

ISOLATION AND CHARACTERISATION OF STINGRAY VENOM TOXIN

Submitted in partial fulfillment of the requirements for the award of Master
of Technology degree in Biotechnology

by

RAGHAVI SURESH



DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119

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This is to certify that this Project Report is the bonafide work of **RAGHAVI SURESH** (41810014) who carried out the project entitled "**ISOLATION AND CHARACTERISATION OF STINGRAY VENOM TOXIN**" under our supervision from January 2023 to May 2023.

Internal Guide

Dr. INDUMATHI S M

External Guide

Dr. KRUPAKAR PARTHASARATHY

Head of the Department

DR. V. RAMESH KUMAR

Submitted for Viva voce Examination held on 05/05/2023

Internal Examiner

External Examiner

DECLARATION

I RAGHAVI SURESH (41810014) hereby declare that the Project Report entitled **“ISOLATION AND CHARACTERISATION OF STINGRAY VENOM TOXIN”** done by me under the guidance of **Dr. Indumathi S M (Internal)** and **Dr. Krupakar Parthasarathy(External)** in Centre for Drug Discovery and Development at Sathyabama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Master of Technology degree in Biotechnology.

DATE: Chennai

PLACE: Chennai



SIGNATURE OF THE CANDIDATE

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ABSTRACT

The presence of marine toxins in seafood poses a health risk to human consumers, which has led to the regulation of the maximum content of marine toxins in seafood in the legislations of many countries. Animal bioassays are used globally to detect the majority of marine toxin groups. The toxins are widely characterized by LC-MS/MS mass spectrometry, and chromatography techniques to isolate compounds separately from the venom mixture. These are then undergone various assays to identify their therapeutic properties. It has become very important to identify and characterize toxins and their components to establish the potential therapeutic properties of these components. In this review, we have discussed various toxins of snake and marine venomous species of different families, the types of classification, and also the methodology used to isolate and characterize the toxin components such as proteins and peptides.

Chapter-1

INTRODUCTION

A toxin is a biologically produced chemical that can cause disease when absorbed by body tissues, some cause mild discomfort and some are lethal. Venom are toxins that are injected by a bite or sting, it is a poisonous liquid secreted by the animal with the help of a specialized gland which is generally associated with the spine, teeth, and sting. The venom's primary purpose is to completely kill or paralyse its prey for defence adaptation. Venoms are concoctions of up to 3000 bioactive substances, such as salts, metabolites, and protein/peptide toxins. Venoms are actively transferred to target organisms via spines, teeth, or modified cell harpoons and are produced in specialised tissues or glands. Most important physiological pathways contain venom targets, which can cause both local (like tissue disruption) and systemic (like paralysis) effects. Venom's various components provide a wealth of novel molecular entities for use in medicine (Kirchhoff et al., 2021).

1.1 Marine envenomation

Marine envenomation is a process in which the victim comes in contact with the toxin-secreting species that results in the venom being released into the tissue. In the case of the marine environment, envenomation occurs as the spine, which causes the mucous sheath to be torn by mechanical pressure, releasing the venom into the wound (Campos et al., 2021). Swimmers, divers, and fishermen are at risk from the venomous water animals (Hornbeak et al., 2017). The marine environment is indeed very dangerous, especially because of the venomous creatures that live in tropical and subtropical regions (Halford et al., 2015). The venom fish make up to 1200 marine fish species. They constitute serious health risks and significant environmental diseases that are very frequently ignored (Thangasami et al., 2018). The sting can be lethal due to the anaphylaxis and acquired hypersensitivity (stings from animals such as jellyfish, ants, wasps, bees, etc.) and direct envenoming (by spiders, jellyfish, Scorpio) (Warrell et al., 2019). Even though marine stings are common not all require medication. Severe envenomation is unusual but includes Irukandji syndrome, stingray, jellyfish stings and blue-ringed octopus envenoming.

Predominantly all marine injuries are caused by jellyfish envenomation (Mubarak et al., 2021).

A toxin is a bioactive compound that is present in venomous animals to protect them from predators and to envenomate the prey. Fish species have been able to adapt to sedentary lives and the decline of squamation thanks to the evolution of venom. They can just raise their poisonous spines in the face of predators rather than running away. The venom must be sufficiently harmful to potential predators to serve as an effective deterrence. The pharmacological research looking at the entire venoms and individual toxins of several fish species demonstrate that fish have therefore evolved extraordinarily strong toxins to work against vertebrate systems. The development of modern techniques helps us in understanding piscine venom (Ziegman et al., 2019). The venom is a mixture of a variety of poisons, enzymes, peptides, and non-proteinaceous substances like serotonin, norepinephrine and acetylcholine (Ziegman et al., 2015).

Even though fish make up more than half of the surviving venomous vertebrates, however only little is known about their venom (Smith et al., 2016). Comparatively, piscine venom is still understudied than those other animal venoms. The most common mode of transfer of venom from fishes is spines which are commonly associated with the dorsal fins, pelvic, anal and pectoral spines (Some species have venomous opercular spines, On either side of their skulls including the stargazer, toadfish, and weever fish. Toadfish and weever fish also maintain poisonous dorsal spines in addition to their opercular spines. The glandular venom tissue is located at the base of these spines in venomous toadfish. All of them are absent in stingrays, which instead develop caudal serrated spines (Ziegman et al., 2019).

Numerous marine toxins from the harmful algae bloom cause the overgrowth of phytoplanktons which contain highly toxic chemicals. These toxins are so-called marine biotoxins. (Botana et al., 2012). The azaspiracid (AZA), brevetoxin (BTX), cyclic imine (CI), domoic acid (DA), okadaic acid (OA), pectenotoxin (PTX), saxitoxin (STX), and yessotoxin (YTX) are eight groups of marine biotoxins that fall based on their unique chemical structures. palytoxin (PITX) and ciguatoxin (CTX) are two other subgroups that are been added (Visciano et al., 2016). Marine toxins have a

profound effect on both humans and marine life. Avian poisonings, fish kills, birth morbidity and death, as well as sanitary ecological, and economic issues are all brought on by the marine toxins. Marine toxins hurt marine mammals as well, often resulting in their deaths, and it has been hypothesised that marine toxins play a crucial ecological role in ensuring their existence (Tubaro and Sosa et al., 2012). The toxin poisoning caused by marine biotoxins is Scombrototoxin fish poisoning, Ciguatera poisoning, Paralytic shellfish poisoning, Neurotoxin shellfish poisoning, Amnesic shellfish poisoning, and Diarrhetic shellfish poisoning (Sobel and Painter 2005).

The cardiovascular consequences which are experienced by the victim who comes in contact with the venom are acute hypotension and cardiac or respiratory failure, which have been blamed for fatalities (Low et al., 1993). During the experimental envenomation change in respiratory and cardiac activity are frequently observed in response to the fish venom. In animal models, it has been demonstrated that the venom of *Scorpaena guttata* and *Scorpaena horrida*. Its reaction has been linked to the activation of secreted endogenous substances at neurokinin receptors (Hopkins et al., 1996).

Venoms' various components provide a rich source of novel molecular entities for use in medicine. Captopril is a drug that was approved in 1981 for the treatment of congestive heart failure and hypertension. There are twelve toxin-based drugs which have been approved for the treatment of cardiovascular diseases, chronic pain, diabetes that are derived from pure venom (snakes, cone snails, and the gila monster). Toxins are basically a research tool to identify new pharmaceutical targets and mechanism of action. The bioactive compound in the animal could help to fill the drug development (Kirchhoff et al., 2022).

Chapter-2

REVIEW OF LITERATURE

2.1 Marine venomous species

Marine toxins have a detrimental effect on marine mammals as well, frequently leading to their demise, and it has been hypothesised that marine toxins play a critical ecological role in maintaining their existence (Winkel and Nimorakiotakis 2003).

Many species of invertebrates and vertebrates have spines that they use as direct protection mechanisms, venom delivery systems for prospective predators and prey, and deterrent defence mechanisms. Conus predatory cone snails have a modified dental component called a radula that rests inside a flexible feeding tube called a proboscis. Both as a self-defence mechanism and to look for prey, the proboscis is expanded. The barbed, harpoon-like radula is launched, enters the tissue, and releases a strong neurotoxic.

The dorsal spines of members of the Scorpaenidae family (stonefish) include venom glands that are utilised to transmit poison. In the case of the stonefish, pressure-sensitive nerves cause the injection of venom via the spine's incision. Stingrays belong to the Chondrichthyes class, and they have modified spines that are found on the distal end of whip-like tails. In most cases, the barbed spine detaches and embeds itself into the victim. Venom is then administered by grooves in the spine's internal anatomy, causing localised tissue destruction and envenomation.

Bite Mechanisms, both invertebrates and vertebrates can use biting mechanisms to deliver venom to potential prey and predators. The Hapalochlaena genus of cephalopods is the most remarkable example of an invertebrate. Through their powerful, bony beaks, blue-ringed octopods can inject potent neurotoxins into a victim's tissues. Sea snakes are elapids that are both poisonous and capable of biting. They do this by injecting venom from glands in their skulls through grooves

in their small, fixed front fangs, which resemble those of terrestrial cobras, kraits, and mambas (Spyres et al., 2020).

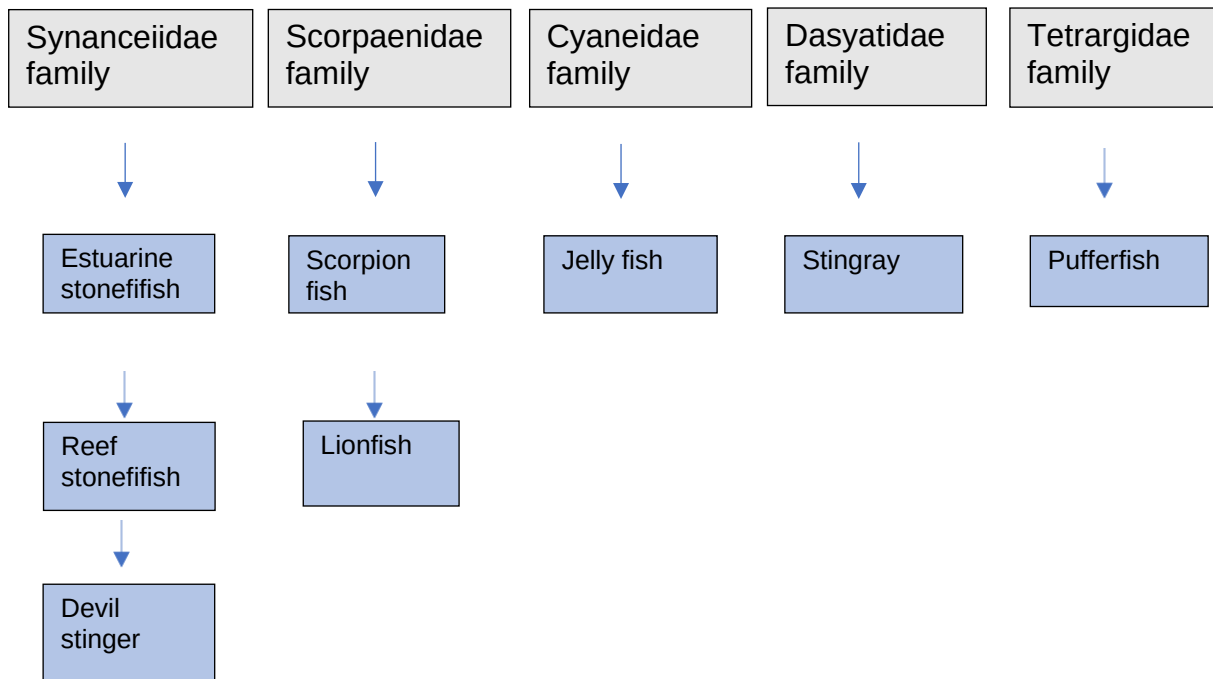


Figure 1.1: Venomous fishes families (Campos et al., 2021)

The scorpionfish, lionfish, and stonefish are members of the huge family of poisonous marine fish known as the Scorpaenidae. Although the majority of stonefish are restricted to the Indo-Pacific, scorpionfish are found throughout the world's tropics, and two species of lionfish from the Indo-Pacific were unintentionally introduced into the Eastern Atlantic.

2.1.1 Stonefish

The stonefish, the most dangerous fish in the world, is a member of the Scorpaenidae, a vast family of marine fish (*Synanceia* spp.) (Diaz 2015). This critically important group of fish are known to poison humans with severe and fatal envenomations. One of the most poisonous fish in the world is the stonefish. When the fish is agitated, up to 15 dorsal fin spines in some *Synanceia* species stand up (i.e. the venom is expelled when pressure is placed on them) (Saggiomo et al., 2021). From the venom glands of the dorsal spines of stonefish specimens gathered

from coral reefs(the most common area the sample is collected), crude venom was recovered (Khalil et al., 2018). Hyaluronidase and Stonustoxin (SNTX), a major pore-forming toxin, are the only known venom components. Only 0.4% of the 170,000 contigs generated by the transcriptomic sequencing of the venom gland were homologous to putative venom proteins (Ziegman et al., 2019).

Although the precise mechanism of action of the venom and each of its constituent parts is still completely unclear, the cytolytic activity of stonefish venom appears to be the result of pore-forming action on cell membranes. They are commonly present in the Indo-Pacific and Northern Australian regions. Common symptoms that are recorded are excruciating pain, rapid swelling, tissue death, muscular weakness, temporary paralysis and very rare case of death. In 2008, one-third of all fish envenomation in Australia was caused by stonefish, which were also the second-most prevalent fish to envenomate humans in Hong Kong (Saggiomo et al., 2021).

Divers encountering stonefish in the Indo-Pacific region first reported cases of Scorpaenidae envenomation in tourists; later, beachcombers discovering shallow tidal marine pools were affected (Diaz 2015).

2.1.2 Scorpionfish

The lionfish and the scorpionfish are both members of the subfamily Scorpaeninae, which is endemic to the Indo-Pacific coastal waters but also extends east and west from the coasts of North Carolina and California, through the Caribbean, and down to Brazil. scorpionfish are less violent than lionfish. The venoms produced by the family Scorpaenidae are heat-labile, nondialyzable, and vary in strength but are quite similar in composition. The following large proteins make up the toxins: hyaluronidase, a factor that causes pain, and affects capillary permeability, and toxic factors that are particular to certain species (Rensch et al., 2021). *Scorpaena plumieri*, venomics has made it possible to correlate the biological activity of the toxins in the venom with skin mucus (Borges et al., 2018). Five components have been identified by chemical analysis of the hemagglutinating fraction of *Scorpaena plumieri* venom c-type isolectin from (SP CL 1-5) (Andrich et al., 2015). The spotted scorpionfish is the most dangerous of the many venomous fish species found along

the Brazilian coast. Fish from the genus *Scorpaena plumieri* make up more than half of all poisonous vertebrates (Campos et al., 2017).

2.1.3 Jellyfish

Each year, jellyfish stings account for over 150 million marine envenomations. Few people need anything more than simple first aid in terms of medical care. However, there are known poisonous jellyfish that are important to human health (Mubarak et al., 2021). When a jellyfish's tentacles come into touch with skin, it stings. When a jellyfish is touched, the millions of microscopic stinging cells (nematocytes) lining its tentacles begin to discharge venom. Nematocysts, also known as cnidocysts, are present in these cells and serve as the venom delivery system. Thousands of cnidocytes can be found inside each cnidarian tentacle. A coiled harpoon covered in venom will be released by the nematocyst in reaction to osmotic pressure or chemical changes. The envenomation is caused by the harpoon's penetration of the target's epidermis, dermis, or infrequently capillaries (Anderson et al., 2009). A mechanism like a harpoon is used to inject the venom into the dermis and release the poison (Berling and Isbister 2015). Traditionally, the bodies of cnidaria were made of a jelly-like substance that encased the internal systems and dangled structures called tentacles from it. The poisonous effect of the venom is mostly responsible for the local and quick skin reaction, which happens between minutes to hours at the location of the sting. The most frequent manifestation of jellyfish envenomation is this reaction. A few days later, the lesions may turn vesicular, occasionally hemorrhagic, and in some cases, as in the case of stings from *Chironex fflleckeri*, even necrotic or ulcerative (Lakkis et al., 2015). Cell damage is caused by cnidarians from the classes Anthozoa, Hydrozoa, Cubozoa, and Scyphozoa creating pores on the cell membrane (Jouiaei et al., 2015).

2.1.4 Weever fish

There are nine living species of poisonous benthic fish in the family Trachinidae, which is divided into two genera (Trachinus and Echiichthys). The bigger weever (*T. draco*) and the lesser weever are the two primary poisonous weever fish species (*E. vipera* - also referred to as *Trachinus vipera*). Weevers are usually found in temperate areas throughout the Mediterranean, North, Celtic, and Irish Seas, reaching in certain cases the Atlantic coast of North Africa. This contrasts with other poisonous marine species, which are found in the Indo-Pacific (Gorman et al., 2020). Weevers have convergently developed dorsal spines that house several anterolateral grooves with venom glands, lined on the interior of the spine with massive glandular cells, like other venomous fish such as stonefish (Smith et al., 2016).

2.1.5 Pufferfish

Pufferfish, members of the Tetraodontidae family, amass large amounts of the dangerous neurotoxin tetrodotoxin (TTX), which they mostly store in their liver and ovaries. Additionally, paralytic shellfish poisons have reportedly been found in some pufferfish species (PSTs). Arothron pufferfish, which are marine, contain significant quantities of PSTs and TTX. In addition, PSTs are a significant toxin and enough accumulate in freshwater pufferfish of the genus Tetraodon (now the genus *Leiodon* or *Pao*), including *Leiodon cutcutia*, *Pao fangi*, *P. leiurus*, *P. suvattii*, and *P. turgidus*, as well as the genus *Sphoeroides*, to cause food poisoning episodes (Nagashima et al., 2018).

Fish belonging to the family Tetraodontidae, which contains 130 species and is divided into 19 genera, have the potential to carry tetrodotoxins. Tetrodotoxin is a neurotoxin that causes human intoxication and death, frequently after eating puffer fish. Fugu, which is regarded as a culinary delight, is widely consumed by Japanese people. Between 0.18 and 0.2 mg of TTX have been shown in case studies to cause severe symptoms in humans, and a fatality was reported following ingestion of about 2 mg of TTX (Rambla-Alegre et al., 2017).

The history of the deadly neurotoxic principle known as TTX goes back to the very beginning of civilization. When Emperor Shun Nung ruled China, it was first recognised that the parent puffer fish was toxic (2838 B.C. – 2698 B.C.) (Makarova et al., 2019). Since many years ago, tetrodotoxin (TTX) has been recognized as a sodium channel blocker, preventing the generation or spread of action potentiation in nerve cells. In investigations on neurophysiology, TTX is a common research instrument and a local anesthetic. The successful chemical synthesis of TTX and its analogues is due to the growing demand for TTX in research projects (Li et al., 2020).

The consensus is that tetrodotoxin serves as a chemical defence, but as with all traits, natural selection can diversify and increase its adaptive uses. Three broad categories can be used to classify behavioural reactions to TTX. Some animals respond to TTX by becoming attracted to it, while others avoid it or flee from it, and still, others slow down or reduce their activity. In a separate area of biomedical research, TTX has received more attention as a potential treatment for several human diseases [12]. We contend that a better comprehension of the history and ecological functions of TTX could enhance its use as a therapeutic (Bucciarelli et al., 2020).

2.1.6 Stingray

The Dasyatidae family of venomous fish includes marine stingrays, which are primarily found in temperate and tropical regions of the world. One of the most dangerous venomous fish in the world is the stingray. At the base of their tails, they have sharp spines that pierce the victim's body. Venom is released into the sting site by the epidermal covering of the spine, which causes excruciating pain and tissue necrosis. Stingray venom is made up of proteins, serotonin, vasoconstrictor peptides, and several other unidentified substances. It has neurotoxic, cardiotoxic, fibrinogenolytic, and anticoagulant properties (Rajeshkumar et al., 2015).

Because the sting puncture that develops into inflammatory events, including necrosis, in humans, has medical significance, stingray skin secretions are extensively studied. According to the literature, these toxic effects are related to the

biochemistry of the sting mucus. The biological functions of fish skin include controlling the osmotic pressure gradient and providing defence against external forces and microbial infections (Coelho et al., 2019). The current research suggests that *D. sephen* venom induces apoptosis by increasing intracellular ROS, lipid peroxidation, and apoptosis in human cervical (HeLa) cancer cells while decreasing cell proliferation, antioxidant status, and mitochondrial membrane potential (Rajeshkumar et al., 2015). The transcriptomes of the five newly sequenced and three reanalyzed stingray venomous tissues contained numerous phospholipase A2 (PLA2) candidates. The identification of several members of the three-finger toxin (TFT) family, which has so far only been discovered in snake venom, was also studied. The TFT proteins from sea snakes and stingrays exhibited a lot of similarities (Kirchhoff et al., 2021). According to a recent study, a natural peptide can strongly constrict blood vessels in the microcirculatory environment. By combining de novo amino acid sequencing with mass spectrometry, the peptide was fully sequenced and recognised as a novel peptide (Conceição et al., 2006).

There are several potential advantages for basic research, clinical diagnosis, the creation of new therapeutic agents, and the production of antiserum from characterising the peptide content of venoms. Techniques like chromatography and mass spectrometry have been used to thoroughly analyse the peptides and small proteins of crude samples (Conceição et al., 2006).

The venom compounds that are been identified so far from the string ray venom are (Mohebbi et al., 2016):

5-HT, 5 – nucleotidase

Acetylcholine

Phosphodiesterase

Proteolytic enzymes

(against- casein, gelatin, fibrogen)

Toxins:

Cystatins – 13.26 KDa

Galectin – 30 KDa

Peroxiredoxin 6 – 26 KDa

Orpotrin – 1 KDa

Porflan – 2 KDa

Coronin – 42 KDa

Cytochrome C – 12 KDa

Peptides and Proteins:

Alpha subunit haemoglobin – 15.8 KDa

Ganglioside GM2 activator – 1.38 KDa

Glutathione-s-transferase μ – 25.8 KDa

Leukocyte elastase inhibitor – 30 KDa

Transaldolase – 35 KDa

ATP synthase – 25.53 KDa

Hyaluronidase – 79 KDa

Galectin – Diverse functions that include anticoagulant, procoagulant, platelet modulating, myotoxin and hemagglutination activities.

Cystatins – They are present inhibitors of cysteine proteinases, including paprin and cathepsins.

Peroxiredoxin 6 – Hydrolysis lipid through PLA2 activity is one of the most important functions.

Orpotrin and Porflan – Have vasoconstrictive and inflammatory effects.

DELTA-alicitoxin-Pse1b - DELTA-alicitoxin-Pse1b is a pore-forming protein that can provoke hemolysis

The stingray venom is basically broken down into four parts:

Neurotoxin: Lack of coordination is brought on by neurotoxins, which are followed by gradual paralysis and, in the case of severe envenomation, respiratory failure. Neurotoxic venoms take time to enter the body and impact the nerves; as a result, symptoms frequently get worse for several hours to a day before becoming better. This is referred to as "non-wounding" in video game lingo. Fatigue injury starts with DX penalties and progresses to full paralysis and ultimately respiratory failure as more fatigue is accumulated. The term "non-wounding" fatigue refers to the idea that the neurotoxin's cumulative exhaustion has no further effects outside of influencing when the symptoms manifest.

Cardiotoxin: They have an impact on the heart, and too much might result in cardiac failure. Cardiotoxins also require time to work, much like neurotoxins. If the venom damage exceeds 1.5 FP, this is referred to in-game as Fatigue damage with a heart attack as a symptom. For ease of explanation, cardiotoxin is also used to account for the symptom of nausea that appears at 1/3 FP.

Necrotoxin: Necrotoxins cause inflammation, edoema, and a necrotic wound by causing localised tissue death. Necrotoxins, like the first two kinds, take time to harm a person. The initial few hours after an accident are when damage is most acute, but the wound continues to deteriorate for several days. The game treats this as HP damage to the area where the stinger hit.

Immediate pain: A chemical is injected into the wound, stimulating the pain neurons and causing crippling pain that lasts for several hours. In terms of the game, this is immediate non-wounding toxic damage that, if the damage exceeds 1/3 HP, causes extreme discomfort. Because it doesn't scale with the size of the victim, this damage isn't counted as fatigue damage, but instead heals quickly—every ten minutes counts as one day of healing.

Chapter-3

AIM AND OBJECTIVES

AIM:

To isolate and characterize the toxin from stingrays.

OBJECTIVE:

- To isolate the toxin from pufferfish and stingrays collected from Kasimedu, Chennai, Tamil Nadu.
- To carry out the Primary extraction and purification of the toxin from each sample.
- To perform the characterization of the toxin by LC-MS/MS mass spectroscopy MALDI-TOF.
- To identify the property of the compound extracted using various specific assays.

Chapter-4

MATERIALS AND METHODS

4.1 Sample collection

Five Stingray tails were collected from the area of Kasimedu, Chennai, Tamil Nadu (13.1251° N, 80.2955° E). The tails were stored in the ice box and brought back to the laboratory and stored at -20°C in a container until further use.

4.2 Homogenization

The tissue and skin from the Barb region were dissected and cut into minute pieces. They were added to the tissue homogenizer with 2ml of 1X PBS buffer and homogenised for 30 minutes. The homogenized sample is then transferred to the eppendorf and centrifuged at 8000rpm for 10 minutes. The supernatant was collected and the pellet was discarded. The supernatant is then stored at -20°C.

4.3 Protein Estimation

The protein estimation was carried out by Bradford Assay. The BSA standard curve was derived. The protein estimation was then carried out for each sample. The sample was added to the 96 well plate in 5 different concentrations along with Bradford reagent and observed at 570nm.

4.4 SDS-PAGE

SDA-PAGE (12%) was carried out with 40 µl of sample in each well and BSA was used as marker and BioRad protein molecular marker was also used to determine the molecular weight of the band formed.

4.5 Electro-Elution

Electro-elution was carried out on the selected bands (5 bands) to isolate the protein of that particular molecular weight. The electro-elution buffer was prepared (1Xtransfer buffer, 1% SDS). The band were cut from the unstained gel using a sterile slide. These were then cut into fine pieces and added to the dialysis bag (colour coded) that was prepared earlier along with 500 µl of elution buffer. The elution was carried out at 4°C for 2 hours at 100V and reverse current for 5 minutes. The gel pieces were removed and the bags were tied back.

4.6 Dialysis

The dialysis bag was added to the 1XPBS in which the dialysis takes place at 4°C. The buffer was changed every 3 hours interval for 6 cycles. The sample is then transferred to the eppendorf and stored at -20°C. Protein estimation was carried out for each band that was isolated.

4.7 Screening of eluted bands

Protein estimation for each eluted band from each sample was carried out. Further, they were run in SDS(12%) for band conformation. Bands were eluted from Sample 1, Sample 2, and Sample 3. The number of bands varies from each sample.

4.8 Haemolysis Activity

The activity of the sample over the human erythrocytes was calculated by using this haemolytic assay. For performing this assay goat blood was collected in the EDTA tubes. 1ml of blood was centrifuged to remove the plasma layer then PBS wash was given three times. Now the pelleted erythrocytes were mixed with basic saline. From that 50µl of the blood is taken in the tube and 100µl of the sample in different concentrations (1µg/µl, 2 µg/µl, 4 µg/µl, 6 µg/µl, 8 µg/µl) was taken. 0.1% Triton x100 as a positive control and 1X PBS as a negative control. Then the tubes were incubated for one hour at 37 °C. After incubation 850µl of the 1X PBS was added to each tube and centrifuged at 3000rpm for 3min. the supernatant obtained from the

centrifugation was transferred into the 96microtiter well plate and observed under 540nm.

$$\begin{array}{lcl} \text{Percentage of} & = & \frac{\text{ABS of Sample} - \text{ABS of Negative control}}{\text{ABS of Positive control} - \text{ABS of Negative control}} \times 100 \\ \text{Hemolytic activity} & & \end{array}$$

Chapter-5

RESULTS AND DISCUSSION

5.1 Sample collection

Five Stingray were collected from Kasimedu, Chennai, Tamil Nadu (13.1251° N, 80.2955° E). The tails were dissected and stored in an ice box. The other parts of the fish were discarded. The ice box was sealed properly and brought back to the laboratory and stored at -20°C .



Figure 5.1: Five stingray ray tail samples collected in an ice box from Kasimedu, Chennai, Tamil Nadu.

5.2 Homogenization

There were a total of 5 tails that were collected. Tissue samples were dissected individually from each tail and collected separately in eppendorf (Sample 1, Sample 2, Sample 3, Sample 4, Sample 5). The tissue from the barb region was dissected using a sterile dissection kit and weighed. The tissue collected from each tail was homogenized individually in 1X PBS buffer. For each tail, 2 ml of 1X PBS was added for the homogenization process. They were then centrifuged at 8000rpm for 10 minutes. The supernatant was collected separately from each centrifuged sample in a 2 ml eppendorf and labelled as Sample 1, Sample 2, Sample 3, Sample 4, and

Sample 5. All the samples are stored at -20°C until future use. The supernatant contains the bioactive compounds and the pellet are discarded.

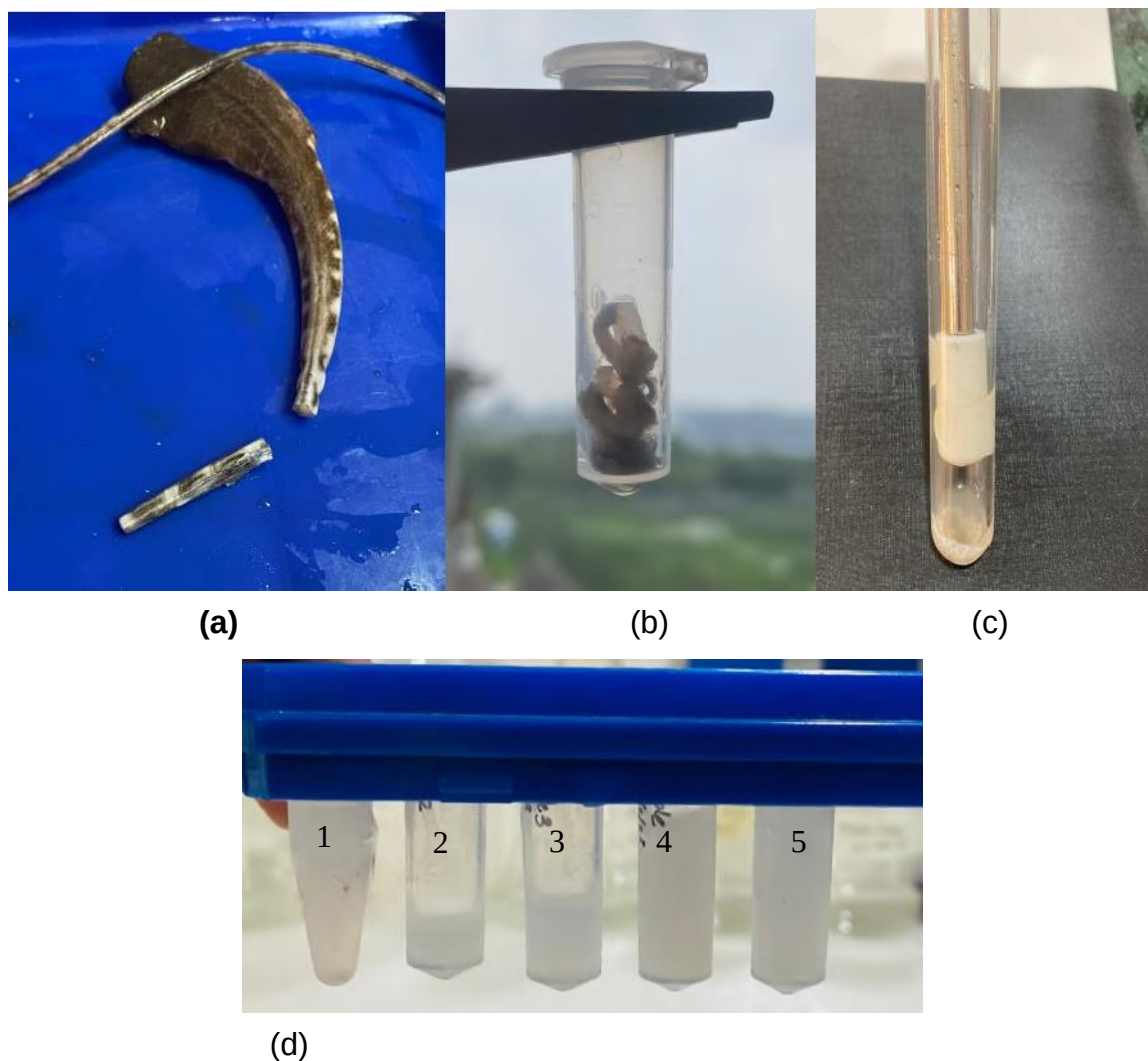


Figure 5.2: (a) Individual tail was cut at the barb region. (b) The tissue from the barb region was dissected. (c) The individual tail tissues are been homogenised in the tissue homogenizer in 1X PBS separately. (d) After centrifugation, the supernatant was collected in separate tubes from each tail sample.

5.3 Protein Estimation

Protein estimation was performed with respect to BSA standard curve. Sample 1, Sample 2, Sample 3, Sample 4, Sample 5 were added at different concentration along with 200 μ l Bradford reagent. Read at OD 570nm.

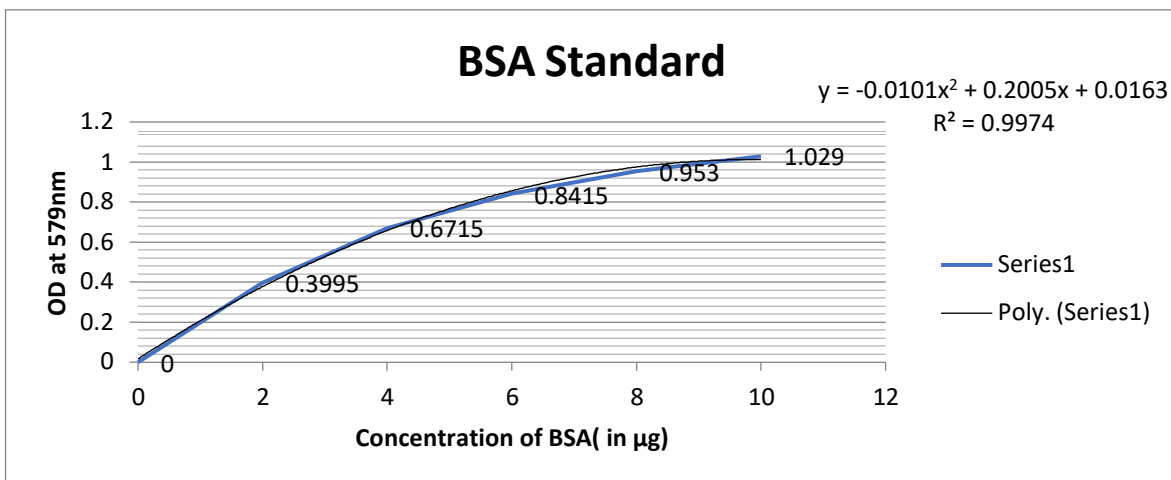


Figure 5.3:BSA standard graph

Table 5.1: BSA protein concentration in various volume.

Volume of BSA	Concentration mg/ml
0	0
2	1.07
4	1.03
6	0.96
8	0.93

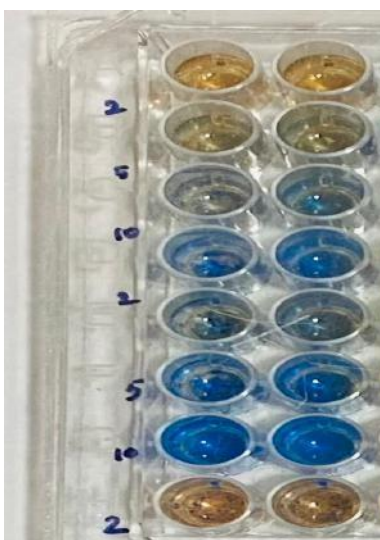


Figure 5.4: Protein estimation for samples 1- 5 in 96 well plate.

Table 5.2: Protein concentration in each sample

Sample	Protein concentration	Available volume
Sample 1	0.37 mg/ml	2 ml
Sample 2	0.86 mg/ml	2 ml
Sample 3	0.54 mg/ml	2 ml
Sample 4	0.92 mg/ml	2ml
Sample 5	0.49 mg/ml	2ml

5.4 SDS- PAGE

SDS gel (12%) was run with Lower range molecular weight, BSA, High range molecular marker along with Sample for band identification for Sample 1 , Sample 2, Sample 3.

Table 5.3: Volume loaded in each lane is for Sample 1 band identification

Lane	Sample	Volume
1.	Lower Molecular-range Marker	5 μ l
2.	BSA	5 μ l
3.	BSA	10 μ l
4.	BSA	5 μ l
5.	BSA	10 μ l
6.	SAMPLE 1	70 μ l
7.	Higher Molecular – range Marker	5 μ l

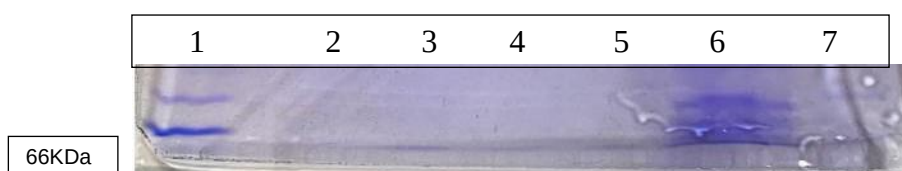


Figure 5.5: Band formation of Sample 1 is observed.

Table 5.4 Volume loaded in each lane for Sample 2 band identification

LANE	SAMPLE	VOLUME
1	BSA	10 μ l
2	Sample -2	30 μ l
3	Sample -2	40 μ l
4	Sample -2	60 μ l

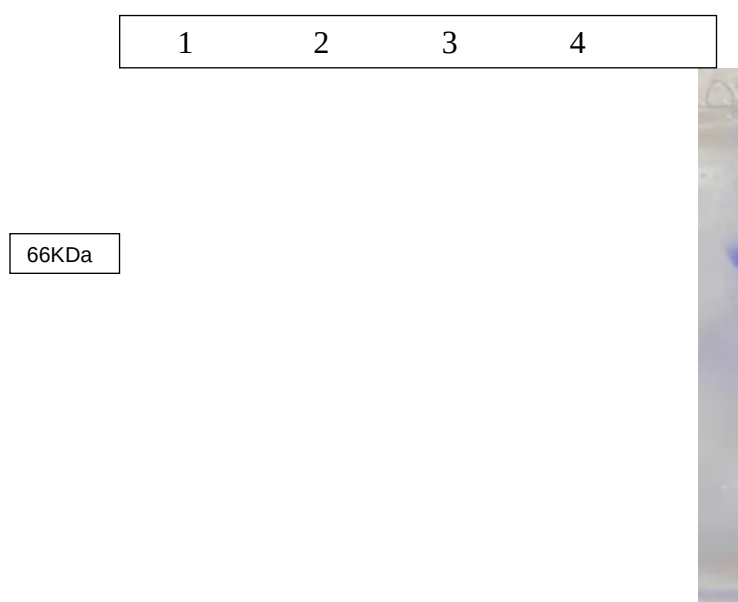


Figure 5.6: Band formation of Sample 2 is observed

Table 5.5 Volume loaded in each lane for Sample 3 band identification

LANE	SAMPLE	VOLUME
1	BSA	10 μ l
2	Sample - 3	30 μ l
3	Sample - 3	40 μ l
4	Sample - 3	60 μ l
5	Lower molecular weight	5 μ l

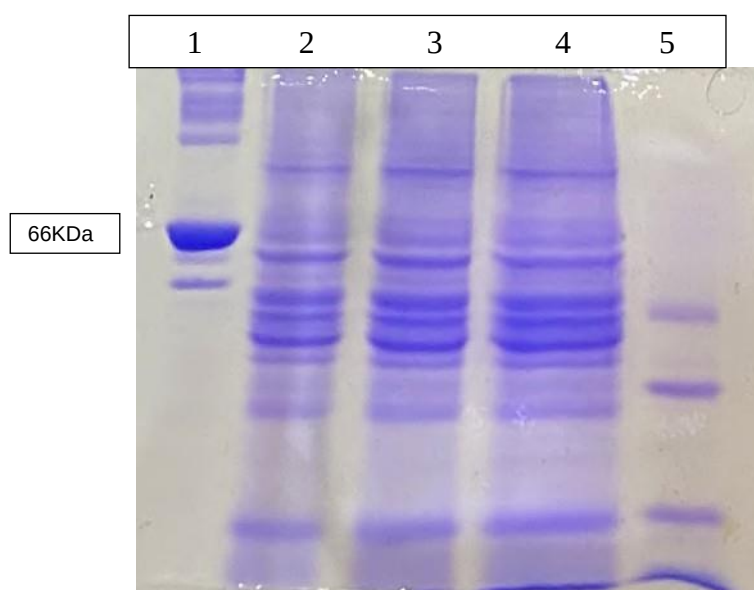


Figure 5.7: Band formation of Sample 3 is observed

5.5 Electro-Elution

In this process from each gel run specific bands are been selected for the elution process. The process was carried out to elute the protein from the gel to the buffer. From Sample 1, 5 bands were selected for elution and were eluted. From Sample 2, there were 3 sets of elution process was performed. Similarly, the process was carried out for Sample 3 with different sets of bands. Elution was carried out in the

dialysis bag where the gel piece and e; elution buffer were tied together. They were run on a transfer buffer where the temperature was maintained to be 4°C. The elution was run for about 2 hrs at 100V and a 5 minutes reverse current was applied such that the protein attached to the wall of the dialysis bag are detached. The dialysis bags are been colour coded for each band. A total of 5 batches of elution were carried out. (Batch1 - Sample 1, Batch 2,3,4 - Sample 2, Batch5 – Sample 3). The gel run and volume of sample loaded in each sample remain the same for all. The elution process is performed on the unstained gel piece that was cut and stored in the cold room. Only the Lane 1 and 2 were cut and allowed for overnight staining, they acted as the indicator where the bands are present for us to cut upon the unstained gel.

Table 5.6: Volume of Sample 1,2 and 3 in each gel run for the elution process

LANE	Sample	Volume
1.	Sample 1/ Sample 2/ Sample 3	40 μ l
2.	Sample 1/ Sample 2/ Sample 3	40 μ l
3.	Sample 1/ Sample 2/ Sample 3	40 μ l
4.	Sample 1/ Sample 2/ Sample 3	40 μ l
5.	Sample 1/ Sample 2/ Sample 3	40 μ l
6.	Sample 1/ Sample 2/ Sample 3	40 μ l
7.	BSA	10 μ l

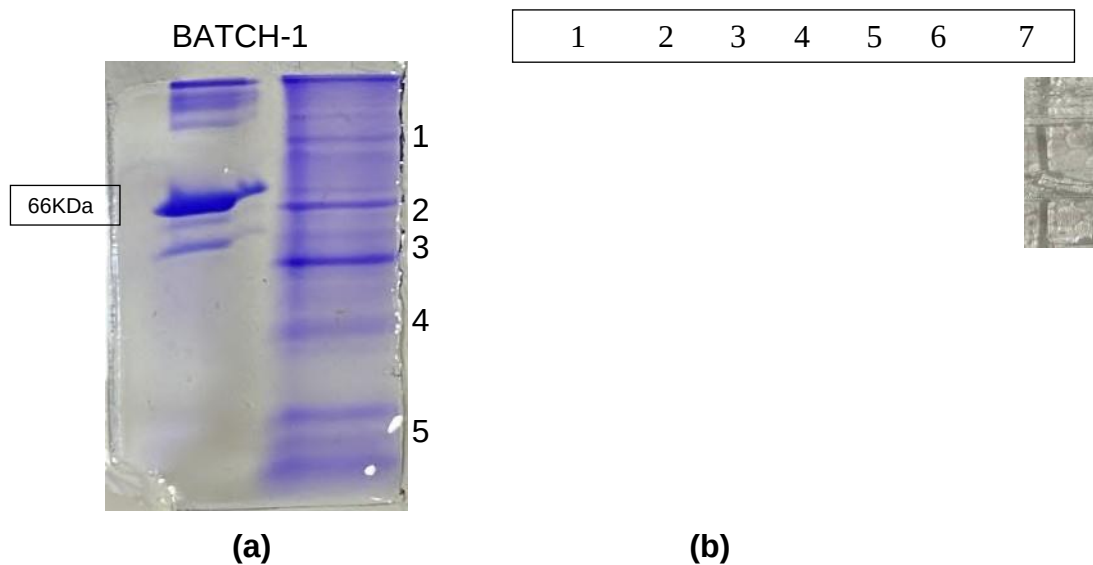


Figure 5.8: (a) Batch 1 of Sample 1, 5 Bands were selected. (b) The selected 5 bands are cut into small pieces for the elution process separately.

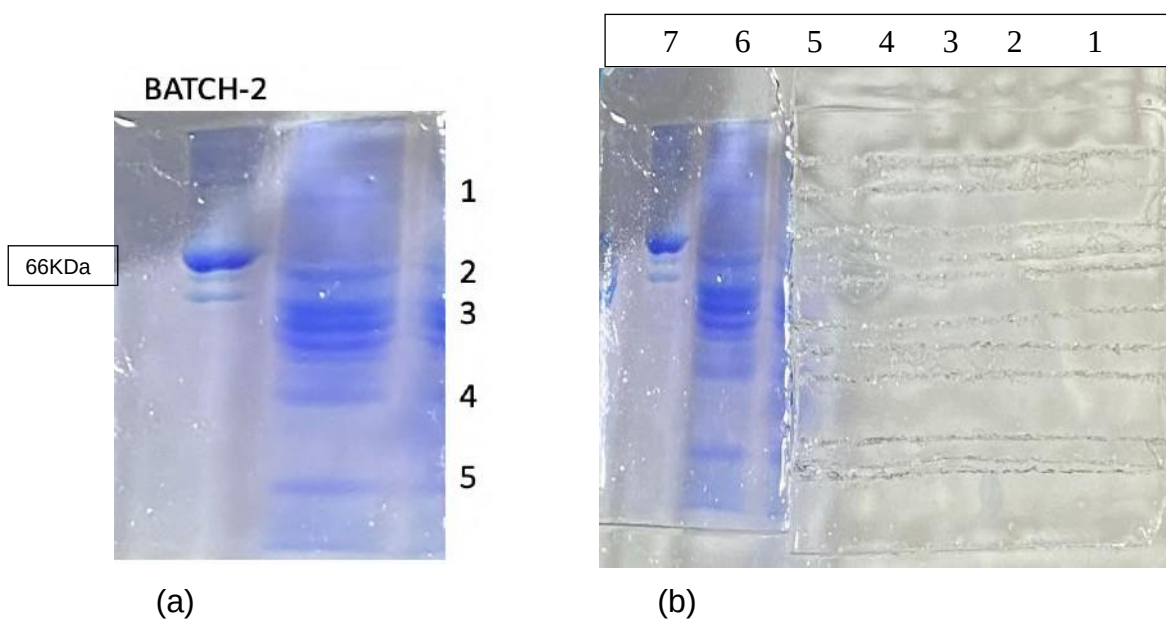


Figure 5.9: (a) Batch 2 of Sample 2, 5 bands were selected for elution. (b) the selected bands are cut separately for the elution process.

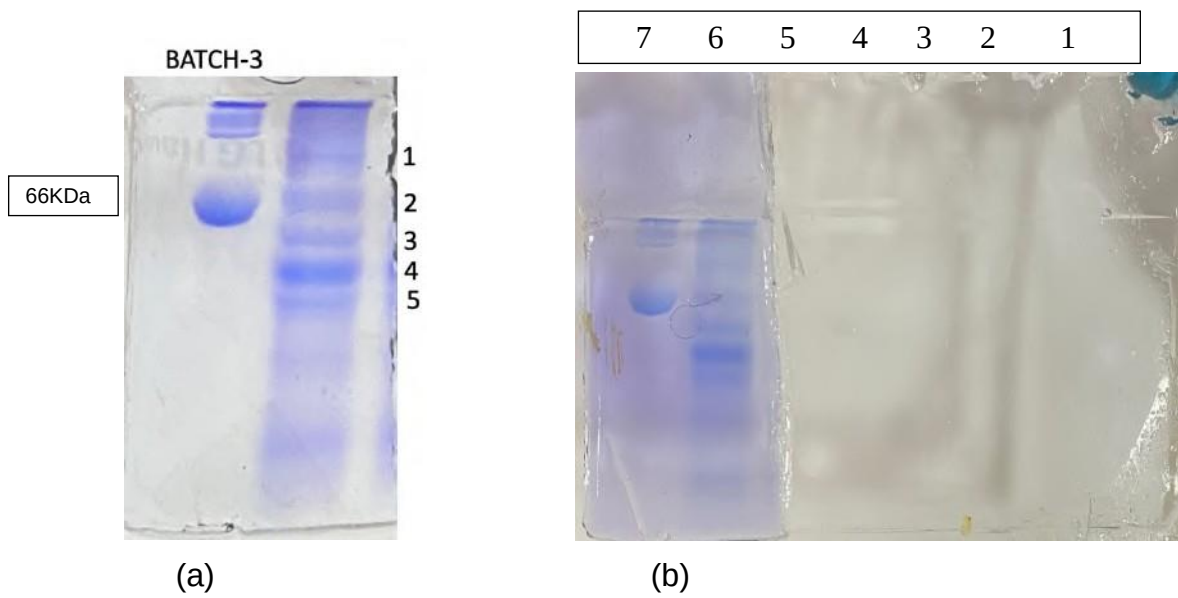


Figure 5.10: (a) Batch 3 of Sample 2,5 bands were selected for elution. (b) the selected bands are cut separately for the elution process.

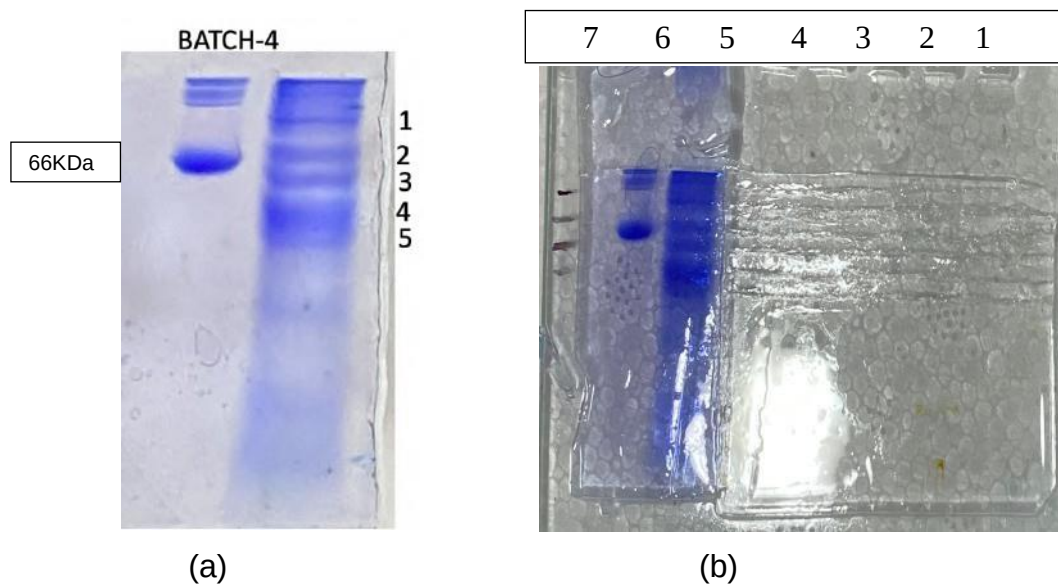


Figure 5.11: (a) Batch 4 of Sample 2,5 bands were selected for elution. (b) the selected bands are cut separately for the elution process.

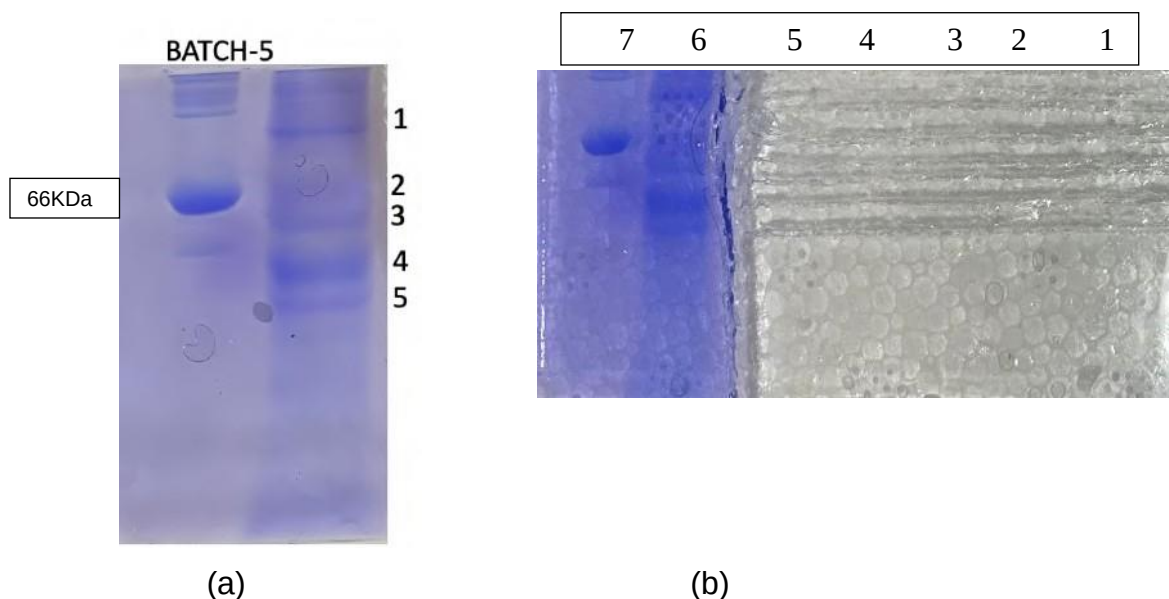


Figure 5.12: (a) Batch 5 of Sample 3,5 bands were selected for elution. (b) the selected bands are cut separately for the elution process.

Figure 5.13: Elution process carried out in the boat with transfer buffer where the dialysis bag consists of the selected bands respectively.

5.6 Dialysis

Once the elution process was complete the gels from the elution bands are allowed for staining overnight for confirmation if the protein is been eluted or not. After the removal of gel pieces, the dialysis bags are tied again and immersed in 1XPBS at 4°C. The buffer was exchanged with fresh 1X PBS buffer for every 3hours. 6 cycles were performed for each elution. Once the dialysis process is complete the elution

buffer in the dialysis bag is exchanged with PBS buffer. They are then transferred to 1.5ml Eppendorf and stored at - 20°C.

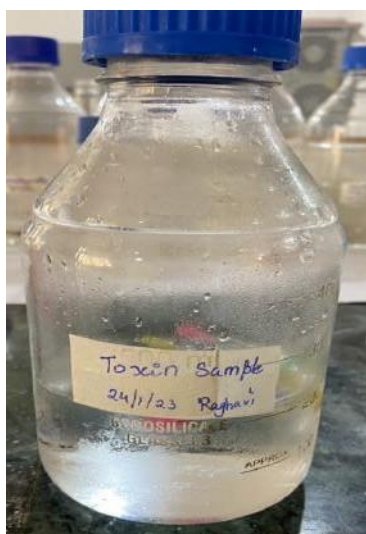


Figure 5.14: Dialysis container with 1X PBS buffer.

5.7 Screening of eluted bands

After the collection of each eluted protein sample protein estimation was performed for each band that was eluted in all 5 batches.

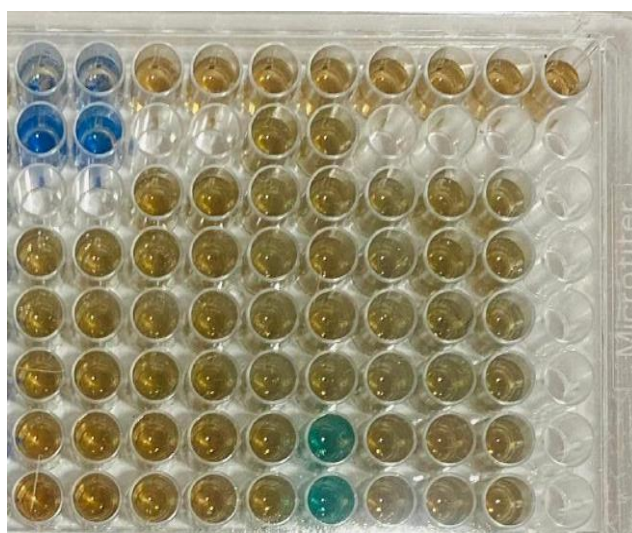


Figure 5.15: Protein estimation of all 5 batches in 96 well plate.

Table 5.7: (a) Protein concentration of Batch 1. (b) Protein concentration of Batch 2. (c) Protein concentration of Batch 3. (d) Protein concentration of Batch 4. (e) Protein concentration of Batch 5.

(a)

Sample	Protein Conc	Volume
Band 1	-0.035 mg/ml	85 μ l
Band 2	-0.051 mg/ml	93 μ l
Band 3	-0.050 mg/ml	97 μ l
Band 4	-0.060 mg/ml	88 μ l
Band 5	-0.032 mg/ml	83 μ l

(b)

Sample	Protein Conc	Volume
Band 1	0.001 mg/ml	248 μ l
Band 2	0.049 mg/ml	238 μ l
Band 3	0.102 mg/ml	193 μ l
Band 4	-0.005 mg/ml	79 μ l
Band 5	0.009 mg/ml	51 μ l

(c)

Sample	Protein Conc	Volume
Band 1	0.023 mg/ml	370 μ l
Band 2	-0.009 mg/ml	478 μ l
Band 3	0.019 mg/ml	500 μ l
Band 4	0.003 mg/ml	500 μ l
Band 5	-0.006 mg/ml	403 μ l

(d)

Sample	Protein Conc	Volume
Band 1	0.038 mg/ml	500 μ l
Band 2	0.389 mg/ml	485 μ l
Band 3	-0.001 mg/ml	434 μ l
Band 4	0.016 mg/ml	457 μ l
Band 5	0.057 mg/ml	434 μ l

(e)

Sample	Protein Conc	Volume
Band 1	-0.045 mg/ml	458 μ l
Band 2	-0.052 mg/ml	600 μ l
Band 3	-0.044 mg/ml	506 μ l
Band 4	-0.022 mg/ml	477 μ l
Band 5	-0.044 mg/ml	597 μ l

Once the protein estimation was processed, Sample 2 eluted Batches were run in 12% SDS gel for observing the band formation.

Table 5.8: (a) Band 1 and 2 gel run. (b) Band 3 and 4 gel run. (c) Band 5 gel run.

(a)

LANE	Sample	Volume
1.	BioRad marker	5 μ l
2.	Band 1 – batch2	40 μ l
3.	Band 1– batch 3	40 μ l
4.	Band 1– batch 4	40 μ l
5.	Band 2 - batch 2	40 μ l
6.	Band 2 - batch 3	40 μ l
7.	Band 2 - batch 4	40 μ l

(b)

LANE	Sample	Volume
1.	BioRad marker	5 μ l
2.	Band 3 –batch 2	10 μ l
3.	Band 3– batch 3	30 μ l
4.	Band 3 –batch 4	30 μ l
5.	Band 4 –batch 2	30 μ l
6.	Band 4– batch 3	10 μ l
7.	Band 4– batch 4	10 μ l

(c)

LANE	SAMPLE	VOLUME
1	BioRad marker	5 μ l
2	Band 5–batch 2	30 μ l
3	Band 5–batch 3	30 μ l
4	Band 5–batch 4	30 μ l

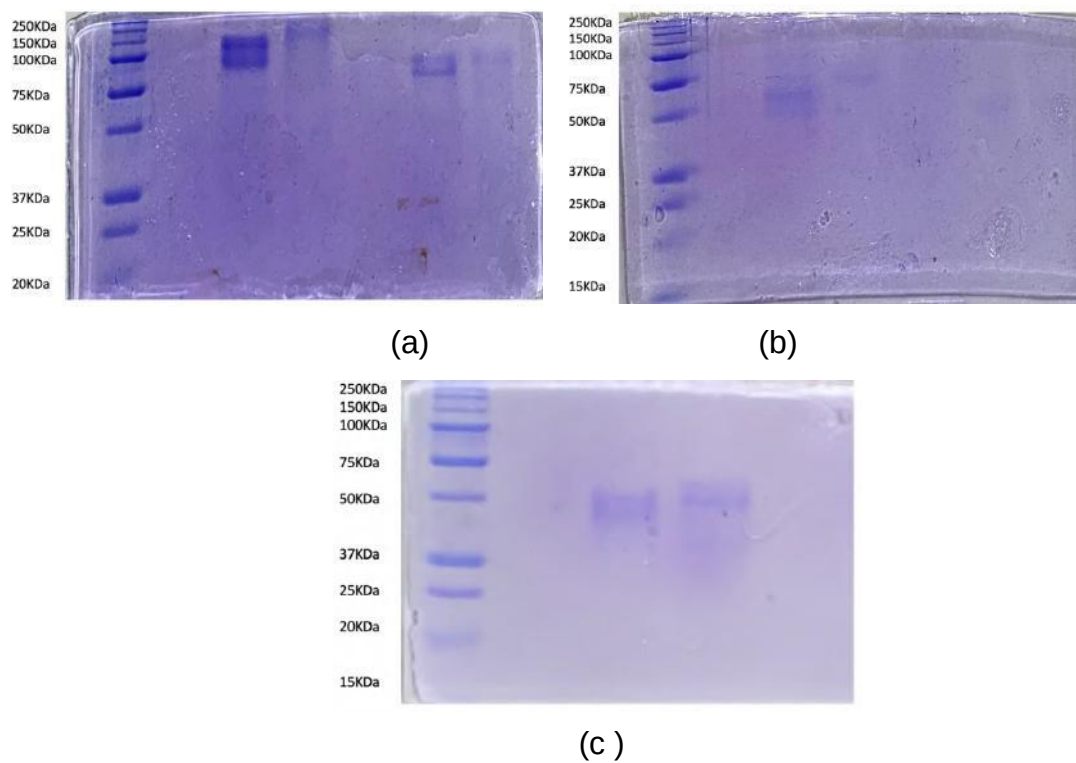


Figure 5.15: (a) gel run of bands 1 and 2 for sample 2 in all 3 batches. (b) gel run of bands 3 and 4 for sample 2 in all 3 batches. (c) gel run of band 5 for sample 2 in all 3 batches.

Chapter-6

CONCLUSION

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