years of outbreak. For example, the aquaculture industry experienced big loss during harvesting season due to low quality of the fish produced. Besides that, the harmful algae can also cause degeneration of human health as results of consuming contaminated shellfish.

Malaysian coastal waters experienced the aggregation of *Alexandrium spp*. where most occurrences are close to the domiciles and aquaculture area. There were severe cases reported since the past decade for poisoning casualty caused by different types of toxic dinoflagelllates such as *Alexandrium minutum* dispersal in Tumpat, Kelantan and *Pyrodinium bahamense* occurrence in Sabah (Lim et al., 2012). Shellfish poisoning related to *Alexandrium spp.* has been occurring in Malaysia since 1990s with the first case of *Alexandrium tamiyavanichii* detected in Sebatu, Malacca followed by the outbreak of *Alexandrium minutum* in Tumpat, Kelantan (Usup et al., 2002; Lim et al., 2006; Lim et al., 2012).

1.2 CATASTROPHIC OUTCOMES OF HAB

There are two major detrimental conditions which prevailed upon blooming of microalgae in Malaysia waters; massive fish killing and shellfish poisonings that can lead to human death. Both toxic and non-toxic microalgae contribute to the damages created out of blooming outbreak.

1.2.1 Enormous Cases of Fish killing

Aquaculture industry is largely affected due to massive fish killings resulted from oxygen depletion and physical infection (Burkholder, Glasgow & Hobbs, 1996). Planktonic dinoflagellates move independently within the water column and commonly assemble at the water surface for maximum UV light exposure. The hypoxia condition induced by excessive growth of microalgae at the water surface leads to the insufficient oxygen supply for fish respiration. Some blooming cases capable of developing ‘dead zone’ where there is low levels of dissolved oxygen which can cause death to aquatic life.

Massive fish killing also resulted from continuous ingestion on the epidermis tissue of the fish by dinoflagellate species like *Pfiesteria piscicida* (Oikonomou et al., 2012). These dinoflagellates are armored with peduncle that helps their attachment to fish organs like gills and fins where ingestion happens gradually. Unfortunately, this will lead to lethal when toxic microalgae attack speeds up the toxin release upon contact with the ruptured gills (Berge et al., 2012). Blooming of *Cochlodinium polykrikoides* had been reported in Sabah where it caused oxygen depletion and also clogging of the gills due to excessive slime production (Wang et al., 2008; Fukuyo et al., 2011; Harun et al., 2015). The same species also responsible for the huge loss of caged finfish in Penang with minimum loss of 6 million USD recorded (Lim et al., 2014). The excess mucus smothers the gills to a point that suffocates fish in the water column. Fish killing cases of species like barramundi, paddletail snapper, estuary cod and fourfinger threadfin were documented in Tanjung Kupang, Johor which caused economical loss to 250 fishermen and fish farmers (Lim et al., 2014; Razali et al., 2015). The causative agent was identified as *Karlodinium australe*. Economical loss due to mass mortality of fish imposed major alarm to the fisheries industry in Malaysia.

1.2.2 Human Health Deterioration

Shellfish poisoning is another outcome of blooming that indirectly affects human health. The byproducts of toxic dinoflagellates are noxious to marine life as well as human being who consumed shellfish such as clams, mussels and oysters. Shellfish like bivalves and molluscs are the potential vector for dinoflagellates poisoning cases. This is because shellfish feed by straining particulate matters suspended in the water column including dinoflagellates (Ferrante et al., 2013). Filter feeders like shellfish naturally insensible to the toxic dinoflagellates.

The toxins synthesized by dinoflagellates are classified according to its symptoms screened on patients which include paralysis, diarrhea and neurological episodes pertaining to the central nervous system as well as gastrointestinal function (Wang, 2008; Gerssen et al., 2010). There is no specific threshold on the cell abundance of toxic microalgae for the outbreak to be considered harmful. This is because different species vary in terms of toxin synthesis regardless of its cell density. *A. tamiyavanichii* from Japan isolates produced high toxins in low cell count (Kon et al., 2015).

In Borneo Sabah, Paralytic Shellfish Poisoning (PSP) cases have been extensively reported since the first outbreak of *Pyrodinium bahamense* in 1976 (Roy, 1977). Two-hundred and two cases of shellfish poisoning with 7 deaths gave a heads-up to researchers in finding the stimulator of the problems. Meanwhile in Peninsular Malaysia, the most frequent cases of HAB being reported were in Tebrau Straits where the water turbulence is highly influencing the rate of blooming in that area (Lim et al., 2014b). In 1999, Singapore, Changi, Punggol and Sungei Buloh of Tebrau Strait experienced blooming of *Dinophysis caudata* that induces toxicity in mussels but no fatality recorded (Lim et al., 2014b).

1.3 FACTORS INFLUENCING THE OVERGROWTH CELLS

A comprehensive understanding on the physiology and external influences on the targeted species outgrowth has been extensively studied. There are many recurring factors that favor the excessive growth and also toxicity attributes of dinoflagellates which include nutrients enrichment and physical oceanography of the coastal water. However, these factors are interchangeable in accordance with the preferences of the dinoflagellates.

1.3.1 Nutrients Enrichment

Sudden blooming of dinoflagellates is highly affected by its natural conditions where surplus nutrients are deposited. Excessive nutrients loading from land support massive blooming of harmful microalgae along the coastline. The nutrient composition however differs according to species (Guisande et al., 2002; Lim et al., 2010). Untreated water discharged from the industrial effluents, agricultural wastes and domestic sewages may provide excess nutrients that could enhance dinoflagellates to grow excessively in response to the high nutrients input (Anderson, Glibert & Burkholder, 2002). Intensive fish farming technique also contributes towards blooming of dinoflagellates due to excessive usage of feed and chemicals (Chinabut et al., 2006).

1.3.2 Physical Oceanographic Changes

Geographical layouts along the coastline are the major factor that favors the excessive growth of both benthic and planktonic dinoflagellates. The most prevalent element is the water dynamic of the assemblage area as the colonization of the dinoflagellates will disperse upon strong wave action (Pistocchi et al., 2011). This is coincides with the fact that most of blooming cases were reported in sheltered area where there is less influence of vertical mixing.

Alexandrium catenella bloom was recorded in Thau Lagoon of Mediterranean Sea where the wave action is restricted hence fostering the growth rate of the microalgae (Vila & Maso, 2005; Anderson, 2007). The lagoon was also holding harbors and jetties that provide full protection of the embayments from tidal actions (Vila & Maso, 2005). Lack of water flowing is one of the conditions that encourage eutrophication to occur.

A study by Hall et al. (2008) claimed that poor mixing of the water column contributed to the stratification of Neuse River Estuary of North Carolina (USA) where dinoflagellate *Karlodinium veneficum* was found dominating the region. All these external circumstances accidentally boost up the cell division where eventually promotes the extensive blooming of dinoflagellates.

1.3.3 Environmental Parameters

Physical variables like water temperature and salinity also support the initiation of blooming and these parameters usually differ according to species. The high temperature is favorable for benthic species and it is further verified when there is no blooming reported during winter (Dagenais-bellefeuille & Morse, 2013). An experimental study by Graneli et al., (2011) concluded that elevated sea water temperature was stimulating the bloom of *Ostreopsis ovata* in Brazil and Italy. The species grew well beyond 26ºC with the influence of other promoting factors. A monitoring study in Golden Horn Estuary, Turkey revealed an attack of blooming species *Prorocentrum minimum* within two consecutive years (Taş & Okuş, 2011). Both incidents were recorded during late spring and summer seasons with tremendous increase of cell counts within weeks of occurrence.

Water salinity varied according to places which harbor different types of HAB species. Previous studies had shown variants in terms of growth capabilities and toxic attributes of *Alexandrium spp*. under wide range of salinities. A study conducted by Sukkainen et al. (2013) showed that a wide range salinity changed the toxins level and growth rate of *A. ostenfeldii* and *A. peruvianum* but not their major toxin profile. The study concluded that toxin productions of the two species are strain-specific.

1.4 *Alexandrium spp*. AS PROMINENT CAUSATIVE OF HAB IN MALAYSIA

Dinoflagellate from genus *Alexandrium* predominate West Coast and East Coast of Malaysia with severe occurrences of PSP cases being reported (Fukuyo et al., 2011). *A. tamiyavanichii* was first discovered in Sebatu, Malacca where green mussel breeding project was established. The outbreak of the microalgae resulted in 3 cases of poisoning (Razali et al., 2015). In Tumpat, the agent of toxicity was *A. minutum* with 6 persons hospitalized and one casualty after consuming benthic bivalve (*lokan)* (Razali et al., 2015).

The genomic of *Alexandrium spp*. is highly confined due to its large genome size which then encourages other method of discovering HAB incidents *in situ* (Yang et al., 2010). The studies on protein profiling of dinoflagellates during HABs occurrence is analytical since proteins indirectly control the biochemical process such as cell growth, proliferation and homeostasis of that microorganism (Yang et al., 2010). Besides, the approach can give simultaneous expression of various proteins under one comprehensive proteomic analysis. The significant of this study may lead to a new discovery of proteomic approaches in order to provide prior knowledge for the study of toxicology properties of HABs. Hence, the analysis of the protein as well as toxin level needs to be considered as an important study to understand the biochemical changes in HABs and also the affected shellfish.

1.5 RESEARCH PROBLEM

The cases reported on blooming of toxic-dinoflagellates increases yet few studies that focused on the internal factors that trigger the outbreaks especially in Malaysia (Latib et al., 2015; Danish-Daniel et al., 2017). Proteomic approaches on *Alexandrium sp.* is very potential to understand more on the biochemical activity between toxic and non-toxic species. Few researches were studied on the effects of toxicity on the growth pattern of the dinoflagellates. Therefore this study would highlight on the study of toxicity and growth abilities of *Alexandrium sp.* from Malaysia waters.

1.6 RESEARCH OBJECTIVES

i. To determine the protein profiles of *A. tamiyavanichii* and *A. leei* at different growth phases

ii. To identify the function of the proteins expressed in relation to the blooming of *A. tamiyavanichii* and *A. leei*

iii. To investigate the effect of *Alexandrium spp*. accumulation on protein profile of oyster

CHAPTER TWO

PROTEIN PROFILING AT DIFFERENT GROWTH PHASES OF *A. tamiyavanichii* AND *A. leei*

2.1 INTRODUCTION

2.1.1 Proteomics of Dinoflagellates

Proteomic is a branch of molecular studies of organism where the protein profiling of the selected species will help researchers to understand the biochemical properties of their intact cells. This is relevant as proteins are important molecules that engaged in various cellular activities like homeostasis and cell growth (Wang et al., 2014). This study will be an advantage for researchers in dinoflagellates team to get the best picture of its molecular changes so that further steps to mitigate and control the effects of HABs can be drafted. A list of proteomics involving HAB species was mentioned in a review by Wang et al. (2014). The protein analysis has been conducted in *Alexandrium spp.* worldwide which gave identifications to most proteins expressed within the same genus (Yang et al., 2010; Wang et al., 2013). However, the proteomic of Malaysia isolates is way behind the development as there is no protein profile of local HABs has been established.

2.1.2 Growth Phases of *Alexandrium spp.*

According to Wang et al. (2014), there are four phases of blooming process which include initiation, development, blooming and dissipation of that large microalgae population. These phases apply to most microalgae including dinoflagellates from