

Partial purification and characterization of a hemolysin (CAH1) from Hawaiian box jellyfish (*Carybdea alata*) venom

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Abstract

We have isolated and characterized a novel hemolytic protein from the venom of the Hawaiian box jellyfish (*Carybdea alata*). Hemolysis of sheep red blood cells was used to quantitate hemolytic potency of crude venom extracted from isolated nematocysts and venom after fractionation and purification procedures. Hemolytic activity of crude venom was reduced or lost after exposure to the proteolytic enzymes trypsin, collagenase and papain. The activity exhibited lectin-like properties in that hemolysis was inhibited by D-lactulose and certain other sugars. Activity was irreversibly lost after dialysis of crude venom against divalent-free, 20 mM EDTA buffer; it was optimal in the presence of 10 mM Ca^{2+} or Mg^{2+} . Two chromatographic purification methods, size fractionation on Sephadex G-200 and anion exchange with quaternary ammonium, provided fractions in which hemolytic activity corresponded to the presence of a protein band with an apparent molecular weight of 42 kDa by SDS-PAGE. We have designated this protein as CAH1. The N-terminal sequence of CAH1 was determined to be: XAADAXSTDIDD/GIIG. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Carybdea alata*; Cnidaria; Coelenterata; Cubozoa; Cubomedusae; Nematocyst; Venom; Hemolysis; Lectin

1. Introduction

Carybdea alata, a cnidarian of the class Cubozoa, the order Cubomedusae, and the family Carybdeidae, inhabits the tropical zones of the Pacific and Atlantic Oceans (Mayer, 1910; Edmondson, 1946; Arneson and Cutress, 1976; Devaney and Eldredge, 1977) and is a member of the same order of cnidaria as the lethal *Chironex fleckeri* (Burnett et al., 1987; Endean et al., 1993). Once infrequently found off the shores of Hawaii (Devaney and Eldredge, 1977), *Carybdea alata* is now regularly observed on leeward Hawaiian beaches 8–10 days after the full moon and is one of the most venomous cnidarian in Hawaii (Thomas and Scott, 1997).

The stings of *Carybdea alata* can induce persistent urticaria, local edema, muscle weakness, paresthesia in extremities, transient dyspnea, and shock (Halstead, 1988; Tamanaha and Izumi, 1996). Such effects arise from the complex mixture of biologically active molecules that

make up cnidarian venoms. Cnidarian venoms may include vasoactive compounds such as 5-hydroxytryptamine (5HT), catecholamines, histamine, and histamine liberators; neuroactive compounds such as quaternary ammonium compounds and certain amino acids and small peptides; and proteins including enzymes, such as proteases, phospholipases, and cytolytic or hemolytic agents (Hessinger and Lenhoff, 1973; Tamkun and Hessinger, 1981; Halstead, 1988; Long and Burnett, 1989; Endean et al., 1993; Macek et al., 1994; Rottini et al., 1995; Gusmani et al., 1997).

Our preliminary characterization of *Carybdea alata* venom indicated the presence of a potent hemolytic protein in addition to several other significant biological activities (Chung et al., 2000). As an initial approach to characterizing this complex venom, we focused on the hemolytic activity for which there is a standardized assay. The hemolytic activity in venom extracted from *Carybdea alata* tentacle nematocysts had properties similar to those ascribed to lectins. We found hemolytic activity was attributable to a protein, which we named CAH1, with an apparent mass of 42 kDa. We present a procedure for the rapid purification, with high recovery, of this protein.

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2. Materials and methods

2.1. Specimen collection and venom preparation

Nearly every month, 8–10 days after the full moon, *Carybdea alata* appeared on the leeward shores of Oahu and could be collected with shallow nets. Nematocysts were harvested by soaking excised tentacles for 3 h in cold (4°C) distilled water, followed by stirring for 10 min and filtration through a plankton net (100–500 µm). The filtrate was then centrifuged up to six times at 500 g for 15 min at 4°C, with microscopic (200x) confirmation of intact nematocysts (Kokelj et al., 1995). Nematocyst yield was monitored at the beginning and end of each extraction procedure by counting the rigid, undischarged, nematocysts using a hemocytometer. Packed nematocyst pellets were stored at 80°C. Following storage or immediately after capture, nematocyst pellets were resuspended to a density of 5.5×10^5 nematocysts/ml and sonicated by Polytron (Brinkman, Westbury, MA) in a buffer consisting of 100 mM Tris base, 100 mM Tricine, 400 mM NaCl, 10 mM CaCl₂, pH 7.0 (nematocyst extraction buffer, NEB). After centrifugation (13,000 g, 15 min), the supernatant (crude venom) was used immediately or stored as aliquots at –80°C. With this procedure, we routinely obtained a yield of 3–10 ml of 1–3 mg/ml crude venom protein from a monthly capture of 100–200 jellyfish. In experiments looking at the role of divalent cations in hemolytic activity, crude venom was subsequently dialysed in 10 mM Tris HCl, 150 mM NaCl, pH 7.4 (TBS) alone or TBS with 10 mM of various salts (KCl, MnCl₂, CaCl₂, MgCl₂, or ZnCl₂).

Crude venom amounts are reported in terms of HU₅₀ units. An HU₅₀ unit is defined as that amount of protein required to lyse 50% of the red blood cells in a 1 ml volume of a 1% blood solution as described below. Though the exact amount varied with monthly jellyfish captures, an HU₅₀ unit typically represented about 200 ng total crude venom protein.

2.2. Hemolysis assay

The hemolysis assay was modified from the protocol of Hessinger and Lenhoff (1973). On a bimonthly basis, vials of heparinized red blood cells (RBC) were acquired from disease-free sheep at the University of Hawaii Livestock Farm (Waialeale, HI). For each experiment an aliquot of the blood was washed three times with phosphate buffered saline (PBS) composed of 136.9 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, and 1.76 mM KH₂PO₄, pH 7.4. Washing was done by low-speed centrifugation (500 g, 2 min) of the blood at 4°C. Washed blood was diluted in PBS to 1% (v/v), approximately 120×10^6 cells/ml. Blood suspensions (0.1 ml, in microcentrifuge tubes) were combined on ice with different amounts of crude venom or fractions and incubated at 37°C for 30 min. Following incubation, the

samples were centrifuged for 2 min at 500 g. The supernatants were transferred to Fisherbrand 96-well microplates (Fisher Scientific, Pittsburg, PA) and absorbance at 414 nm determined by an EIA microplate reader (Bio-Rad, Hercules, CA) to quantify the extent of red blood cell lysis. Reference samples were employed using hypotonic lysis with water as a 100% lysis reference and the 1% blood alone as the 0% reference. Where quantitative measurements of hemolysis are shown, samples were assayed in the same experiment with the same diluted blood and the same batch of isolated venom.

2.3. Protein determination

Sample protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad Protein Assay Kit (Bio-Rad) by comparison with bovine serum albumin (BSA) protein concentration standards.

2.4. Protease activity assay

Protease activity was assayed as in Long-Rowe and Burnett (1994). Crude venom was combined with 1% (w/v) casein in 0.25 M sodium phosphate buffer, pH 7.6 (0.25 M NaH₂PO₄, 0.25 M Na₂HPO₄). As a comparison, collagenase was also combined with casein. Samples were incubated for 1 h at 37°C. Then, unhydrolyzed casein was precipitated with 5% trichloroacetic acid (TCA). The amount of hydrolyzed casein in the supernatant was determined by measuring absorbance at 280 nm with a spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein species were visualized using polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Samples were resuspended in SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS). For SDS-PAGE of reduced proteins, the sample buffer included 5% (v/v) β-mercaptoethanol (β-ME or 2-ME). After heating (95°C for 5 min), samples were loaded on 10–12.5% (w/v) polyacrylamide gels in SDS-PAGE running buffer (24.8 mM Tris-base, 192 mM glycine, 0.5% SDS). Protein electrophoresis was conducted at 15 mA constant current for 1.5 h in an ice bath. Bio-Rad Silver Staining reagent (Bio-Rad) was used to visualize protein bands.

2.6. Anion exchange chromatography

Crude venom was extracted in NEB (see venom preparation section in Methods) and placed into Spectrapor cellulose ester dialysis tubing with a molecular weight cut-off of 3500 Da (Spectrum Laboratories, Rancho Dominguez, CA). Samples were dialyzed with constant stirring against 20 mM Tris-base, 20 mM Tricine, 10 mM CaCl₂, pH 8.9 for the next

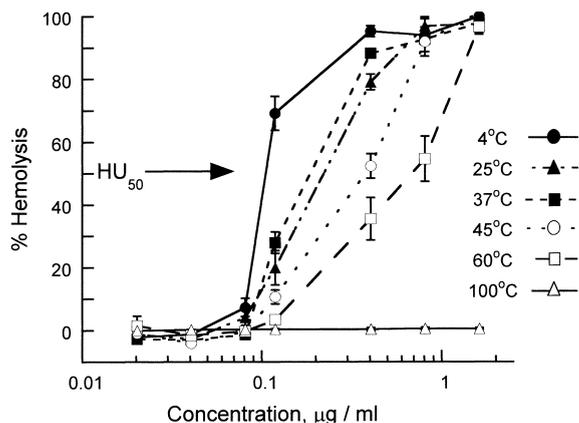


Fig. 1. Hemolytic activity of crude venom after pre-incubation at different temperatures. Plot of percent hemolysis of 1% sheep blood in PBS vs venom protein concentration (log scale). Aliquots of a single extraction of crude venom were held for 30 min at the temperatures indicated, then assayed for hemolytic activity. Arrow indicates 50% hemolysis; the corresponding protein concentration is the HU50. For this and all subsequent figures, experiments were performed in triplicate and mean values with standard errors are shown. Some error bars are within the symbols.

12 h (with one buffer change) at 4°C. Dialyzed crude venom (2–4 mg protein in 5 ml) was syringe-injected into a 5 ml quaternary ammonium (High Q) anion exchange column (Bio-Rad, Hercules, CA). One-ml fractions were collected from the eluent and placed on ice. An additional 5 ml of dialysis buffer was syringe injected and 1-ml fractions were collected and placed on ice. Then 5–10 ml volumes of 100 mM Tricine, 10 mM CaCl₂, pH 8.9, each volume with an increasing amount of NaCl, were successively injected into the column to achieve a step-wise ascending gradient of NaCl as follows: 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 300 mM, and 4 M. One-ml fractions were collected and placed on ice. All fractions were assayed for protein content and hemolytic activity.

2.7. N-Terminal sequencing

The protein to be sequenced was concentrated by fractionation on Sephadex G-200 size-exclusion resin (Amersham Pharmacia Biotech, Piscataway, NJ) that was pre-equilibrated in 0.3 M sodium phosphate buffer, pH 5.5. Crude venom was extracted from nematocysts by sonication in sodium phosphate buffer, pH 5.5. 4.5 mg (1.6 ml) of crude venom protein was loaded onto a 1 × 56 cm Sephadex G-200 column. Fractions were eluted as 1-ml fractions at 6 ml/h at 4°C. The hemolytically active fractions eluted after 5 h and were pooled (BSA standard, molecular weight 66 kDa eluted after 4.5 h). Two µg of the pooled, hemolytically active, fractions were electrophoresed on 10% reducing SDS-PAGE and then transferred at 250 mA for 4 h at 4°C, using 10 mM 3-[cyclohexylamino]-1-propanesulfonic

acid buffer pH 2 (CAPS), to Sequi-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad) for sequencing. The PVDF membrane was stained with 0.5% (w/v) Coomassie R-250 in 40% methanol. The protein band of interest was closely excised. Protein sequence analysis by Edman degradation was provided by The Protein/DNA Technology Center of Rockefeller University (New York, NY).

2.8. Reagents

Unless indicated, chemicals were from Sigma-Aldrich (St Louis, MO) and of high purity. Enzymes were also from Sigma-Aldrich: α-chymotrypsin (EC 3.4.21.1), collagenase IV (EC 3.4.24.3), papain (EC 3.4.22.2), trypsin (EC 3.4.21.4).

2.9. Analysis of data and statistics

The results are presented as mean and standard error of the mean. Two-tailed Student's *t*-test was employed for comparisons between control and experimental conditions. Student's *t*-test was conducted using Sigmaplot 3.06 (Jandel Corporation, San Rafael, CA). A *P* value <0.05 was considered significant.

3. Results

3.1. Hemolytic activity is temperature-sensitive

We found that 1–10 µg of crude venom protein, representing the yield from the nematocysts in 1 cm of tentacle, completely lyse 1 ml of a 1% suspension of sheep blood. For a quantitative assessment, serial dilutions of crude venom were tested for hemolysis. It was apparent that hemolytic activity declined when crude venom was not held on ice before use. We therefore sought to determine (1) if hemolytic activity was proteinaceous and could undergo proteolysis and (2) if crude venom contained proteolytic enzymes.

In order to assess the holding temperature effects on the venom independent of temperature effects on the hemolysis reaction itself, temperature pre-incubation experiments were performed. Fig. 1 shows the percent hemolysis observed in the standard sheep blood assay against the log concentration of crude venom protein, expressed as µg per ml of the hemolysis assay. Each set of symbols represents assays of serially diluted venom held for 30 min at a different temperature. To avoid variations in crude venom potency associated with the monthly jellyfish captures and variations in blood, all the data were obtained using aliquots of the same crude venom extract and the same diluted blood. The curves show a marked rightward shift in the amount of venom necessary for 50% hemolysis in the standard hemolysis assay conditions, indicating reduced venom potency with increased pre-incubation temperature. Hemolytic

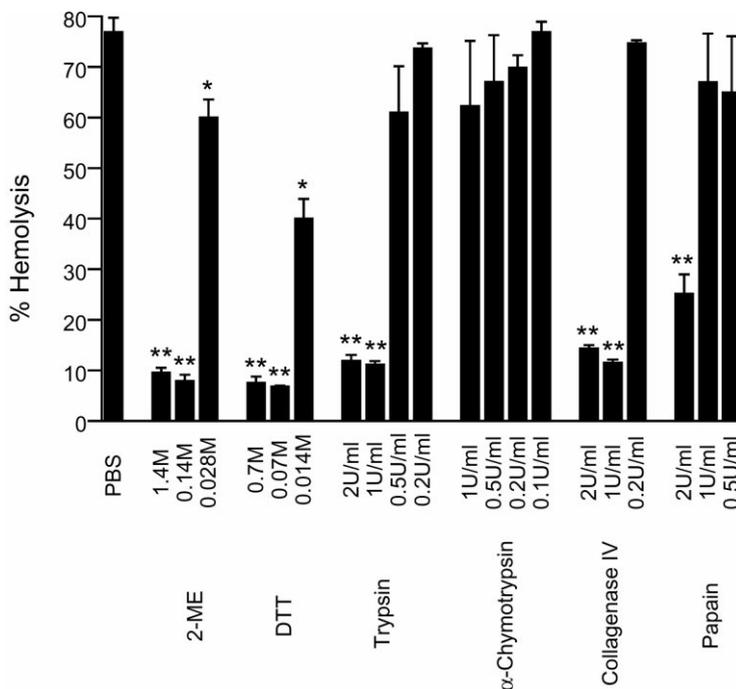


Fig. 2. Effect of reducing agents and proteases on hemolytic activity. Aliquots of crude venom (28 μg protein) were incubated for 15 min at 37°C with 1 ml of trypsin, α -chymotrypsin, collagenase IV, papain, β -Mercaptoethanol (2-ME), or dithiothreitol (DTT) at the concentrations indicated. Then, a 1:10 dilution, corresponding to 1.5 HU_{50} (0.28 $\mu\text{g}/\text{ml}$) of the pre-incubated material, was assayed for hemolytic activity. An asterisk (*) indicates significance at $P < 0.05$ when compared with incubation in buffer only (PBS). Asterisks (**) indicate significance at $P < 0.005$ when compared with incubation in buffer only (PBS).

potency was sharply reduced at 45°C and destroyed by boiling the venom.

3.2. Hemolytic activity is subject to proteolytic degradation

The proteinaceous nature of hemolytic activity was examined by experiments demonstrating degradation of hemolytic activity following crude venom exposure to reducing agents and certain proteolytic enzymes. Fig. 2 presents the results of standard hemolysis assays when 0.28 $\mu\text{g}/\text{ml}$ of crude venom (representing 1.5 HU_{50}) was combined for 15 min at 37°C with various concentrations of these agents as indicated. The effectiveness of the reducing agents, β -mercaptoethanol and dithiothreitol, in degrading hemolytic activity is consistent with their ability to disrupt disulfide bonds and thus alter protein tertiary structure.

Of the proteases, trypsin, collagenase, and to a lesser extent, papain, were highly effective in degrading hemolytic activity. Resistance to degradation by α -chymotrypsin may be due to the lack of its target amino acid bonds or the lack of access to such bonds in the undenatured hemolytic protein.

3.3. Crude venom contains proteases

As suggested in the temperature experiments of Fig. 1, at warm temperatures, hemolytic activity may be degraded by

endogenous proteases. We tested crude venom for proteolytic activity by monitoring its ability to hydrolyse casein as assessed by protein spectroscopy at 280 nm following incubation of venom with casein at 37°C for 1 h. Crude venom was found to have 50–60% of the proteolytic activity against casein as an equal concentration ($\mu\text{g}/\text{ml}$) of collagenase, indicating significant proteolytic activity in crude venom (data not shown).

3.4. Hemolysis is inhibited by carbohydrates

Inhibition of hemolysis by carbohydrates has been reported in other hemolytic toxins (Hatakeyama et al., 1994; Rottini et al., 1995; Gusmani et al., 1997) and is suggestive of a lectin-like protein with binding specificity for carbohydrate moieties. As indicated in Fig. 3, a dramatic and complete absence of hemolysis was observed in the presence of 10 mM D-lactulose ($P < 0.005$ when compared with (+), Student's *t*-test). *p*-Nitrophenyl- α -D-galactopyranoside, *p*-nitrophenyl- β -D-galactopyranoside, and D-galactose were also able to inhibit hemolysis when present at 10 mM ($P < 0.005$ when compared with (+), Student's *t*-test). Phenyl- β -D-galactoside, D-galactosamine, D-raffinose, and D-mannose were weakly inhibitory ($P < 0.05$ when compared with (+), Student's *t*-test). However, some saccharides, such as *N*-acetyl-D-galactosamine, D-glucose,

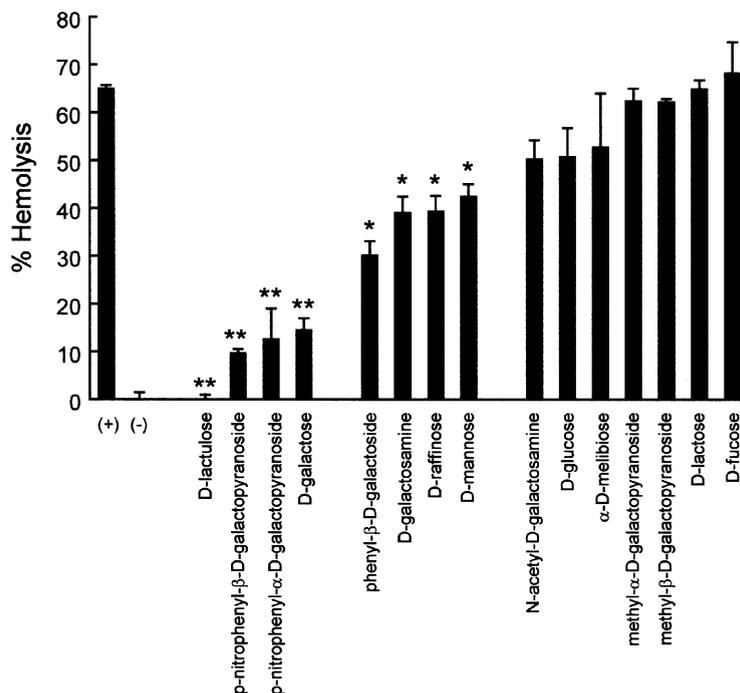


Fig. 3. Carbohydrate inhibition of hemolysis. Approximately 1.5 HU₅₀ (0.28 μg/ml) of crude venom was assayed for hemolytic activity with the standard 1% blood cell suspension, to which 10 mM of a carbohydrate (as indicated) was added. Lysis by venom in the absence of carbohydrates is indicated by (+). In the absence of venom, minimal lysis is observed (-). An asterisk (*) indicates significance at $P < 0.05$ when compared with (+). Asterisks (**) indicate significance at $P < 0.005$ when compared with (+). The seven sugars grouped at the right were NS compared with (+). No hemolysis was observed in the presence of D-lactulose.

α-D-melibiose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, D-lactose, and D-fucose showed no significant inhibition of hemolytic activity ($P > 0.05$ when compared with (+), Student's *t*-test). There is no apparent relationship between the relative sizes of the carbohydrates and their ability to protect against hemolysis.

3.5. The presence of divalent cations is essential for hemolytic activity of crude venom

Observations of enhanced hemolytic activity in the presence of Ca²⁺ from other cnidarian venoms (see Discussion) led us to test the influence of the ionic environment on venom hemolytic potency. Crude venom was extracted from a quantity of nematocysts in NEB in the absence of any exogenous divalent cations. Aliquots of the crude venom were then dialyzed for 4 h at 4°C in TBS or in TBS containing 10 mM of various additional salts as indicated in Fig. 4. After dialysis, the protein content was reassayed and hemolysis assays performed using equal amounts (0.5 μg/ml) of crude venom protein. As seen in Fig. 4, hemolytic activity was reduced following dialysis against TBS alone or TBS with KCl or MnCl₂. In contrast, the activity of venom dialysed against MgCl₂, ZnCl₂, or CaCl₂ was significantly greater than venom dialysed against TBS alone ($P < 0.05$, Student's *t*-test). In addition, crude venom dialysed against

TBS with 20 mM EDTA was found to have lost all hemolytic activity which could not be reversed by either subsequent dialysis with TBS without EDTA or by the reintroduction of buffers containing divalent cations (data not shown).

3.6. Purification and identification of hemolytic activity as a 42 kDa protein

The observations presented above indicate that the hemolytic activity was attributable to a protein or proteins with lectin-like properties requiring the presence of divalent cations. We set about to purify this material with the goal of further characterizing it. Initially, various protein purification strategies including ammonium sulfate precipitation and column chromatography with Sephadex G-200 were utilized. Crude venom was also fractionated using high pressure liquid chromatography, but the resultant loss of hemolytic activity discouraged our continuation of this approach (data not shown). Among these initial methods, G-200 was the most useful in the preparation of hemolytically active fractions. A two-pass G-200 protocol resulted in the isolation of hemolytic activity in a distinct peak in which protein with an apparent molecular weight of 42 kDa appeared as the sole protein species present by SDS-PAGE (Fig. 5(A), G-200).

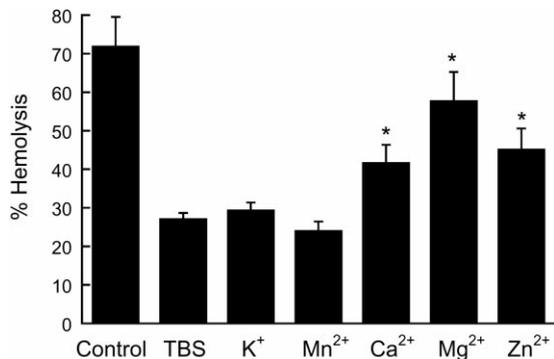


Fig. 4. Divalent cation dependence of hemolytic activity. Crude venom was extracted in NEB (see Methods section). Aliquots of crude venom were then dialysed at 4°C for 4 h. Undialysed crude venom (control, 0.5 µg protein/ml) and venom dialysed in TBS alone (TBS, 0.5 µg protein/ml) were assayed for hemolytic activity. Also, crude venom was dialysed in TBS with the addition of one of the Cl⁻ salts of the cations indicated and assayed for hemolytic activity. An asterisk (*) indicates significance at $P < 0.05$ when compared with TBS alone. Note that the hemolytic activity was greater in venom dialysed against TBS containing the divalent cations Ca²⁺, Mg²⁺, and Zn²⁺ than TBS alone.

To investigate whether this 42 kDa protein was indeed responsible for the hemolytic activity observed, a separate purification protocol was developed utilizing a different principal of separation than the size separation achieved

by Sephadex G-200. Anion exchange chromatography based on quaternary ammonium-bound resin (High Q) provided a high recovery of the hemolytic units present in crude venom. Comparing the hemolytic activity of serial dilutions of crude venom with hemolytically active fractions from G-200 and from anion exchange (Fig. 5(B)), the best recovery of hemolytic activity was obtained by using anion exchange chromatography. The resulting final protocol (Methods) allowed rapid and selective recovery of most of the hemolytic activity (Fig. 6 and Table 1) in two peaks eluted from a High Q anion exchange chromatography column.

As indicated in Table 1, the two peaks (Fig. 6(A), fractions 4–9 and fractions 13–17) represent approximately 70% of the total hemolytic activity of the crude venom. A hemolytically active protein peak eluted in the absence of NaCl (represented by the hemolytic peak at 0 M NaCl in Fig. 6(A)) and another hemolytically active protein peak eluted with 20 mM NaCl. Both peaks had similar hemolytic activity (Table 1) and exhibited approximately 12–15-times more specific activity than crude venom. Analysis of the fractions by SDS–PAGE showed a single protein band with an apparent molecular weight of 42 kDa in the first hemolytic peak (Fig. 6(B), hemolytic peak represented by fraction number 7). There are two additional protein bands, having an apparent molecular weight of 50 kDa each, with the 42 kDa protein in the second hemolytically active peak (Fig. 6(B), fraction number 15).

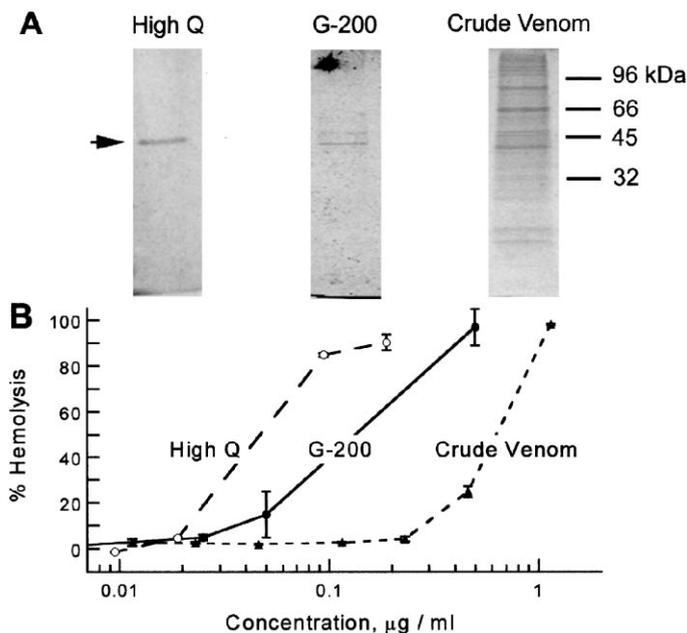


Fig. 5. Comparison of hemolytic fractions separated and purified by size or anion exchange chromatography. (A) Analysis of proteins in crude venom, the hemolytic peak from Sephadex G-200, and the first hemolytic peak from High Q anion exchange (0 mM NaCl). 100 ng of crude venom protein or of peak fractions from G-200 and High Q were subjected to electrophoresis by 12.5% SDS–PAGE (with 2-ME) and silver stained. Note the single protein band (arrow, (A)) with apparent molecular weight of 42 kDa after High Q anion exchange chromatography and the presence of 42 kDa bands in the G-200 fraction and crude venom. (B) Plot of hemolytic activity vs. protein concentration of the material analyzed in (A).

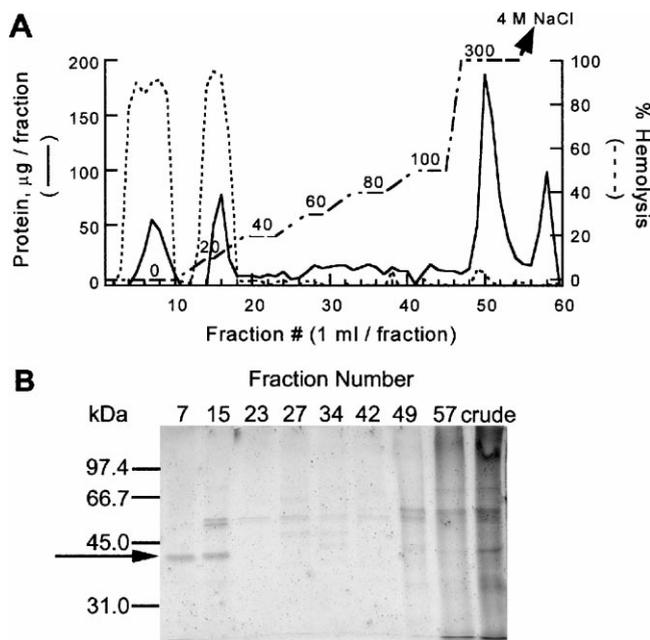


Fig. 6. Anion exchange column chromatography of *Carybdea alata* venom. (A) Crude venom (2.9 mg of protein) was chromatographed on a Bio-Rad High Q anion exchange column. Fractions (1 ml each) were eluted with an ascending step-wise gradient of NaCl (broken line with numbers indicating mM NaCl). Protein content determined for each fraction is plotted (solid line) against the fraction number. The dotted line represents hemolytic activity assessed by hemolysis assay of 2 μ l from each fraction. Hemolytic activity coincided with protein eluting with 0 and 20 mM NaCl. Results in (A) and (B) and Table 1 are from the same experiment and are representative of three separate experiments. (B) 10% SDS-PAGE of anion exchange chromatography fractions. 100 ng of protein from fractions representative of each protein peak in (A) are shown after electrophoresis by 10% SDS-PAGE (with 2-ME). Protein bands in SDS-PAGE were resolved by silver staining. Fraction numbers are those of the chromatograph in (A). Crude venom protein (1 μ g) is represented by “crude”. The position of protein standards run with the fractions is indicated on the far left. Hemolytic activity was present only in fractions showing a 42 kDa protein band (arrow), representing CAH1.

3.7. Partial N-terminal sequence of hemolysin

We used the purification protocols described above to obtain the 42 kDa hemolytic protein for N-terminal sequencing. The hemolytically active fraction resulting after two rounds of G-200 chromatography was run on SDS-PAGE and transferred to PVDF membrane (Fig. 7). The 42 kDa protein band was excised and submitted for N-terminal protein sequencing at the Protein/DNA Technology Center of the Rockefeller University. Analysis of this material provided the sequence: XAADAXSTDIDD/GIIG. The ability to obtain an amino acid sequence from the protein band confirmed that we had isolated a single protein. This sequence of 15 amino acids suggested a general α -helical structure, and contained four aspartate (D) residues. No matching protein sequence was found by a very low stringency comparison against the protein databases available at the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH) in Bethesda, MD using the BLAST search program (Altschul et al., 1997).

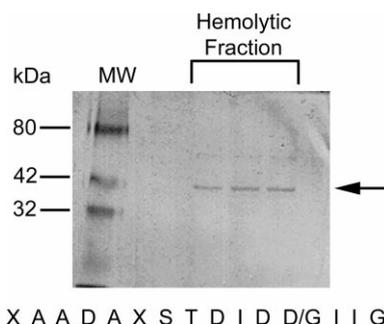


Fig. 7. Analysis of protein submitted for N-terminal sequencing (CAH1). An aliquot having 2 μ g of protein from the hemolytic fractions of Sephadex G-200 was divided and placed into 3 lanes for purification by SDS-PAGE (10% with 2-ME). After transfer to PVDF membrane, the 42 kDa band, observed in each lane by weak Coomassie blue staining (arrow), was excised and provided the amino acid sequence shown. Protein molecular weight standards are shown in the “MW” lane. A, Ala; D, Asp; G, Gly; I, Ile; S, Ser; T, Thr; D/G, Asp or Gly; X, indeterminate.

Table 1
Purification of *Carybdea alata* hemolysin (CAH1) by anion exchange chromatography

Purification step	Total volume ^a (ml)	Protein conc. (mg/ml)	HU50 ($\mu\text{g/ml}$)	Specific activity (units/mg) $\times 10^3$	Total hemolytic units $\times 10^3$	Yield (%)	Purification
Crude venom	4	0.73	0.29	3.5	10	100	1
Dialysed venom	4	0.44	0.22	4.5	9	89	1
First peak (0 mM NaCl)	6	0.01	0.020	50	3	30	15
Second peak (20 mM NaCl)	5	0.02	0.025	40	4	40	12

^a Volumes are approximate.

4. Discussion

In this study, we have focused on a hemolytic component of the nematocyst venom of *C. alata*. The pain and enduring symptoms following stings mentioned in the Introduction leave no doubt that hemolysis represents only one of several actions of this potent venom. In two very different fractionation regimes, only those fractions having a 42 kDa protein (CAH1) had hemolytic activity. The presence of a single protein was confirmed since we were able to obtain an unambiguous sequence of 13 of the N-terminal 15 amino acids. This sequence proved unique. A rough estimate from the amount of protein present in hemolytic fractions indicated that CAH1 may constitute only 5–10% of the total protein in crude venom. However, CAH1 appeared to be responsible for the hemolytic activity present in the venom.

All hemolytic activity was destroyed by boiling (100°C) or treatment of venom with reducing agents or with the proteases trypsin, collagenase or papain. Interestingly, the activity was resistant to α -chymotrypsin. Hemolytic activity was also found to be reduced when pre-incubated at temperatures greater than 25°C. Loss of activity in crude venom may be due to at least two mechanisms. (1) The complete abolishment by heating to 100°C can be attributed to heat denaturation of the hemolytic protein. (2) The loss of activity at 25°C and above may result from temperature-induced changes in molecular structure or from the presence of proteases in the venom. In fact, crude venom was found to contain proteolytic activity against casein although the exact nature of the proteolytic activity in crude venom has yet to be characterized. Protease activity has been identified in the venom of other cnidaria such as *Rhopilema nomadica* (Gusmani et al., 1997).

Hemolytic activity showed lectin-like properties suggesting that hemolysis by CAH1 involves an initial docking or binding with cell surface carbohydrates or phospholipid groups. Evidence for such a property is the ability of certain mono- and oligosaccharides to protect the RBC from lysis by venom. This protection can not be solely explained by an osmotic protective effect provided by large molecules (Rottini et al., 1995) since the inhibition of hemolysis by carbohydrates is not associated with the size of the added carbohydrate. D-lactulose completely prevented hemolysis. *p*-Nitrophenyl- α -D-galactopyranoside and *p*-nitrophenyl- β -D-galactopyranoside also were inhibitory. The carbohydrate groups involved in the postulated binding may be quite specific, as other saccharides, such as *N*-acetyl-D-galactosamine, were found to have very little protective effect. Inhibition of hemolysis by carbohydrates has been observed for other cnidarian hemolysins. The hemolytic activity of *Rhopilema nomadica* was potently inhibited by the carbohydrate *N*-acetylneuraminic acid (Gusmani et al., 1997). The hemolytic activity observed in the venom of the closely related *Carybdea marsupialis* was almost completely inhibited by methyl- β -D-galactopyranoside and *N*-acetylneuraminic acid (Rottini et al., 1995). Methyl- β -D-galactopyranoside did not inhibit hemolysis by *C. alata*

venom (*N*-acetylneuraminic acid has not yet been tested against *C. alata* venom). It is interesting to note the loss of hemolytic activity after chromatography with Sephadex G-200, a carbohydrate-based resin. This may be consistent with the lectin-like properties of CAH1. A low yield of hemolytic proteins from some cnidarian venoms after Sephadex chromatography has been reported by others and has been attributed to an interaction between the hemolytic protein and Sephadex (Long and Burnett, 1989).

The hemolytic activity of crude *Carybdea alata* venom was not reduced following dialysis with Mg^{2+} and Ca^{2+} present but was significantly reduced after dialysis with TBS alone. Further, when crude venom was dialyzed against buffer in which 20 mM EDTA had been added to chelate available divalent cations, hemolytic activity was irreversibly eliminated. These observations suggest the hemolytic activity of *Carybdea alata* crude venom is dependent on the presence of divalent cations. The removal of divalent cations that occurs with dialysis with TBS alone may lead to adverse changes in the protein structure of CAH1. In these studies, crude venom was extracted from nematocysts in nominally divalent cation-free NEB buffer and yet exhibited robust hemolytic activity. This may indicate the presence of Ca^{2+} or Mg^{2+} in the crude venom that is necessary for hemolytic activity but lost during extensive protein purification protocols. In other studies, the hemolytic activity of venom from the cnidaria *Aiptasia pallida* (Hessinger and Lenhoff 1973) and *Actinia equina* (Macek et al., 1994) was found to be enhanced with the addition of calcium. In addition, the hemolytic lectin identified in the holothuridae *Cucumaria echinata* was found to be calcium dependent and is believed to be part of a class of calcium-dependent lectins (C-lectins) (Hatakeyama et al., 1994).

Anion exchange chromatography proved to offer a rapid purification of CAH1 with minimal loss of activity. The presence of hemolytic activity in two distinct peaks, each showing a protein band with an apparent mass of 42 kDa by SDS gel analysis, requires discussion. One peak eluted in the absence of NaCl, indicating the net absence of negative charge on the hemolytic protein at pH 8.9. Thus, this species would be expected to be positively charged or neutral despite an alkaline pH. This is consistent with the high number of aspartate residues in the small portion of sequence we obtained. The second hemolytic peak represents protein eluting in 20 mM NaCl. In the second peak, the 42 kDa parent protein may be stripped of charge-neutralizing divalent cations or may be in a second state of post-translational glycosylation which increases the net negative charge of the molecule.

The fragment of sequence obtained for CAH1 is suggestive of hemolytic activity that is divalent cation-dependent. The aspartate residues seen in the N-terminus sequence could be part of a cation binding site. In addition, examination of the distribution of hydrophobic and hydrophilic residues in the 15 amino acids of the N-terminus seems consistent with an α -helical structure for this segment. An

α -helix is also found at the N-terminus of hemolytic proteins from the cnidaria *Stichodactyla helianthus* and *Actinia tenebrosa* where the N-terminal α -helix is believed to initiate the interaction with lipid membranes that leads to hemolysis (Macek et al., 1994).

5. Note added in proof

Since the submission of this manuscript, Nagai et al. have also reported the presence of a hemolytic protein (CaTX-A, 43 kDa) in *Carybdea alata* venom and have sequenced the cDNA encoding this protein. Our N-terminal sequence for CAH1 compares well with their deduced amino acid sequence at positions 31–45 of CaTX-A. However, their deduced sequence includes a glycine (G) at position 42 of CaTX-A. The amino acid reported by Rockefeller University for our CAH1 was D or G at this position.

Nagai, H., Takuwa, K., Nakao, M., Sakamoto, B., Grow, G.L., Nakajima, T., 2000. Isolation and characterization of a novel protein toxin from the Hawaiian box jellyfish (Sea Wasp) *Carybdea alata*. *Biochem. Biophys. Res. Commun.* 275(2), 589–594.

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