

Characterization, purification and phylogenetic analysis of a cytolysin from the sea anemone *Heteractis magnifica* of the Indian Ocean

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ABSTRACT: It is well established that sea anemones comprise a rich source of cytolytic toxins. The present study reports the isolation and characterization of a cytolysin obtained from the sea anemone *Heteractis magnifica* collected in the Andaman Islands of the Indian Ocean. The crude extract was screened for hemolytic activity by a blood agar plate method and a 6-mm zone of clearance was observed after incubation. The hemolytic property of the crude extract, tested by the microtiter plate method, revealed positive results at concentrations as low as 120 ng/mL. Furthermore, it was favored by alkaline pH and was stable up to 60°C. On the other hand, the hemolytic effect was abolished by the addition of human serum. Purification steps involved ammonium sulfate precipitation and subsequent desalting by dialysis, followed by anion- and cation-exchange chromatographies. The purified fractions displayed the presence of a 19-kDa cytolysin when analyzed by SDS-PAGE. The conserved region of the cytolysin (with 303 bp) was amplified by RT-PCR and was sequenced. The sequence showed maximum homology (97%) with the already reported cytolysins from other sea anemone species.

KEY WORDS: *Heteractis magnifica*, marine toxin, hemolysin, cytolysin.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

The cnidarians comprise species of mostly marine aquatic invertebrates that constitute the phylum Cnidaria represented by sea anemones, corals, sea fans, sea pens, jellyfish and zooanthids, which are known to secrete a variety of cytolytic toxins mainly for defensive purposes (1-5). Four major groups of these cytolysins are found in cnidarians (5, 6). Among them, the 19-kDa pore-forming cytolysins are the most predominant (5-15). In previous studies, the sea anemone *Heteractis magnifica* was subjected to the analysis of different cytolysins including magnificalytins Hmg I, Hmg II and Hmg III (14-18). However, Hmg III is the most potent and active cytolysin, whose molecular mass has been determined as 19 kDa (14-15). The N-terminal amino acid sequences of Hmg III also exhibited homology with other sea anemone hemolysins such as equinatoxins from *Actinia equina*, tenebrosin-C from *Actinia tenebrosa* and cytolysin III from *Stichodactyla helianthus* (19-21).

Besides being known as a pore-forming cytolysin, Hmg III has not been further investigated on its physiological characteristics regarding the cytolytic activity. Hence, the present study has been undertaken on the isolation, physiological characterization and purification of the cytolysin Hmg III from the sea anemone *Heteractis magnifica* employing a human erythrocyte model. Furthermore, the sequence verification has also been performed on this Indian sea anemone species and compared with a Singaporean species of which the sequence has been already known. The sequence comparison among cytolysins of other cnidarians – such as *Stichodactyla helianthus*, *Heteractis crispata*, *Actinia equina* and *Oulactis orientalis* – has also been performed by a phylogenetic analysis for establishing a genetic relationship.

MATERIALS AND METHODS

Collection of the Sea Anemone *Heteractis magnifica*

The sea anemone *Heteractis magnifica* – identified by Fautin and Allen (22) – was collected from Andaman Islands, India, at a depth of 5 m by scuba diving.

Preparation of the Toxin

In the laboratory, the live animal was induced through osmotic thermal stress to eject the epithelial mucus that contains the toxins (8). The cnidarian toxins are stored in the cnida, a capsule within specialized cells that contains a barbed, threadlike tube

that delivers a paralyzing sting when propelled into attackers and prey. Approximately 2 kg of anemone was added to 1 L of warm distilled water (40 to 45°C) for 15 minutes and subject to intermittent stirring to minimize tissue damage. After 15 minutes, the anemone was removed and the solution was filtered. The filtrate was stored in 100-mL aliquots in liquid nitrogen for transportation. Subsequently, the extract was removed from the nitrogen and stored at -70°C until analysis. When required, the aliquots were thawed, concentrated by lyophilization and reconstituted in phosphate buffered saline at pH 7.4.

Protein Assay

Protein concentration was determined by the Lowry et al. method using bovine serum albumin (BSA) as standard (23). The protein concentration during purification studies was measured from the absorbance at 280 nm. One hundred microliters of purified fractions were read at 280 nm using SpectraMax® microplate reader (Molecular Devices, USA).

Hemolytic Activity on Human Blood Agar Plate

Human blood agar plate was prepared by adding 5 mL of human blood to 95 mL of sterile nutrient agar and pouring the mixture immediately on petri plates. After solidification, wells were cut on the agar plate using a corkscrew borer (8 mm diameter). Wells were loaded with 50 µL (1 mg/mL) of protein samples. The plates were observed for hemolysis after overnight incubation at room temperature.

Hemolytic Assay

Hemolytic activity of the toxin was measured quantitatively in terms of attenuation on human red blood cells at room temperature using a microplate reader. Freshly collected human blood with heparin was centrifuged to remove the buffy coat. Afterward, the obtained erythrocytes (RBC) were washed three times in 0.85% saline and stored at 4°C. Toxins at determined concentrations were added in the first well to erythrocyte buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.4) and then subjected to two-fold serial dilution. RBC (100 µL; $D_{630} = 0.5$) in erythrocyte buffer were added to the toxins, and hemolysis was monitored by measuring attenuation at 630 nm for 20 minutes at room temperature. The final volume in all wells was 200 µL. The

percentage of hemolysis was determined at the end of the assay using the following equation of Maček *et al.* (24):

$$\text{Hemolysis (\%)} = (D_{\max} - D_{\text{obs}}) / (D_{\max} - D_{\min}) \times 100$$

in which D_{obs} is the measured attenuation in the well after 20 minutes and D_{\max} is the maximal attenuation by distilled water and D_{\min} is the minimal attenuation by erythrocyte buffer.

Characterization of the Hemolytic Extract

The crude hemolytic extract was characterized with respect to thermal and pH stability, freeze-thaw stability and treatment with human serum.

Effect of temperature and freeze-thaw process

In order to study the stability of the extract under different temperatures, it was exposed to different heat treatments: 25°C (room temperature), 60°C for one hour, 80°C for one hour, 100°C for one hour and 121°C and 15 psi by autoclaving for 15 minutes. The extract was also frozen at –80°C and then quickly thawed to 37°C. The freeze-thaw procedure was repeated three times and the hemolytic activity was assessed.

Effect of pH

The buffer pH of the extract was 9 and was adjusted to 3, 4, 5, 6, 7 and 8 with hydrochloric acid (HCl), then, incubated for one hour at room temperature and assayed.

Effect of human serum

Human serum was added to the extract at a final concentration of 1% and incubated at room temperature for one hour. The mixtures were assessed for hemolytic activity against human RBC, with serum-free extract as control.

Ammonium Sulfate Precipitation

The crude extract was treated with ammonium sulfate (Himedia Laboratories, India) to 20, 40, 60, 80 and 100% saturation according to the Rosenberg table (25). The

mixtures were stirred for 30 minutes at 4°C and centrifuged afterward at 10000 x g for ten minutes at 4°C. The precipitates were resuspended in 10 mM sodium phosphate buffer (pH 7.2) and desalted in a dialyzing tube with a cutoff of 12 to 14 kDa (Himedia Laboratories, India) against 10 mM sodium phosphate buffer at pH 7.2.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE)

Protein samples were analyzed by SDS-PAGE using 5% stacking gel and 12% resolving gels (26). Samples were denatured by boiling in loading buffer containing SDS and β -mercaptoethanol before they were placed on the gel. Thirty micrograms of crude protein and 10 μ g of purified fractions were placed on the gel and underwent electrophoresis. Following electrophoresis at 15 mA for four hours, gels were stained with Coomassie brilliant blue R-250 (Himedia Laboratories, India) and destained in a solution of 50% (v/v) methanol and 10% (v/v) acetic acid.

Purification of Hemolytic Proteins by Ion-Exchange Chromatography

The dialyzed fractions were purified by ion-exchange chromatography. Anion-exchange chromatography was performed using DEAE-cellulose column (1 x 5 cm) at a flow rate of 1 mL/minute (25). The column-stabilizing buffer was 10 mM sodium phosphate buffer, pH 7.0. Two-milliliter (10 mg/mL) of the crude protein extract in stabilizing buffer was placed on the column. Elution of the bound proteins was performed using a linear gradient of sodium chloride (0.1 M, 0.25 M, 0.5 M, 1 M and 1.25 M). Protein concentrations of the collected fractions were measured by absorbance at 280 nm using a microplate reader and fractions showing hemolytic activity were pooled together, lyophilized and dialyzed against 10 mM phosphate buffer overnight at 4°C. The dialyzed fractions were subjected to cation-exchange chromatography using CM-cellulose column and a similar method previously described in this study.

Isolation of RNA

Total RNA was isolated from the tentacles of the sea anemone *H. magnifica* using TRIzol® reagent according to the manufacturer's protocol (ABgene, UK). The quality of the RNA preparation was determined by electrophoresis in agarose gel (1.2%) and the RNA was employed for reverse transcription reaction.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

One microgram of *H. magnifica* RNA was used in the RT-PCR performed with Reverse-iT® One-step RT-PCR kit (ABgene, UK) in a total reaction volume of 20 µL. Gene-specific primers (HMg2F 5P-ATTGATGGTGCAAGTCTGGG-3P, HMg2R 5P-GTTGCTGTACCAGTTATAGTC-3P) were utilized for the amplification reaction. Primer sequences were obtained according to Wang *et. al* (14). Other components of the RT-PCR reaction were 2x RT-PCR master mix (Thermoprime plus DNA polymerase, reaction buffer, 0.2 mM dNTP mix and 1.5 mM MgCl₂ – ABgene, UK) and 50 U/µL of Reverse-iT® RTase blend (ABgene, UK). The mixture was incubated for one hour at 42°C and inactivated by heating at 95°C for five minutes. PCR was performed by denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for five minutes. The PCR product was electrophoresed on 1.2% agarose gel. The PCR band was excised from the agarose gel and eluted and purified using a gel elution kit (Eppendorf, Germany) according to the manufacturer's protocol. The purified PCR product was sequenced.

RESULTS

Hemolytic Assay

The hemolytic activity of the crude extract is shown in the Figure 1. A 6-mm diameter clear zone of lysis was observed around the wells in human blood agar plates when 50 mg of crude extract was added after incubation (Figure 1). The hemolysis induced by hemolysins in red blood cells suspension was concentration-dependent (Figure 2). The crude extract revealed 50% hemolysis at a concentration of ~120 ng/mL by microtiter plate assay (Figure 2).



Figure 1. Hemolytic activity of the crude toxin on blood agar plate.

C: control (PBS buffer, pH-7.4); T: crude toxin.

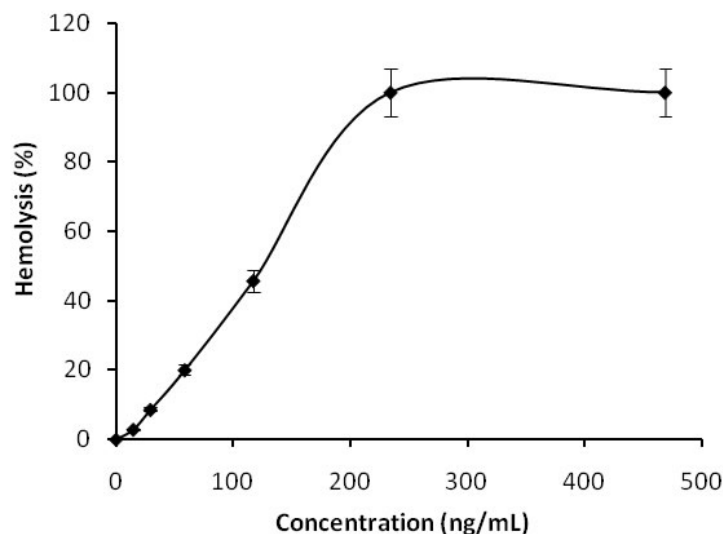
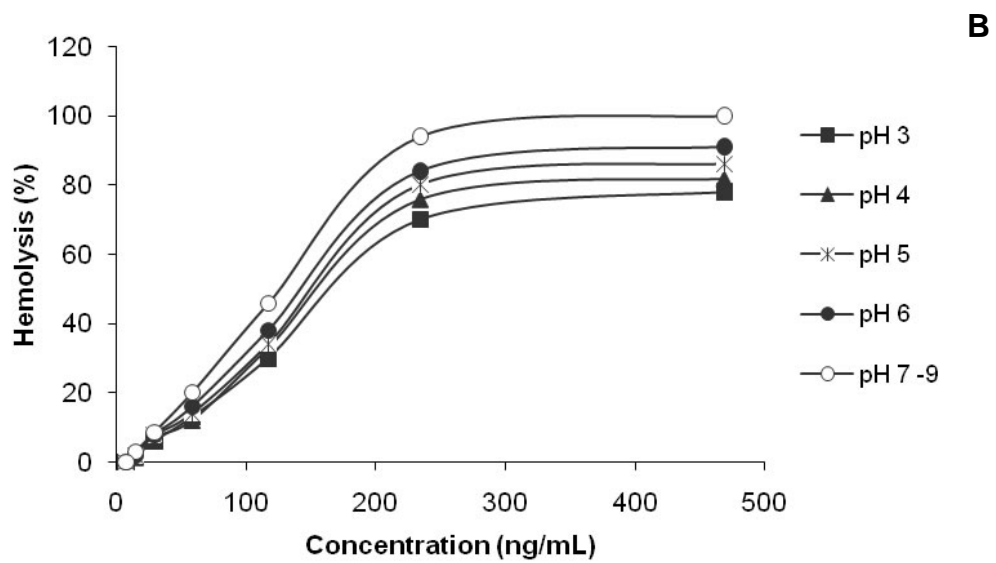
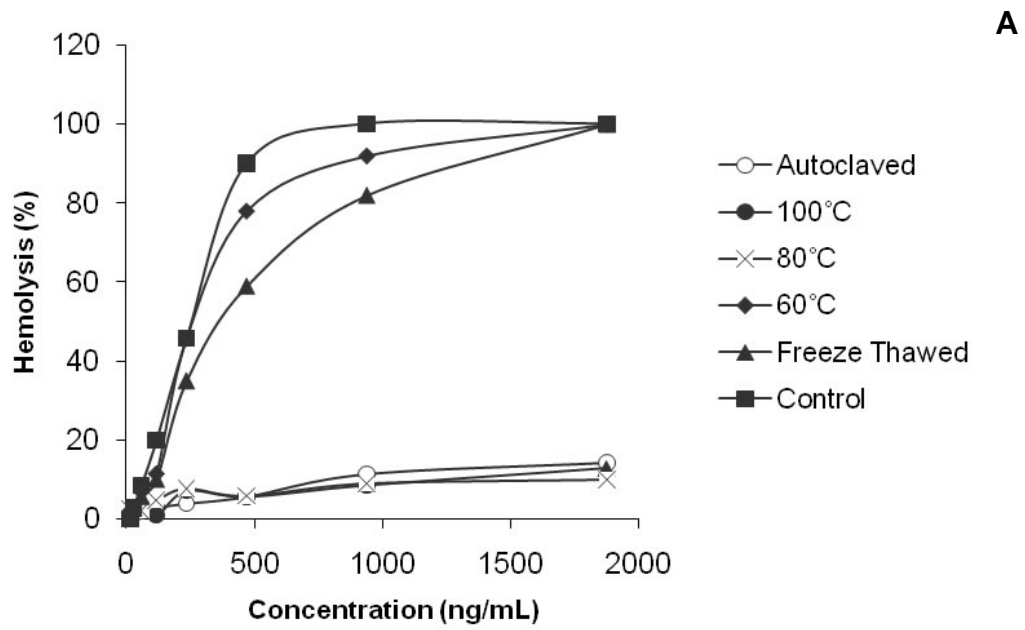


Figure 2. Titration of hemolytic activity. Hemolysis was measured turbidimetrically at room temperature using a microplate reader. The hemolysis percentage was calculated as described in “Materials and Methods”. Data points shown are means of three independent experiments (triplicates).

Physiological Properties

Hemolytic activity peaked at room temperature (25°C) and was partially reduced at 60°C. No activity was observed when the extract was subject to more than 80°C for one hour (Figure 3 – A). Frequent freeze-thaw processes of the stored samples at – 80°C affected considerably the hemolytic activity (Figure 3 - A). The pl of this toxin ranges from 9.0 to 10.0. Therefore, its activity in *in vitro* conditions has also been assumed to occur at this high pH level. Hence, hemolysis was favored in an alkaline pH (Figure 3 – B) whereas activity was reduced in acidic medium. On the contrary, addition of 1% human serum to the extract resulted in neutralization of the hemolytic activity (Figure 3 – C).



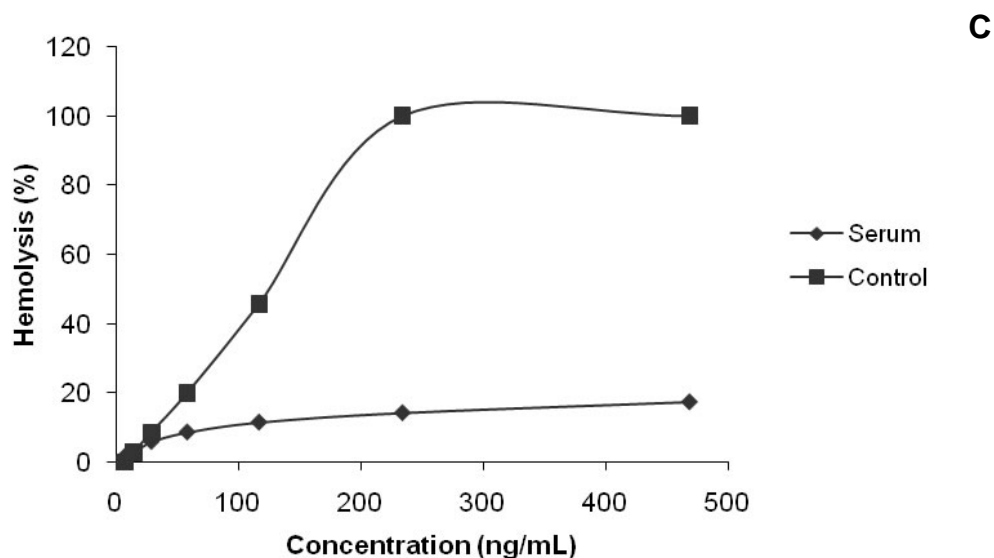


Figure 3. Physiological characteristics of the hemolytic fraction. (A) The effects of temperature, (B) pH and (C) serum on the toxin hemolytic activity. The toxin showed maximum activity at room temperature and in alkaline pH. The hemolytic activity was neutralized after addition of human serum (C). Data points shown are means of three independent experiments (triplicates).

Precipitation of Hemolytic Toxin

Table 1 shows the relative distribution of the hemolytic activity of *H. magnifica* crude extract, precipitated by ammonium sulfate. Crude extract proteins were precipitated in 20%, 40%, 60%, 80% and 100% fractions. All the fractions obtained during salting out process showed hemolytic activity, which indicates that the toxin was precipitated in all fractions (Table 1). However, proteins separated by 60 to 80% ammonium sulfate precipitation were found to be more active. SDS-PAGE analysis of ammonium sulfate precipitated fractions possessing cytolytic activity revealed the presence of a protein band with a molecular weight of 19 kDa (Figure 4).

Table 1. Hemolytic activity of different ammonium sulfate fractions

Ammonium sulfate	Concentrations at which 50% hemolysis was observed (ng/mL)
20%	625
40%	312.5
60%	78.0
80%	39.1
100%	78.0

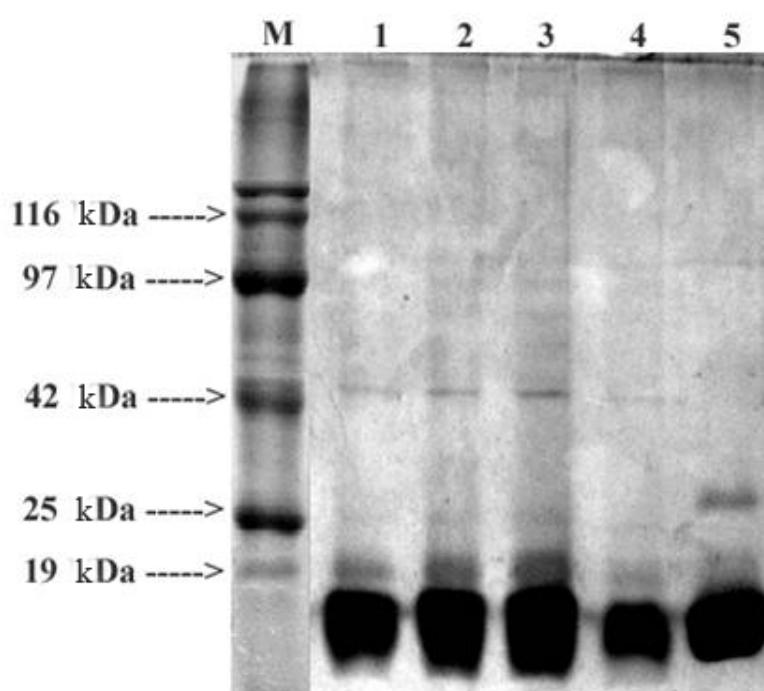


Figure 4. SDS-PAGE analysis of ammonium sulfate fractions. M: marker, lane 1: 100% ammonium sulfate fraction, lane 2: 80% ammonium sulfate fraction, lane 3: 60% ammonium sulfate fraction, lane 4: 40% ammonium sulfate fraction, and lane 5: 20% ammonium sulfate fraction. The 19-kDa hemolytic toxin was precipitated in all fractions but the quantity and activity peaked only in particular fractions (60 to 80%).

Toxin Purification by Ion-Exchange Chromatography

The fractionation of the ammonium sulfate precipitated crude extract of *H. magnifica* is shown in Figure 5 – A, after purification by ion-exchange chromatography. Six major peaks, called A, B, C, D, E and F, were obtained by DEAE-cellulose column chromatography. Peak A fractions were found to exhibit hemolytic activity. These

fractions were pooled, lyophilized and dialyzed against 10 mM phosphate buffer overnight at 4°C. Dialyzed fractions were lyophilized and purified by carboxymethyl-cellulose (CM) cation-exchange chromatography. Subsequently, six major peaks – called A1, A2, A3, A4, A5 and A6 – were obtained by CM-cellulose column (Figure 5 – B) and the fractions under peak A6 showed hemolytic activity. Then, fractions corresponding to peak A6 were pooled together, lyophilized and analyzed by SDS-PAGE.

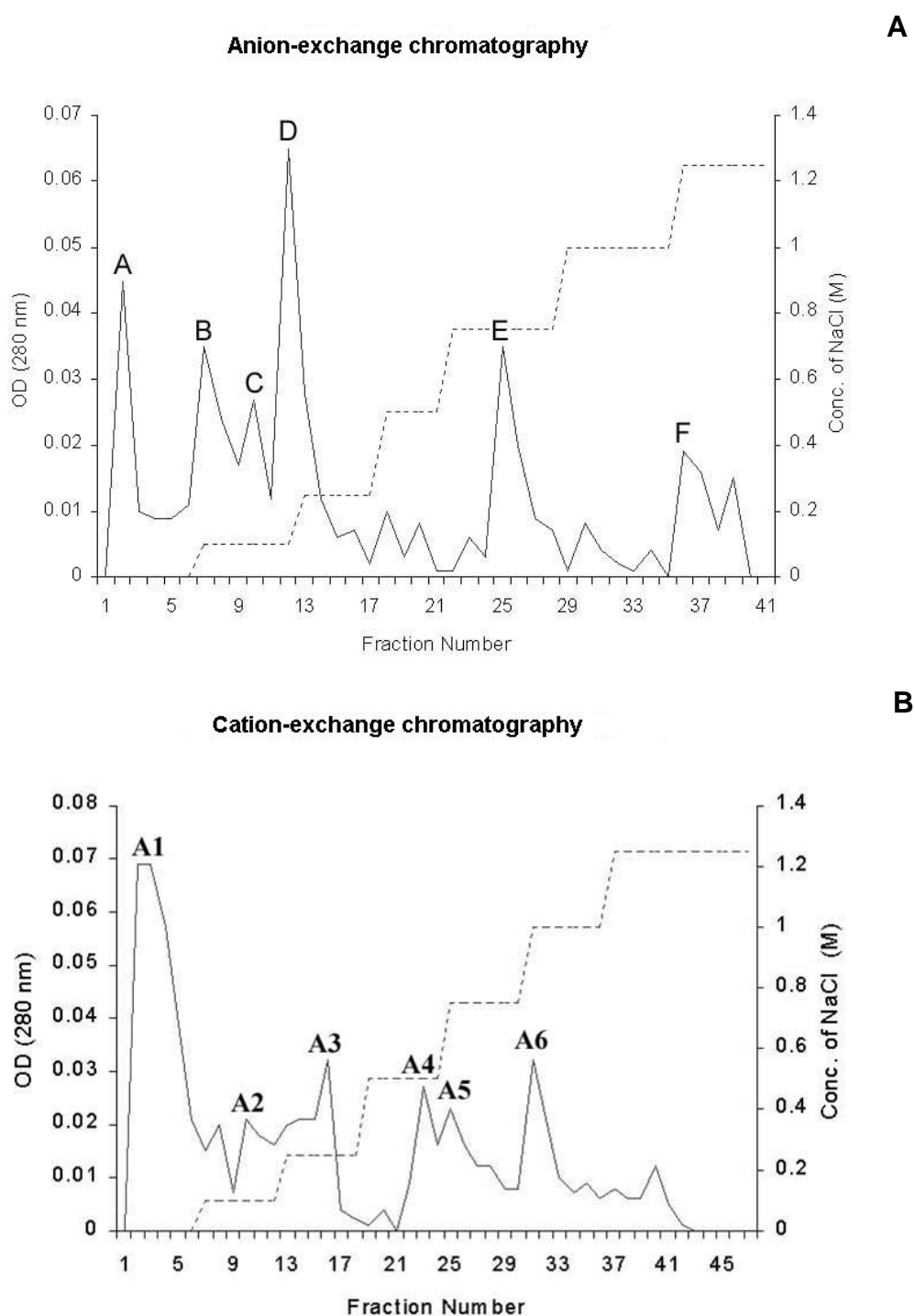


Figure 5. Purification of the hemolytic toxin by ion-exchange chromatography. (A) *Heteractis magnifica* crude protein, dissolved in 10 mM sodium phosphate buffer (pH 7.0), was fractionated by DEAE-cellulose anion-exchange chromatography and fractions under peak A, showed hemolytic activity. (B) Peak A fractions obtained by anion-exchange chromatography were pooled and fractionated by CM-cellulose cation-exchange chromatography. After removal of the unbound components, a linear salt gradient (0-1.25 M NaCl in sodium phosphate buffer, indicated by the dashed line) was added. Fractions from peak A6 showed hemolytic activity.

SDS-PAGE

SDS-PAGE analysis of the active fractions revealed two bands of approximately 14 and 19-20 kDa respectively (Figure 6). The result reveals the presence of the 19 kDa pore-forming cytolyisin in the purified fractions.

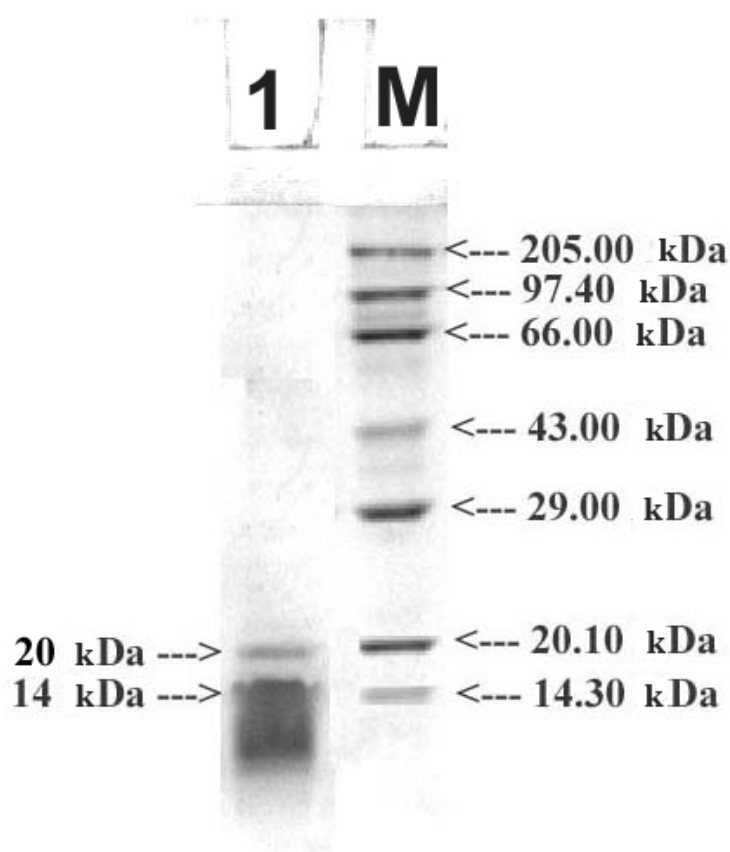


Figure 6. SDS-PAGE analysis of purified fractions.

M: standard protein molecular weight marker. Lane 1: pooled active fractions from the cation-exchange chromatography.

RT-PCR

One hundred micrograms of RNA were extracted from 100 mg of tissue. One microgram of RNA was reverse-transcribed and amplified by RT-PCR. Using PCR primers, HMg2F and HMg2R, a 303 bp PCR product coding for the conserved region of the cytolyisin was obtained (Figure 7) and purified. The purified PCR product was sequenced (accession n. AB254368) and analyzed by BLASTn (Figure 8), BLASTx (Figure 9) and Clustal W (Figure 10).

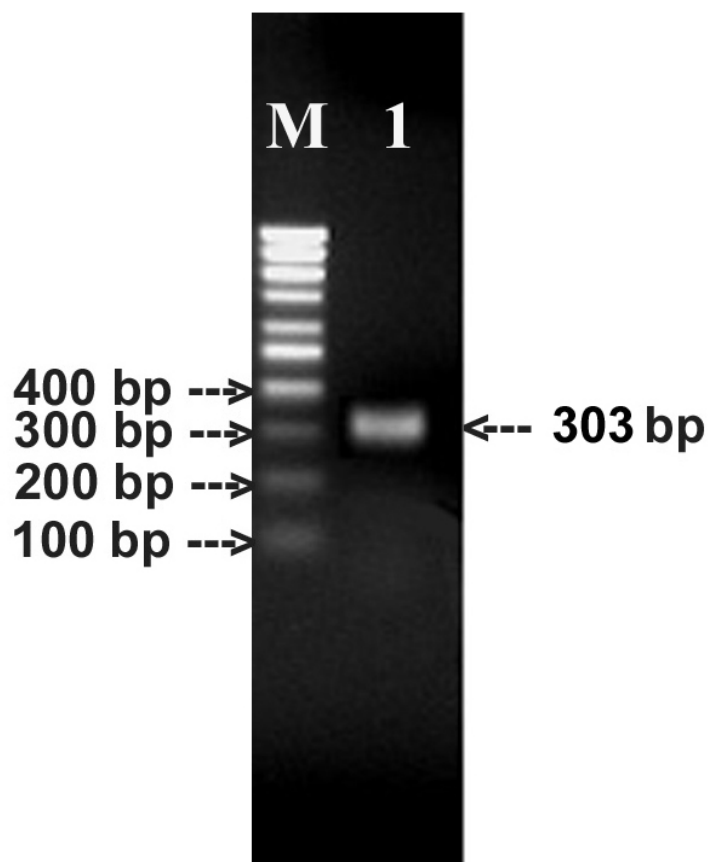


Figure 7. Amplification of the conserved region of the cytolyisin gene by RT-PCR. M: 100 bp DNA ladder; lane 1: 303 bp amplified product code for the conserved region of Hmg III.

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A 18  AAGTCTCGGAGAACTTGGTAAAGTGTCTCGGAAGATTGCTGTTCGGTGTTCGACAACGAGT 77
      |
B 203 AAGTCTCGGAGAACTTGGTAAAGTGTCTCGGAAGATTGCTGTTCGGTGTTCGACAACGAGT 262

A 78  CAGGGGGGTTCATGGACACGATTGAATGCGTATTTCCGTTCTGGTACTACGGACGTCATT 137
      |
B 263 CAGGGGGGTTCGTCGACAGCATTGAATGCGTATTTCCGTTCTGGTACTACGGATGTCATT 322

A 138 TACCAGAGTTTGTCCCAAACCAAAAAGCGCTGCTCTACAGCGGTTCGGAAGGACAGAGGCC 197
      |
B 323 TACCAGAGTTTGTCCCAAACCAAAAAGCGCTACTCTACAGCGGTTCGGAAGGACACAGGCC 382

A 198 CTGTTGCAACGGGGCGCTGTGGCTGCCTTTGCCTATTACATGACCAATGGAAACACTCTTG 257
      |
B 383 CTGTTGCAACGGGGCGCTGTGGCTGCCTTTGCCTATTACATGACCAATGGACACACTCTCG 442

A 258 GGGTTATGTTTCAGCGTTCCCTTTGACTATAAC 289
      |
B 443 GGGTTATGTTTCAGTGTTCCTTTGACTATAAC 474
    
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Figure 8. Comparison of nucleotide sequences of the *Heteractis magnifica* cytolyisin from (A) Indian and (B) Singaporean waters. Sequence comparison showed changes in seven positions (273, 315, 354, 377, 441, 433 and 456).

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A      QVLGELGKVSRKIAVGVNDNESGGSWTALNAYFRSCTTDVILPEFVVPNQKALLYSGRDRG
+VLGELGKVSRKIAVGVNDNESGGSWTALNAYFRSCTTDVILPEFVVPNQKALLYSGRDRG  G
B 55  KVLGELGKVSRKIAVGVNDNESGGSWTALNAYFRSCTTDVILPEFVVPNQKALLYSGRDRG  114

A      PVATGAVAAAFAYYMSNGNTLCVMFSVPPFDYN
PVATGAVAAAFAYYMSNG+TLCVMFSVPPFDYN
B 115 PVATGAVAAAFAYYMSNGHTLCVMFSVPPFDYN  145
    
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Figure 9. Comparison of deduced amino acid sequences of the *Heteractis magnifica* cytolyisin from (A) Indian and (B) Singaporean waters revealed variation in three amino acids (glutamine is replaced by lysine in 55th position, arginine by threonine in 113th position and asparagine by histidine in 132nd position).

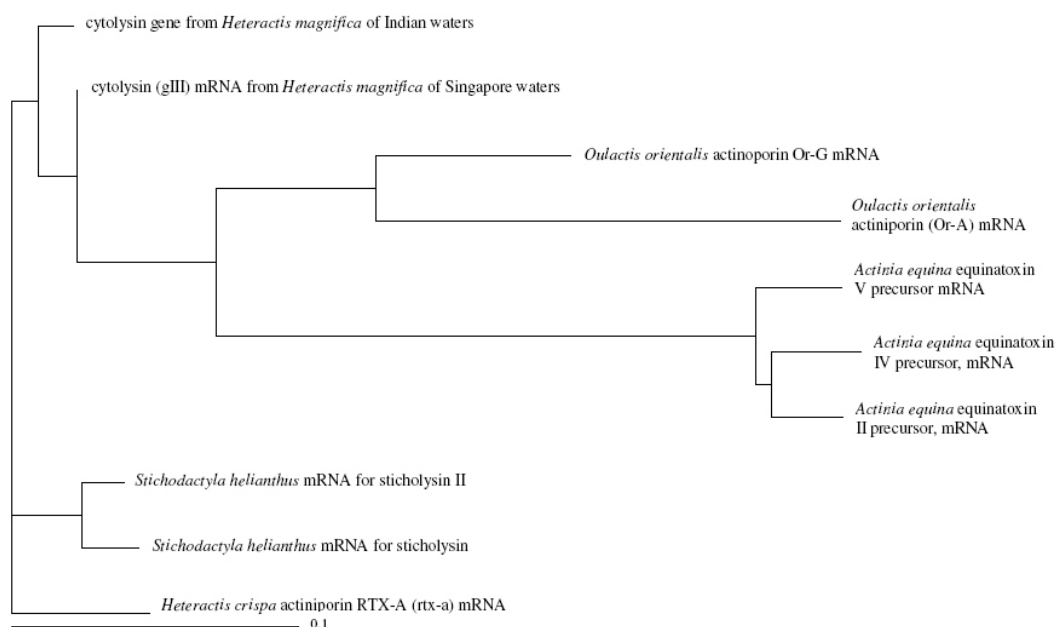


Figure 10. Phylogenetic analysis of the cytolyisin gene of *Heteractis magnifica* with the other reported cytolyisins from sea anemones. The *H. magnifica* cytolyisin showed close relationship with sticholysin and actinoporin RTX-A, rather than equinatoxins.

DISCUSSION

The present study demonstrated the hemolytic activity of a cytolyisin isolated from the sea anemone *H. magnifica*. The isolated toxin required alkaline pH (7 to 9) and a determined temperature (25°C) for its optimal activity. Additionally, this study included cDNA amplification which enabled comparison of the conserved region of this cytolyisin with others.

The purified active protein from ion-exchange chromatography was separated into two bands by SDS-PAGE, one with ~14 kDa and the other with ~19 kDa. The nucleotide sequence of *H. magnifica* cytolyisin was compared with the Hmg III cytolyisin cDNA from the same species of Singaporean waters. Surprisingly, it showed variation in seven positions (273, 315, 354, 377, 441, 433 and 456) of the cDNA bases together with corresponding differences in three amino acids (glutamine is replaced by lysine in 55th position, arginine by threonine in 113th position and asparagine by histidine in 132nd position) in their deduced amino acid sequences.

The cytolyisin sequence from the sea anemone *H. magnifica* was aligned with the reported cytolyisins of other cnidarians. The multiple sequence alignment revealed that *H. magnifica* cytolyisin shares more than 80% homology with the reported cytolyisins from other sea anemones.

BLAST analysis of both this cytolyisin and the cytolyisin of the same Singaporean species revealed 97% (Figure 8) nucleotide homology and 96% homology with deduced protein sequence (Figure 9). Furthermore, it showed up to 95% nucleotide homology with sticholyisin II and 94% with sticholyisin I from the sea anemone *Stichodactyla helianthus* and 94% with actinoporin RTX-A from *Heteractis crispa*. Based on this homology study, a phylogenetic tree was constructed (Figure 10), which indicated a close genetic relationship among *H. magnifica* cytolyisin, sticholyisin and actinoporin RTX-A, rather than connection with equinatoxin.

In the present study, the cytolytic effect has been assessed using human erythrocytes. Interestingly, human serum possesses a total inhibitory effect on the cytolytic activity when added to the incubated erythrocytes. Further studies are necessary on the cytolytic effect of cnidarian cytolyisins on other marine invertebrate, as the body fluid of the latter lacks RBC or molecules of human serum nature.

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