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ENDOGENOUS MUSCLE PROTEASES INVOLVED IN SOFTENING MECHANISMS AND RELEASE OF BIOACTIVE PEPTIDES DURING STORAGE OF UNDERUTILIZED FISH SPECIES

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

Doctor of Philosophy

By

Zeinab Ahmed

College of Health & Biomedicine

Advanced Food Systems Research Unit

2017

I dedicate this thesis to my late parents Saleh Ahmed and Mariam Hamde

&

Safaa, Marwa, Wafaa and Ahmed whose enormous contribution, love, encouragement and support paved the way for my success

CERTIFICATE

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This is to certify that the thesis entitled "**ENDOGENOUS MUSCLE PROTEASES INVOLVED IN SOFTENING MECHANISMS AND RELEASE OF BIOACTIVE PEPTIDES DURING STORAGE OF UNDERUTILIZED FISH SPECIES**" submitted by **Zeinab Ahmed** in partial fulfilment of the requirement for the award of the Doctor of Philosophy in Food Science at Victoria University is a record of the bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Professor Todor Vasiljevic

Date: 31/08/2016

DECLARATION

"I, Zeinab Ahmed, declare that the PhD thesis by publication entitled "**ENDOGENOUS MUSCLE PROTEASES INVOLVED IN SOFTENING MECHANISMS AND RELEASE OF BIOACTIVE PEPTIDES DURING STORAGE OF UNDERUTILIZED FISH SPECIES**" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

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Date: 31/08/2016

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PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	Paper Title	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
2	Calpains- and cathepsins-induced myofibrillar changes in post-mortem fish: Impact on structural softening and release of bioactive peptides	Published	**Published on 17/04/2015 in Trends in Food Science & Technology, SRJ Q1**
3	Proteolytic activities in fillets of selected underutilized Australian fish species	Published	**Published on 21/02/2013 in Food Chemistry; SRJ Q1**
4	Activity of endogenous muscle proteases from 4 Australian underutilized fish species as affected by ionic strength, pH, and temperature	Published	**Published on 21/11/2013 in Journal of Food Science; SRJ Q1**
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6	Endogenous muscle proteases releasing peptides with antioxidative activity during post-mortem storage of silver warehou muscle	In preparation	

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1. Ahmed, Z., Donkor, O., Street, W.A., and Vasiljevic, T.; 2011, Underutilized fish as a source of bioactive peptides, In Proceedings of Australian Institute of Food Science and Technology (AIFST) Food Science Summer School, held on 9th February 2011 at University of Queensland, Brisbane, Australia.

2. Ahmed, Z., Donkor, O., Street, W.A., and Vasiljevic, T.; 2015, Activity of endogenous muscle proteases from 4 Australian underutilized fish species as affected by ionic strength, pH, and temperature, In Proceedings of Australian Institute of Food Science and Technology (AIFST) Food Science Summer School, held on 29th January 2015 at RMIT University, Melbourne, Australia.

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ACE	angiotensin-converting enzyme
ANOVA	analysis of variance
ATP	adenosine triphosphate
CP	creatine phosphate
CA	calpain
CB	cathepsin B
CB+L	cathepsin B and L
CD	cathepsin D
CH	cathepsin H
DTT	dithiothreitol
DH	degree of hydrolysis
DHA	docosahexaenoic acid
dpm	days post-mortem
E-64	[1-(L-trans-epoxysuccinylleucyamino-4-guanidinobutane]
EDTA	Ethylenediaminetetraacetic acid
EPA	eicosapentaenoic acid
FAO	Food and Agriculture Organization of the United Nations
FPH	fish protein hydrolysate
g	gram
h	hour
IS	ionic strength
KCl	potassium chloride
kDa	kilodalton

LHC	light heavy chain
MHC	myosin heavy chain
m	milli
min	minute
ml	millilitre
mM	millimolar
M	molar
MW	molecular weight
n	nano
NaCl	sodium chloride
NaN_3	sodium azide
µ	micro
µg	microgram
µl	microlitre
ω-3	omega-3
PAGE	polyacrylamide gel electrophoresis
pH	hydrogen ion concentration
PMSF	phenylmethyl sulfonyl fluoride
pI	isoelectric point
PUFA	polyunsaturated fatty acids
RP-HPLC	reversed-phase high performance liquid chromatography
rpm	revolution per minute
°C	degree Celsius
RT	room temperature
s	second

SAS	statistical analysis system
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
× g	times gravitational force

ABSTRACT

Texture is one of the most important quality parameters of fish muscle which impacts consumer's acceptance, the market value and the suitability of fish for processing and preservation. Textural changes are rapid in fish muscle leading to unfavourable quality changes such as muscle softening and fillet gaping. Rapid softening of flesh may be one of the main reasons for underutilization of a wide range of bycatch and discarded fishery species. High proteolytic activity of endogenous muscle proteases, calpains and cathepsins, induces myofibrillar breakdown involved in softening of fish during post-mortem storage. In addition, post-mortem proteolysis of fish myofibrils by endogenous enzymes releases polypeptides and oligopeptides, some of which may demonstrate potential bioactive properties. The scope of the present research was to determine the major endogenous enzymes active in post-mortem fish muscle and establish their impact on structural softening and the release of bioactive peptides during storage of underutilized fish species.

The hydrolytic activity of major endogenous enzymes (calpains and cathepsins B, B+L, D, H) was assessed in crude muscle extracted from sixteen Australian underutilized fish. Fish species had a significant effect on the activity of these enzymes with barracouta showing the highest cathepsins B, B+L, D and H activities. Activities of cathepsins B and (or) B+L were higher than cathepsin H for all studied species. Rock ling and tiger flathead demonstrated higher cathepsin B+L activity, whereas gemfish and eastern school whiting showed higher activity towards cathepsin B. All examined species were characterized with lower endogenous activity of cathepsins D and H, suggesting that these enzymes are less likely to be involved in degradation and thus, the softening of fish muscle during *post-mortem* storage. It may be crucial to establish processing and storage conditions that may control the post-mortem

proteolytic activity of cathepsins B and L, in more commercially valued species, to a minimum if these species are to be used as raw materials in other seafood products.

A further study focused on establishing processing and (or) environmental conditions, that may control the proteolytic activity of endogenous calpains and cathepsins B and B+L to reduce their detrimental effects on fish myofibrillar proteins during iced storage. Conditions may thus be selected during fish processing to manipulate the activity of major muscle endogenous proteases. The study determined the effects of ionic strength (0, 500, or 1000 mM NaCl), pH (5.5, 6.0, or 6.5) and temperature (2, 4, or 6 °C) on the proteolytic activity of endogenous calpain-like, cathepsins B and B+L from 4 Australian underutilized fish species, deepwater flathead, ribaldo, ribbonfish and silver warehou. Crude enzymatic mixtures were exposed to different storage conditions upon which proteases activity assays were performed. Deepwater flathead and silver warehou exhibited lower calpain-like and higher cathepsin B+L activity than ribaldo and ribbonfish during various storage conditions. Activity of calpain-like enzymes in three examined species was significantly higher at pH 6.5 than pH 6.0 or 5.5. Raising the reaction temperature significantly increased calpain-like activity in ribaldo. Endogenous activity of cathepsin B in ribbonfish and silver warehou muscles declined significantly with increasing ionic strength. In contrast, activity of cathepsin B in ribbonfish and silver warehou declined significantly with increasing ionic strength at pH 6.5 or 6.0. Processing conditions were shown to influence the proteolytic activity of endogenous calpains and cathepsins which may have implications in controlling tissue softening during post-mortem storage.

The proteolytic mechanisms involved in the degradation of silver warehou, one of the underutilized but important commercial fishery species, during post-mortem storage were investigated. Intact muscle was incubated with various enzyme-inhibitors and (or) activators and the pH was adjusted (6.0, 6.5 or 7.0) to monitor post-mortem weakening of myofibrils

during storage after 1, 3 or 5 days. Changes in muscle pH provided suitable conditions for different proteases to hydrolyse myofibrillar proteins. The hydrolytic activity of endogenous cathepsins B and B+L was maximal at slightly acidic pH. This coincided with accelerated post-mortem autolysis of myofibrils. Antipain, EDTA and E-64 effectively reduced post-mortem degradation of the two most abundant structural proteins, actin and myosin heavy chain. In addition, pepstatin A minimised post-mortem myofibrillar autolysis at pH 6.5 or 7.0. This indicated that mainly cysteine proteases (calpains and cathepsins B and L) and secondary aspartic acid proteases (cathepsin D) are responsible for severe autolysis of muscle proteins associated with tissue softening post-mortem.

The high proteolytic activity of endogenous calpains and cathepsins detected in fish muscle at post-mortem pH may relate to the bioactive properties of peptides liberated during storage. In the final study, the specificity and potential of fish muscle enzymes to release peptides with potent bioactive properties during post-mortem storage of silver warehou muscle was evaluated. Muscle subjected to protease(s) inhibition and (or) activation and changes in pH (6.0, 6.5 or 7.0) during storage after 1, 3 or 5 days was assessed with respect to liberation of peptides with emphasis on antioxidative properties. The peptide profiling of partially hydrolysed muscle was clearly affected by the inhibition and (or) activation of endogenous enzymes, changes in pH and storage time. More peptides were liberated at pH 7.0 than 6.5 or 6.0 in the control as well as in samples treated with E-64, antipain, DTT and EDTA at day 1 and 3. Muscle treatment, changes in pH and storage time significantly influenced the antioxidative properties of liberated peptides. Fewer differences with respect to antioxidative properties of generated peptides were due to increased myofibrillar proteolysis at pH 6.0 and (or) decreased myofibrillar proteolysis at pH 7.0. Enzyme specificity and extensive release of hydrophobic peptides from partially hydrolysed muscle were more likely to be associated with the appreciable antioxidative properties of released peptides. In summary, cysteine (calpains

and cathepsins B and L) and aspartic acid (cathepsin D) proteases accelerated the degradation of myofibrillar proteolysis in silver warehou muscle during post-mortem storage but further work may be required to associate their hydrolytic activity with the bioactive properties of liberated peptides.

CHAPTER 1

Introduction

1.1. Introduction

Fish and seafood products are important components of healthy diets. Fish consumption accounted for about 17 percent of the world population's intake of animal protein in 2013 and continued to increase in 2014 and 2015, probably due to a rise in population and increasing awareness of the health benefits of fish. This has placed pressure on fisheries and fishery producers to effectively sustain current marine resources and meet increasing demand for fish as food. Aquaculture and fisheries are the production sectors responsible for the global utilization and supply of fish that is used for food and nutrition. In recent years, growth and development in aquaculture played a major role in improving the world fish supply. Nevertheless, capture fishery production has been stable since the late 1980s and has not been able to keep pace with the increased demand for fish and fishery products (Food and Agriculture Organization of the United Nations (FAO), 2016).

The number of common fishery resources available for fish supply is declining worldwide due to overexploitation of many commercially important target species. These species are increasingly fished at unsustainable levels since 1990 and thus are becoming exhausted and endangered. This is worsening the global state of marine fisheries. On the other hand, the human population is expected to rise substantially in the next decades, placing an increasing demand for better utilization of current fishery resources. Not all marine captures are adequately utilized for direct human consumption. The incidental capture of non-target fish species is often referred to as bycatch. Some of the bycatch are retained for sale by commercial fishers, but the majority is discarded back to the sea, dead or alive for economic, legal or personal requirements (Food and Agriculture Organization of the United Nations (FAO), 2016). Other reasons for underutilization of bycatch and discarded catch include seasonal variations in catch and composition, undesirable size, colour, flavour, texture, and high fat

content associated with the development of rancidity (Venugopal & Shahidi, 1998). The presence of parasites in certain bycatch species promotes a high proteolytic activity which negatively influence its textural and functional properties (Pacheco-Aguilar, Mazorra-Manzano, & Ramírez-Suárez, 2008). These factors limit the marketability and application of underutilized fish species for food.

Bycatch and discarded fishery resources are not adequately utilized. Proper utilization of bycatch and discarded marine resources may offer environmental and economic advantages, rather than the disposal of these resources which is costly for the processors since refinement of the material is necessary before discarding. Fish processors are prohibited from discarding filleting by-products back into the sea, resulting in costly refinement before discarding the material. Recovery and modification of proteins in fishery by-products to be used as functional food ingredients may be an alternative to discarding. However, the recovery and utilization of fishery by-products has to be more economically feasible than discarding (Kristinsson & Rasco, 2000a). Since global marine catches are declining, competition for common fish stocks is increasing, and the existence of bycatch will continue, effective utilization of these valuable resources is an issue of utmost importance. This may offer a solution for sustaining our marine resources by increasing the number of catches sought for direct human consumption and providing food security and nutrition for the world population (Clucas, 1997).

The most commonly used approach for utilizing bycatch and discarded fishery species is the production of animal feed (Goddard, Al-Shagaa, & Ali, 2008). However, the majority of the otherwise discarded species contain inherent properties that allows them to be utilized for a wide range of applications including food (Figure 1.1). Bycatch and discarded species are highly nutritious (García-Moreno, Pérez-Gálvez, Morales-Medina, Guadix, & Guadix, 2013). Nutritionally, fish is a valuable source of protein, minerals (such as calcium, iron, selenium, zinc and iodine), and vitamins (A, B and D). Consumption of fish proteins has been associated

with many physiological benefits including improved insulin sensitivity (Ouellet, Marois, Weisnagel, & Jacques, 2007), lowered blood pressure (Ait-Yahia et al., 2003), and increased satiation (Uhe, Collier, & O'Dea, 1992). That is probably due to the release of potent bioactive peptides. These peptides, when incorporated in the native protein molecule do not possess physiological properties. Upon enzymatic digestion, these peptides are released from fish proteins to demonstrate a range of potent bioactive properties important in the prevention or treatment of chronic diseases and improvement of physiological functions in the human body (Figure 1.2).

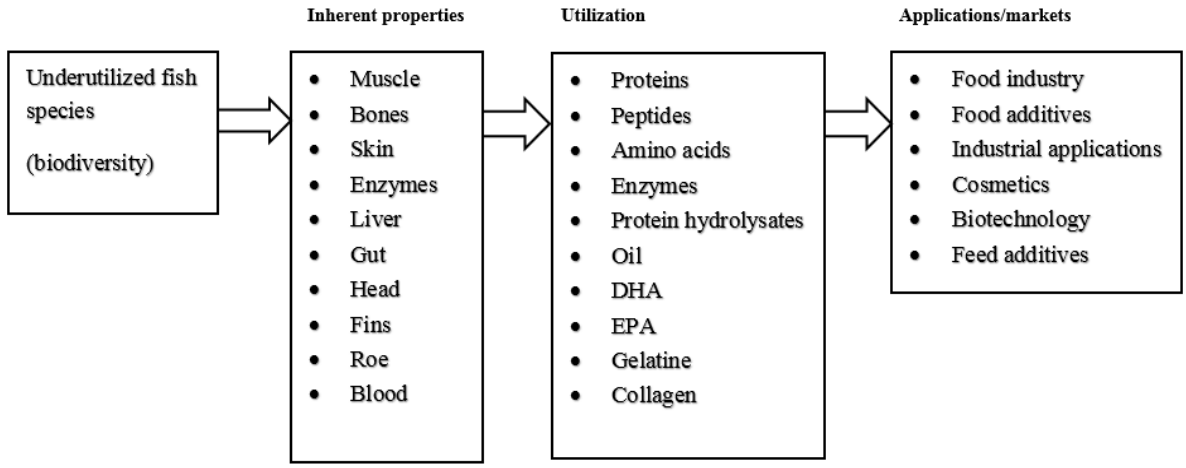

Figure 1.1. Underutilized fishery resources: Properties, utilization and prospects applications (Adapted from (Kim & Venkatesan, 2014)).

In addition, underutilized fishery resources contain high content of essential amino acids which could be added to food and feed products or used for flavour development and sweetness (He, Chen, Sun, Zhang, & Gao, 2006). Fish, especially oily fish, is a rich source of omega-3 (ω-3) polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Sidhu, 2003). These have been implicated in reduced risk of diabetes (Patel et al., 2009), cardiovascular protection (Duda, O'Shea, & Stanley, 2009), anti-inflammatory (Wall, Ross, Fitzgerald, & Stanton, 2010) and

neuroprotective (Delattre, Staziaki, & Ferraz, 2015) activities. Furthermore, fish is a valuable source of endogenous enzymes (viscera, digestive and muscle) which could be extracted and used for food processing. For example, hydrolysates with high nutritious value (Sathivel, Smiley, Prinyawiwatkul, & Bechtel, 2005), excellent functional properties (Yin, Wan, Pu, Bechtel, & Sathivel, 2011), and (or) enhanced bioactive properties (Geirsdottir et al., 2011) could be produced through enzymatic modification of fish proteins. Furthermore, underutilized fishery resources may also be further processed into useful components for various applications in the biomedical, pharmaceutical, cosmetic and food industries. For example, fish skin and fins can be utilized to extract collagen and gelatine. Fish gelatine may be used in microencapsulation of vitamins, colorants, and to microencapsulate food flavours (Ranadheera & Vidanarachchi, 2014). Collagen and gelatine are commonly used for the maintenance of normal bone integrity, treatment for brittle nails and for the nourishment of the scalp hair (Kim & Mendis, 2006). Therefore, there are potential opportunities to use underutilized fish species as a whole, a protein ingredient in human foods, to develop a range of value-added seafood products as well as for other applications (Figure 1.1).

Fish muscle or flesh is the most important product of fish. Freshness of fish is one of the most important quality parameter that determines the consumer's purchasing behaviour of fish and fishery products and the suitability of the raw material for processing and preservation (Haard, 1992). Accelerated softening of fish muscle is a common phenomenon that occurs during post-mortem storage and ultimately leads to loss of fish freshness. Factors such as the rate and extent of rigor mortis, pH decline and myofibrillar degradation influence the texture of fish muscle during storage (Haard, 1992). Nonetheless, post-mortem tissue softening is mainly attributed to the proteolytic degradation of myofibrillar and associated proteins primarily responsible for maintaining the structural integrity of muscle. The amount of endogenous proteases active in post-mortem muscle and their hydrolytic activity determine the

rate and degree of myofibrillar proteolysis and the rate and extent of tissue softening (Koohmaraie & Geesink, 2006). Increased proteolytic activity of endogenous muscle enzymes in post-mortem fish muscle is generally associated with severe breakdown of myofibrillar proteins and associated textural degradation of fish quality (Chéret, Delbarre-Ladrat, Lamballerie-Anton, & Verrez-Bagnis, 2007). At the optimum condition of their activity, these enzymes hydrolyse fish muscle proteins and increase the rate and extent of tissue softening (Figure 1.3).

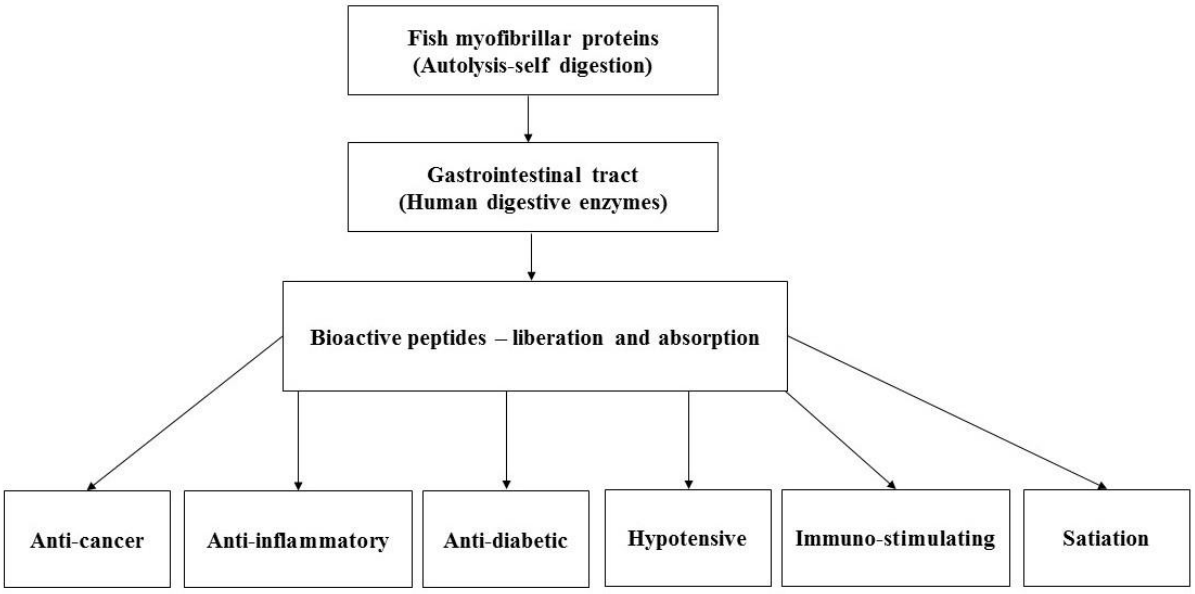

Figure 1.2. Bioactive properties of peptides released during *in vivo* or *in vitro* digestion of fish proteins (Adapted from (Manikkam, Vasiljevic, Donkor, & Mathai, 2016)).

In addition to softening, endogenous muscle proteases may contribute to the bioactive properties of fish-derived peptides (Figure 1.3). Endogenous muscle proteases are specific in nature and thus may hydrolyse different peptide bonds in the polypeptide chain causing limited hydrolysis of fish muscle proteins and generating a large number of oligopeptides and dipeptides which upon further hydrolysis by human digestive enzymes may release peptides with a range of physiological benefits (Figure 1.1). Limited hydrolysis is often necessary to

reduce the size of the original macromolecules and make the peptides bioactive (Cancre et al., 1999). Enzymatic hydrolysis of fish proteins *in vitro* released peptides possessing anticancer (Picot et al., 2006), antioxidative (Samaranayaka & Li-Chan, 2008), immunomodulatory (Yang et al., 2009), and antihypertensive (Jung et al., 2006) properties. The bioactive peptides released during *in vitro* hydrolysis of fish proteins may not necessarily exert the same beneficial effects when consumed by humans. Bioactive peptides must resist the digestive conditions in the gastrointestinal tract, be absorbed through the intestine where they enter the blood circulatory system, interact with appropriate receptors and thus regulate the physiological function within the body (Manikkam et al., 2016). The literature lacks information on clinical studies confirming the physiological effects of fish-derived peptides on humans. Therefore, the biological activity of peptides released during hydrolysis of fish proteins has been examined using animal models (Bauchart et al., 2007), simulated gastrointestinal digestion (Samaranayaka, Kitts, & Li-Chan, 2010) and computer stimulation methods (Darewicz, Borawska, & Pliszka, 2016). There may be significant differences between the peptides generated during *in vivo* digestion and *in vitro* hydrolysis of fish proteins. However, among the wide range of bioactive peptides produced *in vitro*, some of these may be reproducibly observed in the intestinal digesta of humans upon fish consumption (Bauchart et al., 2007). The bioactive properties of fish-derived peptides may be weaker than synthetic drugs commonly used in the treatment or prevention of chronic diseases. However, the peptides derived from fish sources are often consumed in the diet (Darewicz, Borawska, Vegarud, Minkiewicz, & Iwaniak, 2014).

Majority of the research studies documented in the literature relevant to the bioactive properties of fish-derived peptides involved exogenous enzymes. However, often hydrolysis conditions are manipulated for peptide production to determine the optimum degree of hydrolysis of protein substrates using selected exogenous protease(s) based on their specificity

which determines the cleavage pattern of peptide bonds (Shahidi & Zhong, 2008). Less is known about the potential of endogenous muscle proteases in making fish muscle bioactive during the autolytic process post-mortem. Enzymatic activity of fish muscle proteases may be manipulated at different processing conditions in a way that would change the peptide profile and improve the bioactive properties of fish peptides (Figure 1.3). Fisheries producers may select the processing conditions contributing to enhanced biological activities of peptides. This is particularly important considering the consumption of the whole fish as food (i.e. fillet). In contrast, exogenous enzymes may be used to release potent bioactive peptides. However, fish treated with exogenous enzymes are mainly used for research purposes and thus are not sold as fillets in the market.

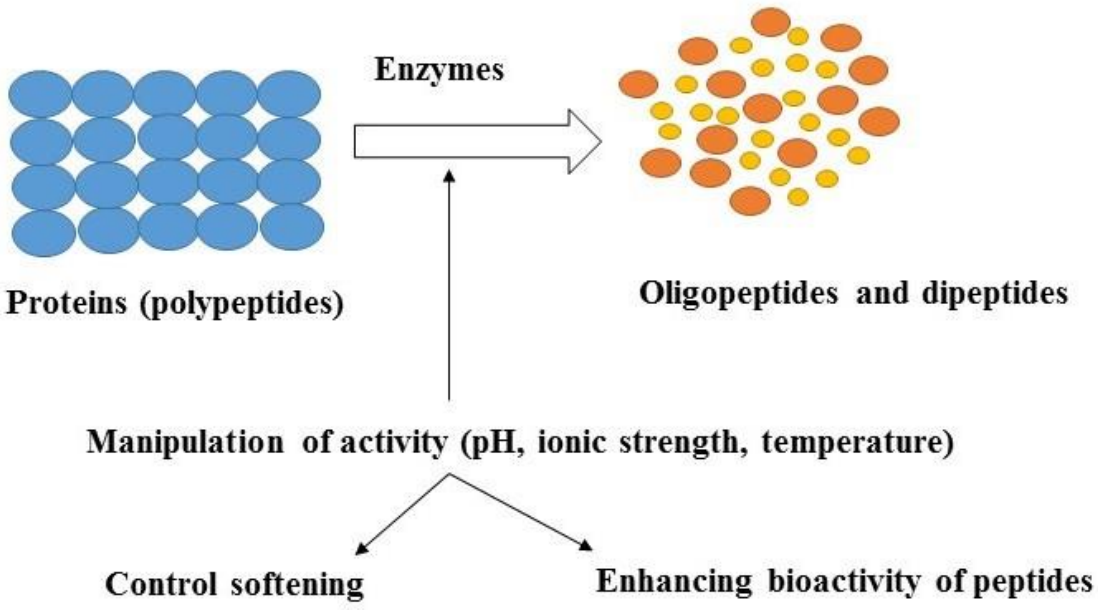

Figure 1.3. Manipulation of the activity of endogenous muscle proteases as a mean to influence softening mechanisms and improve the biological activity of fish-derived peptides.

Application of bycatch and underutilized fishery resources for human foods or as value-added ingredients demand a thorough knowledge about these species. There is a gap in the literature about the major endogenous muscle enzymes active in bycatch and discarded fishery resources. Enzymatic hydrolysis of proteins influences the shelf life, textural, functional and bioactive properties of fish muscle. Different processing and (or) storage conditions may influence muscle proteins and proteolytic enzymes thereby resulting in changes in the quality of the fish product. There is a need to improve our understanding of the enzymatic mechanisms involved in myofibrillar weakening and tissue softening in fish muscle post-mortem. The effect of environmental and storage conditions including ionic strength, pH and temperature on the hydrolytic capacity of endogenous muscle enzymes to weaken the myofibrillar structure during post-mortem storage needs to be established. This would improve our understanding of the conditions that regulate proteolytic activity with respect to controlling post-mortem softening of fish muscle and thus improving its shelf life. Knowledge about how these conditions affect the proteolytic activity of major endogenous enzymes present in underutilized fish muscle post-mortem and the rate and extent of muscle proteolysis may be helpful when processing underutilized fish species for various applications. This may give capture fisheries and producers insight about favourable storage and processing conditions for each species and possibly determine the suitability and application of using underutilized fish species for a range of applications. Additionally, there is a gap in knowledge about the enzymatic mechanisms responsible for post-mortem aging in underutilized fish species. An improved understanding of the underlying mechanisms involved in post-mortem proteolytic degradation of key myofibrillar components may add to existing knowledge on fish muscle proteolysis and concomitant tissue softening.

Enzymatic activity of endogenous muscle proteases may be manipulated using different processing and storage conditions to release peptides with potential bioactive properties. If

underutilized fish species are found to provide health benefits in addition to widely recognised effects arising from the provision of ω-3 fatty acids, this could enlarge the number of fish resources sought for catch and potential food. It may also increase the demand and profitability of bycatch and underutilized fishery resources. Identifying the proteolytic mechanisms associated with the release of bioactive peptides from undervalued fishery species may be useful for the incorporation of these compounds into nutraceutical or pharmaceutical industry. The study is going to address some of these knowledge gap areas and add to existing knowledge on fish endogenous muscle enzymes and their impact on muscle structure and bioactivity of fish peptides focusing on Australian bycatch and discarded marine resources. We focused on the proteolytic enzymes, calpains and cathepsins, due to their high levels in fish, implications in myofibril disintegration and textural degradation of fresh fish post-mortem.

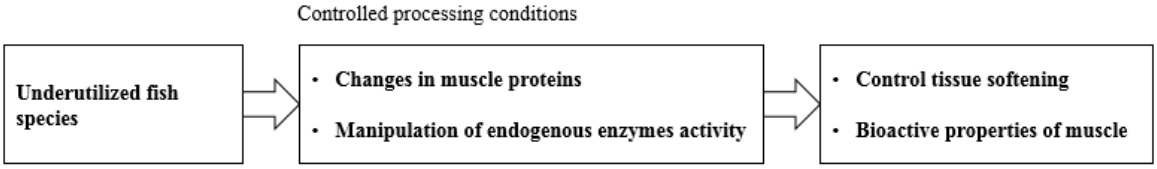

Figure 1.4. Schematic view of the research study illustrating how activity of endogenous muscle enzymes may be manipulated at different conditions to control softening and (or) make fish muscle bioactive.

1.2. Research aims

This research aimed at improving our understanding of endogenous proteolytic enzymes in fish muscle with respect to their: (a) hydrolytic activity profiles during various environmental and processing conditions; (b) impact on the degradation of structural muscle proteins involved in ultimate softening and (c) effect on the bioactivity of peptides released during storage of underutilized fish species (Figure 1.4). Therefore, the knowledge of the major

endogenous muscle enzymes active in underutilized fish species; their properties and hydrolytic mechanisms; the effect of processing and storage conditions on major endogenous enzymes; the proteolytic mechanisms associated with post-mortem myofibrillar weakening and release of peptides with one or more potent bioactive properties during post-mortem storage may contribute to the current literature and may suggest suitable conditions for processing fish and fishery products from underutilized marine resources. The specific objectives were to:

(a) Establish the proteolytic activity of major endogenous muscle enzymes in selected Australian underutilized fish species;

(b) Investigate the effects of ionic strength, pH, and temperature on proteolytic activity of endogenous enzymes;

(c) Identify proteolytic mechanisms involved in post-mortem degradation of muscle implicated in ultimate tissue softening; and

(d) Determine the role endogenous muscle proteases play in releasing peptides with important bioactive properties during post-mortem storage.

1.3. Thesis outline

This thesis is divided into 8 chapters. Chapter 1 is an introduction of the thesis and the proposed research. A thorough literature review of the current scientific knowledge on the scope of the thesis is provided in chapter 2. Chapter 3 focused on establishing knowledge about the post-mortem proteolytic activity of endogenous enzymes in Australian underutilized fishery species. The properties of highly active endogenous muscle enzymes as a function of processing and storage conditions was reported in chapter 4. The enzymatic mechanisms involved in proteolysis of major structural proteins during post-mortem storage of fish muscle was highlighted in chapter 5. This was followed by an examination of the release of peptides

during post-mortem storage of fish muscle and their bioactive properties as influenced by muscle endogenous enzymes. An overall conclusion from the study and future research directions is presented in chapter 7, and all references are listed in chapter 8.

CHAPTER 2

Literature review

Review of the literature is covered in two sections; section 2A and the published section 2B entitled "**Calpains- and cathepsins-induced myofibrillar changes in post-mortem fish: Impact on structural softening and release of bioactive peptides**" by Zeinab Ahmed, Osaana Donkor, Wayne A. Street, W. A. and Todor Vasiljevic in the peer reviewed journal, Trends in Food Science & Technology, 45, 130-146 (2015).

2A. Literature review

2A.1. General trends

2A.1.1. Fish consumption

Consumers are becoming increasingly aware about the health benefits of fish and seafood products and thus are changing their eating habits towards a healthier life. There is a global increase in the intake of fish proteins in recent decades. The world fishery production utilized for human consumption has increased significantly from 67 percent in the 1960s to 87 percent, or more than 146 million tonnes, in 2014. This development in fishery production in the period 1961-2013 was double that of population growth (Figure 2A.1). Fish supply used for non-food products, including fishmeal and fish oil, and direct feeding in aquaculture, comprised the remaining 21 million tonnes in 2014 (Food and Agriculture Organization of the United Nations (FAO), 2016).

Figure 2A.1. Global fish production and utilization for food and non-food purposes (Food and Agriculture Organization of the United Nations (FAO), 2016).

2A.1.2. Fish production and utilization for human consumption

The two sectors responsible for providing the world's population with diversified and nutritious fish are marine fisheries and aquaculture. Marine fisheries resources are collected and managed on a stock by stock basis. The world's marine fisheries expanded continuously from 1954 but exhibited a general declining trend after the 1990s (Figure 2A.2). Over the past 10-15 years, marine fishery production has been stable and have not been able to meet the increased demand for fish supply. On the other hand, rapid development in aquaculture in the last two decades contributed to the growth of fish supply sought for human consumption. Aquaculture resources constitute a wide variety of animals and plants such as fish; crustaceans, molluscs, seaweeds and other aquatic plants. Aquaculture increased the supply of fish utilized for food from 7 percent in 1974, to 26 percent in 1994 and 39 percent in 2004 (Figure 2A.2), with China contributing to over 60 percent of global aquaculture production (Food and Agriculture Organization of the United Nations (FAO), 2016).

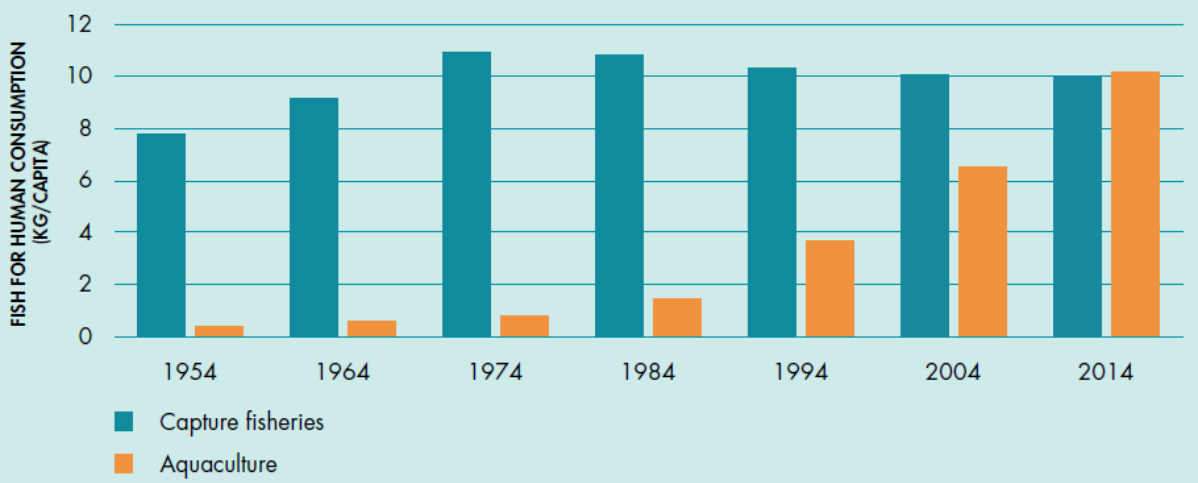

Figure 2A.2. Contribution of aquaculture and capture fisheries to fish for human consumption (Food and Agriculture Organization of the United Nations (FAO), 2016).

2A.1.3. Global state of fisheries and aquaculture

The state of capture fisheries is worsening since 1990. After 1990, there has been an increase in the number of traditionally important marine species fished at unsustainable levels (Figure 2A.3). Approximately 31.4 percent of assessed fish stocks in 2013 were classified as overfished. These stocks are exploited and thus strict management plans must be applied to rebuild stock abundance to full and biologically sustainable levels. Additionally, the number of fully fished stocks declined from 1974 to 1989, and then increased to 58.1 percent in 2013. Fully fished stocks have no potential for increase in production and catch expansion. Therefore, effective management must be followed to sustain their maximum sustainable yield. This may result in limited fish production for human consumption in the next decades. Alternatively, there is a continuous reduction in underfished fishery resources from 1974 to 2013, due to effective fisheries management being enforced to reduce the number of underfished stocks. Underutilized fishery resources are thus subjected to relatively low fishing pressure. However, if these resources are utilized effectively this may increase the number of fishery resources sought for food purposes, thus give a chance to replenish the endangered or overexploited and fully fishery stocks (Food and Agriculture Organization of the United Nations (FAO), 2016).

2A.1.4. Sustainable fisheries for projected world population growth

Sustainability of fisheries production is an important goal of fisheries management and is crucial to food security and nutrition of billions of people. The collapse of overexploited fish populations and the continued increase in fully fished stocks places a global need for effective management of fisheries to restore overfished species and maintain fully fished stocks (Food and Agriculture Organization of the United Nations (FAO), 2016). This is particularly important due to projected substantial growth in the global population in the next decades (Figure 2A.4). In 2015, the world population was estimated at 7.3 billion (United Nations,

2015b) and is anticipated to increase to 8.5 and 9.7 billion by 2030 and 2050, respectively (United Nations, 2015a). Fish may play an essential role in sustaining healthy diets in the future and ensure food security and nutrition for all. With a dramatically increasing world population and the continuation of depleting and exhausting overfished and fully fished marine stocks there is a great urgency to sustainably use underutilized fishery resources for direct human consumption for today, tomorrow and the future.

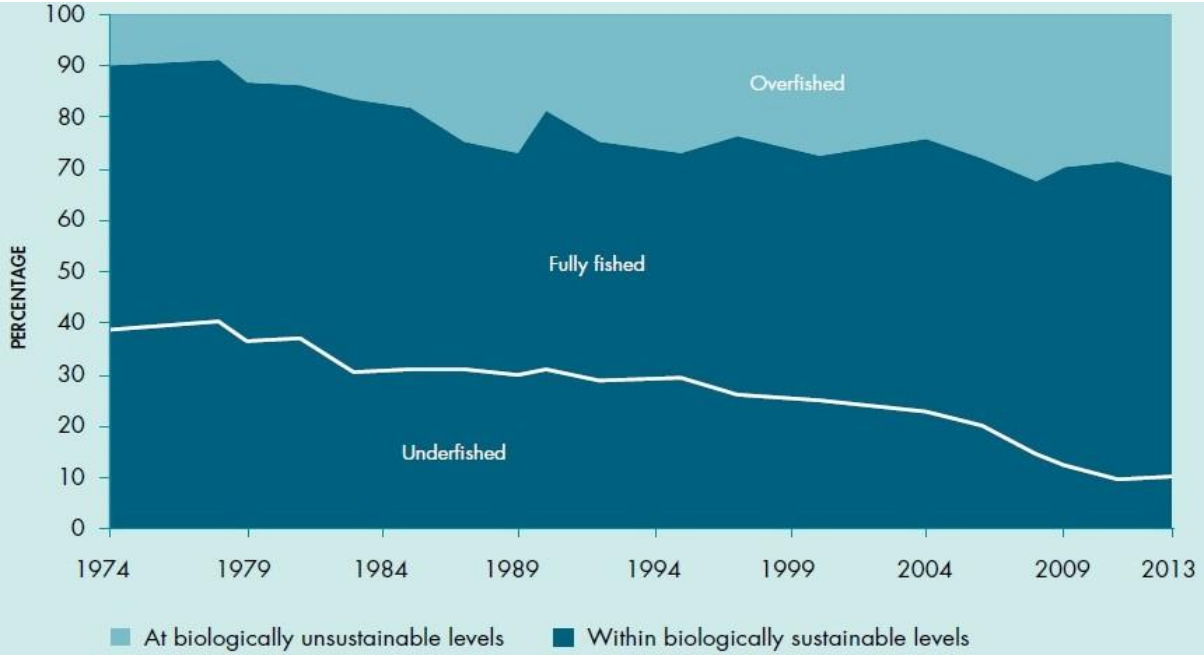

Figure 2A.3. Global trends in the state of marine fishery stocks (Food and Agriculture Organization of the United Nations (FAO), 2016).

2A.2. Fish production and consumption in Australia

Total commercial fisheries production in Australia mimics world trends. Commercial inland fisheries are fully exploited or declining since the early 1990s. Fishery species such as southern Bluefin tuna, gemfish and shark species are exhausted. Although, capture fisheries production has stabilised and there is a moderate increase in aquaculture, there is yet an

increasing demand for fish and fishery products due to population growth, improved product quality and increased awareness of the health advantages of seafood consumption. The total production of fisheries and aquaculture in Australia declined from 2003-2004 up to 2013-2014 (Figure 2A.5). Over the same period, consumption of fish and fishery products increased in Australia in 2003-2004 from 309 718 tonnes to 345 514 tonnes in 2013-2104 (Figure 2A.5). Accordingly, imports of seafood increased during that period to add to existing domestic fish supply and meet increased demand for fish. Australia's population is estimated to increase to 25 million by 2050 which may implicate sustainable use of our marine resource to meet these needs (Kearney, Foran, Poldy, & Lowe, 2003; Savage & Hobsbawn, 2015).

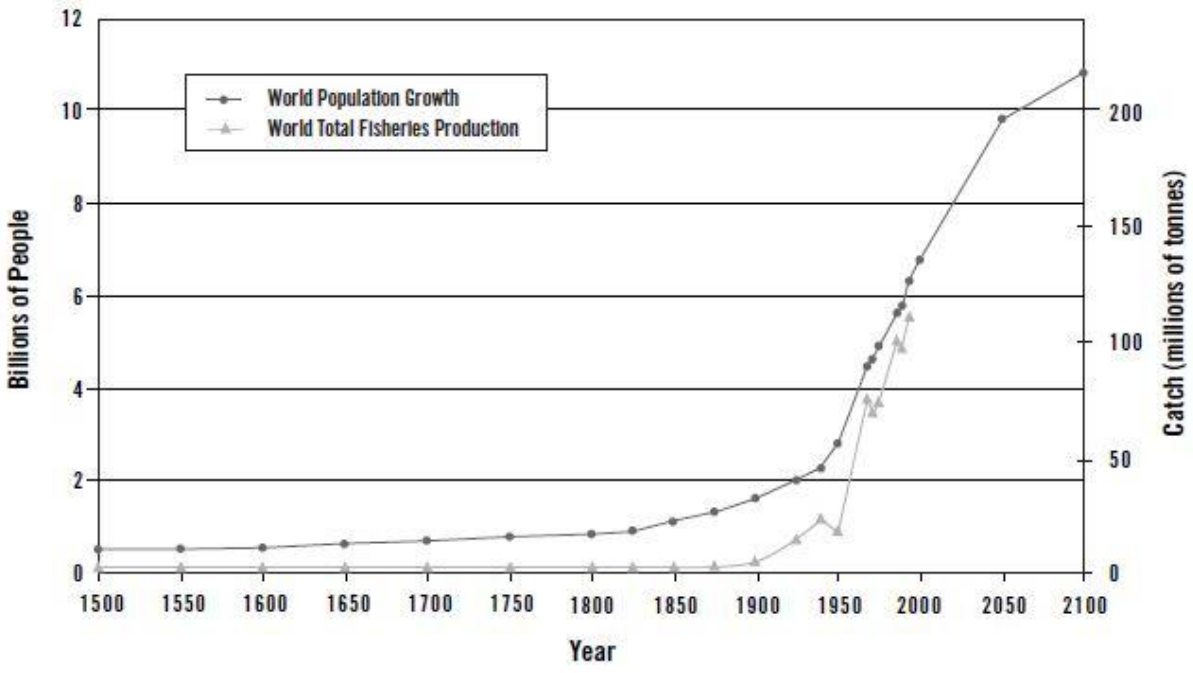

Figure 2A.4. Historic and projected trends in human population growth and fisheries production (Kearney et al., 2003).

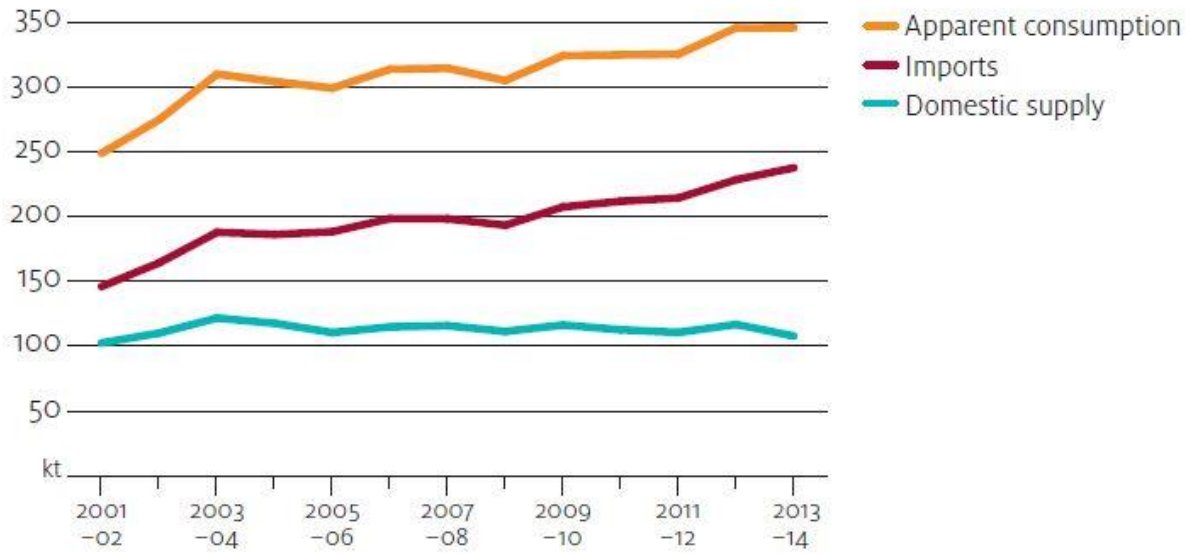

Figure 2A.5. Consumption, supply and imports of fish in Australia (Savage & Hobsbawn, 2015).

2A.3. Underutilization of bycatch and discarded fish species

Not all that is obtained from the sea is adequately used for human foods. Bycatch and discarded marine species are considered underutilized resources. Bycatch refers to the incidental capture of non-target organisms, whereas discards are part of the bycatch that is often returned back to the sea, dead or alive. The fishing operation of targeted species such as shrimp generates high volume of bycatch and discarding. The ratio of shrimp capture to discarded species ranges from 1:5 to 1:15 (Li, Wang, Hardy, & Gatlin, 2004). There is an abundance of underutilized fish species having low economic value. Measures are often taken to reduce bycatch significantly such as the use of bycatch reduction devices, the introduction of bycatch and discard regulations and the improvement in the enforcement of regulatory measures (Blanco, Sotelo, Chapela, & Pérez-Martín, 2007).

Bycatch and discarded species are underutilized for various reasons. Many of these species are characterized with extreme perishable nature making them unfavourable by

consumers and seafood processors. Other causes of underutilization of bycatch and discarded fish species relate to their inherent problems including undesirable size, odour, flavour, texture and high fat content (Venugopal, Shahidi, & Lee, 1995). Croaker (*Pennahia argentata*) is underutilized due to its small size and thus is only converted to low grade surimi (Choi, Hur, Choi, Konno, & Park, 2009). The perishability of bycatch may be one of the main reasons for their underutilization. Pacific whiting muscle is underutilized due to the presence of parasites, namely myxosporidia (*Kudoa paniformis*), which increases its proteolytic activity making it highly perishable and unsuitable for further processing (Mazorra-Manzano, Pacheco-Aguilar, Ramirez-Suarez, & García-Sánchez, 2008). Rapid softening of many underutilized fish species may result in economical losses for fisheries and fishery producers. The majority of these species are discarded back to the sea and may not make it to the shore due to handling and storage expenses, which may be exceed their market value.

Nonetheless, the nutritional composition of the majority of underutilized fish species is comparable to that of prime catch. Nine fish species caught as bycatch during the capture of commercial shrimp demonstrated variable nutrition content of protein (57-77%), lipids (0.6-16%), ash (8-20%) and amino acids. The quality and quantity of fish proteins was reported to be appropriate for use in food products (Córdova Murueta, Navarrete del Toro, & García Carreño, 2007). Underutilized shrimp species, *Acetes chinensis*, contained valuable protein (68.8%), lipid (13.1%), ash (15.1%) and a high index value of essential amino acids (25.77 g/100 g) (He et al., 2006). This suggests that the nutritional components of underutilized fishery resources should not be wasted. Understanding of the processing, handling, treatment and storage conditions that may be used to improve the textural and sensory properties, consumer's acceptance and market value of underutilized fish species may be important.

2A.4. Fish muscle structure and composition

2A.4.1. Structure

Fish flesh or fillet is the most important and highly appreciated part of the edible portion of fish. The body of most fish species consists mainly of skeletal muscle. The skeletal muscles of fish are arranged in segments called myotomes. The fish myotomes are composed of longitudinally oriented muscle fibres, which are the basic elements of the muscle structure. Myotomes are connected to each other by connective tissue layers called myocommata (Figure 2A.6). The connective tissue provides a supporting network throughout the muscle. The term muscle is used to describe the organ in the living animal involved in muscular contraction and relaxation (Dunajski, 1980; Hultin, 1984).

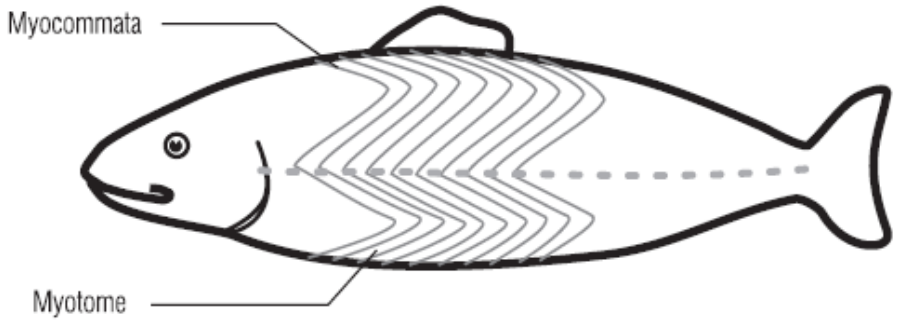

Figure 2A.6. Structural arrangement of muscle tissue in fish (Nielsen & Nielsen, 2012).

2A.4.2. Composition

The chemical composition of fish may vary depending on factors such as environment, season, temperature, spawning, genetic variations, and nutritional status. Most fish species contain approximately 80% water and 18% to 22% protein. Fish species are divided into three categories based on their fat content, lean, moderate fat and fatty fish. Lean fish muscle consists

of less than 2% fat, whereas moderate and fatty fish contain 2% to 5% and 5% to 15% fat, respectively (Venugopal & Shahidi, 1996). Muscle is mainly comprised of water. The majority of this water is located within the myofibrils between the thick and thin filaments and is thus bound to actin and myosin. The content of water in fish muscle is important for the sensory properties such as the colour, juiciness and tenderness of the end product. Water-binding capacity of meat is an important property that influences the yield and quality of the final product. Muscle acidification post-mortem reduces the water-holding capacity of meat. This is significant as the pH approaches the isoelectric point (pI) of major myofibrillar proteins (approximately pH 5.0) (Toldrá, 2003). Lipids is the most variable component in fish. The amount of muscle fat in some species such as mackerel and herring varies from 5 to 23%. In comparison, the total lipid content in species characterized with lean fish muscle is less than 1% (Hultin, 1984). High fat content in fish muscle is associated with an oily appearance and increased susceptibility to oxidation (Toldrá, 2006).

2A.4.2.1. Proteins

Proteins are the second abundant compound in fish muscle. Fish muscle contains a high content of myofibrillar proteins (60-80% of the total protein) as opposed to 40% in mammalian tissues (Delbarre-Ladrat, Chéret, Taylor, & Verrez-Bagnis, 2006). Proteins are important components of foods, both nutritionally and functionally. Nutritionally, proteins are a rich source of energy and essential amino acids important for growth and maintenance. Furthermore, proteins influence the physicochemical and sensory properties of foods and thus contribute to the functionality of the end-products (Korhonen, Pihlanto-Leppäla, Rantamäki, & Tupasela, 1998). Muscle proteins in fish are essential for its structure, function, and integrity. These proteins are involved in: a) the conversion of stiff muscle to meat, (b) changes contributing to tissue softening during storage and further processing, and (c) improvement of the nutritional quality of the meal by increasing the concentration of peptides and free amino

acids. Muscle proteins in fish are grouped into three different classes; connective tissue, sarcoplasmic and myofibrillar proteins (Table 2A.1) (Toldrá, 2006).

Connective tissue proteins constitute approximately 1.3 % of the total weight of muscle proteins in fish muscle (Belitz, Grosch, & Schieberle, 2009). These proteins are insoluble and contribute to the strength, support and framework of the muscle (Toldrá, 2006). Connective tissue proteins are primarily composed of collagen, which contributes partly to the textural properties of muscle tissue. In addition, gelatine is extracted from collagen and applied in many food ingredients characterized with high viscosity and gel-forming capabilities (Hultin, 1984). Generally, the content of connective tissue proteins in fish muscle is lower than that of mammalian muscle (Dunajski, 1980).

Sarcoplasmic proteins make up 20-30% of the total muscle protein. These proteins are soluble in low ionic strength buffers or water. They consist mainly of metabolic enzymes located in the mitochondria, lysosomes, microsomes, nucleus and the cytosol (Toldrá, 2006). In addition, they contain myoglobin and low molecular weight components such as sugar derivative and nucleotides (Hultin, 1984).

Myofibrillar proteins, often referred to as structural or contractile proteins, comprise about 70 to 80% of the total proteins in fish muscle. These proteins participate in muscle contraction and are the proteins which compose the myofibrils within the muscle fibre. The myofibrillar structure is made up of many proteins including actin and myosin as well as tropomyosin, troponins, α-actinin, titin, nebulin and desmin (Hultin, 1984). Actin and myosin are the most abundant myofibrillar proteins in muscle tissue, making up nearly 70% of the proteins in the myofibrils of skeletal muscle. These proteins provide the structural backbone of the myofibril and contribute to muscular contraction in the living tissue, rigor development in post-mortem muscle, as well as the characteristic appearance of muscle (Huff-Lonergan, 2010).

Post-mortem structural changes in actin, myosin and other minor myofibrillar proteins and their relevance to tissue softening are discussed in details in section 2B.

Table 2A.1. Classification and function of muscle proteins (Toldrá, 2006).

Protein	Type	Function
Myosin	Myofibrillar	Contractile
Actin	Myofibrillar	Contractile
Tropomyosin	Myofibrillar	Regulatory
Troponins T, C, I	Myofibrillar	Regulatory
α and β actinin	Myofibrillar	Regulatory
Titin	Myofibrillar	Cytoskeletal
Nebulin	Myofibrillar	Cytoskeletal
Filamin, synemin, vinculin	Myofibrillar	Z-line
Desmin	Myofibrillar	Myofibrils union at Z-line level
Creatine kinase	Myofibrillar	M-line
M protein	Myofibrillar	M-line
Mitochondrial enzymes	Sarcoplasmic	Respiration
Lysosomal enzymes	Sarcoplasmic	Intracellular digestion
Myoglobin	Sarcoplasmic	Natural pigment
Hemoglobin	Sarcoplasmic	Pigment from residual blood
Collagen	Connective	Structure resistance
Elastin	Connective	Structure resistance

2A.5. Textural quality

Fish is highly perishable food and thus spoils rapidly during storage. After fish is harvested, muscle tissue goes through biochemical and structural changes during post-mortem storage as indicated in section 2B, and begins to soften long before they are spoilt. The texture of fish is the most important quality attribute determining consumer's acceptance of fish and fishery products. Post-mortem softening of fish muscle tissue decreases the cohesiveness of the muscle segments in fillets and enhances fillet gaping (Nielsen & Nielsen, 2012). Structural changes in connective tissue and myofibrillar proteins have been linked with textural degradation of fish muscle post-mortem. Collagen, the main constituent of connective tissue

proteins, is important in maintaining muscle cohesiveness. Weakening of collagen during post-mortem storage of sea bream muscle was associated with softening of fillets (Suárez, Abad, Ruiz-Cara, Estrada, & García-Gallego, 2005). However, breakdown of collagen during post-mortem storage occurs at late post-mortem storage and is thus less likely to be the main causative of softening in fish muscle as demonstrated in section 2B. Post-mortem softening in fish muscle has been primarily attributed to the weakening and degradation of myofibrillar proteins. This is described clearly in section 2B. It is crucial to understand softening mechanisms of underutilized fish species. Providing such knowledge may assist with establishing techniques to control tissue softening or use it beneficially for processing these species for a wide range of applications.

Texture of fish muscle may vary depending on the species, age, size and nutritional composition of the fish. Post-mortem factors such as glycolysis, rigor mortis, pH, temperature and proteolysis of muscle proteins influences tissue softening (Huff Lonergan, Zhang, & Lonergan, 2010). These are briefly discussed in section 2B. Textural degradation in post-mortem fish muscle is mostly attributed to the breakdown of structural proteins. Processing and (or) handling conditions (pH, temperature and ionic strength) of fish after harvest may influence the structure and conformation of myofibrillar proteins as well as the activity of endogenous muscle proteases (Dunajski, 1980). Increased storage temperature enhances the activity of endogenous enzymes in post-mortem muscle which consequently induces the disruption of myofibrillar proteins (Zhao et al., 2005). Generally, storing muscle at high temperature causes more drastic proteolytic changes in myofibrillar proteins than storage at low temperature (Yates, Dutson, Caldwell, & Carpenter, 1983). Degradation of myosin heavy chain (MHC) in muscle of Atlantic cod was minor at 0 °C, increased slightly at 6 °C and was severe at 20 °C (Wang, Martinez, & Olsen, 2009). The acidity of muscle is probably the most important factor affecting the activity of endogenous muscle proteases, the structure of

myofibrils and ultimately the textural properties of fish muscle. A low ultimate muscle pH is associated with rapid degradation of muscle proteins and texture during post-mortem storage of fish (Ofstad et al., 1996). In mammalian muscle, weakening of myofibrillar proteins increases at low pH and decreases at high pH in post-mortem muscle (Dutson, 1983). Proteolysis of MHC in muscle of Atlantic cod was rapid at pH 5.5 in comparison to pH 6.3-6.5 (Wang et al., 2009). Furthermore, the activity of endogenous enzymes is influenced by changes in muscle pH. The proteolytic activity of calpains increases at neutral pH whereas the activity of cathepsins increases at low pH (Dutson, 1983). Ionic strength of muscle may influence the activity of endogenous muscle proteases. Increased intramuscular ionic strength and rapid pH decline in muscle post-mortem cause the rupture of the sarcoplasmic reticulum and subsequently leads to the leakage of calcium into the cytosol. This activates calpain in the cytosol which is detrimental to myofibrillar structure (Delbarre-Ladrat et al., 2006). Therefore, physico-chemical changes in post-mortem muscle may impact the textural quality and the consumer acceptability of the end product. An improved understanding of how different processing conditions may influence the proteins and proteolytic enzymes may be prerequisite for manipulating processing conditions to produce optimum quality fish product (Dunajski, 1980).

2A.6. Endogenous muscle proteases

Proteases are specific in their ability to degrade proteins. Endoproteases breakdown the proteins at susceptible peptide bonds distributed along the chain, whereas exoproteases, mainly aminopeptidases and carboxypeptidases, cleave one amino acid unit from the amino- or carboxyl terminal ends of the protein molecule, respectively (Figure 2A.7). Endoproteases are classified into four major classes depending on the chemical group in their catalytic or active site. These include serine, cysteine, aspartic and metalloproteases (Klomklao, Benjakul, &

Simpson, 2012). Proteases are found in the digestive organs of fish. Viscera are considered a valuable source of fish proteases including pepsin and trypsin (Shahidi & Janak Kamil, 2001). Pepsin is found in fish stomach whereas trypsin is highly expressed in pyloric ceca and intestine (Klomklao, 2008). Proteases could also leak from the intestine of fish and cause degradation of surrounding tissue proteins post-mortem (Stoknes, Walde, & Synnes, 2005).

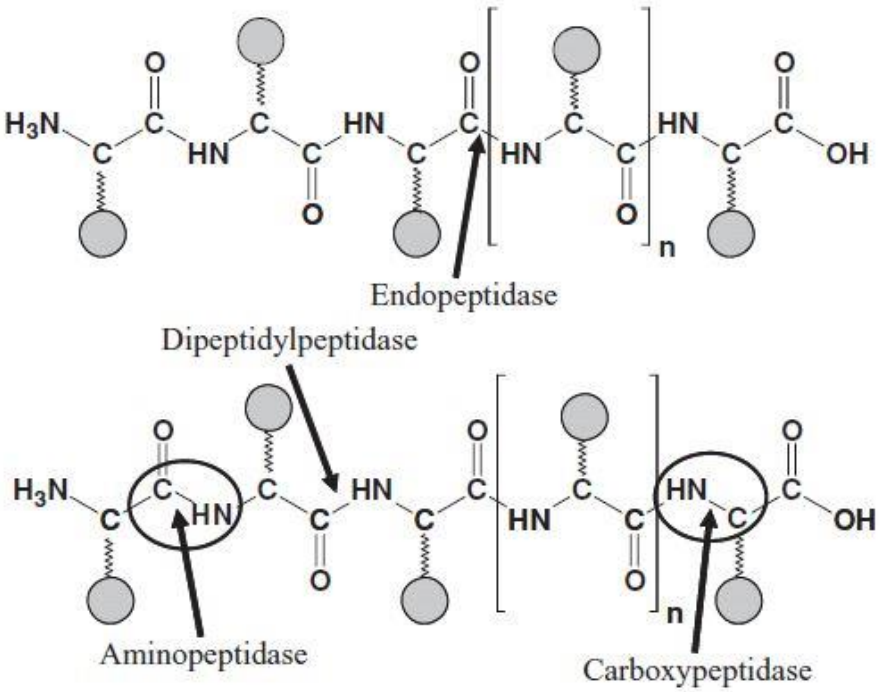

Figure 2A.7. Protein hydrolysis mode of action of muscle proteases (Toldrá, 2012).

Proteases are also endogenous to fish skeletal muscle. Fish muscle is characterized with higher activity of endogenous muscle proteases when compared to bovine muscle (Chéret et al., 2007). Endogenous muscle proteases, mainly calpains and cathepsins, are responsible for the weakening and degradation of key myofibrillar and associated proteins during autolysis and concomitant tissue softening. These enzymes are specific in their hydrolytic nature to degrade myofibrillar proteins. The number of proteases active in post-mortem muscle and their hydrolytic activity determine the rate and degree of myofibrillar proteolysis associated with

ultimate tissue degradation post-mortem (Koohmaraie & Geesink, 2006). Endogenous muscle proteases involved in post-mortem softening of fish muscle are discussed in section 2B.

2A.7. Tissue softening as induced by the activity of endogenous muscle proteases

Although softening mechanisms in fish muscle may not be fully understood, it is well documented in the literature that endogenous muscle proteases are mainly involved in this degradation process. Fish endogenous muscle proteases active during post-mortem storage increase the hydrolysis of structural proteins, weakening of the myofibrillar network and tissue softening (Toldrá, 2006). Endogenous muscle proteases may not necessarily liberate small peptides, but they induce limited hydrolysis of fish muscle proteins to release protein fragments and intermediate size polypeptides. Exoproteases may further breakdown released protein fragments and polypeptides into amino acids and small peptides which have a role in flavour development. Alternatively, these released protein fragments and oligopeptides may be further hydrolysed by human digestive enzymes upon consumption to release a wide range of peptides. Proteolytic-induced degradation of fish muscle proteins during post-mortem storage is described in section 2B and highlighted in chapter 5.

2A.8. Release of bioactive peptides as influenced by myofibrillar proteolysis

The bioactive properties of fish proteins have been an emerging area in scientific research in the last decade. Fish proteins are potential precursors for "bioactive peptides" (Li-Chan, 2015). These peptides, usually composed of 3-20 amino acids, are fragments that are encrypted in the primary sequence of proteins. These peptide sequences, when incorporated in the native protein molecule, do not possess physiological properties. Upon liberation by enzymatic hydrolysis, these peptides demonstrate a range of potent bioactive properties depending on their amino acid composition and sequence which are important for modulating human health. Bioactive peptides derived from fish proteins may be an alternative to

pharmaceutical drugs in the prevention or treatment of chronic diseases (Erdmann, Cheung, & Schröder, 2008).

Enzymatic hydrolysis of fish proteins to release bioactive peptides *in vitro* may be achieved using proteases obtained endogenously (autolysis) or exogenously (commercial enzyme(s) preparation) (Kristinsson & Rasco, 2000b). Autolysis of fish muscle proteins during refrigerated storage liberates polypeptides and oligopeptides fragments depending on the specificity of endogenous muscle enzymes. Hydrolysed peptides may demonstrate bioactive properties either *in vitro* or upon further hydrolysis by human digestive enzymes *in vivo* (Korhonen & Pihlanto, 2006). In some cases, enzymatic digestion of fish proteins by human digestive enzymes may result in further increase in biological properties of released peptides (Samaranayaka et al., 2010). Therefore, bioactive peptides released from hydrolysed fish myofibrillar proteins could potentially survive through the gastrointestinal digestion in the human body (Borawska, Darewicz, Vegarud, & Minkiewicz, 2016). Majority of the research studies on the bioactive properties of peptides released during hydrolysis of fish proteins involved exogenous enzymes. This involves monitoring the degree of proteolysis to cleave peptides with various molecular weights and bioactive properties. Less is known about the potential of endogenous muscle proteases in making fish muscle bioactive during the autolytic process.

Bioactive properties of fish-derived peptides *in vitro* have been identified as described in Figure 2A.8. Studies examining bioactive properties of peptides derived from fish proteins involve enzymatic hydrolysis of fish proteins using endogenous or exogenous enzymes. The resulting crude protein hydrolysate is then subjected to chromatographic separation to yield an enriched bioactive peptide preparation or additional purification steps may be required to isolate single peptides. Separated peptides fractions are collected and screened for biological activity and those having the highest activity are identified using peptide identification

techniques such as mass spectrometry. Once the peptide sequence responsible for biological activity is identified, a confirmation step is usually necessary. The activity of synthetic peptides with amino acid sequences identical to those identified in active fractions is measured and compared for validation (Li-Chan, 2015; Minkiewicz et al., 2008).

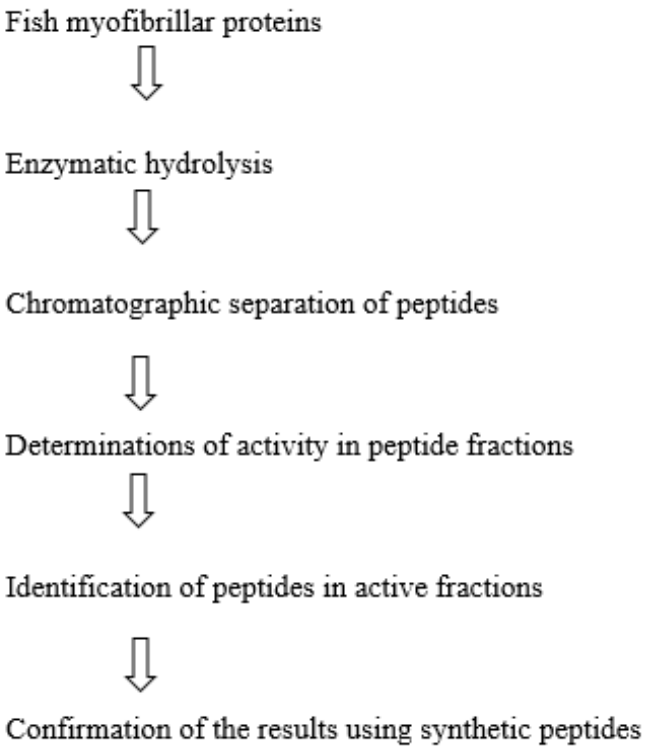

Figure 2A.8. Schematic representation of *in vitro* determination of bioactive peptides (Minkiewicz et al., 2008).

2A.9. Utilization of underutilized fish species

Traditional methods used to produce value-added products from commercially important species may be applied to low value species. These include flavorization, texturization and extraction of edible components. Several edible components such as fish protein hydrolysates (FPHs), protein concentrates and gelatine may be effectively isolated from underutilized fish species (Venugopal & Shahidi, 1998). Enzymatic hydrolysis of underutilized

fish species may be a very promising approach to improve its utilization or produce more profitable end products. Enzymatic modification of muscle proteins changes the conformation of proteins which may consequently influences the functional properties of proteins and their behaviour in food products during preparation, processing and storage (Adler-Nissen, 1982). Functional properties of proteins include solubility, emulsifying, foaming and gelation properties. Nonetheless, the hydrolysis process needs to be controlled by monitoring the degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved in a protein hydrolysate. This allows for the hydrolytic reaction to be monitored closely and terminated by inactivating the enzyme when the desirable DH is reached (Adler-Nissen, 1982). Excessive breakdown of proteins may produce a hydrolysate with a bitter off-flavour and undesirable functional properties. In some instances, limited controlled modification of proteins may bring about more desirable changes in hydrolysates. Nonetheless, monitoring the degree of hydrolysis achieves consistency and reproducibility in the end products.

The high content of muscle proteins and chitin in *A. chinensis* make them suitable for further processing to produce a high value-added product (He et al., 2006). Hydrolysates prepared from underutilized small croacker were comparable to non-muscle protein additives making them suitable as functional ingredients in food (Choi et al., 2009). By-catch and discarded fishery resources may effectively be utilized for the production of fish protein concentrate (Córdova Murueta et al., 2007). Protein concentrates play a significant role in protein fortified foods and beverages, due to their high solubility and digestibility (Venugopal & Shahidi, 1998). In addition, many underutilized fish species may be used as fillets or portions in ready-meals or in marinades preparation (Fagan, Gormley, & Mitchell, 2006).

Enzymatic modification of underutilized fish may be used to improve its bioactive properties. Discarded Mediterranean fish species (García-Moreno et al., 2014) and underutilized silver carp (García-Moreno et al., 2014) demonstrated high antioxidant activity

following enzymatic hydrolysis. Enzymatic hydrolysis of *A. chinensis* generated hydrolysates with potent angiotensin-converting enzyme (ACE)-inhibitory activity (He et al., 2006). ACE plays an important role in the regulation of blood pressure through the production of the potent vasoconstrictor, angiotensin II and the inactivation of the vasodilator, bradykinin. Accordingly, ACE-inhibitors are used for the treatment of hypertension in hypertensive individuals (Murray & FitzGerald, 2007). Enzymatic modification of blue whiting produced a hydrolysate with important functional and ACE inhibitory properties (Geirsdottir et al., 2011). High proteolytic activity, mainly of cathepsin L, present in underutilized Pacific hake fish produced a hydrolysate with high antioxidative potential (Samaranayaka & Li-Chan, 2008) which increased further upon i*n vitro* simulated gastrointestinal digestion (Samaranayaka et al., 2010).

Fish proteins and enzymes from underutilized fishery species have the potential to be used for various food applications, but a thorough knowledge about their properties is needed. The proteolytic activity of endogenous muscle proteases may be manipulated at different processing conditions to control the softening of fish muscle and improve their preservation. In addition, the proteolytic activity of enzymes endogenous to fish skeletal muscle may influence its bioactive properties. Fish muscle primarily consists of actin and myosin and is characterized with high activity of endogenous muscle proteases. Proteolysis of actin and myosin during post-mortem storage may release peptides with important bioactive sequences. Hydrolysis of carp MHC liberated the highest number of sequences with ACE-inhibitory and antioxidant activities (Darewicz et al., 2016). That is probably due to the rich peptide levels in these proteins. The richer the peptide profile of protein in bioactive sequences, the higher the likelihood of those sequences liberated by proteases (Dziuba & Darewicz, 2007). Using *in silico* prediction methods, hydrolysis of carp MHC by human gastrointestinal enzymes resulted in the liberation of the highest number of peptide sequences demonstrating ACE-inhibitory and antioxidant activity (Darewicz et al., 2016). Ingestion of fish proteins in pigs released bioactive

peptides in the duodenum and jejunum, most of which were fragments of actin and myosin (Bauchart et al., 2007). ACE inhibitory activity peptide were identified as Lys-Arg-Val-lle-Gln-Try and Val-Lys-Ala-Gly-Phe on hydrolysates prepared from myosin and actin, respectively (Muguruma et al., 2009). Enzymatically digested crude myosin light chain produced a hydrolysate with ACE inhibitor activity (Katayama et al., 2007).

Limited hydrolysis of primarily actin and myosin by endogenous muscle proteases during post-mortem storage of underutilized fish species may contribute to the release of bioactive peptides. Alternatively, endogenous enzymes may be extracted from underutilized fish muscle and used in processing of other protein hydrolysates (Balti, Nedjar-Arroume, Bougatef, Guillochon, & Nasri, 2010). Production of protein hydrolysates from fishery by-catch and discarded marine species may serve as a rich source of peptides and amino acids. Underutilized fish species are potential sources that are not yet utilized and their utilization for use in human foods is of importance. Many underutilized fish species may yield high quality fillets similar to those obtained from commercial species. However, knowledge about processing and handling conditions adequate to maintain optimum quality of underutilized fish species during storage may aid improve their utilization in the seafood industry.

2B. Published review article

This part of the literature review has been published as the title "**Calpains- and cathepsins-induced myofibrillar changes in post-mortem fish: Impact on structural softening and release of bioactive peptides**" by Zeinab Ahmed, Osaana Donkor, Wayne A. Street and Todor Vasiljevic in the peer reviewed journal, Trends in Food Science & Technology, 45, 130-146.

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Calpains- and cathepsins-induced myofibrillar changes in post-mortem fish: Impact on structural softening and release of bioactive peptides

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Proteolytic activation of endogenous muscle proteases, calpains and cathepsins, contributes to autolysis of fish myofibrils and concomitant softening of fillets during post-mortem storage. Calpains activity causes limited hydrolysis of myofibrils during initial days of post-mortem storage, whereas cathepsins in addition to proteolysis of major myofibrillar and associated proteins have the capacity to breakdown actin and myosin at later stages of post-mortem storage. Proteolysis of fish myofibrils post-mortem releases polypeptides and oligopeptides, some of which may demonstrate potential bioactive properties.

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Introduction

Progressive loss of fish freshness post-rigor is attributed to the complex combinations of biochemical, physical and structural changes in addition to muscle spoilage caused by microbial activity taking place in fish at later stages of refrigerated storage. Proteolytic cleavage of important structural proteins in myofibrils and extracellular matrix, as well as costamere (Z-disk associated structures that connects the myofibrils to the sarcolemma) and intermediate filament proteins (proteins involved in intermyofibrillar linkages) in post-mortem muscle contributes to tissue softening (Delbarre-Ladrat, Chéret, Taylor, & Verrez-Bagnis, 2006). These post-mortem changes in muscle begin from pre-rigor and continue during storage. In their study, Hernández-Herrero, Duflos, Malle, and Bouquelet (2003) demonstrated that fish softening is caused more by the degradation of connective tissue proteins than that of myofibrillar proteins. However, this type of degradation occurs at later stages of post-harvest refrigerated storage in fish muscle. Similarly, structural changes in the intramuscular connective tissue proteins occur slowly after 10 day(s) post-mortem (dpm) with concomitant beef tenderization in extended storage (Nishimura, Liu, Hattori, & Takahashi, 1998). In comparison, proteolysis of key myofibrillar proteins accelerates the softening process observed in fish muscle during early post-mortem storage period (Taylor, Fjaera, & Skjervold, 2002). The degradation of sarcoplasmic proteins, which are rich sources of glycolytic enzymes, is also common in post-mortem fish muscle (Hernández-Herrero *et al*., 2003).

Endogenous muscle proteases reported to be involved in post-mortem fish softening include the cytosolic calpains, lysosomal cathepsins, and connective tissue proteases comprising of elastase and collagenase. With respect to textural deterioration in fish tissue during initial period of post-mortem storage, particular importance is given to endogenous cytoplasmic calpains (both μ- and m-calpains) and lysosomal cathepsins B, H, and L as well as the aspartic cathepsin D. In conjugation, post-mortem physicochemical conditions including low temperature, reduced pH and relatively increased muscle ionic strength may act in synergy with endogenous muscle proteolytic enzymes to modify the interaction and conformation of proteins (Delbarre-Ladrat *et al*., 2006).

Calpains and cathepsins are widely studied with respect to their role in proteolysis of myofibrillar components and

tenderization of muscle foods post-mortem. In addition, endogenous muscle proteases may contribute to the generation of peptides with one or more bioactive properties. The biological activities of peptides released during processing of fish proteins are not widely studied in comparison to those obtained from milk proteins. *In vivo* hydrolysis of fish proteins generated peptides possessing bioactive sequences (Bauchart *et al.*, 2007). Using simulated gastrointestinal digestion Medenieks and Vasiljevic (2008) reported the release of fish peptides demonstrating antihypertensive properties. Documented physiological properties of peptides released from fish muscle may be related to the autolytic process. Autolysis of fish myofibrils post-mortem increases the release of polypeptide fragments and oligopeptides due to the activation of endogenous muscle proteases (Morzel, Verrez-Bagnis, Arendt, & Fleurence, 2000; Verrez-Bagnis, Ladrat, Noëlle, & Fleurence, 2002). The literature lacks knowledge on the role endogenous muscle proteases play in the release of peptides during post-mortem storage of fish muscle. The aim of this paper was to review current literature and provide information on the changes in fish muscle leading to activation of endogenous muscle calpains and cathepsins, proteolytic degradation of structural muscle proteins, and possible release of bioactive peptides from muscle during post-mortem storage.

Post-mortem changes in fish muscle

Early intracellular events leading to fragmentation of myofibrils

Myofibrillar fragmentation at early stages of post-harvest storage followed by bacterial proliferation leads to spoilage of the final product (Fig. 1). Within few minutes post-mortem, a few biochemical changes occur in muscle. Oxygen concentration declines rapidly and predisposes the muscle to anaerobic conditions. Biochemical changes reported in fish muscle post-harvest include a rapid reduction in creatine phosphate (CP) and glycogen content, increase in lactic acid content, reduction of adenosine triphosphate (ATP), and slow increase in trimethylamine content (Nazir & Magar, 1963; Pawar & Magar, 1965). In addition, lipids oxidize, and the concentrations of inosine and hypoxanthine increase (Delbarre-Ladrat *et al.*, 2006).

The most significant change that fish muscle undergoes a few hours after harvest is the onset of rigor mortis. The rigor mortis process is divided into pre-rigor, rigor mortis, and post-rigor stages. During the pre-rigor phase, the muscle remains extensible for some hours and uses CP and glycogen stores to maintain constant ATP supply. Declining ATP levels, particularly when intracellular level is below 2 μM, initiates the rigor mortis process in muscle. Permanent bonds between actin and myosin are formed causing the muscle to lose its ability to shorten or extend and ultimately results in muscle stiffening (Delbarre-Ladrat *et al.*, 2006). Resolution of rigor in the post-rigor stage increases muscle tenderization mainly due to hydrolysis of muscle proteins by endogenous calpains and cathepsins (Ayala *et al.*, 2010). A contradicting study reported that post-mortem fish softening proceeded independently of the resolution of rigor mortis (Ando, Toyohara, Shimizu, & Sakaguchi, 1991).

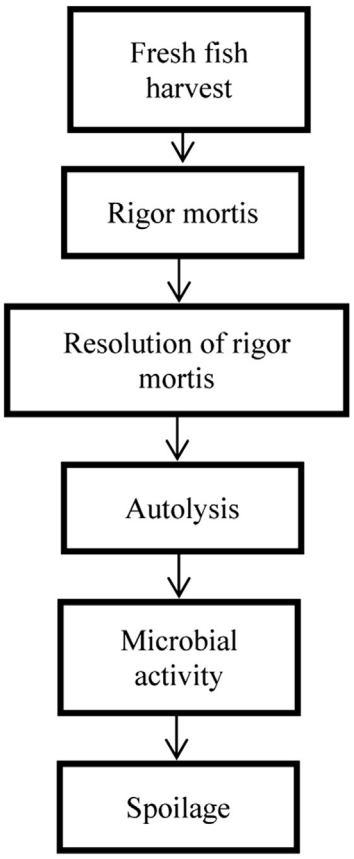

Fig. 1. Post-mortem changes in muscle leading to spoilage of fresh fish (adapted from (Delbarre-Ladrat *et al.*, 2006)).

Activation of endogenous muscle proteases

Biochemical changes are accompanied with increase in muscle osmotic pressure and rapid reduction in muscle pH during post-harvest storage (Wang, Tang, Correia, & Gill, 1998). Changes in pH and intramuscular ionic strength of muscle post-mortem may activate or inhibit protease(s) activity as well as modulate the conformation of myofibrillar proteins in a way that increases its susceptibility to proteolytic cleavage (Yates, Dutson, Caldwell, & Carpenter, 1983). Muscle pH decreases slowly from neutral pH after harvesting the fish to ultimate post-mortem muscle pH of 6 or below (Delbarre-Ladrat *et al.*, 2006). Intramuscular ionic strength in mammalian muscle increases from approximately 165 mM NaCl in ante-mortem to 275–295 mM NaCl post-mortem (Winger & Pope, 1981). These conditions cause the disturbance of sarcoplasmic reticulum, mitochondrial, and lysosomal membranes. The rise in sarcoplasmic calcium ions activates μ-and m-calpain depending on the concentration of calcium ions. The

disruption of the lysosomal membranes due to muscle acidification (pH 6.0–5.0) increases the leakage of cathepsins into the cytosol (Dutson, 1983). Activation of these proteases or their synergistic actions results in autolysis of fish myofibrils and concomitant softening of fish flesh postmortem (Delbarre-Ladrat *et al.*, 2006).

Endogenous muscle proteases involved in myofibrils degradation

The calpains

The calpains protease system consists of μ-calpain, m-calpain and the endogenous inhibitor calpastatin (Fig. 2). Both isoforms of calpains share similar biochemical properties except for their calcium ion requirement. Micromolar (μM) levels of calcium ions are required for activation of μ-calpain *in vitro*, as opposed to m-calpain, which requires milli-molar (mM) levels of calcium ions for its activity (Saido, Sorimachi, & Suzuki, 1994). The sensitivity of both calpains to calcium ions in fish muscle is lower than those of mammalian calpains (Toyohara & Makinodan, 1989). However the calcium requirement of both calpains differs between fish species. Approximately 0.1, 0.5, and 2.5 mM of Ca^{2+} were required for half maximal activation of m-calpain in tilapia (Jiang, Wang, & Chen, 1991), salmon (Geesink, Morton, Kent, & Bickerstaffe, 2000), and trout (Salem, Kenney, Killefer, & Nath, 2004) muscles, respectively.

The two isoforms of calpains are heterodimers composed of an identical small subunit (28 kDa) and a similar large (80 kDa) subunit. The small subunit of both calpains consists of two domains (V and VI) from the N-terminus and C-terminus, respectively (Fig. 2). Domain V of the small subunit is rich in glycine and is also identified as the site of phospholipid binding. Domain VI of the 28 kDa subunit contains five Ca^{2+}-binding sites also referred to as EF-hand motifs. The large subunit of both calpains contains four domains (Domain I–IV). Domain II identified as the catalytic protease domain is made up of subdomains (IIa and IIb) containing a cysteine and histidine residues. Domain III binds to Ca^{2+} and phospholipids. Domain IV like domain VI contains five EF-hand calcium binding sites. The first four EF-hands in domain IV of the 80 kDa subunit also contain Ca^{2+}-binding sites. Nonetheless, the fifth EF-hand molecule in the carboxyl-end of IV and VI domains (Fig. 2) cannot bind Ca^{2+} but interact with each other and form a heterodimer of the two subunits of calpain (Huff Lonergan, Zhang, & Lonergan, 2010; Suzuki, Hata, Kawabata, & Sorimachi, 2004).

Calpastatin

Calpain activity is mainly regulated through calcium concentration and its specific endogenous inhibitor calpastatin. Calpastatin inhibits the activity of both μ-and m-calpains through preventing the auto-proteolytic activation, membrane binding, and the expression of catalytic activity of calpain (Huff Lonergan *et al.*, 2010). Four calpain-inhibiting domains (I, II, III, and IV) and an alkaline N-terminal tail identified as domain L make up the calpastatin molecule (Fig. 2). Calpain-calpastatin binding is calcium dependent. Binding of calcium ions to calpains induces conformational changes in the calpain molecule, specifically in domains I–IV of the catalytic subunit and domain VI of the regulatory subunit. Region A in the calpastatin molecule binds to domain IV and region C binds to calpain domain VI. This binding allows calpastatin inhibitory

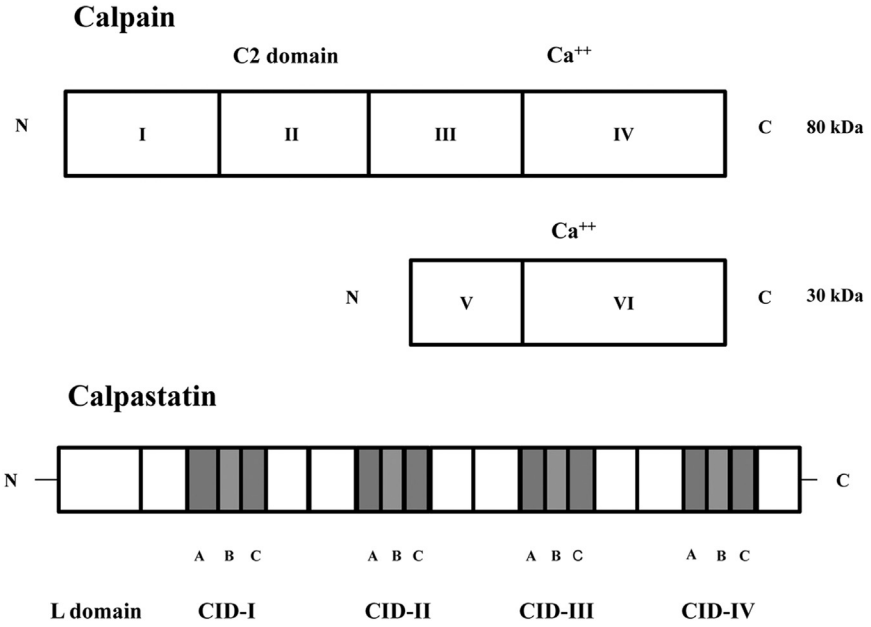

Fig. 2. A schematic diagram showing calpain structure (adapted from (Kawasaki & Kawashima, 1996; Mellgren, 2008)).

domain region B to make several contacts with domains I–III regions of calpains thus blocking its active site and inhibiting its activity (Huff Lonergan *et al.*, 2010; Kawasaki & Kawashima, 1996). This central inhibitory sequence in the calpastatin molecule is identified as TIPP-XYR (Kawasaki & Kawashima, 1996).

Role of calpains activity in myofibrillar proteolysis

Like any protease, calpains have the capacity to cleave proteins *in vitro* and *in vivo*. Calpains demonstrate preference for hydrophobic amino acids particularly leucine, isoleucine and valine. Enzyme specificity may be explained using the model developed by Schechter and Berger (1967), in which the active site of the papain enzyme consists of seven subsites, four of which are amino-terminal to the cleavage site (S1–S4) and the remaining (S1′–S3′) are on the carboxyl-terminus. These subsites can each bind specifically to one amino acid residue of the substrate identified as P1–P4 and P1′–P3′. A hydrophobic amino acid residue at the P2 position and P1 position with another basic amino acid at the P1′ position were preferred sites for calpains on oxidized insulin chain B (Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2002). Substrates rich in proline, glutamate, serine, and threonine referred to as the PEST sequence and/or exhibiting calmodulin-binding domain are also susceptible to calpains hydrolysis (Carafoli & Molinari, 1998). An *in vitro* study demonstrated that the PEST sequence may be potential targets for μ-calpain cleavage (Shumway, Maki, & Miyamoto, 1999). In contradiction, Carillo *et al.* (1996) reported that PEST peptide sequences are not necessary for specifying degradation of proteins by calpains.

Calpains activity results in limited proteolysis of proteins during protein turnover in physiological conditions and in post-mortem muscle. During protein turnover, calpains release myofilaments from myofibrils. Similarly, calpains activity in post-mortem muscle leads to the weakening and destabilisation of the myofibrillar structure by producing some of the structural changes mentioned in Table 1. That increases the susceptibility of released polypeptides to further attack by other proteases (Goll, Thompson, Taylor, & Ouali, 1998). Myofibrillar proteins degraded in muscle under normal post-mortem ageing conditions as a result of calpain activation may not be the same as specific muscle proteins degraded *in vitro*. Nonetheless, myofibrils obtained from naturally aged muscles and isolated myofibrils hydrolysed with μ-calpain under post-mortem like conditions demonstrated similar degradation patterns (Huff-Lonergan *et al.*, 1996). Similarly, a study by Goll, Thompson, Taylor, and Christiansen (1992) reported that both μ- and m-calpains degraded the same myofibrillar proteins that were hydrolysed during post-mortem muscle storage with the exception of major myofibrillar proteins such as actin and myosin.

The lysosomal cathepsins

The lysosomal cathepsins are acid proteases usually located in the lysosomes of living muscle, but become released in post-mortem muscle. Cathepsins differ depending on their active site (aspartic, cysteine, serine proteases), their substrate specificity and inhibitor sensitivity. Lysosomes harbour at least 13 cathepsins including cathepsin A, B, C, D, H and L. Among these, cathepsins B, D, H and L play an important role in post-mortem proteolysis of myofibrils and concomitant meat softening. The endogenous inhibitor cystatin regulates the activity of cathepsins B, H and L *in vivo*. Most cathepsins are endoproteases, but in addition, cathepsin B exhibits a strong dipeptidyl carboxypeptidase (peptidyl-dipeptidase) activity, whereas cathepsin H demonstrates a strong aminopeptidase activity and a limited endopeptidase activity (Goll *et al.*, 1983).

Role of cathepsins in myofibrillar proteolysis

The activity of cathepsins in post-mortem muscle increases significantly with post-mortem time (Gaarder, Bahuaud, Veiseth-Kent, Mørkøre, & Thomassen, 2012).

Table 1. *In vitro* proteolysis of key myofibrillar proteins post-mortem by calpains.

Protease(s)	Substrate	Hydrolysis conditions	Protein changes	Reference
Porcine μ-calpain (1 U)	Myofibrils (porcine muscle)	pH 5.8; 4 °C; 2 or 4 d	Degradation of desmin, actin, myosin heavy chain (MHC), myosin light chain (MLC), troponin T, tropomyosin, & CapZ	Lametsch, Roepstorff, Møller, and Bendixen (2004)
Endogenous m-calpain	Myofibrils (sea bass muscle)	pH 7.0; 25 °C; 0–120 min	Partial degradation of MHC Loss of tropomyosin	Verrez-Bagnis *et al.* (2002)
Purified μ-calpain	Myofibrils (bovine muscle)	pH 5.6; 4 °C; 100 μM $CaCl_2$; 0–120 min	Hydrolysis of nebulin, connectin, filamin, desmin, & troponin T	Huff-Lonergan *et al.* (1996)
Purified m-calpain & commercial cathepsins	Myofibrils (sea bass muscle)	m-calpain (pH 7; 25 °C; 2 h) & cathepsins (pH 5.5; 25 °C; 20 h)	Breakdown of MHC, α-actinin, desmin, actin, troponin, & tropomyosin	Delbarre-Ladrat, Verrez-Bagnis, Noël, and Fleurence (2004)
Calpain	Myofibrils (sea bass white muscle)	pH 7.0; 6 mM Ca^{2+}	Complete loss of costameres & intermediate filaments Z-disks weakening	Taylor *et al.* (1997)
μ-calpain	Bovine skeletal myofibrils	pH 5.5–5.8; 5 °C; 90 min	Loss of desmin & troponin T α-actinin release	Koohmaraie, Schollmeyer, and Dutson (1986)

These enzymes are generally highly active at acidic pH. Cathepsins B, H, and L may be irreversibly inactivated at pH above 7.0. High activity of cathepsins B, D, H, and L in post-mortem fish muscles increased myofibrillar proteolysis in salmon muscle (Yamashita & Konagaya, 1990a). The hydrolytic activity of cathepsins results in weakening and degradation of a number of structural muscle proteins (Table 2) leading to tissue softening. Cathepsins has the capacity to breakdown proteins and release peptides, oligopeptides and smaller polypeptide fragments (Reddi, Constantinides, & Dymsza, 1972). The major lysosomal proteases in post-mortem fish muscle were identified as cathepsins B, D, H and L (Yamashita & Konagaya, 1990a). These cathepsins may act individually or synergistically to hydrolyse post-mortem muscle during refrigerated storage (Table 2).

Cathepsin B

The molecular weight of cathepsin B varies with species. The enzyme has been purified and characterized in skeletal muscles from tilapia (23.5 kDa) (Sherekar, Gore, & Ninjoor, 1988), silver carp (29 kDa) (Liu, Yin, Zhang, Li, & Ma, 2008b), mackerel (*Scomber australasicus*) (28 kDa) (Jiang, Lee, & Chen, 1994), mackerel (*Scomber japonicus*) (23 kDa) (Matsumiya, Mochizuki, & Otake, 1989) and salmon (28 kDa) (Yamashita & Konagaya, 1990c).

Cathepsin B cleaves several synthetic substrates and has the capacity to degrade Z-Arg-Arg-MCA and Z-Phe-Arg-MCA in the presence of SH-containing compounds at about pH 6.0. Nonetheless, the enzyme demonstrated high affinity towards Z-Arg-Arg-MCA (Jiang *et al*., 1994). The hydrophobic S2 subsite of cathepsin B allows the hydrolysis of substrates such as Z-Phe-Arg-MCA with Phe in the P2 position. In addition, Glu_{205} in the S2 position can interact with polar amino acid residues in the P2 position to cleave substrates such as Z-Arg-Arg-MCA. The specificity of cathepsin B on the S3 and S1′ subsites shows a preference for Tyr in the P3 position (Kirschke, Barrett, & Rawlings, 1998) and large hydrophobic residues in the P1′ position (Trp, Tyr, Phe, Leu) over residues such as Asn and Gln (Ménard *et al*., 1993). The peptidyl–dipeptidase activity of cathepsin B results in the sequential cleavage of the C-terminal dipeptides from some proteins and polypeptides (Barrett & Kirschke, 1981).

The activity of cathepsin B was reported to be 29.7 times higher in fish muscle than in bovine meat implicating the important role that this enzyme play in textural changes in fish tissue (Chéret, Delbarre-Ladrat, Lamballerie-Anton, & Verrez-Bagnis, 2007). Maximal proteolytic activity of cathepsin B against Z-Arg-Arg-MCA and Z-Phe-Arg-MCA was detected at various pH and temperature conditions. Cathepsin B from mackerel (*S. australasicus*) muscle maximally hydrolysed Z-Phe-Arg-MCA at pH 6.5 and 55 °C (Jiang *et al*., 1994). In comparison, cathepsin B from hepatopancreas of carp hydrolysed Z-Arg-Arg-MCA at the optimum pH of 6.0 and 45 °C (Aranishi, Hara, Osatomi, & Ishihara, 1997a). The optimum pH for hydrolysis of Z-Arg-Arg-MCA by cathepsin B in silver carp (Liu *et al*., 2008b) and mackerel (*S. japonicus*) muscles has been reported to be 5.5 (Matsumiya *et al*., 1989), whereas the optimum pH for skeletal muscle from mackerel and tilapia were 6.5 and 6.0, respectively (Jiang *et al*., 1994; Sherekar *et al*., 1988). Generally, the proteolytic activity of cathepsin B against most substrates is maximal at pH 6.0, and decreases at neutral pH due to irreversible enzyme inactivation (Sherekar *et al*., 1988). This makes the contribution of cathepsin B to myofibrillar degradation minimal at high pH due to stability of the lysosomal membranes under this condition (Ertbjerg, Henckel, Karlsson, Larsen, & Møller, 1999). Carp cathepsin B resulted in rapid breakdown of myosin heavy chain (MHC) at 50 °C but purified

Table 2. Cathepsins-induced degradation of myofibrillar proteins in post-mortem muscle.

Cathepsin(s)	Hydrolysis conditions	Protein changes	Reference
Carp cathepsin L	Carp myofibrils (pH 5.0–7.0; 37 °C; 20 h)	Degradation of myosin, α-actinin, & troponins T and I	Ogata, Aranishi, Hara, Osatomi, and Ishihara (1998)
Commercial cathepsins B & L	Sea bass myofibrils (pH 5.5; 25 °C; 22 h)	MHC breakdown Hydrolysis of troponin T, tropomyosin, desmin, & actin	Ladrat, Verrez-Bagnis, Noël, and Fleurence (2003)
Herring cathepsin D	Herring myofibrils (pH 4.5; 5 °C; 2 d)	Hydrolysis of myosin, actin, & tropomyosin	Nielsen and Nielsen (2001)
Mackerel cathepsins L, L-like, & B	Various post-mortem like conditions	Cleavage actin & myosin	Jiang, Lee, and Chen (1996)
Bovine cathepsin D	Bovine myofibrils (pH 5.5; 37 °C; 120 min)	Degradation of MHC & titin Limited hydrolysis of actin, tropomyosin, troponins T and I, & MLC	Zeece, Katoh, Robson, and Parrish (1986)
Salmon cathepsin L	Carp myofibrillar fragments (pH 6.5; 20 °C; 60 min)	Hydrolysis of connectin, nebulin, myosin, collagen, α-actinin, & troponins T and I	Yamashita and Konagaya (1991)
Salmon cathepsin B	Carp myofibrillar fragments (pH 6.5; 20 °C)	Cleavage of connectin, nebulin, & myosin	Yamashita and Konagaya (1991)
Cathepsin L (rabbit muscle)	Myofibrils (rabbit muscle)	Degradation of MHC, α-actinin, actin, & troponins T and I	Matsukura, Okitani, Nishimuro, and Kato (1981)

carp cathepsin B from hepatopancreas demonstrated maximal hydrolytic activity towards Z-Arg-Arg-MCA at 35 °C. These studies indicate that the optimum pH and temperature required for the hydrolysis of muscle proteins by cathepsin B may differ from that required to degrade most synthetic substrates, likely due to effects of pH and temperature on substrates.

Cathepsin D

Cathepsin D is a lysosomal aspartic protease of the pepsin family. Its native molecular weight (estimated in skeletal muscles from herring (*Clupea harengus*) to be 38–39 kDa) varies in species and tissues (Nielsen & Nielsen, 2001). Others reported include carp (*Cyprinus carpio*) (41 kDa) (Makinodan, Akasaka, Toyohara, & Ikeda, 1982), and chum salmon (*Oncorhynchus keta*) (50 kDa) (Yamashita & Konagaya, 1992). Unlike the lysosomal cysteine proteases, cathepsin D exhibits a restricted activity on proteins (Barrett & Kirschke, 1981) and can be inhibited by pepstatin. Its catalytic site consists of Asp_{33} and Asp_{231} and is 40–48% homologous with other aspartic proteases such as pepsin, renin, and chymosin (Minarowska, Karwowska, & Gacko, 2009). Hydrophobic amino acid residues are preferential cleavage sites for cathepsin D. Treatment of herring cathepsin D with the bovine β-insulin as a substrate preferentially degraded Leu_{15}, Tyr_{16} and Ala_{14} with the relative ratio 3:2:1 (Nielsen & Nielsen, 2001).

Post-mortem pH and temperature play an important role in the hydrolytic activity of cathepsin D. The enzyme is generally active within the acidic pH range (3.0–5.0). The ultimate post-mortem conditions existing in muscle during normal ageing of meat from mammalian tissues may not be similar to those in fish with reference to cathepsin D activity towards myofibrillar components. Bovine cathepsin D at post-mortem pH (5.1–5.3) induces alteration in Z-disk myofibrils, hydrolyses myosin heavy and light chains, and introduces changes in the troponin-tropomyosin complex (Robbins, Walker, Cohen, & Chatterjee, 1979). In post-mortem mammalian muscle, cathepsin D results in maximal activity against myofibrillar proteolysis at acidic pH (5.5) and higher temperature (45 °C). The enzyme activity decreases considerably at lower temperature and is not detected at 5 °C (Zeece & Katoh, 1989). Its activity is reported to be 1.4 times higher in meat than in fish muscle (Chéret *et al*., 2007). Post-mortem activity of cathepsin D has been detected in the skeletal muscle of carp (Makinodan *et al*., 1982), rainbow trout (Godiksen, Morzel, Hyldig, & Jessen, 2009), sea bass (Chéret *et al*., 2007), and herring muscle (Nielsen & Nielsen, 2001). The ultimate pH of fish muscle is approximately 6.3–6.6 and may not reach low pH optimum for cathepsin D. The enzyme has the capacity to cause structural changes to fish myofibrils at low pH but not above pH 6.0. Thereby cathepsin D is less likely to be involved in post-mortem autolysis of fish myofibrils (Makinodan *et al*., 1982; Yamashita & Konagaya, 1992). Contradictory results reported by Godiksen *et al*. (2009) demonstrated the capacity of cathepsin D to cause structural changes in trout muscle at postmortem pH, probably due to the presence of other cathepsins important for the initial activation of cathepsin D.

Cathepsin H

The molecular weight of cathepsin H purified from rat liver lysosomes was determined to be 28 kDa. The enzyme shows maximum activity at pH 6.0 (Kirschke *et al*., 1977). The activity of cathepsin H could be specifically assayed using the synthetic substrate Arg-MCA. Due to its aminopeptidase activity, cathepsin H releases single amino acids, preferentially large hydrophobics from the NH-terminal end of peptides. Thus, like cathepsin L, cathepsin H generates large peptide fragments requiring further degradation by other proteases (McGrath, 1999). Studies on the purification and identification of cathepsin H in fish and bovine muscles is limited in comparison to cathepsins B, D, and L, probably due to low levels of this enzyme in post-mortem muscle (Chéret *et al*., 2007). Similarly, lower activity of cathepsin H in comparison to that of cathepsin B was detected in muscles from steer carcasses (Wu, Dutson, Valin, Cross, & Smith, 1985). The contribution of cathepsin H to post-mortem softening of fish muscle appears to be negligible (Chéret *et al*., 2007).

Cathepsin L

Endogenous muscle cathepsin L purified from chum salmon (*O. keta*) was characterized with a molecular weight of 30 kDa (Yamashita & Konagaya, 1990d), which agreed well with that isolated from blue scad (*Decapterus maruadsi*) (Zhong *et al*., 2012), silver carp (*Hypophthalmichthys molitrix*) (Liu, Yin, Zhang, Li, & Ma, 2006), and mackerel (*S. australasicus*) (Lee, Chen, & Jiang, 1993). Cathepsin L is highly sensitive towards the synthetic substrate Z-Phe-Arg-MCA (Barrett & Kirschke, 1981). Cathepsin L was maximal at pH 5.0 in mackerel (Lee *et al*., 1993), and silver carp muscles (Liu *et al*., 2006). Activity of cathepsin L was maximal in the pH range (5.0–5.5) in arrowtooth flounder muscle (Visessanguan, Benjakul, & An, 2003), whereas in carp hepatopancreas it was reported at pH 5.5–6.0 (Aranishi, Ogata, Hara, Osatomi, & Ishihara, 1997b). These studies indicate that cathepsin L is optimally active in the pH range (5.0–6.0), above which the activity declines and may be negligible at pH 7.0 (Zhong *et al*., 2012). The optimum temperature for cathepsin L was determined at 50 °C (Aranishi *et al*., 1997b), 55 °C (Zhong *et al*., 2012), and 60 °C (Visessanguan *et al*., 2003), indicating the heat stability of cathepsin L. Differences in optimal pH and temperature conditions for the activity of endogenous enzymes in different fish species may be due to indigenous variations between species and partly, differences in the experimental conditions used for the measurement of activity (Gomez-Guillen & Bautista, 1997).

Cathepsin L has the capacity to digest various proteins. It favours hydrophobic amino acid in the P2 and P3 position (Yamashita & Konagaya, 1990d). It also favours amino acids with short (Ala, Ser) or long, non-branched (Asn, Gln, Lys) side chains. Cathepsin L degrades a wide variety of structural myofibrillar proteins (Table 2). Activity of cathepsin L was four times higher in sea bass muscle than bovine muscle (Chéret *et al*., 2007). It was reported to be responsible for textural changes and degradation of myofibrillar proteins in post-mortem muscle of mackerel (Aoki & Ueno, 1997), chum salmon (Yamashita & Konagaya, 1990b), Pacific whiting (An, Weerasinghe, Seymour, & Morrissey, 1994), and silver carp (Liu *et al*., 2008b). It has also been shown to demonstrate higher hydrolytic potential with regards to the myofibrillar fragment than cathepsin B (Aoki & Ueno, 1997; Liu *et al*., 2008b), probably due to its higher endopeptidase activity (Barrett & Kirschke, 1981).

The caspases

Caspases are a novel family of cysteine proteases activated by the events that initiate apoptosis (Goll, Neti, Mares, & Thompson, 2008). Thus, caspases may be the first active endogenous proteases in post-mortem muscle immediately following animal bleeding (Herrera-Mendez, Becila, Boudjellal, & Ouali, 2006). These proteases cleave many key proteins probably through specific cleavage at Asp residues (Cryns & Yuan, 1998). Their activation results in the proteolysis of many myofibrillar proteins such as titin, nebulin, desmin, troponin I, troponin T, actin, myosin light chain and α-actinin in porcine (Kemp & Parr, 2008), beef (Huang, Huang, Zhou, Xu, & Xue, 2011) and chicken (Huang, Huang, Xue, Xu, & Zhou, 2011) skeletal muscles. In contrast, there is limited knowledge in the literature with regards to the effect of caspases in post-mortem degradation of fish muscle and concomitant softening of flesh.

The proteasome

The 26S proteasome complex is a non-lysosomal protease. It is involved in the breakdown of hormones, antigens, transcription factors, ubiquitin-conjugated or oxidized proteins (Dahlmann & Kuehn, 1995). Fish muscle proteasome is characterized with its strong endoproteases activity in comparison to mammalian muscle (Kinoshita, Toyohara, & Shimizu, 1990). Limited information is however available with respect to its direct role in post-mortem proteolysis of fish myofibrils and associated softening of flesh. In bovine muscle, the proteasome is involved in the proteolysis of nebulin, actin, myosin, tropomyosin and desmin (Robert, Briand, Taylor, & Briand, 1999; Taylor *et al*., 1995b), although these structural changes may be attributed to a sequential effect of calpains and the proteasome (Houbak, Ertbjerg, & Therkildsen, 2008). The proteasome requires activation to induce limited hydrolysis of myofibrillar proteins *in vitro* (Matsuishi & Okitani, 1997), which also indicates that myofibrils are poor substrates for the proteasome (Thomas, Gondoza, Hoffman, Oosthuizen, and Naudé (2004). Up to date, calpains and cathepsins appear to be the proteolytic systems mainly involved in post-mortem breakdown of fish myofibrils, which however should not exclude the role of other endogenous muscle proteases in post-mortem softening of fish flesh.

Major post-mortem structural changes in muscle

Post-mortem storage provides for proteolytic degradation of key myofibrillar and associated proteins important for maintaining the fine structure of muscle. These changes may include the weakening of Z-disks, release of costameres, breakdown of desmin and dystrophin, loss of α-actinin, proteolysis of nebulin, titin and troponin-T, as well as myosin and actin degradation, and tropomyosin delocalization. Collectively, these post-mortem changes lead to fragmentation and weakening of myofibrils, loss in muscle cell integrity and ultimately tenderization of fish muscles during storage (Delbarre-Ladrat *et al*., 2006). A number of structural muscle proteins are modified during post-mortem storage.

The Z-disks

The Z-disks link neighbouring sarcomeres in vertebrate skeletal muscle (Fig. 3). Its integrity is important for the interaction of the actin and myosin filaments of individual sarcomeres (Takahashi, 1996). Due to its localization at the periphery of the Z-disks and at N_2-line region of myofibrils, calpains activity increases the loss in Z-disks integrity and the degree of myofibril fragmentation post-mortem (Raynaud *et al*., 2005; Zeece, Robson, Lee Lusby, & Parrish, 1986). One of the most important components of the Z-disks is α-actinin. It anchors actin filaments to the Z-disks (Fig. 3). The loss and cleavage of α-actinin post-mortem results in the weakening of Z-disks in myofibrils (Papa, Alvarez, Verrez-Bagnis, Fleurence, & Benyamin, 1996). It was reported as early as day one post-harvest in the ordinary muscle of cultured red sea bream suggesting the proteolytic contribution of calpains (Tachibana *et al*., 2001). In their study, Goll, Dayton, Singh, and Robson (1991) reported that calpains released α-actinin from the Z-disks of myofibrils even without weakening the Z-disks or without influencing its ability to bind to actin. In contradiction, Taylor, Geesink, Thompson, Koohmaraie, and Goll (1995a) observed that calpains caused weakening of the thin filament/Z-disk interaction rather than direct Z-disk degradation during the first 72–96 h post-mortem. Proteases other than calpains may also be involved in the degradation of Z-disks. Cathepsin L causes preferential degradation of the Z-disks proteins and disintegration of myofibrils at the Z-disks (Mikami, Whiting, Taylor, Maciewicz, & Etherington, 1987).

CapZ is another protein of the Z-disk involved in anchorage of actin filaments at the Z-disks. It is specifically involved in binding to the barbed ends of actin at the Z-disk of myofibrils (Casella, Craig, Maack, & Brown, 1987). It

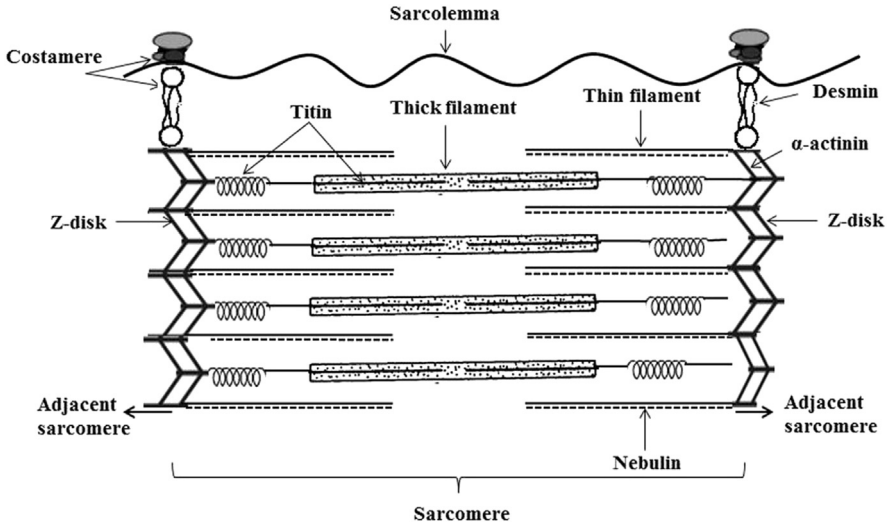

Fig. 3. A schematic diagram of the major myofibrillar component (adapted from (Delbarre-Ladrat *et al.*, 2006)).

has been identified as a myofibrillar substrate for μ-calpain in a study by Lametsch *et al.* (2004) indicating that it may also be involved in post-mortem degradation of the Z-disk.

Costamere and intermediate filaments

Costameres and intermediate filaments anchor structural cell components. Costameres are the cytoskeletal structures connecting peripheral myofibrils to the sarcolemma at the level of Z-disks (Huff-Lonergan & Lonergan, 1999). Proteins constituting the costameres include spectrin, vinculin, α-actinin, dystrophin, talin and filamin. Intermediate filaments consisting primarily of desmin and are involved in linking the myofibrils at the Z-disks levels. Desmin (MW 212 kDa) consists of four subunits having a molecular weight of 53 kDa. It surrounds the Z-disks in skeletal muscle and links adjacent myofibrils at their Z-disks levels (Huff-Lonergan & Lonergan, 1999).

Degradation of dystrophin and desmin is often observed at early stages of fish post-mortem storage. Dystrophin was rapidly lost from 2 to 4 dpm in sea bream muscle and increased the release of the sarcolemma from the myofibrils (Caballero *et al.*, 2009). Bonnal *et al.* (2001) observed the complete loss of dystrophin in sea bass muscle between 2 and 3 days of storage at 4 °C. Calpains activity post-mortem contributes to the degradation of dystrophin and release of products of different molecular weights (Cottin *et al.*, 1992). Similarly, breakdown of desmin increases the gaps between adjacent myofibrils and results in disintegration of myofibrils (Offer & Trinick, 1983). Proteolysis of desmin was detected early during post-mortem storage in sea bass muscle (Delbarre-Ladrat *et al.*, 2004; Verrez-Bagnis *et al.*, 2002), probably due to calpains activation (Delbarre-Ladrat *et al.*, 2004). Calpain-induced degradation of desmin may occur at specific structural sites. Treatment of intact desmin with either m-calpain or μ-calpain resulted in an identical pattern of peptides (Baron, Jacobsen, & Purslow, 2004). The same cleavage sites were also reported in calpain-treated desmin in an earlier study by Croall and DeMartino (1991). Desmin does not possess the PEST domain, yet it was effectively cleaved by calpains suggesting that PEST domain was not necessarily a requirement for calpain-induced proteolysis. Nonetheless, Caballero *et al.* (2009) observed that desmin in sea bream muscle was unaffected before 7 dpm and that it continued to degrade until 14 dpm, probably due to the activity of cathepsins (Lomiwes, Farouk, Wu, & Young, 2014). Cathepsin B was also effective in desmin degradation post-mortem. Unlike calpain, cathepsin B due to its dipeptidyl dipeptidase activity resulted in the cleavage of successive dipeptides from the carboxyl-end of the desmin molecule (Baron *et al.*, 2004). This is in agreement with Lomiwes *et al.* (2014) study that correlated increasing cathepsin B levels in low pH meat with rapid degradation of desmin. Therefore, although calpain activity in high pH muscle may initiate desmin degradation, cathepsins activity may result in desmin proteolysis at later stages of post-mortem storage.

Connectin filaments

Connectin (also called titin) is an elastic myofibrillar protein with a chain weight of approximately 3000–3700 kDa (Huff Lonergan *et al.*, 2010). It accounts for approximately 13% of the total myofibrillar proteins in fish muscle (Seki & Watanabe, 1984). It consists of doublet components, α-connectin (titin 1) and β-connectin (titin 2). It exists as a very thin elastic filament that links the Z-disks at the NH_2-terminal end to the myosin filament at the COOH-terminal region (Fig. 3) (Maruyama, 1997). In living skeletal muscle, titin filaments are responsible for approximately 30% of the total elasticity of myofibrils

(Takahashi & Saito, 1979). Structural changes in connectin were reported during post-mortem storage of fish muscle (Kasamatsu, Kimura, Kagawa, & Hatae, 2004; Mitsuhashi, Kasai, & Hatae, 2002; Seki & Watanabe, 1984). Increasing degradation of connectin post-mortem, results in decreased elasticity of aged muscle and quality changes in fresh fish. In post-mortem fish muscles, connectin was highly sensitive to proteolysis during early stages of post-harvest storage. Approximately 80% of connectin was reduced after 24 h post-mortem storage in fish muscle (Mitsuhashi *et al*., 2002). Storage of squid muscle beyond 7 h post-harvest resulted in reduced α-connectin with concomitant texture softening (Kasamatsu *et al*., 2004).

The splitting of α-connectin occurs specifically at the PEVK segment near N_2-line region of titin, where the filaments are highly elastic (Tanabe, Tatsumi, & Takahashi, 1994). A few research studies reported that elevated levels of calcium ions in post-mortem muscle induced the fragmentation of connectin (Takahashi, Hattori, Tatsumi, & Takai, 1992; Tanabe *et al*., 1994). In contradiction, Kimura, Maki, and Maruyama (1993) reported that post-mortem proteolysis of connectin was induced by calpain rather than the concentration of 0.1 mM $CaCl_2$. Incubation of isolated myofibrils with 0.1 mM calpastatin inhibited fragmentation of connectin. Most studies related connectin degradation during post-mortem storage to meat tenderization. However, Suzuki, Sawaki, Hosaka, Ikarashi, and Nonami (1985) reported no significant change of the content and the electrophoretic pattern of the connectin in chicken skeletal muscle indicated that connectin is unlikely to be responsible for post-mortem decrease in elasticity of muscle and improvement in meat tenderness. A similar study reported no relationship between cod softening and titin degradation during iced storage (Hernández-Herrero *et al*., 2003).

Nebulin filaments

Nebulin is another high molecular weight protein (600–900 kDa) located in the I-band. It accounts for approximately 3–4% of the total myofibrillar proteins. Nebulin filaments attach at one end to the Z line and are in parallel with titin filaments (Fig.3). These filaments may participate in maintenance and regulation of actin filaments (Huff Lonergan *et al*., 2010). Fragmentation of nebulin post-mortem weakens the thin filament and may also modify actin-myosin interactions in post-mortem muscle (Huff Lonergan *et al*., 2010). Like titin filaments, nebulin filaments are also released post-mortem in the presence of free calcium ions (Takahashi *et al*., 1992; Tanabe *et al*., 1994). Calpains may also be responsible for this type of degradation early post-mortem, particularly since calpain activity decreases at later stages of post-mortem storage. Calpains specifically result in the dissociation of the N_2-lines, the area where titin and nebulin filaments coalesce to the Z-disks of myofibrils (Taylor *et al*., 1995a). In fish muscle, the weakening of nebulin begins after 6 h and decreases continuously after 24 h of refrigerated storage (Mitsuhashi *et al*., 2002). Fragmentation of nebulin filaments post-mortem may be initiated by the activation of cysteine proteases and/or by the rise in calcium concentration.

Troponin and tropomyosin

The major regulatory proteins of myofibrils are tropomyosin and troponins (Tn-T, Tn-C, and Tn-I). These proteins associate with actin in the thin filament. Degradation of these proteins post-mortem is associated with the weakening of the thin filament and/or changes within actin and myosin filaments interactions (Goll *et al*., 1998). Calpains are involved in the cleavage of troponin-T in fish (Delbarre-Ladrat *et al*., 2004) and mammalian muscles (Geesink, Taylor, Bekhit, & Bickerstaffe, 2001). It has been reported to cleave rabbit skeletal troponin-T at Thr_{45}-Ala_{46}, Leu_{69}-Met_{70}, Met_{151}-Gly_{152}, Asn_{188}-Ile_{189}, Glu_{220}-Lys_{221}, Lys_{223}-Arg_{224}, Asn_{231}-Val_{232}, Arg_{233}-Ala_{234}, and Ala_{240}-Lys_{241} (Hughes, Geary, Dransfield, McSweeney, & O'Neill, 2001). The loss of troponin-T and the release of polypeptides in the 30 kDa region during ageing improved meat tenderness. It occurs due to the effect of the rise of free sarcoplasmic calcium ion concentration post-mortem on the calpain system rather than the direct effect from calcium on troponin-T (Geesink *et al*., 2001). Cathepsins may also be involved in release of troponin-T at low pH conditions. Cathepsin B contributed to proteolysis of troponin-T (Matsuishi, Matsumoto, Okitani, & Kato, 1992), whereas cathepsin L resulted in the hydrolysis of troponins T and I in carp myofibrils (Yamashita & Konagaya, 1991). Like troponins, release of tropomyosin is common during post-mortem storage of fish muscle. Isolated myofibrils from sea bass muscle treated with endogenous m-calpain resulted in the degradation of tropomyosin from the myofibrillar structure (Verrez-Bagnis *et al*., 2002). On the other hand, Delbarre-Ladrat *et al*. (2004) reported that tropomyosin was unaffected by m-calpain activity but was susceptible to the combined effect of cathepsins B, D and L. Treatment of myofibrils from herring muscle with cathepsin D degraded tropomyosin at pH 4.23 (Nielsen & Nielsen, 2001). Post-mortem degradation of troponin-T and tropomyosin contributed to post-mortem degradation of myofibrils and associated meat tenderization (Delbarre-Ladrat *et al*., 2006).

Actin and myosin filaments

The thick and thin filaments are the two major structural proteins in fish skeletal muscle. These proteins are involved in contraction–relaxation cycle in living and post-mortem skeletal muscle. Thin filaments are a composite filament of actin bound to regulatory proteins tropomyosin and troponin C, T and I and with nebulin (Fig. 3). It is also bound to other proteins including dystrophin, filamin or α-actinin. In post-mortem muscle, degradation of actin

and myosin has been related to softening of meat during ageing storage (Taylor *et al*., 1997).

Actin (approximately 43–48 kDa) is the second abundant myofibrillar protein accounting for about 15–30% of the total muscle protein (Hultin, 1984). It is the primary protein in the thin filaments. In skeletal muscle tissue actin is most likely to exist as fibrous actin (F-actin) (Hultin, 1984). Maintaining the intact structure of actin filament is important to the toughness of fish muscle (Godiksen *et al*., 2009). Minor changes to actin has been reported during post-mortem storage of meat (Lametsch *et al*., 2004). It has remained intact in myofibrils isolated from sea bass muscle when incubated with calpain (Delbarre-Ladrat *et al*., 2004). Similarly, Olson, Parrish, Dayton, and Goll (1977) determined that calpain did not cause any changes to the actin molecule during post-mortem storage of bovine muscle. Furthermore, actin in sea bass muscle was detected as late as 10 dpm (Caballero *et al*., 2009) suggesting that this type of degradation is likely to occur during late post-harvest storage due to the activity of proteases other than calpains. Contradictory to these studies, Lametsch *et al*. (2004) reported the cleavage of actin by μ-calpain. The rate and extent of actin degradation in post-mortem muscle may increase in low pH meat due to cathepsins activity. Cathepsins B, D and L in sea bass muscle resulted in actin proteolysis (Delbarre-Ladrat *et al*., 2004). Cathepsin D activity resulted in actin degradation in herring muscle at pH 4.23 (Nielsen & Nielsen, 2001). Cathepsin B from mackerel muscle caused actin hydrolysis at pH 4.0–5.0, but not structural changes to the actin molecule at pH 5.5–6.5 (Jiang *et al*., 1996). These studies indicate that actin proteolysis is more intense in fish muscle at later stages of post-mortem storage due to the hydrolytic potential of cathepsins.

Myosin is the major constituent of the thick filament representing about 50–60% of the total weight of myofibrillar proteins. Myosin molecule of approximately 470 kDa is made up of two heavy chains (MW 220 kDa) and four light chains (16–26 kDa) (Hultin, 1984). It contributes to the structural integrity of muscle and the tensile strength of meat (Huff Lonergan *et al*., 2010). Myosin proteolysis during cold storage of fish muscle contributes to tissue softening. Calpains are less likely to cause these structural changes (Olson *et al*., 1977). Generally, myosin degradation increases at low pH and may be inhibited at high pH (Dutson, 1983), probably due to release of cathepsins. A correlation between higher levels of cathepsins activity and degradation rate of MHC was reported in a study by Jiang *et al*. (1996). Hydrolysis of MHC was attributed to the activity of cathepsin D in herring muscle (Nielsen & Nielsen, 2001), but in mackerel and salmon muscles to cathepsin L (Jiang *et al*., 1996; Yamashita & Konagaya, 1991). In Pacific whiting muscle, approximately 45% of the original content of MHC was hydrolyzed within 8 days of chilled storage (Benjakul, Seymour, Morrissey, & An, 1997). Cathepsin B activity resulted in the degradation of MHC from mackerel (Jiang *et al*., 1996) and silver carp (Liu, Yin, Li, Zhang, & Ma, 2008a) muscles. Delbarre-Ladrat *et al*. (2004) reported the synergistic effect of cathepsins B, D and L to be responsible for myosin proteolysis in sea bass muscle. Furthermore, myosin degradation reduces the strength and elasticity of surimi gels thereby influencing the textural quality of surimi and surimi products (An, Peters, & Seymour, 1996).

Post-mortem textural changes of fish muscle

Proteolysis of muscle proteins post-mortem is significant to the development of the textural quality of muscle foods during refrigerated storage (Goll *et al*., 1983). Several research studies demonstrated that textural degradation of muscle is clearly influenced by the structural modifications occurring in muscle tissue during post-mortem storage. A significant correlation between the enzymatic activity of cathepsins B and L and muscle degradation was observed in Atlantic salmon (Bahuaud *et al*., 2008). These findings are in agreement with Ayala *et al*. (2010) study that demonstrated that the textural quality of sea bream flesh decreased from pre-rigor until 5-10 dpm simultaneously with the loss of attachment of myofibres from the sarcolemma. Early after death, post-mortem muscle changes result in the loss of the mitochondria and sarcoplasmic reticulum, thereby increasing the intermyofibrillar spaces. During subsequent storage, structural changes observed in the muscle include the enzymatic-induced weakening of the proteins constituting the costamere as well as other major myofibrillar proteins. The calpains and cathepsins act in a complementary way and in synergy to contribute to rapid proteolysis of muscle proteins and associated flesh softening (Ayala *et al*., 2010). Increased softening in sea bream muscle after 4 dpm corresponded to the detachment between myofibres and the myocommata as a result of calpain induced dystrophin degradation (Caballero *et al*., 2009). Similarly, in beef muscle a negative correlation was observed between muscle toughness and initial levels of calpains, and cysteine and serine protease inhibitors (Zamora *et al*., 1996). Higher endogenous activity of calpains post-mortem increased muscle softening with increased degradation of the Z-disks as well as high molecular weight proteins such as troponin, tropomyosin, α-actinin (Yu & Lee, 1986), titin, nebulin, and filamin (Lomiwes *et al*., 2014). The major contractile proteins, actin and myosin are not susceptible to calpains cleavage. Calpains are active during initial days of post-mortem storage in high pH muscle whereas cathepsins activity increases throughout the storage period in low pH muscle. Calpains activity declines with post-mortem time during early period of storage of meat muscle (Li, Xu, & Zhou, 2012), and may become inactive in low pH (5.5–5.8) muscle (Kanawa, Ji, & Takahashi, 2002). The rate and extent of calpain-induced myofibrillar proteolysis may differ from species depending on the post-mortem conditions (Ahmed, Donkor, Street, & Vasiljevic, 2013b). The relative proportion of calpain to calpastatin activity

may be more important with respect to the tenderization of fish muscles than calpain activity alone. Calpastatin activity was approximately 4 folds higher in sea bass than in bovine muscle, although similar levels of calpain activities were detected in both muscles (Chéret *et al*., 2007). Similarly, the calpain to calpastatin ratio in rainbow trout muscle was approximately 1:3 (Saito, Li, Thompson, Kunisaki, & Goll, 2007). This suggests that high calpastatin-to-calpain ratio detected in fish muscles reduces the activity of calpain and inhibits the rate and extent of myofibrillar proteolysis post-mortem associated with rapid textural softening. Nonetheless, Geesink and Koohmaraie (1999) reported that high level of calpastatin activity may not completely inhibit calpains activity. These findings implicate a secondary role for calpains in myofibrillar fragmentation and tenderization of fish muscle post-mortem (Chéret *et al*., 2007).

Cathepsins play a significant role in the tenderization of muscle taking place at later stages of post-mortem meat conditioning. In salmon muscle, a significant increase in cathepsin activity was observed with time post-mortem (Gaarder *et al*., 2012). These high amounts of cathepsins activity are detrimental to the texture of fish fillets (Bahuaud, Gaarder, Veiseth-Kent, & Thomassen, 2010). The study found a direct association between cathepsin L activity and fillet firmness. Cathepsins B and L contributed to the degradation of mackerel surimi and associated gel softening during surimi setting (Ho, Chen, & Jiang, 2000). Cathepsins B, D, or L degraded MHC, α-actinin, desmin, actin, troponin-T and tropomyosin in sea bass muscle (Ladrat *et al*., 2003). The same proteins were also degraded when myofibrils were hydrolysed first with m-calpain following further hydrolysis by commercial cathepsins mixture (cathepsins B, D, and L) in sea bass muscle (Delbarre-Ladrat *et al*., 2004), indicating that calpains are not involved in proteolysis of myofibrils post-mortem. Cathepsins are specific to actin and myosin proteolysis in post-mortem muscle.

Biological properties of fish-derived peptides

In addition to developing meat tenderness, proteolytic degradation of proteins may also contribute to the release of bioactive peptides in fish muscle (Samaranayaka, Kitts, & Li-Chan, 2010). Digestion of fish proteins *in vivo* has been associated with important physiological activities including cholesterol-lowering (Wergedahl *et al*., 2004), enhanced satiation (Bougatef *et al*., 2010), antihypertensive (Ait-Yahia *et al*., 2003), and antihyperglycemic effects (Ouellet, Marois, Weisnagel, & Jacques, 2007). In addition, *in vivo* immune-modulating properties of fermented fish protein powders has also been reported (Duarte, Vinderola, Ritz, Perdigón, & Matar, 2006). Similarly, hydrolysis of fish proteins *in vitro* resulted in the release of peptides with a range of physiologically important activities on the cardiovascular, gastrointestinal, immune, and nervous systems to modulate human health (Table 3). The bioactive sequences encrypted within a polypeptide chain are responsible for the biological properties of fish and fish products. Bioactive peptides are released from their precursor protein following enzymatic hydrolysis of fish proteins in the gastrointestinal (GIT) tract and/or food processing. In the GIT tract and upon consumption, bioactive peptides may be partially or totally resistant to hydrolysis by human digestive enzymes and either: (1) produce local effects directly in the GIT tract, or (2) are transported into the bloodstream to exert physiological activities at the site of action (Shahidi & Zhong, 2008).

In addition, peptides may be released during processing of fish muscle post-mortem and are thus consumed as food components (Meisel & Bockelmann, 1999). Mechanism responsible for the release of bioactive peptides from fish muscle may be related to the hydrolytic action of: (1) endogenous muscle proteases on muscle proteins, and/or (2) exoproteases on protein fragments and polypeptides. During initial days of post-mortem storage, muscle endogenous calpains and cathepsins B, D, H and L hydrolyse major myofibrillar proteins to release a range of polypeptide fragments and consequently improve meat tenderness. The later stages of proteolytic degradation involve the action of muscle exoproteases, aminopeptidases and peptidases, which further break down protein fragments and polypeptides to release a great number of small peptides and free amino acids responsible for flavour development in meat (Fig. 4). The rate of protein degradation during storage may vary depending on processing condition, type of muscle and the level of active endogenous muscle enzymes (Toldrá, 2008). Muscle exoproteases may contribute to the release of small peptides exhibiting bioactive sequences. However less is documented in the literature about the role these proteases play in improving the bioactive properties of peptides released during post-mortem storage and processing of meat. Recent research studies reported the contribution of endogenous muscle enzymes to the production of bioactive fish protein hydrolysates (Table 3). Activation of endogenous muscle proteases post-mortem causes limited breakdown of fish muscle proteins and releases a mixture of polypeptides and oligopeptides. Human digestive enzymes may further hydrolyse these fragments to produce a range of small peptides possessing important physiological properties (Manikkam, Vasiljevic, Donkor, & Mathai, 2015; Meisel & Bockelmann, 1999). Short peptides (<2000 Da) enriched with proline residues demonstrated bioactive properties *in vivo* (Bauchart *et al*., 2007).

Physiological properties of peptides released during post-mortem storage of fish muscle may be influenced by factors including species, type of proteases present, the level of enzymatic activities and the storage conditions (Medenieks & Vasiljevic, 2008). Fish species significantly influenced the activity of endogenous calpains and cathepsins (Ahmed, Donkor, Street, & Vasiljevic, 2013a). Calpains and cathepsins have different specificity on muscle proteins and release a number of polypeptides and

Table 3. Documented bioactive properties of FPHs.

Marine protein	Protease(s)	Biological activities	Beneficial effect	Reference
Sardine (*S. pilchardus*) processing waste	Alcalase®	Secretagogue[a] Growth factor-like CGRP-like	Cardiovascular system regulation through vasodilation Inhibition of cell proliferation	Ravallec-Plé *et al.* (2001)
Cod (*G. morhua*) muscle	Alcalase 2.4L	Secretagogue Growth factor-like	Protein synthesis stimulation Control intestinal mobility Release of digestive enzymes	Ravallec-Plé, Gilmartin, Van Wormhoudt, and Le Gal (2000)
Pacific hake (*M. productus*) muscle	Endogenous	Antioxidative	Retard protein and lipid oxidation	Samaranayaka and Li-Chan (2008)
Tuna cooking juice	Protease XXIII (PR)/Orientase (OR)	DPP-IV inhibition	Antihyperglycemic	Huang, Jao, Ho, and Hsu (2012)
By-products and by-catch	Controlled hydrolysis	Antiproliferative	Anticancerogenic	Picot *et al.* (2006)
E. volitans backbone	Papain, Pepsin, or Trypsin	Antiproliferative Antioxidant Antimicrobial	Antimicrobial peptides contribute to innate immunity	Naqash and Nazeer (2011)
Mackerel (*S. austriasiaus*) muscle	Protease N./Endogenous	Antioxidative	Prevent protein & lipid oxidation	Wu, Chen, and Shiau (2003)
Sardinelle (*S. aurita*) by-products	Endogenous	ACE inhibition	Prevention &/or treatment of hypertension	Bougatef *et al.* (2008)
Pacific hake (*M. productus*) muscle	Endogenous	Antioxidative	Retard protein & lipid oxidation	Samaranayaka *et al.* (2010)
Blue whiting (*M. poutassou*) and brown shrimp (*P. aztecus*)	Controlled hydrolysis	CCK release	Short-term satiation effect & weight control	Cudennec, Ravallec-Plé, Courois, and Fouchereau-Peron (2008)
Smooth hound (*M. mustelus*) muscle	Endogenous	Satiety-enhancing	Suppressed appetite & reduced weight	Bougatef *et al.* (2010)
Flounder (*P. olivaceus*) muscle	α-chymotrypsin	Antioxidative	Retard protein & lipid oxidation	Ko, Lee, Samarakoon, Kim, and Jeon (2013)
Tilapia protein isolates	Flavourzyme/Cryotin	ACE inhibition	Antihypertensive effect	Raghavan and Kristinsson (2009)
Australian salmon, Barracouta, & Silver warehou	Endogenous	ACE inhibition α-amylase inhibition Trypsin inhibition	Diabetes preventions Stimulation of CCK release	Medenieks and Vasiljevic (2008)

[a] Secretagogue—gastrin-and cholecystokinin-like, CGRP—calcitonin gene-related peptide, DPP-IV—Dipeptidyl-peptidase IV, ACE—angiotensin-I- converting enzyme, CCK — cholecystokinin.

oligopeptides. The concentration of active endogenous enzymes acting on myofibrils post-mortem may influence the rate and extent of muscle proteolysis. Raghavan and Kristinsson (2009) reported the improvement of the biological activities of fish protein hydrolysates (FPHs) with increasing rate of myofibrillar proteolysis. Similarly, the presence of highly active endogenous proteases in Pacific hake muscle enhanced its bioactive properties (Samaranayaka & Li-Chan, 2008). These studies postulate that important levels of endogenous calpains and cathepsins activity in post-mortem muscle may lead to higher peptide content in the hydrolysate and ultimately improve the biological activities of released peptides (Wu *et al.*, 2003). Storage conditions influenced the proteolytic activities of calpains, cathepsins in fish species which may also affect the release of bioactive peptides during processing (Ahmed *et al.*, 2013b). Although increased activity of endogenous calpains and cathepsins may contribute to the release of bioactive peptides in fish muscle, it may also accelerate the softening of fish tissue. Future research work should identify the fish species demonstrating bioactive properties during processing and storage. Further research should also examine the bioactive properties of peptides released during autolysis of fish muscle post-mortem.

Conclusions

Post-mortem proteolysis of structural muscle proteins in fish contributes to muscle softening during refrigerated storage. Calpains initiate limited hydrolysis of high molecular weight proteins early post-mortem. Activity of cathepsins results in the degradation of low molecular weight proteins during late post-mortem storage of muscle. Ultimately proteolysis of fish myofibrils post-mortem increases the release of polypeptide fragments and oligopeptides. Some of these peptides may demonstrate one or more

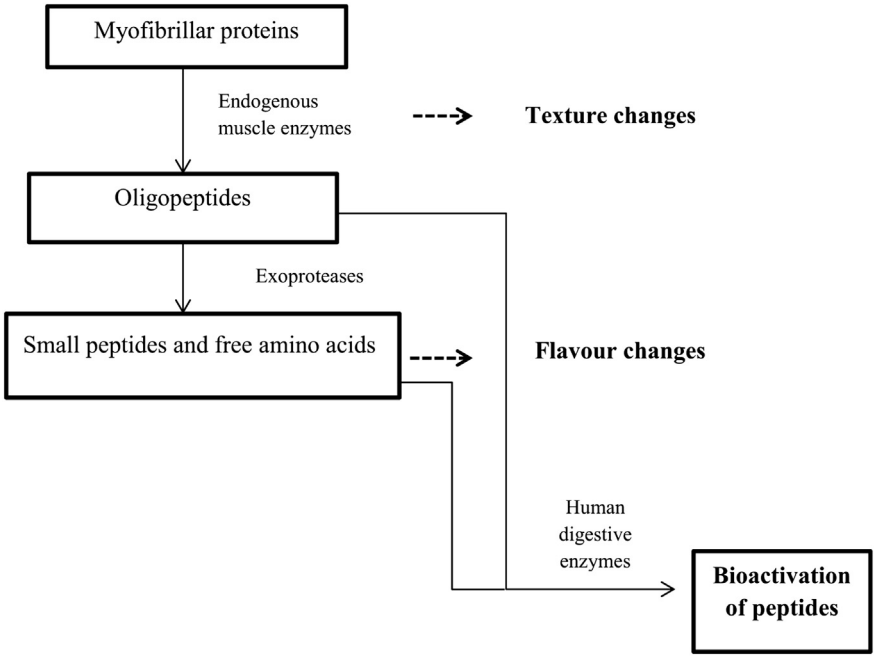

Fig. 4. Schematic representation of the post-mortem proteolysis of myofibrillar proteins and the release of bioactive peptides in meat and meat products (adapted from (Toldrá, 2008)).

bioactive properties important for modulating human health. Future studies should study the relationship between the hydrolysis of myofibrillar proteins post-mortem and the release of potent peptides with important physiological properties upon consumption of fish fillets.

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CHAPTER 3

Proteolytic activities in fillets of selected underutilized Australian fish species

This chapter has been published as a research paper entitled "**Proteolytic activities in fillets of selected underutilized Australian fish species**" by Ahmed, Z., Donkor, O., Street, W. A. and Vasiljevic, T. in the peer reviewed journal, Food Chemistry, 140, 238-244 (2013).

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Proteolytic activities in fillets of selected underutilized Australian fish species

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ABSTRACT

The hydrolytic activity of major endogenous proteases, responsible for proteolysis of myofibrillar proteins during *post-mortem* storage, may be an indicator of the textural quality of fish which influences consumer purchasing behaviour and thus market value of the final product. Furthermore, it may also influence the type and bioactive properties of the peptides released during *post-mortem* proteolysis of myofibrillar proteins. This study compared the activities of cathepsins B, B+L, D, H and calpain-like enzymes in crude muscle extracted from 16 Australian underutilized fish species. Fish species had a significant effect on the activity of these enzymes with barracouta showing the highest cathepsins B, B+L, D and H activities. Activities of cathepsins B and B+L were higher than cathepsin H for all studied species. The more commercially important rock ling and tiger flathead demonstrated higher cathepsin B+L activity, whereas gemfish and eastern school whiting showed higher activity towards cathepsin B. Underutilized fish species showing higher endogenous protease activities may be suitable for fish sauce production, whereas those with lower protease activities for surimi processing.

1. Introduction

The world's fisheries production increased from 16.8 million tonnes in 1950 to 86.4 million tonnes in 1996, and then declined to 77.4 million tonnes in 2010. The stocks of many traditional marine species have diminished worldwide over the last few years and the proportion of overexploited marine stocks increased, which demonstrates that the state of world marine fisheries is worsening (Food and Agriculture Organization of the United Nations (FAO), 2012). Furthermore, a rise in population and improved consumer awareness towards the health benefits of seafood increased the demand for fish and fishery products (He, Chen, Sun, Zhang, & Gao, 2006). Nonetheless, while target fish species continue to be exploited, many non-target species of almost no commercial interest still remain underutilized (Huang & Liu, 2010). Majority of these underutilized species are discarded due to certain technological inconveniences, which include poor sensory quality and/or functional attributes, making a large number of these species mainly utilised for animal feed and/or fertilizers (Geirsdottir et al., 2011; Mazorra-Manzano, Pacheco-Aguilar, Ramirez-Suarez, & García-Sánchez, 2008). The Food and Agriculture Organization of the United Nations (FAO) estimates that about 7.3 million tonnes of fish was discarded annually by marine fisheries throughout the world in the 1992–2001 period (Zeller & Pauly, 2005). Discarded marine species create a substantial waste of valuable living resources, which may be better utilised for human consumption, for economic and environmental reasons. For instance, undervalued species may be formulated and used to develop various high value-added seafood products, particularly since some of these species have nutritional (He et al., 2006), physiological (Medenieks & Vasiljevic, 2008) and functional properties (Geirsdottir et al., 2011) which may be comparable to those of traditional target species.

Enzymatic hydrolysis of fish proteins may be used to improve utilization of currently available underutilized fish resources for human consumption. The hydrolytic process releases a mixture of peptides depending on the enzyme specificity and the extent of protein hydrolysis (Geirsdottir et al., 2011). It involves using endogenous digestive enzymes and/or exogenous enzymes to hydrolyse specific peptide bonds in the protein molecule (Khalil, Metwalli, & El-Sebaiy, 1987) producing protein hydrolysates with enhanced physiological and/or functional properties. Higher autolytic activity of major muscle endogenous proteases induces hydrolysis of key myofibrillar proteins, and thus contributes to weakening of the myofibril structure during *post-mortem* storage. This accelerates textural deterioration in fish. The main proteolytic systems involved in hydrolysis of myofibrillar proteins during *post-mortem* storage of meat from mammalian and fish muscles are the cytoplasmic calpains and the lysosomal cathepsins (Yamashita & Konagaya, 1991). Calpains are optimally active at neutral pH, whereas cathepsins are optimally active in acidic environments

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(Stagg, Amato, Giesbrecht, & Lanier, 2012). The main cathepsins involved in proteolysis of key myofibrillar proteins are cathepsins B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15), cathepsin H (EC 3.4.22.16) and cathepsin D (EC 3.4.22.5) (Aoki & Ueno, 1997). Generally, higher activity of endogenous muscle proteases during initial days of refrigerated storage may be an indicator of texture-associated degradation (Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004).

The contribution of proteolysis to textural properties of fish fillets during iced storage has been widely studied, but there exists a gap in knowledge regarding products of proteolysis during *post-mortem* ageing or storage and cooking, especially small peptides (Bauchart et al., 2007) which may possess different biological effects *in vitro* and *in vivo*. Proteolysis of fish proteins is an important process to liberate peptides and enhance the bioactivity of the protein source (Korhonen & Pihlanto, 2003). Peptides can be present in fish muscle *intra vitam* (e.g. anserine, glutathione) and/or could be generated during *post-mortem* storage via proteolytic digestion of fish myofibrillar proteins (Bauchart et al., 2007). Endogenous muscle proteases (cathepsins B, D, H, and L, and calpains) may initially act on myofibrillar proteins during meat tenderization process to produce a great number of oligopeptides (Arihara, 2006), small peptides and free amino acids (Toldrá and Flores, 1998). In addition, enzymatic digestion of fish muscle was reported to release peptides with improved bioactivity (Bauchart et al., 2007; Hasan et al., 2006; Samaranayaka, Kitts, & Li-Chan, 2010). Fish protein hydrolysates were reported to release peptides that exhibit antioxidative (Je, Qian, Byun, & Kim, 2007; Ovissipour et al., 2012; Wu, Chen, & Shiau, 2003), anti-cancer effects (Picot et al., 2006) and have the capacity to lower plasma cholesterol level (Wergedahl et al., 2004). These studies indicate that fish proteins may be partially degraded during *post-mortem* storage by endogenous muscle proteases releasing a mixture of polypeptides and oligopeptides which, due to the action of human digestive enzymes upon oral administration, may generate a range of small peptides that may possess physiologically important activities. Limited information is available in the literature on the physiological benefits upon gastrointestinal digestion of fresh fish as a whole meal. Thus, the results from the current study may pave the way for future studies in examining the feasibility of fish autolytic process as a means to release bioactive peptides, and improve the value of underutilized fish species.

Medenieks and Vasiljevic (2008) assessed the bioactive potential of three underutilized Australian fish species: silver warehou, barracouta and Australian salmon by *in vitro* assays. Processing conditions and seasonal variations affected the peptide profiles, and consequently the biological activity of the peptides released. Fewer peptides were produced following simulated gastrointestinal digestion of fish harvested in winter than those harvested in summer. The higher proteolytic activities of endogenous enzymes in the summer-harvested species contributed to greater release of polypeptide products and oligopeptides. The study demonstrated that the physiological properties of bioactive peptides released from myofibrillar proteins of these underutilized fish species, was affected by the fish species and most importantly catch season, which may have been influenced by the activity of endogenous proteases. Therefore, the proteolytic activity of major muscle endogenous proteases may affect the biological properties of the peptides released, as a result of proteolytic degradation of myofibrillar proteins from underutilized fish species. Other factors that influence the bioactive properties of the peptides released include the type of protease(s) used, processing conditions and the molecular size of the resulting peptides, which greatly affects their absorption across the gastrointestinal tract and bioavailability in target tissues (Udenigwe and Aluko, 2012). Freshness of the raw material is a critical parameter during processing of seafood products, for example, raw materials showing minimum *post-mortem* deterioration effects are selected for surimi processing (Martín-Sánchez, Navarro, Pérez-Álvarez, & Kuri, 2009). The use of underutilized Australian fish species as fresh fillets or as ingredients to incorporate into value-added seafood products may be restricted. This could be due to the lack of knowledge on the activity of the major proteases which participate to a different extent to the degradation of myofibrillar proteins and thus, influence the bioactive properties of the proteolytic products released. Therefore, the objective of this study was to establish the proteolytic activity of cathepsins B, D, H, and L as well as calpain-like enzymes in sixteen underutilized Australian fish species. This knowledge may be particularly useful when considering the feasibility of using these species as raw materials, during processing of different seafood products, that may be dependent on their endogenous protease activities to improve their commercial value.

2. Materials & methods

2.1. Materials

Underutilized Australian fish species used for this study included Australian salmon (*Arripis trutta*), barracouta (*Thyrsites atun*), bight redfish (*Centroberyx gerrardi*), deepwater flathead (*Neoplatycephalus conatus*), eastern school whiting (*Sillago flindersi*), gemfish (*Rexea solandri*), jackass morwong (*Nemadactylus macropterus*), mirror dory (*Zenopsis nebulos*), nannygai (*Centroberyx affinis*), ocean perch (*Helicolenus barathri*), ribbon fish (*Lepidopus caudatus*), rock ling (*Genypterus tigerinus*), silver trevally (*Pseudocaranx dentex*), silver warehou (*Seriolella punctata*), tiger flathead (*Neoplatycephalus richardsoni*) and yellowspotted boarfish (*Paristiopterus gallipavo*) (Yearsley, Last, & Ward, 1999). The samples were kindly supplied by Barwon Foods (Geelong North, VIC, Australia) and obtained through their supply chain. Fish were delivered fresh within 24 h from catch to our laboratory and stored on ice in a cold room. The samples were processed immediately upon receipt, usually within a couple of hours. Each species of fish was obtained on two separate occasions and assessed individually forming the two replicates for each species.

2.2. Assay of enzymatic activities

2.2.1. Activity measurements of calpain

The fish were filleted and minced for 30–60 s on ice using a food processor. Crude extract for calpain-like activity assay was obtained by homogenising the minced fish (30 g in 75 ml cold extraction buffer) in Tris-buffered saline (100 mM Tris–HCl, 145 mM sodium chloride (NaCl), 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.3) for 1 min on ice. The homogenate was centrifuged (Beckman J2-HS centrifuge, JA-20 rotor, Palo Alto, CA, USA) at 17,000×*g* for 45 min at 4 °C. The supernatant was filtered (Whatman, Qualitative 185 mm, # 1001185, Maidstone, UK), preserved with 5% trehalose and stored at −20 °C until further analysis.

In this study, the enzymes obtained in the crude extracts were not purified. Thus, the detected activity in the assay for calpain is reported as calpain-like (CA-like). The activity determination of calpain-like enzyme was performed using L-Methionine 7-amido-4-methylcoumarin trifluoroacetate salt from Sigma–Aldrich (St. Louis, MO, USA) as the substrate (Lakshmanan, Patterson, & Piggott, 2005) with slight modifications. Enzyme extract (1.0 ml) and 1.0 ml of substrate solution (0.125 mM L-Methionine-7-amido-4-methylcoumarin triflouroacetic salt in 100 mM Bis–Tris, 5 mM calcium chloride ($CaCl_2$), pH 6.5) were incubated for 10 min at 25 °C. By adding 2.0 ml of stopping reagent, consisting of 100 mM sodium monochloroacetate, 70 mM acetic acid and 30 mM sodium acetate at pH 4.3, the reaction was arrested. A

sample blank was prepared by adding calpain extraction buffer instead of the crude extract to the reaction mixture and was used to zero a Shimadzu spectrophotofluorometer (model RF-5301, Kyoto, Japan). Increase in fluorescence intensity of the mixture was measured at an emission wavelength of 510 nm after excitation at 350 nm. One unit of CA-like activity was defined as the amount of enzyme causing an increase in fluorescence intensity per min. CA-like activity was expressed in FU (units of fluorescence) per minute per mg of protein in extract.

2.2.2. Activity measurement of lysosomal enzymes

Minced fish (30 g) was homogenised in 75.0 ml cold extraction buffer (50 mM sodium acetate buffer pH 5.0 containing 1 mM EDTA, and 0.2% v/v Triton X-100) at 4 °C for 1 min. The procedure was repeated for all cathepsin crude extracts. The homogenate was removed and centrifuged at 17,000×*g* for 45 mins at 4 °C. The supernatant was filtered and stored at -20 °C prior to analysis. The activity of cathepsins was directly measured in the crude extract using specific synthetic substrates.

Cathepsin B (CB), Cathepsin B and L (CB+L), and cathepsin H (CH) L activities were measured according to Hagen, Solberg, and Johnston (2008) with slight modifications. *Z*-Arg-Arg-7-amido-4-methylcoumarin hydrochloride, *Z*-Phe-Arg-7-amido-4-methylcoumarin hydrochloride, and L-arginine-7-amido-4-methylcoumarin hydrochloride (Sigma–Aldrich) were used as the substrates for CB, CB+L, and CH, respectively. Since *Z*-Phe-Arg-7-amido-4-methylcoumarin hydrochloride is hydrolysed by both cathepsins B and L, the activity of this substrate was reported as cathepsin B+L (Porter, Koury, & Stone, 1996). In all assays, incubation time was 10 min and the release of 7-amino-4-methylcoumarin (AMC) was measured with an excitation wavelength set at 350 nm and an emission wavelength of 510 nm. A standard curve was constructed with known concentrations of AMC (Sigma–Aldrich) in the stop buffer.

Enzyme activation was performed by incubating 0.5 ml of extract and 1.25 ml of assay buffer for 2 min at 30 °C, before adding 0.25 ml of 20 μM substrate solution. The activation buffers used for CB and CH contained 200 mM sodium phosphate, 2 mM EDTA, 0.05% (w/v) 3-(3(cholamidopropyl)-dimethyl-ammonio)-1-propanesulfonate (CHAPS) (G-Biosciences, St. Louis, USA), and 4 mM dithiothreitol (DTT) (Sigma–Aldrich) of pH 6.0 and 6.6 respectively, and that for CB+L of pH 6.0 contained 200 mM sodium acetate, 2 mM EDTA, 0.05% CHAPS and 4 mM DTT. The reaction was halted after 10 min by adding 2.0 ml of stopping reagent pH 4.3 before measuring the fluorescence intensity of the resulting solution. One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolyses the substrate and releases 1 μM AMC per min at 30 °C. Cathepsins activities were expressed as U/mg of protein. Fluorescence was measured by zeroing the instrument against a sample blank which contained the extraction buffer instead of the crude extract.

Cathepsin D (CD) activity was estimated using acid-denatured bovine haemoglobin (Sigma–Aldrich) as the substrate using the method described previously (Minarowska, Karwowska, & Gacko, 2009) with slight modifications. Briefly, the enzymatic assay involved incubation of 0.1 ml of 2.5% (w/v) acid-denatured haemoglobin (pH 3.5) and 0.4 ml of sarcoplasmic protein extract at 37 °C. The reaction was stopped after 3 h incubation by adding 0.5 ml of 5% trichloroacetic acid (TCA) containing 2.5 M urea, and the absorbance of the solution was measured spectrophotometrically (Bio-Rad SmartSpec™ Plus Spectrophotometer, Bio-Rad Laboratories, CA, USA) at 750 nm. The sample blank was prepared by adding the enzyme solution at the end of the reaction immediately following TCA addition. The activity of CD was calculated by the difference in absorbance of the enzyme sample and the blank. One unit of CD enzymatic activity was defined as an increase of absorbance per hour per mg of protein at 37 °C.

2.3. Assay for determination of protein concentration

The protein content of supernatants was determined by Bradford assay (Bradford, 1976), using Bio-Rad protein reagent (Bio-Rad Laboratories) and bovine serum albumin as the standard. Absorbance was read at 595 nm using a Bio-Rad iMark microplate absorbance reader (10094, Bio-Rad Laboratories, Tokyo, Japan). The values were means of three measurements for each sample.

2.4. Statistical analysis

Results were analysed using a General Linear Model (GLM) procedure of the Statistical Analysis System (SAS) with fish species and replications as the main factors. The effect of fish species on the activity of proteolytic enzymes was considered to be significant at $P \leqslant 0.05$.

3. Results and discussion

3.1. Activity of calpains

The hydrolytic activity of calpain-like enzyme varied among the underutilized fish species. In our study, examined fish species had a significant effect on the activity of calpain-like enzyme ($P < 0.05$). Fig. 1 illustrates the activity of calpain-like enzyme among the sixteen underutilized Australian fish species. Silver trevally was observed to exhibit the highest endogenous activity of calpain-like enzyme followed by mirror dory, jackass morwong and eastern school whiting. Higher calpain-like activity observed by these species may cause rapid softening during chilled storage of fillets, which may affect their textural quality and thus be one of the reasons for their underutilization. Nontheless, high endogenous activity of calpain-like enzyme may also indicate that more proteolytic peptides are released as a result of *post-mortem* proteolysis. Calpains are thought to cause the weakening and disintegration of the Z-line of fish myofibrils (Lakshmanan et al., 2005), and therefore cause limited proteolysis of myofibrillar proteins, making them susceptible to further degradation by other proteases (Delbarre-Ladrat et al., 2004; Hultmann & Rustad, 2007). As a result, controlling the activity of calpain to a minimum during handling and *post-mortem* storage may improve the textural quality of fish species characterised with high endogenous calpain activity.

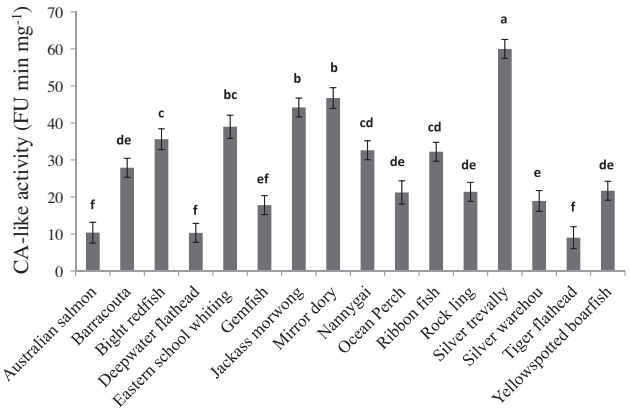

Fig. 1. Calpain-like activity among 16 underutilized Australian fish species. Results are expressed as mean of three observations. The error bars present standard error of the mean (SEM), different annotated letters indicate a significant difference ($p < 0.05$).

Calpains play an important role in the degradation of myofibrillar proteins during the initial 24 h *post-mortem* (Sárraga, Gil, & García-Regueiro, 1993). Calpains are classified into μ-calpain and m-calpain, which differ in sensitivity to calcium ions required upon activation. Calpastatin is the endogenous specific inhibitor of calpain (Chéret, Delbarre-Ladrat, Lamballerie-Anton, & Verrez-Bagnis, 2007). Often, the inhibition potential of calpastatin is greater in fish muscle than mammalian muscles. Chéret and others (2007) reported that the ratio of calpastatin/calpain was 3.6 times higher in sea bass white muscle than in bovine muscle, despite the similarity of calpain contents in fish and meat muscles. Similarly, calpain activity was lower in salmon than mammalian muscle, although calpastatin activity was relatively high in both muscles (Geesink, Morton, Kent, & Bickerstaffe, 2000). These studies suggest that calpain may play a secondary role in *post-mortem* softening of fish flesh when compared to other endogenous muscle proteases, such as cathepsins B and L, due to the high inhibitor/enzyme ratio commonly observed in fish muscle when compared to mammalian muscle. Eastern school whiting, gemfish, rock ling and tiger flathead (Smith & Wayte, 2005) have more commercial value than the other examined underutilized fish species. In our study, eastern school whiting and rock ling exhibited higher calpain-like activity than gemfish and tiger flathead, which may have detrimental effects on textural quality and thus consumer acceptance of these undervalued species. In contrast, the higher calpain-like activity examined in these species may lead to the generation of more peptides with various biological activities. Further studies may be undertaken to assess the contribution of calpain to *post-mortem* proteolysis of fish muscle, and its effect on the textural quality and physiological properties of eastern school whiting, gemfish, rock ling and tiger flathead, or any other species of commercial interest, and establish processing conditions that control calpain activity during initial days in refrigerated storage, to maintain their textural quality and bioactive properties during cold storage to further improve their commercial value.

3.2. Activity of lysosomal enzymes

Comparison of the activity of cathepsin B in the crude enzymatic extracts among the sixteen underutilized Australian fish species is shown in Fig. 2. All examined species had a significant effect on the endogenous activity of CB ($P < 0.05$). Barracouta, silver trevally, gemfish and jackass morwong demonstrated higher endogenous activity of cathepsin B. In contrast, rock ling did not have a significant effect on the endogenous activity of CB

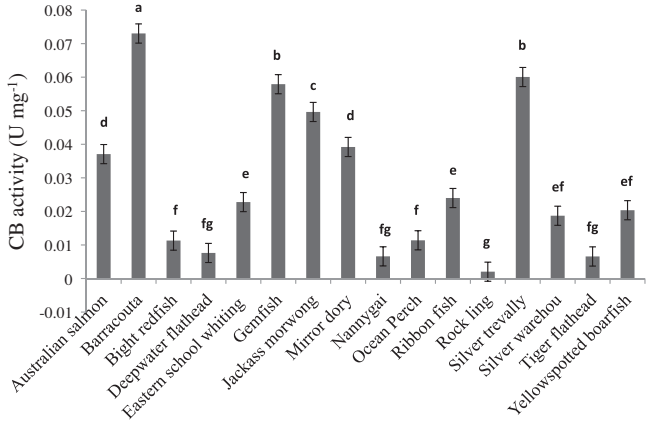

Fig. 2. Cathepsin B activity among 16 underutilized Australian fish species. Results are expressed as mean of three observations. The error bars present SEM, different annotated letters indicate a significant difference ($p < 0.05$).

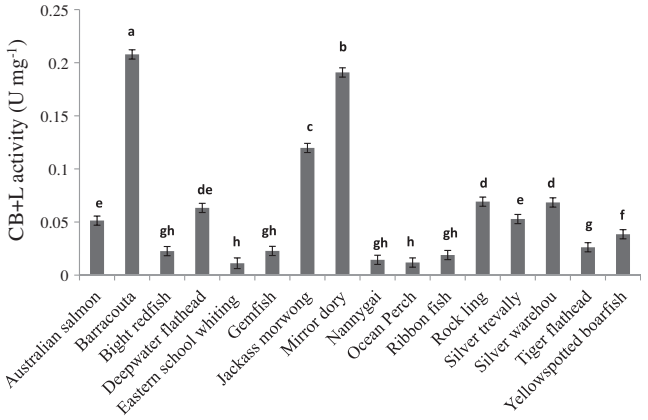

Fig. 3. Cathepsin B+L activity among 16 underutilized Australian fish species. Results are expressed as mean of three observations. The error bars present SEM, different annotated letters indicate a significant difference ($p < 0.05$).

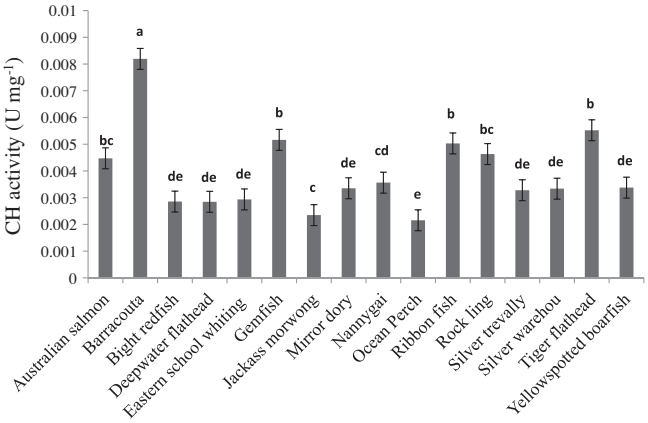

Fig. 4. Cathepsin H activity among 16 underutilized Australian fish species. Results are expressed as mean of three observations. The error bars present SEM, different annotated letters indicate a significant difference ($p < 0.05$).

($P > 0.05$). Fig. 3 illustrates the activity of CB+L in underutilized Australian fish species used for this study. Overall, examined species again had a significant effect on the endogenous activity of cathepsin B+L ($P < 0.05$). The highest level of its activity was observed in barracouta followed by mirror dory and jackass morwong, whereas ocean perch and eastern school whiting demonstrated lower activity. The endogenous activity of cathepsin H has been measured in the examined sixteen underutilized fish species (Fig. 4). Barracouta, tiger flathead, gemfish, ribbon fish, rock ling, and Australian salmon demonstrated higher proteolytic activity of cathepsin H. Selected species used in our study and replication, had a significant effect on the activity of CH ($P < 0.05$). Cathepsin D activity was estimated among the sixteen underutilized fish species (Fig. 5). Barracouta, followed by ribbon fish, Australian salmon and silver warehou were observed to contain higher activity of endogenous cathepsin D when compared to the other examined species. Overall, species and replication had a significant effect on the activity of cathepsin D ($P < 0.05$).

Comparison of the activities of cathepsins B, B+L, and H among the studied underutilized fish species is shown in Fig. 6. Activity of endogenous CH was the lowest in the crude enzymatic extracts obtained from the examined species. Wang and Xiong (1999) reported that cathepsin H was unlikely to cause gel weakening of beef heart surimi, due to its low activity in the crude enzymatic mixture when compared to cathepsins B and L. Cathepsin D may

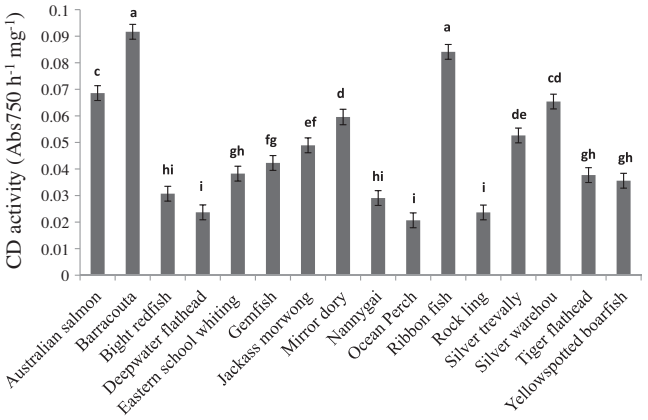

Fig. 5. Cathepsin D activity among 16 underutilized Australian fish species. Results are expressed as mean of three observations. The error bars present SEM, different annotated letters indicate a significant difference ($p < 0.05$).

also play a less significant role in proteolysis of fish proteins, since it hydrolyses myofibrils at an optimum pH, generally between 3 and 4 (Makinodan, Akasaka, Toyohara, & Ikeda, 1982). Furthermore, Porter and co-workers (Porter et al., 1996) reported low levels of cathepsin D activity in all four species of Pacific fish. Therefore, proteolytic activity of cathepsin D and cathepsin H may be less detrimental to the textural quality, and the extent of proteolytic degradation, of underutilized fish species when compared to cathepsins B and/or L.

Post-mortem textural deterioration of fish fillets is also due to the endogenous autolytic activity of cathepsins B and L (Chéret et al., 2007; Yamashita & Konagaya, 1991). Cheret and others (2007) determined that the activity of cathepsins B and B+L was 29.7 and 4.7 times higher in sea bass muscle than bovine muscle, respectively. Their study emphasises the detrimental effect of these enzymes on the degradation of fish muscle as compared to mammalian muscle. In addition, cathepsin L plays a major role in muscle breakdown due to its remarkable endopeptidase activity for a variety of proteins (Ogata, Aranishi, Hara, Osatomi, & Ishihara, 1998). It was found to be the major protease involved in *post-mortem* degradation of muscle obtained from mackerel (Aoki & Ueno, 1997), sea bass (Chéret et al., 2007) and salmon (Yamashita & Konagaya, 1991). For example, mackerel cathepsin L hydrolysed myosin, troponin T and tropomyosin (Aoki & Ueno, 1997). Yamashita and Konagaya (1991) demonstrated that cathepsin L hydrolysed the major muscle structural proteins, such as connectin, nebulin, myosin, collagen, α-actinin and troponins T and I. In addition, cathepsin B and L activities increased significantly during *post-mortem* storage in both the ice-stored and the super-chilled Atlantic salmon muscles (Gaarder, Bahuaud, Veiseth-Kent, Mørkøre, & Thomassen, 2012). These studies suggest that the principal cause of *post-mortem* degradation of fish muscle is due to the hydrolytic nature of cathepsins B and L, and that more proteolytic peptides may be released as a result of such hydrolysis. However, the effect of endogenous cathepsins B and L activities on the textural quality and bioactive properties of the final product, needs to be assessed before such conclusions can be made.

Knowledge on the proteolytic activity of major muscle endogenous proteases may demonstrate which underutilized fish species are suitable for functional foods or as raw materials for further seafood processing. The manufacture of surimi may be used as an approach to improve the utilization of underutilized fish species with low endogenous proteolytic activity. Thus, less fresh water may be needed to remove sarcoplasmic proteins during surimi processing (Martín-Sánchez et al., 2009). Generally, high endogenous proteolytic activity of fish mince produce low-quality surimi products due to degradation of myosin needed to form a surimi gel, the main determinant for surimi quality and price (An, Peters, & Seymour, 1996). In our study, the high level of CB+L activity demonstrated by barracouta, mirror dory and jackass morwong (Fig. 6) may indicate that these species would soften at a faster rate during cold storage, making them unsuitable for surimi production. Thus, these species may be more suitable for fish sauce production which requires the presence of more active endogenous proteases to hydrolyse muscle proteins during fermentation (Klomklao, Kishimura, Benjakul, & Simpson, 2009). Furthermore the highly characterized activity of cathepsin B+L in these species may increase the rate and extent of myofibrillar degradation and the release of biologically active peptides. In contrast, Ocean perch, nannygai, eastern school whiting, bight redfish, ribbon fish, tiger flathead, yellowspotted

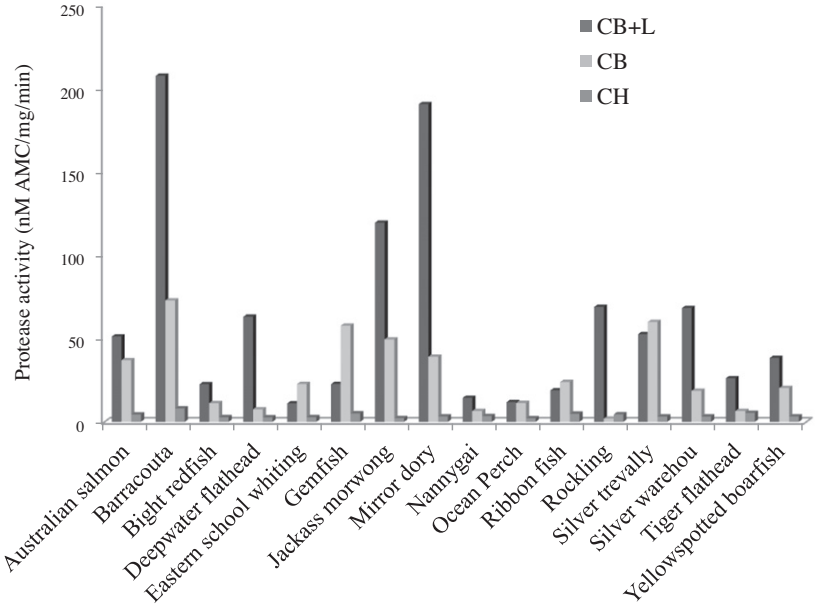

Fig. 6. Comparison of cathepsins B, B+L, and H activities among the examined underutilized fish species.

boarfish and Australian salmon may be suitable for surimi production due to their lower endogenous activity of cathepsins B+L and L. On the basis of commercial importance, rock ling and tiger flathead exhibited higher CB+L activity, whereas the activity of endogenous cathepsin B was higher in gemfish and eastern school whiting (Fig. 6). Processing conditions that control the activity of these major endogenous proteases during *post-mortem* storage, in more commercially valued species, need to be established to improve their textural quality or functional properties if they are to be used as raw materials in other seafood products. Further studies will focus on characterising the properties of cathepsins B and L to establish processing and/or environmental conditions, that may control their detrimental effects on fish myofibrillar proteins during iced storage. The information obtained from this study, together with future work, may provide an insight on how to maintain the textural quality and improve the functional and physiological properties of underutilized marine resources increasing its market value.

4. Conclusion

The activity of endogenous cathepsins B, D, H, L and calpain-like enzymes clearly was species dependant, which implies that the handling and application of these species may differ substantially. Barracouta was shown to have higher endogenous activity of cathepsins B, D, H, and L when compared with the other underutilized fish species used in this study. Activity of cathepsins B and B+L was found to be higher than that of cathepsin H in the crude enzymes extracted from the muscle of sixteen underutilized Australian fish species. Furthermore, lower endogenous activity of cathepsin D and H in all the examined species used in the study, suggest that these enzymes may not be one of the major muscle proteases causing the degradation and thus, softening of fish muscle during *post-mortem* storage. The underutilized fish species with low endogenous proteolytic activities may be suitable for surimi production, and those exhibiting high endogenous protease activities may be used for fish sauce production. It may be crucial to establish effective procedures to control the activity of major fish endogenous proteases, particularly cathepsins B and L, to a minimum to improve the textural quality and physiological properties of underutilized fish species with more commercial importance. In addition, further studies may examine the properties of peptides released during *post-mortem* autolytic process, establish the effects of the proteolytic activity of major muscle endogenous proteases and processing conditions on the bioactivity of Australian underutilized fish species.

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CHAPTER 4

Activity of endogenous muscle proteases from 4 Australian underutilized fish species as affected by ionic strength, pH and temperature

This chapter has been published as the title "**Activity of endogenous muscle proteases from 4 Australian underutilized fish species as affected by ionic strength, pH and temperature**" by Zeinab Ahmed, Osaana N. Donkor, Wayne A. Street, W. A. and Todor Vasiljevic in the peer reviewed journal, Journal of Food Science, 78, C1858-1864 (2013).

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CHAPTER 5

Mechanisms involved in post-mortem proteolytic degradation of silver warehou myofibrils

This chapter entitled "**Mechanisms involved in post-mortem proteolytic degradation of silver warehou myofibrils**" by Zeinab Ahmed, Osaana Donkor, Wayne A. Street, and Todor Vasiljevic has been submitted to Food Chemistry journal. Manuscript ID – FOODCHEM-S-16-04748.

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5.1. Introduction

Texture is an important quality characteristic of meat obtained from fish muscle that determines the acceptability of fish as food. The term "meat" refers to the converted muscle tissue that has undergone rigor mortis followed by the proteolytic breakdown of specific bonds in the myofibrillar structure during post-mortem storage. Proteolysis of myofibrillar proteins leads to weakening of the myofibrillar network, loss of muscle cell integrity and ultimate tissue softening (Delbarre-Ladrat et al., 2006), which is rapid in fish. The loss of α-actinin, involved in the anchorage of actin filaments to the Z-line, increases the weakening of Z-disks of myofilaments (Papa et al., 1995). This is followed by degradation of other key myofibrillar proteins such as titin, nebulin as well as troponin-T and tropomyosin during subsequent post-mortem storage. Actin and myosin are the major forces of muscle contraction and the genesis of muscle stiffening. Myosin consists of two components - heavy chains (MHC) and light chains (MLC) (Huff Lonergan et al., 2010). Actin is bound to many proteins including troponins and tropomyosins, dystrophin and α-actinin. Unlike, other myofibrillar proteins, post-mortem breakdown of actin and myosin defines the mechanism of softening in fish muscle (Godiksen, Morzel, Hyldig, & Jessen, 2009).

Ultrastructural post-mortem changes in fish myofibrillar proteins have been often attributed to the endogenous activity of metalloproteinases (Kubota et al., 2001), cysteine proteases, and serine proteases, in particular myofibril-bound serine protease (MBSP) (Zhong et al., 2012), as well as aspartic proteases with particular reference to cathepsin D (Godiksen et al., 2009). A gap in the literature exists on type and mode of enzymatic action involved in post-mortem degradation of muscle of Australian underutilized fish species. In general, special attention has been given to cysteine proteases, in particular calpains and cathepsins, with respect to inducing post-mortem myofibrillar proteolysis responsible for improving tenderness

of muscle foods during refrigerated storage (Chéret et al., 2007). Calpains may act on fish muscle during initial days of post-mortem storage causing limited proteolysis of myofibrillar proteins and increasing its susceptibility to attack by other proteases. The activity of endogenous cathepsins increases with muscle acidification, and some of these may be activated at very acidic pH conditions (Ahmed, Donkor, Street, & Vasiljevic, 2015). The hydrolytic activity of cathepsins B and L in fish muscle at post-mortem conditions induces exhaustive breakdown of myofibrillar proteins (Liu, Yin, Li, Zhang, & Ma, 2008; Yamashita & Konagaya, 1991), which is probably due to the endopeptidase and exopeptidase activities of cathepsin B and endopeptidase properties of cathepsin L (Rawlings & Barrett, 1994). These endogenous proteases are present at greater levels in fish skeletal muscle when compared to bovine muscle (Chéret et al., 2007). This may contribute to the enhanced post-mortem softening of tissue commonly observed in fish as previously determined (Godiksen et al., 2009). In addition, other endogenous proteases may also be active.

Different strategies are often used when examining the proteolytic degradation occurring in intact fish muscle. Most of the research studies examined the proteolytic degradation of fish myofibrillar proteins using isolated and purified muscle proteases. A major drawback of some of these strategies is that proteolysis observed may be different from those observed *in situ* (Godiksen et al., 2009). Often conditions to study enzyme properties are highly optimized and may not be reflective of the *in situ* conditions in muscle. The literature lacks knowledge of mechanisms involved in autolysis of myofibrillar proteins with special reference to Australian underutilized fish species. Our study used an approach adapted by Wang, Vang, Pedersen, Martinez, and Olsen (2011) to identify proteolytic mechanisms involved in post-mortem degradation of silver warehou, one of the underutilized but important commercial fishery species.

Our study aimed at investigating the effect changes in muscle pH and treating silver warehou muscle with protease(s) inhibitors and (or) activators has on post-mortem degradation of myofibrillar components with emphasis on actin and MHC. This research may improve our understanding of proteolytic mechanisms involved in post-mortem degradation of silver warehou myofibrillar proteins resulting in a loss of its textural quality and add to existing knowledge of fish muscle proteolysis in general.

5.2. Materials and methods

5.2.1. Materials

The study used fresh silver warehou provided by a local supplier (Barwoon Foods, Geelong North, VIC). Fish species were delivered on ice within 24 h from harvest to our laboratory (Victoria University, Werribee), stored in a cold room and handled within 1-2 h of dispatch. All chemicals and reagents were of the highest grade commercially available.

5.2.2. Adjusting muscle pH

The pH was adjusted following Wang et al. (2011) with slight modifications. Approximately 2 g of fish muscle cubes were incubated at room temperate (RT) in 8 ml of 200 mM sodium phosphate (Merck, Darmstadt Germany) buffers, pH 5.5, 6.5 or 7.0, with 0.02% sodium azide (NaN_3) (Sigma Aldrich Inc., St. Louis MO) included. After 0, 15, 30, or 60 min and after 1, 3 or 5 days of incubation, muscle cubes were homogenized 1:1 (w/v) in 150 mM potassium chloride (KCl) (Merck) for 30 s. The pH of the homogenate was measured after homogenization to monitor changes in muscle pH.

5.2.3. Muscle treatment with protease(s) inhibitors and (or) activators

Fish muscle cubes were stored in the absence or presence of protease(s) inhibitors and (or) activators (Wang et al., 2011) as described in section 2.2. Protease(s) inhibitors used were antipain (Sigma Aldrich) at 0.1 mM, E-64 [1-(L-trans-epoxysuccinylleucyamino-4-guanidinobutane] (Santa Cruz Biotechnology) at 0.1 mM, Ethylenediaminetetraacetic acid (EDTA) (Merck) at 5 mM, pepstatin A (Santa Cruz Biotechnology, Dallas Texas) at 0.1 mM, phenanthroline (Merck) at 1 mM and phenylmethyl sulfonyl fluoride (PMSF) (Merck) at 1 mM. The cysteine protease activator, dithiothreitol (DTT) (Sigma Aldrich) was used at 5 mM. Crude extracts and myofibrils were isolated from the muscle after the desired incubation period.

5.2.4. Preparation of crude extracts and myofibrils

Isolation of crude extracts and myofibrils followed a method by Wang et al. (2011) with slight modifications. Fish muscle (6 g) from section 2.3 was homogenized in 24 ml of 50 mM phosphate buffer (pH 7.5), containing 0.02% NaN_3. The homogenate was centrifuged at 2,684 g, 4 °C for 25 min (Sorval RT7 centrifuge, Thermo Scientific, Rockford, IL). Following this, the supernatant, regarded as a crude extract, was frozen at -20 °C. The pellet was resuspended in the same buffer and washed three times. The resulting pellet of the last wash was suspended in 50 mM phosphate buffer at the pH of the incubation solution, containing 0.02% NaN_3 and 500 mM sodium chloride (NaCl) and the sample volume was doubled with 10% sodium dodecyl sulphate (SDS) (Merck). This suspension regarded as myofibrils was stored at -20 °C freezer for further analysis. Protein concentration in the crude extracts and myofibrils was determined using the BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific) and Libra S11 UV/Visible Spectrophotometer (Biochrom Ltd, Cambridge, United Kingdom) to measure the absorbance of the resulting solution at 562 nm.

5.2.5. Cathepsins activity

The proteolytic activity of cathepsins B and B+L in the supernatants was estimated as described previously (Ahmed, Donkor, Street, & Vasiljevic, 2013a). Proteases activation involved incubating 0.5 ml of crude extract with 1.25 ml of activation buffer (pH 6.0) for 2 min at 30 ℃. The activation buffer used for cathepsin B contained 200 mM sodium phosphate, 2 mM EDTA, 0.05% CHAPS, and 4 mM DTT, and that for cathepsin B+L contained 200 mM sodium acetate, 2 mM EDTA, 0.05% CHAPS and 4 mM DTT. The reaction was terminated after 10 min and the fluorescence intensity of the resulting solution was measured using the spectrophotofluorometer. Cathepsins activity in the crude extracts were expressed as U/mg of protein.

5.2.6. One-dimensional SDS-electrophoresis and immunoblot analysis

Protein samples and marker proteins (Precision Plus protein unstained standards, Bio-Rad Laboratories, Hercules, CA) were separated on 4-20% Mini-PROTEAN® TGX Stain-Free™ gels (Bio-Rad). Extracted myofibrils and actin from bovine muscle (Sigma Aldrich), mixed with 2× Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol) (Bio-Rad), were heated at 95-100 °C for 5 min prior to electrophoresis. Approximately 0.5 µg of actin and 0.5 µg total protein of each sample were loaded on gels. Proteins were separated at 100 V for 95 min using a Bio-Rad Mini-Protean Tetra Electrophoresis System connected to a PowerPac Basic apparatus (300V) at RT. Stain-Free (SF) imaging of gels was performed using a ChemiDoc MP imager and Image Lab 5.2 software (Bio-Rad) with a 5-min activation time. All images of the SF gels were analysed using Image Lab 5.2 software.

Myofibrillar proteins from the SF activated gels were transferred to 0.2 µm polyvinylidene fluoride (PVDF) membranes in 7 min using a Trans-Blot Turbo Transfer

System (Bio-Rad) with Trans-Blot Turbo Midi PVDF Transfer Packs (Bio-Rad). Immediately after transfer, the blot was washed 2 × 5 min in TBS buffer (20 mM Tris, pH 7.4 containing 500 mM NaCl) (Bio-Rad) containing 0.05% Tween 20 (TBST) and nonspecific binding sites were blocked with 1× TBS 1% casein blocker (Bio-Rad) for 1 h. The blot was then incubated for 2 h with rabbit polyclonal antibody to alpha skeletal muscle actin (GeneTex Sapphire Bioscience) at a dilution of 1:2500 in blocking buffer and washed 5 × 5 min in TBST. After that the membrane was incubated in a mixture containing goat anti-rabbit IgG H&L (HRP) (abcam, Cambridge, UK) at a dilution of 1:2000 in TBST for 1 h and washed 5 × 5 min in TBST. Finally, the membrane was incubated with Clarity Western ECL Substrate (Bio-Rad) for 5 min and the chemiluminescent signals on the blot was captured using Bio-Rad ChemiDoc MP Imaging System and analysed (Image Lab 5.2 software, Bio-Rad). All washing and incubation steps involved gentle agitation at RT.

5.2.7. Statistical Analysis

Cathepsins activity assay was replicated three times and subsampled twice. Results were analysed using the General Linear Model (GLM) developed by the Statistical Analysis System (SAS). Statistically significant level was defined at $P \leq 0.05$. The model included effects of muscle treatment (presence of protease(s) inhibitors and (or) activators) as the main plot, pH (6.0, 6.5 or 7.0), incubation time in days (1, 3 or 5), and their interactions presented as subplots.

5.3. Results and discussion

5.3.1. Altering the pH in intact fish muscle samples

Adjusting the pH may provide suitable conditions for different proteases to act on muscle proteins. Muscle cubes were incubated in phosphate buffers with pH 5.5, 6.5 or 7.0 at

RT for 1, 3 or 5 days. These conditions are close to post-mortem muscle pH. Initial pH of samples was approximately 6.4. Incubation of muscle for the first hour at different pH levels resulted in a marked increase in tissue pH in all samples during the first 15 minutes followed by subsequent stabilization of pH for up to 5-days (results not shown). Intact muscle incubated in buffer (pH 5.5) reached an ultimate tissue pH of 6.0 by the end of 5-day period. The buffer (pH 5.5) was ineffective at reducing muscle pH further. Samples incubated in pH 6.5 and 7.0 buffers for 5-days maintained an ultimate pH of 6.5 and 7.0, respectively.

5.3.2. Effect of muscle treatment and changes in pH on cathepsins activity

The study measured the endogenous activity of cathepsins B and L using synthetic substrates for cathepsins B and B+L, respectively. The hydrolytic activity of cathepsin B+L in control samples was higher than that of cathepsin B. Changes in muscle pH significantly ($P \leq 0.05$) influenced the activity of endogenous cathepsins (Table 5.1). Proteolytic activity of endogenous cathepsins in the control increased linearly with muscle acidification. Cathepsin B activity increased slightly as pH declined from neutral to a slightly acidic level (Table 5.2). Cathepsin B+L activity was substantially higher at pH 6.0 or 6.5 than pH 7.0 (Table 5.3). In addition to pH, incubation time significantly influenced the activity of endogenous cathepsins (Table 5.1), demonstrating changes in cathepsins activity during post-mortem storage.

Treating silver warehou muscle with protease(s) inhibitors and (or) activators influenced the activity of endogenous cathepsins (Table 5.1). Protease inhibitors blocks the reactive amino group in the active site of the enzyme or chelate cations involved in the cleavage of peptide bonds thus interfering with and reducing or abolishing enzymatic activity. We measured the activity of endogenous cathepsins B and B+L as an indication of cysteine proteases activity in post-mortem muscle. The hydrolytic activity of serine and cysteine proteases against myofibrillar proteins was inhibited in the presence of antipain (Otto &

Schirmeister, 1997). Nonetheless, our results were interpreted in such a way that antipain inhibited mainly the activity of cysteine proteases including calpains and cathepsins B, H and L as previously reported (Jiang, Lee, & Chen, 1994). DTT has the capacity to activate cysteine proteases including cathepsins (Kirschke, Barrett, & Rawlings, 1998). However, the presence of DTT in our study limited cathepsins activation. The highly selective inhibitor of many cysteine proteases, E-64, was used to inhibit the activity of cathepsins B and L (Matsumoto et al., 1999) and calpains (Jiang & Chen, 1999). Endogenous activity of calpains was previously detected in silver warehou muscle (Ahmed, Donkor, Street, & Vasiljevic, 2013b). EDTA inhibits the activity of metalloproteases requiring divalent cations for activity and other proteases such as calpains requiring extracellular calcium for activity (Satish, Blair, Glading, & Wells, 2005). In our study, antipain, DTT, E-64 and EDTA substantially inhibited the endogenous activity of cathepsins at pH of 6.0 or 6.5, suggesting effective inhibition of cysteine proteases.

Table 5.1. Analysis of variance (ANOVA) depicting significance of the main effects of muscle treatment, changes in pH (6.0, 6.5 or 7.0), incubation time in days (1, 3 or 5) and their interactions on the activity of endogenous cathepsins B and B+L.

Source	*p*-value	
	Cathepsin B	Cathepsin B+L
Treatment	<0.0001	<0.0001
pH	<0.0001	<0.0001
Time	<0.0001	0.0042
Treatment × pH	<0.0001	<0.0001
Treatment × Time	<0.0001	<0.0001
Treatment × pH × Time	<0.0001	<0.0001

Table 5.2. Means of the endogenous activity of cathepsin B as influenced by muscle treatment, adjustment of muscle pH, and incubation time.

Treatment	Cathepsin B activity (nM AMC/min/mg protein)								
	pH 6.0			pH 6.5			pH 7.0		
	Time (days)			Time (days)			Time (days)		
	1	3	5	1	3	5	1	3	5
Control	8.49^{cC}	8.70^{dC}	7.94^{bcBC}	6.06^{cAB}	7.05^{cB}	7.75^{cBC}	4.98^{cA}	5.25^{bcAB}	5.33^{bAB}
Antipain	5.08^{bB}	2.76^{aA}	4.93^{aB}	4.64^{bB}	2.81^{aA}	2.80^{aA}	3.11^{abAB}	4.33^{bB}	4.32^{abB}
DTT	3.71^{abB}	5.53^{bB}	4.52^{aB}	2.77^{aAB}	2.69^{aAB}	3.12^{abAB}	2.08^{aA}	2.84^{aAB}	3.14^{aAB}
E-64	4.00^{abAB}	7.02^{cC}	4.27^{aAB}	4.66^{bcAB}	5.92^{cB}	3.48^{abA}	3.55^{bA}	4.13^{abAB}	5.15^{bB}
EDTA	2.99^{aA}	4.62^{bB}	5.40^{aB}	5.14^{bcB}	4.33^{bAB}	3.23^{abAB}	3.21^{abA}	3.28^{abAB}	4.27^{abAB}
PMSF	15.06^{eE}	13.48^{fD}	9.00^{cA}	14.42^{eD}	13.00^{dCD}	10.46^{dB}	12.56^{dCD}	11.96^{dC}	9.84^{cAB}
Pepstatin A	11.76^{dC}	12.84^{fC}	7.06^{bB}	5.19^{bcA}	6.06^{cAB}	5.00^{bA}	5.39^{cA}	6.13^{cAB}	4.77^{bA}
Phenanthroline	8.11^{cC}	10.74^{eD}	10.34^{cD}	8.04^{dC}	13.36^{dE}	11.51^{dD}	6.16^{cB}	3.93^{abA}	8.55^{cC}
SEM					0.001				

[A-E]Different uppercase superscripts in the same row indicates the significant difference between means for muscle samples receiving the same treatment at different pH and incubation time ($P \leq 0.05$).

[a-f]Different lowercase superscripts in the same column demonstrates the significant difference between means for muscle samples subjected to different treatment at the same pH and incubation time ($P \leq 0.05$). Results are expressed as mean of 3 trials. SEM = Standard error of the mean.

Table 5.3. Means of the endogenous activity of cathepsin B+L as influenced by muscle treatment, adjustment of muscle pH, and incubation time

Treatment	Cathepsin B+L activity (nM AMC/min/mg protein)								
	pH 6.0			pH 6.5			pH 7.0		
	Time (days)			Time (days)			Time (days)		
	1	3	5	1	3	5	1	3	5
Control	15.63^{cD}	16.00^{dD}	16.70^{dD}	8.56^{cBC}	10.28^{cC}	11.62^{cC}	4.45^{bA}	7.38^{bB}	8.10^{cB}
Antipain	6.27^{bB}	4.22^{aA}	6.67^{bB}	4.49^{abA}	5.20^{aAB}	4.43^{abA}	3.47^{abA}	6.55^{bB}	6.26^{bB}
DTT	5.56^{abB}	12.10^{cC}	16.18^{dD}	3.36^{aAB}	4.70^{aB}	5.33^{bB}	2.53^{aA}	4.59^{cB}	5.63^{abB}
E-64	5.74^{bBC}	8.04^{bC}	3.42^{aA}	6.08^{bBC}	7.40^{bC}	3.37^{aA}	5.63^{bB}	7.69^{bC}	4.21^{aAB}
EDTA	3.83^{aA}	8.17^{bB}	7.15^{bB}	3.25^{aA}	7.79^{bB}	4.53^{abA}	4.30^{bA}	4.89^{abAB}	6.63^{bcB}
PMSF	31.45^{fE}	14.91^{dB}	10.02^{cA}	28.82^{eD}	13.24^{dB}	11.42^{cA}	20.88^{eC}	9.79^{cA}	13.74^{dB}
Pepstatin A	24.98^{eD}	41.60^{eE}	46.29^{fF}	10.15^{cA}	12.90^{dB}	9.96^{cA}	13.24^{dB}	10.28^{cA}	16.34^{eC}
Phenanthroline	18.99^{dE}	11.30^{cC}	25.56^{eF}	16.67^{dD}	12.81^{dC}	11.59^{cC}	8.78^{cB}	5.42^{abA}	6.48^{bcA}
SEM	0.001								

[A-F] Different uppercase superscripts in the same row indicates the significant difference between means for muscle samples receiving the same treatment at different pH and incubation time ($P \leq 0.05$).

[a- f] Different lowercase superscripts in the same column demonstrates the significant difference between means for muscle samples subjected to different treatment at the same pH and incubation time ($P \leq 0.05$). Results are expressed as mean of 3 trials. SEM = Standard error of the mean.

Proteases other than cysteine proteases may contribute to post-mortem autolysis of silver warehou myofibrillar proteins. Pepstatin A inhibited the endogenous activity of aspartic acid proteases including cathepsin D (Marciniszyn, Hartsuck, & Tang, 1976). The activity of metalloproteases and serine proteases was inhibited in the presence of phenanthroline (Mumford et al., 1980) and PMSF (Sharma & Radha Kishan, 2011), respectively. In addition to limiting the activity of specific proteases, pepstatin A, phenanthroline and PMSF substantially induced significant changes in cathepsins activity depending on pH and incubation time (Table 5.1). Incubation of muscle pieces with pepstatin A activated cathepsin B+L at pH 6.0 or 7.0 (Table 5.3). The activity of cathepsin B at pH 6.5 was enhanced in the presence of phenanthroline (Table 5.2). Furthermore, treating muscle with PMSF activated cathepsins at pH 7.0.

5.3.3. Autolysis of myofibrillar components as influenced by muscle treatment and changes in pH

Electrophoretic pattern of the control and treated samples varied substantially with pH and incubation time (Figure 5.1). Typical bands of actin and MHC in addition to other proteins and their degradation products were observed in all samples. The reduction of the protein bands intensity over time serves as an indication of protein degradation. MHC having a typical band of approximately 220-200 kDa was degraded into smaller fragments with a molecular weight of 190-125 kDa. Additionally, faint bands were observed in the area of 100-55 kDa in all samples. These bands may be characterized as α-actinin and desmin, respectively (Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2003). Actin exhibited a single band with an apparent molecular weight of 39-42 kDa. A band in the area of (~17-18 kDa) relating to MLC was detected in all samples.

In situ weakening of silver warehou myofibrils was pH-dependent (Figure 5.1). The degree of actin and MHC proteolysis in the control was greater at pH 6.0 than at pH 6.5 or 7.0. Incubation time significantly influenced myofibrillar components (Figure 5.1). MHC hydrolysis increased substantially with incubation time at pH 6.0 or 7.0 in control samples. Complete disappearance of MHC was observed by the end of 5-day period at pH 6.0. The band intensity of MHC initially detected after 1-day in the control was reduced by 52% and 80% by the end of storage at pH 6.5 and 7.0, respectively. Our results indicate that slightly acidic pH condition in post-mortem muscle accelerated the extent of actin and MHC hydrolysis during storage. Acidic ultimate muscle pH increases myofibrillar weakening associated with textural degradation of fish quality post-mortem (Ofstad et al., 1996). Increased progression of actin and MHC proteolysis post-mortem at low muscle pH may relate to proximity to their isoelectric points (pI). A significant correlation exists between the degradation rates of proteins and their isoelectric points. At their pI, repulsion between structural proteins within the myofibril is reduced and protein-protein interactions are more favourable (Huff-Lonergan, 2009). Modulation of the conformation of proteins at or close to their pI may increase the formation of protein-enzyme complexes thereby increasing the susceptibility of proteins to proteolysis. Actin and myosin have isoelectric points of 5.5 (Foegeding, Lanier, & Hultin, 1996) and 5.4 (Zechel & Weber, 1978), respectively. Therefore, post-mortem actin and MHC breakdown increases as pH approaches 5.4 - 5.5.

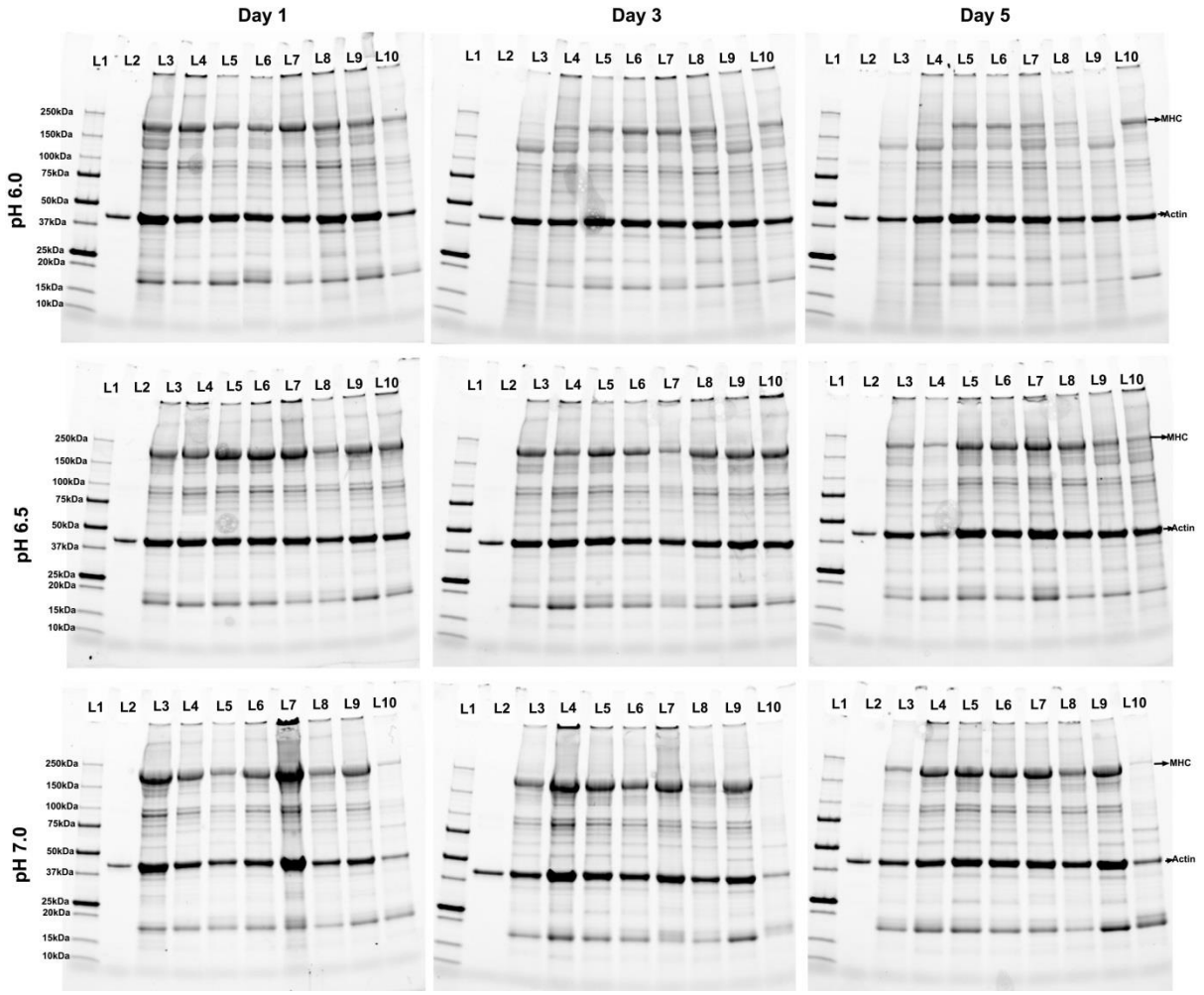

Figure 5.1. Changes in myofibrillar components isolated from silver warehou muscle incubated at three different pH values in the presence or absence of protease inhibitors and (or) activators for 1, 3 or 5 days before isolation of myofibrils. Type of protease inhibitors and (or) activators used is: Lane 1: Standard Molecular Weight Marker, Lane 2 (L2): actin, Lane 3 (L3): control, Lane 4 (L4): 5 mM DTT, Lane 5 (L5): 0.1 mM E-64, Lane 6 (L6): 0.1 mM antipain, Lane 7 (L7): 5 mM EDTA, Lane 8 (L8): 0.1 mM pepstatin A, Lane 9 (L9): 1 mM phenanthroline, Lane 10 (L10): 1 mM PMSF. MHC, myosin heavy chain.

Endogenous proteases active at post-mortem pH may be associated with degradation of muscle proteins. Inhibition and (or) activation of selected proteases influenced myofibrillar components (Figure 5.1). EDTA and DTT minimised, whereas PMSF accelerated MHC breakdown after 1-day of incubation at pH 6.0 in comparison to the control. Antipain, EDTA, E-64, phenanthroline, DTT and PMSF reduced weakening of MHC band after 1-day incubation

at pH 6.5. EDTA inhibited MHC degradation after 1-day incubation at pH 7.0. The increased breakdown of MHC in the presence of EDTA by the end of 3-days in comparison to 1- or 5-days at pH 6.5 may be due to increased chelation of divalent cations associated with stabilising muscle structure (Wang et al., 2011). Substantial inhibition of myofibrillar weakening by the end of 5-days period was mainly attributed to inhibiting the activity of cysteine proteases. Antipain, E-64 and EDTA effectively reduced actin and MHC breakdown at pH 6.0, 6.5 or 7.0 (Figure 5.1). In addition, inhibition of aspartic acid proteases in the presence of pepstatin A reduced proteolysis of actin and MHC by the end of storage at pH 6.5 or 7.0. Limiting the activity of metalloproteases in the presence of phenanthroline decreased actin and MHC hydrolysis at pH 6.5. In addition, phenanthroline inhibited actin breakdown at pH 7.0 (Figure 5.1). Although PMSF inhibited *in situ* weakening of MHC at pH 6.0, it was ineffective in solutions at pH 6.5 or 7.0.

5.3.4. Immunoblot analysis of actin as influenced by muscle treatment and changes in pH

Immunoblotting using antibodies against skeletal actin was used to specifically monitor actin proteolysis and identify proteases mainly involved in its degradation post-mortem. Actin degradation was substantially affected by pH, incubation time and muscle treatment (Figure 5.2). Hydrolysis of actin was significantly greater at pH 6.0 or 6.5 than 7.0. Proteolysis of actin in control samples increased significantly with incubation time at pH 6.0. Enzyme-inhibitor reactions indicated the hydrolytic activity of each class of proteases on intact actin. Muscle treatment substantially accelerated actin degradation over the 3-days incubation period at pH 6.0 in comparison to the control as indicated by faded actin bands. Enhanced intensity of actin band observed in the presence of several protease(s) inhibitors after 3-days incubation at pH 6.0 (Figure 5.2) may be due to interference with the degradation products of higher molecular

weight proteins such as titin and nebulin as indicated by bands with molecular mass greater than 250 kDa (Figure 5.1). Our results indicate that antipain, E-64 and EDTA were the most effective at preventing *in situ* hydrolysis of actin at pH 6.0 or 6.5 over the storage period (Figure 5.2), supporting the cleavage of actin by mainly cysteine proteases. The inhibition of aspartic acid proteases in the presence of pepstatin A effectively reduced actin degradation by the end of 5 -days incubation at pH 6.5 or 7.0. DTT minimised the hydrolysis of actin over the 5-days storage at pH 6.0 or 7.0, probably due to its inhibition of endogenous cathepsin activity (Table 5.3). Limiting the activity of serine proteases in the presence of PMSF reduced *in situ* breakdown of actin at pH 6.0. Similarly, inhibiting the activity of metalloproteases in the presence of phenanthroline inhibited actin proteolysis at pH 6.0 or 7.0 (Figure 5.2).

Rapid autolysis of actin and MHC at slightly acidic pH and significant inhibition of muscle degradation in the presence cysteine protease(s) inhibitors indicate the involvement of calpains and cathepsins in the degradation process post-mortem. Lysosomal cathepsins have optimum activity at slightly acidic to very acidic pH (Toldrá & Reig, 2012). Release of these proteases from the lysosomes with muscle acidification and at their optimum level of hydrolytic activity may cause detrimental effects on myofibrillar structure post-mortem. We have previously detected important levels of cathepsin B+L activity as opposed to cathepsin B (Ahmed et al., 2013b). Endogenous activity of cathepsins B and B+L was maximal at slightly acidic conditions. Cathepsin D was active under very acidic pH conditions in silver warehou muscle (Ahmed et al., 2013a). These proteases may become inactivated at high pH (Kirschke et al., 1998). High endogenous activity of cathepsins determined in silver warehou muscle may be directly associated with increased proteolysis of actin and MHC at pH 6.0. Optimal conditions for MHC breakdown by mackerel cathepsin L or L-like enzymes are in the range pH 5.5-7.0 (Ho, Chen, & Jiang, 2000). Silver carp cathepsin B increased proteolysis of MHC at pH 5.5 (Liu et al., 2008). Mackerel cathepsins B and L at pH 6.5-7.5 caused maximum

breakdown of MHC (Jiang, Lee, Tsao, & Lee, 1997). In the current study, actin was highly resistant to proteolytic cleavage in comparison to MHC in silver warehou muscle, similar to a previous report (Jiang, Lee, & Chen, 1996). Other fish minor myofibrillar proteins including β-tropomyosin/troponin-T were also more susceptible to proteolytic cleavage than actin (An, Weerasinghe, Seymour, & Morrissey, 1994). This also suggests that actin breakdown in fish muscle is likely to occur during prolonged post-mortem storage (Caballero et al., 2009). Enhanced breakdown of actin during late post-mortem storage suggests that cathepsins are mostly involved in post-mortem degradation of actin in silver warehou muscle. Mackerel cathepsin B caused rapid proteolysis of actin at pH 4.0-5.0 (Jiang et al., 1996). Similarly, cathepsin D from herring muscle demonstrated optimum hydrolytic activity against actin at pH 4.23 (Nielsen & Nielsen, 2001). The hydrolytic activity of sea bass cathepsin L enhanced breakdown of actin (Ladrat et al., 2003). Calpains may also be involved in post-mortem proteolysis of actin (Lametsch, Roepstorff, Møller, & Bendixen, 2004). Antipain, E-64 and EDTA were the most effective in reducing *in situ* degradation of myofibrillar components at pH 6.0 or 6.5. In addition to their effect on calpains, antipain, E-64 and EDTA inhibited the activity of cathepsins B and B+L. Therefore, our study indicates that cysteine proteases, including cathepsins and calpains, are the main types of endogenous muscle proteases involved in rapid autolysis of silver warehou myofibrils at slightly acidic pH.

Inhibition of actin and MHC breakdown *in situ* in the presence of PMSF at pH 6.0 indicates the contribution of serine proteases to post-mortem degradation of myofibrils. Nonetheless, acceleration of *in situ* myofibrillar proteolysis at pH 6.5 or 7.0 in the presence of PMSF suggests that endogenous serine proteases are less likely to be involved in post-mortem breakdown of muscle proteins at slightly acidic to neutral H. The degree of *in situ* proteolysis of MHC was greater at pH 7.0 in comparison to that at pH 6.5. Inhibition of endogenous cysteine proteases caused significant reduction of myofibrillar degradation at neutral pH in our

study. This is probably due to the hydrolytic activity of calpains rather than cathepsins. Calpains activation at pH 7.0-7.5 increased the breakdown of mackerel MHC (Ho et al., 2000). In addition to cysteine proteases, aspartic acid proteases were also mainly involved in post-mortem breakdown of myofibrils at pH 6.5 or 7.0. The activity of endogenous muscle metalloproteases was less likely involved in post-mortem breakdown of actin at pH 6.5. However, at neutral pH metalloproteases enhanced actin degradation.

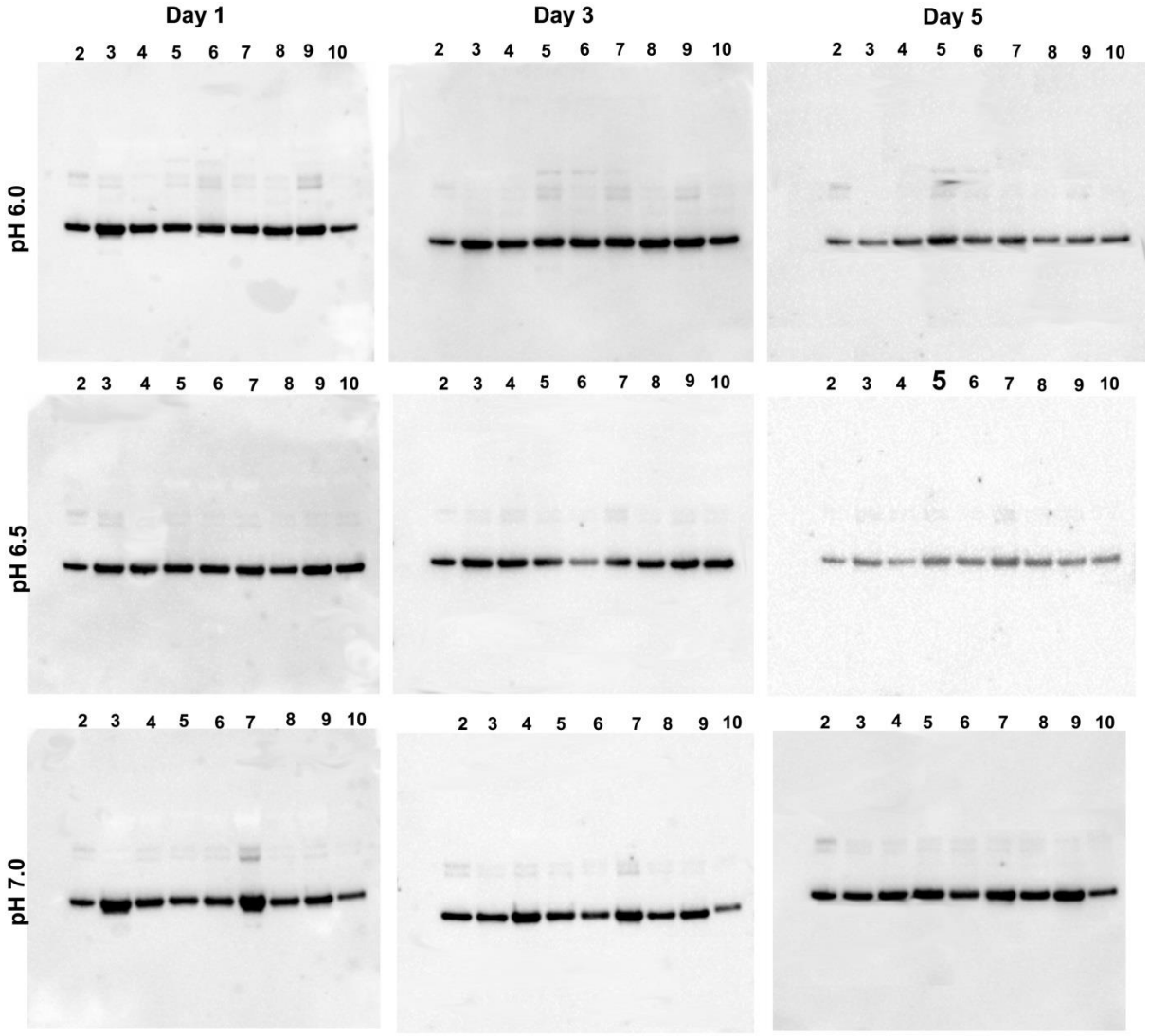

Figure 5.2. Immunoblots of myofibrillar proteins isolated from silver warehou muscle incubated in the presence or absence of protease inhibitors and (or) activators for 1, 3 or 5 days at three different pH values before isolation of myofibrils. Type of protease inhibitors or activators used is: Lane 1: Standard Molecular Weight Marker, Lane 2 (L2): actin, Lane 3 (L3): control, Lane 4 (L4): 5 mM DTT, Lane 5 (L5): 0.1 mM E-64, Lane 6 (L6): 0.1 mM antipain, Lane 7 (L7): 5 mM EDTA, Lane 8 (L8): 0.1 mM pepstatin A, Lane 9 (L9): 1 mM phenanthroline, Lane 10 (L10): 1 mM PMSF.

Inhibition of one class of proteases increased the susceptibility of myofibrillar degradation by other types of proteases. Our results indicated that inhibition of mainly cysteine and secondary aspartic acid proteases reduced *in situ* myofibrillar degradation. The study also supports the contribution of proteases other than cysteine to post-mortem autolysis of myofibrillar proteins. These endogenous proteases however are mostly active at their optimum pH where their reaction mechanisms commence to weaken the myofibrillar structure. Given that the ultimate pH of fish muscle may fall in the range of 6.5-5.0 post-mortem, which presents the optimum for activity of cysteine and aspartic acid proteases. Therefore, activity calpains and cathepsins may be more involved in causing exhaustive breakdown of silver warehou muscle responsible for tissue softening post-mortem. In our study, we also acknowledged the contribution of serine and metalloproteases to post-mortem autolysis of myofibrils.

Less information is provided in the literature about the proteolytic mechanisms involved in post-mortem weakening of fish myofibrils and associated tissue softening. Endogenous calpains and cathepsins, active in post-mortem muscle, may degrade actin and MHC at different locations releasing a range of polypeptides and dipeptides fragments with varying molecular weight and relative intensities on the basis of the proteases active site (Wang et al., 2009). The *MEROPS* database was used to predict sites, at which endogenous cathepsins may cleave actin (MEROPS the peptidase database, 2016a, 2016b, 2016c). Protease specificity prediction server (PROSPER) was used for identifying possible calpain cleavage sites on actin (Song et al., 2012). Actin may be susceptible to cleavage by calpains at the N^{282}+S (asparagine and serine residues, respectively) and the K^{361}+Q (lysine and glutamine residues, respectively) peptide bonds (Figure 5.3). Cathepsin B may target the actin molecule and cleave it at the Met^{301}+Ser peptide bond and result in the release of oligopeptide fragments during post-mortem storage of silver warehou muscle (Figure 5.3). In comparison, cathepsin L may cleave actin at two sites: at the Met^{301}+Ser peptide bond and the Thr^{306}+Met peptide bonds (Figure

5.3). Cathepsin D may attack silver warehou actin at three sites: at the Leu^{106}+Leu, Met^{229}+Ala and Met^{301}+Ser peptide bonds (Figure 5.3). The cleavage of actin by these proteases increases the weakening of actin complex and thus myofibrillar disintegration responsible for tissue softening. In addition, the cleavage of actin by a particular protease may release peptides with one or more potent bioactive properties. Proteolytic degradation of myofibrillar proteins releases peptides with varying biological activities depending on the site cleaved by the protease (Manikkam et al., 2016). Some of these proteases may also modulate the conformation of proteins during post-mortem storage in a way that influences the functional properties of underutilized fish species. Nonetheless, further work may examine proteolytic mechanisms involved in modulating the bioactive and functional properties of Australian underutilized fish muscles to increase its profitability.

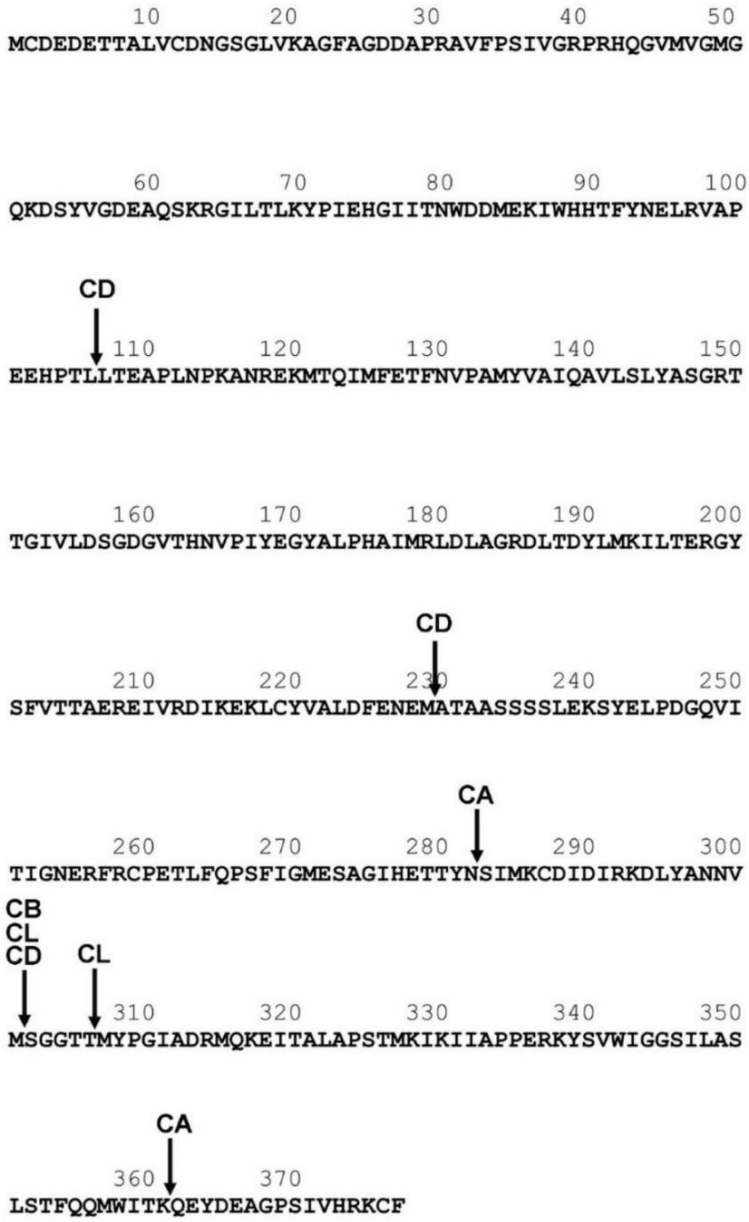

Figure 5.3. Postulated mechanisms responsible for proteolytic degradation of actin during post-mortem degradation of silver warehou muscle. The complete sequence of 377 residues of actin, alpha skeletal muscle (*Homo Sapiens*) substrate was used to identify proteolytic cleavage sites based on MEROPS database (MEROPS the peptidase database, 2016a, 2016b, 2016c) and PROSPER (Song et al., 2012). Arrows indicate cleavage sites by proteases: CA, calpains; CB, cathepsin B; CD, cathepsin D and CL, cathepsin L.

5.4. Conclusion

Post-mortem muscle pH significantly influenced different proteolytic activity and the way these enzymes act on silver warehou myofibrillar proteins. The degree of myofibrillar

weakening post-mortem varied depending on optimum conditions related to each type of proteases during storage. Actin was more resistant to proteolytic cleavage than MHC in silver warehou muscle. Degradation of actin and MHC increased substantially at slightly acidic pH. Cysteine proteases including calpains and cathepsins B and L are mainly responsible for post-mortem autolysis of myofibrils. In addition, aspartic acid proteases (mainly cathepsin D) are also responsible for post-mortem degradation of muscle proteins. Our study did not rule out the involvement of other proteases.

CHAPTER 6

Endogenous muscle proteases releasing peptides with antioxidative activity during post-mortem storage of silver warehou muscle

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GRADUATE RESEARCH CENTRE

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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Todor Vasiljevic	10	Concept development; experimental design; support and technical expertise; statistical analysis	[redacted] Todor Vasiljevic 2016.08.30 09:28:34 +10'00'	30/08/2016

6.1. Introduction

Fish proteins are a potential source of unique proteinaceous fragments often referred to as bioactive peptides (Rasika, Ranadheera, & Vidanarachchi, 2013). These peptides usually consist of 3-20 amino acids and contain sequences which are inactive when encrypted in the native protein molecule. Once liberated, these peptides demonstrate biological activities beneficial for modulating human health. Bioactive peptides are released from their food protein precursors during: (a) *in vitro* hydrolysis with endogenous or exogenous enzymes; (b) *in vivo* digestion by human enzymes in the gastrointestinal tract; and (c) fermentation and maturation (Shahidi & Zhong, 2008). Proteolysis of fish proteins liberate peptides with important physiological benefits including antihypertensive (Borawska, Darewicz, Vegarud, Iwaniak, & Minkiewicz, 2015), antioxidant (Klompong, Benjakul, Kantachote, & Shahidi, 2007), antimicrobial (Jemil et al., 2014), antitumor (Wang et al., 2010) and immunoenhancing (Duarte, Vinderola, Ritz, Perdigón, & Matar, 2006) activities.

Exogenous enzyme(s) preparation are widely used to produce bioactive fish protein hydrolysates (FPHs). These enzymes are selected on the basis of their specificity to hydrolyse specific peptide bonds in the polypeptide chain. Hydrolysis conditions are often manipulated for optimum activity of enzymatically-induced degradation reactions to liberate peptides of interest and the enzymatic activity in the hydrolysate is terminated once a desirable degree of hydrolysis is achieved. The bioactive peptides released after *in vivo* or *ex vivo* hydrolysis of fish myofibrillar proteins may differ from those obtained using *in vitro* laboratory hydrolysis. Carp myofibrillar proteins obtained after *ex vivo* and *in vitro* digestion demonstrated differences in the percentage of depolymerized proteins, bioactive properties and sequences of liberated peptides (Borawska et al., 2016). *In vitro* proteolysis of fish proteins using selected commercial enzymes to yield bioactive peptides may be of increasing importance for the

pharmaceutical industry. This cannot be compared to the properties of peptides released following gastrointestinal digestion in humans. Less is known about the specificity and potential of fish endogenous muscle enzymes to release peptides with potent bioactive properties during post-mortem storage. This is particularly of interest considering the consumption of whole fish in the form of fillet.

Endogenous proteases, mainly calpains and cathepsins, are active in post-mortem fish muscle and mostly involved in tissue softening (Chéret et al., 2007). In addition, these proteases may be responsible for the release of bioactive peptides from fish myofibrillar proteins during post-mortem storage. Endogenous muscle proteases active at post-mortem pH cause limited hydrolysis of fish myofibrillar proteins and generate a number of polypeptides and oligopeptides (Ahmed et al., 2015). High activity of endogenous muscle proteases accelerates the rate and extent of fish myofibrillar proteolysis and increases the levels of oligopeptides released during post-mortem storage (Nishimura, Rhue, Okitani, & Kato, 1988). These oligopeptides and small peptides may demonstrate important biological properties *in vitro* and/or upon further digestion by human digestive enzymes in the gastrointestinal tract. However, high level of peptides released may or may not indicate that liberated peptides are demonstrating bioactive properties. Alternatively, enzymatic digestion of fish proteins by human digestive enzymes may result in further increase in bioactivity of released peptides (Samaranayaka et al., 2010).

Hydrolysis of muscle proteins derived from underutilized fishery resources may yield a mixture of peptides with potent physiological benefits such as antioxidative properties (García-Moreno et al., 2014; Samaranayaka & Li-Chan, 2008). If underutilized fishery species were found to have physiological benefits in addition to their nutritional composition, this may improve their utilization for human foods. The literature lacks knowledge with respect to the role endogenous muscle proteases play in releasing peptides with potent bioactive properties

during post-mortem storage of underutilized fishery species. Storage conditions affected the endogenous activity of calpain-like, cathepsins B and B+L in Australian underutilized fish species (Ahmed et al., 2013b). Furthermore, endogenous enzymes active in silver warehou muscle significantly influenced the extent of myofibrillar proteolysis at post-mortem pH (Ahmed, Donkor, Street, & Vasiljevic, 2016). Silver warehou muscle was characterized with high endogenous activity of calpains and cathepsins B and B+L at post-mortem pH of muscle (5.5, 6.0 or 6.5) (Ahmed et al., 2013b). Using this knowledge, particular pH (6.0, 6.5 or 7.0) levels were selected in the current study to reflect post-mortem pH of muscle associated with increased activity of calpains and cathepsins and determine the feasibility of producing bioactive peptides during post-mortem storage without the need to use commercial enzymes.

The aim of the current study was to establish the role endogenous muscle proteases play in: (a) releasing oligopeptides during post-mortem storage of silver warehou muscle; and (b) contributing to the DPPH radical scavenging activity of liberated peptides. This was achieved by examining the effect enzyme-inhibitors and (or) activators and changes in pH has on the release of peptides with DPPH radical scavenging activity from myofibrillar proteins post-mortem. This research would provide an insight about the role endogenous muscle proteases play in making underutilized fish muscle bioactive during post-mortem storage.

6.2. Materials and methods

6.2.1. Materials

Fresh silver warehou provided by a local supplier (Barwoon Foods, Geelong North, VIC) was used in the study. Fish samples were transported on ice within 24 h from harvest to our laboratory (Victoria University, Werribee) and stored in a cold room for further processing. Samples were processed within 1-2 h of dispatch. All chemicals and reagents were of the highest grade commercially available.

6.2.2. Adjusting pH of samples during storage

The pH was adjusted following Ahmed et al. (2016). Samples were incubated for 0, 15, 30, or 60 min and for 1, 3 or 5 days. Following incubation, samples were homogenized in potassium chloride (KCl) (Merck) (30 s each over 1 min interval) and their pH was measured.

6.2.3. Muscle treatment with protease(s) inhibitors and (or) activators during storage

Fish muscle samples (6 g) were stored in the absence or presence of protease(s) inhibitors and (or) activators as described by Ahmed et al. (2016). Protease(s) inhibitors used were antipain (Sigma Aldrich) at 0.1 mM, E-64 [1-(L-trans-epoxysuccinylleucyamino-4-guanidinobutane] (Santa Cruz Biotechnology, Dallas Texas) at 0.1 mM, Ethylenediaminetetraacetic acid (EDTA) (Merck) at 5 mM, pepstatin A (Santa Cruz Biotechnology) at 0.1 mM, phenanthroline (Merck) at 1 mM and phenylmethyl sulfonyl fluoride (PMSF) (Merck) at 1 mM. The cysteine protease activator, dithiothreitol (DTT) (Sigma Aldrich) was used at 5 mM. No protease(s) inhibitors nor activators were added to the control. After the end of the desired incubation period, crude extracts were isolated.

6.2.4. Preparation of crude extracts

Stored samples from section 6.2.3 were homogenized with four volumes of ice-cold 50 mM phosphate buffer (pH 7.5), containing 0.02% NaN_3 on ice using a homogenizer twice for 30 s. The homogenate was centrifuged (Sorval RT7, Thermo Scientific, Rockford, IL) at 2,684 g (4 °C for 25 min) and the supernatant, identified as crude extracts, was filtered using 0.45 µm filter and frozen at -20 °C for further analysis.

6.2.5. Protein concentration

The BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific) was used to determine the protein concentration in the crude extracts and the absorbance of the solution was measured at 562 nm (Libra S11 UV/Visible Spectrophotometer, Cambridge, United Kingdom).

6.2.6. HPLC analysis of soluble polypeptides released from partially hydrolysed muscle

High performance liquid chromatography (HPLC) (Prominence – I LC -2030C, Shimadzu Corp., Model: Prominence–*i* LC-2030C, Kyoto, Japan) was used to separate soluble polypeptides (crude extracts from section 6.2.4) by a linear gradient from 100% to 0% solvent A (0.1% triflouroacetic acid (TFA) in deionised water) and solvent B (0.1% TFA in acetonitrile) over 90 min at a flow rate of 0.75 ml min^{-1} (Donkor, Henriksson, Singh, Vasiljevic, & Shah, 2007). Samples (20 µl per injection) were injected into a reversed-phase (RP-HPLC) column (C18, 250 mm × 4.6 mm, 5 µm, Grace Vydac, Hesperia CA) and eluted peaks were detected at 214 nm. All samples and solvents were filtered through a 0.45 µm membrane filter and runs were performed at 4 °C.

6.2.7. DPPH radical scavenging activity of liberated peptides

The radical scavenging activity of peptides released during autolysis was measured against 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich) free radicals using a previously reported method (Sah, Vasiljevic, McKechnie, & Donkor, 2015) with slight modification. Briefly, exactly 40 µl of the crude extract sample (0.1 mg protein/ml) was added to 2 ml of 0.05 mM DPPH dissolved in 95% ethanol. The solution was kept in the dark for 30 mins and then centrifuged (2055 × g for 5 min). The absorbance of the solution was measured at 517 nm (Libra S11 UV/Visible Spectrophotometer). For the blank, Milli Q water was used instead of

sample. Radical scavenging capacity of the samples was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{(\text{Absorbance of blank} - \text{Absorbance of sample})}{\text{Absorbance of blank}} \times 100$$

6.2.8. Statistical analysis

DPPH radical scavenging activity was replicated three times and analysed using the General Linear Model (GLM) developed by the Statistical Analysis System (SAS). Statistically significant level was defined at $P \leq 0.05$. The model included effects of muscle treatment (presence of protease(s) inhibitors and (or) activators) as the main plot, pH (6.0, 6.5 or 7.0), incubation time in days (1, 3 or 5), and their interactions presented as subplots.

6.3. Results and discussion

6.3.1. Muscle pH adjustment during storage

Samples were incubated in phosphate buffers with pH 5.5, 6.5 or 7.0 at RT for 1, 3 or 5 days to adjust muscle pH during storage. Initial pH of samples was approximately 6.4. Samples incubated in buffers with pH 5.5, 6.5 or 7.0 reached an ultimate pH of 6.0, 6.5 or 7.0, respectively within the first 15 mins of incubation which remained stable until the end of the storage period. These conditions are relative to post-mortem pH of fish muscle and also correspond to activation of endogenous calpains and cathepsins in silver warehou muscle (Ahmed et al., 2013b). The previous study did not include pH 7.0, however it is included in the current study to establish the effects of calpains and other proteases having neutral pH optima on the release of peptides during post-mortem storage.

6.3.2. Release of oligopeptides from partially hydrolysed muscle as influenced by treatment, changes in pH and storage time

In the current study, RP-HPLC was used to separate soluble oligopeptides from partially hydrolysed muscle stored at different pH in the presence or absence of enzyme(s) inhibitors and (or) activators. In RP-HPLC, peptides are eluted based on their differences in hydrophobic properties in order of increasing overall hydrophobicity and decreasing hydrophilicity (Mant & Hodges, 2008). The total hydrophobic amino acid content increases with retention time when peptides are eluted from the column (Girgih, Udenigwe, Hasan, Gill, & Aluko, 2013). The qualitative RP-HPLC profiles of liberated oligopeptides from the control and treated samples subjected to a varying pH condition throughout post-mortem storage are illustrated in Figures 6.1-6.3. A number of different peaks were eluted between 4 and 50 min. Differences in the release of hydrophobic peptides (between 38 and 50 min) were more prominent than hydrophilic peptides (between 4 and 15 min) in the control and treated samples, with the exception of PMSF. More hydrophilic peptides and less or negligible hydrophobic peptides were released in samples treated with PMSF.

The peptide profiling of partially hydrolysed muscle was clearly affected by the inhibition and (or) activation treatment of samples relative to the control (Figures 6.1-6.3). Our results indicate that endogenous muscle proteases also influenced the concentration of peptides generated during storage. Muscle treatment caused differences in the peptide profiles of partially hydrolysed muscle possible due to protease specificity. The presence of E-64 strongly inhibits the activity of cysteine proteases, including calpains and cathepsins (Matsumoto et al., 1999). Antipain inhibits the activity of cysteine and serine proteases (Otto & Schirmeister, 1997). The activity of serine proteases is limited in the presence of PMSF (Sharma & Radha Kishan, 2011). DTT acts as an activator of cysteine proteases. Although our previous study

demonstrated inhibiting general trend of cathepsins B and B+L activity in the presence of DTT, however calpains activity was not measured in the study. Calpains activity is strongly stimulated in the presence of DTT (Shoshan-Barmatz, Weil, Meyer, Varsanyi, & Heilmeyer, 1994). Pepstatin A inhibits the activity of aspartic acid proteases including cathepsin D (Marciniszyn et al., 1976). EDTA acts as a metal chelator and thus inhibits the activity of calpains and metalloproteases requiring metals for their activity (Angel et al., 2002), whereas phenanthroline limits the activity of metalloproteases (Day & Chen, 1998).

Changes in pH substantially influenced the profile of oligopeptides liberated from the control and treated samples. The release of hydrophobic peptides in the control (A) substantially increased with increasing pH at day 1 (Figure 6.1). Similarly, more hydrophobic peptides were liberated at pH 7.0 in comparison to pH 6.5 or 6.0 in samples treated with DTT (B), E-64 (C), antipain (D) and phenanthroline (G) at day 1 (Figure 6.1). Furthermore, more peptides were generated in the control and samples treated with phenanthroline at day 5 at pH 7.0 in comparison to slightly acidic pH (Figure 6.3).

Muscle pH significantly influenced the proteolytic activity of endogenous calpains and cathepsins in fish muscle. Silver warehou muscle was characterized with high endogenous activity of calpains and cathepsins B and B+L at post-mortem pH of muscle (5.5, 6.0 or 6.5) (Ahmed et al., 2013b). Endogenous activity of calpain-like enzymes significantly declined with reducing pH condition (Ahmed et al., 2013b), whereas cathepsins activity increased with muscle acidification in silver warehou muscle (Ahmed et al., 2016). The high endogenous activity of calpains and cathepsins detected in fish muscle at post-mortem pH may relate to the extent of myofibrillar proteolysis and peptides liberation during storage. Increased activity of endogenous muscle proteases at pH 6.0 relative to pH 6.5 or 7.0 accelerated myofibrillar proteolysis (Ahmed et al., 2016). However, our results indicate that increased activity of endogenous muscle enzymes at pH 6.0 did not correlate with the number of peptides released

during post-mortem storage. The general trend observed in our study was that more peptides were liberated at pH 7.0 than 6.5 or 6.0 in the control as well as in samples treated with E-64, antipain, DTT and EDTA at day 1 and 3.

The liberation of oligopeptides in the control as well as treated samples was affected to a certain extent by storage time (Figures 6.1-6.3). More peptides were generated in the control at pH 6.0 with increasing storage time. In comparison, the concentration of peptides released at pH 6.5 or 7.0 in the control and samples treated with E-64, pepstatin A and phenanthroline was higher at day 1 than subsequent storage. Samples treated with DTT liberated more peptides within days 1-3 at pH 6.0. Lower peptides were generated from EDTA-treated muscle with post-mortem time at pH 6.5 or 7.0. The concentration of peptides decreased markedly with post-mortem time in phenanthroline-treated muscle at pH 6.0. The concentration of hydrophilic peptides released in the presence of PMSF were unaffected by storage time.

The degree of proteolysis (Sah et al., 2015) and the levels of oligopeptides released during hydrolysis increases during storage (Nishimura et al., 1988). This suggests that peptides are increasingly generated with storage time due to continuation of proteolytic activity until storage conditions result in inactivation of endogenous enzymes. However, endogenous enzymes active in fish muscle during post-mortem storage may compete to digest the myofibrillar proteins substrates. If post-mortem conditions in muscle are optimal for the proteolytic activity of a particular class of enzymes that may have an impact on the extent of myofibrillar degradation, the release of peptides and possibly their bioactive properties. The type of endogenous muscle proteases active in muscle and their level of hydrolytic activity at defined conditions may influence the peptide profiling of partially hydrolysed muscle. This is possible due to the specificity and mode of hydrolytic action of each class of muscle proteases as well as changes in proteolytic activity due to pH variations. Inhibition of a particular class of proteases probably increases the likelihood of other proteases to compete at digesting

myofibrillar proteins at specific cleavage sites and release different peptides in comparison to those released by other proteases. For instance, inhibition of the activity of serine proteases through the use of PMSF increased the concentration of hydrophilic peptides in comparison to other treated samples and the control whilst limiting the release of hydrophobic peptides in comparison to the control.

6.3.3. Antioxidative properties of peptides released from partially hydrolysed myofibrillar proteins

The presence of antioxidative compounds in foods preserves the quality and increases the product stability by preventing oxidative deterioration of carbohydrates, lipids and proteins. In addition, the consumption of natural foods demonstrating antioxidant properties can protect the human body against oxidative stress-related degenerative diseases including inflammatory, diabetes, brain dysfunction, cardiovascular and cancer by scavenging free radicals and reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, superoxide anion and hydroxyl radicals (Choe & Min, 2009; Ngo, Vo, Ngo, Wijesekara, & Kim, 2012).

Antioxidants are naturally present in foods, added or formed during processing. Fish protein hydrolysates with antioxidant activities have been isolated from Pacific hake (Samaranayaka & Li-Chan, 2008), yellow strip trevally (Klompong et al., 2007), salmon (Girgih et al., 2013), carp (Borawska et al., 2016) and mackerel (Wu, Chen, & Shiau, 2003) muscles. These studies assessed the antioxidant properties of FPHs using exogenous enzymes under controlled hydrolysis condition.

High endogenous activity of muscle proteases in underutilized fish species may be an asset to produce a hydrolysate with potent biological activities (Samaranayaka & Li-Chan, 2008) without the need to add exogenous enzymes. However, this needs to be evaluated. In our

study, we evaluated the estimate and not the total of the antioxidant activity of hydrolysed silver warehou muscle using DPPH, a stable free radical (Sharma & Bhat, 2009).

The presence of protease(s) inhibitors and (or) activators in our study significantly ($P \leq 0.05$) influenced the antioxidant properties of peptides released in partially hydrolysed muscle (Table 6.1). The control (A) demonstrated different levels of antioxidant activity in comparison to treated samples (Figure 6.4). Changes in pH and storage time significantly influenced the antioxidant activity of the peptides released during storage (Table 6.1). Collectively, muscle treatment, changes in pH and storage time resulted in significant alteration of the DPPH radical scavenging properties of released peptides (Table 6.1). The antioxidant activity of peptides released in the control increased markedly with increasing muscle pH at day 1. Adjusting muscle pH to 6.0, 6.5 or 7.0 resulted in changes in the levels of antioxidant activity measured in samples treated with protease(s) inhibitors and (or) activators during post-mortem storage (Figure 6.4). This is probably due to the effect muscle pH has on the activity of endogenous muscle proteases. Some endogenous muscle proteases are activated with muscle acidification post-mortem while others become limited. The DPPH radical scavenging capacity of peptides liberated at day 1 in samples treated with EDTA (E) was significantly higher than control at pH 6.0 and 7.0. In contrast, peptides generated from partially hydrolysed muscle treated with antipain (D), DTT (B), E-64 (C), phenanthroline (G) and PMSF (H) did not demonstrate any DPPH radical scavenging activity within the first day of post-mortem storage in comparison to the control (Figure 6.4), suggesting that more levels of prooxidants than antioxidants are present in these samples relative to those found in EDTA and the control.

Table 6.1. Analysis of variance (ANOVA) demonstrating significance of the effects of muscle treatment (presence or absence of protease(s) inhibitors and (or) activators), pH (6.0, 6.5 and 7.0), and storage time (1, 3 and 5 days) and their interactions on the DPPH scavenging activity of liberated peptides.

Source	*p*-value
	DPPH radical scavenging activity
Treatment	<0.0001
pH	<0.0001
Time	<0.0001
Treatment × pH	<0.0001
Treatment × Time	<0.0001
Treatment × pH × Time	<0.0001

Storage time significantly influenced the antioxidant properties of the peptides released from partially hydrolysed muscle (Table 6.1). Increased generation of peptides possessing antioxidative properties was observed at day 3 in comparison to day 1 in the control and samples treated with antipain, DTT, E-64, pepstatin A and phenanthroline (Figure 6.4). It is possible that more important levels of peptides characterized with the capacity to entrap free radicals and reactive oxygen species were generated at day 3 (Tutel'yan, Klebanov, Il'ina, & Lyubitskii, 2003). In comparison, fewer peptides with DPPH radical scavenging properties were released at day 5 relative to day 3 in the control and samples treated with antipain, DTT, E-64, pepstatin A and phenanthroline.

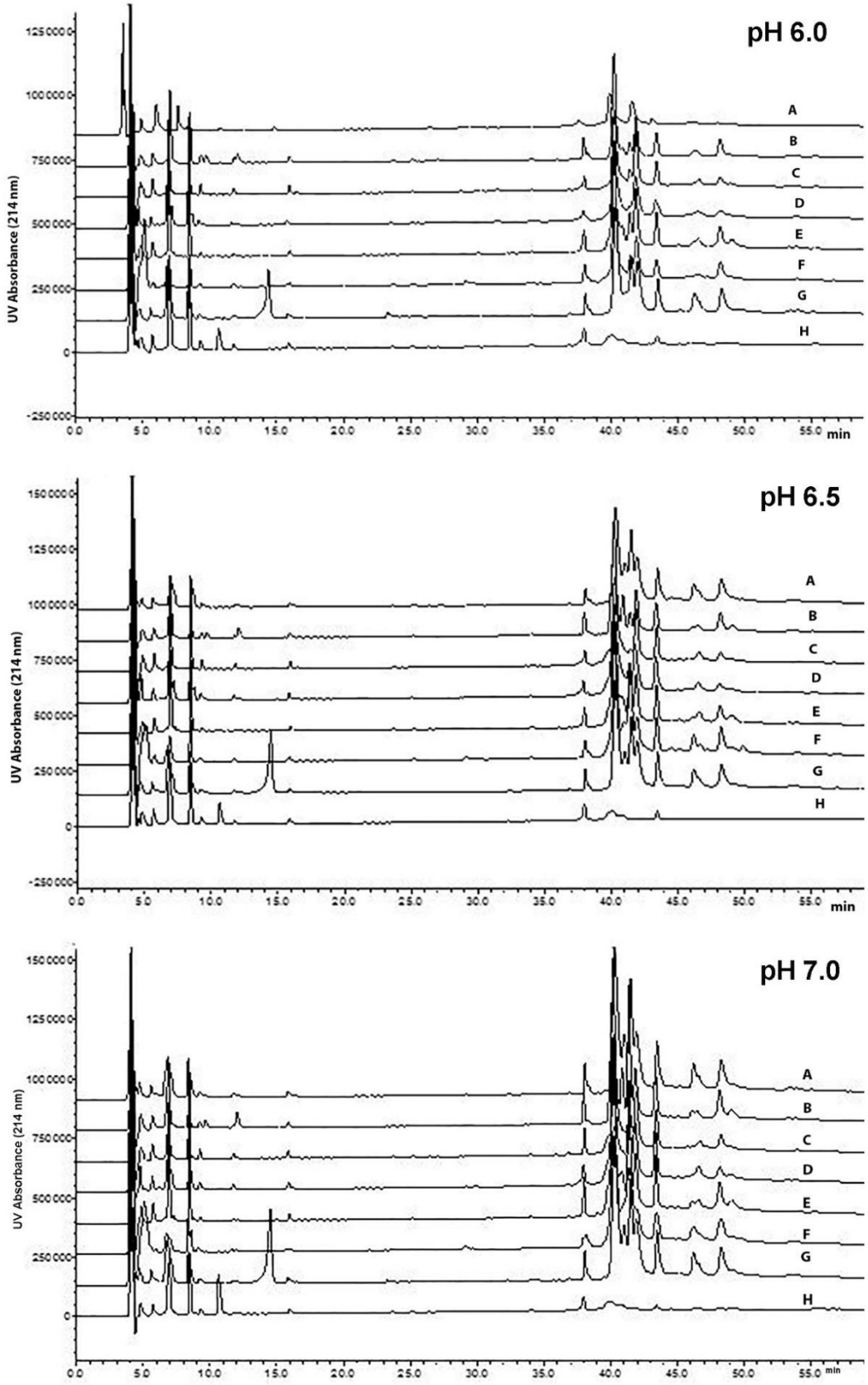

Figure 6.1. Peptide profiling of partially hydrolysed muscle as influenced by treatment (A: control, B: DTT, C, E-64, D: antipain, E: EDTA, F: pepstatin A, G: phenanthroline, and H: PMSF) and changes in pH at day 1.

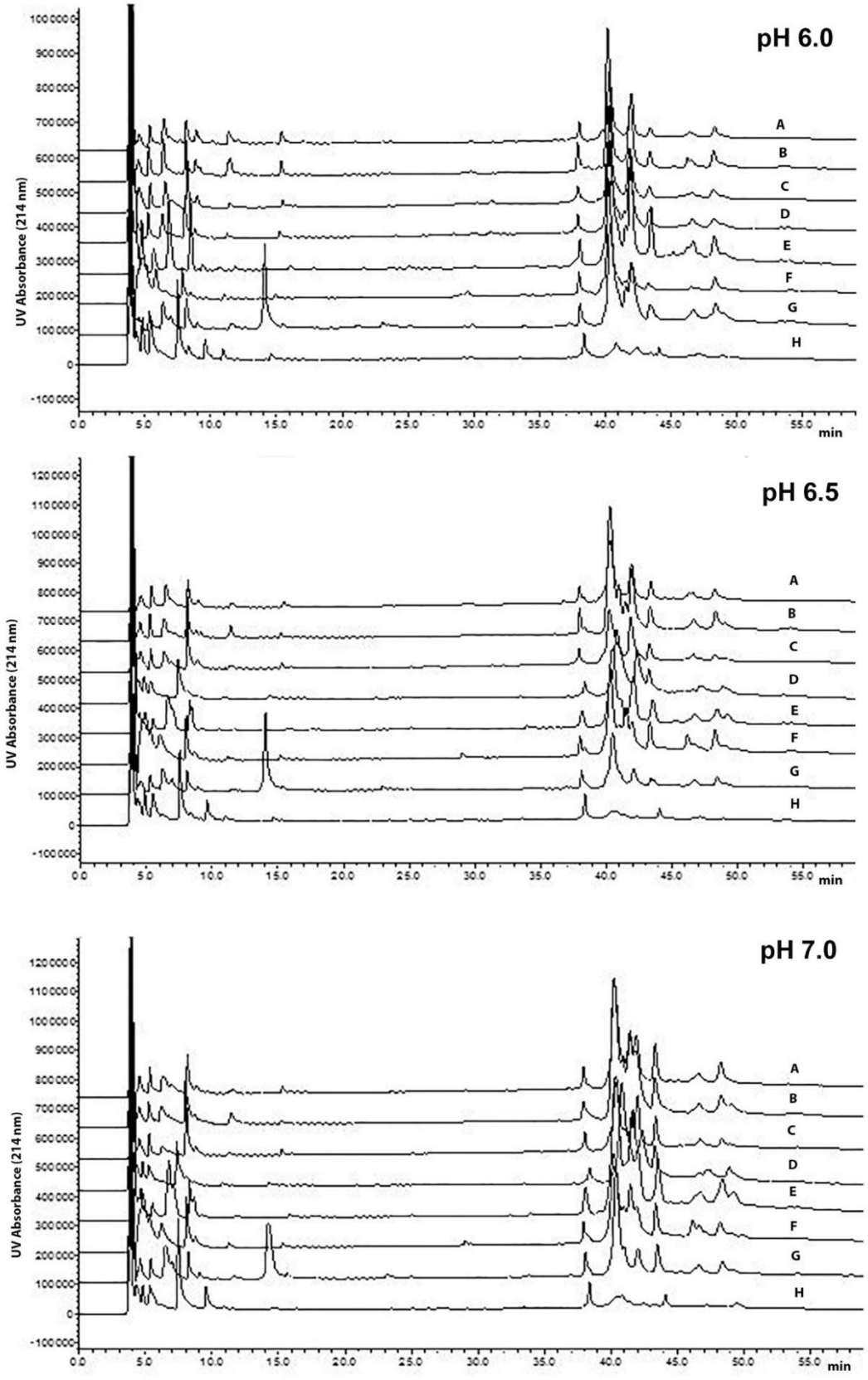

Figure 6.2. Peptide profiling of partially hydrolysed muscle as influenced by treatment (A: control, B: DTT, C, E-64, D: antipain, E: EDTA, F: pepstatin A, G: phenanthroline, and H: PMSF) and changes in pH at day 3.

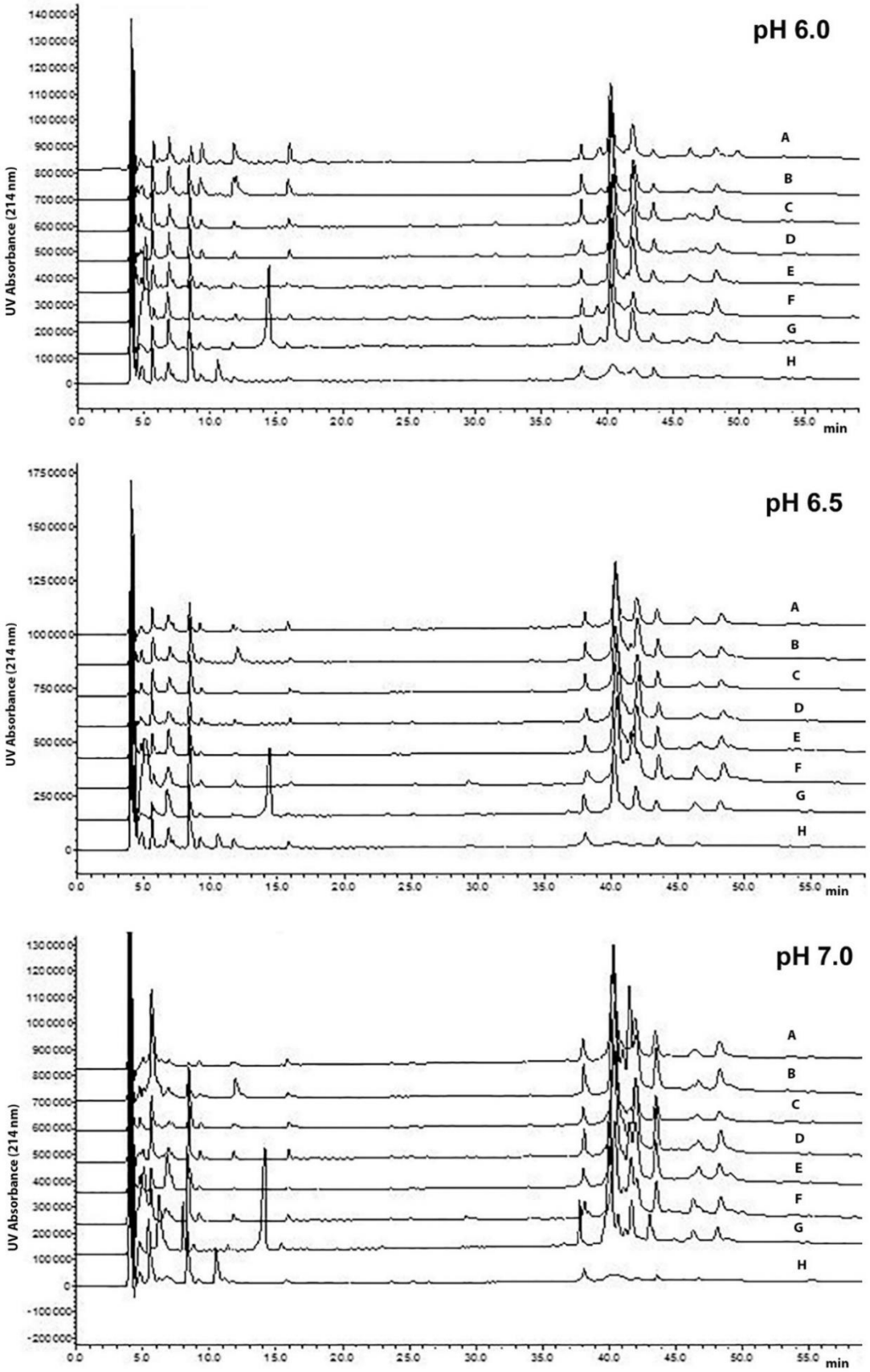

Figure 6.3. Peptide profiling of partially hydrolysed muscle as influenced by treatment (A: control, B: DTT, C, E-64, D: antipain, E: EDTA, F: pepstatin A, G: phenanthroline, and H: PMSF) and changes in pH at day 5.

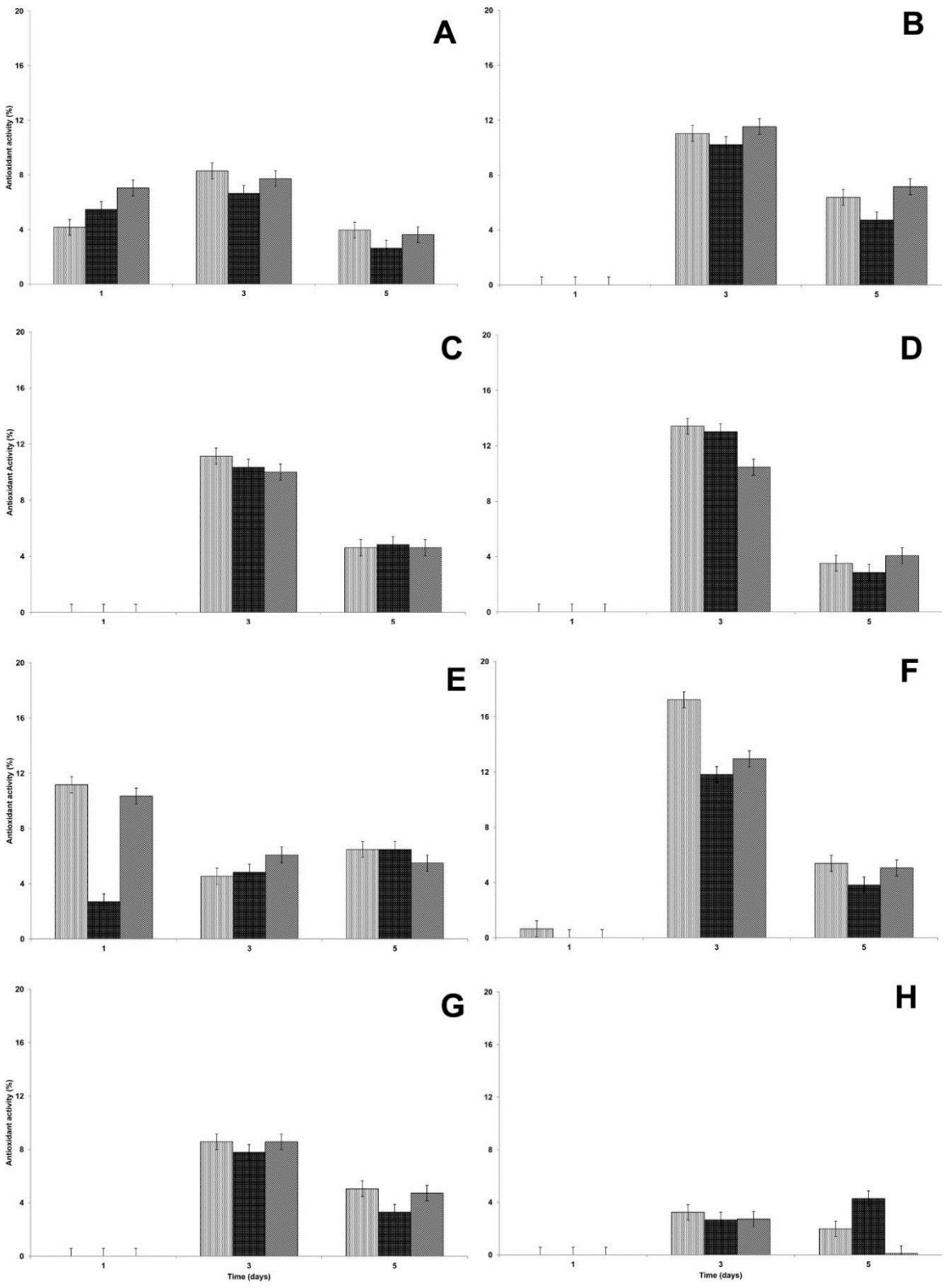

Figure 6.4. Antioxidative activity of peptides released from silver warehou muscle as influenced by muscle treatment during storage after 1, 3 or 5 days at different pH levels (6.0, 6.5 or 7.0); A: control, B: DTT, C: E-64, D: antipain, E: EDTA, F: pepstatin A, G: phenanthroline, and F: PMSF.

A few factors may influence the antioxidative properties of peptides liberated during hydrolysis of fish muscle. These include: (a) the nature and specificity of enzymes used; (b) their amino acid composition and sequence; (c) degree and condition of hydrolysis; (d) number of peptides released; and (e) molecular weight of released peptides. Enzymes with broader specificity release a high number of polypeptides and oligopeptides fragments (Darewicz et al., 2016), the majority of which may exhibit potent biological properties. The molecular weight of released peptides may also influence the bioactive properties of the resultant hydrolysates. Peptides having low molecular weight peptides may demonstrate improved bioactive properties in comparison to high molecular weight peptides (Raghavan & Kristinsson, 2009). A direct relationship may also exist between the degree of proteolysis and antioxidative properties of FPH (Klompong et al., 2007). Antioxidant activity of protein hydrolysates increased with increasing degree of protein hydrolysis (Liu, Kong, Xiong, & Xia, 2010). However, a good correlation does not always exist between the degree of protein hydrolysis and antioxidant activity of the resultant hydrolysate. The high degree of carp muscle tissue hydrolysis did not favour an increase in antioxidant activity (Borawska et al., 2015). The antioxidant activity of hydrolysates may be associated with the number of peptides released during post-mortem storage or FPH production. The highest peptides level detected in mackerel hydrolysate was associated with the most potent antioxidative activity (Wu et al., 2003). In our study, it was difficult to correlate the number of peptides released during post-mortem storage of silver warehou muscle with its DPPH radical scavenging capacity. Another factor that could influence the antioxidant properties of the protein hydrolysates is the molecular weight of liberated peptides. Peptide fractions with molecular weight lower than 3 kDa possessed the highest antioxidant activity in hydrolysates from underutilized silver carp muscle (Malaypally et al., 2015).

Degree of myofibrillar hydrolysis and the number of peptides released were less likely to be involved in improving the DPPH radical scavenging properties of released peptides. No correlation was observed between increased peptides release at pH 7.0 and enhanced antioxidant activity of fish muscle in the control and treated samples. Fewer differences with respect to bioactivity were observed with increased myofibrillar proteolysis at pH 6.0 and decreased myofibrillar proteolysis at pH 7.0.

The DPPH radical scavenging activity of peptides liberated from partially hydrolysed muscle in our study may be related to the specificity of the enzymes involved in post-mortem degradative reactions. The comparisons of the level of antioxidative activity of peptides released during storage of silver warehou muscle treated with protease(s) inhibitors and (or) activators (Figure 6.4) suggest that the same protein can be a source of different antioxidant peptides as previously determined by Borawska et al. (2016) and (or) different levels of antioxidative properties. Therefore, the differences in the levels of antioxidant activity released in post-mortem silver warehou muscle as influence by the presence of protease(s) inhibitors and/or activators may be due to the different specificities of the endogenous muscle proteases most active at post-mortem pH and to a minor extent due to spontaneous amino acid modifications.

Furthermore, antioxidant activity of peptides depends on its amino acids composition and their peptide sequences. Important levels of peptides with sulphur-containing amino acids, cysteine and methionine, improved antioxidant activity in FPHs. These compounds enhance the concentration of glutathione, a major component of cellular antioxidant system, which consequently reduce ROS and free radicals in the cell (Malaypally et al., 2015). Histidine-containing peptides also demonstrated high antioxidant properties (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). Hydrophobic amino acids at the N-terminal position

and in particular rich in leucine residues increased the antioxidant activity of peptides (Park, Jung, Nam, Shahidi, & Kim, 2001). The free radical scavenging activity of the peptides released from salmon protein hydrolysate increased with increased peptide hydrophobic character, except for hydroxyl radicals (Girgih et al., 2013). Similarly, hydrophobic amino acids present in the peptide sequence of jumbo squid contributed substantially to the antioxidant properties of the resultant hydrolysate (Mendis, Rajapakse, Byun, & Kim, 2005). Accordingly, extensive release of hydrophobic peptides from partially hydrolysed silver warehou muscle in our study is mainly associated with the appreciable DPPH radical scavenging activity.

In addition, the enhanced DPPH radical scavenging activity of peptides released from muscle treated with DTT, E-64 and antipain, pepstatin A in the third day of storage relative to the control (Figure 6.4) indicates that inhibition of cysteine and aspartic acid proteases improved the antioxidant properties of released peptides. This is mainly due to that the nature and specificity of enzymes in determining the antioxidative properties of peptides released from fish myofibrils as previously reported (Girgih et al., 2013). However, further studies may examine the contribution of endogenous cysteine and aspartic acid proteases in releasing peptides with important bioactive properties during post-mortem storage of fish muscle. Hydrolytic fragments of mainly actin and myosin are responsible for the improved antioxidant activity of the hydrolysate (Darewicz et al., 2016). Antioxidant peptides with sequences (YA, PR, HH, EL, VKV and KD) are encrypted in the structure of actin and myosin in sardine muscle (García-Moreno et al., 2014).

Important levels of peptides demonstrating DPPH radical scavenging capacity liberated in partially hydrolysed silver warehou muscle during storage could be beneficial. This property may be particularly useful for preventing oxidation of fish species during processing and

storage and improving its shelf life. Antioxidants can be used as prevention agents of adverse changes of texture before the occurrence of microbial spoilage in fish. Another application for FPH with important antioxidative properties is that it could be used to reduce oxidative processes *in vivo* and improve human health. Hydrolysates possessing antioxidative properties could be used in the development of functional foods and simultaneously improve the shelf-life of food products.

6.4. Conclusion

Endogenous muscle proteases cause limited hydrolysis of myofibrillar proteins and yield a number of oligopeptides. Muscle proteases active in post-mortem muscle influenced the peptide profiling of hydrolysed muscle and its DPPH radical scavenging capacity. It appears that the same protein may release different antioxidative peptides and (or) activities. This is mainly due to the differences in the specificity of proteases and their hydrolytic capacity to breakdown myofibrillar proteins at specific cleavage sites and result in release of peptides demonstrating the ability to entrap DPPH radicals. Inhibition of cysteine and aspartic acid proteases improved the DPPH radical scavenging activity of liberated peptides slightly. In addition, increased generation of hydrophobic peptides during post-mortem storage of muscle was associated with enhanced DPPH radical scavenging activity of peptides. This study suggests that cysteine and aspartic acid proteases accelerate the degradation of myofibrillar proteolysis and tissue softening in fish muscle but are less likely to improve the bioactive properties of fish-derived peptides. However, further work may be needed to relate the activity of these proteases in post-mortem fish muscle to other important bioactive properties such as antihypertensive, antimicrobial and immunomodulation.

CHAPTER 7

Conclusions and future research recommendations

7.1. Conclusions

Endogenous muscle proteases, calpains and cathepsins, were involved in proteolysis of major structural muscle proteins associated with tissue softening and generation of peptides with appreciable bioactive properties during post-mortem storage of underutilized fishery species. Calpains initiate limited hydrolysis of high molecular weight proteins early post-mortem. Activity of cathepsins results in the degradation of low molecular weight proteins during late post-mortem storage of muscle. Ultimately, proteolysis of fish myofibrils post-mortem increases the release of polypeptide fragments and oligopeptides. Some of these peptides may demonstrate one or more bioactive properties important for modulating human health.

Knowledge on the proteolytic activity of calpains and cathepsins in underutilized fish species generated in this research may be used to demonstrate which underutilized fish species are suitable as raw materials for further seafood processing. The activity of endogenous cathepsins B, L, D, H and calpain-like enzymes in sixteen Australian underutilized fish clearly was species dependant, which implies that the handling and application of these species may differ substantially. Barracouta was shown to have higher endogenous activity of cathepsins B, D, H and L when compared with the other underutilized fish species. Activity of cathepsins B and B+L was found to be higher than that of cathepsin H in the crude enzymes extracted from the muscle of sixteen Australian underutilized fish species. Furthermore, lower endogenous activity of cathepsin D and H in all the examined species, suggested that these enzymes may not be one of the major muscle proteases causing the degradation and thus, the softening of fish muscle during post-mortem storage. The underutilized fish species with low endogenous proteolytic activities may be suitable for surimi production, and those exhibiting high endogenous protease activities may be used for fish sauce production. High level of cathepsin

B+L activity demonstrated by barracouta, mirror dory and jackass morwong may indicate that these species would soften at a faster rate during cold storage, making them unsuitable for surimi production or any other application demanding low endogenous protease activity. However, these species may be more suitable for fish sauce production or any other application which require the presence of more active endogenous enzymes to hydrolyse muscle proteins during fermentation. Fish species such as Japanese sandfish, characterized with high endogenous proteases activity, are commonly used in the production of fish sauce. Accordingly, Ocean perch, nannygai, eastern school whiting, bight redfish, ribbon fish, tiger flathead, yellowspotted boarfish and Australian salmon may be suitable for surimi production due to their low endogenous activity of cathepsins B and L.

Processing and (or) storage conditions that control the activity of major endogenous enzymes during post-mortem storage, in more commercially valued species were established to minimise their detrimental effects on fish myofibrillar proteins during iced storage and possibly guarantee optimum quality for some fishery products when these species are used as raw materials. Ionic strength (0, 500, or 1000 mM NaCl), pH (5.5, 6.0, or 6.5), and temperature (2.0, 4.0, or 6.0 °C) clearly individually and in combination influenced the activity of these enzymes. Salting of silver warehou muscle inhibited the hydrolytic activity of cathepsin B at pH 6.5 to 6.0, which may consequently slow its rate of softening during storage. Similarly, salting during refrigerated storage (4 °C) of ribaldo (pH 6.0) and ribbonfish (pH 6.5) muscles may reduce the softening rate and influence their shelf life due to calpain-like inhibition effect. Therefore, through processing conditions the activity of endogenous muscle enzymes in underutilized fish species may be manipulated to control the softening process and (or) enhance myofibrillar proteolysis for release of peptides which may be important for flavour development and (or) as bioactive components. This may potentially diversify utilization of underutilized fish species.

Post-mortem muscle pH (6.0, 6.5 or 7.0) significantly influenced different proteolytic activity and the way these enzymes act on silver warehou myofibrillar proteins. The degree of myofibrillar weakening post-mortem varied depending on optimum conditions related to each type of proteases during storage. Actin was more resistant to proteolytic cleavage than MHC in silver warehou muscle. Degradation of actin and MHC increased substantially at slightly acidic pH. Cysteine proteases including calpains and cathepsins B and L accelerated post-mortem autolysis of myofibrils during storage. In addition, aspartic acid proteases (mainly cathepsin D) were also responsible for post-mortem degradation of muscle proteins. Other proteases were also involved in post-mortem hydrolysis of myofibrils, however to a less extent.

Endogenous muscle proteases cause limited hydrolysis of myofibrillar proteins and yield a number of oligopeptides. Endogenous muscle enzymes active in post-mortem silver warehou muscle influenced the peptide profiling of hydrolysed muscle and its DPPH radical scavenging capacity. It appears that the same protein may release different antioxidative peptides and (or) activities. This was mainly due to the differences in the specificity of proteases and their hydrolytic capacity to breakdown myofibrillar proteins at specific cleavage sites and result in release of peptides demonstrating the ability to entrap DPPH radicals. Inhibition of cysteine (calpains and cathepsins B and L) and aspartic acid (mainly cathepsin D) proteases improved the antioxidative activity of liberated peptides slightly, however this may be an area to be explored in future studies. In addition, increased generation of hydrophobic peptides during post-mortem storage of hydrolysed muscle was associated with enhanced antioxidative activity of peptides.

7.2. Future research recommendations

Activity of endogenous enzymes in post-mortem fish muscle appeared to be manipulated using processing conditions. An understanding of how the proteolytic activity of endogenous enzymes in the catch can be manipulated and treated may be useful for the design of proper handling and storage procedures for maintaining optimum quality of fish and fishery products. Established effects of storage conditions on the activity of endogenous muscle proteases in Australian underutilized fish species may have implications for processing low value species for a wide range of fishery products and likely improve their market value. Our research provided information about the activity of major endogenous in underutilized fish species which may determine their suitability as raw materials during processing of different seafood products. Storage conditions may be changed during processing to manipulate the proteolytic activity of calpains, cathepsins B and B+L enzymes post-mortem. Conditions which maintain endogenous proteases activity may be used during processing to reduce the rate of softening. In contrast, fish may be stored in an environment that supports enzymatic activity to possibly increase the rate of proteolysis and liberation of peptides with functional and (or) bioactive properties. This may potentially diversify utilization of underutilized fish species. Furthermore, marine fisheries and aquaculture should work closely with the seafood processing sector of the seafood industry to make sustainable use of our marine resources and thus incorporate low value species in seafood products manufacturing. Future work should examine the applicability of using underutilized fish species for a wide range of seafood applications. In addition, further studies may be required to identify practical use of underutilized fish species for other applications. For instance, the applicability of extracting valuable components such as amino acids, ω-3 polyunsaturated fatty acids, enzymes, peptides, collagen and gelatine from underutilized fish muscle to incorporate as ingredients into value-added food products need to be explored.

Future research may consider using enzymatic modifications of underutilized fish species to improve the functional properties of muscle and its utilization for many applications in the food industry. In addition, fish muscle from underutilized fishery resources may be used as a substrate for release, separation and concentration of peptides with potent physiological effects important in pharmaceutical applications. Nonetheless, the potential of using fish endogenous muscle enzymes for harvesting bioactive peptides from muscle proteins should be closely examined. Future studies may determine the relationship between the activity of endogenous proteases in post-mortem muscle and the bioactive properties of fish proteins *in vivo*. Further work may be needed to relate the activity of these proteases in post-mortem fish muscle to important bioactive properties such as antihypertensive, antimicrobial and immunomodulation. Nonetheless, more work may be needed to develop a large-scale fractionation of peptides with enriched bioactive properties to be used as nutraceutical additives in functional foods.

CHAPTER 8

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