ISOLATION AND PARTIAL CHARACTERISATION OF THREE LETHAL AND HEMOLYTIC TOXINS FROM THE SEA ANEMONE Actiniaria cari

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ABSTRACT

Three lethal and hemolytic toxins, caritoxin I, II and III were isolated from the sea anemone Actinia cari. Following controlled autolysis, tentacles were strained through a nylon sieve and thus a crude extract was obtained. The toxins were further purified by Sephadex G-75 gel chromatography. All three toxins which were eluted at the same peak, were separated by ion-exchange chromatography on CM-cellulose. Disc-PAGE revealed electrophoretic homogeneity of caritoxin II. Caritoxin I was obtained in pure form following rechromatography on CM-cellulose. Purified toxins were stable in the pH range 3 – 8. The preliminary determined molecular weight of the toxins was 20 – 25 000 daltons. All three toxins are basic proteins. Isoelectric point of caritoxin I was determined as 10.7. The isolated toxins are lethal (LD 50 of caritoxin I is 22 μg/kg mice, i.v.) and indicate high hemolytic activity with a pH optimum between 8 and 9. Sphingomyelin specifically and irreversibly inhibits the hemolytic activity of caritoxins as well as some other sea anemone cytolytic toxins.

KEYWORDS

Actinia cari; sea anemone; lethal and hemolytic toxin; cytolytic toxin; inhibition by sphingomyelin.

INTRODUCTION

Relatively little is known about the structure and the mode of action of the sea anemone and other Coelenterate cytolytic toxins. One of the main problems seems to be the isolation of sufficient quantities of fully active proteins from nematocysts or tissue homogenates containing nematocysts. Only a few cytolytic toxins have been isolated from sea anemones (Hessinger and Lenhoff, 1973; Ferlan and Lebez, 1974; Bernheimer and Avigad, 1976, 1978) although cytolytic activity has been demonstrated for a number of different species (Calton and others, 1978; Mebs and Gebauer, 1980; Bernheimer and Avigad, 1981). Ferlan and Lebez (1974) isolated from the sea anemone Actinia equina a lethal and cytolytic toxin, equinatoxin. Since the species Actinia cari belongs to the same genus it was presumed that it might contain a toxin or toxins similar to equinatoxin.

METHODS AND RESULTS

Specimens were collected at the Nort Adriatic Coast and kept frozen until use. Cut tentacles were kept at 4°C for 3 – 4 days in a minimal amount of sea water. Following filtration through a nylon net the filtrate was homogenized and thoroughly centrifuged. Higher hemolytic activities were obtained by adding, before homogenization, CaCl₂ and Α-mercaptoethanol at final concentrations of 0.25 M and 10 mM, respectively. Since fractionary precipitations led to considerable losses of hemolytic activity the centrifuged extract was separated on a 2 x 64
cm column filled with Sephadex G-75, which was washed with 10 mM β-mercaptoethanol in 0.05 M ammonium acetate, pH 7.0 (Fig. 1). Only fraction B demonstrated hemolytic and lethal activity. Therefore, it was further purified with a 1.5 x 15 cm column filled with CM-cellulose. The ion-exchanger was equilibrated with 0.05 M ammonium acetate, pH 7.5, and the proteins eluted with a NaCl linear gradient in the same buffer (Fig. 2). The hemolytically active fractions C1, C2 and C3 were analysed electrophoretically according to Reisfeld and others (1962). Fraction C2 was electrophoretically homogenous, while C1 and C3 contained some impurities. The electrophoretic mobility of all three fractions was equal to that of equinatoxin, under the conditions employed (Fig. 4). Fraction C1 was further purified by rechromatography on CM-cellulose (Fig. 3). The isolated fractions C1, C2 and C3 were named caritoxins (abb. CT I, CT II, CT III).

All three toxins when incubated at room temperature with different buffers were stable in the pH range between 3 and 8. In all hemolytic assays, 3.5 ml suspensions of sheep erythrocytes in 0.15 M NaCl - 10 mM Tris-HCl, pH 7.4 were used giving absorbancy of 0.500 at 540 nm following total hemolysis.

During purification the total and specific hemolytic activities were followed. One hemolytic unit was determined as the amount of protein which brought about 50 % hemolysis in 20 minutes at 37°C. Total yields for the individual toxins
were determined to be about 20%. With gel chromatography the molecular weight was preliminarily estimated. The molecular weight of caritoxin I was determined to be between 20 - 25 000 daltons, similarly as equinatoxin's. The behaviour of caritoxin I on CM-cellulose and the determination of isoelectric point indicated, that it is a basic protein with an $I_p$ about 10.7.

![Graph](image)

**Fig. 5.** Dose dependent hemolytic activity of caritoxin I.

It was observed that all three toxins strongly adsorb to glass and exhibit pronounced denaturation on interfaces. Toxin solutions, especially when diluted to a few micrograms per milliliter, lost all hemolytic activity in several minutes if they were bubbled with oxygen, nitrogen or if they were overlaid with cyclohexane. $\beta$-mercaptoethanol and dithiothreitol did not prevent losses of hemolytic activity.

![Graph](image)

**Fig. 6.** Inhibitory effect of sphingomyelin on the hemolytic action of caritoxin I. Hemolytic assays were performed following a 5 minute preincubation period of toxin with different amounts of sphingomyelin.

The LD50 of caritoxin I was determined on mice, following the method of Reed and Muench (1938). It's high lethality of 22 $\mu$g/kg, i.v., can be compared with that of equinatoxin determined to be 33 $\mu$g/kg rat, i.v. Similarly as in the case of equinatoxin it was observed that sublethal doses of caritoxin I brought about no noticeable pathological alterations, while lethal ones killed instantly or at the most in 3 - 5 minutes, if the toxin was applied intravenously. It was proposed that the toxin probably affects the cardiovascular system, as equinatoxin (Sket and others, 1974). All three toxins are hemolytically very potent even in ngs per 3.5 ml of erythrocytes suspension, as can be seen in Fig. 5 for caritoxin I. Similarly as for equinatoxin (Maček and Lebez, 1981)
caritoxins pH optimum of hemolytic activity was between 8 and 9. Following pre-incubation of individual toxins with suspensions of pure phospholipids prepared according to the method of Kremer and others (1977), only sphingomyelin inhibited hemolytic activity. Inhibition by sphingomyelin depends upon the weight ratio sphingomyelin/caritoxin (for caritoxin I see Fig. 6.). Phosphatidylserine, phosphatidylethanolamine and phosphatidyl choline had no effect even in milligram amounts.

DISCUSSION

Methods previously for the isolation of sea anemone citolytic toxins confirm, that there is no uniform procedure of purification. Even though most authors use in the first purification steps fractionary precipitation (Bernheimer and Avigad, 1978; Ferlan and Lebez, 1974; Mebs and Gebauer, 1980) it could not be applied to our case due to great losses in activity. The inclusion of β-mercaptoethanol into the extraction medium and the eluent during gel chromatography reduced loss of activity. This led us to the presumption that we are dealing with a thiol-activated type of hemolysin as is for example metridiolysin (Bernheimer and Avigad, 1978). Further experiments with purified toxins demonstrated, that neither β-mercaptoethanol nor dithiothreitol in no way influenced activity, but did influence to a great extent toxins yield. Incubation of hemolysins with H2O2 or 5,5'-dithiobis-(2-nitrobenzoic acid) confirmed, that these are not thiol-dependent proteins. Inactivation of caritoxins following bubbling with O2 is not the result of oxidation of eventual SH groups, but the result of denaturation on interfaces. This is also confirmed by inactivation following bubbling with pure nitrogen or on the interface water-cyclohexane. The preliminarily determined molecular weight of caritoxin I is about 20 000 and I10.7 which is similar to that of Stoichactis citolysin with a molecular weight of 16 000 and I10.8 (Bernheimer and Avigad, 1976) and equinatoxin with a molecular weight of 20 000 and I12.5 (Perlan and Lebez, 1974). Even though only a rough comparison of the lethal dose and hemolytic activity is possible, caritoxin I can be placed among the most lethal and hemolytic citolysins of Coelenterate origin. Previous work concerning sea anemone citolytic toxins has shown, that they act directly and that their mode of action is not enzymatic. An exception is the Aiptasia pallida venom in which phospholipase A has been detected (Hessinger and Lenhoff, 1973). With regard to the fact, that isolated citolysins can be inhibited by cholesterol (Bernheimer and Avigad, 1978) or by sphingomyelin, it has been presumed, that these lipids are receptor sites (Linder and others, 1977). Sphingomyelin is also the only pure phospholipid which specifically and completely inhibits the hemolytic activity of caritoxins. Regardless of smaller differences in molecular weight and I, caritoxins, equinatoxin and Stoichactis citolysin form a group of sea anemone citolysins inhibited by sphingomyelin.

REFERENCES


