IN-VITRO PHYSIOLOGICAL ACTIVITIES OF PEPTIDES DERIVED FROM UNDERUTILISED AUSTRALIAN FISH SPECIES

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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B.Sc. Hons. (Nutrition, Food & Health Sciences)

August 2016

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Victoria University, Werribee Campus, VIC, Australia
I dedicate my PhD thesis to my late
grand-father, Mr. Soopaya Manikkam
&
to my parents for their deep belief in
education.
ABSTRACT

Australian underutilised fish species, such as silver warehou (*Seriollela punctata*) and eastern school whiting (*Sillago flindersi*) as well as fish by-products may be potent sources of bioactive peptides. These species and/or their by-products are often wasted due to their poor technological and textural properties, and not acceptable for consumption by the Australian consumers. Interestingly, these fish wastes possess important nutritional value and physiological benefits, owing to their high protein content. Technological processing and the presence of endogenous enzymes in the fish muscle have the potential to release the so-called bioactive peptides during storage or digestion in the gastrointestinal tract. In the new era of the field of food science and technology, the production of bioactive peptides released from fish wastes is becoming increasingly important to preserve the marine sustainable environment and develop essential functional food to maintain human health as well as preventing the risks of developing certain types of metabolic diseases, like obesity and/or metabolic syndrome.

As a result, the main focus of this project was to investigate the controlled hydrolysis of fish muscle proteins from by-catch fish species as a means of producing bioactive peptides with beneficial physiological properties. Important *in-vitro* bioactivities investigated could be related to the prevention of obesity and associated health complications. They include i) angiotensin-converting enzyme (ACE – hypertension) inhibition, ii) trypsin inhibition (satiety-induced) and iii) anti-oxidative (oxidative stress and inflammation-related conditions) activity.

In our introductory study, the physicochemical and biofunctional properties of two types of fish protein hydrolysates, derived from fish scales were investigated in the presence of digestive enzymes; namely, pepsin, trypsin and pancreatin. This study demonstrated that enzymatic hydrolysis can release physiologically active peptides,
which can vary according to the processing conditions. Powdered hydrolysate (PH) exhibited higher biological activities, ACE inhibitory activity of 73.65% compared to agglomerated hydrolysate (AH). The protein content of PH determined the higher biological activities; the higher the protein content, the greater the solubility. This study provides a sound example for the development of protein hydrolysates from Australian fish fillets. However, the processing methods must be designed carefully to achieve optimal recovery of peptides as well as their optimum bioactivities.

Australian underutilised fish species may serve as a potential source of valuable proteins and potent bioactive peptides. This novel research was the first to investigate the effects of storage-processing conditions and an in-vitro pepsin-pancreatin simulated gastrointestinal digestion (SGID) on bioactive peptides’ release during storage of fish fillet, derived from Australian silver warehou (SW). In-vitro bioactivities including ACE and trypsin inhibitory and antioxidant activities were analysed. The antioxidant power was evaluated by DPPH free radical scavenging activity, copper ions chelating and ferric ions reducing abilities. Fillets were stored at chilled (4 and 6°C) and freezing (-18°C) temperatures for 7 and 28 days, respectively. Results indicated that during postmortem storage, endogenous enzymes from fillets released an array of polypeptides over storage time. The demonstrated physiological activities were further increased (p<0.05) during SGID. Bioactivities were greater at 4°C, increasing over 7 days (p<0.05) as compared to 6 and -18°C. An increase by 2°C for chilled temperature was enough to cause significant changes (p<0.05) in activities. The crude extracts obtained by pancreatin treatment demonstrated highest metal chelating activities at 4°C (86.3± 0.1% on day 7). Physiological potency, especially metal chelating activity, of fillets obtained from SW may be manipulated by storage conditions that would consequently be further enhanced during digestion.
Similarly, underutilised Australian eastern school whiting (ESW) fish was investigated for *in-vitro* bioactivities, after exposure to fish endogenous and gastrointestinal (pepsin and pancreatin) enzymes. The study comprised of storing fish at chilled (4 and 6°C) and freezing (-18°C) temperatures for 7 and 28 days, respectively. Hydrolysis by endogenous enzymes only, resulted in increased bioactivities for the 4°C samples, whereas significant decreases (p<0.05) were observed for the 6 and -18°C samples. However, bioactivities of these samples increased significantly (p<0.05) after further hydrolysis under simulated digestion conditions. Proteolysis by digestive enzymes, mainly pancreatin considerably enhanced the antioxidant activities. To benefit from the health properties of ESW fish, it is suggested to consume the fish fresh. The intent is to enhance full use of fish and not certain parts such as fish oil. For proper utilisation and sustainability, whole fish must be used.

As a whole, the production of bioactive peptides or hydrolysates derived from marine wastes may deliver *in-vitro* physiological activities, whilst minimising aquatic wastes and maintaining an ecological marine environment.

The significance of the research corresponds to the utilisation of Australian commercial inferior marine bio-resources, which are perceived as by-catch or wasted fish species. The main innovative strategies of this project was to:

i) manipulate fish fillet by storage and processing conditions to maximise the production of biological active peptides

ii) understand the mechanisms by which endogenous fish muscle hydrolytic enzymes from underutilised fish species liberate bioactive peptides

iii) increase and improve the use and value of Australian bio-resources, that is, novel fish species for the development of human food but processed...
in ways that is intended to improve human health whilst at the same time enhancing the biodiversity of our marine ecosystem by decreasing economic pressure on existing species.
CERTIFICATE

Associate Professor Michael L. Mathai, PhD
Centre for Chronic Diseases
College of Health and Biomedicine
Victoria University, St Albans Campus
Victoria, Australia

This is to certify that the thesis entitled “IN-VITRO PHYSIOLOGICAL ACTIVITIES OF PEPTIDES DERIVED FROM UNDERUTILIZED AUSTRALIAN FISH SPECIES” submitted by Vasambal Manikkam in partial fulfilment of the requirement for the award of the Doctor of Philosophy with specialization in Food Science and Technology at Victoria University is a record of bonafide research work carried out by her under my guidance and supervision and the thesis had not previously formed the basis for the award of any degree, diploma or other similar title.

Associate Professor Michael L. Mathai
(Principal Supervisor)
Date: 31st August 2016
DECLARATION

"I, Vasambal Manikkam declare that the PhD Thesis by Publication entitled "IN-VITRO PHYSIOLOGICAL ACTIVITIES OF PEPTIDES DERIVED FROM UNDERUTILIZED AUSTRALIAN 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."

Vasambal Manikkam
College of Health and Biomedicine
Victoria University, Werribee Campus, VIC, Australia
Date: 31st August 2016
ACKNOWLEDGEMENTS

“There were days of happiness, there were days of sadness, there were days of despair, there were days of belief and disbelief, there were months of lightness, there were months of darkness, there were months of laughter, there were months of dedicated hours in the lab, there were months of full commitment, there were some laid-back days, there were months of fears and tears, with many sleepless nights, there were finally the moments where I could see achievements, with the eventual moments of success...Reflecting on this long and unique journey of mine, it has been a massive roller coaster and learning curve...yet extremely rewarding experience to be cherished...”

-Vasambal Manikkam-

The above quote best encapsulates my long stint as a PhD scholar at Victoria University. The compilation of this thesis would have never been possible without the guidance, help, support, patience, assistance and endless encouragement of countless number of people who have constantly been by my side throughout this memorable journey.

First and foremost, I would like to express my sincere gratitude and thanks to my academic Sherpas. I deeply acknowledge the ongoing guidance, support, discussions of my principal supervisor, Associate Professor Michael L. Mathai throughout the duration of my PhD. I gained valuable insights on renin-angiotensin system (RAS) and its correlation with obesity, body fat and hypertension.

My sincere appreciation goes to my co-supervisors Professor Todor Vasiljevic (Leader, Advanced Food Systems Research Unit, College of Health and Biomedicine) and Dr. Osaana Donkor (Senior Lecturer, College of Health and Biomedicine) for their extensive
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I extend my gratefulness to the Australian Research Council (ARC) – Industry grant for the funding of my research. I am also thankful to the College of Health and Biomedicine, Victoria University, for the financial support. In addition, I extend my appreciation to Geelong Food Co-Clusters (GFCC) for provision of fish samples to conduct my experimental work.

I further acknowledge the laboratory manager, Mr. Nikola Papovik and dedicated technical staff at Werribee campus for their help in materials procurement and technical/instrumental assistance; mainly Mr. Joe Pelle, Mrs. Stacey Lloyd, Mrs. Mary Marshall, Mrs. Charmaine DiQuattro, Mrs Min Thi Nguyen and Dr. Sarah Fraser. Moreover, Dr. Nicolas Milne, Research Fellow from the Institute for Sustainability (ISI), Victoria University, is also recognised for training and assistance with scanning electron microscope.

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An abundance of sincere thanks are expressed to my friends Corrine and Glyn for their whole-hearted and immense support. Many thanks to Freda, Henri, Ramsay, Rivana, Dr. Marta Siegel and Dr. Teresa DeFazio for believing in me, continuing console, source of strength, great sense of humour and especially Norbert for his patience and assistance with document formatting. They all form a big part of my life, inspiring me to strive towards my goals during my PhD endeavour.

Words cannot explain how appreciative I am of my dearest mother and father for all their love, support and sacrifices they have made to provide me with the best education and resources possible. They have always supported me in all of my pursuits. I really hope I have made you very proud parents.
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.

<table>
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<th>Paper Title</th>
<th>Publication Status</th>
<th>Publication Title and Details</th>
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<td>2B</td>
<td>A review of potential marine-derived hypotensive and anti-obesity peptides</td>
<td>Published</td>
<td>Critical Reviews in Food Science and Nutrition; SRJ Q1</td>
</tr>
<tr>
<td>3</td>
<td>Biofunctional and physicochemical properties of fish scales collagen-derived powders</td>
<td>Published</td>
<td>International Food Research Journal; SRJ Q2</td>
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<td>4</td>
<td>Sustainable use of Silvor Warehou (Seriola punctata): Effects of storage, processing conditions and simulated gastrointestinal digestion on selected in-vitro bioactivities</td>
<td>Accepted for Publication Manuscript ID: JFST-0-15-02026R3</td>
<td>Journal of Food Science and Technology; SRJ Q2</td>
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<td>5</td>
<td>Australian Eastern School Whiting (Sillago flindersi): a potential source of metal reducing agents and free radical scavengers</td>
<td>Accepted for Publication Manuscript ID: IFRJ 18324.R1</td>
<td>International Food Research Journal; SRJ Q2</td>
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Declaration by [candidate name]: Vasambal Manikkam

Signature: 

Date: 31/08/2016


Presentations

CHAIRED ORAL PRESENTATION

Vasambal Manikkam, Todor Vasiljevic, Wayne Street, Osaana N. Donkor and Michael L. Mathai (2015). Physiological functionality of Australian silver warehou (Seriolella punctata) changes with storage temperature. Postgraduate Student Research Conference 2015, St Albans Campus, Victoria University, Melbourne, Australia.

CHAIRED POSTER PRESENTATION

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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AACE</td>
<td>American Association of Clinical Endocrinologists</td>
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<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
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<td>AH</td>
<td>Agglomerated Hydrolysate</td>
</tr>
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<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CCK-RP</td>
<td>Cholecystokinin Releasing Peptide</td>
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<tr>
<td>CHO</td>
<td>Carbohydrate</td>
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<td>CNNHS</td>
<td>China National Nutrition and Health Survey</td>
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<td>CVD</td>
<td>Cardio Vascular Diseases</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-Diphenyl-1-Picryl Hydrazyl</td>
</tr>
<tr>
<td>EGIR</td>
<td>European Group for the Study of Insulin Resistance</td>
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<tr>
<td>ESW</td>
<td>Eastern School Whiting</td>
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<td>GI</td>
<td>Glycemic Index</td>
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<td>GIT</td>
<td>Gastro Intestinal Tract</td>
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<td>HC</td>
<td>High Carbohydrate</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HF</td>
<td>High Fat</td>
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<tr>
<td>HP</td>
<td>High Protein</td>
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<td>IDF</td>
<td>International Diabetes Federation</td>
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<tr>
<td>IOTF</td>
<td>International Obesity Task Force</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Resistance</td>
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<tr>
<td>MCA</td>
<td>Metal Chelating Activity</td>
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<tr>
<td>MNCDS</td>
<td>Mauritius Non-Communicable Diseases Study</td>
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<tr>
<td>MS</td>
<td>Metabolic Syndrome</td>
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<tr>
<td>NCEP</td>
<td>National Cholesterol Education Program</td>
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<tr>
<td>PH</td>
<td>Powdered Hydrolysate</td>
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<tr>
<td>RAS</td>
<td>Renin Angiotensin System</td>
</tr>
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<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RP</td>
<td>Released Peptides</td>
</tr>
<tr>
<td>SGID</td>
<td>Simulated Gastro-Intestinal Digestion</td>
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<td>SOD</td>
<td>Superoxide Dismutase</td>
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<td>SW</td>
<td>Silver Warehouse</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>T2DM</td>
<td>Type II Diabetes Mellitus</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-alpha</td>
</tr>
<tr>
<td>WP</td>
<td>Whey Protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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CHAPTER 1

Introduction to the thesis
With marine species comprising approximately one half of the global biodiversity, the ocean offers to the world a wonderful resource for novel compounds, which may serve in improving the health of the worldwide population. Unfortunately, not all of the sea resources are adequately used. Many commercially important fish species are being overfished and existing valued species are becoming exhausted. The unintentional capture of non-target fish species is a well-known fisheries practice, termed as ‘by-catch’ (Korhonen and Pihlanto, 2006). While some of these species are retained for sale, others are thrown back into the sea, primarily due to their low-value market properties and unacceptance to consumers. Considered as wastes, by-catch species are converted into commercial and low-value products such as fertilisers, fish silage, and baits, amongst others (Blanco et al., 2007).

Notably, much of these wasted and discarded fish stocks are highly nutritious, comprising of high-quality protein sources and important novel compounds, the bioactive peptides (Kim and Mendis, 2006). These may thus constitute an incipient industry for valuable human food. Consequently, there is a significant potential for the Australian seafood industry to better utilise these underutilised fish species or fish wastes and convert them into useful consumables (Kim and Mendis, 2006), which may be sustainable to the marine environment and profitable to the fish industry.

Novel enzymatic hydrolytic technology is widely used to convert fish wastes proteins, sourced mainly from the myofibrillar proteins, into more marketable, functional and value-added products (Manikkam et al., 2016; Nurdiani et al., 2015) by commercial proteases. Thus, a number of enzymes have been used, including pepsin, trypsin and pancreatin (Manikkam et al., 2016), chymotrypsin, alcalase (Zhu et al., 2008), papain, to name a few. These enzymes enhance the release of bioactive peptides, which are usually encrypted within the parent protein (Möller et al., 2008). Bioactive peptides have been
identified as specific proteins or amino acid fragments, of size 2-20 amino acids and molecular masses below 6000 Da. They have a positive impact on body functions and may ultimately influence human health positively (Kim and Wijesekara, 2010). Based on their structural properties and amino acid composition and sequences, these peptides may affect one or more physiological functions, acting as antioxidative, anti-obesity, anti-thrombic or anti-hypertensive concomitantly (Möller et al., 2008). Scientific studies suggest that bioactive peptides derived from fish wastes, such as scales (Fahmi et al., 2004; Manikkam et al., 2016b), fillets (Gaarder et al., 2012), amongst others, are inversely related to the risk of hypertension and body fat mass accumulation by inhibiting the activity of ACE (Darewicz et al., 2014; Mathai et al., 2008) and inflammation or formation of free radicals (Lin et al., 2014). In addition, possibly controlling hunger by inhibiting the activity of trypsin enzyme to activate cholecystokinin (CCK), assisting in satiety (Medenieks and Vasiljevic, 2008). However, one potential barrier to usage of these peptides is the bitter taste that is sometimes generated depending on the enzymes used (Johanna et al, 2007).

Generating bioactive peptides by fish endogenous or commercial enzymes can be challenging to the fish industry as maintaining freshness and balance of amino acids is of great importance. Chilling and freezing are two major ways of preserving the freshness of fish, due to their perishable nature. However, these preservation techniques, mainly freezing and frozen storage of fish muscles, may lead to denaturation and aggregation of especially myofibrillar proteins (Tejada, 2001). These biochemical changes may essentially alter biofunctional properties of fish peptides. No studies have yet investigated the effects of chilled and frozen storage on the release of bioactive peptides in the presence of endogenous and digestive enzymes, which characterise this novel project.
The main goal of this research was to understand the inherent properties of fish muscle from Australian fish wastes in relation to the release of functional compounds, mainly, bioactive peptides during storage conditions and human digestion, with important in vitro physiological properties, such as: i) ACE and trypsin inhibitory as well as antioxidant activities (DPPH radical scavenging, metal chelating and reducing power).

The specific objectives were to:

1. Examine the physicochemical and biological properties of fish protein hydrolysates derived from fish scales, as an example to illustrate that a similar production strategy could be adopted within the Australian fish industry

2. Investigate the release of biologically active peptides from the Australian fish fillets during storage conditions (temperature and time) in the presence of muscle endogenous enzymes only

3. Determine the effects of in-vitro simulated gastrointestinal digestion (pepsin-pancreatin) on important physiological activities of released bioactive peptides

4. Assess the ability of released bioactive peptides from the fillets of SW and ESW to act simultaneously as an ACE and/or trypsin inhibitor and anti-oxidant or free radical scavenger

Chapter 2A provides a review of the current literature about the historical background and pathophysiology of metabolic syndrome. It also highlights the prevalence and causes of obesity. Moreover, it focuses on the metabolic regulators of appetite control and the roles of proteinaceous foods on satiety, a mechanism involved in obesity prevention. The correlations among CCK, trypsin inhibitors, food intake and obesity are also briefly explored. Furthermore, the roles of antioxidative fish peptides as free radical scavengers and metal chelating agents in the obesity prevention are also discussed.
Chapter 2B, covering the review paper entitled as ‘A review of potential marine-derived hypotensive and anti-obesity peptides’ presents the concept of bioactive peptides, the techniques for their release, production, isolation and characterisation.

Chapter 3 highlights the physicochemical characteristics and the effects of simulated gastrointestinal digestion on the bio-functional properties (DPPH-radical scavenging properties, ACE- and trypsin- inhibitory activities) of peptides released from hydrolysates prepared from fish scales-derived collagen.

Chapter 4 reports on the effects of storage, processing conditions and simulated digestion on selected in-vitro biological activities of Australian silver warehou fillets in the presence of endogenous enzymes.

Chapter 5 indicates that Australian eastern school whiting, another type of underutilised fish species, could be a potential source of antioxidants and metal reducing agents.

The overall conclusions and future research directions are presented in Chapter 6 and references are listed in Chapter 7.
CHAPTER 2

Review of literature
Chapter 2 presents a comprehensive review of scientific knowledge on metabolic syndrome and the concept of bioactive peptides, focusing on ACE/trypsin inhibitory and anti-oxidant peptides released from Australian fish proteins and their impact on health. Divided into Chapters 2A and 2B, it introduces the major concepts that led to the hypotheses for this research project.

Chapter 2A: Supplementary literature review covers the following topics:

- Metabolic syndrome
- Prevalence and causes of obesity
- Preventative strategies of obesity
  - Metabolic regulators of appetite control (CCK and trypsin inhibitors)
  - Antioxidative peptides
2A.1 Historical background of metabolic syndrome

The term metabolic syndrome (MS) was first conceptualised over 90 years ago, by the Swedish physician Kylin, in 1923, as an assembly of hypertension, hyperglycemia and hyperuricemia (gout) (Zimmet et al., 2005). Twenty years later, Vague argued that upper body fat accumulation, also referred to as android or male-type obesity (Alberti et al., 2006) was the primary cause for the aetiology of metabolic disturbances as seen in Type II diabetes mellitus (T2DM), hypertension and atherosclerosis (Alberti et al., 2006). About 40 years later, Reaven, an American endocrinologist achieving significant nobility with his 1988 Banting Lecture (organised by the American Diabetes Association), ascertained the clinical importance of MS by incorporating insulin resistance (IR) as the fundamental pathophysiological component in his definition, and termed the condition ‘Syndrome X’, obesity being exclusive at that particular era (Reaven, 2003).

Whilst Kaplan, in 1989 renamed the concept as ‘The Deadly Quartet’, other research fellows termed it ‘The Insulin Resistance Syndrome’ (Alberti et al., 2006; DeFronzo and Ferrannini, 1991). Descovich et al. (1993) named it as ‘The Plurimetabolic Syndrome’. In 2000, a new version of the definition was introduced as ‘Hypertriglyceridemic Waist’ and ‘Dysmetabolic Syndrome’ by Lemieux et al. (2000) and Groop and Orho-Melander (2001), respectively. It can thus, be noted that there existed controversial issues to the appropriate designation of this cluster of metabolic, genetic and hormonal phenomenon. Interestingly, one common observation central to their findings was obesity (Table 2A.1), the influencing factor for onset of MS and related health-threatening conditions. Table 2A.1 highlights the yearly classification of the deleterious medical condition, nowadays characterised as ‘Metabolic Syndrome’.
<table>
<thead>
<tr>
<th>Year</th>
<th>Given term</th>
<th>Definition</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1923 | Metabolic disturbances      | Hypertension  
Hyperglycemia  
Hyperuricemia                                                | (Zimmet et al., 2005)         |
| 1947 | Male type obesity           | **Upper body fat accumulation**  
T2DM  
Hypertension  
Atherosclerosis            | (Zimmet et al., 2005)         |
| 1988 | Syndrome X                  | Importance of IR  
Exclusion of obesity                                      | (Reaven, 1988)                |
| 1989 | The deadly quartet          | **Upper body obesity**  
Glucose intolerance  
Hyper-triglyceridemia  
Hypertension                | (Kaplan, 1989)                |
| 1991 | The insulin resistance      | NIDDM  
**Abdominal obesity**  
Hypertension  
Lipid abnormalities  
ASCVD                                    | (DeFronzo & Ferrannini, 1991) |
| 1993 | The Plurimetabolic syndrome | Diabetes  
**Abdominal obesity**  
Hypertension                                      | (Descovich et al., 1993)      |
| 2000 | Hypertriglyceridemic waist  | Hyper-apolipo-proteinemia  
Hyper-insulinemia  
Hyper-cholesterolemia                        | (Lemieux et al., 2000)        |
| 2001 | The Dysmetabolic syndrome   | **Abdominal obesity**  
Insulin resistance  
Hypertension  
Dyslipidemia  
Microalbuminuria                  | (Groop & Orho-Melander, 2001) |
| 2003 | Insulin resistance syndrome | Hypertension  
Insulin resistance  
**Abdominal obesity**                                | (Einhorn et al., 2003)        |
| 2005 | Metabolic syndrome          | **Abdominal obesity**  
Diabetes                                                          | (IDF, 2005)                   |
Pre-diabetes
High cholesterol
High blood pressure

2013 Metabolic syndrome **Visceral adiposity**
Insulin resistance
Hypertension
Elevated TG
Low HDL

(Mitchell et al., 2013)

<table>
<thead>
<tr>
<th>Year</th>
<th>Definition</th>
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<tr>
<td>2013</td>
<td><strong>Visceral adiposity</strong></td>
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Table 2A.1: The yearly classification of the definitions of metabolic syndrome

[T2DM – Type 2 Diabetes Mellitus; IR – Insulin Resistance; NIDDM – Non-Independent Diabetes Mellitus; ASCVD – Atherosclerosis Cardiovascular Diseases; CHD – Coronary Heart Diseases; TG – Triglycerides; HDL – High Density Lipoprotein]

The controversial issues presented by metabolic, hereditary and hormonal interactions as well as increasing obesogenic society, have stipulated urgent need for establishment of international committees to put forward a unifying definition for MS. The International Obesity Task Force (IOTF), the European Group for the Study of Insulin Resistance (EGIR), the National Cholesterol Education Program - Third Adult Treatment Panel (NCEP ATP III), the American Association of Clinical Endocrinologists (AACE) and the International Diabetes Federation (IDF), are amongst the various international committees created (Reaven, 2003).

In Australia, Obesity Australia, Shape Up Australia, Obesity Prevention Australia Inc., Obesity Policy Coalition, Diabetes Australia and Preventative Health Taskforce are some of the establishments committed to reversing the obesity and inactivity rife that is debilitating the Australian nation and driving changes on the public perceptions of obesity.

A syndrome is defined as a constellation of two or more symptoms or signs that are indicative of initiation of a typical disease. Appearing concomitantly, a syndrome occurs due to disordered functions of several body organs owing to anatomical, physiological or
biochemical changes in the human body. A syndrome thus comprises of a host of complex pathophysiological events as in MS.

Accordingly, a universally accepted definition of MS may encompass five key important metabolic components, including abdominal obesity, high blood pressure, glucose intolerance, dyslipidemia and IR (Eckel, 2007), in addition to T2DM and CVD (Grundy et al., 2005). Other health complications implicated in the progression of MS and obesity are illustrated in Figure 2A.1.

Body mass index (BMI) $\geq 30$ kg/m$^2$ is clinically defined as obese. Obesity is physiologically characterised as a chronic imbalance between energy intake and energy expenditure, resulting in extreme build-up of fat in the skeletal muscle, liver, adipose tissue or abdomen (World Health Organization, 2016).

Persistent body fat accumulation is the principal etiological dysregulation factor, leading to the progression of multitude metabolic, hormonal and physiological aberrations (Alberti et al., 2006). Several epidemiological studies correlated metabolic risks and abdominal obesity among different races, and hence, abdominal obesity was further defined by the IDF in terms of ethnicity (International Diabetes Institute, 2006).
Figure 2A.1: Obesity and its associated adverse health and psychosocial consequences
2A.2 Pathophysiology of metabolic syndrome

Pathophysiology is the study of biological and physical manifestations of a disease as they correlate with underlying abnormalities and physiological disturbances. As the worldwide population is becoming more obese, the prevalence of pathophysiological events associated with MS, including IR and oxidative stress, is proportionally increasing. Hypertension has already been described by Manikkam et al. (2015).

2A.2.1 Insulin resistance

In a healthy human body, insulin controls the amount of glucose entering and leaving the bloodstream, reduces glucose production in the liver and increases the rate of glucose uptake, primarily into skeletal muscle and adipose tissue (Shulman, 2000). Conversely, insulin is a fat-storing hormone. When cells lack glucose, insulin uses fat instead and converts the latter into glucose. Thus, from a lipocentric viewpoint, insulin affects lipid metabolism by increasing lipid synthesis in the liver and adipocytes. This eventually results in decreased fatty acid release from the adipose tissue (Sesti, 2006). Figure 2A.2 designates the healthy functioning of insulin.

Metabolic or hormonal disturbances due to excessive visceral fat may however disrupt the insulin’s functions. Consequently, resulting in weight gain and obesity, the sensitivity of muscle tissue gradually declines. To counterbalance impairment of glucose transport in skeletal muscle/fat cells while maintaining normal glucose levels, pancreatic β-cells over-secrete insulin; a condition termed insulin resistance (IR) (Sesti, 2006). Obese individuals become insulin resistant, due to the adverse effects of excessive formation and accumulation of fatty acids, resulting in impaired action of insulin for both glucose and lipid metabolisms (Saltiel, 2000). Formation of fatty acids in obesity interferes with glucose transport enzyme 4, leading to β-cells dysfunction, thus,
decreasing insulin sensitivity (Boden and Shulman, 2002). A closer relationship between hypertension and IR is also established (Figure 2A.3).
Figure 2A.3: Causes of insulin resistance in obese individuals [TG: triglycerides; FFA: free fatty acids; ISP: insulin secretion pathway; NO: nitric oxide; RAS: Renin-angiotensin system; SNS: sympathetic nervous system; TNF: tumour necrosis factor (Henriksen et al., 2011; Mathai et al., 2008; Nieto-Vázquez et al., 2008)]
Other predisposing factors for IR development include hereditary features, aging and sedentary lifestyles. The rise in obesity and IR proportionally increases the risk of diabetes and CVD. In addition, obesity-induced IR is not only typically associated with an abnormality in the fatty acid or cholesterol profile, but also related to the overexpression of inflammatory markers in the adipose tissue, due to oxidative stress (Meigs et al., 2007; Henriksen et al., 2011).

The molecular association between obesity-linked inflammation and IR began more than 20 years ago when an inflammatory marker, tumour necrosis factor (TNF)-α, was discovered to be overexpressed in fat tissues of obese rodent models (Hotamisligil et al., 1993). The cytokine, TNF-α, is generally secreted due to expansion of fatty tissue mass in obese individuals. From experimental models, TNF-α is overproduced in fat and muscle tissues of obese subjects. Northern blot analysis showed that obese women expressed 2.5 fold more TNF-α mRNA in fat tissue compared to lean controls (Hotamisligil et al., 1995). Similarly, ELISA assay indicated increased adipose production of TNF-α protein in obese subjects, strongly correlated with hyperinsulinemia; an indirect measure of IR (Kern et al., 1995).

On the other hand, deletion of functional TNF-α or TNF receptors protected obese mice from obesity-induced IR (Uysal et al., 1997). Decreased adiposity is often associated with body weight reduction, followed by a decline in TNF-α production; eventually preventing inflammatory-induced IR. Body weight reduction in obese women, associated with a decline in TNF-α mRNA expression (45%, $P<0.001$) in adipose tissue, significantly improved insulin sensitivity (Hotamisligil et al., 1995). Besides impairing insulin receptor signalling, TNF-α exerts the following functions: i) inhibiting activity of lipoprotein lipase; ii) stimulating lipolysis in adipocytes; iii) decreasing expression of glucose transported Glut4; and iv) increasing hormone-sensitive lipase (Kern et al.,
1995); all of which may promote lipid accumulation in adipocyte. Figure 2A.3 summarises the metabolic causes of IR.

2A.2.2 Oxidative stress – inflammation

Oxidative stress is defined as an excessive formation and/or insufficient removal of highly reactive molecules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Lobo et al., 2010). ROS can be classified as oxygen-centred radicals and non-radicals (Figure 2A.5). They can be formed either from normal essential physiological processes in the human body (lipid oxidation, stress, inflammation, glycoxidation) or from environmental sources by chain reactions. Initiation, propagation and termination are the three distinct processes of the chain reactions, via enzymatic or non-enzymatic reactions (Figure 2A.4).

As depicted in Figure 2A.5 and observed in obese individuals, reactions of free radicals are associated with a number of chronic degenerative maladies. Certain inflammatory markers, such as coagulating factors (fibrinogen and plasminogen activator inhibitor type-1), serum amyloid A, TNF-α, interleukin-1β, C-reactive protein and chemokines are over expressed in the presence of free radicals and oxidative stress (Lobo et al., 2010). They are also elevated in obese and diabetic patients and shown to be reduced when the patients were more engaged in an active and healthy lifestyle, targeting weight loss (Esser et al., 2014).

Moreover, an excess of ROS can oxidise macromolecules, mainly, proteins and lipids, which are linked with conformational and functional changes, resulting in DNA damage and mutations. The combined reaction of ROS and trace metal ions, Fe²⁺ and Cu²⁺, are oxidatively damaging to specific amino acids, mainly lysine, proline, histidine and arginine (Nimse and Pal, 2015). Crude peptide mixtures obtained from simulated digestion of Australian underutilised fish species, SW and ESW, appeared to be effective
in scavenging DPPH as antioxidant and acted as a reducing agent against iron and copper ions (Manikkam et al., 2016; Manikkam et al., 2016a).
Figure 2A.4: Lifestyle and environmental parameters involved in the production of unpaired electrons via enzymatic and non-enzymatic pathways (Lobo et al., 2010; Valko et al., 2007)
Figure 2A.5: Oxidative stress and related health consequences (Lobo et al., 2010; Uttara et al., 2009; Valko et al., 2007)
2A.3 The global obesity pandemic

2A.3.1 Pervasiveness of obesity

The transition from our ancient generations to modern civilizations has accelerated the incidence of obesity at an alarming rate in both the western industrialised countries (America, Canada, England, Europe and Japan) and developing nations (Africa, China, India). Obesity rates among the Australian population also continue to skyrocket, overtaking USA, as the ‘fattest nation’; imposing severe financial burden on the Australian economy. It was estimated that the overall obesity cost to the Australian government was $58.2 billion in 2008 (National Health Survey, 2008).

To assess whether central obesity precedes subsequent metabolic changes, the data from two major longitudinal studies, namely, The Australian Diabetes, Obesity and Lifestyle Study (AusDiab) and the Mauritius Non-Communicable Diseases Study (MNCDS) were used (Cameron et al., 2008). The prevalence of central obesity played a fundamental role in the advancement of MS and appeared to precede the appearance of other MS components, including diabetes, IR, and abnormal lipid plasma levels (Cameron et al., 2008). The multi-cultural island of Mauritius, with a total population of about 1 281 000, constitutes of different ethnic groups has undergone rapid industrialisation and economic growth over the past several decades. This has greatly impacted on the lifestyles and disease pattern of the country. In 2009, a survey was conducted in collaboration with the Ministry of Health & Quality of Life, Mauritius, the Baker IDI Heart and Diabetes Institute, Melbourne, Australia and other departments located in Sweden, Finland and United Kingdom. The frequency of obesity was 11.3 % for men and 20.5 % for women, whilst overweight prevalence in men was 34.7 % and 35.1 % in women. Therefore, as per the MNCDS (2009), an estimation of 477 000 people between 25 and 74 years of age were obese in Mauritius, which is alarming and immediate actions are required.
In addition, the obesity epidemic is certainly not affecting only adulthood, but is progressively expanding among the young generations, guarantying a growing obese society with a myriad of severe health consequences. In Australia, the occurrence of childhood obesity between 2004 and 2005 increased by 20% by an estimate of 5% in the 1960s (National Health Survey, 2008). A similar trend among European children was observed and reported by the European Association for the Study of Obesity (Lobstein et al., 2005). Correspondingly, the 2002 China National Nutrition and Health Survey (CNNHS) reported more than three million Chinese adolescents have MS. The study also observed the correlation between urbanisation and the greater prevalence of MS in Chinese adolescents (Li et al., 2008).

Universally, WHO (2016) reported that in 2014, more than 1.9 billion adults (18 years and over) were overweight. Of these 600 million were obese. According to the latest report, 41 million children under the age of 5 were overweight or obese in 2016 (WHO 2016). Based on the current trends, 6.9 million Australians will be obese by 2025 (Preventative Health Taskforce, 2015). Such rising levels of overweight and obesity around the globe signals the exigent need to act to reverse this secular trend.
2A.3.2 Aetiology of obesity

Aetiology is the science that deals with the causative factors and predisposes to the development of a certain disease or disorder. The underlying reasons for the rapid expansion of global pandemic of obesity are very complex. It can occur from the interplay between the susceptible ‘thrifty genes theory’ and an industrialised, modern and affluent society. The origin of obesity can be explained by nutritional, demographic, epidemiological and socio-economic evolutionary changes rapidly occurring globally. Our pre-human hominid ancestors, a population of hunter gatherers appeared around 4 million years ago actively searched for their foods, leading a healthy and dynamic lifestyle.

In contrast, current modern environment has exposed populations to abundance of convenient, relatively affordable, highly palatable energy-dense and high-glycemic foods (Brand-Miller et al., 2003). Willett et al. (2002) reported a strong association among foods with high glycemic index (GI), IR, obesity and T2DM. Moreover, the amount of energy consumed daily and diets’ nutritional composition, appearing to affect satiety and appetite of an individual (Benelam, 2009), may also play a major role in causative obesity. Furthermore, restricted consumption of fibre-rich food items; an inadequate intake of fruits and cruciferous vegetables; in addition to high intake of fatty diets, are the most likely culprits for the growing frequency of overweight and obesity in both developed and developing countries. Dietary changes by replacing the intake of refined products with whole grains have been associated with a lower risk of CVD (Willett et al., 2002; Harris and Kris-Etherton, 2010).

Genetic factors have also been determined as a contributing feature. However, genetic makeup of individuals has not substantially changed during the past decades and, therefore the culprit may not be only genetics but rather environmental and urbanization
as well as food abundance and sedentary lifestyle or physical inactivity (Roche et al., 2005).

A study conducted from 1994 to 2006, examined the correlation between active transports; walking, bicycling and public transits, against obesity rates in Europe, North America and Australia. An inverse correlation between obesity rates and active transports in the USA, Australia and Canada was observed; residents using cars as a major means of transportation exhibited the highest obesity rates (Bassett et al., 2008). A similar trend was observed in China (Bell et al., 2002). Furthermore, the increased prevalence of such a metabolic disorder in rural areas of developing countries could possibly be due to poverty, education and social status (Babio et al., 2009). Innate gastrointestinal microbiota was found to be another relevant factor of obesity (Das, 2010). Figure 2A.6 schematically reviews the possible risk factors of obesity.

The vast contributing factors to the obesity epidemic and its associated health consequences make it harder for authorities to develop strategic prevention mechanism. Currently, there is no scientific agreement of the effective methods for the prevention of obesity. One natural plausible way to preserve a healthy body weight is by coupling the intake of a balanced meal with daily physical activities. Although strategies to prevent obesity are in place and the fact that obesity is largely viewed as a lifestyle-dependent condition, obesity may likely be prevented through three main approaches; i) increased satiety by consumption of proteinaceous foods, ii) free radicals scavenging and iii) controlled renin-angiotensin system, the latter being pedantically enlightened by Manikkam et al. (2015). The following sections will discuss the satiety and free radicals scavengers.
Figure 2A.6: Aetiology of obesity

- Central Obesity
  - Adult obesity
  - Childhood/adolescent obesity
- Early nutritional environment
  - Exclusion of breastfeeding or fast food consumption during pregnancy and lactation
- Psychological stress - emotional, cognitive, trauma
- Stress
  - Anger and frustration
- Depression
- Sedentary lifestyle

- Genetic factors & family history
  - Polycystic ovary syndrome
  - Inscita
  - Vitamin D deficiency
  - Acute phase reaction
  - Hypertension
- Hormonal imbalance
  - Polycystic ovary syndrome
  - Inscita
  - Vitamin D deficiency
  - Acute phase reaction
  - Hypertension
- Cardiovascular disease
  - Hypertension
  - Insulin resistance
- Emotional imbalance
  - Anger and frustration
  - Depression
  - Sedentary lifestyle

- Stress
  - Anger and frustration
  - Depression
  - Sedentary lifestyle
2A.4 Metabolic regulators of appetite control – innovative approach in the prevention of obesity occurrence

In view of worldwide escalating prevalence of obesity, the challenges to explicate the mechanisms involved in human food consumption, energy expenditure, balance between energy intake and expenditure, the regulation of body weight, and the conservation of healthy body weight after subsequent weight loss, has indeed established serious scientific debate. The ability to maintain a balance between food consumption and energy output and avoidance of sedentary lifestyle is crucial to prevent excessive body weight gain.

However, there is an important physiological mechanism that is also involved; the appetite management. It is often monitored by two major factors, i) satiety related hormones, biologically released upon presence of foods in stomach; and ii) macronutrient compositions (proteins, fats and carbohydrates) of the daily diet. An in-depth review on the impacts of three major macronutrients on satiety and satiety-related hormones has already been presented (Benelam, 2009; Karhunen et al., 2008). A hierarchal observation stated that the three macronutrients differ in their extent of suppressing hunger and energy intake, in the order of protein > carbohydrate > fat; protein the most satiating (De Graaf et al., 2004).

2A.4.1 Influences of food proteins on satiety

Proteins, derived from the Greek word ‘proteios’, meaning first, have often been considered as the most quenching macronutrient, when consumed at moderate levels. As discussed further, innumerable reports have fully supported this notion whilst others revealed little difference between the effects of protein and carbohydrates (CHO) on food intake and satiety. For instance, the effects of consuming isocaloric preloads with different macronutrient contents on satiety in 10 healthy, non-dieting women indicated
no significant influence on the pleasantness of foods and concluded that satiety was not macronutrient-specific (Rolls et al., 1988). In contrast, proteins, fats and CHO had similar effects on appetite (De Graaf et al., 1992). Similarly, satiety was more affected by sensory characteristics of the test foods rather than macronutrient composition. However, high-protein (HP) or high-carbohydrate (HC) preload decreased hunger more than high-fat (HF) preload.

Nonetheless, various epidemiological studies conducted since the 1950s established that increased satiety, reduced hunger and subsequently energy intake, are positively correlated with HP diets. A literature search has retrieved the earliest research articles, as of the 1970s. Human subjects indicated hunger suppression and lowered feeling to eat after a HP meal than CHO- or fat-rich meal (Booth et al., 1970; Hill and Blundell, 1986). The intent of food intake at an evening meal (4 hours after lunch) was observed after consuming cooked lunch meals with HP (43% energy – meat casserole) of HC (69% energy – vegetable casserole). Food intake during evening meal was significantly reduced by 12% after the HP meal, demonstrating HP foods were the most satiated meals (Barkeling et al., 1990). In another study carried out on 13 healthy women, mycoprotein (fungal-derived protein), compared with chicken protein reduced the desire to eat 3h after a test meal; decreasing energy intake during the rest of the day (Turnbull et al., 1993). Furthermore, comparing the effects of HP test meal (strawberry yoghurt containing whey protein isolate or ham sandwich) with lower-protein (LP) meal (LP strawberry yoghurt or bacon sandwich) indicated that HP meals significantly reduced hunger and produced greater fullness than LP (Vanderwater and Vickers, 1996).

Besides the effects of protein quantity in a particular food on satiety, energy intake or hunger, it has also been hypothesised that protein sources are crucial determinant of satiety efficiencies. In this regard, the satiety power of 6 different protein sources; egg
albumin, casein, gelatin, soy, pea protein and wheat gluten on 12 healthy subjects was investigated (Lang et al., 1998). No significant effect on hunger, satiety and pleasantness ratings, macronutrients and energy intake over the 24h post lunch period was observed; in disagreement with other reports (Lang et al., 1998). Consumption of a HP snack delayed hunger at dinner time by 60min, in comparison to fat- or CHO-rich snack which delayed dinner intake by 25 and 34min, respectively (Marmonier et al., 2000).

Similarly, in 8 out of 10 preload studies, energy intake in subsequent meal was much lower after a HP preload than the LP (Eisenstein et al., 2002). In a randomized single-blind study, pre-loads of HP-yoghurt produced greater satiety and reduced food intake after a fixed time interval in all participants compared to CHO and fat food. Protein is considered to possess greater satiating power (Vozzo et al., 2003).

In one of two studies performed on a group of 16 lean healthy volunteers, 1700 kJ liquid preload containing 48g whey was found to significantly reduce energy intake ($P<0.05$) by 19%, 90min after ingestion compared with isocaloric casein preload. It can be explained on the basis of Mellinkoff’s original amino-static concept, indicating a greater concentration of amino acids would increase satiety (Hall et al., 2003).

Whey protein (WP) isolates and soy protein significantly suppressed hunger and satiety energy intake in comparison to egg albumin and water (control) (Anderson et al., 2004). Furthermore, in one study performed on a group of 6 lean male subjects, fish proteins were more satiating than beef or chicken proteins (Uhe et al., 1992). An egg-based breakfast induced greater satiety and significantly reduced ($P<0.0001$) short-term food intake in overweight and obese individuals as compared to a bagel-based breakfast (Vander Wal et al., 2005).

In another study, 18 subjects enrolled in a 5-month study of 8 week controlled food intake followed by 12 weeks ad libitum intake. The treatment diets included i) control
diet (55% CHO, 15% protein, 30% fat); ii) mixed protein (40% CHO, 30% protein, 30% fat) and iii) whey protein (40% CHO, 15% mixed protein, 15% WP, 30% fat). In summary, increased WP intake did not result in statistically significant differences in weight loss or in total fat loss, but substantial differences in regional fat loss and decreased blood pressure as observed in the WP group (Aldrich et al., 2011). In another study, HP afternoon yoghurt snack improved appetite control, satiety and reduced subsequent food intake compared to other commonly-consumed, energy dense, high fat snacks in 20 healthy women in a randomized crossover design study (Ortinau et al., 2014).

So far, a strong scientific body of emerging evidence has confirmed the efficacy of protein-rich foods to enhance satiety and reduce hunger or appetite; the mechanisms of which can be postulated as i) the Mellinkoff’s theory, ii) gluconeogenesis, iii) protein-derived thermogenesis and iv) effects on satiety markers (Halton and Hu, 2004). Nonetheless, among most of the protein sources examined, there is, currently, a lack of information correlating impacts of fish protein consumption on satiety, body weight loss, RAS activity, body composition and appetite control. Hitherto, merely three reports have been uncovered, assessing the effects of fish consumption on satiety.

The first study, conducted in the early 1990s, compared the effects of three types of meat proteins (beef, chicken and fish) on satiety and amino acid profiles in lean male subjects (Uhe et al., 1992). Results suggested that satiety was greater after a fish compared to beef or chicken protein meals. The second investigation showed a protein-rich lunch meal with fish reduced subsequent energy intake in comparison to a beef protein meal in normal weight young men (Borzoei et al., 2006). The third study explored the effects of fish protein hydrolysates derived from blue whiting (Micromesistius poutassou) and brown shrimp (Penaeus aztecus) on CCK secretion, a mechanism involved in inducing satiety and controlling energy intake (Cudennec et al., 2012; Cudennec et al., 2008).
The mechanisms for increased satiety, hunger reduction and successive energy intake and the roles of satiety hormones (CCK) upon fish proteins consumption remain elusive. There is therefore an urgent need to scrutinize this particular area in the field of food science and nutrition. Our research at Victoria University might enable the Australian food and/or fish industry to maximise the potential use of underutilised fish species, in the development of healthy functional food to promote health and/or prevent the emergences of chronic diseases, such as obesity.

2A.4.2 Cholecystokinin, trypsin inhibitors, food intake and obesity

Originally purified from porcine intestine, as a 33-amino acid peptide, cholecystokinin (CCK) was discovered in 1928 by Ivy and Oldberg (Liddle, 1997). An enzymatic post-translational modification of CCK pre-pro-peptide (pre-pro-CCK), consisting of 115 amino acids is associated with the production of multiple bioactive forms of CCK, with amino acid chain length of 8-33 amino acids (Rehfeld, 2004). The major circulating forms of CCK, mainly CCK-8 and CCK-33, are mainly derived from I cells in the duodenal and jejunal mucosa of the small intestine. CCK-8 is mainly secreted in the GIT in response to luminal nutrients, principally proteins and lipids.

The conceptual establishment of CCK as a satiety hormone was instigated by Gibbs and colleagues, more than 4 decades ago, when they demonstrated the ability of exogenously administered CCK to inhibit food intake in rats (Gibbs et al., 1973). Since that era, CCK has been recognised as the ‘intestinal satiation peptide’ (Cummings and Overduin, 2007). By enhancing gastric distention and slowing gastric emptying, CCK allows food to be digested slowly and remains in the stomach for a longer period of time resulting in feeling of fullness for longer. Protein-rich foods are the strongest stimulus for CCK release in the presence of foods in the gut. Proteins may stimulate CCK release via the inhibition of trypsin digestion of intestinal CCK releasing peptides (CCK-RP) (Hall
et al., 2003). Therefore, the trypsin inhibitory potential of protein-rich food sources is important to be assessed to decide which food will be more satiated in terms of CCK release. Soybean has been the best candidate as a trypsin inhibitor (Nishi et al., 2003). Very limited information is available on the effects of fish proteins on trypsin inhibition and CCK release (Cudennec et al., 2012; Cudennec et al., 2008; Medenieks and Vasiljevic, 2008; Manikkam et al., 2016b).

2A.5 Fish peptides, free radical scavengers and metal chelating agents

An antioxidant is defined as any substance that significantly delays or prevents oxidation of a particular substrate (for example lipids) when present at low concentrations. In terms of the effects in the human body, an antioxidant is a substance in foods that significantly decreases the adverse effects of reactive species, such as ROS, on normal physiological functions (Lobo et al., 2010). Antioxidants can be classified as inhibitors of free-radical oxidation reactions, metal chelators, reducing agents or inhibitors of pro-oxidative enzymes. There is a growing interest in natural antioxidants found in plants as well as from marine sources, from a safety perspective. Some antioxidant evaluation methods include DPPH radical scavenging assay, reducing power assay, metal ion chelating assay, hydroxyl radical scavenging assay, β-carotene-linoleic acid assay, superoxide radical scavenging assay, amongst others. In our research project, the former three assays were used to evaluate the antioxidative activities of the fish peptides.

2A.5.1 Antioxidants and its mechanisms

Antioxidant may act as a free radical scavenger, quenchers of singlet oxygen, and inactivators of ROS, such as peroxides or inhibitors of pro-oxidative enzymes. Bivalent
transition metal ions, Fe\textsuperscript{2+}, in particular can catalyse oxidative processes, leading to the formation of hydroxyl radicals, and can decompose hydro-peroxides via the so-called Fenton reactions (He et al., 2012). Among the ROS family, hydroxyl radical exhibits the strongest oxidative activity and induced severe damage to the biomolecules including lipids, proteins and nucleic acids, promoting the onset of human diseases such as obesity, atherosclerosis, diabetes, cancers and ageing (He et al., 2012). Together with scavenging free radicals, antioxidants may entrap metals and prevent them from participating in the reactions associated with generating free radicals; chelating these metals can effectively reduce oxidation (He et al., 2012).
Chapter 2B: A review of potential marine-derived hypotensive and anti-obesity peptides

Chapter 2B presents a comprehensive review on current scientific knowledge on renin-angiotensin system (RAS) and mechanisms of bioactive peptides to inhibit angiotensin-converting enzyme (ACE). It also discusses the recent advances in the development, bioavailability, isolation and characterisation of bioactive peptides from underutilised fish species and/or fish wastes.

GRADUATE RESEARCH CENTRE

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION:
PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each jointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

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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.

Vasambal Manikkam

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;

2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit;
5. The original data will be held for at least five years from the date indicated below and is stored at the following location(s):

College of Health and Biomedicine, Victoria University, Werribee campus, Melbourne, VIC, Australia.

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A Review of Potential Marine-derived Hypotensive and Anti-obesity Peptides

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A Review of Potential Marine-derived Hypotensive and Anti-obesity Peptides

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Bioactive peptides are food derived components, usually consisting of 3-20 amino acids, which are inactive when incorporated within their parent protein. Once liberated by enzymatic or chemical hydrolysis, during food processing and gastrointestinal transit, they can potentially provide an array of health benefits to the human body. Owing to an unprecedented increase in the worldwide incidence of obesity and hypertension, medical researchers are focusing on the hypotensive and anti-obesity properties of nutritionally derived bioactive peptides. The role of the renin-angiotensin system has long been established in the etiology of metabolic diseases and hypertension. Targeting the renin-angiotensin system by inhibiting the activity of angiotensin-converting enzyme (ACE) and preventing the formation of angiotensin II can be a potential therapeutic approach to the treatment of hypertension and obesity. Fish-derived proteins and peptides can potentially be excellent sources of bioactive components, mainly as a source of ACE inhibitors. However, increased use of marine sources poses an unacceptable burden on particular fish stocks, i.e., the underutilized fish species and by-products can be exploited for this purpose. This paper provides an overview of the techniques involved in the production, isolation, purification, and characterization of bioactive peptides from marine sources, as well as an evaluation of the ACE inhibitory (ACE-I) activity and bioavailability.

Keywords: Angiotensin-converting enzyme, angiotensin II, bio-functional peptides, bioavailability, renin-angiotensin system, anti-obesity peptides

INTRODUCTION

Dietary proteins have been known for their wide range of nutritional, functional and biological properties. Nutritional, proteins are recognized for their ample supply of amino acids that are primarily essential for physical development, body growth, maintenance and repair as well as for the proper functioning of body organs and cells. The nutritional quality of proteins depends on the structural configuration, amino acid content, and the physiological utilization of the specific amino acids after digestion and absorption (Pfohlman, 1996). Whilst from a functional point of view, proteins contribute efficiently to the physicochemical and sensory properties of various protein rich foods (Korhonen and Pihlanto, 2003), their biological properties relate to their satiating power (the feeling of fullness) (Astrup, 2005) and their ability to release bio-functional peptides during food processing or gastrointestinal transit. These peptides may serve as potential ingredients in functional

or health-promoting foods (Korhonen and Pihlanto, 2003; Korhonen and Pihlanto, 2006; Shabili and Zhong, 2008).

During the last 20 years, there has been an increasing scientific and commercial interest in the field of food-derived bioactive peptides owing to their ability to positively affect the major body systems, notably, the cardiovascular, digestive, endocrine, immune and nervous systems as well as minimizing the developmental risks of chronic diseases. Bioactive peptides, defined as food derived peptides (metabolites), which will provide a health benefit to the host beyond nutritional value, have been known for their myriad of bio-functionabilities, such as hypotensive, anti-oxidative, anti-microbial, and opioid activities (Hartmann and Meinild, 2007; Möller et al., 2008), amongst other properties. Remarkably, within the parent protein matrix, bioactive peptides (usually of short-chain length of 3-20 amino acid residues) are inactive. Thus, in order to exert a beneficial health effect, they must be released, resist the digestive conditions of the gastrointestinal tract (GIT) and subsequently be absorbed through the intestine where they enter the blood circulatory system and reach their site of action (Vermeseno et al., 2010; Möller et al., 2008).

In view of the worldwide high occurrence of obesity and hypertension, changes in lifestyle, dietary approaches, and
pharmacological treatments, such as weight loss supplements and anti-obesity drugs, are broadly applied to treat and/or prevent the onset of these two phenomena. However, the available synthetic products, such as sibutramine (reductil), which is an appetite suppressant (Tzianabos et al., 2009) have often been associated with undesirable side effects, such as increased blood pressure, constipation, headaches, acute liver failure, and insomnia (Slovacek et al., 2008; Thuvairajah et al., 2005). Therefore, efforts are being emphasized on the production of functional foods with anti-obesity and anti-hypertensive properties. It has been shown that the hyperactivity of the renin–angiotensin system (RAS) plays an important role in the onset of obesity, metabolic diseases, and hypertension (Weisberg et al., 2007). The most significant regulatory component of the RAS is angiotensin-converting enzyme (ACE), which converts angiotensin I (ANG I) to angiotensin II (ANG II). The latter is a physiologically important constituent that performs a number of functions, such as (i) the secretion of aldosterone; (ii) increase in renal sodium reabsorption; which ultimately results in an increase in the systolic blood pressure (Inagami, 1994) and (iii) growth of adipose tissue (Goosens et al., 2003), which affects the endocrine and metabolic systems. An increase in adipocyte angiotensinogen (AGT) was observed in obese subjects in the study of Van Harmelen and colleagues (2009). Moreover, it was shown that ANG II may promote adipocyte hypertrophy and increasing mRNA expression of fatty acid synthase (FAS) within fat cells (Jones et al., 1997).

Therefore, inhibiting the enzyme ACE may be therapeutically useful in lowering blood pressure, reducing body fat mass, and improving overall body composition. Indeed, several studies have demonstrated the ability of bioactive peptides or ACE inhibitors to perform these functions, both in vitro and in vivo studies (Ohta et al., 1997; Li et al., 2007; Mathai et al., 2008; Lee et al., 2010). Moreover, different synthetic ACE inhibitors, such as captopril, enalapril, and perindopril are extensively used as anti-hypertensive drugs in addition to reducing body fat mass (Mathai et al., 2008). However, due to their side effects, researchers are focusing more on the development of nutritionally derived ACE inhibitors (ACE-I peptides), from various plant sources, such as apricot (Zhu et al., 2010), wheat (Matsui et al., 1999), rice (Li et al., 2007), garlic (Saraswati, 1998), soy (Lo and Li-Chun, 2002), and mushroom (Lee et al., 2004). Animal sources of ACE-I peptides include dried bonito (Yokoyama et al., 1992; Fujita et al., 1995), oyster proteins (Wang et al., 2009), egg white proteins (Lee et al., 2010; Yu et al., 2011), beef hydrolysates (Jung and Lee, 2005) and fish proteins (Fujita and Yoshizawa, 1999; Jung et al., 2006) as well as dairy milk proteins (Doksor et al., 2007).

This article aims to discuss the enzymatic cascade involved in the renin–angiotensin system and provides an overview of the techniques involved in the production, isolation, and purification of bioactive peptides from marine sources as well as evaluation of the ACE-I activity and bioavailability.

OVERVIEW OF REIN-ANGIOTENSIN SYSTEM

In 1984, Robert Tigerstedt, a notable physiologist and his student, Per Bergman made an incredible discovery of the enzyme renin, which has led to the extensive research of the RAS and remains a landmark in its history (Marks and Manwell, 1979). The RAS has long been identified as a regulatory system that is involved in a multitude of physiological and pathophysiological functions, including body fluid regulation, cardiovascular functions, sodium re-absorption from the kidney, increasing vascular tone and aldosterone secretion (Fragani, 1994: Goosens et al., 2003; Weisberg et al., 2007; all of which are contributing factors for hypertension and cardiovascular diseases (CVD). Recently, the RAS present in various tissues such as the adrenal cortex, kidney, heart, liver, retina, pancreas, vascular smooth muscle, brain and most importantly, the adipose tissue (Weisberg et al., 2007; de Kloet et al., 2010), has also been implicated in the etiology of obesity (Goosens et al., 2003; Weisberg et al., 2007; de Kloet et al., 2010). The cascade of enzymatic reactions that occurs in the classical pathway of the RAS, as depicted in Figure 1, is further described.

The initial enzymatic reaction occurring in RAS involves the cleavage of the liver-generated glycogen peptide, angiotensinogen into ANG I, by the enzyme renin (EC 3.4.23.25), secreted by the juxtaglomerular cells of the kidney (Li et al., 2009). The decapeptide ANG I (10 amino-acid sequence: DRVYIHPHEIL) reaches the lungs via circulation, where it is converted into the biologically active ANG II by the dipeptidyl carboxypeptidase, ACE (EC 3.4.15.1). The enzymatic cleavage occurs from the C-terminus and His-etyl-Leucine (HLE) peptide to produce the octapeptide ANG II, which is the central component of RAS. Besides the ACE-dependent pathways, alternate enzymes such as the serine proteases (Chymase) or Cathepsin D and G may also assist in the formation of ANG II and therefore may contribute to heart failure or high blood pressure (Karlisson et al., 1998). Even though many studies have linked the formation of ANG II primarily by the catalytic action of ACE, it still remains unclear whether the synthesis of ANG II is due to the ACE-dependent pathways, the ACE-independent pathways or a combination of the two. As illustrated in Figure 1, ANG II is further cleaved by amino-peptidases into degradation products, such as angiotensin III (active 2–8 fragment of ANG II) and angiotensin IV (active 3–8 fragment of ANG II), which are predominantly involved in brain functions, particularly attributed to learning and memory, modulation of behavior and neuronal development (Goosens et al., 2003; de Kloet et al., 2010).

The complexity of RAS (Figure 2) was further realized in 2000, when a new human homologue of ACE, known as angiotensin-converting enzyme 2 (ACE2) was discovered from S′ sequencing of a human heart failure ventricle cDNA library (Domonhac et al., 2003; Timpins et al., 2000). ACE2, a monopeptidase, hydrolyses the carboxyl terminal Leucine.
from ANG I to liberate angiotensin-(1–9) [ANG-(1–9)], which is then converted into angiotensin-(1–7) [ANG-(1–7)] by ACE2. ACE2 can also convert ANG II directly into angiotensin-(1–7) by cleaving the Phe²-Pro³ amino acid bond of the octapeptide to produce heptapeptide ANG-(1–7), which is then converted into angiotensin-(1–5) [ANG-(1–5)] by the zinc metallopeptidase ACE, due to the enzymatic degradation of the His³-Pro⁴ dipeptide (Donoghue et al., 2000; Ferrario, 2006). The amino acid composition of the RAS components and the enzymatic cleavage occurring during the pathway are demonstrated in Figure 2. The synthesis of ANG-(1–7) is physiologically beneficial since it opposes many of the ANG II-mediated deleterious properties via the G-protein coupled receptor (GPCR) Mas.

**Hypertension**

Systolic and diastolic blood pressures (SBP and DBP) are the clinical parameters involved in the measurement of BP of an individual. An optimal BP of a healthy person is around 120/80 mmHg systolic pressure over 80 mmHg diastolic pressure. A chronic elevation in arterial pressure would result in high blood pressure (HBP), clinically termed as hypertension (≥140/90 mmHg) (Grundy et al., 2005). It is well established that overweight and/or obese individuals tend to develop an increase in BP. The mechanisms, however, remain elusive. Besides blocking the vasoconstrictor effects of ANG II from the RAS, current pharmacotherapy for hypertension includes (i) beta-blockers, which reduce contractile force, (ii) calcium-channel antagonists, which inhibit vascular
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Figure 2. The complexity of the renin-angiotensin system, the renin acid composition and enzymatic cleavage of each angiotensin peptide. Angiotensin-Converting Enzyme 2 (ACE2), a novel homologue of the classical ACE, converts ANG II into angiotensin-(1-7), which acts as a vasodilator through the Mas receptor, via the nitric oxide pathway; release of prostaglandins; inhibition of non-epinephrine release and the activation of the vasodilator Bradykinin. ANG-(1-7) can also be indirectly formed from ANG I by the catalytic actions of the neutral Endopeptidases (NEPs) (Dungan et al., 2000, Ferrario et al., 2000). This diagram illustrates the amino acid sequence of each of the angiotensin peptide involved in the functioning of the renin-angiotensin system. The Leu-Val dipeptide is cleaved by the enzyme renin to form ANG I. The His-Pro bond of ANG I is cleaved by ACE to form the octapeptide ANG II. ACE2 cleaves ANG II at the Pro-Phe bond to synthesise ANG-(1-7). ACE then degrades the His-Pro dipeptide bond from ANG-(1-7) to produce ANG(1-5) (Ferrario, 2006, Fishman & Apfeld, 2000).

Smooth muscle contraction, and (ii) diuretics, which reduce blood volume, thus reducing blood pressure (Staessen et al., 2001). It has also been postulated that an excessive body fat causes an imbalance between the proper functioning of adipose and lean tissues in the liver or skeletal muscle (Luger, 2003). This disordered functioning, eventually results in (i) the production of cytokines (inflammatory markers), adipokines and angiotensin from adipocytes; (ii) an increased activity of sympathetic nervous
system (SNS), and (iii) overexpression of the components of RAS, such as AGT, ACE, and ANG II. These three parameters, consequently, cause sodium retention, extracellular fluid volume expansion, and increased cardiac input; all of which are fundamentally linked to the development of hypertension and are frequently observed in obese persons (Geissens et al., 2003).

Furthermore, the obesity-related activation of SNS may be partly mediated by the adipocyte-derived hormone, leptin (Hall et al., 2001), which increases proportionally with the degree of adiposity. An increase of leptin in hypertensive individuals is associated with elevated plasma renin activity, aldosterone, and angiotensin concentrations (Sohr et al., 1996). On the other hand, weight reduction by as little as 5% can potentially decrease RAS activation (Engel et al., 2005), and reduce the level of hypertension. The link between adipose tissue RAS, blood pressure, and obesity is well documented in animal models (Alvarez-Gallego et al., 1996; Bouatry et al., 2004).

Moreover, obese patients will often have a high plasma level of triglycerides (TG) or cholesterol, which may contribute to plaque formation or constrict arteries, thereby promoting the development of CVD (Eckel, 2007). Another potential cause of hypertension is the abnormality in insulin production.

Besides its role in blood glucose management, insulin, a vasodilator, prevents sodium re-absorption in the kidney of healthy individuals (Reaven, 2003). However, in obese patients, insulin loses its vasodilatory effect and leads to excessive sodium and water re-absorption in the kidney, a causative factor for hypertension. An increased plasma insulin level seems to increase atherogenic risk factors including plasma levels of low density lipoproteins (LDL), triglycerides, and elevated SBP (Reaven, 2003). Obesity-induced hypertension is well associated with the onset of various types of CVD, such as strokes, myocardial infarction, heart failure, and kidney diseases (DeFronzo and Ferrannini, 1991). An in-depth review on the mechanisms involved in the obesity-linked hypertension is provided by Rahmoosi and colleagues (2005). The possible mechanisms of obesity-induced hypertension are schematically illustrated in Figure 3.

**Correlation of RAS with Obesity and Body Fat**

Many studies have correlated the different components (renin, AGT, ANG II, ACE, and ANG II receptors) of RAS with body fat.

![Figure 3: Mechanisms implicated in the onset of hypertension. Obesity increases the activity of the SNS, which results in an increase in sodium (Na+) ions and water in the kidney. The components of RAS, such as AGT, ACE, and ANG II, are overproduced and may act upon (i) the arteries to cause vasodilatation; (ii) the heart to contract the vascular smooth muscle, which result in coronary heart disease; and (iii) the adrenal cortex to secrete aldosterone, which increases the reabsorption of sodium and fluid in the kidney, resulting in high blood pressure. Moreover, the excessive body fat in obese individuals results in the hyperproduction of renin, which increases sodium and fluid retention. Furthermore, the active components of RAS activate the sympathetic nerve terminals to release norepinephrine, which causes vasoconstriction to raise blood pressure.](image-url)
and adipose tissue, which is markedly expanded in obesity. For example, the study of Suzuki et al. (2002) established the fact that adipocytes from obese Zucker rats displayed a significant increase in the content of adipose tissue, which was measured in culture medium under fat cells. Their study reported that the over-secretion of insulin decreased adipocyte hypertrophy (Haimaiti et al., 2002). Other studies have revealed that administration of high-fat diet on the level of RAS components in the adipose tissue. Male Sprague-Dawley rats gained weight upon consumption of high-fat diet, and were assessed for RAS expression in their adipose tissue and its plasma concentration (Blumberg et al., 2004). Real-time polymerase chain reaction demonstrated a 2.5-fold increase in AGT mRNA in subcutaneous adipose tissue in obesity-prone rats compared to obesity-resistant rats. Moreover, an increase in plasma AGT concentration was observed in obesity-prone rats (Blumberg et al., 2004).

Similarly, Van Harmelen et al. (2006a) studied the association of adipocyte AGT genes expression with human obesity. The reverse transcription polymerase chain reaction was used to measure the levels of subcutaneous adipose AGT mRNA and 18S ribosomal RNA in the obese participants. Their study concluded that AGT mRNA expression was about 2 times elevated in the obese men. Moreover, Northern blot analyses demonstrated AGT expression in abdominal subcutaneous adipose tissue of nine obese subjects, aged 40-62 years old (Karlsson et al., 1998). Furthermore, Yasue and team (2010) demonstrated that both AGT and adipose tissue AGT was greatly increased in the obese human group. The above studies provide strong evidence for the notion that adipocytes expressing AGT may affect adipogenesis, which is well correlated with the severity of obesity.

Decades ago, the presence of ACE in adipose tissue was debatable. Seye et al. (1993) showed that ACE inhibition failed to prevent ANG II production by isolated adipocytes, suggesting that an alternate enzyme produced the ANG peptides. However, a later experiment demonstrated the presence of ACE mRNA in both human adipose tissue and in cultured adipocytes (Ingel et al., 1999). Later on, more studies confirmed that the inhibition of ACE prevented the formation of ANG II. In a recent study of Iyengar et al. (2008), mice lacking ACE gene have improved energy expenditure, with reduced fat mass as well as improved glucose clearance. Likewise, Carter et al. (2004) hypothesized that ACE inhibition in aged rats improved their body composition and physical performance compared to control rats. Moreover, ACE inhibitors administered to hypertensive rats reduced their blood pressure considerably (Ld et al., 2007). These studies confirmed the importance of ACE inhibition in relation to the prevention of obesity and obesity-related diseases.

**Physiological Roles of ANG II in Obesity**

Indeed, most of the biological effects of RAS are mediated by the peptide ANG II, by binding to either the angiotensin type 1 or type 2 receptors (AT1R and AT2R), in target tissues or organs (Wessendorf et al., 1990). In certain cases, it was reported that most of the functional actions of ANG II are mediated by the AT1R via intracellular activation of phospholipase, inhibition of adenylate cyclase, and stimulation of tyrosine phosphorylation (Sharma et al., 2001). Conversely, the physiological properties of AT2R remain less well-defined; nonetheless, it is generally hypothesized that its activation opposes the effects transmitted by AT1R activation in different aspects (Yasue and Okamoto, 2005). Briefly, some of the reported functions of ANG II include increased blood pressure, increased synthesis of glucocorticoid hormones, and decreased glucose uptake. Moreover, it acts as a potent vasodilator by binding to specific cell surface receptors to produce contractile proteins and to promote sodium and fluid retention, which results in an increase in BP (Eriksson et al., 2002). The activity of ACE leads to production of ANG II and the degradation of bradykinin, a vasodilator, resulting in promotion of hypertension (Eriksson et al., 2002).

Furthermore, ANG II has the potential to stimulate the synthesis of inflammatory cytokines and growth factors (e.g. tumor necrosis factor alpha, IL-1, PAF, etc.), reactive oxygen species (ROS) and coagulation factor (PAI-1) via the AT1 receptor, which increases cellular insulin signaling (Subotic et al., 2008) and vascular damage (Viridis et al., 2011). Most importantly, in the adipose tissue, ANG II enhances the accumulation of fatty acids and triglycerides within the body and promotes cell proliferation, which is a common causative factor for obesity and metabolic syndrome (Mathai et al., 2008; de Kloet et al., 2010).

**Adipocyte Differentiation**

Adipocyte tissue, a passive reservoir for energy storage, in the form of triacylglycerol, has been an important source for ANG II secretion, which plays a major role in adipocyte growth and differentiation (Darmon et al., 1994; Saint-Marc et al., 2001). While adipogenic differentiation is defined as the formation of new fat cells from precursor cells, adipocyte hyper trophy is the terminology used to describe an increase in the size of adipocytes due to excessive fat storage. ANG II also has been long recognized as a growth factor in a variety of cells and tissues (Sil and Sen, 1997). The mechanisms by which ANG II actually stimulates adipocyte differentiation remain complex and elusive; although, some suggestions have been made. Prostaglandin (PGI2), a major metabolite of arachidonate and an adipocytic acid in adipocyte tissue, has been associated with the onset of adipocyte differentiation. In vitro studies demonstrated that ANG II stimulated the release of PGI2 from adipocytes, via the AT3 receptor (Darmon et al., 1994; Saint-Marc et al., 2001).
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et al., 2001). Moreover, evidence suggests that overexpression of ANG II increases the triglycerides content of adipocytes. This was demonstrated in 3T3-L1 and human adipose cells in the study of Jones and colleagues (1997). According to Weisberger et al. (2007), the mechanism by which ANG II increases triglycerides content of fat cells, is by increasing the activity of fatty acid synthase, an enzyme that is involved in lipogenesis and which catalyzes the synthesis of palmitate from acetyl CoA and malonylCoA in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the rate-limiting enzymes for triglyceride synthesis in adipose tissue. Furthermore, the stimulation of the so-called peroxisome proliferator-activated receptors (PPAR) by ANG II can also result in lipogenesis and differentiated cells (de Kriet et al., 2013).

**Body Weight Regulation**

The impact of ANG II on body weight regulation has been broadly investigated in animal models. Young rats receiving a 1-week intracerebro-ventricular (ICV) infusion of ANG II, for a period of 10 days, were monitored for body weight change and food intake on a daily basis (Porter et al., 2003). It was observed that the ANG II-infused rats exhibited a decreased body weight gain and food intake compared to the control animals (Porter et al., 2003). These results were in line with the study of Bink et al. (1996), in which male Sprague-Dawley rats treated with ANG II infusion lost 18–26% of body weight by 1 week. Bink and colleagues (1996) postulated that ANG II produced weight loss through a pressure-independent mechanism, which included both marked anorexia and metabolic effects. Conversely, other studies have reported weight loss, overall change in body composition and improvement in glucose intolerance with the administration of ACE inhibitors, which blocks the synthesis of ANG II (Carter et al., 2003; Mulhau et al., 2008; Weisberger et al., 2003). The role of ANG II in body weight regulation remains controversial; however, there is enough scientific evidence to confirm the implication of ANG II in increasing body weight, food intake and energy expenditure as well as the potential of bioactive peptides to inhibit the formation of ANG II.

**BIOACTIVE PEPTIDES**

The extensive research and development in the field of bioactive peptides began since the 1970s when Mellander proposed that casein-derived phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants (Pihlanto-Legrand, 2000) and the peptides were named caseinophosphopeptides (CPPs) (Korhonen and Pihlanto, 2003). Since that era, milk and milk-derived products have been the primary focus for the characterization of bioactive peptides (Korhonen and Magnussen, 2005). However, with increasing knowledge on the functionality of the biologically active peptides, intense efforts have been made in the last two decades, to identify and quantify functional peptides from diverse plant and animal sources, with various physiological functions, as illustrated in Tables 1 and 2, respectively. A number of commercial functional foods containing bioactive peptides are presently available on the market. Some examples include (i) Calpis®, which is a Japanese product fermented with bacterial culture of Lactobacillus helveticus and Saccharomyces cerevisiae and contains bioactive peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) derived from milk caseins, which have been claimed to exert antihypertensive properties (ii) Eivolut®, developed in Finland, contained peptides VPP and IPP, which corresponds to β-casein and κ-casein fragments (Hartmann and Mielä, 2007); (iii) Collective®

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Primary functional properties</th>
<th>Rat study model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Pro</td>
<td>Inhibition of angiotensin-converting enzymes (anti-hypertensive)</td>
<td>Male Sprague-Dawley rats</td>
<td>(Lee et al., 2004)</td>
</tr>
<tr>
<td>Ile-Pro-Pro</td>
<td>Inhibition of angiotensin-converting enzymes (anti-hypertensive)</td>
<td>Male spontaneously hypertensive rats</td>
<td></td>
</tr>
<tr>
<td>Gly-Pro-Pro</td>
<td>Inhibition of angiotensin-converting enzymes (anti-hypertensive)</td>
<td>Male spontaneously hypertensive rats</td>
<td></td>
</tr>
<tr>
<td>Val-Pro-Pro</td>
<td>Antioxidant effects</td>
<td>Male Sprague-Dawley rats</td>
<td>(Niki et al., 2007)</td>
</tr>
<tr>
<td>Ile-Pro-Pro</td>
<td>Antioxidant effects</td>
<td>Male Sprague-Dawley rats</td>
<td>(Low et al., 2000)</td>
</tr>
</tbody>
</table>

*: Not specified

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Table 2. Examples of animal food-derived bioactive peptides and their potential physiological activities

<table>
<thead>
<tr>
<th>Animal source</th>
<th>Amino acid sequences</th>
<th>Bio-functional properties</th>
<th>Study model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried hamfish</td>
<td>Th-Lys-Pro</td>
<td>Inhibition of angiotensin-2-converting enzyme (angiotensin-converting enzyme)</td>
<td>Male Wistar rats</td>
<td>(Yokoyama et al., 1997)</td>
</tr>
<tr>
<td>Chicken bone protein</td>
<td>—</td>
<td>-</td>
<td>In vitro study</td>
<td>(Chen et al., 2008)</td>
</tr>
<tr>
<td>Beef by-products</td>
<td>Val-Leu-Ala-Glu-Tyr-Lys</td>
<td>Inhibition of angiotensin-1-converting enzyme</td>
<td>In vitro study</td>
<td>(Zhang and Liu, 2005)</td>
</tr>
<tr>
<td>Egg white protein</td>
<td>TADERYTL</td>
<td>Anti-oxidant capacity</td>
<td>In vitro study</td>
<td>(Davidson et al., 2004)</td>
</tr>
<tr>
<td>Fish protein hydrolysate</td>
<td>—</td>
<td>Anti-proliferative activity (HeLa cells)</td>
<td>In vitro study</td>
<td>(Post et al., 2009)</td>
</tr>
<tr>
<td>Shrimp head</td>
<td>Gly-Tyr-Cys-Asp-Leu-Lys</td>
<td>Angiogenic activity</td>
<td>STC-1 cells</td>
<td>(Cudmore et al., 2006)</td>
</tr>
<tr>
<td>Australian stinger</td>
<td>Glu-Tyr-Cys-Asp-Leu-Lys</td>
<td>Anti-microbial activity</td>
<td>In vitro study</td>
<td>(Bhattacharyya et al., 2008)</td>
</tr>
<tr>
<td>Rabbit skin gelatin</td>
<td>—</td>
<td>Anti-oxidant capacity</td>
<td>In vitro study</td>
<td>(Liu and Li, 2008)</td>
</tr>
</tbody>
</table>

* Not specified

Marine source of collagen and elastin can be used as an anti-wrinkle ingredient; and (iv) Natripeptin™, another marine-derived bioactive compound, has been found to be effective in increasing satiety and weight loss response (Pauzura, 2009). Calpis®, Evolva® and Natripeptin™ are commercial products with Food for Specified Health Use (FOSHU) claims (Shimizu, 2012).

Bioactive peptides are food-derived functional protein sequences that, beyond their basic intrinsic nutritional value, confer a physiological effect on one or more body organs, when administered in adequate amounts (Müller et al., 2008). One of the main criteria for their application is that they must be safe for human consumption. These peptides, generally inactive when encrypted in the primary sequence of a large protein molecule, will exert their biological functions only after being liberated from their parent protein (Kotomori and Philiasto, 2003). The release of the physiological peptides from the intact proteins may be achieved by three major ways, including (i) in vivo during gastrointestinal transit by digestive (e.g. trypsin) or microbial enzymes, (ii) in vitro during food processing such as ripening or fermentation by isolated microbial enzymes (e.g. Lactobacillus helveticus), and (iii) through the actions of enzymes derived from proteolytic microorganisms (Kotomori and Philiasto, 2003; Shahidi and Zhong, 2008).

Upon release as their individual entities, these short-chain bioactive peptides may act as potent metabolism modulators (Kotomori and Philiasto, 2003), regulatory compounds with hormone-like functionalities (Shahidi and Zhong, 2008) or play an important role in pathogenesis (Shahidi et al., 2004). Depending on their specific amino acid sequences, various food-based functional peptides may exert multiple physiological properties, indicating that specific peptide sequences may exhibit two or more different biological effects. The well-established physiological activities of the biofunctional peptides are illustrated in Figure 6. However, there are a few factors that may affect their proper functionality, namely, the innate amino acid composition, structural conformation, their amino acid sequence as well as their bioavailability or bio-accessibility (Vermoesen et al., 2004). In order to stimulate a biological response, the peptides must be bioavailable, i.e., following digestion, they must be able to (i) cross the intestinal epithelial cells and enter the blood circulation system; (ii) bind directly to specific epithelial cell-surface receptor sites; and (iii) produce local effects in the GIT (Kotomori and Philiasto, 2003).

Techniques for the Release of Bioactive Peptides

Peptides with ACE-I activity have already been isolated from different food proteins (Yamamoto, 1997). A potentially successful food protein source to generate bio-functional peptides is expected to meet at least two major selection criteria, including (i) the potential of use of underutilized proteins and/or peptides (e.g. underutilized fish species and their scales, skins, etc.), and (ii) utilization of identified peptide sequence of specific pharmaceutical interest. These two criteria can be implemented to generate high yields of characterized potent peptide with one or multiple bioactivities.

Bioactive peptides can be produced through enzymatic hydrolysis to yield digestive enzymes of the whole protein molecules. Simulated gastrointestinal digestion techniques, which can be a cost-effective strategy, have been so far, the most common means of investigating the effects of digestive enzymes (pepsin, trypsin, pepsin, and other proteases) on the delivery of potential biologically active peptides, such as the ACE-I peptides (Lo and Li-Chan, 2005; Kotomori and Philiasto, 2006). Different combinations of proteases including chymotrypsin, papain, and thermolysin; commercial enzymes, such as Alcalase, Flavourzyme, and Neutrase; enzymes from bacterial and fungal sources; gene expression approaches; and chemical hydrolysis involving the applications of acids and/or alkali are potential technologies that have been used to produce bioactive peptides from various proteins as reviewed in

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recent articles (Kocheran and Piliarto, 2006; Shahidi and Zhong, 2008). Furthermore, parameters such as degree of protein hydrolysis, the duration of hydrolysis, enzyme-substrate ratios, and the pre-treatment of the protein prior to hydrolysis (Rohder et al., 2010), are important to be considered in the production of bioactive peptides.

Isolation and Characterization Techniques of Bioactive Peptides

Prior to isolating and characterizing particular bioactive peptides, the selected protein food source must primarily be hydrolysed, for example, by enzymatic hydrolysis. This hydrolysis process allows the release of a crude peptide mixture, which should then be analyzed for different bioactivities, in particular interest to the investigators. After determination of the bio-functionalities, the released peptides or the hydrolysates are then fractionated mainly by ultrafiltration, which is based on peptide size (Kocheran and Piliarto, 2006); or preparative HPLC (Vermassen et al., 2008). The fraction demonstrating the highest bioactivity is further purified to isolate an individual peptide using different separations techniques, principally reverse-phase high-performance liquid chromatography (RP-HPLC) or gel permeation chromatography (Jung and Lee, 2005; Jung et al., 2006; Donkor et al., 2007; Wang et al., 2008; Cao et al., 2010). Purification may lead to more active fractions. Moreover, the characterization of individual peptide fractions is achieved using the combined techniques of mass spectrometry and protein sequencing. For instance, matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS) was used to
analyze the sequence of the purified ACE inhibitor derived from rice hydrolysate (Li et al., 2007).

Moreover, because bioavailability is a crucial determinant of the potency of this ACE inhibitor, it is therefore crucial to determine whether the peptides are absorbed within the gut and transported into blood circulation. The most widely used assay to investigate the bioavailability and transport of purified ACE inhibitor involved the Caco-2 cell monolayer, which can potentially mimic the human small intestinal epithelial cells (Sanke et al., 2002; Vemalakulor et al., 2004). Even a complete sequence of partial ACE-I peptide, automated Edman degradation on peptide/protein sequencer or MSMS (Dionex et al., 2007) is usually applied. The flow diagram for the production, isolation, purification, identification and development of ACE-I peptides from various protein food sources is presented in Figure 5. Should the compound indicate effective in vitro bio-functionalities, some researchers will then investigate the effects of the derived compound in animal models to study the in vivo physiological aspects of the bioactive constituent. For example, the peptides are administered in spontaneously hypertensive rats (SHR) to investigate whether the potent ACE inhibitor will influence BP and cardiovascular health in vivo (Ramcharan and Shah, 2011).

Industrial Techniques Used for the Generation and Purification of Bioactive Peptides

Recently, food proteins-derived bioactive peptides have become a new growing area of potential candidates for the various health-promoting functional foods. At present, milk- and marine-derived proteins are the best known source of such physiological ingredients; and they present promising areas for developing new value-added food products. Owing to the increasing health-conscious consumers’ interest in the consumption of these biological food components/products and commercialization’s perspective, there has lately been an emergence for the production of such peptides on an industrial or plant scale, rather than only laboratory scale. This can be exemplified by the study of Wang and others (2003). Oysters-derived oligopeptide-enriched hydrolysates, produced from the protease of Bacillus sp. SM8011 at laboratory level, and eventually scaled up to pilot (100 L) and plant (1000 L) levels with the same conditions, were found to exert antimicrobial activity and immunostimulating effects when investigated in BALB/c mice (Wang et al., 2010). However, until recently, this general industrial scale production of bioactive peptides has been challenging owing to expensive production/processing cost, costly methods, such as chromatography techniques, complex processing step, and limiting appropriate large-scale technologies (Korhonen and Pihlanto, 2005; Pouliot et al., 2006; Korhonen and Pihlanto, 2007).

It is important to note that from an industrial point view, the development of functional foods with added physiological value depends on (i) cost-effective isolation and purification techniques with the capacity to yield bioactive peptides with specific molecular weight range and with particular health benefits, (ii) enrichment of bioactivity of the characterized peptide to such a level that when the product is consumed, a minimal active amount is enough to deliver its bioactivity to the target site, as well as (iii) safety and organoleptic characteristics of the final product (Kim and Mendis, 2006; Korhonen and Pihlanto, 2006; Pouliot et al., 2006).

Pressure-driven membrane-based separation techniques have been, by far, the best potential available technology, that have been extensively used in the concentration of milk- and marine-derived bioactive peptides (Kim and Mendis, 2006; Pouliot et al., 2006; Murray and Fitzgerald, 2007). Examples of these separation methods comprise of microfiltration (MF), ultrafiltration (UF), reverse osmosis (RO), and diafiltration (DF) (Pouliot et al., 2006; Korhonen and Pihlanto, 2007; Saxena et al., 2009; Ayyel and Dorozdji, 2011), of which, the latter three, are now industrially involved in the production of whey powder and whey protein concentrates (WPCs), yielding a protein content of 35–80% (Korhonen and Pihlanto, 2007).

In view to the isolation and enrichment of marine peptides exerting high ACE-I and antioxidative activities, UF has been preferably used, using molecular weight cut-off membranes ranging from 1 to 10 kDa (Jeon et al., 1999; Fujita et al., 2001; Je et al., 2005; Rapcak et al., 2006; He et al., 2006). Some of the proposed advantages of UF include: (i) cost-effectiveness (ii) ease to scale-up for commercial production, (iii) high thermal stability and chemical sensitivity, (iv) improvement of the efficiency of membrane-coupled biocatalysis (v) increase of production yield, and (vi) yield and productivity of each membrane molecular weight range (Korhonen and Pihlanto, 2006; Saxena et al., 2009). Additionally, the so-called membrane biocatalytic technology, which involves the combination of enzymatic hydrolysis of marine proteins and peptide separation by UF, is currently considered as another potential processing scheme for the generation and isolation of marine-derived bioactive peptides, with beneficial health attributes (Bryan and Kim, 2011; Kim and Mendis, 2006; Gokarn and Kim, 2010).

According to Pouliot and colleagues (2006), in order to obtain specific fractions, which is rich in particular peptides of interest from the crude mixture, the coupling of membrane-processes with chromatographic techniques, may often deemed necessary. Such chromatographic methods may include (i) ion-exchange, (ii) hydrophobic interaction, (iii) size-exclusion, and (iv) affinity chromatography. However, on an industrial scale, the processing costs and processing steps involved are two crucial factors that must be considered prior to industrializing these techniques. Consequently, semi- and preparative-scale chromatography may only be implied when high purity of the active peptides is critical for commercialization (Pouliot et al., 2006). Importantly, it is worthwhile taking into account that the potency of the targeted bioactive component will
Figure 5: Diagrammatical representation of the processes involved in the production, isolation, purification, identification and characterization of bioactive peptides from animal protein food sources. Food proteins are hydrolyzed by selected proteases, such as pepsin to form the hydrolysate or to isolate the peptides. The latter is then assessed for potential differential bioactivities, which are then purified and isolated. Isolated peptides are eventually re-assayed for the specific bioactivities and the most active peptide is sequenced by either MALDI-TOF or peptide sequencer to determine the amino acid sequence of the physiologically active peptide. Furthermore, the synthesized peptide undergoes a bioavailability assay, most commonly using the Caco-2 cell monolayer to investigate whether the peptides can be absorbed within the GIT. Some in vivo studies are also involved prior to development of the bioactive peptides as a food ingredient to be incorporated into functional foods or in a nutraceutical.
eventually designate the extent of purification that is required, prior to food formulation.

Subsequently, commercialization of these industrially derived functional products can only be achieved if the latter has been claimed to exhibit a specific health effect by certain regulatory health claim policy. Japan, the birthplace of functional foods and the first country to adopt a legal system in relation to allowing claims on functional foods, introduced, in 1991, the Food for Specified Health Use (FOSHU) licensing system, which was under the regulatory system called “Foods with Health Effects” (SHF). By 2002, six ACE inhibitors, e.g. Ramipril, were marketed and are marketed under this category. As reported by Siril and others (2008), these ACE inhibitors were all marketed under this category. The group of products that are marketed under this category include (i) ACE inhibitors with a specific health claim, e.g. Ramipril, (ii) ACE inhibitors with a specific health claim for the prevention of cardiovascular disease, e.g. Ramipril, (iii) ACE inhibitors with a specific health claim for the prevention of osteoporosis, e.g. Ramipril, (iv) ACE inhibitors with a specific health claim for the prevention of cancer, e.g. Ramipril, (v) ACE inhibitors with a specific health claim for the prevention of diabetes, e.g. Ramipril, (vi) ACE inhibitors with a specific health claim for the prevention of neurodegenerative disease, e.g. Ramipril, (vii) ACE inhibitors with a specific health claim for the prevention of respiratory disease, e.g. Ramipril, and (viii) ACE inhibitors with a specific health claim for the prevention of kidney disease, e.g. Ramipril.

These products are available on the Japanese, American, and European markets.

**ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES**

**Mode of Actions of ACE Inhibition**

ACE primarily deactivates the vasoconstrictor bradykinin, while at the same time catalyzes the conversion of inactive ANG I into a potent vasodilator, ANG II. Several studies have investigated the beneficial roles of bradykinin, which are predominantly mediated by the bradykinin type 2 receptor (B2R) (Eridos et al., 1997). In specific tissues and organs, bradykinin (i) contracts the uterine and ileal smooth muscle, (ii) enhances vascular permeability, (iii) increases mucous secretion, (iv) induces vasodilatation by the nitric oxide-mediated pathways and by inhibiting the angiotensin II converting enzyme (ACE), (v) improves glucose utilization and insulin sensitivity (Dula et al., 2001). Therefore, in order to prevent the occurrence of deleterious health effects due to the degradation of bradykinin, ACE must be inhibited.

Certainly, ACE inhibition has played a fundamental role in the therapeutic treatment of hypertension and normal heart function. Recent studies have also demonstrated the role of ACE inhibition in adipogenesis inhibition, body weight control in addition to improvement of body composition, glucose intolerance and lipid metabolism (Jayasothy et al., 2008; Mathai et al., 2008; Weissinger et al., 2008). In regard to hypotension, ACE inhibitors will strictly maintain for balance between the vaso-constrictive and vaso-dilatatory effects of bradykinin. This balance is particularly maintained by blocking or diminishing the production of ANG II whilst simultaneously reducing the degradation of bradykinin (Brown and Vaughan, 1998).

**Classification of ACE Inhibitors**

Depending on their inhibitory activity following pre-incubation with ACE, the ACE-I food peptides can be divided into three major groups. The first category is known as the "true inhibitor type" peptides since their IC50 value is not affected by pre-incubation with ACE. The second group are the second group of peptides that are hydrolyzed by ACE resulting in weak inhibition. The third group includes the "pro-drug type" peptides, which are converted to "true inhibitor type" peptides by ACE or enzymes (proteases) of the digestive tract. In this regards, in vivo studies have demonstrated that only peptides belonging to the groups of true inhibitor or pro-drug type reduce SBP of SHR (Iyoyukihito et al., 2000).

**Structure-activity Correlation of ACE Inhibitory Peptides**

The inhibition modes of ACE-I peptides have been determined by Lineweaver-Burk studies of enzymatic inhibition (Hai-Lun et al., 2006). These plots have shown that there are two types of inhibition: competitive and non-competitive inhibition. For instance, in the study of Hai-Lun and team (2006), the Lineweaver-Burk plot indicated that the three novel ACE-I peptides derived from Acesta chilenensis, which is a Chinese underutilized marine shrimp species, were all competitive inhibitors. Competitive inhibition involves the binding of the inhibitor to the active site of the enzyme (ACE) to block its activity, while preventing the binding of the substrate (HHL) to the active site of the enzyme (Li et al., 2004). Interestingly, the presence of tyrosine (Tyr), tyrosine (Tyr), proline (Pro) or phenylalanine (Phe) amino acids in the C-terminal of the peptide and branched-chain aliphatic amino acids at the N-terminal is usually suitable for the peptides to act as competitive inhibitors by binding with ACE (Cashman and Chrousos, 1971).

Conversely, a non-competitive inhibitory mechanism, defined as the mechanism in which the inhibitor and the substrate may both be bound to the enzyme's active site, has also been observed in some peptides (Wang et al., 2008; Lee et al., 2010). A non-competitive inhibitor indicates that the inhibitor has an equal affinity for the enzyme and the enzyme-substrate complex. Moreover, ACE inhibition is also expressed as the IC50 value, which is the protein concentration in the sample (μg/mL) required to inhibit 50% of the ACE activity (Donker et al., 2007). Several structural features, such as molecular mass, amino acid chain length, shape, hydrophobicity, and charge can potentially influence the efficiencies of ACE-I peptides released from food protein sources (Meisel, 1997). It is also important to note that the active site of ACE, usually, cannot accommodate large peptide molecules. Furthermore,
the overall hydrophobicity of the peptide is also critical for its bioactivity. Due to these reasons, the inhibition of ACE activity is highly desirable. In vitro ACE-1 activity is generally measured by observing the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors. The widely used method has always been based on the availability of the enzyme's active site. However, in practice, the enzyme can be inhibited by various means, including the formation of a complex with a specific substrate or the addition of a co-factor or activator. In vitro ACE-1 activity is generally measured by observing the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors. The widely used method has always been based on the availability of the enzyme's active site. However, in practice, the enzyme can be inhibited by various means, including the formation of a complex with a specific substrate or the addition of a co-factor or activator.

Bioavailability of ACE Inhibitory Peptides

Although the ACE-1 potential of bioactive peptides can be measured in vitro, it is often a challenge to extrapolate a direct relationship between ACE-1 activity in vitro and anti-hypertensive activity or anti-obesity (for e.g., body composition) in animal or human studies. In this regard, bioavailability is a crucial factor for the potency of the ACE-1 peptide. This indicates that for the latter to exert its physiological effects in vivo, it must reach the target organ(s) in an intact form. Bioavailability of peptides refers to the total amount of amino acids that can be absorbed and utilized by the cells. This implies that the functional components must remain active and resistant to (i) digestive conditions of the GIT, (ii) brush border membrane peptidases, and (iii) absorption through the intestinal epithelium where they enter the blood circulatory system. The resistance to degradation by ACE-1 can be confirmed by (i) hydrolysis with pepsin and pancreatin to mimic the GIT digestion process (chemically known as simulated artificial digestion) and (ii) the epithelial intestinal cells (commonly termed as the Caco-2 cell monolayer).

Caco-2 cell monolayers, derived from a human colon carcinoma, exhibit characteristics that resemble the human intestinal epithelial cells, such as a polarized monolayer, well-defined brush border on the apical surface and inter-cellular junctions. This cell line has been used to assess transport of the bioactive compound in both directions (apical to basolateral and vice versa) across the cell monolayer. The peptides are rapidly metabolized by amino-peptidases into their constituent amino acids. However, the bioavailability studies have demonstrated that certain peptides are resistant to these physiological processes and can reach the circulation intact. This could possibly be due to the fact that several peptides are prone to ACE inhibition in vivo. It is possible that the potential to resist digestion conditions can be exemplified by the peptide LILP, which was hydrolysed to a shorter active form, HILP, by cellular peptidases prior to transport and absorption across the intestinal epithelial cells in a concentration-dependent manner. This suggests that the peptide HILP is readily absorbed and is resistant to proteases. It therefore may offer the possibility for an alternative peptide in the treatment or prevention of hypertension in humans.

In Vitro Assay for Evaluation of ACE Inhibitory Activity

To study and develop effective ACE inhibitors from natural sources, a simple, rapid, sensitive, and reliable analytical method as a means to monitor and pre-screen the enzyme activity in vitro is desirable and crucial. In vitro ACE-1 activity is generally measured by observing the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors. The commonly used method has always been based on the availability of the enzyme's active site. However, in practice, the enzyme can be inhibited by various means, including the formation of a complex with a specific substrate or the addition of a co-factor or activator. In vitro ACE-1 activity is generally measured by observing the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors. The commonly used method has always been based on the availability of the enzyme's active site. However, in practice, the enzyme can be inhibited by various means, including the formation of a complex with a specific substrate or the addition of a co-factor or activator.

Sources of ACE Inhibitory Peptides

A myriad of potent ACE inhibitors are available and are usually categorized into two major types: the synthetic and the nutritionally derived ACE inhibitors. Pharmacological inhibitors are biochemically based on the structure of the amino acid sequence, including three major categories: (i) sulfhydryl-containing groups; (ii) dicarboxyl-containing groups, and (iii) phosphorus-containing groups (Cushman and Cushman, 1999). These anti-hypertensive drugs, such as captopril, enalapril, and lisinopril, have effectively been used in the treatment of hypertension, coexistent heart failure, and diabetic neuropathy. Moreover, ramipril and quinapril exert effects such as plaque stabilization. However, the effects of nitric oxide and prostaglandins, a mediator of vascular smooth muscle proliferation, decreased coronary vasculoreactivity and anti-angiogenic function. These beneficial effects are due to ACE inhibition (Weng et al., 2004). Some of the latest ACE inhibitors are disease-oriented and may also occur due to drug-drug and/or drug-nutrient interactions. Although their established therapeutic efficiency, their inherently associated side effects necessitate the urgent need for the research and...
development of innovative, safer, economical, and natural alternatives.

MARINE-DERIVED ACE INHIBITORS

The discovery of ACE inhibitors from marine organisms started in the early 1990s, when dried bonito (Katsuwonus pelamis), a traditional Japanese seasoning made of bonito muscle, was examined for its potential to inhibit the activity of ACE. Yokoyama and colleagues (1992) hydrolyzed dried bonito by various proteases and the in vitro ACE-I activity of the hydrolysates was measured. Their results indicated that amongst all the other digests, the dried bonito hydrolyzed by thermolysin, of which 8 inhibitory peptides were identified by HPLC, demonstrated the highest in vitro ACE-I activity. However, in an in vivo study, a digest of dried bonito by gastrointestinal proteases failed to lower BP after a single oral administration in spite of a fairly high in vitro ACE inhibition ($IC_{50} = 41 \mu g/mL$) (Fujita et al., 1995). As mentioned earlier, the parent peptide must be hydrolyzed to exert a health beneficial effect. This was confirmed in the in vivo study of Fujita and Yoshikawa (1999) where the efficiency of the isolated ACE-I peptide LKFPNM (parent peptide) and LKP was compared after oral administration in SHR. It was found that LKFPNM and captopril showed a maximum decline in BP after 4 h of administration, whereas LKP reduced BP after 2 h, indicating a more potent inhibition of ACE than the parent peptide (Fujita and Yoshikawa, 1999). From their study, it can be deduced that ACE-I peptides isolated from the dried bonito exerted remarkably higher hypotensive activity in vivo but weaker activities in vitro, which was ascertained by using captopril as the reference drug. It is known that BP is regulated by the contraction of the vascular smooth muscle and endothelial function. The effect of dried bonito on rat isolated aortas was investigated (Ocione et al., 2003). The study demonstrated that ACE inhibition can have a direct action on vascular smooth muscle, involved in vasodilator activity and regulation. Besides dried bonito, a variety of other fish species have been investigated for their potential and hypotensive bioactivity.

With marine species comprising approximately one-half of the global biodiversity, the ocean offers a wonderful resource for novel compounds, which may serve in improving health of the worldwide population. Unfortunately, not all of the sea resources are adequately used. In recent years, over-exploitation of fishery resources has become a major concern worldwide. Many commercially important fish species are being overfished and existing valued species are becoming exhausted. According to the Food and Agriculture Organization (FAO), approximately 77% of the 143.6 million tons of fish and shellfish caught in 2006 was used for human consumption (Wilson et al., 2011). For remaining processing, such as skin, scales, fins, frames, viscera, shells, trimmings, are either thrown back in the sea or retained for production of fishmeal, fish oil, fertilizer, fish silage, and animal feed (Kohonen and Pihlanto, 2006). The annual unintentional catch and discards have been reported to be 73.9 trillion (Food and Agriculture Organization, 2005), presenting a serious marine conservation problem (Blanco et al., 2007). Notably, much of these wasted and discarded stocks are highly nutritious, comprising of high-quality proteins, vitamins, omega-3 fatty acids, and important novel compounds, the bioactive peptides. These wasted species may thus, constitute an incipient industry for valuable human food.

Consequently, there has been growing interest in exploring the possible uses of the fish by-products or the remaining raw materials, so that they can potentially be utilized rather than posing a waste and sustainability problem. For instance, sea bream scales were used to reproduce ACE-I peptides, which were analyzed for their ability to exert their hypotensive effects in SHR. The amino-acid sequences of the potential inhibitors were determined to be Gly-Tyr, Val-Tyr, Gly-Phe, and Val-Ile-Tyr (Palma et al., 2004). Similarly, Je and co-workers (2004) hydrolyzed Alaska Pollock (salt water fish of the Cod family), which is usually discarded as an industrial by-product in the Korean fish processing plant, with papain to purify a novel ACE-I peptide, of amino acid sequence of Pro-Gly-Ala-Arg-Tyrgly-Ala-Arg-Ala. Another study, involving the utilization of Atlantic Pollock skin, was carried out to investigate the relationship between ACE-I activity of the hydrolysates and their structure (Byun and Kim, 2002). Theodore and Kristensson (2007) investigated the potential of 7.9 hydrolysates made from extracted codfish muscle proteins. Their results indicated that hydrolysates prepared from pure and non-enzymatic oxidations do show a potential for ACE inhibition, which may find a use as bioactive ingredients. Tilapia, an important aquaculture, and economically important aquatic species, was used as a potential source of bioactive compounds because of the large quantities of waste generated, on a yearly basis. Raghavan and Kristensson (2003) used tilapia protein hydrolysate to evaluate its in vitro ACE inhibition capability and the outcomes of their study suggested that optimizing enzymes can be used to obtain peptides from tilapia with a high level of ACE inhibition.

Due to the fact that hypertension is affecting more than 15–20% of the adult population worldwide, researchers are attempting to use the most natural resources, such as seaweed frames, sponges, or sharks, to develop anti-hypertensive bioactive compounds. Moreover, in order to exert an anti-hypertensive effect in vivo, the neurotropically derived ACE inhibitor must be absorbed in their intact form from the intestine and further be resistant to plasma peptidases degradation to reach the target sites after oral or intravenous administration. Yellowfin sole frame protein, normally discarded as an industrial waste in the process of fish manufacturing, has been hydrolyzed by digestive enzymes (such as $a$-chymotrypsin) to generate a potent ACE inhibitor (Jung et al., 2006). The purified non-competitive inhibitor, comprising of 11 amino acids, naturally hydrophobic, was then orally administered in SHR. Systolic blood pressure was measured at 1, 2, 3, 6, and 9 h and a significant reduction of 22 mmHg at 3 h after administration was
<table>
<thead>
<tr>
<th>Source</th>
<th>Treatment</th>
<th>Peptide sequence</th>
<th>IC₅₀ value (µM)</th>
<th>Type of inhibition</th>
<th>Physiological activities</th>
<th>Study model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alaska Pollock fatty tissue</td>
<td>Pepsin</td>
<td>Pro-Cys-Glu-Ala-Nor-Thr-Arg-Gly-Ala</td>
<td>14.7</td>
<td>Non-competitive</td>
<td>ACE inhibition</td>
<td>In vitro</td>
</tr>
<tr>
<td>Yellow flounder (Lampetra plana)</td>
<td>a-chymotrypsin</td>
<td>Met-Asp-Phi-Pro-Gly-Ala-Gly-Arg-Leu</td>
<td>28.7</td>
<td>Non-competitive</td>
<td>Hypotensive</td>
<td>In vivo</td>
</tr>
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<td>Tuna frame protein hyaluronate</td>
<td>Gly-Asp-Leu-Arg-Lys-Thr-Thr-Arg-Glu-Leu</td>
<td>11.28</td>
<td>Non-competitive</td>
<td>Hypotensive</td>
<td>In vivo</td>
<td>(Lee et al., 2010)</td>
</tr>
<tr>
<td>Oysters (Crassostrea gigas)</td>
<td>Pepsin</td>
<td>Val-Val-Tyr-Pro-Arg-Thr-Glu-Ala</td>
<td>56.8</td>
<td>Non-competitive</td>
<td>Hypotensive</td>
<td>In vivo</td>
</tr>
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<td>Pacific red (Ulua maori)</td>
<td>Pepsin</td>
<td>Leu-Leu-Met-Asp-Arg-Asp-Glu-Pro</td>
<td>35.7</td>
<td>Non-competitive</td>
<td>ACE inhibition</td>
<td>In vivo</td>
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<tr>
<td>Strips (Acorus calamus)</td>
<td>Pepsin</td>
<td>Leu-His-Pro</td>
<td>1.6</td>
<td>Competitive</td>
<td>Hypotensive</td>
<td>In vivo</td>
</tr>
<tr>
<td>Rainbow trout (Oncorhynchus mykiss)</td>
<td>Pepsin</td>
<td>Lys-Val-Asp-Glu-Pro-Ala-Met-Str-Pro-Ala</td>
<td>63.9</td>
<td>Competitive</td>
<td>ACE inhibition</td>
<td>In vivo</td>
</tr>
<tr>
<td>Edible (Brachyura marina)</td>
<td>Alcalase</td>
<td>Arg-Asp-Thr-Glu-Ala-Asp-Arg-Glu-Pro</td>
<td>9.6</td>
<td>Competitive</td>
<td>ACE inhibition</td>
<td>In vivo</td>
</tr>
<tr>
<td>Grass carp pivosomes</td>
<td>Pepsin</td>
<td>Gly-Tyr</td>
<td>0.0005±±±±±</td>
<td>Competitive</td>
<td>ACE inhibition</td>
<td>In vivo</td>
</tr>
<tr>
<td>Chum salmon muscle hyaluronate</td>
<td>Thrombolysin</td>
<td>Leu-Phe</td>
<td>30.2</td>
<td>Competitive</td>
<td>ACE inhibition</td>
<td>In vivo</td>
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<tr>
<td>Hard clam (Malleus baronia)</td>
<td>Pepsin</td>
<td>Tyr-Aib</td>
<td>51</td>
<td>Mixed-type</td>
<td>ACE inhibition</td>
<td>In vivo</td>
</tr>
<tr>
<td>Sea cucumber (Acisalpa nigra)</td>
<td>Bovine trypsin</td>
<td>Met-Asp-Glu-Glu-Glu-Glu-Glu-Glu-Glu</td>
<td>15.9</td>
<td>Pro-drug</td>
<td>Hypotensive</td>
<td>In vivo</td>
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<td>Shrimp muscle hydroclastin</td>
<td>Pepsin</td>
<td>Glu-Tyr</td>
<td>2.68</td>
<td>ACE inhibition</td>
<td>In vivo</td>
<td>(Wu et al., 2006)</td>
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<tr>
<td>Chum salmon heart</td>
<td>Pepsin</td>
<td>Gly-Asp-Gly</td>
<td>730</td>
<td>Hypotensive</td>
<td>2 µg/kg</td>
<td>Male mice</td>
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<tr>
<td>Sea bass scales</td>
<td>Pepsin</td>
<td>Val-Glu-Tyr</td>
<td>7.5</td>
<td>Hypotensive</td>
<td>2 µg/kg</td>
<td>Male mice</td>
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<td>Bigeye tuna dark muscle</td>
<td>Pepsin</td>
<td>Tyr-Pro-Glu-Ala-Ala-Glu-Leu-Met-Glu-Val-Arg-Pro</td>
<td>21.6</td>
<td>ACE inhibition</td>
<td>300 mg/kg</td>
<td>Male mice</td>
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<tr>
<td>Squidward squidfish muscle</td>
<td>Alcalase</td>
<td>The-Pro-Asp-Asp-Glu-Pro</td>
<td>0.02±±±±±</td>
<td>ACE inhibition</td>
<td>In vivo</td>
<td>(Wijesinana et al., 2011)</td>
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<td>Literature Review</td>
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<tr>
<td><strong>Wakeren</strong> (Umbria phalensis)</td>
<td>Pretaza5 (Atropa Schoenus)</td>
<td>Val-Tyr</td>
<td>1.41***</td>
<td>—</td>
<td>ACE inhibition</td>
<td>In vivo</td>
</tr>
<tr>
<td></td>
<td>Be-Tyr</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Hypertensiv</td>
<td>In vivo</td>
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<tr>
<td></td>
<td>Ala-Tyr</td>
<td>14.1</td>
<td>—</td>
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<td></td>
<td>Pro-Tyr</td>
<td>42.3</td>
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<td>Val-Tyr</td>
<td>3.3</td>
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<tr>
<td></td>
<td>Leu-Tyr</td>
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<td>—</td>
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<tr>
<td></td>
<td>Lys-Tyr</td>
<td>23.6</td>
<td>—</td>
<td>—</td>
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<td><strong>Carthia</strong> (Dactylocoronaria protein)</td>
<td>Bacterial protease (Bacillus polymyxa)</td>
<td>Ala-Pro</td>
<td>0.12</td>
<td>—</td>
<td>ACE inhibition</td>
<td>In vivo</td>
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<tr>
<td></td>
<td>Alkali, pepsis</td>
<td>Gly-Pro-Gly-Leu-Leu-Gly-Pro-Leu-Leu-Cys-Leu-Ser</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
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<tr>
<td><strong>Atlantic salmen</strong> (Salmo salar)</td>
<td>Alkali, pepsis</td>
<td>Ala-Pro</td>
<td>6.000***</td>
<td>—</td>
<td>ACE inhibition</td>
<td>In vivo</td>
</tr>
<tr>
<td><strong>Jellyfish</strong> (Mastigias sp) (protein)</td>
<td>Pepsin, pepsis</td>
<td>Val-Tyr</td>
<td>6.300***</td>
<td>—</td>
<td>ACE inhibition</td>
<td>In vivo</td>
</tr>
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<td></td>
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<td>—</td>
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<tr>
<td><strong>Chalone muscle</strong></td>
<td>Thermolysin</td>
<td>—</td>
<td>2.59**</td>
<td>—</td>
<td>Anti-hypertensive</td>
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<td>ACE inhibition</td>
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<td>200 mg/kg, 400 mg/kg and</td>
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<td><strong>Salmon muscle</strong> (Onchorynchus gorbuscha)</td>
<td>Pepsin</td>
<td>—</td>
<td>2.5</td>
<td>—</td>
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<td><strong>Tilapia</strong> (C. coriacea)</td>
<td>Pepsin</td>
<td>—</td>
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<td><strong>Sardine</strong> (Sardinella brasiliensis)</td>
<td>Skeletal muscle alkaline protease</td>
<td>—</td>
<td>0.24***</td>
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* p<0.05
** p<0.01
*** p<0.001
— Not specified
Literature Review

observed. This is an indication that the isolated ACE-1 peptide from yellowtail saithe frame protein might be added as a food ingredient to develop functional food or may be used as a nutraceutical, with the potential of lowering BP (Yang et al., 2006).

From the coastal waters of China derive chinon, a marine shrimp, which is highly harvested annually. However, it is currently utilized for its high protein value due to its small size, but rarely used as flavoring agent in shrimp sauce. Cao and team (2010) endeavored to prepare ACE-4 peptides from this shrimp's protein hydrolysate using fractional precipitation (pH 3.3) to examine whether the peptides will be resistant to the digestive enzymes. The fractionated peptide, Lys-His-Pro, exhibiting 32.7% ACE inhibition in vitro, was investigated for its in vivo anti-hypertrophic activity in male SHR, as evaluated by tail-cuff method. Their findings demonstrated that after an oral administration of a high dose of 6 mg/kg body weight of the synthetic peptide, BF of SHR was significantly reduced by 31 ± 3.1% (P < 0.01) at 1 hour, 30 ± 3.4% (P > 0.05) at 6 hour, and 29 ± 3.5% (P < 0.01) at 8 hour (Cao et al., 2010).

In the study of Wang and colleagues (2008), an ACE-I peptide was derived from yeast proteins by papain treatment. After separation and purification by gel filtration chromatography, RP-HPLC, and LC-MS, the peptide sequence, which was determined to be a nonapeptide of Val-Val-Lys-Pro-Arg-Tyr-Glu-Asp-Asp, was assayed for heat, pH, and digestive enzymes stability. The peptide indicated a yield of 8.6%, with good heat and pH stability, as well as strong enzyme-resistance properties against digestive enzymes. This further showed the excellent in vivo anti-hypertensive effect when orally administrated in SHR at a dose of 20 mg/kg. This could therefore, be served as a natural, cheap, safe, and innovative source of hypotensive peptide (Wang et al., 2008), Table 3 lists the ACE-I peptides from different marine organisms and the treatment used to isolate these peptides.

ANTI-OBESEITY PEPTIDES

Increasing epidemiological evidence is linking the prevalence of obesity to dietary factors. It is well established that high carbohydrate and fat diets, coupled with a sedentary lifestyle, are the major lifestyle factors associated with a disordered lipid metabolism, which often results in high plasma levels of LDL, HDL, atherogenic and dyslipidemia of glucose. It has been recently recognized that protein is the most valuable macronutrient. Moderate intake of protein diet plays a crucial role in body weight loss and weight maintenance (Gosev et al., 2004). The proposed mechanisms for weight loss include increased satiety and thermogenesis, suppression of fat free mass and lowering total caloric, resulting in decreased body weight (Weltman et al., 2006). However, little information is available on the mechanistic roles of nutritionally derived ACE-I peptides in controlling food intake, lowering body weight, and improving glucose tolerance. As discussed earlier, RAS blockers have been linked not only to hypotensive action but also to improvements in abnormal glucose metabolism, which may result in diabetes.

In two regards, Okada and colleagues (2009) used the stroke-prone SHR, a well-characterized animal model of essential hypertension with high incidence of stroke, cardiac hypertrophy, kidney dysfunction, and hyperglycemia, to investigate the effects of a Sulfophr-derivatized ACE-I peptide (SP-A) exhibiting an ACE inhibitory concentration of KI value of 67.4 μg/ml, on blood glucose levels and blood pressure (Okada et al., 2009). SP-A treated SHR animals showed (1) a decreased ACE activity in the kidney, aorta, and mesenteric and lowered BP, (2) reduced glucose levels after glucose loading, but no effect on insulin secretion, which suggested that SP-A administration improved glucose tolerance and insulin sensitivity in the stroke-prone animals. Fish protein hydrolysate, not examined for their ACE inhibition potential, have been demonstrated to reduce plasma total proportion of LDL, cholesterol and lower ApoB100, cholesterol apoprotein B-100 activity in Zucker rats (Wernigold et al., 2004). While many pharmacological studies have been done, no naturally derived ACE-I peptides have been tested as possible treatments for obesity and the complications of metabolic diseases.

CONCLUSION

The unprecedented occurrence in the food-related diseases, such as hypertension and obesity globally, has increased our awareness of nutrition and health. The implementation of nutritionally derived functional food products that not only offer basic nutritional value but also physiological effects that can potentially prevent or treat these detrimental health states. The RAS has long been recognized for its role in the pathogenesis of known primary health disorders. Many different studies have been conducted to examine the effects of the RAS blockers and diet on blood pressure. It has been shown that diet can lower blood pressure and that ACE-I peptides may have similar effects. ACE-I peptides have been shown to lower blood pressure in animal models and human studies.

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Literature Review

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Literature Review

Review of Anti-obesity Peptides


Literature Review


CHAPTER 3

Biofunctional and physicochemical properties of fish scales collagen-derived protein powders
Chapter 3 describes the physicochemical properties as well as the potential of fish protein hydrolysates derived from fish wastes (scales) to act as an ACE and trypsin inhibitor and an anti-oxidant or free radical scavenger. The effects of digestive proteases involved in an *in-vitro* simulated digestion on the bio-functionalities of the hydrolysates were also evaluated.

GRADUATE RESEARCH CENTRE

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION:
PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each co-authored publication and placed at the beginning of the thesis chapter to which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

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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.

Vasambal Manikkan

Signature Date: 29/04/2016

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;

2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to a) granting bodies; b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
3. The original data will be held for at least five years from the date indicated below and is stored at the following location(s):

College of Health and Biomedicine, Victoria University, Werribee Campus, Melbourne, VIC, Australia

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Updated: June 2015
Biofunctional and physicochemical properties of fish scales collagen-derived protein powders

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Abstract
Fish waste, such as scales, is a rich source of proteins, which can be applied in various commercial applications. Enzymatic hydrolysis for example simulated gastrointestinal digestion (SGID) can release physiologically active peptides with the potential to benefit consumers’ health. Powdered (PH) and agglomerated (AH) hydrolysates prepared from fish scales-derived collagen were investigated for their physiological and biofunctional properties. Having a higher protein and moisture content, PH showed greater solubility and digestibility than AH. In vitro SGID significantly impacted on the studied inhibitory activities. The released peptides (RP) of PH after completed digestion, exhibited higher angiotensin converting enzyme inhibitory (ACE-I) activity (73.5%) compared to AH. Both preparations showed similar trypsin inhibitory (TI) activities, 44.33% and 47.11% respectively. In contrast, the antioxidant activities of the hydrolysates were very low upon SGID. Physicochemical properties of these preparations apparently affected their in vitro physiological properties, which were further modulated through SGID.

Keywords
Angiotensin converting enzyme
Bisacitic peptides
Enzymatic hydrolysis
Fish waste
Particle size
Trypsin inhibitory activity

Introduction
On an annual basis worldwide, more than 50% of the total catch of the 100 million tons of fish harvest is discarded as underutilised fish and fish processing wastes, including scales (Blanco et al., 2007). The waste is created due to poor technological properties, low commercial value and reduced functional features. Such wastes are either thrown back into the sea, causing a serious marine environmental issue (Blanco et al., 2007); or they are rejected as by-products, traditionally being utilized for the production of fertilizer and animal feed (Korhonen and Fihanto, 2006). Importantly, these discarded materials consist of nutritionally valuable compounds, in terms of their protein and mineral composition. Thus, these natural marine resources could effectively be developed into novel compounds, such as proteinaceous hydrolysates or bioactive peptides, by employing advanced food technologies, such as enzymatic hydrolysis (Möller et al., 2008). Manikkam et al. (2015) highlighted that human gut enzymes are able to hydrolyse protein fragments from marine sources to produce a range of small peptides acquiring essential biofunctionalities. Therefore, this process of enzymatic hydrolysis is a potential improvement in the area of fish science and sustainability as it firstly, aids in minimising marine wastes and maintaining a sustainable aquatic environment, and secondly, enables the production of food hydrolysates with physiological functions, which may aid in the prevention and/or management of human diseases.

The development of enzymatic hydrolysates, with potential ACE-I activity (Fahani et al., 2004) and antioxidant capacity (Lin and Li, 2006) from fish wastes of different fish species had led to maximizing protein recovery. Moreover, fish wastes, such as scales are indeed valuable sources of collagen. Marine scales-derived collagen has been widely known for its cosmetic, anti-aging and food technological applications (Ogawa et al., 2004). Besides, the collagen-derived hydrolysates produced by commercial enzymes exerted high anti-free radical activity and showed greater potential to decrease blood pressure (Morrison et al., 2002). Furthermore, Zhu et al. (2010) concluded that dietary supplementation with marine collagen derived peptides could offer protection against diabetes and hypertension; and could supply novel bioactive peptides in functional foods.

Metabolic diseases, such as obesity and its related comorbidities, like hypertension, diabetes,
cardiovascular diseases, amongst others, are current medical concerns that are reaching pandemic levels worldwide. Obesity, a life-style-dependent condition, may be prevented by increasing satiety and monitoring the renin-angiotensin system (Mathai et al., 2008). Captopril and periopril are speimics of synthetic ACE inhibitors that have successfully aided in reducing fat mass, body weight and blood pressure of rats (Mathai et al., 2008). However, their side effects provide important substituions to naturally derived ACE inhibitors from food sources, since the latter are natural, safer as well as fairly inexpensive; and may form part of daily balanced diet.

Alternatively, the inhibition of certain enzymes involved in nutrients metabolism may fundamentally assist in the management of obesity. One plausible approach could include trypsin inhibition since it may assist in the production of cholecystokinin (CCK), induced by a trypsin-sensitive CCK-releasing peptide to control appetite (Herzig et al., 1996). A current lack of information relevant to TI potency of fish hydrolysates and their RP upon digestion requires further attention. Obesity is also a risk factor for releasing reactive oxygen species, oxidising lipids within the body and causing progressive aging, inflammation and increased risk of cancers, such as prostate and breast carcinomas (Giovanucci, 2007). Therefore, a need to explore the anti-oxidant activity of fish scales derived hydrolysates, as a very important property to maintain health, is necessary.

Currently on the market, more than 500 commercial health products with claimed bioactive peptides are available. Two of which include 1) Nutripeptin, a cod hydrolysate for lowering glycemic index and 2) Fortidiun LIQUAMEN; a fish autolysate exhibiting multiple effects, such as, reducing oxidative stress, lowering glycemic index and anti-stress (Gudrard et al., 2010). There is a need to use marine waste to develop more commercial health products to combat metabolic diseases. Owing to the ongoing wastage of fish scales within the fish industry, there has recently been a mounting interest in exploring their possible uses as functional food ingredients or nutraceuticals, aiming at beneficially influencing human health. For instance, sea bream scales were used to produce ACE-I peptides, with amino acid sequences of Gln-Tyr, Val-Tyr, Gly-Phc and Val-Ile-Tyr. They were analysed for their hypotensive effects in spontaneously hypertensive rats (Fahmi et al., 2004). However, there is limited information about the ACE-I peptides derived from fish scales collagen.

Therefore, this study aimed at investigating the physicochemical properties as well as the potential of two fish protein powders, derived from the collagen of fish scales, to simultaneously act as i) an ACE inhibitor, ii) a trypsin enzyme inhibitor, and iii) an anti-oxidant or free radical scavenger. The effects of the digestive proteases involved in an in vitro simulated digestion on the bio-functionalities of these powders were also evaluated.

Materials and Methods

Materials
Angiotensin-I-converting enzyme from rabbit lung (A6778), Hippuryl-Histidyl-Leucine (HHL), captopril, trifluoroacetic acid (TFA), trichloroacetic acid (TCA), acetonitrile, peptin (from porcine stomach mucosa), bile extract (porcine), pancreatin (porcine pancreas), trypsin (Type II-S from Porcine pancreas), Nε-Benzyloxycarbonyl-arginine-nitroanilide hydrochloride (BAPNA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), vitamin C, Bradford Reagent and bovine serum albumin (BSA) were all purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Ethyl acetate, glacial acetic acid and dimethyl sulfoxide were from Merek Pty Ltd (Darmstadt, Germany). All the other chemicals used for the preparation of buffering solutions were of analytical grade. Fish scale collagen-derived protein powders were kindly supplied by Eastern P/L (Geelong, Victoria, Australia).

Scanning electron microscope (SEM) analysis
Prior to the SEM analysis of the powders' morphology, a representative amount of each product was placed on a two-sided adhesive carbon tape to dry in a vacuum oven overnight. Desiccated samples were then gold-coated (Neo Coater MP - 19020NCTR, JEOL, Japan) for 8 and 10 min for PH and AH respectively, to generate a gold plasma around the samples. Imaging was carried out using a bench top SEM (Neo Scope JCM – 5000, JEOL, Japan).

Protein and moisture content determination
The protein content was analysed by the Kjeldahl method (AOAC 955.04) and the moisture content was assayed by oven drying at 105°C (AOAC 990.19).

Particle size distribution (PSD) pattern analysis
A 0.1% (w/w) dispersion was prepared from each sample. The refractive index of the hydrolysate dispersions was measured by a refractometer (ATAGO Co. Ltd, Tokyo, Japan). The dispersions were left hydrated overnight with constant stirring on a magnetic stirrer (Industrial Equipment & Control Pty. Ltd, Australia) in a cold room (4°C). The PSD patterns of the hydrolysates were then determined
using a Malvern Zetasizer Nano ZS Instrument (Malvern Instruments, Worcestershire, UK) at 25°C.

**Solubility**
The solubility of the undigested samples was determined according to Dissanayake et al. (2012). Briefly, a 0.1% solution (w/w), prepared from each product was vortex-mixed (Chiltern Scientific Instrumentation Ltd., United Kingdom) and immediately centrifuged at 16,850 g (Servall® R77) for 15 min. Protein contents of the supernatants were determined by the Kjeldahl method. The % solubility was calculated as follows,

\[
\text{Solubility, } \% = \left( \frac{\text{Protein content in supernatant}}{\text{Protein content of original sample}} \right) \times 100
\]

**Digestibility**
The protein digestibility assay was conducted according to Folk et al. (2011), with slight modifications. Powders, approximately 10 mg, were digested in 5 ml trypsin solution (0.1 mg/ml in 100 mM Tris-HCl buffer, pH 7.6). The suspension was incubated at 37°C for 2 h and the process was stopped by adding 30 ml of 0.5% TCA. The mixture was allowed to stand for 30 min in a cold room at 4°C and centrifuged at 16,850 g (Servall® R77) for 20 min. The resultant precipitate was dissolved in 2.5 ml NaOH. Protein content was determined by the Kjeldahl method. Protein digestibility was determined using equation 2:

\[
\text{Protein digestibility, } \% = \left( \frac{\text{Protein content in digested sample}}{\text{Protein content in TCA precipitate}} \right) \times 100
\]

**Simulated gastrointestinal digestion (SGID)**
The in vitro SGID process was a modified version of Medenikos and Vasiljevic (2008). After the peptic digestion, the pH was increased to 8.0 with 1 M NaHCO₃ before the addition of 5 ml trypsin enzyme solution (EC 2.3.2.5) (0.2 g trypsin in 10 ml 0.1 M phosphate buffer), followed by shaking incubation (Inova™ 4250, New Brunswick Scientific, Edison, NJ, USA) at 37°C for 1 h at 100 rpm. To simulate the small intestinal digestive conditions, the pH of the partially digested samples was adjusted to 6.3 with 1 M HCl, after which, 3 ml pancreatin/bile solution was added, followed by 2 h shaking incubation at 37°C at 100 rpm. To monitor the bioactivities after each digestive treatment, an aliquot of digest was removed, submerging into a boiling water bath (RATEK Instruments) for 15 min to halt all enzymatic reactions. After cooling on ice, all samples were centrifuged at 4,000 g (Beckman J2-HS centrifuge, JA-20 rotor, Palo, Alto, CA, USA) for 15 min, and the supernatant fractions were filtered using a 0.45 μm pore size filter (Schleicher & Scuell GmbH, Germany) into clean tubes and were stored at -18°C for further analyses.

**Determination of ACE inhibitory activity**
The ACE-I activity, involving the application of ACE (1 unit/10 ml) and its substrate HHL (5 mM), was spectrophotometrically assayed according to Donkor et al. (2007). The resulting residue of hippuric acid was dissolved in 1 ml deionised water and its concentration was determined by measuring the absorbance at 228 nm, using UV/Visible spectrophotometer against MilliQ water as the blank. The extent of inhibition was calculated using equation 3:

\[
\text{ACE inhibitory activity, } \% = \left( 1 - \frac{A - B}{C - D} \right) \times 100
\]

Where,

A = Absorbance in the presence of ACE and without the ACE-I component
B = Absorbance without the ACE-I component
C = Absorbance with ACE and the ACE-I component
D = Absorbance without ACE and with the ACE-I component

The ACE inhibition was also expressed in terms of the IC₅₀ value, representing the protein concentration (mg/ml) in the sample required to inhibit 50% of the ACE activity.

**Determination of tryptin inhibitory (TI) activity**
The TI activity of the undigested powders and their digest was determined according to Medenikos and Vasiljevic (2008). Briefly, 250 μl of samples were pre-incubated at 37°C for 10 min with 625 μl of BAPNA solution. This was followed by the addition of 250 μl trypsin enzyme solution before incubating for 10 min at 37°C. The reaction was terminated by adding 250 μl of 30% glacial acetic acid and vortex-mixed. The absorbance of each sample was read at 410 nm. The extent of inhibition was calculated using Equation 3.

**DPPH radical scavenging activity (RSA)**
The antioxidant capacity of the products and their digest was evaluated by measuring the free RSA according to Donkor et al. (2012), with slight modifications. Briefly, 4.0 ml DPPH solution (0.075 mM in methanol) was added to 0.1 ml diluted (in 1 ml methanol) undigested sample and the digests followed by 30 min incubation in the dark, after which, the absorbance was read at 517 nm with a Pharmacia UV spectrophotometer. The antioxidant activity was calculated as percent inhibition, using
Equation 4:

Inhibition, % = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}}\right) \times 100$

**Reverse-phase HPLC analysis of released peptides**

The non-digested powders and the peptides released upon the SGID were profiled using a Varian HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a reverse-phase C18 monomeric column - 5 μm, 300Å, 250mm x 4.6mm and a guard column (Grace Vylea, Hopkoria, CA, USA) (Dunkor et al. 2007). Samples were applied using a 10 μl injection loop. The peptides were eluted by a linear gradient from 100% to 0% solvent A [0.1% TFA in deionised water] in solvent B (0.1% TFA in 90%, v/v, acetonitrile in deionised water) over 90 min. All samples and mobile phase solvents were filtered through a 0.45 μm membrane filter. Peptide separations were conducted at a flow rate of 0.75 ml/min. The eluted peptides were monitored at 214 nm using a Varian 9050 variable wavelength UV/Vis detector.

**Statistical analysis**

The experiments were arranged to explore the influence of three digestive enzymes, namely peptic, trypsin and pepsin on the inhibitory activities of the two fish protein powders. The *in vitro* gastrointestinal digestion of each sample was conducted in triplicate. The readings of the inhibitory activities of each replicate were taken three times, indicating that the mean of nine readings was considered. The results were analysed using a General Linear Model (GLM) procedure by the Statistical Analysis System (SAS). All data were analysed by a two-way ANOVA using the SAS software. The level of significance was set to $P < 0.05$.

**Results and Discussion**

**Morphology of fine (PH) and granulated (AH) fish powders**

Figure 1 illustrates the electronmicrographs obtained for each sample. The PH particles demonstrated highly porous spherical shapes with uneven surfaces (Figure 1A). The appearance of cracks and holes on the surfaces was also obvious. In comparison to AH, which showed irregular network of structures (Figure 1B), PH displayed a smoother matrix. The differences observed could be due to processing treatments employed during their manufacture. There is currently very little information relevant to the structural conformation or morphology of hydrolysates produced from fish scales collagen. Thus, comparing our results with previous studies is difficult since our study is the first to look into the morphological properties of fish scales collagen-derived powders.

However, in the study of Foh et al. (2011), fresh minced meat and hot water dip hydrolysates exhibited smoother matrix compared to their concentrates, showing aggregates of packed flake-like structures. Zhang et al. (2008) revealed a significant difference between the surface morphology of collagen and gelatin. Collagenous samples demonstrated fibril networks with rough membranous structure, whereas the gelatin membrane without fibril networks appeared only as smooth structure on the surface. Such demonstrated differences due to manufacturing of these products may also play a role in their solubility, subsequent digestion and release of the physiologically important peptides.

**Physicochemical properties**

In our study, the physicochemical properties of the fish protein powders have been characterised by their protein and moisture contents, solubility and digestibility, as presented in Table 1, in addition to their PSD pattern. The PH showed protein content of 71.24 ± 0.00% whereas AH contained substantially less proteins (44.52 ± 0.23%). The difference in protein content is likely caused by the processing of these protein powders. Moreover, the high protein content of PH could be a result of the solubilisation of protein during hydrolysis and the removal of insoluble undigested non-protein substances (Benjakul and Morrissey, 1997).

Furthermore, the types of enzymes used for the enzymatic hydrolysis in the production of the fish protein powders might have impacted on the protein
content of the final product. This is due to the fact that proteolytic enzymes are site-specific enzymes, indicating that the types of polypeptide fragments released upon hydrolysis are dependent upon their specificity (Damodaran et al., 2008). For instance, Muzaffar et al. (2012) found that protein content of hydrolysates prepared by Alcalase enzyme (82.66 ± 1.36%) differed from that of Flavourzyme-derived hydrolysate (73.51 ± 3.53%). In our study, the moisture content played no apparent role in higher protein concentration of the samples with PH having a higher moisture content of 7.26 ± 0.08% compared to AH, 6.87 ± 0.14% (Table 1).

From the PSD pattern (Figure not shown) of the fish protein powders, no obvious differences in PSD between the two products were observed, with all particles being below 10 nm in size. It can also be deduced that PH and AH had approximately 15% and 25% of particle size greater than 1 nm, respectively. Moreover, for the certainty of the PSD measurements, the refractive index of the sample dispersions were determined to be 1.38 for both AH and PH. Particle size is an important parameter that governs the functionality of a protein hydrolysate. One such functionality is solubility (Table 1), which differed (P < 0.05) between PH and AH.

Solubility of hydrolysates greatly depends on the water-protein interactions within the matrix. Enzymatic treatment during the production of the hydrolysates may affect water holding capacity as demonstrated by Kristinsson and Rasco (2000). Moreover, Muzaffar et al. (2012) reported significant difference between the solubility of hydrolysates derived from Alcalase and Flavourzyme. Similarly, Damodaran et al. (2008) reported that the types of enzymes used in the production of hydrolysates may impact on their solubility. In our study, after centrifugation of AH and PH, more pellet was

| Table 1. Selected physicochemical properties of fish scale-collagen derived hydrolysates |
| Sample | Protein content, % | Moisture content, % | Solubility, % | Digestibility, % |
| Precipitated hydrolysate | 71.24 ± 0.05 | 2.68 ± 0.06 | 41.15 ± 0.12 | 65.74 ± 0.04 |
| Agglomerated hydrolysate | 49.22 ± 0.02 | 6.17 ± 0.13 | 18.99 ± 0.03 | 22.34 ± 0.03 |

Values are represented as the mean ± standard deviation. Small letters within same row represent the significant difference (P < 0.05).

Figure 2. RP-HPLC profiles of peptides released by SGID from the fish-scales collagen-derived powders. (A) peptide profiling of undigested samples; (B) peptide profiling of released peptides after simulated digestion observed for AH, an indication of lower solubility of AH compared to PH. Another factor that might have affected the solubility of our samples is hydrophobicity. Damodaran et al. (2008) mentioned that proteins with higher hydrophobic amino acids will show a lower solubility. Based on this theoretical concept, it can be suggested that in our study, AH is more hydrophobic due to its lower solubility and aggregated (agglomerated) form. Further studies should look into the hydrophobicity of our samples to better understand the change in their solubility.

Whilst digestibility is another important functional property of a fish protein hydrolysate, it is also crucial in the determination of the nutritive quality of a protein, since it affects the bioavailability of its amino acids. In our study, the in vitro protein digestibility of the samples was evaluated by the release of TCA-soluble nitrogen. As indicated in Table 1, AH had a significantly lower digestibility (P < 0.05) than PH, a similar trend to its solubility. Temperature used during enzymatic hydrolysis, one factor that may affect protein digestibility, might have impacted on the digestibility of AH during its manufacturing process (Guo et al., 1999). In contrast, the higher protein solubility of PH might have improved its digestibility. Moreover, protein conformation, the processing techniques, enzymes involved during hydrolysis, and anti-nutritional factors, such as trypsin inhibitors are several factors that may influence the digestibility of hydrolysates (Damodaran et al., 2008).
Table 2. ACE-inhibitory activity and the IC₅₀ values of the undigested hydrolysates, their enzymatic digests and the released peptides after completion of SGID

<table>
<thead>
<tr>
<th>Digestion stages</th>
<th>ACE inhibition (%)</th>
<th>IC₅₀ (mg/mL)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Forwarded hydrolyze</td>
<td>Aggloetemized hydrolyze</td>
</tr>
<tr>
<td>Undigested</td>
<td>25.04²⁵</td>
<td>75.45²⁵</td>
</tr>
<tr>
<td>Peptic digests</td>
<td>33.33²⁵</td>
<td>53.33²⁵</td>
</tr>
<tr>
<td>Trypsin digests</td>
<td>30.71²⁵</td>
<td>65.09²⁵</td>
</tr>
<tr>
<td>Pancreatic digests</td>
<td>65.54²⁵</td>
<td>75.51²⁵</td>
</tr>
<tr>
<td>Fergusons after completion of SGID</td>
<td>73.62²⁵</td>
<td>69.75²⁵</td>
</tr>
<tr>
<td>SEM</td>
<td>1.62</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The means present average of 6 independent observations (±SEM); SEM – pooled standard error of the mean.
Small letters represent the significant differences (P<0.05) within a row for same parameter.
Capital letters represent the significant differences (P<0.001) within a column.

Peptide profiling

The qualitative profiles of the undigested samples and the peptides released upon SGID, as analysed by RP-HPLC, are illustrated in Figure 2A and 2B, respectively. The retention times of undigested PH, AH and captopril were between 40 and 50 minutes (Figure 2A). Similarities in peak shape and retention times were observed for digested captopril and PH (around 40 minutes; Figure 2B), indicating that PH may contain peptides with similar chemical structure to captopril. However, it would require further purification and sequencing to confirm. Moreover, the retention times of the observed peaks for all the undigested (Figure 2A) and digested (Figure 2B) samples were very similar. This could possibly indicate that the types of peptides released could be comparable but differed in concentrations. Further isolation, purification and sequencing techniques should be performed to identify and characterise the potent released peptides.

Bio-functionalities of undigested AH, PH, enzymatic digests and RP after completion of SGID

ACE-I activity

Table 2 presents the extent of in vιro ACE inhibition by undigested PH, AH, captopril, digests and RP. Captopril is a modified proline dipeptide and the first compound developed in the ACE-I class of anti-hypertensive drugs. Undigested AH exhibited a significantly higher ACE-I activity of 75.45% (P < 0.05) compared to PH, 23.04%. Apparently, the low solubility and larger particle size of AH did not impact on ACE-I activity.

Bioactive peptides containing 3-20 amino acid residues per molecule are generally inactive when encrypted in the amino acid sequence within a large protein molecule (Müller et al., 2008). The biologically active peptides will exert physiological functions only after cleavage from the parent protein. Upon enzymatic hydrolysis, such as during gastrointestinal digestion, proteins are broken down into oligopeptides before further conversion into shorter peptides. In this regards, digestion is an important process that possibly releases these bio-functional peptides, acting beneficially on the target organ in the human body, after complete absorption (Verzele et al., 2004).

The ACE inhibition of the crude peptic-digests of PH, AH and captopril were 33.33%, 53.35% and 40.66%, respectively. Moreover, an increase in activity against ACE in the trypsin and pancreatin digests of all samples was observed. Trypsin cleaves peptide bonds in non-polar amino acids. The peptides derived from tryptic hydrolysis can have C-terminus amino acids such as Valine, Alanine, Leucine, Proline, Tyrosine, Phenylalanine, Histidine and Tryptophan. Trypsin-derived peptides may inhibit ACE with a high affinity to substrates having C-terminus amino acids such as Alanine, Histidine, Leucine, Proline and Valine (Jung et al., 2006).

The ACE inhibition by RP from the PH and AH obtained after complete digestion were 73.65% and 69.57%, respectively. As depicted in peptide profiling (Fig. 2B), the RP of PH appeared to have a similar retention time as that of captopril. Collagen contains large amounts of hydroxyl-proline as a constituent amino acid (Morimatsu et al., 2002), thereby the strong ACE-I activity of RP from PH in comparison to AH. Moreover, the conditions of the in
Table 3: Trypsin inhibitory activity of the undigested hydrolysates, their enzymatic digests and the released peptides after completion of SGID

<table>
<thead>
<tr>
<th>Digestion Stages</th>
<th>Trypsin Inhibition (%)</th>
<th>IC50 (mg/mL)</th>
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<tr>
<td></td>
<td>Faeured hydrolysatse</td>
<td>Agglomerated hydrolysatse</td>
</tr>
<tr>
<td>Undigested</td>
<td>14.32±25</td>
<td>27.70±39</td>
</tr>
<tr>
<td>Peptid digests</td>
<td>12.11±26</td>
<td>32.9±48</td>
</tr>
<tr>
<td>Trypsidigests</td>
<td>12.74±26</td>
<td>31.4±50</td>
</tr>
<tr>
<td>Pancreatin digests</td>
<td>32.92±26</td>
<td>49.8±40</td>
</tr>
<tr>
<td>Fragments after completion of SGID</td>
<td>44.33±33</td>
<td>47.15±35</td>
</tr>
</tbody>
</table>

SEM: 1.32  0.05

The means present average of 6 independent observations (k=6); SEM - pooled standard error of the mean. Small letters represent the significant differences (P<0.05) within a row for same parameter. Capital letters represent the significant differences (P<0.05) within a column.

Ex vivo digestion process, such as enzyme preparations, temperatures, pH and incubation time might impact the degree of hydrolysis and the resultant ACE-I activity of the samples.

The decline in the inhibitory activity of RP from AH after the pancreatic digests could be due to further degradation by these enzymes (Donkor et al., 2007). Prolin residues are often resistant to degradation and may often pass from the small intestine into the blood circulation as short peptides (Korthoen and Philastre, 2006). Further studies would be necessary to confirm the amino acid sequence of these peptides after purification.

ACE-inhibitory peptides have been identified and isolated from various food sources, such as probiotic yoghurt (Donkor et al., 2007). However, these studies used bacterial and various commercial proteolytic enzymes (such as Alcalase, Flavourzyme, etc.) for the production of ACE-I peptides; thus, comparison between previous reports and the current study is very difficult. Moreover, there is very limited information regarding ACE-I peptides of hydrolysates derived from the collagen of fish scales. Therefore, more research is required to establish these effects in order to maximise the utilisation of these marine wastes.

Trypsin inhibitory activity

The TI potency of the undigested AH and PH were 27.70% and 14.33%, respectively (Table 3). Similar to ACE-I activity, TI was increased by SGID. In general, AH digests showed higher inhibitory activity compared to those of PH. The lower inhibitions of PH digests may be attributed to a different peptide profile and disappearance of bioactive peptides during proteolysis. Likewise, Donkor et al. (2007) reported the appearance and disappearance of some potent peptides during the storage of probiotic yoghurt. Gobet et al. (2000) stated similar findings. However, the current study indicated that there was no significant (P<0.05) difference between the TI activities of the RP of AH and PH (47.11% and 44.33%), respectively.

It has previously been hypothesised that high protein food sources influence the secretion of CCK at a higher rate compared to low protein sources (Blom et al., 2006). The current study showed an inverse correlation between the protein content and trypsin inhibition, which was observed in the case of PH (Table 3). Medeniers and Vaisjes (2008) investigated the TI activity of peptides released by SGID of myofibrillar proteins extracted from fresh and frozen fish. Their study demonstrated that TI activity was greatly affected by state of fish (fresh, frozen) and season of catch (winter, summer). Their findings indicated that environmental factors and processing conditions may impact on peptides release as well as TI activity, which may have also played a major role in the observed differences in our study.

DPPH radical scavenging activity (DPPH RSA)

Our results reported low free DPPH RSA for PH and AH (data not shown). Enzymatic hydrolysates of collagen from fish scales were found to be weak antioxidants in previous studies (Morimura et al., 2002), in line with our observations. Several factors such as protein sources, enzyme specificity, proteolytic activity, structure and molecular weight as well as amino acid composition are known to affect antioxidative activities of protein hydrolysates (Nagai et al., 2014). Peptides containing cysteine amino acids were reported to be responsible for high antioxidant activity. Thus, the low antioxidant activity...
in our study might be due to poor sources of cysteine amino acid. Conversely, tryptic-derived peptides of Hoki (Hokius belangeri) skin hydrolysates exhibited high DPPH RSA [Mendis et al. 2005]. The tryptic gelatin hydrolysates were found to be rich sources of amino acids constituents Gly (37.5%), Pro (9.3%), Glu (8.8%), Ala (8.6%), Arg (7.1%) and Hyp (5.98%). Similarly, croaker (Ochilinus ruber) muscle protein hydrolysate scavenged 59.7% DPPH; and the peptide sequence of the purified fraction responsible for the antioxidant activity contained cysteine. The presence of cysteine could be an associated link to the antioxidant capacity of the peptide [Naezer et al., 2012]. Thus, it appears that antioxidant peptides could be released from fish collagen but additional research should be conducted to explore potential mechanisms for their release.

**Conclusion**

Our study demonstrated that these two fish protein powders were likely prepared by two different processing methods resulting in variations of their protein content and morphology, which may have impacted on their solubility and digestibility. Furthermore, SCASQG has improved the stability of the fish meat and tryptic inhibitory activities of the released peptides of the products, but not the antioxidant capacity. Moreover, the physicochemical properties such as the high protein content, the fine particle sizes as well as the corresponding high solubility and digestibility of the PH could have contributed to the significantly high ACE and trypsin inhibitory activities of its released peptides. However, further purification and sequencing of the released peptides of the PH are required to confirm if the latter could possibly be utilised as a potential ACE inhibitor. As a whole, the production of hydrolysates derived from marine wastes such as scales may deliver health benefits to consumers, whilst minimising aquatic wastes and maintaining an ecological oceanic environment. At the same time the processing techniques should be linked to the intended use since the assessed samples showed quite different physiological functionalities.

**Acknowledgements**

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**References**


Recent developments of marine ingredients for food and nutraceutical applications: a review. Journal of Fisheries and Aquatic Sciences 2: 21-27.


Muzaffar, M., Saffari, N. and Zakaria, F. 2012. Production of protein hydrolysates from fish by-product prepared by enzymatic hydrolysis. Aquaculture, Aquaculture,
Justification:

In the study presented in Chapter 3, captopril was selected as the positive reference as compared to other ACE inhibitors (e.g. perindopril, enalapril and zofenopril) for the following reasons:

i) Captopril was the first ACE inhibitor discovered with clinically proven effects on reducing high blood pressure.

ii) For better data comparison with other scientific studies, as most of them used captopril as a positive control; and for reproducibility.

iii) Most peptides and food derived protein hydrolysates exhibit carboxyl groups that are bonded in a similar as captopril compound. Therefore, captopril acts an appropriate positive control.

iv) From a commercial point of view, captopril is readily available on the market and cost-effective (Sigma Aldrich) in comparison to perindopril, for example.
CHAPTER 4

Sustainable use of Silver Warehou (Seriollela punctata): Effects of storage, processing conditions and simulated gastrointestinal digestion on selected in-vitro bioactivities
Chapter 4 investigates the effects of storage conditions (chilling and freezing temperatures) and simulated gastrointestinal digestion on the release of bioactive peptides in the presence of both digestive (pepsin and pancreatin) and endogenous enzymes from Australian silver warehou fillets.

The paper entitled “Sustainable use of silver warehou (Seriollela punctata): effects of storage, processing conditions and simulated gastrointestinal digestion on selected in-vitro bioactivities” by V. Manikkam, M.L. Mathai, W.A, Street, O.N. Donkor, and T. Vasiljevic has been published in the peer-reviewed journal “Journal of Food Science and Technology” (2016), 53(9): 3574-3582.
### GRADUATE RESEARCH CENTRE

#### DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each jointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. **PUBLICATION DETAILS** (to be completed by the candidate)

<table>
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<th>Sustainable use of silver grapefruit (Spondias dulcis): Effects of storage, processing conditions and simulated gastrointestinal digestion on selected in-vitro bioactivities</th>
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<td>Journal of Food Science and Technology</td>
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<td>Date:</td>
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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](http://policy.vu.edu.au).

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Date: 2016 08 05 14:05:45 +1000
Signature

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3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
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the following location(s):

College of Health and Biomedicine, Victoria University, Werribee Campus, Melbourne, VIC, Australia

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From: Narinder Singh <em@editorialmanager.com>
Date: 30 August 2016 at 3:51:16 PM AEST
To: Todor Vasiljevic <todor.vasiljevic@yu.edu.au>
Subject: JFST: Your manuscript entitled Sustainable use of silver warehou (Seriollela punctata) - effects of storage, processing conditions and simulated gastrointestinal digestion on selected in-vitro bioactivities
Reply-To: Narinder Singh <narinders@yahoo.com>

Ref.: Ms. No. JFST-D-15-02026R3 
Sustainable use of silver warehou (Seriollela punctata) - effects of storage, processing conditions and simulated gastrointestinal digestion on selected in-vitro bioactivities
Journal of Food Science and Technology
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With kind regards
Narinder Singh, Dr.
Editor-in-Chief
Journal of Food Science and Technology
Sustainable use of silver warehou (*Seriollela punctata*) - effects of storage, processing conditions and simulated gastrointestinal digestion on selected *in-vitro* bioactivities

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Highlights

- Australian underutilised silver warehou fillets were assessed for their potential bioactivities.

- Fish endogenous enzymes released bioactive peptides with limited breakdown from the proteins.

- Simulated gastrointestinal digestion improved degree of hydrolysis and *in-vitro* bioactivities.

- Storage temperature and times had a significant effect on bioactivities of processed fillets.

- Stored fillets demonstrated high DPPH radical scavenging and metal chelating activities.
Abstract

Australian underutilised fish species may serve as a potential source of valuable proteins and potent bioactive peptides. This novel research is the first to investigate the effects of storage-processing conditions and an *in-vitro* simulated gastrointestinal digestion (pepsin-pancreatin) on bioactive peptides’ release during storage of fish fillet, derived from Australian silver warehou (*Seriolella punctata*). *In-vitro* bioactivities including angiotensin-converting enzyme and trypsin inhibitory and antioxidant activities were analysed. The antioxidant power was evaluated by DPPH free radical scavenging activity, Cu$^{2+}$ chelating and Fe$^{3+}$ reducing abilities. Fillets were stored at chilled (4 and 6°C) and freezing (-18°C) temperatures for 7 and 28 days, respectively. Results indicated that during postmortem storage, endogenous enzymes from fillets released an array of polypeptides over storage time. The demonstrated physiological activities were further increased (p<0.05) during simulated digestion. Bioactivities were greater at 4°C, increasing over 7 days (p<0.05) as compared to 6 and -18°C. An increase by 2°C for chilled temperature was enough to cause significant changes (p<0.05) in activities. The crude extracts obtained by pancreatin treatment demonstrated highest metal chelating activities at 4°C (86.3± 0.1% on day 7). Physiological potency, especially metal chelating activity, of fillets obtained from silver warehou may be manipulated by storage conditions that would consequently be further enhanced during simulated digestion.

**Keywords:** ACE inhibition, by-catch, copper ions chelating activity, DPPH, pancreatin, underutilised fish species
Chapter 4

Introduction

Silver warehou (*Seriolella punctata*) constitutes an important commercial species for the Australian fisheries. It can be captured either intentionally by bottom trawling and retained, or unintentionally and rejected due to lack of profitable interest (Australian Fisheries Management Authority 2015). A large proportion of silver warehou catch is landed during the spawning months, deflating prices, causing financial losses to the seafood industry. Its constant name changing also resulted in poor identification and a lack of acceptance by local and international markets (McLaughlin et al. 2009). Moreover, the poor sensory qualities, principally, its off-white colour after filleting have reduced its consumer demand. Thus, these consequences do not make silver warehou a prime catch, but it is instead considered as by-catch. It is categorized as under-valued fish species (McLaughlin et al. 2009). Considered as wastes, by-catch species are disposed of into the ocean, posing substantial threat to the species and jeopardizing the marine ecosystem (Blanco et al. 2007). Alternatively, they are converted into commercial and low-value products such as fertilizers, fish silage, meals, and baits, amongst others (Blanco et al. 2007).

Interestingly, fish waste, an excellent source of proteins, can be converted into more marketable, functional and health value-added products by novel enzymatic hydrolytic technology (Nurdiani et al. 2015). Enzymatic hydrolysis has been widely used to improve the functional properties (water-holding, emulsification, gelling and solubility) of mainly myofibrillar proteins (Kristinsson and Rasco 2000). Moreover, utilizing intact marine-derived proteins to produce bioactive peptides by enzymatic hydrolysis is increasingly becoming the focus of today’s scientific world. Bioactive peptides, derived from various food sources, display an array of physiological functions,
including anti-hypertensive, anti-oxidative, opioid activities, just to name a few (Kim and Wijesekara 2010).

Since Australian consumers i) have specific fish requirements for consumption, including inexpensive, boneless and skinless white-flesh fillets (Department of Agriculture 2015), ii) prefer domestic seafood and iii) are prepared to pay more for home-branded products (Calogeras et al. 2011), the seafood industry should meet the above requirements to promote sale of silver warehou. Therefore, enzymatic proteolysis could be an effective approach to improve utilization of silver warehou fillets by making it a potential medium for production of bioactive peptides, with prospective physiological properties, which have not been previously documented.

Generating bioactive peptides by silver warehou endogenous and/or external enzymes whilst maintaining freshness and balance of amino acids may be challenging, but of great importance. Chilling and freezing are two major ways to preserve freshness of fish, due to their perishable nature (Garthwaite 1997). However, these preservation techniques may lead to myofibrillar proteins denaturation and aggregation (Tejada 2001). These biochemical changes may essentially alter biofunctional parameters of fish peptides. No study has hitherto investigated the effects of chilled and frozen storage on in-vitro bioactivities of underutilised fish species.

Subsequently, evaluating bioactive peptides release during simulating gastrointestinal digestion (SGID) is vital because it demonstrates the types of peptides produced during digestion, and thus most likely to survive the GI tract (Korhonen and Pihlanto 2006). Because of the importance of maximizing freshness and high quality of silver warehou fillet while simultaneously benefiting from the bioactive peptides liberated upon SGID, the main emphasis of our study was to establish combined effects of storage conditions (temperature, storage time) on the quality-related changes as well as in vitro
biofunctionalities of undigested and digested fillets during storage. Our exploration could provide the Australian seafood industry with a better understanding on the plausible means of developing silver warehou fillets to maximize their use, increase commercial value and enhance consumers’ demand. The study would also provide a foundation for future research in regards to identification of muscular proteins that contain potential bioactive peptides as well as structural characterization of these peptides.

Materials and methods

Samples collection

Silver Warehou was kindly supplied by Barwon Foods (Seafood & Food Service Specialists; North Geelong, Australia). After having caught off the south-eastern coast including Tasmania and Bass Strain on the continental slope, fresh whole silver warehou was transported on ice in a polystyrene foam box to Werribee campus, Victoria University, within 12-24 hours. Upon arrival to our laboratory, fish was immediately stored in the cold room (4°C) and on ice, prior to handling, within 2 hours.

Experimental design

The experimental design, depicted by Figure 1, was set up to investigate the effects of storage and processing conditions on the quality and *in vitro* bioactivities of the commercially important silver warehou fillets. The weight and length of each whole fish were approximately 900 g and 35 cm, respectively. From each fish, two fillets were obtained, weighing almost 150-200 g. On day 0 (the day of samples’ arrival), each fillet portion was individually glad-wrapped, further sealed with aluminium foil, randomly placed into labelled locked containers, and stored at the selected temperatures in temperature-controlled fridges and freezer.
Chilled temperatures of 4 and 6°C were selected for the following reasons: i) home fridges are usually stabilized between 4 and 6°C, depending on the setting. Therefore, it was important to investigate the effects of these 2 primary cold temperatures on the release of bioactive peptides and their \textit{in vitro} bioactivities; ii) fish major endogenous enzymes, such as calpain and cathepsins, fundamentally involved in the hydrolysis of myofibrillar proteins are active at cold temperatures (Ahmed et al. 2013a). Hence, a difference by 2°C would potentially impact on their hydrolytic abilities, thus, deserving attention. The 7 days storage period for chilled samples were appropriate since in real life, fish are not stored in the refrigerator for more than a couple of days, due to rapid microbial growth. In addition, the frozen storage studies for 28 days were appropriate since a decrease in bioactivities were observed over time, and hence the storage period was discontinued.

Two portions of fillets, representing the duplicates, were removed daily for analyses for refrigerated samples and for first week of frozen fillet, with a further weekly analysis, up until 28 days. On each day of analysis, frozen samples were defrosted in the cold room (4°C) for a couple of hours, prior to processing. Fish fillets were also subjected to an \textit{in vitro} simulated digestion, with gastrointestinal enzymes, \textit{viz}, pepsin and pancreatin to investigate the ability of releasing physiologically important peptides. The quality assessments involved macronutrient compositions, pH determination and microbiological analysis. All the 5 \textit{in vitro} bioactivities were monitored during undigested and digested states. The laboratory and all equipment used during the daily processing of fish were aseptically maintained at all times. The procedure was repeated on three different occasions reflecting seasonal differences.
Chemicals

Angiotensin-I-converting enzyme, $N_\alpha$-Benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPNA), copper sulphate, 1,1-Diphenyl-2-picrylhydrazyl, Hippuryl-Histidyl-Leucine, iron (III) chloride, pancreatin (P7545; porcine pancreas), pepsin (P7000; porcine stomach mucosa), potassium ferricyanide, pyridine, pyrocatechol violet, sodium phosphate, trichloroacetic acid and trypsin (Type II-S from Porcine pancreas) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Dimethyl sulfoxide (DMS), ethyl acetate and glacial acetic acid were from Merck Pty Ltd (Darmstadt, Germany). All other chemicals used for chemical compositional analyses and preparation of buffering solutions were of analytical laboratory grade.

Quality assessment

Macronutritional compositional analysis

The protein and fat contents were evaluated by Kjeldahl procedure (AOAC 955.04) and Soxhlet extraction method (AOAC 963.15), respectively. The oven dry method (105°C) was used until a constant weight and the moisture content (AOAC 990.19) estimated. The incineration technique using muffle furnace (550°C) was applied to assess the ash content (AOAC 938.08).

pH determination

Ten grams of fish fillet was homogenised with MilliQ water in the ratio of 1:10 (w/v), and pH of the homogenate measured using a calibrated pH meter (Merck Pty Limited, Germany) at room temperature.
Microbiological analysis

The number of viable cells from the total plate count was determined as colony forming units/g (Log CFU/g). Plate containing 25-250 colonies was considered (AOAC 2000).

Processing conditions

Undigested condition

On each sampling day, 25 g of fish fillets from each selected temperature was minced using a mortar and pestle, followed by addition of 250 ml MilliQ water (MilliQ plus, Millipore Australia) and homogenization. The homogenate was centrifuged at 11,000 x g (JA20 rotor, Beckman Instruments Inc., Palo Alto, CA, USA) for 20 min at 4°C. On each removal day, frozen fillets were defrosted in the cold room (4°C) for 2 h prior to homogenization. The supernatant obtained from each homogenate after centrifugation was filtered into clean tubes as crude protein/peptide extracts, and stored at -20°C until further assayed.

Digestion condition

Fresh (day 0), frozen and chilled samples were subjected to an in vitro pepsin-pancreatin artificial digestion, to investigate the effects of digestive enzymes on silver warehou fillets during storage, according to the method described by Medenieks and Vasiljevic (2008), with modifications. Fillets (25 g) were ground and mixed with 100 ml MilliQ water; this step presented time 0 of the digestion process. All samples were then acidified with 1 M HCL to pH 2 and 5 ml of pepsin (Porcine gastric mucosa) solution was added. The samples were then incubated for 2 h at 37°C at 100 rpm. After pepsin digestion, pH was adjusted to 6.3 with 1 M NaHCO₃ solution and further to 7.5 with 1 M
NaOH, prior to addition of pancreatin solution. The mixture was carefully mixed and incubated at the same above conditions. After 2 h of incubation, the tubes were immersed in boiling water bath for 15 min to halt the enzymatic reactions. They were then cooled on ice for 5-10 mins. The cooled samples were then centrifuged at 1500 x g (Sorvall® RT7 centrifuge, DuPont, Newtown, CT, USA) for 15 min at 4°C). To investigate the changes in bioactivities of digests during digestion, aliquots of digests were removed at 30 min intervals for 4 h. All GI digests were filtered using 0.45 µm membrane filter (Schleicher & Schuell GmbH, Germany) before storing at -20°C for additional analyses.

**In vitro bioactivities**

**ACE inhibitory (ACE-I) activity**

The ACE-I activity was established spectrophotometrically (NovaSpec® - II Spectrophotometer; Pharmacia, Cambridge, UK) assayed by measuring the absorbance at 228 nm. The extent of inhibition was calculated using Equation 1 (Donkor et al. 2007).

\[
\text{ACE inhibitory activity, } \% = \left[ 1 - \left( \frac{C-B}{A-B} \right) \right] \times 100
\]  

(1)

Where,

A = Absorbance in the presence of ACE and without the ACE-I component

B = Absorbance without the ACE-I component

C = Absorbance with ACE and the ACE-I component

D = Absorbance without ACE and with the ACE-I component
Trypsin inhibitory activity

The TIA was determined according to Medenieks and Vasiljevic (2008). A volume of 250 µL of crude extracts was pre-incubated at 37°C for 10 min with 625 µl of BAPNA-DMS-Tris buffer solution. This was followed by the addition of 250 µl trypsin enzyme solution (40 mg trypsin enzyme in 200 ml of 1 mM HCl) before incubating for 10 min at 37°C. The reaction was terminated by adding 250 µl of 30 % glacial acetic acid and vortex-mixed. The absorbance of each sample was read at 410 nm. The inhibitory activity was evaluated using Equation 1.

DPPH free radical scavenging activity (RSA)

The antioxidant capacity of samples was evaluated by measuring the free RSA following the method of Donkor et al. (2012), with slight modifications. Briefly, 4.0 ml DPPH solution (0.075 mM DPPH in methanol) was added to 0.1 ml diluted (in 1 ml methanol) sample followed by 30 min incubation in the dark, after which, the absorbance was read at 517 nm with a Pharmacia UV spectrophotometer (Cambridge, UK). The scavenging activity was calculated as percent inhibition, using Equation 2.

\[
\text{Inhibition, } \% = \left[ 1 - \left( \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}} \right) \right] \times 100
\]  

Metal chelating activity

The chelating activity of crude in vitro fish peptide extracts on pro-oxidative copper ions (Cu^{2+}) was investigated according to (Zhu et al. 2008), with modifications. Briefly, 500 µl of 2 mM CuSO_4 was mixed with 500 µl of pyridine (pH 7.0) and 10 µl of 0.1 % pyrocatechol violet. An aliquot of 500 µl peptide extract was then added and allowed to quiescently stand for 5 min. The disappearance of the blue colour was then recorded by the measuring the absorbance at 632 nm. An equivalent volume of MilliQ
water instead of sample was used as the blank. The Cu$^{2+}$ chelating activity of the crude protein/peptide extracts was calculated as shown below.

$$\text{Cu}^{2+} \text{ chelating activity} = \left(\frac{A_o - A_s}{A_o}\right) \times 100$$ (3)

Where,

$$A_s = \text{absorbance of the sample}$$

$$A_o = \text{absorbance of the blank solution using distilled water instead of sample}$$

**Reducing power assay**

The potential to act as a reducing agent by the ability of donating an electron to Fe$^{3+}$ ions reducing it to Fe$^{2+}$ was determined by investigating the reducing power of fish crude protein/peptide extracts according to (Zhu et al. 2008), with adjustments. Briefly, 1.0 ml of samples was mixed with 1.0 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 1.0 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 30 min. Trichloroacetic acid (10%) was added to the mixture, and centrifuged at 1500 x g (Sorvall® RT7) for 10 min. Finally, the supernatant was mixed with MilliQ water and ferric chloride solution (0.1%). After quiescent standing at room temperature for 10 min, the absorbance was measured at 700 nm. An equivalent volume of MilliQ water instead of the sample was used as the blank. Increase absorbance represented increased reducing power of samples.

**Statistical analysis**

All experimental analyses were conducted using a randomized, split plot in time blocked design. The digestion time was included as an additional factor in a subplot when required. The replications served as the block. The experimental design (Figure 1) was triplicated and subsampled twice resulting in at least 6 independent observations ($n \geq 6$).
Results were analyzed using a General Linear Model (GLM) procedure of the Statistical Analysis System (SAS). The level of significance was preset at $p<0.05$.

**Results and discussion**

**Quality assessment**

The proximate composition of fresh fillets (day 0) was 18.9% protein, 1.8% fat, 77.5% moisture and 1.5% ash. Proximate analyses of fillets were also conducted on respective sampling days to identify changes during storage (data not shown). There were slight but not significant changes in protein and moisture content, but no substantial change in fat and ash content of fish fillets ($p>0.05$); with similar observation made (Gandotra et al. 2012). Similarly, pH of fillet on day 0 was 6.8 and did not change much ($p>0.05$) irrespective of storage temperatures and time (data not shown). On the other hand, an increase by 4 and 5 log cycles in bacterial load in raw fish muscle was observed (Table 1). A total plate count value surpassing 6 logs CFU/g is considered as bacteriological spoilage of fish muscle and inedible for human consumption (International Commission on Microbiological Specifications for Foods 1986). Accordingly, our fillets were still consumable until day 3 when refrigerated at 4°C and day 14 when frozen. After day 3 at 4°C, fish fillets quickly deteriorated, with increased bacterial load, noticeable pungent smell and colour change. From our findings, it is however, not advisable to stow fish muscle at 6°C due to rapid microbial spoilage. Psychro-tolerant Gram-negative bacteria (*Pseudomonas* spp. and *Shewanella* spp.) could grow on chilled fish, above 4°C (Gram and Huss 2000).
**In vitro bioactivities during storage (undigested condition)**

The impact of storage conditions in the presence of endogenous enzymes only, on the biochemical functionalities of crude extracts from fillets were investigated. Results shown in Table 1 indicated that endogenous enzymes from silver warehou fillets liberated polypeptides during *postmortem* storage due to limited breakdown of the protein matrix. As a result, all samples contained peptides that exerted assessed bioactivities to a varying degree (p<0.05). Interestingly, storage temperature exhibited a varying (p<0.05) effect on fillets stored at 4°C, showing increasing bioactivities over a period of 7 days, likely due to relative stability of cathepsins L (Yang et al. 2015). Furthermore, the high levels of cathepsins B+L activities in silver warehou (Ahmed et al. 2013b) likely enhanced the hydrolysis of myofibrillar proteins during storage and consequently improved the physiological properties in *vitro* at 4°C.

Over time, a slight change in reducing power (data not shown) of crude extracts of undigested fillets was observed at 4°C. Specific amino acids Trp, Met, Cys and Lys constituted an important asset for a peptide or functional component to act as a reducing agent, especially in reducing iron (III) to iron (II) (Carrasco-Castilla et al. 2012). Our fillets could be lacking these specific amino acids. However, an amino-acid profile analysis of the crude-extracts would have enabled identification of the types of amino acids present in our samples. Moreover, endogenous cathepsins L is often implicated in the release of peptides during storage at 4°C, as demonstrated in pacific hake muscle (Samaranayaka and Li-Chan 2008), explaining the high DPPH radical scavenging and metal chelating activities of our undigested samples at 4°C.

On the other hand, chilled samples at 6°C showed a decreasing trend in biofunctional activities (Table 1). There is a distinct difference in all 4 assessed bioactivities between storage at 4 and 6°C. The values increased during the 7 days of
storage at 4°C, but decreased significantly (p<0.05) at 6°C. The difference in temperature by only 2°C would have been enough to create favourable conditions within the fillets matrix, increasing microbiological activity. More specifically, psychrotrophic bacteria are able to grow at temperature below 7°C, correlating with our bacterial count (Table 1). Generally, high activity of endogenous muscle proteases during initial days of refrigerated storage may be an indicator of texture-associated degradation (Delbarre-Ladrat et al. 2004). Postmortem conditions such as pH and temperature may influence the level of activity of muscle proteases and consequently impacting on the properties of resulting properties (Ahmed et al. 2013a). Silver warehou exhibited lowest endogenous calpain-like enzymatic activity (Ahmed et al. 2013a), supporting our observed decline in in vitro bioactivities at 6°C during storage.

Similar to 6°C samples, frozen samples (-18°C) showed a decreasing trend in selected in vitro bioactivities (Table 1). Freezing and thawing may result in i) fragmentation of cell membranes and lysis of intra-cellular organelles, ii) decreased water-holding capacity of fish muscle and iii) drop in myosin and actomyosin Ca^{2+}-ATPase activities, resulting in a change in the myosin head (Makri 2010). These changes could result in unfolding tertiary conformation of myosin owing to weakening in intra-molecular bonds (Makri 2010). Reduction in bioactivities during frozen storage could also be explained by instability and sensitivity of fish myosin to denaturation and degradation as well as the cross-linking and aggregation of myofibrillar proteins (Tejada 2001). When water is separated as ice, proteins, mainly myofibrillar proteins, became unstable and protein denaturation began. Therefore, the hydrophobic and hydrophilic amino acid groups normally associated with the interior of a protein molecule became more exposed (Tejada 2001), supporting our low biochemical activities of frozen samples.
In-vitro bioactivities during storage and digestion

Human digestive enzymes, such as pepsin, chymotrypsin and pancreatin, may further hydrolyse polypeptides fragments producing a range of smaller peptides exhibiting important physiological properties (Manikkam et al. 2015). After oral administration, gastrointestinal enzymes may break down the active peptides, thereby increasing or decreasing their functional activities (Vermeirssen et al. 2004). The following section will thus discuss the impact of gut enzymes on the release of bioactive peptides and their bioactivities.

ACE-inhibition of crude extracts

On day 0, fresh fillets exhibited an ACE-inhibition of 70.2% at the end of pancreatin digestion (Figure 2). This effectiveness of pancreatin at increasing ACE-I activity was observed throughout the storage stability experimentation, irrespective of storage temperature and time. Moreover, an overall increase in ACE-I activity of 4°C samples was observed throughout the 1 week (Figure 3), whereas a significant decline (p<0.05) in activity was observed at 6 and -18°C. Di- or tri-peptides, especially proline amino acid residue at the C-terminus are generally resistant to degradation by digestive enzymes, and the most favoured for ACE-I peptides (Vermeirssen et al. 2004). It was also predicted that a C-terminal Gly is not very favourable residue for exhibiting ACE inhibition (Wu et al. 2006), which could possibly have been the case for our 6°C and frozen samples. However, further investigation of the amino acid profiles of the crude extracts is required to fully understand the discrepancy in ACE-I activity of the various samples.
Trypsin inhibition of crude extracts

Digestion also improved the trypsin inhibition by the fish digests. Unlike ACE-I activity, cold storage at 4°C and time did not significantly (p>0.05) impact on trypsin inhibition (data not shown). In general, fresh samples appeared to be a source of more potent peptides with greater trypsin inhibitory activity than the frozen fish, in line with the study of Medenieks and Vasiljevic (2008). During freezing, the formation of ice crystals caused cryo-concentration of solutes, partial dehydration and dislocation of water molecules in the muscle, accelerating protein denaturation and aggregation (Makri 2010), which could have impacted on the release of bioactive peptides and consequently on the trypsin inhibition ability. At the beginning of chilled storage (4°C), trypsin inhibition was not high (p>0.05), reaching to a nearly constant activity at the end of 7 days. It was likely that peptides of interest were not released, in line with Donkor et al. (2007). Similar to ACE-I activity, trypsin inhibition declined during storage at 6 and -18°C (data not shown). It has been very difficult to compare our results with previous studies since no research has been done in this particular area, requiring further investigations.

DPPH free radical scavenging activity

The relatively stable organic radical, DPPH, has been extensively utilized as a free radical to evaluate reducing substances. In regards to 4°C samples, pepsin digestion for 30 min appeared to released peptides with significant (p<0.05) scavenging activity followed by minimal increase thereafter. During pepsin digestion, more hydrophobic amino residue side chain groups were expected to be exposed, which would make the peptides more accessible to the DPPH radicals, allowing them to trap the radical more easily (You et al. 2010). However, an increasing trend in activity during pancreatin digestion was observed, not in line with previous studies (You et al. 2010), whereby a
sharp decrease in activity was observed with pancreatin digestion. Antioxidative properties of peptides are more related to their amino acids composition, structure and hydrophobicity. Small neutral amino acids include alanine; serine and cysteine (Daniel 2004) often play an important role in antioxidant activities. With respect to 6°C and frozen samples, a sharp decrease was again observed (data not shown). This might potentially be due to the initial hydrolysis resulting in the release of oligo-or poly-peptides with lower antioxidant activities. Upon further treatment, either proteolytic activity of the antioxidant peptides or their physical aggregation had weakened the scavenging ability of the DPPH free radical. The obtained results suggest that crude peptide extract at the end of digestion of 4°C samples probably contained peptides, which are electron donors; and could possible react with free radicals, converting into more stable products and terminate the chain reaction (Bougatet et al. 2009).

Cu²⁺ ions chelating activity

The fish digests during in vitro digestion were also evaluated for copper ions chelation, as a measure of antioxidant activity (Figure 4).Irrespective of storage time and temperatures, chelating activity decreased with pepsin treatment, but significantly increased (p<0.05) with pancreatin, in line with You et al. (2010). This may suggest that fewer peptide bonds are broken down with pepsin than with pancreatin. Pancreatin contain many enzymes, including trypsin and additional protease, which could aid in hydrolysing the fish peptides into even more smaller peptides. The decrease in activity during pepsin digestion could be due to specific peptide structure and amino acid side chain groups (Decker et al. 1992). Pepsin treatment might have disrupted the structure of silver warehou peptides and reduced their ability to bind and trap copper ions (Zhu et al.
2008). Among all the bioactivities assessed, metal chelating activity was the highest, irrespective of storage conditions.

*Ferric ions reducing power of crude extracts*

The reducing power, another indicator of antioxidant activity of bioactive compounds, including peptides, is used to evaluate the ability of an antioxidant to donate an electron or hydrogen. The presence of antioxidants in the crude extracts or hydrolysates causes the reduction of the Fe\(^{3+}\)/ferric cyanide (FeSCN) complex to ferrous ion (Fe\(^{2+}\)) and the colour of the solution changes from yellow to green or blue shade, depending on the reducing potential of the compound (Bougatef et al. 2009). No obvious colour change was observed in our samples, corresponding to the low activity. Our fish crude extracts were possibly not effective in producing amino acids that are associated with reducing power, in contrast with previous studies (Bougatef et al. 2009; You et al. 2010).

**Conclusion**

Endogenous enzymes in silver warehou fillets released an array of polypeptides during postmortem storage due to apparent limited breakdown of the protein matrix. The *in vitro* biofunctionalities of native proteins were definitely enhanced when fillets were subjected to simulated gastrointestinal digestion. Samples at 4\(^\circ\)C and pancreatin digests were more effective in producing high bioactivities. The analysed crude extracts demonstrated higher antioxidant activities with respect to DPPH radical scavenging activity and chelating ability of copper ions. However, lower ACE- and trypsin-inhibitory activities were observed. The comparison of our data with other studies was difficult since no investigations had been previously conducted on the effects of storage temperatures
and times on liberated peptides from fish. However, our findings indicated that the underutilised fish analyzed could be valuable food items, containing bioactive peptides. Further studies are needed to identify muscular proteins as the origin of these bioactive peptides as well as primary sequences of these peptides.

Acknowledgement

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References


Figure captions

**Figure 1.** Flowchart depicting the experimental design applied in the current study.

**Figure 2.** Physiological activities of fresh fillets at day 0. Legend: - angiotensin converting enzyme inhibition (ACE-I); - trypsin inhibition (TI); - antioxidative (DPPH) activity; - metal chelating (MC) activity; - reducing power assay (RPA)

**Figure 3** ACE-inhibitory activities of the digests during various stages of the simulated digestion of fillets at 4°C on day 1, day 2, day 3, day 4, day 5, day 6, and day 7.

**Figure 4** Effects of storage temperatures and times on the metal (Cu^{2+}) chelating activity of the digests during various stages of the simulated digestion of silver warehou fillets at chilled temperatures – 4°C (A) and 6°C (B) and during frozen storage at -18°C (C) on day 1, day 2, day 3, day 4, day 5, day 6, day 7, day 14, day 21, and day 28.
Table 1: Microbial count and *in vitro* bioactivities of undigested fillets as influenced by storage conditions

<table>
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<th>Storage temperatures</th>
<th>Microbial count (Log CFU/g)</th>
<th>ACE inhibition (%)</th>
<th>Trypsin inhibition (%)</th>
<th>DPPH RSA (%)</th>
<th>MCA (%)</th>
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<tr>
<td></td>
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<td>6°C</td>
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<td>4.2</td>
<td>3.4</td>
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<td>38.6</td>
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<td>7.2</td>
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The means present the average of 6 independent observations (n ≥ 6); SEM = pooled error of the mean

nd: not determined – microbial count and *in vitro* activities were not determined on these specific days due to practicality reasons

On day 0, the total plate count of fresh fillet was 3.3 Log CFU/g

On day 0, the *in vitro* bioactivities of fresh fillet were as follows: ACE inhibition: 42.1%; Trypsin inhibition: 42.3%; DPPH RSA: 37.2%; MCA: 62.1% and RPA: 18.6%. 

Chapter 4
Figure 1
Figure 2
Figure 3
CHAPTER 5

Australian Eastern School Whiting
(*Sillago flindersi*) – a potential source of metal reducing agents and free radical scavengers
Chapter 5 identifies the potential of Australian underutilised fish species, ESW to release antioxidant peptides in the presence of endogenous and digestive enzymes during chilled and frozen storage.

The paper entitled “Australian Eastern School Whiting (Sillago flindersi) – a potential source of metal reducing agents and free radical scavengers” by V. Manikkam, M.L. Mathai, W.A. Street, O.N. Donkor, and T. Vasiljevic had been peer-reviewed and accepted for publication in the peer-reviewed International Food Research Journal.
GRADUATE RESEARCH CENTRE

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION:
PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each jointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

<table>
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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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In the case of the above publication, the following authors contributed to the work as follows:

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Australian Eastern School Whiting (*Sillago flindersi*) – a potential source of metal reducing agents and free radical scavengers

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Abstract

Underutilised Australian Eastern School Whiting (*Sillago flindersi*) fish was investigated for *in-vitro* bioactivities, after exposure to fish endogenous and gastrointestinal (pepsin and pancreatin) enzymes. The study comprised of storing fish at chilled (4 and 6°C) and freezing (-18°C) temperatures for 7 and 28 days, respectively. Hydrolysis by endogenous enzymes only, resulted in increased bioactivities for the 4°C samples, whereas significant decreases (p < 0.05) were observed for the 6 and -18°C samples. However, bioactivities of these samples increased significantly (p < 0.05) after further hydrolysis under simulated digestion conditions. Proteolysis by digestive enzymes, mainly pancreatin considerably enhanced the antioxidant activities. To benefit from the health properties of eastern school whiting fish, it is suggested to consume the fish fresh. The intent is to enhance full use of fish and not certain parts such as fish oil. For proper utilization and sustainability, whole fish must be used.

Keywords

Antioxidant, free radicals, metal chelating activity, myofibrillar proteins, simulated digestion, reducing power
Introduction

Endemic to South-Eastern Australia, eastern school whiting (*Sillago flindersi*) is one of the most important fish species in Australian fishery, considering the annual landings by the New South Wales Ocean Trawl Fishery exceeding 800 t annually (Wild fisheries Research Program, 2010). However, owing to their small shaped size, delicacy, slight sweetness, bony and fine texture, such species are often discarded, posing a significant sustainability issue and severe economic loss to the seafood industry (Wild fisheries Research Program, 2010).

Fish, a poikilothermic, aquatic chordate, is nutritionally and economically important; and must be utilized efficiently. From a nutritional perspective, fish constitute a rich source of essential fatty acids, vitamin and minerals and majorly, essential amino acids (high protein quality) (Khora, 2013). Besides the recovery of fish oil, with potential therapeutical benefits and to prevent fish wastage, a novel technique; enzymatic hydrolysis, could be developed to also recover proteins or their derivatives peptides (Manikkam *et al.*, 2016a; Nurdiani *et al.*, 2015). Fish is a rich source of easily digestible proteins, which are highly sensitive to proteolytic digestion, with a digestibility of more than 90% (Khora, 2013). This high digestibility could correspond to the high proportion of myofibrillar proteins (60-80%) as compared to collagenous fibres (3-10%) in fish muscle (Delbarre-Ladrat *et al.*, 2006).

Myofibrillar proteins from fish muscle undergo hydrolysis or proteolysis during storage at refrigerated or freezing temperatures by the endogenous enzymes present in the fish muscle. These enzymes are of mainly the calpain and/or the cathepsins family. Their enzymatic activity impacts on the breakdown of the proteins (Delbarre-Ladrat *et al.*, 2004). For instance, eastern school whiting exhibited high endogenous activity of calpain-like enzyme in comparison to silver warehou and other fish species (Ahmed *et al.*, 2013).
This high enzymatic activity may cause rapid softening during chilled storage of fish fillets, affecting textural quality, constituting further reasoning for its low commercial value. Interestingly though, autogenous muscle enzymes, active at post mortem storage conditions, often lead to proteolysis of fish muscle, which may either cause quality losses during cold storage (Ahmed et al., 2013) or release of peptides, with beneficial in vitro bioactivities, such as ACE-inhibitory activity and potential anti-oxidant capacities (Manikkam et al., 2016a). It is therefore essential to identify if eastern school whiting fillets could similarly exert significant in vitro bioactivities, which could be an asset in improving its commercial value.

Moreover, it is equally crucial to explore the stability of these proteins/peptides in the gastrointestinal tract (GIT) upon digestion. In vitro methods, known as the simulated gastrointestinal digestion (SGID) simulate the human GIT and are extensively used, since they are rapid and safe (Medenieks and Vasiljevic, 2008; Samaranayaka and Li-Chan, 2008; You et al., 2010). Therefore, we hypothesized that manipulating the storage conditions of fish fillets could release an array of polypeptides with potential in vitro bioactivities. Hence, the aim of our study was to explore the combined effects of refrigerated and freezing storage on the quality-related changes as well as bioactivities of eastern whiting during human digestion. It is important to maximize the utilization of this species to boost up the seafood industry sustainability.

**Materials and Methods**

*Samples collection*

Barwon Foods (Seafood and Food Service Specialists; North Geelong, Australia) generously provided the fresh eastern school whiting (ESW), which were conveyed on ice in polystyrene box to our Food Science laboratory (Werribee campus, Victoria
University), within 24 h of catch. The fish were processed immediately once arrived to our laboratory.

**Chemicals**

Angiotensin-I-converting enzyme, \( N_\alpha \)-Benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPNA), copper sulphate, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Hippuryl-Histidyl-Leucine, iron (III) chloride, pancreatin (P7545; porcine pancreas), pepsin (P7000; from porcine stomach mucosa), potassium ferricyanide, pyridine, pyrocatechol violet, sodium phosphate, trichloroacetic acid (TCA), and trypsin (Type II-S from Porcine pancreas) were all purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Acetonitrile, dimethyl sulfoxide (DMS), ethyl acetate, glacial acetic acid and trifluoroacetic acid (TFA) were from Merck Pty Ltd (Darmstadt, Germany). All other chemicals used for preparation of buffering solutions were of analytical lab grade.

**Experimental design**

The experimental design (Figure 1) was established to investigate the impact of fish endogenous enzymes and in combination with digestive enzymes on the release of crude peptides from fish fillets during storage at chilled and frozen temperatures. Whole fish, with approximate length of 20-30 cm, were separately packaged, randomly divided, placed in labelled locked containers and stored at 4, 6 (chilled) and -18°C (frozen), for 7 and 28 days respectively. The reasoning for the selection of storage temperatures and times has already been underlined by Manikkam et al., (2016a), as a similar experimental design was developed for silver warehou fish. On day 0, the fish were stored whole at the selected conditions since ESW are small sized and delicate to handle. Sampling small number of fish each day minimized contamination and biased results. On each sampling
day, fish were removed from storage and fillets acquired aseptically to avoid unnecessary contamination. Same procedure was repeated on 3 different occasions, reflecting seasonal differences; and results were presented as means of three replicates and two subsampling.

**Processing of fillets (undigested and digested)**

Ten grams of fish fillets were minced using a mortar and pestle, followed by addition of 100 ml MilliQ water and homogenization. The homogenate was centrifuged at 5600 x g (JA20 rotor, Beckman Instruments Inc., Palo Alto, CA, USA) for 20 min at 4°C. Frozen fillets were defrosted in the cold room for 2-3 hours prior to filleting. The supernatant obtained from each crude homogenate after centrifugation was filtered into clean tubes, which were stored at -20°C until further assayed. Moreover, a simulated GI digestion, described by Manikkam *et al.*, (2016a) was carried out to study the combined effects of endogenous and GI enzymes on generation of bioactive peptides from ESW fillets.

**In vitro bioactivities of fish crude extracts**

In our study, five important *in vitro* bioactivities of the obtained fish crude extracts were analyzed according to Manikkam *et al.*, (2016a). The assayed bioactivities included ACE- and trypsin- inhibitory activities in addition to determination of antioxidant power by i) measuring the free radical DPPH scavenging activity, ii) metal (copper) chelating ability and iii) acting as a reducing agent by donating an electron to Fe (III) ions.

**Reverse-phase HPLC analysis of crude peptide extracts**

Crude peptide extracts before and after simulated digestion were profiled using a Varian HPLC (Varian Analytical Instruments, Walnut Creek CA, USA) equipped with a
reverse-phase C-18 monomeric column - 5µm, 300 Å, 250mm x 4.6mm and a guard column (Grace Vydac, Hesperia, CA, USA) (Donkor et al., 2007). Samples were applied using a 10 µl injection loop. The peptides were eluted by a linear gradient from 100% to 0% solvent (0.1% TFA in deionised water) in solvent B (0.1% TFA in 90% v/v, acetonitrile in deionised water) over 90 min. All samples and mobile phase solvents were filtered through a 0.45 µm membrane filter (Schleicher and Schuell GmbH, Germany). Peptides separation was conducted at room temperature with a flow rate of 0.75 ml/min. The eluted peptides were monitored at 214 nm using a Varian 9050 variable wavelength UV/Vis detector.

Statistical analysis

All experimental analyses were conducted using a randomized, split plot in time blocked design. The digestion time was included as an additional factor in a subplot when required. The replications served as the block. The experimental design was triplicated and subsampled twice resulting in at least 6 independent observations ($n \geq 6$). Results were analysed using a General Linear Model (GLM) procedure of the Statistical Analysis System (SAS). The level of significance was preset at $p < 0.05$.

Results and Discussion

Impact of fish endogenous enzymes and storage conditions on in vitro bioactivities

Table 1 indicates that hydrolysis of fish muscle protein in the presence of endogenous enzymes is possible. However, it is crucial to determine the effects of storage conditions (temperature and time) on the hydrolytic ability of these enzymes in releasing potential bioactive peptides. Storage temperature and time had significant impact ($p < 0.05$) on releasing peptides from fish samples, with varying degree of bioactivities (Table 1).
Chilled samples, at 4°C showed increasing trend of ACE inhibition, DPPH radical scavenging and metal chelating activities from 0-7 days. A similar pattern was observed with crude extracts from Australian silver warehou fish (Manikkm et al., 2016a). Endogenous enzymes may be more active at 4°C than at 6 or -18°C. Moreover, the stability and/or autolytic activity of fish enzymes post mortem are also fish species-specific (Delbarre-Ladrat et al., 2004; Ahmed et al., 2013).

However, trypsin inhibition of 4°C samples declined from 37.47% (day 0) to 36.87% (day 3) and increased slowly from 4-7 days during the cold storage. The rate of hydrolysis/degradation of bioactive peptides by fish endogenous enzymes during the one week of storage likely depended on the type of peptide (amino acid sequence) formed. The end results may indicate that some peptides of interest may disappear and new ones may appear during prolonged storage (Donkor et al., 2007). On the other hand, extended storage of fish fillets at 4°C could have potentially increased the trypsin inhibition of the crude extracts; nonetheless from a consumption viewpoint, fish stored at 4°C for more than 1 week would be drastically loaded with microbial organisms and could be detrimental to human health. But, should the peptides indicate high trypsin inhibition after 1 week of storage at 4°C, fish protein hydrolysates could potentially be developed (Kristinsson and Rasco, 2000; Cudennec et al., 2008).

In contrast, a reduction in bioactivities at 6°C was observed (Table 1). Fish flesh is subjected to spoilage during higher temperatures, with the growth of psychrophilic bacteria (Mol et al., 2007). These bacteria were possibly using fish proteins and peptides as energy source and released amino acids for growth (Kristinsson and Rasco, 2000) hence, reducing the release of potential bioactive peptides from ESW fillets at 6°C. Moreover, as explained by Manikkm et al., (2016a), the difference in chilled
temperatures by only 2°C would have been enough to create favourable conditions within the fillets matrix to promote microbial activity.

Freezing has long been recognised as an effective means of preservation to extend shelf life of fish. Nonetheless, freezing and frozen storage of fish muscle may however lead to negative effects, such as denaturation and aggregation of especially myofibrillar proteins. These conformational changes often result in altered functional properties (solubility, water holding capacity), changed textural attributes (hard, dry texture) and juiciness (Barros et al., 1998). Moreover, freezing-thawing cycle induces biochemical changes owing to mechanical damage, denaturation of muscle proteins and loss of water-holding capacity (Hallier et al., 2008). Furthermore, myosin, the major contractile proteins of skeletal muscle become unstable and easily denatured due to formation of disulphide bonds, as water is separated in the form of disruptive ice crystals (Ramírez et al., 2000). Therefore, hydrophobic amino acid groups normally associated with the interior of a protein molecule become exposed to biochemical damages.

In addition, freezing and thawing may result in i) fragmentation of cell membranes and lysis of intra-cellular organelles, and ii) decline in myosin and actomyosin Ca\(^{2+}\)-ATPase activities, resulting in a change of the myosin head. These changes could result in unfolding tertiary conformation of myosin owing to weakening in intra-molecular hydrophobic bonds (Tejada, 2001). The above explanation supports the significant decline in examined \textit{in vitro} bioactivities (p < 0.05) observed for the frozen samples, in accordance with Manikkam et al., (2016a), whereby a similar tendency was perceived with silver warehou fish.

During muscle storage, cathepsins B and D may be released from the lysosomal matrix into the cytoplasm and intracellular spaces as a consequence of lysosomes breakdown (Bechet et al., 2005). In this regards, ESW demonstrated lower cathepsin D
activity compared to silver warehou (Ahmed et al., 2013), which likely explained the lower bioactivities at 6°C and -18°C in the present study. Additionally, cathepsins B and L activities increased significantly during post mortem storage in both super-chilled and ice-stored Atlantic salmon muscle (Gaarder et al., 2012), suggesting that i) the principal cause of post mortem degradation of fish muscle may be attributed mainly to cathepsins B and L and ii) more proteolytic peptides may be released as a result of such hydrolysis. Consequently, the high antioxidant activities observed with ESW at 4°C, 6°C and -18°C in the current study.

Synergistic effects of endogenous enzymes, pepsin and pancreatin on the bio-functionalities of fillets

Hitherto, our study has elucidated that a mixture of crude polypeptides, oligopeptides and/or peptides can be generated during storage over a period of time, by the hydrolytic nature of endogenous enzymes on fish muscle proteins, correlated with our previous study on silver warehou (Manikkam et al., 2016a). However, released peptides exhibited lower bioactivities; raising concerns of whether further proteolytic processes may promote breaking down of these concentrated mixtures of proteins into simpler/shorter peptides with greater in vitro activities. In this regard, it is valuable to assess the effects of GI enzymes, physiologically active at certain pH, on the release of potent bioactive peptides as well as on their increasing or decreasing functional activities.

Endogenous calpains and cathepsins may act synergistically with post mortem physiochemical conditions including low temperature, reduced pH and relatively increased muscle ionic strength to modify the interaction and structural conformation of proteins. (Delbarre-Ladrat et al., 2006). Biochemically, proteins’ digestion begins in the stomach by action of pepsin at acidic pH 2. In the luminal phase of the small intestine,
the polypeptides are further cleaved by the pancreatin proteases, at more alkaline pH (6.3), resulting in a mixture of oligopeptides and free amino acids (Vermeirssen et al., 2004). In Figure 2, overall significant increase (p < 0.05) in bioactivities of fresh fillets (day 0) was observed at the end of digestion in comparison to undigested crude peptide mixtures. This indicated that SGID with pepsin and pancreatin had influentially impacted on the hydrolytic pathways of muscle proteins into peptides, exhibiting higher activities. The peptide profiling clearly indicated increased peptide content at the end of digestion (240 min) of fresh fillets on day 0 (Figure 4B).

**ACE-I activity of crude extracts**

An overall significant increase (p < 0.05) in ACE inhibition was observed at 4°C from day 0 to 7. No change in ACE-I activity for day 5 samples stored at 4°C, (210 and 240 min). A similar observation was made for day1 samples held at 4°C for 90 and 120 min with pepsin digestion, day7 (6°C) at 180, 210 and 240 min and day14 (-18°C) at 210 and 240 min of pancreatin digestion (data not shown). The unchanged activities observed with the above mentioned samples could be due to i) hydrolysis reaching its maximum and no further break down of peptides or ii) disappearance of peptides during hydrolysis, as observed by Donkor et al., (2007). Furthermore, at the end of frozen storage (day 28), a significant decrease (p>0.05) in ACE-I activity was observed (data not shown). This decline could be explained by the susceptibility of proline amino acids residue at the C-terminus, generally known as the most favoured amino acids for ACE-I peptides, to freezing damage (Vermeirssen et al., 2004, Manikkam et al., 2016b).

Another important observation was that irrespective of storage time and temperature, maximum ACE inhibition was noted with pancreatin digestion. Pepsin and/or pancreatin digestion has mainly been applied to release peptides from plant products, such as soy
protein (Lo and Li-Chan, 2005), peas (Vermeirssen et al., 2003), dairy proteins (Pihlanto-Leppala et al., 2000), and some marine sources (Hai-Lun et al., 2006, Samaranayaka and Li-Chan, 2008, Darewicz et al., 2014), with limited research findings. It is therefore difficult to draw a conclusion on how effective the pepsin-pancreatin digestion released peptides with ACE inhibition activity. However, Based on our findings, peptides with ACE inhibition potential can be derived from ESW fillets when stored at 4°C for a week.

**Trypsin inhibition of crude extracts**

Similar to ACE-I activity, storage conditions (time and temperature) had significant ($p < 0.05$) effect on trypsin inhibition (data not shown). The inhibitory effect of trypsin enzyme in stomach is important to boost CCK production in the presence of food. The satiety hormone CCK controls the amount of food one consumes. High protein content foods, such as soy (Nishi et al., 2003), whey (Zhou et al., 2011) and fish have gained considerable attention in ability to reduce hunger and maintain satiety (Cudennec et al., 2008, Cudennec et al., 2012) by inducing CCK production. In comparison to undigested crude extracts, digestion improved TIA of fish digest. Maximal trypsin inhibition of 62.67% was achieved on day 7 (4°C), at 240 min of digestion, demonstrating that ESW fillet can be a potent trypsin inhibitor; however further investigation need to be carried out to identify peptide sequence associated with the activity.

**Antioxidant activities (DPPH free radical scavenging activity, metal chelating activity and reducing power assay)**

The scavenging abilities of DPPH radicals by crude extracts changed depending on temperature and time (data not shown). An increase in activity from day 1-7 (4°C) and a decline for 6°C and frozen samples was observed. Frozen samples exhibited weak
antioxidant activity, possibly due to physical aggregation of peptides (Ren et al., 2010). In further studies, to better understand the aggregation of peptide and/or protein aggregation during frozen conditions, monitoring the reactive changes in sulphide groups is crucial, since the latter impacts on myosin proteins (Ramírez et al., 2000). Various studies have investigated antioxidant properties of hydrolysates or bioactive peptides from animal sources, mackerel (Wu et al., 2003) and grass carp myofibrillar proteins (Ren et al., 2010) amongst others. Nevertheless, the current study investigated the release of peptides from underutilised Australian fish species, with potent antioxidant activity. Antioxidant properties are more related to the composition, structure and hydrophobicity of proteins or peptides present in original protein sources. Some examples of amino acids that cause antioxidant activity include Tyr, Trp, Met, Lys, Cys and His (Sarmadi and Ismail, 2010). The sulphide group in cysteine has an independently crucial antioxidant action due to its direct interaction with radicals (Sarmadi and Ismail, 2010). Therefore, further studies should focus on the fractionation and purification of peptides to identify the amino acid sequence contributing to the high antioxidant activity of ESW fillets.

The copper ions (Cu$^{2+}$) chelation by ESW crude extracts as affected by storage temperatures, time and digestive enzymes are illustrated by Figure 3. Irrespective of storage conditions (temperature and time), MCA decreased with pepsin digestion, but an upward trend was observed with pancreatin treatment. A similar observation was made with loach protein hydrolysates (You et al., 2010). Enzymatic hydrolysis is indeed an effective technology for utilizing low value fish. However, peptide size can affect chelating capacity of peptides, which is further affected by structure, molecular weight and amino acid composition. The first stage of simulated digestion (pepsin) may have disrupted the structure of ESW peptides and reduce its abilities to bind and trap Cu$^{2+}$ ions; a similar observation was made with loach (You et al., 2010) and silver warehou
peptides. The second pancreatin-involved digestive stage may have increased the degree of hydrolysis and released more free amino acids. Moreover, any high-affinity metal binding groups, such as carboxylic groups (Zhu et al., 2008) become fully exposed or newly formed (You et al., 2010). Further fractionation, purification and identification of peptides associated with high MCA from the ESW crude extracts deemed necessary.

Direct reaction of a substance is not the only mechanism by which antioxidants may display their activity. Secondary antioxidants do not convert free radicals to more stable products but slow rate of oxidation by one important mechanism; chelation of pro-oxidant metals. Transition metals (copper) promote oxidation by acting as a catalyst of free radical reactions (Končić et al., 2011). Chelation of metals by certain antioxidant compounds, such as ESW-derived peptides, decreases their pro-oxidant effect by reducing their redox potentials and stabilising the oxidised form of the metal (Končić et al., 2011). Excess of transition metal ions in human body can result in various anomalies, including neurodegenerative diseases. Based on our results obtained from in vitro study, ESW peptides may serve as efficient and natural metal chelating agent, involved in chelation therapy in the prevention and/or treatment of inflammatory diseases.

The reducing power of crude extracts also changed with respect to storage temperatures, times and digestive treatments (data not shown). A similar trend as the above physiological activities was observed, that is, an increase in activity of 4°C samples, from day 1-7. Pancreatin digests showed higher reducing power, in line with You et al., (2010). The reducing power of a sample or crude extracts of peptides, an indicator of antioxidant activity, is used to evaluate the ability of an antioxidant to donate an electron or hydrogen or acting as a reducing agent. In the presence of an antioxidant
derived from proteinaceous food sources, such as from ESW peptides, ferrous chloride is formed, based on the chemical equation below:

\[
\text{Potassium ferricyanide} + \text{Ferric chloride} \rightarrow \text{Potassium ferrocyanide} + \text{ferrous chloride}
\]

The colour of the solution changed from yellow to green or blue shade depends on the reducing potential of the sample (Jin and Wu, 2015). Several reports have revealed that there is a direct correlation between antioxidant activities and reducing power of bioactive peptides. Sardinella hydrolysates produced with commercial alcalase enzyme showed potent reducing power (Jeevitha et al., 2014). The lowest reducing power of ESW crude peptides could be due to the peptides associated with reducing power have not been produced with pepsin and/or pancreatin enzymatic reactions. Alcalase enzyme cleavage of peptides demonstrated highest reducing power (Jin and Wu, 2015).

**Peptide profiling**

The peptides released upon simulated gut digestion were analysed by RP-HPLC and qualitative profiles are presented in Figure 4. The peptide profile was clearly affected by storage temperature, time and simulated digestion. Season could be another factor impacted on peptide profile (Medenieks and Vasiljevic, 2008), whereby winter fish released fewer peptides compared with summer fish. In this study, peptide content increased at the end of pancreatin digestion (240 min), for 4°C samples (Figure 4B), but decreased for 6°C and -18°C samples (data not shown). This observation correlated with the increased bioactivities of 4°C samples, as explained above. The retention times of observed peaks were similar suggesting that the types of peptides released were similar but their concentrations differ. This implies that some amino acids might have been affected during freezing and cold storage. The lower concentration of peptides released prior to simulated digestion could proportionally relate to the lower proteolytic activity.
of endogenous enzymes. Moreover, the combination of digestive and fish intracellular enzymes might have contributed to better hydrolytic process, releasing more active peptides.

**Conclusion**

Results demonstrated that fish endogenous enzymes can hydrolyse proteins into amino acids, but to a lesser extent, whilst simulated gastrointestinal digestion enhanced the degree of hydrolysis. Interestingly, pancreatin treatment was more effective with greater bioactivities compared to digestion with pepsin enzyme. Cold storage at 4°C produced greater physiological activities, mainly Cu²⁺ chelating activity and DPPH radical scavenging activity. Overall, our findings suggested that the undervalued eastern school whiting fish can constitute of a potential valuable functional food entity. The potency to act as a metal chelating agent and antioxidant by scavenging radicals should be further investigated by fractionation of specific peptides. Moreover, the amino acid sequence responsible for the multi-functionalities of ESW-derived peptides should be determined.

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Table 1. *In vitro* bioactivities of undigested fillets upon storage at chilled and frozen conditions

The means present average of 6 independent observations (≥ 6); SEM: pooled Standard Error of Mean. DPPH RSA: DPPH radical scavenging activity; MCA: Metal chelating activity; RPA: Reducing power assay; nd: not determined. On day 0, the *in vitro* activities of fresh raw fillets were as follows: ACE inhibition: 35.37%; Trypsin inhibition: 37.48%; DPPH RSA: 46.36%; MCA: 56.82%; and RPA: 17.50%.

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<th>Sampling days</th>
<th>Storage temperatures</th>
<th>ACE Inhibition %</th>
<th>Trypsin Inhibition %</th>
<th>DPPH RSA %</th>
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Figure 1. Experimental design
Figure 2. *In vitro* bioactivities of fresh fillets on day 0 during the stages of simulated gastrointestinal digestion. ACE, Angiotensin-converting enzyme; DPPH RSA, DPPH radical scavenging activity; MCA, metal chelating activity; RPA, reducing power assay. Standard error of mean (SEM): ACE inhibition = 0.07; Trypsin inhibition = 0.05; DPPH RSA = 0.00; MCA = 0.04; RPA = 0.00
Figure 3. Effects of storage temperatures and times on the metal (Cu$^{2+}$) chelating activity of the digests during various stages of the simulated digestion of ESW fillets stored at 4°C (A), 6°C (B) and -18°C (C); Standard error of mean (SEM) = 0.04
Figure 4A. Peptide profiling of undigested crude extracts as affected by storage temperatures (4°C) and times by RP-HPLC: day 0-fresh (A), day 3 (B), day 5 (C) and day 7 (D)
Figure 4B. Peptide profiling digested crude extracts as affected by storage temperatures (4°C) and times by RP-HPLC: day 0-fresh (A), day 3 (B), day 5 (C) and day 7 (D).
Justification:

Figure 4A (a-d) showed RP-HPLC peptide profile of undigested fish extracts from fillets stored at 4°C. The chromatograms showed substantially higher levels of liberated peptides over storage time. This alteration in peptide profiles might have contributed to the observed changes in *in-vitro* bioactivity of fish extracts (Table 1). Such changes in peptide profiling could be explained by the release of water soluble peptides eluting predominantly in the intermediate/high hydrophobic region of the chromatogram.
CHAPTER 6

Overall conclusions and future research direction
6.1 Overall conclusions

Fish wastes, such as scales and underutilised fish species, all constitute excellent source of high quality proteins. They can release physiologically active peptides during enzymatic hydrolysis. Hydrolytic processes in the presence of both digestive (pepsin, trypsin and pancreatin) and endogenous enzymes present in fish muscles assist in the proteolytic break down of proteins into smaller units of amino acids. These polypeptides have been demonstrated to possess important in vitro bio-functional activities, for example, ACE inhibitory and antioxidant activities.

Our study demonstrated that the two assessed fish protein powders derived from fish scales were likely prepared by two different processing methods resulting in variations of their protein content and morphology. This may have impacted on their solubility and digestibility. Furthermore, in vitro digestion has improved the ACE and trypsin inhibitory activities of the released peptides of the products, but not the antioxidant capacity. Moreover, the physicochemical properties such as the high protein content, the fine particle sizes as well as the corresponding high solubility and digestibility of the powdered hydrolysate could have contributed to the significantly high ACE inhibitory activities of its released peptides. As a whole, the production of protein hydrolysates derived from marine wastes such as scales may deliver health benefits to consumers, whilst minimising aquatic wastes and maintaining an ecological oceanic environment. At the same time, the processing techniques should be linked to the intended use since the examined samples showed quite different physiological functionalities.

Further studies demonstrated that intrinsic endogenous enzymes present in the muscles of fish fillets of SW and ESW have the potential to release an array of polypeptides during post-mortem storage due to limited breakdown of the protein matrix. In the presence of endogenous enzymes only, released peptides from both fish species
Chapter 6

exhibited *in vitro* bioactivities of ACE and trypsin inhibitory as well as antioxidant activities. These were definitely enhanced when fillets where subjected to simulated gastrointestinal digestion, using pepsin and pancreatin. Interestingly, pancreatin treatment was more effective with greater bioactivities compared to digestion with pepsin enzyme. Amongst the three major assessed bioactivities, both examined fish species revealed greater metal chelating activities. In addition, results also demonstrated that storage temperatures and times significantly impacted on (p<0.05) the evaluated bioactivities. Deterioration of fish fillets over time resulted in a significant reduction (p<0.05) in *in vitro* physiological activities. Fillets stored at 4°C demonstrated greater potential of metal (Cu²⁺) ions chelating capacities in comparison to 6°C and frozen (-18°C) samples.

The comparisons of our data with other studies was difficult since no previous investigations have been conducted on the effects of storage temperatures and times on liberated peptides from underutilised fish. However, our findings indicated that these undervalued fish species can constitute of a potential valuable functional food entity, containing essential bioactive peptides. Owing to the high anti-oxidative activity of released peptides of mainly ESW, it is suggested to consume the fish fresh. Moreover, from a food technological perspective, the peptides, once identified and purified could be employed as a new functional food ingredient in the development of functional foods with the motif of preventing reactive oxidative stress in obese individuals.
6.2 Future research directions and applications

Obesity, already a worldwide epidemic, affecting both childhood and adulthood, is linked to the development of various health conditions, such as diabetes, cardiovascular diseases and oxidative stress (inflammation) related diseases, such as cancers. Various weight loss nutraceuticals and medical treatments are available on the global market to help obese individual lose weight. However, these remedies are costly and pose severe secondary negative effects. There is an urgent need to find affordable alternatives for weight gain prevention. As our results demonstrated, fish fillets possess excellent sources of proteins, which when consumed fresh and digested, are converted into valuable peptides exhibiting physiological bioactivities, mainly anti-oxidative activities. These peptides could provide an important avenue to be incorporated as functional food ingredients with potential health promoting properties. Therefore, certain technological prospects that should be considered and addressed in view of the functionality of fish peptides are as follows:

6.2.1 In vitro assessment of the absorption and bioavailability of the bioactive peptides

It would be helpful to understand whether the bioactive peptides has the potential to be absorbed within the intestinal transport system and into the bloodstream; a factor of physiological requisite in order for the peptide to exert its bioactivity to the target organ. Such assessment is usually performed using the human intestinal Caco-2 cell monolayer as a model of the intestinal epithelium (Satake et al., 2002).
6.2.2 Characterization and quantification of bioactive peptides

Characterisation and quantification of bioactive peptides is a means of determining the known amino acid composition of the active peptides. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) are often the two major techniques that may assist in this phase of future research. The purified and sequenced peptide can be further synthesized in large enough quantities to test for efficacy in animal studies and clinical trials (Vermeirssen et al., 2002; Vermeirssen et al., 2005).

6.2.3 In vivo study

Conducting an animal study, followed by clinical trials would enhance the understanding of the physiological roles of the identified peptide to exhibit anti-obesity properties in vivo. To ensure such a valuable health benefit, the effects of fish consumption on weight gain, blood pressure, glucose levels and body composition could be investigated in obese and control mice.
CHAPTER 7

List of references
**Note:** Chapter 7 presents the references of Chapter 1 (Introduction) and Chapter 2A (Literature review).


Bell, A.C., Ge, K., & Popkin, B.M. (2002). The road to obesity or the path to prevention: motorized transportation and obesity in China. *Obesity Research, 10*, 277-283.


Limitations
This section of the thesis highlights a limited aspect of bioactive peptides and/or fish protein hydrolysates, which requires attention from a food technological perspective. As previously discussed, fish protein hydrolysates exhibit essential physiological and physicochemical properties. Due to the degree of hydrolysis and hydrophobicity, these peptides and/or enzymes may tend to exert certain poor sensory qualities, such as bitterness (Kristinsson and Rasco, 2000). Research demonstrated that the degree of bitterness depends on the enzymatic cleavage and composition of the fish muscle. Moreover, fish muscles are less bitter in comparison to plant- and or meat-based hydrolysates. To improve their level of acceptance more to health-conscious consumers, the pharmaceuticals must consider the ways to reduce and/or completely remove the bitter taste of these hydrolysates. Some of the ways to achieve this technological hurdle, which considering the probable loss of some essential proteins, include:

i) Solvent extraction

ii) Treatment with exopeptidases

iii) Selective extraction with alcohols

iv) Chromatographic removal using different matrices

v) Adsorption of bitter peptides/compunds on activated carbon

vi) Performing hydrolysis at slightly alkaline pH conditions

Reference:

Appendix
Figure 1: Effects of storage temperature and time on ferric ions reducing power of the digests during various stages of the simulated gastrointestinal digestion of silver warehou (SW) fillets stored at 4°C (A), 6°C (B), and -18°C (C). Standard error of mean (SEM) = 0.00.

(A) 4°C, (B) 6°C, (C) -18°C.
Figure 2: Effects of storage temperature and time on trypsin inhibitory activity of the digests during various stages of the simulated gastrointestinal digestion of ESW fillets stored at 4°C (A), 6°C (B) and -18°C (C); Standard error of mean (SEM) = 0.07
Figure 3: Effects of storage temperature and time on DPPH radical scavenging activity (RSA) of the digests during various stages of the simulated gastrointestinal digestion of ESW fillets stored at 4°C (A), 6°C (B) and -18°C (C); Standard error of mean (SEM) = 0.00
Figure 4: Effects of storage temperature and time on DPPH radical scavenging activity (RSA) of the digests during various stages of the simulated gastrointestinal digestion of ESW fillets stored at 4°C (A), 6°C (B) and -18°C (C); Standard error of mean (SEM) = 0.00