An ichthyotoxic protein in the defensive skin secretion of the Red Sea trunkfish Ostracion cubicus

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Abstract By the aid of acetone precipitation and RP-HPLC column chromatography coupled to various bioassays of ichthyotoxicity and cytolysis, a new ichthyotoxin designated ''boxin'' was isolated from the defensive skin secretion of the Red Sea trunkfish *Ostracion cubicus*. Boxin is a stable, heat and proteolysis resistant protein of 18 kDa. Its protein nature was assessed by spectral analysis, strong proteolysis, amino acid analysis and amino acid sequence determination. Similar to pahutoxin (PHN), an organic cationic surfactant derived from the same secretion, boxin is not effective by injection, and its ichthyotoxicity is achieved only upon delivery to the surrounding water. The latter suggests that fish lethality is mediated by externally allocated target sites (receptors). Boxin, however, differs from PHN by (1) possessing a 30 times higher ichthyotoxicity (mole per mole) and (2) being devoid of PHN's capacity to permeabilize biological membranes. From an ecological point of view, it is noteworthy that polypeptides are very useful in fulfilling allomonal functions in the marine environment due to the high information content inherent in their structures and their solubility in seawater.

Introduction

Many species of marine fish have been reported as ichthyocrinotoxic (Halstead 1978), releasing into the surrounding water toxic skin secretions. The chemistry of these secretions was studied in only a few representatives of ''soap'' (Serranidae), ''toad'' (Batrachoididae), ''flat'' (Solidae) and ''trunk'' (box) (Ostraciidae) fishes, and they were shown to be composed substantially of low molecular weight surfactants or detergents (Zlotkin and Barenholz 1983; Tachibana et al. 1984; Tachibana and Gruber 1988).

Trunkfish of the genus *Ostracion* were shown to secrete ichthyotoxic and hemolytic non-substituted or β-substituted choline chloride esters of palmitic acid (Goldberg et al. 1982, 1988; Fusetani and Hashimoto 1987). The first and best studied trunkfish ichthyotoxin was designated ''pahutoxin'' (PHN) (Boylan and Scheuer 1967). PHN is a typical cationic surfactant (Mann and Povich 1969) with a quaternary ammonium headgroup and an acetylated hydrocarbon chain as the hydrophobic portion, the choline chloride ester of β-acetoxy palmitic acid.

The occurrence of proteins in Red Sea trunkfish (*Ostracion cubicus*) secretion was demonstrated by Aknin (1989), and their involvement in ichthyotoxicity has been reported (Kalmanzon et al. 1999). These proteins were shown to fulfill regulatory functions as enhancers and carriers of PHN (Kalmanzon et al. 1999).

However, the present study demonstrates that the Red Sea trunkfish, in addition, possesses an ichthyotoxic protein. The chemoecological significance of this finding is discussed.

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Materials and methods

Crude trunkfish secretion

The toxic secretion was collected by gently agitating the Red Sea trunkfish *Ostracion cubicus* in distilled water, followed by lyophilization of the resulting foamy secretion (Kalmanzon et al. 1999).

Fish toxicity assay

Fries of 150 to 200 mg body weight of commercially bred gilt head sea bream (*Sparus aurata*) were used as test animals. A single fry

was placed in a separate beaker containing the test substance in 10 ml of filtered seawater. Lethality, expressed by loss of balance and the arrest of opercular movements, was recorded after 1 h. Each concentration of test substance was assayed on five to seven fries. The 50% lethal concentration (LC_{50}) was calculated according to Reed and Muench (1938).

In assays based on injection, test substances were dissolved in phosphate buffered saline (PBS) and injected intramuscularly in a volume of 10 µl into the dorsal rear part of the fry. After injection, fries were observed for lethality for a period of 24 h.

Column chromatography

The toxic protein boxin was isolated from the cold acetonic precipitate (Fig. 1) by the aid of a semi-preparative reversed phase (RP) column in a Hewlett-Packard 1050 HPLC system. See legend to Fig. 1 for details.

Proteolysis

Pronase and Trypsin each at 5% (E/S), 37 °C for up to 2 h were used for proteolysis (Fig. 2).

Protein estimation

Proteins were quantitatively determined by the Folin phenol assay of Lowry et al. (1951). The presence of proteins was also indicated by the occurrence of cold acetone precipitation (Fig. 1).

Assays on membrane permeability

Assays on hemolysis were performed according to Primor and Zlotkin (1975) with 20% (v/v) suspensions of washed human red blood cells in PBS, pH 7.4. Liposomal permeability was assessed by monitoring fluorescence release from phosphatidylcholine:cholesterol liposomes loaded with sodium carboxyfluorescein (CF) (Lichtenberg et al. 1981; Kalmanzon et al. 1992). A suspension of small unilamellar liposomes (containing 10% lipid w/v) loaded with 0.1 *M* of CF was placed in the cuvette of a spectrofluorometer (Perkin Elmer LS-50B) and the test substance added. The CF release was monitored by the fluorescence intensity (excitation 490 nm; emission 520 nm).

Results

Ichthyotoxic protein in the trunkfish skin secretion

In our previous study (Kalmanzon et al. 1999) the occurrence of ichthyotoxic proteins in the Red Sea trunkfish *Ostracion cubicus* was indicated by two pieces of information: firstly, by the cold acetone treatment which yielded, in addition to the acetone-soluble, lipophilic ichthyotoxic fraction, also the acetonic precipitate which possessed water-extractable ichthyotoxicity; and, secondly, by a RP-HPLC separation of an ichthyotoxic-hemolytic fraction obtained by gel filtration. The RP-HPLC column yielded two ichthyotoxic fractions, the first, relatively polar, was identified as a protein fraction and the second, highly hydrophobic, was identified as PHN.

In the present study the above acetonic precipitate provides the starting point for the isolation and purification of an ichthyotoxic protein from the trunkfish secretion (Fig. 1).

►

Fig. 1 Isolation and purification of boxin, a new ichthyotoxin from the skin secretion of the Red Sea trunkfish (*Ostracion cubicus*). **A** Main steps of fractionation used to obtain the nonsoluble pellet (protein precipitate). The pellet is insoluble in distilled water but when suspended in seawater shows obvious ichthyotoxicity. **B** The pellet was dissolved in dimethyl sulfoxide (DMSO) (Chang et al. 1991) and loaded on a RP-18 semi-preparative column (10 µm, 22 × 250 mm, Vydac, Hesperia, California) equilibrated by Solvent A (0.1% trifluoroacetic acid; TFA) and a linear gradient, at 1% min^{-1}, of Solvent B (0.1% TFA, in acetonitrile). Flow rate was 6 ml min^{-1}. The cross-hatched peak was ichthyotoxic and used for further fractionation. **C** Of the above ichthyotoxic fraction 1 mg was charged on a Vydac RP-18 analytical column (5 µm, 4.6 × 250 mm) equilibrated by Solvent A (0.1% TFA) and eluted at a flow rate of 0.5 ml min^{-1} by a linear gradient of B (0.1% TFA, in acetonitrile). **D** A 100 µg portion of the main fraction from C was rechromatographed. The column eluant composition and flow rate were the same as for C. The gradient of CH_3CN is graphically presented (*dashed line*). **E** Spectral analysis of the UV absorbance of the peak-fraction shown in D

Isolation and purification of a new ichthyotoxic protein

The main steps of separation are presented in Fig. 1. The insoluble pellet derived from protein precipitate (Fig. 1A) revealed ichthyotoxicity when suspended in seawater. It was dissolved in dimethyl sulfoxide (DMSO) (Chang et al. 1991) and separated by a semi-preparative RP column in an HPLC system. The main fraction (Fig. 1B, cross-hatched peak), possessing 32% of total protein substance charged on the column, was ichthyotoxic. This cross-hatched peak (Fig. 1B) was treated by two successive steps of RP chromatography (Fig. 1C, D). The sharpness and the symmetry of the peak obtained (Fig. 1D) provides the first indication of chemical homogeneity. The substance was designated "boxin". As the first step in characterization, its protein nature was assessed.

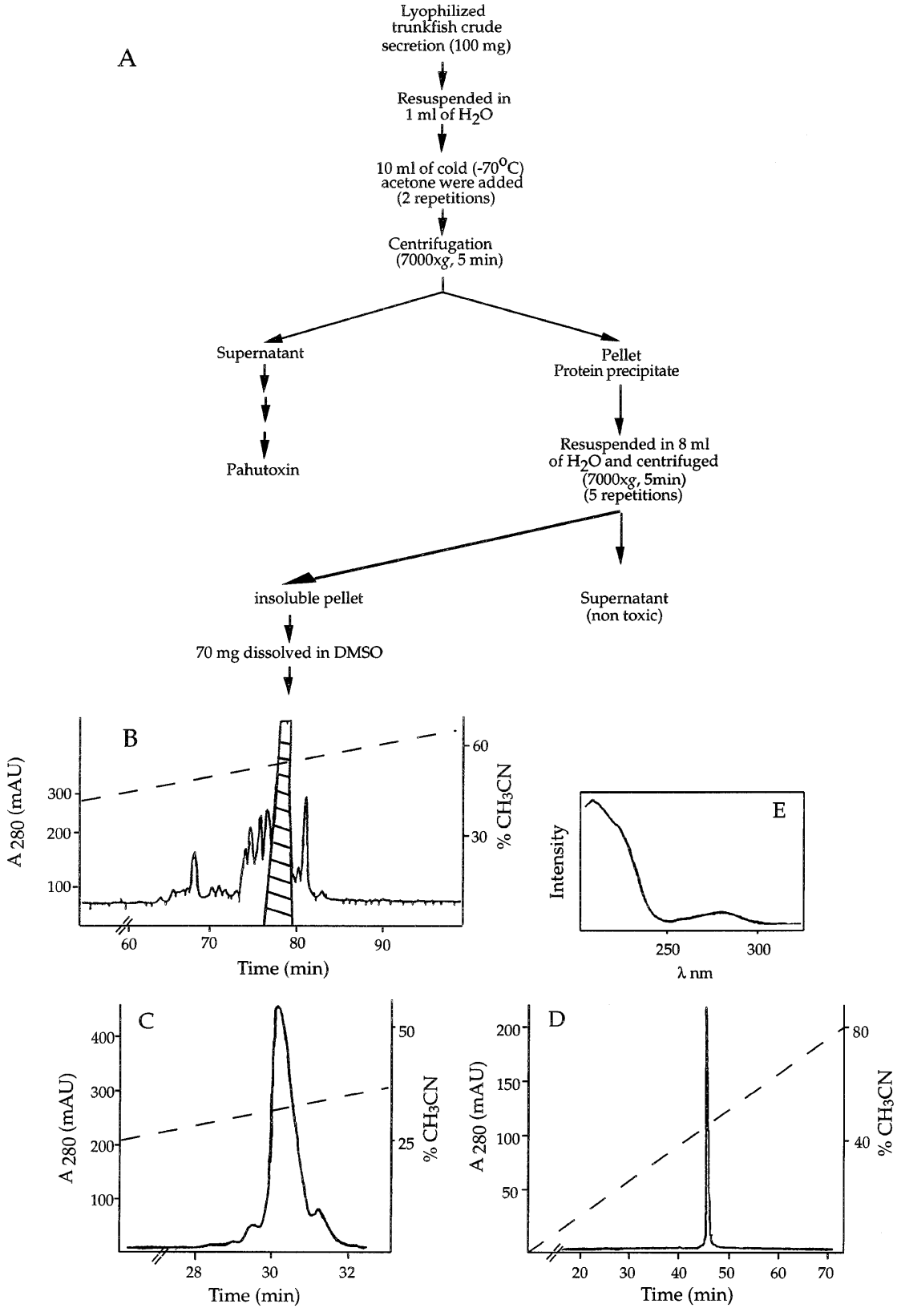
Assessment of protein nature

The protein nature of the final fraction (Fig. 1D) was suggested by the typical UV absorbance pattern shown in Fig. 1E. However, in order to verify that the toxicity of this fraction is due to a protein factor and not simply contamination by pahutoxin remaining from the acetone extraction (Fig. 1A), the fraction was treated with proteolytic enzymes.

The experiment (presented in Fig. 2) examines boxin's vulnerability to proteolytic digestion and heat treatment. As shown, boxin is resistant to Trypsin, withstands boiling, but is destroyed by the potent proteolytic mixture Pronase.

Toxicity

Boxin was shown to possess an LC_{50} value in the standard assay with the *Sparus aurata* fries (see Table 1) of 1.57 µg ml^{-1} (0.088 µ*M*); it corresponds to about 3.5% (by weight) of the entire crude secretion and is responsible for about 3% of the total ichthyotoxicity (data not shown).



Molecular weight

Molecular weight was determined by laser desorption induced time of flight mass spectrometry (LD + TOF-MS) in the laboratory of A. Admon, Department of Biology, Technion, Haifa. In Fig. 3 the signal of 17 876 Da represents the molecular weight of the molecule.

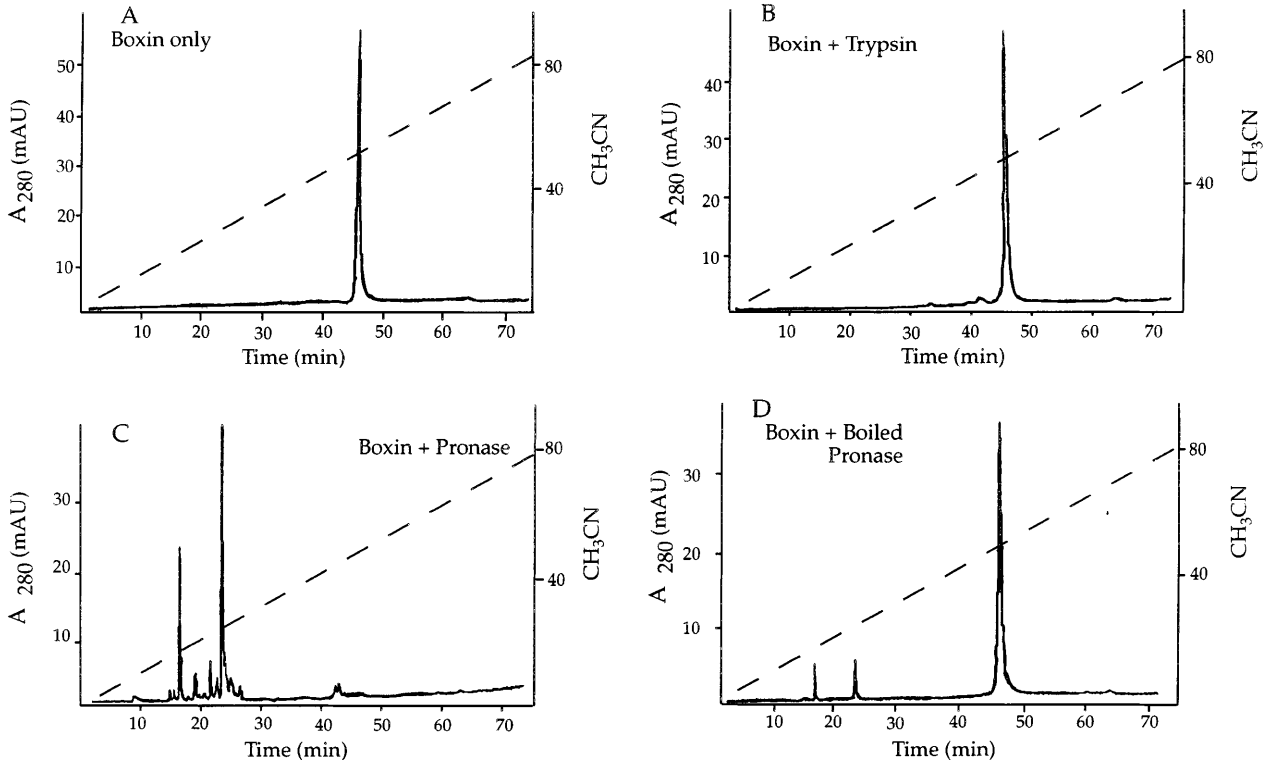


Fig. 2 Proteolysis of boxin. Four portions of boxin, 80 μg each, were placed in separate vials (**A** to **D**). Vial A was the untreated control; Vial B was treated with Trypsin (5%, E/S, 37 °C, 2 h); Vial C was treated with Pronase (5%, E/S, 37 °C, 2 h); and Vial D (with Pronase) was immersed in boiling water for 1 h. When assayed on *Sparus aurata* fries Vials A, B and D were toxic and Vial C was nontoxic

Primary structure

Boxin was shown to possess an amydated N-terminus as revealed by its resistance to the conventional phenyl isothiocyanate cleavage (Edman degradation, Matsudaira 1993). Preliminary amino acid analysis (data not shown) revealed the presence of methionine enabling a CnBr cleavage. The derived fractions were isolated by HPLC (data not shown; Laboratory for Macromolecules, School of Medicine, Hebrew University Jerusalem).

The amino acid sequence of the *N*-terminal segment of the CnBr fragment is presented in Fig. 4. Although the location of the segment in the 18 kDa molecule is presently unclear it supplies supporting evidence for chemical homogeneity [by yielding a single phenylthiohydantoin (PTH)-residue in each degradation step].

Boxin versus pahutoxin

The profound difference in the composition of pahutoxin and boxin (an 18 kDa protein), both present in the trunkfish skin secretion, strongly suggested a comparison of their activities. As shown in Table 1, each of the two substances was assayed for its ability to affect three separate systems, namely the intact animal (fish), cells (human red blood cells) and artificial phospholipid membranes (liposomes). The data presented in Table 1 and Figs. 5 and 6 indicate that:

1. Both substances reveal a similar ichthyotoxicity on the basis of weight; however, on a molar basis, boxin is 33 times more potent.
2. Both substances were not effective by injection. Based on the data in Table 1, if body weight is converted in terms of volume (100 mg = 100 μl) then it appears that boxin and pahutoxin were injected respectively at 350 and 800 times their molar concentrations (LC_{50}) for externally delivered ichthyotoxicity.
3. With pahutoxin the time to lethality reveals a biphasic dose dependence (Fig. 5), and it can be greatly

Table 1 The lethal and lytic action of boxin and pahutoxin (HD_{50} 50% of hemolytic dose)

Effect	Boxin	Pahutoxin
Ichthyotoxicity (LC_{50})	1.57 μg ml^{-1} (0.088 μ*M*)	1.25 μg ml^{-1} (2.87 μ*M*)
Ichthyotoxicity: time to dose dependence	See Fig. 5	See Fig. 5
Injection ichthyotoxicity (LD_{50})	>50 μg 100 mg^{-1} body weight	>100 μg 100 mg^{-1} body weight
Hemolysis	Non-hemolytic	Weakly hemolytic (HD_{50} = 350 μ*M*)
Liposomal permeability	No effect (Fig. 6)	Effective (Fig. 6)

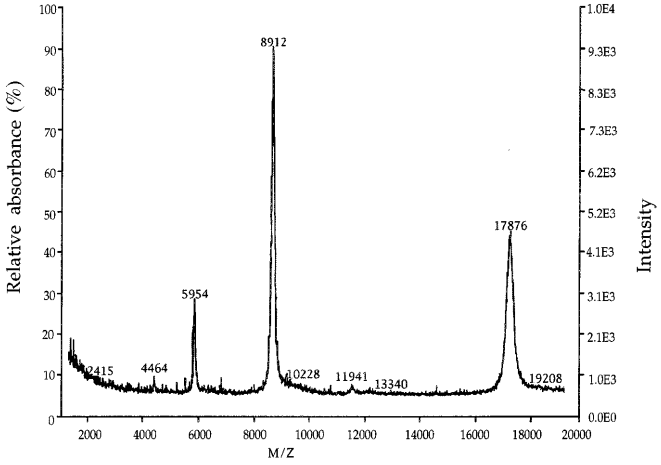


Fig. 3 Analysis of boxin by the method of laser desorption induced time of flight mass spectrometry (LDI + TOF-MS). The signal at 17 876 indicates the molecular weight of boxin. The signal at 8912 indicates half of the boxin mass. The signal at 5954 indicates one-third of boxin mass

Asn-Tyr-Gly-Asp-Trp-Gly-Arg-Trp-Asn-Asn-Tyr-Phe-Cys-Gly-Lys-Thr-Arg-His-Phe-Val-Cys-Ala-Arg-Asn-Thr

Fig. 4 Amino acid sequence of the *N*-terminal section of the fragment of boxin cleaved by cyanogen bromide (CnBr); 300 μg was treated with CnBr, and the solutions were loaded on an Applied Biosystems 1408, HPLC system

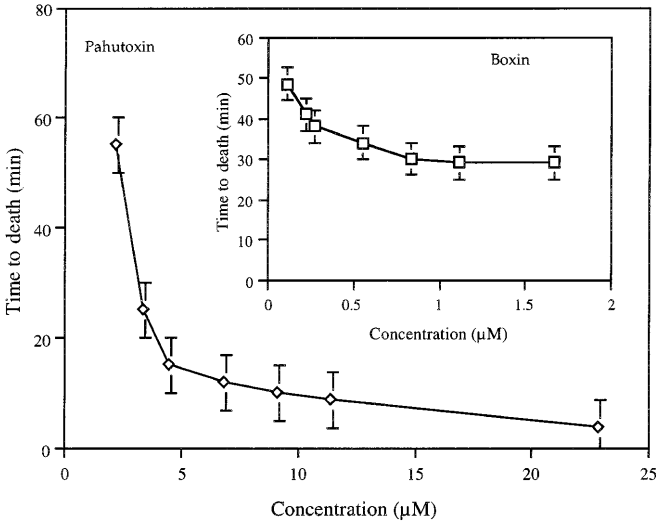


Fig. 5 Lethality time to dose dependence of pahutoxin and boxin. Five to seven *Sparus aurata* fries were used in each point of the decreasing concentrations of pahutoxin or boxin. Death was determined upon cessation of opercular movements. Experiment performed at room temperature

reduced by increasing doses. With boxin, on the other hand, the time to lethality cannot be reduced beyond a certain degree (Fig. 5; see "Discussion").

4. An additional and more striking difference between the two substances is that, in contrast to pahutoxin, boxin is unable to permeabilize cellular or liposomal membranes (see "Discussion").

Discussion

The present study reports the purification and characterization of an ichthyotoxic protein, boxin, derived from a defensive trunkfish (*Ostracion cubicus*) secretion. Boxin was isolated by cold acetone precipitation of the crude secretion (Fig. 1A) and purified by two successive steps of RP-HPLC chromatography (Fig. 1B, C). The protein nature of boxin was demonstrated by spectral analysis (Fig. 1E), proteolysis (Fig. 2), amino acid analysis (data not shown) and sequence determination (Fig. 4). To summarize, boxin is a stable, heat- and proteolysis-resistant protein of 18 kDa molecular weight (see below).

Boxin is not the first ichthyotoxic protein to be found in defensive secretions of fish. Two polypeptides (3 to 4 kDa), pardaxin and grammistin derived from the skin secretion of flat and soap fish, respectively, have been reported by Hashimoto and Oshima (1972) and Lazarovici et al. (1986). Boxin, however, differs from the above amphipathic polypeptides by being devoid of phospholipolytic and cytolytic activity (Table 1). In addition, when compared to the above toxins, boxin is of higher molecular weight (18 kDa instead of 3 to 4 kDa).

When considering the pharmacological specificity and selectivity of proteins in nature, the resemblance between boxin and pahutoxin derived from the same secretion is curious. Both substances affect the fish only through an external application into the surrounding water and are absolutely ineffective by injection. The above mode of externally directed ichthyotoxicity emphasizes the allomonal role of these substances and suggests the possibility that both substances act via specific externally directed receptor molecules, presumably located on the fish gill membranes.

The above avenue of reasoning could lead to the assumption that the two chemically differing substances possess the same mode of action. There are several examples in nature where a simple organic compound such as morphine is mimicked at the receptor level by endorphin polypeptides or where a receptor of a complex polypeptide such as α-bongarotoxin may be occupied by an alkaloid such as tubocurarine. However, the possibility that pahutoxin and boxin act via identical receptors is highly unlikely in view of the fact that boxin, when assessed on a molar basis, is 33 times more potent than pahutoxin (Table 1). Moreover, the distinction between boxin and pahutoxin is made evident by two additional pieces of information: firstly, as shown in Fig. 5, the two toxins reveal a marked difference in their lethality time/dose dependence. Secondly, boxin, in contrast to pahutoxin, appears to be devoid of surfactant activity, as revealed by its inability to permeabilize cellular and liposomal membranes (Table 1; Fig. 6). However, the relation between pahutoxin's surfactivity and ichthyotoxicity has not yet been established. This aspect carries special significance in view of the clear relation which was found between the biological action

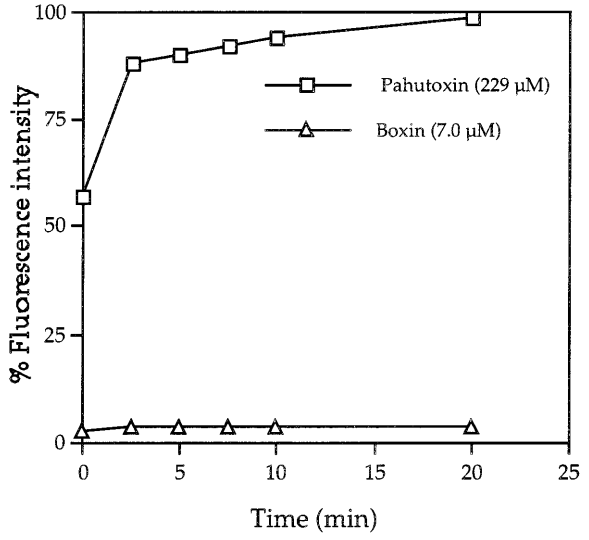


Fig. 6 Effect of pahutoxin and boxin on the release of sodium carboxy fluorescein from liposomes. Technical details are presented in "Materials and methods". Both substances were applied in a concentration which exceeds by 80-fold their ichthyotoxic LC_{50} concentration, namely, pahutoxin 229 μM; boxin 7.0 μM.

of pardaxin and its amphipathic surfactant properties (Bloch-Shilderman et al. 1997).

To summarize, there are, presently, two chemically identified ichthyotoxic substances in trunkfish (*Ostracion cubicus*) secretion, the first is an amphipathic quaternary ammonium detergent-like substance, and the second is an 18 kDa protein. Each substance is responsible for only about 3% of the total ichthyotoxicity of the crude secretion (data not shown). Therefore, the toxicity of the entire secretion can be attributed either to the presence of additional toxins or cooperative interactions among the various constituents. Such cooperativity between pahutoxin and a nontoxic polypeptide substance was recently revealed (Kalmanzon et al. 1999). It thus may be expected that boxin, like pahutoxin, is involved in cooperative interaction. This aspect, as well as the above receptor hypothesis, deserves future study.

However, regardless of the specific role that boxin may fulfill in trunkfish secretion, the very occurrence of an allomonal non-amphipathic protein in the marine environment is attractive from the chemoecological point of view. Two basic properties of boxin, namely its protein nature and chemical stability, are in perfect accordance to its biological role. The protein nature provides boxin with the necessary solubility in seawater and with the pharmacological specificity/selectivity inherent in a protein structure. Its chemical stability enables boxin to withstand the impact (such as degradation or denaturation) of the harmful marine environment.

In summary, in the marine medium, low molecular weight organic allomones, typical for the terrestrial environment, can be replaced by peptides.

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