ANALYSIS OF PROTEOMIC PATTERN OF ALIMENTARY CANAL BETWEEN LARVAE AND ADULT MOTH OF *Conopomorpha cramerella* Snellen

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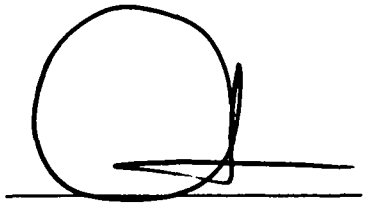

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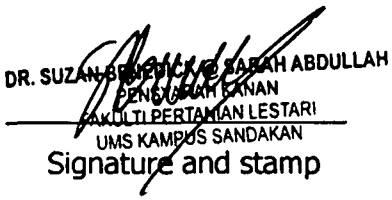

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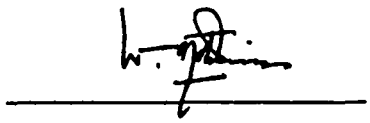

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ABSTRACT

Cocoa Pod Borer is the most serious pest in Southeast Asia. Despite the damages done by the CPB, there is only few proceedings research done in order to cope with this problem. Samples of adult moth and larvae of CPB were collected from the Quoin Hill Research Center, Tawau, Sabah. The alimentary canals were extracted and mixed with lysis buffer (2.5% SDS, 5% β-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8). Differences of the proteins expressions between the alimentary canals of adult moth and larvae of CPB were investigated in effort to compare and determine the protein bands that vary quantitatively and qualitatively between both the adult moth and larvae. The protein bands were separated by using 12.5% SDS-PAGE which were stained with Coomassie Brilliant Blue (CBB) and silver staining. The protein bands were observed and analyzed with Bio-Rad Gel Imager and Image Lab software version 4.1 by Bio-Rad at default 70% sensitivity. From the SDS-PAGE, there were various differences between adult moth and larvae in terms of their protein expression. The adult moth consisted more of low molecular weight protein bands ranging from 50 – 80 kDa compared to the larvae which have more protein bands that were high in their molecular weight ranging 80 – 250 kDa. From this research, there could be a possibility that the proteins and enzymes expressed were used in the food digestion of the CPB. Other than that, the proteins expressed could be involved in the restructuring of the guts of the CPB larvae to the adult moth.

PERBANDINGAN ANALISA CORAK PROTEOMIK SALURAN PENCERNAAN ANTARA PERINGKAT LARVA DAN KUPU-KUPU DEWASA *Conopomorpha cramerella*

ABSTRAK

Ulat Pengorek Buah Koko (UPBK) adalah serangga perosak yang paling serius di Asia Tenggara. Walaupun UPBK memberi banyak kerosakan, hanya terdapat beberapa penyelidikan yang membuat kajian untuk menghadapi masalah ini. Sampel kupu-kupu dewasa dan larva telah dikumpulkan dari Pusat Penyelidikan Quoin Hill, Tawau, Sabah. Saluran pencernaan telah diekstrak dan dicampurkan dengan *lysis buffer* yang mengandungi 2.5% SDS, 5% β-mercaptoethanol, dan 62.5 mM Tris-HCl, pH 6.8. Perbezaan ekspresi protein saluran pencernaan antara kupu-kupu dewasa dan larva telah dikaji untuk membandingkan dan mengenalpasti jalur protein yang berbeza secara kuantitatif dan kualitatif antara kedua-duanya. Jalur-jalur protein telah dipisahkan menggunakan 12.5% SDS-PAGE yang kemudiannya diwarnakan dengan Coomassie Brilliant Blue (CBB) dan pewarnaan silver. Pemerhatian dan analisis telah dijalankan ke atas jalur-jalur protein dengan menggunakan Bio Rad Gel Imager dan perisian Image Lab versi 4.1 daripada Bio-Rad pada ketetapan sensitiviti 70%. Daripada SDS-PAGE, terdapat pelbagai perbezaan antara kupu-kupu dewasa dan larva dari segi ekspresi protein. Kupu-kupu dewasa mempunyai jalur protein yang lebih rendah dari segi berat molecular iaitu daripada julat 50 – 80 kDa berbanding larva yang mempunyai jalur protein yang lebih berat iaitu 80 – 250 kDa. Daripada kajian ini, terdapat kemungkinan bahawa protein dan enzim tersebut digunakan dalam pencernaan makanan UPBK. Selain itu, protein yang ditunjukkan mungkin terlibat dalam penyusunan semula komponen saluran pencernaan larva kepada kupu-kupu dewasa.

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LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

%	Percentage
cm	centimeter
Ft	feet
Ha	Hectare
In	inch
kDa	Kilodalton
L	Liter
nm	nanometer
M	Molar
mL	Milliliter
mM	Milimolar
t	Tonnes
µl	Microliter
µg	Microgram
V	Voltant
°C	Degree celcius
1-DE	One dimensional
APN	Aminopeptidase N
APS	Ammonium persulfate
BPB	Bromophenol Blue
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
CPB	Cocoa Pod Borer
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
MCB	Malaysian Cocoa Board
MW	Molecular weight
SDS-PAGE	Sodium-dodecyl sulfate Polyacrylamide Gels
SPSS	Statistical Package of Social Science
SSA	School of Sustainable Agriculture
TEMED	Tetramethylethylenediamine
UMS	Universiti Malaysia Sabah
WCF	World Cocoa Foundation

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Theobroma cacao or commonly called as cocoa means 'food of the gods' in Latin. Back then, the Mayans used cocoa to create a ritual beverage that was shared during betrothal and marriage ceremonies, giving off the idea of linkage between chocolate and romance (WCF, 2014). It is until nowadays, that cocoa is regarded as the main component of the world's favourite – chocolates.

Figure 1.1 Cocoa tree (*Theobroma cacao) (source: dropdata)*

About 90-95% of all cocoa production comes from smallholder farmers. Most of the production of cocoa is in the West African countries of Ghana, Ivory Coast, Nigeria and Cameroon. These countries collectively accounted for 68% of world production, followed by Indonesia (13%) and Brazil (5%). In contrast, Malaysia is not among the world's top cocoa producers. However, Malaysia is a major cocoa processor (Shapiro *et. al.*, 2008).

Cocoa was introduced into Malaysia for commercial planting in 1950's under the crop diversification program of the National Agricultural policy (MCB, 1991). It is after then, that cocoa industry grew to become the third major commodity crop in Malaysia after oil palm and rubber. However, the cocoa planting industry dramatically declined to 45,365 ha and 36,236 t dried cocoa beans in 2003 after reaching the peak of 414,236 ha in 1989 and 247,000 t dried cocoa beans in 1990. This is caused by the problems that are rising in every single country's cocoa production including Malaysia.

The problems influencing the poor production of cocoa in Malaysia was affected by the competition among commodities such as oil palm and rubber. Other than that, the cocoa production was also forced to drop due to the infestation of diseases such as black pod and vascular streak dieback. The environmental concerns of the soil fertility and its sustainability are also contributing to the challenges of cocoa planting in Malaysia. However, the major problem is the pest and disease attack with estimation 30-40% of the crop is lost to pests and disease (WCF, 2014).

The most devastating pest of cocoa remains the cocoa pod borer (CPB) *Conopomorpha cramerella* (Lepidoptera: Gracillariidae) causing a considerable yield lost in Malaysia and also the South East Asian region. The CPB larvae cause losses when they tunnel directly into the pod and feed on the placenta, the pulp surrounding the beans. This will cause the pulp to harden and difficult to be extracted and reduced the bean size (Azhar, 1986).

Figure 1.2 The internal infestation of cocoa by CPB (*source: dropdata*)

The infestation of the CPB will cause heavy losses or even a major collapse in cocoa industry in our country if effective control measure is not taken. Since the occurrence of this insect in cocoa, much research on taxonomy, economic importance, ecology and biology of this pest, and control measures to suppress its populations has been undertaken. However, there are no control methods whether the cultural, physical, chemical or biological control that seems to work singly.

1.2 Justification of the study

The infestation of CPB causes the uneven ripening of cocoa pods and low quality of beans. This is due to the burrowing and tunneling of the larvae throughout the cocoa pulp. It is also reported by Rita *et. al.* in 1989 that CPB was also hosting other fruits such as nam-nam, rambutan, lychees and pulasan. These fruits have obvious differences in terms of physical appearance. Therefore, they must have different sets of enzymes and proteins. In order to digest these fruits, there must be a diverse set of enzymes and proteins of the CPB.

Out of all the stages of the life cycles of CPB, it is the larvae that inflict the most damages. According to a research done by Lim (1982), the degree of damage cocoa depends on the age of pods, the total number of larvae and the period of larval feeding. This shows how the damage of cocoa production was majorly inflicted by larvae and not the adult moth. Undeniably, it is due to the feeding habit of the larvae from eating the cocoa placenta and pulp.

In order to cope with this problem, there is a great need to understand the biological characteristics of CPB. Therefore, this research was done to give early insight on the protein expressions of adult moth and larvae of the CPB as well as for us to further understand the proteins and enzymes included in the CPB larvae and adult moth. By then, it can also be look up to as basis of the information for future research.

1.3 Objectives of the study

This study aims to achieve several objectives which are:

i. To extract the total protein from larvae and adult moth of CPB
ii. To compare the proteome pattern of the larvae and adult moth of CPB's alimentary canals
iii. To determine the protein bands that vary quantitatively and qualitatively between CPB alimentary canals of larvae and adult moth

1.4 Hypothesis of the study

H_o : There were no significant differences between the protein expression of the larvae and adult moth of *Conopomorpha cramerella*

H_1 : There were significant differences between the protein expression of the larvae and adult moth of *Conopomorpha cramerella*

CHAPTER 2

LITERATURE REVIEW

2.1 Scenario of Cocoa Industry

2.1.1 Worldwide

Cocoa is very important and vital for both producer and industrial countries as an international commodity. The biggest producer of cocoa worldwide is the tropical countries that are centered in the West Africa; Ghana, Ivory Coast, Nigeria and Cameroon as the main distributer of 68% of world production. A step behind are the Indonesia and Brazil with respectively 13% and 5% (WCF, 2014).

2.1.2 Malaysia

Malaysia has been commercially started its cocoa industry in between the 1953 to 1959. Starting from there, Malaysia has been able to raise their accelerated growth through the high prices of cocoa in the 1970s and 1980s demanding the country to expand the cultivated area of cocoa to its optimum, peak area of 414,236 ha by 1989.

Production of cocoa beans in Malaysia was according to the flow of the cocoa cultivated area. By reaching the peak in 1990 at 247,000 t, lower cultivation forces the production of cocoa beans to start decreasing. However, even though the hectareage in 2006 and through 2007 continued to decline, the production of cocoa beans increased slightly. Overall, Peninsular contributed 21,871 t (62.2%) of the total production, Sabah with 11,474 t (32.6%) and Sarawak producing 1,835 t (5.2%).

Since the very first start of the cocoa grinding industry in Malaysia in 1973, it has expanded rapidly and successfully in the 1980s. By this current time, Malaysia possesses 10 local grinding plants with an annual processing capacity of 300,000 t. Malaysia continued importing cocoa beans from Indonesia, Ghana and Cote d'Ivore, about 438,956 t in 2007, with a slight increase of 8.7% from 2006 (MCB, 2014).

2.1.3 Cocoa Production in Malaysia

The Malaysian cocoa sector has undergone dramatic changes during the last few decades. Previously, with low production costs and efficient marketing structure, cocoa production was a profitable venture in Malaysia and the sector maintained an upward development in the area. However, since then declining world prices, higher labour costs, loss of production due to pests and diseases, which is the main reason of economic decay in most producing countries along with a switch in the relative competitiveness of other crops (particularly oil palm) have transposed the previous trend, when production grew at a rapid rate.

This in turn was affecting the production of the cocoa in Malaysia. Since the last few years, Malaysia had been producing less and lesser cocoa each region. However, this does not apply to the world, or even Malaysian consumption. The unstable production and consumption of the cocoa pose an impact that forced our country to import cocoa beans and cocoa products from other country even though we are among the largest cocoa producer in Asia and Oceania.

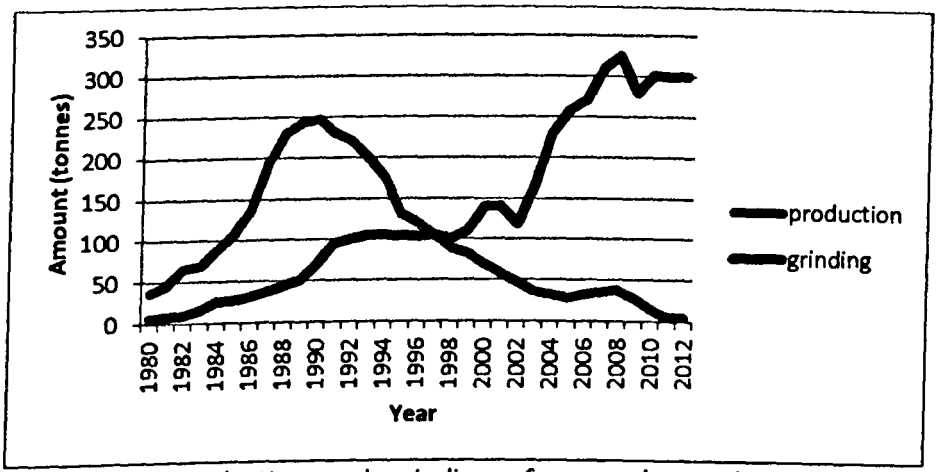

Figure 2.1 The production and grinding of cocoa beans in Malaysia (Source: MCB,2014)

2.2 Cocoa (*Theobroma cacao*)

2.2.1 Taxonomy and morphology of cocoa

Cocoa is a native of Amazon region of South America. With the scientific name of *Theobroma cacao* L., it is a member of the *Sterculiaceae* or *Sterculia* family, a medium sized family with 50 genera and 750 species. More recent DNA evidence reclassifies the genus under the *Malvaceae* family. The genus *Theobroma* consists of 22 species, including the Brazilian species *Theobroma cupuacu*, which provides a bitter-sweet pulp widely used in juice drinks in the Amazon (www.kew.org). Mostly, it is produced in the tropical areas of the continent of Africa.

The cocoa tree is an evergreen that grows to about 15 to 25 ft. The flowers and fruits of cocoa grow directly out of its trunk. The fruit of cocoa tree is oblong-shaped, commonly called a pod. It can be four to 12 inch long. When the pod is young it is green in color, but will change to red, yellow or purple when they are fully ripe. Each pod contains 20 to 60 reddish-brown cocoa beans that are an inch long. The cocoa beans are surrounded and coated by a sugary pulp and are usually arranged in five rows.

2.3 Cocoa Pod Borer (CPB)

2.3.1 Taxonomy of CPB

The scientific name of Cocoa Pod Borer (CPB) is *Conopomorpha cramerella* Snellen. The change of generic name for the cocoa pod borer from *Acrocercops cramerella* to *Conopomorpha cramerella* was made by Bradley (1985). The order name of CPB is Lepidoptera and the family is *Gracillariidae*.

2.3.2 Morphology and Life Cycle of CPB

2.3.2.1 Egg

Lepidopterans oviposit singly or in clusters; most of the butterflies lay solitary eggs. Egg clustering often results in larval aggregation in the beginning of the development

and larvae that live in groups may or may not live isolated at the end of development. Most species attach their eggs to the vegetation that will serve as the food plant for the larvae. Female cocoa pod borer laid eggs on the pod surface (Lim, 1992).

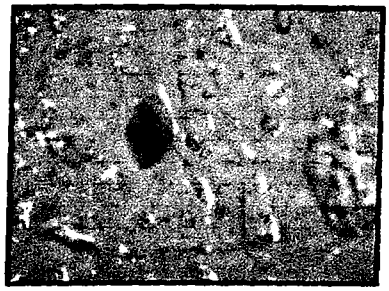

Figure 2.2 The egg of CPB

2.3.2.2 Larvae

Larvae develop in the egg and then emerge through the eggshell, which they sometimes eat. They increase in size each time they moth or shed their skins. The period between molts is termed an instar and typically a caterpillar passes through five instars as it eats and grows.

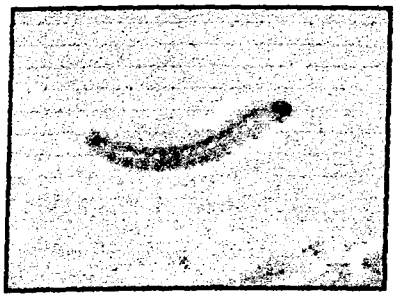

Figure 2.3 The early instar larvae of CPB after hatching

Figure 2.4 The middle instar larvae of CPB (source: MCB)

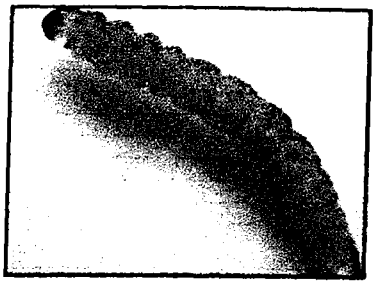

Figure 2.5 The late instar larvae of CPB

2.3.2.3 Pupae

Metamorphosis occurs inside the pupae. The pupae may be covered in silk, or naked, and can be encased in rolled foliage or in the soil. Once a larvae has attained a critical size, it changes behavior and stops feeding and begins searching for or creating a site to pupate. Pupation can be quick, lasting 2 to 3 weeks, or prolonged, lasting more than one year.

Figure 2.6 The early stage of CPB pupae

Figure 2.7 The late stage of CPB pupae

2.3.2.4 Adult Moth

Moths are sexually mature adult life stage of Lepidoptera. Many of them feed on nectar or a liquid sugar source for energy required for flight. Moths with wings have two pairs which is a pair of forewings and a pair of hindwings. The forewings are attached to the second thoracic segment, the mesothorax. The hindwings are attached to the third thoracic segment, the metathorax.

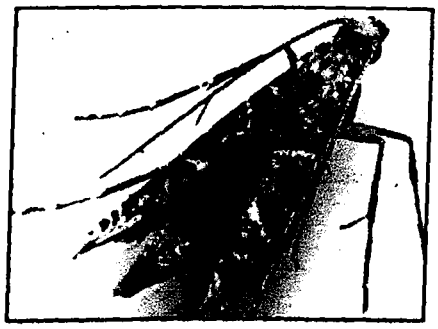

Figure 2.8 The adult moth of CPB

2.3.3 Behavior of CPB

2.3.3.1 Time of Emergence of Adult Moth

CPB is a nocturnal insect. Time for emergence of adult moths was observed emerging between 1900 to 2100 hours. No emergence was observed before 1800 hours (Lim, 1992).

2.3.3.2 Mating

Mating started at between 1800 and 0700 hours and peaked at 0400-0500 hours. They were usually ready to mate on the third night after emergence. Female month appear to mate more than once, sometimes up to four times, based on the maximum number of spermatophores found in a female (Lim, 1992).

2.3.3.3 Time of Tunneling Out and Pupation Site

Mature larvae will tunnel out from the pod between the times 0800 to 1900 hours. The activities reach to its peak time at 2000 to 2100 hours. After that, no more larvae was seen and found after 0800 hours. The cocoon was often found in the sunken spot of the leaves and there is membrane that uses to cover these spot on the leaves. The preferred sites for cocoa pod borer were mainly on the dried leaves, on pods, tree trunks, and tree branches. Pupation was also found on the green leaves (Lim, 1992).

2.4 Internal Composition of Lepidoptera

2.4.1 Midgut of Lepidoptera

The midgut, one of the largest insect organs, is derived from the endoderm. The larval midgut is formed on an epithelial cell monolayer composed of columnar, goblet, and stem cells (Goldsmith *et. al.*, 2010). The columnar cells are mainly responsible for food digestion and nutrition absorption. Digestion is usually controlled by the digestive enzyme and is always dependent on their localization on gut (Terra *et. al.*, 2005). The apical membrane of columnar cell is characterized by a well-developed brush border. Goblet cells are involved in the ionic regulation in gut, and the regenerative cells have the responsibility in renewal of the epithelial cells during metamorphosis (Martins *et. al.*, 2006).

Midgut is also a barrier to the foreign substances during food digestion. It has been early discovered that midgut is one of the important targets for insect control. One successful example is the transgenic crops that produce *B. thuringiensis* crystal endotoxins. These toxins cause the death of the insect by binding to their receptors and thus forming a prepore oligometric structure through which cell content are leaking.

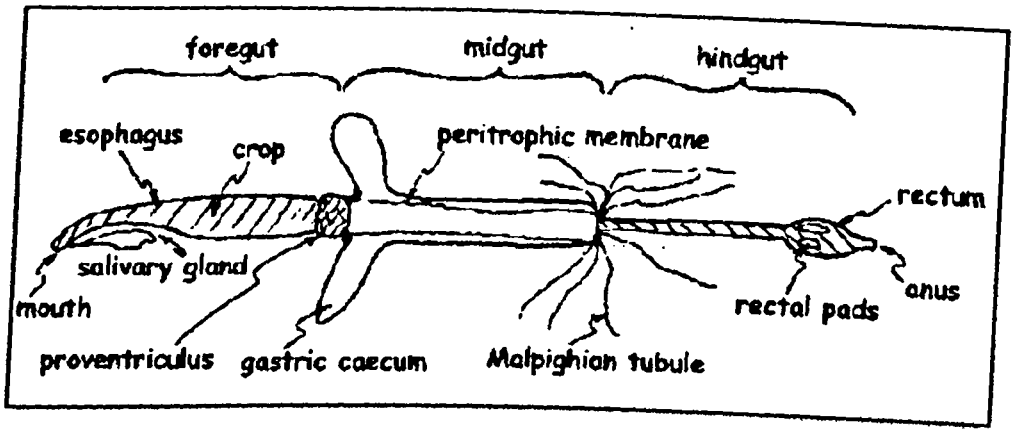

Figure 2.9 The general insect alimentary canal (www.entomology.oso.edu)

2.4.2 Peritrophic Membrane (PM) of Lepidoptera

The peritrophic membrane (PM) of insect in the alimentary canal is an invertebrate-unique semi-permeable that lines the midgut of an insect. It is similar to the mucous lining of vertebrate gut, separating midgut epithelium and intestinal contents as well as the protective lining for the epithelium. Lyonet found a sheath encasing the food bolus in lepidopteran larvae in 1762.

The larval PM is transparent, colorless and semi-permeable with a certain toughness and elasticity. The main component of insect PM is chitin nanofibers embedded in the matrix of proteins, proteoglycans and glycoproteins. However, the functions and the chemical components of the matrix remain rudimentary. The main biological function of the PM include the protection from the toxins ingested through oral feeding, the spatial organization of digestion, and serves as a physical barrier to pathogens. As the PM plays an important role in facilitating food digestion, it can also be made into a significant target for insect control.

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