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DETECTION OF ESTERASE ACTIVITY CONVERTING CYTOTOXIC RENIERAMYCIN M TO JORUNNAMYCIN A IN THE CRUDE ENZYME OF THE NUDIBRANCH *JORUNNA FUNEBRIS*

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INTRODUCTION

JorunnamycinA (JA) is a renieramycin member of the marine tetrahydroisoquinolinequinone alkaloids. JA has been recently used as an important starting material to prepare various 22-*O*-acyl derivatives of renieramycin M (RM) for improving its potent cytotoxicity against cancer cell lines as well as studying structure-cytotoxicity relationships.[1] RM is a major cytotoxic component, previously isolated from a Thai blue sponge *Xestospongia* sp. pretreated with potassium cyanide.[2] To chemically synthesize JA, the angeloyl group at C-22 of RM must be removed by cleavage of the ester bond to yield a free alcohol moiety. However, the routine acid and alkaline hydrolysis reactions were not successful to cleave this ester bond. The chemical transformation was performed by the 3-step reaction involving hydrogenation, hydride reduction and oxidation as shown in Figure 1.[1] Interestingly, JA was recently isolated in a good yield from a marine nudibranch *Jorunna funebris* pretreated with potassium cyanide.[3] This shell-shed gastropod mollusk is a sponge-eating animal, which specifically feeds on the sponge *Xestospongia* and accumulates RM and JA in both visceral part and mantle. The absence of JA in the sponge *Xestospongia*, therefore, led to a hypothesis that the reaction catalyzed by specific enzyme(s) in the nudibranch tissue might be involved in the conversion of RM to JA. Considering the structures of these two related alkaloids, the enzyme involved in the hydrolysis of the ester bond at C-22 is possibly an esterase type. In this communication, our preliminary study aiming to clarify esterase activity in the crude protein extract prepared from *J. funebris* is designed to support the hypothesis. The discovery of this specific enzyme activity will be an important step for the future development to utilize the specific esterase enzyme in replacing harmful reagents for such specific chemical reactions.

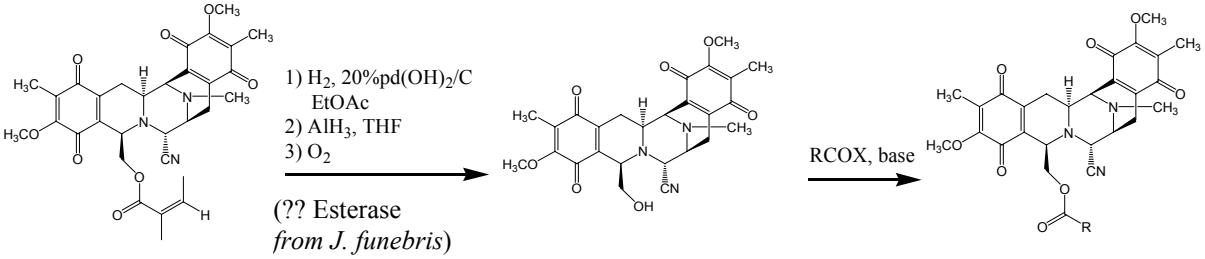

Figure 1 Steps in preparation of 22-*O*-acyl renieramycin M derivatives.

MATERIALS AND METHODS

Materials The nudibranch *J. funebris* samples were collected in 2012 at Sichang islands, Chonburi, Thailand. The samples were stored at -80°C until they were used for analysis of RM/JA contents and preparation of crude enzyme extracts.

Determination of RM/JA contents in J. funebris tissues *J. funebris* samples were dissected to separate the visceral and mantle parts. A pool of each part was ground into homogeneous paste under liquid nitrogen. Each part (500 mg) was accurately weighed and pretreated with 500 µL of 10 mM KCN in 50 mM phosphate buffer (pH 7.0) for 5 h. The sample suspension was macerated with 2 mL of methanol for 24 h and subsequently adjusted to 5 mL by brine solution. The resulting mixture was further partitioned with 5 mL of EtOAc. The EtOAc layer (1 mL) was evaporated to dryness and re-dissolved with 1 mL methanol to yield the EtOAc extract. The EtOAc extract was subjected to HPLC (Shimadzu SPD-M20A SIL-20A, Japan) using a LiChrospher 100 RP-18 HPLC column (4 x 125 mm, 5 µm), 20 µL of sample

volume, 70% MeOH in water as a mobile phase at a flow rate of 1.0 mL/min, detection with a UV detector (Shimadzu,SDD-M10A PDA) at λ 270 nm. Retention times and UV spectra of the HPLC chromatographic peaks responsible for RM and JA presenting in the EtOAc extract were compared with those of the RM and JA reference standards.

Crude protein preparation Each visceral and mantle part of *J. funebris* samples was pooled and accurately weighed for 1 g. Each part was homogenized with 4.0 mL of 50 mM Tris-HCl buffer (pH 8.0) then centrifuged at 5,000g 4°C for 15 min to remove cell debris. The supernatant was added by ammonium sulfate to bring up salt concentration to 70% and stirred at 4°C for 20 min. The mixture was centrifuged at 15,000g, 4°C for 20 min. The pellet obtained (the 0-70% fraction) was re-dissolved in 2 mL of 50mM Tris-HCl buffer (pH 8.0) and stored at -80°C. Desalting process of the crude protein by a PD-10 column (8.3 mL of Sephadex G-25 Medium, GE healthcare, Sweden) eluted by 50 mM Tris-HCl buffer (pH 8.0) was performed and the crude protein was collected at 3.5-5.5 mL of the eluate.

Partial purification of the crude protein The crude protein from the visceral part of *J. funebris* was partially purified by sequential ammonium sulfate precipitation. The visceral part was treated with the process as mentioned above. The initial pellet of proteins from 20% ammonium sulfate concentration was discarded. The remaining supernatant was sequentially precipitated by higher concentrations (40–100%) of ammonium sulfate with 5% increment interval. The protein pellet from each precipitation was desalted by a PD-10 column prior to the esterase activity assay, as previously described.

Total protein assay Total soluble proteins were quantified by the Bradford assay[4] using Bio-Rad protein assay dye reagent (Bio-Rad product 500-0006) and the standard protocol for 96-well microplates. The absorbance at 595 nm of the standard protein BSA and its corresponding concentrations (0.125–1.0 mg/mL) were plotted as the protein concentration standard curve.

Esterase activity assay of the prepared proteins The assay for esterase activity of the crude proteins and the partially purified proteins was modified from the anti-lipase activity assay.[5] In brief, the reaction was carried out in a 200-µL volume comprising 20 µL of RM (50 µM RM solution in 10% DMSO in MeOH), 0.4 mg of the prepared protein, and 50 mM Tris-HCl buffer (pH 8.0) and incubated at 25°C for 90 min. Then, the reaction mixture was partitioned with200 µL of EtOAc and 100 µL of the EtOAc layer was transferred to evaporate to dryness. The residue was dissolved with 100 µL of MeOH to give the extract for determination of RM/JA contentsby HPLC as previously described. The enzyme activity (U/mg) was calculated from enzyme unit (U) divided by the protein content in each fraction. Enzyme unit (U) was defined as the amount of enzyme required to produce 1 µmol of JA per min.

RESULTS

Determination of RM/JA contents in J. funebris tissues To detect the RM and JA contents in the mantle and the visceral parts of *J. funebris*, the EtOAc extracts from both tissue parts were prepared and subjected to quantitative analysis by HPLC (Figure 2). The retention times in the HPLC chromatograms of the references RM and JA were at 7.77 and 2.90 min, respectively and both compounds showed the similar UV spectra with λ_{max} at 270 nm (Figure 2A and B). The results showed that both RM and JA were presented in the mantle and visceral parts by comparison their HPLC retention times and UV spectra to those of RM and JA reference standards (Figure 2C and D).

Crude protein preparation and partial purification of the crude protein The crude proteins from the mantle and the visceral tissues were prepared by precipitation with 70% ammonium sulfate to obtain 1.4%mg and 3.2%mg w/w proteins of the tissue wet weight, respectively. Partial purification of the crude protein from the visceral part was further prepared through sequential precipitations with different ammonium sulfate concentrations (20–100%) by 5% increasing interval (Figure 3). The proteins were initially precipitated at the 40–45% fraction and completely precipitated at the 80–85% fraction. The 50–55%, 55–60%, and 60–65% fractions significantly yielded higher protein contents than the others, with 0.62%mg - 0.72%mg protein of the visceral part wet weight.

Esterase activity of the protein/enzyme preparations To detect the esterase activity of the prepared proteins, RM was used as the enzyme substrate and JA was the expected final product. HPLC was performed to detect the presence of RM and JA from the reaction mixtures. Preliminary results showed that RM was converted to JA by the proteins only from the visceral part (Figure 4B and C). Sequential precipitation of the visceral proteins by ammonium sulfate showed that the protein of the 65-70% fraction presented the highest esterase activity (Figures 3 and 4D). In addition, the neighboring fractions (55-60%, 60-65%, and 70-75%) also exhibited comparatively high activity. Reduction in the activity of the other fractions was apparently observed (Figure 3).

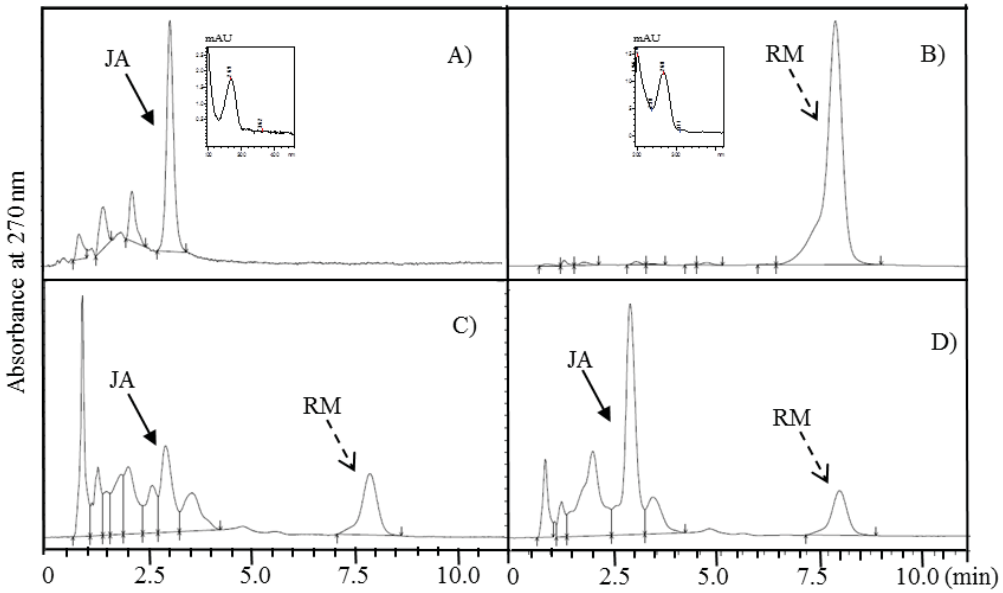

Figure 2 Detection of jorunnamycin A (JA) and renieramycin M (RM) in *J. funebris* tissues. The HPLC chromatograms of A) the reference JA, B) the reference RM, C) the EtOAc extract of the mantle part of *J. funebris*, and D) the EtOAc extract of the visceral part of *J. funebris*. The inserts show the UV spectra of JA and RM

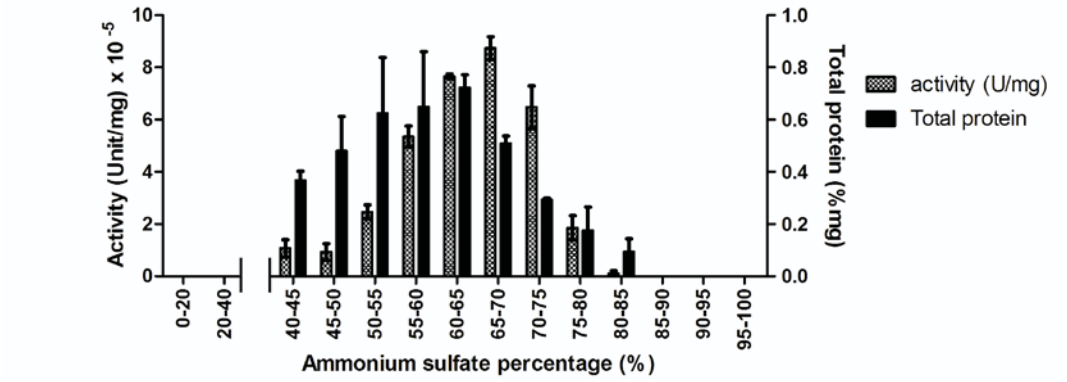

Figure 3 Esterase activity for RM hydrolysis and total protein content of the partially purified crude proteins by sequential ammonium sulfate precipitation.

DISCUSSION

From the results, an accumulation of RM/JA in *J. funebris* body suggested that these organisms certainly contained the secondary metabolites of interest. The conversion of RM to JA was found in the crude and partially purified enzymes from the visceral part of *J. funebris.* Thus, it could be inferred that the esterase enzyme involved in converting RM to JA was extracted into both enzyme preparations. Since there was a limitation of starting materials, we tried to achieve the suitable percentage of ammonium sulfate in our preliminary experiment. Total proteins were 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[6], we expected that the protein fractions at different percentages of ammonium sulfate would show various degrees of activity. Interestingly, the 65-70% fraction showed the highest esterase activity whereas the 60-65% fraction possessed the largest amount of total protein content. It suggested that the 65-70% fraction certainly contained the highest concentration of the esterase for RM hydrolysis. The 55-75% fractions revealed a range of well-detected esterase activity, therefore, the protein fraction precipitated by 55-75% ammonium sulfate was selected for further study. This partially-purified enzyme from the visceral part will be used for further optimization study to investigate effects of different parameters and substrate specificity on its esterase activity. Further studies are required to purify and characterize the enzyme.

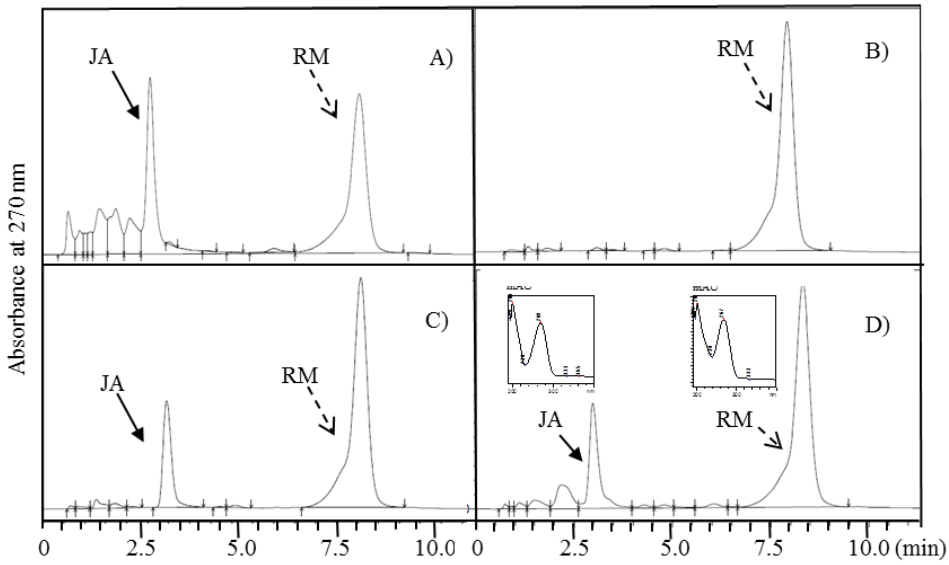

Figure 4 Detection of jorunnamycin A (JA) produced from renieramycin M (RM) by esterase activity of the crude enzymes from *J. funebris* tissues. The HPLC chromatograms of A) a mixture of the references JA and RM, B) and C) the EtOAC extracts of the enzymatic reactions of the mantle and the visceral crude enzymes, respectively, and D) the EtOAC extract of the enzymatic reaction of the 65-70% ammonium sulfate fraction of the visceral crude enzyme. The inserts show the UV spectra of JA and RM.

CONCLUSION

In this study, the esterase activity producing JA from RM of the crude enzyme prepared from the visceral part of the nudibranch *J. Funebris* was revealed. The enzyme activity was demonstrated to catalyze the hydrolysis reaction cleaving the angeloyl moiety of RM to produce JA. Finally, the 55-75% ammonium sulfate was suitable to partially purify the enzyme with the desirable esterase activity after initial precipitation of the visceral proteins with 55% ammonium sulfate.

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