CHAPTER IV

RESULTS AND DISCUSSION

4.1 Determination of renieramycin M and jorunnamycin A contents in *J. funebris* tissues by HPLC

In this study, the crude protein from *Jorunna funebris* was investigated for the esterase activity involving in renieramycin M hydrolysis reaction. The tested tissue samples were analyzed by HPLC and found to accumulate the two secondary metabolites of interest, renieramycin M and jorunnamycin A. It implied that the enzyme(s) with the activity in converting renieramycin M to jorunnamycin A presented in the selected tissues.

The HPLC chromatograms of both mantle and visceral tissues showed the peaks of jorunnamycin A and renieramycin M. These compounds were initially identified by comparing with the HPLC chromatograms of the references jorunnamycin A and renieramycin M together with their characteristic UV spectra having maximum absorption at 270 nm detected by a Photodiode array detector (Figure 7).

From the results, the accumulations of renieramycin M and jorunnamycin A presented in *J. funebris* tissue samples suggested that these organisms certainly contained the secondary metabolites of interest and suited for next steps of this study.

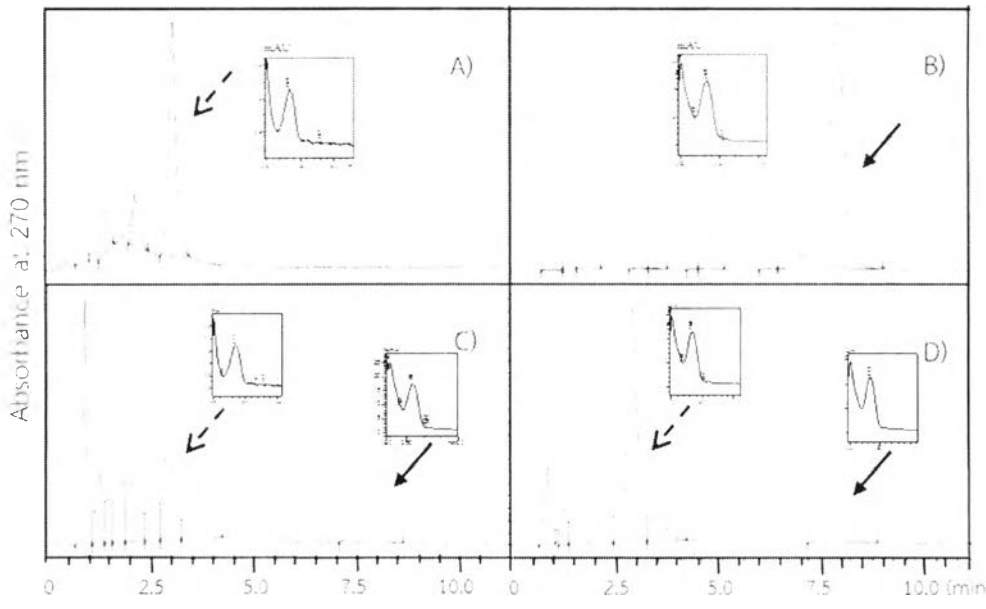


Figure 7. Detection of jorunnamycin A (JA) and renieramycin M (RM) obtained from *J. funebris* tissues. The HPLC chromatograms of **A)** the reference jorunnamycin A, **B)** the reference renieramycin M, **C)** the EtOAc extract of the mantle part of *J. funebris*, and **D)** the EtOAc extract of the visceral part. Dashed arrows represent jorunnamycin A and black arrows represent renieramycin M and inserts are UV spectra.

4.2 Preparation of the crude enzyme extracts

The extraction method for the proteins from the selected specimens followed a previous study with some modifications by using ammonium sulfate precipitation instead of polyethyleneglycol (PEG) for fractionation (MacKenzie *et al.* 2012). Because of using PEG at high concentration may cause the interference with Bradford assay (Andrews *et al.* 1989). The esterase activity for renieramycin M hydrolysis of the crude protein/enzyme extract was determined by analyses of the jorunnamycin A product using HPLC-UV and HPLC-QTOF methods.

4.2.1 Preparation of the crude proteins by ammonium sulfate

The ammonium sulfate precipitation technique was employed due to the fact that this is a low heat solubilization technique leading to prevention of protein

denaturation (Harris and Angal 1989). Since the particular concentration at 70% ammonium sulfate almost reached saturation which allowed precipitation of most proteins (King 1972), it was initially selected to precipitate proteins containing enzyme(s) of interest from both visceral and mantle parts. The results showed that the crude proteins precipitated at 70% ammonium sulfate from the visceral and mantle parts were obtained in 3.24 and 1.40 % w/w of tissue wet weight, respectively.

The obtained crude proteins were further subjected to be determined their catalytic activity for renieramycin M hydrolysis by the HPLC method as mentioned above. The HPLC chromatograms (Figure 8) showed that the jorunnamycin A product was detected only from the reaction mixture catalyzed by the crude protein from the visceral part but not from the mantle tissues.

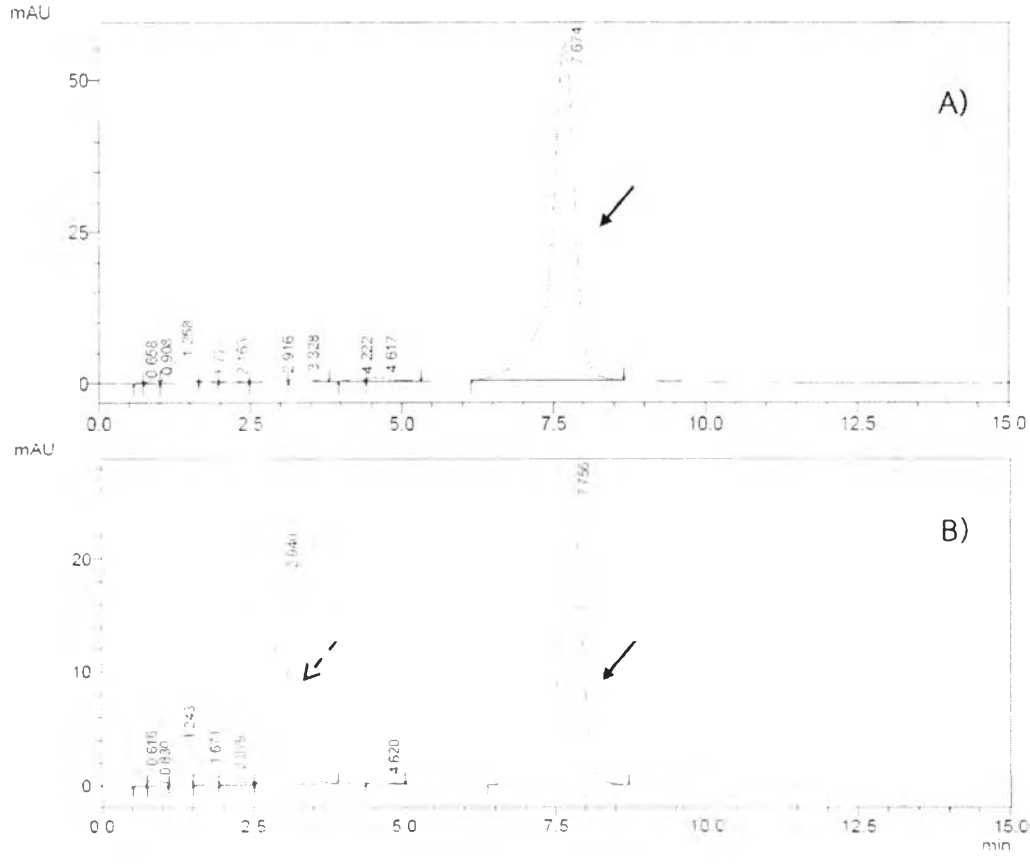


Figure 8. The HPLC chromatograms of the ErOAc extracts from the reaction mixtures catalyzed by the crude enzymes from **A)** mantle part and **B)** visceral part of *J. funebris* precipitated at 0-70% ammonium sulfate concentration. Dashed arrows represent jorunnamycin A and black arrows represent renieramycin M.

The crude protein homogenates from the visceral part *of J. funebris* tissues exhibited the enzymatic renieramycin M hydrolysis in accordance with the detection of the jorunnamycin A peak presenting in the HPLC chromatogram. However, the activity of interest was not found in the crude protein homogenates from the mantle part. According to the previous report of Charupant *et al.* (2007) and the obtained data in this study, jorunnamycin A was found in the mantle tissues. These findings possibly inferred that jorunnamycin A was produced by the enzymatic reaction abundantly occurred in visceral organs including digestive tract and reproductive organs and subsequently transported to the mantle part as being a chemical defense substance.

4.2.2 Optimal ammonium sulfate concentration for crude protein precipitation

Since there was a limitation of starting materials, we tried to achieve the suitable percentage of ammonium sulfate in order to precipitate most proteins exhibiting the high esterase activity for renieramycin M hydrolysis. Total proteins were initially precipitated at 0-70% ammonium sulfate concentration where the activity of interest was detected. Because different proteins are precipitated in different concentrations of ammonium sulfate, fractionation can be used for semi-purification of the proteins according to the difference in their solubility. It was expected that the protein fractions at different percentages of ammonium sulfate concentration would show various degrees of protein concentration and enzyme activity. Crude protein fractionation can be achieved by adding various quantities of ammonium sulfate into protein solutions. In this study, working concentrations of ammonium sulfate ranged from 40 to 100 % with 5% interval were used to stepwise fractionate the crude proteins (Figure 9). After total protein content of each fraction was quantified, the resulting precipitated protein in individual fraction was examined the esterase activity for renieramycin M hydrolysis. The activity was observed by HPLC analysis and calculated for the amount of jorunnamycin A equivalent to renieramycin M using the renieramycin M standard curve. The results are shown in Figure 10.

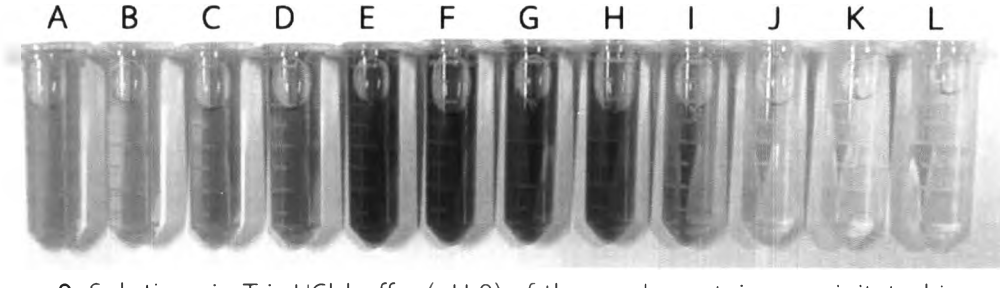


Figure 9. Solutions in Tris-HCl buffer (pH 8) of the crude proteins precipitated in different ammonium sulfate concentrations (40-100%). **A)** 40-45% **B)** 45-50% **C)** 50-55% **D)** 55-60% **E)** 60-65% **F)** 65-70% **G)** 70-75% **H)** 75-80% **I)** 80-85% **J)** 85-90% **K)** 90-95% **L)** 95-100% of ammonium sulfate.

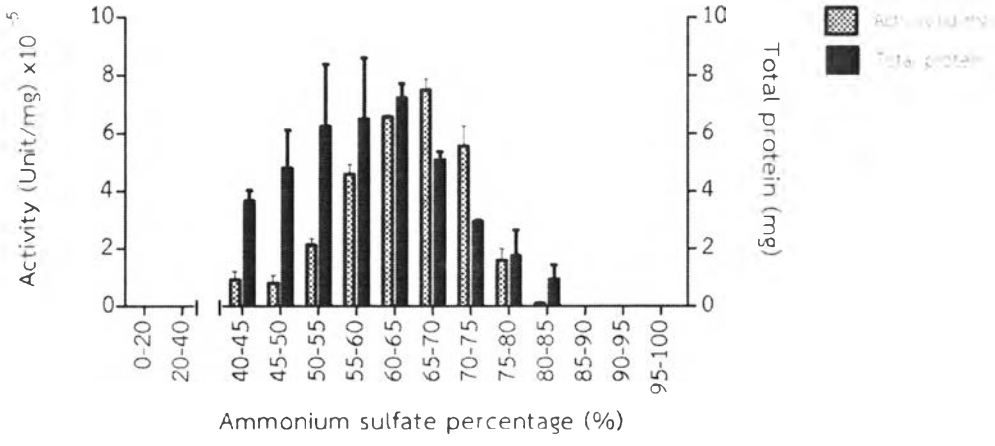


Figure 10. The esterase activity for renieramycin M hydrolysis and total protein content of the partially purified crude enzymes prepared by ammonium sulfate fractionation. Each bar represents the means ± SD (n = 3)

Interestingly, the 65-70% ammonium sulfate fraction showed the highest esterase activity (7.50±0.38 x10^{5} U/mg) whereas the 60-65% ammonium sulfate fraction possessed the largest amount of total protein content (7.24±0.48 mg). It suggested that the 65-70% fraction certainly contained the maximal concentration of highly possible esterase(s) for renieramycin M hydrolysis. Interestingly, the solution colors of this most active fraction and the active fractions nearby were the same blue color as the sponge *Xestospongia* sp. The protein fractions precipitated by 55-75% ammonium sulfate revealed a range of well-detected enzymatic activity (4.6-7.5 x10^{5} U/mg), therefore the ammonium sulfate concentration range of 55-75% was selected to precipitate the desired crude enzyme after 50% ammonium sulfate precipitation for the followings studies.

4.2.3 Identification of jorunnamycin A and renieramycin M by HPLC-QTOF mass spectrometry

As mention earlier, jorunnamycin A and renieramycin M were identified mainly by the HPLC-UV method, we also employed the HPLC-QTOF mass spectrometry to confirm their identity. The accurate masses for the protonated

molecular ions of jorunnamycin A ($C_{26}H_{27}N_3O_7$ +H) and renieramycin M ($C_{31}H_{33}N_3O_8$ +H) was calculated as 494.1922 and 576.2340, respectively. The EtOAc extracts of the reaction mixtures catalyzed by the active crude enzymes were subjected to HPLC-QTOF measurement. After chromatographic process by HPLC and detection by Q-TOF MS, the focused peaks were detected at the retention times of 5.19 minutes and 12.96 minutes with the molecular mass 494.1928 and 576.2355, respectively. (Figure 11)

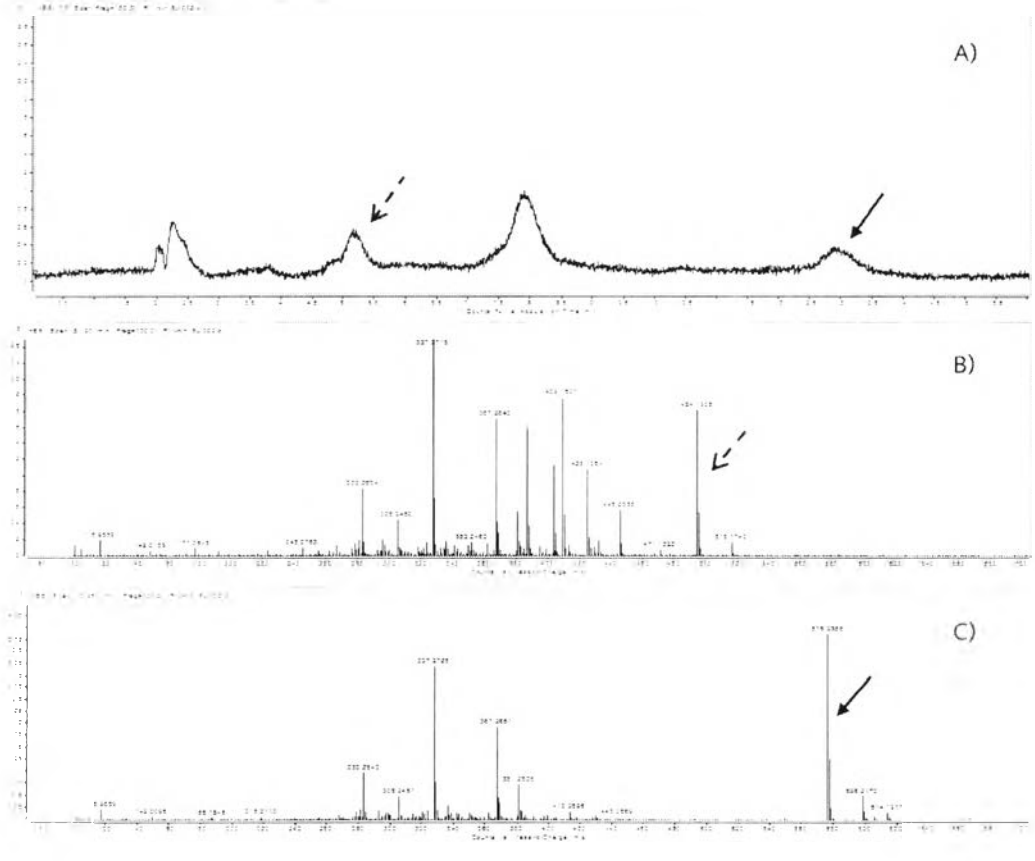


Figure 11. HPLC-QTOF mass measurement for the EtOAc extract of the reaction mixture catalyzed by the crude enzyme precipitated from 55-75% ammonium sulfate. **A)** HPLC chromatogram (the dashed and black arrows show the peak of jorunnamycin A and renieramycin M, respectively), **B)** Mass spectrum of jorunnamycin A peak (5.19 min). The dashed arrow shows the protonated molecular ion at m/z 494, **C)** Mass spectrum of renieramycin M peak (12.96 min). The black arrow shows the protonated molecular ion at m/z 576.

From the data, the peak presented at 5.19 minutes with the protonated molecular ion at m/z 494.1928 certainly confirmed the presence of jorunnamycin A product and the peak presented at 12.96 minutes with the protonated molecular ion at 576.2355 was renieramycin M. There were other two peaks with high intensity presented in the chromatogram. The peak at 2.36 minutes did not showed the same UV spectral pattern as renieramycin M and jorunnamycin A at 270 nm in the HPLC-UV analysis thus this peak was not representing the related compound of interest. The molecular mass data of the peak presented at 7.89 minutes suggested that its molecular formula was supposed to be $C_{24}H_{30}O_6$ which was also not the related compound of interest. These additional two peaks were most likely to be some organic compounds that were probably ionized very well due to their intensity levels in the HPLC chromatogram.

4.2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the crude enzyme extracts

The preliminary experiment to determine esterase(s) converting renieramycin M to jorunnamycin A was carried out by using SDS-PAGE (Figure 12).

From the above results, the esterase activity of the fraction 65-70% ammonium sulfate precipitation exhibited the highest activity while the fractions 55-60%, 60-65%, and 70-75% demonstrated the lower levels of the activity. The correlation between the activity and the intensity of the band specifically occurred only from the proteins with an approximate size of 40 kDa presented in SDS-PAGE. In fact, the general size of esterases has very wide range of molecular weights from 30 kDa to 100 KDa (Bornscheuer 2002; Choresh *et al.* 2004; MacKenzie *et al.* 2012). This result suggested that proteins with the approximate size of 40 kDa in well-detected fractions might be esterase enzyme(s) responsible for the conversion of renieramycin M to jorunnamycin A. The findings may be useful for further study to purify and characterize specific enzyme(s) of interest.

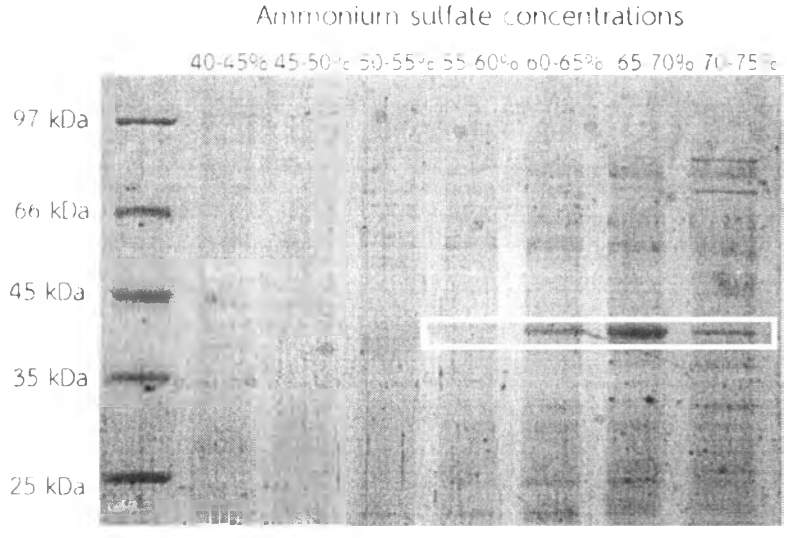


Figure 12. SDS-PAGE of the crude enzymes partially purified by different ammonium sulfate concentrations. Bands of the expected protein with the approximate size of 40 kDa are showed in the white box.

4.3 Determination of parameters of the esterase activity of the crude enzyme extract

The crude enzyme from the visceral part precipitated by 55-75% ammonium sulfate was used to study particular parameters essential for its catalytic activity of renieramycin M hydrolysis including temperature, pH, and buffer. The aim of this study was to achieve the optimal condition for the greatest enzymatic activity.

4.3.1 Determination of time and substrate concentration for the standard protocol of the esterase activity assay

In this study, the standard protocol to detect the esterase activity of the crude enzyme prepared from the visceral part of *J. funebris* was followed the lipase activity assay protocol (McDougall *et al.* 2009) using Tris-HCl buffer (pH 8.0) at 25 °C, was selected as an example to follow. The amount of jorunnamycin A produced from the reaction was monitored every 30 minutes (30, 60, 90, and 120 minutes). Concentrations of renieramycin M including 0.025, 0.05 and 0.1 mM were used. The

crude enzyme final concentration used in each experiment was 2 mg/mL. As expected, either the higher concentration or the longer time of incubation was applied to the experiment, the greater amount of jorunnamycin A product was obtained. It was noted that the esterase activity of the crude enzyme at the concentration 2 mg/mL saturated after 90 minutes of reaction period when 0.025 mM renieramycin M was used as the substrate (Figure 13). However, the amount of jorunnamycin A product still increased when the higher concentrations of renieramycin M at 0.05 and 0.1 mM were used. Concerning the limited amount of available renieramycin M and the sensitivity to detect jorunnamycin A produced from the reaction, the concentration of renieramycin M at 0.05 mM and the incubation time at 90 minutes were selected as the standard protocol for following experiments. This particular condition (2 mg/mL of the crude enzyme in Tris-HCl buffer, pH 8.0, incubation time 90 minutes, temperature 25 °C, and 0.05 mM renieramycin M) was appropriate for this study under researcher's rationale due to available amount of renieramycin M and the obviously detectable peaks of renieramycin M substrate and jorunnamycin A product in HPLC chromatograms within optimal period of time.

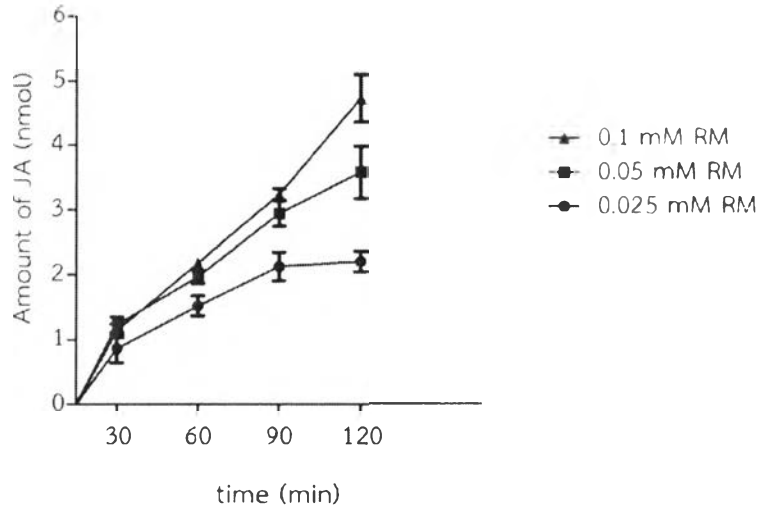


Figure 13. Production of jorunnamycin A (JA) by the crude enzyme from *J. funebris* at different incubation times and renieramycin M (RM) concentrations. The concentration of the crude enzyme was 2 mg/mL. Each bar represents the mean ± SD (n = 3).

4.3.2 The effect of temperature

The enzymatic activity was assayed at temperature ranging from 20 to 60 °C. From the result, the activity was enhanced when the temperature (Figure 14). At 50 °C, the activity initially decreased and dramatically reduced afterward. The maximal activity of the crude enzyme occurred at 45 °C (11.4±0.44 x10^5 U/mg) and lasted in a good level at 50 °C (10.5±0.70 x10^5 U/mg). The activity was significantly decreased after 50 °C and barely detected at 60 °C (0.96±0.11 x10^5 U/mg).

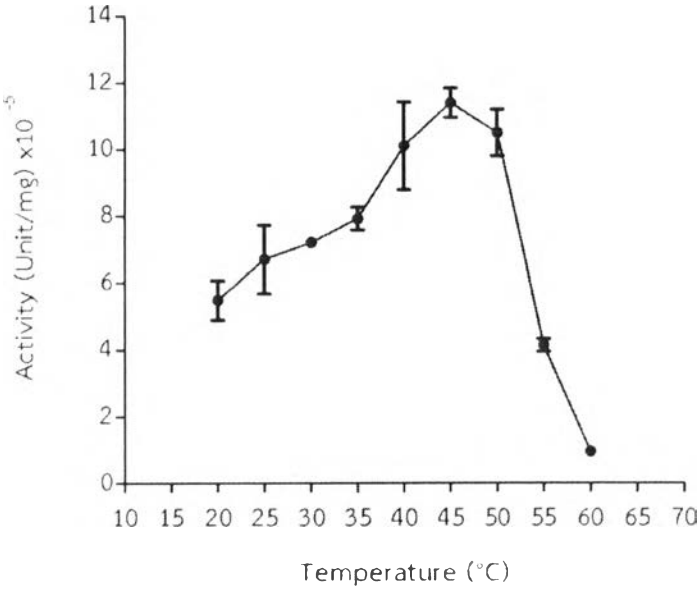


Figure 14. The esterase activity for renieramycin M hydrolysis of the crude enzyme at different temperatures. Each bar represents the mean ± SD (n = 3).

The average activity from the standard condition at 25 °C was at the level of 6.70±1.02 x10^5 U/mg whereas the activity at 45 °C was 11.4±0.44 x10^5 U/mg. Therefore, the crude enzyme was able to work in a wide range of temperature, from 20 °C to 50 °C. It appeared that temperature not more than 50 °C was an adjustable parameter for the enzyme activity to produce jorunnamycin A with properly-required quantity. The stability property under a wide range of temperature is actually beneficial for enzymatic study in laboratory and viable for industrial enzymatic operation in the future

4.3.3 Effect of pH

The effect of pH on the reaction was examined by varying the pH value of Tris-HCl buffer from pH 7 to pH 11.5 with 0.5 interval. The result showed that the activity started to obviously increase at pH 9.5 ($14.0\pm0.33 \times 10^{5}$ U/mg) and exhibited the highest activity at pH 10 ($15.2\pm0.65 \times 10^{5}$ U/mg) (Figure 15). According to the results, the activity of the crude enzyme gradually decreased when the pH value was greater than 10 but it was still active until the pH of 11.5. Although reaction by esterase normally worked best under condition of pH 5.5-8.5 (Fojan *et al.* 2000; Neves Petersen *et al.* 2001), this observation suggested the ability of this crude enzyme to catalyze the reaction under such an extreme basic condition. Indeed, the majority of visceral tissues of *J. funebris* include digestive tract and reproductive organs where the enzymes, especially in intestine, favorably work in such a basic condition (pH 7.5-8) (Cockburn and Reid 1980). Previous study reported that the proteinases isolated from the digestive tract of some marine invertebrates showed the optimal pH in alkaline region (DeVillez and Buschlen 1967; Kozlovskaya and Vaskovsky 1970). Thus, the greatest activity present in basic condition might be due to the property of the crude enzyme previously functioning in visceral organs (Kozlovskaya and Vaskovsky 1970). Nevertheless, the experiment to optimize pH value for renieramycin M hydrolysis in this study was not appropriately designed because the buffering capacity of Tris-HCl did not cover the range of testing pH values. The next experiment would require several buffers exhibiting buffering capacity which covers all tested pHs.

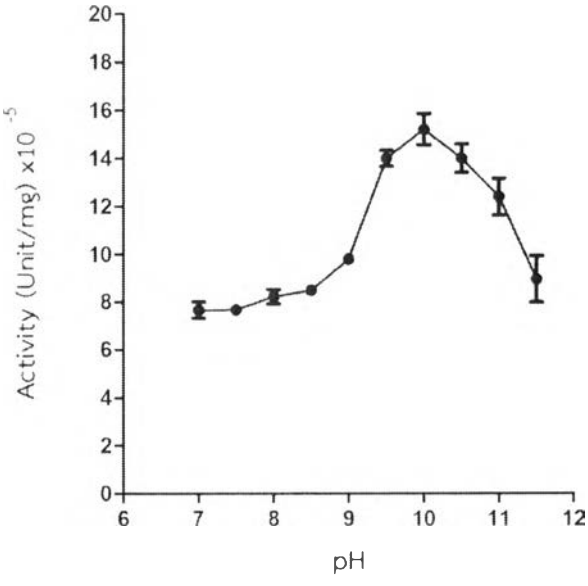


Figure 15. The esterase activity for renieramycin M hydrolysis of the crude enzyme in different pH values. Each bar represents the mean ± SD (n = 3).

Since the highest activity was observed at high pH value, it was important to verify whether jorunnamycin A was produced from the enzymatic reaction but not from the general alkaline hydrolysis. The experiment was carried out by comparing the reaction mixture (50 mM Tris-HCl, pH 10.0 incubated with 0.05 mM renieramycin M substrate) with and without the addition of the crude enzyme. The HPLC chromatogram of the reaction without the crude enzyme showed the absence of jorunnamycin A while the reaction with the crude enzyme showed the greatly produced jorunnamycin A. It was confirmed that the jorunnamycin A product detected from the reaction under the condition of pH 10.0 was in fact from the enzymatic reaction (Figure 16).

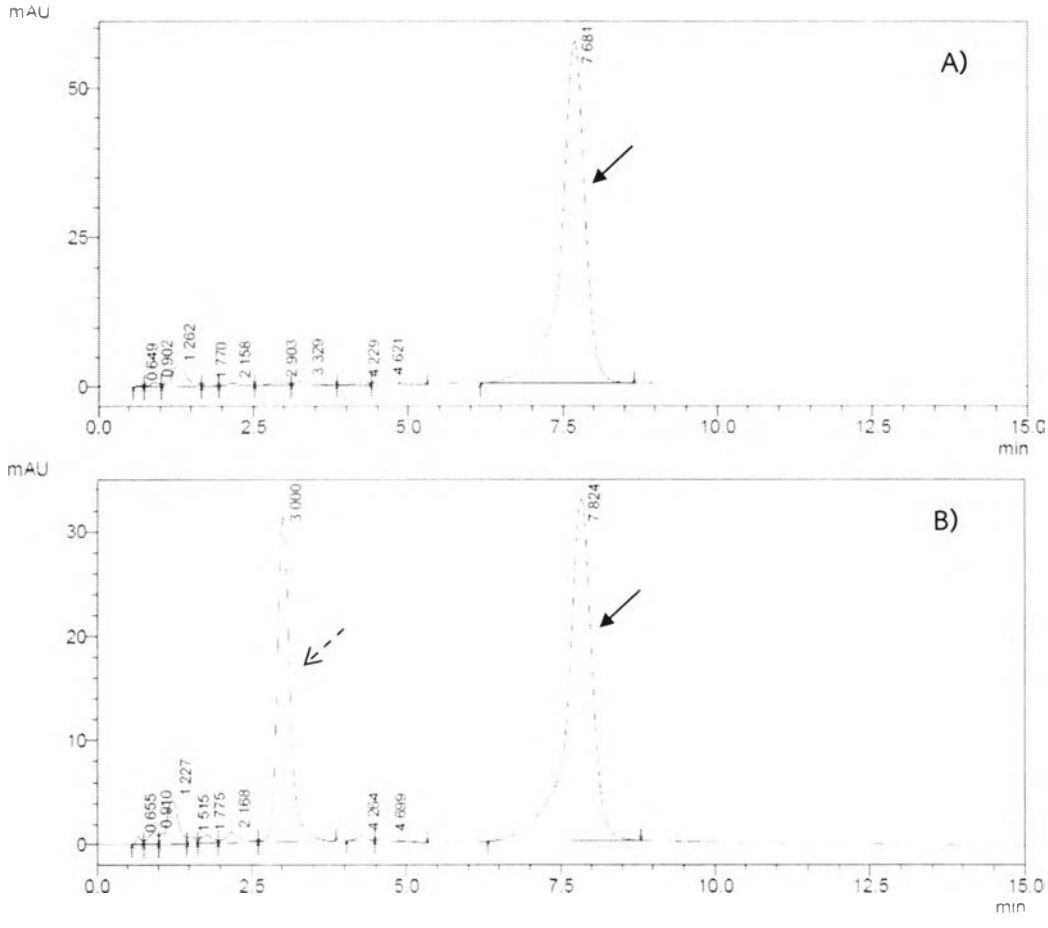


Figure 16. The HPLC chromatograms of the EtOAc extracts from the reaction mixtures under the condition of pH 10.0 **A)** The reaction mixture without the crude enzyme. **B)** The reaction mixture with the crude enzyme. Dashed arrows represent jorunnamycin A and black arrows represent renieramycin M.

4.3.4 Effect of buffer

The effect of different buffers was investigated to determine whether there was a variation in activity when different buffers at the same pH were used in the reaction. From the literature review, esterases commonly work at pH 5.5-8.5 as mentioned before (Fojan *et al.* 2000; Neves Petersen *et al.* 2001). Three buffers at pH 8.0 including Tris-HCl, Tricine, and phosphate buffer solution (PBS) were used for this experiment. The highest activity at 25.2±1.53 x10^5 U/mg was from the reaction with Tricine buffer. The reactions with Tris-HCl and PBS buffers exhibited similar levels of

the activity at 8.86±1.50 x10^{5} U/mg and 11.6±0.11 x10^{5} U/mg, respectively. A comparison of examined activities was shown in Figure 17.

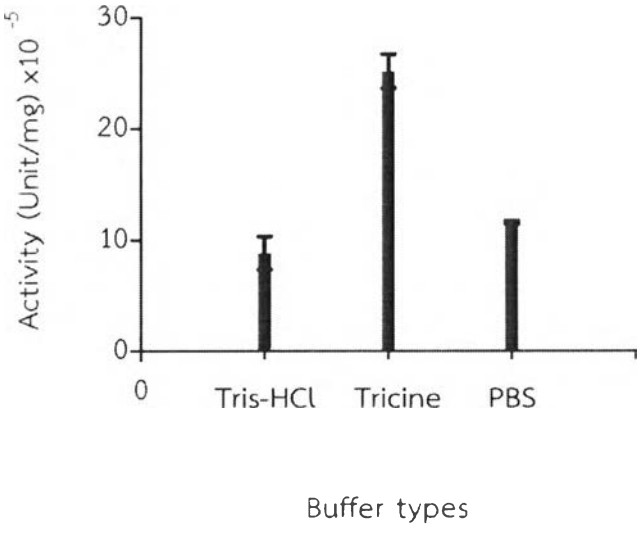


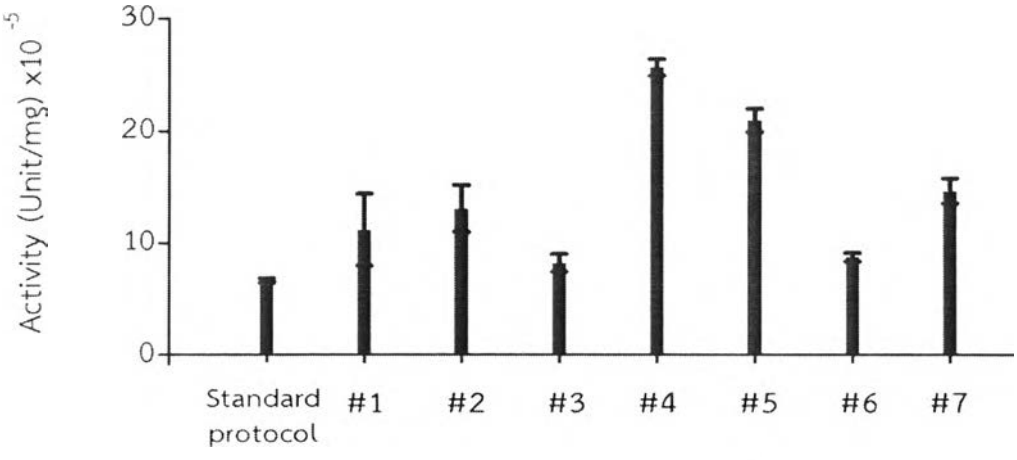
Figure 17. The esterase activity for renieramycin M hydrolysis of the crude enzyme in different buffers.

These three buffers effectively maintain the same buffering range which is about pH 7-9 (Bates *et al.* 1973) and available in the laboratory. Thus, these buffers at pH 8.0 were considered to be suitable for the assay. The activity from the reaction containing Tris-HCl buffer and PBS showed the comparable levels of the activity while the reaction with Tricine presented the significantly higher level of the activity. Tris-HCl and PBS are common buffers used in many experiments to study proteins from marine organisms (Metcalf *et al.* 1972; Bocquené *et al.* 1990; Montella *et al.* 2012) because they are commercially available, inexpensive, freely soluble in water and inert in many enzymatic systems (Good *et al.* 1966). Tricine is moderately soluble in water and normally used as a buffer in tissue culture media (Gardner 1969; Spendlove *et al.* 1971) and gel electrophoresis (Schägger and Von Jagow 1987). It is not completely explicable why Tricine buffer enhanced the activity of the crude enzyme in converting renieramycin M to jorunnamycin A. Due to the fact that Tricine

has higher negative charges than Tris and phosphate (in PBS) (Schägger and Von Jagow 1987), this might be one of the factors to promote the renieramycin M hydrolysis reaction. Further study to illustrate this phenomenon should be performed.

4.3.5 Combination of the optimal parameters used in renieramycin M hydrolysis reaction

From the previous results, every parameters including temperature, pH and buffer type affected the crude enzyme activity. Individual parameter which yielded the best activities was serially combined to give a superlative reaction. The condition #7 which was the combination of the best parameters (50 mM Tricine buffer, pH 10.0, at 45 °C) was expected to yield the highest activity. The results showed that all modified conditions exhibited higher esterase activity than the standard protocol. Surprisingly, the best condition for renieramycin M hydrolysis by the visceral crude enzyme of *J. funebris* was condition #4 (50 mM Tricine buffer, pH 8.0 at 25 °C) which exhibited the greatest activity at 25.7±0.74 x10^{5} U/mg or 3-fold higher activity than the standard protocol (Figure 18).



Buffer	Tris-HCl				Tricine			
pH	8		10		8		10	
Temperature (°C)	25	45	25	45	25	45	25	45

Figure 18. The esterase activity of the crude enzyme from visceral part of *J. funebris* for renieramycin M hydrolysis under various conditions. Each bar represents the mean ± SD (n = 3).

From overall results, all conditions containing Tricine buffer except condition #6 generally showed the enzyme activity superior to the comparable conditions containing Tris-HCl buffer. Considering the conditions containing Tris-HCl, the enzyme activity did not significantly differ under the given different pH values and temperatures. The conditions with Tricine buffer (#4, #5 and #7) appeared to give obviously elevated levels of the activity as compared to the standard protocol.

The activities from the conditions with the same buffer and pH value but different temperatures, e.g. the standard protocol and condition #1, were at well detectable levels with slight difference. This observation inferred that the crude enzyme was active in a wide range of temperatures (25 or 45 °C). This property of the crude enzyme might be beneficial for further investigation because there will be more options for available scientific equipment and suitable accessibilities of necessary items.

When compared the yield of jorunnamycin A from two different reactions, the chemical method gave 45-50% yield of jorunnamycin A by the 3-step reactions as previously mentioned (Charupant *et al.* 2009) whereas the enzymatic reaction under the standard protocol gave approximately 23% yield. Remarkably, condition #4, which exhibited the greatest activity, produced jorunnamycin A nearly 90% yield (Table 4).

Table 4. Percentage yield of produced jorunnamycin A from the reaction under various conditions.

Assay condition	Parameters			Average yield Percentage ± SD
	Buffer type	pH	Temperature (°C)	
Standard protocol	50 mM Tris-HCl	8.0	25	23.1±0.69
#1	50 mM Tris-HCl	8.0	45	38.8±11.2
#2	50 mM Tris-HCl	10.0	25	45.3±7.24
#3	50 mM Tris-HCl	10.0	45	28.5±2.74
#4	50 mM Tricine	8.0	25	88.8±2.55
#5	50 mM Tricine	8.0	45	72.5±3.56
#6	50 mM Tricine	10.0	25	30.3±1.36
#7	50 mM Tricine	10.0	45	50.7±3.81

The enzymatic reaction under the standard protocol gave approximately 23% yield which was less than the chemical method. An explanation for this evidence is that the crude enzyme had not yet reached the saturation stage under the standard protocol at the renieramycin M concentration of 0.05 mM and 90-minute incubation. It is highly expected that jorunnamycin A will be exceedingly produced until desirable amount of jorunnamycin A product is reached under the longer incubation time.

Although the standard enzymatic condition yielded less amount of jorunnamycin A product than the chemical method, the increment of the yield was possible by changing the condition either pH or temperature. For conditions #1 (50

mM Tris-HCl, pH 8, 45 °C) and #2 (50 mM Tris-HCl, pH 10.0, 25 °C) gave approximately 46% and 50% yields, respectively. These two conditions produced jorunnamycin A with the amount nearly equal to the yield from the chemical reaction. Alternatively, conditions #4 and #5 which contained Tricine buffer at pH 8.0 were in the optimal pH range (pH 7-9) of Tricine buffering capacity and yet gave high yields of jorunnamycin A at 88.8% and 72.5%, respectively. Tricine cost is slightly more expensive than Tris-HCl but it may produce jorunnamycin A with more cost-effectiveness. Thus, Tricine may be the suitable buffer for the enzymatic reaction. Besides, if there is no limitation of chemical reagent accessibility, condition #4 (50 mM Tricine, pH 8.0, 25 °C) is possibly the best condition to produce jorunnamycin A under restricted time.

Additionally, the data from this study showed that the renieramycin M hydrolysis by the crude enzyme from *J. funebris* is an environment-friendly reaction in term of green chemistry by less usage of toxic chemicals and reduction in number of steps of the chemical synthesis for renieramycin M derivatives.

4.3.6 Stability of the crude enzyme

For the study of enzyme stability during storage in various temperatures, the intact tissues of organisms were stored in -80 °C before extraction. In this study, five batches of tissues were collected at different times (Table 5). The activity of the crude enzyme under the standard protocol from each batch was determined at the same time in July 2013. The most recent batch (#5 collected in June 2013) exhibited the highest activity ($7.11 \pm 0.10 \times 10^{5}$ U/mg) as expected. The activity of the crude enzyme was steadily decreased over the storage time of the animal tissues.

Table 5. The collection time and activity of five tissue sample batches.

Tissue sample batch	Collection time (Month/Year)	Storage time (months)	Average activity ± SD (U/mg) x10^5
#1	October/2011	21	2.94±0.26
#2	May/2012	14	3.70±0.10
#3	November/2012	8	4.61±0.03
#4	January/2013	6	5.06±0.33
#5	June/2013	1	7.11±0.10

The tissue samples from batch #5 (June/2013) were selected for investigating the stability of the crude enzyme after extraction and storage in Tris-HCl buffer solution. The crude enzyme solutions were stored at 3 different temperatures (4 °C, -20 °C and -80 °C) and the activity was consequently determined. The examination of individual sample was performed every two months. The results showed that the activity of the crude enzyme which was stored at 4 °C considerably reduced to the level of 56.9% and 42.3% after two and four months of storage, correspondingly (Table 6). It is expected that the activity of the crude enzyme stored at 4 °C would be eventually eradicated if the crude enzyme was still kept at this temperature. The crude enzyme stored at -20 °C exhibited a slight reduction in activity to the level of 85.0% and decreased to the level of 73.9% within four months. Interestingly, the activity of the crude enzyme stored at -80 °C exhibited high activity and remained the activity almost at the same level within four months (Figure 19).

Table 6. Activity and relative activity (%) of the crude enzyme in Tris-HCl buffer solution stored at different temperatures.

temp / Months	4 °C		-20 °C		-80 °C	
	Average activity ±SD (U/mg) x10^{5}	Relative activity (%)	Average activity ±SD (U/mg) x10^{5}	Relative activity (%)	Average activity ±SD (U/mg) x10^{5}	Relative activity (%)
0	5.31±0.40	100±7.60	5.47±0.32	100±5.78	5.30±0.37	100±6.91
2	3.03±0.10	56.9±1.68	4.65±0.29	85.0±5.28	5.16±0.36	93.8±0.84
4	2.25±0.10	42.3±1.69	4.04±0.23	73.9±4.26	4.92±0.08	92.8±1.51

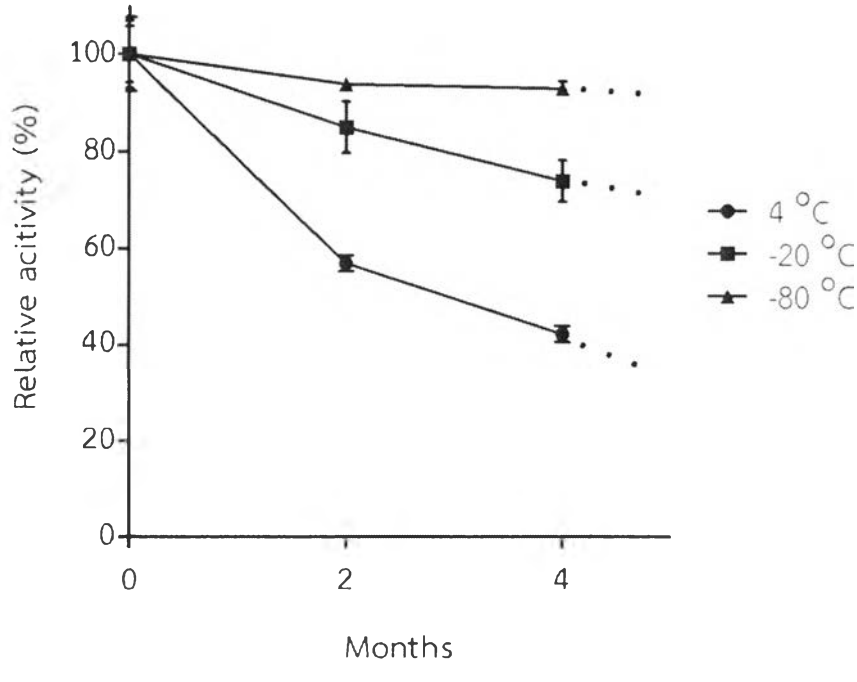


Figure 19. The relative activity of the crude enzyme in Tris-HCl buffer solution stored at different temperatures and storage times. Each bar represents the mean ± SD (n = 3).

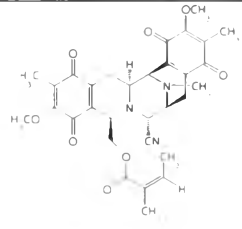
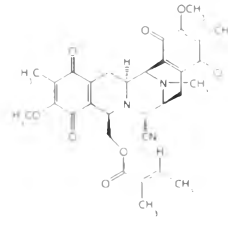
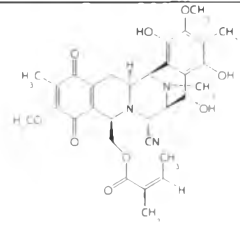
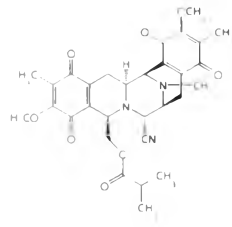
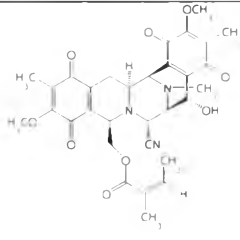
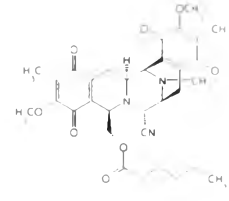
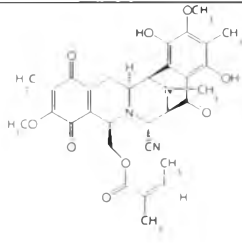
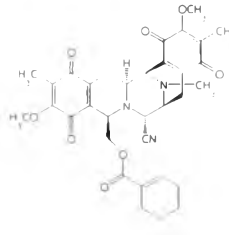
To summarize, it can be inferred that the storage temperature at -80 °C is suitable for the crude enzyme solution. In addition, the temperature at -20 °C is an alternative to store the enzyme solution with remaining good activity at least for 4

months. The temperature at 4 °C should not be used for enzyme storage because of the dramatically decreased activity of the enzyme overtime.

4.4 Substrates specificity

The experiment was aimed to determine whether the crude enzyme was able to convert renieramycin M related compounds to their corresponding products. The 55-75% ammonium sulfate precipitation of the crude enzyme extract from the visceral part of *J. funebris* was used for studying substrate specificity. The renieramycin series e.g. renieramycin M (RM), renieramycin N (RN), renieramycin Q (RQ) and renieramycin O (RO) and four synthesized renieramycin M derivatives (**1**-**4**) from the previous study (Charupant *et al.* 2009) were used in this experiment. All of selected compounds share the *bis*-tetrahydroisoquinoline core structure. Compared to renieramycin M structure, the renieramycins series, RN, RO and RQ, contain the same substitution but different substitutions at various positions of the core structure whereas the synthesized renieramycin M derivatives (**1**-**4**) share the same core structure but different 22-*O*-acyl substitutions. The crude enzyme activity for each different renieramycin substrate was comparatively investigated under the standard protocol (Tris-Hcl buffer, pH8, 25 C, 0.05 mM renieramycin derivative) as shown in Table 7.

Table 7. The esterase activity of the crude enzyme toward various renieramycin substrates.

Compound	Average activity ±SD (U/mg) x10^{-1}	structure	Compound	Average activity ±SD (U/mg) x10^{-1}	structure
RM	6.70±0.40		**1**	0.31±0.03	
RN	ND		**2**	37.4±0.33	
RO	ND		**3**	5.03±0.21	
RQ	ND		**4**	ND	

*ND; Not detactable

Surprisingly, among four renieramycins sharing the same 22-*O*-angeolyl ester side chain (renieramycins M, N, O, and Q), renieramycin M was the only substrate for the crude enzyme from *J. funebris* to catalyze the 22-*O*-angeloyl hydrolysis. The

corresponding deangeolyl renieramycin products were completely not produced from the reaction mixtures using renieramycins N, O, and Q as substrates. The results suggested that the esterase enzyme presenting in the crude protein from the nudibranch is highly specific to renieramycin M core structure. The core structures of renieramycins M and O are chemically defined as the fused two tetrahydroquinolinequinone moieties while those of renieramycins O and Q consist of a tetrahydroquinonlinequinone fused with a tetrahydroisoquinolinehydroquinone. In addition, renieramycins N, O, and Q share the oxygenated substituents (either a carbonyl or a hydroxyl) at C-14. With limited numbers of renieramycin derivatives, the esterase activity-substrate core structure relationships could be drawn as the follows: a) the quinone moiety at the east part of the core structure is essentially required for the esterase activity and b) the oxygenated substituents at C-14 completely irradiate the esterase activity. This implied that the active size of the enzyme pocket might be very specific to the structure of renieramycin M. The position C-15 of renieramycin M is a free quinone carbonyl group whereas that of renieramycins N, O, and Q is able to generate a hydrogen bond with the oxygenated substituents at C-14. This change may affect the binding of the substrate to the binding site within the enzyme pocket.

We also paid our attention to investigate the 22-*O*-acyl specificity to the esterase enzyme. Four renieramycin M derivatives (**1**-**4**) with different 22-*O*-acyl moieties were selected as substrates for the crude enzyme from *J. funebris*. The reaction containing compound **1** as a substrate showed a small peak overlapping other peak(s) and the small peak possibly corresponded to jorunnamycin A The esterase activity for compound **1** was determined as $0.31\pm0.03 \times 10^{5}$ U/mg. Alternatively, compounds **2** and **3** were converted to jorunnamycin A by the enzymatic hydrolysis at the activity levels of $37.4\pm0.33 \times 10^{5}$ U/mg and $5.03\pm0.21 \times 10^{5}$ U/mg, respectively. The hydrolysis activity of the crude enzyme for compound **4** was not detected.

The results of the 22-*O*-acyl derivatives (**1**-**4**) hydrolysis indicated that the conjugated double bond at C-25 was not important for the activity since **2** and **3** were 22-*O*-aliphatic acyl renieramycin M derivatives. In addition, it is noticeable that

the enzyme activity would not be active if the C-25 double bond was *cis*- (**1** and **4**). In contrast, the appreciated level of enzyme activity was detected when the double bond at C-25 was *trans*- as in renieramycin M. In some studies, the stereochemistry of the substrate affected to the enzyme activity (Jao and Casida 1974; Soderlund *et al.* 1982). Lastly, the crude enzyme was likely to be more specific to the short acyl aliphatic chain than the long chain according to the greatly improved hydrolysis activity for **2** as compared to the activity for **3** and renieramycin M. The size of the substituent influenced the ability of the enzyme at the binding site to react with the substrate. The number of carbons in the substituent group is critical. If the smaller group than angeloyl group (5 carbon atoms) was substituted, the better esterase activity was found. The bigger substituent group than angeloyl group was possibly not well fit to the enzyme pocket, resulting in low or undetectable activity as expected.

Furthermore, an experiment was performed to investigate whether renieramycin M was able to be converted to jorunnamycin A by other enzymes such as lipase for ester hydrolysis. Since lipase is the enzyme commonly found in most organisms (Schmid and Verger 1998) and able to catalyze various types of substrates (Schmidt and Bornscheuer 2005), it was utilized for this investigation. From the preliminary screening, jorunnamycin A was not found in the reaction composing renieramycin M substrate and lipase as shown by the HPLC chromatogram (Figure 20). This finding suggested that general enzyme lipases were unable to catalyze the renieramycin M hydrolysis reaction and a specific esterase enzyme was required for the conversion of renieramycin M to jorunnamycin A. Besides, it is inferred that there may be an evolution of the esterase enzyme from the general one which is restricted to the renieramycin M hydrolysis. The renieramycin M-specific esterase might only exist in the nudibranch *J. funebris* which usually feed on the renieramycin-producing sponge *Xestospongia* sp. Renieramycin M or the corresponding renieramycin E was the main constituents presenting in extremely high quantity in the sponge tissues. The nudibranch might utilize the specific esterase enzyme to convert the highly toxic renieramycin to the less toxic one.

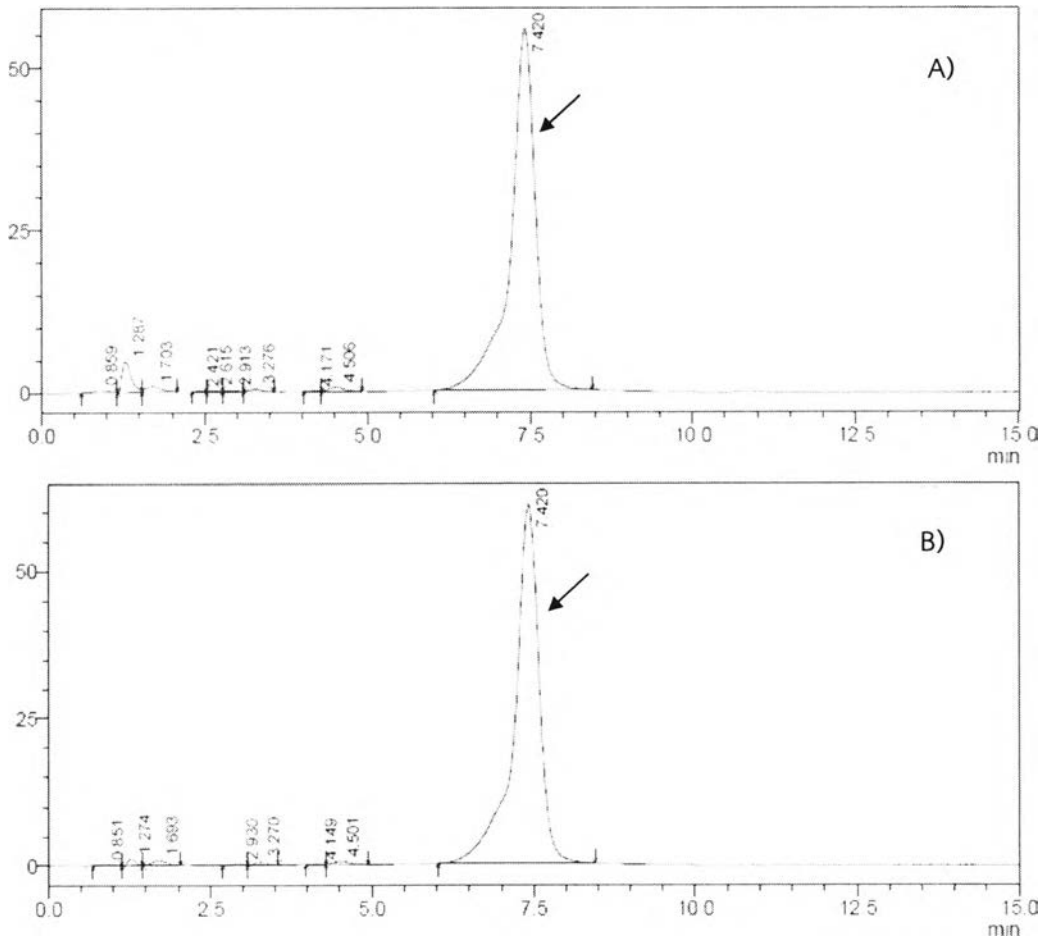


Figure 20. The HPLC chromatograms of EtOAc extracts from the reaction mixtures catalyzed by the crude enzymes in the **A)** absence and **B)** presence of lipase. Black arrows represent the peak of renieramycin M.