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Factors affecting the protease activity of venom from jellyfish *Rhopilema esculentum* Kishinouye

Cuiping Li,[a,b] Huahua Yu,[a] Song Liu,[a] Ronge Xing,[a] Zhanyong Guo[a] and Pengcheng Li[a,*]

[a]*Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China*
[b]*Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*

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Abstract—In this paper, the effects of some chemical and physical factors such as temperature, pH values, glycerol, and divalent metal cations on the protease activity of venom from jellyfish, *Rhopilema esculentum* Kishinouye, were assayed. Protease activity was dependent on temperature and pH values. Zn^{2+}, Mg^{2+}, and Mn^{2+} in sodium phosphate buffer (0.02 M, pH 8.0) could increase protease activity. Mn^{2+} had the best effects among the three metal cations and the effect was about 20 times of that of Zn^{2+} or Mg^{2+} and its maximal protease activity was 2.3×10^5 U/mL. EDTA could increase protease activity. PMSF had hardly affected protease activity. *O*-Phenanthroline and glycerol played an important part in inhibiting protease activity and their maximal inhibiting rates were 87.5% and 82.1%, respectively.

The jellyfish, *Rhopilema esculentum* Kishinouye, a cnidarian of the class Scyphozoa, the order Rhizostomeae, and the family Rhopilema, is distributed widely off the coasts of China, Japan, and Korea, and is abundant in late summer to early autumn.[1] Painful *R. esculentum* Kishinouye stings to swimmers and fishermen are common and result in local edema, tingle, and paresthesia in extremities. Such effects arise from the complex mixture of biologically active molecules that make up jellyfish venoms.[2]

Proteinous venom from jellyfish nematocyst has a unique structure and many bioactivities such as cardiac toxicity, netro-toxicity, protease activity, hemolysis, hepatocyte toxicity, myotoxicity, and antioxidant activity.[3–8] The bioactivities of *Chironex fleckeri* and *Chrysaora quinqueeirrha* are studied more than other jellyfish.[9–13] Ramasamy reported *C. fleckeri* produced a transient hypertensive response followed by hypotension and cardiovascular collapse within 4 min administration in anaesthetized rats.[7] *C. quinqueeirrha* nematocyst venom which had netro-toxicity was lethal to rainbow killifish when injected intraperitoneally or topically applied to the exposed brain or denuded epithelium.[13] Scholars have studied on biochemistry, pharmacology, and toxicology of jellyfish venom since 1960s, and tried to extract its active components as a new source of medicine. It is reported that jellyfish venom can have promising applications in cardiovascular medicine and target medicine of nerve molecular biology.[14] So, it is useful to study jellyfish venom to benefit human health. However, further study on jellyfish venom has been complicated by many factors including thermal instability, the aggregation of heterogeneous proteins and peptides, and the presence of protease during purification.[15]

Protease can degrade some active proteins resulting in the loss of bioactivities of jellyfish venom.[16] Choosing optimal conditions such as temperature, pH values, additives, and so on to inhibit protease activity is very necessary for enhancing the stability of the active components and simplifying its purification. Many proteases isolated from bacterium had already been investigated, but the study on the protease activity of jellyfish was comparatively deficient.[17–20] Long-Rowe reported the effects of pH on protease activity of *C. quinqueeirrha* venom and employed boronic acid column to excise protease. *O*-Phenanthroline and PMSF could inhibit the protease activity and 0.5% EDTA could enhance the protease activity.[15] Gusmani reported 5 mM EDTA and 2 mM PMSF could inhibit the protease activity of *Rhopilema nomadica* venom.[5] But there is no report about the

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* Corresponding author. Tel.: +86 532 82898707; fax: +86 532 82968951; e-mail: pcli@ms.qdio.ac.cn

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protease activity of venom from jellyfish *R. esculentum* Kishinouye and the effects of glycerol on protease activity of the venom from jellyfish. Furthermore, they only researched the effects of additives of one concentration on protease activity, which couldn't choose the optimal concentration of protease inhibitor to inhibit protease activity. Moreover, jellyfish venoms clearly vary in activity and composition, and different results of some factors affecting protease activity were obtained because of species differentia.[21] In the present study, protease activity of venom from jellyfish *R. esculentum* Kishinouye and the effects of temperature, pH values, additives including glycerol, metal cations, PMSF, *O*-phenanthroline and EDTA on it were first investigated.

Crude protein was prepared as follows. The jellyfish *Rhopilema esculentum* Kishinouye (*R. esculentum*) was collected in the Shazikou Bay in Qingdao, Shandong Province, China, in August 2004. The oral arms with tentacles were manually excised in vivo, packed in polythene bags, and frozen immediately at −20 °C. The frozen oral arms were autolyzed in cold (4 °C) seawater for 2 days. After filtering some residual tentacles, they were sonicated 15 times for 3 s each time at 100 mV. The resultant fluids were clarified by centrifugation at 13,000 rpm for 20 min at 4 °C and used as crude protein (CP). Sample protein concentrations were determined by the method of Bradford,[22] using bovine serum albumin (BSA) as a standard.

L-Tyrosine standard curve was protracted: 1 mL L-tyrosine of different concentrations (10–60 μg/mL) was incubated with 3 mL of 0.55 M Na_2CO_3 and 1 mL Folin-phenol solution at 37 °C for 20 min and then the absorbance at 640 nm was determined.

Protease activity was carried out by the method of Folin-phenol of Bakhtiar with a slight modification.[23] Briefly, 1 mL of 1% (w/v) casein in sodium phosphate buffer (PBS, 0.02 M) and 1 mL CP were pre-incubated at 37 °C, respectively. After 5 min, the casein was incubated with the CP at 37 °C for 10 min, and then unhydrolyzed protein was precipitated with 3 mL of 10% trichloroacetic acid (TCA) and softly shaken. After centrifugating at 13,000 rpm for 10 min, 3 mL Na_2CO_3 was added to 1 mL supernatant followed by 1 mL Folin-phenol reagent and then immediately shaken up. The reaction mixture was allowed to stand for 20 min at 37 °C before measuring the absorbance at 640 nm by a spectrophotometer against blank samples. One unit of protease activity was defined as 1 ng tyrosine released from casein hydrolyzed by protease of 1 mL CP at 37 °C, pH 8.0, for 1 min. Inhibiting rate $I\% = (1 - A_i/A_o) \times 100\%$, where A_i is the absorbance of inhibitor added and A_o is the absorbance of inhibitor unadded.

The effects of temperature on protease activity were assayed as follows. 1 mL of 1% casein in PBS (0.02 M, pH 8.0) was incubated with 1 mL CP at 4, 30, 37, 50, and 60 °C for 10 min, respectively, and then the protease activity of venom was determined according to the method described above.

The effects of pH on protease activity were assayed as follows. 1 mL of 1% casein in pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5 PBS, respectively, was incubated with 1 mL CP at 37 °C for 10 min, and then the protease activity of venom was determined according to the method described above.

The effects of additives on protease activity were assayed as follows. 1 mL additive of different concentrations was added to 1 mL CP before pre-incubation and then the mixture was incubated with 1 mL of 1% casein in PBS (0.02 M, pH 8.0) at 37 °C for 10 min. Then the protease activity of venom was determined according to the method described above.

Figure 1 shows that protease activity was obviously influenced by temperature. When the temperature changed from 4 to 37 °C, protease activity was increased quickly and reached the maximum at 37 °C, so it was regarded as the optimum reactive temperature of protease. Protease activity was markedly reduced at temperature over 50 °C. Carrette reported that at temperatures greater than or equal to 43 °C, venom lost its lethality more rapidly,[24] so it should be at lower temperature to research other characters of *R. esculentum* venom, in order to reduce the degradation effect of the protease to protein. The effects of temperature on protease activity could be explained as follows: when the temperature was below 50 °C, activity energy was consequently decreased with the incubation temperature increasing, inducing the reactive rate, namely protease activity enhancement. As the nature of protease was protein, when the temperature was above 50 °C, protease was denatured with the incubation temperature increasing, resulting in the rate of reaction getting decreased.[25] Protease activity was consequently decreased.

Protease activity was influenced by pH and showed an obvious peak (Fig. 2), implying that at least one protease was present in CP.[26] At pH 7.5, protease activity was minimal and *R. esculentum* venom had a higher hemolytic activity at this pH, confirming that protease activity could affect hemolytic activity.[27] Protease activity was maximal at pH 8.0 and apart from incubation at

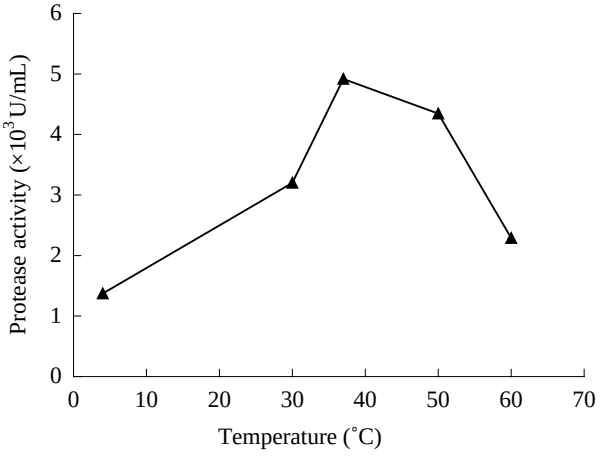

Figure 1. The effects of temperature on protease activity.

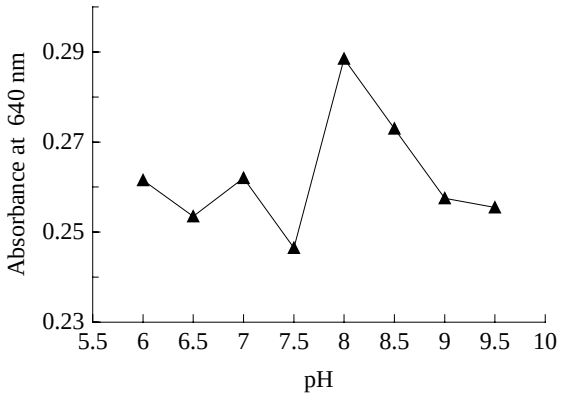

Figure 2. The effects of pH on protease activity.

pH 8.0, protease activity obviously decreased. This finding was in accordance with Lane's research showing that the optimum pH of protease from jellyfish *Physalia* venom was 7.8–8.0.[28] According to the research, PBS at pH 7.5 should be chosen to make jellyfish venom stable when studying other activities of jellyfish *R. esculentum* venom. The reasons that pH influenced protease activity may be explained as follows: (1) more acid or alkali could damage the spatial structure of protein with changing the proteinaceous conformation, so protease activity got reduced. (2) When pH values did not change greatly, protease did not denature, but its activity was influenced, since pH values could affect the dissociated state of substrates, intermediate complexes, and some groups of protease active sites. (3) pH values influenced the decomposition of the relative groups which kept spatial structure of protease molecular stable, resulting in the conformation of protease active sites, so protease activity was affected consequently.[25]

As shown in Figure 3, protease activity was inhibited by glycerol. when the concentration was 1%, the inhibitory effect was the greatest and the inhibiting rate was 82.1%. When the concentration was higher than 1%, the inhibitory effect began to decreased and protease activity consequently improved. The inhibitory effect was the lowest at the concentration of 6% glycerol which could induce the subunit of protease to aggregate,[29] destroying the active site of protease resulting in protease activity decrease. Su investigated that glycerol could effectively reduce the hydrolyzation of protease.[30] However,

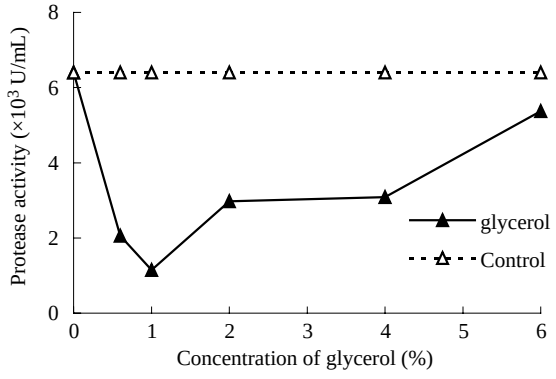

Figure 3. The effects of glycerol on protease activity.

glycerol is a co-solvency and it can change the thermodynamic feature of solution. A balance can be established when hydration of protein surface completes and combines the co-solvent entirely. The balance would stabilize the protein. When the concentration was above 1%, the effect of stabilization on protease may be higher than that of 1%. So, protease activity gradually increased compared to the activity at 1%.

Three familiar metal cations Zn^{2+}, Mg^{2+}, and Mn^{2+} in animals were chosen in this work to study their effects on protease activity of jellyfish *R. esculentum*. As shown in Figure 4, the presence of Zn^{2+}, Mg^{2+}, and Mn^{2+} could enhance protease activity. Zn^{2+} had hardly any effect on protease activity when the concentration was below 0.1 mM. When the concentration changed from 0.1 to 0.2 mM, protease activity went from 5.5×10^3 to 1.2×10^4 U/mL. Protease activity reached the maximum 1.4×10^4 U/mL at 0.5 mM. When the concentration was above 0.5 mM, with the concentration of Zn^{2+} increasing, protease activity consequently decreased. Mg^{2+} could increase protease activity, but the effects were very minimal and had little concentration-dependence. Mn^{2+} could significantly activate the protease and had the greatest effect among the three metal cations; at 8 mM, protease activity (2.3×10^5 U/mL) was about 40 times greater than the control (6.0×10^3 U/mL). Protease activity had a better linear relation with the concentration of Mn^{2+}. The effects of Zn^{2+}, Mg^{2+} were similar and the effects of Zn^{2+} were greater than those of Mg^{2+}. With regard to metal requirements of protease activity, on the basis of the data obtained from addition of metal cations, we would predict the relative metal ion requirements to be $Mn^{2+} >> Zn^{2+} > Mg^{2+}$. The results suggested that Zn^{2+} and Mg^{2+} had broad, but weak, metal ion binding capacity and a probable participation of these cations in jellyfish *R. esculentum* protease activity, similar to what was observed in the sea urchin embryo.[31] Mn^{2+} was probably tightly bound at the active site and was required to stabilize the protease from jellyfish *R. esculentum*.[32] Metal cations were necessary for activity of a part of enzyme, and Zn^{2+}, Mg^{2+}, and Mn^{2+} were usually used as activators of enzyme.[25,33] It was consistent with the results of this experiment.

PMSF was a serine protease inhibitor and it had been reported that PMSF could inhibit protease activity of jellyfish *C. quinqueeirrha*.[2,15] However, in this study, PMSF had hardly affected protease activity (Fig. 5). This result might be explained by the fact that there was no serine protease in jellyfish *R. esculentum* venom or PMSF was decomposed under the condition.

Protease activity was evidently influenced by *O*-phenanthroline (Fig. 6) and it could inhibit the protease activity. When the concentration of *O*-phenanthroline was below 0.5%, protease activity reduced with the concentration increasing and there was a linear relation between them. 0.5% *O*-phenanthroline had a strong inhibitory effect and the inhibiting rate reached the maximum (87.5%). The results showed that the CP probably contained metal-activated proteases, since *O*-phenanthroline was the inhibitor of metaloprotease.[15] Metalo-

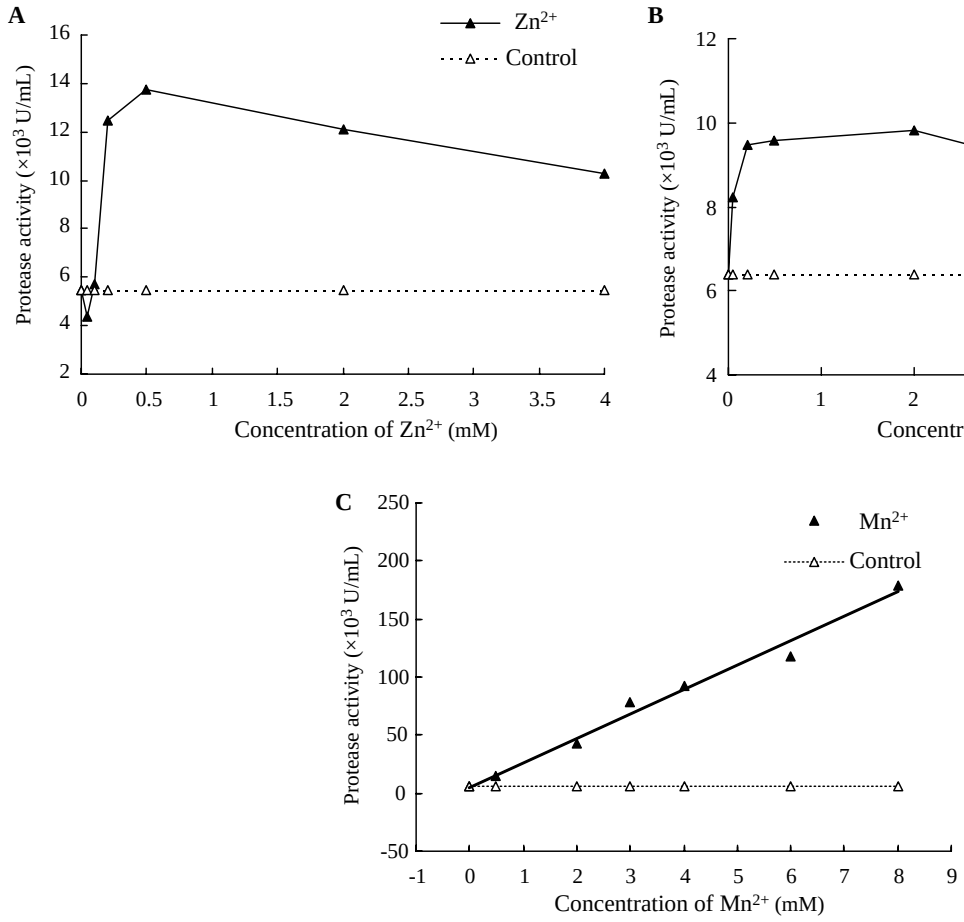

Figure 4. The effects of bivalent metal cations on protease activity. (A) Zn^{2+}. (B) Mg^{2+}. (C) Mn^{2+}.

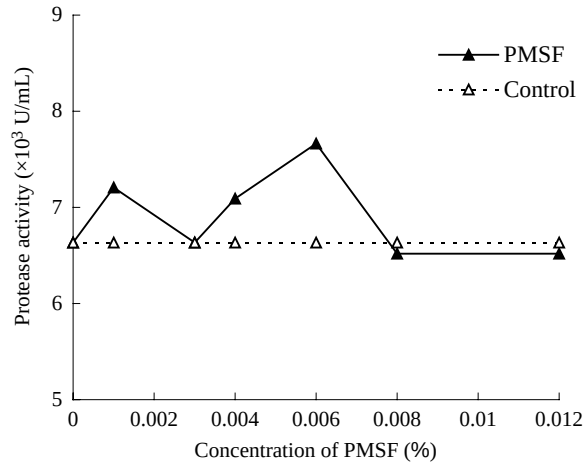

Figure 5. The effects of PMSF on protease activity.

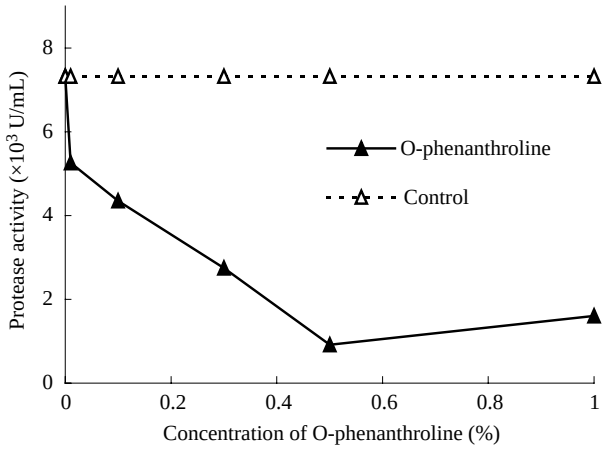

Figure 6. The effects of *O*-phenanthroline on protease activity.

protease could utilize a metal ion such as the above-mentioned Mn^{2+}, Zn^{2+}, and Mg^{2+} as a coordinator to exert bond cleavage resulting in protein hydrolysis.

Figure 7 shows that EDTA could increase the protease activity. When the concentration of EDTA was 1%, protease activity was improved by 3.74 times. As EDTA was a chelator, it could chelate some heavy metal cations such as Fe^{2+}, Hg^{2+}, and Pb^{2+} to reduce the inhibitory effects on protease activity.[25] It was reported that when the concentration of EDTA was higher than 2 mM, the hemolytic activity of *R. esculentum* venom reduced greatly,[28] because EDTA could make protease activity increase and protease could degrade the protein with hemolytic activity.[2] On the other hand, EDTA could also chelate some metal cation activating protease or metaloprotease. Maybe the effect of enhancement on protease activity was stronger than that of inhibition, so it showed that EDTA made protease activity increase.

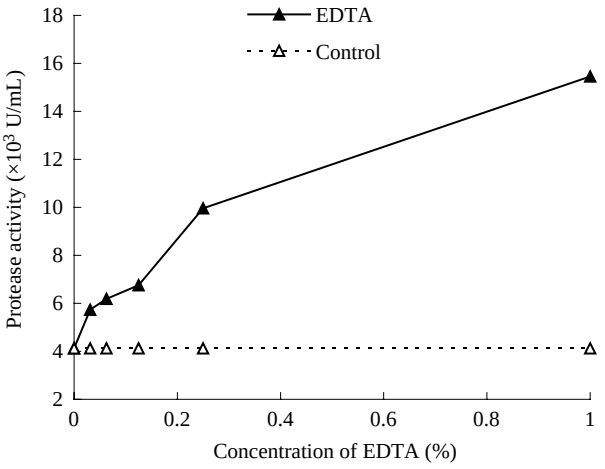

Figure 7. The effects of EDTA on protease activity.

In conclusion, the results of this study showed that the jellyfish *R. esculentum* had protease activity and was affected by some factors. Protease activity was dependent on temperature and pH values. At 37 °C, protease activity reached the maximum and protease activity was maximal at pH 8.0. Zn^{2+}, Mg^{2+}, and Mn^{2+} in sodium phosphate buffer (0.02 M, pH 8.0) could increase protease activity. Mn^{2+} had the best effects among the three metal cations and the maximal effect was about 20 times of that of Zn^{2+} or Mg^{2+} and it could make protease activity reach 2.3×10^5 U/mL. EDTA could increase protease activity. PMSF had hardly any effect on protease activity. *O*-Phenanthroline and glycerol could significantly inhibit protease activity and their maximal inhibiting rates were 87.5% and 82.1%, respectively. According to the study, it is useful to inhibit protease activity using optimal methods to make other active proteins stable and it is significant to study the bioactivities of the venom from jellyfish *R. esculentum*. On the other hand, protease has some toxicological actions inducing hemagglutination holdback, local capillary vessel, and the organization damaging.[34] These investigations can give us important references to research the venom of the jellyfish *R. esculentum* in the future.

Acknowledgments

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