Original Research Paper

Isolation and Physical Characterization of Hydrophobin-Like Proteins (HLP) from Aerial Conidia of *Metarhizium Anisopliae* Var. *Anisopliae*

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Abstract: The aim of this study was to isolate and physically characterized Hyrophobin-Like Proteins (HLP) from the aerial conidia of two local isolates of *Metarhizium anisopliae* var. *anisopliae*, namely, TA and LR2. The protein samples were isolated based on their insolubility in hot Sodium Dodecyl Sulfate (SDS). The SDS-insoluble proteins were then purified in cold 98% formic acid and performic acid. The molecular weight of the Formic Acid Extracted (FAE) proteins fell in the range of 13-17 kDa with the contents of 3.80 and 3.56 mg mL^{-1} for TA and LR2, respectively. Due to the stringent protocol of isolation and purification, the FAE proteins can be verified as Hydrophobin-Like Proteins (HLP). Both the HLP samples isolated from *M. anisopliae* isolates TA and LR2 have low contact angles of 57.06±0.38 and 58.43±0.25°, respectively. The HLP also revealed good emulsification effect in the oil-water phase. Conidia showed good dispersion in aqueous solution with the application of HLP. Paper precoated with HLP resisted wetting by water for up to 439 sec. 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.

Keywords: Hydrophobin-Like Proteins *Metarhizium Anisopliae*, Formic Acid Extracted Proteins

Iintroduction

Hydrophobins are small (~10 kDa) and the most surface active proteins known to date produced only by filamentous fungi (Linder *et al*., 2005; Wösten, 2001). The functional features of hydrophobins are their ability to self-assemble at the hydrophobic-hydrophilic interfaces and *vice versa*, lowering down the water surface tension and configuration of coatings (Wösten *et al*., 1993; 1999; Lugones *et al*., 1999). They are highly insoluble even in 2% of hot Sodium Dodecyl Sulfate (SDS) and only can be dissolved in concentrated acid such as 100% trifluoroacetic acid or 98% formic acid (de Vries *et al*., 1993; Wessels *et al*., 1991). 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-Setälä *et al*., 1997; Talbot, 1997).

Theentomopathogenic fungus *Metarhizium anisopliae* is an important fungal biocontrol agent in controlling ticks, termites, mites, mosquitoes and other arthropods (de Faria and Wraight, 2007). The initial stage of pathogenesis involved the attachment of the conidia to the host cuticle (Bidochka *et al*., 1997). This non-specific hydrophobic attachment of the conidia to the host cuticle is also found to involve the mediation of the Hydrophobin-Like Proteins (HLP) from the conidia onto the hydrophobic surfaces of the cuticle (Boucias *et al*., 1988). The formic-acid extractable proteins have been proved as HLP in the conidia of *Beauveria bassiana* (Jeffs *et al*., 1999). The hydrophobin gene *ssga* of *M. anisopliae* has also been studied in the molecular basis (Bidochka *et al*., 2001). However, little effort has been done on the physical characterization and the potential applications of the HLP.

In this study, the isolation of the FAE proteins from the conidia of two local isolates *M. anisopliae* were determined and physically characterized. The potential application methods of HLP with the conidia suspensions have also been examined in the laboratory scale.

Science Publications

Materials and Methods

Fungal Culture Preparation

Two virulent isolates TA and LR2 of *Metarhizium anisopliae* var. *anisopliae* were obtained from the Entomology Research Laboratory of Universiti Putra Malaysia Bintulu Sarawak Campus, Malaysia (Hoe *et al.*, 2009). The fungi were grown on SabouraudDextrose Agar (Merck, Germany) plates supplemented with 1% of yeast extract (Merck, Germany) at $25\pm2°C$ for 2 weeks. The conidia from the culture were gently scrapped off and collected aseptically into sterile 1.5 mL microcentrifuge tubes and stored at -20°C for isolation of the Formic Acid Extractable (FAE) proteins.

Isolation of SDS-Insoluble Proteins

Isolation of the SDS-insoluble proteins from the aerial conidia of two *M. anisopliae* isolates, TA and LR2 were done according to the protocol described by (Jeffs *et al.*, 1999) with slight modification. Conidia at 50 mg were placed into a 1.5 mL microcentrifuge tube and added with 1 mL of 2% Sodium Dodecyl Sulfate (SDS) (Merck, Germany) buffer containing 5% of 2-mercaptoethanol. The samples were boiled for 10 min in boiling water bath (100°C). After centrifugation for 10 min at 7400 g, the SDS-soluble proteins were discarded leaving the reserved sample of SDS-insoluble proteins. The isolation above was repeated for four cycles and finally rinsed twice with ultrapure water.

Purification by Formic Acid

The reserved samples were then suspended in 1 mL of 98% formic acid (Merck, Germany) under sonicating water bath maintaining at not more than 10°C for 2 h. Subsequently, 2 mL of performic acid (Wessels *et al.*, 1991) was added to the supernatant and stood for 4 h on ice. The purified proteins were further added with 2 mL of 45% sodium hydroxide (NaOH) and 1 mL of ultrapure water and left to stand overnight at 4°C for neutralization. After standing overnight, the purified samples were centrifuged at 3000 *g* for 5 min to get the precipitates. The precipitates were then rinsed twice with 1 mL of isopropyl alcohol (alcohol: Ultrapure water 3:1) and then diluted to the desired concentration for further analysis.

Quantification of Formic Acid Extracted (FAE) Proteins

The concentrations of the Formic Acid Extracted (FAE) proteins were determined by Bradford Method (Daniel and Stuart, 1991). Bovine serum albumin served as standard.

SDS-PAGE Analysis

The FAE protein samples were analyzed with SDS-PAGE buffer system (Laemmli, 1970). The proteins were boiled for 3 min before loading on the Tris-glycine gels (15% separating gel and 5% stacking gel). The gel was run at 100 V for 1 h using BioRad electrophoresis system. After electrophoresis, the gel was stained with 1% Coomassie Brilliant Blue R-250 for 10 min and then destained with destaining solution (10% methanol and 10% glacial acetic acid) until the band can be visualized.

Contact Angle Determination of HLP

Each 10 μL of aqueous samples was pipetted onto sterilized Parafilm (Pechiney, Chicago) surface and stabilized for 1 min. The 10 μL ultrapure water was used as negative control and 2% of Sodium Dodecyl Sulfate (SDS) as positive control in this experiment. Contact angles were measured by Low-Bond Axisymmetric Drop Shape Analysis (LB-ASDSA) using Image J plugin software (Stalder *et al.*, 2010; Williams *et al.*, 2010). The digital images of the sample droplets were captured by a digital single-lens reflex camera (Sony A200). The images were then processed by Irfan View Image software for the droplet edge detection before the LB-ASDSA was carried out. All measurements were carried out at $25\pm2°C$ with 10 replicates.

Wetting Ability Determination of HLP on Hydrophilic Surface

Paper with density of 70 gm^{-2} was used as hydrophilic surface. The paper squares (2×2 cm) were coated with FAE proteins for 10 sec and let dried for another 30 min. Water drop at 10 μL each of was pipetted onto the surface of FAE proteins coated and non-coated paper. The time used for wetting the paper was recorded. The experiment was performed at $25\pm2°C$ with 3 replicates and was repeated three times.

Emulsifying Property of HLP

Ultragrade mineral oil (Edward, Germany) was emulsified in equal volume of the HLP aqueous solutions using ultrasonic dispenser (Wise Clean, Germany) at 100% relative frequency for 5 min and 24 h. Similar mixture without HLP served as control. After homogenization, the emulsion phases were sampled and examined under an optical microscope (Leica, Germany).

Determination of Aqueous Dispersion of Conidia

Two application methods with treatment of conidia suspension at 1×10^7 conidia mL^{-1} were used in this experiment. The first involved pre-coating the HLP samples (1 mg mL^{-1}) onto the inner surface of a microcentrifuge tube for 30 min and let dried for another 30 min before treatment. The second method was premixing the HLP samples with conidia suspension in a non-coated microcentrifuge tube. Similar treatment with no HLP applied served as control. The mixtures were later pipetted and loaded into a Neubaueur haemocytometer for observation of conidia dispersion.

Statistical Analysis

All data were analyzed by SAS software version 9.0 and treatment means were separated by Tukey Test.

Results

SDS-PAGE Profiles and Qantification of FAE Proteins

The FAE proteins profile revealed similar molecular mass (Fig. 1) with little intraspecific variability. This also revealed slight difference in composition of extracted proteins between the two isolates. Both FAE proteins revealed in the profile consisted of four conspicuous bands with molecular weights of 13.0, 15.0, 16.5 and 17.0 kDA. The FAE protein of isolate TA has a major band with slightly lower molecular mass (16.5 kDA) as compared to FAE protein of isolate LR2 with molecular mass of 17.0 kDA. Bands below 13.0 kDA were not detected for both isolates.

The content of FAE protein of isolate TA (3.85 mg mL^{-1}) was significantly higher than that of isolate LR2 (3.56 mg mL^{-1}) (Table 1).

Contact Angle Measurement

The contact angles of HLPTA (58.43±0.25°) and HLPLR2 (57.06±0.38°) were not significantly different. The high mean contact angle (108.57±4.47°) of water and low mean contact angle of SDS (41.54±1.01) were both regarded as negative and positive controls, respectively (Table 2).

Wetting Ability of the HLP on the Hydrophilic Surface

The water droplet took longer time to wet the surface of the HLP coated paper as compared to the non-coated paper (100 sec). However, there was no significant different for water to wet the HLP coated papers between the HLPTA (430.67 sec) and HLPLR2 (439.00 sec) proteins (Table 3).

Emulsifying Property of HLP

Both HLPTA and HLPLR2 were able to stabilize the emulsion of water and oil. Figure 2 showed the evenly distributed air vesicles after 5 min of ultrasonic treatments (Fig. 2A and 2B). After 24 h, the fine, stable and uniform air vesicles are formed (Fig. 2C and 2D). The droplets without the application of HLP aggregated immediately and completely separated into the water and oil phase within 24 h (Fig. 2E).

Determination of Aqueous Dispersion of Conidia

The conidia were distributed evenly in the suspension in HLP-coated tube initially, with most of the conidia precipitated onto the bottom of the tube after being kept in static condition for more than 1 h (Fig. 3B and 3C). All the conidia floated to the surface when the HLP samples were premixed with the conidia suspension (Fig. 3D and 3E). In the control, some conidia were precipitated while most floated to the water surface (Fig. 3A).
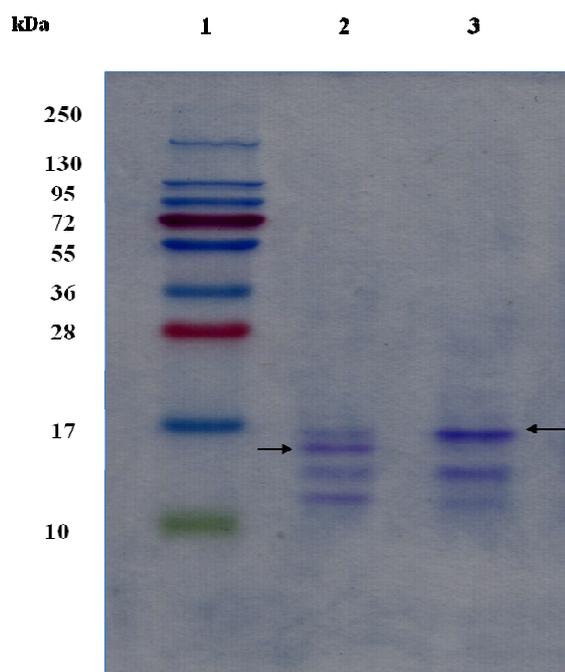

Fig. 1. SDS-PAGE (15% Tris-glycine gel) profiles of FAE proteins from aerial conidia of *M. anisopliae* isolate TA and LR2; Lane 1: Protein ladder, Lane 2: FAE protein of *M. anisopliae* isolate TA, Lane 3: FAE protein of *M. anisopliae* isolate LR2 Note: Arrows indicate the major bands represented by FAE

Table 1. Comparison of means concentration of FAE proteins isolated from two isolates of *M. anisopliae*

Samples	Means concentration (mgmL^{-1})
HLPTA	3.85±0.02[a]
HLPLR2	3.56±0.02[b]

Means with different alphabet indicate significant difference at p<0.05 by T Test

Table 2. Means comparison of the contact angle of formic-acid extracted protein isolated from two isolates of *M. anisopliae*

Treatments	Means contact angle (°)
Ultrapure water	108.57±4.47a
HLPLR2	57.06±0.38b
HLPTA	58.43±0.25b
SDS	41.54±1.01c

Means concentration with different alphabet indicate significant differences at p<0.05 by Tukey Test

Table 3. Means comparison of the wetting time of water on HLP coated and non coated paper

Treatments	Means time for wetting (sec)
HLPTA	430.67±42.85[a]
HLPLR2	439.00±20.52[a]
Ultrapure water	100.00±0.00[b]

Means time with different alphabet indicate significant differences at p<0.05 by Tukey Test
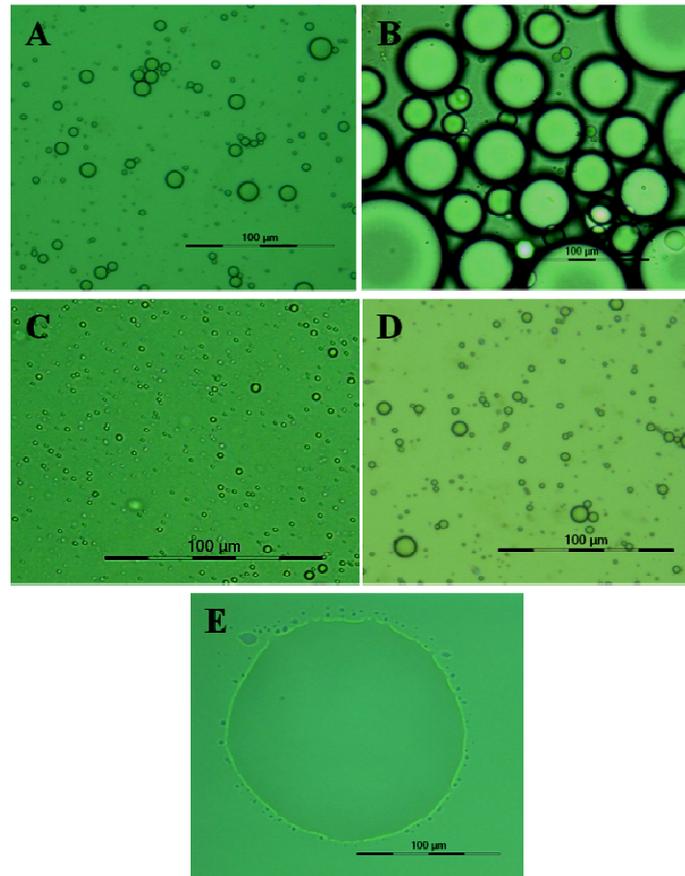
Fig. 2. Optical micrograph of 100 μg mL^{-1} of FAE proteins from *M. anisopliae* isolates TA and LR2 emulsified in water and mineral oil phase; (A) HLPTA in water-oil phase after 5 min of sonication, (B) HLPLR2 after 5 min of sonication, (C) HLPTA after 24 h of sonication, (D) HLPLR2 after 24 h of sonication, (E) Control after 24 h of sonication. Bar = 100μm
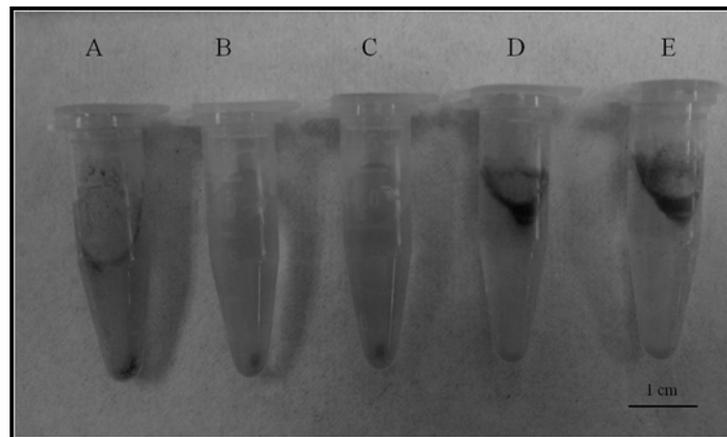

Fig. 3. (A) TA spore suspension as control, (B) Conidia suspension in HLPTA pre-coated tube, (C) Conidia suspension in HLPLR2 pre-coated tube, (D) Conidia suspension of isolate TA premixed with HLPTA, (E) Conidia suspension of isolate LR2 premixed with HLPLR2. Bar = 1 cm

When observed under the microscope, the mixing of HLP and conidia suspension of *M. anisopliae* portrayed clumpy conidia (Fig. 4C). The conidia were distributed evenly (Fig. 4B) when the hydrophobic surface of the tube was coated with the HLP. In untreated suspension, the conidia appeared mostly in chains but not in clumps (Fig. 4A).
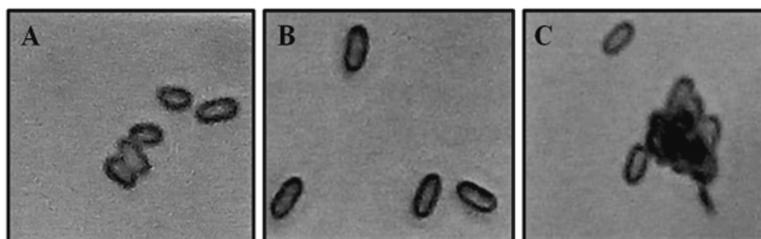
Fig. 4. Aerial conidia of *M. anisopliae* isolate TA under 400X optical microscope after 30 min of vortex, (A) TA conidia suspension alone, (B) TA conidia suspension in the HLP coated tube, (C) HLPTA premixed with TA conidia suspension, Note: Scaling at 0.0025 μm^2

Discussion

The isolation of FAE proteins from *M. anisopliae* var. *anisopliae* involved stringent procedures in that only Hydrophobin-Like Proteins (HLP) would be retained at the end of the purification steps. The unique biochemical extractions of the FAE proteins from the aerial conidia (SDS-insoluble and formic acid soluble) have led to the identification of HLP. Hydrophobins are highly insoluble in hot SDS. These proteins can only be solubilised when treated with concentrated cold formic acid (Bidochka *et al.*, 1995; de Vries *et al.*, 1993; Wessels *et al.*, 1991) and dissociated into monomers only after oxidizing all the eight cysteine residues to cysteic acid by the performic acid (Wessels *et al.*, 1991) which only then can be observed in SDS-PAGE protein profile. The range of molecular masses observed from both of the FAE proteins isolated from *M. anisopliae* isolates in this study fell within the range of 13.0-17.0 kDA (Fig. 1) indicating very similar molecular masses compared to those of the other entomopathogens, like *Beauveria bassiana* (12.0-15.0 kDA) and *Paecilomyces fumosoroseus* (15.0 and 17.5 kDA) (Ying and Feng, 2004).

Contact angle analysis was used to prove the biophysical property of the HLP after purification (Scholtmeijer *et al.*, 2002; Linder *et al.*, 2002). In this study, a liquid is considered to be hydrophobic if its contact angle is more than 90° and hydrophilic if the contact angle is less than 90° (Njobeunwu *et al.*, 2007; Lumsdon *et al.*, 2005). The FAE proteins in our study were characterized as Hydrophobin-Like Proteins (HLP) because of the very low contact angles and their unique property that can alter the hydrophobic surface into hydrophilic surface.

Paper surface is hydrophilic but becomes hydrophobic when coated with HLP thereby causing the paper to resist wetting for an extended period. This water proofing properties could be harnessed for industrial and medical application. Hydrophobins have also been reported previously by Wösten *et al.* (1994) to effectively stabilize various emulsions. In this study, the oil was emulsified in the HLP aqueous solutions and the emulsion remained stable for a long time. This enhances potential of HLP as emulsifying agents.

When HLP was introduced into conidia suspension, the conidia aggregated and clumped causing them to float to the surface. The aerial conidia have hydrophobic, basic monopolar surface and were negatively charged in neutral condition (Munoz *et al.*, 1995). Thus, the conidia are repulsive and will aggregate when suspended in the water (Holder *et al.*, 2007). The addition of HLP into the conidia suspension further increased the hydrophobicity of the suspension causing the conidia to be more repulsive and hence formed clump more tightly and floated to the surface.

However, when conidia suspension was loaded into a plastic tube previously coated with HLP the conidia became evenly dispersed with no clumping. The coating of HLP on the hydrophobic surface of the plastic tube enables the proteins to reassemble to form a hydrophilic monolayer on the hydrophobic surface. This effectively prevent clumping and aggregation of the conidia when the aqueous suspension was loaded into a HLP-coated tube due to the hydrophobic nature of the conidia. This phenomenon of hydrophobins that can self-assemble to form amphiphillic monomers and reverse the assemblage of the interfaces was explained by Wösten and Wessels (1997).

Conclusion

HLP proteins which were successfully isolated from the aerial conidia of *M. anisopliae* possessed unique physical characteristics. The remarkable characteristics associated with these proteins auger well for potential industrial applications as surfactants in liquid formulation, emulsifiers in food processing technology, in surface coating of biomaterials and electronic applications. However, more understanding of the structural and functional relationships at the molecular level will be needed to further exploit the potential of these unique hydrophobin-like proteins.

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Author's Contributions

D.S.B. Kiong: Experimental work and data analysis; preparation of draft manuscript.

Choon Fah J. Bong: Supervisor of first author in this research; planning, design and funding of the research; participated in all parts of the research work; preparation of final manuscript.

P.J.H. King: Participated in isolation and characterization work; data analysis. Review of manuscript.

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