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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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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- μ L volume comprising 20 μ L of RM (50 μ M 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 with 200 μ L of EtOAc and 100 μ L of the EtOAc layer was transferred to evaporate to dryness. The residue was dissolved with 100 μ L of MeOH to give the extract for determination of RM/JA contents by 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 μ mol 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).

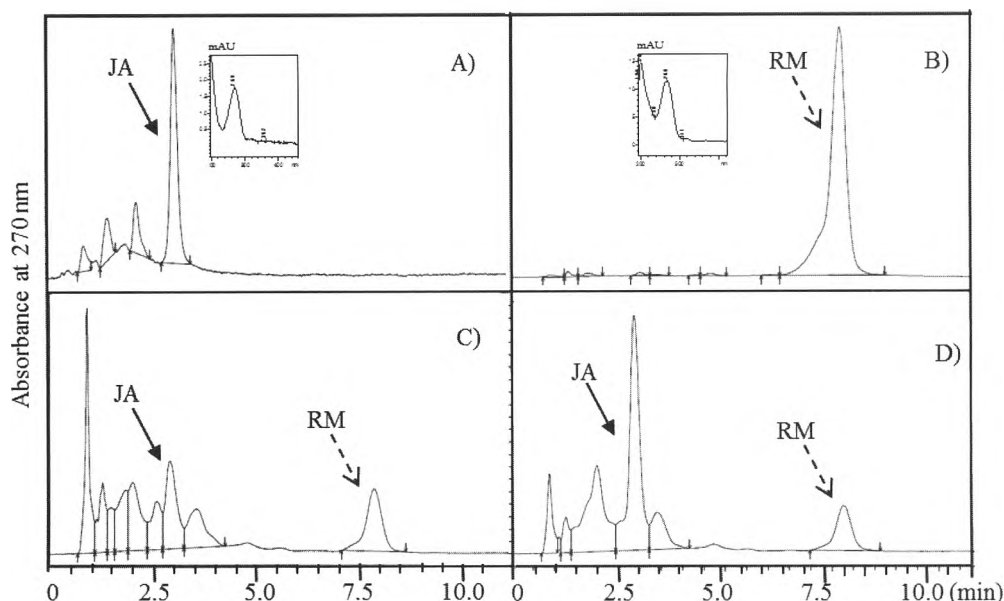


Figure 2 Detection of jorunnamycin A (JA) and renieramycin M (RM) in *J. funebris* tissues. The HPLC chromatograms of A) the reference JA, B) the reference RM, C) the EtOAc extract of the mantle part of *J. funebris*, and D) the EtOAc extract of the visceral part of *J. funebris*. The inserts show the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

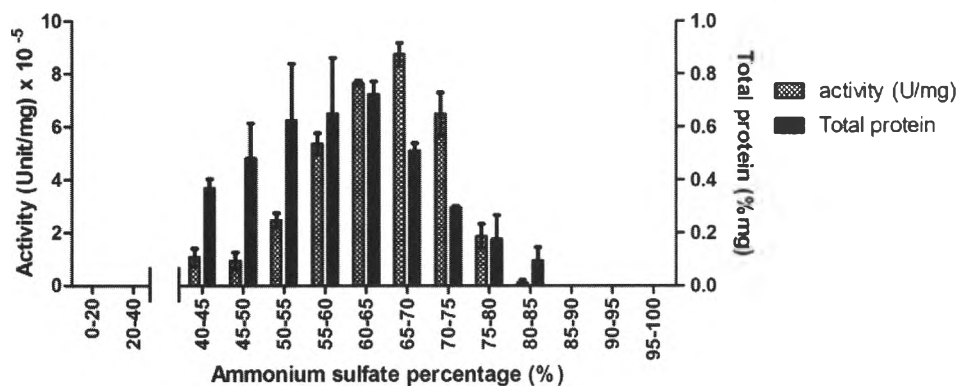


Figure 3 Esterase activity for RM hydrolysis and total protein content of the partially purified crude proteins by sequential ammonium sulfate precipitation.

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.

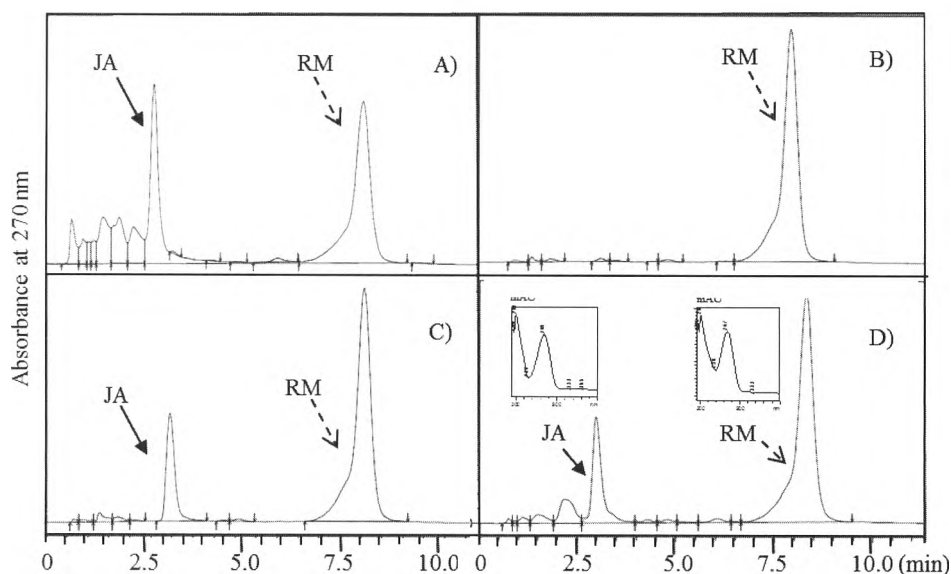


Figure 4 Detection of jorunnamycin A (JA) produced from renieramycin M (RM) by esterase activity of the crude enzymes from *J. funebris* tissues. The HPLC chromatograms of A) a mixture of the references JA and RM, B) and C) the EtOAC extracts of the enzymatic reactions of the mantle and the visceral 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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