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## BOXIN – AN ICHTHYOTOXIC PROTEIN FROM BOXFISHES

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## ABSTRACT

The occurrence of toxic proteins in various sources like microorganisms, snake venoms, fish venoms etc have created an intense curiosity and formulation of research to study them. Boxin is one such toxic protein that is less widely known and appreciated. Boxin is a stable, heat and trypsin resistant toxic protein isolated from the crude defensive skin secretions of boxfishes by cold acetonic precipitation and RP-HPLC chromatography. The molecular weight of boxin is reported to be 18 kDa which is known from laser desorption induced time of flight mass spectrophotometry. Boxin has an UV absorbance at 254 nm and 280 nm. In ichthyotoxic front, boxin is 33 times more potent than pahutoxin, which is associated with boxin in the skin secretions. Boxin is known to be the representative of protein fractions found in the toxic mucus of boxfishes. It contributes about 3% of the total ichthyotoxicity to the skin secretion. Apart from boxin, the other protein fractions in the secretions are chemically distinct entities which are reported to be the enhancers of ichthyotoxicity and chelators of pahutoxin performing allomonal roles in the marine environments, due to their high chemical stability and protein nature which ensures them resistance in harmful environments and solubility in sea water. In short, these proteins are ideal candidates which can replace other allomones for cooperative interactions with functional significance.

**Keywords:** Boxin, Pahutoxin, Ichthyotoxicity, RP-HPLC, Allomones

## INTRODUCTION

Proteins are enzymes which catalyze several biochemical reactions in cellular metabolism with few having structural and mechanical functions. Even though proteins are regarded as beneficiary substances forming a major source of diet, deadly properties of protein toxins and venoms are less widely known. Among the marine protein toxins, the venoms of cone shells, box jellyfish, lionfishes, stone fishes, stingrays etc are the most studied. A complex mixture of toxic proteins are found in venoms of several species of box jellyfish family which are life threatening to humans [1]. Conotoxins are powerful neurotoxins isolated from the venom of the marine cone shells. Stonustoxin, a multifunctional lethal protein with hemolytic activities found in the venom of stone fishes, and proteinaceous venom from lionfishes are well acknowledged among fish protein toxins [1]. Stingrays produce venom that is largely a protein toxin which is sensitive to higher temperatures. Stingrays sting with its barb, but it is rarely fatal.

Many species of marine fishes are reported to be ichthyocinotoxic that releases toxic skin secretions into the surrounding water [2]. The chemistry of these secretions are studied only in few representatives of the family Batrachoididae, Solidae, Ostracidae. They were shown to be substantially composed of low molecular weight surfactants or detergents. Members of the family Ostracidae exude an ichthyotoxic, hemolytic substance in their skin's mucous secretions when they are under stress [3, 4]. This toxic secretion in crude form is referred to as Ostracitoxin which are choline chloride esters of palmitic acid. Despite that Ostracitoxin is considered to be non-proteinaceous over the last few years, the occurrence of proteins in it is been demonstrated and its ichthyotoxicity is reported since 1989 [5].

The presence of heat-precipitated proteinaceous mucoid material in the toxic skin secretions of boxfish species is reported by few researchers so far [6, 7]. The pharmacological and chemoecological significance of this soluble protein fraction (SPF) is also confirmed and reported [2, 6]. This protein is known for its regulatory functions as enhancers and carriers of ostracitoxin. In addition, it is also reported to show no ichthyotoxicity. On the other hand, the toxic protein fraction of ostracitoxin is named "Boxin" as produced by the boxfishes [2, 7]. It is by the aid of acetone precipitation and RP-HPLC column chromatography coupled to various bioassays of ichthyotoxicity and cytotoxicity, boxin is isolated from the defensive skin secretion of the boxfishes [2]. Boxin is a stable, heat and proteolysis resistant protein of 18 kDa. Spectral analysis, strong proteolysis, amino acid analysis and amino acid sequence

determination are various methods applied for assessing the protein nature of boxin [2].

Similar to ostracitoxin, Boxin is not efficacious by injection, nevertheless its ichthyotoxicity is achieved only on its release to the surrounding water [2]. This fact propounds that lethality to fishes is mediated by externally located targeted sites called receptors. But still, Boxin is known to have few properties that makes it distinct from ostracitoxin. A remarkable feature is that polypeptides are highly useful in accomplishing allomonal functions in the marine environment due to the high information inherent in their structures and their solubility in seawater and this fact is exemplified by boxin [2, 6].

## Origin of boxin

The members of the family Ostracidae are distributed in shallow waters through the tropical and subtropical seas of the world. They are classified under the order Tetraodontiformes and suborder Balistoidei. Indo-Pacific and Atlantic oceans are regions where these fishes are restricted to. Thirty species under thirteen genera are seen worldwide [8]. However, only six species from four genera are reported in Indian waters [9]. But still, Boxin is reported to be found only in the toxic skin secretions of the yellow boxfish *Ostracion cubicus* (Linnaeus, 1758) till now (Figure 1). Its presence in skin secretions of other members of the family Ostracidae is not reported yet for which more studies are demanded.



Fig. 1: Yellow Boxfish *Ostracion cubicus*

### Isolation methods of boxin

The occurrence of ichthyotoxic proteins in the defensive skin secretion of boxfishes is indicated by two evidences, primarily by the cold acetone treatment which yields an acetonic precipitate with water-extractable ichthyotoxicity and an acetone-soluble, lipophilic, ichthyotoxic fraction, secondly by RP-HPLC separation that separates the ichthyotoxic, hemolytic fraction through gel filtration [6]. The RP-HPLC column yields two ichthyotoxic fractions, the first being relatively polar protein fraction and the second being the highly hydrophobic pahutoxin (pure form of ostracitoxin). The yellow boxfish *Ostracion cubicus* is agitated in a beaker with distilled water of around 30-50 ml which produces the toxic mucus that is lyophilized into a powdered form [10].

The lyophilized crude boxfish skin secretion is resuspended in 1 ml of water to which 10 ml of cold acetone (-70° C) is added twice. This mixture is centrifuged at 7000 rpm for 5 minutes which produces

the supernatant and the pellet [6]. The supernatant is filtered, evaporated which yields a lipophilic fraction that is extracted with chloroform:methanol and in turn is centrifuged at 13000 rpm. The supernatant obtained is separated by RP-HPLC C-18 column which yields the pahutoxin (Figure 2).

The pellet obtained from the centrifuged crude secretion is resuspended in 8 ml of water and centrifuged at 7000 rpm for 5 minutes and repeated 5 times [6]. This produces the supernatant which can be lyophilized to produce the soluble protein fraction. And the insoluble pellet is in turn dissolved in DMSO (Dimethyl sulfoxide) and loaded on a RP-18 semi-preparative column [2]. The main fraction possessing 32% of the total protein substance is ichthyotoxic [2]. This cross-hatched peak is treated by two successive steps of RP-HPLC chromatography which ultimately leads to the separation of Boxin (Figure 2). Chemical homogeneity is first indicated by the sharpness and the symmetry of the peak obtained [2].

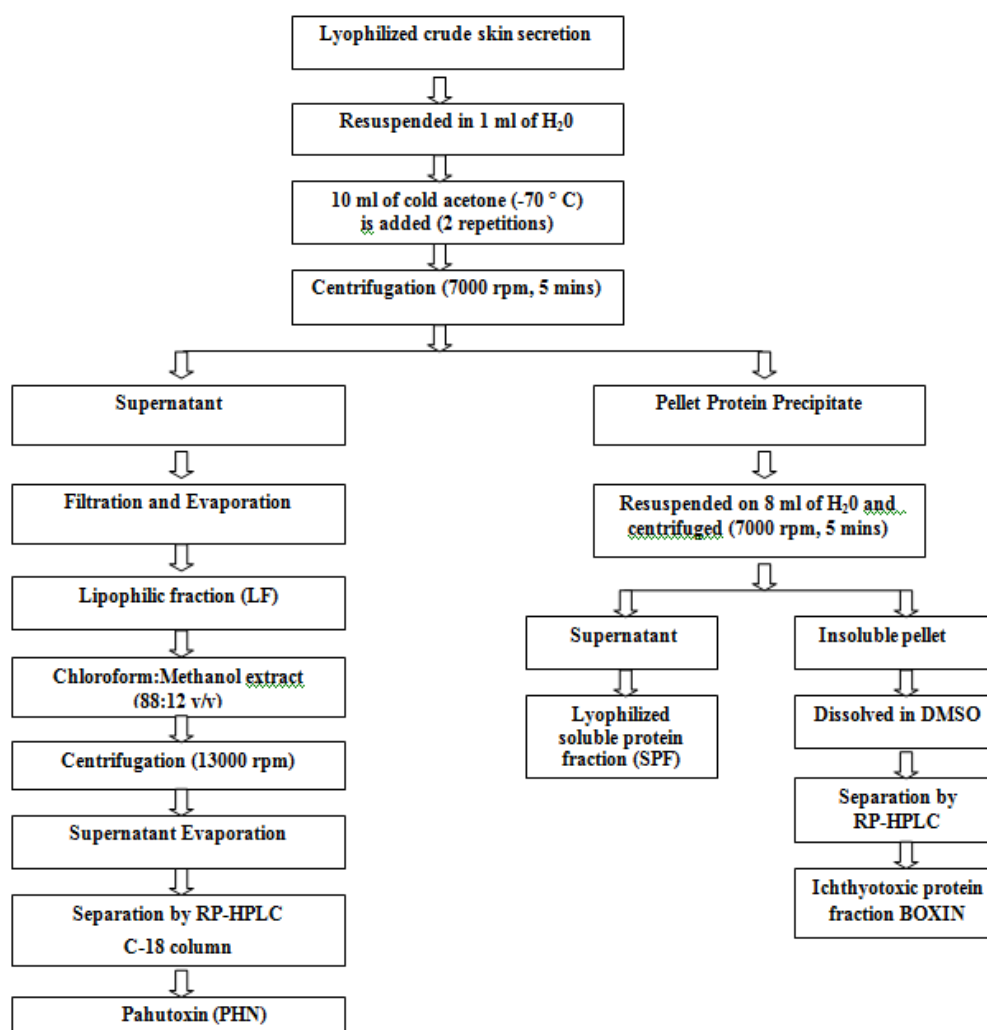


Fig. 2: Isolation of Boxin [6]

### Primary structure of boxin

Due to its resistance to the conventional phenyl isothiocyanate cleavage in Edman degradation, boxin is proved to possess an amidated N-terminus [2]. The presence of methionine which enables the cleavage by cyanogen bromide (CnBr) is revealed by preliminary amino acid analysis [2]. The molecular weight of boxin is determined by laser desorption induced time of flight mass spectrometry (LD+TOF-MS) which represents that boxin is an 18 kDa molecule [2]. The amino acid sequence of the N-terminal segment of boxin which is cleaved by CnBr is determined as:

"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".

### Protein nature of boxin

Assessment of protein nature of the fractions obtained from chromatography is highly essential to prove that pahutoxin is associated with proteins in the toxic mucus. Subjecting to heat treatments or enzymatic proteolysis are the most common ways to ascribe the biological activity of any polypeptide [7]. The soluble protein fraction (SPF) resisted heat at 95°C for 60 minutes and

showed resistance to trypsin at 5% E/S at 37°C for 2 hours. However, it loses its ichthyotoxicity wholly on incubation with the potent proteolytic mixture pronase at 5% E/S at 37°C for 2 hours due to proteolysis. This gives a considerable evidence for the presence of proteins in the crude defensive skin secretions [7]. The presence of proteins can also be affirmed through quantitative assays viz, the Folin phenol assay of Lowry *et al.*, (1951) and the Protein-dye binding assay of Bradford (1976) using the bovine serum albumin (BSA) as the standard. Qualitative assessments of protein employing the non-fluorescent reagent Fluorescamine (Udenfriend *et al.*, 1972) forms highly fluorescing compounds on reaction with primary amines.

The occurrence of proteins is also signified by the formation of trichloroacetic acid (TCA) and ice-cold acetic precipitates. The protein nature of the final fraction is implied by the typical UV absorbance pattern in spectrophotometry. The absorbance at 280 nm and 254 nm as well, denotes that presence of proteins [6]. Amino acid analysis and amino acid sequence determination are other methods to assess the proteins from crude secretions. These techniques are extremely beneficial in understanding that proteins exist in boxfish skin secretions and function as ichthyotoxins or as chelators of pahutoxin [2, 7].

#### Ichthyotoxic property of boxin

No ichthyotoxicity was found in the lyophilized soluble protein fraction (SPF) in contradiction to the lipophilic fraction (LF) [6]. The lipophilic fraction of the toxic mucus has shown notable ichthyotoxicity whereas the soluble protein fraction (SPF) does not (Table 1). Henceforth, it is noted that the soluble protein factor produces a synergic effect i.e., potentiates the ichthyotoxicity of the lipid factor and pahutoxin (PHN) [6]. Concisely, SPF is known to be a chelator for PHN which increases the ichthyotoxic property of PHN (Table 1).

**Table 1: Effects of various substances in different concentrations [6]**

Substances	Concentrations( $\mu\text{g ml}^{-1}$ )	Effects
SPF	500	NA
LF + SPF	3 + 40	Lethal within 10 min
PHN + SPF	0.9 + 50	Lethal within 10 min
LF + BSA	3 + 40	NA
LF + SPF (p)	3 + 40	NA
LF + SPF (t)	3 + 40	NA
LF + SPF (b)	3 + 40	NA

Note: NA – Not active, BSA – Bovine serum albumin, p - Pronase, t - Trypsin, b – A bath of boiling water

Boxin shows an  $\text{LC}_{50}$  value of  $1.57 \mu\text{g ml}^{-1}$  ( $0.088 \mu\text{M}$ ) in the fish toxicity assay using *Sparus aurata* fries [2]. It is confirmed that proteins correspond to about 15% of the dry weight of entire crude secretion in which Boxin forms 3.5% [2]. In fact, boxin is reported to be responsible for about 3% of the total ichthyotoxicity possessed by the crude toxic mucus secretion.

#### Comparison of boxin and pahutoxin

The comparative studies about the activities of boxin and pahutoxin strongly suggests the intense distinction between them (Table 2). The capability of boxin and pahutoxin to affect three separate systems namely whole animal like fish, cells (human red blood cells) and the artificial phospholipid membranes (liposomes) are analysed and compared for determining their dissimilarities [2]. In spite of showing similar ichthyotoxicity on basis of weight by both the substances, boxin differs by revealing 33 times more potency on a molar basis [2]. The lethality of pahutoxin is a biphasic dose dependent one i.e., the time to produce the effect (lethality) in test animals can be highly reduced by increasing the dose concentrations [2]. But that is not the case of boxin where the time to lethality cannot be reduced beyond a certain level.

A phenomenal difference between both the substances is that, in opposition to pahutoxin, boxin is not able to permeabilize the cellular or liposomal membranes [2]. Pahutoxin shows effective hemolysis but boxin produces no prominent hemolysis.

Spectrophotometry unfolds the difference between boxin and pahutoxin through UV absorbance patterns [2]. The protein fraction is absorbed at 280 nm and 254 nm, whereas pahutoxin is absorbed only at 254 nm. Quantitative assays like Folin phenol assay (Lowry *et al.*, 1951) and Protein-dye binding assay (Bradford, 1976) and the qualitative assay using Fluorescamine are used to detect proteins. The Dragendorff assay (Boylan and Scheuer, 1967) detects the quaternary amines thereby identifying pahutoxin. Though various criteria are put forth to distinguish boxin and pahutoxin, there is one particular factor which is analogous in both of them [2, 7]. The two substances are absolutely ineffective by injection and notable ichthyotoxicity is produced on external application into the surrounding water (Table 2) [2, 7].

**Table 2: Contrasting features of Boxin and pahutoxin [2]**

Effects/Methods	Boxin	Pahutoxin
Ichthyotoxicity ( $\text{LC}_{50}$ )	$1.57 \mu\text{g ml}^{-1}$ ( $0.088 \mu\text{M}$ )	$1.25 \mu\text{g ml}^{-1}$ ( $2.87 \mu\text{M}$ )
Ichthyotoxicity: Time to Dose Dependence	No reduction after certain level	Increased doses reduces lethality
Injection	$>50 \mu\text{g}$ $100 \text{ mg}$ $1$ body weight	$>100 \mu\text{g}$ $\text{mg}$ $1$ body weight
ichthyotoxicity	Non-hemolytic	Weakly hemolytic
Hemolysis	No effect	Effective
Liposomal permeability	Positive	Negative
Folin phenol assay	Positive	Negative
Bradford assay	Positive	Negative
Dragendorff assay	Negative	Positive

#### Association of protein and pahutoxin

The occurrence of ichthyotoxic proteins in crude skin secretions are demonstrated by RP-HPLC fraction obtained through gel filtration chromatography and by the acetone-extraction pellet containing active proteins [6]. An intriguing query which arises here is that whether the protein fractions from boxfish secretions are amphipathic surfactant polypeptides alike pardaxin or grammistin isolated from skin secretions of flatfish and soapfish [11-13]. The association of proteins with the low molecular weight lipophilic fraction is showed by the hexane-propanol extract of the protein fraction. It is significant to note that protein-PHN association discloses a substantial degree of specificity which evidences that there may be certain functional importance but this is not yet proved till now [6]. But the striking pharmacological facet pertaining to the proteins is the “phenomenon of potentiation”. Because the soluble protein factor (SPF) as proved non-ichthyotoxic by itself is able to enhance the ichthyotoxicity of both the lipophilic factor and pahutoxin. It is reported that such a phenomenon was not observed previously revealing a novel aspect in the detergent-protein interactions pharmacology [6, 7]. Chemical characterization of the polypeptides is extremely necessary to clarify the molecular basis behind this potentiation if any. It is also ambiguous that whether this potentiation is an outcome of synchronous but independent actions of PHN and proteins on separate target sites, or it is due to the pre-formed protein-PHN associative complexes. This assumption about association is of notable importance as it presents a good deal of pharmacokinetic significance to the binding and enhancing roles of proteins in boxfish secretions [6]. Precisely, during the protein-PHN association, the protein functions during the first stage of intoxication as an “affinity probe” helping by targeting the detergent at critical sites especially on the fish gill membranes [2]. It may also aid in preventing intoxication of the boxfish itself. If it is proved that protein is not responsible for pahutoxin's action, an alternative concept is put forward that the receptors absent in the boxfishes mediates the process of intoxication [6]. Another critical question is that whether the proteins that produce potentiation are identical or different entities. But as far as it is studied, these proteins depict chemically distinct substances.

#### CONCLUSION

Boxin is a representative of the proteins found in boxfish secretions. Spectral analysis, enzymatic proteolysis, amino acid analysis and

sequence determination methods help assess the protein nature of boxin. Boxin is reported to be highly stable, heat and trypsin resistant protein which is subjected to proteolysis by the action of pronase. The molecular weight of boxin is known to be 18 kDa which is higher compared to other toxic proteins. It is not the first ichthyotoxic protein to be found from defensive skin secretions of fish because pardaxin and grammistin are found earlier. Boxin is a non-amphipathic polypeptide devoid of phospholytic and cytolytic activities. Both Boxin and pahutoxin are completely ineffective by injections, but highly toxic to fishes if externally applied into the surrounding water. Boxin is 33 times more potent than pahutoxin in its ichthyotoxicity. The protein fraction in the skin secretions of boxfish are responsible for about 15% total ichthyotoxicity of the entire crude secretion in which boxin contributes 3% ichthyotoxicity. The associative hypothesis actually defines the co-operative interaction of boxin with pahutoxin and interprets the functional role of proteins in the skin secretions. From the chemoecological aspect, it is very attractive to note that proteins are perfect candidates to fulfil the allomonal role in the marine environment notwithstanding the specific action that boxin plays in the boxfish secretions. The high chemical stability offers boxin to resist the impacts of the harmful marine environments. And the protein nature of boxin enables required solubility in sea water due to the high information content intrinsic in its structure. Briefly, proteins or peptides similar to boxin could be replaced for other allomones in the marine environment.

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