Development of bioactive bread enriched with a seaweed peptide fraction with potential heart-health effects

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Signature ________________________ Date____________________

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TABLE OF CONTENTS

General aims...21

CHAPTER 1. General Introduction...22

1.1 Aims 23

1.2 Functional foods and health 24

1.3 Bioactive compounds in marine macroalgae.......................................26

1.4 Red algae as an alternative source of protein 28

1.5 Epidemiology of hypertension and Cardiovascular disease (CVD)................29

1.6 Protease enzymes as targets for the prevention of hypertension..................31

1.6.1 Aspartic proteases 31

1.6.2 ACE-I inhibitors...32

1.6.3 The enzyme renin and its mechanismof action in prevention of hypertension...34

1.6.4 Types of inhibitors in the RAAS...36

1.6.5 Development of renin inhibitors 37

1.6.6 Renin inhibitory compounds and peptides from plant sources 38

1.6.7 The RAAS: a potential therapeutic target in the treatment of diabetic kidney disease and other disease treatments..38

1.6.8 Platelet-activating factor acetylhydrolase (PAF-AH) and heart health.....40

1.6.9 Darapladib and the development of commercial PAF-AH inhibitors.......40

1.7 Bioactive peptides previously isolated from macroalgae..........................42

1.8 Effects of food processing on bioactive peptides....................................45

1.9 Bioavailability of bioactive peptides and survival in the gastrointestinal tract (GI tract). 47

1.10 Suitable food vehicles for delivery of bioactive peptides 49

1.10.1 Bread as a carrier for bioactive compounds 52

1.11 Final remarks 53

CHAPTER 2. Isolation and characterisation of bioactive pro-peptides with *in vitro r*enin inhibitory activities from the macroalga *Palmaria palmata* (Linnaeus) Weber and Mohr 57

2.1 Aims 58

2.2 Introduction 59

2.3 Materials and Methods 61

2.3.1 Materials and Reagents 61

2.3.2 Extraction of *Palmaria palmata* (Linnaeus) Weber and Mohr protein 61

2.3.3 Enzymatic Hydrolysis of the *Palmaria palmata* (Linnaeus) Weber and Mohr Protein Extract 63

2.3.4 Protein quantification and amino acid profiling 63

2.3.5 Fractionation of the seaweed hydrolysate using RP-HPLC 64

2.3.6 *In vitro* renin inhibitory assay 64

2.3.7 Peptide identification by tandem mass spectrometry 67

2.3.8 Database Search, confirmation of sequences, and *de novo* sequencing of peptides.. 68

2.3.9 Microwave-assisted solid phase peptide synthesis 68

2.3.10 *In silico* prediction of peptide availability 69

2.4 Results 70

2.4.1 Protein quantification 70

2.4.2 RP-HPLC enrichment of the seaweed hydrolysate 72

2.4.3 *In vitro* inhibitory effects of *P. palmaria p*apain hydrolysate on renin 74

2.4.4 Identification of renin inhibitory peptides from *P. palmata* protein hydrolysate 76

2.4.5 In silico analysis of the tridecopeptide IRLIIVLMPILMA 79

2.5 Discussion 80

CHAPTER 3. Development of a seaweed derived platelet activating factor acetylhydrolase (PAF-AH) inhibitory hydrolysate, synthesis of inhibitory peptides and assessment of their toxicity using the Zebrafish larvae assay........ 84

3.1 Aims 85

3.2 Introduction 87

3.3 Materials and Methods 89

3.3.1 Materials and Reagents 89

3.3.2 *Palmaria palmata* protein extraction and hydrolysis with Papain........ 89

3.3.3 PAF-AH inhibitor screening assay........ 89

3.3.4 Fractionation of *Palmaria palmata* hydrolysate using RP-HPLC 92

3.3.5 Peptide identification by tandem mass spectrometry........ 92

3.3.6 Database search, confirmation of sequences, and de novo sequencing 92

3.3.7 Microwave-assisted solid phase peptide synthesis 92

3.3.8 Zebrafish larvae assay 92

3.4 Results 94

3.4.1 PAF-AH inhibitory activity of *P. palmata* hydrolysates and fractions........ 94

3.4.2 PAF-AH inhibition of synthesised peptides........ 96

3.4.3 Toxicity of *P. palmata* hydrolysate on Zebrafish larvae........ 98

3.5 Discussion 101

CHAPTER 4. Identification of an active seaweed derived renin inhibitory dipeptide and confirmation of a hypotensive effect in spontaneously hypertensive rats following simulated gastrointestinal digestion……………………………………………….…..105

4.1 Aims 106

4.2 Introduction 107

4.3 Materials and Methods 109

4.3.1 Materials..109

4.3.2 *In silico* analysis of the renin inhibitory peptide IRLIIVLMPILMA 109

4.3.3 Simulated Gastric Digestion of the peptide IRLIIVLMPILMA 109

4.3.4 Removal of polyethylene glycols from *Palmaria palmata* protein digested samples using a titanium dioxide (TiO_2) cleanup procedure. 112

4.3.5 Tandem mass spectrometry analysis of the hydrolysate 113

4.3.6 In vivo determination of the hypotensive effect of the *P. palmata* protein hydrolysate and the tridecapeptide IRLIIVLMPILMA in spontaneously hypertensive rats (SHRs)..113

4.3.7 Statistics 114

4.4 Results 115

4.4.1 *In silico* analysis of IRLIIVLMPILMA 115

4.4.2 ESI-Q-TOF analysis of the *in vitro* GI simulated digestion of IRLIIVLMPILMA 115

4.4.3 Short term hypotensive effects of *P. palmata* protein hydrolysate and IRLIIVLMPILMA in SHR 117

4.5 Discussion 119

CHAPTER 5. Increasing the health benefits of bread: Assessment of the physical and sensory qualities of bread formulated using a renin inhibitory *Palmaria palmata* protein hydrolysate.……………………………………………………………..….122

5.1 Aim..123

5.2 Introduction 124

5.3 Materials and Methods 126

5.3.1 *Palmaria palmata* protein extraction and hydrolysis with Papain.......... 126

5.3.2 Bread ingredients 126

5.3.3 Preparation of breads.. 126

5.3.4 Bread evaluation – loaf volume, weight and loaf specific volume calculations.. 128

5.3.5 Heavy metal and Iodine analysis of seaweed bread product.................. 129

5.3.6 Sensory analysis 129

5.3.7 Renin inhibitory assay 129

5.3.8 Microscopy... 129

5.3.9 Statistics 131

5.4 Results 132

5.4.1 Loaf volume and crust/crumb colour 132

5.4.2 Digital image analysis of bread crumb 133

5.4.3 Texture profile analysis (TPA) of bread crumb 134

5.4.4 Brightfield light microscopy of bread samples 136

5.4.5 Confocal scanning laser microscopy of bread samples.......................... 137

5.4.6 Heavy metal and Iodine analysis of the bread product 140

5.4.7 Sensory analysis of breads 140

5.4.8 Renin inhibitory activity of bread samples 141

5.5 Discussion 143

CHAPTER 6. General discussion and conclusion……………………………..148

TABLE OF FIGURES

Chapter 1

Figure 1.1: The Renin Angiotensin Aldosterone System..30

Figure 1.2: The structure of renin.. 35

Figure 1.3: Sites of inhibition of the RAAS...36

Figure 1.4: Expansion of the necrotic core due to PAF-AH activity. 42

Figure 1.5: Intestinal transport mechanisms for peptides. 47

Chapter 2

Figure 2.1: Schematic representation of the bioassay guided isolation and characterisation 62

Figure 2.2: Renin inhiition assay. 66

Figure 2.3: ESI-Q-TOF system for identifying renin inhibitory peptides. 67

Figure 2.4: RP-HPLC chromatogram of a papain hydrolysate of *P. palmata* protein....73

Figure 2.5: Renin inhibitory assay. 75

Chapter 3

Figure 3.1: The PAF-AH inhibition assay. 91

Figure 3.2: PAF-AH inhibitory activities of seven, chemically synthesized peptides isolated from *Palmaria palmata* protein. 97

Figure 3.3: Survival of zebrafish larvae over 48 hrs 99

Figure 3.4: Images of the larvae after 24 h exposure (A) and 48 h exposure (B).........100

Chapter 4

Figure 4.1: Schematic of simulated *in vitro* gastric digestion.....................................111

Figure 4.2: UPLC chromatograms of the hydrolysed analytes. 116

Figure 4.3: Nano-ESI-MS spectra data of the hydrolysed peptide 117

Figure 4.4: SBP of SHRs over 24hrs 118

Chapter 5

Figure 5.1: Images of all four bread samples examined. 127

Figure 5.2: Texture profile analysis and moisture content...135

Figure 5.3: Light micrograph images of bread samples..137

Figure 5.4: Confocal micrographs of bread samples 139

Figure 5.5: Sensory analysis. 141

Figure 5.6: Renin inhibitory assay of breads. 142

Annex

Figure 8.1: Purification of IRLIIVLMPILMA..184
Figure 8.2: Purification of NIGK.. 185
Figure 8.3: Non linear regression of IRLIIVLMPILMA concentration versus percentage inhibition. 186
Figure 8.4: Non linear regression of NIGK 187
Figure 8.5: PAF-AH assay of HPLC fractions 10-25 188

TABLE OF TABLES

Chapter 1

Table 1.1: Bioactive peptide containing products available commercially 51

Chapter 2

Table 2.1: Amino acid composition of protein extracted from *Palmaria palmata* 71

Table 2.2: Peptides identified in RP-HPLC Fr-25 enriched from the *P. palmata* papain hydrolysate. 78

Table 2.3: Cleavage of the peptide IRLIIVLMPILMA 79

Chapter 3

Table 3.1: Peptides identified by tandem mass 95

Chapter 4

Table 4.1: Concentrations of electrolytes used to create the simulated saliva fluid (SSF) simulated gastric fluid (SGF) and simulated duodenal fluid (SDF). 110

Table 4.2: Cleavage points of the peptide IRLIIVLMPILMA 115

Chapter 5

Table 5.1: The bread formulations used in this study 128

Table 5.2: Volume and colour analysis of the control 132

Table 5.3: Image analysis results 133

ABBREVIATIONS

Δ SBP – change in systolic blood pressure

a* – redness/greenness

ACE-I – angiotensin converting enzyme I

AChe – acetylcholinesterase

APP – amyloid precursor protein

ARA – aldosterone-receptor antagonists

ARB – angiotenisn receptor blocker

ATP – adenosine triphosphate

B – buckwheat bread

b* – brightness

BACE1 – β-secretase

BCA – bicinchoninic acid

BH – buckwheat bread containing 4% *P. palmata* hydrolysate

BMI – body mass index

BPM – beats per minute

BSA – bovine serum albumin

C – control wheat bread

CH – control wheat bread containing 4% *P. palmata* hydrolysate

COPD – chronic obstructive pulmonary disease

CVD – cardio-vascular disease

DBP – diastolic blood pressure

DDA – data dependent acquisition

DHA – docosahexaenoic acid

DMSO – dimethyl sulfoxide

DRI – direct renin inhibitor

DTNB – 5,5’ Dithio-bis (2-nitrobenzoic acid)

EC – enzyme commission number

EDANS – 5-(2-aminoethylamino)naphthalene-1-sulfonic acid

EPA – eicosapentaenoic acid

ESI-Q-TOF MS – electro spray ionization-quaternary-time of flight mass spectrometry

FA – formic acid

FB – fluorescent brightener

FDA – food and drug administration

FG – fast green

FITC – fluorescein iso-thiocynate

Fr – fraction

gACE – germinal angiotensin converting enzyme

GalNAc – *N*-Acetylgalactosamine

GI – gastro-intestinal

Glu-Fib – glufibrinopeptide

GRAS – generally recognized as safe

GSK – GlaxoSmithKline

HIV – human immunodeficiency virus

HMPB – 4-hydroxymethyl-3-methoxyphenoxybutyric acid

HNA – 2″-Hydroxynicotianamine

HPLC – high performance liquid chromatographyhrs – hours

IC_{50} –The half maximal inhibitory concentration

IOP – intraocular pressure

L* – lightness

LAB – lactic acid bacteria

LDL – low density lipoprotein

LHRH – luteinizing hormone-releasing hormone

LPC – lysophosphatidylcholine

Lp-PLA2 – lipoprotein-associated phospholipase A_2

m/z – mass/charge

MAFP – methyl arachidonyl fluorophosphates

MALDI-TOF – Matrix-assisted laser desorption/ionization-time of flight

Mpa – megapascal

MSG – monosodium glutamate

MWCO – molecular weight cut-off

MW-SPPS – microwave assisted solid phase peptide synthesis

NCBI – National Center for Biotechnology informationoxNEFA – oxidized non-esterified fatty acids

PAF-AH – platelet activating factor acetylhydrolase

PBS – phosphate buffered saline

PLGS- protein lynx global server

PUFAs – poly-unsaturated fatty acids

RAAS – Renin angiotensin aldosterone system

RIP – type-2 ribosome inactivating proteins

RP-HPLC – reverse phase- high performance liquid chromatogrphy

rpm – revolutions per minute

sACE – somatic angiotensin converting enzyme

SBP – systolic blood pressure

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SHR – spontaneous hypertensive rats

TAG – tumour associated glycoprotein

TFA – trifluoroacetic acid

TOF – time of flight

TPA – texture profile analysis

TRH – Thyrotropin Releasing Hormone

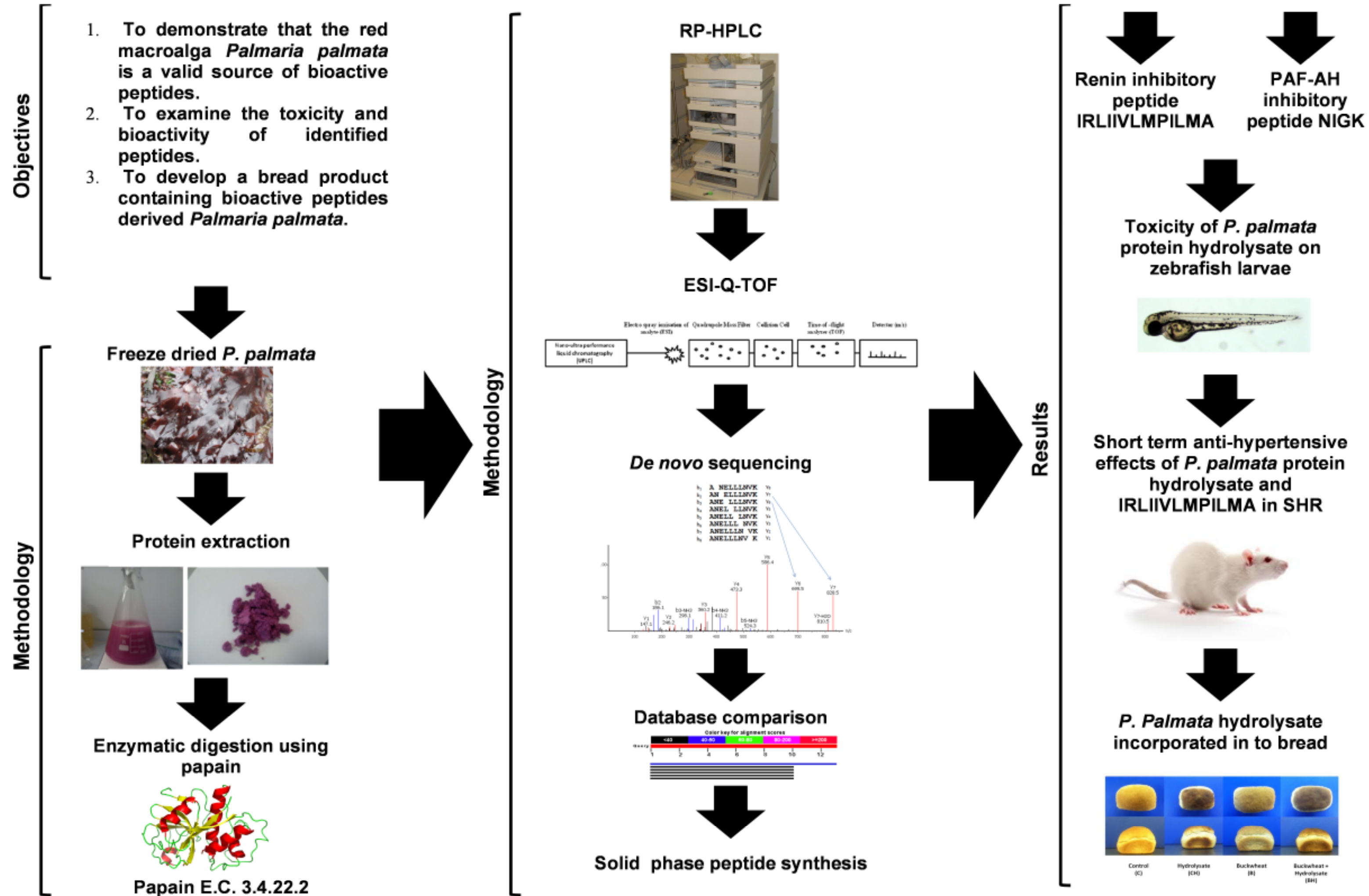
Tris – tris(hydroxymethyl)aminomethane

UPLC – Ultra High Performance Liquid Chromatography

UV – ultra violet

VLDL – very low density lipoprotein

GRAPHICAL ABSTRACT



LIST OF PUBLICATIONS WHICH RESULTED FROM THIS PH.D THESIS

(1) **Fitzgerald, C**, Gallagher, E., O'Connor, P., Prieto, Soler-Vila, A., Grealy, M. (2013), Development of a seaweed derived Platelet Activating Factor Acetylhydrolase (PAF-AH) inhibitory hydrolysate, synthesis of inhibitory peptides and assessment of their toxicity using the Zebrafish larvae assay. *Peptides*, In press.

(2) **Fitzgerald, C**., Mora-Soler, L., Gallagher, E., O'Connor, P., Prieto, J., Soler-Vila, A., Hayes, M. (2012), Isolation and characterisation of bioactive pro-peptides with *in vitro* renin inhibitory activities from the macroalga *Palmaria palmata*. *J. Agricultural and Food Chemistry*, 60, 30, 7421-7427.

(3) **Fitzgerald, C**., Gallagher, E., Tasdemir, D., Hayes, M. (2011), Heart health peptides from macroalgae and their potential use in functional foods, *Journal of Agricultural food chemistry*, 16, 6829-6836.

(4) **Fitzgerald, C.,** Gallagher, E., Doran, L., Auty, M., Prieto, J., Hayes, M. (2014), Increasing the health benefits of bread: Assessment of the physical and sensory qualities of bread formulated using a renin inhibitory *Palmaria palmata* protein hydrolysate. *LWT - Food Science and Technology*, 56, 2, 398-405.

(5) **Fitzgerald, C.,** Aluko, R., Hossain, M., Rai, D., Hayes, M. (2014), Degradation of a macroalgal derived tridecapeptide by gastrointestinal digestion liberates a dipeptide which produces an antihypertensive effect in spontaneously hypertensive rats. *Journal of Agricultural food chemistry*, *In press*.

CONFERENCE ATTENDED

(1) Presented a poster entitled "Isolation of heart healthy peptides derived from *Palmaria palmata* and incorporation of these peptides in bread" at Euro Food Chem XVI held in Gdansk in July 2011. This poster received the young researcher award sponsored by CRC press for best poster presentation.

(2) Presented a poster entitled "Isolation and characterisation of bioactive peptides with *in vitro* renin inhibitory activities from the macroalga *Palmaria palmata*" at the NutraMara 2012 conference held in Dublin from the 25th to the 26th of April 2012. This poster received the best poster award sponsored by United Fish Industries and Springer, New York.

(3) Presented a 15 minute talk entitled "Isolation and characterisation of bioactive peptides with *in vitro* renin inhibitory activities from the macroalga *Palmaria palmata.*" at the 10th Nordic Nutrition Conference in Reykjavik on 4th of June 2012.

(4) Gave an oral presentation entitled "Dulse seaweed proteins and peptides: Potential as heart health ingredients and their delivery in bread products" at the annual Walsh Fellowship seminar held in the RDS Dublin on the 22nd of November 2012.

(5) Presented a poster entitled "Simulated digestion of a novel renin inhibitor isolated from the macroalgae *Palmaria palmata*"at the 2nd International Conference on Food Digestion (INFOGEST) which ran from the 6th -8th of March 2013 at CIAL and ICMAT, Madrid, Spain.

(6) Gave an oral presentation of my PhD research entitled "Dulse seaweed proteins and peptides: Potential as heart health ingredients and their delivery

in bread products" at the annual student research day at UCL, London on the 20th of September 2013.

(7) Presented a poster and oral presentation entitled "Development of a bioactive bread enriched with seaweed protein and peptide fractions with potential heart-health effects" at the NutraMara student event entitled "The World Is Your Oyster: How You Eat It Is Up To You" on October 31st 2013 at Teagasc Food Research Centre, Ashtown.

ABSTRACT

Cardiovascular disease (CVD) is currently a global epidemic and is now the leading cause of mortality worldwide. The two major approaches for the prevention of CVD in the developed world are public health based Policies and clinical based Strategies focusing on high-risk individuals. Pharmaceutical companies have developed a range of treatments to tackle the causes of CVD and these include the development of anti-inflammatory, anti-hypertensive and anti-cholesterol drugs. However, unpleasant side-effects often exist with prescription drug-taking. Sourcing of natural food components from foods to provide protection against the development of CVDis a useful strategy to help combat illness.

This thesis aims to utilise Irish macroalgae as a source of bioactive compounds which can be delivered in the food vehicle Bread to confer a Heart health effect to the consumer. Hypertension, one of the major risk factors associated with CVD may be controlled by inhibiting enzymes of the reninangiotensin aldosterone System (RAAS). Inhibition of the enzyme renin is an important strategy for the control of hypertension as renin is the initial and rate limiting enzyme of the RAAS. Inhibition of the circulating enzyme platelet activating factor acetylhydrolase (PAF-AH) is also important in the control of atherosclerosis development. PAF-AH generates two pro-inflammatory mediators lysophosphatidylcholine (LPC) and oxidized non-esterified fatty acids (oxNEFAs). Both of these mediators are involved in promotion of atherosclerotic plaque which may be lead to high blood pressure development.

Macroalgae are part of the regular diet of many Asian cultures and have a tradition of being consumed in many coastal regions of the Western World. Regular consumption of macroalgae is associated with a decline in the prevalence of breast cancer and diabetes

mellitus development. Species of macroalgae belonging to the group known as *Rhodophyta* or the red macroalgae are known to contain levels as high as 47 % protein. Several bioactive peptides, including ACE-Iinhibitory peptides were isolated previously from macroalgae protein extracts and hydrolysates.

In this thesis the extraction and isolation of renin and PAF-AH inhibitory peptides from the macroalgae *Palmaria palmata* was carried out. The effectiveness of the isolated renin inhibitory tridecapeptide IRLIIVLMPILMA was further explored in terms of its capacity to survive gastrointestinal (GI) digestion and its ability to lower blood pressure *in vivo* in spontaneously hypertensive rats (SHRs). The *Palmaria palmata* protein hydrolysate from which these peptides were identified was subsequently incorporated in to bread and the effects of its addition were observed in terms of volume, colour, texture profile, moisture, crumb structure, sensory attributes and renin inhibitory activity.

GENERAL AIMS

This project examines the feasibility of creating a bioactive bread product containing renin and PAF-AH inhibitory peptides derived from the Red seaweed *Palmaria palmata* (Linneaus) Weber and Mohr. The aims of this project were:

(1) To demonstrate that the red macroalgae *Palmaria palmata* is a valid source of renin and platelet activating factor acetylhydrolase inhibitory peptides.

(2) To examine the functionality of the bioactive peptides and the protein hydrolysates generated by studying their bioavailability, toxicity and bioactivity *in vivo* through the use of simulated gastrointestinal digestion, zebrafish larvae assays and *in vivo* analysis using spontaneously hypertensive rats (SHRs).

(3) To develop a bread product containing renin and PAF-AH inhibitory peptides derived from the red macroalga *Palmaria palmata* and to assess if this bread maintains its renin inhibitory activity following the baking process.

CHAPTER 1. GENERAL INTRODUCTION

Parts of this chapter were published as a review paper in the Journal of Agriculture and Food Chemistry: Ciarán Fitzgerald, Eimear Gallagher, Deniz Tasdemir, and Maria Hayes, Heart health peptides from macroalgae and their potential use in functional foods, (2011), 59, 6829-683.

1.1 Aims

The aim of this introductory chapter is to highlight the potential of bioactive peptides with health benefits derived from macroalgae and to discuss the feasibility of delivering these peptides in baked goods and other food products. It also aims to give an overview of the topics listed below:

(1) Functional Foods.

(2) Marine derived bioactive compounds and peptides.

(3) The epidemiology of cardiovascular disease (CVD).

(4) Protease inhibition and its role in CVD prevention.

1.2 Functional foods and health

Research has shown that diet, nutrition and health are intimately linked [1]. A functional food may be defined as a food that imparts a health benefit to the consumer that goes beyond basic human nutrition. The concept of functional foods as a means to protect the health of consumers was developed at the beginning of the 1980s in Japan as a way to reduce the high health costs of a population with long life expectancy projections [2]. According to the American Academy of Nutrition and Dietetics, all foods provide some level of physiological function but the term functional foods is reserved for foods along with fortified, enriched, or enhanced foods that have a potentially beneficial effect on health when consumed as part of a varied diet, on a regular basis, at effective levels based on significant standards of evidence [3]. A simple example of a functional food readily available on the market at the moment would be bread enriched Omega-3 fatty acids [4].

Peptides may be either protein derived molecules or exist endogenously within a system and are the most diverse and most widely studied food derived biomolecules [3]. A peptide is generally defined as a molecule 2-30 amino acids long linked by amide bonds. Bioactive peptides are amino acid sequences of between 2-30 amino acids in length that impart a positive, “hormone-like” response to the consumer with actual health benefits *in vivo*. These peptides play an important role in living body systems by directing and coordinating intra and inter-cellular communications and cellular functions. Peptides have greater bioavailability than proteins or free amino acids and peptides with low molecular weights are less allergenic than native proteins [1]. Indeed, protein hydrolysates containing peptides are often used in hypoallergenic infant food formulations for this reason [5]. Moreover, in the United States, there has been a 1300%

increase in the number of new peptide chemical entities entering clinical study since the 1970s.

One advantage of food-derived peptide candidates is that they are not anticipated to have unforeseen side-effects as proteins and peptides have a long history of use and are generally regarded as safe (GRAS)[6]. However, there are a number of problems associated with the use of peptides including the short half-life of peptides and their effective delivery to the target site [7]. Bioactive peptides are known to have a number of therapeutic applications including their use as antimicrobial agents [8], cytomodulatory [9] and immunomodulatory agents [10], derma-pharmaceutical applications [11], antioxidant [12] and heart health activities [13]. Peptides derived from dairy [14], soy [15] and terrestrial based plant materials [16] are also known to have positive effects against diseases associated with the development of metabolic syndrome; diabetes [17], hypertension and stroke [18] and obesity [19]. From 2011 onward the functional foods market is expected to reach U.S. $167 billion with a yearly growth potential of 10% [20]. This predicted increased demand for functional foods may be due to increased healthcare costs coupled with a steady increase in life expectancy and a desire for an improved quality of life in advancing years.

This thesis focuses on the generation, isolation and characterisation of renin and platelet activating factor acetylhydrolase (PAF-AH) inhibitory peptides from the red seaweed *Palmaria palmata* (Linnaeus) Weber and Mohr. It describes chemical synthesis and survival of a renin inhibitory peptide assessed using a simulated gastrointestinal digestion model. Peptides identified in the seaweed hydrolysate were also tested for their ability to inhibit the enzyme platelet activating factor acetylhydrolase (PAF-AH). Spontaneously hypertensive rats were used to assess the effects of the renin peptide and the hydrolysate on blood pressure *in vivo*. Finally, the peptide containing hydrolysate

was formulated into a bread product and renin activity was measured. Sensory analysis on the final bread product using semi trained taste panels is also shown.

1.3 Bioactive compounds in marine macroalgae

Algae may be defined as oxygenic photosynthesisers other than that of terrestrial plants [21]. Marine macroalgae, or seaweed, are plant-like algae that generally live attached to rock or other hard substrata in coastal areas [21]. Marine organisms including seaweeds and microalgae, as a result of their exigent, competitive, and aggressive surroundings produce specific and active biomolecules and secondary metabolites [22]. These secondary metabolites help to protect seaweeds from the harsh conditions in which they exist, including extremes of salinity and temperature and ultra violet (UV) irradiation, coupled with nutrient deficiencies [23]. Until recently, seaweeds or macroalgae were primarily used as a source of functional and technological ingredients in the food industry for use as emulsifying agents and to enhance viscosity and gelation in food formulations, pharmaceuticals, and cosmetics [24]. In addition to their technological properties, macroalgae exhibit original and interesting nutritional properties [25]. Edible marine algae are regularly consumed among the East Asian populations of China, Korea, and Japan, [26] and they are a rich source of polysaccharides, dietary fibre, minerals, and proteins [25].

Seaweeds consumed in their whole form can have beneficial physiological effects, [27, 28] and many bioactive compounds including peptides, carbohydrates, and fats (more importantly polyunsaturated fats PUFAs) sourced from macroalgae have been identified [29, 30]. Seaweeds are viewed as "natural" by consumers, who favour natural rather than chemically synthesised ingredients [31].

Edible macroalgae, including algae from the Protista orders, Phaeophyta (brown algae), Chlorophyta (green algae), and Rhodophyta (red algae), have a long history of use in the

human diet [32]. The Japanese are the main consumers of macroalgae, eating 1.6 kg (dry weight) per capita per year [32]. In Japanese and Korean cuisine, red algae consumed include "Nori" (or "Kim") and "Laver" from *Porphyra* species. Additionally, the red alga *Palmaria palmata*, known as "Dulse", has a long tradition of consumption in coastal European and North American regions [33]. Brown kelp is also consumed in Japan. For example, "hijiki" (*Hijikia fusiformis (Harvey*) *Okomura*), "Wakame" (*Undaria pinnatifida* (Harvey) *Suringar*), and "Makonbu" (*Laminaria japonica Areschoug*) and species of *Laminaria* are also eaten in China, where it is referred to as "hai dai". Furthermore, green algae from *Ulva* species are consumed as part of a traditional Hawaiian cuisine which is known as "Limu palahalaha" [33]. From a nutritional point of view, edible macroalgae are a low-calorie containing food, with a high concentration of minerals, vitamins, and proteins that have low lipid content. Macroalgae are an excellent source of vitamins A, C, D, and E along with the B vitamins including riboflavin, niacin, pantothenic acid, and folic acid. They also contain minerals including calcium (Ca), phosphorus (P), sodium (Na), and potassium (K) [25]. Macroalgae are rich in polysaccharides, notably cell wall, structural polysaccharides. Most of these polysaccharides, which include the agars, carrageenans, ulvans, and fucoidans, cannot be digested in the human gastrointestinal tract and therefore may be regarded as a good source of dietary fibre and a potential source of prebiotics [34]. Prebiotics are selectively fermented ingredients that allow specific changes in both the composition and/or activity of the gastrointestinal microflora that confers benefits upon the host's well-being and health [34]. Regular consumption of macroalgae in the whole form is associated with many positive health benefits. For example, a recent epidemiological study of 362 women aged 30-65 years old suggested that daily consumption of "gim" (*Porphyra* species), an edible macroalga traditionally eaten in Korea, was inversely associated with the risk of breast cancer development [27]. Similar

research suggests that alga consumption may decrease the risk of diabetes mellitus in Korean men [28].

1.4 Red algae as an alternative source of protein

Red algae, which belong to the phylum Rhodophyta, are an ancient group of eukaryotic organisms that are mainly derived from the marine environment. They account for the vast majority of seaweed species currently known and represent the dominant group in terms of biodiversity in all seaweed floras of the world [35]. The red seaweed used in this study, *Palmaria palmata* (Linneaus) Weber and Mohr is a reddish brown, membranous or leathery macroalga with flattened fronds of between 50-300 (1000) mm long, arising from a discoid base, usually with a small stipe expanding gradually to form simple or dichotomously and palmately divided fronds, often with characteristic marginal leaflets [36]. Traditionally, this seaweed was consumed as a food in the Republic of Ireland, Scotland, Iceland, Norway and France. It has the non-scientific and common names *Dillisk, Dulse* or *Creathnach* in Irish and is still consumed dried or cooked. *Palmaria palmata* occurs in the North East Atlantic but has also been successfully cultivated in Ireland and Germany and represents a suitable candidate source of proteins for this reason. Depending on the season and geographical location, the protein content of *P. palmata* can vary between nine and twenty-five percent peaking during the winter months and lowest during the warmer months of summer [32]. This fluctuation in protein is mostly caused by the amount of sunlight hours and the need to create more pigments to up-regulate photosynthesis [37]. Moreover, *P. palmata* protein contains all of the eight essential amino acids required by humans. The essential amino acid content in *P. palmata* represents 26-50% of total protein content which is similar to egg protein or proteins from legumes such as soya [38].

1.5 Epidemiology of hypertension and cardiovascular disease (CVD)

Cardiovascular disease claims more lives each year than the five next leading causes of death combined, namely, cancer, influenza, chronic obstructive pulmonary disease (COPD), accidents, and diabetes mellitus [39]. Currently in the United States, an estimated 81 million adults (1 in 3) have one or more symptoms of CVD, and of this figure, an estimated 74,500,000 people suffer from high blood pressure or hypertension [40]. Hypertension or high blood pressure is one of the major, yet controllable, risk factors in the development of CVD [41]. It is defined as systolic blood pressure (SBP) above 140 mmHg and/or diastolic blood pressure (DBP) above 90 mmHg [42]. To combat hypertension, various stages of the renin angiotensin aldosterone system (RAAS) which is shown in Figure 1.1 can be positively affected. This system is responsible for the control of blood pressure and fluid balance in humans. The RAAS system involves the enzymes renin (Enzyme Commission number (E.C.) 3.4.23.15) and angiotensin- I-converting enzyme (ACE-I) (E.C. 3.4.15.1) and is capable of stimulating atherosclerosis by triggering basic reactions that ultimately lead to growth, instability, and rupture of atherosclerotic plaques and facilitation of thrombosis [43]. Two ways of inhibiting this system include firstly blocking the formation of angiotensin-II by the enzyme ACE-I from angiotensin I and secondly by inhibiting the conversion of angiotensinogen into angiotensin-I by the enzyme renin[43].

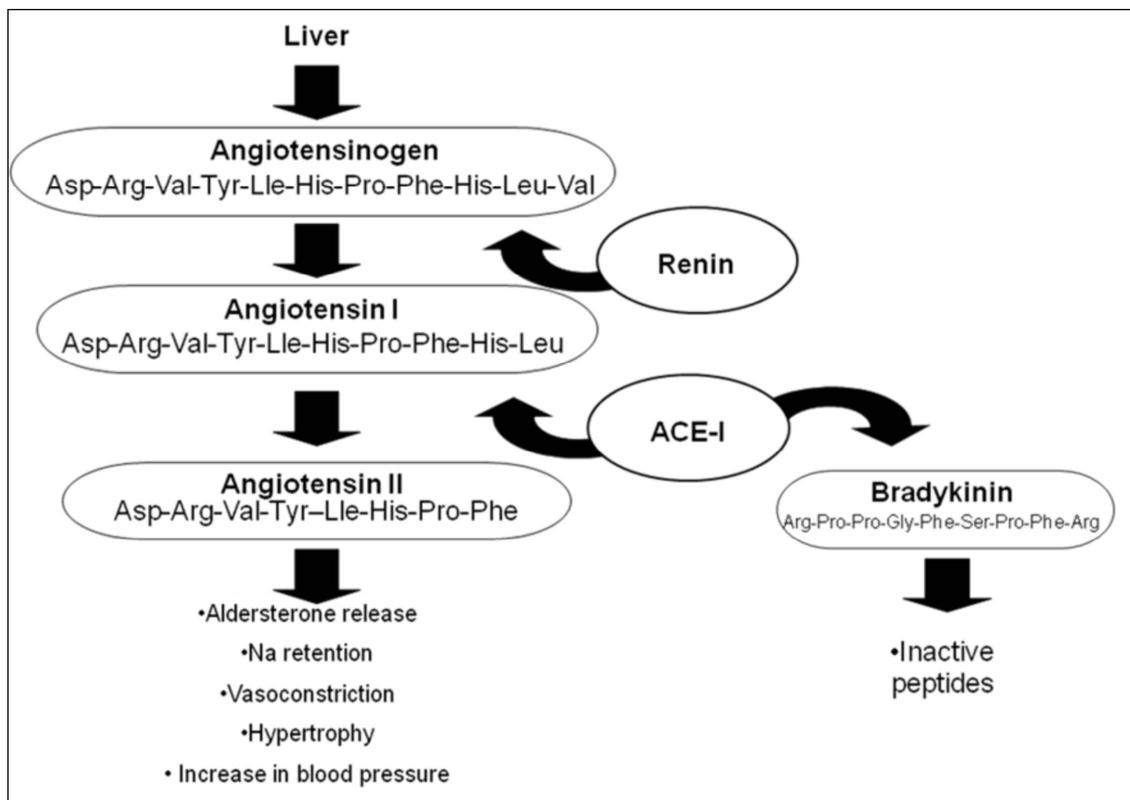


Figure 1.1: The Renin angiotensin aldosterone system.In the initial and rate-limiting reaction of the RAAS, renin hydrolyses angiotensinogen, its' only known substrate to form angiotensin-I. ACE-I in turn hydrolyses the decapeptide angiotensin-I to form the octapeptide angiotensin-II, a potent vaso-constrictive molecule. ACE-I also acts on the vasodilatory peptide bradykinin, inactivating it, which in turn further raises blood pressure.

1.6 Protease enzymes as targets for the prevention of hypertension

Proteases are proteolytic enzymes that catalyse the hydrolysis of the peptide bond in peptides and proteins [44]. Proteases are classified based on the key residue they use to catalyze the hydrolysis of the peptide bond. Hydrolysis can occur either at the N- or C-terminus of the peptide chain, catalysed by proteins referred to as exopeptidases, which are divided into two groups; the aminopeptidases and the carboxypeptidases respectively. Serine, cysteine and threonine proteases bind their substrates covalently, whereas aspartic, metallo and glutamic proteases act through a general acid-base mechanism [43]. Proteases and modified protease activities in disease states are targets for multiple diseases and hence are potential bioactive peptide drug or functional food targets. Human immunodeficiency virus protease (HIV-protease), high blood pressure and thrombosis are examples of diseases where the disease related protease is the therapeutic target [44, 45]. Recently, three new enzyme targets have been validated by the Food Development Authority (FDA) approval of new enzyme inhibitor drugs. These include mitogen-activated protein kinase, renin, and dipeptidyl peptidase IV [46].

1.6.1 Aspartic proteases

A minor protease class, aspartic proteases use the general acid-base hydrolysis mechanism of action. Most proteases belong to either the A1 family of pepsin-like proteases or the A2 retroviral family [47]. Aspartic proteases have been identified in plants, fungi, mammals, viruses and protozoa [48]. In humans, members of the aspartic protease class form part of the digestive system with non-specificity for peptide substrates as is the case with pepsin (EC 3.4.23.2) and gastricin (EC 3.4.23.3). However, in humans, it also includes proteases with a single substrate such as renin with its substrate angiotensinogen, and β-secretase (EC 3.4.23.46) (BACE1) that hydrolyses the amyloid precursor protein (APP) as its only substrate. Aspartic proteases

can be expressed in a single, cellular compartment as is the case with cathepsin D which is expressed in the lysosomes [49]. Alternatively, it can be secreted with pepsin and gastricin, or can be expressed in specific tissues that restrict their activities, such as BACE1 in the brain [50].

1.6.2 ACE-I inhibitors

In mammals, two forms of ACE-I exist; one expressed in somatic tissue (sACE, 1306 residues), which has two active sites and the other ACE-I which is expressed in germinal cells in the male testes (gACE, 732 residues) which has one active site. sACE is a translated tandem duplication. This duplicated structure produces a protein with two domains, the N-domain and the C-domain [51]. Despite having around 60% sequence homology with each other, studies have highlighted the unique physiological roles of the N- and C- domains of ACE-I [52]. The principle functional unit in each domain is the M2-type zinc metallopeptidase motif, an His-Gluxx-His with a Glu positioned 23-24 residues further towards the C-terminus, these residues are ligands for the zinc cofactor required for the peptidase catalytic activity [51]. Through its role in the RAAS, ACE-I plays a key role in the regulation of blood pressure and electrolyte homeostasis [53]. It carries out this function by hydrolysing peptides through the removal of a dipeptide from the C-terminus, as is the case in the conversion of angiotensin I to angiotensin II, or the degradation of bradykinin. It can also act as an endopeptidase, shown by cleavage of peptides with amidated C-termini [54].

Inhibition of ACE-I is considered to be a useful therapeutic approach in the treatment of hypertension [55]. The first ACE-I inhibitor was discovered when Ferreira (1964) discovered a "bradykinin potentiating factor" in the venom of the snake *Bothrops jararaca* [56]. Many studies have been attempted regarding the synthesis of ACE-I inhibitors such as captopril, enalapril, alacepril and lisinopril, which are currently used

in the treatment of essential hypertension and heart failure in humans (marketed under the commercial names of Capoten ®, Vasotec ®, Alcacepril ® and Prenevil ® respectively) [55]. Clinical studies have revealed that ACE-I inhibitors significantly reduce the morbidity and mortality of patients with myocardial infarction or heart failure. These inhibitors can bind to the active site to block it, or to an inhibitor binding site that is remote from the active site to alter the enzymes conformation such that the substrate no longer binds to the ACE-I active site [55]. However, chemically synthesized ACE-I inhibitors do have side effects. Chronic cough is a well described non-dose dependent effect of the ACE-I inhibitor drugs which is reported in 5-35% of patients who are prescribed the agents [57]. Despite the promise of ACE-I inhibitors nearly 70% of patients still fail to achieve adequate blood pressure control, and the desired improvements in cardiovascular morbidity and mortality have also failed to materialize. Both ACE-I inhibitors and angiotensin receptor blockers (ARBs) interrupt the negative feedback loop of the renin system, and angiotensin-I can still be released.

Given that ACE-I inhibitors were first isolated from snake venom [56] it is no surprise that many other natural sources yield ACE-I inhibitory compounds. To date, the majority of ACE-I inhibitory peptides have been sourced from Milk protein [41]. For example, the hypotensive peptides IPP and VPP were first derived from their parent proteins κ-casein and β-casein using enzymes from the bacteria *Lactobacillus helveticus*[58]. Other food sources of ACE-I inhibitors include eggs. The ACE-I inhibitory peptides IEW, IAT, LKP were isolated from ovatransferrin hydrolysates by the enzymes pepsin and thermolysin (EC 3.4.24.27) [42].

ACE-I inhibitors were identified from multiple natural marine sources such as yellow fin sole, shrimp, clam, and sea cucumber as outlined in a recent review by Wijesekara and Kim [55]. For example, the ACE-I inhibitory pentapeptide LKPNM was isolated from the traditional Japanese food Katsuo-bushi made from dried bonito fish hydrolysed

with thermolysin [59]. Suetsano et al. (2000) discovered four tetrapeptides (AITL, TLTT, LPTG and TALL) with ACE-I inhibitory activity from a peptic digest of the seaweed *Undaria pinnatifida* [29]**.** Indeed, over 556 ACE inhibitory peptides derived from natural sources are reported in the peptide database BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep)**.**

1.6.3 The enzyme renin and its mechanism of action in prevention of hypertension

Inhibition of the renin enzyme was first reported in 1898 by Tigerstedt and Bergman who observed that an extract from rabbit kidney was sufficient to increase blood pressure in living rabbits. They called this vasoconstrictive substance renin[60]. Renin has the EC number 3.4.23.15 and is a member of the aspartic protease family, which also includes the enzymes pepsin, cathepsin, and chymosin [61]. Renin is a monospecific enzyme that displays remarkable specificity for its only known substrate, angiotensinogen [61] and indeed, renin is also known as angiotensinogenase. The most important source of circulating renin is the granular cells of the juxtaglomerular apparatus situated in the macula densa mechanism of the kidneys. Renin is produced in response to three main stimuli; (1) Decrease in arterial blood pressure; (2) Decrease in sodium chloride (NaCl) levels in the ultrafiltrate of the nephron in the kidneys and (3) sympathetic nervous system activities which also control blood pressure levels. Renin is produced through the activation of pro-renin, the enzymatic precursor of renin. Pro-renin is inactive due to a 43 amino acid N-terminal pro-peptide that covers the active site and blocks access of the active site to angiotensinogen. It is activated either through proteolytic cleavage of the pro-peptide chain or by non-proteolytic activation in the juxtaglomerular cells by the unfolding of the proteolytic propeptide, which is how the majority of circulating renin is produced [61]. The structure of renin shown in Figure

1.2 consists of two homologous lobes with the active site residing in the deep cleft between them [61]. The monospecific catalytic activity of its active site is due to two aspartic acid residues, one located in each lobe of the molecule. Due to this high specificity, renin has only one known function and is exclusive in this ability to convert angiotensinogen to angiotensin-I by cleaving the di-peptide LV within angiotensinogen [61].

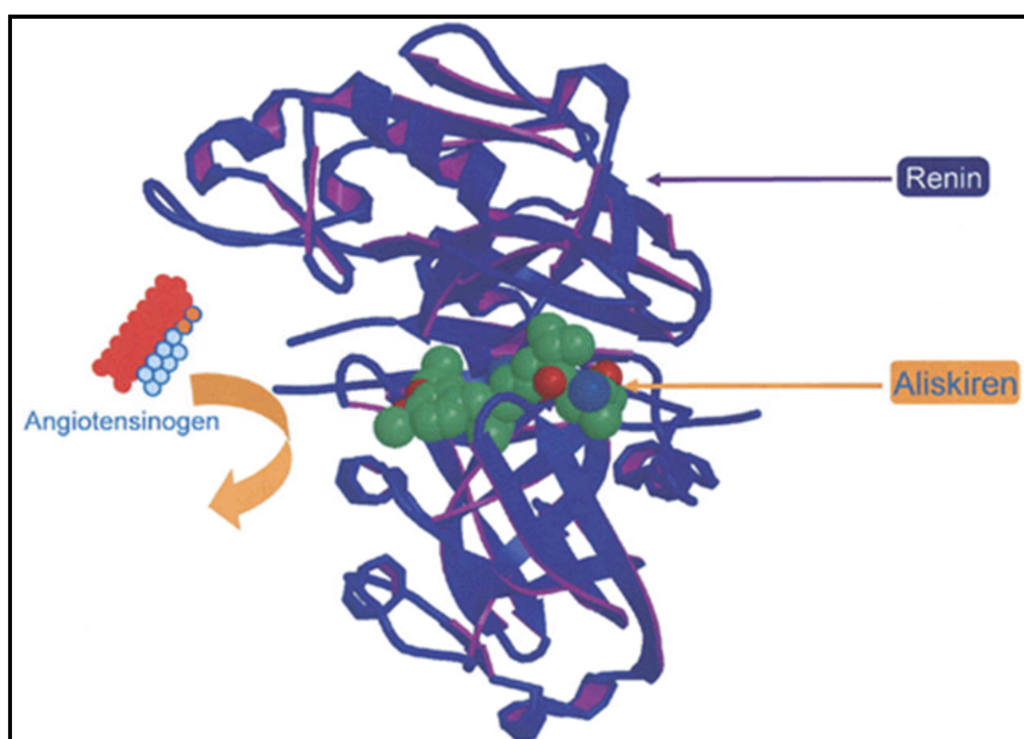


Figure 1.2: The structure of renin.**Source:** Gradman, A. H., Kad, R (2008), J. Am. Coll. Cardiol., 51, 5, 519-528.

As early as the 1950s, renin was recognised as the initial and rate-limiting substance involved in the RAAS, and the approach of inhibiting circulating renin was suggested as the most likely approach to succeed in pharmacological inhibition of the RAAS [62].

1.6.4 Types of inhibitors in the RAAS

Bioactive peptides and drugs which target the RAAS regulatory pathway can be classified by which part of the RAAS they modify. Direct renin inhibitors (DRIs) include β-andrenoceptor blockers which target renin, the ACE inhibitors, the AT_1 blockers (ARBs), the aldosterone-receptor antagonists (ARAs) and combined ACE and neutral endopeptidase inhibitors called vasopeptidase inhibitors [63]. The sites of where enzymes may be inhibited within the RAAS are shown in Figure 1.3.

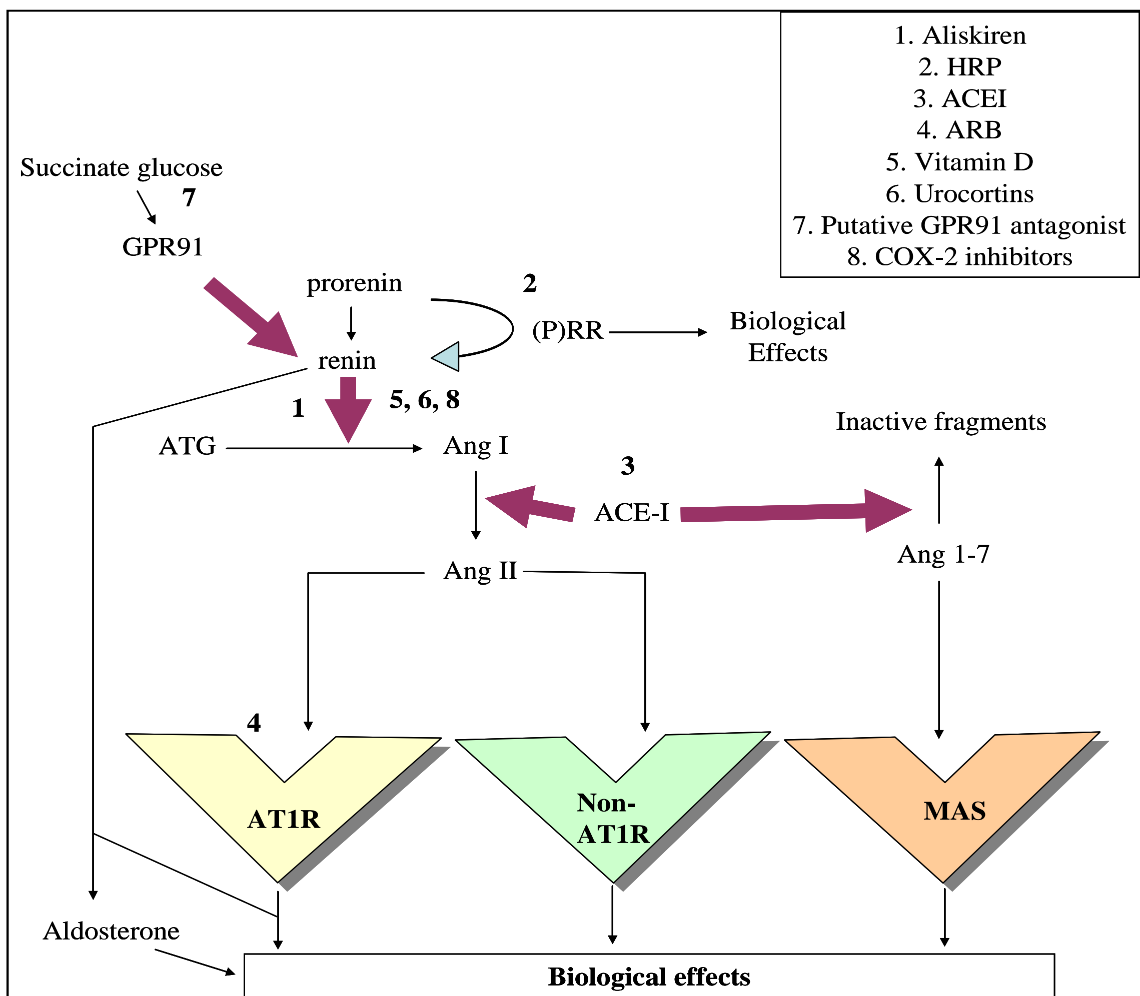


Figure 1.3: Sites of inhibition of the RAAS. Black arrows represent activation steps; Purple arrows denote enzyme actions. The sites of actions of RAAS inhibitors are indicated by numbers 1-8. Adapted from: Komers, R (2013) Clinical Science, 124, 553-566.

1.6.5 Development of renin inhibitors

Pepstatin was the first synthesised renin inhibitor. However, it was initially described as a pepsin inhibitor derived from various species of the bacterial genus *Actinomyces* in 1970 by Umezawa et al [64]. Pepstatin is a hexapeptide containing the unusual amino acid statine [65]. Pepstatin was considered as a renin inhibitor due to its potency as an inhibitor of aspartyl proteases. However, its activity on renin was found to be several orders of magnitude lower in terms of potency compared to its pepsin inhibitory activity [65]. The next generation of renin inhibitors came in the form of analogues of the 45 amino acid long pro-renin segment. This segment shields access to the catalytic site of renin in its inactive form. Indeed, four peptides from this segment were described to have potent *in vitro* renin inhibitory effects in the past but ultimately showed poor drug-like properties [66]. Another strategy used to develop effective renin inhibitory drugs looked at improving the specificity, stability and potency of inhibitors by developing peptidometric inhibitors with improved structures [67]. This approach led to the development of remikiren and zankiren. These drugs never proceeded past pre-clinical trials due to their poor oral bioavailability resulting in a decreased hypotensive effect [67]. Aliskiren was the first orally active non-peptide renin inhibitor on the market. Developed by Novartis and marketed under the names Rasilez® and Tekturna®, aliskiren inhibits renin by occupying the S3sp sub-pocket of renin's active site which is not exploited by peptide-like inhibitors[68].Figure 1.2 shows the inhibition of renin by the pharmaceutically designed molecule aliskiren. The alkylether aromatic side chains interact with the S3sp subpocket of the renin active site blocking access to angiotensinogen [61].

1.6.6 Renin inhibitory compounds and peptides from plant sources

Renin inhibitory peptides were identified previously from plant sources including soybean [69], hemp seed [70] and the plant *Scutellaria baicalensis*[71]. Previously, Li and Aluko (2010) used the enzyme alcalase to hydrolyse pea protein and produced three dipeptides: Isoleucine-Arginine, Lysine–Phenlyalanine, and Glutamine-Phenylalanine, each with potent renin inhibitory activity [72]. In addition, Agomuoh et al. (2010) identified renin inhibitors from the plant *Nauclea latifolia.* Studies were carried out regarding the renin inhibitory activity of various foods [73]. Some studies have equally looked at the inhibitory activities of some synthetic peptides [74-76]. Screening for renin inhibition using synthetic analogues of plant compounds has also been studied previously [77, 78].

1.6.7 The RAAS: a potential therapeutic target in the treatment of diabetic kidney disease and other disease treatments

One of the risk factors associated with the development of CVD includes high blood pressure, and this may be controlled by inhibition of a number of enzymes in the RAAS and the human body that are known to increase blood pressure and atherosclerosis. Angiotensin converting enzyme I (ACE-I; EC 3.4.15.1) is one such enzyme. ACE-I is a zinc metalloprotease that plays an important role in RAAS and the control of blood pressure and fluid regulation [51]. Inhibition of ACE-I is a well-established approach in the treatment of hypertension. ACE-I removes a dipeptide from the C-terminus of angiotensin I, converting it to angiotensin II, a potent vasoconstrictor. Chemically synthesized ACE-I inhibitors including captopril (marketed as Capoten®), enalapril (marketed as Vasotec®), alcacepril (marketed as Alaceril®), and lisinopril (marketed as Prinivil®) are ordinarily prescribed for the treatment of high blood pressure [55]. ACE-I

is also involved in the degradation of bradykinin, which is a vasodilator [54]. ACE-I also acts as an endopeptidase, shown by cleavage of peptides with amidated C-termini, as seen in the cleavage of Histidine -Leucine in the RAAS [51]. Bradykinin-potentiating peptides prevent the hypertensive effect of angiotensin II and potentiate the hypotensive effect of the circulating vasodilatory peptide bradykinin by also inhibiting ACE-I (as shown in Figure 1.1) [54].

As mentioned, renin inhibition may prevent the development of high blood pressure. However, the blood pressure lowering effect of renin inhibition is not the sole gain of the therapy. The RAAS is documented as playing a pivotal role in the prevention and treatment of diabetic nephropathy and some other proteinuric kidney diseases [79]. RAAS inhibition can slow the progressive decrease in glomerular filtration rate, reduce proteinuria and microalbuminuria, and reducing cardiovascular mortality and morbidity in diabetic patient [79]. Use of renin inhibitors over ACE-I inhibitors in the treatment of kidney diseases has a number of advantages. Directly inhibiting renin completely halts the production of angiotensin peptides, whereas ACE-I inhibition merely reduces the production. A phenomenon known as "ACE escape" or "aldosterone breakthrough" occurs where angiotensin-II gets converted independent of the RAAS by circulating chymases [80]. Therefore even with ACE-I inhibition many patients will still experience renal failure, renin inhibition offers a more complete blockade of the RAAS and thus may offset renal failure further [79].

Renin inhibitors may also be used in the treatment of glaucoma. Apart from cataracts, glaucoma is the leading cause of sight loss world-wide [81]. Glaucoma is a condition where optic neuropathy occurs due to intraocular pressure (IOP). Diet, ethnic and genetic factors all play a role in the etiology of the condition, however the biggest cause of IOP is Hypertension. An active RAAS has been described within the eye, and studies

now show that drugs such as renin inhibitors may relieve IOP and in turn offset the development of glaucoma [81].

1.6.8 Platelet-activating factor acetylhydrolase (PAF-AH) and heart health

Another enzyme that is associated with the risk of cardiac events is the enzyme platelet-activating factor acetylhydrolase also known as lipoprotein associated phospholipase A_2 (Lp-PLA_2)[82]. A proatherogenic role has been postulated for this enzyme as it generates two key pro-inflammatory mediators [82]. This observation has led to suggestions of a causative role for PAF-AH in the development of atherosclerosis [82]. PAF-acetylhydrolase is a circulating enzyme produced and secreted by inflammatory cells centrally involved in atherosclerosis [83]. It is bound predominately to apolipoprotein B-containing lipoproteins and is highly expressed in the necrotic core of atherosclerotic lesions [83]. It generates two key pro-inflammatory mediators, lysophosphatidylcholine (LPC) and oxidized non-esterified fatty acids (oxNEFAs). Evidence exists for a regulatory role of these lipids in promoting atherosclerotic plaque development that can ultimately lead to the formation of a necrotic core, a key determinant in atherosclerotic plaque vulnerability, as illustrated in Figure 1.4 [82]. Mayer et al. [79] discovered a variety of compounds from chlorophyta, phaeophyta, and rhodophyta that have inhibitory properties against bee-derived phospholipase A_2 [84]. The discovery of natural PAF-AH inhibitors and their inclusion in the treatment of CVD and incorporation into functional foods has high potential and these inhibitors are yet to be fully exploited.

1.6.9 Darapladib and the development of commercial PAF-AH inhibitors

Chemically synthesised darapladib became the lead compound amongst a group of substituted pyrimidones observed to have inhibitory activity towards PAF-AH *in vitro*.

Darapladib was shown previously to prevent necrotic core expansion, a key determinant in atherosclerotic plaque vulnerability [83].However, a recently completed double blinded trial by GSK using 15,828 patients with stable coronary heart disease administered 160 mg of darapladib per day showed no significant reduction the risk of the primary composite end point of cardiovascular death, myocardial infarction, or stroke. It is possible though that the coronary risk among patients in this study may already have been minimized by concurrent therapy [85]. An on-going GSK trial called the stabilization of plaques using darapladib thrombolysis in myocardial infarction (SOLID-TIMI 52) aims to determine the clinical benefit of direct inhibition of PAF-AH activity with darapladib in patients after an acute coronary syndrome is expected to finish in 2015 [86].Furthermore the anti-atherosclerosis activity of varespladib an inhibitor of several sPLA2s is currently under investigation by the pharmaceutical company Anthera [87].

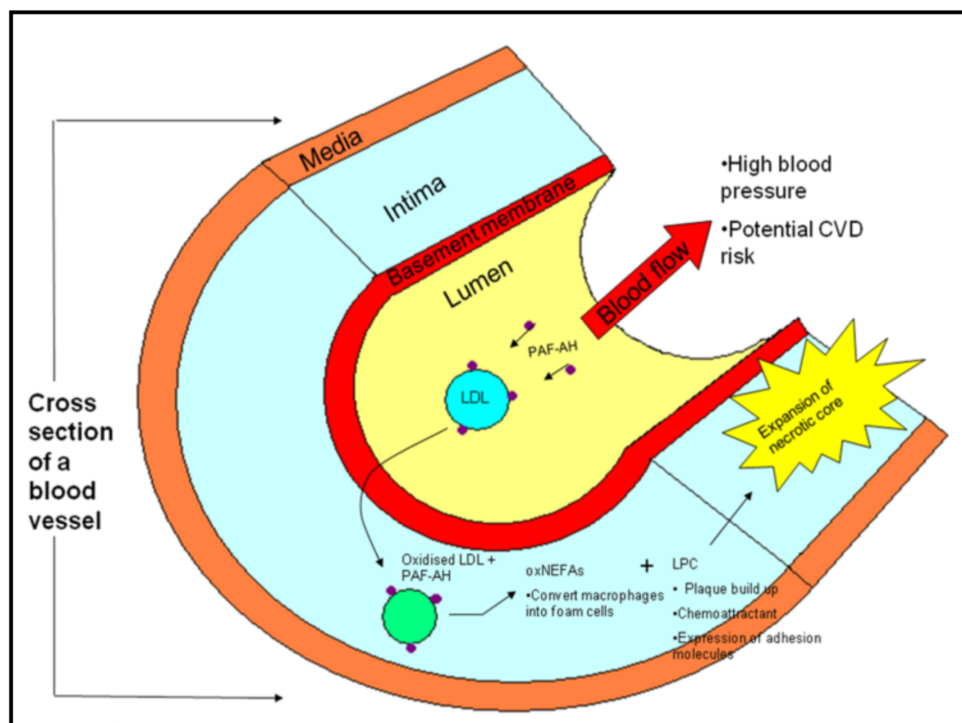


Figure 1.4: Expansion of the necrotic core due to PAF-AH activity. In blood plasma, 80% of PAF acetylhydrolase is bound to low-density lipoproteins (LDL). As LDL is transported into the intima from the lumen, it is oxidized; PAF-acetylhydrolase then generates two pro-inflammatory mediators, lysophosphatidylcholine (LPC) and oxidized nonesterified fatty acids (oxNEFAs). LPC increases plaque build-up as it is an important chemo-attractant for macrophages and increases the expression of adhesion molecules. oxNEFAs convert the attracted macrophages into foam cells, which agglutinate in the lumen and constrict blood vessels, which may lead to high blood pressure and potentially CVD.

1.7 Bioactive peptides previously isolated from macroalgae

In the sourcing of bioactive peptides from macroalgae, it is important to take into account differences in protein content between species and also within species collected at different locations and during different seasons [32]. Generally, the protein fraction of brown macroalgae is low (3-15% of dry weight) compared with that of the green (10-26% of dry weight) or red macroalgae (35-47% of dry weight) [32]. As mentioned previously, the season in which the macroalga is harvested influences the protein content. Glycoproteins known as lectins are also found in macroalgae. Lectins may be divided into four main subgroups, namely, legume lectins, chitin binding lectins, monocot mannose binding lectins, and type-2 ribosome inactivating proteins (RIP) [88].

The main characteristic of this class of protein is their ability to interact specifically with carbohydrates and to combine with the glycol components of the cell surface [89]. Macroalgal lectins have been detected and isolated from several Rhodophyta [90]. Lectins have biotechnological applications in several scientific and medicinal fields of research including biology, cytology, biochemistry, and medicine [88]. However, lectins may present a problem if used for human food use as glycoproteins are known to be responsible for allergic reactions and in some instances anaphylactic shock [91]. Macrolalgal and many other plant-derived lectins have been used for clinical blood typing in medicinal biology. For example, lectins derived from the green alga *Codium fragile* have been shown to recognize GalNAc, an antigen for blood group A [92]. They also have a number of valuable bioactive properties. For example, lectins from *Perocladiella capillacea* demonstrated analgesic and anti-inflammatory properties in rodent model [88]. Holanda et al. showed that a lectin extracted from the red alga *Solieria filiformis* had antibacterial activity against six pathogenic Gram-negative species including *Serratia marcescens*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Proteus* sp., and *Pseudomonas aeruginosa*[90]. The lectin known as amansin isolated from the red alga *Amansia multifida* is used as a mitogen, which is a substance that encourages cell division, for human lymphocytes, and it therefore has potential for use in anticancer therapies [86]. Phycobiliproteins found in macroalgae are usually divided into three separate groups on the basis of their colour and absorption properties, namely, phycoerythrin, phycocyanin, and allophycocyanin [93]. They are components of the macromolecular light harvesting complex of macroalgae called the phycobilisome [94]. In many red algae, phycoerythrin, the most abundant phycobiliprotein found in the Rhodophyta, is the major soluble protein of the cell [94]. The primary potential of these molecules seems to be as natural dyes, but an increasing number of investigations have shown their health-promoting properties and

broad range of pharmaceutical applications [95]. Phycoerythrin is a powerful and highly sensitive fluorescent reagent, which can serve as a label for antibodies, receptors, and other biological molecules in a fluorescence- activated cell sorter, and phycobiliproteins, in general, are used in immunolabelling experiments and fluorescence microscopy and diagnostics [95]. These macromolecules also have potent bioactivities such as antioxidant, antidiabetic, and anticancer properties. Bermejo et al. demonstrated the antioxidant capabilities of the phycobiliprotein phycocyanin isolated from a protein extract of the green microalga *Spirulina platensis* and suggested these bioactive capabilities are attributed to the ability of the protein to chelate metal and to scavenge free radicals [96]. Furthermore, purified C-phycoerythrin was shown to relieve the symptoms of diabetic complications in rats through significant reductions in oxidative stress and oxidized LDL-triggered atherogenesis [97]. Following administration of 25 and 50 mg/kg per body weight per day over 28 days, C-phycoerythrin decreased food intake, organ weight, serum concentration of glucose, cholesterol, tumour associated glycoprotein (TAG), very low density lipoprotein (VLDL)-cholesterol creatine, uric acid, and thiobarbituric acid reactive substances in the rats, suggesting the possible therapeutic role in human diabetes of C-phycoerythrin [97]. Purified recombinant allophycocyanin was found to have a significant inhibitory effect on S-180 carcinoma in mice with inhibition rates ranging from 7.9 to 61.9% with doses ranging from 4.65 to 18.6 mg/(kg day) [97]. Other peptides include a hexapeptide from *Ulva* species, which displays mitogenic properties, and agglutinin glycoprotein from *Soleria robusta* and *Eucheuma serra*, which displays mitogenic, cytotoxic, and anticancer properties [98].

Digestion of macroalgal proteins with proteolytic enzymes has led to the discovery of many bioactive peptides. ACE-I inhibitory peptides were released from *Undaria pinnatifida* proteins using enzymes including pepsin [29] and from the parent proteins

of *Polysiphonia urceolata* using the enzymes protamex, alcalase, and flavourzyme [92]. Peptide fractions isolated from the red macroalga *Porphyra yezeonsis* ("nori") were found to have a hypotensive effect when administered orally to SHR models [99].

1.8 Effects of food processing on bioactive peptides

Food processing provides an additional value to foods by improving food safety, shelf life, palatability, nutritive, and functional values [100]. However, depending on the processing technique employed, food processing may be detrimental to the survival of bioactive peptides [100]. Changes in the molecular structure of an amino acid may lead to changes in the bioactivity and absorption of the peptide of interest. Heat, the most common and oldest form of food processing, modifies the food proteins to make them more edible in terms of texture and flavour [101]. Heat can be used to enhance functional properties of proteins. Denaturation of proteins for example improves the water binding and emulsification properties. Heat also decreases protein solubility due to aggregation and coagulation [101]. During heating, the lysine residues of proteins may react with reducing carbohydrates in the same food system, resulting in the maillard or non-enzymatic browning reaction [101]. This can reduce the nutritional value of proteins as the bioavailability of lysine is reduced [101]. Heat may also destroy the bioactivity of peptides. However, it has been shown that some ACE-I inhibitory peptides can retain their activity when heated to temperatures of 70ºC and 100 °C for 20 min [102].

Other food production processes may facilitate the release of bioactive peptides from their parent proteins. For example, during milk fermentation, lactic acid bacteria (LAB) hydrolyse caseins into peptides and amino acids [100]. High hydrostatic pressure also promotes the proteolysis and release of bioactive peptides. Quiros et al. showed that proteolysis of ovalbumin with pepsin for the production of ACE-I inhibitory peptides

was accelerated under pressures of 200-400 MPa [103]. Another factor to consider when bioactive peptides are incorporated into a food matrix is the often bitter taste of hydrolysates, which is attributed to the formation of low molecular weight peptides composed mainly of hydrophobic amino acids [100]. This can limit the use of some bioactive peptides that possess proven bioactivities [100]. Strategies to de-bitter protein hydrolysates include treatment with activated carbon, extraction with alcohol, isoelectric precipitation and chromatographic separation. Masking the taste of these peptides can be achieved through the use of ingredients including monosodium glutamate (MSG) and also by enzymatic hydrolysis of the proteins/peptides [104]. The latter method has limited capability due to the enzymatic hydrolysis of the active peptides themselves, which may render the bioactive peptides inactive [100].

Microencapsulation is a technology that has solved many problems that limit the use of bioactive peptides and additives in foods, as it can mask undesirable flavours and reduce volatility, hygroscopicity, and reactivity [105]. Furthermore, microencapsulation improves the stability of the products under adverse environmental conditions and provides controlled liberation of the encapsulated material under pre-established conditions [105]. For example, the oral delivery of tuna-derived antihypertensive oligopeptides composed of hydrophilic peptides to consumers was achieved by encapsulating them in liposomes [106]. Moreover, antimicrobial lysozymes were previously encapsulated using zein protein derived from maize[107] to minimize binding between antimicrobials and the food matrix [107]. In addition, casein hydrolysates were previously encapsulated in maltodextrin to mask the off-flavour of the hydrolysate, which facilitated its inclusion in a protein bar [105].

1.9 Bioavailability of bioactive peptides and survival in the gastrointestinal tract (GI tract).

Following consumption, the gastrointestinal epithelium acts as both a physical and a biochemical barrier to absorption of food derived bioactive peptides and drugs. The physical barrier is represented by the impermeable gastrointestinal epithelium and the biochemical barrier consists of enzymatic peptidases [108]. In order for a bioactive peptide to be delivered to its active site it is necessary to fully understand these barriers. The GI tract shown in Figure 1.5 has site specific absorption based on the peptide/drug consumed and based on regional differences in pH, enzyme activity, thickness of mucosa and residence time and surface area [108]. The pH of the GI tract varies from 1-7. The bioavailability of protein and peptides depends on their ability to cross the intestinal mucosa and reach the systemic circulation.

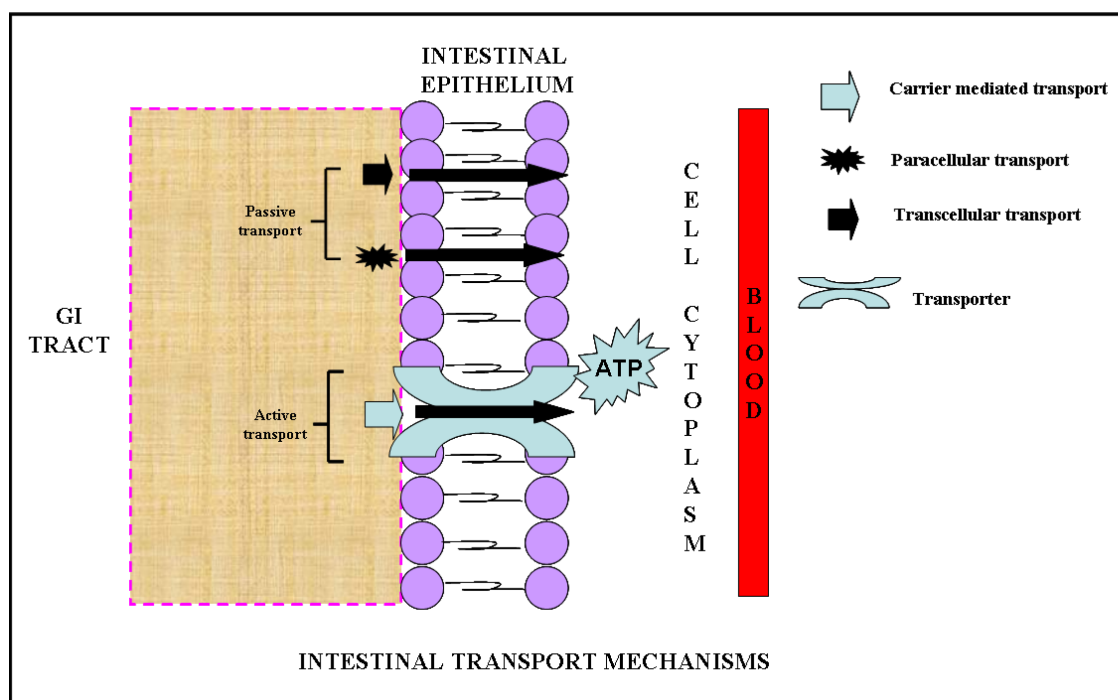


Figure 1.5: Intestinal transport mechanisms for peptides.**Adapted from:**Renukuntla, J., Dutt Vadlapudi, A., Patel, A., Boddu, S. H. S., and Mitra, A. K.International Journal of Pharmaceutics, 447, (2013), 75-93 [107].

Transport across the intestinal epithelium may be either active or passive and the mechanisms of transport depend on the physicochemical properties of the peptide and on the length of the peptide in question. Active transport of peptides involves movement from low to high concentrations by transmembrane proteins and energy in the form of ATP is used. Passive transport involves diffusion of drug molecules in the direction of the concentration gradient [108]. Carrier mediated transport involves the movement of molecules via transporters [108]. Detailed understanding of the structural features of a bioactive peptide is needed to target these transports for efficient delivery of the peptide to the target sites.

Bioactive peptides may have to interact with target sites at the luminal side of the intestinal tract. Furthermore, they have to be absorbed in order to reach the target organ [109]. In addition to being resistant to further enzymatic digestion by endogenous gut enzymes, bioactive peptides must also show the ability to cross the intestinal epithelium to reach target sites [110]. Previous studies have used Caco-2 cell monolayers to assess the ability of bioactive peptides isolated from milk to cross the intestinal epithelium [111]. While the exact route of transportation for bioactive peptides is not fully understood some peptides cross via paracellular diffusion and transcytosis [111].Different formulation strategies may be used to increase the oral bioavailability of peptides and these include methods such as use of absorption enhancers [112], enzyme inhibitors [113], hydrogels [114], liposomes [115], cyclodextrins [116] and nanoparticles [117].

Factors such as peptide length also affect the bioavailability of the molecules. It was once believed that dietary proteins were completely digested to amino acids after ingestion, but, it is now evident, that about 30 - 50 % of dietary protein gets absorbed in the form of small peptides [118]. Previously, it was proven that peptides in the range of 3 to 51 amino acid residues in length can be absorbed by the gastrointestinal tract and

may incur a physiological response [118]. However, increased length of the peptide chain does diminish the amount of the molecule being absorbed. For example the tri-peptide thyrotropin releasing hormone (TRH) has the same hormonal response when administered orally as intravenously, whereas the decapeptide luteinizing hormone-releasing hormone (LHRH) had half the maximal response when it was administered orally than when administered intravenously [118]. Peptides consisting of two or three amino acids are absorbed intact across the brush border membrane by the peptide transporter PepT1 using a transmembrane electrochemical proton gradient as the driving force and this transporter has broad specificity. In this instance they absorbed more rapidly than free amino acids [119]. In terms of amino acid composition, peptides containing hydrophobic residues such as proline and valine are more easily transported across the GI tract [119].

1.10 Suitable food vehicles for delivery of bioactive peptides

There are many products already on the market, which utilise bioactive peptides as functional components. For example, there are two products available that contain the bioactive peptides IPP and VPP, namely, Calpis© and the Ameal-S drink developed in Japan and Evolus developed by Valio Ltd. a Finnish company. Both products were generated from fermented milk products and have ACE-I inhibitory actions and are proven antihypertensive agents in spontaneously hypertensive rats (SHRs) and in human clinical trials [120]. Furthermore, a thermolysin digest of dried bonito containing antihypertensive peptides is used in a soup product in Japan and has claimed antihypertensive effects [120, 121]. Table 1.1 shows commercially available products that contain bioactive peptides with different health-promoting properties. However, there is some controversy over the effect of lactotripeptides on blood pressure as recent studies found no statistically significant differences between the groups that consumed

peptides and the placebo groups [17]. This highlights the importance of the bioactive peptides being capable of reaching the bloodstream and careful selection of a test population.

Table 1.1: Bioactive peptide containing products available commercially

Effect	Product	Pepitde(s)	Company	Source/Food Matrix
Heart Health				
Hypotensive	Ameal ®	VPP,IPP	Calpis	Milk
Hypotensive	Evolus®	VPP, IPP	Valio Ltd.	Milk
Hypotensive	Peptide soup	LKPNM (katsuobushi oligopeptide)	Nippon Supplement, Inc.	katsuobushi (dried bonito)
Hypotensive	Casein DP ®	FFVAPFPEVFGK	Kanebo Ltd.	Milk
Hypotensive	C12 peptide ®	FFVAPFPEVFGK	DMV (De Melkindustrie Veghel) International	Milk
Hypotensive	Valtyron ®	VT	Senmi Ekisu Co. Ltd.	Sardine
Hypotensive	Biozate ®	Whey-derived peptides	Davisco	Milk
Cholesterol lowering	CSPHP ®	Enzymatically decomposed lecithin bound to hydrolysed isolated soy protein (ratio of 80:20)	Kyowa Hakko	Soy
Other				
Energy Boost	Cysteine peptide peptide supplement	Mixture of whey protein derived peptides comprising atleast 6.5 wt % cysteine	DMV International	Milk
Satiety	Glycomacropeptide	Casein-derived whey peptide	Davisco	Milk

1.10.1 Bread as a carrier for bioactive compounds

Bread is one of our most ancient processed foods and archaeological evidence of bread baking dates back to 10,000 years BC [122]. The basic components of bread are water, flour, yeast (*Saccharomyces cerevisiae*) and salt. However, bread ingredients differ across the globe. Seaweed breads are already in existence. For example, *Porphyra* seaweeds are traditionally used in Wales to make bread known as 'Laver' bread [123]. As bakery and pasta products are widely consumed food products [124], bread is an ideal vehicle for delivery of bioactive compounds [124]. Previously Prabhasankar et al. incorporated wakame (*Undaria pinnatifida*) into pasta to improve its bio-functional properties and found that incorporation of up to 20% wakame enhanced interactions between starch granules and the protein matrix [124]. This resulted in an improved pasta textural quality. In an attempt to increase the amount of fibre in the form of alginate, Hall et al. previously demonstrated that the macroalga *Ascophyllum nodosum*, when incorporated in amounts of between 5 and 20 g per 400 g loaf of bread, had no significant difference in acceptability to the consumer under the terms of appearance, aroma, flavour, after-taste, and texture when compared to a non-macroalgae enriched control bread [125]. This was assessed using a taste panel of 79 un-trained sensory panelists using visual analogue scales (1-9; extremely unacceptable to extremely acceptable) [125]. The introduction of alginate into a diet is thought to slow gastric emptying and create a prolonged sense of satiety [126]. Inclusion of brown macroalgae (10-40% of which is alginate) into food products, is also thought to increase the anti-obesity and anti-diabetic properties of the food [126]. Microencapsulated polyunsaturated fatty acids (PUFA) derived from tuna were incorporated into bread and this was proven to increase PUFA levels in human blood plasma [4]. PUFAs are known to lower blood pressure, reduce serum triglyceride levels, have anti-inflammatory

activities, and decrease the risk of cardiac events [127]. Macroalgae and microalgae are the nutritional source of PUFAs for many fish, hence, macroalgae are also a viable source for these bioactive molecules in human nutrition [127]. Moreover, certain components of marine algae appear to improve the rheological properties of flour dough and the quality of the finished products made from it [90]. Oxidoreductase derived from the algae *Chondrus crispus* when added to flour dough increased resistance to extension and extensibility by at least 10% compared to similar dough that did not contain oxidoreductase. It also increased the final volume of a finished baked product by 20%, compared to that of a similarly baked product not containing oxidoreductase [128].

1.11 Final remarks

Every year around 4.3 million Europeans die from the effects of heart disease resulting in €196 billion being spent per year on its treatment [129]. Coronary heart disease remains the Western Worlds' biggest killer. However these numbers are decreasing, this is mainly due to scientific advances that have arrested and indeed reversed the growth in mortality due to CVD [129]. High blood pressure is the highest risk factor associated not only with cardiac related death but with mortality in general as it exacerbates practically all human health issues [130]. Some of the main scientific advancements in curtailing CVD include better diagnostic tools and new interventional procedures but the most significant of these have been the emergence of families of drugs such as ACE-I inhibitors, statins, beta blockers and ARBS [129]. Western society has a number of rising statistics that will make the advancement of CVD treatment even more crucial. One of these is the rapidly ageing population. By 2050, the number of people over the age of 50 is expected to rise by 35% and the number of people over 80 is expected to rise by 300% [129]. The recent rise in the incidence of obesity and with that, type II diabetes, will also increase the significance of furthering CVD treatment research [129].

With these issues in mind new strategies to prevent heart health issues are constantly sought. While inhibition of the RAAS is not a new concept in combating hypertension, the approach of inhibiting it by debilitating its initial and rate limiting enzyme renin is emerging again as a pharmaceutical strategy [131]. Recently, ACE-I inhibitory compounds were isolated and characterised from natural food products and incorporated into functional food products [120]. Very few natural renin inhibitory compounds are present in the literature, but with the inherent advantages of renin inhibitors over ACE-I inhibition [61] they are potentially a better candidate for inclusion in blood pressure lowering functional foods. Similarly with PAF-AH inhibition, while this strategy is only on the cusp of being realised as a viable treatment for atherosclerotic plaque, natural PAF-AH inhibitors as functional food ingredients would be a feasible adjunct therapy for at risk individuals.

In the past, the primary source of bioactive peptides was dairy products [41]. The variety of macroalga species, the environments in which they are found, and their ease of cultivation make macroalgae a relatively untapped source of new bioactive compounds [2] and more efforts are needed to fully exploit their potential for use and delivery to consumers in food products. Red seaweed such as *Palmaria palmata* have for centuries been part of traditional diets of Atlantic coastal countries so it is already in the public consciousness as being a nutritious food product. Its' abundance around Irish coasts combined with its rich protein content make it an ideal candidate for exploration for bioactive peptides [32].

The idea of using bread as a vehicle for bioactive compound delivery is a natural one; bread is one of the mostly widely consumed, processed foods globally and therefore can be included in a candidates diet without augmenting it substantially. Previous methods to introduce functional ingredients such as folate [132], Probiotics [133] and

microencapsulated marine lipids [134] have taken advantage of the ubiquitous consumption of bread to deliver positive health results to the consumers.

The scientific rationale for the approach used in this work was to use protein material isolated from the red seaweed *Palmaria palmata* to generate peptides and to screen these peptides for their ability to inhibit two enzymes important in heart health and blood circulation, namely renin and PAF-AH. This rationale was adopted as the red seaweed *Palmaria palmata* is known to be rich in protein but was not examined previously as a resource of bioactive peptides. Furthermore, renin and PAF-AH inhibitory peptides were not previously identified from seaweeds and were only recently identified from pea protein source. Within this work, a hydrolysate was generated and peptides purified that exhibited both PAF-AH and renin inhibitory activities. These peptides were synthesised to confirm the bioactivity. The ability of the hydrolysate (from which these peptides were derived) to survive in the food vehicle bread was also examined. This approach was used as the aim of the thesis was to develop a food ingredient with heart and circulatory health benefits so the delivery of this ingredient in bread was required. The thesis did not look at the possibility of using the chemically synthesised peptides for pharmaceutical purposes and the work carried out reflects this. This thesis strives to increase the understanding of how inhibition of various proteases involved in cardiovascular health may be achieved through examining natural sources of bioactive peptides. It expands on previous research carried out in the areas of food proteomics and functional food. While there are many references to pharmaceutical studies throughout this thesis, the research within focuses on food applications according to the remit of the department of Food Biosciences at Teagasc Ashtown Food Research Centre.

This thesis specifically aims to widen the scope of heart health peptide research to include renin and PAF-AH inhibitory peptides derived from novel marine resources specifically from the red seaweed *Palmaria palmata* (Linneaus) Weber and Mohr. Furthermore, the feasibility of introducing bioactive hydrolysates and peptides isolated from *Palmaria palmata* in to a baked product is explored along with the potential bioavailability of seaweed derived hydrolysed proteins and peptides. Toxicity analysis using a zebrafish larvae model are looked at along with the effects on the blood pressure of spontaneously hypertensive rats (SHRs) following consumption of both the *Palmaria palmata* protein hydrolysate and an identified renin inhibitory peptide.

CHAPTER 2. ISOLATION AND CHARACTERISATION OF BIOACTIVE PRO-PEPTIDES WITH *IN VITRO*RENIN INHIBITORY ACTIVITIES FROM THE MACROALGA *PALMARIA PALMATA* (LINNAEUS) WEBER AND MOHR

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2.1 Aims

The aim of this Chapter was to isolate and characterise renin inhibitory peptides fromthe red macroalga *Palmaria palmata which was assigned the reference number ISCG0022.* This species was collected and identified by a trained phycologist within the Irish Seaweed Centre Galway (ISCG) and a voucher specimen of this seaweed is maintained at the ISCG at NUI Galway under the code ISCG0122. The main objectives of this chapter were to:

(1) Extract protein from *Palmaria palmata* (Linnaeus) Weber and Mohr and hydrolyse it with the food grade enzyme papain.

(2) Use *in vitro* bioassay guided fractionation to identify renin inhibitory hydrolysates and RP-HPLC fractions.

(3) Identify and confirm active peptides using purification techniques including nano-electrospray ionization quadropole-time of flight mass spectrometry (ESI-Q-TOF MS), *de novo* sequencing and solid phase peptide synthesis.

2.2 Introduction

Macroalgae represent a rich and sustainable source of nutritional protein [25]. The Rhodophyta, or red seaweeds, in particular, can have a protein content of up to 47% of their dry weight in certain species belonging to the taxon Porphyridiales. This is considerably higher than the protein content obtained from the green macroalgae (Chlorophyta). Chlorophyta can contain between 10 to 26% protein in terms of their dry weight.The brown macroalgae (Phaeophyceae) contain between 3 to 15% protein of their dry weight [32]. The red macroalga *Palmaria palmata* (*Linnaeus*) Weber and Mohr, colloquially known as Dulse or Dillisk is already part of the diet of several Atlantic coastal countries including Ireland, Wales and Iceland [33]. Previous seasonal variation studies carried out on *P. palmata* sourced from the French coast found that it had a protein content of between 9 and 25% of its dry weight [38]. The protein content of *Palmaria palmata* is highest in the winter and spring months and decreases during the summer months [38]. This increased protein content is thought to be caused by the decrease of sunlight hours during winter and early spring months and the need of the seaweed to create more pigments to up-regulate photosynthesis [37].

Pro-peptides can be defined as peptide sequences of between 2-30 amino acids in length which form part of a protein sequence but that can be cleaved by enzymes to become bioactive. The bioactivities associated with pro-peptides and peptides include antimicrobial, antioxidant, angiotensin-I-converting Enzyme (ACE-I) inhibitory and anti-inflammatory actions. ACE-I inhibitory peptides have been isolated from numerous different natural food sources such as meat [135], vegetables [136] and marine sources such as fish [59] and indeed macroalgae [29]. ACE-I (E.C. 3.4.15.1) along with the enzyme renin (E.C 3.4.23.15) play an important role in the control and regulation of blood pressure and salt water balance and, ACE-I inhibitors can prevent

the development of high blood pressure and have been identified from a number of food sources previously. More recently, renin inhibitory compounds from plant sources including peas [72], soybean [69], and the herb baicailin were identified [71]. Furthermore, structure−activity relationship modelling of renin inhibitory di-peptides derived from these sources was carried out previously [137, 138]. However, Li and Aluko (2011) are the only group to date to characterise and synthesize renin inhibitory peptides from a plant protein source to date [72].

The objective of this chapter was to generate, enrich, isolate, and characterize renin inhibitory peptides from *P. palmata* protein by hydrolysis of the protein with the food grade enzyme papain. Reverse phase-high performance liquid chromatography (RP-HPLC) was subsequently employed to enrich for peptides with renin inhibitory activities. Further characterisation using electrospray ionization quadropole time-of-flight mass spectrometry (ESI-Q-TOF MS) and *de novo* sequencing was used to characterise peptides within the renin inhibitory RP-HPLC fractions. Renin inhibitory peptides were synthesized using microwave assisted solid phase peptide synthesis (MW-SPPS) and tested again for inhibitory activity using the renin inhibitory assay to confirm bioactivity. The peptides identified in this study were not, to the best of the authors' knowledge, reported previously in peptide databases such as BIOPEP (www.BIOPEP.com) or other peptide databases [139, 140]. However, di-peptides that correspond to amino acids found within the identified bioactive peptide sequence were reported previously in the literature as renin inhibitory, antioxidant, and ACE-I inhibitory peptides [72, 141].

2.3 Materials and Methods

2.3.1 Materials and Reagents

P. palmata was collected at Spiddal, Co. Galway, Ireland, on the 4th of November 2010. Ammonium sulfate, papain from *Carica papaya*(Catalogue number 76216, Activity Units 20.7 U/mg protein), and the specific renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, which was used as a positive control, were supplied by Sigma Aldrich (Steinheim, Germany). The renin inhibitory screening assay kit was supplied by Cambridge BioSciences (Cambridge, England, U.K.). Acetonitrile and water were supplied by Romil Ltd., (Cambridge, England,U. K.). All other chemicals used were of analytical grade.

2.3.2 Extraction of *Palmaria palmata* (Linnaeus) Weber and Mohr protein

The process used for bioassay guided fractionation of *P. palmata* protein hydrolysates coupled with ESI-Q-TOF MS is summarized in Figure 2.1. Protein was extracted using the method previously described by Galland-Irmouli et al. (1999) [38]. Briefly, 10 g of dried *P. palmata* was suspended in 1 L of ultrapure water. Following ultra-sonication for 1 h (Branson® 2510 Ultrasonic Bath, Branson Ultrasonics, Danbury, USA) the seaweed solution was left to stir overnight on a magnetic stirrer plate (C-MAGHS 7KAMAG, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 4 °C. The solution was then centrifuged at 10, 000g for 1 h and the supernatant decanted. The pellet fraction was suspended in 200 mL of ultrapure water and subjected to a second extraction procedure as described above. Both supernatants were pooled together and subsequently brought to 80% Ammonium sulfate saturation, stirred for an hour at 4 °C on a magnetic

stirrer plate (C-MAGHS 7KAMAG, IKA-Werke GmbH & Co. KG, Staufen, Germany), and centrifuged at 20, 000g for 1 h to precipitate the protein fraction. The precipitates were subsequently dialysed using 3.5 KDa MWCO dialysis tubing (Fisher Scientific, New Hampshire State, USA) against ultrapure water at 4 °C overnight. The precipitates were subsequently freeze-dried and stored at −80 °C until further use.

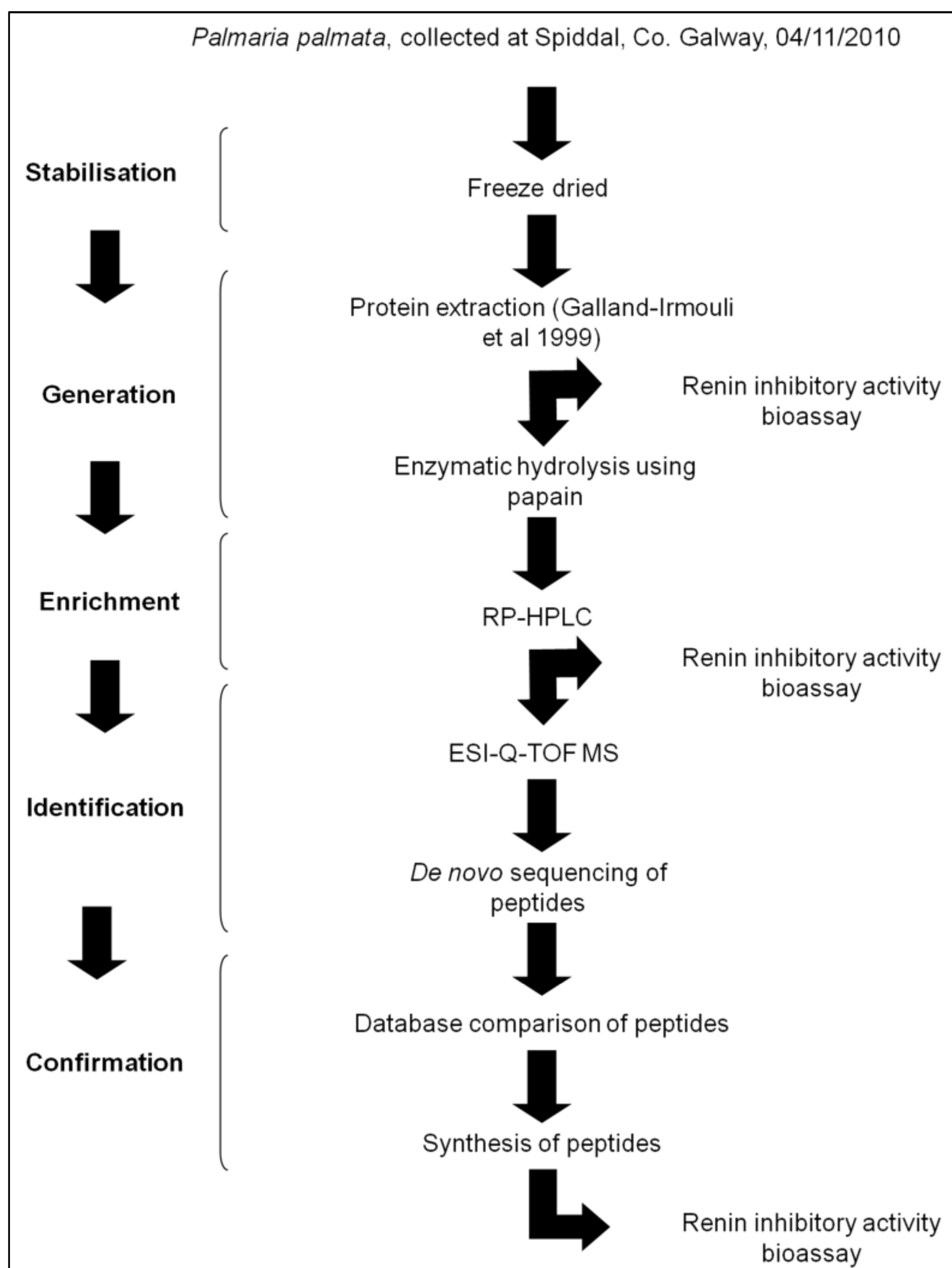

Figure 2.1: Schematic representation of the bioassay guided isolation and characterisation approach used in this chapter to isolate renin inhibitory peptides from a *P. palmata* protein hydrolysate with the enzyme papain.

2.3.3 Enzymatic Hydrolysis of the *Palmaria palmata* (Linnaeus) Weber and Mohr Protein Extract

Papain hydrolysates (X 3) of the *P. palmata* protein were prepared using a New Brunswick 1.5 L bioreactor (Cambridge, U.K.) with temperature and pH control. The protein was dispersed in Romil HPLC grade water at a concentration of 0.015 g/mL at a total volume of 1 L. The temperature was adjusted to 60 °C and the pH to 6.0. The pH was regulated using 0.01 M NaOH. Once the appropriate conditions were achieved, the enzyme papain was added at a concentration of 20.7 U/mg of protein to initiate hydrolysis. Temperature and pH were kept constant for 24 h, and the hydrolysis of *P. palmata* protein was carried out using the New Brunswick bioreactor (Cambridge, U.K.) at 300 rpm. Following hydrolysis, the enzyme papain enzymes was deactivated by heating the mixture at 95 C for 10 min in a water bath.

2.3.4 Protein quantification and amino acid profiling

The concentration of the *P. palmata* protein was quantified using the Quanti-Pro bicinchoninic acid (BCA) assay kit according to the manufacturers' instructions (Sigma, Saint Louis, MO, USA). This method is commonly called the Lowry method and detects protein in a reaction catalyzed by copper, a component of the Folin phenol component of the assay kit. The chemical reaction detects peptide bonds and is also sensitive to some amino acids such as Tyrosine and Tryptophan [142]. Unknown protein content was quantified using a bovine serum albumin (BSA) standard curve. The amino acid composition was then calculated by hydrolysing the protein in 6M HCl at 110 ºC for 23 hours and analysing the amino acid profile using the free amino acid method [143]. This method separates the amino acids by HPLC, each amino acid is

identified by their retention times and quantified by measuring area under the peak, comparison with known standards yields the amount of sample.

2.3.5 Fractionation of the seaweed hydrolysate using RP-HPLC

The *P. palmaria*papain hydrolysates were further purified using the Varian Pro-Star Reverse-phase high performance liquid chromatography (RP-HPLC) system coupled to a photodiode array detector (SpectraLab Scientific Inc., Ontario, Canada). Prior to analysis, samples were filtered using 0.22 µm filters (Millipore, Billerica, Massachusetts, USA) and a volume of 1 mL at a concentration of 10 mg/mL was injected onto a reverse phase Phenomenex (Torrance, California, USA) C18 column with 5 µm particle size (100 µm × 21.2 mm). The column was equilibrated with TFA/H_2O (0.1% v/v) at a flow rate of 1.0 mL/min. Trifluoroacetic acid (TFA)/acetonitrile (0.1% v/v) was used as the mobile phase and the concentration of the eluting solvent was raised from 0% to 100% over 60 min. Chromatogram peaks were detected at an absorbance of 214 nm. Fractions eluted were collected every minute. This was repeated three times before corresponding fractions were pooled together. Acetonitrile and TFA were then removed under nitrogen and the fractions subsequently freeze-dried.

2.3.6 *In vitro* renin inhibitory assay

The renin inhibitory bioassay (Figure 2.2) utilises a renin-based synthetic peptide substrate first synthesised by Wang et al. (1993) [144] which incorporates the fluorophore 5-(2-aminoethylamino)-naphthalene-1-sulfonic acid (EDANS) at one end and an EDAN-quenching molecule (Dabcyl) at the other end. After cleavage by renin, the peptide-EDANS product is released yielding bright fluorescence which can be easily analysed using excitation wavelengths of 335-345 nm and emission wavelengths of

485-510 nm. The assay was carried out in accordance with the manufacturers' instructions. Briefly, prior to carrying out the assay, the renin assay buffer was diluted by adding 3 mL of 50 mM Tris-HCl, pH 8.0 buffer to 27 mL of HPLC grade water (Sigma Aldrich, Dublin) to give a final assay buffer concentration of 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. The renin protein solution was diluted 20-fold with assay buffer before use, and the assay buffer was pre-heated to 37 °C in accordance with the manufacturers' instructions. Background wells were prepared by adding 20 µL of substrate, 160 µL of assay buffer, and 10 µL of HPLC-grade water. The negative control well was prepared by adding 20 µL of substrate, 150 µL of assay buffer, and 10 µL of HPLC grade water to wells. Inhibitor wells were prepared by adding 20 µL of substrate, 150 µL of assay buffer, and 10 µL of sample together. The reaction was initiated by adding 10 µL of renin to the control and sample wells. Fluorescence intensity was recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. All macroalgal extracts were assayed at a concentration of 1 mg/mL in triplicate. The known specific renin inhibitor, Z-R-R-P-F-H-Sta-I-H-K-(Boc)-OMe, was used as a positive control. Z-R-R-P-F-H-Sta-I-H-K-(Boc)-OMe works by mimicking the amino acid sequence around the cleavage site of angiotensinogen. It is a proven *in vitro* renin inhibitor and has demonstrated prolonged renin inhibition in marmosets [145]. Percentage inhibition was calculated using the following equation:

$$\% \text{ Renin inhibition} = ((100\% \text{ initial activity (AF)} - \text{inhibitor (AF)}) \div 100\% \text{ initial activity (AF)}) \times 100$$

where; AF is the average fluorescence and Initial activity was the absorbance measured in the absence of an inhibitor or test agent. Data were compared using the Students t-test and considered significantly different if $P < 0.05$. The inhibitory concentration that

reduced renin activity by 50% (IC_{50}) was determined by nonlinear regression from a plot of peptide concentration versus percentage inhibition.

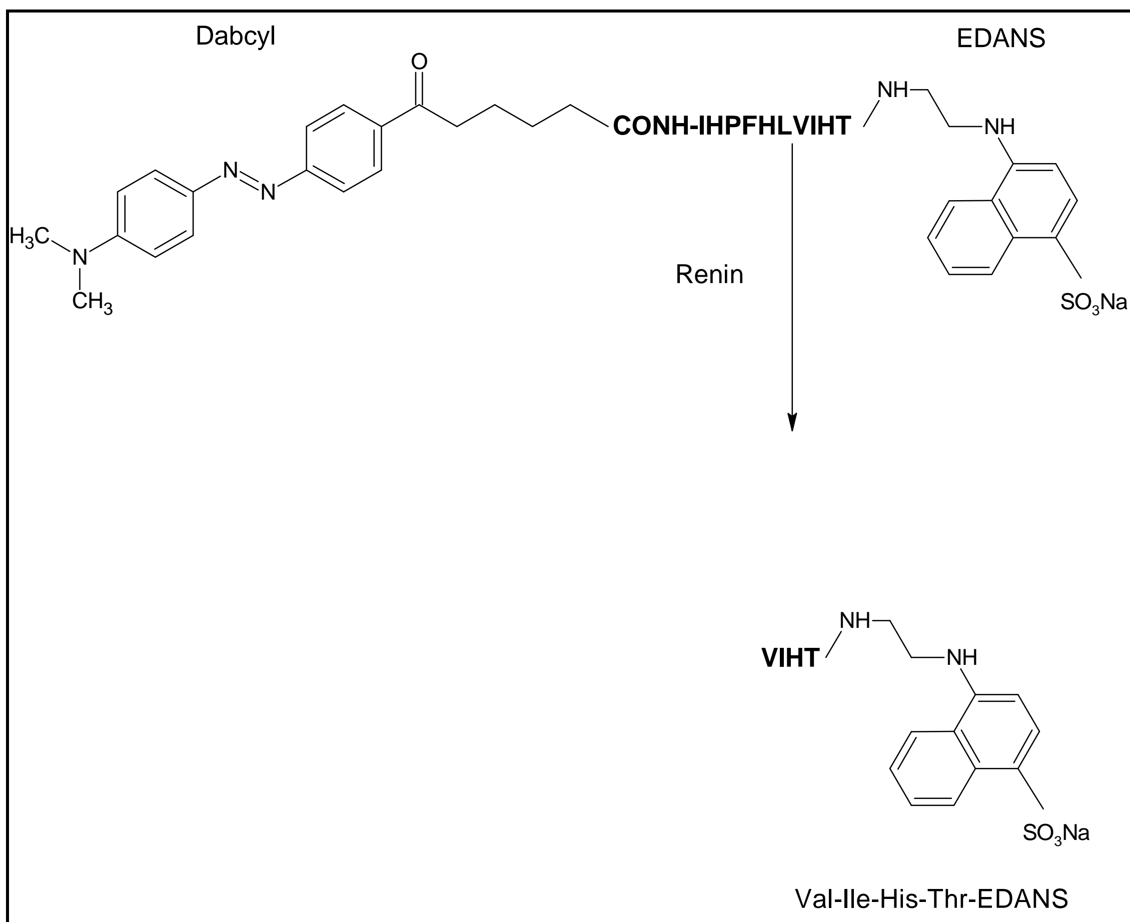

Figure 2.2: Renin enzyme activity is measured when the renin cleaves the synthesized substrate at renins active site (LV) and releases the fluorescent VIHT-EDANS cleavage product. Adapted from Wang, G. T. Chung, C. C. Holzman, T. F. Krafft, G. A. Analytical Biochemistry, 210, (1993), 351-359 [144].

2.3.7 Peptide identification by tandem mass spectrometry

Fractions and peptides were analysed using an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer coupled to a nano-ultra performance liquid chromatography system (Waters Corporation, Milford, MA, USA) using positive ionization mode as illustrated in Figure 2.3 The freeze-dried sample was dissolved in Water/Acetonitrile (90:10, v/v) with 0.1% formic acid (FA) and filtered through a 0.22 μm syringe filter (Millipore). After filtering, 1 μL of the re-dissolved fraction was loaded onto a Nanoacquity ™ ultra high performance liquid chromatography (UPLC) column BEH130 C18 (100 μm × 100 mm, 1.7 μm particle size), preceded by a Symmetry C18 (180 μm × 20 mm, 5 μm particle size) trapping column. Mobile phases consisted of solvent A, which contained 0.1% FA in water, and solvent B, which contained 0.1% FA in Acetonitrile. Trapping of the peptides was achieved using a loading time of 3 min at a flow rate of 5 μL/min with 97% of solvent A and 3% of solvent B and then elution onto the analytical column at 250 nL/min. Chromatographic conditions consisted of 95% of solvent A and 5% of solvent B isocratically for 3 min, followed by a linear gradient from 95 to 50% of solvent A over 48 min. Mass spectral data were acquired in MS^e mode with collision energy for a full mass scan of 6 V and a collision energy ramp of 15−35 V. In the data dependent acquisition (DDA) mode, a 1 s TOF MS scan from m/z 100 to m/z 1500 was performed. The Q-TOF was calibrated externally using glufibrinopeptide (Glu-Fib) for the mass range m/z 100 to 1500.

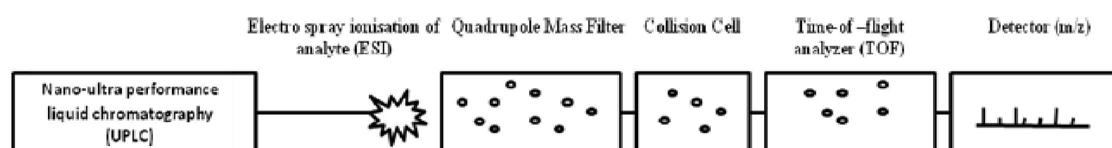

Figure 2.3: ESI-Q-TOF system for identifying renin inhibitory peptides.

2.3.8 Database search, confirmation of sequences, and *de novo* sequencing of peptides

Automated spectra processing and peak list generation was performed using the software Protein Lynx Global Server (PLGS), v2.4 (Waters Corporation), the MaxEnt™ algorithms within PLGS work by collapsing multiple isotopes and charge states from the raw MS data into monoisotopic peptide peaks. Database searches were performed using Mascot interface 2.2 in combination with the Mascot Daemon interface 2.2.2 (Matrix Science, Inc., Massachusetts, USA) (http://www.matrixscience.com), against the UniProt and NCBI non-redundant databases. Mascot searches were done with non-enzymatic specificity and with a tolerance on the mass measurement of 100 ppm in the MS mode and 0.6 Da for MS/MS ions. Oxidation of Methionine (Met) was used for variable modification. Mascot uses a probabilistic scoring algorithm for protein identification. Comparison between the sequences of proteins to determine the protein origin of peptides was done using the UniProtKB/TrEMBL database. As there was no prior knowledge of the amino acid sequence *de novo* sequencing was required to identify the peptides. Peptides are realized through *de novo* sequencing by using the mass difference between two fragment ions to calculate the mass of an amino acid residue on the peptide backbone.

2.3.9 Microwave-assisted solid phase peptide synthesis

All synthesized peptides described in this thesis were produced following our instruction by Paula O'Connor at Teagasc Food Research Centre Moorepark. The bioactive peptides were synthesized using microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a Liberty CEM microwave peptide synthesizer (Mathews, North Carolina, USA). Peptides were synthesized on H-Ala-HMPB-

ChemMatrix and H-Ile- HMPB-ChemMatrix resins (PCAS Biomatrix Inc., Quebec, Canada).Solid phase peptide synthesis was first described by Merrifield (1963) who was later awarded the Nobel prize for this work in 1984 [146]. It involves the sequential attachment of protected amino acid to an insoluble support resin. Synthetic peptides were purified using RP-HPLC on a Semi Preparative Jupiter Proteo (4u, 90A) column (Phenomenex, Cheshire, U.K.) developed in a gradient of 30−58% acetonitrile/0.1% TFA over 35 min for the peptide IRLIIVLMPILMA. Fractions containing the desired molecular mass were identified using MALDI-TOF mass spectrometry and were pooled and lyophilized on a Genevac HT 4X (Genevac Ltd., Ipswitch, U.K.) lyophilizer. The process of bioassay guided fractionation of *P. palmata* protein hydrolysates coupled with ESI-Q-TOF MS is summarized in Figure 2.1.

2.3.10 *In silico* prediction of peptide availability

The most active peptide sequences were assessed for potential cleavage by gastrointestinal tract (GI) enzymes using the program Expasy Peptide Cutter (http://ca.expasy.org/cgi-bin/peptidecutter/peptidecutter.pl) [147]. The peptides were evaluated against a number of enzymes that are found in the GI tract using by cleaving the peptide *in silico* with enzymes including pepsin (pH > 1.3 and pH 2.0), chymotrypsin, and trypsin.

2.4 Results

2.4.1 Protein quantification

Protein extracts were obtained using the method of Galland- Irmouli et al. (1999) [38]. The extraction procedure outlined in this study yielded protein levels of 10.01% (±0.24) protein on a dry weight basis. This is in close correlation to data found by Galland-Irmouli et al. (1999) in their seasonal variation study of the protein content of *P. palmata* harvested in late October, where *P. palmata* harvested off the west coast of France was found to have protein levels of approximately 10% of the total dry mass of *P. palmata*[38]. All of the eight essential amino acids were found in this protein extract. This compares favourably with protein extracted from other plant and animal protein sources (Table 2.1).

Essential amino acids	***Palmaria palmata***	**Egg**	**Soya**	**Yellow fin tuna**
Ile	4.8	5.4	5.1	4.0
Leu	7.2	8.6	7.6	6.9
Lys	4.5	7.0	6.1	8.1
Met	2.1	5.7	2.7	2.5
Cys	1.8	-	-	.4
Phe	4.7	9.3	8.4	3.3
Tyr	3.4	-	-	-
Thr	5.2	4.7	4.1	3.8
Val	8.1	6.6	5.2	4.5
Non-essential				
His	2.1			
Asp	12.3			
Glu	11.6			
Pro	3.4			
Ser	6.1			
Gly	6.1			
Ala	8.4			
Arg	6.6			

Table 2.1: Amino acid composition of protein extracted from *Palmaria palmata* compared to protein isolated from egg [147], soya [148] and yellow fin tuna [149].

2.4.2 RP-HPLC enrichment of the seaweed hydrolysate

To further isolate renin inhibitory peptides released during papain hydrolysis of *P. palmata* protein, RP-HPLC was performed and resultant fractions collected every minute were tested for their renin inhibitory activities. Figure 2.4 shows the RP-HPLC chromatogram obtained for the papain hydrolysate of the *P. palmata* protein. The fractions (Fr) observed between minute ten and minute twenty-five were collected and assayed for renin inhibition. The fraction collected at minute 25 (Fr-25) gave the highest renin inhibitory activity of all fractions tested and inhibited renin by 58.97% (±1.26) at a concentration of 1 mg/mL compared to the positive control Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe which inhibited renin by 94.71% (±0.87) at a concentration of 10 μM.

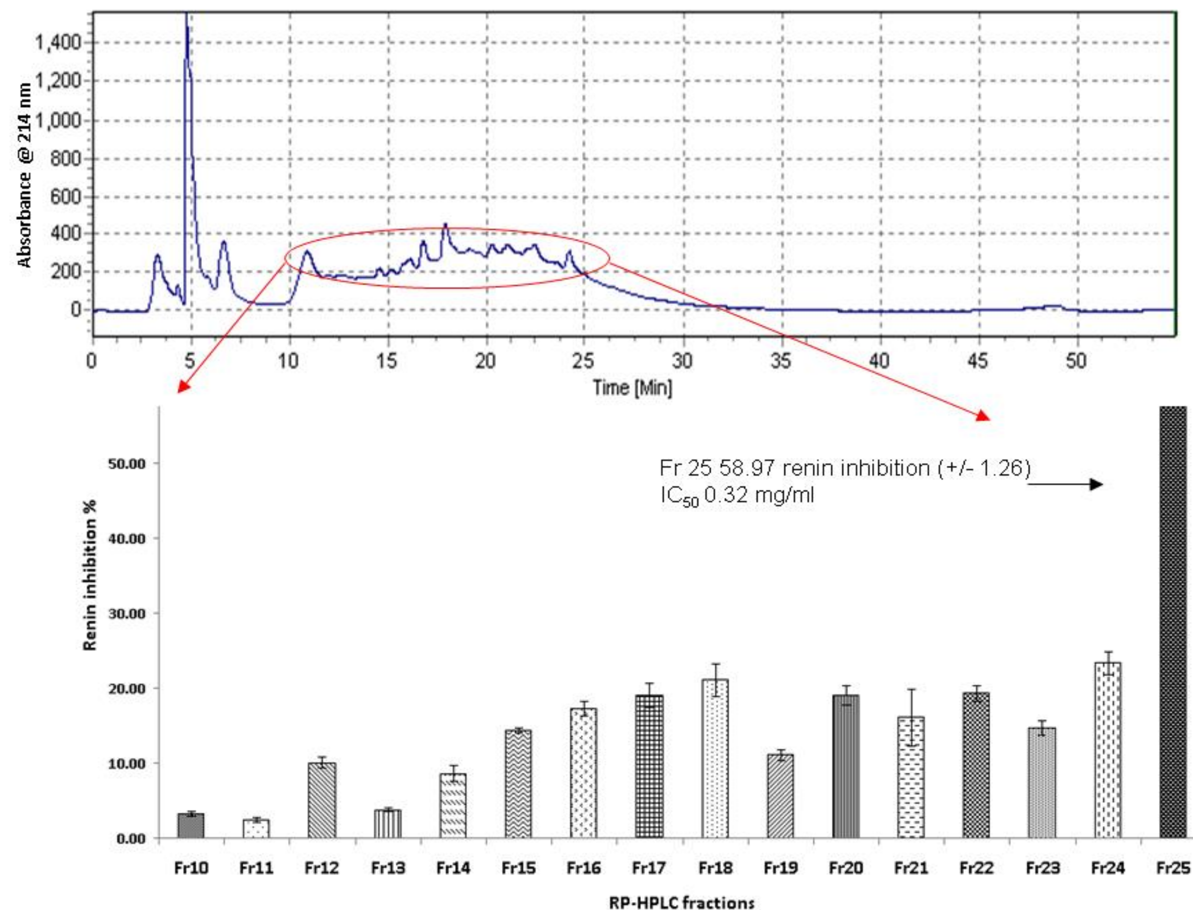

Figure 2.4: RP-HPLC chromatogram of a papain hydrolysate of *P. palmata* protein. Absorbance was plotted at 214 nm. The renin inhibitory results obtained from the RP-HPLC fractions collected between minute ten to twenty-five (Fr10−Fr25) of the *P. palmata* papain protein hydrolysate are shown. Samples were tested at a concentration of 1 mg/mL. Data is displayed as the percentage (%) inhibition values. The values obtained are mean ± SEM (n = 3).

73

2.4.3 *In vitro* inhibitory effects of *P. palmaria* papain hydrolysate on renin

A papain hydrolysate of the *P. palmata* protein extract was generated in order to release renin inhibitory peptides from the parent proteins. When assayed for renin inhibition, the papain hydrolysed protein extract inhibited renin by 41.89% (±3.22) compared to the specific renin inhibitor, Z-AAPFH-Sta-IHK-(Boc)-OMe, which was used as the positive control. This positive control was tested at two different concentrations: 1 µM (half the maximal inhibitory concentration (IC_{50}) and 10 µM, respectively. These concentrations were selected due to previous studies where the half maximal inhibitory concentration (IC_{50}) value for Z-AAPFH-Sta-IHK-(Boc)-OMe was obtained and also where this compound demonstrated >90% inhibition of renin at a concentration of 10 µM[145]. In this study, Z-AAPFH-Sta-IHK-(Boc)-OMe, assayed at 10 µM and 1 µM, inhibited renin by 94.71% (±0.87) and 57.35% (±0.91), respectively, as shown in Figure 2.5.

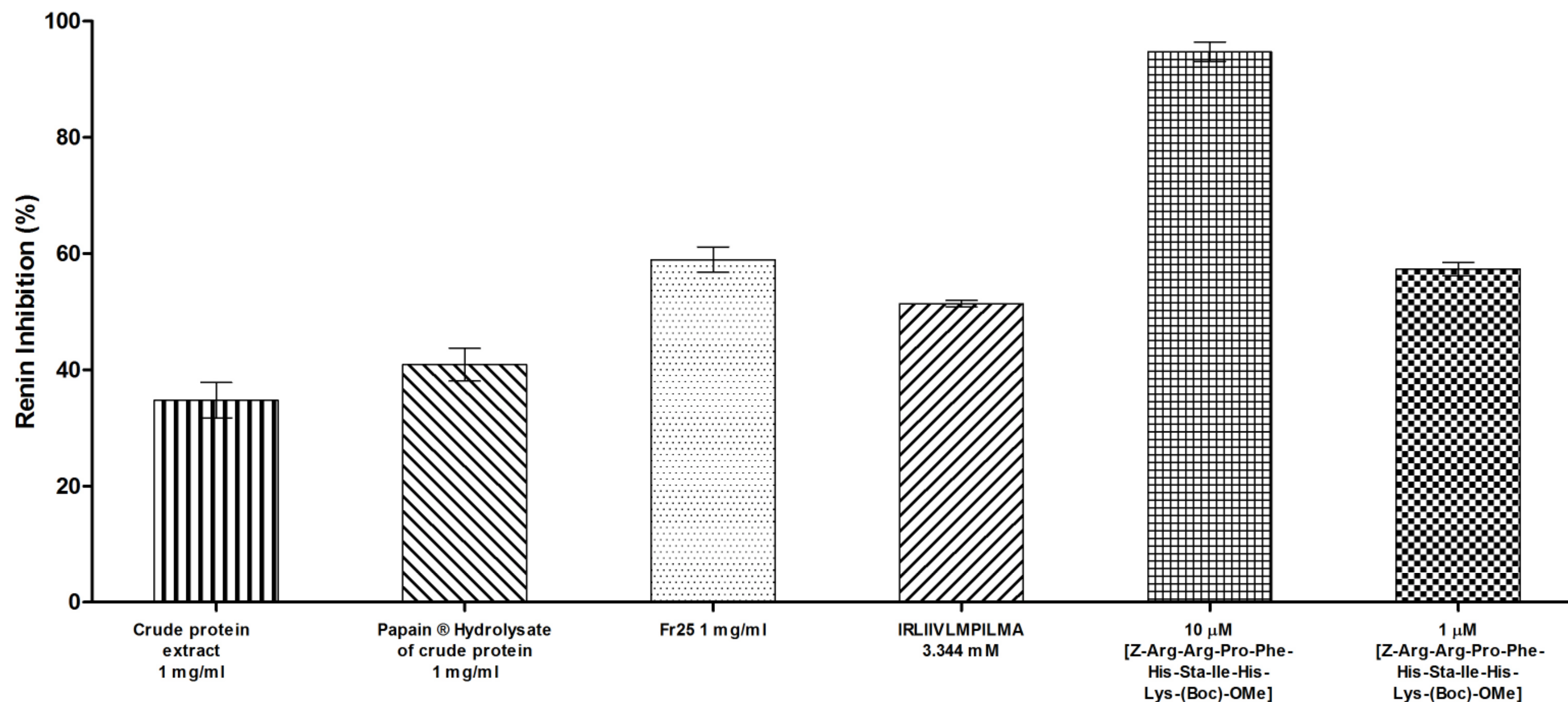

Figure 2.5: The percentage renin inhibitory results obtained for the protein, the papain hydrolysed protein, and Fraction (Fr) Fr-25, when assayed at a concentration of 1 mg/mL. Also shown is the chemically synthesised peptide IRLIIVLMPILMA assayed at the IC_{50} value of 3.344 mM (±0.31) and the positive control Z-AAPFH-Sta-IHK-(Boc)-OMe which was assayed at 1 μM and 10 μM. Values are mean ± SEM (n = 3).

2.4.4 Identification of renin inhibitory peptides from *P. palmata* protein hydrolysate

To identify the renin inhibitory peptides from Fr-25, ESI-QTOF MS was performed and eleven peptides were elucidated using *de novo* sequencing (Table 2.2). All eleven peptides identified had no homology to previously sequenced proteins from *P. palmata*. However, only 35 proteins from *P. palmata* have been characterised to date and all peptides identified from Fr-25, when aligned withBasic Local Alignment Search Tool(BLAST), show 100% homology with sections of the proteins; Photosystem II protein Y from *Cyanidium caldarium*; *Galdieria sulphuraria*, Photosystem I reaction center subunit VII from *Chlorella vulgaris*; and Cytochrome b6-f subunit 7 from *Cyanidioschyzon merolae*. These proteins are found in seaweed species closely related to *P. palmata*, including *Cyanidioschyzon merolae*, *Cyanidium caldarium*, *Galdieria sulphuraria*, and the microalga *Chlorella vulgaris*. For example, the protein Cytochrome c oxidase (accession number D5I1R8) isolated from *P. palmata* has 80.50% homology with Cytochrome c oxidase (accession number Q9ZZQ9) isolated from *Cyanidioschyzon merolae.* Furthermore, the protein Photosystem II D2 (accession number Q52W49), identified in *P. palmata* previously, is 93.20% homologous, with the same protein (accession number P28253) isolated from *Cyanidium caldarium* and *Galdieria sulphuraria* (accession number Q9TM47). Finally, the protein Photosystem I P700 chlorophyll A apoprotein (accession number Q8MAG9) isolated from *P. palmata* is 85.30% homologous, with the same protein isolated from *Chlorella vulgaris* (accession number P56341).

The chemically synthesized peptides listed in Table 2.2 were tested for renin inhibitory activities as described. Of the eleven chemically synthesised peptides, the tridecapeptide

corresponding to the amino acid sequence IRLIIVLMPILMA had a renin inhibitory IC_{50} value of 3.344 mM (±0.31).

Sequence	Phylum	Protein (assession number)	Species sharing 100% homology	Observed mass	Calculated mass	Charged state
D.IRLIIVLMPILMA.A	Rhodophyta	Photosystem II protein Y (O19893)	*Cyanidium caldarium,Galdieria sulphuraria*	1494.93	499.29	(+3)
MNEIVALMI.I	Rhodophyta	Cytochrome b6-f complex (Q85FX8)	*Cyanidioschyzon merolae*	1032.53	517.26	(+2)
P.ILMA.A	Rhodophyta	Photosystem II protein Y (O19893)	*Cyanidium caldarium,Galdieria sulphuraria*	446.25	447.1235	(+1)
I.LMAASWAIY.N	Rhodophyta	Photosystem II protein Y (O19893)	*Cyanidium caldarium,Galdieria sulphuraria*	1024.5	1025.48	(+1)
Q.ILPSILVPLV.G	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	1062.7	532.32	(+2)
L.PSIL.V	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	428.26	429.09	(+1)
I.LVPLVGLV.F	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	808.54	809.4	(+1)
V.PLVGLVFPAI.A	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	1024.63	1025.48	(+1)
L.VFPAIAM.A	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	747.39	748.4	(+1)
V.FPAI.A	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	446.25	447.12	(+1)
F.PAIA.M	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	370.22	371.11	(+1)

78

Table 2.2: Peptides identified in RP-HPLC Fr-25 enriched from the *P. palmata*papain hydrolysate**.** ., denotes the point of cleavage by the enzyme papain.

2.4.5 *In silico* analysis of the tridecapeptide IRLIIVLMPILMA

The tridecapeptide IRLIIVLMPILMA identified in this work is longer in length than most previously identified heart health peptides, such as IPP and VPP, and may be considered a pro-peptide. Therefore, this peptide was subjected to *in silico* analysis using the online computer tool Expasy peptide cutter (http://ca.expasy.org/cgi-bin/peptidecutter/peptidecutter.pl) [147] to determine potential cleavage peptides and amino acids that may result from potential digestion of the peptide *in vivo* with enzymes and acids. This tool is cost effective and provides a guide for researchers prior to *in vivo* animal studies which can be expensive. The option of all enzymes and chemicals from the list displayed in Expasy peptide cutter was chosen, and the results of cleavage analysis are shown in Table 2.3.

Enzyme	Cleavages	Position of cleavages
Arg-C proteinase	1	2
CNBr	2	8 12
Chymotrypsin-low specificity (C-term to [FYWML], not before P)	4	3 7 11 12
Clostripain	1	2
Pepsin (pH1.3)	3	3 6 11
Pepsin (pH>2)	3	3 6 11
Proteinase K	9	1 3 4 5 6 7 10 11 13
Thermolysin	9	2 3 4 5 6 9 10 11 12
Trypsin	1	2

Table 2.3: Cleavage of the peptide IRLIIVLMPILMA by proteolytic enzymes and acids using the programme Expasy Peptide Cutter

2.5 Discussion

The protein content obtained in this study were similar to those obtained in the compositional study carried out previously by Galland-Irmouli et al. (1999). However, seaweed collected in Ireland was found to contain more of the essential amino acids isoleucine (4.8 g/ 100g of amino acid) and threonine (5.2 g/ 100g of amino acid) compared to 4.6 and 0 g/100g of isoleucine and threonine for protein isolated from *P. palmata* collected in France.Furthermore, the *P. palmata* hydrolysate was found to contain higher levels of threonine and valine than the content of these amino acids found in eggs [148] and soya [149]. In addition, the seaweed hydrolysate generated in this work had higher levels of isoleucine, leucine, cysteine, phenylalanine, threonine and valine than that found in the protein fraction of yellow fin tuna[150]. The *Palmaria palmata* protein was also found to contain a substantial level of the essential amino acid lysine (4.8 g/ 100g of amino acid). These results suggest that *P. palmata* protein is of high nutritional value and may be a potential alternative source of proteins rich is essential amino acids.

Inhibition of the renin angiotensin aldosterone system (RAAS) is a well-established approach in the treatment of hypertension andthe generation of renin inhibitory bioactive peptides from food protein sources has been reported previously[72]. In this study, the enzyme papain was used to release bioactive peptides from *P. palmata* protein. Papain was chosen to perform hydrolysis, as it has generally recognized as safe (GRAS) status. Furthermore, previous literature detailed the peptide generating capacity of papain, and it was used previously to generate peptides from algal protein waste capable of reducing high blood pressure in the RAAS through ACE-I inhibition [151]. Another reason why papain was chosen as a hydrolytic enzyme is that it has a broad substrate specificity but also exhibits specific substrate preferences for bulky

hydrophobic or aromatic residues [152], which may correlate with renin inhibitory peptides [72]. Inhibition of the enzyme ACE-I, with chemically synthesized ACE-I inhibitors including captopril (Capoten ®), enalopril, alcacepril, and lisinopril is well-known [55]. However, inhibition of renin is thought to have several health advantages over ACE-I inhibition. Renin is the only known enzyme that converts angiotensinogen to angiotensin I. In the phenomenon known as “ACE escape”, angiotensin II levels increase in blood plasma due to other enzymes converting angiotensin I to angiotensin II during ACE-I inhibition [80]. Renin inhibition in turn eliminates angiotensin I from the bloodstream, preventing the formation of angiotensin II. Another advantage of renin inhibition over ACE-I inhibition is that renin inhibitors do not affect kinin metabolism and, hence, are not expected to cause the side effects associated with ACE-I inhibition including dry cough or angioneurotic edema [131].

While ACE-I inhibitory peptides were isolated from the macroalga*Undaria pinnatifida*before [29], this study is, to the best of the authors knowledge, the first to identify renin inhibitory activities for peptides isolated from *P. palmata*, or indeed any macroalga. Papain hydrolysed *P. palmata* protein inhibited renin by 41.89% (±3.22). It is well documented that hydrolysis with proteolytic enzymes such as papain is used frequently as a method to liberate bioactive peptides from plant and other proteins [72, 153]. In this study, hydrolysis of *P. palmata* protein with papain and further fractionation using RP-HPLC was found to enhance the renin inhibitory activity of the protein. Fraction twenty-five (Fr-25) inhibited renin activity by 58.97% (±1.26) at a concentration of 1 mg/mL compared to the chemically synthesized positive control, Z-Arg-Arg-Pro-Phe-His-Sta-Ile- His-Lys-(Boc)-OMe, which inhibited renin by 50% at a concentration of 1 μmol/mL (Figure 2.5).

In order to confirm the renininhibitory activity of Fr-25, the peptides within this fraction were identified and characterised using ESI-Q-TOF mass spectrometry, and elucidated using *de novo* sequencing. Eleven peptides (Table 2.1) were chosen for chemical synthesis based on their amino acid sequences. Sequences with amino acids containing bulky side chains at the C-terminus and hydrophobic amino acids including isoleucine and arginine at the N-terminus were chosen for chemical synthesis, as peptides containing these amino acid sequences were documented previously as potential enzyme (ACE-I and renin) inhibitors [72, 154] In order to inhibit ACE-I and renin, aromatic or branched side chain residues are preferred in the amino acid sequence of the peptide. Aliphatic, basic and aromatic residues are preferred in the penultimate positions and aromatic, proline and aliphatic residues are preferred in the ultimate position for ACE-I inhibitory peptides [155]. In this study, chemical synthesis of the peptides was necessary in order to confirm the observed renin inhibitory activities of Fr-25. Table 2.1 displays the eleven peptides identified in Fr-25. It also shows the parent macroalgal proteins that contain the amino acid sequences elucidated for the identified peptides. These proteins were not characterised for *P. palmata*. The peptides elucidated in this study do not share homology with any peptides discovered in *P. palmata*, as to date there are only 35 proteins completely sequenced from *P. palmata* [152]. However, the eleven peptides identified in Fr-25 were found to correspond to the amino acid sequences of proteins found in seaweed species closely related to *P. palmata*. *P. palmata* proteins are closely related to proteins sequenced from the seaweed species *Cyanidioschyzon merolae*, *Cyanidium caldarium*, *Galdieria sulphuraria*, and *Chlorella vulgaris*. Inherent protein sequence homologies exist between *P. palmata* and these macroalgae species.

In this work, all chemically synthesized peptides were assayed for their abilities to inhibit renin, and the tridecapeptide IRLIIVLMPILMA (Figure 2.5) inhibited reninby

50% at a concentration of 3.344 mM (±0.31). This compared favourably with the positive control, which was tested at 10 μM and 1 μM, respectively, and also to previously isolated peptidic renin inhibitors from plant pea sources that displayed IC_{50} values between 9.2 mM (± 0.18) and 22.66 mM (± 1.71) [72, 153].

Several published works explore the potential of *in silico* methods for screening bioactive peptides [72, 154]. In this study, the propeptide IRLIIVLMPILMA identified in Fr-25 and confirmed to inhibit renin*in vitro* using the Cayman renin inhibitory bioassay method was subjected to *in silico* cleavage analysis using the computer program Expasy peptide cutter. Enzymes found in the gastrointestinal (GI) tract were chosen for cleavage analysis. Enzymes that may cleave the propeptide IRLIIVLMPILMA potentially release di- and tri-peptides. For example, pepsin (pH 1.3 and pH > 2) is predicted to cleave this propeptide at positions 3, 6, and 11, releasing the dipeptide IR and the tripeptide IIV and the di-peptide MA. It also may release the pentapeptide LMPIL. In addition, the enzymes trypsin, thermolysin, and arg-C-proteinase were predicted to release the dipeptide IR from IRLIIVLMPILMA (Table 2.3). Overall, the N-terminal amino acids of the propeptide remained largely uncleaved, with only proteinase K capable of cleaving the peptide in the first position. These results suggest that this propeptide and the di- and tripeptides released from it may cross the lumen into the bloodstream and potentiate an antihypertensive effect. Indeed, certain peptides including IR and IRL were previously documented as having renin, antioxidant, and ACE-I inhibitory activities [72, 141]. The peptide IR has a documented IC_{50} value of 9.2 mM for renin [72].Furthermore the peptide IRLIIVLMPILMA is quite hydrophobic due to the isoleucine, leucine and valine residues. Hydrophobic peptides were shown previously to be considerably bioavailable with the capabilities to cross the gut mucosa and to enter the blood stream more efficiently [110].

CHAPTER 3. DEVELOPMENT OF A SEAWEED DERIVED PLATELET ACTIVATING FACTOR ACETYLHYDROLASE (PAF-AH) INHIBITORY HYDROLYSATE, SYNTHESIS OF INHIBITORY PEPTIDES AND ASSESSMENT OF THEIR TOXICITY USING THE ZEBRAFISH LARVAE ASSAY.

This chapter was published as a research article in the Journal Peptides: Ciarán Fitzgerald, Eimear Gallagher, Paula O'Connor, José Prieto, Leticia Mora-Soler, Maura Grealy, Maria Hayes, "Development of a seaweed derived platelet activating factor acetylhydrolase (PAF-AH) inhibitory hydrolysate, synthesis of inhibitory peptides and assessment of their toxicity using the zebrafish larvae assay", (2013), 50, 119-124.

3.1 Aims

Dietary modifications for heart health continue to rise in popularity with consumers. While nine in 10 (87%) persons with chronic high blood pressure and/or cholesterol depend heavily on prescriptions to treat their condition, 50 % are also using diet to manage their condition. Circulatory health ingredients are popular with consumers and up and coming heart markets include stroke, atherosclerosis, minimizing blood plaque and improving circulation. As well as targeting the enzyme Renin, this thesis examined the use of the red seaweed as a source of PAF-AH inhibitory peptides. A PAF-AH inhibitory peptide NIGK was generated from a hydrolysate of *P palmata* protein using the enzyme papain and screened for its ability to inhibit PAF-AH. This work was pursued as the vascular inflammatory role of platelet activating factor acetylhydrolase (PAF-AH) is thought to be due to the formation of lysophosphatidyl choline and oxidized non-esterified fatty acids. This enzyme is considered a promising therapeutic target for the prevention of atherosclerosis and there is a need to expand the available chemical templates of PAF-AH inhibitors. The prevention of atherosclerosis through diet is an interesting area of scientific research and one which this thesis explores. Within this chapter, PAF-AH inhibitory peptides were isolated and characterized from the red macroalgae *P. palmata*. Furthermore, it examines the toxicity of a *P. palmata* protein hydrolysate using a zebrafish larvae assay. The main objectives of this chapter were to:

(1) Examine *Palmaria palmata* protein hydrolysate for peptides with the ability to inhibit the action of the enzyme PAF-AH which is important in circulation health

(2) Use *in vitro* bioassay guided fractionation to identify PAF-AH inhibitory hydrolysates and RP-HPLC fractions.

(3) Identify and sequence PAF-AH inhibitory peptides using nano- ESI-Q-TOF MS, *de novo* sequencing and solid phase peptide synthesis.

(4) Examine the toxicity of the *P. palmata* protein hydrolysate using a zebrafish larvae assay as an experimental model

3.2 Introduction

Atherosclerosis is an inflammatory disease and the most common cause of stroke and Cardiovascular disease worldwide [156] . Recent studies suggest that the enzyme lipoprotein-associated phospholipase A_2 (Lp-PLA2) or platelet activating factor acetylydrolase (PAF-AH) plays an active role in atherosclerotic development and progression [82, 157]. Indeed, in 2008, Wilensky and colleagues showed that selective inhibition of PAF-AH prevented progression to advanced coronary atherosclerotic lesions and confirmed the role of vascular inflammation, independent from hypercholesterolemia, in the development of lesions implicated in the pathogenesis of myocardial infarction and stroke [82].

As previously stated, PAF-AH is secreted from multiple inflammatory cells but is highly expressed in the necrotic core of atherosclerotic lesions [83, 158]. PAF-AH is predominately bound to apolipoprotein containing lipids and 80% of PAF-AH in blood plasma is bound to low density lipoproteins (LDL) [83, 158]. LDL is oxidised following its transportation from the lumen to the intima of the cell [83]. This facilitates hydrolysis of LDL by the enzyme PAF-AH into two pro-inflammatory mediators; lysophosphatidylcholine (LPC) and oxidised non-esterified fatty acids (oxNEFAs) [82, 159].

Growing evidence supports a pro-atherogenic role of PAF-AH in blood circulation. Pharmaceutical companies including GlaxoSmithKline (GSK) have invested financially in the development of synthetic compounds to combat atherosclerosis through PAF-AH inhibition. For example, GSK are in phase three clinical trials with the substituted pyrimidone darapladib, a synthetic, non-peptidic PAF-AH inhibitor [160]. However, very few natural inhibitors of PAF-AH are known to date and a patent search regarding PAF-AH inhibitors derived from plant and natural resources yielded only a few studies

regarding uncharacterized "crude" extracts. For example, Yu et al. identified PAF-AH inhibitors from extracts generated from 224 terrestrial plants [160]. Methanolic extracts were generated from these plants and were further partitioned with n-hexane, chloroform and ethyl acetate [161]. Seven methanol extracts were found to inhibit PAF-AH by 50% at a concentration of 100 mg/ml and two ethyl acetate extracts were found to inhibit PAF-AH activity at the same concentration [161]. In another study, synthetic analogs of cyclic enol-carbamates isolated from the bacteria *Pseudomonas fluorescens* were identified as potent inhibitors of PAF-AH [162]. Drugs that influence lipoprotein concentration were studied previously for their potential inhibitory effects on PAF-AH. Drugs examined to date included niacin, statins and finofibrate [162].

In this chapter, the amino acid composition of Irish sourced *P. palmata* was determined. Furthermore a PAF-AH inhibitory tetrapeptide with the amino acid sequence Asparagine-Isoleucine-Glycine-Lysine (NIGK) was generated, enriched for and isolated from the red seaweed *P. palmata* by hydrolysis of this seaweed protein with the food grade enzyme papain. This enzyme was chosen as it was used successfully in previous studies to release bioactive peptides from protein. Papain is also a food grade cysteine protease with known proteolytic capabilities [153, 163]. The peptide NIGK was chemically synthesized and its PAF-AH inhibitory activity confirmed using an *in vitro* bioassay method. In addition, the toxicity of the seaweed hydrolysate was assessed using the zebrafish larvae bioassay. Two-day-old zebrafish larvae were exposed to 1, 5, and 10 mg/ml concentrations of the hydrolysate over a 48 hr period to examine any toxic effects the hydrolysate may have in a living model.

3.3 Materials and Methods

3.3.1 Materials and Reagents

The PAF-AH inhibitor screening kit was supplied by the Cayman Chemical Company (Ann Arbor, MI, USA). Acetonitrile and water were supplied by Romil Ltd. (Cambridge, England, United Kingdom). Dimethyl sulfoxide (DMSO), Ammonium sulphate and the specific platelet activating factor (PAF) inhibitor methyl arachidonyl fluorophosphates (MAFP), which was used as a positive control, were supplied by Sigma–Aldrich (Steinheim, Germany). H-Ala-HMPB-ChemMatrix and H-Ile-HMPB-ChemMatrix resins were supplied by Produits Chimiques Auxiliaires et de Syntheses(PCAS) Biomatrix Inc. (Quebec, Canada). All other chemicals used were of analytical grade. Instant Ocean ® was acquired from Spectrum Brands (Ontario, Canada)

3.3.2 *Palmaria palmata* protein extraction and hydrolysis with papain

Protein was extracted from *P. palmata* following the protocol of Galland-Irmouli et al. [38] as described in section 2.2.2. The hydrolysis process used was the same as the process used to isolate renin inhibitory peptides in section 2.2.3.

3.3.3 RP-HPLC analysis

RP-HPLC analysis was carried out according to the method described in Section 2.3.5. The same RP-HPLC chromatogram was generated.

3.3.4 PAF-AH inhibitor screening assay

This assay was carried out using a PAF-AH inhibitor kit supplied by Cayman Chemical Company in accordance with the manufacturers' instructions. Briefly, prior to carrying out the assay, the PAF-AH inhibitor assay buffer was diluted by adding 27 ml of HPLC

grade water to 3 ml of 0.1 M Tris–HCl (pH 7.2). The substrate, 2-thio PAF was reconstituted in 12 ml of diluted assay buffer to achieve a concentration of 400 µM. Background wells were prepared by adding 10 µl of assay buffer, 200 µl of 2-thio PAF substrate and 10 µl of solvent (in this case DMSO/water 5:95, v/v) together in a well (×3). Initial activity wells were prepared by adding 200 µl of the 2-thio PAF substrate solution and 10 µl of the solvent DMSO/water (5:95, v/v) together in a well (X3). Inhibitor wells were prepared by adding 200 µl of the 2-thio PAF substrate solution and 10 µl of the test fraction. The reaction was initiated by adding 10 µl of human plasma PAF-AH to all wells except the background wells. The plate was then covered and incubated for 20 min at 25 ℃. After incubation, 10 µl of 5, 5' dithio-bis (2-nitrobenzoicacid) (DTNB) was added to all wells to develop the reaction. All potential inhibitors were assessed in triplicate. The PAF-AH inhibition assay uses 2-thio PAF as a substrate for PAF-AH. The acetyl thioester bond is cleaved at the sn-2 position by PAF-AH [164]. The sulphur bond of the DTNB molecule is then broken due to the available ionic sulphur on the substrate leaving the 5-thio-2-nitrobenzoic acid which can be detected at an absorbance of 414nm (as illustrated in Figure 3.1). Methyl arachidonyl fluorophosphonate (MAFP), a known PAF-AH inhibitor was used as a positive control. The inhibitory concentration that reduced PAF-AH activity by 50% (IC_{50}) was determined by nonlinear regression from a plot of peptide concentration versus percentage inhibition.

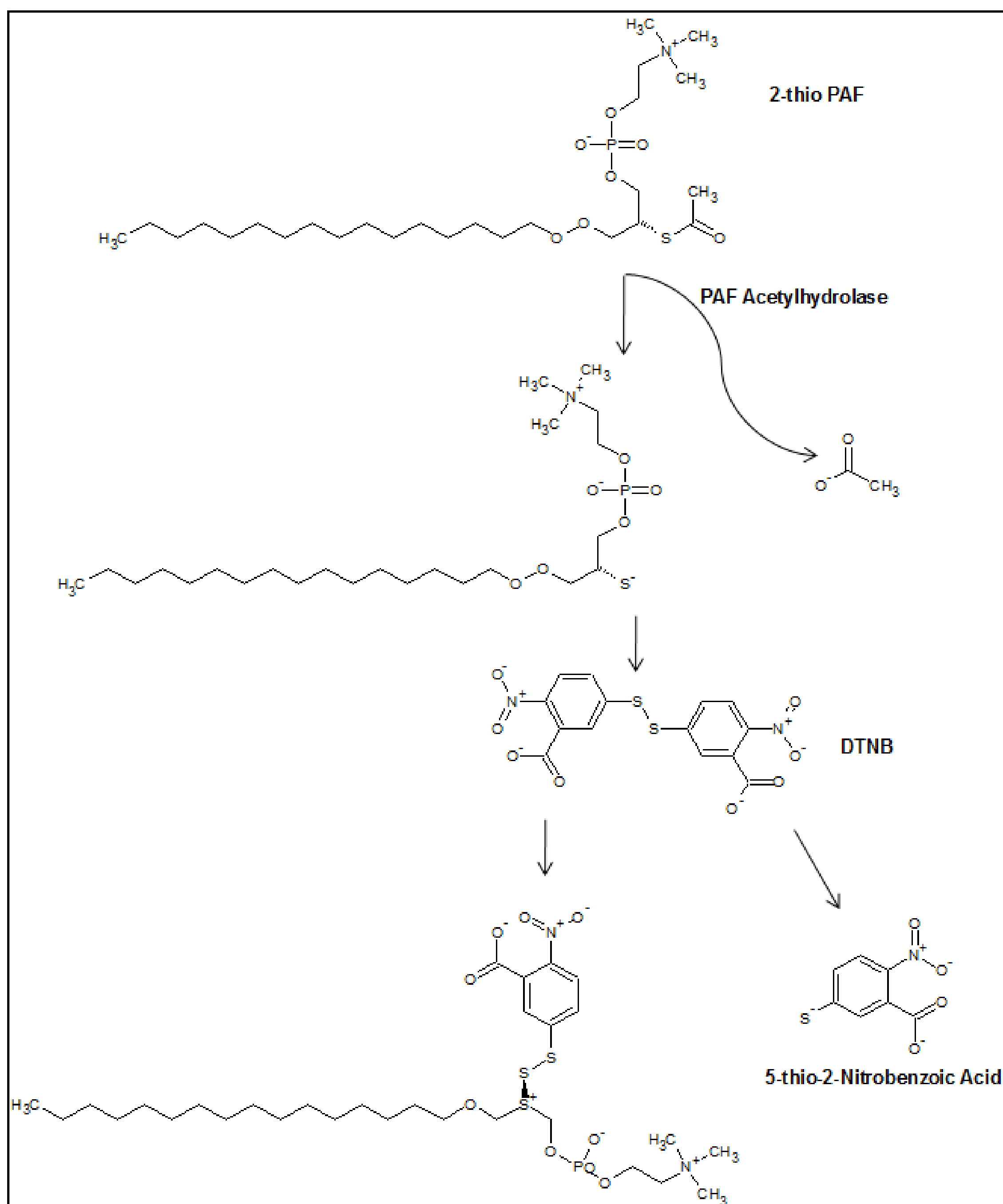

Figure 3.1: The PAF-AH inhibition assay. PAF-AH enzyme activity is assessed by measuring the amount of 5-thio-2-Nitrobenzoic Acid produced after PAF-AH cleaves the acetyl thioester bond at the *sn*-2 position of the 2-thio PAF substrate in the presence of DTNB.

3.3.5 Fractionation of *Palmaria palmata* hydrolysate using RP-HPLC

The *P. palmaria* papain hydrolysate was enriched further as described in section 2.2.5.

3.3.6 Peptide identification by tandem mass spectrometry

Peptides were identified using the method described in section 2.2.7.

3.3.7 Database search, confirmation of sequences, and de novo sequencing

Sequencing and confirmation of peptide sequences were carried out by the same method as described in 2.2.8.

3.3.8 Microwave-assisted solid phase peptide synthesis

Peptides were sequenced using the method described in section 2.2.9.

3.3.9 Zebrafish larvae assay

The protocols for the maintenance and care of zebrafish (*Daniorerio*) were carried out according to guidelines published by Westerfield (1993) [165]. The fish were kept in a room at 28 ºC with a light cycle of 14:10 light:dark and at a stocking density of 20 fish per 20 L tank. On the evening before spawning, two male fish were placed in spawning trays (20 cm × 9 cm) with a female separated from the males by a mesh insert. Upon entering the light cycle the following morning, the fish were put together. Post spawning, embryos were gathered from the base of the spawning trays and cultured in filter-sterilized egg water (60 µg/ml "Instant Ocean" aquarium salts in distilled water) at 28 ºC. To assess the effects of the *P. palmata* protein hydrolysate on the zebrafish larvae, 48 h old larvae were placed in a 12 well plate (ThermoFisher Scientific, MA,

USA). Ten zebrafish larvae were present in each well of this plate. The hydrolysate was tested in triplicate (3 wells containing 10 zebrafish per well) at 1, 5, and 10 mg/ml concentrations along with a control of distilled water. The heart rate and survival of the larvae were then monitored over a 48 h period by visually counting the heart beats over the course of a minute. Images were captured on a Nikon DXM1200C Digital camera at a magnification of 4 and an exposure of 1/300 s using the program Nikon ACT-1C (Nikon, Tokyo, Japan).

3.4 Results

3.4.1 PAF-AH inhibitory activity of *P. palmata* hydrolysates and fractions

The PAF-AH inhibitory peptides identified in this study were found to correspond to peptide sequences found in proteins characterised from macroalgal species closely related to *P. palmata*. The amino acid sequences of peptides identified in this study using de novo sequencing are presented in Table 3.1. All peptides presented are between four and 13 amino acids in length. When aligned using BLAST, these peptides share 100% sequence homology with the protein Photosystem II protein Y from *Cyanidium caldarium* and the protein Photosystem I reaction subunit VII from *Chlorella vulgaris*. The peptide NIGK corresponded to the amino acid sequence 22–25 of the protein Photosystem II Protein Y found in the red algae *C. caldarium* and *Galdieria sulphuraria*.

Sequence	Protein (assession number)	Species sharing 100% homology	Start-End	Calculated mass	Observed mass	Charged state
D.IRLIIVLMPILMA.A	Photosystem II protein Y (O19893)	*Cyanidium caldarium, Galdieria sulphuraria*	3 - 15	1494.93	499.29	(+3)
F.PAIA.M	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	19 - 22	370.22	371.11	(+1)
V.FPAI.A	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	18 - 21	446.25	447.12	(+1)
P.ILMA.A	Photosystem II protein Y (O19893)	*Cyanidium caldarium, Galdieria sulphuraria*	12 - 15	446.25	447.1235	(+1)
L.VFPAIAM.A	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	17 - 23	747.39	748.4	(+1)
S.AQILP.S	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	3 - 7	540.32	541.35	(+1)
Y.NIGK.A	Photosystem II protein Y (O19893)	*Cyanidium caldarium, Galdieria sulphuraria*	22 - 25	430.25	431.09	(+1)

Table 3.1: Peptides identified by tandem mass spectrometry enriched from the *P. palmata* papain hydrolysate.., denotes point of cleavage by the enzyme papain.

3.4.2 PAF-AH inhibition of synthesised peptides

The PAF-AH inhibitory values are presented in Figure 3.2. All synthesised peptides and the *P. palmata* protein hydrolysate were tested at 1 mg/ml while the known PAF-AH inhibitor MAFP (thepositive control) was tested at a concentration of 259.35 ng/ml. This concentration was chosen as previous studies reported this as the concetration at which 90% of the PAF-AH enzyme was inhibited [166]. The peptide with the amino acid sequence NIGK was the strongest peptidic inhibitor observed, inhibiting PAF-AH by 50.74% (± 2.12) at a concentration of 1 mg/ml. The protein hydrolysate from which these peptides were derived inhibited PAF-AH by 30.37% (± 1.97) at the same concentration compared to the positive control MAFP which inhibited PAF-AH by 80.56% (±1.84) at a concentration of 259.35 ng/ml.

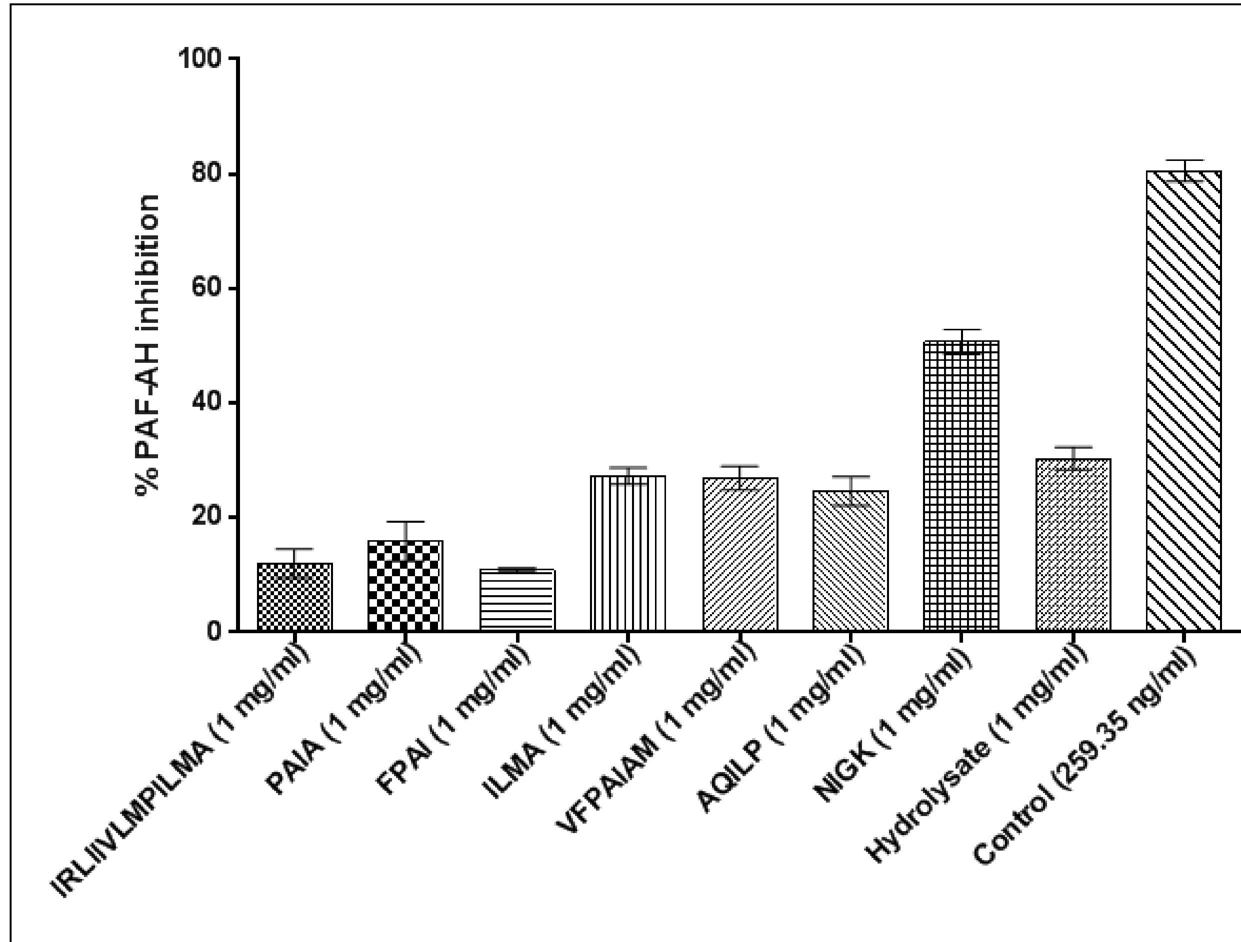

Figure 3.2: PAF-AH inhibitory activities of seven, chemically synthesized peptides isolated from *Palmaria palmata* protein; The *P. palmata* hydrolysate and as a control, the known PAF-AH inhibitor methyl arachidonyl fluorophosphates (MAFP). Values are mean ± SEM (n = 3).

3.4.3 Toxicity of *P. palmata* hydrolysate on zebrafish larvae

Figure 3.3.A shows the survival of the larvae over a 48 h period. A significant toxic effect was observed when the larvae were subjected to a dose of 10 mg/ml of the *P. palmata* hydrolysate. Less than 20% of the larvae survived after 24 h and none after 30 h of exposure. This effect was also apparent when the heart rate of the larvae was monitored (Figure 3.3.B). The heart rate of the larvae dropped to 46 beats per minute (BPM) compared to the control which had a heartbeat of 122.66 BPM after 24 h of contact with the hydrolysate. When the larvae were exposed to 5 mg/ml of the hydrolysate, no significant drop in percentage survival rates of the larvae or drop in heart rates were observed after 24 h of exposure as shown in Figure 3.3.A and B. However, after 48 h of contact with the hydrolysate at a concentration of 5 mg/ml, survival rates of the larvae were down to 30% and the BPM of the surviving larvae was 113 BPM compared to the control BPM of 133.3. No adverse effects were seen in terms of percentage survival or BPM of the larvae exposed to a concentration of 1 mg/ml of the *P. palmata* hydrolysate. All larvae survived after 48 h exposure and the heart rate of these larvae was not significantly different to that of the larvae used in the control wells.

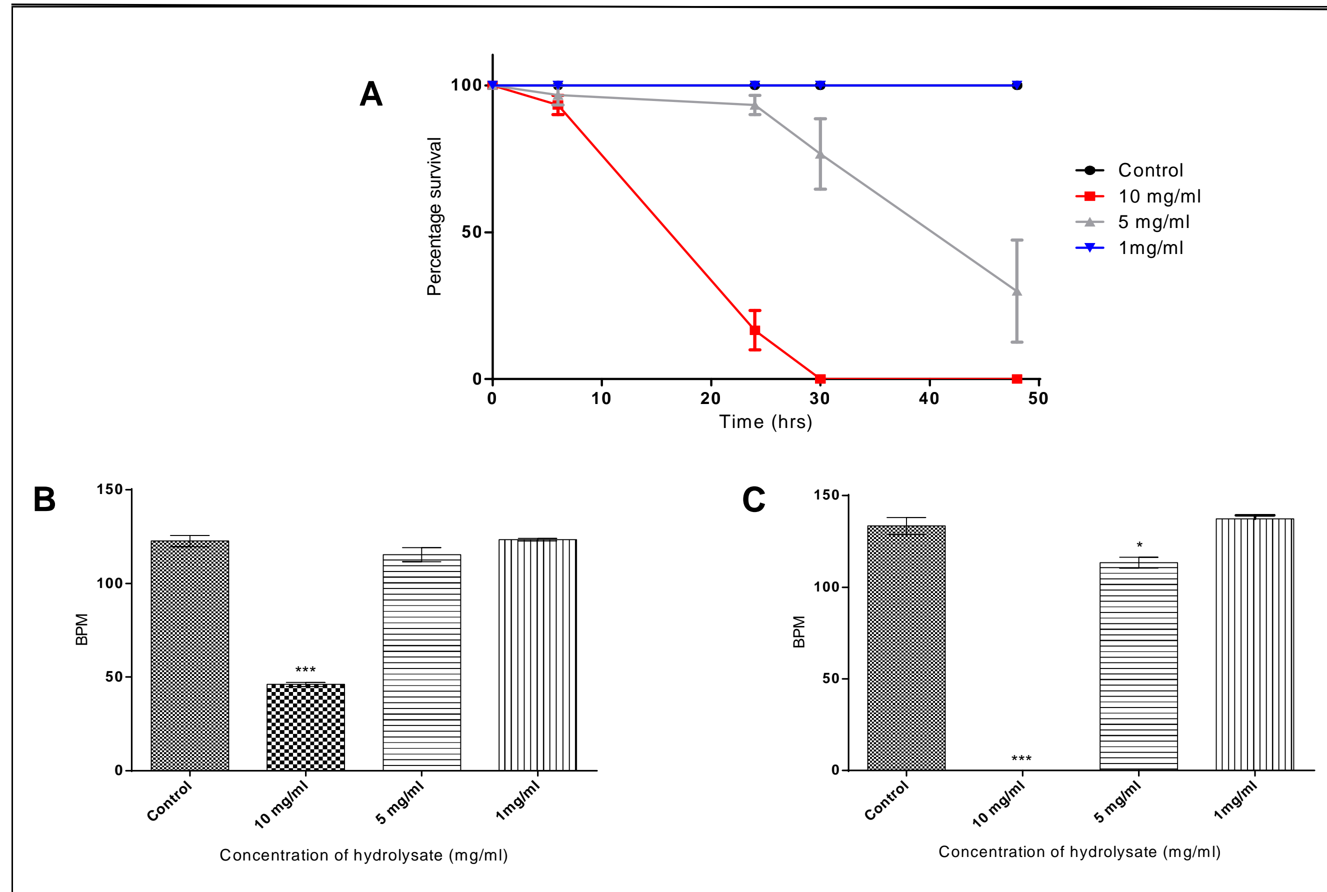

Figure 3.3: Survival of zebrafish larvae over 48 h when exposed to the control and 10, 5 and 1 mg/ml of the *P. palmata* hydrolysate can be seen in A. Values are mean ± SEM (n = 3).B and C shows the heart rate in beats per minute (BPM) of the larvae when exposed to the control and 10, 5 and 1 mg/ml of the hydrolysate after 24 h contact and 48 h contact, respectively. Values are mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control.

After 24 hr (Figure 3.4.A) of exposure to the hydrolysate at concentrations of 1 mg/ml and 5 mg/ml, the larvae were found to have no significant morphological differences when compared to the control larvae with the exception of a slightly swollen yolk sack in larvae exposed to 5 mg/ml of the hydrolysate. The larva exposed to 10 mg/ml of the hydrolysate (Figure 3.4.A) developed severe curvature of the spine and a deformed yolk sack. After 48 h, (Fig 14.B) the larvae exposed to 1 mg/ml of the hydrolysate were still morphologically identical to that of the control while the larvae exposed to 5 mg/ml appeared to have swollen yolk sacks and also developed curved spines.

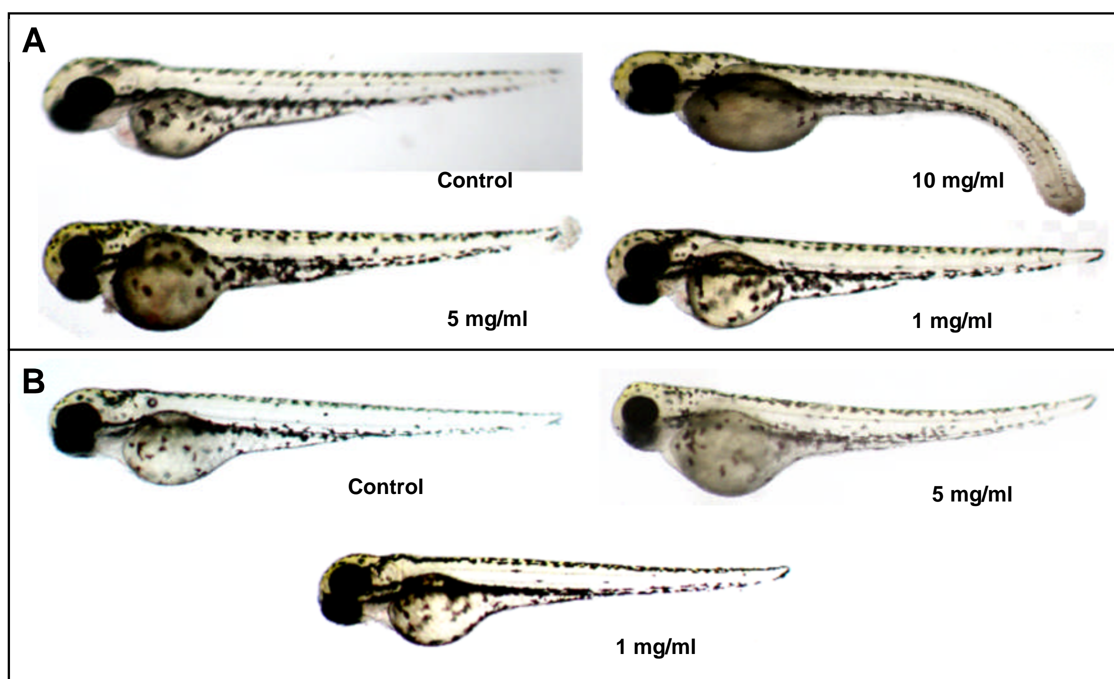

Figure 3.4: Images of the larvae after 24 h exposure (A) and 48 h exposure (B) to the control and 10, 5 and 1 mg/ml of the hydrolysate.

3.5 Discussion

RP-HPLC was used to further fractionate potential PAF-AH inhibitory peptides from the *P. palmata* hydrolysate. Sixty fractions were generated and screened for PAF-AH inhibitory activities [164]. Fraction twenty-five was further purified using ESI-Q-TOF MS. All peptides identified and selected for chemical synthesis, with the exception of NIGK, were hydrophobic in nature and had either an aromatic or a hydrophobic residue at their N-terminus. For example, the peptide FPAI possessed the aromatic residue phenylalanine while the hydrophobic residue proline was present in the peptide PAIA. Previous studies have linked heart health bioactivity (ACE-I and renin enzyme inhibition) with hydrophobic or aromatic amino acids at the N-terminus of the peptide [72, 167]. The peptide NIGK was chosen for chemical synthesis and further bioactivity assessment due to its water soluble nature as water soluble peptides are easily included in food and pharmaceutical products and are suitable for large scale production. In addition, the positive charge associated with both the arginine and lysine residues at the N- and C-terminal ends of the peptide sequence are associated with the characteristics of heart health peptides identified previously [83]. As discussed in Chapter two, a problem that was encountered during this work was that only 35 *P. palmata* proteins were fully characterised and accessible in the PubMed database (http://www.ncbi.nlm.nih.gov). The peptides identified in this study and shown in Table 3.1 were found to correspond to peptide sequences found in proteins characterised from macroalgal species closely related to *P. palmata* including *C. caldarium*, *G. sulphuraria* and *C. vulgaris*. In the absence of an entirely sequenced proteome for *P. palmata* this homology verifies the origins of these peptides along with e-values where available using BLAST (http://blast.ncbi.nlm.nih.gov/Blast). The e-values obtained for the peptides identified in this study ranged from 1.1 to 4.0×10^{-2} when assessed using

BLAST. Furthermore, the sequences identified were not found in the peptide database BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). However, the di-peptides NI and GK that could potentially be generated from peptide NIGK were identified in the sequences of other peptides found within BIOPEP. Figure 3.2 shows the percentage PAF-AH inhibitory values of the seven peptides identified from the *P. palmata* papain hydrolysate that were assayed for PAF-AH inhibition at a concentration of 1 mg/ml. All synthesized peptides and the *P. palmata* protein hydrolysates were tested at 1 mg/ml as this concentration was shown to be non-toxic when tested on zebrafish larvae (Figures 3.3 and 3.4). Furthermore, where enzyme inhibition by peptides generated from food sources was studied previously, concentrations of 1 mg/ml were used [72].

The concentration at which the tetrapeptide NIGK exhibited half the maximal inhibitory concentration (IC_{50}) was 2.32 mM (±2.12) compared to the positive control MAFP which inhibited PAF-AH by 80.56% (± 1.84) at a concentration of 700 nM. The positive control MAFP compound is a proven inhibitor of PAF-AH in endothelial cells [166].

The PAF-AH inhibitory drug darapladib operates through inhibition of azetidinone [168]. In this chapter, the mechanism of action of PAF-AH inhibition by the tetrapeptide NIGK and whether it inhibits in a competitive or non-competitive manner was not examined. The catalytic site of PAF-AH contains a Serine residue at position 273 [168]. Azetidinones target the serine residue in the active site of enzymes [168]. Inhibition of PAF-AH by the peptide NIGK may be due to salt bridge formation between the Lysine residue at the C-terminus of NIGK and the Serine residue of the active site of PAH-AH, which in turn, would incapacitate the active site [169]. The inhibitory concentrations of NIGK and the *P. palmata* papain protein hydrolysate are

much higher than those reported for the crude plant extract PAF-AH inhibitory activities reported by Yu et al., where extracts inhibited PAF-AH by 50% at a concentration of 100 mg/ml [161]. However, these extracts were not purified and the active components were not identified.

While seaweed and seaweed products have, for centuries, been consumed as part of a normal diet in numerous countries across the globe, it was important to examine any toxicological effects this particular hydrolysate may have *in vivo.* Sensory analysis on a bread product containing this hydrolysate was carried out in a future Chapter (Chapter 5). These concerns were addressed by use of the zebrafish larvae assay. Zebrafish embryos and larvae make for excellent models when screening compounds, where only a small quantity of test material is available for use. Furthermore, zebrafish survive for days in low volumes of water [170]. Results obtained in Figures 3.3 and 3.4 demonstrated that the zebrafish larvae exposed to 1 mg/ml of the *P. palmata* protein hydrolysate were not adversely affected by this concentration of the PAF-AH inhibitory hydrolysate. This assay was carried out in triplicate (3 $\times$ 10 zebrafish larvae). *P. palmata* hydrolysate concentrations of 5 and 10 mg/ml however were toxic to the zebrafish larvae assayed. Use of the zebrafish larvae assay as a model system goes back to the early 1970s when George Streisinger selected zebrafish larvae to develop the first Vertebrate assay enabling forward genetic screening [171, 172]. Zebrafish were mainly used to study organ development. Indeed, zebrafish are seen as a relevant model for human disease and pharmaceutical research [173, 174]. An advantage of the zebrafish model is that they can be used in medium and large scale screening procedures as they are small, robust and survive hostile conditions [175]. Other advantages for using zebrafish larvae include; a clear embryo which permits unobstructed observation of developmental morphology, the high rate of this development, high fecundity, and develpment that is similar to that of mammals [176]. The main limitation with the use of

zebrafish larvae in assessing potential safety issues, as is the case in this study, is the lack of extensive validation with diverse and comprehensive pharmacological compound sets to adequately understand the advantages and limitations of the zebrafish larve model [177]. Another drawback of the zebrafish larvae assays is that uptake of compounds into the zebrafish larvae can vary and they should be measured for accurate interpretation of results, thereby avoiding false negatives [177]. Furthermore, the avoidance of a false negative result in terms of the toxicity of the peptide and hydrolysate was overcome in Chapter four where the toxicity of the hydrolysates was assessed by Eurofin Foods Ireland Ltd, (Finglas, Dublin 11, Ireland). Despite these limitations, the zebrafish larvae model was chosen here as the author deduced that it offered a novel method for partially predicting and assessing the safety liabilities of the hydrolysate from which the tetrapeptide NIGK was derived. The effects of this hydrolysate were also assessed in an animal rodent model in Chapter five of this thesis.

This chapter identified the amino acid sequence of a peptide isolated from a macroalga that displayed *in vitro* PAF-AH inhibitory activity. The non-toxic effects displayed by the hydrolysate at a concentration of 1 mg/ml coupled with its PAF-AH inhibitory activity at this concentration, suggests it has a potential role as a functional food ingredient for maintenance of heart health. As the peptide identified in this study, NIGK, targets the enzyme PAF-AH, it could potentially be used with ACE-I or renin inhibitors to prevent atherosclerosis and high blood pressure development; both risk factors for the development of stroke and cardiovascular disease.

CHAPTER 4. IDENTIFICATION OF AN ACTIVE SEAWEED DERIVED RENIN INHIBITORY DIPEPTIDE AND CONFIRMATION OF A HYPOTENSIVE EFFECT IN SPONTANEOUSLY HYPERTENSIVE RATS FOLLOWING SIMULATED GASTROINTESTINAL DIGESTION.

This work is under review as a research article for publication in theJournal of Agriculture and Food Chemistry: Ciarán Fitzgerald, Rotimi Aluko, Mohammad Hossain, Dilip Rai, José Prieto and Maria Hayes. Identification of an active seaweed derived renin inhibitory dipeptide and confirmation of a hypotensive effect in spontaneously hypertensive rats following simulated gastrointestinal digestion.

Animal studies performed in section 4.3.6 were carried out by the staff of the Department of Human Nutritional Sciences at the University of Manitoba closely following the authors' experimental design.

4.1 Aims

In order to exert a bioactive effect *in vivo*, oral delivery of the renin inhibitory peptide identified in Chapter two and the hydrolysate are necessary. Oral delivery of bioactive peptides and proteins face immense challenges due to the gastrointestinal environment. After consumption, the gastrointestinal epithelium acts as a physical and biochemical barrier for absorption which may result in low bioavailability. An ideal bioactive peptide candidate should be able to reach the site of absorption intact. Alternatively, the active component of the bioactive peptide should survive GI digestion and should be able to exert a beneficial effect at the active site and a positive *in vivo* effect should be observed.

The aims of this chapter were:

(1) To determine potential cleavage of the renin inhibitory peptide with the amino acid sequence IRLIIVLPILMA using *in silico* analysis. This aim provides a guide for future assessment in animal models.

(2) To assess if the renin inhibitory tridecapeptide could survive gastrointestinal digestion and to determine the resulting dipeptides using mass spectral (MS) analysis. This was assessed using an *in vitro* simulated gastrointestinal digestion process and Nano-ESI-MS mass spectral (MS) analysis.

(3) To determine if the hydrolysate and the renin inhibitory tridecapeptide had any hypotensive effect in an animal model. Spontaneously hypertensive rats (SHRs) were chosen as a suitable model for this work. SHRs were used as the animal group of choice previously for determination of the hypotensive effect of bioactive peptides of both pharmaceutical and food origin. SHR rats are recognized as a suitable animal model [178].

4.2 Introduction

To reach their proposed target, naturally occurring bioactive peptides intended for inclusion in functional foods must first survive food processing conditions. Furthermore, they must be capable of enduring gastrointestinal digestive enzymes. Indeed, past studies have shown that ACE-I inhibitory peptides with inherent *in vitro* activity exhibited poor activity *in vivo* due to inactivation during gut transit [179].Conversely, previous studies have also shown that bioactive peptides that inhibit the enzymes of the RAAS survived cleavage by digestive enzymes and increased activity of the peptides post cleavage was reported [101, 119]. When investigating if a compound with apparent *in-vitro* bioactivity can overcome the conditions of the GI tract, simulated gastrointestinal digestion is a useful methodology that can be used prior to animal experimentation in order to determine the *in vivo* effect of a compound identified *in vitro* as bioactive [180]. There is great variety in the literature concerning *in vitro* digestion studies. These varieties relate to the enzymes used, the conditions of hydrolysis used and the type of analysis performed on the resultant products [181]. However, in general, most methods attempt to mimic the gastric phase through digestion with pepsin at pH 2-3 and subsequent duodenal phase digestion using pancreatic proteases such as trypsin and chymotrypsin at a pH of between 7 and 8 [181].

Spontaneously hypertensive rats (SHR) have been used as a model for the measurement of hypertension since the late 1960's [178]. SHR rats are not the only animal model used to study the effects of hypertension *in vivo*. Other animal models used to study hypertension include the Dahl salt-sensitive rats derived by Dahl from the Sprague-Dawley stock. This breed was developed in order to have a suitable model for monitoring hypertension caused by a high sodium chloride (NaCl) diet [182]. Other animal models include renovascular hypertension rats [182]. However, due to their

broad use to date, far more data has been obtained with SHR and it is therefore easier to compare results generated with SHRs to previous findings [178].Indeed, to date, two studies have used the SHR rat model to verify the effect of naturally occurring renin inhibitory peptides *in vivo*[74, 183]. In addition, our study wanted to assess the effects of the peptide hydrolysate on hypertensive subjects.

In this work, the tridecapeptide IRLIIVLMPILMA identified in Chapter two was subjected to a simulated gastrointestinal digestion procedure to assess if it could survive GI transit and potentially be used to positively affect hypertension. *In silico* cleavage analysis coupled with a simulated gastrointestinal digestion method were developed and used to assess survival of this peptide *in vivo* using SHRs. The peptide hydrolysate from which the renininhibitory peptide was derived was also observed in all models. The effect on the blood pressure of the animals was observed over a 24 hour period.

4.3 Materials and Methods

4.3.1 Materials

The tridecapeptide IRLIIVLMPILMA was synthesized by GL Biochem Ltd., (Shanghai, China). All enzymes used for the simulated gastric digestion of the tridecapeptide including; pepsin from porcine gastric mucosa, amylase, pancreatin and porcine bile extract were obtained from Sigma Aldrich (Dublin, Ireland). All reagents used in the formulation of the gastric fluids including, potassium chloride, monobasic potassium phosphate, sodium bicarbonate, NaCl and magnesium chloride hexahydrate were also sourced from Sigma Aldrich (Germany).

4.3.2 *In silico* analysis of the renin inhibitory peptide IRLIIVLMPILMA

The programme Expasy peptide cutter (http://web.expasy.org/peptide_cutter/) was used to predict where the main proteases of the gastrointestinal (GI) tract could cleave the renin inhibitory tridecapeptide IRLIIVLMPILMA.

The online tool ToxinPred (http://crdd.osdd.net/raghava/toxinpred/index.html) was used to predict the toxicity of the peptide and its fragments after hydrolysis[147]. In addition, the hydrolysate was assessed for its' heavy metal and Iodine content by Eurofin Foods Ireland (Finglas, Dublin 11, Ireland) as described in Chapter two.

4.3.3 Simulated Gastric Digestion of the peptide IRLIIVLMPILMA

The simulated gastric digestion method was carried out according to the procedures developed by the European Cooperation in Science and Technology (COST) funded group INFOGEST (http://www.cost-infogest.eu/). Simulation of the gastric digestion process was carried out in three phases as shown in Figure 3.5. Initially, an oral phase

containing 1 g of the synthesised peptide was mixed with 1 ml of simulated salivary fluid (SSF) (Table 4.1) and 150 U/ml of amylase. This mixture was left to stir in a Water bath (Stuart Shaking Water Bath, Staffordshire, UK) at 37 ºC for 2 minutes. The second phase of the simulation was the gastric phase. The oral phase was mixed with 2 ml of the prepared simulated gastric fluid (SGF) (Table 4.1) along with pepsin at a concentration of 1000 U/ml of SGF and 0.0375. The pH was adjusted to pH 3 using 1M hydrochloric acid (HCL) and left to stir in a Water bath (Stuart Shaking Water Bath, Staffordshire, UK) at 37 ºC for two hours. The final stage of the digestion process was the duodenal phase. For this phase, 4 ml of the simulated duodenal fluid (SDF) (Table 4.1) was added to the SGF along with pancreatin at a concentration of 100U/ml of trypsin activity. Porcine bile extract was also added at a concentration of 2.4 mg/ml of the total volume. The mixture was adjusted to pH 7 using 1 M NaOH and left to stir in a water bath at 37 ºC for 2 hours before deactivation by heating at 90 ºC for 20 min and subsequent freeze-dried.

Simulated Saliva Fluid (SSF)		**Simulated Gastric Fluid (SGF)**		**Simulated Duodenal Fluid (SDF)**	
Compound	**Concentration**	**Compound**	**Concentration**	**Compound**	**Concentration**
KCl	12.50 mM	KCl	35.08 mM	KCl	6.91 mM
KH_2PO_4	14.02 mM	KH_2PO_4	0.63 mM	KH_2PO_4	0.56 mM
$NaHCO_3$	7.99 mM	$NaHCO_3$	25.98 mM	$NaHCO_3$	43.00 mM
NaCl	4.10 mM	NaCl	41.00 mM	NaCl	32.85 mM
$MgCl_2(H_2O)_6$	0.29 mM	$MgCl_2(H_2O)_6$	0.59 mM	$MgCl_2(H_2O)_6$	0.32 mM

Table 4.1: Concentrations of electrolytes used to create the simulated saliva fluid (SSF) simulated gastric fluid (SGF) and simulated duodenal fluid (SDF).

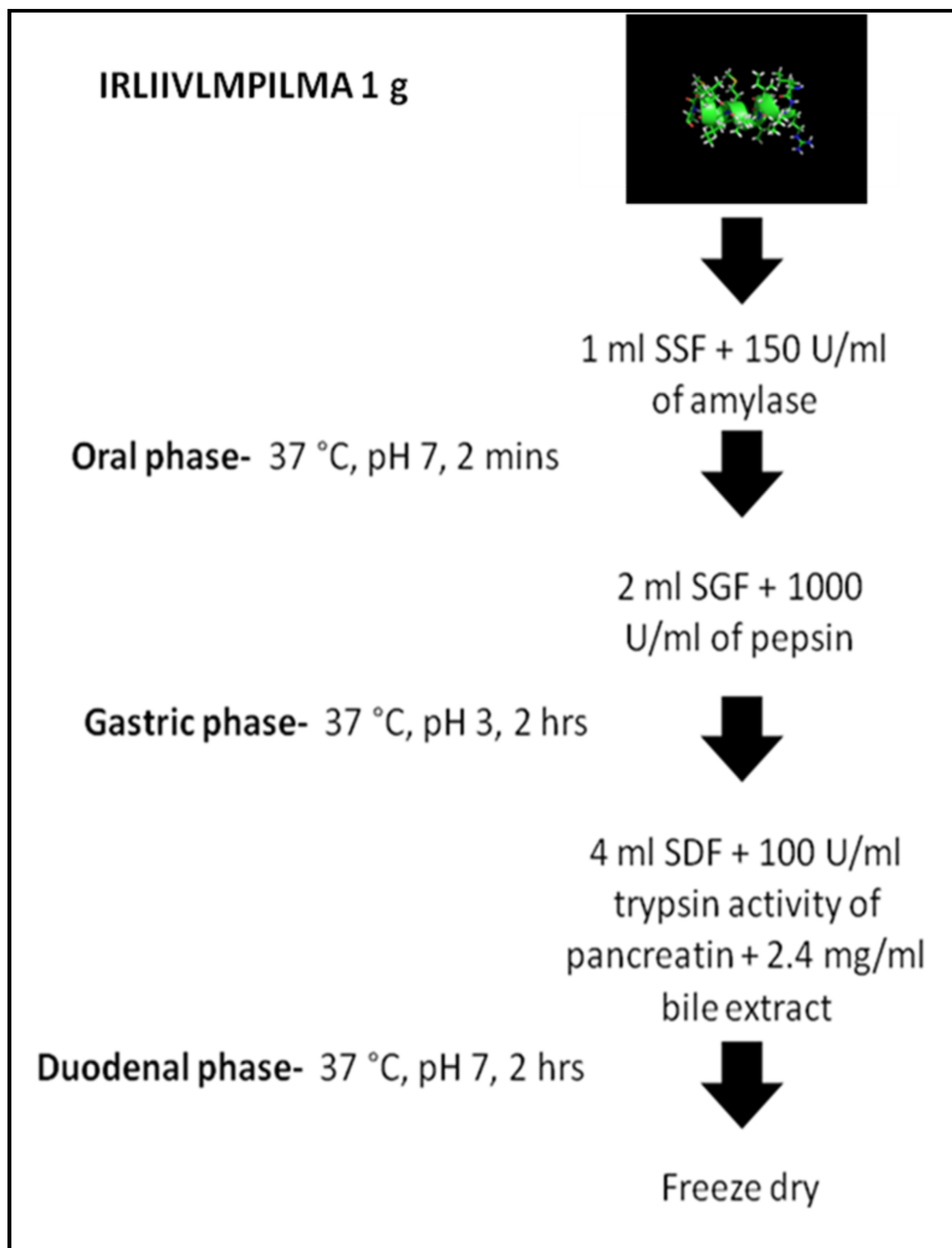

Figure 4.1: Schematic of simulated *in vitro* gastric digestion. The simulation was carried out in three stages; 1) Oral stage 2) Gastric stage 3) Duodenal stage. The 3D image of IRLIIVILMPILMA was generated using primary sequence information and the programme Peplook.

4.3.4 Removal of polyethylene glycols from *Palmaria palmata* protein digested samples using a titanium dioxide (TiO2) cleanup procedure.

Digested samples were cleaned-up for mass spectrometry analysis and determination of the peptide/peptide fragments using Pierce™ Graphite spin Columns which were supplied as part of the Pierce™ TiO_2phospopeptide enrichment and clean-up kit[184]. The graphite spin columns were used according to the manufacturers' instructions. Briefly, the column was prepared by placing it in a 2 mL eppendorf tube followed by centrifugation at 2000 × *g* for 1 minute to remove storage buffer. The column was then rinsed twice with 100 µL of NH_4OH and subsequently centrifuged at 2000 ×*g*. The graphite was activated by adding 100 µL of acetonitrile followed by centrifugation at 2000 × *g* for 1 minute before adding 100 µL of 1 % trifluoroacetic acid (TFA) in water (1:99 v/v) followed again by centrifugation of the column at 2000 × *g* for 1 minute. After column preparation, 50 mg of the hydrolysate was added and allowed to bind to the column for 10 minutes with periodical vortexing. The column was then centrifuged at 1000 × *g* for 3 minutes and the flow through discarded. The column was subsequently placed in a new tube and washed with 200 µL of 1% TFA followed by centrifugation at 2000 × *g* for 1 minute. The column was then placed in a new tube and spun at 2000 × *g* for 1 minute following addition of 100 µL of 0.1% formic acid in a 50:50 acetonitrile:water (v/v) ratio. This step was repeated three times and the resulting elution was freeze-dried. This method demonstrated that the TiO_2microcolumn can rapidly remove polyethylene glycol (PEG) contaminants from protein and phosphorylated samples improving the quality of extracts for MS analysis [185].

4.3.5 Tandem mass spectrometry analysis of the hydrolysate

The enriched, digested fraction generated from the renin inhibitory peptide which was identified in Chapter two from a *Palmaria palmata* protein papain hydrolysate was further analysed using an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer coupled to a nano-ultra performance liquid chromatography system (Waters Corporation, Milford, MA, USA) used in positive ionisation mode. The sample was dissolved in water:acetonitrile80:20(v/v). The re-dissolved hydrolysate was injected onto a nano-UPLC Acquity column BEH130 C_{18} (100 µm × 100 mm, 1.7 µm particle size) preceded by a Symmetry C_{18} (180 µm × 200 mm, 5 µm particle size) trapping column. Mobile phases consisted of solvent A, which contained 0.1% formic acid (FA) in water, and solvent B, which contained 0.1% FA in acetonitrile. Trapping of the peptide/peptide fragments was achieved using a loading time of 3 minutes at a flow rate of 5 µL/min with 98% solvent A and 2% of solvent B and then elution onto the analytical column at 300 nL/min. Chromatographic conditions consisted of 95 % of solvent A and 5% of solvent B isocratically for 2 min, followed by a linear gradient from 85 to 20% of solvent A over 63 min. Mass spectral data were acquired in MS^e mode with collision energy for a full mass scan of 6eV and a collision energy ramp of 15-35eV for a mass range *m/z* 155 to *m/z* 1600. The Q-TOF was calibrated externally using MS/MS fragment ions of glu-fibrinopeptide (Glu-fib).

4.3.6 In vivo determination of the hypotensive effect of the *P. palmata* protein hydrolysate and the tridecapeptide IRLIIVLMPILMA in spontaneously hypertensive rats (SHRs).

Animal studies performed in section 4.3.6 were carried out by the staff of the Department of Human Nutritional Sciences at the University of Manitoba closely

following the authors' experimental design. Animal experiments were carried out in accordance with the guidelines from the University of Manitoba Animal Protocol and Management Review Committee. Adult (20-week old) Spontaneously hypertensive rats (SHR), weighing between 360-400 g were kept under a 12 hour, day/night cycle at 21°C and fed a standard chow diet and water *ad libutum*. The SHR were split into four groups consisting of four rats in each group and administered the following treatment: (a) *P. palmata* hydrolysate dissolved in phosphate buffer saline (PBS, pH 7.2) at 50 mg/kg body weight (b) the synthesised tridecapeptide IRLIIVLMPILMA dissolved in PBS at 50 mg/kg body weight (c) the positive control Captopril ® dissolved in PBS at 10 mg/kg body weight and (d) PBS only. Each group received a 1 ml single dose of each treatment *via* oral gavage. Before blood pressure was determined, rats were first anesthetized in a chamber at 40 °C with 4% isofluorane for 4 min. The systolic blood pressure (SBP) of SHR was measured at 0, 2, 4, 6, 8 and 24hrs by the tail cuff method while the rats were in an unconscious state using the Mouse Rat Tail Cuff Blood Pressure System (IITC Life Sciences, Woodland Hills, CA, USA). The change in SBP mmHg (Δ SBP) was determined by subtracting the SBP at time *n* (where *n* is equal to 2, 4, 6, 8, 24hrs) from the SBP at time 0.

4.3.7 Statistics

The student's *t*-test and ANOVA analysis were performed using GraphPad Prism version 5.04 for Windows, GraphPad Software, (La Jolla, California, USA).

4.4 Results

4.4.1 *In silico* analysis of IRLIIVLMPILMA

Table 4.2 shows the resultant peptide fragments obtained from the peptide IRLIIVLMPILMA following *in silico* analysis using the online tool Expasy Peptide Cutter. The tridecapeptide was cleaved at position 3, 6, and 11 by pepsin at both pH 1.3 and pH > 2. The enzyme trypsin cleaves the peptide further at position 2 at the carboxy end of the amino acid arginine releasing the Di-peptide, amino acid sequence IR.

Name of enzyme	No. of cleavages	Positions of cleavage sites
Pepsin (pH1.3)	3	3, 6, 11
Pepsin (pH>2)	3	3, 6, 11
Trypsin	1	2

Table 4.2: Cleavage points of the peptide IRLIIVLMPILMA by the main enzymes of GI tract using the online tool Expasy Peptide Cutter.

4.4.2 ESI-Q-TOF analysis of the *in vitro* GI simulated digestion of IRLIIVLMPILMA

Figures 4.2 and 4.3present the UPLC chromatograms and MS traces obtained following MS analysis of the tridecapeptide following *in vitro* simulated gastrointestinal digestion according to the INFOGEST procedure and the corresponding Nano-ESI-MS spectra data. The base peak intensity chromatogram (Figure 4.2.a.) displays the peaks of all hydrolysed analytes. The extracted chromatogram in Figure 4.2.b. displays doubly charged $[M+2H]^{2+}$ ions at *m/z* 748.43 representing the whole, un-cleaved tridecapeptide IRLIVLMPILMA (theoretical mass = 1494.94). The corresponding Nano-ESI-MS spectral data in Figure 4.3.a. shows both the protonated molecular ions for IRLIIVLMPILMA at *m/z* 1495.8 and the doubly protonated at *m/z* 748.4. Figure 4.3.c.

displays the extracted chromatogram highlighting the peaks corresponding to the $[M+H]^+$ ions at *m/z* 1226.66 representing the cleaved fragment LIIVLMPILMA (theoretical mass = 1225.75). Figure 4.3.b. displays the resultant Nano-ESI-MS spectra data hydrolysed peptide fragment LIIVLMPILMA at *m/z* 1226.7. Figure 4.3.d. shows the extracted ion chromatogram displaying $[M+H]^+$ ions at *m/z* 288.25 the expected observed mass of the peptide IR (theoretical mass = 287.20) now cleaved from the parent peptide. The consequential Nano-ESI-MS spectral data in Figure 4.3.c. shows the dipeptide IR at *m/z* 288.3.

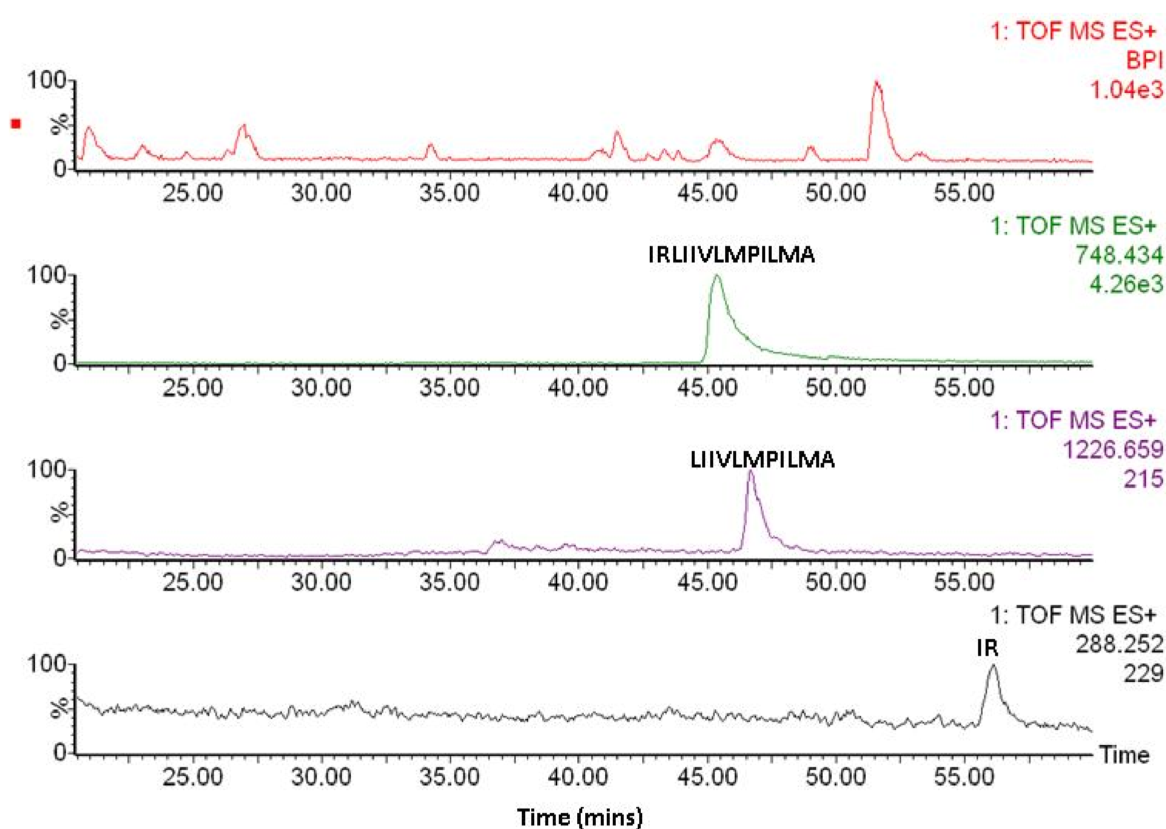

Figure 4.2: a) Base peak intensity (BPI) chromatogram of the hydrolysed analytes. b) Extracted ion chromatograms (XIC) for $[M+2H]^{2+}$ ions at *m/z* 748.43. c) $[M+H]^+$ ions at *m/z* 1226.66. d) $[M+H]^+$ ions at *m/z* 288.25.

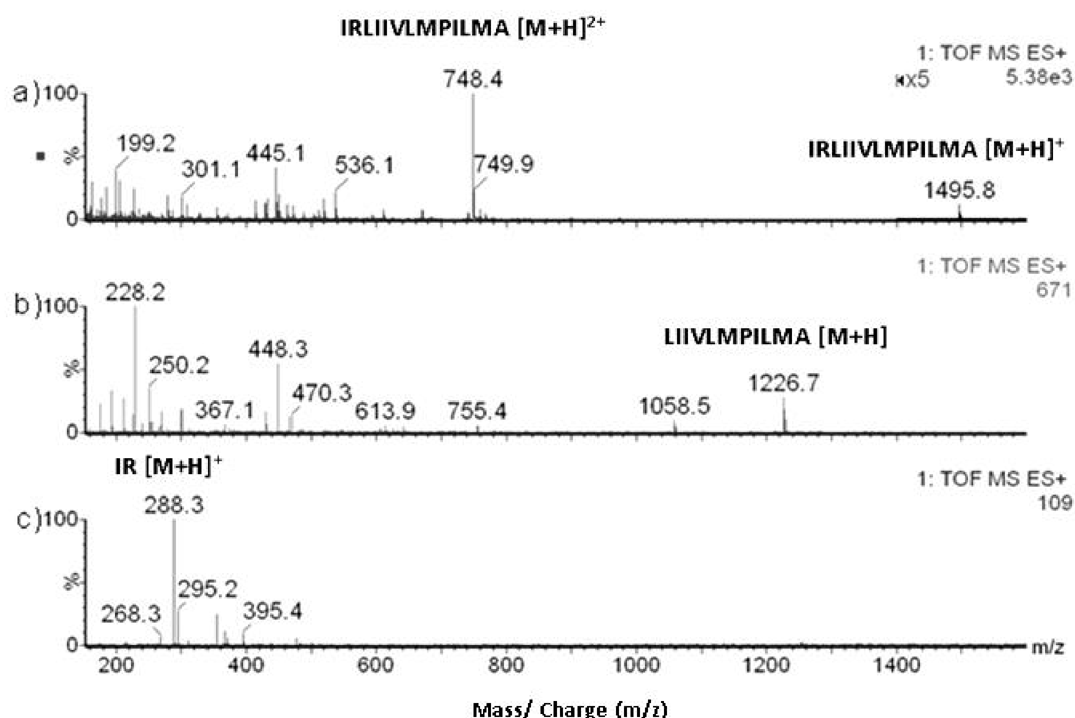

Figure 4.3: Nano-ESI-MS spectra data showing singly protonated molecular ions for a) peptide IRLIIVLMPILMA at *m/z* 1495.8 and doubly protonated at *m/z* 748.4, b) hydrolysed peptide fragment LIIVLMPILMA at *m/z* 1226.7 and c) dipeptide IR at *m/z* 288.3.

4.4.3 Short term hypotensive effects of *P. palmata* protein hydrolysate and IRLIIVLMPILMA in SHR

Figure 4.4 shows the SBP over a 24 hour period in spontaneously hypertensive rats (SHR) following administration of the control (saline), the known blood pressure lowering drug captopril (3 mg/kg body weight) as a positive control, the *P. palmata* protein hydrolysate administered at 50 mg/kg body weight and the synthesized peptide IRLIIVLMPILMA administered at 3 mg/kg body weight. After 2hrs, the rat group that were fed with captopril recorded a 29 mmHg drop in SBP from 184.5 (+/- 0.28) mmHg to 156 mmHg (+/- 1.08). Likewise, the group fed the *P. palmata* protein hydrolysate recorded a drop of 34 mmHg in SBP from 187.75 (+/-0.25) to 153.5 (+/- 0.64) mmHg

SBP, while the group fed the tridecapeptide IRLIIVLMPLIMA presented a drop of 33 mmHg in blood pressure from 187.5 (+/-0.95) to 154.75 (+/-0.94) mmHg SBP. After 24hrs, the effect of all blood pressure lowering agents was a drop in mmHg SBP of 21, 16 and 17 mmHg respectively compared to the SBP recorded at 0hrs. ANOVA analysis of the data shows that there is no statistical difference between the SBP of rats administered the captopril, *P. palmata* protein hydrolysate or the peptide IRLIIVLMPILMA over the 24hr period. However, all three are statistically different to that of the saline control ($P < 0.001$) over the test period.

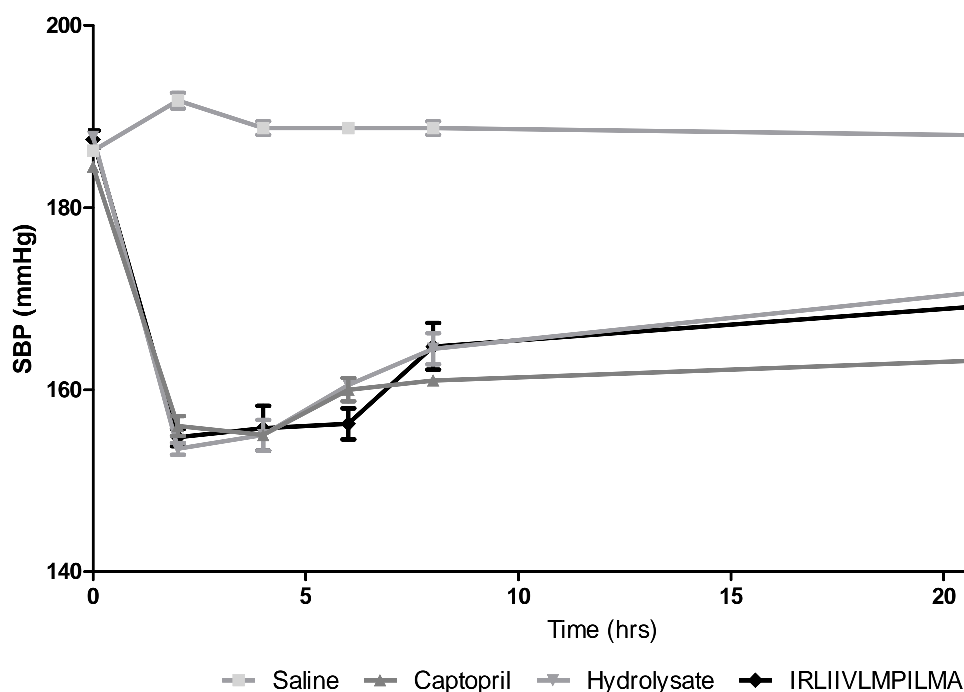

Figure 4.4: SBP of SHRs over 24hrs after oral administration of saline, captopril (10 mg/kg body weight, *P. palmata* protein hydrolysate (50 mg/kg body weight) and the synthesised peptide IRLIIVLMPILMA (50 mg/kg body weight). Values are mean ± SEM (n = 3).

4.5 Discussion

The feasibility of functional food or pharmaceutical application of bioactive peptides depends on overcoming several challenges and one important aspect of use is oral bioavailability. Bioactive peptide action is often hampered by proteolytic enzyme attack in the gastrointestinal tract. In this work, the survival of a renin inhibitory peptide with the amino acid sequence IRLIIVLMPILMA was assessed using a combination of *in silico* analysis followed by a simulated gastrointestinal digestion procedure and subsequent *in vivo* administration of the peptide and hydrolysate to SHRs.

The use of *in silico* methods to predict the cleavage sites of potential bioactive peptides that are to be ingested is an important preliminary step when assessing their bioavailability. If the active sequence of a bioactive peptide is predicted to be cleaved *in silico* by the enzymes of the GI tract this would rule out further costly *in vitro* and subsequent *in vivo* analysis. Previously, *in silico* methods were used to predict the formation of ACE-I inhibitory peptides during gastrointestinal digestion [186]. The fact that trypsin cleaved the peptide at position 2 (between the residues R-L) at the carboxy end of the amino acid is particularly relevant from a renin inhibition point of view. Previous work showed that this di-peptide inhibited renin, and ACE-I activities at concentrations of 1 mg/ml when isolated from a pea protein hydrolysate [72].

In vitro digestion models are widely used to study the structural changes, digestibility, and release of food components under simulated gastrointestinal conditions [187]. While not being as accurate as *in vivo* methods, these models are a less time consuming and less costly precursor method [187]. Previous studies examined the ACE-I inhibitory activity of pea and whey protein digests using *in vitro* digestion but chose to analyse the resulting hydrolysates with SDS-PAGE and HPLC [136]. To confirm the results obtained using *in silico* analysis, the bioactive tridecapeptide identified in this study was

subjected to the *in vitro* simulated gastric digestion outlined in Figure 4.1. In addition, the sample was analysed using UPLC-MS in order to identify the breakdown products and the dipeptide IR.

The MS data obtained and shown in Figures 4.2 and 4.3confirms the *in silico* cleavage analysis results which predicted that the renin inhibitory di-peptide IR remains intact during normal digestive processes. In this study, ESI-Q-TOF mass spectrometry was used in tandem with UPLC due to the high sensitivity of peptide detection that ESI-Q-TOF offers along with the rapid and high degree of separation offered by UPLC [188].

While *in vitro* and *in silico* methods are powerful, predictive tools, the likely mode of action of a molecule within a living system cannot be fully mapped out due to the myriad of complex reactions a molecule will encounter within an organism. Therefore, animal models are necessary in functional food research as well as pharmaceutical research [189]. Rats are a strong medical model for humans as almost all human genes known to be associated with disease have orthologues in the rat genome [190]. The results shown in Figure 4.4 present the short term changes in SBP observed over a 24 hour period in Spontaneously Hypertensive Rats (SHR) fed a diet of the seaweed protein papain hydrolysate; the synthesized tridecapeptide IRLIIVLMPILMA; a positive control (Captopril®); and a Saline solution (negative control). The ACE-I inhibitor Captopril was used as an anti-hypertensive positive control as we were unable to obtain a sample of the renin inhibitor aliskiren. Interestingly, both the hydrolysate and the tridecapeptide IRLIIVLMPILMA displayed very similar trends in lowering the SBP of the rats over a 24 hour period (Figure 4.4). Previous papers examined the blood pressure lowering effects of a pea protein hydrolysate in SHR rats [183]. However, the pea protein hydrolysates were administered at a concentration of 200mg/kg of body weight and this dose resulted in a 19 mmHg drop in systolic blood pressure in SHRs.

Likewise, SHRs treated with an antihypertensive buckwheat hydrolysate recorded an initial decrease in SBP of 27.0 ± 7.6 mmHg after 2 hrs but only when treated with 100 mg/kg body weight [191], twice the concentration of the *P. palmata* protein hydrolysate and the tridecapeptide IRLIIVLMPLIMA used in this study. Further studies should be carried out to assess the long-term effects of the hydrolysate and IRLIIVILMPILMA to ensure that the decrease in SBP is sustained, and also to ensure that there are no negative effects due to prolonged exposure. *In silico* analysis of the peptide using the online tool ToxinPred also showed that the peptide was non-toxic, which indicates that it may be suitable for use in functional foods and pharmaceutical agents. Furthermore, some toxicological parameters were assessed in chapter 3 through the use of a zebrafish larvae assay and heavy metal and Iodine content.

This Chapter demonstrated that the tridecapeptide IRLIIVLMPLIMA is a renin inhibitory pro-peptide with the ability to impart a hypotensive effect *in vivo* confirmed by monitoring changes in SBP in SHRs over a 24 hour period. The dipeptide IR is the active sequence of the tridecapeptide IRLIIVLMPILMA and this was confirmed here using the simulated digestion of the tridecapeptide and MS analysis of the breakdown products. The renin inhibitory activity of the dipeptide IR was not assessed in this study but was previously determined [183]. Both the peptide and the *P. palmata* protein papain hydrolysate may have potential for use as functional ingredients in the growing nutraceutical market for the prevention of hypertension.

CHAPTER 5. INCREASING THE HEALTH BENEFITS OF BREAD: ASSESSMENT OF THE PHYSICAL AND SENSORY QUALITIES OF BREAD FORMULATED USING A RENIN INHIBITORY *PALMARIA PALMATA* PROTEIN HYDROLYSATE.

This chapter was published as a research harticle in LWT-The Science of Food and Technology The paper is entitled: Ciarán Fitzgerald, Eimear Gallagher, Linda Doran, Mark Auty, José Prieto, Maria Hayes "Increasing the health benefits of bread: Assessment of the physical and sensory qualities of bread formulated using a renin inhibitory Palmaria palmata protein hydrolysate." (2013), 56, 398-405.

5.1 Aim

For centuries, bread has been consumed as a staple of the human diet. The four aims of this chapter were:

(1) To assess whether a seaweed protein bioactive hydrolysate identified as having renin inhibitory properties could be incorporated into this common food vehicle without adverse physical and sensory effects.

(2) To assess if the renin inhibitory protein hydrolysate generated from *Palmaria palmata* could survive the bread baking process and if this product still possessed renin inhibitory activities following the baking process.

(3) To determine any changes in the bread product as a result of addition of the generated protein hydrolysate to the bread product and to provide a description of these changes through the use of semi-trained consumer panelists.

(4) To confirm an increase in the protein and essential amino acid content of the bread by the addition of the seaweed hydrolysate.

5.2 Introduction

Baked products are the most widely consumed foods in the world and therefore have great potential as vehicles for bioactive ingredient delivery [124]. Indeed, many bread companies routinely enrich their baked products with folic acid [192]. Increasing the protein, nutritional and health benefits of bread is one strategy for improving consumer nutrition and this is not a novel concept. The literature conveys how breads have been improved by the addition of skim milk powder and soy proteins to breads[193]. Furthermore, numerous Omega-3 products have been launched in the United States, with Omega-3 enriched bread being used as a delivery vehicle for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This is delivered in the form of microencapsulated Tuna oil at levels of 10 mg of long chain n-3 polyunsaturated fatty (PUFA) oils per slice [4]. Baked products can provide consumers with high quality Omega-3 breads with higher contents of EPA and DHA than the average functional food product found in the US. Furthermore, the Norwegian bakery company Bakers, launched omega-3 fortified bread in 2009 for school children. Other common cereal based bioactive products include biscuits enriched with Omega-3 fatty acids to impart health benefits to the consumer [4].

Addition of alternative protein sources to bread increases the nutritional profile of bread, but can also be used to enhance the potential health benefits. It is well documented how wheat protein is deficient in some essential amino acids, in particular lysine, which is the first limiting amino acid in wheat [149]. Lysine deficiency leads to the poor utilization of protein and thus results in protein malnutrition [194]. Attempts to improve the nutritional profile of breads are not new. For example, fish protein concentrates were added to wheat bread previously to increase the lysine content of the bread[195]. Indeed, previous work demonstrated that during the month of November, *P.*

palmata seaweed can contain levels of lysine of 5.9 g/100 g of total amino acids[38]. For centuries, red seaweed species were incorporated into traditional laver breads across the United Kingdom (UK) and Ireland [123], however the health benefits of this practice were not examined.

This work examined the textural and sensory attributes of a wheaten, yeast raised bread formulated using the *P. palmata* protein hydrolysate described in Chapters one and two and compared this potentially bioactive bread to control wheat breads. Four formulations were examined; a wheat flour control bread, a control bread containing 4% *P. palmata* protein hydrolysate, a buckwheat bread consisting of a blend of 70% wheat : 30% buckwheat, and a fourth formulation 70% wheat : 30% buckwheat containing 4% *P. palmata* hydrolysate. Buckwheat was used as previous studies have described the antihypertensive effects of this cereal [191, 196]. The volume, colour, texture profile, moisture, crumb structure, sensory attributes and renin inhibitory activity of all four bread formulations were assessed. Brightfield light microscopy and confocal scanning laser microscopy images using a new triple labelling protocol were also obtained to visualise the main ingredients of the breads.

5.3 Materials and Methods

5.3.1 *Palmaria palmata* protein extraction and hydrolysis with papain

Protein was extracted from *P. palmata* following the protocol of Galland-Irmouli et al. [38] as described in Chapter one. The hydrolysis process used was the same as that used to isolate renin and PAF-AH inhibitory peptides.

5.3.2 Bread ingredients

Standard Baker's wheat flour (63 % water absorption, 13.5% protein) was supplied by James Neills Ltd., Belfast, Northern Ireland. Buckwheat flour (13% protein) was supplied by Ziegler and Co., Wunsiedel, Germany and dried Yeast was supplied by Doves Farms Ltd., UK. The Dough improver was supplied by Fermex International Ltd, Worcester, UK and salt was obtained from Ineos Enterprises, Cheshire, UK. Emulsified bread fat was purchased from Irish Bakels Ltd. (Ireland).

5.3.3 Preparation of breads

The four bread formulations used in this study are presented in Table 5.1. Previous sensory studies where seaweeds were used as food ingredients incorporated the brown seaweed *Ascophyllum nodosum* into bread at concentrations of up to 4%[125]. The three bread test formulations used in this work were:

1. C: the Control.
2. CH: the Control containing 4% of the *P. palmata* hydrolysate generated in Chapter one.
3. B: a blend of 70% wheat: 30% Buckwheat.

4. BH: a blend of 70% Wheat: 30% Buckwheat containing 4% of the *P. palmata* hydrolysate.

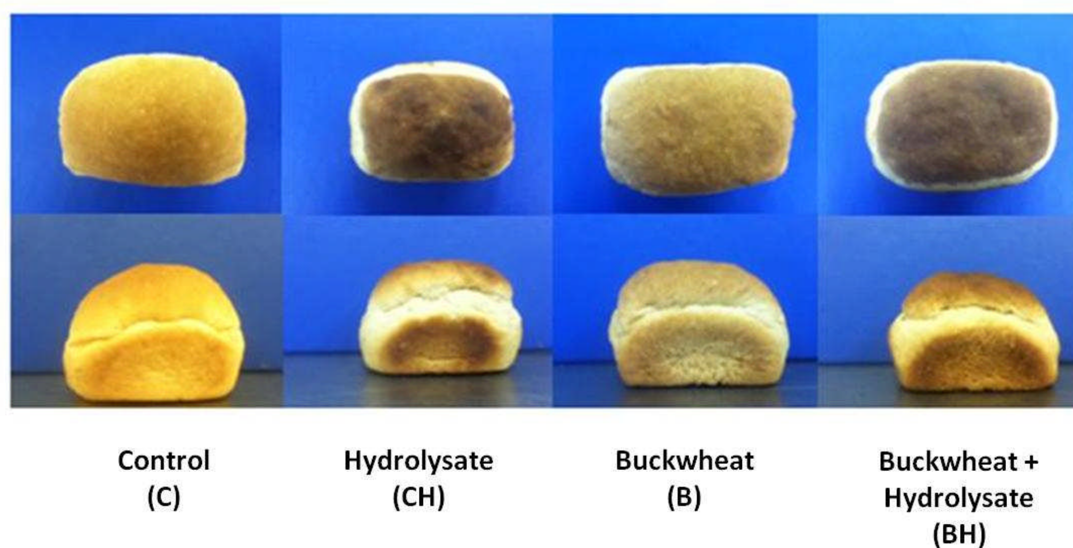

Figure 5.1: Images of all four bread samples examined.

Buckwheat was chosen in this study due to its high content of essential amino acids and as it has a well-balanced total amino acid content [197]. Previous studies have also highlighted the ability of buckwheat proteins to inhibit the enzymes of the renin angiotensin aldosterone system (RAAS)[197]. Inclusion levels of 4% hydrolysate was chosen as previous sensory studies have shown that it is acceptable to incorporate seaweed (*Ascophyllum nodosum*) into bread at concentrations of up to 4%[125]. The dry ingredients were mixed in a Kenwood Chef mixer (Kenwood Ltd, Hampshire, UK) at minimum speed for one minute. Water and yeast were added and a dough mixture was prepared by mixing at high speed for a further 2.5 minutes. The resulting dough was then rested in a proofer (Koma, Roermond, the Netherlands) for 15 minutes at 35 ºC and 80 % relative humidity. The dough was divided into four 60 g pieces which were hand moulded, placed in tins and subsequently proofed for 45 mins at 35 ºC and 80 % relative humidity. Loaves were baked at 220 ºC for 20 mins in a deck oven (Tom

Chandley Ltd., Manchester, UK) and subsequently cooled at room temperature for 2 hrs and stored in polyethylene bags (two loaves per bag). Four loaves were produced for each formulation. Two of these loaves were evaluated after 24hrs and the remaining two after 120hrs storage at room temperature. Each bake was performed in triplicate.

Ingredient (g/100 g of flour)	**Control (C)**	**Control + Hydrolysate (CH)**	**Buckwheat (B)**	**Buckwheat + Hydrolysate (BH)**
Wheat Flour	100	100	70	70
Buckwheat	-	-	30	30
Fat	1	1	1	1
Yeast	1.5	1.5	1.5	1.5
Salt	2	2	2	2
Improver	0.5	0.5	0.5	0.5
Water	63	63	62	62
Hydrolysate	-	4	-	4

Table 5.1: The bread formulations used in this study; Ingredients are presented as g / 100g of the wheat/buckwheat flour base

5.3.4 Bread evaluation – loaf volume, weight and loaf specific volume calculations

After 24 hrs, the loaf volume for all formulations was measured using a volume meter (TexVol BVM-L30, Sweden). Loaf weight was recorded and the loaf specific volume (ml/g) was calculated. Moisture was calculated following a procedure based on the International Association of Cereal Chemists (ICC) method 110/1 [198]using a Brabender moisture oven (Brabender, Duisberg, Germany). Crust and crumb colour were measured using an Ultra-Scan Pro colour meter (Hunter Associates Laboratory Inc., Reston, Virginia, USA) and results expressed using the Hunter crust colour L* (lightness), a* (redness/greenness) and b* (yellowness/blueness). The crumb structure of the centre slices of each bread formulation was evaluated using the C-Cell bread Imaging System (Calibre Control International Ltd., UK). Crumb texture was assessed by conducting a texture profile analysis (TPA) on 10 mm thick slices using a Texture Analyser (TA-XT2*i*, Stable Micro Systems, Surrey, UK) equipped with a 10 mm

perspex probe and using the Texture Exponent Software. Moisture and TPA was repeated after 120hrs on the two remaining loaves from each bread formulation. All bread formulations were tested in triplicate.

5.3.5 Heavy metal and Iodine analysis of seaweed bread product

The total cadmium (Cd), lead (Pb), methyl-mercury ($[CH_3Hg]^+$), arsenic (As) and iodine I_2levels of the formulated bread were assessed by Eurofins Food Ireland Ltd., (Finglas, Dublin 11, Ireland).

5.3.6 Sensory analysis

Sensory analysis was conducted on all four bread formulations 24hrs post-baking using a sensory panel consisting of 12 semi-trained consumers. Using a hedonic scale, panellists were asked to assess the breads under the characteristics of appearance, texture, flavour and general acceptability by placing a mark on a 6 cm line (0 = unacceptable, 6 = very acceptable)[199].

5.3.7 Renin inhibitory assay

This assay was carried out on bread samples (1 mg/ml) from each of the four formulations according to the manufacturer instructions (Cayman chemical Company, Michigan, USA) and as outlined previously in Chapter two.

5.3.8 Microscopy

For examination by brightfield light microscopy and confocal scanning laser microscopy, bread samples were resin embedded, sectioned and stained as described below. Samples of bread approximately 3 mm cubed in size were cut from the middle of

each loaf using a razor blade and placed in a fixative which consisted of 2.5% w/w glutaraldehyde in 80% aqueous ethanol. After 2hrs, the fixative was replaced with 80%, 90%, 95% and 100% ethanol in sequential steps for 1hr each. Fixed bread cubes were transferred to flat-ended Beem capsules containing LR White polymethacrylate resin (Agar Scientific Ltd., Stansted, UK) and allowed to infiltrate at room temperature overnight. The resin was replaced with fresh resin and capsules containing the fixed bread cubes were cured at $60^{o}C$ overnight in an oven. Semi-thin sections of 5 µm thickness were then cut from the resin blocks on a Leica EM UC7 ultramicrotome (Leica Microsystems, Mannheim, Germany) and transferred to light microscope slides prior to imaging by conventional optical or confocal microscopy as described below. Sections were cut from two replicate blocks.

5.3.8.1 Brightfield light microscopy

Resin sections were double stained with one drop of an aqueous solution of 0.1 % Iodine in 0.2 % w/w potassium iodide to stain starch for 5s. The stain was then drained off and one drop of 0.1 % w/w aqueous acid fuschin added. A coverslip was placed on top and the sections were imaged using an Olympus BX51 light microscope (Mason Technology, Dublin, Ireland) using brightfield illumination. Digital images (8 bit, TIFF, 2048 x 1024 pixels) were taken using a ProgRes CT3 camera (Jenoptik, Germany) using 10x, 20x or 60x objectives.

5.3.8.2 Confocal scanning laser microscopy

Resin sections of the fixed bread cubes were triple labelled to show all major ingredients. One drop of 0.1 % w/w ethanolic solution of fluorescein iso-thiocynate (FITC) was added to the resin section to label starch. After 10 s, the FITC was drained off and replaced with one drop of aqueous fluorescent brightener 28 (FB28) at a

concentration of 0.125 % (w/w) to label cellulosic material. Subsequently, one drop of 0.1 w/w aqueous fast green (FG) was added to label proteins. The sections were rinsed gently with running water and a cover slip placed on top. Stained sections were imaged using a Leica SP5 confocal scanning laser microscope (Leica Microsystems, Mannheim, Germany) fitted with 20x 0.7 NA and 63x 1.4 NA oil immersion objectives. Sequential images were acquired using triple-channel imaging: a 405 nm blue diode laser was used to excite the fluorescent brightener; a 488nm argon laser was used for excitation for FITC and a 633 nm helium-neon laser for rhodamine B excitation. Emission signals for FB28, FITC and FG were sequentially collected using band pass filters 450 – 490 nm; 510 – 550 nm and 650 – 700 nm, respectively. Digital 8-bit images (1024 x 1024 pixels) were obtained for each separate excitation wavelength and channels were combined and pseudo-coloured to show cellulosic material as blue, starch as green and protein as red.

5.3.9 Statistics

The student's *t*-test was performed using GraphPad Prism version 5.04 for Windows, GraphPad Software, (La Jolla, California, USA).

5.4 Results

5.4.1 Loaf volume and crust/crumb colour

Table 5.2 shows the volume and crust/crumb colour of the four bread formulations. Compared to the control bread formulation, all other bread formulations resulted in a reduced loaf volume (*P* <0.001). The breads most notably reduced in terms of loaf volume were the breads containing the hydrolysate (CH) (2.98 ± 0.02 mg/L) and the buckwheat and hydrolysate fraction (BH) combination (2.54 ± 0.02 mg/L). The loaves containing buckwheat only (B) as a flour replacement differed slightly and had a final specific volume of 3.10 ± 0.06 mg/L.

Formulation	**Specific Volume (ml/g)**	**Crust L***	**Crumb L*/b***
Control (C)	3.30 ± 0.05	58.42 ± 1.47	7.70 ± 0.13
Control + Hydrolysate (CH)	2.68 ± 0.02 ***	53.02 ± 2.54	5.39 ± 0.22 **
Buckwheat (B)	3.10 ± 0.06 *	58.21 ± 0.37	6.98 ± 0.25
Buckwheat + Hydrolysate (BH)	2.54 ± 0.02 ***	44.95 ± 2.00 **	5.37 ±0.26 **

Table 5.2: The results obtained following volume and colour analysis of the control (C), control and hydrolysate containing bread (CH), the buckwheat bread (B) and the buckwheat and hydrolysate containing (BH) breads. Values obtained were mean values ± S.E.M. (*n*=3). *P<0.05, **P<0.01 and ***P<0.001 compared with control.

Concerning the crust colour (*L**) of the loaves, the only bread sample which differed from the control (C) was the BH formulation (*P*< 0.01). All other breads did not significantly differ from the control bread. Bread manufactured by including the hydrolysate and buckwheat fractions (BH) as a replacement for flour resulted in a significantly darker coloured crust, as evidenced by the lower *L** value of 44.95 (± 2.00).

The crumb colour of bread loaves was altered by the addition of the *P. palmata* hydrolysate ($P < 0.01$). Both the CH and BH bread loaves recorded similar L^*/ b^* (lightness/yellowness ratio) values of 5.39 ± 0.22 and 5.37 ± 0.26 respectively, in comparison to the control (7.70 ± 0.13).

5.4.2 Digital image analysis of bread crumb

The results of the crumb grain analysis of the bread formulations are presented in Table 5.3. The inclusion of buckwheat appeared to reduce the number of cells in the bread slices significantly, as shown in bread formulation B (1462 ± 50) and bread formulation BH (1496 ± 22) compared to the control formulation (C) (1760 ± 22).

The cell wall thickness and cell volume were not significantly affected by the inclusion of the *P. palmata* hydrolysate (CH), buckwheat (B) or a combination of the two (BH), indicating that the general coarseness of the slice texture remained unchanged.

Formulation	Number of cells	Cell Wall thickness	Cell volume
Control (C)	1760 ± 22	0.430 ± 0.003	6.44 ± 0.19
Control + Hydrolysate (CH)	1640 ± 46	0.416 ± 0.006	6.59 ± 0.80
Buckwheat (B)	1462 ± 50**	0.443 ± 0.006	7.49 ± 0.44
Buckwheat + Hydrolysate (BH)	1496 ± 22**	0.426 ± 0.003	7.53 ± 0.61

Table 5.3: Image analysis results obtained for the control bread (C), control and hydrolysate bread (CH), buckwheat bread (B) and buckwheat and hydrolysate containing (BH) breads. Values are mean ± S.E.M. (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared with control.

5.4.3 Texture profile analysis (TPA) of bread crumb

The results for the crumb TPA and crumb moisture content are presented in Figure 5.2. Compared to the control bread sample, the crumb texture of all three test breads was found to be significantly firmer. For example, the BH bread was three times as firm (828g ± 26g) as that of C (275g ± 10g). Wheat bread containing the hydrolysate (CH) showed similar hardness properties to that of the buckwheat bread (B) (573g ± 25g) and (506g ± 19g) respectively. Similarly, in terms of cohesiveness, the BH formulation presented the least cohesive crumb. In addition, the CH and B bread formulations both appeared to have higher cohesive values and these cohesive values were lower than those of the control bread (Figure5.2.b). Likewise, the springiness properties of each bread demonstrated a comparable trend to values obtained for cohesiveness with the control formulation showing the highest result for this parameter ($P<0.001$). The combined BH bread formulation had a lower springiness quality compared to the CH and B formulations. The moisture content of the crumb in this bread was not significantly affected by the addition of the hydrolysate or buckwheat.

The effect of storage time (120 hrs) on the bread qualities was also assessed and these results areshown in Figure 5.2. As expected, crumb hardness increased for all four bread formulations. The most significant increase, however, was observed with the BH bread. The crumb hardness of the BH bread almost tripled from 828g (± 26g) to 2459g (± 315g) over the 120hrs period of storage. Crumb cohesiveness and springiness were also greatly affected by storage time. The BH bread sample presented the most significant changes over the testing period. Interestingly, the only formulation not to significantly lose crumb moisture during the storage period was the buckwheat bread (B).

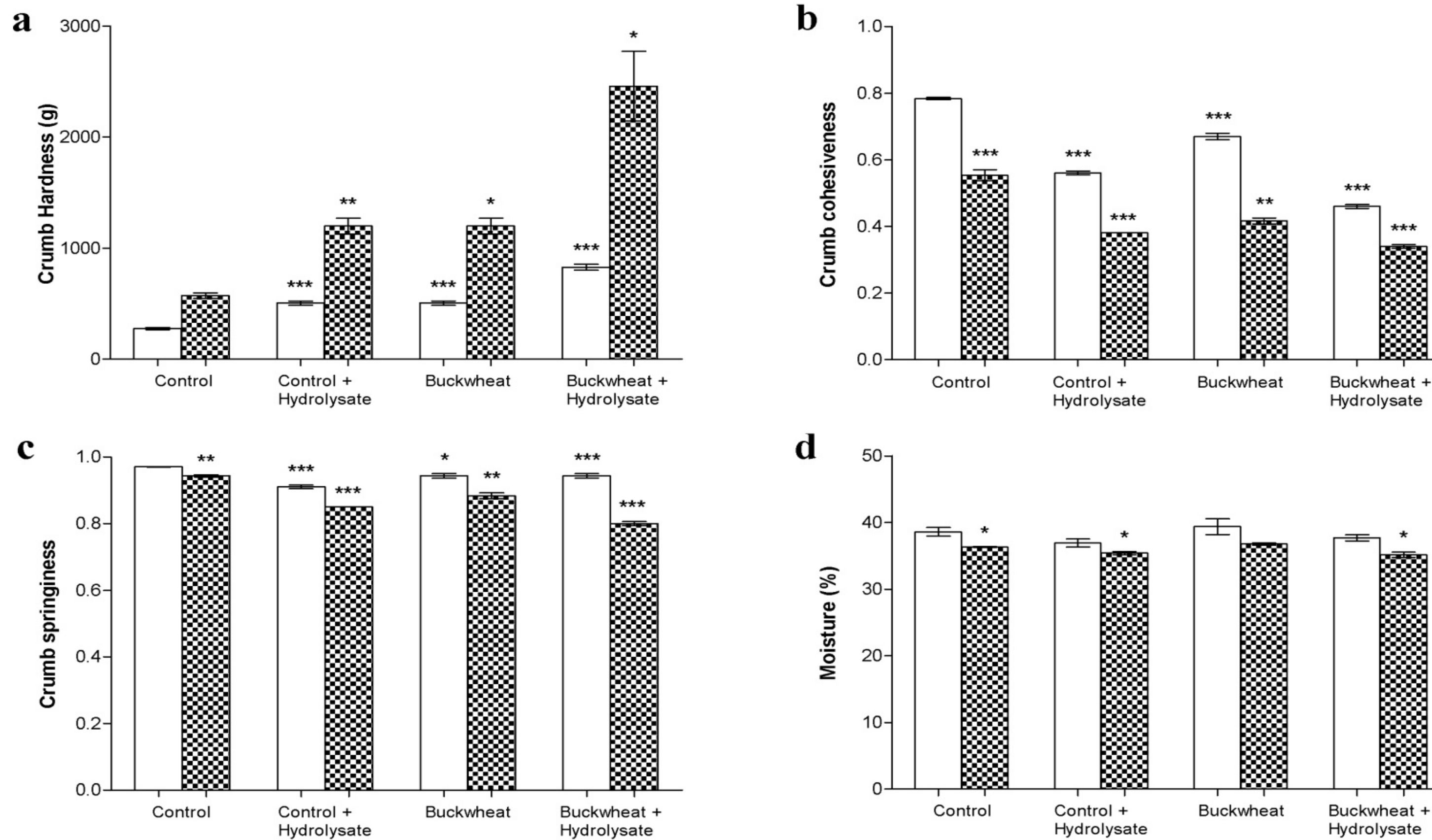

Figure 6: Texture profile analysis and moisture content. Crumb hardness (a), cohesiveness (b), springiness (c) and moisture percentage (d), of the control (C), control + hydrolysate (CH), buckwheat (B) and buckwheat + hydrolysate (BH) breads. [white box] = 24hrs of storage. [checkered box] = 120hrs of storage. Values are mean ± S.E.M. (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared with control.

5.4.4 Brightfield light microscopy of bread samples

All microscopy carried out in this thesis was carried out by the staff of the National Food Imaging Centre, at Teagasc Moorepark Food Research Centre. Light micrographs of stained sections of the different bread formulations are shown in Figure 5.3. Starch grains appeared as a dark blue colour, whilst protein was stained pink/red. Light microscopy showed some differences between the four bread samples. In Figure 5.3.a the control bread (C) showed partially gelatinised wheat starch granules of between 10 – 50 µm in diameter. Larger starch granules were elongated and irregularly shaped, whilst smaller granules were more spherical and rounded. The pink stained protein appeared as a semi-continuous network, often surrounding individual starch grains. Figure 5.3.b, displays microscopic images of the bread containing 4% seaweed protein hydrolysate (CH). This image shows similar starch granulation properties and a protein network similar to that of the control. However, the starch granules appeared more evenly distributed and less swollen. The buckwheat bread (B) in Figure 5.3.c also shows a wheat starch and protein profile similar to the control bread sample. In addition, there are large clusters of partially gelatinised, close-packed polyhedral starch granules of between 5 – 10 µm in diameter found in the buckwheat bread (B). These starch clusters are characteristic of buckwheat flour particles (results not shown). The BH bread shown in Figure 5.3.d demonstrates features of both the hydrolysate (CH) and buckwheat bread (B), with some large protein regions and close-packed polyhedral starch grains. These properties are characteristic of buckwheat flour.

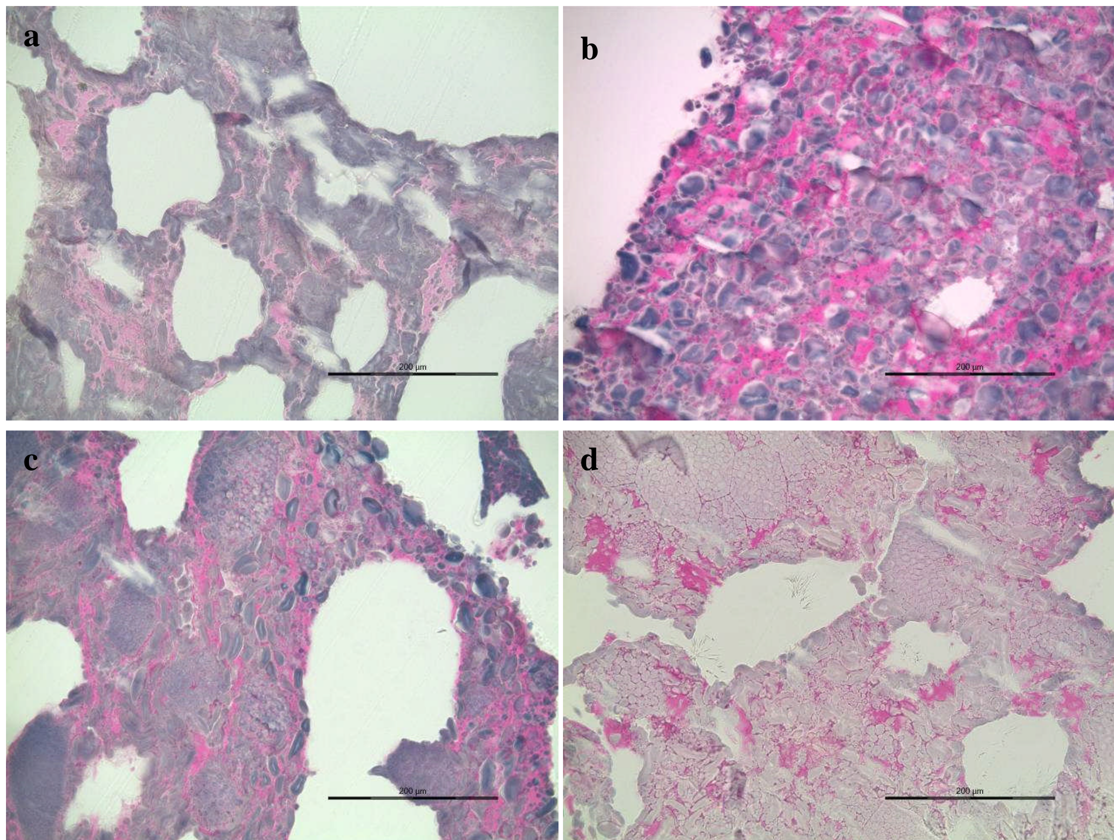

Figure 5.3: Light micrograph images of the control (a), control and hydrolysate (b), buckwheat(c) and buckwheat and hydrolysate (d) breads (x 20 obj). Starch grains appeared as dark blue, whilst protein is shown as pink/red colour.

5.4.5 Confocal scanning laser microscopy of bread samples

Confocal microscopy of resin embedded sections of bread was carried out using a new triple labelling protocol which effectively localised the main ingredients of the breads. Representative images are shown in Figure 5.4. Plant cell wall material (blue), starch (green) and protein (red) were imaged using fluorescent brightener (FB), fluorescein iso-thiocynate (FITC) and fast green (FG) labelling, respectively. The location of starch and protein correlated well with the light microscopy results, providing further validation of the new confocal staining procedure. The control (C) sample showed swollen starch granules with a semi-continuous protein network and cell wall fragments randomly scattered throughout the resin section (Figure5.4.a). Figure 5.4.b displays

microscopic images of the bread containing 4 % of the seaweed protein hydrolysate (CH), appeared similar to the control bread except that the starch granules were less swollen and more evenly dispersed. Figure 5.4.c contained buckwheat starch (B) and had a similar, swollen starch granule image to the control (C). In addition, this bread had close-packed, clusters of polyhedral starch grains. Figure 5.4.d (BH) contained similar clusters of polyhedral grains to those in the buckwheat bread formulation. However, the wheat starch in this formulation appeared less swollen than in the hydrolysate containing bread formulation.

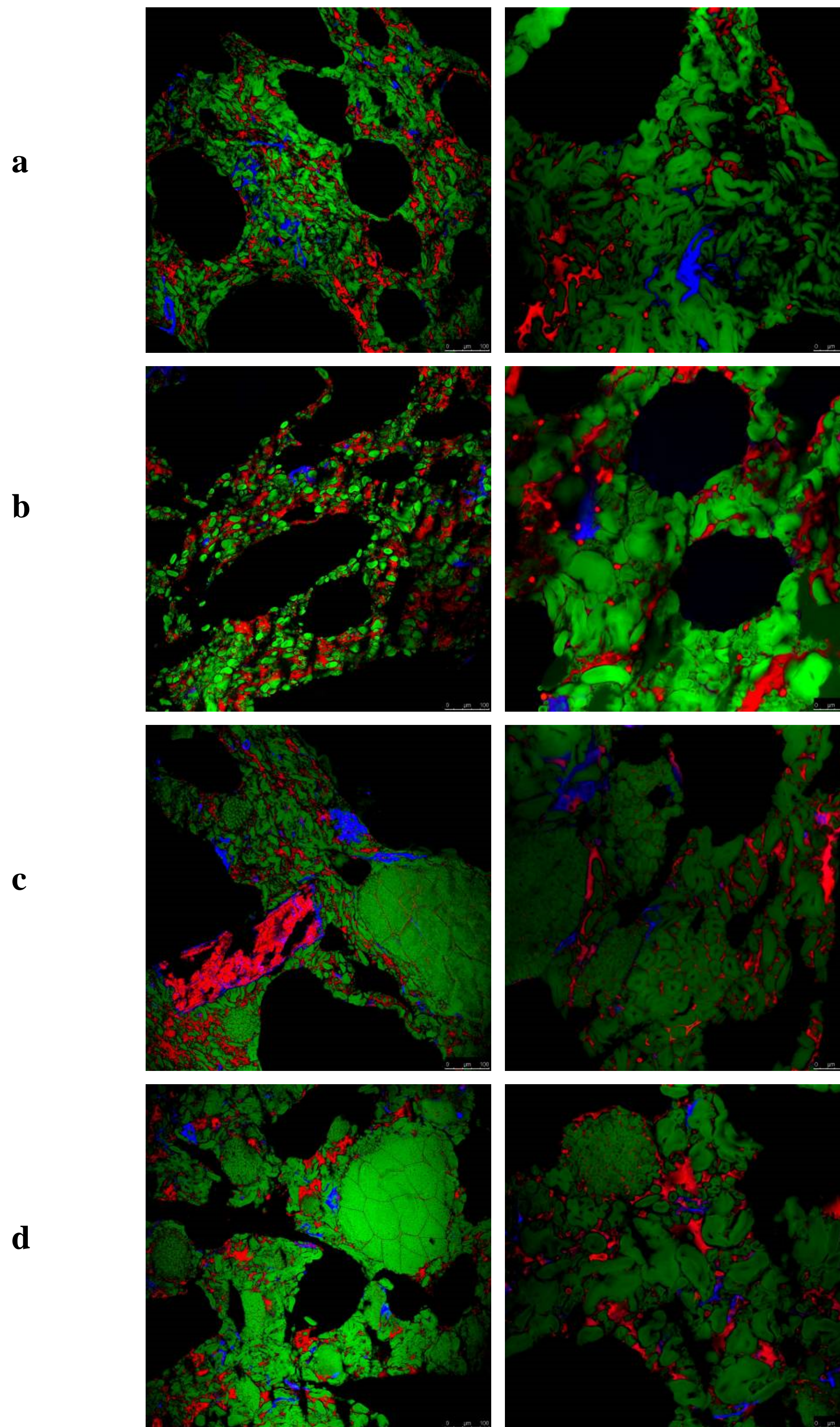

Figure 5.4: Confocal micrographs of control (a), control and hydrolysate (b), buckwheat (c) and buckwheat and hydrolysate (d) breads. Images on left are at x20 obj. (oil) and images on the right are at x63 obj. (oil). Plant cell wall material appears blue, starch as green and protein content is shown in red.

5.4.6 Heavy metal and Iodine analysis of the bread product

The level of cadmium, lead, arsenic and methyl-mercury detected in the bread product was 0.024, 0.047, 0.032 and 0.002 µg/g of freeze dried bread respectively. The iodine level was recorded as 1.19 µg per gram of bread tested.

5.4.7 Sensory analysis of breads

The appearance, texture, flavour and general acceptability scores are displayed in Figure 5.5. These results show the average score which the sensory panel assigned breads regarding the sensory characteristics of appearance, texture flavour and general acceptability as expressed by the panel member grading the bread from one to six. A distinctive trend can be observed with all four breads in terms of their sensory attributes. All three test breads scored lower than the wheat control (C) which scored just below five out of six regarding the sensory attributes which included appearance, texture flavour and general acceptability. The second favourite bread formulation was identified as the buckwheat bread formulation (B). The test panel consistently scored this bread four out of six for appearance, texture flavour and general acceptability. The third favourite bread formulation was the CH bread formulation which scored three for texture, flavour and general acceptability. It received a score of 3.58 (± 0.61) for appearance. The BH bread was the least favourite bread and scored low for flavour (1.54 ± 0.72) and general acceptability (1.56 ± 0.65). It scored similar results to the CH bread in terms of texture. Many of the panel members commented that the BH bread gave a strong, bitter after-taste.

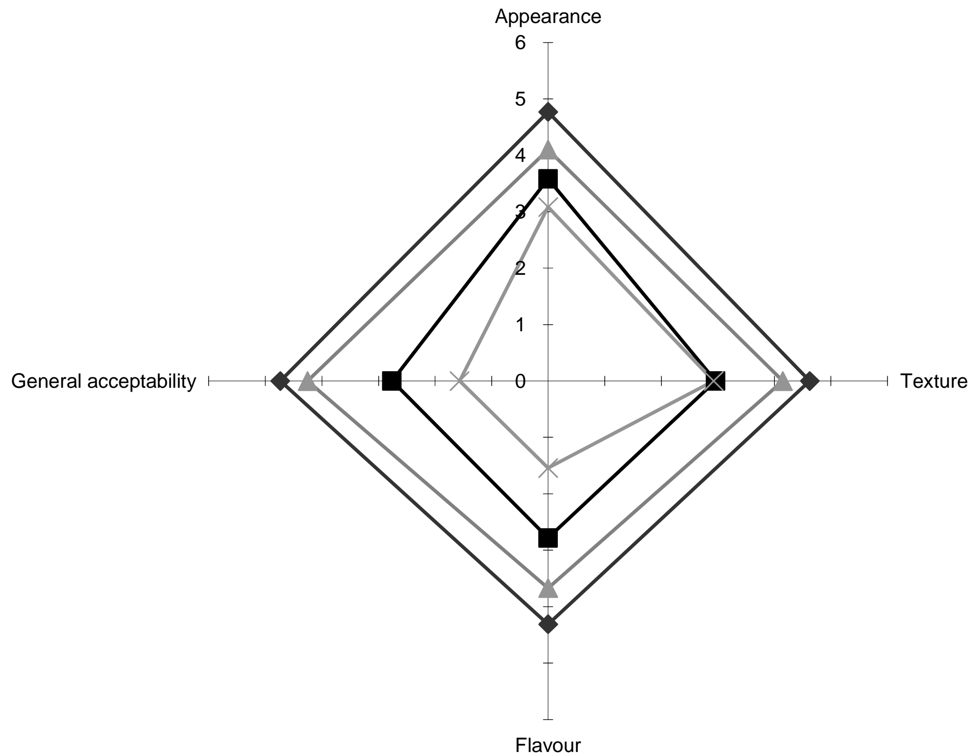

Figure 5.5: Sensory analysis. A group of 12 panellists were asked to rate the control (C), control + hydrolysate (CH), buckwheat (B) and buckwheat + hydrolysate (BH) breads under the appearance, texture, flavour and overall acceptability. Control (C) = ♦. Control + hydrolysate (CH) = ■. Buckwheat (B) = ▲. Buckwheat + hydrolysate (BH) = x. Values are mean (n=12).

5.4.8 Renin inhibitory activity of bread samples

The percentage inhibition of all four breads is shown in Figure 5.6. All three test breads demonstrated significantly higher renin inhibitory activity than that of the control bread (P<0.01). The *P. palmata* papain protein hydrolysate bread (CH) inhibited renin by 11.21% (± 0.77) when tested at a concentration of 1mg/ml compared to the positive control which was higher than the buckwheat bread formulation (B) which had a renin inhibitory value of 9.546% (± 0.48). Not surprisingly, combining the buckwheat and

seaweed protein hydrolysate in bread formulation increased the renin inhibitory activity to the bread to 14.92 % (± 1.88).

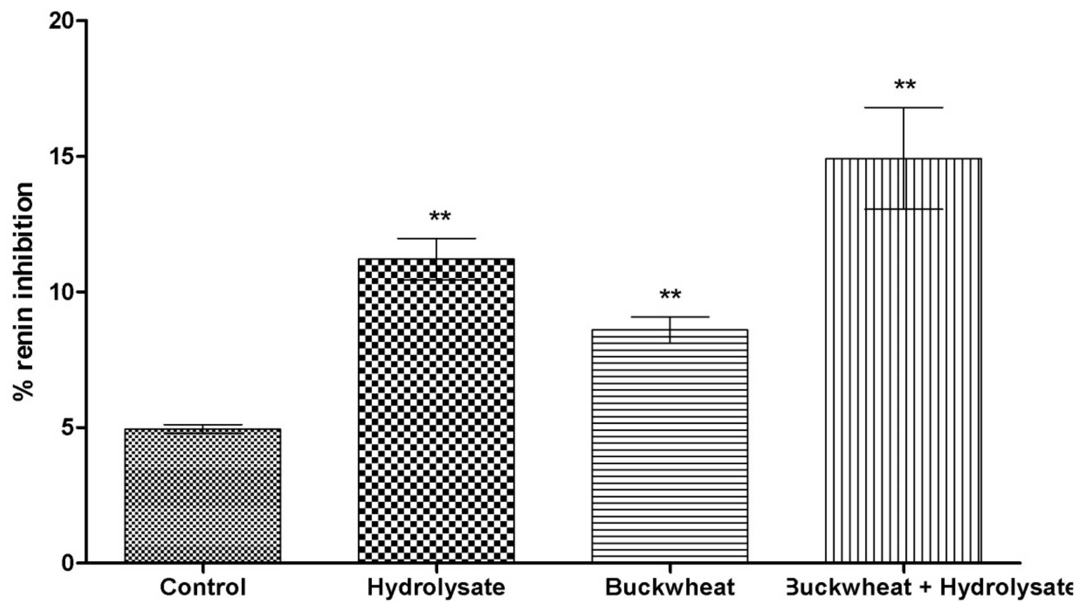

Figure 5.6: Renin inhibitory assay of the control (C), control + hydrolysate (CH), buckwheat (B) and buckwheat + hydrolysate (BH) breads. Values are mean ± S.E.M. (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared with control.

5.5 Discussion

This chapter confirmed that a renin inhibitory hydrolysate generated from *P. palmata* protein using the food grade enzyme papain retained bioactivity following the bread-making process. Furthermore, the textural and sensory attributes of the formulated breads containing the hydrolysate were found to be acceptable by a semi-trained, sensory panel using the hedonic scale [199].

Loaf volume is important in terms of consumer acceptance of bread products as it is an indicator of the density of the bread product and affects bread texture. The addition of 4% of a seaweed protein hydrolysate to wheat breads resulted in a decreased loaf volume. This decrease may be due, in part, to the additional protein added to the bread formulation which may compete with the wheat starch for free moisture, thereby restricting the hydration and swelling of starch granules. (Figures 5.3.b and 5.4.b). The starch grains shown in Figure 5.3.b and 5.4.b appeared less swollen and were evenly distributed compared to the control (C) and buckwheat (B) breads. This indicates that inhibition of the gelation process took place. A lower loaf volume is a common effect of protein addition to bread[200]. In a previous study, Moroni, et al. (2011)[201] demonstrated that the inclusion of buckwheat at 10% increased the loaf volume but the addition of 20% buckwheat had no effect on breadloaf volume. The findings from this work are in agreement with this observation as the addition of 30% buckwheat decreased loaf volume significantly. The inclusion of coarse buckwheat flour may have interfered with the elastic nature of the gluten network and may have contributed to a decreased loaf volume. The combination of buckwheat and the *P. palmata* papain protein hydrolysate (BH) resulted in greatly decreased loaf volume.

The crust and crumb colour of the bread darkened with the addition of the *P. palmata* hydrolysate. This result was expected as the browning of bread is due to non-enzymatic,

Maillard reactions[202]. The lower volume of the BH bread may have contributed to the darker crumb colour observed. Lower loaf volume, resulted in a lower surface area and therefore a dense and darker crumb appearance. The colour of the loaf greatly affects consumer perception of the quality of the bread, previous studies have suggested that bread color had a relatively greater impact upon consumer choice than the price and nutrition information combined [203].

Crumb structure of bread is an important factor in terms of the sensory aspects of bread, and directly affects the texture and staling properties [202]. The addition of the renin inhibitory hydrolysate had no effect on the number of cells, cell wall thickness or cell volume of internal bread crumb. The addition of buckwheat decreased the number of cells in the crumb in B bread (1462 ± 50) and the BH bread (1496 ± 22). Addition of buckwheat had no effect on wall thickness or the cell volume of the bread (Table 5.3). This result is positive in terms of the sensory acceptance of the bread product.

Addition of the hydrolysate which was identified as having a lysine content of 4.5 g per 100g of amino acid in chapter two improved the nutritional parameters of the bread product. Furthermore, the iodine content of the bread was assessed. Fortification of bread with iodine from marine sources including seaweed is a potentially attractive option for bakeries and food scientists. Dulse bread is known to the consumer and, in addition, Iodine is required by consumer groups including pregnant women [204]and those suffering from thyroid-gland dysfunction (under active thyroid gland)[205]. The level of iodine detected in the bread product was 1.19 µg per gram of bread tested. This is within the amount recommended by the FDA. The iodine-replete healthy adult has about 15–20 mg of Iodine, 70%–80% of which is contained in the thyroid[206].Median urinary iodine concentrations of 100–199 µg/L in children and adults, 150–249 mg/L in pregnant women and >100 µg/L in lactating women indicate iodine intakes are adequate

[207]. Iodine values lower than 100 µg/L in children and non-pregnant adults indicate insufficient iodine intake, although iodine deficiency is not classified as severe until urinary iodine levels are lower than 20 µg/L. Non-fortified bread ordinarily contains 0.39 µg iodine per gram of bread [208] therefore the bread containing *P. palmata* protein hydrolysate may be seen as a good source of iodine. The levels of heavy metals identified within the hydrolysate product were within the limits recommended [209] and below the levels found in other cereal products [210].

Sensory analysis (Figure 5.5) correlated well with the texture profile analysis results obtained (Figure 5.2), except where the panelists rated the texture of the CH and the BH breads similarly. TPA results showed the BH bread to be almost twice as hard as the CH bread. However, the crumb cohesive and springiness properties were similar, which translated into a similar mouth feel and texture when chewed by the panelist. Previous studies where sensory analysis was carried out on bread products that contained protein from plant sources reported a similar trend in terms of texture [200, 211]. The flavour characteristics of the two breads formulated using the renin inhibitory hydrolysate (CH and BH) were rated significantly lower by the sensory panelists than the control bread (C). Panelists described these breads as having a disagreeable and bitter flavour. A bitter flavour is often attributed to peptide content [100].This bitterness is also associated with Buckwheat flour, due to the high Tannin composition of this grain [212]. The use of semi trained sensory panels and a hedonic scale is a common method of assessing the sensory attributes of bread [213].

A number of different strategies could be employed to de-bitter protein hydrolysates. These include masking the bitter taste with various flavour agents[104], further enzymatic treatment of the protein[104]or, by the introduction of bitter inhibiting compounds such as neodiosimine and cellotrioside into the food matrix[214, 215]. The

panel preference for bread appearance was observed in decreasing order of C bread >B bread >CH bread>BH bread. Again, due to the added protein in the BH and CH breads, it is thought that a greater number of maillard reactions occurred which, resulted in, a darker bread appearance than the colour of the control bread (C) or buck wheat bread (B) formulations.

The renin inhibition assay showed that the renininhibitory properties of the *P. palmata* protein hydrolysate were retained during the baking process. Our previous study showed at a concentration of 1 mg/ml the hydrolysate can inhibit renin*in vitro* by 41.89% (±3.22)[163]. Similar baking processes have recorded crumb temperatures of 98 ºC [216]and it has been shown that bioactive peptides, such as ACE-I inhibitors retained their bioactivities following heating at 70 and 100 ºC for 20 minutes[102]. Starch is gelatinised in wheat breads and proteins are denaturated during the baking process[217]. Denatured proteins release water while the gelatinisation of starch absorbs water [217]. From the light and confocal micrograph images obtained in this work of the CH bread, this phenomenon of starch gelatinisation in tandem with protein denaturation is not visible. Therefore, it can be said that the renin inhibitory activity obtained in a previous study was not deleted due to the baking process, as the confocal micrograph images obtained showed that the seaweed protein was not denatured during the baking process. While the renin inhibitory effect of the buckwheat bread was not further investigated, buckwheat is known to have a wide range of bioactive compounds[218], including compounds that inhibit the RAS such as 2″-Hydroxynicotianamine (HNA) [196].

Seaweed enriched breads have already been developed with the potential to reduce habitual energy intake. The bread, which was manufactured using *Ascophyllum nodosum* could positively affect body mass index (BMI) and body composition[125]. In this present study, the renin inhibitory bioactivity of a *P. palmata* papain hydrolysate

was maintained when bread was used as a food carrier and the baking process was not found to inhibit renin inhibition. Furthermore, the bread developed using the seaweed hydrolysate did not greatly affect the sensory attributes of the CH bread and this bread could be developed further as a bioactive and consumer accepted product. Further work will include assessment of the bioavailability of the renin inhibitory peptides within the *P. palmata* hydrolysate bread in accepted cell models or *in vivo.* Also, the nutritional composition of the breads will be further examined.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion and conclusion

To survive the harsh and ever-changing environment that is the ocean, macroalgae produce distinctive biomolecules and secondary metabolites that have different properties to those found in terrestrial plant life [23]. These attributes make macroalgae an exciting and interesting source of bioactive compounds [2]. The overall aim of this Ph.D thesis was to investigate the hypothesis that the Irish seaweed *Palmaria palmata*(Linnaeus) Weber and Mohris a viable source of protein from which bioactive peptides with renin and PAF-AH inhibitory activities could be generated using food grade enzymes. These bioactivities could potentially have beneficial heart health effects, once consumed and this thesis aimed to determine these beneficial effects if present. One of the main aims of this work was to prove this hypothesis. *Palmaria palmata* is well known and is distributed across most European, Atlantic coastal Shores. It is also found on the coastline of Canada, Korea and Japan [219]. The broad distribution of this seaweed species, coupled with the fact that aquaculture of this species has recently occurred successfully made it an ideal choice of seaweed for this work.

The thesis also examined the potential survival of these bioactive peptides in the functional food delivery vehicle bread and a baked bread product containing the heart healthy hydrolysate was made. Survival of the renin inhibitory properties of the hydrolysate was also confirmed following a simulated gastrointestinal digestion. Indeed, this research aimed to prove that these peptides could provide a beneficial hypotensive effect once consumed. This was also demonstrated through the use of an animal model – spontaneously hypertensive rats (SHRs).

An important consideration in this study was the choice of seaweed species. *Palmaria palmata* (Linnaeus) Weber and Mohr was chosen for a number of reasons. Firstly, *Palmaria palmata* is known to have a high content of protein (as high as 25% according

to some studies) [32]. This makes *P. palmata* a more efficient starting material than seaweed species known to have low protein content such as the brown seaweeds [32] and increases the probability of recovering active peptides from its protein extract. However the sustainability of the plant source is an important concern when considering utilising it for industry. In Ireland, seaweed harvesting rights are traditionally held by families and this makes industrial harvesting of seaweed a contentious issue. Sustainability of the seaweed protein supply for bioactive peptide generation cannot therefore be guaranteed. However, recent research carried out at NUI Galway and Bord Iascaigh Mhara (BIM) in Ireland managed to successfully cultivate *Palmaria palmata* through an aquaculture process [220].

From a biochemical point of view, a benefit of using seaweed derived protein from *P. palmata* to generate bioactive peptides is that this protein source is rich in peptide sequences that have both hydrophobic and bulky side chain containing amino acids present [38]. These amino acids are often implicated in inhibiting enzymes of importance in the control of blood pressure such as the ACE-I enzyme. Indeed, it has been suggested that peptides containing bulky side chain amino acids and amino acids including proline, isoleucine and leucine can inhibit enzymes such as ACE-I, acetylcholinesterase (AChe) and others [119]. Finally, *P. palmata* has a long tradition of use in bread products in countries including Ireland, Denmark and Wales [123]. Furthermore, it is consumed on a daily basis in Japan and under EU regulations *P. palmata* is recognised as a sea vegetable. Therefore, for many consumers, the inclusion of this Seaweed in a commercial bread product would not be seen as peculiar and consumer acceptance of the bread product is likely.

The enzyme papain was used in this study in order to liberate bioactive peptides from the parent seaweed proteins of *Palmaria palmata*. Papain was the enzyme of choice, as

a food grade enzyme was required for the hydrolysis process and papain has a broad specificity for different substrates. The hydrolysate was intended for food use and consumption [152]. In addition, papain was used previously to generate hypotensive peptides from food matrices [151].

Effective and direct separation of the *P. palmata* protein hydrolysate was required in this study to identify the bioactive peptides responsible for both renin and PAF-AH inhibitory activities and indeed those peptides that survived gastrointestinal transit. [221]. The approach used in this work is commonly termed bioassay guided fractionation and was used previously to generate bioactive peptides for pharmaceutical applications [222]. ESI-Q-TOF was used along with *de novo* sequencing and database comparison in order to identify the peptides. This method is quicker and more accurate than previously documented bioassay guided fractionation techniques such as RP-HPLC. The renin and PAF-AH inhibitory peptides were compared to bioactive peptides previously identified and found in databases such as BIOPEP and others [139] and were chemically synthesized to ensure that the peptides identified were indeed the active agents.

The findings of Chapters two and three support the hypothesis that the Irish seaweed *P. palmata* is a valid source of heart health peptides with renin and PAF-AH inhibitory activities that could reduce high blood pressure and artherosclerosis. We have reported the discovery of the renin inhibitory tridecapeptide IRLIIVLMPILMA. This peptide inhibited renin by 50% at a concentration of 3.344 mM in an *in-vitro* assay. This is the first reported study that showed the generation of a renin inhibitory peptide from *Palmaria palmata*. Furthermore, the peptide identified is, to the authors' knowledge, the most potent renin inhibitory peptide derived from a food or a plant source to date. Furthermore we characterized and isolated a PAF-AH inhibitory peptide from the same

source. The peptide NIGK was identified and this peptide demonstrated substantial PAF-AH inhibitory activity. NIGK inhibited PAF-AH and had an IC_{50} value of 2.32 mM. This is the first report of a PAF-AH inhibitory seaweed derived peptide to date.

We also looked at the toxicity of the hydrolysate generated which contained both the PAF-AH and renin inhibitory peptides using zebrafish larvae assay. The toxic effects of the hydrolysate, at three different concentrations, were examined by determining the percentage survival of the zebrafish larvae and the heart rate of the zebrafish larvae over a 48 hr period. Previous studies where zebrafish larvae were used to examine the toxic effects of pharmaceutical drugs demonstrated a close correlation with results subsequently obtained from trials carried out through use of human clinical trials [175]. The zebrafish larvae assay was used as it generates results rapidly while being a cost efficient method which requires minimal economic resources [175]. In addition, Chapter three is the first report where a potential functional food peptide was assessed using this model. Following testing of the hydrolysate generated, at three different concentrations of 1, 5, and 10 mg/ml, it was determined that the percentage survival of the zebrafish larvae decreased along with the heart rate of the zebrafish when the hydrolysate was fed at concentrations of either 5 or 10 mg/ml. However, when larvae were fed concentrations of the hydrolysate up to 1 mg/ml over a 48hr period, no toxic effects were determined in terms of percentage survival, heart rate or morphological anomalies of the zebrafish larvae when compared to the control group. The main issue with the use of zebrafish in assessing potential safety issues is the lack of extensive validation with diverse and comprehensive pharmacological compound sets to adequately understand the advantages and limitations of the zebrafish larvae assay [177].

The safety of the hydrolysate when incorporated in a bread product was also assessed in terms of heavy metal and Iodine content; all of which were shown to be within advised

parameters set by the FDA and EFSA [194]. Macroalgae in the past have shown the ability to bio-accumulate heavy metals such as lead, mercury, arsenic and cadmium [223]. Almost all seaweeds are rich in Iodine [224], an important nutrient in metabolic regulation [14]. However, consumption of large quantities of iodine can have adverse effects such as decreasing thyroid hormone synthesis [225]. With this in mind, the bread developed and enriched with *P. palmata* protein hydrolysate was assessed for iodine, lead, mercury, arsenic and cadmium. Chapter four demonstrated that the levels of iodine and heavy metals in the bread product are comparable to other common food stuffs. For instance, the levels of lead, mercury, arsenic and cadmium are comparable to that found in other wheaten breads [210] while still being within the EU regulatory guidelines [209] and much lower than the main source of these toxins which is fish and shellfish [210]. The level of iodine found in the bread containing the hydrolysate was 1.19 µg/g, which is almost three times the level displayed in other non-fortified breads which displayed levels of 0.39 µg/g in previous studies, yet not as high as the levels found in marine fish which can contain 2.11 µg/g of the element [208]. Therefore the bread containing the *P. palmata* hydrolysate can be seen as a good source of iodine which is essential for thyroid metabolism while still being within FDA recommendations [204].

Bread was chosen as the delivery vehicle in this work as it is produced and consumed globally, particularly in the Western World where CVD is the most prominent cause of death [124]. This thesis aimed to develop a heart healthy bread that could be used as a substitute for everyday bread and which could serve as a functional food for the prevention of CVD. The addition of the *P. palmata* hydrolysate also boosted the nutritional quality of the bread as it increased the Protein content of the bread by 4 % and more specifically the lysine content. This is important as wheat bread is known to be deficient in this amino acid [149]. Results from the *in vitro* renin inhibitory assay of the four different bread formulations tested found that that the renin inhibition

previously observed in the *P. palmata* protein hydrolysate survived the baking process. Microscopy analysis of the four breads developed in Chapter four support the hypothesis that the bioactive, peptide-containing hydrolysate would not be denatured during the bread baking process. Texture profile, colour and sensory analysis results obtained suggested that the addition of the seaweed hydrolysate to the bread product did not adversely affect the organoleptic characteristics of the bread product. Panelists described the bread formulations with the seaweed hydrolysate as having a bitter taste, a characteristic associated with peptide content [100] and also with buckwheat breads due to high tannin composition of the flour [212].

The *in vitro* digestion study carried out in Chapter 5 was used to test the theory that the tridecapeptide IRLIIVILMPILMA could also survive proteolysis by GI tract enzymes and that the active dipeptide IR would be released from this peptide to ensure a hypotensive effect. The *in vitro* model is a fast and inexpensive method and it does not carry with it the ethical constraints associated with animal studies [181]. In the context of this PhD thesis, it was used to justify the eventual use of spontaneously hypertensive rats (SHRs) as an *in vivo* model for testing the hypotensive effect of the renin inhibitory tridecapeptide IRLIIVILMPILMA and the *P. palmata* protein hydrolysate. The mass spectral (MS) data generated after *in vitro* gastric digestion of the tridecapeptide revealed that this peptide was cleaved in two, liberating the previously identified renin inhibitory dipeptide IR [72]. This peptide was previously isolated from pea protein hydrolysates where it displayed a renin inhibitory IC_{50} of 9.2 mM [72]. This pea protein hydrolysate subsequently displayed a hypotensive effect in SHR rats [183].

While *in silico* and *in vitro* assay methods are useful tools that may be used to predict the presence of a bioactive peptide in a protein source and its potential bioactivity, the likely mode of action of a molecule within a living system cannot be determined

without the use of animal and human models. This is due to the myriad of complex reactions a molecule will encounter within an organism, making animal research necessary in determining health beneficial effects in pharmaceutical and functional food research areas [189]. In this work, SHRs were used. This rat type is specifically bred to be hypertensive and is a well-known animal model for determining bioactive agents with potential for use in human clinical intervention trials. Consumption of both the renin inhibitory peptide IRLIIVLMPILMA and the *P. palmata* protein hydrolysate by SHR rats resulted in a substantial drop in blood pressure compared to the control.

This thesis demonstrated that the Irish seaweed *P. palmata* is a source of protein which could be used to generate bioactive renin and PAF-AH inhibitory peptides through the use of the food grade enzyme papain. Positive, hypotensive effects were demonstrated both *in vitro* and in SHR animal models. Bread was also shown to be a suitable carrier for these bioactive peptides and the hydrolysate from which the peptides were generated. Future work in this area could include further pre-clinical trials to observe the long term effect of the *P. palmata* protein hydrolysate and the peptide IRLIIVLMPILMA followed by human clinical intervention studies. However, further preclinical and clinical trials were outside the scope of this project for both economic and resource reasons. Subsequent studies could assess if consumption of the seaweed bread product would affect human blood pressure positively. In addition, further augmentation of the bread formulation could improve the flavour and texture characteristics of the end product.

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ANNEX

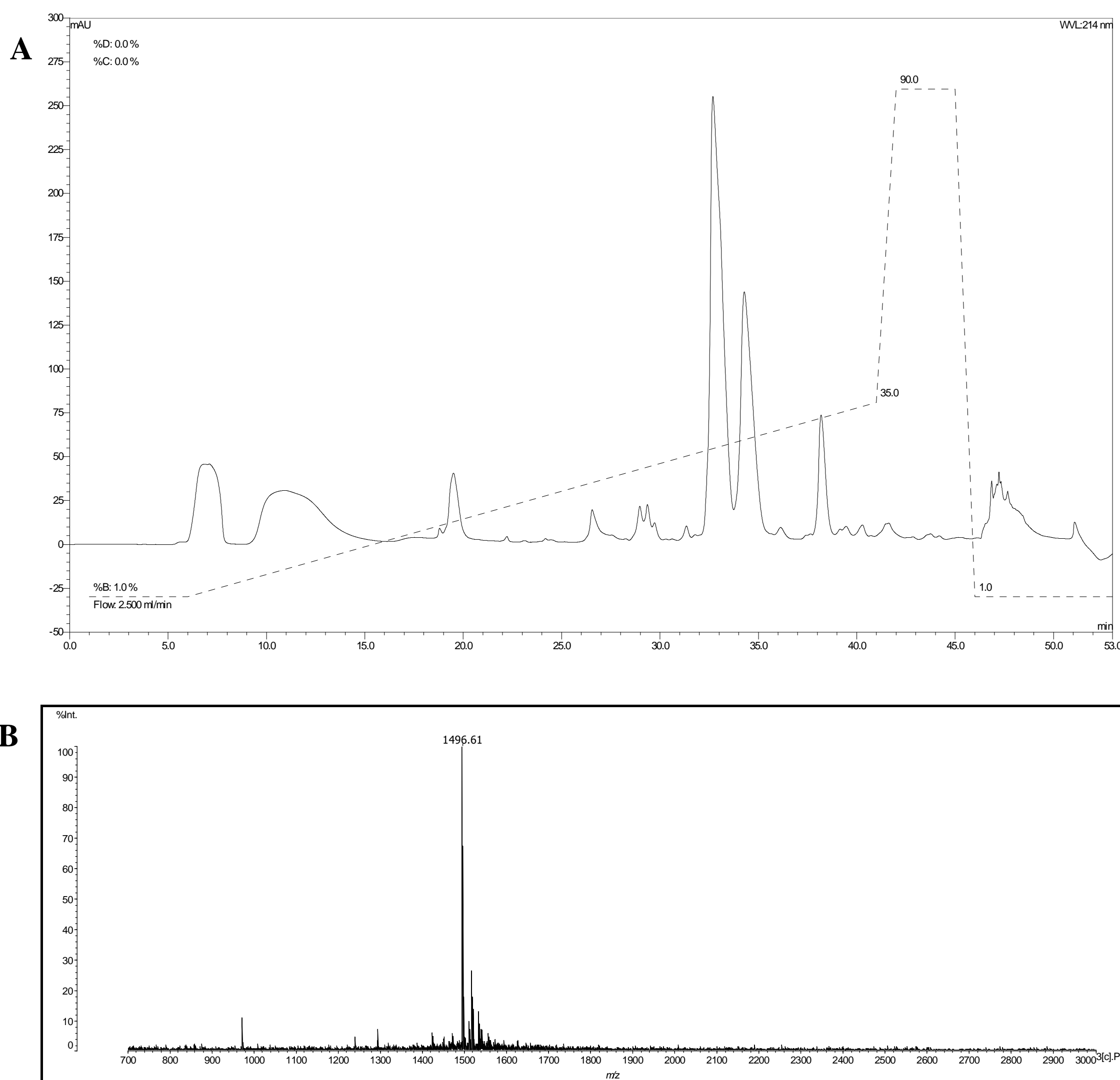

Figure 8.1: Purification of IRLIIVLMPILMA using RP-HPLC. Synthetic Peptides were purified using RP-HPLC. RP-HPLC chromatogram for the renin inhibitory Peptide IRLIIVLMPILMA is shown in figure 25 A. Fractions containing the desired molecular mass were identified using MALDI-TOF mass spectrometry. The MS spectrum for IRLIIVLMPILMA is shown in figure 25 B.

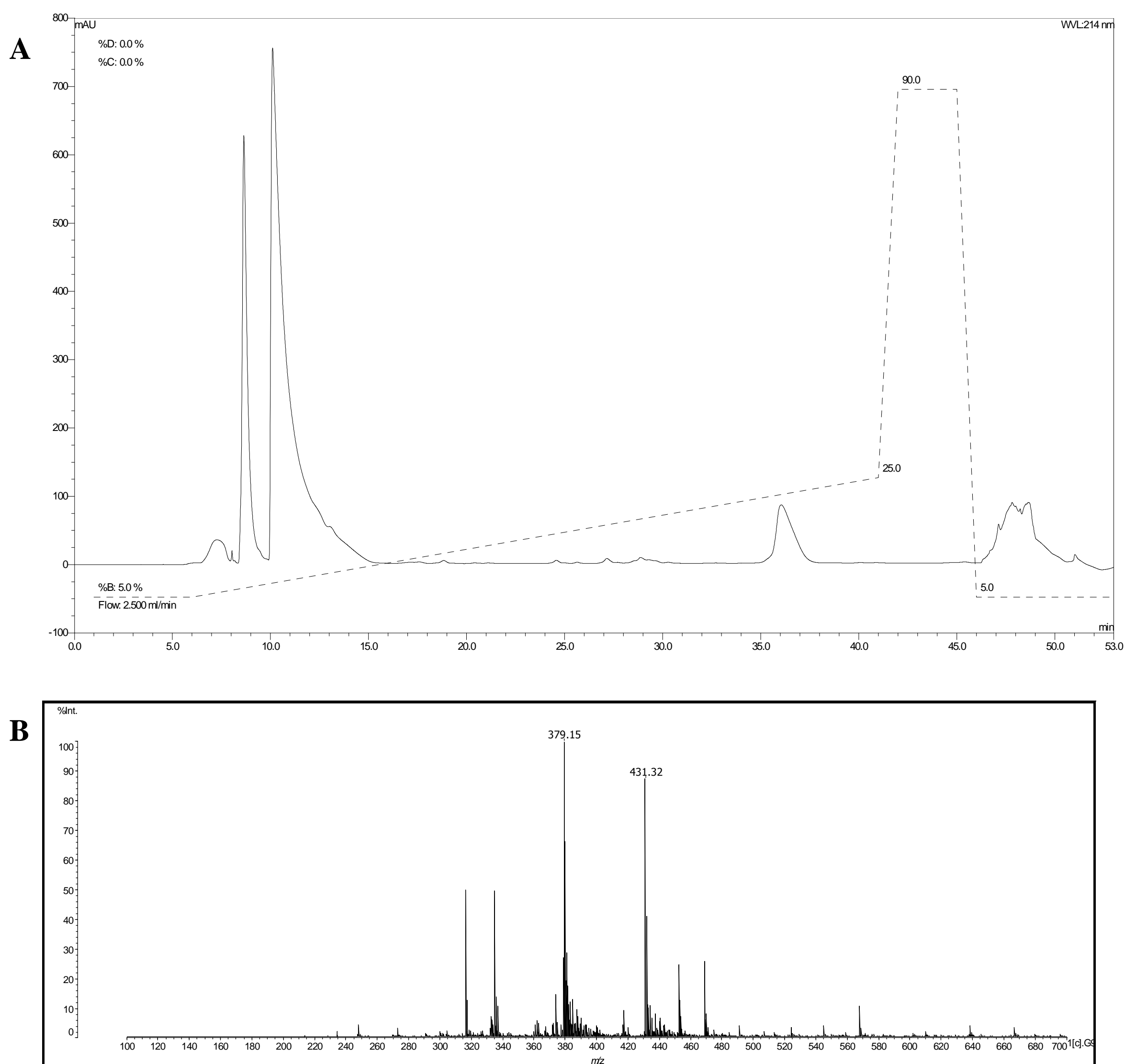

Figure 8.2: Purification of NIGK. Synthetic Peptides were purified using RP-HPLC. RP-HPLC chromatogram for the PAF-AH inhibitory Peptide NIGK is shown in figure 26 A. Fractions containing the desired molecular mass were identified using MALDI-TOF mass spectrometry. The MS spectrum for NIGK is shown in figure 26 B.

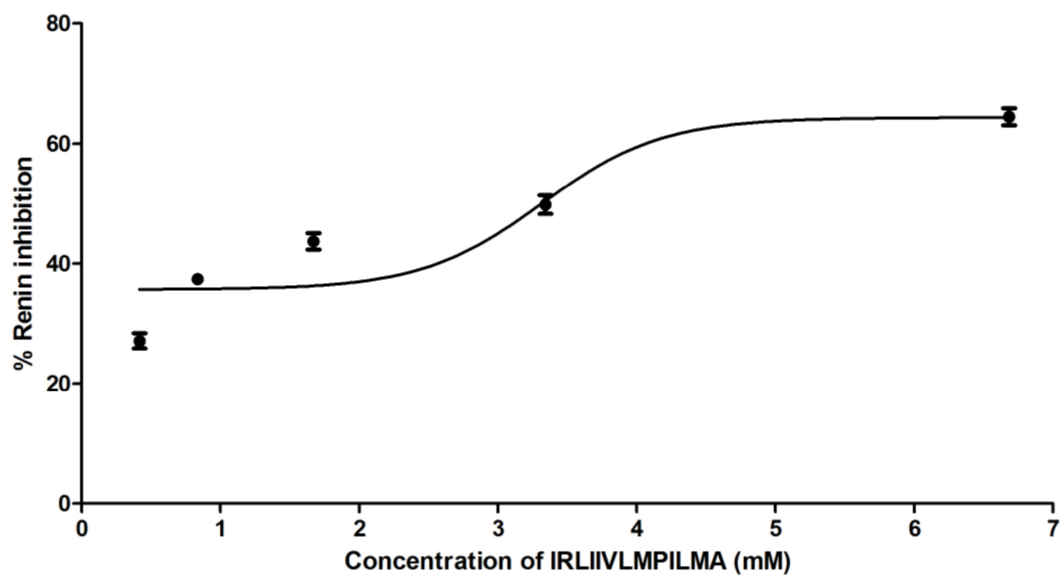

Figure 8.4: Non-linear regression of IRLIIVLMPILMA concentration versus percentage inhibition.

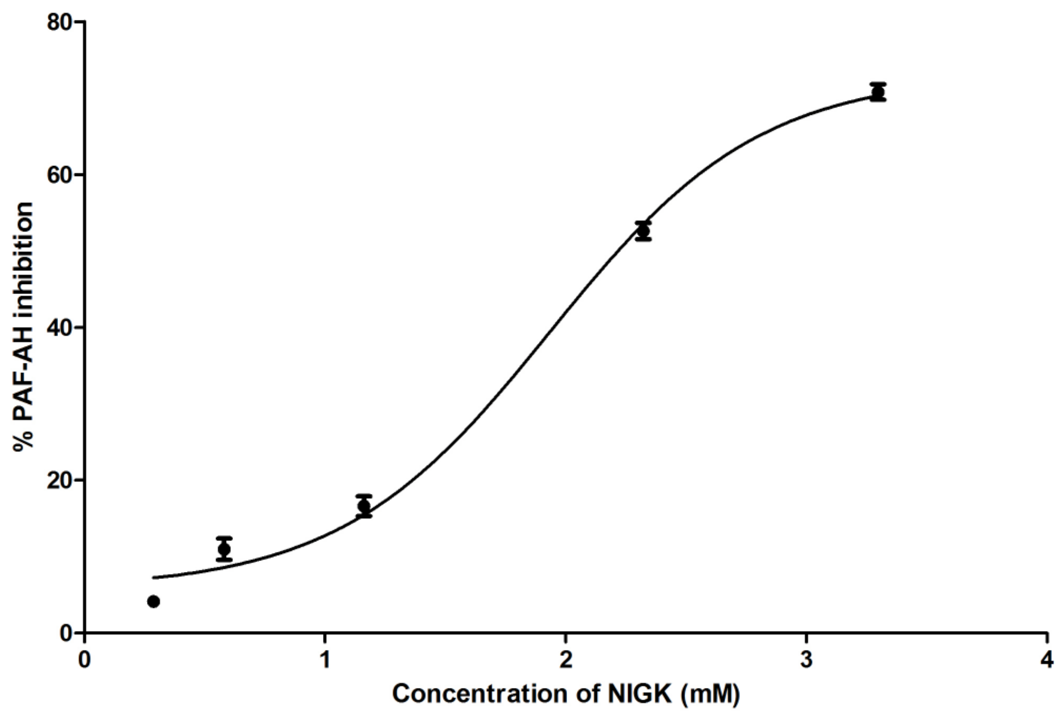

Figure 8.4: Non-linear regression of NIGK concentration versus percentage inhibition.

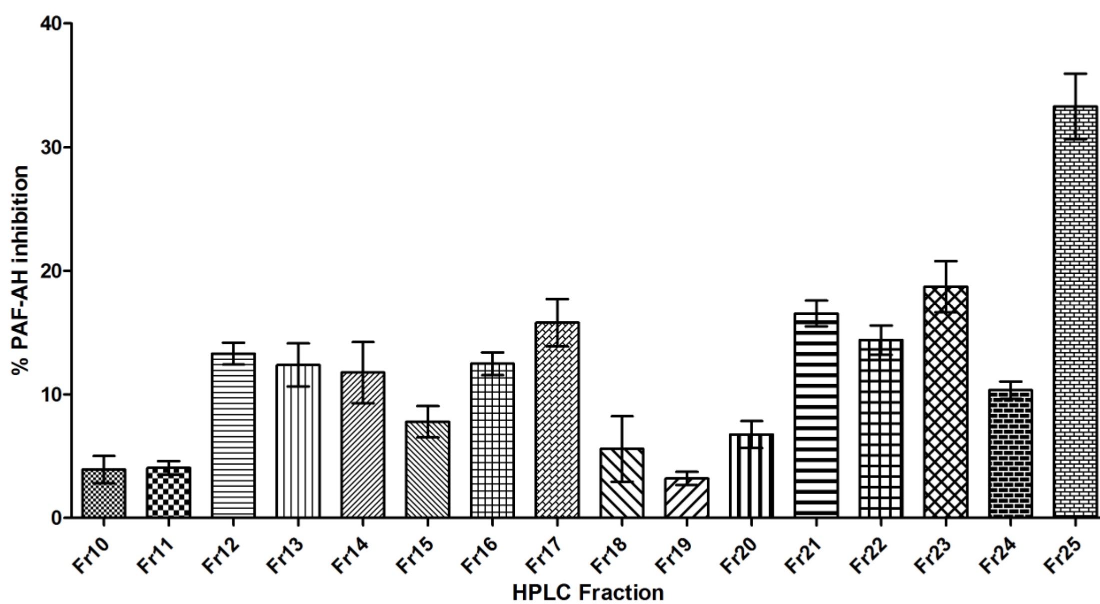

Figure 8.5: PAF-AH assay of HPLC fractions 10-25 of the *P. palmata* papain protein hydrolysate. Samples were tested at a concentration of 1 mg/mL. Data is displayed as % inhibition. Values are mean ± SEM (n = 3).