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Hemolytic toxin from the soft coral *Sarcophyton trocheliophorum*: isolation and physiological characterization

Karthikayalu S (1, 2, 3), Rama V (2), Kirubagaran R (1), Venkatesan R (1)

(1) National Institute of Ocean Technology, Chennai, Tamil Nadu, India; (2) Department of Industrial Biotechnology, Dr. M.G.R. University, Maduravoyal, Chennai, Tamil Nadu, India; (3) AxioGen Biotech Private Limited, Pondicherry, India.

ABSTRACT: The unifying characteristic of cnidarians is the production of protein and polypeptide toxins. The present study describes the identification of a hemolytic toxin from the soft coral *Sarcophyton trocheliophorum*. The crude extract was highly cytotoxic (EC_{50} = 50 ng/mL) against human erythrocytes. It was also tested for hemolytic activity by the blood agar plate method, resulting in a hemolytic halo of 12 mm with 50 µg of protein. The stability of the venom under different physiological conditions was analyzed. The venom hemolytic activity was augmented by alkaline and neutral pH whereas it was reduced in acidic pH. The activity was stable up to 60°C. The hemolytic activity was completely abolished by the addition of serum and reduced significantly during frequent freezing-thawing cycles. Toxin purification was performed by ammonium sulfate precipitation and subsequently desalted by dialysis against 10 mM sodium phosphate buffer (pH 7.2), followed by anion exchange chromatography on DEAE cellulose column and gel filtration chromatography using Sephadex G-50 matrix. The purified active fractions possessed a prominent protein of approximately 45 kDa, as revealed by SDS-PAGE.

KEY WORDS: *Sarcophyton trocheliophorum*, cnidarian toxin, cytolysin.

CONFLICTS OF INTEREST: There is no conflict.

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CORRESPONDENCE TO:

KARTHIKAYALU SUBBRAYALU, Department of Industrial Biotechnology, Dr. M.G.R. Educational and Research Institute, Dr. M.G.R. University, Maduraoyal, Chennai 600095, India. Phone: +91 44 23782176. Fax: +91 44 23783165. Email: karthikayalu@gmail.com.

INTRODUCTION

The phylum Cnidaria includes the following benthic and pelagic animal classes: anthozoa, scyphozoa, cubozoa and hydrozoa. They are diploblastic (ectoderm and endoderm) in their cellular organization with a homogeneous elastic material (mesoglea) between these two layers. Soft corals (anthozoa) are found worldwide in tropical environments. They are sessile, colonial forms having soft, fleshy and fragile tissues without any physical defense capability against their potential predators. However, they can survive in such competitive environments because of their chemical defense strategy (1).

The soft coral *Sarcophyton trocheliophorum* (common name: toadstool coral or leather coral) resembles a toadstool and presents a fine carpet of polyps, a light-brown color and is found predominantly in the shallow tropical waters. Its buccal polyps are covered by a ring of tentacles. The tentacles are armed with nematocysts. *S. trocheliophorum* was also covered with a layer of mucus that functions as an energy carrier (2). When stressed the coral release toxins that get trapped in the mucus and function as first line of defense. Moreover, toxin production, a common feature of cnidarians, allows them to produce a variety of peptides and proteins that act as either neurotoxins or cytolysins (3-6). Toxins are produced by specialized stinging cells, the nematocysts. These nematocysts contain fine harpoon-like microscopic structures (cnida) that penetrate the surface layer of the victim and deliver a mixture of highly toxic substances. The role of toxins includes the capturing and killing of prey as well as digestion and protection from predators**.**

The toxins from sea anemones are better characterized than the other groups of cnidarians. Despite the belief that all cnidarians are venomous, the extant biochemical and functional studies have focused only on a few groups and very little research has been done on the toxicology of soft corals. But soft coral stinging nematocysts contain active proteinaceous venoms, while the extra-nematocyst tissues possess many other biologically and pharmacologically active metabolites (1-7). Hence, the present study was undertaken to identify the cytolytic toxins from the soft coral *Sarcophyton trocheliophorum*. Furthermore, the study expands on the physiological characterization and purification of the cytolytic toxin.

MATERIALS AND METHODS

Collection of Soft Coral *Sarcophyton trocheliophorum*

Soft coral *S. trocheliophorum* was collected from Chidiatappu (latitude N: 11°29'493"; longitude E: 92°42'483"), South Andaman region of Andaman Islands, India. The soft coral was collected by scuba diving at a depth of 5 m.

Toxin Extraction

The live coral was subjected to osmotic thermal stress, by spraying warm distilled water at 45°C, to induce the secretion of toxin (8). The soft coral was always covered with thin layer of mucus. Hence, the secreted toxin and the mucus mixed together to form a thick slimy layer on the coral surface. The mucus layer was filtered through a sieve and stored in liquid nitrogen during transportation and at –70°C in the laboratory. When required, the aliquots were thawed and concentrated by lyophilization and reconstituted in phosphate buffered saline (PBS, pH 7.4).

Protein Assay

Concentrations of the proteins were determined by the method of Lowry *et al*. (9) using bovine serum albumin (BSA) as standard. The concentrations of proteins during purification studies were measured at 280 nm. Purified fractions of 100 µL were read at 280 nm using a microtiter plate reader (Molecular Devices, USA).

Ammonium Sulfate Precipitation

The crude extract was treated with ammonium sulfate (Hi-media Labs, India) to 100% saturation as per Rosenberg Table (10). The mixture was stirred for 30 minutes at 4°C and later centrifuged at 10,000 x g for ten minutes at 4°C. The precipitate was resuspended in 10 mM sodium phosphate buffer (pH 7.2) and desalted by dialysis through a dialysing tube with a cut-off at 12,000-14,000 Da (Hi-media Labs, India) against 10 mM sodium phosphate buffer at pH 7.2.

Hemolytic Assay

Hemolytic activity was measured as the attenuation of human red blood cells at ambient room temperature using a microtiter plate reader (Molecular Devices, USA). Freshly collected human blood with heparin was centrifuged to remove the buffy coat, and the erythrocytes (RBC) obtained were washed three times in 0.85% saline

and stored at 4°C. Desired concentrations of protein solution were added in the first well to erythrocyte buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.4), and then serially diluted 2-fold with erythrocyte buffer. RBCs (100 µL; D630 = 0.5) in erythrocyte buffer were added to the protein solution. The final volume in all wells was 200 µL. Hemolysis was monitored by measuring attenuation at 630 nm for 20 minutes at room temperature. The percentage of hemolysis was determined at the end of the assay using the following equation of Maček *et al.* (11):

Hemolysis (%) = $(D_{max} – D_{obs})/(D_{max} – D_{min})$ x 100

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

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 aseptically, with the result poured immediately onto the Petri dishes. After solidification, wells were cut into 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.

Characterization of the Hemolytic Fraction

The crude extract was characterized with respect to thermal and pH stability, stability during freezing-thawing cycles and treatment with human serum.

Effect of temperature

In order to study the effect of temperature on hemolytic activity, the extracted proteins were exposed to various heat treatments: 25°C (room temperature), 37°C for one hour, 60°C for one hour, 80°C for one hour, 100°C for one hour and by autoclaving at 121°C and 15 psi for 15 minutes.

Effect of freezing-thawing procedure

The extract was frozen at –80°C and then thawed quickly to 37°C. The freezing-thawing procedure was repeated three times and the hemolytic activity was assessed.

Effect of pH

The extract buffer was adjusted to pH 3, 4, 5, 6, 7, 8 and 9 with hydrochloric acid (HCl), incubated for 1 h 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 RBCs, with serum-free extract as the control.

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 60 mL/hour (10). The column-stabilizing buffer was 10mM sodium phosphate, pH 7. One milliliter of extracted proteins in the stabilizing buffer was loaded on the column (10 mg/mL). Elution of the bound proteins was done by using a linear gradient of sodium chloride (0.1 M, 0.25 M, 0.5 M, 1 M and 1.25 M) in stabilizing buffer. The protein concentrations in the collected fractions were measured at 280 nm by using a microtiter plate reader. All the fractions were tested for hemolytic activity and fractions showing hemolytic activity were pooled together, lyophilized and dialyzed overnight against 10 mM Tris buffer, pH 7.0 at 4°C.

Gel Filtration Chromatography

The dialyzed fractions were subjected to gel filtration chromatography in a Sephadex G-50 column (1.5 x 60 cm) pre-equilibrated with 10 mM Tris-HCl; pH 7.0 at a flow rate of 40 mL/hour (10). One milliliter (25 mg/mL) of anion-exchange chromatography proteins purified in the running buffer was loaded on the column. Chromatography was performed at 4°C. The fractions collected were tested for hemolytic activity and the fractions showing activity were pooled, lyophilized and analyzed by SDS-PAGE.

SDS-PAGE

Purified protein was analyzed by SDS-PAGE (12). SDS-PAGE was performed using 5% stacking gel and 10% resolving gels. Samples were denatured by boiling in loading buffer containing SDS and β-mercaptoethanol prior to loading onto the gel.

Following electrophoresis at 30 mA for four hours, gels were stained by silver staining (10).

RESULTS

Toxin production by cnidarians is an important aspect of their defense. In the present investigation we report the presence of hemolytic toxin for the first time from the soft coral *S. trocheliophorum*. The live coral was stressed to secrete toxin and the secreted toxin got trapped in the mucus. Moreover, corals slough off large amounts of mucus when stressed (13). Then 450 mL of mucus containing the toxin was collected. The protein concentration was 0.46 mg/mL in the extracted fraction and 30 mg/mL in the ammonium sulfate-precipitated fraction.

Hemolytic activity of crude extract is shown in Figure 1. The hemolysis induced by hemolysin in red blood cells was concentration-dependent and 50% hemolysis was observed at a concentration of ~50 ng/mL (Figure 1). The hemolytic activity was further confirmed on blood agar plate, while 50 µg of crude extract produced a hemolytic halo 12 mm in diameter (Figure 2).

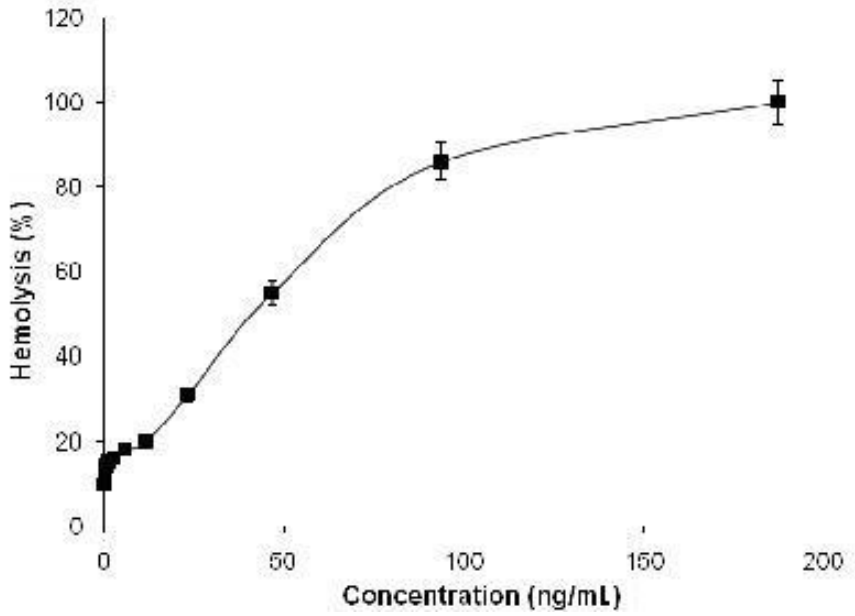

Figure 1. Titration of hemolytic activity. Hemolysis was measured turbidimetrically at room temperature using a microplate reader. The percentage of hemolysis was calculated as described in the Materials and Methods section. Data points shown are means of three independent experiments (triplicates).

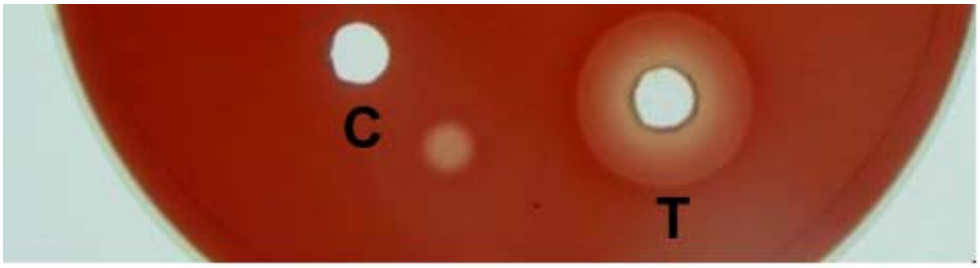

Figure 2. Hemolytic activity of the crude extract on blood agar plate.

C: control (PBS buffer, pH 7.4), T: crude extract.

Although the hemolytic assay of the extract provided an excellent response, it was dependent on temperature (Table 1) and pH (Table 2). Hemolytic activity peaked at room temperature (25°C) and 37°C. The activity was partially reduced at 60°C and no activity was observed when the crude extract was treated at 80°C, 100°C and autoclaved (Table 1), indicating that the toxin responsible for hemolytic effect is heat labile. Hemolysis was favored by alkaline and neutral pH (Table 2) whereas this activity was reduced in acidic medium (Table 2). When 1% human serum was added to the extract, the hemolytic activity on human RBCs was neutralized (Figure 3). Activity was reduced by one-third during frequent freezing-thawing cycles, indicating the unstable nature of the toxin (Figure 3).

Table 1. Effect of temperature on the hemolytic activity of the crude extract

Temperature (^{o}C)	% hemolysis (SEM)
25	48.2 (0.46)
37	51.8 (0.40)
60	42.2 (0.72)
80	14.6 (0.20)
100	5.7 (0.06)
121	5.2 (0.02)

Data points shown are means of three independent experiments (triplicates).

Table 2. Effect of pH on the hemolytic activity of the crude extract

pH	% hemolysis (SEM)
3	–
4	35.2 (1.90)
5	46.1 (0.55)
6	52.6 (0.20)
7	58.4 (0.15)
8	58.8 (0.10)
9	58.6 (0.17)

At pH 3 RBCs were lysed due to very low pH, hence effects of pH cannot be calculated at pH 3. Data points shown are means of three independent experiments (triplicates).

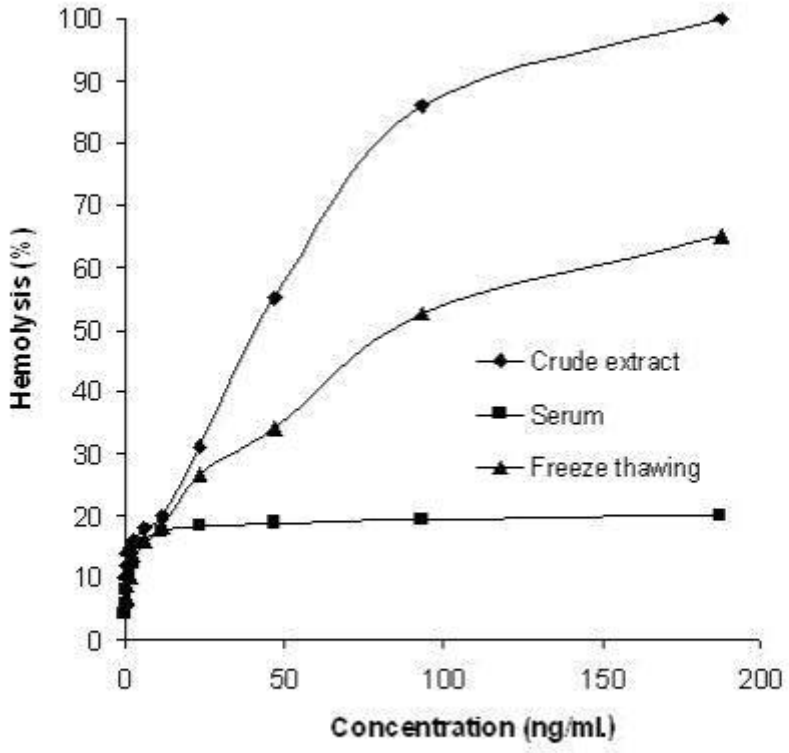

Figure 3. Effects of serum and freezing-thawing on hemolytic activity.

The crude extract of *S. trocheliophorum* was fractionated by anion exchange chromatography. Six peaks, labeled A, B, C, D, E and F were obtained by DEAE-Cellulose column chromatography (Figure 4). Peak A exhibited hemolytic activity and the fractions under peak A were pooled, lyophilized and dialyzed against 10mM phosphate buffer. Subsequent gel filtration chromatography of the pooled sample

using Sephadex G-50 produced seven peaks (A1, A2, A3, A4, A5, A6 and A7). The highest peak, A1, showed hemolytic activity (Figure 5). SDS-PAGE analysis of the fractions under the peak A1 displayed a prominent protein band with a molecular weight of approximately 45 kDa (Figure 6). Due to the low yield and unstable nature of the purified toxin, the activity level (EC_{50}) and yield of the pure protein could not be calculated.

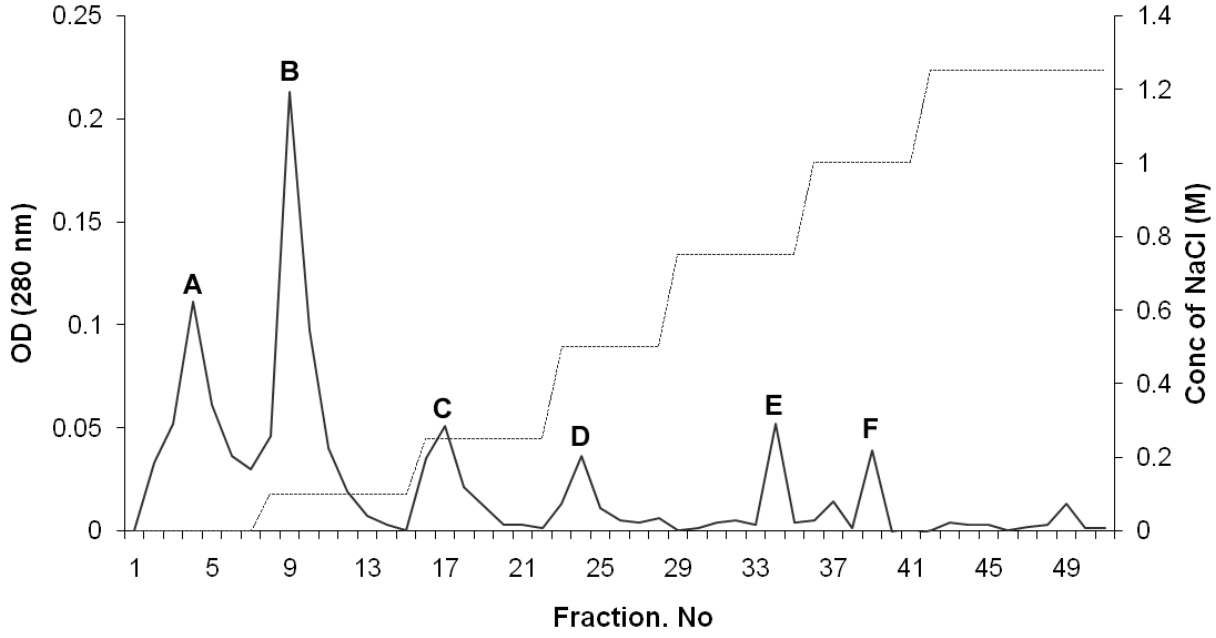

Figure 4. Fractionation of hemolytic crude extract by anion exchange chromatography. *S. trocheliophorum* crude extract, dissolved in 0.01 M phosphate buffer (pH 7.0), was fractionated by DEAE cellulose anion-exchange chromatography using a flow rate of 1 mL/minute. Five-milliliter fractions were collected and fractions under peak A correspond to washout fractions, shown as hemolytic activity.

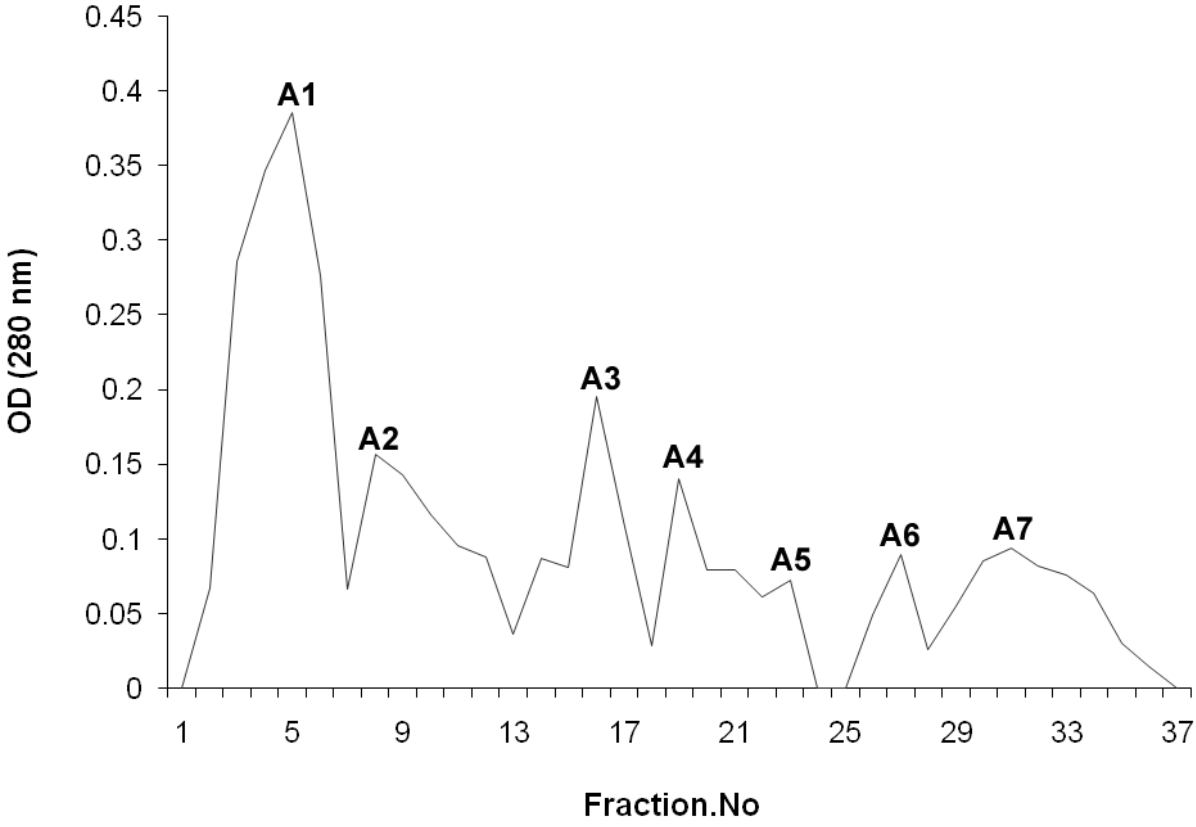

Figure 5. The fractions under A in the anion-exchange chromatography were pooled and fractionated by gel filtration chromatography at a flow rate of 40 mL/hour. Five-millimeter fractions were collected. Fractions under peak A1 exhibited hemolytic activity.

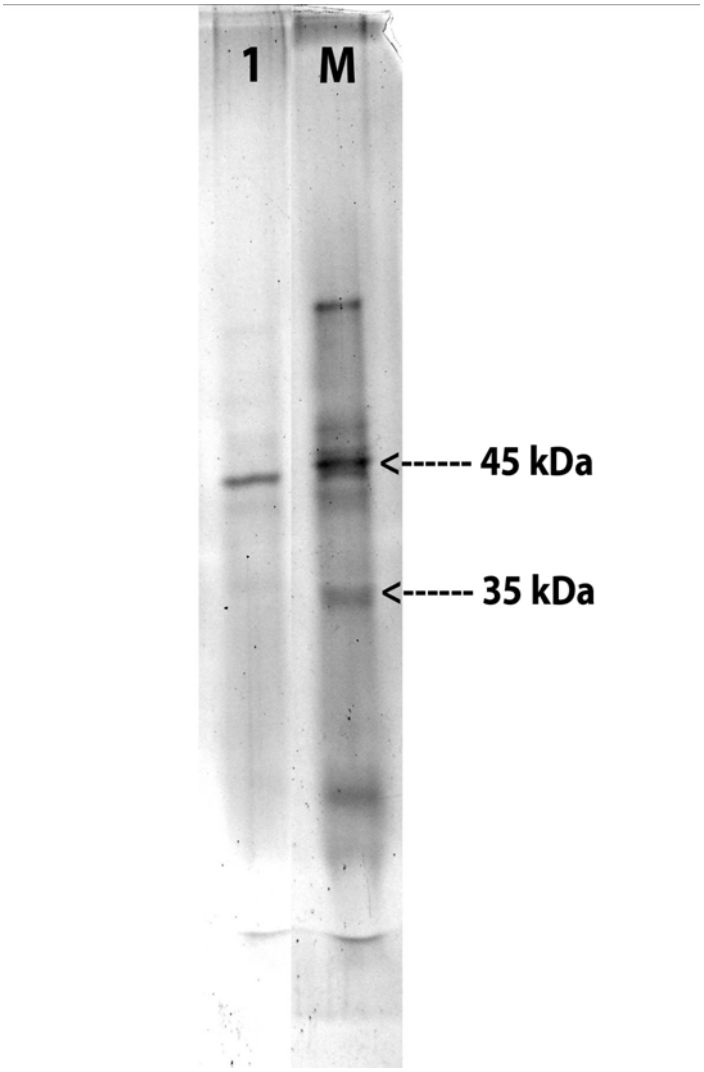

Figure 6. SDS-PAGE analysis of purified fractions.

M: standard protein molecular weight marker (Fermentas, Canada). Lane 1: pooled active fractions from gel filtration chromatography.

DISCUSSION

Cnidarians, in general, are venomous and contain one or more cytolytic and neurotoxic peptides or proteins. Cytolysins, ranging in size from short peptides (5-8 kDa) to larger proteins (98 kDa), were reported from different species of cnidarians (5). Based on the size, 30-45 kDa toxins were classified into a group of cytolysins (5) and were detected in toxin preparations obtained from sea anemones, jelly fishes, soft corals, sea fans and sea pens (5, 14-17). Hemolytic toxins of 30, 31, 32.5 and 35 kDa were detected from the different species of the fire coral *Millepora* (class: Hydrozoa) (18-20). Venoms isolated from the red sea soft corals *Nephthea* sp., *Dendronephthya* sp. and *Heteroxenia fuscescens* were found to posses hemolytic

toxins (7). Nematocyst venom with phospholipase A_2 activity was reported from the tissue homogenates of the soft corals *Alcyonium digitatum, Sinularia flexibilis, Sarcophyton elegans* and *Dendronephthya* sp. (17). The hemolytic toxin from the sea anemone *Aiptasia pallida* exists in two isoforms α and β of 45 and 43 kDa, respectively (15, 16). In this study, SDS-PAGE analysis of the purified fractions showed a prominent protein band of ~45 kDa. Based on this weight it is believed that this toxin may also belong to the group of cytolysins of 30 to 45 kDa.

This paper reports the presence of hemolytic toxin from the soft coral *S. trocheliophorum*, and is the first work to identify peptide toxin from this species. The cytolytic effect has been assessed using human erythrocytes. Interestingly, human serum exerts a total inhibitory effect on the cytolytic activity of the toxin. The elimination of hemolytic activity by the serum is probably due to the antagonistic effects of serum proteins. Significant reduction in the hemolytic activity was observed during storage even at –70°C, thus indicating the unstable nature of the hemolytic protein. The result of freezing-thawing experiments also confirms the unstable nature of the toxin. Based on the experiment data it is evident that the soft coral *S. trocheliophorum* secretes cytolytic toxin when stressed. The toxin is cytolytic in nature and is used for the host defense. Moreover, mucus secretion, a characteristic of corals, forms a coating over the polyps and is implicated in a number of physiological functions. Given that coral mucus serves as an energy carrier, it is rich in nutrients and diverse in bacterial populations. It has been hypothesized that the microbial communities found on the coral surface may also play a role in coral defense. Hence, further investigation on the identification and characterization of the mucus-associated bacteria would be helpful in understanding the role of the toxins in host defense and in the symbiotic relationship.

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