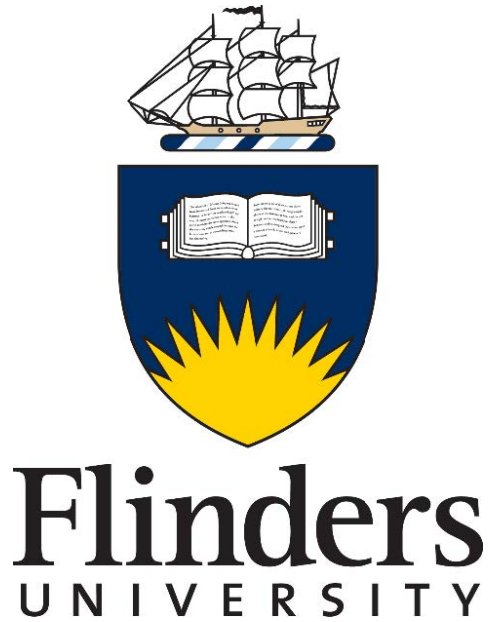
Developing a Yeast Based Screening System for Sea Anemone Actinoporins

Ly Kim

28 June 2021



School of Biological Sciences

College of Science and Engineering

Flinders University, South Australia

A thesis submitted in fulfilment of the requirements for the degree of

Master of Biotechnology

Declaration

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

Ly Kim

28 June 2021

Acknowledgements

Firstly, I would like to express my deepest gratitude to my supervisor Professor Catherine Abbott for providing me an opportunity to undertake the research in her laboratory. I am very grateful for her time and effort in guiding me and continual laboratory support throughout this research project. I would like to thank Dr. Sunita Ramesh, my co-supervisor for providing me the yeast strains and her advice on how to grow the yeast. I also thank Cassie Hoepner, a Ph.D. student in the lab for her anemone venom samples that I used throughout this project. Special thanks to Mr. Robert Qiao, a Ph.D. student for analysis of the transcriptomic data. I am also thankful for my classmate Mona Kaspal for her encouragement when I faced difficult times. This thesis would not have been possible without the Australia Awards Scholarship. I would like to express my sincere thanks towards to the Australia government for providing this prestige scholarship for me to study a Master's degree in Australia. Finally, I would like to thank my friends and family for emotional support and encouragement during my study.

Table of Contents

List of Tables

List of Figures

Abstract

Type II cytolysins, also known as actinoporins, are abundant in sea anemone venom. Due to their ability to form a cation-selective conduction pathway which allows ions or molecules to move in and out of cells to make an osmotic imbalance which can lead to cell death, actinoporins are of great interest for development as cancer therapeutics. This research firstly aimed to validate the partial actinoporin sequence from the sea anemone *Entacmae quadricolor*. Secondly, the research aimed to develop and assess a *Saccharomyces cerevisiae* yeast screening system for evaluating actinoporins from *E. quadricolor* and other anemone species and to compare this assay to the established haemolysis assay. This research hypothesized that yeast would be an effective system that could be used for screening potential actinoporins for development as anti-cancer therapeutic drugs.

Multiple PCR products representing the *E. quadricolor* gene were obtained and purified. Sanger sequencing of these products was not successful, but this was due to the PCR products containing more than one sequence/gene. A Blast search of the newly assembled *E. quadricolor* transcriptome identified the possible presence of 14 highly similar actinoporin genes, some of which contain multiple isoforms. Venoms from *E. quadricolor* were collected at three time points, before hosting a Pairs of *Amphiprion percula* anemonefish, four weeks after hosting the fish, and four weeks after the fish were removed. These venoms were used to test the haemolysis and yeast screening assay.

The sheep erythrocyte haemolysis assay showed that all venom samples tested at concentration 0.5 μg/mL to 8 μg/mL could lyse the cells but there was a lot of variation in EC_{50} at 415 nm of samples collected at the three time points tested. The yeast assay was developed using two different wild type *Saccharomyces cerevisiae* strains, BY4741 and Invsc2. Anemone venom was able to inhibit yeast growth up to 61% in a dose dependent manner at protein concentrations of 100 μg/mL. However, the yeast assay was unable to discriminate between

the potency of the different venoms in the same manner as the hemolysis assay. The determination of the *E. quadricolor* genome and cloning of individual actinoporin genes products is required to validate the number of actinoporin genes expressed in the *E. quadricolor* transcriptome. The variability of the results observed in the haemolysis assay could be improved if a method for fractionating and obtaining a pure 15- 20 kDa fraction were developed using HLPC. Once a purer actinoporin containing fraction is obtained from the venom, it could be used in the yeast assay to determine the role of the actinoporin in slowing yeast growth. Hence, the yeast screening assay could be used for screening actinoporins for development as anticancer drugs.

Abbreviations

2DE	two-dimensional gel electrophoresis
3D	three-dimensional
ANOVA	analysis of variance
An	anemone
aa	amino acid
BCA	bicinchoninic acid
BME	2-mercaptoethanol
BUSCO	benchmarking universal single copy orthologs
bp	base pair
BSA	bovine serum albumin
Cat no.	catalogue number
cDNA	complementary deoxyribonucleic acid
°C	degree Celsius
EDTA	ethylenediaminetetraacetic acid
g	gram
x g	times gravity
GC	Guanine cytosine
h	hour
IBC	Institutional Biosafety Committee

HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
Kb	kilobase
KCl	potassium chloride
kDa	kilo Dalton
KH_2PO_4	potassium dihydrogen phosphate
L	litre
M	molar
mg	milligram
mins	minutes
mL	millilitre
mM	millimolar
MW	molecular weight
µg	microgram
µL	microliter
µm	micrometre
µM	micromolar
NaCl	sodium chloride
Na_2HPO_4	disodium phosphate
NaOH	sodium hydroxide

NB	note
ng	nanogram
nM	nanomolar
NS	not significant
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ref	reference
RNA	ribonucleic acid
RIN	RNA integrity number
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	seconds
TAE	Tris-acetate-EDTA
Tm	Melting temperature
V	volt
v/v	volume per volume
w/v	weight per volume

WT	wild type
U	unit

Chapter 1. Introduction

Cancer is one of the world's major causes of death. There were 9.6 million deaths globally in 2018 due to cancer (World Health Organization, 2018). Cancer is the abnormal growth of cells in the body and malignant cancer cells can invade adjacent tissues and spread to other organs through blood and lymphatic systems (Cooper & Hausman, 2000). The most common cancer types are breast, lung, colorectal, and prostate cancer (Nagai & Kim, 2017). As a result of the war on cancer, many treatment options are available such as surgery, chemotherapy, and radiation therapy. However, these treatments are not efficient enough to eliminate all cancers and they are associated with many side effects (Hanahan, 2014). Therefore, there is a continuing demand for new therapeutic drugs for cancer. Marine resources have been explored as a source of naturally derived compounds for development of cancer therapeutics. Sea anemones are of great interest because of their complex venoms. There are about 12,000 species of sea anemones which can be divided into 46 families (Madio et al., 2019). Sea anemones are from the phylum Cnidaria. Venom is produced by specialized cells called nematocysts (Madio et al., 2017). Venom is used by sea anemones to catch prey, defend themselves against predators, and acquire food (Sanderson et al., 2016). The venom of sea anemones is a mixture of toxins. There are over 1500 toxin-like sequences and they are grouped mainly into 38 protein families (Madio, 2018). These proteins are categorized into four main functional groups: phospholipase A2, cytolysins, neurotoxins, and non-proteinaceous compounds (Madio et al., 2019). The main components in the venom are cytolysins and neurotoxins (Madio et al., 2017). Cytolysins are pore forming toxins and there are five types: cytolysin I - V. The most abundant cytolysins from sea anemones are type II cytolysins which are commonly known as actinoporins (Madio et al., 2019). Actinoporins bind to the cell membrane by sphingomyelin and form a cation-selective conduction pathway which allows ions or molecules to move in and out of the cell leading to osmotic imbalance and cell

cytotoxicity (Ramírez-Carreto et al., 2020). Due to these effects, actinoporins may be used to induce apoptosis in cancer cells. Actinoporin cytotoxic action in combination with anticancer medications has been investigated in tumor cell lines, and they have been shown to improve the efficacy of certain anticancer drugs (Soletti et al., 2008) . Thus, the venom from sea anemones is a novel untapped source for anticancer molecules. However due to the number of sea anemones species, further development in this field will only be possible if we have an effective system for high throughput screening of multiple candidates.

1.1 Cancer and apoptosis

Cancer is a state of disease in which cells are undergoing uncontrolled proliferation. Cancer can be triggered by the genetic alteration in somatic cells, except some cancers that are caused by lifestyle and environmental factors (Ponder, 2001). The genetic alteration or epigenetic changes lead to changes of specific region in the genetic code that result in cell cycle deregulation and tumor formation. Genetic alterations include amplification of oncogenes and mutation or malfunction of tumor suppressor genes (Beckmann et al., 1997). Carcinogenesis is a multistep process that is divided into three stages. The first stage is initiation, which involves irreversible mutation in the cell. The second stage is promotion, which is the expansion of a mutated cell into a tumor. The third stage is progression, whereby the benign lesion or preneoplastic cells goes through inheritable changes to become a malignant neoplasma (Barrett & Wiseman, 1987). Vogelstein and Kinzler (1993) also assert that mutation in one cell can proceed to a second mutation in the cell. The cells become malignant as they accumulate mutations and have the ability to invade surrounding tissues and organs. (Vogelstein & Kinzler, 1993).

There are six essential hallmarks of cancer. Cancer cells acquire capabilities such as; self-sufficiency in growth signals, evading apoptosis, insensitivity to growth signals, sustaining angiogenesis, limited replicative potential, and tissue invasion and metastasis (Hanahan &

Weinberg, 2000). One of the most important pathways targeted in cancer treatment is apoptosis, also known as programmed cell death. Apoptosis is triggered by the intrinsic and extrinsic pathways, which activate the caspase pathway and the mitochondria-mediated pathway in response to physiological and pathological stimulation. (Johnstone et al., 2002; Elmore, 2007). Apoptosis has been targeted in some cancers therapies including irradiating tumors and treated tumors with cytotoxins (Gerl & Vaux, 2005). Apoptosis studies have helped researchers to accumulate knowledge about how cancer progresses and to develop therapeutic strategies. Most chemotherapy drugs use the apoptosis pathway to target cancer cells for cell death (Johnstone et al., 2002). However, cancer cells can develop drug resistance by several mechanisms, including efflux drug pumps, inhibition of drug activity, damaged DNA repair, changing drug target, inhibition of cell death and the epithelial-mesenchymal transition (Housman et al., 2014). Moreover, chemotherapy or radiation therapy often result in side effects. Therefore, it is essential to look for new compounds that could directly induce apoptosis and are also less harmful to normal cells.

1.2 Marine bioactives as anticancer drugs

For hundreds of years, natural compounds have played an essential part in medicine. Many therapeutics are sourced from plant, animals, microbes, and marine species (da Rocha et al., 2001). For example, a plant-derived compound, toxol showed an anticancer effect against various types of cancer (Wani et al., 1971); the immunosuppressant cyclosporine A was discovered from a microbe (Flechner, 1983), and Ecteinascidin 743 from the marine tunicate *Ecteinascidia turbinate* has demonstrated antitumor activities (Rinehart, 2000; Erba et al., 2001).

Natural products can be found in abundance in marine organisms. There are about one to two millions marine species (Simmons et al., 2005). More than 10,000 compounds have been identified during the past decades (Sithranga Boopathy & Kathiresan, 2010). With the

development of newer collection techniques, structure determination and molecular and compound synthesis techniques, more bioactive products from marine sources have been studied and discovered to enable new drugs candidates with antibacterial, antiviral and anticancer effects in the treatment of various diseases (Jha & Zi-Rong, 2004).

In 2015, there were 13 marine-derived products in clinical trials, and nine are in use as medicines, four of which are for cancer therapy (Rangel & Falkenberg, 2015). Cytarabine (Ara-C), a nucleoside derived from the Caribbean sponge *Cryptotheca crypta*, was the first anticancer drug to be approved in 1972 for the treatment of leukemia and lymphoma. (McConnell et al., 1994). Another drug of marine origin for cancer therapy is Eribulin a synthetic derivative of a polyether macrolide (Halichondria B) derived from the marine sponge *Halichondria okadai* that was licensed by the Food and Drug Administration (FDA) in 2010 for treatment of metastatic breast cancer (Hirata & Uemura, 1986; Liu et al., 2012a). Trabectedin is an alkaloid derived from the tunicate *Ecteinascidia turbinata*. In 2015, it was approved for the treatment of soft tissue sarcoma and ovarian cancer patients. (Galmarini et al., 2014; Khalifa et al., 2019). Brentuximab vedotin is an antibody-drug conjugate that was approved in 2011 to treat Hodgkin lymphoma and systemic anaplastic large cell lymphoma, and it was approved in 2018 to treat peripheral T-cell lymphoma in combination with chemotherapy. (Richardson et al., 2019). It is a synthetic analog of the sea hare *Dolabella auricularia's* monomethyl auristatin E. (Doronina et al., 2003). As a result, marine organisms are a promising future source for drug discovery. With an increase in the number of compounds isolated each year, it is expected that there will be more anticancer drugs derived from the marine sources approved for use in cancer treatment.

1.3 Sea anemone and symbiosis

Sea anemone or flower of the sea is in the phylum Cnidaria, class Anthozoa, order Actiniaria (Frazao, 2012; Madio et al., 2017) . It is one of the most venomous animals on earth. There are approximately 12,000 sea anemone species and they are classified into 46 families (Madio et

al., 2019). Sea anemones are marine invertebrates that lack the free-swimming medusa stage unlike other cnidarias. Sea anemones are a single polyp that attaches to the sea floor, but some other species are found in soft sediments and a few float on the water surface (Thangaraj et al., 2019). They are found in all depths and altitudes of marine habitats (Daly et al., 2008). Sea anemones can reproduce sexually or asexually (Galliot & Schmid, 2003). Sea anemones are also considered as predatory animals. They use venom in order to defend against predators and to capture prey (Frazao, 2012). Venoms are recognized as the most important aspect of a sea anemone for its survival in the marine environment and its ability to adapt to the changes in their habitats. Venom of sea anemones are of high interest for researchers to explore their pharmacological compounds and properties.

Some sea anemones form a symbiotic relationship with zooxanthellae. Sea anemones use the oxygen and foods from the photosynthesis from the algae, in return, sea anemones provide protection and exposure to sunlight (Dubinsky & Stambler, 2010). Only 10 species of sea anemones have found to host the anemonefish which are in the families of Actiniidae, Stichodactylidae and Thalassianthidae (Fautin et al., 1992). The symbiotic interaction between sea anemones and anemonefish has received little research, to date the relationship between hosting fish and venom production has not been studied, but it is believed that the selectivity of sea anemones for anemonefish host is based on the range of venom toxicity (Nedosyko et al., 2014). This mutualistic relationship provides benefits to both sea anemones and anemonefish, where sea anemones are protected from the predatory reef fish by hosting anemonefish (Allen, 1975; Fautin, 1991), and anemonefish and their eggs are protected from predators thus increasing their life span (Buston & García, 2007; Elliott et al., 1994; Arvedlund et al., 2000). A study by Mebs (1994) indicated that anemonefish are resistant to the toxin of sea anemones because of the mucus secreted from their skin layer.

Entamae quadricolor is known as the bubble-tip anemone (Figure 1). It is a universal sea anemone that can be found throughout the Indo-Pacific (Nanninga et al., 2014). *E. quadricolor* hosts the most anemonefishes and there is a competition among anemonefishes for this sought after species. This sea anemone possesses intermediate toxicity which is suitable for anemonefishes to survive (Nedosyko et al., 2014). It also exhibits biofluorescence including green fluorescent protein and red fluorescent protein (Wiedenmann et al., 2005). The phylogenetic analysis of *E. quadricolor* DNA sequence demonstrated that it is in the phylum cnidaria which is divided into two groups Anthozoa, containing Hexacorallia and Octocorallia subclasses, and Medusozoa, including Staurozoa, Cubozoa, Scyphozoa, and Hydrozoa. (Chen et al., 1995; Rivera-de-Torre et al., 2020).

Figure 1.1 Rainbow bubble-tip anemone (*Entacmaea quadricolor*)

1.4 Sea anemone toxin structure

There have been considerable investigations into the toxins of sea anemones in the past two decades. A previous study on the structure of sea anemone toxins found that the toxins are produced by the specialized stinging cells called nematocytes (Anderluh & Maček, 2002; Moran et al., 2013). Each nematocyte contains a coiled hollow tube filled with venom. When discharged, it expels the venom in a harpoon-like fashion (Beckmann & Özbek, 2012). Sea

anemones use their nematocysts for prey capture, defense, competition and sourcing food (Kass-Simon & Scappaticci, 2002).

A few species of sea anemone can be very hazardous but most are less harmful to humans. They contain highly toxic venom that can cause lethal effects. A sea anemone sting can induce allergies or severe pain (Mizuno et al., 2012). For example, it is reported that the sea anemone *Anemonia viridis* causes a severe toxic skin reaction in humans (Tezcan & Gözer, 2015). Venom of sea anemone *Stichodactyla haddoni* has caused organ failure and lethal shock in humans (Nagata et al., 2006; Garcia et al., 1994). Stinging by sea anemone *Phyllodiscus semoni* induces chronic skin lesions and acute renal failure in the rat (Mizuno et al., 2000; Nakamoto, 1998). Nedosyko et al. (2014), who studied venom from nine sea anemones that host anemonefish found that the most toxic sea anemones are *Cryptdendrum adhaesivum, S. haddoni, Heteractis aurora, and E. quadricolor*, while the least toxic are *Heteractis malu* and *Macrodactyla doreensis*.

A number of studies have investigated the composition of sea anemone venom. Venom from sea anemones are a mixture of bioactives and toxic compounds. To date, about 250 compounds have been isolated. They are mainly composed of proteins and peptides which exhibit cytolytic or neurotoxic effects (Frazao, 2012). The toxins in sea anemone venom have been characterized and classified into four groups, phospholipase A2s (PLA2s), neurotoxins, cytolysins, and non-proteinaceous compounds (Madio et al., 2019). Within each group, there are multiple types of proteins and peptides. Phospholipase A2 degrades glycerolphospholipids of cell membrane into lysophospholipid and fatty acids. PLA2 can cause membrane damage, inflammation and blocks neurotransmission (Nevalainen et al., 2004); PLA2s are low molecular weight of 13–15 kDa and classified into 15 groups and numerous subgroups (Murakami et al., 2010). The type of sea anemone toxins that has received the most attention is neurotoxins. Neurotoxins interact

with various ion channels, including sodium and potassium channels. These toxins disrupt the ion efflux pumping in and out of the cell (Honma & Shiomi, 2006). Currently, nine more novel structural folds of neurotoxins have been identified (Madio et al., 2019). Cytolysins are toxins that cause osmotic imbalance and cell lysis by forming pores in cell membranes (Tejuca et al., 2009a; Anderluh & Maček, 2002). Cytolysins are classified into five types (I-V) based on their primary structure and functional properties (Madio et al., 2019). Non-proteinaceous compounds (e.g., purines, biogenic amines) are also present in venom and are thought to cause pain during envenomation (Madio et al., 2019). Despite some research on caissarone, a small molecule from the sea anemone *Buodosoma caissarum*, little is known about this class of toxins (de Freitas & Sawaya, 1986).

1.5 Mechanism of actinoporins

Research has focused on cytolysins for their ability to form pores in the membrane which can result in cell death. The most characterized cytolysins are type II cytolysins which are also known as actinoporins (Šuput, 2009). This protein has attracted much interest as a lead drug against cancer. Actinoporins have a molecular weight around 20 kDa and are synthesized as about 170 amino acids without cysteine residues (Črnigoj Kristan et al., 2009; Anderluh & Macek, 2003). The three dimensional (3D) structure of actinoporin have been identified by X-ray crystallography from the two most studied cytolysins; Equinatoxin II (EqtII) from the sea anemone *Actinia equina* and Sticholysin II (StII) from *Stichodactyla helianthus* (Lanio et al., 2001; Anderluh et al., 1999). These studies showed that these proteins have highly conserved regions in primary and tertiary structure. The 3D structure reveals that actinoporins consist of

a compact β-sandwich core flanked on two sides by α-helices as shown in figure 1.2 (García-Linares et al., 2013; Mechaly et al., 2011).

Image removed due to copyright restriction.

Figure 1.2 3D structures of actinoporins as exemplified by (A) Δ-stichotoxin-She1a (Sticholysin I; PDB accession code 2KS4) and (B) Δ-actitoxin-Aeq1a (Equinatoxin II; PDB 1KD6). Image is from (Madio et al., 2019).

The pore formation process of actinoporins has been shown to be a multistage process. Initially, the aromatic site phosphocholine (POC) of actinoporins binds to the sphingomyelin of the cell membrane, then the N-terminal of the α-helical region is transferred to the lipid-water interface. Finally, oligomerisation of the actinoporin on the membrane surface occurs and the α-helices of three or four monomers insert into the membrane and form the ion conductive pathway which result in colloid-osmotic shock that leads to cell death as in Figure 1.3 (Črnigoj Kristan et al., 2009; Macrander & Daly, 2016).

Image removed due to copyright restriction.

Figure 1.3 Scheme showing different steps of the most accepted mechanism of pore formation by actinoporins. MS: water soluble monomer. TS: water soluble tetramer. The amount of monomers forming the pore, as well as the particular step where oligomerization occurs, are still matter of study. Mm: monomer bound to the membrane. T1 and T2: tetrameric non-conductive lipid-bound forms. P: tetrameric pore. The conformational changes involving detachment and extension of the N-terminal α-helix would take place along the Mm↔T1↔T2 transitions. Image is from (García-Ortega et al., 2011).

Recent omic-studies of actinoporins from sea anemones suggest that actinoporins are multigene families consisting of many different isoforms with similar sequence. For instance, 47 actinoporins from *Heteractis crispa* were cloned and sequenced (Leychenko et al., 2018); 52 actinoporins were identified as magnificalysins from *Heteractis magnifica* (Wang et al., 2008) ; five isoforms of equinatoxins from *A. equine* (Anderluh et al., 1999); and two sticholysins from *S. helianthus* (Lanio et al., 2001) have been described. This variation, and gene expansion in actinoporins is hypothesized to be due to gene duplication and divergence of sequences for wider interaction with prey and contributes to the defence mechanism of sea anemones (Valle et al., 2015).

Various investigations have been directed to evaluate the cytotoxic action of numerous sea anemone venom on various malignant cell types. For instance, Soletti *et al.* (2008) showed that the cytolysins, toxin Bc2 isolated from the sea anemone *Bunodosoma caissarum* and recombinant equinatoxin-II from sea anemones *A. equina* potentiate cytotoxicity against human glioblastoma cell lines U87 and A172. When combined with anticancer drugs, 10-300 fold less drugs are needed to kill the cancer cells. Thus, candidate venom proteins from sea anemone may be combined with existing anticancer drugs, to result in synergetic effects. For example actinoporins may facilitate the access of chemotherapy reagents by binding to spingilipids, and forming pores which enable the drugs to get into the cytosol of cancer cells thus improving chemotherapy delivery. In addition, purified actinoporin RTX-A from sea

anemone *H. crispa* exhibits anticancer effects through inducing the p-53 apoptosis pathway and inhibiting the expression of the oncogenes AP-1 and NF-$_k$B (Fedorov et al., 2010). In a different investigation, researchers demonstrated the cytotoxicity of the venom from *H. magnifica* on human breast cancer cell lines (T47D and MCF7) and the lung cancer cell line (A549) (Ramezanpour et al., 2014). Cytolysins have been shown in several studies to cause more damage in cancer cells than in normal cells (Tejuca et al., 2009a; Tejuca et al., 2009b). When compared to normal cells, the cytoplasmic membrane of tumor cells has a markedly different composition, with higher concentrations of sphingomyelin, phosphatidylinositol, and cholesterol (Ros et al., 2011). Actinoporin has a high affinity for lipids including sphingomyelin so it targets cancer cells more than normal cell. Other examples of cytoxicity of sea anemones venoms are listed in table 1.1. Combined, all these investigations demonstrate the high capability of cytotoxic cytolysins isolated from sea anemones to be successful anticancer drugs for various sorts of cancer as a monotherapy or to be blended with other existing anticancer medications in order to improve their efficacy and thus be less toxic to normal cells.

Table 1.1 Cytotoxicity of compounds extracted from sea anemones to different cell lines, Table taken from (Mariottini & Pane, 2013).

Species	**Compound or material**	**Cells**	**Tissue/organ/histology**	**Organism**	**IC_{50}–ED_{50}**	**Ref.**
Actinia equina	Equinatoxin II	V-79-379 A	Normal lung fibroblasts	Chinese hamster	8.8×10^{-10} mol/L	(Batista et al., 1990)
Actinia equina	Equinatoxin II-I18C mutant	MCF 7	Breast adenocarcinoma	Human	0.2–0.3 µg/mL	(Potrich et al., 2005)
		ZR 751	Breast carcinoma	Human	5.8 µg/mL	
		HT 1080	Fibrosarcoma	Human	14.2 µg/mL	
		A172	Glioblastoma	Human		
Aiptasia mutabilis	Crude venom	Vero	Normal kidney cells	Monkey	2000 nematocysts/mL	(Marino et al., 2004)
		Hep-2	Epithelial carcinoma	Human	Not indicated	
Heteractis crispa	Actinoporin RTX-A	HL-60	Promyelocytic leukemia	Human	1.06 nM/mL	(Fedorov et al., 2010)
		HeLa	Cervix carcinoma	Human	2.26 nM/mL	
		THP-1	Monocytic leukemia	Human	1.11 nM/mL	

		MDA-MB-231	Breast cancer	Human	4.64 nM/mL	
		SNU-C4	Colon cancer	Human	4.66 nM/mL	
		Cl 41	Epidermal cells	Mouse	0.57 nM/mL	
Sagartia rosea	Acidic actinoporin Src I	U251	Glioblastoma	Human	3.5 µg/mL	(Jiang et al., 2003)
		NSCLC	Non-small cell lung carcinoma	Human	2.8 µg/mL	
		BEL-7402	Liver carcinoma	Human	3.6 µg/mL	
				Human	7.4 µg/mL	
		BGC-823	Stomach adenocarcinoma	Mouse	3.4 µg/mL	
		NIH/3T3	NIH Swiss embryo			
Urticina piscivora	Crude extract	KB	Epidermoid carcinoma	Human	6.54 µg/mL	(Cline et al., 1995)
		HEL299	Embryonic lung	Human	10.07 µg/mL	
		L1210	Lymphocytic leukemia	Mouse	2.34 µg/mL	

1.6 Toxin screening assay

To assess the toxicity of venoms, several biological assays have been performed. The haemolysis assay on red blood cells from various sources such as sheep, chicken, human etc. Haemolysis assay is one of the most widely used assay (Marino et al., 2004; Lanio et al., 2001). Tests for neurotoxicity (Nedosyko et al., 2014), and cytotoxicity are also used (Ramezanpour et al., 2014; Fedorov et al., 2010). The cytotoxicity assays often use animals or cell lines. These assays are all designed to assess biological activity of venom at particular targets of interest, while each technique has limitations in terms of assay versatility, throughput, amount of toxin needed, and specificity. Thus, developing an assay to identify the effects of venoms that can be used in a high throughput setup would be an extremely useful tool for the future venom research.

1.7 A yeast-based screening assay

Due to the existence of multigene families of actinoporins that consist of many isoforms, there is a need for an effective screening system to examine a large collection of potential compounds. Therefore, a yeast screening assay could be a suitable vector for a fast and successful platform. Yeast-based screening has been used for many years for high throughput screening of active compounds for discovery of pharmaceuticals for treatment of diseases and

to study mechanisms of action of potential molecules (Liu et al., 2012b; Galao et al., 2007). *Saccharomyces cerevisiae* has been used for high throughput screening of aquaporin inhibitors and cytochrome P450 whole cell activity (Wu et al., 2008; Chen & Morgan, 2006). In this study, *Saccharomyces cerevisiae* has been selected. Yeast is a single celled eukaryote with a size of 10-20 μm. It is easy to grow and has fast proliferation and requires inexpensive material and equipment for culturing (Galao et al., 2007). It can be genetically manipulated and contains eukaryotic post-translation modifications (Schmidt, 2004). The cell wall of *S. cerevisiae* contains 7% Sphingolipids of the mass of the plasma membrane. Sphingolipid metabolism in *S. cerevisiae*, comprises metabolites, enzymes, and their genes. Sphingolipids can form microdomains or lipid rafts when cholesterol is presented demonstrating a high degree of lateral movement. Sphigolipid also has a role in the regulation of signal transduction pathways (Dickson, 2010). Thus, the presence of sphingolipids in the cell wall, should allow actinoporins to assemble in the yeast cell wall. Furthermore, it can be used to achieve high levels of recombinant protein expression and is a generally recognized as a safe (GRAS) organism (Huang et al., 2014). Therefore, yeast *S. cerevisiae* is an ideal organism for developing a screening assay for anemone venoms.

1.8 Hypotheses and Aim

This research aimed to validate a partial sequence of an actinoporin from the sea anemone *E. quadricolor*. In addition, this research aimed to develop *S. cerevisiae* as a screening system for venoms containing actinoporins from *E. quadricolor* and other anemones. The yeast screening system was compared to the established haemolysis assay. This research hypothesized that *E. quadricolor* would have a family of actinoporin genes and yeast would be an effective system that could be used for screening potential actinoporins for development as anticancer drugs.

1.9 Biotechnology significance

This project will be the first study to identify a full-length sequence for the actinoporin gene from the sea anemone *E. quadricolor*. The identification and cloning of the full-length sequence is necessary in future for studying and manipulation of the gene and it would be beneficial for comparing other isoforms of the actinoporin and to study protein-membrane interactions. Furthermore, the development of the yeast screening assay using *S. cerevisiae* will enable the establishment of a high throughput screening system for actinoporins from multiple sea anemone species. In addition, as actinoporin appear to be a multigene family, the yeast expression system could be used in future if candidate actinoporins were discovered, but they would require an inducible promoter to express actinoporin gene or multiple genes from any one anemone species to select the most potent actinoporins that could be developed into a drug for the treatment of cancers.

Chapter 2. Materials and Methods

2.1 Bioinformatics

Only a single partial nucleotide sequence of sea anemone *E. quadricolor* actinoporin (GenBank accession number KX947313.1) was available in the GenBank nucleotide database at the National Center for Biotechnology Information (NCBI). Basic Local Alignment Tool (BLAST) was used to search for and gather similar sequences of actinoporins from six other sea anemone species using this sequence (Table 2.1). Multiple sequence alignments were conducted using ClustalX2 (Larkin et al., 2007) and edited in Genedoc (Nicolase & Hugh, 1997). A maximum likelihood phylogenetic tree was constructed using MEGA X to examine the evolution between sea anemone *E. quadricolor* actinoporin protein and other sea anemones.

Table 2.1 List of sea anemone's cytolysin sequences obtained from NCBI database.

Number	Organism	Length	Acession #	Source	Sequence	Ref.
1	*Heteractis magnifica* (gIII)	752 bp	AF170706.1	mRNA	Complete	(Wang et al., 2000)
		211 aa	AAF06362.1			
2	*Oulactis orientalis*	591 bp	AY861662.1	mRNA	Partial	(Il'ina et al., 2005)
		173 aa	AW47579.1			
3	*Heteractis crispa* (rtx-a)	594 bp	AY855350.1	mRNA	Partial	(Monastyrnaia et al., 1999)
		171 aa	AAW47930.1			
4	*Heteractis crispa* (hct-S3)	603 bp	MG887786.1	mRNA	Partial	(Leichenko et al., 2014)
		201 aa	AXN75556.1			
5	*Heteractis crispa* (hct-S15)	603 bp	MG887797.1	mRNA	Partial	(Leychenko et al., 2018)
		201 aa	AXN75564.1			
6	*Heteractis crispa* (hct-A)	627 bp	MG887805.1	mRNA	Partial	(Leychenko et al., 2018)
		209 aa	AXN75572.1			
7	*Heteractis crispa* (hct-A2)	597 bp	MG887781.1	mRNA	Partial	(Leichenko et al., 2014)
		199 aa	AXN75548.1			
8	*Entacmaea quadricolor*	588 bp	KX947313.1	mRNA	Partial	(Macrander & Daly, 2016)
		196 aa	APQ32085.1			
9	*Stichodactyla helianthus* (StnII)	528 bp	AJ005038.1	mRNA	Partial	unpublished
		175 aa	CAC20912.1			
10	*Stichodactyla helianthus* (StnIII)	639 bp	MH327769.1	mRNA	Complete	(Rivera-de-Torre et al., 2018)
		212 aa	AWM11685.1			
11	*Stichodactyla gigantea*	588 bp	JQ353486.1	mRNA	Partial	(Hu et al., 2011)
		195 aa	AFC17962.1			
12	*Actinia equina* (EqtII)	877 bp	U41661.1	mRNA	Complete	(Anderluh et al., 1996)
		214 aa	AAC47005.1			
13		847 bp	AF057028.1	mRNA	Complete	(Pungercar, Strukelj &

	Actinia equina (EqtIV)	214 aa	AAD39836.1			Anderluh 2014)

2.2 Sample collection

Six healthy *E. quadricolor* anemones were purchased from the aquarium livestock trader (Marine plus, Adelaide, Australlia) and transported to the Animal House facility at Flinders University, South Australia. The anemones were acclimatised in the aquaria for two weeks. Pairs of *Amphiprion percula* anemonefish was assigned to each anemone in Week 1 in October 2019. The venom used in this work was collected at three time points; from each anemone before they hosted fish, four weeks after hosting fish and then four weeks after fish were removed. Seawater was filtered from a sump to ensure all six linked tanks maintained a controlled and constant environment (25 ◦C ± 2, salinity 33 ± 2, pH 8.3 ± 0.2). Anemonefish and water quality were monitored daily, and food was provided to the anemones in the form of a small piece of whitebait on a weekly basis by a Ph.D. student Cassie Hoepner.

2.3 RNA isolation

All RNA samples used in this study were isolated by a Ph.D. student, Cassie Hoepner. Total mRNA was extracted from the tentacles of sea anemone *E. quadricolor* using the RNeasy mini Kit (Qiagen, Germany) as per manufacturer's instructions. Briefly tissue samples from three tentacles were disrupted using a mortar and pestle and ground to a fine powder under liquid nitrogen and then added to the lysate buffer RLT and homogenised. The samples were then transferred to the mini spin column. Total RNA (including mRNA) binds to the RNeasy membrane. The column was washed three times with wash buffer by centrifugation to wash away the contaminants and total RNA was eluted from the column with RNAse-free water (Appendix 6.4). All RNA samples were stored at -80°C.

2.4 Complementary DNA (cDNA) synthesis

The total RNA concentration extracted from the pooled tentacles was measured using the NanoDrop™ One Micro volume UV-Vis Spectrophotometer (Cat. no. 840274100, Thermo Scientific™). The genomic DNA was eliminated using gDNA wipeout buffer (Qiagen, Germany) before cDNA synthesis. cDNA was synthesized from 0.5 µg of total RNA using the Quantitect Reverse transcription kit (Qiagen, Germany) with 1 µL of Reverse Transcriptase master mix and Reverse Transcriptase primer mix in a total 20 µL as per the manufacturer's instructions (Appendix 6.5). The cDNA was aliquoted neat and also as a 1:2 dilution using double autoclaved water and stored at -20°C. All the PCR products purified and sequenced were made from cDNA aliquot diluted 1:2.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 Primer design

The primers used in this study were generated from the nucleotide sequence of *E. quadricolor* actinoporin (KX947313.1). The forward primer and reverse primer were designed to amplify from the beginning and the end of the sequence, respectively. The expected product size was 588 bp (Figure 2.1). The tool used to design primers was OligoEvaluator™ (Oligo analyser tool, Sigma-Aldrich) with a primer size of 20 bp, 45% GC content, and the melting temperature of both primers was checked using the NEB Tm Calculator- https://tmcalculator.neb.com/#!/main (Table 2.2). The AnemocytII PCR primer mix contained 10 µL of 100 µM AnemocytII forward primer (Sigma-Aldrich), 10 µL of 100 µM AnemocytII reverse primer (Sigma-Aldrich) and 80 µL of double autoclaved water. The primer mix was stored at -20 °C.

Table 2.2 The detail of primers used in this project.

Name	Sequence	Length (bp)	Product size (bp)	Secondary structure	GC content	Tm	NEB Tm
AnemocytII forward primer	TCTTTGAGGAAAGACACCAC	20	588	Weak	45%	59.3 °C	63°C
AnemocytII reverse primer	CTGGAGAAATGGACGATTGT	20		None	45%	61.5 °C	

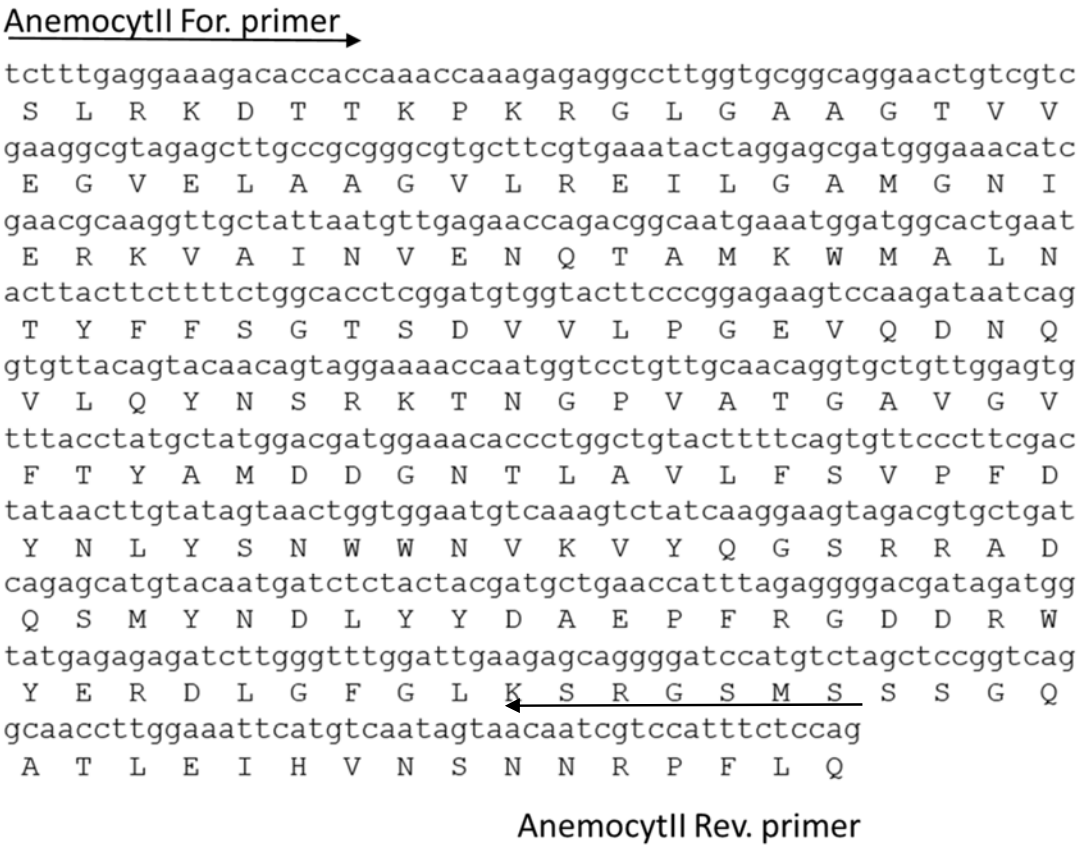


Figure 2.1 The nucleotide sequence of *E. quadricolor* actinoporin (KX947313.1) and its translated amino acid sequence. The nucleotide sequences corresponding to the AnemocytII forward and reverse primers are indicated by arrows.

2.5.2 PCR

PCR amplification was performed using the AnemocytII forward and reverse primer mix and cDNA as a template. Both the high fidelity NEB Next® Ultra Q5 Master mix (Cat. no. M0544S, New England Biolabs) and the simpler and cheaper Gotaq polymerase (Cat. no. M829A, Promega, USA) were initially used and compared. For NEB Next® Ultra Q5 Master mix, each PCR reaction contained 12.5 µL (1X) of NEB Next® Ultra Q5 Master mix, 1.25 µL (0.5 µM) of primer mix, 10.25 µL of double autoclaved water and 1 µL of cDNA to make the total reaction of 25 µL. For Gotaq polymerase, each PCR reaction contained 1X of 5X green buffer, 2.5 mM of $MgCl_2$, 0.5 µM of dNTPs, 1.25 U/µL of Gotaq polymerase, double autoclaved water, and cDNA to the total reaction of 25 µL. PCR reactions were run in a PX2 thermocycle (Thermofisher) following the cycling conditions of 1 cycle of 98°C for 30 sec for initial denaturation; followed by 35 cycles of 98 °C for 15 sec (denaturation), 63°C for 30 sec (annealing), and 72°C for 30 sec (extension); followed by 1 cycle of 72°C for 5 mins for final extension. PCR products were stored at -20°C and analysed using gel electrophoresis.

Gradient PCR was performed using NEB Next® Ultra Q5 Master mix in attempt to separate the multiple bands that appeared at 63°C annealing. Gradient PCR reactions were run in Mastercycler Nexus gradient (Eppendorf) following the same cycling condition as normal PCR except the annealing temperature ranged from 52.1 °C to 62.9 °C. All PCR products were stored at -20°C.

2.5.3 Gel electrophoresis

The PCR products were analysed using gel electrophoresis. The 2% gel was made up of 0.6 g of Nusieve 3: 1 agarose (Cat. no. 50090, FMC Bioproducts) and 30 mL of 1X TAE running buffer (Appendix 6.1). The gel was visualized using 0.5 µL of GelRed® Nucleic Acid Gel Stain (Cat. no. 41003, Biotium). The gel was run at 100 V for 40 mins with the PCR marker (Cat. G316A, Promega) and imaged using the Gel Doc EZ imager (Biorad, USA).

2.6 PCR purification

DNA PCR products were purified directly or from a gel slice using the Wizard® SV Gel and PCR Clean-Up System (Cat. no. A9285, Promega) as per manufacturer's instruction (Appendix 6.6). Gel electrophoresis was used with 5 µL PCR reaction to confirm the required amplicon size, and either the product of the appropriate size was excised from the gel with a scalpel under UV and dissolved in membrane-binding solution, or PCR products was mixed directly with the membrane-binding solution as directed by the manufacturer. The purified DNA (PCR products) were stored at -20 °C.

2.7 Sequencing

The Purified DNA (PCR product) template was pre-mixed with either the AnemocytII forward primer or AnemocytII reverse primer used for PCR for sequencing (AGRF, Purified DNA-PD service). The recommended amount for sequencing PD samples was 6-12 ng in 12 µL for PCR product 200 to 400 bp and 18-30 ng in 12 µL for PCR product 600 bp to 800 bp. The estimated quantities for the samples sequencing reaction for this project is detailed in the appendix 6.7.

2.8 Preparation of RNA libraries and transcriptomic sequencing

RNA was extracted from three pooled tentacles from six anemone specimens, 0 and 72 hrs after milking anemone, before and after hosting fish, using a RNeasy mini kit (Qiagen Venlo, Netherlands), these 24 total RNA samples were sent to the Flinders University Genomics Facility for library preparation and sequencing. RNA was quality controlled and quantified via LabChip (Perkin Elmer) and Qubit 2.0 (ThermoFisher Scientific). All 24 RNA samples had RIN values between 7 and 9.2 and were thus used for library preparation. The TruSeq stranded mRNA library prep kit (Illumina) was used to create each library starting with between 200 ng and 1 µg RNA as per standard protocol. Pooled equimolar libraries were quality checked and

sequenced on the NovaSeq S4 flow cell, paired end 2x150 bp, to achieve an average coverage of 20 million reads per sample.

2.9 Transcriptome analysis

The transcriptome analysis was conducted by a Ph.D. student Robert Qiao. An *E. quadricolor* transcriptome was assembled with Trinity (Haas et al., 2013) utilising all sequences obtained from the Illumina RNA sequencing of the 24 library samples after first being quality controlled with AdaptorRemover (Lindgreen, 2012) and Trimmomatic on default settings. The resulting transcriptome consisted of 589,808 trinity-genes with 868,797 trinity contigs. The quality of the *de novo* assembly was assessed by multiple matrices including contig Ex90N50 (approximately 1.5 Kb), BUSCO completeness (97.7% complete on 255 BUSCO groups searched). A Blast analysis utilising the default setting was conducted using the *E. quadricolor* actinoporin partial DNA sequence (KX947313.1) as the query to reveal conserved genes in the *E. quadricolor* assembled transcriptome.

2.10 Venom collection

Venoms were extracted from sea anemones using a milking technique in 2019 by Ph.D. student Cassie Hoepner as previously described in Sencic & Macek (1990) before fish were introduced to the anemone, after four weeks hosting the fish and four weeks after the fish were removed. The venom was extracted by gently massaging the tentacles of the anemone inside a plastic aquarium bag. Crude venom samples were placed in −80 °C freezer, lyophilised in 5 mL aliquots using Christ Beta 2–8 Freeze Dryer (Martin Christ, Osterode, Germany). After lyophilisation, samples from each individual anemone were pooled and mixed using mortar and pestle before being aliquoted in 1.5 mL protein lobind tubes (Cat. no. 022431081, Eppendorf, USA) and stored at −80 °C until needed for the assays. Crude venom was dissolved initially in phosphate buffered saline (PBS), but it was discovered that venom was more soluble

in autoclaved Milli-Q water. Samples were resuspended routinely as 50 -100 mg of dry crude venom in 300-1000 µL of Milli-Q water (Millipore Milli-Q Biocel water purifier, Cat. no. ZMQ550F01).

2.11 Bicinchoninic acid (BCA) protein assay

The protein concentration of reconstituted crude venom was determined by the Pierce™ BCA Protein Assay (Cat. no. 23227, Thermo Scientific, USA) before being utilized in the haemolysis and yeast assay. A serial dilution of bovine serum albumin (BSA) was used to make a BCA standard curve. The working solution was prepared by mixing reagent A and B at the ratio 5:1. 25 µL of standards and crude venoms were added in triplicate to a F- bottom 96-well plate (Cat. no. 655180, Cellstar). 200 µL of working reagent was added into the standards and samples wells. The plate was incubated at 37 °C for 30 min and then cooled down at RT before measuring the absorbance at 562 nm using a microplate reader FLUOstar Omega (BMG Labtech, Germany) (Appendix 6.8). The protein concentration of each reconstituted venom sample was determined from the BSA standard curve (Figure 2.2).

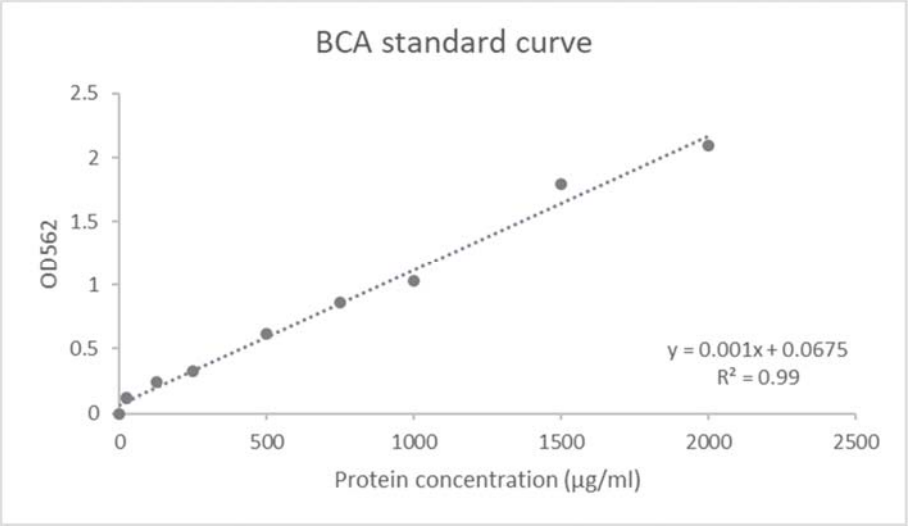


Figure 2.2 A typical BCA standard curve used for calculating protein concentration. The graph was made in Excel.

2.12 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to visualize proteins/peptides present in the venom samples. Samples containing 15-20 µg protein were mixed with 10 µL of 6X sample buffer (Cat. no. B6105, Tokyo Chemical Industry, USA) containing 0.05% 2-mercaptoethanol and heated at 95 °C for 5 mins in the heat block to denature proteins. The samples were run on a 4 -20% Mini-PROTEAN TGX Gels (Cat. no. 4561094, BioRad, USA) at 170 V for 40 mins using 1X running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3). Molecular masses were determined using 5 µL of Precision Plus Protein Dual Xtra Standard (Cat. no. 161-0377, BioRad, USA). Following electrophoresis, gels were fixed in 40% (v/v) ethanol and 10% (v/v) acetic acid solution for 15 minutes and then washed with deionized water before staining with QC Colloidal Coomassie stain (Cat. no. 1610803, BioRad, USA) for 24 h and then destained for 3 h with deionized water (Appendix 6.9)

2.13 Haemolysis assay

A haemolysis assay was performed to assess the toxicity of venom samples. This assay used five mL of sheep blood (CPDA-1/ACD) less than four weeks old, acquired from Applied Biological Products Management (Adelaide, Australia). Five mL of erythrocyte pellet was re-suspended in 50 mL of PBS at pH 7.4 and washed three times at 1500 xg for 10 mins at 4°C (Appendix 6.1) as previously described in Evans et al. (2013). 100 µL of erythrocyte suspension was added to reconstituted venom samples at protein concentrations ranging from 0.5 µg/mL to 8 µg/mL according to each individual venom toxicity and concentration in triplicate in a V-bottom 96-well plate (Cat. no. 3396, Costar, Corning, USA). PBS was added to make up the total volume to 200 µL. The plate was incubated at 37 °C for 40 mins. Samples were centrifuged at 500 xg for five mins to pellet cells, 100 µL of supernatant was transferred

into another F-bottom 96-well plate (Cat. no. 655180, Cellstar) and used to measure the concentration of hemoglobin released at absorbance 415 nm using the microplate reader FLUOstar Omega (BMG Labtech, Germany). 0.1% of Triton X-100 and PBS were used in each run of the assay as a positive control and negative control, respectively (Bailey et al., 2005) (Appendix 6.10). The percentage haemolysis was determined by the equation; Haemolysis (%) = [(OD sample – OD negative control)/ (OD positive control – OD negative control)] x 100. The haemolysis percentage was used to plot and create a dose response curve that was used to determine the EC_{50} of each reconstituted venom sample (effective concentration to achieve 50% lysis).

2.14 Yeast screening assay

Two wild type *Saccharomyces cerevisiae* yeast strains, BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and Invsc2 (MATa his-Δ1 leu2 trp1-289 ura3-52), were kindly provided by Dr. Sunita Ramesh (Flinders University, Australia). The yeast strains were grown in the Yeast peptone dextrose (YPD) agar plates containing 1% (w/v) yeast extract (Cat. no. 91099871, BD), 2% (w/v) peptone (Cat. no. 353664, Chem-supply), but 2% (w/v) dextrose was replaced by D-Glucose (Cat. no. 101174, AnalaR), 2% (w/v) Bacto agar (Cat. no. 0184001, Difco) at 30 °C for three to five days. The plates were sealed with Parafilm and stored at 4 °C for screening assay and fresh working plates were steaked from the glycerol stock every four weeks.

For the yeast screening assay, a single yeast colony was grown overnight in five mL of liquid YPD media at 30 °C with shaking at 140 rpm. The pre-grown cells were harvested by centrifugation at 3,000 xg for five mins and washed two times with five mL of autoclaved water. The cells were then diluted 1:100 in fresh YPD media. The diluted cells were added in triplicate to a 96-well plate (Cat. no. 655180, Cellstar). Each well contained 150 µL of yeast

cells and various concentration of venom 25 µg/mL, 50 µg/mL, and 100 µg/mL to make up the total volume to 250 µL. The positive control was metformin 100mM as described in Li, Wang and Snyder (2019) and the negative control was the yeast cells only. Cell growth was measured at the OD 600 nm every 15 mins for 24 h at 30 °C using a FLUOstar Omega microplate reader (BMG Labtech, Germany) to determine the yeast growth curve.

2.15 Statistical analysis

The data were expressed as mean ± SD. The statistical significance was analysed using one-way analysis of variance (ANOVA) with Post Hoc Tukey test via software IBM SPSS statistics (version 27). Differences were considered statistically significant at 95% confidence level.

Chapter 3. Results

3.1 Bioinformatics

There was only one partial nucleotide sequence for an *E. quadricolor* actinoporin gene (KX947313.1) available in the database in September 2020. This sequence was used to design the forward and reverse primers at the start and the end of the nucleotide sequence and used to amplify the partial sequence which was 588 bp in length (Figure 2.1).

The translated *E. quadricolor* actinoporin amino acid sequence was compared with 12 other sea anemone actinoporin sequences as listed in table 2.1. Amino acid residues that are 100% conserved are highlighted in orange, 80% in yellow, and 60% in blue. The alignment shows that although the nucleotide sequence did not contain the start nor stop codon for this protein, as it extends beyond the C- terminus of the other actinoporins, it may encode the C-terminus of the *E. quadricolor* actinoporin.

The *E. quadricolor* actinoporin amino acid sequence (Seq_8) is very similar to the other sequences, with some regions being high conserved sharing 100% and 80% amino acid identity. The highly conserved phosphocholine (POC) binding site residues (Ser52, Val85, Ser103, Pro105, Tyr111, Tyr131, Tyr135, and Tyr136 in Sticholysin II) which are important for pore formation are marked with black triangles and a conserved motif RGD is marked with a black star (Figure 3.1).

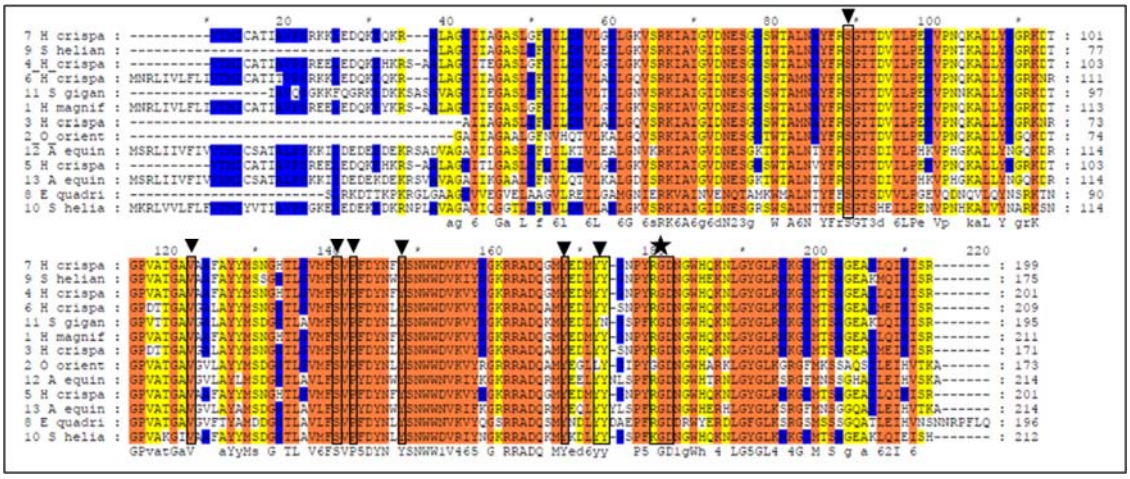


Figure 3.1 Multiple sequence alignment of 13 amino acid actinoporin sequences from the species listed in table 2.1. Orange shading (100% identity), Yellow (80% identity) and blue (60% identity). A POC binding site and RGD motif are framed in black and triangles and star, respectively. The multiple sequence alignment was performed using ClustalX2 (Larkin et al., 2007). Editing and annotating of multiple sequence alignment was performed in GeneDoc (Nicolase & Hugh, 1997)

The multiple sequence alignment of actinoporin amino acid sequence was used to construct a phylogenetic tree using the maximum parsimony method with the bootstrap consensus from 1000 replicates. The actinoporin sequences fall into four clusters. Cluster I contains actinoporins from the Actiniidae family. Cluster II, III and IV all contain members of Stichodactylidae family. The actinoporin of *E. quadricolor* was grouped in Actiniidae family with Equinatoxin II and IV from *A. equina* and *O. orientalis* with a bootstrap value of 97 (Figure 3.2).

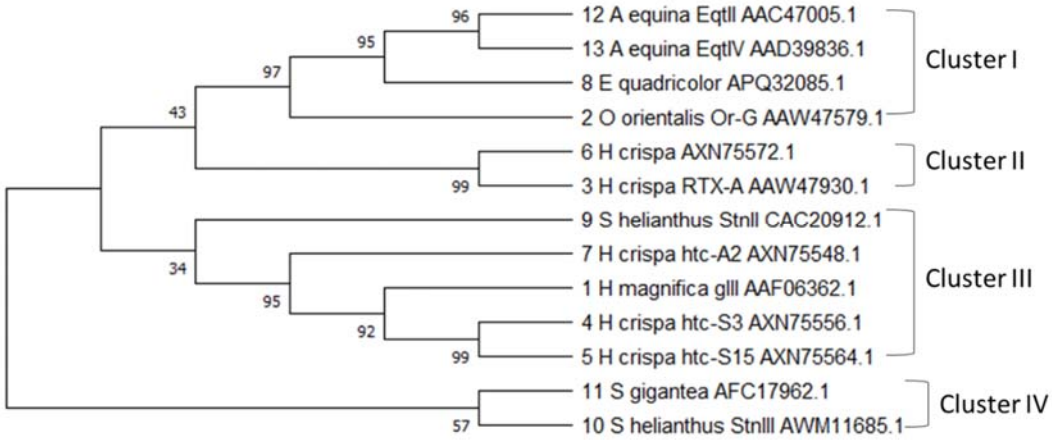


Figure 3.2 Phylogenetic tree of actinoporin amino acid sequences from sea anemones in table 2.1. The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 13 amino acid sequences. There was a total of 171 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Tamura et al., 2013).

3.2 PCR and Sequencing

The mRNA in the total RNA extracted from the sea anemone *E. quadricolor* tentacles was reverse transcribed using Quantitect Reverse transcription and PCR was performed using two different Taq polymerase reagents. The NEB Next ultra II Q5 master mix produced two bands of about 500 bp and 750 bp while the Gotaq polymerase produced only one band (Figure 3.3A). Therefore, NEB Next ultra II Q5 master mix which is a high fidelity polymerase and more suitable for sequencing was used for the remaining PCR reactions. To separate the bands observed, gradient PCR was performed with the annealing temperature ranging from 52.1°C

to 62.9°C. There were three bands of 200 bp, 300 bp and 750 bp produced at annealing temperatures 52.1°C, 53.4°C, 55.1°C, and 56.7°C. Only one band of about 750 bp appeared at 59.9°C and 61.6°C, and one band approximately 600 bp at 62.9°C (Figure 3.3B).

A number of bands were excised and purified or double purified using the Wizard® SV Gel and PCR Clean-Up System. Purified products of about 200 bp, 300 bp, 600 bp and 750 bp were initially isolated (Figure 3.3C). However, as the products at 600 bp and 750 bp appeared as two bands, they were excised and repurified again to get single bands (Figure 3.3 D).

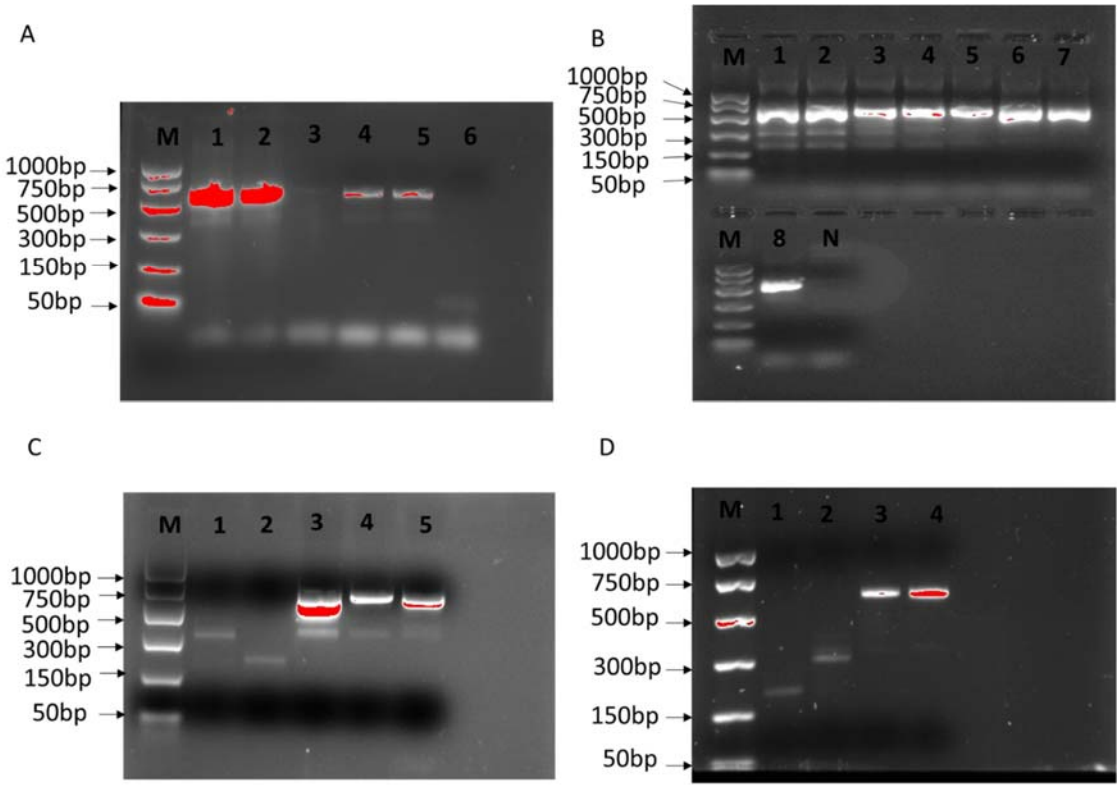


Figure 3.3 PCR products obtained with AnemocytII primers. The PCR products were electrophoresed on 2% agarose gels and run at 100 V for 40 mins. The gels were visualized using GelRed and the Gel Doc EZ imager. (A) Trial PCR with Go taq polymerase and NEB Next ultra II Q5 master mix, M: PCR Marker (Promega), 1: neat cDNA with NEB Next ultraII Q5 master mix, 2: 1:2 cDNA with NEB Next ultra II Q5 master mix, 3: negative control, 4: neat cDNA with Gotaq polymerase, Gotaq polymerase 5: 1:2 cDNA with Gotaq polymerase, 6: negative control. (B) Gradient PCR with NEB

Next ultra II Q5 master mix, M: PCR Marker (Promega), 1: 52.1°C, 2: 53.4°C, 3: 55.1°C, 4: 56.7°C, 5: 58.3°C, 6: 59.9°C, 7: 61.6°C, 8: 62.9°C, N: negative control. (C) Gel purified PCR products, M: PCR Marker (Promega), 1: 300 bp, 2: 200 bp, 3: 600 bp, 4: 750 bp, 5: 750 bp. (D) Double purified PCR products, M: PCR Marker (Promega), 1: 200 bp, 2: 300 bp, 3: 600 bp, 4: 750 bp.

The purified PCR products were sent for Sanger sequencing. However, the sequences returned contained multiple n residues due to the poor sequencing quality from these purified products. The best sequence was 8-Cyto-4-REV-750-A08 and this matched 84% with the available sequence of *E. quadricolor* actinoporin in the database (Table 3.1). To get the better quality sequencing, cloning of individual PCR purified products is required. However, this process requires IBC approval for a Notifiable Low Risk Dealing due to the PCR products being toxin genes and this approval would take a while. Thus, due to time constraints, cloning of the PCR products was not conducted and it was decided to wait for the results of a transcriptomics experiment being conducted concurrently in the group.

Table 3.1 Summary of data from Sanger sequencing.

Sample name	**Q20 Bases**	**Intensity**
1-Cyto-1-FOR-200-A01	133	187
2-Cyto-1-REV-200-A02	125	192
3-Cyto-2-FOR-300-A03	133	73
4-Cyto-2-REV-300-A04	252	353
5-Cyto-3-FOR-600-A05	115	687
6-Cyto-3-REV-600-A06	166	834
7-Cyto-4-FOR-750-A07	102	345
8-Cyto-4-REV-750-A08	360	1940

NB:

Kb Q20 Bases: Number of quality bases (>=Q20) detected

Intensity: Sequence signal intensity (Signals less than 700 may be affected by background and adjacent sequence. Signals over 6000 may produce overloaded/poor reads).

3.3 Actinoporin transcriptomic data

A BlastN of the partial actinoporin *E. quadricolor* nucleotide sequence into the assembled *E quadricolor* transcriptome revealed 30 trinity contigs that had between 38 to 96% identity with the *E. quadricolor* nucleotide sequence (KX947313.1) (Table 3.2). The 30 contigs could be broken down into 14 different genes and some genes had between one and 11 isomers/isoforms. The nucleotide sequences were translated into amino acid sequences ranging from 42 to 224 aa which shared between 64 to 91 % sequence identity with the partial *E. quadricolor* actinoporin protein sequence in NCBI (APQ32085) (Table 3.2). It appeared that not all of the contigs assembled represent full-length cDNAs as they lack either a start codon, a stop codon or both.

Table 3.2 Information on the *E. quadricolor* actinoporin sequence (KX947313.1) from the NCBI database, the 30 trinity contigs that matched this reference sequence, and full-length sequences of actinoporin from three other species are included for comparison. Name: the DN value represents a single gene and the numbering/letters after this indicate they are isoforms. I to XIV indicate the 14 genes identified in the analysis.

Number		Name	Nucleotide length (bp)	Protein length (aa)	Reverse compliment
1		*Entacmaea quadricolor* (APQ32085)	588	196	
2	I	TRINITY_DN1815_c0_g1_i3	2086	224	
3	I	TRINITY_DN1815_c0_g1_i1	1860	224	
4	I	TRINITY_DN1815_c0_g1_i5	1860	224	
5	I	TRINITY_DN1815_c0_g1_i14	1679	224	
6	I	TRINITY_DN1815_c0_g1_i11	1678	224	

7	I	TRINITY_DN1815_c0_g1_i15	1683	224	
8	I	TRINITY_DN1815_c0_g1_i12	1679	224	
9	I	TRINITY_DN1815_c0_g1_i2	1499	224	
10	I	TRINITY_DN1815_c0_g1_i9	1678	224	
11	I	TRINITY_DN1815_c0_g1_i10	1108	201	
12	I	TRINITY_DN1815_c0_g1_i4	1499	224	
13	II	TRINITY_DN21422_c0_g1_i1	952	121	Yes
14	III	TRINITY_DN33127_c1_g1_i2	736	200	
15	IV	TRINITY_DN80030_c0_g1_i2	760	123	Yes
16	IV	TRINITY_DN80030_c0_g1_i1	825	123	Yes
17	V	TRINITY_DN83764_c0_g1_i1	603	42	
18	III	TRINITY_DN33127_c1_g1_i1	659	183	
19	VI	TRINITY_DN117687_c0_g1_i2	334	111	Yes
20	VII	TRINITY_DN34620_c0_g1_i1	392	113	Yes
21	VIII	TRINITY_DN77109_c0_g1_i6	450	96	Yes
22	IX	TRINITY_DN117687_c0_g1_i1	324	108	Yes
23	X	TRINITY_DN187603_c0_g1_i2	308	103	Yes
24	X	TRINITY_DN187603_c0_g1_i1	280	93	Yes
25	XI	TRINITY_DN203865_c0_g1_i1	271	90	
26	XII	TRINITY_DN242801_c0_g1_i1	517	62	Yes
27	XIII	TRINITY_DN312098_c0_g1_i1	671	87	
28	XIII	TRINITY_DN312098_c0_g1_i2	899	87	
29	XIV	TRINITY_DN350625_c0_g1_i4	291	82	
30	XIV	TRINITY_DN350625_c0_g1_i2	299	92	
31	XIV	TRINITY_DN350625_c0_g2_i1	265	53	
32		*Heteractis magnifica (gIII)* (AF170706.1)	752	211	
33		*Actinia equina (EqtIV)* (AF057028.1)	847	214	
34		*Stichodactyla helianthus (StnIII)* (MH327769.1)	639	212	

The multiple sequence alignment of the nucleotide sequences of the 30 contigs showed that the primers originally designed from the partial sequence of *E. quadricolor* actinoporin (KX947313.1) could possibly anneal to 17 of these contig sequences with the forward primer and 20 contig sequences with the reverse primer (Figure 3.4). Due to this reason, the purified products sent for sequencing probably contain multiple products from the contigs shown here. The presence of multiple genes and isoforms that could have been amplified by the PCR

primers, explains the poor sequencing result and each of our PCR products probably contained multiple products of very similar size, such they could not be separated by gel electrophoresis.

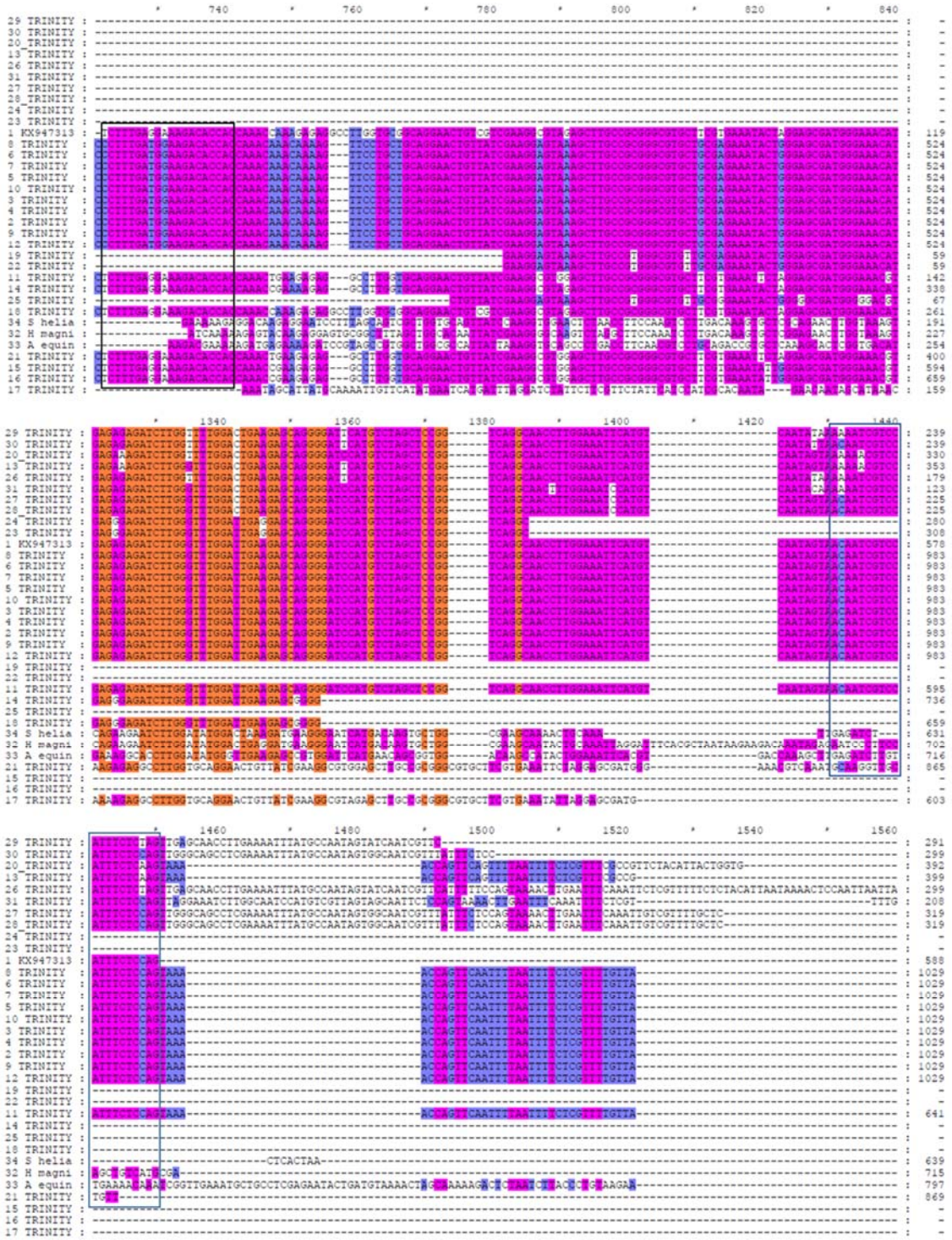


Figure 3.4 Multiple sequence alignment of the nucleotide sequences in table 3.2. Shading in orange indicates 70% identity, pink (50% identity) and blue (25% identity). The regions where the AnemocytII forward and reverse primer will align are in black and blue rectangles, respectively. Multiple sequence alignment was performed using ClustalX2 (Larkin et al., 2007). Editing and annotating of multiple sequence alignment by GeneDoc (Nicolase & Hugh, 1997)

The multiple sequence alignment of amino acid sequences showed that some contigs encode full-length actinoporins while others appear to be partial sequences that do not encode the N or the C-terminus. Ten of the 30 contigs shared up to 89 to 91% identity with original partial *E. quadricolor* sequences. The POC bind site and RGD motif are conserved (50% - 70%) in the contig sequences (Figure 3.5)

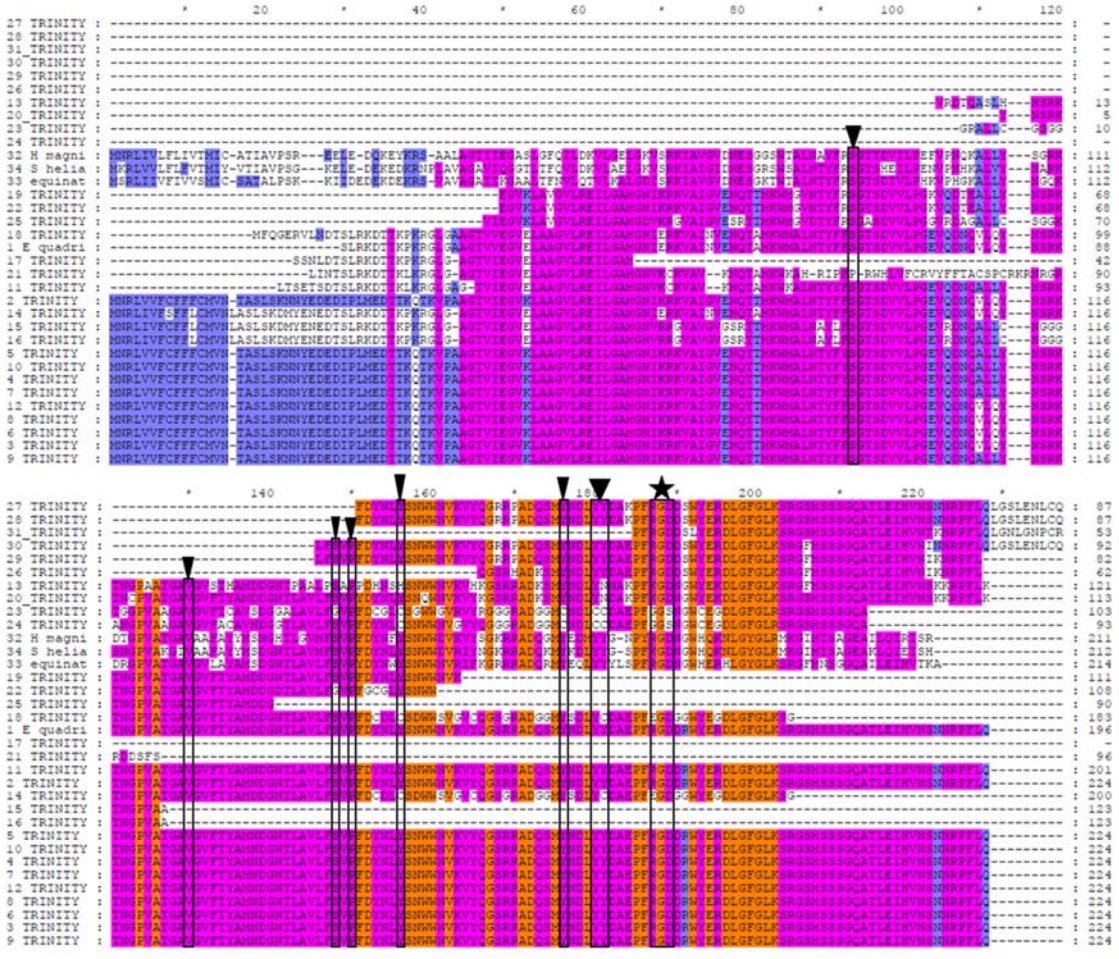


Figure 3.5 Multiple sequence alignment of amino acid sequences listed in table 3.2. Shading in orange indicates 70% identity, pink (50% identity) and blue (25% identity). A POC binding site and RGD motif are framed in black triangles and star, respectively. Multiple sequence alignment was performed using ClustalX2 (Larkin et al., 2007). Editing and annotating of multiple sequence alignment by GeneDoc (Nicolase & Hugh, 1997)

Phylogenetic analysis of the amino acid sequences of assembled contigs revealed that the contigs are homologous to *E. quadricolor* actinoporin sequence and the 10 possible full-length 224 aa sequences were grouped together with the bootstrap value of 99. However, the analysis was unable to separate the 14 genes into individual clades, and most of bootstrap values in the consensus tree are not significant (<80) indicating the program was not able to separate the actinoporin genes reliably. The full-length actinoporin sequences from *H. magnifica, A. equina and S. helianthus* act as an outgroup and separate from all of the *E. quadricolor* sequences (Figure 3.6).

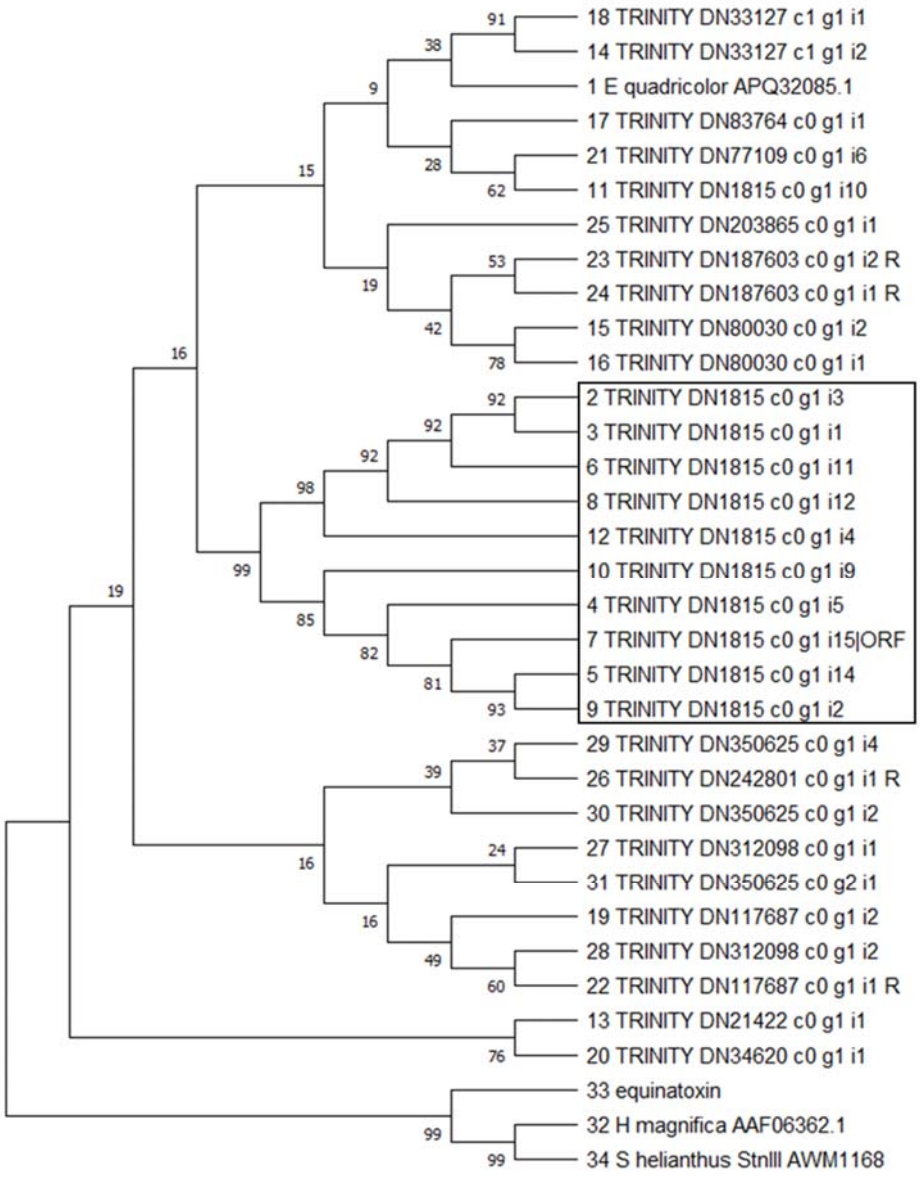


Figure 3.6 Phylogenetic tree of amino acid sequences from assembled trinity contigs described in table 3.2. The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences

(10 replicates). This analysis involved 34 amino acid sequences. There are a total of 237 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Tamura et al., 2013). The 10 possible full-length sequences of actinoporin are framed in a box.

3.3 Protein profile of venom from *E. quadricolor*

The protein concentrations of each reconstituted venom samples are detailed in table 3.3 and ranged from 0.36 µg/mL to 1.31 µg/mL. The venom samples obtained from anemones hosting fish showed multiple bands of low and high molecular weight. Although equal amounts of 15 µg protein were loaded into each well as estimated from the BCA assay, bands were barely visible in the Anemone (An) 3 sample (Figure 3.7A). Venom samples collected after the anemone had been hosting a fish pair for four weeks and from the anemone four weeks after the fish were removed also showed multiple bands (Figure 3.7B and C). Three main bands (indicated by the three arrows) of differing intensity were visualised in the 15 to 20 kDa range where type II cytolysins/actinoporins are expected.

Venom samples from An1 and An6 before, with and after hosting fish were loaded in the same gel to identify if there were any differences in band intensity during the different treatment periods. The before hosting fish, after hosting fish and after fish were removed venom samples appeared to contain proteins around the same size with some slight variations in intensities observed (Figure 3.7D).

Table 3.3 Protein concentration of all venom samples determined by BCA assay. 50 mg of dry crude venom was reconstituted in 300 to 500 µL of Milli-Q water, enough water was added to solubilize the sample.

Before fish samples	Protein concentration (µg/µL)	With fish samples	Protein concentration (µg/µL)	After fish samples	Protein concentration (µg/µL)
An1	0.54	An1	0.73	An1	0.39

An2	0.60	An2	0.89	An2	0.37
An3	0.74	NA	NA	An3	0.52
An4	0.81	NA	NA	An4	0.52
An5	0.67	An5	0.60	An5	0.56
An6	1.31	An6	0.84	An6	0.36

NA: An3, An4 after hosting fish samples were not available.

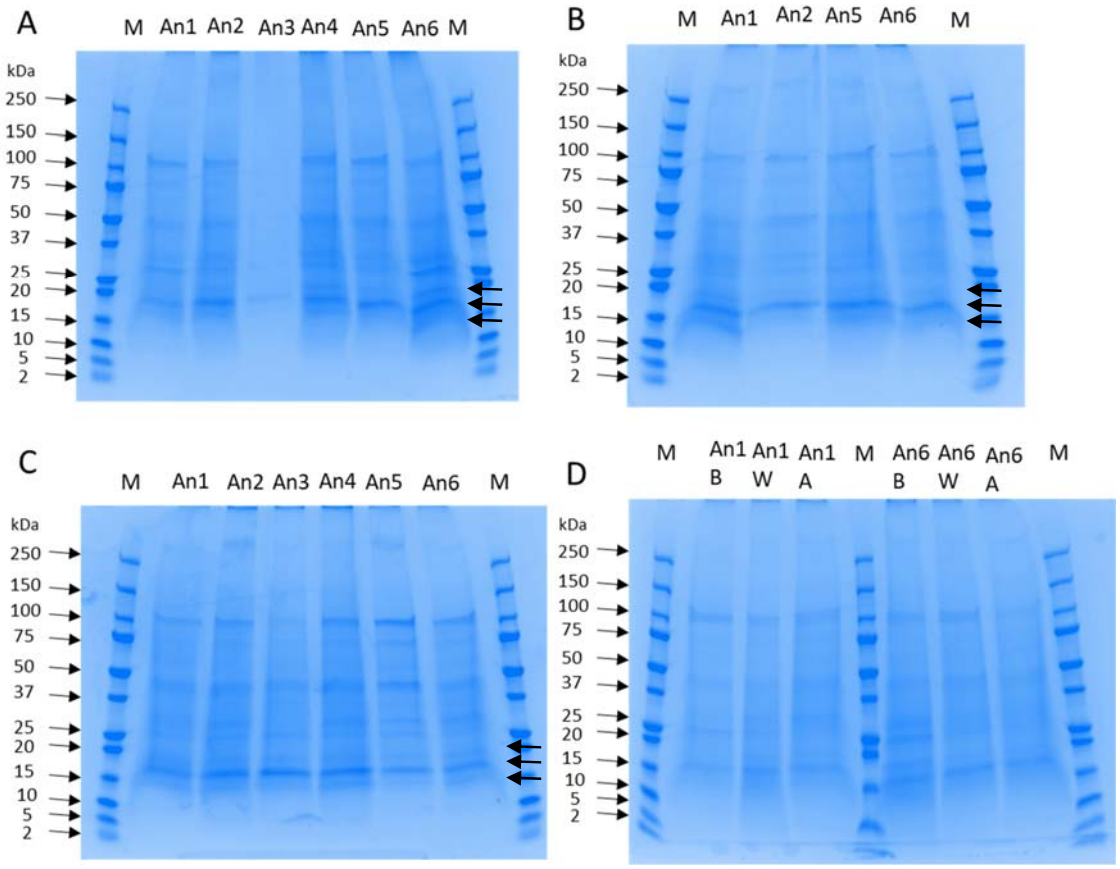


Figure 3.7 SDS-PAGE of reconstituted anemone venom. (A) venom before hosting fish (B) venom after hosting fish for four weeks. (C) venom four weeks after fish were removed. (D) Before, with and after samples in one gel. Lane M indicated molecular weight marker. 15 µg of reconstituted venom sample was loaded into 4-20% Mini-PROTEAN® TGX Protein Gel and stained with QC Colloidal Coomassie stain. Left head arrows indicate the bands at 15 to 20 kDa range.

3.4 Haemolysis assay

Due to the degraded quality of the protein sample from An3 in the before hosting fish treatment group that was observed in the SDS-Page gel (Figure3.7A), the haemolysis results from this sample are not included here. Initially the EC_{50} of each venom sample was calculated at 415 nm and then averaged for the three time points. Using this method, the average EC_{50} for each venom sample collected from anemone before hosting fish was 3.07 µg/mL. The highest percentage haemolysis observed at this period was from An6 venom which gave 100% lysis at protein concentration 1.75 µg/mL and then the lowest haemolysis observed was from An1 venom which only gave 80% lysis at the highest protein concentration tested 8 µg/mL (Figure 3.8A). Venom collected from the same anemones after they hosted fish for four weeks and four weeks after fish were removed showed less variation in the haemolysis results observed for each anemone. For venom collected from anemones four weeks after hosting fish, the samples An3 and An4 were not available for this assay. The average EC_{50} of this group was 3.26 µg/mL (Figure 3.8B), while the average EC_{50} for all the venoms collected four weeks after the fish were removed was 1.65 µg/mL (Figure 3.8C). However, there were no significance differences between the three time points ($p>0.05$).

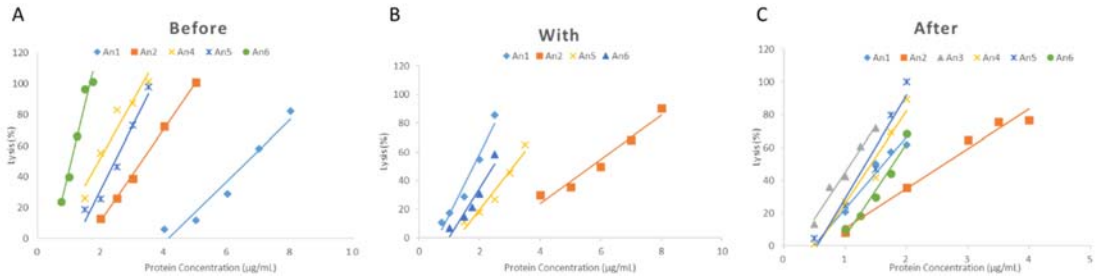


Figure 3.8 Haemolytic activity of venom samples at different protein concentrations. (A) Before hosting fish. (B) Four weeks after hosting fish. (C) Four weeks after fish were removed.

A range of sample concentrations was trialled in order to calculate an EC50 for each anemone, but to more accurately compare venom samples, they were all tested using the same protein

concentrations 1.2 µg/mL, 1.6 µg/mL, 3.2 µg/mL and 4.8 µg/mL. Again, venom collected from anemones before hosting fish showed high variability and very high lysis at the concentration 4.8 µg/mL for An4, An5, and An6, while less lysis was observed for An1 and An2 (Figure 3.9A). Venoms collected after four weeks hosting fish from An1, An5 and An6 also exhibited high lysis at this higher protein concentration (4.8 ug/mL) but less lysis was observed from venom collected from An2 at this concentration (Figure 3.9B). Venoms collected four weeks after the fish were removed from the anemone demonstrated less variation among the six samples, and lysis ranged from 60% to 100% at 4.8 µg/mL protein (Figure 3.9C). However, no significance difference was found between the venom collected at the three time points ($p>0.05$).

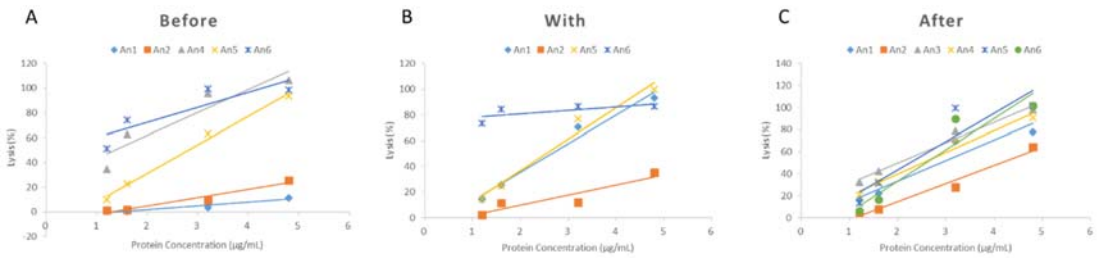


Figure 3.9 Haemolytic activity of venom samples collected from anemones at protein concentrations of 1.2 µg/mL, 1.6 µg/mL, 3.2 µg/mL and 4.8 µg/mL (A) Before hosting fish. (B) four weeks after hosting fish. (C) Four weeks after fish were removed.

The EC_{50} was also calculated by averaging each haemolysis result observed at the four protein concentrations and plotting these averaged values to make an EC_{50} curve. Utilising this method, the EC_{50} of venom collected before hosting fish was 3.33 µg/mL, while the EC_{50} of venom from anemone hosting fish was lower at 2.87 µg/mL and this stayed low at 2.67 µg/mL in venom collected four weeks after the fish were removed (Figure 3.7B). Among the three periods examined, venom collected before anemones hosted fish showed the highest variation

compared to the other treatment periods when utilising venom samples at the same concentration at 1.2 µg/mL, 1.6 µg/mL, 3.2 µg/mL and 4.8 µg/mL (Figure 3.7A).

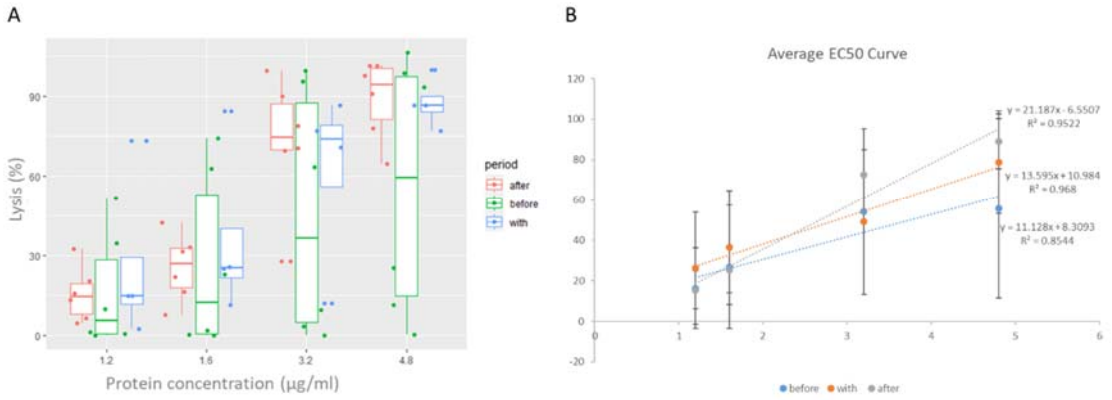


Figure 3.10 (A) The variation box and whisker plot of percentage lysis results of venom collected from anemones before hosting fish (green), four weeks after hosting fish (blue) and four weeks after fish were removed (red). The graph was made in R. (B) The EC_{50} curve calculated from averaging haemolysis results for venom collected at each time point; before hosting fish (blue), four weeks after hosting fish (red), and four weeks after fish were removed (grey). The data were presented in mean ± SD (n=6).

3.5 Yeast screening assay

One of the major aims of this thesis was to see if a toxicity screening system could be established in yeast. For establishing this assay only venom samples collected from *E. quadricolor* sea anemones before hosting fish and then after hosting fish for four weeks were tested on the wild type *Saccharomyces cerevisiae* strains BY4741 and Invsc2. Venom samples collected from anemones before hosting fish and four weeks after hosting fish were able to inhibit the growth of both wild type yeast strains at protein concentrations of 25 µg/mL and greater, but had no effect at lower doses ranging from 0.5 µg/mL to 10 µg/mL (Appendix 6.14). As the protein concentration of venom increased, yeast growth decreased. Yeast growth decreased significantly in WT BY4741 strains at the highest venom protein concentration trialled 50 µg/mL and 100 µg/mL, while in WT Invsc2 yeast showed significant decrease at 25

µg/mL, 50 µg/mL and 100 µg/mL (Figure 3.11 to 3.14). Furthermore, the yeast strain WT Invsc2 showed significance difference ($p<0.001$) in cell growth compared to yeast strain WT BY4741. The two yeast strains reached their stationary phase at about 16 hrs. At this time point, all venom at 100 µg/mL protein concentration on average decreased yeast cell growth by 29% in WT BY4741 strain, while in the WT Invsc2 strain a 46% decrease in growth was observed (Figure 3.15). No significance differences in effects of individual venoms collected from anemones before fish and four weeks after hosting fish were observed within or between samples ($p>0.05$).

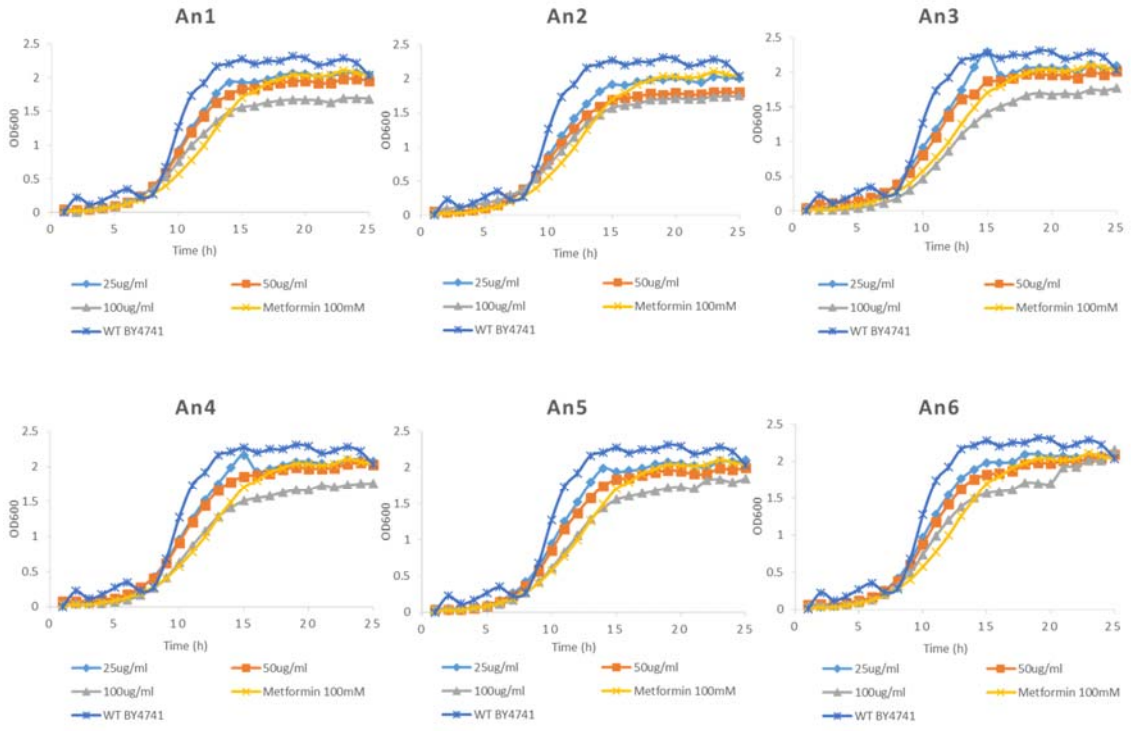


Figure 3.11 The effect of venom collected from anemones before hosting fish on yeast WT BY4741growth.

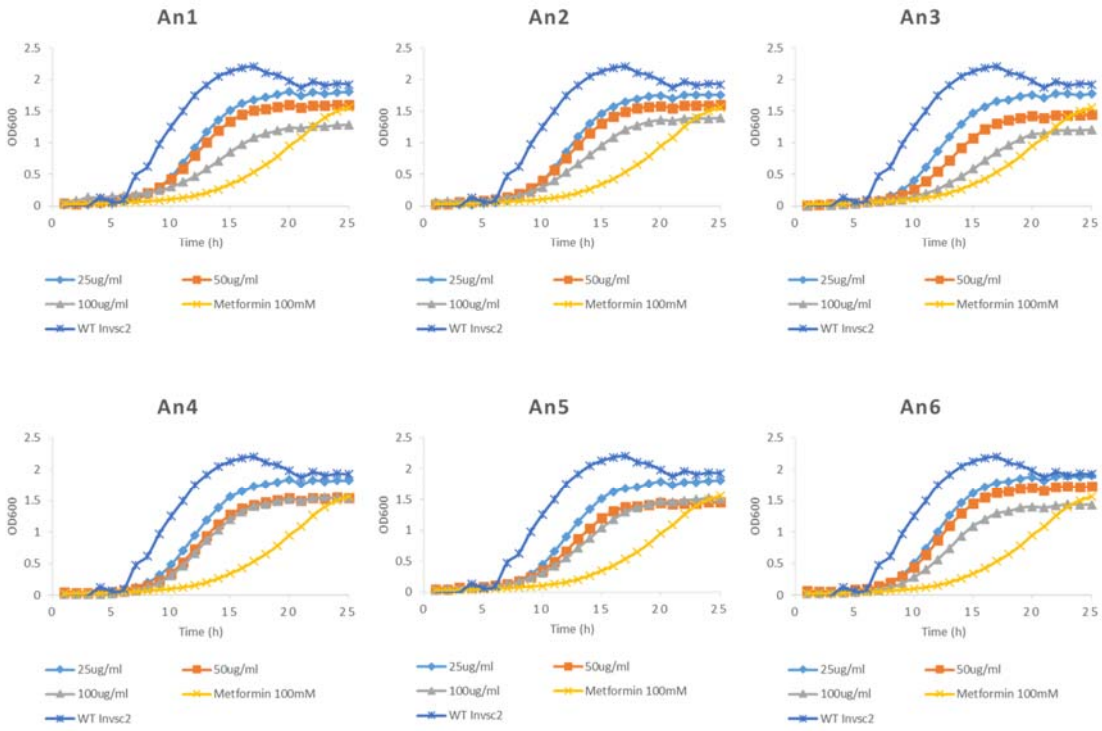


Figure 3.12 The effect of venom collected from anemones before hosting fish on yeast WT Invsc2 growth.

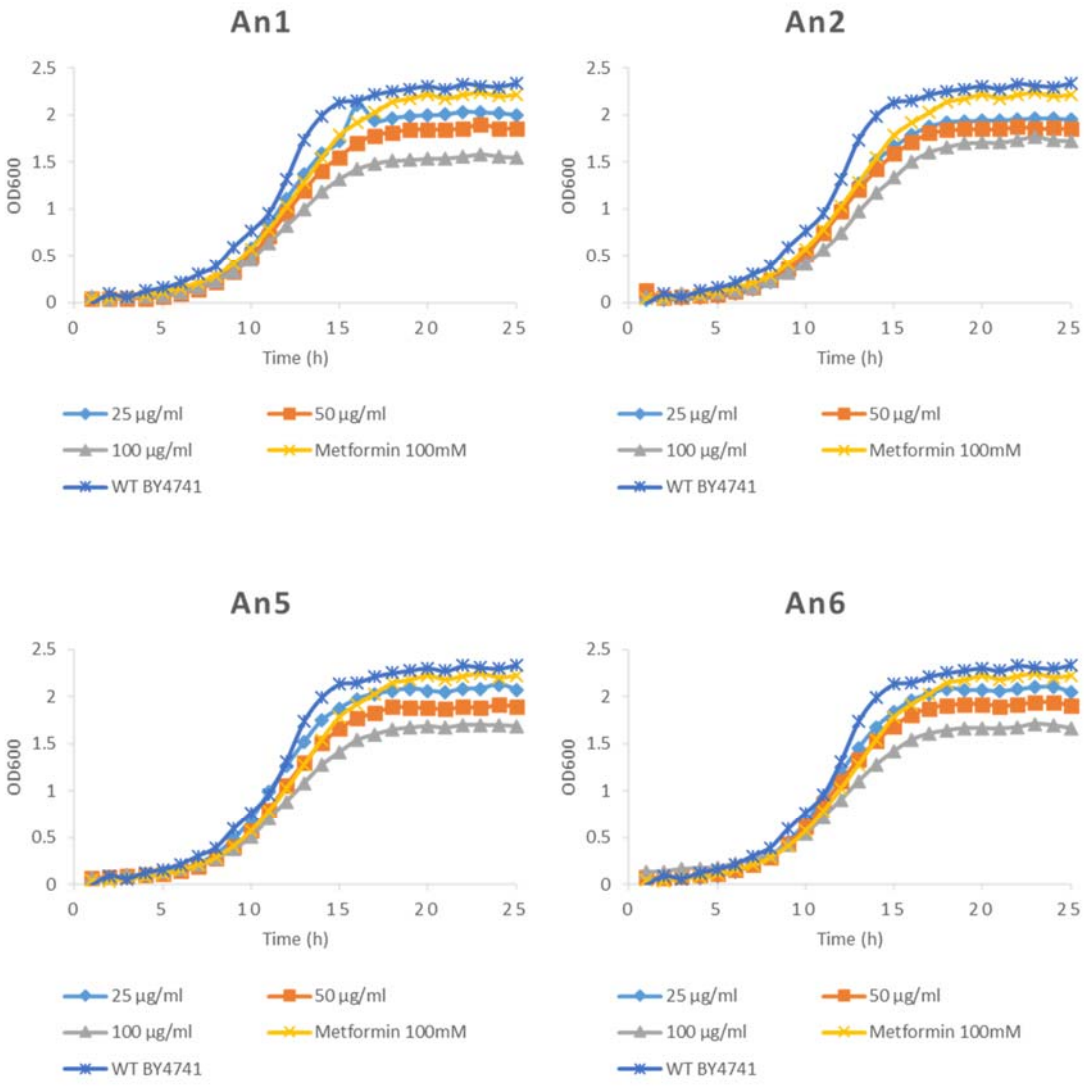


Figure 3.13 The effect of venom collected from anemones hosting fish for four weeks on yeast WT BY4741 growth.

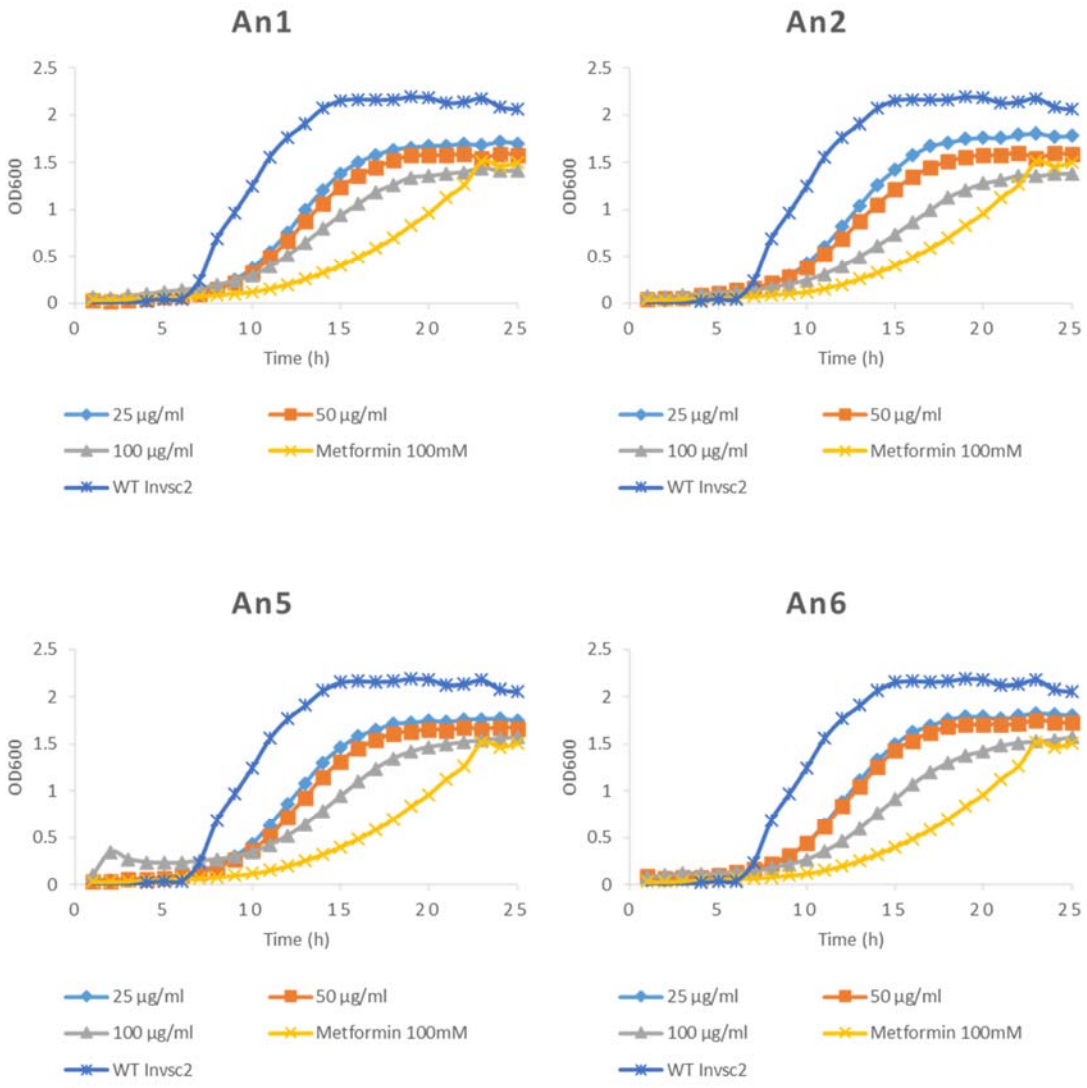


Figure 3.14 The effect of venom collected from anemones hosting fish for four weeks on yeast WT Invsc2 growth.

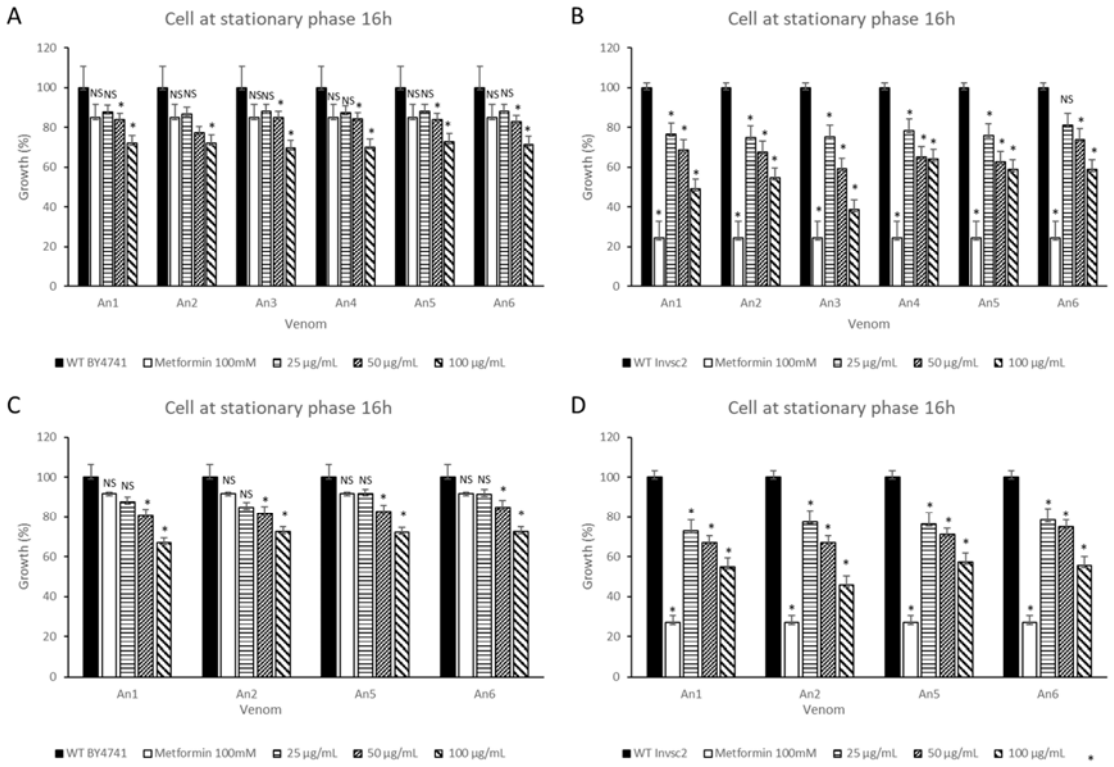


Figure 3.15 The percentage growth of yeast cells at the stationary phase 16 h after the treatment with venom collected before and after hosting fish for four weeks. (A) Venom from anemones before hosting fish with yeast strain WT BY4741, (B) Venom from anemones before hosting fish with yeast strain WT Invsc2, (C) Venom from anemones four weeks after hosting fish with yeast strain WT BY4741. (D) Venom from anemones four weeks after hosting fish with yeast strain WT Invsc2. The data were presented in mean ± SD (n=3), NS: not significant, *: significant difference from negative control ($p<0.05$).

Chapter 4. Discussion

Sea anemone venom contains actinoporins which are some of the most promising compounds present in venom with potential for development as new anticancer drugs. This study aimed to validate a partial sequence for an actinoporin and to develop a yeast-based assay for screening potential candidates from sea anemone venoms. Transcriptomics was also conducted on RNA expressed in *E. quadricolor* tentacles and identified up to 30 different actinoporin transcripts were expressed. While venom was able to reduce yeast growth at high concentrations in a dose-dependent manner, the yeast assay was unable to differentiate the variation in toxicity observed from individual venom samples in the traditional haemolysis assay.

4.1 Bioinformatics

The multiple sequence alignment showed high amino acid similarity between the partial *E. quadricolor* actinoporin protein sequence and those collected from other sea anemones species found in the database including Equinatoxin II from the sea anemone *A. equina* and Sticholysin II from *S. helianthus* (Anderluh et al., 1999; Lanio et al., 2001). The actinoporin of *E. quadricolor* contains the POC binding site and RGD motif that are highly conserved in the actinoporin family. Previous studies have suggested that the POC binding site is one of the important regions in the functional pore formation of actinoporins because of its affinity towards sphingomyelin. The POC binding site together with other important regions such as a cluster of aromatic residues, an array of amino acid and N-terminal α-helix, are said to initiate the pore formation by binding to sphingomyelin and transferring the N-terminus of the protein into the cell membrane (Črnigoj Kristan et al., 2009). Schon et al. (2008) propose actinoporin toxins are able to use the sphingomyelin in two ways, firstly as a specific receptor to attack cells and secondly as a promoter of membrane organization once bound to elicit actinoporin action. Garcia-Linares et al. (2014) also demonstrated that the conserved integrin binding motif RGD found in actinoporins, plays a role in sustaining the oligomerisation of actinoporin. When

one of the residues in this motif is mutated the haemolytic activity is significantly reduced, and the oligomerization behaviour is changed.

4.2 PCR and Sequencing

Sequencing results revealed that the initial PCR products from *E quadricolor* contained more than one sequence when AnemocytII primers were used for amplification. Moreover, the transcriptomic data assembled into 30 trinity contigs, some encoding partial and some encoding full-length proteins ranging from 42 to 224 aa that share 64-92 % sequence identity with the *E. quadricolor* actinoporin protein sequence (APQ32085.1) in the database. Looking at the DNA alignment for these 30 contigs, it is clear that the primers designed from the partial sequence of actinoporin could possibly amplify up to 17 contig sequences with the forward primer and 20 contig sequences with the reverse primer from the total assembled sequences. There were 10 transcripts that encoded proteins of 224 aa that could possibly be full-length *E. quadricolor* actinoporins which share between 89 to 91% sequence identity to the partial actinoporin protein sequence in the NCBI database. These results provide strong evidence that multigene actinoporin families have been recorded in other sea anemone species, where each protein encoded has a different isoelectric point, molecular weight, and cytolytic activity (Valle et al., 2015). Wang et al. (2008) demonstrated that Heteractis magnificalysins (HMgs) from *H. magnifica* are encoded by a multigene family with highly homologous members. The authors were able to clone 52 DNA sequences from sea anemone mRNA with each encoding a different HMgs isoforms with the DNA sequences showing subtle sequence variation (62–100%). Similarly, *H. crispa* actinoporins are encoded by a multigene family containing 47 representatives (Leychenko et al., 2018). The multiple actinoporin genes present in anemone genomes could arise from gene duplication, gene divergence or mutation in order to widen interaction with prey, respond to a broad range of predators or as a defence mechanism (Wang et al., 2008).

4.3 Protein profile of venom

SDS-PAGE is a commonly used method for the separation of proteins/peptides according to their molecular weight. During this study, firstly the precast gel 12% Mini-PROTEAN TGX gels and homemade gels were used to visualise the proteins present in venom and stained with Coomassie Brilliant Blue R 250 stain, and also a silver stain was tested, but all of these methods did not produce clear visible bands (Appendix 6.12). The precast 4-20% Mini-PROTEAN TGX Gels SDS-PAGE gels were then trialled with QC colloidal Coomassie stain which enabled visualization of proteins more clearly. The venom collected from anemones before hosting fish, four weeks after hosting fish, and four weeks after fish were removed contained multiple proteins which ranged from 15 to 260 kDa. This verifies that venoms are a complex mixture of proteins. Hoepner et al. (2019) also ran *E. quadricolor* venom samples (10 µg of protein) on 12% Mini-PROTEAN® TGX Stain-Free™ Protein Gels and stained with Coomassie Brilliant blue R 250 stain. Utilising this method, they visualised more than 20 protein bands in each sample. Moreover, Madio et al. (2017) revealed a complex set of low and high molecular weights using 2DE gel and using proteomic and transcriptomic techniques, they identified 131 coding sequences from tentacles of sea anemone *S. haddoni*, only 33 of those were known protein toxins. Therefore, not all of the mRNA transcripts expressed by the tentacles were present in milked venom. One of the most intense bands observed in our venom samples was seen at approximately 18 kDa. Previous work has identified that actinoporins from cnidarians typically have a molecular weight of about 20 kDa, (Frazao, 2012). Thus, it is possible that this 20 kDa band observed contains *E. quadricolor* actinporin proteins. Hu et al. (2011) also demonstrated that the *S. gigantea* anemone contained an actinoporin with molecular weight of about 19 kDa which was purified using cation-exchange chromatography. Two other cytolysins, Sticholysin I and Sticholysin II, purified from the anemone *S. helianthus* were each around 20 kDa in size (Lanio et al., 2001). However, it is well known that significant biological

activities are typically found in the low molecular weight range (Bulati et al., 2016). In this thesis, Tris-Glycine gels were used and proteins of low molecular weight under 15 kDa were not visualised in gels. This could be due to degradation of small molecular weight peptides or proteins or that gel separation was not effective at this range. The gel electrophoresis method needs further optimisation to better separate and discriminate bands in the venom. Tris-Tricine SDS-PAGE is recommended for the detection of low molecular weight proteins ranging from 1 to 100 kDa (Schägger, 2006). In addition, it has been known that venom contains about 250 compounds of bioactive and toxin (Frazao, 2012). Therefore, when visualising the proteins present in venom in the gel, multiple compounds could present in one band. Further characterisation of the bands seen in this study samples are needed. Two dimensional gels, fractionation of crude venom by chromatography or mass spectrometry proteomics techniques could be used to identify all of the proteins/peptides present in the *E. quadricolor* venom samples.

4.4 Haemolytic activity of the venom

Analysis of the haemolytic activity of the venoms collected at the three different time points showed that different protein concentrations of each venom were required to reach 50% lysis (EC_{50}), and large variations occurred within treatment groups and between treatment groups. Initially the protein concentrations needed to be optimised for each individual venom sample to generate an EC_{50} value for each sample due to the variation in haemolysis observed. Following on from this, a protein concentration that could potentially work on all samples was determined and then haemolysis values for each individual in a group at each protein concentration were averaged and used to obtain one EC_{50} value for each treatment group. The EC_{50} of venom obtained before anemones hosted fish, after hosting fish for four weeks, and then four weeks after fish were removed were 3.07 µg/mL, 3.26 µg/mL and 1.65 µg/mL, respectively. The EC_{50} of venom samples collected from the anemones after fish were removed

for four weeks was lower than before hosting fish and after hosting fish, which indicated that these venom samples were more toxic than those collected before anemone hosted fish. This suggested that the anemone venom may change in composition when the anemone and fish live together. However, when each individual venom was tested at the same concentration 1.2 µg/mL, 1.6 µg/mL, 3.2 µg/mL and 4.8 µg/mL, the EC_{50} for venoms obtained before anemones hosted fish, after hosted fish for four weeks, and then after fish were removed were 3.33 µg/mL, 2.87 µg/mL and 2.67 µg/mL, respectively. There is clearly a difference in the EC_{50} values obtained from the two methods. The variation in haemolysis results was high in the venoms collected from anemones before hosting fish, but when venom was collected from the same anemones after four weeks hosting fish and four weeks after fish were removed, there was less variation in toxicity between samples. This could be due to the acclimation period after the anemones arrived in the Animal House not being long enough. Venom was extracted from anemones before hosting fish just two weeks after the anemones arrived into the animal house, it is not known how long these anemones were housed with the local Adelaide supplier and how much time had lapsed since they were collected and shipped from their natural environment. The observed variation between samples seemed to lessen the longer the anemones were housed at Flinders under the same conditions. Although, it appeared that the venom extract was more potent after hosting fish this data did not meet statistical significance. This result needs to be verified using fractionated venom samples before it can have published.

A prior study also demonstrated the large variations in toxicity between anemone individuals during a bleaching event (Hoepner et al., 2019) and a study on jellyfish toxin also discovered that dilution of the toxin caused variation in lysis between the samples (Bailey et al., 2005). Nedosyko et al. found the EC_{50} of *E. quadricolor* to be at a much lower protein concentration of 0.62 µg/mL (Nedosyko et al., 2014) which is much lower than the EC_{50} found in this study. Gigantoxin-4 which was purified from sea anemone *Stichodactyla gigantea* was able to induce

lysis of human erythrocytes with an EC_{50} as low as 40 ng/mL (Hu et al., 2011). Similarly, the purified Sticholysin I and Sticholysin II from *S. helianthus* has an EC_{50} for haemolysis of about 40-45 ng/mL protein (Lanio et al., 2001). Moreover, when the haemolytic activity observed from crude venom is at a higher EC_{50}, it is possibly due to the fact that crude venom contains a variety of proteins/peptides with various potency and mechanisms compared to when using a purified toxin (Bliss, 2008). The variation in the percent haemolysis observed at different concentrations could also be due to the use of erythrocytes of different species. Haemolysis assays have been performed using both human and other animal erythrocytes (Marino et al., 2004; Bailey et al., 2005). The haemolytic activity appears to differ greatly between erythrocytes from different species. The most susceptible erythrocytes were found to be from sheep blood with a EC_{50} at 10.7 µg/mL and this is the species used in this thesis, whereas the human erythrocyte haemolytic activity EC_{50} was at 25.6 µg/mL from sea anemone *Anthopleura dowii* venom (Rami-rez-Carreto et al., 2019). Previous research has shown that differences in haemolytic activity could be attributed to the lipid composition of the erythrocyte membrane from different species (GarciMia-Linares et al., 2016). The high content of sphingomyelin in the lipids of erythrocyte membranes affects the haemolytic effect of the actinoporin (Hu et al., 2011), and the erythrocytes from sheep contain the highest amount of sphingomyelin (Rami-rez-Carreto et al., 2019).

4.5 Yeast screening assay

The yeast assay was also established for screening actinoporins from sea anemones in regard to its ease of culture and cheap cost. In addition, this assay is suitable for experimentation in a 96 well-plate which increases the throughput of screening. In the initial experiment, the yeast *Saccharomyces cerevisiae* strain BY4741 was grown overnight in YPD media and the pre-grown yeast was diluted to the cell density of 0.1 OD_{600} in 200 µL of liquid culture. Then, the venom at the concentration from 0.5 µg/mL to 8 µg/mL was added, but there was no effect on

the yeast growth. Li, Wang and Snyder (2019) demonstrated that metformin suppresses heme production by 50% in yeast strain BY4741and BY4743 at concentrations 10mM to 200mM. Thus, metformin was chosen as a positive control for our study and tested with our study strains, WT BY4741 and Insvc2 along with sitagliptin, a drug which works as type 2 diabetes medication to decrease blood glucose (Appendix 6.13B and D). Only metformin showed a decrease in the yeast growth in both stains at concentrations ranging from 10 mM to 200 mM (Appendix 6.13A and C). As a result, metformin was used as a positive control and yeast cells only as a negative control in this experiment. Yeast cells were grown and the venom was initially added at the same concentrations used in the haemolysis experiments. Yeast cell growth was not affected by the venom at these low doses, however metformin decreased the yeast growth by around 20% at concentration 200mM (Appendix 6.14A). Next, the venom protein concentration was increased from 5 µg/mL to 100 µg/mL. However, it still did not affect yeast growth (Appendix 6.14B). This may have been due to starting with too many yeast cells at the beginning of the assay. The assay was therefore modified to dilute the pre-grown yeast to 100-fold in liquid YPD media, and the venom at protein concentrations 25 µg/mL, 50 µg/mL and 100 µg/mL were added. Under these conditions the venom decreased yeast growth in both strains in a dose-dependent manner. However, the main limitation in this assay, was unlike the haemolysis assay the yeast assay is unable to differentiate the effects of each individual venom. Consequently, using the pre-grown yeast dilution to the density of 0.1 OD or 100-fold dilution of the pre-grown yeast, the venoms at low concentration seemed not to impact the yeast growth. It is possible that the actinoporins or other components of the venom were unable to enter the yeast cell, however at high concentrations of venom yeast cell growth was affected. Different fractions of the venom would need to be applied to yeast, to see which venom components can enter yeast via the cell wall. The fact that the yeast cell wall contains 7% spingolipid suggested that actinoporins would be able to target the cell wall.

Further investigation should be conducted to optimise conditions such that effects on yeast growth can be measured. In previous studies, crude venoms were fractionated to get potential fractions containing the actinoporins and other toxins before testing in the cytotoxic and/or haemolysis assays (Moghadasi et al., 2020; Bulati et al., 2016; Rami-rez-Carreto et al., 2019). In addition, the yeast was grown to the mid-log phase, because at this phase yeast has high proliferative potential. Yeast cells can be sensitive to toxic substances at the mid-log phase because of its higher metabolism (Gong et al., 2020). In order to develop the yeast system more fully as a high throughput system, we would need to develop a simple and cheap fractionation pipeline to enhance the actinoporin containing fraction in the venom. Hence, the next experiment should fractionate the crude venoms before examining the effect of venoms on yeast at the mid-log phase.

4.6 Conclusion and future direction

In conclusion, this study demonstrated that there are possibly 14 actinoporin genes each with isoforms present in the sea anemone *E. quadricolor* genome using a transcriptomics study. As observed for other species, these isoforms may have different properties and different toxicities. Thus, their sequences need to be validated. Whole genome assembly of the *E. quadricolor* genome will provide further evidence for each member of this gene family, however an assembled and annotated genome is not yet available. The contigs that contain transcripts with a start and a stop codon could be used to design primers for further PCR reactions from tentacle RNA. Once IBC approval for this project has been obtained these PCR products could be cloned to capture single gene transcripts and then cloned DNA could be sequenced to confirm each of the genes and isoforms observed. In addition, this study showed there were higher variations in the haemolysis assay in the venom samples before hosting fish than after hosting fish and after fish were removed. This could be hypothesized that anemone venoms would change in composition when the anemone and fish live together, but looking at

venom profile in the yeast assay, it did not support this. In addition, the yeast screening assay could be utilised to screen the effects of venoms on yeast growth and there were significant decreases in growth at concentration 50 μg/mL and 100 μg/mL in both strains. However, one limitation of this method is that it was unable to differentiate the effects of individual venoms on yeast cell proliferation, while the traditional haemolysis assay could. Future research should be conducted to further develop and confirm these initial findings. The major improvement that could be made to this work would be to fractionate venom samples and then to use these different fractions in the yeast and/or haemolysis assay. Proteomic techniques such as mass spectrometry could be used to identify and confirm the presence of actinoporins in bioactive fractions. Alternately proteomics methods could be used to identify all of the proteins present in the venom. With all of these improvements, the yeast screening assay could be optimised and used for high throughput screening of actinoporins from *E. quadricolor* and other species for development as anticancer therapeutics.

Chapter 5. References

Alajlani, M, Göransson, U & El-Seedi, HR 2019, Marine natural products: a source of novel anticancer drugs, *Marine drugs*, vol. 17, no. 9.

Allen, G. 1975. The anemonefishes: classification and biology. New Jersey: TFH publications.

Anderluh, G., Križaj, I., Štrukelj, B., Gubenšek, F., Maček, P. & Pungerčar, J. 1999. Equinatoxins, pore-forming proteins from the sea anemone *Actinia equina*, belong to a multigene family. *Toxicon,* 37**,** 1391-1401.

Anderluh, G. & Macek, P. 2003. Actinoporins, pore-forming toxins of sea anemones (Actiniaria). *Pore-forming Peptides and Protein Toxins.* London: Taylor and Francis.

Anderluh, G. & Maček, P. 2002. Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria). *Toxicon,* 40**,** 111-124.

Anderluh, G., Pungercar, J., Strukelj, B., Macek, P. & Gubensek, F. 1996. Cloning, sequencing, and expression of equinatoxin II. *Biochem Biophys Res Commun,* 220**,** 437-42.

Arvedlund, M., Mccormick, M. & Ainsworth, T. 2000. Effects of photoperiod on growth of larvae and juveniles of the anemonefish Amphiprion melanopus.

Bailey, P.M., Bakker, A.J., Seymour, J.E. & Wilce, J.A. 2005. A functional comparison of the venom of three Australian jellyfish—*Chironex fleckeri*, *Chiropsalmus* sp., and *Carybdea xaymacana*—on cytosolic Ca2+, haemolysis and *Artemia* sp. lethality. *Toxicon,* 45**,** 233-242.

Barrett, J.C. & Wiseman, R.W. 1987. Cellular and molecular mechanisms of multistep carcinogenesis: relevance to carcinogen risk assessment. *Environmental Health Perspectives,* 76**,** 65-70.

Batista, U., Macek, P. & Sedmak, B. 1990. The cytotoxic and cytolytic activity of equinatoxin II from the sea anemone *Actinia equina*. *Cell Biol Int Rep,* 14**,** 1013-1024.

Beckmann, A. & Özbek, S. 2012. The nematocyst: a molecular map of the cnidarian stinging organelle. *International Journal of Developmental Biology,* 56**,** 577-582.

Beckmann, M.W., Niederacher, D., Schnürch, H.-G., Gusterson, B.A. & Bender, H.G. 1997. Multistep carcinogenesis of breast cancer and tumour heterogeneity. *Journal of Molecular Medicine,* 75**,** 429-439.

Bliss, C.I. 2008. The toxicity of poisons applied jointly. *Annals of Applied Biology,* 26**,** 585-615.

Bulati, M., Longo, A., Masullo, T., Vlah, S., Bennici, C., Bonura, A., Salamone, M., Tagliavia, M., Nicosia, A., Mazzola, S., Colombo, P. & Cuttitta, A. 2016. Partially purified extracts of sea anemone *Anemonia viridis* affect the growth and viability of selected Tumour cell lines. *Biomed Res Int,* 2016**,** 3849897-3849897.

Buston, P.M. & García, M. 2007. An extraordinary life span estimate for the clown anemonefish Amphiprion percula. *Journal of Fish Biology,* 70**,** 1710-1719.

Chen, C., Odorico, D., Tenlohuis, M., Veron, J. & Miller, D. 1995. Systematic relationships within the Anthozoa (Cnidaria: Anthozoa) using the 5′-end of the 28S rDNA. *Molecular Phylogenetics and Evolution,* 4**,** 175-183.

Chen, H. & Morgan, J.A. 2006. High throughput screening of heterologous P450 whole cell activity. *Enzyme and Microbial Technology,* 38**,** 760-764.

Cline, E.I., Wiebe, L.I., Young, J.D. & Samuel, J. 1995. Toxic effects of the novel protein UPI from the sea anemone Urticina piscivora. *Pharmacol Res,* 32**,** 309-314.

Cooper, G.M. & Hausman, R.E. 2000. The development and causes of cancer. *The Cell: A molecular Approach,* 2.

Črnigoj Kristan, K., Viero, G., Dalla Serra, M., Maček, P. & Anderluh, G. 2009. Molecular mechanism of pore formation by actinoporins. *Toxicon,* 54**,** 1125-1134.
Da Rocha, A.B., Lopes, R.M. & Schwartsmann, G. 2001. Natural products in anticancer therapy. *Current Opinion in Pharmacology,* 1**,** 364-369.
Daly, M., Chaudhuri, A., Gusmão, L. & Rodríguez, E. 2008. Phylogenetic relationships among sea anemones (Cnidaria: Anthozoa: Actiniaria). *Molecular Phylogenetics and Evolution,* 48**,** 292-301.
De Freitas, J. & Sawaya, M.I. 1986. Anomalies in sea-urchin egg development induced by a novel purine isolated from the sea anemone *Bunodosoma caissarum*. *Toxicon,* 24**,** 751-755.
Dickson, R.C. 2010. Roles for sphingolipids in Saccharomyces cerevisiae. *Advances in experimental medicine and biology,* 688**,** 217-231.
Doronina, S.O., Toki, B.E., Torgov, M.Y., Mendelsohn, B.A., Cerveny, C.G., Chace, D.F., Deblanc, R.L., Gearing, R.P., Bovee, T.D. & Siegall, C.B. 2003. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nature Biotechnology,* 21**,** 778-784.
Dubinsky, Z. & Stambler, N. 2010. *Coral reefs: an ecosystem in transition*, Springer Science & Business Media.
Elliott, J., Mariscal, R. & Roux, K. 1994. Do anemonefishes use molecular mimicry to avoid being stung by host anemones? *Journal of Experimental Marine Biology and Ecology,* 179**,** 99-113.
Elmore, S. 2007. Apoptosis: a review of programmed cell death. *Toxicologic Pathology,* 35**,** 495-516.
Erba, E., Bergamaschi, D., Bassano, L., Damia, G., Ronzoni, S., Faircloth, G. & D'incalci, M. 2001. Ecteinascidin-743 (ET-743), a natural marine compound, with a unique mechanism of action. *European Journal of Cancer,* 37**,** 97-105.
Evans, B.C., Nelson, C.E., Shann, S.Y., Beavers, K.R., Kim, A.J., Li, H., Nelson, H.M., Giorgio, T.D. & Duvall, C.L. 2013. Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. *Journal of Visualized Experiments***,** e50166.
Fautin, D.G. 1991. Review article the anemonefish symbiosis: what is known and what is not. *Symbiosis*.
Fautin, D.G., Allen, G.R., Allen, G.R., Naturalist, A., Allen, G.R. & Naturaliste, A. 1992. Field guide to anemonefishes and their host sea anemones.
Fedorov, S., Dyshlovoy, S., Monastyrnaya, M., Shubina, L., Leychenko, E., Kozlovskaya, E., Jin, J.-O., Kwak, J.-Y., Bode, A.M. & Dong, Z. 2010. The anticancer effects of actinoporin RTX-A from the sea anemone *Heteractis crispa* (= Radianthus macrodactylus). *Toxicon,* 55**,** 811-817.
Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* 39**,** 783-791.
Flechner, S. 1983. Cyclosporine: a new and promising immunosuppressive agent. *The Urologic Clinics of North America,* 10**,** 263.
Frazao, B. 2012. Sea anemone (Cnidaria, Anthozoa, Actiniaria) toxins: an overview. *Marine Drugs,* 10**,** 1812-1851.
Galao, R.P., Scheller, N., Alves-Rodrigues, I., Breinig, T., Meyerhans, A. & Díez, J. 2007. Saccharomyces cerevisiae: a versatile eukaryotic system in virology. *Microbial Cell Factories,* 6**,** 32.
Galliot, B. & Schmid, V. 2003. Cnidarians as a model system for understanding evolution and regeneration. *International Journal of Developmental Biology,* 46**,** 39-48.

Galmarini, C.M., D'incalci, M. & Allavena, P. 2014. Trabectedin and plitidepsin: drugs from the sea that strike the tumor microenvironment. *Marine Drugs,* 12**,** 719-733.
García-Linares, S., Castrillo, I., Bruix, M., Menéndez, M., Alegre-Cebollada, J., Martínez-Del-Pozo, Á. & Gavilanes, J.G. 2013. Three-dimensional structure of the actinoporin sticholysin I. Influence of long-distance effects on protein function. *Archives of Biochemistry and Biophysics,* 532**,** 39-45.
Garci-a-Linares, S., Richmond, R., Garci-a-Mayoral, M.a.F., Bustamante, N., Bruix, M., Gavilanes, J.G. & Marti-Nez-Del-Pozo, A. 2014. The sea anemone actinoporin (Arg-Gly-Asp) conserved motif is involved in maintaining the competent oligomerization state of these pore-forming toxins. *FEBS Journal,* 281**,** 1465-1478.
García-Ortega, L., Alegre-Cebollada, J., García-Linares, S., Bruix, M., Martínez-Del-Pozo, Á. & Gavilanes, J.G. 2011. The behavior of sea anemone actinoporins at the water–membrane interface. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1808**,** 2275-2288.
Garcia, P.J., Schein, R.M. & Burnett, J.W. 1994. Fulminant hepatic failure from a sea anemone sting. *Annals of Internal Medicine,* 120**,** 665-666.
Garciмia-Linares, S., Rivera-De-Torre, E., Morante, K., Tsumoto, K., Caaveiro, J.M.M., Gavilanes, J.G., Slotte, J.P. & Martiminez-Del-Pozo, A. 2016. Differential effect of membrane composition on the pore-forming ability of four different sea anemone actinoporins. *Biochemistry,* 55**,** 6630-6641.
Gerl, R. & Vaux, D.L. 2005. Apoptosis in the development and treatment of cancer. *Carcinogenesis,* 26**,** 263-270.
Gong, L., Yang, G., Yang, B. & Gu, J. 2020. Development of the yeast *Saccharomyces cerevisiae* as a biosensor for the toxicity detection of toxic substances. *bioRxiv***,** 2020.01.07.898106.
Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., Macmanes, M.D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C.N., Henschel, R., Leduc, R.D., Friedman, N. & Regev, A. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols,* 8**,** 1494-1512.
Hanahan, D. 2014. Rethinking the war on cancer. *The Lancet,* 383**,** 558-563.
Hanahan, D. & Weinberg, R.A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.
Hirata, Y. & Uemura, D. 1986. Halichondrins-antitumor polyether macrolides from a marine sponge. *Pure and Applied Chemistry,* 58**,** 701-710.
Hoepner, C.M., Abbott, C.A. & Burke Da Silva, K. 2019. The ecological importance of toxicity: sea anemones maintain toxic defence when bleached. *Toxins,* 11**,** 266.
Honma, T. & Shiomi, K. 2006. Peptide toxins in sea anemones: structural and functional aspects. *Marine Biotechnology,* 8**,** 1-10.
Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N. & Sarkar, S. 2014. Drug resistance in cancer: an overview. *Cancers,* 6**,** 1769-1792.
Hu, B., Guo, W., Wang, L.H., Wang, J.G., Liu, X.Y. & Jiao, B.H. 2011. Purification and characterization of gigantoxin-4, a new actinoporin from the sea anemone *Stichodactyla gigantea*. *Int J Biol Sci,* 7**,** 729-39.
Huang, M., Bao, J. & Nielsen, J. 2014. Biopharmaceutical protein production by *Saccharomyces cerevisiae*: current state and future prospects. *Pharmaceutical Bioprocessing,* 2**,** 167-182.
Il'ina, A.P., Monastyrnaya, M.M., Isaeva, M.P., Guzev, K.V., Rasskazov, V.A. & Kozlovskaya, E.P. 2005. Primary structures of actinoporins from sea anemone *Oulactis orientalis*. *Russian Journal of Bioorganic Chemistry,* 31**,** 320-324.

Jha, R.K. & Zi-Rong, X. 2004. Biomedical compounds from marine organisms. *Marine Drugs,* 2**,** 123-146.
Jiang, X., Peng, L., Yang, W., Tang, X., Liu, W. & Xu, A. 2003. Expression and purification of Src I from sea anemone Sagartia rosea as a recombinant non-fusion protein. *Protein Expression and Purification,* 32**,** 161-166.
Johnstone, R.W., Ruefli, A.A. & Lowe, S.W. 2002. Apoptosis: a link between cancer genetics and chemotherapy. *Cell,* 108**,** 153-164.
Kass-Simon, G. & Scappaticci, J., Aa 2002. The behavioral and developmental physiology of nematocysts. *Canadian Journal of Zoology,* 80**,** 1772-1794.
Khalifa, S.a.M., Elias, N., Farag, M.A., Chen, L., Saeed, A., Hegazy, M.-E.F., Moustafa, M.S., Abd El-Wahed, A., Al-Mousawi, S.M., Musharraf, S.G., Chang, F.-R., Iwasaki, A., Suenaga, K., Alajlani, M., Göransson, U. & El-Seedi, H.R. 2019. Marine natural products: a source of novel anticancer drugs. *Marine Drugs,* 17.
Lanio, M.a.E., Morera, V., Alvarez, C., Tejuca, M., Gómez, T., Pazos, F., Besada, V., MartíNez, D., Huerta, V., Padrón, G. & Chávez, M.a.D.L.A. 2001. Purification and characterization of two hemolysins from *Stichodactyla helianthus*. *Toxicon,* 39**,** 187-194.
Larkin, M., Blackshields, G., Brown, N., Chenna, R., Mcgettigan, P., Mcwilliam, H., Valentin, F., Wallace, I., Wilm, A., Lopez, R., Thompson, J., Gibson, T. & Higgins, D. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics,* 23**,** 2947-2948.
Leichenko, E.V., Monastirnaya, M.M., Zelepuga, E.A., Tkacheva, E.S., Isaeva, M.P., Likhatskaya, G.N., Anastyuk, S.D. & Kozlovskaya, E.P. 2014. Hct-a is a new actinoporin family from the *Heteractis crispa* sea anemone. *Acta Nature,* 6**,** 89-98.
Leychenko, E., Isaeva, M., Tkacheva, E., Zelepuga, E., Kvetkina, A., Guzev, K., Monastyrnaya, M. & Kozlovskaya, E. 2018. Multigene family of pore-forming toxins from sea anemone *Heteractis crispa*. *Marine Drugs,* 16.
Li, X., Wang, X. & Snyder, M.P. 2019. Metformin affects heme function as a possible mechanism of action. *G3 (Bethesda, Md.),* 9**,** 513-522.
Lindgreen, S. 2012. AdapterRemoval: easy cleaning of next-generation sequencing reads. *BMC Research Notes,* 5**,** 337.
Liu, K.K.-C., Sakya, S.M., O'donnell, C.J., Flick, A.C. & Ding, H.X. 2012a. Synthetic approaches to the 2010 new drugs. *Bioorganic & Medicinal Chemistry,* 20**,** 1155-1174.
Liu, Z., Tyo, K.E.J., Martínez, J.L., Petranovic, D. & Nielsen, J. 2012b. Different expression systems for production of recombinant proteins in Saccharomyces cerevisiae. *Biotechnology and Bioengineering,* 109**,** 1259-1268.
Macrander, J. & Daly, M. 2016. Evolution of the cytolytic pore-forming proteins (actinoporins) in sea anemones. *Toxins,* 8**,** 368.
Madio, B. 2018. *Evolution of the sea anemone venom arsenal.* Doctor of Philosophy, The University of Queensland.
Madio, B., King, G. & Undheim, E.a.B. 2019. Sea anemone toxins: a structural overview. *Marine Drugs,* 17.
Madio, B., Undheim, E.a.B. & King, G.F. 2017. Revisiting venom of the sea anemone *Stichodactyla haddoni*: omics techniques reveal the complete toxin arsenal of a well-studied sea anemone genus. *Journal of Proteomics,* 166**,** 83-92.
Marino, A., Musci, G. & La Spada, G. 2004. Hemolytic effects of crude venom from *Aiptasia mutabilis* nematocysts. *Chemistry and Ecology,* 20**,** 451-459.
Mariottini, G.L. & Pane, L. 2013. Cytotoxic and cytolytic cnidarian venoms. A review on health implications and possible therapeutic applications. *Toxins,* 6**,** 108-151.

Mcconnell, O., Longley, R. & Koehn, F. 1994. The discovery of marine natural products with therapeutic potential. *Biotechnology (Reading, Mass.),* 26**,** 109.
Mebs, D. 1994. Anemonefish symbiosis: vulnerability and resistance of fish to the toxin of the sea anemone. *Toxicon,* 32**,** 1059-1068.
Mechaly, A.E., Bellomio, A., Gil-Cartón, D., Morante, K., Valle, M., González-Mañas, J.M. & Guérin, D.M. 2011. Structural insights into the oligomerization and architecture of eukaryotic membrane pore-forming toxins. *Structure,* 19**,** 181-191.
Mizuno, M., Ito, Y. & Morgan, B.P. 2012. Exploiting the nephrotoxic effects of venom from the sea anemone, *Phyllodiscus semoni*, to create a hemolytic uremic syndrome model in the rat. *Marine Drugs,* 10**,** 1582-1604.
Mizuno, M., Nishikawa, K., Yuzawa, Y., Kanie, T., Mori, H., Araki, Y., Hotta, N. & Matsuo, S. 2000. A case report of acute renal failure following a sting presumedly by a sea anemone. *Am. J. Kidney Dis,* 36**,** E10.
Moghadasi, Z., Shahbazzadeh, D., Jamili, S., Mosaffa, N. & Pooshang Bagheri, K. 2020. Significant anticancer activity of a venom fraction derived from the persian gulf sea anemone, *Stichodactyla haddoni*. *Iran J Pharm Res,* 19**,** 402.
Monastyrnaia, M.M., Zykova, T.A. & Kozlovskaia, E.P. 1999. Isolation and characteristics of high molecular weight cytolysins from the sea anemone *Radianthus macrodactylus*. *Bioorg Khim,* 25**,** 733-41.
Moran, Y., Praher, D., Schlesinger, A., Ayalon, A., Tal, Y. & Technau, U. 2013. Analysis of soluble protein contents from the nematocysts of a model sea anemone sheds light on venom evolution. *Marine Biotechnology* 15**,** 329-339.
Murakami, M., Taketomi, Y., Girard, C., Yamamoto, K. & Lambeau, G. 2010. Emerging roles of secreted phospholipase A2 enzymes: lessons from transgenic and knockout mice. *Biochimie,* 92**,** 561-582.
Nagai, H. & Kim, Y.H. 2017. Cancer prevention from the perspective of global cancer burden patterns. *Journal of Thoracic Disease,* 9**,** 448-451.
Nagata, K., Hide, M., Tanaka, T., Ishii, K., Izawa, M., Sairenji, T., Tomita, K. & Shimizu, E. 2006. Anaphylactic shock caused by exposure to sea anemones. *Allergology International,* 55**,** 181-184.
Nakamoto, M. 1998. Stings of box jellyfish and sea anemones. *Rinsho Hifuka,* 52**,** 29-33.
Nanninga, G.B., Saenz-Agudelo, P., Manica, A. & Berumen, M.L. 2014. Environmental gradients predict the genetic population structure of a coral reef fish in the Red Sea. *Molecular Ecology,* 23**,** 591-602.
Nedosyko, A.M., Young, J.E., Edwards, J.W. & Da Silva, K.B. 2014. Searching for a toxic key to unlock the mystery of anemonefish and anemone symbiosis. *PLoS One,* 9**,** e98449.
Nei, M. & Kumar, S. 2000. *Molecular Evolution and Phylogenetics,* New York, Oxford University Press.
Nevalainen, T.J., Peuravuori, H.J., Quinn, R.J., Llewellyn, L.E., Benzie, J.A., Fenner, P.J. & Winkel, K.D. 2004. Phospholipase A2 in cnidaria. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 139**,** 731-735.
Nicolase, K.B. & Hugh, B.J. 1997. GenDoc: a tool for editing and annotating multiple sequence alignments.
Ponder, B.a.J. 2001. Cancer genetics. *Nature,* 411**,** 336-341.
Potrich, C., Tomazzolli, R., Dalla Serra, M., Anderluh, G., Malovrh, P., MačEk, P., Menestrina, G. & Tejuca, M. 2005. Cytotoxic Activity of a Tumor Protease-Activated Pore-Forming Toxin. *Bioconjugate Chem,* 16**,** 369-376.

Ramezanpour, M., Da Silva, K.B. & Sanderson, B.J. 2014. The effect of sea anemone (H. magnifica) venom on two human breast cancer lines: death by apoptosis. *Cytotechnology,* 66**,** 845-852.
Ramírez-Carreto, S., Miranda-Zaragoza, B. & Rodríguez-Almazán, C. 2020. Actinoporins: from the structure and function to the generation of biotechnological and therapeutic tools. *Biomolecules,* 10**,** 539.
Rami-Rez-Carreto, S., Perez-Garci-A, E.I., Salazar-Garci-A, S.I., Bernaldez-Sarabia, J., Licea-Navarro, A., Rudino-Pinera, E., Perez-Marti-Nez, L., Pedraza-Alva, G. & Rodri-Guez-Almazan, C. 2019. Identification of a pore-forming protein from sea anemone *Anthopleura dowii* Verrill (1869) venom by mass spectrometry. *The Journal of Venomous Animals and Toxins Including Tropical Diseases,* 25.
Rangel, M. & Falkenberg, M. 2015. An overview of the marine natural products in clinical trials and on the market. *J. Coast. Life Med,* 3**,** 421-428.
Richardson, N.C., Kasamon, Y.L., Chen, H., De Claro, R.A., Ye, J., Blumenthal, G.M., Farrell, A.T. & Pazdur, R. 2019. FDA approval summary: brentuximab vedotin in first-line treatment of peripheral T-cell lymphoma. *The Oncologist,* 24**,** e180-e187.
Rinehart, K.L. 2000. Antitumor compounds from tunicates. *Medicinal Research Reviews,* 20**,** 1-27.
Rivera-De-Torre, E., Martínez-Del-Pozo, Á. & Garb, J.E. 2018. *Stichodactyla helianthus*' de novo transcriptome assembly: discovery of a new actinoporin isoform. *Toxicon,* 150**,** 105-114.
Rivera-De-Torre, E., Palacios-Ortega, J., Slotte, J.P., Gavilanes, J.G., Martínez-Del-Pozo, Á. & García-Linares, S. 2020. Functional and structural variation among sticholysins, pore-forming proteins from the sea anemone *Stichodactyla helianthus*. *International Journal of Molecular sciences,* 21**,** 8915.
Ros, U., Pedrera, L., Dr-Az, D.N., De Karam, J.C., Sudbrack, T.P., Valiente, P.A., Martr-Nez, D., Cilli, E.M., Pazos, F., Itri, R., Lanio, M.E., Schreier, S. & Γŕlvarez, C. 2011. The membranotropic activity of N-terminal peptides from the pore-forming proteins sticholysin I and II is modulated by hydrophobic and electrostatic interactions as well as lipid composition. *J Biosci,* 36**,** 781-791.
Sanderson, B.J.S., Da Silva, K.B. & Ramezanpour, M. 2016. How venom from the magnificent sea anemone, *Heteractis magnifica*, kills breast and lung cancer cells. *In:* GOFFREDO, S. & DUBINSKY, Z. (eds.) *The Cnidaria, Past, Present and Future: The world of Medusa and her sisters.* Cham: Springer International Publishing.
Schägger, H. 2006. Tricine–SDS-PAGE. *Nature Protocols,* 1**,** 16-22.
Schmidt, F. 2004. Recombinant expression systems in the pharmaceutical industry. *Applied Microbiology and Biotechnology,* 65**,** 363-372.
Schön, P., García-Sáez, A.J., Malovrh, P., Bacia, K., Anderluh, G. & Schwille, P. 2008. Equinatoxin II permeabilizing activity depends on the presence of sphingomyelin and lipid phase coexistence. *Biophysical Journal,* 95**,** 691-698.
Sencic, L. & Macek, P. 1990. New method for isolation of venom from the sea anemone *Actinia cari*. Purification and characterization of cytolytic toxins. *Comparative Biochemistry and*

Physiology. B, Comparative Biochemistry, 97**,** 687-693.
Simmons, T.L., Andrianasolo, E., Mcphail, K., Flatt, P. & Gerwick, W.H. 2005. Marine natural products as anticancer drugs. *Molecular Cancer Therapeutics,* 4**,** 333.
Sithranga Boopathy, N. & Kathiresan, K. 2010. Anticancer drugs from marine flora: an overview. *Journal of Oncology,* 2010.

Soletti, R.C., De Faria, G.P., Vernal, J., Terenzi, H., Anderluh, G., Borges, H.L., Moura-Neto, V. & Gabilan, N.H. 2008. Potentiation of anticancer-drug cytotoxicity by sea anemone pore-forming proteins in human glioblastoma cells. *Anti-Cancer Drugs,* 19.
Šuput, D. 2009. In vivo effects of cnidarian toxins and venoms. *Toxicon,* 54**,** 1190-1200.
Tamura, K., Stecher, G., Peterson, D. & Filipski, A. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution,* 30**,** 2725-2729.
Tejuca, M., Anderluh, G. & Dalla Serra, M. 2009a. Sea anemone cytolysins as toxic components of immunotoxins. *Toxicon,* 54**,** 1206-1214.
Tejuca, M., Pérez-Barzaga, V., Pazos, F., Álvarez, C. & Lanio, M. 2009b. Construction of sea anemone cytolysin-based immunotoxins for selective killing of cancer cells. *Revista Cubana de Fisica,* 26**,** 15-22.
Tezcan, Ö.D. & Gözer, Ö. 2015. Severe toxic skin reaction caused by a common anemone and identification of the culprit organism. *Journal of Travel Medicine,* 22**,** 269-271.
Thangaraj, S., Bragadeeswaran, S. & Gokula, V. 2019. Bioactive compounds of sea anemones: a review. *International Journal of Peptide Research and Therapeutics,* 25**,** 1405-1416.
Valle, A., Alvarado-Mesén, J., Lanio, M., Álvarez, C., Barbosa, J. & Pazos, I. 2015. The multigene families of actinoporins (part I): isoforms and genetic structure. *Toxicon,* 103**,** 176-187.
Vogelstein, B. & Kinzler, K.W. 1993. The multistep nature of cancer. *Trends in Genetics,* 9**,** 138-141.
Wang, Y., Chua, K.L. & Khoo, H.E. 2000. A new cytolysin from the sea anemone, Heteractis magnifica: isolation, cDNA cloning and functional expression. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology,* 1478**,** 9-18.
Wang, Y., Yap, L.L., Chua, K.L. & Khoo, H.E. 2008. A multigene family of *Heteractis magnificalysins* (HMgs). *Toxicon,* 51**,** 1374-1382.
Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P. & Mcphail, A.T. 1971. Plant antitumor agents. VI. isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *Journal of the American Chemical Society,* 93**,** 2325-2327.
Wiedenmann, J., Vallone, B., Renzi, F., Nienhaus, K., Ivanchenko, S.V., Roecker, C. & Nienhaus, G.U. 2005. Red fluorescent protein eqFP611 and its genetically engineered dimeric variants. *Journal of Biomedical Optics,* 10**,** 014003.
World Health Organization. 2018. *Cancer* [Online]. World Health Organization. Available: https://www.who.int/news-room/fact-sheets/detail/cancer [Accessed 10 Auguest 2020].
Wu, B., Altmann, K., Barzel, I., Krehan, S. & Beitz, E. 2008. A yeast-based phenotypic screen for aquaporin inhibitors. *Pflügers Archiv-European Journal of Physiology,* 456**,** 717-720.

Chapter 6. Appendices

Appendix 6.1 Buffer and solution used in this project

1. Buffer for running gel electrophoresis
    a. 50X Tris-Acetate-EDTA buffer (TAE) stock

        Tris-base: 242 g

        Acetic acid: 57.1 mL

        0.5M EDTA (pH 8.0): 100 mL

        Dissolve Tris-base in 800mL of Milli-Q water and add acetic acid and EDTA, then add Milli-Q to make 1L

    b. 1X TAE buffer

        50X TAE buffer: 20 mL

        Milli-Q water: 980 mL

2. Buffer and solution for running SDS-PAGE
    a. 10X Tris-Glycerin buffer (250mM Tris, 192mM Glycine, pH 8.3)

        Tris-base: 30.285g

        Glycine: 144.13g

        Were dissolved in 800 mL of Milli-Q water and adjust pH to 8.3 using NaOH or HCl, then add Milli-Q water to make 1L

    b. 10% SDS

        SDS: 10g

        were dissolved in 100mL Milli-Q water

    c. 1X running buffer

        10X Tris-Glycerin buffer: 100 mL

        10% SDS: 10 mL

        Milli-Q water: 890 mL

d. Fixing solution (40% ethanol and 10% acetic acid)

   Ethanol: 40 mL

   Acetic acid: 10 mL

   Milli-Q: 50 mL

3. Buffer for haemolysis assay

   a. 1X PBS

      NaCl: 8g

      KCl: 0.2g

      Na2HPO4: 1.44g

      KH2PO4: 0.24g

      Were dissolved in 800 mL of Milli-Q and adjust pH to 7.4 using NaOH or HCl, then add Milli-Q water to make 1 L.

Appendix 6.2 Standard Operating Procedure for safe handling of anemone venom

Safety is of paramount focus during the undertaking of experiments involving anemones and the extraction and subsequent assaying of venom.

Safe Anemone Handing Practices

• Always wear gloves when touching and/or moving anemones

• Safety glasses MUST always be worn during extraction of venom

• Two people MUST be present for the entire venom extraction period

Anemone Venom

• Lab coat, safety glasses, enclosed shoes and gloves MUST be worn when handling venom

• Venom MUST be held within double containments during transport

• Venom MUST be stored within double containment at -80°C when not in use

• **DISPOSAL**: all pipette tips, 96-well plates, 5 mL tubes and 1.6 mL cryotube MUST be collected for disposal in a purple cytotoxic bin. ALL liquid and powder venom MUST be placed in a purple cytotoxic bin, if not available please freeze with double containment before disposing in the purple bin

The purple cytotoxic bin MUST be disposed of in appropriate waste stream

Haemolysis Assay

• Lab coat, safety glasses, enclosed shoes and gloves MUST be worn when handling venom and blood

• **DISPOSAL**: all pipette tips, 50 mL tubes and 96-well plates must be disposed of in appropriate yellow autoclave bags. Blood waste MUST be deactivated using bleach before

disposal down the sink. If blood waster has been mixed with venom it must be disposed in the purple bin waste stream.

Yeast Screening Assay

• Lab coat, gloves, safety glasses, enclosed and shoes MUST be worn when handling yeast

• **DISPOSAL**: all pipette tips and 96-well plates in contact with yeast MUST be collected and disposed of in appropriate waste stream. If yeast has been in contact with venom, it must be placed in the purple bin waste stream.

Appendix 6.3 Venom milking and preparation procedure

	Flinders University School of Biological Sciences **Standard Operating Procedures (SOP) for Venom Milking and Preparation.**	
Personnel Hazards & Safety	   	

SOP Number	RA Number	RA Score	Date:
			29/08/20
Contact Person	**SOP prepared by**		**Review Date :**
	Catherine Abbott/ Ly Kim		29/08/23

General

This Standard Operating Procedures (SOP) is to utilise a non-lethal method of venom extraction from the anemone and prepare samples into dry crude venom for toxicology analysis.

Materials and Equipment

1. Sea anemones
2. Aquarium bag
3. Freeze dryer
4. Mortar and pestle
5. 5 mL tubes
6. 1.6 mL cryotubes
7. -20ºC and -80ºC Freezer

Procedure

1. Milking is to be done using sterile techniques.

2. Transfer anemone from tank into a clean plastic aquarium bag, avoiding excess water that will dilute sample.

Refer to SOP for safe anemone handling

3. Massage the tentacles of the anemone collecting the venom extracted in the aquarium bag (< 1 minute until no more liquid is released).

4. Transfer anemone back into aquaria and monitor for recovery (anemones should reopen 30mins-1hour).

5. Aliquot collected venom into 5mL labelled tubes (2.5-3 mL in each) and store at -20°C (24 hours) and then -80°C (48 hours) until ready to lyophilise samples (measure total volume in measuring cylinder as liquid is transferred from aquarium bag to tubes).

6. Repeat for all anemones once per month.

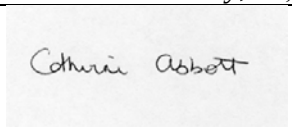
7. Samples are lyophilised (Chris Beta 2-8 Freeze Dryer) (4-6 holes in lid) to obtain a dry crude venom sample.

8. Dry crude venom for each individual at each milking will be pooled and homogenised (mortar and pestle) then weighted and stored at -80°C in 1.6 mL cryotubes until required.

Reference:

Nedosyko, A.M., Young, J.E., Edwards, J.W. & Da Silva, K.B. 2014. Searching for a toxic key to unlock the mystery of anemonefish and anemone symbiosis. *PLoS One,* 9**,** e98449.

Sencic, L. & Macek, P. 1990. New method for isolation of venom from the sea anemone Actinia cari. Purification and characterization of cytolytic toxins. *Comparative Biochemistry and physiology. B, Comparative Biochemistry*, 97**,** 687-693.

Authorised by Supervisor:	Catherine Abbott *Name*	 *Signature*	21/06/21 *Date*

Appendix 6.4 Purification of total RNA from anemone tissues

	Flinders University School of Biological Sciences **Standard Operating Procedures (SOP) for Purification of Total RNA from Anemone Tissues**	
Personnel Hazards & Safety	   	

SOP Number	RA Number	RA Score	Date:
			20/11/20
Contact Person	**SOP prepared by**		**Review Date :**
	Catherine Abbott/ Ly Kim		20/11/23

General

This Standard Operating Procedures (SOP) is for purifying RNA from animal tissue using RNeasy Mini Kit.

Materials and Equipment

1. Animal tissue samples
2. RNeasy Mini Kit.
3. 14.3 M β-mercaptoethanol (β-ME)
4. 70% ethanol
5. Liquid nitrogen
6. Centrifuge
7. Pipettes
8. Pipette tips
9. forceps
10. -80ºC Freezer

Procedure

1. Excise the tissue sample from the animal or remove it from storage. Tissue should be stored in an agent like RNAlater or protect for stablisation of the RNA. Remove stabilised tissues from the reagent using forceps. Determine the amount of tissue. Do not use more than 30 mg. Weighing tissue is the most accurate way to determine the amount.
2. If using only a portion of the tissue, cut it on a clean surface. Weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenisation.

   Proceed to step 3. RNA in RNA protect stabilised tissues is protected during cutting and weighing of tissues at ambient temperature (15–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNA protect Tissue Reagent. Previously stabilised tissues can be stored at −90 to −65°C without the reagent.
3. Disrupt the tissue (do not use more than 30 mg tissue).

   Disruption and homogenisation using a motor–pestle homogeniser: Place the weighed RNA protect stabilised tissue in a mortar and grind by pestle in liquid nitrogen. Immediately disrupt and homogenise the tissue and add the homogenised tissue into 600 µL Buffer RLT. Proceed to step 4.

   Note: Incomplete homogenisation leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column.

   Note: Ensure that β-ME is added to Buffer RLT before use. Add 10 µL β-ME per 1 mL Buffer RLT
4. Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to a new microcentrifuge tube (not supplied). Use only this supernatant (lysate) in subsequent steps.

   In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.
5. Add 1 volume of 70% ethanol* to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.

   Note: The volume of lysate may be less than 350 µL or 600 µL due to loss during homogenisation and centrifugation in steps 3 and 4.

   Note: Precipitates may be visible after the addition of ethanol. This does not affect the procedure.

6. Transfer up to 700 µL of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Reuse the collection tube in step 7. If the sample volume exceeds 700 µL, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.
7. Add 700 µL Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 8.
8. Add Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 9.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use. Add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
9. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

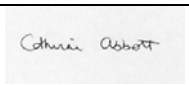
   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
10. **Optional:** Place the RNeasy spin column in a new 2 mL collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 12.
11. Place the RNeasy spin column in a new 1.5 mL collection tube (supplied). Add 25 µL RNAse-free water directly to the spin column membrane. Close the lid gently, wait for a minute and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
12. Since the expected RNA yield from tentacles will be low, repeat step 14 using another 25 µL RNAse-free water. Reuse the collection tube from step 14.

If using the eluate from step 14, the RNA yield will be 15–30% less than that obtained using the second volume of RNAse-free water, but the final RNA concentration will be higher.

Reference:

RNeasy Mini Handbook 10/2019 (Qiagen)

Authorised by Supervisor:	Catherine Abbott *Name*	 *Signature*	20/11/20 *Date*

Appendix 6.5 Reverse transcription with elimination of genomic DNA for quantitative PCR procedure

	Flinders University School of Biological Sciences **Standard Operating Procedures (SOP) for Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR.**	
Personnel Hazards & Safety		

SOP Number	RA Number	RA Score	Date:
			29/01/21
Contact Person	**SOP prepared by**		**Review Date :**
	Catherine Abbott/ Ly Kim		29/01/24

General

This Standard Operating Procedures (SOP) is for cDNA (cDNA) synthesis from total RNA with integrated removal of genomic contamination for use in real-time PCR.

Materials and Equipment

1. RNA
2. Quantitect Reverse transcription kit
3. Heat block
4. Pipettes
5. Pipette tips
6. -20ºC Freezer and 4ºC fridge

Procedure

1. Thaw template RNA on ice. Thaw gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water at room

temperature (15–25°C). Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes, and then store on ice.

2. Prepare the genomic DNA elimination reaction on ice according to Table below. Mix and then store on ice.

Table: Genomic DNA elimination reaction components

Component	Volume/reaction	Final concentration
gDNA Wipeout Buffer, 7x	2 µl	1x
Template RNA	Variable (up to 1 µg*)	
RNase-free water	Variable	
Total volume	**14 µl**	–

* This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, and regardless of the primers used or cDNA analyzed.

3. Incubate for 2 min at 42°C. Then place immediately on ice.

   Note: Do not incubate at 42°C for longer than 10 min.

4. Prepare the reverse-transcription master mix on ice according to Table 2. Mix and then store on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed.

   Note: The protocol is for use with 10 pg to 1 µg RNA. If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 40 µL reaction volume.

Table: Reverse Transcription reaction components.

Component	Volume/reaction	Final concentration
Reverse-transcription master mix		
Quantiscript Reverse Transcriptase*	1 µl	
Quantiscript RT Buffer, 5x†‡	4 µl	1x
RT Primer Mix‡	1 µl	
Template RNA		
Entire genomic DNA elimination reaction (step 3)	14 µl (add at step 5)	
Total volume	**20 µl**	–

* Also contains RNase inhibitor.

† Includes Mg^{2+} and dNTPs.

‡ For convenience, premix RT Primer Mix and 5x Quantiscript RT Buffer in a 1:4 ratio if RT Primer Mix will be used routinely for reverse transcription. This premix is stable when stored at –20°C. Use 5 µl of the premix per 20 µl reaction.

5. Add template RNA from step 3 (14 µL) to each tube containing reverse-transcription master mix. Mix and then store on ice.
6. Incubate for 15 min at 42°C. In some rare cases (e.g., if the RT-PCR product is longer than 200 bp or if analysing RNAs with a very high degree of secondary structure), increasing the incubation time up to 30 min may increase cDNA yields.
7. Incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase.
8. Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix. Store reverse-transcription reactions on ice and proceed directly with real-time PCR, or for long-term storage, store reverse-transcription reactions at –20°C.

Reference:

QuantiTect Reverse Transcription Handbook 03/2009 (Qiagen)

Authorised by Supervisor:	Catherine Abbott *Name*	Catherine Abbott *Signature*	29/01/2021 *Date*

Appendix 6.6 DNA purification using Wizard SV gel and PCR clean-up system procedure

	Flinders University School of Biological Sciences **Standard Operating Procedures (SOP) for Wizard® SV Gel and PCR Clean-Up System**	
Personnel Hazards & Safety	   	

SOP Number	RA Number	RA Score	Date:
			20/11/20
Contact Person	**SOP prepared by**		**Review Date :**
	Catherine Abbott/ Ly Kim		20/11/23

General

This Standard Operating Procedures (SOP) is for purifying DNA using Wizard® SV Gel and PCR Clean-Up System.

Materials and Equipment

1. PCR Products in gel slice or in liquid
2. Wizard® SV Gel and PCR Clean-Up System kit
3. Centrifuge
4. 1.5 mL microcentrifuge tube
5. Pipettes
6. Pipette tips
7. Vortex
8. Scalpel
9. UV machine
10. -20ºC Freezer and 4ºC fridge

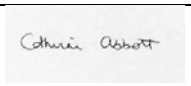
Procedure

1. **A. Dissolving the Gel Slice** - Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5mL microcentrifuge tube. Add 10µL Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

   B. Processing PCR Amplifications - Add an equal volume of Membrane Binding Solution to the PCR amplification.
2. Insert SV Minicolumn into Collection Tube and transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × g for 1 minute. Discard flow-through and reinsert Minicolumn into Collection Tube.
4. Add 700µL Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flow-through and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500µL Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.
6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
7. Carefully transfer Minicolumn to a clean 1.5mL microcentrifuge tube.
8. Add 50µL of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute. Discard Minicolumn and store DNA at 4°C or –20°C.

Reference:

Wizard® SV Gel and PCR Clean-Up System instructions (Promega)

Authorised by Supervisor:	Catherine Abbott *Name*	 *Signature*	20/11/20 *Date*

Appendix 6.7 DNA sample preparation for AGRF sanger sequencing service

1. Purified DNA (PD) Service: Purified DNA template (plasmid or PCR product) is pre-mixed with the appropriate primer, and submitted for BDT labelling, purification and sequencing.
2. Templates should be resuspended and submitted only in water.
3. To ensure good DNA quality, templates should be analysed by both:

   a. Agarose gel electrophoresis using a known mass standard where a visible band should be present on the gel at the expected quantitated level.

   b. Spectrophotometer to ensure OD_{260}/OD_{280} range is between 1.8 and 2.0

   $OD_{260}/OD_{280} < 1.8$ may indicate protein contamination

   $OD_{260}/OD_{280} > 2.0$ may indicate RNA contamination
4. The template and sequencing primer (0.8 pmol/µL) (one primer per submission) and miliQ water (if necessary) should be pre-mixed in a total volume of 12µL, according to Table below.
5. Preferred tube: 1.5mL flip-cap tube (snap lock and boil proof are preferred -use clean DNase free tubes that have NOT been autoclaved).

Table A. Recommended amounts of template and primer for sequencing reactions.

Template	Recommended Quantity for PD Samples (in 12µL)
PCR Product 100 – 200 bp	3 - 8 ng
PCR Product 200 – 400 bp	6 - 12 ng
PCR Product 400 – 600 bp	12 - 18 ng
PCR Product 600 – 800 bp	18 - 30 ng
PCR Product >800 bp	30 - 75 ng
Plasmid, Single-stranded	150 - 300 ng
Plasmid, Double-stranded	600 - 1500 ng
Primer Quantity (one primer per reaction)	10pmol* (0.8 pmol/µl)

Table B. Calculated amounts of template for sequencing reaction in this project.

	1	2	3	4	5	6	7	8
Name	Cyto-1-FOR-200	Cyto-1-REV-200	Cyto-2-FOR-300	Cyto-2-REV-300	Cyto-3-FOR-600	Cyto-3-REV-600	Cyto-4-FOR-750	Cyto-4-REV-750
DNA	1.3 µL	1.3 µL	6.3 µL	6.3 µL	9 µL	9 µL	5.8 µL	5.8 µL
Primer	2 µL FOR	2 µL REV	2 µL FOR	2 µL REV	2 µL FOR	2 µL REV	2 µL FOR	2 µL REV
Water	8.7 µL	8.7 µL	3.7 µL	3.7 µL	1 µL	1 µL	4.2 µL	4.2 µL
Total	12 µL	12 µL	12 µL	12 µL	12 µL	12 µL	12 µL	12 µL

Appendix 6.8 Pierce Bicinchoninic Acid Protein assay procedure

	Flinders University School of Biological Sciences **Standard Operating Procedures (SOP) for Pierce Bicinchoninic Acid Protein Assay (BCA).**	
Personnel Hazards & Safety	   	

SOP Number	RA Number	RA Score	Date:
			20/10/20
Contact Person	**SOP prepared by**		**Review Date :**
	Catherine Abbott/ Ly Kim		20/10/23

General

This Standard Operating Procedures (SOP) is to determine the protein content of the anemone venom (per mg of dry weight) using Bovine Serum Albumin (BSA) as a standard.

Materials and Equipment

1. Venom samples
2. Pierce™ BCA Protein Assay kit
3. 37 ºC incubator
4. Pipettes
5. Pipette tips
6. 96-well plate

Procedure

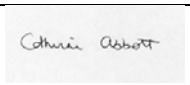
1. Dilute the contents of one albumin standard (BSA) stock ampule (2mg/mL) into 9 tubes (BCA kit, Thermoscientific, Cat no. TA262192)
2. Make serial dilutions as following:

Tube	Volume of Milli-Q (µL)	Volume and source of BSA (µL)	Final BSA concentration (µg/mL)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	200 of stock	1000
D	175	175 of B	750
E	325	325 of C	500
F	325	325 of E	250
G	325	325 of F	125
H	400	100 of G	25
I	400	-	0 (Blank)

3. Calculate working reagent (WR)
4. Total WR volume = (#standards + #samples) x (#replicates) X (volume of WR per sample, 200ul)
5. Prepare WR by mixing 50:1 of reagent A and B
6. Resuspend 50 mg of anemone venom for each sample in 500 µL of sterile Milli-Q water
7. Pipette 25 µL of each sample and standards into 96 wells plate
8. Add 200 µL of the WR to each well and mix plate thoroughly
9. Cover plate and incubate at 37°C for 30mins
10. Cool plate at RT
11. Measure absorbance at 562nm using Fluostar Omega
12. Make a graph of BSA standard absorbance vs. concentration and fit a linear line of best fit
13. Determine unknown venom concentration from standard curve graph

Reference:

User guide: Pierce™ BCA Protein Assay Kit (Thermo Scientific)

Authorised by Supervisor:	Catherine Abbott *Name*	 *Signature*	29/01/2021 *Date*

Appendix 6.9 SDS-PAGE electrophoresis procedure

	Flinders University School of Biological Sciences **Standard Operating Procedures (SOP) for SDS-PAGE Electrophoresis.**	
Personnel Hazards & Safety	   	

SOP Number	RA Number	RA Score	Date:
			20/10/20
Contact Person	**SOP prepared by**		**Review Date :**
	Catherine Abbott/ Ly Kim		20/10/23

General

This Standard Operating Procedures (SOP) is for separation of proteins using SDS-PAGE electrophoresis.

Materials and Equipment

1. Venom samples
2. 6x sample buffer (Cat no. 3SBFI, TCI)
3. 10x tris-glycine buffer (Tris-base, Glycine)
4. 10% SDS
5. 2- mercaptoethanol
6. 1x running buffer
7. Heat block
8. Pipettes
9. Pipette tips
10. Precision plus protein dual extra standards (Cat. no. 161-0377, BioRad)
11. 4-20 % Mini-PROTEAN® TGX Precast Protein Gels (Cat. no. 4561094, BioRad)
12. Tetra cell (Biorad)
13. Power pack

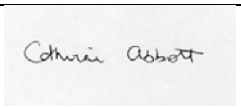
14. Fixing solution: Ethanol and Acetic Acid
15. QC Colloidal Coomassie Blue (Cat. no. 1610803, BioRad)

Procedure

1. Make 10x tris-glycine buffer (30.285g tris base, 144.134g glycine in 800 mL, pH 8.3-9, add Milli Q to make 1L) and 1X running buffer (100 mL of 10x tris-glycine buffer, 10mL 10% SDS, 890 mL Milli Q).
2. Following the instructions for 4-20 % Mini-PROTEAN® TGX Precast Protein Gels 10-well, 50 µL (Cat no. 4561094, BioRad) assemble the gel in the Mini-PROTEAN® tetra cell. (http://www.biorad.com/webroot/web/pdf/lsr/literature/Bulletin_1658100.pdf)
3. Fill the buffer chambers with 1x running buffer and wash sample wells with running buffer.
4. Make loading dye (70 µL of 6X sample buffer (TCI, Cat no. 3SBFI), 30 µL of 2-mercaptoethanol)
5. Add 15 µg of samples and 10 µL of loading dye in a microcentrifuge tube.
6. Incubate at 95 ^{o}C for 5mins.
7. Load precision plus protein dual extra standards (5 µL) into lane 1 and 50 µL of samples into the remaining lanes.
8. Run gel at 170 v for 40 mins by attaching tetra cell to a consort power pack.
9. Remove gel from the cell when the last bands of marker have reached the reference line.
10. Rinse gel once with deionized water.
11. Fix gel in fixing solution (40% ethanol and 10% acetic acid) for 15 minutes with gentle agitation.
12. Rinse gel once with deionized water.
13. Add QC Colloidal Coomassie Blue (BioRad) to the gel and stain for 24 hours with gentle agitation.
14. Destain the gel in deionized water. Destain for 3 hours with gentle agitation. Change the water at least three times.
15. Load the stained gel into Gel Doc EZ and run the coomassie blue protocol.

Reference:

LaemmLi, UK 1970, 'Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4', Nature, vol. 227, no. 5259, pp. 680-5.

Authorised by Supervisor:	Catherine Abbott *Name*	 *Signature*	20/10/20 *Date*

Appendix 6.10 Haemolysis assay procedure

	Flinders University School of Biological Sciences **Standard Operating Procedures (SOP) for Haemolysis Assay.**	
Personnel Hazards & Safety	   	

SOP Number	RA Number	RA Score	Date:
			29/1/21
Contact Person	**SOP prepared by**		**Review Date :**
	Catherine Abbott/ Ly Kim		29/1/24

General

This is a Standard Operating Procedures (SOP) to determine the protein concentration of anemone venom required to the haemolyse 50% of sheep erythrocytes (to work out the EC_{50} value of a venom sample). The original procedure was done in 1 mL tube and the protocol was converted to a 96-well plate for high throughput assay.

Hazards

1. Expose to hazardous substances (venom)
   - Use appropriate PPE
   - Clean up spills immediately
   - Dispose all hazardous substances appropriately
2. Expose to biological materials (blood)
   - Use appropriate PPE
   - Clean up spills and store or dispose of all biological materials appropriately
   - See notes about immunisation against Q fever present in sheep.
3. Centrifuge
   - Ensure that you have balanced the tubes before you centrifuge
   - Never attempt to open the lid of a centrifuge or slow the rotor by hand or open the lid while rotor is in motion as serious injuries may be incurred.

Materials and Equipment

1. Anemone venom samples
2. Sheep blood (CPDA-1/ACD)
3. Phosphate Buffered Saline (PBS), pH 7.4
4. Sterile Milli-Q water
5. Pipettes
6. Pipette tips
7. Centrifuge
8. Microplate reader
9. Flat Bottomed 96 well plates (Cat. no. 655180, Cellstar)
10. V Bottomed 96 well plates (Cat. no. 3396, Costar)

Procedure

1. Immunisation for Q-fever was obtained at Occmed SA Clinic 29/10/2020.

2. Resuspend 50 mg venom in 350-500 µL of sterile Milli-Q water and run a BCA assay

3. Place 5 mL of Sheep Blood (CPDA-1/ACD) (obtained from Applied Biological Products Management) in 50 mL centrifuge tube on ice (blood stored at 4°C for up to 3 weeks). **Refer to SOP for safe handling of anemone venom and blood.**

4. Centrifuge blood for 10 minutes at 1500 xg at 4°C.

5. Remove serum and white buffy layers using a 10 mL Stripette (Sigma Aldrich CLS4492).

6. Resuspend the pellets of erythrocytes in 50 mL of Phosphate Buffered Saline (PBS) (800mL milli-Q, 8g NaCl, 0.2g KCl, 1.44g Na2HPO4, 0.24g KH2PO4 make up to 1L with milli-Q) buffer, pH 7.4.

7. Repeat steps 4 -6 twice.

8. Adjust the volume of erythrocytes used, so that the 100% lysed positive control can be read at chosen wavelength (415nm)- max absorbance < 5.4 OD; (540nm) -max absorbance <1 OD
• Complete lysis is achieved using positive control Triton X-100 (0.1%).

9. Pipette 100 µL of erythrocytes into each well of a V-bottom 96-well plate as needed. Add venoms to erythrocyte suspensions at various concentrations in triplicate.

    • This is done by adding venom dilutions to the 100 µL of erythrocytes and adding PBS to make a total volume of 200 µL

10. Include a negative control (PBS) to test background haemolysis and a positive control to test 100% haemolysis, both in triplicate.

11. Incubate at 37°C for 40 minutes on a stirrer then centrifuge plate for 5 minutes at 500 xg at 4°C.

12. Transfer 100 µL of each supernatant to a well in a flat bottom 96-well plate.

13. Read absorbance at 415 nm using a plate reader spectrometer.

14. Calculate percentage lysis as:

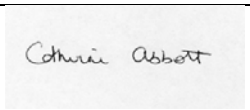
(AbsSample – AbsNegative)/ (AbsPostive-AbsNegative) x100

15. Use Excel to create an EC50 curve for effective concentration of 50% lysis

Reference:

Bailey, P.M., Bakker, A.J., Seymour, J.E. & Wilce, J.A. 2005. A functional comparison of the venom of three Australian jellyfish—Chironex fleckeri, Chiropsalmus sp., and Carybdea xaymacana—on cytosolic Ca2+, haemolysis and Artemia sp. lethality. *Toxicon,* 45**,** 233-242.

Evans, B.C., Nelson, C.E., Shann, S.Y., Beavers, K.R., Kim, A.J., Li, H., Nelson, H.M., Giorgio, T.D. & Duvall, C.L. 2013. Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. *JoVE (Journal of Visualized Experiments)***,** e50166.

Authorised by Supervisor:	Catherine Abbott *Name*	 *Signature*	21/3/21 *Date*

Appendix 6.11 Yeast screening assay procedure

	Flinders University School of Biological Sciences **Standard Operating Procedures (SOP) for Yeast Screening Assay.**	
Personnel Hazards & Safety	   	

SOP Number	RA Number	RA Score	Date:
			29/1/21
Contact Person	**SOP prepared by**		**Review Date :**
	Catherine Abbott/ Ly Kim		29/1/24

General

This Standard Operating Procedures (SOP) is for screening of cytotoxic effects of sea anemone venom on the wild type yeast *Saccharomyces cerevisiae* strains.

Hazards

1. Expose to hazardous substances (venom)
   - Use appropriate PPE
   - Clean up spills immediately
   - Dispose all hazardous substances appropriately in the purple bin waste stream
2. Expose to biohazard (yeast)
   - Use appropriate PPE
   - Clean up spills and store or dispose of all biological materials appropriately
3. Centrifuge
   - Ensure that you have balanced the tubes before you centrifuge
   - Never attempt to open the lid of a centrifuge or slow the rotor by hand or open the lid while rotor is in motion as serious injuries may be incurred.

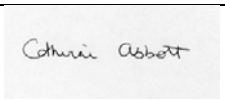
Materials and Equipment

1. Anemone venom samples

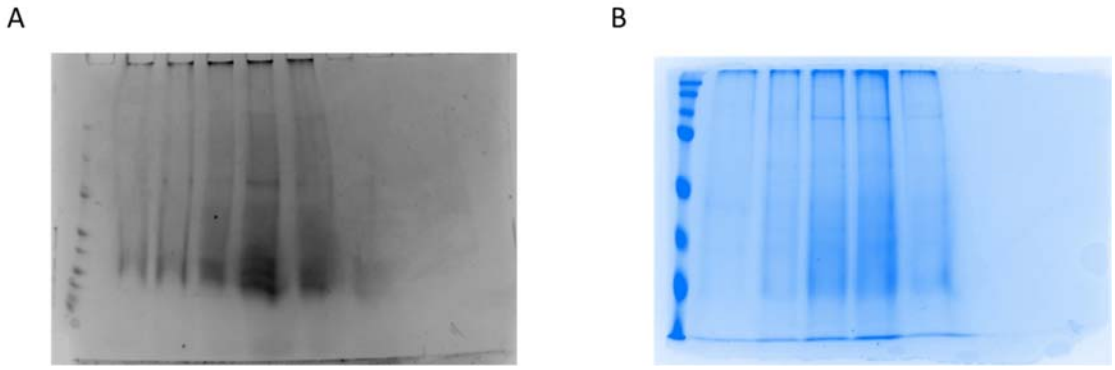
2. Wild type yeast *S. cerevisiae* strains
3. Phosphate Buffered Saline (PBS), pH 7.4
4. Sterile Milli-Q water
5. Pipettes
6. Pipette tips
7. Flat bottomed sterile 96-well plates (Cat. no. 655180, Cellstar)
8. Centrifuge
9. Shaking incubator
10. Microplate reader
11. -80ºC Freezer

Procedure

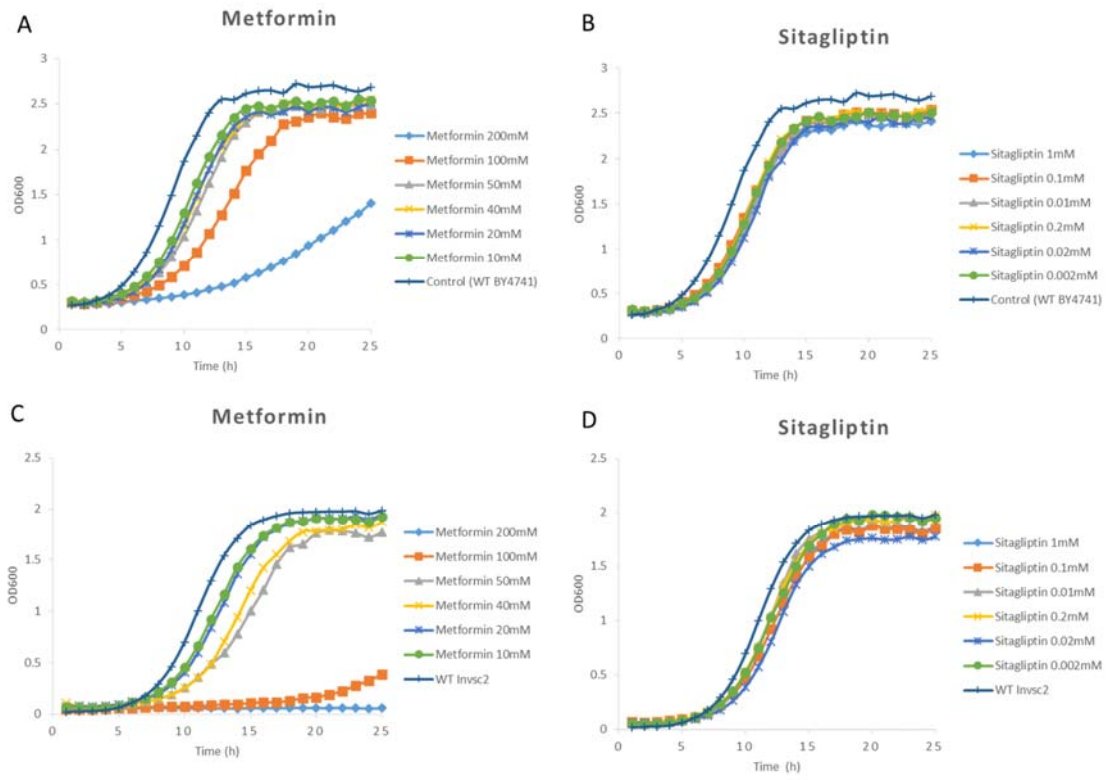
1. Pick a single wild type yeast *S. cerevisiae* colony less than one month old and grow in 5 mL Yeast Peptone Dextrose (YPD) media
2. Incubate cells at 30 ^{0}C with shaking at 140 rpm overnight
3. Spin cell at 3000 xg for 5 minutes at 4^{0}C
4. Remove supernatant and add 5mL of autoclaved water and vortex
5. Repeat step 3-4, twice
6. Re-suspend cells with 5mL of water
7. Dilute the pre-grown yeast cells to 1:100 (100 µL of culture + 9,900 µL of YPD media)
8. Add 150 µL of 1/100 yeast cells in 96-well plate in triplicate
9. Add venom at final concentration of 25 µg/mL, 50 µg/mL, 100 µg/mL in YPD media
10. Add YPD media to make up to 250 µL
11. Add 50 µL of Metformin 500 mM to positive control samples in triplicate, only 100 µL of yeast cell as negative control
12. Read absorbance at OD 600nm every 15 minutes for 24h at 30^{0}C.
13. Use Excel to make yeast growth curve

Authorised by Supervisor:	Catherine Abbott *Name*	 *Signature*	29/1/21 *Date*

Appendix 6.12 Gel images of trial SDS-PAGE. A. 4 -20% Mini-PROTEAN TGX Gel with silver stain. B. Home-made 10 % SDS-PAGE gel with Coomassie Brilliant Blue R 250 stain.



Appendix 6.13 Yeast growth curve with metformin and sitagliptin treatment. (A) Yeast strain WT BY4741 and metformin. (B) Yeast strain WT BY4741 and sitagliptin. (C) Yeast strain WT Invsc2 and metformin. (D) Yeast strain WT Invsc2 and sitagliptin.



Appendix 6.14 Low venom protein concentrations do not affect yeast cell growth. (A) Yeast WT BY4741 and venom at protein concentration 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, and 8 µg/mL. (B) Yeast WT By4741 and venom at protein concentration at 5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL.