Angiotensin-I-converting enzyme and prolyl endopeptidase inhibitory peptides from marine processing by-products

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Declaration

I hereby declare that the work herein, submitted for the degree of Master of Science in Letterkenny Institute of Technology is the result of my own investigation, except where reference is made to published literature. I also certify that the material submitted in this thesis has not been previously submitted for any other qualification.

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Date: 26/10/11

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Abstract

Like many natural resource-based processing industries, the seafood processing sector gives rise to a significant volume of organic waste. Environmental issues, economic concerns and legal restrictions regarding the disposal of processing wastes have led to increased research in the discovery of alternative value-added products, such as bioactive peptides from these waste streams. Bioactive peptides have various physiological functionalities in the human body following consumption and these include antihypertensive, anti-amnesiac, mineral-binding, immunodulatory, antioxidative and antithrombotic activities. The main focus of this thesis was to examine whether mackerel and whelk fisheries processing by-products and mackerel meat tissues are suitable sources for the isolation of angiotensin-I converting enzyme (ACE-I) and prolyl endopeptidase (PEP) inhibitory peptides. This thesis outlines the steps involved in the preparation of fish waste hydrolysates using enzymes, development of bioassays to monitor ACE-I and PEP inhibitory activities, application of capillary electrophoresis for ACE-I inhibitory activity and the concentration of peptidic fractions using membrane filtration techniques.

Ecostone®A200, Corolase® N, Corolase® PP, Corolase® LAP, Pepsin and Thermolysin were used as suitable hydrolytic enzymes for the production of ACE-I and PEP inhibitory peptides. Whelk waste consisting of shell discards were hydrolysed using the Ecostone®A200 polysaccharide hydrolytic enzyme. Mackerel waste which consisted of white meat and brown meat tissues and head, tail and skin discard portions were hydrolysed using the Corolase® N, Corolase® PP, Corolase® LAP, Pepsin and Thermolysin proteolytic enzymes. ACE-I and PEP bioassays identified potential ACE-I inhibitory activity of mackerel samples with inhibition values of up to 39% (± 7.02) while PEP inhibitory activity occurred for all hydrolysates and ranged from 35 (± 1.35) to 78% (± 1.88) inhibition. ACE-I inhibitory assays results were confirmed using a purified ACE-I enzyme source and a developed capillary electrophoresis ACE-I inhibitory assay protocol. ACE-I inhibitory activities for the same hydrolysates used in the ACE-I bioassay increased to 100% inhibition with positive ACE inhibitory activity ranging from 71 – 100% depending on the mackerel tissue source and hydrolytic enzyme employed. Two of the ACE-I inhibitory samples from different mackerel tissues were further concentrated using membrane filtration techniques through molecular weight cutoff (MWCO) filters (10 kDa and 3 kDa). The purpose of this was to ascertain if there were potentially a number of various sized peptide inhibitors present and to

assess if the hydrolytic conditions resulted in partial hydrolysis of the inhibitory peptides present in the samples. Results indicated that the experimental strategy employed and the utilisation of marine waste may serve as an approach to identifying novel bioactive peptides.

List of Abbreviations

ACE-I: Angiotensin-I-converting enzyme

BGE: Background electrolyte

BIM: Bord Iascaigh Mhara (Irish Sea Fisheries Board)

CE: Capillary electrophoresis

DAD: Diode array detector

FAO: Food and Agriculture Organisation

FOSHU: Foods for Specific Health Use

HA: Hippuric acid

HGG: Hippuryl- glycine- glycine

HPLC: High performance liquid chromatography

HHL: Hippuryl-L-histidyl-L-leucine

HL: Histidyl- leucine

ICES: International Council for the exploration of the Seas

id: Internal diameter

kDa: kilo Dalton

LIF: laser-induced fluorescence

LOD: Limit of detection

LOD: Limit of quantification

MALDI-TOF: Matrix assisted laser desorption/ionisation- time of flight

MS: Mass spectrometer

NEA: Northeast Atlantic mackerel

PB: phosphate buffer

PEP: prolyl endopeptidase

PO: prolyl oligopeptidase

PPT: parts per trillion

PSI: pounds per square inch

RP: Reverse phase

RPM: revolutions per minute

RSD: Relative standard deviation

SD: Standard deviation

SHRs: spontaneously hypertensive rats

WHO: World Health Organisation

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Wilson, J., Hayes, M., Carney, B. 2011. Angiotensin-I-converting enzyme and prolyl endopeptidase inhibitory peptides from natural sources with a focus on marine processing by-products. *Food Chemistry*, **129**, 235-244.

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1. Literature review

1.1 Introduction

In recent years, over-exploitation of fishery resources has become a major concern worldwide. According to data published by the Food and Agriculture Organisation (FAO, 2008), approximately 77% of the 143.6 million tons of fish and shellfish caught in 2006 was used for human consumption. The remaining processing leftovers which, includes trimmings, fins, frames, heads, shells, skin and viscera are normally used for the production of fishmeal, fish oil, fertiliser, fish silage and animal feed. These by-products may be identified as processing leftovers that are not normally saleable but which can be recycled after treatment. Annual discard from marine capture and aquaculture fisheries worldwide is estimated to be approximately 20 million tonnes per year (FAO, 2008). In Ireland, figures from An Bord Iascaigh Mhara (BIM; The Irish Sea Fisheries Board), estimated that 63,786 tonnes of marine processing waste were produced nationally in 2000, 45% of which arose from the northwest region (Pfeiffer, 2003). European legislation, Council Directive 1999/31/EC of 26 April 1999 on the landfill of waste, has set specific targets for the amount of municipal waste allowed for disposal at landfills. Landfill is also a costly waste disposal option for the seafood industry. Generation of substantial quantities of marine processing by-products is therefore both an environmental and economic concern for the seafood processing sector (Kim and Mendis, 2006). As a result, there has been increased interest in exploring the possible uses of fish by-products, or rest raw material, so that they become viewed as a potential resource due to their valuable protein and lipid content and sugars and minerals instead of a waste problem. (Akihisa *et al.*, 2004; Bruyere *et al.*, 2004; Kelley, 2001; Larsen *et al.*, 2000; Yokoyama *et al.*, 1992).

Proteins from food are sources of numerous bioactive peptides with anti-hypertensive, opioid, anti-thrombotic and immune-modulating activities. The use of fish processing by-products as substrate provides a novel approach for the potential discovery of high-value bioactive products. In the past twenty years, an emerging area of research is the identification of bioactive compounds that may be present in marine by-products. Bioactive compounds are described as 'food-derived components' that, in addition to their nutritional value, exert a physiological effect in the body' (Vermeissen *et al.*, 2004).

This thesis reviews current information on ACE-I-inhibitory and PEP inhibitory peptides. It also details the potential health benefits of these peptides and their potential for use as functional food components. The thesis examines the isolation and characterisation strategies used to obtain these inhibitors from natural sources, with a focus on marine substrates. Emphasis for this thesis is placed on identification of bioactive peptides from mackerel and whelk waste, two products identified by a local large scale fish processing company (Per. comm. Errigal Fish Company Ltd., Carrick, County Donegal). The development of these technologies in search of novel bioactive peptides for human exploitation from marine processing wastes will bring increased value to what is today considered an environmental waste problem.

1.2 Mackerel and Whelk life history and habitats

Atlantic Mackerel (*Scomber scombrus*) is an abundant, fast swimming, migratory pelagic schooling species found on both sides of the North Atlantic Ocean. The mackerel in the eastern Atlantic has traditionally been divided into three spawning groups named according to their spawning areas: Southern (Gibraltar to the Bay of Biscay), Western (Bay of Biscay to Scotland), and North Sea and these are managed as one stock; the Northeast Atlantic Mackerel (NEA). The Northeast Atlantic (NEA) mackerel supports a very valuable fishery, with landings that have ranged between 470,000 and 820,000 tons since the mid-1990s (ICES, 2008). Mackerel processing and waste data supplied by the Errigal Fish Company Ltd., Carrick, Co. Donegal up to the end of 2008 detailed annual processing input of 7,000 metric tons with 2,800 metric tons of waste generated as a result.

Mackerel are abundant in cold and temperate shelf areas with a rapid growth cycle and a life span of approximately 17 years. They are opportunistic feeders and prey most heavily on crustaceans such as copepods, krill, and shrimp. They also eat squid, and some fish and ascidians. Mackerel also play an important ecological role by feeding on zooplankton and on the pelagic juvenile and larval stages of a number of commercially important fish stocks. Mackerel may grow to a maximum length of 66 cm, although fish >50 cm are uncommon; they reach reproductive maturity when they are between two and three years old. Mackerel are batch spawners with females shedding their eggs in some twenty batches during the course of the spawning season which is between February and July. Eggs generally float in the surface water and hatch in four to seven days, depending on water temperature (ICES, 1990).

Atlantic mackerel are iridescent blue green above with a silvery white underbelly (Figure 1.1). They have twenty to thirty wavy black bars that run across the top half of their body, and there is a narrow dark streak that runs along each side from pectoral to tail fin below the bars. Their body is spindle shaped, tapering at both ends. They have two separate large dorsal fins that are grey or dusky. The pectoral fins are black or dusky at the base, and the tail fin is grey or dusky.

Figure 1. 1. *Scomber scombrus*, Atlantic mackerel.

Mackerel are preyed upon heavily by dolphins, whales, dogfish, hake, Atlantic cod, bluefish, and striped bass, swordfish, red hake, Atlantic bonito, blue-fin tuna, blue shark, sea lamprey, and short-fin mako and thresher sharks (ICES, 1990).

The common whelk, *Buccinum undatum* L. 1758, is a large edible gastropod mollusc (Figure 1.2) common in the sub-tidal range to 200m depth in on both sides of the Atlantic, from Canada south to New Jersey in North America and in Europe from Norway to south- west France. Fahy *et al.* (2000) reported the onset of maturation in Irish Sea whelk at between 60 and 70 mm total length and around 6-8 yrs. The shell has 7-8 whorls with spiral ridges and is yellowish brown with irregular light and dark spiral areas. The aperture is broadly oval tapering to a point with a short wide siphonal canal leading from the base of aperture. Whelk shell structure alters in response to a variety of factors that include latitude, environment, sex of the individual and predation pressure. Shell thickness in *Buccinum* alters in response to the occurrence of large decapod predators. Irish inshore waters support morphologically easily distinguishable stocks of whelk and the more heavily armoured ones co-occur with high densities of brown crab (Fahy *et al.*, 2005). Predators of *B. undatum* include several invertebrate species such as crabs, lobsters, and starfish.

The major European countries exploiting whelk include Belgium, France, Iceland, Ireland,

and the UK with the main traditional markets in Europe found in England, France, and the Netherlands (Nasution and Roberts, 2004). The fishery for this species boomed in the 1990s with an increase in demand from the Far East, mainly South Korea (Fahy *et al.*, 2005). In 2003, 672 tons of whelks were landed from the Cape grounds in Co. Donegal in six locations of which the largest were Malin and Greencastle. Landing data was provided by Errigal Fish Company Ltd., Carrick, Co. Donegal and in the period 2008 to present whelk input for processing by the company was 4,000 metric tons per annum which results in the generation of 1,700 metric tons of waste material.

Figure 1. 2. *Buccinum undatum* the common Irish whelk

1.3 Function of ACE-I and PEP inhibitory peptides

Hypertension is one of the most common cardiovascular diseases worldwide. Data gathered by Kearney *et al.* (2005) demonstrated that over 25% of the adult population nearly 1 billion people worldwide suffer from hypertension. Hypertension is a risk factor for arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (Kearney *et al.*, 2005). The prevalence of high blood pressure increases with age, affecting approximately 65% of the population in developed nations within the age group 65-74 years (Duprez *et al.*, 2002). The World Health Organisation (WHO) estimates that by 2020, heart disease and stroke will have surpassed infectious diseases to become the leading cause of death and disability worldwide (Lopez and Murray, 1998). Diet therapy and lifestyle modifications are the two most important tools commonly employed to effectively lower blood pressure (Hermansen, 2000).

Therefore, any food component that has the ability after digestion to reduce blood pressure in humans is a potential candidate for use in the prevention and/or treatment of cardiovascular disease.

Angiotensin-I converting enzyme (ACE, EC 3.4.15.1) belongs to a class of zinc proteases and is widely distributed in mammalian tissues. It is predominantly found as a membrane-bound ecto-enzyme in the vascular endothelial cells of the lungs and other organs such as the brain, heart, liver, kidney, intestine and placenta as well as in several other cell types including absorptive epithelial, neuroepithelial, and male germinal cells (Riordan, 2003). ACE-I catalyses the formation of the potent vasoconstrictive and salt-retaining octapeptide angiotensin II with the amino acid sequence Asp-Arg-Val-Try-Ile-His-Pro-Phe, from the decapeptide angiotensin I, Asp-Arg-Val-Try-Ile-His-Pro-Phe-His-Leu, by liberating the C-terminal di-peptide His-Leu in the Renin-Angiotensin system (Yang *et al.*, 1971) (Figure 1.3). ACE-I also inactivates the vasodilative peptide bradykinin in the kallikrein-kinin system (Meisel *et al.*, 2006) (Figure 1.3). Additionally ACE-I functions as a stimulant for the release of aldosterone in the adrenal cortex (Silvestre *et al.*, 1999). Therefore ACE inhibitors can be expected to reduce and regulate blood pressure and fluid and salt balance in mammals (Figure 1.3).

Prolyl endopeptidase (PEP, EC 3.4.21.26) also known as prolyl oligopeptidase (POP or PO), is a highly conserved serine protease enzyme that cleaves peptide bonds at the carboxyl side of Proline residues in proteins with a relatively small molecular weight (30 amino acids in size) containing the recognition sequence X-Pro-Y, where X is a peptide or protected amino acid and Y is either an amide, a peptide, an amino acid, an aromatic amine or an alcohol (Walter *et al.*, 1980). PEP is widely distributed in mammalian tissues and was found in over 20 human tissue types with the highest activity found in skeletal muscle and in the human brain (Kalwant and Porter, 1991). In the brain, the highest PEP activity was found in the cortices (Irazusta *et al.*, 2002). PEP has also been isolated from various tissues such as rat (Rupnow *et al.*, 1979), lamb (Yoshimoto *et al.*, 1981) and bovine brain (Yoshimoto *et al.*, 1983) as well as pig liver (Moriyama *et al.*, 1988). PEP has also been found in the bacterium *Flavobacterium meningosepticum* (Chevallier *et al.*, 1992) and in the mushrooms *Lyophilium cinerascens* (Yoshimoto *et al.*, 1988) and *Agaricus bisporus* (Sattar *et al.*, 1990) as well as Spinach thylakoids (Kuwabara, 1992).

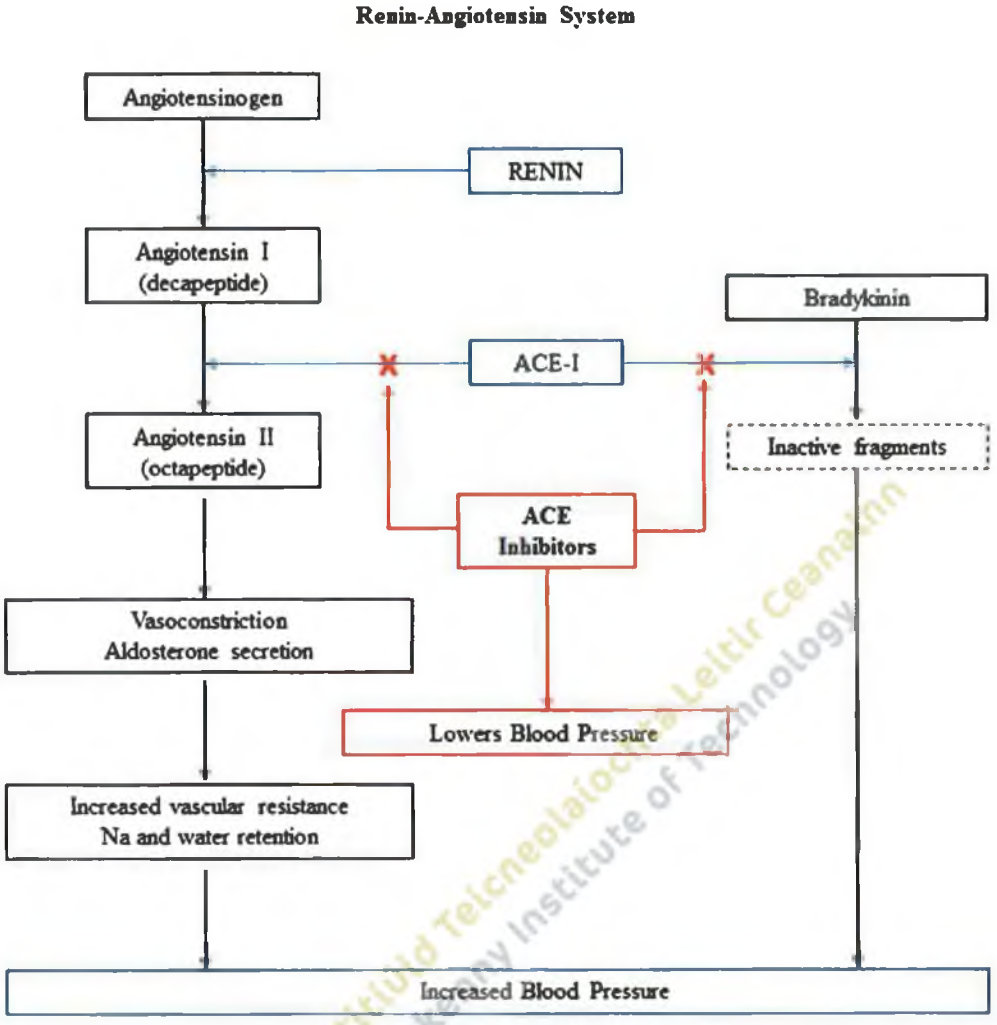


Figure 1. 3. A product of renin-cleaved angiotensinogen is the decapeptide Angiotensin I. When Angiotensin I is cleaved by Angiotensin-I-converting enzyme (ACE-I) which is present on the surface of vascular endothelial cells, Angiotensin II is formed. This is responsible for arteriolar vasoconstriction, which increases blood pressure. Bradykinin, a known vasodilator is also degraded by ACE and this also contributes to an increase in vasoconstriction. Control of ACE by ACE-I inhibitors therefore increases bradykinin and prevents Angiotensin II formation which lowers blood pressure.

PEP was first isolated as an oxytocin-inactivating enzyme from the human uterus and was found to hydrolyse the prolyl-leucyl bond in oxytocin (Walter *et al.*, 1971). *In vitro*, PEP plays an important role in the degradation of biologically active peptide hormones and neuropeptides that contain proline and PEP is known to cleave the peptides angiotensin I and II, neurotensin, vasopressin, bradykinin, thyroid releasing hormone and substance P (Burbach *et al.*, 1983, De Weid *et al.*, 1984, Rennex *et al.*, 1991, Welches, *et al.*, 1993, Yoshimoto *et al.*, 1983).

Previous studies suggested that prolyl endopeptidase could be related to neurodegeneration and disturbances in memory and cognition (Husain and Nemeroff, 1990, Rossor *et al.*, 1986). Recently, research has suggested that PEP functions in relation to mood disorders by acting as a regulator of inositol phosphate (InsP) signalling thereby modulating the effect of inositol depleting drugs such as Lithium in the treatment of bipolar disorder (BD) (Williams, 2004). Amyloid β plaques are commonly found in the brain of Alzheimer's disease patients. Roβner et al. (2005) studied the expression of PEP in adult and aged transgenic mice, which expressed amyloid β plaques. In the hippocampus of adult mice studied, PEP expression was increased in parallel with memory deficits but before appearance of the amyloid β plaques. Altered serum PEP activity has also been reported in many psychiatric disorders, and abnormal levels of PEP activity have been found to be significantly higher in Alzheimer's patient brains than in control patients (Aoyagi *et al.*, 1990, Ichai *et al.*, 1994, Maes *et al.*, 1995). In addition, lowered serum PEP activity was identified in bulimia nervosa and anorexia nervosa patients (Maes *et al.*, 2001). Furthermore, PEP activity was increased by antidepressants and increased serum PEP activity was found in patients with biopolar disorder (manic) and in schizophrenic patients and persons with stress induced anxiety such as Post- Traumatic Stress Disorder (PTSD) (Maes *et al.*, 1998, Maes *et al.*, 1999). Altered PEP activity has also been observed in autism spectrum disorders (Momeni *et al.*, 2005). PEP-like immunoreactivity has also been detected in the hippocampus of senescence-accelerated mice (Fukunari *et al.*, 1994).

Inhibitors of PEP (POP) Tc80 have potential as anti-protozoal drugs specifically against the agent of Chagas disease, *Trypanosoma cruzi* (Bastos *et al.*, 2005) which is prevalent in Latin America. Inhibitors of PEP may improve memory by blocking the metabolism of endogenous neuropeptides (Tezuka *et al.*, 1999). There are two known kinds of PEP inhibitors, peptidic inhibitors and non-peptidic inhibitors (Amor *et al.*, 2004). Peptidic inhibitors contain an electrophilic centre such as an alpha-keto beta-amide group and this group is considered to be an active centre. Non-peptidic inhibitors contain either a catechol or pyrogallol group which is believed to be responsible for PEP inhibition (Fan *et al.*, 1999). As PEP inhibitors have been identified in natural plant extracts and wine and green tea PEP inhibitors from natural sources could help in modulating PEP activity through dietary intake and the mental disorders associated with PEP.

1.4 Sources of ACE-I and PEP inhibitory peptides

ACE-I- inhibitory peptides have been isolated from various marine proteins such as Heshiko, a fermented mackerel product (Itou and Akahane, 2004), skipjack tuna muscle (Kohama *et al.*, 1988), sardine muscle (Bougatef *et al.*, 2008), shark meat (Wu *et al.*, 2008), Alaskan Pollack skin (Byun and Kim, 2001), marine shrimps (Wang *et al.*, 2008a), pacific hake (Cinq-Mars and Li-Chan, 2007) and salmon chum (Ono *et al.*, 2005) (Table 1.1). Theodore *et al.* (2007) generated a catfish protein hydrolysate containing ACE-I-inhibitory activity using the enzyme preparation Protamex®. This study found that protein hydrolysates of purified catfish protein had strong ACE-I-inhibitory activity. Suetsuna (2002) identified the ACE-I-inhibitory peptides Ile-Ala-Glu, Ile-Val-Glu, Phe-Glu, and Ala-Leu-Ala-Phe-Glu from pearl oyster and clam. Jae-Young *et al.* (2005) identified the ACE-I-inhibitory peptide Glu-Val-Met-Ala-Gly-Asn-Leu-Tyr-Pro-Gly from *Mytilus edulis*. A number of ACE-I inhibitory peptides have also been isolated from soy and chicken breast proteins (Ariyoshi, 1993, Ringseis *et al.*, 2005, Saiga *et al.*, 2006), garlic (Suetsuna, 1998) and wine (Pozo-Bayón *et al.*, 2007). Eggs have also been found to contain compounds with antihypertensive functions such as ovokinin, derived from an enzymatic digest of ovalbumin (Fujita *et al.*, 1995, Matoba *et al.*, 1999). Also hydrolysis of α-zein, which is a maize endosperm protein, by thermolysin yields 3 strong ACE inhibitors (Miyoshi *et al.*, 1995). Additionally potatoes and their by-products/wastes have also been found to contain high potency ACE-I inhibitors after controlled hydrolysis (Pihlanto *et al.*, 2008).

Synthesised chemical drugs such as Captopril®, Enalapril®, Alacepril® and Lisinopril® are extensively used medications in the treatment and prevention of hypertension. However, these drugs often cause side-effects in the patient, such as a persistent dry cough, angio-oedema, taste disturbances, increased potassium levels, reduced renal function and skin rashes (Atkinson and Robertson, 1979, Dicpinigaitis, 2006). Naturally sourced ACE-I-inhibitors raise the possibility that hypertension could be modulated through dietary intake.

Few PEP inhibitors are from natural plant sources and they are usually chemically synthesised structures. However, naturally occurring PEP inhibitors have been identified in red wine (Yanai *et al.*, 2003), plant extracts used in herbal or traditional medicines (Fan *et al.*, 2001), green tea (Kim *et al.*, 2001), and peptides isolated from bovine brain (Ohmori, *et al.*, 1994). The inhibitory activities of plant sourced PEP inhibitors are low and their binding modes are generally not known. A wide variety of PEP inhibitors have been described in

scientific literature and patents (Jarho, 2007). Most of the published inhibitors are chemically synthesised, substrate-like inhibitors that are based on the N-acyl-L-prolyl-pyrrolidine structure and their binding mode is known.

A study by Amor *et al.* (2004) isolated PEP inhibitory compounds from *Syzygium samarangense* (Blume) Merr & Perry (Myrtaceae) which is a plant native to the Philippines and known as "makopa" (Amor *et al.*, 2004). Furthermore, *Ginkgo biloba* leaves were examined for their anti-amnesic constituents/PEP inhibitors and results showed significant PEP inhibition. PEP activity-guided fractionation and column chromatography of the MeOH extracts of G. biloba leaves resulted in the isolation of 6-(10'Z-heptadecenyl) salicylic acid (Lee *et al.*, 2004). The Tibetan folk medicine *Rhodiola* Radix originates from several alpine *Rhodiola (R.)* plants and is used as a haemostatic, tonic and contusion. The anti-fatigue, anti-anoxia effects of this plant have also been reported. Fan *et al.* 2001 found that methanol extracts of *Rhodiola sachalinensis* also showed potent PEP inhibitory activity. The PEP inhibitory activities of Bangladeshi Indigenous Medicinal plants including *Emblica officinalis*, *Zingiber officinale* and *Myristica malabarica* were examined by Khanom *et al.* (2000). Tezuka, *et al.* (1999) examined the PEP inhibitory activities of a number of crude Chinese and Indian drug extracts. This study found that water-extracts of *Rhodiola sacra* (IC_{50}, 0.77 μg/ml) and the methanol-extracts of *Lycopodium clavatum* (IC_{50}, 1.3 μg/ml), *Paeonia lactiflora* var. *trichocarpa* (IC_{50}, 5.7 μg/ml), *Paeonia veitchii* (IC_{50}, 2.4 μg/ml) and *R. sacra* (IC_{50}, 0.67 μg/ml) showed strong PEP inhibitory activities. In addition, Tezuka *et al.* (1999) examined the PEP inhibitory activity of eleven compounds from *Salvia deserta*, and found that in addition to a catechol group alpha-hydroxy-para-quinone group may be related to the PEP inhibition. The PEP inhibitory activity of unsaturated fatty acids was also examined by Park *et al.* (2006). This study looked at the effects on PEP activity of mono- and poly-unsaturated fatty acids found in vegetable seeds and fatty fish.

A number of PEP inhibitors have been chemically synthesised as anti-amnesiac drugs and an example of one such inhibitor is S 17092 which can inhibit both chemically induced amnesia and spontaneous memory deficits in humans (Morain *et al.*, 2002). PEP inhibitory peptides have been found in a variety of food sources as outlined in Table 1.2. PEP inhibitory peptides associated with red wine produced from Cabernet Sauvignon grapes were characterised by Yanai *et al.* (2003). Two peptides were separated by reverse phase High Pressure Liquid Chromatography and subsequently purified using gel filtration techniques

and their amino acid structures determined as Val-Glu-Ile-Pro-Glu and Tyr-Pro-Ile-Pro-Phe (Yanai *et al.*, 2003). PEP inhibitory peptides have also been identified from sake and sake cake (Saito *et al.*, 1997), salmon, cod, trout and cheese hydrolysates (Sorensen *et al.*, 2004, Yoshikawa *et al.*, 2000) and also from synthetic peptide fragments of human β-casein (Asano *et al.*, 1991). PEP inhibitory compounds have also been found in sulphated chitooligosaccharides (Je *et al.*, 2007), in a methanolic extract of green tea (Kim *et al.*, 2001), and in the roots of the plant *Lindera strychnifolia* F. Vill (Kobayashi *et al.*, 2002).

Natural plant derived ACE-I-inhibitors and PEP inhibitors have lower bioactivities *in vivo* than synthetic PEP and ACE-I-inhibitory drugs but also display no harmful side-effects (Riordan, 2003) and are generally lower in cost (Vermeirssen *et al.*, 2004). As a result, the search for bioactive peptides from a variety of different food sources has become a major area of research with potential for the functional foods sector.

Products exploiting the potential of bioactive peptides are already on the market or under development by food companies. Examples of food products on the market containing ACE-I inhibitory peptides are Ameal S (Calpis Co., Ltd., Japan), a sour-milk product in tablet form which contains 2 potent ACE-I inhibitors, VPP [β-casein f(84-86)] and IPP [β-casein f(74-76)] (Nakamura *et al.*, 1995) and Peptide Soup (The Nippon Synthetic Chemical Industry Co., Ltd., Japan), which is a Katsuobushi oligopeptide obtained from thermolysin digested Katsuobushi (dried bonito) (Yokoyama *et al.*, 1992).

1.5 Derivatisation of ACE-I and PEP inhibitory peptides

The experimental strategy for the derivatisation of ACE-I and PEP inhibitory peptides is outlined in Figure 1.4. This approach employs the proteolysis of a protein source using bioassay-guided fractionation and separation of peptides followed by chemical elucidation and *in vivo* assays of active peptide fractions. Three key factors are important in relation to the release of peptides with bioactivities such as ACE-I and PEP inhibition. These three factors are (a) the nature of the matrices, (b) the choice of protease and (c) the extent of hydrolysis (Guerard *et al.*, 2010).

Guerard *et al.* (2010) extensively reviewed this topic and concluded that numerous substrates are potential candidates to be transformed into bioactive hydrolysates containing bioactive

Table 1.1. Examples of ACE inhibitory peptides derived from marine sources.

Origin	Hydrolysis	Peptide Sequence	IC_{50} (μM)	Reference
Sardine muscle	Alkaline protease	Lys-Trp	1.03	Matsufuji *et al.* (1994)
Dried Skipjack tuna muscle	Thermolysin	Leu-Lys-Pro-Met-Asn	2.4	Fujita & Yoshikawa (1999)
Skipjack Tuna muscle	Acid extract	Pro-Thr- His-Ile-Lys-Trp-Gly-Asp	2.0	Kohama *et al.* (1988)
Salmon chum muscle	Thermolysin	Phe-Leu Leu-Phe	13.6 383.2	Ono *et al.* (2005)
Shark meat	Protease SM98011	Cys-Phe Glu-Try Phe-Glu	1.96 2.68 1.45	Wu *et al.* (2008)
Shrimp *Acetes chinensis*	*L. fermentum* SM 605	Asp-Pro Gly-Thr-Gly Ser-Thr	2.15 5.54 4.03	Wang *et al.* (2008b)
Oyster protein	Pepsin	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	66.00	Wang *et al.* (2008a)
Alaska Pollack skin	Alcalase, pronase E & collagenase	Gly-Pro-Met Gly-Pro-Leu	17.13 2.60	Byun and Kim (2001)
Anchovy fermented fish sauce	Unknown	Lys-Pro	22.00	Ichimura *et al.* (2003)
Algae protein waste	Pepsin	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	29.60	Sheih *et al.* (2009)
Wakame (*Undaria pinnatifida*)	Protease S "Amano"	Ile-Tyr Val-Trp Ile-Trp	6.10 3.30 1.50	Sato *et al.* (2002)

Table 1.2. Examples of PEP inhibitory peptides derived from various sources.

Origin & hydrolysis	Peptide Sequence	IC_{50} (μM)	Reference
Cod, salmon and trout hydrolysed with porcine trypsin	N/A[a]	N/A	Sorensen *et al.* (2004)
Cabernet Sauvignon red wine	Val-Glu-Ile-Pro-Glu	17.00	Yanni *et al.* (2003)
	Try-Pro-Ile-Pro-Phe	87.80	
γ-zein hydrolysed by subtilisin (Carlsberg)	His-Leu-Pro-Pro-Pro-Val	80.00	Maruyama *et al.* (1992)
Synthetic peptide fragments of human β-casein (region 49-59)	Ile-Tyr-Pro-Phe-Val-Glu-Pro-Ile	8.00	Asano *et al.* (1991)
Sake cake hydrolysate by pepsin	Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala	42.80	Saito *et al.* (1997)
	Leu-Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala	29.00	
	Leu-Leu-Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala	24.30	
Sake hydrolysate by pepsin	pGlu-Leu-Phe-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Pro	24.30	Saito *et al.* (1997)
	pGlu-Leu-Phe-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Pro-Arg	14.10	
	pGlu-Leu-Phe-Gly-Pro-Asn-Val-Asn-Pro-Trp-His-Asn-Pro-Arg	11.80	
Bovine brain	Met-Pro-Pro-Pro-Leu-Pro-Ala-Arg-Val-Asp-Phe-Ser-Leu-Ala-Gly-Ala-Leu-Asn	38.40	Ohmori *et al.* (1994)

[a] Not available

peptides as long as they are available in sufficient quantities, are of good quality in terms of freshness and weak microbial contamination (Guérard *et al.*, 2008). They also characterised the most popular proteases for use in by product solubilisation and found that Alcalase®, Flavourzyme®, Neutrase®, and 258 Protamex TM are the most commonly used enzymes for by-product solubilisation (Guérard *et al.*, 2008).

The isolation of bioactive peptides typically involves the hydrolysis of the protein of choice with different proteolytic enzymes alone or in combination with GRAS micro-organisms. Hydrolysates can then be pre-screened for bioactivity using standard *in vitro* inhibition studies. The most commonly used method for the detection of ACE I-enzyme inhibitory activity is based on the Cushman and Cheung protocol (Cushman & Cheung, 1971), whereas for PEP inhibitor identification the methods employed are generally based on a protocol developed by Yoshimoto *et al.* (1979). Positive hydrolysates can then be sub-fractionated using a range of fractionation and chromatographic techniques to isolate, purify and concentrate the bioactive peptide fraction with activity determined by *in vitro* assays. The peptide structure can then be elucidated using Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) elucidation methods. *In vivo* assays are then used to determine the bioavailability of the isolated peptide fraction in an animal and subsequently a human subject.

Proteases of microbial origin can potentially release bioactive peptides (Gobbetti *et al.*, 2002). Given the highly proteolytic nature of LAB such as *Lactococcus lactis* (Matar *et al.*, 1996) and *Lactobacillus helveticus* it is not surprising that their use as microbial catalysts for the generation of bioactive peptides has been investigated (Rokka, 1997). Previously ACE-I inhibitory peptides were released from whey and casein following fermentation with different strains of lactic acid bacteria (LAB) followed by hydrolysis with digestive enzymes (Mizuno *et al.*, 2004). Screening for proteolytic activity from a bacterial culture collection is performed using suitable agar plates supplemented with skim milk (Pailin *et al.*, 2001, Van der Berg *et al.*, 1993). Proteolytic strains are recognised by zones of clearing using this method. Other methods used to identify GRAS proteolytic strains include HPLC breakdown analysis of milk proteins such as whey and casein compared to standards such as bovine serum albumin (BSA) (Minervini *et al.*, 2003).

ACE-I and PEP inhibitory activity of bioactive peptides is first determined using *in vitro* inhibition studies (Cushman & Cheung, 1971, Yoshimoto *et al.,* 1979) in order to obtain lead candidates for inhibitory activity. ACE-I and PEP inhibitory activity is determined by reference to positive inhibitors such as Captopril®, a synthetic ACE-I inhibitor, or Val-Glu-Ile-Pro-Glu, a known PEP inhibitor. The Cushman and Cheung assay protocol for ACE-I inhibition measurement is based on the hydrolysis of hippuryl-histidyl-leucine (HHL) by ACE-I to give hippuric acid (HA) and histidyl-leucine as products and subsequent quantification of HA release. The HA is extracted into ethyl acetate and quantified by measuring the absorbance in a spectrophotometer at 228 nm (Roy *et al.*, 2000). This method requires several steps and the HA can be contaminated with ethyl acetate which also absorbs strongly at 228 nm unless fully evaporated using nitrogen evaporation.

Alternative rapid and sensitive High Pressure Liquid Chromatography (HPLC) methods for direct analysis of the ACE-I reaction mixture through complete separation of HHL and HA have been developed (Wu *et al.*, 2002). Furthermore, various high throughput and rapid screening capillary electrophoresis (CE) methods to determine the ACE-I-inhibitory values for bioactive peptides have also been developed (He *et al.*, 2007).

The CE and HPLC methods were compared and the CE method was found to be faster, more automated and needed fewer samples, substrates and other accompanying reagents which suggested that the CE method was more suitable for high throughput screening of protein hydrolysates with ACE-I-inhibitory activity. The HPLC method is thought to have better reproducibility than the CE technique. A high-throughput method was recently developed for screening ACE-I inhibitors, where the free HA is mixed with pyridine and benzene sulfonyl chloride. This mixture produces a yellow colour with a λ_{max} at 410 nm, which is directly proportional to the released HA. This investigation found that there was good correlation between the IC_{50} values of Captopril® obtained by the newly developed colorimetric method and the commonly used HPLC technique (Jimshena & Gowda, 2009).

Bioactive peptides are typically concentrated by molecular weight cutoff (MWCO) and/or ultra-filtration through 3 and 10kDa membrane filters as ACE-I-inhibitory peptides are usually between 2-30 amino acids in size (Erdmann *et al.*, 2008) and most PEP- inhibitory peptides discovered to date are usually between 4- 18 amino acids in size. Synthesis and application of the first fluorogenic substrate, *N*- carbobenzoxyglycylprolyl-4-

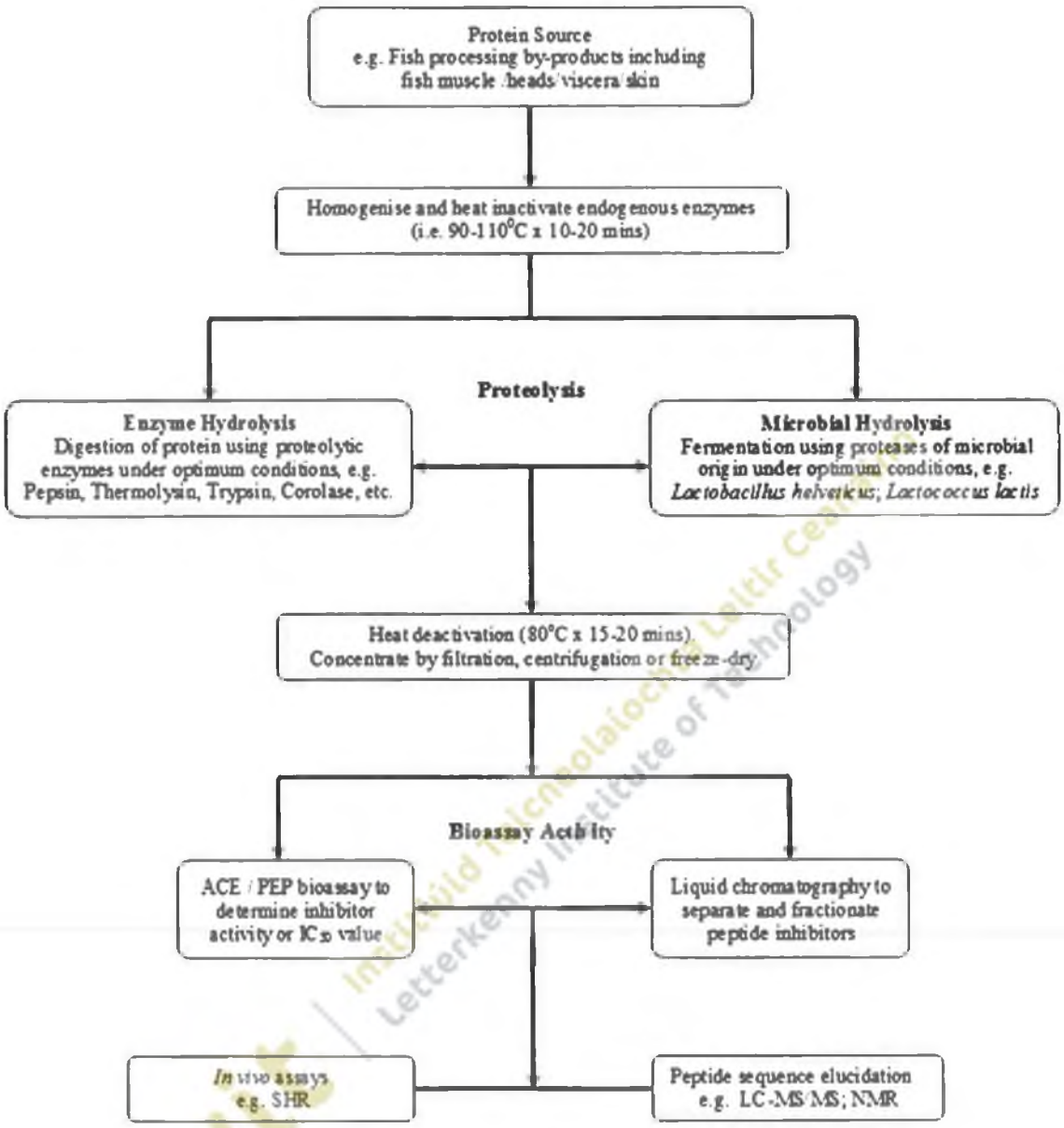


Figure 1.4. Experimental strategy for the derivatisation of ACE-I and PEP inhibitory peptides.

methylcoumarinyl amide (Z-Gly-Pro-MeCouNH) for the determination of PEP was first reported by Yoshimoto *et al.* (1979). *In-vivo* testing demonstrated that high PEP activity levels were observed in the testis, liver and skeletal muscle of rats. Activity in human body fluids was also tested for levels of PEP activity and semen was found to possess the highest cleaving activity (Yoshimoto *et al.*, 1979).

In- vitro PEP inhibitory activity may be determined by the assay method described by Yanai *et al.* (2003). This is a modified version of the PEP assay described by Yoshimoto *et al.* (1979). A phosphate buffer (pH 7.0), PEP from *Flavobacterium meningosepticum* and the test sample in methanol are mixed and incubated at 30°C for 5 min, and the reaction started by the addition of 2 mM *Z*-Gly-Pro-*p*NA (in 40% 1,4-dioxane). After incubation at 30°C for 10 minutes, a stop solution (1 M HCl) is added and the absorbance of the solution is measured at 410 nm. Suitable positive and negative controls are used.

1.5.1 Principles of capillary electrophoresis (CE)

Capillary electrophoresis techniques are routinely employed to separate molecules with high resolution and the method has been extended to a large diversity of chemical compounds as the separation is based on the number of theoretical plates, which may be in excess of 1,000,000 (Lauer and Rozing, 2010). Other advantages of CE are its low consumption of samples and reagents, use of aqueous solutions, little sample pre-treatment required, compatibility with auto-samplers, easy cleaning and conditioning of capillaries. There are a number of different modes of electrophoretic separation that may be coupled to diverse detection methods in the same equipment. These methods include UV detection, laser-induced fluorescence (LIF) excitation, electrochemical detection and mass spectrometry.

Capillary electrophoresis is a technique in which molecules are separated in narrow capillaries under an applied electric field (Lauer and Rozing, 2010). The electric field rather than gas or solvent flow moves the molecules through the capillary. Molecules in solution will then be separated based on their electrophoretic mobility. The electrophoretic mobility of an object in an applied electric field is determined by the charge on the molecule, the frictional coefficient of the molecule, which depends on size and shape, and the viscosity of the solvent:

$$\mu_e = \frac{q}{(6\pi\eta r)}$$

where μ_e = the velocity of the particle in an applied field, q = ion charge, η = solution viscosity, r = ion radius.

The velocity of the particle in an applied field is μ_e times E, where E is the applied field. Hence small highly charged species have high mobilities and large minimally charged

species have low mobilities. The simplest CE method is capillary zone electrophoresis (CZE), a method by which molecules ions or particles are separated solely by their electrophoretic mobility. The simplification that holds true for this technique is that the velocity is proportional to the charge to mass ratio. The capillaries are usually made of silica. In uncoated capillaries at pH greater than 4 the SiOH groups are ionized to SiO^-. This leads to a phenomenon called electroosmotic flow (EOF). An overview of a CE system illustrating a cross-section of the capillary is shown in Figure 1.5.

A fundamental constituent of CE operation is electroosmotic, or electroendosmotic flow (EOF). EOF is the bulk flow of liquid in the capillary and is a consequence of the surface charge on the interior capillary wall. The EOF results from the effect of the applied electric field on the solution double-layer at the wall (Figure 1.6). The EOF controls the amount of time solutes remain in the capillary by superposition of flow on to solute mobility. This can have the effect of altering the required capillary length, but does not affect selectivity. Under aqueous conditions most solid surfaces possess an excess of negative charges. This can result from ionization of the surface (that is, acid-base equilibria) and/or from adsorption of ionic species at the surface. For fused silica both processes occur, although the EOF is most strongly controlled by the numerous silanol groups (SiOH) that can exist in anionic form (SiO^-) (Figure 1.6). Counterions (cations, in most cases), which build up near the surface to maintain charge balance, form the double layer and create a potential difference very close to the wall. This is known as the zeta potential. When the voltage is applied across the capillary the cations forming the diffuse double-layer are attracted toward the cathode. Because they are solvated their movement drags the bulk solution in the capillary toward the cathode. The zeta potential is essentially determined by the surface charge on the capillary wall. Since this charge is strongly pH dependent, the magnitude of the EOF varies with pH. At high pH, where the silanol groups are predominantly deprotonated, the EOF is significantly greater than at low pH where they become protonated. Depending on the specific conditions, the EOF can vary by more than an order of magnitude between pH 2 and 12 (Lauer and Rozing, 2010).

A unique feature of EOF in the capillary is the flat profile of the flow. Since the driving force of the flow is uniformly distributed along the capillary (that is, at the walls) there is no pressure drop within the capillary, and the flow is nearly uniform throughout. The flat flow

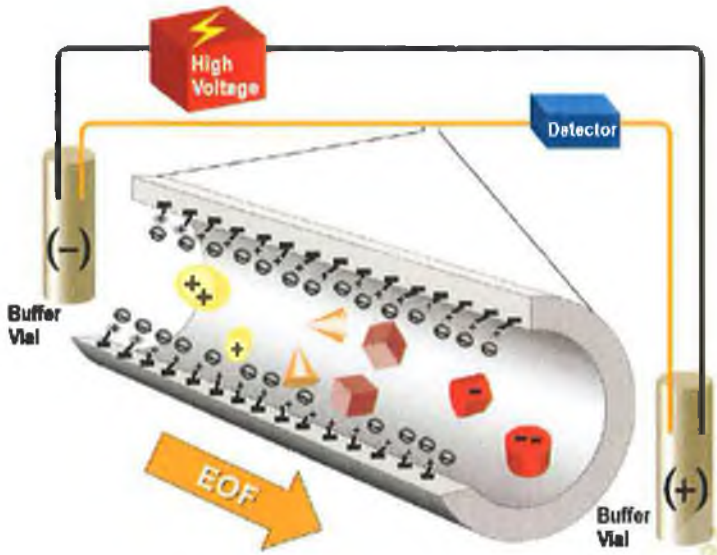


Figure 1. 5. An overview of a CE system with capillary cross-section. (Source: http://www.beckmancoulter.co.jp/product/product01/cap_zone.html)

profile is beneficial since it does not directly contribute to the dispersion of solute zones. This is in contrast to that generated by an external pump which yields a laminar or parabolic flow due to the shear force at the wall. Another benefit of the EOF is that it causes movement of nearly all species, regardless of charge, in the same direction. Under normal conditions (negatively charged capillary surface), the flow is from the anode to the cathode. Anions will be flushed towards the cathode since the magnitude of the flow can be more than an order of magnitude greater than their electrophoretic mobilities. Thus cations, neutrals, and anions can be electrophoresed in a single run since they all "migrate" in the same direction. This process is depicted in Figure 1.5. Here, cations migrate fastest since the electrophoretic attraction towards the cathode and the EOF are in the same direction, neutrals are all carried at the velocity of the EOF but are not separated from each other, and anions migrate slowest since they are attracted to the anode but are still carried by the EOF toward the cathode (Lauer and Rozing, 2010).

In CZE, the whole system is filled with a background electrolyte (BGE), whose main purposes are to transport the electrical current when a voltage is applied over the system, to provide the electrical field strength and to facilitate the separation of the analytes. Another important purpose of the BGE is pH regulation in order to keep the migration velocity of the analytes and the velocity of EOF constant. In this way, a stable and reproducible migration behavior of the sample components can be obtained (Lauer and Rozing, 2010).

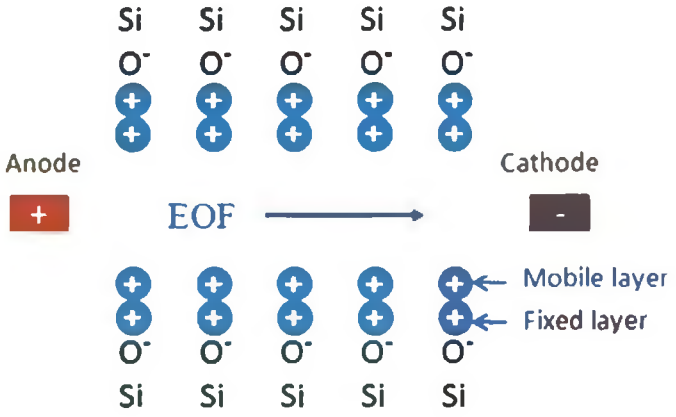


Figure 1. 6. Ionised silica capillary walls illustrating EOF direction.

The concentration of the BGE plays an important role in separation resolution in electrophoresis. If a constant voltage is applied over the capillary, the electric current will increase at higher BGE concentrations and this can cause extra peak broadening due to a strong increase in Joule heat. Provided that the generated Joule heat is not too high to affect peak efficiency, a higher buffer concentration can produce better peak shapes at high analytic concentrations leading to improved peak reproducibility. Ideal separation in CZE is usually obtained by using a voltage as high as possible in order to obtain the best separation in the shortest time with the best efficiency. However, high voltage leads to difficult heat dissipation during the electrophoretic separation due to Joule heat, which is known to affect the EOF, diffusion of analytes, and therefore the efficiency and reproducibility of the separation, for this reason it is important to control this phenomenon (Xuan *et al.*, 2006).

In CE, proteins can adsorb to the capillary wall causing alterations of the EOF and band broadening. With the use of non-coated fused-silica capillaries this can lead to a very poor migration time and low plate number. In order to minimise such effects, the separation of protein mixtures is run at extreme pH values, by which either the dissociation of the silanol groups of the capillary wall is minimised (at acid pH) or the dissociation of free amino groups of the proteinous solutes is decreased (at high pH values as in the method used, pH 9.18) (Lauer and Rozing, 2010).

1.5.2 Principles of high performance liquid chromatography (HPLC)

The goal and purpose of liquid chromatography is to physically resolve the individual components of a sample from each other so that they may be quantified or identified. A high

performance liquid chromatography instrument has a number of components. These include a solvent reservoir, a pump, a sample injection system, a column and a detection system. The mobile phase (solvent) is a liquid used to carry the sample through the column (where separation occurs) and on to the detector (where data is generated). Compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC has been applied to food, nutraceuticals, cosmetics, pharmaceuticals, environmental matrices, forensic samples and industrial chemicals.

The choice of the mobile phase is dependent on the type of chromatography to be used and because of this the mobile phase can be used as a tool in the optimisation process. A high pressure pump is used to push the mobile phase (and sample) through the system. The pump is capable of pushing a fixed volume of mobile phase in a fixed time. When the sample is introduced into the system, it is carried by the mobile phase onto the column. It is here that separation occurs. Columns are typically steel tubes with a variety of length, diameters and packing materials. Again, the choice of stationary phase is based on the type of compounds to be separated. Detection systems are located after the column. When the separated compounds leave the column, they pass through the detector where individual signals are recorded for each compound. Analytes may be detected by various means such as refractive index, electrochemical or ultraviolet-absorbance. The amount of analyte leaving the column will determine the intensity of the signal produced in the detector. In reversed phase (RP) chromatography, hydrophobic interactions are the primary retention mechanism. The analyte is attracted to the hydrophobic surface of the particle in the stationary phase. Polar interactions and primary hydrogen bonding are an important secondary retention mechanism. The level of hydrophobic and polar interaction forces between solute and stationary phase will determine the overall solute retention time (Kellner *et al.*, 1998).

Preparative HPLC is used for the isolation and purification of a product with the sample being collected by a fraction collector after identification at required absorption maximum wavelength. The amount of compound required to isolate or purify differs dramatically as μg quantities are required for isolation of enzymes while quantities from one to a few mg are required for identification and structure elucidation of unknown compounds. Larger amounts, in gram quantity, are necessary for standards, reference compounds and compounds for toxicological and pharmacological testing (Huber and Majors, 2007).

1.5.3 Principles of mass spectrometry (MS)

Mass spectrometry is based on the generation of gaseous ions from analyte molecules, the subsequent separation of these ions according to their mass-to-charge (*m/z*) ratio, and the detection of these ions. The resulting mass spectrum is a plot of the relative abundance of the ions produced as a function of the *m/z* ratio. MS actually measures the relative molecular mass M_r of a compound in atomic units or Daltons (Da) (Kellner *et al.*, 1998).

MS instruments consist of five parts; sample introduction, analyte ionisation, mass analysis, ion detection, and data handling. MS is a combined separation and detection technique and is widely applied especially in four major fields; analysis of organic compounds, analysis of inorganic compounds, analysis of surfaces and organic mass spectrometry. MS is the most sensitive of the spectrometric techniques for molecular analysis compared to other techniques such as Nuclear Magnetic Resonance (NMR) and infrared spectrometry (Kellner *et al.*, 1998). In Matrix-assisted laser desorption/ionisation (MALDI) analysis the sample is mixed with an appropriate matrix-solution, e.g., containing sinapinic acid and deposited onto a stainless- steel target. Upon drying, crystallisation occurs and the crystals are bombarded with photons, the frequency of which corresponds to the absorption maximum of the matrix molecules, sample ions are generated which can be mass analysed by a time-of-flight (TOF) mass spectrometer. This technique is especially useful for the analysis of bio-macromolecules as compounds with molecular masses up to 300kDa can be detected. A TOF instrument consists of a pulsed ion source, an accelerating grid, a field-free flight tube and a detector (Kellner *et al.*, 1998).

1.6 Structural properties involved in ACE-I and PEP inhibitory activities of peptides

It has been demonstrated that di- or tri-peptides, especially those with C-terminal proline or hydroxyproline residues, are generally resistant to degradation by digestive enzymes (Matsufuji *et al.*, 1994, Vermeirssen *et al.*, 2004). In addition, short peptides consisting of two or three amino acids are absorbed more rapidly than free amino acids (Gardner, 1988; Webb, 1990). The ACE-I inhibitory tripeptides IPP and VPP, for example, were detected in the aorta of Spontaneously Hypertensive Rats (SHR), following oral administration of fermented milk (Masuda *et al.*, 1996). Larger peptides (10–51 amino acids) present in the diet can also be absorbed intact through the intestine and produce biological effects, although the potency of the peptides decreases as the chain length increases (Roberts *et al.*, 1999).

However as binding to ACE appears to be strongly influenced by the C-terminal sequence of the peptides, it has been suggested that proline, lysine or arginine are the preferred amino acids at the C-terminal residue and thus contribute to ACE-I-inhibitory potency (Meisel, 1997). Furthermore, studies in SHR revealed that di-peptides with a C-terminal tyrosine residue produced a slow but prolonged decrease in systolic blood pressure compared to di-peptides with phenylalanine at the C-terminal. In contrast, di-peptides with a C-terminal phenylalanine caused a more rapid reduction in systolic blood pressure and a shorter duration of action (Suetsuna, 1998). Figure 1.7. details information concerning the structural conformation of peptides that may have ACE-I-inhibitory activities.

It has been established that ACE-I inhibitors exhibit antihypertensive activity *in vivo* following administration to Spontaneously Hypertensive Rats (SHR) (Itou and Akahane, 2004) and hypertensive human patients (Mizuno *et al.*, 2005, Takano, 1998). Some peptides are susceptible to degradation or modification in the gut, the vascular system and the liver. Several ACE-I-inhibitory peptides with weak *in vitro* activity produce a strong antihypertensive effect *in vivo* while others lose their activity. An example of this is the peptide Leu-Lys-Pro-Asn-Met, derived from a thermolysin digest of dried bonito, with an *in vitro* activity of (IC_{50} = 2.4 μmol/L), but after hydrolysis *in vivo*, its inhibitory activity was 8 times higher at (IC_{50} = 0.32 μmol/L) (Fujita & Yoshikawa, 1999). *In vivo* activation or loss of activity is perhaps due to further endogenous enzymatic cleavage (Meisel *et al.*, 2006). Interestingly, *in vivo* comparative studies with Captopril® have shown that ACE-I- inhibitory peptides exhibit higher *in vivo* activity than would be expected from their *in vitro* activity. Fujita and Yoshikawa (1999) found that bioactive peptides have higher tissue affinities and are subject to a slower elimination from the body than Captopril®.

The ability of prolyl residues to protect peptides has led to the evolution of proline specific peptidases such as PEP. Proline has a cyclic structure, which prevents free rotation around the φ-bond and, in a peptide sequence Proline does not have a main chain NH proton that could form intramolecular hydrogen bonds. Therefore, Proline is unique amongst the 20 natural α-amino acids. Furthermore it does not participate in hydrogen bonding and can prevent hydrogen bonding of neighbouring residues. Prolyl residues protect biologically active peptides from non-specific degradation. The majority of published PEP inhibitors are substrate-like inhibitors that are based on the *N*-acyl-*L*-prolyl-pyrrolidine structure and their binding mode to PEP is known. Most PEP inhibitors have a pyrrolidine or a substituted

pyrrolidine ring at the P1 site and if the ring size of pyrrolidine is decreased or increased by one methylene group the inhibitory activity decreases. In addition, pyrrolidine can be replaced by other five membered rings including isoxazolidine, thiazolidine, pyrrole, 2,3-dihydropyrrole and 2,5-dihydropyrrole but ring opening abolishes PEP inhibitory activity. The inhibitory activities of atypical plant derived PEP inhibitors are unknown to date.

1.7 Bioactive peptides as functional foods

Functional Foods are regulated by Regulation (EC) No. 1924/2006, of the European Parliament and of the Council, December 20, 2006: nutrition and health claims made on foods. This legislation regulates nutritional and or health claims proposed for new products, including their presentation, labelling and promotion. Japan was the first country to adopt a legal system in relation to allowable health claims on functional foods through the introduction of the FOSHU (Foods for Specific Health Use) licensing system in 1991. Advertising approval is granted by the Japanese Ministry of Health to companies' who can prove that a health claim connected to a product has been scientifically evidenced. Between 2005- 2007, 537 FOSHU products with an estimated value of US$ 6.3 billion have been approved (Hartmann & Meisel, 2007).

During the production of functional foods containing bioactive peptides, methods must be developed to enhance their bioavailability. Functional or novel foods are created by fortifying or adding enriched fractions of the bioactive peptide to the product. Bioactive peptides may be produced from precursor protein substrates by a number of methods such as using specific Generally Recognised as Safe (GRAS) proteolytic bacterial enzymes (Hayes *et al.*, 2007; Wang *et al.*, 2008b), or enzymatic hydrolysis with digestive enzymes such as trypsin and pepsin, (Byun & Kim, 2001; Miguel *et al.*, 2009). Other methods include endogenous microbial activity of fermented foods such as cheese and yogurt (Je *et al.*, 2005). Also genetically modified proteins, such as the soybean proglycin A1aB1b, can be designed to carry multiple copies of bioactive sequences (Prak *et al.*, 2006).

Bioactive peptides may be latent (or encrypted) within the primary or parent proteins and in order to exert a physiological response on the various systems in the body proteolysis is required for their release and activation (Gobbetti *et al.*, 2004). Some of these peptides may

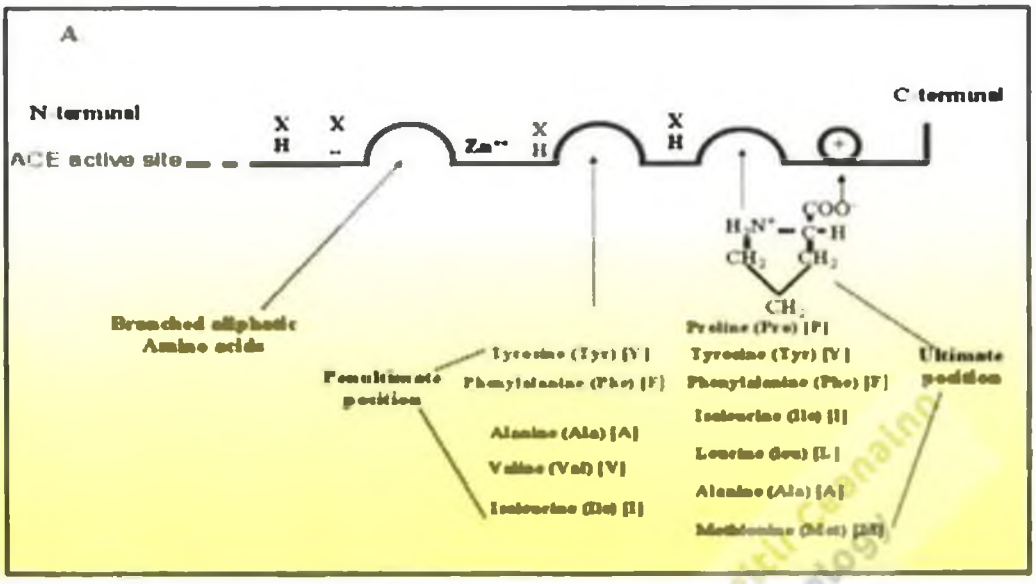
also act as bio-carriers by sequestering calcium and other minerals and thereby may enhance their bioavailability (Silva & Malcata, 2005). Bioactive peptides usually contain between two to thirty amino acid residues per molecule (Erdmann *et al.*, 2008).

Digestion of proteins starts in the stomach by the action of pepsin at acidic pH 2-3. In the more alkaline conditions of the small intestine, the polypeptides are further cleaved by the pancreatic proteases trypsin, a-chymotrypsin, elastase and carboxypeptidase A and B. This then results in a mixture of oligopeptides and free amino acids, of which the free amino acids are absorbed into the enterocytes across the brush border membrane via distinct amino acid transport systems. The oligopeptides undergo further hydrolysis by the action of a number of brush border peptidases, resulting in a mixture largely consisting of free amino acids and di- and tri-peptides (Vermeissen *et al.*, 2004). Following digestion, bioactive peptides can either be absorbed through the intestine to enter the blood circulation intact and exert systemic effects, or produce local effects in the gastrointestinal tract. Depending on the sequence of amino acids, these peptides can exhibit diverse activities, including opioid agonist and antagonist activities, mineral-binding, immunodulatory, antioxidative, antiamnesiac, antithrombotic and antihypertensive activities (Hartmann & Meisel, 2007; Kitts & Weiler, 2003). Some peptides are multifunctional and can exert more than one of the effects mentioned (Meisel, 2004). The IC_{50} value (the inhibitor concentration leading to 50% inhibition) is used to estimate the effectiveness of different inhibitory peptides.

1.7.1 Survival of bioactive peptide inhibitors *in vivo*

Due to the unknown and often incomplete bioavailability of ACE-I or PEP inhibitory peptides following oral administration, there is not necessarily an *in vivo* effect after the identification of an ACE-I/PEP-inhibitory peptide *in vitro* so it is necessary to perform *in-vivo* animal studies using animal models. In order to determine ACE-I-inhibition *in vivo* Spontaneously Hypertensive Rats (SHRs) are often used. *In vivo* studies are subsequently followed by human dietary intervention studies following the identification of positive hits with *in vivo* animal trials. An *in vitro* study using *Dictyostelium discoideum* or cellular slime mould is used to study the involvement of PEP in mental health disorders such as biopolar disorder. *D. discoideum* has the advantage of a haploid genome, which enables genes to be knocked out easily. Using this organism PEP was found to function as a modulator of inositol phosphate signalling a system proposed to be the target of drugs used to treat bipolar disorder (Williams, 2004).

A: Binding to ACE is strongly influenced by the hydrophobicity of the 3 C-terminal amino acid residues with aromatic or branched side chain residues being preferred.



B: Observed trends in the hydrophobicity for the *L*-amino acids are shown.

Figure 1.7. Structure activity correlation between C-terminal tri-peptide sequences of ACE-I-inhibitory peptides and the ACE enzyme. **A:** Binding to ACE is strongly influenced by the hydrophobicity of the 3 C-terminal amino acid residues. Aromatic or branched side chain residues are preferred. Aliphatic, basic and aromatic residues are preferred in the penultimate positions and aromatic, proline and aliphatic residues are preferred in the ultimate position. The positive charge of Arginine (R) also contributes to the ACE-I-inhibitory potency of several peptides. A C-terminal lysine (K) with a positive charge on the ε-amino group also contributes to ACE-I-inhibition. **B:** Trends in hydrophobicity of the *L*-amino acids. Phenylalanine (F) is the most hydrophobic of the *L*-amino acids and is preferred as one of the C-terminal amino acid residues. The branched aliphatic amino acid residues are preferred at the N-terminal end of the ACE-I-inhibitory peptide with the exception of Arginine (R). Underlined sequences have previously been identified as ACE-I-inhibitors. (Source: Hayes *et al.*, 2007)

In order to produce antihypertensive or anti-amnesiac effects *in vivo*, the peptides must be absorbed intact through the intestine and reach the target organ/ system in an active form. In this regard, specific structural properties play an important role.

1.8 Aims and objectives

Studies on PEP and ACE-I inhibitory peptides derived from fish waste by-products such as waste muscle, fins, head and scales are limited with the notable exception of enzyme isolation from fish viscera. This project will address waste remediation by examining the potential for the production of high-value products other than enzymes from the waste stream, rather than regarding the waste as a disposal problem or for the manufacture of low economic value fertilizers and animal feedstuffs. This project will establish if fish waste from mackerel and whelk species is an optimal source for the generation of ACE-I and PEP inhibitory peptides through controlled hydrolysis. Moreover, it will establish what subsequent downstream processing methods are necessary for the production of a high-value peptide product with enhanced health benefits and increased economic value.

This project will examine the steps involved in the preparation of fish waste hydrolysates using proteolytic enzymes, the development and implementation of bioassays to monitor ACE-I and PEP inhibitory activities and the concentration of the active peptide fractions using membrane filtration techniques.

Specific objectives of this thesis are:

- Can commercially available proteolytic enzymes generate ACE-I and PEP inhibitory peptides through the hydrolysis of fish waste from mackerel and whelk species?
- Which hydrolytic enzymes are more suitable in the generation of the ACE-I and PEP inhibitory peptides?
- What specific fish waste source (waste muscle, head, skin and tail) is likely to generate these bioactive peptides?
- Development and implementation of suitable bioassays to pre-screen marine hydrolysate samples for ACE-I and PEP inhibitory activity
- Develop and validate a CE-ACE for the analysis of ACE-I inhibitiory peptides from marine hydrolysates.

- Ascertain if active ACE-I inhibitory hydrolysates can be fractionated using MWCO filtration
- Foster a collaborative industrial link with the Errigal Food Company Ltd., Carrick, Co. Donegal.

2. Materials and Methods

2.1 General materials and methods

2.1.1 Chemicals and reagents

Angiotensin-converting enzyme (ACE-I) assay reagents of purified ACE-I enzyme (EC 3.4.15.1), crude ACE-I enzyme extract from acetone dehydrated rabbit lung, Captopril®, hippuric acid (HA), hippuryl-L-histidyl- L-leucine (HHL), 2,4,6 trinitrobenzene sulfonic acid (TNBS), trifluoroacetic acid (TFA), HEPES and sodium tungstate were purchased from Sigma Aldrich Ireland Ltd., Ireland. The substrate hippuryl-glycyl-glycine (Hip-Gly-Gly) was supplied by Bachem Holding AG, Bubendorf, Switzerland. Ammonium hydroxide (NH_4OH), hydrochloric acid (HCl 37%), sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Lennox Laboratories (Dublin, Ireland). Potassium di-hydrogen phosphate (KH_2PO_4) was purchased from BDH Laboratory Supplies (Poole, England). De-ionised water was used throughout and was obtained using a Millipore Direct-Q system (ASTM Type 1 18.2 MΩ). All other reagents were of analytical grade.

Purified ACE-I was supplied as a 0.5 mg lyophilised powder containing 5.5 U of enzyme/ mg and was reconstituted in 1.375 mL sterile deionised H_2O to give final enzyme concentration of 2 mU/µL. Aliquots (25µl) of purified ACE were stored at -20°C for up to 3 months and thawed on ice prior to use. Stock solutions of purified ACE-I enzyme were further diluted tenfold in sterile de-ionised water (225 µL) to yield a final working concentration of 5 mU/25 µL per enzyme reaction. Stock and working solutions of crude ACE-I extract from rabbit lung dehydrated by acetone were prepared as previously described (Serra *et al.* 2005). The stock solution was prepared by dissolving 2 g of the powder in 10 mL of phosphate buffer (50 mM, pH 8.3). The solution was soaked overnight under refrigeration (2-6°C) then centrifuged for 60 minutes at 13,500 rpm and 4°C. The supernatant was collected and the stock solution could be stored for up to 3 months under refrigeration (2-6°C). The working solution (1 g/10 mL) was prepared freshly before each assay by diluting the stock solution in phosphate buffer (pH 8.3).

Two concentrations of Captopril® were prepared. For the colorimetric ACE-I assay: a Captopril® stock solution of 1.0 mg/ mL was prepared by dissolving 0.025g in 25 mL of de-ionised water. This stock was then diluted to a test concentration of 0.008 mg/ mL using de-ionised water as the diluent. For the CE- ACE assay: a Captopril® stock solution (0.1 mg/10 mL) was prepared by dissolving 0.001g in 10 mL of 100 mM sodium borate buffer at (pH

8.3). This stock was then diluted to the test concentration of 0.002 mg/ mL using de-ionised water as the diluent. The hippuryl-L-histidyl-L-leucine (HHL) substrate solution (1 mM) was prepared fresh for each purified ACE-I enzyme assay by dissolving 0.0025 g in 5.0 mL of 100 mM sodium borate buffer (pH 8.3). The solution was kept on ice until required. The substrate solution of Hip-Gly-Gly (100 mM) was prepared by dissolving 100 mg in 2 mL of 1 mM ammonium hydroxide solution. After complete dissolution, the volume was increased to 3.4 mL with de-ionised water and stored on ice until required.

Ammonium hydroxide solution (1 mM) was prepared by adding 5.5 mL ammonium hydroxide to 94.5 mL de-ionised water. Sodium tungstate solution was freshly prepared in a final concentration of 1 g/10 mL in de-ionised water. A TNBS solution (6 mM) was prepared by dissolving 175 µL of TNBS (5%) in 5 mL de-ionised water. Aliquots of 300 µL were stored at -20^{0}C and used within 3 months. TFA solution (0.1%) was prepared by dissolving 100 µL in 10 mL de-ionised water in a 10 mL volumetric flask.

2.1.2 Buffer preparation

The HEPES assay buffer (50 mM), pH 8.3 was prepared by dissolving 1.3015 g HEPES salt and 1.7532 g of sodium chloride in de-ionised water. The pH was adjusted to 8.3 using a few drops of I M HCl and volume adjusted to 100 mL, the solution was then vacuum filtered through a 0.2 µm polyethersulfone (PES) filter (Pall Life Sciences, Cork, Ireland).

Phosphate buffer (100 mM), pH 8.3 was prepared by dissolving 340.2 mg of potassium di-hydrogen phosphate in 20 mL of de-ionised water; pH was adjusted to pH 8.3 using a 10% NaOH solution and volume adjusted to 25 mL. This solution was then used to produce a 50 mM phosphate buffer, pH 8.3 by taking 12.5 mL of the buffer and adding 12.5 mL de-ionised water. Both buffers were vacuum filtered through a 0.2 µm PES filter.

Capillary electrophoresis running buffer (background electrolyte (BGE)) of 20 mM boric acid borate buffer at pH 9.18 was prepared as follows; 0.247 g boric acid was dissolved in approximately 160 mL de-ionised water, when fully dissolved 1.526 g of sodium borate was added, solution was stirred until fully dissolved, the pH was then adjusted using a number of drops of 10% NaOH to pH 9.18. Solution was transferred to a volumetric flask and the final volume adjusted to 200 mL with de-ionised water and then vacuum filtered through a 0.2 µm PES.

The 100 mM sodium borate buffer at pH 8.3 containing 300 mM NaCl was prepared as follows: 7.6284 g sodium borate was dissolved in approximately 160 mL de-ionised water, when fully dissolved 3.506 g of sodium chloride was added, solution was stirred until fully dissolved, the pH was then adjusted using a number of drops of 1 M HCl to pH 8.3. Solution was transferred to a volumetric flask and the final volume adjusted to 200 mL with de-ionised water, the solution was then filtered through a 0.2 µm PES filter.

2.1.3 Bradford protein assay

The Bio-Rad protein assay (Alpha Technologies Ltd., Wicklow, Ireland) based on the method of Bradford (1976) was used for the determination of the concentration of solubilised protein in samples. The assay involves the addition of an acidic dye to a protein solution, and subsequent colorimetric measurement of the coloured product formed. The absorbance maximum for an acidic solution of coomassie brilliant blue G-250 dye shifts from 465 nm to 595 nm when protein binding occurs and a differential colour change is observed in response to various concentrations of protein. Comparison to a standard curve provides a relative measurement of protein concentration in test samples. This method was employed to determine the protein content of both purified ACE enzyme, rabbit lung and hydrolysate samples.

A stock solution of 0.2 mg/ mL of bovine serum albumin (BSA) (Sigma Aldrich Ireland Ltd., Ireland) was freshly prepared and kept on ice. A dilution series of 2.0, 4.0, 6.0, 8.0, 10.0, 15.0 and 20.0 µg/mL was prepared from the stock solution using de-ionised water as the diluent. Each standard and test sample solution (160 µL) was pipetted into separate microtitre plate wells and a reference blank solution of 160 µl de-ionised water was also included. Protein solutions were assayed in triplicate. When required test samples were diluted using de-ionised water to ensure that their absorbance would fall within the linear working range of the standard curve. Bio Rad dye reagent concentrate (40 µl) was then added to each well using a multi-channel pipette. Samples and reagent were mixed thoroughly and incubated at room temperature for 10 minutes. Absorbance values measured at 595 nm using a microtitre plate reader (Spectrostar Omega, BMG Labtech with Omega software version 1.2) were recorded for each standard and test sample. The protein concentration of test samples was determined using the equation of a line obtained from the standard calibration plot.

2.2 Enzyme hydrolytic studies

Table 2.1 lists the samples and sample sources used in this study. The main focus of this thesis was to examine whether fisheries processing byproducts are utilizable sources of ACE-I and PEP inhibitory peptides. The following sections outline the steps involved in the preparation of fish waste hydrolysates using enzymes, development of bioassays to monitor ACE-I and PEP inhibitory activities, application of capillary electrophoresis for ACE activity and the concentration and purification of individual peptides using developed chromatographic techniques.

2.2.1 Sample pre-treatments

Mackerel and whelk samples from the Cape grounds in Co. Donegal were kindly provided by Errigal Fish Company Ltd., in Carrick, Co. Donegal. Mackerel were provided as whole fresh fish samples while whelk samples were shell discards from pre-cooked (140°C) processed samples and contained some attached cooked meat. Both mackerel and whelk marine samples were stored frozen at -20°C.

Whole mackerel samples were de-frosted overnight at 4°C prior to enzymatic hydrolytic studies. Mackerel tissues consisted of white meat, brown meat from the lower dorsal side or mackerel head, tail meat and skin portions. Initial studies were performed on mackerel white meat samples and the other mackerel tissue sources were examined after optimal enzyme hydrolysis conditions were established.

Mackerel white meat tissue samples (approximately 10-20 g portions) were harvested from either side of the backbone and brown meat tissues were harvested from the lower dorsal side using a scalpel and cut into 0.5 cm pieces, 100-300 mL of de-ionised water was added and the sample was stomached (Lab blender 400, VWR, Dublin, Ireland) for 40 seconds then autoclaved at 105°C for 10 minutes to heat inactivate endogenous enzymes. Mackerel head, tail and skin portions were pre-treated in a similar manner but homogenisation of the tissue was performed using an Ultra Turrax homogeniser (IKA, T50 basic Ultra Turrax, VWR, Dublin, Ireland). Homogenised and heat inactivated samples were then transferred to a sterile 500 mL Infors reaction vessel(s) (Infors UK, Reigate, Surrey, England) under aseptic conditions using a Class II biological safety cabinet (Microflow, AGB Scientific Ltd.). Enzymatic hydrolysis of tissue samples were performed as described below.

Table 2. 1. List of samples and sample sources used in this study.

Samples	Purpose	Source
Marine samples		
Mackerel	Isolation of ACE and PEP inhibitors	Errigal Fish Company Ltd., Co. Donegal
Whelk	Isolation of ACE and PEP inhibitors	Errigal Fish Company Ltd., Co. Donegal
Hydrolytic enzymes		
Ecostone® A200	Carbohydrate hydrolysis	AB Enzymes GmbH, Darrmstadt, Germany
Corolase® LAP	Protein hydrolysis	AB Enzymes GmbH, Darrmstadt, Germany
Corolase® N	Protein hydrolysis	AB Enzymes GmbH, Darrmstadt, Germany
Corolase® PP	Protein hydrolysis	AB Enzymes GmbH, Darrmstadt, Germany
Thermolysin	Protein hydrolysis	Sigma Aldrich Ireland Ltd
Pepsin	Protein hydrolysis	Fischer Scientific Ireland Ltd
ACE enzymes		
Crude ACE enzyme (rabbit lung extract)	Colorimetric ACE assay	Sigma Aldrich Ireland Ltd
Purified ACE enzyme (EC 3.4.15.1)	Capillary electrophoresis ACE assay	Sigma Aldrich Ireland Ltd
ACE substrates		
Hippuryl-glycyl-glycine (Hip-Gly-Gly)	Colorimetric ACE assay	Bachem Holding AG, Switzerland
Hippuryl-L-histidyl-L-leucine (Hip-His-Leu)	Capillary electrophoresis ACE assay	Sigma Aldrich Ireland Ltd
PEP enzyme source		
Bovine calf serum	PEP bioassay	Queens University Belfast
Prolyl synthetic substrate		
Z-glycyl-prolyl-AMC	PEP bioassay	Queens University Belfast

Whelk shell samples were retrieved from the -20°C freezer and placed into 500 mL beaker, covered and left overnight to thaw. The shell was then ground using a mortar and pestle into small pieces (< 1 cm). The shell pieces were rinsed in tap water, drained and finally rinsed in de-ionised water to provide a crude cleaning of samples. Fifty grams (wet weight) of washed, crushed shell was placed in a 500 mL beaker with 200 mL of de-ionised water. The mixture was heated to 50°C for 20 minutes on a hotplate with stirring and was subsequently transferred to the Infors reaction vessel and autoclaved at 121°C for 15 minutes to sterilise and heat-inactivate endogenous enzymes. Enzymatic hydrolysis of whelk shell was performed as described below.

2.2.2 Hydrolytic enzymes and hydrolytic reactions

All enzyme hydrolysis conditions were selected based on the manufacturer specification data sheets and the final hydrolytic conditions are provided in Table 2.2. Ecostone®A200 (EcoT) is an alpha-amylase preparation produced from a non-pathogenic strain of *Bacillus* species and hydrolyses polysaccharides within an optimum pH range is 6-8. Corolase® N (CorN) is a proteolytic enzyme product which contains exclusively endo-proteinase activities. It performs best at neutral pH and is obtained from *Bacillus subtilis* cultures. Corolase® PP (CorP) is another proteolytic enzyme product which contains both endo-proteinase activities and various amino- and carboxy-peptidases. It performs best at neutral and slightly alkaline pH 7-9 and is obtained from pig pancreas glands. Corolase® LAP (CorL) is a proteinase product which contains exclusively exo-peptidase activity. It performs best at slightly acid and alkaline pH 6-9 and is obtained from *Aspergillus sojae* cultures. All of the above enzymes were kindly supplied by AB Enzymes (AB Enzymes GmbH, Darmstadt, Germany). Pepsin (Pep) is obtained from porcine gastric mucosa and performs best at acid pH 2-4, and was purchased from Fischer Scientific Ltd. Ireland. Thermolysin (TherM) is a thermostable (thermophilic) extracellular metallo-endopeptidase containing four calcium ions. Cofactors are zinc and calcium. It hydrolyses protein bonds on the N-terminal side of hydrophobic amino acid residues. The pH optimum is 8.0 and it was obtained from *Bacillus thermoproteolyticus* which was purchased from Sigma Aldrich Ireland Ltd.

An Infors Multifors HT Fermenter controlled by the Infors AG, XDDC system control

software (Infors UK, Reigate, Surrey, England) was used for all enzyme hydrolysis reactions. All glassware, equipment and de-ionised water used in this section were sterilised at 121^{0}C for 15 minutes and Infors fermentation reaction vessels (500 mL capacity) were sterilised at 121°C for 30 minutes prior to use.

Table 2.2. Hydrolytic enzymes and optimal hydrolytic conditions selected.

Enzyme	Code	Hydrolytic conditions[1]				
		% Enz. conc.[2]	pH	Temp	Agitation	Time
Ecostone®A200	EcoT	5.0%	7.5	60°C	400 rpm	0.3 hours
Corolase® N	CorN	4.0%	7.0	55°C	250 rpm	1.0 hours
Corolase® PP	CorP	0.5%	8.5	45°C	250 rpm	24.0 hours
Corolase® LAP	CorL	0.6%	9.0	60°C	250 rpm	20.0 hours
Pepsin	Pep	0.1 - 1.2%	2.5	35°C	250 rpm	2.5 hours
Thermolysin	TherM	0.25 - 0.35%	8.0	55°C	250 rpm	18.0 hours

1: Reaction conditions were selected based on manufacturer specifications. 2: Final percentage enzyme assay concentrations were determined based on the initial weight of tissue samples (g/g of sample). All hydrolytic reactions were de-activated by treatment of 80°C for 15 minutes prior to storage at -20°C or -80°C.

Infors Multifors HT Fermenter system was switched on and reactions parameters selected based on optimal hydrolytic conditions (Table 2.2). Reaction vessels with marine samples were allowed to equilibrate to selected hydrolytic conditions of pH, temperature and agitation. When all parameters had reached the set conditions for the respective hydrolytic enzyme the required amount of the enzyme was dissolved in 2.0 mL sterile de-ionised water and injected into the reaction vessel via the sample port. Each hydrolytic reaction was run for the specified time period (Table 2.2) and reactions were terminated by heat de-activation. For heat de-activation, reaction vessels were removed to the Class II biological safety cabinet and hydrolysates was decanted into sterile labelled 50 mL plastic falcon tubes. The tubes were placed in a water bath at 80°C for 15 minutes to heat de-activate the enzyme reaction. The tubes were removed, left to cool and finally stored at -20°C and -80°C.

In the case of a double hydrolytic enzyme reaction (e.g. Pep and TherM or Pep and CorL) the hydrolytic enzyme with the lower pH and temperature parameters (i.e. Pep)

was run first for the set conditions and the conditions were then raised to the requirements of the second hydrolytic enzyme (i.e. TherM or CorL). Reactions were terminated by heat de-activation as described above.

2.3 Colorimetric ACE-I inhibition bioassay

Preliminary screening of hydrolysates for ACE-I inhibition was performed using a modified ACE-I enzyme assay employed by Serra *et al.* 2005. The method is based on the cleavage of the substrate hippuryl-glycyl-glycine (HGG) by ACE-I and subsequent reaction with trinitrobenzenesulfonic acid (TNBS) to form 2, 4, 6-trinitrophenyl-glycyl-glycine, whose absorbance was determined at 415 nm in a microtitre plate reader.

Marine hydrolysates were retrieved from -20°C storage and thawed at 4°C overnight. ACE-I enzyme reactions were established as follows: 20 µL of an crude ACE-I enzyme extract from dehydrated rabbit lung (1 g/10 mL) was added to a 1.5 mL eppendorf tube containing 20 µL of mackerel or whelk extract solution, or 30 µL of 50 mM phosphate buffer, pH 8.3 (negative control) or 10 µL of Captopril® solution (0.008 mg/ mL) (positive control). Samples were centrifuged for 30 seconds at 1000 rpm and pre-incubated for 5 minutes at 37°C. The enzymatic reaction was started by the addition of 80 µL of the HEPES assay buffer and 25 µL of the substrate solution Hip-Gly-Gly. After centrifugation at 1000 rpm for 30 seconds to mix contents, the samples were incubated for 35 minutes at 37°C. The reaction was stopped by the addition of 100 µL of sodium tungstate solution (100 g/L) and 100 µL of sulphuric acid (0.33 mM) and contents mixed following the addition of 1000 µL of de-ionised water. The sample was centrifuged at 1000 rpm for 30 seconds and a 75 µL aliquot of the supernatant was placed in a microtitre plate (Costar 96-well flat-bottomed plate). To assay for Gly-Gly product formation 100 µL of phosphate buffer (100 mM, pH 8.3) and 5 µL of TNBS (6 mM) solution were added. Microtitre plates were incubated in the dark at room temperature for 30 minutes and absorbance (415 nm) readings were determined using a microtitre plate reader (Spectrostar Omega, BMG Labtech with Omega software Version 1.2). A blank solution was also prepared using 70 µL of 50 mM phosphate buffer with addition of all components except the rabbit lung. Assays were performed in triplicate.

Calculation of ACE-I inhibition, on percentile basis, was performed using the following equation:

$$\text{Inhibition}(\%) = 100 - \left(\left(\frac{A_S - A_B}{A_E - A_B} \right) x100 \right)$$

where A_S is the measured absorbance at 415nm in the presence of an inhibitor, A_B is the absorbance of the blank solution and A_E is the absorbance of the rabbit lung without inhibitor.

2.4 Capillary electrophoresis ACE-I inhibition assay

The enzymatic reaction was based on the liberation of Hippuric acid (HA) from hippuryl-L-histidyl-L-leucine (HHL) substrate catalysed by purified ACE-I enzyme (EC 3.4.15.1). ACE-I inhibitory activity was determined by a CE modification of the Cushman and Cheung (1971) method. This method was employed in order to verify the possible ACE-I inhibitors found using the ACE-I colorimetric assay and also to accurately quantify the degree of inhibition present in the samples.

For direct CE analysis, the total reaction volume was 100 µL, including 25 µL of 1mM HHL, 25 µL of 5.0 mU ACE-I and 25 µL of Captopril (0.002 mg/mL) (positive control), 100 mM buffer (negative control) or mackerel/whelk marine hydrolysates (test samples). Reactions were prepared in 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl. All reactions were allowed to incubate at 37°C for 30 minutes. The enzymatic reactions were terminated by the addition of 25 µL of 0.1% TFA. All hydrolysate samples were analysed in triplicate. The 100 µL reaction solution was then applied to the CE system for separation and quantification of HA. The amount of HA liberated from HHL per minute in the absence of ACE-I inhibitor was defined as 100% ACE activity. The IC_{50} value was defined as the amount of inhibitor required to inhibit 50% of the baseline uninhibited ACE activity. The CE run buffer (BGE) consisted of 20 mM boric acid– borate buffer, pH 9.18. The percentage ACE-I inhibition was calculated using the following formula:

$$\%\ \text{ACE-I Inhibition} = 100 - \left(\frac{\text{Test Sample HA peak area}}{\text{HA peak area for ACE enzyme without inhibitor}} \times 100 \right)$$

All CE analysis were performed using a Beckman Coulter P/ACE ™ MDQ Capillary Electrophoresis System equipped with a PDA detector and controlled by means of the CE System software 32 Karat, version 5.0 (Beckman Coulter Ltd., High Wycombe, UK). The uncoated fused-silica capillary with an internal diameter of 75 µm and total length of 43 cm, 32 cm from inlet to detector (Composite Metal Services Ltd., Shipley, UK) was maintained at 25 ^{0}C. To activate a new capillary it was rinsed with methanol at 0.5 pounds per square inch (psi) for 10 minutes, followed by 1 N NaOH at 0.5 psi for 5 minutes, de-ionised water at 0.5 psi for 10 minutes and finally with the BGE at 0.5 psi for 20 minutes, to ensure a clean capillary surface. Daily, before the first sample injection, the capillary was flushed with BGE buffer for 3 minutes at 5.0 psi and after each sample injection and separation the capillary was rinsed with 0.1 N NaOH at 5.0 psi for 3 minutes followed by de-ionised water at 5.0 psi for 3 minutes. Samples were introduced from the anodic end of the capillary by hydrodynamic injection for 8 seconds at 0.1 psi. The PDA was set at an acquisition range from 190 to 300 nm at an acquisition rate of 4 scans per second (4 Hz). The detection wavelength was set at 228 nm and the electrophoretic separations were performed at 20 kV for a run time of 5.5 minutes at a capillary temperature of 25°C. The system was run in the normal polarity mode. Data were collected and peak area, height and migration time were analysed by the 32 Karat, version 5.0 software. Prior to use, all buffers and solutions used in the assay were filtered through a 0.2 µm PES filter.

Weekly the capillary was regenerated by performing a rinse cycle which consisted of flushing the capillary with 0.1N NaOH at 1.0 psi for 3 minutes in triplicate, followed by de-ionised water at 1.0 psi for 10 minutes, and then with BGE at 1.0 psi for 15 minutes.

2.4.1 Validation and enzyme kinetic studies for CE method

A set of working HA standards were prepared and used for calculation of the HA concentration. A stock standard solution of HA was prepared by dissolving HA in 100 mM sodium--borate buffer (pH 8.3) containing 300 mM NaCl to a concentration of 2 mM and stored at 4°C. A series of 10 mL working standards of HA were obtained from the stock standard solution by dilution with the same buffer over the range 0.01,

0.025, 0.05, 0.075, 0.10, 0.15 and 0.20 mM. Injections of standards were performed in triplicate in order of increasing concentration. A calibration curve was obtained by plotting the area of the HA peak on the obtained electropherogram against it's concentration.

The precision of a method is determined by the extent to which the test results of multiple injections of standards agree. The precision of the CE method used was expressed in terms of relative standard deviation (RSD). Linearity is the ability to show that the results are directly proportional to the analyte concentrations in samples within a given range. This was demonstrated for the CE method employed by analysing HA standards in triplicate in the range 0.01mM – 0.20 mM and plotting peak area versus HA concentration, a linear trend-line was added to determine the degree of correlation achieved. Values closer to 1.0 show a greater degree of correlation.

The limit of detection (LOD), defined as the lowest concentration of an analyte that the analytical procedure can reliably differentiate from background noise, this was evaluated based on a signal-to-noise ratio (S/N) of 3. The limit of quantification (LOQ) is defined as the level at, or above, in which the measurement precision is satisfactory for quantitative analysis. In this case, the LOQ was evaluated based on an S/N ratio of 10.

A kinetic study of ACE was performed at various substrate concentrations ranging from 0 to 8.0 mM, all in the presence of 2.0 mU of the enzyme to verify the above described method for ACE assay. One unit (U) of ACE activity is defined as the amount of enzyme required to catalyse formation of 1 mM of HA from HHL per minute at 37°C under the given conditions. The ACE activity of each sample applied to the CE system could be calculated by quantifying the amount of the HA formed in the enzymatic reaction. The velocities of the ACE-catalysed reactions were calculated from the amounts of HA generated in the enzymatic reaction. The initial velocities at various substrate concentrations were plotted as a function of the HHL concentrations. Kinetic data was analysed by linear regression analysis using Lineweaver-Burk plots (Whitford, D. 2005).

2.5 Prolyl endopeptidase activity and PEP inhibition assay

Prolyl endopeptidase activity from bovine calf serum was determined based on the enzyme's catalytic ability to cleave the prolyl synthetic substrate Z-glycyl-prolyl-AMC with the liberation of the fluorophore 7-amino-4-methylcoumarin (AMC) (Goossens *et al.*, 1992). Prolyl endopeptidase catalytic activity could then be calculated based on the amount of AMC fluorophore product formed. The PEP assay and inhibition studies were performed at Queen's University Belfast (QUB), UK with permission from Dr Brian D. Green from the School of Biological Sciences, and the kind assistance of Danielle Calderwood who supplied all necessary reagents.

Prior to analysis and transport to QUB, all hydrolysate samples were freeze-dried using a Christ alpha 1-4 freeze-drier with Vacubrand vacuum pump (SciQuip Ltd., Shropshire, UK). Samples were reconstituted in HEPES buffer (50 mM, pH 7.4) to a concentration of 100 mg/ mL (0.025 g in 250 µL buffer). Bovine calf serum was used as the enzyme source (*Flavobacterium meningosepticum* is often used as the PEP-specific source but as this is pathogenic it was not used). A 50mM Z-Gly-Pro-AMC was used as the fluorogenic substrate for the determination of post-proline cleaving enzyme (prolyl endopeptidase). Berberine (13.3 µM) (Sigma Aldrich Ireland Ltd., Ireland) was used as the PEP-specific positive inhibitor. HEPES buffer (50 mM) was used as a negative control. Greiner black transparent bottomed 96 half well plates (Sigma Aldrich Ireland Ltd.) were used in the assay.

PEP activity was assayed as follows; 20µL of bovine calf serum was added to each microtitre well, 20 µL of each sample or 20 µL of berberine or HEPES buffer was then added. The assay was initiated by adding 20 µL of Z-Gly-Pro-AMC to all wells. The plate was then incubated with shaking for 1 hour at 37^0C. The enzyme reaction was terminated by adding 100 µL of acetic acid (3 mM) to each well. All samples and controls were run in triplicate. As the test samples were coloured 20 µL of each sample with no reagents added were run to in order to obtain background colour readings. The microtitre plate was then read in a Tecan Safire^2 plate reader, model IS89 (AQS Manufacturing Ltd., West Sussex, UK) at excitation wavelength 351 nm and emission wavelength 430 nm.

The sample background readings were subtracted from the fluorescence readings and the degree of inhibition (%) was then calculated using the following:

$$\text{PEP inhibition (\%)} = \frac{\text{control} - (\text{sample} - \text{background})}{\text{control}} * 100$$

2.6 Purification of ACE-I inhibitory samples

Hydrolyate samples that strongly inhibited the ACE-I enzyme were selected and further purified using a Pall Minimate Tangential flow Filtration system (Fisher Scientific Ireland, Dublin, Ireland) equipped with molecular weight cut off (MWCO) capsules of 3kDa and 10kDa. Two samples of mackerel were selected, sample M-CorN-4 (white muscle meat) and M-CorN-6 (head, tail and skin). Also one sample of whelk W-EcoT-1 which was found to be a potential PEP-inhibitor but negative for ACE-I inhibition was selected. Each sample was centrifuged at 2000 rpm for 4 minutes and the supernatant passed through the 10kDa MWCO capsule and the filtrate collected, a sample of filtrate was analysed for protein content and ACE-I inhibitory activity by CE. The remainder of the filtered samples were stored in acid-washed glass bottles at -20^{0}C. For both the mackerel samples, a portion of the filtrate prior to freezing was further filtered through the 3kDa MWCO capsule and the filtrate collected, a sample of filtrate was again analysed for protein content and ACE-I inhibitory activity by CE. Between sample filtrations the capsules were thoroughly rinsed and cleaned using large volumes of de-ionised water followed by 0.1 N NaOH.

3. Results and discussion

3.1 Preparation of ACE-I inhibitory peptides from mackerel and whelk waste

Previous studies have demonstrated that marine sources are suitable for the isolation of ACE-I inhibitory peptides (Section 1.4, Table 1.1), however no standard protocol is recommended. In this study, mackerel and whelk hydrolysates were prepared by means of hydrolysis with commercial enzymes including Ecostone®A200 (EcoT), Corolase® N (CorN), Corolase® PP (CorP), Corolase® LAP (CorL), Pepsin (Pep) and Thermolysin (TherM). The hydrolysis was necessary in order to release ACE-I inhibitory peptides from the inactive forms of intact mackerel and whelk sources. Initial enzyme hydrolysis involved the use of mackerel white meat tissue and crushed whelk shell as potential sources of ACE-I inhibitors. The following six hydrolytic reactions were established:

- Whelk shell with EcoT (W-EcoT-1)
- Mackerel white meat with Pep (M-Pep-1)
- Mackerel white meat with CorN (M-CorN-1)
- Mackerel white meat with CorP (M-CorP-1)
- Mackerel white meat with Pep and CorL (M-PepCorL-1)
- Mackerel white meat with Pep and TherM (M-PepTherM-1)

Sample preparation and hydrolytic conditions are described in section 2.2.1 & 2.2.2 and all hydrolysates were stored at -20°C. Mackerel white meat tissue were digested with single proteolytic enzymes or in combination with two proteolytic enzymes to examine if double enzyme digests were capable of generating stronger ACE-I inhibitors than single enzyme hydrolysis studies. The alpha-amylase EcoT hydrolytic enzyme was only used to examine carbohydrate whelk shell hydrolysis and proteolytic enzymes were not investigated as whelk shell waste contained little or no meat tissue.

Hydrolysate samples were retrieved from the -20°C and pre-screened for ACE-I inhibitory activity. Initially, hydrolysates were analysed based on the Cushman and Chung method (1971) however ACE assays were not reproducible (data not shown). A modified alternative colorimetric ACE-I bioassay developed by Serra *et al.* (2005)

was employed to screen hydrolysate samples. The method is based on the cleavage of the substrate hippuryl-glycyl-glycine (HGG) by ACE-I and subsequent reaction with trinitrobenzenesulfonic acid (TNBS) to form 2, 4, 6-trinitrophenyl-glycyl-glycine, whose absorbance was determined at 415nm using a microtitre plate reader (Figure 3.1). A crude ACE-I enzyme from rabbit lung dehydrated by acetone was employed as the enzyme source and Captopril® was used as a positive ACE-I inhibitor control (Section 2.3).

The colorimetric ACE-I bioassay proved successful and reliable at identifying potential ACE-I inhibitors from test samples. Using this assay method positive ACE-I inhibition for Captopril® ranged from forty to fifty per cent inhibition. Table 3.1 provides a summary of the percentage ACE-I inhibition from marine hydrolysate

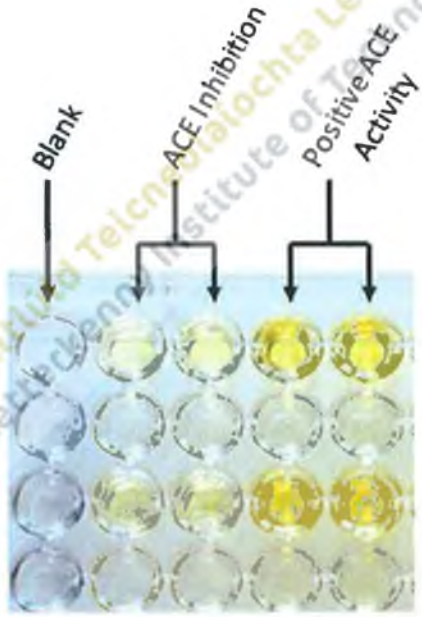


Figure 3.1. Microtitre plate assay for colorimetric ACE-I bioassay.

samples. Whelk hydrolysate (W-EcoT-1) did not possess ACE-I inhibitory peptides since all three replicate samples demonstrated no inhibition of ACE-I enzyme activity. Conversely, mackerel white meat hydrolysates all demonstrated some level of ACE-I inhibitory activity with the exception of hydrolysate M-CorP-1. The percentage inhibition observed ranged from an average of twenty six percent to thirty nine percent inhibition. The hydrolysate M-CorN-1 exhibited the highest degree of

potential ACE-I inhibition with a mean value of thirty nine percent. Mackerel white meat hydrolysates with double enzyme digests did not demonstrate increased ACE-I inhibition with the percentage inhibition ranging from twenty six to thirty two percent for both samples analysed (Table 3.1).

The percentage RSD calculated for each set of triplicate samples per analysis ranged from eleven to thirty four per cent indicating that the accuracy and precision of the method was highly variable. Possible reasons for this degree of variation between samples and replicates may be due to the enzyme source used and interference from other marine proteins in the samples. The ACE-I enzyme employed was a crude extract from dehydrated rabbit lung and other components present in the enzyme supernatant may have possibly interfered with the enzymatic reaction.

Table 3.1. Percentage ACE-I inhibition for whelk (W) and mackerel (M) hydrolysates determined by ACE-I colorimetric bioassay. Sample ID codes are described in text.

Sample ID	% ACE-I inhibition[1]				
	Rep 1	Rep 2	Rep 3	Mean ± SD	% RSD
W-EcoT-1	00.0	00.0	00.0	-	-
M-Pep-1	28.0	29.0	41.0	32.67 ± 7.23	22.15
M-CorN-1	38.0	32.0	46.0	38.67 ± 7.02	18.16
M-CorP-1	0.0	0.0	0.0	-	-
M-PepCorL-1	23.0	27.0	29.0	26.33 ± 3.06	11.60
M-PepTherM-1	24.0	27.0	44.0	31.67 ± 10.79	34.06

[1] Calculation of % ACE-I inhibition is described in Section 2.3

In order to confirm initial observations, three of the initial hydrolysis conditions were selected and repeated. Frozen tissue samples were recovered from the -20°C and hydrolytic conditions were established as described in Section 2.2. In addition, a duplicate sample of mackerel white meat using CorN was established to examine reproducibility. The repeat hydrolytic reactions established were:

- Whelk shell with EcoT (W-EcoT-2)
- Mackerel white meat with CorN (M-CorN-2)
- Mackerel white meat with CorN (M-CorN-3)
- Mackerel white meat with Pep and TherM (M-PepTherM-2)

Results from the repeated hydrolysis for potential ACE-I inhibitory samples by the ACE-I colorimetric bioassay confirmed the initial results (Table 3.1 & Table 3.2). As observed previously, the whelk hydrolysate W-EcoT-2 did not demonstrate ACE-I inhibition activity. Both mackerel white meat hydrolysate samples (M-CorN-2 & M-CorN-3) indicated the release of ACE-I inhibitory peptides with percentage inhibition ranging from twenty five and thirty seven per cent (Table 3.2). Although both repeat samples were lower than the initial thirty nine per cent observed for M-CorN-1 (Table 3.1) the level of ACE-I inhibition was considered an acceptable result for reproducibility under test conditions employed. Sample M-PepTherM-2 resulted in twenty nine per cent potential ACE-I inhibition compared to the initial result of thirty two per cent which also demonstrated good reproducibility.

Table 3.2. Repeat hydrolysis of whelk (W) and mackerel (M) samples to confirm ACE-I inhibition activity. Percentage ACE-I inhibition was determined by ACE colorimetric bioassay. Sample ID codes are described in text.

Sample ID	% ACE Inhibition				
	Rep 1	Rep 2	Rep 3	Mean ± SD	% RSD
W-EcoT-2	00.0	00.0	00.0	-	-
M-CorN-2	17.0	25.0	34.0	25.33 ± 8.50	33.57
M-CorN-3	31.0	33.0	48.0	37.33 ± 9.29	24.89
M-PepTherM-2	24.0	26.0	38.0	29.33 ± 7.57	25.81

The percentage RSD's calculated for each set of triplicate samples per analysis ranged from twenty five to thirty four per cent indicating that the accuracy and precision of the method was variable as discussed previously. Regardless, the method identified potential ACE-I inhibitory peptides from marine hydrolysates and was therefore considered suitable for pre-screening samples for ACE-I inhibition. More

importantly, the conditions established for hydrolysis of mackerel samples clearly indicated the potential of this tissue source for the isolation of ACE-I inhibitory peptides. All hydrolysate samples were maintained in storage at -20°C prior to further analysis to confirm and quantify their ACE-I inhibitory potential.

3.2 Confirmation of ACE-I inhibitory activity by capillary electrophoresis ACE assay

Although the colorimetric ACE-I bioassay was suitable for pre-screening marine hydrolysates to identify potential ACE-I inhibitory peptides the method was not accurate in quantifying the percentage ACE-I inhibition in samples. A more robust capillary electrophoresis ACE (CE-ACE) assay method was developed to accurately quantify the percentage inhibition associated with marine hydrolysates. The enzymatic reaction was based on the liberation of Histidyl-Leucine (HL) and Hippuric acid (HA) from Hippuryl-L-Histidyl-L-Leucine (HHL) as a substrate catalysed by purified ACE-I enzyme with the resultant HA quantified by CE. Experimental details for the CE-ACE assay are described in Section 2.4.

Initial studies involved determining ACE-I assay conditions and investigating the linear working range of the CE-ACE assay. The optimum capillary electrophoretic conditions for the ACE reaction mixture were determined based on the methods reported by Zhang *et al.* (2000) and He *et al.* (2007). In 20 mM boric acid–borax buffer, pH 9.18, satisfactory separation of the reactants and products of the reaction, including HHL, HL and HA was achieved (Figure 3.2). The electropherogram illustrates the separation process involved in the CE-ACE assay where the substrate HHL control produced a large peak area and height, when the ACE-I enzyme was added to the assay reaction this resulted in a reduction in size of the HHL substrate peak (Table 3.3) as it was catalysed by the ACE-I enzyme to yield HL and HA. The HL peak generated from this ACE-I catalysed reaction appears smaller than the HA peak as the HL structure does not contain a chromophore (a chemical group capable of selective light absorption) but the HA structure does so it therefore results in larger absorbance values for both area and height. The inclusion of the positive ACE-I inhibitor Captopril® (0.002mg/mL) to catalytic ACE-I enzyme reactions of HHL results in a reduced HA peak area indicating positive inhibition of ACE-I enzyme

(Table 3.3). The percentage ACE-I inhibition was calculated using the following formula:

$$\% \text{ ACE-I Inhibition} = 100 - \left(\frac{\text{Test Sample HA peak area}}{\text{HA peak area for ACE enzyme without inhibitor}} \times 100 \right)$$

The average percentage ACE-I inhibition by Captopril® was calculated at 57.2 ± 4.7% for seven individual CE-ACE assays indicating that the assay developed was sensitive and reliable for ACE-I inhibition studies.

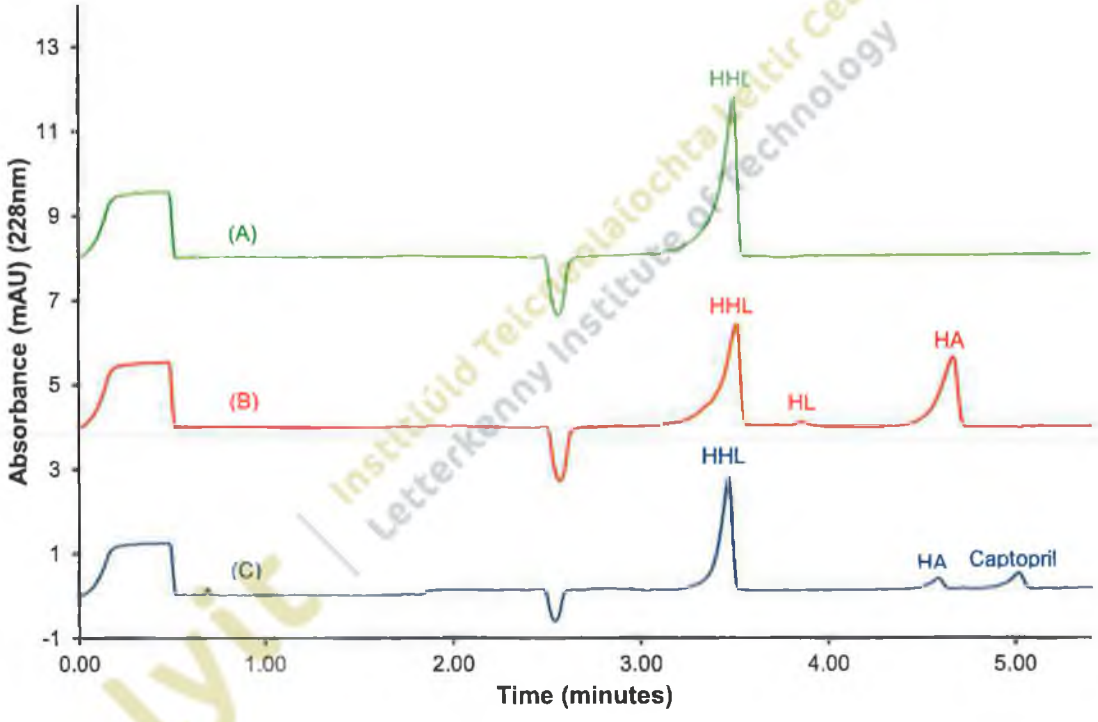


Figure 3.2. Typical CE electropherograms for (A); HHL substrate, (B); purified ACE-I enzyme HHL catalytic reaction resulting in two products HL and HA and (C); ACE enzyme reaction with positive ACE inhibitor Captopril®.

Table 3.3. Average CE peak areas and percentage ACE-I inhibition for HHL substrate, purified ACE-I enzyme HHL catalytic reaction and ACE-I enzyme assay with positive ACE inhibitor Captopril®. Peak areas are a mean of seven individual CE runs for each sample. Typical CE electropherograms are shown in Figure 3.2.

Sample	Average peak areas		% Inhibition
	HHL	HA	
HHL substrate	19,641 ± 2,908	n/a	n/a
ACE-I assay	11,936 ± 2,596	4,483 ± 976	n/a
Captopril®	10,653 ± 2,392	1,917 ± 459	57.2 ± 4.7

n/a = not applicable

There was some variation in migration time for the analytes throughout and between analyses. The HA is the most anionic component with an average migration time of approximately 4.7 minutes while less anionic HHL and HL migrated at approximately 3.6 and 3.8 minutes respectively. Captopril® which had an average migration time of approximately 5.1 minutes and was used as a positive ACE-I inhibitor control (Figure 3.2). The average migration time for HHL was calculated to be 3.56 minutes and all analysis times were between ± five per cent of this time (3.38 – 3.72 minutes). The average migration time for HL was calculated to be 3.78 minutes and all analysis times were between ± five per cent of this time (3.66 – 3.90 minutes). The average migration time for HA was calculated to be 4.75 minutes, all analysis times were between ± five per cent of this time (4.53 – 4.99 minutes).

This variation may have been caused by temperature fluctuations occurring with the run buffer and samples as the sample chamber was not temperature controlled and the temperature in the room did vary quite considerably. Temperature variations with corresponding changes in run buffer viscosity have been determined to be detrimental to obtaining reproducible results, with a change in viscosity of two to three per cent for every 1°C temperature fluctuation (Lauer and Rozing, 2010). Another possible reason for the variation in analyte migration time may be due to siphoning from the capillary buffer inlet to outlet reservoir if liquid levels are not kept level. It has been shown that a height difference of 2 mm between buffer reservoirs results in a two to three per cent shift in migration time for a 50 μm internal diameter (id) capillary

increasing to 10% for a 100 µm id capillary (Lauer and Rozing, 2010). The capillary id used for analysis was a 75 µm id capillary so this may explain the ± 5% variation found.

To determine the linear working range of the CE-ACE assay a standard calibration curve was established for working concentration standards of HA (Figure 3.3). A strict linear correlation between HA concentration and its peak area was obtained. A fresh set of HA working standards were prepared weekly from the stock standard and analysed prior to sample analysis. A regression equation was calculated for each analysis with a minimum correlation coefficient of greater than 0.999 (n =7) obtained within the HA concentration range from 0.01 to 0.20 mM. Linearity in the HA concentration range indicated no interaction between HA and the electrophoretic capillary under the conditions employed. By exploiting the ultraviolet absorbance maximum of HA at 228 nm, the LOD for HA was calculated to be 7.0 µM and the LOQ was calculated to be 24 µM. During sample analyses, standards at four different concentration levels (0.2 mM, 0.1 mM, 0.075 mM and 0.01 mM) were also analysed throughout the run to ensure correct calculation of analyte concentration and also to verify the retention time of analyte(s).

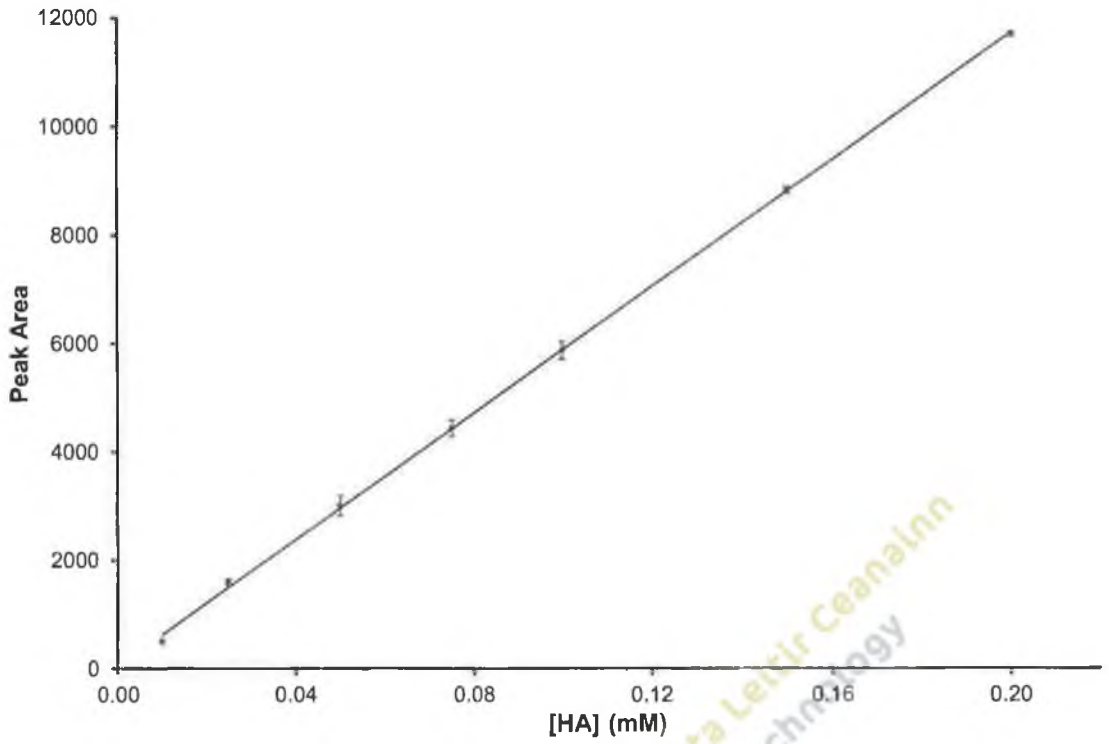


Figure 3.3. Calibration curve for HA concentration standards from CE electropherograms. Correlation co-efficient (R^2) of 0.9997 was achieved and the equation of the line was: $Y = 58402\ X + 41.555$.

3.2.1 ACE enzyme kinetic analysis by CE-ACE assay

ACE kinetics was studied by changing the amount of the substrate HHL, in the presence of a fixed amount (2 mU) of enzyme. The initial velocity for different concentrations of HHL (0-8 mM) was determined by plotting the HA peak area obtained (enzyme velocity) against the substrate concentrations to obtain information on the substrate saturation of enzyme. The kinetic data was analysed by Lineweaver-Burk plots resulting in a *K*m value for purified ACE enzyme of 1.2 mM. The value of *K*m obtained in this study was lower than those reported previously for purified rabbit lung ACE, which were 2.60 mM by Cushman *et al.* (1971) and 2.30 mM by Das *et al.* (1975) however it was much closer to that of 1.45 mM as reported by Zhang *et al.* (2000). It is likely that the higher values of *K*m obtained by Cushman and Das arose from the incomplete separation of HA from the substrate by solvent extraction using ethyl acetate and as it also absorbs at 228 nm. The difference in *K*m may also be possibly attributed to the different buffer systems used in enzyme assays, with boric acid–borate buffer used in this study and by Zhang *et al.* (2000) while previous reports used phosphate assay buffers.

3.2.2 ACE-I inhibitory activity for marine hydrolysates by CE-ACE assay

Whelk and mackerel white meat hydrolysates were tested for ACE-I inhibitory activity using the developed CE-ACE assay. Hydrolysates were retrieved from -20°C storage and ACE assay conditions were established as described in Section 2.4. Analysis of ACE activity was determined by CE and percentage ACE-I inhibition activity is summarised in Table 3.4. A typical CE electropherogram for one of the mackerel hydrolysates is provided in Figure 3.4. In all cases the level of ACE-I inhibition increased when assayed by the CE-ACE assay, with the exception of the whelk sample hydrolysate. The increase in ACE-I inhibition activity is presumably due to the use of a purified ACE-I enzyme source aligned with the use of the more sensitive CE analytical method.

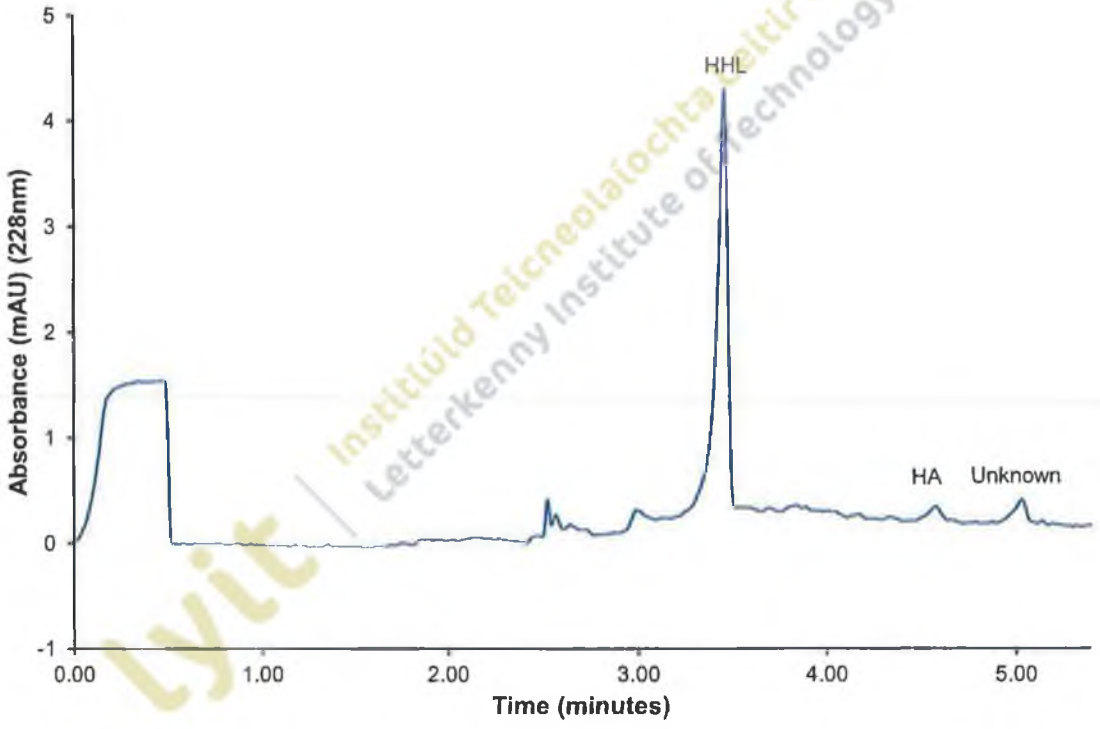


Figure 3.4. Typical electropherogram of a sample hydrolysate (M-PepTherM-1). A number of un-identified peaks were observed and are attributed to unknown components in the hydrolysate sample.

Table 3.4. Percentage ACE-I inhibition for whelk (W) and mackerel (M) hydrolysates determined by CE-ACE assay. Sample ID codes are described in text.

Sample ID	Sample No.	% ACE-I inhibition				
		Rep 1	Rep 2	Rep 3	Mean ± SD	% RSD
W-EcoT-1	1	00.0	00.0	00.0	-	-
	2	00.0	00.0	00.0	-	-
	3	00.0	00.0	00.0	-	-
M-Pep-1	1	100.0	100.0	100.0	-	-
	2	100.0	100.0	100.0	-	-
	3	100.0	100.0	100.0	-	-
M-CorN-1	1	47.0	47.0	43.0	45.67 ± 2.31	5.06
	2	60.0	69.0	72.0	67.00 ± 6.24	9.32
	3	100.0	100.0	100.0	-	-
M-CorP-1	1	93.0	94.0	92.0	93.00 ± 1.00	1.08
	2	93.0	93.0	91.0	92.33 ± 1.15	1.25
	3	93.0	91.0	89.0	91.00 ± 2.00	2.20
M-PepCorL-1	1	100.0	100.0	100.0	-	-
	2	100.0	100.0	100.0	-	-
	3	100.0	100.0	100.0	-	-
M-PepTherM-1	1	83.0	86.0	84.0	84.33 ± 1.53	1.81
	2	81.0	87.0	82.0	83.33 ± 3.21	3.86
	3	86.0	80.0	85.0	83.67 ± 3.21	3.84
M-CorN-2	1	95.0	91.0	93.0	93.00 ± 2.00	2.15
	2	94.0	95.0	94.0	94.33 ± 0.58	0.61
	3	93.0	94.0	94.0	93.67 ± 0.58	0.62
M-CorN-3	1	100.0	100.0	100.0	-	-
	2	100.0	100.0	100.0	-	-
	3	100.0	100.0	100.0	-	-
M-PepTherM-2	1	76.0	76.0	76.0	76.00 ± 0.00	0.00
	2	72.0	71.0	73.0	72.00 ± 1.00	1.39
	3	78.0	80.0	75.0	77.67 ± 2.52	3.24

CE-ACE assay confirmed that the whelk hydrolysate W-EcoT-1 sample did not contain ACE-I inhibitory peptides as no inhibition of ACE-I was observed. However sample M-CorP-1 which was negative for potential ACE-I inhibition when tested by the colorimetric bioassay showed a high degree of ACE-I inhibition in the CE based assay with samples exhibiting inhibition in the range of eighty nine to ninety four per cent. All the other samples analysed exhibited ACE-I inhibition by the CE method, the percentage of ACE-I inhibition was higher with this method than the results obtained in the colorimetric bioassay in all cases. The % RSD obtained for all but one set of triplicate samples analysed by the CE method were all below five per cent indicating a high degree of accuracy and precision with this method especially when compared to the % RSD's obtained with the colorimetric bioassay method.

The ACE-I enzyme source used in the colorimetric bioassay, the acetone dehydrated rabbit lung was a crude preparation compared to the lyophilized purified ACE-I enzyme employed by the CE- ACE assay. In the colorimetric bioassay which was developed and verified by Serra *et al.* (2005) the ACE enzyme was extracted from the rabbit lung into the supernatant by spinning in an ultracentrifuge for 40 minutes at 40 000 x g whereas in this study the rabbit lung was centrifuged at 13,500 rpm (17523 x g) for 1 hour as an ultracentrifuge was not available resulting in a less pure or crude supernatant enzyme source. This was possibly the reason why triplicate sample results showed a degree of variation. Although there was some variation in the results obtained by the colorimetric bioassay, the method was found to be reproducible with the repeated hydrolysate analysis which means that it was found to be a valid technique for pre-screening of samples for possible ACE-I inhibitory activity. There are a number of advantages in using this technique in that it is less time-consuming than the CE assay method and employs only inexpensive reagents, enzyme source and a relatively inexpensive microtitre plate reader capable of analysing 96-well plates leading to rapid throughput of samples.

The three repeat hydrolysate samples of mackerel white tissue meat hydrolysed with Corolase® N (M-CorN-1, -2 and -3) all exhibited a high degree of ACE-I inhibition with the CE-ACE method with the first sample average being seventy one per cent and the subsequent two samples each recording averages of ninety four and one

hundred per cent respectively. Sample M-CorN-1, the first Corolase® N hydrolysate was the only sample that exhibited any inconsistency between the set of three triplicate samples tested with an ACE-I inhibitory range of forty six to one hundred per cent. This was the first sample investigated using this set of hydrolysis conditions so was stored for a slightly longer period of time than the two repeat samples possibly leading to degradation of the sample or an experimental error may have occurred during the assay procedure as one of the three samples resulted in one hundred per cent ACE-I inhibition. This may have been a contributing factor to the lower percentage ACE-I inhibition found in the CE-ACE assay as this sample in the initial pre-screen was found to have the highest potential ACE-I inhibition when analysed by the colorimetric bio-assay.

The CE-ACE assay results demonstrated that the hydrolysate sample M-Pep-1 was a strong ACE-I inhibitor with one per cent inhibition recorded. This sample was hydrolysed with pepsin employing conditions analogous to those in the human stomach. The purpose of this selection of hydrolysis conditions was to determine if consumption of mackerel white tissue meat would result in natural generation of ACE-I inhibitors in the body. The results found from both ACE assays indicate that this possibility is indeed feasible and would suggest that the consumption of mackerel white tissue meat may be beneficial to the diet, especially for those who suffer from hypertension.

3.3 ACE-I inhibitory peptides from different mackerel tissue sources

Having established that mackerel white meat tissue was a suitable source for the isolation of ACE-I inhibitory peptides other mackerel tissue sources were examined. Particular emphasis was placed on discard sources such as head, skin and tail portions. Corolase® N was selected as a suitable hydrolytic enzyme since it had previously demonstrated its repeatability to generate ACE-I inhibitory peptides from mackerel white meat tissue. The following hydrolytic reactions were established and sample preparation and hydrolytic conditions are described in section 2.2.1. & 2.2.2:

- Mackerel white meat with CorN (M-CorN-4)
- Mackerel brown meat with CorN (M-CorN-5)
- Mackerel head, skin and tail with CorN (M-CorN-6)

Hydrolysates were pre-screened for ACE-I inhibition activity using the ACE-I colorimetric bioassay (Table 3.5) and assayed by the CE-ACE method to accurately determine the percentage of ACE-I inhibitors present (Table 3.6).

Table 3.5. Percentage ACE-I inhibition for different mackerel tissue hydrolysates determined by ACE-I colorimetric bioassay. Sample ID codes are described in text.

Sample ID	% ACE- Inhibition				
	Rep 1	Rep 2	Rep 3	Mean ± SD	% RSD
M-CorN-4	30.00	36.00	39.00	35.00 ± 4.58	13.09
M-CorN-5	24.00	35.00	37.00	32.00 ± 7.00	21.88
M-CorN-6	26.00	23.00	45.00	31.33 ± 11.93	38.08

Table 3.6. Percentage ACE-I inhibition for different mackerel tissue hydrolysates determined by CE-ACE assay. Sample ID codes are described in text.

Sample ID	Sample No.	% ACE-Inhibition				
		Rep 1	Rep 2	Rep 3	Mean ± SD	% RSD
M-CorN-4	1	100.00	100.00	100.00	-	-
	2	100.00	100.00	100.00	-	-
	3	100.00	100.00	100.00	-	-
M-CorN-5	1	100.00	100.00	100.00	-	-
	2	100.00	100.00	100.00	-	-
	3	100.00	100.00	100.00	-	-
M-CorN-6	1	95.00	97.00	95.00	95.67 ± 1.15	1.21
	2	96.00	90.00	93.00	93.00 ± 3.00	3.23
	3	94.00	94.00	94.00	94.00 ± 0.00	0.00

The hydrolysis of mackerel white tissue meat with Corolase® N sample M-CorN-4 resulted in thirty five per cent potential ACE-I inhibition by the ACE colorimetric bioassay method. This result is almost identical to that obtained with the previous samples indicating excellent reproducibility with the repeated hydrolysis conditions.

This completed a quadruple set of identical hydrolysis conditions employed for this sample of mackerel white tissue meat.

Sample M-CorN-5, the brown meat tissue sample resulted in thirty two per cent potential ACE-I inhibition by the ACE-I colorimetric bioassay method. Sample M-CorN-6, the head, tail and skin sample resulted in thirty one per cent potential ACE-I inhibition by the ACE colorimetric bioassay method. All three hydrolysate samples were similar for ACE-I inhibition activity which demonstrated a similar degree of ACE-I inhibitor generation by the Corolase® N hydrolysis for different mackerel tissue sources. The percentage RSD's obtained for the triplicates of each sample again varied from thirteen to thirty eight per cent and this variation has previously been discussed.

As previously observed the CE-ACE assay was more sensitive and accurate at quantifying ACE-I inhibitory activity. The percentage RSD's for all triplicate samples analysed by the CE-ACE method were all less than three point two per cent and the majority of replicate samples had zero per cent RSD indicating a high degree of accuracy and precision with this method (Table 3.6). Hydrolysates M-CorN-4, the white meat tissue sample, and M-CorN-5, the brown meat tissue sample, resulted in one hundred per cent ACE-I inhibition by the CE ACE assay method. Sample M-CorN-6, the head, tail and skin sample resulted in an average of ninety four per cent ACE-I inhibition by this method. These results indicate that Corolase N is a suitable proteolytic enzyme for the release of potential ACE inhibitory peptides from different mackerel tissues sources. In addition, the fact that discard mackerel portions of head, tail and skin portions could be utilised as a novel tissue sources for the discovery of potent ACE-I inhibitory peptides might be economically beneficial to the seafood processing sector.

3.4 Purification of ACE-I inhibitory peptides from mackerel hydrolysates

Previous studies have indicated that ACE-I inhibitory peptides are usually between 2-30 amino acids (Table1.1) and that these bioactive peptides can be concentrated by molecular weight cut off (MWCO) and/or by ultra-filtration through 3 and 10KDa membrane filters. Three samples were chosen for purification based on their ACE-I

inhibitory peptide activity. Two mackerel samples, M-CorN-4, a white meat tissue sample and M-CorN-6, a head, tail and skin sample were selected. Both these samples were found to contain potent ACE-I inhibitors and as the samples were taken from different parts of the mackerel there was a possibility they may have contained different ACE-I inhibitory peptides. The third sample selected was the whelk sample W-EcoT-1 which was included as negative control for ACE-I inhibition. All three hydrolysate samples were retrieved from the -20°C freezer and defrosted before filtering through a Pall Minimate 10KDa MWCO capsule filter and samples were subsequently filtered through a second Pall Minimate 3KDa MWCO capsule filter as described in Section 2.6.

Protein content for each filtered sample was determined by the Bio-Rad protein assay based on the method of Bradford (1976) and was used for determination of the concentration of solubilised protein in samples. The assay involves the addition of an acidic dye to a protein solution, and subsequent measurement with a microtitre plate reader. A differential colour change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. Comparison to a standard curve provides a relative measurement of protein concentration. Each of the three selected samples was analysed in triplicate to determine their total protein content as per the method described in Section 2.1.3. The corresponding MWCO filtered samples were also analysed to determine if the filtration process resulted in a reduction in protein content (Table 3.7).

A standard curve was used to determine the concentration of protein present in the samples by substitution of the sample absorbance into the equation of the line generated (Figure 3.5).

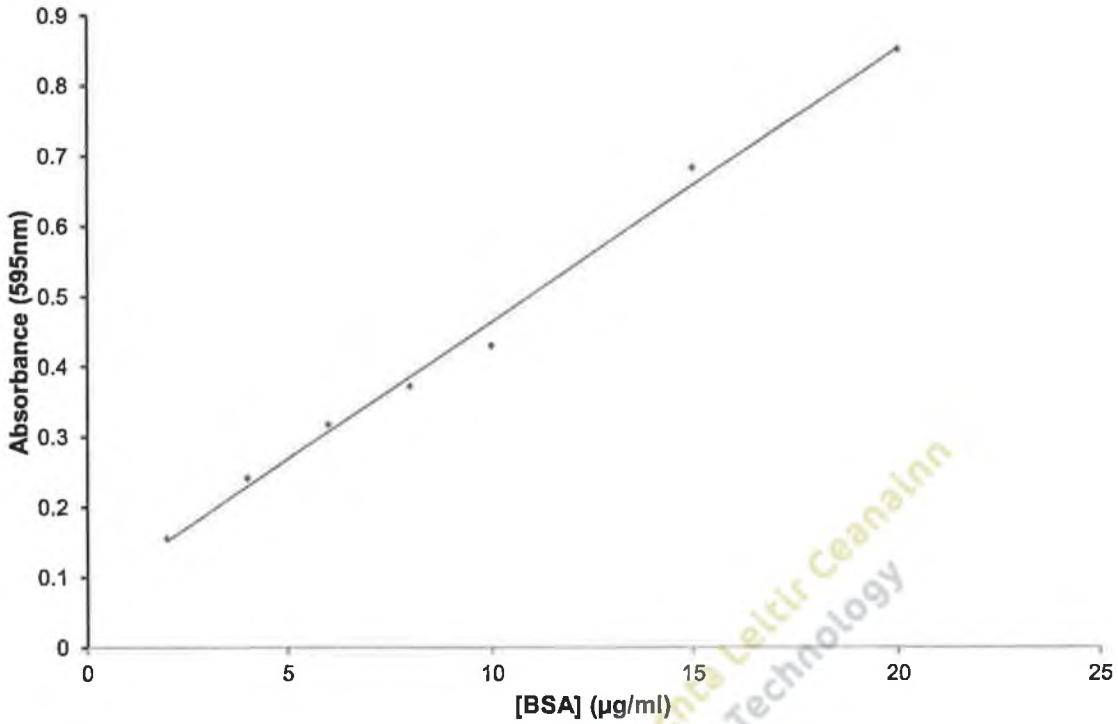


Figure 3.5. BSA calibration curve for protein determination. Correlation coefficient (R^2) = 0.9941 (n = 7) was achieved and the equation of the line was: Y = 0.0389X + 0.0741.

Table 3.7. Bio-Rad protein assay for 10kDa and 3kDa MWCO filtration samples.

Sample ID	Total protein (µg/ml)				
	Rep 1	Rep 2	Rep 3	Mean ± SD	% RSD
W-EcoT-1	79.20	68.50	82.60	76.77 ± 7.36	9.59
W-EcoT-1 10kDa	0.00	0.00	0.00	-	-
M-CorN-4	140.00	177.65	188.00	168.55 ± 25.26	14.99
M-CorN-4 10kDa	39.00	45.00	53.00	45.67 ± 7.02	15.38
M-CorN-4 3kDa	4.80	3.40	5.80	4.67 ± 1.21	25.83
M-CorN-6	113.40	125.60	117.48	118.83 ± 6.21	5.23
M-CorN-6 10kDa	10.94	9.55	11.35	10.61 ± 0.94	8.89
M-CorN-6 3kDa	5.62	5.57	5.60	5.60 ± 0.03	0.45

The total protein content of all the three samples tested was reduced considerably by the filtering process. As each sample was successively filtered through smaller

MWCO capsules the amount of protein present also decreased. In the case of the whelk shell sample W-EcoT-1 all protein was removed when the sample was filtered through the 10kDa MWCO capsule. Conversely, both mackerel hydrolysates retained protein fractions when filtered through the 3kDa MWCO capsule (Table 3.7).

After filtration, each sample was analysed in triplicate using the CE-ACE assay method to quantify ACE-I inhibitory activity and to determine if the filtered fractions still retained ACE-I inhibition activity or if it was removed by the filtering process (Table 3.8). Stacked electropherograms of the three samples showing their filtered and unfiltered profiles were compared to confirm that the samples appeared more purified after each filtration step illustrated by the reduction in the number of peaks in their profile (Figures 3.6, 3.7 and 3.8).

As previously observed W-EcoT-1 hydrolysate was inactive for ACE-I inhibition and the 10kDa filtered samples which contained no protein fraction were also inactive (Table 3.8, Figure 3.8). Filtered mackerel hydrolysates still retained positive ACE-I inhibition activity suggesting that ACE-I inhibitory peptides were less than 10kDa in size (Table 3.8, Figures 3.6 & 3.7). Hydrolysate sample M-CorN-4 prior to filtration possessed an average of one hundred per cent ACE-I inhibition (Table 3.6). After sample filtration through the 10kDa MWCO capsule the average ACE-I inhibition percentage remained at one hundred per cent. After filtration through the 3kDa capsule it was found to be ninety one per cent a reduction of nine per cent with percentage RSD's of less than one point two per cent for each set of triplicates. It was concluded that the active ACE-I inhibitory component in this filtered hydrolysate was smaller or equal in size to 3kDa which would account for only the slight reduction in activity by the second filtration step.

Hydrolysate sample M-CorN-6 prior to filtration possessed an average of ninety four per cent ACE-I inhibition (Table 3.6). After sample filtration through the 10kDa MWCO capsule the average ACE-I inhibition percentage was determined to be eighty two per cent a reduction of twelve per cent with percentage RSD's of less than two per cent for each set of triplicates.

Table 3.8. Percentage ACE-I inhibition for 10kDa and 3kDa MWCO filtration of selected marine hydrolysates. ACE inhibition activity was determined by CE-ACE assay. Sample ID codes are described in text.

Sample ID	Sample No.	% ACE-Inhibition				
		Rep 1	Rep 2	Rep 3	Mean ± SD	% RSD
W-EcoT-1 10kDa	1	0.0	0.0	0.0	-	-
	2	0.0	0.0	0.0	-	-
	3	0.0	0.0	0.0	-	-
M-CorN-4 10kDa	1	100.0	100.0	100.0	-	-
	2	100.0	100.0	100.0	-	-
	3	100.0	100.0	100.0	-	-
M-CorN-4 3kDa	1	92.0	93.0	92.0	92.33 ± 0.58	0.63
	2	92.0	91.0	92.0	91.67 ± 0.58	0.63
	3	87.0	89.0	88.0	88.00 ± 1.00	1.14
M-CorN-6 10kDa	1	82.0	82.0	82.0	82.00 ± 0.00	0.00
	2	75.0	73.0	73.0	73.67 ± 1.15	1.57
	3	91.0	92.0	92.0	91.67 ± 0.58	0.63
M-CorN-6 3kDa	1	0.0	0.0	0.0	-	-
	2	65.0	50.0	64.0	59.67 ± 8.39	14.06
	3	0.0	0.0	0.0	-	-

After filtration through the 3kDa capsule it was found to be zero per cent (in two of the three samples). It was concluded that the active ACE-I inhibitory component was larger than 3kDa but smaller or equal in size to 10kDa which would explain why there was no inhibition with the smaller size fraction. It appeared that a possible experimental error occurred in sample two as only this one sample of the three analysed exhibited an average of sixty per cent inhibition. The percentage RSD obtained was also very high for the triplicates of this sample at fourteen per cent. It was most likely that the full aliquot of ACE enzyme, 25 µL was not added to this sample due to a pipetting error resulting in less HA production which appeared as inhibition. The decision to analyse three samples in triplicate each time for this assay method due to the small aliquots being measured was verified by highlighting this type of error.

The filtration process identified possible different ACE-I inhibitory peptides associated with mackerel hydrolysates. Considering that both mackerel hydrolysates were digested under the same conditions and using the same Corolase® N proteolytic enzyme the difference in potential ACE-I inhibitory peptides might be assumed due to differences from various mackerel tissue sources i.e. white meat tissues (M-CorN-4) as opposed to head, skin and tail portions (M-CorN-6). Alternatively, the ACE inhibitory peptide associated with each mackerel hydrolysate may be similar and incomplete of partial hydrolysis by Corolase® N may not have released the active 3kDa fraction from mackerel head, tail and skin portions (M-CorN-6). Further studies would have to clarify if the amino acid sequence is similar for both 10kDa and 3kDa fractions.

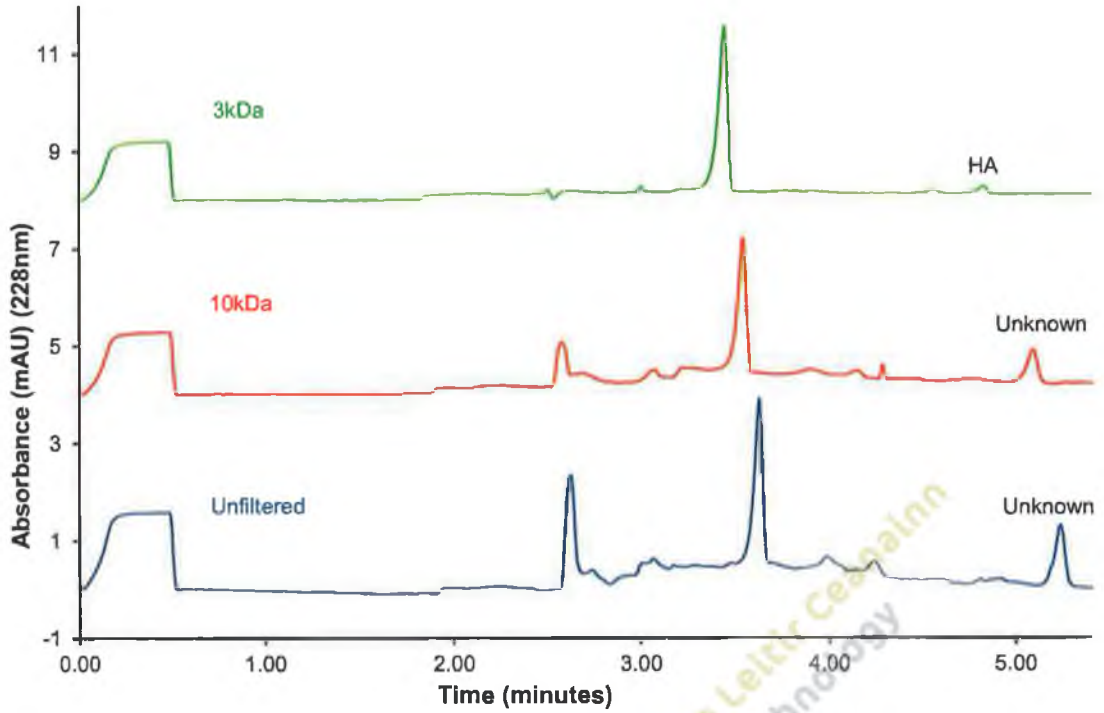


Figure 3.6. Stacked electropherogram for unfiltered, 10KDa and 3KDa filtered M-CorN-4 hydrolysate. ACE inhibition activity was determined by CE-ACE assay as described in Section 2.4

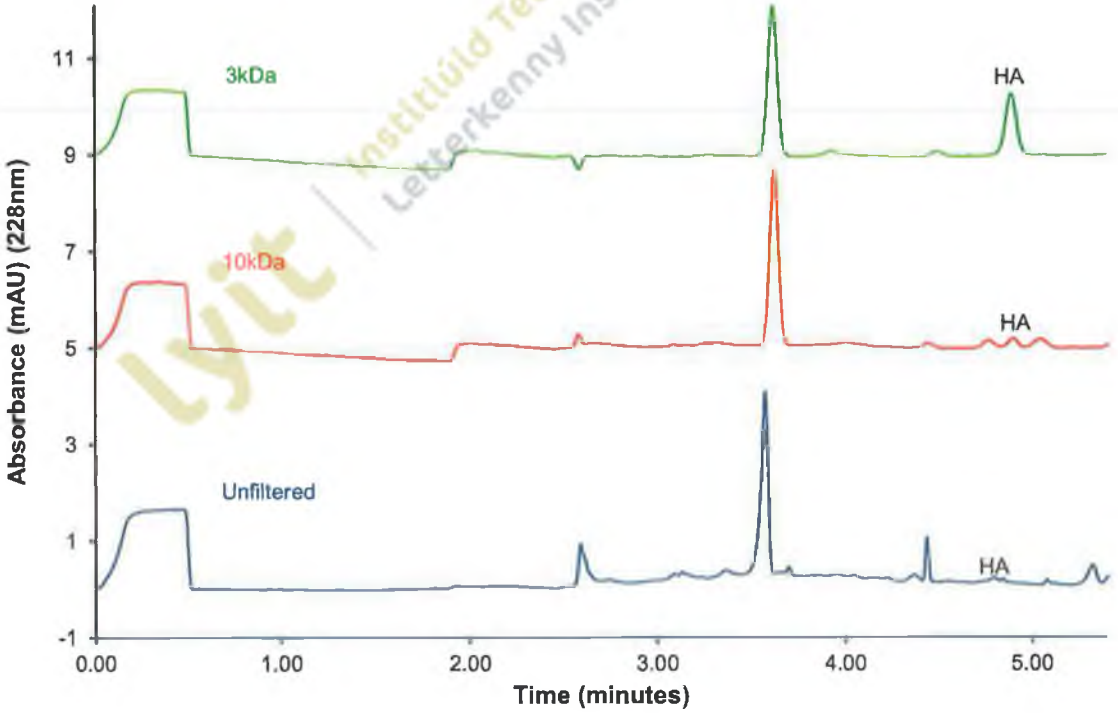


Figure 3.7. Stacked electropherogram for unfiltered, 10KDa and 3KDa filtered M-CorN-6 hydrolysate. ACE inhibition activity was determined by CE-ACE assay as described in Section 2.4

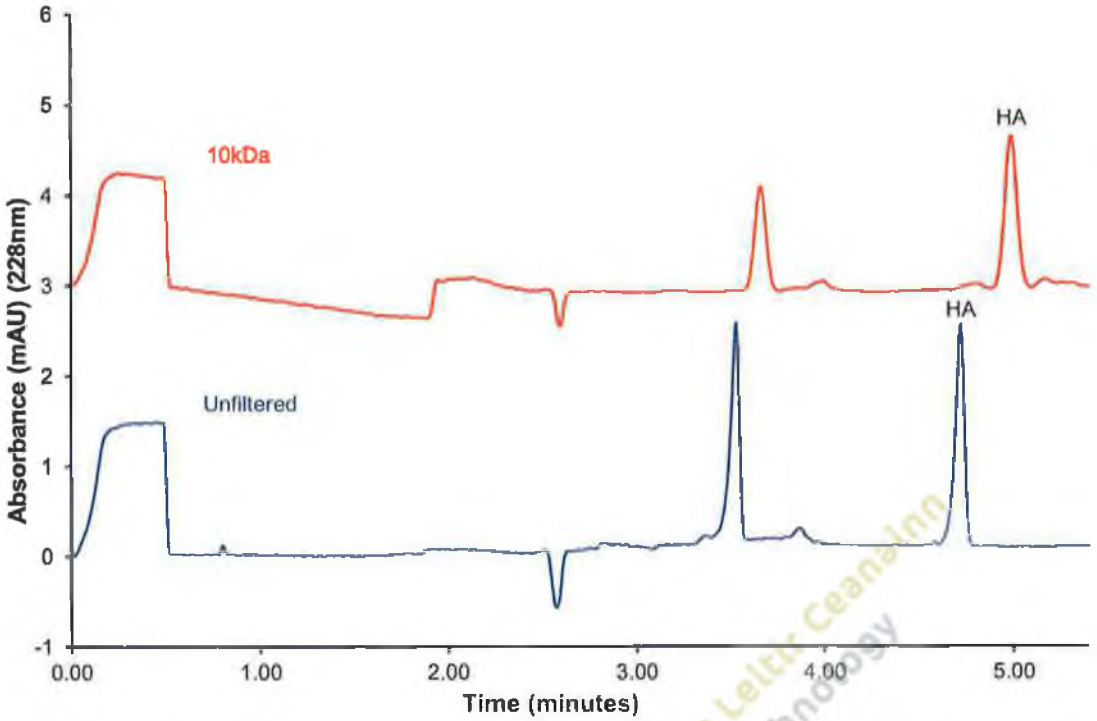


Figure 3.8. Stacked electropherogram for unfiltered and 10KDa filtered W-EcoT-1 hydrolysate. ACE inhibition activity was determined by CE-ACE assay as described in Section 2.4

3.5 Preliminary screening of marine hydrolysates for PEP inhibitory activity

A wide variety of PEP inhibitors have been described in scientific literature (Jarho, 2007) with several PEP inhibitors chemically synthesised (Morain *et al.*, 2002). In addition, PEP inhibitory peptides have been found in a variety of food sources as outlined in Table 1.2. As part of this study it was decided to investigate if marine hydrolysates generated for ACE inhibitory peptide studies were active for PEP inhibitory peptides. The PEP assay chosen for this preliminary screening of marine hydrolysates was prolyl endopeptidase activity from bovine calf serum. Prolyl endopeptidase activity from bovine calf serum was determined based on the enzyme's catalytic ability to cleave the prolyl synthetic substrate Z-glycyl-prolyl-AMC with the liberation of the fluorophore 7-amino-4-methylcoumarin (AMC) (Goossens *et al.* 1992). Prolyl endopeptidase catalytic activity was then calculated based on the amount of AMC fluorophore product formed. All the hydrolysed samples were pre-screened for possible PEP inhibitory activity using this assay method and assays were performed at Queens University Belfast as described in Section 2.5.

The positive PEP- inhibitor berberine inhibited the bovine calf serum enzyme source by an average of forty six per cent. Unexpectedly, all marine hydrolysates analysed were positive for inhibition of bovine calf serum with inhibitory activity ranging from thirty four to eighty per cent inhibition depending on the hydrolysate examined (Table 3.9). All samples were run in triplicate and there was excellent consistency between the triplicates as all % RSD's recorded were all below eight per cent, demonstrating a high degree of accuracy and precision was obtained with the analysis method. The average PEP inhibitory activity for the whelk hydrolysate W-EcoT-1 was thirty five per cent which was less than the berberine positive control. The average PEP inhibitory activity for all other hydrolysates were greater than the berberine positive control suggesting that these samples were potential novel sources of PEP inhibitory peptides. There was only slight difference between mackerel tissue sources and proteolytic enzyme employed in determining PEP inhibitory activity, however M-CorN-6 yielded higher PEP inhibitory activity (>76%) for all three replicate samples. This observation might suggest that head, tail and skin mackerel portions may be a suitable target for future PEP inhibitory studies.

These preliminary observations only demonstrate the potential for identifying PEP inhibitory peptides from marine waste sources. Future work could explore the use of a more specific PEP enzyme source from *Flavobacterium meningosepticum* (Yanai *et al.* 2003) however on this occasion time did not permit.

Table 3.9. Percentage PEP inhibiton for whelk (W) and mackerel (M) hydrolysates determined by PEP fluorogenic assay. Sample ID codes are described in text.

Sample ID	% PEP-Inhibition				
	Rep 1	Rep 2	Rep 3	Mean ± SD	% RSD
W-EcoT-1	34.28	34.85	36.85	35.33 ± 1.35	3.82
M-Pep-1	69.60	63.90	61.11	64.87 ± 4.33	6.67
M-CorN-1	47.77	53.36	55.88	52.34 ± 4.15	7.94
M-CorP-1	77.01	72.49	68.43	72.64 ± 4.29	5.91
M-PepCorL-1	71.27	71.25	75.24	72.72 ± 2.54	3.49
M-PepTherM-1	63.62	64.46	68.60	65.46 ± 2.78	4.24
M-CorN-2	69.18	69.96	68.37	69.17 ± 0.79	1.14
M-CorN-3	64.71	62.05	61.46	62.74 ± 1.73	2.76
M-PepTherM-2	69.07	69.81	69.83	69.57 ± 0.43	0.62
M-CorN-4	63.42	65.03	61.67	63.37 ± 1.68	2.65
M-CorN-5	67.46	66.97	69.63	68.02 ± 1.42	2.08
M-CorN-6	77.06	76.51	80.00	77.86 ± 1.88	2.41

4 Conclusions and future work

Like many natural resource-based processing industries, the seafood processing sector gives rise to a significant volume of organic waste, an estimated 45% of which arises in the northwest region of Ireland (Pfeiffer 2003). Environmental issues, economic concerns and legal restrictions regarding the disposal of processing wastes have led to increased research in the discovery of alternative value-added products, such as bioactive peptides from these waste streams. Maximising profitability from the available supply of raw material is recognised as a priority for the marine processing sector (Marine Institute's Sea Change Strategy 2007-2013). This necessitates increasing the proportion of the catch destined for human consumption and secondly extracting high value products such as pharmaceuticals and functional food ingredients from the waste stream. Bioactive compounds of both scientific interest and commercial value may be available for extraction directly from marine processing waste or alternatively may be formed from components present in the waste by microbial fermentation and/or enzyme hydrolysis steps before extraction is undertaken. Identification of bioactives of natural origin is a growing field and the use of fish processing by-products as substrates to generate high-value bioactive products is a new approach with potential for the functional foods sector. The main focus of this thesis was to examine whether mackerel and whelk fisheries processing by-products and mackerel meat tissues are suitable sources for the isolation of angiotensin-I-converting enzyme (ACE-I) and prolyl endopeptidase (PEP) inhibitory peptides.

The initial problem in the isolation of bioactive peptides from natural sources is the establishment of proteolytic hydrolysis conditions. In this study, mackerel and whelk hydrolysates were prepared by examining different commercial enzymes including Ecostone®A200, Corolase® N, Corolase® PP, Corolase® LAP, Pepsin and Thermolysin. All enzyme hydrolysis conditions selected were based on the manufacturer specifications and the final hydrolytic conditions are provided in Table 2.2. Hydrolytic reactions were controlled by use of an Infors Multifors HT Fermenter which allowed for standardisation of reaction conditions and offered the ability to repeat hydrolytic conditions when necessary. The approach of using different hydrolytic enzymes was to identify a suitable enzyme source for the releases of bioactive peptides. Preliminary screening of marine hydrolysates using either single or double enzyme digests indicated that this approach was suitable for the release of potential ACE-I and PEP inhibitory peptides (Section 3.1 & 3.5). Future studies could

explore altering the hydrolytic parameters such as pH, temperature and incubation period of a single selected hydrolytic enzyme in order to optimise enzyme activity and release of bioactive peptides.

A fundamental aspect in the characterisation of bioactive peptides is the development of suitable and reliable assays for the detection of the target peptide. Issues of cost, speed, specificity, sensitivity and reliability are major concerns in the selection of suitable assays. In addition, bioassays have the ability to pre-screen samples in order to determine if candidates are selected for further studies. Since ACE-I and PEP inhibitory peptides were the bioactive peptides of interest, suitable bioassays for these peptides were examined for pre-screening of marine hydrolysates.

Prolyl endopeptidase activity and inhibition studies were determined by the method of Goossens *et al.* (1992) and this bioassay method suggested that all marine hydrolysate samples contained potential PEP inhibitory peptides (Table 3.9). This bioassay was simple and rapid to use and had the only special requirement a Tecan Safire^2 microtitre plate reader. The other advantage of this bioassay was the ability of the assay to simultaneously screen multiple samples using the microtitre plate reader. Although, all marine hydrolysate samples were positive for potential PEP inhibitory peptides the assay should be repeated to confirm initial observations and future work should explore the use of a more specific PEP enzyme source from *Flavobacterium meningosepticum* (Yanai *et al.*, 2003). These preliminary observations only demonstrate the potential for identifying PEP inhibitory peptides from marine waste sources.

Cushman & Cheung (1971) is the standard method for determining ACE-I inhibitory activity and the assay relies on the release of HA from HHL substrate using a purified ACE enzyme source. The method requires several steps and the HA can be contaminated with ethyl acetate which also absorbs strongly at 228 nm unless fully evaporated using nitrogen evaporation (Roy *et al.*, 2000). Although the method was attempted in this laboratory using Captopril® as a positive ACE-I inhibitory control the method proved unreliable under test conditions (data not shown). An alternative and validated colorimetric ACE-I bioassay by Serra *et al.* (2005) was employed to screen marine hydrolysate samples (Section 2.3 & 3.1). This ACE-I colorimetric assay was suitable for pre-screening samples for ACE-I inhibitory activity since the positive Captopril® ACE inhibitory control yielded forty to fifty per cent inhibition and as

reported in this study the mean percentage ACE-I inhibition for positive marine hydrolysates ranged from twenty five to thirty nine per cent inhibition (Table 3.1; Table 3.2 & Table 3.5). These percentage ACE-I inhibition values correspond well with the reported values of 8% to 57% for plant extracts by Serra *et al.* (2005) using the same method. The particular advantages of the colorimetric ACE bioassay were the use of inexpensive reagents and crude ACE enzyme extract, the short assay time and the ability to simultaneously screen multiple samples using the BMG Labtech microtitre plate reader capable of analysing 96-well plates leading to rapid throughput of samples. The disadvantages included the poor sensitivity of the assay (see below), the variability in accuracy and precision of the assay (Section 3.1) and the requirement for a microtitre plate reader.

Although the colorimetric ACE-I bioassay was suitable for pre-screening marine hydrolysates to identify potential ACE-I inhibitory peptides the method was not accurate in quantifying the percentage ACE-I inhibition in samples. A more robust capillary electrophoresis ACE (CE-ACE) assay method was developed to accurately quantify the percentage inhibition associated with marine hydrolysates. ACE activity was determined by a CE modification of the Cushman and Cheung (1971) method and optimum capillary electrophoretic conditions were determined based on the methods reported by Zhang *et al.* (2000) and He *et al.* (2007) (Section 2.4). The CE method developed in this study was extremely sensitive (Section 3.2.2), had a rapid analysis time (< 5minutes) and was accurate to less than five per cent RSD for all positive ACE-I inhibitory marine hydrolysates (Table 3.4 & 3.6). In addition the method could be automated with the inclusion an auto-sampler to the Beckman Coulter P/ACE ™ MDQ Capillary Electrophoresis system. Moreover the method requires much less sample, substrate and other reagents and has high reproducibility (Zhang *et al.* 2000). This approach should prove to be valuable in developing diagnostic methods of ACE activity assay and screening for ACE inhibitory peptides.

As the majority of ACE-I inhibitors discovered to date are peptidic in origin, the main focus for determination of possible ACE-I inhibitors was reserved for the mackerel tissues. It was found that commercially available proteolytic enzymes, in particular Corolase® N were capable of generating novel ACE-I inhibitory peptides through the controlled hydrolysis of fish waste and meat tissue from mackerel. Mackerel body parts such as white muscle tissue, head, skin and tail were found to generate active ACE-I inhibitory bioactive peptides.

Positive ACE-I inhibitory activity as determined by CE-ACE bioassay ranged from seventy one to one hundred per cent depending on the mackerel tissue source and hydrolytic enzyme employed (Table 3.6).

Based on the ACE-I inhibitory studies of mackerel tissue two ACE-I inhibitory mackerel tissue samples were selected for further concentration and purification. Sample M-CorN-6, the head, tail and skin sample prior to concentration possessed an average of ninety four per cent ACE-I inhibition. After sample filtration through the 10kDa MWCO capsule the average ACE-I inhibition percentage was determined to be eighty two per cent a reduction of twelve per cent. After filtration through the 3kDa capsule it was found to be zero per cent. It was concluded that the active ACE-I inhibitory component was larger than 3kDa but smaller or equal in size to 10kDa which would explain why there was no inhibition with the smaller size fraction (Table 3.8). Sample M-CorN-4, a mackerel white tissue sample, prior to concentration possessed an average of one hundred per cent ACE-I inhibition. After filtration through the 10kDa MWCO capsule the average ACE-I inhibition percentage remained at one hundred per cent. After further filtration through the 3kDa capsule it was found to be ninety one per cent a reduction of only nine per cent (Table 3.8). It was concluded that the active ACE-I inhibitory component in this sample was smaller or equal in size to 3kDa which would account for only the slight reduction in activity by the second filtration step.

Results indicated that the experimental strategy employed and the utilisation of marine waste from mackerel and whelk species may serve as an approach to identifying bioactive peptides capable of generation of ACE-I and bovine calf serum PEP inhibitors through controlled hydrolysis. The potential for development of these techniques in search of bioactive peptides for human exploitation from marine processing wastes would bring increased value to what is today considered an environmental waste problem. As two mackerel hydrolysates M-CorN-4 3kDa and M-CorN-6 10kDa were generated from different mackerel tissue sources (Section 3.4) there is the possibility that these hydrolysates may possess different ACE-I inhibitory peptides and may be of novel origin. Future work would include further purification and characterization of the concentrated ACE-I inhibitory samples using RP-HPLC followed by peptide sequence elucidation using MALDI-TOF MS analysis or Edman degradation studies.

This project addressed the issue of waste remediation by examining the potential for the production of high-value products other than enzymes from the waste stream, rather than regarding the waste as a disposal problem or for the manufacture of low economic value

fertilizers and animal feedstuffs. Advances in bioprocess engineering technologies such as more efficient design and development of manufacturing equipment and processes such as bioreactors, together with novel use of nanotechnology methods such as using chitin, chitosan and crab shell waste fermentation products to encapsulate functional food ingredients (Srinivosa and Tharanatham, 2007) will mean that fish processing wastes in the future may serve as inexpensive raw materials in the generation of high-value bioactive compounds which may have a variety of applications (Kim and Mendis, 2006). Development of these technologies will ensure the exploitation of potential added-value products from this waste stream and will present unique challenges and opportunities for the seafood industry worldwide. As ACE-I and PEP inhibitors can be sourced from naturally occurring plants and herbal remedies the possibility that hypertension and PEP associated disorders may be modulated through dietary intake is a real possibility.

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