VENOM CHARACTERIZATION AND TOXICOLOGY OF BOX JELLYFISH *Chironex indrasaksajiae*

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ลักษณะสมบัติของพิษและพิษวิทยาของแมงกะพรุนกล่อง *Chironex indrasaksajiae*

น.ส.ชนิกานต์ หยงสตาร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ชนิกานต์ หยงสตาร์ : ลักษณะสมบัติของพิษและพิษวิทยาของแมงกะพรุนกล่อง *Chironex indrasaksajiae*. (VENOM CHARACTERIZATION AND TOXICOLOGY OF BOX JELLYFISH *Chironex indrasaksajiae*) อ.ที่ปรึกษาหลัก : ดร.ศุภณัฐ ไพโรหกุล, อ.ที่ปรึกษาร่วม : รศ. ดร.ไทยถาวร เลิศวิทยาประสิทธิ,พญ. ดร.นวลกันยา สถิรพงษ์สุทธิ

แมงกะพรุนกล่องจัดเป็นหนึ่งในสิ่งมีชีวิตที่มีพิษร้ายแรงที่สุดในโลก ในประเทศไทยสำรวจพบแมงกะพรุนกล่องหลายสายสกุล *Chironex* (*C. indrasaksajiae)* ซึ่งมีพิษร้ายแรงถึงขั้นทำให้ผู้สัมผัสพิษเสียชีวิตได้ภายในเวลาไม่กี่นาที การศึกษาครั้งนี้จึงมีวัตถุประสงค์ที่จะระบุลักษณะสมบัติของพิษแมงกะพรุนกล่อง *C. indrasaksajiae* โดยใช้วิธีการทางโปรตีโอมิกส์ ผลการศึกษาพบกลุ่มโปรตีนทั้งหมด 532 กลุ่ม ประกอบไปด้วย พิษและโปรตีนที่มีความสำคัญสำหรับการพัฒนาของกระเปาะพิษและการสร้างกระเปาะพิษ (nematogenesis) โดยกลุ่มโปรตีนพิษที่มีมากที่สุดใน *C. indrasaksajiae* คือ CfTX-1 และ CfTX-2 ที่มีลำดับเพปไทด์ครอบคลุม 39.3% และ 34.8% ตามลำดับ โดยโปรตีนที่มีลักษณะคล้าย CfTX อยู่ในกลุ่มของพิษที่พบได้ใน Cnidaria เท่านั้น ซึ่งมีคุณสมบัติ cytolytic, hemolytic, dermonecrotic, ทำให้เกิดการอักเสบและเป็นอันตรายถึงชีวิต การศึกษานี้ให้ข้อมูลเชิงลึกเกี่ยวกับความหลากหลายของพิษโปรตีนที่พบในแมงกะพรุนกล่อง *C. indrasaksajiae* และเป็นการศึกษาแรกที่มีการศึกษาโปรตีโอมิกของพิษแมงกะพรุนกล่อง *C. indrasaksajiae* นอกจากนี้ผลการศึกษากิจกรรมของพิษ แสดงให้เห็นว่าพิษของแมงกะพรุนกล่อง *C. indrasaksajiae* มีฤทธิ์ทำให้เม็ดเลือดแตกได้ ซึ่งมีความรุนแรงมากกว่า *C. fleckeri* ถึง 100 เท่า ซึ่งมีรายงานผลการศึกษาพบว่า *C. fleckeri* เป็นสัตว์ที่มีพิษอันตรายที่สุดในปัจจุบัน การรักษาคนที่โดนแมงกะพรุนต่อยจะมุ่งไปที่การบรรเทาอาการ ดังนั้นจึงมีความจำเป็นที่จะต้องหาวิธีรักษาที่มีประสิทธิภาพเพื่อลดอัตราการเสียชีวิตจากการสัมผัสพิษแมงกะพรุนกล่อง ผลการศึกษาในครั้งนี้พบว่า Copper gluconate มีฤทธิ์ในการยับยั้งการแตกของเม็ดเลือดแดงที่ได้รับพิษจาก *C. indrasaksajiae* ได้ แสดงให้เห็นว่า ยารักษาที่มีส่วนผสมของ Copper gluconate เป็นหลักจะสามารถยับยั้งการแตกของเซลล์เม็ดแดงที่เกิดจากพิษของแมงกะพรุนได้ นอกจากนี้อุณหภูมิยังส่งผลต่อกิจกรรมการแตกของเม็ดเลือดที่เกิดจากพิษ *C. indrasaksajiae* พบว่าที่อุณหภูมิ 37°C และ 45°C สามารถลดการแตกของเม็ดเลือดแดงได้อย่างมีนัยสำคัญ และที่ 60°C ไม่พบการแตกของเซลล์เม็ดเลือดแดง การศึกษาในครั้งนี้ให้ความรู้ ความเข้าใจที่ดีขึ้นเกี่ยวกับพิษของแมงกะพรุนกล่อง *C. indrasaksajiae* ซึ่งสามารถนำไปศึกษาเปรียบเทียบกับพิษของแมงกะพรุนชนิดอื่น ๆ ในอนาคต การวิเคราะห์วิวัฒนาการของพิษ หรือการค้นพบยาใหม่ ๆ

สาขาวิชา	วิทยาศาสตร์ทางทะเล	ลายมือชื่อนิสิต .....
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Chanikarn Yongstar : VENOM CHARACTERIZATION AND TOXICOLOGY OF BOX JELLYFISH *Chironex indrasaksajiae*. Advisor: Supanut Pairohakul, Ph.D. Co-advisor: Assoc. Prof. Thaithaworn Lirdwitayaprasit, Ph.D., Nuankanya Sathirapongsasuti, M.D., Ph.D.

Box jellyfish is considered one of the most venomous animals. The genus *Chironex* (*C. indrasaksajiae*) is a multi-tentacle box jellyfish found in Thai waters that are dangerous to humans, particularly the venom that could cause death within minutes. This study aimed to perform a proteomic approach to characterize the protein components of *C. indrasaksajiae* venom. A total of 532 unique protein groups were identified, including toxins and proteins essential for nematocyte development and nematocyst formation (nematogenesis). Toxins CfTX-1 and CfTX-2, with sequence coverage of 39.3 percent and 34.8 percent, respectively, are the most abundant in the venom of *C. indrasaksajiae*. The CfTX-like proteins are members of a group of potent toxins unique to Cnidaria. These toxins are linked with cytolytic, hemolytic, inflammatory, dermonecrotic, and deadly activities. This work is the first to describe the cubozoan jellyfish venom proteome and sheds light on the range and diversity of protein toxins produced by this dangerous box jellyfish. Furthermore, our findings indicated that the venom had strong hemolytic activity that demonstrated 100-fold more potently hemolytic activity (HU_{50}) than *C. fleckeri* which is currently mentioned as the most lethal animal on the planet. Current therapies focus on symptom alleviation after envenomation. Therefore, effective treatments are required to lower the death rates associated with cnidarian envenomation. Copper gluconate showed the potent inhibitory effect on the hemolysis induced by *C. indrasaksajiae* venom. The considerable inhibition of hemolytic activity following cubozoan stinging by copper gluconate-based products represents a novel and effective therapeutic approach for the treatment of cnidarian envenomation. Heat also influenced the hemolytic activity induced by *C. indrasaksajiae* venom. At 37°C and 45 °C, the temperatures appropriate for such inactivation of the venom activity. This work provides an enhanced understanding of *C. indrasaksajiae* box jellyfish venom that can be used for future comparative studies, toxin evolution analysis, and drug discovery.

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CHAPTER I
Introduction

1. Research background and rationale

In recent years, the incidences of marine envenomation appear to be increasing worldwide due to global warming and the rising frequency of human encounters with venomous marine creatures (Needleman et al., 2018; 2018). Many humans experience jellyfish stings, particularly along with coastal areas of the ocean where numbered millions of stings occur annually (Fenner, 1997a). By stinging, many jellyfish species have been known to cause injuries or even death (Cegolon et al., 2013; Fenner and Williamson, 1996; Mubarak et al., 2021; Tibballs, 2006b). Such serious reactions which can be more than 100 fatal cases per year (Pirkle and Yanagihara, 2019), are consequences of stings inflicted by Australian box jellyfish *Chironex fleckeri*. This species is considered as one of the most venomous marine creatures in the world (Williamson et al., 1996). A meter of tentacle contact can occasionally result in death attributed to acute cardiovascular collapse within minutes after stinging (Yanagihara and Shohet, 2012).

Thailand locates in the Indo-pacific region, where it is the hotspot zone for box jellyfish envenomation (Gershwin et al., 2010; Gershwin et al., 2013) (Figure 1). The medical records showed the envenomation cases of box jellyfish (both Thai and foreigners) in Thai water since 1998 (Thaikruea and Siriarayapon, 2014). However, many people do not believe that box jellyfish can kill humans, so they dismiss and neglect this problem. Thus, the knowledge about box jellyfish biodiversity or envenomation has been limited in Thailand. Until now, there have been six fatal cases of box jellyfish envenomation occurring on the islands of Samui and Pha-ngan in the Gulf of Thailand (Thaikruea and Siriarayapon, 2016). All these fatal cases may be caused by *Chironex indrasaksajiae* which has recently been described as a new species of box jellyfish from the Gulf of Thailand (Sucharitakul et al., 2017). *C. indrasaksajiae* envenomations occur each year, typically from August to November (Thaikruea and Siriarayapon, 2016).

Numerous biological activities are related to cubozoan venoms. Particularly, *Chironex* sp. entire tentacle and nematocyst extracts produce lethal, dermonecrotic, nociceptive, cytotoxic, neurotoxic, myotoxic, cardiotoxic, and hemolytic effects (Tibballs, 2006b). Nevertheless, despite the clinical and pharmacological relevance of box jellyfish venoms to humans, their compositions have not been thoroughly studied. This thesis described the proteomic characterization of *C. indrasaksajiae* venom to identify proteins that may contribute to the deleterious effects of box jellyfish stings in humans. To date some known components in toxins, including porin, proteases, lipases, small molecular weight compounds, and lipids have been formally identified. Of these, a porin or a pore-forming protein is the fast-acting component in the venom. This protein can create pores on the membrane of human red blood cells, making them leak, release large amounts of potassium, and may cause cardiac arrest and death (Yanagihara and Shohet, 2012). Therefore, this study focused on porins activities. Copper gluconate and heat were also tested in the study because these two factors have been reported that the treatments can perturb the self-assembly of porins (Avigad and Bernheimer, 1976; Melo et al., 2016; Yanagihara, 2019). This therapy is limited and transient to support cardiovascular hemodynamics. It may allow for more effective treatment.

2. Objectives

1.2.1 Characterization of the proteins in *C. indrasaksajiae* venom

1.2.2 Effect of the venom from *C. indrasaksajiae* on sheep RBCs

1.2.3 Effect of copper gluconate and heat on the activity of venom

3. Expected beneficial outcomes

The results of this study will clear up the details of biochemical components in *Chironex indrasaksajiae* venom together with mechanistic and kinetic studies of the venom on human red blood cells using the hemolytic assay. Moreover**,** this study will provide important insights to suggest primary therapeutic approaches.

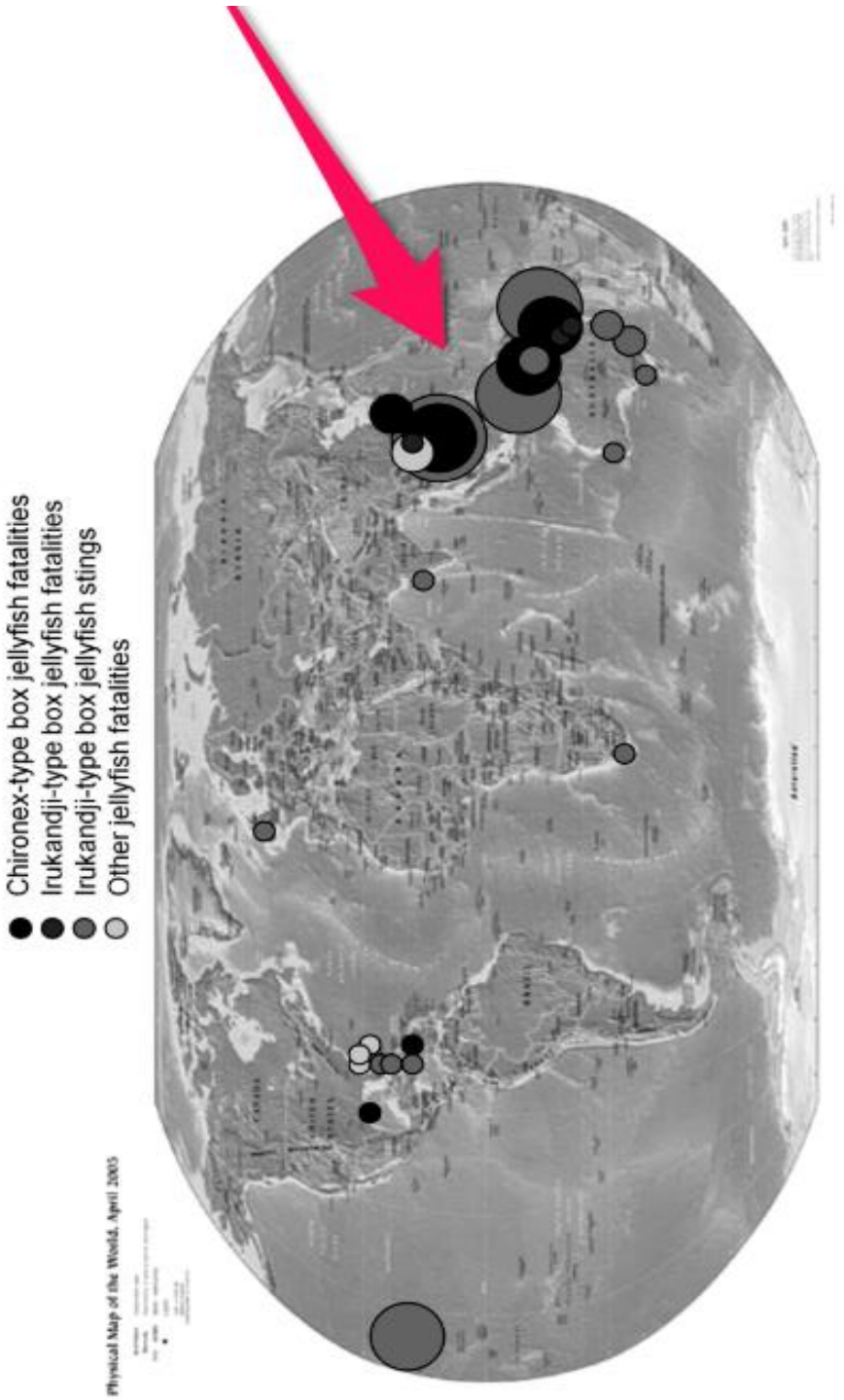

Figure 1 Pattern of worldwide deaths and envenomation attributed to jellyfish. Red arrow shows the hot spot zone of box jellyfish envenomation. Source : Gershwin et al. (2013)

CHAPTER II
Literature review

1. Introduction

Marine venomous creatures have been identified in various phyla throughout the animal kingdom, such as Cnidaria, Platyhelminthes, Nematoda, Mollusca, and Echinodermata (Williamson et al., 1996). Among the members of these divisions, the Phylum Cnidaria is the most abundant and diverse group comprising more than 10,000 living species (Zhang, 2011, p. 9). Cnidarians appear to have diverse morphologies, including five main classes: Hydrozoa (hydroids, hydras, siphonophores), Anthozoa (hard and soft corals, sea anemones), Scyphozoa (true jellyfish), Cubozoa (box jellyfish), and Staurozoa (stalked jellyfish) (Figure 2), but all members of this phylum universally possess stinging capsules, known as nematocysts or cnidae, which give the phylum its name. These stinging organelles produce specialized toxic secretory products for capturing prey. However, the nematocysts of some groups are highly dangerous. More than 100 species are toxic to humans, and contact with these toxins causes a wide range of conditions, from cutaneous rashes to cardiovascular and respiratory collapse (Fernandez et al., 2011). The most dangerous group is the Cubozoa, commonly called "box jellyfish." Although Cubozoa is the smallest class of Cnidaria, comprising some 50 described box jellyfish species (Bentlage, 2012), they comprise the most highly specialized class of the Cnidaria phylum. They account for the majority of deaths among jellyfish-stung victims worldwide (Fenner and Williamson, 1996).

1.1 General characteristics of box jellyfish

Box jellyfish is the common name for the members of the class Cubozoa. One of its distinctive characteristics is the cubic shape of their bells, as opposed to crowns or dome-shaped bodies (Bentlage et al., 2010). It is for this reason that they are known as “Box Jellyfish.” (Brinkman and Burnell, 2008) Some species can have up to 15 tentacles growing from the corners of the cube-shaped bell, and all

jellies share the same origin of attachment sites. These tentacles can be up to 10 feet in length and hold up to 5,000 stinging cells per cm^2 (Hartwick, 1991b; Williamson et al., 1996). The venom contained in nematocysts is among the deadliest in the world. Box Jellyfish venom contains toxins that could attack the heart, skin cells, and nervous system (Brinkman and Burnell, 2008; Carrette and Seymour, 2006; Jouiaei, Yanagihara, et al., 2015; Winkel et al., 2005). Patients stung by box jellyfish could go into shock and experience heart failure, resulting in death within minutes (Ramasamy et al., 2004; Sharmaine Ramasamy et al., 2005; S. Ramasamy et al., 2005a, 2005b, 2005c). Members of the box jellyfish have clusters of eyes on each of the four sides of the jellyfish's bell. They can produce images with the refractive index gradient of their lenses. It aids them in locating prey and avoiding predators (Nilsson et al., 2005). Besides, box jellies can also swim vigorously. For example, *Chironex fleckeri* can swim at a rate of about 4 knots, whereas most jellyfish can only keep themselves in the current and merely drift with the current. As a result of these features, the box jellyfish is able to hunt prey while avoiding predators (Hamner et al., 1995; Shorten et al., 2005). Last but not least, box jellyfish has transparent-blue bodies. As a result, distinguishing between them underwater is extremely difficult, resulting in human damage (Cegolon et al., 2013; Suntrarachun et al., 2001).

Box jellyfish are classified into two distinct orders based on the number of tentacles that arise from each corner: order Chirodropida and order Carybdeida

1.1.1 Order Chirodropida (multiple tentacles group)

They are distinguishable from other box jellyfish by the presence of branching muscular bases at the corners of their cubic umbrellas and small saccules linked with the stomach cavity. They generally have multiple tentacles at each corner. Each year, members of this group kill a large number of people, typically in the tropical Indo-Pacific area. Approximately 7 chirodropid genus worldwide have fast-acting venom (Fenner et al., 2010). The most well-known genus *Chironex*, sometimes known as "sea wasps," is infamous for its capacity to cause lethal stings or severe systemic symptoms (Endean et al., 1991; Fenner, 2005; Fenner and Williamson, 1996; Learmont, 2006; Lippmann et al., 2011).

1.1.2 Order Carybdeida (single tentacle group)

They have unforked pedalia, with only one tentacle attaching to each corner of the bell. They range in size from a few millimetres to 500 mm in bell height. A large number of the species in order Carybdeida may cause the Irukandji syndrome. The main genus is *Alatina, Carukia, Malo,* and *Morbakka*. The consequences and symptoms of envenomation are quite delayed, appearing 25-40 minutes after being stung by a jellyfish (Carrette et al., 2012; Gershwin et al., 2013).

1.2 The genus *Chironex*: taxonomy and distribution

Although the box jellyfish has been considered as "the world's most venomous creature", it has been reported that only a few species in the class are involved in human deaths, specifically members of the genus *Chironex*. From the latest revision of the phylogeny of the genus *Chironex,* there are currently three recognized species of the genus *Chironex* in the world (WoRMS database: Collins and Jarms, 2021). First is *Chironex fleckeri* Southcott, 1956 (Figure 3a). This species is the largest species of cubozoa, with an averaging bell height of about 25 cm (almost the same size as a basketball ball) when mature (Kingsford and Mooney, 2014). It caused fatalities (at least 68 deaths since the first report in 1883) in Australia (Fenner, 2005; Lumley et al., 1988; Williamson et al., 1996). Second, *Chironex yamaguchii* Lewis & Bentlage, 2009, commonly known as habu-kurage (Figure 3b). This species caused several deaths (at least-three confirmed deaths) in Japanese waters (Lewis and Bentlage, 2009). The last one, *C. indrasaksajiae* , Sucharitakul, 2017, a new species of box jellyfish from the Gulf of Thailand, has killed six people since 1998 (Sucharitakul et al., 2017; Thaikruea and Siriarayapon, 2014) (Figure 3c).

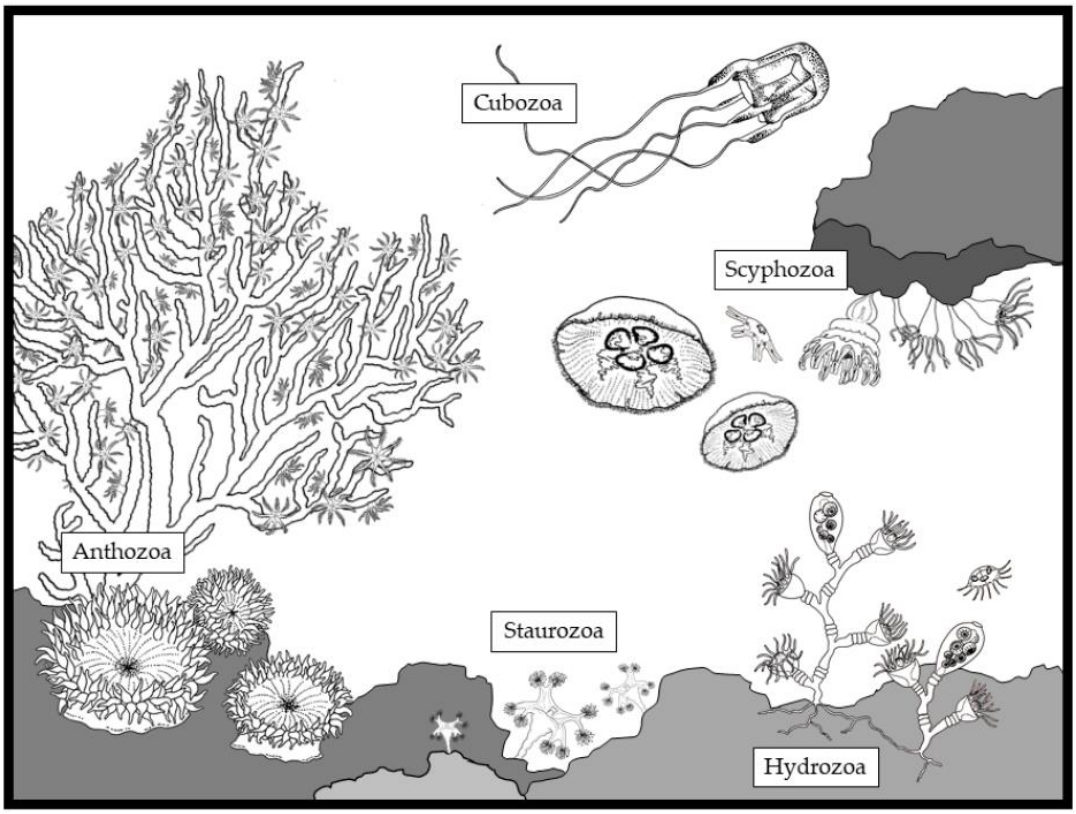

Figure 2 The diverse organisms belonging to the phylum Cnidaria within the main classes: Hydrozoa (hydroids, hydras, siphonophores), Anthozoa (hard and soft corals, sea anemones), Scyphozoa (true jellyfish), Cubozoa (box jellyfish), and Staurozoa (stalked jellyfish). Source : D'Ambra and Lauritano (2020)

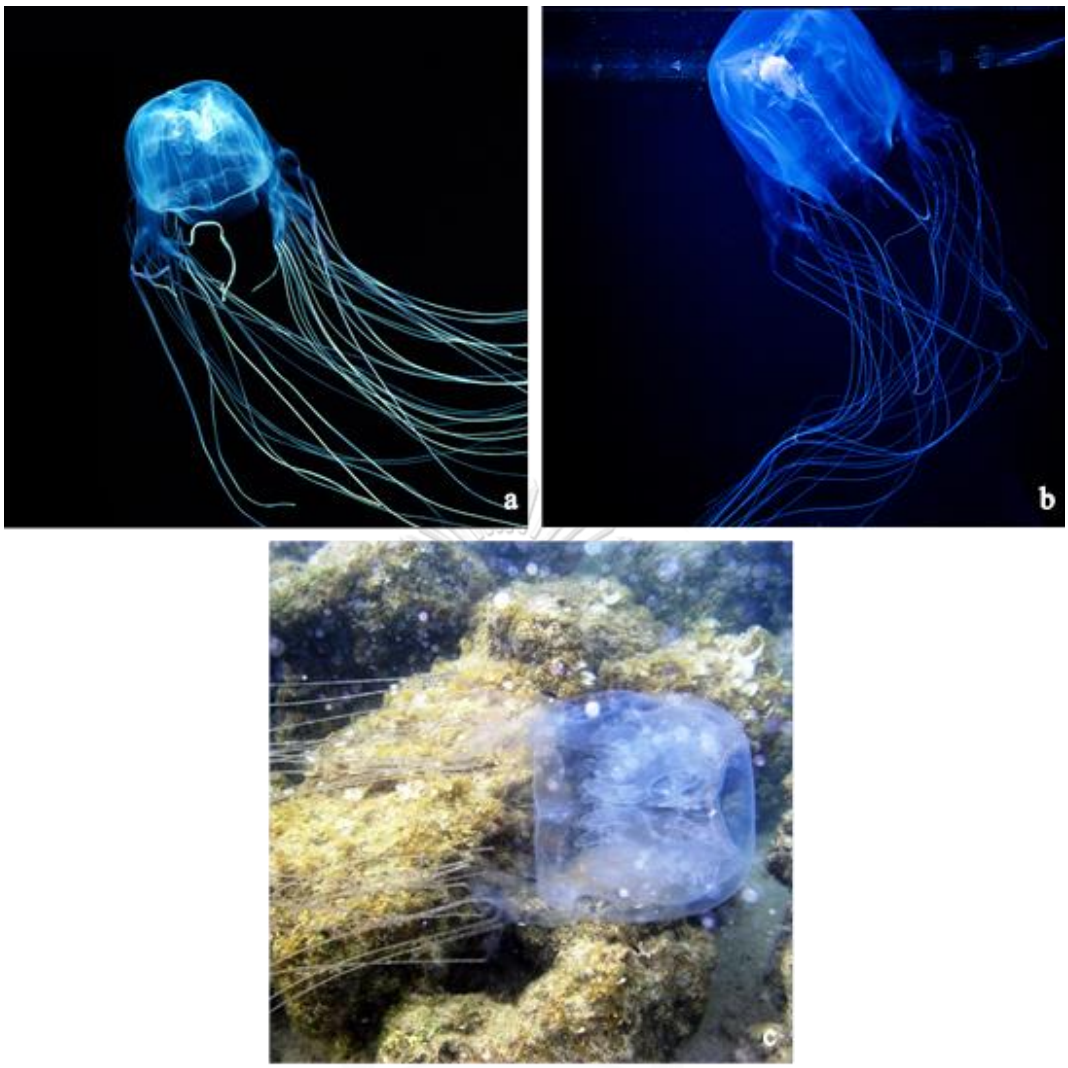

Figure 3 Morphological Variation between the members of the genus *Chironex*. (a) *Chironex fleckeri* (Photographs by Gary Bell), (b) *Chironex yamaguchii* (Photographs by Shawn Miller) and (c) *Chironex indrasaksajiae* (Photographs by Sakanan Plathong)

1.3 The genus *Chironex*: life cycle

During the mating season, sea wasps have a natural tendency to aggregate in a coastal, estuary, or enclosed waters to produce their offspring, increasing the danger of unavoidable stinging to swimmers or fisherman, which many negative impacts on tourism and fishery, and among other human activities. *Chironex* has a polymorphic life history in which it may reproduce sexually with gametic meiosis as well as asexually, or without sex, like the other class members. To begin, the mature jellyfish swims to a nearby freshwater river (estuarine) in quest of a suitable mating. According to Hartwick (1991), the estuaries and exposed coastline provide suitable environmental conditions for polyps and medusae. Cubomedusae that have reached maturity release their gametes into the water column, where they are fertilized and grow into planula larvae. After spawning, they die and do not participate in raising their descendants. Planulae spend a few days swimming in the water column before settling on the substrate. After settlement, the planulae grow into polyps. The polyp stage is a tiny, sessile predator that ambushes prey. While they are developing, these polyps' major food sources are zooplankton and phytoplankton. This stage undergoes asexual budding to take immediate advantage of local increases in the food supply. The minimal requirements of the medusae stage include a stable hard substrate for attachment that allows it to maintain its population until the environment favors the survival of the medusa. The polyps are developed after a few months of feeding. The polyps then transform into a single juvenile medusa. Afterwards, juvenile medusas grow up into adult medusae form. (Gordon and Seymour, 2012; Hartwick, 1991a; Kingsford and Mooney, 2014). A summary of cubozoa life history is provided in Figure 4.

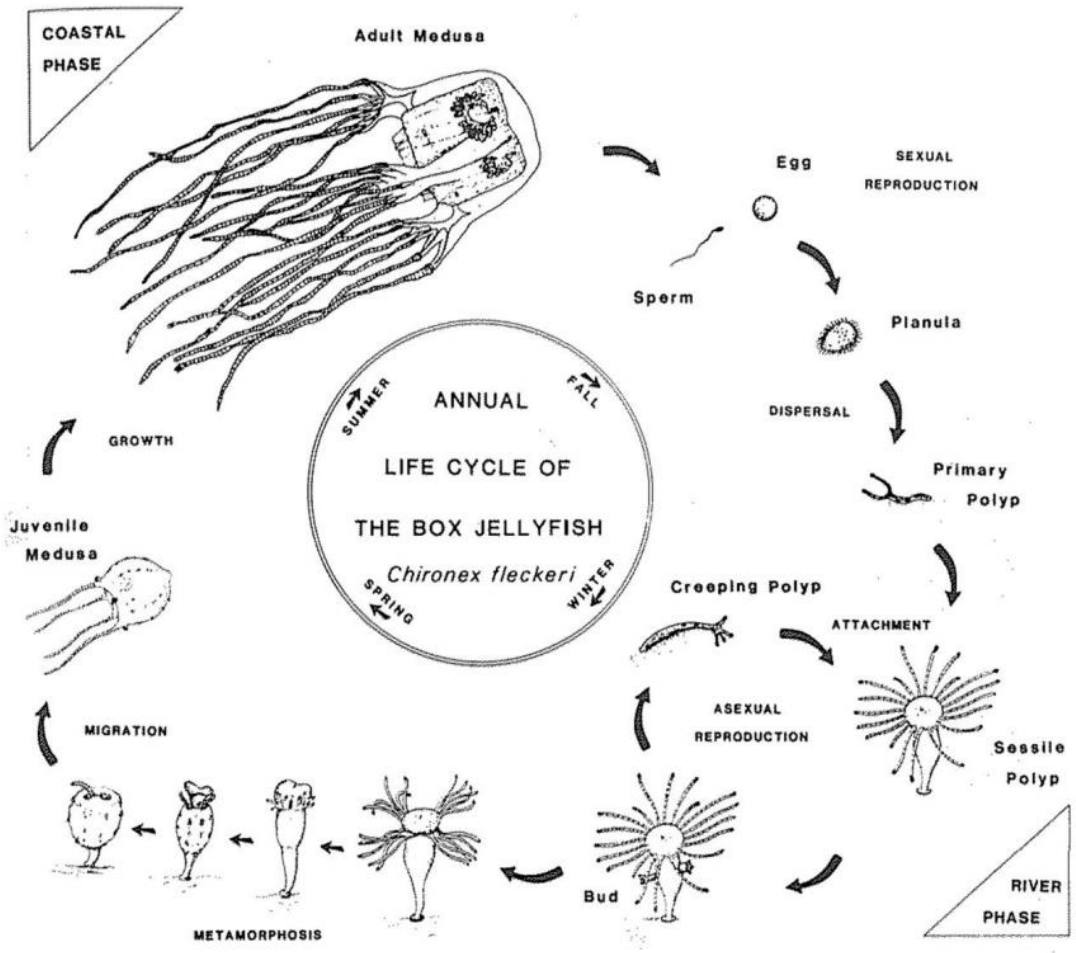

Figure 4 The *Chironex fleckeri* life cycle shows an alternation in both generations (sexual medusa phase to asexual polyp phase) as well as between the estuarine and coastal habitats Source : Gordon (2014))

1.4 The genus *Chironex*: anatomy

In general, the medusa form of this genus has a cubic shape or box-shape bell (figure 5). From each of the four lower corners of the bell hangs a short stalk called claw-like shaped pedalium that bears multiple long, slender tentacles. There are no nematocyst warts on the outer surfaces of the bell. Nematocysts are usually only found on their tentacles. The margin of the bell is folded inwards to form a shelf. It is called "velarium," which restricts the bell's cavity and creates a powerful movement when the bell pulsates (Schierwater and DeSalle, 2021, p. 161). As a result, the ability to locomote box jellyfish is more rapid than that of other jellyfish. The speeds that have been recorded can be up to six meters per minute (Shorten et al., 2005). At the centre of the bell is a mobile appendage called the "manubrium," which looks like an elephant's trunk. The tip of the manubrium is the mouth. The gastrovascular cavity that is known as the interior of the bell is divided by four septa into a central stomach and four gastric saccules with V-shaped gastric phacellae. The shape of the gastric saccules is the key characteristic of this genus. The distinctive gastric saccules of *Chironex* members have been described as cock's comb-like or grape cluster-like (Gershwin, 2005b; Lewis and Bentlage, 2009).

The nervous system of box jellyfish is more developed than other jellyfish. They have a nerve ring around the base of the bell that can control coordinates their pulsing movements. Box jellyfish are unique in the property of true complete eyes. There are retinas, corneas, and lenses. Their eyes, known as "rhopalia," are clusters of six eyes located in pockets called "dome-shaped rhopalial niche ostia in each half of the bell's outer surfaces" (Lewis and Bentlage, 2009; Ruppert et al., 2004; Southcott, 1956). Box jellyfish contains a total of twenty-four eyes, including twenty ocelli (simple eyes). Instead of just differentiating between light and dark, the jellyfish can detect specific spots of light and can produce images with the refractive index gradient of their lenses. Statoliths beneath the rhopalia sense gravity pull and facilitate the jellyfish in orienting itself (Nilsson et al., 2005; Ueno et al., 1995).

A fully grown *Chironex* can measure up to 20 cm along each box side (or 30 cm in diameter) (Kingsford and Mooney, 2014), and the tentacles can grow up

to 3 m in length. Its weight can reach 2 kg. There are about 15 tentacles on each corner ((Hartwick, 1991b; Tibballs, 2006b). Each tentacle contains bands or buttons of thousands of densely packed nematocysts that line the epithelial surfaces of tentacles that can inject venom into the victim (Rifkin and Endean, 1983; Williamson et al., 1996). This is a deadly organ that can cause serious damage, and many different types of nematocysts are found in this species (Gershwin, 2006).

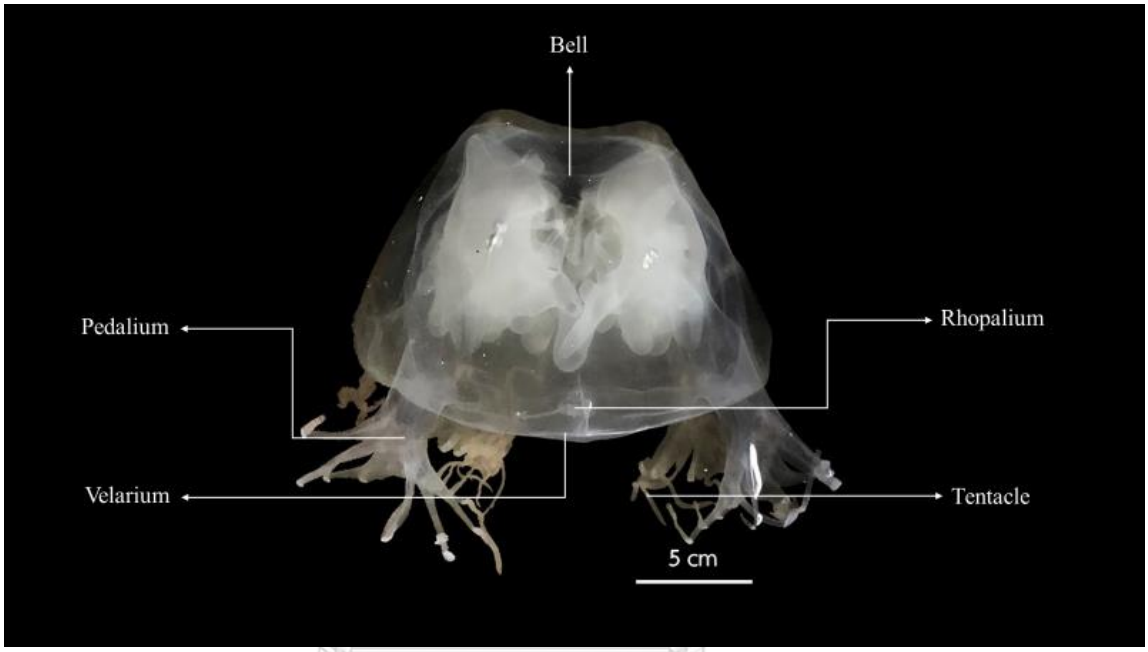

Figure 5 Morphology of a box jellyfish (*Chironex* sp.) (Photographs by Chanikarn Yongstar)

2. Cnidae

Cnidocyte is a unique and distinguishing feature of all Cnidarians. It is a remarkable, specialized, explosive organelle formed in a giant post-Golgi vesicle by a sequential accumulation of proteins from the Golgi apparatus. It comprises a collagen-walled capsule that undergoes further structural modifications in the extracellular matrix before migration to the tentacle surface (Beckmann and Özbek, 2012a; Jouiaei, 2016; Özbek et al., 2009). Each cnidocyte holds “a cnida” filled with fluid and contains a long tubular invagination of the capsule wall. Depending on the type of cnidae, the everted tubule may sting or paralyze prey by penetrating the epidermis and

releasing toxins, sticks to the prey's or substrate's surface, or fulfilling other functions. (Peach and Pitt, 2005).

Cnidae have been classified into three main types: penetrant nematocysts, the volvent spirocyst (Mariscal, McLean, et al., 1977) and the glutinant ptychocysts (Mariscal, Conklin, et al., 1977). In cubozoa, nematocysts are the only type of cnidae that comprise the cnidome, and three main categories of nematocysts are commonly found: mastigophore, euryteles, and isorhizas (Gershwin, 2006; Jouiaei, 2016; Östman, 2000). The study of cnidomes, and also information upon morphology of cnidae, are now regarded as an important part of jellyfish species identification (Acuna et al., 2003; Conklin et al., 1977; Östman, 1982; Ryland et al., 2004). Additionally, the function of each type of nematocyst has been described based on the structure (Rifkin and Endean, 1983). Consequently, the toxin should differ amongst nematocyst types (Carrette et al., 2002). Subsequently, the nematocyst ratio variation could reveal each species' venom level (Killi et al., 2020).

For Chirodropids, cnidae occur only in the gastrodermis or epidermis of jellyfish tentacle. The mature nematocyst capsule contains (1) a rapidly eversible penetrate of the non-penetrate tubule, (2) a complex mixture of proteins (often proteins), and (3) other small molecular weight compounds (Özbek et al., 2009; Yanagihara, Kuroiwa, Oliver, Chung, et al., 2002; Yanagihara, Kuroiwa, Oliver, and Kunkel, 2002).

Upon the nematocyst discharged, the initiation of the exocytosis process is stimulation of the cnidocilia (a hair-like trigger) on the outer surface of the nematocysts (Figure 6). The discharge process is one of the fastest events known in the animal kingdom. It can expel a tubule 200–800 microns in length at speed up to 18.6 m/s with an acceleration of more than 5,000,000 g and create pressure up to 7.7 GPa at the site of impact, allowing the tubule to penetrate human skin and a thick-cuticle of a prey crustacean (Beckmann and Özbek, 2012a; Nüchter et al., 2006; Özbek et al., 2009; Tardent, 1995).

Upon contact with human skin, thousands of tubules then evert themselves and drive through the skin within milliseconds. The first part of the tube has a set of spines that lock into the epidermis, providing the tube with a stable base, followed by the rest of the tubule extending into the dermis. The venom inside the cell is promptly

passed down via the tube and deposited in the dermis. The length of the penetrant tubules renders possibly the direct deposition of toxin into pierced capillaries, thus explaining the rapid onset of toxicity in humans (Figure 7) (Law, 2018; Rifkin and Endean, 1983; Tibballs, 2006b; Tibballs et al., 2011).

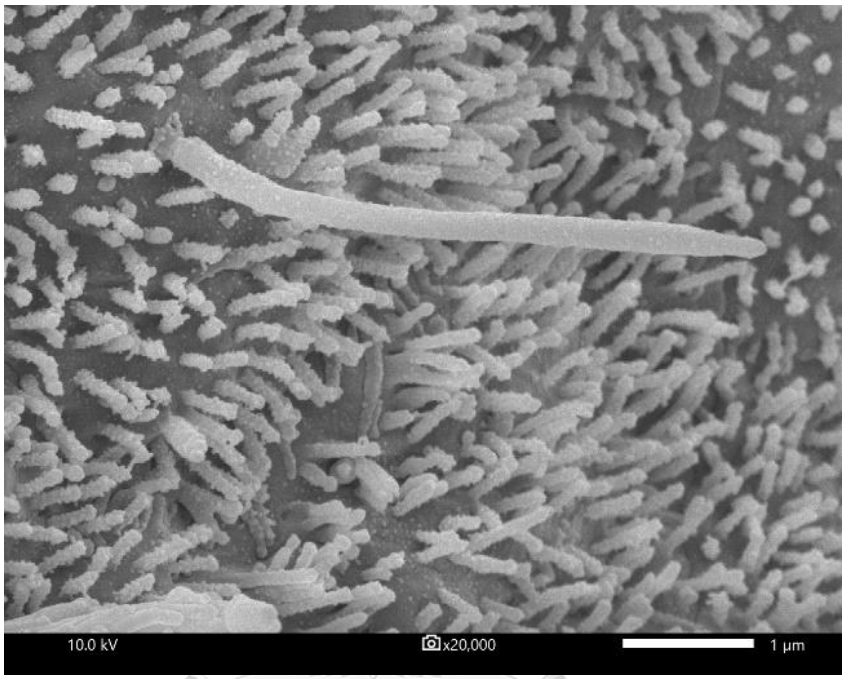

Figure 6 The sensory cilium of the nematocysts is mechanoreceptive may be, non-motile and stiff and is called Cnidocilia. (Photographs by Chanikarn Yongstar)

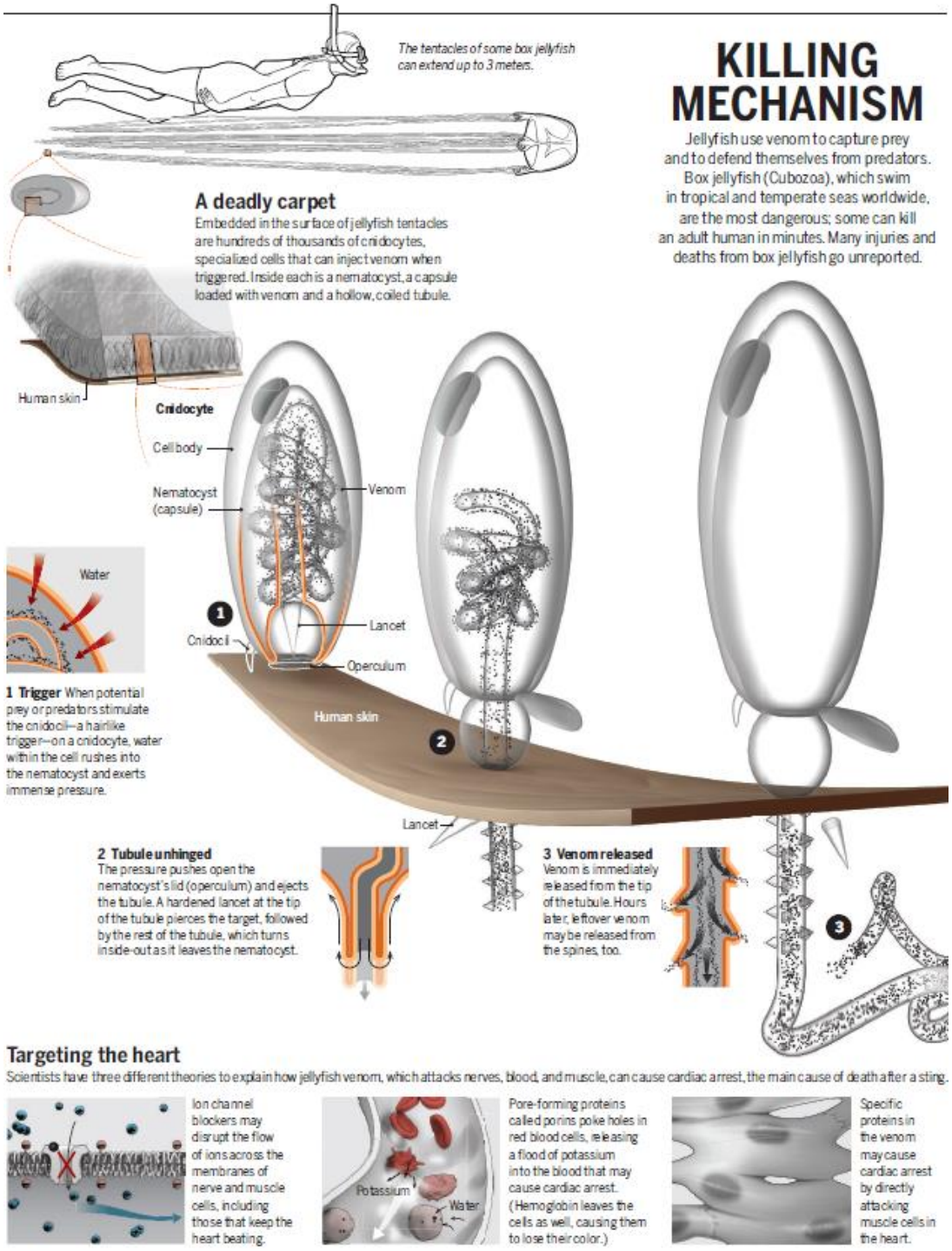

Figure 7 Killing mechanism of a box jellyfish (*Chironex* sp.) Source : Law (2018)

3. Box jellyfish in Thai waters

Box jellyfish are found mostly in tropical latitudes. They are especially abundant in the Indo-Pacific area, which is regarded as a hotspot zone for box jellyfish envenomation (Gershwin et al., 2013). Every year, thousands of severe stings and potentially hundreds of fatal envenomations were reported (Fenner, 1997b). Occasions of these cases were commonly reported in Northern Australia, and others were reported from tropical regions such as the Philippines, Borneo, Japan, Malaysia, and Thailand. (Fenner, 2005; Fenner et al., 2010; Fenner and Williamson, 1996; Fernandez et al., 2011; N.-S. Lee et al., 2001; Lippmann et al., 2011; Pirkle and Yanagihara, 2019; Suntrarachun et al., 2001; Thaikruea and Siriarayapon, 2018; Thaikruea et al., 2012b; Tibballs, 2006b; Tiemensma et al., 2021; Vimpani and Harris, 1988; Williamson et al., 1980)

After the Ministry of Public Health (MOPH) reported the first suspected box jellyfish envenomation in Thailand, when two foreign tourists died on Pha-Ngan Island, Surat Thani province. The investigation discovered clinical symptoms compatible with envenomation by multi-tentacled box jellyfish. At the time, no government authorities in Thailand had heard of box jellyfish murdering people. There was a strong belief that the fatalities were caused by Caucasian people's hypersensitivity to local jellyfish toxins. After a Swedish girl died at a beach on Lanta Island in 2008 with anaphylactic shock from jellyfish contact. Moreover, she had extensive tentacle marks all over her legs (Thaikruea et al., 2012a). This incident prompted a more extensive response by the MOPH and marine biological team. However, one of the most often asked topics by local government officials and tour operators was whether or not venomous jellyfish constitute a significant concern in Thailand.

According to the box jellyfish survey, the information was collected by the marine and coastal resources department. Ten box jellyfish species were found in Thai water (the Andaman Sea and the Gulf of Thailand), including single-tentacle and multi-tentacles (Aungtonya and Chanachon, 2012; PMBC Database, 2020). Among these box jellyfish, the genus *Chironex* is infamous for its toxin-causing deadly instances worldwide. In Thailand, *C. indrasaksajiae* which has recently been

described as a new species of box jellyfish from the Gulf of Thailand is the most dangerous species (Figure 8b) (Sucharitakul et al., 2017). Another two unnamed species are *Chironex* sp.A found in the Andaman Sea (Figure 8a), and *Chironex* sp.C found in the western part of the Gulf of Thailand (Figure 8c).

Over the last fifteen years, there has been an increase in fatal and near-fatal box jellyfish envenomation cases in Thailand. Most of these cases were from medical records and patients from 1998 onwards. There were approximately 918 cases of envenomation which 8 case reports of death (Suntrarachun et al., 2001; Suriyan et al., 2019; Thaikruea and Siriarayapon, 2014, 2018; Thaikruea et al., 2012a; Thaikruea et al., 2012b). Six out of eight occurred on Pha-ngan and Samui islands, with the highest incidence of jellyfish-related deaths in Thailand (Thaikruea and Siriarayapon, 2016, 2018). According to the results of DNA analysis from tentacles and clinical features from the victims, 2 cases were confirmed to have been caused by *C. indrasaksajiae,* which was recently described as a new species of box jellyfish from the Gulf of Thailand (Sucharitakul et al., 2017).

Moreover, the usual envenomation cases occur each year, typically from August to November (Thaikruea and Siriarayapon, 2016). It correlated with the occurrence period of *C. indrasaksajiae* around this area, suggesting environmental conditions favor *C. indrasaksajiae*. Several mechanisms may be likely to contribute to more medusa abundance, such as more suitable environmental factors (Canepa et al., 2017), the effects of eutrophication (Ishii et al., 2008), overfishing (Lo et al., 2008), habitat modification for aquaculture and climate change (Dong et al., 2010). Another possibility is that it is a high tourist season on the islands. Logically, the more tourists there are on the islands, the more likely the jellyfish will come into contact. The full moon parties, which take place on Rin beach on Pha-ngan island every month on the night of the full moon, gather the largest crowds. Parties have increased in recent years, including black moon and half-moon parties. More importantly, the period that *C. indrasaksajiae* was found to be abundant was the Southwest Monsoon period, suggesting hazard management should readily cover this period. Since deaths related to *C. indrasaksajiae* envenomation have been reported in the Gulf of Thailand. However, the understanding of the specific toxicity and biology of this new species *C. indrasaksajiae* is awaiting the outcome of this study.

Figure 8 The members of the genus *Chironex* in Thailand. (a) *Chironex* sp.A, (b) *Chironex indrasaksajiae*, and (c) *Chironex* sp.C
Source: Central Database System and Data Standard for Marine and Coastal Resources (https://km.dmcr.go.th/th/c_1/s_266/d_14616#.WFjlN1WLSUk)

4. Box jellyfish venom

The complex mixture of bioactive compounds (venoms) that box jellyfish produced, was stored and delivered by cnidae. One form of cnidae, which is found in all cnidarians, is nematocyst (stinging cell) (Beckmann and Özbek, 2012a). The large portions of nematocyst tubules can penetrate the human dermis and inject the capsule's venom (Kitatani et al., 2015). Furthermore, when the nematocyst discharged (exverted or inside out), the venoms that have accumulated on the outside of the tubules are discharged as well (Rifkin and Endean, 1983). As a result, venom may be injected continually, rather than just when the tube is completely everted. Venom would be run off the tubules and passed through capillaries, adding to the quickness with which the envenomation occurs.

Cnidarian venoms have been studied extensively since the early twentieth century, and their toxicological variety has been demonstrated by various clinical observations and studies. The venoms of cnidarians are not well understood, however recently over 150 toxins have been found. They seem to be generally composed of a variety of protein enzymes (e.g. phospholipases A_2 and metalloproteases), pore-forming toxins, neurotoxins, and other non-protein bioactive compounds (e.g. serotonin, histamine, bunodisine, and caissarone) (Jouiaei, 2016).

Cubozoa includes various species of box jellyfish, which are generally classified into two distinct groups, that are harmful to humans. The morphological

differences between chirodropids and carybdeids indicate that the larger multi-tentacled chirodropids may be capable of injecting venom dosages far greater than those injected by the four-tentacled carybdeids. The venoms are stored in nematocysts and include a mixture of bioactive compounds. These compounds are either cytolytic, cytotoxic, inflammatory, or lethal. (Brinkman and Burnell, 2009). As of now, only a few individual venom proteins have been thoroughly investigated. However, increasing evidence suggests that cubozoan jellyfish produce at least one unique group of homologous bioactive proteins that are labile, basic, hemolytic, and similar in size (40–46 kDa) (Brinkman and Burnell, 2007; Nagai, 2003; Nagai, Takuwa, et al., 2000a; Nagai et al., 2002; Nagai, Takuwa, Nakao, Sakamoto, et al., 2000). Several *in vitro* and *in vivo* studies confirmed that these novel toxins are also potentially fatal. Moreover, they may produce cutaneous pain, inflammation, and necrosis in animals, which is comparable to the symptoms reported in envenomed humans (Auerbach et al., 2019b; Bailey et al., 2005; Bueno et al., 2021; Ramasamy et al., 2003; Ramasamy et al., 2004; S. Ramasamy et al., 2005a, 2005b; Winkel et al., 2005; Winter et al., 2007; Winter et al., 2008). Secondary structural studies and protein homology predictions indicate that the novel toxins may function as pore-forming toxins (Brinkman and Burnell, 2007; Parker and Feil, 2005). Despite the fact that pore-forming (cell-destroying) toxins are likely to present in all cnidarian venoms, they have been identified principally in the venoms of cubozoan. The biological activities of porins include hemolytic, myotoxic, cardiotoxic, and dermonecrotic effects (Jouiaei, 2016; Tibballs, 2018).

However, cubozoan venoms are typically similar in their biological activities, but the wide range and severity of the effects caused by different species suggest that their venoms differ in their protein composition, biological activity, and potency. This study reviewed the isolation of bioactive venom protein components, biological activity, biochemical characterization of cubozoan venoms, a particularly focusing on the genus *Chironex* which was responsible for the most deaths from jellyfish stings.

4.1 Isolation of jellyfish venom

Chironex venoms have historically been extracted from either entire tentacles or isolated nematocysts. These samples were extracted as either fresh, frozen, or lyophilized, with the latter (isolated nematocysts) being the most common method (Table 1) (e.g. Baxter et al., 1968; Bloom et al., 1998; Cantoni et al., 2020; Carrette and Seymour, 2004; Crone and Keen, 1969; Endean, 1987; Endean et al., 1969; Endean and Rifkin, 1983; T. E. B. Keen, 1971; Olson et al., 1984; Othman and Burnett, 1990; Wiltshire et al., 2000; Yanagihara and Shohet, 2012). Active venoms have been prepared and extracted using different techniques, including electrical discharge of nematocysts through human amnion (Crone and Keen, 1971), or mortar-and-pestle homogenization (Comis et al., 1989) and sonication of whole tentacles (Othman and Burnett, 1990). The use of entire tentacle extraction, on the other hand, has been questioned since jellyfish are never injected with tentacle tissue except for the components of the nematocysts.

In 1969, Endean *et al.* were the first researcher's group that achieved *Chironex* venom from isolated nematocysts by gently grinding thawed tentacles in a mortar and pestle to get crude nematocyst contents, then letting the mixture autolyze in sea water at 4 °C for 5 days to remove tentacle debris (Endean et al., 1969). Later, in 1998, Bloom *et al.* developed a subsequent modification of the venom preparation method in which whole tentacles were autolyzed in sea water at 4 °C for several days, followed by lyophilization of the isolated nematocyst precipitants. This method was an effective approach for preserving intact nematocysts containing bioactive components for more extended periods of time (Bloom et al., 1998), and after that, it has been routinely used by several jellyfish researcher groups (e.g. Bailey et al., 2005; Brinkman and Burnell, 2007; Carrette and Seymour, 2004; S. Ramasamy et al., 2005a). Afterwards, in 2012, Yanagihara and Shohet developed a new method of venom preparation from isolated nematocysts. The citrate solution, an acidic compound, was used to dislodge the nematocysts without breaking them. At 4 °C, this method can keep nematocysts intact for several years (Yanagihara and Shohet, 2012).

For extracting venom from the nematocysts, several protocols can be used. Venom can be obtained utilizing high pressure to force nematocyst disruption

(Yanagihara and Shohet, 2012), grinding with micrometer-size glass beads (Carrette and Seymour, 2004), or sonication of the nematocyst suspension in various buffers (Helmholz et al., 2007; Nagai, Takuwa, et al., 2000a; Reinicke et al., 2020; So et al., 2016). Afterwards, the sample is centrifuged to remove any remaining capsules, tubules, or tissue debris. Depending on the protocol, the venom sample contains varying amounts of proteins and peptides from nematocysts and varies in amounts of proteins and peptides from the tentacle debris. Accumulated research suggests that the most effective venom extraction method from nematocysts is using glass beads in homogenization (Frazao and Antunes, 2016).

Table 1 Summary of partially purified bioactive proteins from the box jellyfish, genus *Chironex*. (Adapted from Brinkman and Burnell, 2009)

Reference	Source of extracts [a]	Purification method [b]	Estimated Molecular Mass (kDa)		Biological Activities [d]
			Native	Denatured [c]	
Crone and Keen (1969a, 1971), Freeman and Tumer (1971), and Freeman (1974)	T, M	SE, CEX, AEX	(1) 150 (2) 70		L,C L,C,H,D
Baxter and Marr (1969)	N, M	SE	(1) 350 (2) 10-30 (3) 1.8		L,H
Olson et al. (1984)	M	IA (*α-Physalia*), SE	65	**20**+ Several bands	L,C,P+
Catlon and Burnett (1986)	T	IA (CSL antivenom)		150,50	L,C
Naguib et al. (1988)	T	IA (*α-Chironex*)		120 70 14.5 130,115,90,80,75,52,29	L,H,D L,D L Ambiguous activities
Othman and Burnett (1990)	T_L	CEX, AEX		50,24	L
	T_L	HIC		24,45-50	L
	T_L	IA (*α-Chironex*)		**24**+ Several bands	L
Colllins et al. (1993)	T_L	mAb (*α-Chironex*)		50	H
Endean (1987) and Endean et al.(1993)	N_M, N	SE	(1) 600 (2) 150 (3) 70 (4) <14	59,43,25,**18** 59,43,25,**18** 26,10	L,M L,M L,H
Bloom et al. (1998)	N_L	SE, NPAGE	438,225,207,45,22		L
	N_L	SDS-CGE		201,**174**,112,71,39,31	

Reference	Source of extracts [a]	Purification method [b]	Estimated Molecular Mass (kDa)		Biological Activities [d]
			Native	Denatured [c]	
Bloom et al. (2001)	N_L	PEG-CE, SDS-CGE		(1) 200	Hepatotoxic
				(2) **173**,163,45,39,41	(F4>F1>F2>F3)
				(3) 39,14	
				(4) <14	L,H,IR
Brinkman and Burnell (2008)	N	SE, CEX	(1) >600	**160**,45,43,41,39	
			(2) 370	**45,43**	H (CfTX-1 and 2)
			(3) 145	45,43,**41,39**	H
			(4) 90	Several bands	
			(5) 70	Not analyzed	H
			(6) 55	Several bands	
			(7) 27	Several bands	

[a] Sources of extracts include tentacle extract (T), milked venom (M) and nematocyst venom (N); subscripts L and M indicate lyophilized sources and venom isolated specifically from microbasic mastigophore nematocysts, respectively.

[b] Purification methods include size-exclusion (SE), cation-exchange (CEX), anion-exchange (AEX), and immunoaffinity (IA) chromatography; mAb indicates monoclonal antibodies; antibodies raised for IA and mAb studies are indicated in brackets; electrophoretic methods include native PAGE (NPAGE), PEG–capillary electrophoresis (PEG–CE) and SDS–capillary gel electrophoresis (SDS–CGE).

[c] The molecular mass of denatured proteins was determined by SDS-PAGE, unless SDS–CGE is specified; major proteins are shown in bold.

[d] Biological activities: lethal (L), cardiotoxic (C), hemolytic (H), dermonecrotic (D), pore-forming in lipid membranes (Pþ), musculotoxic (M), induction of immunological response (IR) and hepatotoxic.

4.2 Bioactivity of the genus *Chironex* venom

Following five decades of research, the genus *Chironex* (especially *C. fleckeri*) has become the most extensively studied cubozoan jellyfish. Early studies on whole tentacle and nematocyst extracts have reported several biological activities associated with *C. fleckeri* venom proteins, including cardiotoxic, hemolytic, dermonecrotic, neurotoxic and immunological effects (Baxter et al., 1968; Bueno et al., 2021; Crone, 1976; Crone and Keen, 1969; Endean et al., 1969; Endean et al., 1993; Shirley E. Freeman, 1974; Shirley E Freeman and Turner, 1969; Horiike et al., 2015; Piontek et al., 2020)

4.2.1 How box jellyfish venom kills, stopping the heart in a short time in five minutes : the cardiovascular effects and hemolytic toxic effects

The difficulty of investigating the lethality of *Chironex* venom was differences in the source of jellyfish venom and the units of reported lethal dose that depend on each research group. However, extensive experiments of envenomed on different animals, which include fish, prawns, crayfish and mice, rats, rabbits, guinea pigs, sheep, rabbits, and monkeys revealed that venom caused the heart to fail to relax progressively and it became paralyzed in systole (Brinkman and Burnell, 2009; Endean et al., 1969). Currently, there appear to have two scientific hypotheses of lethal effect that support the idea of how box jellyfish venom kills and stops the heart within minutes: the cardiovascular effects and the hemolytic effects.

Firstly, the cardiovascular effects that the venom acts directly on the heart make the vasoconstrictor effects on other parts of your body less effective. Moreover, Ramasamy et al. in 2004, indicated that acute cardiovascular effects from *Chironex* venom are more likely to cause rapid death than respiratory arrest (Ramasamy et al., 2004). Despite many decades of investigation, the mechanism behind the fatal impact of *Chironex* venom has remained unknown. The prevailing hypothesis was that the culprits are ion channel blockers, molecules that interfere with the flow of ions into and out of cells. As a result, nerve and muscle cells cannot work properly, including those that control the heart pumping due to these

blockages. Early studies suggested that venom-induced calcium ion (Ca^{2+}) transport across muscle fibre membranes plays an important role in muscle contracture and paralysis (Joseph W Burnett and Calton, 1983; Endean, 1987; Endean and Henderson, 1969). The study by Mustafa and colleagues found that the toxin increases sodium influx into the cells and subsequently increases calcium via the sodium/calcium ion exchange mechanism resulting in calcium overload. This may induce arrhythmias and lead to sudden death (Mustafa et al., 1995; Vassalle and Lin, 2004). Afterwards, Cheng claimed that the toxin might comprise fibrinolysins, phospholipases, proteases, hyaluronidases, collagenases, kinins, vasoactive amines (such as histamine), and catecholamines. The toxin acts directly on the myocardial, neurologic and hepatic tissue, disrupting cell membranes and releasing inflammatory mediators. However, this assumption is not supported by research or pathophysiological explanations (Learmont, 2006). Moreover, Bailey et al. (2005) suggested that the rapid influx of Ca^{2+} into cardiac cells could be attributed to the indiscriminate entry of ions via large pores in the cell membrane formed after exposure to *Chironex* venom rather than via Ca^{2+} channels. Another study about a direct action of venom on cardiac and vascular muscle was observed by Hughes et al. (2012). They treated cardiac and vascular tissues of rats with unpurified extracts from nematocysts. It caused atrial standstill and negative inotropy that were unaffected by propranolol, atropine, or CGRP8-37. In addition, it caused contraction of small mesenteric arteries that was unaffected prazosin, bosentan, or tetrodotoxin. Therefore, the investigators concluded that the venom had no effect on autonomic nerves, postsynaptic α1or β1-adrenoreceptors, muscarinic, endothelin, or CGRP receptors. However, the venoms may involve direct effects on cardiac and vascular muscle. More recently, Pereira and Seymour (2013) showed in vitro that *C. fleckeri* venom, in contrast to *Carukia barnesi* venom, is highly toxic to human cardiac and skeletal muscle cells. All these studies suggest that severe hypotension caused by the venom is due to direct cardiotoxicity, which overwhelms any peripheral vasoconstrictor effect.

Secondly, the hemolytic toxic effects of pore-forming protein on red blood cells or porin could be considered a candidate for the fatalities. According to Yanagihara's theory, the venom of box jellyfish contains proteins that break down red blood cells and allow potassium to be released. The release of K+

causes a disruption in the electrical rhythms that keep the heart pumping, which ultimately results in the heart suspending its contractions. Not only ion channel blockers in venom, but also many “porins”: proteins that puncture cells, allowing their contents to leak out (figure 9). In 2012, Yanagihara and a colleague reported that venom of *Chironex fleckeri*, rapidly punctures red blood cells, causing them to leak a huge amount of potassium ions. A high level of potassium in the blood, or hyperkalemia, causes cardiac arrest, and when the mice was injected with high doses of venom, their hearts quickly stopped. The same happened when the mice was injected only the porins from the venom. This study also agreed with Yanagihara's theory that suspected porins might be the lethal components by causing the destruction of red blood cells.

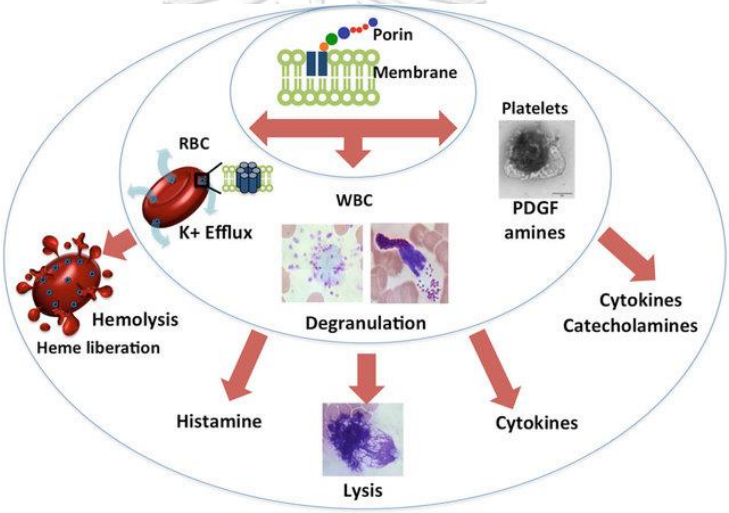

Figure 9 Schematic of the hypothesis of venom-porin initiated mechanism of potassium, catecholamine and cytokine release. Cubozoan venom porin injected into the blood stream inserts and self assembles to form pores on target cell plasma membranes causing target cell effects. Source : Yanagihara, Wilcox, Smith, et al. (2016)

4.2.1.1 Pore-forming protein toxin (PFTs)

Chironex venom contains potent pore-forming toxins (PFTs), also known as porin, cytolysin or hemolysin. Early studies of box jellyfish venom suggested that hemolysins have not been considered to be lethal (Bailey et al., 2005). However, it has been discovered that a catastrophic hyperkalemic state precedes clinically measurable hemolysis and that this is caused explicitly by the cubozoan venom PETs (Yanagihara and Shohet, 2012). These toxins appear to function simply by forming pores in cell membranes, disrupting the permeability barrier and leading eventually to cell death.

Generally, there are three major steps in the generation of pores by PFTs, summarized in figure 10 (Anderluh and Lakey, 2008; Parker and Feil, 2005). The organisms synthesize PFTs as soluble units (mostly monomers, dimers or oligomers). Second, upon soluble binding units to lipid membranes, they form oligomers at the surface of the membrane (self-assembly). In most cases, these membrane-bound complexes form an ordered complex called a pre-pore, which is not yet functional. Finally, these membrane-bound complexes undergo conformational changes, and become inserted into the lipid membrane bilayer, forming a functional pore. The binding to lipid membranes sometimes depends on the presence of specific receptors, such as lipids (sphingomyelin, cholesterol, etc.), glycolipids, or glycoproteins (Podobnik and Anderluh, 2017).

PFTs can be classified into two families, depending on secondary structure as the α-helical or β-barrel structural features of their transmembrane channel (as described by Gouaux, 1997). The α-PFTs are predicted to form pores using helices. These toxins tend to be highly α-helical, with the larger toxins having pore-forming domains consisting of a three-layer structure of up to ten α-helices sandwiching a hydrophobic helical hairpin in the middle of the structure. This hairpin is thought to drive the initial steps of the insertion process. Second group, the β-PFTs are named because of their structural characteristics composed mostly of β-barrel domains. These toxins tend to be rich in β-sheet (Parker and Feil, 2005).

Full amino acid sequence analysis and molecular modelling, show that the porin has structural motifs familiar with self-assembling bacterial porins, such as anthrolysin O and streptolysin O (Bernheimer et al., 1979). Afterwards, Chung et al. (2001) reported on the first hemolytic porin from *Alatina* sp. (previously reported as *Carybdea alata*). Since then, all cubozoan venom has been found to have close homologous proteins. In *C. fleckeri* venom, there are two different isoforms of this porin (MW 43 and 45 kDa) (Brinkman and Burnell, 2009; Nagai, Takuwa, Nakao, Sakamoto, et al., 2000; Yanagihara, Kuroiwa, Oliver, Chung, et al., 2002). Moreover, secondary structure analysis and remote protein homology predictions suggest that the family of box jellyfish toxins (CfTXs, CqTX, CrTX and CaTX) may act as α-PFTs, with three-domain Cry-like toxins. These domains were estimated to play an essential role in membrane penetration and pore-forming after binding to the specific receptors (Brinkman and Burnell, 2009; Podobnik and Anderluh, 2017). Thus, the fact that porins are conserved across all cnidarian species supports the concept that these proteins are key venom components and play an important role in the clinical symptoms of cubozoan stings (Yanagihara, Wilcox, Smith, et al., 2016).

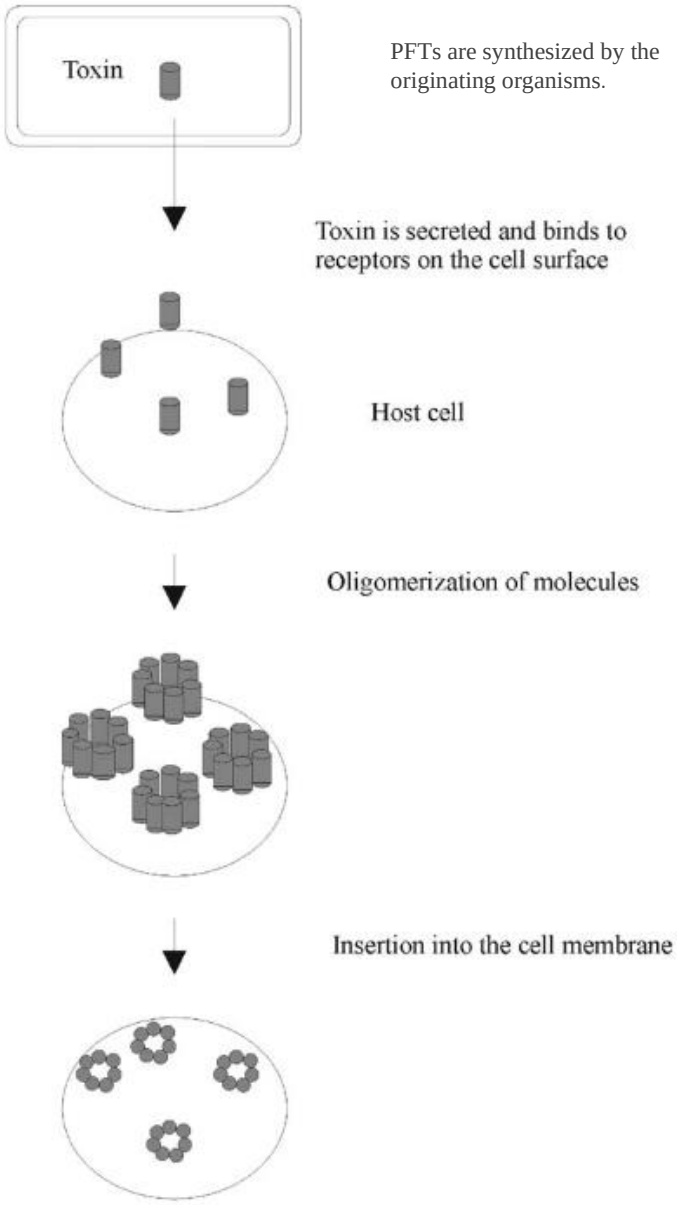

Figure 10 The 3 major steps involved in membrane pore formation generated by PFTs. Source : Parker and Feil (2005)

4.2.2 Severe pain and dermatotoxic effects

The studies by Shirley E Freeman and Turner (1969) and T. E. B. Keen (1971) showed that a dermal necrosis factor isolated from the venom caused rapid skin death in experimental animals. This symptom was similar to that seen in human skin too. For human dermal necrosis, the lesions appear to whip-like with a purple to brown color; the wounds are associated with erythema, oedema, and vesicle formation and may progress to full-thickness necrosis within the first two weeks after stinging. Long-term complications may include keloids, granulomas, hyperpigmentation, fat atrophy, and muscle contractures (Figure 11) (Thaikruea and Siriarayapon, 2015a).

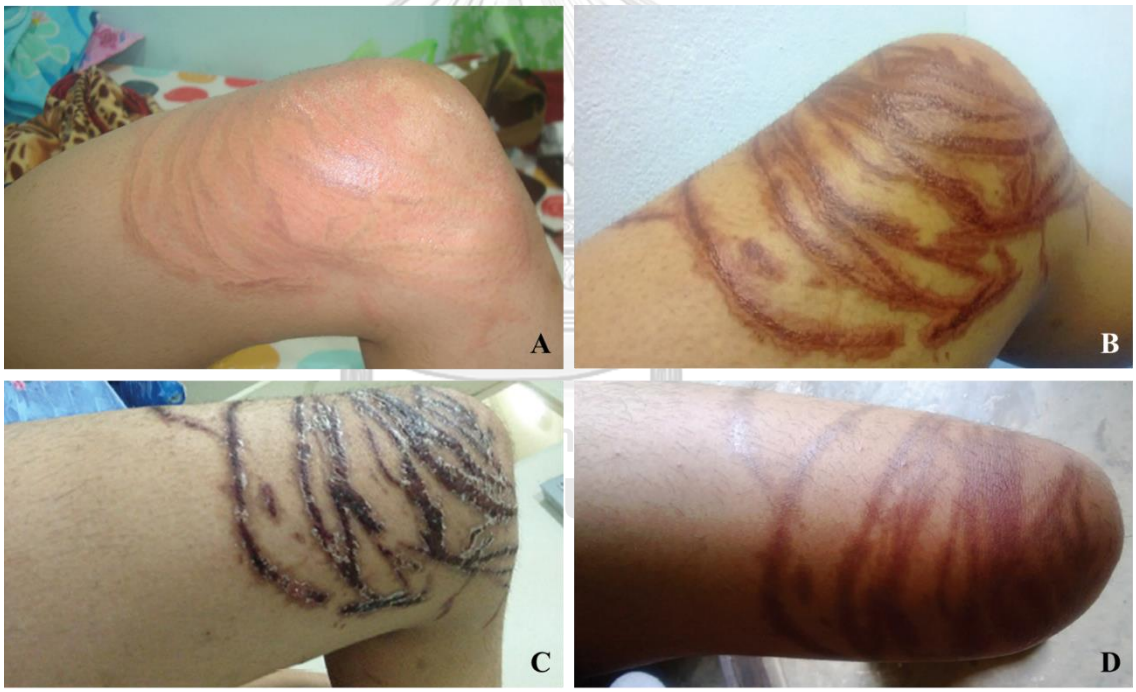

Figure 11 Wound characteristics; (A) on the sixth day, (B) on the fifth day with small blisters developed, (C) on the 14th day with turned wound to crusts with moderate to severe itching, and (D) on the 61st day, dark brown tentacle arks remained on patient's knee with smooth skin. Source : Thaikruea and Siriarayapon (2015a)

Several researchers have attempted to elucidate the mechanism underlying the severe pain and inflammation caused by *C. fleckeri* venom. Inflammatory pain is mediated by several chemical components, which act on pain receptors (nociceptors) directly or indirectly via complex signaling pathways (figure 12)(Baxter et al., 1968; Brinkman, 2008; Shirley E Freeman and Turner, 1969; T. Keen and Crone, 1969). In a recent study involving *Chironex* tentacle extracts, the burning sensation associated with cnidarian envenomation was linked to the activation of transient receptor potential vanilloid-I (TRPV1) channels in nociceptive neurons (Cuypers et al., 2006). Moreover, CfTX-1 and -2 proteins isolated from *C. fleckeri* venom, are thought to be responsible for skin inflammation and necrosis (Auerbach et al., 2019a).

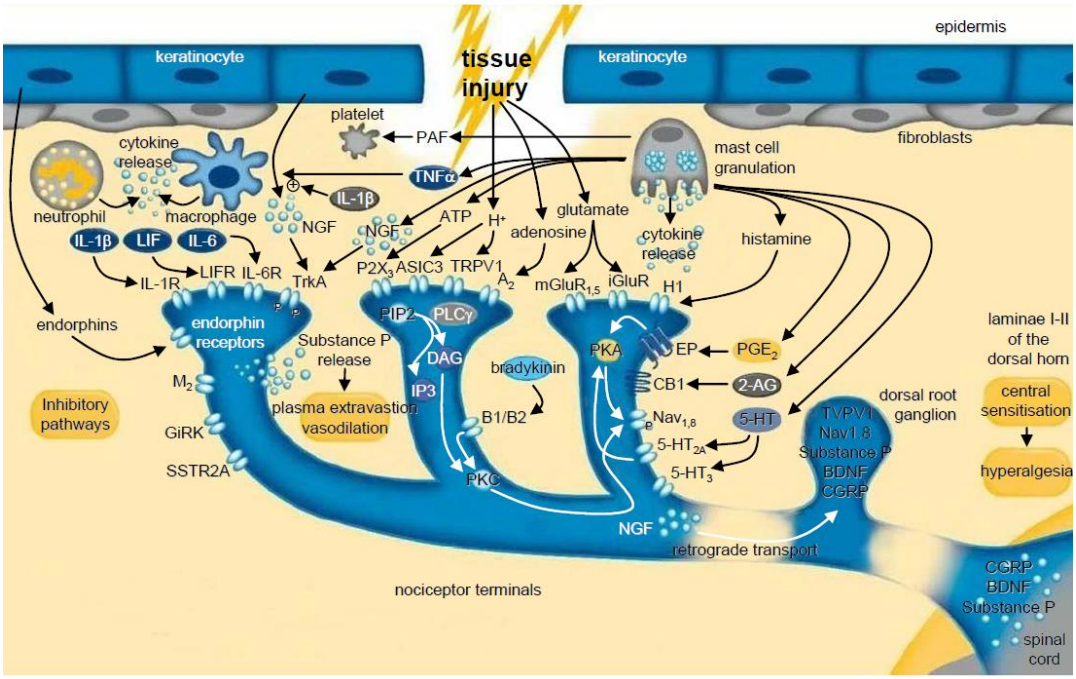

Figure 12 Mediators and signaling pathways involved in inflammation and pain. Source : Brinkman (2008)

4.2.3 Neurotoxic effects

Endean et al. (1993) isolated a neurotoxin (T4; 150 kDa) that blocked conduction in the isolated sciatic nerve of the toad and produced a progressive reduction in the response of the rat diaphragm to phrenic nerve stimulation. In 2003, Ramasamy and colleagues also investigated the neurotoxicity of the *C. fleckeri* venom on the chick biventer cervicis nerve muscle. Venom (50 mg/ml) significantly inhibited indirect and direct twitches of the biventer nerve muscle. However, the isolated neurotoxic proteins of this study may be assumed to be different toxins producing the neurotoxic effects observed in the study of Endean. They suggested that the differences were because of venom concentration differences or inactivation during the venom extraction process (Ramasamy et al., 2003).

4.2.4 Immunological effects

Immunological issues of *C. fleckeri* envenomation and other jellyfish envenomation have not been given much attention. With so many foreign proteins and bioactive components being deposited into the human dermis and blood during envenomation, it's no surprise that immune reactions are common (Tibballs, 2018). According to O'Reilly et al. (2001) study, of 19 patients of *C. fleckeri* envenomation, 11 patients (58 %) had delayed hypersensitivity responses that healed either spontaneously or with an oral antihistamine and a topical corticosteroid.

Dermatitis caused by jellyfish stings can be characterized as 3 types of immune reaction, i.e., immediate allergic, late-phase reaction and relapsing allergic response, which correspond to clinical reactions (Asztalos et al., 2014). According to Horiike et al. (2015) study, the isolated CqTX-As (45 kDa) from *Chironex yamaguchii* venom were use as allergens for the prick test. Only an immediate allergic response, such as itching and erythema, was detected in the test. Moreover, they detected the IgG-binding acidic glycoprotein (of 30 and 66 kDa) in the nematocyst capsule wall and spine, and also identified CqTX-A protein the nematocyst. They found that this protein, the major toxic protein, is also a heat-stable

IgE-binding allergen. The detection of these proteins might explain why some people have both immediate allergy-toxic and persistent allergic reactions.

5. Clinical effects of *Chironex* envenomation

5.1 Symptoms

Extreme pain, impaired consciousness, dyspnoea (breathing difficulty), cardiac dysfunction, pulmonary oedema, shock, hypertension and hypotension, rapid acute cutaneous inflammation (dermonecrosis), and permanent scarring are some of the clinical symptoms of *Chironex* envenoming. Respiratory and cardiac failure may occur within a few minutes in the most severe cases (Beadnell et al., 1992; Brinkman and Burnell, 2009; Lumley et al., 1988; Williamson et al., 1984a).

Millions of cnidae are discharged into the skin when a Chironex tentacle touches a victim's skin. The tubules of the discharged nematocysts penetrate the epidermis and into the underlying vascular and nerve-rich dermis, delivering venom into the surrounding tissue (Figure 13) (Yanagihara, Wilcox, Smith, et al., 2016). Lymphatic and capillary vessels easily absorb venom, although the absorption rate depends on the venom dosage and peripheral circulation, which is regulated mainly by the muscular activity of surrounding tissues (Brinkman, 2008; Joseph W. Burnett, 1991; Fenner, 1991; Williamson et al., 1996). Envenomation is especially dangerous for children because of their greater surface area to mass ratio and absence of body hair, which causes the tentacles to contact into direct touch with their skin (Fenner and Harrison, 2000). Moreover, many factors affect the pathophysiology of a jellyfish sting. These depend on the number and type of nematocysts, the wound site, the skin thickness, the patient's health, and the condition of envenomated tissue (Yanagihara, Wilcox, Smith, et al., 2016).

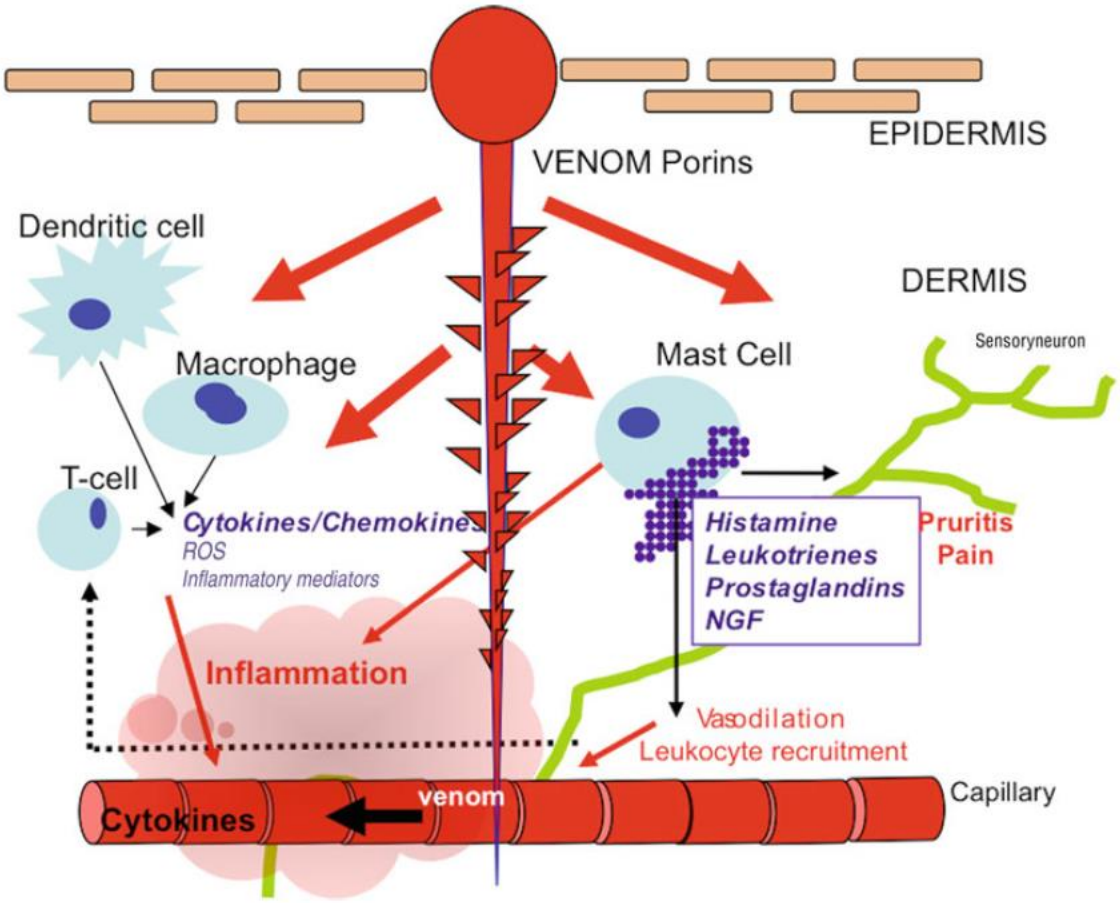

Figure 13 Illustration of discharged eurytele nematocyst structures. Source : Yanagihara (2016)

Porin-based puncture of erythrocytes causes a rapid and potentially lethal due to leakage of potassium ions from red blood cells into the plasma before the pore on erythrocyte expansion is enough to enable the release of the big tetrameric haemoglobin molecule. Moreover, when porins are exposed to the system, the platelets may be ruptured and the resulting foam cells can develop, causing an explosion of histamine, inflammatory cytokines, and other chemokines to life-threatening levels (Yanagihara, Wilcox, Smith, et al., 2016).

At the envenomation site, severe pain, oedema, and linear wheals with white ischaemic centers surrounding vivid erythematous flares have occurred (Joseph W. Burnett, 1991). These symptoms can last for days or even weeks. As a result of this, Yanagihara, Wilcox, Smith, et al. (2016) suspect that leakage of white blood cells and platelets that are the source of catecholamines, biogenic amines, and

cytokinesis caused these symptoms of envenomation. More than 300 different chemicals are stored in platelets and their granules, which are released uncontrolled when porin is operated (Ren et al., 2008; Whiteheart, 2011). This group of compounds includes small biogenic amines (like histamine and serotonin) and cytokines (like platelet-derived growth factor (PDGF), tumor necrosis factor-alpha (TNF-), interleukin-7 (IL-7), and endothelial growth factor (EGF), as well as small peptides (like dopamine and serotonin) (EGF). In addition, white blood cells have a lot of inflammatory cytokines and other biogenic amines that can be released when porins start breaking down (Yanagihara, Wilcox, Smith, et al., 2016). Similar to the study of Williamson et al. (1984b), they suggested that the acute inflammatory response evoked by *C. fleckeri* venom may be caused by the release of compounds such as histamine, serotonin, bradykinin, and perhaps prostaglandins. As a result of these findings, it seems that porin is both the fastest-acting fraction and sufficiently deadly to be the most significant venom component in terms of clinical symptoms, and thus porin blockage should be a primary aim for treatment.

5.2 Treatments

5.2.1 First aid with vinegar

Contacting jellyfish tentacles with human skin causes thousands of nematocyst tubules to be injected into the epidermis and dermis, causing both local and systemic injury (Tibballs et al., 2011). The goals of first aid are to reduce the amount of venom released from the nematocysts that have not been discharged and to relieve pain. Avoid rubbing the wound, and immediately rinse the stinging area with vinegar for at least 30 seconds to deactivate any remaining undischarged nematocysts. Freshwater should not be applied since it prompts the nematocysts to discharge. Seawater should be utilised if vinegar is unavailable (Lakkis et al., 2015; Premmaneesakul and Sithisarankul, 2019). According to the protocol of expert opinions on jellyfish envenomation especially box jellyfish envenomation, after vinegar pouring jellyfish tentacles often detach on their own. If

the skin remains adhered, it should be pulled off with forceps, but only after the vinegar has been applied (Thaikruea et al., 2020).

5.2.2 Antivenom

The only jellyfish antivenom manufactured worldwide is *Chironex fleckeri* antivenom, which has been used since 1970 (Tibballs, 2018; Winkel et al., 2003). In severe cases of *C. fleckeri* envenomation, administering the commercially available CSL box jellyfish antivenom is recommended (Tibballs, 2006a). CSL box jellyfish antivenom prepared from the sera of sheep that have been hyperimmunized with *C. fleckeri* milked venom (Barnes, 1967). It significantly reduces the acute inflammatory response of the skin at the site of envenomation; however, it is not known whether its effect lessens long-term scarring (Williamson et al., 1984b). Each ampoule of antivenom contains sufficient activity to neutralize 20,000 intravenous LD_{50} mouse doses. The recommended dose is three or more ampoules on theoretical and experiential grounds for resuscitation, analgesia, and skin-sparing cosmetic effects (Sutherland, 1992). However, the use of antivenom has minor side effects, including cutaneous rashes. Therefore, administration of the antivenom is indicated only in significant envenomations, such as those associated with cardiorespiratory instability or severe pain, and its use is restricted to trained personnel (Bailey et al., 2003). Afterwards, co-administration of CSL box jellyfish antivenom with verapamil (calcium channel blockade) and magnesium sulphate ($MgSO_4$), was a more effective therapy in countering the actions of venom (Ramasamy et al., 2004). However, there is no clearly effective specific therapy available. The current antivenom is used without clinical validation and has been linked to negative outcomes of envenomations. Moreover, clinical evidence for using *C. fleckeri* antivenom in other species is limited, and it is not available in Thailand (Premmaneesakul and Sithisarankul, 2019; Suntrarachun et al., 2001).

5.2.3 Zinc and copper gluconate

Pore-forming toxins (PFTs), or porins, are the most rapidly acting and lethal components. This protein is the most significant venom component in terms of clinical symptoms. Thus, effective therapeutics for cnidarian envenomation will target the blockade molecules of these pore-forming toxins (PFTs), as well as other membrane perturbance (MPs) (Yanagihara, Wilcox, Smith, et al., 2016). Porin insertion affects the permeability barrier of the cell membrane, allowing monovalent ions to flow. In particular, the efflux of K^+, the influx of Na^+, the influx of Ca^{2+}, and the efflux of CI^- all result in depolarization of the cells due to the rapid equilibration of the internal and external ionic solutions. With enough size and duration of open time, bigger molecules such as intermediates in metabolism (e.g., nucleotides and sugar phosphates) would be allowed to flow out of the pores and cause cell death, also known as pyroptosis, which is represented as a loss of cellular function. Consequently, large proteins such as haemoglobin and lysosomal enzymes leak from red blood cells (RBC), resulting in hemolysis and tissue necrosis (Bashford et al., 1985).

Pore-forming toxins are classified into broad classes based on their secondary structure, with some requiring Ca^{2+} for self-assembly polymerization to form functional transmembrane pores. Because of this, some divalent cations, including Zn^{2+}, Cu^{2+}, and Mg^{2+}, may be able to competitively bind to calcium-binding sites and lead to inhibiting calcium-dependent porin proteins from self-assembling into polymeric pores. In addition, zinc has been reported to have membrane-protective functions in the presence of membrane-disrupting molecules and toxins (MPs) (Yanagihara, 2013).

According to Yanagihara (2019), copper gluconate alone is significantly more effective at reducing venom-induced cell hemolysis than zinc gluconate alone or combined with copper gluconate. In the study, human RBCs were incubated with 5 units/mL/% RBC *Alatina alata* venom, zinc gluconate, and copper gluconate at increasing doses for 1 hour at 37 °C. Per cent hemolysis was determined by OD_{405}. As a result, it was determined that copper gluconate is active at approximately 30 µM or greater for box jellyfish venom hemolysis inhibition,

whereas zinc gluconate is active at approximately 300 μM or greater. Copper gluconate is approximately ten times more potent than zinc gluconate alone in inhibiting box jellyfish venom. Herein, copper gluconate was used to investigate hemolysis inhibition of the *C. indrasaksajiae* venom.

5.2.4 Hot water immersion

Immersion in hot water (45 °C) is a well-established and widely recommended treatment for marine envenomations, including severe pain from echinoderm and venomous fish stings (Buckley and Isbister, 2012; Ongkili and Cheah, 2013). Immersing the sting location with hot water is considered to deactivate key components of marine venoms, although the exact mechanism of action remains unclear. Other researchers suggested that the temperatures required for such inactivation are significantly higher (60 °C), and that the pain relief experienced during hot-water immersion is the result of physiological responses to the heat application rather than a chemical event (such as increased subcutaneous blood flow) (Wilcox and Yanagihara, 2016). The widespread hypothesis is that the temperatures were used to inactivate venom components. Several in vitro and in vivo investigations indicate that cnidarian venoms from all classes within the phylum are heat-labile (Table 2). Another possibility is that hot-water immersion has a direct potential therapeutic impact on pain receptors, decreasing perceived pain (Muirhead, 2002). Herein, temperatures were utilized in this study to determine the inactivation of *C. indrasaksajiae* venom components.

Table 2 Published evidence of heat inactivation of cnidarian venoms or venom components at temperatures (Adapted from Wilcox and Yanagihara, 2016)

Study	Species	Methods	Results
Baxter & Marr (1969)	*Chironex fleckeri* (Cubozoa)	Venom heated to 30, 40, 50, 60, or 70 °C for 10 min Rapid exposure: venom brought "quickly" to 44, 47, 50, 53, 56, or 59 °C and immediately chilled	10 min exposure: activity lost between 40 and 50 °C Rapid exposure: activity lost at >53 °C
Endean & Henderson (1969)	*Chironex fleckeri* (Cubozoa)	Venom incubated at 37, 42 or 45 °C for 5, 10, 15 or 20 min	Activity lost between 40–42 °C Barnacle muscle contraction (all activity lost if heated at 42 °C for 5 min, but activity remained after heating at 40 °C for 20 min)
Chung et al. (2001)	*Alatina alata* (formerly *Carybdea alata*; Cubozoa)	Venom serially diluted and maintained at 4, 25, 37, 45, 60 or 100 °C for 30 min	Venom potency decreased when exposed to temperatures above 25 °C; activity was "sharply reduced" at 45 °C, and abolished entirely at 100 °C
Monastyrnaya et al. (2002)	*Radianthus macrodactylus* (Anthozoa)	Isolated cytolysins heated briefly to 35, 45, 60, and 80 °C	Activity decreased linearly with increasing temperature; at 35, 45, 60, and 80 °C caused the loss of 6, 18, 95, and 100% of their initial hemolytic activity.
Carette et al. (2002)	*Chironex fleckeri* (Cubozoa)	Venom incubated at 4, 21.5, 33, 39, 43, 48, 53, or 58 °C for 2, 5, or 20 min	Activity decreased overall with increased temperature and duration of exposure. All activity lost at 20 min of 43 °C or 5 min of 50 °C
Koyama et al. (2003)	*Chiropsalmus quadrigatus* (Cubozoa)	Venom heated to 40 °C for 10 min	Significant attenuation of activity
Marino et al. (2004)	*Aiptasia mutabilis* (Anthozoa)	Venom incubated at 4 °C, 20 °C, 40 °C and 60 °C for 5, 30 or 60 min	All activity lost at 60 °C; activity reduced in a time- and dose-dependent manner at lower temperatures
Noguchi et al. (2005)	*Chiropsalmus quadrigatus* (Cubozoa)	Venom heated to 50 °C for 10 min	All activity lost
Kang et al. (2009)	*Nemopilema nomurai* (Scyphozoa)	A: venom incubated at 4, 20, 40, 60 and 80 °C for 60 min B: venom incubated at 40 °C for 0, 10, 30, 120 or 360 min	A: all activity lost at temperatures > 60 °C, attenuation of activity in 40 °C incubated venom B: activity reduced in a time- and dose-dependent manner
Feng et al. (2010)	*Cyanea nozakii* (Scyphozoa)	Venom incubated at 35, 50, 65 and 80 °C for 20 or 40 min	All activity lost at temperatures > 65 °C; activity reduced in time- and dose-dependent manner
Cuiping et al. (2011)	*Cyanea nozakii* (Scyphozoa)	Venom incubated at 20, 40, 60, and 80 °C for 10, 30, or 50 min	Nearly all activity lost at 60 °C for even 10 min; activity lost with increased temperature and incubation time
Pereira & Seymour (2013)	*Chironex fleckeri* (Cubozoa)	Venom incubated at 24, 37, 44, 46, 48, 50, 60, or 100 °C for 20 min	All activity lost at 44 °C for cardiomyocytes and at 48 °C for skeletal myocytes (80% survival at 44 °C)
Li et al. (2013)	*Stomolophus meleagris* (Schyphozoa)	Isolated venom toxin (SmTX) incubated at 4, 15, 25, 37, 45 and 60 °C for 30 min	Activity decreased with temperature above 37 °C
García-Arredondo et al. (2014)	*Millepora complanata* (Hydrozoa)	Venom incubated at 4, 25, 37, 45, 60 and 100 °C for 30 min	Activity decreased at temperatures ě 45 °C in a dose-dependent manner
Hernández-Matehuala (2015)	*Millepora alcicornis* (Hydrozoa)	Venom incubated at various temperatures between 0 and 100 °C for 60 min	Activity sharply decreased when incubated abouve 40 °C, with >50%

CHAPTER III

Cnidae characterization and optimization of venom extracted from Thai multi-tentacle box jellyfish (*Chironex indrasaksajiae*) by bead homogenization

1. Introduction

Box jellyfish envenomation is becoming an important public health concern in Thailand due to many fatal and severe cases caused by these creatures. *Chironex indrasaksajiae* and *Chiropsoides buitendijki* are multi-tentacle box jellyfish in Thai waters (PMBC Database, 2020). According to the fatal cases, *Chironex indrasaksajiae* is considered as the most venomous box jellyfish in Thai waters that caused death 7 out of 8 cases with dying 2-5 minutes after being stung (Fenner et al., 2010; Sonthichai et al., 2016; Thaikruea and Siriarayapon, 2016; Thaikruea et al., 2012a; Thaikruea et al., 2012b). According to the box jellyfish survey, *Chiropsoides buitendijki* is the most abundant species of box jellyfish in Thai water and can be found 100-200 individuals per net per hour in the blooming season of jellyfish (Yongstar, 2017). There are no reports of fatal cases from *C. buitendijki* in Thai waters. Although *Chiropsoides* envenomation is rare to be as severe as *Chironex*, it can cause severe pain, redness, and swelling in the stinging area of the skin (Thaikruea and Siriarayapon, 2018).

Box jellyfish belong to class Cubozoa, phylum Cnidaria which their cube-shaped medusae can distinguish. Their defining feature of this phylum is a stinging organelle called “cnidae” which are specialized membrane-enclosed cellular organelles or secretions of the Golgi apparatus capable of explosive discharge on the activation of cnidocytes (Beckmann and Özbek, 2012b; Fautin, 2009; Özbek, 2011). Cnidae have been distinguished into three broad types: penetrant nematocysts, the volvent spirocyst (Mariscal, McLean, et al., 1977) and the glutinant ptychocyst (Mariscal, Conklin, et al., 1977). In cubozoa, nematocysts are the only type of cnidae that comprise the cnidome and three main categories of nematocyst are commonly found: mastigophore, euryteles and isorhizas (Gershwin, 2006; Jouiaei, 2016; Östman, 2000). The study of cnidome, and also information upon morphology of cnidae, are now regarded as an important part of jellyfish species identification

(Acuna et al., 2003; Conklin et al., 1977; Östman, 1982; Ryland et al., 2004). Moreover, each type of nematocysts has been described based on the structure of each type (Rifkin and Endean, 1983). Consequently, the toxin should differ amongst nematocyst types (Carrette et al., 2002). Subsequently, the variation in nematocysts ratio could reveal the level venom variation of each species (Killi et al., 2020). This is the first time that the cnidomes of *C. indrasaksajiae* and *C. buitendijki* have been found.

Moreover, box jellyfish venom has attracted researchers because of its toxic properties that cause death within minutes. The biologically active components in the venom of these Thai multi-tentacle box jellyfish have not been isolated, and the mechanism of their toxicity has not been studied yet. The crucial and primary step of venomics is to optimize the conditions and methods of venom extraction for maximum recovery of venom. For decades, acquiring pure box jellyfish venom stored in millions of cnidae has been a major challenge for researchers. Due to the difficulty of obtaining high quantities of venom from these organelles, various novel venom extraction techniques have been developed. Initial venom extraction experiments, for example, focused on natural nematocyst discharge, which required an electrical simulation to rupture the cnidae membrane of the fresh tentacles placed over a human amniotic membrane (Barnes, 1967). The possibility of sample contamination from the amniotic membrane led to efforts to improve mechanical disruption techniques. After that, mortar and pestle grinding in ice and filtered seawater (Endean et al., 1969; Endean and Rifkin, 1983; Endean et al., 1991) were used to extract the venom. Tentacle homogenization followed by low-temperature centrifugation was also used to extract the venom. Sonication with high sound frequencies to rupture nematocysts was also used (Bloom et al., 1998; Mustafa et al., 1995). Poor volume recovery and low yields of venom proteins were problems with these procedures, and venom extracts mixed with extraneous proteins from tentacle rather than pure cnidae content (Cantoni et al., 2020). In 2004, Carrette and Seymour presented a protocol for an extraction procedure that was both quick and repeatable, resolving issues that had arisen in the past. With glass beads and a bead beater, freshly isolated nematocysts (Bloom et al., 1998) were manually burst in MilliQ water at 4°C to avoid heat built-up. By using centrifugation, the venom was isolated from the cnidae structural

components and the tentacle debris. This approach has been applied in numerous box jellyfish venom research due to the rapid and repeatability of the procedure (Carrette and Seymour, 2004). In 2012, Yanagihara and Shohet developed methods of venom preparation and extraction to recover venom yields with high specific activity (Yanagihara and Shohet, 2012). With the use of trisodium citrate to cytolyze the tentacles that were freshly excised from the beachside. This technique gets maximizes recovery of intact cnidae from tentacles (>90% of all cnidae) compared to the venom preparation method of Bloom et al. (1998).

Further research is required to understand the mechanisms of venom activity and the appropriate treatments for envenomation. The isolation of bioactive jellyfish venom is required as one of the initial steps in this procedure. Thus, this chapter aimed to optimize the venom extraction protocol from the cnidae isolated from the whole tentacle to acquire the highest protein yield to be researched further for the effects of these box jellyfish according to Yanagihara and Shohet (2012) procedure of venom preparation and Carrette and Seymour (2004) for venom extraction method by using bead homogenization.

2. Materials and methods

2.1 Jellyfish collection

The animal collection was done according to the approved protocol by the Mahidol University-Institute Animal Care and Use Committee and the Mahidol University Biosafety Committee (see appendix Biohazard Control Plan). The matured specimen of *C. indrasaksajiae* was captured near the coastal zone at Chaloklum Bay in Koh Pha-Ngan (Suratthani, the Gulf of Thailand) in August 2019 (Figure 14) that is the seasonal of jellyfish occurring in this area (see appendix Figure A5). The green spotlight was used to look at them at night and picked the jellyfish up by using a fishing net that had a plastic bag inside to prevent the attachment of the tentacles to the net. (Figure 15B)

During the survey and collection of box jellyfish samples at Libong Island and Sikao Bay in September 2020 (Trang, the Andaman Sea, Thailand) (Figure

14), *C. buitendijki* was found that is also the seasonal of jellyfish occurring in this area (see appendix Figure A6). This is considered a good opportunity to represent the less venomous multi-tentacle box jellyfish compared to the deadly venomous ones (*C. indrasaksajiae*). A total of 102 *C. buitendijki* samples were collected by using the shrimp trammel net on transects along with the water current for 1 hour (Figure 15A). The net was 140 meters long and 1.4 meters wide. The trammel net was composed of 3 layers, with the outer layers of the net (14–26 cm mesh size) covering the inner layer (4 cm mesh size).

After taking the box jellyfish out of the water, the whole tentacles from live jellyfish samples of both species were manually excised from the beachside and preserved in 1 M tri-sodium citrate solution (1 volume of tentacles: 9 volumes of citrate solution) at 4 °C. After that, the samples were transported to the lab, always kept at 4 °C, and frequently shaken for the cytolysis process.

For examining the distribution of cnidae, a centimeter of live jellyfish tentacle was excised and preserved in chilled glutaraldehyde solution for analysis with Light Microscopy (LM), Scanning Electron Microscopy (SEM), and Transmission Electron Microscopy (TEM).

For species identification, box jellyfish species were examined for morphological identification using photographs taken by a camera, bell height measuring, and branching pedalia counting. In addition, molecular identification was performed, including DNA extraction, PCR amplification, and sequencing, following the optimized protocol (Sathirapongsasuti et al., 2021). The resulting sequences were compared with the Thai box jellyfish database (https://med.mahidol.ac.th/transmed/en/Research/NS/Research/TBJ_Database) using the Basic Local Alignment Search Tool (BLAST) (http://bl-ast.Ncbi.nlm. nih.gov).

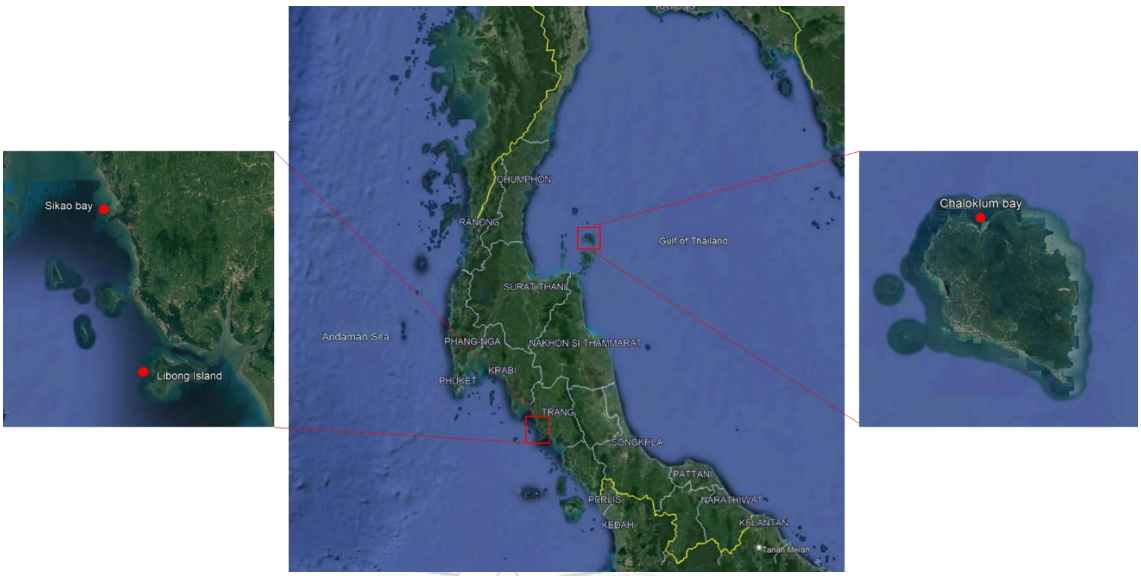

Figure 14 Map showing the sampling sites in the Gulf of Thailand and the Andaman Sea. (Left) The sampling location in the Gulf of Thailand is Chaloklum bay at Koh Pha-ngan (Red circle). (Right) Sampling locations in the Andaman Sea are Libong Island and Sikao bay in Trang province (Red circle). Source : Satellite imagery (September 2020) courtesy of Google Earth.

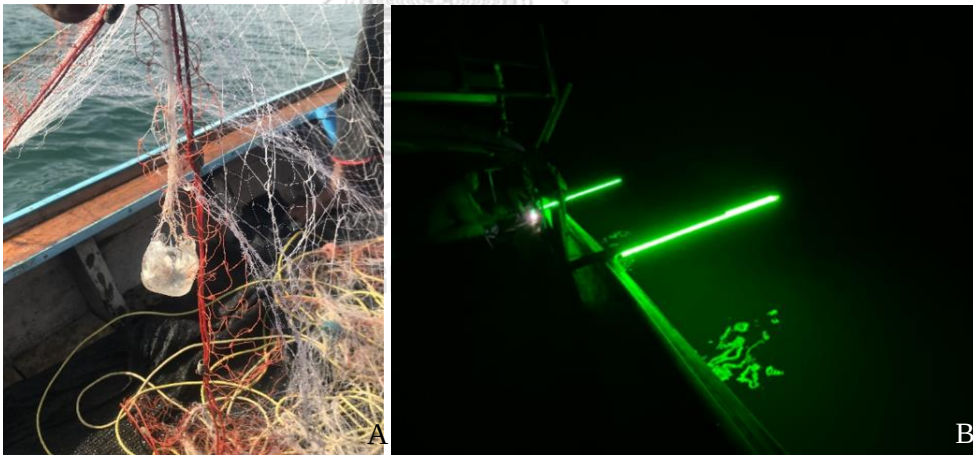

Figure 15 shows the various sampling techniques (A) The trapped jellyfish in the net that is used on the transect. (B) The green spotlight was used to find the jellyfish.

2.2 Nematocysts isolation and washing

The tentacle solutions (Figure 16A) were gentle rotation at 4 °C until less than 10-20 percent debris was observed under microscope. In this cytolysis period, the tentacles were frequently checked for % recovery of cnidae and then sieved through a 0.5-mm mesh size. (Figure 16B). Filtered solution (cnidae solution) was continually shaken. After approximately 90% recovery of cnidae, the solutions were sieved, and the filtered solutions were collected. Cnidae were kept using two different methods.

The first one, cnidae pellets were stored at -80 °C. Initially, the samples were centrifuged at 400 g at 4 °C for 20 min. Next, undischarged cnidae pellets were gently resuspended in chilled 1 M trisodium citrate solution at 1:20 (v:v) and separated them into 2 ml tubes. Afterward, the samples were washed twice with chilled 1 M trisodium citrate solution and centrifuged at 250 g for 20 min. The supernatant was removed, and the pellets were collected for morphological observation using the light microscope and then stored at -80 °C until use. (Figure 17)

In the second one, cnidae were maintained as cnidae solution (citrate solution) at 4 °C. The step started with two techniques of cnidae precipitation. In the first technique, the cnidae solutions were centrifuged at 4 °C at 400 g for 20 min. Undischarged cnidae pellets were resuspended in chilled 1 M tri-sodium citrate at 1:20 (v:v). The second technique, the cnidae solution, was continually precipitated at 1 g at 4 °C for a week. It would be separated into three layers (Figure 18). Then, each layer was collected into a separate tube for the cnidae pellets' checking before elimination. The precipitated pellets were resuspended in chilled 1 M tri-sodium citrate at a ratio of 1:20 (v:v). After the resuspension process from these two techniques of cnidae precipitation, the samples were given a gentle shake. Take 7 µl of samples to the Fast Read 102® plate for the quality control process by debris checking (debris refers to cytolyzed tentacle tissue). Next, the samples were washed at 250 g for 20 min, followed by debris checking and backwashing until the solutions ran clear or acceptable. During the process of the cytolysis method, the light microscope was used for the observation of nematocyst morphology.

Figure 16 (A) Tentacles from live jellyfish were preserved in chilled 1 M tri-sodium citrate solution at 4 °C. (B) The solutions were sieved through a 0.5-mm mesh size funnel filter. (Photographs by Chanikarn Yongstar)

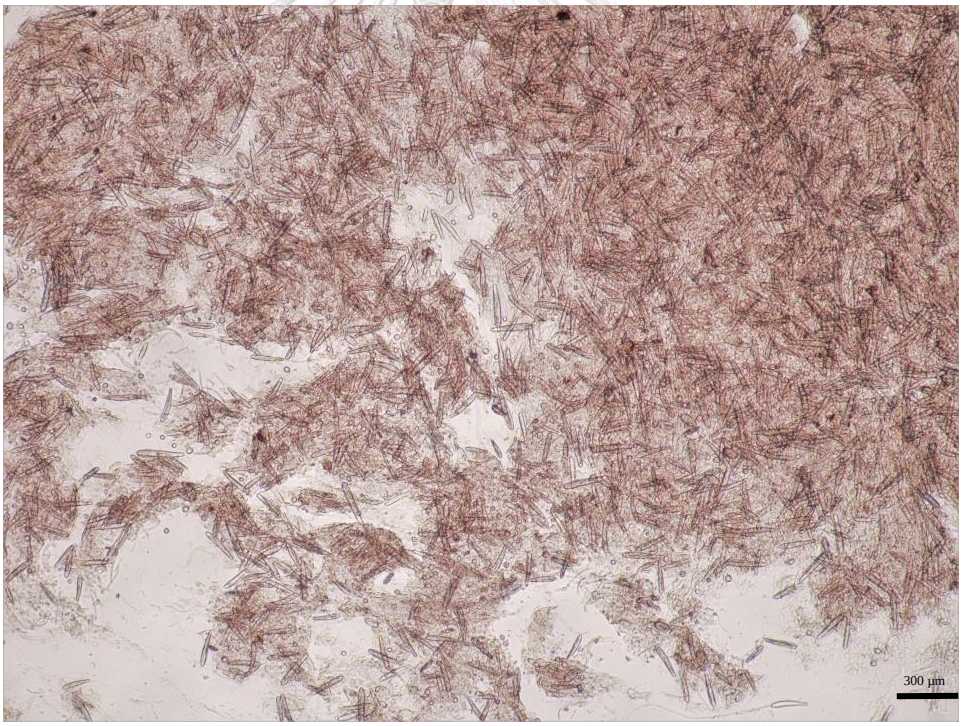

Figure 17 The undischarged cnidae pellets on the process of morphology observation under the light microscope. (Photographs by Chanikarn Yongstar)

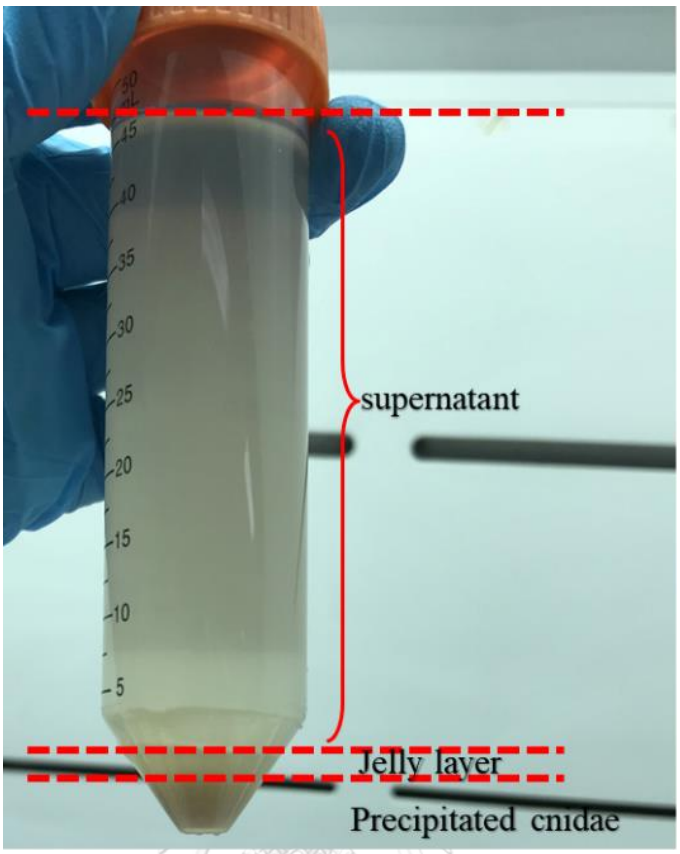

Figure 18 Three layers of the sample after leaving it on precipitation at 1 g in 4 °C for a week. (Photographs by Chanikarn Yongstar)

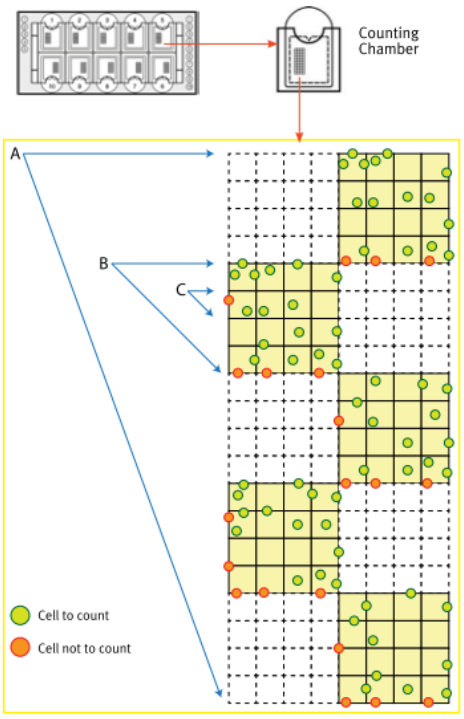

Figure 19 Fast Read 102 cell count: a disposable plastic device that is a slide divided into 10 independent chambers, each containing a standard volume of 7 µl. The sample was introduced into the chamber using a pipette and then examined the area of the grid under the light microscope. After the counting chamber was filled, the cells distributed in the 5 squares (black lines) were counted. Considering (A) each chamber contained a grid with a volume of 1 µl. (B) Each grid was divided into 10 squares. (C) Each square has a dimension of 1 x 1 mm, a depth of 0.1 mm, and a volume of 0.1 µl. Each square was subdivided into 16 smaller squares (called "sectors"). Considering the cells on the edges, it was only counted the upper and right sides of each square to avoid the risk of overestimation and underestimation.

2.2.1 Estimating the number of cnidae in a sample

The Fast Read 102® was used to count and estimate the number of cnidae present in the trisodium citrate solution (Figure 19). The amount of tissue debris and cnidocilia were counted and the cnidae concentration (cnidae/ml) was determined by the formula:

$$\text{Cnidae } \left(\frac{\text{cnidae}}{\text{ml}}\right) = \frac{\sum \text{cnidae counted in 5 square}}{5} \times 10^4$$

In counting, the types of cnidae were additionally classified based on the structure of the tubule, armature, and cnidae nomenclature with LM techniques according to Östman criteria. (Gershwin, 2006; Jouiaei, Casewell, et al., 2015; Östman, 2000).

2.2 Optimization of the conditions for maximum recovery of venom

This part aims to optimize the conditions for venom extraction from cnidae solutions. Preliminary tests with ultrasonication demonstrated in our hands its limited applicability to rupture the nematocyst cells. A lot of cnidae remained intact. I, therefore, explored in detail a bead mill homogenizer (Precellys 24 homogenizer, Bertin Technologies SAS, France). Moreover, different homogenization tubes with various beads in various sizes are available for this instrument. In this experiment, four types of homogenization tubes with various beads were used, including ceramic beads with a diameter of 1.4 mm, 2.8 mm, and a mix of 1.4 mm and 2.8 mm beads, as well as glass beads with a diameter of 0.5 mm. According to the manufacturer, tubes with ceramic beads with a diameter of 1.4 mm are recommended for soft animal tissue. Tubes with ceramic beads (2.8 mm in diameter) and mixed beads of both sizes are recommended for hard or vegetal tissues. They suggest that a mix of beads makes it possible to grind heterogeneous samples. The last type, glass beads with a diameter of 0.5 mm, is adapted for mechanical lysis of cells or bacteria. Besides the parameters of bead types, I also tried different three periods of homogenization intervals (40s,

45s, and 60s) and two levels of homogenization speed (5000 rpm and 6000 rpm). *C. buitendijki* samples were used for the first optimizing extraction method before going on to *C. indrasaksajiae* samples. According to estimating the number of cnidae in a sample, the proportion between cnidae and tissue debris is acceptable for extraction when the percentage of undischarged cnidae is greater than 60%.

2.3 Venom extraction

Prior to any process of extraction, a 7 µl sample (of each vial) of the fluid was placed on a Fast Read 102® cell counting slide to determine the number of whole cnidae in the solution. The extraction was performed one replicate due to the limitation of sample size with one sample. Approximately 750,000 cnidae for *C. indrasaksajiae* and 4 million cnidae for *C. buitendijki* were suitable amounts per extraction tube. This amount of cnidae was ~200 mg in wet weight. Then the amount of calculated chilled cnidae solution samples was transferred to chilled 2 ml homogenizing tubes containing various types of beads. These beads included ceramic beads with a diameter of 1.4 mm, 2.8 mm, and a mix of 1.4 mm and 2.8 mm beads, and glass beads with a diameter of 0.5 mm using a serological pipette. The samples were centrifuged at 4°C, 10,000g for 10 minutes. (Note: make sure that all cnidae were precipitate, not still suspended in solution.) After that, the supernatant was removed and discarded. For the extraction solvent, 2% SDS was used by adding up to 1.4 ml for each tube (the recommended maximum volume of homogenizing tubes). Cnidae pellets were resuspended by pipetting up and down gently. Then, the solutions in homogenizing tubes containing beads were shaken in Precellys 24 homogenizer at 5000 and 6500 rpm for 10-s , 15-s and 20-s intervals, repeat 4-6 cycles. (after 40 s approximately 95% of cnidae were ruptured for *Chironex* and 75% for *Chiropsalmus* (Carrette and Seymour, 2004)). Furthermore, the cnidae mixture was held in an ice bucket for two minutes before disruption started and for an additional one minute after each sequential disruption event. The percentage of discharged cnidae was monitored microscopically. Venom extracts were centrifuged at 10,000g at room temperature for 15 min to separate the protein dissolved in the solution. The supernatant was then aliquoted and immediately stored at -30 °C until use.

2.4 Quality control method

The Quality Control for samples includes measuring protein concentration and estimating per cent disruption. Firstly, the protein concentration was measured triplicate using a BCA protein assay kit. It was assumed that protein concentration was directly proportional to venom concentration (Carrette and Seymour, 2004). This BCA method was described by Smith et al. (1985). This study utilized albumin as a protein standard at concentrations of 0, 25, 125, 250, 500 to 1000 g/ml, and 1000 g/ml. The working reagent was prepared by mixing reagent A with reagent B (50:1). Then, the working reagent (200 µl) was mixed with the sample (25 µl) and incubated at 37 °C for 30 minutes. The peptide bond formed by the proteins can reduce Cu^{2+} in the working reagent to Cu^{+}, resulting in a change in the color of the solution from green to purple. The changing color can be measured using a microplate reader at 562 nm in Infinite M200 PRO microplate reader (Tecan, Grödig, Austria). The protein concentration of the sample can be calculated from protein standard curve. Another Quality Control process is calculating % disruption. The rest of sample at the bottom of each homogenized tube were taken 7 µl to the Fast Read chamber, then determined the number of wholes, and disrupted cnidae in the mixture using LM.

2.5 Statistic analysis

Due to the limitation of sample size with one sample, triplicate in this part were mentioned as replication of precision in each step of venom extraction. All results were expressed as means. The quantity of venom extracted from different conditions were evaluated by using compare means via SPSS software (version 24).

3. Results and discussion

3.1 Observations of cnidae

The cnidome, or census of cnidae in a species, was microscopically examined during cytolysis. In addition, induced discharge of cnidae was performed for explicit characterization. A total of 6 nematocyst types: large microbasic mastigophore, medium microbasic mastigophore, small microbasic mastigophore, trirhopaloid, small microbasic eurytele and ellipsoidal isorhiza, were identified in *C. indrasaksajiae* sample (Figure 20), whereas six nematocyst types: medium microbasic mastigophore, hockey-stick-form microbasic mastigophore, trirhopaloid, small microbasic eurytele, holotrichous isorhiza and ellipsoidal isorhiza, were observed in *C. buitendijki* (Figure 21). Interestingly, *C. buitendijki* and *C. indrasaksajiae* contained four main types of nematocysts: mastigophores, oval trirhopaloid, euryteles and isorhizas but there are some different in size, form, and proportion (Figure 22). Only holotrichous isorhiza and hockey-stick-form microbasic mastigophores were found in *C. buitendijki* but not *C. indrasaksajiae*.

The cnidae abundance was further examined as a percentage of the total count of each cnidae type. In *C. buitendijki* the proportion of mastigophores was less than that in *C. indrasaksajiae*. As a result of the proportion of euryteles and isorhizas, *C. buitendijki* had a higher proportion than *C. indrasaksajiae*. Finally, there is no difference in the proportion of oval trirhopaloid.

The proportions of these cnidae indicate the effect of box jellyfish species and medusa size on the proportion of cnidae. An increase in the proportion of mastigophores in the cnidome of *C. indrasaksajiae* may also be responsible for why *Chironex* species has caused numerous human fatalities, while the *Chiropsoides* species has not. McClounan and Seymour (2012) study showed that the proportion of mastigophore is presumably the lethal venom component. Similarly, the species and size of the medusae affected the proportion of euryteles and isorhizas that *C. buitendijki* had a higher proportion of euryteles and isorhizas than *C. indrasaksajiae*. According to Endean et al. (1969) and Carrette et al. (2002), the proportion of mastigophores in the genus *Chironex* increases rapidly from juvenile to mature. It

seems like the change in cnidae proportion of *Chironex* sp. fluctuates in response to its food. Moreover, an increase in the overall proportion of mastigophores indicates an increase in the amount of venom produced per animal required to paralyze or kill prey. Similarly, the variation in the proportions of euryteles and isorhizas emphasizes the hypothesis of changing cnidae proportions for different types of prey. According to the study by Rifkin and Endean (1983), the functions of isorhiza may be to grapple and hook to the surface of the prey by entanglement and adhesion. Moreover, this difference in the total proportion of mastigophores between *C. indrasaksajiae* and *C. buitendijki* could perhaps explain the differences in human fatalities related to both species. According to Williamson et al. (1996), mastigophores contain the venom associated with death. Thus, the proportion of mastigophores was formerly thought to be the reason for variation in fatal envenomation between these two species. The difference in fatalities encounters may be due to the percentage of mastigophores, with an increase in *C. indrasaksajiae* proving to be enough to give the lethal dosage as the results of several fatal cases in Thailand whereas *C. buitendijki* has not. (Fenner et al., 2010; PMBC Database, 2020; Sonthichai et al., 2016; Suntrarachun et al., 2001; Suriyan et al., 2019; Thaikruea and Siriarayapon, 2016; Thaikruea et al., 2012a; Thaikruea et al., 2012b)

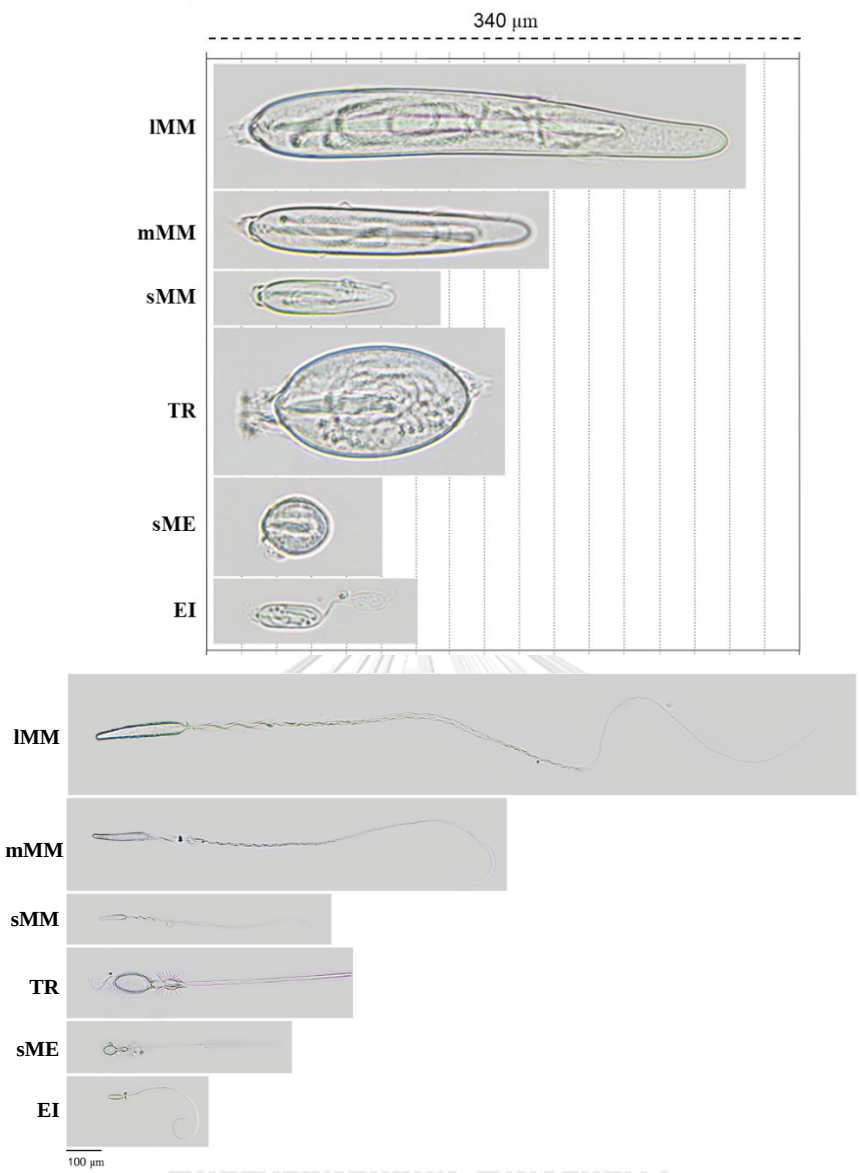

Figure 20 Representative undischarged and discharged cnidae of *C. indrasaksajiae*. Letters on left refer to the different types of undischarged cnidae, as follows: lMM = large microbasic mastigophore; mMM = medium microbasic mastigophore; sMM = small microbasic mastigophore; TR = trirhopaloid; sME = small microbasic eurytele; EI = ellipsoidal isorhiza. On the right side represent discharged cnidae; (A) discharged large microbasic mastigophore; (B) discharged medium microbasic mastigophore; (C) discharged small microbasic mastigophore; (D) discharged trirhopaloid; (E) discharged small microbasic eurytele; (F) discharged ellipsoidal isorhiza. Scale bars = 100 µm.

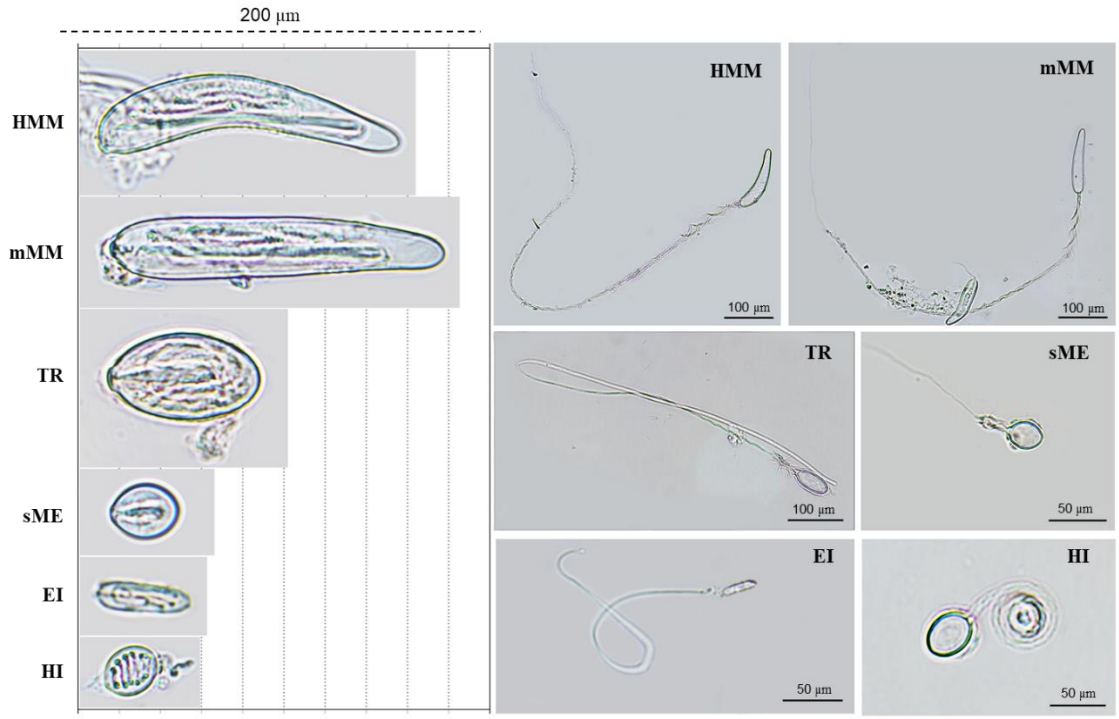

Figure 21 Representative undischarged and discharged cnidae of *C. buitendijki*. Letters on left refer to the different types of undischarged cnidae, as follows: mMM = medium microbasic mastigophore; HMM = hockey-stick-form microbasic mastigophore; TR = trirhopaloid; sME = small microbasic eurytele; HI = holotrichous isorhiza; EI = ellipsoidal isorhiza. On the right side represent discharged cnidae; (A) discharged medium microbasic mastigophore; (B) discharged banana-form microbasic mastigophore; (C) discharged trirhopaloid; (D) discharged small microbasic eurytele; (E) discharged holotrichous isorhiza; (F) discharged ellipsoidal isorhiza. Scale bars of HMM, mMM, TR = 100 µm and sME, EI, HI = 50 µm.

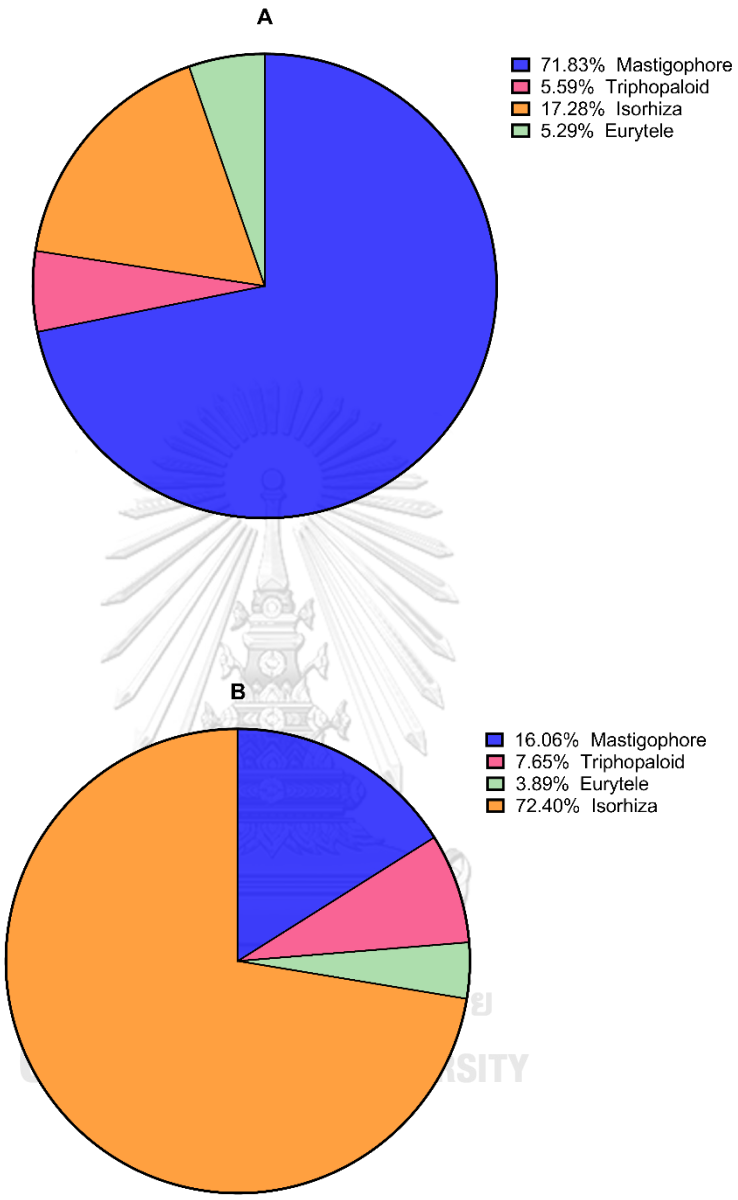

Figure 22 represents the proportion of multi-tentacle box jellyfish cnidae observed in (A) *C. buitendijki* and (B) *C. indrasaksajiae*. The pie chart represents the percentage of each type of cnidae in the cnidome present in each species.

3.2 Optimization of venom extraction using bead homogenization

This part aims to optimize the conditions of venom extraction from cnidae for maximum recovery of *C. indrasaksajiae* venom yields. The elucidation of the mechanism of action of these venoms has been hindered by several technological restrictions in venom preparation. Cnidarian envenomations here include the penetration of prey by myriad tiny, specialized penetrant cnidae, or nematocysts, each holding just picolitres of venom. Aqueous extract preparations of whole tentacles or partially purified cnidae provide only an incomplete proportion of aqueous soluble venom components that are contaminated with other tentacular and structural components, including cnidae capsule- wall collagens.

Cnidae extraction can be accomplished using several methods. Our preliminary tests with ultrasonication demonstrated the limited applicability of ultrasonication to rupture the nematocyst cells. The majority of the cnidae remained intact after ultrasonication. Therefore, one of the most commonly used methods for jellyfish venom extraction which is bead homogenization was considered to utilize in this process. Carrette and Seymour (2004) proposed that the method is quick, reproducible, and has little heat built up. In this study, I explored a Precellys 24 homogenizer by utilizing different types of bead homogenization tubes, different homogenization speeds, and different homogenization times. Experiments were first performed using *C. buitendijki* because the specimens were readily available with many samples for optimization and a greater quality of % cnidae recovery after the cytolysis process than with *C. indrasaksajiae* according to Yanagihara and Shohet (2012) procedure of venom preparation (Appendix figure A1-4). This is the newly developed method of osmotic effect on venom preparation. Trisodium citrate solution as strong as 1 M succeeded in obtaining a nice preparation of isolated cnidae by acting like a hypotonic solution for cnidae to lightly emission from cnidocyte without prompting the cnidae to discharge.

Step 1: Selection of beads; the results of the protein concentration and the percentage of cnidae disruption from the use of different types of beads with a setting homogenization speed of 5,000 rpm and a homogenization time of 40s based on the protocol of Carrette and Seymour (2004). This study clearly indicated that the

ceramic bead with a mix of 1.4 mm and 2.8 mm bead size is the most suitable for homogenizing cnidae (Table 3). According to the manufacturer, this bead is recommended for elastic tissue (Yu, 2016). Of these, it is proper for the cnidae that the first structural nematocyst molecules identified in isolated capsules were collagenous and termed (Beckmann and Özbek, 2012a).

Step 2: Selection of homogenized conditions; The highest quantity of protein concentration was obtained when the homogenization speed was set to 5000 rpm. and the homogenization time was set to 45 s (15 s-intervals with 3 cycles) (Table 4). The result showed the homogenization speed have effect on venom protein concentration. According to Carrette and Seymour (2004), increasing in speed of homogenization cause more heat buildup in tube lead to the possibility of venom denaturing. For the homogenization time, the highest quantity of protein concentration was obtained when the homogenization time was set to 45 s (15 s-intervals with 3 cycles) (Table 4). According to Carrette and Seymour (2004), after 40 s approximately 95% of nematocysts were ruptured for *Chironex* sp. and 75% for *Chiropsalmus* sp. The results of *Chiropsoides* revealed that increasing homogenization time increased yield. However, homogenizing for too long reduces yield because it allows more heat to accumulate during homogenization. It is another possibility of venom denaturing. Then, the homogenized tube with mixed size of ceramic beads were applied to homogenize at the condition of 5000 rpm in speed and 15 s intervals in time for *C. indrasaksajiae* venom extraction. However, the protein concentration and percentage of cnidae disruption results didn't turn out the way of expectation. Consequently, suitable conditions were then examined for this species. The most appropriate conditions for *C. indrasaksajiae* species were 0.05 mm glass beads with a homogenization speed of 5000 rpm and a homogenization time of 15 s–intervals.

Table 3 The average of protein concentrations released in cnidae disruption at homogenization speed of 5000 rpm and homogenization time of 40s for different types of beads.

	Protein concentration (µg/ml)	
Type of beads	Protein concentration (µg/ml)	% cnidae disruption
Glass beads (0.5 mm)	124.162	63.33
Ceramic beads (1.4 mm)	145.445	80.67
Ceramic beads (2.8 mm)	147.617	70.67
Ceramic beads (mixed of 1.4 mm, 2.8 mm)	159.113	85.00

Table 4 The average of protein concentrations obtained by different techniques of extracting venom from box jellyfish venom

	Protein concentration (µg/ml)	
Type of beads	*C. buitendijki*	*C. indrasaksajiae*
Glass beads (0.5 mm)	124.162	70.784
Ceramic beads (1.4 mm)	145.445	-
Ceramic beads (2.8 mm)	147.617	-
Ceramic beads (mixed of 1.4 mm, 2.8 mm)	159.113	24.213
Homogenization speed	*C. buitendijki*	*C. indrasaksajiae*
5000 rpm	187.021	70.7835
6500 rpm	156.286	19.2834
Total Homogenization time	*C. buitendijki*	*C. indrasaksajiae*
40s	142.593	-
45s	199.714, 401.278*	101.7165*
60s	187.021	-

Initial mass of cnidae 50 mg and *200 mg

CHAPTER IV

Venom proteome of the Thailand sea wasp, *Chironex indrasaksajiae* (cnidaria: cubozoa)

1. Introduction

A large number of humans experience jellyfish stings, particularly along with coastal areas of the ocean where numbered millions of stings take place annually worldwide (Fenner, 1997a). By stinging, many jellyfish species have been known to cause injuries or even death (Cegolon et al., 2013; Fenner and Williamson, 1996; Mubarak et al., 2021; Tibballs, 2006b). Such serious reactions which can be more than 100 fatal cases per year (Pirkle and Yanagihara, 2019), are consequences of stings inflicted by box jellyfish which the infamous *Chironex fleckeri* (Australian box jellyfish) is considered as one of the most venomous marine creatures in the world (Williamson et al., 1996). A meter of tentacle contact can occasionally result in death attributed to acute cardiovascular collapse within minutes after stinging (Yanagihara and Shohet, 2012).

Thailand is located in the Indo-Pacific region, the hotspot zone for box jellyfish envenomation (Gershwin et al., 2010; Gershwin et al., 2013). The medical records showed numerous envenomation cases of box jellyfish (both Thai and foreigners) in Thai water since 1998 (Thaikruea and Siriarayapon, 2014). However, many people do not believe that box jellyfish can kill humans, so they dismiss and neglect this problem. Thus, the knowledge about box jellyfish biodiversity or envenomation has been limited in Thailand. Currently, twelve species of jellyfish in Class Cubozoa are found in Thai waters (PMBC Database, 2020; Toshino et al., 2019). Among all species found in Thai waters, Chirodropids, or multi-tentacled box jellyfish, are believed to pose a serious danger to humans which have been referred from the injuries and fatal case reports of box jellyfish envenomation in Thailand (Thaikruea and Siriarayapon, 2015b, 2016, 2018; Thaikruea et al., 2012a; Thaikruea et al., 2012b). Until now, regarding the animal distribution and seasonal, *C. indrasaksajiae* is expected to be a cause of seven fatal cases in the central Gulf of Thailand, the islands of Samui and Pha-ngan (Thaikruea and Siriarayapon, 2016).

This species has recently been described as a new species of box jellyfish from the Gulf of Thailand (Sucharitakul et al., 2017). The envenomation of the genus *Chironex* occasionally results in death attributed to acute cardiovascular collapse within minutes (Tibballs, 2006b).

The entire tentacle and nematocyst extracts of *Chironex* have been linked to several biological activities, including fatal, dermonecrotic, nociceptive, cytotoxic, neurotoxic, myotoxic, and cardiotoxic effects (Brinkman and Burnell, 2009). Even though *C. indrasaksajiae* venoms have a significant medical and pharmacological impact on humans owing to the high number of fatalities in Thai water and because it is a newly described species in Thailand; thus, their compositions have not yet been studied. This is the first time that the proteomic characterization of the venom of *C. indrasaksajiae* has been done. This may be valuable information in understanding the venom compositions and their modes of action and also helpful for treating stung patients.

2. Materials and methods

2.1. Jellyfish collection

The animal collection was done according to the approved protocol, Mahidol University-Institute Animal Care and Use Committee, and Mahidol University Biosafety Committee. *C. indrasaksajiae* was captured near the coastal zone at Chaloklum bay of Koh Pha-ngan (Suratthani, the Gulf of Thailand) in August 2019. Whole tentacles from live jellyfish samples of these two species were manually excised and preserved in 1 M tri-sodium citrate solution (1 volume of tentacles: 9 volumes of citrate solution) and kept at 4 °C, then vigorously shaken for the cytolysis process.

For species identification, box jellyfish species were examined for morphological identification using photographs taken by a camera, bell height measuring, and branching pedalia counting. In addition, molecular identification was performed, including DNA extraction, PCR amplification, and sequencing, following the optimized protocol (Sathirapongsasuti et al., 2021). The resulting sequences were

compared with our Thai box jellyfish database (https://med.mahidol.ac.th/transmed-/en/Research/NS/Research /TBJ_Database) using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm. nih.gov).

2.2 Venom extraction for *Chironex indrasaksajiae*

Box jellyfish venom was extracted using a bead mill homogenizer (Precellys 24 homogenizer, Bertin Technologies SAS, France) according to the manufacturer's protocol and the method described by Carrette and Seymour (2004) with some modifications. Briefly, 200 mg of cnidae (approximately 750,000 cnidae of *C. indrasaksajiae*) were aliquoted to a Precellys 24 VK05 lysing kit 2.0-ml tube with 0.5 mm glass beads and 1.4 ml of 2% SDS were then added to the tube. Samples were processed in 6 cycles, each consisting of a bead beating at 5,000 rpm for 15 seconds. Tubes were rested on ice for at least 1 minute between each cycle. Lysates of venom extract were cleared by centrifugation at 10,000g at 4°C for 15 minutes. Supernatants were aliquoted to a protein LoBind 1.5 ml tubes and stored at -30°C until used. The percentage of discharged cnidae was monitored microscopically.

BCA protein assay was used to measure protein concentration. It was assumed that protein concentration was correlated with venom concentration (Carrette and Seymour, 2004). Measurement of protein using bicinchoninic acid was described by Smith et al. (1985). Albumin was used for protein standards at concentrations of 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml. The samples were read at 562 nm in Infinite M200 PRO microplate reader (Tecan, Grödig, Austria). Prior to the venom activity assay, frozen venom samples were thawed and are referred to as "thawed" venom.

2.3 SDS-PAGE

The venom proteins from box jellyfish cnidae were separated by Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins according to their sizes and charges. Twenty microliters of venom solutions were prepared, consisting of the sample (13 µl), loading buffer (5 µl), and 100 mM

DTT (2 µl). The solution was then heated at 95 °C for 3 minutes, followed by a 30-second spin-down step. Next, the venom solutions were loaded into a Run-Blue™ 4–12% bis-tris precast gel (Expedeon, San Diego, CA, USA). The gel was placed in a RunBlue MES buffer system and ran at a constant voltage of 100 V for 45 minutes. After protein separation, protein bands were visualized using InstantBlue® protein stain for 2 hours, and the size of protein bands was compared with the protein marker (Enzmart Biotech, Bangkok, Thailand).

2.4 In-gel tryptic digestion

The protein bands were excised from a gel into small pieces (5 fragments) and placed in Eppendorf tubes for in-gel digestion following the protocol from Shevchenko et al. (2006) with modifications. Gel fragments were destained using 500 µl of 100 mM ammonium bicarbonate and acetonitrile (1:1 vol/vol), incubated and vortexed for 30 minutes. After 30 minutes, the solution was removed, and acetonitrile (200 µl) was added to shrink the gel. The protein in the gel was then reduced with 10 mM DTT (200 l) and incubated for 30 minutes at 56 °C. After that, acetonitrile (200 µl) was added after 10 minutes of incubation. Then 55 mM chloroacetamide solution (200 µl) was added to alkylate the proteins and incubated for 20 minutes in the dark. The gels were shrunk with acetonitrile solution for about 10 minutes. 13 ng/µl of trypsin (150 µl) were added to the gel pieces and incubated on ice for 120 minutes to saturate the gel. Then the trypsin solution was removed before adding 100 mM ammonium bicarbonate (100 µl) to cover the gel pieces. The proteins were then digested and incubated overnight at 37 °C. After that, peptides were extracted by extraction buffer (5% formic acid and acetonitrile, 1:2 vol/vol) and incubated at 37 °C for 15 minutes. Then peptide solution was collected and lyophilized by the evaporator. Then, peptides were resuspended with 100 µl of 0.1% formic acid for LC-MS/MS analysis.

2.5 Peptides preparation for LC-MS/MS analysis

Prior to LCMS/MS analysis, the peptide samples were desalted using C_{18} StageTips (Rappsilber et al., 2007). Briefly, microcolumns for peptide desalting were prepared from 200-µL pipette tips and a C_{18} membrane disk. Two mg/mL of Jupiter® C_{18} beads (15 µm, Phenomenex, Torrance, CA, USA) suspended in acetonitrile was packed on the C_{18} membrane disk by centrifugation at 3000g for 3 minutes. The spin column was equilibrated with 0.1% formic acid, and the peptide samples were applied to the sorbent. Bound peptides were washed with 0.1% formic acid and eluted by 0.1% formic acid in 70% acetonitrile. The solvent was evaporated using vacuum centrifugation, and dried peptides were stored till LC-MS/MS analysis at –80°C.

The venom peptides samples were resuspended in 0.1% formic acid. They were then subjected to a Pierce™ peptide quantification assay using fluorescence detection following the protocol suggested by the manufacturer for venom peptide quantification. Briefly, albumin peptides were used as the standard solution at concentration of 0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 µg/ml. The standard and sample solutions (10 µl) were incubated with 70 µl of fluorometric peptide assay buffer and 20 µl of fluorometric peptide assay reagent for 5 minutes. The fluorescence of sample was measured at the excitation wavelength of 390 nm and emission wavelength of 475 nm using a fluorescence spectrophotometer (Agilent Cary Eclipse fluorescence spectrophotometer, Agilent).

2.6 LC-MS/MS analysis

Dried venom peptides were resuspended in 0.1% formic acid. Each 400 ng of the resuspended peptides was injected into a C18 analytical column for low-pH reverse phase separation using an Acclaim™ PepMep™ 100, nanoViper C18 column (75 µm × 2 cm, 3 µm, Thermo Fisher Scientific) coupled to an EASY-nLC 1000 ultra-high pressure liquid chromatography system (Thermo Fisher Scientific). Peptides were separated by a gradient concentration mixture of mobile phase buffer (solvent A: 0.1% formic acid and 99% Type I distilled water) and eluting buffer

(solvent B: 0.1% formic acid and 99.9% acetonitrile) shown in table 5. The solvent A and B were run over 60 min at a flow rate of 300 nL/min. After separation, eluted peptides from the column were ionized by nano-electrospray ionization before being transmitted to the mass spectrometer. The Easy-nLC 1000 was connected online with a Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific) using an EASY-Spray ion source (Thermo Fisher Scientific) as an interface. The ions spray voltage was set to 2000 V. The mass spectrometer was operated in the Data Dependent Acquisition (DDA) mode in which a full MS scan (the mass range of precursor ions: 350-1600 m/z; the resolution of orbitrap mass analyzer: 70,000; the precursor AGC: $3x10^6$) was followed by the 10 most intense precursor ions being subjected to Higher-energy Collisional Dissociation (HCD) fragmentation. The precursor ions with a charge of > +1 were selected for fragmentation. HCD fragmentation was performed and acquired the automatic gain control (AGC) target of $5x10^4$, maximum injection time of 100 milliseconds and an isolation window of 2.0 m/z. The m/z of product ions was scanned at a resolution of 17,500. All data were acquired using Thermo Xcalibur 2.2 software (Thermo Fisher Scientific).

Table 5 Concentration of mobile phase buffer (solvent A) and eluting buffer (solvent B) were used to elute peptides for 60 minutes.

Time (minutes)	% Solvent A	% Solvent B
0	95	5
43	80	20
53	60	40
55	5	95
60	5	95

2.7 Spectral searches and bioinformatics analysis

The raw MS/MS spectral data from each LC-MS/MS run was searched against the custom cnidarian proteins and toxins databases using MaxQuant software (Galaxy Version 1.6.10.43) with the Andromeda search engine in the Galaxy bioinformatics platform (Cox et al., 2011; Tyanova et al., 2016). Default settings were used, with "match between runs", "iBAQ (intensity-based absolute-protein-quantification)" and "PTXQC (proteomics quality control of MaxQuant search)" turned on. For protein identification, a minimum peptide length of 7 amino acids was required. The peptide and protein false-discovery rate was set at 1%. Enzyme specificity was set to trypsin, allowing 2 missed cleavages. For the first peptide identification search, the mass tolerance was 20 ppm. After time and mass-dependent peptide mass recalibration, a peptide mass tolerance in the main search of 4.5 ppm was used. For the MS/MS, a fragment mass tolerance was set to FTMS 20 ppm, and ITMS 0.5 Da. For all searches, cysteine carbamidomethylation was set as a fixed modification and oxidation of methionine and N-terminal acetylation as variable modifications. The quality of the generated results by MaxQuant was further verified by the PTXQC option in the Galaxy platform (Bielow et al., 2016). For protein quantification, the iBAQ value, where the sum over all the peptides of a protein is divided by the number of theoretically observable peptides, was used to estimate an approximate measure of absolute protein abundance (Schwanhäusser et al., 2011; Tyanova et al., 2016). Then, the proteins were assigned to different protein groups, and the percentage of each protein group was calculated by dividing the summed iBAQ values of proteins assigned to the group by the summed iBAQ values of all quantified proteins identified in the sample.

Spectral data were searched against four databases: 1) a custom database created from UniProtKB/Swiss-Prot entries retrieved from separated taxonomy searches for "Cnidarian" (263,816 entries), 2) a custom database created from UniProtKB/Swiss-Prot entries retrieved from separate keyword searches for "toxin", "venom", "hemolysin" or "porin" (191,347 entries), 3) a custom database created from entries retrieved from the annotated Tox-Prot program (https://www.uniprot.org/pro-gram/Toxins; 7,437 entries) (Jungo et al., 2012), and 4)

a custom database created from GenBank (NCBI) entries retrieved from separated keyword searches for “hemolysin” or “hemolysis” or “pore-forming protein” or “porins” (156,104 entries).

Perseus version 1.6.15.0 (available online at https://maxquant.net/perseus/) was used for MS/MS data processing and visualization. Many protein IDs can be allocated to the same protein groups because a single peptide might be linked to multiple proteins (such as in the case of razor proteins). In order to avoid redundant proteins in analysis while still maintaining the maximum information, a leading protein-based protein identification technique was introduced. In brief, an initial table was constructed that contained the protein group information. Leading proteins and majority proteins were combined after the proteins tagged (+) with "only identified by site," "reverse," and "contaminant" were excluded from the initial table. The final table was improved by correcting the leading protein sequences, removing the comprehensive contaminants, and recalculating the sequence length and iBAQ. The proteins (leading and majority) were annotated with the molecular functions and biological process of identified proteins using the batch retrieval tool on the PIR database (https://proteininformation-resource.org/) (Ashburner et al., 2000; Wu et al., 2003). All peptide contig sequences of potential box jellyfish toxin families, which were retrieved from the identified proteins in "protein groups.txt", were manually constructed for each toxin by mapping the start and stop positions on the reference sequence. Multiple Sequence Alignment was performed using Clustal Omega (Madeira et al., 2019) and visualized using Jalview software (Waterhouse et al., 2009). Sequence similarity of proteins was analyzed by EMBOSS Needle (Madeira et al., 2019). A full table of mass spectrometric and database search features for each identified protein (accession numbers, sequences, Gene Ontology (GO) annotations, and InterPro results) can be found in Appendix: Dataset 1. Additionally, all peptides that were identified by MaxQuant in “peptide.txt” were tabulated, with duplicate sequences removed, keeping only the highest scoring peptide among the duplicates. All peptides scoring “NaN” (not a number) were also removed. GO annotations of all sequences from these filtered peptides were done by eggNOG-mapper using the default setting. For quantitative data and graphs were performed using the software

GraphPad Prism (version 8). The area proportional Venn diagrams were obtained using the freely available online tool BioVenn (Hulsen et al., 2008).

3. Results and discussion

3.1 Venom profiling

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of *C. indrasaksajiae* venom proteins is shown in figure 23. The molecular weights of proteins varied from <20 kDa to 200 kDa. The *C. indrasaksajiae* venoms contained only one condensed protein band at approximately 50 kDa and two minor protein bands at about 60 kDa and 150 kDa.

After decades of research on the proteins in box jellyfish venom, several studies have shown that the SDS-PAGE profiles of nematocyst venom or tentacle extracts are dominated by one or two major proteins with similar molecular weights (42–46 kDa) (Brinkman and Burnell, 2008; Brinkman and Burnell, 2007, 2009; Nagai, Takuwa, et al., 2000a; Nagai, Takuwa, Nakao, Sakamoto, et al., 2000; Wiltshire et al., 2000). There is no significant amino acid sequence homology between the box jellyfish toxins and other known proteins, suggesting that the toxins belong to a unique family of bioactive proteins (Brinkman and Burnell, 2007; Nagai et al., 2002). Toxins produced by box jellyfish are typically labile, basic proteins with molecular weights ranging from 42 to 46 kDa capable of producing severe hemolytic activity. The results of bioactivity assays for selected toxins also indicate that the proteins are lethal and induce localized pain, inflammation, and dermonecrosis (Nagai, Takuwa, et al., 2000a; Nagai et al., 2002; Nagai, Takuwa, Nakao, Sakamoto, et al., 2000). According to secondary structural studies and remote protein homology predictions, the box jellyfish venom may act as a pore-forming toxin (Parker and Feil, 2005). However, major protein bands of *C. indrasaksajiae* venoms had a molecular weight of between 47 and 57 kDa, which is a larger size than a novel family of bioactive box jellyfish venom proteins (Table 6). In addition, our LC-MS/MS observation showed that major protein bands of *C. indrasaksajiae* venoms could have resulted from a pore-forming toxin, porins, hemolysin, and basic cytolysins.

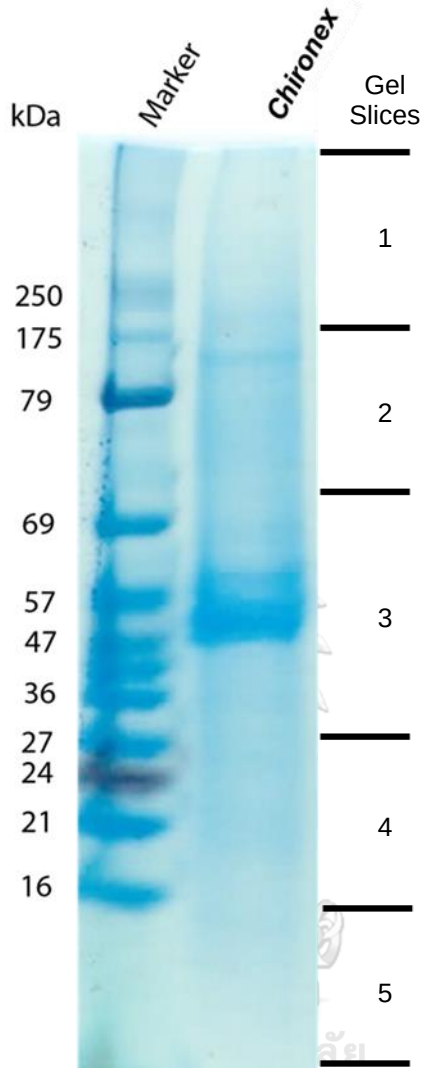

Figure 23 Gel electrophoresis profile of venom proteins from *C. indrasaksajiae* venom sample, using 4-12% Bis-Tris gel. Sizes of 5 gel slices for the in-gel digestion experiment were indicated on the right.

Table 6 Comparison *C. indrasaksajiae* venom proteins from novel family of bioactive box jellyfish proteins

Species	Toxin Name	Exp. MW (kDa)	Accession No.	Reference
C. rastoni	CrTX-A	43	AB015778	Nagai, Takuwa, et al. (2000b)
	CrTX-B	46	-	
C. alata	CaTX-A	43	AB036714	Nagai, Takuwa, Nakao, Sakamoto, et al. (2000)
	CaTX-B	45	-	
	CAH2	42	-	(Chung et al., 2001)
C. yamaguchii	CqTX-A	44	AB045319	(Nagai et al., 2002)
C. fleckeri	CfTX-1	43	EF636902	(Brinkman and Burnell, 2007)
	CfTX-2	45	EF636903	
C. indrasaksajiae	CfTX-like (CiTX)	50	-	This study

3.2 Proteomic analysis of *C. indrasaksajiae* venom

3.2.1 Overall composition of peptide and protein identification from *C. indrasaksajiae* cnidae venom

To identify proteins in *C. indrasaksajiae* venom, crude venom was fractionated using SDS-PAGE and peptides from in-gel tryptic digests of 5 gel fragments (Figure 23) were analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS). For screening proteins in *C. indrasaksajiae* venom, the spectral data were searched against all Cnidarian entries presented in the Swiss-Prot database with the significance threshold set as P-value < 0.05 to ensure the exclusive inclusion of the statistically significant protein matches as described in section 2.7. Any contaminants or false positive hits were removed during this filtering process. As the results of protein identification based on an MS/MS ion search with 3.9 % MS/MS identified, a total of 532 unique protein groups and a total of 1,241 unique peptides were identified from in-gel digestion experiment (Appendix: Dataset A1). Based on functional annotation, identifications included proteins associated with cellular and metabolic processes, structural components, and potential toxins. Uncharacterized and predicted proteins were excluded for de novo sequencing in further analysis due to not having been annotated in the PIR database.

The most commonly identified proteins were structural from the annotated proteins, reflecting the composition of the cnidae capsule, primarily

composed of minicollagens (Özbek et al., 2009), and the tubule in which tubulin is a major component. Molecular components of the nematocyst capsule body is primarily composed of minicollagens (Özbek et al., 2009), and the actin and tubulin are major components in the tubule, spines, as well as proteins associated with cnidocilia of nematocyte (Beckmann and Özbek, 2012a) that were incomplete cytolyzed in venom preparation process. A detection of identified structural proteins in *C. indrasaksajiae* venom, including actin, tubulin beta chain, tubulin alpha chain etc., have a similar result in the nematocyst proteomes of *C. fleckeri* in which non-secreted proteins were hypothesized to play an important role in nematogenesis, to be products of unusual secretion pathways or truncated protein products (Brinkman et al., 2012).

When categorized into functional groupings, the most abundantly identified proteins reflect the known composition of the cnidae, with toxins, collagens, and proteins that play important roles in basal metabolisms such as actin, tubulin, histones and ATPase (Figure 24, Table 7). Other categorized protein groups were the cytoskeleton components, membrane-bound enzyme, mitochondrial gene products, muscular and non-muscular components, photoprotein, a signaling protein, heat shock protein, transcription and translation factors, ATP-binding protein, enzymes and cellular oxido-reductive proteins. All of these proteins resemble the jellyfish in the class Scyphozoa, Cubozoa, Hydrozoa, Ctenophores, and most of them are similar to the proteins derived from the same genus in the venom proteomes *C. fleckeri* (Brinkman et al., 2012).

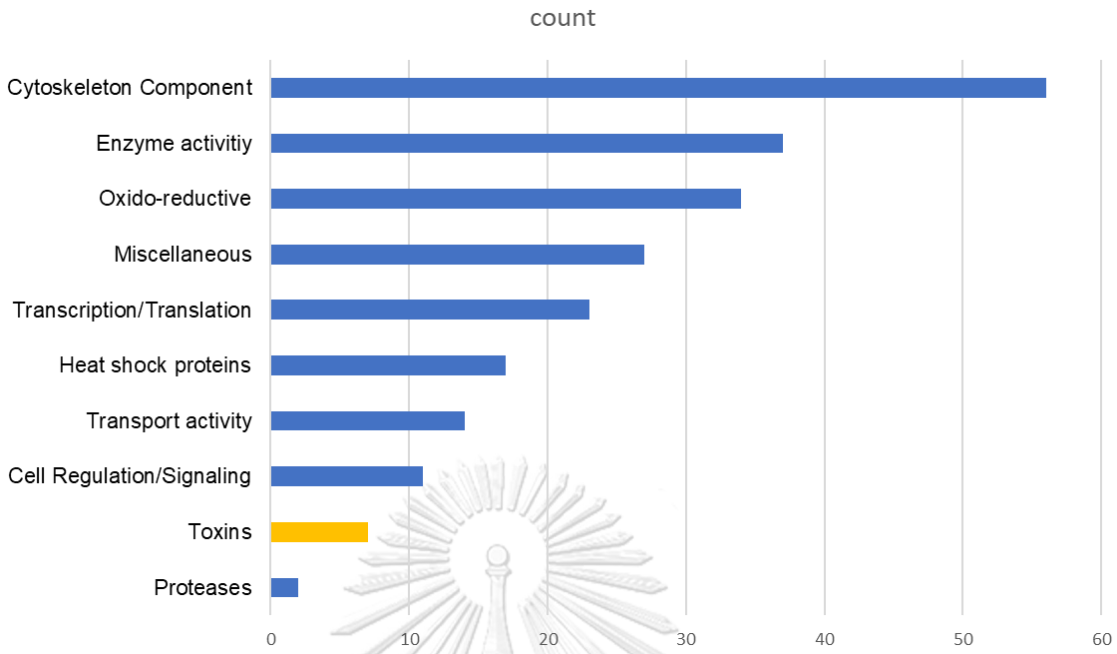

Figure 24 Venom proteome of *C. indrasaksajiae*: Functional annotation of proteins identified in proteomics experiments.

Table 7 Protein identification is categorized into functional groupings using MAXQUANT and ANDROMADA

Accession Number	Description	USC	CO	score
Cell Regulation/Signaling				
A0A7M5XJN9	Major vault protein	4	5.8	38.938
P62184	Calmodulin	5	67.1	82.955
Q6VQ14	Dickkopf-3 related protein	4	25	39.374
A0A6S7H750	Prohibitin	2	16.9	16.365
T2MD34	Serine/threonine protein phosphatase 2A regulatory subunit	2	6.2	12.226
Cytoskeleton Component				
A0A0C2N961	Actin	1	35.4	32.968
Q8IAC3	Actin	2	58.2	33.502
P41113	Actin-3	24	64.9	323.31
T2MHI5	Alpha-actinin-1	4	6	39.677
Q8I8C8	Beta actin	2	38.4	11.075
Q8WRT4	Beta catenin	2	6.3	17.834
A0A515L5S5	Beta tubulin	2	52.3	12.781
T2MEN5	Clathrin heavy chain	4	2.7	39.547
T2MHI3	Flotillin-1 (Fragment)	3	6.5	29.176
A0A6S7H3J5	Gelsolin 2	1	5.9	135.99
A0A7D9KLB0	Histone H2A	3	51.7	43.52
A0A7D9E3N3	Histone H2B	1	12	80.219
A0A7D9DZX8	Histone H3, partial	1	6.8	32.689
A0A2B4R9Q9	Histone H4	16	41	191.64
A0A6P8HH48	myoferlin-like isoform X3	2	1.2	17.309

Accession Number	Description	USC	CO	score
T2MG36	Myosin-10	3	1.6	18.181
T2MEA3	Myosin-Ie (Fragment)	2	2.8	37.944
D1FX76	Thypedin (Fragment)	6	28.8	45.261
A7RUT1	Tubulin alpha chain	27	71	323.31
A0A7M5WUU7	Tubulin beta chain	25	64.3	323.31
A7SSE3	Tubulin gamma chain	2	7.8	28.655
Heat shock proteins				
A0A7D9DUK8	60 kDa heat shock	1	6	8.7908
A0A7M5UYR8	CCT-alpha	5	12.5	56.179
T2MEY3	CCT-beta	2	4.7	12.174
T2MG46	CCT-epsilon	2	4.2	15.475
A0A3M6TE72	CCT-theta	1	2.9	6.054
A0A2B4RPD3	Endoplasmin	1	4.3	5.6868
Q967E1	Heat shock protein 90	2	8.6	13.159
D1FX74	HSP70-1	15	27.9	323.31
Oxido-reductive				
A0A7M5VCV6	3-hydroxyisobutyrate dehydrogenase	1	4.6	8.0621
A0A2B4T1C4	ATP synthase subunit alpha	10	23.9	323.31
A7SPP0	ATP synthase subunit beta	7	23.8	168.15
A0A7M5XDK4	Catalase	1	3	15.448
A0A516MVE8	Cytochrome c oxidase subunit 2	1	8	23.328
A0A6S7LBX5	Cytosolic 10-formyltetrahydrofolate dehydrogenase-like	1	11.2	9.9495
A0A3M6U8Q7	Electron transfer flavoprotein subunit alpha	3	13.6	36.359
D1FNZ5	Ferritin	1	7.1	8.5634
T2MH20	Glutamate dehydrogenase	2	5.4	19.885
A0A7D9DQ40	Glyceraldehyde-3-phosphate dehydrogenase	2	4	19.267
A0A6S7GM63	Glycine cleavage system P protein	2	3.7	11.509
A0A7M5V6Q3	H(+)-transporting two-sector ATPase	7	15	70.351
A0A6S7KB29	Malate dehydrogenase	2	13	15.717
A0A7M5XAK5	Methionine adenosyltransferase 2 subunit beta	1	4.1	8.388
T2ME72	Methylmalonate-semialdehyde dehydrogenase [acylating]	1	3.4	5.6789
T2MIQ9	NADH-ubiquinone oxidoreductase 49 kDa subunit	2	4	12.408
A0A6S7INI4	Peroxiredoxin-1-like isoform X2	3	19.6	20.168
A0A3M6UTM8	Proton-translocating NAD(P)(+) transhydrogenase	2	2.5	13.634
T2M830	Pyrroline-5-carboxylate reductase	1	3.3	6.6201
A0A0C2JYJ1	Succinate dehydrogenase	1	1.7	5.81
A0A6P8IZ04	Superoxide dismutase [Cu-Zn]	1	9.9	79.776
A0A7D9DBH9	Thioredoxin domain-containing protein 17	1	7.3	5.6889
A0A2B4S736	Vacuolar proton pump subunit B	5	16.9	57.517
A0A7D9LK55	V-type proton ATPase 16 kDa proteolipid subunit	1	23.1	14.759

Table 8 Top 20 hits lists of peptide identification in *C. indrasaksajiae* venom

Most abundance peptide (Top20)	Length	score	%iBAQ	Accession no.	Protein Name
VMGAIGSLGTAIGK	14	130.01	33.0159	A7L036	Toxin CfTX-2 precursor; (Short=Toxin 2)
AVQEQSDQELQEALYGVK	18	282.26	14.4451	A7L035	Toxin CfTX-1 precursor; (Short=Toxin 1)
KWPHNFAYSQK	11	183.95	10.5900	A7L036	Toxin CfTX-2 precursor; (Short=Toxin 2)
AIQEQSDQELQEALYGVK	18	287.04	9.4208	A7L036	Toxin CfTX-2 precursor; (Short=Toxin 2)
KAVQEQSDQELQEALYGVK	19	464.9	7.2593	A7L035	Toxin CfTX-1 precursor; (Short=Toxin 1)
NEESDLRPTEVSALAANIPVYQGVR	25	472.72	4.2224	A7L036	Toxin CfTX-2 precursor; (Short=Toxin 2)
IENPPENGIR	10	179.68	4.1712	A0A7M5X1G2	Lactamase_B domain-containing protein
EVTSLGREEYQR	12	300.35	1.0493	A7L036	Toxin CfTX-2 precursor; (Short=Toxin 2)
ADKEEHVYMAK	11	160.91	0.8831	A7S4S2	Predicted protein
SGDGVSHTVPIYEGYALPHAILR	23	188.26	0.7846	A0A6G3MNH4	Actin (Trinotate prediction) (Fragment)
GIYETPGGEILR	12	135.55	0.7287	T2MHY4	Argininosuccinate synthase (Fragment); (EC=6.3.4.5; AltName: Full=Citrulline--aspartate ligase)
LLGSVTIAQGGVLPNIQAVLLPK	23	201.65	0.5614	A0A6P8J5L9	Histone H2A
VETGIIKPGMVVHFSPANITTEVK	24	185.71	0.5581	D1FX72	Elongation factor 1-alpha
LSEQFTAMFR	10	223.83	0.4763	A0A6S7I6R8	Tubulin beta chain
SYELPDGQLITIGNER	16	293.31	0.4377	A0A0C2N961	Actin, cytoplasmic type 5
MVASGASVDLIDER	14	70.1	0.3848	A0A2B4SIU8	Uncharacterized protein K02A2.6
LVLEVAQHLGENVVR	15	231.06	0.3470	A0A0C2N354	ATP synthase subunit beta; (EC=7.1.2.2)
EVEACDCLQGFQLTHSLGGGTGSGMGTLLISK	32	214.62	0.2607	A0A6G3MGA1	Tubulin beta chain (Fragment)
LVIIANNTPQLR	12	226.47	0.2173	A7RQD8	60S ribosomal protein L30
KAVQEQSDQELQEALYGVKR	20	262.37	0.2119	A7L035	Toxin CfTX-1 precursor; (Short=Toxin 1)

Table 8 showed that the most abundant peptides making the top six hit list were matched with *C. fleckeri* toxin-1 (CfTX-1) and toxin-2 (CfTX-2). These abundant peptides reflect the amount of toxin in *C. indrasaksajiae* cnidae venom, suggesting these toxins play an important functional role in box jellyfish envenomation. CfTX-1 and CfTX-2 were also the major toxin protein components in *C. fleckeri* venom with molecular masses ~43 and 45 kDa, respectively (Brinkman, 2008; Brinkman et al., 2012; Brinkman and Burnell, 2008; Brinkman and Burnell, 2007, 2009; Brinkman et al., 2015).

In the screening for toxins in the cnidarian database, the results of toxin protein identification in *C. indrasaksajiae* venom, only 7 toxin proteins were found. (Appendix: dataset 2) Due to the limited number of annotated toxin proteins available in the cnidarian database used for the search, the cnidarian database provided 304 toxin proteins, but only 8 of those toxin proteins were annotated in the box jellyfish group. To maximize toxin identifications, spectral data were also compared against other publicly available toxin databases as noted in Section 2.7 from Tox-Prot program and custom databases with keyword searched for “toxin” or “venom” or “hemolysin” or “hemolysis” or “pore-forming protein” or “porins”. The protein matches from all custom toxin database searches were pooled together for qualitative analysis, resulting in the identification of 74 potential toxin groups (Table 9). The vast majority of them such as Jellyfish toxin family (JFTs) (Klompen et al., 2021), pore-forming protein (PFTs) (Mesa-Galloso et al., 2021; Parker and Feil, 2005; Podobnik and Anderluh, 2017), porin family (Jeanteur et al., 1994; Sardiello et al., 2003; Yanagihara and Shohet, 2012) and hemolysin (Chung et al., 2001; Crone and Keen, 1969) contained the toxins that contributed to hemolysis (Figure 25). Hemolytic toxic effects that the destruction of red blood cells by pore-forming protein or porin might be suspected as the fatal mechanism of box jellyfish envenomation (Mesa-Galloso et al., 2021; Yanagihara and Shohet, 2012; Yanagihara, Wilcox, Smith, et al., 2016). Other toxins were also detected in venom proteome of *C. indrasaksajiae*, comprising the toxic components including hemostasis impairing toxin, oxidoreductase, enzyme (hydrolase, protease), metalloproteinase, signaling protein, cytolytic toxin, neurotoxin and miscellaneous (Brain-derived neurotrophic factor). This pattern of toxin family distribution is similar to the venom proteomes of

other medusozoans, including *C. fleckeri* (Brinkman et al., 2012), in which the most abundant venom proteins identified were pore-forming toxins.

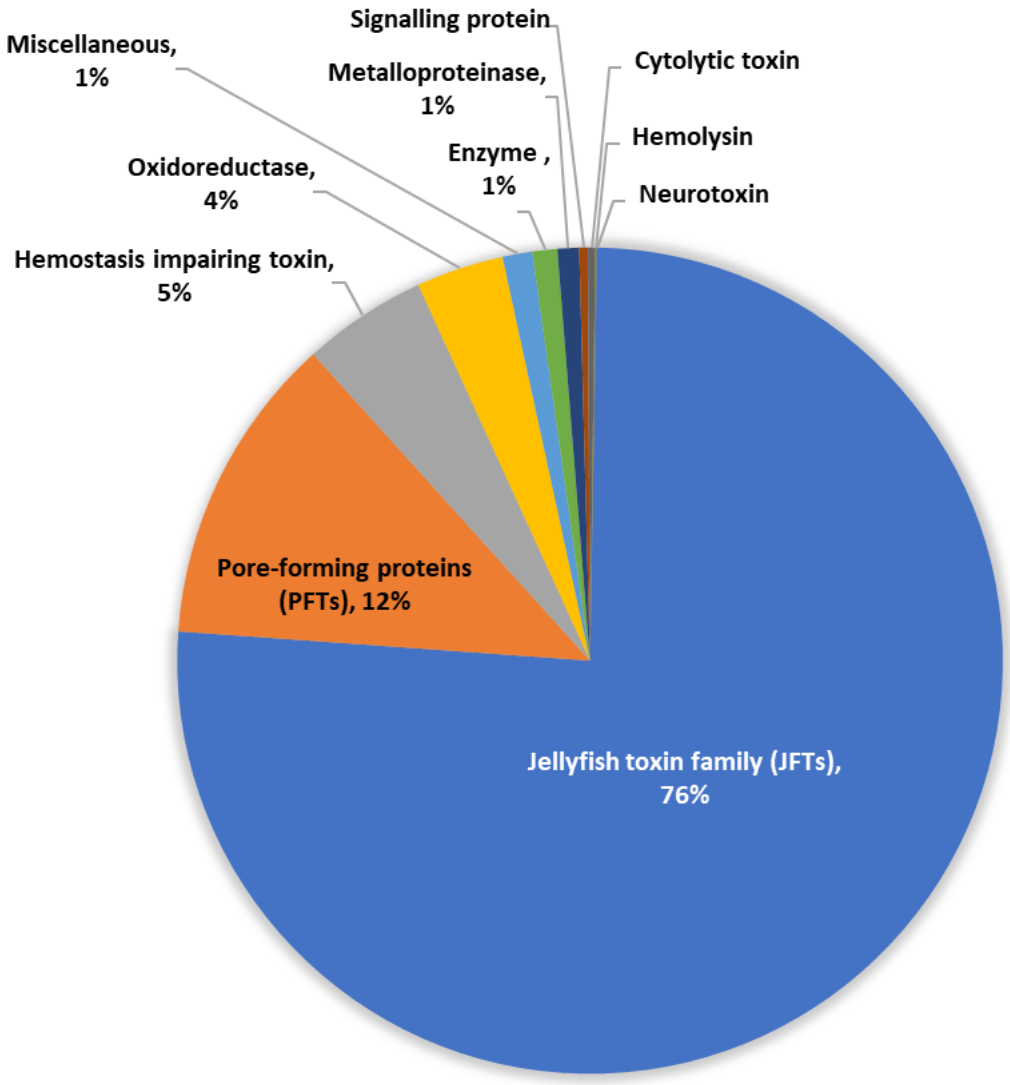

Figure 25 Abundance of each protein family estimated by iBAQ values:

Table 9 Venom proteome of the toxic component in *C. indrasaksajiae* cnidae venom

Accesion number	Protein name	Protein type	Organism	Sequence coverage [%]	Mol. weight [kDa]	%iBAQ
A7L035	Toxin CfTX-1	Jellyfish toxin family (JFTs)	*Chironex fleckeri* (Australian box jellyfish)	39.3	51.39	49.7451
A7L036	Toxin CfTX-2	Jellyfish toxin family (JFTs)	*Chironex fleckeri* (Australian box jellyfish)	34.8	51.684	16.3708
Q03105.1	V-type proton ATPase 16 kDa proteolipid subunit	Hemostasis impairing toxin	*Torpedo marmorata* (marbled electric ray)	11.7	15.511	4.1321
WP_195824412.1	Outer membrane porin A	Porins family	*Rahnella laticis*	47.2	38.205	3.7760
A0A6M2B2L8	Outer membrane porin A	Porins family	*Rahnella* sp.	41.7	38.137	3.1087
P0CV91	Peroxiredoxin-4	Oxidoreductase	*Crotalus atrox* (Western diamondback rattlesnake)	58.3	4.1407	1.9502
A0A5N5NKB0	Voltage-dependent anion-selective channel protein 3	Porins family	*Pangasianodon hypophthalmus* (Striped catfish)	8.6	33.697	1.1599
A0A1B3B2Q3	Brain-derived neurotrophic factor	Miscellaneous	*Aspidelaps scutatus* (Shield-nose snake)	6.8	23.098	1.0116
A8GBU5.1	DNA protection during starvation protein	Oxidoreductase	*Serratia proteamaculans*	13.8	18.823	0.8590
S4YJ80	Maltoporin	Porins family	*Serratia plymuthica*	7.1	46.577	0.7774
T1PRE3	Toxin CfTX-A	Jellyfish toxin family (JFTs)	*Chironex fleckeri* (Australian box jellyfish)	21.6	50.485	0.5611
KXJ13572	Zinc metalloproteinase nas-15	Metalloproteinase	*Exaiptasia diaphana*	0.7	34	0.5518
WP_208714547.2	Outer membrane porin A	Porins family	*Pantoea cypripedii*	12.4	38.157	0.4803
A0A1B9C210	Toxin VapC	Hydrolase Enzyme	*Acidithiobacillus ferrivorans*	7.1	15.878	0.4082
P58762	Toxin CqTX-A	Jellyfish toxin family (JFTs)	*Chiropsoides quadrigatus* (Box jellyfish)	32.3	51.801	0.4076
P62184	Calmodulin	Signalling protein	*Renilla reniformis*	20.3	16.706	0.3787
CAH20400.1	Outer membrane porin A	Porins family	*Yersinia pseudotuberculosis*	16.1	18.132	0.2945
WP_211461371.1	Porin	Porins family	*Collimonas silvisoli*	2.9	40.35	0.2773
T1PQV6	Toxin CfTX-B	Jellyfish toxin family (JFTs)	*Chironex fleckeri* (Australian box jellyfish)	15	51.116	0.2754
A0A2L0NG73	Cytotoxin (Fragment)	Cytolytic toxin	*Bacillus thuringiensis*	13.4	10.998	0.2513
Q2NUI3.1	DNA protection during starvation protein	Oxidoreductase	*Sodalis glossinidius*	12	18.752	0.2368
A0A5C9C1A7	Outer membrane porin A-F	Porins family	*Chitinophagaceae bacterium*	1.5	50.495	0.2226
A0A6M2B081	Outer membrane porin C	Porins family	*Rahnella* sp.	24.4	40.316	0.2180
A0A2D4KTV9	Rab GDP dissociation inhibitor	Hemostasis impairing toxin	*Micrurus paraensis*	15.2	14.018	0.1950
A0A5E4SH76	Protein CyaE	Pore-forming proteins (PFTs)	*Pandoraea morbifera*	2.2	55.501	0.1586
A0A670I9L0	Peptidase M12B domain-containing protein	Metalloproteinase	*Podarcis muralis* (Wall lizard)	4.2	19.084	0.1558
Q6EH50	Heterotepalin-4	Hydrolase Enzyme	*Phytolacca heterotepala* (Mexican pokeweed)	7.7	35.153	0.1275
A0A2R6EAJ7	Putative toxin VapC	Hydrolase Enzyme	*Halobacteriales archaeon*	14.8	14.54	0.1010
WP_164101151.1	Pore-forming outer membrane protein	Pore-forming proteins (PFTs)	*Candidatus Laterigemmans baculatus*	2.6	39.96	0.0845
6EF2	Chain B, Proteasome subunit alpha type-2	Enzyme	*Saccharomyces cerevisiae*	5.2	27.048	0.0683
WP_049125341.1	Outer membrane porin K36	Porins family	*Klebsiella pneumoniae*	4.3	27.606	0.0593
AHG06297.1	TX-like toxin	Jellyfish toxin family (JFTs)	*Chironex fleckeri* (Australian box jellyfish)	27	32.129	0.0531
PHY01340.1	Enoyl-CoA hydratase	Enzyme	*Rhodospirillaceae bacterium*	7.4	27.78	0.0503
A0A3M6T9I2	Metalloendopeptidase	Metalloproteinase	*Pocillopora damicornis* (Cauliflower coral)	2.4	38.395	0.0473
A0A7H1N0E6	Toxin VapC	Hydrolase Enzyme	*Defluviicoccus vanus*	5	15.118	0.0400
WP_177051734.1	Colicin-like pore-forming protein	Pore-forming proteins (PFTs)	*Pseudomonas reactans*	3.2	37.567	0.0333
A0A3A3DU41	Calcium binding hemolysin protein	Hemolysin	*Paracoccus* sp.	1.3	99.898	0.0324
A0A7W0QSA7	Toxin VapC	Hydrolase Enzyme	*Chloroflexia bacterium*	0.6	15.688	0.0323
KXJ15976.1	Serine/threonine-protein phosphatase PP1-beta	Hydrolase Enzyme	*Exaiptasia diaphana*	8.5	37.479	0.0254
A0A6H6F6Q5	Fibronectin-binding outer membrane protein CadF	Porins family	*Campylobacter jejuni*	2.5	35.937	0.0249
SFV70780.1	Type II secretion outermembrane pore forming protein (PulD)	Pore-forming proteins (PFTs)	*hydrothermal vent metagenome*	1.3	67.024	0.0230
XP_022786707.1	Allene oxide synthase-lipoxygenase protein-like	Miscellaneous	*Stylophora pistillata* (Smooth cauliflower	1.6	76.789	0.0210

			coral)			
WP_186663430.1	Alpha-xenorhabdolysin family binary toxin subunit B	Pore-forming proteins (PFTs)	*unclassified Pseudomonas*	3.3	33.97	0.0171
WP_037523409.1	TonB-dependent receptor	Porins family	*Sphingobium yanoikuyae*	2.7	114.06	0.0154
A0A3N5D2S8	Phosphoporin PhoE	Porins family	*Buttiauxella warmboldiae*	12.5	38.606	0.0152
CAE1143962.1	Outer membrane porin N	Porins family	*Serratia* sp.	3.3	40.146	0.0119
A0A196QEF2	Outer membrane porin A-like	Porins family	*Marinobacter* sp.	3.1	38.924	0.0101
E3D1S1	Toxin CdiA; Putative hemagglutinin	Pore-forming proteins (PFTs)	*Neisseria meningitidis*	1	144.37	0.0097
A0A4U3FJA4	Outer membrane porin A	Porins family	*Erwinia persicina*	16.1	38.199	0.0090
Q25338	Delta-latroinsectotoxin-Lt1a	Neurotoxin	*Latrodectus tredecimguttatus* (Mediterranean black widow spider)	0.9	135.82	0.0088
A0A7X4LNM1	Porin	Porins family	*Vibrio* sp.	0.3	50.548	0.0076
A0A6J4E9K9	TonB-dependent receptor	Porins family	*Pseudomonas* sp.	2.4	63.32	0.0060
A0A1I8HRR7	Mitogen-activated protein kinase	Neurotoxin	*Macrostomum lignano*	0.7	447.21	0.0051
PFX13481.1	Phosphopyruvate hydratase	Enzyme	*Stylophora pistillata*	0.6	188.02	0.0050
A0A7V8BS80	Porin	Porins family	*Burkholderiaceae bacterium*	3.3	35.511	0.0047
A0A2I7KCU5	Hemolysin-type calcium-binding protein repeat protein	Hemolysin	*Phaeobacter inhibens*	0.9	98.71	0.0046
A0A193PK31	Transient receptor potential channel A1 isoform a	Neurotoxin	*Tropilaelaps mercedesae*	2.4	131.170	0.0045
A0A6G1PY87	Peptidase C81	Hydrolase, Protease Enzyme	*Channa argus* (northern snakehead)	1.5	208.749	0.0042
XP_022796828.1	Polycystic kidney disease protein 1-like 2	Pore-forming proteins (PFTs)	*Stylophora pistillata*	1.1	243.56	0.0041
A0A6G1SKA8	Ankyrin repeat and SAM domain-containing protein 1A	Neurotoxin	*Aceria tosichella* (wheat curl mite)	0.4	222.985	0.0032
PFX18590.1	Polycystic kidney disease protein 1-like 2	Pore-forming proteins (PFTs)	*Stylophora pistillata*	1.7	142.41	0.0028
V7IBB3	Toxin CdiA; Haemagg_act domain-containing protein	Pore-forming proteins (PFTs)	*Eikenella corrodens*	0.6	345.5	0.0028
H0KH93	Hemolysin A	Hemolysin	*Aggregatibacter actinomycetemcomitans RhAA1 []*	1.6	113.438	0.0026
WP_047006348.1	TonB-dependent receptor	Porins family	*Aurantiacibacter gangjinensis*	0.6	123.76	0.0025
A0A1M6H3Y0	Ca2+-binding protein, RTX toxin-related	Hemolysin	*Maribius salinus*	0.3	322.006	0.0025
F1CLH1	Putative RTX-toxin (Fragment)	Hydrolase, Protease Enzyme	*Vibrio vulnificus*	1	169.516	0.0025
QIM62508.1	Porin	Porins family	*Pasteurellaceae bacterium*	3.6	41.401	0.0022
A0A1G3GTZ6	Porin	Porins family	*Pseudomonadales bacterium*	3.4	54.278	0.0015
WP_210641556.1	Flp pilus assembly complex ATPase component TadA	Cytolytic toxin	*Gallibacterium anatis*	1.2	116.49	0.0014
A0A1E1X276	Putative ankyrin	Neurotoxin	*Amblyomma aureolatum*	0.6	373.213	0.0008
A0A1N7JGU6	Thrombospondin type 3 repeat-containing protein	Pore-forming proteins (PFTs)	*Kaistella chaponensis*	3	53.512	0.0005
A0A2D3TFQ5	Peptidase C80	Hydrolase, Protease Enzyme	*Candidatus Hamiltonella*	1.2	198.59	0.0002
A0A6P9C1G7	disintegrin and metalloproteinase domain-containing protein	Metalloproteinase	*Pantherophis guttatus* (Corn snake)	5	85.916	0.0001
A0A3D9CPX6	Outer membrane porin A	Porins family	*Epilithonimonas hispanica*	4.5	52.317	0.0001

3.2.2 Potential toxin proteins identified in the *C. indrasaksajiae* venom proteome

As the results of toxin protein identification in *C. indrasaksajiae* venom that were searched against Tox-Prot program and custom toxin databases in table 9, it showed that the most abundant protein making the top hit list were matched with *C. fleckeri* toxin-1 (CfTX-1) and toxin-2 (CfTX-2) which accounted for 50% and 16% of the total toxin protein content, respectively. These highly abundant venom proteins belong to a family of taxonomically restricted novel cnidarian toxins (42– 46 kDa) that includes CqTX-A, CrTX-A, and CaTX-A from box jellyfish species *Chironex yamaguchii* (Nagai et al., 2002) (as *Chiropsalmus quadrigatus* (Lewis and Bentlage, 2009)), *Carybdea rastonii* (Nagai, Takuwa, et al., 2000a), and *Alatina moseri* (Nagai, Takuwa, Nakao, Sakamoto, et al., 2000) (as *Carybdea alata* (Gershwin, 2005a)), respectively, as well as other representatives from Cubozoa, Scyphozoa, and Hydrozoa. Recently, proteins with sequence homology to the box jellyfish toxins have also been identified in the venoms of Scyphozoa, such as *Cassiopea xamachana* (Ames, 2016), *Cyanea capillata* (Lassen et al., 2011), *Nemopilema nomurai* (Li et al., 2020), *Chrysaora fuscescens* (Ponce et al., 2016) and the hydrozoan *Hydra magnipapillata* (Balasubramanian et al., 2012), inferring the toxin family is present throughout Cnidaria. Members of this toxin family are potently hemolytic and cause pain, inflammation, dermonecrosis and death in experimental animals (Hornbeak and Auerbach, 2017; Tibballs, 2018; Tiemensma et al., 2021; Yanagihara, Wilcox, Smith, et al., 2016), suggesting the toxins play an important functional role in jellyfish envenoming. In cubozoans, computational analyses of this toxin family have been linked to the presence of a specific family of porin family or pore forming toxins (PFTs), described as the most potent cnidarian toxin families currently known (D'Ambra and Lauritano, 2020; Jayathilake and Gunathilake, 2020; Jouiaei, Yanagihara, et al., 2015; Podobnik and Anderluh, 2017). The apparent restriction of a cnidarian-specific PFT family to medusozoan venoms resulted in the current nomenclature of “jellyfish toxins (JFTs)” (Jouiaei, Yanagihara, et al., 2015; Klompen et al., 2021; Surm and Moran, 2021).

Putative pore-forming toxins were the second most abundant toxin group, accounting for 12% of the total toxin protein content. Using a combined database, there were 9 PFTs proteins identified, comprising polycystic kidney disease protein 1-like 2, type II secretion outer membrane pore-forming protein (PulD), alpha-xenorhabdolysin family binary toxin subunit B, colicin-like pore-forming protein, pore-forming outer membrane protein, protein CyaE, thrombospondin type 3 repeat-containing protein, toxin CdiA and porin family.

Full amino acid sequence analysis and molecular modelling, show that the porin has structural motifs familiar with self-assembling bacterial porins, such as anthrolysin O and streptolysin O (Bernheimer et al., 1979). Afterwards, Chung et al. (2001) reported on the first hemolytic porin from *Alatina* sp. (previously reported as *Carybdea alata*). Since then, all cubozoan venom has been found to have close homologous proteins. In *C. fleckeri* venom, there are 2 different isoforms of this porin (MW 43 and 45 kDa) (Brinkman and Burnell, 2009; Nagai, Takuwa, Nakao, Sakamoto, et al., 2000; Yanagihara, Kuroiwa, Oliver, Chung, et al., 2002). Moreover, secondary structure analysis and remote protein homology predictions suggest that the family of box jellyfish toxins (CfTXs, CqTX, CrTX and CaTX) may act as α-PFTs, with three-domain Cry-like toxins. These domains were estimated that might play an essential role in membrane penetration and pore-forming after binding to the specific receptors (Brinkman and Burnell, 2009; Podobnik and Anderluh, 2017). Thus, the fact that porins are conserved across all cnidarian species supports the concept that these proteins are key venom components and play an important role in the clinical symptoms of cubozoan stings (Yanagihara, Wilcox, Smith, et al., 2016).

According to Yanagihara's theory, the venom of box jellyfish contains proteins that break down red blood cells and allow potassium to be released. The efflux of K^+ causes the electrical rhythms that keep the heart pumping to be disrupted and the heart to stop beating. Not only ion channel blockers in venom but also many “porins”: proteins that puncture cells, allowing their contents to leak out. In 2012, Yanagihara and a colleague reported that the venom of *Chironex fleckeri* rapidly punctures red blood cells, causing them to leak a huge amount of potassium ions. A high level of potassium in the blood, or hyperkalemia, causes cardiac arrest,

and when the mice were injected with high doses of venom, their hearts quickly stopped. The same happened when the mice were injected only the porins from the venom that porins were suspected might be the lethal components by destroying red blood cells.

In the venoms, metalloproteases were the predominant protease enzymes. Mainly, they were homologous to zinc metalloproteinase-disintegrin and metalloproteinase domain-containing protein, which were also identified in the proteome of the venom of other cnidarians, including Lion's mane jellyfish *C. capillata*, sea wasp *C. fleckeri*, Pacific sea nettle *C. fuscescens*, ghost jellyfish *Cyanea nozakii*, *Cyanea sp.*, Nomura's jellyfish *N. nomurai*, cannonball jellyfish *S. Meleagris* , and starlet sea anemone *Nematostella vectensis* (Leung et al., 2020). It has been suggested that metalloprotease induces protease-mediated tissue damage by the degradation of the extracellular matrix, which eventually results in necrosis, oedema, and haemorrhage (H. Lee et al., 2011). Correspondingly, the skin and tissue necrosis caused by *C. indrasaksajiae* envenomation (Tibballs, 2018) is most likely associated with the highly represented metalloproteases in its venom. However, the functional role of proteases in jellyfish venom is not well understood. At the same time, based on observations in other venomous animals, the results suggested that the proteases in the venom may be responsible for promoting the spreading and activation of other toxins.

Jellyfishes are exposed to continuous environmental changes, such as salinity, temperature, pollution, solar radiation, microorganisms and pathogens. This can cause the activation of inner defence responses, namely the production of reactive oxygen species (ROS). Although ROS are needed in the organism, an excess of these chemical species could cause a metabolic balance disorder, damaging cellular lipids, proteins and DNA. Catalase, glutathione peroxidase, superoxide dismutase, thioredoxin and peroxiredoxins are proteins with powerful antioxidant properties. *C. indrasaksajiae* showed to possess peroxiredoxin. These antioxidant proteins are likely just a few of many other possible molecules of *C. indrasaksajiae* with powerful antioxidant properties and therefore constitute a natural resource of antioxidant and anti-UV radiation agents (Ruan et al. 2014).

CHAPTER V

The toxic effects of *Chironex indrasaksajiae* (cnidaria: cubozoa) venoms and therapeutic approaches by using copper-gluconate and heat as the treatment and/or inhibitor of venom toxins on sheep red blood cells

1. Introduction

Chironex indrasaksajiae, a chirodropid cubozoan jellyfish that inhabits ocean areas in the Gulf of Thailand, contains cardiovascular, hemolytic, dermonecrotic, immunological, and lethal effects in its venom (Thaikruea and Siriarayapon, 2015b, 2018). It is estimated that approximately seven human fatalities have occurred due to *C. indrasaksajiae* envenomation over the past 15 years. According to case reports, the causes of death are cardiovascular collapse, respiratory failure, or a combination of the two (Thaikruea and Siriarayapon, 2018). The mechanisms behind these phenomena are not yet elucidated. Moreover, box jellyfish venom has developed much attraction for researchers because of its toxic properties that cause death within minutes. The biologically active components of *C. indrasaksajiae* (Thai multi-tentacles box jellyfish) venom and the mechanism of its toxicity have not been studied yet. However, jellyfish venoms are known to be complex mixtures that store in cnidae with several bioactivities such as hemolytic activity, cardiovascular activity, neurotoxic activity and dermonecrotic activity. (Bailey et al., 2005; Bonnet, 1999; Brinkman, 2016; Chung et al., 2001; D'Ambra and Lauritano, 2020; Ramasamy et al., 2003; Ramasamy et al., 2004) Of these all activities, hemolytic activity is considered to play the most basic damage mechanism, and almost all studied jellyfish venoms exhibit strong hemolytic toxicity (Mariottini, 2014). Therefore, this activity is an initial approach to purification or characterization of the venom due to rapid, easily reproducible, and quantifiable procedures.

In this study, hemolytic activity assays were used to test the toxic effects of the venom extracted from isolated cnidae of *C. indrasaksajiae* on sheep red blood cells. Some potential venom components were examined to associate with these biological activities. Due to the detection of protein bands in the molecular mass range of CfTX toxins, I aim to investigate if similar proteins were present in the

venom of *C. indrasaksajiae* using liquid chromatography with tandem mass spectrometry. The CfTX-like proteins were identified in *C. indrasaksajiae* venom that may have similar roles as the CfTXs in *C. fleckeri* venom and explain in part the cytotoxic and cytolytic effects of this box jellyfish venom.

Due to CfTX-like proteins in *C. indrasaksajiae* venom have similar functions to the CfTXs in *C. fleckeri* venom as highly potent and rapid-acting pore-forming toxins (PETs). Box jellyfish venom, according to Yanagihara's hypothesis, includes proteins that break down red blood cells and allow potassium to leak out. As a result of K^+ efflux, the heart's electrical rhythms are disturbed, resulting in cardiac arrest (Law, 2018; Yanagihara and Shohet, 2012; Yanagihara, Wilcox, Smith, et al., 2016). This mechanism that red blood cells being punctured by porin might be the lethal mechanism. Thus, finding the method and blocking composition for treatment and/or inhibiting porin protein should be a primary aim for treatment.

Pore-forming toxins, or porins, are a problematic and fast-acting component of cnidarian venom. Pore-forming toxins are classified broadly according to their secondary structure, with some requiring calcium for self-assembly polymerization to construct functional transmembrane pores (Parker and Feil, 2005). Thus, some divalent cations, including Zn^{2+}, Cu^{2+}, and Mg^{2+}, may compete for calcium-binding sites and disrupt the self-assembly of calcium-dependent porin proteins into functional polymeric pores (Yanagihara, 2013). According to Yanagihara (2019), copper gluconate is ten times more efficient than zinc gluconate alone or in conjunction with copper gluconate in reducing box jellyfish venom-induced cell hemolysis. This study evaluated hemolysis inhibition in *C. indrasaksajiae* venom using copper gluconate.

Furthermore, the widespread hypothesis about how heat can treats cnidarian envenomations is that the temperatures are used to inactivate venom components. Several in vitro and in vivo investigations indicate that cnidarian venoms from all classes within the phylum are heat-labile (Wilcox and Yanagihara, 2016). In this study, *C. indrasaksajiae* venom components were also tested for their inactivation using heat. These findings may have clinical relevance for the treatment of box jellyfish envenomation.

2. Experimental section

2.1 Venom collections

The animal collection was done according to the approved protocol, Mahidol University-Institute Animal Care and Use Committee, and Mahidol University Biosafety Committee. *C. indrasaksajiae* was captured near the coastal zone at Chaloklum bay of Koh Pha-ngan (Suratthani, the Gulf of Thailand) in August 2019. Whole tentacles from live jellyfish samples of mature *C. indrasaksajiae* were manually excised and preserved in 1 M tri-sodium citrate solution (1 volume of tentacles: 9 volumes of citrate solution) and kept at 4 °C, then vigorously shaken for the cytolysis process.

2.2 Venom extraction

Intact cnidae of *C. indrasaksajiae* were isolated and cleaned, similar to the methods described by Yanagihara and Shohet (2012). Excised tentacles were gently rotated in 1 M tri-sodium citrate solution at 4° C until approximately 70% cnidae were recovered by spontaneous release from cytolyzed tentacle tissue. The tentacle solutions were sieved, and the filtered solutions were collected. Sieved (0.5-mm mesh) cnidae solutions were centrifuged at 4 °C at 400 g for 20 min. Undischarged cnidae pellets were resuspended in chilled 1 M tri-sodium citrate at 1:20 (v:v) and washed twice at 400 g for 20 min. The cnidae pellets were then gently transferred to pre-chilled Precellys 24 CKmix lysing kit 2.0-ml tube for venom extraction.

C. indrasaksajiae venom was extracted from cnidae pellets using a bead mill homogenizer (Precellys 24 homogenizer, Bertin Technologies SAS, France) according to the manufacturer's protocol and the method described by Carrette and Seymour (2004) with some modifications. Briefly, 200 mg of cnidae (approximately 750,000 cnidae of *C. indrasaksajiae*) were aliquoted to a pre-chilled Precellys 24 CKmix lysing kit 2.0-ml tube with the mixed of 1.4 mm and 2.8 mm ceramic beads, and 1.4 ml of chilled-sterile water were then added to the tube. Samples were

processed in 6 cycles, each consisting of a bead beating at 5,000 rpm for 15 seconds. Tubes were rested on ice for at least 1 minute between each cycle. Lysates of venom extract were cleared by centrifugation at 10,000g at 4°C for 15 minutes. The viscous supernatant (venom) was snap-frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined using a BCA protein assay. It was assumed that protein concentration was correlated with venom concentration (Carrette and Seymour, 2004). Measurement of protein using bicinchoninic acid was described by Smith et al. (1985). Albumin was used for protein standards at concentrations of 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml. The samples were read at 562 nm in Infinite M200 PRO microplate reader (Tecan, Grödig, Austria). Prior to the venom activity assay, frozen venom samples were thawed and are referred to as "thawed" venom.

2.3 Hemolytic assays

Sheep red blood cells were used to evaluate the hemolytic activity of the *C. indrasaksajiae* venom. Sheep whole blood in EDTA was purchased from Salaya home animal hospital, Thailand. The packed red blood cells were isolated by centrifugation at 3,500 g at 4°C for 10 minutes. The packed red cells were then repeatedly washed 4-6 times with phosphate buffer saline (PBS) and recovered by centrifugation (3500 x g, 4 °C, 10 min). Finally, RBCs were resuspended in PBS to make a 1% RBC solution for hemolysis experiments. Hemolytic activity assays were performed quadruplicate in 96-well v-bottom microplates. A serial three-fold dilutions of total venom were applied to the erythrocyte suspensions in each well and incubated at 37°C for 1 hour, then immediately centrifuged at 1,000 g for 4 min at 4°C. Next, 150 µL of supernatants will be transferred to 96-well flat-bottom microplates. The released haemoglobin concentration was spectrophotometrically measured at 405 nm and 540 nm using an Infinite M200 PRO microplate reader. Hemoglobin possesses isosbestic points for both its oxygenated and deoxygenated forms, with the large variation in light absorption between the these two forms (Uyuklu, 2011). Total hemolysis was achieved with 5% Triton X-100 as a positive control (100% hemolysis). A negative control (0% hemolysis) was achieved with unexposed 2%

RBC supernatant in PBS. One hemolytic unit (HU_{50}) was defined as the concentration of protein sample required to cause 50 % hemolysis of sheep RBC in 1 mL of a 1% RBC solution at 37°C in 1 hr. The Hemolytic activity (% hemolysis) was calculated using the following equation:

$$\text{Hemolytic activity (\%)} = \frac{OD_{sample}\text{-}OD_{0\%}}{OD_{100\%}\text{-}OD_{0\%}} \times 100$$

Where is the absorbance of the reaction system with venom proteins, $OD_{100\%}$ is the absorbance of the 100% hemolysis control and $OD_{0\%}$ is the absorbance of the 0% hemolysis control.

A significant difference between treatments was analyzed by determining the curved relationship reflecting dose-dependent effects of the two venoms using the GraphPad Prism statistical package version 5.00 (GraphPad Software, USA). The HU_{50} of each species was estimated by fitting log(agonist) vs. response using non-linear regression analysis and was expressed as mean ± SEM (n = 4).

2.4 Ex vivo hemolytic assays to evaluate therapeutic approaches

2.4.1 Using copper gluconate

In 96-well v-bottom microtiter plates, hemolytic activity assays will be performed by adding washed 1 per cent red blood cells (RBC) derived from sheep blood. After thawing frozen *C. indrasaksajiae* venom, three-fold dilutions of total venom were applied to the erythrocyte suspensions in each well. Each well was treated simultaneously with a 1/5 total volume dose of 100 mM copper gluconate to achieve a total final concentration of 30 μM for each well of the treated experiment. After an hour of incubation at 37°C, the microplate was immediately centrifuged at 1,000xg for 4 minutes at 4°C before being analyzed. Next, 150 μL of supernatants will be transferred to new 96-well flat-bottom microplates. The concentration of released haemoglobin was measured spectrophotometrically at 405 and 540 nm using

an Infinite M200 PRO microplate reader. The hemolytic activity, expressed as a percentage of total hemolysis, was determined using the method outlined in section 2.3.

2.4.2 Using heat

Hemolytic activity assays will be performed in 96-well v-bottom microtiter plates by adding washed 1% red blood cells (RBC) prepared from sheep blood. Frozen *C. indrasaksajiae* venom samples were thawed and exposed under different temperature ranges from 0°C (ice bucket), 25°C (room temperature), 37°C (water bath), 45°C (water bath), 60°C (hot plate), and 100°C (boiled water). A serial three-fold dilutions of incubated venom were applied to the erythrocyte suspensions in each well. The microplate was incubated at 37°C for 1 hour before being centrifuged at 1,000 g for 4 minutes at 4°C. The next step is to transfer 150 µL of the supernatants to new 96-well flat-bottom microplates. The concentration of released haemoglobin was measured spectrophotometrically at 405 nm and 540 nm using an Infinite M200 PRO microplate reader. The hemolytic activity (% hemolysis) was calculated as described in section 2.3.

2.4.3 Statistic analysis

The program Prism version 5.00 (GraphPad Software, USA) was used for all analysis of data and statistics. Each concentration-response curve was plotted in triplicate using erythrocytes from one animal. For each turn, the HU_{50} was estimated by fitting log(agonist) vs. response using non-linear regression analysis. The HU_{50} of each experimental condition was expressed as mean ± SEM (n = 4), and multiple comparisons were made by one-way analysis of variance followed by Tukey's test. All tests were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1 *C. indrasaksajiae* venom activity on RBCs

The protein concentration of *C. indrasaksajiae* venom from bead mill homogenization of cnidae was determined by the method of BCA protein assay and compared with BSA protein concentration standards. The results showed that 200 mg of cnidae (approximately 750,000 cnidae of *C. indrasaksajiae*) homogenized in 1 mL of sterile water contained about 10.67 µg of proteins. The hemolytic effects of *C. indrasaksajiae* venom were examined in the microplate hemolytic assays using sheep RBC. These assays allowed quantitative comparison of toxic venom potency by calculation of HU50 values through colorimetric detection of viable cells and haemoglobin release after venom exposure, respectively. Figure 26 shows dose-response curves of the hemolytic activity of *C. indrasaksajiae* venom. The venom of *C. indrasaksajiae* (0.0003 – 19.7 ng/ml) produced concentration-dependent hemolysis on erythrocytes. Minimum *C. indrasaksajiae* venom induced complete RBC lysis is at 6.58 µg/ml. From the concentration-response curve, the HU_{50} value of *C. indrasaksajiae* venom was calculated as 1.32 ng/ml for 405 nm and 1.96 ng/ml for 540 nm.

Hemolysis is one of the key factors and important effects of cnidarian stinging. It involves the breaking down of red blood cells (RBCs) with the consequent release of haemoglobin (Mariottini, 2014). Our result showed that the venom from the cnidae of *C. indrasaksajiae* multi-tentacle box jellyfish from Thai waters exhibits potent hemolytic activity. Interestingly, in our bioassay, the venom of *C. indrasaksajiae* demonstrated 100-fold more potently hemolytic activity than *C. fleckeri* (HU_{50}) (Brinkman et al., 2014) that, which is currently mentioned as the most lethal animal on the planet (Jouiaei, Yanagihara, et al., 2015; Williamson et al., 1996)

The venom protein components were characterized and identified in chapter IV to find potential toxin proteins that could be linked to the toxic effects. Biochemical analysis of *C. indrasaksajiae* venom proteins revealed that the venoms contained major protein bands of molecular weight between 47 and 57 kDa, which is a larger size than a novel family of bioactive box jellyfish venom proteins (ranging

from ~43 to 51 kDa) (Brinkman et al., 2015). The visualization of protein bands within the molecular range of CfTX proteins suggested suspecting the presence of similar CfTX proteins in the venom of *C. indrasaksajiae*. In *C. indrasaksajiae* venom, the most abundant toxins are *C. fleckeri* toxin-1 (CfTX-1) and toxin-2 (CfTX-2). These abundant peptides reflect the amount of toxin in *C. indrasaksajiae* cnidae venom, suggesting these toxins play an important functional role in box jellyfish envenomation. CfTX-1 and CfTX-2 were also the major toxin protein components in *C. fleckeri* venom (Brinkman, 2008; Brinkman et al., 2012; Brinkman and Burnell, 2008; Brinkman and Burnell, 2007, 2009; Brinkman et al., 2015).

CfTXs are cytotoxic to sheep and human erythrocytes, as well as the smooth muscle cells of the rat aorta. Furthermore, CfTXs have the potential to cause acute cardiovascular collapse and death in experimental animals (Brinkman and Burnell, 2008; Brinkman et al., 2014; Yanagihara and Shohet, 2012). Moreover, there is a significant structural similarity between the N-terminal domains of CfTXs and the N-terminal domains of pore-forming 3d-Cry toxins from various strains of the bacterium *Bacillus thuringiensis*. This suggests that the box jellyfish toxins may have a similar pore-forming mechanism of action in susceptible cells such as cardiomyocytes and erythrocytes (Brinkman and Burnell, 2007; Brinkman et al., 2014; Klompen et al., 2021; Mesa-Galloso et al., 2021). Furthermore, electron microscopy has shown that partially extracted CfTXs from *C. fleckeri* venom cause human red blood cells to develop transmembrane pores (Yanagihara and Shohet, 2012).

Proteins structurally similar to CfTX toxins have been found in the venoms of other jellyfish species belonging to the class Cubozoa. These proteins include CaTX-A from *Alatina moseri* (Nagai et al., 2000a), which was formerly known as *Carybdea alata* Gershwin (2005), CrTX-A from *Carybdea brevipedalia* (Nagai, 2009) and CqTX-A from *Chironex yamaguchii*, formerly known as *Chiropsalmus quadrigatus* (Lewis and Bentlage, 2009). Moreover, homologous proteins to the CfTXs have also been found or predicted in other Scyphozoa and Hydra species (e.g. CcTX-1 from *Cyanea capillata* (Lassen et al., 2011), TX-1 and TX-2 from *Aurelia aurita* (Killi et al., 2020), and CaTX-like from *Hydra magnipapillata (Sher et al., 2005)*). This indicated that homologous proteins to the

CfTXs have been linked to the presence of a specific family of pore-forming toxins (PFTs), described as the most potent cnidarian toxin families currently known (Jouiaei, Yanagihara, et al., 2015). However, some cnidarian jellyfish have no CfTX-like proteins, which may indicate that other venom components such as serine protease inhibitors or metalloproteinases make up most of the venom component in some cnidarians (Banagouro et al., 2022; Yang et al., 2022).

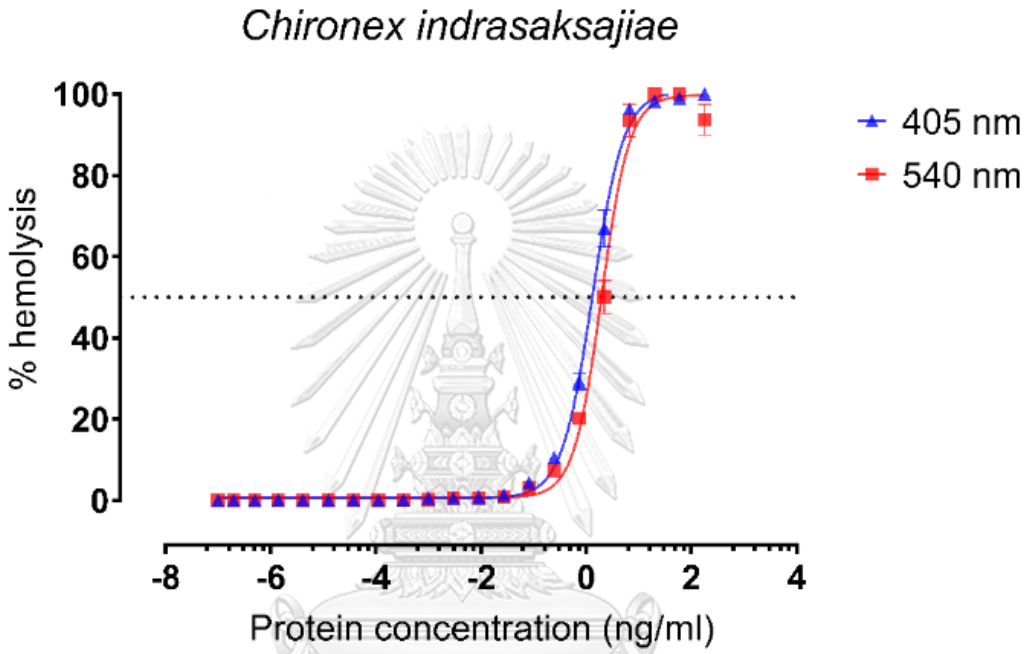

Figure 26 Representative concentration-response curve of the hemolytic proteins. Concentration-response curve of *Chironex indrasaksajiae* venom. All results were representative of quadruplicate experiments and expressed as mean±S.E.M. (n = 4)

3.2 Copper gluconate inhibition of *C. indrasaksajiae* venom

Sheep-washed red blood cells (RBCs) were subjected to *C. indrasaksajiae* venom at a starting concentration of 19.7 ng/ml. These cells were treated simultaneously with a 1/5 total volume dose of 100 mM copper gluconate to achieve a total final concentration of 30 µM. After an hour of incubation at 37°C, the concentration of released haemoglobin was measured spectrophotometrically. As shown in Figure 27, copper gluconate showed the potent inhibitory effect on the hemolysis induced by *C. indrasaksajiae* venom in 1% RBCs. The copper gluconate

inhibited hemolysis of RBCs by a maximum of 81.28 % at the most concentrated dose of 19.7 μg/ml.

The hemolytic toxins (porins) of class Cubozoa are potent proteins that share homology of protein structures with a group of self-assembling bacterial pore-forming toxins that has the ability to alter cell membrane permeability (Yanagihara, 2013). Yanagihara and Shohet (2012) found that the venom of *C. fleckeri* contains proteins that break down red blood cells and allow potassium to be released. The proteins in venom were not only ion channel blockers but also many "porins": proteins that puncture cells, allowing their contents to leak out. The efflux of intracellular potassium as a consequence of hemolysis appears to be the role of a high level of potassium in the blood, or hyperkalemia, that causes the electrical rhythms that keep the heart pumping to be disrupted and the heart to stop beating quickly in mice when injected with high doses of venom. The same happens when mice have only been injected with the porins from the venom. Thus, hemolytic toxins (porins) were suspected as the fatal mechanism.

Bioinformatic analyses revealed that the *C. fleckeri* toxins, CfTX-1 and CfTX-2, which were the most abundant toxin protein found in C. indrasaksajiae venom, belong to a family of potent cnidarian pore-forming toxins. Pore-forming toxins (PFTs), or porins, are the most rapidly acting and lethal components. This protein is the most significant venom component in terms of clinical symptoms. Thus, effective therapeutics for cnidarian envenomation will target the blockade molecules of these pore-forming toxins (PFTs), as well as other membrane perturbance (MPs) (Yanagihara, Wilcox, Smith, et al., 2016). Porin insertion affects the permeability barrier of the cell membrane, allowing monovalent ions to flow. In particular, the efflux of K^+, the influx of Na^+, the influx of Ca^{2+}, and the efflux of CI^- all result in depolarization of the cells due to the rapid equilibration of the internal and external ionic solutions. With enough size and duration of open time, bigger molecules such as intermediates in metabolism (e.g., nucleotides and sugar phosphates) would be allowed to flow out of the pores and cause cell death, also known as pyroptosis, which is represented as a loss of cellular function. Consequently, large proteins such as haemoglobin and lysosomal enzymes leak from red blood cells (RBC), resulting in hemolysis and tissue necrosis (Bashford et al., 1985).

Pore-forming toxins are classified into broad classes based on their secondary structure, with some requiring Ca^{2+} for self-assembly polymerization to form functional transmembrane pores. Because of this, some divalent cations, including Zn^{2+}, Cu^{2+}, and Mg^{2+}, may be able to competitively bind to calcium-binding sites and lead to inhibiting calcium-dependent porin proteins from self-assembling into polymeric pores. In addition, zinc has been reported to have membrane-protective functions in the presence of membrane-disrupting molecules and toxins (MPs) (Yanagihara, 2013).

According to Yanagihara (2019), copper gluconate alone is significantly more effective at reducing venom-induced cell hemolysis than zinc gluconate alone or combined with copper gluconate. In this study, human RBCs were incubated with 5 units/mL/% RBC *Alatina alata* venom, zinc gluconate, and copper gluconate at increasing doses for 1 hour at 37 °C. As a result, it was determined that copper gluconate is active at approximately 30 µM or greater for box jellyfish venom hemolysis inhibition, whereas zinc gluconate is active at approximately 300 µM or greater. Copper gluconate is approximately 10 times more potent than zinc gluconate alone in inhibiting box jellyfish venom. Herein, 30 mM of copper gluconate was also active for hemolysis inhibition of the *C. indrasaksajiae* venom. Moreover, copper gluconate was use as the active ingredients of commercially first-aid treatment for Sting No More™ that was newly developed ex vivo experimental assays for first-Aid approaches by demonstrating that vinegar inhibits nematocyst discharge and that 30 mM copper gluconate-based products inhibit hemolytic activity (Yanagihara, 2013, 2019; Yanagihara and Shohet, 2012; Yanagihara, Wilcox, King, et al., 2016; Yanagihara and Wilcox, 2017). The significant inhibitory effects of copper gluconate-based products on hemolytic activity after cubozoan stinging event represent a novel and effective therapeutic approach for the treatment of cnidarian envenomation.

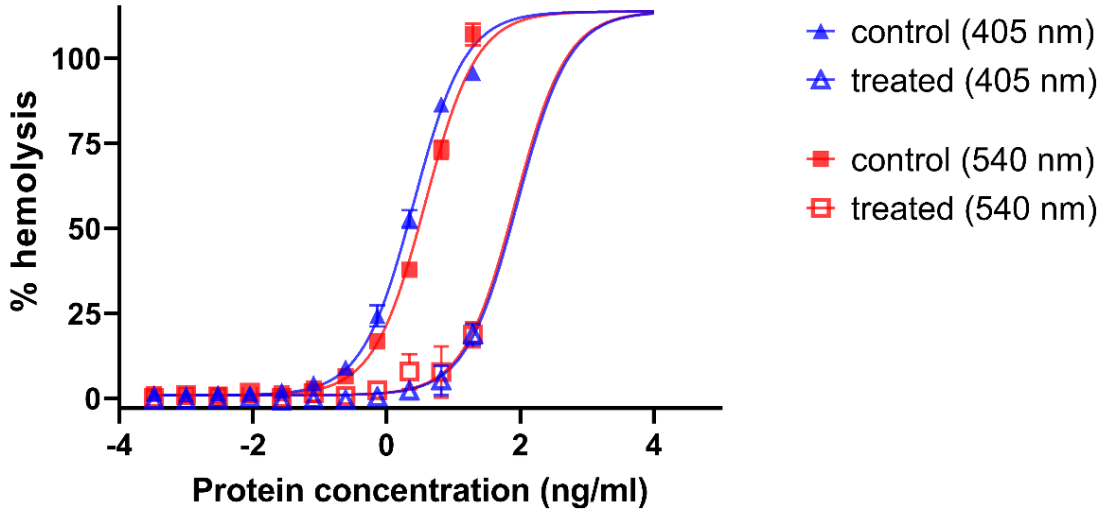

Figure 27 Representative concentration-response curves of hemolysis induced by C. indrasaksajiae venom on red blood cells after treated with copper gluconate

3.3 Effect of heat on the biological activity of *C. indrasaksajiae* venom

Herein, temperatures were utilized in this study to determine the inactivation of *C. indrasaksajiae* venom components. 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. The hemolysis induced by *C. indrasaksajiae* venom in red blood cells of the control group was concentration-dependent and 50% hemolysis was observed at a concentration of ~1.9 ng/mL (section 3.1).

Figure 28 shows the per cent hemolysis observed in the standard sheep blood assay against the log concentration of crude venom protein expressed as ng per ml of the hemolysis assay. Each set of symbols represents assays of serially diluted venom held for 1 hour at a different temperature. The hemolytic activity induced by *C. indrasaksajiae* venom was affected by temperature. According to the graphs, the amount of venom required to cause 50% hemolysis under standard hemolysis assay conditions has changed significantly to the right, indicating that venom potency decreases with higher pre-incubation temperatures.

Hemolytic activity peaked at 0°C and room temperature (25°C). These results indicated applying ice-cold water or an ice pack as a first-aid treatment for *C. indrasaksajiae* envenomation in order to relieve pain or denature the toxin protein by low temperature is not an appropriate approach to handle the situation. Cryotherapy, or immersing envenomed areas in ice-cold water, was previously recommended to improve outcomes in fatal envenomations by delaying the venom's movement into the heart. However, experimental evidence does not support cryotherapy or immersion in cold water for lowering pathology or enhancing clinical efficacy, and this therapy is no longer advised (Frank, 1971; Wilcox and Yanagihara, 2016). According to Yanagihara and Wilcox (2017), heat treatment significantly reduced the size of the hemolytic area in *C. fleckeri* stings, but not as effectively as in *A. alata* stings, suggesting that the venom toxins of the two species have distinct temperature tolerances. Even in the absence of scraping, the application of ice did not reduce the sting area; rather, it dramatically increased its severity, more than doubling the size of the hemolytic zone at 18 hours. Moreover, further research on the impact of ice on

chirodropid stinging is important because there have been documented deaths in situations in which ice was used. (Thaikruea and Siriarayapon, 2016, 2018).

As the results are shown in figure 28, the hemolytic activity was significantly reduced at 37°C and 45°C, but not abolished. At the temperature of 37°C, that is, human body temperature can reduce the activity of venom, indicating after the victim was stung by *C. indrasaksajiae*, our body can partly reduce the hemolytic activity. According to Yanagihara and Wilcox (2017), they defined the term "venom load" as the total amount of venom discharged into sting-site tissues. Due to the fact that less than one per cent of cnidae discharge venom at initial tentacle contact, the researcher has to find a way to remove the attached tentacles, stop the discharged cnidae, reduce the symptoms and the first-aid that are concern in the treatment when dealing with potentially lethal cubozoan stings. Immersion in hot water (45 °C) is a well-established and widely recommended treatment for a wide range of marine envenomations, including severe pain from echinoderm and venomous fish stings (Buckley and Isbister, 2012; Ongkili and Cheah, 2013). Immersing the sting location with hot water is considered to deactivate key components of marine venoms, although the exact mechanism of action remains unclear. Another possibility is that hot-water immersion has a direct potential therapeutic impact on pain receptors, decreasing perceived pain (Muirhead, 2002).

In addition, no activity was observed when the *C. indrasaksajiae* venom was treated at 60°C and 100°C (Table 11), indicating that the toxin responsible for the hemolytic effect is heat-labile. Many researchers suggested that the temperatures required for such inactivation are significantly higher (60 °C), and that the pain relief experienced during hot-water immersion is the result of physiological responses to the heat application rather than a chemical event (such as increased subcutaneous blood flow) (Wilcox and Yanagihara, 2016). Additionally, the widespread hypothesis on how heat treats cnidarian envenomations is that the temperatures were used to inactivate venom components. Several in vitro and in vivo investigations indicate that cnidarian venoms from all classes within the phylum are heat-labile .

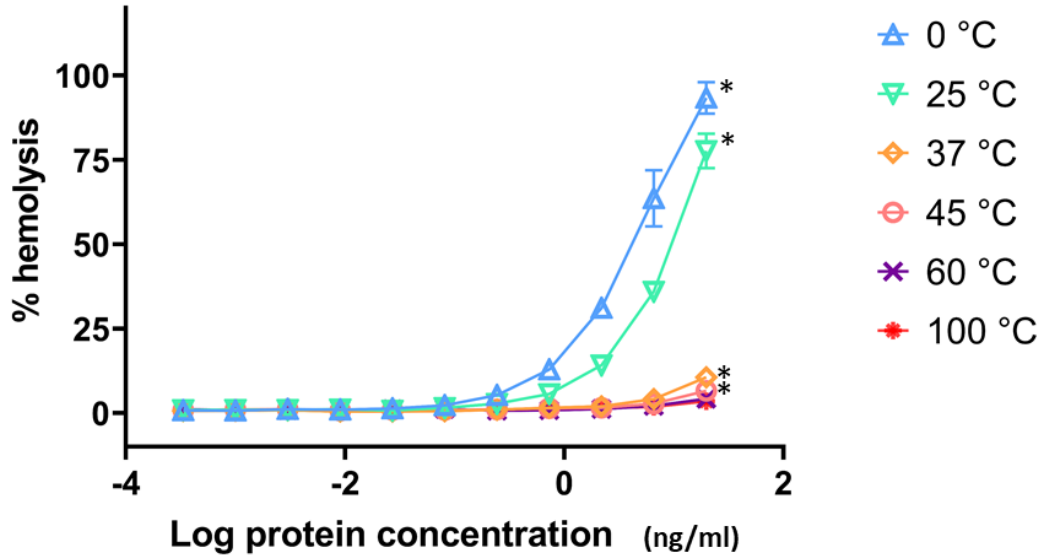

Figure 28 Hemolytic activity of crude venom after pre-incubation at different temperatures. Plot of per cent hemolysis of 1% sheep blood in PBS vs venom protein concentration (log scale). Aliquots of a single extraction of crude venom were held for 30 min at the temperatures indicated, then assayed for hemolytic activity. Arrow indicates 50% hemolysis; the corresponding protein concentration is the HU_{50}. 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.

CHAPTER VI
General discussion

1. Introduction

Venomous jellyfish such as the Thai multi-tentacled box Jellyfish, *Chironex indrasaksajiae*, which was just recently described as a new species of box jellyfish from the Gulf of Thailand, inflict painful stings that have the potential to become life-threatening within minutes. These responses are particularly associated with hemolytic and cytolytic reactions. *Chironex* venoms have been shown in several studies to have a wide range of harmful effects on a vast wide range of physiological systems and types of cells. Despite these results, our knowledge of the molecular composition of *C. indrasaksajiae* venoms remains incomplete. This thesis uses proteomics and toxicological bioassays to help clarify some of the present questions surrounding the composition and toxicity of jellyfish venom. Moreover, our knowledge about the known components in *Chironex* species, a porin, a pore-forming protein, is the fast-acting component in the venom. This protein can create pores on the membrane of human red blood cells, making them leak. This leak can release large amounts of potassium ions and may cause cardiac arrest, leading to death. Therefore, this study will be focused on porins activities. Copper gluconate and heat will be tested in the study because these two factors have been reported that the treatments can perturb the self-assembly of porins. This therapy is limited and transient to support cardiovascular hemodynamics. It may allow for more effective therapy.

2. Key findings and their significance

2.1 The cnidome, or census of cnidae in Thai multi-tentacle box jellyfish

C. buitendijki and *C. indrasaksajiae* contain four main types of nematocysts: mastigophores, oval trirhopaloid, euryteles, and isorhizas. However, there are some differences in size, form, and proportion in different species of jellyfish. Moreover, there are differences in the cnidae ratios between two species of Thai multi-tentacle box jellyfish. In *C. buitendijki*, the proportion of mastigophores was less than that in *C. indrasaksajiae*. The proportion of mastigophore is presumably the lethal venom component (McClounan and Seymour, 2012). Mastigophore in *C. indrasaksajiae* shows a remarkable massive to that of *C. buitendijki*. Because of this, the volume of venom content in mastigophores of *C. indrasaksajiae* were higher than that of *C. buitendijki*. Moreover, the length of the mastigophores tubule in *C. indrasaksajiae* was long enough to penetrate the human dermis. Venom would be run off the tubules and passed through capillaries, adding to the quickness with which the envenomation occurs. Thus, the proportion of mastigophores was formerly thought to be the reason for the variation in fatal envenomation between these two species. The difference in fatalities encountered may be due to the percentage of mastigophores, with an increase in *C. indrasaksajiae* proving to be enough to give the lethal dosage as the result of several fatal cases in Thailand, whereas *C. buitendijki* has not.

2.2 Venom extraction for *C. indrasaksajiae*

The schematic design of the extraction procedure for *C. indrasaksajiae* venom is shown in figure 29. Findings from these experiments suggest that although both species are multi-tentacle box jellyfish, the appropriate extraction techniques are different. The possible explanation was that the varying proportions of cnidae types in each species (Figure 20) appeared to result in different venom mixtures that caused the similar extraction process to be inapplicable. Therefore, an appropriate protocol for the extraction of venom from cnidae should be optimized whenever a new species is investigated.

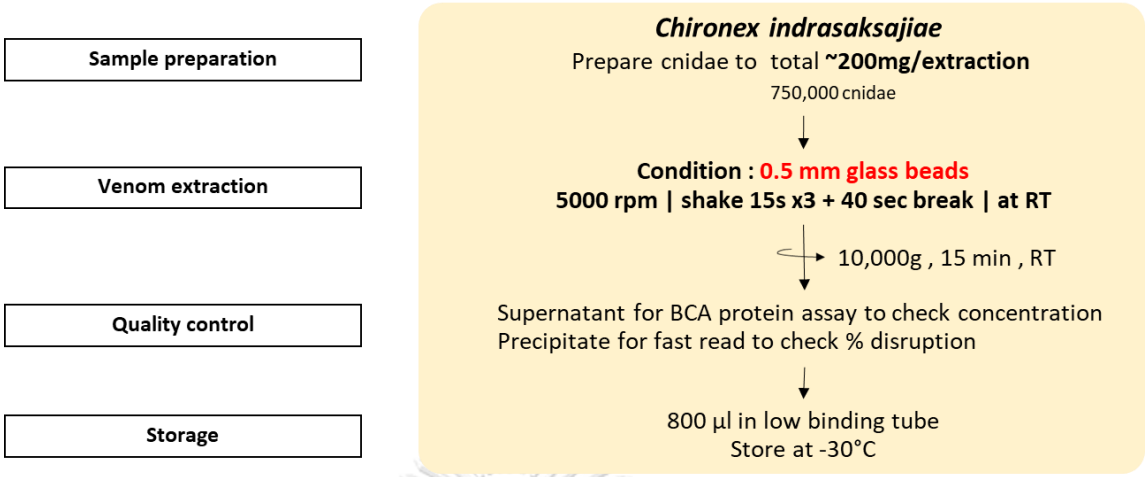

Figure 29 Schematic design of the extraction procedure for *C. indrasaksajiae* venom

2.3 *C. indrasaksajiae* venom composition

The principal aim of this study was to identify proteins in the venom of *C. indrasaksajiae* that could be responsible for its wide range of bioactivities and debilitating effects in humans. As our results show, the venom proteome of *C. indrasaksajiae* contains a diverse array of proteins dominated by toxins and proteins involved in nematogenesis. Due to the lack of genomic and transcriptomic sequences currently available for *C. indrasaksajiae,* the limited cnidarian toxin on the database and *C. indrasaksajiae* is a new species of box jellyfish found in Thai water; it caused the low protein identification rate. The most abundant toxins in *C. indrasaksajiae* venom are CfTX-1, CfTX-2, with sequence coverage of 39.3% and 34.8%, respectively. The CfTX-like proteins belong to a family of potent toxins only found in Cnidaria. Toxicological data and bioinformatic information suggest that this expanding toxin family plays a significant functional role in envenoming. The vast majority of them contained the toxins that contributed to hemolysis. Hemolytic toxic effects that the destruction of red blood cells by pore-forming protein or porin might be suspected as the fatal mechanism (Mesa-Galloso et al., 2021; Yanagihara and Shohet, 2012; Yanagihara, Wilcox, Smith, et al., 2016). Further research is necessary to elucidate the actions of these toxins at the molecular level. Thus, CfTX-like toxin

or C. *indrasaksajiae* toxins (CiTXs) were further studied in genomic and transcriptomic sequences to understand better the molecular, structural and functional diversity of this important toxin family. It will lead to a better understanding of and improvements in medical treatments of cnidarian stings.

2.4 Toxic effects of *C. indrasaksajiae* venoms and therapeutic approaches

Hemolysis is one of the key factors and important effect of cnidarian stinging. It involves the breaking down of red blood cells (RBCs) with the consequent release of haemoglobin (Mariottini, 2014). Our result showed that the venom from the cnidae of *C. indrasaksajiae* multi-tentacle box jellyfish from Thai waters exhibits potent hemolytic activity. Interestingly, in our bioassay, the venom of *C. indrasaksajiae* demonstrated 100-fold more potently hemolytic activity than *C. fleckeri* (HU_{50}) (Brinkman et al., 2014) that, is currently mentioned as the most lethal animal on the planet (Jouiaei, Yanagihara, et al., 2015; Williamson et al., 1996). The toxic effects of *C. indrasaksajiae* have been primarily attributed to CfTX-like toxins, which are highly abundant in the venom. This suggests that these proteins may also be responsible for the toxic effects observed in our *in vitro* assays. Our current understanding of the structure of the venom produced by box jellyfish C. indrasaksajiae is augmented by the discovery of these CfTX-like proteins, despite the fact that further characterization of these proteins is required.

There is no clearly effective specific therapy available. Current treatments are directed at relief of symptoms or, in serious cased, support of cardiovascular integrity after envenomation. Thus, there is a need for effective therapies to reduce the mortality outcomes for cnidarian envenomation. Copper gluconate showed a potent inhibitory effect on the hemolysis (key factors of fatal mechanism) induced by *C. indrasaksajiae* venom in 1% RBCs. The significant inhibitory effects of copper gluconate-based products on hemolytic activity after a cubozoan stinging event represent a novel and effective therapeutic approach for the treatment of cnidarian envenomation.

The hemolytic activity induced by *C. indrasaksajiae* venom was affected by temperature. The amount of venom required to cause 50% hemolysis

under standard hemolysis assay conditions has changed significantly to the right, indicating that venom potency decreases with higher pre-incubation temperatures. The temperatures required for such inactivation are significantly higher (60 °C). Nevertheless, hot-water immersion at 60 °C are unable to apply because the temperature is too high for the skin, and the application of heat might enhance subcutaneous blood flow, which in turn accelerates the spread of venom to the core (heart).

3. Limitations and suggestions for future research

Researchers have developed an interest in the venom of box jellyfish due to its lethal properties, which cause death within minutes. The important and essential phase of venomics is to optimize venom extraction conditions and methods for optimal venom recovery. Researchers have struggled for decades to acquire pure box jellyfish venom stored in millions of cnidae. Due to the difficulties of collecting large volumes of venom from these organelles, several novel approaches for venom extraction have been established. In this study, the approach of Yanagihara and Shohet (2012) was utilized for the isolation and cleaning of cnidae, in which tentacle solutions are gently rotated for several months. The solutions were sieved, and the filtered solutions were collected after around 90% recovery of cnidae. However, cnidocilia attracted to cnidae were still present in the cnidae solution after the fifth cleaning, suggesting that the cytolyzed duration should be extended for all components except cnidae pellets. Cnidae pellets were used for venom extraction using a slightly modified Carrette and Seymour (2004) approach. The outcomes of the experiments that measured the protein concentration of C. indrasaksajiae venom and the percentage of cnidae disruption did not go according to expectations. According to the findings, further investigations into the optimization of venom extraction should involve a variety of parameters in order to get the highest recovery of *C. indrasaksajiae* venom yields. Because *C. indrasaksajiae* is a newly described species of box jellyfish found in Thai waters and there are no specific annotated toxin proteins for this species. Therefore, it is recommended that further research be conducted to construct de novo tentacle transcriptomes in tandem with venom proteomics. Due to

the limited number of annotated toxin proteins in the cnidarian database utilized for the search, the cnidarian database provided 304 toxin proteins, but only eight of those toxin proteins were annotated in the box jellyfish group. These factors contributed to the poor peptide identification rate from LC-MS/MS (Appendix : PTXQC).

Cnidarian envenomations are an important public health problem, responsible for more deaths than shark attacks annually. For this reason, optimization of first-aid care is essential. The findings showed that copper gluconate effectively inhibited the hemolysis generated by *C. indrasaksajiae* venom. However, the copper gluconate concentration utilized in this experiment was too high for intravenous injection. Further study should have been performed on the intravenous injection concentration in order to improve or develop antivenom for preventing deaths from *C. indrasaksajiae* envenomations. According to the findings, *C. indrasaksajiae* venoms and toxins are heat labile at temperatures safe for human application, which supports the use of hot-water immersion of the sting area(s). However, ice packs are often recommended and used by emergency personnel. After performing the experiments, the results do not support for the use of ice in the treatment of C. indrasaksajiae envenomations. This study supports the use of hot-water immersion is a safe and effective method of reducing pain from cnidarian envenomations and is also associated with improved clinical outcomes. These findings may possibly have a role in the treatment of jellyfish stings, however the therapeutic potential for applying heat to treat *C. indrasaksajiae* envenoming is limited. First, these stings can result in death within minutes, and second, heat can produce vasodilation and increase the transport of venom into the circulatory system. However, heat application may be beneficial for other box jellyfish stings in which venom distribution and systemic effects tend to develop more slowly. In the case of Irukandji syndrome, which is produced by some tropical carybdeids, systemic symptoms may not appear until 20–40 minutes after the sting.

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APPENDIX

Biohazard Control Plan

The safety precaution or program designed to protect personnel (employees, students), the community and the environment from biohazard.

Safety program includes the following:

- Proper training for personnel
- Provision of equipment for personal protection
- Assign emergency operator and decontamination plan

I. Proper training for personnel

No one is allowed to work with box jellyfish toxin without having prior training by the Principle Investigator who supervised their work.

II. List of primary safety equipment and personnel protective equipment requirements

The following PPE must be worn when working with box jellyfish and its venom, depending on where and which procedures are conducted.

-Field work/underwater work

2mm neoprene thickness full length wetsuit, 3mm neoprene thickness gloves or rubber/silicone scrubbing gloves, 3mm neoprene thickness boots, face shield

-Laboratory

long sleeves lab coat, gloves, surgical mask and eye protection or face shield is required any time because there is a risk of a splash of box jellyfish cnidae/venom outside BSC.

III. Biosafety requirements and procedures

In general, work with box jellyfish must be performed in a BSL2 laboratory. This includes but is not limited to a room suitable for tissue culture with negative pressure and a closing door, and equipped with a certified Class II Biosafety Cabinet (BSC), and a dedicated tissue culture incubator. During

work with box jellyfish toxin, a warning sign must be posted on the door alerting personnel of the presence of box jellyfish venom.

IV. Storage

- Storage of box jellyfish cnidae must be in leak-proof secondary containers at 4˚C.
- Storage of box jellyfish venom must be in leak-proof secondary containers at -80˚C.
- Both cnidae and venom must be clearly marked with a warning label to indicate toxin is present.

V. Waste decontamination and disposal

Decontamination

The box jellyfish tissue or extracted venom will be detoxified/decontaminated by using concentrated detergent/3-5% vinegar/heat then disposed as biohazardous waste. Moreover, we will rigorously inactivate of contaminated surfaces and objects using concentrated detergent.

-Solid waste

Everything that contacts box jellyfish or box jellyfish venom must be decontaminated or contained before disposal. Solid waste can be collected in a biohazard bag. At the end of the work session, the biohazard bag will be closed, sprayed with 3-5% vinegar, and deposited into a biohazardous waste container.

-Liquid waste

Liquid that contacts box jellyfish or box jellyfish venom will be aspirated into container containing concentrated detergent/vinegar. Allowing a minimum time of 30 minutes to inactivate venom. A simple 500 ml bottle with 100 ml concentrated detergent/3-5% vinegar may be suitable to collect liquid waste.

VI. Accidents and spills

- Small spills (<25 ml) can be decontaminate by layering paper towels soaked in 3-5% vinegar on top of the spill, allowing 20 minutes to inactivate venom, then depositing the paper towels in the solid waste bag.
- Large spills (>25 ml) should be treated more cautiously. Warning sign should be post on a door advising personnel not to enter. Notify the PI. In this case, extensive decontamination must be carried out using concentrated detergent.

VII. List of procedures if any accident, injury or illness occurs

1. call for help, if outside the hospital call 1669, if inside the hospital call emergency room
2. pour vinegar over the wound or affected area for at least 30 seconds
3. if unconscious and not breathing/beating, start CPR before pouring vinegar
4. if medical treatment and management is needed follow the Protocol: Expert opinions on jellyfish envenomation especially box jellyfish envenomation (attachment3)

VIII. List specific treatment provision for accidental exposure

- vinegar for decontamination
- copper gluconate spray or cream for specific treatment

  For detailed treatment please see Protocol: Expert opinions on jellyfish envenomation especially box jellyfish envenomation (attachment-2)

Figure A1: example of *C. indrasaksajiae* cnidae counting grid for calculating % cnidae disruption

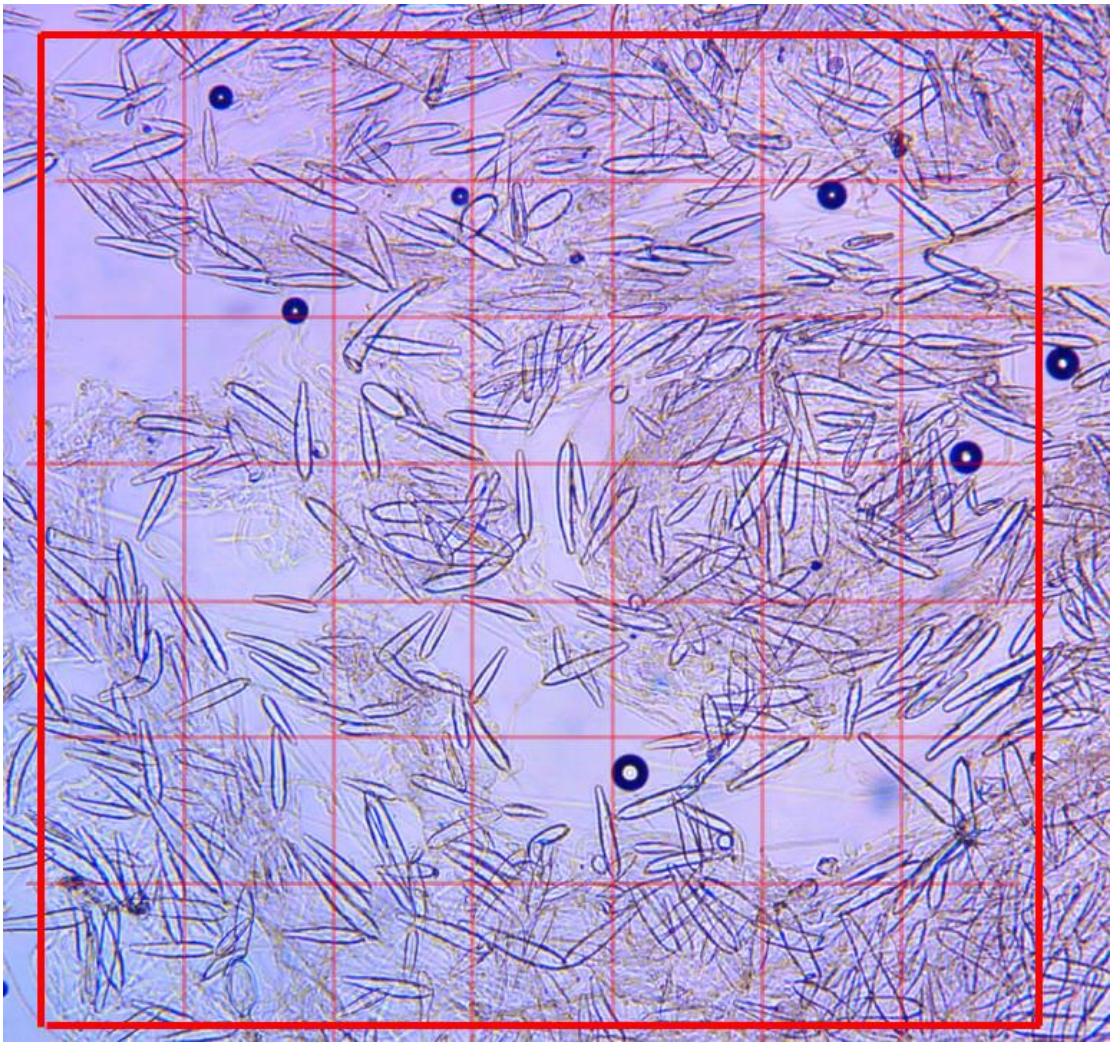


Figure A2: % cnidae disruption of *C. indrasaksajiae* cnidae for each type

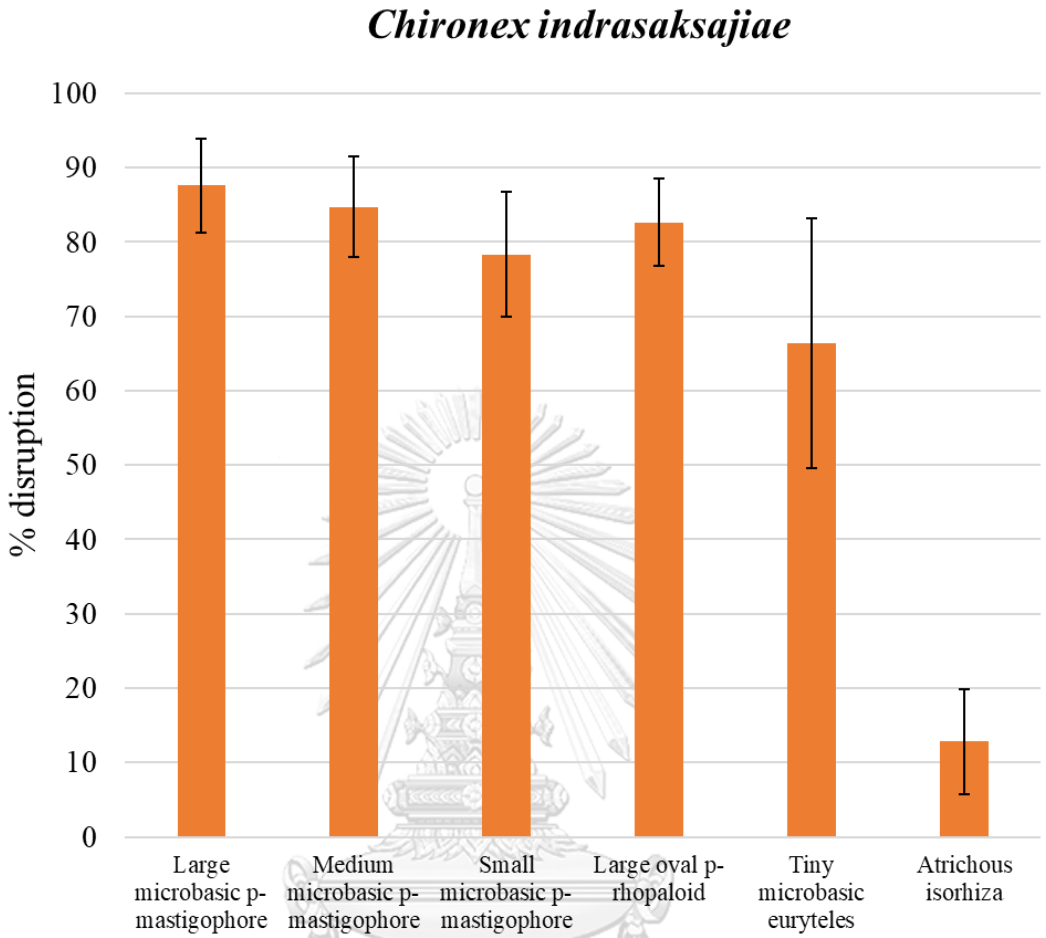


Figure A3: example of *C. buitendijki* cnidae counting grid for calculating % cnidae disruption

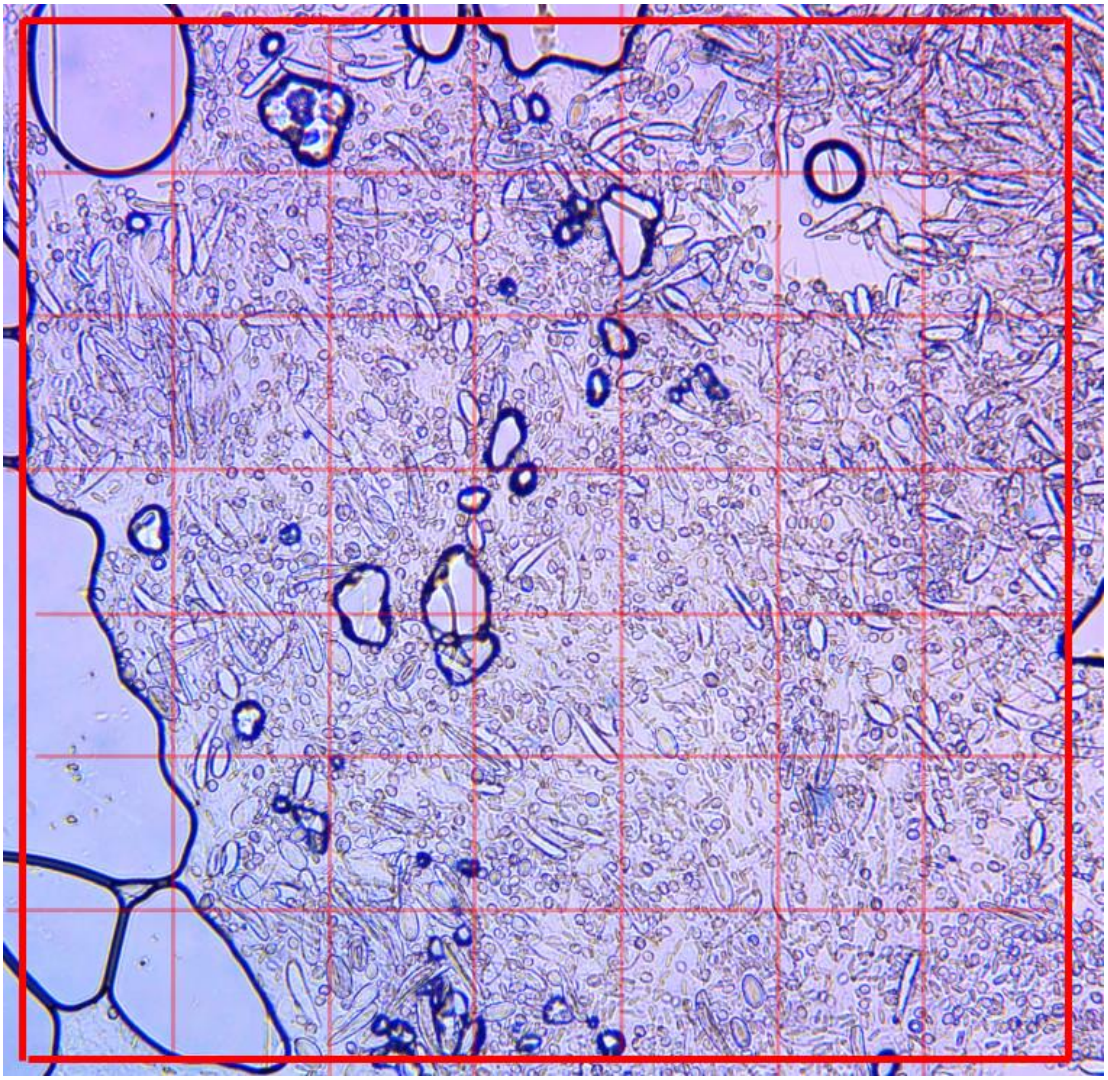


Figure A4: % cnidae disruption of *C. buitendijki* cnidae for each type

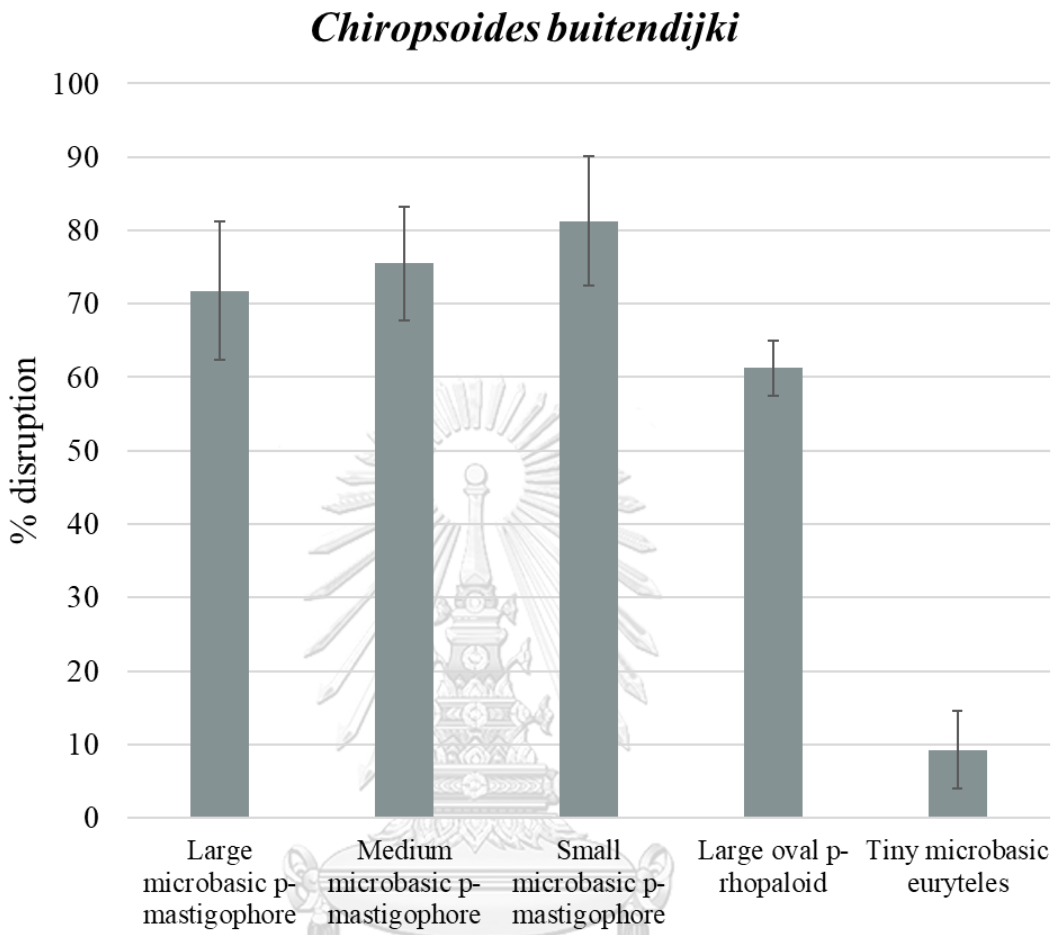
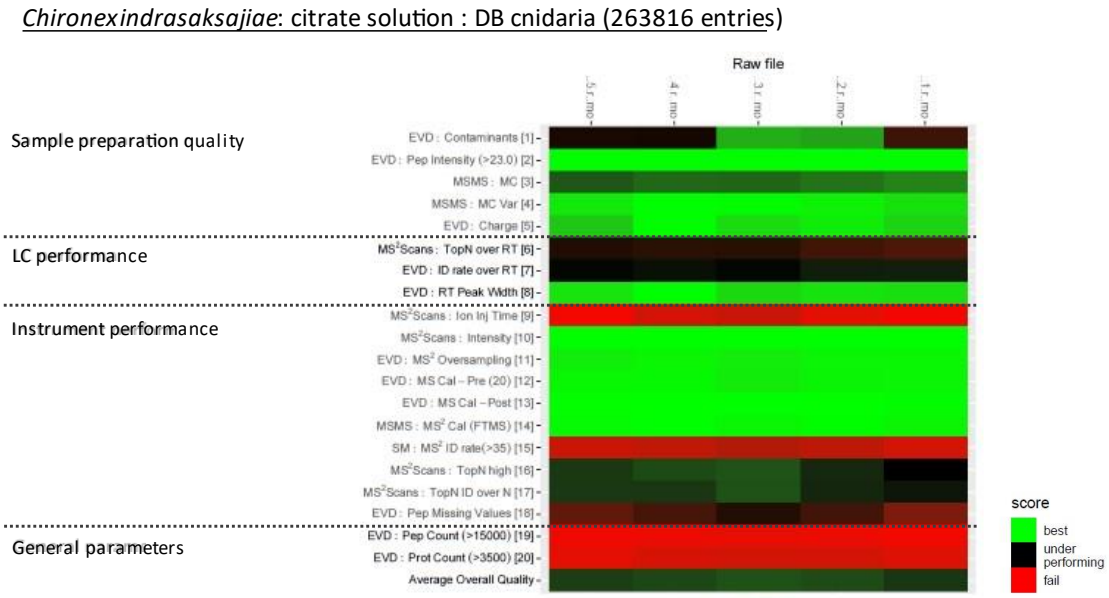


Figure A5 Species distribution of toxic jellyfish in the coastal area of Suratthani province, Thailand (Source: https://km.dmcr.go.th/c_1/s_369/d_19235)

Figure A6 Species distribution of toxic jellyfish in the coastal area of Trang province, Thailand (Source: https://km.dmcr.go.th/c_1/s_368/d_19233)

PTXQC Figure 1 Heat map represents the quality of LC-MS/MS analysis



Dataset A1 List of all protein groups identified from *C. indrasaksajiae* venom by LC-MS/MS from the SDS-PAGE gel. Proteins are organized according to the platform LC-MS/MS. All proteins have the percentage of identity in Blast search, Mass (KDa), protein score and the amino acid sequence with the corresponding ion score.

Accession Number	Protein Identify	Protein family	Protein type	InterProScan Protein Function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Peptide sequences	Razor + unique peptides	Sequence coverage [%]	Score	%iBAQ
P41113	Actin-3	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Podocoryna carnea*	Hydrozoa	41.826	AGFAGDDAPR AVFPSIVGR AVFPSIVGRPR CPETLFQPAFIGMESAGIHETTYNSIMK DLTDYMMK DLYANTVLSGGTTMFPGIADR DSYVGDEAQSK DSYVGDEAQSKR EISSLAPPTMK EKMTQIMFETFNSPAMYVAIQAVLSLYASGR FRCPETLFQPAFIGMESAGIHETTYNSIMK GYSFTTTAER HQGVMVGMGQK HQGVMVGMGQKDSYVGDEAQSK HQGVMVGMGQKDSYVGDEAQSKR IWHHTFYNELR KDLYANTVLSGGTTMFPGIADR KYSVWIGGSILASLSTFQQMWISK MTQIMFETFNSPAMYVAIQAVLSLYASGR SYELPDGQVITIGNER VAPEEHPVLLTEAPLNPK VAPEEHPVLLTEAPLNPKANR YPIEHGIVTNWDDMEK YSVWIGGSILASLSTFQQMWISK	24	64.9	323.31	33.0159
A0A7M5WUU7	Tubulin beta chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Clytia hemisphaerica*	Hydromedusan	49.876	ALTVPELTQQMFDAK AVLVDLEPGTMDSVR EEYPDRIMNTFSVVPSPK EIVHLQAGQCGNQIGAK EVDEQMLNVQNK FPGQLNADLR FPGQLNADLRK FWEVISDEHGIDPTGTYHGDSDLQLER GHYTEGAELVDSVLDVVR GHYTEGAELVDSVLDVVRK IMNTFSVVPSPK INVYYNEATGGK KLAVNMVPFPR LAVNMVPFPR LHFFMPGFAPLTSR LTTPTYGDLNHLVSATMSGVTTCLR MREIVHLQAGQCGNQIGAK MSMKEVDEQMLNVQNKNMMAACDPR NSSYFVEWIPNNVK SGPFGQIFRPDNFVFGQSGAGNNWAK TAVCDIPPR YLTVAAMFR	25	64.3	323.31	14.4451
A7RUT1	Tubulin alpha chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Nematostella vectensis*	Sea anemone	50.202	AFVHWYVGEGMEEGEFSEAR AFVHWYVGEGMEEGEFSEAREDLAALEK AVCMLSNTTAIAEAWAR AVFVDLEPTVVDEVR AYHEQLTVAEITNACFEPANQMVK DVNAAIATIK DYEEVGVDSVEGEGEEEEGEEY EDAANNYAR	27	71	323.31	10.5900

Accession Number	Protein Identify	Protein family	Protein type	InterProScan Protein Function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Peptide sequences	Razor + unique peptides	Sequence coverage [%]	Score	%iBAQ
									EDLAALEKDYEEVGVDSVEGEGEEEEGEEY EIVDLVLDR FDGALNVDLTEFQTNLVPYPR IHFPLATYAPVISAEK LADQCTGLQGFLIFHSFGGGTGSGFSSLLMER LDHKFDLMYAK LIGQIVSSITASLR NLDIERPTYTNLNR PTYTNLNR QLFHPEQLITGK QLFHPEQLITGKEDAANNYAR RAFVHWYVGEGMEEGEFSEAR RNLDIERPTYTNLNR RTIQFVDWCPTGFK TIGGGDDSFNTFFSETGAGK TIGGGDDSFNTFFSETGAGKHVPR TIQFVDWCPTGFK VGINYQPPTVVPGGDLAK YMACCLLYR				
A7L035	Toxin CfTX-1 precursor; (Short=Tox in 1)	cubozoan protein toxin (cpt) family	pore-forming toxin	toxin activity	hemolysis in other organism ion transport	*Chironex fleckeri*	Box jellyfish	51.39	AEESVGSVVKK AFLDGVR AKVMGAIGSLSTAVGK AVQEQSDQELQEALYGVK AVQEQSDQELQEALYGVKR DLILLDLYQLVATPGHSPNIASGIK ETYLFLSYLYPR;EVSNLGREEYK FIAMVVQR;FSPIFSILSLVVGLFSGTK IASGCLDILVGISSVLK IASGCLDILVGISSVLKDFAK KAVQEQSDQELQEALYGVK KAVQEQSDQELQEALYGVKR MLTMLELFTDLCSLR VMGAIGSLSTAVGK	16	36	323.31	9.4208
A0A2B4R9Q9	Histone H4	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Stylophora pistillata*	Coral	23.67	DAVTYTEHAKDNIQGITKPAIREIAQDFKTDLRG VLKVFLENVIRISGLIYEETRISGLIYEETRGVLK KTVTAMDVVYALKKTVTAMDVVYALKRRISG LIYEETRRISGLIYEETRGVLKSTELLIRKTVTAM DVVYALKTVTAMDVVYALKRVFLENVIRVFLE NVIRDAVTYTEHAKYRPGTVALR	16	41	191.64	7.2593
Q8IAC3	Actin	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Galaxea fascicularis*	Coral	41.724	AGFAGDDAPR AVFPSIVGR AVFPSIVGRPR DLTDYLMKILTER DSYVGDEAQSK DSYVGDEAQSKR EIAALAPPTMK GYSFTTTAER HQGVMVGMGQK HQGVMVGMGQKDSYVGDEAQSK HQGVMVGMGQKDSYVGDEAQSKR IWHHTFYNELR KYSVWIGGSILASLSTFQQMWISK LCYVALDFEQEMNTAASSSSLEK SYELPDGQVITIGNER TTGIVLDSGDGVSHTVPIYEGYALPHAILR VAPEEHPVLLTEAPLNPK VAPEEHPVLLTEAPLNPKANR YPIEHGIVTNWDDMEK YSVWIGGSILASLSTFQQMWISK	2	58.2	33.502	4.2224
A7L036	Toxin CfTX-2 precursor; (Short=Tox in 2)	cubozoan protein toxin (cpt) family	pore-forming toxin	toxin activity	hemolysis in other organism ion transport, cardiotoxin	*Chironex fleckeri*	Box jellyfish	51.684	AEESVSSVVTK AFLDGVR AFLDGVRNEESDLRPTEVSALAANIPVYQGVR AIQEQSDQELQEALYGVK AIQEQSDQELQEALYGVKRA	13	31.8	323.31	4.1712

Accession Number	Protein Identify	Protein family	Protein type	InterProScan Protein Function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Peptide sequences	Razor + unique peptides	Sequence coverage [%]	Score	%iBAQ
									KVMGAIGSLGTAIGK EVTSLGREEYQR FIAMVVQR KWPHNFAYSQK NEESDLRPTEVSALAANIPVYQGVR TANGLLSILKDVPSK TDDEETFLFLSYLYPK VFEDLLKTDDEETFLFLSYLYPK VMGAIGSLGTAIGK WPHNFAYSQK				
A0A6P8HZX0	Tubulin alpha chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Actinia tenebrosa*	Sea anemone	50.159	AFVHWYVGEGMEEGEFSEAR AFVHWYVGEGMEEGEFSEAREDLAALEK AVCMLSNTTAIAEAWAR AVFVDLEPTVVDEVR DVNAAIATIK EDAANNYAR ELIDLVLDR FDGALNVDLTEFQTNLVPYPR GHYTVGKELIDLVLDR IHFPLATYAPVISAEK LADQCTGLQGFLIFHSFGGGTGSGFSSLLMER LDHKFDLMYAK LIGQIVSSITASLR NLDIERPTYTNLNR PTYTNLNR QLFHPEQLITGK QLFHPEQLITGKEDAANNYAR RAFVHWYVGEGMEEGEFSEAR RNLDIERPTYTNLNR RTIQFVDWCPTGFK TIGGGDDSFNTFFSETGAGK TIGGGDDSFNTFFSETGAGKHVPR TIQFVDWCPTGFK VGINYQPPTVVPGGDLAK YMACCLLYR	2	62.5	30.247	1.0493
A0A6S7H3J5	Gelsolin 2	Villin/gelsolin family	Cytoskeleton Component	actin filament binding	molecular function	*Paramuricea clavata*	Sea-whip	31.428	TVELDTLLDDKPVQHR	1	5.9	135.99	0.8831
A0A2B4T1C4	ATP synthase subunit alpha	ATPase alpha/beta chains family	Oxido-reductive	Produces ATP from ADP in the presence of a proton gradient across the membrane	ATP synthesis coupled proton transport	*Stylophora pistillata*	Coral	59.646	AVDSLVPIGR EAYPGDVFYLHSR EVAAFAQFGSDLDAATQSLLNR GIRPAINVGLSVSR GMALNLEPDNVGVVVFGNDK HALIIYDDLSK STVAQLVK TAIAIDTIINQK VLSIGDGIAR VVDALGNPIDGK	10	23.9	323.31	0.7846
A0A7M5VD25	Tubulin beta chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	regulation of cellular response to heat	*Clytia hemisphaerica*	Hydromedusan	49.892	ALTVPELTQQMFDAKAVLVDLEPGTMDSVREE YPDRIMNTFSVVPSPKEIVHLQAGQCGNQIGAK EVDEQMLNVQNKFPGQLNADLRFPGQLNADLR KFWEVISDEHGIDPTGTYHGDSDLQLERGHYTE GAELVDSVLDVVRGHYTEGAELVDSVLDVVRK IMNTFSVVPSPKINVYYNEATGGKKLAVNMVPF PRLAVNMVPFPRLHFFMPGFAPLTSRLTTPTYG DLNHLVSATMSGVTTCLRMREIVHLQAGQCGN QIGAKMSATFIGNSTAIQELFKMSATFIGNSTAIQ ELFKRMSMKEVDEQMLNVQNKNMMAACDPR NSSYFVEWIPNNVKSGPFGQIFRPDNFVFGQSGA GNNWAKTAVCDIPPRYLTVAAMFR	2	64.3	138.98	0.7287
D1FX74	HSP70-1	Heat shock protein 70 family	Heat shock proteins	ATP binding ATP hydrolysis activity	Cellular response to heat stress	*Chironex fleckeri*	Box jellyfish	71.391	AKFEELNGDLFR ATAGDTHLGGEDFDNR EIHDIVLVGGSTR FEELNGDLFR IQQLLSDTFNGK	15	27.9	323.31	0.5614

Accession Number	Protein Identify	Protein family	Protein type	InterProScan Protein Function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Peptide sequences	Razor + unique peptides	Sequence coverage [%]	Score	%iBAQ
									MKEVAEAYLGK NCQNVVVTVPAYFNDSQR NQVALNPTNTVFDAK NQVALNPTNTVFDAKR NSLESYAYQMK RFDDAGAQADMK SFYAEEISSMVLTK SINPDEAVAYGAAVQAAILQGDK TTPSYVAFTDTER VEIIANDQGNR				
D1FX72	Elongation factor 1-alpha	GTPase family	Miscellaneous	Elongation factor	Protein biosynthesis, Transcription regulation	*Chironex fleckeri*	Box jellyfish	50.895	AVAFVPISGWHGDNMLEPTER EHALLAYTLGVK FQEIEKEVSAYLK IGGIGTVPVGR SGDAALIVLVPSKPMCVEPYSAYPPLGR TLFEALDSILPPQRPTDK TLFEALDSILPPQRPTDKPLR VETGIIKPGMVVHFSPANITTEVK	8	28	86.072	0.5581
A7SPP0	ATP synthase subunit beta	ATPase family	Oxido-reductive	Produces ATP from ADP in the presence of a proton gradient across the membrane	oxidative phosphorylation, ion transport, cellular localization	*Nematostella vectensis*	Sea anemone	44.84	AHGGYSVFAGVGER FTQAGSEVSALLGR IPSAVGYQPTLATDMGTMQER SLQDIIAILGMDELSEDDK SLQDIIAILGMDELSEDDKLTVAR TVLIMELINNVAK VALTGLTVAEYFR	7	23.8	168.15	0.4763
Q64FW3	Tubulin beta chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Scleronephthya gracillimum*	Coral	49.913	ALTVPELTQQMFDAK AVLVDLEPGTMDSVR EIVHLQAGQCGNQIGAK EVDEQMLNVQNK FPGQLNADLR FPGQLNADLRK FWEVISDEHGVDPTGTYHGDSDLQLER GHYTEGAELVDSVLDVVR GHYTEGAELVDSVLDVVRK INVYYNEATGGK KLAVNMVPFPR LAVNMVPFPR LHFFMPGFAPLTSR LTTPTYGDLNHLVSATMSGITTCLR MREIVHLQAGQCGNQIGAK MSMKEVDEQMLNVQNK NMMAACDPR NSSYFVEWIPNNVK TAVCDIPPR YLTVAAMFR	2	50.3	73.909	0.4377
A0A7M5X1G2	Lactamase_B domain-containing protein	Metallo-beta-lactamase	Hydrolase	hydroxyacylglutathione hydrolase activity, metal ion binding	breakdown of antibiotics by antibiotic-resistant bacteria	*Clytia hemisphaerica*	Hydromedusan	51.21	IENPPENGIR RIENPPENGIR	2	2.5	12.107	0.3848
A7S7W1	Actin-related protein 2	Actin family	Cytoskeleton Component	actin binding, ATP binding	organelle organization, regulation of biological process, protein polymerization	*Nematostella vectensis*	Sea anemone	44.819	DLMVGDEASQLR GYAFNHTADFETVR HVWDYTFGESK LALETTVLVEQYTLPDGR LCYVGYNIEQEQK YMLEVNYPMDNGIVR	6	21.1	118.94	0.3470
A0A7D9KLB0	Histone H2A	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Paramuricea clavata*	Sea-whip	12.563	LLSGVTIAQGGVLPNIQAVLLPK LLSGVTIAQGGVLPNIQAVLLPKK NDEELNKLLSGVTIAQGGVLPNIQAVLLPK VGAGAPVYLAAVLEYLSAEILELAGNAAR	3	51.7	43.52	0.2607
A0A6S7J4V0	Elongation factor 1-alpha	GTPase family	Miscellaneous	Elongation factor	Protein biosynthesis	*Paramuricea clavata*	Sea-whip	50.423	EHALLAYTLGVK IGGIGTVPVGR LHINVVIGHVDSGK	2	10.8	28.784	0.2173

Accession Number	Protein Identify	Protein family	Protein type	InterProScan Protein Function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Peptide sequences	Razor + unique peptides	Sequence coverage [%]	Score	%iBAQ
									YYVTVIDAPGHR				
A0A6G3MNH4	Actin	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Henneguya salminicola*	Myxozoa	11.556	GYSFTTTAERSGDGVSHTVPIYEGYALPHAILRS YELPDGQVITIGNER	1	47.6	20.785	0.2119
A0A7D9E3N3	Histone H2B	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Paramuricea clavata*	Sea-whip	13.86	AMNIMNSFVNDIFER	1	12	80.219	0.2117
T2MHY4	Argininosuccinate synthase	Argininosuccinate synthase	Proteases	argininosuccinate synthase activity, ATP binding	arginine biosynthetic process, urea cycle	*Hydra vulgaris*	Hydra	47.732	GIYETPGGEILR	1	2.9	6.1161	0.2081
A0A6P8J5L9	Histone H2A	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Actinia tenebrosa*	Sea anemone	13.481	LLGSVTIAQGGVLPNIQAVLLPK NDEELNKLLGSVTIAQGGVLPNIQAVLLPKK	2	24.8	27.755	0.1950
A0A6S7I6R8	Tubulin beta chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Paramuricea clavata*	Sea-whip	50.389	EIVHVQVGQCGNQIGAK FPGQLNADLR FPGQLNADLRK KLAVNMVPFPR LAVNMVPFPR LSEQFTAMFR MREIVHVQVGQCGNQIGAK NMMAACDPR NSSYFVEWIPNNVK RLSEQFTAMFR	4	16.5	41.752	0.1899
A0A6S7INI4	Peroxiredoxin-1-like isoform X2	Thioredoxin family	Oxido-reductive	peroxidase activity, peroxiredoxin activity	response to oxidative stress	*Paramuricea clavata*	Sea-whip	18.783	GLFIIDDKGILR LVQAFQFTDK QITVNDLPVGR	3	19.6	20.168	0.1758
A7RQD8	60S ribosomal protein L30	Ribosomal protein	Ribosomal protein	RNA binding structural constituent of ribosome	cytosolic large ribosomal subunit, Ribosome	*Nematostella vectensis*	Sea anemone	12.902	KSEIEYYAMLAK LVIIANNTPQLR LVIIANNTPQLRK TGVHHYTGNNIELGTACGK	4	36.4	40.517	0.1679
A7SS49	40S ribosomal protein S9	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Nematostella vectensis*	Sea anemone	22.194	HIDFSLNSPYGGGR HIDFSLNSPYGGGRPGR LDYVLGLR LFEGNALLR MKLDYVLGLR	5	18.9	39.169	0.1642
T2MHV8	ATP synthase subunit beta	ATPase family	Oxido-reductive	Translocase, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis, Hydrogen ion transport, Ion transport	*Hydra vulgaris*	Hydra	60.223	AHGGYSVFAGVGER FTQAGSEVSALLGR GSITSVQAIYVPADDLTDPAPATTFAHLDANTVLSR IMNVIGEPIDER IPSAVGYQPTLATDMGTMQER KGSITSVQAIYVPADDLTDPAPATTFAHLDANTVLSR VALTGLTVAEYFR	3	19.9	75.233	0.1500
T2MER0	40S ribosomal protein S14	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Hydra vulgaris*	Hydra	16.117	IEDVTPIPSDSTR IEDVTPIPSDSTRR TKTPGPGAQSALR TPGPGAQSALR	4	17.9	27.638	0.1498
A0A6P8HB76	Actin-related protein 2	Actin family	Cytoskeleton Component	actin binding, ATP binding	Arp2/3 complex-mediated actin nucleation, organelle organization, protein polymerization	*Actinia tenebrosa*	Sea anemone	44.858	HIVLSGGTTMYPGLPSR HVWDYTFGESK LCYVGYNIEQEQK VLLTEPPMNPMK YMLEVNYPMDNGIVR	1	17.3	5.9163	0.1485
T2MHT5	Heat shock protein HSP 90-alpha	Heat shock protein 90 family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding, stress response	*Hydra vulgaris*	Hydra	82.611	ADLVNNLGTIAK APFDLFENKK DIYYITGESK ELISNASDALDK ELISNASDALDKIR GVVDSEDLPLNISR	7	8.4	47.031	0.1462

Accession Number	Protein Identify	Protein family	Protein type	InterProScan Protein Function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Peptide sequences	Razor + unique peptides	Sequence coverage [%]	Score	%iBAQ
									RAPFDLFENKK				
A0A3M6TW19	Sodium/potassium-transporting ATPase subunit alpha	ATPase family	Cytoskeleton Component	ATP binding, metal ion binding, P-type potassium transmembrane transporter activity	Ion transport, Potassium transport	*Pocillopora damicornis*	Coral	116.61	ADIGVAMGIAGSDVSK GFKVDNSSLTGESEPQSR VAEIPFNSTNK VDNSSLTGESEPQSR VIMVTGDHPITAK	5	5.5	78.812	0.1416
A0A7M5V6Q3	H(+)-transporting two-sector ATPase	ATPase family	Oxido-reductive	Translocase, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis, Hydrogen ion transport, Ion transport	*Clytia hemisphaerica*	Hydromedusan	69.114	FKDPVKDGEAK LAANHPLLTGQR LAEMPADSGYPAYLGAR RTALVANTSNMPVAAR TALVANTSNMPVAAR TGKPLSVELGPGIMGSIFDGIQRPLK VGHSELVGEIIR	7	15	70.351	0.1399
A0A0C2N961	Actin	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Thelohanellus kitauei*	Myxozoa	42.354	AGFAGDDAPR AVFPSIVGR AVFPSIVGRPR DLTDYLMKILTER DSYVGDEAQSK DSYVGDEAQSKR GYSFTTTAER HQGVMVGMGQK HQGVMVGMGQKDSYVGDEAQSK HQGVMVGMGQKDSYVGDEAQSKR IWHHTFYNELR KYSVWIGGSILASLSTFQQMWISK SYELPDGQLITIGNER YPIEHGIVTNWDDMEK YSVWIGGSILASLSTFQQMWISK	1	35.4	32.968	0.1384
A0A6S7KB29	Malate dehydrogenase	LDH/MDH superfamily	Oxido-reductive	L-malate dehydrogenase activity	carbohydrate metabolic process, malate metabolic process, tricarboxylic acid cycle	*Paramuricea clavata*	Sea-whip	22.775	AGAGSATLSMAYAGAR IQNAGTEVVNAK	2	13	15.717	0.1377
T1PRE3	Toxin CfTX-A precursor	cubozoan protein toxin (cpt) family	pore-forming toxin	toxin activity	hemolysis in other organismion transport	*Chironex fleckeri*	Box jellyfish	50.485	ETIDFLHNMEAKLSDTLGILESRRTFNDVIAFLK SALEELKTEVSASPSTVSQVSEVVNTVGSSLTKT EVSASPSTVSQVSEVVNTVGSSLTK	5	14.8	117.44	0.1322
A0A3M6UPR6	Ornithine carbamoyltransferase	ATCase/OTCase family	Transferase	amino acid binding, carboxyl- or carbamoyltransferase activity	urea cycle, cellular amino acid metabolic process	*Pocillopora damicornis*	Coral	41.665	SLVWQEAENR SLVWQEAENRK	2	2.9	12.59	0.1277
A0A0C2N354	ATP synthase subunit beta	ATPase family	Oxido-reductive	Translocase, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis, Hydrogen ion transport, Ion transport	*Thelohanellus kitauei*	Myxozoa	57.605	FTQAGSEVSALLGR IPSAVGYQPTLATDMGTMQER LVLEVAQHLGENVVR VALTGLTVAEYFR	1	11.8	32.29	0.1265
A0A3M6UZF7	Phosphate carrier protein	Mitochondrial carrier family	transport protein	inorganic phosphate transmembrane transporter activity	mitochondrial phosphate ion transmembrane transport	*Pocillopora damicornis*	Coral	39.414	IQTQPGWANTLR LNNDVGSTPIQAAR	2	7.3	43.086	0.1219
A0A6G3MGA1	Tubulin beta chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Henneguya salminicola*	Myxozoa	39.212	EVEACDCLQGFQLTHSLGGGTGSGMGTLLISK FPGQLNADLR FPGQLNADLRK KLAVNMVPFPR LAVNMVPFPR	1	15.2	50.449	0.1217
A0A6P8IU89	Peroxiredoxin-6-like	Thioredoxin family	Oxido-reductive	peroxidase activity, antioxidant activity, oxidoreductase activity	response to oxidative stress	*Actinia tenebrosa*	Sea anemone	24.15	VIDSLQLTATK	1	5	8.9566	0.1213
T2MFK0	IDP	Isocitrate and isopropylmalate dehydrogenases family	Oxido-reductive	isocitrate dehydrogenase (NADP+) activity, magnesium ion binding, NAD binding	glyoxylate cycle, isocitrate metabolic process, tricarboxylic acid cycle	*Hydra vulgaris*	Hydra	53.105	IKVENPVVELDGDEMTR TIEAEAAHGTVTR	2	6.4	19.04	0.1094
A0A2B4S736	Vacuolar proton pump	ATPase family	Oxido-reductive	ATP binding	ATP metabolic process, proton transmembrane transport	*Stylophora pistillata*	Coral	54.799	AVVQVFEGTSGIDAK GFPGYMYTDLATIYER IPIFSAAGLPHNDIAAQICR	5	16.9	57.517	0.1093

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	subunit B								IYPEEMIQTGISAIDTMNSIAR NFITQGTYENR				
A0A7D9LK55	V-type proton ATPase 16 kDa proteolipid subunit	ATPase family	Oxido-reductive	ATP binding	ATP metabolic process, proton transmembrane transport	*Paramuricea clavata*	Sea-whip	7.8483	SGTGIAAMSVMRPELIMK	1	23.1	14.759	0.1077
P58762	Toxin CqTX-A precursor	cubozoan protein toxin (cpt) family	pore-forming toxin	toxin activity	hemolysis in other organism ion transport	*Chiropsoides quadrigatus*	Box jellyfish	51.801	AEESVGSVVKK AFLDGVR AKVMGAIGSLSTAVGK DLILLDLYQLVATPGHSPNIASGIK ETYLFLSYLYPR EVSNLGREEYK FFDLMK FIAMVVQR FSPIFSILSMVVGLFSGTK IASGCLDILVGISSVLK IASGCLDILVGISSVLKDFAK TNDKETYLFLSYLYPR VMGAIGSLSTAVGK	3	30.3	18.191	0.1051
T2MEY5	Actin-related protein 2/3 complex subunit 4	Actin family	Cytoskeleton Component	actin binding, ATP binding	organelle organization, regulation of biological process, protein polymerization	*Hydra vulgaris*	Hydra	19.503	AATLKPYLNAVR	1	7.1	7.9366	0.1030
A0A3M6THH7	Actin-related protein 2	Actin family	Cytoskeleton Component	actin binding, ATP binding	Arp2/3 complex-mediated actin nucleation, organelle organization, protein polymerization	*Pocillopora damicornis*	Coral	48.575	GYAFNHTADFETVR HIVLSGGTTMYPGLPSR HMVFLGGAVLADIMK LALETTVLVEQYTLPDGR LCYVGYNIEQEQK YMLEVNYPMDNGIVR	2	21.2	26.951	0.0979
A0A2B4S643	Doublesex- and mab-3-related transcription factor A2	DMRT family	Transcription factors	metal ion binding, sequence-specific DNA binding	regulation of transcription, DNA-templated	*Stylophora pistillata*	Coral	56.634	QPALADNYDEYTDSHK RQPALADNYDEYTDSHK	2	3.3	15.176	0.0967
Q56GW1	60S ribosomal protein L12	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Hydractinia echinata*	Hydroid	9.5452	VVGGEVGATSSLAPK	1	16.5	7.4003	0.0963
T2MEW2	Actin-related protein 3	Actin family	Cytoskeleton Component	actin binding, ATP binding	organelle organization, regulation of biological process, protein polymerization	*Hydra vulgaris*	Hydra	46.783	AEPEDHYFLLTEPPLNTPENR DITFFIQQLLK HGIVEDWDLMER NVVLSGGSTMFR	4	13.5	30.191	0.0926
T2M5M8	Oxysterol-binding protein	OSBP family	lipid-binding protein	lipid transporter activity, sterol binding	lipid transport, part of intracellular membrane-bounded organelle, membrane and cytosol	*Hydra vulgaris*	Hydra	110.34	VQLQEEAEKR	1	1	5.6573	0.0918
A0A6P8ICY6	ATP synthase subunit alpha	ATPase alpha/beta chains family	Oxido-reductive	Produces ATP from ADP in the presence of a proton gradient across the membrane	ATP synthesis coupled proton transport	*Actinia tenebrosa*	Sea anemone	59.691	AVDSLVPIGR EAYPGDVFYLHSR GIRPAINVGLSVSR HALIIYDDLSK STVAQLVK TGAIVDVPVGEELLGR TSVKEPMLTGIK VLSIGDGIAR	1	17	41.86	0.0917
T2MEP2	40S ribosomal protein S18	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Hydra vulgaris*	Hydra	18.05	AGELTDEEVER RAGELTDEEVER	2	7.7	13.223	0.0875
D1FX76	Thypedin (Fragment)	Thymosin beta family	Cytoskeleton Component	actin monomer binding	actin filament organization	*Chironex fleckeri*	Box jellyfish	75.952	EVIQEEAADSR EVIQEEATDSR EVIQEEATDSRAEVK HVETQEKNELPTPQMLK LEMKPDELPDVSEVNKFDTGK	6	28.8	45.261	0.0862

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									LEMKPDELPDVSEVSK				
A0A7M5XJN9	Major vault protein	Major vault protein family	scaffold/adaptor protein	protein binding	regulation of several cellular processes including transport mechanisms, signal transmission and radiation responses	*Clytia hemisphaerica*	Hydromedusan	95.405	AIPLDANEGVYVR HEADRLEQEAR IEGEAAVQQAQLK VPHNAAVQIYDYK	4	5.8	38.938	0.0832
A0A7D9DW98	RING finger 151-like	Kelch	unknown	zinc ion binding	Ubiquitin mediated proteolysis	*Paramuricea clavata*	Sea-whip	70.768	ELDGKVEAVKNSQDQIK	1	2.7	6.2695	0.0782
T2ME82	Peroxiredoxin-4	Thioredoxin family	Oxido-reductive	peroxidase activity, peroxiredoxin activity	response to oxidative stress	*Hydra vulgaris*	Hydra	28.734	GLFIIDDKGILR LVQAFQYTDK QITMNDLPVGR	2	13	11.419	0.0774
A0A6P8IJ56	60S ribosomal protein L40	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Actinia tenebrosa*	Sea anemone	14.745	ESTLHLVLR IQDKEGIPPDQQR TITLEVEPSDTIENVK	3	29.7	44.728	0.0762
A0A6P8HNP3	RNA helicase	Helicases	Enzymes	RNA helicase activity, hydrolase activity, ATP binding, translation initiation factor activity	Protein biosynthesis	*Actinia tenebrosa*	Sea anemone	46.876	GIYAYGFEKPSAIQQR GRDVIAQAQSGTGK VLITTDLLAR	3	9.7	49.297	0.0747
P38984	40S ribosomal protein SA	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Hydra viridissima*	Hydra	32.145	FTPGTFTNQIQK KSDGIHIINLR	2	7.8	18.634	0.0708
A0A7M5WRU5	PHB domain-containing protein	Band 7/mec-2 family	Cytoskeleton Component	ubiquitin protein ligase binding	transcription by RNA polymerase II, cellular response to stress, positive regulation of transcription by RNA polymerase II, signal transduction	*Clytia hemisphaerica*	Hydromedusan	27.122	VIAAEGEMNAAR YLQTLQTISAEK	2	9.9	16.355	0.0704
A0A515L5S5	Beta tubulin	Tubulin	Cytoskeleton Component	GTP binding, structural constituent of cytoskeleton	microtubule-based process	*Porites lutea*	Coral	12.445	FPGQLNADLR FPGQLNADLRK IMNTFSVVPSPK KLAVDMVPFPR LAVDMVPFPR LTTPTYGDLNHLVSATMSGVTTCLR	2	52.3	12.781	0.0697
A0A6B2G3U8	ATP synthase subunit beta	ATPase family	Oxido-reductive	Translocase, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis, Hydrogen ion transport, Ion transport	*Myxobolus squamalis*	Myxozoa	21.388	IMNVIGEPIDER VALTGLTVAEYFR VSLVYGQMNEPPGAR	1	19.9	20.433	0.0690
A0A6P8IZ41	mitochondrial-processing peptidase subunit beta-like	Peptidase family	Metalloendopeptidases	metal ion binding	proteolysis	*Actinia tenebrosa*	Sea anemone	54.487	IVLAAAGGVDHDELVK	1	3.3	6.1715	0.0671
A0A6P8I3C7	60S ribosomal protein L13	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Actinia tenebrosa*	Sea anemone	24.256	STESLQANVQR	1	5.2	15.925	0.0662
A5PGP4	Beta tubulin	Tubulin	Cytoskeleton Component	GTP binding, structural constituent of cytoskeleton	microtubule-based process	*Psammocora contigua*	Coral	16.452	FPGQLNADLR FPGQLNADLRK IMDTFSVVPSPK KLAVNMVPFPR LAVNMVPFPR LHFFMPGFAPLTSR LTTPTYGDLNHLVSATMSGVTTCLR	1	49.3	5.8261	0.0645
A0A7D9HDQ5	Carboxypeptidase D-like	Peptidase family	Proteases	metallocarboxypeptidase activity, zinc ion binding	peptide metabolic process, protein processing	*Paramuricea clavata*	Sea-whip	37.044	YVGNMHGNEVVGR	1	4	17.329	0.0643
A0A7D9DQ40	Glyceraldehyde-3-phosphate dehydrogenase	glyceraldehyde-3-phosphate dehydrogenase family	Oxido-reductive	calcium ion binding, glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity, NAD/NADP	oxidation-reduction process, glycolytic process, glucose metabolic process	*Paramuricea clavata*	Sea-whip	77.255	LVSWYDNEYGYSHR VPVPDVSVVDLTVK	2	4	19.267	0.0636

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				binding									
A0A2B4RPD3	Endoplasmin	Heat shock protein 90 family	Heat shock proteins	unfolded protein binding, ATP binding	protein folding, stress response	*Stylophora pistillata*	Coral	98.525	ELISNSSDALDKIR GVVDSDDLPLNVSR VLFETATLR	1	4.3	5.6868	0.0609
A0A0C2M5P0	Outer membrane protein A (OmpA)	PsbO family	Toxin	Porin activity	ion transport	*Thelohanellus kitauei*	Myxozoa	38.15	SDVLFNFNK	1	2.5	5.9302	0.0584
A0A0C2N4J3	Cleavage stimulation factor 50 kDa subunit	CstF1	cleavage stimulation factor	forming the mature 3' end of an mRNA molecule	mRNA 3'-end processing	*Thelohanellus kitauei*	Myxozoa	35.818	MTSDILNER	1	2.8	6.1197	0.0581
A0A7M5UEI8	ATP synthase subunit alpha	ATPase alpha/beta chains family	Oxido-reductive	Produces ATP from ADP in the presence of a proton gradient across the membrane	ATP synthesis coupled proton transport	*Clytia hemisphaerica*	Hydromedusan	59.1	AVDSLVPIGR EAYPGDVFYLHSR GIRPAINVGLSVSR GMALNLEPDNVGVVVFGNDK HALIIYDDLSK STVAQLVK TAIAIDTIINQK TSVKEPMLTGIK VVDALGNPIDGK	1	20.4	5.7641	0.0575
A0A6P8IZ04	Superoxide dismutase [Cu-Zn]	Copper/zinc superoxide dismutase	Oxido-reductive	metal ion binding, superoxide dismutase activity	cellular response to oxidative stress, response to inorganic substance, cellular response to oxygen-containing compound, cellular metabolic process, response to toxic substance	*Actinia tenebrosa*	Sea anemone	15.66	GGHELSLTTGNAGGR	1	9.9	79.776	0.0559
A0A6S7HIX8	Ras-related rab7	Ras family	Miscellaneous	GTPase activity, GTP binding	protein transport	*Paramuricea clavata*	Sea-whip	23.04	DPENFPFVVLGNKVDLENR FQSLGVAFYR LVTMQIWDTAGQER TLDSWRDEFLIQASPR TSLMNQYVNK TSLMNQYVNKK	6	34.1	112.89	0.0554
T2M644	Microtubule-associated proteins 1A/1B light chain 3C	ATG8 family	non-motor microtubule binding protein	ubiquitin protein ligase binding	autophagosome assembly cellular response to starvation autophagy of mitochondrion proteolysis	*Hydra vulgaris*	Hydra	14.534	FLVPQELTMSQFVTIIR MSLAPTQSFYLIVNNK RDEVAGIR TKFLVPQELTMSQFVTIIR	4	34.4	60.593	0.0549
W0K4S7	TX-like toxin (Fragment)	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Chironex fleckeri*	Box jellyfish	32.129	ETLLTQLILFTK FENYANGISASIDANK SIVDEAFKR SSSEINAEIDGLIQQLTTVNADTK	4	20.6	120.88	0.0512
T2MEK8	Ras-related protein Rab-1A	Ras family	Miscellaneous	GTPase activity, GTP binding	membrane trafficking control	*Hydra vulgaris*	Hydra	22.496	FADDTYTESYISTIGVDFKLLLIGDSGVGKMNPE YDYLFKNATNVEQAFMTMAAEIK	4	28.2	104.96	0.0502
A0A2B4SV07	Tubulin beta chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Stylophora pistillata*	Coral	50.114	ALTVPELTQQMFDAK EEYPDRIMNTFSVVPSPK EIVHLQAGQCGNQIGAK EVDEQMLNVQNK FPGQLNADLR FPGQLNADLRK FWEVISDEHGIDPTGTYHGDSDLQLER GHYTEGAELVDSVLDVVR GHYTEGAELVDSVLDVVRK IMNTFSVVPSPK INVYYNEATGGK KLAVNMVPFPR LAVNMVPFPR LHFFMPGFAPLTSR LTTPTYGDLNHLVSATMSGVTTCLR MREIVHLQAGQCGNQIGAK MSMKEVDEQMLNVQNK MSSTFIGNSTAIQELFK MSSTFIGNSTAIQELFKR	2	58.5	38.043	0.0496

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									NMMAACDPR NSSYFVEWIPNNVK SGPFGQIFRPDNFVFGQSGAGNNWAK TAVCDIPPR				
T2MHI3	Flotillin-1 (Fragment)	Flotillin family	Cytoskeleton Component	protease binding	membrane trafficking, phagocytosis, and epidermal growth factor receptor signaling	*Hydra vulgaris*	Hydra	49.104	AIMGTMTVEEIYQDR AIMGTMTVEEIYQDRK VILEAEAEAESIR	3	6.5	29.176	0.0492
A0A6P8IV21	ATP synthase subunit beta	ATPase family	Oxido-reductive	Translocase, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis, Hydrogen ion transport, Ion transport	*Actinia tenebrosa*	Sea anemone	59.552	AHGGYSVFAGVGER FTQAGSEVSALLGR GIAELGIYPAVDPLDSISR IPSAVGYQPTLATDMGTMQER SLQDIIAILGMDELSEDDK TVLIMELINNVAK	1	18	7.705	0.0490
A0A6S7I385	Ras-related protein Rab-14	Ras family	Miscellaneous	GTPase activity, GTP binding	regulators of intracellular membrane trafficking	*Paramuricea clavata*	Sea-whip	24.185	GAAGALMVYDITR STYNHLSSWLTDAR TGENVEEAFLETAK TGENVEEAFLETAKK	4	19.4	31.273	0.0486
T2MG36	Myosin-10	Myosin family	Cytoskeleton Component	actin filament binding, ATP binding, motor activity	organelle transport, morphogenesis and functioning of the cell	*Hydra vulgaris*	Hydra	224.54	IQQLSAIR LEVNMQAQK LRLEVNMQAQK RIQQLSAIR VIQYLTAIAGR	3	1.6	18.181	0.0483
A0A7M5X8H3	PHB domain-containing protein	Band 7/mec-2 family	Cytoskeleton Component	ubiquitin protein ligase binding	transcription by RNA polymerase II, cellular response to stress, positive regulation of transcription by RNA polymerase II, signal transduction	*Clytia hemisphaerica*	Hydromedusan	36.213	SVQTTLQTDEVK	1	3.8	7.5469	0.0473
A0A0C2MW98	Carboxypeptidase D	Peptidase family	Metallopeptidase	metallocarboxypeptidase activity, zinc ion binding	peptide metabolic process, proteolysis	*Thelohanellus kitauei*	Myxozoa	22.589	LNANGVDLNR	1	5.1	6.5614	0.0465
A0A2B4RVA0	Tubulin alpha chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Stylophora pistillata*	Coral	50.114	AFVHWYVGEGMEEGEFSEAR AFVHWYVGEGMEEGEFSEAREDLAALEK EDAANNYAR LDHKFDLMYAK LIGQIVSSITASLR NLDIERPTYSNLNR RAFVHWYVGEGMEEGEFSEAR RTIQFVDWCPTGFK TIQFVDWCPTGFK VGINYQPPTVVPGGDLAK	1	24.2	20.624	0.0463
A0A2B4R9T9	Chaperone protein DnaK	Heat shock protein 70 family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding, stress response	*Stylophora pistillata*	Coral	79.179	IINEPTAAALAYGIDKK	1	2.3	7.6965	0.0448
A7SQP1	40S ribosomal protein S13	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Nematostella vectensis*	Sea anemone	17.134	GLTPSQIGVILR KGLTPSQIGVILR	2	8.6	18.348	0.0437
P62184	Calmodulin	EF-hand	Signalling protein	calcium ion binding	calcium-mediated signaling	*Renilla reniformis*	Sea pansy	16.839	ADQLTEEQIAEFK EADIDGDGQVNYEEFVK EAFSLFDKDGDGTITTK SLGQNPTEAELQDMINEVDADGDGTIDFPEFLT MMAR VFDKDGDGFISAAELR	5	67.1	82.955	0.0408
A0A6S7HAR0	Vesicle-fusing ATPase	ATPase family	Miscellaneous	ATP binding, ATP hydrolysis activity	involved in cellular and subcellular movement	*Paramuricea clavata*	Sea-whip	89.77	ELQELVQYPVEHPEK EVDIGIPDATGR IVSQLLTLMDGLK KYEMFAQTLQQSR LDQLIYIPLPDEPSR LIVDEAVNDDNSVVTLSQAK MDELQLFR	7	11.8	70.652	0.0406
Q6VQ14	Dickkopf-3	Dickkopf-like	Signalling protein	signaling receptor binding	negative regulation of canonical	*Hydra vulgaris*	Hydra	21.221	AGVSEGQPGTFCDR	4	25	39.374	0.0406

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	related protein	protein family		receptor regulator activity signaling receptor activity	Wnt signaling pathway canonical Wnt signaling pathway				AGVSEGQPGTFCDRHEDCAGEGK MLAPGYYSQECNAHK NVYVGAQVQK				
A0A0C2MT43	HSP 90-alpha 1	Heat shock protein 90 family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding, stress response	*Thelohanellus kitauei*	Myxozoa	82.08	ELLSNSSDALDK	1	1.7	7.5403	0.0399
T2MEH6	Dynein light chain Tctex-type 1	dynein light chain family	Enzymes	acts as the force generating protein of eukaryotic cilia and flagella	microtubule-based process	*Hydra vulgaris*	Hydra	12.487	ESIEGAIGGNAYQHNK VNQWTSSVVEQCLNQLTK	2	30.1	31.916	0.0399
Q6PTK5	S-adenosylmethionine synthase	AdoMet synthase family	Enzymes	ATP binding, metal ion binding, methionine adenosyltransferase activity	one-carbon metabolic process, S-adenosylmethionine biosynthetic process	*Obelia sp.*	Hydrozoan	34.832	IIVDTYGGWGAHGGGAFSGKKIIVDTYGGWGA HGGGAFSGKSLVAAGLAR	3	9.3	17.798	0.0386
B5MG02	Beta tubulin	Tubulin	Cytoskeleton Component	GTP binding, structural constituent of cytoskeleton	microtubule-based process	*Cirrhipathes sp.*	Coral	16.158	EEYPDRIMNTFSVVPSPK IMNTFSVVPSPK KLAVNMVPFPR LAVNMVPFPR LHFFMPGFAPLTSR LREEYPDR LTTPTYGDLNHLVSATMSGVTTCLR	1	48.6	7.9334	0.0381
A0A7M5UKE9	GOLD domain-containing protein	EMP24/GP25L family	Cytoskeleton Component	assembly of membrane-associated complexes, regulating assembly of cargo into membranous vesicles	endoplasmic reticulum to Golgi vesicle-mediated transport, intracellular protein transport, Golgi organization	*Clytia hemisphaerica*	Hydromedusan	25.117	DKLTELQLR QLLDQIDQITK	2	9.3	14.775	0.0380
Q0H8V3	Heat shock protein 70 (Fragment)	Heat shock protein 70 family	Heat shock proteins	ATP binding	stress response	*Myxobolus squamalis*	Myxozoa	28.063	IINEPTAAALAYGLDK STAGDTHLGGEDFDNR	1	12.5	7.2087	0.0374
T2MHE8	Aspartate-tRNA ligase	Class-II aminoacyl-tRNA synthetase family	Enzymes	catalytic activity, acting on a tRNA RNA binding, ligase activity	tRNA metabolic process, translational elongation, cellular amino acid metabolic process	*Hydra vulgaris*	Hydra	64.623	GEEILSGAQR IISAASEGGANVFK	2	4.2	14.545	0.0368
A0A6P8HKX7	T-complex protein 1 subunit zeta-like	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding	*Actinia tenebrosa*	Sea anemone	57.775	DGNVLLHEMQIQHPTASLIAR VATAQDDITGDGTTSNVLIIGELLK	2	8.7	29.764	0.0363
A0A3M6V529	Ribosomal_L23eN domain-containing protein	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Pocillopora damicornis*	Coral	19.313	LAPDYDALDVANK	1	7.5	6.4591	0.0363
A7RIF0	T-complex protein 1 subunit eta	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding	*Nematostella vectensis*	Sea anemone	59.602	GGAEQFMEETER NDAVVAGGGAIEMELSK TFSYAGFEMQPK	3	7.5	21.162	0.0363
T2MES1	40S ribosomal protein S15a	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Hydra vulgaris*	Hydra	14.89	HGYIGEFEIIDDHR	1	10.8	16.51	0.0362
A0A6S7H9G6	78 kDa glucose-regulated	Heat shock protein 70 family	Heat shock proteins	ATP binding	stress response	*Paramuricea clavata*	Sea-whip	87.458	ITITNDQNR NELESYAYSLK VEIIANDQGNR	2	4	14.338	0.0354
A0A3M6TL43	AAA domain-containing protein	AAA ATPase family	miscellaneous	ATP binding, proteasome-activating activity	proteolysis, macromolecule catabolic process, response to stimulus	*Pocillopora damicornis*	Coral	47.184	FSEGTSADREIQR IIMATNRPDTLDPALLRPGR TLMELLNQMDGFDALGQVK VALDMTTLTIMR	4	15.4	24.619	0.0354
A0A2B4SDD9	Transient receptor potential cation channel subfamily	Ankyrin repeat family	Receptor	ion channel activity	protein folding process	*Stylophora pistillata*	Coral	127.15	GAIVELLLNR	1	0.9	6.305	0.0352

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	A member 1												
A0A6S7HLY3	Leucine-rich repeat and coiled-coil domain-containing 1	leucine-rich repeat		protein binding	cell cycle, cell division	*Paramuricea clavata*	Sea-whip	118.45	ITVVEGLESLR	1	1.1	5.8908	0.0350
T2MH20	Glutamate dehydrogenase	Glu/Leu/Phe/Val dehydrogenases family	Oxido-reductive	glutamate dehydrogenase (NAD+/NADP+) activity	cellular amino acid metabolic process	*Hydra vulgaris*	Hydra	59.53	DIVHSGLEFTMER IIAEGANGPTTPGADK	2	5.4	19.885	0.0349
A0A6S7H484	Ras-related Rab-5C	Ras family	Miscellaneous	GTPase activity, GTP binding	protein transport	*Paramuricea clavata*	Sea-whip	31.498	TAMNVNEIFLAIAK YHSLAPMYYR	2	8.5	12.837	0.0349
T2MDB0	RNA helicase	Helicases	Enzymes	RNA helicase activity, hydrolase activity, ATP binding, translation initiation factor activity	Protein biosynthesis	*Hydra vulgaris*	Hydra	46.404	GYDVIAQAQSGTGK VLITTDLLAR	1	5.9	15.658	0.0341
A7RME1	Adenosylhomocysteinase	Adenosylhomocysteinase family	Enzymes	hydrolase activity, adenosylhomocysteinase activity	one-carbon metabolic process, organic substance metabolic process, sulfur compound metabolic process	*Nematostella vectensis*	Sea anemone	47.941	ESLVDGLKR	1	2.1	5.8888	0.0321
A0A6P8HXS0	Actin	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Actinia tenebrosa*	Sea anemone	22.874	AGFAGDDAPR AVFPSIVGR AVFPSIVGRPR DSYVGDEAQSK DSYVGDEAQSKR HQGVMVGMGQK HQGVMVGMGQKDSYVGDEAQSK HQGVMVGMGQKDSYVGDEAQSKR IWHHTFYNELR TAPEEHPILLTEAPLNPK YPIEHGIVTNWDDMEK YSVWIGGSILASLSTFQQMWISK	1	54.4	5.9126	0.0319
Q9Y206	G protein a subunit 4 (Fragment)	G-protein family		G-protein beta/gamma-subunit complex binding, GTPase activity, GTP binding	adenylate cyclase-modulating G protein-coupled receptor signaling pathway, cell communication, regulation of biological process, response to stimulus	*Hydra vulgaris*	Hydra	34.701	IGAPDYVPTQQDVLR TTGIVETHFVFK	2	9	12.377	0.0317
A0A7D9D6W9	Endothelin-converting enzyme 1-like	Zinc Metalloprotease	Metalloprotease	metal ion binding, metalloendopeptidase activity	involved in many physiological and pathological processes, including blood pressure and inflammatory response	*Paramuricea clavata*	Sea-whip	67.458	ITLGENIADNGGTK	1	2.4	15.51	0.0313
A0A7M5WSW0	26S proteasome non-ATPase regulatory subunit 6	Proteasome family	Proteases	enzyme regulator activity	proteasome-mediated ubiquitin-dependent protein catabolic process	*Clytia hemisphaerica*	Hydromedusan	45.443	VGGIVETNRPDSK	1	3.3	6.974	0.0313
A0A6S7GLJ8	ADP/ATP translocase	ADP,ATP carrier protein	Transfer/carrier protein	ATP:ADP antiporter activity	mitochondrial ADP/ATP transmembrane transport	*Paramuricea clavata*	Sea-whip	31.55	GVAGAGVLAGFDK GVAGAGVLAGFDKFK	2	5.2	11.691	0.0309
A0A516MVE8	Cytochrome c oxidase subunit 2	Cytochrome c oxidase subunit 2 family	Oxido-reductive	copper ion binding, transporter activity, cytochrome-c oxidase activity	Electron transport, Respiratory chain	*Halipteris finmarchica*	Coral	28.281	VLVTGADVLHSFALPSLGIK	1	8	23.328	0.0302
T2M913	Programmed cell death 6-interacting protein	Vacuolar protein-sorting protein Bro1-like family	Cytoskeleton Component (membrane traffic protein)	sorting of cargo proteins of the multivesicular body (MVB) for incorporation into intralumenal vesicles	multivesicular body sorting pathway	*Hydra vulgaris*	Hydra	96.133	LPIAEDQIR	1	1.1	5.7578	0.0300
A0A7M5WK83	Acyl-coenzyme A oxidase	Acyl-CoA oxidase family	Oxido-reductive	oxidoreductase activity, acting on the CH-CH group of donors, flavin	lipid homeostasis, fatty acid beta-oxidation	*Clytia hemisphaerica*	Hydromedusan	75.095	MISNIK	1	0.9	6.069	0.0299

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				adenine dinucleotide binding, lipid binding, carboxylic acid binding									
T2MHZ3	Ornithine-oxo-acid aminotransferase	ATCase/OTCase family	Transferase	amino acid binding, carboxyl- or carbamoyltransferase activity	urea cycle, cellular amino acid metabolic process	*Hydra vulgaris*	Hydra	48.105	DNGLLAKPTHGDIIR LRDNGLLAKPTHGDIIR	2	3.9	12.469	0.0290
A0A6S7IPB5	Ribosomal protein L19	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Paramuricea clavata*	Sea-whip	22.569	LLSDQAEAR	1	4.7	5.9288	0.0289
T2MEY3	CCT-beta	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding	protein folding	*Hydra vulgaris*	Hydra	60.015	GATQQILDEAER LKGSGNLDAIQIIK	2	4.7	12.174	0.0285
T2MHI5	Alpha-actinin-1	Aequorin family	Cytoskeleton Component	actin binding, calcium ion binding	bioluminescence	*Hydra vulgaris*	Hydra	101.47	EGLLLWCQR EMADTDSAEQVMESFR ILAGDKPYITADELRR NVNVQNFHMSFK	4	6	39.677	0.0281
T2MEN5	Clathrin heavy chain	Clathrin	Cytoplasmic protein	polymerizes into a polyhedral lattice on intracellular membranes to form protein-coated membrane vesicles	intracellular protein transport, vesicle-mediated transport	*Hydra vulgaris*	Hydra	192.02	IVLENSVFSDHR IYIDANNNPER TLQIFNIEMKVVGAMQLYSIER	4	2.7	39.547	0.0280
T2MGD9	Proteasome subunit beta	Peptidase family	Enzymes	peptidase activity	proteolysis, macromolecule catabolic process	*Hydra vulgaris*	Hydra	31.69	HGVIVAVDSR LLANMVYQYK	2	7	12.386	0.0272
T2MFG0	Transmembrane emp24 domain-containing protein 10	EMP24/GP25L family	Cytoskeleton Component	assembly of membrane-associated complexes, regulating assembly of cargo into membranous vesicles	endoplasmic reticulum to Golgi vesicle-mediated transport, intracellular protein transport, Golgi organization	*Hydra vulgaris*	Hydra	26.178	EEVHKDVLVTGEYR	1	6.2	10.663	0.0269
A0A7M5VCV6	3-hydroxyisobutyrate dehydrogenase	HIBADH-related family	Oxido-reductive	3-hydroxyisobutyrate dehydrogenase activity, NAD/NADH binding	cellular amino acid metabolic process, branched-chain amino acid catabolic process, valine catabolic process	*Clytia hemisphaerica*	Hydromedusan	30.352	DLGLAQNASTATK	1	4.6	8.0621	0.0268
A0A7M5X2E8	Fumarylacetoacetase	FAH family	Enzymes	fumarylacetoacetase activity, metal ion binding	L-phenylalanine catabolic process, tyrosine catabolic process	*Clytia hemisphaerica*	Hydromedusan	46.091	ASSVIVSGTPVR DHATNVGTMFR	2	5.5	11.149	0.0266
A0A7M5V1M4	AAA domain-containing protein	AAA ATPase family	miscellaneous	ATP binding, proteasome-activating activity	proteolysis, macromolecule catabolic process, response to stimulus	*Clytia hemisphaerica*	Hydromedusan	46.579	ADTLDPALLRPGR EAVELPLTHFELYK EYLHAQEEVKR MNLSDEVDLEDYVARPDK RFDAQTGADREVQR	5	16.9	39.31	0.0262
A0A3M6TH93	Peptidase A1 domain-containing protein	Peptidase family	Enzymes	aspartic-type endopeptidase activity	play an important role in the host invasion process	*Pocillopora damicornis*	Coral	42.443	VVFDTGSSNLWVPSKK	1	4.1	7.1932	0.0254
A0A6S7LBX5	Cytosolic 10-formyltetrahydrofolate dehydrogenase-like	Aldehyde dehydrogenase family	Oxido-reductive	oxidoreductase activity, detoxification of exogenously and endogenously generated aldehydes	intermediate structures can be toxic, and health problems arise when those intermediates cannot be cleared. (facial flushing, lightheadedness, palpitations, nausea)	*Paramuricea clavata*	Sea-whip	24.415	MAPLLAAGNTVVLKPAQVTPMTALK	1	11.2	9.9495	0.0245
A0A7M5X6I9	Rab GDP dissociation inhibitor	Rab GDI family	G-protein	enzyme regulator activity	protein transport, cell communication, regulation of biological process, response to stimulus	*Clytia hemisphaerica*	Hydromedusan	50.026	EALASSLMGIFEK GRDWNVDLIPK SPYLYPLYGLGELPQGFAR	3	9.7	27.808	0.0238
A0A6S7H750	Prohibitin	Prohibitin family	Regulatory protein	DNA synthesis inhibiting	regulation of cell proliferation	*Paramuricea clavata*	Sea-whip	16.365	AIVAQFDASELITQR DLQNVNITLR	2	16.9	16.365	0.0238
A0A2B4RAR4	60S ribosomal protein L4	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	Protein biosynthesis (translation)	*Stylophora pistillata*	Coral	38.808	LAGHQTSAESWGTGR	1	4.3	26.353	0.0237
A0A6S7GI07	PHB domain-containing protein	Band 7/SPFH domain	Cytoskeleton Component	unknown	protein localization to plasma membrane	*Paramuricea clavata*	Sea-whip	46.384	AILGTLTVEEIYK AILGTLTVEEIYKDR DADIGVAEANR IAAEVAAPLAK	5	11.2	46.827	0.0237

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									MGIEILSFTIK				
T2MFY6	Hippocalcin-like protein 1	Aequorin family	Photoprotein	calcium ion binding, intracellular Ca2+ indicator	bioluminescence	*Hydra vulgaris*	Hydra	21.897	IYGNFFPYGDAAK LSLAEFIEGAK	2	12.6	11.637	0.0236
A0A7D9IWZ5	Ras-related Rab-10-like	Ras family	Miscellaneous	GTPase activity, GTP binding	regulators of intracellular membrane trafficking	*Paramuricea clavata*	Sea-whip	11.25	FHTITTSYYR LLLIGDSGVGK	1	21.2	10.726	0.0231
A0A7M5X1E6	HATPase_c domain-containing protein	Heat shock protein 90 family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding, stress response	*Clytia hemisphaerica*	Hydromedusan	82.627	ADLVNNLGTIAK APFDLFENKK DIYYITGESK ELISNSSDALDKIR GVVDSEDLPLNISR RAPFDLFENKK	1	8.5	8.1284	0.0225
A0A2B4RHG2	H(+)-transporting two-sector ATPase	ATPase family	Oxido-reductive	Translocase, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis, Hydrogen ion transport, Ion transport	*Stylophora pistillata*	Coral	68.375	DMGYNVSMMADSTSR GNEMSEVLR GSLAEADKITLEVAK LAEMPADSGYPAYLGAR RTALVANTSNMPVAAR TALVANTSNMPVAAR VGHSELVGEIIR	3	13.6	18.847	0.0225
T2MEV1	Tubulin beta chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Hydra vulgaris*	Hydra	50.296	ALTVPELTQQMFDSKAVLVDLEPGTMDSVREE YPDRIMNTFSVVPSPKEVDEQMLNVQNKFPGQL NADLRFPGQLNADLRKIMNTFSVVPSPKKLAVN MVPFPRLAVNMVPFPRLHFFMPGFAPLTSRMRE IVHVQAGQCGNQIGAKMSATFIGNSTAIQELFK MSATFIGNSTAIQELFKRMSMKEVDEQMLNVQ NKNMMAACDPRNSSYFVEWIPNNVKTAVCDIP PRYLTVAAMFR	2	39.3	13.905	0.0222
A0A7M5XHZ1	Calcium-transporting ATPase	Cation transport ATPase family	transport protein	ATP binding, P-type calcium transporter activity	Calcium transport, ion transport	*Clytia hemisphaerica*	Hydromedusan	123.97	ADVGFAMGIQGTDVAK EIVAVTGDGTNDGPALK EIVAVTGDGTNDGPALKK KADVGFAMGIQGTDVAK	4	3	51.329	0.0216
T2M830	Pyrroline-5-carboxylate reductase	Pyrroline-5-carboxylate reductase family	Oxido-reductive	pyrroline-5-carboxylate reductase activity	L-proline biosynthetic process	*Hydra vulgaris*	Hydra	32.565	LMTNTPVQYR	1	3.3	6.6201	0.0216
A0A2B4RI99	PC3-like endoprotease variant B	Peptidase family	Miscellaneous	serine-type endopeptidase activity	Probably involved in the processing of hormone and other protein precursors at sites comprised of pairs of basic amino acid residues.	*Stylophora pistillata*	Coral	95.388	ASYDFNDMDADPKPR GDVSIDLFSPSGTR LAQEALMQGALK RGDVSIDLFSPSGTR	4	4.9	28.235	0.0213
A7SMZ7	Dynein light chain roadblock	dynein light chain family	Enzymes	acts as the force generating protein of eukaryotic cilia and flagella	microtubule-based process	*Nematostella vectensis*	Sea anemone	10.835	DIDPQNDLTFLR SEVEETLKR	2	21.9	14.751	0.0213
T2M388	Calcium/calmodulin-dependent protein kinase	Protein kinase superfamily	Enzymes	ATP binding, calmodulin binding, calmodulin-dependent protein kinase activity, protein serine kinase activity, protein threonine kinase activity	protein phosphorylation	*Hydra vulgaris*	Hydra	54.309	DLKPENLLLASK GAILTTVFAR	2	4.6	14.093	0.0213
A0A7D9DUK8	60 kDa heat shock	Heat shock protein 60 family	Heat shock proteins	ATP binding, Chaperone	protein folding	*Paramuricea clavata*	Sea-whip	61.214	GYISPYFINTAK VGGSSEVEVNEK VGGSSEVEVNEKK VTDALNATR	1	6	8.7908	0.0208
A0A3M6UET3	Dynein light chain	dynein light chain family	Enzymes	acts as the force generating protein of eukaryotic cilia and flagella	microtubule-based process	*Pocillopora damicornis*	Coral	10.307	FNIEKDIAAYIK NFGSYVTHETK YNPTWHCIVGR	3	38.2	18.474	0.0204
A0A6S7FCE1	WD repeat-containing 27-like	WD40-repeat	Signalling protein	protein binding	transduction and transcription regulation to cell cycle control and apoptosis	*Paramuricea clavata*	Sea-whip	126.57	KLEQEIEIGR	1	0.9	5.9177	0.0202

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A0A6G3MHL3	40S ribosomal protein S14	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Henneguya salminicola*	Myxozoa	16.15	ADRDESSPYAAMLAAQDVAAR DESSPYAAMLAAQDVAAR IEDVTPIPSDSTR	2	22.5	15.161	0.0202
A0A2B4RKT3	26S proteasome non-ATPase regulatory subunit 2	Proteasome family	Multiprotein complex	enzyme regulator activity	protein catabolic process, regulation of biological process including cell cycle progression, apoptosis, or DNA damage repair.	*Stylophora pistillata*	Coral	100.69	ASTSSMTSVPKPLK VGQAVDVVGQAGKPK	2	3.2	12.732	0.0199
A0A7M5X4L7	SAM domain-containing protein	SAM (sterile alpha motif) domain	Signalling protein	protein binding	Phosphatidylinositol signaling system	*Clytia hemisphaerica*	Hydromedusan	30.219	VTGQGVFYR	1	3.4	6.7571	0.0197
A0A0C2IX80	Actin-3	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Thelohanellus kitauei*	Myxozoa	41.88	AGFAGDDAPR AVFPSIVGR AVFPSIVGRPR DLTDYLMKILTER DSYVGDEAQSK DSYVGDEAQSKR EKMTQIMFETFNSPAMYVAIQAVLSLYASGR GYSFTTTAER;HQGVMVGMGQK HQGVMVGMGQKDSYVGDEAQSK HQGVMVGMGQKDSYVGDEAQSKR IAPEEHPVLLTEAPLNPK IWHHTFYNELR KYSVWIGGSILASLSTFQQMWISK MTQIMFETFNSPAMYVAIQAVLSLYASGR SYELPDGQVITIGNER YPIEHGIVTNWDDMEK YSVWIGGSILASLSTFQQMWISK	1	48.7	6.0739	0.0190
A7RV73	Vacuolar protein sorting-associated protein 29	VPS29 family	vesicle coat protein	Acts as component of the retromer cargo-selective complex (CSC). The CSC is believed to be the core functional component of retromer or respective retromer complex variants acting to prevent missorting of selected transmembrane cargo proteins into the lysosomal degradation pathway.	intracellular protein transport, retrograde transport, endosome to Golgi	*Nematostella vectensis*	Sea anemone	20.713	GDFDENLSYPEQK	1	7.1	6.6078	0.0187
Q967E1	Heat shock protein 90	Heat shock protein 90 family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding, stress response	*Dendronephthya klunzingeri*	Soft coral	84.318	ADLVNNLGTIAKDIYYITGESKEGLELPEDEEEK EGLELPEDEEEKKELISNSSDALDKIRGVVDSED LPLNISR	2	8.6	13.159	0.0187
A0A7M5V8K6	Tubulin alpha chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Clytia hemisphaerica*	Hydromedusan	50.396	AFVHWYVGEGMEEGEFSEAR EDAANNYAR LDHKFDLMYSK NLDIERPSYTNLNR QLFHPEQLITGK QLFHPEQLITGKEDAANNYAR RAFVHWYVGEGMEEGEFSEAR RTIQFVDWCPTGFK TIQFVDWCPTGFK YMACCLLYR	1	20	5.8338	0.0186
A0A6P8H2U9	EH domain-containing protein 3-like	EH domain	Cytoskeleton Component (membrane traffic protein)	ATP binding, GTP binding, calcium ion binding	cellular localization, vesicle-mediated transport, endocytic recycling	*Actinia tenebrosa*	Sea anemone	61.866	LDISDEFQR SLSSFGNAFLNR	2	3.9	15.424	0.0180
A0A6P8HRF6	Electron transfer flavoprotein subunit beta	EFT family	Oxido-reductive	electron transfer activity	Electron transport	*Actinia tenebrosa*	Sea anemone	27.973	EIDGGLETINVK	1	4.7	5.7883	0.0175

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A0A6P8HBY3	Gamma-cystathionase	PLP-dependent transferases	Toxin	cystathionine gamma-lyase activity, L-cysteine desulfhydrase activity, L-cystine L-cysteine-lyase, pyridoxal phosphate binding	amino-acid biosynthesis, transsulfuration pathway , In human - Abnormality of serum amino acid level	*Actinia tenebrosa*	Sea anemone	37.646	LGISDTLIR	1	2.6	6.5684	0.0171
A0A7M5XH86	Annexin	Annexin family		calcium-dependent phospholipid binding, calcium ion binding	Phospholipid metabolism and transport	*Clytia hemisphaerica*	Hydromedusan	58.72	FNVILASR	1	1.5	5.7339	0.0171
A7SFJ3	26S proteasome regulatory subunit 7	AAA ATPase family	Regulatory protein	ATP binding, proteasome-activating activity, TBP-class protein binding	positive regulation of RNA polymerase II transcription preinitiation complex assembly, RNA polymerase II preinitiation complex assembly	*Nematostella vectensis*	Sea anemone	48.455	FDDGAGGDNEVQR FVVDLGDQVAPTDIEEGMR	2	7.4	41.323	0.0168
A0A6P8IXQ3	golgin subfamily B member 1-like isoform X2	Golgin family	Cytoskeleton Component	RNA binding, sequence-specific DNA binding	Golgi organization, protein localization to pericentriolar material, regulation of transcription, DNA-templated	*Actinia tenebrosa*	Sea anemone	636.89	AEQLEQELKK	1	0.2	6.0525	0.0167
T2MEG5	Casein kinase II subunit beta	Casein kinase 2 subunit beta family	kinase modulator	kinase activity, protein kinase regulator activity	Core component of nucleosome	*Hydra vulgaris*	Hydra	26.037	IHPMSYQIQAQAAANAR	1	7.6	11.255	0.0167
A0A6P8IDS4	argininosuccinate lyase-like	Fumarate lyase family	Enzymes	argininosuccinate lyase activity	cellular amino acid metabolic process	*Actinia tenebrosa*	Sea anemone	52.685	VNVLPLGSGALAGNPFGIDR	1	4.3	16.541	0.0165
D1FNZ2	40S ribosomal protein S4-like protein	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Carukia barnesi*	Box jellyfish	25.638	HPGSYDIVHIK	1	4.8	6.42	0.0164
A0A7M5UGB8	RNA helicase	Helicases	Enzymes	RNA helicase activity, hydrolase activity, ATP binding, translation initiation factor activity	Protein biosynthesis	*Clytia hemisphaerica*	Hydromedusan	46.611	GIYAYGFERPSAIQQR GRDVIAQAQSGTGK KLDYGQHIVSGTPGR	1	11.1	7.1591	0.0164
A0A2B4SYV9	Small nuclear ribonucleoprotein Sm D1	snRNP core protein family	Ribosomal protein	processing of pre-mRNAs to mature mRNAs, and are a major component of spliceosome	spliceosomal snRNP assembly, mRNA processing, mRNA splicing	*Stylophora pistillata*	Coral	13.181	LSHETVTIELK	1	9.2	5.6831	0.0155
A0A7D9D9T9	Elongin-C	Elongin-C	Transcription factors	ubiquitin-protein transferase activity	positive regulation of transcription elongation from RNA polymerase II promoter, ubiquitin-dependent protein catabolic process, transcription elongation from RNA polymerase II promoter	*Paramuricea clavata*	Sea-whip	12.631	AMLSGPGQFSENETNEVHFR	1	17.7	55.996	0.0155
A0A6S7J7Q5	60S ribosomal L23a	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Paramuricea clavata*	Sea-whip	14.485	TNKLDQFAIIK	1	8.5	6.4673	0.0152
D1FX73	D-3-phosphoglycerate dehydrogenase	D-isomer specific 2-hydroxyacid dehydrogenase family	Oxido-reductive	NAD binding, phosphoglycerate dehydrogenase activity	cellular amino acid metabolic process, L-serine biosynthetic process	*Chironex fleckeri*	Box jellyfish	54.938	ADLTIKR;AGTGVDNIDLK FQGGNINLLNAPSVAK	3	6.5	30.117	0.0147
T2M7J1	Vacuolar protein sorting-associated protein 35 (Fragment)	VPS35 family	Cytoskeleton Component (membrane traffic protein)	Acts as component of the retromer cargo-selective complex (CSC). The CSC is believed to be the core functional component of retromer or respective retromer complex variants acting to prevent missorting of selected transmembrane cargo proteins into the lysosomal degradation pathway.	intracellular protein transport, retrograde transport, endosome to Golgi	*Hydra vulgaris*	Hydra	92.3	LFEIFSQQVAHVIQSR LFLQGALAADQQR VLEYTEEIFSK	3	5	19.064	0.0147

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A0A6B2G8U1	V-type proton ATPase subunit B, brain isoform	ATPase family	Oxido-reductive	ATP binding	ATP metabolic process, proton transmembrane transport	*Myxobolus squamalis*	Myxozoa	14.796	YAEIVTLTLPDGTVR	1	10.9	10.148	0.0147
A0A7D9M9T4	Tricarboxylate transport protein	Mitochondrial carrier family	transport protein	transfer molecules across the membranes of the mitochondria	Energy transport across the membranes of the mitochondria	*Paramuricea clavata*	Sea-whip	16.126	FIHDQTQPNPK GLSSLLYGSIPK	1	15.4	6.6974	0.0145
T2MEB6	26S protease regulatory subunit 8	ATPase family	Proteases	peptidase activity, proteasome-activating activity	macromolecule catabolic process, protein metabolic process	*Hydra vulgaris*	Hydra	48.337	EVIELPVKHPELFEALGIAQPKIAETMPGASGAEI KKIAETMPGASGAEIKTMLELLNQLDGFEPTQNI KVPDSTYEMIGGLDK	5	16.3	34.502	0.0143
A0A7M5V6M5	Eukaryotic translation initiation factor 3 subunit I	eIF-3 subunit I family	Translation factors	translation initiation factor activity	formation of cytoplasmic translation initiation complex, Protein biosynthesis	*Clytia hemisphaerica*	Hydromedusan	36.624	TERPVNSAAVSPIR	1	4.3	5.7725	0.0142
A0A7M5UYR8	CCT-alpha	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding	protein folding	*Clytia hemisphaerica*	Hydromedusan	59.354	EQLAIAEFANSLLVIPK FATEAAITILR ICDDELILIK LHPTSIISGYR MLVDDIGDVTITNDGATILK	5	12.5	56.179	0.0140
A0A7M5XC96	14_3_3 domain-containing protein	14-3-3 family	scaffold/adaptor protein	protein binding	stress response, regulation of signal transduction pathway	*Clytia hemisphaerica*	Hydromedusan	28.321	DSTLIMQLLR LGLALNYSVFHYEIK	1	10	8.1226	0.0138
A7S3G2	Tubulin alpha chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Nematostella vectensis*	Sea anemone	50.218	AFVHWYVGEGMEEGEFSEAR AFVHWYVGEGMEEGEFSEAREDLAALEK AVCMLSNTTAIAEAWAR AVFVDLEPTVVDEVR DVNAAIATIK EDAANNYAR FDGSLNVDLNEFQTNLVPYPR IHFPLATYAPVISAEK LDHKFDLMYAK NLDIERPSYTNLNR QLFHPEQLITGK QLFHPEQLITGKEDAANNYAR RAFVHWYVGEGMEEGEFSEAR RTIQFVDWCPTGFK TIQFVDWCPTGFK VGINYQPPTVVPGGDLAK	2	41.2	11.404	0.0137
A0A6S7GJD9	Pyridoxal 5-phosphate synthase subunit SNZ ERR-like	PdxS/SNZ family	Enzymes	catalytic activity	pyridoxal phosphate biosynthetic process	*Paramuricea clavata*	Sea-whip	34.315	TKGEAGTGDVAEAVR	1	4.7	5.7767	0.0137
T2MG46	CCT-epsilon	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding	protein folding	*Hydra vulgaris*	Hydra	55.285	KDVDFELIK KQQISLATQLVR	2	4.2	15.475	0.0136
A0A7M5UT44	MHD domain-containing protein	Adaptor complexes medium subunit family	Cytoskeleton Component (membrane traffic protein)	clathrin binding	intracellular protein transport, vesicle-mediated transport	*Clytia hemisphaerica*	Hydromedusan	48.47	ILQEFITQQSHK	1	2.9	5.9959	0.0136
A0A7M6DS06	40S ribosomal protein S9	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Clytia hemisphaerica*	Hydromedusan	26.248	KQLVNVPSFLVR LFEGNALLR QLVNVPSFLVR	2	9.2	12.906	0.0135
A0A3M6U8Q7	Electron transfer flavoprotein subunit alpha	EFT family	Oxido-reductive	electron transfer activity, flavin adenine dinucleotide binding	Electron transport, oxidation of fatty acids	*Pocillopora damicornis*	Coral	35.208	AAVDAGYVPNDMQVGQTGK DPEAPIFQVADYGLVADLFK TIVAINKDPEAPIFQVADYGLVADLFK	3	13.6	36.359	0.0134

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T1PQV6	Toxin CfTX-B precursor	cubozoan protein toxin (cpt) family	pore-forming toxin	toxin activity	hemolysis in other organism ion transport	*Chironex fleckeri*	Box jellyfish	51.116	ETLLTQLILFTK FENYANGISASIDANK SIVDEAFKR SWHNNPGLQGHWK	1	10.8	5.8486	0.0132
A0A3M6TYB6	Protein disulfide-isomerase	Thioredoxin family	Oxido-reductive	protein disulfide isomerase activity	response to oxidative stress	*Pocillopora damicornis*	Coral	57.901	MDSTANEVESVK	1	2.3	9.504	0.0131
A0A0C2JEY5	6-phosphogluconate dehydrogenase	6-phosphogluconate dehydrogenase family	Oxido-reductive	phosphogluconate dehydrogenase (decarboxylating) activity, NADP binding	pentose phosphate pathway, D-gluconate metabolic process	*Thelohanellus kitauei*	Myxozoa	50.35	DYFGAHTYKR SAVLPANLIQAQR	2	5	13.698	0.0128
P29146	PC3-like endoprotease variant A precursor	Peptidase family	Miscellaneous	serine-type endopeptidase activity	Probably involved in the processing of hormone and other protein precursors at sites comprised of pairs of basic amino acid residues.	*Hydra vulgaris*	Hydra	88.84	VQVNAGYENPR	1	1.4	8.2104	0.0127
A0A7M5XM42	26S proteasome non-ATPase regulatory subunit 8	Proteasome family	Proteases	maintenance of protein homeostasis by removing misfolded or damaged proteins	proteolysis, proteasome-mediated ubiquitin-dependent protein catabolic process	*Clytia hemisphaerica*	Hydromedusan	31.324	ELQENVYIR	1	3.3	5.8753	0.0125
D1FNZ5	Ferritin	Ferritin family	Oxido-reductive	ferric iron binding, ferroxidase activity	cellular iron ion homeostasis, iron ion transport	*Carukia barnesi*	Box jellyfish	19.447	VGPGLGEFQFDK	1	7.1	8.5634	0.0125
T2MEG1	Cell division control protein 42 homolog	Rho family	small GTPase	anion binding, carbohydrate derivative binding, purine nucleotide binding, GTPase activity	cell division, small GTPase mediated signal transduction	*Hydra vulgaris*	Hydra	21.158	NVFDEAILAALEPPEQPK TPFLLVGTQVDLR	2	16.2	18.781	0.0124
A0A7M5XLH2	HATPase_c domain-containing protein	Heat shock protein 90 family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding, stress response	*Clytia hemisphaerica*	Hydromedusan	94.51	ADKDNHVLHITDTGIGMTK ELISNSSDALDKIR	1	4	5.6484	0.0124
A7S494	40S ribosomal protein S27	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Nematostella vectensis*	Sea anemone	9.5893	LVQSPNSYFMDVK	1	15.5	6.4563	0.0123
A0A3M6UMV8	Aconitate hydratase	Aconitase	Catalyzes	4 iron, 4 sulfur cluster binding, aconitate hydratase activity, citrate dehydratase activity, metal ion binding	tricarboxylic acid cycle	*Pocillopora damicornis*	Coral	80.637	SLFTVTPGSEQIR VGLIGSCTNSSYEDMTR	2	4	21.987	0.0123
A0A7D9DZX8	Histone H3, partial	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Paramuricea clavata*	Sea-whip	97.851	EIAQDFKTDLRFQSSAVMALQEASEAYLVGLFE DTNLCAIHAKSTELLIRKYRPGTVALR	1	6.8	32.689	0.0122
T2ME39	Dynamin GTPase	Dynamin family	Cytoskeleton Component (membrane traffic protein)	microtubule binding GTPase activity	organelle fission	*Hydra vulgaris*	Hydra	93.953	GMEDLIPIVNK KFFLSHPAYR SSVLENFVGR	3	3.7	17.329	0.0122
T2M2W0	Transmembrane emp24 domain-containing protein 7	EMP24/GP25L family	Cytoskeleton Component	assembly of membrane-associated complexes, regulating assembly of cargo into membranous vesicles	endoplasmic reticulum to Golgi vesicle-mediated transport, intracellular protein transport, Golgi organization	*Hydra vulgaris*	Hydra	24.56	VIIDYQTHHR	1	4.8	6.7453	0.0119
A0A6P8I146	Vacuolar proton pump subunit B	ATPase family	Oxido-reductive	ATP binding	ATP metabolic process, proton transmembrane transport	*Actinia tenebrosa*	Sea anemone	55.195	AVVQVFEGTSGIDAK GFPGYMYTDLATIYER IPQSTLAEFYPR IYPEEMIQTGISAIDTMNSIAR TVSGVNGPLVILDNVK	2	16.2	15.159	0.0117
C1IS28	Clathrin heavy chain	Clathrin	Cytoplasmic protein	polymerizes into a polyhedral lattice on intracellular membranes to form protein-coated membrane vesicles	intracellular protein transport, vesicle-mediated transport	*Carukia barnesi*	Box jellyfish	31.75	IHDGSEEPATHNALAK IVLENSVFSDHR IYIDANNNPER	1	14	6.4339	0.0117
T2MHV0	Aldehyde dehydrogen	Aldehyde dehydrogenase	Oxido-reductive	oxidoreductase activity, acting on the aldehyde or	cellular amino acid catabolic process	*Hydra vulgaris*	Hydra	51.471	ELGEYGLQQYSEVK	1	3	27.065	0.0116

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	ase	family		oxo group of donors, NAD or NADP as acceptor									
T2M9U0	Coiled-coil domain-containing protein 13	CCDC13 family	Cytoskeleton Component	protein binding	ciliogenesis, genome stability, primary cilia formation	*Hydra vulgaris*	Hydra	66.394	SSFVTQIEEK	1	1.8	6.7435	0.0116
A0A3M6T8Y2	Histone H2A	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Pocillopora damicornis*	Coral	13.575	LLAGVTIAQGGVLPNIQAVLLPK NDEELNKLLAGVTIAQGGVLPNIQAVLLPK VGAGAPVYLAAVLEYLSAEILELAGNAAR	2	46.8	36.056	0.0116
A0A6P8J7X2	3-ketoacyl-CoA thiolase	Thiolase family	Enzymes	acyltransferase activity, transferring groups other than amino-acyl groups	fatty acid metabolic process	*Actinia tenebrosa*	Sea anemone	41.768	LVSYGIAGVEPSIMGIGPVPAIK	1	5.8	9.6712	0.0116
T2MHJ7	60 kDa heat shock protein	Heat shock protein 60 family	Heat shock proteins	ATP binding, Chaperone	protein folding	*Hydra vulgaris*	Hydra	62.435	GKNVLIEQSFGGPK GYISPYFINTAK LVQDVANNTNEEAGDGTTTATVLAR VTDALNATR	1	10.1	5.6993	0.0115
A0A7M6DKY4	HP domain-containing protein	Villin/gelsolin family	Cytoskeleton Component	protein binding, actin filament binding	organelle organization	*Clytia hemisphaerica*	Hydromedusan	93.026	AASLNSNDVFLMK	1	1.6	7.957	0.0115
A7SGX3	Histone H2B	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Nematostella vectensis*	Sea anemone	13.104	KESYLTYIYK	1	8.4	6.791	0.0113
A0A7M5VD94	Signal sequence receptor subunit gamma	TRAP-gamma family	Cytoskeleton Component	SRP-dependent cotranslational protein targeting to membrane	protein transport, cellular localization	*Clytia hemisphaerica*	Hydromedusan	20.883	LTQEEELLLQDFSR	1	7.6	48.838	0.0112
A0A7M5WQL4	Septin-type G domain-containing protein	Septin family	GTPase	GTP binding	cell cycle	*Clytia hemisphaerica*	Hydromedusan	40.522	EAELQRMQQMLEAMR	1	4.3	5.7905	0.0112
T2MF33	Delta-1-pyrroline-5-carboxylate synthase	Glutamate 5-kinase family	Enzymes	ATP binding, glutamate 5-kinase activity, glutamate-5-semialdehyde dehydrogenase activity	Amino-acid biosynthesis, Proline biosynthesis	*Hydra vulgaris*	Hydra	85.385	GPVGVEGLLTTK LRSELSMQQTMR	2	3.1	11.356	0.0112
A0A7M5TTE7	Glutamyl-tRNA synthetase	Prolyl-tRNA synthetase family	Enzymes	catalytic activity, acting on a tRNA, ligase activity	tRNA metabolic process, translational elongation, cellular amino acid metabolic process	*Clytia hemisphaerica*	Hydromedusan	150.79	AIQGATSHHLGQNFSK	1	1.2	5.8481	0.0111
A0A7M5WMC3	Sulfoacetaldehyde acetyltransferase	TPP enzyme family	Enzymes	magnesium ion binding, sulfoacetaldehyde acetyltransferase activity, thiamine pyrophosphate binding	organic acid metabolic process, sulfur compound metabolic process, xenobiotic metabolic process, nitrogen compound metabolic process	*Clytia hemisphaerica*	Hydromedusan	68.3	GPTQLNIPR	1	1.4	6.2496	0.0110
A0A6P8ID21	26S proteasome regulatory subunit 4	AAA ATPase family	Oxido-reductive	ATP binding, proteasome-activating activity	protein catabolic process	*Actinia tenebrosa*	Sea anemone	49.195	AVANQTSATFLR ESVELPLTHPELYEEMGIKPPK TMLELLNQLDGFDSK	3	11.1	42.63	0.0110
T2MFI6	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit; (Short=Oligosaccharyl transferase 48 kDa subunit)	DDOST 48 kDa subunit family	Transferase	dolichyl-diphosphooligosaccharide-protein glycotransferase activity, metal ion binding	protein glycosylation, protein N-linked glycosylation via asparagine	*Hydra vulgaris*	Hydra	48.658	TLVLLDNASIK	1	2.5	6.8071	0.0110
A0A6P8IS66	acetyl-CoA acetyltransferase	Thiolase family	Transferase	acyltransferase activity, transferring groups other than amino-acyl groups	Lipid metabolism	*Actinia tenebrosa*	Sea anemone	44.746	VNIHGGAVSLGHPIGMSGAR	1	4.8	6.5147	0.0106
T2M1Q9	NADH-	Complex I 49	Oxido-reductive	NAD binding,	Transport	*Hydra vulgaris*	Hydra	56.839	LLNIQIPER	2	4	12.408	0.0106

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	ubiquinone oxidoreductase 49 kDa subunit	kDa subunit family		oxidoreductase activity, acting on NAD(P)H, quinone binding					TYLQALPYFDR				
A0A6P8HPQ4	ADP-ribosylation factor-like protein 3	GTPase Family	Miscellaneous	GTPase activity, GTP binding	positive regulation of microtubule polymerization	*Actinia tenebrosa*	Sea anemone	20.453	ILLLGLDNAGK LNVWDIGGQR	2	11.6	16.99	0.0105
A0A2B4R9G5	Histone H2B	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Stylophora pistillata*	Coral	16.673	KESYSLYIYK RKESYSLYIYK	2	7.3	10.85	0.0103
T2MF68	Small nuclear ribonucleoprotein E	snRNP core protein family	Ribosomal protein	nucleic acid binding	RNA splicing, via transesterification reactions, protein-containing complex assembly	*Hydra vulgaris*	Hydra	10.263	VMVQPINLIFR	1	12.6	6.8975	0.0102
T2M8M2	V-type proton ATPase subunit a	ATPase family	Oxido-reductive	ATP binding	ATP metabolic process, proton transmembrane transport	*Hydra vulgaris*	Hydra	92.423	DLNPDVNAFQR EMAIGVMTR	2	2.5	11.753	0.0102
A0A3M6TK98	CCT-epsilon	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding	protein folding	*Pocillopora damicornis*	Coral	59.823	HKLDVTSVEDYQK KDVDFELIK	1	4	5.6459	0.0098
T2MGR5	60S ribosomal protein L7	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Hydra vulgaris*	Hydra	30.257	LPGVPETILK	1	3.8	6.0903	0.0098
A0A6B2GAS2	V-type proton ATPase subunit C	ATPase family	Oxido-reductive	ATP binding	ATP metabolic process, proton transmembrane transport	*Myxobolus squamalis*	Myxozoa	11.76	VFVESVLR	1	8.1	5.839	0.0096
A0A7M5UZ04	Proteasome subunit alpha type	Peptidase family	Enzymes	creating an electrochemical gradient over the inner membrane	proteolysis, macromolecule catabolic process	*Clytia hemisphaerica*	Hydromedusan	25.624	LVQIEYALAAVAAGGPSVGLK YSFSLTTFSPSGK	2	14.5	19.582	0.0095
T2MEP0	26S protease regulatory subunit 10B	ATPase family	Proteases	peptidase activity, proteasome-activating activity	macromolecule catabolic process, protein metabolic process	*Hydra vulgaris*	Hydra	44.394	FSEGTSADREIQR IIMATNRPDTLDPALLRPGR KIHIDLPNEQGR VALDMTTLTIMR	1	14.5	6.5068	0.0094
T2MD34	Serine/threonine protein phosphatase 2A regulatory subunit	phosphatase 2A regulatory subunit family	Regulatory protein	protein phosphatase regulator activity	cell communication, regulation of biological process, response to stimulus, signal transduction	*Hydra vulgaris*	Hydra	54.465	DHWNQTIVALVYNVLK VLESPEFQPTIAK	2	6.2	12.226	0.0093
A0A6P8I6V2	NADH-ubiquinone oxidoreductase 75 kDa subunit	NADH:ubiquinone oxidoreductase	Transmembrane protein	oxidoreductase activity, NADH dehydrogenase (ubiquinone) activity	aerobic respiration	*Actinia tenebrosa*	Sea anemone	73.788	LNEDVNEEWISDK TATYVNTEGR	2	3.4	18.062	0.0092
A0A7D9ENF0	Endoplasmin	Heat shock protein 90 family	Heat shock proteins	unfolded protein binding, ATP binding	protein folding, stress response	*Paramuricea clavata*	Sea-whip	106.82	ELISNASDALDK ELISNASDALDKIR GVVDSDDLPLNVSR LGVIEDHSNR	2	4.1	26.299	0.0091
A0A3M6UTM8	Proton-translocating NAD(P)(+) transhydrogenase	AlaDH/PNT family	Oxido-reductive	oxidoreductase activity	proton transmembrane transport	*Pocillopora damicornis*	Coral	113.19	AVIEAANLFGR TLGTGYADVDNPVFYK	2	2.5	13.634	0.0090
T2MH17	Pyruvate carboxylase	Biotin carboxylation domain	Enzymes	ATP binding, metal ion binding, pyruvate carboxylase activity	gluconeogenesis, pyruvate metabolic process	*Hydra vulgaris*	Hydra	131.69	EHDVDAIHPGYGFLSER LLLTDTTFR	2	2.2	13.344	0.0087
A7RJL4	Histone H2A	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Nematostella vectensis*	Sea anemone	13.641	LLSGVTIAQGGVLPNIQSVLLPK VGAGAPVYMAAVLEYLSAEILELAGNAAR	1	41.3	10.754	0.0087
A7SR65	Alpha-1,4 glucan phosphoryl	Glycogen phosphorylase	Enzymes	small molecule binding anion binding transferase activity,	glycogen metabolic process cellular carbohydrate catabolic process	*Nematostella vectensis*	Sea anemone	91.821	VLYPNDNFFEGK	1	1.5	6.2916	0.0086

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	ase			transferring hexosyl groups heterocyclic compound binding organic cyclic compound binding	macromolecule catabolic process								
G9IT37	Cytochrome c oxidase subunit 2	Cytochrome c oxidase subunit 2 family	Oxido-reductive	copper ion binding, transporter activity, cytochrome-c oxidase activity	Electron transport, Respiratory chain	*Chiropsoides quadrigatus*	Box jellyfish	27.502	LLYLMDEVIEPSITIK	1	6.5	20.028	0.0085
A0A7M5WLW3	AP domain-containing protein	Peptidase family	metalloprotease	manganese ion binding, metalloaminopeptidase activity	the processing and regular turnover of intracellular proteins	*Clytia hemisphaerica*	Hydromedusan	52.887	GVTYDTGGADVK	1	2.5	6.1097	0.0085
A0A2B4RI23	GTP-binding protein SAR1	GTPase Family	Miscellaneous	GTPase activity, GTP binding	protein transport, cellular localization, vesicle-mediated transport	*Stylophora pistillata*	Coral	18.072	LVFLGLDNAGK	1	6.8	5.9415	0.0084
T2MEK4	60S ribosomal protein L8	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Hydra vulgaris*	Hydra	27.996	GAPLAVVVFR GAPLAVVVFRDPYK	2	5.4	11.469	0.0082
A0A7M5X9R0	Ribos_L4_asso_C domain-containing protein	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Clytia hemisphaerica*	Hydromedusan	40.123	LDSIYGTWR	1	2.5	5.6833	0.0082
A0A2B4S5E4	ADP-ribosylation factor 6	GTPase Family	Lipoprotein	GTPase activity, GTP binding	cell spreading, Rac-induced formation of plasma membrane ruffles, cell migration, and Fc-mediated phagocytosis	*Stylophora pistillata*	Coral	20.024	FNVWDVGGQDK QDLPDAMKPHEVQEK	2	14.9	12.834	0.0081
A0A7M5WHZ5	Clathrin heavy chain	Clathrin	Cytoplasmic protein	polymerizes into a polyhedral lattice on intracellular membranes to form protein-coated membrane vesicles	intracellular protein transport, vesicle-mediated transport	*Clytia hemisphaerica*	Hydromedusan	193.05	ALEHYTDIYDIKR TLQIFNIEMK	1	1.4	6.8929	0.0079
A0A6S7GQM4	Cytoskeletal actin 1	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Paramuricea clavata*	Sea-whip	41.658	AGFAGDDAPRAVFPSIVGRAVFPSIVGRPRDDDVAALVVDNGSGMCKDLTDYLMKILTERDSYVGDEAQSKSYVGDEAQSKRGYSFTTTAERHQGVMVGMGQKHQGVMVGMGQKDSYVGDEAQSKHQGVMVGMGQKDSYVGDEAQSKRIWHHTFYNELRKYSVWIGGSILASLSTFQQMWISKVAPEEHPVLLTEAPLNPKVAPEEHPVLLTEAPLNPKANRYPIEHGIVTNWDDMEKYSVWIGGSILASLSTFQQMWISK	1	41.6	22.187	0.0079
A0A6S7H104	Trifunctional enzyme subunit beta	Thiolase family	Enzymes	acyltransferase activity, transferring groups other than amino-acyl groups	Lipid metabolism	*Paramuricea clavata*	Sea-whip	36.302	DQLLLGPAYATPK	1	3.8	6.8576	0.0078
T2MCX1	10-formyltetrahydrofolate dehydrogenase	Aldehyde dehydrogenase family	Oxido-reductive	formyltetrahydrofolate dehydrogenase activity, hydroxymethyl-, formyl- and related transferase activity, oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	10-formyltetrahydrofolate catabolic process, biosynthetic process, one-carbon metabolic process	*Hydra vulgaris*	Hydra	101.71	EESFGPIMIISR SPLIIFGDCDMDK	2	2.7	14.604	0.0077
A0A0C2M3X2	Citrate synthase	Citrate synthase family	Transferase	transferase activity, Ligand for members of the frizzled family of seven transmembrane receptors.	citrate metabolic process, carbo, Tricarboxylic acid cyclehydrate metabolic process	*Thelohanellus kitauei*	Myxozoa	55.861	LPVIAGLIYR	1	2	6.2679	0.0075
A0A6S7I699	Growth factor receptor-bound 2	SH2/SH3 domain	Receptor	protein binding	regulation of intracellular signalling pathways	*Paramuricea clavata*	Sea-whip	24.555	FNSLNELVDYHR	1	5.7	9.343	0.0074
A7S9Y7	Proteasome subunit alpha type	Peptidase family	Enzymes	creating an electrochemical gradient over the inner membrane	proteolysis, macromolecule catabolic process	*Nematostella vectensis*	Sea anemone	26.609	GPQLFHLDPSGTFIQYDAK	1	7.9	52.086	0.0074

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A0A7M6DPA8	26S proteasome non-ATPase regulatory subunit 13	Proteasome family	Proteases	maintenance of protein homeostasis by removing misfolded or damaged proteins	ubiquitin-dependent protein catabolic processu proteasome assembly	*Clytia hemisphaerica*	Hydromedusan	42.958	FYDLSSNYSK	1	2.7	6.1523	0.0073
A7T498	Coatomer subunit delta	Coatomer family	Cytoplasmic protein	structural molecule activity	intracellular protein transport, vesicle-mediated transport	*Nematostella vectensis*	Sea anemone	43.042	ENVNLAQIR	1	2.4	5.8753	0.0073
T2MGR0	60S ribosomal protein L17	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Hydra vulgaris*	Hydra	21.267	GLDVDSLVIDHIQVNR YSTDPENPTK	2	14.2	35.457	0.0072
A0A3M6T4R3	CSD domain-containing protein	Cold shock domain	Cold shock proteins	nucleic acid binding	response to low temperature	*Pocillopora damicornis*	Coral	34.852	GLEAANVTGPDGSPVQGSK	1	6.4	6.4244	0.0072
A7RIC3	Ornithine--oxo-acid aminotransferase	ATCase/OTCase family	Transferase	amino acid binding, carboxyl- or carbamoyltransferase activity	urea cycle, cellular amino acid metabolic process	*Nematostella vectensis*	Sea anemone	47.118	VLPMNTGVEGGETACK	1	3.7	8.079	0.0070
A0A6B2G6E6	Spliceosome RNA helicase DDX39B	Helicases	Enzymes	helicase activity, ATP binding, nucleic acid binding	Protein biosynthesis	*Myxobolus squamalis*	Myxozoa	26.596	ILVATNLFGR	1	4.4	9.2388	0.0070
A0A7D9EFJ8	26S proteasome non-ATPase regulatory subunit 2	Proteasome family	Proteases	endopeptidase activity, enzyme regulator activity	proteasome-mediated ubiquitin-dependent protein catabolic process, Ubiquitin proteasome pathway	*Paramuricea clavata*	Sea-whip	9.6589	TITGFQTHTTPVLLAHGER VGQAVDVVGQAGKPK	1	38.6	5.8806	0.0069
A0A6S7GW03	Histone-lysine N-methyltransferase PRDM9-like	Villin/gelsolin family	Cytoskeleton Component	transferase activity, actin filament binding	metabolic process	*Paramuricea clavata*	Sea-whip	33.149	IFETLNDDLANISVWMAANK	1	6.9	5.6954	0.0068
A0A3M6T7R7	Small nuclear ribonucleoprotein Sm D2	snRNP core protein family	Ribosomal protein	processing of pre-mRNAs to mature mRNAs	mRNA processing, mRNA splicing	*Pocillopora damicornis*	Coral	13.404	GDSVILVLR HCNMVLENVK	2	16.1	11.339	0.0068
A0A7M5UM43	40S ribosomal protein S17	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Clytia hemisphaerica*	Hydromedusan	15.356	ICEEIAIIPSKK	1	9.1	5.6568	0.0067
A0A0C2IER9	RNA helicase	Helicases	Enzymes	RNA helicase activity, hydrolase activity, ATP binding, translation initiation factor activity	Protein biosynthesis	*Thelohanellus kitauei*	Myxozoa	46.747	DVLAQAQSGTGK	1	2.9	6.3323	0.0067
A0A6B2FYE2	T-complex protein 1 subunit alpha	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding	*Myxobolus squamalis*	Myxozoa	36.077	IALLDFSLQK	1	3	6.0816	0.0067
A0A7D9HS87	40S ribosomal protein S23	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Paramuricea clavata*	Sea-whip	15.892	SGHAVGDIPGVR	1	8.4	8.9617	0.0066
T2M4N0	Ras-related GTP-binding protein C	GTR/RAG GTP-binding protein family	small GTPase	GTPase activity	cellular response to organonitrogen compound, cellular response to starvation positive regulation of TOR signaling, cellular response to oxygen-containing compound, autophagy, regulation of autophagy, TOR signaling	*Hydra vulgaris*	Hydra	42.746	VDGLTDDHKIETQR	1	3.7	7.254	0.0064
T2MFZ0	60S ribosomal protein L3	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Hydra vulgaris*	Hydra	46.115	NNASTDYDLTEK	1	3	7.6637	0.0064
A0A6S7FT97	AP complex	Adaptor complexes	Cytoskeleton Component	clathrin binding	intracellular protein transport, vesicle-mediated transport	*Paramuricea clavata*	Sea-whip	86.285	ASMIWIIGEYAERKGEIFELK	2	2.7	14.86	0.0064

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	subunit beta	large subunit family	(membrane traffic protein)										
A0A6P8J123	protein lin-9 homolog	Lin-9 family	Cytoskeleton Component (DNA metabolism protein)	odorant binding	cell cycle, regulation of cell cycle, transcription by RNA polymerase II, regulation of transcription by RNA polymerase II	*Actinia tenebrosa*	Sea anemone	59.967	MAEEESSAEALVSLK	1	2.8	5.8864	0.0064
A0A6S7J2P0	Tubulin alpha-3 chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Paramuricea clavata*	Sea-whip	30.896	AFVHWYVGEGMEEGEFTEAR DVNAAIATIK FDGALNVDLTEFQTNLVPYPR LDHKFDLMYAK;LIGQIVSSITASLR NLDIERPTYTNLNR PTYTNLNR RNLDIERPTYTNLNR RTIQFVDWCPTGFK TIQFVDWCPTGFK VGINYQPPTVVPGGDLAK	1	44.6	6.1516	0.0064
A0A7D9MJP5	26S proteasome regulatory subunit RPN11	Peptidase family	Proteases	metallopeptidase activity, isopeptidase activity, Lys63-specific deubiquitinase activity	protein-containing complex intracellular	*Paramuricea clavata*	Sea-whip	13.731	LINPNMMVLGQEPR	1	11.6	5.6599	0.0062
A0A2B4RHH5	Enoyl-CoA hydratase	Enoyl-CoA hydratase/isomerase	Enzymes	catalytic activity	fatty acid beta-oxidation pathway	*Stylophora pistillata*	Coral	34.416	FGQPEILLGTLPGAGGTQR	1	6	6.8763	0.0061
A0A0C2JYJ1	Succinate dehydrogenase	FRD/SDH	Oxido-reductive	succinate dehydrogenase (ubiquinone) activity, flavin adenine dinucleotide binding	electron transport chain, tricarboxylic acid cycle, cellular respiration	*Thelohanellus kitauei*	Myxozoa	71.036	AAFGLASEGFK	1	1.7	5.81	0.0060
A7SKY1	40S ribosomal protein S25	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Nematostella vectensis*	Sea anemone	13.025	DKLNNLVLFDK	1	9.5	6.5735	0.0060
T2M6B6	CTP:phosphoethanolamine cytidylyltransferase	Cytidylyltransferase family	Enzymes	transferase activity, ethanolamine-phosphate cytidylyltransferase activity	Lipid metabolism, phosphatidylethanolamine biosynthesis process	*Hydra vulgaris*	Hydra	42.071	TEGVSTTNLVGR	1	3.2	6.6868	0.0060
T2MH90	26S protease regulatory subunit 6A	ATPase family	Proteases	peptidase activity, proteasome-activating activity	macromolecule catabolic process, protein metabolic process	*Hydra vulgaris*	Hydra	47.831	EKLPAIIFIDELDAIGTK LPAIIFIDELDAIGTK VIAATNRVDILDPALLR	3	8.2	19.961	0.0058
T2M338	Stomatin-like protein 2	Band 7/mec-2 family	Cytoskeleton Component	ubiquitin protein ligase binding	positive regulation of cell junction assembly, positive regulation of cell-cell adhesion mediated by cadherin, positive regulation of endocytosis, protein kinase C signaling, protein localization to plasma membrane, regulation of receptor internalization	*Hydra vulgaris*	Hydra	40.629	ESMQMQVEAER FVPQQEAWIVER	2	6.3	11.143	0.0057
A0A6P8H3F4	methylcrotonoyl-CoA carboxylase beta chain	ClpP/crotonase	Enzymes	ligase activity, Biotin carboxylase activity	In human -Abnormality of acid-base homeostasis, Acidosis, Aminoaciduria, coma	*Actinia tenebrosa*	Sea anemone	62.001	ALYGDTLVTGFAR IVDGSKFDEFK	2	4.2	12.445	0.0055
A0A2B4RCT7	4-hydroxyphenylpyruvate dioxygenase	4HPPD family	Oxido-reductive	4-hydroxyphenylpyruvate dioxygenase activity, metal ion binding	aromatic amino acid family metabolic process	*Stylophora pistillata*	Coral	28.503	MPLNEPAPGKR SIVVTNWEETIK	2	9.3	13.173	0.0055
A0A7M5XDK4	Catalase	Catalase family	Oxido-reductive	oxidoreductase activity, acting on peroxide as acceptor, heme binding	hydrogen peroxide catabolic, response to oxidative stress	*Clytia hemisphaerica*	Hydromedusan	56.481	FSTVGGESGSADTAR	1	3	15.448	0.0053
T2MFP1	Glucosamine-6-phosphate isomerase	Glucosamine/galactosamine-6-phosphate isomerase family	Enzymes	glucosamine-6-phosphate deaminase activity	carbohydrate metabolic process, malate metabolic process, tricarboxylic acid cycle	*Hydra vulgaris*	Hydra	30.307	YFVLGLPTGSTPLGMYK	1	6.3	6.5839	0.0052

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A0A7D9DX54	Serine/threonine-protein phosphatase	PPP phosphatase family	Enzymes	Catalytic activity, protein serine/threonine phosphatase activity	protein dephosphorylation, cellular protein modification	*Paramuricea clavata*	Sea-whip	35.403	SPDTNYLFMGDYVDR YSFLQFDPAPR	2	8.4	24.22	0.0052
A0A6S7FUZ2	U6 snRNA-associated Sm LSm6	snRNP Sm proteins family	RNA splicing factor	RNA binding	mRNA splicing, via spliceosome	*Paramuricea clavata*	Sea-whip	8.9722	GNNVLYISTQK	1	13.9	5.8567	0.0052
A7SH82	40S ribosomal protein S18	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Nematostella vectensis*	Sea anemone	17.856	IPDWFLNR	1	5.2	5.8787	0.0051
A0A6P8H4T2	Tubulin beta chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Actinia tenebrosa*	Sea anemone	55.257	FPGQLNADLR FPGQLNADLRK KLAVNMVPFPR LAVNMVPFPR LREEYPDR YLTVASMFR	1	7.7	6.2441	0.0051
T2M6K3	Phosphatidylinositol 4-kinase type 2	PI3/PI4-kinase family	Enzymes	transferase activity, 1-phosphatidylinositol 4-kinase activity, ATP binding	Inositol phosphate metabolism, Metabolic pathways, Phosphatidylinositol signaling system	*Hydra vulgaris*	Hydra	55.132	IYQGSSGSYFVR	1	2.5	6.1817	0.0051
A0A6P8IB06	H(+)-transporting two-sector ATPase	ATPase family	Oxido-reductive	Translocase, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis, Hydrogen ion transport, Ion transport	*Actinia tenebrosa*	Sea anemone	68.449	FKDPVKDGEAK GNEMSEVLR LAEMPADSGYPAYLGAR LLKDDFLQQNGYTPYDR RTALVANTSNMPVAAR TALVANTSNMPVAAR TGKPLSVELGPGIMGSIFDGIQRPLK	1	15.5	5.8042	0.0051
A0A6P8HH48	myoferlin-like isoform X3	Ferlin family	Cytoskeleton Component (membrane traffic protein)	metal ion binding	plasma membrane organization	*Actinia tenebrosa*	Sea anemone	229.68	DDLIGETIVDLENRSLDGEGNFNWR	2	1.2	17.309	0.0050
A0A7D9F1Z3	Tubulin alpha chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Paramuricea clavata*	Sea-whip	50.277	AFVHWYVGEGMEEGEFSEAR ALYVDLEPTVIDEVR AVCMLSNTTAIAEAWAR DVNAAIATIK;EDAANNYAR FDGALNVDLTEFQTNLVPYPR IHFPLATYAPVISAEK LDHKFDLMYAK LIGQIVSSITASLR RAFVHWYVGEGMEEGEFSEAR RTIQFVDWCPTGFK TIQFVDWCPTGFK VGINYQPPTVVPGGDLAK YMACCLLYR	1	38.7	19.495	0.0050
A0A3M6UKC8	Proteasome subunit alpha type	Peptidase family	Enzymes	creating an electrochemical gradient over the inner membrane	proteolysis, macromolecule catabolic process	*Pocillopora damicornis*	Coral	28.497	AITIFSPDGHLFQVEYAQEAVK	1	8.6	10.139	0.0050
A0A7D9E1X0	uncharacterized protein K02A2.6-like	DNA/RNA polymerase superfamily	Oxido-reductive	oxidoreductase activity	DNA integration	*Paramuricea clavata*	Sea-whip	95.68	ISLDLSNQR	1	1.1	6.2866	0.0049
A0A6P8HCJ6	40S ribosomal protein S2	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Actinia tenebrosa*	Sea anemone	30.061	TPYQEFTDYLAK	1	4.3	9.2201	0.0049
A0A6S7LSY9	60S ribosomal L26	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Paramuricea clavata*	Sea-whip	7.4226	KAHFTAPSSVR	1	17.5	7.161	0.0049
A0A7M5X7K7	Adenosine kinase	Carbohydrate kinase PfkB family	Enzymes	adenosine kinase activity	AMP salvage, purine ribonucleoside salvage	*Clytia hemisphaerica*	Hydromedusan	38.981	SLVANLAAANHFK	1	3.7	5.8522	0.0049
A0A7M5VFT9	AAA domain-containing protein	AAA ATPase family	miscellaneous	ATP binding, proteasome-activating activity	proteolysis, macromolecule catabolic process, response to stimulus	*Clytia hemisphaerica*	Hydromedusan	41.08	AVANQTSATFLR ESVELPLTHPELYEEMGIKPPK IDSLDPALIRPGR	1	12.8	6.4988	0.0048
A0A3M6V300	Proteasome subunit beta	Peptidase family	Enzymes	peptidase activity	proteolysis, macromolecule catabolic process	*Pocillopora damicornis*	Coral	31.086	RGPGLYYVDSDGTR	1	5	9.7309	0.0048

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A0A7M5X813	Eukaryotic peptide chain release factor subunit 1	Eukaryotic release factor 1 family	Translation factors	translation release factor activity	Protein biosynthesis	*Clytia hemisphaerica*	Hydromedusan	49.557	LSVLGAITSVQQR	1	2.9	7.8057	0.0047
A0A7M5VHF3	4-hydroxyphenylpyruvate dioxygenase	4HPPD family	Oxido-reductive	4-hydroxyphenylpyruvate dioxygenase activity, metal ion binding	aromatic amino acid family metabolic process	*Clytia hemisphaerica*	Hydromedusan	43.938	FWSVDDSQIHTEYSALR	1	4.4	7.1567	0.0047
A0A6P8HFB4	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	2-oxoacid dehydrogenase family	Toxin	acyltransferase activity	https://supfam.org/SUPERFAMILY/cgi-bin/scop.cgi?ipid=SSF47005	*Actinia tenebrosa*	Sea anemone	50.804	MAAFIGNLFCRISSLYGVK	1	4.1	5.7348	0.0047
W6A2X0	RNA helicase	Helicases	Enzymes	RNA helicase activity, hydrolase activity, ATP binding, translation initiation factor activity	Protein biosynthesis	*Nanomia bijuga*	Siphonophore	63.482	DLMACAQTGSGK MLDMGFEPQIR VGHTGLATSFFNEK VGSTSENITQK	4	8.5	22.766	0.0046
A0A3M6UDL6	Dolichol-phosphate mannosyltransferase subunit 1	glycosyltransferase 2 family	Transferase	dolichyl-phosphate beta-D-mannosyltransferase activity	Cellular protein modification process, macromolecule biosynthetic process, protein glycosylation	*Pocillopora damicornis*	Coral	29.39	GANYLTQVLLRPGASDLTGSFR YSILLPTYNER	2	12.7	13.453	0.0045
A0A0C2MUS2	Chaperonin (Cpn)	Heat shock protein 60 family	Heat shock proteins	ATP binding, Chaperone	protein folding	*Thelohanellus kitauei*	Myxozoa	40.732	AIAQVGTISANSDETVGK AVAAGMNPMDLKR EIELEDKFENMGAQMVK	3	12.4	51.415	0.0045
A0A7M5TZB5	GOLD domain-containing protein	EMP24/GP25L family	Cytoskeleton Component	assembly of membrane-associated complexes, regulating assembly of cargo into membranous vesicles	endoplasmic reticulum to Golgi vesicle-mediated transport, intracellular protein transport, Golgi organization	*Clytia hemisphaerica*	Hydromedusan	23.551	KLEDLSEAIVNDFAYMR LEDLSEAIVNDFAYMR	2	8.3	12.354	0.0045
A0A7D9HLI8	Guanine nucleotide-binding G(Q) subunit alpha	GTP-binding protein	G-protein	protein-containing complex binding, G protein-coupled receptor binding, GTPase activity	cell communication, regulation of biological process, response to stimulus	*Paramuricea clavata*	Sea-whip	34.863	APTTGIIEYPFDLDTIIFR	1	6.4	30.3	0.0044
A7SAZ3	Acetyl-coenzyme A synthetase	ATP-dependent AMP-binding enzyme family	Enzymes	acetate-CoA ligase activity, AMP/ATP binding	acetyl-CoA biosynthetic process from acetate, organic acid metabolic process, cofactor metabolic process	*Nematostella vectensis*	Sea anemone	66.752	INQFYGAPTAIR	1	2	6.5954	0.0044
A0A6S7JBV3	Isocitrate lyase	Isocitrate lyase/Mulate synthase	Enzymes	isocitrate lyase activity, metal ion binding	organic acid metabolic process	*Paramuricea clavata*	Sea-whip	40.917	FQFVTLAGFHSLNHAMFK	1	4.9	5.8235	0.0043
A0A0C2M5R5	26S protease regulatory subunit 8	ATPase family	Proteases	peptidase activity, proteasome-activating activity	macromolecule catabolic process, protein metabolic process	*Thelohanellus kitauei*	Myxozoa	25.926	IDILDPALLRPGR TMLELLNQLDGFEPTQNIK	1	13.7	6.9785	0.0043
A7RVC0	Clathrin heavy chain	Clathrin	Cytoplasmic protein	polymerizes into a polyhedral lattice on intracellular membranes to form protein-coated membrane vesicles	intracellular protein transport, vesicle-mediated transport	*Nematostella vectensis*	Sea anemone	191.23	HLPLVKPYLR IVLENSVFSDHR TLQIFNIEMK	1	1.9	5.8545	0.0042
A0A7M6DQ59	DUF4062 domain-containing protein	DUF family	Unknown	Unknown	Unknown	*Clytia hemisphaerica*	Hydromedusan	188.13	VSQYIESLPNTITDLFSFLLDEWSEK	1	1.6	5.8733	0.0042
A7SSE3	Tubulin gamma chain	Tubulin	Cytoskeleton Component	structural constituent of cytoskeleton, GTP binding	microtubule-based process	*Nematostella vectensis*	Sea anemone	52.524	EDIFKENLDELDNSR YPGYMNNDLIGLIASLIPTPR	2	7.8	28.655	0.0042

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A0A7M5UMD5	cGMP-dependent protein kinase	Protein kinase	Enzymes	ATP binding, cGMP binding, cGMP-dependent protein kinase activity, protein serine kinase activity, protein threonine kinase activity	protein phosphorylation	*Clytia hemisphaerica*	Hydromedusan	81.611	ALLGEDLRASVHSDPQFVR	2	2.6	10.79	0.0042
D7F487	Actin	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Porites compressa*	Coral	7.3162	AVFPSIVGR AVFPSIVGRPR DSYVGDEAQSK DSYVGDEAQSKR GFAGDDAPR HQGVMVGMGQK HQGVMVGMGQKDSYVGDEAQSK HQGVMVGMGQKDSYVGDEAQSKR YPIEHGIVTNWDDMEK	1	88.1	6.4217	0.0042
A0A7D9DBH9	Thioredoxin domain-containing protein 17	Thioredoxin family	Oxido-reductive	controlling of cellular redox signalling pathways	maintaining cellular redox homeostasis via a thiol-disulfide reductase activity	*Paramuricea clavata*	Sea-whip	13.824	LTGIPTLIK	1	7.3	5.6889	0.0042
A0A2B4S907	Heat shock cognate 71 kDa protein	Heat shock protein 70 family	Heat shock proteins	ATP binding	stress response	*Stylophora pistillata*	Coral	70.882	TFYAEEISSMVLTK TTPSYVAFTDTER VEIIANDQGNR	1	5.9	6.6181	0.0040
A0A7M5V566	Sodium/potassium-transporting ATPase subunit alpha	Cation transport ATPase family	transport protein	ATP binding, metal ion binding, P-type potassium transmembrane transporter activity	Ion transport, Potassium transport	*Clytia hemisphaerica*	Hydromedusan	113.61	ADIGVAMGIAGSDVSK GVGIISEGNETVEDIAQR VDNSSLTGESEPQSR VIMVTGDHPITAK	1	6	39.896	0.0040
A7SFA3	ATP-citrate synthase	Succinate/malate CoA ligase family	Enzymes	ATP binding, ATP citrate synthase activity, metal ion binding	acetyl-CoA biosynthetic process, citrate metabolic process, fatty acid biosynthetic process	*Nematostella vectensis*	Sea anemone	120.42	IGNTGGMLDNILASK RGGPNYQEGLR	2	2.4	13.568	0.0040
A0A2B4RE01	RNA helicase	Helicases	Enzymes	RNA helicase activity, hydrolase activity, ATP binding, translation initiation factor activity	Protein biosynthesis	*Stylophora pistillata*	Coral	47.595	GFKDQIYDIFR GIYAYGFEKPSAIQQR VLITTDLLAR	1	8.9	15.3	0.0039
A0A6S7HIH5	Transport Sec61 subunit alpha	SecY family	Transporter	protein transmembrane transporter activity	translational elongation, intracellular protein transmembrane transport, protein targeting to membrane, protein targeting to ER	*Paramuricea clavata*	Sea-whip	46.105	AFSPATVNTGR GTEFEGAVIALFHLLATR GTLMELGISPIVTSGLIMQLLAGAK	3	12.8	23.832	0.0038
A0A7M5XIK0	Serine/threonine-protein phosphatase	PPP phosphatase family	Enzymes	Catalytic activity, protein serine/threonine phosphatase activity	protein dephosphorylation, cellular protein modification	*Clytia hemisphaerica*	Hydromedusan	37.554	ELFLSQPILLELEAPLK IKYPENFFLLR	2	8.5	37.109	0.0038
A0A1U9XQR0	Fructose-bisphosphate aldolase	Glycolytic family	Enzymes	lyase activity, fructose-bisphosphate aldolase activity	glycolytic process, response to cadmium ion	*Aurelia sp.*	Scyphozoa	39.413	KPWALSFSYGR	1	3	11.784	0.0038
A7T660	Glutamate dehydrogenase	Glu/Leu/Phe/Val dehydrogenases family	Oxido-reductive	glutamate dehydrogenase (NAD+/NADP+) activity	cellular amino acid metabolic process	*Nematostella vectensis*	Sea anemone	40.173	ASNAGGVAVSGLEMSQNSLR	1	5.5	7.3286	0.0037
A0A3M6TG32	Peptidase S53 domain-containing protein	Peptidase family	Enzymes	metal ion binding, serine-type endopeptidase activity	Probably involved in the processing of hormone and other protein precursors at sites comprised of pairs of basic amino acid residues.	*Pocillopora damicornis*	Coral	63.069	IPVPGVMGTSASTPTVAGIISLVNDAR	1	4.7	19.735	0.0036
A0A3M6U683	AAA domain-containing protein	AAA ATPase family	miscellaneous	ATP binding, proteasome-activating activity	proteolysis, macromolecule catabolic process, response to stimulus	*Pocillopora damicornis*	Coral	49.287	ESVELPLTHPELYEEMGIKPPK TMLELLNQLDGFDSK VAEEHAPSIVFIDEIDAVGTK	1	13.2	7.9255	0.0035
T2MH44	Vacuolar proton pump subunit B	ATPase family	Oxido-reductive	ATP binding	ATP metabolic process, proton transmembrane transport	*Hydra vulgaris*	Hydra	57.734	AVVQVFEGTSGIDAK GFPGYMYTDLATIYER GPPVLAEDFLDIMGQPINPWSR IPIFSAAGLPHNDIAAQICR IYPEEMIQTGISAIDTMNSIAR	1	18.3	33.33	0.0035
A0A7D9I	ATP	ATPase family	Oxido-reductive	Translocase, proton-	ATP synthesis, Hydrogen ion	*Paramuricea*	Sea-whip	45.546	AHGGYSVFAGVGER	1	21.4	157.53	0.0034

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TV7	synthase subunit beta			transporting ATP synthase activity, rotational mechanism	transport, Ion transport	*clavata*			DQEGQDVLLFIDNIFR FTQAGSEVSALLGR IPSAVGYQPTLATDMGTMQER TVLIMELINNVAK VALTGLTVAEYFR				
A0A7M5V0D8	40S ribosomal protein S15	Ribosomal protein	Ribosomal protein	structural constituent of ribosome	translation	*Clytia hemisphaerica*	Hydromedusan	22.247	GVDLDQLLDLSSEQLMDLVHAR	1	11.2	7.1758	0.0033
T2M4I5	Ras-related protein Rab-18	Ras family	Miscellaneous	GTPase activity, GTP binding	membrane trafficking control	*Hydra vulgaris*	Hydra	22.704	LALWDTAGQER	1	5.4	5.9484	0.0033
T2MEH3	Casein kinase II subunit alpha	Protein kinase superfamily	Enzymes	ATP binding, protein serine/threonine kinase activity	Apoptosis, Cell cycle, Transcription, Transcription regulation	*Hydra vulgaris*	Hydra	41.759	FNDILGR VYADVNTHRPR	2	5.1	13.15	0.0033
A7SHH5	Protein disulfide-isomerase	Thioredoxin family	Oxido-reductive	protein disulfide isomerase activity	response to oxidative stress	*Nematostella vectensis*	Sea anemone	53.433	QLAPIWDQLGEK	1	2.5	7.6273	0.0033
A0A7M5V2U8	Histone H2B	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Clytia hemisphaerica*	Hydromedusan	14.446	SMSIMNSFVNDIFER	1	11.4	19.946	0.0033
A0A2B4SGK7	T-complex protein 1 subunit alpha	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding	*Stylophora pistillata*	Coral	54.348	EQLAIAEFANSLLVIPK FATEAAITILR GANDFMLDEMER	1	8	6.8541	0.0032
A0A3M6TE72	CCT-theta	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding	protein folding	*Pocillopora damicornis*	Coral	59.026	IVAFSCPLDAMTTETK	1	2.9	6.054	0.0032
D1FX75	Coronin	Coronin family	Cytoskeleton Component	actin filament binding	actin filament organization	*Chironex fleckeri*	Box jellyfish	56.717	NEVALFYR	1	1.6	5.945	0.0032
A0A3M6UGG9	Vesicle-fusing ATPase	ATPase family	Miscellaneous	ATP binding, ATP hydrolysis activity	involved in cellular and subcellular movement	*Pocillopora damicornis*	Coral	89.409	EVDIGIPDATGR;IVSQLLTLMDGLK KYEMFAQTLQQSR LGDIVSVQACPDVK NVFIIGATNRPDIIDPAILRPGR	2	9.3	12.88	0.0032
A0A2B4RAG5	Arginyl-tRNA synthetase	tRNA ligase family	Catalyse	arginine-tRNA ligase activity, ATP binding	Protein biosynthesis (translation), cellular amino acid metabolic process, RNA metabolic process	*Stylophora pistillata*	Coral	26.822	LLEFAGHDVLR	1	4.5	5.6473	0.0031
A0A6P8HJY7	Spectrin beta chain	Spectrin family	Cytoskeleton Component	actin binding, phospholipid binding, structural constituent of cytoskeleton	actin filament capping	*Actinia tenebrosa*	Sea anemone	269.89	DALLLWCQSK NVTVTNFTTSWR	2	0.9	14.794	0.0030
A0A7M5WHY1	Imidazolon epropionate hydrolase	Urocanase family	Enzymes	urocanate hydratase activity, hydro-lyase activity	heterocycle catabolic process, aromatic amino acid family catabolic process, cellular nitrogen compound catabolic process, alpha-amino acid metabolic process	*Clytia hemisphaerica*	Hydromedusan	75.577	VFVTAGLGGMSGAQPK	1	2.3	6.273	0.0030
A0A6P8J9G7	spectrin alpha chain, non-erythrocytic 1-like	Spectrin family	Cytoskeleton Component	actin binding, calcium ion binding, calmodulin binding	organelle organization, regulation of biological process, protein polymerization, protein depolymerization, regulation of biological quality	*Actinia tenebrosa*	Sea anemone	279.89	DLSSVQTLITK	1	0.5	6.0477	0.0030
T2MCX5	Vacuolar protein sorting-associated protein 26B (Fragment)	VPS26 family	Cytoskeleton Component (membrane traffic protein)	Acts as component of the retromer cargo-selective complex (CSC). The CSC is believed to be the core functional component of retromer or respective retromer complex variants acting to prevent missorting of selected transmembrane cargo proteins into the lysosomal degradation pathway.	intracellular protein transport	*Hydra vulgaris*	Hydra	37.907	LFLSGYELTPTMK	1	4	6.4505	0.0029

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T2MFU6	26S proteasome non-ATPase regulatory subunit 7	Peptidase family	Signalling protein	isopeptidase activity, metallopeptidase activity	proteasome regulatory	*Hydra vulgaris*	Hydra	36.955	VVVHPLVLLSVVDHFNR	1	5.2	5.9735	0.0029
A0A6P8IQL3	Tricarboxylate transport protein	Mitochondrial carrier family	transport protein	transfer molecules across the membranes of the mitochondria	Energy transport across the membranes of the mitochondria	*Actinia tenebrosa*	Sea anemone	35.51	GLSSLLYGSIPK TFIIGGIAGAASVFGNTPVDVVK	2	10.7	20.965	0.0029
A0A6P8HUS3	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	FRD/SDH	Oxido-reductive	flavin adenine dinucleotide binding, succinate dehydrogenase (ubiquinone) activity	electron transport chain, tricarboxylic acid cycle, cellular respiration	*Actinia tenebrosa*	Sea anemone	73.19	DPIPVLPTVHYNMGGIPTNYK	1	3.2	7.9644	0.0028
A0A6B2G5S4	Cytosol aminopeptidase	Peptidase family	Metalloprotease	manganese ion binding, metalloaminopeptidase activity, catalytic activity, acting on a protein	proteolysis	*Myxobolus squamalis*	Myxozoa	12.888	TVEVDNTDAEGR	1	9.8	7.4808	0.0028
A0A7M5X4Q9	Ras-related protein Rab-14	Ras family	Miscellaneous	GTPase activity, GTP binding	response to stimulus, multi-organism process, vesicle-mediated transport, cellular localization, organelle organization	*Clytia hemisphaerica*	Hydromedusan	23.986	AAGPYNYSYIFK GAAGALMVYDITR STYNHLSSWLTDAR	1	18.1	6.1319	0.0028
A0A3M6U3P0	Dolichyl-diphosphooligosaccharide--protein glycotransferase	STT3 family	Transferase	dolichyl-diphosphooligosaccharide-protein glycotransferase activity, metal ion binding	protein glycosylation	*Pocillopora damicornis*	Coral	80.922	FGSVYTEQGKPTGYDR	1	2.3	6.9657	0.0028
A0A2B4SUQ0	S-phase kinase-associated protein 1	SKP1 family	Enzymes	kinase activity	cellular protein modification process, proteolysis, macromolecule catabolic process	*Stylophora pistillata*	Coral	14.985	NDFTPEEEEQVR NDFTPEEEEQVRK	2	10	23.456	0.0028
T2MHJ0	Non-specific serine/threonine protein kinase	Protein kinase superfamily		ATP binding, protein serine kinase activity, protein threonine kinase activity	Protein phosphorylation	*Hydra vulgaris*	Hydra	56.891	ALYLIATNGTPELQHPER	1	3.5	6.1049	0.0027
A0A6P8IQ88	Isocitrate dehydrogenase	Isocitrate/isopropylmalate dehydrogenase	Enzymes	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	glyoxylate cycle, isocitrate metabolic process, tricarboxylic acid cycle https://supfam.org/SUPERFAMILY/cgi-bin/scop.cgi?sunid=53659	*Actinia tenebrosa*	Sea anemone	50.557	FKDIFQEIYER	1	2.5	6.3951	0.0027
A0A7M5XFH8	Macro domain-containing protein	Histone H2A		DNA binding, protein heterodimerization activity	chromatin organization	*Clytia hemisphaerica*	Hydromedusan	44.614	AISNYFVTVMASSLK	1	3.6	6.3231	0.0026
A0A2B4SFI2	Glutamine amidotransferase	GMP synthetase	Metabolic enzymes	ATP binding, GMP synthase (glutamine-hydrolyzing) activity, pyrophosphatase activity	cellular amino acid metabolic process, GMP biosynthesis, Purine biosynthesis	*Stylophora pistillata*	Coral	70.19	VAILDAGAQYGK	1	1.9	6.5816	0.0026
A0A7D9IY68	Unconventional myosin-Ib-like	Myosin family	Motor protein	actin binding, ATP binding, motor activity	actin filament organization, cellular localization, protein transport	*Paramuricea clavata*	Sea-whip	59.802	YLGLLENVR	1	1.8	5.6502	0.0026
A7TCL7	Histone H2A	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Nematostella vectensis*	Sea anemone	20.084	DAVTYTEHAK;DNIQGITKPAIR GVLKVFLENVIR;ISGLIYEETR ISGLIYEETRGVLK KTVTAMDVVYALK KTVTAMDVVYALKR RISGLIYEETR RISGLIYEETRGVLK TVTAMDVVYALK TVTAMDVVYALKR	1	48.9	16.524	0.0026

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									VFLENVIR VFLENVIRDAVTYTEHAK VGAGAPVYLAAVLEYLSAEILELAGNAAR				
A0A2B4RLL7	Small monomeric GTPase	small GTPase	Miscellaneous	G protein activity, GTP binding	cell communication, Rab protein signal transduction, response to stimulus	*Stylophora pistillata*	Coral	23.556	GAAGALLVYDISSR	1	6.6	10.091	0.0026
A0A6S7GM63	Glycine cleavage system P protein	GcvP family	Oxido-reductive	oxidoreductase activity, heterocyclic compound binding, cation binding amino acid binding, organic cyclic compound binding	glycine catabolic process, alpha-amino acid metabolic process, cellular amino acid catabolic process	*Paramuricea clavata*	Sea-whip	100.91	FGVPLGYGGPHAGFFAIR SAHGTNPASAQMAGFK	2	3.7	11.509	0.0025
T2MGJ0	Polyadenylate-binding protein	Polyadenylate-binding protein type-1 family		RNA binding	regulation of mRNA metabolism processes	*Hydra vulgaris*	Hydra	70.76	SLGYAYVNFQQPADAER	1	2.7	49.582	0.0025
Q8WRT4	Beta catenin	Armadillo family	Cytoskeleton Component	cadherin binding	cell adhesion	*Nematostella vectensis*	Sea anemone	48.876	LIILASGGPAELVR NEGIAAYAAAVLFR	2	6.3	17.834	0.0024
A7T5I4	6-phosphogluconate dehydrogenase	6-phosphogluconate dehydrogenase family	Oxido-reductive	NADP binding oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	oxidation-reduction process monocarboxylic acid catabolic process generation of precursor metabolites and energy cellular carbohydrate catabolic process glucose 6-phosphate metabolic process NADP metabolic process	*Nematostella vectensis*	Sea anemone	36.892	GLLFVGSGVSGGEEGAR	1	5.1	6.284	0.0024
A0A7D9M457	Glutamine--tRNA ligase	class-I aminoacyl-tRNA synthetase family	Enzymes	aminoacyl-tRNA ligase activity, ATP binding	translation, cellular amino acid metabolic process, RNA metabolic process	*Paramuricea clavata*	Sea-whip	23.42	LFTLTALR	1	3.8	5.7609	0.0024
A0A6S7FNG6	Mitogen-activated kinase 1	Protein kinase	Enzymes	ATP binding, protein kinase activity	protein phosphorylation	*Paramuricea clavata*	Sea-whip	16.055	YIHSANVLHR	1	7.4	5.8272	0.0024
Q8I8C8	Beta actin	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Myxobolus squamalis*	Myxozoa	42.069	AVFPSIVGR AVFPSIVGRPR DLTDYLMKILTER DSYVGDEAQSK DSYVGDEAQSKR GYSFTTTAER HQGVMVGMGQK HQGVMVGMGQKDSYVGDEAQSK HQGVMVGMGQKDSYVGDEAQSKR WHHTFYNELR KYSVWIGGSILSSLSTFQQMWISK SYELPDGQVITIGNER VAPEEHPVLLTEAPLNPK VAPEEHPVLLTEAPLNPKANR YPIEHGIVTNWDDMEK YSVWIGGSILSSLSTFQQMWISK	2	38.4	11.075	0.0024
A7T288	T-complex protein 1 subunit delta	Chaperonin family	Heat shock proteins	unfolded protein binding, ATP binding, Chaperone	protein folding	*Nematostella vectensis*	Sea anemone	52.476	VVSQYSNLLAPISVDAVLK	1	3.9	6.5718	0.0023
A0A3M6UHN4	Coatomer subunit zeta	Coatomer family	Cytoplasmic protein	coating Golgi-derived transport vesicles	toxin transport, protein transport, vesicle-mediated transport, Golgi to endoplasmic reticulum	*Pocillopora damicornis*	Coral	19.916	MDSAVLEASLYTVK	1	7.9	7.3506	0.0023
A0A0C2N200	Ras-related protein RABA2a	Ras family	Miscellaneous	GTPase activity, GTP binding	cell proliferation	*Thelohanellus kitauei*	Myxozoa	23.291	AQLWDTAGQER VVLIGDSGVGK	1	10.5	6.8319	0.0023
A0A2B4RKX8	Ras-related protein Rab-30	Ras family	Miscellaneous	GTPase activity, GTP binding	regulators of intracellular membrane trafficking	*Stylophora pistillata*	Coral	24.164	LQVWDTAGQER	1	5.2	5.9427	0.0022

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T2M2X1	Adenylate kinase isoenzyme 5	Adenylate kinase family	Enzymes	ATP binding, nucleobase-containing compound kinase activity	nucleobase-containing compound metabolic process	*Hydra vulgaris*	Hydra	106.47	GFLIDGFPR	1	1.9	5.7019	0.0022
A0A7M5XAK5	Methionine adenosyltransferase 2 subunit beta	dTDP-4-dehydrorhamnose reductase family	Oxido-reductive	Acting on the CH-OH group of donors with NAD+ or NADP+ as acceptor	S-adenosylmethionine biosynthetic process	*Clytia hemisphaerica*	Hydromedusan	32.845	VLITGASGLLGR	1	4.1	8.388	0.0022
A7S7R3	Peptidylprolyl isomerase	Peptidylprolyl isomerase family	Enzymes	peptidyl-prolyl cis-trans isomerase activity	chaperone-mediated protein folding	*Nematostella vectensis*	Sea anemone	14.172	LEDGTEFDSSIPR	1	9.9	35.519	0.0022
Q6PTK3	Triosephosphate isomerase	Triosephosphate isomerase family	Enzymes	triose-phosphate isomerase activity	gluconeogenesis, glycolytic process	*Obelia sp.*	Hydrozoan	23.116	VIACIGELLSER	1	5.7	7.6857	0.0022
A0A3M6UAJ0	Autophagy_act_C domain-containing protein	Ubiquitin-like-conjugating enzyme	Enzymes	ubiquitin-like protein transferase activity	autophagocytosis	*Pocillopora damicornis*	Coral	25.371	FVQAVIPTIEYDYTR	1	6.7	6.2445	0.0022
A0A6S7IAM5	Coatomer subunit beta	Coatomer family	Cytoplasmic protein	structural molecule activity	intracellular protein transport, vesicle-mediated transport	*Paramuricea clavata*	Sea-whip	20.84	LVEKPSPILLGPHDFSNIK	1	10	7.4539	0.0022
A0A7D9JSB7	Flagellar-associated, partial	EF-hand	Calcium-binding protein	calcium ion binding, catalytic activity	Ca2+ messenger system	*Paramuricea clavata*	Sea-whip	56.937	DAEELLSIAFR QYPDVKDAEELLSIAFR	2	3.4	17.453	0.0021
B3EZK6	Ribosomal protein S5	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Cyanea capillata*	Scyphozoa	19.095	SIAECLADELINASK	1	8.8	52.342	0.0021
A0A3M6UHA6	Eukaryotic translation initiation factor 5A	eIF-5A family	Translation factors	ribosome binding, translation elongation factor activit	translation, regulation of biological process, cellular component disassembly	*Pocillopora damicornis*	Coral	17.394	VHLVGLDIFTGK	1	7.6	16.938	0.0021
A0A6S7H7V7	26S proteasome regulatory subunit 6B	Proteasome family	Proteases	ATP binding, proteasome-activating activity	Ubiquitin proteasome pathway	*Paramuricea clavata*	Sea-whip	33.2	EAVELPLTHFELYK IQSVPLVIGQFLEAVDQNTGIVGSTTGSNYYVR RFDAQTGADREVQR	1	20.5	18.498	0.0020
A0A6P8J2Y0	unconventional myosin-Id-like	Myosin family	Cytoskeleton Component	actin binding, ATP binding, motor activity	actin filament organization, cellular localization, protein transport	*Actinia tenebrosa*	Sea anemone	116.66	NKDTLYQDFKR	1	1.1	6.0005	0.0020
A0A3M6TAB1	Cation_ATPase_N domain-containing protein	ATPase family	Cytoskeleton Component	ATP binding, P-type calcium transporter activity	cellular calcium ion homeostasis	*Pocillopora damicornis*	Coral	130.95	EIVAVTGDGTNDGPALK EIVAVTGDGTNDGPALKK VDESSLTGESDLVK	1	2.7	6.1783	0.0020
A0A2B4SZL5	Radixin	ERM family	Cytoskeleton Component	protein binding	linking proteins in the plasma membrane to the actin cytoskeleton	*Stylophora pistillata*	Coral	48.857	IGFPWSEIR	1	2.2	5.7572	0.0020
A0A6S7J8T1	Unconventional myosin-VI-like	Myosin family	Cytoskeleton Component	actin filament binding, ATP binding, motor activity	organelle transport, morphogenesis and functioning of the cell	*Paramuricea clavata*	Sea-whip	31.647	IVEANPLLESFGNAK	1	5.3	10.449	0.0020
A0A6S7FHP2	Splicing factor 3B subunit 1	SF3B1 family	RNA splicing factor	mRNA binding	mRNA processing, mRNA splicing, spliceosomal complex assembly	*Paramuricea clavata*	Sea-whip	124.69	AIVNVIGMNK VVDDLKDENEQYRK	2	2.1	11.733	0.0019
T2M8E4	RNA helicase	Helicases	Enzymes	RNA helicase activity, hydrolase activity, ATP binding, translation initiation factor activity	Protein biosynthesis	*Hydra vulgaris*	Hydra	73.345	APILIATDVASR MLDMGFEPQIR	1	3.5	6.052	0.0019
A0A3M6UPC1	Cupin_8 domain-containing protein	jumonji family	Transcription factors	metalloenzymes that adopt the cupin fold and are candidates for enzymes that regulate chromatin remodelling	histone demethylation mechanism	*Pocillopora damicornis*	Coral	49.214	HSLEVIQNYPDPK	1	3.1	5.8899	0.0019
A0A6S7FJL3	Acid-sensing ion channel 1 isoform X2	Epithelial sodium channel family	Cytoskeleton Component	sodium channel activity, ligand-gated ion channel activity	sodium ion transmembrane transport	*Paramuricea clavata*	Sea-whip	45.327	MLFPAITVCNYNPIR	1	3.8	5.6475	0.0019
A0A6P8J5R1	Arp2/3 complex 34	ARPC2 family	Cytoskeleton Component	protein binding, actin binding	organelle organization, protein polymerization, regulation of	*Actinia tenebrosa*	Sea anemone	34.145	VTVVFSTVFK	1	3.4	5.9804	0.0019

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	kDa subunit				actin filament polymerization								
A0A7M5XCK8	40S ribosomal protein S2	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Clytia hemisphaerica*	Hydromedusan	34.829	SLEQIYLYSLPIK	1	4	6.2554	0.0019
T2MHV5	Dolichyl-diphosphoo ligosacchari de--protein glycosyltra nsferase subunit 1	OST1 family	Transferase	transferase activity	protein glycosylation	*Hydra vulgaris*	Hydra	67.759	FIDHVFDDFVIDK	1	2.2	6.8172	0.0018
A0A6S7HCN4	Myoferlin-like isoform X2	Ferlin family	Cytoskeleton Component	receptor trafficking	plasma membrane organization	*Paramuricea clavata*	Sea-whip	34.476	SLNGEGNFNWR	1	3.7	6.0651	0.0018
T2M6Z7	26S proteasome non-ATPase regulatory subunit 1	Proteasome family	Proteases	enzyme regulator activity, endopeptidase activity	macromolecule catabolic process, protein metabolic process, regulation of biological process	*Hydra vulgaris*	Hydra	109.03	FGAILAQGIIDAGGR	1	1.5	6.2499	0.0018
A0A7M5U820	PHB domain-containing protein	Flotillin family	Cytoskeleton Component	protease binding	membrane trafficking, phagocytosis, and epidermal growth factor receptor signaling	*Clytia hemisphaerica*	Hydromedusan	29.546	AILGTLTVEEIYK AILGTLTVEEIYKDR GVAVTVTGVAQVK MGIEILSFTIK	1	14.6	5.7066	0.0018
A0A6P8HBR1	acyl-coenzyme A synthetase ACSM3	ANL superfamily	Toxin	ATP binding, fatty acid ligase activity, fatty-acyl-CoA synthase activity, metal ion binding	acyl-CoA metabolic process, lipid metabolism, regulation of blood pressure	*Actinia tenebrosa*	Sea anemone	69.412	ADDVILSSGYR	1	1.8	6.346	0.0017
A0A2B4RKG6	U6 snRNA-associated Sm-like protein LSm8	snRNP Sm proteins family	Ribosomal protein	RNA binding	RNA splicing, via transesterification reactions	*Pocillopora damicornis*	Coral	8.124	GFDQTVNLILDESHER	1	21.3	7.0851	0.0017
T2MGG5	Proteasome subunit alpha type	Peptidase family	Enzymes	creating an electrochemical gradient over the inner membrane	proteolysis, macromolecule catabolic process	*Hydra vulgaris*	Hydra	29.356	LGFQLYQSDPSGNYSGWK	1	6.9	22.4	0.0017
A0A6S7KFV7	Serine threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	Armadillo repeat	Enzymes	protein-protein interaction surfaces	intracellular transport processes	*Paramuricea clavata*	Sea-whip	18.578	KLSTIALALGVER	1	7.7	5.8622	0.0017
A0A2B4R5X1	Receptor protein-tyrosine kinase	Receptor tyrosine kinases (RTKs)	Receptor	transmembrane receptor protein tyrosine kinase activity	cellular processes including growth, motility, differentiation, and metabolism.	*Stylophora pistillata*	Coral	57.35	MVLIQELEMLRK	1	2.4	5.7294	0.0016
A0A6S7FYI7	Phosphoenolpyruvate carboxykinase	Phosphoenolpyruvate carboxykinase family	Enzymes	GTP binding, kinase activity, metal ion binding, phosphoenolpyruvate carboxykinase (GTP) activity	glucose homeostasis, glucose metabolic process, fatty acid catabolic process, response to lipid, cellular carbohydrate biosynthetic process, cellular response to oxygen-containing compound, alcohol biosynthetic process, cellular chemical homeostasis, cellular response to organic substance, triglyceride biosynthetic process, response to starvation	*Paramuricea clavata*	Sea-whip	71.857	FLWPGFGENSR	1	1.7	6.3165	0.0016
T2MGT3	Calcium-transporting ATPase	Cation transport ATPase family	transport protein	ATP binding, P-type calcium transporter activity	Calcium transport, ion transport	*Hydra vulgaris*	Hydra	110.46	TGTLTTNQMSVNK	1	1.3	6.3692	0.0015
A0A6P8IBR2	Coatomer subunit beta	Coatomer family	Cytoplasmic protein	structural molecule activity	intracellular protein transport, vesicle-mediated transport	*Actinia tenebrosa*	Sea anemone	106.92	LKEPELLEPLMPAIR	2	2.7	11.353	0.0014

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									VLQELVMDILR				
A0A7M6DLD4	Phosphoenolpyruvate phosphomutase	Phosphoenolpyruvate mutase family	Enzymes	catalytic activity, phosphoenolpyruvate mutase activity	organic phosphonate metabolic process	*Clytia hemisphaerica*	Hydromedusan	35.265	VEAFIAGWGLEEALKR	1	5.1	6.1202	0.0014
A0A7D9HE72	Cytoplasmic dynein 1 heavy chain 1	dynein heavy chain family	Motor protein	ATP binding	cytoplasmic microtubule organization	*Paramuricea clavata*	Sea-whip	374.45	FTQDMQPHYIYSPR	1	0.4	5.8077	0.0014
A0A7D9I9R3	Betaine-homocysteine S-methyltransferase 1	Homocysteine S-methyltransferase	Enzymes	betaine-homocysteine S-methyltransferase activity, zinc ion binding	methionine biosynthetic process, methylation	*Paramuricea clavata*	Sea-whip	38.847	QGFIDLPEFPFALEPR	1	4.6	25.639	0.0013
A0A7M6DM89	Prohibitin	Prohibitin family	Regulatory protein	DNA synthesis inhibiting	regulation of cell proliferation	*Clytia hemisphaerica*	Hydromedusan	12.804	SVVAQFNASQLITMR	1	13.3	6.6772	0.0013
A0A7D9HQD3	Poly(U)-specific endoribonuclease	ENDOU family	Enzymes	endoribonuclease activity, metal ion binding, RNA binding	response to cellular stress	*Paramuricea clavata*	Sea-whip	33.183	AFIALLDNYER	1	3.9	7.4884	0.0013
A0A7M5VG37	Coatomer subunit beta	Coatomer family	Cytoplasmic protein	structural molecule activity	intracellular protein transport, vesicle-mediated transport	*Clytia hemisphaerica*	Hydromedusan	108.21	HSEILQANLK	1	1	13.573	0.0012
A0A6S7IIE5	methylcrotonoyl-CoA carboxylase beta chain	ClpP/crotonase	Enzymes	ligase activity, Biotin carboxylase activity	Biotin transport and metabolism	*Paramuricea clavata*	Sea-whip	93.345	ISVMGGEQAATVLATITK NIPLIFLQNITGFMVGR	2	4.1	15.868	0.0012
A0A3M6TZW6	Tr-type G domain-containing protein	GTPase Family	Miscellaneous	GTPase activity, GTP binding	Translation	*Pocillopora damicornis*	Coral	93.474	GTVGFGSGLHGWAFTLK	1	2	7.2115	0.0012
A0A2B4SU39	Unconventional myosin-VI	Myosin family	Cytoskeleton Component	actin filament binding, ATP binding, motor activity	organelle transport, morphogenesis and functioning of the cell	*Stylophora pistillata*	Coral	115.82	QSQSLIVSGESGAGK	1	1.5	14.405	0.0011
A3F9E8	Histone H2B	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Anacropora matthai*	Coral	12.869	VLKQVHPDTGLSSK	1	12	6.1316	0.0011
A0A6P8I0E1	RNA helicase	Helicases	Enzymes	RNA helicase activity, hydrolase activity, ATP binding, translation initiation factor activity	Protein biosynthesis	*Actinia tenebrosa*	Sea anemone	49.077	TAAYLIPLLER	1	2.5	5.8925	0.0010
A0S5U7	40S ribosomal protein S7	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Hydra vulgaris*	Hydra	13.343	TLTAVHDAILEDLCFPSEIVGK	1	19.1	6.4388	0.0010
A0A7M5V9W6	Glutaminyl-tRNA synthetase	class-I aminoacyl-tRNA synthetase family	Enzymes	ATP binding, glutamine-tRNA ligase activity	glutaminyl-tRNA aminoacylation, translation, cellular amino acid metabolic process, RNA metabolic process	*Clytia hemisphaerica*	Hydromedusan	93.307	SEIDMQLLDLLGPK	1	1.7	6.1466	0.0010
T2MEV0	Small monomeric GTPase	small GTPase	Miscellaneous	G protein activity, GTP binding	cell communication, Rab protein signal transduction, response to stimulus	*Hydra vulgaris*	Hydra	26.787	ETYNTLTTWLTDAR	1	5.8	16.474	0.0010
A0A7D9HEZ4	ADP-ribosylation factor 1	ARF family	GTP-binding protein	GTPase activity, GTP binding	regulation of vesicular traffic and actin remodelling	*Paramuricea clavata*	Sea-whip	12.641	ILILGLDGAGK	1	9.8	5.7443	0.0009
T2MEA3	Myosin-Ie (Fragment)	Myosin family	Cytoskeleton Component	actin filament binding, ATP binding, motor activity	organelle transport, morphogenesis and functioning of the cell	*Hydra vulgaris*	Hydra	112.19	NFVGDYIGLDDNPALR NPESLFLLEELR	2	2.8	37.944	0.0009
A0A6S7JHC0	Cullin-associated NEDD8-dissociated 1	CAND family	Enzymes		protein ubiquitination, protein-containing complex assembly	*Paramuricea clavata*	Sea-whip	29.144	HTVDDGLDIRK	1	4.2	5.9562	0.0009
T2M767	Calcium-binding protein p22	Aequorin family	Photoprotein	calcium ion binding, intracellular Ca2+ indicator	bioluminescence	*Hydra vulgaris*	Hydra	22.274	IPELAINPLGER	1	6.2	5.7901	0.0009
T2MEP4	Small nuclear	snRNP core protein family	Ribosomal protein	processing of pre-mRNAs to mature mRNAs, and	spliceosomal snRNP assembly, mRNA processing, mRNA	*Hydra vulgaris*	Hydra	18.233	NGTVAHGTITGVDMSMNTHLK	1	12.4	7.2816	0.0008

Accession Number	Protein Identify	Protein family	Protein type	InterProScan Protein Function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Peptide sequences	Razor + unique peptides	Sequence coverage [%]	Score	%iBAQ
	ribonucleoprotein Sm D1			are a major component of spliceosome	splicing								
A0A2B4SSG8	Actin	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Stylophora pistillata*	Coral	26.77	AVFPSIVGR AVFPSIVGRPR DLYANTVLSGASTMYPGIADR GYSFTTTAER	1	17.3	5.7348	0.0008
A0A7M5X1K0	Proteasome subunit alpha type	Peptidase family	Enzymes	creating an electrochemical gradient over the inner membrane	proteolysis, macromolecule catabolic process	*Clytia hemisphaerica*	Hydromedusan	26.945	LGSTAIGIQTSEGVVLAVER	1	8.2	5.9844	0.0008
A0A6P8HKU3	28S ribosomal protein S23	Ribosomal protein	Ribosomal protein	rRNA binding, structural constituent of ribosome	translation	*Actinia tenebrosa*	Sea anemone	19.615	PVWFDVVEAFPPIVETK	1	10	6.5247	0.0007
A0A6S7GD58	Ubiquilin-4	UBA/Ubiquitin family		identical protein binding	cellular response to DNA damage stimulus	*Paramuricea clavata*	Sea-whip	60.544	ALSNLESLPGGFNALR	1	2.8	6.5631	0.0006
A7RFQ0	Histone H2A	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Nematostella vectensis*	Sea anemone	15.067	NDEELNKLLQGVTIAQGGVLPNIQAVLLPK	1	21.4	8.8995	0.0006
A0A1V0IGH5	Histone H2B	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Hydra vulgaris*	Hydra	14.635	AMSIMNSFVNDIFER	1	11.2	10.792	0.0006
A0A6P8HM54	cAMP-dependent protein kinase catalytic subunit 1-like isoform X2	protein kinase	Enzymes	ATP binding, protein serine/threonine kinase activity	protein phosphorylation	*Actinia tenebrosa*	Sea anemone	40.203	DNSNLYMVLEFVPGGEMFSHLR	1	6.4	5.6697	0.0006
A7SMT0	Ras-related protein Rab-21	Ras family	Miscellaneous	GTPase activity, GTP binding	membrane trafficking control	*Nematostella vectensis*	Sea anemone	24.221	DSNGAILVYDITDEDSFLK	1	8.8	22.131	0.0006
A0A6P8II37	protein dpy-30 homolog	dpy-30 family	Chromatin regulator	protein binding, histone methyltransferase complex, protein homodimerization activity	transcription, DNA-templated, chromatin silencing at telomere, histone H3-K4 methylation	*Actinia tenebrosa*	Sea anemone	12.738	AYLDQTVVPILLQGLATLSK	1	17.4	6.9151	0.0005
A0A7M5XFC1	PIPK domain-containing protein	Phosphatidylinositol-4-phosphate 5-kinasse family	Cytoskeleton Component (membrane traffic protein)	ATP binding, phosphatidylinositol phosphate kinase activity	phosphatidylinositol metabolic process	*Clytia hemisphaerica*	Hydromedusan	74.941	EATFLQQLLPGYYMNLHQNPR	1	3.2	6.0794	0.0005
A0A6P8IXB5	uncharacterized protein LOC116306116	EF-hand	Calcium-binding protein	calcium ion binding, catalytic activity	Ca2+ messenger system	*Actinia tenebrosa*	Sea anemone	64.12	KEEYMSAVGLALLFDR	1	2.9	5.8844	0.0004
T2MDH6	Tight junction protein ZO-1	Tight junction protein family	tight junction	cell adhesion molecule binding	cell-cell adhesioncell-cell junction organizationprotein localization to cell junction	*Hydra vulgaris*	Hydra	187.56	AYQVTDDTVSVLVDHF	1	1	6.3327	0.0004
T2M3H0	26S proteasome non-ATPase regulatory subunit 13	Proteasome family	Proteases	maintenance of protein homeostasis by removing misfolded or damaged proteins	ubiquitin-dependent protein catabolic processu proteasome assembly	*Hydra vulgaris*	Hydra	43.512	ITLLSLMELTFK	1	3.2	6.824	0.0004
A0A6B2G0C0	Clathrin heavy chain	Clathrin	Cytoplasmic protein	polymerizes into a polyhedral lattice on intracellular membranes to form protein-coated membrane vesicles	intracellular protein transport, vesicle-mediated transport	*Myxobolus squamalis*	Myxozoa	29.118	NLQNLLLLTAIK	1	4.7	5.6471	0.0004
A0A7M5WTA2	Septin	Septin family	GTPase	GTP binding	cell cycle	*Clytia hemisphaerica*	Hydromedusan	48.653	GLENYVGFANLPNQVFR	1	4	7.2302	0.0004
A0A7D9D847	Histone H2A	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Paramuricea clavata*	Sea-whip	14.511	LLSGVTIAQGGVLPNIQAVLLPK LLSGVTIAQGGVLPNIQAVLLPKK NDEELNKLLSGVTIAQGGVLPNIQAVLLPK VGAGAPVYMAAVLEYLSAEILELAGNAAR	1	43.8	9.139	0.0004

Accession Number	Protein Identify	Protein family	Protein type	InterProScan Protein Function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Peptide sequences	Razor + unique peptides	Sequence coverage [%]	Score	%iBAQ
A0A7M5VA08	Histone deacetylase	Histone deacetylase family	Enzymes	deacetylase activity	chromatin regulator	*Clytia hemisphaerica*	Hydromedusan	67.678	FNLPLLILGGGGYTIR	1	2.6	5.8663	0.0004
A0A6P8HB84	Arginyl-tRNA synthetase	tRNA ligase family	Catalyse	arginine-tRNA ligase activity, ATP binding	Protein biosynthesis (translation), cellular amino acid metabolic process, RNA metabolic process	*Actinia tenebrosa*	Sea anemone	82.111	MLDDKGNTAVYLLYALTR	1	2.5	6.3369	0.0003
A0A6S7I0Z1	Radixin	ERM family	Cytoskeleton Component	protein binding	linking proteins in the plasma membrane to the actin cytoskeleton	*Paramuricea clavata*	Sea-whip	65.994	FFPEDVSEELIQEVTQR	1	3	6.5575	0.0003
A0A6S7FUV7	Coiled-coil domain-containing 22 homolog	CCDC22 family	Cytoskeleton Component	protein binding	ubiquitin-dependent protein catabolic process, positive regulation of ubiquitin-dependent protein catabolic process	*Paramuricea clavata*	Sea-whip	60.813	MEEVDNIIIHTLR	1	2.4	6.3751	0.0003
Q6WBY4	Beta actin	Actin family	Cytoskeleton Component	ATP-binding, Nucleotide-binding	cellular component organization	*Exaiptasia diaphana*	Sea anemone	41.824	AVFPSIVGR AVFPSIVGRPR CPEALFQPSFLGMESAGIHETTYNSIMK DLTDYLMKILTER DSYVGDEAQSK DSYVGDEAQSKR EKMTQIMFETFNSPAMYVAIQAVLSLYASGR GYSFTTTAER;HQGVMVGMGQK HQGVMVGMGQKDSYVGDEAQSK HQGVMVGMGQKDSYVGDEAQSKR IWHHTFYNELR KYSVWIGGSILASLSTFQQMWISK MTQIMFETFNSPAMYVAIQAVLSLYASGR SYELPDGQVITIGNER VAPEEHPVLLTEAPLNPK VAPEEHPVLLTEAPLNPKANR YPIEHGIVTNWDDMEK YSVWIGGSILASLSTFQQMWISK	1	54.5	5.8683	0.0003
A0A248SNM5	NADH-ubiquinone oxidoreductase chain 1	NADH:ubiquinone oxidoreductase	Transmembrane protein	oxidoreductase activity, NADH dehydrogenase (ubiquinone) activity	aerobic respiration	*Zignisis sp.*	Sea-whip	6.6872	KGPNVVGVYGLLQPLADGLK	1	32.3	5.8811	0.0003
T2ME72	Methylmalonate-semialdehyde dehydrogenase [acylating]	Aldehyde dehydrogenase family	Oxido-reductive	oxidoreductase activity, malonate-semialdehyde dehydrogenase (acetylating) activity, methylmalonate-semialdehyde dehydrogenase (acylating) activity	pyrimidine nucleobase catabolic process, alpha-amino acid metabolic process, cellular amino acid catabolic process	*Hydra vulgaris*	Hydra	57.144	EMTLNQLVGAAFGAAGQR	1	3.4	5.6789	0.0002
A0A3M6U657	G_PROTEIN_RECEP_F1_2 domain-containing protein	G-protein coupled receptor family	Receptor	G protein-coupled receptor activity	signal transduction pathways	*Pocillopora damicornis*	Coral	32.856	MDISFFIFARK	2	3.8	9.3775	0.0002
A0A6P8HLH0	Histone H2B	Histone	Cytoskeleton Component	nucleic acid binding protein binding	DNA damage, transcriptional activation, heterochromatinisation process	*Actinia tenebrosa*	Sea anemone	15.897	AMGIMNSFVNDLFER	1	10.4	6.245	0.0001

Dataset 2 Toxin protein identification in *C. indrasaksajiae* venom from cnidarian database.

Accession Number	Protein Identify	Protein type	Protein function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Sequence coverage [%]	Score
A7L035	Toxin CfTX-1 precursor; (Short=Toxin 1)	Pore-Forming toxin	- Hemolytic activity (potent) - Cardiotoxin	hemolysis in other organism, ion transport, effects on the cardiovascular system	*Chironex fleckeri*	Box jellyfish	51.39	36	323.31
A7L036	Toxin CfTX-2 precursor; (Short=Toxin 2)	Pore-Forming toxin	- Hemolytic activity (potent) - Cardiotoxin	hemolysis in other organism, ion transport, effects on the cardiovascular system	*Chironex fleckeri*	Box jellyfish	51.684	29.4	323.31
T1PRE3	Toxin CfTX-A precursor; (AltName: Full=Toxin A; Short=TX-A)	Pore-Forming toxin	- Hemolytic activity (potent) - minor effects on the cardiovascular system	Cytolysis, Hemolysis	*Chironex fleckeri*	Box jellyfish	50.485	17	126.65
P58762	Toxin CqTX-A precursor; (Short=CQT-A; Short=Toxin A)	Pore-Forming toxin	- Hemolytic activity (potent) - Critical allergen - Causes cutaneous inflammation	hemolysis in other organism, ion transport, Cytolysis, effects on the cardiovascular system, Heat-stable protein that continues to cause erythematous skin eruption in the patient and to recognize IgE after heating.	*Chiropsoides quadrigatus*	Box jellyfish	51.801	30.3	22.178
T1PQV6	Toxin CfTX-B precursor; (AltName: Full=Toxin B; Short=TX-B)	Pore-Forming toxin Delta-Endotoxin (insectocide)	- Hemolytic activity (potent) - minor effects on the cardiovascular system	Cytolysis, Hemolysis	*Chironex fleckeri*	Box jellyfish	51.116	13	24.962
A0A1I8HRR7	Mitogen-activated protein kinase; (EC=2.7.11.24)	Neurotoxin	ATP binding DNA binding G protein-coupled peptide receptor activity MAP kinase activity metal ion binding protein serine kinase	DNA integration DNA recombination	*Macrostomum lignano*	Flatworm	447.21	0.7	14.842

Accession Number	Protein Identify	Protein type	Protein function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Sequence coverage [%]	Score
			activity protein threonine kinase activity						
A0A2D4KTV9	Rab GDP dissociation inhibitor (Fragment)	Hemostasis impairing toxin	- GTPase activator activity - L-amino-acid oxidase activity - Rab GDP-dissociation inhibitor activity - GTPase activation	- protein transport - small GTPase mediated signal transduction	*Micrurus paraensis*	Snake	14.018	15.2	48.809
A0A2N1A2B1	MARTX multifunctional-autoprocessing repeats-in-toxin holotoxin RtxA		- calcium- and calmodulin-responsive adenylate cyclase activity - cysteine-type peptidase activity - metal ion binding - toxin activity	Virulence	*Psychromonas sp.*	Bacteria	472.83	0.5	6.2411
A0A670I9L0	Peptidase M12B domain-containing protein	Zinc metalloprotease	metalloendopeptidase activity toxin activity	proteolysis, play roles in cell signaling, cell fusion, and cell-cell interactions	*Podarcis muralis*	Lizard	19.084	4.2	6.7832
A0A0Q4MSR4	Haemagg_act domain-containing protein (Fragment)	Haemolysin	- Cysteine endopeptidases - carbohydrate-dependent haemagglutination activity	Virulence, Polysaccharide metabolism and transport	*Erwinia sp.*	Bacteria	256.14	0.3	6.5401
A0A6N6VWA2	Endotoxin_N domain-containing protein		toxin activity	cytolysis by symbiont of host cells, sporulation resulting in formation of a cellular spore	*Silvanigrella paludirubra*	Bacteria	62.571	1.8	5.7896
A0A2R6EAJ7	Ribonuclease VapC; (Short=RNase VapC; EC=3.1.-.-; AltName: Full=Toxin VapC)	Hydrolase	- magnesium ion binding - ribonuclease activity - toxin activity - Hydrolases; Acting on ester bonds	Toxic component of a toxin-antitoxin (TA) system	*Halobacteriales archaeon*	Bacteria	14.54	14.8	7.844

Accession Number	Protein Identify	Protein type	Protein function	Biological process	Species of Closet	Organism group	Mol. weight [kDa]	Sequence coverage [%]	Score
A0A6P6YCL4	26S proteasome regulatory subunit 6A-B-like	Neurotoxin, Presynaptic neurotoxin	ATP binding, proteasome-activating activity, toxin activity	exocytosis, protein catabolic process	*Dermatophagoides pteronyssinus*	Dust mite	86.209	2.2	6.432
A0A1B1LHQ5	Putative insecticidal binary-like toxin		- Insecticidal crystal toxin - toxin activity	Virulence, modulation by symbiont of host defense response	*Bacillus thuringiensis*	Bacteria	41.341	1.7	8.1739
V7IBB3	Toxin CdiA	Haemolysin Polymorphic toxins	- toxin activity	Virulence	*Eikenella corrodens*	Bacteria	345.5	0.6	7.2283
A0A2D3TFQ5	Peptidase C80 domain-containing protein	Metallopeptidases	- cysteine-type peptidase activity - metal ion binding - autolytic activity - toxin activity	Autolysis, Virulence, Toxin causes actin crosslinking which leads to depolymerization and rounding of cells	*Candidatus Hamiltonella*	Bacteria	198.59	1.2	8.0899
A0A6J1TPU2	F-actin-monooxygenase MICAL2	Hemostasis impairing toxin	actin binding, FAD binding, L-amino-acid oxidase activity, metal ion binding, monooxygenase activity, NADPH:sulfur oxidoreductase activity	actin filament depolymerization, positive regulation of transcription via serum response element binding	*Notechis scutatus*	Snake	209.12	0.4	6.0442

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และ Chiropsoides buitendijki

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Senior Project

Environmental Factors Affecting the Species and Abundance of Box Jellyfish at Libong Island, Trang Province

The Degree of Bachelor of Science in Computational Science Walailak University 2017