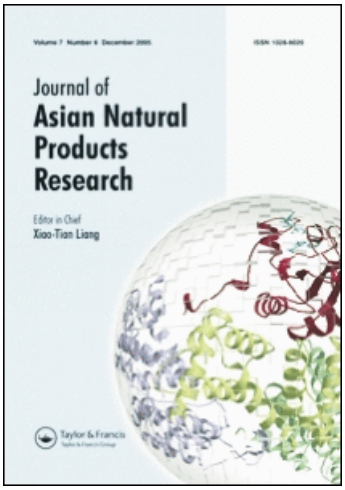

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Postantibiotic effect of purified melittin from honeybee (*Apis mellifera*) venom against *Escherichia coli* and *Staphylococcus aureus*

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To cite this Article Han, Sangmi , Yeo, Joohong , Baek, Haju , Lin, Shin-Min , Meyer, Stacy and Molan, Peter(2009) 'Postantibiotic effect of purified melittin from honeybee (*Apis mellifera*) venom against *Escherichia coli* and *Staphylococcus aureus*', Journal of Asian Natural Products Research, 11: 9, 796 — 804
To link to this Article: DOI: 10.1080/10286020903164277
URL: http://dx.doi.org/10.1080/10286020903164277

Journal of Asian Natural Products Research
Vol. 11, No. 9, September 2009, 796–804

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(*Received 14 April 2009; final version received 6 July 2009*)

Since the ancient times, the antibacterial application of honeybee venom (BV) has been practised and persisted. We investigated the antibacterial activity of whole BV and purified melittin against *Escherichia coli* and *Staphylococcus aureus* by the minimum inhibitory concentrations (MICs) and the postantibiotic effects (PAEs). The *in vitro* PAEs of whole BV and isolated melittin were determined using *E. coli* and *S. aureus*. The PAEs of whole BV against *E. coli* and *S. aureus* were 0.15 h (1 × MIC), 2.4 h (5 × MIC), and 3.45 h (1 × MIC), respectively. The PAEs of melittin against *E. coli* and *S. aureus* were 0.1 h (1 × MIC), 3.2 h (5 × MIC), and 4.35 h (1 × MIC), respectively. These results suggest that whole BV and melittin will be developed as a novel antibacterial drug.

Keywords: bee venom; melittin; minimum inhibitory concentration; postantibiotic effect

1. Introduction

In recent years, much attention has been given to find new antibiotics to which bacteria cannot develop resistance. Antimicrobial peptides have emerged as good candidates. These peptides have been isolated from natural sources, including mammals, insects, and plants. Especially, some venoms were reported to have antibacterial activity [1,2]. The venom of cobra *Hemachatus haemachatus* has been reported to show antibacterial property [3]. Various peptides, derived from scorpion venom, also have antimicrobial activity against bacteria and fungi [4]. Other than the venoms from snakes and scorpions, honeybee venom (BV; *Apis mellifera*) has been known to have a natural antimicrobial effect [5]. The polypeptide melittin is the main component of BV [6]. It dominates more than 40% of dry venom. Melittin has a moderate antibacterial and antifungal activity against many kinds of organisms [5,7].

The postantibiotic effect (PAE) is a pharmacodynamic parameter that may be considered in choosing antibiotic dosing regimens [8]. It is defined as the length of time the bacterial growth is suppressed following a brief exposure to an antibiotic [9,10]. Based on the results of our previous studies, we propose that whole BV should have the strong antibacterial effect against *Escherichia coli* and *Staphylococcus aureus* isolated from mastitis dairy cows [11].

Here, we study the antibacterial continuance of the main component of BV in order to use the mastitis drug on the

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020903164277
http://www.informaworld.com

mastitis dairy cows. Gel chromatography and reverse-phase liquid chromatography were used to successfully isolate and purify melittin from the whole BV. We examined the PAE of whole BV and melittin isolated from BV against *E. coli* and *S. aureus*.

2. Results and discussion

2.1 Separation, purification, and identification of melittin

Figure 1(a) shows five fractions separated from the whole BV by Superdex peptide column chromatography. Each fraction was measured by a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay (MALDI-TOF). The molecular weight of the major fraction I corresponded to the molecular weight of melittin [6]. Fraction I was further purified using PepRPC HR 10/10 column (Figure 1(b)). The peak of melittin was indicated by an arrow in Figure 1(b) and the final product was shown by a single peak (Figure 2(a)). To identify the melittin, the purified melittin was shown by a single band on 20% SDS-PAGE (Figure 2(b)). The molecular mass and purity of purified melittin were analyzed by a MALDI-TOF mass spectrometry, giving a molecular mass of 2845.931 (Figure 2(c)). The amino acid sequence determination of purified melittin was performed by chemically assisted fragmentation (CAF)-MALDI-TOF. The fragment masses of the CAF-labeled peptides are shown in Figure 2(d) and amino acid sequence is shown in Figure 2(e), and the following 23 amino acid sequence was obtained: GIGAVLKVLTT-GLPALISWIKRK. The purified melittin was identified as melittin by a homology search using PepFrag. Melittin is the principal toxic component in the venom of the European honeybee *A. mellifera* and is a cationic, hemolytic peptide. Melittin is a small linear peptide composed of 26 amino acid residues (NH_2-GIGAVLKVL-TTGLPALISWIKRKRQQ-$CONH_2$) in which the amino-terminal region (residues 1–20) is predominantly hydrophobic, whereas the carboxy-terminal region (residues 21–26) is hydrophilic due to the presence of a stretch of positively charged amino acids [12].

2.2 Susceptibility and PAE

In order to investigate antibacterial activity, the susceptibility of *E. coli* and *S. aureus* for whole BV and melittin on *E. coli* and *S. aureus*, the minimum inhibitory concentration (MIC) was estimated as shown in Table 1. The MIC of whole BV was 0.25 μg/ml and that of melittin was 0.125 μg/ml for *E. coli*. The MIC of whole BV and melittin was 0.06 μg/ml similar to that for *S. aureus*. Both whole BV and melittin for the susceptibility of *S. aureus* were higher than those for *E. coli*.

For *E. coli*, the mean PAEs of whole BV were 0.15 h at 1 × MIC and 2.4 h at 5 × MIC, respectively (Table 1; Figure 3(a)), and those of melittin were 0.1 h at 1 × MIC and 3.2 h at 5 × MIC (Table 1; Figure 4(a)). At 10 × MIC, no *E. coli* could be detected (lower limit of counting 100 cfu/ml) within 1 h. Regrowth was not observed as early as 18 h (Figures 3(a) and 4(a)). The exposure time was 1 h so that whole BV and melittin has enough time to kill *E. coli* at concentrations up to 10 × MIC. For *S. aureus*, the mean PAEs of whole BV and melittin were 3.45 and 4.35 h at 1 × MIC, respectively (Table 1; Figures 3(b) and 4(b)). *E. coli* and *S. aureus* were completely killed by melittin at 10 × MIC (Figures 3(b) and 4(b)). At 5 and 10 × MIC, no *S. aureus* could be detected within 1 h. Regrowth was not observed as early as 18 h (Figures 3(b) and 4(b)).

2.3 Discussion

The MIC is the main microbiological parameter used to predict the efficacies of antibiotics. However, it is well known that

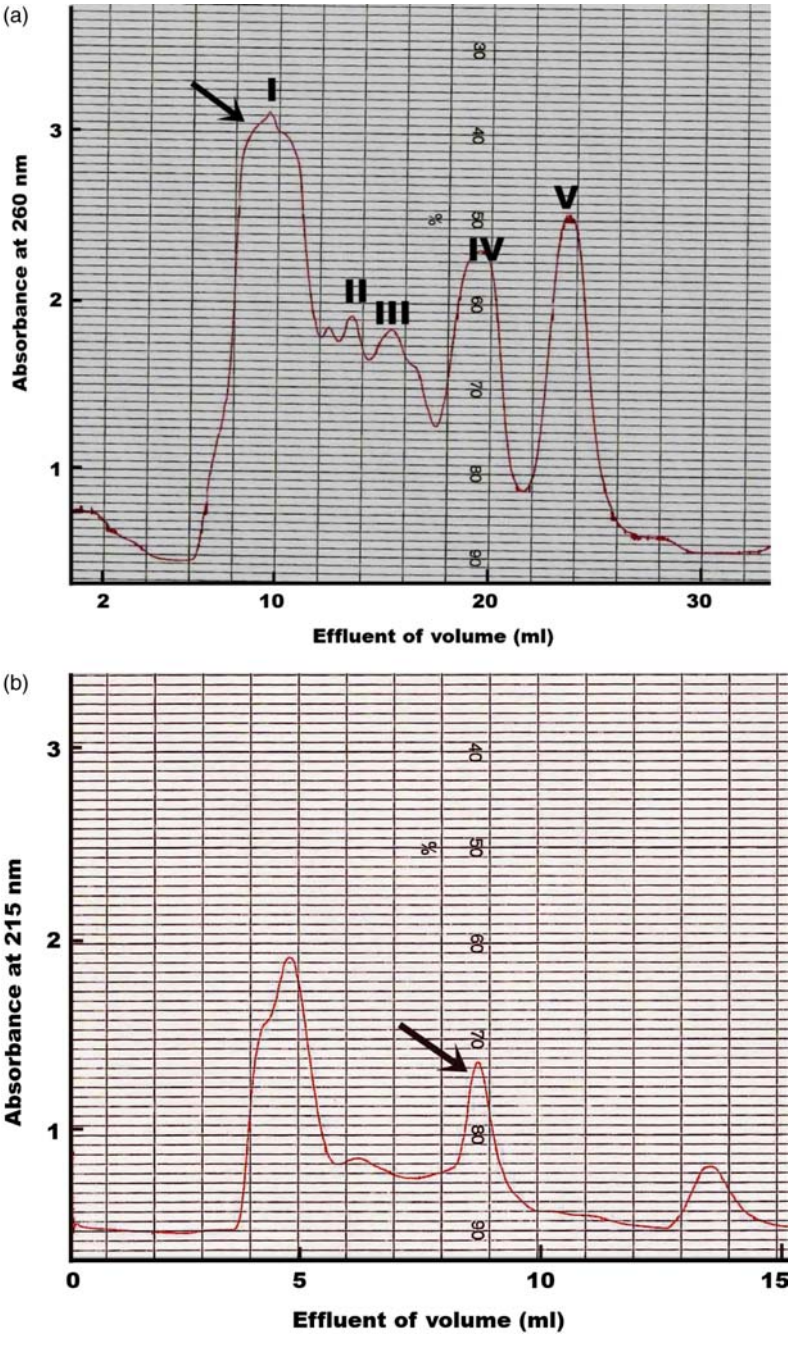
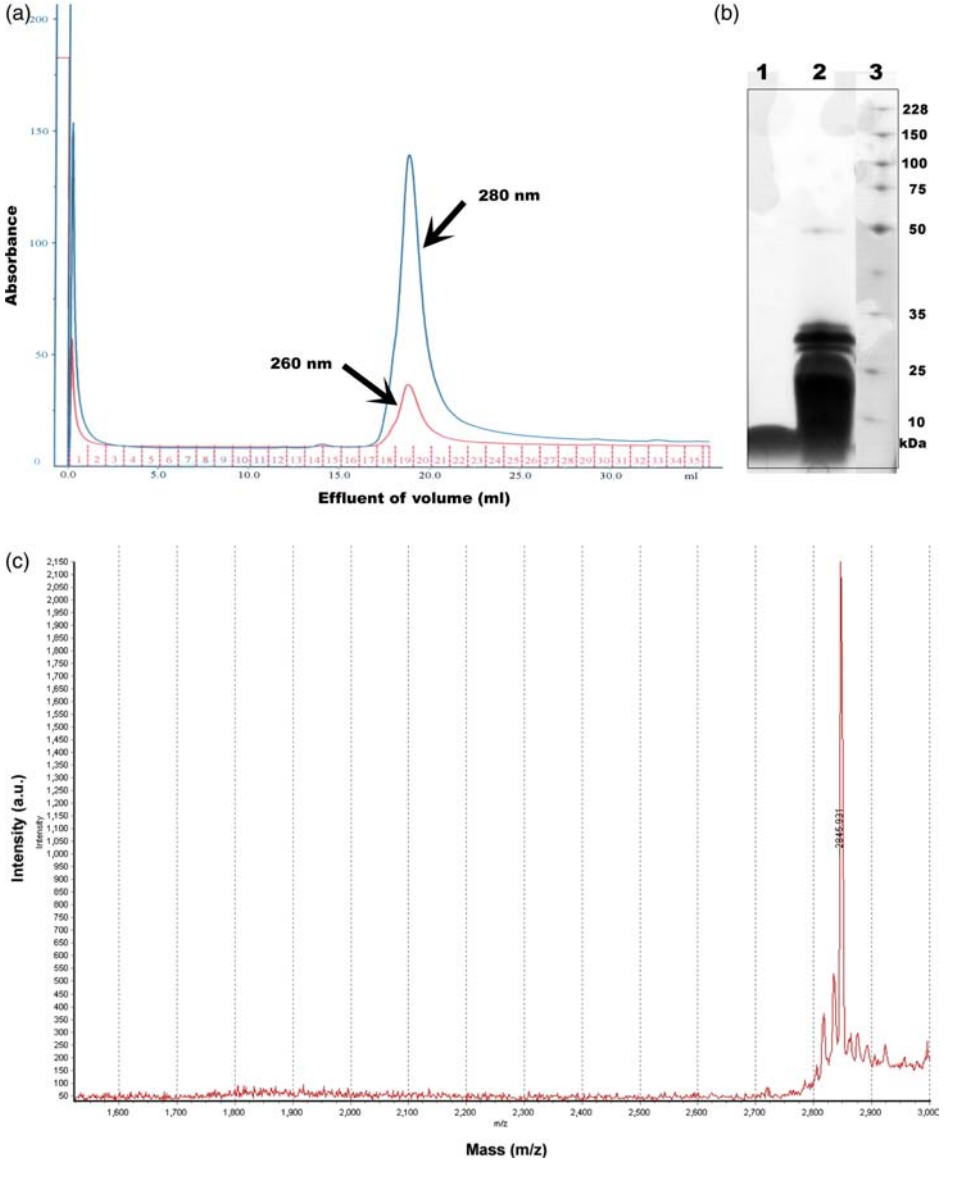


Figure 1. (a) Separation of 100 mg of the components of honeybee crude venom on a Superdex peptide column. (b) Separation of fraction 1 from the Superdex peptide column on a PepRPC HR 10/10 column. Arrow indicates the peak of melittin.

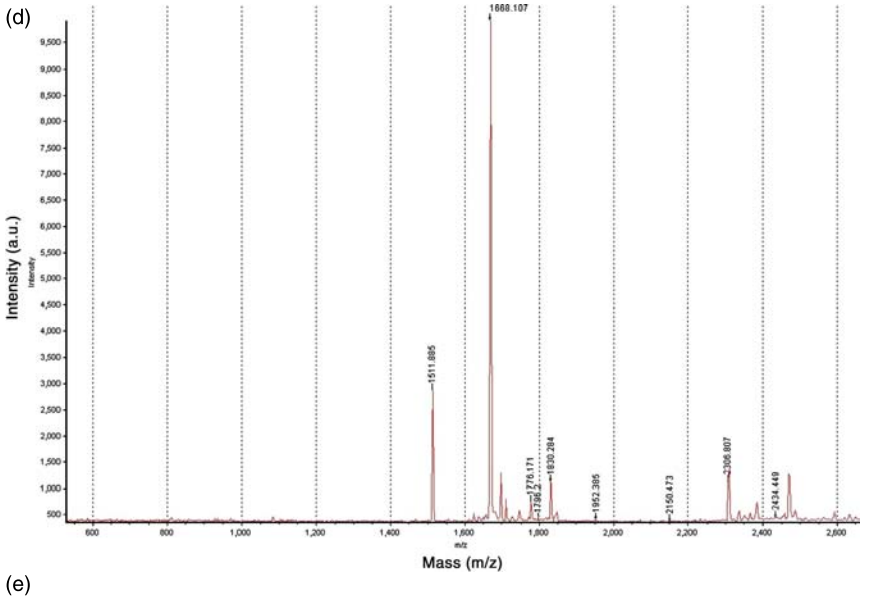
MIC may vary according to the inoculum size used [13,14]. Recently, to make up for the weak points in the MIC, PAE has been used as a reliable antimicrobial parameter. The PAE, the phenomenon of continued bacterial growth inhibition after exposure to an antimicrobial agent, has become a subject of clinical interest because the presence of a PAE may be an important consideration in designing antibiotic dosage regimens [15,16].

BV is a treatment modality which may be thousands of years old [3,17]. BV was said to contain enzymes, non-peptide components, and several peptides such as melittin, apamin, and mast cell degranulating peptide [6,17]. The BV has been studied to determine its antibacterial effect. It has been reported that the BV contains melittin which is active against bacteria [6,18].

For the pharmacological action of the BV and purified melittin, antibacterial



(d)



(e)

ProFound- Search Result Details The Rockefeller University Edition

Measured Mass (M)	Avg/ Mono	Computed Mass	Error (%)	Residues		Missed Cut	Peptide sequence
				Start	To		
1510.877	M	1510.911	−0.002	8	21	0	VLTTGLPALISWIK
1667.099	M	1667.013	0.005	8	22	1	VLTTGLPALISWIKR
1795.192	M	1795.108	0.005	8	23	2	VLTTGLPALISWIKRK
1951.377	M	1951.208	0.009	8	24	3	VLTTGLPALISWIKRKR
2149.465	M	2149.323	0.007	1	21	1	GIGAVLKVLTTGLPALISWIK
2305.799	M	2305.424	0.016	1	22	2	GIGAVLKVLTTGLPALISWIKR
2433.441	M	2433.519	−0.003	1	23	3	GIGAVLKVLTTGLPALISWIKRK

Figure 2. (a) Identification of 10 mg purified melittin on a Superdex 75 column. (b) Twenty percent SDS-PAGE analysis of purified melittin stained with Coomassie blue R-250. Lane 1, purified melittin; lane 2, honeybee crude venom; lane 3, protein marker. (c) Mass spectrometry of purified melittin. (d) Fragment masses of purified melittin from CAF-MALDI-TOF. (e) Peptide sequence of purified melittin was determined in melittin using PepFrag.

activity was observed by MIC and PAE against *E. coli* and *S. aureus.* Whole BV and melittin for *S. aureus* were more sensitive than those for *E. coli*. Both whole BV and melittin have been active in the initial killing at 10 × MIC. In this study, at 10 × MIC, the regrowth of *E. coli* was not observed at 18 h. Both whole BV and melittin were very active in the initial killing. Even with 1 × MIC, the $\log_{10}$ cfu/ml decreased to about 1 within 1 h. At 5 × and 10 × MIC of both whole BV and melittin, the *S. aureus* regrowth was not observed at 18 h.

Therefore, this study suggests that PAE for whole BV and melittin will be clinically achievable concentrations as antibacterial drug for the treatment of infections caused by *E. coli* or *S. aureus.* The excellent antibacterial activity observed in these results led to the development of whole BV and melittin containing drug as a novel

Table 1. MIC and PAE of whole BV and melittin against *E. coli* and *S. aureus*.

	Micro-organisms			
	E. coli		*S. aureus*	
Sample	MIC (μg/ml)	PAE (h)	MIC (μg/ml)	PAE (h)
Whole BV	0.25	0.15[a]/2.4[b]	0.06	3.45[a]
Melittin	0.125	0.1[a]/3.2[b]	0.06	4.35[a]

Notes: [a]Exposure to 1 × MIC of whole BV or melittin for 1 h.
[b]Exposure to 5 × MIC of whole BV or melittin for 1 h.

natural antibacterial agent for the treatment of infections caused by *E. coli* and *S. aureus*.

3. Experimental

3.1 Collection of BV

Experimental colonies of honeybees (*A. mellifera*) were maintained at the National Academy of Agricultural Science, Korea. BV was collected by a bee venom collector (ChungJin Biotech Co., Ltd, Korea). The collected BV was diluted in cold water and then centrifuged at 10,000 *g* for 5 min at 4°C to discard residues from the supernatant. BV was lyophilized by a freeze dryer and stored in a refrigerator for later use.

3.2 Isolation, purification, and identification of purified melittin from BV

The whole BV was dissolved in a 0.1 M ammonium formate buffer and loaded onto a Superdex peptide HE 10/30 column (GE healthcare, Uppsala, Sweden) equilibrated with the same buffer. The purification of fractions was performed by a PepRPC HR 10/10 column equilibrated with a 30% (v/w) acetonitrile buffer in water and a Superdex 75 10/300 GL column (GE healthcare) equilibrated with a 0.03 M ammonium acetate buffer in water. To identify the purity and molecular weight of the isolated melittin, the final component was subjected to electrophoresis on SDS-PAGE gel and then stained with Coomassie blue. The molecular mass of the purified peptide was determined by MALDI-TOF-MS (Reflex III, Bruker, Germany). For protein identification, the purified melittin spot was enzymatically digested in-gel in a manner similar to that previously described by Shevchenko *et al.* [19] and using modified porcine trypsin. The derivation reactions of the tryptic peptides were performed using the instructions and reagents in an Ettan CAF-MALDI sequencing kit [20]. The additional reagents included C_{18} ZipTip (Millipore, Billerica, MA, USA). All the spectra were obtained using the ion gate set for the precursor ion in the post source decay analysis mode. The fragment masses of the CAF-labeled peptides were determined using the Ettan MALDI-TOF software and the purified melittin was identified using PepFrag (http://prowl.rockefeller.edu/prowl/pepfrag.thml).

3.3 Bacterial and culture conditions

E. coli (ATCC 25922) and *S. aureus* (ATCC 9144) were obtained from the Honey Research Unit, Waikato University, Hamilton, New Zealand. The cultures were inoculated overnight on trypticase soy (TS; Difco Laboratories, Detroit, MI, USA) agar plates and incubated at 37°C.

3.4 MIC determination

The MICs were determined by the broth dilution method for whole BV and melittin isolated from BV [21]. The whole BV and melittin were dissolved in sterile water and filtered using a 0.22 μm syringe filter. Two hundred microliters of bacteria were added to

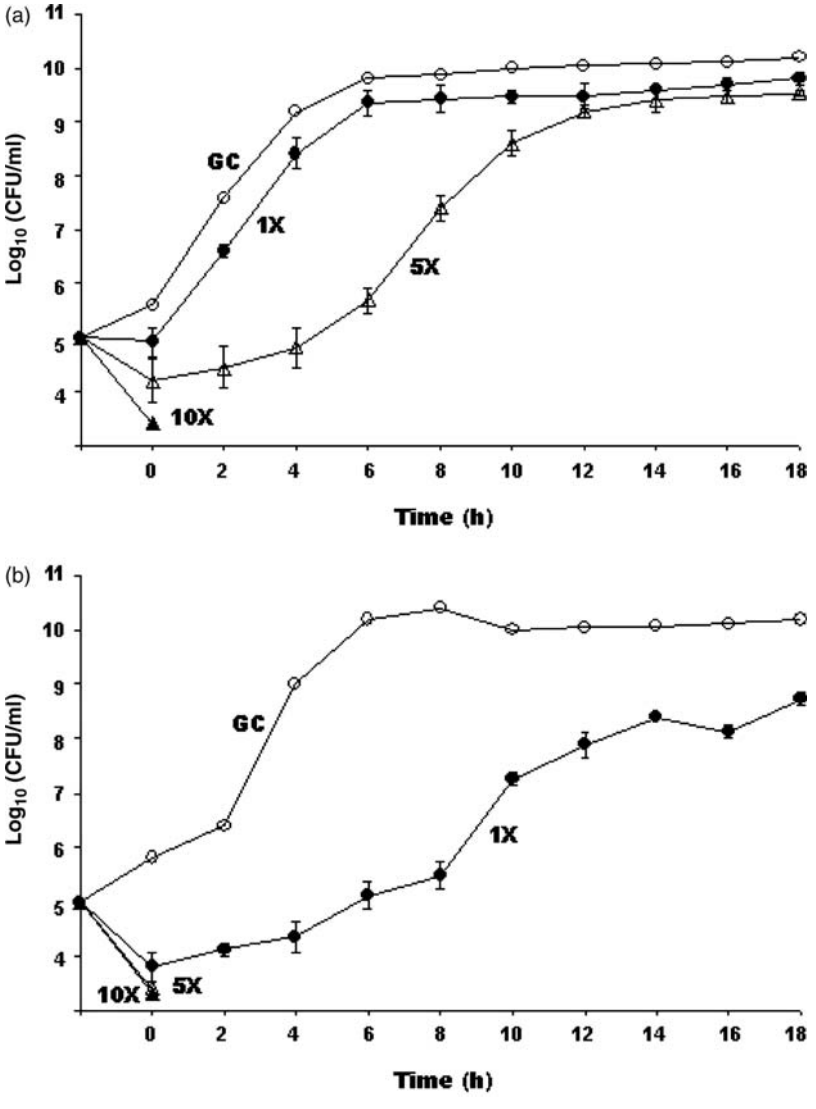


Figure 3. Concentration-dependent PAE of whole BV for *E. coli* (a) and *S. aureus* (b) measured at 1 ×, 5 ×, and 10 × MIC. PAE induced by 1 h exposure of the bacteria to whole BV, using rapid whole BV removal by repeated washing and centrifugation. GC is the growth control of the bacteria. The values are expressed as the means ± SE ($n = 3$) from three independent experiments.

20 μl of diluted samples in 96-well plates. Three independent experiments were performed as replicates. The plates were incubated at 37°C for 24 h. The inhibition of bacterial growth was determined by the absorbance using a microplate reader (BMG FLUO star, OPTIMA, Auckland, New Zealand) at 405 nm. Results were expressed as MIC, the lowest concentration of whole BV and melittin that inhibits the growth of the *E. coli* and *S. aureus* [22].

3.5 PAE determination

The PAE was determined by the equation $T - C$, where T is the time required for viability counts of an antibiotic exposed

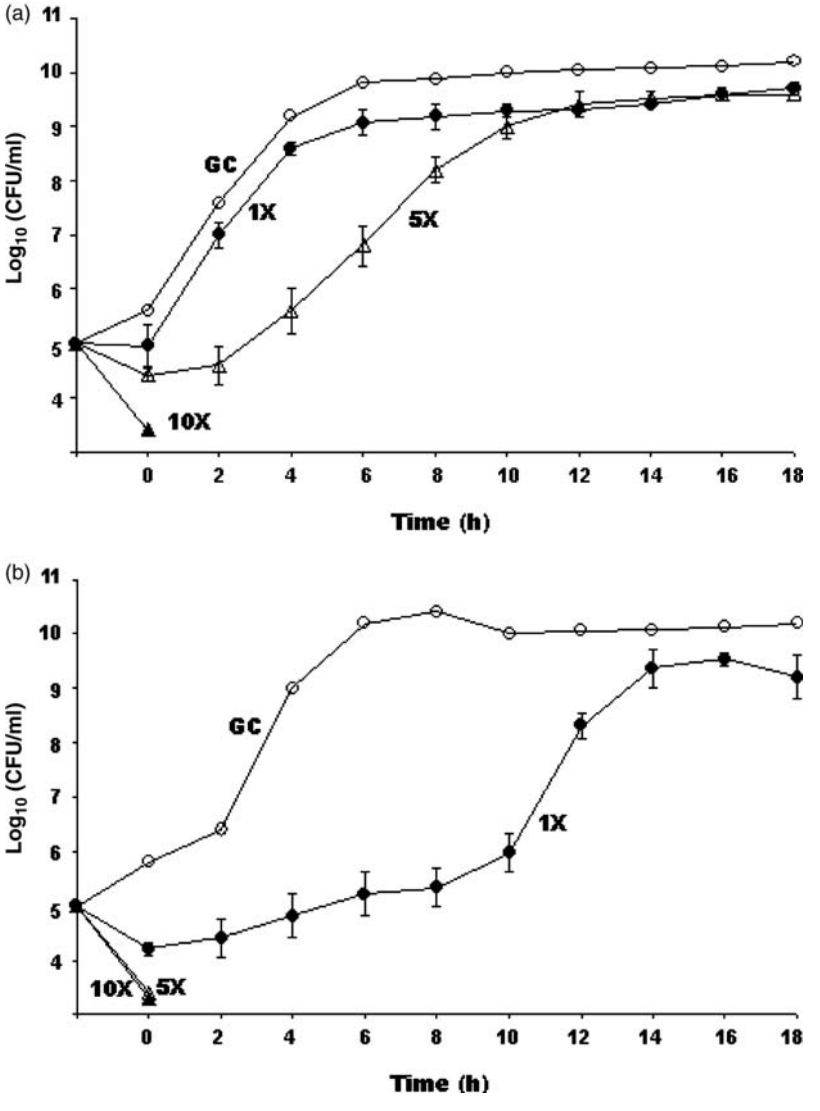


Figure 4. Concentration-dependent PAE of purified melittin from honeybee crude venom for *E. coli* (a) and *S. aureus* (b) measured at 1 ×, 5 ×, and 10 × MIC. PAE induced by 1 h exposure of the bacteria to melittin, using rapid melittin removal by repeated washing and centrifugation. GC is the growth control of the bacteria. The values are expressed as the means ± SE ($n = 3$) from three independent experiments.

culture to increase by 1 $\log_{10}$ above counts immediately after dilution, and C is the corresponding time for growth control [10,23]. For PAE testing, inocula were prepared by suspending growth from an overnight TS agar plate in broth. Tubes containing 3 ml broth with whole BV or melittin were inoculated with approximately 5×10^6 cfu/ml controls with only inoculum being included in each experiment. The PAE was induced by exposure to 1 ×, 5 ×, and 10 × MIC of whole BV or melittin for 1 h at 37°C. At the end of the exposure period, cultures were harvested

by centrifugation (8500 *g*; 3 min) and washed with prewarmed water by centrifugation and resuspension. Tested samples were diluted to 1:1000 in prewarmed broth to remove the whole BV and melittin and then incubated at 37°C for 18 h. Viability counts were determined before exposure and immediately after dilution.

3.6 Statistical analysis

All experiments were performed in triplicate. Data were presented as mean ± SE.

Acknowledgements

This work was supported by a grant (Code #; 20070301-034-001-01) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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