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Marine Cod (*Gadus morhua*) Head Hydrolysates

in vitro ACE inhibitory activity and
investigation of structure-activity relationship

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Preface

This thesis marks the completion of the master's degree in biotechnology (MBIOT5) at the Department of Biotechnology and Food Science (IBM) at the Norwegian University of Science and Technology (NTNU). The thesis was conducted in collaboration with SINTEF Ocean in connection with the project HEADS UP.

First of all, I would like to thank my supervisor professor Turid Rustad for all her knowledge, guidance and feedback throughout the whole process of this master thesis. I also wish to thank Veronica Hjellnes at the food chemistry lab for all help and support during the laboratory work, fellow master student Marte Vilde Monslaup for good company during analyses, and Siri Stavrum for running the HPLC analyses.

I send my deepest gratitude to my family for motivation and encouragement. And last, but not least, I would like to thank my fellow students at NTNU for all support throughout this thesis and for making these five years in Trondheim unforgettable.

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Abstract

Marine by-products contain valuable nutrients and there is an increasing interest in production of value-added products for human consumption.

Cod (*Gadus morhua*) head hydrolysates produced by SINTEF Ocean in the pilot project HEADS UP were the base for analyses in this thesis. The hydrolysates were produced from cod harvested in Tufjord in February, March or May of 2017 with varying processing conditions, namely proteolytic enzymes (Bromelain + Papain or Protamex), concentration of enzymes and amount of water. One hydrolysate was produced with stick water, and two of the hydrolysates were added antioxidants. The thesis aimed to measure *in vitro* ACE inhibitory activity of the hydrolysates, and further investigate structure-activity relationship.

All the cod head hydrolysates expressed ACE inhibitory activity *in vitro*, and IC_{50} values ranged from 2.60 to 4.91 mg protein/mL. The inhibitory effect exerted by one of the hydrolysates was misleading and was therefore not included in this range. The ACE inhibitory activities of the hydrolysates were found to be relatively low compared to the antihypertensive drug Captopril (IC_{50} = 0.097 mg/mL) and relatively high compared to inhibitor peptides from other origins than cod heads.

Four of the hydrolysates were fractionated by ultrafiltration. The fractions enriched in small peptides were found to exert less potent ACE inhibitory activity than the unfractionated hydrolysates. It is therefore suggested that fractionation is unnecessary to increase antihypertensive effects of the cod head hydrolysates.

The hydrolysates were composed of amino acids that have been found to contribute to inhibition of ACE, including leucine, isoleucine, valine, phenylalanine and methionine, in addition to a balanced distribution and high content of essential amino acids (average content 38.8 ± 11%). The hydrolysates contained peptides with broad molecular weight distribution and contained low amounts of free amino acids. No correlation was found between degree of hydrolysis and IC_{50}.

No correlations were found between season of harvest of the cod and chemical composition of the cod head hydrolysates. Additionally, no correlations were found between processing parameters and ACE inhibitory activity and molecular properties of the hydrolysates. The use of stick water in the production of one hydrolysate did affect the sensory aspects, but not molecular properties.

Cod head hydrolysates were found to be a promising potential food ingredient with antihypertensive effects, high solubility, no bitter taste and good nutritional value due to low amounts of free amino acids and high amounts of essential amino acids.

Sammendrag

Marine biprodukter inneholder verdifulle næringsstoffer, og det er en økende interesse for å produsere videreforedlede produkter til humant konsum.

Torskehodehydrolysater produsert av SINTEF Ocean i pilotprosjektet HEADS UP ble brukt som basis for alle analyser utført i denne oppgaven. Hydrolysatene ble produsert fra torsk (*Gadus morhua*) innhentet i Tufjord i februar, mars og mai 2017 med varierende produksjonsbetingelser, henholdsvis proteolytiske enzymer (Bromelain + Papain eller Protamex), konsentrasjon av enzymene og mengde vann. Et av hydrolysatene ble produsert med limvann, og to av hydrolysatene ble tilsatt antioksidanter. Målet i denne oppgaven var å måle *in vitro* ACE inhiberende aktivitet i hydrolysatene, og videre undersøke sammenhenger mellom kjemisk struktur og inhiberende aktivitet.

Alle hydrolysatene utøvde ACE inhiberende effekt *in vitro*, og IC_{50}-verdiene ble beregnet til 2,60 – 4,91 mg protein/mL. Den inhiberende effekten til ett av hydrolysatene var villedende, og ble derfor ikke inkludert. De ACE-inhiberende effektene til hydrolysatene ble vurdert til å være relativt lave sammenlignet med den blodtrykkssenkende medisinen Captopril (IC_{50} = 0,097 mg/mL), og relativt høye sammenlignet med inhiberende peptider isolert fra andre opphav enn torskehoder.

Fire av hydrolysatene ble fraksjonert ved hjelp av ultrafiltrering. Fraksjonene anriket med små peptider ble funnet til å utøve svakere ACE inhiberende aktivitet sammenlignet med de ufraksjonerte hydrolysatene. Det er derfor foreslått at fraksjonering er unødvendig for å øke antihypertensive effekter utøvet av torskehodehydrolysatene.

Hydrolysatene ble bestemt til å inneholde aminosyrer som er bevist involvert i inhibering av ACE, inkludert leucin, isoleucin, valin, fenylalanin og metionin. Det ble i tillegg bestemt at hydrolysatene inneholdt en balansert fordeling og høy andel av essensielle aminosyrer (gjennomsnittlig innhold 38,8 ± 11 %). Hydrolysatene bestod av peptider med en bred fordeling av molekylvekter, og inneholdt små mengder med frie aminosyrer. Ingen sammenheng ble funnet mellom hydrolysegrad og IC_{50}.

Sesongvariasjon og produksjonsbetingelser ble funnet til å ikke påvirke den kjemiske sammensetningen av hydrolysatene. Bruk av limvann i produksjonen av et av hydrolysatene påvirket dets sensoriske kvalitet, men ikke kjemiske sammensetning.

Torskehodehydrolysat er en lovende potensiell matingrediens med antihypertensive effekter, høy løselighet, ingen bitter smak og høy næringsverdi grunnet små mengder frie aminosyrer og høye mengder essensielle aminosyrer.

Abbreviations

ACE	**A**ngiotensin I-**C**onverting **E**nzyme
ACEI	**A**ngiotensin I-**C**onverting **E**nzyme **I**nhibition
BSA	**B**ovine **S**erum **A**lbumin
CHH	**C**od **H**ead **H**ydrolysate
DH	**D**egree of **H**ydrolysis
FPH	**F**ish **P**rotein **H**ydrolysate
FPLC	**F**ast **P**rotein **L**iquid **C**hromatography
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
IC_{50}	**I**nhibitor **C**oncentration that inhibits **50**% of ACE activity
SHR	**S**pontaneously **H**ypertensive **R**ats

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Chapter 1: Introduction

1.1. Background and motivation

Global fish resources are limited, and the worldwide population is continually growing. Utilization of marine resources to their full potential is therefore of great interest (Rustad *et al.*, 2011). By-products generated from fish processing have large potential for utilization and economic benefits for the fish industry. By-products can be defined as fish products that are unsuitable for direct human consumption, including viscera, cut-offs, heads, frame and skin (Rustad *et al.*, 2011). By-products are further described in section 1.2.1. Globally, marine nutrients provide only 2% of the daily calorie intake and 15% of animal protein intake (Keskitalo *et al.*, 2017). An increased utilization of by-products for human consumption is therefore of interest.

In 2014, the global amount of harvested wild fish exceeded 90 million tons, and more than 70 million tons of farmed fish were produced (FAO, 2016). Post-harvest losses have been estimated by FAO to account for 27% of all landed fish, and 50% of round fish is commonly discarded during processing of sea food (FAO, 2016; Guérard *et al.*, 2005). By-products can account for two third of the weight of round cod when viscera are included (Mackie, 1974; Šližytė *et al.*, 2005; Falch *et al.*, 2006). In Alaska, 300 000 tons of Pacific salmon (*Oncorhynchus* sp.) is annually harvested, which generate 50 000 tons of heads and 30 000 tons of viscera (Sathivel *et al.*, 2005).

According to the annual analysis report of marine by-products by SINTEF Ocean, approximately 3.3 million tons of fish and crustaceans were produced from live catch and farmed seafood in Norway in 2016, of which 910 000 tons (28%) were considered by-products. Approximately 76% (688 000 tons) of the by-products from all sectors were utilized (Richardsen *et al.*, 2017). Demersal fisheries accounted for the largest fraction of unexploited by-products; only 56% of the demersal fish by-products were utilized. This high number is mainly due to lack of technology for onboard processing on trawlers and fishing fleets in open waters, as well as lack of economic incentives for the fishing fleet to transport the by-products ashore.

Only 28% of the by-products from landed crustaceans were utilized in Norway in 2016. In contrast, up to 91% of the by-products from aquaculture were utilized, and 100% from the pelagic fish sector (Richardsen *et al.*, 2017). Aquaculture plants are located close to shore; thus, the fish can be processed shortly after harvesting. Pelagic fish are caught off-shore but are stored in refrigerated sea water systems (RSW) upon transport to shore-based processing plants.

By-products are normally processed for production of fish meal, silage, fertilizer and animal feed, which are considered low market-value products (Hsu, 2010). Fish by-products include valuable fractions of nutrients; especially proteins and oils, but also vitamins and minerals. Considering the content of these, production of high-value products containing for example protein hydrolysates, fish blood and marine lipids, for example omega-3 fatty acids, may give great potentials for human consumption (Rustad *et al.*, 2011). Other advantages include economic benefits for the fish industry and environmental benefits due to a decrease in disposal of waste (Chalamaiah *et al.*, 2012).

Marine by-products contribute to an important value-adding resource for the Norwegian fisheries and aquaculture industry. An increased focus on utilizing the by-products has led to good exploitation of these resources by several companies. There are however great potentials in increasing the degree of exploitation, especially in the demersal fisheries (Richardsen *et al.*, 2017).

1.2. Fish by-products

1.2.1. Definition of by-products

The most used nomenclature for the excess raw materials from marine harvest and catch is *by-products*. However, several terms have previously been used, including waste, co-streams and co-products. Some of these may induce negative associations as they potentially undermine the potential utilization of these valuable raw materials. *Rest-raw materials* is a term that might be used to indicate the potentials of these products, but *by-products* is the most widely and commonly used expression (Rustad *et al.*, 2011). This term will thus be used throughout this thesis.

There are several definitions of by-products, one of which describes them as products that are unsuitable for direct human consumption, including heads, viscera, cut-offs, bone and skin, as well as bycatch (Rustad *et al.*, 2011). Regulatory papers distinguish between by-products that can be processed for human consumption and parts that cannot, including waste and discards (Rustad, 2003).

A definition developed in the Norwegian fish industry distinguishes between by-products and waste. *By-products* are in this definition described as products that are not considered the main saleable products (fillet, round, eviscerated or beheaded fish), but products that can be recycled after treatment. *Waste* is in this definition described as raw materials that are not suitable for consumption or value-added products, and is burned, destroyed or composted (Bekkevold and Olafsen, 2007).

1.2.2. Applications and biochemical properties of by-products

The applications of marine by-products in the Norwegian industry per 2016 are presented in figure 1.1.

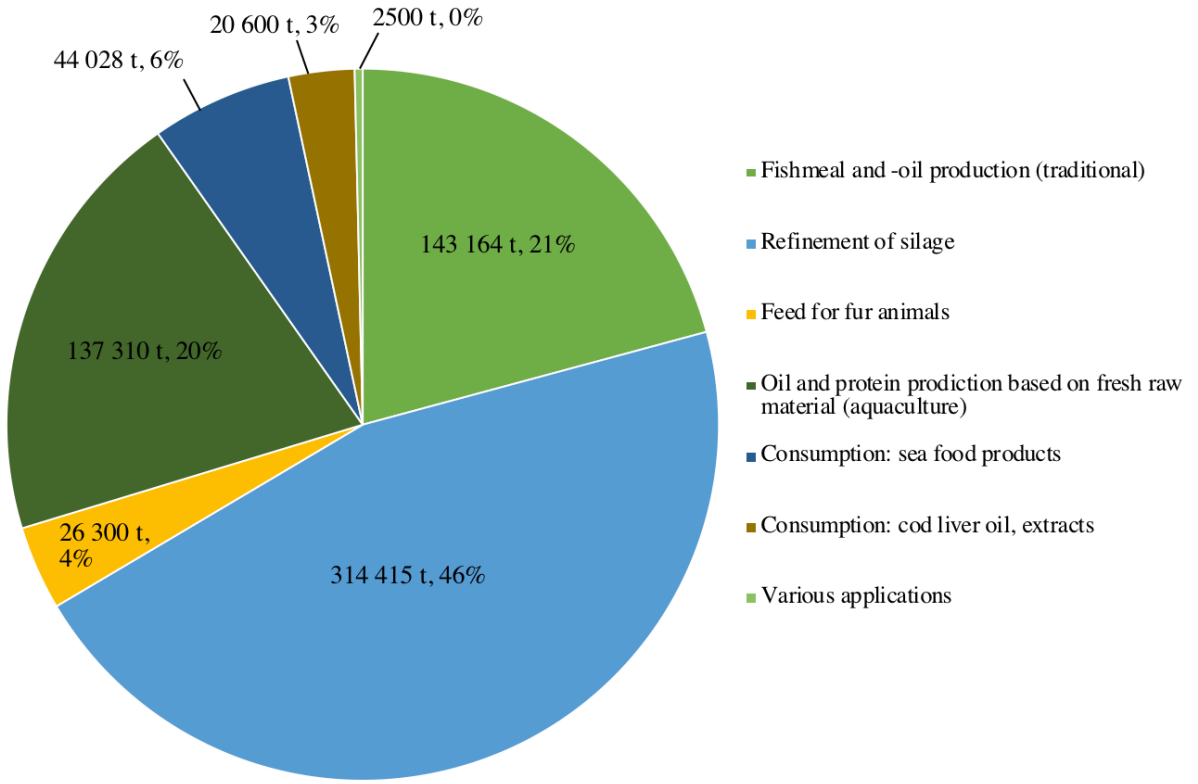

Figure 1.1: Applications of by-products in Norwegian industry in 2016 (measured in tons (t) and %). Figure modified from "Analysis of marine by-products, 2016" by SINTEF Ocean (Richardsen *et al.*, 2017).

In 2016, approximately 10% of marine by-products were consumed directly by humans. This includes dried fish heads, roe, liver and tongues, while 3% were consumed as cod liver oil and protein extracts. The category "various applications" includes chitin/chitosan for use in cosmetics, among other things (figure 1.1) (Richardsen *et al.*, 2017).

According to the analysis report by SINTEF Ocean, the biggest fractions of unexploited by-products in Norway in 2016 were heads, viscera, liver and roe from demersal fisheries (Richardsen *et al.*, 2017). These fractions contain valuable nutrients, as shown for heads from cod, saithe and haddock in table 1.1.

Table 1.1: Chemical composition of heads from cod, saithe and haddock (weight%) (Økland and Kjerstad, 2002).

	Water (%)	**Protein (%)**	**Lipids (%)**	**Ash (%)**
Cod	79.3	14.2	4.1	6.4
Saithe	77.9	16.6	< 1	5.3
Haddock	78.9	13.1	< 1	7.0

The numbers show that the fish heads are relatively lean, and the potential for using the protein fraction enables production of for example protein hydrolysate or fish gelatin, which are considered value-added products (Økland and Kjerstad, 2002).

1.2.3. Utilization of cod heads

The work conducted in this thesis was done in relation to the project HEADS UP by SINTEF Ocean, which was a pilot project for production hydrolysates from cod (*Gadus morhua*) heads. The project was carried out in collaboration with the Norwegian Seafood Research Fund (FHF) and Fjordlaks AS. Cod heads from Norway have traditionally been dried and exported to Nigeria and Asia. However, this is a resource intensive production which is time and labor consuming, and the market for dried cod heads is unpredictable. Fjordlaks has, like many others in conventional industry, dried and exported cod heads. In that occasion, SINTEF Ocean started the pilot project in order to investigate the possibilities for production of high quality protein hydrolysates from cod heads aimed for human consumption (FHF, 2018; FHF, 2016).

1.2.4. Stability and quality of by-products

Successful utilization of by-products highly depends on the initial quality of the raw material and the following quality of the by-products. By-products can be divided into two major groups based on the possible degradation and spoilage: easy degradable and relatively stable, respectively. The easy degradable by-products contain fractions with high content of endogenous enzymes (digestive enzymes naturally present in the tissue), for instance viscera, whereas the second group contain more stable by-products such as head, bones and skin (Rustad *et al.*, 2011).

Industrial separation of by-products breaks down the biological unit of the cells, causing release of endogenous enzymes and free access to oxygen, which lead to increased rate of degradation (Rustad *et al.*, 2011). The release of endogenous enzymes initiates autolysis, which is the degradation process of proteins, carbohydrates, lipids and nucleic acids in tissues (Thorkelsson *et al.*, 2009). To ensure high quality of the products produced, by-products must be processed or chilled rapidly after production (Rustad *et al.*, 2011). The rate of enzymatic degradation in by-products depends on amount and types of endogenous enzymes, but also fish species, season, gender and how the raw material is handled and processed.

By-products are susceptible for microbial spoilage. By-product fractions such as intestines and gills contain high numbers of bacteria, and fractions with high content of blood are highly susceptible to microbial spoilage. Spoilage caused by bacteria can be limited by handling the by-products hygienically to prevent introduction and contamination of spoilage bacteria from equipment or people, as well as separating easily degradable fractions from the more stable ones (Thorkelsson *et al.*, 2009; Rustad *et al.*, 2011).

Stability of proteins

Proteolysis is highly affecting the stability of protein fractions in by-products during storage. In addition, reaction products from the lipid fraction of fatty fish may affect the stability of and yield of protein fractions, for instance by reactions between proteins and products from lipid oxidation. These reactions may alter the amino acid composition and molecular weight of the proteins, which may negatively affect the nutritional value of the protein fractions. Altered protein conformation may additionally impact functional properties of the by-product and sensory aspects, including texture and color (Thorkelsson *et al.*, 2009).

1.2.5. Challenges in utilizing by-products

One challenge in using by-products may include establishment of stable and sustainable supplies of acceptable by-products. The suppliers have to deliver by-products of constant defined and stable quality. (Thorkelsson *et al.*, 2009). This challenge might limit large-scale production of value-added products.

Another challenge might be that utilization of processed marine by-products in food systems can introduce skepticism among people (Rustad *et al.*, 2011). This however depend on the market.

1.3. Hydrolysis and fish protein hydrolysate (FPH)

Hydrolytic modification of food proteins is a widely utilized method for improving sensory characteristics and the stability of available protein sources (Adler-Nissen, 1986). Protein hydrolysate is the product obtained through breakdown of native proteins into peptides of various sizes through enzymatic or chemical processing (Chalamaiah *et al.*, 2012; Skanderby, 1994). Enzymatic hydrolysis of proteins is carried out by endogenous or exogenous proteolytic enzymes or a combination to cleave peptide bonds, whereas chemical hydrolysis is conducted by cleaving peptide bonds with acid or base.

Chemical hydrolysis has been widely used industrially because it is relatively inexpensive and easy to conduct. However, this method introduces some limitations to food ingredients. Chemical hydrolysis can be difficult to control and tends to result in hydrolysates with varying chemical composition and functional properties. Additionally, this process is often performed at extreme temperatures and pH, which leads to products with reduced nutritional qualities and poor functionality (Kristinsson and Rasco, 2000b). Enzymatic hydrolysis is carried out under milder conditions than chemical hydrolysis. The use of enzymes allows good control of the hydrolysis and the properties of the resulting products (Kristinsson and Rasco, 2000b).

The peptides resulting from hydrolysis are relatively small, which enables easy digestion and absorption in the body (Chalamaiah *et al.*, 2012). Protein hydrolysates are produced from a variety of raw materials, including soy (Tsumura *et al.*, 2005), dairy products (Peñas *et al.*, 2006) and fish (Šližytė *et al.*, 2005).

The product obtained from hydrolysis of fish by-products is referred to as fish protein hydrolysate (FPH), and raw materials typically used for production of FPH are fish skin, head, muscle, viscera, liver, bone, frame and roe or egg (Chalamaiah *et al.*, 2012). Production of FPH is considered a beneficial process for extracting proteins from by-products.

1.3.1. Enzymatic hydrolysis

The steps in an enzymatic hydrolysis of by-products is presented in figure 1.2. Several variations are used in industry and research, but this flowchart shows the general steps of the process.

After by-product fractions are generated or received, they are minced or homogenized. Water and proteolytic enzymes are added, and the hydrolysis is carried out until desired time or degree of hydrolysis is reached. Termination of the hydrolysis is carried out by inactivation of the enzymes by low pH or high temperatures. The resulting mixture after hydrolysis, which is referred to as the slurry, is separated into fractions by centrifugation. The fractions obtained are sludge on the bottom, aqueous phase in the middle (also referred to as the supernatant, containing the FPH), and, depending in the lipid content of the raw material, a lipid-protein fraction between the aqueous phase and the sludge, an oil-water emulsion and an oil layer on the top (Cui, 1996). The aqueous phase is then separated and either concentrated or dehydrated by evaporation or freeze or spray drying (Kristinsson and Rasco, 2000b).

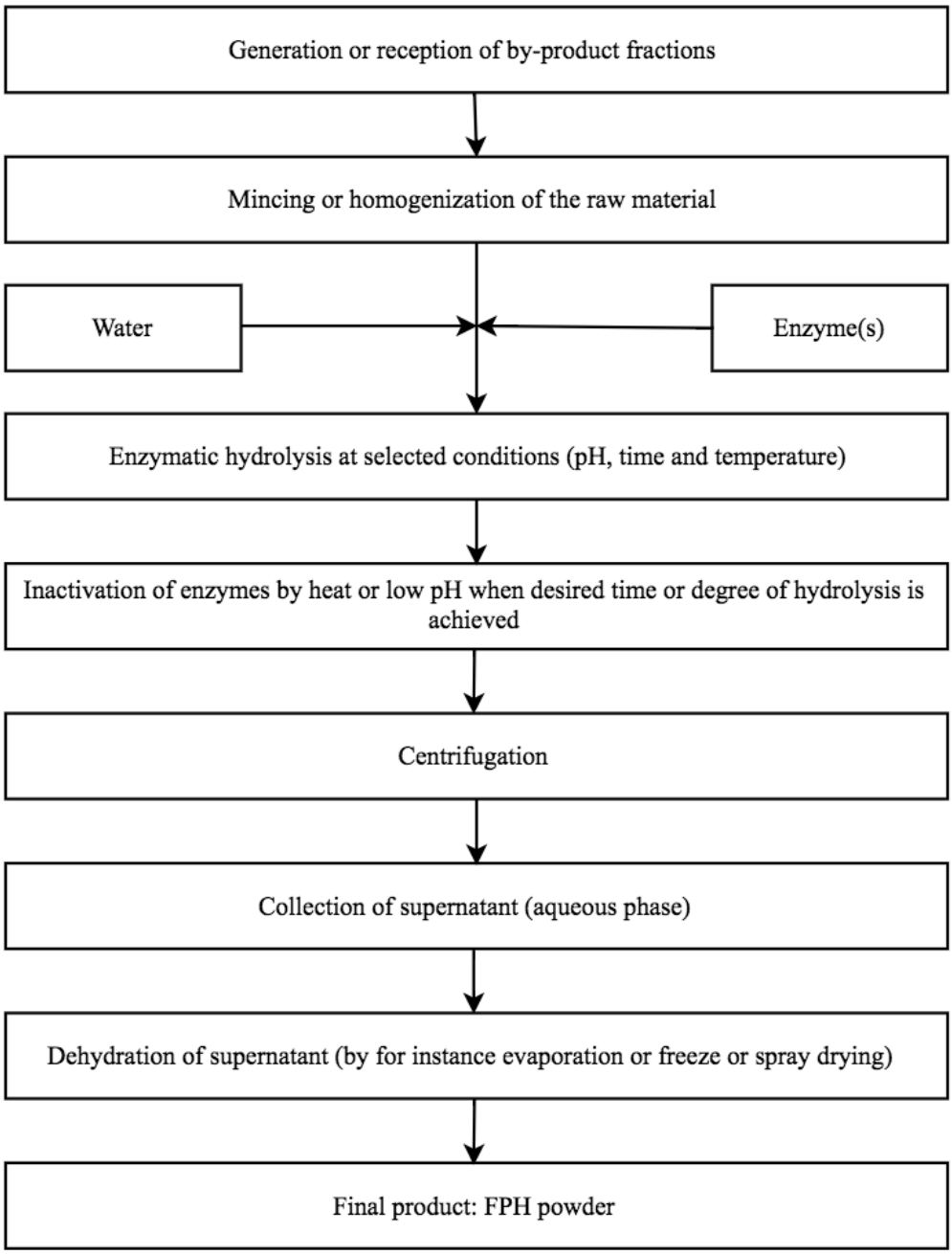

Figure 1.2: Flowchart describing the general steps in an enzymatic hydrolysis for production of FPH from marine by-products. The figure is modified from Kristinsson and Rasco (2000b) and Benjakul *et al*. (2014).

Proteolytic enzymes

Enzymatic hydrolysis of proteins is performed by proteolytic enzymes, also called peptidases or proteases. These enzymes possess varying substrate specificities (Lahl and Braun, 1994), and are divided into two groups based on their hydrolysis mechanism: endopeptidases and exopeptidases, respectively. Endopeptidases hydrolyze peptide bonds within protein molecules,

resulting in peptides of bigger size and a small amount of free amino acids. Exopeptidases cleave terminal peptide bonds at either the N-terminus (aminopeptidases) or the C-terminus (carboxypeptidases), resulting in a systematic release of terminal amino acids (Kristinsson and Rasco, 2000b). An illustration of the proteolytic attack is shown in figure 1.3.

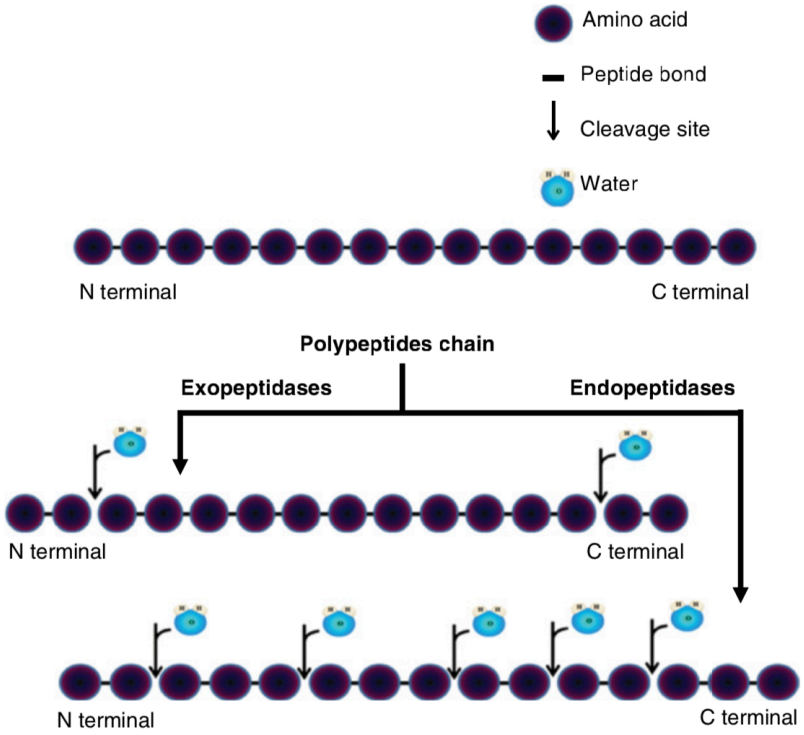

Figure 1.3: Illustration of the proteolytic degradation of a polypeptide. Exopeptidases hydrolyze the protein from either the N-terminus (aminopeptidases) or the C-terminus (carboxypeptidases), and the endopeptidases cleave peptide bonds within the protein (Benjakul *et al.*, 2014).

Endogenous proteases

Autolytic hydrolysis is carried out by endogenous enzymes (Kristinsson and Rasco, 2000b). This hydrolytic activity depends on the concentration of endogenous enzymes in the raw material. Viscera and kidneys contain high amounts of these enzymes, whereas for example heads and cut-offs contain low amounts (Søvik, 2005). The autolysis might be difficult to control due to a complex and varying composition of endogenous enzymes, and the resulting hydrolysates will have varying compositions and properties (Kristinsson and Rasco, 2000b).

Exogenous proteases

In order to control and accelerate the hydrolysis, exogenous proteolytic enzymes are usually added. Inactivation of endogenous enzymes in certain raw materials (e.g. viscera) might be carried out before hydrolysis in order to further control the process. A wide range of exogenous enzymes from microbial, plant and animal origin are used for production of food hydrolysates (Benjakul *et al.*, 2014). Among the most utilized are the microbial derived Alcalase, Flavourzyme, Neutrase, Protamex, Corolase 7089 and Corolase PN-L, the plant derived Papain, ficin and Bromelain, and the animal derived trypsin, pepsin and pancreatin (Kristinsson and Rasco, 2000b; Kristinsson and Rasco, 2000c; Liaset *et al.*, 2003). The choice of enzyme has considerable impact on the yield and properties of the final product (Shahidi *et al.*, 1995), and overall, the hydrolysis process must be adapted to the substrate (raw material, e.g. cod heads), the exogenous enzymes and the desired product (Adler-Nissen, 1986).

1.3.2. Properties and applications of FPH

The properties of the FPH are determined by several factors, including the initial quality and composition of the by-product, the pretreatment of the by-products, the activity of endogenous and exogenous enzymes and their specificity and concentration, peptide sizes, as well as the processing conditions of the hydrolysis, including pH, temperature and time of hydrolysis (Benjakul *et al.*, 2014; Mahmoud *et al.*, 1992; Opheim *et al.*, 2015).

The content of proteins and lipids in the by-product affects the properties of the hydrolysate. Low amounts of lipids in the raw materials are important for stability and sensory properties of the FPH. High content of lipid makes the hydrolysate susceptible to oxidation due to the polyunsaturated lipids chains (Rustad *et al.*, 2011). This might affect the oxidative stability and potentially reduce the sensory properties and quality of the FPH (Opheim *et al.*, 2015). Hydrolysate containing a high amount of proteins and low amount of lipids is characterized as a high quality hydrolysate (Šližytė *et al.*, 2005).

The peptide sizes in the hydrolysate highly affect the properties of the FPH. The degree of hydrolysis (DH %) is a parameter utilized in order to evaluate the distribution of peptide sizes in a hydrolysate (Kristinsson and Rasco, 2000b). The DH describes the ratio between the amount of broken peptide bonds and the total amount of peptide bonds present in a quantity of hydrolysate. Hydrolysates with low DH consist of larger peptides with higher molecular

weights, whereas hydrolysates with high DH contains shorter peptides with lower molecular weights.

Numerous peptide properties are enhanced or altered upon hydrolysis compared to their native equivalents (Atef and Ojagh, 2017). Some of the properties of FPH are listed below.

Sensory properties

Bitter taste is a challenge in FPH and might reduce the sensory properties of the hydrolysate as a food additive. It has been suggested that small peptides containing hydrophobic amino acids, especially on the C-terminus, might cause the bitterness (Saha and Hayashi, 2001; Otagiri *et al.*, 1985). DH between 4 and 40% indicates a high concentration of small peptides in the hydrolysate, which may induce bitterness (Dauksas *et al.*, 2004). Reducing the DH to 3-5% might contribute to low degree of bitterness due to high presence of free amino acids (Adler-Nissen, 1984).

Various methods have been suggested to reduce the bitterness, some of which include coating or encapsulating the peptides responsible for bitterness, or incorporation of glutamic acid, glutamyl-rich peptides, polyphosphates, gelatin or glycine in the products (Kristinsson and Rasco, 2000a). It has also been suggested that Protamex produces hydrolysates with low amount of bitter peptides (Liaset *et al.*, 2003). A possible explanation is that Protamex lacks specificity towards hydrophobic amino acids, and thus reduces the amount of hydrophobic amino acids exposed to its surroundings.

Additionally, the FPH may have a fish off-flavor, which might reduce sensory quality if the FPH is incorporated in non-fish food systems.

Nutritional properties

Short peptides, typically di- or tripeptides, are more easily absorbed than free amino acids and large peptides in the intestine, and the DH thus affects the nutritional value of the FPH. The control and knowledge of the peptide sizes in a hydrolysate is therefore important to obtain a physiologically active hydrolysate (Siemensma *et al.*, 1993).

The relative concentration of dietary essential amino acids is the main factor determining the nutritional value of food protein (Shiau, 1994). Amino acids that are essential, conditionally essential and nonessential in humans are presented in table 1.2. Essential amino acids are not synthesized in the body and are thus required in dietary proteins. Conditionally essential amino acids are synthesized in the body, but they are required in larger amounts during certain stages of human lives, for instance at young age, during growth and sometimes during illness. During these stages, the amino acids must thus be supplemented through diet. Nonessential amino acids are at all times synthesized in sufficient amounts in the body (Nelson and Cox, 2013).

Table 1.2: The nonessential, conditionally essential and essential amino acids in humans (Nelson and Cox, 2013).

Nonessential	Conditionally essential	Essential
Alanine (Ala)	Arginine (Arg)	Histidine (His)
Asparagine (Asn)	Cysteine (Cys)	Isoleucine (Ile)
Aspartate (Asp)	Glutamine (Gln)	Leucine (Leu)
Glutamate (Glu)	Glycine (Gly)	Lysine (Lys)
Serine (Ser)	Proline (Pro)	Methionine (Met)
	Tyrosine (Tyr)	Phenylalanine (Phe)
		Threonine (Thr)
		Tryptophan (Trp)
		Valine (Val)

Fish muscle contains a balanced composition of dietary essential amino acids and is a good source of nutritive and easily digestible proteins (Friedman, 1996; Venugopal *et al.*, 1996; Yanez *et al.*, 1976). Production of FPH preserves the content of essential amino acids (Atef and Ojagh, 2017).

Functional properties

Hall and Ahmad defined functional properties as "the overall physiochemical behavior or performance of proteins in food systems during processing, storage, and consumption" (Hall and Ahmad, 1992). Hydrolysis of fish by-products affects physiochemical properties, including water holding capacity, oil absorption capacity, protein solubility, gelling activity, foaming and emulsifying capacity (Chalamaiah *et al.*, 2010). These properties highly depend on the DH of the hydrolysate.

Balti *et al*. found that water holding capacity increases proportionally with increasing DH due to an augmented number of polar side groups of the terminal amino acids (amino and carboxyl ends). Additionally, it was found that solubility of protein hydrolysates increases with increasing DH, whereas foaming capacities, fat absorption and emulsifying properties decrease (Balti *et al.*, 2010). Foaming and emulsifying capacities are found to be best at low DH, preferably lower than 10% (Damodaran, 1997). These properties are reduced at higher DH.

Addition of FPH to food formulations can contribute to enhanced or altered functional properties (Chalamaiah *et al.*, 2010), for example as stabilizers in beverages and as flavor enhancers in confectionary products. FPH can provide protein supplements for people suffering from protein deficiency (Kristinsson and Rasco, 2000b).

Bioactive properties

Fish derived proteins and peptides may possess health promoting properties, including antihypertensive, antioxidative, immunomodulatory, antithrombic and cholesterol-lowering effects (Kim and Mendis, 2005; Hartmann and Meisel, 2007). These health benefits are caused by bioactive peptides, which have been defined as "food derived peptides that in addition to their nutritional value exert a physiological effect in the body" (Vermeirssen *et al.*, 2004). The bioactive peptides may thus exert a beneficial effect upon human health, affecting some of the major physiological body systems, including the cardiovascular, digestive, nervous, endocrine and immune system (Hernàndez-Ledesma *et al.*, 2011; Erdmann *et al.*, 2008).

Bioactive peptides are inactive within the sequence of their primary protein structure, but can be released during food processing, for example by enzymatic hydrolysis or fermentation, or by digestive enzymes during gastrointestinal digestion (Erdmann *et al.*, 2008; Hernàndez-Ledesma *et al.*, 2011). A bioactive peptide molecule is usually composed of between 2 and 20 amino acid residues. Upon digestion, the peptides can either be absorbed in the intestine and enter the blood circulation and exert systemic effects in its intact form, or induce effects locally in the gastrointestinal tract (Erdmann *et al.*, 2008). In addition to marine species, bioactive peptides have been found in proteins from milk, egg, meat and several plants, including soy and wheat (Erdmann *et al.*, 2008).

Bioactive peptides have been shown to have an inhibitory effect on Angiotensin I-converting enzyme (ACE), which act as an important contributor in elevating the blood pressure in human. Bioactive peptides can therefore be antihypertensive (Je *et al.*, 2004). This will be further described below.

1.4. Antihypertensive effects of FPH

One of the documented effects of bioactive peptides is lowering of the blood pressure in humans. Hypertension has been estimated to affect one third of the Western population (Kearney *et al.*, 2005), and is associated with several cardiovascular diseases, including stroke, coronary heart disease and peripheral heart disease (Hernàndez-Ledesma *et al.*, 2011). Prevention and treatment of hypertension reduce the risk of cardiovascular diseases, and nutritional factors have been shown to have a considerable impact in the prevention or treatment of hypertension. Applications of bioactive peptides for production of antihypertensive foods can thus enhance these effects (Hernàndez-Ledesma *et al.*, 2011).

1.4.1. Angiotensin I-converting enzyme

Angiotensin I-converting enzyme (ACE) is essential for the regulation of blood pressure in mammals, and is found in all mammalian tissue and organs (Je *et al.*, 2004). The renin-angiotensin system is important in maintaining homeostasis in both blood pressure, fluid and salt balance in mammals (Turner and Hooper, 2002; Weinberg *et al.*, 2000). In this system, ACE catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II. ACE is also involved in the kinin-kalicrein system, where it hydrolyzes the vasodilator bradykinin. Thus, through its actions, ACE contributes to elevate blood pressure by formation of the vasoconstrictor angiotensin II and inactivation of the vasodilator bradykinin. Inhibition of ACE is considered an efficient approach for controlling hypertension (Je *et al.*, 2004; Hartmann and Meisel, 2007; Hernàndez-Ledesma *et al.*, 2011; Erdmann *et al.*, 2008). The effects of ACE in the renin-angiotensin and the kinin-kalicrein systems are shown in figure 1.4.

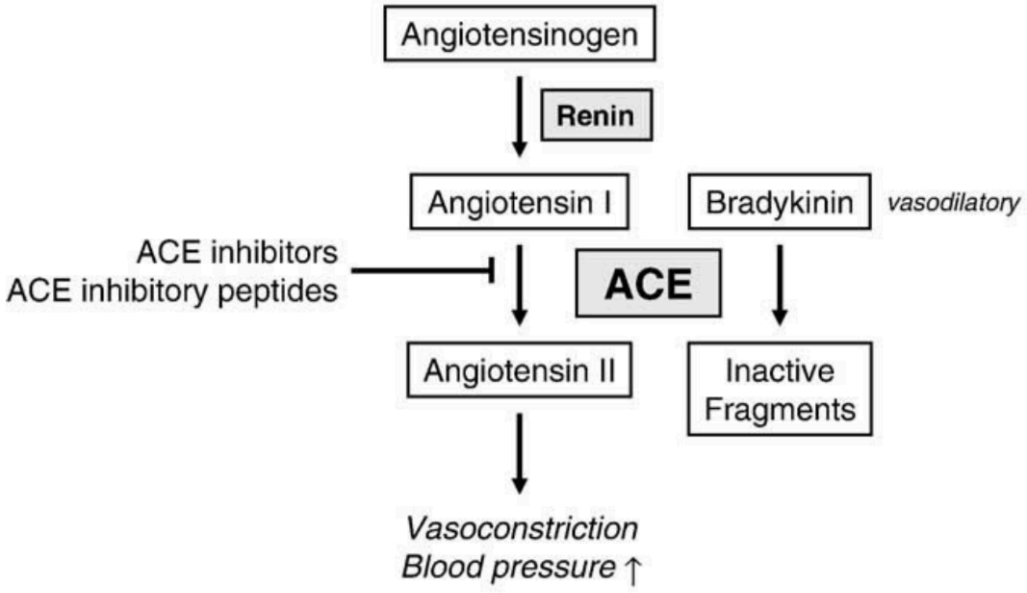

Figure 1.4: Overview of the catalyzing effects of ACE in the renin-angiotensin and kinin-kalicrein systems. ACE catalyzes the conversion of angiotensin I to form the vasoconstrictor angiotensin II, as well as hydrolyzing the vasodilator bradykinin. Inhibition of ACE prevent increase of blood pressure (Erdmann *et al.*, 2008).

1.4.2. Inhibition of ACE

ACE (EC 3.4.15.1) is a dipeptidyl carboxypeptidase in the zinc protease class. It is an exopeptidase which is cleaving and releasing dipeptides from the C-terminal of oligopeptides (Je *et al.*, 2004). ACE cleaves the decapeptide angiotensin I and forms the octapeptide angiotensin II upon releasing a dipeptide (Hartmann and Meisel, 2007). Synthetic ACE inhibitors have for decades been used as antihypertensive drugs by blocking these processes (Erdmann *et al.*, 2008).

A variety of synthetically produced ACE-inhibitors, such as Captopril, Enalapril and Lisinopril, are commonly used worldwide for treatment of hypertension (Hernàndez-Ledesma *et al.*, 2011). These drugs are efficient, but intake is associated with side effects such as taste disturbances, cough and skin rashes (Atkinson and Robertson, 1979). Food derived antihypertensive peptides could potentially reduce the need for synthetic drugs and increase the demand for value added by-products.

Characteristics of ACE inhibitory peptides

For ACE inhibitory peptides to express antihypertensive effects *in vivo*, they must be absorbed intact through the intestine and reach the target tissue (i.e. the cardiovascular system), in an active form. There are several characteristics that must be fulfilled for the bioactivities to be existent. With the proper size, typically between 2 and 20 amino acids, the peptides are less susceptible for gastrointestinal breakdown, and transport to and absorption in target tissues are enabled (Vermeirssen *et al.*, 2004). *In vitro* studies investigating the resistance of inhibitory peptides against gastrointestinal degradation have been performed, exposing inhibitory peptides to sequential hydrolysis with pepsin and pancreatic extracts for simulation of the gastrointestinal conditions. The studies showed that several peptides, including the tripeptides Ile-Pro-Pro and Val-Pro-Pro, were resistant to the degradation, indicating that the peptides would reach the circulation and eventually the target tissues *in vivo* (Ohsawa *et al.*, 2008; Foltz *et al.*, 2008).

Even though the substrate specificity of the ACE active site is not fully determined, there are several suggestions as to what influences the degree of interaction with substrates. It has been shown that the binding to ACE strongly depend on the C-terminal tripeptide of the inhibitory peptide (substrate). ACE appear to favor substrates with C-terminus tripeptide composed of hydrophobic amino acids with aromatic or branched side chains (Vermeirssen *et al.*, 2004). Investigations have shown that the most potent ACE inhibitory peptides has Trp, Pro, Tyr or Phe at the C-terminus (Hernàndez-Ledesma *et al.*, 2011). Finally, it has been shown that the ACE inhibitory effect is strongly increased by the presence of Leu within the C-terminus (Gómez-Ruiz *et al.*, 2004).

A suggested interaction mechanism between substrate and ACE active site was presented by Ondetti and Cushman in 1982. The mechanism proposed interaction between the C-terminal tripeptide of the substrate and three subsites within the active site, creating an inhibitory effect. The model of interaction was based on an ACE inhibitory peptide from snake venom, which was the first potent ACE inhibitor to be discovered (Ondetti *et al.*, 1977). The discoveries of this inhibitor led to the development of the antihypertensive drug Captopril, which is a synthetic analogue to the snake venom peptide (Ondetti and Cushman, 1982). The model as it was presented in the article is shown in figure 1.5.

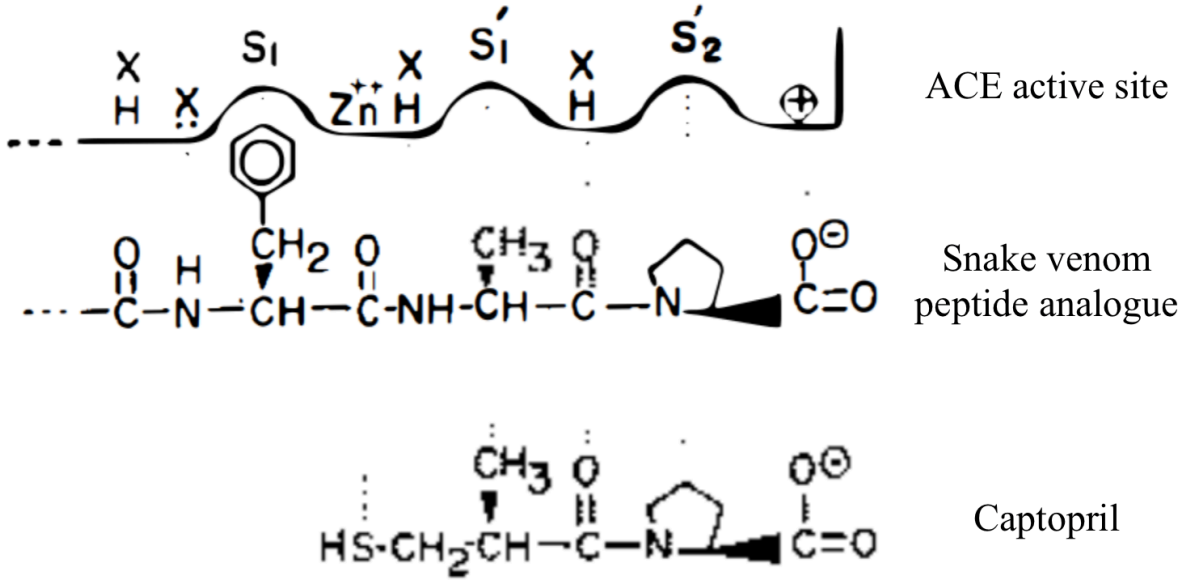

Figure 1.5: Proposed interaction between protein and synthetic substrate and ACE active site. Figure modified from Ondetti and Cushman (1982).

Evaluation of ACE inhibitory effect

The ACE inhibitory effect is most often evaluated by the parameters *IC_{50}* and *ACEI* (%). IC_{50} is defined as the concentration of inhibitor required to inhibit 50% of the ACE activity (Je *et al.*, 2004), and is the most commonly used measure of inhibitory effect. The parameter is often measured in μM or mg inhibitor/mL, and a low IC_{50} indicates that the inhibitor is potent. The ACEI (ACE inhibition, %) of an inhibitor measures the percentage of ACE activity inhibition.

ACE inhibitory activities have been investigated for a variety of food derived peptides, including peptides from cheese whey (Abubakar *et al.*, 1998), casein (Maeno *et al.*, 1996), wheat (Motoi and Kodama, 2003) and soybean (Chiang *et al.*, 2006) as well as a variety of fish proteins (He *et al.*, 2007). Some fish derived inhibitory peptides are listed below.

He *et al*. (2007) produced hydrolysates from twelve different marine by-products (including cod frame and skin, mackerel head, oyster and shark meat) using four different exogenous proteases (Protamex, Alcalase, Flavourzyme and SM98011). The ACE inhibitory properties of the hydrolysates were investigated, resulting in IC_{50} values ranging from 0.17 – 501.7 mg/mL. The results showed that peptides rich in amino acids with branched or aromatic side chains expressed low IC_{50} (e.g. shark meat), whereas the IC_{50} of peptides containing fewer of these amino acids exceeded 100 mg/mL (e.g. oyster).

An ACE inhibitory peptide was isolated from Alaska pollock frame protein hydrolysate with the amino acid composition Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala (IC_{50} = 14.7 μM) (Je *et al.*, 2004).

Isolation of peptides from seaweed has resulted in a number of ACE inhibitory dipeptides (Sato *et al.*, 2002), including Val-Tyr (IC_{50} = 35.2 μM), Ile-Tyr (6.1 μM), Ala-Trp (18.8 μM), Phe-Tyr (42.3 μM), Val-Trp (3.3 μM), Ile-Trp (1.5 μM) and Leu-Trp (23.6 μM). Lee *et al.* (2010) isolated a potent ACE inhibitory peptide from tuna frame hydrolysate, composed of 21 amino acids; Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro (IC_{50} = 11.28 μM). This peptide was tested *in vivo* in spontaneously hypertensive rats (SHR), resulting in a significant decrease in systolic blood pressure.

Hooper and Turner (1987) investigated the ACE inhibitory effect performed by the synthetic ACE inhibitors Captopril, Lisinopril and Enalapril upon inhibition of ACE from porcine kidney. IC_{50} values were determined to 0.021 ± 0.013 μM for Captopril, 0.011 ± 0.004 μM for Lisinopril and 0.037 ± 0.010 μM for Enalapril, respectively. Fujitaa and Yoshikawab (1999) determined the *in vivo* ACE inhibitory effect of Captopril in SHR rats to IC_{50} = 0.022 μM.

The IC_{50} of the mentioned synthetic ACE inhibitors indicates that these drugs are considerable more potent than most of the previously mentioned food derived ACE inhibitory peptides. However, *in vivo* comparative studies with fish derived ACE inhibitory peptides and Captopril (Fujitaa and Yoshikawab, 1999) showed that the inhibitory peptides exerted higher activity than what was expected from their *in vitro* activity. In this study, fish derived peptides were determined to have higher tissue affinities and were degraded slower than Captopril. The same study (Fujitaa and Yoshikawab, 1999) showed that Leu-Lys-Pro-Asn-Met (IC_{50} = 2.4 μM) was cleaved by ACE to Leu-Lys-Pro (IC_{50} = 0.32 μM), and exerted antihypertensive activities almost equivalent to Captopril on a molar basis after single oral administration in SHR. In addition, the peptide possessed a longer-lasting antihypertensive activity compared to Captopril.

Health claims for ACE inhibitory peptides

Health claims are approved statements about a relationship between food and health. Till now, health claims of antihypertensive effects exerted by marine bioactive peptides are not approved in Europe. In order to approve health claims on antihypertensive effect, *in vivo* tests have to be carried out in order to establish documented health beneficial effect of specific peptides. Health claims are authorized by the European Commission when scientific evidence *in vivo* can be provided. Authorization also requires that the effect can be easily understood by the consumer (EC, 2018). Antihypertensive peptides may however be sold and marketed as food supplements. However, the antihypertensive effect of a katsuobushi oligopeptide produced from hydrolysis of dried bonito has been authorized by Japanese authorities. This peptide is marketed as PEPTIDE ACE3000 in Japan, Vasotensin® in the United States and as PeptACE™ and Levenorm™ in Canada (Thorkelsson *et al.*, 2009).

1.5. Aims of the thesis

The first aim of the thesis was to measure *in vitro* ACE inhibitory activity in hydrolysates produced from cod by SINTEF Ocean in the project HEADS UP.

This thesis additionally aimed to determine structural properties of the hydrolysates in order to establish a correlation between the chemical composition and ACE inhibitory activity. The amino acid composition, molecular weight distribution, degree of hydrolysis and composition of free amino acids were determined to investigate correlations to the ACE inhibitory activity.

The final aim of the thesis was to fractionate the cod head hydrolysates based on molecular weight and measure ACE inhibitory activity in the low molecular weight fraction.

Chapter 2: Materials and methods

2.1. Overview of the work performed

The laboratory work conducted in this thesis was performed during the fall of 2017 and spring of 2018. An overview of the experiments and progress is presented in figure 2.1. More detailed descriptions of the analytical methods are presented in section 2.3.

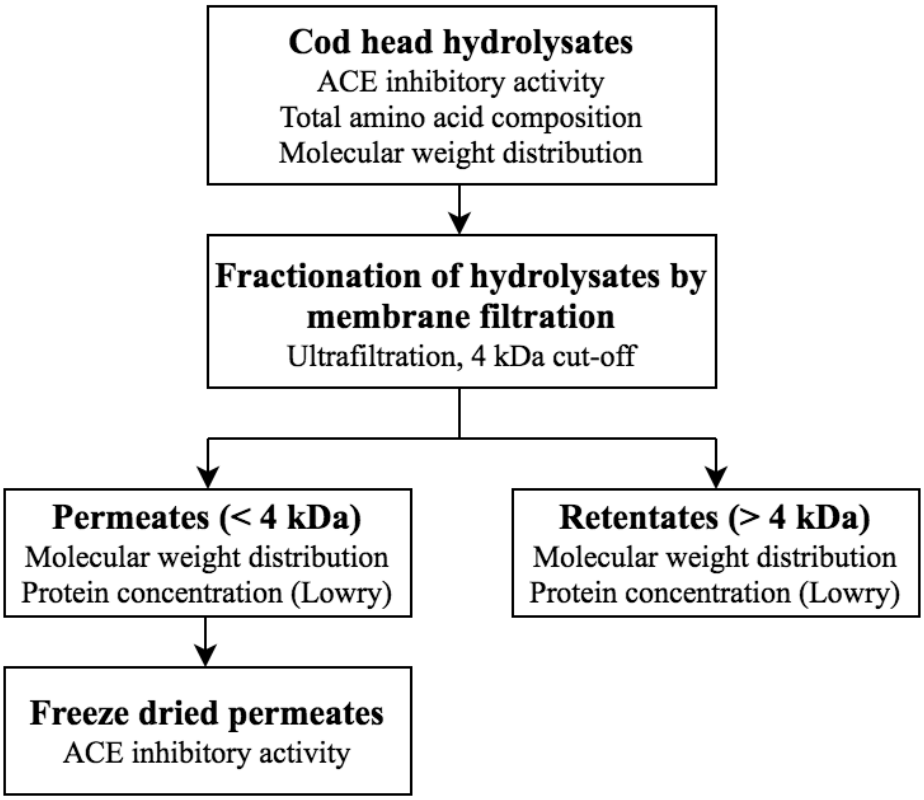

Figure 2.1: Overview of the experiments conducted in this thesis.

Cod head hydrolysates (CHHs) were the base of all experiments performed. Analyses were initially performed on the original CHHs, of which ACE inhibitory activity, total amino acid composition and molecular weight distribution were determined. Membrane filtration was carried out to fractionate the hydrolysate (in solution) based on peptide size. Four CHHs were chosen for fractionation. Molecular weight distribution and protein concentration were determined for the fractions obtained from membrane filtration; the retentates (peptides larger than 4 kDa) and the permeates (peptides smaller than 4 kDa). Finally, the permeates were freeze dried, and the ACE inhibitory activity of these fractions were investigated.

All analyses were carried out in the food chemistry laboratory at the Department of Biotechnology and Food science at NTNU campus Gløshaugen. The membrane filtration was conducted at the food science laboratory at NTNU campus Kalvskinnet.

2.2. Cod head hydrolysates

The hydrolysates were produced by SINTEF Ocean in the pilot project HEADS UP, which was carried out in Tufjord in the north of Norway. Heads from cod were used as raw material for the preparation of protein hydrolysate. 13 hydrolysates were produced. The variables studied were month of catch, type and concentration of proteolytic enzyme, amount of water and antioxidants added. The processing parameters are presented in table 2.1.

Table 2.1: Processing parameters for hydrolysis of cod heads. Hydrolysis was carried out at 50°C and 400 kg of raw material were utilized per experiment.
(* 200 L of stick water from the oil factory in Tufjord was used)

Experiment number	Month of harvest (2017)	Water (kg)	Proteolytic enzyme (and antioxidant)	Enzyme concentration (% w/w raw material)
1	February	400	Papain + Bromelain	0.1
2	February	300	Papain + Bromelain	0.1
3	February	300	Protamex	0.05
4	March	300	Papain + Bromelain	0.1
5	March	200	Papain + Bromelain	0.1
6	March	100	Papain + Bromelain	0.1
7	March	200	Protamex	0.1
8	March	300	Protamex	0.1
9	March	200*	Papain + Bromelain	0.1
10	May	300	Papain + Bromelain	0.1
11	May	300	Papain + Bromelain (and citric acid)	0.1
12	May	300	Papain + Bromelain	0.1
13	May	300	Papain + Bromelain (and sodium sulphite)	0.1

As presented in table 2.1, the cod were harvested in February, March and May of 2017, respectively. The amount of water used to carry out the hydrolysis varied from 100 – 400 kg, and for one of the experiments (number 9), stick water was utilized instead of pure water. The proteolytic enzymes utilized were either Protamex or a combination of Papain and Bromelain. In addition, in experiment number 11 and 13 antioxidants were added; citric acid and sodium sulphite, respectively.

The hydrolysis was carried out in Tufjord by utilizing the SINTEF Mobile Sea lab, which is a mobile hydrolysis plant. After production, the hydrolysates were frozen and transported to the lab facilities of SINTEF Ocean in Trondheim. The hydrolysates were freeze dried at NTNU campus Gløshaugen.

The CHH powders were relatively fine-grained, had a neutral to beige color and a slight fish odor. The hydrolysates were denoted T1-H, T2-H, …, T13-H. Figure 2.2 shows the appearance of the hydrolysates.

Figure 2.2: The cod head hydrolysates; top row: T1-H – T4-H, middle row: T5-H – T8-H, bottom row: T9-H – T13-H.

The powders were stored in zip-lock bags in a freezer (-20°C) in the food chemistry laboratory at NTNU campus Gløshaugen. They were removed for short periods of time to take out samples for analysis.

2.3. Analysis of the cod head hydrolysates

The analytical methods used to investigate ACE inhibitory activity and structural properties of the CHHs are presented in this section.

2.3.1. Investigation of ACE inhibitory activity

ACE inhibitory activity expressed by the CHHs was investigated by using the method of Sentandreu and Toldrá. The method is based on the reaction between ACE from rabbit lung and the substrate Abz-Gly-Phe(NO_2)-Pro. Through enzymatic catalysis, ACE cleaves the substrate, resulting in formation of Abz-Gly and Phe(NO_2)-Pro. ACE can however be inhibited, in this case by bioactive peptides in the cod head hydrolysate (figure 2.3). Abz-Gly is a fluorescent molecule, and the amount of it can be detected fluorimetrically by using appropriate emission and excitation wavelengths. Absence of fluorescence thus indicates inactivity of ACE.

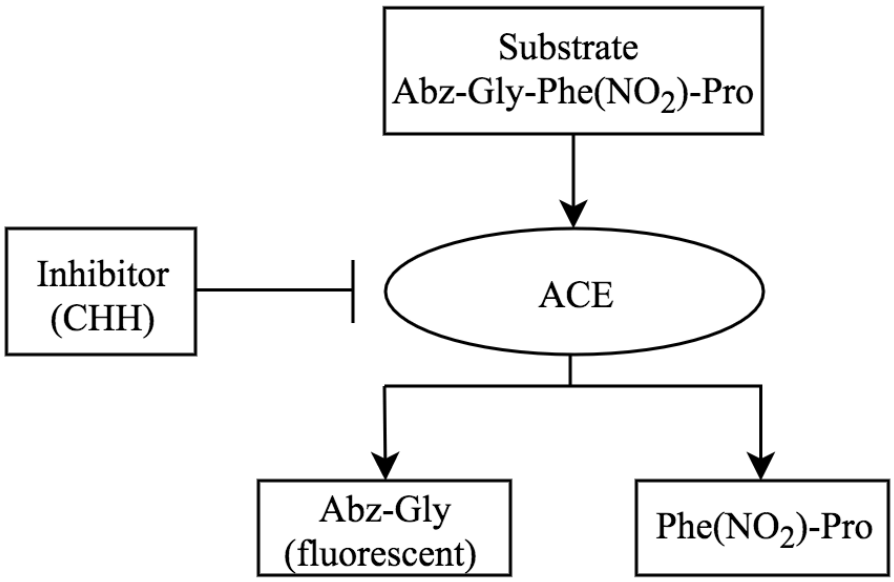

Figure 2.3: Illustration of the reaction between ACE and the substrate Abz-Gly-Phe(NO_2)-Pro. By enzymatic cleavage of the substrate, the fluorescent residue Abz-Gly is generated. However, in presence of an inhibitor (i.e. CHH), the substrate is not cleaved, and thus fluorescence is absent.

The following reagents were used to determine ACE inhibitory activity:

Enzyme: 7.5 μg/mL (~ 3 mU/mL) ACE from rabbit lung (Sigma-Aldrich, cat. no. A-6778) in Tris-Base buffer (150 mM) at pH 8.3 (buffer B). This solution is denoted "ACE working solution".

Substrate: 0.45 mM o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe(NO_2)-Pro) (Bachem, cat. no. M-1100) in Tris-Base buffer (150 mM) containing 1.125 M NaCl at pH 8.3 (buffer C). This solution is denoted "substrate working solution".

Inhibitor: varying concentrations of CHH (powder). Powder dissolved in 10 mL of buffer B to give different concentrations.

The procedure for preparation of the reagents is presented in appendix A. The experimental procedure is presented in figure 2.4. The fluorometer used was TECAN Spark ® multimode microplate reader.

As described in figure 2.4, the inhibitor (CHH) was pre-incubated with the enzyme (ACE), followed by addition of substrate. Different concentrations of inhibitor were investigated to measure varying degrees of ACE inhibition. A negative control (without inhibitor) was included in the measurements. It was expected that fluorescence exerted by the negative control increased steadily throughout the time of measurement, whereas the amount of fluorescence decreased proportionally with augmenting concentrations of inhibitor. It was thus expected that if the enzyme was fully inhibited, no fluorescence would be detected.

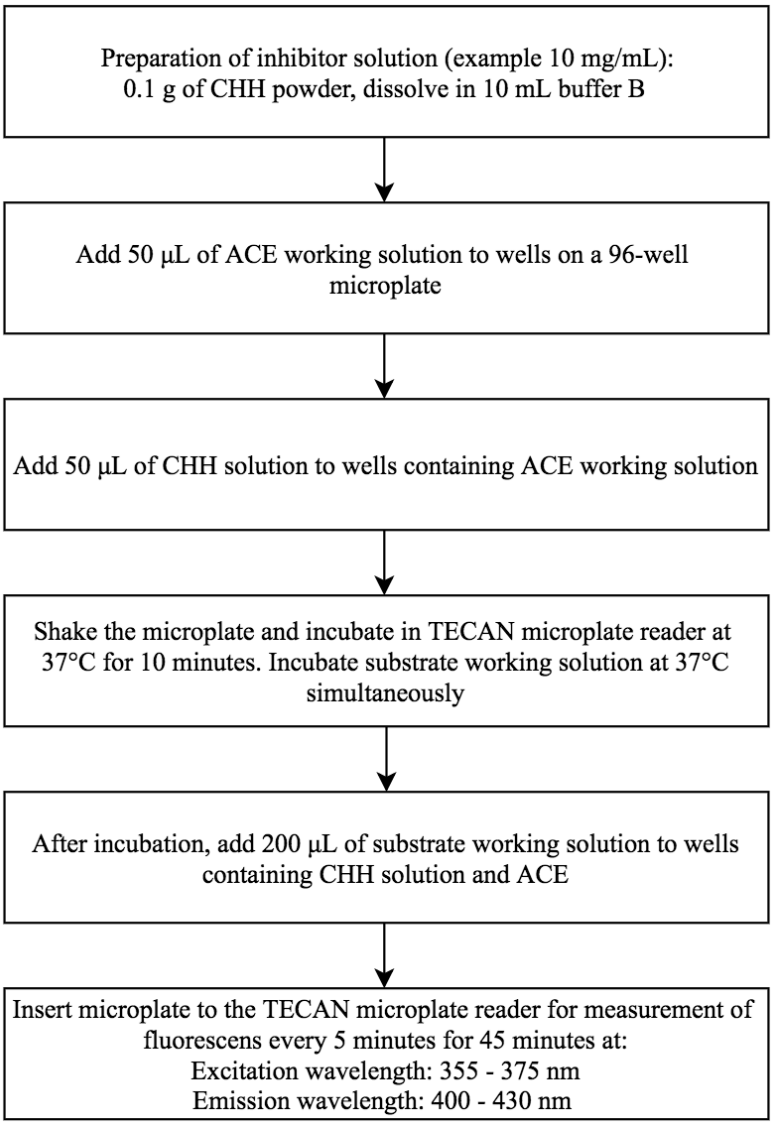

Figure 2.4: Flowchart describing the experimental procedure for measuring the ACE inhibitory activity of CHHs (Sentandreu and Toldrá, 2006).

Fluorescence in the samples was measured by the TECAN microplate reader every fifth minute for 45 minutes. The raw data obtained in the measurements included the fluorescence detected at each measurement for each concentration investigated, including the control. By plotting fluorescence against time and applying linear regression, a slope was obtained for each sample. The ACEI (%) could thereafter be plotted for each concentration. Equation 2.1 was utilized for calculation of ACEI (%).

$$ACEI\ (\%) = \left(1 - \frac{slope\ of\ sample}{slope\ of\ control}\right) \times 100\% \qquad (2.1)$$

ACEI (%) was plotted against respective concentrations (mg/mL) to further determine IC_{50}. Finally, the protein contents of the hydrolysates (appendix H) were utilized, and the resulting

IC_{50} values were given by mg protein/mL. The calculations are further explained in appendix B. IC_{50} was determined for each of the CHHs (T1-H – T13-H) and for the permeates from membrane filtration.

2.3.2. Determination of total amino acid composition

The total amino acid compositions of the CHHs were determined by hydrolyzing CHH samples according to the flowchart presented in figure 2.5, and thereafter analysis using High Performance Liquid Chromatography (HPLC) (Waters Nova-Pak C18 reversed phase column, 4 μm). The procedure is based on the method developed by Blackburn (Blackburn, 1978).

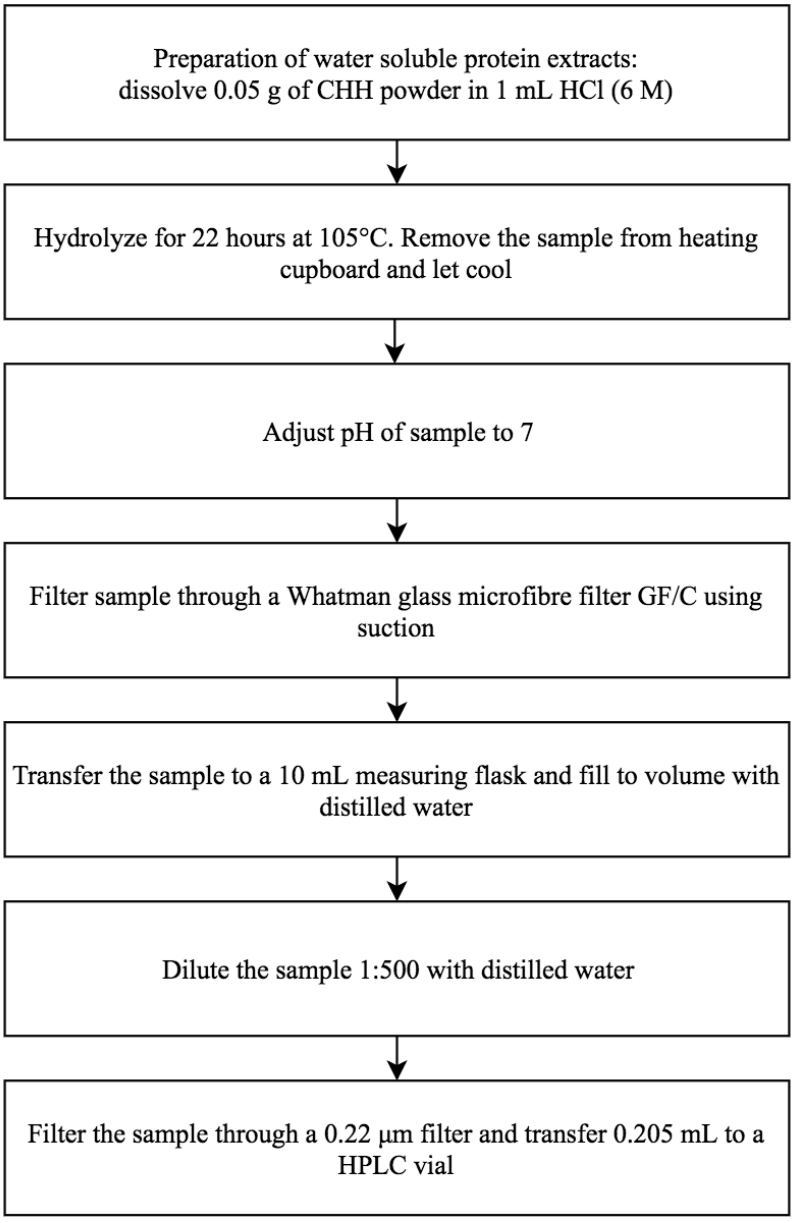

Figure 2.5: Flowchart presenting the steps in preparing a sample for determination of the total amino acid composition.

The HPLC analyses were performed by Siri Stavrum at the Department of Biotechnology and Food Science at NTNU. The chromatogram obtained from HPLC displayed the amino acids detected in the samples and the amount of them (nmol/mL). Calculations were carried out in order to determine the total amount of amino acids in the sample (mg amino acids/g CHH quantified). The raw data and calculations are presented in appendix C. All the CHHs (T1-H – T13-H) were analyzed.

2.3.3. Fractionation of cod head hydrolysates: membrane filtration

Membrane filtration separates peptides based on their molecular weight and was conducted at NTNU campus Kalvskinnet. The analysis was carried out in collaboration with fellow master student Marte Vilde Monslaup. The fractionation was carried out by utilizing the MMS AG Membrane Systems.

Hydrolysates T1-H, T5-H, T9-H and T13-H were chosen for analysis. Hydrolysate solutions (1 g CHH/100 mL distilled water) were prepared and centrifuged using an Eppendorf Centrifuge 5804 R (4500 rcf, 15 minutes). The supernatant was filtrated through a Whatman glass microfibre filter GF/C using suction (pore size 0.4 μm). The filtrates were stored in centrifuge tubes in the freezer (-20°C) until transportation to campus Kalvskinnet. 150 – 200 mL of filtrate was prepared for each hydrolysate.

The membrane filtration was carried out using ultrafiltration in flat sheet parallel mode, and the membranes utilized had a molecular weight cut-off of 4 kDa. The hydrolysate samples were separated into two fractions; permeates and retentates. The retentates consisted of peptides larger than 4 kDa, other large molecules and water. The permeates were composed of peptides that penetrated the membrane (< 4 kDa), small molecules and water. The fractions were stored in centrifuge tubes in the cold room (2°C) in the lab at Gløshaugen until further analyses. The resulting volumes were approximately 100 mL of retentate and 50 mL of permeate.

The ACE inhibitory activities of the permeates were measured. The permeates were freeze dried in order to concentrate the peptides. Samples were then prepared in the same manner as presented in section 2.3.1. by quantifying freeze dried powder to reach determined concentrations. The ACE inhibitory activities of the concentrations were then investigated by following the procedure in figure 2.4.

2.3.4. Determination of molecular weight distribution

The distribution of peptide molecular weights in the permeates, retentates and their respective original CHHs (T1-H, T5-H, T9-H and T13-H) were determined by using gel filtration in an ÄKTA Fast Protein Liquid Chromatography (FPLC) system. The analysis was carried out in collaboration with fellow master student Marte Vilde Monslaup. The CHH samples were prepared by dissolving powder (0.1 g) in the elution buffer, a sodium acetate buffer (4 mL, 0.05 M, pH 5, filtrated through 0.2 μm filter), followed by filtration through a sterile filter (0,2 μm). The permeates and retentates (liquid, 1 mL) were directly filtrated (through a 0.2 μm filter). The samples were injected (0.8 – 0.9 mL) into the FPLC. 100 μL of the sample was thereafter eluted in the column (Superdex Peptide 10/300 GL). The total volume of the column was 24 mL. Peptides in the column were detected using wavelength 280 nm. The flow rate was 0.5 mL/minute. The column separates peptides with molecular weights ranging from 100 to 7000 Da.

The results were displayed as peaks eluting at varying elution volumes (mL). The peaks represent peptides present in the sample. The heights of the peaks depend on the absorbance at 280 nm and thus the concentration of the peptides is the sample.

Standard proteins were utilized in order to establish a relationship between elution volume and molecular weight. The relationship was thereafter used for determination of the molecular weights of the proteins in the CHH samples. The proteins utilized as references and their respective molecular weights are listed in table 2.2.

Table 2.2: Protein standards utilized to determine a relationship between elution volume and molecular weight of peptides detected by gel filtration.

Standard	Molecular weight (Da)
Vitamin B12	1 356
Aprotinin	6 512
Cytochrome C	12 384
Pepsin	34 000

The relationship between the standard proteins and their elution volumes was determined in appendix D.

2.3.5. Determination of free amino acids

The amounts of free amino acids in hydrolysates T1-H, T5-H, T9-H and T13-H were determined by fellow master student Marte Vilde Monslaup. The procedure was carried out according to the method of Osnes and Mohr (Osnes and Mohr, 1985), and the raw data and calculations are presented in appendix E.

2.3.6. Determination of degree of hydrolysis

The degree of hydrolysis of the CHHs was determined by fellow master student Ayat Asfour. The analysis was performed by using formol titration according to the method of Taylor (Taylor, 1957). The raw data and calculation of DH (%) are presented in appendix F.

2.3.7. Determination of protein concentration: Lowry protein assay

The concentrations of proteins in the retentates and permeates were determined by using the Lowry method (Lowry *et al.*, 1951). The analysis was carried out in collaboration with fellow master student Marte Vilde Monslaup. The experimental procedure is presented in appendix G.

Determination of the protein concentration was initially performed to enable investigation of the ACE inhibitory activity expressed by the peptides in the permeate. However, freeze drying the permeates and thereafter following the procedure presented in section 2.3.1. was found to be a better approach to obtain correct concentrations of inhibitory agent.
The protein concentrations still however provide useful information about the protein recovery from membrane filtration, and a protein mass balance was calculated. This is presented in appendix G.3.

Chapter 3: Results and discussion

In this thesis, the ACE inhibitory activity was measured, and structural properties were investigated in thirteen CHHs. The hydrolysates are referred to as T1-H – T13-H in this chapter, and the processing parameters of the hydrolysates are given in table 3.1.

Table 3.1: Processing parameters for hydrolysis of cod heads. The hydrolysis resulted in hydrolysates denoted T1-H – T13-H **(*** 200 L of stick water from the oil factory in Tufjord was used).

Hydrolysate	**Month of harvest (2017)**	**Water (kg)**	**Proteolytic enzyme (and antioxidant)**	**Enzyme concentration (% w/w raw material)**
T1-H	February	400	Papain + Bromelain	0.1
T2-H	February	300	Papain + Bromelain	0.1
T3-H	February	300	Protamex	0.05
T4-H	March	300	Papain + Bromelain	0.1
T5-H	March	200	Papain + Bromelain	0.1
T6-H	March	100	Papain + Bromelain	0.1
T7-H	March	200	Protamex	0.1
T8-H	March	300	Protamex	0.1
T9-H	March	200*	Papain + Bromelain	0.1
T10-H	May	300	Papain + Bromelain	0.1
T11-H	May	300	Papain + Bromelain (and citric acid)	0.1
T12-H	May	300	Papain + Bromelain	0.1
T13-H	May	300	Papain + Bromelain (and sodium sulphite)	0.1

3.1. ACE inhibitory activity

The first aim of this thesis was to measure the *in vitro* ACE inhibitory activity of the CHHs. All the 13 hydrolysates showed ACE inhibitory activity, and the calculated IC_{50} values ranged from 2.6 to 4.9 mg protein/mL (figure 3.1).

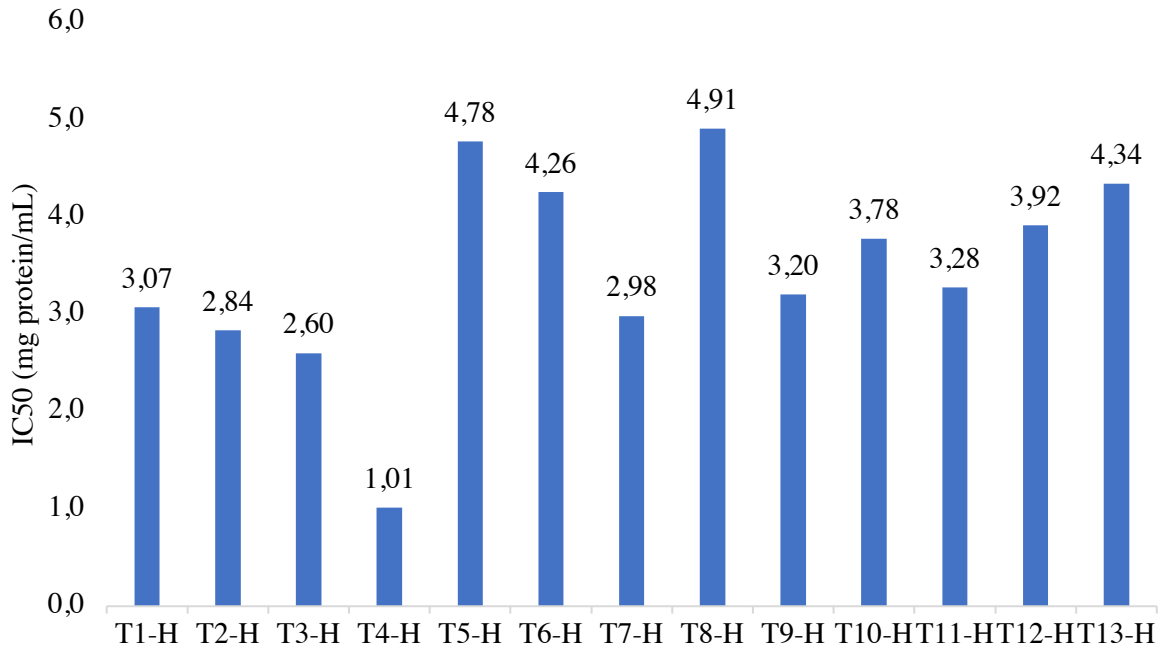

Figure 3.1: Calculated IC_{50} values (mg protein/mL) of the CHHs.

Slight differences in the ACE inhibitory effects were observed in terms of the calculated IC_{50} values. The IC_{50} of T4-H was found to be considerably lower than of the other hydrolysates. More concentrations of T4-H should have been investigated in order to enable determination of the true ACE inhibitory activity. The unreliable IC_{50} of T4-H was most likely due to poor experimental results, limiting the utilization of logarithmic analysis.

Peptides with more and less potent activity than the CHHs have been discovered. Peptides from fermented soy beans hydrolyzed with chymotrypsin and pepsin expressed *in vitro* ACE inhibitory activity of IC_{50} = 0.019 mg/mL. Other potent food peptides have been isolated from pea protein hydrolysate (IC_{50} = 0.07 mg/mL, pepsin and chymotrypsin), squid gelatin (0.340 mg/mL, Alcalase) and sea squirt (*Styela clava*) (1.023 mg/mL, Protamex) (Aluko, 2012). Other peptides have been found to exert less potent inhibitory activity than the CHHs, including peptides from scallop, herring skin and cod skin, with IC_{50} values > 10 mg/mL (He *et al.*, 2007).

The ACE inhibitory activities of the CHHs were considerably lower than for Captopril, IC_{50} = 0.097 mg/mL (0.021 μM (Hooper and Turner, 1987), M_W = 217.3 g/mol), which was expected because Captopril is a drug especially developed for treatment of hypertension.

Hydrolysates T3-H, T7-H and T8-H were produced by Protamex, whereas the remaining hydrolysates were produced with Papain and Bromelain (table 3.1). Papain and Bromelain are selective endoproteases. The preferred cleaving site of Papain is between arginine or lysine and amino acids that are not valine (Sigma-Aldrich/Merck). Bromelain cleaves peptide bonds between lysine, alanine or tyrosine and any amino acid (Sigma-Aldrich/Merck). The specificity of Protamex is not specified by the manufacturer Novozymes. However, this proteolytic enzyme is widely used because of its ability to produce hydrolysates without bitter peptides (Nguyen *et al.*, 2011; Liaset *et al.*, 2002). As presented earlier, bitterness is associated with the presence of a hydrophobic amino acid at the C-terminus of a peptide (Otagiri *et al.*, 1985), and it can be assumed that Protamex does not cleave the carboxyl end of hydrophobic amino acids.

Hydrophobic amino acids in the C-terminal tripeptide sequence have been shown to contribute to ACE inhibitory effect (Li *et al.*, 2004). Thus, based on the theoretical sites of cleavage of the proteolytic enzymes utilized, hydrophobic amino acids were assumed to be present in the C-terminus of peptides in the CHH, which thus may have contributed to ACE inhibitory effect. Amount of water and type and concentration of proteolytic enzyme used in the production (table 3.1) did not impact the ACE inhibitory activities of the CHHs.

The ACE inhibitory potency of protein fractions is influenced by numerous factors, including processing conditions of the FPH. The final composition of hydrolysates depends on the specificity of proteolytic enzymes, the protein primary structure in the raw material and how available the peptide bonds are towards degradation of the enzymes. Additionally, temperature during hydrolysis highly affects the activity of enzymes and the native proteins. These matters were illustrated when skate fish was hydrolyzed with four different proteolytic enzymes, namely trypsin, Alcalase, Neutrase and pepsin. The ACE inhibitory potency of the hydrolysates differed, with IC_{50} values of 1.13, 1.89, 3.55 and 6.19 mg/mL, respectively (Aluko, 2012). Thus, the ACE inhibitory activity of hydrolysates produced from the same source of native proteins may differ.

Effect of CHH peptide size on ACE inhibitory activity

Earlier studies have shown that the size of peptides have considerable impact on the ACE inhibitory activity (Lee *et al.*, 2010; Je *et al.*, 2004). In order to investigate the effect of peptide sizes on ACE inhibitory activity, membrane filtration was carried out to fractionate hydrolysates T1-H, T5-H, T9-H and T13-H, and the ACE inhibitory activities of the fractions of small peptides (permeates) were measured. The IC_{50} values of the four permeates are presented in table 3.2.

Table 3.2: The ACE inhibitory activity of the permeates (P) obtained from membrane filtration measured in IC_{50} (mg permeate/mL).

	T1-H P	**T5-H P**	**T9-H P**	**T13-H P**
IC_{50} (mg P/mL)	7.96	9.04	7.66	3.89

The ACE inhibitory activities of the permeates were measured to be lower in T1-H, T5-H and T9-H and higher in T13-H compared to the original hydrolysates (figure 3.1). The results were not as expected but suggest that fractionating the CHH in order to obtain a fraction enriched in ACE inhibitory peptides might be unnecessary. Fractionation of proteins is time consuming and relatively costly and could involve challenges in realization of large scale production of bioactive hydrolysates.

Food protein hydrolysates contain a complex variety of peptide sizes and structures. Aluko suggested that the antihypertensive effects exerted by food protein hydrolysates is caused by interactions between different peptide components (Aluko, 2012). This may explain why the original hydrolysates expressed more potent ACE inhibitory activities than the permeates.

3.2. Structure and composition of the cod head hydrolysates

This thesis aimed to determine structural properties of the hydrolysates in order to evaluate a relationship between these and ACE inhibitory activities of the CHHs. The structural results and their correlations with inhibitory activity are presented below.

3.2.1. Total amino acid composition

The total amino acid compositions of the CHHs were determined by analyzing acid hydrolyzed samples of CHH by HPLC. The distribution and total content of amino acids in the hydrolysates are presented in this section, and the correlation between amino acid content and ACE inhibitory effect is discussed. The nutritional value of the hydrolysates based on amino acid content is also presented.

Distribution and total content of amino acids

The amino acids detected in hydrolysates T1-H, T5-H, T9-H and T13-H and the amount of them (mg amino acid/g CHH) are presented in figure 3.2. These hydrolysates were chosen as examples. The amino acid compositions of all the hydrolysates are given in appendix C.

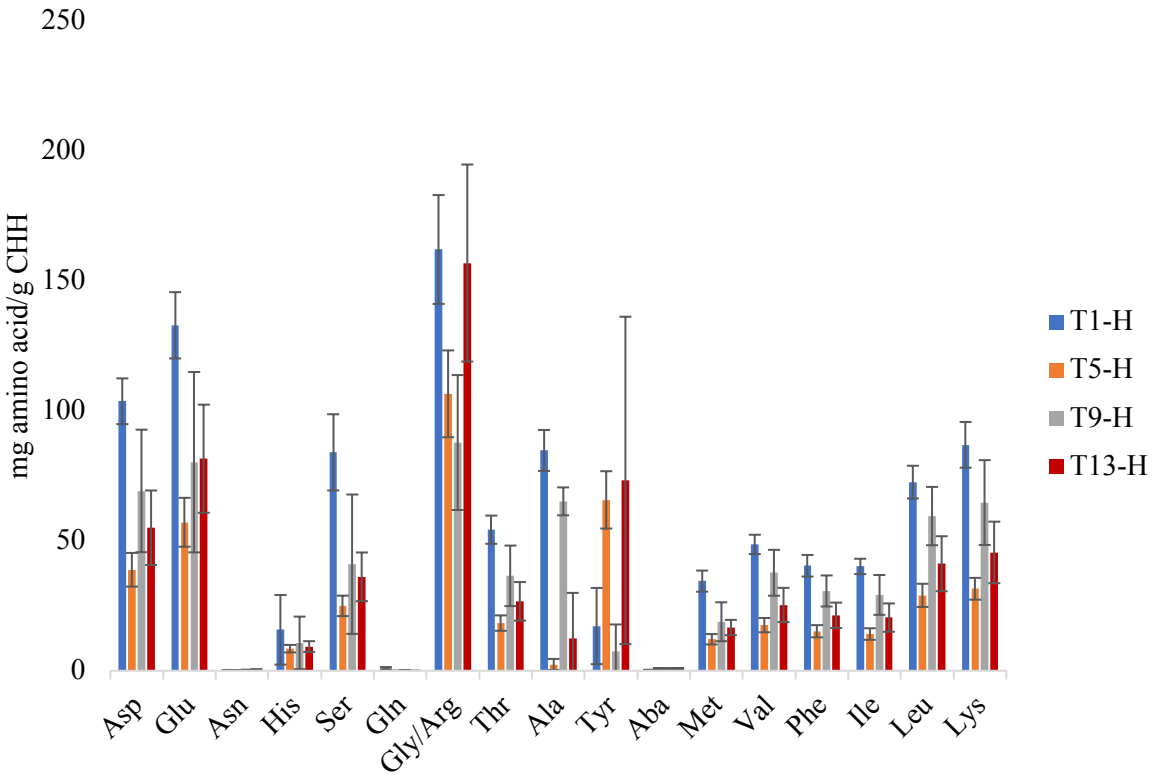

Figure 3.2: Amino acid composition (mg amino acid/g CHH) in hydrolysates T1-H, T5-H, T9-H and T13-H (mean ± SD, n = 3).

The distribution of amino acids was found to be relatively uniform in hydrolysates T1-H, T3-H, T5-H and T13-H. This trend is overall repeated in all the hydrolysates (appendix C). The most prominent amino acids were found to be aspartate, glutamine, glycine and arginine (detected together by the HPLC), alanine, leucine and lysine.

Several amino acids were not detected by the HPLC, namely cysteine, proline and tryptophan. Tryptophan is degraded during acid hydrolysis (Kristinsson and Rasco, 2000b), whereas proline is not detected by the utilized HPLC method. Glutamine, asparagine and aminobutyric acid were detected in small amounts.

Amino acid composition: ACE inhibitory activity and nutritional value of the hydrolysates

The composition of amino acids can be utilized for evaluation of which amino acids contribute to ACE inhibitory effect as well as the nutritional value of the hydrolysates.

The discussion of amino acids involved in ACE inhibitory activity and nutritional value is further based on the percentage distribution of amino acids, which is presented in table 3.3.

Table 3.3: Percentage distribution of amino acids in the CHHs. Essential amino acids are highlighted in italics.

	T1-H	T2-H	T3-H	T4-H	T5-H	T6-H	T7-H	T8-H	T9-H	T10-H	T11-H	T12-H	T13-H
Asp	10.59	10.21	10.30	10.49	8.77	10.88	8.64	10.64	10.81	8.50	11.44	12.05	8.84
Glu	13.57	13.32	13.23	12.19	12.89	12.83	12.73	13.55	12.54	12.74	13.74	13.61	13.10
Asn	0.03	0.01	0.02	0.03	0.02	0.04	0.02	0.03	0.04	0.02	0.05	0.04	0.05
His	*1.61*	*2.31*	*2.23*	*2.00*	*1.92*	*0.89*	*1.95*	*0.82*	*1.68*	*1.83*	*0.13*	*0.24*	*1.50*
Ser	8.58	8.83	9.11	6.47	5.63	7.90	5.46	7.83	6.41	5.51	7.52	7.62	5.80
Gln	0.10	0.14	0.11	0.02	0.00	0.07	0.00	0.06	0.02	0.00	0.01	0.04	0.00
Gly/Arg	16.55	17.09	18.08	14.05	24.05	15.87	23.76	16.80	13.72	24.66	16.04	16.37	25.18
Thr	*5.54*	*5.53*	*5.31*	*5.63*	*4.14*	*5.55*	*4.10*	*5.40*	*5.71*	*3.96*	*5.78*	*5.92*	*4.29*
Ala	8.65	8.65	9.28	10.26	0.55	9.66	0.83	9.57	10.19	0.68	9.60	9.75	1.99
Tyr	1.76	1.97	2.18	1.37	14.84	0.68	15.10	0.86	1.16	14.99	0.04	0.03	11.75
Aba	0.04	0.04	0.04	0.12	0.20	0.07	0.19	0.05	0.12	0.15	0.04	0.04	0.14
Met	*3.52*	*3.59*	*3.38*	*2.99*	*2.74*	*2.97*	*2.76*	*3.19*	*2.95*	*2.67*	*2.97*	*2.85*	*2.67*
Val	*4.96*	*4.73*	*4.69*	*5.78*	*3.96*	*5.43*	*3.96*	*5.08*	*5.89*	*3.89*	*5.51*	*5.66*	*4.06*
Phe	*4.13*	*3.98*	*3.94*	*4.76*	*3.43*	*4.46*	*3.49*	*4.15*	*4.80*	*3.40*	*4.42*	*4.37*	*3.43*
Ile	*4.10*	*3.82*	*2.37*	*4.45*	*3.19*	*4.36*	*3.27*	*4.21*	*4.56*	*3.12*	*4.49*	*2.78*	*3.28*
Leu	*7.41*	*7.13*	*6.38*	*9.19*	*6.55*	*8.53*	*6.58*	*8.27*	*9.30*	*6.47*	*8.64*	*8.89*	*6.61*
Lys	*8.87*	*8.66*	*9.36*	*10.19*	*7.12*	*9.82*	*7.16*	*9.51*	*10.11*	*7.39*	*9.60*	*9.74*	*7.31*
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

ACE inhibitory activities exerted by protein hydrolysates are dependent on the composition of amino acids in the peptide sequences in addition to the peptide size. Hydrophobic amino acids with branched or aromatic side chains have been shown to favor inhibition of ACE, particularly when present in the three C-terminal positions (Vermeirssen *et al.*, 2004). Hydrophobic branched amino acids are leucine, isoleucine, alanine, methionine and valine, and hydrophobic aromatic amino acids are phenylalanine, tyrosine and tryptophan. As presented in table 3.3, leucine and isoleucine were present in the hydrolysates (6.4 – 9.3% and 2.4 – 4.6%). The total amounts of isoleucine and leucine were plotted against IC_{50} of the hydrolysates, but no correlation was observed. Alanine (0.6 – 10.3%), methionine (2.7 – 3.6%), valine (3.9 – 5.9%), phenylalanine (3.4 – 4.8%) and tyrosine (0.03 - 15.1%) were detected in varying amounts but were present in the hydrolysates. Since tryptophan is degraded during acid hydrolysis, the content and influence on ACE inhibitory activity of this amino acid could not be determined.

As presented earlier, Papain hydrolyzes peptide bonds resulting in peptides with arginine or lysine at C-terminal position. Bromelain produces peptides with lysine, alanine or tyrosine at C-terminal position, and Protamex might produce peptides with non-hydrophobic amino acids at C-terminal position. Of these amino acids, alanine and tyrosine are hydrophobic amino acids. Alanine is present in relatively high amounts, whereas tyrosine is present in varying, and in some hydrolysates, low amounts. The relatively high amount of hydrophobic amino acids in the CHHs indicate potential presence in peptides that exert ACE inhibitory activity (Li *et al.*, 2004). Peptides produced by Protamex does not possess hydrophobic amino acid at the C-terminal position. However, peptides might exert ACE inhibitory activities when hydrophobic amino acids are present in the three C-terminal positions.

ACE inhibitory peptides have previously been produced by using the same proteolytic enzymes as for the CHHs. Even though the specificity of Protamex is not known, several antihypertensive peptides have been produced by using this proteolytic enzyme: Tyr-Asn from hard clam (IC_{50} = 51 μM) (Tsai *et al.*, 2008), Ala-Tyr from corn gluten meal (14.2 μM) (Yang *et al.*, 2007) and Met-Leu-Leu-Cys-Ser from *Styela plicata* (24.7 μM) (Ko *et al.*, 2011). Several ACE inhibitory peptides that fit into the proteolytic characteristics of Bromelain and Papain have been isolated from marine raw materials. These include Val-Tyr (IC_{50} = 35.2 μM), Ile-Tyr (6.1 μM), Phe-Tyr (42.3 μM) produced from seaweed (Sato *et al.*, 2002), and Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala (14.7 μM) produced from Alaska pollock (Je *et al.*, 2004).

Based on these findings, it could be speculated that the proteases produced peptides with lysine, alanine, tyrosine, arginine or non-hydrophobic amino acids (Protamex) on the C-terminus of the peptides. In addition, it could be suggested that presence of branched or aromatic hydrophobic amino acids in the tree C-terminal positions contributed to the ACE inhibitory activities. However, this needs to be further studied.

Nutritional value of the hydrolysates

The essential amino acids detected in the CHHs are highlighted in italics in table 3.3. Of all the essential amino acids, tryptophan was absent, whereas leucine and lysine were most prominent (6.4 – 9.3 and 7.1 – 10.2%) in the CHHs. The remaining essential amino acids were present in varying amounts. The combined average amount of essential amino acids in the hydrolysates was found to be 38.8 ± 11%. Of the conditionally essential amino acids, glycine and arginine were present, and, in varying amounts, tyrosine. As previously mentioned, the amount of essential amino acids is preserved upon enzymatic hydrolysis (Atef and Ojagh, 2017), and the CHHs were found to contain a balanced distribution and high content of essential and conditionally essential amino acids.

3.2.2. Molecular weight distribution

The molecular weight distribution of peptides in the retentates, permeates and their respective original hydrolysates were investigated with gel filtration by FPLC. During gel filtration, peptides of small molecular weight are fitted inside the pores of the column (stationary phase). Peptides too large to fit in the pores will thus elute before the smaller peptides because of less delay through the column. It was expected that the peptides in the retentates would elute in lower volumes than in the permeates, and peptides in the original hydrolysates would elute throughout the whole chromatogram. The analysis was performed to investigate a possible correlation between peptide molecular weight and ACE inhibitory activity.

In order to establish a relationship between elution volume (mL) and molecular weight of peptides detected, four proteins with known molecular weights were analyzed by gel filtration. The proteins utilized were pepsin (M_W = 34 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa) and vitamin B12 (1.4 kDa). Their chromatograms are included in appendix D. A linear relationship between elution volume (mL) and molecular weight was established based on the standards. The relationship is described in equation 3.1.

$$log_{10}(M_w) = -2.2124 \times K_{av} + 4.9272 \quad (3.1)$$

Equation 3.1 enabled calculation of molecular weights of the peptides in the samples detected by gel filtration. The derivation of equation 3.1 is presented in appendix D.

The column utilized, Superdex Peptide 10/300 GL, separates peptides with molecular weights ranging from 100 to 7000 Da. The average molecular weight of one amino acid is 110 Da, thus peptides containing between ~1 and 60 – 70 amino acids are detected in the valid range of the column. The valid range of elution volumes was determined to be between 15.83 and 29.17 mL by utilizing equation 3.1. The calculations of molecular weights are most trustworthy within this range. Determination of peptide sizes outside of this range introduce uncertainties and might be misleading.

The molecular weight distributions of the peptides in hydrolysates T1-H, T5-H, T9-H and T13-H are presented in figure 3.3.

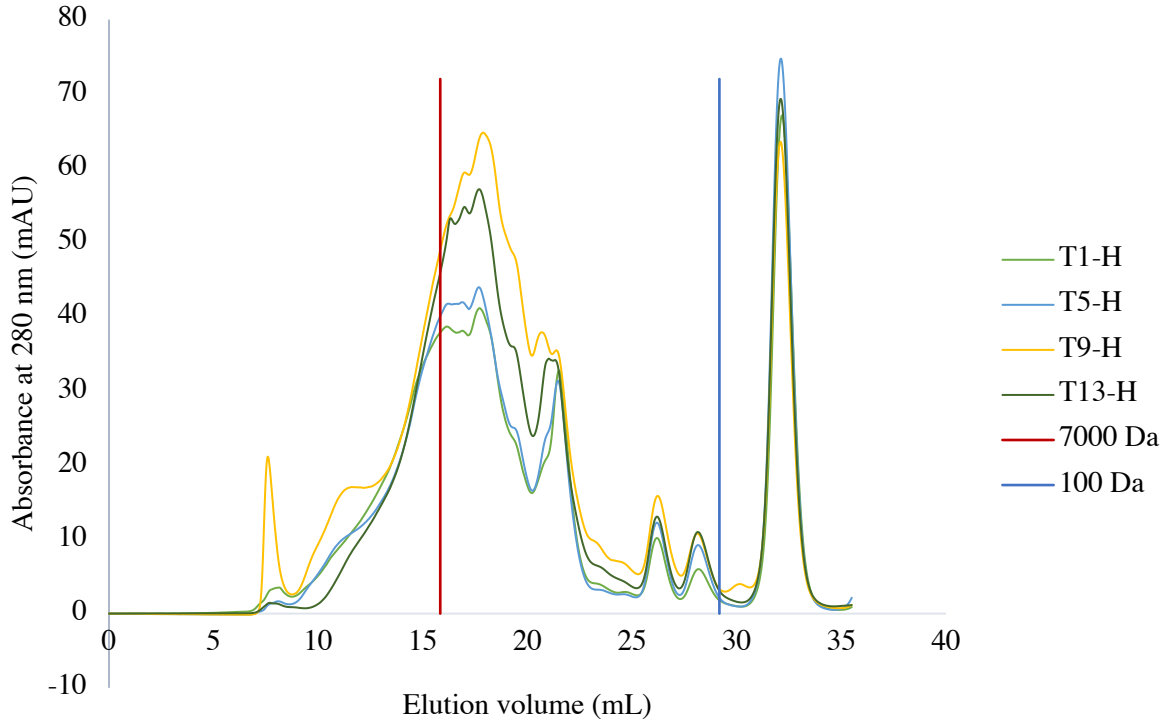

Figure 3.3: Chromatogram from gel filtration of T1-H, T5-H, T9-H and T13-H analyzed by FPLC using the column Superdex Peptide 30/100 GL. The valid range of the column is indicated as lines (peptide sizes 100 – 7000 Da).

The molecular weight distribution of peptides in hydrolysates T1-H, T5-H, T9-H and T13-H are as indicated in figure 3.3 relatively uniform and indicates that the hydrolysates contain peptides with a wide range of molecular weights.

Five distinct peaks were detected in the chromatogram of original hydrolysates. The first at 17.7 mL contained peptides of ~3900 Da. Peptides of ~1200 Da were detected at 21.4 mL, and peptides of 260 Da and 140 Da were detected at 26.2 and 28.2 mL, respectively. The last distinct peak eluted at 32.1 mL contained peptides < 110 Da.

The molecular weight distributions of the retentates and permeates are presented in figure 3.4 and 3.5, respectively.

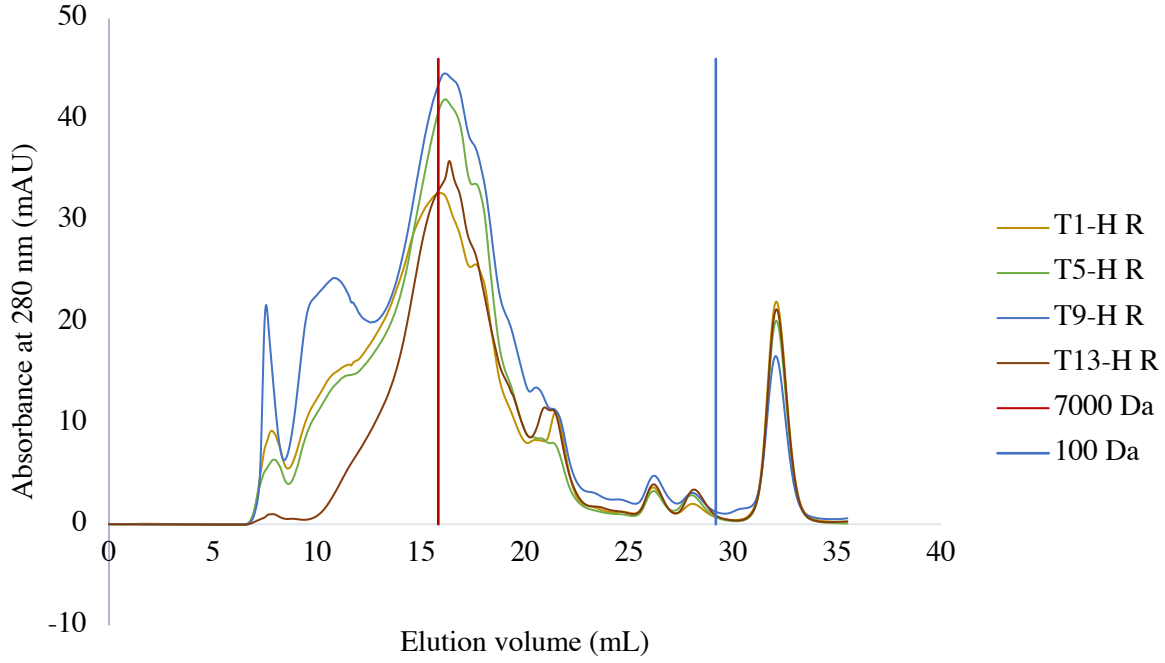

Figure 3.4: Chromatogram from gel filtration of T1-H, T5-H, T9-H and T13-H retentates (R) analyzed by FPLC using the column Superdex Peptide 30/100 GL. The valid range of the column is indicated as lines (peptide sizes 100 – 7000 Da).

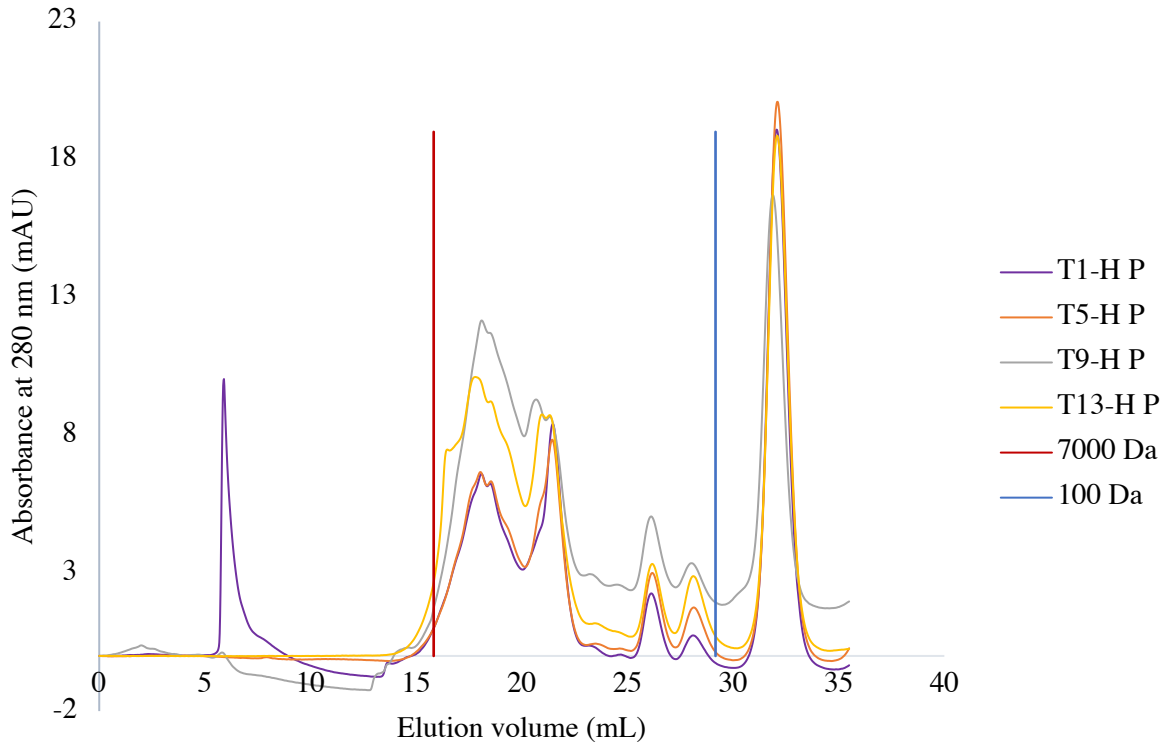

Figure 3.5: Chromatogram from gel filtration of T1-H, T5-H, T9-H and T13-H permeates (P) analyzed by FPLC using the column Superdex Peptide 30/100 GL. The valid range of the column is indicated as lines (peptide sizes 100 – 7000 Da).

Five prominent peaks were detected in the chromatograms of the permeates and the retentates. The chromatogram of the retentates indicates, correspondingly to the original hydrolysates, presence of peptides with a broad specter of molecular weights. The permeates contained peptides with lower molecular weights than the original hydrolysates and retentates.

The molecular weight distributions of retentates and permeates combined are presented in figure 3.6.

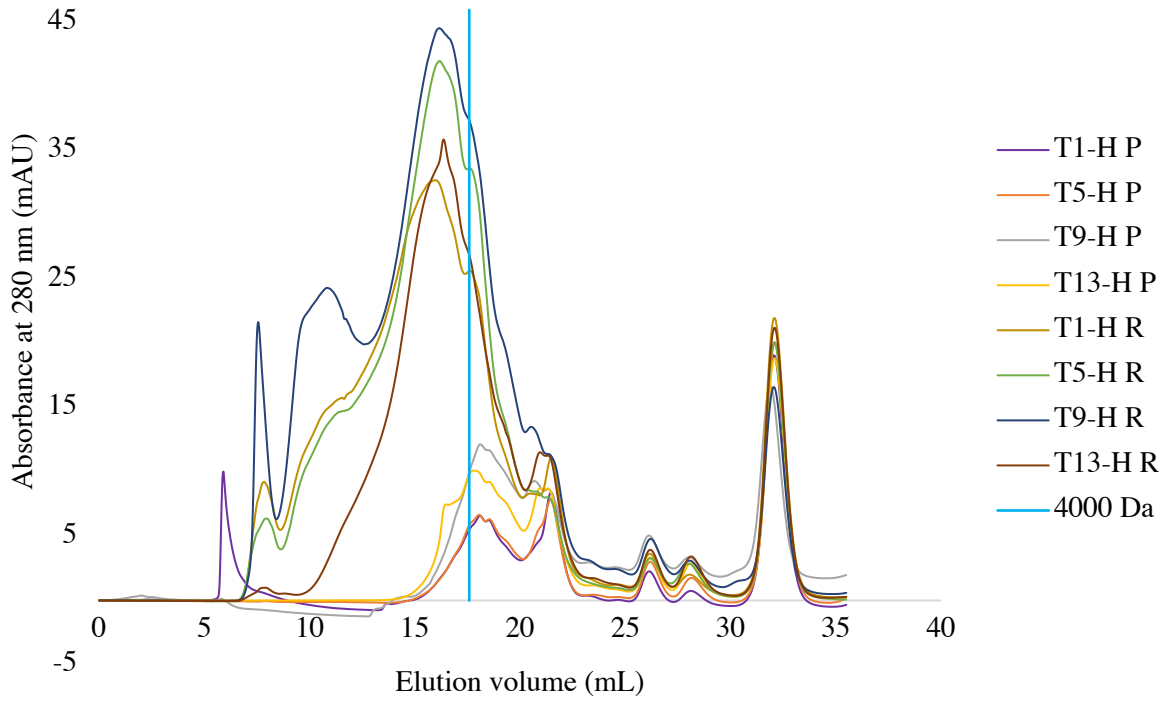

Figure 3.6: Chromatogram from gel filtration of T1-H, T5-H, T9-H and T13-H permeates (P) and retentates (R) analyzed by FPLC using the column Superdex Peptide 30/100 GL.
The membrane filter cut-off (4000 Da) is indicated as a line at elution volume 17.6 mL.

The permeates were composed almost exclusively of peptides smaller than 4000 Da. The retentates were expected to consist solely of peptides larger than 4000 Da (figure 3.6). However, 50 mL of each unfiltered CHH solution was retained as dead volume in order to prevent desiccation of the tank of the membrane filtration apparatus. The retentate was returned to the tank after fractionation from the permeate. The solution withdrew from the tank was therefore composed of retentate and unfiltered dead volume, and the retentate contained peptides smaller than 4000 Da.

Some unidentified peaks were detected in the chromatograms of retentates and permeates. These may have been caused by changes in peptide composition upon membrane filtration, maybe formation of peptide complexes, as they were not detected in the original hydrolysates. Additionally, a peak eluted at 7.5 mL from original hydrolysates and retentates of T9-H was assumed to originate from the stick water utilized in the production of this hydrolysate.

The broad peak eluted at 16 – 17 mL in the original hydrolysates and retentates was absent in the permeates. This peak was calculated to consist of peptides of ~ 4000 Da (30 - 40 amino acids). As previously presented in section 3.1, the ACE inhibitory activities expressed by the original hydrolysates were more potent than the permeates. It could be assumed that peptides of ~ 4000 Da enhanced the ACE inhibitory activity. These peptides were fractionated into the retentate, which may explain the less potent inhibitory activity of the permeates. However, the permeate still exerted ACE inhibitory activity. This might indicate that the ACE inhibitory activity was expressed by small peptides, but that the effect was amplified by larger peptides. The ACE inhibitory activity of the retentates could have been measured in order to investigate if this fraction exerted a more potent inhibitory activity than the permeates.

Although smaller peptides (2 – 20 amino acids) have been found be highly ACE inhibitory (Je *et al.*, 2004; Lee *et al.*, 2010), larger peptides might contribute to this inhibitory effect by exerting effects themselves, or by interactions with smaller peptides to amplify their effects (Aluko, 2012). Paiva *et al*. investigated *in vitro* ACE inhibitory properties of ultrafiltrate fractions with different peptide molecular weight ranges (<1, 1 – 3 and ≥ 3 kDa) of *Fucus spiralis* protein hydrolysate. The fraction with peptides ≥ 3 kDa showed significantly higher ACE inhibitory activity than the two other fractions (Paiva *et al.*, 2017), which suggest that peptides of relatively large sizes might possess or amplify ACE inhibitory activity.

Uncertainties in the standard equation and the method

Equation 3.1 was derived based on standard proteins with molecular weights ranging from 1400 to 34000 Da. Thus, the elution volumes corresponding to molecular weights lower than 1400 Da were not validly covered by the equation. The molecular weights of peptides detected at the highest elution volumes were therefore assumed to be wrongly calculated. Additionally, the column is not separating peptides effectively down to the lower detection limit (100 Da), and the method is based on the assumption that all peptides have the same affinity for the column. In addition to size, the peptides may carry electrical charges that might impact the separation and detection using gel filtration.

Evaluation of the membrane filtration and the molecular weight distribution

The chromatograms in figure 3.6 show a distinct difference between permeates and retentates in peaks eluted around 17 – 18 mL (4000 Da). This indicates that the membrane filtration resulted in a relatively clean cut-off, besides the fact that retentates also contain the dead-volume. In addition, the line indicating the cut-off indicates that the standard equation was valid within the range of standard proteins (1400 – 34000 Da) due to the visibly clean cut-off between retentates and permeates at this particular elution volume.

The four CHHs that were membrane filtered and analyzed for molecular weight distribution were produced with the same proteolytic enzymes (Papain and Bromelain). The molecular weight distributions were thus expected to be uniform. It could however be interesting to analyze the CHHs produced with Protamex (T3-H, T7-H or T8-H) to determine the distribution of peptide sizes and how these may have affected the ACE inhibitory activity.

3.2.3. Free amino acids

The content of free amino acids in hydrolysates T1-H, T5-H, T9-H and T13-H are given in table 3.4. The calculations are presented in appendix E.

Table 3.4: Amount of free amino acids (mg) detected per gram of CHH in hydrolysates T1-H, T5-H, T9-H and T13-H (mean ± SD, n = 3, n = 1 for T13-H).

	T1-H	**T5-H**	**T9-H**	**T13-H**
mg free aa/g	11.46 ± 0.86	21.29 ± 1.1	13.32 ± 0.13	25.44

The composition of free amino acids in the remaining nine hydrolysates were determined by Ayat Asfour and are presented in table 3.5.

Table 3.5: Amount of free amino acids (mg) detected per gram of CHH. The analysis was performed by Ayat Asfour.

	T2-H	**T3-H**	**T4-H**	**T6-H**	**T7-H**	**T8-H**	**T10-H**	**T11-H**	**T12-H**
mg free aa/g	22.15	27.77	27.20	14.58	9.53	12.24	15.46	8.08	7.47

The amounts of free amino acids in the hydrolysates were found to be relatively low. This indicates that Protamex, Papain and Bromelain mostly cleave peptide bonds endoproteolytically, and thus produced dipeptides or larger. The amounts of free amino acids

however deviated slightly. This might be due to structural differences in the cod heads, which impacts the specificity of the proteolytic enzymes. No correlations were found between the amount of free amino acids and amount of water utilized in hydrolysis and the season of catch of the cod.

The relative concentration of free amino acids in hydrolysates can be investigated by comparison with the total amount of amino acids. The total amounts of amino acids and free amino acids (mg aa/g CHH) in hydrolysate T1-H parallel 1 are given in table 3.6.

Table 3.6: The total amount of amino acids (mg aa/g CHH) and the amount of free amino acids (mg free aa/g CHH) in hydrolysate T1-H (parallel 1).

Amino acid	Total amount of aa (mg/g CHH)	Amount of free aa (mg/g CHH)
Asp	98.09	0.46
Glu	123.96	0.94
Asn	0.23	0.02
His	0.49	0.31
Ser	71.27	1.15
Gln	0.45	0.33
Gly/Arg	144.32	1.35
Thr	49.52	0.63
Ala	79.48	1.46
Tyr	0.37	0.46
Aba	0.36	0.02
Met	31.21	0.81
Val	46.76	0.58
Phe	36.76	0.54
Ile	38.72	0.35
Leu	68.39	1.45
Lys	80.69	1.26
Total	871.06	12.12

The comparison provided in table 3.6 indicates that the amount of free amino acids in hydrolysate T1-H was low (1.4% free amino acids). The low content of free amino acids emphasizes that the hydrolysates exert ACE inhibitory activity, because inhibitory peptides are

composed of at least two amino acids. Additionally, low amounts of free amino acids are beneficial for the nutritional value of the CHHs. Free amino acids are poorly absorbed *in vivo* compared to di or tripeptides (Siemensma *et al.*, 1993).

3.2.4. Degree of hydrolysis

The degree of hydrolysis (%) of the hydrolysates was determined to range from 10.87 to 15.85% (figure 3.7). The calculations are presented in appendix F.

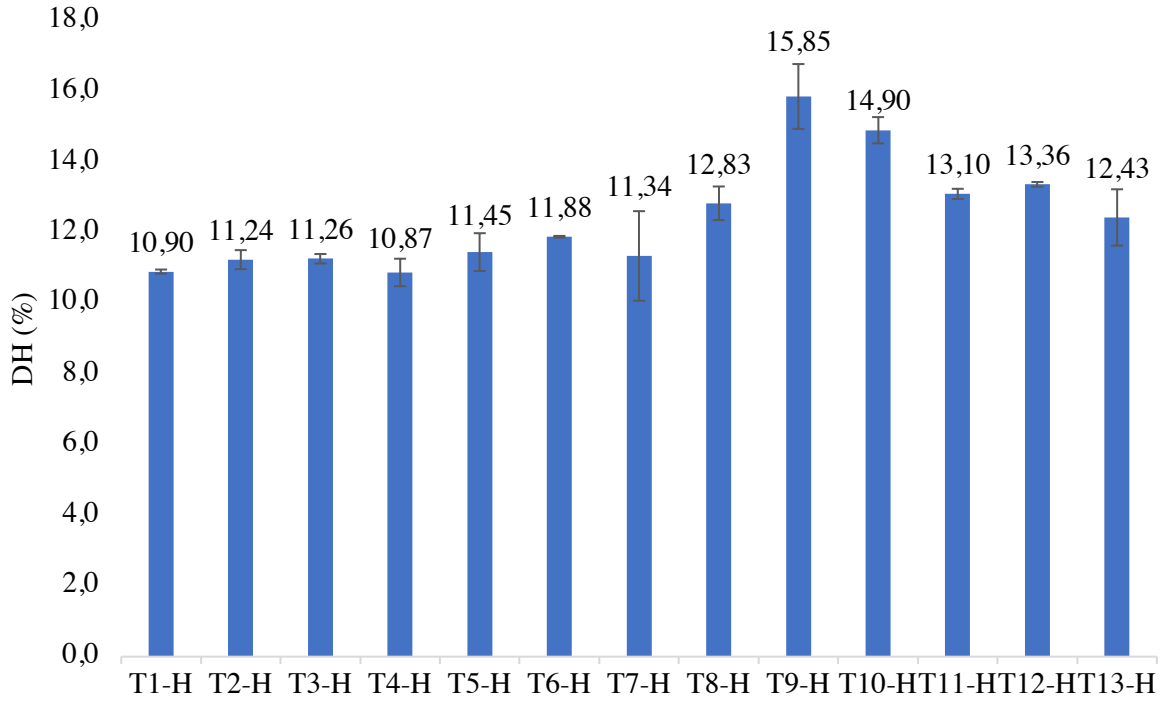

Figure 3.7: Degree of hydrolysis (%) of the CHHs. Analysis performed by Ayat Asfour (mean ± SD, n = 2).

As previously presented, the DH is directly correlated to the peptide sizes in a protein hydrolysate. The molecular weight distribution (figure 3.3) indicated that the CHHs are composed of peptides with a wide range of molecular weights. The DH values of the CHHs are thus indicating a complex composition of small and large peptides. Although four out of the thirteen hydrolysates were analyzed by gel filtration, it was assumed that the remaining hydrolysates produced by Papain and Bromelain, but also those produced by Protamex, were composed of peptides with a wide range of molecular weights based on their DH values.

DH and ACE inhibitory activity

Balti *et al*. investigated possible correlations between DH (%) and ACE inhibitory activities in hydrolysates from cuttlefish. Three hydrolysates with varying DH were investigated, namely 5, 10 and 13.5%. The hydrolysate with DH = 13.5 % displayed the most potent ACE inhibitory activity (IC_{50} = 1.0 mg/mL). The IC_{50} values of the other hydrolysates were not given, but these exerted lower antihypertensive activities (Balti *et al.*, 2010). These findings indicate that the DH may impact the ACE inhibitory activity of a protein hydrolysate. This can be explained by the fact that ACE inhibitory peptides generally consist of between 2 and 20 amino acids, which are considered relatively small.

A graphical presentation is provided in figure 3.8 in order to investigate a potential correlation between the ACE inhibitory activity and DH of the CHHs.

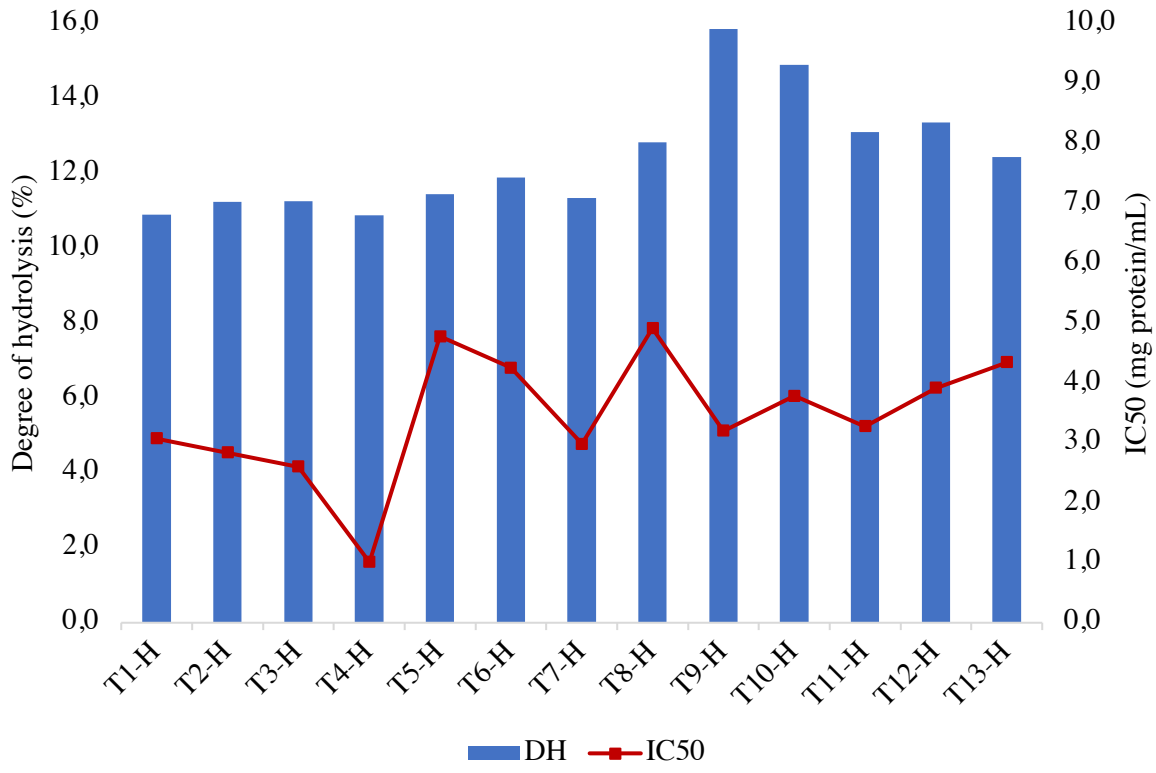

Figure 3.8: The DH (%) and IC_{50} (mg protein/mL) of the 13 CHHs. The DH is given by the blue poles whereas the IC_{50} is given by the red line.

Although it was expected, no significant correlation was observed between DH and IC_{50} in figure 3.8.

Šližytė *et al*. suggested that a degree of hydrolysis of approximately 25% can be considered as high, in which a considerable number of small peptides has been produced (Šližytė *et al.*, 2009). In order to investigate whether increasing the DH impacts the ACE inhibitory activity, the IC_{50} values of the CHHs were plotted against DH and presented in figure 3.9.

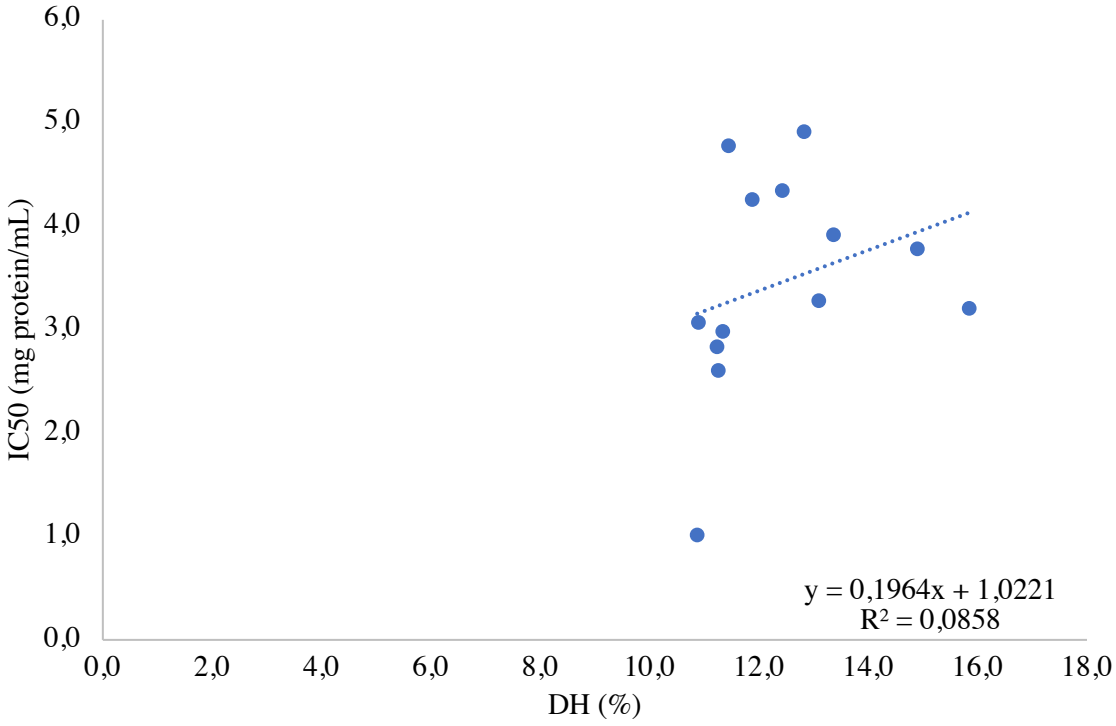

Figure 3.9: Linear relationship between DH (%) and IC_{50} (mg protein/mL).

Based on figure 3.9, increasing the DH would not affect the ACE inhibitory activities of the CHHs. Additionally, an augmented DH would negatively affect functional properties of the hydrolysate, including foaming capacities, fat absorption and emulsifying properties, and an increased number of small peptides would contribute to an increased bitter taste.

Peptides smaller than 6000 Da containing hydrophobic amino acids are found to have bitter taste (FitzGerald and O'Cuinn, 2006). Considering the molecular weight distributions of the CHHs (section 3.2.2.), peptides smaller than 6000 Da were highly present in the hydrolysates. However, investigations done by SINTEF Ocean have shown that the hydrolysates do not have bitter taste (FHF, 2018), which could be explained by absence of hydrophobic amino acids on the peptide C-terminals.

3.3. Evaluation of the methods

The methods for measuring the ACE inhibitory activity (section 2.3.1) and total amino acid composition (section 2.3.2) were easily conducted and gave good results.

To enable more accurate determination of molecular weights in protein hydrolysates (section 3.2.2), more standard proteins could have been used so that the standard equation would validly cover the range of peptide sizes investigated.

Membrane filtration of the hydrolysates resulted in relatively good protein recovery (appendix G.3). However, the fractionation was considered unnecessary to obtain fractions with more potent ACE inhibitory activity, though fractionation gave good results for antioxidative properties of the CHHs, which was investigated by a fellow master student.

3.4. Conclusions in the HEADS UP project

SINTEF published a report in relation to the termination of the HEADS UP pilot project. This report included observations, conclusions and future aims and challenges regarding the CHH produced in Tufjord (Remme, 2017).

A series of conclusions were provided. The cod head hydrolysates were water soluble and did not have bitter taste, and were therefore considered suitable as food additives (FHF, 2018). The proteolytic enzyme had the largest impact on yield, odor and taste of the hydrolysates compared to the other processing parameters. The amount of water utilized in the production had a small impact on the yield and chemical composition, but reduced amount of water resulted in higher yield of proteins. Seasonal variations of the cod had no considerable impact on the chemical composition of the hydrolysates, and the yield of dried hydrolysate from raw material was determined to be approximately 10%. Finally, it was determined that stick water from the oil factory in Tufjord was not suitable for use in the hydrolysis process (Remme, 2017).

These conclusions provided by SINTEF Ocean correspond to the findings in this thesis. The amount of water utilized in the production had no considerable effect on the ACE inhibitory activity, nor the structural properties of the hydrolysates. Seasonal variations of the cod did not impact ACE inhibitory activities or structural properties of the hydrolysates. Additionally,

hydrolysate T9-H, which was produced with stick water, had considerably more potent fish off-flavor. However, the stick water did not affect the ACE inhibitory activity and the structural properties, but the sensory aspects reduces the quality of this hydrolysate as a potential food ingredient.

Among the future aims and challenges presented in this report were further refinement of the product to decrease the amount of fat, salt and taste. It was suggested that investigations can be performed to determine the stability of the product, and methods for drying and freezing the product can be developed to preserve the quality and stability. The final aim and challenge was full scale production of cod head hydrolysates in the future, which is already being investigated in the project HEADS UP II (FHF, 2018), which was started 1st of April 2018 and aims for investigation of large-scale production of hydrolysates from cod heads, saithe and redfish.

Chapter 4: Conclusion

The objectives of this thesis were to evaluate *in vitro* ACE inhibitory activities and investigate structure-activity relationships of cod head hydrolysates.

All the cod head hydrolysates expressed ACE inhibitory activity *in vitro*, and IC_{50} values ranged from 2.60 – 4.91 mg protein/mL. The inhibitory effect exerted by one of the hydrolysates was misleading and was therefore not included in this range. The ACE inhibitory activities of the hydrolysates were found to be relatively low compared to the antihypertensive drug Captopril (IC_{50} = 0.097 mg/mL) and relatively high compared to inhibitor peptides from other origins than cod heads.

Four of the hydrolysates were fractionated by ultrafiltration. The fractions enriched in small peptides were found to exert less potent ACE inhibitory activity than the unfractionated hydrolysates. It is therefore suggested that fractionation is unnecessary to increase antihypertensive effects of the cod head hydrolysates.

The hydrolysates were composed of amino acids that have been found to contribute to inhibition of ACE, including leucine, isoleucine, valine, phenylalanine and methionine, in addition to a balanced distribution and high content of essential amino acids (average content 38.8 ± 11%). The hydrolysates contained peptides with broad molecular weight distribution and had low amounts of free amino acids. No correlation was found between degree of hydrolysis and IC_{50}.

No correlations were found between season of harvest of the cod and chemical composition of the cod head hydrolysates. Additionally, no correlations were found between processing parameters and ACE inhibitory activity and molecular properties of the hydrolysates. The use of stick water in the production of one hydrolysate did affect the sensory aspects, but not molecular properties.

Cod head hydrolysates were found to be a promising potential food ingredient with antihypertensive effects, high solubility, no bitter taste and good nutritional value due to low amounts of free amino acids and high amounts of essential amino acids.

Chapter 5: Suggestions for further work

In order to further investigate the antihypertensive properties of the CHHs, ACE inhibitory peptides can be purified from the hydrolysates, and the sequences can be determined, for example by using mass spectrometry. The CHHs can additionally be fractionated into several fractions by using varying peptide size cut-offs, and the ACE inhibitory activities of the fractions can be measured individually.

Additionally, the *in vivo* activity of the ACE inhibitory peptides can be investigated, for example by analyzing effects *in vitro* with gastrointestinal enzymes to determine possible degradation of the peptides, or by investigating the effect of the peptides on blood pressure in SHR.

Other proteolytic enzymes than Protamex, Papain and Bromelain can be utilized for production of cod head hydrolysates, and the antihypertensive potency can be determined to investigate impact of proteolytic enzymes. Finally, the functional and sensory properties of the cod head hydrolysates must be tested by incorporation in food systems.

References

Abubakar, A., Saito, T., *et al*. 1998. Structural Analysis of New Antihypertensive Peptides Derived from Cheese Whey Protein by Proteinase K Digestion. *Journal of Dairy Science,* 81**,** 3131 - 3138.

Adler-Nissen, J. 1984. Control of the Proteolytic Reaction and of the Level of Bitterness in Protein Hydrolysis Processes. *Journal of Chemical Technology and Biotechnology,* 34**,** 215 - 222.

Adler-Nissen, J. 1986. *Enzymatic Hydrolysis of Food Proteins,* Barking, UK, Elsevier Applied Science Publishers.

Aluko, R. E. 2012. Bioactive Peptides. *Functional Foods and Nutraceuticals*. New York: Springer

Atef, M. & Ojagh, S. M. 2017. Health Benefits and Food Applications of Bioactive Compounds from Fish Byproducts: A review. *Journal of Functional Foods,* 35**,** 673 - 681.

Atkinson, A. B. & Robertson, J. I. S. 1979. Captopril in the Treatment of Clinical Hypertension and Cardiac Failure. *The Lancet,* 314**,** 836 - 839.

Balti, R., Ali, N. E.-H., *et al*. 2010. Influence of Degree of Hydrolysis on Functional Properties and Angiotensin I-Converting Enzyme-Inhibitory Activity of Protein Hydrolysates from Cuttlefish (*Sepia officinalis*) By-Products. *Journal of the Science of Food and Agriculture,* 90**,** 2006 - 2014.

Bekkevold, S. & Olafsen, T. 2007. *Marine biprodukter: Råvarer med muligheter,* Trondheim, Stiftelsen RUBIN.

Benjakul, S., Yarnpakdee, S., *et al*. 2014. Fish Protein Hydrolysates: Production, Bioactivities, and Applications. *In:* Kristinsson, H. G. (ed.) *Antioxidants and Functional Components in Aquatic Foods*. 1st ed. Reykjavik, Iceland: WILEY Blackwell.

Blackburn, S. 1978. *Amino Acid Determination: Methods and Techniques,* New York, Marcel Dekker.

Chalamaiah, M., Dinesh Kumar, B., *et al*. 2012. Fish Protein Hydrolysates: Proximate Composition, Amino Acid Composition, Antioxidant Activities and Applications: A review. *Food Chemistry,* 135**,** 3020 - 3038.

Chalamaiah, M., Narsing, G. R., *et al*. 2010. Protein Hydrolysates from Meriga (*Cirrhinus mrigala*) Egg and Evaluation of their Functional Properties. *Food Chemistry,* 120**,** 652 - 657.

Chiang, W.-D., Tsou, M.-J., *et al*. 2006. Angiotensin I-Converting Enzyme Inhibitor Derived from Soy Protein Hydrolysate and Produced by Using Membrane Reactor. *Food Chemistry,* 98**,** 725 - 732.

Cui, H. 1996. *The Influence of Degree of Hydrolysis on the Structural and Functional Properties of Fish Protein Peptic Hydrolysates*. Masters thesis, University of Washington.

Damodaran, S. 1997. *Food Proteins and Their Applications,* New York, CRC Press.

Dauksas, E., Šližytė, R., *et al*. 2004. Bitterness in Fish Protein Hydrolysates and Methods for Removal. *Journal of Aquatic Food Product Technology,* 13**,** 101 - 114.

EC. 2018. *Health Claims* [Online]. Food: Nutrition and Health Claims. Available: https://ec.europa.eu/food/safety/labelling_nutrition/claims/health_claims_en [Accessed 30.04.2018].

Erdmann, K., Cheung, B. W. Y., *et al*. 2008. The Possible Roles of Food-Derived Bioactive Peptides in Reducing the Risk of Cardiovascular Disease. *The Journal of Nutritional Biochemistry,* 19**,** 643 - 654.

Falch, E., Rustad, T., *et al*. 2006. Geographical and Seasonal Differences in Lipid Composition and Relative Weight of By-products from Gadiform Species. *Journal of Food Composition and Analysis,* 19**,** 727 - 736.

FAO 2016. The State of World Fisheries and Aquaculture 2016. Contributing to food secutirty and nutrition for all. Rome.

FHF. 2016. *Restråstoff: Anvendelser av hoder til humant konsum (HEADS UP)* [Online]. Available: http://www.fhf.no/prosjektdetaljer/?projectNumber=901308 [Accessed 11.05.2018].

FHF. 2018. *Kvalitetsprotein fra torskehoder (HEADS UP II)* [Online]. Available: http://www.fhf.no/prosjektdetaljer/?projectNumber=901499 [Accessed 11.05.2018].

FitzGerald, R. J. & O'Cuinn, G. 2006. Enzymatic Debittering of Food Protein Hydrolysates. *Biotechnology Advances,* 24**,** 234 - 237.

Foltz, M., Cerstiaens, A., *et al*. 2008. The Angiotensin Converting Enzyme Inhibitory Tripeptides Ile-Pro-Pro and Val-Pro-Pro Show Increasing Permeabilities with Increasing Physiological Relevance of Absorption Models. *Peptides,* 29**,** 1312 - 1320.

Friedman, K. 1996. Nutritional Value of Proteins from Different Food Sources: a review. *Journal of Agricultural and Food Chemistry,* 44**,** 6 - 29.

Fujitaa, H. & Yoshikawab, M. 1999. LKPNM: A Prodrug-Type ACE-Inhibitory Peptide Derived from Fish Protein. *Immunopharmacology,* 44**,** 123 - 127.

Gómez-Ruiz, J. A., Ramos, M.*, et al*. 2004. Angiotensin Converting Enzyme-Inhibitory Activity of Peptides Isolated from Manchego Cheese. Stability under Simulated Gastrointestinal Digestion. *International Dairy Journal,* 14**,** 1075 - 1080.

Guérard, F., Sellos, D.*, et al*. 2005. Fish and Shellfish Upgrading, Traceability. *Adv Biochem Engin/Biotechnol,* 96**,** 127 - 163.

Hall, G. M. & Ahmad, N. H. 1992. Functional Properties of Fish Protein Hydrolyzates. *In:* Hall, G. M. (ed.) *Fish Processing Technology*. New York, US: Blackie Academic & Professional.

Hartmann, R. & Meisel, H. 2007. Food-Derived Peptides with Biological Acitivity: from Research to Food Applications. *Current Opinion in Biotechnology,* 18**,** 163 - 169.

He, H.-L., Chen, X.-L.*, et al*. 2007. High Throughput and Rapid Screening of Marine Protein Hydrolysates Enriched in Peptides with Angiotensin-I-Converting Enzyme Inhibitory Activity by Capillary Electrophoresis. *Bioresource Technology,* 98**,** 3499 - 3505.

Hernàndez-Ledesma, B., del Mar Contreras, M.*, et al*. 2011. Antihypertensive Peptides: Production, Bioavailability and Incorporation into Foods. *Advances in Colloid and Interface Science,* 165**,** 23 - 35.

Hooper, N. M. & Turner, A. J. 1987. Isolation of Two Differentially Glycosylated forms of Peptidyl-Dipeptidase A (Angiotensin Converting Enzyme) from Pig Brain: A Re-Evaluation of their Role in Neuropeptide Metabolism. *Biochemical Journal,* 241**,** 625 - 633.

Hsu, K.-C. 2010. Purification of Antioxidative Peptides Prepared from Enzymatic Hydrolysates of Tuna Dark Muscle By-Product. *Food Chemistry,* 122**,** 42 - 48.

Je, J.-Y., Park, P.-J.*, et al*. 2004. A Novel Angiotensin I Converting Enzyme Inhibitory Peptide from Alaska Pollack (*Theragra chalcogramma*) Frame Protein Hydrolysate. *Journal of Agricultural and Food Chemistry,* 52**,** 7842-7845.

Kearney, P. M., Whelton, M.*, et al*. 2005. Global Burden of Hypertension: Analysis of Worldwide Data. *The Lancet,* 365**,** 217 - 223.

Keskitalo, C., Bujnicki, J.*, et al*. 2017. *Food From the Oceans,* Luxembourg, Scientific Advice Mechanism (SAM) (European Commision).

Kim, S.-K. & Mendis, E. 2005. Bioactive Compounds from Marine Processing Byproducts. *Food Research International,* 39**,** 383 - 393.

Ko, S.-C., Kang, M.-C.*, et al*. 2011. Effect of Angiotensin I-Converting Enzyme (ACE) Inhibitory Peptide Purified from Enzymatic Hydrolysates of *Styela plicata*. *European Food Research and Technology,* 233**,** 915 - 922.

Kristinsson, H. G. & Rasco, B. A. 2000a. Biochemical and Functional Properties of Atlantic Salmon (*Salmo salar*) Muscle Proteins Hydrolyzed with Various Alkaline Proteases. *Journal of Agricultural and Food Chemistry,* 48**,** 657 - 666.

Kristinsson, H. G. & Rasco, B. A. 2000b. Fish Protein Hydrolysates: Production, Biochemical, and Functional Properties. *Critical Reviews in Food Science and Nutrition,* 40**,** 43 - 81.

Kristinsson, H. G. & Rasco, B. A. 2000c. Kinetics of the Hydrolysis of Atlantic Salmon (*Salmo salar*) Muscle Proteins by Alkaline Proteases and a Visceral Serine Protease Mixture. *Process Biochemistry,* 36**,** 131 - 139.

Lahl, W. J. & Braun, S. D. 1994. Enzymatic Production of Protein Hydrolysates for Food Use. *Food Technolocy Chicago,* 58**,** 68 - 71.

Lee, S.-H., Qian, Z.-J.*, et al*. 2010. A Novel Angiotensin I Converting Enzyme Inhibitory Peptide from Tuna Frame Protein Hydrolysate and its Antihypertensive Effect in Spontaneously Hypertensive Rats. *Food Chemistry,* 118**,** 96 - 102.

Li, G.-H., Le, G.-W.*, et al*. 2004. Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Food Proteins and their Physiological and Pharmacological Effects. *Nutrition Research,* 24**,** 469 - 486.

Liaset, B., Julshamn, K.*, et al*. 2003. Chemical Composition and Theoretical Nutritional Evaluation of the Produced Fractions from Enzymatic Hydrolysis of Salmon Frames with Protamex™ . *Process Biochemistry,* 38**,** 1747 - 1759.

Liaset, B., Nortvedt, R.*, et al*. 2002. Studies on the Nitrogen Recovery in Enzymatic Hydrolysis of Atlantic Salmon (*Salmo salar*, L.) Frames by Protamex ™ Protease *Process Biochemistry,* 37**,** 1263 - 1269.

Lowry, O. H., Rosebrough, N. J.*, et al*. 1951. Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry,* 193**,** 265 - 275.

Mackie, I. M. 1974. Proteolytic Enzymes in Recovery of Proteins from Fish Waste. *Process Biochemistry,* 9**,** 12 - 14.

Maeno, M., Yamamoto, N., *et al*. 1996. Identification of an Antihypertensive Peptide from Casein Hydrolysate Produced by a Proteinase from *Lactobacillus helveticus* CP790. *Journal of Dairy Science,* 79**,** 1316 - 1321.

Mahmoud, M. I., Malone, W. T., *et al*. 1992. Enzymatic Hydrolysis of Casein: Effect of Degree of Hydrolysis on Antigenicity and Physical Properties. *Journal of Food Science,* 57**,** 1223 - 1229.

Motoi, H. & Kodama, T. 2003. Isolation and Characterization of Angiotensin I-Converting Enzyme Inhibitory Peptides from Wheat Gliadin Hydrolysate. *Molecular Nutrition & Food Research,* 47**,** 354 - 358.

Nelson, D. L. & Cox, M. M. 2013. *Lehninger Principles of Biochemistry,* New York, W. H. Freeman and company.

Nguyen, H. T. M., Sylla, K. S. B., *et al*. 2011. Enzymatic Hydrolysis of Yellowfin Tuna (*Thunnus albacres*) By-Products Using Protamex Protease. *Food Technololgy and Biotechnology,* 49**,** 48 - 55.

Ohsawa, K., Satsu, H., *et al*. 2008. Producibility and Digestibility of Antihypertensive β-casein Tripeptides, Val-Pro-Pro and Ile-Pro- Pro, in the Gastrointestinal Tract: Analyses Using an *in vitro* Model of Mammalian Gastrointestinal Digestion. *Journal of Agricultural and Food Chemistry,* 56**,** 854 - 858.

Økland, H. M. W. & Kjerstad, M. 2002. Potensiale for ilandføring og utnyttelse av fiskehoder. Ålesund: Møreforskning.

Ondetti, M. A. & Cushman, D. W. 1982. Enzymes of the Renin-Angiotensin System and their Inhibitors. *Annual Review of Biochemistry,* 51**,** 283 - 308.

Ondetti, M. A., Rubin, B., *et al*. 1977. Design of Specific Inhibitors of Angiotensin-Converting Enzyme: New Class of Orally Active Antihypertensive Agents. *Science,* 196**,** 441 - 444.

Opheim, M., Larssen, E., *et al*. 2015. Hydrolysis of Atlantic Salmon (*Salmo salar*) Rest Raw Materials - Effect of Raw Material and Processing on Composition, Nutritional Value, and Potential Bioactive Peptides in the Hydrolysate. *Process Biochemistry,* 50**,** 1247 - 1257.

Osnes, K. K. & Mohr, V. 1985. Peptide Hydrolases of Antarctic Krill, *Euphausia superba*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 82**,** 599 - 606.

Otagiri, K., Nosho, Y., *et al*. 1985. Studies on a Model of Bitter Peptides Including Arginine, Proline and Phenylalanine Residues. I. Bitter Taste of Di- and Tripeptides, and Bitterness Increase of the Model Peptides by Extension of the Peptide Chain. *Agricultural and Biological Chemistry,* 49**,** 1019 - 1026.

Paiva, L., Lima, E., *et al*. 2017. Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity, Antioxidant Properties, Phenolic Content and Amino Acid Profiles of *Fucus spiralis* L. Protein Hydrolysate Fractions. *Marine drugs,* 15.

Peñas, E., Restani, P., *et al*. 2006. Evaluation of the Residual Antigenicity of Dairy Whey Hydrolysates Obtained by Combination of Enzymatic Hydrolysis and High-Pressure Treatment. *Journal of Food Protection,* 69**,** 1707 - 1712.

Remme, J. F. 2017. HEADS UP Kvalitetsproteiner fra torskehoder. SINTEF Ocean.

Richardsen, R., Nystøyl, R., *et al*. 2017. Analyse Marint Restråstoff, 2016. Tromsø: SINTEF Ocean, Kontali Analyse AS.

Rustad, T. 2003. Utilisation of Marine By-Products. *Electronic Journal of Environmental, Agricultural and Food Chemistry (EJEAFChe),* 2**,** 458 - 463.

Rustad, T., Storrø, I., *et al*. 2011. Possibilities for the Utilisation of Marine By-Products. *International Journal of Food Science & Technology,* 46**,** 2001 - 2014.

Saha, B. C. & Hayashi, K. 2001. Debittering of Protein Hydrolyzates. *Biotechnology Advances,* 19**,** 355 - 370.

Sathivel, S., Smiley, S., *et al*. 2005. Functional and Nutritional Properties of Red Salmon (*Oncorhyncus nerka*) Enzymatic Hydrolysates. *Journal of Food Science,* 70.

Sato, M., Hosokawa, T., *et al*. 2002. Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Wakame (*Undaria pinnatifida*) and their Antihypertensive Effect in Spontaneously Hypertensive Rats. *Journal of Agricultural and Food Chemistry,* 50**,** 6245 - 6252.

Sentandreu, M. À. & Toldrá, F. 2006. A Fluorescence-Based Protocol for Quantifying Angiotensin-Converting Enzyme Activity. *Nature Publishing Group,* 1**,** 2423-2427.

Shahidi, F., Han, X.-Q., *et al*. 1995. Production and Characteristics of Protein Hydrolysates from Capelin (*Mallotus villosus*). *Food Chemistry,* 53**,** 285 - 293.

Shiau, S.-Y. 1994. Seafood Protein in Human and Animal Nutrition. *In:* Sikorski, Z. E., Pan, B. S. & Shahidi, F. (eds.) *Seafood Proteins*. New York, US: Springer US.

Siemensma, A. D., Weijer, W. J., *et al*. 1993. The Importance of Peptide Lengths in Hypoallergenic Infant Formulae. *Trends in Food Science & Technology,* 4**,** 16 - 21.

Sigma-Aldrich/Merck. *Bromelain (E.C. 3.4.22.32)* [Online]. Available: https://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=16410479 [Accessed 29.04.2018].

Sigma-Aldrich/Merck. *Papain (E.C. 3.4.22.2)* [Online]. Available: https://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=16410606 [Accessed 29.04.2018].

Skanderby, M. 1994. Protein Hydrolysates: their Functionality and Applications. *Food Technol. Int. Eur*, 141 - 144.

Šližytė, R., Mozuraityte, R., *et al*. 2009. Functional, Bioactive and Antioxidative Properties of Hydrolysates Obtained from Cod (*Gadus morhua*) Backbones. *Process Biochemistry,* 44, 668 - 677.

Šližytė, R., Rustad, T., *et al*. 2005. Enzymatic Hydrolysis of Cod (*Gadus morhua*) By-products: Optimization of Yield and Properties of Lipid and Protein Fractions. *Process Biochemistry,* 40, 3680 - 3692.

Søvik, S. L. 2005. *Characterization of Enzymatic Activities in By-products from Cod Species: Effect of Species, Season and Fishing Ground*. Doctoral thesis, Norwegian University of Science and Technology.

Taylor, W. H. 1957. Formol Titration: An Evaluation of its Various Modifications. *Analyst,* 82, 488 - 498.

Thorkelsson, G., Šližytė, R., *et al*. 2009. Fish Proteins and Peptide Products: Processing Methods, Quality and Functional Properties. *In:* Luten, J. B. (ed.) *Marine Functional Food*. Wageningen: Wageningen Academic Publishers.

Tsai, J. S., Chen, J.-J., *et al*. 2008. ACE-Inhibitory Peptides Identified from the Muscle Protein Hydrolysate of Hard Clam (*Meretrix lusoria*). *Process Biochemistry,* 43, 743 - 747.

Tsumura, K., Saito, T., *et al*. 2005. Functional Properties of Soy Protein Hydrolysates Obtained by Selective Proteases. *LWT - Food Science and Technology,* 38, 255 - 261.

Turner, A. J. & Hooper, N. M. 2002. The Angiotensin-Converting Enzyme Gene Family: Genomics and Pharmacology. *Trends in Pharmacological Sciences,* 23, 177 - 183.

Venugopal, V., Chawla, S. P., *et al*. 1996. Spray Dried Protein Powder from Threadfin Bream: Preparation, Properties and Comparison with FPC type-B. *Journal of Muscle Foods,* 7, 55 - 71.

Vermeirssen, V., Van Champ, J., *et al*. 2004. Bioavailibility of Angiotensin I Converting Enzyme Inhibitory Peptides. *British Journal of Nutrition,* 92, 357 - 366.

Weinberg, M. S., Weinberg, A. J., *et al*. 2000. Effectively Targetting the Renin-Angiotensin-Aldosterone System in Cardiovascular and Renal Disease: Rationale for using Angiotensin II Receptor Blockers in Combination with Angiotensin-Converting Enzyme Inhibitors. *Journal of the Renin-Angiotensin-Aldosterone System,* 1**,** 217 - 233.

Yanez, E., Ballester, D., *et al*. 1976. Enzymatic Fish Protein Hydrolyzate: Chemical Composition, Nutritive Value and use as a Supplement to Cereal Protein. *Journal of Food Science,* 41**,** 1289 - 1292.

Yang, Y., Tao, G., *et al*. 2007. Peptide with Angiotensin I-Converting Enzyme Inhibitory Activity from Hydrolyzed Corn Gluten Meal. *Journal of Agricultural and Food Chemistry,* 55**,** 7891 - 7895.

Appendix A: Preparation of solutions

A.1 Preparation of solutions for the ACE inhibitory experiments

The solutions were prepared according to the protocol of Sentandreu and Toldrá:

Buffer B: 150 mM Tris-base buffer (pH 8.3)

Buffer B was prepared by dissolving Tris-base buffer (Sigma-Aldrich) (18.71 g) in 1000 mL of distilled water. The pH was adjusted to 8.3 using HCl (6 M).

Buffer C: 150 mM Tris-base buffer (pH 8.3) with 1.125 M NaCl

Buffer C was prepared by dissolving Tris-base buffer (Sigma-Aldrich) (18.71 g) and NaCl (65.75 g) in 1000 mL of distilled water. The pH of the buffer was afterwards adjusted to 8.3 by adding HCl (6 M).

Substrate stock solution

Stock solution of the substrate (Abz-Gly-Phe(NO_2)-Pro) had previously been prepared and was used in experiments until the 23rd of October 2017. New stock solution was prepared by dissolving substrate (Bachem, cat. no. M-1100, 483.48 g/mol) (48.2 mg) in 9.969 mL of buffer C. Final concentration of substrate was 10 mM. The stock solution was afterwards distributed into Eppendorf tubes (1 mL) and stored at -20°C.

Substrate working solution

The working solution (0.45 mM) was prepared by diluting substrate stock solution (10 mM) in buffer C. The final volume was dependent on the number of parallels to be analyzed. 200 μL was utilized for each parallel. The substrate working solution was prepared each day of the experiment.

ACE stock solution

The ACE stock solution had already been prepared, and was stored in Eppendorf tubes (1 mL) at -20°C. According to the protocol (Sentandreu and Toldrá, 2006), the stock solution was prepared by diluting lyophilized ACE in glycerol at 50% with buffer A (300 mM Tris-base buffer (pH 8.3) with 2 μM $ZnCl_2$, previously prepared) to reach an enzyme concentration of approximately 150 μg/mL.

ACE working solution

The working solution (7.5 μg/mL) was prepared by diluting ACE stock solution (150 μg/mL) in buffer B. The final volume was dependent on the number of parallels to be analyzed. 50 μL was used for each parallel. The ACE working solution was made fresh each day of the experiment.

Inhibitor solution

The inhibitor solution was prepared by dissolving CHH in buffer B to a total volume of 10 mL. The amount of CHH to be quantified was dependent on the final concentration of the inhibitor solution. 1, 5 and 10 mg/mL were for instance investigated. To achieve these concentrations, 0.01, 0.05 and 0.1 grams were quantified and dissolved with buffer B to a final volume of 10 mL.

A.2. Preparation of solutions for the Lowry-assay

The solutions were prepared according to the protocol of Lowry *et al*:

Solution A: 2% Na_2CO_3 in NaOH (0.1 M)

Dissolve 20 g of Na_2CO_3 in 1000 mL NaOH (0.1 M)

Solution B: 1% $CuSO_4$

Dissolve 1 g of $CuSO_4$ x 5 H_2O in 100 ml water.

Solution C: 2 % Potassium Sodium Tartrate

Dissolve 2 g of potassium sodium tartrate in 100 mL of distilled water.

Solution D: Alkaline copper reagent

1 mL of solution B + 1 mL of solution C + 100 mL of solution A.

Prepared the day of the experiment.

Appendix B: ACE inhibitory experiments

Calculation of ACE inhibitory activity

Concentrations of inhibitor (CHH) were chosen to enable calculation of IC_{50}, thus concentrations that gives inhibitory activity of 0 to 80 – 90% of inhibition were chosen. The concentrations found to be inhibitory within this range was 0.5, 1, 5, 10, 20 and 30 mg CHH/mL. These concentrations were thus investigated for all the hydrolysates. Inhibitor and ACE was pre-incubated, and substrate was added. The fluorescence was subsequently measured every fifth minute for 45 minutes by TECAN Spark ®. Investigation of the ACE inhibitory activity of hydrolysate T1-H is used to exemplify the calculation process.

Processed data from T1-H are presented in figure B.1, in which fluorescence is plotted versus time for all concentrations investigated, and linear regression have been applied.

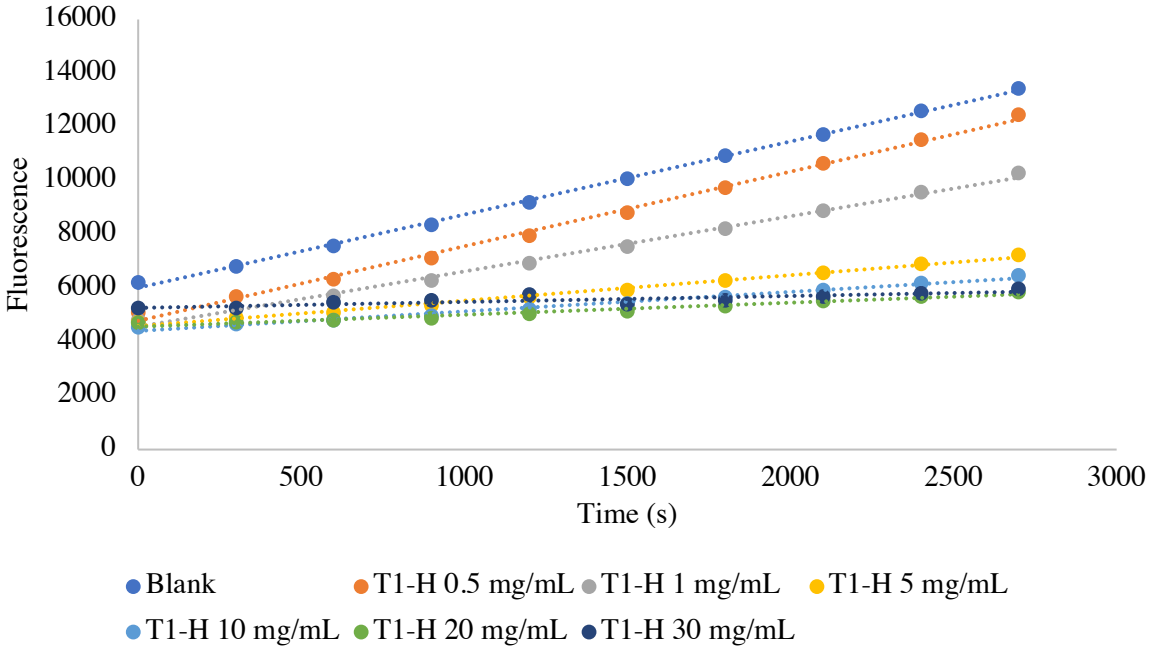

Figure B.1: Fluorescence plotted against time (seconds) for concentrations of hydrolysate T1-H investigated for ACE inhibitory activity. The negative control is denoted "blank".

Application of linear regression enabled calculation of ACEI (%) for all the concentrations investigated. The calculation of ACEI (%) was performed according to equation B.1.

$$ACEI(\%) = \left(1 - \frac{slope\ of\ sample}{slope\ of\ control}\right) \times 100 \qquad \text{(B.1)}$$

The equations obtained from linear regression of the concentrations of T1-H are presented in table B.1.

Table B.1: Equations obtained from linear regression of fluorescence as a function of time (s) for each concentration investigated for ACE inhibitory activity of hydrolysate T1-H.

Concentration of T1-H	Equation
Control (0 mg/mL)	y = 2.7205x + 6020.5
0.5 mg/mL	y = 2.775x + 4789.7
1 mg/mL	y = 2.0548x + 4572.6
5 mg/mL	y = 0.9485x + 4590.4
10 mg/mL	y = 0.7257x + 4413.9
20 mg/mL	y = 0.2212x + 5271.2
30 mg/mL	y = 0.4476x + 4569.3

Equation B.1 was utilized for calculation of the ACEI (%) values as exemplified in equation B.2 for 1 mg T1-H/mL.

$$ACEI(\%) = \left(1 - \frac{2.0548}{2.7205}\right) \times 100 = 24.47\% \quad \text{(B.2)}$$

The calculated ACEI (%) values for all concentrations investigated are presented in table B.2.

Table B.2: ACE inhibitory activity (ACEI %) expressed by concentrations of T1-H.

Concentration (mg CHH/mL)	ACEI (%)
0.5	-2.00%
1	24.47%
5	65.14%
10	73.32%
20	91.87%
30	83.55%

The ACE inhibitory effect (ACEI %) was plotted as function of the inhibitor concentration, and logarithmic regression was applied. The correlation between ACE inhibitory effect and concentration of hydrolysate is presented in figure B.2.

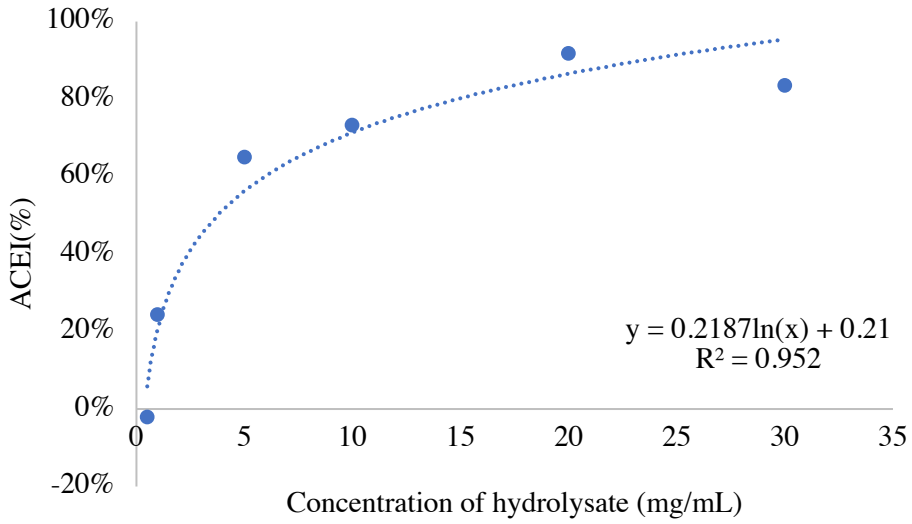

Figure B.2: Logarithmic correlation between ACE inhibiting effects (ACEI %) and investigated concentrations of inhibitor (mg CHH/mL).

The equation obtained from logarithmic regression expresses the relationship between the ACE inhibiting effect and the concentrations investigated in this assay as indicated in equation B.3.

$$ACEI\ (\%) = 0.2187 \cdot \ln(inhibitor\ concentration) + 0.21 \qquad (B.3)$$

Equation B.3 enabled calculation of IC_{50}, which corresponds to the inhibitor concentration expressing 50% inhibition (ACEI = 50%). This calculation was carried out by inserting 0.5 for ACEI (%) and solving the equation for Inhibitor concentration. The calculation of IC_{50} expressed by hydrolysate T1-H is shown in equation B.4.

$$IC_{50} = exp\left(\frac{0.5-0.21}{0.2187}\right) = 3.77\ mg/mL \qquad (B.4)$$

Thus, the IC_{50} expressed by hydrolysate T1-H was calculated to be 3.77 mg CHH/mL, which correspond to 3.07 mg protein/mL as hydrolysate T1-H contains 81.57% protein (appendix H). This process was applied for calculation of IC_{50} for all the cod head hydrolysates.

Inhibitory activity of the permeates from membrane filtration were investigated for different concentrations than the original CHHs. One stock solution was prepared for each of the four permeates by dissolving freeze dried powder in buffer B to obtain a final concentration of 10 mg permeate/mL. The concentrations investigated were 1, 2, 4, 6, 8 and 10 mg/mL. These were prepared from the stock solution and analyzed in the same manner as described above.

Appendix C: Total amino acid composition

The total amino acid composition of CHHs was determined after HPLC analysis. Three parallels were analyzed for each hydrolysate. The raw data from HPLC was processed by using equation C.1. Utilization of this equitation provided the concentration of the amino acids in each sample.

$$mg\ aa/g = \frac{nmol\ aa/mL \times M_w\ aa\ (g/mol) \times volume\ extract\ (mL) \times dilution}{1 \times 1000 \times 1000 \times sample\ weight\ (g)} \quad \text{(C.1)}$$

In which amino acid is denoted *aa*, *nmol aa/mL* was detected by HPLC and M_w of amino acid is the molar weight when bound in protein.

The raw data and calculation of amino acid content in hydrolysate T1-H parallel 1 is provided as example in table C.1, and an example of calculation (Asp) is provided in equation C.2.

Table C.1: Raw data and calculated amounts of amino acids in hydrolysate T1-H parallel 1. Amount of hydrolysate powder quantified was 0.0507 g, the volume was extracted to 10 mL and diluted 1:500. *nmol/mL* for each amino acid was detected by HPLC.

aa detected by HPLC	M_w (g/mol)	nmol/mL	mg aa/g
Asp	115	8.649	98.09
Glu	129	9.744	123.96
Asn	114	0.021	0.23
His	137	0.036	0.49
Ser	87	8.307	71.27
Gln	128	0.035	0.45
Gly/Arg	98	14.933	144.32
Thr	101	4.971	49.52
Ala	71	11.352	79.48
Tyr	163	0.023	0.37
Aba	85	0.043	0.36
Met	131	2.415	31.21
Val	99	4.789	46.76
Phe	147	2.536	36.76
Ile	113	3.475	38.72
Leu	113	6.137	68.39
Lys	128	6.392	80.69
Total		83.858	871.06

$$mg\ Asp/g = \frac{8.649\ nmol/g \times 115\ g/mol \times 10\ mL \times 500}{1 \times 1000 \times 1000 \times 0.0507\ g} = 98.09 \qquad \text{(C.2)}$$

The average amount of amino acids detected in hydrolysates T1-H – T4-H are presented in table C.2, T5-H – T8-H in table C.3 and T9-H – T13-H in table C.4. The calculations were performed in similar manner as described in table C.1 and equation C.2.

Table C.2: Average amount of amino acids (mg amino acid/g CHH) calculated from HPLC for hydrolysates T1-H – T4-H (mean ± SD, n = 3).

Amino acid	T1-H	T2-H	T3-H	T4-H
Asp	103.61 ± 8.8	103.74 ± 7.0	111.56 ± 14.1	56.72 ± 28.3
Glu	132.81 ± 12.7	135.31 ± 10.0	143.35 ± 16.9	65.90 ± 39.6
Asn	0.27 ± 0.0	0.15 ± 0.1	0.19 ± 0.0	0.19 ± 0.1
His	15.74 ± 13.4	23.48 ± 1.8	24.10 ± 3.2	10.83 ± 10.3
Ser	83.97 ± 14.6	89.70 ± 6.5	98.64 ± 15.1	34.96 ± 30.6
Gln	0.97 ± 0.5	1.40 ± 0.7	1.24 ± 0.3	0.09 ± 0.1
Gly/Arg	161.96 ± 20.9	173.64 ± 13.2	195.87 ± 23.9	75.93 ± 41.6
Thr	54.23 ± 5.4	56.16 ± 4.6	57.49 ± 7.9	30.42 ± 15.7
Ala	84.69 ± 7.9	87.86 ± 6.5	100.54 ± 12.3	55.45 ± 22.5
Tyr	17.19 ± 14.6	20.00 ± 9.8	23.58 ± 3.3	7.40 ± 10.7
Aba	0.35 ± 0.0	0.43 ± 0.0	0.46 ± 0.1	0.65 ± 0.4
Met	34.47 ± 4.1	36.46 ± 2.0	36.61 ± 5.1	16.13 ± 10.1
Val	48.58 ± 3.7	48.03 ± 3.5	50.82 ± 6.6	31.25 ± 13.4
Phe	40.38 ± 4.2	40.40 ± 3.1	42.65 ± 5.3	25.70 ± 10.9
Ile	40.12 ± 2.9	38.77 ± 3.0	25.64 ± 22.4	24.06 ± 10.5
Leu	72.52 ± 6.3	72.45 ± 5.6	69.12 ± 19.7	49.68 ± 20.6
Lys	86.86 ± 8.8	87.98 ± 7.0	101.34 ± 12.9	55.08 ± 27.4
Total	978.73 ± 122.2	1015.95 ± 83.6	1083.21 ± 105.5	540.45 ± 282.8

Table C.3: Average amount of amino acids (mg amino acid/g CHH) calculated from HPLC for hydrolysates T5-H – T8-H (mean ± SD, n = 3).

Amino acid	T5-H	T6-H	T7-H	T8-H
Asp	38.84 ± 6.5	112.73 ± 3.3	49.25 ± 15.9	106.72 ± 9.6
Glu	57.08 ± 9.4	132.88 ± 9.9	72.55 ± 23.9	135.91 ± 15.5
Asn	0.09 ± 0.0	0.38 ± 0.3	0.13 ± 0.1	0.31 ± 0.2
His	8.49 ± 1.4	9.24 ± 14.9	11.12 ± 3.4	8.25 ± 11.0
Ser	24.95 ± 3.9	81.87 ± 13.2	31.14 ± 7.6	78.57 ± 16.7
Gln	0.0 ± 0.0	0.75 ± 0.8	0.0 ± 0.0	0.63 ± 0.3
Gly/Arg	106.47 ± 16.7	164.40 ± 18.1	135.37 ± 42.8	168.54 ± 21.6
Thr	18.33 ± 3.0	57.51 ± 2.1	23.37 ± 7.3	54.16 ± 6.0
Ala	2.43 ± 2.1	100.05 ± 6.3	4.72 ± 1.1	96.00 ± 8.7
Tyr	65.70 ± 11.0	6.99 ± 9.5	86.01 ± 26.5	8.61 ± 11.6
Aba	0.88 ± 0.1	0.67 ± 0.3	1.08 ± 0.2	0.48 ± 0.1
Met	12.11 ± 2.0	30.77 ± 5.2	15.72 ± 4.9	31.97 ± 5.0
Val	17.55 ± 2.7	56.19 ± 2.1	22.54 ± 6.9	50.92 ± 4.8
Phe	15.20 ± 2.3	46.15 ± 2.9	19.87 ± 5.8	41.60 ± 3.9
Ile	14.12 ± 2.2	45.17 ± 1.9	18.65 ± 5.8	42.29 ± 4.2
Leu	29.00 ± 4.5	88.35 ± 3.0	37.47 ± 11.7	82.93 ± 8.2
Lys	31.53 ± 4.2	101.71 ± 7.3	40.79 ± 12.0	95.41 ± 12.6
Total	442.76 ± 68.1	1035.82 ± 89.6	569.75 ± 175.7	1003.29 ± 115.3

Table C.4: Average amount of amino acids (mg amino acid/g CHH) calculated from HPLC for hydrolysates T9-H – T13-H (mean ± SD, n = 3, n = 1 for T10-H).

Amino acid	T9-H	T10-H	T11-H	T12-H	T13-H
Asp	69.13 ± 23.6	39.93	86.65 ± 20.8	81.95 ± 24.1	55.00 ± 14.3
Glu	80.19 ± 34.7	59.86	104.06 ± 29.3	92.55 ± 32.4	81.52 ± 20.8
Asn	0.26 ± 0.2	0.08	0.39 ± 0.2	0.30 ± 0.2	0.32 ± 0.3
His	10.72 ± 10.1	8.58	0.99 ± 0.6	1.63 ± 2.4	9.32 ± 2.1
Ser	40.98 ± 26.8	25.90	56.94 ± 23.1	51.85 ± 21.1	36.11 ± 9.4
Gln	0.10 ± 0.1	0.00	0.04 ± 0.1	0.29 ± 0.1	0.0 ± 0.0
Gly/Arg	87.74 ± 25.9	115.91	121.49 ± 35.7	111.3 ± 25.9	156.75 ± 37.9
Thr	36.49 ± 11.6	18.63	43.74 ± 12.0	40.25 ± 12.2	26.69 ± 7.4
Ala	65.13 ± 5.4	3.22	72.68 ± 20.2	66.30 ± 18.2	12.39 ± 17.6
Tyr	7.39 ± 10.4	70.45	0.28 ± 0.2	0.19 ± 0.1	73.17 ± 62.9
Aba	0.76 ± 0.3	0.73	0.28 ± 0.1	0.26 ± 0.1	0.89 ± 0.2
Met	18.84 ± 7.5	12.55	22.48 ± 6.6	19.36 ± 10.0	16.63 ± 2.9
Val	37.67 ± 8.8	18.27	41.72 ± 10.6	38.51 ± 10.9	25.30 ± 6.6
Phe	30.68 ± 6.0	16.00	33.46 ± 8.4	29.73 ± 8.8	21.33 ± 4.9
Ile	29.16 ± 7.7	14.68	33.98 ± 8.8	18.93 ± 18.0	20.44 ± 5.4
Leu	59.47 ± 11.2	30.41	65.42 ± 17.7	60.48 ± 17.7	41.16 ± 10.6
Lys	64.67 ± 16.3	34.75	72.74 ± 22.5	66.21 ± 20.2	45.50 ± 11.8
Total	639.38 ± 191.6	469.94	757.35 ± 214.3	680.07 ± 211.3	622.49 ± 174.7

The total amount of amino acids detected in the cod head hydrolysates are presented in figure C.1.

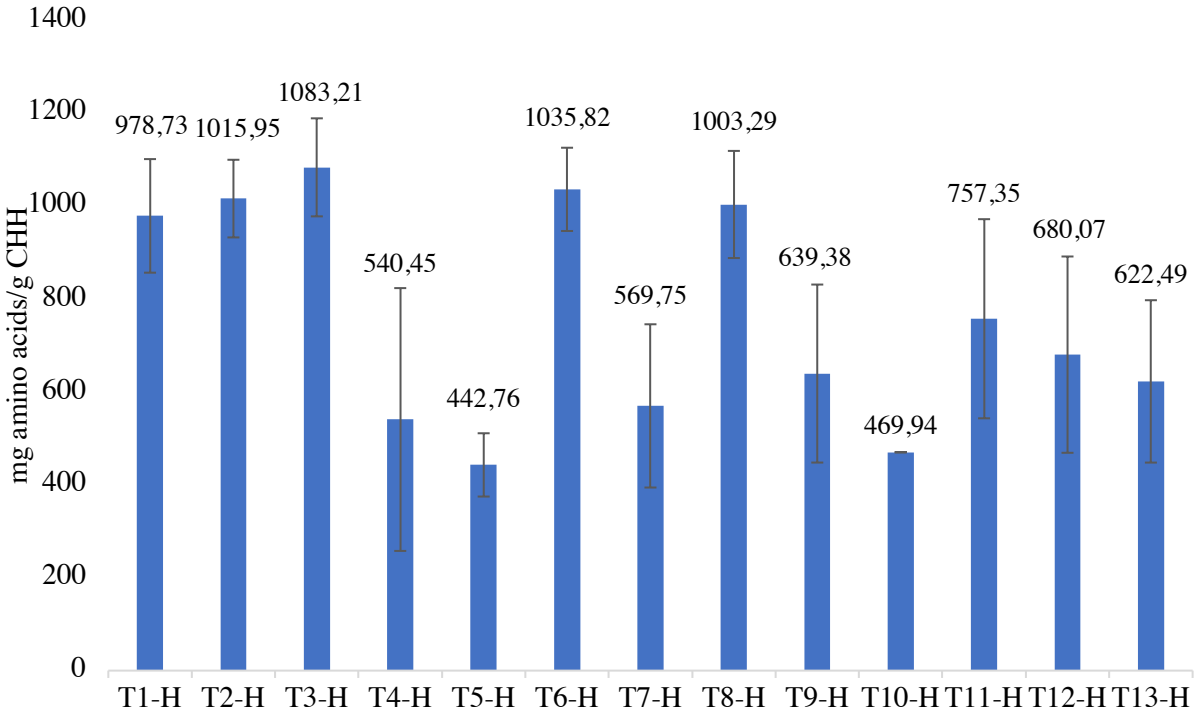

Figure C.1: The total amino acid content (mg amino acids/g CHH) in the cod head hydrolysates (mean ± SD, n = 3. n = 1 for T10-H).

Appendix D: Molecular weight distribution

The molecular weight distributions of CHHs, retentates and permeates were analyzed by using gel filtration on a FPLC system as described in chapter 2. The peptides were separated based on molecular weight and were detected as peaks eluted throughout the chromatograms.

D.1. Chromatograms of standard proteins

Four standard proteins were utilized for determination of a relationship between molecular weight and elution volume; pepsin, cytochrome C, aprotinin and vitamin B12. Analysis of the standards was performed by Veronica Hjellnes at the food chemistry lab at NTNU campus Gløshaugen. The chromatograms of the standards are presented in figure D.1, D.2, D.3 and D.4, respectively.

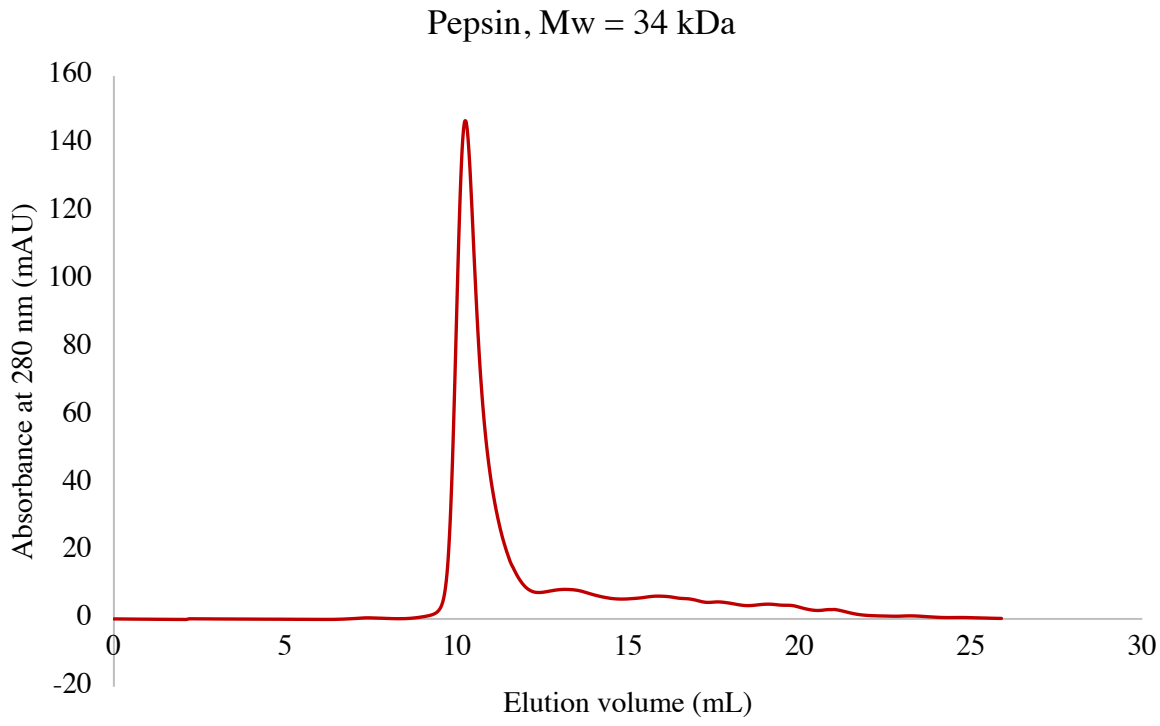

Figure D.1: Chromatogram from gel filtration of pepsin analyzed by using FPLC and the column Superdex Peptide 30/100 GL. Pepsin has molecular weight 34 000 Da, and the peak was detected at elution volume 10.29 mL.

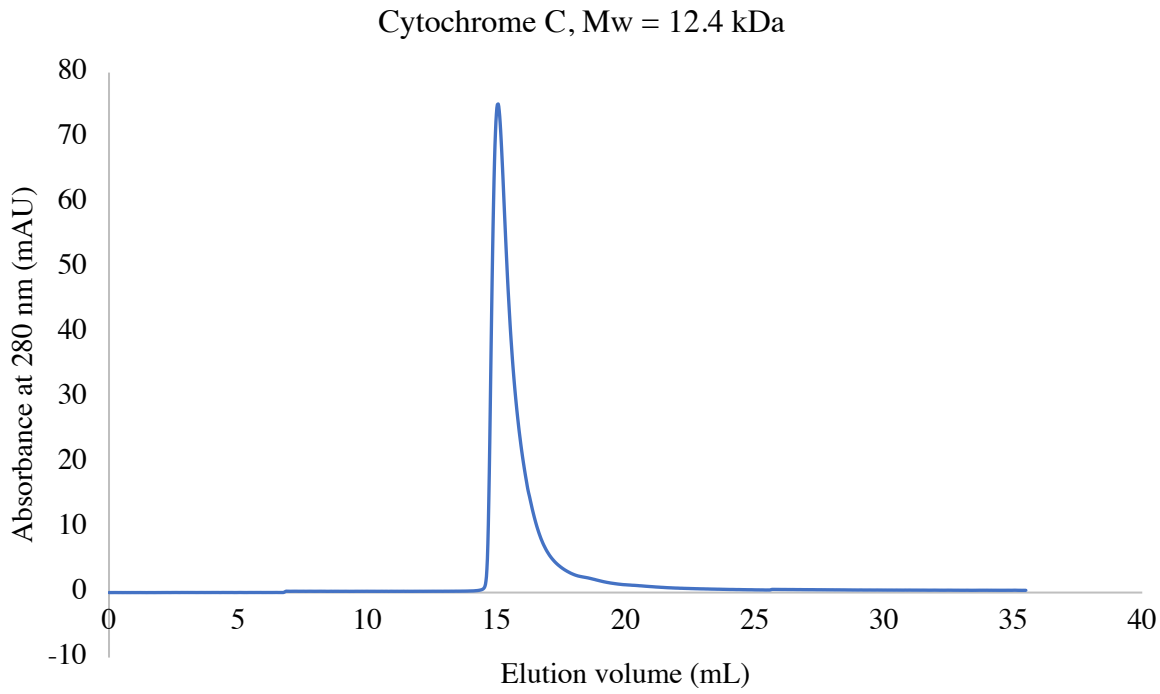

Figure D.2: Chromatogram from gel filtration of cytochrome C analyzed by FPLC using the column Superdex Peptide 30/100 GL. Cytochrome C has molecular weight 12 384 Da, and the peak was detected at elution volume 15.1 mL.

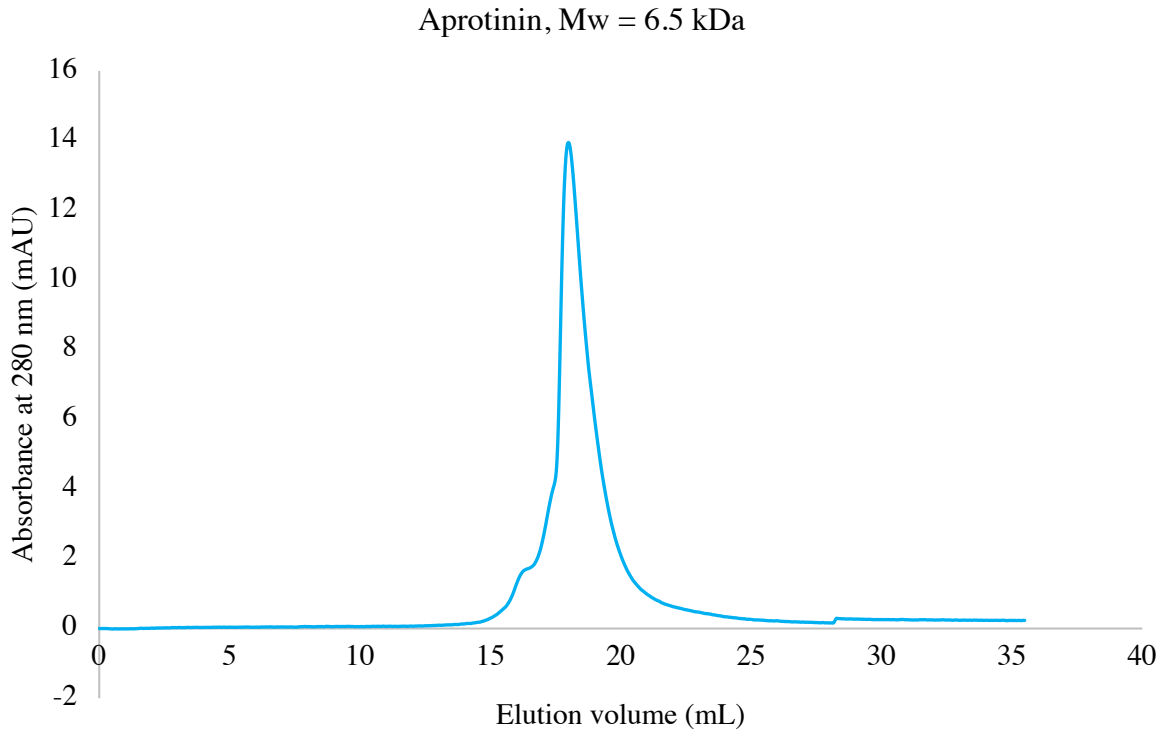

Figure D.3: Chromatogram from gel filtration of aprotinin analyzed by FPLC using the column Superdex Peptide 30/100 GL. Aprotinin has molecular weight 6 512 Da, and the peak was detected at elution volume 18.0 mL.

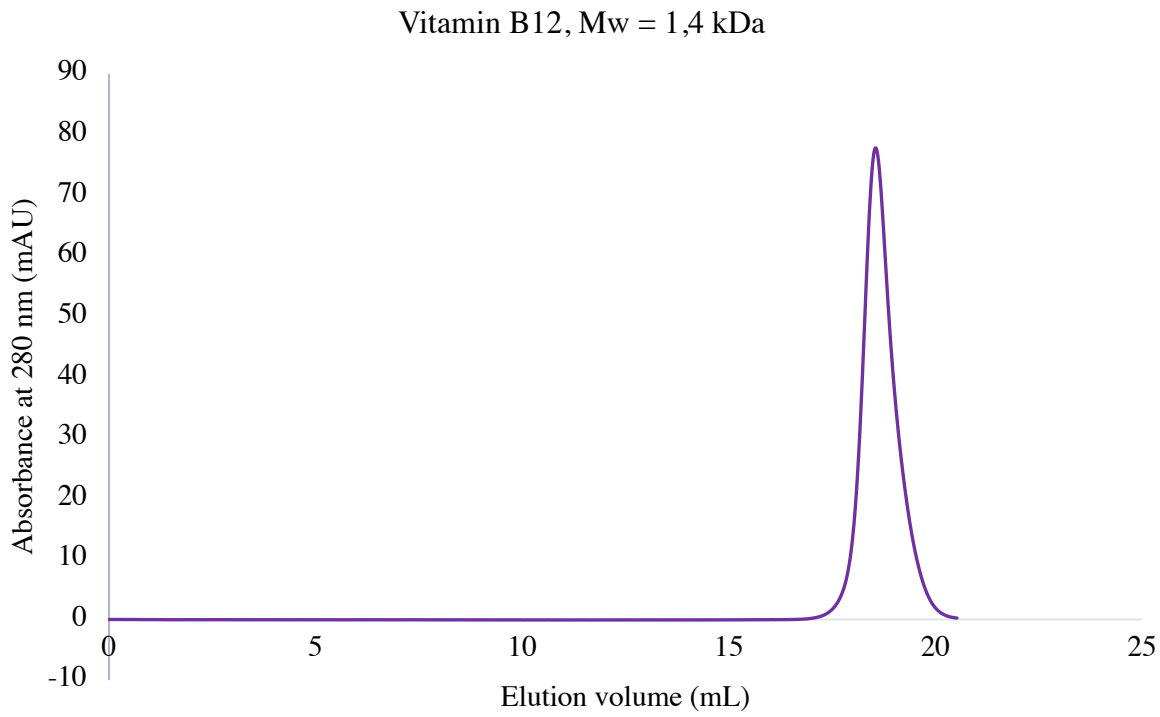

Figure D.4: Chromatogram from gel filtration of vitamin B12 analyzed by FPLC using the column Superdex Peptide 30/100 GL. Vitamin B12 has molecular weight 1 356 Da, and the peak was detected at elution volume 18.54 mL.

D.2. Calculation of peptide molecular weights

To enable determination of the peptide sizes based on the elution volumes in the chromatograms, a relationship between the known molecular weights of the standard proteins and their elution volumes was determined. This relationship was established by calculating a partition coefficient K_{av} for each of the standards. This calculation was carried out using equation D.1,

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad \text{(D.1)}$$

In which V_e is the elution volume of the protein, V_t is the total bed volume (24 mL for the particular column utilized) and v_0 is the void volume (8 mL).

Molecular weights, elution volumes, K_{av} and logarithmic molecular weights of all standard proteins are presented in table D.1. K_{av} and $\log_{10}(M_w)$ were further utilized to determine the relationship.

Table D.1: The molecular weights, elution volumes, partition coefficients K_{av} and logarithmic molecular weights of the standard proteins pepsin, cytochrome C, aprotinin and vitamin B12. K_{av} was calculated by using equation D.1 and applying the elution volumes of the respective proteins. K_{av} and $\log_{10}(M_w)$ were further utilized for determining the relationship between molecular weight and elution volume.

Standard	M_w (Da)	V_e (mL)	K_{av}	$\log_{10}(M_W)$
Pepsin	34 000	10.29	0.1431	4.53147
Cytochrome C	12 384	15.1	0.4438	4.09286
Aprotinin	6 512	18.0	0.6250	3.81371
B12	1 356	18.54	0.6588	3.13226

The partition coefficients K_{av} were plotted against the respective logarithmic molecular weights as presented in table D.1. By applying linear regression of the graph, a standard curve of the relationship was obtained. The standard equation given in equation D.2.

$$log_{10}(M_w) = -2.2124 \times K_{av} + 4.9272 \quad \text{(D.2)}$$

Equation D.2 was used for calculating molecular weights of peptides in the hydrolysates based on elution volumes of the peaks detected by gel filtration (referred to as equation 3.1 in chapter 3).

Appendix E: Free amino acids

The total amount of free amino acids in four of the hydrolysates (T1-H, T5-H, T9-H and T13-H) were detected by HPLC, and the raw data was processed by using equation E.1, in which the amount of free amino acids were determined.

$$mg\ free\ aa/g = \frac{nmol\ aa/mL \times M_w aa\ (g/mol) \times 1{,}25 \times volume\ extract\ (mL) \times dilution}{1 \times 1000 \times 1000 \times sample\ weight\ (g)} \qquad \text{(E.1)}$$

In equation E.1, amino acids are denoted aa, nmol aa/mL was detected by HPLC and M_W aa is molar weight of amino acids bound in protein. The composition of free amino acids in hydrolysate T1-H parallel 1 is provided as example in table E.1.

Table E.1: Raw data from HPLC and calculated amounts of free amino acids in hydrolysate T1-H parallel 1. Amount of FPH quantified was 0.02 g, the volume was extracted to 10 mL and diluted 1:25. *nmol/mL* for each amino acid was detected by HPLC.

Aa detected by HPLC	M_W aa (g/mol)	nmol aa/mL	mg free aa/g FPH
Asp	115	0.3183	0.46
Glu	129	0.5821	0.94
Asn	114	0.0120	0.02
His	137	0.1818	0.31
Ser	87	1.0535	1.15
Gln	128	0.2075	0.33
Gly/Arg	98	1.1017	1.35
Thr	101	0.5020	0.63
Ala	71	1.6429	1.46
Tyr	163	0.2251	0.46
Aba	85	0.0223	0.02
Met	131	0.4975	0.81
Val	99	0.4683	0.58
Phe	147	0.2944	0.54
Ile	113	0.2470	0.35
Leu	113	1.0236	1.45
Lys	128	0.7889	1.26
Total			12.12

The relative percent amounts of free amino acids in the FPH T1-H, T5-H, T9-H and T13-H are presented in table E.2.

Table E.2: The distribution of free amino acids (%) detected in hydrolysates T1-H, T5-H, T9-H and T13-H.

Free aa	T1-H	T5-H	T9-H	T13-H
Asp	3.7	3.4	3.5	4.1
Glu	7.9	6.5	7.2	7.2
Asn	0.1	0.1	0.1	0.1
His	2.5	2.9	2.7	1.8
Ser	9.0	6.4	7.7	6.0
Gln	2.9	3.2	3.0	2.1
Gly/Arg	11.0	10.4	10.7	11.7
Thr	5.2	5.7	5.5	4.8
Ala	11.9	11.1	11.5	11.2
Tyr	3.8	4.2	4.0	4.5
Aba	0.6	0.5	0.6	0.4
Met	6.7	7.3	7.0	5.5
Val	4.8	6.0	5.4	5.6
Phe	4.8	6.3	5.6	6.5
Ile	3.0	3.6	3.3	3.4
Leu	11.9	13.5	12.7	14.7
Lys	10.2	8.9	9.6	10.5
Total	100.00	100.00	100.00	100.00

Appendix F: Degree of hydrolysis

The raw data utilized for calculation of degree of hydrolysis is presented in table F.1.

Table F.1: The data utilized for calculation of DH (%) for the cod head hydrolysates. Raw data and the calculated DH (%) is provided for the first parallel of each hydrolysate. The average DH (%) is presented in figure 3.7.

Hydrolysate	Sample weight (g)	Amount of NaOH (mL)	Free amino groups (%)	% N (total in sample)	DH (%)
T1-H	1.50	15.30	1.43	13.1	10.94
T2-H	1.50	15.41	1.44	13.0	11.04
T3-H	1.50	15.35	1.43	12.8	11.17
T4-H	0.50	5.42	1.52	13.6	11.15
T5-H	0.50	5.30	1.48	13.4	11.07
T6-H	0.50	5.68	1.59	13.4	11.87
T7-H	0.50	5.00	1.40	13.4	10.44
T8-H	0.50	6.12	1.71	13.7	12.49
T9-H	0.50	6.14	1.72	11.3	15.20
T10-H	0.50	6.33	1.77	12.1	14.63
T11-H	0.50	5.62	1.57	12.1	12.99
T12-H	0.50	5.79	1.62	12.1	13.41
T13-H	0.50	5.61	1.57	12.1	12.99

Equation F.1 was utilized for calculation of free amino groups.

$$Free\ amino\ groups\ (\%) = \frac{Amount\ of\ NaOH\ (mL) \times [NaOH](M) \times 14.007 g/mol}{sample\ weight\ (g) \times 1000} \times 100 \quad (F.1)$$

In which the concentration of NaOH utilized was 0.1 M and 14.007 g/mol is the molar weight of nitrogen. The degree of hydrolysis was thereafter calculated by using equation F.2,

$$DH(\%) = \frac{Free\ amino\ groups\ (\%)}{\%\ N} \times 100 \quad (F.2)$$

In which %N was calculated by dividing the total amount of protein in the given sample by 6.25. The amount of proteins in the hydrolysates was determined by SINTEF Ocean (appendix H).

Appendix G: Determination of protein concentrations

G.1. Experimental procedure of the Lowry protein-assay

The concentration of proteins in the fractions obtained from membrane filtration were investigated using the Lowry method. The procedure is summarized in figure G.1.

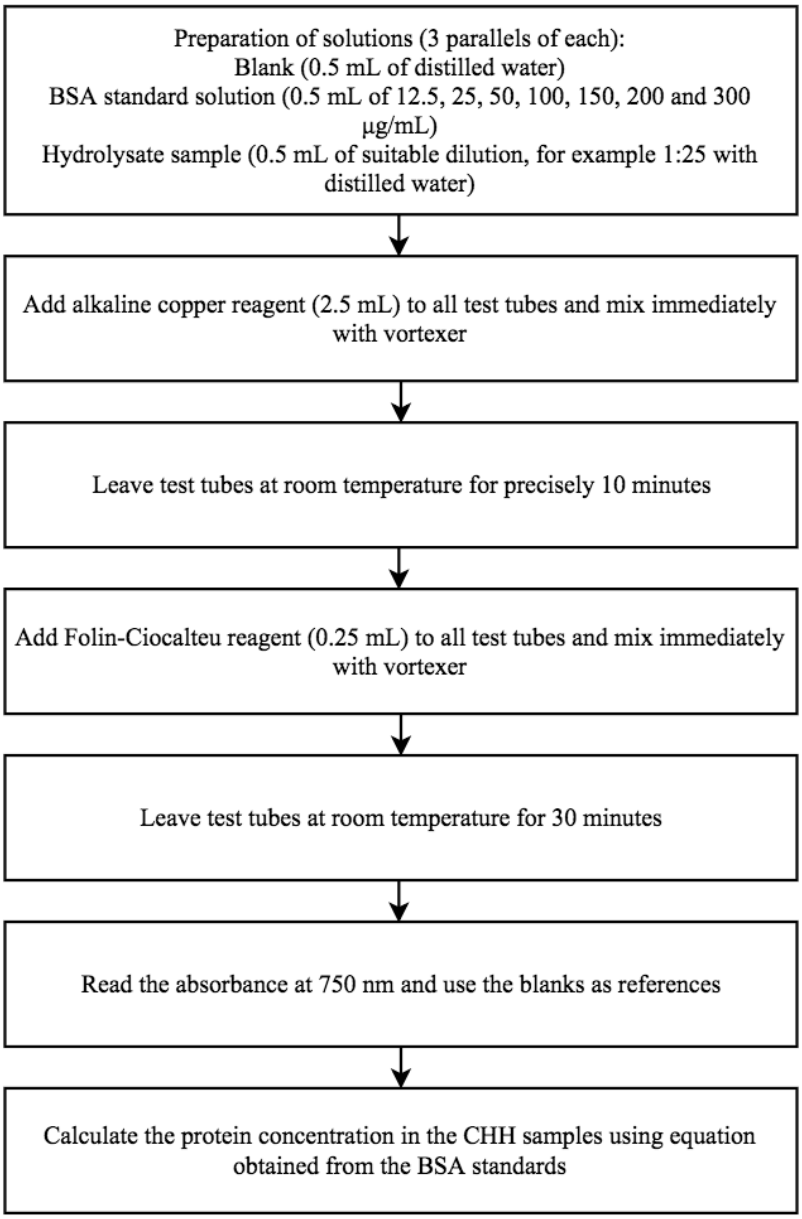

Figure G.1: Flowchart describing the procedure of determining the protein concentration in retentates and permeates (referred to as "hydrolysate sample") (Lowry *et al.*, 1951).

G.2. Standard curves

In order to establish a relationship between absorbance and protein concentration, bovine serum albumin (BSA) was utilized. The concentrations 12.5, 25, 50, 100, 150, 200 and 300 µg BSA/mL were used for generation of a linear relationship, which is presented in equation G.1.

$$Absorbance\ at\ 750\ nm = a\ \cdot Protein\ content\ (\mu g/mL) + b \qquad \text{(G.1)}$$

In which *a* is the slope of the standard curve and *b* is the incline. The standard curve equations were solved with respect to protein content upon filling in the average absorbance based on three parallels of the sample. The protein content was obtained in µg/mL. Thereafter the dilution of the sample was corrected, and the concentration was converted to mg/mL.

The analysis was performed five separate times. Hence five standard curves were generated. Their respective equations are presented in table G.1.

Table G.1: The equations of the five standard curves generated based on concentrations of BSA and absorbance at 750 nm.

Analysis number	Standard curve equation	R^2
1	y = 0.002x + 0.0236	0.9887
2	y = 0.0018x + 0.0325	0.9870
3	y = 0.0018 x + 0.0215	0.9883
4	y = 0.0019 x + 0.0239	0.9861
5	y = 0.002 x + 0.0209	0.9843

The raw data generated from measuring absorbance of the standard solutions was thereafter used for generation of the first standard curve is presented in table G.2 below. The standard curve is presented in figure G.2. Finally, the procedure for calculating the protein concentrations for the permeate samples analyzed in attachment to this standard curve is shown.

Table G.2: The absorbance of seven concentrations of BSA at 750 nm. The average absorbance of each concentration was utilized for determination of the standard curve.

	Absorbance at 750 nm				
BSA (µg/mL)	Parallel 1	Parallel 2	Parallel 3	Average	SD
12.5	0.030	0.031	0.032	0.0310	0.001
25	0.056	0.062	0.066	0.0613	0.005
50	0.127	0.128	0.128	0.128	0.001
100	0.222	0.229	0.224	0.225	0.004
150	0.342	0.342	0.353	0.346	0.006
200	0.448	0.453	0.451	0.451	0.003
300	0.593	0.577	0.593	0.588	0.009

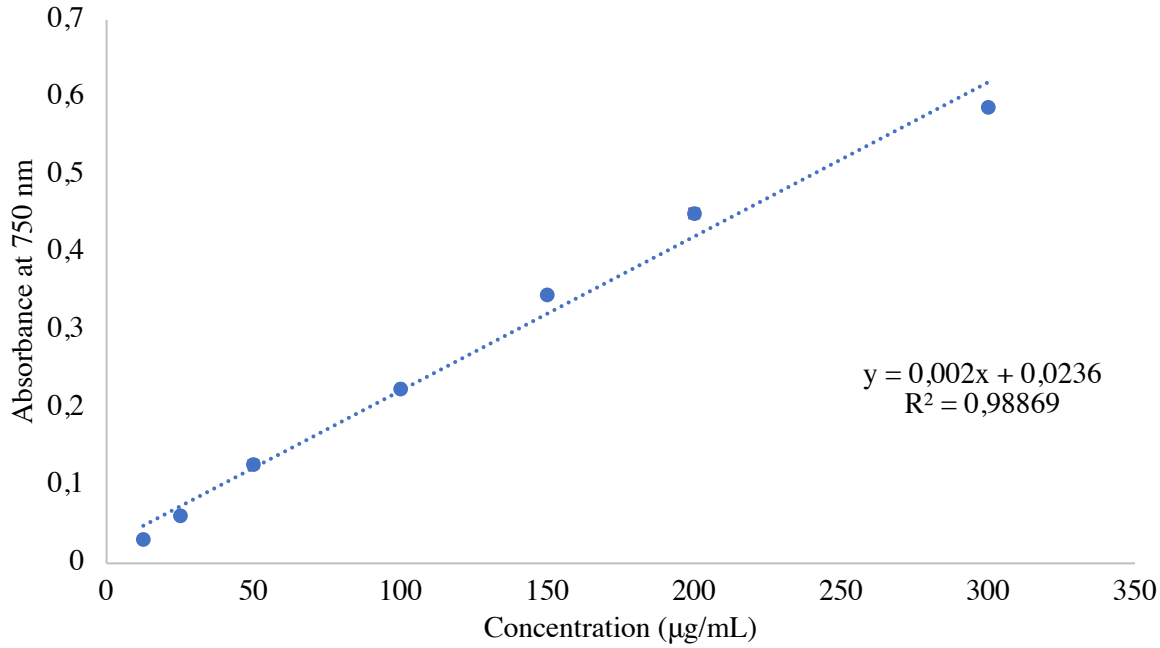

Figure G.2: The standard curve generated from linear relationship between the BSA concentrations and absorbance at 750 nm. The standard curve was utilized in the first of five Lowry assays, in which the permeates were analyzed.

The permeates of hydrolysate T1-H, T5-H, T9-H and T13-H were diluted 1:25 and prepared according to the procedure in figure G.1. The raw data from calculations of the concentrations are presented in table G.3.

Table G.3: The absorbance of T1-H P, T5-H P, T9-H P and T13-H P at 750 nm. The average absorbance of each sample was utilized for calculations of the protein concentrations.

		Absorbance at 750 nm			
	Dilution	Parallel 1	Parallel 2	Parallel 3	Average
T1-H P	0.04	0.169	0.169	0.169	0.169
T5-H P	0.04	0.167	0.161	0.159	0.162
T9-H P	0.04	0.153	0.152	0.155	0.153
T13-H P	0.04	0.179	0.179	0.189	0.182

Based on the average absorbance at 750 nm for all of the samples, the concentration of proteins in the permeates were calculated by using the equation obtained from the standard curve number one (table G.1). Equation G.2 presents how the protein content of sample T1-H P was calculated.

$$0.169 = 0.002 \cdot Protein\ content\ (\mu g/mL) + 0.0236 \qquad \text{(G.2)}$$

Equation G.2 was thereafter solved with respect to protein content, the dilution (1:25) was corrected, and the concentration was altered from μg/mL to mg/mL.

G.3. Mass balance: protein recovery from membrane filtration

The concentrations of proteins in the permeates and retentates were determined by using the method described in section G.2. The calculated protein concentrations from Lowry are presented in table G.4.

Table G.4: Concentration of proteins (mg protein/mL) in the permeates and retentates of T1-H, T5-H, T9-H and T13-H determined by the Lowry method (mean ± SD, n = 5 for permeates, n= 3 for retentates).

	Permeate (mg protein /mL)	**Retentate (mg protein/mL)**
T1-H	1.77 ± 0.16	11.34 ± 0.54
T5-H	1.77 ± 0.17	11.02 ± 0.88
T9-H	2.23 ± 1.09	8.31 ± 0.29
T13-H	2.11 ± 0.14	9.63 ± 0.75

The recovery of proteins in retentates and permeates from the unfiltered solution of CHHs was calculated by using the volumes of unfiltered CHH solution, retentates and permeates, and the protein content in the hydrolysates. The exact volumes of CHH solution, retentates and permeates were not measured, but should have been done. Approximately 150 mL of CHH solution was membrane filtered, which yielded approximately 50 mL of permeate and 100 of retentate. The protein contents of the hydrolysates (appendix H) were used to calculate the protein recovery. The protein contents in unfiltered CHH solutions were not determined by Lowry but should have been done. An alternative method was used, and the total amounts of proteins in the unfiltered CHH solutions was calculated as presented in equation G.3 (for T1-H).

$$Proteins\ in\ T1-H\ = 1\ g/100\ mL \times 150\ mL\ \times 0.8157 = 1.224\ g = 1224\ mg \qquad (G.3)$$

The amounts of proteins in the retentates and permeates were calculated by using the concentrations determined by Lowry (table G.4) and the volumes of the solutions. Calculations of the protein contents in T1-H permeate (T1-H P) and retentates (T1-H R) are provided as examples in equation G.4 and G.5.

$$Proteins\ in\ T1-H\ P = 50\ mL\ \times\ 1.77\ mg/mL = 88.5\ mg \qquad (G.4)$$

$$Proteins\ in\ T1-H\ R = 100\ mL\ \times\ 11.34\ mg/mL = 1134\ mg \qquad (G.5)$$

The same procedure was followed for calculation of the protein contents in unfiltered solutions, permeates and retentates of hydrolysate T5-H, T9-H and T13-H. The amount of proteins in the solutions and the calculated amounts of proteins lost during membrane filtration are presented in table G.5.

Table G.5: Calculated amounts of protein (mg) in the unfiltered CHH solutions, permeates and retentates. The amounts of proteins lost (mg) are also included.

Hydrolysate	CHH (mg)	Permeate (mg)	Retentate (mg)	Protein loss (mg)
T1-H	1224	88.5	1134	1.5
T5-H	1256	88.5	1102	66
T9-H	1260	111.5	831	318
T13-H	1135	105.5	963	67

Appendix H: Protein content in the cod head hydrolysates

The protein contents of the cod head hydrolysates were determined by SINTEF Ocean and are presented in table H.1.

Table H.1: Protein content (%) in the cod head hydrolysates, determined by SINTEF Ocean.

Hydrolysate	Protein content (%)
T1-H	81.57
T2-H	81.40
T3-H	80.20
T4-H	85.00
T5-H	83.75
T6-H	83.75
T7-H	83.75
T8-H	85.63
T9-H	70.63
T10-H	75.63
T11-H	75.63
T12-H	75.63
T13-H	75.63