UNIVERSITI PUTRA MALAYSIA

PHYSICAL CHARACTERIZATION OF HYDROPHOBIN-LIKE PROTEINS FROM AERIAL CONIDIA OF ENTOMOPATHOGENIC FUNGUS (Metarhizium anisopliae var.anisopliae) AND THEIR ROLE IN CONTROLLING TERMITE (Coptotermes curvignathus)

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Hydrophobin-like protein (HLP) was found in the aerial conidia of this fungus and play a role in attachment on the cuticle surface of the termite during pathogenesis. The aim of this study was to isolate and physically characterized HLP from the aerial conidia of two local isolates of *M. anisopliae*, also to evaluate the interaction effect between HLP and *M. anisopliae* conidia suspension on the mortality of *C. curvignathus*. The protein samples were isolated based on their insolubility in hot sodium dodecyl sulfate (SDS). The SDS-insoluble proteins were then purified by three purification methods namely formic acid only, formic acid followed by performic acid, and trifluoroacetic acid. The isolated and purified protein samples were further quantified and characterized as HLP by studying the contact angle when applied on the hydrophobic surface, wetting ability when applied on hydrophilic surface and emulsifying properties. Virulence test of termite bioassay was performed. The HLP samples were verified by their low molecular weight and unique characteristics. Due to the stringent protocol of isolation and purification, the formic acid followed by performic acid purified proteins showed four conspicuous highly intensified bands (13.5 kDa, 15.0 kDa, 16.5 kDa and 17.0 kDa). This method of purification produced highest concentration of HLP (17.82 µg mL^{-1} and 15.64 µg mL^{-1} for both isolates TA and LR2, respectively) among the three purification methods. The contact angle measurement showed that both protein isolates HLPTA (58.43° ± 0.25°) and HLPLR2 (57.06°± 0.38°) have the contact angle of < 90°when applied on the hydrophobic surface as compared to the water (108.37° ± 0.50°). When hydrophilic paper was coated with a layer of HLP solution, the paper resisted wetting by water up to 439 ± 20.52 seconds. These results indicated that the HLP is able to convert hydrophilic surface into hydrophobic property and *vice versa*. This was due to the unique characteristic of HLP which can self-assemble themselves and configure the adsorption at the hydrophobic-hydrophilic interfaces. The HLP

also revealed good emulsification effect in the oil-water phase. Aerial conidia of *M*. *anisopliae* showed good dispersion in aqueous solution with the application of pre-coated HLP prior to the mixing of conidia suspension. Two application methods were used to test the efficacy and pathogenicity of *M. anisopliae* with HLP samples. The termite, *C*. *curvignathus* showed the fastest trend of mortality (100%) by the pre-coated method with HLPTA prior to the conida topical application on the Day 4. Similar trend of mortality was observed for the same method of application for HLPLR2. HLP treatment alone on termite did not revealed any toxic effect. In conclusion, the most applicable purifying agent used for the purification process of hydrophobin-like proteins (HLP) samples was 98% formic acid followed by perfomic acid. This study showed the strain specificity of the HLP on its application and was found to have enhancing effect but non-toxic when applied alone on *C. curvignathus*. These unique properties of HLP from local isolates of *M. anisopliae* var. *anisopliae* are of great potential to be used in wide range of industrial applications especially in enhancing the formulation of the biological control agent in controlling insect pests.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN PROTEIN ERTI HIDROFOBIN SECARA FIZIKAL DARIPADA KONIDIA KULAT ENTOMOPATOGEN (*Metarhizium anisopliae* var. *anisopliae*) DAN FUNGSINYA DALAM PENGAWALAN ANAI-ANAI (*Coptotermes curvignathus*)

Oleh

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Protein erti-hidrofobin (HLP) telah ditemui pada permukaan konidia kulat ini dan memainkan peranan dalam perlampiran pada permukaan kutikula serangga perosak sebelum penembusan berlaku. Tujuan kajian ini adalah untuk memencil dan menciri HLP secara fizikal daripada dua isolat *M*. *anisopliae* tempatan juga untuk menilai kesan interaksi antara pertambahan HLP dan konidia *M*. *anisopliae* atas kematian *C*. *curvignathus*. Sampel kajian protein dipencilkan berdasarkan sifat tidak keterlarutan mereka dalam sodium dodecyl sulfat (SDS) yang panas. Protein SDS-larut kemudian ditulenkan dengan tiga kaedah penulenan iaitu asid formik sahaja, asid formik diikuti oleh asid performic, dan asid trifluoroasetik. Sampel protein hasil penulenan telah dikesan dan disahkan sebagai HLP daripada keberatan molekul yang rendah dan ciri-ciri fizikal mereka yang unik. Sampel protein terpencil dan tulen telah dilanjutkan dengan analysis kuantitinya dan dicirikan sebagai HLP dengan mengkaji sudut kenalan apabila digunakan pada permukaan hidrofobik, keupayaan membasah apabila disalut pada permukaan hidrofilik dan sifat pengemulsian. Ujian keracunan terhadap anai-anai, *C*. *curvignathus* telah dilakukan. Sampel HLP telah dikesan dan disahkan oleh keberatan molekul yang rendah mereka dan ciri-ciri yang unik. Protokol pengasingan dan penulenan yang lasak daripada asid formik diikuti oleh asid performik, protein tulen menunjukkan empat jalur yang paling terang dan jelas (13.5 kDa, 15.0 kDa, 16.5 dan 17.0 kDa). Kaedah penulenan ini menghasilkan HLP dengan kepekatan yang tertinggi (17.82 μg mL^{-1} dan 15.64 μg mL^{-1} untuk kedua-dua isolat TA dan LR2, masing-masing) di antara ketiga-tiga kaedah penulenan. Kedua-dua sampel HLP ini yang dipencilkan dari konidia *M*. *anisopliae* isolat-isolat TA dan LR2 masing-masingnya mempunyai sudut perhubungan yang rendah pada permukaan hidrofobik, 57.06° ± 0.38° dan 58.43° ± 0.25°, masing-masing berbanding dengan air (108.37° ± 0.50°). HLP juga mendedahkan kesan pengemulsian antara fasa minyak-air dengan baik. Kertas (sebagai permukaan hidrofilik) yang disalut dengan selapis cecair yang

mengandungi HLP boleh bertahan pembasahan daripada setitik air sehingga 439 ± 20.52 saat berbanding dengan kertas yang tidak disalut dengan HLP. Keputusan ini menunjukkan bahawa HLP memaparkan bahagian sifat hidrofiliknya apabila dihadapi dengan permukaan hidrofobik dan sebaliknya. Ini disebabkan oleh ciri-ciri unik yang boleh HLP sendiri memasang diri mereka sendiri dan mengkonfigurasi penjerapan antara muka hidrofobik-hidrofilik untuk mengubah keadaan permukaan hidrofilik kepada hidrofobik dan sebaliknya. The HLP juga mendedahkan kesan pengemulsian baik dalam fasa minyak-air. Konidia menunjukkan kesan penyebaran yang baik dalam cecair yang mengandungi sampel HLP. Dua kaedah telah digunakan untuk menguji keberkesanan dan pathogenicity *M*. *anisopliae* dengan sampel HLP. Anai-anai, *C*. *curvignathus* menunjukkan tren kematian yang paling cepat (100%) pada hari ke-4 dengan kaedah pra-bersalut dengan HLPTA sebelum conidia diaplikasikan secara topical pada *C*. c*urvignathus*. Tren kematian yang sama telah diperhatikan bagi kaedah yang sama bagi HLPLR2. Hasil kajian menunjukkan bahawa permohonan HLP sampel sebelum konidia meningkat kesan jangkitan *M*. *anisopliae*. Rawatan HLP sahaja pada anai-anai tidak mendedahkan sebarang kesan toksik kepada anai-anai. Analisi interaksi menunjukkan terdapat kesan interaksi yang signifikasi pada HLP kaedah pra-bersalut sebelum larutan konidia diaplikasikan. Kesimpulannya, agen penulenan yang paling sesuai digunakan untuk proses pemencilan protein-erti hidrofobin (HLP) dari konidia adalah 98% asid formik diikuti oleh asid perfomic. Kajian ini juga menunjukkan keunikan HLP pada isolat-isolat *M*. *anisopliae* yang tertentu. Sifat unik HLP dari isolat tempatan *M*. *anisopliae* var. *anisopliae* mempunyai potensi yang besar untuk digunakan dalam pelbagai jenis aplikasi industri. Ini juga telah mewujudkan peluang untuk meningkatkan faedah performulaan agen kawalan biologi untuk mengawal anai-anai dan juga serangga perosak lain.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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aa	Amino acid
ANOVA	Analysis of variance
APS	Ammonium persulphate
BSA	Bovine serum albumin
cm	Centimeter
conidia mL^{-1}	Conidia per mL
g	Gram
G	Gravity
g m^{-2}	Gram per meter square
HLP	Hydrophobin-like protein
HLPTA	Hydrophobin-like protein isolated and purified from aerial conidia of *Metarhizium anisopliae* isolate TA
HLPLR2	Hydrophobin-like protein isolated and purified from aerial conidia of *Metarhizium anisopliae* isolate LR2
kDa	KiloDalton
LB-ASDSA	Low-Bond Axisymmetric Drop Shape Analysis
M	Molar
mg mL^{-1}	Milligram per milimeter
mil ha	Million hectares
mL	Mililiter
mm	Milimeter
mmol L^{-1}	Milimole per liter
mN m^{-1}	Mili Newton per meter square
NaOH	Sodium hydroxide
NaCl	Sodium chloride
nm	Nanometer
SAS 9.2	Statistical Analysis Software 9.2
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate –polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
µL	Microliter
µg	Microgram
v/v	Volume per volume
w/v	Weight per volume
var	Variance
θ	Degree of contact angle
°	Degree
°C	Degree Celcius
%	Percent

CHAPTER 1

INTRODUCTION

1.1 Problem statement

Currrent trend of agriculture has expanded to the peat soil area. Expansion of land use on peat land especially for oil palms cultivation has attracted pest and diseases especially termite's occurrence (Bong *et al*., 2012; Kon *et al*., 2012). Termites are now found to be the most destructive insect pest that caused huge damage in most monoculture plantations especially that of the Acacia and oil palm. Current controls of termite in most of the plantations are still relying on the applications of termiticides (Sukartana *et al*., 2009; Sun *et al*., 2002). Implementation of biological control agent in termite's control is an alternative for chemical control. However, the cryptic lifestyle and natural defensive behaviour of this subterranean termite against the fungal epizootic infection has rendered entomopathogens rather difficult to be applied in field condition (Chouvenc *et al*., 2011). Thus, the prospect of using biological control agent as a control strategy in termite management would require the enhancement of the virulent agent's mechanism. In recent studies, hydrophobins isolated from variable entomopathogens have been reviewed to be essential for the fungi to attach onto the host cell surfaces prior to infestation (Ali *et al*., 2012; Askolin *et al*., 2005; Wösten *et al*., 1994).

1.2 Background

Oil palm (*Elaeis guineensis*) is an important industrial crop in Malaysia and Indonesia which produce 86% of the world's palm oil. Malaysia currently accounts for 39% of world palm oil production and 44% of world exports (MPOB, 2015). Being one of the biggest producers and exporters of oil palm and palm oil products, Malaysia has an important role to play in fulfilling the growing global need for oils and fats sustainably. Until the year of 2014, total oil palm planted area in Malaysia has reached 5.39 million hectares with annual production of 19.7 million tonnes crude palm oil. However, expansion of oil palm cultivation to fulfil world's oil demand has led to suitable soil for planting oil palm diminished. This has led to the expansion of current cultivation of oil palms to the peat land.

Termites are the most common organic matter decomposer in nature and play a role in the forest nutrient recycling (Robert *et al*., 2007). Termites from the genus *Coptotermes*, notably *Coptotermes curvignathus*, is one of the major species that occur on peat cultivated with oil palms especially in Indonesia and Malaysia (Bong *et al*., 2012; Kon *et al*., 2012). Due to environmental concern on the negative effects of applying chemical pesticides, an alternative method by using the entomopathogenic fungus *Metarhzium anisopliae* has gained its

prominence as a biological control agent for termites (Khan *et al*., 2012; Hoe *et al*., 2009; Andrew, 2000).

There are several processes involved during the mode of action and virulence of *M. anisopliae* such as adhesion, germination, differentiation and penetration (Shahid *et al*., 2012). Interaction between the conidia of *M*. *anisopliae* and their host cuticles depend on the occurrence of nonspecific and specific mechanisms (Wang *et al*., 2002). The adhesion of fungus conidia to the host's hydrophobic cuticle surface followed by excretion of a series of enzymes for penetration into the host's cells is the main mechanism involved in killing the insect pests. Among the series of mechanisms, adhesion of conidia to the host cuticle was found to be the most important initial step during pathogenesis (Zhang *et al*., 2011; Cho *et al*., 2006; Holder and Keyhani, 2005).

Hydrophobins are small amphiphilic proteins produced only by filamentous fungi (Wösten, 2001). In nature, hydrophobins play an important role during the fungal development such as formation of aerial hyphae, protection coat for the spores, and mediate the surface tension of the hydrophobic surface of the host cuticle during pathogenesis (Wösten and Wessels, 1997; Nakari-Setala *et al*., 1997; Tablot, 1997). They have the ability to initiate the adhesion process in the initial step of pathogenesis (van Wetter *et al*., 2000; Tablot *et al*., 1993; Wösten *et al*., 1993).

With respect to fungal pathogens of insects, the initial attachment of the host surfaces involves hydrophobic interactions between the conidia and waxy cuticular surface of the insects (Boucias *et al*., 1988). The most unique characteristic of hydrophobins are their ability to self-assemble themselves in hydrophobic-hydrophilic interfaces and *vice-versa*. This unique characteristic of hydrophobin tends to lower down the cuticle surface tension of the host to ease the attachment of the fungal conidia (Wösten *et al*., 1999; Tablot, 1997). Based on this mechanism, hydrophobins from these entomopathogenic fungi have created an interesting role in the initial step of fungi attachment and also in the pathogenesis.

1.3 Objectives

The objectives of this study are to:

(1) Isolate and physically characterize, hydrophobin-like proteins from the aerial conidia of *M*. *anisopliae*,

(2) Evaluate the effects and different application methods of hydrophobin-like proteins with the conidia of *M*. *anisopliae* in controlling termite, *C. curvignathus*

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BIODATA OF STUDENT

Diana Kiong Siaw Boon, born in 26 July 1986, originated from Kuching, studied in SRB Chung Hua No.4 in 1993. After completing her primary school, she studied in Sekolah Menengah Kebangsaan Green Road in 1999. She furthered her study and successfully obtained a Second-Upper class in Bachelor of Science Bioindustry from Universiti Putra Malaysia in 2010. She was assigned to Plantation, Conservation Department and Nursery Department in Grand Perfect Sdn. Bhd. Plantation, Bintulu, Sarawak, Malaysia during her internship. She is currently undertaking Degree of Master of Science in Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus. During her master study, she attended 2010 15th Biological Sciences Congress.

LIST OF PUBLICATIONS

(a) Papers Submitted or Published

Kiong, D.S.B., Bong, C.F.J. and King, P.J.H. (2014). Isolation and physical characterization of hydrophobin-like proteins (HLP) from aerial conidia of *Metarhizium anisopliae* var. *anisopliae*. *American Journal of Biochemistry and Biotechnology.* Published online first on 29th January 2015.

(b) Presented Works

Kiong, D.S.B., Bong, C.F.J. and Wong, SK. (2010) Isolation and purification of *Pleurotus florida* hydrophobin. *15th Biological Sciences Graduate Congress*. *University of Malaya*. *In press*