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Biochemical and physiological analyses of a hemolytic toxin isolated from a sea anemone *Actineria villosa*

Gen-ichiro Uechi[a,*], Hiromu Toma[a], Takeshi Arakawa[b], Yoshiya Sato[a]

[a]*Division of Tropical Parasitology, Unit of Social and Environmental Medicine, School of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan*
[b]*Division of Molecular Microbiology, Center of Molecular Biosciences, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan*

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Abstract

A species of venomous sea anemone *Actineria villosa* was recently found inhabiting the coastal areas of Okinawa, Japan. This marine animal produces various proteinous toxins, so that a local health organization was called for medical treatment for those who had accidental contact with this animal. In this study we analyzed the biochemical and physiological properties of hemolytic protein from *A. villosa*. The toxin purified from the tentacles of the animals was found to be a protein with a molecular weight of approximately 19 kDa. We named this newly found hemolytic toxin of *A. villosa*, Avt-I. Incubation of the toxin with sphingomyelin inhibited hemolytic activity by up to 85%, showing that Avt-I may target sphingomyelin on the erythrocyte membrane. The hemolytic activity was stably maintained at temperatures below 45 °C, however, a sharp linear decrease in heat stability was observed within the range of 45–55 °C. Our results provide the first evidence that *A. villosa* produces a toxin with strong hemolytic activity similar in biochemical and physiological properties to other members of actinoporin family previously isolated from related species of sea anemones.

Keywords: Sea anemone; *Actineria villosa*; Avt-I; Hemolysin; Toxin; Peptide

1. Introduction

Sea anemones are known to produce various bioactive substances for self-defense against predators as well as for food predation. Several distinct types of protein toxins have been isolated from several species of sea anemones: (i) neurotoxins of 46–49 amino acid residues cross-linked by three intramolecular disulfide bonds having specific affinity for sodium channels associated with synaptic nerve terminals (Schweitz et al., 1981, 1985; Vincent et al., 1980); and (ii) hemolysins, basic and cysteine-less polypeptides. Biochemical analyses of these hemolytic polypeptides indicated that the majority of these toxins have molecular weight of approximately 20 kDa and these polypeptides bind to erythrocyte cells by specifically recognizing sphingomyelin molecules embedded within lipid bilayers and act as potassium channel (Bernheimer and Avigad, 1976; Varanda and Finkelstein, 1980; Ales et al., 2000). In addition to this activity, cardiostimulatory activity has also been reported for some toxins from sea anemones (Norton et al., 1990; Thomson et al., 1987; Simpson et al., 1990).

Actineria villosa, which has recently been reported inhabiting the coastal areas of the Okinawan islands (Oshiro et al., 2001), is a sea anemone species relatively close to *Phillodiscus semoni*. The species is attracting medical precaution due to its strong toxic activity and the increasing number of inflicted injury cases on humans. *A. villosa* is an

* Corresponding author. Tel.: +81 98 895 1129; fax: +81 98 895 1409.
E-mail address: k018761@eve.u-ryukyu.ac.jp (G.-i. Uechi).

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important species of sea anemone calling for increased awareness of its highly poisonous properties. Severe skin damages were caused by contact with venoms from globular vesicles that located on the animal's tentacles. Recently a major perforin like toxin, AvTX60, has been purified from the nematocysts of *A. villosa*, and characterized with a potent toxicity on mice (Oshiro et al., 2004). In the present study, we isolated a novel hemolytic toxin from globular vesicles of tentacles and found that it has biochemically and physiologically similar properties to those of hemolysins from other species of sea anemone (Nagai et al., 2002; Macek and Lebez, 1988; Kem and Dunn, 1988; Simpson et al., 1990; Wang et al., 2000).

2. Materials and methods

2.1. Animals and sample collection

A. villosa was collected at Odo coast along Itoman-city, Okinawa, Japan. Globular vesicles and surrounding nematocysts were removed from the surface of the body using forceps and immediately submerged in phosphate buffer (10 mM sodium phosphate buffer, pH 6.0) in centrifuge tubes.

2.2. Purification of hemolytic toxins from A. villosa

Toxic protein compounds were discharged from the globular vesicles by vigorously shaking the centrifuge tubes. Tubes were centrifuged at 10,000*g* for 30 min and the supernatant fluid was recovered as a crude toxin. The supernatant was diluted with 10 volumes of phosphate buffer and filtrated through a 0.45-μm membrane prior to application to a CM-sephadex column (Amersham Pharmacia Biotech) equilibrated with the phosphate buffer. The column was washed with the phosphate buffer, and the crude venom was eluted sequentially with the buffer containing various concentrations of NaCl (0–1.0 M). The fractions with hemolytic activity to sheep erythrocytes were pooled and concentrated by using an Amicon Ultra (Millipore). The concentrated fractions were further purified using a TSKgel CM-5PW column (7.5×75 mm; Tosoh) equilibrated with the phosphate buffer. The adsorbed proteins in the column were eluted using a liner gradient of NaCl in the same buffer at a flow rate of 1.0 ml/min. A hemolytic fraction was collected and two hundred microliters of the concentrated toxins were further applied to a Superdex75HR column (10×300 mm; Amersham Pharmacia Biotech) equilibrated with the dilution buffer containing 0.15 M NaCl. The concentrated eluates were finally purified with the LC-10A HPLC system (Shimadzu) at a flow rate of 0.5 ml/min, and the fractions were collected by monitoring the absorbance at 210 and 280 nm. The protein concentrations of the hemolytic toxins as finally purified were determined by the BCA protein assay (Pierce) by using bovine serum albumin as the standard and were used in the present experiments.

2.3. Biochemical analyses of hemolytic toxin

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to estimate molecular size of the hemolytic toxin according to the method of Laemmli (1970). The proteins electrophoresed by SDS-PAGE were visualized by Coomassie Brilliant Blue staining method. The isoelectric point of the isolated hemolytic toxin was determined using PhastGel IEF 3–9 (Amersham Pharmacia Biotech), a homogeneous polyacrylamide gel containing Pharmalyte carrier ampholytes. For the determination of the presence of intra- or intermolecular disulfide bonds, the purified toxin was denatured by boiling for 5 min in the presence of 10 mM DTT in 20 μl of 10 mM sodium phosphate buffer (pH 6.0) containing 0.3 M NaCl.

2.4. Hemolysis assays

To determine the hemolytic activity of the purified toxin, fish (red sea bream) and mammalian (sheep, mouse and human) erythrocytes were used as target cells. Various concentrations of the hemolytic toxin in a volume of 5 μl were added to 1 ml of 1% erythrocyte suspensions, and the mixtures were incubated at 37 °C for 1 h. The samples were centrifuged at 3000*g* for 5 min and measured by its absorbance at 540 nm. For determination of the relative hemolytic activity of the toxin, 0.1 mg/ml of saponin was used as a positive control.

2.5. Hemolytic activity inhibition assays with sphingomyelin

Ten milligrams of sphingomyelin (Sigma) micelles were prepared in distilled water by sonicating the mixture three times for 20 s and the suspension was used. One hundred nanograms of the toxin were mixed with 1 mg of sphingomyelin in a volume of 20 μl buffer and allowed to stand for 5 min at room temperature. The hemolytic activity of the mixture was measured as described above. Unrelated protein, bovine serum albumin (BSA) was used as the negative control.

2.6. Thermostability assays of the hemolytic toxin

Aliquotes of the purified hemolytic protein were incubated at temperatures ranging from 40 to 80 °C for 5 min in preheated test tubes. The relative hemolytic activity of the toxin to 0.01% saponin was determined by measuring the absorbance at 540 nm as described above.

2.7. N-terminal amino acid sequence determination

The hemolytic toxin was separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE),