ANTI-CARCINOGENIC PEPTIDES DERIVED FROM FISH BY-PRODUCTS

A thesis submitted to Victoria University for the degree of Doctor of Philosophy

by

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I dedicated this thesis to My Beloved Parents

ABSTRACT

Fish by-products can account up to 75% of total weight of fish catch, harvested or processed. Despite their high economic value, by-products have been used mainly for the production of low-value products resulting in a low profit for the fish industry. Thus, it is imperative to develop efficient and effective technologies for the recovery of valuable ingredients from fish by-products. The main focus of this thesis was to develop a simple hydrolysis process to extract valuable compounds and liberate bioactive peptides from fish by-products of fish species endemic to Australia as well as exploring the essential physiological properties of these bioactive peptides, especially their potent use for colon cancer treatment.

Four Australian fish species (Atlantic salmon (*Salmo salar*), Barramundi (*Lates calcarifer*), Flathead (*Platycephalus fuscus*), and Silver warehou (*Seriolella punctata*) by-products were used in the initial stage of the study. The fish compositional differences were estimated and optimum condition (pH, temperature and fish:water ratio) for the extraction of valuable compounds were proposed. The chemical composition of four fish species differed significantly ($p < 0.05$) with the protein content ranging from 14.7 ± 0.09 to 16.8 ± 0.41%. Adjusting pH to 2.5 yielded two times more of extracted oil than at pH 4.5. Salmon and Barramundi oils contained high amounts of monounsaturated fatty acids (MUFAs) (39-50%) while Silver warehou and Flathead oils contained 46-49% of saturated fatty acids (SFAs). The particle size of the protein fractions was small, ranging from 126.9 to 489.5 nm. Molecular weight of extracted proteins was fish species dependent - Silver warehou and Barramundi samples contained proteins of 250, 120 and 100 kDa while these bands were absent from Salmon and Flathead samples.

In the second study, extraction process involving the use of endogenous proteases and/or addition of exogenous enzyme preparation resulted in proteinaceous hydrolysates. The extracts from Salmon, Flathead, Silver warehou and Barramundi by-products were evaluated for selected physiological and physical properties. Fish by-products were subjected to four different treatments, with or without the addition of acid fungal protease (AFP). The peptides produced in fish protein hydrolysate (FPH) were examined for bioactive properties based on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and angiotensin I-converting enzyme (ACE) inhibitory activities. Furthermore, dried FPHs were also examined for properties such as colour, solubility, heat stability, emulsion activity, and rheological properties. Hydrolysis duration and addition of AFP to FPHs, influenced the degree of hydrolysis (DH), DPPH scavenging and ACE inhibition activities. Salmon FPH treated with AFP showed the highest DH (43.86 %) and ACE inhibitory activity (95.50 %). High antioxidant activity was observed for Flathead and Barramundi FPHs with DPPH scavenging activity of 45.86 % and 43.03%, respectively. The solubility of dried FPHs ranged from 93 to 100% and increased with decreased DH. Emulsifying capacity of FPH for Barramundi was the highest, whereas FPH for Silver warehou showed the highest emulsion stability. Heat induced the gelation of Barramundi and Silver warehou FPHs but not for Salmon and Flathead FPHs. These results have implications for the use of protein hydrolysate from fish by-products in food formulation technology and serve as important sources of bioactive compounds.

Since Flathead protein hydrolysate showed the highest antioxidant activity, it was further fractionated and purified. Peptide fractions extracted from Flathead by-products were then evaluated for *in vitro* free radical scavenging and cancer cell cytotoxic activities. The degree of hydrolysis (DH), presence of protease and molecular weight (MW) influenced the DPPH (2,2-diphenyl-1-pycryl-hydrazyl) and ABTS (2,2-Azino-bis-3-ethylbenzothiazoline-6-

sulfonic) radicals scavenging activities (RSA) of Flathead peptide fraction. Low MW peptides (< 3 kDa), obtained from overnight incubation with added an exogenous protease, significantly inhibited free radicals and showed the highest RSA of 94.03 % and 82.89 % against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively. The presence of bioactive peptides during H_2O_2 exposure increased viability of T4056 normal cells. Furthermore, peptide fractions < 3 kDa inhibited the growth of HT-29 colon cancer cells up to 91.04 %, although the activity was found to be non-selective. Further purification revealed a novel peptide, Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg, with RSA properties. The results indicated that peptides extracted from Flathead by-products have a potential to be used as natural antioxidants and/or chemo-protective agents.

In order to evaluate the efficacy of identified peptide, the peptide was assessed using simulated gastrointestinal digestion. The results showed that the identified peptide retain anti colon cancer activity before, during and after gastrointestinal (GI) digestion. Three proline residues identified in the peptide sequence were likely responsible for its resistance to pepsin digestion. The growth of HT-29 cells was significantly inhibited up to 28.89% and 29.68% by undigested and pepsin digested peptides, respectively. Further digestion using pancreatin cleaved the peptide into smaller peptides and slightly increased the cytotoxicity of the peptide on HT-29 colon cancer cell lines resulting in lower cell viability (61.7 ± 0.00 %). The peptide was also fairly selective maintaining higher viability of the T4056 normal cells. These findings suggest that the peptide can maintain its activity during GI digestion and has potency to be used as peptide-based cancer drug via oral administration.

CERTIFICATE

Todor Vasiljevic (M.Sc., PhD)
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This is to certify that the thesis entitled "ANTI-CARCINOGENIC PEPTIDES DERIVED FROM FISH BY-PRODUCTS" submitted by Rahmi Nurdiani in partial fulfillment of the requirement for the award of the Doctor of Philosophy with specialization in Food Sciences and Technology at Victoria University is a record of bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Werribee, Australia

(Prof. Todor Vasiljevic)

Thesis supervisor

Date: 31/8/2016

DECLARATION

"I, Rahmi Nurdiani, declare that the PhD thesis entitled "ANTI-CARCINOGENIC PEPTIDES DERIVED FROM FISH BY-PRODUCTS" is no more than 100,000 words in length including quote 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".

Signature: Date: 31/8/2016

Rahmi Nurdiani

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Bismillāhirrahmānirrahīm. In the name of Allah, Most Gracious and Most Merciful

Alhamdu lillāhi rabbil-ʿālamīn. All the praises be to God, the Lord of the 'Alamîn.

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List of Publications during Candidature

Peer reviewed publications

1. Nurdiani, R., Dissanayake, M., Street, W. E., Donkor, O. N., Singh, T. K., & Vasiljevic, T. (2015). Sustainable use of marine resources – turning waste into food ingredients. *International Journal of Food Science & Technology, 50* (11): 2329–2339. doi: 10.1111/ijfs.12897
2. Nurdiani, R., Dissanayake, M., Street, W. E., Donkor, O. N., Singh, T. K., & Vasiljevic, T. (2016). In vitro study of selected physiological and physicochemical properties of fish protein hydrolysates from 4 Australian fish species. *International Food Research Journal, 23* (5): 2042-2053
3. Nurdiani, R, Vasiljevic, T, Yeager, T, Singh, TK & Donkor, ON 2016, 'Bioactive peptides with radical scavenging and cancer cell cytotoxic activities derived from Flathead (*Platycephalus fuscus*) by-products', *European Food Research and Technology*, vol. (published online).
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Posters Presentation

Nurdiani, R., Donkor, O. N., Singh, T. K., & Vasiljevic, T. Production of Pink Ling (*Genypterus blacodes*) Head Hydrolysate using Acid Fungal Protease. The 47th Annual AIFST Convention. 22 – 24 June 2014. Melbourne Convention Centre, Melbourne.

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PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

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

Item/ Chapter No.	**Paper Title**	**Publication Status** (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	**Publication Title and Details** (e.g. date published, impact factor etc.)
2	Bioactive peptides from fish by-products with anticarcinogenic potential (Review)	Accepted for publication	International Food Research Journal (Rank Q2)
3	Sustainable use of marine resources – turning waste into food ingredients	Published	International Journal of Food Science and Technology (Volume 50, Issue 11, pages 2329–2339, Rank Q1)
4	In vitro study of selected physiological and physicochemical properties of fish protein hydrolysates from 4 Australian fish species	Published	International Food Research Journal (IFRJ Volume 23 Issue 5 2016, pages 2042-2053, Rank Q2)
5	Bioactive peptides with radical scavenging and cancer cell cytotoxic activities derived from Flathead (Platycephalus fuscus) by-products	Published online	European Food Research and Technology (Rank Q1)

Declaration by [candidate name]: Rahmi Nurdiani **Signatur** [redacted] **Date: 31/8/2016**

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List of Abbreviations and Acronyms

ABTS	2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic
ACE	Angiotensin I-converting enzyme
AFP	Acid fungal protease
DH	Degree of hydrolysis
DHA	Docosahexaenoic acid
DPPH	2,2-diphenyl-1-pycryl-hydrazyl
EPA	Eicosapentaenoic acid
FAO	Food Agricultural Organisation
FPH	Fish protein hydrolysate
GI	Gastrointestinal
kDa	Kilo Dalton
MUFA	Monounsaturated fatty acids
MW	Molecular weight
MWCO	Molecular weight cut off
ON	Incubated overnight without addition of AFP
ONE	Incubated overnight with addition of AFP
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RSA	Radical scavenging activity
SFA	Saturated fatty acids
UF	Unfractionated
UH	Unhydrolysed/Control

CHAPTER 1

INTRODUCTION

Chapter 1. Introduction

1.1. Background

Global population is expected to reach 9.7 billion by 2050. This creates a daunting challenge for researcher, technical experts and the world leaders on how to feed the world's growing population without damaging precious natural resources, including aquatic environment (Msangi et al. 2013). According to the 2016 Food Agricultural Organisation (FAO) Fisheries and Aquaculture Department's report, world per capita fish consumption increased significantly from an average of 9.9 kg in the 1960's to 19.7 kg in 2013 with preliminary estimates for 2015 pointing towards beyond 20 kg (FAO 2016). Significant growth in fish consumption means greater volumes of fish by-products are generated.

Fish by-products, defined as leftovers after handling and processing, include viscera, heads, cut-offs, bone, skin, fins, roes and frames and can account for up to 75% of total weight of fish harvested or catch (Rustad, Storrø & Slizyte 2011). By-products have been used mainly for the production of low-value products such as fertilizer, silage, pet food, and fish meal, resulting in a low profit for the fish industry. Therefore, it is important for seafood processing industry to develop technologies that allow the recovery of valuable ingredients from fish by-products, as it can potentially generate additional income as well as reduce disposal costs (Sanmartín et al. 2009)

Fish by products contain valuable lipid and protein fractions as well as essential compounds such mineral and vitamins (Rustad 2003). Many researchers have attempted to recover these valuable compounds by chemical and biochemical processes or the combination of both (Kristinsson & Rasco 2000a; Ovissipour et al. 2012). Fish by-products are commonly converted into fish protein hydrolysate (FPH) before being separated into

various desired fractions. FPH possess desirable functional properties such as good water holding capacity, texture, gelling, foaming and emulsifying capacity (Rustad, Storrø & Slizyte 2011). In addition, FPH is also a source of bioactive peptides with various activities, such as antioxidant, anticancer and ACE inhibitor (Bougatef et al. 2008; Je et al. 2009; Naqash & Nazeer 2011).

Bioactive peptides usually consists of small fragments of peptides that contain 2 - 20 amino acids though some have been reported to be more than 20 residues (Ryan et al. 2011). Bioactive peptides are inactive within the sequence of the parent proteins and may become active after undergoing hydrolysis or digestion (Sarmadi & Ismail 2010). Due to its advantages, enzymatic hydrolysis has been widely employed in extraction and isolation of bioactive peptides from fish by-products (Diniz & Martin 1997). The quality or properties of peptides liberated after enzymatic hydrolysis is dependent on the type of proteases and experimental conditions during hydrolysis (Mendis, Rajapakse & Kim 2005; Nazeer & Anila Kulandai 2012; See, Hoo & Babji 2011). After digestion and being absorbed in the intestines, bioactive peptides enter the blood stream and reach the target site to exert the bioactivities (Erdmann, Cheung & Schröder 2008).

Recently, bioactive peptides are gaining increasing attention as alternative drug for cancer treatments. Peptide-based drug therapies are known for their strong specificity, tumor penetrating ability, low toxicity profiles and small size (Barras & Widmann 2011; Rodrigues et al. 2009). Many papers have reported the isolation and identification of anticarcinogenic peptides from milk (Gill & Cross 2000; Sah et al. 2015) and marine species (Suarez-Jimenez, Burgos-Hernandez & Ezquerra-Brauer 2012). Recent reports also demonstrated that fish by-products can be used as valuable sources of anticancerogenic peptides (Alemán, Pérez-Santín, Bordenave-Juchereau et al. 2011; Picot et al. 2006). Due to the biodiversity of the

marine environment, however, further research and exploration of anticancer peptides isolated from by-products of different fish species is necessary.

1.2. Aims of the thesis

The aims of this research were to develop a simple hydrolysis process for liberation of bioactive peptides from fish by-products of fish species endemic to Australia, to explore the essential physiological and chemical properties of these bioactive peptides and to improve our understanding of the mechanism and application of these peptides in the treatment of colon cancer.

The specific objectives of this research were:

1. to establish the compositional differences in valuable compounds from fish by-products and to propose the optimum condition (pH, temperature and fish:water ratio) for extraction of the valuable components from four selected Australian fish species Atlantic salmon (*Salmo salar*), Barramundi (*Lates calcarifer*), Flathead (*Platycephalus fuscus*), and Silver warehou (*Seriolella punctata*).
2. to observe the effect of various incubation time and addition of enzyme on selected physiological activities (bioactivities) and physical functionality of fish protein hydrolysate extracted from four selected Australian fish species (Atlantic salmon (*Salmo salar*), Barramundi (*Lates calcarifer*), wild caught Flathead (*Platycephalus fuscus*), and Silver warehou (*Seriolella punctata*).
3. to examine the potency of antioxidative and anticancer peptides in Flathead hydrolysate by examining the radical scavenging properties and cancer cell cytotoxic effect based on different *in vitro* cell assays based test.
4. to purify and characterize liberated Flathead peptides with the greatest radical scavenging and cancer cell cytotoxic effect.

5. to evaluate anticancer activity of identified peptide and its stability during simulated gastrointestinal digestion

1.3. Thesis outline

Chapter two of this thesis presents a thorough literature review of the current scientific knowledge on the proposed subject. The production of bioactive peptides from fish by-products was discussed. The recent advances in utilization of fish by-products as sources of antioxidative and anticarcinogenic peptides were also described.

Chapter three focuses on the exploration of compositional differences and extraction process of valuable compounds from fish by-products of four selected Australian fish species Atlantic salmon (*Salmo salar*), Barramundi (*Lates calcarifer*), Flathead (*Platycephalus fuscus*), and Silver warehou (*Seriolella punctata*). The extraction conditions for the best separation of major components were optimized in the first phase. The optimum condition (pH, temperature and fish;water ratio) obtained were then utilized to recover valuable fatty acids and soluble fish protein from fish by-products.

Chapter four discusses whether the addition of an exogenous protease, Acid Fungal Protease (AFP), and different incubation time affects the production of bioactive peptides and the functional properties of FPH produced from fish by-products of four selected Australian fish species (Atlantic salmon (*Salmo salar*), Barramundi (*Lates calcarifer*), Flathead (*Platycephalus fuscus*), and Silver warehou (*Seriolella punctata*).

Chapter five reports on the potency of antioxidant peptides isolated from Flathead (*Platycephalus fuscus*) by-products. Flathead hydrolysate was produced by enzymatic hydrolysis and fractionated based on different molecular weight cut off (>10, 10 – 3 and < 3 kDa). The fractions were tested for radical scavenging and anticancer properties based on

several *in vitro* and cell based assays. Fractions with the highest bioactivity were further purified and the peptides were identified.

Chapter six deals with the anticancer properties of identified peptides isolated from Flathead (*Platycephalus fuscus*) and its stability during simulated gastrointestinal digestion. Cell based assays as well as apoptosis analysis was conducted to examine the activities of identified peptides.

Chapter 7 provides the summary of the results of all experiments carried out and future research directions to further explore the sustainable use of fish by-products for human health benefits.

CHAPTER 2

A. LITERATURE REVIEW
B. REVIEW ARTICLE

Chapter 2A. Literature Review

2.1 Fish consumption and fishery by-products

Over the past five decades, world per capita fish consumption has been increasing steadily, from an average of 9.9 kg in the 1960s to 11.5 kg in the 1970s, 12.5 kg in the 1980s, 14.4 kg in the 1990s and reaching 20.1 kg in 2014 (Table 2.1) (FAO 2009; FAO 2016). Moreover, fish provided almost 20% of the global population's intake of animal protein (FAO, 2016). It is evident that fish provides a good source of high quality protein which has greater satiety effect than other sources of animal proteins like beef and chicken (Uhe, Collier & O'Dea 1992). In addition, fish also provides essential unsaturated fatty acids (e.g. EPA and DHA), vitamins (A, B and D) and minerals (including calcium, iodine, zinc, iron and selenium) (FAO 2016).

Table 2.1 World fisheries and aquaculture production and utilization (Adapted from FAO 2016)

	2009	**2010**	**2011**	**2012**	**2013**	**2014**
Total capture (million tonnes)	90.2	89.1	93.7	91.3	92.7	93.4
Total aquaculture (million tonnes)	55.7	59.0	61.8	66.5	70.3	73.8
Human consumption (million tonnes)	123.8	128.1	130.8	136.9	141.5	146.3
Per capita food fish supply (kg)	18.1	18.5	18.6	19.3	19.7	20.1

An increase in fish production as a consequence of a rise in demand for fisheries products means that the volume of fish by-products will also increase (Sanmartín et al. 2009). By definition, fish wastes or fish by-products are leftovers after treatment or processing (Kim & Mendis 2006). According to Irianto, Dewi and Giyatmi (2014), there are five types of fish wastes or fishery by-products, including 1) by-products resulted from the catching of non-target fish species or so called by-catch; 2) by-products as leftovers from seafood industry's processing which includes viscera, heads, cut-offs, bone, skin, fins and frames; 3) by-products obtained as secondary products after fish meal processing (e.g. fish oil); 4) by-products resulted from a surplus during fishing season due to the limitations of industry's capacity to utilize raw materials; and 5) by-products resulted from inadequate distribution, marketing and storage handling (e.g. rotten fish during distribution or storage). Undoubtedly, fish by-products present a huge problem for the environment and the seafood industry. The amount of by-products discarded by the seafood industries may constitute as much as 70% of fish and shellfish harvested (Olsen, Toppe & Karunasagar 2014). In addition, fish meat and oil left over following filleting typically accounted for 20–30% and 5–15% of whole fish weight, respectively (Gehring et al. 2011).

In 2005, Food and Agriculture Organization (FAO) reported significant decrease of fishery discards due to the implementation of more selective fishing technologies and increased utilization of unwanted by-products (Kelleher 2005). The utilization of fish by-products can potentially generate additional income for seafood processing industries as well as reduce disposal costs of the waste (Arvanitoyannis & Kassaveti 2008). Most fish by-products are commonly used for the production of livestock feed, silage, fish meal, and fertilizer (Jaczynski 2008). With proper handling and processing, fish by-products can be regarded as sources of valuable proteins and peptides for promoting human welfare.

Converting fish by-products into fish protein hydrolysates (FPH) is regarded as the most common approach to utilize these valuable resources (Chalamaiah et al. 2012).

In order to develop efficient technologies to recover and utilize fish by-products, it is essential to understand fundamental properties of the raw materials. By understanding the properties, it may be possible to manipulate the behaviour of bio-molecules such as fish proteins or lipids that eventually increase recovery yields (Gehring et al. 2011).

Water is one of the major components of fish. Depending on the species, the water content of fish can reach up to 90%. Water provides a reaction medium for other components such as proteins and lipids. It is, therefore, important to be familiar with how water interacts with other components. Water molecules, which contain electro-negative oxygen and electro-positive hydrogen atoms, have electrostatic charges on their surface that allow interaction between water dipoles and other charged molecules such as proteins (Fig 2.1.). Proteins or other food components should also electrostatically charged in order to bond with water dipoles. This interaction is essential for solubility property of food products (Jaczynski 2008).

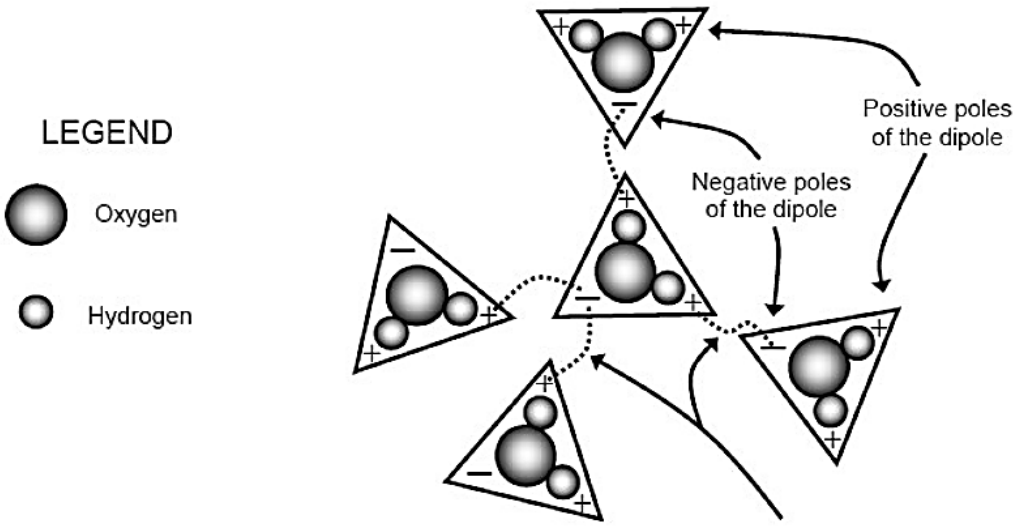

Figure 2.1 Water dipoles are responsible in creating weak bonds between each other or other charged molecules (e.g. proteins) (Jaczynski 2008)

Protein of fish can be categorized into three main groups, i.e sarcoplasmic, myofibrillar and stromal proteins. Sarcoplasmic proteins are present in the sarcoplasm, which sometimes termed as 'water soluble proteins' because they can be dissolved at low ionic strength (Strasbourg, Xiong & Chiang 2008). The myofibrillar proteins, constituting 50-60% of muscle proteins, are referred as 'salt soluble fraction' because they require high salt concentration for solubilisation. Myofibrillar proteins are responsible for the contractile apparatus that allows movement (Neves, Harnedy & FitzGerald 2016). Myosin and actin comprise about 65% of the total myofibrillar content and about 40% of the total muscle protein content. The stromal proteins form the connective tissue which present in the skin, bone, swim bladder and myocommata in muscle. They consist mostly of collagen which largely related to muscle toughness or tenderness (Strasbourg, Xiong & Chiang 2008).

Fish proteins are also consist of essential amino acids that required for human nutrition. Each amino acid contain amino and acid/carboxyl group that are bonded to a central carbon atom. The side chain of each amino acid is unique that may differentiate properties of protein. Hydrophobic side chains may result in limited water solubility if the protein while polar side chain may result in protein-water interaction via the hydrogen bond (Jaczynski 2008). Good protein-water interaction is essential for protein solubility and water holding capacity. The side chains can also assume different electrostatic charges depending on the condition or treatments subjected to fish protein (Gehring et al. 2011). Negative charges of protein will interact with positive hydrogen ions if acid is added into a protein solution. On the other hand, by adding bases to a solution, the positive charges of protein will interact with negative charges of bases and the protein becomes negatively charged (Fig 2.2). When the charge equilibrium is reached, the protein molecule assumes a zero net electrostatic charge, which is called the isoelectric point (p*I*). The p*I* of each protein is very specific. The knowledge of p*I* of fish protein is essential in order to be able to separate protein from other components,

which eventually optimize the protein recovery yields. Hultin and Kelleher (1999) published a patented process to isolate protein from fish muscle with concurrent separation of lipids. The process is called isoelectric solubilisation/precipitation (ISP), which has been widely used to recover proteins and lipids, from fish by-products (Batista 1999; Batista, Pires & Nelhas 2007; Chen & Jaczynski 2007; Chen, Tou & Jaczynski 2007; Nurdiani et al. 2015). The lipids liberation from fish tissue is important during protein isolate production as it reduces the susceptibility of protein isolate to lipid oxidation (Nolsøe & Undeland, 2009).

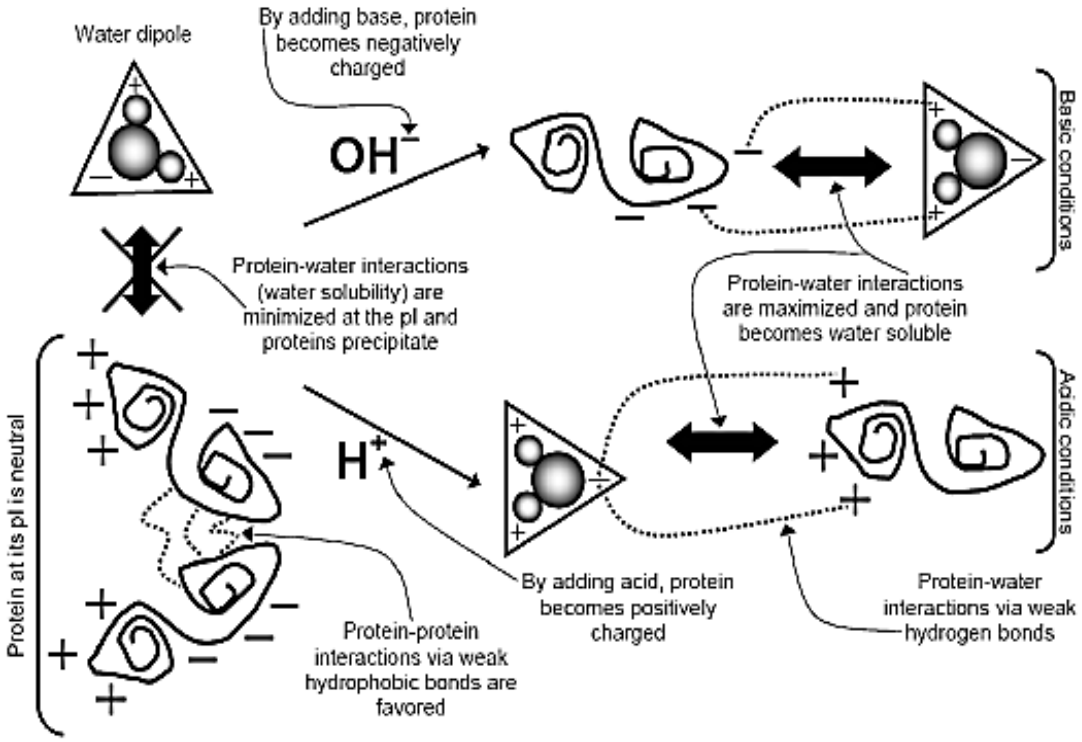

Figure 2.2 The principle of isoelectric precipitation or solubilisation (ISP) of protein (Jaczynski 2008).

Fish lipids are known to have higher level of unsaturation compare to fats of terrestrial animals. They have low melting point so that commonly referred as fish oils. Most marine oils are good sources of eicosapentaenoic acid (EPA; 20:5ω-3) and docosahexaenoic acid (DHA; 22:6ω-3). The ω-3 polyunsaturated fatty acids (PUFA) in fish oils have been shown to

be beneficial for human health, such as reducing the risk of cardiovascular health (Chen, Tou & Jaczynski 2007), reducing the incidence and severity of arrhythmias, alleviating mental disorders, type-2 diabetes, inflammatory diseases and immuno-modulatory effects (Shahidi & Finley 2001), and as cancer chemopreventive agents (Rose & Connolly 1999) among others. The fatty acids composition of fish is largely dependent on the species, sex, age, seasonal variation, fish origin, fatty acid composition of fish feed and anatomical location of fish muscle (Alasalvar et al. 2002; Bae & Lim 2012; Huynh et al. 2007; Nurdiani et al. 2015; Tanakol et al. 1999).

Fish oils are hydrophobic and have lower specific density than water that makes fish oil float in the water. They also do not have charges present in the surface and cannot bond with water. Researchers are usually separate fish oil from other components in fish isolate solution by centrifugation with sufficiently high *g* force (Batista et al. 2010; Beaulieu et al. 2009; Jaczynski 2008).

2.2 Fish protein hydrolysate (FPH)

Fish protein hydrolysates are defined as fish proteins that are enzymatically or chemically broken down into smaller peptides or free amino acids with varying sizes (Kristinsson & Rasco 2000a). FPH has been known to possess desirable functional properties as it can contribute to water-holding, texture, gelling, foaming and emulsification properties in different food systems (Diniz & Martin 1997; Rustad, Storrø & Slizyte 2011). FPH, therefore, can be used as readily available sources of protein for animals and humans (Neklyudov, Ivankin & Berdutina 2000).

FPH can be categorized based on its raw materials, which can be differentiated based on the possible spoilage and degradation levels. Fish parts which contain various concentration of endogenous enzymes i.e: viscera, liver, roe, are grouped as an easily degradable by-

products. Skin, bones, frame and heads, on the other hand, are considered as relatively more stable by-products (Rustad, Storrø & Slizyte 2011) .

2.2.1 Fish visceral protein hydrolysate

Fish viscera are a rich source of protein and polyunsaturated lipids but have low storage stability if not frozen or preserved (Ovissipour et al. 2009). Viscera can encompass up to 25% of the weight of Sturgeon (Ovissipour et al. 2012). During Sardinella processing, solid wastes including heads and viscera constitute 30% of original material (Bougatef et al. 2008). The production of FPH from viscera have been conducted using several proteolytic enzymes including Protamex, Alcalase, Neutrase, Flavorzyme, trypsin, pepsin, α-chymotrypsin (Batista et al. 2010; Bhaskar et al. 2008; Bougatef et al. 2010; Ovissipour et al. 2009). Several studies also reported the use of endogenous enzyme from viscera to produce FPH (Aspmo, Horn & Eijsink 2005; Ben Khaled et al. 2011; Bougatef et al. 2010; Klomklao, Benjakul & Kishimura 2013).

FPH derived from heads and viscera of *Sardinella aurita* showed high ACE inhibitory activity (63.2 ± 1.5% at 2 mg/ml) (Bougatef et al. 2008) and high radical scavenging activities (Barkia et al. 2010; Souissi et al. 2007). Some functional properties of FPH from viscera have also been studied. Souissi et al. (2007) reported that the solubility of Sardinella visceral hydrolysate increased with the increased of degree of hydrolysis (DH) while its emulsifying capacity and whippability decreased. Besides DH, pH was another factor to influence the functional properties of FPH. Lower emulsifying and foaming capacity of FPH, extracted from horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*) was noticed at pH 4 and a gradual increase in these properties observed with the increasing pH (Sampath Kumar, Nazeer & Jai Ganesh 2012).

2.2.2 Fish egg and roe protein hydrolysate

Fish roe of salmon, cod and sturgeon have been known to have high economic value and sold under commercial name 'caviar'. Roe or egg from many other fish species, however, are underutilized or considered as by-products. In order to recover valuable components from fish roes, several researcher studied their chemical compositions and characteristics. Intarasirisawat, Benjakul and Visessanguan (2011) reported that fish roes, which are usually removed from several tuna species during canning, contain considerable amount of protein and have a high content of polyunsaturated fatty acid. Fish roes of white sea bream contain 4.91–6.73% EPA and 23.09–27.50% DHA (Cejas et al. 2003) while Pacific herring contain 13.72% EPA and 21.65% DHA (Huynh et al. 2007). Roes from several fresh water species including catla, carp, rohu and murrel contain protein in the range of 16.6-28.2% (Balaswamy et al. 2009).

Conversion of fish roes into protein hydrolysates using Alcalase was reported by several authors. Galla, Pamidighantam, Akula, and Karakala (2012) hydrolysed roes from *Channa striatus* and *Labeo rohita* in order to improve their functional properties and antioxidative activity. Both hydrolysates showed lower molecular weight than the protein concentrates. Low molecular weight peptides present in the hydrolysates might be responsible for the exhibited antioxidant activity. They also found higher protein solubility at pH 12 compared to a lower pH. Similarly, low solubility was recorded from defatted skipjack (*Katsuwonous pelamis*) roe hydrolysate at pH 3-4 (Intarasirisawat et al. 2012). This may be associated with the isoelectric point (p*I*) of phosvitin, a protein in egg yolk, which has a pI of 4.0 (Ternes 1989). Further purification of skipjack roe hydrolysates showed that the purified peptides possessed high ACE inhibitory and antioxidative activities (Intarasirisawat et al. 2013). Recently, Yang et al. (2016) reported that ultrafiltrated roe hydrolysates (URH) derived from giant grouper (*Epinephelus lanceolatus*) induced apoptosis on oral cell cancer. These studies

indicated that peptides prepared from fish roes could be further employed as a functional food ingredient.

2.2.3 Fish liver protein hydrolysate

Fish lipid is rich with long-chain n-3 (omega-3) PUFA, particularly EPA (C20:5 n-3) and DHA (C22:6 n-3). Consumptions of these PUFAs have been considered to be essential for human nutrition, health and disease prevention. Commercial fish liver oils are mostly obtained from cod and shark livers. Tuna liver, on the other hand, is mostly utilize to produce fish meal and animal feed or is directly discarded as by-product (Ahn, Lee & Je 2010). To maximize the economic benefit of underutilized fish liver; Ahn, Lee and Je (2010) hydrolysed tuna liver using Flavourzyme, Alcalase, Neutrase and Protamex. The hydrolysates showed excellent antioxidant activities against DPPH, hydrogen peroxide and hydroxyl radical scavenging, and reducing power. In addition, the acetylcholinesterase inhibition activity of the hydrolysate showed its potency as natural drug to treat Alzheimer's disease. Similar experiment conducted by Je et al. (2009) showed the protection ability of the hydrolysates toward hydroxyl radical-induced oxidative DNA damage. Furthermore, tuna liver hydrolysates had ACE-inhibitory properties which suggest that tuna liver hydrolysates may be a beneficial ingredient to be used as antihypertensive agents.

2.2.4 Fish skin protein hydrolysate

Fish skin is a major by-product of the fish processing industry that can provide valuable source of collagen and gelatin. Due to the bovine spongiform encephalopathy (BSE) disease and for religious and social reasons, fish skin has gained importance as an alternative source of gelatin and collagen in recent years (Badii & Howell 2006). Gelatin and collagen from fish skin were mainly extracted using acids or bases treatment (Giménez et al. 2005; Gómez-

guillœn & Montero 2001; Tabarestani et al. 2010; Takeshi & Suzuki 2000). Many papers have reported the biochemical properties of gelatin and collagen extracted from marine and freshwater animals, such as dusky spinefoot, sea chub, eagle ray, red stingray, and yantai stingray (Bae, et al. 2008), Atlantic salmon (Arnesen & Gildberg 2007), seabass (Sinthusamran, Benjakul & Kishimura 2013), striped catfish (Singh et al. 2011), cod and hake (Fernández-Díaz, Montero & Gómez-Guillén 2001), Nile perch (Muyonga, Cole & Duodu 2004), bigeye snapper and brownstripe red snapper (Jongjareonrak et al. 2006), farmed giant catfish (Jongjareonrak et al. 2010) and smooth hound (Bougatef et al. 2012). Extracted fish gelatin has been used as natural biopolymer for the production of biodegradable film (Arfat et al. 2014). Several authors compared the characteristics and qualities of fish gelatin with mammalian gelatin (Chiou et al. 2008; Gómez-Estaca et al. 2009; Nur Hanani, Roos & Kerry 2012) and found that fish gelatin has good film-forming ability. The film-forming ability depends mainly on the physical and chemical characteristics of the gelatin, especially the amino acid composition, which is highly species specific, and the molecular weight distribution, which depends mainly on processing conditions.

Production of fish skin protein hydrolysate was conducted either directly using fish skin as the raw material or using prepared gelatin/collagen. Kim et al. (2001) isolated antioxidative peptides from gelatin hydrolysate of Alaska Pollack skin with serial digestions in the order of Alcalase, Pronase E, and collagenase using a three-step recycling membrane reactor. The isolated peptides, containing a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp, showed antioxidative activity on peroxidation of linoleic acid and enhanced the viability of cultured liver cells. The Alaska Pollack skin digested with Protamex also showed DPPH radical scavenging activity (IC_{50} value of 2.5 mg/mL) and reducing power (0.14 at 1 mg/mL, 53.8% of that of reduced glutathione at the same concentration) (Jia et al. 2010).

Many papers reported that protein hydrolysate isolated from fish skin/gelatin/collagen using Alcalase showed high bioactivities (Alemán, Giménez, Montero et al. 2011; Cai et al. 2015; Chi, Wang, Hu et al. 2015; Giménez et al. 2009; Ngo, Ryu & Kim 2014) that indicated the ability of this endopeptidase to produce shorter peptide sequences as well as terminal amino acid sequences responsible for various health beneficial bioactivities. Other enzymes used to isolate bioactive peptides from fish skin/gelatin/collagen are including papain, bromelain, pancreatin, neutrase, pepsin, trypsin and α-chymotrypsin (Fu & Zhao 2013; Nazeer & Anila Kulandai 2012; Ngo et al. 2011; Yang et al. 2008), whereas the use of endogenous protease was limited (Karnjanapratum & Benjakul 2015).

2.2.5 Fish head protein hydrolysate

Head waste is the most important waste materials in shrimp processing industries as it accounts for approximately 35-45% of the whole shrimp waste (Cao et al. 2009). Several papers described efficient and economical method for extracting proteins from shrimp head waste by autolysis method (Cao et al. 2008; Cao et al. 2009) while others used Alcalase to isolate essential amino acids from head waste (Gildberg & Stenberg 2001; Mizani, Aminlari & Khodabandeh 2005).

Alcalase, which is known for its broad specificity, has also been used to hydrolyse head waste from several other fish species including herring (Sathivel et al. 2003), sardine (Barkia et al. 2010; Souissi et al. 2007), and tuna (Yang et al. 2011). Gbogouri et al. (2004) optimized the use of Alcalase using response surface methodology to digest salmon heads and studied functional properties of resulted hydrolysate. They reported that hydrolysates with high DH had the best solubility while emulsifying capacity, emulsion stability, and fat absorption capacities were better when DH was low. Similarly, Sathivel et al. (2005) reported increased solubility of red salmon head hydrolysate as DH increased.

Beside physicochemical properties, several authors were also reported the bioactivities of fish head protein hydrolysates. Ohta et al. (1997) investigated the antihypertensive activity of protein hydrolysates derived from chum salmon head by oral administration to spontaneously hypertensive rats (SHR), and found that isolated peptides Asp-Trp (IC_{50} = 13μM) was one of the strongest ACE inhibitory dipeptides ever reported in food protein hydrolysates. The antioxidant activities of tuna head protein hydrolysate (THPH) were evaluated by Yang et al. (2011). THPH showed high radical scavenging activities and oxidation inhibitory effect. Recently, Chi, Wang, Wang et al. (2015) isolated three antioxidant peptides, i.e. Trp-Glu-Gly-Pro-Lys (WEGPK), Gly-Pro-Pro (GPP), and Gly-Val-Pro-Leu-Thr (GVPLT), from protein hydrolysate of bluefin leatherjacket (*Navodon septentrionalis*) heads. They concluded that the molecular size of peptides and the present of hydrophobic and/or aromatic amino acids, such as Trp, Gly, Pro, Val, Leu, in their sequences could have contributed to the antioxidant activities of the three isolated peptides.

2.2.6 Fish bone and frame protein hydrolysate

Fish backbone and frame are major fractions of wastes from seafood processing industries (Gildberg, Arnesen & Carlehög 2002). Fish bones contain around 30% protein (Je et al. 2007) and have been reported to contain high level of calcium (Jung, Shahidi & Kim 2009; Malde et al. 2010). In addition, fish bones are promising alternative source of gelatin and collagen (Koli et al. 2012; Takeshi & Suzuki 2000).

In order to make value-added products from fish backbones and frames, several researchers attempted to prepare protein hydrolysates and analyzed their biochemical and functional properties. Gildberg, Arnesen and Carlehög (2002) evaluated the sensory attributes of cod (*Gadus morhua*) backbones hydrolysate digested with bacterial enzymes (Alcalase, Neutrase and Protamex) and trypsin. They found that best recoveries of protein hydrolysates

were obtained with the bacterial enzymes, but these hydrolysates were bitter than the hydrolysates obtained by tryptic digestion. Cod backbones hydrolysed using Protamex, however, had potential to enhance product stability by preventing oxidative deterioration and have radical scavenging activity showed due to the ability to scavenge lipid radicals. The present of gastrin/CCK- and CGRP-like peptides could make the cod hydrolysates useful for incorporation in functional foods (Šližytė et al. 2009).

Backbones of tuna were hydrolysed using various proteases (alcalase, α-chymotrypsin, neutrase, papain, pepsin and trypsin) for production of antioxidant peptides. Antioxidative peptide Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser (1519 Da) was isolated from peptic hydrolysate and reported to have no cytotoxic effect when tested on human lung fibroblast and human endothelial cells (Je et al. 2007). The peptic protein hydrolysate from flying fish, seela and ribbon backbones also showed high radical scavenging activities (Nazeer & Anila Kulandai 2012; Nazeer et al. 2011). Shabeena and Nazeer (2011) further purified flying fish backbone hydrolysate and identified Leu-Glu-Val-Lys-Pro (596.9 Da) as active peptide responsible for antioxidative activity. Frame hydrolysates extracted from various fish species have also showed high antioxidant activities (Fan et al. 2012; Je, Park & Kim 2005; Jun et al. 2004), immunomodulatory properties (Hou et al. 2012) and angiotensin I-converting enzyme inhibitory (Jung et al. 2006; Lee, Qian & Kim 2010).

2.2.7 Fish dark muscle protein hydrolysate

Fish have two types of muscle, i.e. white or light muscle and red or dark muscle (Fig. 2.3). White fish such as cod and haddock only have a small strip of dark muscle whereas fatty fish such as herring, tuna or mackerel have much larger portion of dark muscle (Murray &

Burt 2001). Fish dark muscles have limited use because they contain higher amount of lipid and pro-oxidant metal which make them more prone to oxidation and off-flavour (Bae et al. 2011). Interestingly, fish species with dark muscle make up to 40% of total fish catch in the world consist of fish species with dark muscles (Hultin & Kelleher 2000). Therefore, it is essential to produce value-added products from fish dark muscles.

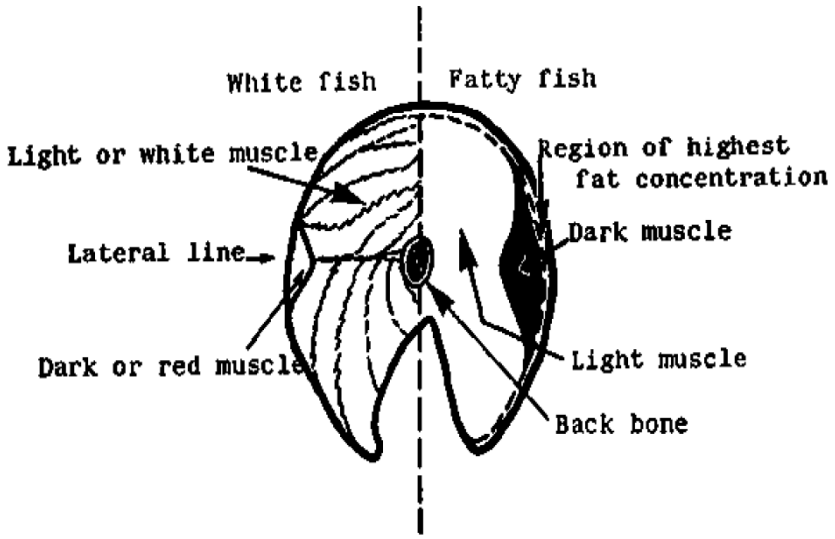

Figure 2.3 Structure of fish muscle (Murray & Burt 2001)

Fish species with large portion of dark muscles has become alternative raw material for surimi production (Chaijan et al. 2004) and the technology has been well studied (Hultin & Kelleher 2000). Dark muscles were also used to produce hydrolysates with certain bioactivities. Qian, Je and Kim (2007) prepared hydrolysate from tuna dark muscle using Alcalase, neutrase, pepsin, papain, α-chymotrypsin, and trypsin and tested the angiotensin I converting enzyme (ACE) inhibitory properties of resulted hydrolysates. The pepsin-derived hydrolysate exhibited the highest ACE-I inhibitory activity and the peptide was identified to be Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro (1581 Da; IC_{50} = 21.6 μM). The same enzymes were employed to hydrolyse tuna dark muscle and antioxidative peptide

Leu-Asn-Leu-Pro-Thr-Ala-Val-Tyr-Met-Val-Thr (MW 1222 Da) was isolated (Je et al. 2008).

In order to enhance the bioactivities of isolated peptides, hydrolysate from tuna dark muscle was fractionated using membrane separation technology (Saidi et al. 2014a; Saidi et al. 2014b; Saidi et al. 2013). The membrane fractionation influenced the molecular weight distribution of the peptide fractions and their bioactivities. Low molecular size peptide fractions that contained amino acids such as Tyr, Phe, Pro, Ala, His, and Leu showed better radical scavenging activities than the larger size ones. The result was in agreement with Hsu (2010) who isolated two antioxidative peptides Leu–Pro–Thr–Ser–Glu–Ala–Ala–Lys–Tyr (978 Da) and Pro–Met–Asp–Tyr–Met–Val–Thr (756 Da); and two antiproliferative peptides Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr (1206 Da) and Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr (1124 Da) from tuna dark muscle hydrolysates (Hsu, Li-Chan & Jao 2011). These results show that tuna dark muscle hydrolysates may be useful ingredients in food and nutraceutical applications.

2.3 Production of fish protein hydrolysate

Fish protein hydrolysate can be produced by hydrolysing fish muscle or body parts using chemicals (acid or alkaline), or biochemical (microbial enzymes, digestive enzymes) added at appropriate levels in controlled systems (Ovissipour et al. 2012). Fish protein can either be hydrolysed in sequence releasing one peptide at a time, or through the formation of intermediates that are further hydrolysed to smaller peptides. The latter is called ‘zipper mechanism’ (Sila et al. 2013). The properties of peptides liberated by FPH are highly dependent on the type of proteases or chemicals, pH, temperature and time implemented during hydrolysis (Mendis, Rajapakse & Kim 2005; Nazeer & Anila Kulandai 2012; See, Hoo & Babji 2011).

2.3.1 Chemical hydrolysis

Hydrolysis of fish protein using chemicals is widely used for the rapid transformation of fish waste or by-products. Fish proteins are broken down into variable sizes of peptides under either acidic (Gao et al. 2006) or alkaline conditions (Batista 1999). Chemical hydrolysis is a relatively inexpensive process, characterized by the use of high temperature, extreme pH and strong chemicals (Kristinsson & Rasco 2000a). In spite of some advantages including high recovery yields and fast process, chemical hydrolysis may decrease protein functionality, and generate bitter hydrolysates with heterogeneous quality (Sanmartín et al. 2009). These characteristics may limit the use of the final products as food ingredients and nutraceutical products.

2.3.1.1 Acid and alkali hydrolysis

Early method for acid hydrolysis involved boiling the protein with 6 N sulphuric acid (H_2SO_4) for 18 to 24 hours. Since there was complexity of removing H_2SO_4 after hydrolysis, hydrochloric acid (HCl) was used instead which had the advantage of readily removed from the hydrolysis mixture under vacuum conditions (Pickering & Newton 1990). A disadvantage of this process however, may be due to contamination, incomplete hydrolysis and degradation of the product. Furthermore destruction of tryptophan, an essential amino acid, is another major drawback of acid hydrolysis (Kristinsson & Rasco 2000a; Pickering & Newton 1990). Consequently, scientists continued to develop advance methods for acid hydrolysis by: 1) introducing vapour-phase hydrolysis system to counter contamination; 2) addition of protectant agents to suppress degradation 3) use of microwave to generate high temperature to accelerate and ensure complete hydrolysis; and 4) the use of a microcapillary tube for the recovery of tryptophan (Adebiyi et al. 2005; Pickering & Newton 1990; Tsugita et al. 1987).

The use of alkali, either sodium or potassium hydroxides, for hydrolysis was introduced by Amos Herbet through his US patent in 1888 (Jones 2010). The implementation of high temperature, ranged from 100ºC to 180ºC or higher, was intended for rapid dissolution and hydrolysis of protein into small peptides and amino acids in the form of sodium or potassium salt. According to Kaye, Weber and Wetzel (2004), alkaline hydrolysis breaks nearly 40% of all peptide bonds in protein. The majority of the products of this process are single amino acids or small peptides in the 2-5 residue range (nearly 98% of hydrolysate) (Kaye, Weber & Wetzel 2004). Several adverse reactions may occur during hydrolysis, initiated by hydrogen abstraction from the alpha carbon of an amino acid and racemisation of L-amino acid into D-amino acids, which are not absorbed by humans (Kinsella & Melachouris 1976).

2.3.1.2 pH-adjustment method

In order to improve the yield and functional properties of fish protein isolate, pH adjustment method was developed in late 1990's by Hultin and Kelleher (1999) at University of Massachussetts Marine Station, USA. The process, also known as pH-shift or isoelectric/precipitation method, involves the use of high and low pH followed by isoelectric precipitation. Essentially, by treating fish protein with pH above or below its isoelectric point (pI), the yield of soluble protein will increase. Since the method was introduced, several authors reported higher protein yields and lower level of membrane lipids in recovered materials compared to traditional surimi production (Batista, Pires & Nelhas 2007; Kristinsson & Liang 2006; Kristinsson & Hultin 2003). In addition, this method has been shown to be a feasible process for recovering valuable fish oil (Jaczynski 2008; Nurdiani et al. 2015; Okada & Morrissey 2007).

Despite the fact that pH adjustment method was aimed at application on fish muscles, several studies have shown that the method was also applicable for the recovery of proteins from fish by-products (Batista et al. 2006; Bechtel, Sathivel & Oliveira 2005; Bechtel,

Sathivel & Oliveira 2006). The yield of protein recovered varied among fish species and pH used (Chen & Jaczynski 2007). Further study by Chen, Tou and Jaczynski (2007) showed that isoelectric solubilisation/precipitation method allowed recovery of high quality protein and lipids suitable for human consumption.

2.3.2 Biochemical hydrolysis

Biochemical hydrolysis to produce fish protein hydrolysate can be performed by using commercial proteases or utilizing proteolytic enzymes present in fish muscle or viscera (Kristinsson & Rasco 2000a). Diniz and Martin (1997) reported the advantages of enzymatic hydrolysis to include: a) the possibility to control the characteristics of end product; b) to conduct under mild conditions avoiding extreme pH and temperature fluctuation which may affect the quality of FPH; c) the unnecessary removal of enzyme which is deactivated by heating; d) end products possess attractive functional properties; and e) no destruction of amino acids. Also the enzyme hydrolyses specific bonds result in consistent FPH products (Berg, Tymoczko & Stryer 2002).

Enzymes from plant, animal, and microbial origins have been used for the hydrolysis of fish. There are two kinds of proteolytic enzymes employed for FPH production, namely endogenous enzymes and commercial proteases. Autolysis using endogenous enzymes from Pacific hake (*Merluccius productus*), boosted by *Kudoa paniformis* parasitic infection, for FPH production has been reported by Samaranayaka and Li-Chan (2008). In addition, endogenous enzymes obtained from viscera (Aspmo, Horn & Eijsink 2005; Shahidi, Han & Synowiecki 1995) and hepatopancrease (Balti, Nedjar-Arroume et al. 2010) have shown significant activities. Recently, Ahmed et al. (2013), investigate the activity of endogenous protease from four Australian fish species in correlation with storage conditions. It was

suggested that the activity of calpain and cathepsin, two main endogenous proteases found in fish muscle, were significantly affected by temperature and pH during storage.

Enzymes of microbial origin used in FPH production offered some advantages including a wide variety of available catalytic activities, greater pH and temperature stabilities (Diniz & Martin 1997). Several researchers have utilized enzymes produced from *Bacillus* and *Aspergillus* strains for the production of FPH with potential bioactivities (Benjakul & Morrissey 1997; Ovissipour et al. 2012; Qian, Je & Kim 2007; Thiansilakul, Benjakul & Shahidi 2007). *Aspergillus niger* var. are known to produce acid and semi-alkaline proteases (Barthomeuf, Pourrat & Pourrat 1988; Jarai & Buxton 1994; O'Donnell et al. 2001; Pourrat et al. 1988); nevertheless, reports on the use of protease from *A. niger* for FPH production were limited (Nurdiani et al. 2016a). Alcalase, pepsin, papain, trypsin, chemotrypsin, pancreatin, bromelain, neutrase and Flavourzyme are among commercial proteases that have been widely used for the production of fish hydrolysate with potential bioactivities (Table 2.2). Proteases are categorized according to the sources and the optimum conditions required for enzyme activity.

2.4 Degree of Hydrolysis

Degree of hydrolysis (DH) may be defined as the number of broken peptide bonds in a protein hydrolysate (Rutherfurd 2010). It measures the extent of hydrolysis degradation of protein and is the most widely used indicator to compare hydrolysis rate of different protein hydrolysates (Bougatef et al. 2008). Since there is no standard method in determining degree of hydrolysis of fish hydrolysate, various techniques have been implemented by several authors (Table 2.3).

Table 2.2 Documented commercial proteases used for the conversion of fish by-products into FPH

Enzyme	Sources	pH range	Temperature range (ºC)	Bioactivities of FPH	References
Alcalase	*Bacillus licheniformis*	6 - 8	50 – 70	Antioxidant	Ahn, Lee & Je (2010) Alemán, Giménez, Montero et al. (2011) Barkia et al. (2010) Intarasirisawat et al. (2012) Je et al. (2007) Je et al. (2009) Jun et al. (2004) Ngo et al. (2011) Wiriyaphan, Chitsomboon & Yongsawadigul (2012) Zhong et al. (2011)
Neutrase	*Bacillus subtilis*	7.0	50 – 55	Antioxidant	Ahn, Lee & Je (2010) Fan et al. (2012) Fu & Zhao (2013) Je et al. (2007)

					Je et al. (2009) Jia et al. (2010) Jun et al. (2004) Ngo et al. (2011) Wang et al. (2014) Zhong et al. (2011)
Pronase	*Streptomyces griseus*	8.0	50	Antioxidant	Jun et al. (2004) Kim et al. (2001)
Orientase	*Bacillus subtilis*	7.0	50	Antioxidant	Hsu (2010) Hsu, Lu & Jao (2009)
Protease XXIII	*Aspergillus melleus*	7.5	37	Anticancer, Antioxidant	Hsu (2010) Hsu, Li-Chan & Jao (2011)
Flavourzyme	*Aspergillus oryzae*	7	50	Antioxidant	Ahn, Lee & Je (2010) Fan et al. (2012) Je et al. (2009) Zhong et al. (2011) Zhuang, Li & Zhao (2009)
Protamex	*Bacillus* protease complex	5.5 7.5	35 – 60	Antioxidant	Ahn, Lee & Je (2010) Alemán, Pérez-Santín, Bordenave-Juchereau et al. (2011)

					Je et al. (2009) Šližytė et al. (2009) Zhong et al. (2011)
Collagenase	*Clostridium histolyticum*	7.5 - 7.6	37	Antioxidant	Alemán, Giménez, Montero et al. (2011) Kim et al. (2001)
Pepsin	Bovine/porcine	2 - 2.2	37 - 40	Antioxidant, immunomodulator antiproliferative, antimicrobial	Alemán, Giménez, Montero et al. (2011) Fan et al. (2012) Hou et al. (2012) Je et al. (2007) Jun et al. (2004) Mendis et al. (2005) Mendis, Rajapakse & Kim (2005) Naqash & Nazeer (2011) Nazeer & Anila Kulandai (2012) Nazeer et al. (2011) Ngo et al. (2011) Qin et al. (2011) Sampath Kumar, Nazeer & Jaiganesh (2011) Wiriyaphan, Chitsomboon & Yongsawadigul (2012)

					Zhong et al. (2011)
Trypsin	Bovine/porcine	7.5 - 8.5	37-- 50	Antioxidant, immunomodulator	Alemán, Giménez, Montero et al. (2011a) Fan et al. (2012) Hou et al. (2012) Je et al. (2007) Jun et al. (2004) Mendis et al. (2005) Mendis, Rajapakse & Kim (2005) Naqash & Nazeer (2011) Nazeer & Anila Kulandai (2012) Nazeer et al. (2011) Ngo et al. (2011) Qin et al. (2011) Sampath Kumar, Nazeer & Jaiganesh (2011) Wiriyaphan, Chitsomboon & Yongsawadigul (2012) Yang et al. (2008) Zhong et al. (2011)
α- chymotrypsin	Bovine/porcine	7.5-8	37 – 40	Antioxidant	Fan et al. (2012) Je et al. (2007)

					Jun et al. (2004) Mendis et al. (2005) Mendis, Rajapakse & Kim (2005) Nazeer & Anila Kulandai (2012) Ngo et al. (2011) Sampath Kumar, Nazeer & Jaiganesh (2011)
Pancreatin	Bovine/porcine	7 – 9	37	Anticancer, Antioxidant	Kannan et al. (2011) Yang et al. (2008)
Bromelain	Pineapple	5 – 8	45 – 60	Antioxidant	Yang et al. (2008) Hou et al. (2012a)
Papain	Papaya	5 – 9	40 – 80	Antioxidant, antiproliferative, antimicrobial	Fan et al. (2012) Je et al. (2007) Jun et al. (2004) Naqash & Nazeer (2011) Nazeer & Anila Kulandai (2012) Nazeer et al. (2011) Ngo et al. (2011) Qin et al. (2011) Yang et al. (2008) Zhong et al. (2011)

One of the earliest methods used is the pH-stat method introduced by Jacobsen et al. (2006). Where the pH-stat method is not suitable, DH may be determined using soluble-nitrogen tricholoacetic acid (SN-TCA) method (Kristinsson & Rasco 2000a). Another well-known method used for DH estimation is the trinitro-benzene-sulfonic acid (TNBS). This method was developed by Satake et al. (1960) but most researcher consult modified TNBS method proposed by Adler-Nissen (1979).

Formol titration method has also been used to estimate DH by measuring titration reaction between amino acids and formaldehyde in the presence of sodium or potassium hydroxide. It was observed that the increasing number of free amino acid was associated with increasing DH of hydrolysate (Nilsang et al. 2005). The presence of free amino acids can be traced also by a colorimetric method using ninhydrin and o-phtalaldehyde (OPA) method (Nielsen, Petersen & Dambmann 2001; Roth 1971; Roth & Hampaĭ 1973). The OPA method, however, was known to be a more sensitive test for amino acid in determining DH (Roth 1971).

The DH of protein hydrolysate is influenced by several factors, including duration of hydrolysis and temperature (Ovissipour et al. 2009), enzyme type and concentration (Batista et al. 2010; Gbogouri et al. 2004; Jun et al. 2004), and substrate/buffer ratio (Benjakul & Morrissey 1997). Generally, increasing incubation time will increase the DH. Persian sturgeon viscera hydrolysed with Alcalase 2.4L showed increasing DH as incubation time increased (Ovissipour et al. 2009). Several studies however, have shown that the rate of hydrolysis decreases after 15 to 60 minutes of hydrolysis (Bougatef et al. 2008; Nasri et al. 2013a). The rate of hydrolysis of Sardinella by-products was high for the first 60 minutes before it subsequently decreased to steady state (Bougatef et al. 2008). Similarly, hydrolysis of goby muscle protein occurred at a higher rate for the first 30 minutes and slowed down afterward (Nasri et al. 2013a). Typical hydrolysis curves were also reported for capelin

(Shahidi, Han & Synowiecki 1995), Pacific whiting waste (Benjakul & Morrissey 1997), salmon waste (Gbogouri et al. 2004), yellow strip trevally (Klompong et al. 2007), herring (Liceaga-Gesualdo & Li-Chan 1999), sardine (Ben Khaled et al. 2011; Quaglia & Orban 1987), goby fish (Nasri et al. 2012), toothed pony fish (Klomklao, Benjakul & Kishimura 2013), silver carp (Dong et al. 2008) and zebra blenny (Ktari et al. 2012). The reduction rate of hydrolysis might have resulted from exhaustion of substrate, enzyme inhibition and enzyme deactivation or autolysis (Guerard, Guimas & Binet 2002).

Increasing reaction temperature has been reported to increase DH significantly and has been shown to relate to the optimum temperature of the enzyme used (Ovissipour et al. 2009). Benjakul and Morrissey (1997) observed high Alcalase activity at 55-70°C and a considerable decrease in the hydrolysis rate was observed at above 70°C. Furthermore, the authors observed that waste:buffer ratio significantly affected hydrolysis rate and nitrogen recovery ($p<0.05$). As mentioned earlier, enzyme shows specificity in cleaving peptide bonds. Several studies showed that enzyme type and concentration also have great influence on the DH (Aspmo, Horn & Eijsink 2005; Baek & Cadwallader 1995; Barkia et al. 2010; Benjakul & Morrissey 1997; Jun et al. 2004; Raghavan & Kristinsson 2008).

DH determination is important in relation to bioactivity and functional properties of fish protein hydrolysates and it significantly influences antioxidant activity of fish protein hydrolysates, including the ones extracted from marine fish skin gelatin (Alemán, Giménez, Montero et al. 2011), scad muscle (Thiansilakul, Benjakul & Shahidi 2007), alkali-treated tilapia (Raghavan & Kristinsson 2008), black scabbardfish (Batista et al. 2010), mackerel (Wu, Chen & Shiau 2003), and yellow stripe trevally (Klompong et al. 2007). DH of protein hydrolysates has also been known to be associated with other bioactivity such as ACE inhibition (Balti, Bougatef et al. 2010; Bougatef et al. 2008; Cao et al. 2010; Nasri et al. 2013b; Nasri et al. 2014). However, no correlation between DH of fish hydrolysates and

antiproliferative activity was observed in studies by Hsu, Li-Chan and Jao (2011) and Picot et al. (2006).

On the other hand, varying results have been reported on the influence of DH on functional properties of fish protein hydrolysates as specified below. Liceaga-Gesualdo and Li-Chan (1999) stated that herring protein hydrolysates have excellent solubility with a high degree of hydrolysis. Balti, Bougatef et al. (2010) reported that solubility, emulsifying and water-holding capacities of cuttlefish hydrolysate increased while foaming capacity decreased with increasing DH. Similarly, Klompong et al. (2007) observed that higher DH resulted in higher solubility yet lower emulsion activity index, emulsion stability index, foaming capacity, and foam stability. Since excessive hydrolysis has adverse effect on particular functional property (Kristinsson & Rasco 2000a), a controlled hydrolysis reaction is necessary.

The effect of DH on bioactivity and functional properties of fish protein hydrolysates is likely related to the molecular weight as well as the amino acid composition of resulting peptides (Faithong et al. 2010; Hou 2012; Wu, Chen & Shiau 2003). Thiansilakul, Benjakul and Shahidi (2007) reported that DH greatly influences the peptide chain length as well as the exposure of the terminal amino groups of products obtained. Low molecular weight peptides from smooth hound (Bougatef et al. 2009), mackerel (Wu, Chen & Shiau 2003), and Alaska Pollack frame (Je, Park & Kim 2005) showed high antioxidant activities. High solubility of fish egg protein hydrolysate was reported to be due to its molecular size reduction, formation of smaller and more hydrophilic peptide units (Chalamaiah et al. 2010). Smaller peptides possess more polar residues with ability to form hydrogen bonds with water and thus increase solubility (Gbogouri et al. 2004). The presence of low molecular weight peptides of fish hydrolysates, however has less gelling and emulsifying properties (Eysturskarð et al. 2010;

Liu et al. 2010; Quaglia & Orban 1990). For peptides to have good emulsifying properties, peptide length should have a minimum >20 residue (Lee et al. 1987).

Table 2.3 Methods for determining degree of hydrolysis of FPH

Methods	Principles	Advantages and/or Problems	Fish species	References
pH-stat	Monitors the DH by adding a base to keep the pH constant during the hydrolysis. The amount of base consumed is related to the number of peptide bonds hydrolysed and then used in an equation to estimate the DH.	Problems: Limitation of using this method for pH condition higher than around Excessive amount of base may be undesirable in the end product	Trevally Yellowfin tuna Atlantic salmon Pacific whiting Shark muscle Cuttlefish Tuna, Halibut, Jumbo flying squid	Klompong et al. (2007) Guerard et al. (2001) Kristinsson & Rasco (2000b); Kristinsson & Rasco (2000c) Benjakul & Morrissey (1997) Diniz & Martin (1998) Balti, Bougatef et al. (2010) Alemán, Giménez, Montero et al. (2011)
SN-TCA	By calculating the ratio of percent 10% TCA soluble nitrogen in the hydrolysate compared to the total amount of protein in sample.	Advantage: This method is simple and useful when working within the pH of 3 to 7 range	Herring Persian sturgeon Tuna, Jumbo flying squid	Hoyle & Merritt (1994) Ovissipour et al. (2012) Alemán, Giménez, Montero et al. (2011)

TNBS	Based on reaction of TNBS with N-terminal group or primary amines at slightly alkaline conditions.	Advantage: Short reaction time and simple spectrophotometric method Problem: The presence of insoluble proteinaceous material can be problematic for spectrophotometric analysis.	Pacific whiting Cod Shrimp	Benjakul & Morrissey (1997) Himonides, Taylor & Morris (2011) Huang, Ren & Jiang (2011); Huang, Zhao & Jiang (2011)
Formol titration	DH is calculated from ratio of a-amino nitrogen (AN) and total nitrogen (TN).	Advantage: The increasing number of free amino acid was associated with increasing DH of hydrolysate (depend on enzyme used).	Cod	Šližytė et al. (2005)
Colometric (using ninhydrin)	Based on the production of intense colors which serve as the basis of numerous analytical procedures, both qualitative and quantitative	Problems: Less sensitive than OPA	Alaskan Pollock	Hou (2012)

OPA	Based on the reaction between amino acid groups with OPA in the presence of beta-mercaptoethanol forming a coloured compound at 340 nm using spectrophotometer.	Problems: OPA reagent does not react or react poorly with proline and cysteine.	Black scabbardfish	Batista. et al. (2010)

2.5 Purification of bioactive peptides derived from fish protein hydrolysate

Since most biologically active peptides occur naturally in relatively low concentrations, they may not be concentrated enough to produce beneficial effects *in vivo*. In addition, the present of organic solvents with strong acids or alkaline may denature and inactivate desired peptides (Kristinsson & Rasco, 2000a). Therefore, it is essential to develop appropriate technologies to concentrate and enrich bioactive peptides in active forms (Pihlanto & Korhonen 2003). Membrane separation techniques are now widely applied to purify, concentrate and separate bioactive peptides from unhydrolyzed proteins or any other unwanted materials (Bourseau et al. 2009). It involves the use of pressure to force a mixture through a porous membrane, hence the name pressure-driven membrane-based separation (Pihlanto & Korhonen 2003). Microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) can be used after hydrolysis to increase the specific activities of FPH (Amado et al. 2009, 2013; Chabeaud et al. 2009; Shahidi & Zhong 2008). Combination of serial enzymatic hydrolysis and multistep recycling membrane reactor technology is being considered as a potential method for the generation of bioactive peptides with neutraceutical value (Kim & Wijesekara 2013).

Generally, fish-derived bioactive peptides are separated into fractions using membranes in the range 1–50 kDa (Chi, et al. 2015; Ding et al. 2011; Raghavan & Kristinsson 2009; Taheri et al. 2014). Fractions with the highest activity are then further purified to separate individual peptides using gel permeation chromatography and reverse performance liquid chromatography (RP-HPLC) (Ko et al. 2013; Liu et al. 2010). In order to identify the individual peptide fractions, the combine techniques of mass spectrometry including Matrix Assisted Laser Deionisation Time of Flight (MALDI–TOF), ESI (electrospray ionisation) and Edman degradation were used (Bernardini et al. 2011). A synthetic version of the peptide is then synthesized and the assay is repeated to verify bioactivity (Ryan et al. 2011). Procedure

for the isolation and identification of bioactive peptides from fish by products is presented in Fig 2.4.

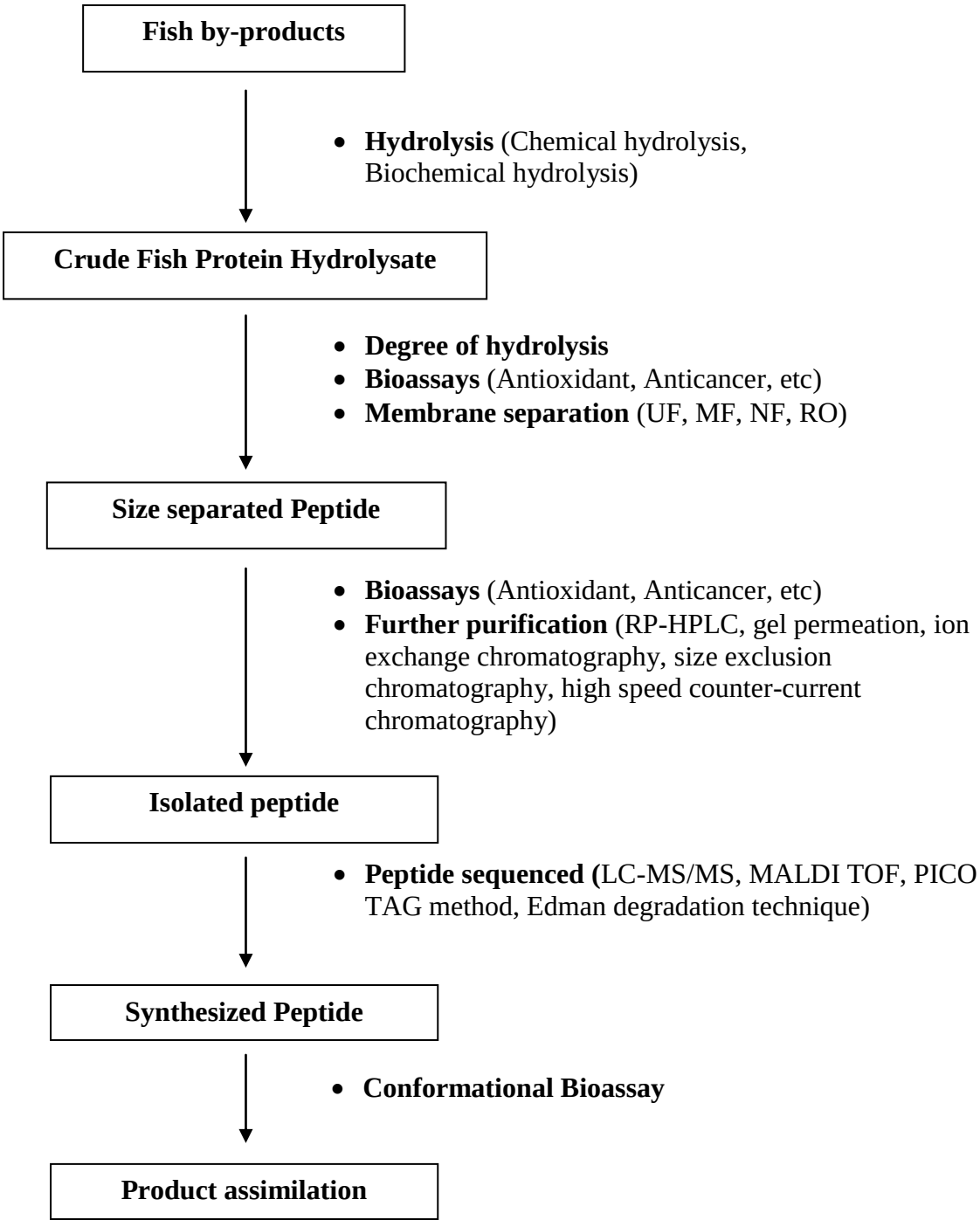

Figure 2.4 Procedure for the isolation and identification of bioactive peptides from fish-by-products (adapted from Ryan et al., 2011, Bernardini et al., 2011).

2.6 Functional properties of FPH derived from fish by-products

Functional properties of FPH are particularly important if they are used as ingredients in food products. Functional properties of food proteins are defined as "those functional and chemical properties which affect the behaviour of proteins in food systems during processing, storage, preparation and consumption" (Kinsella & Melachouris 1976). The physical and chemical properties of food that determine protein functionality include size, shape, amino acid composition and sequence, hydrophobicity/hydrophilicity ratio, peptide structure, molecular flexibility/rigidity, net charge and distribution of charges, and the ability to interact with other food components (Damodaran 2008). Solubility and emulsifying properties are among the most reported functional properties exhibited by FPH. Reports on heat stability, rheological and sensory properties of FPH, on the other hand, are limited. .

2.6.1 Solubility

Solubility can be considered as the most important functional properties of FPH as other properties such as foaming and emulsification are affected by solubility (Wilding, Lillford & Regenstein 1984). High solubility is a desirable attribute of FPH as low solubility may cause a sandy mouth feel and unattractive final products (Petersen 1981). The solubility of FPH is determined by interaction of hydrophobic and/or ionic groups. While hydrophobic interaction may decrease solubility, ionic interaction promotes solubility. Hydrolysis process enhances solubility by converting hydrophobic groups to hydrophilic groups (by generating two end carbonyl and amino groups) although it also exposes some of the hydrophobic groups to the surface. Smaller peptides generated after hydrolysis have more polar residues with increased ability to form hydrogen bonds with water (Kristinsson & Rasco 2000a). This explains why

increased degree of hydrolysis result in increased solubility of FPH. However, extensive hydrolysis could have negative effects on other functional properties. Influence of degree of hydrolysis on the solubility of FPH have been investigated and reported (Balti, Bougatef et al. 2010; Foh et al. 2010; Gbogouri et al. 2004)

Solubility of FPH is also affected by the p*I* of fish protein. As the charges on a protein's surface diminish, so do the protein-water interaction. Chen and Jaczynski (2007) investigated the effect of pH and ionic strength (IS) on the solubility of rainbow trout FPH. The solubility was minimum at pH 5.5. However when IS = 0.2, the minimum solubility shifted toward more acidic pH (Fig 2.5.). Similarly, tilapia hydrolysate solubility also increased rapidly at acidic (pH<3) and basic (pH>6) and lowest at its p*I* (Foh et al. 2011).

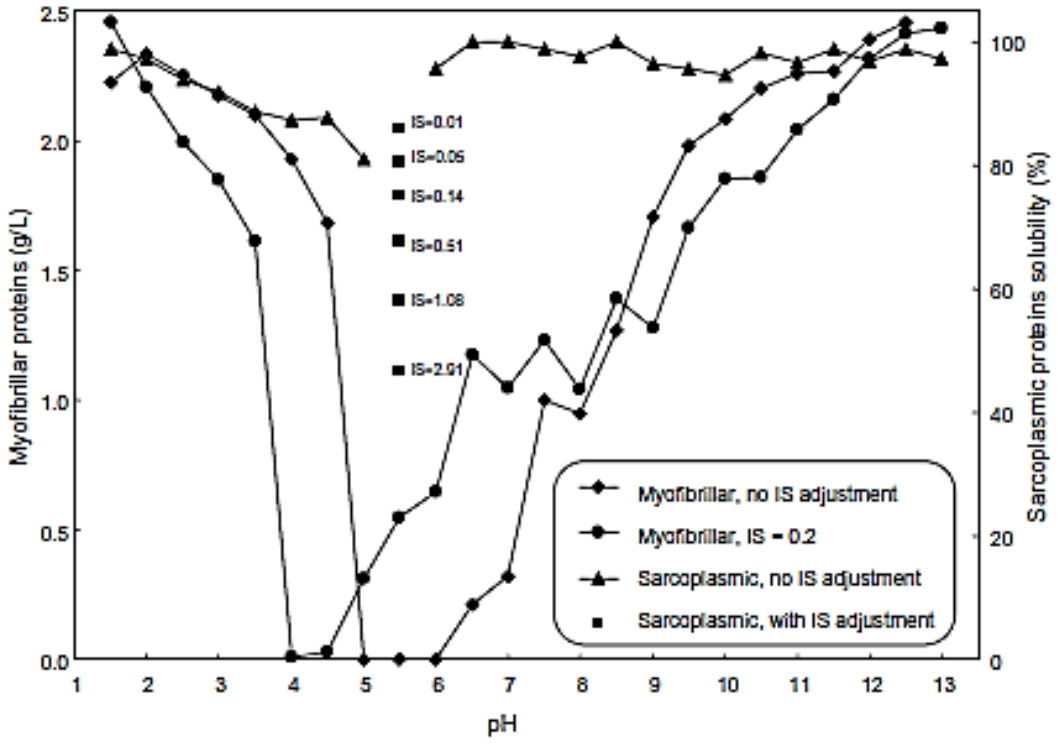

Figure 2.5 Solubility of myofibrillar and sarcoplasmic proteins as a function of pH and ionic strength (IS) (Gehring et al. 2011)

2.6.2 Emulsifying properties

Emulsifying properties of FPH is dependent on how effective the hydrolysate lowers the interfacial tensions between hydrophobic and hydrophilic components in food (Kristinsson & Rasco 2000a). Like solubility, the emulsifying properties are influenced by the extent of hydrolysis. Extensive hydrolysis is not recommended as low molecular peptides with had low amphiphilicity, which is required for producing an emulsion. Small peptides are less efficient in reducing the interfacial tension because they may not readily agglomerate to produce a fat-globule membrane due to charge repulsions (Turgeon, Gauthier & Paquin 1991). Lee et al. (1987) stated that protein hydrolysates have to be composed of a minimum length of 20 amino acids in order to possess a good emulsifying capacity. Apart from molecular size of the peptides, Kato et al. (1985) suggested that the flexibility of protein or peptide structure may also be a vital factor governing the emulsifying properties.

Low emulsifying capacity was reported for sardine protein hydrolysate (Quaglia & Orban 1990), salmon protein hydrolysate (Gbogouri et al. 2004), and yellow stripe trevally (Klompong et al. 2007) as DH increased. In contrary, cuttlefish by-product hydrolysate with the lowest DH 5% exhibited lower emulsifying capacity than the other hydrolysates (DH 10% and DH 13.5%) even though it contained more hydrophobic and larger peptides (Balti, Bougatef et al. 2010). The medium molecular weight size liberated during limited hydrolysis, at DH 10% and DH 13.5%, might have enhanced the flexibility of the peptides at the oil/water interface, resulting in a larger surface area and, consequently, greater emulsion formation.

In a recent review, Halim, Yusof and Sarbon (2016) reported the emulsifying activity and stability of FPHs, extracted from various fish species, were highly vary from 25.16 to 270 m^2/g and 0.144 to 130%, respectively. Several factors contributing to the properties are

solubility, molecular size, amino acid sequence of the peptide, degree of hydrolysis, acetylation of the peptide, types of proteases used and the extraction solvent. The pH of the solution during hydrolysis also affected emulsifying properties. The pH range 6-10 produced the highest emulsifying activity index (EAI) while pH 4 produced the lowest (Taheri et al. 2013).

2.6.3 Heat stability

Heat stability is rarely reported as functional properties of FPH. Instead, several papers discussed the influence of heat on fish or shrimp muscles for its implication during processing (Huang & Ochiai 2005; Sriket et al. 2007; Thanonkaew, Benjakul & Visessanguan 2006). Heat has a major influence on myofibrillar proteins (myosin and actin), connective tissue proteins and sarcoplasmic proteins (Kong et al. 2008). Application of heat may cause denaturation, dissociation of myofibrillar proteins, transversal and longitudinal shrinkage of muscle fibre (Kong et al. 2007). Since heat stability indicates good ability of FPH to withstand heat during high temperature processing or food formulation, research on the effect of heat on the quality of FPH is encouraged.

Recently, Nurdiani et al. (2016a) studied the heat stability of freeze dried FPH extracted from by-products of 4 Australian fish species. Heat stability of fish protein was species specific. Relatively low heat stability of Barramundi and Silver warehou hydrolysates might be affected by the amount of myosin heavy chains, which are more unstable and easily denatured. Poulter et al. (1985) stated that environment temperature also influenced the heat stability of fish protein. Fish live in the waters with higher ambient temperatures showed more stable proteins.

Chapter 2B. Review Article: Bioactive peptides from fish by-products with anticarcinogenic potential

The paper entitled "**Bioactive peptides from fish by-products with anticarcinogenic potential**" by Nurdiani, R., Vasiljevic, T., Singh, T.K. and Donkor, O.N. has been accepted for publication in *International Food Research Journal* (Manuscript ID: IFRJ16198.R2).

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Declaration by [candidate name]: Rahmi Nurdiani

Signature: [redacted]

Date: 20/05/2016

Paper Title:
Bioactive peptides from fish by-products with anticarcinogenic potential

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

Name	Contribution %	Nature of Contribution
Rahmi Nurdiani	80%	Idea, hypothesis, conception, execution
Todor Vasiljevic	7 %	Critical appraisal, conception
Tanoj K. Singh	5%	Critical appraisal, conception
Osaana N. Donkor	7%	Critical appraisal, conception and submission to Journal

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Bioactive peptides from fish by-products with anticarcinogenic potential by *R. Nurdiani, T. Vasiljevic, T.K. Singh, and O.N. Donkor* was published in the peer review journal, *International Food Research Journal, 24/5, 1840-1849,2017*.

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CHAPTER 3

Chapter 3. Sustainable use of marine resources – turning waste into food ingredients

The paper entitled "**Sustainable use of marine resources – turning waste into food ingredients**" by Nurdiani, R, Dissanayake, M., Street, W. E., Donkor, O.N., Singh, T. K. and Vasiljevic, T. has been published in *International Journal of Food Science and Technology* 50: 2329–2339. doi:10.1111/ijfs.12897

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Paper Title:

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Muditha Dissanayake	4 %	Critical appraisal, execution
Wayne E. Street	2 %	Critical appraisal, conception
Osaana N. Donkor	6 %	Critical appraisal, conception
Tanoj K. Singh	4 %	Critical appraisal, conception
Todor Vasiljevic	6 %	Critical appraisal, conception and submission to Journal

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Signature 6		06/06/2016

Sustainable use of marine resources-turning waste into food ingredients vitro study of selected physiological and physicochemical properties of fish protein hydrolys by *R. Nurdiani, M. Dissanayake, W.E. Street, O.N. Donkor, T.K. Singh, and T. Vasiljevic* was published in the peer review journal, *International Journal of Food Science & Technology, 50/11, 2329-2339, 2015*.

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CHAPTER 4

Chapter 4. In vitro study of selected physiological and physicochemical properties of fish protein hydrolysates from 4 Australian fish species

The paper entitled "***In vitro* study of selected physiological and physicochemical properties of fish protein hydrolysates from 4 Australian fish species**" by Nurdiani, R, Dissanayake, M., Street, W. E., Donkor, O.N., Singh, T. K. and Vasiljevic, T. has been published in *International Food Research Journal*. IFRJ 23(5): 2042-2053.

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

Name	Contribution %	Nature of Contribution
Rahmi Nurdiani	78 %	Idea, research question, research design, results and discussion, conception, execution, interpretation
Muditha Dissanayake	5 %	Critical appraisal, execution
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In vitro study of selected physiological and physicochemical properties of fish protein hydrolysates from 4 Australian fish species by *R. Nurdiani, M. Dissanayake, W.E. Street, O.N. Donkor, T.K. Singh, and T. Vasiljevic* was published in the peer review journal, *International Food Research Journal, 23/5, 2029-2040, 2016*.

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CHAPTER 5

Chapter 5. Bioactive peptides with radical scavenging and cancer cell cytotoxic activities derived from Flathead (*Platycephalus fuscus*) by-products

The paper entitled **"Bioactive peptides with radical scavenging and cancer cell cytotoxic activities derived from Flathead (*Platycephalus fuscus*) by-products"** by Rahmi Nurdiani, Todor Vasiljevic, Thomas Yeager, Tanoj K. Singh and Osaana N. Donkor has been accepted for publication in *European Food Research Journal* (Manuscript ID: EFRT-16-0343.R1) DOI: 10.1007/s00217-016-2776-z

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Declaration by [candidate name]: Rahmi Nurdiani

Signature: [redacted]

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Paper Title:
Bioactive peptides with radical scavenging and cancer cell cytotoxic activities derived from Flathead (Platycephalus fuscus) by-products

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

Name	Contribution %	Nature of Contribution
Rahmi Nurdiani	80%	Idea, research question, research design, results and discussion, conception, execution, interpretation
Todor Vasiljevic	6%	Critical appraisal, conception
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Tanoj K. Singh	4%	Critical appraisal, conception
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ORIGINAL PAPER

Bioactive peptides with radical scavenging and cancer cell cytotoxic activities derived from Flathead (*Platycephalus fuscus*) by-products

Rahmi Nurdiani[1,2] · Todor Vasiljevic[1] · Thomas Yeager[3] · Tanoj K. Singh[4] · Osaana N. Donkor[1]

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Abstract Peptide fractions extracted from Flathead by-products were evaluated for in vitro free radical scavenging and cancer cell cytotoxic activities. The degree of hydrolysis (DH), presence of protease and molecular weight (MW) influenced the 2,2-diphenyl-1-pycryl-hydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic (ABTS) radical scavenging activities (RSA) of Flathead peptide fraction. Low MW peptides (<3 kDa), obtained from overnight incubation with added an exogenous protease, significantly inhibited free radicals and showed the highest RSA of 94.03 and 82.89 % against $DPPH^{\cdot}$ and $ABTS^{\cdot+}$, respectively. The presence of bioactive peptides during H_2O_2 exposure increased viability of T4056 normal cells. Furthermore, peptide fractions <3 kDa inhibited the growth of HT-29 colon cancer cells up to 91.04 %, although the activity was found to be non-selective. Further purification revealed a novel peptide, Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg, with RSA properties. These results indicated that peptides extracted from Flathead by-products have a potential to be used as natural antioxidants and/or chemo-protective agents.

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Keywords Flathead (*Platycephalus fuscus*) · Protein hydrolysate · Peptide · Fraction · Radical scavenging activity (RSA) · Cancer cell cytotoxic activity

Abbreviations

ABTS	2,2-Azino-bis-3-ethylbenzothiazoline-6-sulphonic
AFP	Acid fungal protease
DH	Degree of hydrolysis
DPPH	2,2-Diphenyl-1-pycryl-hydrazyl
FPH	Fish protein hydrolysate
MW	Molecular weight
MWCO	Molecular weight cut off
ON	Incubated overnight without the addition of AFP
ONE	Incubated overnight with the addition of AFP
ROS	Reactive oxygen species
RSA	Radical scavenging activity
UF	Unfractionated
UH	Unhydrolysed/Control

Introduction

Formation of free radicals is an inevitable consequence during respiration. As these radicals are very unstable and react rapidly with other groups or substances in the body, they may cause cell or tissue injury [1]. Reactive oxygen species (ROS), such as hydroxyl radicals and hydrogen peroxide (H_2O_2), can act as both initiators and promoters of tumours by damaging cellular macromolecules such as DNA, proteins and lipids and by acting as cell-signalling molecules (as nitric oxide does) [2]. In addition, H_2O_2 can increase cancer cell proliferation and migration leading to metastasis [3]. Although the human body has innate defence mechanisms to counter ROS in the form of

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antioxidant enzymes, consumption of food with antioxidant properties may strengthen the antioxidant balance of the body [4].

There is increasing evidence that proteins are prone to oxidation and free radicals [5]. Nevertheless, the ability of proteins to interact with free radicals could also lead to the development of natural sources of antioxidants [6]. Numerous studies have revealed potential antioxidant activities of peptides derived from aquatic species [7, 8]. Recent evidence suggests that fish by-products can also be used as valuable sources of bioactive peptides with antioxidant and anticancer activities [9, 10]. Bioactive peptides are commonly incorporated into nutraceutical products by transforming them into active form after gastrointestinal digestion. The peptides are then absorbed by the intestine and transported by the circulatory system to reach the target site and exert bioactivities [11].

Free radical scavenging activity and mechanism of proteins are dependent on amino acid composition. Activity is suppressed if the free radical scavenging amino acids are located in the protein interior and inaccessible to pro-oxidants [6]. One approach to increase the exposure of antioxidative amino acids in protein is by enzymatic hydrolysis. Enzymatic hydrolysis, conducted under controlled conditions, has produced fish protein hydrolysate (FPH) with potential free radical scavenging activities [12]. Exogenous enzymes are preferred over endogenous enzymes in producing antioxidative peptides, due to the consistency of peptide composition obtained and shorter hydrolysis time to obtain similar degree of hydrolysis [13]. Nevertheless, several studies have reported the use of various endogenous enzymes that produced potent antioxidative fish protein hydrolysates [14, 15].

The scavenging activity of hydrolysates is likely dependent on molecular weight distribution [1, 14]. Pihlanto and Korhonen [16] stated that in order to obtain more peptides with desired range of molecular weights as well as remove undigested polypeptides and an enzyme at the end of the process, various techniques involving reverse osmosis, ultra-filtration and diafiltration membrane with defined nominal molecular weight cut-offs (MWCO) are now industrially applied [17].

Flathead is a bottom-dwelling marine fish, mainly caught with gillnets or bottom trawlers [18]. In Australia, Flathead is sold as fillet while remaining body parts (skins, frames and heads) are treated as waste. Flathead has been used as bio indicator of environmental health [19], yet research on the utilization of Flathead by-products as a potential source of bioactive peptides is limited. Our previous study showed that Flathead protein hydrolysate exhibited high radical scavenging activity [20]. Therefore, in order to further investigate the potency of antioxidative peptides in Flathead hydrolysate, this study was set out to: (a) produce Flathead protein hydrolysate by employing endogenous and exogenous protease, using simple extraction method, (b) isolate different fractions of peptides from Flathead protein hydrolysate by means of centrifugal filter units, (c) examine radical scavenging properties based on different in vitro assays, (d) evaluate cancer cell cytotoxic effect of the fractions and (e) identify peptides possessing radical scavenging and cancer cell cytotoxic properties.

Materials and methods

Materials

Flathead (*Platycephalus fuscus*) by-products (heads, backbones and frames) were kindly provided by Barwon Foods, North Geelong, Victoria, Australia. By-products were collected after processing and kept in ice during transporting to the laboratory. In the laboratory, the samples were then immediately processed into FPH as described in sample preparation section below.

Food-grade acid fungal protease (AFP, activity 2000 SAPU/g), obtained by controlled fermentation of *Aspergillus niger* var (*A. niger*), was donated by Enzyme Solutions Pty. Ltd (Croydon South, VIC, Australia). 2,2-Diphenyl-1-pycryl-hydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic (ABTS) acid, trichloroacetic acid (TCA), trifluoroacetic acid (TFA) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Acetonitrile was purchased from Merck (Darmstadt, Germany). All other reagents were of the highest grade commercially available.

Preparation of Flathead protein hydrolysate

Heads, backbones and frames of Flathead were minced, without adding water, using a laboratory mincer (MG-22SS, Handy Imports, Smithfield, NSW, Australia). Approximately 300 g of minced sample was then mixed with an appropriate volume of 1 % sulphuric acid to adjust pH to 2.5 and obtained fish-to-water ratio of 1:1 [21]. Subsequently, the mixture was divided into three equal parts and processed as: (a) 0 h incubation and without enzyme addition (unhydrolysed/control) (UH), (b) overnight incubation (18 h) at room temperature without enzyme addition (ON); and (c) overnight incubation (18 h) at room temperature with the addition of AFP (ONE). AFP is a food-grade enzyme with an effective pH range from 2.5 to 3.5 and a temperature tolerance of up to 50 °C [21]. AFP, also known as aspergillopepsin I, has a broad specificity, which enables the enzyme to easily and efficiently hydrolyse most soluble proteins but generally favours hydrophobic residues in P1 and P1′ (http://www.brenda-enzymes.org/enzyme.

Table 1 Fractions of Flathead protein hydrolysate obtained after ultra-filtered centrifugation

Treatments	Fractions			
	Unfractionated (UF)	>10 kDa	Between 3 and 10 kDa	<3 kDa
Unhydrolysed/control (UH)	UH UF	UH > 10	UH 3–10 kDa	UH < 3 kDa
Incubated overnight without the addition of AFP (ON)	ON UF	ON > 10 kDa	ON 3–10 kDa	ON < 3 kDa
Incubated overnight with the addition of AFP (ONE)	ONE UF	ONE > 10 kDa	ONE 3–10 kDa	ONE < 3 kDa

php?ecno=3.4.23.18). The enzyme activity of the commercially prepared protease added was 50 SAPU/g of sample. According to the manufacture protocol, one Spectrophotometric Acid Protease Unit (SAPU) is the amount of the enzyme that liberates one micromole of tyrosine per minute under the condition of the assay.

After each treatment, the samples were centrifuged at 2055×*g* for 10 min at 20 °C using a Sorvall centrifuge (RT-H750 swing bucket rotor, RT model, DuPont Company, Newtown, CT, USA). Upon centrifugation, five distinct layers were formed in the tube [20]. For this study, only the soluble protein/peptide extract layers were used for analysis while other layers were discarded. 20 mL of the soluble proteins was immediately stored at −80 °C to be used for analysis of degree of hydrolysis (DH). The remaining soluble protein layers were ultra-filtered sequentially using an Amicon Ultra-15 Centrifugal Filter Devices (10 and 3 kDa MWCO) (Merck Millipore Ltd., Kilsyth, Victoria, Australia). The samples were centrifuged at 2055×*g* for 30 min at 10 °C using Sorvall centrifuge. Upon centrifugation, four fractions were obtained from each treatment, namely unfractionated sample (UF); fraction >10 kDa, proteins retained by the 10 kDa membrane; fraction between 3 and 10 kDa, oligopeptides permeating through the 10 kDa membrane but not the 3 kDa membrane; and a fraction below 3 kDa, peptides permeating through the 3 kDa membrane (Table 1). All 12 fractions were then stored at −80 °C and lyophilized to obtain FPH powders. The protein content (mg/mL) of each fraction was assayed using Bradford reagent according to Bradford [22] using bovine serum albumin (0.1–1.3 mg/mL) as standard.

Evaluation of degree of hydrolysis

The degree of hydrolysis (DH) of control and hydrolysed soluble protein/peptide extract was assessed based on a previously published method [23] with minor modifications. An aliquot of 2 mL of soluble extract was mixed with an equal volume of 20 % TCA followed by centrifugation at 8161×*g* for 20 min at room temperature (Eppendorf Centrifuge 5415C, Crown Scientific Pty Ltd, Moorebank, NSW, Australia). The supernatant was collected and degree of hydrolysis was established by determining the nitrogen content using Kjeldahl method [24] and expressed as:

$$\%\mathrm{DH} = \left(\frac{10\ \%\ \text{TCA soluble nitrogen in the sample}}{\text{Total nitrogen in the sample}}\right) \times\ 100\ \% \quad (1)$$

Peptide profiling by reversed-phase high-performance liquid chromatography (RP-HPLC)

The peptides in fish soluble extracts were profiled using RP-HPLC. Lyophilized sample (100 mg), dissolved in Milli Q water (1 mL), was centrifuged at 29,416×*g* using JLA-16.250 rotor in Avanti J-26S XPI High-Performance Centrifuge (Beckman Coulter Inc., Brea, CA) for 30 min at 4 °C and filtered through 0.22-µm membrane filter (Schleicher & Schuell GmbH, Germany) into HPLC sample vials. Peptides were separated on a Shimadzu HPLC system (Shimadzu Model LC-2030, Shimadzu Corporation, Kyoto, Japan) equipped with Vydac Everest C18 column (250 mm × 4.6 mm, particle size 5 µm, pore size 300 Å; Grace Davison Discovery Sciences, Rowville, VIC, Australia). The peptides were eluted by a linear gradient of 0–100 % of solvent B (0.1 % TFA in acetonitrile) in solvent A (0.1 % TFA in deionized water) over 90 min. The flow rate was maintained at 0.75 mL/min, and eluted peptides were detected at 215 nm. All solvents were filtered through a 0.45-µm membrane filter prior to use.

Radical scavenging activities of peptide fractions

DPPH radical scavenging assay

The scavenging effect of peptide fractions on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was measured according to previously described method [25] with some modifications. Peptide sample (0.04 mL; 0.01 mg protein/mL) was mixed with 0.05 mM DPPH dissolved in 95 % ethanol (2 mL). The mixture was then allowed to stand in the dark for 30 min. The mixtures were then clarified by centrifuging at 2055×*g* for 5 min (Sorvall centrifuge). The absorbance of the resulting solution was recorded at 517 nm (Libra S12 UV/Visible Spectrophotometer; Biochrom Ltd, Cambridge, United Kingdom). Milli Q water was used a blank. The scavenging effect was expressed as shown in the following equation:

$$\text{Scavenging activity } (\%) = \left[\frac{(\text{Blank absorbance} - \text{Sample absorbance})}{\text{Blank absorbance}}\right] \times 100 \quad (2)$$

$ABTS^{\cdot+}$ radical scavenging activity

The activity of peptides to scavenge $ABTS^{\cdot+}$ radicals was examined based on the modified method of Ozgen et al. [26]. Briefly, 0.5 mL of ABTS stock solution (7.4 mM ABTS in sodium acetate buffer, pH 4.5) was mixed with 0.5 mL of 2.6 mM potassium persulphate to obtain $ABTS^{\cdot+}$ working solution. This $ABTS^{\cdot+}$ solution was allowed to stand for 12–16 h at room temperature in the dark. Afterwards, the solution was further diluted with 20 mM acetate buffer to obtain an absorbance of 0.700 ± 0.02 at 734 nm.

Diluted samples (10 µl; 0.01 mg protein/mL) was added to 1 mL of $ABTS^{\cdot+}$ solution. The mixtures were then incubated at 30 °C for 6 min in the dark. The absorbance of the mixture was measured at 734 nm. Milli Q water was used a blank. The antioxidant capacity of samples was determined by using Eq. (2).

Hydroxyl radical scavenging activity assay

The scavenging activity of peptide fractions for hydroxyl radical was assayed according to the previously described method [25]. Briefly, 20µL of sample (at 0.01 mg protein/mL) was mixed with 500 µL of 2 mM ferrous sulphate and 100 µL of 2 mM hydrogen peroxide. After incubation at room temperature for 10 min, 500 µL of 2.5 µL salicylic acid was added. The mixture was then incubated at 37 °C for 30 min before being subjected for absorbance measurement at 510 nm. Results were determined using the following equation:

$$\text{Hyrdoxyl radical scavenging activity } (\%) = \left[\frac{1 - (As - Ab)}{(Ac)}\right] \times 100 \quad (3)$$

where A*s* is the absorbance of the sample, A*b* is the absorbance of a blank solution in the absence of salicylic acid and A*c* is the absorbance of the control solution using Milli Q water instead of the sample.

The IC_{50} value (µg/mL), expressed as a concentration of proteins in the crude extract containing an antioxidant substance required to remove 50 % of radicals, was determined for a peptide fraction <3 kDa, which showed the highest radical scavenging activity.

Culture of cells

Normal colon (T4056) cells were obtained from the Applied Biological Materials Inc. (Richmond, USA) while colon cancer (HT-29) cells were obtained from American Type Culture Collection (Manassas, VA. USA). The cells were cultured in RPMI 1640 medium (Life Technologies Australia Pty Ltd, Victoria Australia) with 10 % FBS in and incubated at 37 °C under 5 % CO_2. Passage 16–20 was used in all monolayer employed in the oxidative stress and cancer cell cytotoxic experiments. The medium was changed three times per week.

Assessment of oxidation-induced cell viability

Cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay using a commercial kit (Promega, Madison, Wis., USA), as described previously [27] with some modifications. Viable T4056 cells (2.5×10^3) were seeded into each well of 96-well plate containing 100 µL medium and incubated at 37 °C in 5 % CO_2 for 24 h. After the removal of spent media, cells were coincubated with samples (at 0.0005 mg protein/mL) and/or H_2O_2 (250 µM) in RPMI for 24 h. At the end of incubation, a 20 µL of a fresh mixture of MTS (Promega, Madison, WI) were added to each well. Fresh medium was used as control instead of samples. After incubating at 37 °C for 60 min, the absorbance at 490 nm was measured by a microplate reader (Bio-Rad Imark Microplate Reader; Bio-Rad Laboratories Pty., Ltd, NSW, Australia) and cell viability was recorded.

Measurement of cancer cell cytotoxic effect of peptide fractions

The inhibition rate of cancer cell lines (HT-29) after treatment with peptide fractions was also determined using MTS assay. Viable HT-29 cells (2.5×10^3) were seeded into each well of 96-well plate containing 100 µL medium and incubated at 37 °C in 5 % CO_2 for 24 h. After the removal of spent media, HT-29 cells were then treated with samples (at 0.005 mg protein/mL) or water (control) for 24 h. Afterwards, MTS solution (20 µL) was added to the wells at the end of incubation period. The absorbance was measured at 490 nm using a microplate reader.

Morphology of treated cells were assessed by an inverted microscope (Motic AE2000 Trinocular, Motic Incorporation Ltd, Hong Kong) and photographed via a Moticam camera (Motic Incorporation Ltd, Hong Kong) attached to the microscope.

Identification of bioactive peptides

The peptide fraction that showed the highest radical scavenging was further fractionated using Varian Pro Star Preparatory HPLC (Varian Analytical Instruments, California,

USA). Fractions were then analysed by LC–MS/MS using a quadrupole TOF mass spectrometer (MicroTOFq, Bruker Daltonics, Bremen, Germany) coupled online with HPLC (Monash Biomedical Proteomic Facility, Melbourne). Peptides were separated on a Dionex pepmap100, 75 µm id, 100 Å pore size, reversed-phase nano-column. The peptides were eluted over a 30-min gradient from 5 to 55 % of a 0.1 % formic acid in 90 % acetonitrile buffer. Data from LC/MS/MS run were exported in Mascot generic file format (*.mgf) and searched against the Swiss-Prot and NCBInr databases using the MASCOT search engine (version 2.4, Matrix Science Inc., London, UK). The following search parameters were used: missed cleavages, 1; peptide charge, 2+ and 3+; fixed modifications, nil; variable modification, oxidation (Met).

Statistical analysis

All analyses were done in triplicate, and data were averaged. Data obtained were subjected to one-way analysis of variance using SAS statistical software. P value < 0.05 was used for significance using least significance difference (LSD) procedures.

Results and discussion

Preparation of Flathead protein hydrolysate

Enzymatic hydrolysis is a common method to produce bioactive peptides from whole protein molecules, employing endogenous and/or exogenous enzymes derived from microorganism, plant and animal. In this study, endogenous enzyme and AFP were used to produce protein hydrolysate under acidic conditions (pH 2.5). The resulting soluble protein fractions of hydrolysate were further fractionated and tested for antioxidant activities.

The extent of protein hydrolysis of Flathead hydrolysate was estimated by calculating the ratio of the percentage of 10 % TCA soluble nitrogen in the hydrolysate compared to the total amount of protein in sample [23]. The degree of hydrolysis of UH, ON and ONE soluble protein were significantly different ($p < 0.05$), with ONE showed the highest DH (48.16 ± 0.82), followed by ON (43.37 ± 0.57) and UH (18.50 ± 0.10). The results showed that control sample (UH) underwent hydrolysis as well, but at a significantly lower rate. The hydrolysis process, executed by endogenous enzymes, might have started once the by-products were minced and acidified using 1 % H_2SO_4. Cytoplasmic calpains and lysosomal cathepsins are two main endogenous proteolytic enzymes which are responsible for hydrolysing fish proteins [28]. Our previous study showed that Flathead protein was easily hydrolysed and did not produce any visible bands when analysed using SDS-PAGE [21]. Longer hydrolysis time and addition of AFP significantly increased the degree of hydrolysis. The degree of hydrolysis of ONE was significantly higher than ON indicating that addition of protease further accelerated the hydrolysis. The degree of hydrolysis as well as molecular weight of peptides produced is dependent on the type of substrate, a type of protease, protease specificity and physicochemical conditions employed during hydrolysis [29].

The soluble peptides of hydrolysed Flathead proteins were confirmed using RP-HPLC (Fig. 1) with most of the peaks appearing in the first 25 min of elution, which is identified as the hydrophilic region. Chromatograms of UH (Fig. 1a–d) showed fewer liberated peptides, compared to ON (Fig. 1e–h) and ONE (Fig. 1i–l). The addition of AFP and longer hydrolysis time resulted in an apparent rise of peaks assigned to smaller peptides (Fig. 1i–l). The differences in peptide profile pattern of sample hydrolysed with AFP (ONE) and endogenous enzymes (UH and ON) might be due to differences in the properties of enzyme cleaving sites as well as the accessibility of peptide bonds to each protease. The fractionation steps separated larger peptides resulting in peptide fractions <3 kDa (Fig. 1d, h, l). Although similar pattern was observed for <3 kDa fractions, ONE showed higher concentration of small peptides (Fig. 1l).

Radical scavenging activities of Flathead peptide fractions

Free radical scavenging is a primary mechanism by which antioxidants inhibit oxidative processes. However, there is no single method that is able to provide an accurate profile of antioxidant activity. Therefore, in order to provide reliable information about antioxidant capacity of Flathead peptides, three different in vitro assays were performed to confirm this activity using DPPH, ABTS and hydroxyl radical scavenging.

DPPH scavenging activity

DPPH scavenging activities of fractionated Flathead peptides are summarized in Fig. 2. Overall, fractions with peptides of MW between 3 and 10 kDa (Fig. 2C) and <3 kDa (Fig. 2D) showed higher DPPH scavenging activity than fractions of larger-sized peptides (>10 kDa) (Fig. 2B) and UF (Fig. 2A). These results are in agreement with those of Yang [30], who reported that peptides from tilapia retorted skin gelatin hydrolysates with MW below 6.5 kDa were associated with greater antioxidative activities. Peptide fraction of cod hydrolysate below 3 kDa (1.7 mg protein/mL), which was prepared using commercial protease, also showed high DPPH radical scavenging activity (74.1 %). The result

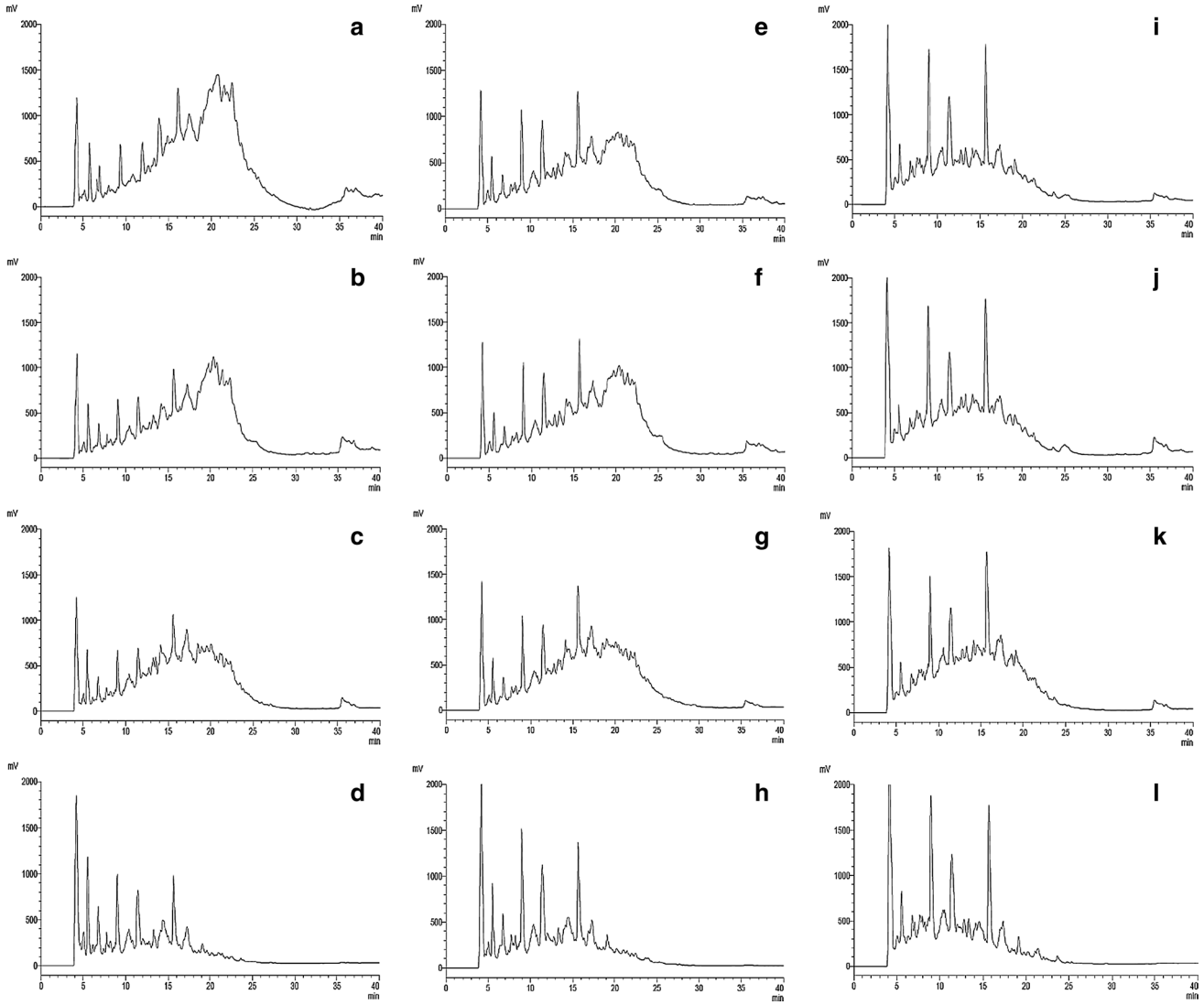

Fig. 1 Peptide profiles of Flathead fractions. **a** UH UF, **b** UH > 10 kDa, **c** UH 3–10 kDa, **d** UH < 3 kDa, **e** ON UF, **f** ON > 10 kDa, **g** ON 3–10 kDa, **h** ON < 3 kDa, **i** ONE UF, **j** ONE > 10 kDa, **k** ONE 3–10 kDa, **l** ONE < 3 kDa

was similar to that of the synthetic antioxidant BHT (79.8 %) at 0.2 mg/mL concentration [31]. Ranathunga et al. [32] stated that low MW peptides were more effectively interact with radicals, thus interfering the oxidation process.

The UF and >10 kDa peptide fractions obtained from different treatments showed similar scavenging capacity ($p > 0.05$), indicating that smaller peptides (3–10 kDa and <3 kDa) were responsible for exerted activity ($p < 0.05$). For a fraction consisting of <3 kDa peptides, the highest DPPH radical scavenging activity (94 ± 0.05 %; IC_{50} = 5 µg of protein/mL) was exhibited by the sample obtained from overnight incubation with AFP (ONE) (Fig. 2D). Wu et al. [33] also reported higher level of peptides with antioxidant activity obtained from protease activity than from autolysis. More importantly when our results are compared to previous studies, much lower IC_{50} was obtained in the current study than that of loach protein hydrolysate (IC_{50} = 2.42 mg/mL) [34], tilapia frame protein hydrolysate (IC_{50} = 1.92 mg/mL) [35] or reduced glutathione (GSH), a known antioxidant, with IC_{50} of 25 µg/mL [36]. This indicates that the fraction of Flathead protein hydrolysate <3 kDa contained a peptide or peptides with a high potential DPPH scavenging activities.

ABTS scavenging activity

The activities for DPPH and ABTS scavenging followed a similar trend suggesting that the two antioxidant mechanisms might be related. As shown in Fig. 2, decreasing Flathead molecular weight peptides increased $ABTS^{\cdot+}$ radical

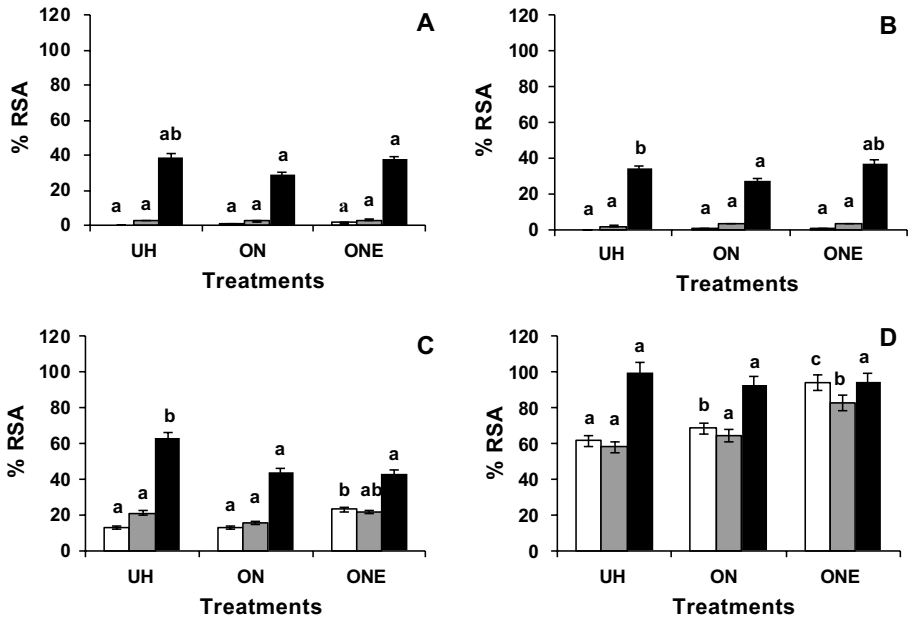

Fig. 2 Radical scavenging activity (RSA) of peptide fractions. **A** Unfractionated (UF), **B** >10 kDa, **C** 3–10 kDa, **D** <3 kDa. *White square* DPPH radical scavenging, *grey square* ABTS radical scavenging, *black square* hydroxyl radical scavenging. *UH* Flathead peptide 0 h incubation, *ON* Flathead peptide incubated overnight without the addition of AFP, *ONE* Flathead peptide incubated overnight with the addition of AFP. Results represent mean ± SD. Values with the *same lower cases* (a, b, ab, c) within the same radical scavenging analysis indicate no significant difference ($P > 0.05$)

scavenger activity. There was very low scavenging activity observed from UF (Fig. 2A) and >10 kDa (Fig. 2B) peptide fractions ($p < 0.05$) despite treatment differences. The ability of <3 kDa peptides fraction to quench ABTS radicals was twofold higher than that of 3–10 kDa peptide fractions. Contrary to our result, Alemán et al. [37] reported that lower MW showed lower ABTS activities than unfractionated sample. Large number of free amino acid and small peptides without antioxidant capacity might present in the lower MW fractions. Also, other factors, such as substrate and peptide composition might also influence the radical scavenging activity and should not be disregarded [38].

The highest ABTS scavenging activity was obtained from ONE <3 kDa fraction (82.89 ± 1.93 %; $IC_{50} = 6$ µg/mL) (Fig. 2D). Cheung et al. [38] reported that ABTS radical activity of FPH hydrolysed with exogenous enzymes were higher than the autolysate and the standard (trolox at 16.7 µM). It was suggested that the addition of enzyme further increased the antioxidant activity of FPH. In the current study, the IC_{50} value for the ABTS radical scavenging activity of ONE <3 kDa peptide fraction was lower than that of defatted shrimp processing by-product hydrolysate and BHT with IC_{50} values of 7.4 and 7.2 µg/mL, respectively [39]. The ABTS radical scavenging activity of Flathead peptide fraction may also significantly be influenced by the DH of sample as it was shown in the study involving tilapia hydrolysates [40].

Hydroxyl scavenging activity

The scavenging activity of Flathead peptide fraction against hydroxyl radicals ranged from 28.39 to 100 % (Fig. 2). It is worth noting that at very low concentration (0.01 mg protein/mL), Flathead peptide fractions with molecular size <3 kDa could scavenge up to 100 % of hydroxyl radicals ($IC_{50} = 5$ µg protein/mL) (Fig. 2D). Although lower than our result, Ren et al. [41] reported strong hydroxyl RSA (IC_{50} values of 1.68 ± 0.34 mg/mL) from grass crap (*Ctenopharyngodon idellus*) muscle hydrolysate with MW <3 kDa. This indicated that FPH fractions with MW <3 kDa might contain peptides with specific amino acid sequences and molecular weight ranges, which are more easily accessible to the hydroxyl radicals and allow the peptides to trap the radicals [35]. Our <3 kDa peptide fraction showed higher hydroxyl radical scavenging activity, thus a lower IC_{50} value than purified peptides derived from tilapia frame hydrolysate (Asp-Cys-Gly-Tyr, $IC_{50} = 27.6$ µg/mL and Asn-Tyr-Asp-Glu-Tyr, $IC_{50} = 38.4$ µg/mL), or glutathione (GSH) ($IC_{50} = 0.24$ mg/mL) [35]. Since chemical activity of hydroxyl radical was greatest among ROS, hydroxyl scavenging activity of Flathead peptides was a good indicator of its potency.

Oxidation-induced cell viability

Oxidative stress is known to be responsible for cell death via apoptosis or programmed cell death [42]. In order to study the effect of reactive oxygen species on viability of normal cells and how peptides protect these cells, T4056 normal colon cells were induced with hydrogen peroxide and the cells were in turn treated with peptides for 24 h. Although hydrogen peroxide is a reactive non-radical compound, it could be easily converted into more reactive species such as singlet oxygen and hydroxyl radicals, which

can then induce toxic effects in cells [43]. Figure 3A shows that at 0.0005 mg protein/mL, Flathead peptides hardly showed cytotoxic effect on T4056 cells. When exposed to H_2O_2, the viability of T4056 was greatly decreased by 46.07 % (Fig. 3B). The presence of peptides during H_2O_2 exposure increased the viability of the cells. Our data clearly showed that peptides were able to prevent cell injury and death from radical exposure. Similarly, Zhong et al. [27] reported that low molecular size peptides from silver carp (*Hypophthalmichthys molitrix*) by-product protein hydrolysates could act as biological antioxidant by increasing the viability of Caco-2 cells against H_2O_2.

Cancer cells cytotoxic effect of peptide fractions

Colon cancer, one of the most common forms of cancer and second leading cause of cancer death in the world, is mostly treated by surgery, or in some cases combined with chemotherapy and radiotherapy [44]. Since such therapies often are associated with deleterious effects caused by drug-induced damage to healthy cells and tissue, Rodrigues et al. [45] suggested that bioactive peptides with lack of toxicity to healthy cells would be a promising candidate for anticancer treatment. In this study, HT-29 colon cancer cells were treated with different fractions of Flathead peptides at 0.005 mg protein/mL. The low molecular size fractions were found to possess stronger cytotoxicity in cancer cell (Fig. 4A c, d) than that of high molecular size fractions (Fig. 4A a, b). The <3 kDa peptide fractions inhibited the growth of HT-29 colon cancer cells up to 87.54, 88.98 and 91.04 % for ONE, ON and UH, respectively. Similar cytotoxic effect, however, was shown as T4056 normal colon cells were treated with the same dose of peptides (Fig. 4B). Low viability of T4056 cells (4.86–8.3 %) was observed in the <3 kDa MW fractions. The present results indicate the non-cell selective nature of the peptides. It is necessary to further purify and identify Flathead peptides that may cause apoptosis on colon cancer cells but non-toxic to normal colon cells. Purified peptides (Trp-Pro-Pro) from blood clam (*Tegillarca granosa*) muscle showed strong cytotoxicity towards PC-3, DU-145, H-1299 and HeLa cancer cell lines in a dose-dependent manner, yet non-toxic on normal mouse fibroblast cell line NIH3T3 [46]. Other option for utilizing non-selective anticancer peptides is by delivering or injecting the peptides to target (cancer cells) sites. Very potent anticancer substances with small molecular size can

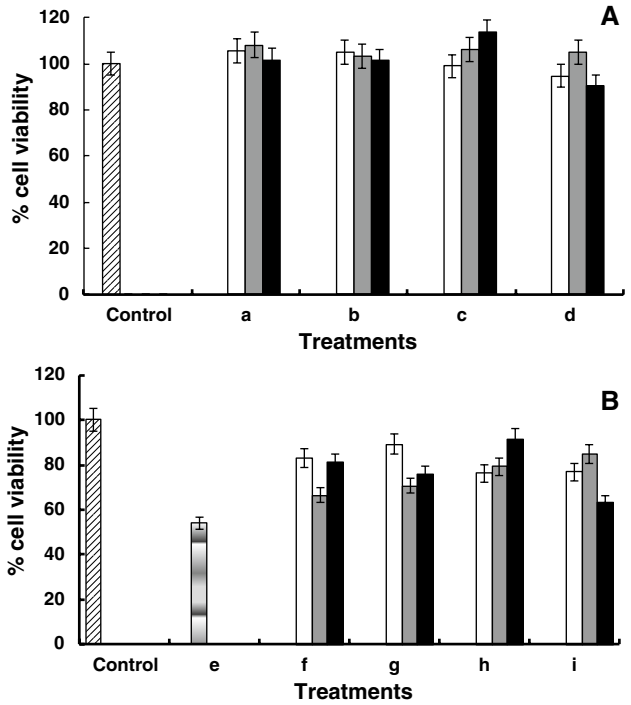

Fig. 3 Cell viability of T4056 normal colon cells treated with Flathead peptide fractions (**A**) and after oxidative stress induced by H_2O_2 (**B**). *a* T4056 cells treated with UF peptides, *b* T4056 cells treated with >10 kDa peptides, *c* T4056 cells treated with 3–10 kDa peptides, *d* T4056 cells treated with <3 kDa peptides, *e* T4056 cells exposed to 0.25 mM H_2O_2, *f* T4056 cells cotreated with UF peptides, *g* T4056 cells cotreated with >10 kDa peptides, *h* T4056 cells cotreated with 3–10 kDa peptides, *i* T4056 cells cotreated with <3 kDa peptides. *Square with upper right to lower left fill* control T4056 cells, *square with shaded vertical line* cells exposed to 0.25 mM H_2O_2, *white square* cells treated with UH, *grey square* cells treated with ON, *black square* cells treated with ONE

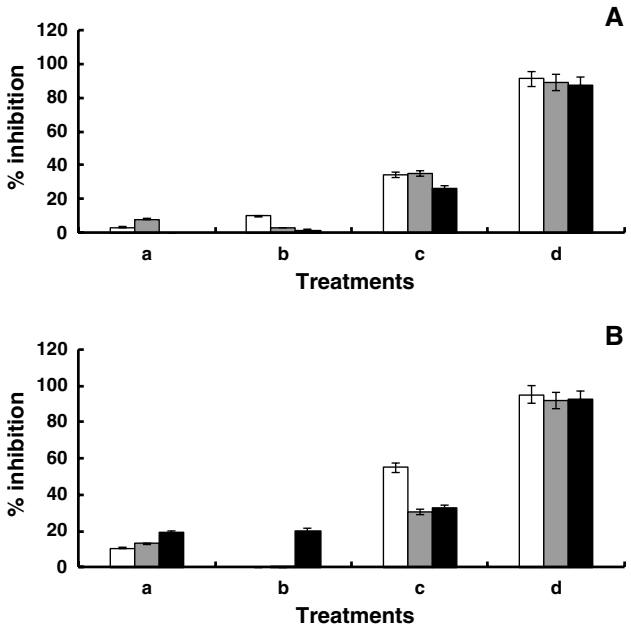

Fig. 4 Cytotoxicity of peptide fractions against HT-29 colon cancer cell line (**A**) and T4056 normal colon cells (**B**). *White square* cells treated with UH, *grey square* cells treated with ON, *black square* cells treated with ONE. *a* Unfractionated (UF), *b* >10 kDa, *c* 3–10 kDa, *d* <3 kDa

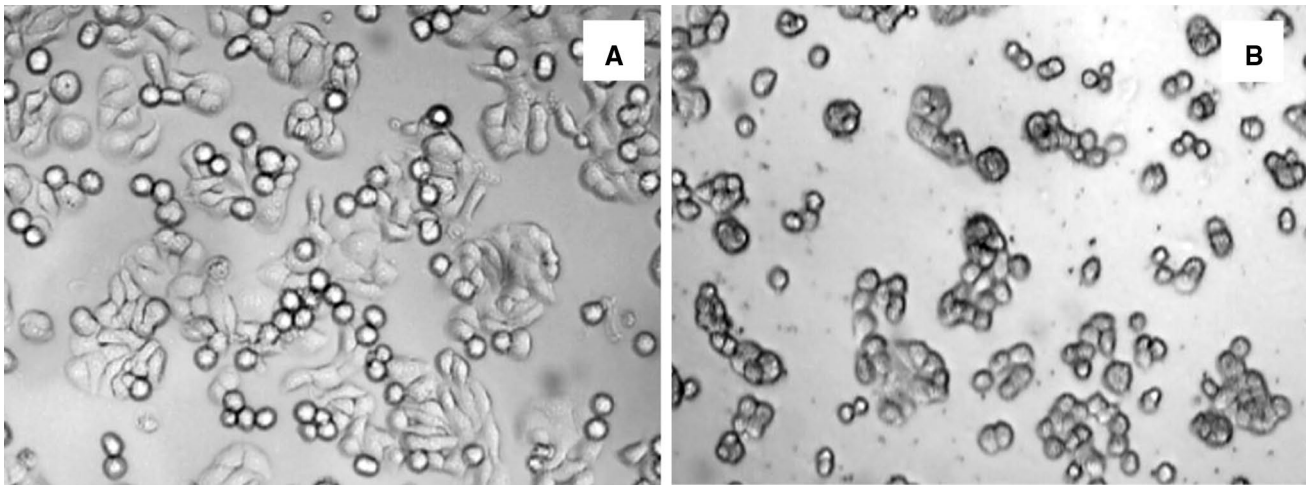

Fig. 5 Cell morphology of HT-29 colon cancer cells. **a** Control HT-29 cells, **b** treated with peptide fraction <3 kDa at 0.005 mg protein/mL

be developed as targeted drugs that exploit the particular genetic dependencies and vulnerabilities of cancer cells [47].

The morphological changes in HT-29 colon cancer cells after 24-h exposure to <3 kDa peptides were noted as depicted in Fig. 5. HT-29 cells began to show cell shrinkage and fragmentation, typical appearance of apoptotic cells (Fig. 5b) compared to untreated cells (Fig. 5a). Further *cell based* study is essential to observe the mechanistic pathways and structure/function relationship of peptides in stimulating apoptosis.

Identification of bioactive peptides

The obtained results indicated that ONE <3 kDa peptide fraction contained the highest radical scavenging ability. In order to measure the contributions of small peptides to the RSA, this fraction was further separated and analysed. Nine fractions (fractions 1–9) with different elution times were selected (Fig. 6a). These fractions were pooled, freeze-dried and assayed for DPPH scavenging activity at a concentration of 2.0 mg/mL. Figure 6b shows all nine fractions exhibited DPPH scavenging activities which varied from 1.06 ± 0.66 to 23.32 ± 0.65 %. Consequently, all fractions were subjected to peptide identification using a quadrupole TOF mass spectrometer.

The search (fractions 1–9) against Swiss-Prot and NCBI database resulted in many matches relating to peptides (amino sequences) identified in different organisms other than fish. However, a peptide (from fraction 9) with 15 amino acid bases (Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg; Mw = 1337.51 Da) matched a fish species from the database. Physiochemical properties of the peptide include: (1). Iso-electric point at pH 6.61; (2) Net charge at pH 7 = 0; and (3) Good water solubility

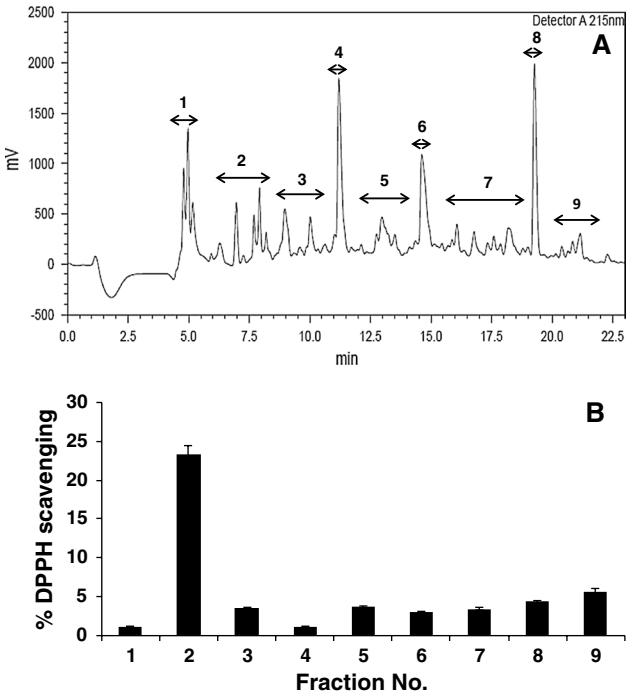

Fig. 6 **a** Fractionation by Preparatory RP-HPLC. Collected fractions are numbered from 1 to 9. **b** DPPH scavenging activity of the collected fractions, at the concentration of 2 mg/mL. Data were presented as mean ± standard deviation

(www.pepcalc.com). Based on AntiCP prediction, the peptide has anticancer properties (http://crdd.osdd.net/raghava/anticp/submit_prot.php).

Regarding relationship between structure and activity of radical scavenging peptides, it has been reported that peptides with high free radical scavenging activity contain amino acids with aromatic side chains (Trp, Tyr, His and Phe), sulphur-containing side chains (Cys and Met) or

hydrophobic amino acids (Val, Leu and Ala) [48, 49]. The identified peptide has Met, Leu and Ala as well as being rich in Pro and Gly. Several authors reported that peptides contained Leu, Pro and Gly reacted with free radicals and convert them to more stable products, terminating the radical chain reaction [50, 51].

Conclusion

Flathead peptides fraction obtained in this study exhibited noticeable antioxidant activities in different test models in vitro. The radical scavenging activities of peptides were related to degree of hydrolysis, presence of protease and molecular weight. Low molecular size peptides (<3 kDa) showed the highest free radical scavenging activity in vitro. In addition, Flathead peptides could protect the normal colon cells from cell injury and death during H_2O_2 exposure. At 0.005 mg protein/mL, small peptides also showed strong cytotoxic activity against HT-29 colon cancer cells although this activity was found to be non-selective. Further fractionation and purification stages generated a novel peptide that was identified as Met-Gly-Pro–Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg. Peptide extracted from Flathead by-products may act as potent free radical scavengers and cancer cells cytotoxic agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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CHAPTER 6

Chapter 6. Anticancer properties of a peptide isolated from Flathead (*Platycephalus fuscus*) by-products and its stability during *in vitro* gastrointestinal digestion

6.1. Introduction

Colorectal cancer is the second most commonly diagnosed in females and third in males with an estimated 1.4 million cases and 693,900 deaths occurring in 2012 worldwide. Australia is one of the countries with the highest incidence rates (≥ 31.0 per 100,000 populations) of colorectal cancer for both males and females (American Cancer Society 2015). The increasing incidents of colocteral cancer are associated with unhealthy diet, obesity and smoking habits (Doubeni et al. 2012). While the most common treatment for colorectal cancer is surgery combined with chemotherapy by cytotoxic drugs and radiation, this therapy is just moderately successful especially in the late stages of cancers (Hajiaghaalipour et al. 2015). Furthermore, such therapies often are associated with deleterious effects caused by drug-induced damage to healthy cells and tissue (Hubenak et al. 2014). Thus discovery of new safe cancer drugs from natural products becomes an important goal of research in biomedical sciences.

Recently, an increasing number of new anticancer compounds have been identified from the marine environment (Jimeno et al. 2004; Lin 2014; Simmons et al. 2005). Furthermore, protein hydrolysates and/or peptides from seafood also showed effective anticancer activities (Chen, Lin & Lin 2009; Chi et al. 2015; Ding et al. 2011). Several authors have also reported anti-colorectal cancer properties of peptides isolated from fish protein hydrolysate (FPH). Leu-Ala-Asn-Ala-Lys (MW= 515.29 Da) isolated from oyster hydrolysate had anticancer activity against human colon carcinoma (HT-29) cell lines

(Umayaparvathi et al. 2014). Kannan et al. (2011) reported that peptide fractions extracted from shrimp by-products inhibited the growth of human colon epithelial cancer cell line Caco-2 by 60% after 72 h of exposure. Similarly, loach peptide fractions inhibited the proliferation of human Caco-2 colon cancer cells (You et al. 2011). In a previous study by Nurdiani et al. (2016b), low molecular weight (< 3kDa) peptide fractions isolated from Flathead (*Platycephalus fuscus*) by-products were reported to show strong antioxidant and anticancer activities against HT-29 cell lines. Purification and identification stage revealed a novel peptide with 15 amino acid bases (Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg; Mw = 1337.51Da), predicted to have anticancer properties based on AntiCP prediction (http://crdd.osdd.net/raghava/anticp/submit_prot.php). In the present study, therefore, the intent was to establish potency of this peptide as an anticancer agent.

One of the greatest challenges in developing fish peptides as functional food ingredients is proving their efficacy as bioactive components such as anticancer agents. The potential effect of the peptides depends on their capacity to reach the target organs. Gastrointestinal l (GI) conditions tracts may influence the primary structure and thus intended functions of the peptides before they reach the required target sites (Segura-Campos et al. 2011). Several fish bioactive peptides with ACE inhibitor and antioxidative properties have been assessed for their stability during gastrointestinal digestion (Chen, et al. 2012; Jensen et al. 2009; Samaranayaka, Kitts & Li-Chan 2010); yet, not much information on stability of anticancer peptides during GI digestion. The objective of this study, therefore, was to evaluate the impact of simulated GI digestion on peptide structure by means of reversed phase high performance liquid chromatography (RP-HPLC) and to analyze the anticancer capacity of the peptides during and after GI digestion.

6.2. Materials and methods

6.2.1. Materials

The peptide structure (H- Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg -OH) was determined in the previously reported work (Nurdiani et al., 2016b). The peptide was synthesized by Mimotopes (Clayton, VIC, Australia) at >95% purity. Staurosporine solution (from *Streptomyces* sp), trifluoroacetic acid (TFA), and pepsin (from porcine gastric mucosa) were obtained from Sigma-Aldrich Pty. Ltd. (Castle Hill, Australia). Pancreatin Amylase and Protease was purchased from U.S. Pharmacopeia (Rockville MD, USA). Acetonitrile was purchased from Merck (Darmstadt, Germany). All other reagents were of the highest grade commercially available.

6.2.2 Stability of peptide against gastrointestinal (GI) protease

Stability of the peptide against the gastrointestinal proteases was assayed using *in vitro* pepsin–pancreatin hydrolysis according to a method of Nalinanon et al. (2011), with slight modifications. Exactly 30 mg of peptide was diluted in 10 ml KCl-HCl buffer. The pH was adjusted to 2.0 with a drop-wise addition of 1 M HCl. Pepsin was then added (E/S 1:35 w/w), and the mixture was incubated under continuous shaking in an incubator shaker (Innova 4200, New Brunswick Scientific GmbH, Germany) for 60 min at 37°C. An aliquot (2 mL) was sampled at the end of this period to establish effects of pepsin digestion on the peptide stability and its anticarcinogenic properties. The pH of the solution was then adjusted to 5.3 with 1 M $NaHCO_3$ solution and further to pH 7.5 with 1 M NaOH. Afterwards, pancreatin (E/S 1:25 w/w) was added, and the mixture was further incubated with continuous shaking for 3 h at 37°C. The digestion was terminated by submerging the solution in boiling water for 10 min. As soon as the GI digest was cooled to room temperature, it was

centrifuged at 12,000 *x g* for 25 min (Eppendorf Centrifuge 5415C, Crown Scientific Pty Ltd, Moorebank, NSW, Australia). The supernatant was then collected and used for analysis.

6.2.3. Peptide profiling by reversed-phase high performance liquid chromatography (RP-HPLC)

To establish whether the stimulated gastrointestinal digestion affected the structure of peptide, the undigested and GI digested peptide solutions were profiled using an RP-HPLC. Synthetic (undigested) peptide (4 mg) was dissolved in Milli Q water (1 mL) prior centrifugation. All peptide solutions were centrifuged at 8161 *x g* for 30 min at room temperature (Eppendorf Centrifuge). All solvents were filtered through a 0.45 µm membrane filter prior to use. Peptides were separated on a Shimadzu HPLC system (Shimadzu Model LC-2030, Shimadzu Corporation, Kyoto, Japan) equipped with Vydac Everest C18 column (250 mm x 4.6 mm, particle size 5 µm, pore size 300Å; Grace Davison Discovery Sciences, Rowville, VIC, Australia). The peptides were eluted by a linear gradient of 0% to 100 % of solvent B (0.1 % TFA in acetonitrile) in solvent A (0.1 % TFA in deionized water) over 25 min. The flow rate was maintained at 0.75 mL/min and eluted peptides were detected at 215 nm.

6.2.4. Anticancer and cell cytotoxic effects of peptides

Two types of colon cell lines were grown and cultured separately in RPMI 1640 medium (Life Technologies Australia Pty Ltd, Victoria Australia) with 10 % FBS and incubated at 37 °C under 5 % CO_2. Normal colon (T4056) cells were obtained from the Applied Biological Materials Inc. (Richmond, USA) while colon cancer (HT-29) cells were purchased from American Type Culture Collection (Manassas, VA. USA). Passage 16-20

was used in normal and cancer cell cytotoxic experiments. The medium was changed three times per week.

The anticancer activity and cell cytotoxicity effects of peptides on HT-29 and T4056 was determined using an MTS assay based on the previously published method (Nurdiani et al. 2016b). Briefly, viable HT-29 cells or T4056 (2.0×10^3) were seeded into each well of a 96-well plate containing 100 μL of the medium and incubated at 37°C in 5 % CO_2 for 24 h. After incubation, the spent media was removed and cells were then treated with samples (at 0.25 mg/mL). Unstimulated cells were used as a negative control, while Staurosporine (at 0.25 μM) was used as a positive control. After 72 h of cell exposure to the samples, MTS reagent (20 μL) was added to the wells and incubated for an additional 1 h under the same conditions. The absorbance was measured at 490 nm using a microplate reader (iMark Bio-rad, Bio-Rad Laboratories Pty., Ltd., New South Wales, Australia). The experiments were repeated three times. The determination of a peptide concentration used in this study was based on the preliminary experiment (data not shown). As there was no significant difference on the viability of cells treated with different peptide concentrations (at 0.025, 0.05, 0.1 or 0.25 mg/mL), the highest concentration (0.25 mg/mL) was chosen.

6.2.5. Morphology of cells

In order to observe the changes on morphology of cells after treatments, the treated cells were assessed by an inverted microscope (Motic AE2000 Trinocular, Motic Incorporation Ltd, Hong Kong). Briefly, viable HT-29 cells or T4056 (2.0 x 103) were seeded into each well of a 96-well plate as described in Section 6.2.4. After 72 h incubation, the plate was mounted to the microscope and viewed with a magnification of 40x. The cells were photographed via a Moticam camera (Motic Incorporation Ltd, Hong Kong) attached to

the microscope. The images of cells were analysed using software Motic Image Plus 2.0 (Motic Incorporation Ltd, Hong Kong).

6.2.6. Extent of cell apoptosis

The degree of apoptosis of cell population is an important parameter of cell health. For apoptosis experiment, a Muse Cell Analyser (Merck Millipore, Australia) was used. In brief, HT-29 cells were plated in 12-well plates at a density of 2.0 x 10^3 containing 500 µL medium and incubated at 37 °C in 5 % CO_2 for 24 h. After 24 h of incubation, the spent media were removed and replaced with peptides solution or Staurosporine (Section 6.2.4). The cells were incubated for 72 h before being harvested for the apoptosis experiment. The final concentration of harvested cells used in the assays was between 1 x 10^5 to 1 x 10^7 cells/mL. The staining and the assay protocol was performed based on the manufacture's guide. Briefly, exactly 100 µL of Muse Annexin V & Dead Cell reagent (Merck Millipore, Australia) was added to 100 µL cells in suspension and gently mixed by pipetting up and down or vortexing at a medium speed for 3 to 5 second. The mixture was incubated for 20 min at room temperature in the dark before it was analysed using the cell analyser.

6.2.7. Statistical analysis

Data presented are the means ± SD of results from a minimum of three independent experiments with similar patterns unless otherwise mentioned. Statistical analysis was performed using one-way ANOVA. A p value of less than or equal to 0.05 was considered as significant.

6.3. Results and Discussion

6.3.1 Stability of peptide against gastrointestinal (GI) protease

After oral administration, peptides have to survive a series of possible hydrolysis by gastrointestinal protease before they reach target sites and become functionally active. In this work, identified peptide Met_1-Gly_2-Pro_3-Pro_4-Gly_5-Leu_6-Ala_7-Gly_8-Ala_9-Pro_{10}-Gly_{11}-Glu_{12}-Ala_{13}-Gly_{14}-Arg_{15} was subjected to *in vitro* digestion using pepsin and pancreatin proteases. The change of peptide structure during and after GI digestion was observed (Fig 6.1).

Figure 6.1B showed that pepsin only slightly hydrolysed the peptide which indicates that the purified peptide was resistant to this gastric protease. It has been suggested that the peptides ability to resist enzymatic attack is due to their amino acid composition. Peptides with proline residues are generally able to resist degradation by digestive enzymes (Sarmadi & Ismail 2010). Three out of fifteen residues in the identified peptide were proline residues which is likely responsible for its resistance to pepsin digestion. A desktop based enzymatic digestion exercise was performed on above identified peptide as substrate, using online tool "PeptideCutter" (Swiss Institute of Bioinformatics; http://web.expasy.org/cgi-bin/peptide_cutter/peptidecutter.pl). Peptidecutter tool identified one potential pepsin cleavage site, namely Leu_6-Ala_7. The experimental data presented in Fig 6.1B showed only limited hydrolysis of peptide with pepsin under the experimental conditions, which clearly means that the hydrolysis of potential pepsin site only occurs at extremely slow rate.

Further digestion using pancreatin, which mimics the digestion process in the intestine, resulted in the hydrolysis of parent molecule yielding smaller peptides (Fig 6.1C).

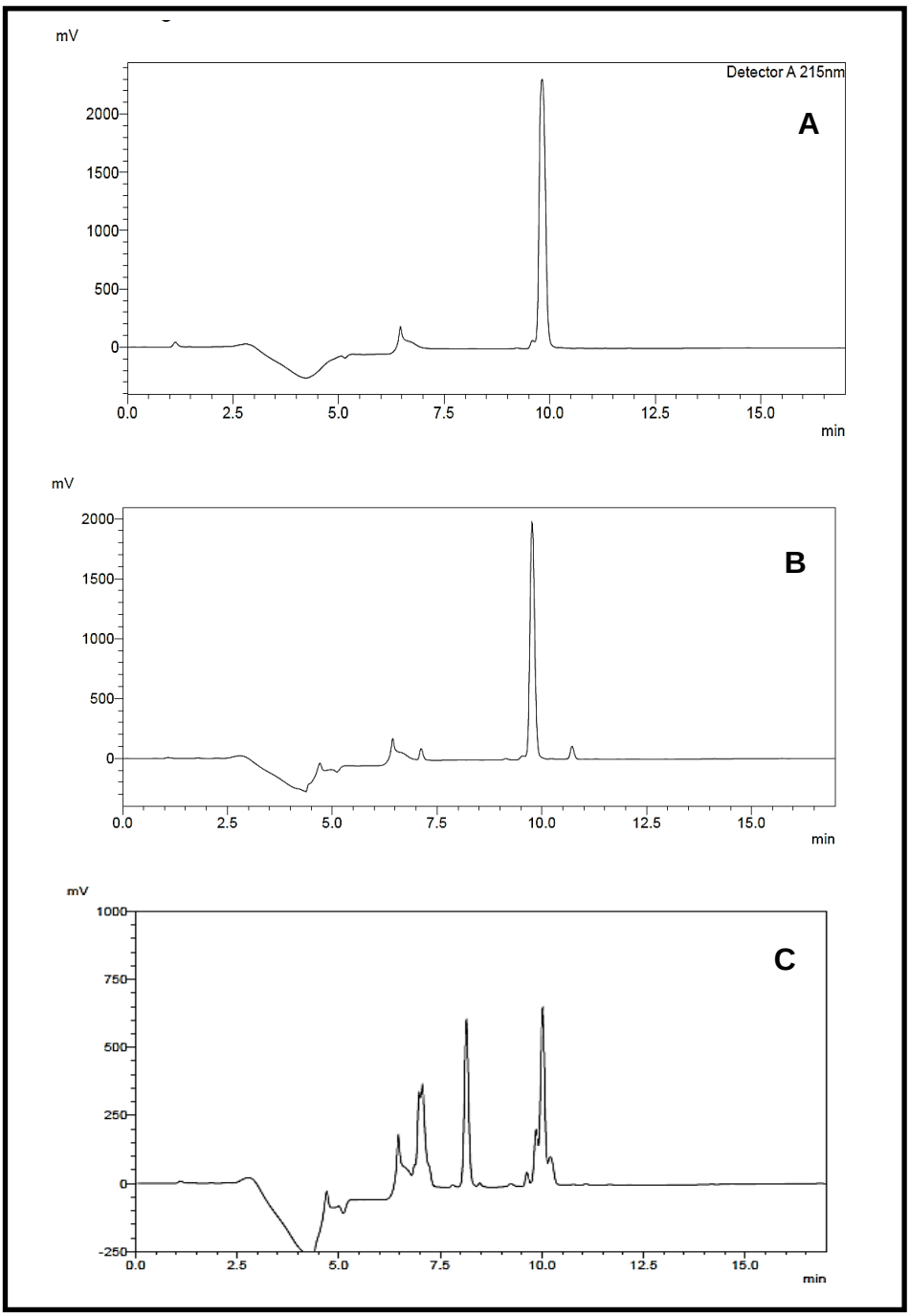


Figure 6.1. Reverse-phase HPLC profile of undigested peptide (A); pepsin digested peptide (B) and pepsin and pancreatin digested peptide (C).

Pancreatin preparation is known to contain numerous enzymic activities, including amylase, lipase, and proteases (Mullally et al. 1994). In addition to trypsin (cleaves peptide bonds such as Lys-X and Arg-X residues), pancreatin also contained chymotrypsin which is known to hydrolyse peptide bonds involving hydrophobic amino acid residues such as Tyr, Trp, Phe and Leu. Once again the digestion of identified peptide was attempted using PeptideCutter tool but with the selection of enzymes such as chymotrypsin and trypsin. This exercise yielded two potential hydrolysis sites on the peptide, namely Met_1-Gly_2 and Leu_6-Ala_7. This information might explain experimental data on hydrolysis of fifteen amino acid residue peptide with chymotrypsin summarized in Fig 6.1C. No trypsin hydrolysis site was identified as expected in light of its known extremely narrow specificity mentioned above.

6.3.2. Anticancer activity and cell cytotoxicity of peptide

Many papers have reported the anticancer activity of purified peptides isolated from fish by-products (Ding et al. 2011; Doyen et al. 2011; Umayaparvathi et al. 2014). In the current study, the anticarcinogenic activity of identified peptide isolated from Flathead (*Platycephalus fuscus)* by-products was evaluated. Its stability/sensitivity against GI digestion was also determined. The HT-29 cell line was incubated with undigested synthetic peptide, pepsin digested peptide and pepsin and pancreatin digested peptide at a concentration of 0.25mg/mL. All peptides including Staurosporine showed anticancer activity by inhibiting the growth of HT-29 during 72 h incubation. Staurosporine, isolated from *Streptomyces staurospores*, showed very strong anticancer activity by reducing the cell viability to 19.4 %. The undigested and pepsin digested peptides (at 0.25 mg/mL) were found to have a similar anticancer activity (Fig 6.2.). The growth of HT-29 cells was significantly inhibited up to 28.89% and 29.68% by undigested and pepsin digested peptides, respectively. At the same concentration, the peptide digested with both pepsin and pancreatin proteases

showed higher HT-29 growth inhibition activity resulting in lower cell viability (61.7 %) than that prior to digestion with pancreatic enzymes. These results indicated that not only the identified parent peptide but the smaller peptides also showed anticancer activity and may in fact possess higher anticancer activity. Umayaparvathi et al. (2014) reported that Leu-Ala-Asn-Ala-Lys (515.29 Da), a purified peptide from oyster hydrolysate, had a strong cytotoxic activity on the HT-29 colon cancer cells with IC_{50} = 60.21 ± 0.45 µg/mL. The size of the peptide as well as its amino acids composition obviously may affect its anticancer activity. However the authors did not test resistance and behavior of this peptide during the GI digestion.

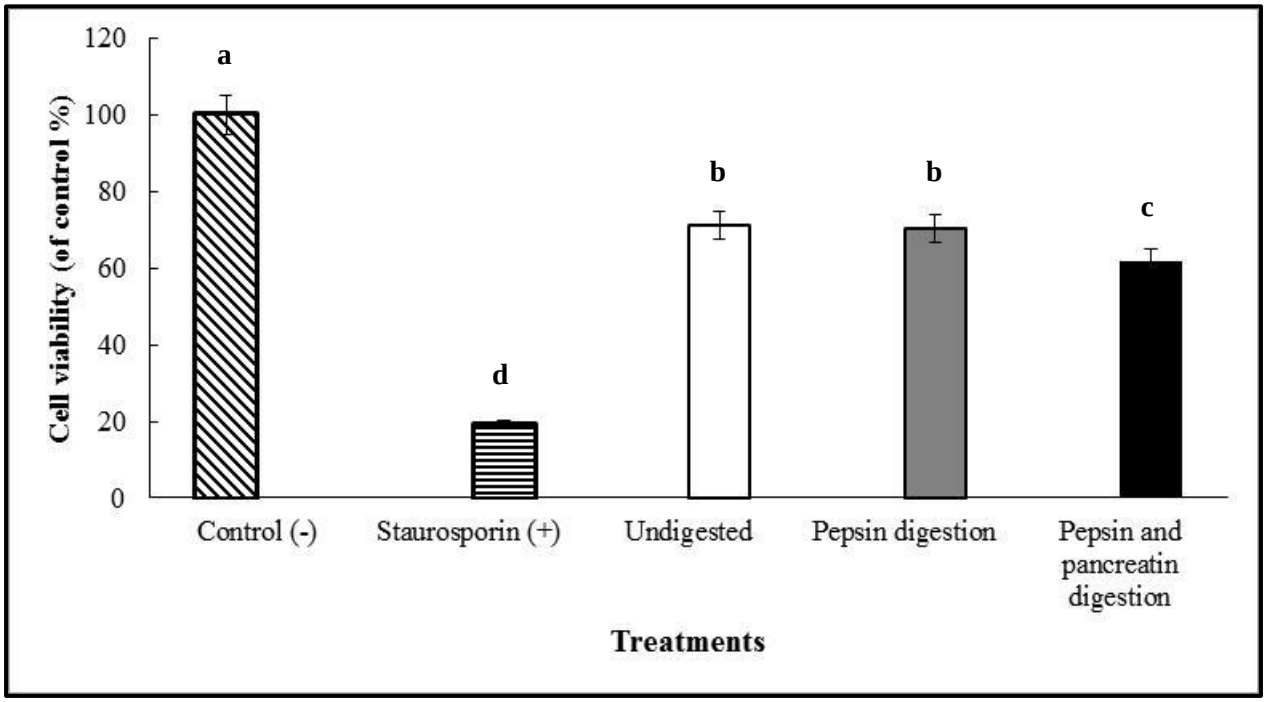


Figure 6.2. Anticancer activity expressed as cell viability of the peptide (at 0.25 mg/mL) subjected to the simulated GI digestion against HT-29 colon cancer cell lines. Staurosporin (at 0.25 µM) was used as a positive control. Values with the different lowercase letter (a, b, c, d) indicate significant difference ($P < 0.05$)

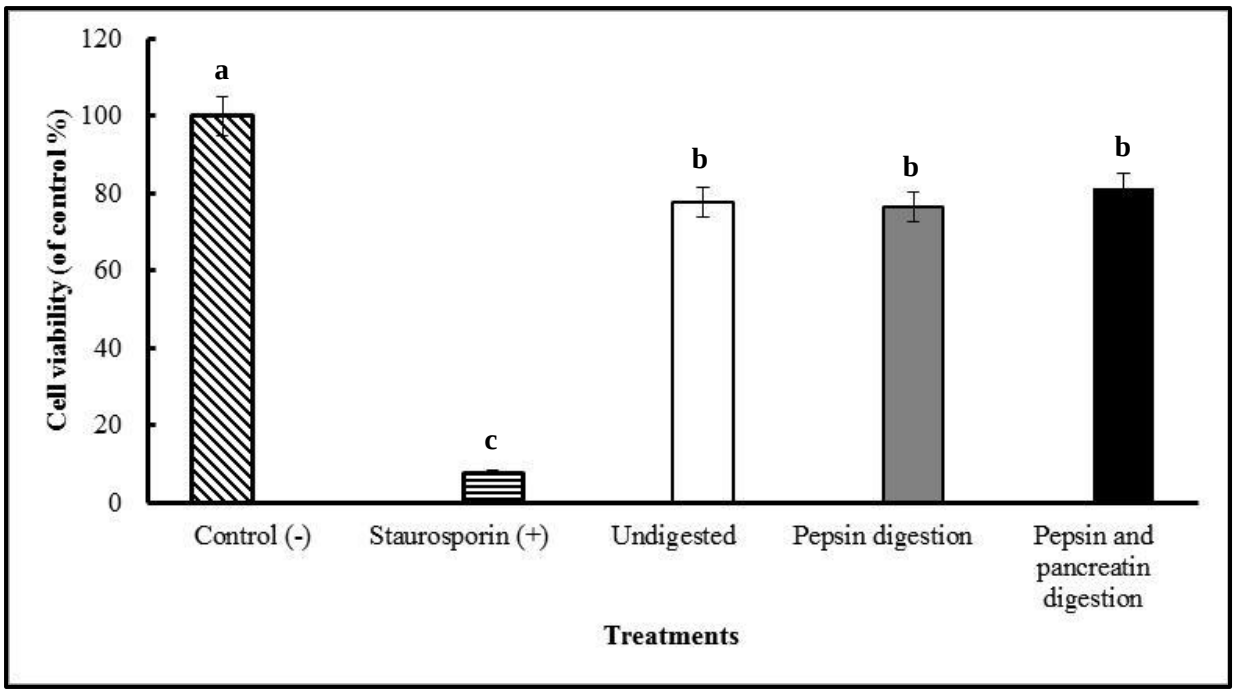


Figure 6.3. Cell viability of T4056 normal colon cells treated with undigested and GI digested peptides. Staurosporin (at 0.25 µM) was used as positive control. Values with the different lowercase letter (a, b, c) indicate significant difference ($P < 0.05$).

Cells cytotoxicity of the peptide was evaluated by exposing T4056 normal colon cell lines to the samples of the undigested and GI digested peptide for 72 hr. At 0.25 µM, Staurosporine showed a high cytotoxicity towards T4056 cells with only 7.93 % viability remaining after 72 hr of exposure indicating non-selective mode of action of this drug (Fig 6.3.). On the other hand, the samples of the fish derived peptide had resulted in a similar ($p>0.05$) effect on the cell viability. Interestingly a slight increase of the cell viability was observed for the cells treated with the sample obtained after pancreatic digestion. These results indicated that the peptide was fairly selective maintaining higher viability of the normal cells. Furthermore, as the cell viability increased upon the complete GI passage, this would confirm that the peptide digestion resulted in a release of a more potent peptide or peptides. Similarly, Chi et al. (2015) reported that a purified peptide (Trp-Pro-Pro) isolated

from blood clam (*Tegillarca granosa*) muscle exerted strong cytotoxicity toward PC-3, DU-145, H-1299 and HeLa cancer cell lines in a dose-dependent manner, yet it was non-toxic to a normal mouse fibroblast cell line NIH3T3.

6.3.3. Assessment of cell morphological changes

Apoptosis, a form of programmed cell death, normally occurs in multicellular organisms which enables them to control cell number and eliminates cells that threaten their survivals. This mechanism is characterized by a series of morphological changes such as chromatin condensation, cell shrinkage, membrane blebbing, packing of organelles, the formation of apoptotic bodies, internucleosomal DNA fragmentation and the eventual cell death (Gerl & Vaux 2005). In the present study, observation using inverted microscope revealed that peptide treatments induced apoptosis in HT-29 cells (Fig 6.4). Staurosporine (Fig 6.4B) and peptide treatments (Fig 6.4C; 6.4D; 6.4E) caused substantial apoptotic morphological changes in HT-29 cells compared to the control (Fig 6.4A).

The peptide treatments also induced morphological changes in T4056 cells (Fig. 6.5). The morphological features of apoptosis observed, include apoptotic bodies and cells shrinkage. At 0.25 µM, Staurosporine was too toxic to the normal colon cells T4056 (Fig 6.5B). It destroyed the cells membranes and resulted in massive cell death (Fig. 6.3). Staurosporine, a protein kinase C (PKC) inhibitor with a broad spectrum of activity, is an alkaloid isolated from the culture broth of *Streptomyces staurospores*. It has been known to inhibit cell cycle progression of several cell lines (Abe et al. 1991; Crissman et al. 1991). Staurosporine also showed strong cytotoxic effect on the growth of HeLa S3 cells, with an IC_{50} value of 4×10^{-12}M after 72 h exposure (Tamaoki et al. 1986).

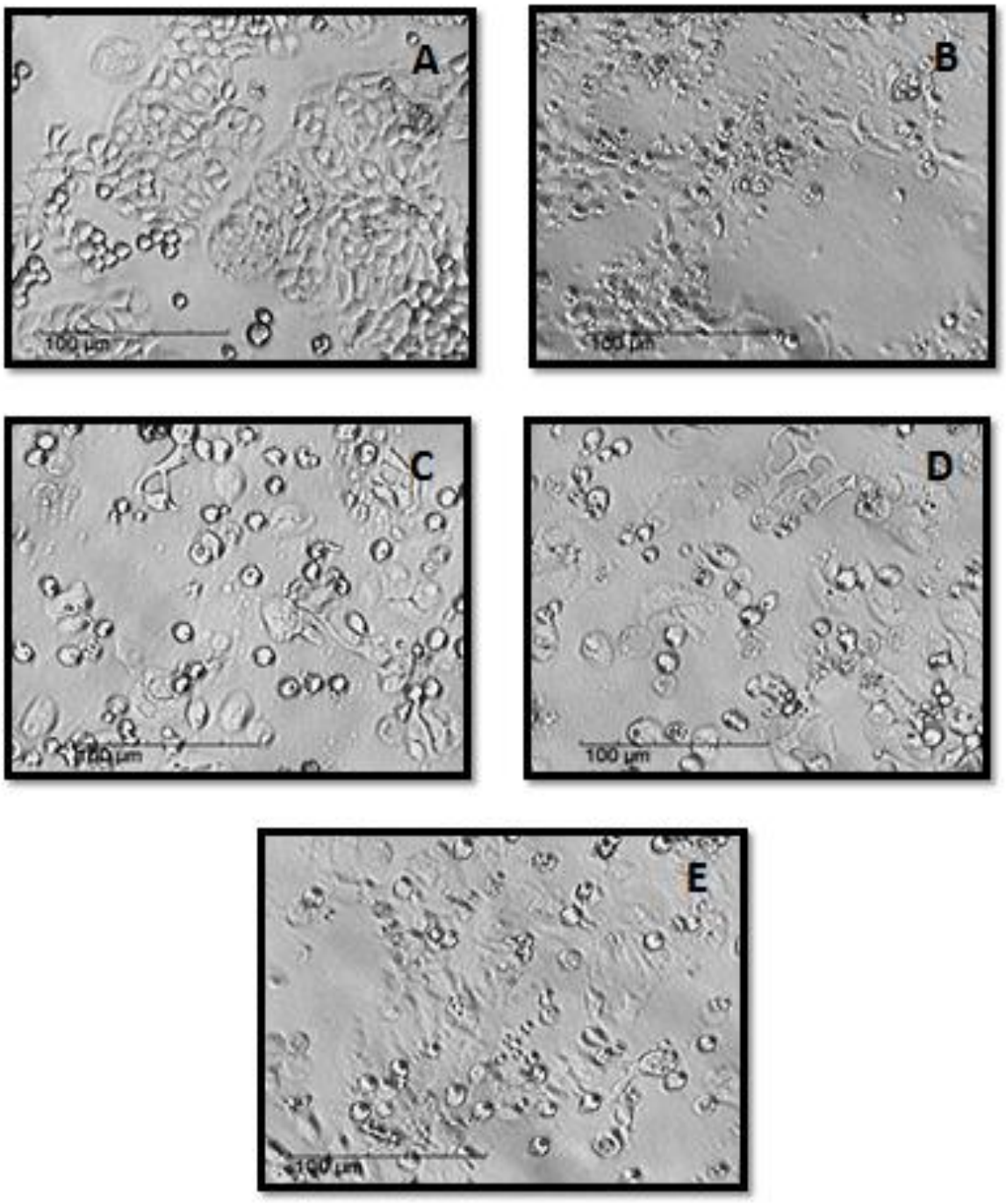


Figure 6.4. Morphological changes of HT-29 cancer cells after 72 h incubation with media/control (A); staurospoprine at 0.25 µM (B); undigested peptide (C); pepsin digested peptide (D); and pepsin and pancreatin digested peptide (E).

Morphologically, the exposure of the T4056 cells to undigested and digested peptides (Fig 6.5C; 6.5D; 6.5E) during 72 h incubation had less impact than on HT-29 cells (Fig 6.4C; 6.4D; 6.4E). T4056 showed less susceptibility to lysis as a result of peptide treatment. Wang et al. (2008) reported that cell selectivity and susceptibility to lysis were determined by the composition of cell membrane bilayers and the distribution of phospholipids. The amount of phosphatidylserine (PS) located in the outer leaflets of cancer cells is 3–7 times that in the inner leaflets of normal cells in the membranes (Leuschner & Hansel 2004). The identified peptide, Met_1-Gly_2-Pro_3-Pro_4-Gly_5-Leu_6-Ala_7-Gly_8-Ala_9-Pro_{10}-Gly_{11}-Glu_{12}-Ala_{13}-Gly_{14}-Arg_{15}. is composed of several hydrophobic amino acids including Met, Gly, Pro, Leu, and Ala, which could lead to enhanced interactions between the peptide and the outer leaflets of cancer cell membrane bilayers that have high anionic phospholipid contents. Huang et al. (2011) stated that the hydrophobic properties of peptides could play important roles in their anticancer activities. This may also support the cell selectivity properties of identified peptide.

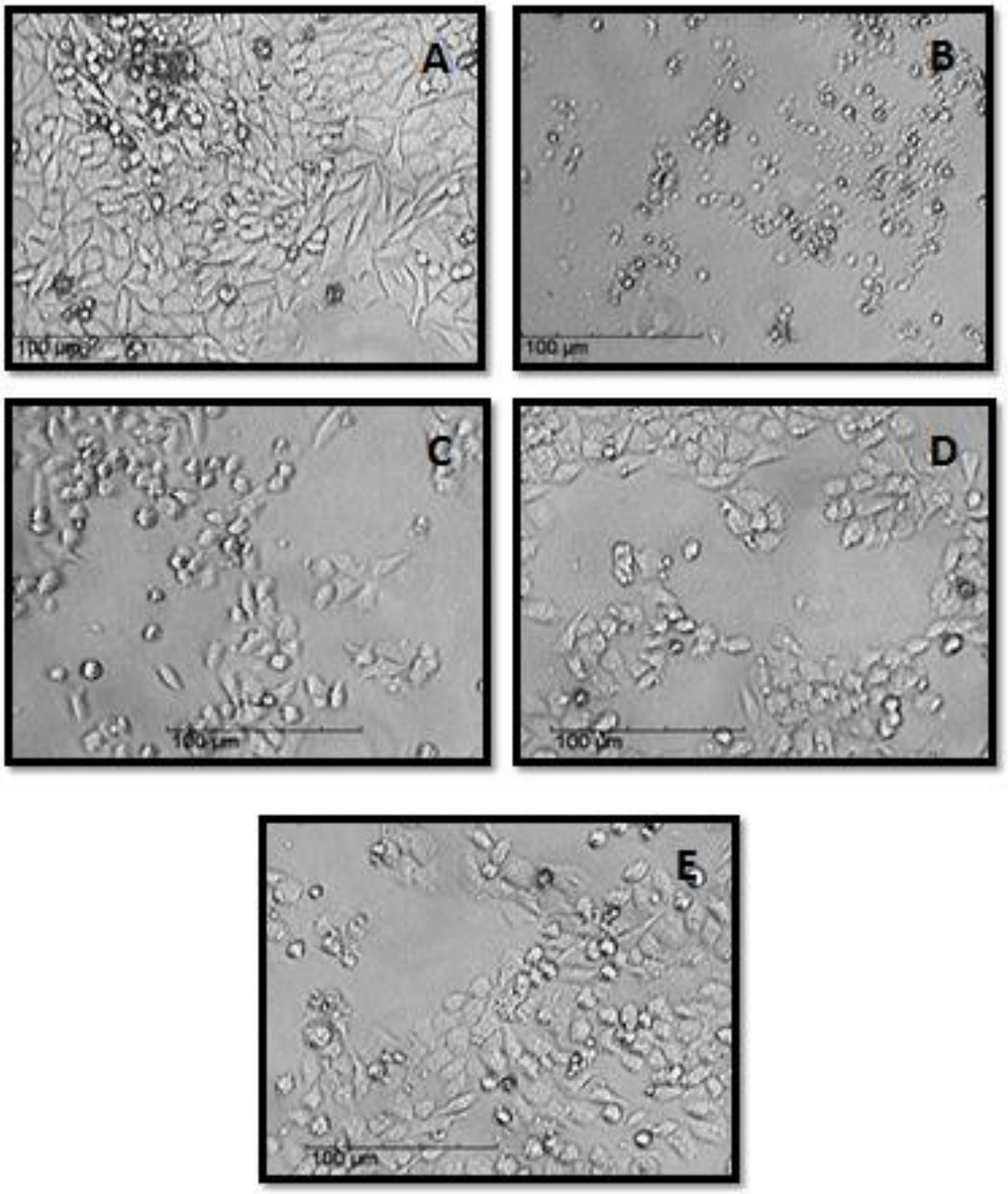
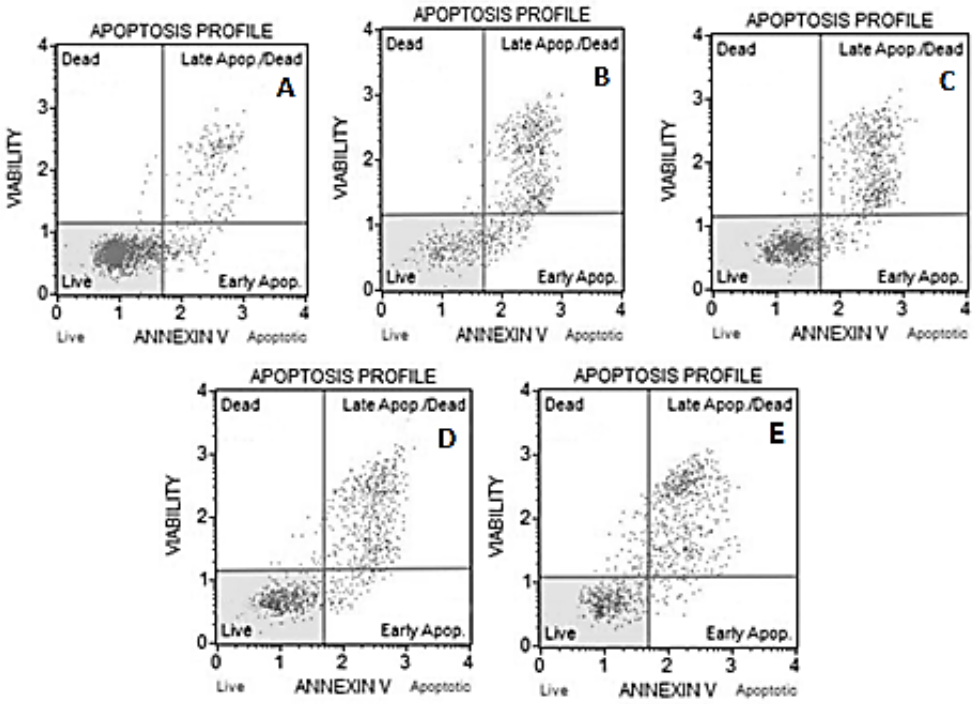


Figure 6.5. Morphological changes of T4056 normal colon cells after 72 h incubation with media/control (A); staurospoprine at 0.25 µM (B); undigested peptide (C); pepsin digested peptide (D); and pepsin and pancreatin digested peptide (E).

6.3.4. Degree of apoptosis of HT-29 colon cancer cell lines

In order to quantitatively illustrate the apoptotic process of HT-29 cells induced by staurosporine, undigested and GI digested peptides, Muse Annexin V & Dead Cell Assay was carried out. The assay is based on the detection of phosphatidylserine (PS) on the surface of apoptotic cells.



	Live (%)	Early apoptosis (%)	Late Apoptosis (%)	Dead (%)
A	83.41 ± 1.31	6.73 ± 0.49	9.41 ± 0.67	0.45 ± 0.22
B	23.78 ± 2.57	15.71 ± 0.54	59.81 ± 3.16	0.7 ± 0.26
C	48.60 ± 4.44	7.29 ± 0.94	42.94 ± 3.16	1.17 ± 0.57
D	43.2 ± 3.11	7.92 ± 0.95	47.34 ± 2.26	1.54 ± 0.03
E	38.88 ± 4.17	4.53 ± 0.98	53.06 ± 3.10	3.54 ± 2.30

Figure 6.6. Apoptosis profile of HT-29 treated with media/control (A); Staurosporine at 0.25 µM (B); undigested peptide at 0.25 mg/mL (C); pepsin digested peptide at 0.25 mg/mL (D); and pepsin and pancreatin digested peptide at 0.25 mg/mL (E) for 72 h. All data are presented as the mean ± SD of triplicate results.

As depicted in Fig. 6.6, the ratio of the apoptotic cells of the treated group (B, C, D, E) is higher than that of the control (A), confirming that peptides induced apoptosis of HT-29

cells. HT-29 cells treated with Staurosporine (B) showed the lowest number of live cells (23.78 ± 1.31%), followed by treatment E (pepsin and pancreatin digested peptide), D (pepsin digested peptide). and C (undigested peptide). The cells treated with pepsin and pancreatin digested peptide showed the highest number of dead cells (3.54 ± 2.30%). Similar ratio of live, apoptotic and dead cells was observed for treatment C and D. After 72 h of exposure, treated HT-29 cells were mostly observed in the late apoptotic stage indicated early apoptotic stage may occur earlier. The effect of different exposure period, therefore, will be the subject of further research.

6.4. Conclusion

Bioactive peptide Met_1-Gly_2-Pro_3-Pro_4-Gly_5-Leu_6-Ala_7-Gly_8-Ala_9-Pro_{10}-Gly_{11}-Glu_{12}-Ala_{13}-Gly_{14}-Arg_{15} isolated from Flathead hydrolysate showed anticancer activity before, during and after gastrointestinal (GI) digestion. Slightly higher activity was shown by peptides that underwent complete GI digestion using pepsin and pancreatin protease. These results indicated that the peptide can maintain its activity during GI digestion and has potency to be used in cancer therapy via oral administration. Anticancer properties of the identified peptide against various different cancer cells may need to be investigated.

CHAPTER 7

Chapter 7. General conclusions and Future Research Direction

7.1. General conclusions

By-products from four fish species endemic to Australia, namely aquacultured Atlantic salmon (*Salmo salar*), Barramundi (*Lates calcarifer*), wild caught Flathead (*Platycephalus fuscus*), and Silver warehou (*Seriolella punctate*), contained valuable compounds that have potential applications in formulated food systems and pharmaceutical industry. The recovery of valuable compounds was influenced by several factors including pH, temperature and ratio of added water and substrate. Using pH adjustment method, optimum oil and protein recovery from fish by-products was observed at low pH (2.5). Compared to Flathead and Silver warehou, Salmon and Barramundi by-products were better sources of fish oil with high amounts of MUFAs and PUFAs. Flathead and Salmon extracted protein consisted of lower molecular weight than Silver warehou and Baramundi. The molecular size of protein of extracted protein may influence the functional properties and their potential application in food formulation.

Fish by-products undergone autolysis or hydrolysis using exogenous protease showed potent as natural sources of antioxidants and ACE-inhibitory peptides. Hydrolysis duration and the addition of exogenous enzymes affected the degree of hydrolysis and bioactivity of peptides isolated. Flathead and Salmon showed the highest and lowest antioxidant activity, respectively. Flathead and Barramundi by-products are promising sources of ACE inhibitory peptides as no previous studies have been done on these two species. The functional properties of FPH did not entirely relate to degree of hydrolysis. Other factors such as fish species and balance between hydrophilic and hydrophobic forces of fish proteins appeared to

be involved. Salmon hydrolysate showed relatively high emulsification capacity, HCT and heat stability which makes it suitable as additive for foods that undergo high temperature processing. Silver warehou and Baramundi FPHs formed heat-induced gels, important for food structuring.

Flathead protein hydrolysate, which showed high antioxidant activity, underwent further fractionation and purification. The peptide fractions obtained exhibited noticeable radicals scavenging activities of peptides that were related to degree of hydrolysis, presence of protease and molecular weight. Low molecular size peptides (< 3 kDa) showed the highest free radicals scavenging activity *in vitro*. Flathead peptides were able to protect normal colon cells from cell injury and death during H_2O_2 exposure. < 3 kDa peptides showed strong cytotoxicity activity against HT-29 colon cancer cells although this activity was found to be non-selective. Further fractionation and purification stages generated a novel peptide that was identified as Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg.

In order to evaluate its efficacy, the identified peptide was hydrolysed using pepsin and pancreatin proteases. The identified peptide showed anticancer activity before, during and after simulated gastrointestinal (GI) digestion. As peptides that underwent complete GI digestion showed higher anticancer activity, it indicated that the parent peptide can maintain its activity during GI digestion and the resulting smaller peptides were also biologically active. Peptide, such as the one identified in the present study, and numerous others isolated from fish by products has the potential to be developed as preventative health care products in cancer therapy via oral administration.

7.2. Future Research Direction

The results showed that fish by products can be utilized and converted into protein hydrolysate with desirable properties. There are several aspects that should be considered in

order to maximize the potency of FPH. First, the implementation of simple scalable extraction technology should consider the species of fish used as it will affect the quality and functional properties of recovered compounds. Second, the use of microbial proteases is encouraged as it effectively hydrolysed fish proteins. The results from this thesis, however, also showed that endogenous enzymes efficiently hydrolysed fish by-products and produced good quality FPHs. Further studies need to be completed on physicochemical properties of FPH since there is lack of research on heat stability and rheological properties of FPH. FPH and isolated peptides obtained in this thesis may possess other biofunctional properties such as antidiabetic, antithrombotic, hypocholesterolemic, opioid, antiappetizing, immunomodulatory and antimicrobial activities. Further analyses, therefore, are expected.

Novel peptide, Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg, identified during the present study, was stabile during the simulated gastrointestinal digestion and showed favourable anti colon cancer activity. Due to its increasing activity, further purification and identification of smaller peptides produced after simulated GI digestion is recommended. Further work in the development of the identified peptide as a candidate for a cancer therapy may include: 1) detailed work on digestion of peptides in more rigorous simulated gastrointestinal digestion models; 2) transport mode of peptides across the gut wall or uptake in the circulatory system; 3) efficacy of peptide against various cancer types, e.g. breast, leukemia, colon, prostate, etc., using cell culture and animal models; and 4) substantiation of the findings from above mentioned into actual human clinical trials. Finally, the identification of peptide sources from fish was fairly challenging as limited information is available in the database; thus development of fish peptide database with the help of modern 'omics' tools, e.g. genomics, proteomics and metabolomics is urged.

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