MOLECULAR CHARACTERIZATION OF CALPONIN-2, AN IMMUNE-RELATED GENE IN SNAKEHEAD FISH, *CHANNA STRIATA*

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ABSTRACT

Channa striata, locally known as "snakehead fish" is an important commercial freshwater fish in Southeast Asia. While highly immune to common diseases affecting commercial fishes, recent reports have shown that *C. striata* are prone to the disease Epizootic Ulcerative Syndrome (EUS). Control of EUS is associated with immune responsive genes. In this study, it is suggested that calponin-2 is a potential immune-related gene (designed as *Cs*Calp) that plays a functional role during EUS infection in snakehead fish. The full-length cDNA of the *Cs*Calp is 1114 base pairs (bp) long, containing a 5′ untranslated region (UTR) of 104 bp, a 3‟ UTR of 56 bp including a stop codon (TAA), and an open reading frame (ORF) of 954 bp encoding 317 amino acids with the predicted molecular mass of 35.14 kDa and an estimated isoelectric point of 6.15. Sequence analysis showed that calponin has a calponin homology domain and calponin-like repeats. Quantitative real-time PCR analysis showed ubiquitous expression of *Cs*Calp in all the *C. striata* tissues tested with the highest expression level in the blood. Preliminary analysis of *Cs*Calp expression in blood challenged with *Aeromonas hydrophila* showed significant ($P<0.05$) changes in the gene regulation. As time progressed, the expression level of *Cs*Calp increased significantly and reached the maximum at 24 h after the *A. hydrophila* challenge. The results indicated that *Cs*Calp gave positive response towards bacterial infection in *C. striata*. *Cs*Calp was then directly cloned into the TOPO expression vector, pTrcHis® TOPO in the *E. coli* system. This construct, however, failed to give significant expression of recombinant *Cs*Calp. Nevertheless, these findings should be further investigated for downstream functional studies of calponin-2 in the snakehead fish immune system.

ABSTRAK

Channa striata, lebih dikenali sebagai "haruan" ialah ikan air tawar komersial yang penting di Asia Tenggara. Walaupun amat imun kepada penyakit-penyakit yang biasa menjangkiti ikan-ikan komersial, laporan baru-baru ini telah membuktikan bahawa *C. striata* cenderung kepada penyakit Sindrom Ulcer Epizotik (EUS). Kawalan EUS dikaitkan dengan gen-gen responsif imun. Dalam kajian ini, dicadangkan calponin-2 berpotensi sebagai satu gen responsif imun (direka sebagai *Cs*Calp) yang berperanan semasa jangkitan EUS dalam ikan haruan. Panjang keseluruhan cDNA *Cs*Calp ialah 1114 pasangan bes (pb) termasuk satu 5‟ rantau tidak diterjemah (UTR) 104 pb, satu 3‟ rantau 56 pb termasuk kodon penamat (TAA), dan satu rangka bacaan terbuka (ORF) 954 pb yang mengekod 317 asid amino dengan jangkaan jisim molekul 35.14 kDa dan satu titik isoelektrik dianggarkan 6.15. Analisis jujukan menunjukkan bahawa calponin mempunyai satu domain homologi calponin dan ulangan seperti calponin. Analisis kuantitatif masa-nyata PCR menunjukkan bahawa *Cs*Calp diekspresikan dalam semua tisu *C. striata* yang diuji dengan tahap ekspresi tertinggi dalam darah. Analisis awal ekspresi *Cs*Calp dalam darah yang dicabar dengan *Aeromonas hydrophila* menunjukkan perubahan yang signifikan ($P<0.05$) dalam kawal aturan gen. Apabila masa berlalu, tahap ekspresi *Cs*Calp telah meningkat dengan nyata dan sampai di tahap maksimum pada 24 jam selepas cabaran *A. hydrophila*. Keputusan ini menunjukkan bahawa *Cs*Calp memberi gerak balas positif terhadap jangkitan bakteria dalam *C. striata*. *Cs*Calp kemudian diklonkan secara langsung ke dalam vektor ekspresi TOPO, pTrcHis® TOPO di dalam sistem *E. coli*. Konstruk ini, bagaimanapun, gagal untuk memberi ekspresi rekombinan *Cs*Calp yang signifikan. Walau bagaimanapun, penemuan-penemuan ini seharusnya disiasat lebih lanjut untuk kajian fungsi-fungsi calponin-2 dalam sistem imun ikan haruan.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celcius
µl	:	microlitre
µM	:	microMolar
Ar	:	Argon
bp	:	base pair
$CaCl_2$	:	calcium chloride
cDNA	:	complementary DNA
CFU/ml	:	colony-forming unit per mililitre
cm	:	centimeter
DEPC	:	diethylpyrocarbonate
DMSO	:	dimethyl sulfoxide
DNA	:	deoxyribonucleic acid
dNTP	:	deoxyribonucleoside triphosphate
E. coli	:	*Escherichia coli*
EDTA	:	etylenediaminetetraacetic acid
ESI-MS/MS	:	Electrospray ionization tandem mass spectrometry
et al.	:	*et alii* (and other people)
EtBr	:	ethidium bromide
g	:	gravity
GC	:	guanine-cytosine
h	:	hour
IPTG	:	isopropyl β-D-1-thiogalactopyranoside
kb	:	kilobase

kDa	:	kilo Dalton
kV	:	kilo Volt
LB	:	Luria Bertani
lb/sq	:	pounds per square foot
M	:	Molar
m/z	:	mass-to-charge ratio
mbar	:	milibar
MeOH/D.W	:	methanol-to-distilled water ratio
$MgCl_2$	:	magnesium chloride
min	:	minute
ml	:	milliliter
mM	:	milliMolar
mRNA	:	messenger RNA
NaOH	:	sodium hydroxide
ng	:	nanogram
nl	:	nanolitre
OD	:	optical density
ORF	:	open reading frame
PAGE	:	polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
psi	:	pounds per square inch absolute
RNA	:	ribonucleic acid
RNase	:	ribonuclease
rpm	:	rotation per minute

RT-PCR	:	reverse-transcriptase polymerase chain reaction
s	:	seconds
SDS	:	sodium dodecyl sulfate
Taq	:	*Thermus aquaticus*
TBE	:	tris borate EDTA
TEMED	:	tetramethyl-ethylenediamine
Tris	:	tris(hydroxymethyl)amino methane
U	:	Unit
UTR	:	untranslated region
UV	:	ultraviolet
V	:	Volt
v/v	:	volume per volume
w/v	:	weight per volume
X-gal	:	bromo-chloro-indolyl-galactopyranoside

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Channa striata also known as "haruan" or snakehead murrel, is a prominent tropical freshwater fish that has been extensively used for medicinal and pharmaceutical purposes (Mat Jais *et al.*, 1994; Michelle *et al.*, 2004). It is an important commercial freshwater fish in Southeast Asia. However, the infectious disease, epizootic ulcerative syndrome (EUS) has resulted in high mortality and caused severe economic losses in the snakehead fish farming (Lilley *et al*., 1997). The available literature reported that infection by pathogenic agents like bacteria (*Aeromonas hydrophila*) (Austin & Adams, 1996; Roberts, 1997), fungus (*Aphanomyces invadans*) and viruses can cause severe mortality in the *C. striata* (Dhanaraj *et al*., 2008). Consequently, snakehead fish rely on their innate immune components to fight against these infections.

Understanding different components of the snakehead fish immune mechanisms is required to improve fish health. Thus, one alternative is to: identify, characterize, determine the changes in the expression and investigate the roles of immune-related genes. Some of the immune molecules that have been characterized in *C. striata* includes chemokine receptors (Bhatt *et al*., 2014), thioredoxin (Palanisamy *et al*., 2014), serine protease (Arockiaraj *et al*., 2014), cathepsin (Kumaresan *et al*., 2014) and tumor necrosis factor receptor (Palanisamy *et al*., 2015).

Calponin is a family of actin-associated proteins first found in smooth muscle cells (Takahashi *et al*., 1986). It has three isoforms (h1-, h2- and acidic calponins) which encoded by three homologous genes. Generally, this gene plays a major role in smooth muscle contraction as calponin is highly expressed in muscle cells. Basic calponin specifically expressed in differentiated smooth muscle cells and has been extensively studied for its role in the regulation of smooth muscle contractility (Winder

et al., 1998; Small & Gimona, 1998; Morgan & Gangopadhyay, 2001). Acidic calponin has been reported in nervous tissues and implicates in neuronal regeneration and growth (Ferhat *et al*., 1996; Ferhat *et al*., 2001). However, h2-calponin or calponin-2 is found in both smooth muscle and certain non-muscle cells such as human epidermal keratinocytes (Fukui *et al*., 1997), with its mRNA detected in endothelial cells (Sakihara *et al*., 1996) and fibroblasts (Masuda *et al*., 1996). It has also been reported that calponin-2 significantly expressed in peripheral blood cells of myeloid lineage with an up-regulation during monocyte to macrophage differentiation (Huang *et al*., 2008). These accumulating evidences clearly indicate the function of calponin-2 in multiple cellular activities via regulation of actin cytoskeleton.

Indeed, calponin-2 was found to be inhibitor in myeloid cell proliferation, migration and phagocytosis when functional characterization of macrophages from calponin-2 knock-out mice was constructed (Huang *et al*., 2008). In addition, forced expression of calponin-2 in cells lacking endogenous calponin results in an association with the actin stress fibers and a decrease in the rate of cell proliferation (Hossain *et al*., 2003). These accumulated results suggest a microfilament-associated activity of calponin-2, which may regulate the function of actin cytoskeleton.

The actin cytoskeleton plays a major role in cell motility that is essential for macrophage locomotion, phagocytosis and endocytosis (Beningo & Wang, 2002). These activities are essential in defensive and autoimmune responses. Elsewhere, the regulation and function of calponin-2 have been reported in zebrafish (Tang *et al*., 2006). At present, the role of calponin-2 in freshwater fish has yet to be clearly described. The gene regulation and function of calponin-2 are largely unknown as the majority of previous studies of calponin are focussed on mammalian h1-calponin. This

calls for more investigations to be conducted to facilitate a better understanding on the role of calponin-2 particularly in non-muscle cells.

The present study aims to isolate and characterize calponin-2 gene from *C. striata* followed by *Cs*Calp mRNA profiling at varied time point upon *A. hydrophila* infection. This study also aims to elucidate possible functional role of *Cs*Calp from the subsequent result. Knowing the role of *Cs*Calp gene will be useful in understanding the fish immune system. Finally, *Cs*Calp expression data upon *A. hydrophilla* challenge may underlie its role upon infection that can be used as a tool to overcome disease problem caused by this bacteria.

Objectives

The specific objectives of this study are listed as follows :

i. To isolate and characterize the calponin-2 gene in *C. striata*;

ii. To investigate the mRNA expression of calponin-2 during infection of *A. hydrophila*;

iii. To elucidate the possible role of calponin-2 in *C. striata* upon challenge with *A. hydrophila*.

CHAPTER 2: LITERATURE REVIEW

2.1 *Channa striata*

2.1.1 Zoology of *Channa striata*

Channa striata (Bloch, 1793) belongs to the family of Channidae that was taxonomically classified (Figure 2.1). This species is commonly known as snakehead murrel or serpent-headed fish. In Malaysia, this predaceous fish is locally known as "haruan".

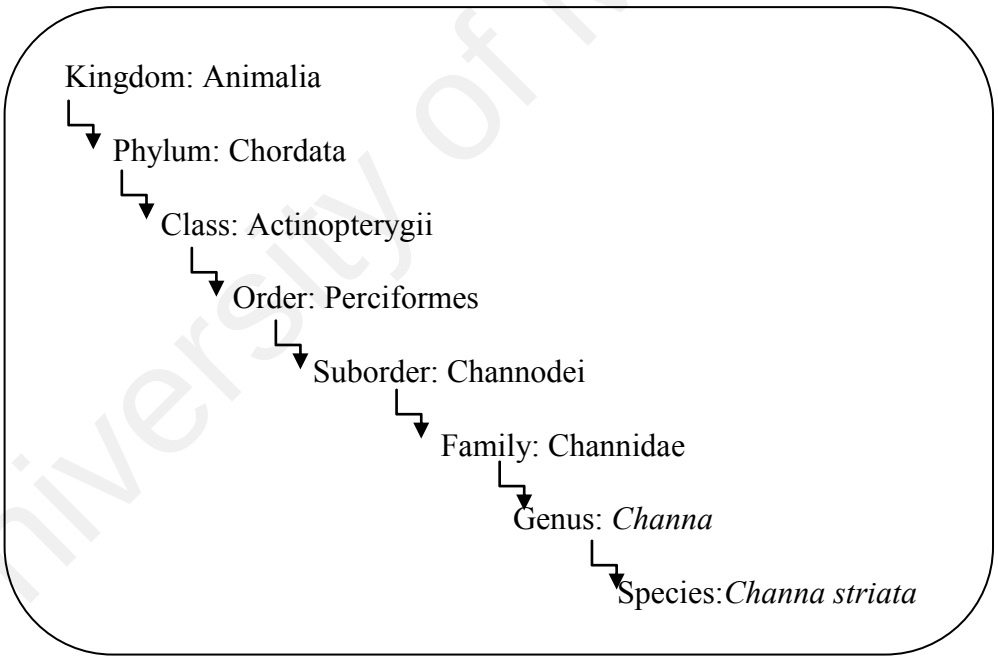


Figure 2.1: Taxonomic classification of *C. striata* (Bloch, 1793).

Generally, the sizes of these species vary from moderate to large. They have some distinguishing features such as its large head resembles that of a snake‟s as described in its given name- snakehead (Figure 2.2). The murrel has an elongated

cylindrical body shape with elongated dorsal and anal fins. Generally, it is tan with dark brown mottling.

In a recent study, it was found that *C. striata* have a diploid (2n) chromosome number of 42, where the karyotype composed of 6 metacentric, 2 acrocentric and 34 telocentric chromosomes. Meanwhile, there is no heteromorphic sex chromosome found in the karyotype (Supiwong *et al.*, 2009).

Figure 2.2: *Channa striata.*
(Source: fishbase.org)

2.1.2 Habitat and distribution

C. striata is the most widely distributed snakehead species, found in the water that spans from Pakistan and India to Southeast Asia and Southern China (Mohsin & Ambak, 1983; Hossain *et al*., 2008). This freshwater fish species can be found in wide ranges of habitats such as rivers, streams, lakes, ponds, reservoirs, ditches, irrigation canals, mining pools, swamps, earthen ponds and paddy fields (Ambak *et al*., 2006; Jamaluddin *et al*., 2011; Lee & Ng, 1994; Musikasinthorn, 2003).

Additionally, their habitats are often covered by thick aquatic vegetation which expands over the entire water surface (Gam *et al*., 2006). Snakehead murrel also seem equally proficient in stationary as well as slow flowing waters (Peter & Kelvin, 1990).

2.1.3 Economic and medical importance

In Malaysia, snakehead fish is abundant in nature, thus it is normally marketed alive, fresh from the catch. This predaceous species is valuable for its therapeutic properties as well as nutritive qualities. Many people have consumed this fish as an alternative remedy for wound-healing following clinical operations, road accidents and caesarians (Barman *et al*., 2014). Consequently, the farming of snakehead fish is carried out commercially by many parties because of their high demands in dietary, clinical and research areas (Gam *et al*., 2006).

C. striata is a prominent tropical freshwater fish widely used for medicinal and pharmaceutical purposes (Mat Jais *et al.*, 1994; Michelle *et al.*, 2004). Many researchers believed that the flesh of this fish is able to rejuvenate due to its recovering elements and hence is given to elderly and those who are in convalescence (Mat Jais, 2007; Kumar *et al*., 2008). The analysis of amino acid composition in snakehead fish demonstrated its role in the process of wound healing (Mat Jais *et al*., 1994). The effectiveness of snakehead fish in the healing of wounds has been proved experimentally (Gam *et al*., 2006).

There are many products available in the markets which contain the extracts of snakehead fishes. For example, the production of cream that contains arachidonic acid which acts as a precursor of prostaglandin that may initiate blood clotting by inducing platelet aggregation and adhesion in endothelial tissue, and thus encourages tissue growth (Mat Jais *et al*., 1994).

2.2 Epizootic Ulcerative Syndrome in Fish

Epizootic ulcerative syndrome (EUS) is one of the most devastating diseases affecting over 100 species of freshwater fish as well as estuarine finfish since 1971 (Saikia & Kamilya, 2012). It was first reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Japan (Egusa & Masuda, 1971) and was later reported in estuarine fish, grey mullet in eastern Australia (Mckenzie & Hall, 1972). EUS is also recognized as Red Spot Disease (RSD) in Australia and mycotic granulomatosis (MG) in Japan.

The dreadful fish disease, EUS has spread across Southeast Asia, India and later to Pakistan and Africa. EUS is defined as seasonal epizootic since it occurs mostly during periods of low temperatures (18 – 22 °C) and after periods of heavy rainfall. EUS is characterized by the occurrence of severe, open haemorrhagic or necrotic ulcerative lesions on the head, mid-body, and dorsal regions of the fish (McGarey *et al.*, 1991). EUS is transmitted from one fish to another through the water supply. The susceptible life stages of the fish are usually juvenile and young adults. To date, there is no report of EUS being found in fish fry or fish larvae.

The aetiology of EUS is very complex and the specific causative agent of this disease remained unclear. Earlier studies showed the role of *A. invadans* in the spread of EUS (Ahmed & Hoque, 1999; Lilley & Roberts, 1997; Vishwanath *et al*., 1998; Saylor *et al*., 2010). However, the fungus alone cannot invade and induce the disease as it is unable to break the skin barrier of fish (Willoughby *et al*., 1995). In addition, it may not be considered as the primary aetiology in view of the fact that, they always been associated with fishes in water without causing any harm (Kar *et al*., 2000).

On the other hand, many studies have reported the association of bacterium *A. hydrophila* and the pseudofungi *Aphanomyces sp.* with EUS-affected fishes (Llobrera & Gacutan, 1987; Boonyaratpalin, 1989; Costa & Wejeyaratne, 1989; Torres, 1990; Lio-Po *et al*., 1992; Roberts *et al*., 1993; Pathiratne *et al*., 1994; Angka *et al*., 1995; Callinan *et al*., 1995; Karunasagar *et al*., 1995; Willoughby *et al.*, 1995; Lilley & Roberts, 1997; Iqbal *et al*., 1999; Rahman *et al*., 1999). Besides, experimental infections using *A. hydrophila* isolated from EUS infected fishes were found to induce EUS-like lesions in fish (Angka, 1990; Karunasagar, 1994; Lio-Po *et al*., 1992; Lio-Po *et al*., 1998).

Since 1983, several types of viruses have been isolated that showed viral association in EUS infected fish (Frerichs, 1995). These viral isolates were mainly rhabdoviruses and birnaviruses. It has also been reported that fish rhabdoviruses as a tertiary causative agent of EUS (Kasornchandra *et al*., 1992; Dhanaraj *et al*., 2008).

Example of fish species that commonly affected by this syndrome are snakeheads *C. striata*, catfish *Clarias batrachus*, sea bass *Lates calcarifer,* swamp eel *Monopterus albus,* gourami *Trichogaster pectoralis*, gobies *Glossogobius giurus*, grey mullet *Mugil cephalus* and barbs *Puntius* spp. There is no effective treatment of EUS infected fish since the primary pathogen of EUS has not yet been identified. Control of EUS in natural water bodies may not be possible. In outbreaks occurring in small, closed water bodies, liming water and improving water quality, together with removal of infected fish, is often effective in reducing mortalities.

2.3 *Aeromonas hydrophila* Causing Disease

2.3.1 Background

A. hydrophila is a ubiquitous Gram-negative bacteria commonly found in aqueous environments (Hazen *et al*., 1978). Aeromonads are facultative; they are capable of utilizing nutrients present in water and surviving for long periods in the absence of host fish (Camus *et al*., 1998). These rod shaped bacteria are widespread in fresh, brackish, estuarine and marine water (Carnahan & Altwegg, 1996). They are frequently isolated from both healthy and diseased fish as well as from other aquatic animals (Rahman *et al.*, 2004).

A. hydrophila can often be isolated from ulcers or internal organs of EUS-affected fish (Llobrera & Gacutan, 1987; Pal & Pradhan, 1990) (Figure 2.3). Conflicting views have been expressed over the precise role of *A. hydrophila* as a fish pathogen (Heuschmann-Brunnerr, 1965; Eurell *et al*., 1978; Michel, 1981). Aeromonads are considered to be opportunistic pathogens or as secondary invader of compromised or stressed hosts (Jeney & Jeney 1995).

Figure 2.3: *C. striata* infected with *A. hydrophila.*
(Source: Mydeen & Haniffa, 2011)

2.3.2 Pathology

A. hydrophila is considered to be one of the most important bacteria among the aetiological agents of fish diseases (Paniagua *et al*., 1990). Since its initial recognition as the causal agent of haemorrhagic septicaemia (Haley *et al*., 1967; Austin & Austin, 2007), *A. hydrophila* has been recovered as a pathogen from a wide variety of freshwater fish species, including ornamental fish (Hettiarachchi & Cheong, 1994) and occasionally from marine fish (Larsen & Jensen, 1977).

This opportunistic pathogen has been associated with tail and fin rot, haemorrhagic septicaemia and EUS (Autin & Adams, 1996; Roberts, 1997). Haemorrhagic septicaemia is recognized by the presence of small surface lesions (which lead to the sloughing off of scales), local haemorrhages particularly in the gills and vent, ulcers, abscesses, exophthalmia and abdominal distension. Llobrera and Gacutan (1987) described the presence of necrotic ulcers in a variety of fish from the Philippines. Internally, there may be accumulation of ascetic fluid, anaemia and damage to the organs, notably kidney and liver (Huizinga *et al*., 1979; Miyazaki & Kaige, 1985).

Also, redsore disease in bass has been credited to *A. hydrophila* (Hazen *et al*., 1978). This condition, which may reach epizootic proportions, is characterized by erosion of the scales and pin-prick haemorrhages, which may cover up to 75 % of the body surface. There is often a high mortality rate. Hettiarachchi and Cheong (1994) reported that *A. hydrophila* as the cause of disease in freshwater ornamental fish in Sri Lanka, with disease signs including the presence of eroded fins, haemorrhages on the skin and at the base of the caudal fin, sloughing scales and haemorrhaging in the intestinal wall.

To date, disease control strategies for this pathogen have centered on the use of antimicrobial compounds and to some extent vaccination (Adams *et al*., 1995; Austin & Austin, 2007). With the success of probiotics (Newaj-Fyzul, *et al*., 2007) for the control of motile *Aeromonas* infections, attention has focused on immunostimulants and plant products.

2.4 Fish Immune System

2.4.1 Introduction

Fish has various mechanisms that protect them from infections caused by foreign pathogens. Unlike higher vertebrate, fish is a free-living organism from early embryonic stages of life. The fish immune system is made of layered defense mechanisms to fight against any infections efficiently. Consequently, they depend on their innate immune system for survival, beginning at the early stages of embryogenesis (Rombout *et al*., 2005). Fish possess both innate and adaptive immunity. The components of the innate immune response include physical barriers, cellular and humoral factors.

In addition, innate immune system plays a major role in both the acquired immune response as well as homeostasis through a system of receptor proteins. Conversely, the adaptive immune system develops after its exposure toward infectious pathogen. It has the advantage of being antigen-specific. Yet, the development of acquired immune response takes a while after the first exposure to the antigen. However, during subsequent exposures the responses are rapid and higher magnitude than initial response (Wiley *et al.*, 2011; Adedeji *et al.*, 2012). This specific immune system includes lymphocytes, immunoglobulins, T-cell receptor and major

histocompatibility complex receptors (Rauta *et al*., 2012). The complex network of these elements is necessary to respond specifically to antigens.

Adaptive immunity thought to have arisen early in vertebrate evolution, between the divergence of cyclostomes (lampreys) and cartilaginous fish (sharks) around 450 million years ago in an evolutionary time period estimated to be less than 20 million years (Marchalonis & Schluter, 1998) (Figure 2.4).

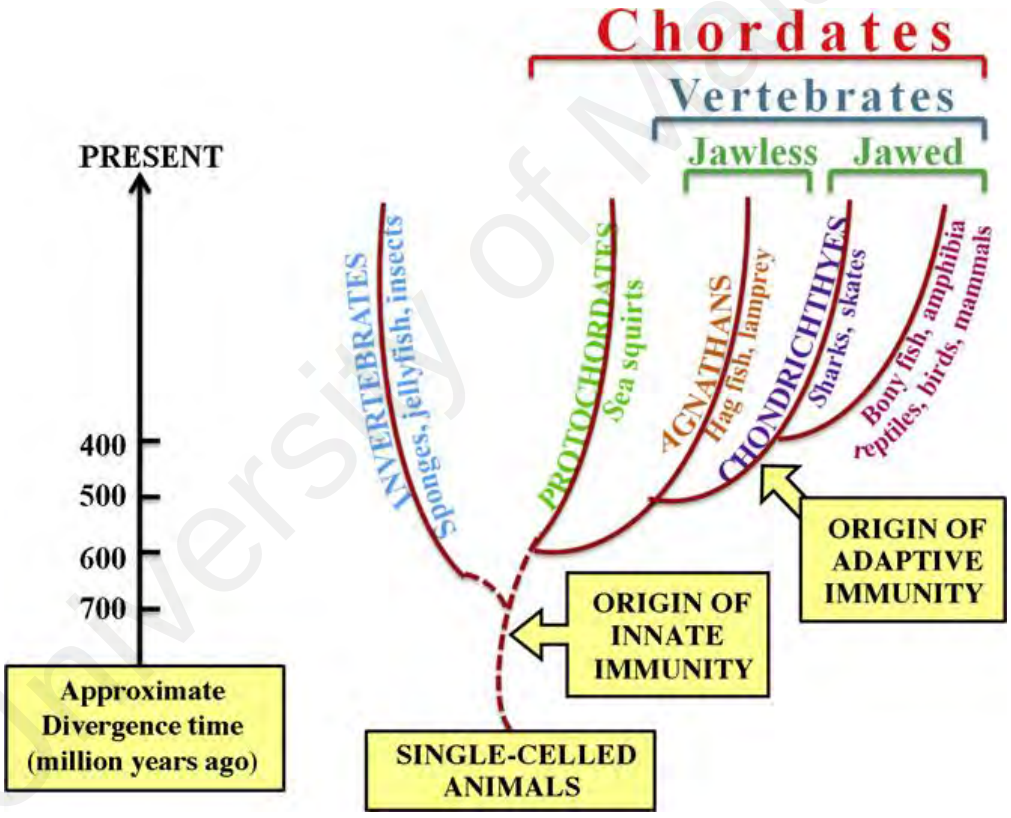


Figure 2.4: Origins and evolutionary relationships between the innate and adaptive immunity.

(Source: Rauta et al., 2012)

2.4.2 The Innate Immune system

With the limitations of the acquired immune system, poikilothermic nature, limited repertoire of antibodies and the slow proliferation, maturation and memory of lymphocytes, the innate response has been considered as essential component in invading pathogens in fish (Whyte, 2007). The physical barrier includes epithelial and mucosal barrier of the skin, gills and alimentary tract, is an extremely important to prevent pathogens from entering the organism (Magnadottir, 2010). In fish, the innate defense mechanisms involve the production of broad-spectrum antibacterial peptides, lysozyme, lectins, acute phase protein, complement system, release of cytokines, inflammation and phagocytosis (Rauta *et al*., 2012).

2.4.2.1 Physical barrier

Fish scales, skin mucous, gills and epidermis act as the first barrier to infection (Ellis, 2001; Ingram, 1980; Shephard, 1994). The important protective role of skin mucous has been widely studied in several fish species such as rainbow trout (*Salmo gairdneri*), carp (*Cyprinus carpio*), Japanese eel (*Anguilla japonica*) and Atlantic salmon (*Salmo salar)* (Hjelmeland *et al*., 1983; Rombout *et al*., 1993; Aranishi & Nakane, 1997; Rombout & Joosten, 1998; Fast *et al*., 2002). Fish mucous functions to trap and slough the pathogen from attaching to the epithelium of fish.

Besides, mucous also contains lectins, lysozymes, pentraxins, complement proteins, antibacterial peptides and IgM (Alexander & Ingram, 1992; Rombout *et al*., 1993; Aranishi & Nakane, 1997; Boshra *et al*., 2006; Saurabh & Sahoo, 2008). In addition, the epidermis or skin constitutes one of the major physical barriers in fishes as it prevents the entry of pathogens into the host via thickening, cellular hyperplasia and its integrity (Hibiya, 1994).

2.4.2.2 Inflammatory Response

Inflammation is a vital non-specific reaction of fish immune system. This internal response is induced by the entry of a pathogen and any traumatic occurrence in the host tissues. This event provides the host good opportunity to maintain stability and recover from injury but could be harmful to host tissue if chronic (Talaro, 2005).

Once a pathogen penetrated the host tissue, mediator factors are released in order to expand and make blood capillaries more permeable. Eventually, the blood flow increased that allowing the migration of the defense cells. The granulocytes are the first cell type to arrive at the inflammation area to attack the foreign pathogens. The remaining pathogenic cells and cellular debris are phagocytosed by macrophages (Magnadottir, 2006).

Inflammation is mediated by a variety of molecules including cytokines such as interleukins and the Tumour Necrosis Factor (TNF). The previous studies have reported the inflammatory response of rainbow trout following the injection of bacteria in detail (Afonso *et al*., 1997; Afonso *et al*., 1998a; Afonso *et al*., 1998b; Afonso *et al*., 2000). After 24 –48 h bacteria injection, there was a marked increase (500-fold) in the number of neutrophils in the peritoneal cavity compared with resting cavity. By 48 h, neutrophils were about five times more numerous than macrophages. The authors speculated that the macrophages could make use of myeloperoxidase and glycogen granules derived from the neutrophils to enhance their bactericidal activity.

2.4.2.3 Phagocytosis

Phagocytosis is one of the most important processes in fish as it is a poikilothermic organism. Changes in temperature however do not influence the phagocytic activities (Blazer, 1991; Lange & Magnadottir, 2003; Magnadottir *et al*.,

2005). The main phagocytic cells in fish include neutrophils and macrophages (Secombes & Fletcher, 1992). These cells produce reactive oxygen species (ROS) during a respiratory burst to remove bacteria (Secombes, 1996; Lamas & Ellis, 1994). In addition, both neutrophils and macrophages possess lysozyme and other lytic enzymes in their lysosomes (Fisher *et al*., 2006; Uribe *et al*., 2011).

2.5 Immunogene or immune-related gene

Immune-related genes are responsible in regulating the immune system. Many studies have documented the immune responses toward fish‟s diseases as fish‟s immune system is critical to their ability to survive and reproduce. Manning (1998) reported that the underlying mechanisms leading to the regulation of gene transcription of both the innate and adaptive immune systems represent the interactions that allow the recognition and the elimination of pathogens.

Immune-related genes are vital to provide essential amino acids and polyunsaturated fatty acids that are needed throughout a healing process (Ghassem, 2003). Jais (1994) has described that the healing process is helped by polypeptide formation with glycine, aspartic acid and glutamic acid that will increase the tensile strength to treat wounds. There is also a study that explained the mechanism that related to the immune related genes. For example, Baie (2000) reported that cells will secrete collagen into the wound site and the collagen was then cross-linked, forming fibers that will eventually heal the wound. The muscle tissue of *C. striata* is rich in angiotensin-converting-enzyme (ACE) inhibitor peptides, suggesting that this species could be able to serve as a formulation of functional foods to prevent hypertension (Ghassem, 2011).

There is vast development of the discovery and functional studies of immune-related genes in fish since the emergence of zebrafish (*D. rerio*) as a model organism and the advancement in genome sequencing technology and bioinformatics (Lieschke & Trede, 2009; Trede *et al*., 2004; Van Muiswinkel, 2008). Indeed, many immune-relevant genes for both innate and acquired immunity, including those encoding cytokines, complements, lectins, immunoglobulins, and certain cell surface molecules, have been characterized from various fish species.

2.6 Calponin

2.6.1 Background of Calponin

Calponin is an actin filament-associated protein of 34–37 kDa (292–330 amino acids) found in smooth muscle (Takahashi *et al*., 1986) as well as non-muscle cells (Hossain *et al*., 2005; Hossain *et al*., 2006). It was first isolated from chicken gizzard and bovine aorta (Takahashi *et al*., 1986; Takahashi *et al*., 1988). Three distinct calponin isoforms namely basic (calponin-h1), neutral (calponin-h2) and acidic calponin have been identified based on isoeletric point (Tang *et al*., 2006).

Calponin-1 is specific to smooth muscle cells and function as a marker for smooth muscle cell differentiation (el-Mezgueldi *et al*., 1996). In contrast to basic calponin, calponin-2 is found in both smooth muscle and non-muscle cells such as epidermal keratinocytes, fibroblasts, lung alveolar epithelial cells and peripheral blood cells of myeloid lineage (Hossain *et al*., 2005; Hossain *et al*., 2006; Masuda *et al*., 1996; Huang *et al*., 2008).

The acidic calponin has been found in smooth muscle (Applegate *et al*., 1994) and brain (Trabelsi-Terzidis *et al*., 1995) and its function remains to be investigated. Most of previous structural and functional studies of calponin were attained from chicken gizzard calponin that is equivalent to the mammalian basic calponin. The neutral calponin is the focus of this study.

2.6.2 Function of Calponin

Calponin has a regulatory role in smooth muscle contraction due to its *in vitro* inhibitory properties (Winder *et al*., 1990; Abe *et al*., 1990). Calponin is associated in the regulation of smooth muscle contraction through its interaction with F-actin and inhibition of the actin-activated MgATPase activity of phosphorylated myosin (Tang *et al*., 2006).

Both properties are lost following phosphorylation (primarily at serine 175) by protein kinase C or calmodulin-dependent protein kinase II. Through high affinity binding to F-actin, calponin inhibits the actin-activated myosin MgATPase (Winder *et al*., 1993) and motor activity (Walsh, 1991).

Over-expression of basic (Jiang *et al*., 1997) or neutral (Hossain *et al*., 2003) calponin inhibits the rate of cell proliferation, suggesting its role in modulating actin-cytoskeleton during cytokinesis (Hossain *et al*., 2003). There is also a report that shows calponin-1 has a significant role in agonist-induced signal transduction in smooth muscle cells (Je *et al*., 2001). On the other hand, calponin-3 has been reported in nervous tissues and implicates in neuronal regeneration and growth (Ferhat *et al*., 1996; Ferhat *et al*., 2001)

Calponin-2 has been shown to function in cytoskeletal organization (Fukui *et al*., 1997). Indeed, it stabilizes actin filaments and inhibits actin cytoskeleton-related cellular functions, such as cytokinesis, migration, and phagocytosis (Hossain *et al*., 2005; Hossain *et al*., 2006; Huang *et al*., 2008). The function of calponin-2 in regulating the actin cytoskeleton of non-muscle cells suggests its role in multiple cellular activities.

Previous studies suggested that calponin-2 may play a role in the organization of actin cytoskeleton (Danninger & Gimona, 2000) and in cytokinesis (Hossain *et al*., 2003). This hypothesis is supported by the observation that calponin-2 is expressed at significant levels in epidermal keratinocytes, lung alveolar epithelial cells, and fibrolasts (Hossain *et al*., 2003). The forced over-expression of calponin-2 results in inhibition the rate of smooth muscle cell proliferation (Hossain *et al*., 2003).

Tang *et al*. (2006) have demonstrated that calponin-2 expression is limited to vasculature from 16 to 30 hours after fertilization during zebrafish (*D. rerio*) development. *In vitro* studies in zebrafish showed that basic fibroblast growth factor-induced human umbilical vein endothelial cell migration was down-regulated by knockdown of calponin-2 expression using an antisense adenovirus (Tang *et al*., 2006).

The over-expression of calponin-2 enhanced the migration and hastened wound healing (Tang *et al*., 2006). These events are correlated with activation of mitogen-activated protein kinase. Besides that, inhibition of this pathway blocked the promigratory effect of calponin-2. These data suggest that calponin-2 shows an important role for migrating endothelial cells both *in vivo* and *in vitro* and its expression is necessary for appropriate vascular development (Tang *et al*., 2006) (Figure 2.5).

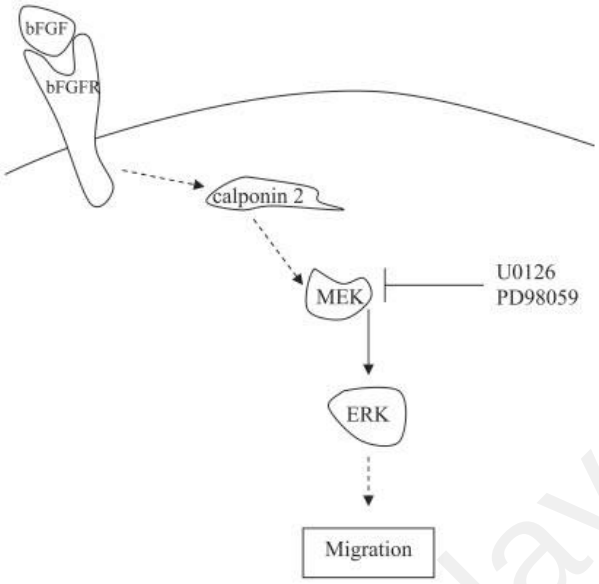


Figure 2.5: Model for role of calponin-2 in endothelial cell migration.
(Source: Tang et al., 2006)

It has also been reported that calponin-2 is significantly expressed in peripheral blood cells of myeloid lineage (Huang *et al*., 2008) with an up-regulation during monocyte to macrophage differentiation (Huang *et al*., 2008). These accumulating evidences clearly indicate the function of neutral calponin in multiple cellular activities via regulation of actin cytoskeleton. Indeed, calponin-2 was found to be an inhibitor in myeloid cell proliferation, migration and phagocytosis (Huang *et al*., 2008).

These results suggest a microfilament-associated activity of calponin-2, which may regulate the function of actin cytoskeleton (Huang *et al*., 2008). The actin cytoskeleton plays a major role in cell motility that is essential for macrophage locomotion, phagocytosis and endocytosis (Beningo & Wang, 2002). These activities are essential in defensive and autoimmune responses. At present, the role of calponin-2 in freshwater fish has yet to be clearly described.

CHAPTER 3: METHODOLOGY

3.1 Identification of calponin-2

Calponin-2 gene sequence was identified from *C. striata* transcriptome unigenes obtained by Illumina‟s Solexa sequencing technology that was retrieved from transcriptome database of *C. striata* (Bhassu *et al*., unpublished). This unigene was assembled from Illumina Solexa short reads of RNA sequencing from the muscle of *C. striata.* It was identified as calponin gene through BLAST homology search against the NCBI database (http://blast.ncbi.nlm.gov/Blast).

3.2 Sample collection for cDNA cloning of calponin-2 and Tissue Distribution

Healthy *C. striata* (average body weight of 25 g) were purchased live from a commercial farm, Rotomas Technology (M) Sdn. Bhd. (Farm), based in Kajang, Selangor. The animals were treated in accordance to ethical procedures. The authority that permitted the study is part of the collaborative team and specific permission was obtained from the owner. The work has passed through Institutional Animal Care and Use Committee, University of Malaya. Fishes were maintained in flat-bottomed glass tank (300 l) with aerated and filtered freshwater at 29 ± 2 ºC in the laboratory. All fishes were acclimatized for 1 week prior to start of experiments. The fishes were fed three times daily with commercial pellets and the water was changed daily during acclimatization period. Maximum of 5 fishes per tank were maintained during the experiment. Muscle tissue from *C. striata* fish was used for cDNA cloning of *Cs*Calp gene. Tissues including blood, eyes, hearts, kidney, gills, and muscles pooled from three adults were used for *Cs*Calp gene expression profile in *C. striata*.

3.3 RNA purification and cDNA synthesis

Total RNA was extracted from the fish using a modified single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction developed by Chomczynski and Sacchi (2007). One to 2 g of pooled tissues were added to about 100 ml of liquid nitrogen, and further grounded using mortar and pestle.

The ground muscle tissue was transferred into a 1.5 ml Eppendorf tube containing 1 ml of Trizol reagent (Life Technologies, Carlsbad, CA), vortexed for 30 seconds and left for 5 minutes at room temperature. Two hundred microlitres of chloroform was added to the homogenized sample, before being mixed by shaking it vigorously for about 15 seconds. The mixture was then incubated at room temperature for 3 minutes. Then, the tube was centrifuged at 13,000 × g for 30 minutes at 4 ºC to obtain colorless aqueous solutes. Centrifugation step was made by using Tabletop High-speed Micro Centrifuge machine (Hitachi, Japan).

Five hundred microlitres of the top most solutes was pipetted into a microcentrifuge tube containing 500 µl ice-cold isopropyl alcohol. To mix the solution, the tube was inverted for a few times before incubating it for 15 minutes at room temperature. Another centrifugation step was performed after the incubation at 13,000 × g with the same duration and temperature as before where it lead to production of RNA pellet at the bottom of the tube with liquid supernatant.

After discarding the supernatant carefully, the RNA pellet was washed with 1 ml of 75 % ethanol. The tube was again centrifuged at 12,000 × g for 15 minutes at 4 ºC. The supernatant was again discarded and the pellet was air-dried for 15 minutes. Following this procedure, the RNA pellet obtained was resolved in 50 µl of DEPC-treated water. The isolated RNA was stored at -80 ºC for long term storage. The total RNA concentration was measured spectrophometrically (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, England). Quantification of the sample was

made by using absorption of light at Abs 260/280 where the good quality RNA will have a ratio of 1.6 to 2.1.

From the isolated total RNA, first strand synthesis of cDNA was made by reverse transcription using M-MLV reverse transcriptase (Promega, USA) with oligo-dT (20) VN primer following the manufacturer‟s instruction manual. Briefly, in a 200 µl PCR tube, RNA template (1 µg/reaction), 0.025 µg/µl oligo-dT (20) VN primers and nuclease-free water were mixed to a total volume of 5 µl. The tube was then incubated in a thermocycler at 70 ºC for 5 minutes and chilled immediately at 4 ºC for another 5 minutes. Then, reverse transcription mix was prepared in a 200 µl PCR tube by mixing nuclease-free water, ImProm-II™ 5× Buffer, 25 mM $MgCl_2$, 10 mM dNTPs mix and ImProm-II™ reverse transcriptase in a final volume of 15 µl. The 15 µl of RT-mix were then added to the 5 µl of RNA-primer mixture. The tube with a total volume of 20 µl was incubated in a thermocycler at 25 ºC for 5 minutes, 42 ºC for 60 minutes and then 70 ºC for 15 minutes. The cDNA was kept in -20 ºC for later use.

For quantification of the amount of DNA, readings in NanoVue Plus Spectrophotometer were taken at 260 nm and 280 nm, which allowed calculation of the concentration of nucleic acids in the sample. An OD of 1 corresponds to approximately 50 µg/ml for double stranded DNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimation of the purity of the nucleic acid.

$$DNA\ conc.(\mu g/\mu l) = (OD_{260} \times 100\ (dilution\ factor) \times 50\mu g/ml)/1000$$

3.4 Polymerase Chain Reaction

3.4.1 Primer design

Unigene of *Cs*Calp sequence was obtained from *C. striata* transcriptome database which had been generated using Illumina‟s Solexa sequencing technology, GenBank accession number JN104634. Possible longest open reading frame (ORF) was identified using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

The primers pair sequences were designed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 3.1).

Table 3.1: Specific Primers for Calponin-2.

Name	Sequence (5' to 3')	Melting Temperature (°C)
CALP-F	TCCTCAGCTTTTCAACTCAGCTCCT	57.88
CALP-R	GGCTTCCTTCAACAAAGGTCCAGCC	60.17

3.4.2 PCR Mastermix

In brief, the mastermix cocktail was prepared by adding the following reagents to the final volume of 20 µl; 1.5× PCR buffer, 3.75 mM $MgCl_2$, 0.25 mM of each dNTP, 0.25 µM of each forward and reverse primer and 0.15 U *Taq* polymerase. Sterile distilled water was added to make the final volume up to 20 µl. The mixture was then mixed and aliquoted into labeled 0.2 ml PCR tube. Then, approximately 50 ng of purified DNA was pipetted into each PCR tube. Additional reactions were included in the mastermix calculation and prepared to serve as negative control. The complete recipe of PCR mastermix is presented as Appendix A.

3.4.3 Amplification by PCR

PCR was carried out as per the following profile: one denaturation step at 94 °C for 3 minutes followed by 35 cycles of 30 seconds of denaturation at 94 °C, 30 seconds of primer annealing at 63.3 °C and one minute of extension at 72 °C. This was followed by a 5 minutes final extension step at 72°C and an incubation step at 10 °C for another 5 minutes. PCR products were then retrieved and either analyzed immediately by gel electrophoresis or stored at 4 °C for later use.

3.5 Gel Electrophoresis

The PCR products were separated on 1 % agarose gels at 80 volts in 1× Tris-borate-EDTA buffer for about an hour. Gels were stained in ethidium bromide and visualized under a 365 nm UV light and documented using a gel documentation machine, AlphaImager (Alpha Innotech, CA, USA).

3.6 Molecular Cloning of *Cs*Calp cDNA

DNA purification was carried out using NucleoSpin Extract II (Machery-Nagel, Germany) according to manufacturer‟s instructions. PCR products containing same sample replicates were pooled together into a single tube; further 2 volumes of Buffer NT i.e. 30 µl PCR product with 60 µl Buffer NT; were added to sample.

A NucleoSpin Extract II column was placed into a 2 ml collection tube and the sample was loaded. The sample was then centrifuged for 1 minute at 11,000 × g. Flow-through was discarded and the column was placed back into the collection tube. In order to wash the silica membrane, 700 µl Buffer NT3 was added to the column and was centrifuged with the same setting as previously described. The flow-through was discarded. Centrifugation step at 11,000 × g for 3 minutes was done to remove Buffer

NT3 completely. This is because residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for about 5 minutes at 70 ºC prior to elution.

Then, the NucleoSpin Extract II column was placed into a fresh 1.5 ml microcentrifuge tube and 30 µl Buffer NE was added. The sample was then incubated at room temperature for about 5 minutes and centrifuged for another 2 minutes to elute the DNA. The eluted DNA was checked for its quality by gel electrophoresis and stored at -20 ºC for future use.

3.6.1 Preparation of bacterial growth broth and plated media

For cDNA cloning, Luria Bertani (LB) growth media was used to grow bacterial cells. Preparation of LB agar plates were done by first dissolving 7 g of LB agar powder mix (Lennox, Laboratorios CONDA, Spain) in 200 ml of distilled water in a 1000 ml conical flask. The mixture was mixed well using a magnetic stirrer. Then, the flask which containing media was closed with aluminum foil, labeled and sterilized by autoclaving for 20 minutes (121 ºC, 151 lb/sq). After the sterilization step was completed, the LB media was cooled off under running tap water until the temperature reaches approximately 50 ºC.

Then, 50 µg/ml of ampicillin (Bio Basic Inc., Canada) was added into the media before it was mixed by gentle swirling. The LB media was then poured into labeled sterile disposable plastic Petri dishes until approximately half-full and left to solidify at the ambient temperature for about two hours. The LB agar media that have hardened were kept in 4 ºC for future use.

LB broth was prepared by first dissolving 20 g of LB broth powder (Lennox, Laboratorios CONDA, Spain) in 900 ml distilled water in a clean universal bottle. The solution was mixed well by using a magnetic stirrer. Then, the mixture‟s volume was

adjusted to 1,000 ml by adding appropriate volume of distilled water. Then, the media broth was autoclaved at the same setting as described previously. Autoclaved LB broth was kept at room temperature for future use. Both LB broth and agar media were prepared fresh before being used in subsequent transformation and plasmid isolation steps.

3.6.2 yT&A cloning vector-based DNA cloning

The PCR-amplified products from section 3.4 were cloned into yT&A® *E. coli* vector (vector map in Appendix B) using yT&A® cloning kit (Yeastern Biotech, Taiwan). Briefly, the PCR amplicon was first ligated to the plasmid vector before the vector was transformed into competent bacterial cells. The bacteria were then grown on a growth media overnight and screened for positive transformant the next day. All the aforementioned cDNA cloning steps were carried out according to the protocol provided by the manufacturer and are described in details in the following subsections.

3.6.2.1 Ligation

A ligation reaction was set up according to the recommendations in the instruction manual supplied with the kit. The yT&A vector and ligation buffers were thawed on ice and briefly centrifuged prior to use. For a single 10 µl reaction, the ligation mix was prepared by mixing 2 µl of yT&A cloning vector, 3 µl of purified PCR product, 1 µl 10× Ligation Buffer A, 1 µl 10× Ligation Buffer B, 1 µl YEA T_4 DNA Ligase (2 unit/µl) and sufficient volume of sterile distilled water were then added to the mixture to top the reaction volume up to 10 µl. The reaction was spun briefly to collect any droplets on the tube wall to the bottom of the tube. The mixture was then incubated at 22 ºC for 20 minutes and the DNA ligase was inactivated for 10 minutes at 65 ºC.

3.6.2.2 Transformation

Five microlitres of the ligation product were transferred into a pre-chilled 1.5 ml microcentrifuge tube containing 50 µl of *E. coli* competent cells (ECOS™ 101 competent cells strain: DH5α, Yeastern Biotech, Taiwan). The mixture was then incubated on ice for 30 minutes. The bacteria mixture was heat shocked at 42 ºC for 90 seconds and snap-chilled on ice immediately for another 2 minutes to let the cells recover from the heat stress. The bacterial mixture was added with 500 µl of LB broth and mixed briefly by pipetting. The mixture was then incubated at 37 °C for 90 minutes with agitation at 220 rpm. This step allows the bacteria to recover from the heat shock and express the antibiotic resistance genes as encoded in the yT&A® cloning vector.

Then, 100 µl of sterilized water and 20 µl of (0.012 mg/ml of LB agar) X-Gal (5 prime, Hamburg) dissolved in DMSO were pipetted onto the LB media containing ampicillin. The LB media stored at room temperature until all the liquid is dried. Then, bent glass rod was sterilized by dipping into the ethanol, flamed on a Bunsen burner and cooled to room temperature.

The bacterial culture was retrieved from the shaker after the incubation and was spun down at 100 × *g* for 10 minutes to concentrate the cell to the bottom of the tube. About 100 µl of the cell suspension was then transferred onto a dry LB agar plate and was spread evenly on the agar surface using a sterile glass rod spreader. This step was carried out in an aseptic condition to avoid contamination by other microorganisms. The plate was then placed in an incubation oven set at 37 °C to allow the bacteria to grow overnight. The remainder of the bacterial culture was stored at 4 °C if re-plating is required later on.

3.6.2.3 Colony Selection by PCR

The grown *E. coli* colonies observed on the plate the next day would have transformed into ampicillin-resistance strains based on blue/white selection. A single white colony was gently isolated from the LB agar surface using a sterile wire loop. A portion of the isolated colony was first transferred onto a 6 × 6 gridded LB agar plate to serve as a library plate. The plate was then inverted and incubated at 37 °C for 16 hours. While the toothpick was then placed into 0.2 ml PCR tubes containing the reagents for colony PCR and the reaction was performed immediately to validate the presence of foreign inserts in the transformed colonies. These steps were repeated with the other positive colonies until the required number of isolates was obtained. This PCR screening step was carried out as described in section 3.4 with the annealing temperature set at 63.3 °C for optimal specific primer binding. The PCR products were then analyzed by agarose gel electrophoresis as described in section 3.5 and colonies carrying the desired inserts were identified based on the presence of cDNA bands that corresponded to the length of the inserted fragment.

3.6.2.4 Plasmid isolation from transformed *E. coli* DH5α

Bacterial colonies that have been positively identified to carry the correct inserts were isolated from the library plate and inoculated into 10 ml of LB broth media supplied with 50 µg/ml of ampicillin. The inoculants were cultured at 37 °C for 16–18 hours with agitation at 220 rpm. A 900 µl aliquot of the cell suspension was transferred into a labeled 1.5 ml microcentrifuge tube containing 100 µl of glycerol once overnight culture was complete. The mixture was mixed by pipetting and then stored at -80 °C as glycerol stock.

The rest of the cell suspension was transferred into a labeled 1.5 ml tube and centrifuged at 4,260 × g for 15 minutes. The supernatant formed was decanted entirely without disturbing the pellet. The pelleted cells were then treated with High Yield Plasmid Kit (Yeastern Biotech, Taiwan) according to the manufacturer‟s protocols and the isolated plasmids were kept in -20 ºC until use.

3.6.2.5 DNA sequencing of isolated recombinant plasmids

Prior to DNA sequencing, the recombinant plasmids were checked again for the presence of inserts by PCR amplification using Calp-F and Calp-R primers. Then the isolated recombinant plasmids were sent for sequencing at both forward and reverse direction using universal primers M13F (-20) and M13R (-20). The nucleotide sequences of the cloned cDNAs were determined using the automated DNA sequencer from Applied Biosystem (ABI, USA).

3.7 Sequence characterization using biological computational tools

The sequencing results obtained were in the forward and reverse direction and they were further aligned using MEGA 5 (Tamura *et al*., 2011). The non-overlapped region at the 5' and 3' of the sequence were deleted and the overlapped regions were subjected to online software Vecscreen (http://www.ncbi.nlm.nih.gov/VecScreen.html) to screen the presence of yT&A vector sequences. The DNA sequences were edited to remove the vector sequences. The protein coding sequences represented by the longest open reading frame, ORF were searched in the sequence using online software ORF Finder (http://www.ncbi.nlm.nih/gorf). The amino acid sequence translated from the ORF was used as a query sequence in a similarity search using BlastP against UniProt Knowledgebase (Swiss-Prot+TrEMBL) at http://web.expasy.org/blast/.

By using amino acid sequences, the structure of the protein was also being determined. The analysis used a specific website, where motif analysis, signal peptide, transmembrane and 3D structures of the protein was identified. The physico-chemical characterization of *Cs*Calp was computed using the ExPASy PROTPARAM bioinformatics portal (http://web.expasy.org/protparam/) (Gasteiger, 2005). Signal peptide was determined using the SignalP (http://www.cbs.dtu.dk/services/ SignalP/). The presumed tertiary structures were established for calponin using the SWISS-MODEL prediction algorithm (http://swissmodel.expasy.org/) (Arnold *et al*., 2006).

The multiple sequence alignment using MEGA 5 program based on ClustalW algorithm were performed on the amino acid sequence together with other homologues of calponin-2 amino acid sequences. Based on this alignment, the phylogenetic relationship of the calponin-2 was determined using the neighbour-joining (NJ) method with a cut-off point of 50 % and the reliability of the tree was assessed by bootstrapping at 5,000 replication (Kumar *et al*., 2004).

3.8 Bacterial culture of *Aeromonas hydrophila*

A. hydrophila was isolated from the muscle sample of EUS infected *C. striata* on tryptone soy agar (Oxoid), identified biochemically and by DNA sequence homology, and maintained as stocks in 15 % glycerol at -70 ºC. For routine use, cultures were grown on TSA at 28 ºC. Authenticity was verified after Austin & Austin (2007). Broth cultures were prepared in tryptone soy broth (Oxoid) with overnight incubation at 28 ºC. Then, the broths were centrifuged at 3,000 × g for 10 min at 4 ºC, before the cells were washed twice in phosphate-buffered saline (PBS; Oxoid) pH 7.4, and the pellets resuspended in fresh buffer. The bacterial concentration was estimated by counting the

colonies grown in tryptic soy agar with a guide plate ruled in square centimeters. Inoculum concentration was adjusted to 5×10^6 CFU/ml.

3.9 Immune challenge and tissue collection

Healthy *C. striata* (average body weight of 25 g) were purchased live from the same commercial farm in Selangor, Malaysia. A total of 36 snakehead fishes were used for pathogen exposure and tissue collections. Fishes were maintained in flat-bottomed glass tank (300 l) with aerated and filtered freshwater at 29 ± 2 ºC. All fishes were acclimatized for 1 week before being challenged with *A. hydrophila*. A maximum of 5 fishes per tank were maintained during the experiment.

The immune challenge was conducted by intraperitoneal injection using *A. hydrophila* (5×10^6 CFU/ml) suspended in 1× phosphate buffered saline (PBS, pH = 7.4). Tested fishes were injected with 100 µl of bacteria solution, and controls were injected with 100 µl PBS instead of stimulant. All tissue samples were collected at pre-injection 0 h and post-injection at standard time intervals of 3 h, 6 h, 12 h, 24 h, and 48 h. Tissues including skin, heart, muscle, gill, kidney, liver, blood and spleen were isolated and collected. Using a sterilized syringe, the blood (0.5 – 1.0 ml per fish) was collected from the fish caudal vein puncture and immediately centrifuged at 4,000 × g for 10 min at 4 °C to allow blood cell collection for total RNA extraction. Successively, all different tissues were immediately snap-frozen in liquid nitrogen and stored at -80 ºC for total RNA isolation.

Total RNA were isolated from all tested tissues from *C. striata* using TRIZOL Reagent following the manufacturer‟s protocol (Life Technologies, Carlsbad, CA). The total RNA was treated with RNase free DNA set (5 Prime GmbH, Hamburg, Germany) to remove contaminating DNA. The concentration of RNA was measured

spectrophometrically (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, England). First-strand cDNA was synthesized from total RNA by using M-MLV reverse transcriptase (Promega, USA) following the manufacturer‟s instruction manual with oligo-dT (20) VN primer as described in section 3.3.

3.10 Quantitative real-time PCR analysis of *Cs*Calp mRNA expression

The expression patterns of *Cs*Calp mRNA in different tissues were determined using quantitative real-time PCR (qRT-PCR) in an ABI 7500 Real-time Detection System (Applied Biosystems, USA) with β-actin gene as the internal reference gene. After challenge with *A. hydrophilla,* the *Cs*Calp mRNA expression was also determined. The qRT-PCR was performed in a reaction mixture of 20 µl which was composed of 4 µl of 50 ng/µl cDNA template, 1× SYBR® Green PCR Master Mix (Applied Biosystems, USA) and 0.3 µM of each primers. The qRT-PCR cycle profile was 1 cycle of 95 °C for 10 s, followed by 35 cycles of 95 °C for 5 s, 58 °C for 10 s and 72 °C for 20 s and finally 1 cycle of 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. The real-time primers were designed using IDT SCI TOOLS programs (http://www.idtdna.com/SciTools/SciTools.aspx). β-actin of *C. striata* primers were designed from the sequence of GenBank Accession No. EU570219. The primer details of gene specific primer (*Cs*Calp) and internal control (β-actin) are provided in Table 3.2.

Table 3.2: Primer set designed for real-time PCR.

Primer	**Sequence 5' - 3'**
*Cs*Calp Reverse	TGACACACTGTCCAGCCTTCATCT
*Cs*Calp Forward	GCAACATGACGCAGGTCCAAACTA
β-actin Forward	TCTTCCAGCCTTCCTTCCTTGGTA
β-actin Reverse	GACGTCGCACTTCATGATGCTGTT

After the PCR program, data were analyzed with ABI 7500 SDS software (Applied Biosystems, USA). To maintain consistency, the baseline was set automatically by the software. The comparative C_T ($2^{-\Delta\Delta CT}$) methodology was used to analyze the expression level of *Cs*Calp. All samples were analyzed as triplicates and the results are expressed as relative fold of mean ± standard deviation as reported by Livak and Schmittgenm (Livak & Schmittgenm, 2001). The qPCR results using $2^{-\Delta\Delta CT}$ methodology can be found in Appendix C. For comparison of relative *Cs*Calp mRNA expression, statistical analysis was performed using one-way ANOVA and mean comparisons were performed by Tukey"s Multiple Range Test using SPSS 11.5 at 5 % significant level.

3.11 Expression of recombinant calponin

Primers for the amplification of the *Cs*Calp gene were designed with FastPCR 6.1, with and without the N-terminal signal sequence (Table 3.3). The PCR reaction was set up in a total volume of 25 µl as follows: 2 µl of template (100 ng), 1 µl of forward and reverse primer (10 pM), 8.5 µl of molecular water and 12.5 µl of PCR mastermix as described in section 3.4.2. Initial activation of the *Taq* was performed for 10 min at 95 °C, followed by 25 cycles as follows: 95 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min, followed by a final extension at 72 °C for 8 min and holding of samples at 4 °C.

Table 3.3: Primer set designed for expression of recombinant protein. Restriction sites are underlined.

Primer	**Sequence 5' - 3'**
CalpEP Forward	GGAGTCGGATCCATGGCTTCCTTCAACA
CalpEP Reverse	CTCCGGCAAGCTTTTAGTAATCTGTTCCA

After PCR, the products were verified by electrophoresis on a 1 % agarose gel using a 100 bp DNA ladder (Promega, USA). The band of interest was cut out with a sterile razor blade and the DNA eluted using the MinElute Gel Extraction kit (Qiagen, Crawley, UK). The expression of the *Cs*Calp was then undertaken using the TOPO® pTrcHis Expression System (Invitrogen, USA) following the supplier‟s protocol. The PCR product was ligated to the pTrcHis TOPO vector (vector map in Appendix B) and introduced into *E. coli* TOP10 cells (Invitrogen, USA). Twelve colonies for each transformation were picked for secondary screening and their insert was analyzed for size and orientation by tip-dip PCR using the gene specific forward primer and the vector specific pTrcHis reverse primer (5‟-GAT TTA ATC TGT ATC AGG-3‟).

Protein expression was accomplished by growing and inducing 50 ml of cells as follows: 2 ml of LB broth containing 50 μg/ml ampicillin were inoculated with a single colony and grown overnight at 37 °C with agitation at 180 rpm. Subsequently, 50 ml of LB broth containing 50 μg/ml ampicillin were inoculated with 1 ml of the overnight culture and grown until mid-log. The culture was then induced with IPTG to a final concentration of 1 mM and the culture grown at 37 °C with shaking at 100 rpm for 5 hours. The cells were then harvested by centrifugation at 3,000 × *g*, 10 min, 4 °C, and the pellets stored at -80 °C before proceeding to SDS-Page prior to protein identification.

Pellets were prepared for SDS-PAGE gel according to the protocol from Maniatis *et al*., 1989 and Sambrook *et al*., 2001. The complete recipe of SDS-PAGE is presented as Appendix D. One to 15 μl of the samples were loaded on 12 % SDS-PAGE gels. The gels were run at 90 V constant current for about 2 hours and stained with Coomasie blue for protein bands were observed after destaining solution overnight.

Protein molecular weight markers (Fermentas, USA) were used to identify the molecular weights of expressed proteins observed on the gel.

3.12 Protein Identification by Q-TOF MS

3.12.1 In-gel digestion

Proteins were subjected to in-gel trypsin digestion (Shevchenko *et al*., 1996). Excised gel spots were destained with 100 µl of destain solution (50 % MeOH/ D.W.) with shaking for 5 min. After removal of the solution, gel spots were incubated with 200 mM ammonium bicarbonate for 20 min. The gel pieces were dehydrated with 100 µl of acetonitrile and dried in a vacuum centrifuge. The above procedure was repeated three times. The dried gel pieces were rehydrated with 20 µl of 50 mM ammonium bicarbonate containing 0.2 µg modified trypsin (Promega, USA) for 45 min on ice. After removal of solution, 70 µl of 50 mM ammonium bicarbonate was added. The digestion was performed overnight at 37 ^{o}C. The peptide solution was desalted using C18 nano column (in house).

3.12.2 Desalting and concentration

Custom-made chromatographic columns were used for desalting and concentration of the peptide mixture prior to mass spectrometric analysis. A column consisting of 100–300 nl of Poros reverse phase R2 material (20–30 µm bead size, PerSeptive Biosystems) was packed in a constricted GELoader tip (Eppendorf, Hamburg, Germany). A 10 ml syringe was used to force liquid through the column by applying a gentle air pressure. Thirty microliters of the peptide mixture from the digestion supernatant was diluted 30 µl in 5 % formic acid, loaded onto the column, and washed with 30 µl of 5 % formic acid. For analyses by MS/MS, peptides were eluted

with 1.5 µl 50 % methanol/ 49 % H_2O/ 1 % formic acid directly into a pre-coated borosilicate nanoelectrospray needle (EconoTipTM, New Objective, USA).

3.12.3 ESI-MS/MS

MS/MS of peptides generated by in-gel digestion was performed by nano-ESI on a MicroQ-TOF III mass spectrometer (Bruker Daltonics, 255748 Germany). The source temperature was room temperature. A potential of 1 kV was applied to the pre-coated borosilicate nanoelectrospray needles (EconoTipTM, New Objective, USA) in the ion source combined with a nitrogen back-pressure of 0–5 psi to produce a stable flow rate (10–30 nl/min). The cone voltage was 800 V. The quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The collision gas was Ar at a pressure of 6^{-7} x 10^{-5} mbar and the collision energy was 15–40 V. Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a micro-channel plate detector and a time-to-digital converter. The data were processed using a peptide sequence system.

3.12.4 Bioinformatics

Identified proteins were searched against UniProt (Swiss-Prot/TrEMBL) Knowledge base to determine their main biological functions in the cell. Protein-protein interactions were predicted using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database v9.0 (www.string-db.org/).

CHAPTER 4: RESULTS

4.1 Isolation of calponin-2 from *C. striata*

In the present study, a novel calponin-2 gene was isolated for the first time in *C. striata* (*Cs*Calp). This was validated using the specific primer designed from the unigenes belonging to calponin-2 that was retrieved from the transcriptome database of *C. striata* (Table 3.1). These primers were optimized, and the best annealing temperature for amplification was found to be at 63.3 ^{o}C (Figure 4.1). Upon validation this product was used for cloning and sequencing. There were seven colonies with foreign inserts in the plasmid. Positive clones containing the expected-size inserts were screened by colony PCR and then subjected for DNA sequencing (Figure 4.2). The expected size was 983 bp. All samples contained the insert with expected sizes of bands except for the sample in lane 2. The obtained sequence was used to determined bioinformatics characterization using biological computational tools, gene expression analysis and protein expression.

4.2 *C. striata* calponin Sequence Analysis

A full length cDNA of *Cs*Calp was identified from the constructed *C. striata* cDNA library (Figure 4.3).

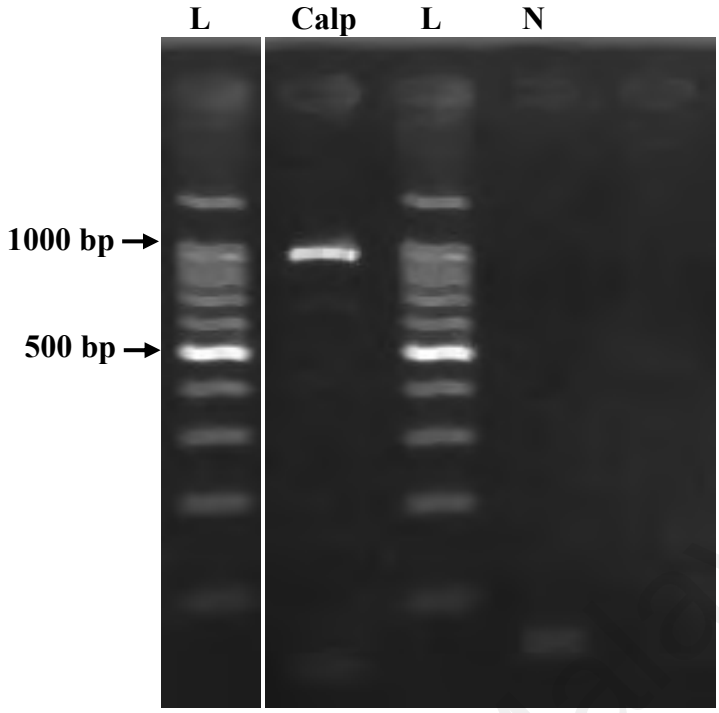


Figure 4.1: Gel image showing validation of optimized annealing temperature (63.3 °C) using cDNA sample of *C. striata*. The expected size of PCR amplicons is 983 bp. [Calp: *Cs*Calp; L: Promega 100 bp DNA ladder; N: negative control].

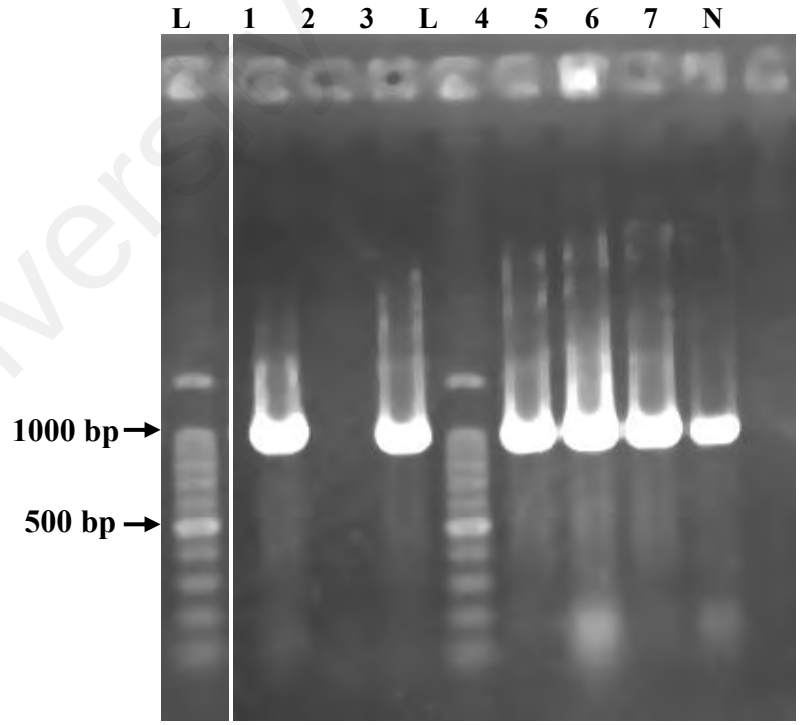


Figure 4.2: Gel image showing the colony PCR of seven colonies that had foreign insert in the plasmid. The expected size of PCR amplicons is 983 bp. [L: Promega 100 bp DNA ladder; 1–7: Number of seven colonies; N: negative control].

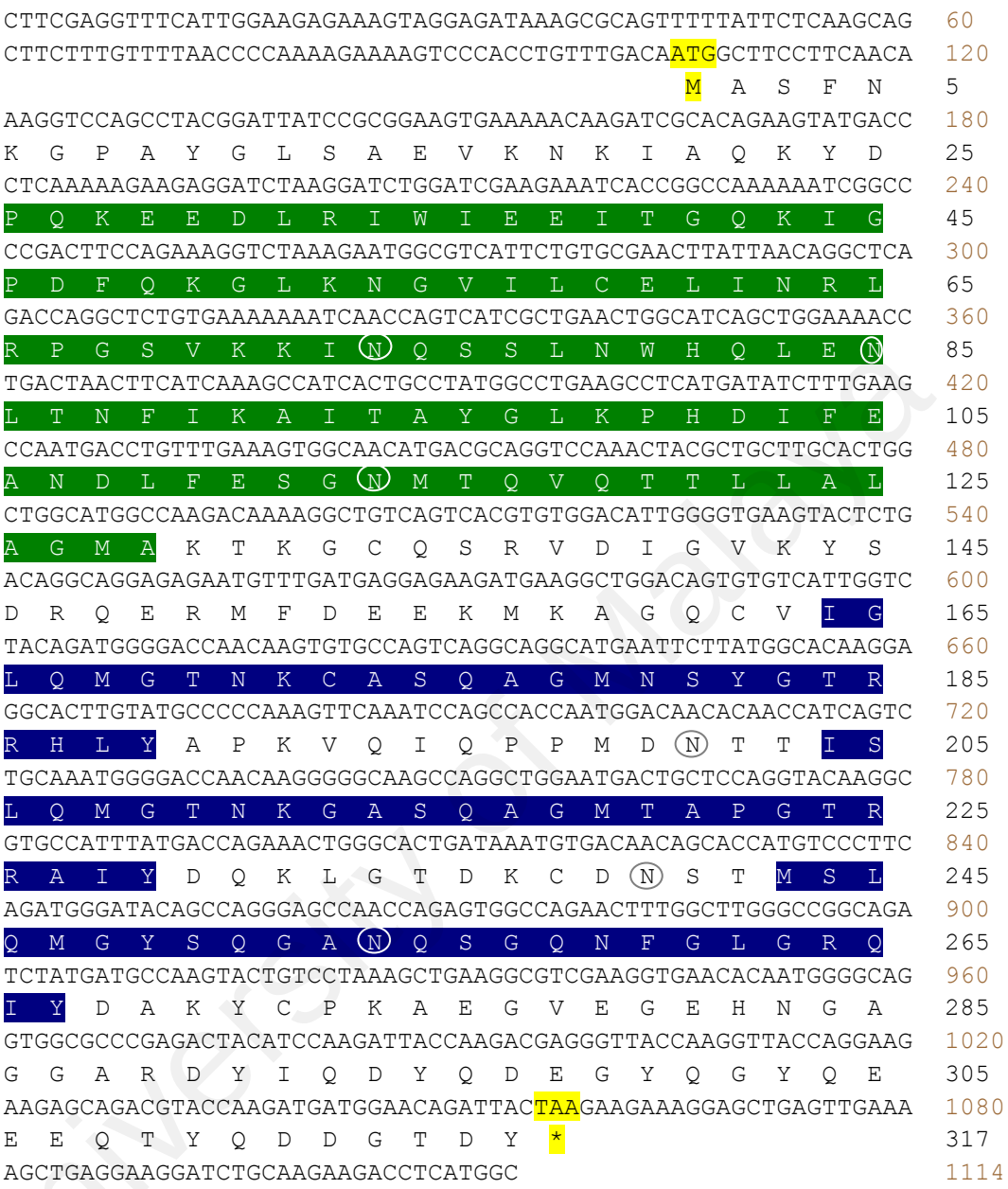


Figure 4.3: Nucleotide and deduced amino acid sequences of *Cs*Calp from *C. striata*. The brown color numbers indicated on the right of each row represents the position of nucleotide and the black color numbers represent the amino acid position. The nucleotide sequence is numbered from 5‟ end and the single letter amino acid code is shown below the corresponding codon. The calponin homology (CH) is highlighted in green, and the calponin family repeats are highlighted in blue. The N-glyco motif sites are indicated in circle. The start and stop codons are highlighted in yellow.

The isolated full-length of *Cs*Calp was 1114 bp long, containing 954 bp open reading frame (ORF) encoding 317 amino acids. The cDNA consisted a 5′ untranslated region (UTR) of 104 bp, a 3‟ UTR of 56 bp incuding a stop codon (TAA). This *Cs*Calp amino acid sequence neither has a signal peptide nor a transmembrane region. The calculated molecular mass was 35.14 kDa.

The computed pI value of *Cs*Calp is 6.15, less than 7 (pI<7) indicates that this protein were considered as acidic. The computed isoelectric point (pI) will be useful in developing buffer system for purification by isoelectric focusing method. Expasy‟s ProtParam computes the extinction coefficient at 280 nm that is favoured because proteins absorb light strongly at that wavelength while other substances commonly in protein solutions do not.

Extinction coefficient of *Cs*Calp at 280 nm was 35215 M^{-1} cm^{-1} with respect to the concentration of Cys, Trp and Tyr. This high extinction coefficient indicates presence of high concentration of Cys, Trp and Tyr. The computed extinction coefficients help in the quantitative study of protein-protein and protein-ligand interactions in solution. The instability index is indicative of the stability of the protein under *in vitro* conditions. There are certain dipeptides, the occurrence of which is significantly different in the unstable proteins compared with those in the stable ones. This method assigns a weight value of instability. Using these weight values it is possible to compute an instability index (II). The *in vivo* instability of proteins is possibly determined by the order of certain amino acids in its sequence. A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable (Guruprasad *et al*., 1990). The instability index value for the *Cs*Calp was found to be 39.94. This classifies the protein as stable.

4.3 Bioinformatics analysis of *Cs*Calp

The structural features of *Cs*Calp are predicted by the SMART program (Figure 4.4). Calponin homology domain profile is shown in yellow where there are 98 amino acids long ranging from 28 to 125 amino acids. There are also three subunits of calponin domains predicted by Pfam presented in grey. The position of amino acid that encode for these domains lie between 164 to 267 amino acids with each of subunits with 26 amino acids long. The low complexity region is shown in pink, between 293 to 316 amino acids.

The amino acid sequences of *Cs*Calp are visualized by the Protter software (Omasits *et al*., 2014) from N-terminal to C-terminal. This analysis explains that *Cs*Calp is an intracellular protein with no signal peptide and contains 6 N-glyco motifs cysteine in calponin domain as shown in Figure 4.5. SWISS-MODEL (ExPASy) was used to build the predicted 3D model of calponin-2 gene that used protein sequence as the query. The structure of *Cs*Calp protein exhibited α-helices and β-pleated sheet (Figure 4.6). There are four major α-helices structures which are built according to calponin homology and calponin-like repeats.

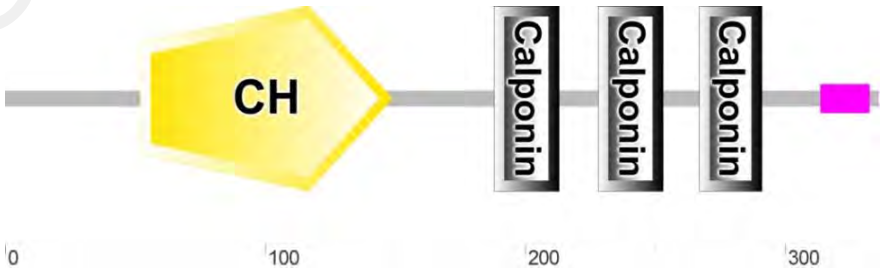


Figure 4.4: Structural features of *Cs*Calp. Conserved domains of *Cs*Calp are predicted by SMART program. Calponin homology domain was shown in yellow, the three calponin domains predicted by Pfam are presented in gray and low complexity region shown in pink.

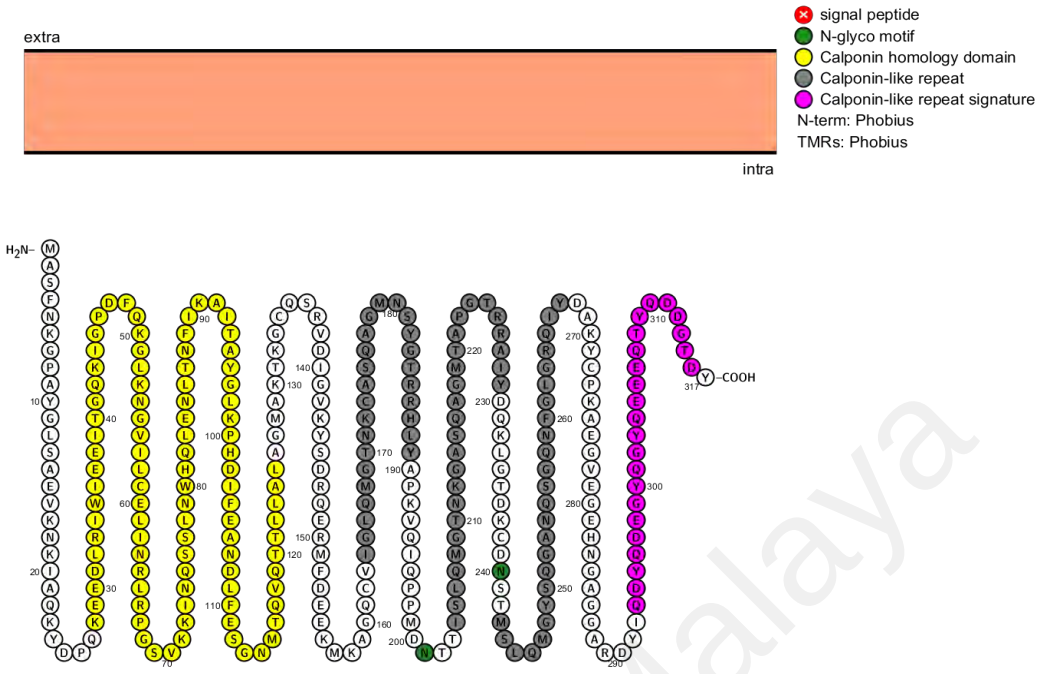


Figure 4.5: Topological illustration of the *Cs*Calp. Illustration generated with the Protter tool (Omasits *et al*., 2014).

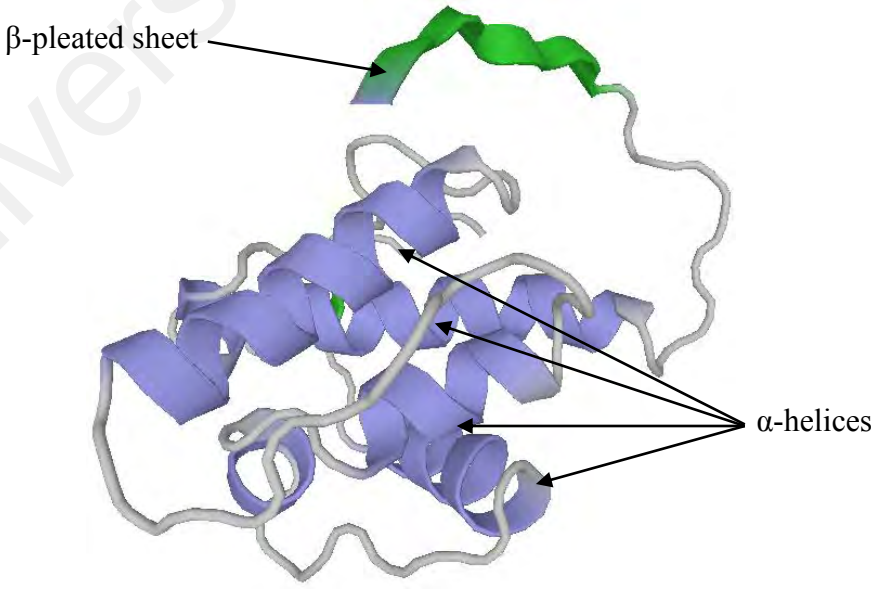
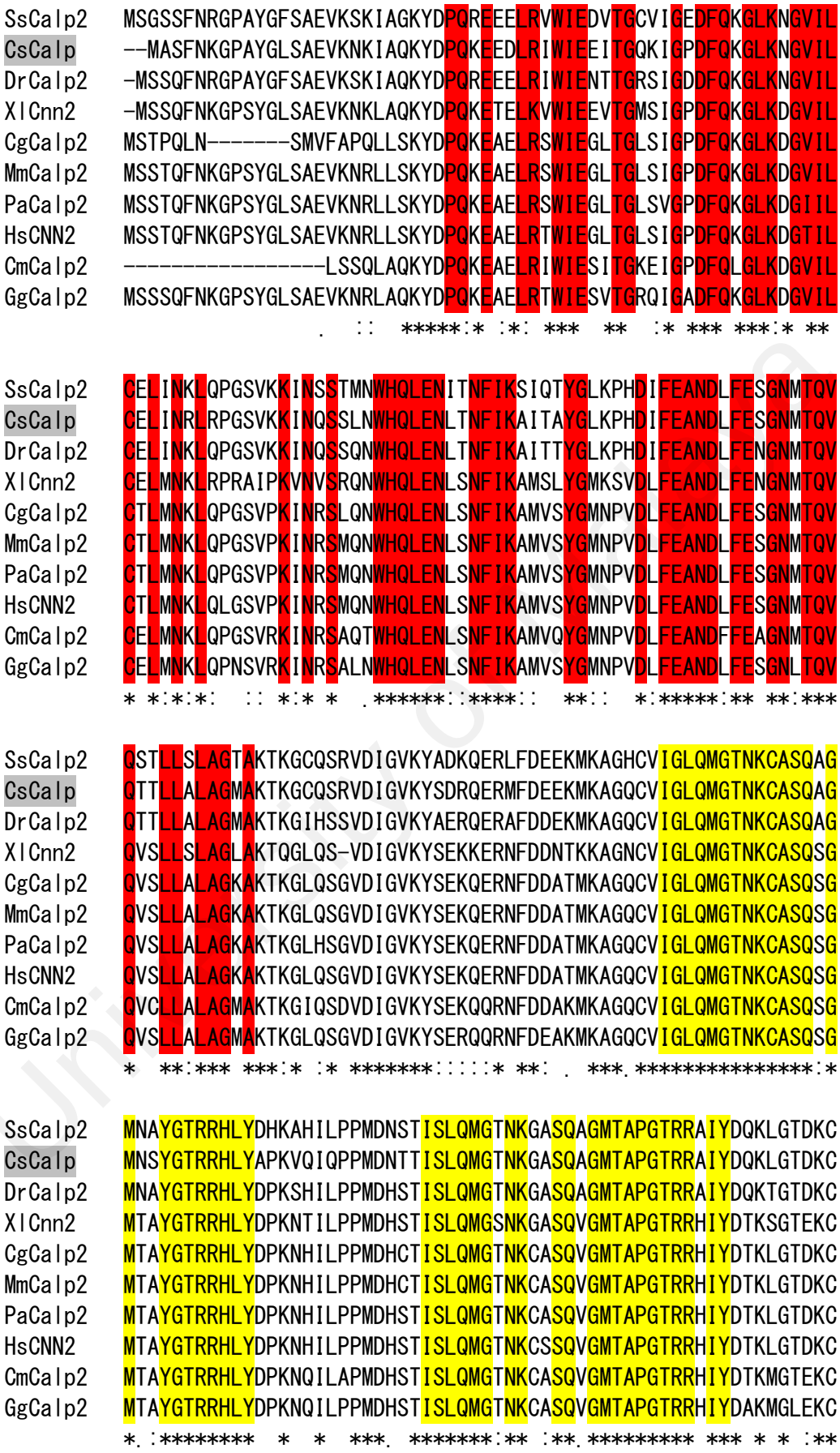


Figure 4.6: Predicted 3D structure of calponin-2 gene in *C. striata* by using SWISS-MODEL (Arnold *et al*., 2006). The structure of calponin shows the quaternary structure that built as a monomer.

Sequence analysis with the BLASTP program revealed that the deduced amino acid sequence of *Cs*Calp exhibited similarities with calponin-2 of other vertebrates. It displayed highest identity with calponin-2 of Nile tilapia (*Oreochromis niloticus)* which is 89 %. Besides, it also shows high similarity with calponin-2 of zebrafish *(Danio rerio)* and Atlantic salmon (*Salmo salar)* in which both exhibit 83 % and 81 % of identity respectively. The *Cs*Calp has also shown identities with higher group of vertebrates. This includes cow (*Bos Taurus)* and human (*Homo sapiens)*, with 77 % and 71 % of identity.

Multiple amino acid sequence alignment of the calponin-2 gene was performed against fishes *Salmo salar*, *Danio rerio*, calponin-2 from higher vertebrate family group chicken (*Gallus gallus),* mouse *(Mus musculus),* African clawed frog *(Xenopus laevis),* Chinese hamster (*Cricetulus griseus),* black flying fox (*Pteropus alecto),* green turtle *(Chelonia mydas)* and human *(Homo sapiens)* (Figure 4.7). The result revealed that *C. striata* has the longest amino acid sequence, 317 amino acids.

Even though conserved motifs were observed among the sequences, the length of the amino acids varied from species to species. The conserved sites are 176 out of 320 sites while the variable sites in the alignment are 140 sites. There are 90 out of 320 are the Parsimony-Informative sites. The Parsimony-Informative sites are significant to infer the phylogenetic relationship. This shows that the homologous of calponin-2 gene is quite conserved among different species.


SsCalp2 MSGSSFNRGPAYGFSAEVKSKIAGKYDPQREEELRVWIEDVTGCVIGEDFQKGLKNGVIL
CsCalp --MASFNKGPAYGLSAEVKNKIAQKYDPQKEEDLRIWIEEITGQKIGPDFQKGLKNGVIL
DrCalp2 -MSSQFNRGPAYGFSAEVKSKIAQKYDPQREEELRIWIENTTGRSIGDDFQKGLKNGVIL
XlCnn2 -MSSQFNKGPSYGLSAEVKNKLAQKYDPQKETELKVWIEEVTGMSIGPDFQKGLKDGVIL
CgCalp2 MSTPQLN-------SMVFAPQLLSKYDPQKEAELRSWIEGLTGLSIGPDFQKGLKDGVIL
MmCalp2 MSSTQFNKGPSYGLSAEVKNRLLSKYDPQKEAELRSWIEGLTGLSIGPDFQKGLKDGVIL
PaCalp2 MSSTQFNKGPSYGLSAEVKNRLLSKYDPQKEAELRSWIEGLTGLSVGPDFQKGLKDGIIL
HsCNN2 MSSTQFNKGPSYGLSAEVKNRLLSKYDPQKEAELRTWIEGLTGLSIGPDFQKGLKDGTIL
CmCalp2 ----------LSSQLAQKYDPQKEAELRIWIESITGKEIGPDFQLGLKDGVIL
GgCalp2 MSSSQFNKGPSYGLSAEVKNRLAQKYDPQKEAELRTWIESVTGRQIGADFQKGLKDGVIL
SsCalp2 CELINKLQPGSVKKINSSTMNWHQLENITNFIKSIQTYGLKPHDIFEANDLFESGNMTQV
CsCalp CELINRLRPGSVKKINQSSLNWHQLENLTNFIKAITAYGLKPHDIFEANDLFESGNMTQV
DrCalp2 CELINKLQPGSVKKINQSSQNWHQLENLTNFIKAITTYGLKPHDIFEANDLFENGNMTQV
XlCnn2 CELMNKLRPRAIPKVNVSRQNWHQLENLSNFIKAMSLYGMKSVDLFEANDLFENGNMTQV
CgCalp2 CTLMNKLQPGSVPKINRSLQNWHQLENLSNFIKAMVSYGMNPVDLFEANDLFESGNMTQV
MmCalp2 CTLMNKLQPGSVPKINRSMQNWHQLENLSNFIKAMVSYGMNPVDLFEANDLFESGNMTQV
PaCalp2 CTLMNKLQPGSVPKINRSMQNWHQLENLSNFIKAMVSYGMNPVDLFEANDLFESGNMTQV
HsCNN2 CTLMNKLQLGSVPKINRSMQNWHQLENLSNFIKAMVSYGMNPVDLFEANDLFESGNMTQV
CmCalp2 CELMNKLQPGSVRKINRSAQTWHQLENLSNFIKAMVQYGMNPVDLFEANDFFEAGNMTQV
GgCalp2 CELMNKLQPNSVRKINRSALNWHQLENLSNFIKAMVSYGMNPVDLFEANDLFESGNLTQV
SsCalp2 QSTLLSLAGTAKTKGCQSRVDIGVKYADKQERLFDEEKMKAGHCVIGLQMGTNKCASQAG
CsCalp QTTLLALAGMAKTKGCQSRVDIGVKYSDRQERMFDEEKMKAGQCVIGLQMGTNKCASQAG
DrCalp2 QTTLLALAGMAKTKGIHSSVDIGVKYAERQERAFDDEKMKAGQCVIGLQMGTNKCASQAG
XlCnn2 QVSLLSLAGLAKTQGLQS-VDIGVKYSEKKERNFDDNTKKAGNCVIGLQMGTNKCASQSG
CgCalp2 QVSLLALAGKAKTKGLQSGVDIGVKYSEKQERNFDDATMKAGQCVIGLQMGTNKCASQSG
MmCalp2 QVSLLALAGKAKTKGLQSGVDIGVKYSEKQERNFDDATMKAGQCVIGLQMGTNKCASQSG
PaCalp2 QVSLLALAGKAKTKGLHSGVDIGVKYSEKQERNFDDATMKAGQCVIGLQMGTNKCASQSG
HsCNN2 QVSLLALAGKAKTKGLQSGVDIGVKYSEKQERNFDDATMKAGQCVIGLQMGTNKCASQSG
CmCalp2 QVCLLALAGMAKTKGIQSDVDIGVKYSEKQQRNFDDAKMKAGQCVIGLQMGTNKCASQSG
GgCalp2 QVSLLALAGMAKTKGLQSGVDIGVKYSERQQRNFDEAKMKAGQCVIGLQMGTNKCASQSG
SsCalp2 MNAYGTRRHLYDHKAHILPPMDNSTISLQMGTNKGASQAGMTAPGTRRAIYDQKLGTDKC
CsCalp MNSYGTRRHLYAPKVQIQPPMDNTTISLQMGTNKGASQAGMTAPGTRRAIYDQKLGTDKC
DrCalp2 MNAYGTRRHLYDPKSHILPPMDHSTISLQMGTNKGASQAGMTAPGTRRAIYDQKTGTDKC
XlCnn2 MTAYGTRRHLYDPKNTILPPMDHSTISLQMGSNKGASQVGMTAPGTRRHIYDTKSGTEKC
CgCalp2 MTAYGTRRHLYDPKNHILPPMDHCTISLQMGTNKCASQVGMTAPGTRRHIYDTKLGTDKC
MmCalp2 MTAYGTRRHLYDPKNHILPPMDHCTISLQMGTNKCASQVGMTAPGTRRHIYDTKLGTDKC
PaCalp2 MTAYGTRRHLYDPKNHILPPMDHSTISLQMGTNKCASQVGMTAPGTRRHIYDTKLGTDKC
HsCNN2 MTAYGTRRHLYDPKNHILPPMDHSTISLQMGTNKCSSQVGMTAPGTRRHIYDTKLGTDKC
CmCalp2 MTAYGTRRHLYDPKNQILAPMDHSTISLQMGTNKCASQVGMTAPGTRRHIYDTKMGTEKC
GgCalp2 MTAYGTRRHLYDPKNQILPPMDHSTISLQMGTNKCASQVGMTAPGTRRHIYDAKMGLEKC

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SsCalp2    DNSTMSLQMGSNAGANQSGQNFGLGRQIYDAKYCPKNEEGV-----NGAGADYVADYQDE
CsCalp     DNSTMSLQMGYSQGANQSGQNFGLGRQIYDAKYCPKAEGVEGEHNGAGGARDYIQDYQDE
DrCalp2    DNSTMSLQMGYSQGANQSGQNFGLGRQIYDAKYCPKGGAVAAGE----GNDGQYTDYQDE
XlCnn2     DNSSMSLQMGYTQGANQSGQIFGLGRQIYDPKYCPTGNRDDLPHDENEQE----------
CgCalp2    DNSSMSLQMGYTQGANQSGQVFGLGRQIYDPKYCPQGPAADGAPVSGD---G--HGEGPE
MmCalp2    DNSSMSLQMGYTQGANQSGQVFGLGRQIYDPKYCPQGSAADGAPAGDG------QGEAPE
PaCalp2    DNSSMSLQMGYTQGANQSGQVFGLGRQIYDPKYCPQGSVADGASGAAGDCPS--PGAAPE
HsCNN2     DNSSMSLQMGYTQGANQSGQVFGLGRQIYDPKYCPQGTVADGAPSGTGDCPD--PGEVPE
CmCalp2    DNTSMSLQMGSNQGANQSGQIFGLGRQIYDPKYCPQGNTGDVANAVYD------QSTDPP
GgCalp2    DNSSMSLQMGSNQGANQSGQVFGLGRQIYDPKYCPQGTPGDAANAAGE------PGADPP
           **::****** . ******* ********* ****

SsCalp2    GYQEYKDDTVPVYQEEGTDY
CsCalp     GYQGYQEEE-QTYQDDGTDY
DrCalp2    GYQGYQDDG-QDY-------
XlCnn2     --Q-YQQDF-----------
CgCalp2    YLAYCQEEAGY---------
MmCalp2    YLAYCQEEAGY---------
PaCalp2    YPPYYQEEAGY---------
HsCNN2     YPPYYQEEAGY---------
CmCalp2    EYHYYREEEGY---------
GgCalp2    GYHYYHQEESC---------
               :::
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Figure 4.7: Multiple Sequence Alignment of calponin-2 in *C. striata* with ten other homologous calponin amino acid sequences. Abbreviations are as follows: SsCalp2, *S. salar* calponin2 (NP001133873); DrCalp2, *D. rerio* calponin2 (AAZ14863); XlCnn2, *X. laevis* calponin2 (AAH46257); CgCalp2, *C. griceus* calponin2 (EGV99480); MmCalp2, *M. musculus* calponin2 (NP031751); PaCalp2, *P. alecto* calponin2 (ELK19295); HsCNN2, *H. sapiens* calponin2 (CAG46630); CmCalp2, *C. mydas* calponin2 (EMP24163); GgCalp2, *G. gallus* calponin2 (NP001135728). Deletions are indicated by dashes. „*“ indicates conservative and „:“ & „.“ indicate semi conservative. Conserved site of calponin homology domain is highlighted with red, whereas conserved site of calponin-like repeat is yellow highlighted.

The phylogenetic tree was constructed to gain insight into evolutionary relationship of deduced calponin-2 of *C. striata* with calponin from other species (Figure 4.8). The results from phylogenetic tree showed two distinct clades that grouped fishes into one group and higher vertebrates to another group. Calponin of *C. striata* is closely clustered with calponin homology of Nile tilapia (*Oreochromis niloticus)* and zebrafish (*Danio rerio)*. Besides, the calponin homology of *Homo sapiens* was closely related with calponin homology of rhesus monkey (*Macaca mulatta)* with bootstrap value of 68 %. The evolution of calponin homology of higher verterbrates evolved earlier than calponin homology of the fish group.

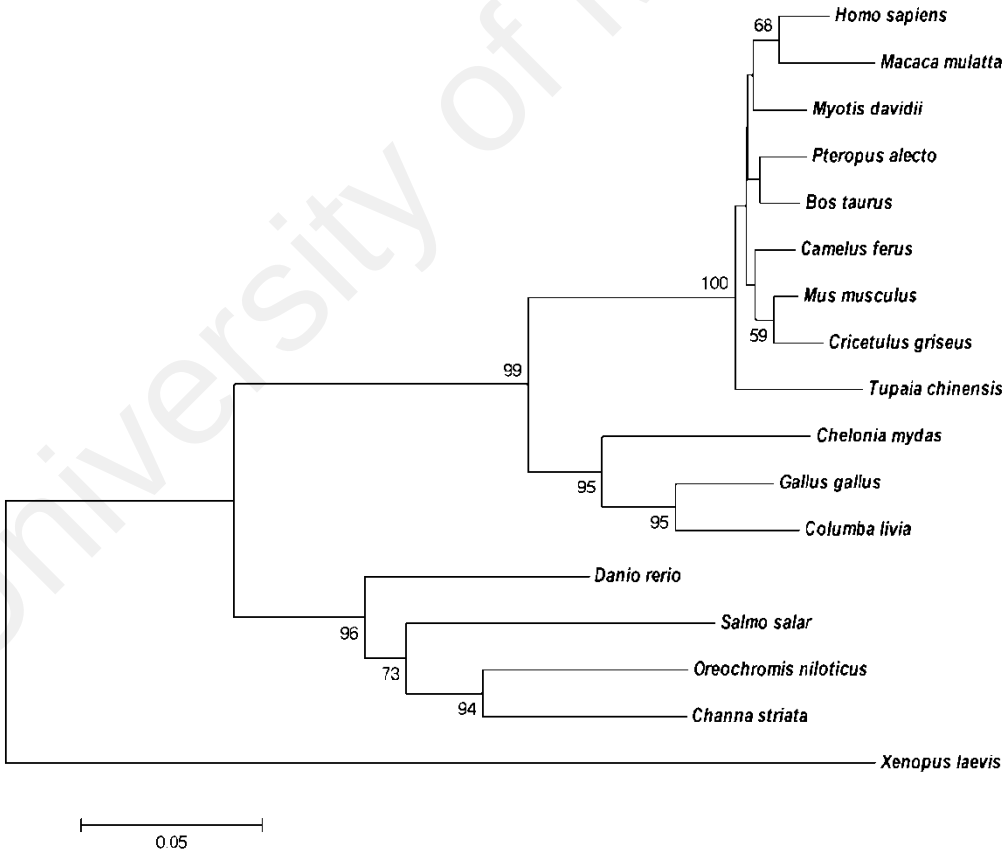

Figure 4.8: A phylogenetic tree of *C. striata* calponin with other homologous calponin protein sequence was reconstructed by Neighbour-joining method. The tree is based on alignment corresponding to full length amino acid sequences, using MEGA 5 software. The numbers shown at the branches denote the bootstrap majority consensus values of 5,000 replicates. GenBank accession number of species tested can be found in Appendix F.

4.4 Tissue expression of *Cs*Calp

Quantitative real-time RT-PCR was used to investigate the distribution of *Cs*Calp mRNA in different tissues. The mRNA transcripts of *Cs*Calp could be detected in all the examined tissues with different expression levels including spleen, gill, kidney, muscle, heart, liver, blood and skin (Figure 4.9). The highest expression was found in blood followed by kidney, liver, spleen, muscle, heart, gill and skin. Statistical analysis showed that *Cs*Calp mRNA expression was significantly higher ($P<0.05$) in the blood. Therefore, blood was selected as a target to further quantify *Cs*Calp mRNA transcript in *C. striata* challenged with *A. hydrophila*.

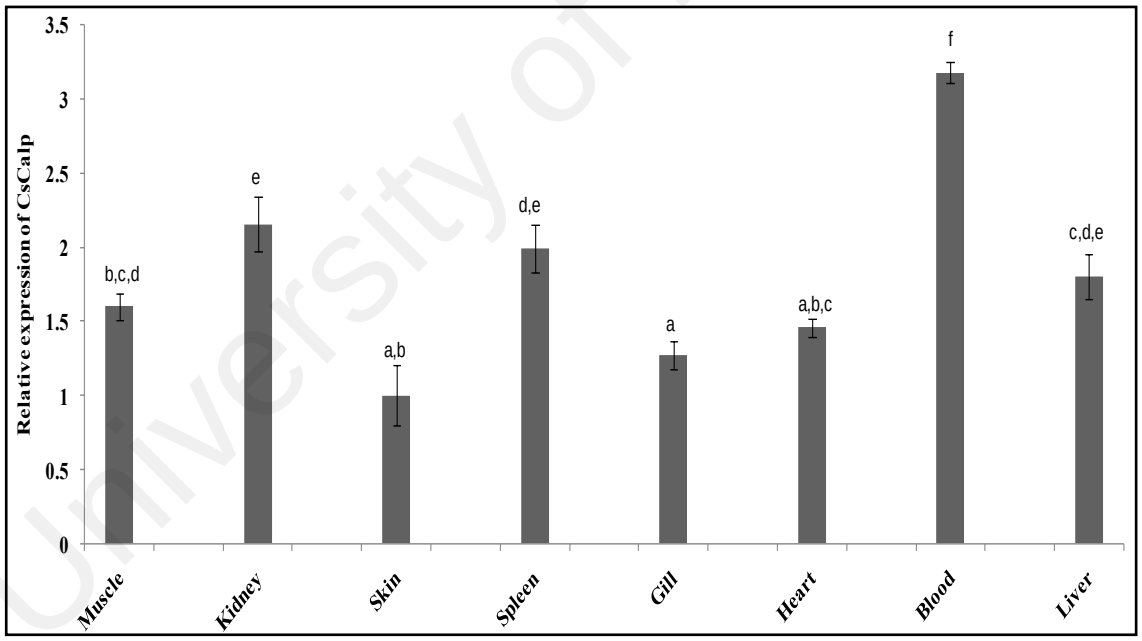

Figure 4.9: Gene transcript patterns of *C. striata* calponin in different tissues by real-time PCR. Data are expressed as a ratio to *Cs*Calp mRNA transcription in skin. Means with different letters are significantly different at $P<0.05$ level by one-way ANOVA and Tukey's Multiple Range Test. Vertical bars represent the mean ± SD (N = 3).

4.5 *Cs*Calp gene expression after *A. hydrophila* challenge

The mRNA expression levels of *Cs*Calp in blood of *C. striata* after *A. hydrophila* challenge were quantified by real-time PCR with β-actin gene as internal control (Figure 4.10). For both *Cs*Calp and β-actin genes, there were only one peak at the corresponding melting temperature in the dissociation curve analysis, indicating that the PCR was specifically amplified. Compared to the control, the expression of *Cs*Calp in *A. hydrophila* challenged group increased significantly and reached to the maximum at 24 h after challenge, which was about 120-fold respectively of control group ($P<0.05$). Afterwards, the expression of *Cs*Calp decreased gradually, and at 48 h after challenge, which was 60-fold of that in the control group ($P<0.05$). Then, the expression level of *Cs*Calp further decreased to a 16-fold during 72 h post-induction.

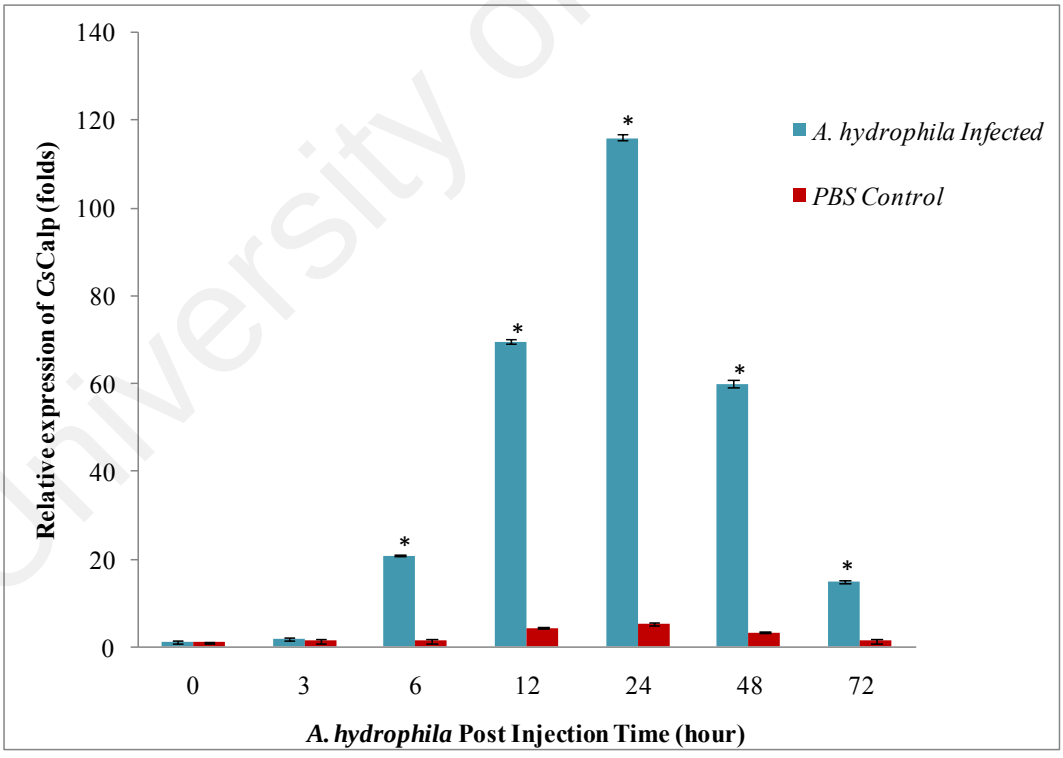

Figure 4.10: The temporal expression patterns of *Cs*Calp injected with *A. hydrophila* and PBS (control) in *C. striata* by qRT-PCR. The statistical analysis was based on the comparison of the relative expression ratio of *Cs*Calp to the β-actin by calculating $2^{-\Delta\Delta Ct}$ values. Data are expressed as a ratio to *Cs*Calp mRNA expression at 0 hour. The significant difference at $P<0.05$ level by one-way ANOVA and Tukey"s Multiple Range Test of *Cs*Calp expression between the challenged and the control group are indicated with asterisk (*). Vertical bars represent the mean ± SD (N = 3).

4.6 Expression of Recombinant *Cs*Calp

In order to subclone the *Cs*Calp coding region corresponding to 317 amino acids into the pTrcHis TOPO vector, two primers were designed to amplify only the ORF region, with respective primer sequences and the restriction sites used (Table 3.3). The PCR amplification was carried out to optimize and validate the primer pairs. Figure 4.11 shows the best optimized annealing temperature at 51 ^{o}C. Upon validation this product were used for cloning and expression in *E. coli*. The 983 bp product was successfully cloned into pTrcHis to obtain recombinant plasmid, pTrcHis-*Cs*Calp.

The pTrcHis-*Cs*Calp was transformed into *E. coli* and 12 clones were selected by growth in ampicillin and glucose-containing medium. The clones containing the construct were identified by colony PCR, using specific primers and then confirmed by nucleotide sequencing. The length of all PCR products was approximately 983 bp. Seven out of twelve clones‟ investigated contained DNA inserts compatible with the size of the ORF of the calponin-2 of *C. striata* (Figure 4.12).

Small-size cultures (10 ml) of the positive clones of pTrcHis-*Cs*Calp were subjected to IPTG induction to identify clones which capable of expressing high levels of recombinant protein. Positive clone should showed expression of the unique predicted 40 kDa protein after IPTG induction. The *Cs*Calp production increases in parallel with the duration of induction where the optimal induction at 5 hours post-induction (Figure 4.13).

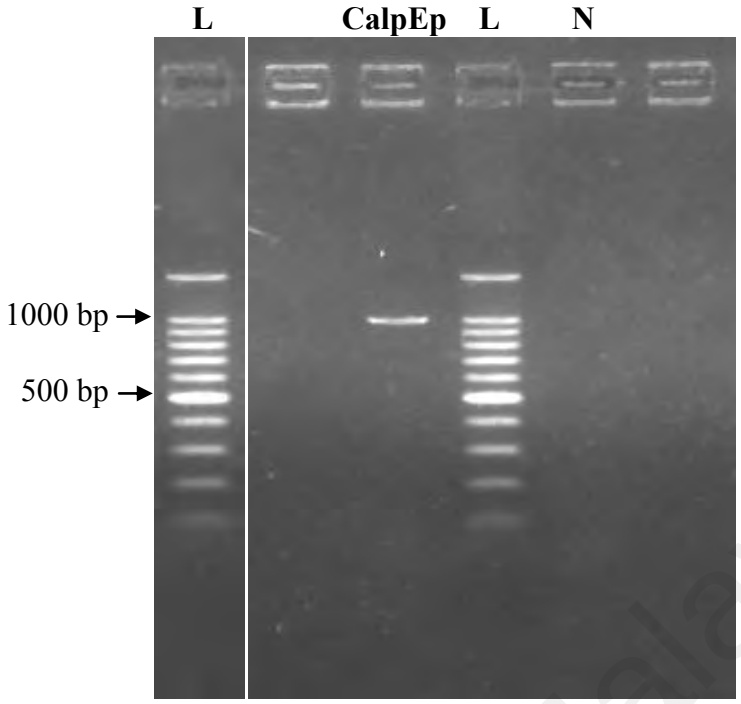

Figure 4.11: Gel image showing validation of optimized annealing temperature (51 °C) using cDNA sample of *C. striata*. The expected size of PCR amplicons is 983 bp. [CalpEP: *Cs*Calp_Protein expression; L: Promega 100 bp DNA ladder; N: negative control].

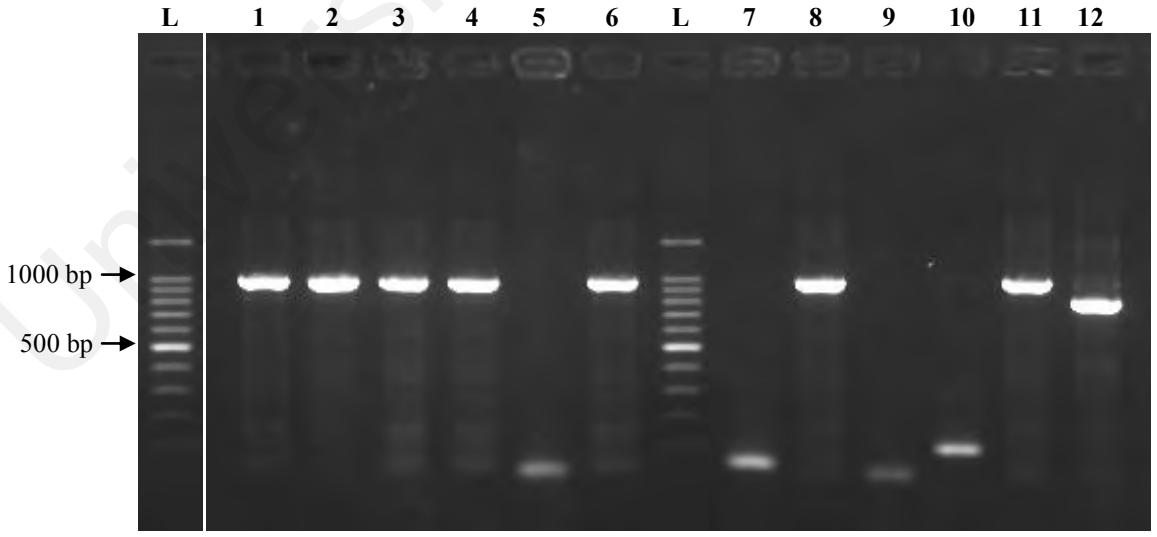

Figure 4.12: Colony PCR. Gel image showing the colony PCR of twelve colonies. The expected size of positive recombinant is about 1000 bp. [L: Promega 100 bp DNA ladder; 1-12: Number of colonies].

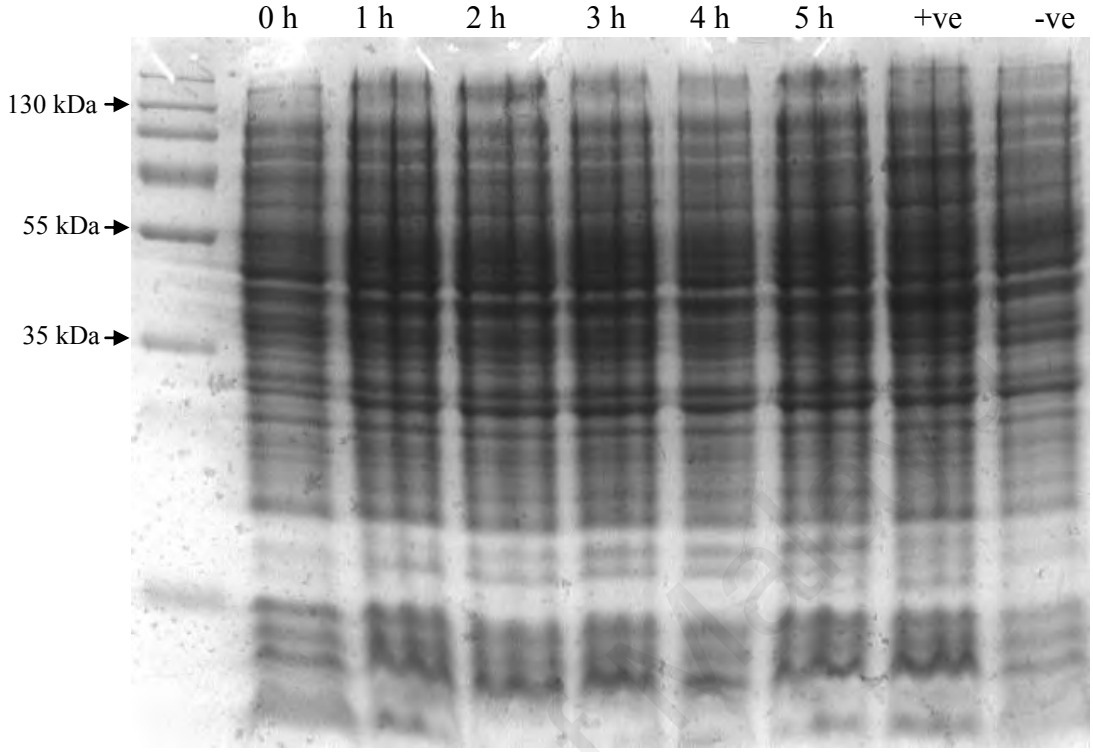

Figure 4.13: One-dimensional SDS-Page gel showing temporal induction of IPTG on positive clone. Positive control is TOP10 cells containing pTrcHis-TOPO while negative control is TOP10 cells only.

4.7 Protein Identification by nano-ESI Q-TOF MS/MS

Protein identification of predicted recombinant protein was performed using q-TOF analysis to determine the expressed *Cs*Calp protein. Triplicates of expected size 40 kDa bands were excised and sent for amino acid determination. Protein band from gel was digested with trypsin. The digested gel was then subjected to nanoelectrospray quadrupole-TOF tandem mass analysis. The generated peptides were analyzed by q-TOF mass spectrometer had resulted in good readings. Then, the generated mass spectra of the peptides were analyzed using Mascot and homology search database for searching a protein sequence collections with peptide mass maps (Figure 4.14).

However, none of the replicates were identified as calponin-2. Protein band identified two different proteins: monomeric lac repressor (LacI) protein and phosphoglycerate kinase. Both proteins were detected in *E. coli* species. It can be deduced that construct with this expression vector was obtained with pTrcHis TOPO direct expression vector. This construct, however, failed to give significant expression of recombinant *Cs*Calp.

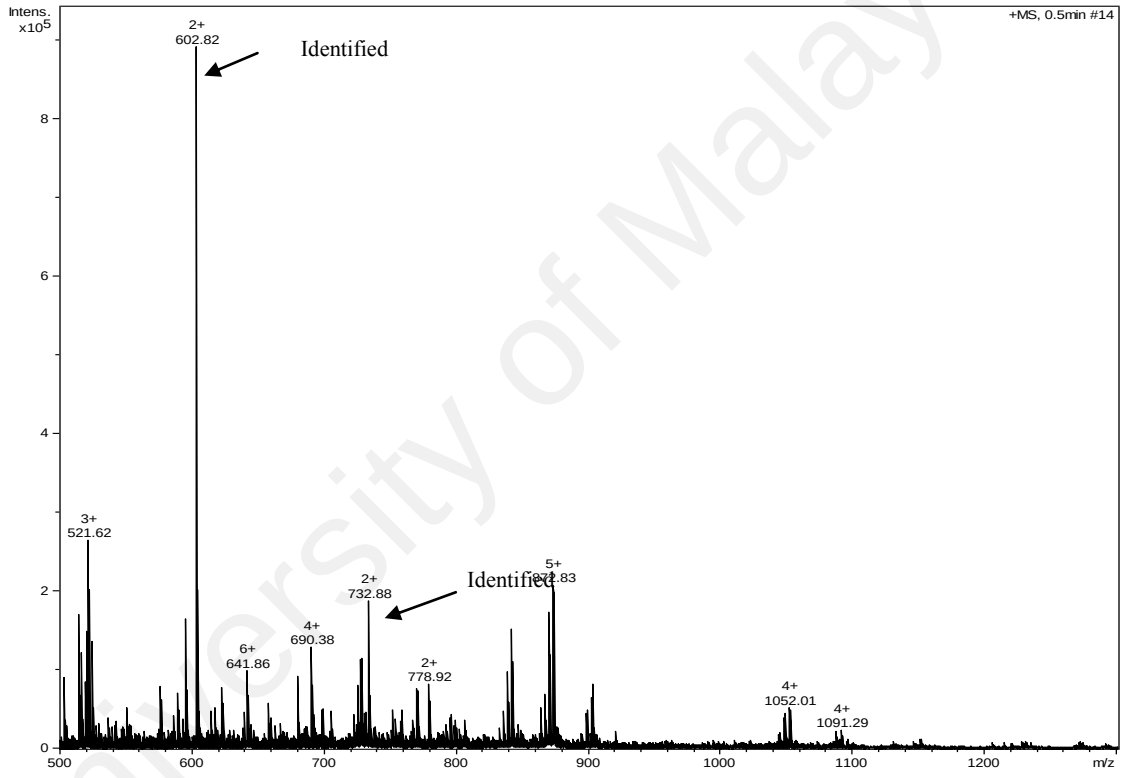

Figure 4.14: Q-TOF mass spectra of protein sample. Q-TOF MS spectrum of tryptic peptides from 40 kDa predicted pTrcHis-*Cs*Calp. The spectrum shows all the peptide ions present in the tryptic digest. A representative product ion spectrum of a doubly charged precursor ion at m/z 602.82 and its matched amino acid sequence are shown there is no insert. MS/MS spectrum of a doubly charged peptide at m/z 732.88 has also been identified and its matched amino acid sequence is not shown any insert of calponin-2. The complete series of y-ions lead to confident interpretation of the spectrum.

CHAPTER 5: DISCUSSION

Calponin-2 gene has been studied in zebrafish (*D. rerio*) showing the important role of calponin-2 in vascular development. Calponin-2 gene is involved in cytoskeletal organization, endothelial cell migration, and signal transduction (Tang *et al*., 2006). Besides calponin-2 was found to be an inhibitor in myeloid cell proliferation, migration and phagocytosis (Huang *et al.*, 2008). Calponin-2 gene is conserved among different species. In this present study, a novel calponin-2 gene (*Cs*Calp) was cloned from *C. striata*. Prior to this study, to the author‟s knowledge, no full-length cDNA of *Cs*Calp has been isolated in *C. striata*, and this study is the first report on cloning of the full-length cDNA of *Cs*Calp from *C. striata*.

5.1 Sequence analysis of calponin in *Channa striata*

An untranslated region is present in the calponin-2 gene isolated with a 5′ untranslated region (UTR) of 104 bp, and a 3‟ UTR of 56 bp incuding a stop codon (TAA). The sequences encode in this region do not include the open reading frame. Thus, probably this non-coding sequence can be used to do a comparative study. Besides, it could serve as the attachment site for the transcription factors. Unfortunately, no study is carried out to prove the function of the non-coding sequences of calponin-2 as they have never been expressed.

Homology analysis revealed that the deduced amino acid sequence of *Cs*Calp had more than 50 % similarity with calponin of other animals (89 % with *O. niloticus*, 83 % with *D. rerio*, 81 % with *S. salar*, 73 % with *C. livia*, 77 % with *B. taurus* and 71

% with *H. sapiens*). The domain analysis indicates that the CsCalp comprises of conserved calponin homology (CH) and three calponin repeats as similar with mammalian calponin-2 (Gimona & Small, 1996; Morgan & Gangopadhyay, 2001). Briefly, calponin homology domain profile is a number of actin-binding proteins, including spectrin, α-actinin and fimbrin, contain a 250 amino acid stretch called the actin binding domain (ABD).

The ABD has probably arisen from duplication of a domain which is also found in a single copy in a number of other proteins like calponin or the vav proto-oncogene and known as the calponin homology (CH) domain (Stradal *et al*., 1998; Keep *et al*., 1999). The Vav proto-oncogene is a member of the VAV gene family that is essential in hematopoiesis. The CH domain is present in a large number of actin binding proteins including filamin, α-actinin, utrophin and dystrophin (Gimona *et al*., 2002). The function of the CH domain in these protein is unclear, but for calponin there is evidence that demonstrated the direct interaction of CH domain with the MAP kinases ERK1 and ERK2 (Leinweber *et al*., 1999) and for Vav it was shown that the CH domain is important for the RhoGEF function of this protein (Walsh & Doherty, 1997; Jögi *et al*., 2002).

A detailed analysis of the CH domain-containing proteins has shown that they can be divided into three groups (Stradal *et al*., 1998). The first group is the fimbrin family of monomeric actin cross-linking molecules containing two ABDs. Second group consists of dimeric cross-linking proteins (α-actinin, β-spectrin, filamin, etc.) and monomeric F-actin binding proteins (dystrophin, utrophin) each containing one ABD. The third group is proteins that containing only a single amino terminal CH domain.

The resolution of the 3D structure of various CH domains show that the conserved core consists of four major α-helices (Keep *et al*., 1999). The predicted 3D structure of *Cs*Calp also depicted this conserved characteristic of calponin family protein. The amino acid sequences of both calponin homology (CH) and calponin-2 repeats contributed to the construction of this model.

Calponin-2 contains three repeats of a well conserved motif of about 26 amino acids. Such a domain is also found in a number of other proteins whose physiological role is not yet established such as mammalian transgelin 2, *Drosophila synchronous* flight muscle protein SM20 and rat neuronal protein NP25 (Ren *et al*., 1994). The calponin-like repeat is a short actin-binding module. Actin-binding sites formed by either CH domains or calponin-like repeats occupy non-competing binding sites along the actin filament (Lener *et al*., 2004). Both pattern and profile for the calponin-like repeat were developed together. The signature pattern corresponds to the first 20 residues, whereas the profile covers the entire calponin-like repeat.

The molecular domain structure of the calponin reveals the modular construction which also consistent with molecular domain structure of calponin-2 in *C. striata* (Figure 4.6 and Appendix E). The most prominent structural element is the N-terminal calponin homology (CH) domain, a predominantly helical globular structure that gave name to a large superfamily of protein-protein interaction modules. The second modular component is the short tandem sequence repeats in the C-terminal half of calponin. Both, the highly basic, 23-residue repeat sequences and the acidic intervening sequences follow the consensus of a type I intrinsically unstructured protein which may account for the flexible folding properties of the calponin molecules.

The extreme C-terminal of the calponin contain the isoform-specific tail sequences (Rozenblum & Gimona, 2008). Documented interactions with CH domains range from actin, microtubules (MTs) and intermediate filaments (IFs), to mitogen-activated protein kinases (MAPKs), Zn^{2+} fingers, Ca^{2+}-binding proteins of the S100 family, and phospholipids. The involvement of the CH domain in actin binding, however, remains controversial.

Comprehensive deletion and domain swap experiments have revealed that the unique tail sequences of all three calponin isoforms negatively regulate their F-actin binding (Burgstaller *et al*., 2002), and *in vitro* binding studies have demonstrated that the region spanning amino acids 142-163 (ABS1) also mediates actin binding and contains the myosin ATPase inhibitory peptide (residues 142‑147).

A multiple sequence alignment (MSA) is a sequence alignment of three or more biological sequences, generally protein, DNA, or RNA. In this study, ten different protein sequences were aligned to detect the conserved sequences and region in term of different species. The position of 282 to 301 of amino acid showed highest variable of amino acid sequences among ten different species.

MSA analysis revealed that all domains are highly conserved in all of calponin investigated, which are calponin homology domain and calponin family repeat. Calponin homology domain is an actin-binding domain which may be present as a single copy or in tandem repeats (which increases binding affinity). The CH domain is found in cytoskeletal and signal transduction proteins, including actin-binding proteins like spectrin, alpha-actinin, dystrophin, utrophin, and fimbrin, proteins essential for regulation of cell shape (cortexillins), and signaling proteins (Marchler-Bauer *et al*., 2013).

The phylogenetic tree was built to identify the relationship of 17 different species in term of homologous calponin-2 gene. There are two distinct clades. First clade consists of fish species which are zebrafish (*D. rerio*), Nile tilapia (*O. niloticus*), Atlantic salmon (*S. salar*) and snakehead murrel (*C. striata*) (Figure 4.8). While the other clade consists of higher vertebrates such as house mouse (*M. musculus),* pig *(Sus sucrofa),* human (*H. sapiens)* and so on. As a general rule, if the bootstrap value for a given interior branch is 95 % or higher, then the topology at that branch is considered "correct". *Cs*Calp is group together with other calponin-2 of fish species with convincing bootstrap value of 96 %.

5.2 Quantitative Real-time PCR

The quantitative real-time RT-PCR revealed that *Cs*Calp was expressed in all the tested tissues with the highest expression level occurred in the blood. This result is consistent with a previous study that demonstrated significant levels of calponin-2 in peripheral blood cells of myeloid lineage (Huang *et al*., 2008). The myeloid lineage includes monocytes (macrophages) and granulocytes. Fish blood is essential for transportation of defender cells to attack foreign pathogens during inflammatory response. The differential expression of *Cs*Calp expressed in different tissues indicated that it could potentially be involved in different physiological process, such as cytoskeletal organization (Fukui *et al*., 1997), regulating macrophage motility and phagocytosis (Huang *et al*., 2008), cell proliferation (Jin *et al*., 2003) and signal transduction (Leinweber *et al*., 1999, 2000).

Various diseases, which mostly caused by bacteria and viruses, have affected the commercial fish aquaculture. *A. hydrophila* is the extremely virulent pathogen prevalent causing mass mortalities and economic losses in fish aquaculture (Joseph & Carnahan, 1994). This Gram-negative bacteria are widely distributed in water environments

including well water and heavily polluted waters. In the present study, *A. hydrophila* was chosen to challenge the snakehead fish to understand the time-course of *Cs*Calp gene expression upon pathogen exposure. Calponin-2 have been suggested to play a part in the immune response to bacterial infections in fish.

In our study, *Cs*Calp expression in *C. striata* was up-regulated at an earlier time point (6 h) and reached to highest level at 24 h with *A. hydrophila* challenge. The results showed that *Cs*Calp might be involved in a transient systemic immune response to the *A. hydrophila* stimulation. After 6 h challenge with *A. hydrophila*, the transcripts level of *Cs*Calp was significantly higher than that in the control group, it can be deduced that the extra calponin translated from more calponin transcripts would give positive response towards *A. hydrophila* infection. As time progressed, the expression of *Cs*Calp dropped to a low level at 72 h, which might caused by the excessive expression of *Cs*Calp from the previous response.

The rapid and dynamic temporal expression profile of *Cs*Calp upon *A. hydrophila* stimulation might be correlated with the numbers of myeloid cells in blood. Huang *et al* (2008) reported that calponin-2-free mice had reduced numbers of peripheral blood neutrophils and monocytes. Neutrophils and macrophages are the main components that involved in phagocytosis in fish (Secombes & Fletcher, 1992). Previous studies demonstrated that calponin-2 play multiple roles in regulation macrophage function such as regulation of myeloid cell proliferation and negative regulator on macrophage migration that is essential for phagocytosis (Huang *et al*., 2008).

5.3 Over-expression of Recombinant CsCalp

Although *Cs*Calp is a eukaryotic gene, *E. coli* was the choice for expression system in our study because it is the most common organism for convenient and high level production of recombinant proteins.

Here, *Cs*Calp was introduced directly into the pTrcHis TOPO expression vector. This is designed for cloning the PCR product immediately after the reaction using the inherent topoisomerase activity of the vector. The insertion and direction of the *Cs*Calp insert in this vector was determined by PCR amplification using one of the sequencing primers supplied by the kit and a complement insert specific primer. This result was also confirmed by sequencing. Construct with this direct expression vector was obtained. This construct, however, failed to give significant expression of recombinant *Cs*Calp.

The results showed that not every gene can be expressed using this particular vector in this organism. This may be due to the unique structural features of the gene sequence, stability and translational efficiency of mRNA, protein misfolding, degradation by the host cell proteases, major differences in codon usage between the foreign gene and native *E. coli*, potential toxicity of the protein for the host cell. However, there is no such study that have been reported the over-expression of calponin-2 in this particular expression vector. In other words, there is no conclusive experimental evidence showing the presence of recombinant calponin-2 protein in *E. coli*. Perhaps different strategies by using different cloning strategies will provide further information for this study (Figure 5.1).

Addition of an affinity tag such as the His-tag has been widely used for facilitated purification of recombinant proteins (Terpe, 2003). However, there is a possibility for

the tag structure to modify the overall conformation of calponin that is a potentially allosteric regulatory protein and has been shown with conformational changes sensitive to local structural modification (Jin *et al*., 2000). Therefore, we recommend expressing and purifying *Cs*Calp as non-fusion protein for a more reliable functional characterization.

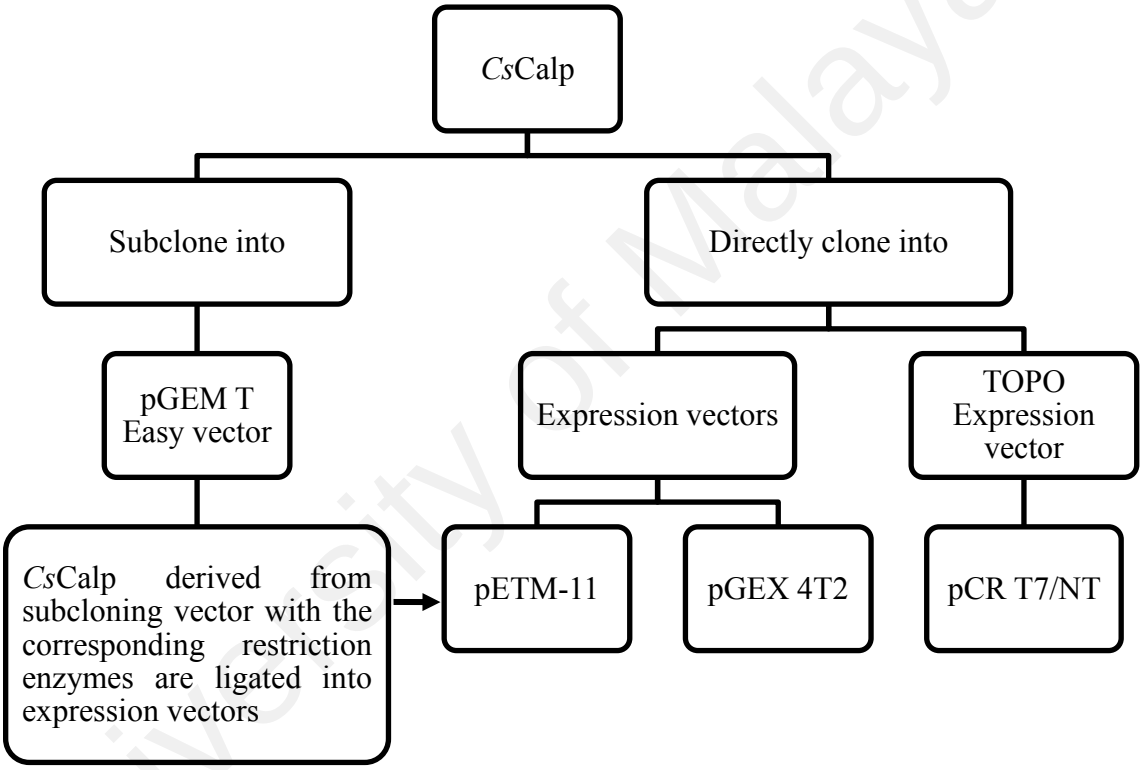

Figure 5.1: Cloning strategies of *Cs*Calp into different cloning vectors for future recommendation.

CHAPTER 6: CONCLUSION

In conclusion, a novel calponin cDNA (*Cs*Calp) was successfully isolated and cloned from *C. striata*. The obtained sequence was characterized at molecular level using various bioinformatics tools. *CsCalp* was found to be constitutively expressed in many tissues tested, but the highest expression was observed in blood. The rapid and dynamic expression profiles of mRNA *Cs*Calp challenged with *A. hydrophila* indicated that *Cs*Calp was perhaps involved in the innate immune response against bacterial infection. In the presence of high expression of *Cs*Calp, it is believed to correlate with the numbers of neutrophil and monocytes in blood. However, the changes in *Cs*Calp activity at the protein level and blood count should be performed to support its role. Further analysis and comparison of temporal expression patterns of *Cs*Calp mRNA after different pathogen challenges (i.e. virus, fungi, and Gram-positive bacteria) is warranted in order to gain a comprehensive understanding of the roles of calponin in the resistance against pathogen. The recombinant product of *Cs*Calp was over-expressed using *E. coli* expression vector system and further identified using Q-TOF MS. This construct, however, failed to give significant expression of recombinant *Cs*Calp. This outcome supports the need to substantiate the results obtained with comparative analysis on different strategies of prokaryotic expression of *Cs*Calp. These findings are expected to provide basic knowledge on *Cs*Calp. In addition, future work should emphasize on the connection between phagocytosis and calponin-2 expression in *C. striata*.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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