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Antinematodal Effect of Antimicrobial Peptide, PMAP-23, Isolated from Porcine Myeloid against *Caenorhabditis elegans*

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Abstract: The antinematodal activity and mechanism of a 23-mer antimicrobial peptide, PMAP-23, derived from pig myeloid was investigated. PMAP-23 displayed a strong antinematodal activity against the eggs and worms of *Caenorhabditis elegans*. To investigate the antinematodal mechanism of PMAP-23, fluorescence activated flow cytometry and confocal laser scanning microscopy were performed. *C. elegans* treated with PMAP-23 showed higher fluorescence intensity by propidium iodide (PI) staining than normal cells. Confocal microscopy showed that the peptide was localized in the egg's shell and cell membrane. The action of the peptide against *C. elegans* membranes was examined by testing the membrane disrupting activity using liposome (PC/PS; 3 : 1, w/w). The result suggests that PMAP-23 may exert its antinematodal activity by disrupting the structure of the cell membrane via pore formation or via direct interaction with the lipid bilayers.

Keywords: *Caenorhabditis elegans*; antinematodal activity; PMAP-23

INTRODUCTION

Nematodes are worldwide in their distribution and are known to attack a wide variety of crop plants [1]. During the past few decades, nematode control has been based mostly on the use of chemicals. However, because of environmental toxicity and antibiotic resistance, other control techniques have been investigated [2].

Recently, antimicrobial peptides have been recognized to play important roles in the innate host defence mechanisms of most living organisms including plants, insects, amphibians and mammals [3–8], and are known to possess potent antibiotic activity against bacteria, fungi and even certain viruses [9–13]. Therefore the possibility of antinematodal activity of the antimicrobial peptides was investigated.

In addition, the antinematodal mechanism of the peptides was studied to determine whether they act like known cationic antimicrobial peptides. Plant antifungal proteins, such as chitinases [14], glucanases [15], lipopeptide echinocandin [16] and peptide-nucleoside nikkomycin [17], are known to exert their activities by disruption of the cell wall

Abbreviations: DCC, dicyclohexylcarbodiimide; HOBt, N-hydroxybenzotriazole; MALDI, matrix-assisted laser desorption ionization; MBHA resin, 4-methyl benzhydrylamine resin; PBS, phosphate-buffered saline.

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structure or by interference with cell wall synthesis. With the exception of these plant antifungal proteins and lipopeptides, most antimicrobial proteins are known to adopt the pore-formation mechanism as their mode of action [18].

PMAP-23 peptide was identified by cDNA cloning and shown to possess potent antibacterial activity with chemically synthesized peptide [19,20]. Although PMAP-23 had already shown antibacterial activity, its antibiotic effect against nematodes and its mode of action are not yet known.

Therefore, in this study the antinematodal activity of PMAP-23 on *C. elegans* was investigated. The nematicidal mechanism of PMAP-23 on *C. elegans* was investigated by fluorescence activated flow cytometric analysis and confocal laser scanning microscopy. Also discussed is whether PMAP-23 is important for phospholipid–vesicle interaction.

MATERIALS AND METHODS

Peptide Synthesis

PMAP-23 peptide was synthesized by the solid phase method using Fmoc(9-fluorenyl-methoxycarbonyl)-chemistry [21]. Rink amide 4-methyl benzhydrylamine (MBHA) resin (0.55 mmol/g) was used as the support to obtain a *C*-terminal amidate peptide. Fmoc-amino acid coupling was performed with N-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were protected as follows: *tert*-butyl (Asp), trityl (Gln), *tert*-butyloxycarbonyl (Lys). Deprotection and cleavage from the resin were achieved with a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol and triisopropylsilane (88 : 2.5 : 2.5 : 2.5 : 2.5 : 2.0, v/v) for 2 h at room temperature. The crude peptide was then repeatedly washed with diethylether, dried in vacuum and purified by reversed-phase preparative HPLC on a Waters 15 μm Deltapak C_{18} column (19 × 30 cm). The peptide purity of the peptide was determined by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (Beckman, USA), 4.6 × 25 cm. Purified peptides were hydrolysed with 6 N HCl at 110 °C for 22 h, and dried in a vacuum. The residues were dissolved in 0.02 N HCl and subjected to amino acid analysis (Hitachi Model, 8500 A, Japan). Peptide concentrations were determined by amino acid analysis. The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization (MALDI) mass spectrometer [22].

Preparation of Eggs and Worms of *C. elegans*

Escherichia coli OP 50 was grown in LB medium supplemented with streptomycin (10 μg/ml) at 37 °C with shaking. The *C. elegans* strain was cultivated on a NGM (3 g NaCl, 2.5 g tryptone, 1 ml of 1 M $CaCl_2$, 1 ml of 5 mg/ml cholesterol in EtOH, 1 ml of 1 M $MgSO_4$, 25 ml of 1 M KPO_4 buffer, 17 g agar per liter) plate covered with a lawn of *E. coli* (OP 50). The worms were rinsed off the agar plate with M9 buffer (0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.5% NaCl, 1 mM $MgSO_4$) at room temperature, washed twice by centrifugation at 1000 × **g** for 2 min and then resuspended in M9. Then 10 ml hypochlorite solution (2.4% NaOCl, 0.5 M NaOH) was added to the worms in 1 ml of M9 buffer and the tube was vortexed or shaken for a few seconds. The shaking/vortexing was repeated every 2 min for 20 min. The reaction was stopped with 20 ml of M9, mixed, and centrifuged at 10 000 × **g** for 5 min. Most of the liquid was removed and 20 ml of M9 mix was added, then centrifuged at 10 000 × **g** for 5 min. Aliquots of the egg suspension (150 μl, approximately 100 eggs) were placed onto a 96-well plate.

Antinematodal Activity

One ml of the eggs or freshly hatched juvenile suspension (10–15 larvae/ml) was transferred to each sample vial. Peptides were added to aliquoted eggs or a hatched juvenile suspension of *C. elegans* and incubated at 25 °C. The dead eggs or juveniles were counted to 60 h under an inverted microscope.

FACScan Analysis

To determine membrane integrity after peptide treatment, the *C. elegans* cells were harvested and mixed with peptides to a final concentration of 200 μM. They were then incubated for up to a further 60 h at 25 °C with constant shaking (140 rpm). The potent antimicrobial peptide, melittin, was used in this study as a positive control, which has a well known strong antimicrobial effect. After incubation, the cells were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS), pH 7.4. Permeabilization of the cell membrane was detected by incubating peptide treated cells in propidium iodide (PI, 50 μg/ml final concentration)

for 1 h at 4 °C followed by the removal of unbound dye by excessive washing with PBS. Flow cytometry was performed using a FACScan analyser (Becton Dickinson, San Jose, CA).

Confocal Laser Scanning Microscopy (CLSM)

Intracellular localization of the fluorescein isothiocyanate (FITC)-labelled PMAP-23 in the eggs or worms of *C. elegans* was analysed by confocal laser scanning microscopy. FITC-PMAP-23 was added to 100 μl of the cell suspension at hatching (200 μM) and the motility rate (25 μM), and the cells were incubated at 24 °C for 15 min. The cells were pelleted by centrifugation at 10 000 rpm for 5 min and washed three times with ice-cold PBS buffer. Intracellular localization of FITC-PMAP-23 was examined by a Leica TCS 4D connected to a Olympus IX 70 upright microscope (Olympus, Japan).

Carboxyfluorescein Leakage Measurement

Carboxyfluorescein (CF)-encapsulated large unilamellar vesicles (LUV) composed of phosphatidylcholine (PC) and phosphatidylserine (PS) (3 : 1, w/w) were prepared by reverse-phase ether evaporation methods [23] using 100 mM CF in phosphate-buffered saline, pH 7.4. The initially formed vesicles were extruded through a Nucleopore filter of 0.1 μm. To remove free CF dye, the vesicles were passed through a Bio-Gel A 0.5 m (Bio-Rad, Richmond, USA) column (1.5 × 30 cm) using the eluting buffer. The separated LUV fractions, after appropriate dilution to a final concentration of 6.36 μM phosphate, were mixed with PMAP-23 and filled with buffer to 2 ml in a cuvette at 25 °C. The time-course leakages of CF from LUV were monitored by measuring fluorescence intensity at 520 nm excited at 490 nm on a Shimadzu RF-5000 spectrofluorometer (Tokyo, Japan). The apparent percent leakage value at fluorescence intensity, F, was calculated by the following equation:

$$\% \text{ leakage (apparent)} = 100 \times (F - F_0)/(F_t - F_0)$$

F_t denotes the fluorescence intensity corresponding to 100% leakage after the addition of 20 μl of 10% Triton X-100. F_0 represents the fluorescence of the intact vesicle.

RESULTS AND DISCUSSION

PMAP-23 was chemically synthesized as a 23-residue peptide amidated at the *C*-terminus. The correct peptide was obtained in greater than 90% yield and with a measured mass of 2962.0 vs a calculated mass of 2962.5 Da, and was homogeneous after purification, as confirmed by the mass. The amino acid sequences of PMAP-23 and melittin are summarized in Table 1.

In a previous study, the peptide was shown to possess potent antibacterial activity against Gram-positive and -negative strains [20]. These results suggested that the antimicrobial peptide, PMAP-23, has activity in bacteria. However, the nematicidal activity and mechanism(s) of action of PMAP-23 are not thoroughly understood.

A honeybee venom toxin, melittin, used as a comparison, has been reported to possess a potent antimicrobial activity with a broader spectrum, while exhibiting a strong haemolytic activity [9].

The *in vitro* antinematodal activity of PMAP-23 was measured against the eggs and worms of *C. elegans* cells, and expressed as the hatching rate and the percent motility, respectively. As shown in Figures 1 and 2, over 20 h the peptide did not show any activity on eggs while it showed activity on worms in a dose-dependent manner. However, when incubated for a longer period of time (60 h), the peptide showed activity on eggs (Figure 3). This result may be due to the chitinous layer in the egg shell [24] and these rigid layers may be affected by hatching rate in PMAP-23. Although the hatching rate of eggs was not decreased in the 200 μM range (Figure 1), the percent mobility of the worms tested was highly susceptible to PMAP-23 even in the 25 μM range (Figure 2). The results showed that PMAP-23 had a remarkable antinematodal activity in worms.

Table 1 Amino Acid Sequence of PMAP-23 and Melittin, and Molecular Weight Determination by MALDI-MS

Peptide	Sequence	Calculated value	Observed value
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR-NH_2	2962.5	2962.0
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2847.4	2850.6

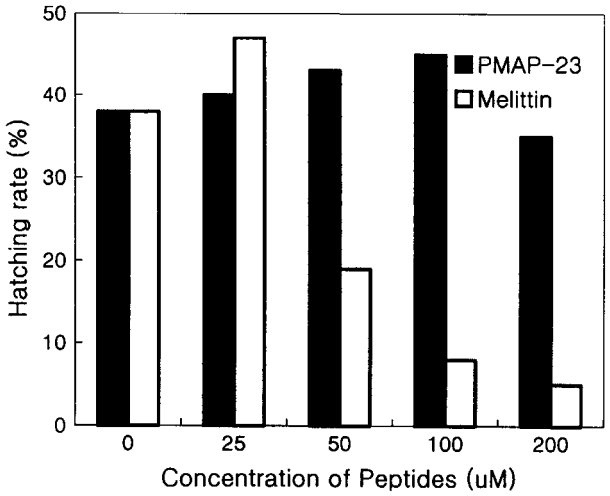

Figure 1 Antinematodal activity of PMAP-23 against the eggs of *C. elegans*. One ml of the eggs was transferred to each sample vial. Peptides were added to aliquots of eggs of *C. elegans* and incubated at 25 °C. The dead eggs were counted after incubation under an inverted microscope.

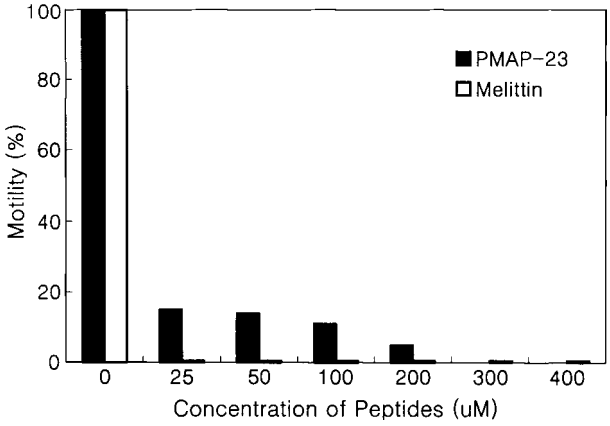

Figure 2 Antinematodal activity of PMAP-23 against the worms of *C. elegans*. One ml of the freshly hatched juvenile suspension (10–15 larvae/ml) was transferred to each sample vial. Peptides were added to aliquots of worms of *C. elegans* and incubated at 25 °C. The dead worms were counted on an inverted microscope.

Although the overall antibiotic mechanism(s) of antibiotic peptides have not been clearly elucidated, disruption of the cell structure by pore formation [25] or ion channel generation seems to be the most likely mechanism [26]. To determine the nematicidal effect of PMAP-23 whether by the disintegration of the cell membrane or by an effect on the cell physiology, the cells were incubated with the DNA intercalating dye propidium iodide (PI). In this study,

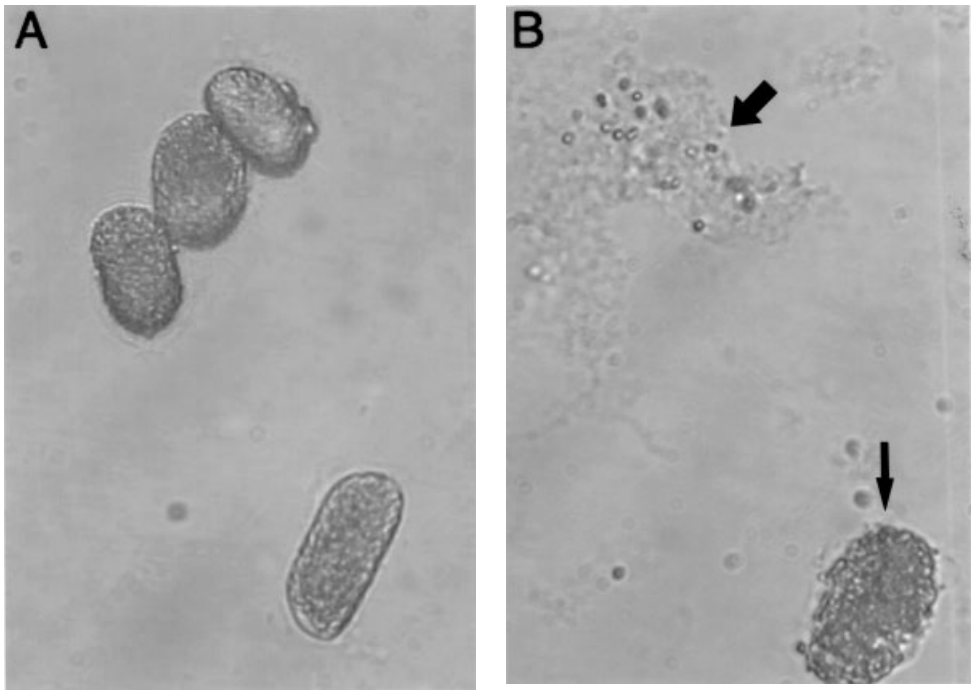

Figure 3 Effect of PMAP on the eggs of *C. elegans*. Peptide was added to a final concentration of 300 μM. The reaction mixture was incubated with concentrations of PMAP for 60 h in S media. A: No peptide treatment on *C. elegans*; B: After treatment of PMAP-23 on *C. elegans*.

PI staining of the peptide treated cells was expected to give further information about the cytotoxic mechanism of PMAP-23. That is, in the case of the disruption of the *C. elegans* cell membrane, the peptide would be permeabilized and therefore would allow the free diffusion of small dyes, such as PI, into the cytoplasm. Detection of internal PI was analysed on single cells by FACS analysis. The results indicated that while normal cells showed no PI fluorescence activity signal, the fluorescence histogram of cells treated with PMAP-23 and melittin at a concentration of 300 μM and 200 μM, showed a total shift of the peak to the right (Figure 4). The fluorescence intensity of these cells serves as a marker for the relative amount of internalized PI in the case of a permeabilized cell membrane like that of disintegrating cells.

To examine the target sites of PMAP-23 in *C. elegans*, they were labelled with FITC and visualized under confocal laser scanning microscopy. FITC did not show any effect on the antinematodal activity of PMAP-23 (data not shown). The FITC-labelled PMAP-23 penetrated the cell membrane and accumulated in the egg's shell and plasma membrane immediately after addition to the *C. elegans* (Figures 5 and 6). This result suggested that the major target site of PMAP-23 is the plasma membrane of the nematode.

The antinematodal mechanisms of the peptide have not been clearly elucidated, but the disruption

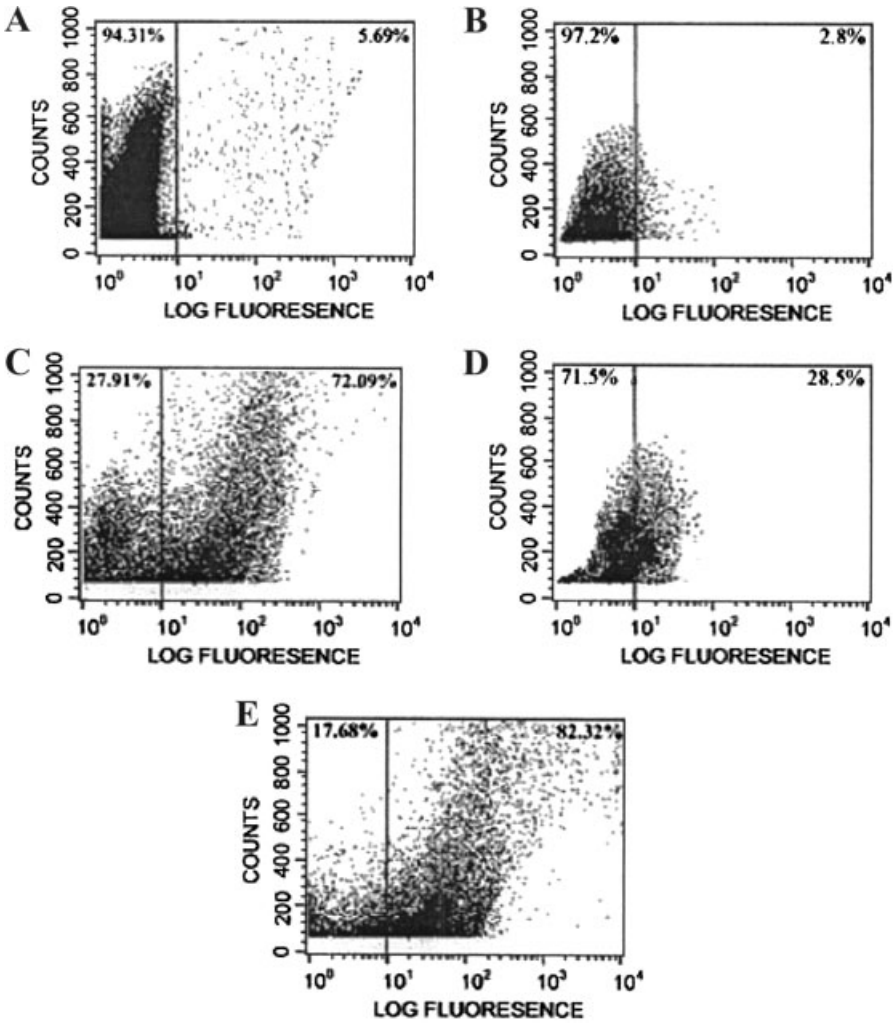

Figure 4 FACScan analysis of propidium iodide staining in *C. albicans*. Histograms show the fluorescence intensity of internalized PI after peptide treatment of *C. elegans*. A: control staining without any peptide treatment; B: indicates PI staining of PMAP-23 treated cells for 20 h; C: PMAP-23 treated cells for 60 h; D: melittin treated cells for 20 h; E: melittin treated cells for 60 h.

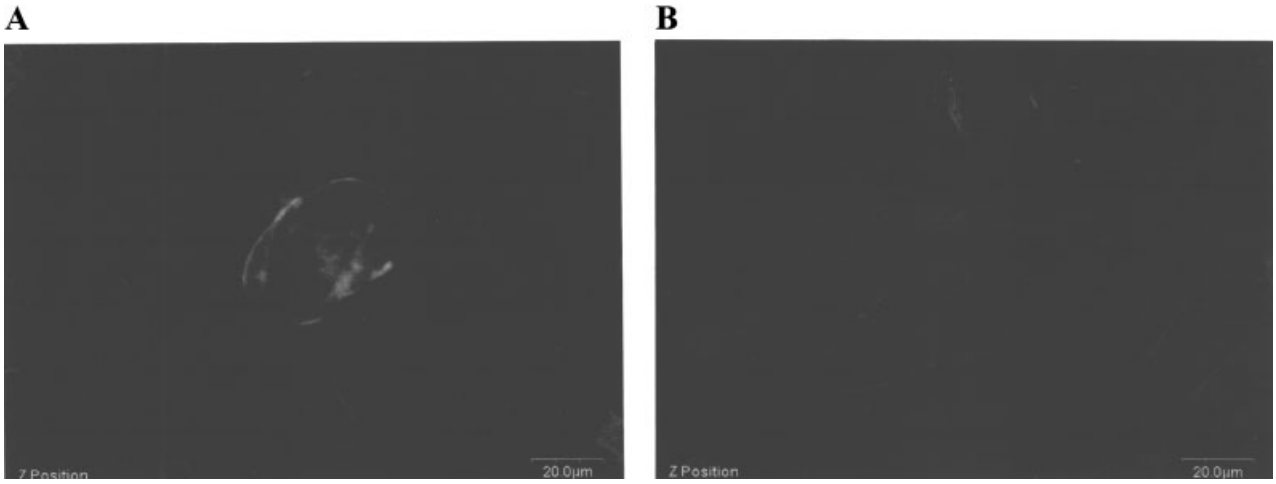

Figure 5 Distribution of PMAP-23 in the eggs of *C. elegans* as visualized by confocal laser scanning microscopy. A: The eggs of *C. elegans* incubated with FITC labelled PMAP-23. B: The eggs of *C. elegans* incubated without peptide.

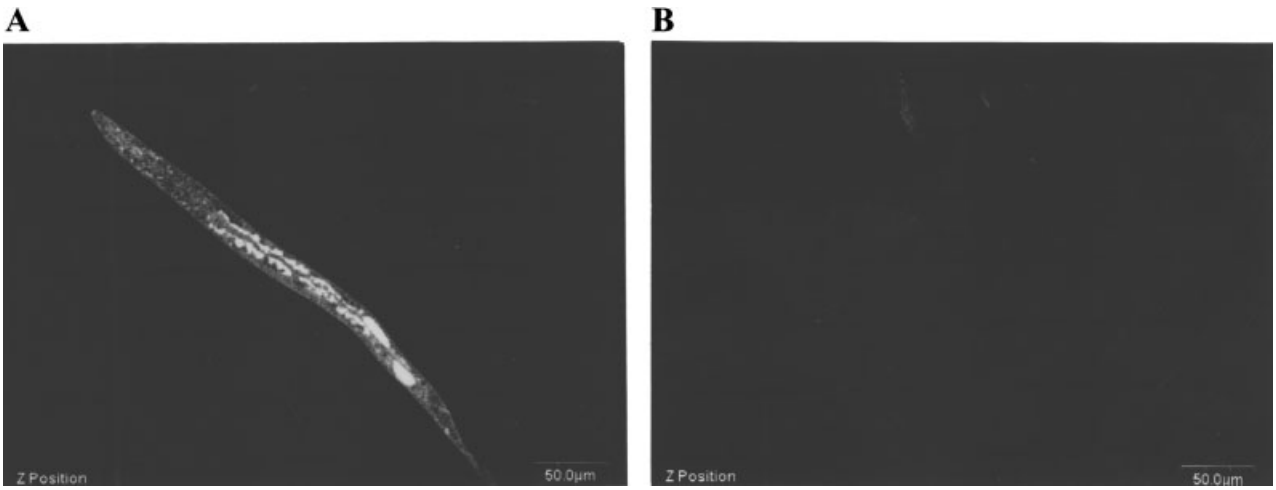

Figure 6 Distribution of PMAP-23 in the worm of *C. elegans* as visualized by confocal laser scanning microscopy. A: The worms of *C. elegans* incubated with FITC labelled PMAP-23. B: Worms of *C. elegans* incubated without peptide.

of the cell structure by pore formation [27,28] or by ion channel generation seems to be the most likely mechanism [29]. Therefore, the antinematodal effects of synthetic peptides were further investigated by testing the lipid membrane disrupting activity using a CF-entrapped artificial liposomal vesicle (PC/PS; 3 : 1, w/w). The negatively charged artificial membrane liposome, which mimicked the biological membrane, was monitored by the leakage of carboxyfluorescein (CF) dye trapped in the liposome during incubation with PMAP-23. The result showed that CF was released from LUV (large unilamellar vesicle) after treatment of 1.25 μM and 20 μM in a dose-dependent manner (Figure 7).

CONCLUSION

The synthetic peptide, PMAP-23, derived from pig myeloid was investigated. PMAP-23 displayed a strong antinematodal activity against the eggs and worms of *Caenorhabditis elegans*. This PMAP-23, which exhibits potent antinematodal activity, may be useful as a template or lead compound for the development of a novel therapeutic agent.

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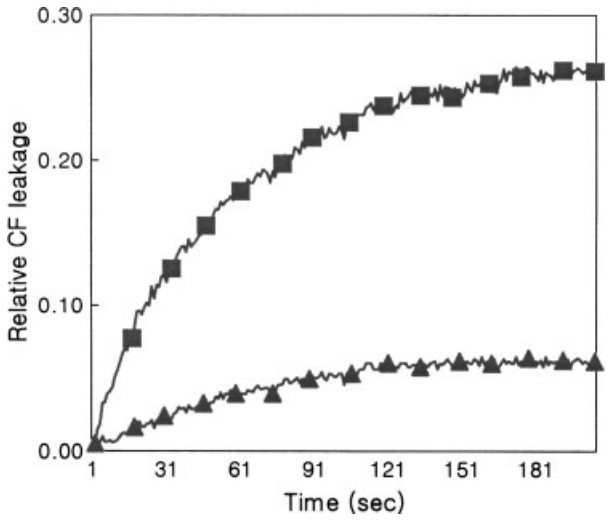

Figure 7 Interaction of PMAP-23 with large unilamellar vesicles (LUV). The PC/PS (3 : 1) vesicle containing CF was incubated with PMAP-23 in the phosphate-buffered saline (pH 7.4). The CF release was monitored after the addition of PMAP-23. 20 μM of PMAP-23 (■), 1.25 μM of PMAP-23 (▲).

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