CHAPTER IV

PURIFICATION AND CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES FROM THE SANDWORM *Perinereis nuntia* Savigny

4.1 Introduction

From last few years, the reports of discovered and characterized of low – molecular – mass antimicrobial peptides (AMPs) from a wide variety of organisms especially in invertebrates have been accumulating in a rapid rate. On the other hand, the resistance to antibiotics of microorganism has risen and the resistance to most or all available agents has appeared in the clinic over the past decade. There is a growing need to discover and introduce new drugs, and AMPs provide new candidates for screening of new antibiotics. Because of their biochemical diversity, broad spectrum against microorganisms and rarely induce microbe resistance (Walsh, 2000).

It is well known that invertebrates lack an acquired immune system and their defense mechanisms rely on innate immune responses which include a cellular response mediated by haemocytes, humeral immune response and AMPs to lyse invading microorganisms. Therefore, many AMPs have been identified from a variety of invertebrate source including insects, ascidians, chelicerates, mollusk and annelids. Among invertebrates, annelids are an important group of investigation due to their extensive inhabitation and enormous commercial significance. Perinerin, a 51 amino acid residues AMP was isolated from sandworm *Perinereis aibuhitensis* (Pan *et al.*, 2004). Two small peptides, arenisin 1 and 2 were isolated from coelomocytes from lugworm *Arenicola marina* by Ovchinnikova (2004) while Tasiemski (2007) reported AMP named hedistin which containing bromotryptophan was extracted from coelomocytes of clamworm *Nereis diversicolor*. Furthermore, terrestrial and parasite

annelids were also isolated AMPs. The well–known terrestrial annelid, earthworm, *Lumbricus rubellus* was discovered proline rich with 62 amino acids AMP named lumbricin I (Cho *et al.*, 1998) while another earthworm, *Eisenia fetda* was isolated vermipeptide family from tissue and coelomic fluid and the activities of vermipeptide family was shown antimicrobial, antiviral and antitumer (Wang *et al.*, 2007). Two AMPs was isolated from leech *Theromyzon tessulatum*, an ectoparasite of aquatic bird which cystine rich AMP, theromacin and anionic AMP, theromyzin from body fluid of leech (Tasiemski *et al.*, 2003).

Sandworm, *Perienreis nuntia* Savigny is a marine annelid which used as live feed in aquatic industry. According to the literature reviews and introduction, no previous report of AMPs from *P. nuntia*, hence it was interested as source of AMPs. In the present study, AMPs from *P. nuntia* were purified, characterized and evaluated for antimicrobial activity.

4.2 Materials and methods

4.2.1 Equipments

Autoclave	Hirayama, Japan
Auto pipette	Gilson, USA
Fraction collector	LKB Bromma, Australia
Freeze dryer	Eyela FD550, Japan
HPLC	ThermoFinnigan, USA
- Semi-preparative column reverse phase C18 125 x 8.0 mm, 5 µm 300 Å	VDS optilab, Germany
- Analytical column reverse phase	Phenomenex, USA

Protein/peptide C18 , 250 x 4.60 mm, 5 µm 300 Å

Lamina flow	International Scientific supply, Thailand
Micro centrifuge	Tomy MC-15A, Japan
Orbital shaker	HT Infors, USA
Refrigerated centrifuge	Kubota KR 20000T, Japan
Spectrophotometer	Synergy HT, USA
Solid phase extraction (SPE) C18 reverse phase	Phenomenex, USA
Syringe filter PVDF, 0.45 µm	Vertical, USA
Vortex	Scientific Industries, USA

4.2.2 Chemicals

Acetic acid	AR grade	Merck, USA
Acetonitrile	HPLC grade	Merck, USA
Agar	-	Difco, USA
Bacitracin	AR grade	Fluka, USA
BCA protein assay kit	-	Pierce, USA
Beef extract	-	Difco, USA
Bio-Gel P-10	AR grade	BIO-RAD, USA
Bovine serum albumin	AR grade	Sigma, USA
Colistin	AR grade	Sigma, USA
Copper sulphate	AR grade	Merck, USA
Ethanol	AR grade	Merck, USA
Glucose	AR grade	Sigma, USA

Glycerol	AR grade	Merck, USA
Malt extract	-	Difco, USA
Peptone	-	Difco, USA
Pepstatin A	AR grade	Sigma, USA
Petroleum ether	AR grade	Merck, USA
Potassium sulphate	AR grade	Merck, USA
Sodium chloride	AR grade	Merck, USA
Sodium hydroxide	AR grade	Merck, USA
Sulfuric acid	AR grade	Sigma, USA
Trifluoroacetic acid	HPLC grade	Sigma, USA
Yeast extract	-	Difco, USA

4.2.3 Organisms

4.2.3.1 *Farmed sandworm* (*P. nuntia*)

Four months old farmed sandworm (average weight 0.73 g/worm) was obtained from sandworm farm Samutsongkram Thailand. Upon arrival, live farmed sandworm was grown in laboratory and fed with a small size commercial fish diet once a day until finished experiment. After challenged, they were random sampling, washed with normal sea water then put on ice and frozen at -70 °C until use.

4.2.3.2 *Microorganisms*

- Tested microorganisms

: *Bacillus subtilis* ATCC 6633 (Gram positive bacteria)

: *Staphylococcus aureus* ATCC 25923(Gram positive bacteria)

: *Escherichia coli* ATCC 25922 (Gram negative bacteria)

: *Vibrio harveyi* strain 104 (Gram negative bacteria)

: *Candida albicans* ATCC 10231 (fungi)

- Challenged microorganism

: *Vibrio harveyi* strain 104 (Gram negative bacteria)

- Growth of microorganisms

B. subtilis, *S. aureus*, and *E. coli* were grown on nutrient broth (NB) and nutrient agar (NA, Appendix A) at 37 °C and were maintained in NA at 4 °C. *V. harveyi* was grown on NB and NA (with salt) at 30 °C and was maintained in NA (with salt) at 4 °C. Their stocks were kept in NB (or NB with salt for *V. harveyi*) with 10% sterile glycerol at -70 °C.

C. albicans was grown on yeast-malt medium (YM) and yeast-malt broth (YMB) at 30 °C. It was maintained and kept as above with YM and YMB with 10% sterile glycerol.

4.2.4 Isolation of antimicrobial peptides from sandworm *P. nuntia*

4.2.4.1 *Challenged sandworm with V. harveyi*

A . Growth of *V. harveyi*

V. harveyi from stock was streaked on NA with salt and incubated at 30 °C for 24 hr. Then picked up 5 – 6 fresh colonies into NB with salt and grown them in shaking flask at 100 rpm, 30 °C for 16 hr. After that collected cell, washed with sterile normal saline and adjusted cell concentration to 0.5 Mc Farland standard with 3% NaCl sterile solution.

B. Challenged sandworms

Farmed sandworm was normal growth in laboratory for 3 days before challenged (not stravated). Bacteria suspension from A was added to sandworms in sea water and concentration of *V. harveyi* was 10^7 cfu/worm. Sandworm was challenged by incubation with *V. harveyi* for 24 hr. When 24 hr, all challenged sandworm was washed with sterile 3% NaCl, transfer to new tank with normal sea water and grow them as 4.2.3.1. After that, challenged 10 sandworms were randomly collected from the tank at 0 hr (immediately after wash), 12, 24, 48, 72, 96 and 120 hr respectively and kept in -70 °C until acid extraction and antimicrobial activity was tested as describe below. Non-challenged sandworm was a control group. The highest antimicrobial activity period was repeated in 250 worms and extracted in further step.

4.2.4.2 *Acid extraction*

The whole body challenged sandworm was homogenized with an equal weight of 0.1% acetic acid containing pepstatin A 0.1 mg/ml. The homogenate was kept in ice for 2 hr then centrifuged at 10 °C, 10,000 rpm for 10 min. The supernatant was recovered and the pellet was re-extracted under the same conditions. First and second supernatants were combined as crude extract, lyophilized and tested for antimicrobial activity.

4.2.4.3 *Purification of AMPs by gel-filtration*

The lyophilized crude extract was dissolved in distilled water and adjusted concentration of protein approximately to 40 mg/ml (protein determination by BCA assay kit, Appendix B). The 15 ml of crude extract was applied on a column

(2 x 60 cm.) of Bio-Gel P-10 gel-filtration column equilibrated with 0.1% acetic acid. Then the crude extract was loaded gently onto the surface of the column. After absorption, 0.1% acetic acid was added, gently washing the wall without disturbing the column bed. The elution was performed at a flow rate of 20 ml/hr. Fractions of 10 ml were collected and aliquot 2 ml. The aliquots were lyophilized, adjusted concentration to 20 fold or 100 fold and assayed antimicrobial activity. The absorbance of eluted was monitored at 280 nm. The fractions containing antimicrobial activity were pooled for next purification.

4.2.4.4 *Purification by solid phase extraction (SPE)*

The 15 ml of pooled active fractions after purification by Bio-Gel P-10 filtration were further purified using solid phase extraction (SPE) with Phenomenex C18 cartridges (C18 reverse phase). Steps-wise elutions were performed with 15 ml of 10%, 50% and 80% acetonitrile. The 4 eluted fractions (non bound, 10%, 50% and 80% acetonitrile fractions) were aliquot, evaporated and lyophilized. The aliquot samples were dissolved in 0.1% acetic acid to concentration 50 fold and assayed antimicrobial activity. The active eluted fractions from SPE were future purified by reversed - phase HPLC.

4.2.4.5 *Purification by Reverse-phase HPLC (RP-HPLC)*

The lyophilized 50% ACN eluted fractions from SPE (and called 50 ACN) showed antimicrobial activity. Thus, the 50 ACN fractions were dried, dissolved in 5% ACN to concentration 20 fold and subjected to reverse-phase HPLC as following steps.

First step – The 50 ACN fraction was subjected to reverse-phase HPLC on a VDS optilab C18 semi-preparative column (125 x 8.0 mm, 5 µm and 300 Å). The mobile phase consisted of 90% acetonitrile in 0.1% trifluoroacetic acid (solution A, Appendix A) and 0.1% trifluoroacetic acid (solution B). The elution was performed with a linear gradient of 10% to 70% of solution A for 60 min at a flow rate of 1.0 ml/min and increased 1% of solution A/1 min. The absorbance of peptides were monitored by UV 6000 at 215 nm. The eluted 1 ml fractions were collected manually in polypropylene tubes. Each fraction was evaporated, lyophilized dissolved in 0.1% acetic acid to concentration 50 fold and tested for antimicrobial activity.

Second step – The fraction number 5, 15 and 40 from first step were showed antimicrobial activity and called F5, F15 and F40 respectively. They were further separated by HPLC on Phenomenex C18 reverse–phase analytical column (250 x 4.60 mm, 5 µm and 300 Å). The dried F5 was resuspended in 5% acetonitrile to concentration 50 fold then subjected to RP-HPLC and the mobile phase system consisted of solution A and B was followed as first step. The elution of F5 was performed with a linear gradient of 5 to 15% solution A over 25 min at 1.0 ml/min and increased 1% of solution A/1 min. While F15 and F40 were resuspended in 10% and 25% acetonitrile, performed with a linear gradient of 15 to 40% solution A over 25 min and 40 to 65% solution A over 30 min respectively at the same flow rate. The 0.5 ml fractions were hand collected evaporated, lyophilized, dissolved in 0.1% acetic acid to concentration 100 fold and tested for activity. The active fractions were submitted for purity measurements by HPLC. The purified peptides were tested antimicrobial activity and molecular weight of AMP.

4.2.5 Antimicrobial activity assay by paper disc method

The antimicrobial activity of crude extract and fraction of each steps of purification were determined by paper disc method as following;

4.2.5.1 ***Preparation of paper discs***

: extracted sandworm solution (crude extract and purified fraction)

: positive control solutions

- for Gram positive bacteria : 0.45 units/disc or 0.045 units/disc of bacitracin
- for Gram negative bacteria : 5000 units/disc or 500 units/disc of colistin
- for fungi : 0.1 mg/ml or 0.01 mg/ml of ketoconazole

: negative control solutions : 0.1% acetic acid

All of test solutions and control solutions were dropped 80 µl on sterile blotting thick paper discs (∅ 0.8 cm) or 45 µl on small thin paper disc (∅ 0.6 cm) for the small among of sample. Paper discs were dried under room temperature before placed on microorganisms culture as describe in 4.2.5.3.

4.2.5.2 ***Preparation of microorganisms***

B. subtilis, *S. aureus* and *E. coli* from stock were streaked on NA and incubated at 37 °C for 24 hr. Then picked up 5 – 6 fresh colonies, inoculated into 50 ml of fresh NB and shaking flask at 250 rpm, 37 °C for 6 hr.

V. harveyi from stock was streaked on NA with salt and incubated at 30 °C for 24 hr. After that, picked up 5 – 6 fresh colonies, inoculated into 50 ml NB with salt and shaking flask at 100 rpm, 30 °C for 18 hr.

C. albicans was grown as above on YM at 30 °C for 24 hr and in 50 ml YMB which shaking flask at 250 rpm, 30 °C for 6 hr.

4.2.5.3 *Antimicrobial activity assay*

Then 200 µl of 10^6 cfu/ml of log phase microorganisms culture were spreaded on NA (for *B. subtilis*, *E. coli* and *S. aureus*), NA with salt (for *V. harveyi*) and YM (for *C. albicans*). Extracted sandworm, positive control and negative control were prepared as describe in 4.2.5.1 on paper discs and placed on NA, NA with salt and YM in the same plate. Plates were incubated at suitable temperature for each microbe for 24 hr. An activity was determined by observing the clear zone of suppression of microbial growth around the blotting paper disc.

4.2.6 Molecular weight determination

The exact molecular mass of purified peptides were determined by matrix-assisted laser desorption ionization time of flight mass spectromrtry (MALDI-TOF) at Bio Service Unit (BSU) of National Science and Technology Development Agency, Thailand.

4.3 Results and discussion

4.3.1 Challenged sandworm with *Vibrio harveyi* strain 104

The farmed sandworm was challenged with *V. harveyi* for 24 hr. After challenged, it was washed, grow as normal condition and collected at the period of time 0, 12, 24, 48, 72, 96 and 120 hr respectively. The non-challenged sandworm was used as a control. The whole bodies of non-challenged and challenged sanworms were

homogenated and extracted with 0.1% acetic acid. The crude extract samples were tested for antimicrobial activity against microorganisms; *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. The antimicrobial activity was measured the clear zone or inhibiting zone. In the results, all crude extracts showed the activity against only *B. subtilis* and no activity was observed in other microorganisms (Figure C1 to C3, Appendix C). Moreover, the crude extract from challenged sandworm found the highest antimicrobial activity in the sample after challenged 24 hr and kept them immediately and the activity was decreased during the time pass (Table 4.1 , Figure C1). From these results, the 250 sandworms were challenged and collected at 24 hr after challenged for next experiment.

Table 4.1 Antimicrobial activity against *B. subtilis* of challenged sandworm

Period	Diameter of clear zone (cm) *
Control (non-challenged)	1.02
After challenge 24 hr	1.38
After challenge 24 hr + 12 hr	1.12
After challenge 24 hr + 24 hr	1.18
After challenge 24 hr + 48 hr	1.10
After challenge 24 hr + 72 hr	1.06
After challenge 24 hr + 96 hr	1.08
After challenge 24 hr + 120 hr	1.08

* Diameter of thick paper disc is 0.8 cm

Comparison of non-challenged and challenged sandworms found that *V. harveyi* infection of the sandworm could induce the sandworm to produce antimicrobial molecule more than non-challenged of the sandworm. As in mussle, *Mutilus galloprovincialis* was challenged by marine bacteria *Vibrio splendidus* by injected 100 µl of heat – killed bacteria (10^7 cfu) into the adductor muscle. The AMP, defensin in mussle was strongly enhanced and was highest after 6 hr of challenged (Mitta *et al.*, 2000). Tasiemskit (2004) also reported the challenged leech (annelid) *Theromyzon tessulatum*. The transcripts level of AMPs, theromacin and theromyzin were enhanced rapidly in 6 hr after bacteria challenge. In the contrary, the other annelid, clamworm *Nereis diversicolor* was challenged by heat kill of *E. coli* and *Micrococcus luteus* (10^7 cfu). The RNA level of AMP, hedistin was analyzed at 0, 3, 6 and 24 hr after injection. But no enhancement of the basal transcription level was observed on hedistin gene expression (Tasiemskit *et al.*, 2007). The researchers of both annelids were the same group and they explained the different of the results. In annelids, two modes of fighting infectious by different AMPs i) rapid transcriptional upregulation of the gene coding for AMPs mainly in a specific tissue, after septic injury and rapid release into the haemolymph of the antimicrobial compounds in annelid. This mode AMPs was increased when they were challenged. ii) Constitutive production and storage of the antimicrobial substances, particularly in coelomocytes and release of the peptides into the coelomic fluid after immune challenge in annelids. These AMPs are maintained in body fluid (Tasiemskit *et al.*, 2007). As describe above, AMPs from sandworm in this experiment may be rapid transcriptional of the gene coding for AMPs after challenged as in mode 1. Because they were increased when sandworms were challenged.

4.3.2 Peptide purification by gel-filtration

The crude extract of 250 challenged sandworms were lyophilized, dissolved in distilled water and adjusted to around 40 mg/ml protein concentration. The 15 ml concentrated crude extract was loaded 9 times to a gel filtration column with Bio-Gel P-10, cut off more than 20 kDa and eluted with 0.1% acetic acid. The 10 ml fraction was collected and tested antimicrobial activity (see Scheme 1). The each fraction was tested antimicrobial activity against all microorganisms. First time, the concentrated 20 fold of gel-filtration fractions were found activity against only *B. subtilis* (Figure 4.1 and Figure C4). Then gel-filtration fractions were concentrated to 100 fold (or 5 fold from *B. subtilis*) and tested activity against *S. aureus*, *E. coli*, *V. harveyi* and *C. albicans*. The activities of 100 fold concentration were found against *S. aureus*, *E. coli* and *V. harveyi* but inactive against *C. albicans* (Table 4.2 and Figure C5). From these results, as from concentration of fraction and diameter of clear zone showed that *B. subtilis* was most sensitive to gel-filtration fraction and followed by *V. harveyi*, *S. aureus* and *E. coli* respectively. Thereafter, *B. subtilis* was used to follow activity in future purification step because it was most sensitive to AMPs and used low concentration of purified fraction for testing.

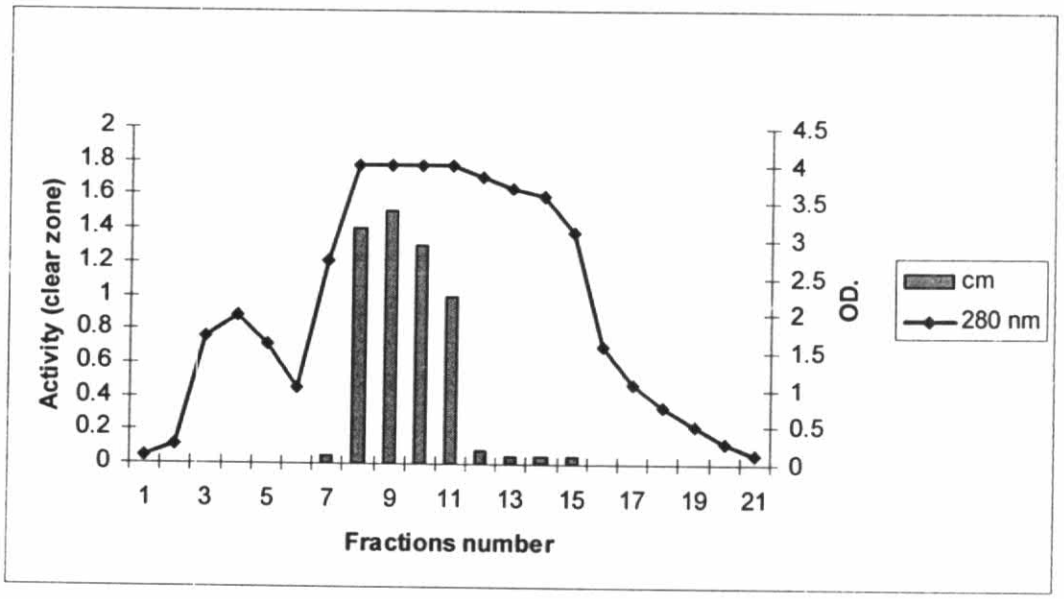
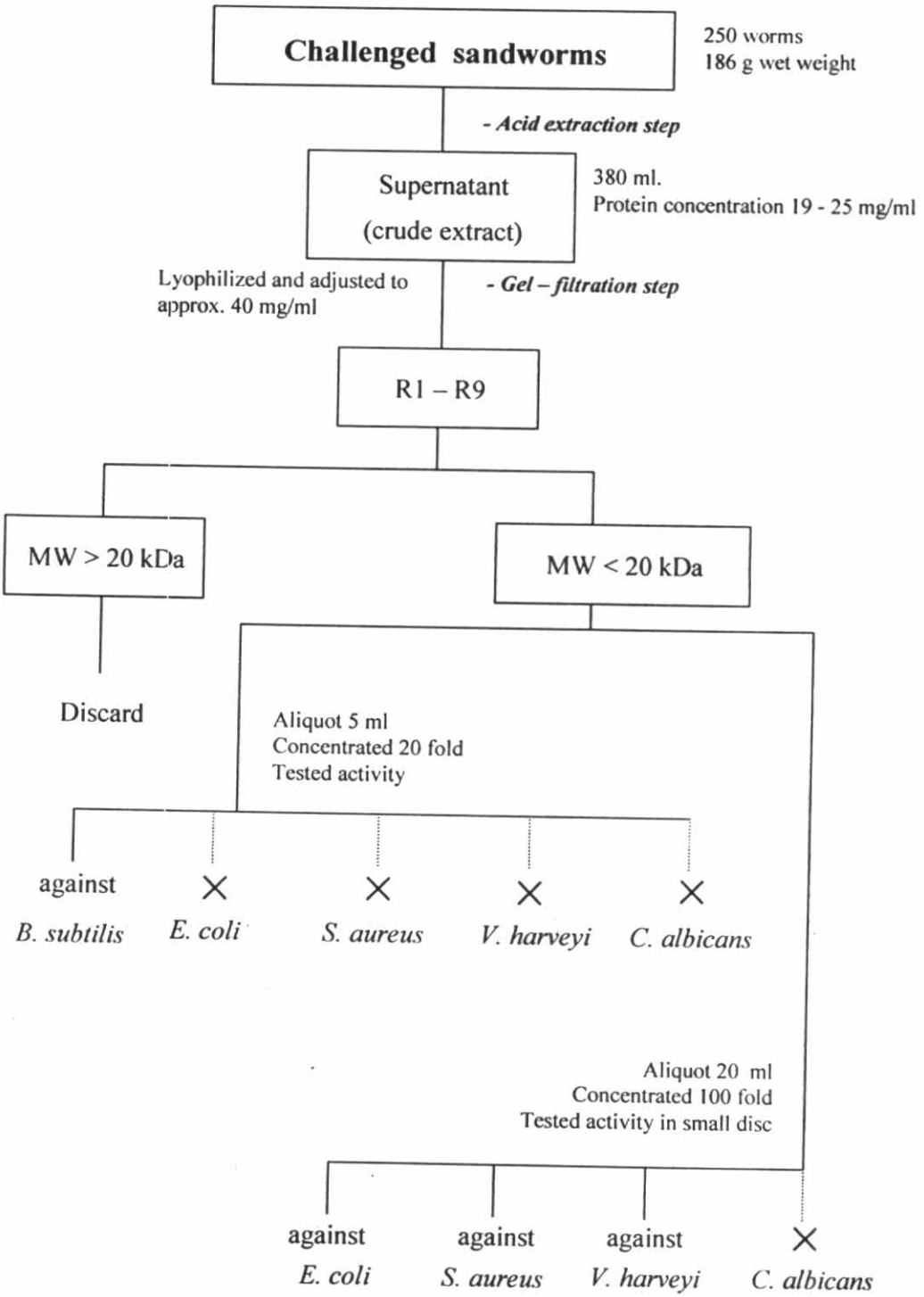


Figure 4.1 Protein absorbance and antimicrobial activity against *B. subtilis* of each fraction from gel-filtration step



Scheme 1 Peptide purification by gel-filtration

Table 4.2 Antimicrobial activity of gel-filtration fraction of challenged sandworm

Microorganisms	Concentrated fraction	Diameter of clear zone (cm) *
B. subtilis (G+)	20 fold	0.8
S. aureus (G+)	100 fold	1.1
E. coli (G-)	100 fold	0.8
V. harveyi (G-)	100 fold	1.5
C albicans (fungi)	100 fold	-

* Diameter of thin paper disc is 0.6 cm

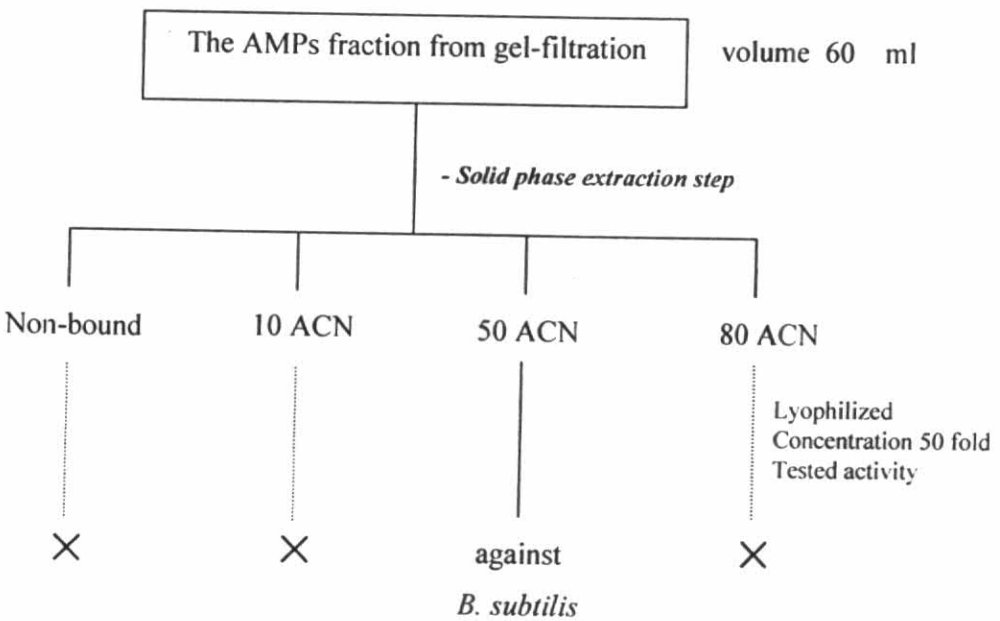
Typically, most AMPs are shown activity against Gram positive bacteria but eluted fraction from gel-filtration of this experiment were shown activity against *V. harveyi* higher than other Gram positive bacteria *S. aureus*. This could be attributable of *V. harveyi* is a challenged bacteria in this experiment. Furthermore, *Vibrio* sp. is capacity to extensively degrade native cuticle collagen of marine polychaetes as in *Nereis* sp. (Tasiemski *et al.*, 2007). Vibrio collagenase would help bacteria entrance into the worm body, making mechanical defense barrier of the cuticle in efficient against Vibrio invasion.

4.3.3 Peptide purification by solid phase extraction (SPE)

The eluted fractions from gel–filtration which showed antimicrobial activity were pooled and loaded to SPE C18 reverse phase cartridge. Non-bound fraction was collected then SPE was eluted with 10%, 50% and 80% ACN respectively. The non-bound fraction and eluted fraction were called non-bound, 10, 50 and 80 ACN respectively (see Scheme 2). All fractions were evaporated, lyophilized and resuspened with 0.1% acetic acid for concentrated to 50 fold before testing for

antimicrobial activity against *B. subtilis*. The results indicated that only 50 ACN fraction showed antimicrobial activity (Figure C6).

The solid phase extraction method separated compounds according to their hydrophobicity. As all AMPs were detected in range of 10 – 60% acetonitrile (Haug *et al*., 2002; Stensvag *et al*., 2007; Tasiemski *et al*., 2004, 2007). This suggests that AMPs are same polarity range and may be a basic character of AMPs for attachment to microbial cell membrane.



Scheme 2 Peptide purification by solid phase extraction

4.3.4 Peptide purification by Reversed phase HPLC (RP-HPLC)

4.3.4.1 *Purification by semi-preparative column*

This step was purified by semi-preparative reverse phase C18 column. The dried 50 ACN fraction was dissolved in 5% ACN to concentrated 20 fold. The 100 µl concentrated solution was loaded to column and separated by gradient 10 – 70% of solution A (or 9 – 63% ACN) containing 0.1% TFA (see Scheme 3). All fractions with shown protein or peptide peak were collected, 1 ml per fraction (Figure 4.2). The same fraction number was pooled and aliquot. The aliquots were evaporated, lyophilized, dissolve in 0.1% acetic acid to concentrated 50 fold to tested activity. The antimicrobial activity was found activity against *B. subtilis* in fraction number 5, 15 and 40 with diameter of clear zone 0.70, 0.75 and 0.65 cm respectively (Figure C7). They were eluted by 13.5%, 22.5% and 45% acetonitrile and the fraction number 5,15 and 40 were called F5, F15 and F40. The F5, F15 and F40 fraction were purified in next step.

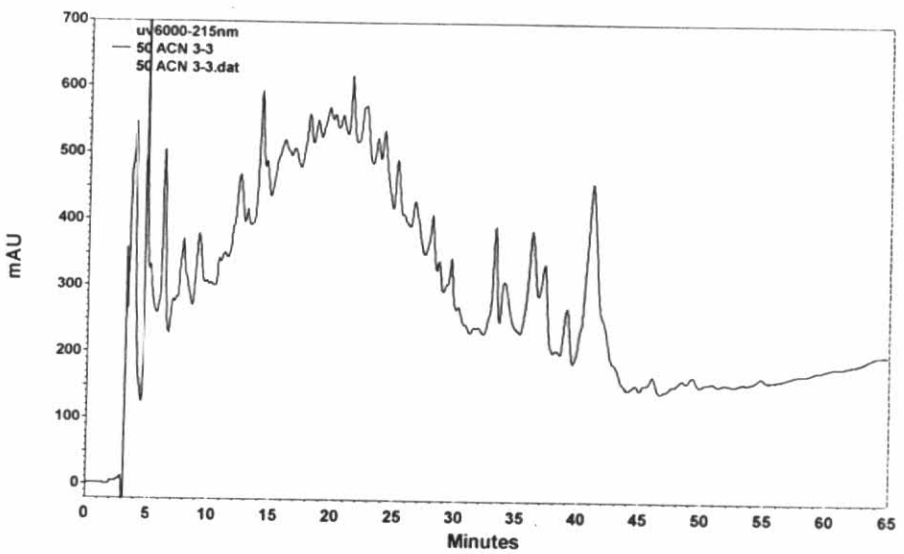
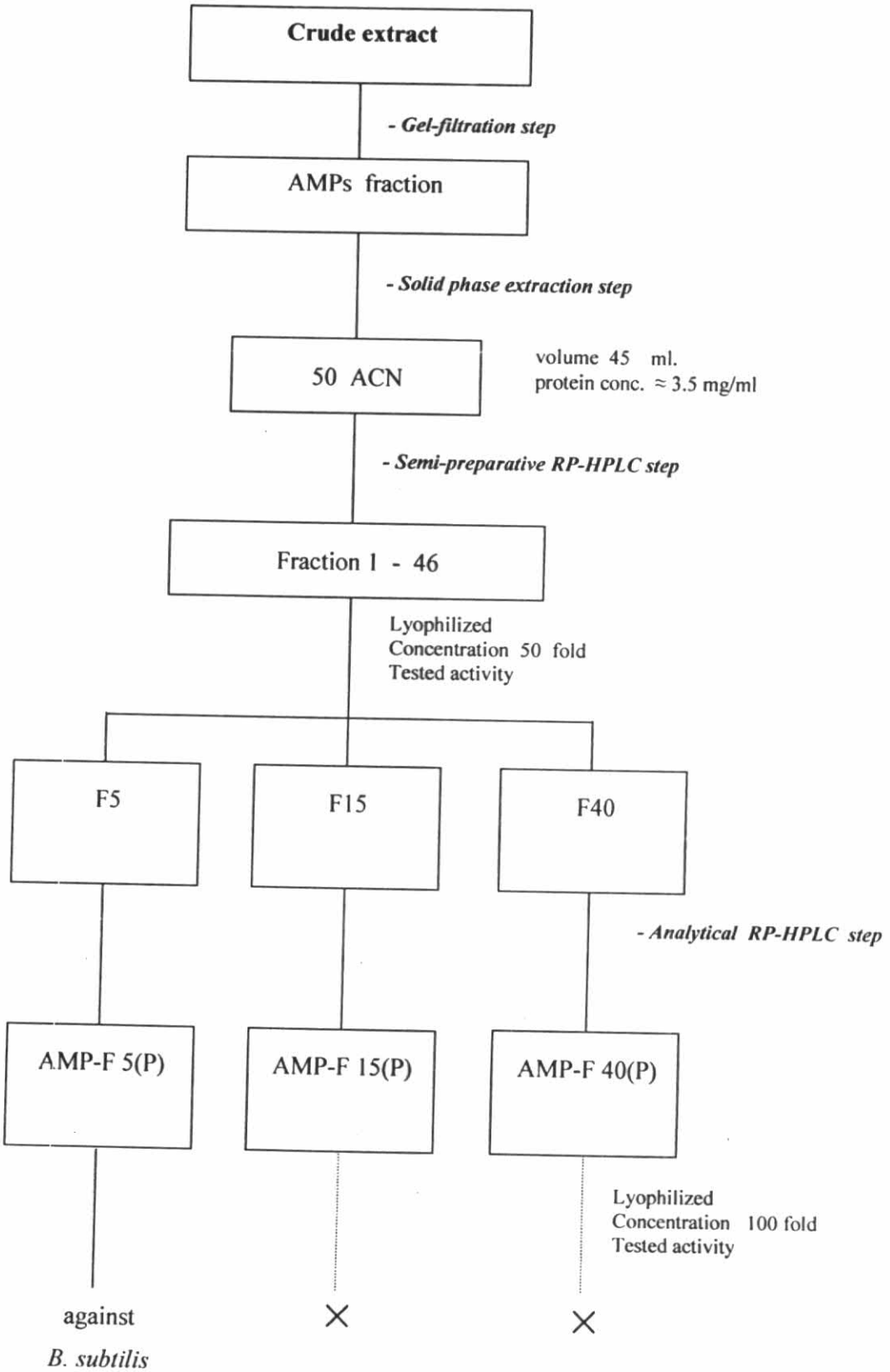


Figure 4.2 RP-HPLC chromatogram of 50 ACN fraction by semi-preparative column with a gradient of 9 - 63% ACN containing 0.1% TFA



Scheme 3 Summary of AMPs purification step

4.3.4.2 *Purification by analytical column*

Analytical reverse phase protein/peptide C18 column was used in this step. The dried F5, F15 an F40 were dissolved in 5%, 15% and 40% acetonitrile respectively and adjusted to concentrated 50 fold (see Scheme 3). The F5 solution was loaded to column and separated by gradient from 5 - 15% of solution A (or 4.5 – 13.55% ACN) with 0.1% TFA (figure 4.2). The F15 and F40 were separated as F5 but gradient eluent was 10 – 25% A (9 – 22.5% ACN) and 35 – 50% A (31.5 – 45% ACN) (Figure 4.3 and 4.4). The results showed that purified peptide separating from F5 fraction was eluted at 8.25% ACN and called AMP-F5(P) and showed antimicrobial activity with diameter of clear zone 0.7 cm. While the purified peptide from F15 and F40 fraction were eluted at 15.75% and 40.95% ACN respectively but no activity was detected in the AMP F15(P) and AMP-F40(P).

The activity of AMP-F15(P) and AMP-F40(P) were lost during purification by analytical reverse phase HPLC column. They were very low concentration and/or may loss some require molecules for their activity. Straus (2006) reported that AMP, daptomycin are absolutely required calcium for activity. Because daptomycin display activity by binding to the cytoplasmic membrane in a calcium-dependent manner. Therefore, AMP-F15(P) and AMP-F40(P) may need some molecule such as co-factor to express their activities.

Moreover, we calculated among of AMP(P), it was 0.26 µg from 1 g wet weight of fresh sandworm. As in perinerin, AMP from *Perinereis aibuhitensis* was found 0.3 µg from 1 g of worm (Pan *et al.*, 2004).

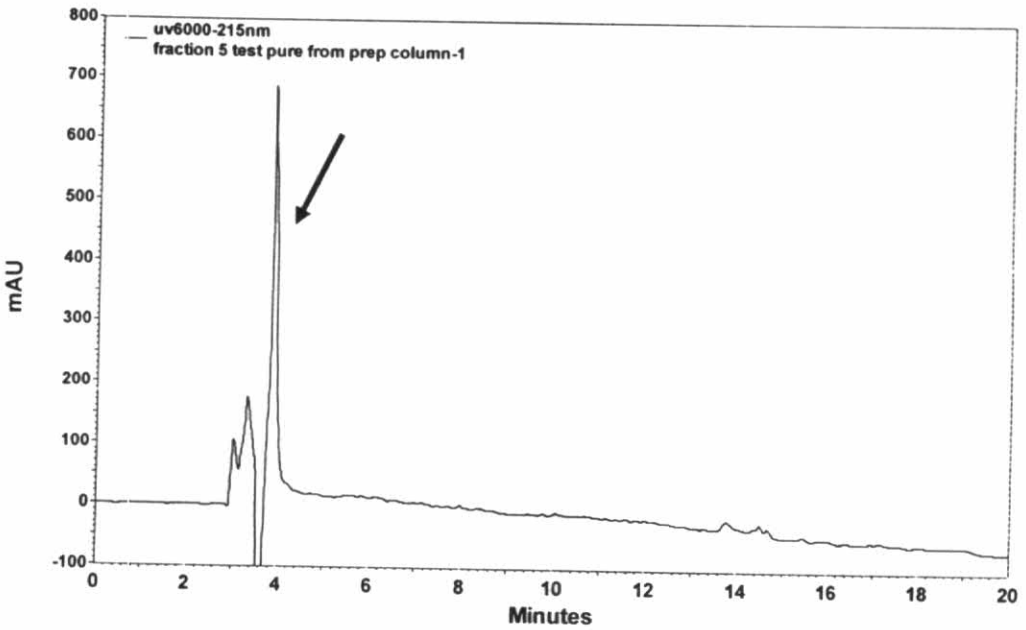


Figure 4.3 RP-HPLC chromatogram of AMP-F5(P) fraction by analytical column with a gradient of 4.5 – 13.5% ACN containing 0.1% TFA

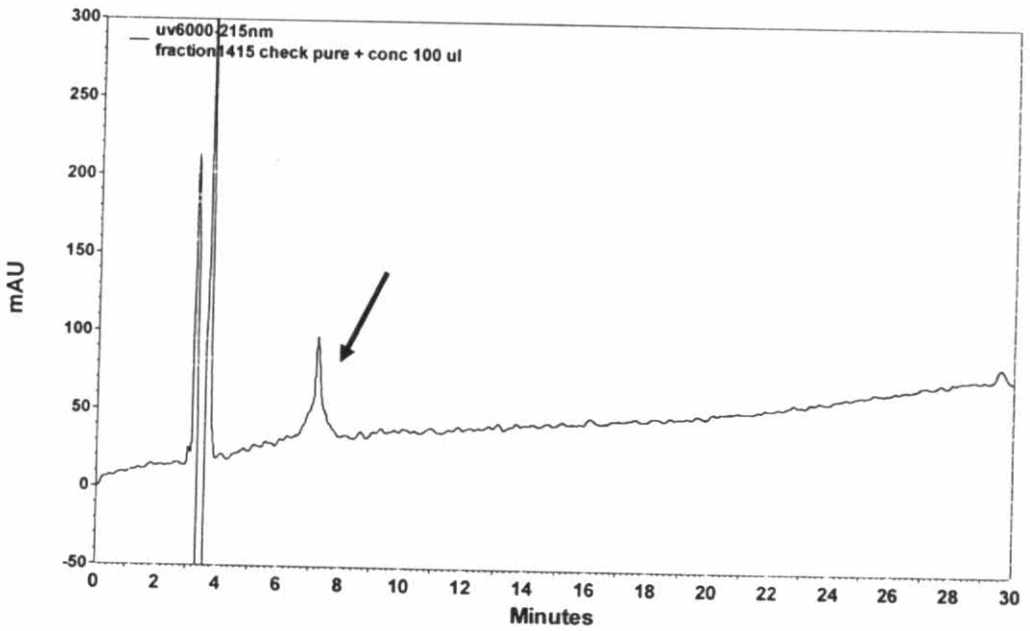


Figure 4.4 RP-HPLC chromatogram of AMP-F15(P) fraction by analytical column with a gradient of 9 – 22.5% ACN containing 0.1% TFA

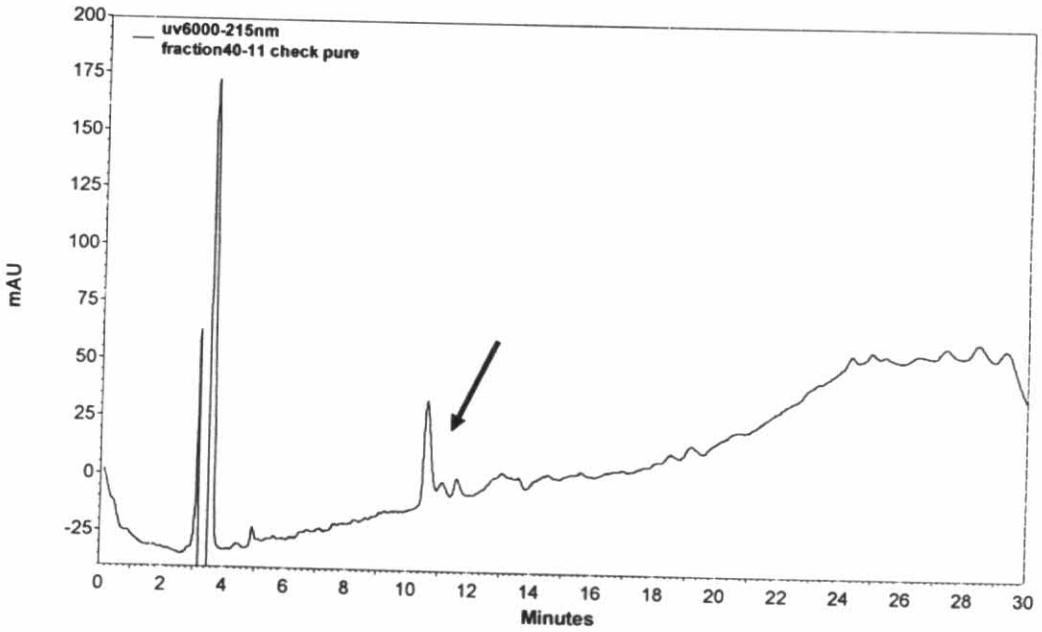


Figure 4.5 RP-HPLC chromatogram of AMP-F40(P) fraction by analytical column with a gradient of 31.5 – 45% ACN containing 0.1% TFA

4.3.5 *Molecular weight determination*

The molecular weight of AMP-F5(P), AMP-F15(P) and AMP-F40(P) was determined by MALDI-TOF mass spectroscopy. The results showed that AMP-F5(P), AMP-F15(P) and AMP-F40(P) were mixed peptides and molecular weight of major peptide in AMP-F5(P) is 2461.797 Daltons, AMP-F15(P) is 8564.595 Daltons and AMP-F40(P) is 2459.779 Daltons (Figure 4.6 to 4.8).

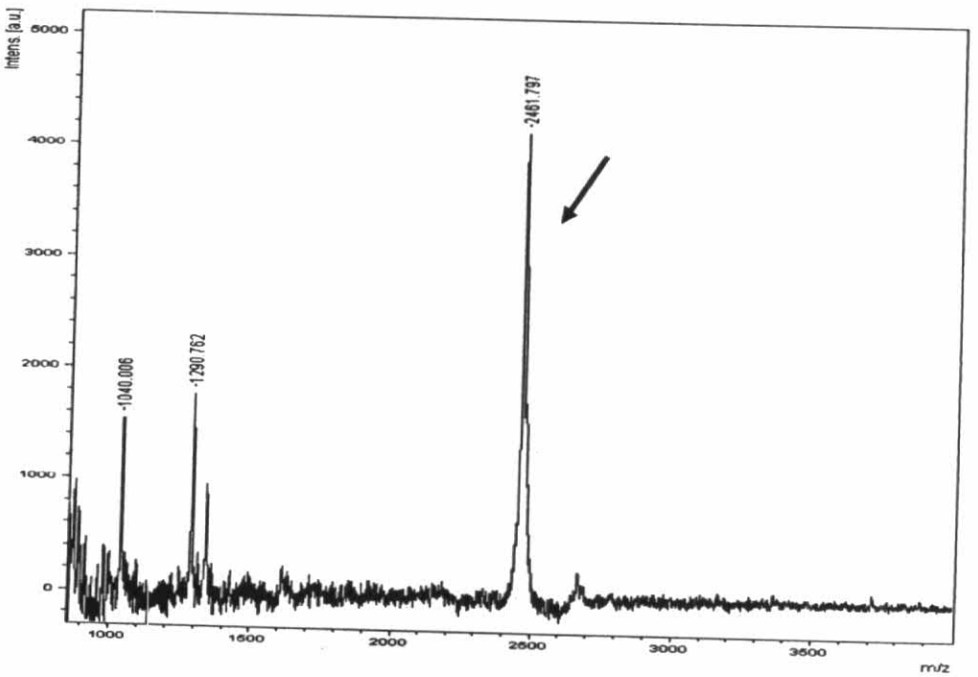


Figure 4.6 MALDI-TOF spectrum of AMP – F5(P)

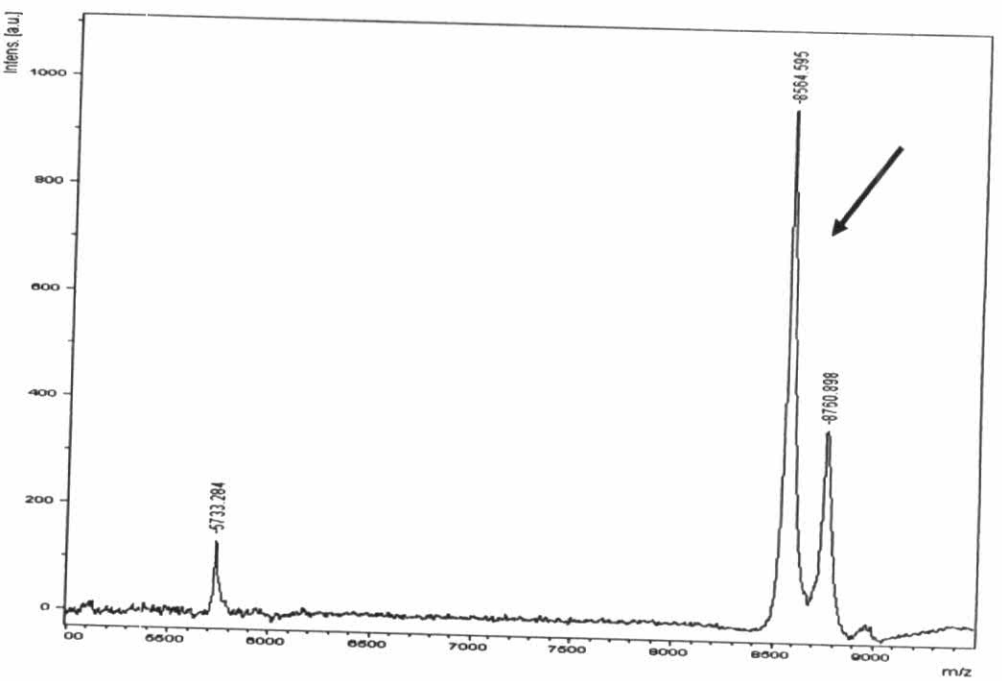


Figure 4.7 MALDI-TOF spectrum of AMP – F15(P)

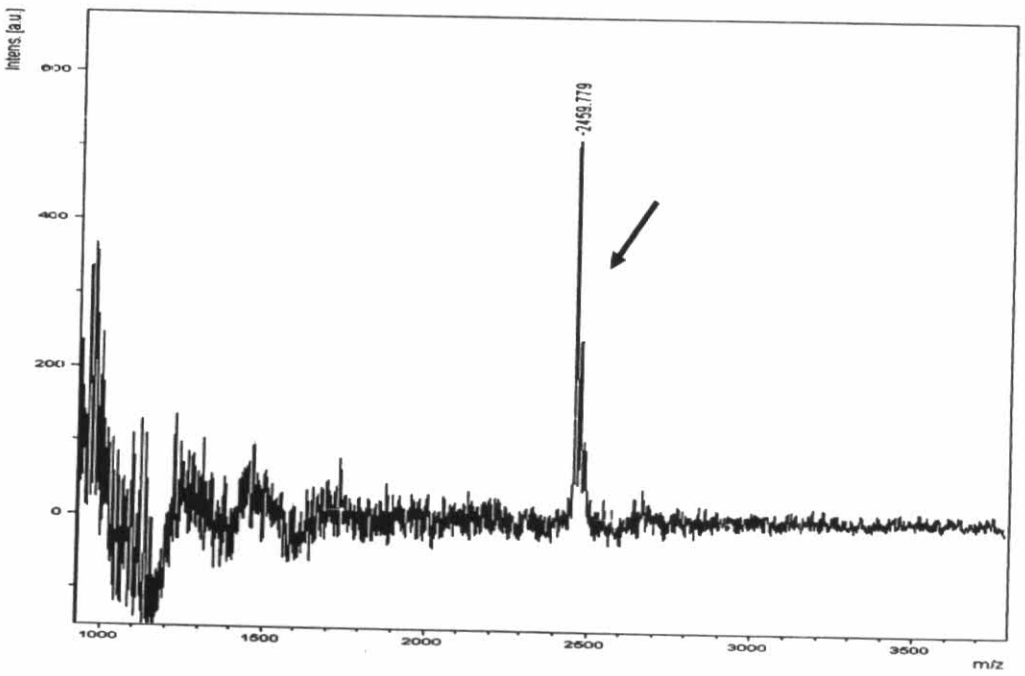


Figure 4.8 MALDI-TOF spectrum of AMP – F40(P)

4.4 Conclusion

Three AMPs were isolated from sandworm *P. nuntia*. AMP-F5(P) showed activity against *B. subtilis* while AMP-F15(P) and AMP-F40(P) were not. The molecular weight of major peptide in AMP-F5(P) is 2461 Dalton. AMP-F5(P) was found to contain 0.26 μg/1 g wet weight of fresh sandworm. The unstable activity of AMP-F15(P) and AMP-F40(P) may come from the very low concentration and/or loss of important molecule during purification. The AMP-F5(P) is apparently a worthy of further investigations due to its constant activity.