Chapter 3

Results

The rubber trees are regularly wounded by a systematic "tapping" procedure, in which the bark is shaved to a thin layer. A fresh wound is being created on the tapping site. This could open up an opportunity for the microbial such as bacterial and fungus to invade the tissue. The rubber tree must be well equipped with antimicrobial compounds in order to kill or sustain the invaders. We report the screening, purification and characterization of antimicrobial proteins from fresh *Hevea* latex in part A and a proposed defense function of small rubber particles in sustaining the microbial invaders including an enhancing effect of the rubber particle proteins on the antifungal activity of a commercial antibiotic in Part B.

Part A: Antifungal protein from *Hevea* latex

1. Screening and purification of antimicrobial protein in the fresh *Hevea* latex

1.1 Screening of antimicrobial protein from fresh *Hevea* latex

Fresh natural rubber latex derived from the freshly collected latex by tapping the rubber trees, *H. brasiliensis*. It contains about 0.95-1.20% protein by volume of the latex. Distribution ratio of the latex proteins in different fractions of the centrifuged fresh latex are 27.2% in the top rubber layer fraction, 47.5% in the middle aqueous cytosolic serum phase (designated as C-serum) and the remaining 25.3% is in the sediment membrane-bound organelles bottom fraction (BF). These sediment membrane-bound organelles BF comprise lutoid particles, Frey-Wyssling complex and also possibly minute small tiny rubber particles. The aqueous cytosolic C-serum contains various distinct several proteins types of different nature and catalytic functions, which are active in the overall latex functions and of various metabolic processes (d' Auzac, 1989; Premakumari, 1992; Wititsuwannakul, 2001). The soluble contents of the membrane-bound organelles bottom fraction (BF) contain organic compounds and also with

several types of proteins of different nature and catalytic functions (collectively designated as the B-serum) (Hasma, 1992). Both of the soluble aqueous sera (B-serum and C-serum) are major parts of non-rubber latex constituents, which contain most of the proteins as part of the composite mixtures with rubber particles in the rubber latex. The localization and the presence of antimicrobial proteins in the aqueous sera (B-serum and C-serum) were therefore investigated.

Screening for the presence of antimicrobial protein was performed on B-serum and C-serum fractions obtained by step-wise acetone precipitation in increments of 0-20%, 20-40%, 40-60%, 60-80% and 80-95%. Each fraction was centrifuged at 10,000 x g and resuspended in 50 mM Tris-HCl, pH 7.4. Assays of antimicrobial activity of the isolated protein fractions were screened by broth microdilution method. Only the 60-80% B-serum fraction was found to be effective against tested microbial species as shown in Table 5. Neither of the acetone fractions from C-serum (data not shown) nor the rest of the B-serum fractions showed any antimicrobial activity. The most sensitive fungal strain to the 60-80% B-serum fraction was *C. albicans* ATCC 90028 (MIC 112 μg/ml) while *Cryptococcus neoformans* was not sensitive (MIC >1.8 mg/ml). Higher MIC (896 μg/ml) was obtained in most of the Gram-negative bacteria tested, except for insensitive *Pseudomonas aeruginosa* species.

The antimicrobial activity of protein was expressed as minimal inhibitory concentration (MIC). From preliminary result, the 60-80% acetone fraction was further fractionated to 60-70% and 70-80%. The results showed that the 70-80% acetone fraction contained the highest specific antimicrobial activity (lowest MIC) as showed in Fig. 7A. The SDS-PAGE analysis of serial acetone fractioned proteins represented in Fig. 7B and the 70-80% acetone fraction revealed only two major protein bands of 10 kDa and 28 kDa. The 70-80% acetone fraction was screened for antifungal activity against *C. albicans* of 28 clinical isolates and obtained the MICs ranged from 8.6 to 137.8 μg/ml and two standard strains (*C. albicans* ATCC 10231 and ATCC 90028) showed the MICs of 56 and 112 μg/ml, respectively (Table 6).

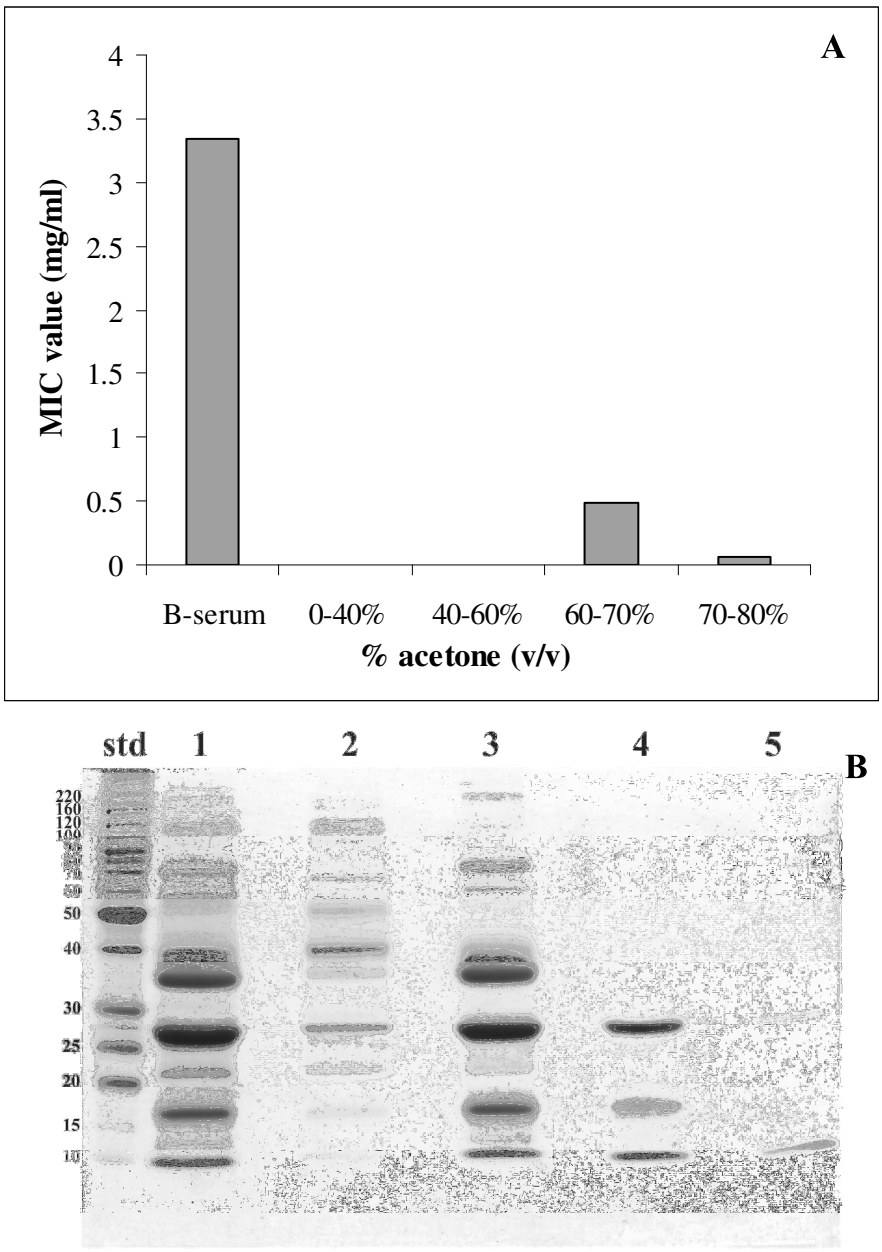

Fig. 7 (A) Antifungal activity of acetone precipitated B-serum protein fractions. Proteins of each fraction were tested for antifungal activity against *C. albicans* ATCC 10231 with broth microdilution method as described in Materials and Methods. The results are represented as minimal inhibitory concentration (MIC, mg/ml)

(B) SDS-PAGE separation of acetone fractionated B-serum proteins (12% gel). Lanes Std, Standard proteins marker; lane 1, B-serum; lane 2-5, 0-40%, 40-60%, 60-70%, 70-80% acetone precipitates, respectively.

Table 5 Screening of a crude 60-80% acetone precipitated fraction from the *H. brasiliensis* against various potential microbial pathogens of the oral cavity and respiratory tract.

Microbial	MIC (μg/ml)
Gram negative bacteria	
Porphyromonas gingivalis W50	896
Porphyromonas gingivalis 381	896
Porphyromonas gingivalis ATCC 33277	896
Prevotella intermedia 25611	896
Tannerella forsythia ATCC 43037	896
Aggregatibacter actinomycetemcomitans ATCC 33384	896
Pseudomonas aeruginosa ATCC 27853	>1,800
Gram positive bacteria	
Streptococcus mutans ATCC 25175	>1,800
Lactobacillus fermentum ATCC 14931	>1,800
Fungi	
Candida albicans ATCC 90028	112
Cryptococcus neoformans ATCC 90112	>1,800
Cryptococcus neoformans ATCC 90113	>1,800

*Controls were cell suspensions added to broth without hevein.

Table 6 Antifungal activity of 60-80% acetone precipitated fraction against *C. albicans* (28 clinical isolates and 2 ATCC strains). The activity represented as minimal inhibitory concentration (MIC)

Clinical strains	MIC (μg/ml)	Clinical strains	MIC (μg/ml)
A001/1	8.6	A030/1	68.8
A002/4	34.4	A030/4	137.8
A003/1	68.8	A031/3	137.8
A007/2	68.8	A033/2	137.8
A016/5	68.8	A034/4	137.8
A017/5	68.8	A035/4	68.8
A018/2	68.8	A036/4	68.8
A019/3	68.8	A037/4	68.8
A020/1	137.8	A039/4	137.8
A023/2	68.8	A044/1	137.8
A024/1	68.8	A045/5	68.8
A025/3	68.8	A046	34.4
A026/1	137.8	A052/3	137.8
A026/2	137.8	ATCC 10231	56
A027/3	137.8	ATCC 90028	112

1.2 Purification and characterization of antimicrobial protein from B-serum

The B-serum was step-wise fractionated by acetone to separate the active antimicrobial protein fractions in increments of 0-40%, 40-60%, 60-70% and 70-80%. Each fraction was centrifuged at 10,000xg and resuspended in 50 mM Tris-HCl, pH 7.4. The most effective fraction (70-80%) was further purified by DEAE-Sepharose Fast Flow chromatography. The elution profile screen with the present of three major protein peaks, only the first peak exhibited anti-*Candida* activity (Fig. 8). The first peak fractions were pooled and its yield was calculated to be 0.3 mg per 1 liter of fresh latex. Upon SDS-PAGE, the first peak fraction contained only a single protein band (Fig. 9A) with M_r *ca* 10 kDa. Tricine-SDS-PAGE, commonly employed for low MW proteins analysis, was further carried out to improve accuracy on the M_r determination. Similar to the SDS-PAGE, Tricine-SDS-PAGE, the profile with a single protein band with M_r around 9-10 kDa was revealed (Fig. 9B). The precise MW of this active antifungal protein was then sought from MALDI-TOF mass spectrometer analysis. The active protein exhibited a major peak corresponding to M_r of 4,717 Daltons (Fig. 10). Its N-terminal sequence (11 residues) and amino acid composition were determined (Fig. 11 and Table 7). The data obtained in M_r value, N-terminal sequence residues and amino acid composition, indicates that the purified antifungal protein is hevein, a previously known chitin binding protein (Archer, 1960; Walujono *et al.*, 1975). The purification of the hevein antifungal protein was accordingly summarized in Table 8.

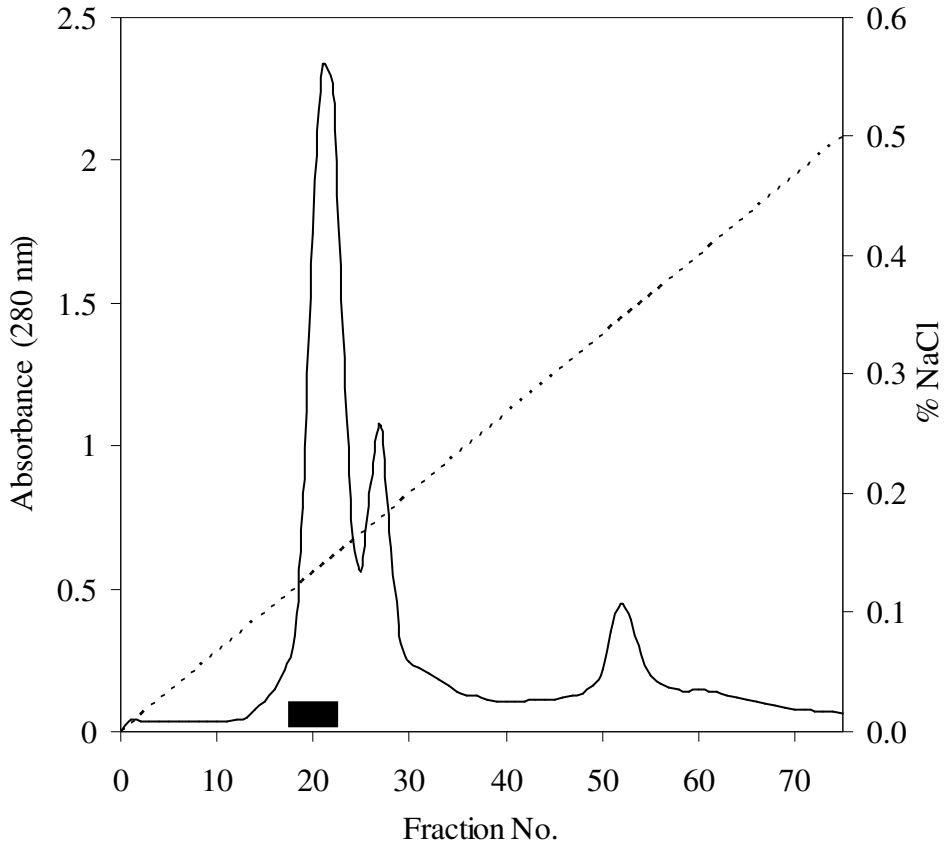

Fig. 8 Chromatographic profile of hevein from DEAE Sepharose Fast Flow column chromatography.

Column was equilibrated in 50 mM Tris-HCl, pH 7.4 and eluted with a gradient of 0-0.5 M NaCl in the same buffer. The absorbance of eluting protein was determined at 280 nm and tubes with anti-*Candida* activity against *C. albicans* ATCC 10231 were marked with black bar area.

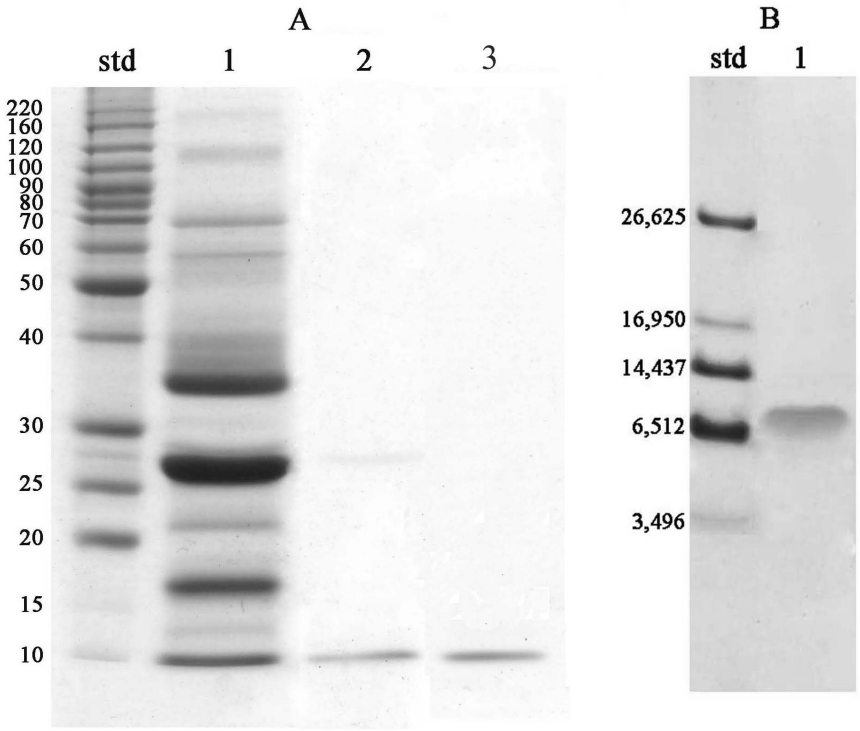

Fig. 9 (A) Analysis of proteins by 12% SDS-PAGE and protein bands were stained with Coomassie Brilliant Blue R-250. Lane std, molecular mass marker; lane 1, B-serum; lane 2, an 70-80% acetone precipitate from B-serum; lane 3, purified hevein from DEAE Sepharose Fast Flow column.

(B) Tricine SDS-PAGE (16.5% gel) of purified hevein. Lane std, molecular mass marker, lane 1 purified hevein from DEAE Sepharose Fast Flow chromatography fractionation

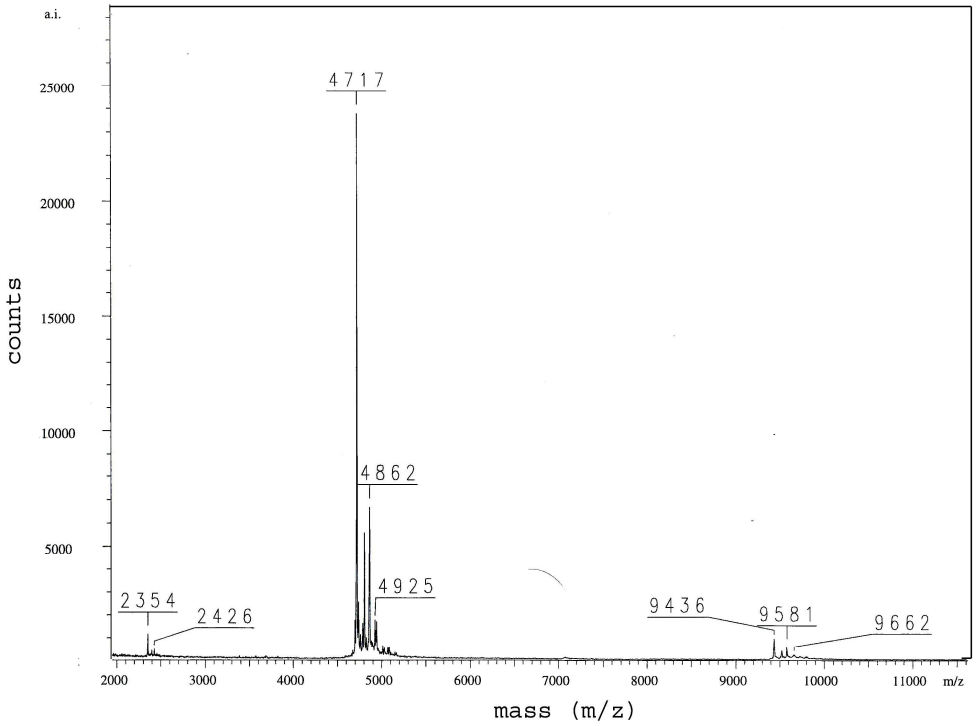

Fig. 10 Mass spectrum analysis of purified antifungal protein by MALDI-TOF mass spectrometer

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CLUSTAL W (1.82) multiple sequence alignment


hevein          EQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKD 43

unknown         EQCGRQAGGKL---------------------------------- 11
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Fig. 11 N-terminal sequencing analysis of purified hevein

11 residues of purified antifungal protein were aligned with hevein and show 100% identity to hevein from previous study (Walujono *et al*., 1975).

Table 7 Amino acid composition of hevein and the purified antifungal protein

Amino acid	1	2	3	4
Ala	1.3	0.8	1	**1**
Cys	8	8	7.9	**8**
Asp	7.6	5.1	6.2	**6.4**
Glu	6	3.9	5.5	**5.6**
Phe	0.1	0	0	**0**
Gly	5.8	3.9	4.9	**5**
His	0.9	1.4	1.4	**0.9**
Ile	0.2	0	0	**0.01**
Lys	2.1	2.1	2	**2.8**
Leu	2.3	2.1	1.9	**2**
Met	0	0	0	**0**
Pro	2.5	2.1	1.9	**2.1**
Arg	1.2	1.9	1	**0.9**
Ser	3.5	5	3.7	**3.4**
Thr	1.1	0.9	0.9	**0.9**
Val	0.2	0.7	0	**0**
Try	1.1	1.9	1	**0.9**
Total	43.9	39.8	39.3	**39.91**

1 = Composition of hevein (Van Parijs, 1991)

2 = Composition according to Archer,1960.

3 = Composition according to Walujono *et.al.*, 1975.

4 = Composition of antifungal protein purified by 70-80% acetone fractionation and DEAE Sepharose, Fast Flow chromatography.

Table 8 Purification of antimicrobial protein (hevein)

Purification step	Protein conc. (mg/ml)	Total protein (mg)	MIC (µg/ml)	Yield (%)
B-serum	3.46	173.0	-	100
60-70%acetone fractionation	3.88	46.6	485	27
70-80%acetone fractionation	0.76	3.8	64	2.2
DEAE column chromatography	0.24	0.5	12.5	0.3

* 1 L of fresh latex yield 0.3 mg of hevein

2. Biochemical properties of the hevein-antifungal protein

2.1 Thermal stability of hevein-antifungal protein

The thermal stability of hevein was examined by preincubating hevein at various temperatures range from 4, 50, 60, 70, 80, 90°C and in boiling temperature for 0.5, 1 and 1.5 h before employed in the antifungal susceptibility testing. The results shown in Fig. 12 indicated that hevein was very stable to the extreme temperature, and was probably the most stable protein present in the latex B-serum. The antifungal activity of hevein fully retained up to 30 min in boiling temperature. Its activity decreased by 50% and 75% after boiling for 1 h and 1.5 h, respectively (Fig. 12).

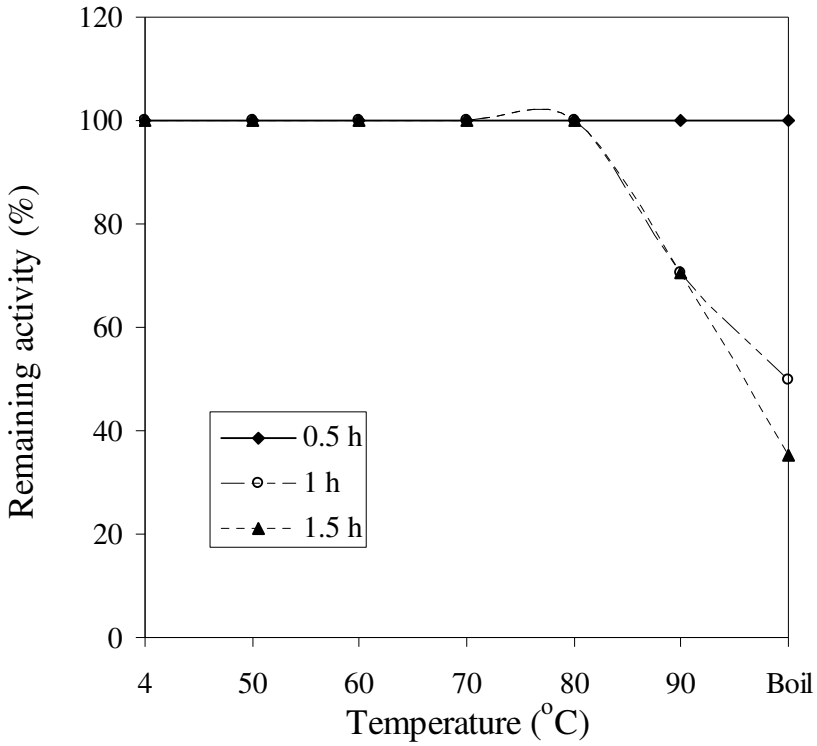


Fig. 12 Thermal stability of hevein-antifungal protein

Hevein was heated in 50 mM Tris-HCl, pH 7.4 for 0.5, 1.0 and 1.5 h at the indicated temperatures, cooled in ice bath rapidly before assayed. The results were expressed as the percentage of remaining activity when compared to the control hevein (kept at 4°C before assayed) was used as 100% antifungal activity.

2.2 pH stability of hevein-antifungal protein

In addition to the thermal stability assay, pH stability was another criterion to be characterized for the purified hevein. Pretreatment of the purified hevein was carried out in the pH ranges of 2 up to 9. This is to check if the purified hevein can withstand the extreme pH, and also to see the pH effect on the antifungal activity. The results were shown in Fig. 13, depicting the pH effect on the remaining antifungal activity upon the preincubation or pretreatment of hevein at various pH prior to the determination of MIC. It was clear from the Fig. 13 results that hevein was quite pH stable. Hevein also have quite broad range pH stability; 100% stable in pH 2-9 at 4°C for 24 h**.**

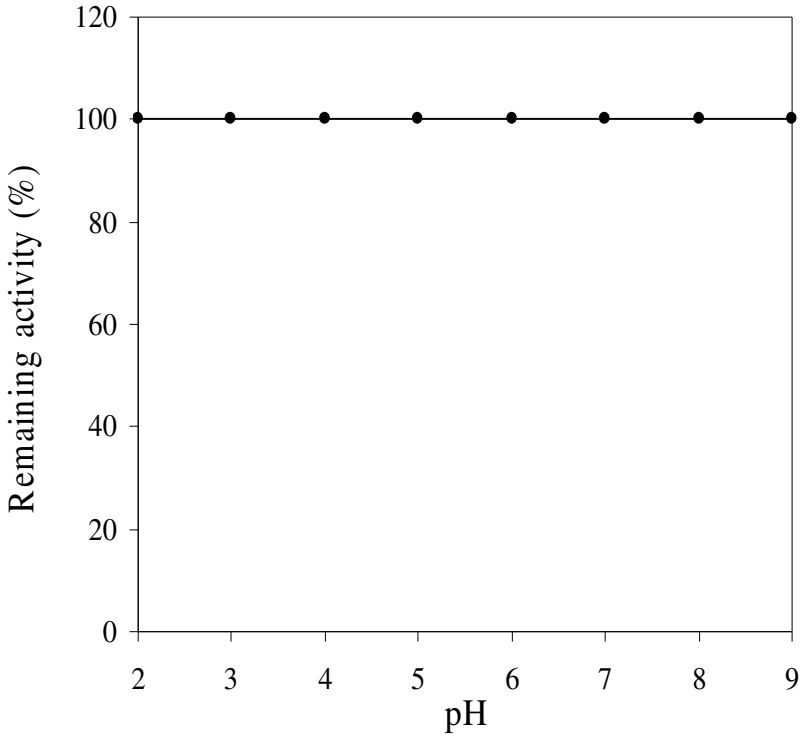


Fig. 13 pH stability of hevein- antifungal protein.

Hevein was incubated in buffers of indicated pH for 24 h at 4°C and readjusted to pH 7.4 before antifungal activity testing. Antifungal activity was assayed according to broth microdilution method as described in Materials and Methods. The results were elucidated as the remaining activity when compared to the control which was suspended in 50 mM Tris- HCl, pH 7.4 and stored at 4°C before assayed.

3. Anti-*Candida* activity of hevein and its effect by *Hevea* protease inhibitor (HPI)

The antifungal activity of hevein was investigated against ATCC strains of *Candida* spp. including *C. albicans* ATCC 10231 and ATCC 90028, *C. tropicalis* ATCC 750, ATCC 66029 and ATCC 13803, *C. krusei* ATCC 34135 and ATCC 6258 and *C. glabrata* ATCC 90030 and ATCC 66032 by the broth microdilution method. The concentration of protein required for 80% growth inhibition (MIC_{80}) was used as a measure of the inhibitory potency of hevein on these fungi. Hevein was able to inhibit the growth of *C. tropicalis* strains in suspension cultures at as little as 12 μg/ml. *C. tropicalis* strains were the most sensitive species of *Candida* tested. Strains of *C. albicans*, *and C. krusei* were moderately sensitive while those of *C. glabrata* were the least sensitive with MIC values of >190 μg/ml (Fig. 14)

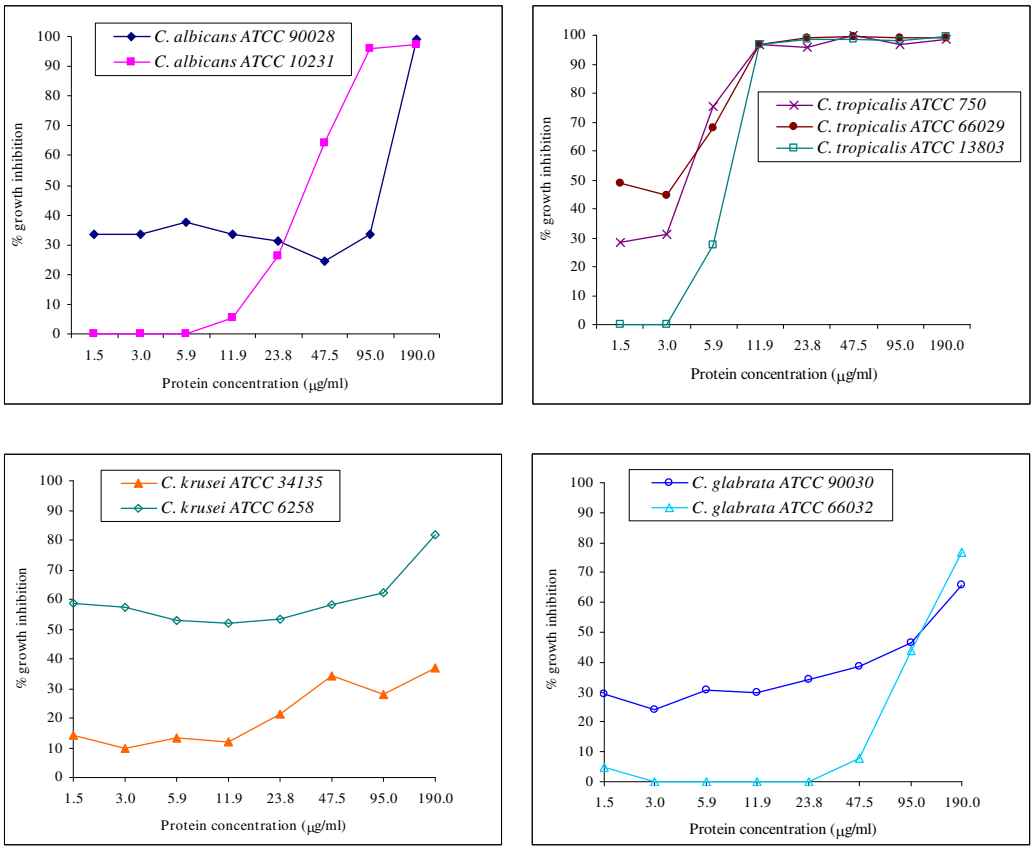
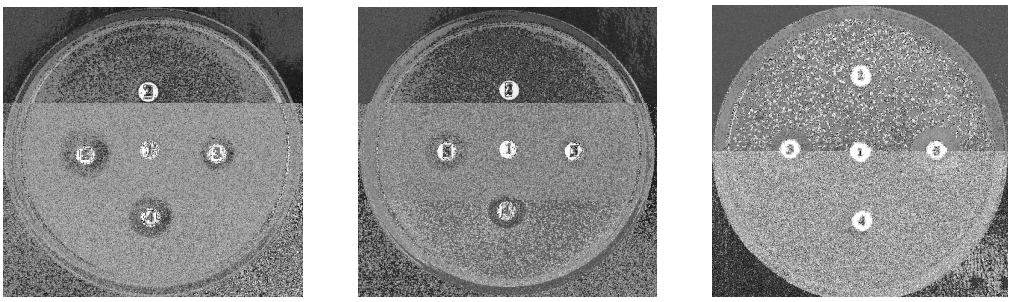


Fig. 14 Anti-*Candida* activity of purified hevein. The percentage of growth inhibition was measured at varying concentrations of hevein with the following test organisms: *C. albicans* ATCC 90028 (◆), ATCC 10231 (■), *C. tropicalis* ATCC 750 (✱), ATCC 66029 (●), ATCC 13803 (□), *C. krusei* ATCC 34135 (▲), ATCC 6258 (◇), *C. glabrata* ATCC 90030 (○), ATCC 66032 (△). The growth inhibition is represented as percentage of the growth inhibition of control cultures.

Similarly, the disk diffusion assay also demonstrated inhibition of growth of *C. tropicalis* ATCC 750 (Fig. 15 A1) and *C. albicans* ATCC 10231 (Fig. 15 A2) by hevein, added to the disc at a concentration of as little as 125 μg/ml. At each concentration the inhibition zone was bigger with *C. tropicalis* ATCC 750. Moreover, an enhancing effect of HPI on the antifungal activity of hevein was also revealed from the bigger clear zone diameter size obtained upon combination with hevein (Fig. 15 B). The HPI (at 400 μg) when tested alone showed no antifungal activity as no clear zone could be seen from disc 2. However, when it (at either 200 or 400 μg) was combined with 40 μg of hevein, the clear zone diameter obtained (discs 4 or 5) was significantly bigger than that of the hevein control, disc 3.



(A1) (A2) (B)

Fig. 15 (A) Growth inhibition (clear zone) of hevein against *C. tropicalis* ATCC 750 (A1) and *C. albicans* ATCC 10231 (A2) and (B) the enhancing effect on antifungal activity of hevein against *C. albicans* ATCC 10231 by *Hevea* protease inhibitor (HPI). Discs in both A1 and A2 contained 40 µl hevein solution added at different concentration, where disc 1 is the negative control containing 40 µl of 20 mM Tris-HCl buffer, disc 2-5 contained 40 µl of hevein solution at 25, 125, 250, 750 µg/ml or 1, 5, 10 and 30 µg/disc, respectively. For the discs in B, disc 1, negative control containing 40 µl of 20 mM Tris-HCl buffer; disc 2, 400 µg of HPI; disc 3, 40 µg of hevein; disc 4-5, 40 µg of hevein combined with 200 and 400 µg of HPI, respectively.

4. Inhibitory effect of chitotriose on the antifungal activity of hevein

The cell wall of fungi and yeasts are composed of chitin which is a minor (0.6 to 9%) but important component of the yeast cell wall, particularly of the septa between independent cell compartments, budding scars, and the ring around the constriction between mother cell and bud. Hevein is a well known chitin-binding protein (Broekaert, 1990). Our results showed that the fungal growth inhibitory effect of hevein was decreased, in a dose dependent manner, upon addition of N, N′, N″-triacetylchitotriose (chitotriose). The antifungal activity was completely abolished in a presence of 10 mM chitotriose (Table 9). This confirms that the binding between hevein and fungal chitin is essential for the fungus growth inhibition as well as mediated by N-acetylglucosamine oligomers.

Table 9 Inhibitory effect of chitotriose on the antifungal activity of hevein

Sample	Fungal growth inhibition (%)
1. Hevein*	100
2 = 1+ Chitotriose addition (1 mM)	50
3 = 1+ Chitotriose addition (5 mM)	25
4 = 1+ Chitotriose addition (10 mM)	0

* Hevein concentration = 500 μg/ml = 106 μM

5. Hevein in inducing yeast cell aggregation : calcium requirement

Hevein has previously been shown to aggregate rubber particles in a lectin-like manner. The polybridging between hevein and rubber particle was mediated by N-acetylglucosamine (Gidrol *et al*., 1994). Similarly, our result demonstrated the ability of hevein in inducing yeast cell aggregation in a lectin-like manner. $CaCl_2$ was also shown to enhance the aggregation with optimum concentration of 0.5 mM (Fig. 16). Morever, EDTA at 10 mM was able abolish the induction of yeast cell aggregation by hevein (Fig. 17). This suggests that the aggregation between hevein and yeast cells is calcium dependent.

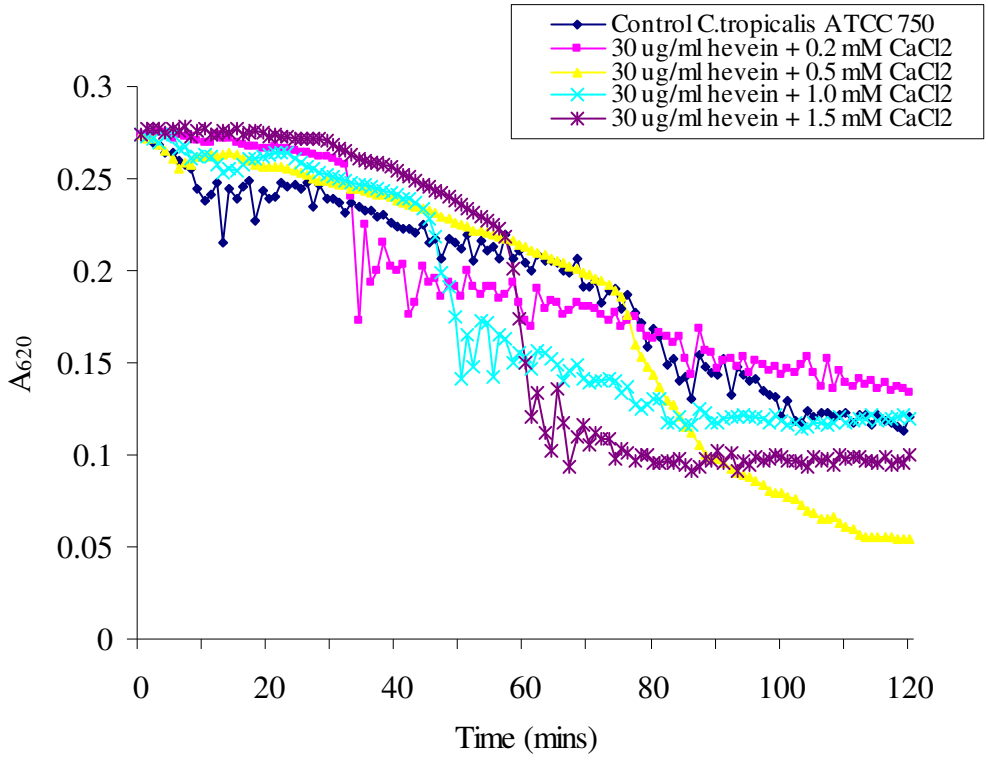


Fig. 16 Effect of Ca^{2+} on *C. tropicalis* ATCC 750 aggregation induced by hevein.

Yeast cells were suspended in different concentration of calcium-containing buffer at room temperature. The suspension was monitored at absorbance 620 nm (A_{620}) to the appropriate dilution and hevein was added to a final concentration of 30 μg/ml. Buffer was added to the negative control sample. The A_{620} of the suspensions was monitored every minute for 2 h after protein supplement.

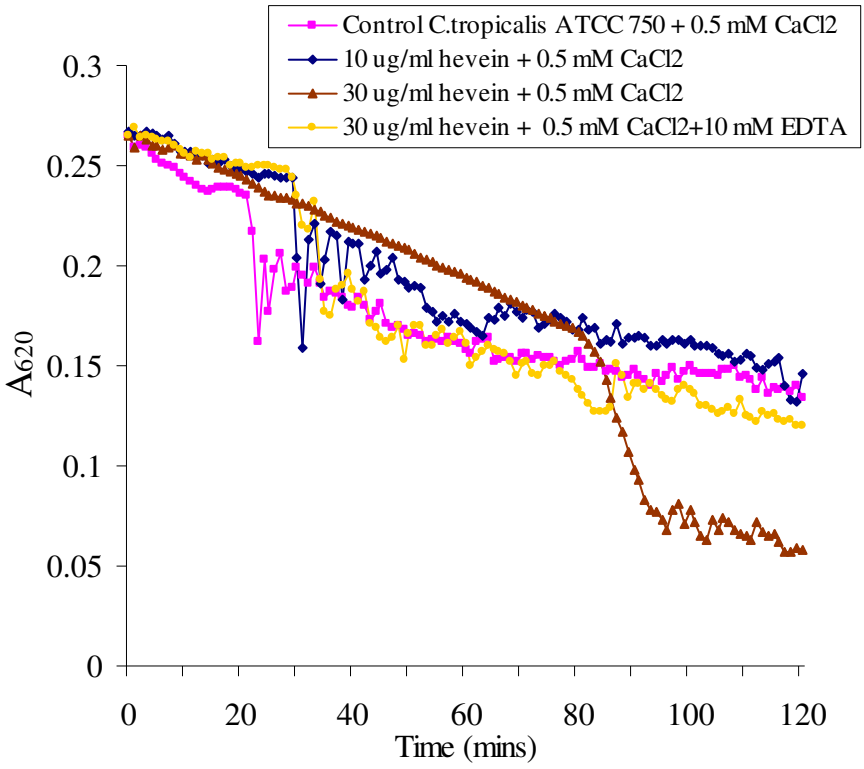


Fig. 17 Effect of EDTA on *C. tropicalis* ATCC 750 aggregation induced by hevein.

Yeast cells were suspended in calcium-containing buffer with or without 10 mM EDTA at room temperature.

Part B: Small rubber particles and their enhancing effect on the antifungal drug

1. Yeast cell aggregation induced by small rubber particles (SRP)

The SRP are highly abundant in rubber latex and account for more than 95% of total rubber particle population (Gomez and Hamzah, 1989). SRP had recently been shown to play an important role in latex coagulation (Wititsuwannakul *et al*., 2007). A possible association of antifungal factor has also been proposed for the SRP. A supportive form of evidence on this regard is revealed from this finding. The aggregation of yeast cells was shown to be induced by washed SRP (Fig. 18). Although further study on specific interaction between localized receptor on the SRP and yeast lectin is still needed. This result, however, suggests a physiological role of the colloidal SRP in sustaining the microbial invaders.

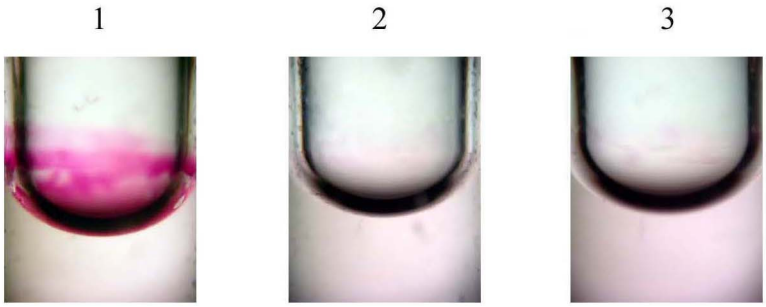


Fig. 18 Yeast cells aggregation by small rubber particle (SRP) after 30 min-incubation (1), yeast cells control (2) and SRP control (3). The assay mixtures were stained with basic Fuchsin. The yeast cells aggregate was separated by centrifugation and viewed with a light microscope.

2. An enhancing effect of SRP and SRP proteins (SRPP) on the activity of an antifungal drug

Amphotericin B (AMB), a polyene, is fungicidal in vitro and in vivo against *C. albicans* and *Aspergillus fumigatus*. It has been used as the standard treatment for invasive aspergillosis, particularly for severe and life-threatening infections. While the AMB is associated with a number of adverse effects or reactions, in many cases the efficacy of drug outweighs the risk. In this experiment, when washed SRP was added to dilution series of AMB to assess their effects on the MIC of AMB against *C. albicans* ATCC 10231. The addition of SRP significantly reduced the MIC of AMB from 0.5 μg/ml (when tested alone) to 0.125 μg/ml when combined with SRP as low concentration at 2.18 μg/ml or with four-fold decrease of AMB as shown in Table 10 whereas no intrinsic antifungal effect was found with SRP. Similar to the SRP, the SRPP, isolated and purified from SRP, also exhibited an enhancing effect towards the AMB antifungal activity (Table 10).

Table 10 Enhancing affects of small rubber particles (SRP) and small rubber particle proteins (SRPP) in combination with amphotericin B against *C. albicans* ATCC 10231

Sample	protein conc. (μg/ml)*	AMB (μg/ml)	% AMB reduction
1. AMB	-	0.5	-
2. AMB + SRP	2.18	0.125	75
3. AMB + SRPP	0.40	0.125	75

*Small rubber particle (SRP) and small rubber particle proteins (SRPP) at indicated protein concentration were the concentration which combined to AMB and conserved 80% growth inhibition but not active alone. MIC of AMB alone is 0.5 μg/ml.

Moreover, SRPP showed enhancing effect on AMB against all eight strains tested when added at indicated concentration (0.07-0.4 μg/ml). The *C. albicans* ATCC 90028, *C. albicans* ATCC 10231 and *C. tropicalis* ATCC 66029 were found to be among the most susceptible strains. In the presence of the SRPP at concentration ≥ 0.40 μg/ml, the MIC_{80}s of AMB for *C. albicans* ATCC 90028, *C. albicans* ATCC 10231 and *C. tropicalis* ATCC 66029 were reduced four-fold (Table 11). A lower (two-fold) reduction level of the MIC_{80}s of AMB for *C. krusei* ATCC 34135, *C. tropicalis* ATCC 750, *C. tropicalis* ATCC 13803, *C. glabrata* ATCC 90030 and *C. glabrata* ATCC 66032 was also observed in the presence of SRPP at concentration ranged from ≥ 0.07 μg/ml to ≥ 0.40 μg/ml.

Table 11 Enhancing affect on antifungal activity of SRPP combined with amphotericin B (AMB) against *Candida* spp.

Strain	MIC_{80} (μg/ml)		% AMB reduction
	AMB alone	AMB + SRPP	
C. albicans ATCC 10231	0.5	0.125 + ≥ 0.40	75
C. albicans ATCC 90028	0.25	0.0625 + ≥ 0.40	75
C. krusei ATCC 34135	2	1 + ≥ 0.10	50
C. tropicalis ATCC 750	1	0.5 + ≥ 0.14	50
C. tropicalis ATCC 13803	1	0.5 + ≥ 0.07	50
C. tropicalis ATCC 66029	1	0.25 + ≥ 0.40	75
C. glabrata ATCC 90030	1	0.5 + ≥ 0.07	50
C. glabrata ATCC 66032	0.5	0.25 + ≥ 0.40	50

3. SRPP in inducing yeast cell aggregation

The SRPP were shown to play the active role in inducing yeast cell aggregation (Fig. 19). The results showed that upon incubation of the SRPP with *Candida* cell suspensions for 30-min, the cell aggregates formed were separated into the top of the hematocrit tube upon centrifugation. The increase in the size of the aggregate was directly proportional to the amount of SRPP (10, 20, 40, 80 μg/ml) added to the incubation mixtures (Fig. 19 A1-4) when compared to control of the SRPP at indicated concentration without yeast cells (Fig. 19 B1-4). Other proteins including bovine serum albumin (BSA), served as a control, was unable to induce yeast cell aggregation (data not shown). A direct involvement of SRPP in inducing yeast cell aggregation was thus demonstrated. However, chitinase-treated SRPP and trypsinized SRPP failed to agglutinate yeast cells due to no visible aggregation was observed (Table 12). From the light microscope with dark-field illumination, the agglutination of yeast cells was strongly induced by the SRPP at a concentration as low as 6.25 μg/ml (Fig. 20).

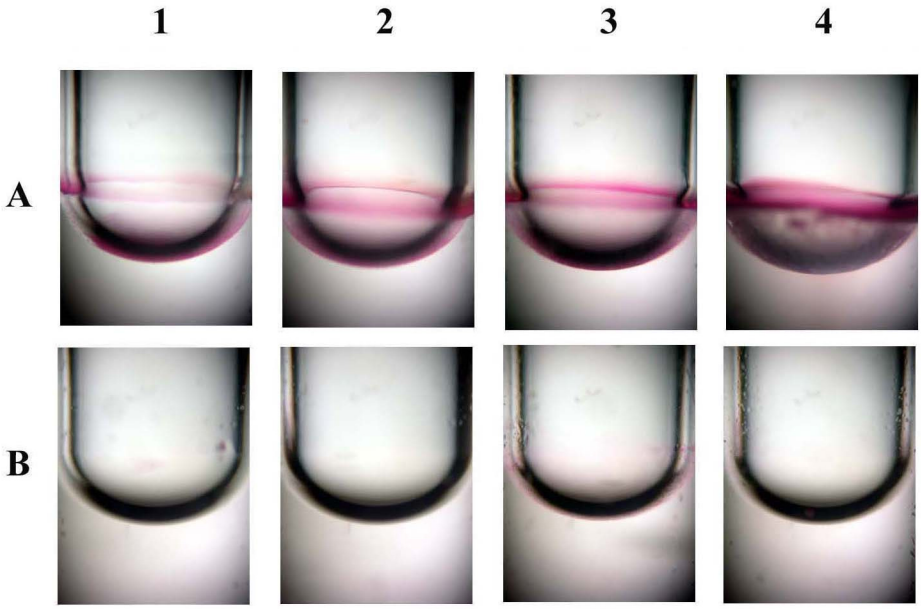


Fig. 19 Yeast cells aggregation by small rubber particle proteins (SRPP).

1-4 indicated SRPP at concentrations of 10, 20, 40, 80 μg/ml, respectively; A, SRPP control in buffer (without yeast cells); B, yeast cells aggregated with SRPP. The yeast cells aggregation assay was tested as described in the Methods.

Table 12 Effect of different SRPP fractions on the aggregation of *C. albicans* ATCC 10231

Sample	protein concentration (μg/ml)											
	0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100
SRPP	0	0	0	0	0	0	0	+	+	+	+	+
Chitinase-treated SRPP	0	0	0	0	0	0	0	0	0	0	0	0
Boiled SRPP	0	0	0	0	0	0	0	+	+	+	+	+
Trypsinized SRPP	0	0	0	0	0	0	0	0	0	0	0	0

Key: 0 = no visible aggregation or settling.

\+ = maximum visual aggregation with settling.

C. albicans ATCC 10231 growth at exponential phase (8 h) and incubated with SRPP for 1 h. The experiments were repeated twice.

* SRPP (200 μg/ml) treated with 0.1 mg/ml trypsin for 15 min and treated with 0.15 U chitinase and boiled for 10 min to terminate trypsin activity. Boiled 0.1 mg/ml trypsin and 0.15 U chitinase also tested as a control and did not induce yeast aggregation.

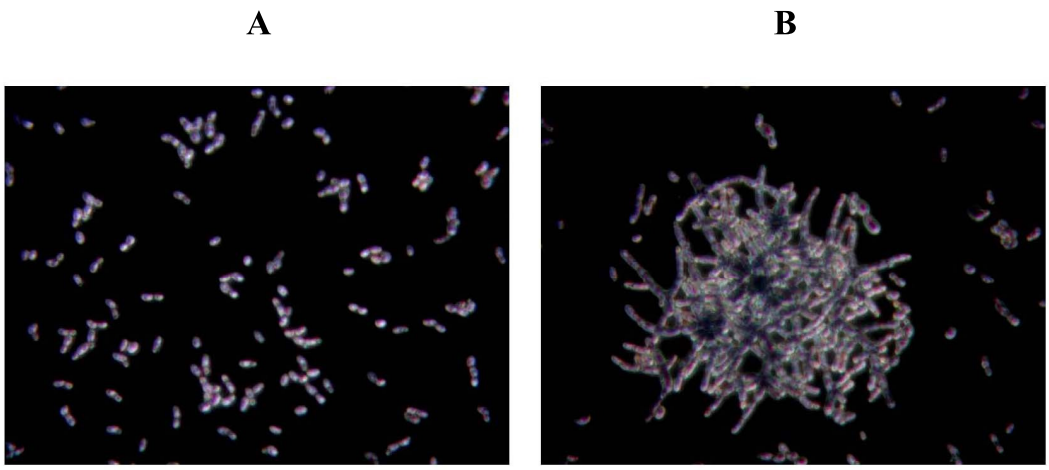


Fig. 20 Agglutination of SRPP against *C. albicans* ATCC 10231

The yeast cells were incubated in TBS (A) and with SRPP (B).

4. Characterization of rubber particle proteins

The rubber particle bound proteins were extracted from both washed mature rubber particles (Moir's zone 1) and small rubber particles (Moir's zone 2) in 0.2% Triton X-100 and analyzed under Tricine-SDS-PAGE. As shown in Fig. 21, the purified SRPP from Moir's zone 2 contains two major proteins of 14 and 24 kDa. This is different from those of zone 1 which contains only one major 14 kDa protein.

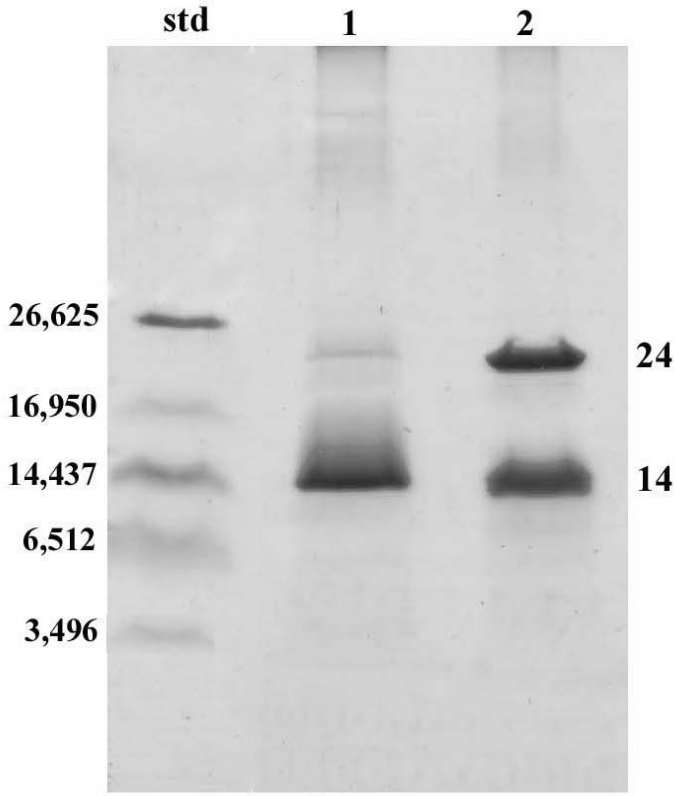


Fig. 21 Tricine SDS-PAGE (16.5% gel) of rubber particle proteins.

Rubber particle proteins from Moir's zone 1 (lane 1) and Moir's zone 2 (lane 2). Lane std is molecular mass marker.

Between the two proteins (14 and 24 kDa) of SRPP, only the 24 kDa protein was detected to be a glycoprotein that stained by GelCode® Glycoprotein Staining Kit as described in Materials and Methods (Fig. 22).

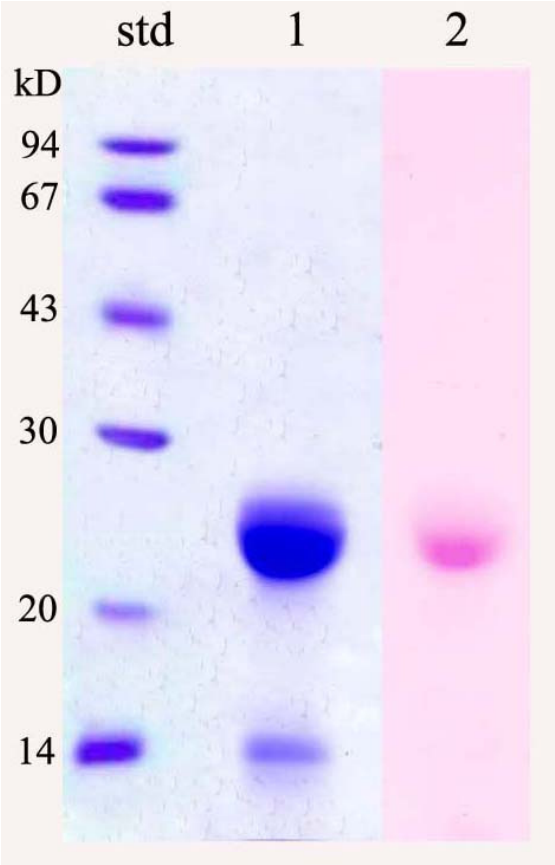


Fig. 22 Glycoprotein staining of SRPP.

10 μg of protein samples and standard were loaded on 12% SDS-PAGE. Lane std, molecular mass marker; Small rubber particle proteins from Moir's zone 2 stained with Coomassie brilliant blue R-250 (lane 1) and GelCode® Glycoprotein Staining Kit (lane 2).

5. Identification of the 14 kDa and 24 kDa -SRPP

The electrophoretic band corresponding to the 14 kDa and 24 kDa were excised and digested with trypsin. Peptide mass fingerprints (PMF) were produced by analyzing the digested protein with a Bruker Biflex III MALDI-TOF mass spectrometer. The resulting PMF were compared with the calculated masses of all tryptic peptides that can be theoretically produced from the sequences corresponding to all *H. brasiliensis* proteins in SWISS-PROT and NCBI public protein databases. For 14 kDa protein band, the masses of all tryptic peptides, both theoretical and experimental, were calculated and was compared (Table 13). The mass spectrum of 14 kDa was shown in Fig. 23. The protein that yielded the best match between the theoretical and experimental mass values was confirmed to be the same protein formerly defined as a rubber elongation factor protein, REF (Dennis, 1989) and as the Hev b 1 latex allergen (Yeang *et al*., 1996). The amino acid sequence of REF and their tryptic cleavage sites were shown in Fig. 24. Similarly, the mass spectrum of 24 kDa was shown in Fig. 25. The protein that yielded the best match between the theoretical and experimental mass values was identified to be a small rubber particle protein, SRPP (Oh *et al.,* 1999) and as the Hev b 3 latex allergen (Yeang *et al.,* 1996; Yeang *et al.,* 1998). The amino acid sequence and tryptic of the 24 kDa-SRPP was shown in Fig. 26. The amino acid composition of theoretical masses of trypsinized 14 kDa (REF) and 24 kDa-SRPP were shown in Table 13 and 14, respectively. More than 50% hydrophobic amino acid contents were found among most of (>70%) the tryptic peptides derived from REF and SRPP. This supports the unique lipid soluble property of the rubber particle proteins/peptides.

Table 13 Peptide sequences, amino acid compositions and calculated experimental and theoretical masses of tryptic 14 kDa-derived peptides.

Experimental mass	Theoretical mass	Peptide sequence	Amino acid composition (%)		
			hydrophobic	neutral	hydrophilic
-	2689.3548	YLGFVQDAATYAVTTF SNVY LFAK	54.17	25	20.83
1621.87	1621.8795	FVDSTVVASVTIIDR	53.33	26.67	20
-	1617.6987	AEDEDNQQGQGEGLK	33.33	26.67	40
1605.88	1605.8846	SGPLQPGVDIIEGPVK	68.75	12.5	18.75
1158.65	1158.6477	DASIQVVSAIR	54.55	27.27	18.18
1109.58	1109.5989	FSYIPNGALK	60	20	20
1088.59	1088.5946	SLASSLPGQTK	45.45	45.45	9.09
1045.60	1045.5789	NVAVPLYNR	55.56	22.22	22.22
-	753.4869	SLPPIVK	71.43	14.29	14.29
-	728.325	VFYGEN	50	16.67	33.33
-	685.3627	AAPEAAR	71.43	0	28.57
-	444.318	ILAK	75	0	25
-	262.1397	DK	0	0	100

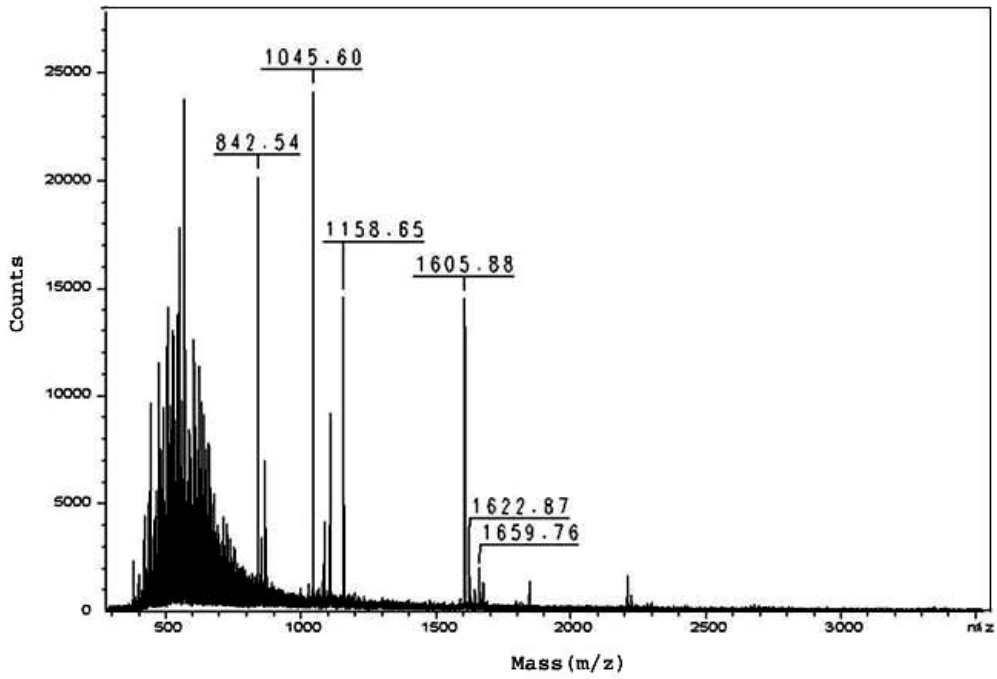


Fig. 23 The 14 kDa tryptic digest mass spectrum from peptide mass fingerprinting analysis by MALDI -TOF mass spectrometer

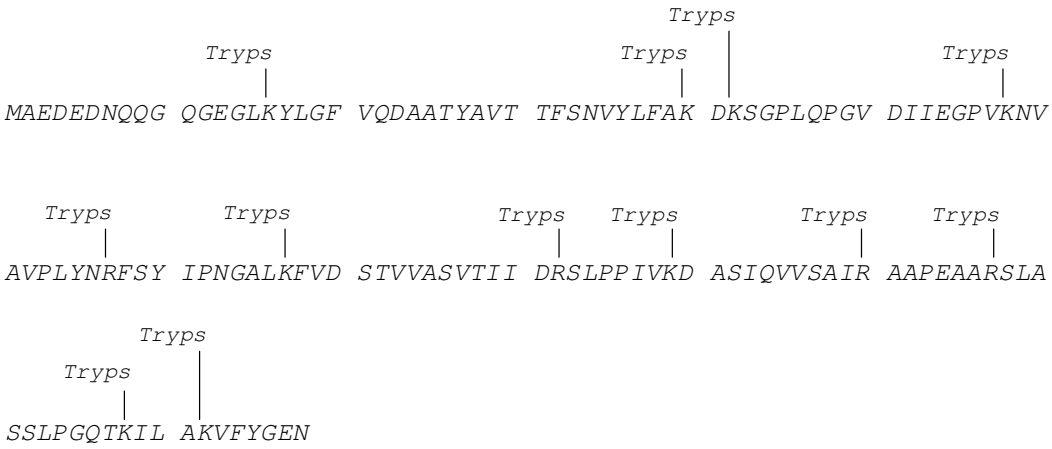


Fig. 24 The Amino acid sequence and their tryptic cleavage site of the 14 kDa (REF).

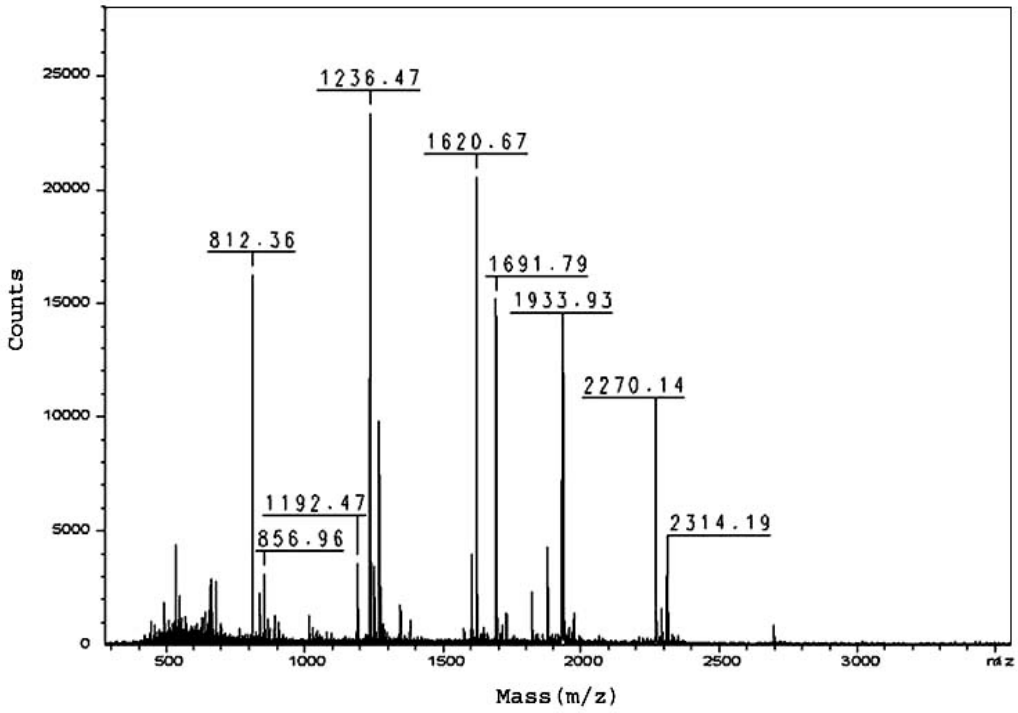


Fig. 25 The 24 kDa tryptic digest mass spectrum from peptide mass fingerprinting analysis by MALDI -TOF mass spectrometer

Table 14 Peptide sequences, amino acid compositions and calculated experimental and theoretical masses of tryptic 24 kDa-derived peptides.

Experimental mass	Theoretical mass	Peptide sequence	Amino acid composition (%)		
			hydrophobic	neutral	hydrophilic
2270.10	2270.2794	LPLVPQVANVVVPTAVYFSEK	66.67	19.05	14.29
1938.89	1938.9847	AAGVYAVDSFSTLYLYAK	55.56	16.67	27.78
1933.93	1934.0229	IVLDVASSVFNTGVQEGAK	57.89	26.32	15.79
1880.93	1881.0327	DISGPLKPGVDTIENVVK	55.56	16.67	27.78
1824.91	1825.0316	TVDVSVTSLDGVVPPVIK	61.11	22.22	16.67
1691.79	1691.9618	TVVTPVYYIPLEAVK	60	13.33	26.67
1620.67	1620.7976	QVSAQTYSVAQDAPR	40	40	20
1346.58	1346.7566	VSSYLPLLPTEK	50	25	25
1236.47	1236.6371	AEQYAVITWR	50	20	30
-	1121.4779	MAEEVEEER	33.33	0	66.67
1018.46	1018.5567	ALYANLEPK	55.56	11.11	33.33
-	911.4217	GTTEQGYR	25	37.5	37.5
812.36	812.4301	YLDFVR	50	0	50
-	765.389	YNDVVR	33.33	16.67	50
-	724.3148	VFGDEAS	57.14	14.29	28.57
-	508.2765	FVDK	50	0	50
-	445.2769	ALNK	50	25	25
-	361.2445	ITK	33.33	33.33	33.33
-	260.1968	LK	50	0	50

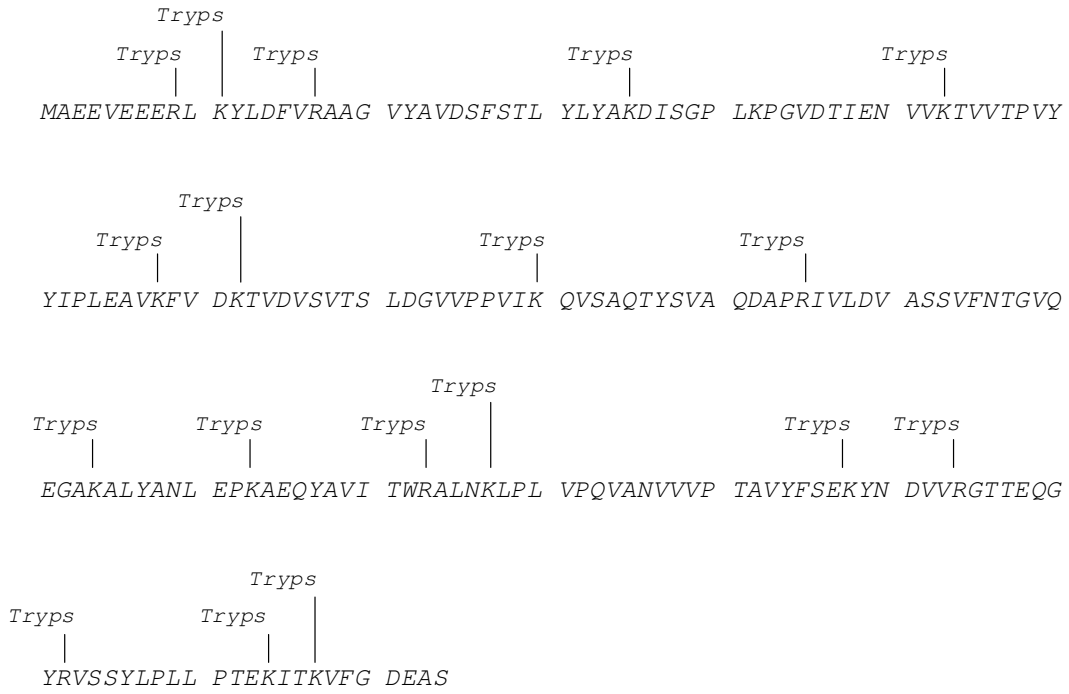


Fig. 26 The amino acid sequence and tryptic cleavage sites of the 24 kDa-SRPP

After the SRPP was incubated with trypsin as described in Methods. Amino acid sequence was analyzed by MALDI-TOF spectrometry.

6. The enhancing effect of trypsinized SRPP on MIC of AMB

Although the SRPP pretreated with trypsin were unable to induce yeast cell aggregation. However, they still showed full capacity in reducing the MIC_{80}s of AMB at the same potency level as that of native SRPP (Table 15). The Tricine-SDS-PAGE analysis (Fig. 27) of the tryptic derived peptides indicated complete digestion within 15 min, showing a smeared band of molecular weight size lower than 6.5 kDa. The amount of tryptic peptide mixture protein required to give a four-fold reduction of the MIC_{80} of AMB is equal to that of the native SRPP (≥ 0.4 μg/ml).

Moreover, when tryptic peptides were fractionated by partitioning in a lipid solvent, chloroform/methanol (2:1, v/v), the tryptic peptides recovered from the chloroform phase were four-fold more effective in reducing the MIC_{80} than those from the methanol aqueous phase (Table 16). The chloroform phase accounts for 87.6% of the total tryptic peptide protein. This result agrees well with the very high level (88%) of hydrophobic tryptic peptide content, composing of ≥ 50% hydrophobic amino acids, derived from trypsinized 14 and 24 kDa proteins as summarized under Table 13 and 14, respectively.

Table 15 Enhancing effect of the trypsinized SRPP on the antifungal activity of AMB against *C. albicans* ATCC 90028

Sample	AMB concentration (μg/ml)
1) AMB alone	0.25
2) AMB + SRPP (0.4 μg/ml)	0.0625
3) AMB + trypsinized SRPP (0.4 μg/ml)*	0.0625
4) AMB + boiled trypsin (0.05 mg/ml)	0.25

* SRPP was trypsinized for 15, 30, 45 and 60 min. Control trypsinized SRPP at 0.4 μg/ml and 0.1 mg/ml trypsin control could not inhibit fungal growth when tested alone.

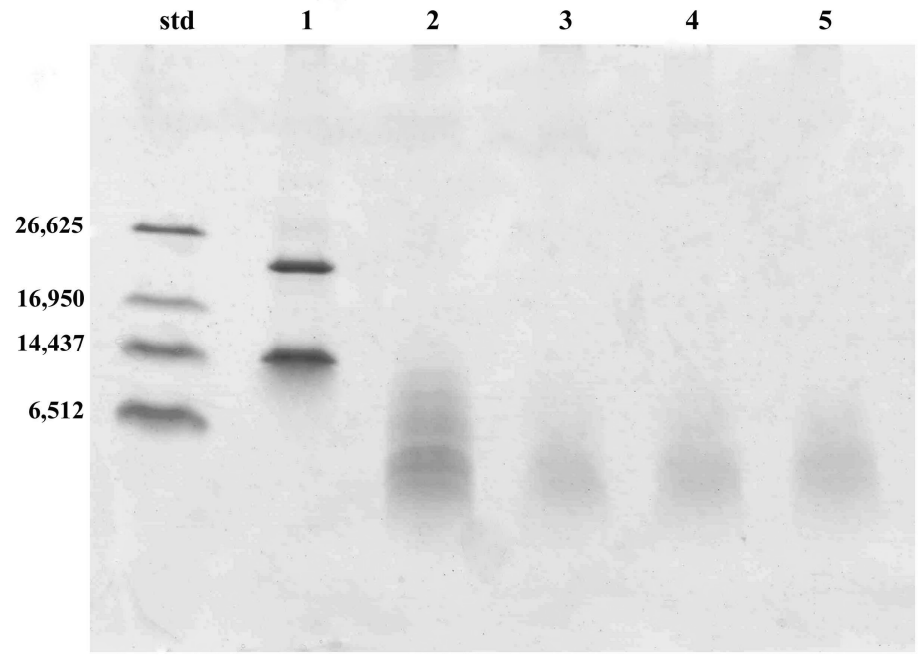


Fig. 27 Tricine SDS-PAGE (16.5% gel) of trypsinized SRPP.

Small rubber particle proteins were treated with 0.1 mg/ml trypsin at various time periods as indicated. Lane std, molecular mass marker; Lane 1, untreated SRPP; Lane 2-5, SRPP treated with trypsin for 15, 30, 45 and 60 min, respectively.

Table 16 MIC_{80} values of AMB against *C. albicans* ATCC 10231 with and without trypsinized SRPP proteolipid fractions

Sample	AMB concentration (μg/ml)
1) AMB alone	0.5
2) AMB + trypsinized SRPP (0.4 μg/ml)	0.125
3) AMB + methanol phase trypsinized SRPP (0.4 μg/ml)	0.125
4) AMB + chloroform phase trypsinized SRPP (0.1 μg/ml)	0.125

*Small rubber particle proteins (SRPP) and trypsinized SRPP at indicated protein concentrations were the concentration which combined to AMB and remain the 80% growth inhibition but not active alone. MIC of AMB alone is 0.5 μg/ml.

The experiments were repeated twice.