![UiT The Arctic University of Norway]

The Norwegian College of Fishery Science

# Enzymatic protein hydrolysis of residual raw material from Atlantic cod

Selectivity of proteases, outcome and bioactivities

Liudmila Sorokina

Master's thesis in Marine Biotechnology   BIO-3901   August 2020

# Foreword

The practical work connected with this master thesis was conducted at the lab facilities of the research institute Nofima and at Marbio. Over the last five years, I have enjoyed my time working on both my bachelor and master theses at Nofima. I am grateful to Jan Arne Arnesen and Birthe Vang for helping me find a project for my master thesis, and for helping me find a great team of supervisors. I would like to thank Diana Lindberg and Nils Kristian Afseth for letting me be a part of the project Notably, and providing me with guidance at their lab in Ås.

I want to express my gratitude to my supervisors for their support and guidance. I want to thank my main supervisor Klara Stensvåg for teaching me how to improve my writing and for helping me with data presentation and analysis. I would like to thank my supervisor Jan Arne Arnesen for teaching and helping me with all the methods I used, for helping me analyze the results and for reviewing my numerous texts. I would also like to thank my co-supervisor Jeanette Hammer Andersen for helping me plan the work at Marbio, and for reviewing my results and analysis. I am grateful to Runar Gjerp Solstad for teaching me how to perform SEC and for fixing the machine after I used it. I want to thank Kjersti Lian for teaching me how to perform SDS-PAGE and for helping with the analysis. I would also like to thank Diana Lindberg for the help with SDS-PAGE. I am grateful to Kirsti Helland and Marte Albrigtsen for teaching me how to perform different bioassays and for their willingness to answer all my questions. I would also like to thank Birthe Vang for helping me with the project during the first months and for reviewing my thesis even when she is on maternity leave. I would like to express my gratitude to Nils Kristian Afseth for reviewing my thesis. I am grateful to everyone for creating such a supportive environment! I have learnt a lot despite the coronavirus outbreak that made me have to adjust some of my plans.

I would like to thank my husband for his support, patience and proofreading, and my family and friends for their support and encouragement.


Liudmila Sorokina

Tromsø, August 2020

# Abstract

Global trends show that interest in fish products and fish consumption are increasing, while marine fishery resources are decreasing. Fish processing industry produces a high amount of residual raw materials that have nutritious proteins and other valuable compounds. An optimization of residual raw materials' utilization can help meet the growing demand for fish products and help reduce environmental problems. A promising valorization method is enzymatic protein hydrolysis. In this project, enzymatic protein hydrolysis was performed to produce protein hydrolysates from complex material of Atlantic cod heads. Three types of material from cod heads (muscle, skin and bone) were hydrolyzed by 23 different proteases. The produced hydrolysates were analyzed and evaluated based on yield, molecular weight (determined by SEC and SDS-PAGE), selectivity of proteases towards collagen and myofibrillar proteins (selectivity ratio), and bioactivity properties (anti-proliferative, antioxidant and anti-inflammatory). It was determined that the highest yield from muscle was produced by Tail 191, from skin by Tail 194, and from bone by Tail 190. Different proteases produced hydrolysates with different average MW. Notably, Tail 189 produced hydrolysates with the lowest average MW from all three types of raw material. The SDS-PAGE patterns of the hydrolysates from skin indicated that Tail 193 and Endocut 01 might have selectivity towards peptide bonds they cleave. The selectivity ratio identified that Endocut 01 had the highest selectivity towards myofibrillar proteins and Flavourzyme was the only enzyme selective to collagen. The results of bioactivity assays showed no antiproliferative or anti-inflammatory activity of the hydrolysates, however, all hydrolysates demonstrated antioxidant activity. The hydrolysates made from muscle showed higher antioxidant activity than the hydrolysates prepared from skin and bone. Based on the results, conditions for a scale-up experiment (from 5 g of raw material to 250 g) were suggested, which included recommendation of several enzymes per material, adjustment of temperature to optimal for each enzyme, monitoring of hydrolysis process and determination of ash content in the product.

# Table of Contents

# List of Tables

## List of Figures

# 1 Introduction

Regular fish consumption is recommended by WHO and FAO for a healthy diet (WHO & FAO, 2003). In the last years, consumers have expressed increasing interest in fish and fish products, influenced by a global trend of eating healthy food (Korczek et al., 2018). According to FAO (2018), global consumption of fish increases by a yearly average of 3.2% (calculated between 1961 and 2016) and was at 151.2 million tonnes in 2016. However, marine resources are limited (Tahergorabi et al., 2011); and according to FAO (2018) monitoring, marine fishery resources continue to decline. FAO (2018) reported that the fraction of marine fish stock caught within biologically sustainable levels was reduced from 90% in 1974 to 66.9 % in 2015.

The fish processing industry produces a large amount of residual raw material, accounting for 50-70% of the original raw material weight (Liu et al., 2015). *Residual raw material* is defined as discarded body parts of commercial fish (heads, trimmings, frames, skin, bones, gills, fins, viscera, blood, and roes) and bycatch (Vidanarachchi et al., 2014). The research institute SINTEF defines *marine residual raw material* as "the non-primary products obtained from the use of a marine raw material" (Richardsen et al., 2019). The residual raw material can be further separated into several groups depending on origin and handling: residual raw material and by-product. When residual raw materials are handled in accordance with the hygiene regulations, they are called *residual raw materials*, and can be used for human consumption or feed. When residual raw materials are handled in accordance with the by-product regulations, they are called *by-products*, and are not allowed to be used for human consumption (Richardsen et al., 2019). According to the EU regulations, by-products are divided into three categories based on their potential risk to human and animal health: category I (very high risk material), category II (high risk),  and category III (low risk) (EU Parliament & Council of the EU, 2009).

Residual raw materials contain proteins, lipids and other valuable compounds such as calcium, astaxanthin, etc. (Rustad & Hayes, 2012). A small amount of residual raw material is used for human consumption, while the rest is used for production of animal feed, fishmeal and silage (Rustad, 2006). For example, in Norway 3.57 million tonnes of seafood was produced in 2018, which created 954 000 tonnes of residual raw materials, and 82 % of this residual raw material was utilized (Richardsen et al., 2019). These residual raw materials were used to produce a variety of products in different market areas (Figure 1). However, as Figure 1 shows, human consumption constitutes only 13%, where 11% is direct consumption (e.g. cod tongues, heads, roe) and 2% indirect consumption, which includes cod liver oil, ingredients for functional food

and flavoring additives in foods (extracts). Pharmaceutical products and supplements are also produced from Norwegian-based residual raw materials, but in very small amounts (Richardsen et al., 2019).
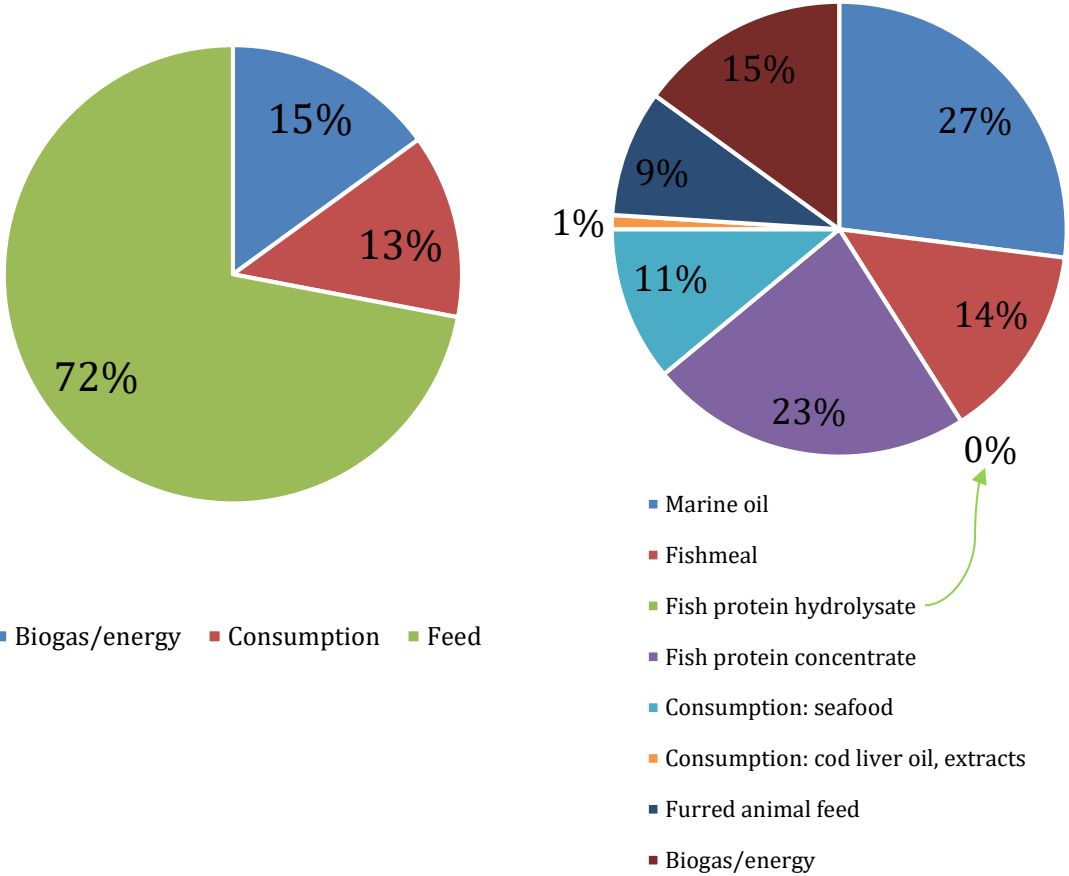


**A**                                                                 **B**

*Figure 1 – Utilization of the residual raw materials in Norway in 2018: according to (A) the market areas and (B) product groups* (Richardsen et al., 2019).

Large quantities of residual raw materials and growing demand for fish products can be solved by development of new technologies to better utilize fish processing residual raw materials. A considerable amount of research and effort has been conducted to optimize use of residual raw material and develop technologies to recover usable ingredients (Vidanarachchi et al., 2014). Currently, in Norway several companies are researching and developing methods to produce marine protein hydrolysates for human nutrition (Richardsen et al., 2019). Such optimization can help avoid future environmental challenges, produce value-added products and create new business opportunities (Liu et al., 2015). Seafood residual raw materials are considered to have potential not only by food industry but also by nutraceutical industry (Vidanarachchi et al., 2014).

## 1.1 Background

### 1.1.1 Atlantic cod and residual raw material

Atlantic cod (*Gadus morhua*) is distributed across the Northern Atlantic from Atlantic Canada to Northern Europe (Figure 2 A) (Johansen et al., 2009). It is a fish with great economical value; according to Statistics Norway (2020), 327 648 tonnes of Atlantic cod was landed in 2019. The production of cod fillets can render up to 60% of the fish as residual raw materials (Gildberg et al., 2002). Atlantic cod can grow up to 1.8 m long and 55.6 kg (Moen & Svensen, 2004), and the head constitutes about 20% of the fish weight. Cod head (Figure 2 B) is a complex material containing muscle (55%), bones (20%), gills (15%), skin (5%) and eyes (4%) with average protein content of approximately 15% (Arnesen & Gildberg, 2006).



| **A** | **B** |
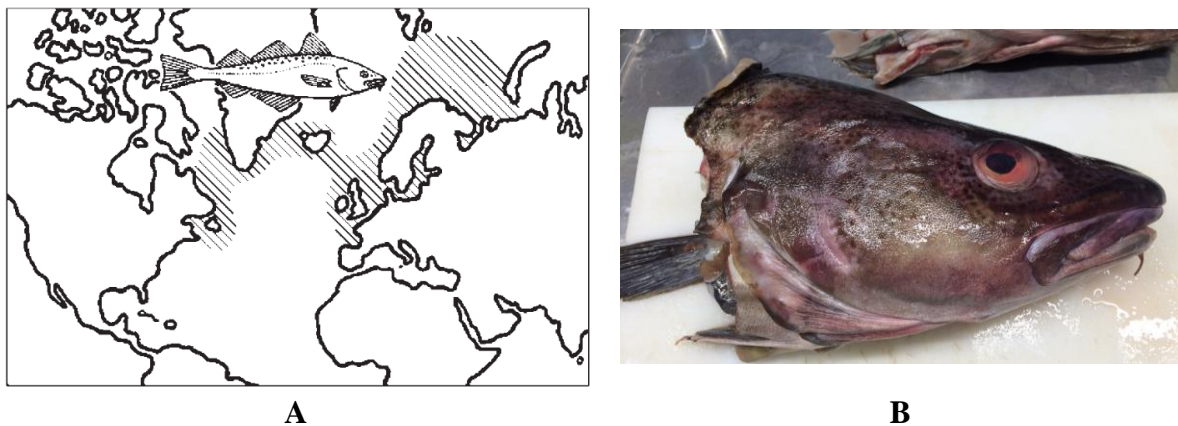
*Figure 2 – Atlantic cod. (A) Distribution in the Northern Atlantic. Figure taken from* (King, 2007). *(B) Atlantic cod head. Photo taken by L. Sorokina.*

### 1.1.2 Proteins – versatile macromolecules

Proteins belong to a complex and diverse group of macromolecules. Based on function, proteins can be classified into nine major categories, which are presented in Table 1. *Proteins* are "polymers of amino acids, with each amino acid residue joined to its neighbor by a peptide bond" (Nelson & Cox, 2013). Proteins are also called polypeptides, and the difference between protein and peptide is in the number of amino acids in the chain. Generally, peptides have molecular weight below 10 kDa, while proteins have higher molecular weights (Nelson & Cox, 2013).

Table 1 – *Major categories of proteins. Table made based on* (Hardin et al., 2016).

| Class of proteins | Function |
|---|---|
| Enzymes | catalysts that greatly increase the rates of chemical reactions |
| Structural proteins | provide physiological support and shape to cells and organelles |
| Motility proteins | have important roles in the contraction and movement of cells and intracellular structures |
| Regulatory proteins | control and coordination of cellular functions, ensuring that cellular activities are regulated |
| Transport proteins | involved in the movement of other substances into, out of, and within the cell |
| Hormonal proteins | mediate communication between cells in distant parts of an organism |
| Receptor proteins | enable cells to respond to chemical stimuli from their environment |
| Defensive proteins | provide protection against disease |
| Storage proteins | serve as reservoirs of amino acids |

*Amino acids* are building blocks of proteins, and 20 common amino acids are involved in protein synthesis (Hardin et al., 2016). All 20 amino acids share common structural features: an amino group, a carboxyl group, a hydrogen atom and an R group (or side chain). As shown in Figure 3, all of the groups are connected to the central carbon atom – alpha carbon (Hardin et al., 2016). Amino acids have different chemical characteristics due to differences in side chains; side chains vary in size, structure, and electric charge. In addition to 20 common amino acids there are many uncommon ones, which are modified after protein synthesis (post-translational modifications) or are present in living organisms but are not part of proteins (Nelson & Cox, 2013).
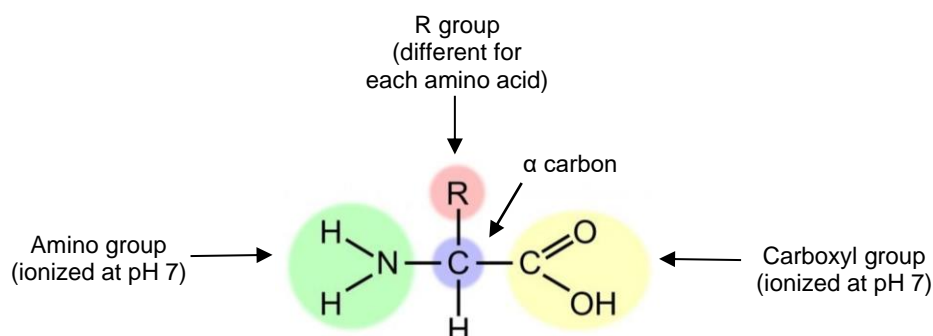


Figure 3 – *Schematic structure of an amino acid. Figure taken from* (Hardin et al., 2016)

In proteins and peptides, amino acids are covalently joint by *peptide bonds*. In the cell, the peptide bond formation and hydrolysis are controlled enzymatically. Peptide bond is formed between a carboxylic acid and an amino group (Petsko & Ringe, 2009) with release of a water molecule, as shown in Figure 4.
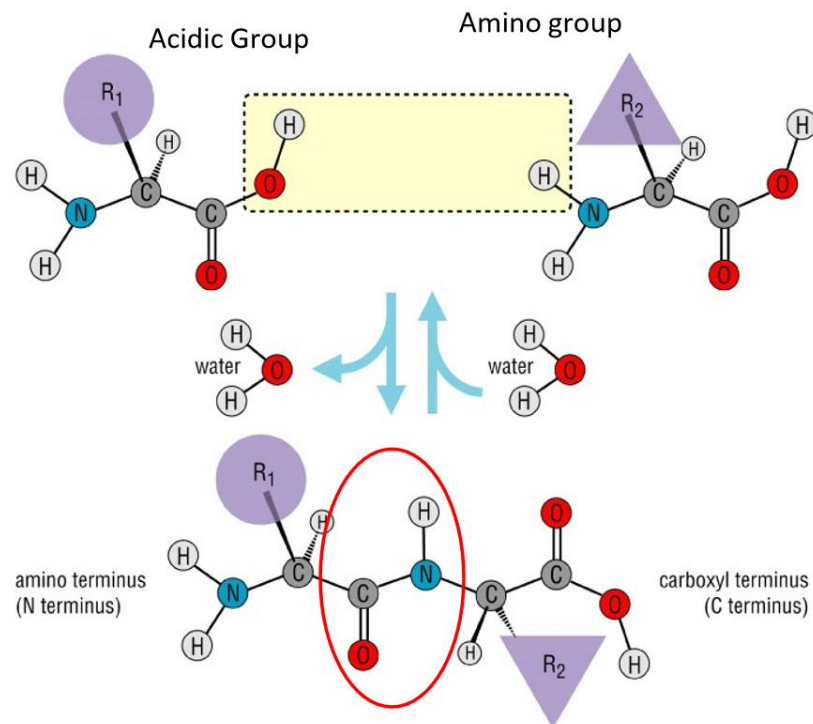


*Figure 4 – Peptide bond formation and hydrolysis. Peptide bond is a bond formed between a carboxylic acid and an amino group by the loss of a water molecule. R1 and R2 represent different side chains. Figure taken from* (Petsko & Ringe, 2009)

*Protein structure* is commonly characterized by four levels: primary structure, secondary, tertiary and quaternary (Figure 5). *Primary structure* is the sequence of amino acids in a protein, which determines how the protein folds into higher-level structures. *Secondary structure* is either alpha helices or beta stands; they are local region of structure formed by hydrogen bonding between NH and CO groups of the polypeptide backbone. *Tertiary structure* is the overall three-dimensional arrangement of all atoms in a protein, and it depends on interactions between various R-groups. *Quaternary structure* describes proteins, which consist of two or more polypeptide chains, and characterizes three-dimensional arrangements of these complexes (Hardin et al., 2016; Nelson & Cox, 2013).

A number of chemical interactions stabilize polypeptides, such as covalent bonds, disulfide bonds, salt bridges, hydrogen bonds, long-range electrostatic interactions and van der Waals interactions (Petsko & Ringe, 2009). Noncovalent bonds and interactions are weaker than

covalent but are numerous and important in folding and maintaining secondary, tertiary and quaternary structures (Nelson & Cox, 2013).
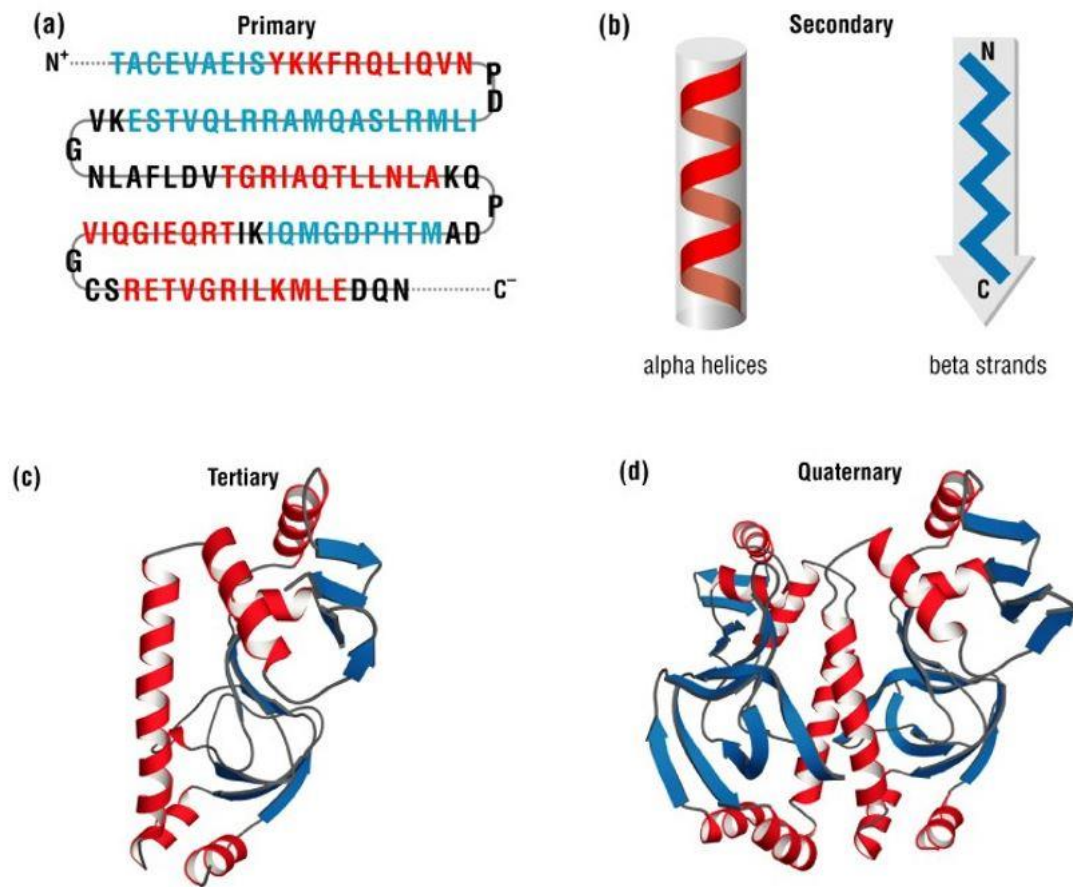


*Figure 5 – Levels of protein structure. (a) Primary structure: The linear amino acid sequence of the polypeptide chain including post-translational modifications and disulfide bonds. (b) Secondary structure: Local structure of linear segments of the polypeptide backbone atoms without regard to the conformation of the side chains. (c) Tertiary structure: The three-dimensional arrangement of all atoms in a single polypeptide chain. (d) Quaternary structure: The arrangement of separate polypeptide chains (subunits) into the functional protein. Figure taken from* (Petsko & Ringe, 2009).

Proteins can be denatured by increased temperature, extremes of pH, certain miscible organic solvents, certain solutes, or detergents. All these denaturing agents have different modes of action, which disrupt noncovalent interactions within a protein. Denaturation cause formation of protein aggregates due to association of exposed hydrophobic surfaces, and denaturation can lead to protein precipitation (Nelson & Cox, 2013).

### 1.1.2.1 Collagen in fish skin and bone

Fish skin and organic matrix of bones consist predominantly of collagen (Harnedy & Fitzgerald, 2012). *Collagen* is a fibrous protein, which consists of three α-chains intertwined into a right-handed triple superhelical structure (collagen molecule), and each α-chain forms a left-handed helix (Gomez-Guillen et al., 2011). Depending on collagen type and source, three α-chains can

be identical or different; for example, type I collagen has two identical $\alpha_1$-chains and one $\alpha_2$-chain and the size of each chain is ~100 kDa (Liu et al., 2015). Collagen molecules assemble into fibrils and fibrils form collagen fiber (Figure 6 A) (Hardin et al., 2016).

The amino acid sequence of collagen is characterized by Glycine-X-Y repeating units (Figure 6 B), where X is predominantly proline (Pro) and Y is hydroxyproline (Hyp). Presence of Glycine (Gly) at every third residue is essential for the formation of the collagen helical structure; since Gly is the smallest amino acid (has only a hydrogen as its R-group), it can fit into the center of the superhelix without any steric hindrance (Liu et al., 2015). Gly, Pro and Hyp make up approximately half of the amino acid residues in each $\alpha$-chain, therefore, the other half of amino acids contribute to formation of different collagen types (Cui et al., 2007). To date, 29 types of collagen have been identified with type I being the major fibrillar collagen in most fish organs (Liu et al., 2015). Collagen types differ in amino acid composition, sequence, structural and functional properties (Pal & Suresh, 2017).
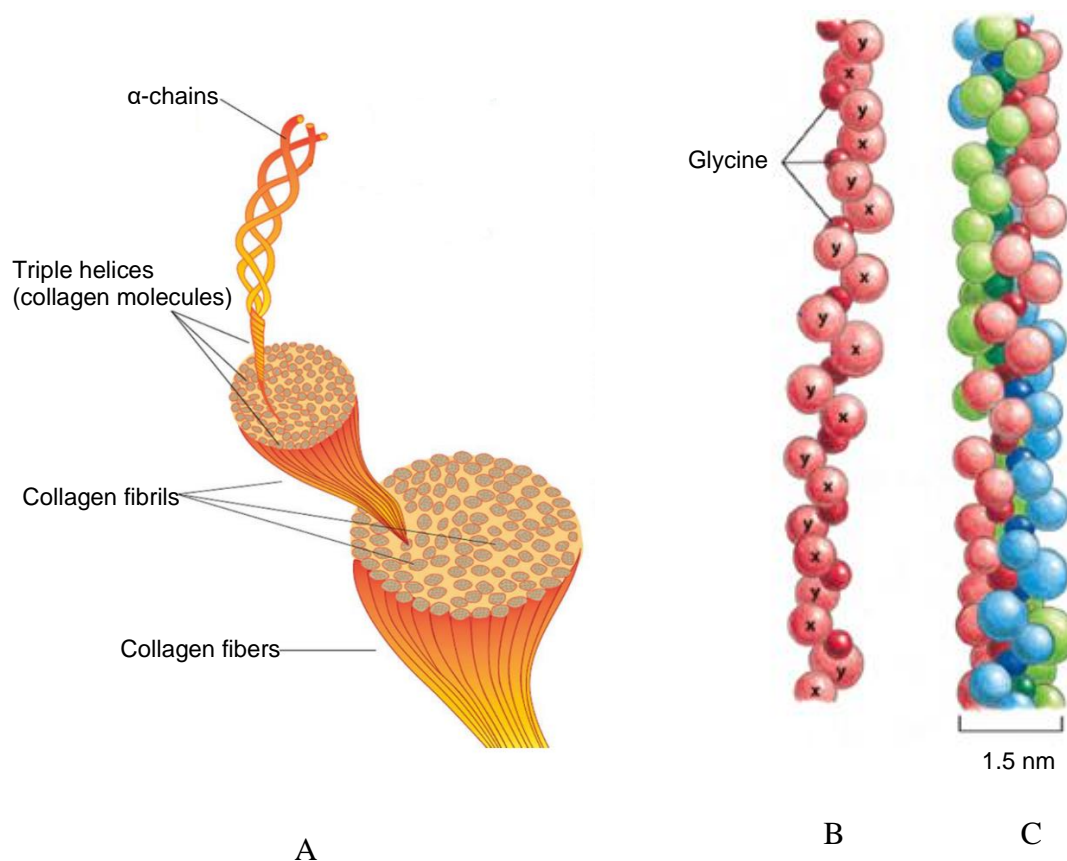


*Figure 6 – Collagen structure. (A) Structure of the collagen fibers. Figure is taken from* (Hardin et al., 2016). *(B) An α-chain, which has triplet of amino acids with glycine at every third position, X and Y. X position usually has proline and Y has 4-hydroxyproline. (C) A triple helix formed by tightly packed α-chains. Glycine is positioned in the center of the helix. The diameter of a collagen of the triple helix is 1.5 nm. Figure is taken from* (Alberts et al., 2015).

## Composition of fish skin

Fish skin consists of several layers, as shown in Figure 7 A: epidermis, dermis and hypodermis (Burton & Burton, 2018). Collagenous fibers are found in the lower layer of dermis, *stratum compactum*, (Figure 7 B) and scales (Hawkes, 1974). The majority of collagen in fish skin belongs to type I, but some amount of type III is also present (Babel, 1996). According to work of Gordon & Lorimer (1960), skin from Atlantic cod consists of 75% collagen, 10% other proteins, 2.5% peptides and free amino acids, 0.6% mucopolysaccharide, 1% lipid and 12% ash.
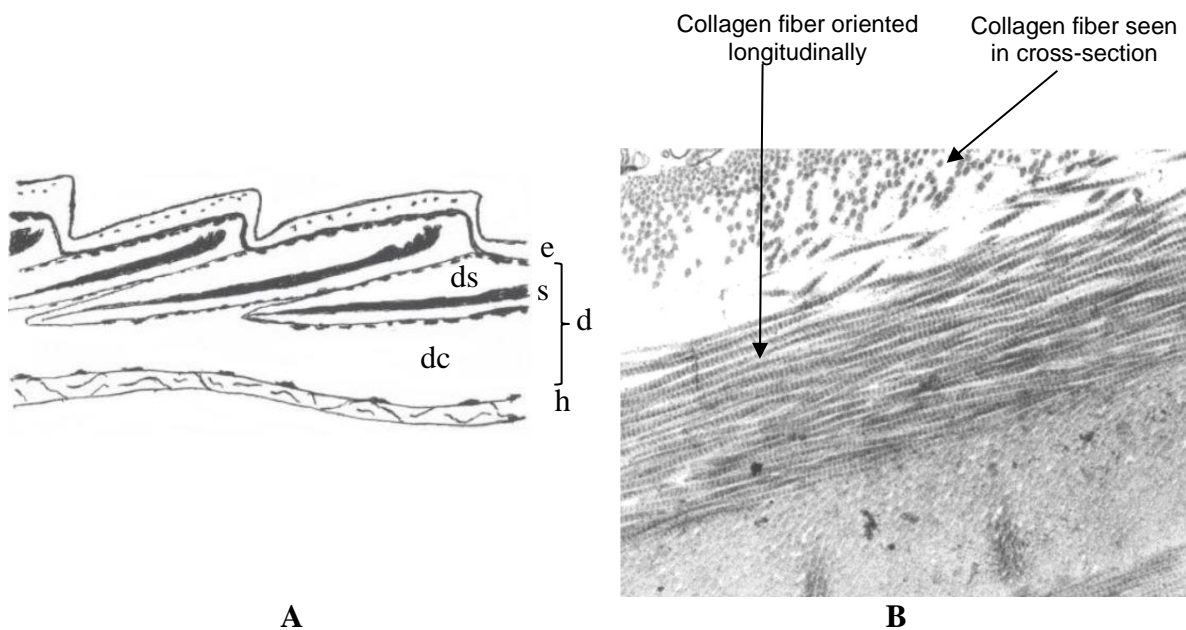


*Figure 7 – Fish skin. (A) Layers of fish skin: e – epidermis; s – scale; d – dermis; ds – stratum spongiosum; dc – stratum compactum; h – hypodermis. (B) Collagen fibers in the stratum compactum of winter flounder skin. Figures taken from (Burton & Burton, 2018).*

## Fish bone structure

Bones constitute approximately 10-15% of total fish biomass (Toppe et al., 2007). Bones are composed of mineral crystals (70%), extracellular organic matrix (20%) and cells (10%) (Heo et al., 2018). Collagen type I (90%) is a major component of the organic matrix, whereas the inorganic portion is primarily composed of hydroxyapatite (HA) crystals (S. Kim & Jung, 1996). Hydroxyapatite crystals are embedded into the organic matrix, where functional groups of collagen interact with HA (Stock, 2015). The nucleation and growth of HA crystals can occur within the channels and gaps of collagen molecules and on the surface of the collagen fibrils (Cui et al., 2007). Bone tissue is highly complex and ordered mineral-organic composite material. This composite material is organized into layers (lamellae) and, depending on the

bone type, the layers are arranged into higher order structures (Cui et al., 2007). The basic building blocks of the zebrafish skeletal bone are shown in Figure 8.
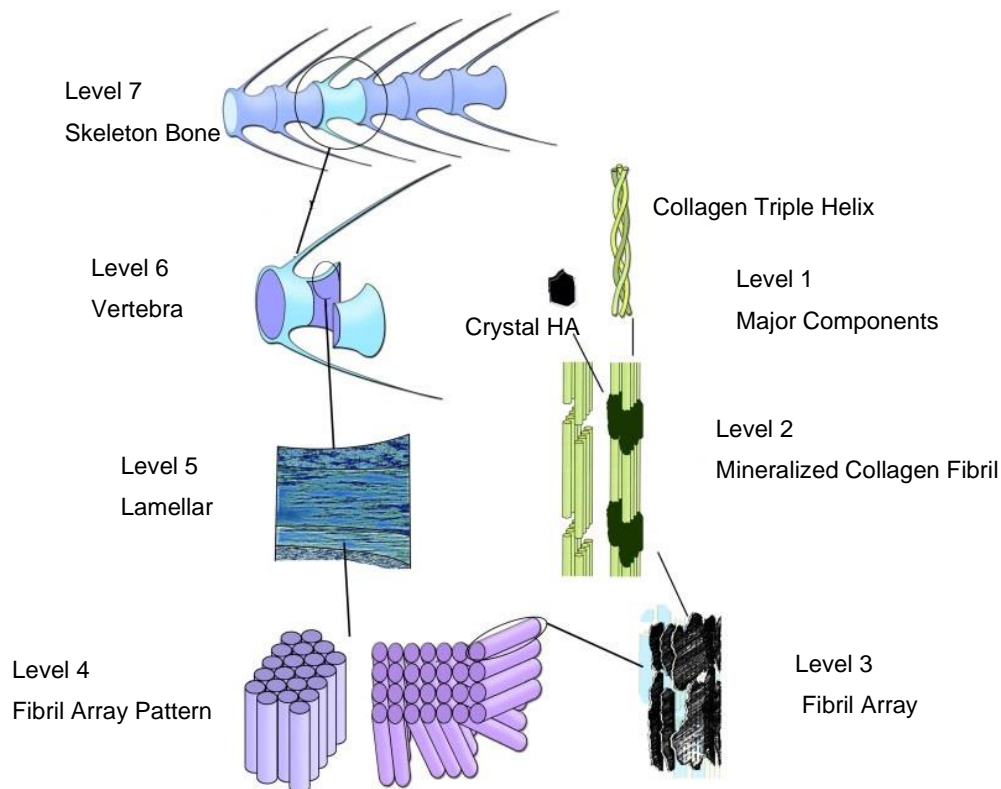


Figure 8 – Hierarchical organization of the zebrafish skeleton bone. Level 1: HA crystals and collagen fibrils. Level 2: Mineralized collagen fibrils – the basic building blocks. Level 3: The array of mineralized collagen fibrils. Level 4: Two common fibril array patterns: arrays of parallel fibrils or a plywood-like structure. Level 5: The lamellar structure in one vertebra. Level 6: A vertebra. Level 7: Skeleton bone. Figure taken from (Cui et al., 2007).

### 1.1.2.2 Myofibrillar proteins in fish muscle

Fish skeletal muscle has white and dark muscle; and white muscle is usually more abundant than dark muscle (Kristinsson & Rasco, 2000). The proportion depends on the nature of the fish species, e.g. tuna (strong swimming fish) has more dark muscle than cod (a slow-moving fish). Dark muscle is enriched with oxygen-carrying haem proteins and has higher amount of lipids (Hall, 2011).

Fish muscle consists of fibers, which are formed by many myofibrils, and myofibrils are made of myofibrillar proteins. In the space between myofibrils sarcoplasmic proteins are found. Fibers are bound together by connective tissue (stroma proteins) (Tahergorabi et al., 2011). Myofibrillar proteins constitute 65-75% (w/w) of total proteins in fish muscle, while sarcoplasmic proteins account for 15-35% (w/w) and stroma proteins make up on average 3% (w/w) (Harnedy & Fitzgerald, 2012). Myofibrillar proteins consist of myosin (main component

of thick filament), actin (main component of thin filament), and regulatory proteins such as tropomyosin, troponin and actinin (Figure 9). Sarcoplasmic proteins consist of myoglobin, hemoglobin, globins, albumins, and some enzymes. While stroma proteins consist of collagen and elastin (Tahergorabi et al., 2011).
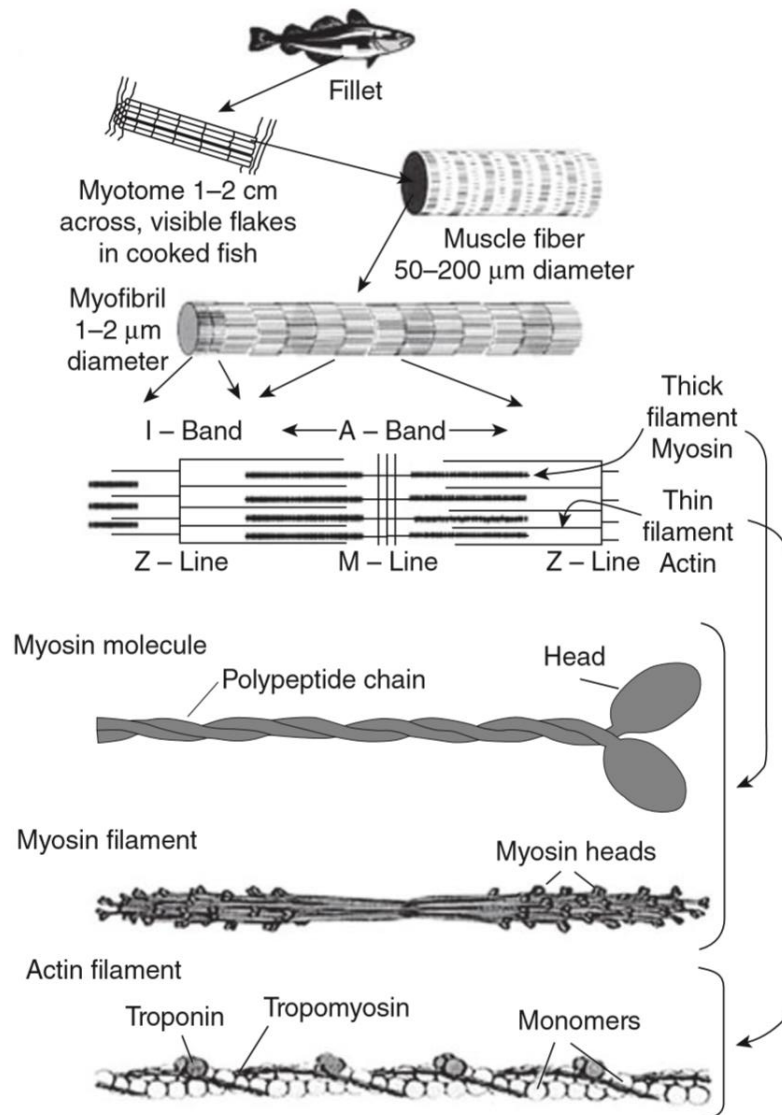


*Figure 9 – Structure of myofibrillar proteins (muscle tissue). White muscle consists of separate units called myotomes. Figure taken from* (Tahergorabi et al., 2011).

### 1.1.2.3 Proteolytic enzymes

*Enzyme* is a biological catalyst, which "accelerates the rate of a chemical reaction without itself becoming permanently altered in the process" (Petsko & Ringe, 2009). Most enzymes are proteins with the exception of a small group of catalytic RNA molecules (Nelson & Cox, 2013). *Proteolytic enzymes* or *proteases* "catalyze the cleavage of peptide bonds in protein-based substrates" (Walsh, 2014b), as shown in Figure 10 A. Proteases belong to a large and diverse

group of enzymes (Ward, 2011). Most proteases show some selectivity towards the peptide bond they hydrolyze (Walsh, 2014b). Specificity of enzymatic reaction is connected to active site structure and characteristics of substrate (e.g. structure, shape and electrical complementarities) (Parkin, 1993b).

Proteolytic enzymes can be classified based on several criteria such as source of enzymes (microbial, plant, animal), catalytic action (endopeptidase or exopeptidase) and characteristics of the active site (Adler-Nissen, 1993). Characterization as exopeptidases or endopeptidases is based on the position of the peptide bond in a substrate (Figure 10 B), which is hydrolyzed by an enzyme. *Exopeptidases* cleave peptide bond positioned on either the N terminus (aminopeptidases) or the C terminus (carboxypeptidases) of the protein-substrate, whereas *endopeptidases* hydrolyze peptide bone found internally in a protein (Ismail et al., 2019). Endopeptidases are further divided into several classes based on which amino acid residues or co-factors are essential in a catalytic site. The major classes are: serine proteases (e.g. trypsin), cysteine proteases (e.g. papain, bromelain), aspartic proteases (e.g. pepsin), metalloproteases (e.g. thermolysin) and threonine proteases (e.g. proteasome) (Clark & Pazdernik, 2016).



| **A** | **B** |
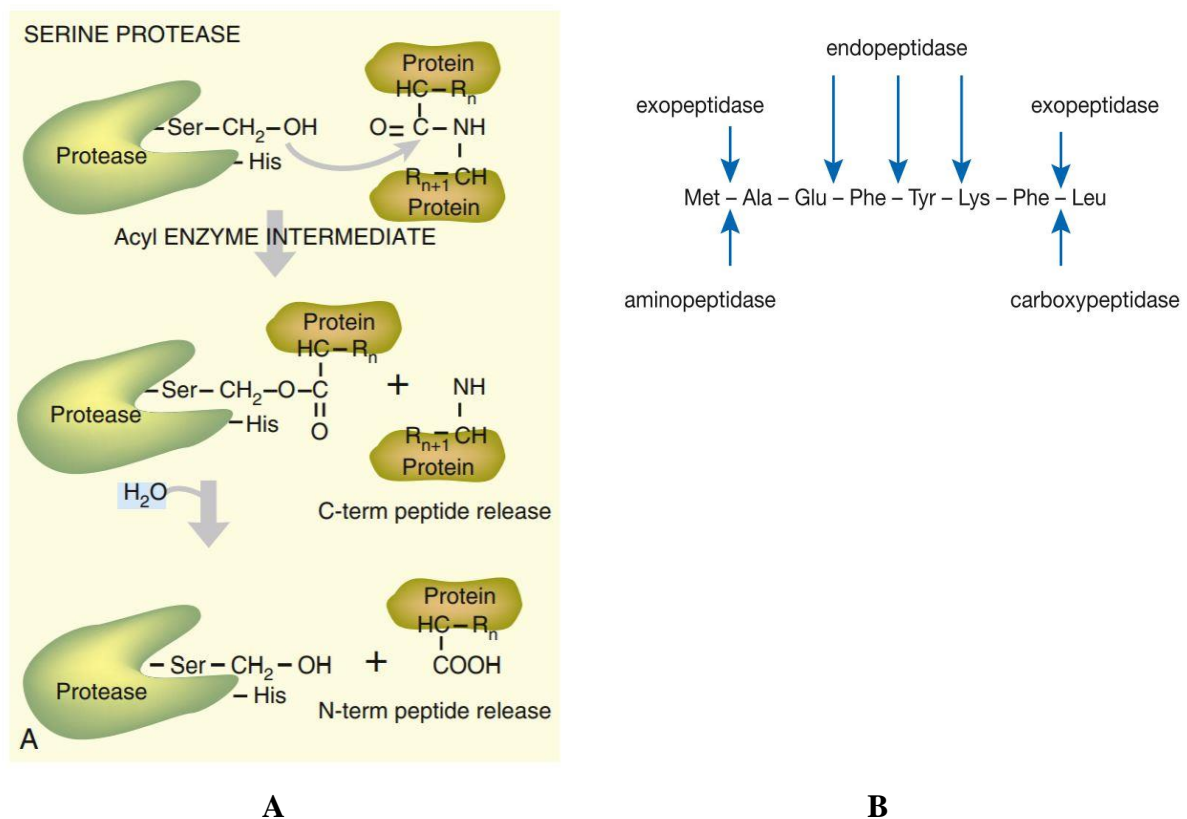
*Figure 10 – Proteolytic enzymes. (A) Serine protease mechanism of action. Figure taken from* (Clark & Pazdernik, 2016). *(B) Cleavage site specificity of proteases. Figure taken from* (Hooper, 2002)*.*

Reactions catalyzed by enzymes are influenced by environmental conditions. In food processing applications enzymatic reactions encounter a broad spectrum of conditions. Therefore, how specific enzymatic reactions are affected by its environment is essential knowledge in order to control and optimize the process. The most dominant factors are considered to be pH, temperature and water availability (Parkin, 1993a).

Industrial proteases are used, for example, in food industries, e.g. brewing (malting), baking (texture improvement), meat (meat tenderization), seafood (deskinning, fish protein hydrolysate) (Ismail et al., 2019). Industrial enzymes are produced in large quantities and are purified to a limited degree because economical considerations (e.g. production costs) are often essential for commercial success (Walsh, 2014a). Therefore, often detailed composition of industrial proteases is not available. Characterization of several commercial proteases, often used for hydrolysis of food proteins, have shown that e.g. Alcalase 2.4 L has three proteases in its composition, Corolase 2TS has two, while Flavourzyme 1000L has ten proteases (Merz et al., 2016).

## 1.1.3 Valorization of residual raw material by enzymatic hydrolysis

*Enzymatic protein hydrolysis (EPH)* is a process where "enzymes cleave the peptide bond between two amino acids" (Vang et al., 2018) in protein based substrates; during this process the molecular weight of proteins and peptides is decreased, the number of ionizable groups is increased and hydrophobic groups are exposed (He et al., 2013). In recent years, EPH have gained significant attention as a versatile processing technology. It is currently the most common method to produce hydrolysates from fish residual raw materials (Halim et al., 2016). EPH is used to recover a lipid phase, a soluble peptide fraction, and a non-soluble sediment (Aspevik et al., 2017; Böcker et al., 2017; Wubshet et al., 2017, 2018). EPH has replaced chemical hydrolysis because chemical hydrolysis has some drawbacks. These drawbacks include difficulty to control the process and products' properties; harsh processing conditions; reduced nutritional quality of the product due to destruction of some amino acids; presence of large amounts of salts (formed as a result of neutralization process); residual organic solvents and toxic chemicals in the final product (Kristinsson & Rasco, 2000). Whereas EPH is performed under mild processing conditions (temperature, pH and pressure) (He et al., 2013). Application of commercial proteases for hydrolysis offers possibilities to control the characteristics of the product by the choice of enzyme, reaction conditions and time of hydrolysis (Rustad & Hayes, 2012).

The extracted protein hydrolysate is often used as a feed ingredient, while there is an interest in retargeting this product for human consumption. Fish residual raw material is a complex material for hydrolysis and the challenge is to produce hydrolysates with reproducible properties (Rustad & Hayes, 2012). Current biotechnological processes make it challenging to utilize the full potential of complex raw materials, creating a demand for further development of biotechnological processes (Nofima, 2018).

### 1.1.4  Bioactive peptides in fish hydrolysates

*Bioactive peptides* are peptides which exhibit biological activity (Halim et al., 2016), and this activity affects physiological functions of the organism (Hayes & McKeon, 2014). Generally marine bioactive peptides can be classified into three broad groups: naturally active peptides (can be directly extracted); peptides that can be produced by hydrolysis of parent proteins with the use of enzymes; and peptides produced by fermentation (Sable et al., 2017).

In the last decades considerable research has been conducted to find bioactive peptides in fish protein hydrolysates. Studies show that fish proteins are an interesting source of bioactive peptides. Peptides from fish protein hydrolysates demonstrate a number of bioactive properties such as antioxidative, antihypertensive properties, antithrombic activity, immunomodulatory effect, anticancer activity, antimicrobial, and body weight reduction effect (Alemán & Martínez-Alvarez, 2013; Meram Chalamaiah et al., 2018; Cicero et al., 2017; N. R. A. A. Halim et al., 2016; Harnedy & Fitzgerald, 2012; Ishak & Sarbon, 2018; S. K. Kim & Wijesekara, 2010; Korczek et al., 2018; Lordan et al., 2011; Ngo et al., 2012; Rocha et al., 2018; Sila & Bougatef, 2016). However, only a few studies investigated bioactive peptides in protein hydrolysate from Atlantic cod. The reported bioactivates are antioxidant activity (Farvin et al., 2014, 2016; Girgih et al., 2015; Godinho et al., 2016; Jamnik et al., 2017; I. Jensen & Mæhre, 2016; Pampanin et al., 2016; Slizyte et al., 2009), antiproliferative activity on cancer cell lines (Picot et al., 2006), ACE inhibitory activity (Dragnes et al., 2009; Godinho et al., 2016; Jeon et al., 1999), immunomodulatory effect (Y. Chen et al., 2019), and regulation of food intake (Cancre et al., 1999; Dale et al., 2018; Fouchereau-Peron et al., 1999; Slizyte et al., 2009).

### 1.1.5  From laboratory to industry: challenges of upscaling

Practical scale-up of biochemical and chemical processes is complicated and a case specific procedure (Piccinno et al., 2016). Therefore, understanding the nature of the process and the reasoning behind the process is essential (Shinnar, 2004). Characterization of enzymatic hydrolysis of residual raw material is a difficult task because it is "a combination of parallel

and consecutive occurring reactions" (Himonides et al., 2011). The main complicating factors are natural presence of enzymes inhibitors in the raw material and different susceptibility of diverse bonds in the proteins to different enzymes (Himonides et al., 2011). The final aim of the scale-up process is to deliver a product which fulfills quality specifications at the required production rate and yield on an industrial level (Shinnar, 2004). It is considered challenging to produce hydrolysates with the same quality specifications from residual raw materials on an industrial scale due to biomass complexity (different components in the mixture) and its variation in quality (oxidation state of protein components and oil, presence of microorganisms in the material, and activity of spoilage enzymes) (Vang et al., 2018).

Enzymatic hydrolysis at a larger scale (> 2000 L) or industrial scale does not proceed the same way as it does at a laboratory scale (Vang et al., 2018). Scaling up is challenging because larger volume causes change in mixing, mass and heat transfer, and shear rate (Acosta-Pavas & Ruiz-Colorado, 2020; Ehly et al., 2007; Shinnar, 2004). The most commonly used approach to control a scale-up process is to identify key factors and their impact on the process (Shinnar, 2004). Identification of these factors is easier if the scale-up process is divided into several steps by increasing volume, e.g. by 10-fold each step. Another option is to closely monitor the process of hydrolysis, which will allow to control and adjust processing settings (Vang et al., 2018).

Infrastructure is another factor influencing the scale-up process; industrial scale technologies are often different from laboratory equipment (Piccinno et al., 2016). The equipment that was used in the laboratory cannot always be applicable for a larger scale or can be difficult/expensive to get for industrial use (Vang et al., 2018). For example, lab scale centrifuges, which are used for separation of different phases after hydrolysis in the laboratory, are changed to a decanter or tricanter centrifuge on an industrial scale; or freeze-drying of the hydrolysate often used in the laboratory is changed to spray-drying on an industrial scale. It is also worth mentioning that economic factors should be taken into consideration from the early investigative stage in the laboratory (Shinnar, 2004).

### 1.1.6 Research project Notably

As mentioned earlier, in Norway several companies are involved in research and development of new methods to produce protein hydrolytes for human consumption (Richardsen et al., 2019). One of these is the research institute Nofima, which coordinates the research project Notably (Novel cascade technology for optimal utilization of animal and marine by-products). The aim of Notably is to develop new technological solutions where processing of cod and chicken residual raw materials takes place in several different stages, so that multiple high-value components can be extracted from the same rest raw material. Better biotechnological solutions have to be developed to achieve this goal; and enzymes play a central role in the solution. The traditional EPH leaves several valuable components such as minerals and non-soluble collagen in sediment, and therefore the idea is to use more specific enzymes, which can release connective tissue proteins and myofibrillar proteins in separate stages. Better understanding of the enzyme's action on different types of residual raw materials will enable future development of a multistep process. This multistep process, or cascade, will constitute a combination of several different processing steps, each one aimed at giving separate products with the highest possible yield and quality. The intention is that the cascade bioprocessing will result in better utilization of residual raw materials and increased value for food industry (Nofima, 2018).

## 1.2 Methodological framework

### 1.2.1 Enzymatic hydrolysis

The method for enzymatic hydrolysis of fish meat was developed more than 70 years ago by Canadian researches (Gildberg, 1993) and today enzymatic hydrolysis "is one of the well-recognized technologies in valorization of residual raw materials" (Wubshet et al., 2018). The main steps of enzymatic hydrolysis of fish material are shown in Figure 11. The process can be divided into three steps: pre-treatment, hydrolyzation and recovery.

1) Pre-treatment step focuses on preparation of the substrate. Raw material is prepared for hydrolysis by making a homogenized water-mince mixture. The material is washed, ground or cut into small pieces, and then the minced material is mixed with water usually in 1:1 ratio (He et al., 2013).

2) The next step is hydrolyzation – the selected enzyme is added into the homogenous mixture with the material. Selection of enzyme is based on the characteristics of the substrate and

desired functionalities of the final product. The temperature and pH are adjusted depending on the optimal conditions of an enzyme (He et al., 2013). However, pH adjustment is not recommended because use of acid or base, as mentioned earlier, can lead to reduction of nutritional value due to destruction of some amino acids and high levels of salt in the product (Aspevik et al., 2017). The enzyme/substrate ratio and processing time are determined according to the desired functionalities and yield of the final product. The hydrolysis process is terminated by heat inactivation of enzyme (90-95 °C for 10-30 min) (He et al., 2013).

3) Recovery is the final step of the process. After the end of hydrolysis, the crude hydrolysate is separated into water phase, sediment phase and oil phase by centrifugation or three-phase decanter. The water phase, which contains dissolved protein hydrolysate, is freeze-dried or spray-dried, and can also be up-concentrated by evaporator before drying (Aspevik et al., 2017).
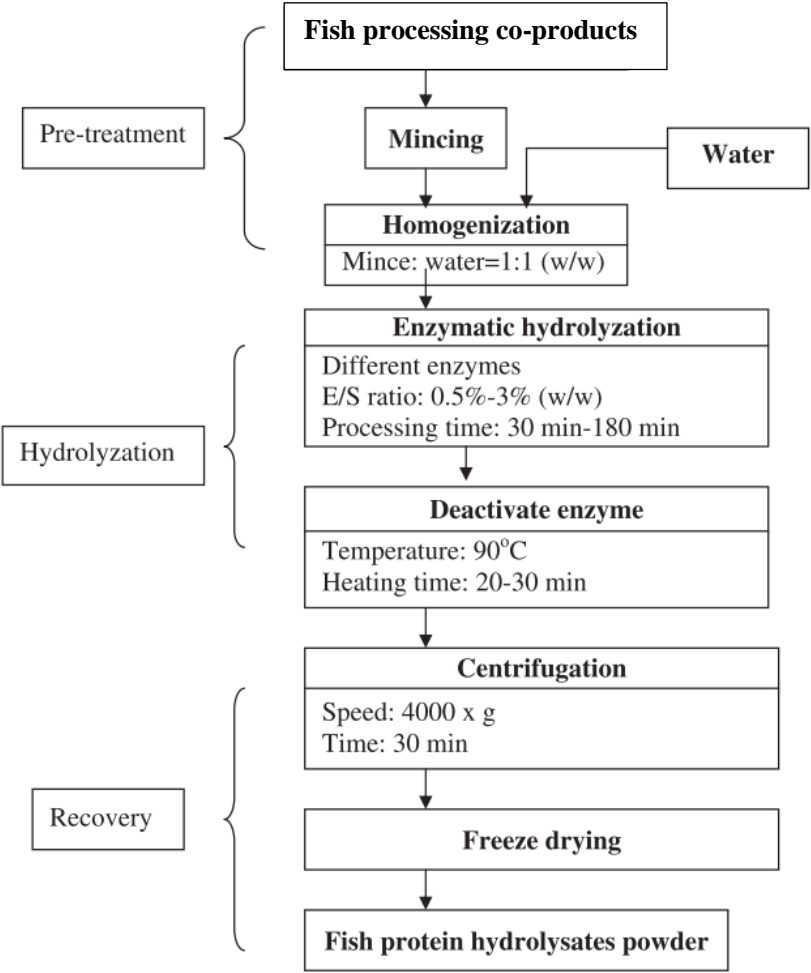


*Figure 11 – General workflow of enzymatic processing method to produce fish protein hydrolysate. Figure adapted from* He et al. (2013)*.*

## 1.2.2 Principles of size exclusion chromatography

Size exclusion chromatography (SEC) is a "liquid column chromatographic technique that sorts molecules according to their size in solution" (Striegel et al., 2009). Currently SEC is the most popular method to determine molar mass distributions (Podzimek, 2010).

Molecular weight (MW) determination by SEC starts with dissolution of a sample in a solvent (mobile phase) followed by injection of the solution into a column. The column is packed with porous particles and filled with a mobile phase. The mobile phase is going through the column at a fixed flow rate and thus creating a pressure gradient along the column. As a result of the pressure gradient, the sample molecules pass through the column. Figure 12 demonstrates how separation occurs based on a mixture of two groups of macromolecules with different sizes. The smaller molecules are able to get into the pores and are retained longer in the column, while bigger molecules are not able to fit into the pores and are going straight through the column. At the column outlet a detector generates a signal proportional to the concentration of eluting molecules (Malawer & Senak, 2004; Podzimek, 2010). SEC is "a relative and not an absolute molecular weight technique" and, therefore, calibration of columns should be performed with standards of known molecular weight (Mori & Barth, 1999). Since MW of peptides are important for their functionalities, SEC is widely used to determine the MW distribution of peptides in hydrolysates.
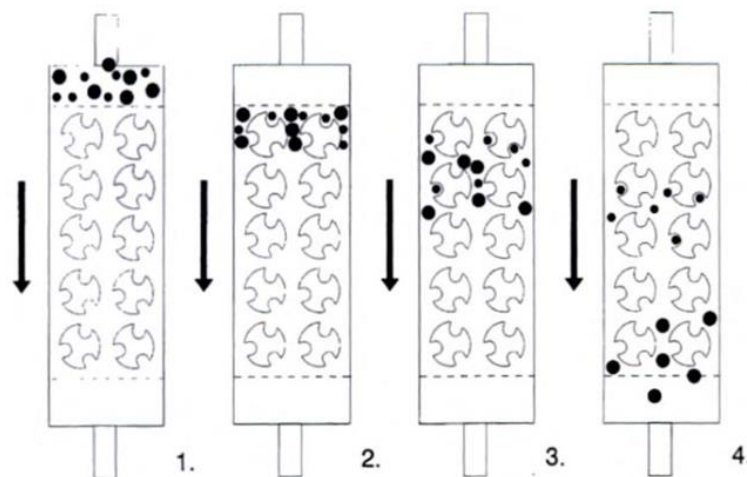


*Figure 12 – Separation of two macromolecular sizes by SEC: (1) sample mixture immediately after injection, before entering the column packing; (2) sample mixture enters the head of the column packing; (3) start of separation by size; and (4) complete resolution* (Malawer & Senak, 2004)*.*

## 1.2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is another analytical method for separating proteins by size. It is the most common electrophoretic method applied to proteins (Walsh, 2014c). Electrophoresis is an "analytical technique that separates analytes from each other on the basis of charge" (Walsh, 2014c). SDS is a negatively charged detergent that binds to proteins at a ratio of ~1.4 g SDS per gram of protein, unfolds proteins and contributes to the overall negative charge of the protein-SDS complex (Bio-Rad Laboratories, 2017). The polyacrylamide gel functions as a molecular sieve; it regulates the migration of proteins in proportion to their charge-to-mass ratio (Nelson & Cox, 2013). Gels have different concentrations of acrylamide; and gels with higher acrylamide concentration have smaller pore size (Invitrogen, 2016). When an electric field is applied, negatively charged protein-SDS complexes will migrate to anode based on their size (Figure 13). Molecular weight markers, containing a mixture of several known proteins of known molecular weight, are used to assess the relative sizes of the proteins in a sample (Invitrogen, 2016). After electrophoresis, protein bands are often visualized for analysis; and visualization is done by protein stains. Examples of protein stains include Coomassie stains, fluorescent stains and silver stains (Bio-Rad Laboratories, 2017; Invitrogen, 2016).
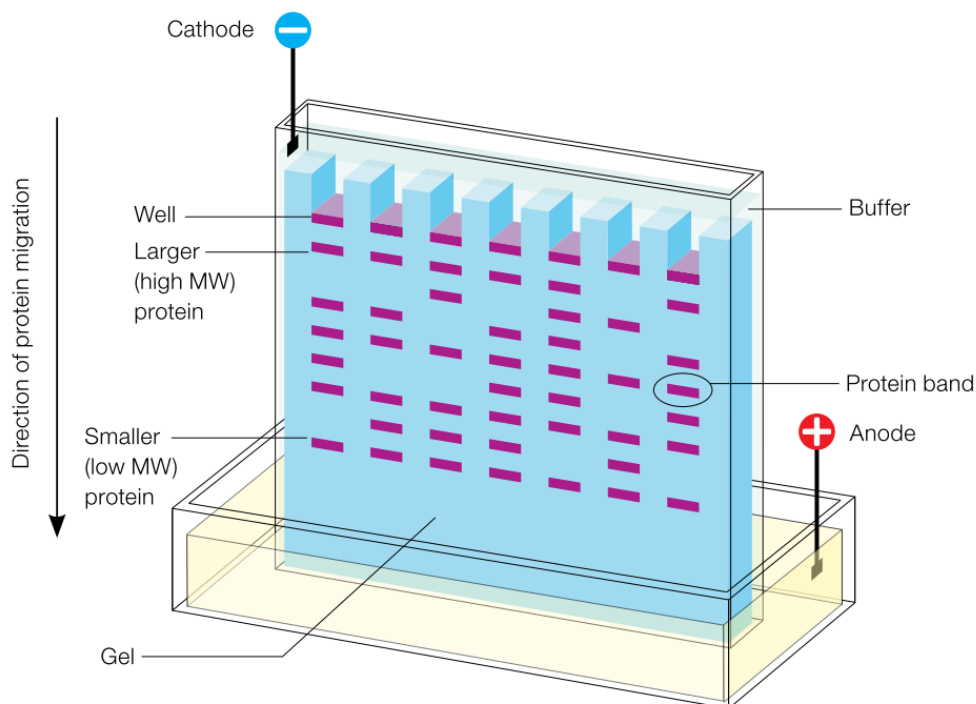


*Figure 13 – Schematic of electrophoretic protein separation in a polyacrylamide gel. Figure taken from* (Bio-Rad Laboratories, 2017).

## 1.2.4 Principles of *in vitro* bioactivity assays

The following bioactivity assays are presented below: Aqueous One Solution Cell Proliferation assay (viability assay), oxygen radical absorbance capacity assay (antioxidant assay) and anti-inflammatory assay.

### 1.2.4.1 Aqueous One Solution Cell Proliferation assay

Cancer is one of the major causes of death worldwide with an estimated 9.6 million deaths only in 2018 (WHO, 2018). Cancer is "an abnormal growth and proliferation of cells in the body" (Meram Chalamaiah et al., 2018). Cell division is a physiological process, which is tightly regulated under normal conditions. However, certain mutations can disrupt the regulation process and normal cells can be transformed into cancer cells; cancer cells start to divide uncontrollably and spread to surrounding tissues (Suarez-Jimenez et al., 2012). Thus, an important strategy for treating tumors is inhibition of deregulation of cell proliferation (Meram Chalamaiah et al., 2018). Cancer is a group of diseases, which can originate in almost any organ or tissue; and more than a hundred different types of cancer are characterized based on the site of origin and the specific cell type involved (Hardin et al., 2016).

Cell-based assays are used to evaluate cell proliferation activity of a test compound. One of the common methods is tetrazolium reduction, where tetrazolium compound (e.g., MTS) is used to detect viable cells (Riss et al., 2016). Aqueous One Solution Cell Proliferation assay is a "colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays" (Promega, 2012). The principle of the assay is based on bio-reduction of tetrazolium compound (MTS) by metabolically active cells into a colored formazan product. The formazan product is soluble across cell membranes and is present in culture medium. The number of living cells in culture is directly proportional to the amount of formazan product that was formed. Color intensity (formazan product) is measured by recording absorbance at 490 nm and compared to cells in negative control wells (Promega, 2012).

Various cancer cell lines (e.g., human liver cancer, human monocytic leukoma, human cervical cancer, human breast cancer) are used to investigate antiproliferative properties of test molecules. Different cell lines have their own morphology and tumor characteristics due to different origin, and thus have different sensitivities to test compounds (Meram Chalamaiah et al., 2018).

### 1.2.4.2 Oxygen radical absorbance capacity assay

An inevitable part of aerobic metabolism is the formation of free radicals, which are highly reactive due to unstable unpaired electrons (He et al., 2013). Therefore, the human defense mechanism includes several antioxidant systems to prevent free radicals from causing damage (I. Jensen & Mæhre, 2016). However, under certain conditions the defense system cannot remove all free radicals. Free radicals cause cellular damage by oxidizing lipids, DNA and proteins (Alemán & Martínez-Alvarez, 2013), which can initiate diseases such as cancer, diabetes mellitus, atherosclerosis, arthritis (He et al., 2013), neurodegenerative disorders, inflammation (Girgih et al., 2015), cardiovascular disease, and aging (Cheung et al., 2015). Furthermore, oxidation of lipids during food processing and storage cause quality deterioration of food (Ahn et al., 2012) and is, therefore, an important issue in the food industry (Nasri, 2017). Oxidation of unsaturated lipids in food results in formation of off-flavours and undesirable odours, but also decrease the nutritional quality and lead to formation of potentially toxic compounds (Farvin et al., 2014). Synthetic antioxidants are used today, but their use is under strict regulation because of their potential health risks (Farvin et al., 2014) connected with toxicity, protein and DNA damage and side effects (Chi et al., 2015). Therefore, a search for new and safe antioxidants from natural origin has gained great interest in recent years (Sila & Bougatef, 2016).

Currently there is no single assay that is able to evaluate the overall antioxidative potential of a compound, because oxidative processes are complex and different mechanisms can protect biological system from free radicals (Sveinsdottir et al., 2014). Sveinsdottir et al. (2014) classify these mechanisms into five categories:

(1) inhibition of generation and scavenging properties against free radicals,
(2) reducing ability,
(3) metal-chelating capacity,
(4) activity as antioxidative enzymes,
(5) inhibition of oxidative enzymes.

Over 20 assays have been developed to evaluate the antioxidant activities of compounds (Hayes & McKeon, 2014). Among them the most often methods used to evaluate the antioxidant potential of marine food compounds are oxygen radical absorbance capacity (ORAC) assay, 2,2-diphenyl-1-picrylhydraxyl (DPPH) radical-scavenging capacity assay, Trolox equivalent antioxidant capacity (TEAC) assay, ferric-reducing antioxidant power (FRAP) assay, and total radical-trapping antioxidant parameter (TRAP) assay (Sveinsdottir et al., 2014).

ORAC assay is the most common assay in research, clinical and food laboratories. In the ORAC assay a component is tested on its ability to quench free radical by hydrogen donation to form a stable compound and thus stop radical chain reaction (Sveinsdottir et al., 2014). The main principles of the ORAC assay were developed by Glazer (1988) and later the assay was improved by Ou, Hampsch-Woodill, & Prior (2001) who proposed to use fluorescein as the fluorescent probe. The ORAC assay measures antioxidant scavenging activity against peroxyl radical induced by AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride). Caused by the exposure to free radicals, fluorescent molecule fluorescein losses fluorescence. Antioxidants can protect fluorescein from damage and the protective effect is evaluated based on a fluorescence intensity measured by a fluorometer (Ou et al., 2001). The ORAC assay measures antioxidant capacity of substrates and combines inhibition time and degree of inhibition into a single measurement (Huang et al., 2002).

### 1.2.4.3 Anti-inflammatory assay

Inflammation is a reaction of the innate defense system to tissue injury caused by a pathogen or wound. During the acute inflammation (the immediate response to cell death or injury) the cells from the injured tissue initiate a cascade of events by releasing inflammatory mediators. Chronic inflammation can develop if inflammatory pathways are stimulated for a long period of time (Willey et al., 2014). However, uncontrolled and chronic inflammation can lead to development of diseases, such as rheumatoid arthritis, cardiovascular diseases, asthma (Ahmad et al., 2019), diabetes, atherosclerosis, and cancer (Subhan et al., 2017). Steroidal and non-steroidal anti-inflammatory drugs are used for management of inflammation, but both types of drugs can cause serious side effects (Ahmad et al., 2019). Therefore, there is a focus to find new anti-inflammatory compounds.

Cell-based assays are used to evaluate anti-inflammatory properties of a test compound. Macrophages play an important role in host defense systems and activated macrophages regulate inflammation by inflammatory cytokines (e.g., TNF-α and interleukins (IL)) (Joshi et al., 2016). Thus, one of the potential approaches to treat inflammatory diseases is by inhibition of production of pro-inflammatory mediators (Subhan et al., 2017). Macrophages activated by lipopolysaccharides (LPS) are used to study inflammation *in vitro* (Rocha et al., 2018).

Anti-inflammatory assay can evaluate the ability of a test compound to inhibit production of TNF-α by LPS activated macrophages. First, Thp-1 cells are stimulated by PMA (phorbol 12-myristate 13-acetate) to differentiate from monocytes to macrophages. Then the cells are

incubated with the test compound followed by addition and incubation with LPS. The amount of TNF-α produced can be detected and quantified by Sandwich ELISA (enzyme-linked immunosorbent assay).

The principle of ELISA technique is based on the the binding affinity and specificity of antibodies (Nelson & Cox, 2013). Sandwich ELISA is a several stage process. First, ELISA plates are coated with human TNF-α antibodies (capture antibodies) followed by addition of test samples (antigen) and standards. Then biotin conjugated human TNF-α Ab (detection antibodies) binds to a test sample or standard forming a antibody-antigen-antibody sandwich. Afterwards, biotin binds to Extravidin, which is congugated to alkaline phosphatase (enzyme). When alkaline phosphatase reacts with chromogenic substrate pNPP, yellow color is generated, thus detecting TNF-α presence (Figure 14). The color intensity is measured by spectrophotometer at 405 nm (Biosciences, n.d.; Crowther, 2001).
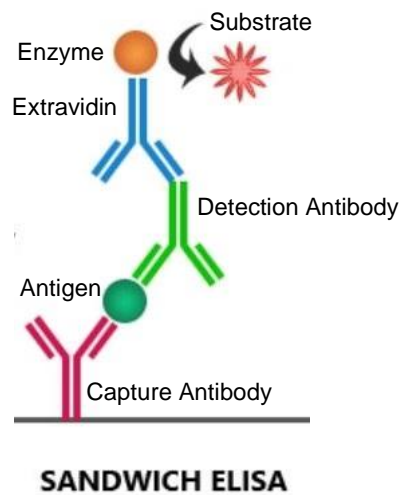


*Figure 14 – Sandwich ELISA principle. Figure taken from (Boster Biological Technology, n.d.)*

## 1.3  Aim of the project

The main aim of the project is to evaluate enzymatic hydrolysis of and hydrolysates from Atlantic cod (*G. morhua*) heads.

Sub-goals are:

1) Evaluate and compare the yield and molecular weight of peptides of the individual hydrolysates from muscle, skin and bone from cod heads by using 23 different proteases.
2) Identify proteases that are selective towards myofibrillar proteins or collagen, by calculating selectivity ratio based on hydrolysate yield.
3) Evaluate and document the bioactivities of the hydrolysates based on antioxidative, viability, anti-inflammatory *in vitro* assays.
4) Propose conditions for small upscaling (e.g. enzyme, temperature, pH).

# 2  Materials and methods

## 2.1  Raw material

Heads from Atlantic cod (*Gadus morhua*) obtained from Brødrene Karlsen AS (Husøy, Senja, Norway), were used as raw material. The fish was caught in spring 2019. The cod heads were transported and stored frozen (-24℃) until start of the experiments.

## 2.2  Enzymes and other chemicals

Enzymes (all proteases), their optimal working conditions and their price range are listed in Table 2. All other chemicals used in the experiments were of analytical grade and are listed in Appendix A 1 Table 6.

*Table 2 – Proteolytic enzymes used for hydrolysis, optimal working conditions\*, price range\*\* and their manufacturers.*

| Enzyme | pH | Temperature (℃) | Price range | Manufacturer |
|---|---|---|---|---|
| **Alcalase AF 2.4 L** | 8.0-9.5 | 65-75 | Low | Novozymes A/S, Bagsvaerd, Denmark |
| **Bromelain** | 4.0-8.0 | 45-60 | Medium | Gunung Sewu Group, Jakarta, Indonesia |
| **Corolase 2TS** | 7-8 (6-9) | 65 | Medium | AB Enzymes GmbH, Darmstadt, Germany |
| **Corolase 7090** | 6.5-7.5 | 45-70 | Low | AB Enzymes |
| **Tail 10** | 9 (5-9) | 30-65 | Low | Tailorzyme ApS, Søborg, Denmark |
| **Tail 189** | 8 (5-9) | 45 | High | Tailorzyme |
| **Tail 190** | 9 (5-9) | 30-60 | High | Tailorzyme |
| **Tail 191** | 7 (6-8) | 55 | High | Tailorzyme |
| **Tail 192** | 7 (6-8) | 30-55 | High | Tailorzyme |
| **Tail 193** | 8 (5-9) | 45 | High | Tailorzyme |
| **Tail 194** | 7 (6-9) | 60 | High | Tailorzyme |
| **Tail 197** | 7 | 50 | High | Tailorzyme |
| **FoodPro 30L** | 8.0-9.5 | 45-65 | n.d.a.\*\*\* | DuPont Danisco, Copenhagen, Denmark |
| **FoodPro 51FP** | 8.0-10.0 | 45-60 | High | DuPont Danisco |
| **FoodPro PNL** | 6.5-7 | 55-65 | Low | DuPont Danisco |
| **Veron L10** | 5.5-8.0 | 55-65 | n.d.a. | AB Enzymes |
| **Flavourzyme** | 5.0-7.0 | 50 | High | Novozymes |
| **Protamex** | 5.00-11.0 | 60 | Medium | Novozymes |
| **Promod 144GL** | 5.0-7.0 | 50-60 | n.d.a | Biocatalysts Ltd., Cardiff, UK |
| **Promod P950L** | 5.0-7.0 | 50-60 | n.d.a | Biocatalysts |
| **Endocut 01** | 6.0-8.0 | 45-55 | Low | Tailorzyme |
| **Endocut 02** | 9-10 | 60 | Low | Tailorzyme |
| **Endocut 03** | 7-10 | 55-70 | Low | Tailorzyme |

## 2.3  Workflow of the experiment

This master thesis is part of the Notably project. The main contribution of this thesis is the investigation of the enzymes that are specific for myofibrillar proteins in muscle tissue and collagen in skin and bones, which is the first step in development of cascade technology. Bioactivity testing of the hydrolysates is an additional contribution. The general workflow of the experiment is visualized in Figure 15.
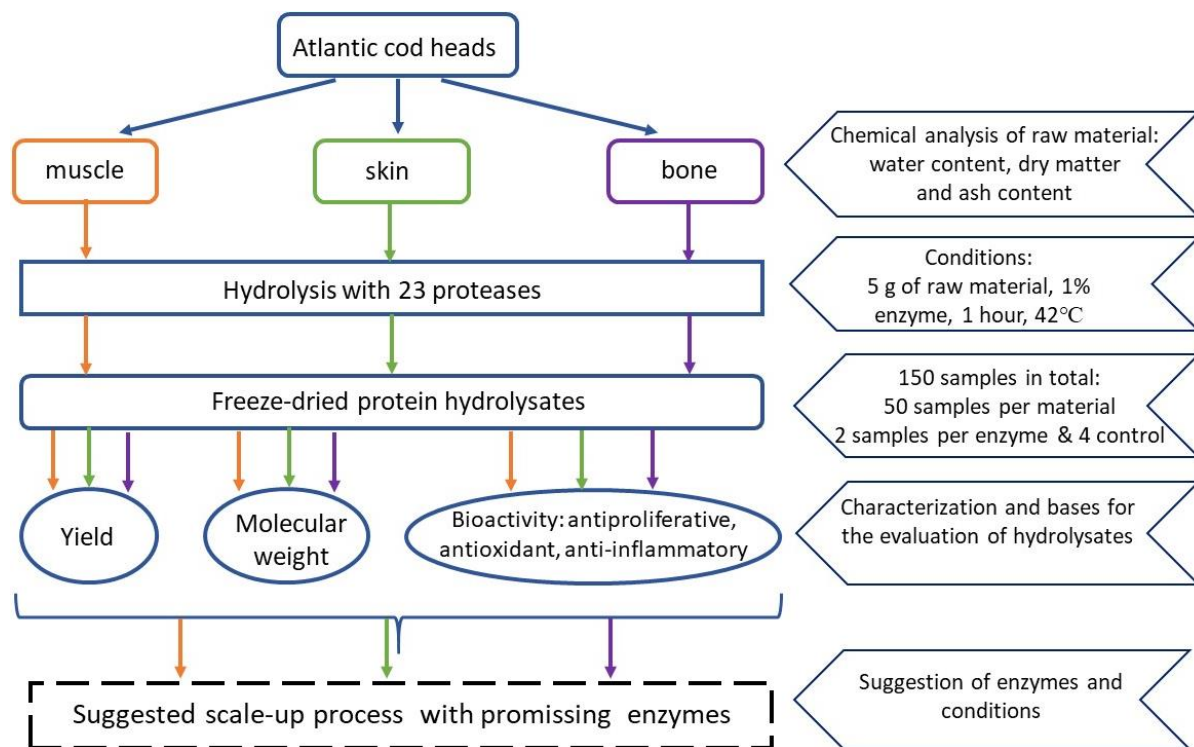


*Figure 15 – Schematic workflow of the experiment.*

## 2.4  Preparation of cod heads

Frozen cod heads, with a weight of 950 g to 2600 g, were thawed overnight in a cold room (12℃) and then washed in cold water. Three types of material were prepared: skin, muscle and bone. Skin was cleaned from scales, muscle and connective tissue, then washed in cold water and cut into small pieces (ca. 0.5×0.9 cm) using scissors. Muscle was removed from heads, washed in cold water and then cut into small pieces (ca. 0.5×0.9 cm) with a knife. Bones were

25

cleaned from muscle and connective tissues with a knife and a brush, washed with cold water and cut into smaller pieces with the help of scissors and pliers. The precut pieces of bone were stored frozen (-24°C) until they were crushed using a commercial blender (Waring Commercial Inc., Torrington, USA). The precut skin, muscle and crushed bones (5±0.2 g) were weighed into 25 mL tubes. The tubes were stored frozen at -24°C until hydrolysis.

## 2.5 Chemical analysis of the raw material

The chemical composition of muscle, skin and bone from the cod heads was determined by measurement of dry matter and ash content. The measurements were performed in quadruplicate (ap. 5 g of material per replicate). First, dry matter was identified by drying the muscle, skin and bone in a heating cabinet at 110°C until a stable weight was reached. Then the samples were placed into a high temperature oven overnight at 500°C. Dry matter weight and ash weight were recorded and difference in weight between dry matter and ash, wet weight and dry weight was calculated.

## 2.6 Preparation of the hydrolysates

Three types of material (skin, muscle, bone) were hydrolyzed using 23 protease enzymes, and a control treatment without addition of enzyme (Milli-Q water instead of enzyme) was performed. The procedure of hydrolysis was performed in the same way for each sample. Hydrolysis was performed in duplicate and control treatment was made in quadruplicate.

Approximately 5 g of material in 25 mL tubes (Sarstedt, Mawson Lakes, Australia) were thawed in a cold-water bath for ca. 1 hour. Afterwards, each tube was filled with Milli-Q water (21.1 or 21.2 mL, volume was dependent on enzyme). The total liquid volume of water and enzyme was 21.25 mL. Each tube was shaken to evenly distribute the material in the water. The tubes were placed in a warm water bath (HETO-DAN ApS, Broenderslev, Denmark) at 45°C for 22 min to prepare the samples for hydrolysis. Afterwards, enzyme (1% w:v or w:w) was added into each tube: for enzymes in liquid form – 50 µL and in powder form – 150 µL (due to dilution of 0.05 g in 150 µL of Milli-Q water). Vortex was used to help dissolve powder enzymes in water. The tubes with enzymes were placed onto a nutating mixer (VWR International, Radnor, USA) in a heating cabinet (42°C). The hydrolysis proceeded for one hour followed by enzyme inactivation by brief microwaving (7 sec) and then hot water bath (ca. 95°C) for 15 min. Immediately after inactivation, the tubes with samples were centrifuged for 10 min at 4°C and 3000 rpm, to achieve separation of sediment and the liquid phase

(hydrolysate). The hydrolysates were filtered using Whatman No. 41 filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK), weighed, placed into a freezer (first at -24°C for storage and later at -78°C as preparation for freeze drying) and then freeze-dried in a freeze dryer FreeZone 12 (Labconco, Kansas City, USA). The weight of the freeze-dried hydrolysates was registered. The remaining sediment in the tubes and on filter papers were placed into heating cabinets for drying at 110°C and 62°C respectively. When stable weight was achieved the weight of dried material was registered. For analysis and comparison of the results, the weights were adjusted per 5 g (an example of calculation is presented in Appendix A 2.1.). Calculations of total yield and hydrolysate yield were done using the formulas below (an example is presented in Appendix A 2.2.).

(1) control treatment yield or background reaction (protein dissolved in water; no enzyme added) $= \left( \frac{freeze-dried\ hydrolysate\ weight\ for\ control\ (g)}{dry\ matter\ of\ the\ material\ (g)} \right) \times 100\%$

(2) total yield $= \left( \frac{freeze-dried\ hydrolysate\ weight\ (g)}{dry\ matter\ of\ the\ material\ (g)} \right) \times 100\%$

(3) hydrolysate yield (%) = total yield (%) – background reaction (%)

Also, the theoretical maximum hydrolysate yield was calculated for each type of raw material using the formulas for the hydrolysate yield. In the calculations, the amount of organic matter in the raw materials was considered as 'freeze-dried hydrolysate weight' and an assumption was made that all organic matter consisted only of proteins.

Calculations of sediment reduction due to enzymatic hydrolysis were done using the formulas below (an example is presented in Appendix A 2.3.).

(1) control treatment sediment or background reaction sediment $= \left( \frac{sediment\ weight\ for\ control\ (g)}{dry\ matter\ of\ the\ material\ (g)} \right) \times 100\%$

(2) total sediment $= \left( \frac{sediment\ weight\ (g)}{dry\ matter\ of\ the\ material\ (g)} \right) \times 100\%$

(3) sediment reduction (%) = total sediment (%) – background reaction sediment (%)

Based on hydrolysate yields, skin/muscle and bone/muscle selectivity ratios were calculated (an example is presented in Appendix A 2.4.).

## 2.7 Determination of molecular weight

### 2.7.1 Size exclusion chromatography

The molecular weight of peptides in the hydrolysates were determined by size exclusion chromatography (SEC). SEC was performed using Shimadzu Prominence high-performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan). LabSolutions GPC software (Shimadzu) was used to calculate weight average MW of the hydrolysates, an example of calculation of weight average MW is shown in Appendix A 2.5. Freeze-dried hydrolysates were dissolved in a mobile phase (Acetonitrile: $H_2O$: Trifluoroacetic acid in the ratio 30:70:0.05) to get concentration at 20 mg/mL followed by centrifugation for 1 min. Subsequently the solutions (100 µL) were pipetted into vials and placed into a sampler. The injection volume was 10 µL and the separation was performed using a BioSepTM 5 µm SEC-S2000 145 Å LC column 300×7.8 mm. Every 7$^{th}$ sample was a blank to control for possible cross-contamination of the samples.

### 2.7.2 SDS-PAGE

The molecular weight of the hydrolysates and of the control treatment made from skin was also estimated by SDS-PAGE. First, an appropriate concentration of freeze-dried hydrolysates for running of SDS-PAGE was determined by testing several concentrations on three samples (control, hydrolysates with high and low average MW). The protein amount tested with hydrolysates were: 50 µg, 100 µg, 200 µg, 250 µg and 500 µg per well; and with the control treatment were: 25 µg, 50 µg, 100 µg and 200 µg per well. Electrophoresis was carried out using a precast NuPAGE 12% Bis-Tris gel (Invitrogen, ThermoFisher Scientific, USA), and premixed buffers: MOPS SDS Running buffer (Invitrogen) and NuPAGE LDS Sample Buffer (Invitrogen). Two MW markers were used to determine approximate molecular weight of the samples: HiMARK Pre-stained Protein standard (Invitrogen) and SeeBlue Plus2 Prestained standard (Invitrogen). The freeze-dried samples were diluted in distilled water and 20% of the sample buffer to get a concentration at 25 mg/mL for hydrolysates (~250 µg protein per well) and 5 mg/mL for the control treatment (~25 µg protein per well). The resulted mixtures were briefly vortexed and heated to 50℃ for 10 min on a heated block (Gant, Kisker Biotech, Germany). Afterwards, the samples and MW markers were loaded on the gel. Electrophoresis was performed for 50 min at 1.5 Ampere and 200 Volt using XCell SureLock Mini-Cell XCell4 chamber system (Invitrogen). After electrophoresis, the gels were washed with water, placed into a container filled with water and heated in the microwave for ca. 30 sec followed by changing of water (3 times in total). Then the gels were covered with a staining reagent

SimplyBlue SafeStain (Invitrogen), heated in the microwave for ca. 30 sec and placed onto a rocking platform (VWR) for 1 hour at 40 rpm followed by washing the gels in water and keeping them in water overnight. Afterwards, photos of the gels were taken for analysis.

## 2.8 Bioactivity testing

An evaluation of the bioactivity of the freeze-dried hydrolysates made from muscle, skin and bone was performed. Three assays were made to test for antiproliferative, antioxidant and anti-inflammatory properties. The test concentration for each assay was determined based on a literature review and consideration that crude hydrolysates were tested. A preliminary concentration test of three hydrolysates per material at several concentrations (0.1, 1, 10 and 100 µg/mL) was done for the antioxidant assay; and a preliminary testing of two hydrolysates per material at several concentrations (25, 50, 100, 250 and 500 µg/mL) was done for the anti-inflammatory assay.

### 2.8.1 Viability assay

An Aqueous One Solution Cell Proliferation assay was performed to evaluate antiproliferative properties of the hydrolysates against human melanoma cells.

**Cell culture maintenance: splitting and seeding**

Human melanoma cells (cell line A2058) were grown at 37°C and 5% $CO_2$. First, cell culture was prepared for seeding. Growth medium (DMEM supplemented with 1% L-alanyl-L-Glutamin, 10% FBS and 0.1% Gentamycin), phosphate buffered saline (PBS) and trypsin were pre-warmed to 37°C. Growth media was removed, and cells were washed with PBS for ca. 1 min followed by trypsinization. Afterwards, cells were resuspended in growth media (ca. 10 mL). Then 1 mL of the cell suspension was pipetted into a new flask and placed into a Heracell VIOS 160i $CO_2$ incubator (Thermo Fisher Scientific, MA, USA) at 37°C and 5% $CO_2$ for culture maintenance and the remaining volume was used for seeding. Cell number in the cell suspension for seeding was estimated by mixing Trypan blue (100 µL) with the cell suspension (100 µL). Bürker counting chamber was used to count cells in 10 µL under microscope (Leica Microsystems GmbH, Germany) followed by calculation of cell suspension volume and volume of media to get the desired cell density (2000 cells per well for 72-hour incubation) and volume for seeding. Cell suspension (100 µL) was added to each well in 10 microtiter plates (Nunclon Delta Surface, Thermo Fisher Scientific). Afterwards, the plates were placed into Heracell VIOS 160i $CO_2$ incubator at 37°C and 5% $CO_2$ for 24 hours.

**Cell Proliferation assay, reading and results evaluation**

Freeze-dried hydrolysates (150 in total) were diluted in distilled water a day before addition and were stored at 4°C; start concentration was 10 mg/mL making the final concentration 1 mg/mL. After 24-hours incubation, the cells were ready for addition of the hydrolysate solutions. First, the cells were inspected under the microscope, growth media was removed and fresh media (90 µL) was added. Then the hydrolysate solutions (tested at 1 mg/mL; 10 µL) were added in triplicate. Growth media was negative control and 10% DMSO was positive control. After the addition, the microtiter plates were incubated for 72 hours at 37°C and 5% $CO_2$.

After 72-hour incubation, Aqueous One Solution (10 µL) was added into each well followed by 1-hour incubation at 37°C and 5% $CO_2$. Then the plates were ready for measurement of absorbance at 485 nm by the DTX 880 Multimode Detector (Beckman Coulter, Brea, California, USA). The results were analyzed in Excel. Percent of survival was calculated as

$$survival\ \% = \frac{(OD\ test\ well - OD\ positive\ control)\ \times 100}{OD\ negative\ control\ - OD\ positive\ control}$$

## 2.8.2  Antioxidant assay

Antioxidant properties of the hydrolysates were analyzed using the oxygen radical absorbance capacity assay.

All reagents were diluted in 75 mM phosphate buffer (pH 7.4), except for freeze-dried hydrolysates, which were diluted in Milli-Q water. The assay was performed using black MaxiSorp™ plates (VWR International, Radnor, USA). The final assay mixture volume was 210 µL. First, the hydrolysate samples were pipetted into the wells (21 µL, start concentration 500 µg/mL) followed by distilled water (4 µL). Wells with Trolox standard (25 µL; standard curve 18 – 12.5 – 6.25 – 3.13 – 1.57  – 0 µM) and blank wells (210 µL phosphate buffer) were prepared as well. Then fluorescein (125 µL, 55 nM) was added into each well. The plates were incubated for 15 min at 37°C. After incubation, AAPH (60 µL, 54 mg/mL) was added and the plates were placed into a plate reader Victor 3 (PerkinElmer, Waltham, MA, USA), which recorded 25 measurements of fluorescence at 1 min intervals. All reaction mixtures were prepared in duplicate. A template in Excel was used to analyze the raw data. First, fluorescence measurements were normalized to the blank measurements. Then the area under the fluorescence curve was calculated. The hydrolysate sample values were expressed as Trolox

equivalents, calculated based on the Trolox standard curve calculated for each plate. Formulas are presented in Appendix A 2.6.

### 2.8.3  Anti-inflammatory assay

Anti-inflammatory activity of the hydrolysates was evaluated based on the anti-inflammatory assay using LPS stimulated human macrophages and measuring the expression of TNF-α. The amount of TNF-α in the samples were determined by ELISA.

**Cell culture: splitting and seeding**

Human monocyte cell line THP-1 was grown at 37 ℃ and 5% $CO_2$ in RPMI media supplemented with 10% FBS (ultralow endotoxin). Cell culture from Nunc Easy flask (Thermo Fisher Scientific) was transferred into 50 mL centrifuge tube and centrifuged for 5 min at 150 g. Afterwards, the old media was removed and fresh pre-warmed (37℃) media (25 mL) was added. Cell density was calculated using Bürker counting chamber: first, cell suspension (50 µL) was mixed with trypan blue (450 µL) and then 10 µL was pipetted onto a counting chamber. Afterwards, volumes of growth media and cell suspension were calculated and mixed together to get cell density of $10^6$ cells per mL. PMA (50 ng/mL) was added to the cell suspension to stimulate differentiation of monocytes into macrophages. Cell suspension (100 µL) was pipetted into separate wells of the microtiter plates (VWR International) and the plates were placed into an incubator at 37℃ and 5% $CO_2$ for 48 hours. After 48-hour incubation, cells were inspected under microscope followed by removal of old media, then cells were washed with pre-warmed PBS and fresh media was added. The plates were placed back into an incubator for another 24 hours.

**Addition of test samples**

After the incubation, cells were ready for addition of the hydrolysates. The freeze-dried hydrolysates were diluted in distilled water to make start concentration at 1 mg/mL, making final concentration at 100 µg/mL. Old media was removed and 80 µL of fresh media was added in the test wells, 90 µL of media was added into the wells for LPS control and 100 µL of media – into the wells with cell control. The hydrolysate solutions (10 µL) were added into the test wells in duplicate and incubated for 1 hour at 37℃ and 5% $CO_2$. Then LPS solution (10 µL; cells were exposed to 10 ng/mL LPS) was added to each well, except for the cell control.

Following a 6-hours incubation at 37℃ and 5% $CO_2$, the plates were stored at -80℃ until analysis with ELISA.

**Evaluation of TNF-α expression with ELISA**

Nunc Maxisorp 96F-well ELISA plates (VWR International) were coated with antibodies 'Anti-Human TNF alpha Purified' diluted to 2 µg/mL in 10 mM TBS pH 7.4. Antibody solution (100 µL) was added into each well followed by overnight incubation at 4°C.

The next day washing buffer, blocking buffer, assay diluent and human TNF-α solution (1000 pg/mL, diluted in assay diluent) were prepared (Appendix A 1: Table 7). The coated plates were washed (4 times) with washing buffer using Aquamax 2000 microplate washer (VWR International) followed by addition of blocking buffer (200 µL) and incubation for 1 hour at room temperature and shaking (300 rpm). The plates were washed again 4 times with washing buffer and were then ready for addition of the test samples from the anti-inflammatory assay plates. The total volume in each well was 100 µL: wells with test samples and LPS control had 1:20 dilution in assay diluent from the respective wells of the anti-inflammatory assay; wells for cell control had 1:2 dilution in assay diluent; wells with TNF-α standard had a human TNF-α dilution series (standard curve 1000 – 500 – 250 – 125 – 62.5 – 31.3 – 15.6 – 0 pg/mL). Then the plates were incubated for 2 hours at room temperature and shaking (300 rpm) followed by washing of the plates by Aquamax 2000.

After the wash, 100 µL of Anti-Human TNF alpha Biotin (diluted in assay diluent to 3 µg/mL) was pipetted into each well and the plates were incubated for 1 hour at room temperature and shaking (300 rpm). The plates were washed by Aquamax 2000. Then 100 µL of ExtrAvidin-Alkaline Phosphatase solution (1:20000 dilution in assay diluent) was added into each well and the plates were incubated for 30 min at room temperature and shaking (300 rpm). After the incubation, the plates were washed by Aquamax 2000 using program 'ELISA soak wash', where plates were soaked for 30 sec in each wash step. Then 100 µL of pNPP substrate solution was added into each well. The plates were incubated for 45 min at room temperature. After the incubations, the plates were placed into DTX880 plate reader (Beckman Coulter) to measure OD values at 405 nm. The results were calculated using the equation for the standard curve in Excel and analyzed using the following cut-off values: Active > 50% inhibition; 40% < Questionable < 50% inhibition and Inactive < 40% inhibition.

## 2.9 Scale-up of the hydrolysis process: theoretical approach

Promising proteases were selected for hydrolysis on a larger scale based on the hydrolysis results of 23 protases. Three enzymes per material were chosen based on the selectivity ratio, the highest hydrolysate yield and the highest hydrolysate yield among non-Tail enzyme (due to economic considerations). The antioxidant activity of the hydrolysates was also taken into consideration. There were enzymes with high antioxidant activity among the selected enzymes for muscle and bone, but not for skin. Therefore, an additional enzyme was chosen for skin, which had high results in antioxidant activity and good hydrolysate yield. The hydrolysis with 250 g of raw material was only planned but not performed.

# 3  Results

The project's focus is selective utilization of myofibrillar proteins and collagen from residual raw material of Atlantic cod heads. The effectiveness of 23 proteases to hydrolyze proteins in Atlantic cod heads (skin, muscle and bones) were evaluated and compared based on yield, weight of sediment, selectivity ratio, average MW and MW distribution of some hydrolysates. The bioactive properties of the hydrolysates were also evaluated by viability, antioxidative and anti-inflammatory assays.

## 3.1  Chemical composition of muscle, skin and bone

The chemical composition of three main materials of Atlantic cod heads is presented in Table 3. As the table shows, skin and muscle had similar proportions of water, dry matter and ash content. As expected, bone had a considerably lower percentage of water and dry matter but a higher percentage of ash content in comparison to skin and muscle. Skin had the highest organic matter content at 20%, while bone had 18.5% and skin only 16%.

*Table 3 – Chemical composition of raw material: skin, muscle and bone. The percentage is an average of quadruplicate and the weight of raw material was adjusted per 5 g for comparison.*

| Material | Water (%) | Dry matter (%) | Ash content (%) | Organic matter* (%) |
|----------|-----------|----------------|-----------------|---------------------|
| **Skin** | 78.60 | 21.40 | 1.26 | 20.14 |
| **Muscle** | 83.11 | 16.89 | 0.92 | 15.96 |
| **Bone** | 47.47 | 52.53 | 34.03 | 18.50 |

* contains mostly protein but also low amounts of lipids and carbohydrates

## 3.2  Yield of enzymatic hydrolysis

Hydrolysate yield from three types of material is presented in Figure 16. The hydrolysates made from muscle had higher *hydrolysate yield* compared to the hydrolysates produced from skin or bone; and the hydrolysates from bones had the lowest yield (based on the *total yield* hydrolysates from skin had the highest yield, see Appendix A 3). The weight of freeze-dried hydrolysate, total yield and hydrolysate yield can be found in Appendix 3 (for muscle Table 10, skin Table 11 and bone Table 12). The theoretical maximum hydrolysate yield for the three types of material was calculated to 79.4% for muscle, 41.6% for skin and 32.8% for bone respectively.

Among the hydrolysates from muscle, the highest result was 50% made by Tail 191 and the lowest result was 20.3% (Flavourzyme). Three hydrolysates with the highest yield were very close to each other (50%, 48.6% and 47.6%) and the gap between the third place and the fourth was 8.5%. When sorted based on percentage of yield, all 20 hydrolysates starting from the fourth place had small differences between two adjacent positions (from 0.08% to 2.7%).

Among the hydrolysates made from skin, the highest hydrolysate yield was 29.5% (Tail 194) and the lowest yield was 6.1% (Endocut 01). When sorted according to the yield percentages, no big gaps (0.01-2.45%) were observed between two adjacent positions among the top 20 hydrolysates. The difference between position 20 (Promod 144GL) and 21 (Veron L10) was 5.5% and between 22 (Bromelain) and 23 (Endocut 01) – 7.8%.

Small variation was observed among the hydrolysates made from bones. The highest hydrolysate yield was 5.4% (Tail 190), while the lowest was 3% (Veron L10). When sorted according to the yield percentage, the difference between the adjacent positions was 0-0.57%.
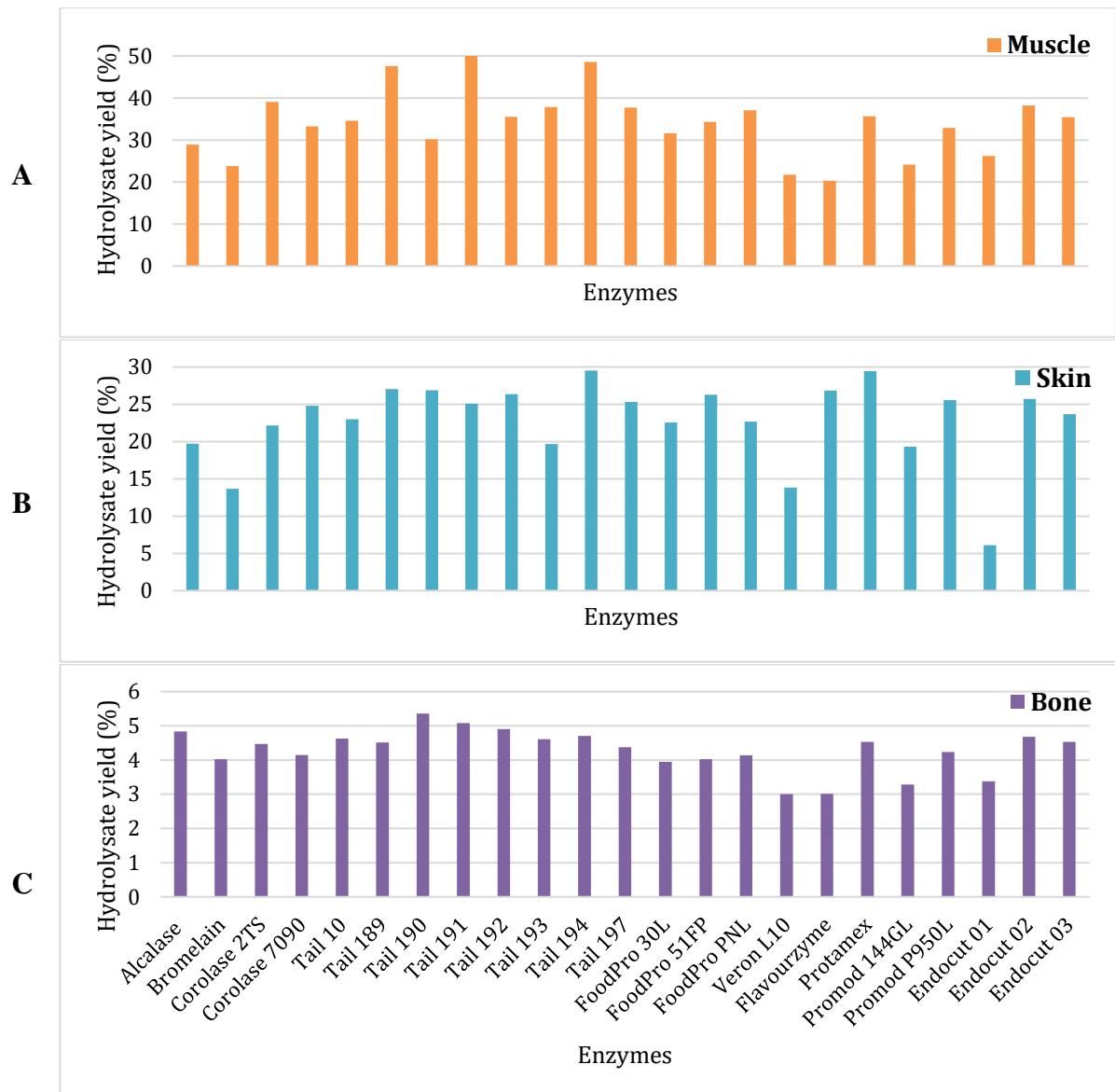
*Figure 16 – Hydrolysate yield for (A) muscle, (B) skin and (C) bone. The amount of protein hydrolyzed by an enzyme; the background reaction\* is subtracted from the total yield. Results are based on duplicates and the weight of raw material was adjusted per 5 g for comparison.*
*\*proteins solubilized in the control reaction (without addition of enzyme).*

## 3.3  Sediment reduction by enzymatic hydrolysis

The amount of sediment reduced by enzymatic treatment in comparison to the control treatment is presented in Figure 17. The highest reduction of sediment was shown by enzymatic hydrolysis of muscle and the lowest – by hydrolysis of bones. The sediment weight is presented in Appendix A 3 (Table 10, Table 11, Table 12).

Variation was observed among the sediments from the hydrolysis of muscle: the highest reduction of sediment was 46.3% (Tail 191) and the lowest was 19.9% (Flavourzyme). After hydrolysis of skin sediment reduction varies from 18% (Corolase 2TS) to 7.1% (Veron L10).

Whereas after hydrolysis of bone the sediment reduction varied from 5.9% (Tail 192) to 0.28% (Endocut 01).
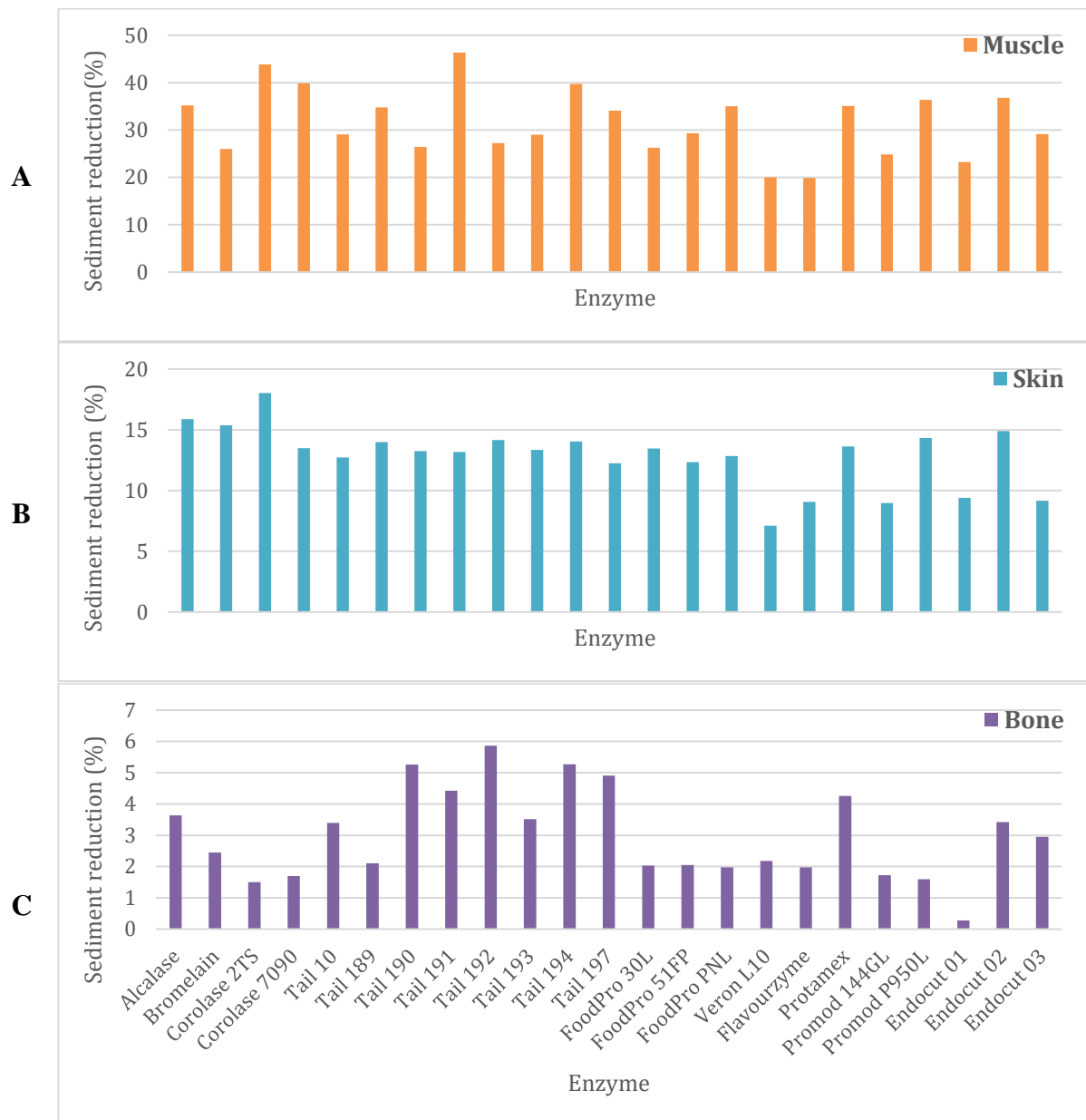


*Figure 17 – Sediment reduction due to enzymatic hydrolysis: (A) muscle, (B) skin, (C) bone. Sediment is a sum of sediment in the tubes after hydrolysis dried at 110℃ until stable weight and residue on the filter paper dried at 62℃ until stable weight. Results are based on duplicates and the weight of raw material was adjusted per 5 g for comparison.*

## 3.4 Selectivity ratio towards collagen and myofibrillar proteins

The selectivity ratio towards collagen and myofibrillar proteins of the proteases (Table 4) was calculated based on the comparison of hydrolysate yields produced from muscle (rich in myofibrillar proteins) and skin (rich in collagen), as well as from muscle and bones (rich in collagen).

The skin/muscle ratio of 22 enzymes was below 1 and the range was from 0.23 to 0.89. Thus, most proteases had selectivity (higher hydrolysate yield) towards myofibrillar proteins and Endocut 01 showed the highest selectivity ratio. Even though Endocut 01 had produced hydrolysates with low yield both from muscle (19th place) and skin (23rd place) (Figure 16), the relative difference between the two yields was the biggest in comparison to other samples. Only Flavourzyme demonstrated selectivity towards collagen (1.32) due to the lowest yield for muscle and 5th highest yield for skin.

The bone/muscle ratio demonstrated that all ratios were below 1, ranging from 0.09 to 0.18. This indicated that all proteases work best on myofibrillar proteins.

*Table 4 – Selectivity ratio of proteases: skin/muscle and bone/muscle.*

| Enzyme | Ratio | |
|---|---|---|
| | Skin/muscle | Bone/muscle |
| Alcalase | 0.68 | 0.17 |
| Bromelain | 0.58 | 0.17 |
| Corolase 2TS | 0.57 | 0.11 |
| Corolase 7090 | 0.75 | 0.12 |
| Tail 10 | 0.67 | 0.13 |
| Tail 189 | 0.57 | 0.09 |
| Tail 190 | 0.89 | 0.18 |
| Tail 191 | 0.50 | 0.10 |
| Tail 192 | 0.74 | 0.14 |
| Tail 193 | 0.52 | 0.12 |
| Tail 194 | 0.61 | 0.10 |
| Tail 197 | 0.67 | 0.12 |
| FoodPro 30L | 0.71 | 0.12 |
| FoodPro 51FP | 0.77 | 0.12 |
| FoodPro PNL | 0.61 | 0.11 |
| Veron L10 | 0.64 | 0.14 |
| Flavourzyme | 1.32 | 0.15 |
| Protamex | 0.83 | 0.13 |
| Promod 144GL | 0.80 | 0.14 |
| Promod P950L | 0.78 | 0.13 |
| Endocut 01 | 0.23 | 0.13 |
| Endocut 02 | 0.67 | 0.12 |
| Endocut 03 | 0.67 | 0.13 |

## 3.5 Molecular weight of the hydrolysates

**Average molecular weight determined by SEC**

Molecular weight of the peptides in the hydrolysates were determined by SEC. As Figure 18 shows, the average MW of hydrolysates produced both from muscle and from bone were lower than the average MW of hydrolysates from skin.

Average MW of hydrolysates produced from muscle ranged from 3564 Da (Flavourzyme) to 954 Da (Tail 189). When the hydrolysates were sorted according to their average MW, the hydrolysate produced by Flavourzyme stood out due to a big difference in comparison to the adjacent hydrolysate produced by Endocut 03 (2348 Da).

Average MW of the hydrolysates produced from skin ranged from 13037 Da (Veron L10) to 1198 Da (Tail 189). The hydrolysates produced by Veron L10 and Promod 144GL (11230 Da) stood out from the other hydrolysates as having peptide with high average MW. The hydrolysate that had the next highest average MW was made by Flavourzyme (5803 Da).

Average MW of the hydrolysates produced from bones ranged from 9731 Da (Veron L10) to 1065 Da (Tail 189). When the hydrolysates were sorted according to their average MW, the hydrolysates produced by Veron L10 and Promod 144GL (6111 Da) differed from the other hydrolysates. Both had considerably higher average MW, than the hydrolysate with the third highest average MW of 2353 Da (Corolase 2TS).

The average MW of protein fragments produced by control treatment was also determined by SEC. Protein fragments in the control sample from skin had the highest average MW (77309 Da) in comparison to the control samples from muscle and bone; protein fragments in the control sample from muscle had the lowest average MW (17544 Da); whereas protein fragments in the control sample from bone had average MW of 39059 Da. The molecular weight distribution of the fragments are presented in Appendix A 4 (muscle Table 16, skin Table 17 and bone Table 18).
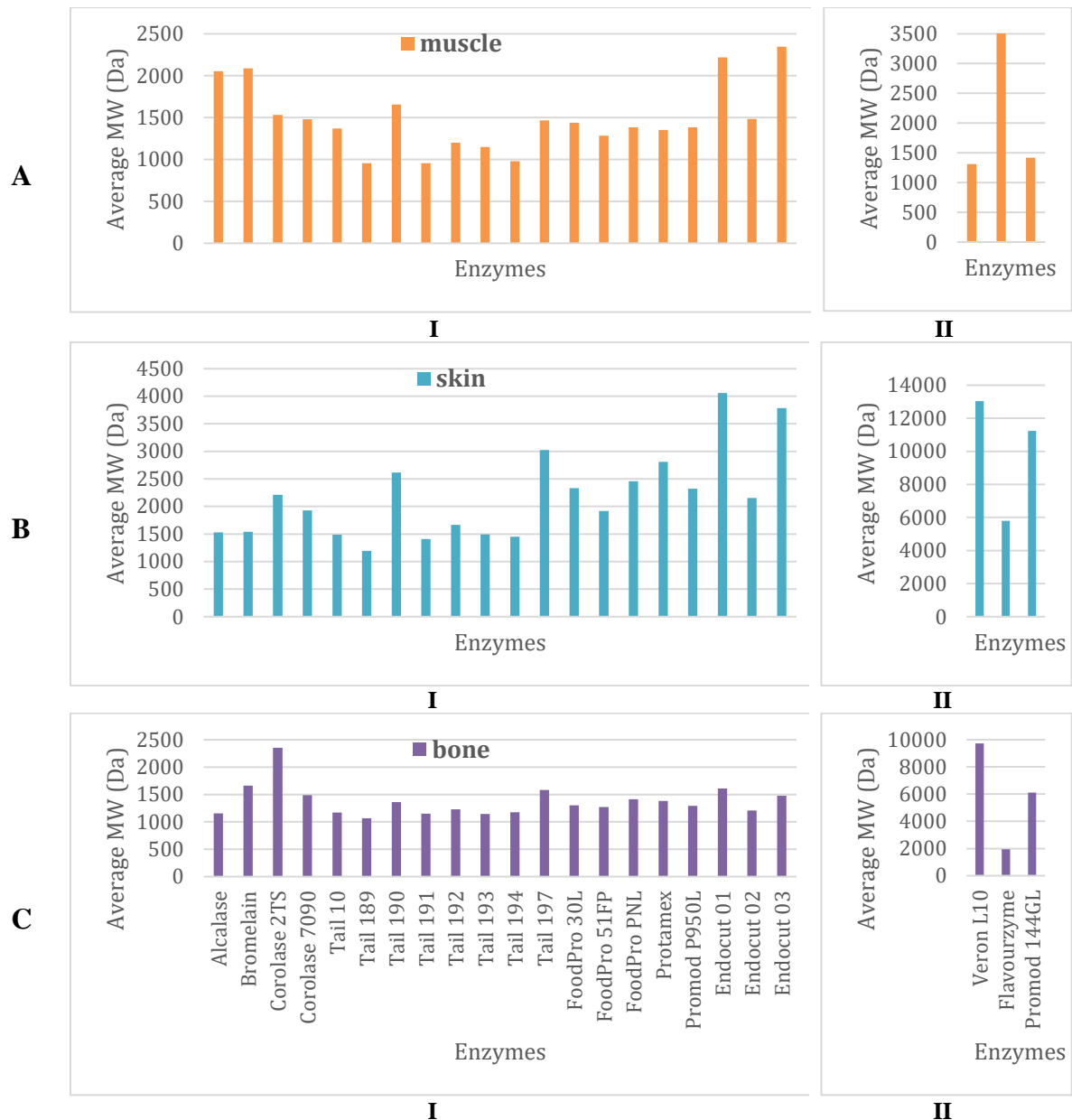
*Figure 18 – Average molecular weight of peptides determined by SEC: (A) muscle (B) skin, (C) bone; (I) 20 enzymes, (II) 3 enzymes that produced hydrolysates with higher average MW than others. Peptides are produced by 23 enzymes from muscle, skin and bone of cod heads (control treatments without addition of enzyme are not present due to high MW values). Results are based on duplicates.*

**Molecular weight distribution of some hydrolysates determined by SEC**

Molecular weight distribution of the following hydrolysates was analyzed: the hydrolysates with the highest yield, the hydrolysates with the highest yield among non-Tail enzymes (due to economic considerations), and the hydrolysates with the highest and the lowest average MW.

The MW distribution of some hydrolysates from muscle are presented in Appendix A 4, Table 13. Comparison of the hydrolysate with the highest yield (Tail 191) and hydrolysate with the lowest average MW (Tail 189) showed rather similar MW distribution of the peptides: the hydrolysate made by Tail 189 had slightly over 80% of the fragments with MW under 1000

Da, while the hydrolysate made by Tail 191 had slightly under 80%. The hydrolysate produced by Corolase 2TS (the highest yield among not Tail enzymes) had only around 60% of the peptides with MW under 1000 Da. The hydrolysate produced by Flavourzyme (with the lowest yield and the highest average MW) also had 60% of the peptides with MW under 1000 Da, however, it also had ~17% fragments with MW around 15000-16000 Da.

The MW distribution of some hydrolysates from skin are presented in Appendix A 4, Table 14. Comparison of the MW distribution of the hydrolysate with the highest yield (Tail 194) and hydrolysate with the lowest average MW (Tail 189) demonstrated relatively similar MW distribution: the hydrolysate made by Tail 189 had almost 80% of the fragments with MW under 1000 Da, while the hydrolysate made by Tail 191 had 70%. The hydrolysate produced by Protamex (the highest yield among not Tail enzymes) had only 24% of the peptides with MW under 1000 Da with the majority peptides (~70%) in the range of 1010 Da – 5100 Da. The hydrolysate produced by Endocut 01 (lowest yield) had only ~20% of the peptides with MW under 1000 Da, most peptides (~67%) had MW in the range from 1020 Da to 5000 Da and ~11% of peptides had MW around 15000 Da. The hydrolysate produced by Veron L10 (the highest average MW) had almost 60% of fragments with MW around 20500 Da and 33% peptides in the range from 1050 Da to 4100 Da.

The MW distribution of some hydrolysates from bone are presented in Appendix A 4, Table 15. Comparison of the MW distribution of the hydrolysate with the highest yield (Tail 190) and hydrolysate with the lowest average MW (Tail 189) showed some similarities: the hydrolysate made by Tail 189 had almost 60% of the fragments with MW under 1000 Da, while the hydrolysate made by Tail 190 had 40%; both hydrolysates had peptides with 1020 Da – 1030 Da (Tail 189: 24% and Tail 190: 31%). The hydrolysate produced by Alcalase (the highest yield among not Tail enzymes) had 78% of peptides with MW under 1003 Da. The hydrolysate produced by Veron L10 (the lowest yield and the highest average MW) had around 50% of peptides with MW of 17100-18000 Da and ~37% of peptides in the range from 1000 Da to 4100 Da. The hydrolysate produced by Flavourzyme (the lowest yield) had 61% of peptides with MW in the range from 1030 Da to 6820 Da and 22% of peptides with MW around 600 Da.

**Molecular weight distribution of the hydrolysates made from skin determined by SDS-PAGE**

The hydrolysates prepared from skin had the highest average MW in comparison to hydrolysates from muscle and bone (Figure 18). SDS-PAGE was done using the hydrolysates and the control treatment made from skin because the SEC conditions used, with the chosen column and calibration proteins (proteins with the highest MW were bovine serum albumin – 66.5 kDa and albumin from chicken egg white – 42.7 kDa), do not separate peptides with high MW into distinct groups.

The SDS-PAGE showed that most of the hydrolysates had similar patterns with three clearly visible bands: one over 200 kDa and two around 100 kDa (Figure 19). The size of the bands corresponded to α chains of collagen type I: $\alpha_1$ and $\alpha_2$ have MW of ~100 kDa; and a β component (dimer) with MW of ~200 kDa. However, three hydrolysates made by Corolase 2TS, Veron L10 and Promod 144GL did not have any visible bands. All hydrolysates showed 'smear' (protein fragments with gradually increasing MW) with different color intensity at different MW; for example, the hydrolysate prepared by Corolase 2TS had 'smear' below 51 kDa, while the hydrolysate prepared by Flavourzyme had 'smear' within the whole lane and had a stronger color intensity. The hydrolysates made by Tail 193, Flavourzyme and Endocut 01 showed several additional bands. The control treatment showed a different pattern in comparison to the hydrolysates; it had the highest color intensity in the top of the lane (above 64 kDa) and had one visible band around 100 kDa.
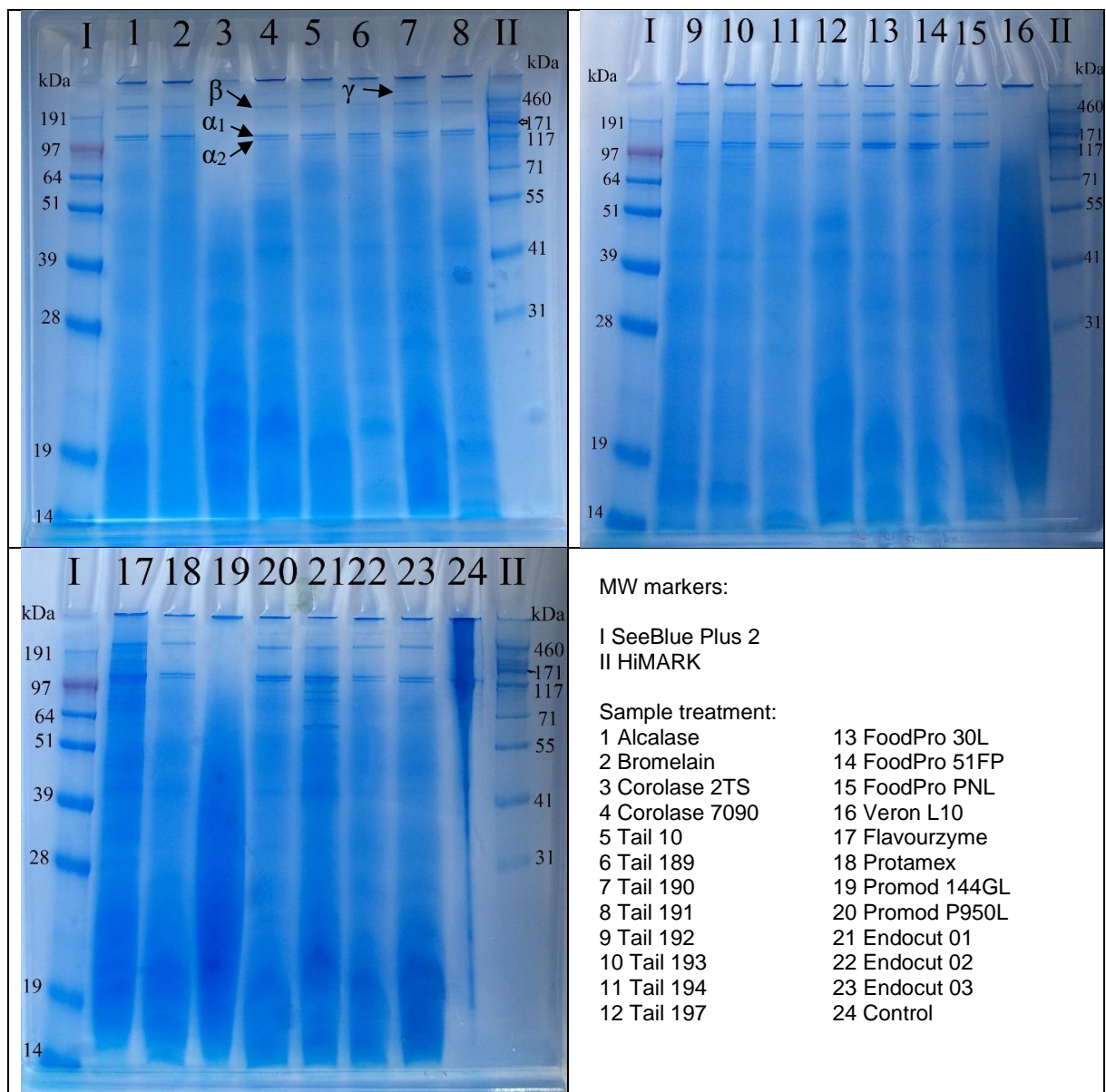
*Figure 19 – SDS-PAGE patterns of fish skin hydrolysates obtained by different protease treatments and control treatment (without addition of enzyme). α-chains, β component, γ component and MW standards from two MW markers (SeeBlue Plus2 and HiMARK) are noted. Electrophoresis was carried out with 12% Bis-Tris gel, MOPS SDS Running buffer and NuPAGE LDS Sample Buffer.*

## 3.6 Bioactivities of the hydrolysates

The hydrolysates' bioactivities were evaluated in three assays: viability, antioxidant and anti-inflammatory.

### 3.6.1 Antiproliferative properties of the hydrolysates

No significant inhibition of human melanoma cells' growth was observed at test concentration of 1 mg/mL (Appendix A 5.3, Table 21). A slight difference was observed among the hydrolysates prepared from muscle, skin and bone. In comparison to the negative control (growth media) hydrolysates from muscle showed a 109-95% survival rate; hydrolysates from skin demonstrated 105-84% and hydrolysates from bone – 113-74%.

### 3.6.2 Antioxidant properties of the hydrolysates

The test concentration for the assay was chosen based on the preliminary testing of several concentrations (results are shown Appendix A 5.1 Table 19). Based on the ORAC assay results at test concentration of 50 µg/mL, the hydrolysates produced from muscle have higher antioxidant activity than the hydrolysates from skin and bone (Figure 20). Antioxidant activity of the protein fractions produced by the control treatment was also evaluated. The control treatment from bone had the highest antioxidant activity (1.15 µM TE) in comparison to skin (0.82 µM TE) and muscle (0.55 µM TE).

Among the hydrolysates made from muscle, the hydrolysate produced by Tail 194 (4.95 µM TE) had the highest activity, while the hydrolysate made by Endocut 01 (2.42 µM TE) demonstrated the lowest activity. Among the hydrolysates prepared from skin, the hydrolysate made by Tail 189 (2.43µM TE) showed the highest antioxidant activity and the hydrolysate prepared by Promod 144GL (0.82µM TE) had the lowest activity. Among the hydrolysate made from bones, the hydrolysate prepared by Alcalase (2.36µM TE) demonstrated the highest antioxidant activity, and the hydrolysate produced by Promod 144GL (1.03µM TE) showed the lowest activity.
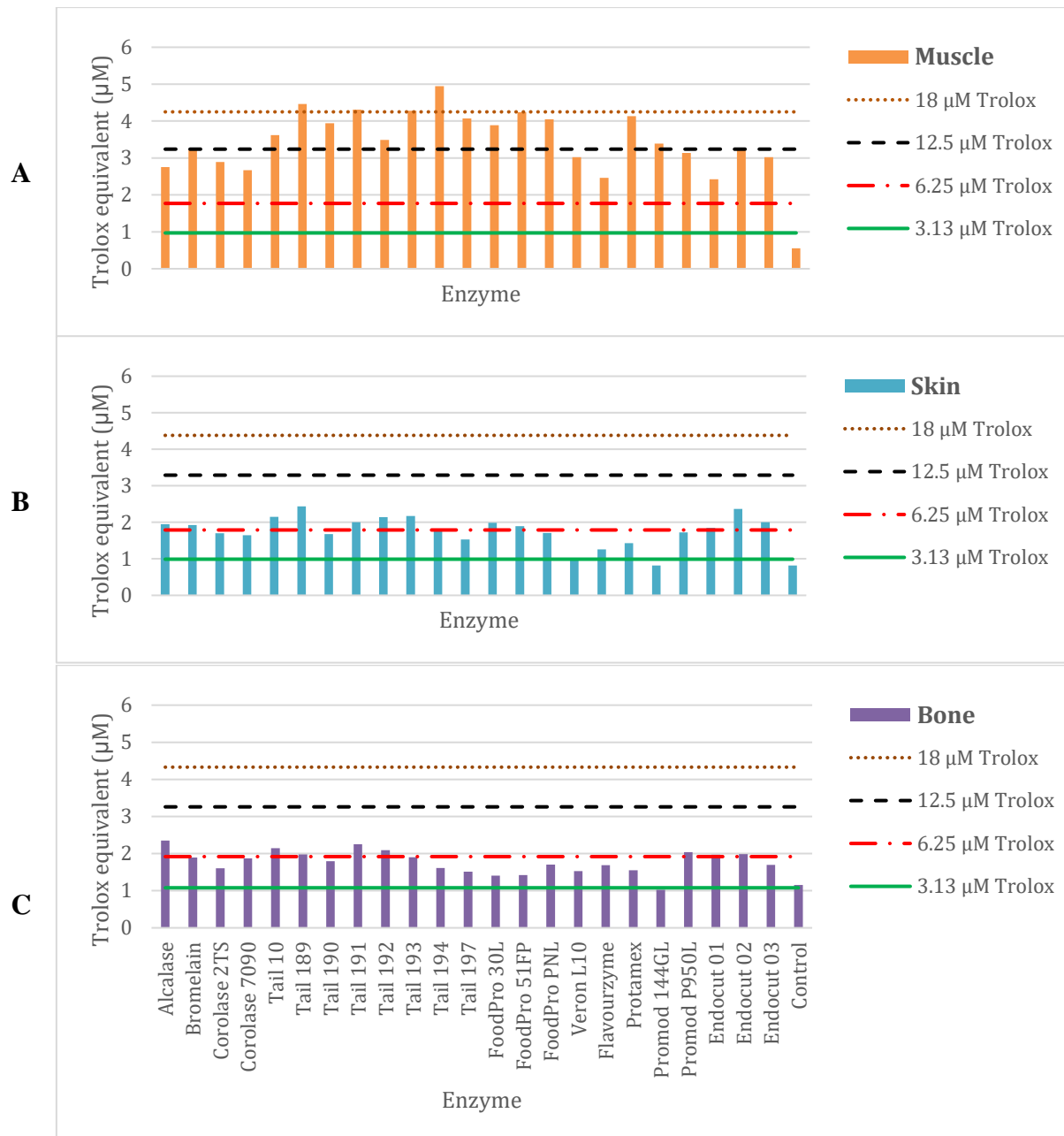
*Figure 20 – Oxygen radical absorbance capacity of hydrolysates produced by 23 enzymes and one control treatment (without addition of enzyme): (A) muscle, (B) skin, (C) bone. Results are expressed as Trolox equivalents (TE). Concentrations of Trolox used to plot standard curve and their equivalents in TE are shown for reference. Test concentration was 50 µg/mL.*

### 3.6.3 Anti-inflammatory properties of the hydrolysates

The test concentration for the assay was chosen based on the preliminary testing of several concentrations (results are shown in Appendix A 5.2, Table 20). The anti-inflammatory properties of the hydrolysates were evaluated based on the expression of TNF-α by LPS stimulated human macrophages. No anti-inflammatory properties were detected at a test concentration of 100 µg/mL.

All hydrolysates from Atlantic cod demonstrated inhibition lower than 30% (Appendix A 5.4, Table 22). The cut-off values for anti-inflammatory activity of a sample were: >50% inhibition – active sample, 40% inhibition – questionable and <30% inhibition – inactive sample. Due to natural variation 20% and -20% inhibition can be observed in wells without any active compounds, thus, interpreted as 0% inhibition.

## 3.7  Suggested method for scale-up of the hydrolysis process

This part gives a theoretical presentation of how the enzymatic hydrolysis process can be scaled up in the lab from 5 g of raw material to 250 g.[1] Based on the results of small-scale hydrolysis (5 g raw material), three enzymes were chosen for a large-scale hydrolysis (250 g raw material). The enzyme #1 was the enzyme chosen based on the selectivity ratio (Table 4), #2 was the enzyme with the highest hydrolysate yield (Figure 16) and #3 was the enzyme with the highest hydrolysate yield among non-Tail enzymes. When looking at the antioxidant activity (Figure 20) of the hydrolysates made by these enzymes, the hydrolysate produced from muscle by Tail 191 had the 3rd highest antioxidant activity, while the hydrolysate from bone made by Alcalase had the highest antioxidant activity among the hydrolysates from bone. However, the selected enzymes for scale-up for skin made hydrolysates with low antioxidant activity; therefore, an additional enzyme was chosen with the 2nd highest results in antioxidant activity and good hydrolysate yield (Table 5).

*Table 5 – Enzymes chosen for scale-up, conditions for the experiment and price range of the enzymes.*

| Raw material | # | Enzyme | Temperature (℃) | pH | Price range* |
|---|---|---|---|---|---|
| **Muscle** | 1 | Endocut 01 | 45 | not adjusted | Low |
| | 2 | Tail 191 | 55 | not adjusted | High |
| | 3 | Corolase 2TS | 65 | not adjusted | Medium |
| **Skin** | 1 | Flavourzyme | 50 | not adjusted | High |
| | 2 | Tail 194 | 60 | not adjusted | High |
| | 3 | Protamex | 60 | not adjusted | Medium |
| | 4 | Endocut 02 | 60 | not adjusted | Low |
| **Bone** | 1 | Flavourzyme | 50 | not adjusted | High |
| | 2 | Tail 190 | 45 | not adjusted | High |
| | 3 | Alcalase | 65 | not adjusted | Low |

*as defined in Table 2

---

[1] The scale-up was not performed in the laboratory due to the restrictions imposed by corona virus outbreak.

The general workflow of this scale-up hydrolysis process (Figure 21) is the same as presented for a smaller scale. The main differences are that hydrolysis will be performed at an optimum temperature of an enzyme and that the raw material/water proportion will be 1:1 (w/w). Additional measurements such as monitoring of the hydrolysis process by SEC at different time points of hydrolysis and determination of ash content in freeze-dried hydrolysates and sediments will be made. The increase of the raw material only up to 250 g still allows to perform the hydrolysis at a laboratory scale, however, some of the equipment will be different.
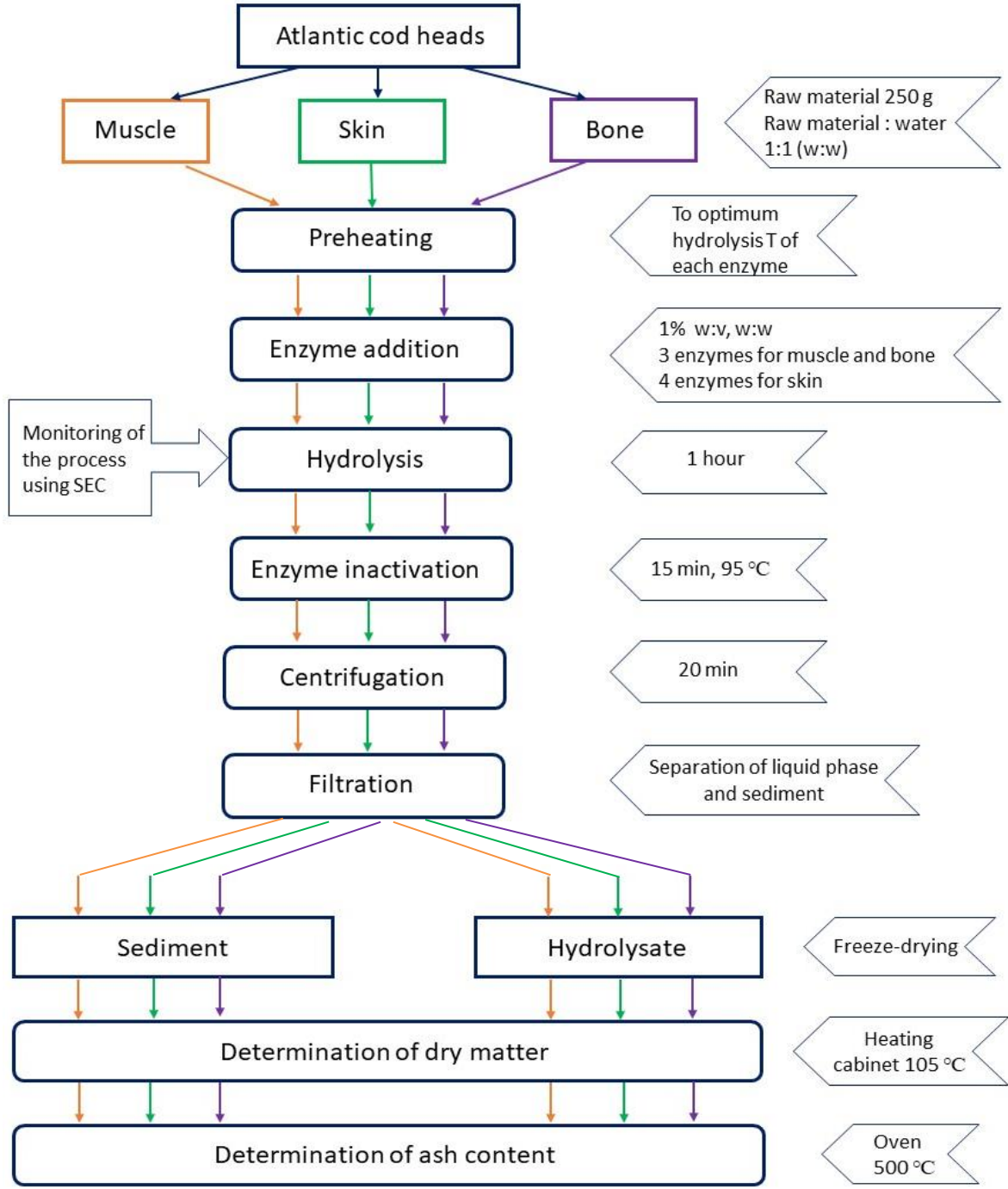


*Figure 21 – Suggested workflow of a hydrolysis process scale-up.*

# 4 Discussion

In this project a small-scale hydrolysis of residual raw material from Atlantic cod heads using 23 proteases was performed. The main aim was to evaluate proteases used to produce hydrolysates from different types of raw material of Atlantic cod heads: muscle, skin and bone. The evaluation was done based on the hydrolysates' yield, weight of remaining sediment, molecular weight of the hydrolysates, selectivity ratio as well as bioactive properties. Based on the evaluation of enzymes, optimum conditions for hydrolysis were suggested for a larger-scale hydrolysis process.

The results showed that the three types of raw material from cod heads had different chemical composition, thus different amount of protein for hydrolysis. The hydrolysis with Tail enzymes produced the highest hydrolysate yield from all types of raw material. Based on the hydrolysis yield and amount of sediment left after hydrolysis, no clear differences were observed in performance of most of the enzymes. The selectivity ratio showed that Flavourzyme was the only enzyme with selectivity towards collagen, while Endocut 01 had the highest selectivity towards myofibrillar proteins. Hydrolysis with different proteases produced hydrolysates with different MW profiles. Notably, Tail 189 produced hydrolysates with the lowest average MW from all types of raw material. In general, the hydrolysates made from muscle had the smallest average MW, while the hydrolysates from skin had the largest average MW. The SDS-PAGE results of the hydrolysates from skin indicated that Tail 193 and Endocut 01 might have selectivity towards peptide bonds they cleave, and that Corolase 2TS might be an effective protease for cod skin hydrolysis. The bioactivity testing demonstrated that the hydrolysates had no antiproliferative or anti-inflammatory activity. However, all hydrolysates demonstrated antioxidant activity. The hydrolysates produced from muscle showed higher antioxidant activity than the hydrolysates prepared from skin and bone. Also, some variation of the activity was observed in the hydrolysates made from the same material but with different enzymes. Suggestions for a larger-scale experiment of raw material include: to use several enzymes, the choice was based on the selectivity ratio, the highest yield among all enzymes and among non-Tail enzymes (due to economic considerations) and antioxidant activity; to use optimum temperature for each individual enzyme; to monitor progression of the hydrolysis process by checking MW of hydrolysates at different time points; and to determine ash content of freeze-dried hydrolysates and sediment.

## 4.1 Evaluation of proteases

### 4.1.1 Material from cod heads and yield after hydrolysis

Atlantic cod head is a complex material, where muscle, skin and bone share a total weight of 80% (Arnesen & Gildberg, 2006). The evaluation of chemical composition of muscle, skin and bone showed that the materials have different characteristics (Table 3). It is also known that the materials have different protein composition (Harnedy & Fitzgerald, 2012) and different proteins' accessibility for the enzymes (Arnesen & Gildberg, 2006; Cui et al., 2007). Chemical composition of muscle found in this project corresponds well with the results for muscle reported by I.-J. Jensen and colleagues (2013) and Shahidi and colleagues (1991). Dry matter and water content found in this project for skin are similar to the values reported by Skierka & Sadowska (2007) and Arnesen & Gildberg (2007). However, the chemical composition of bone determined in this project differed significantly to the chemical composition reported by Toppe and colleagues (2007). This variation can be explained by the difference in treatments of bones prior to analysis; in the work of Toppe and colleagues (2007) bone pretreatment included manual cleaning with knife, boiling to remove remaining muscle and freeze-drying.

**Hydrolysate yield**

Muscle, skin and bone from cod heads were hydrolyzed by 23 proteases under the following conditions: 1% enzyme, 1 hour, 42°C, pH not adjusted. Under these hydrolysis conditions, the highest hydrolysate yield was produced by Tail 191 from muscle, Tail 194 from skin and Tail 190 from bone.

Among the materials (muscle, skin and bone), three general trends were observed: only a slight difference in yield produced by the 23 different proteases tested (Figure 16); only a slight difference in the amount of sediment left after hydrolysis (Figure 17); and different positions (when ranked) of the same samples based on the yield and amount of sediment. These trends might indicate that either the testing methods/conditions used are not accurate enough to determine differences among the performance of proteases or there are no clear differences among the tested proteases. Several possible explanations for the observed results are discussed in the following paragraphs.

One possible explanation is that enzymes work similar in their ability to hydrolyze raw material from cod heads *under the conditions used*. According to the product information of the protases, these conditions are not optimal for 20 out of 23 proteases (Table 2). For example, temperature

during hydrolysis was 42°C, which is lower than optimal for 20 out of 23 proteases. Only Tail 10 (30-65°C), Tail 190 (30-60°C) and Tail 192 (30-55°C) have an optimal temperature range which includes 42°C. However, seven proteases have an optimal temperature range starting from 45°C. Among the remaining proteases, four have an optimum temperature from 50°C, another four from 55°C, three from 60°C and two from 65°C.

Another possible explanation is – *the presence of ash in the freeze-dried hydrolysates*, because some part of ash is water soluble. The amount of ash in each hydrolysate was not determined due to the low weight of the samples. However, it is expected that ash content varies from hydrolysate to hydrolysate even from the same type of raw material, thus causing overestimation of protein content in the hydrolysates. The suggestion that water soluble mineral compounds positively influence the yield of hydrolytes was earlier made by Slizyte and colleagues (2005b). They estimated that freeze-dried hydrolysates prepared from a mixture of backbones and viscera from Atlantic cod contain 10.6% of ash when prepared by Flavourzyme and 9.7% when made by Neutrase (Slizyte et al., 2005b).

An explanation could be that *the exact amount of starting raw material* has a significant influence when the weight is as low as 5 g. Since raw material is washed in water before placement into tubes for hydrolysis, some extra amount of water will negatively influence the weight of the raw material. This results into smaller amount of protein substrate for enzymes. Also, *the determination of sediment weight* included several changes of tare and several weighings, which might have caused some errors in the final results. Results can also be influenced by *activity of endogenous enzymes* (Hayes & McKeon, 2014; Muralidharan et al., 2013) and *protease inhibitors* present in the raw material (Aspevik et al., 2017; Kristinsson & Rasco, 2000).

When comparing the three materials, bone has the lowest hydrolysate yield (Figure 16). The explanation might be that bone has high ash content (~34%), as shown in Table 3, and that the proteins in the bone are not easily accessible for the proteases due to structural characteristics of bones. This has also been pointed out earlier by Arnesen and Gildberg (2006).

A comparison of the theoretical maximum hydrolysate yields calculated for each type of raw material (Section 3.2) and the obtained hydrolysate yields (Figure 16) indicated that not all proteins were extracted from all types of raw materials. Along with not optimal hydrolysis conditions, other possible explanations can be that some proteins were denatured by heating

and denatured proteins are considered to be resistant to enzymatic breakdown; also hydrophobic interactions among peptides cause aggregations, which have reduced accessibility for enzymatic breakdown (Benjakul et al., 2014; Slizyte et al., 2005a). Skipnes and colleagues (2008) determined that the denaturation temperature of collagen in cod skin starts from 32℃, whereas cod muscle denaturation starts from 38℃ for myosin, from 57℃ for sarcoplasmic proteins and from 76℃ for actin. Slizyte and collegues (2005a) studied hydrolysis of cod residual raw materials and reported that the amount of hydrophobic amino acids in non-solubilized sediment fraction is higher than in the protein hydrolysate fractions, which, they suggested, supported the hypothesis that during hydrolysis hydrophobic amino acids are less accessible for enzymatic breakdown due to aggregation.

## 4.1.2  Size of peptides in different hydrolysates

Analysis of average MW (Figure 18) reveals similar tendencies among the hydrolysates produced from all three raw materials. The same enzyme (Tail 189) produced hydrolysates with the lowest average MW from all materials. This observation could indicate that Tail 189 is a universal enzyme with broad specificity. Tail 189 produced hydrolysates with high yield from muscle (2nd) and skin (3rd), but not from bone (11th place). The hydrolysates with the highest MW also had very low yield for all materials. This could indicate that the enzymes have narrow specificity, because proteases have different specificities (Kristinsson & Rasco, 2000), or that the hydrolysis conditions were far from optimum.

In this project, proteases produced peptides with a variety of MW from different raw materials. The average MW of the hydrolysates ranged from 954 Da to 3564 Da for muscle, from 1198 Da to 13037 Da for skin and from 1065 Da to 9731 Da for bone (Figure 18). The variation in average MW of hydrolysates produced by different enzymes was expected, because each protease has its selectivity towards peptide bonds they can cleave (Walsh, 2014b). It is well-known that the MW of peptides influences functional properties (Kristinsson & Rasco, 2000) and bioactive properties (Fernandes, 2016; Jeon et al., 1999). Therefore, the choice of the desired MW of peptides is done based on the desired functionalities of the final product. For example, peptides with antioxidant properties have been reported to have MW in the range <500 Da up to 1500 Da (Kumar et al., 2012), while the emulsifying and foaming properties of peptides increase with the increase of peptides' MW (Slizyte et al., 2009). The MW of the peptides in the hydrolysate depends on the substrate, protease and processing condition (Kristinsson & Rasco, 2000; Pal & Suresh, 2016).

The hydrolysates and control treatment made from skin were further studied using SDS-PAGE because the hydrolysates from skin had the highest average MW in comparison to muscle and bone. SDS-PAGE allowed to analyze the MW distribution of protein fragments with high MW (>14 kDa). The estimated MW of the bonds present in most of the hydrolysates and the control treatment (Figure 19) corresponds well with the MW of α-chains ($\alpha_1$ and $\alpha_2$) of collagen type I (ap. 100 kDa each) and higher MW components, possibly γ component (trimer) and β component (dimer). The structure of collagen type I – $[\alpha_1(I)]_2 \alpha_2(I)$ (Liu et al., 2012) – allows the distinction between α-chains: $\alpha_1$ has two-fold band intensity comparing to $\alpha_2$. These findings are in agreement with previous research on fish skin collagen (J. Chen et al., 2016; Li et al., 2013; Liu et al., 2012; Woo et al., 2008; Yu et al., 2018). Analysis of the SDS-PAGE patterns of the hydrolysates might indicate that proteases Tail 193 and Endocut 01 have selectivity towards peptide bonds they can cleave, because the hydrolysates have several visible bands below 97 kDa (Figure 19). While 'smear' might indicate that a protease has a broad range of peptide bonds it can cleave.

Three hydrolysates made by Corolase 2TS, Veron L10 and Promod 144GL did not have visible bands that corresponds to α-chains. This might indicate that these proteases are more effective than other proteases and that they have cleaved all substrate into peptides; or that they have hydrolyzed only readily available protein fragments dissolved in the water. Corolase 2TS demonstrated the highest reduction of sediment (Figure 17). This observation could indicate that Corolase 2TS might be the most effective out of the enzymes tested, however, Corolase 2TS had low hydrolysate yield (Figure 16). Veron L10 and Promod 144GL produced hydrolysates with low yield and demonstrated low sediment reduction. These observations could indicate that these proteases are not optimal for hydrolysis of cod skin. The difference in color intensity between the hydrolysate made by Corolase 2TS and the hydrolysates made by Veron L10 and Promod 144GL might be explained by the ability of Corolase 2TS to cut substrate into low MW peptides (washed away from the gel), while Veron L10 and Promod 144GL produce more high MW fragments (present in the gel and caused the lane to expand). This observation is supported by the average MW of the hydrolysates determined by SEC, where the hydrolysates made by Veron L10 and Promod 144GL have the highest and second highest average MW respectively (Figure 18).

### 4.1.3 Determination of proteases' selectivity ratio

The selectivity ratio was calculated based on the hydrolysate yield (Table 4). The highest and lowest ratio showed the biggest relative difference in yield between two substrates. The skin/muscle ratio was chosen to determine the enzymes' selectivity towards collagen and myofibrillar proteins. The bone/muscle ratio was not chosen because the limited accessibility of collagen in bone for proteases resulted in all ratios being favorable towards myofibrillar proteins in muscle.

Based on the selectivity ratio, Endocut 01 was identified as the enzyme with the highest selectivity towards myofibrillar proteins. According to the manufacturer's product information, Endocut 01 is an endo-protease with a broad specificity, and is characterized as a protease that efficiently hydrolyzes fish proteins including collagen (Tailorzyme, 2016). Flavourzyme showed the highest selectivity ratio towards collagen. Flavourzyme is characterized by the manufacturer as a protease with exopeptidase as a key activity which cleaves amino acids at N-terminal (Novozymes A/S, 2017).

In general, the skin/muscle selectivity ratio showed that most of the proteases (22 out of 23) had selectivity towards myofibrillar proteins. A possible explanation might be in the nature of proteins, since myofibrillar proteins and collagen have different amino acids composition and different protein structure (accessibility to possible site of peptide bond cleavage) (Parkin, 1993b). Although, some ratios (e.g. Tail 190, Protamex, Promod 144GL) are close to 1, indicating that the activity is similar and independent of the protein nature, which suggests that those proteases have broad activity. Another explanation might be in processing conditions (Parkin, 1993a), because they were not optimal for the majority of the enzymes and may have influenced the performance of enzymes differently. Also, factors that might have influenced the hydrolysate yield (discussed earlier in section 4.1.1) consequently could have influenced the calculated ratios.

The biggest relative difference in yield is one approach to determine selectivity. And the highest yield can be an alternative approach to look at the selectivity of proteases. The highest yield from the three types of raw material was produced by different enzymes, however, all of them were Tail enzymes. This finding suggests that Tail enzymes are efficient enzymes in comparison to other proteases. However, Tail enzymes have high prices per kg (Table 2) and little is known about these enzymes. Therefore, it is also interesting to look at the enzymes with known characteristics and with lower prices, which have the highest yield after Tail enzymes.

## 4.2  Assessment of bioactivity

### 4.2.1  Antiproliferative properties against cancer cells

No significant inhibition of human melanoma cells' growth was observed at test concentration 1 mg/mL (Appendix A 5.3, Table 21). However, antiproliferative activity of protein hydrolysates from Atlantic cod on human breast cancer cell lines has been reported by Picot et al. (2006). The difference can be explained by several factors. The first factor is that different cell lines were used for testing. And different types of cancer cells may vary in cell membrane composition, fluidity and surface area (Song et al., 2011). The second factor is that hydrolysates were prepared by different processing methods; as Hsu et al. (2011) reported, hydrolysis for different time periods resulted in different antiproliferative activity (including no activity) on human breast cancer cell lines. Another factor is batch to batch variation in raw material. Picot et al. (2006) observed antiproliferative activity in three out of five hydrolysates produced from blue whiting (hydrolysates produced by the same method).

A number of research articles report antiproliferative activity of fish protein hydrolytes on different cancer cell lines (Halim et al., 2018; Hsu et al., 2011; Naqash & Nazeer, 2010; Pan et al., 2016; Song et al., 2014; Yaghoubzadeh et al., 2020) where a correlation was observed between antioxidant and anticancer properties: some peptides with antioxidant properties also possess antiproliferative properties (Nwachukwu & Aluko, 2019). Since the hydrolysates demonstrated antioxidative properties in this project several additional factors could have contributed to the negative antiproliferative results against cancer cells. These factors could be: testing of crude hydrolysate and not purified fractions as was done by e.g. Naqash & Nazeer (2010) or Yaghoubzadeh et al. (2020); or low test concentration. In this project test concentration was as high as 1 mg/mL, while the test concentration used by e.g. Song et al. (2011) was ranging from 5 to 40 mg/mL. However, high concentrations of any compound can disrupt the cell functionality and be cytotoxic (Kisitu et al., 2019) (Cronk, 2013), thus giving a false positive result (not caused by a peptide with antiproliferative activity).

As other bioactivity, the antiproliferative properties of protein hydrolysates are considered to be influenced by MW and amino acid composition (Pan et al., 2016). Cationic amino acids are reported to be crucial for the antiproliferative activity of peptides because cationic peptides can interact strongly with the anionic components on cell membranes and thus would disrupt tumor cells (Song et al., 2014).

### 4.2.2 Antioxidant properties

The hydrolysates from all three materials and from the control treatments (without addition of enzyme) demonstrated antioxidant activity (Figure 20). These results are in accordance with previous research (Farvin et al., 2014, 2016; Girgih et al., 2015; Godinho et al., 2016; Jamnik et al., 2017; I. Jensen & Mæhre, 2016; Pampanin et al., 2016; Slizyte et al., 2009). The exact mechanism of antioxidant activity of some peptides is not fully understood, however, there are several hypotheses that try to explain the antioxidant activity of protein hydrolysates (Alemán & Martínez-Alvarez, 2013). These hypotheses can be summarized to:

(1) presence of specific amino acids: such as amino acids with aromatic side chains (tryptophan, tyrosine, phenylalanine, histidine), sulfur-containing side chains (cysteine and methionine), hydrophobic amino acids (valine, leucine, alanine), proline, and lysine (M. Chalamaiah et al., 2012; Chi et al., 2015; Sarmadi & Ismail, 2010)

(2) abundance of specific amino acids, for example glycine and proline in collagen;

(3) position of certain amino acids within the sequence;

(4) hydrophilic and hydrophobic partitioning in the peptide sequence;

(5) molecular weight of the peptides;

(6) peptide conformation (Alemán & Martínez-Alvarez, 2013).

Results show that the hydrolysates from muscle demonstrate higher antioxidant activity than the hydrolysates from skin and bone. The difference can be explained by different amino acid composition in myofibrillar proteins (muscle) and in collagen (skin and bone). A variation in the antioxidant activity among hydrolysates from the same material was also observed. This observation can be explained by the MW of the peptides (longer peptides can fold and bury hydrophobic amino acids inside) and enzyme specificity (where enzyme cuts and which amino acids are exposed). The results also show that hydrolyzed protein have higher activity than non-hydrolyzed (control treatment). This observation can be explained by exposure of some amino acids in peptides, which are usually packed inside proteins (Elias et al., 2008).

### 4.2.3 Anti-inflammatory properties

When the anti-inflammatory properties of the hydrolysates were evaluated, no significant decrease in TNF-α expression by LPS stimulated human macrophages was observed at test concentration of 100 µg/mL (Appendix A 5.4, Table 22). This result corresponds well with the results obtained by Halldorsdottir et al. (2014), who also did not observe an anti-inflammatory effect in hydrolysates produced from white muscle of Atlantic cod. However, anti-

inflammatory peptides have been identified and purified from different protein sources (Ahn et al., 2015) including fish: salmon (Ahn et al., 2012), tuna (Cheng et al., 2015), sandfish (Jang et al., 2017) and Argentine croaker (Rocha et al., 2018). Studies have identified amino acids that have anti-inflammatory properties: glycine, histidine, cysteine, glutamine and tryptophan (Joshi et al., 2016).

No observed decrease in production of proinflammatory cytokines in the present study cannot exclude a hypothesis that there were one or several peptides with anti-inflammatory properties in the testing samples or that it is possible to produce anti-inflammatory peptides from Atlantic cod. Besides the amino acid composition of the raw material, several factors can influence the result such as processing method (enzyme, temperature, pre-treatment, duration of hydrolysis, pH, etc.); the test concentration of hydrolysates used in the assay; or testing of crude hydrolysate instead of fractioned hydrolysates.

**Suggested further experiments**

Based on the results and their evaluation, some follow up experiments can be suggested. Protein hydrolysates produced in this project were aimed for human consumption; thus, negative results in viability assay against cancer cell line became a good indication that the hydrolysates are not cytotoxic. However, a viability assay against non-cancerous human cell line should be performed. Antioxidant activity evaluated by ORAC assay was performed using crude hydrolysates. Crude hydrolysate is a complex mixture containing peptides of various size; therefore, the next step could be to fractionate hydrolysates based on the MW and test these fractions to identify MW of peptides with the highest activity. Since the antioxidant compound can have different mechanisms of action (Sveinsdottir et al., 2014), it could also be interesting to perform other antioxidant assays that detect other antioxidative mechanisms, e.g. ferrozine assay (measures metal-chelating capacity). ORAC is a chemical based assay, while cell-based assays are considered to be more biologically relevant because they can "address some issues of uptake, distribution, and metabolism" (Wolfe & Rui, 2007) of a test compound. Therefore, it is relevant to verify the antioxidant potential of the hydrolysates using a cell-based assay such as cellular antioxidant activity (CAA) assay.

## 4.3 Suggested scale-up process

This project was a pilot project using small amounts of material from cod heads to evaluate different enzymes. The reason for doing it this way was to keep the amount of material and enzymes as low as possible to save money and time. This is very often a general approach (K.-K. Cheng et al., 2007; Neubauer et al., 2013) before suggesting an upscaling process in a research lab or in an industrial context. In this project, experiments were performed using 5 g of raw material in each reaction, and these small-scale experiments made the basis for suggestions for an upscaling to 250 g. The pilot experiment is used to document which enzymes to choose for an upscaled process.

Two main points can be used to explain the need to perform a larger scale lab experiment: (1) a deeper understanding of the process, and (2) economic considerations connected with the highest yield and prices of the enzymes. Both points are crucial according to the theory of scale-up processes published by Shinnar (2004).

The choice of enzymes for an upscaled hydrolysis was based on considerations of selectivity ratio, hydrolysate yield, bioactivity and economic considerations. The results made it difficult to choose one enzyme per material. Therefore, three enzymes were chosen for muscle and bone, and four enzymes for skin (Table 5). Initially, it was planned to use enzymes for a scale-up based only on selectivity ratio. However, the selectivity ratio revealed that enzymes selective only to collagen or myofibrillar proteins have relatively low hydrolysate yield. Therefore, the decision was made to increase the number of enzymes proposed for a scale-up. Notably, Tail enzymes were observed to produce the highest yield from different types of raw material. However, the chosen Tail enzymes have high prices per kg (Table 2), and price is also an important factor when economic viability of the project is assessed. Therefore, Alcalase, Protamex and Corolase 2TS, which are often used for hydrolysis of food proteins (Ahn et al., 2014; Meinlschmidt et al., 2016; Merz et al., 2016), and which also have lower prices and demonstrated high hydrolysate yield in this project, were also chosen for a scale-up. The enzymes selected for a scale-up for muscle and bone, based on the hydrolysate yield and the selectivity ratio, also showed high results in the antioxidant assay. However, the enzymes selected for skin based on the same criteria did not have high antioxidant results. Therefore, an additional enzyme (Endocut 02) was chosen, which produced the hydrolysate with high result in antioxidant assay and have low price per kg. Thus, larger scale experiment is intended to help chose one enzyme per material.

An overall aim is a hydrolysis process that is economically viable. Yield has high importance in this connection; therefore, enzymes should be tested at their optimum temperature to work at their optimum activity. However, pH will not be adjusted because pH adjustment with acid or base might result in high levels of salt in the final hydrolysate reducing the nutritional value of the product (Aspevik et al., 2017). The state of proteins in the raw material is influenced by the temperature (Skipnes et al., 2008), which might influence the enzymes' accessibility to the substrate (Slizyte et al., 2005a). Therefore, any possible reduction in yield has to be identified at a laboratory scale.

For several reasons it is expected that a scale-up from 5 g to 250 g will allow a more accurate and detailed understanding of the process. First, a 50-fold increase in the amount of raw material will result in a more homogenous sample, which will make the yield less influenced by any small variations in each sample. Second, a more nuanced understanding of the process will be achieved by monitoring the hydrolysis process at different timepoints, determination of ash content in freeze-dried hydrolysates and determination of dry matter as well as ash content of the sediment (Figure 21).

**Suggested further experiments**

Additional experiments can be considered to get a better understanding of the hydrolysis process and further analyze the hydrolysates. It could be interesting to investigate the proteases used for hydrolysis and identify why Tail enzymes produced hydrolysates with higher yield from all three types of raw material. A characterization of proteases could be done by measurement of protease activity, for example using azo-casein assay. Another option is to determine the composition of the enzymes' preparations by e.g. mass spectrometric analysis. Also, determination of collagen content in the hydrolysates could be done to identify the purity of the raw materials. This could be done by determination of L-hydroxyproline concentration. An alternative to SEC monitoring of the hydrolysis process could be a Fourier transform infrared (FTIR) spectroscopy. An advantage of FTIR is shorter analysis time in comparison to SEC, which allows for near real-time monitoring of the hydrolysis process (Vang et al., 2018). Protein hydrolysates are being developed for human consumption; therefore, the sensory properties of the products are important and could be evaluated by for example a sensory panel.

# 5 Conclusion

The hydrolysates produced by 23 proteases from different materials of Atlantic cod heads (muscle, skin and bone) were evaluated and compared based on several criteria such as yield, selectivity towards different substrates, MW and bioactive properties. Further, based on the results, conditions for a scale-up experiment (from 5 g of raw material to 250 g) were proposed. The hydrolysate yield and the amount of sediment left after hydrolysis revealed that there were no clear differences among the majority of hydrolysates produced from the same type of material. The highest hydrolysate yield was produced by Tail enzymes from all types of material: from muscle by Tail 191, from skin by Tail 194, and from bone by Tail 190. As expected, different proteases produced hydrolysates with different average MW. However, the same enzyme (Tail 189) produced hydrolysates with the lowest average MW from all three types of raw material. Type of material also contributed to the variation in average MW; the average MW of hydrolysates produced from skin was higher than the average MW of hydrolysates from muscle and from bone. The SDS-PAGE patterns' analysis of the hydrolysates from skin indicated that Tail 193 and Endocut 01 might have selectivity towards peptide bonds they cleave. Another observation from SDS-PAGE patterns was that Corolase 2TS might be the most effective protease for cod skin hydrolysis out of the proteases tested. The selectivity of the proteases towards collagen or myofibrillar proteins were determined based on the yield ratio, which showed that only Flavourzyme demonstrated selectivity towards collagen, and that Endocut 01 had the highest selectivity towards myofibrillar proteins. The bioactivity assays showed that the hydrolysates did not have antiproliferative or anti-inflammatory properties under the conditions used; however, antioxidant properties were observed in all hydrolysates. The suggestions for a scale-up experiment include recommendation of several enzymes per material based on hydrolysate yield, selectivity ratio, antioxidative properties and consideration of price; hydrolysis conditions (optimal temperature and not adjustment of pH); monitoring the progression of hydrolysis by taking test samples for evaluation of MW distribution and determination of the amount of protein content and ash content in the product and sediment.

# Works cited

Acosta-Pavas, J. C., & Ruiz-Colorado, A. A. (2020). Approximation of Scale-Up of Enzymatic Hydrolysis Process from Phenomenological-Based Semiphysical Model and Control Theory Tools. *Industrial & Engineering Chemistry Research*, *59*, 8046–8065. https://doi.org/10.1021/acs.iecr.9b06912

Adler-Nissen, J. (1993). Proteases. In T. Nagodawithana & G. Reed (Eds.), *Enzymes in Food Processing* (3rd ed., pp. 148–204). Academic Press.

Ahmad, T. B., Rudd, D., Kotiw, M., Liu, L., & Benkendorff, K. (2019). Correlation between Fatty Acid Profile and Anti-Inflammatory Activity in Common Australian Seafood by-Products. *Marine Drugs*, *17*(155), 1–20. https://doi.org/10.3390/md17030155

Ahn, C.-B., Cho, Y.-S., & Je, J.-Y. (2015). Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate. *Food Chemistry*, *168*, 151–156. https://doi.org/10.1016/j.foodchem.2014.05.112

Ahn, C.-B., Je, J.-Y., & Cho, Y.-S. (2012). Antioxidant and anti-inflammatory peptide fraction from salmon byproduct protein hydrolysates by peptic hydrolysis. *Food Research International*, *49*(1), 92–98. https://doi.org/10.1016/j.foodres.2012.08.002

Ahn, C.-B., Kim, J.-G., & Je, J.-Y. (2014). Purification and antioxidant properties of octapeptide from salmon byproduct protein hydrolysate by gastrointestinal digestion. *Food Chemistry*, *147*, 78–83. https://doi.org/10.1016/j.foodchem.2013.09.136

Alberts, B., Johnson, A. D., Lewis, J., Morgan, D., Raff, M., Roberts, K., & Walter, P. (2015). *Molecular Biology of the Cell* (6th ed.). Garland Science.

Alemán, A., & Martínez-Alvarez, O. (2013). Marine collagen as a source of bioactive molecules. A review. *The Natural Products Journal*, *3*(2), 105–114.

Arnesen, J. A., & Gildberg, A. (2006). Extraction of muscle proteins and gelatine from cod head. *Process Biochemistry*, *41*, 697–700. https://doi.org/10.1016/j.procbio.2005.09.001

Arnesen, J. A., & Gildberg, A. (2007). Extraction and characterisation of gelatine from Atlantic salmon (*Salmo salar*) skin. *Bioresource Technology*, *98*(1), 53–57. https://doi.org/10.1016/j.biortech.2005.11.021

Aspevik, T., Oterhals, Å., Rønning, S. B., Altintzoglou, T., Wubshet, S. G., Gildberg, A., Afseth, N. K., Whitaker, R. D., & Lindberg, D. (2017). Valorization of Proteins from Co- and By-Products from the Fish and Meat Industry. In C. S. K. Lin (Ed.), *Chemistry and Chemical Technologies in Waste Valorization* (pp. 123–150). Springer. https://doi.org/10.1007/978-3-319-90653-9

Benjakul, S., Yarnpakdee, S., Senphan, T., Halldorsdottir, S. M., & Kristinsson, H. G. (2014). Fish protein hydrolysates: production, bioactivities, and applications. In H. G. Kristinsson (Ed.), *Antioxidants and Functional Components in Aquatic Foods* (1st ed., pp. 237–282). Wiley Blackwell.

Bio-Rad Laboratories. (2017). *A Guide to Polyacrylamide Gel Electrophoresis and Detection* (pp. 1–92).

Biosciences. (n.d.). *Cytokine ELISA Protocol*. Retrieved April 12, 2020, from https://www.bdbiosciences.com/us/applications/s/cytokineelisa

Böcker, U., Wubshet, S. G., Lindberg, D., & Afseth, N. K. (2017). Fourier-transform infrared spectroscopy for characterization of protein chain reductions in enzymatic reactions. *Analyst*, *142*, 2812–2818. https://doi.org/10.1039/c7an00488e

Boster Biological Technology. (n.d.). *What is ELISA?* Retrieved April 12, 2020, from https://www.bosterbio.com/protocol-and-troubleshooting/elisa-principle

Burton, D., & Burton, M. (2018). The integument. In D. Burton & M. Burton (Eds.), *Essential Fish Biology: Diversity, Structure and Function* (pp. 27–46). Oxford University Press.

Cancre, I., Ravallec, R., Wormhoudt, A. Van, Stenberg, E., Gildberg, A., & Gal, Y. Le. (1999). Secretagogues and Growth Factors in Fish and Crustacean Protein Hydrolysates. *Marine Biotechnology*, *1*(5), 489–494.

Chalamaiah, M., Dinesh, B., Hemalatha, R., Jyothirmayi, T., Dinesh Kumar, B., Hemalatha, R., Jyothirmayi, T., Dinesh, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. *Food Chemistry*, *135*(4), 3020–3038. https://doi.org/10.1016/j.foodchem.2012.06.100

Chalamaiah, Meram, Yu, W., & Wu, J. (2018). Immunomodulatory and anticancer protein hydrolysates (peptides) from food proteins: A review. *Food Chemistry*, *245*, 205–222. https://doi.org/10.1016/j.foodchem.2017.10.087

Chen, J., Li, L., Yi, R., Xu, N., Gao, R., & Hong, B. (2016). Extraction and characterization of acid-soluble collagen from scales and skin of tilapia (*Oreochromis niloticus*). *LWT - Food Science and Technology*, *66*, 453–459. https://doi.org/10.1016/j.lwt.2015.10.070

Chen, Y., Xie, W., Qu, C., Zheng, J., Jin, S., Li, H., Li, H., Jin, G., Meng, F., & Jin, D. (2019). Immunoenhancement of dried cod skin collagen: Oligo-peptides on cyclophosphamide-induced immunosuppression in mice. *International Journal of Clinical and Experimental Medicine*, *12*(6), 7047–7055.

Cheng, K.-K., Zhang, J.-A., Liu, D.-H., Sun, Y., Liu, H., Yang, M.-D., & Xu, J.-M. (2007). Pilot-scale production of 1,3-propanediol using Klebsiella pneumoniae. *Process Biochemistry*, *42*, 740–744. https://doi.org/10.1016/j.procbio.2007.01.001

Cheng, M.-L., Wang, H.-C., Hsu, K.-C., & Hwang, J.-S. (2015). Anti-inflammatory peptides from enzymatic hydrolysates of tuna cooking juice. *Food and Agricultural Immunology*, *26*(6), 770–781. https://doi.org/10.1080/09540105.2015.1036352

Cheung, R. C. F., Ng, T. B., & Wong, J. H. (2015). Marine Peptides: Bioactivities and Applications. *Marine Drugs*, *13*, 4006–4043. https://doi.org/10.3390/md13074006

Chi, C., Wang, B., Wang, Y., & Zhang, B. (2015). Isolation and characterization of three antioxidant peptides from protein hydrolysate of bluefin leatherjacket (*Navodon septentrionalis*) heads. *Journal of Functional Foods*, *12*, 1–10. https://doi.org/10.1016/j.jff.2014.10.027

Cicero, A. F. G., Fogacci, F., & Colletti, A. (2017). Potential role of bioactive peptides in prevention and treatment of chronic diseases: a narrative review. *British Journal of Pharmacology*, *174*, 1378–1394. https://doi.org/10.1111/bph.13608

Clark, D. P., & Pazdernik, N. J. (2016). Proteomics. In D. P. Clark & N. J. Pazdernik (Eds.), *Biotechnology* (2nd ed., pp. 295–333). Elsevier Inc. https://doi.org/10.1016/B978-0-12-385015-7.00009-0

Cronk, D. (2013). High-throughput screening. In R. Hill & H. Rang (Eds.), *Drug Discovery and Development: Technology in Transition* (Second, pp. 95–117). Elsevier Ltd. https://doi.org/10.1016/B978-0-7020-4299-7.00008-1

Crowther, J. R. (2001). *The ELISA Guidebook*. Humana Press.

Cui, F. Z., Li, Y., & Ge, J. (2007). Self-assembly of mineralized collagen composites. *Materials Science and Engineering R: Reports*, *57*(1–6), 1–27. https://doi.org/10.1016/j.mser.2007.04.001

Dale, H. F., Jensen, C., Hausken, T., Lied, E., Hatlebakk, J. G., Brønstad, I., Lihaug Hoff, D. A., & Lied, G. A. (2018). Effect of a cod protein hydrolysate on postprandial glucose metabolism in healthy subjects: a double-blind cross-over trial. *Journal of Nutritional Science*, *7*(33), 1–9. https://doi.org/10.1017/jns.2018.23

Dragnes, B. T., Stormo, S. K., Larsen, R., Ernstsen, H. H., & Elvevoll, E. O. (2009). Utilisation of fish industry residuals: Screening the taurine concentration and angiotensin converting enzyme inhibition potential in cod and salmon. *Journal of Food Composition*

*and Analysis*, *22*, 714–717. https://doi.org/10.1016/j.jfca.2009.01.020

Ehly, M., Gemperline, P. J., Nordon, A., Littlejohn, D., Basford, J. K., & Cecco, M. De. (2007). Scale-up of batch kinetic models. *Analytica Chimica Acta*, *595*, 80–88. https://doi.org/10.1016/j.aca.2007.02.040

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant Activity of Proteins and Peptides. *Critical Reviews in Food Science and Nutrition*, *48*(5), 430–441. https://doi.org/10.1080/10408390701425615

EU Parliament, & Council of the EU. (2009). *REGULATION (EC) No 1069/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL (Animal by-products Regulation)* (1069/2009; Issue October).

FAO. (2018). *The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals.* http://www.fao.org/3/i9540en/i9540en.pdf

Farvin, K. H. S., Andersen, L. L., Otte, J., Nielsen, H. H., Jessen, F., & Jacobsen, C. (2016). Antioxidant activity of cod (*Gadus morhua*) protein hydrolysates: Fractionation and characterisation of peptide fractions. *Food Chemistry*, *204*, 409–419. https://doi.org/10.1016/j.foodchem.2016.02.145

Farvin, K. H. S., Lystbæk, L., Hauch, H., Jacobsen, C., Jakobsen, G., Johansson, I., & Jessen, F. (2014). Antioxidant activity of Cod (*Gadus morhua*) protein hydrolysates: In vitro assays and evaluation in 5 % fish oil-in-water emulsion. *Food Chemistry*, *149*, 326–334. https://doi.org/10.1016/j.foodchem.2013.03.075

Fernandes, P. (2016). Enzymes in Fish and Seafood Processing. *Frontiers in Bioengineering and Biotechnology*, *4*(July), 1–14. https://doi.org/10.3389/fbioe.2016.00059

Fouchereau-Peron, M., Duvail, L., Michel, C., Gildberg, A., Batista, I., & Gal, Y. (1999). Isolation of an acid fraction from a fish protein hydrolysate with a calcitonin-gene-related-peptide-like biological activity. *Biotechnology and Applied Biochemistry*, *29*, 87–92.

Gildberg, A. (1993). Enzymic Processing of Marine Raw Materials. *Process Biochemistry*, *28*, 1–15.

Gildberg, A., Arnesen, J. A., & Carlehog, M. (2002). Utilisation of cod backbone by biochemical fractionation. *Process Biochemistry*, *38*, 475–480.

Girgih, A. T., He, R., Hasan, F. M., Udenigwe, C. C., Gill, T. A., & Aluko, R. E. (2015). Evaluation of the in vitro antioxidant properties of a cod (*Gadus morhua*) protein hydrolysate and peptide fractions. *Food Chemistry*, *173*, 652–659. https://doi.org/10.1016/j.foodchem.2014.10.079

Glazer, A. N. (1988). Fluorescence-based species: assay for reactive oxygen a protective role for creatinine. *The FASEB Journal*, *2*(9), 2487–2491. doi.org/10.1096/fasebj.2.9.3371593

Godinho, I., Pires, C., Pedro, S., Teixeira, B., Mendes, R., Nunes, M. L., & Batista, I. (2016). Antioxidant Properties of Fish Protein Hydrolysates Prepared from Cod Protein Hydrolysate by *Bacillus sp. Applied Biochemistry and Biotechnology*, *178*, 1095–1112. https://doi.org/10.1007/s12010-015-1931-5

Gomez-Guillen, M. C., Gimenez, B., Lopez-Caballero, M. E., & Montero, M. P. (2011). Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids*, *25*(8), 1813–1827. https://doi.org/10.1016/j.foodhyd.2011.02.007

Gordon, E., & Lorimer, J. W. (1960). The acid-soluble collagen of cod skin. *Archives of Biochemistry and Biophysics*, *88*, 373–381.

Halim, N. R. A., Azlan, A., Yusof, H. M., & Sarbon, N. M. (2018). Antioxidant and anticancer activities of enzymatic eel (*monopterus sp*) protein hydrolysate as influenced by different molecular weight. *Biocatalysis and Agricultural Biotechnology*, *16*, 10–16.

https://doi.org/10.1016/j.bcab.2018.06.006

Halim, N. R. A., Yusof, H. M., & Sarbon, N. M. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science and Technology*, *51*, 24–33. https://doi.org/10.1016/j.tifs.2016.02.007

Hall, G. M. (2011). Surimi and Fish Mince Products. In G. M. Hall (Ed.), *Fish Processing – Sustainability and New Opportunities* (pp. 98–111). Wiley Blackwell.

Halldorsdottir, S. M., Sveinsdottir, H., Freysdottir, J., & Kristinsson, H. G. (2014). Oxidative processes during enzymatic hydrolysis of cod protein and their influence on antioxidant and immunomodulating ability. *Food Chemistry*, *142*, 201–209. https://doi.org/10.1016/j.foodchem.2013.07.053

Hardin, J., Bertoni, G., & Kleinsmith, L. J. (2016). *Becker's World of the Cell. Technology Update* (8th Editio). Pearson.

Harnedy, A., & Fitzgerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods*, *4*, 6–24. https://doi.org/10.1016/j.jff.2011.09.001

Hawkes, J. W. (1974). The structure of fish skin. *Cell and Tissue Research*, *149*(712), 147–158. https://doi.org/10.1007/BF00222271

Hayes, M., & McKeon, K. (2014). Advances in the Processing of Marine Discard and By-products. In S. K. Kim (Ed.), *Seafood Processing By-Products. Trends and Applications* (pp. 125–144). Springer.

He, S., Franco, C., & Zhang, W. (2013). Functions, applications and production of protein hydrolysates from fish processing co-products (FPCP). *Food Research International*, *50*, 289–297. https://doi.org/10.1016/j.foodres.2012.10.031

Heo, S.-Y., Ko, S.-C., Nam, S. Y., Oh, J., Kim, Y.-M., Kim, J.-I., Namwon, K., Myunggi, Y., & Jung, W.-K. (2018). Fish bone peptide promotes osteogenic differentiation of MC3T3-E1 pre-osteoblasts through upregulation of MAPKs and Smad pathways activated BMP-2 receptor. *Cell Biochemistry & Function*, *36*, 137–146. https://doi.org/10.1002/cbf.3325

Himonides, A. T., Taylor, A. K. D., & Morris, A. J. (2011). Enzymatic Hydrolysis of Fish Frames Using Pilot Plant Scale Systems. *Food and Nutrition Sciences*, *2*, 586–593. https://doi.org/10.4236/fns.2011.26082

Hooper, N. M. (2002). Proteases: a primer. *Biochemical Society*, *38*, 1–8. https://doi.org/10.1042/bse0380001

Hsu, K.-C., Li-Chan, E. C. Y., & Jao, C.-L. (2011). Antiproliferative activity of peptides prepared from enzymatic hydrolysates of tuna dark muscle on human breast cancer cell line MCF-7. *Food Chemistry*, *126*, 617–622. https://doi.org/10.1016/j.foodchem.2010.11.066

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *Journal of Agricultural and Food Chemistry*, *50*, 4437–4444. https://doi.org/10.1021/jf0201529

Invitrogen. (2016). *Protein gel electrophoresis technical handbook* (pp. 1–87). ThermoFisher Scientific.

Ishak, N. H., & Sarbon, N. M. (2018). A Review of Protein Hydrolysates and Bioactive Peptides Deriving from Wastes Generated by Fish Processing. *Food and Bioprocess Technology*, *11*, 2–16. https://doi.org/10.1007/s11947-017-1940-1

Ismail, B., Mohammed, H., & Nair, A. J. (2019). Influence of Proteases on Functional Properties of Food. In *Green Bio-processes* (pp. 31–53). https://doi.org/10.1007/978-981-13-2354-6

Jamnik, P., Istenič, K., Koštomaj, T., Wulff, T., Geirsdóttir, M., Almgren, A., Jónsdóttir, R.,

Kristinsson, H. G., & Undeland, I. (2017). Bioactivity of Cod and Chicken Protein Hydrolysates before and after in vitro Gastrointestinal Digestion. *Food Technology and Biotechnology*, *55*(3), 360–367.

Jang, H. L., Liceaga, A. M., & Yoon, K. Y. (2017). Isolation and Characteristics of Anti-Inflammatory Peptides from Enzymatic Hydrolysates of Sandfish (*Arctoscopus japonicus*) Protein. *Journal of Aquatic Food Product Technology*, *26*(2), 234–244. https://doi.org/10.1080/10498850.2016.1221015

Jensen, I.-J., Larsen, R., Rustad, T., & Eilertsen, K.-E. (2013). Nutritional content and bioactive properties of wild and farmed cod (*Gadus morhua* L.) subjected to food preparation. *Journal of Food Composition and Analysis*, *31*, 212–216. https://doi.org/10.1016/j.jfca.2013.05.013

Jensen, I., & Mæhre, H. K. (2016). Preclinical and Clinical Studies on Antioxidative, Antihypertensive and Cardioprotective Effect of Marine Proteins and Peptides — A Review. *Marine Drugs*, *14*(211). https://doi.org/10.3390/md14110211

Jeon, Y.-J., Byun, H.-G., & Kim, S.-K. (1999). Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. *Process Biochemistry*, *35*, 471–478.

Johansen, S. D., Coucheron, D. H., Andreassen, M., Karlsen, O., Breines, R., Furmanek, T., Jørgensen, T. E., Nordeide, J. T., Moum, T., Nederbragt, A. J., Stenseth, N. C., & Jakobsen, K. S. (2009). Large-scale sequence analyses of Atlantic cod. *New Biotechnology*, *25*(5). https://doi.org/10.1016/j.nbt.2009.03.014

Joshi, I., Sudhakar, S., & Nazeer, R. A. (2016). Anti-inflammatory Properties of Bioactive Peptide Derived from Gastropod Influenced by Enzymatic Hydrolysis. *Applied Biochemistry and Biotechnology*, *180*, 1128–1140. https://doi.org/10.1007/s12010-016-2156-y

Kim, S., & Jung, W. (1996). Fish and bone as a calcium source. In *Maximising the value of marine by-products* (pp. 328–339). Woodhead Publishing Limited. https://doi.org/10.1016/B978-1-84569-013-7.50015-6

Kim, S. K., & Mendis, E. (2006). Bioactive compounds from marine processing byproducts - A review. *Food Research International*, *39*(4), 383–393. https://doi.org/10.1016/j.foodres.2005.10.010

Kim, S. K., & Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: A review. *Journal of Functional Foods*, *2*(1), 1–9. https://doi.org/10.1016/j.jff.2010.01.003

King, M. (2007). *Fisheries Biology, Assessment and Management* (2nd ed.). Blackwell Publishing Ltd.

Kisitu, J., Bennekou, S. H., & Leist, M. (2019). Chemical concentrations in cell culture compartments (C5) – Concentration definitions. *ALTEX. Alternatives to Animal Experimentation*, *36*(1), 154–160. https://doi.org/10.14573/altex.1901031

Korczek, K., Tkaczewska, J., & Migdał, W. (2018). Antioxidant and Antihypertensive Protein Hydrolysates in Fish Products – a Review. *Czech Journal of Food Sciences*, *36*(3), 195–207.

Kristinsson, H. G., & Rasco, B. A. (2000). Fish Protein Hydrolysates: Production, Biochemical, and Functional Properties. *Critical Reviews in Food Science and Nutrition*, *40*(1), 43–81. https://doi.org/10.1080/10408690091189266

Kumar, N. S. S., Kumar, N. S. V., & Jaiganesh, R. (2012). Therapeutic Drugs: Healing Power of Marine Fish. In S.-K. Kim (Ed.), *Marine Medicinal Foods. Implications and Applications: Animals and Microbes* (pp. 269–286). Elsevier Science & Technology. https://doi.org/10.1016/B978-0-12-416003-3.00018-4

Li, Z.-R., Wang, B., Chi, C., Zhang, Q.-H., Gong, Y., Tang, J.-J., Luo, H., & Ding, G. (2013).

Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel (*Scomberomorous niphonius*). *Food Hydrocolloids*, *31*(1), 103–113. https://doi.org/10.1016/j.foodhyd.2012.10.001

Liu, D., Liang, L., Regenstein, J. M., & Zhou, P. (2012). Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). *Food Chemistry*, *133*(4), 1441–1448. https://doi.org/10.1016/j.foodchem.2012.02.032

Liu, D., Nikoo, M., Boran, G., Zhou, P., & Regenstein, J. M. (2015). Collagen and Gelatin. *Annual Review of Food Science and Technology*, *6*(1), 527–557. https://doi.org/10.1146/annurev-food-031414-111800

Lordan, S., Ross, R. P., & Stanton, C. (2011). Marine bioactives as functional food ingredients: Potential to reduce the incidence of chronic diseases. *Marine Drugs*, *9*, 1056–1100. https://doi.org/10.3390/md9061056

Malawer, E. G., & Senak, L. (2004). Introduction to Size Exclusion Chromatography. In C.-S. Wu (Ed.), *Handbook of Size Exclusion Chromatography* (2nd ed., pp. 1–24). Marcek Dekker.

Meinlschmidt, P., Sussmann, D., Schweiggert-Weisz, U., & Eisner, P. (2016). Enzymatic treatment of soy protein isolates: effects on the potential allergenicity, technofunctionality, and sensory properties. *Food Science and Nutrition*, *4*(1), 11–23. https://doi.org/10.1002/fsn3.253

Merz, M., Claaßen, W., Appel, D., Berends, P., Rabe, S., Blank, I., Stressler, T., & Fischer, L. (2016). Enzymatic Characterization of commercially available peptidases in respect of the production of protein hydrolysates with defined compositions using a three-step methodology. *Journal of Molecular Catalysis B: Enzymatic*, *127*, 1–10. https://doi.org/10.1016/j.molcatb.2016.02.002

Moen, F. E., & Svensen, E. (2004). *Marine fish and invertebrates of Northen Europe*. KOM.

Mori, S., & Barth, H. G. (1999). *Size exclusion chromatography*. Springer.

Muralidharan, N., Shakila, R. J., Sukumar, D., & Jeyasekaran, G. (2013). Skin, bone and muscle collagen extraction from the trash fish, leather jacket (*Odonus niger*) and their characterization. *Journal of Food Science and Technology*, *50*(6), 1106–1113. https://doi.org/10.1007/s13197-011-0440-y

Naqash, S. Y., & Nazeer, R. A. (2010). Antioxidant Activity of Hydrolysates and Peptide Fractions of *Nemipterus japonicus* and *Exocoetus volitans* Muscle. *Journal of Aquatic Food Product Technology*, *19*(3–4), 180–192. https://doi.org/10.1080/10498850.2010.506256

Nasri, M. (2017). Protein Hydrolysates and Biopeptides: Production , Biological Activities , and Applications in Foods and Health Benefits. A Review. In F. Toldrá (Ed.), *Advances in Food and Nutrition Research* (pp. 109–159). Elsevier Science & Technology.

Nelson, D. L., & Cox, M. M. (2013). *Principles of Biochemistry. Lehninger* (6th ed.). W. H. Freeman and Company.

Neubauer, P., Cruz, N., Glauche, F., Junne, S., Knepper, A., & Raven, M. (2013). Consistent development of bioprocesses from microliter cultures to the industrial scale. *Engineering in Life Sciences*, *13*, 224–238. https://doi.org/10.1002/elsc.201200021

Ngo, D.-H., Vo, T.-S., Ngo, D.-N., Wijesekara, I., & Kim, S.-K. (2012). Biological activities and potential health benefits of bioactive peptides derived from marine organisms. *International Journal of Biological Macromolecules*, *51*(4), 378–383. https://doi.org/10.1016/j.ijbiomac.2012.06.001

Nofima. (2018). *Cod heads and chicken carcasses rise in value*. https://nofima.no/en/nyhet/2018/12/cod-heads-and-chicken-carcasses-rise-in-value/

Novozymes A/S. (2017). *Product Data Sheet: Flavourzyme 1000 L* (pp. 1–2).

Nwachukwu, I. D., & Aluko, R. E. (2019). Anticancer and antiproliferative properties of food-derived protein hydrolysates and peptides. *Journal of Food Bioactives*, *7*, 18–26. https://doi.org/10.31665/JFB.2019.7194

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *Journal of Agricultural and Food Chemistry*, *49*, 4619–4626. https://doi.org/10.1021/jf010586o

Pal, G. K., & Suresh, P. V. (2017). Comparative assessment of physico-chemical characteristics and fibril formation capacity of thermostable carp scales collagen. *Materials Science and Engineering C*, *70*, 32–40. https://doi.org/10.1016/j.msec.2016.08.047

Pal, G. K., & Suresh, P. V. (2016). Sustainable valorisation of seafood by-products: Recovery of collagen and development of collagen-based novel functional food ingredients. *Innovative Food Science and Emerging Technologies*, *37*, 201–215. https://doi.org/10.1016/j.ifset.2016.03.015

Pampanin, D. M., Haarr, M. B., & Sydnes, M. O. (2016). Natural peptides with antioxidant activity from Atlantic cod and Atlantic salmon residual material. *International Journal of Applied Research in Natural Products*, *9*(2), 1–8.

Pan, X., Zhao, Y.-Q., Hu, F.-Y., Chi, C.-F., & Wang, B. (2016). Anticancer Activity of a Hexapeptide from Skate (*Raja porosa*) Cartilage Protein Hydrolysate in HeLa Cells. *Marine Drugs*, 1–11. https://doi.org/10.3390/md14080153

Parkin, K. L. (1993a). Environmental Effects on Enzyme Activity. In T. Nagodawithana & G. Reed (Eds.), *Enzymes in Food Processing* (3rd ed., pp. 39–70). Academic Press.

Parkin, K. L. (1993b). General Characteristics of Enzymes. In T. Nagodawithana & G. Reed (Eds.), *Enzymes in Food Processing* (3rd ed., pp. 7–38). Academic Press.

Petsko, G. A., & Ringe, D. (2009). *Protein Structure and Function*. Oxford University Press.

Piccinno, F., Hischier, R., Seeger, S., & Som, C. (2016). From laboratory to industrial scale: a scale-up framework for chemical processes in life cycle assessment studies. *Journal of Cleaner Production*, *135*, 1085–1097. https://doi.org/10.1016/j.jclepro.2016.06.164

Picot, L., Bordenave, S., Didelot, S., Fruitier-Arnaudin, I., Sannier, F., Thorkelsson, G., Berge, J. P., Guerard, F., Chabeaud, A., & Piot, J. M. (2006). Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. *Process Biochemistry*, *41*, 1217–1222. https://doi.org/10.1016/j.procbio.2005.11.024

Podzimek, S. (2010). Size Exclusion Chromatography. In S. Podzimek (Ed.), *Light Scattering, Size Exclusion Chromatography and Asymmetric Flow Field Flow Fractionation*. Wiley.

Promega. (2012). *CellTiter 96® AQueous One Solution Cell Proliferation Assay* (pp. 2014-12–15). www.promega.com/protocols/

Richardsen, R., Myhre, M., Nystøyl, R., Strandheim, G., & Marthinussen, A. (2019). *Analyse marint restråstoff 2018* (pp. 1–35).

Riss, T. L., Moravec, R. A., Niles, A. L., Benink, H. A., & Worzella, T. J. (2016). Cell Viability Assays. In G. S. Sittampalam (Ed.), *Assay Guidance Manual* (pp. 1–31). Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Rocha, M. Da, Alemán, A., Baccan, G. C., Elvira, M., Gómez-guillén, C., Montero, P., & Prentice, C. (2018). Anti-Inflammatory, Antioxidant, and Antimicrobial Effects of Underutilized Fish Protein Hydrolysate. *Journal of Aquatic Food Product Technology*, *27*(5), 592–608. https://doi.org/10.1080/10498850.2018.1461160

Rustad, T. (2006). Physical and chemical properties of protein seafood by-products. In F. Shahidi (Ed.), *Maximising the Value of Marine By-Products* (pp. 3–21). Woodhead Publishing.

Rustad, T., & Hayes, M. (2012). Marine Bioactive Peptides and Protein Hydrolysates: Generation, Isolation Procedures, and Biological and Chemical Characterizations. In M. Hayes (Ed.), *Marine Bioactive Compounds: Sources, Characterization and Applications* (pp. 99–114). Springer.

Rustad, T., Storrø, I., & Slizyte, R. (2011). Possibilities for the utilisation of marine by-products. *International Journal of Food Science and Technology*, *46*(10), 2001–2014. https://doi.org/10.1111/j.1365-2621.2011.02736.x

Sable, R., Parajuli, P., & Jois, S. (2017). Peptides, peptidomimetics, and polypeptides from marine sources: A wealth of natural sources for pharmaceutical applications. *Marine Drugs*, *15*(4). https://doi.org/10.3390/md15040124

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides*, *31*, 1949–1956. https://doi.org/10.1016/j.peptides.2010.06.020

Shahidi, F., Naczk, M., Pegg, R. B., & Synowiecki, J. (1991). Chemical Composition and Nutritional Value of Processing Discards of Cod (*Gadus morhua*). *Food Chemistry*, *42*, 145–151.

Shinnar, R. (2004). A Systematic Methodology for the Design Development and Scale-up of Complex Chemical Processes . The Role of Control and Concurrent Design. *Industrial & Engineering Chemistry Research*, *43*, 246–269. https://doi.org/10.1021/ie0304715

Sila, A., & Bougatef, A. (2016). Antioxidant peptides from marine by-products: Isolation, identification and application in food systems. A review. *Journal of Functional Foods*, *21*, 10–26. https://doi.org/10.1016/j.jff.2015.11.007

Skierka, E., & Sadowska, M. (2007). The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod (*Gadus morhua*). *Food Chemistry*, *105*, 1302–1306. https://doi.org/10.1016/j.foodchem.2007.04.030

Skipnes, D., Plancken, I. Van Der, Loey, A. Van, & Hendrickx, M. E. (2008). Kinetics of heat denaturation of proteins from farmed Atlantic cod (*Gadus morhua*). *Journal of Food Engineering*, *85*, 51–58. https://doi.org/10.1016/j.jfoodeng.2007.06.030

Slizyte, R., Dauksas, E., Falch, E., Storrø, I., & Rustad, T. (2005a). Characteristics of protein fractions generated from hydrolysed cod (*Gadus morhua*) by-products. *Process Biochemistry*, *40*, 2021–2033. https://doi.org/10.1016/j.procbio.2004.07.016

Slizyte, R., Dauksas, E., Falch, E., Storrø, I., & Rustad, T. (2005b). Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (*Gadus morhua*) by-products. *Process Biochemistry*, *40*, 1415–1424. https://doi.org/10.1016/j.procbio.2004.06.033

Slizyte, R., Mozuraityte, R., Martínez-Alvarez, O., Falch, E., Fouchereau-Peron, M., Rustad, T., Martinez-Alvarez, O., Falch, E., Fouchereau-Peron, M., & Rustad, T. (2009). Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (*Gadus morhua*) backbones. *Process Biochemistry*, *44*, 668–677. https://doi.org/10.1016/j.procbio.2009.02.010

Song, R., Wei, R., Luo, H., & Yang, Z. (2014). Isolation and identification of an antiproliferative peptide derived from heated products of peptic hydrolysates of half-fin anchovy (*Setipinna taty*). *Journal of Functional Foods*, *10*, 104–111. https://doi.org/10.1016/j.jff.2014.06.010

Song, R., Wei, R., Zhang, B., Yang, Z., & Wang, D. (2011). Antioxidant and Antiproliferative Activities of Heated Sterilized Pepsin Hydrolysate Derived from Half-Fin Anchovy (*Setipinna taty*). *Marine Drugs*, *9*, 1142–1156. https://doi.org/10.3390/md9061142

Statistics Norway. (2020). *Fisheries*. https://www.ssb.no/en/jord-skog-jakt-og-fiskeri/statistikker/fiskeri

Stock, S. R. (2015). The Mineral–Collagen Interface in Bone. *Calcified Tissue International*,

*97*(3), 262–280. https://doi.org/10.1007/s00223-015-9984-6.The

Striegel, A. M., Yau, W. W., Kirkland, J. J., & Bly, D. D. (2009). *Modern Size-Exclusion Liquid Chromatography* (2nd ed.). Wiley.

Suarez-Jimenez, G.-M., Burgos-Hernandez, A., & Ezquerra-Brauer, J.-M. (2012). Bioactive Peptides and Depsipeptides with Anticancer Potential: Sources from Marine Animals. *Marine Drugs*, *10*, 963–986. https://doi.org/10.3390/md10050963

Subhan, F., Kang, H. Y., Lim, Y., Ikram, M., Baek, S.-Y., Jin, S., Jeong, Y. H., Kwak, J. Y., & Yoon, S. (2017). Fish Scale Collagen Peptides Protect against CoCl$_2$/TNF- α-Induced Cytotoxicity and Inflammation via Inhibition of ROS , MAPK , and NF- κB Pathways in HaCaT Cells. *Oxidative Medicine and Cellular Longevity*, 1–17.

Sveinsdottir, H., Hamaguchi, P. Y., Bakken, H. E., & Kristinsson, H. G. (2014). Methods for assessing the antioxidative activity of aquatic food compounds. In H. G. Kristinsson (Ed.), *Antioxidants and Functional Components in Aquatic Foods* (pp. 151–174). Wiley Blackwell.

Tahergorabi, R., Hosseini, S. V, & Jaczynski, J. (2011). Seafood proteins. In G. O. Phillips & P. A. Williams (Eds.), *Handbook of Food Proteins* (pp. 116–149). Woodhead Publishing.

Tailorzyme. (2016). *Product Data Sheet: TailorFood Endocut-01L.*

Toppe, J., Albrektsen, S., Hope, B., & Aksnes, A. (2007). Chemical composition, mineral content and amino acid and lipid profiles in bones from various fish species. *Comparative Biochemistry and Physiology Part B*, *146*, 395–401. https://doi.org/10.1016/j.cbpb.2006.11.020

Vang, B., Altintzoglou, T., Måge, I., Wubshet, S. G., Afseth, N. K., & Whitaker, R. D. (2018). Nofima: Peptide Recovery and Commercialization by Marine Biomass. In G. de Gonzalo & P. D. de María (Eds.), *Biocatalysis: An Industrial Perspective* (pp. 459–476). Royal Society of Chemistry.

Vidanarachchi, J. K., Ranadheera, C. S., Wijerathne, T. D., Udayangani, R. M. C., Himali, S. M. C., & Pickova, J. (2014). Applications of Seafood By-products in the Food Industry and Human Nutrition. In S. Kim (Ed.), *Seafood Processing By-Products. Trends and Applications* (pp. 463–528).

Walsh, G. (2014a). Industrial enzymes: an introduction. In *Proteins: Biochemistry and Biotechnology* (2nd ed., pp. 311–326). John Wiley & Sons, Ltd.

Walsh, G. (2014b). Industrial enzymes: proteases and carbrohydrases. In *Proteins: Biochemistry and Biotechnology* (2nd ed., pp. 327–369). John Wiley & Sons, Ltd.

Walsh, G. (2014c). Proteins and proteomics. In *Proteins: Biochemistry and Biotechnology* (2nd ed., pp. 1–22). Blackwell Publishing Ltd.

Ward, O. P. (2011). Proteases. In M. Moo-Young (Ed.), *Comprehensive Biotechnology* (2nd ed., pp. 571–582). Elsevier. https://doi.org/10.1016/B978-0-08-088504-9.00222-1

WHO. (2018). *Cancer.* https://www.who.int/news-room/fact-sheets/detail/cancer

WHO, & FAO. (2003). *Diet, Nutrition and the Prevention of Chronic Diseases.* http://www.fao.org/3/AC911E/ac911e00.htm

Willey, J. M., Sherwood, L. M., & Woolverton, C. J. (2014). *Prescott's Micobiology* (9th ed.). Mc Graw Hill Education.

Wolfe, K. L., & Rui, H. L. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*, *55*(22), 8896–8907. https://doi.org/10.1021/jf0715166

Woo, J.-W., Yu, S.-J., Cho, S.-M., Lee, Y.-B., & Kim, S.-B. (2008). Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin. *Food Hydrocolloids*, *22*(5), 879–887. https://doi.org/10.1016/j.foodhyd.2007.04.015

Wubshet, S. G., Måge, I., Bocker, U., Lindberg, D., Knutsen, S. H., Rieder, A., Rodriguez, D.

A., & Afseth, N. K. (2017). Analytical Methods FTIR as a rapid tool for monitoring molecular weight distribution during enzymatic protein hydrolysis of food processing by-products. *Analytical Methods*, *9*, 4247–4254. https://doi.org/10.1039/c7ay00865a

Wubshet, S. G., Wold, J. P., Afseth, N. K., Böcker, U., Lindberg, D., Ihunegbo, F. N., & Måge, I. (2018). Feed-Forward Prediction of Product Qualities in Enzymatic Protein Hydrolysis of Poultry By-products : a Spectroscopic Approach. *Food and Bioprocess Technology*, *11*, 2032–2043. https://doi.org/10.1007/s11947-018-2161-y

Yaghoubzadeh, Z., Peyravii, F., Hami, G., Reza, K., & Esmail, S. (2020). Antioxidant Activity and Anticancer Effect of Bioactive Peptides from Rainbow Trout (*Oncorhynchus mykiss*) Skin Hydrolysate. *International Journal of Peptide Research and Therapeutics*, *26*, 625–632. https://doi.org/10.1007/s10989-019-09869-5

Yu, F., Zong, C., Jin, S., Zheng, J., Chen, N., Huang, J., Chen, Y., Huang, F., Yang, Z., Tang, Y., & Ding, G. (2018). Optimization of extraction conditions and characterization of pepsin-solubilised collagen from skin of giant croaker (*Nibea japonica*). *Marine Drugs*, *16*(1), 1–12. https://doi.org/10.3390/md16010029

# Appendix

## A 1 List of chemicals

*Table 6 – List of chemicals used in the project.*

| Type of chemical | Product ID | Manufacturer |
|---|---|---|
| Acetonitrile | | Merck, Darmstadt, Germany |
| Trifluoroacetic acid | | Merck, Darmstadt, Germany |
| Human melanoma A2058 | ATCC CRL-11147 | LGC Standards, Teddington, UK |
| CellTiter 96® AQueous One Solution Reagent | G3581 | Promega Biotech AB, Madison, WI, USA |
| DMSO (Dimethyl sulfoxide) | D4540 | Sigma-Aldrich, St. Louis, USA |
| Trypan Blue 0.4 % | T8154 | Sigma-Aldrich, St. Louis, USA |
| Trypsin | X0930 | Biowest, Nuaillé, France |
| Dulbecco's Modified Eagle's Medium (DMEM) | D6171 | Sigma-Aldrich, St. Louis, USA |
| L-alanyl-L-Glutamine | K0302 | Biochrom Ltd., Cambridge, UK |
| Fetal Bovine Serum (FBS) | S1810-500 | Biowest, Nuaillé, France |
| Gentamycin | A2712 | Biochrom Ltd., Cambridge, UK |
| di-Sodium hydrogen phosphate dihydrate (Na2HPO4 × 2H2O) | | Merck, Darmstadt, Germany |
| 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) | 44091-4 | Sigma Aldrich, St. Louis, MO, USA |
| Fluorescein | 46960 | Sigma Aldrich, St. Louis, MO, USA |
| Trolox | 238813 | Sigma Aldrich, St. Louis, MO, USA |
| THP-1 Human monocyte | ATCC TIB-202 | LGC Standards, Teddington, UK |
| RPMI 1640, low endotoxin | FG1385 | Biochrom Ltd., Cambridge, UK |
| Fetal Bovine Serum (FBS), Ultralow endotoxin | S1860-500 | Biowest, Nuaillé, France |
| Phosphate Buffered Saline (PBS) | D8537 | Sigma-Aldrich, St. Louis, USA |
| Trypan blue 0.4% | | Sigma-Aldrich, St. Louis, USA |
| PMA, stock solution 1mg/mL | P1585 | Sigma-Aldrich, St. Louis, USA |
| LPS | L2630 | Sigma-Aldrich, St. Louis, USA |
| TRIZMA base | 93352 | Sigma-Aldrich, St. Louis, USA |
| NaCl | S5886 | Sigma-Aldrich, St. Louis, USA |
| Tween20 | P1379 | Sigma-Aldrich, St. Louis, USA |
| BSA | A2153 | Sigma-Aldrich, St. Louis, USA |
| pNPP substrate 5mg | S0942 | Sigma-Aldrich, St. Louis, USA |
| pNPP substrate 40mg | P5994 | Sigma-Aldrich, St. Louis, USA |
| Diethanolamine | D8885 | Sigma-Aldrich, St. Louis, USA |

| Extravidin-alkaline phosphate | E2636 | Sigma-Aldrich, St. Louis, USA |
|---|---|---|
| Anti-Human TNF alpha Purified | 14-7348-85 | eBioscience, San Diego, CA, United States |
| Anti-Human TNF alpha Biotin | 13-7349-85 | eBioscience, San Diego, CA, United States |
| Human TNF alpha recombinant protein | 14-8329-63 | eBioscience, San Diego, CA, United States |

*Table 7 – Chemical composition of solutions used in ELISA.*

| Solutions | Chemicals |
|---|---|
| **10 × TBS pH 7.4** | TRIZMA base 12.1 g<br>NaCl 88 g<br>MilliQ water 1 L<br>HCl for pH adjustment to 7.4 |
| **1 M diethanolamine buffer pH 9.8** | $MgCl_2$ 100 mg<br>Diethanolamine 97 mL<br>MilliQ water 1 L<br>HCl for pH adjustment to 9.8 |
| **Washing buffer** | 1× TBS (10 mM) pH 7.4<br>0.05% Tween 20 |
| **Blocking buffer** | 1× TBS (10 mM) pH 7.4<br>2% BSA |
| **Assay diluent** | 1× TBS (10 mM) pH 7.4 with 1% BSA |
| **pNPP substrate solution** | pNPP substrate 20mg/mL<br>1 M diethanolamine buffer pH 9.8  20 mL |

# A 2 Calculations

## A 2.1. Adjustment of weight to 5 g for comparison

Hydrolysis was performed in duplicate. Raw material weigh was ca. 5 g. Adjustments of results to 5 g were done to compare treatments with different enzymes.

*Table 8 – Weight of raw material and sediment. Example with skin as raw material and Bromelain.*

| Replicate | Weight of the raw material (g) | Average weight of the raw material (g) | Sediment (g) | Average sediment (g) |
|---|---|---|---|---|
| I | 5.0124 | $\frac{(5.0124+5.0052)}{2} = 5.0088$ | 0.1386 | 0.14465 |
| II | 5.0052 | | 0.1507 | |

Calculation of how much sediment/freeze-dried hydrolysate is produced from 5 g of raw material ($x$):

First, the percentage that 5 g constitutes relative to the average weight of raw material (y) was calculated:

$$\frac{y}{100\ \%} = \frac{5\ g}{5.0088\ g}$$

$$y = \frac{5\ g \times 100\ \%}{5.0088\ g} = 99.82\ \%$$

Then based on the percentage the weight per 5 g was calculated:

$$\frac{x}{0.14465\ g} = \frac{99.82\ \%}{100\ \%}$$

$$x = \frac{99.82\ \% \times 0.14465\ g}{100\ \%} \approx 0.1444\ \text{g}$$

## A 2.2. Calculation of hydrolysate yield

Example for muscle and Alcalase

1) control treatment yield (muscle) $= \left(\frac{0.127\ g}{0.844\ g}\right) \times 100\% = 15.11\%$

2) total yield (muscle, Alcalase) $= \left(\frac{0.371g}{0.844g}\right) \times 100\% = 43.99\%$

3) hydrolysate yield (muscle, Alcalase) = 43.99% - 15.11% = 28.88%

## A 2.3. Sediment reduction due to enzymatic hydrolysis

Example for muscle and Alcalase

1) control treatment sediment (muscle) = $\left(\frac{0.733g}{0.844g}\right) \times 100\% = 86.86\%$

2) total sediment (muscle, Alcalase) = $\left(\frac{0.436g}{0.844g}\right) \times 100\% = 51.67\%$

3) sediment reduction (muscle, Alcalase) = 86.86% - 51.67% = 35.19%

## A 2.4. Calculation of selectivity ratio

Example for skin/muscle ratio and Alcalase

Selectivity ratio (Alcalase) $= \frac{Hydrolysate\ yield\ skin\ (\%)}{Hydrolysate\ yield\ muscle\ (\%)} = \frac{19.7\%}{28.9\%} = 0.68$

## A 2.5. Weight average MW

First, total mass of each type of molecule is calculated using the formula $N_iM_i$, where $N_i$ is the number of molecules of weight $M_i$

Then, the total molecular weight of the sample is calculated using the formula: $\sum N_iM_i$

Afterwards, the weight fraction of each type of molecule ($W_i$) is calculated using the formula: $W_i = N_iM_i/ \sum N_iM_i$

Weight average molecular weight is calculated using the formula below

Weight average MW $= \sum W_iM_i = 490\ 805.8$

Table 9 – Calculation of weight average MW.

| Number of molecules ($N_i$) | Mass of each molecule ($M_i$) | Total mass of each type of molecule ($N_iM_i$) | Weight fraction type of molecule ($W_i$) | $W_iM_i$ |
|---|---|---|---|---|
| 2 | 800 000 | 1 600 000 | 0.11 | 84 544.25 |
| 5 | 650 000 | 3 250 000 | 0.21 | 139 531 |
| 20 | 420 000 | 8 400 000 | 0.55 | 233 025.1 |
| 7 | 270 000 | 1 890 000 | 0.12 | 33 705.42 |

**A 2.6. ORAC**

Dilution series of Trolox was used to plot a standard curve to which measured values were related to. First, data was normalized to the well Trolox 0 µM (phosphate buffer, fluorescein and AAPH):

Areal in between curves = Areal under curve$_{sample}$ – Areal under curve$_{Trolox\ 0}$

The equation for trend line was made based on the normalized data. Trolox equivalent (µM) was calculated using this equation:

$y = ax + b, => x = (y – b)/a$,

where y is Areal under curve$_{sample}$

# A 3 Hydrolysis

*Table 10 – Hydrolysis results from muscle: weight of freeze-dried hydrolysate (adjusted per 5 g for comparison) with standard deviation, percentage of total yield (relative to the dry weight of the material) and hydrolysate yield; weight of sediment (adjusted per 5 g for comparison) with standard deviation, percentage of sediment (relative to the dry weight of the material) and percentage of sediment reduction due to enzymatic hydrolysis.*

| Enzyme | Freeze-dried hydrolysate weight (g) | Standard Deviation (g) | Total yield (%) | Hydrolysate yield (%) | Sediment weight (g) | Standard Deviation (g) | Sediment relative to dry matter (%) | Sediment reduction due to enzymatic hydrolysis (%) |
|---|---|---|---|---|---|---|---|---|
| Alcalase | 0.37 | 0.003 | 44.0 | 28.9 | 0.44 | 0.004 | 51.7 | 35.2 |
| Bromelain | 0.33 | 0.011 | 38.9 | 23.8 | 0.51 | 0.022 | 60.8 | 26.0 |
| Corolase 2TS | 0.46 | 0.020 | 54.2 | 39.1 | 0.36 | 0.038 | 43.0 | 43.8 |
| Corolase 7090 | 0.41 | 0.008 | 48.3 | 33.2 | 0.40 | 0.005 | 47.0 | 39.8 |
| Tail 10 | 0.42 | 0.001 | 49.7 | 34.5 | 0.49 | 0.002 | 57.8 | 29.1 |
| Tail 189 | 0.53 | 0.012 | 62.7 | 47.6 | 0.44 | 0.002 | 52.1 | 34.8 |
| Tail 190 | 0.38 | 0.014 | 45.3 | 30.2 | 0.51 | 0.016 | 60.4 | 26.4 |
| Tail 191 | 0.55 | 0.004 | 65.1 | 50.0 | 0.34 | 0.003 | 40.5 | 46.4 |
| Tail 192 | 0.43 | 0.005 | 50.6 | 35.5 | 0.50 | 0.005 | 59.6 | 27.2 |
| Tail 193 | 0.45 | 0.010 | 52.9 | 37.8 | 0.49 | 0.015 | 57.8 | 29.0 |
| Tail 194 | 0.54 | 0.014 | 63.7 | 48.6 | 0.40 | 0.010 | 47.1 | 39.7 |
| Tail 197 | 0.45 | 0.003 | 52.8 | 37.7 | 0.45 | 0.004 | 52.8 | 34.1 |
| FoodPro 30L | 0.39 | 0.016 | 46.7 | 31.6 | 0.51 | 0.016 | 60.6 | 26.3 |
| FoodPro 51FP | 0.42 | 0.023 | 49.4 | 34.3 | 0.49 | 0.004 | 57.6 | 29.3 |
| FoodPro PNL | 0.44 | 0.019 | 52.2 | 37.1 | 0.44 | 0.016 | 51.9 | 35.0 |
| Veron L10 | 0.31 | 0.004 | 36.8 | 21.7 | 0.56 | 0.003 | 66.8 | 20.0 |
| Flavourzyme | 0.30 | 0.013 | 35.4 | 20.3 | 0.57 | 0.017 | 67.0 | 19.9 |
| Protamex | 0.43 | 0.013 | 50.7 | 35.6 | 0.44 | 0.012 | 51.8 | 35.1 |
| Promod 144GL | 0.33 | 0.002 | 39.3 | 24.2 | 0.52 | 0.015 | 62.0 | 24.9 |
| Promod P950L | 0.41 | 0.002 | 48.0 | 32.9 | 0.43 | 0.008 | 50.5 | 36.4 |
| Endocut 01 | 0.35 | 0.007 | 41.3 | 26.2 | 0.54 | 0.001 | 63.6 | 23.2 |
| Endocut 02 | 0.45 | 0.002 | 53.3 | 38.2 | 0.42 | 0.016 | 50.1 | 36.8 |
| Endocut 03 | 0.43 | 0.021 | 50.5 | 35.4 | 0.49 | 0.015 | 57.7 | 29.1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Control** | 0.13 | 0.008 | 15.1 | 0.0 | 0.73 | 0.030 | 86.9 | 0.0 |

*Table 11 – Hydrolysis results from skin: weight of freeze-dried hydrolysate (adjusted per 5 g for comparison) with standard deviation, percentage of total yield (relative to the dry weight of the material) and hydrolysate yield; weight of sediment (adjusted per 5 g for comparison) with standard deviation, percentage of sediment (relative to the dry weight of the material) and percentage of sediment reduction due to enzymatic hydrolysis.*

| Enzyme | Freeze-dried hydrolysate weight (g) | Standard Deviation (g) | Total yield (%) | Hydrolysate yield (%) | Sediment weight (g) | Standard Deviation (g) | Sediment relative to dry matter (%) | Sediment reduction due to enzymatic hydrolysis (%) |
|---|---|---|---|---|---|---|---|---|
| **Alcalase** | 0.77 | 0.080 | 72.2 | 19.7 | 0.22 | 0.015 | 20.9 | 15.9 |
| **Bromelain** | 0.71 | 0.018 | 66.2 | 13.7 | 0.23 | 0.009 | 21.4 | 15.4 |
| **Corolase 2TS** | 0.80 | 0.057 | 74.6 | 22.1 | 0.20 | 0.020 | 18.8 | 18.0 |
| **Corolase 7090** | 0.83 | 0.011 | 77.3 | 24.8 | 0.25 | 0.007 | 23.3 | 13.5 |
| **Tail 10** | 0.81 | 0.066 | 75.5 | 23.0 | 0.26 | 0.015 | 24.1 | 12.7 |
| **Tail 189** | 0.85 | 0.054 | 79.5 | 27.0 | 0.24 | 0.017 | 22.8 | 14.0 |
| **Tail 190** | 0.85 | 0.015 | 79.4 | 26.9 | 0.25 | 0.007 | 23.6 | 13.2 |
| **Tail 191** | 0.83 | 0.038 | 77.6 | 25.1 | 0.25 | 0.024 | 23.6 | 13.2 |
| **Tail 192** | 0.84 | 0.008 | 78.9 | 26.4 | 0.24 | 0.001 | 22.6 | 14.2 |
| **Tail 193** | 0.77 | 0.007 | 72.2 | 19.7 | 0.25 | 0.008 | 23.5 | 13.3 |
| **Tail 194** | 0.88 | 0.026 | 82.0 | 29.5 | 0.24 | 0.003 | 22.8 | 14.0 |
| **Tail 197** | 0.83 | 0.002 | 77.8 | 25.3 | 0.26 | 0.012 | 24.6 | 12.2 |
| **FoodPro 30L** | 0.80 | 0.002 | 75.1 | 22.6 | 0.25 | 0.004 | 23.3 | 13.5 |
| **FoodPro 51FP** | 0.84 | 0.006 | 78.8 | 26.3 | 0.26 | 0.006 | 24.5 | 12.3 |
| **FoodPro PNL** | 0.80 | 0.026 | 75.2 | 22.7 | 0.26 | 0.009 | 24.0 | 12.8 |
| **Veron L10** | 0.71 | 0.010 | 66.3 | 13.8 | 0.32 | 0.014 | 29.7 | 7.1 |
| **Flavourzyme** | 0.85 | 0.017 | 79.3 | 26.8 | 0.30 | 0.004 | 27.7 | 9.1 |
| **Protamex** | 0.88 | 0.003 | 81.9 | 29.4 | 0.25 | 0.000 | 23.2 | 13.6 |
| **Promod 144GL** | 0.77 | 0.015 | 71.8 | 19.3 | 0.30 | 0.006 | 27.8 | 9.0 |
| **Promod P950L** | 0.83 | 0.020 | 78.0 | 25.5 | 0.24 | 0.006 | 22.5 | 14.3 |
| **Endocut 01** | 0.63 | 0.007 | 58.6 | 6.1 | 0.29 | 0.003 | 27.4 | 9.4 |
| **Endocut 02** | 0.84 | 0.002 | 78.2 | 25.7 | 0.23 | 0.010 | 21.9 | 14.9 |
| **Endocut 03** | 0.81 | 0.067 | 76.2 | 23.7 | 0.30 | 0.006 | 27.6 | 9.2 |
| **Control** | 0.56 | 0.046 | 52.5 | 0.0 | 0.39 | 0.031 | 36.8 | 0.0 |

*Table 12 – Hydrolysis results from bone: weight of freeze-dried hydrolysate (adjusted per 5 g for comparison) with standard deviation, percentage of total yield (relative to the dry weight of the material) and hydrolysate yield; weight of sediment (adjusted per 5 g for comparison) with standard deviation, percentage of sediment (relative to the dry weight of the material) and percentage of sediment reduction due to enzymatic hydrolysis.*

| Enzyme | Freeze-dried hydrolysate weight (g) | Standard Deviation (g) | Total yield (%) | Hydrolysate yield (%) | Sediment weight (g) | Standard Deviation (g) | Sediment relative to dry matter (%) | Sediment reduction due to enzymatic hydrolysis (%) |
|---|---|---|---|---|---|---|---|---|
| Alcalase | 0.19 | 0.005 | 7.2 | 4.8 | 2.40 | 0.001 | 91.3 | 3.6 |
| Bromelain | 0.17 | 0.002 | 6.4 | 4.0 | 2.43 | 0.004 | 92.5 | 2.4 |
| Corolase 2TS | 0.18 | 0.001 | 6.9 | 4.5 | 2.45 | 0.002 | 93.5 | 1.5 |
| Corolase 7090 | 0.17 | 0.005 | 6.5 | 4.1 | 2.45 | 0.070 | 93.3 | 1.7 |
| Tail 10 | 0.18 | 0.007 | 7.0 | 4.6 | 2.40 | 0.029 | 91.6 | 3.4 |
| Tail 189 | 0.18 | 0.002 | 6.9 | 4.5 | 2.44 | 0.001 | 92.9 | 2.1 |
| Tail 190 | 0.20 | 0.000 | 7.8 | 5.4 | 2.36 | 0.001 | 89.7 | 5.3 |
| Tail 191 | 0.20 | 0.001 | 7.5 | 5.1 | 2.38 | 0.004 | 90.5 | 4.4 |
| Tail 192 | 0.19 | 0.001 | 7.3 | 4.9 | 2.34 | 0.017 | 89.1 | 5.9 |
| Tail 193 | 0.18 | 0.001 | 7.0 | 4.6 | 2.40 | 0.011 | 91.4 | 3.5 |
| Tail 194 | 0.19 | 0.005 | 7.1 | 4.7 | 2.36 | 0.038 | 89.7 | 5.3 |
| Tail 197 | 0.18 | 0.003 | 6.8 | 4.4 | 2.37 | 0.001 | 90.1 | 4.9 |
| FoodPro 30L | 0.17 | 0.003 | 6.3 | 3.9 | 2.44 | 0.027 | 92.9 | 2.0 |
| FoodPro 51FP | 0.17 | 0.003 | 6.4 | 4.0 | 2.44 | 0.005 | 92.9 | 2.1 |
| FoodPro PNL | 0.17 | 0.006 | 6.5 | 4.1 | 2.44 | 0.014 | 93.0 | 2.0 |
| Veron L10 | 0.14 | 0.002 | 5.4 | 3.0 | 2.44 | 0.005 | 92.8 | 2.2 |
| Flavourzyme | 0.14 | 0.006 | 5.4 | 3.0 | 2.44 | 0.033 | 93.0 | 2.0 |
| Protamex | 0.18 | 0.003 | 6.9 | 4.5 | 2.38 | 0.005 | 90.7 | 4.3 |
| Promod 144GL | 0.15 | 0.002 | 5.7 | 3.3 | 2.45 | 0.016 | 93.2 | 1.7 |
| Promod P950L | 0.17 | 0.003 | 6.6 | 4.2 | 2.45 | 0.036 | 93.4 | 1.6 |
| Endocut 01 | 0.15 | 0.000 | 5.8 | 3.4 | 2.49 | 0.017 | 94.7 | 0.3 |
| Endocut 02 | 0.19 | 0.001 | 7.1 | 4.7 | 2.40 | 0.006 | 91.5 | 3.4 |
| Endocut 03 | 0.18 | 0.002 | 6.9 | 4.5 | 2.42 | 0.018 | 92.0 | 2.9 |
| Control | 0.06 | 0.001 | 2.4 | 0.0 | 2.49 | 0.015 | 95.0 | 0.0 |

# A 4 Molecular weight distribution

*Table 13 – Molecular weight distribution of some hydrolysates produced from muscle. The hydrolysate produced by Tail 189 had the lowest average MW; the hydrolysate produced by Tail 191 had the highest yield, the hydrolysate produced by Corolase 2TS had high yield; the hydrolysate produced by Flavourzyme had the lowest yield and the highest average MW; the hydrolysate produced by Veron L10 had the second lowest yield. SEC results are shown for both replicates.*

| | Tail 189 | | | | Tail 191 | | | | Corolase 2TS | | | | Flavourzyme | | | | Veron L10 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | | II | | I | | II | | I | | II | | I | | II | | I | | II | |
| Peak# | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % |
| Total | 936 | 100 | 973 | 100 | 953 | 100 | 960 | 100 | 1565 | 100 | 1499 | 100 | 3650 | 100 | 3478 | 100 | 1370 | 100 | 1253 | 100 |
| 1 | 6897 | 4.77 | 7283 | 5.1 | 3684 | 8.9 | 3578 | 9.2 | 6628 | 11.5 | 4191 | 21.2 | 16556 | 17.3 | 14746 | 18.5 | 4541 | 12.3 | 4281 | 10.3 |
| 2 | 2063 | 3.16 | 2066 | 3.2 | 1282 | 14.0 | 1287 | 14.2 | 1976 | 10.0 | 1301 | 17.9 | 4219 | 3.3 | 3995 | 3.1 | 1978 | 11.8 | 1976 | 11.2 |
| 3 | 1317 | 8.27 | 1323 | 8.1 | 843 | 29.9 | 847 | 29.8 | 1295 | 17.8 | 867 | 27.7 | 3013 | 4.2 | 2914 | 3.9 | 1293 | 18.7 | 1290 | 18.8 |
| 4 | 850 | 23.18 | 854 | 22.6 | 464 | 40.9 | 467 | 40.5 | 866 | 27.7 | 671 | 1.6 | 2086 | 5.2 | 2022 | 5.3 | 860 | 25.3 | 858 | 26.0 |
| 5 | 659 | 3.73 | 660 | 3.9 | 225 | 2.0 | 225 | 1.8 | 672 | 1.5 | 485 | 23.8 | 1365 | 9.0 | 1321 | 9.2 | 668 | 1.9 | 666 | 2.0 |
| 6 | 478 | 32.73 | 481 | 31.9 | 86 | 0.7 | 86 | 0.6 | 485 | 23.8 | 308 | 2.4 | 878 | 15.2 | 861 | 15.6 | 464 | 22.0 | 460 | 23.2 |
| 7 | 305 | 12.98 | 307 | 13.4 | 17 | 3.2 | 18 | 3.2 | 308 | 2.3 | 225 | 1.4 | 650 | 5.8 | 653 | 4.8 | 221 | 1.1 | 219 | 1.2 |
| 8 | 223 | 6.29 | 225 | 6.9 | 0 | 0.1 | 0 | 0.1 | 224 | 1.3 | 95 | 0.3 | 476 | 15.8 | 476 | 15.6 | 27 | 4.9 | 27 | 5.0 |
| 9 | 84 | 0.44 | 85 | 0.5 | 0 | 0.1 | 0 | 0.1 | 90 | 0.3 | 23 | 2.8 | 305 | 10.2 | 304 | 10.5 | 3 | 0.8 | 3 | 0.8 |
| 10 | 17 | 3.89 | 17 | 3.8 | 0 | 0.1 | 0 | 0.1 | 22 | 2.8 | 2 | 0.5 | 218 | 5.1 | 218 | 5.5 | 0 | 0.4 | 0 | 0.4 |
| 11 | 0 | 0.10 | 0 | 0.1 | 0 | 0.3 | 0 | 0.3 | 2 | 0.4 | 0 | 0.2 | 88 | 0.7 | 88 | 0.5 | 0 | 0.2 | 0 | 0.2 |
| 12 | 0 | 0.05 | 0 | 0.1 | 0 | 0.0 | 0 | 0.0 | 0 | 0.2 | 0 | 0.3 | 23 | 6.0 | 22 | 5.7 | 0 | 0.7 | 0 | 0.7 |
| 13 | 0 | 0.40 | 0 | 0.4 | | | | | 0 | 0.3 | | | 2 | 1.2 | 2 | 0.9 | | | | |
| 14 | | | | | | | | | | | | | 0 | 0.3 | 0 | 0.3 | | | | |
| 15 | | | | | | | | | | | | | 0 | 0.6 | 0 | 0.7 | | | | |

*Table 14 – Molecular weight distribution of some hydrolysates produced from skin. The hydrolysate produced by Tail 189 had the lowest average MW; the hydrolysate produced by Tail 194 had the highest yield, the hydrolysate produced by Protamex had high yield; the hydrolysate produced by Endocut 01 had the lowest yield; the hydrolysate produced by Veron L10 had also the highest average MW. SEC results are shown for both replicates.*

| | Tail 189 | | | | Tail 194 | | | | Protamex | | | | Endocut 01 | | | | Veron L10 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | | II | | I | | II | | I | | II | | I | | II | | I | | II | |
| Peak # | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % |
| Total | 1172 | 100 | 1214 | 100 | 1428 | 100 | 1472 | 100 | 2786 | 100 | 2837 | 100 | 3965 | 100 | 4148 | 100 | 13219 | 100 | 12856 | 100 |
| 1 | 42610 | 0.2 | 43049 | 0.2 | 42456 | 0.2 | 46185 | 0.2 | 15479 | 6.6 | 15932 | 7.0 | 53440 | 1.2 | 54710 | 1.2 | 20850 | 59.3 | 20322 | 59.2 |
| 2 | 5364 | 4.3 | 5472 | 4.9 | 6146 | 7.0 | 6217 | 7.2 | 5108 | 15.6 | 5019 | 15.6 | 15139 | 10.8 | 15323 | 11.6 | 4120 | 8.0 | 4019 | 7.7 |
| 3 | 2500 | 5.4 | 2500 | 6.0 | 2546 | 6.4 | 2578 | 6.6 | 2641 | 10.4 | 2605 | 10.4 | 4963 | 15.2 | 4968 | 15.8 | 2801 | 8.4 | 2773 | 8.4 |
| 4 | 1593 | 10.9 | 1593 | 11.9 | 1600 | 15.4 | 1613 | 15.8 | 1647 | 18.1 | 1629 | 18.1 | 2646 | 11.8 | 2649 | 12.1 | 1775 | 8.8 | 1764 | 9.1 |
| 5 | 992 | 26.8 | 994 | 27.8 | 986 | 31.4 | 990 | 31.8 | 1020 | 24.8 | 1013 | 24.7 | 1641 | 16.1 | 1648 | 15.9 | 1066 | 8.3 | 1059 | 8.4 |
| 6 | 602 | 38.3 | 604 | 38.1 | 601 | 32.8 | 606 | 32.5 | 598 | 20.7 | 594 | 20.6 | 1023 | 24.2 | 1026 | 23.6 | 628 | 5.5 | 625 | 5.5 |
| 7 | 365 | 7.9 | 368 | 6.6 | 378 | 3.1 | 382 | 3.0 | 380 | 1.5 | 376 | 1.5 | 610 | 16.5 | 612 | 15.7 | 387 | 0.2 | 385 | 0.2 |
| 8 | 280 | 2.7 | 281 | 2.1 | 281 | 1.0 | 284 | 0.9 | 275 | 0.4 | 272 | 0.4 | 382 | 1.3 | 383 | 1.3 | 298 | 0.1 | 296 | 0.1 |
| 9 | 125 | 0.5 | 128 | 0.4 | 132 | 0.4 | 134 | 0.3 | 134 | 0.2 | 132 | 0.2 | 277 | 0.5 | 277 | 0.4 | 130 | 0.3 | 128 | 0.3 |
| 10 | 48 | 0.8 | 59 | 0.4 | 59 | 0.4 | 60 | 0.3 | 46 | 0.6 | 45 | 0.6 | 149 | 0.1 | 148 | 0.1 | 43 | 0.4 | 42 | 0.4 |
| 11 | 19 | 0.6 | 36 | 0.1 | 26 | 0.6 | 25 | 0.4 | 7 | 0.6 | 7 | 0.7 | 104 | 0.1 | 107 | 0.1 | 7 | 0.5 | 7 | 0.6 |
| 12 | 6 | 1.3 | 19 | 0.6 | 6 | 1.0 | 7 | 0.8 | 1 | 0.2 | 1 | 0.2 | 44 | 0.6 | 45 | 0.7 | 1 | 0.1 | 1 | 0.1 |
| 13 | 0 | 0.1 | 5 | 1.0 | 1 | 0.3 | 1 | 0.2 | 0 | 0.0 | 0 | 0.0 | 7 | 0.9 | 7 | 0.8 | 0 | 0.0 | 0 | 0.0 |
| 14 | 0 | 0.1 | 0 | 0.1 | 0 | 0.0 | 0 | 0.0 | 0.00 | 0.0 | 0 | 0.0 | 1 | 0.3 | 1 | 0.3 | | | | |
| 15 | 0 | 0.1 | 0 | 0.0 | 0 | 0.0 | | | | | | | 0 | 0.1 | 0 | 0.1 | | | | |
| 16 | | | | | 0 | 0.1 | | | | | | | | | | | | | | |

79

*Table 15 – Molecular weight distribution of some hydrolysates produced from bone. The hydrolysate produced by Tail 189 had the lowest average MW; the hydrolysate produced by Tail 190 had the highest yield, the hydrolysate produced by Alcalase had high yield; the hydrolysates produced by Veron L10 and Flavourzyme had the lowest yield; the hydrolysate produced by Veron L10 had the highest average MW. SEC results are shown for both replicates.*

| | Tail 189 | | | | Tail 190 | | | | Alcalase | | | | Veron L10 | | | | Flavourzyme | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | | II | | I | | II | | I | | II | | I | | II | | I | | II | |
| Peak# | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % | Mw | % |
| Total | 1089 | 100 | 1042 | 100 | 1373 | 100 | 1350 | 100 | 1153 | 100 | 1153 | 100 | 10061 | 100 | 9402 | 100 | 1941 | 100 | 1958 | 100 |
| 1 | 6491 | 4.17 | 6249 | 3.78 | 6522 | 5.63 | 6392 | 5.5 | 4202 | 8.9 | 4448 | 8.6 | 17732 | 51.6 | 17043 | 49.6 | 6578 | 15.3 | 6818 | 14.9 |
| 2 | 2727 | 4.56 | 2722 | 4.37 | 2808 | 7.96 | 2785 | 7.8 | 1645 | 13.5 | 1644 | 13.1 | 4045 | 8.4 | 4083 | 9.1 | 2727 | 11.1 | 2781 | 11.2 |
| 3 | 1701 | 9.03 | 1693 | 8.84 | 1682 | 14.76 | 1673 | 14.9 | 1003 | 31.4 | 997 | 31.2 | 2731 | 9.6 | 2736 | 9.9 | 1680 | 14.4 | 1695 | 14.1 |
| 4 | 1032 | 24.08 | 1029 | 23.67 | 1025 | 31.18 | 1020 | 31.1 | 579 | 39.9 | 573 | 40.3 | 1704 | 9.3 | 1711 | 9.6 | 1029 | 21.0 | 1033 | 20.9 |
| 5 | 590 | 40.81 | 586 | 41.14 | 586 | 34.04 | 583 | 34.2 | 372 | 2.4 | 369 | 2.6 | 1032 | 8.8 | 1037 | 9.2 | 589 | 22.7 | 588 | 22.6 |
| 6 | 368 | 10.09 | 366 | 10.58 | 378 | 2.68 | 375 | 2.7 | 271 | 1.2 | 269 | 1.3 | 694 | 2.1 | 697 | 2.2 | 365 | 8.3 | 365 | 8.4 |
| 7 | 281 | 4.51 | 279 | 4.71 | 275 | 1.25 | 273 | 1.2 | 129 | 0.3 | 129 | 0.3 | 543 | 5.2 | 545 | 5.4 | 273 | 3.7 | 273 | 3.8 |
| 8 | 124 | 0.40 | 122 | 0.42 | 126 | 0.34 | 125 | 0.3 | 42 | 1.4 | 42 | 1.4 | 373 | 0.8 | 377 | 0.8 | 129 | 0.5 | 129 | 0.5 |
| 9 | 49 | 0.88 | 46 | 1.01 | 42 | 1.23 | 41 | 1.3 | 6 | 0.6 | 6 | 0.6 | 278 | 0.6 | 283 | 0.6 | 41 | 1.9 | 42 | 2.1 |
| 10 | 19 | 0.64 | 17 | 0.57 | 6 | 0.59 | 6 | 0.6 | 1 | 0.3 | 1 | 0.3 | 144 | 0.4 | 149 | 0.4 | 5 | 1.0 | 5 | 1.1 |
| 11 | 5 | 0.79 | 5 | 0.79 | 1 | 0.28 | 1 | 0.3 | 0 | 0.2 | 0 | 0.2 | 42 | 1.7 | 43 | 1.7 | 0 | 0.1 | 0 | 0.2 |
| 12 | 0 | 0.05 | 0 | 0.06 | 0 | 0.06 | 0 | 0.1 | | | | | 5 | 1.1 | 5 | 1.1 | 0 | 0.1 | 0 | 0.1 |
| 13 | | | 0 | 0.06 | | | | | | | | | 0 | 0.2 | 0 | 0.2 | | | 0 | 0.1 |
| 14 | | | | | | | | | | | | | | | | | | | | |

*Table 16 – Molecular weight distribution of protein fragments in the control treatment for muscle. All four replicates are presented.*

| | I | | II | | III | | IV | |
|---|---|---|---|---|---|---|---|---|
| **Peak#** | Mw | % | Mw | % | Mw | % | Mw | % |
| **Total** | 20692 | 100 | 22889 | 100 | 13731 | 100 | 12866 | 100 |
| **1** | 60621 | 28.5 | 65583 | 29.5 | 67541 | 11.7 | 68484 | 12.6 |
| **2** | 14493 | 22.3 | 15068 | 22.5 | 16278 | 34.7 | 15878 | 25.4 |
| **3** | 1551 | 0.7 | 1545 | 0.6 | 1504 | 0.8 | 1554 | 0.9 |
| **4** | 936 | 6.4 | 935 | 6.3 | 933 | 4.4 | 933 | 6.5 |
| **5** | 703 | 6.4 | 703 | 6.1 | 691 | 6.0 | 689 | 7.5 |
| **6** | 503 | 12.1 | 503 | 11.7 | 487 | 12.9 | 489 | 15.4 |
| **7** | 367 | 1.2 | 367 | 1.2 | 357 | 1.3 | 359 | 1.3 |
| **8** | 283 | 0.8 | 283 | 0.8 | 273 | 1.0 | 276 | 1.1 |
| **9** | 127 | 3.1 | 128 | 2.8 | 125 | 3.2 | 128 | 4.3 |
| **10** | 39 | 14.3 | 39 | 14.2 | 38 | 19.4 | 40 | 19.7 |
| **11** | 7 | 1.7 | 6 | 1.9 | 5 | 2.5 | 6 | 3.3 |
| **12** | 1 | 0.4 | 1 | 0.4 | 0 | 0.9 | 0 | 1.1 |
| **13** | 0 | 0.8 | 0 | 0.8 | 0 | 1.3 | 0 | 0.9 |
| **14** | 0 | 0.4 | 0 | 0.4 | | | | |
| **15** | 0 | 0.8 | 0 | 0.9 | | | | |

*Table 17 – Molecular weight distribution of protein fragments in the control treatment for skin. All four replicates are presented.*

| | I | | II | | III | | IV | |
|---|---|---|---|---|---|---|---|---|
| **Peak#** | Mw | % | Mw | % | Mw | % | Mw | % |
| **Total** | 74367 | 100 | 76779 | 100 | 80533 | 100 | 77556 | 100 |
| **1** | 408466 | 8.0 | 405978 | 9.7 | 443319 | 8.6 | 454826 | 8.8 |
| **2** | 47727 | 87.4 | 43804 | 85.3 | 48330 | 87.4 | 43051 | 87.2 |
| **3** | 1740 | 1.1 | 1734 | 1.2 | 1734 | 1.0 | 1702 | 1.1 |
| **4** | 1044 | 0.8 | 1039 | 0.9 | 1050 | 0.8 | 1033 | 0.7 |
| **5** | 736 | 0.2 | 733 | 0.2 | 732 | 0.2 | 731 | 0.2 |
| **6** | 619 | 0.3 | 616 | 0.4 | 616 | 0.3 | 613 | 0.3 |
| **7** | 546 | 0.1 | 542 | 0.1 | 542 | 0.1 | 541 | 0.1 |
| **8** | 486 | 0.3 | 484 | 0.3 | 483 | 0.2 | 482 | 0.2 |
| **9** | 392 | 0.1 | 389 | 0.1 | 390 | 0.1 | 390 | 0.1 |
| **10** | 313 | 0.1 | 310 | 0.1 | 312 | 0.0 | 310 | 0.0 |
| **11** | 129 | 0.1 | 127 | 0.1 | 128 | 0.0 | 128 | 0.1 |
| **12** | 45 | 0.4 | 44 | 0.4 | 44 | 0.4 | 44 | 0.4 |
| **13** | 6 | 1.1 | 6 | 1.2 | 7 | 0.7 | 7 | 0.8 |
| **14** | 0 | 0.0 | 0 | 0.0 | 1 | 0.1 | 1 | 0.1 |

*Table 18 – Molecular weight distribution of protein fragments in the control treatment for bone. All four replicates are presented.*

| Peak# | I Mw | I % | II Mw | II % | III Mw | III % | IV Mw | IV % |
|---|---|---|---|---|---|---|---|---|
| **Total** | 42505 | 100 | 41677 | 100 | 36135 | 100 | 35920 | 100 |
| **1** | 50188 | 84.5 | 49914 | 83.3 | 44813 | 80.4 | 44318 | 80.9 |
| **2** | 1664 | 1.3 | 1669 | 1.3 | 1652 | 1.4 | 1645 | 1.4 |
| **3** | 1009 | 1.3 | 1015 | 1.3 | 1013 | 1.3 | 1010 | 1.3 |
| **4** | 693 | 1.7 | 690 | 1.8 | 688 | 2.2 | 690 | 2.2 |
| **5** | 505 | 3.6 | 501 | 4.0 | 496 | 4.8 | 498 | 4.6 |
| **6** | 363 | 0.9 | 363 | 1.0 | 360 | 1.1 | 360 | 1.1 |
| **7** | 275 | 0.7 | 276 | 0.7 | 274 | 0.9 | 273 | 0.9 |
| **8** | 189 | 0.1 | 192 | 0.2 | 189 | 0.2 | 189 | 0.2 |
| **9** | 130 | 0.5 | 130 | 0.5 | 130 | 0.6 | 129 | 0.6 |
| **10** | 40 | 3.2 | 41 | 3.6 | 41 | 4.2 | 40 | 4.2 |
| **11** | 5 | 1.9 | 6 | 2.1 | 6 | 2.5 | 5 | 2.3 |
| **12** | 0 | 0.2 | 0 | 0.2 | 0 | 0.3 | 0 | 0.3 |

# A 5 Bioactivity

## A 5.1. Determination of hydrolysates' test concentration for antioxidant assay.

Based on results shown in Table 19, test concentration of 100 µg/mL was considered to be high relatively to Trolox concentrations used for plotting of the standard curve, while the next tested concentration 10 µg/mL was relatively low. Therefore, the decision was made to test all the hydrolysates at concentration 50 µg/mL.

*Table 19 – Concentration test for antioxidant assay.*

| Enzyme producer of hydrolysate | Test concentration (µg/mL) | Trolox equivalent (µM) | | |
|---|---|---|---|---|
| | | Muscle | Skin | Bone |
| **Alcalase** | 100 | 4.3 | 3.3 | 2.5 |
| | 10 | 0.1 | 0.0 | 0.0 |
| | 1 | -0.7 | -0.8 | -0.9 |
| | 0.1 | -1.0 | -1.0 | -1.0 |
| **Tail 190** | 100 | 4.7 | 2.5 | 2.9 |
| | 10 | 0.2 | -0.1 | -0.1 |
| | 1 | -0.8 | -0.8 | -0.9 |
| | 0.1 | -1.0 | -0.9 | -1.0 |
| **Endocut 02** | 100 | 4.5 | 3.3 | 2.7 |
| | 10 | 0.3 | 0.0 | 0.1 |
| | 1 | -0.7 | -0.9 | -0.7 |
| | 0.1 | -0.9 | -1.0 | -0.8 |

**A 5.2. Determination of hydrolysates' test concentration for anti-inflammatory assay.**

As Table 20 shows, no correlation between concentrations and inhibition was observed, indicating that the assay was not performed correctly. Therefore, due to time limitation, the decision was made to use concentration 100 µg/mL for testing all the hydrolysates as the concentration in the middle of the tested range.

*Table 20 – Concentration test for anti-inflammatory assay.*

| Raw material | Enzyme used to produce hydrolysate | Concentration (µg/mL) | Inhibition (%) |
|---|---|---|---|
| **muscle** | Tail 194 | 500 | 32.2 |
| | | 250 | 69.5 |
| | | 100 | 31.1 |
| | | 50 | 36.7 |
| | | 25 | 32.3 |
| | Promod 144GL | 500 | 63.9 |
| | | 250 | 24.3 |
| | | 100 | 34.2 |
| | | 50 | 34.1 |
| | | 25 | 54.8 |
| **skin** | Corolase 2TS | 500 | 28.2 |
| | | 250 | 64.1 |
| | | 100 | 18.8 |
| | | 50 | 51.1 |
| | | 25 | 59.8 |
| | Tail 189 | 500 | 35.3 |
| | | 250 | 35.9 |
| | | 100 | 18.6 |
| | | 50 | 47.6 |
| | | 25 | 36.8 |
| **bone** | Alcalase | 500 | 8.7 |
| | | 250 | 23.3 |
| | | 100 | 18.7 |
| | | 50 | 11.7 |
| | | 25 | 74.5 |
| | Endocut 02 | 500 | 56.4 |
| | | 250 | 70.2 |
| | | 100 | 65.8 |
| | | 50 | 36.9 |
| | | 25 | 70.4 |

## A 5.3. Survival rate of human melanoma cells

*Table 21 – Survival rate of human melanoma A2058 cells in Aqueous One Solution Cell Proliferation assay for hydrolysates (average of two replicates) from cod muscle, skin and bone produced by 23 enzymes and control (without addition of enzyme). Final concentration of hydrolysates was 1 mg/mL.*

| Enzyme | Survival rate (%) | | |
|---|---|---|---|
| | muscle | skin | bone |
| Alcalase | 114.7 | 94.6 | 102.6 |
| Bromelain | 115.6 | 96.6 | 99.0 |
| Corolase 2TS | 113.1 | 93.2 | 100.9 |
| Corolase 7090 | 113.3 | 97.7 | 99.0 |
| Tail 10 | 95.9 | 99.5 | 89.6 |
| Tail 189 | 106.5 | 97.2 | 90.9 |
| Tail 190 | 96.5 | 102.6 | 95.4 |
| Tail 191 | 96.1 | 96.8 | 86.5 |
| Tail 192 | 117.7 | 96.6 | 82.0 |
| Tail 193 | 108.2 | 97.2 | 81.0 |
| Tail 194 | 110.5 | 99.7 | 84.3 |
| Tail 197 | 109.6 | 95.9 | 81.6 |
| FoodPro 30L | 113.2 | 103.0 | 74.4 |
| FoodPro 51FP | 101.6 | 105.8 | 75.0 |
| FoodPro PNL | 108.3 | 103.0 | 75.4 |
| Veron L10 | 107.2 | 104.5 | 75.1 |
| Flavourzyme | 115.8 | 91.8 | 113.8 |
| Protamex | 113.4 | 92.9 | 107.2 |
| Promod 144GL | 119.9 | 84.6 | 100.3 |
| Promod P950L | 113.7 | 91.1 | 98.3 |
| Endocut 01 | 106.2 | 103.1 | 100.4 |
| Endocut 02 | 107.9 | 97.7 | 93.9 |
| Endocut 03 | 116.8 | 101.4 | 88.0 |
| Control | 104.2 | 98.4 | 75.5 |

# A 5.4. Anti-inflammatory properties of hydrolysates

*Table 22 – Inhibition of TNF-α expression by THP-1 cells stimulated by LPS. Test concentration 100 µg/mL.*

| Enzyme | Inhibition of TNF-alpha production (%) | | |
|---|---|---|---|
| | **Muscle** | **Skin** | **Bone** |
| **Alcalase** | -1.3 | 8.1 | 14.9 |
| **Bromelain** | 1.9 | 6.7 | 13.8 |
| **Corolase 2TS** | -3.7 | -1.5 | 13.3 |
| **Corolase 7090** | -2.0 | -11.2 | 23.7 |
| **Tail 10** | -7.7 | -11.2 | 19.3 |
| **Tail 189** | -0.8 | -10.7 | 23.1 |
| **Tail 190** | 6.6 | -2.8 | 12.6 |
| **Tail 191** | 2.9 | -19.9 | 21.4 |
| **Tail 192** | -9.7 | -12.6 | 14.3 |
| **Tail 193** | 7.1 | -14.5 | 7.1 |
| **Tail 194** | 3.8 | -7.4 | 18.4 |
| **Tail 197** | 0.9 | 19.5 | 8.7 |
| **FoodPro 30L** | 13.2 | -8.7 | 0.9 |
| **FoodPro 51FP** | 15.2 | -12.0 | -2.9 |
| **FoodPro PNL** | 17.9 | 1.7 | 28.8 |
| **Veron L10** | 13.3 | 14.3 | 17.0 |
| **Flavourzyme** | 18.2 | -2.5 | 15.2 |
| **Protamex** | 19.7 | -1.2 | 17.9 |
| **Promod 144GL** | 17.9 | -10.9 | -3.7 |
| **Promod P950L** | 15.4 | 16.1 | 2.4 |
| **Endocut 01** | 1.9 | 1.2 | -5.2 |
| **Endocut 02** | -2.9 | -6.5 | 3.5 |
| **Endocut 03** | 6.9 | -8.7 | 26.0 |
| **Control** | 3.3 | 16.2 | 18.4 |