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Partial characteristics of hemolytic factors secreted from airborne *Aspergillus* and *Penicillium*, and an enhancement of hemolysis by *Aspergillus micronesiensis* CAMP-like factor via *Staphylococcus aureus*-sphingomyelinase

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One of the advantages for initial survival of inhaled fungal spores in the respiratory tract is the ability for iron acquisition via hemolytic factor-production. To examine the ability of indoor *Aspergillus* and *Penicillium* affecting hemolysis, the secreted factors during the growth of thirteen strains from eight species were characterized *in vitro* for their hemolytic activity (HA) and CAMP-like reaction. The hemolytic index of HA on human blood agar of *Aspergillus micronesiensis*, *Aspergillus wentii*, *Aspergillus westerdijkiae*, *Penicillium citrinum*, *Penicillium copticola*, *Penicillium paxilli*, *Penicillium steckii*, and *Penicillium sumatrense* were 1.72 ± 0.34, 1.61 ± 0.41, 1.69 ± 0.16, 1.58 ± 0.46, 3.10 ± 0.51, 1.22 ± 0.19, 2.55 ± 0.22, and 1.90 ± 0.14, respectively. The secreted factors of an *Aspergillus wentii* showed high HA when grown in undernourished broth at 25°C at an exponential phase and were heat sensitive. Its secreted proteins have an estimated relative molecular weight over 50 kDa. Whereas, the factors of *Penicillium steckii* were secreted in a similar condition at a late exponential phase but showed low HA and heat tolerance. In a CAMP-like test with sheep blood, the synergistic hemolytic reactions between most tested mold strains and *Staphylococcus aureus* were identified. Moreover, the enhancement of α-hemolysis of *Staphylococcus aureus* could occur through the interaction of *Staphylococcus aureus*-sphingomyelinase and CAMP-like factors secreted from *Aspergillus micronesiensis*. Further studies on the characterization of purified hemolytic- and CAMP-like-factors secreted from *Aspergillus wentii* and *Aspergillus micronesiensis* may lead to more understanding of their involvement of hemolysis and cytolysis for fungal survival prior to pathogenesis.

***Keywords*:** hemolytic index, CAMP-like, *Aspergillus micronesiensis*, *Aspergillus wentii*, *Staphylococcus aureus*, sphingomyelinase

Introduction

Fungi are ubiquitous in the outdoor and indoor environment. *Aspergillus* and *Penicillium* are commonly found in the indoor air of homes and workplaces. Exposure to these fungi can cause an adverse effect on human health (e.g. allergic reactions) (Eduard, 2009). Most fungi grow well in a wide temperature range and need water for their growth. Besides freely available water, fungi can also use water vapor molecules (e.g. from humid indoor air) for their growth, and especially xerophilic fungi are of interest because those can grow at low relative humidity. Spores of common xerophilic molds (e.g. certain *Aspergillus* and *Penicillium* species) are very small and after inhalation, they can reach the tiniest lung alveoli (Lacey and West, 2006). Indoor mold spores can inhabit at least transient or stable in healthy people as lung mycobiota that may shape the respiratory immune response and may also trigger inflammation or appear to be a cofactor with bacteriobiota in inflammation contributing to lung function and lung disease progression (Nguyen *et al*., 2015). However, the mucociliary lining represents the first clearance for removal of airborne mold from human airways; inflammation may occur as allergic and non-allergic reactions. The toxic effects and irritations probably result from chronic or high exposure to airborne spores, fungal fragments, and their metabolites. Many molds are reported to produce metabolites, including volatile organic compounds and mycotoxins (Eduard, 2009). Some mold may take advantage of producing hemolytic factors for acquisition of iron, which is required for survival and growth. *Stachybotrys chartarum*, a toxigenic fungus, was primarily reported to produce not only mycotoxins but also hemolysin, which caused hemorrhaging in a reddish worm model (Vesper and Vesper, 2002). Hemolytic activities have been reported also in various pathogenic fungi, some endemic fungi and opportunistic fungi (Nayak *et al*., 2013). In an indoor environment, although some airborne mold was reported to be able to lyse erythrocytes as well (Van Emon *et al*., 2003; Donohue *et al*., 2005, 2006; Nayak *et al*., 2013), most hemolytic factors secreted from indoor mold are still under investigation of

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their characteristics. Besides the production of a hemolytic factor, the acquisition of iron from hemolysis could be involved in the cooperation of cohabitating microbiota as synergist or antagonist. *Escherichia coli* Nissle, a probiotic bacterium, could reduce *Salmonella* Typhimurium intestinal colonization by competing for iron (Deriu *et al*., 2013). Additionally, *Staphylococcus aureus* exposed to *Corynebacterium striatum* exhibited decreased hemolytic activity, reflecting an attenuation of virulence (Ramsey *et al*., 2016). Conversely, the synergistic interaction between *S. aureus* and *Propionibacterium acne* resulted in hemolysis enhancement involved in skin invasion was determined by Lo *et al.* (2011) which was used for a choice in the development of an *S. aureus* immunotherapy. In fungi, it was found that most dermatophytes recovered from both symptomatic and asymptomatic lesions had hemolytic activity and CAMP-like reaction with *S. aureus* (Doegen *et al*., 2015). Nevertheless, Schaufuss and colleagues (2005) demonstrated that dermatophytes could trigger CAMP-like reactions with skin bacteria flora not only *S. aureus* but also *S. intermedius* or *Listeria ivanovii*. The synergistic hemolytic reactions between inhaled mold and common bacteria in the respiratory tract might be an alternative support for fungal survival. In this study, the secreted factors, especially proteins, from three *Aspergillus* and five *Penicillium* species were determined and/or partially characterized *in vitro* for their ability of hemolysis in various environmental conditions involving hemolytic activity and/or synergistic interaction between these molds and common bacteria in the respiratory tract, particularly *S. aureus*, during survival and growth.

Materials and Methods

Identification of airborne *Aspergillus* and *Penicillium* isolates

Thirteen *Aspergillus* and *Penicillium* strains collected from indoor air of an academic building in Chiang Mai, Thailand, which had positive hemolytic activity (HA) in a preliminary study, were identified to species level based on morphological characters and nucleotide sequencing. Macro- and microscopic characters were observed on Sabauraud's dextrose agar (SDA), Czapek agar (Cz), and malt extract agar (MEA). The whole-cell DNA was extracted from all tested fungi (Vanittanakom *et al*., 2002) and subjected to PCR analysis. The internal transcribed spacer (ITS) regions including the 5.8S ribosomal RNA gene of five phenotypically distinct *Aspergillus* isolates were amplified and sequenced (Selangor Darul Ehsan) (White *et al*., 1990). A part of the β-tubulin gene of eight distinct *Penicillium* isolates was amplified and sequenced (Houbraken *et al*., 2010). For strain identification, a homology search with the generated sequences against sequences on GenBank was performed.

Hemolytic index determination and OD measurement of hemolysis

To semi-quantify the hemolysis on solid media of airborne *Aspergillus* and *Penicillium*, the isolates which germinated at 25°C for three days and grown continuously upshifted to 37°C for an additional 12 days on Tryptic soy agar supplemented with human blood (HBA) or sheep blood (SBA) had an observed hemolytic zone. Sheep blood was available and common used in hemolytic activity testing. Additionally, the human red blood cell which contained some differences in membrane composition from sheep blood (Christie *et al*., 1944) was used in this experiment. The semiquantitative hemolysis was demonstrated as a hemolytic index (HI) that was calculated from the diameter of the hemolytic zone divided by the diameter of the fungal colony (Luo *et al*., 2001).

In a liquid media experiment, the semiquantitative hemolysis of secreted hemolytic factors was quantified by measurement of the spectrophotometric OD of the released hemoglobin at a wavelength of 540 nm (Malcok *et al*., 2009). Results of OD measurement were calculated into a percentage of hemolysis compared with distilled water when incubation at 4°C for 30 min as 100% hemolysis. Alpha-hemolysin from *S. aureus* (Sigma; ~26 U) was used as positive hemolysis control in all HA determinations.

Fungal cultivation and preparation of secreted hemolytic factors during growth

To determine whether the release of hemolytic factors might depend on the environmental conditions, the hemolytic activities during growth in different medium compositions, temperatures and the presence of red blood cell were observed in an *Aspergillus* and a *Penicillium*. One strain each of *Aspergillus* and *Penicillium* which had low hemolytic activity, particularly on SBA, and/or slow growth during upshift to 37°C were selected to determine whether the release of hemolytic factors might depend on the environmental conditions. Both strains were cultivated to grow in complete medium (Tryptic soy broth; TSB) or undernourished medium (modified *Aspergillus* Minimal Medium; mAMM, using AMM [Barratt *et al*., 1965] supplemented with 0.5% NaCl) at three different temperatures. In each 50 ml liquid media in 125 ml-Erlenmeyer flasks, 10^5 spores of selected mold (*A. wentii*-B1 or *P. steckii*) were added and incubated at 25°C for 0, 3 days and upshifted to 37°C for 3, 6 days compared to at 25°C or 37°C for 0, 3, 6, 9 days on a shaker incubator at 150 rpm. In each culture filtrate of various culture conditions, the enzyme inhibitor-cocktails of 50 mM iodoacetic acid, 50 mM phenylmethanosulfonyl fluoride, and 500 mM EDTA was added in a final concentration of 10 μM, 0.1 mM, and 1 mM, respectively. All were concentrated by being frozen at -20°C and freeze-dried (CHRiST, ALPHA 1-4) and pellets were resuspended in PBS in a concentration of 50 times (50x) to an initial volume. Protein concentration was measured by a colorimetric assay based on the Bradford dye-binding method (Bradford Protein Assay, Bio-Rad Laboratories). All concentrated culture filtrates (CCFs) were kept at 4°C or -20°C before use. Two batches of preparation were done.

In a preliminary experiment, the un-concentrated culture filtrates of *A. wentii*-B1 or *P. steckii* during growth in undernourished liquid medium using mAMM had no hemolytic activity. Thus, to examine whether, in the presence of red blood cells, the hemolytic factors which might be induced to release during growth were observed in mAMM. After each incubation time (3, 6, and 9 days) of growth of those selected *Aspergillus* or *Penicillium* strain, sheep red blood cells were added in the cultures into the final concentration of 1% or

7%, and cultures were incubated continuously for an additional 12 h. The negative control using mAMM or PBS without mold was incubated at 25°C or 37°C for 12 h. Non-lysed red blood cells and fungal cell were removed by centrifugation at 3,000 × *g* for 10 min. The supernatant of each sample was transferred to a flat-bottom 96-well plate for OD measurement. The HA of all supernatants were determined by measurement the spectrophotometric OD of the released hemoglobin. Alpha-hemolysin from *S. aureus* was used as positive hemolysis control in all experiments.

Heat stability test

To determine the heat stability of CCFs, the HA of heated CCFs were incubated at 100°C for ten minutes in a water bath and compared with the original samples. Ten microliters of each two-fold serial dilution was dropped on SBA and incubated at 37°C. Lysis of red blood cells was noted at 24–48 h. Semiquantitative hemolytic activity of CCFs was determined by the final dilution that could lyse red blood cells.

SDS-PAGE and native gel electrophoresis

To prepare CCFs for partial purification and characterization, ultrafiltration membranes were used for fractionation of CCFs based on size. CCFs from 3, 6, and 9 days of growth in batch 1 or batch 2, which had strong hemolytic activity, were pooled and fractionated by ultrafiltration. Centrifugal ultrafiltration with serial fractionation by a molecular weight cut off of 100, 50, 30, and 10 kDa (Vivaspin Spin Concentrator, GE Healthcare) was performed in serial steps into five fractionates of <10, 10–30, 30–50, 50–100, and >100 kDa. All fractionates were separated and/or determined protein profiles by SDS-PAGE or Native gel electrophoresis and observed hemolytic property on SBA plate.

Vertical SDS-PAGE was performed by the discontinuous buffer system of Laemmli (1970) with separating and stacking gel monomer solution containing 0.1% SDS with 10% and 4% acrylamide, respectively. Samples were initially solubilized with sample buffer (625 mM Tris-HCl; pH 6.8, 5% SDS, 10% glycerol, 5% 2-β-mercaptoethanol, and 0.01% bromphenol blue) and denatured at 100°C for 3 min and 10 µl/lane were loaded. Electrophoresis was carried out in electrode reservoir buffer (0.1% SDS, 192 mM glycine, and 25 mM Tris-HCl) at a constant voltage of 100 V (per two gels) for approximately 2 h. Vertical Native gel electrophoresis was performed in a similar manner of SDS-PAGE using separating and stacking gel monomer solution containing 7.5% and 4% acrylamide, respectively and sample buffer, without SDS and/or 2-β-mercaptoethanol. Protein bands were visualized by silver staining (PlusOne Silver Staining Kit, GE Healthcare). To observe which protein band could lyse red blood cells, one non-denatured protein separating gel was also carefully placed on the SBA plate and incubated at 37°C for 24 and 48 h and compared simultaneously with Silver stain separating gel from the same run. To confirm the hemolytic activity of protein band visualized on SBA plate, the protein bands of interest were cut out from gel, soaked in elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA, pH 7.5) and incubated at 25°C overnight on a shaker incubator at 150 rpm. The hemolytic activity of each elution was observed daily on an SBA plate.

CAMP-like test and CAMP diffusion assay

To determine the cooperative hemolytic reactions between airborne mold with common bacteria in the respiratory tract, a CAMP-like test, modified from Schaufuss and colleagues (2005), was performed using thirteen strains of *Aspergillus* or *Penicillium* species, two bacterial strains of *Staphylococcus aureus* (ATCC 25923) or *Streptococcus pneumoniae* (ATCC 49619), and sheep erythrocytes. Briefly, each mold tested was inoculated and incubated at 25°C for 3 days, then each bacterium was streaked in a straight line across the plate at a distance of 15–25 mm from the border of the mold colony and incubated continuously for seven days. A *Rhodococcus equi* (soil isolate), as a known CAMP-like positive reaction with *S. aureus*, was streaked in a straight line of 2–3 cm in length at a right side and incubated additionally for 24 h. The cooperative hemolytic reaction was demonstrated as a hemolytic zone between the mold colony and bacterial colony and observed daily.

To investigate whether the interaction of the cooperative hemolysis of *Aspergillus* or *Penicillium* may be involved with sphingomyelinase activity secreted from *S. aureus*, the CAMP diffusion assay modified from Schaufuss *et al.* (2005) was performed using CCFs produced from *Aspergillus* or *Penicillium* and a purified sphingomyelinase produced from *S. aureus* (Merck). For the preparation of the CCFs, 10^5 spores of each strain of *Aspergillus* or *Penicillium* were incubated in 50 ml of TSB or mAMM for 6 days at 25°C and then filtered and steriled through a 0.2 µm membrane filter. CCFs of *R. equi* obtained from incubation in 50 ml of TSB at 37°C for 18 h and centrifugation at 3,000 × *g* for 10 min was used as a positive control. All filtrates were concentrated to 50× by a freeze-dry method. In a CAMP diffusion assay, 5 µl (0.056 U) of purified sphingomyelinase (171 U/mg P, activity 1393.7 U/ml, and diluted 1:10 in 0.25 mol/L phosphate buffer pH 7.5) or 5 µl of *S. aureus*-cell suspension (ATCC 25923) was dropped on the center of SBA plate, and then 10 or 80 µl of each CCFs from *Aspergillus* or *Penicillium* or *R. equi* was filled in the punched-out hole two cm far from the center of the medium. Firstly, the CCFs from one strain of each species of *Aspergillus* and *Penicillium* was tested for the cooperative interaction with *S. aureus* to confirm the CAMP-like test. Then, the CCFs of tested mold which reacted positively with *S. aureus*-colony were tested further in the same manner with sphingomyelinase of *S. aureus*. The positive reaction with a clear hemolytic zone between each CCF from mold and *S. aureus*-colony and/or purified sphingomyelinase of *S. aureus* when incubated at 37°C within 5 days were observed daily.

The GenBank accession number for nucleotide sequence data of five *Aspergillus* isolates including *A. micronesiensis*, *A wentti* A1, *A. wentti* A2, *A. wentti* B1, *A. westerdijkiae* are MG991937, MG980049, MG991936, MG984573, and MG984589, respectively. The GenBank accession number for nucleotide sequence data of eight *Penicillium* isolates including *P. citrinum* A1, *P. citrinum* A2, *P. citrinum* A4, *P. copticola*, *P. paxilli* A, *P. paxilli* B, *P. steckii*, and *P. sumatrense* are MK397996, MK397997, MK397998, MK398001, MK397999, MK398000, MK398002, MK398003, respectively.

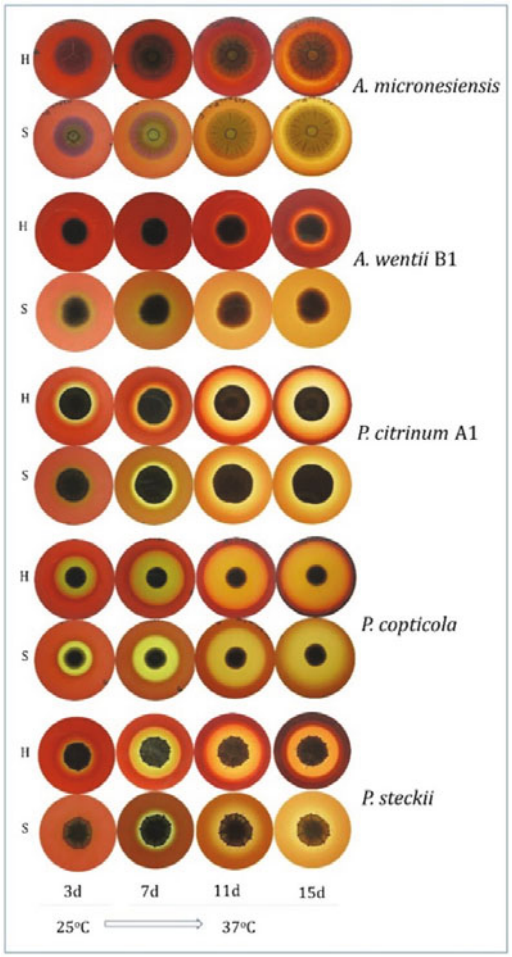

Fig. 1. The differential *in vitro* hemolytic activities of airborne mold. Hemolytic zones shown as complete or incomplete or diffusible hemolysis were triggered by *A. micronesiensis, A. wentti*-B1, *P. citrinum* A1, *P. copticola*, and *P. steckii* during grown on HBA (H) and SBA (S) at 25°C for 3 days and upshifted to 37°C for additional 4, 8, and 12 days.

Table 1. Hemolytic index (HI) of airborne mold. Average HI were obtained from *Aspergillus* and *Penicillium* 15-days-grown at 25°C upshifted to 37°C on solid agar supplemented with human blood (HBA) or sheep blood (SBA).

	HI* (± SD)	
	HBA	SBA
A. micronesiensis	1.43–2.10 (1.72± 0.34)	1.24–1.66 (1.40 ± 0.22)
A. wentii A1	1.24–1.69 (1.50 ± 0.23)	~**
A2	1.25–2.50 (1.84 ± 0.63)	~
B1	1.16–1.92 (1.48 ± 0.39)	~
A. westerdijkiae	1.54–1.85 (1.69 ± 0.16)	0–1.35 (0.84 ± 0.14)
P. citrinum A1	1.74–2.67 (2.08 ± 0.52)	1.30–1.40 (1.36 ± 0.05)
A2	1.04–1.37 (1.25 ± 0.18)	1.10–2.17 (1.50 ± 0.59)
A4	1.12–1.64 (1.42 ± 0.27)	1.22–1.88 (1.58 ± 0.33)
P. paxilli A	1.12–1.55 (1.34 ± 0.22)	~
B	1.00–1.24 (1.10 ± 0.12)	~
P. copticola	2.69–3.67 (3.10 ± 0.51)	2.27–2.50 (2.37 ± 0.12)
P. steckii	2.38–2.80 (2.55 ± 0.22)	1.07–3.00 (1.82 ± 1.04)
P. sumatrense	1.73–2.00 (1.90 ± 0.14)	0–1.48 (0.95 ± 0.06)

*repeated 3 times
** incomplete or diffusible hemolysis

Results

Characteristics of hemolytic activity triggered by airborne *Aspergillus* and *Penicillium*, and their hemolytic index

The five *Aspergillus* strains isolated from indoor air were identified as *A. wentii* (3 strains; A1, A2, and B1) *A. micronesiensis* and *A. westerdijkiae* (one strain of each). The eight *Penicillium* isolates were identified by sequencing a part of the β-tubulin gene as *P. citrinum* (3 strains; A1, A2, and A4), *P. paxilli* (2 strains; A and B), *P. copticola*, *P. steckii*, and *P. sumatrense* (one strain of each).

In a preliminary study, all tested strains grew well during incubation at 25°C on HBA; however, nine of thirteen strains

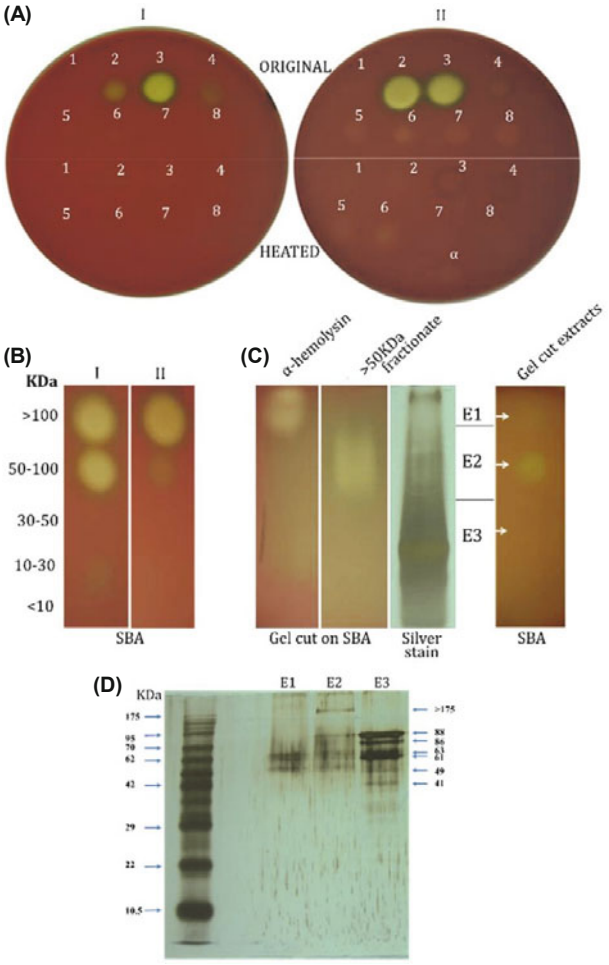

Fig. 2. Characteristics of hemolysis on SBA induced by CCFs of *A. wentii*-B1 in two batches (I, II). (A) hemolytic zones induced by original- and heat-CCFs (50×) (no. 1, 2, 3, and 4 collected during growth at 25°C for 0, 3, 6, and 9 days; no. 5 and 6 collected during growth at 25°C upshift to 37°C for 6 and 9 days; no. 7 and 8 collected during growth at 37°C for 3 and 9 days). (B) hemolytic zones induced by protein fractionate of >50 kDa out of 5 fractionates (MWs between <10 to >100 kDa) which were ultrafiltrated from pooled CCFs of no. 2 and no. 3. (C) hemolytic zone induced by a fractionate of >50 kDa-native gel cut at 37°C for 48 h and α-hemolysin gel cut served as control. Native gel preparation of >50 kDa-fractionate was cut into three pieces (E1, E2, and E3) and eluted proteins from gel cut extract E2 showed lysis of sheep blood. (D) silver-stained protein profiles of native gel cut extracts showed band of >175 kDa in E2 differently from E1 and E3.

could not produce a hemolytic zone. At 37°C, only *A. micronesiensis*, *A. westerdijkiae*, and *P. citrinum* (A2 and A4) grew and produced a hemolytic zone. Although some species of *Aspergillus* and *Penicillium* could not grow at 37°C, all tested species still survived and most of them had a hemolytic zone whenever they could germinate at 25°C before growing continuously at 37°C on blood agar. In Figure 1, during growth at 25°C and upshift to 37°C on HBA and SBA, *Aspergillus* and *Penicillium* had a hemolytic zone in different characteristics and their HI were produced in high variation between strains (Table 1). However, it looks like most strains tested could lyse human blood better than sheep blood. HA on HBA of *Aspergillus* species including *A. wentii* (3 strains; A1, A2, and B1), and *A. micronesiensis* and *A. westerdijkiae* (one strain of each) were identified similarly with HI in average of 1.72 ± 0.34, 1.61 ± 0.41, 1.69 ± 0.16, respectively. Of the *Penicillium* species, HA characteristics and HI showed more differences between species and strains. *P. citrinum* (3 strains; A1, A2 and A4), *P. paxilli* (2 strains; A and B), and *P. copticola*, *P. steckii* and *P. sumatrense* (one strain of each) had HI in average of 1.58 ± 0.46, 3.10 ± 0.51, 1.22 ± 0.19, 2.55 ± 0.22, and 1.90 ± 0.14, respectively. Some species had incomplete and/or diffusible hemolysis on SBA, particularly *A. wentii* and *P. paxilli* whereas some species had high HI and a clear hemolytic zone either on HBA or SBA, such as *P. copticola* (Fig. 1). One strain of *A. wentii* (B1) and *P. steckii* which had low hemolytic activity and/or slow growth during upshift to 37°C, were selected to determine whether the release of hemolytic factors might depend on the environmental conditions.

Hemolytic factors secreted during growth of *A. wentii*-B1 and *P. steckii*, and their partial characteristics

During the growth of *A. wentii*-B1 in complete medium (TSB), no hemolytic activity was observed when tested on SBA, despite that culture filtrates derived from all conditions were concentrated 50 times (CCFs [50×]). In contrast to growth in undernourished medium (mAMM), CCFs (50×) derived from filtrates during the exponential phase of growth (3–9 days) showed visible zones of hemolysis in both batches when grown at 25°C (Fig. 2A). Although hemolytic characteristics of *A. wentii*-B1 on SBA shown in Fig. 1 were incomplete or diffusible, a visible zone of hemolysis could be seen on the first day of incubation at 25°C before hemolytic diffusion. Nevertheless, these hemolytic factors secreted from *A. wentii*-B1 were heat-sensitive (Fig. 2A).

Of *A. wentii*-B1, CCFs derived from 3, 6, and 9 days during growth in mAMM at 25°C in both batches showed strong hemolytic activity when tested on SBA within three days with a peak at 6 days during the exponential phase of growth. The profile of these secreted proteins seen on preliminary silver-stained SDS-PAGE gel demonstrated at least ten bands with relative molecular mass ranging from 74 to 10 kDa (data not shown). CCFs derived from 4 and 6 days were then pooled and fractionated by ultrafiltration of molecular weight cut off between 100 and 10 kDa in serial steps. In Fig. 2B, the protein fractionates of >100 and of 100–50 kDa showed the lysis of sheep blood cells within 48 h, but the fractionate of < 50 kDa had no hemolytic zone. The fractionates of >100 and 100–50 kDa were pooled and run in two separate gels on 7.5% native gel electrophoresis. One gel was stained with silver and another gel was placed on the surface of the SBA plate and then incubated at 37°C. In Fig. 2C, hemolysis demonstrated as a clear green area was induced by fractionate of >50 kDa-native gel at 37°C within 48 h similar to the purified α-hemolysin which used as a positive control. Those bands of hemolysis on the unstained gel were in the same position of protein bands on the silver staining gel. Native gel preparation of >50 kDa-fractionates was cut into three pieces (E1, E2, and E3) and the proteins were eluted from each gel cut. E2 is the area which had a hemolytic zone whereas E1 and E3 are the pieces of above and below E2, respectively. Eluted proteins from gel cut extract E2 showed lysis of sheep blood within 24 hours (Fig. 2C). When separated on SDS-PAGE, E2 showed silver-stained bands with relative molecular mass of 49, 61, 63, 88, and >175 kDa, whereas E1 showed three bands of 49, 61, and 63 kDa and E3 showed five bands of 41, 49, 61, 63, 86, and 88 kDa (Fig. 2D). Protein

Table 2. Characteristics of the cooperative hemolytic reactions between mold and bacteria on SBA

Cooperative interactions between each colony of *Aspergillus* or *Penicillium*, and each common bacterial colony occurred in a CAMP-like test (A), and interactions between the CCFs of *Aspergillus* or *Penicillium*, and *S. aureus*-colony and/or a purified *S. aureus*-sphingomyelinase occurred in a CAMP diffusion assay (B).

(A) CAMP-like test

	S. aureus[a]	*S. pneumoniae*[b]
A. micronesiensis	+	-
A. wentii A1	+	+
A2	+	+
B1	+	+
A. westerdijkiae	+	-
P. citrinum A1	+	v
A2	+	-
A4	+	v
P. copticola	+	+
P. paxilli A	-	v
B	-	v
P. steckii	+	+
P. sumatrense	~	-

(B) CAMP diffusion assay

	S. aureus	Sphingomyelinase
A. micronesiensis	+	+[c]
A. wentii A1	+	~
B1	+	~
P. citrinum A2	-	nd
P. copticola	-	nd
P. paxilli A	-	nd
P. steckii	+	-
P. sumatrense	+	~

[a] repeated 3 times, [b] repeated 4 times, [c] repeated 2 times
+, Hemolysis; -, No hemolysis; ~, Incomplete or diffusible hemolysis; v, Variable; nd, Not done

profiles of native gel cut extracts showed a band of >175 kDa in E2 different from E1 and E3.

The CCFs of *P. steckii* obtained during growth in TSB also had no hemolytic activity in all conditions. Although CCFs obtained during growth in mAMM could lyse sheep erythrocytes on SBA, only CCFs obtained from day nine during late exponential growth at 25°C had a hemolytic zone (data not shown). This factor showed low HA and was heat tolerance.

The release of hemolytic factors secreted by *A. wentii*-B1 and *P. steckii* during growth in mAMM could be slightly induced by the presence of sheep red blood cells. Hemolysis was somewhat increased during the exponential growth phase of *A. wentii*-B1, particularly at the 25°C upshift to 37°C either in batch 1 or batch 2. Nonetheless, the induction of the release of the hemolytic factors of *A. wentii*-B1 neither depended on the amount of 1% nor 7% sheep blood presented. By *P. steckii*, the induction of hemolysis was variable and quite different in batch 1 and batch 2.

Synergistic hemolytic CAMP-like reactions could be triggered by most tested strains of *Aspergillus* or *Penicillium* with *S. aureus* and/or *S. pneumoniae*

A cooperative (CAMP-like) reaction could be developed as a complete hemolytic zone on SBA, but not on HBA. Cooperative complete hemolytic zone shown as half-moon shape occurred when the border of mold colonies such as *P. copticola* [Fig. 3A(1)] touched the zones of the incomplete hemolytic zones of *S. aureus*, whereas a complete hemolytic zone was developed under the colony of *S. pneumoniae*. In Table 2A and Fig. 3A(2), all of the tested species of *Aspergillus* and most of *Penicillium* had synergistically cooperative interactions with *S. aureus*, except *P. paxilli* and *P. sumatrense*, whereas only three tested species including *A. wentii*, *P. copticola*, and *P. steckii* had cooperative interaction with *S. pneumoniae*.

Hemolysis could be enhanced by the cooperation between CAMP-like factors from *A. micronesiensis* and sphingomyelinase from *S. aureus*

The enhancements of hemolysis could be developed a complete hemolytic zone shown as a rugby-shape inner the incomplete hemolysis zone by the interaction between mold-CCFs as a CAMP-like factor and the hemolytic factor secreted during the growth of *S. aureus* and/or a purified *S. aureus*-sphingomyelinase (Fig. 3B). In a preliminary study of CAMP diffusion assay, 10 µl 50×-CCFs derived from six-day-old cultures of most *Aspergillus* or *Penicillium* could not enhance a hemolytic zone on SBA plate except *A. micronesiensis*. Hence, a volume of eight times of each CCFs (80 µl) of all *Aspergillus* or *Penicillium* except *A. micronesiensis* was used in CAMP diffusion assay on SBA plate within five days. In Table 2B, the results of the cooperative hemolytic interaction between mold-CCFs as a CAMP-like factor and the hemolytic factor secreted during the growth of *S. aureus* were positive in tested strains of *A. micronesiensis, A. wentii*, *P. steckii*, and *P. sumatrense*, but not in *P. citrinum*, *P. copticola*, and *P. paxilli*. However, only *A. micronesiensis* had clear hemolytic zone when

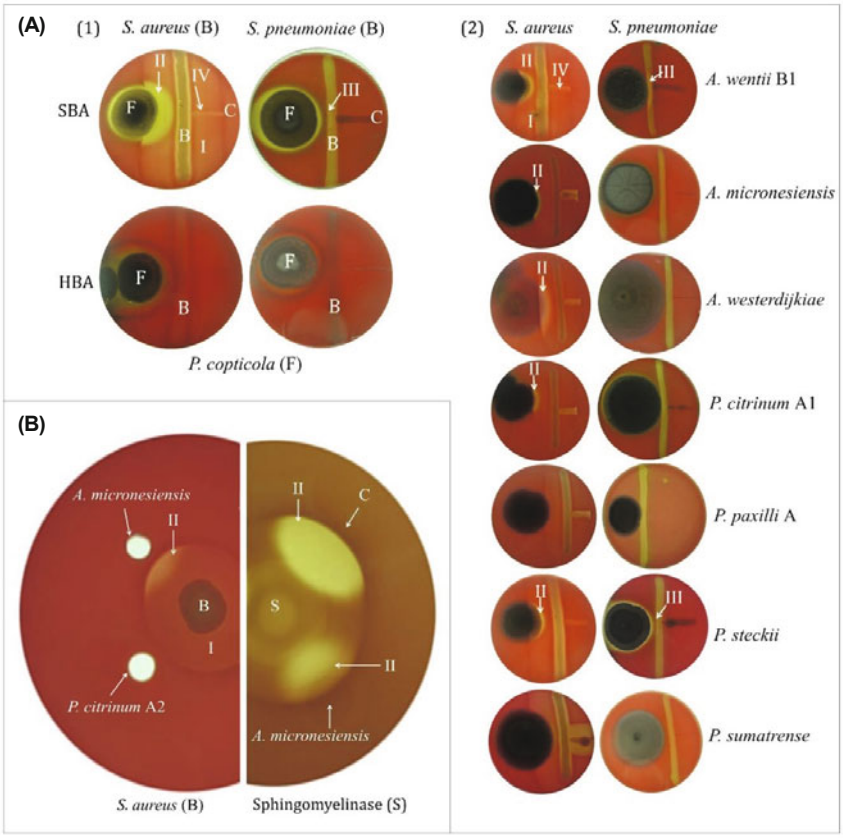

Fig. 3. Synergistic hemolytic reactions of mold and bacteria in a CAMP-like test (A) and a CAMP diffusion assay (B). A (1), synergistic hemolytic interaction between mold (F) such as *P. copticola* and *S. aureus* (B) occurred on SBA inner incomplete hemolytic zone of *S. aureus* (I) as a half-moon shaped (II) whereas the interaction between mold and *S. pneumoniae* (B) occurred beneath bacterial colony (III). No cooperative hemolytic reactions on HBA were seen. A (2), the synergistic hemolytic reactions on SBA with *S. aureus* (II) and/or *S. pneumoniae* (III) could be triggered by isolates of *A. wentii* B1, *A. micronesiensis*, *A. westerdijkiae, P. citrinum* A1 and *P. steckii,* but not by *P. paxilli* A and *P. sumatrense, R. equi* used as control (C) for synergistic hemolytic reaction with *S. aureus* (IV). B, In a CAMP diffusion assay on SBA, a complete hemolytic zone enhanced by the cooperation between the CCF of *A. micronesiensis* with hemolytic factors secreted during growth of *S. aureus* (B) was shown as a rugby-shaped (II) inner incomplete hemolytic zone of *S. aureus* (I) whereas the CCF of *P. citrinum* A2 gave a negative result. The rugby-shaped hemolytic zone also occurred by the cooperation between the CCF of *A. micronesiensis* with a purified sphingomyelinase of *S. aureus* (S). The CCF of *R. equi* used as a positive control (C).

tested with sphingomyelinase. Although 80 µl CCFs of *P. steckii* and *P. sumatrense*, or 160 µl CCFs of *A. wentii*-A1 and -B1 were used when tested with sphingomyelinase in a CAMP diffusion assay, nevertheless, the enhancement of hemolysis could incompletely or weakly occur. In Fig. 3B, the result showed that the enhancement of hemolysis could develop a rugby-shaped complete hemolytic zone by mold-CCFs as a CAMP-like factor, at least CCF from *A. micronesiensis*, via *S. aureus*-sphingomyelinase.

Discussion

The first line of pathogenesis is a microbial entry into the host body and can survive and grow in the host tissue. To achieve these requirements, the ability for the acquisition of iron by producing hemolytic factors is one of the advantages. Various pathogenic fungi have been reported for their hemolytic activity and/or health effects such as *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Malassezia* species, and some endemic or opportunistic fungi (Luo *et al*., 2001; Schaufuss and Steller, 2003; Nayak *et al*., 2013; Juntachai *et al*., 2014). Hemolytic factors produced by *P. chrysogenum* and *A. niger* have been characterized and demonstrated adverse health effects such as activation of macrophage inflammatory protein-2 (MIP-2) production (Donohue *et al*., 2005) and toxicity in mouse neuron cells (Donohue *et al*., 2006), respectively. Our preliminary *in vitro* study showed that many indoor airborne molds could secrete factors during growth into solid agar for direct hemolytic activity and/or synergistic interaction with some common bacteria in the respiratory tract, particularly *S. aureus*, to lyse red blood cells. There are still questions about hemolytic reactions, such as; "How different are any secreted hemolytic factors in quality or quantity or characteristics between strain or species?" or "Could the release of hemolytic factors be dependent on environmental conditions?" or "Which secreted factors are involved in the cooperation between airborne mold and common bacteria when they are the cohabitating partner in the respiratory tract?" The findings presented here are the partial characteristics of the factors affecting hemolysis secreted from eight airborne species including *A. micronesiensis*, *A. wentii*, *A. westerdijkiae*, *P. citrinum, P. copticola*, *P. paxilli*, *P. steckii*, and *P. sumatrense* that may be provided for more understanding of the involvement of these factors on hemolysis.

Asp-hemolysin produced by *A. fumigatus* was often suggested to be involved as fungal virulence (Nayak *et al*., 2013). Although a study of Wartenberg and colleagues (2011) showed that hemolysis was not altered in *A. fumigatus*-mutant lacking aegerolysin gene with no virulence attenuation in the mouse model, however Asp-hemolysin which belongs to aegerolysin family was found as a major secreted protein in various growth conditions. However, the variable numbers of aegerolysin proteins in unrelated fungal taxa were described (Nayak *et al*., 2013). Hemolysin is likely not only to be essential for pathogenicity but may also be used for nutrition acquisition during fungal survival in a host. In this study, most of the thirteen strains tested of *Aspergillus* and *Penicillium* could not survive at 37°C including *A. wentii*, *P. paxilli*, *P. copticola*, *P. steckii*, and *P. sumatrense* (data not shown), but all of them had HA on HBA and/or SBA at initial germination condition of 25°C and could continuously survive at 37°C (Table 1). *A. wentti*-B1 which could not survive at 37°C also secreted hemolytic factors well at 25°C during germination and exponential growth for three to six days in undernourished broth (Fig. 2A) and still survive during an upshifted temperature to 37°C. The ability of hemolytic factor production during spore germination and survival before passing through host airways may take advantages for the growth of these molds in the starvation condition in host tissue. The relative molecular weight of more than 50 kDa of secreted *A. wentti*-B1-hemolytic factors is rather higher than hemolysin in Aegerolysin family which is one of the largest groups of hemolytically active proteins (Nayak *et al*., 2013). Interestingly, a protein band of >175 kDa presented only in eluted protein E2 which had a hemolytic zone, did not present in E1 and E3 which had non-hemolytic zones. Further efforts on the preparation and purification in a large amount of E2 released in the suitable condition is needed to identify the protein involving in hemolysis.

Hemolytic factors released from *A. wentii*-B1 were sensitive at 100°C for 10 min, whereas hemolytic factors of *P. steckii* were heat tolerance. Hemolysins from *Trichophyton mentagrophytes* were not affected by proteinase K treatment, high (100°C) nor low (-20°C) temperatures, which was the characteristic of a small peptide hemolysin (Schaufuss *et al*., 2007). The hemolytic activity has been described to attribute to free fatty acid released through the hyphal breakdown of contaminated sago starch mold, such as *P. citrinum,* which was hypothesized as an etiology of Sago hemolytic disease (SHD) affecting rural Papua New Guineans (Atagazli *et al*., 2010; Shipton *et al*., 2013). Hemolytic activity of *P. steckii* in this study was well-detected during late exponential growth with hyphal autolysis for nine days in undernourished broth at 25°C. It is likely that hemolytic factors secreted from *P. steckii* might be small peptides or non-proteinaceous agents such as free fatty acid. As we found the different hemolysis by *P. steckii* in two batches, it is suggested that hemolytic factors of *P. steckii* may be produced in various amount or involvement with several factors during growth even though in the same condition. However, several secondary metabolites from *P. citrinum*, *P. steckii* as well as *P. sumatrense* have been described (Malmstrom *et al*., 2000). Further efforts on purification and characterization of secreted factors from these species affecting hemolysis are needed.

Synergistic hemolytic reactions between all tested strains of *Aspergillus* or *Penicillium* with *S. aureus* and/or *S. pneumoniae*, could be generated only on SBA (Table 2 and Fig. 3). As previously described by Christie and colleagues (1944), red blood cells could be lysed by CAMP factors that contained a large amount of the sphingomyelin in the cell membrane, and that have been pretreated with *S. aureus* β-toxin (sphingomyelinase). Because the sheep red blood cell membrane contained a greater amount of the sphingomyelin than human blood, the purified sphingomyelinase of *S. aureus* was used to test the possible involvement of these interactions. Using CAMP diffusion assay, CCFs of those *Aspergillus* or *Penicillium* which had CAMP-like reactions on SBA were prepared to be tested with a purified sphingomyelinase of *S.*

aureus. In the preliminary test, many samples of various CCFs did not develop CAMP-like reaction on CAMP diffusion assay. This may be due to the environmental condition for the release of CAMP factors. Contrary to the release of the hemolytic factors, CAMP factors were released from molds cultured in TSB rather than in mAMM. CCFs from mAMM cultures could change the color of sheep red blood agar to green resulting in difficulty interpreting the results. In this study, CCFs released from particularly *A. micronesiensis* that acted as CAMP-like factors enhanced the hemolysis induced by a purified sphingomyelinase from *S. aureus*. These reactions could confirm that amongst the hemolytic factor released from *S. aureus*, sphingomyelinase was necessary for hemolysis enhancement by indoor molds. The results in this study were consistent with previously described cooperative (CAMP-like) lytic processes of group B streptococci (Bernheimer *et al.*, 1979) and dermatophytes (Schaufuss *et al.*, 2005). However, CAMP diffusion assay should be repeated in CCFs prepared from more strains of *A micronesiensis*, *A. wentii*, and *P. sumatrense*. Recently, the CAMP reaction between *S. aureus* and *Propionibacterium acnes*, which resulted in hemolysis enhancement involved in skin invasion, has been demonstrated for development of a potential therapeutic treatment (Lo *et al.*, 2011). The *S. aureus* skin infection in mice was suppressed by a combination of CAMP factor (*P. acnes*) neutralization and β-hemolysin (*S. aureus*) immunization. Thus, the information obtained in this study may lead to more understanding of the involvement of those hemolytic and CAMP-like factors of *Aspergillus* and *Penicillium* in cytolysis for fungal survival prior to pathogenesis.

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Conflict of Interest

The authors declare no conflict of interest.

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