Isolation, hydrolysation and bioactive properties of collagen from cod skin

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Einangrun, vatnsrof og lífvirkni kollagens úr þorskroði

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Abstract

Previous studies have indicated that collagen peptides have a biological activity. Positive results have been obtained from human studies, specifically those who are related to improving joint conditions. Collagen is mainly produced from bovine hides and pigskin but there is a market for collagen from other sources for example marine sources.

The overall aim of the project was to create a valuable product from cod skin. A lot of cod skin and other by-products are thrown away every year so it is important finding a solution to decrease that because of its great deal of variable nutrients.

The aim of this project was divided into three steps. The first step was to find a method to isolate collagen from cod skin with good yields. The second step was to hydrolyse the collagen with various enzymes in order to get different degree of hydrolysis. The third step was measuring the bioactivity of hydrolysed collagen. The aim was seeing what effect different degree of hydrolysis had on the bioactivity of collagen and what affect each enzyme had on the collagen. The enzymes that were selected for the hydrolysis step were: Alcalase, Flavourzyme, Neutrase, Protamex, Tail-37 and TZ-02-L and the bioactivity measurements that were performed on the collagen hydrolysates were: ACE-inhibiting, elastase-inhibiting, Metal Chelating, ORAC and Reducing power.

The isolation step was successful and collagen was isolated from cod skin with good yields. Collagen was hydrolysed with various enzymes and the type and ratio of enzymes effected the outcome of degree of hydrolysis and bioactivity. Values for antioxidant activity of collagen peptides were low. The results from this project indicate that collagen from cod skin may have inhibiting effect on elastase and can be good for skin care.

Key words: Collagen, cod skin, hydrolysis, degree of hydrolysis, bioactivity.

Ágrip

Rannsóknir hafa sýnt fram á að kollagen peptíð hafa ýmsa lífvirkni. Jákvæðar niðurstöður hafa fengist úr rannsóknum á mönnum, sérstaklega tengdar jákvæðum áhrifum á liði. Kollagen er aðallega framleitt úr nauta- og svínaskinni en það er markaður fyrir kollageni úr öðrum afurðum t.d. fiskafurðum.

Heildar markmið verkefnisins var að framleiða verðmæta vöru úr þorskroði. Miklu magni af þorskroði og öðrum hliðarafurðum er hent árlega og er því mikilvægt að finna lausn til að minnka það magn og sérstaklega vegna þess að roðið inniheldur mikið magn næringarefna.

Markmið þessa verkefnisins má skipta upp í þrjú skref. Fyrsta skrefið var að setja upp aðferð til þess að einangra kollagen úr þorskroði með góðum heimtum, annað skrefið var að vatnsrjúfa kollagen með mismunandi ensímum til þess að fá sem hæst DH gildi og þriðja skrefið var að mæla lífvirkni kollagen peptíða. Markmiðið var að athuga hvaða áhrif mismunandi DH gildi höfðu á lífvirknina. Ensímin sem voru valin í vatnsrofið voru: Alcalase, Flavourzyme, Neutrase, Protamex, Tail-37 and TZ-02-L og lífvirknimælingarnar sem voru gerðar: ACE-inhibiting, elastase-inhibiting, Metal Chelating, ORAC and Reducing power.

Einangrunarskrefið var árangursríkt og kollagen var einangrað úr þorskroðinu með góðum heimtum. Kollagenið var vatnsrofið með mismunandi ensímum og tegund og hlutfall ensíma hafði áhrif á stig vatnsrofs og lífvirkni. Niðurstöðurnar sýndu fram á lág gildi í andoxunarmælingum en niðurstöðurnar sýndu einnig fram á að kollagen úr þorskroði geti haft hamlandi áhrif á elastase og haft góð áhrif á húðina.

Lykilorð: Kollagen, þorskroð, vatnsrof, stig vatnsrofs, lífvirkni.

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Table of contents

List of tables

.

List of figures

List of equations

Abbreviations

ACE	Angiotensin converting enzyme
AnV	Anisidine value
ASC	Acid-Soluble Collagen
AUC	Area under the curve
CFF	Cross Flow Filtration
DH	Degree of hydrolysis
DTT	Dithiothreitol
ECM	Extra cellular matrix
EP	Enzyme product
ES	Enzyme substrate
Fe^{2+}	Heme
Fe^{3+}	Hemin
FPH	Fish protein hydrolysates
FPLC	Fast protein liquid chromatography
Gly	Glycine
H_2SO_4	Sulfuric acid
HPLC	High performance liquid chromatography
Hyp	Hydroxyproline
LOX	Lipoxygenase
MC	Metal Chelating
NaOH	Natrium hydroxyde
NFF	Normal Flow Filtration
$^1O_2$	Singlet oxygen
$^3O_2$	Triplet oxygen
OPA	o-phthaldialdehyde
ORAC	Oxygen radical absorbance capacity
Pro	Proline
PSC	Pepsin-Soluble Collagen
ROS	Reactive oxygen species
RP	Reducing Power
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFF	Tangential Flow Filtration

1 Introduction

The Atlantic cod is found in the North Atlantic ocean (Cohen, 1990). For the last 5 years the amount of cod caught in Iceland has increased of approximately 50 tons annually (HagstofaIslands, 2015). That means there will be more by-products from the fish, for example fish skin. Most of the by-products are thrown away or sold at a low price for animal feed (Chalamaiah, Hemalatha, & Jyothirmayi, 2012; Venugopal, 2009).

The biggest part of the cod skin is water, approximately 75%. Proteins are the biggest part of the dry material. Previous studies have shown that the highest yield of collagen from cod skin is 17% (Gudmundsson & Hafsteinsson, 1997). Collagen is a structural protein found in all animals (Shoulders & Raines, 2009).

Various methods have been used to isolate collagen from cod skin and they have different steps with various materials. In order to isolate the collagen from cod skin it is necessary to remove non-collagenous proteins and fat. The colour of collagen is important, and in order to get white collagen it is necessary to remove all the pigments.

Proteins can be broken down into smaller peptides using enzymatic conversion. That process is called protein hydrolysis (Chalamaiah *et al.*, 2012) and is used to improve the functional properties of the protein (Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011).

The overall aim of this project was to create a valuable product from cod skin. A lot of cod skin and other by-products are thrown away annually so it is important to try to find a solution to decrease that. The by-products are rich of variable nutrients so it is a waste to throw it away. The aim of this study can be divided into three steps. The first step was to find a method to isolate collagen from cod skin with good yields. The second step was to hydrolyse the collagen with various enzymes in order to get different degree of hydrolysis. The third step was measuring the bioactivity of hydrolysed collagen. The aim was to see what effect different degree of hydrolysis had on the bioactivity of collagen and what effect each enzyme had on the collagen. The enzymes that were selected for the hydrolysis step were: Alcalase, Flavourzyme, Neutrase, Protamex, Tail-37 and TZ-02-L, and the bioactivity measurements that were performed on the collagen were: ACE-inhibiting, elastase-inhibiting, Metal Chelating, ORAC and Reducing power.

2 Review of the literature

2.1 Cod

The Atlantic cod (*Gadus morhua*) is a teleost- and demersal fish from the *gadidae* family (Cohen, 1990) (Figure 1). The cod can live up to 30 years and weigh up to 50 kg (Matís, 2015). Its color varies depending on its age of the environment it lives in. The young cods are browner while the older ones are gray. The cod has a stripe on its lateral line and dark spots on the dorsal side (Matís, 2015).

Figure 1. Cod (*Gadus morhua*) (Hlíðberg, 2006).

Figure 2 below shows the geographic distribution of cod. It is found in the North Atlantic Ocean, around Iceland, on the east and west coasts of Greenland, the coast of North America, and on the coast of Europe, from the Bay of Biscay and to the Barents Sea (Cohen, 1990).

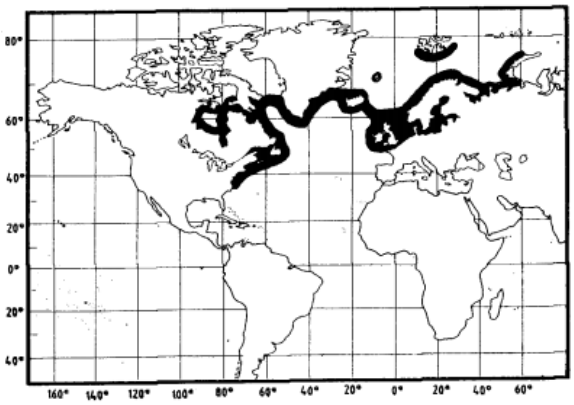


Figure 2. Geographic distribution of Atlantic cod (Cohen, 1990).

Table 1 shows the amount of cod caught in Iceland from 2010 to 2015. For the last four years the catch has amounted to over 200.000 tons each year (HagstofaIslands, 2015) of which 3% is skin (Arason, 2012).

Table 1. Cod caught in Iceland 2010-2015 (tons) (Hagstofalslands, 2015).

Year	Weight (tons)	Skin (tons)
2015	224.657	6.740
2014	239.951	7.199
2013	236.303	7.089
2012	204.955	6.149
2011	182.353	5.471
2010	178.597	5.358

2.2 Protein

The protein molecules are made up of sequences of different amino acids (Lehninger, 2008). The body can choose from 20 different amino acids, they are divided into indispensable amino acids and dispensable amino acids. Nine of those 20 amino acids are indispensable whereas the body cannot make them itself, while 11 of the amino acids are dispensable, meaning that the body can make them if it has enough of nitrogen, carbon, hydrogen and oxygen available (Insel, 2011).

2.2.1 Protein structure

There are four levels of protein structure: primary, secondary, tertiary and quaternary (Lehninger, 2008). The primary structure of a protein is a linear sequence where the amino acids are forming one or more polypeptide chains. The secondary structure occurs when the polypeptide chains are coiling or folding caused by amino acid chains being linked together by hydrogen bonds (Buxbaum, 2015). In the tertiary structure the protein has a three-dimensional shape and side groups are interacting with one another as well as the surrounding fluid environment. Some proteins have a quaternary structure formed by all the polypeptide chains that make the protein, shaping the final three-dimensional structure of the protein (Buxbaum, 2015; Lehninger, 2008).

2.2.2 Amino acids

Amino acids contain amino ($-NH_2$) and carboxyl (-COOH) groups, connected to the same carbon atom. They have side groups which vary between amino acids, defining them (Lehninger, 2008). Peptide bonds are covalent bonds that connect the amino acids together (Alberts, 2002). To make a peptide bond, the amino group and the carboxyl group of two amino acids needs to link together (Insel, 2011). That happens when a nitrogen atom from NH_2 shares electrons with the carbon atom from the other amino acid, a process wherein a water molecule (H_2O) is released (Alberts, 2002). Figure 3 below shows the process. The amino acids are in a specific order within each type of protein, called an amino acid sequence (Bray, 2010).

A dipeptide consists of two amino acids linked by a peptide bond, a tripeptide has three amino acids are connected by peptide bonds, an oligopeptide consists of 4-10 linked amino acids and polypeptide refers to more than 10 amino acids joined by a peptide bonds (Insel, 2011).

Amino acids differ in their interactions with water and in their interaction with each other. This difference affects how they contribute to protein stability as well as protein function (Petsko, 2004). Amino acids can be hydrophobic, hydrophilic and amphipathic (Petsko, 2004).

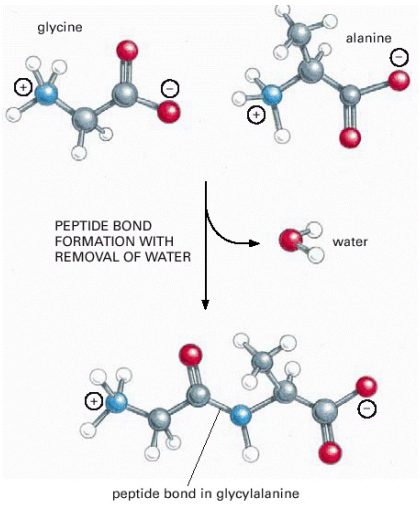


Figure 3. Formation of peptide bond (Alberts, 2002).

2.3 Collagen

Collagen is an structural protein found in all animals, with 28 different types of collagen have been identified (Shoulders & Raines, 2009). It is found in the connective tissue throughout the body, for example in the bones, tendons, cartilage, skin, teeth and in the muscles. Collagen gives viscosity in the muscles (Srinivasan Damodaran, 2008). It forms a triple helix, because of its geometric buildings, and a high molecular weight (Mohammad, 2014).

Type I collagen is the main collagen component of the skin and muscles of marine fish (Sikorski, 1994). Type III can also be found in skin but the quantity varies between persons depending on age (Mohammad, 2014). Collagen is the oldest protein discovered to date, having been found in the soft tissues of the fossilized bones of a 68 million years old Tyrannosaurus rex (Shoulders & Raines, 2009). Table 2 below shows the composition of different collagen.

Table 2. Composition of different collagen (Lodish, 2000).

Type	Structural features	Representative tissues
I	300-nm-long fibrils	Skin, tendon, bone, ligament, dentin, interstitial tissues
II	300-nm-long fibrils	Cartilage, vitreous humor
III	300-nm-long fibrils; often with type I	Skin, muscle, blood vessels
V	390-nm-long fibrils with globular N-terminal domain; often with type I	Similar to type I; also cell cultures, fetal tissues

2.3.1 Structure of collagen

In 1940 the collagen molecule was thought to be comprised of a single extended polypeptide chain and also that all amide bonds were belived to be in cis conformation. But in 1951 a theory was proposed for the correct structure of the α-helix and the β-sheet (Shoulders & Raines, 2009).

The base for the molecular structure of collagen is three intertwining helical polypeptide chains are the base for the molecular structure of collagen (Ramachandran, 1976). Each one of those chains forms an left-handed helix. These three chains coil around each other in a polyproline II-type (PPII) helical conformation, forming a right-handed triple helix. The PPII helices are packed tight within the triple helix, mandating that every third residue is Glycine (Shoulders & Raines, 2009). The pattern is X-Y-Gly and it occurs in all types of collagen. The X and Y can be any amino acid (Berisio, Vitagliano, Mazzarella, & Zagari, 2002) but the most common are proline (28%) and hydroxyproline (38%) (Figure 5). The most common triplet in collagen is ProHypGly (Gomez-Guillen *et al*., 2011; Shoulders & Raines, 2009).

The amino acid composition of collagen is very unique as it has an exclusive degree of symmetry in the general nature. Glycine forms a little less than one third of total number of residues in the chain, 25% of the collagen chain is formed by Proline and Hydroxyproline. Other amino acids present in collagen are Alanine, which counts for approximately 10%, and the amino acids Arginine, Lysine, Aspartic acid and Glutamic acid, which together form 20% of the amino acid residues. Those amino acids play an important role in the inter-triple-chain connections that lead to fibril formation (Ramachandran, 1976). The chemical structure of the amino acids is shown in Figure 4.

Proline and Hydroxyproline have a unique structure, where the C^b atom is connected to the peptide nitrogen by a side chain, forming a 5-membered ring. The peptide unit, connecting to the Proline and Hydroxyproline has a little freedom of rotation about the $N+C^a$ bond. In that way, Proline and Hydroxyproline stabilize the collagen structure (Ramachandran, 1976).

The estimation of the amount of collagen in the animals is usually based on the contents of Hydroxyproline by use of a conversion factor (Sikorski, 1994). Since the amount of Hydroxyproline is different in various species of fish and also in different tissues in the body different conversion factors are used (Sikorski, 1994).

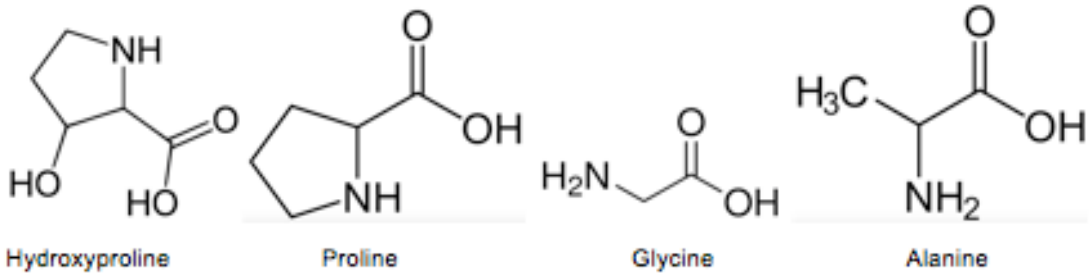


Figure 4. Amino acids: Hydroxyproline, Proline, Glycine and Alanine.

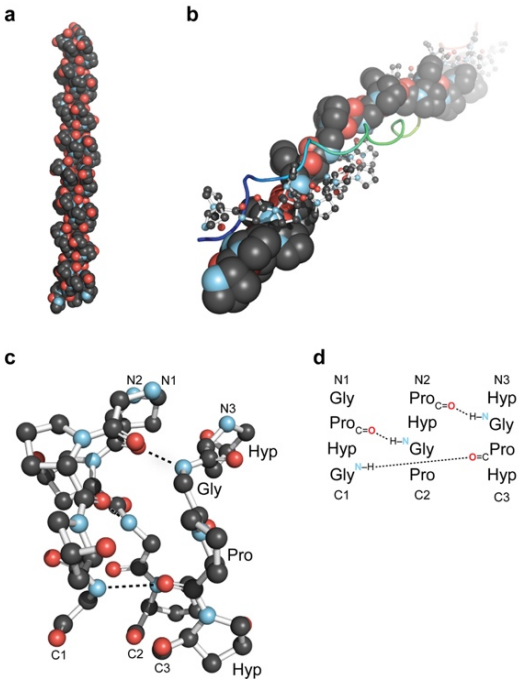


Figure 5. Overview of the collagen triple helix. (a) Crystal structure of collagen triple helix. (b) View of the axis of the triple helix. (c) Segment of the collagen triple helix. (d) Stagger of the segment in panel c of the three strands (Lodish, 2000).

2.3.2 Demand for collagen

In recent years consumers have shown increased interest in the relationship between diet and health (Harnedy & FitzGerald, 2012). Today's consumers are generally aware of the importance of a healthy diet and they prefer their food that have the possibility to promote good health and reduces the risk or delays the beginning of disease (Harnedy & FitzGerald, 2012).

Studies have indicated a biological activity of collagen peptides, both in animal models and human studies. Positive results have been obtained from the human studies, specifically those who are related to improving joint conditions. Based on positive results in that regard, collagen hydrolysates have been used in pharmaceutical and dietary supplements because of their indicated ability as mentioned above to improve joint conditions (Gomez-Guillen *et al*., 2011).

Collagen and hydrolysed collagen are mainly produced from bovine hides and pigskin. For various reasons there is a market for collagen deriving from other sources. For example, some people fear the use of cattle gelatin because of the outbreak of mad cow disease (Sadowska, Kolodziejska, & Niecikowska, 2003). As for pigskin, some religions forbid the consumption of pork (Mohammad, 2014). It would therefore be beneficial to find a source of collagen from seafood that is healthy for consumers, because there is clearly a market for that (Mohammad, 2014).

More than half of the fish catch is disposed of as processing waste or by-product (Je, Qian, Byun, & Kim, 2007). The amount of food that goes to waste increases each year, despite ongoing discussions on how to reduce food waste (Nagai & Suzuki, 2000; Raghavan & Kristinsson, 2009). The by-products from fish processing are heads, backbones, viscera, blood and skin, with most of it being thrown back into the sea or sold to animal feed manufacturers at a low price (Chalamaiah *et al*., 2012; Venugopal, 2009).

Therefore it is important to try to exploit those side streams, using the raw materials to process valuable products (Liceaga-Gesualdo & Li-Chan, 1999; Nagai & Suzuki, 2000).

2.4 Hydrolysis

Proteins can be broken down into smaller peptides, called protein hydrolysis, using enzymatic conversion (Chalamaiah *et al*., 2012). Dietary proteins contain biologically active peptides, which can be set free during gastrointestinal digestion or food processing, but are inactive in the parent protein sequence. When those bioactive peptides are released from the parent protein sequence, they can have an effect on various physiological functions of the organism. Collagen and gelatin have widely been viewed as sources of biologically active peptides, promising health benefits for both pharmaceutical and nutritional applications (Gomez-Guillen *et al*., 2011).

Hydrolysis of protein is used to improve the functional properties of the protein, e.g. solubility, foaming and emulsification (Srinivasan Damodaran, 2008). Hydrolysed collagen has been broken down into smaller peptides, making it easier to digest. Because of its easy absorption it is convenient to use in food, drinks, dietary supplements and many speciality foods (Mohammad, 2014; Srinivasan Damodaran, 2008). In recent years the request for hydrolysed collagen has escalated and it is by now a valuable ingredient in many various products, for example functional foods, dietary foods and cosmetic and pharmaceutical products (Mohammad, 2014). It has also been added to prepared diets for older people, athletes and people who needs to keep their body weight at a preferable level (Neklyudov, Ivankin, & Berdutina, 2000).

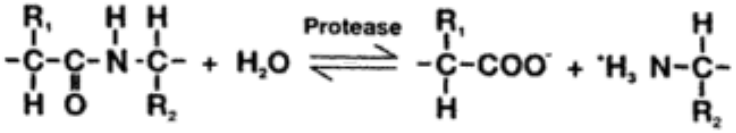


Figure 6. Hydrolysis reaction, protein broken down to peptides with enzymes (Damodaran, 1997).

In the hydrolysis reaction showed in Figure 6, one mole from both the carboxyl group and the amino group is liberated for every peptide bond cleaved by the enzyme. If all the peptide bonds in the protein are broken down, the final product will be a mixture of all amino acids that are constituent of the protein. If some peptides bonds stay unbroken, the product will be a mixture of polypeptides from the original protein (Srinivasan Damodaran, 2008).

2.4.1 Degree of hydrolysis

A degree of hydrolysis (DH) is declared as the ratio of cleaved peptide bonds in the protein hydrolysates process (Rutherfurd, 2010). Equation 1 below shows the formula for DH calculations.

Equation 1. The formula for DH.

$$\%DH = (n/nT) \times 100$$

n: number of moles of peptide bonds cleaved per mole of protein
nT: total number of moles of peptide bonds present in one mole of protein

To measure the DH the OPA method was used (Nielsen, Petersen, & Dambmann, 2001). Different methods can be used to evaluate DH. One of them is the OPA method that was developed and defined for measurement of proteolysis of milk proteins in milk. In the hydrolysis the α-amino groups are released and in the OPA method they react with o-phthaldialdehyde (OPA) and β-mercaptoethanol, together forming a compound that absorbs strongly at 340 nm (Church, Swaisgood, Porter, & Catignani, 1983). Later modifications use dithiothreitol (DTT), instead of β-mercaptoethanol as it is more environmentally friendly (Nielsen *et al.*, 2001).

2.4.2 Enzymes

In every cell there are thousands of different types of enzymes. Enzymes are proteins that all have different purpose. Their main function is to catalyze chemical reactions and they do that without being destroyed (Insel, 2011). In all enzymes there is an active site, in which there are amino acids chains that take part in binding the substrate and catalyze it. When the substrate binds to the enzyme it forms an enzyme-substrate (ES) complex which triggers the change in the enzyme and that makes catalysis possible (Harvey, 2011). At which point the ES will transform into an enzyme-product (EP) which is then cleaved into enzyme and product (Harvey, 2011).

The choice of enzyme used in the production of protein hydrolysates is very important as it affects the results and can determine both the nutritional and the functional properties. Those two factors are very important in the hydrolysis of proteins and therefore enzymatic hydrolysis can be a way to improve the products in those fields (Hrckova, 2002).

Proteases are categorized into two groups, depending on their function: endoproteases and exoproteases (Srinivasan Damodaran, 2008). Endoproteases break the bond within the peptide chains while exoproteases break the bond from either end of the peptide chains. The endoproteases make a range of polypeptides with different molecular weight, depending on the extent of hydrolysis (Hamada, 2000).

Proteases are enzymes that break proteins down into peptides and amino acids (Insel, 2011), hydrolyzing the peptide bonds that connect the amino acids together in the polypeptide chain forming protein. They belong to a certain group of enzymes that can hydrolyze by catalyzing the reaction of hydrolysis of different bonds and the do that with the participation of a water molecule (Novozymes(a),

no date). Peptidases are enzymes that break down peptide bonds in peptide units (Insel, 2011), as discussed previously in Figure 6.

Each enzyme has its own optimal conditions in relation to temperature, pH and ionic strength that differ between enzymes. The order in which enzymes are added to the reaction mixture can change the effects of each individual enzyme. When the first enzyme has brought on their reaction it becomes the substrate of the second enzyme. The order of the enzyme reactions can have an effect on the degree of hydrolysis (Liu, 2012).

In the following chapters properties of the enzymes used in the project will be discussed. The enzymes used in this project are Alcalase, Neutrase, Flavuorzyme, Protamex, Collagenase, Tail37 and TZ-02-L.

2.4.2.1 Neutrase

Neutrase is a zinc metallic endoprotease that hydrolyses internal peptide bonds randomly and it is isolated from *Bacillus amyloiquefaciens.* (Novozymes(a), no date).

2.4.2.2 Alcalase

Serine proteases have a serine group in their active site, which is necessary for them to be able to substrate binding and cleavage. Alcalase is an endoprotease of the serine type (A/S, 2002). Alcalase has been used in many studies when using collagen, because it has a wide specificity, meaning that a high degree of hydrolysis can be accomplished under moderate conditions in a short time (Gomez-Guillen *et al.*, 2011).

2.4.2.3 Protamex

Protamex is a Bacillus protease complex and is used to hydrolyze food proteins (Novozymes, no date). Protamex assisted reactions are popular in the fish industry and they have been used repeatedly for fish hydrolysis, as it can be used to accomplished high degree of hydrolysis in a relatively short time under the right conditions (Molla, 2011). Optimal pH for Protamex is 6.5-7.6 and the optimal temperature is 50-56 °C (Liaset, 2002).

2.4.2.4 Flavourzyme

Flavourzyme has both exopeptidase and endoprotease activity, it is produced by fermenting a picked strain of *Aspergillus oryzae*. It has a higher ability to release more free amino acids than for example endoprotease Alcalase (Hrckova, 2002).

2.4.2.5 Collagenase

Collagenases are enzymes that cleave the major chain of the structural collagen, they are made up by various microorganisms and different animal cells. Collagenase is secreted by the anaerobic bacteria *Clostridium histolyticum* which is the most powerful collagenase (Harper, 1980; Sigma-Aldrich, e.d.).

2.4.2.6 Tail 37 and TZ-02-L

Tail 37 and TZ-02-L are enzymes from a small startup company in Denmark named Tailorzyme (Sydmarken 32E 2860 Søborg Denmark).

2.5 Collagen isolation

Prior to the hydrolysis, collagen is usually isolated. Though different methods can be used they include many of the same steps. Table 3 summaries some of the methods that have been studied (more detailed table found in Appendix I). The first step in the isolation process is to prepare the skins. According to some studies the ideal temperature for preparation is 4°C (Nagai & Suzuki, 2000) (Y. R. Huang, Shiau, Chen, & Huang, 2011; Senaratne, Park, & Kim, 2006; M. Zhang, Liu, & Li, 2009) (Wang *et al.*, 2008) but there are some that prepare the skin at room temperature (Duan, Zhang, Du, Yao, & Konno, 2009; Gudmundsson & Hafsteinsson, 1997; Phanturat, Benjakul, Visessanguan, & Roytrakul, 2010).

Most methods require the skins to be cut into smaller pieces, as they are then easier to work with and the stirring works better. The most common method in removing non-collagenous proteins and pigments from the skin is putting the skin in a NaOH solution. This steps varies between methods, one let the skin soak for 90 minutes (Phanturat *et al*., 2010), another for 6 hours (Duan *et al*., 2009), some for 24 hours (Wang *et al.*, 2008) (M. Zhang *et al.*, 2009) and yet others for 3 days (Y. R. Huang et al., 2011) (Senaratne *et al.*, 2006). The skin:solution ratio varies also between methods, the most common ratio being 1:10 (w/v) but 1:8 (w/v) and 1:20 (w/v) has also been used.

Color, caused by pigments in the fish skin, is one of the most important features in collagen. In order for obtained collagen to become white it is important to use methods that remove those pigments from the skin (Sadowska *et al*., 2003).

Although butyl alcohol is commonly used to remove fat from the cod skin, the concentration varies between methods, from 10% butyl alcohol (Nagai & Suzuki, 2000) (Senaratne *et al*., 2006) (Y. R. Huang *et al*., 2011) to 15% butyl alcohol (M. Zhang *et al.*, 2009) can be used. Some methods do not involve alcohol but instead neutralize the skin after the NaOH step and, when the skin has reached pH 7, soak it in an acid solution. The acids that have been used are sulfuric acid (Gudmundsson & Hafsteinsson, 1997), citric acid (Gudmundsson & Hafsteinsson, 1997) and acetic acid (Phanturat *et al.*, 2010). The skin:solution ratio varies from 1:7 to 1:10.

In order to extract gelatin from the cod skin it can be soaked in tap water over night (Phanturat *et al*., 2010). Most of the methods that use butyl alcohol to remove the fat, also use acetic acid to extract gelatin from the cod skin. Some methods let the skin soak in acetic acid for only 24 hours (Wang *et al*., 2008), while others let the skin soak in the acid for 3 days (Nagai & Suzuki, 2000), (Senaratne et al., 2006), (Duan *et al.*, 2009).

Collagen isolated from cod skin using acid is called asic-soluble collagen (ASC). When the enzyme pepsin is used to increase the extractability of collagen, it is called a pepsin-soluble collagen (PSC) (Matmaroh, Benjakul, Prodpran, Encarnacion, & Kishimura, 2011; M. Zhang *et al*., 2009).

Table 3. Examples of different methods used to isolate collagen from different sources.

	Nagai and Suzuki, 2000	**Senaratne, Park, Kim, 2006**	**Zhang, Liu and Li, 2009**	**Wang, An, Yang *et.al.*, 2008**	**Huang, Shiau, Chen, 2011**	**Duan, Zhang, Du *et.al.*, 2009**	**Phanturat, Benjuakul *et.al.*, 2010**	**Guðmundsson, Hafsteinsson, 1997**
Non-collagenous protein removal	0.1 N NaOH	0.1 N NaOH	0.1 M NaOH 0.5% nonionic detergent	1.0 M NaCl (0.05 M Tris–HCl, pH 7.5)	0.1 M NaOH	0.1 M NaOH	0.1 M NaOH	Sulfuric acid
Fat removal	10% butyl alcohol	10% butyl alcohol	15% butyl alcohol		10% butyl alcohol, 0.5 M acetic acid	1% detergent	0.05 M acetic acid	Citric acid,
Remove pigments			3% H2O2 solution					
Extraction	0.5 M acetic acid	0.5 M acetic acid		0.5 M acetic acid		0.5M acetic acid	dH2O	dH2O
Centrifuge	20000xg 1 hour		9000g, 15 min 4 °C	20000g, 1h		20000xg, 1h	Cheesecloth	Büchner funnel
Supernatant	Salted out	Salted out	Salted out	Salted out	Collected	Salted out		Evaporated
Precipitate	Centrifugation	Centrifugation	Centrifugation	Centrifugation	Centrifugation	Centrifugation	Blender	Air dried
Dialysis	Dialysed	Dialysed	Dialysed	Dialysed	Dialysed	Dialysed		
Pepsin		10% pepsin	1.5% pepsin		1.5% pepsin			

2.6 Bioactive properties of fish proteins

Biologically active peptides can be found in dietary proteins, while they are inactive in the parent protein sequence they can be set free in gastrointestinal digestion, food processing and fermentation (Gomez-Guillen *et al*., 2011). The bioactive peptides that are prepared from food proteins are often small peptides (2-20 amino acids) (Chi, Wang, Wang, Zhang, & Deng, 2015). Studies have shown that bioactive peptides from fish can affect various biological functions including antihypertension (Raghavan & Kristinsson, 2009), immunomodulatory, antithrombotic, antioxidant (Klompong, Benjakul, Kantachote, & Shahidi, 2007), anti-cancer and antimicrobial activities (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). The amino acid composition, sequence, size and configuration of peptides is related to the biological activities of the protein hydrolysates (Aleman, Gimenez, Perez-Santin, Gomez-Guillen, & Montero, 2011).

2.6.1 Antioxidants and oxidation

Lipid oxidation is a sequence of chemical changes that are effected by the interaction of lipids with oxygen. In the lipid oxidation the phospholipids that are found especially in fish, disintegrate and form small volatile molecules that have off-aromas (Srinivasan Damodaran, 2008). The rate of oxidation can be affected by many various factors, like fatty acid composition, the presence and activity of antioxidants, the degree of unsaturation, the partial pressure of oxygen, the surface being exposed to oxygen and the storage conditions of food that contains fat (Belitz, 2009).

In an oxygenated environment, an oxidative stress occurs in all organisms (Srinivasan Damodaran, 2008). Oxidative damage in the body is related to various health disorders, such as diabetes, cancer, neurodegenerative and inflammatory diseases (Aleman *et al*., 2011). Reactive oxygen species (ROS) and free radicals are generated in humans during cellular respiration. They have unpaired electrons that attract electrons from other substances, which can result in oxidative stress in the cells or tissues (Chalamaiah *et al.*, 2012).

Lipid oxidation in the oxidation process can lead to lower food quality, shortening of shelf-life and potentially produce a toxic reaction in products (Aleman *et al*., 2011). In order to limit the loss of quality, the rate of the oxidation process is decreased. It is necessary to know more about the oxidation reaction to be able to take appropriate measures, for example what triggers the start of the oxidation (Belitz, 2009).

2.6.1.1 Oxidation pathway

The pathway of oxidation occurs in three steps: initiation, propagation and termination. Initiation is the first step of the oxidation process. Fatty acids (LH) produce free radicals ($L^{\bullet}$) using hydrogen abstraction (1). Reaction (2) shows how hydroxyperoxides can be separated, using heat, in order to produce alkoxyl radical ($LO^{\bullet}$). Reaction (3) shows how it is separated catalytically by metals, in order to produce alkoxyl radicals, while in reaction (4) it produces peroxyl radicals ($LOO^{\bullet}$) (Frankel, 2007). Free radicals are stabilized when the alkyl radical have been formed, with double bond shifting,

and a conjugated double bond is made in polyunsaturated fatty acids. They can be both *cis* and *trans*, but are more often *trans* and that is because of they are more stable (Srinivasan Damodaran, 2008).

(1) $LH \rightarrow L^{\bullet} + H^{\bullet}$

(2) $LOOH + M^{+} \rightarrow LO^{\bullet} + HO^{-} + M^{++}$

(3) $LOOH + M^{++} \rightarrow LOO^{\bullet} + OH^{+} + M^{+}$

(4) $2LOOH \rightarrow LO^{\bullet} + LOO^{\bullet} + H_2O$

Figure 7. The initiation step of the oxidation pathway.

The next step is propagation and there is a formation of free radicals that can that promote further oxidation. Lipid radicals react with triplet oxygen and produce peroxyl radicals ($LOO^{\bullet}$) (5), when alkyl radical ($L^{\bullet}$) combines with a radical on the triplet oxygen, a covalent bond is made and frees the other radicals on the oxygen. The peroxyl radicals react with fatty acid (LH), when hydrogen is added to peroxyl radicals, it forms primary hydroperoxide (LOOH) products and an alcyl radical ($L^{\bullet}$) (6). It can form a new alkyl radical on another fatty acid, a reaction that can spread between fatty acids (Frankel, 2007; Srinivasan Damodaran, 2008).

(5) $L^{\bullet} + {}^{3}O_2 \rightarrow LOO^{\bullet}$

(6) $LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$

Figure 8. The propagation step of the oxidation pathway.

The last step of the oxidation process is termination. Radicals interact with each other and form a stable molecular product (L-L) (8) (Frankel, 2007). When there are atmospheric conditions the reaction can be between peroxyl and alcoxyl radicals but when the environment is low in oxygen, the reactions can happen between alkyl radicals forming a fatty acid dimer (Srinivasan Damodaran, 2008).

(8) $L^{\bullet} + L^{\bullet} \rightarrow L\text{-}L$

(9) $2LO^{\bullet} \rightarrow LOOL$

(10) $L^{\bullet} + LOO^{\bullet} \rightarrow LOOL$

(11) $LO^{\bullet} + L^{\bullet} \rightarrow LOL$

(12) $LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2$

Figure 9. The termination step of the oxidation pathway.

2.6.1.2 Types of oxidation

There are several types of oxidation, for example photo-oxidation, metal-mediated oxidation and oxidation catalyzed by enzymes. Photo-oxidation is when triplet oxygen ($^{3}O_2$) reacts with radicals and the oxygen becomes single-stated ($^{1}O_2$), a reaction that can be triggered by light (Belitz, 2009; Pokorny, Yanishlieva, & Gordon, 2001). Traces of heavy metals are found in fats, oils and foods. They can be found in raw food but can also come from processing and handling equipment or even from packaging

material. Heavy metals participate in the initiation reaction when hydroperoxides are disintegrated into radicals (Belitz, 2009; Chary, Kamala, & Raj, 2008). Heme (Fe2+) and hemin (Fe3+) are proteins originating from blood, they are widely spread in the food and they affect the aroma defects and rancidity that take place in the storage of fish, poultry and cooked meat (Belitz, 2009). Enzymes can catalyze oxidation reactions, for example lipoxygenase (LOX) can catalyze the oxidation of few unsaturated fatty acids. Reactions that are catalyzed by enzymes are slightly different from the oxidation reactions. Reactions that are catalyzed by lipoxygenase are characterized with all the enzymatic catalysis features, for example substrate specificity, pH optimum, peroxidation selectivity, sensitivity to heat treatment and a high reaction rate in temperatures 0-20°C (Belitz, 2009; Srinivasan Damodaran, 2008).

The products from the oxidation reactions are both primary- and secondary. The primary products are odor- and tasteless with the most common methods to determine them being peroxide value (PV) and conjugated dienes (Belitz, 2009; Nollet & Toldrá, 2009). The secondary oxidation products come from the disintegration of fatty acid hydroperoxides. They have a bad odor as well as a bad taste. Anisidine value (AnV) and TBARS are common methods to determine secondary oxidation products (Nollet & Toldrá, 2009; Srinivasan Damodaran, 2008).

2.6.1.3 Antioxidant

As there are different reasons for oxidation, there are also different types of antioxidants. The activity of antioxidants depends on many different factors, for example the composition of the lipids, antioxidant concentration, oxygen pressure, temperature, and other antioxidants and common food components, such as protein and water, can also have an effect (Pokorny *et al*., 2001).

There are two classes of antioxidants: primary and secondary antioxidants. The primary antioxidants react with the lipid radicals in order to convert them into a more stable product. They are called free radical scavengers (Hudson, 2012; Srivastava, 2013) from the way they can delay the initiation step and also interrupt the propagation step in the oxidation (Srivastava, 2013).

The secondary antioxidants have various mechanisms and they can reduce the rate of the chain initiation (Hudson, 2012). They can chelate pro-oxidant metals and deactivate them, deactivate singlet oxygen, absorb ultraviolet radiation, renovate hydrogen to primary antioxidant, decompose hydroperoxide to non-radical species and act as oxygen scavengers (Akoh & Min, 2008).

To control the pro-oxidant metals chelators or sequestering are used. Chelators can inhibit the pro-oxidant activity of the metals with various mechanism, for example by preventing of metal redox cycling, forming insoluble complexes, occupying all metal coordination sites, and by a steric hindrance of interaction between metals and lipids (Srinivasan Damodaran, 2008).

Because of the antioxidants different activities there are many various methods to measures them. In this project there were three antioxidant measurement methods used metal chelating activity, ORAC value and reducing power. The collagens peptides' ability to chelate metals was measured with the metal chelating method. If the collagen peptides forms a complex with the metal it has inactivates it (Frankel, 2007). The ORAC method is dependent on the formation of free radicals, it measures the ability of sample to absorb oxygen and preventing it to induce oxidation. It is measured with fluorescence and the results from the ORAC assay are relative to standard (Trolox): if there is no oxidation in the

reaction there is an antioxidant activity in the sample. (Alam, Bristi, & Rafiquzzaman, 2013; Cao & Prior, 1999).

Reducing power is a method that measures the ability of a compound to reduce ferric ion (Fe^{3+}) to ferrous iron (Fe^{2+}), the samples are absorbed and if the absorbance is increased there is a greater reducing power (Oyaizu, 1986).

Figure 10 shows the activity of an antioxidant as a radical scavenger.

(1) $LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$

(2) $LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$

(3) $LOO^{\bullet} + AH \rightarrow LOOA$

(4) $LO^{\bullet} + A^{\bullet} \rightarrow LOA$

Figure 10. Activity of an antioxidant as a radical scavenger.

The biological tissues in food contain several antioxidant systems but those antioxidants are often removed in the food process. Therefor it is common to add antioxidant to the food (Srinivasan Damodaran, 2008).

Many studies have been made to measure the antioxidant activity of peptides and various hypotheses been proposed to explain the activity. Peptides have various antioxidant activity and they can be free radical scavengers, lipid peroxidation inhibitors and chelating agents (Alemán & Martínez-Alvarez, 2013).

Studies have shown that the peptide bond, the structural conformation of the peptide and the position of specific amino acids in the sequence of the peptide can affect the antioxidant activity of the peptide (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005).

Protein substrate, conditions in the hydrolysis and the specificity of the enzyme affects the antioxidant activity (Ktari *et al*., 2013) and individual amino acids can also affect the antioxidant activity, for example have Glycine and Proline from collagen showed antioxidant activity (Mendis, Rajapakse, Byun, & Kim, 2005). Hydrophobic amino acids can also affect the antioxidant activity (Mendis *et al*., 2005).

Antioxidant activity has been detected in hydrolysed peptides from various marine species. As shown in Table 4, antioxidant activity has been found in peptides processed from collagen from jumbo squid (Giménez, Alemán, Montero, & Gómez-Guillén, 2009; Mendis *et al*., 2005), Alaska Pollack (Kim *et al*., 2001), Kingfish (Nazeer & Anila Kulandai, 2012), Atlantic salmon (Opheim *et al*., 2015), Tilapia (Ngo, Qian, Ryu, Park, & Kim, 2010; Yang, Liang, Chow, & Siebert, 2009), Croaker (Kumar, Nazeer, & Jaiganesh, 2012), Silver carp (Zhong, Ma, Lin, & Luo, 2011) and Sole (Giménez *et al*., 2009).

2.7 Elastase

With aging comes change in the skin, caused by various interaction with environmental factors, such as UV light and also with genes, hormones and metabolism. Those factors affect the proteins in the extracellular matrix (ECM), for example collagen and elastin (Scharffetter–Kochanek *et al*., 2000; Thring, Hili, & Naughton, 2009).

Elastases are proteinases that belong to the class of serine proteases, cysteine proteinases and metalloproteinases, which can solubilize fibrous elastin. In mammals the elastases are found in the pancreas and the phagocytes (Bieth, 2000). Elastases participate in ECM degradation and they can cleave collagen, fibronectin and elastin (Shamamian *et al*., 2001; Takeuchi, Gomi, Shishido, Watanabe, & Suenobu, 2010).

When the skin is exposed to UV-irradiation it can increase the risk for photo-oxidative damage, which can lead to photo-aging. Photo-aging can be described as wrinkles, and loss of skin tone and resilience (Scharffetter–Kochanek *et al.*, 2000).

2.8 ACE

Angitotensin I-converting enzyme (ACE) plays a big part in controlling the blood pressure. ACE catalysis the transformation from angiotensin I to angiotensin II in the renin-angiotensin system. Angiotensin is a hormone that induces vasoconstriction, which is followed by an increase of blood pressure (Figure 11) (Möller *et al*., 2008). ACE inactivates the bradykinin (Raghavan & Kristinsson, 2009) which is a peptide in the Kallinkrein-kinin system that effects the blood pressure, it is a vasodilator that causes the blood pressure to fall (Figure 11) (Pocock & Richards, 2009).

ACE-inhibitors are therefore a good source for preventing hypertension and reducing the risk of cardiovascular diseases (Möller *et al*., 2008; Raghavan & Kristinsson, 2009). Hypertension has become a worldwide problem that affects 15-20% of all adults (Aleman *et al*., 2011). The inhibition of ACE has been used in the treatment for hypertension and it plays a big role in the development of drugs used to control high blood pressure (Möller *et al*., 2008).

Fish protein hydrolysates, including collagen (Gomez-Guillen *et al*., 2011), have been shown to be a good source for ACE inhibitory, with higher DH increasing the activity (Geirsdottir *et al*., 2011; Gomez-Guillen *et al*., 2011). ACE inhibitory hydrolysates have been obtained from both land-based sources and various marine sources (Gomez-Guillen *et al*., 2011). Studies have shown that peptides from skate (Lee, Jeon, & Byun, 2011), salmon (Gu, Li, Liu, Yi, & Cai, 2011), bovine (Y. Zhang, Olsen, Grossi, & Otte, 2013), tilapia (Roslan, Yunos, Abdullah, & Kamal, 2014) and cod (Himaya, Ngo, Ryu, & Kim, 2012) are among those with ACE inhibitory activity as shown in Table 4.

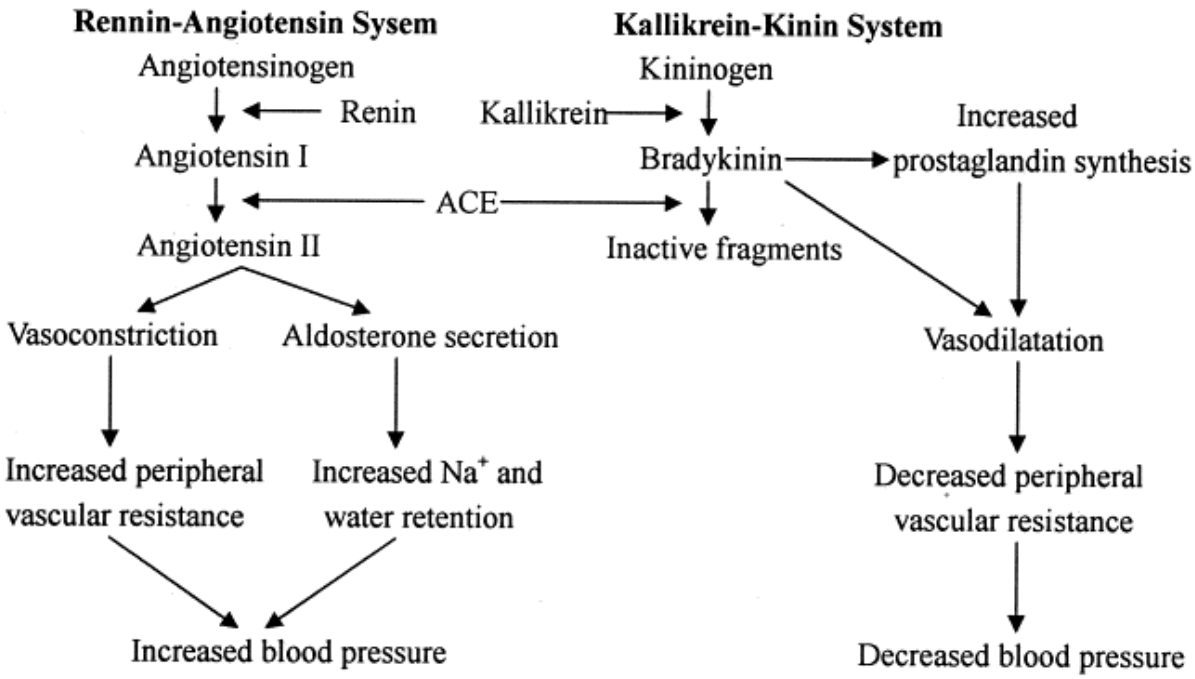


Figure 11. Role of angiotensin converting enzyme (ACE) in the regulation of blood pressure (Li, Le, Shi, & Shrestha, 2004).

Table 4. Summary bioactivity measurements on hydrolysed collagen from different sources.

Authors	Enzymes	Raw material	DH %	Bioactivity
Chi *et al.*, 2015	Trypsin, Flavourzyme, Neutrase, Alcalase	Bluefin leatherjacket by-product		DPPH, HO, and O2 scavenging activities
Giménez *et al.,* 2009	Alcalase	Squid and sole skin	40-50%	MC, FRAP and ABTS assay
Gu *et al.*, 2011	Alcalase, Papain	Atlantic salmon skin		Iron chelating activity
Guo *et al*., 2013	Trypsin	Alaska pollock skin		Iron chelating activity
Himaya *et al.,* 2012	Pepsin, Trypsin and α-chymotrypsin	Pacific cod		ACE
Kim *et al*., 2001	Collagenase,	Alaska pollock skin		DPPH, r-s activity,RPl antioxidant capacity
Ktari *et al*., 2013	Alcalase, Trypsin	Cuttlefish by-products		DPPH, r-s activity,RPl antioxidant capacity
Lee *et al*., 2011	Alcalase, Papain, Pepsin, Neutrase, Trypsin	Skate skin		IC50 for ACE inhibitory activity
Mendis *et al*. 2005	Trypsin, Pepsin	Jumbo squid skin	97	DPPH, RP and Fe2+ chelatingactivity
Nazeer & Anila Kulandai, 2012	Papain, Pepsin	Giant kingfish muscle and skin		DPPH, Hydroxyl r-s and superoxide r-s activity
Ngo *et al*., 2010	Pepsin	Nile tilapia scales		DPPH, ABTS, FRAP,antioxidant activity
Opheim *et al*. 2015	Papain, Bromelain, Protamex, Endogenous	Atlantic salmon	40-50%	DPPH, ABTS, FRAP,antioxidant activity
Phanturat *et al*. 2010	PCE, Alcalase, Neutrase	Bigeye snapper skin	5 - 25%	FRAP, chelating activity, DPPH, hydroxyl r-s activity
Rodriguez-Diaz, Kurozawa, Netto & Hubinger, 2011	Protamex	Blue shark skin	17,30%	Antioxidant activity in meat model system
Roslan *et al*. 2014	Alcalse	Tilapia by-product	20%	Antioxidant activity in meat model system
Kumar *et al*. 2012	Pepsin, Trypsin, α-chromotrypsin	Croaker skin		DPPH,
Yang *et al*., 2009	Acid/thermal hydrolysis	Tilapia skin		ABTS measurement and DPPH
Zhang *et al*., 2013	Alcalase, Collagenase, Thermolysin, Proteinase K, Pepsin, Trypsin	Bovine	12	ACE
Zhong *et al*. 2011	Alcalase, Flavourzyme, Neutrase, Papain, Pepsin, Protamex, Trypsin	Silver carp by-product	20	DPPH, OH r-s, superoxide r-s lipid peroxidation

*r-s = radical-scavenging

2.9 Filtration

Studies have shown that peptides with lower molecular weight tend to have higher bioactivity (Ahn, Lee, & Je, 2010; Jeon, Byun, & Kim, 1999). It is therefore important to think about the molecular weight when producing bioactive peptides in order to get the desired functional properties of the bioactive peptide (Kim & Wijesekara, 2010).

Filtration can be used to isolate the desired molecular size from hydrolysates. Different filtration methods are available, including tangential flow filtration.

2.9.1 Tangential flow filtration

Tangential flow filtration (TFF) is a filtration technique based on pressure differences with the starting solution passing alongside the surface of the filter (GE, 2014). TFF can be used for clarifying, concentrating and purifying proteins (Millipore, 2003).

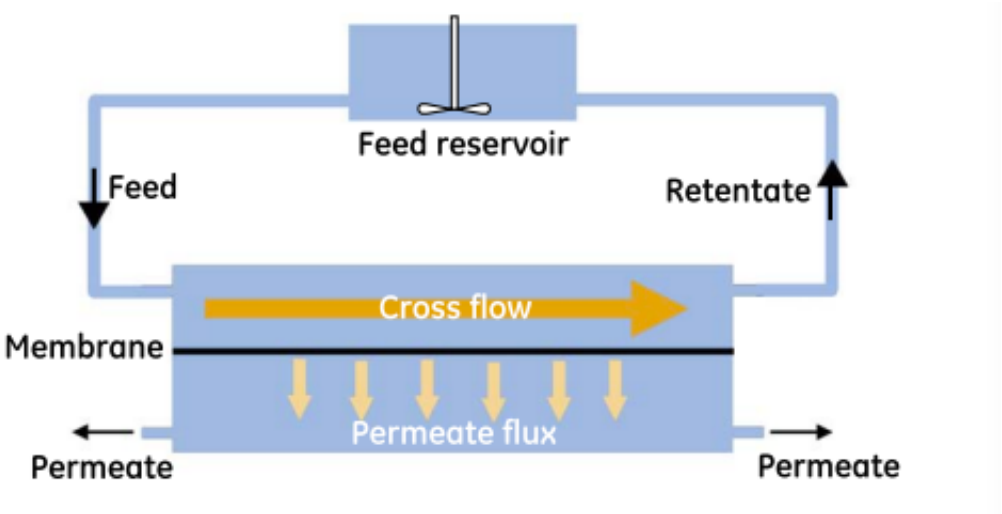


Figure 12. The function of tangential flow filtration (GE, 2014).

Figure 12 shows the function of TFF. The feed passes to the filter, components that are smaller than the filter pore through the filter (permeate) and the components that are larger than the filter are retained in the solution (retentate), where they pass along the membrane surface and goes back to the feed reservoir (GE, 2014).

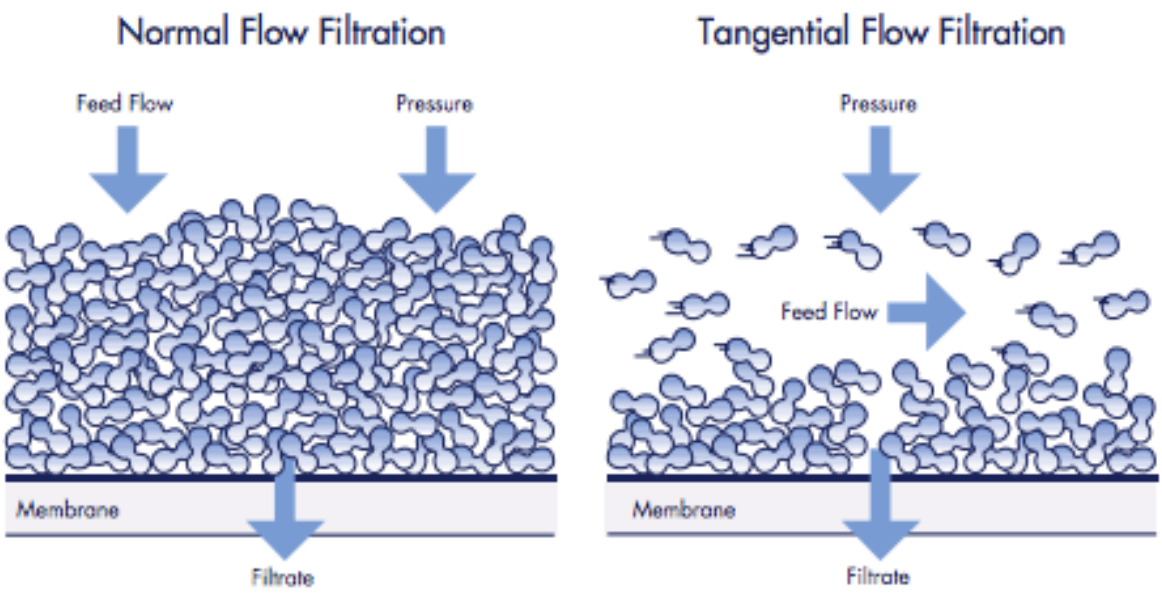


Figure 13. The difference between normal flow filtration and tangential flow filtration. (Millipore, 2003)

Figure 13 shows the difference between normal flow filtration (NFF) and TFF. In NFF the flow goes directly to the membrane, the pressure is applied and all the small components goes straight through the membrane while the molecules that are too large for the filter they are collected on the membrane surface (Millipore, 2003).

The main difference between those two methods is that TFF filters use membrane exclusively and the solution can recirculate, while in the NFF the stream only passes through the filter once (GE, 2014).

2.9.2 SDS-PAGE

Gel electrophoresis is used to get information about the molecular weight of proteins, charges and subunit, and to detect the purity of a particular protein preparation. There are many different techniques of gel electrophoresis. The most common method used for proteins is sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) (Garfin, 2003), which is used in this project.

Electrophoresis in gels that have SDS are an effective tool for the separation of polypeptide chains in complexed samples that are biological. They are used to classify and separate proteins by size (Cleveland, Fischer, Kirschner, & Laemmli, 1977).

3 Methods and materials

The project included process for the isolation, hydrolysis of collagen and further steps Figure 14. The collagen is isolated from the cod skin. Various isolation methods were studied and several of them were tested and different results were shown. The isolated collagen was used in further studies. The collagen was hydrolysed using seven different enzymes and the degree of hydrolysis was measured using the OPA method.

Bioactive measurements were performed on hydrolysed collagen peptides and chemical analysis were performed on both isolated collagen and hydrolysed collagen peptides.

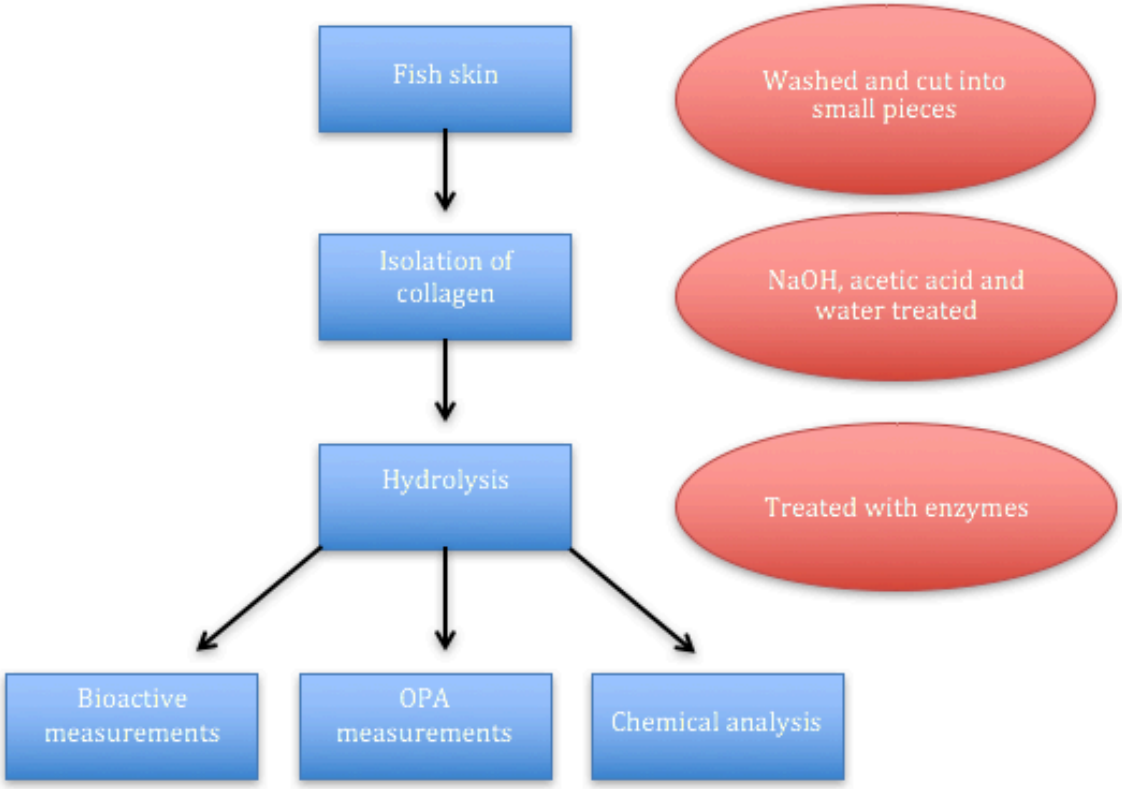


Figure 14. Flowchart of the whole process of the project.

3.1 Chemicals

Table 5. List of chemicals.

Chemicals	Company
AAPH	Sigma Aldrich
ACE from rabbit lung	Sigma Aldrich
Acetic acid	Sigma Aldrich
Alcalase	Sigma Aldrich
Butyl alcohol	Merck
Citric acid	Sigma Aldrich
Collagenase	Sigma Aldrich
Dithiothreitol (DTT) (99%)	Sigma Aldrich
Ethanol (99.5%)	Gamla apótekið
Flavourzyme	Novozymes
Ferrous chloride	Sigma Aldrich
HCl	Sigma Aldrich
H_2SO_4	Sigma Aldrich
NaOH	Sigma Aldrich
Neutrase	Novozymes
o-phthakdialdehyde (OPA) (97%)	Sigma Aldrich
Phosphate buffer	Sigma Aldrich
Protamex	Novozymes
Serine	Sigma Aldrich
sodium-dodecyl-sulfate (SDS)	Sigma Aldrich
Sodium tetraborate decahydrate	Sigma Aldrich
Tail 37	Tailorzyme
Trichloroacetic acid	Sigma Aldrich
Trolox	Sigma Aldrich
TZ-02-L	Tailorzyme

3.2 Devices

Table 6. List of devices.

Devices	Model
Centrifuge	Beckham Coulter
Freeze dryer	Virtis genesis
Incubator shaker	Innova, New Brunswick
Magnet stirrer	RCT basis
pH meter	Portamess, Knick
Spectrophotometer	Ultrospec 3000
Vortex mixer	Heidolph Reax
Waterbath	Julabo

3.3 Isolation of collagen

Three different methods for collagen isolation were tested. Different chemicals were used in each method and the processing steps varied between them. All the methods will be described step by step in the following chapters.

3.3.1 Collagen extraction with 0.1M NaOH and 0.05M acetic acid

Collagen was extracted from cod skin with method according to (Phanturat *et al*., 2010) with slight modifications. Cod skin was obtained from a local fish store Galleri fiskur and cut into small pieces. The skin was soaked in 0.1 M NaOH solution with the skin/solution ratio of 1:10 at room temperature with a gentle stirring every 10 minutes for 30 minutes and repeated three times to remove non-collagenous proteins and pigments. After this 90 minutes process the skins were washed with tap water until the pH was neutral or faintly basic, a tube with running water was put in the glass containing the skin Figure 15.

The pH was measured at room temperature using a portable pH meter (Portamess 913, Knick, Germany). The cod skins were soaked in 0.05M acetic acid with skin/solution ratio 1:10 (w/v) for 3 hours at room temperature, the solution was stirred every 10 minutes to swell the collagenous material in the fish matrix. After 3 hours acid process the cod skins were washed with water until the pH was neutral. The skins were soaked in tap water with the skin/water ratio (1:3) (w/v) at 45°C over night with stirring (100 rpm) in an incubator shaker (Innova 4430, New Brunswick, Canada) to extract gelatin from the skin.

The next day the mixture was filtered through two layers of cheesecloth and weight in aluminum trays and put in the -18° freezer and freeze-dried (Virtis genesis, USA) (Figure 16).

(Protocol of this method found in Appendix III).

Figure 15. Washing cod skin until neutral pH using tube with running water.

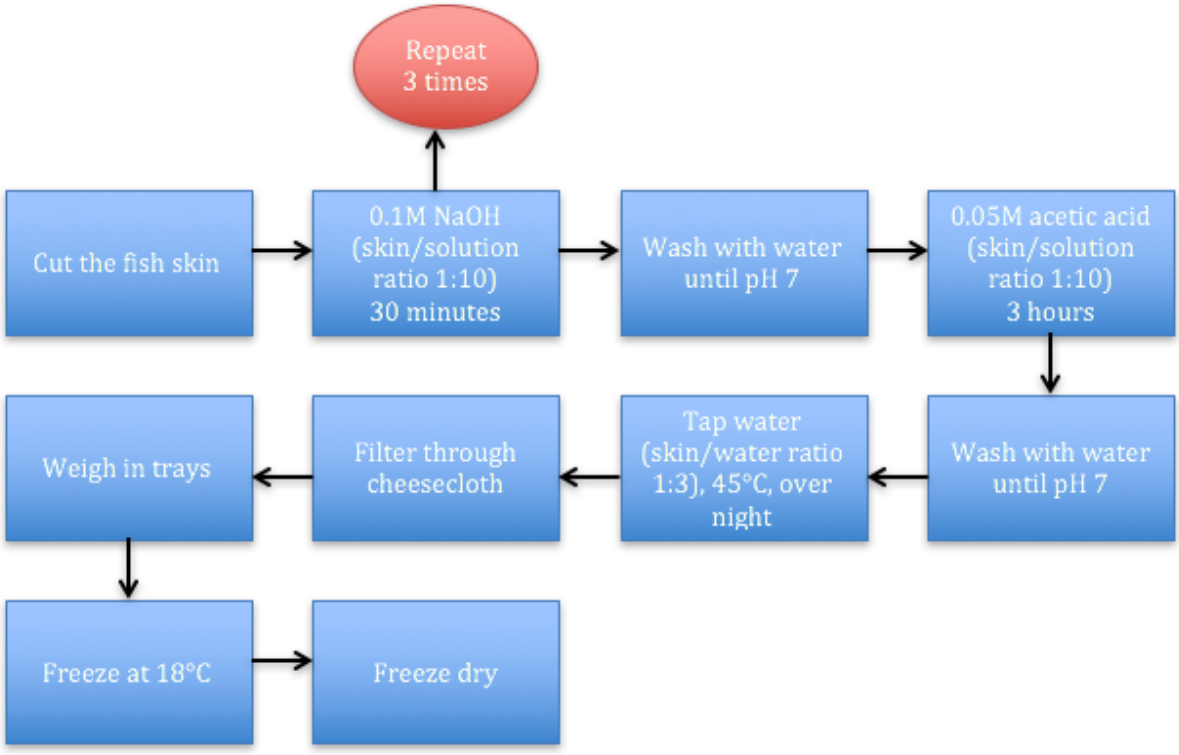


Figure 16. Flowchart of method for collagen extraction with 0.1 M NaOH and 0.05 Acetic acid.

3.3.2 Method using 0.7% Citric acid, 0.1% NaOH and 0.1% H_2SO_4

Fish skin was obtained from a local fish store Galleri fiskur. The ratio used in the next steps was 1 kg of cod skin (wet weight) to 7 l of alkali or acid solution. Tap water was used when mixing the acid and alkali solution but distilled water was used in the extraction step (Gudmundsson & Hafsteinsson, 1997).

The first step was rinsing the fish skin, followed by cutting it into small pieces and then it was treated with NaOH solution (w/v) for 40 minutes and washed with water until the pH was 7. This step was repeated 3 times. Then the skin was treated with sulfuric acid solution (w/v) for 40 minutes and washed with water until the pH was 7, it was repeated 3 times. The skin was then treated with citric acid for 40 minutes and washed with water until the pH was 7 (Gudmundsson & Hafsteinsson, 1997). The pH was measured with a portable pH meter (Portamess 913, Knick, Japan).

After the acid and alkali treatment the cod skin were soaked in water at 45°C over night without stirring. According to (Gudmundsson & Hafsteinsson, 1997) the next step was to filter the remaining solution in a Büchner funnel with Whatman no.4 filterpaper and the extract left was then evaporated under vacuum at 43-45°C and that was done until 85-90% of the water was removed.

The method was performed with slight modification. When the cod skin had soaked in water over night the solution was filtered through cheesecloth, weighed into trays, frozen and freeze-dried (Virtis genesis, USA) Figure 17.

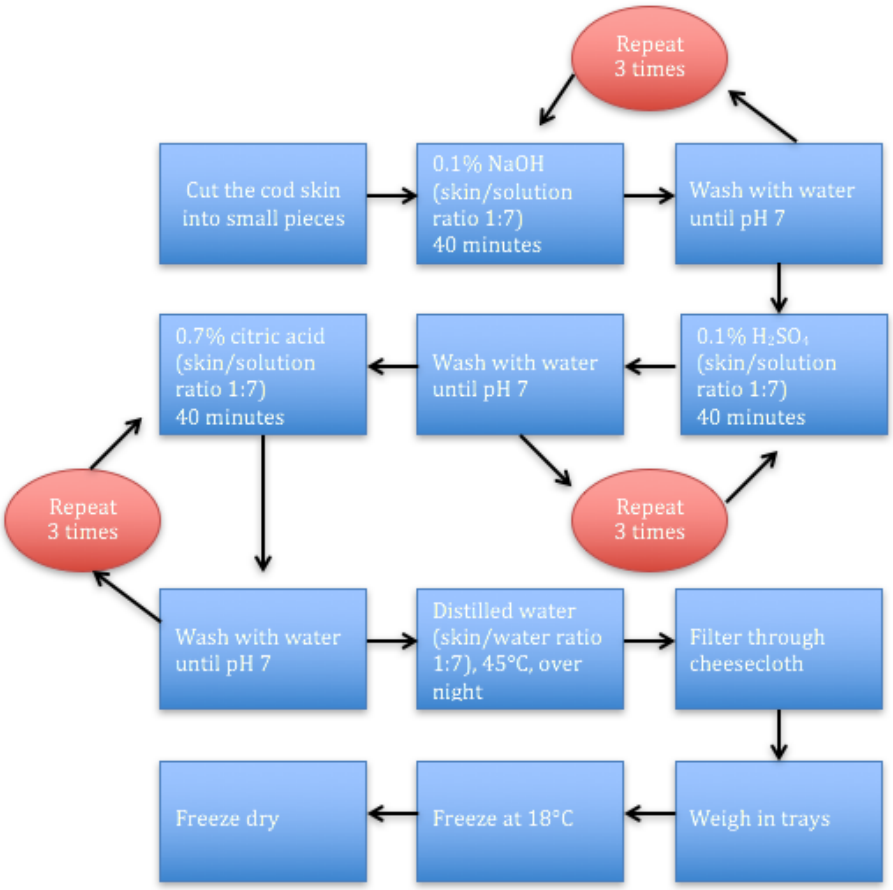


Figure 17. Flowchart of method for collagen extraction with 0.7% Citric acid, 0.1% NaOH and 0.1% H_2SO_4.

3.3.3 Method using 0.1 M NaOH, 10% Butyl alcohol and 0.5 M acetic acid

Cod skin was obtained from fish store Galleri fiskur, rinsed and soaked in 0.1 M NaOH with sample/alkali solution ratio of 1:8 (w/v) in order to remove the non-collagenous proteins. Then it was stirred for 6 hours and changed after 3 hours. After the 6 hours period the cod skin was washed with cold distilled water until the pH was neutral (Duan *et al.,* 2009).

According to (Duan *et al.*, 2009) 1,0% detergent was used to extract fat but the method was made with slight modification. The cod skin was soaked in 10% butyl alcohol over night with the sample/solution ratio 1:10 (w/v) then it was washed with cold water.

Then the cod skin was cut into small pieces so it would be easier to work with. The skin was extracted with 0.5 M acetic acid for 3 days with some stirring, the sample/solution ratio 1:6 was used. The extract from the acid step was centrifuged at 20000xg for 1 hour (Beckham Coulter, USA). NaCl was added to a final concentration of 2.5 M and 0,05 M tris (hydroxymethyl) aminomethane was used to reach pH 7 to the salt-out the supernatant. When 2 l of 0.05 M tris (hydroxymethyl) aminomethane had been mixed with the solution the pH had not yet reach 7. The salting-out step was repeated with 0.5 M tris (hydroxymethyl) aminomethane. The pH reached 7 and was then centrifuged (Beckham Coulter, USA) Figure 18.

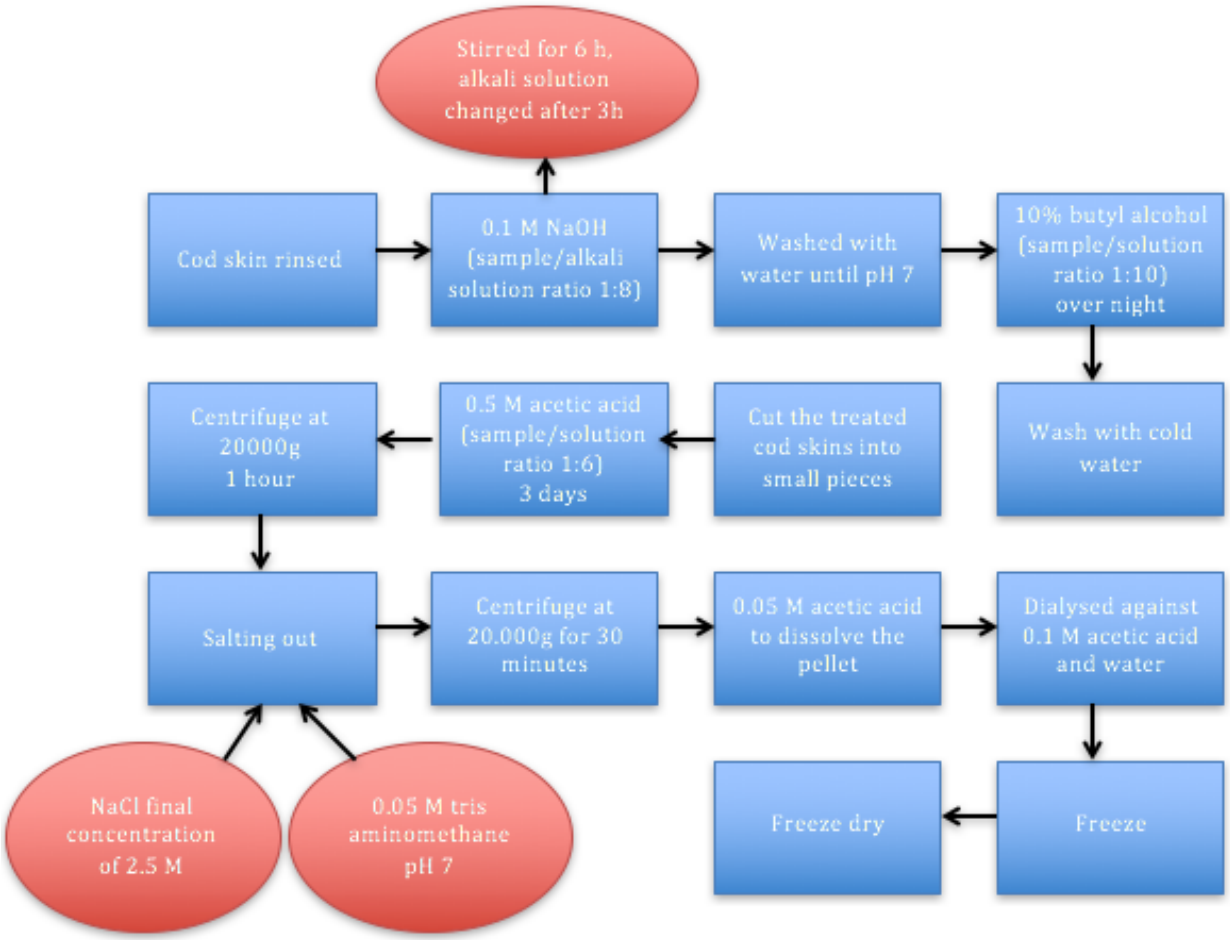


Figure 18. Flowchart of method for collagen extraction with 0.1 M NaOH, 10% Butyl Alcohol and 0.5 M Acetic acid.

3.4 Hydrolysis of collagen

The isolated collagen was hydrolysed with various enzymes. It was decided to keep the same temperature (45°C) and hydrolysis time for all the enzymes (2 hours) except for one time in the pre-trials. Flavourzyme was put in hour later so it was only for 1 hour. Figure 19 shows the hydrolysis process step by step. The protocol used can be found in Appendix IV.

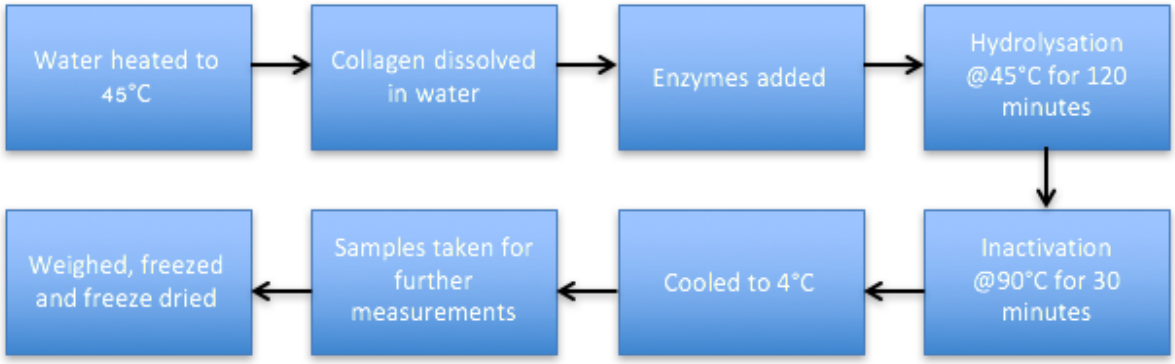


Figure 19. Flowchart of hydrolysis of collagen.

3.4.1 Pre-trial

Two pre-trials were performed. In the first one four enzymes were tested at the same conditions and in the second one four enzymes were tested with different hydrolysis time for one enzyme.

3.4.1.1 Four enzymes at the same conditions

Four different enzymes were tested: Neutrase, Flavourzyme, Protamex and Alcalase with the ratio 1/50. The pH of the solution was 6.85, that is optimal condition for all the enzymes except Alcalase. 0.1 M NaOH was used to adjust the pH to 7.98 in the solution that Alcalase was mixed with. The bottles were put in the incubator shaker (Innova 4430, New Brunswick, Canada) with 100 rpm at 45°C for 120 minutes, followed by inactivation at 90°C for 30 minutes in a waterbath (Julabo) in sealed bags. The bags were then put on ice and the solution was cooled to 4°C and sample taken for OPA measurements. The solution was then centrifuged for 20 minutes at 8000 xG (Beckham Coulter, USA), filtered through cheesecloth, weighed in trays, frozen and then freeze-dried(Virtis genesis, USA).

3.4.1.2 Four enzymes with different hydrolysis time

In pre-trial II four enzymes were tested: Collagenase, Flavourzyme, Protamex and Alcalase. They all had E/S ratio 1/50. Five samples were tested: Collagenase, Protamex+Flavourzyme (put in at the same time), Alcalase+Flavourzyme (put in at the same time), Protamex+Flavourzyme (Flavourzyme put in hour later) and Alcalase+Flavourzyme (Flavourzyme put in hour later). The preparation for they hydrolysis was done with the same method as described above.

All the samples were put in the incubator shaker (Innova 4430, New Brunswick, Canada) with 100 rpm at 45°C for 120 minutes, but the samples that were marked “hour later” were put in the shaker

containing only one of the enzymes that was used in the hydrolysis process. Flavourzyme was then put in the solution hour later. The next steps were as described above.

3.4.2 Trial

Six different enzymes or enzymes mixtures were tested: Protamex, Protamex+Flavourzyme, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L. The pH of the solution varied between 6.5-6.9 and that value was used for all enzymes used. Different enzyme/substrate (E/S) was used: 1/25, 1/50 and 1/100. All enzymes and different ratios were performed twice). The hydrolysis were performed with the same method as used in pre-trial.

3.5 Tangential flow filtration

Hydrolysates were fractionated using Labscale ™ Benchtop TFF System fractionation unit with Pellicon XL module (Millipore, USA). Three filters were used to get different cut off points. First the 30kDa filter was used, then 10kDa and finally 5kDa. Seven different samples were taken and freeze-dried for further analysis taken in seven steps of the process. In every step a sample was put in an aluminum tray for freeze-drying. Also 3.5 ml of the sample was put in red cap tube and 1.5 ml of the sample in an Eppendorf tube for bioactive measurements. In Table 7 below these seven steps are shown.

Protocol for this method can be found in Appendix V.

Table 7. Steps in the filtration where samples were taken.

Step	Size
1	Before filtration
2	> 30 kDa
3	< 30 kDa
4	Between < 30 kDa and > 10 kDa
5	< 10 kDa
6	Between < 10 kDa and > 5 kDa
7	< 5 kDa

3.6 Degree of hydrolysis by OPA (o-phthaldialdehyde) method

Degree of hydrolysis was measured with OPA method according to (Church *et al.*, 1983) and with slight changes made by (Nielsen *et al.*, 2001). The OPA reagent was prepared by dissolving 7.620 g of di-Na-tetraborate decahydrate and 200 mg SDS were dissolved in 150 ml deionized water. Magnet stirrer (RCT basis, IKA) was used for stirring and heating the solution up to 30°C, the reagents need to be dissolved totally before continuing. When the solution is completely dissolved it should be transparent.

The next step was to dissolve 160 mg OPA in 4 ml ethanol and the OPA solution was mixed with the solution mentioned above by rinsing with deionized water. Then 176 mg of DTT was weighed in the solution and rinsed with deionized water and finally the bottle was filled up to the 200 ml mark with

deionized water. A serine standard was prepared by with diluting 50 mg serine in 500 ml of deionized water.

The sample solution was mixed in a 100 ml volumetric flask by weighing 1 g of sample in the flask and filled up to the mark with deionized water. All the spectrophotometer readings were performed at 340 nm and deionized water was used as a control and all the assays were performed at room temperature.

Test tubes were obtained and 3 ml of OPA reagents were added to all the tubes. To do the standard measuring 400 µl standard solution was added to the test tube that had already contained the OPA reagents and mixed for 6 seconds on a vortex mixer (Heidolph Reax top, Schwabach, Germany). After that the mixture was set for exactly 2 minutes before it was put in the spectrophotometer (Ultrospec 3000 pro Amersham pharmacia biotech, Cambridge, England) and read at 340 nm.

Sample and blank (deionized water) were treated the same way. All the measurements were performed in duplicate.

Protocol for this method can be found in Appendix VI.

3.7 Enzymes

The optimal conditions are different between enzymes. Optimum activity for Neutrase is pH 5.5-7.5 and 45-55°C and it needs zinc ions for its activity (Novozymes(a), no date). Alcalase is active between pH 6.5-8.5 and the optimal temperature is 45-65°C. Alcalase has a very wide substrate specificity and it is able to hydrolyse most of the peptide bonds within a protein molecule (A/S, 2002). Optimal pH for Protamex is 6.5-7.6 and the optimal temperature is 50-56 °C (Liaset, 2002). Optimal pH for Flavourzyme is 5-7 and the optimal temperature is 50-55°C (Hoa, 2014). Their optimal conditions are at physiological pH and temperature (Harper, 1980; Sigma-Aldrich, e.d.). Tail 37 and TZ-02-L are enzymes from Tailorzyme. There is no manual for those enzymes and the information about their optimal conditions was obtained by email from Peter Kamp Busk from R&D at Tailorzyme. Optimal pH for tail 37 is 6-9 and temperature 45-55°C and optimal pH for TZ-02-L is 9-10 and temperature 60°C (Table 8).

Table 8. Optimal conditions for enzymes used in the hydrolysation trials.

Enzyme	Optimal pH	Optimal T°C
Neutrase	5.5-7.5	45-55
Alcalase	6.5-8.5	45-65
Protamex	6.5-7.6	50-56
Flavourzyme	5-7	50-55
Collagenase	6.3-8.8	37
Tail 37	6-9	45-55
TZ-02-L	9-10	60

3.8 Bioactivity measurements

All bioactivity measurements were made by the staff of Matís in Sauðárkrókur. The protocols they used are presented below.

3.8.1 Oxygen radical absorbance capacity (ORAC) assay

ORAC was performed according to (Ganske, 2006; D. Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). Following solutions were added in triplicates: 1. 60 µl of 10 nM Fluorescein solution, 2. 10 µl of Trolox dilutions as standard or sample solution. The microplate containing the mixture was incubated for 10 minutes at 37°C. After the incubation 30 µl of 120 mM AAPH solution was added and fluorescence (excitation: 485nm, emission: 520) was recorded, that was done every minute for 100 minutes in a microplate reader (POLARstar Optima, BMG Labtech). The area under the fluorescence curve (AUC) was calculated by the normalized curve using Equation 2.

Equation 2. For calculating the AUC.

$$AUC = (f0/f0+f99/f0) \times 0,5 + (f1/f0+ \ldots + f98/f0)$$

In the equation above f0 stands for the fluorescence reading at the initiation of the reaction and f99 was the last measurement made. To obtain the net AUC it was needed to substract the AUC of the blank from the sample or standard. The value for ORAC was calculated and expressed as micromoles of Trolox equivalents per gram (µmol of TE/g).

3.8.2 Metal chelating

The method of (Boyer, Grabill, & Petrovich, 1988) was used to determine the chelating activity on Fe^{2+} with slight modifications. Samples needed to be dissolved, they were dissolved in purified water in order to give 10 mg/ml solution and then centrifuged for 5 min at 4000 rpm. The samples that had been dissolved in water (100 µL) were mixed with 50 µl of 0.2 mM ferrous chloride and 100 µL of 0.5mM ferrozine for 30 minutes and this was performed at room temperature. The absorbance of the samples was read at 560 nm using a microplate reader (POLARstar OPTIMA, Offenburg, Germany). The metal chelating was calculating using equation 3.

Equation 3. For calculating the metal chelating.

$$\text{Chelating activity (\%)} = \left(\frac{ABlank - (ASample - AControl)}{ABlank}\right) \times 100$$

ABlank, ASample and AControl are the absorbance of the blank. The sample and the control were read at 560 nm.

3.8.3 Reducing power

The reducing power was measured according to (Oyaizu, 1986) and with volumes adapted to microplate format. Samples were dissolved in purified water in order to give 10 mg/ml solution and then centrifuged for 5 min at 4000 rpm. The sample (13µl) was mixed with 63 µl of phosphate buffer (0.2 M, pH 6.6) and 63 µl of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The sample were put in an incubation at 50°C for 30 minutes. After this 30 minutes 63 µl of 10% trichloroacetic acid was added and mixed with 40 µl of ferric chloride solution (0.1%) and the absorbance was measured at 720 nm in a microplate reader (POLARstar BMG Labtech). In the calculations the relative reducing power of the sample was calculated in relation to ascorbic acid standars (0-200 µg/ml) and that is mg of ascorbic acid equivalents per g sample.

3.8.4 Inhibition of ACE

Samples were dissolved in purified water to give a 10 mg/mL solution and centrifuged for 5 min at 4000 rpm. Activity of angiotensin converting enzyme (ACE) was measured following (Sentandreu & Toldrá, 2006) with slight modifications. 25µl of enzyme solution (3.3mU/mL ACE from rabbit lung in 300mM Tris buffer and 2µM $ZnCl_2$, pH 8.3) and 25µl of sample were added to a black 96-well microplate and equilibrated to 37°C for 10 min. Subsequently, 100µl of pre-heated (37°C) substrate solution (0.45mM Abz-Gly-p-nitro-Phe-Pro-OH (Trifluoroacetate salt)) was added to the mixture with an injector (in the microplate reader) to start the reaction just prior to measurement. Fluorescence (ex/em 340/405nm) was recorded every minute for 60 minutes in a microplate reader (POLARstar OPTIMA with incubator system). The ACE inhibitory activity (ACEI) was calculated using Equation 4.

Equation 4. For calculating ACE.

$$ACEI\ [\%] = \left(1 - \frac{\Delta_{sample}}{\Delta_{control}}\right) \cdot 100$$

Where Δ_{sample} is the slope of the sample and $\Delta_{control}$ is the slope of the control sample. The sample concentration required to reduce ACE activity to 50% (IC_{50}) was determined by using a range of sample concentrations and regressing percentage of ACE inhibition percentage on sample concentration.

3.8.5 Elastase inhibition

The samples were dissolved in purified water at a concentration of 10mg/mL and centrifuged for 5 min at 4400 rpm. For elastase inhibition the spectrophotometric method described in (Wittenauer, Maeckle, Sussmann, Schweiggert-Weisz, & Carle, 2015) was used with slight modifications. Porcine pancreatic elastase (PPE, E.C.3.4.21.36) was dissolved in 0.2 mM Tris-HCl buffer (pH 8.0) to a concentration of 0.2 mg/mL. In a clear microplate 115µL of Tris-HCl was mixed with 5 µL of PPE solution and 5 µL of sample dilutions/blank (water)/standard (EGCG) dilutions. The mixture was incubated for 15 minutes at 25°C and the reaction started by adding 125 µL of 1.6 mM N-Succinyl-Ala-Ala-Ala-p-nitroanilide (AAAPVN) substrate.

Absorbance (405nm) was recorded every 60 seconds for 20 minutes in a microplate reader (POLARstar Optima, BMG Labtech).

Calculations:

Enzyme inhibition activity (%) = $[(OD_{Control} - OD_{Sample}) / OD_{Control}] * 100$

Inhibition of both enzyme activities was measured at different sample concentrations and concentration needed to reduce enzyme activity 50% was calculated (IC_{50}) by interpolation from regression analysis.

3.8.6 SDS-PAGE analysis

The first step was to prepare samples, buffer and gel cassette. Samples were prepared in Eppendorf and heated at 95°C for 10 minutes, the running buffer was prepared and put in the fridge, the gel casettes were opened and the gel packaging buffer was drained away and it was rinsed with deionized water. The next step was assembling the XCell sureLock™ Mini-Cell.

Then the samples were loaded in the well. 12 µl of the appropriate concentration of the protein sample were loaded into each well, 15 µg of the collagen hydrolysates samples were put in each well. 5 µl of protein marker is loaded and 2.5 µl of sample buffer is loaded into empty wells to ensure a uniform running of the stacking front.

When the gels are running the lid must be on the Buffer Core. The XCell sureLock™ Mini-Cell was placed in a plastic box and ice around it, that cools the system during the run. The XCell sureLock™ Mini-Cell was then connected to the power supply and the power is turned on and the appropriate running conditions were set.

At the end of the run the power was turned off and the cables were disconnected from the power supply. The lid was removed and the gel cassette was removed from the mini-cell. The gel was then stained.

Protocol for this method can be found in Appendix VII.

3.9 Chemical analysis

Chemical analysis was performed by Matís chemical lab. Measurements were made for water (ISO-6496, 1999), fat (AOCS-Ba-3-38, 1997) salt (AOAC-976.18, 2000), ash (ISO-5984, 2002) and protein (ISO-5983-2, 2005).

3.9.1 Protein analysis

Two different methods were used for protein analysis, Dumas and Modified Lowry Method (MLM).

3.9.1.1 DUMAS

The sample was burned in a combustion tube. Oxygen was injected into the combustion tube and that lead to a degradation of the sample into N_2, NO, vaporous halogen, SO_2, SO_3, H_2O and CO_2. The gas from the sample was transported to the post combustion tube and the reduction tube. After the post combustion tube the gas was pre-dried in a watertight before the rest of the liquid was dried in a drying column that contained Sicapent (ISO-16634-1, 2008).

In the reduction tube the NOx compounds were transferred to tungsten to make N_2 and extra oxygen was tied. Sulphur was also tied with tungsten and the vaporous halogens were tied with silver wool. The nitrogen in the gas stream was measured in a thermal conductivity detector that sends electrical signals to the computer where the results were recorded by a graph and the integral was calculated (ISO-16634-1, 2008). To convert Nitrogen to collagen conversion factor of 5.5 was used (Maeda, Hosomi, Chiba, & Fukunaga, 2013).

3.9.1.2 Modified Lowry Method

The samples were dissolved in purified water at a concentration of 10mg/mL and centrifuged for 5 min at 4400 rpm. A Modified Lowry Protein assay kit (Thermo Scientific, #23240) was used for protein quantification. Instructions of the manufacturer for the microplate procedure was followed. For this 40µL of each standard (BSA) and sample were added into wells and 200µL Modified Lowry Reagent added and mixed. After 10 minute incubation 20µL of 1N Folin-Ciocalteu Reagent was added to each well and incubated for 30min. Absorbance was read at 530 nm in the microplate reader (POLARstar, BMG Labtech). Protein content was calculated based on BSA standard curve by interpolation from linear regression analysis.

3.9.2 Water analysis

The sample was heated in a heating oven at 103°C +/-2°C for four hours and the water corresponds to the weight loss (ISO-6496, 1999).

3.9.3 Fat analysis

The fat sample was extracted with a extraction apparatus (2050 Soxtec Avanti Automatic system) with petroleum ether with the boiling range 40-60°C (AOCS-Ba-3-38, 1997).

3.9.4 Ash analysis

The sample was ashed at 550°C and the residue was weighed (ISO-5984, 2002).

3.9.5 Salt analysis

Sample mixed with water and acidified. Solvable chlorides were titrated with AgNO3. AgNO3 electrode and titrator from Metrohm were used to determine the balance point (AOAC-976.18, 2000)

3.10 Statistical analysis

Microsoft Excel 2016 was used to calculate means, standard deviation (SD) and creating graphs. Statistical analysis was carried out using NCSS (version 2000; NCSS Statistical software, Utah, USA) with one-way ANOVA with Tukey-Kramer Multiple-Comparison test for mean comparison. Significance of differences was defined as the 5% level ($P<0.05$).

4 Results and discussion

4.1 Isolation of collagen

The cod skin used in this project was obtained fresh from fish store and the isolation process was also made with fresh skin. Figure 20 below shows cod skin that was used in the isolation process.

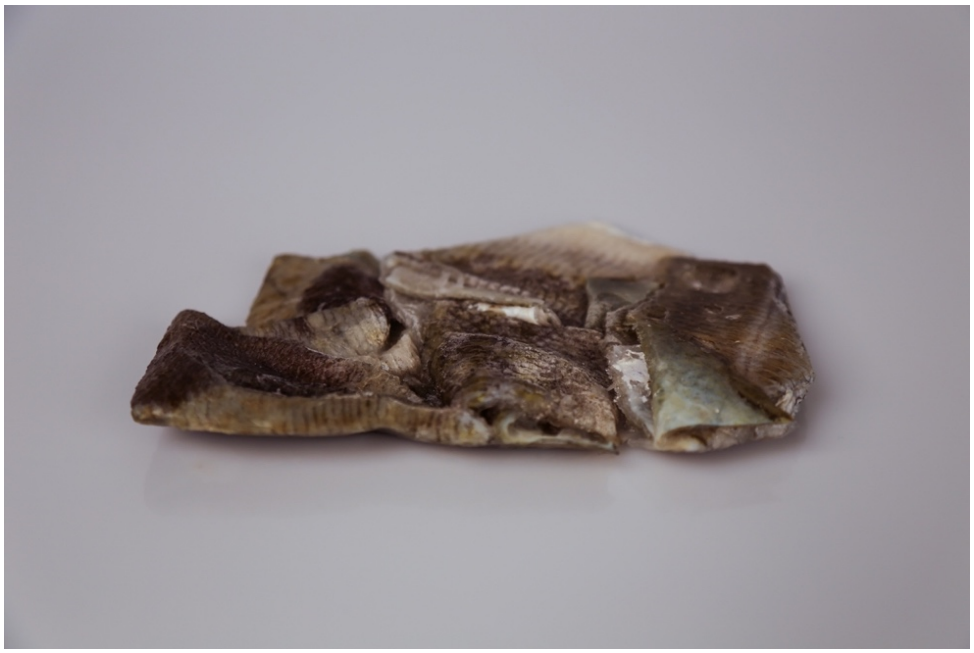


Figure 20. Cod skin used in the isolation process.

Samples of cod skin were send to chemical analysis in order to know the amount of nutrients in the skin. The chemical analysis was performed in the chemical lab at Matís on the 16th June 2015. As Table 9 shows the biggest part of the skin is water (75%) and the amount of protein is also high (24%), the amount of other nutrients was not as high.

Table 9. Chemical content of cod skin used in the isolation process.

Chemical	Amount (%)
Water	75
Protein	24
Fat	0.8
Ash	3.8
NaCl	0.12

As described in the material and methods chapter, three different methods to isolate collagen were tested using: i) NaOH and acetic acid, ii) Citric acid, NaOH and H_2SO_4 and iii) NaOH, butyl alcohol and acetic acid. Each method will be described separately in the following chapters.

4.1.1 Collagen extraction with 0.1M NaOH and 0.05M acetic acid

This method showed the best yields of all the methods that were tried. It was somewhat modified differencing type and amount of water until the best results were obtained. Table 10 shows information about water, alkali and acid used and yields collected from each sample.

In the first two runs distilled water was used both for mixing the acid- and alkali solutions, for washing and in the extraction step in the end. A lot of distilled water was needed and it was difficult to wash the skin whereas a very large amount of water was needed to get the pH near to 7.

The distilled water was replaced with tap water since it is more accessible and easier to use it in the production. As the Table 10 shows the yields were higher when tap water was used. Several factors could be responsible for that. The method had been performed twice before the tap water was used and training and experience in performing the isolation could explain the higher yields. Another factor that could be affected was that there was much easier to wash the skin with tap water to reach neutral pH.

When the method was performed for the first time the ratio of water/skin in the extraction step was 1 part skin against 10 parts water. That is a lot of water so it was decided to reduce the amount of water to be removed with freeze drying and also to reduce the amount of water in the incubator shaker overnight. As Table 10 shows a various water ratios were tested and it did not matter what the ratio was in terms of the yield.

Table 10. Type of water used, pH of base and acid used and yields of collagen isolated from cod skin.

Label	Sample (skin:water ratio)	Type of water	pH alkali	pH acid	Yield (%)
a	220615 (1:10)	Distilled water	13.3	2.64	7.72
b	020715 (1:10)	Distilled water	13.2	2.87	4.63
c	070715 (1:10)	Tap water	13.3	2.94	9.95
d	140715 (1:10)	Tap water	13.2	2.99	10.2
e	160715 (1:10)	Tap water	13.4	2.96	-
f	220715 (1:3)	Tap water	13.4	3.01	15
g	220715 (1:5)	Tap water	13.4	3.01	14.7
h	220715 (1:7)	Tap water	13.4	3.01	14.7
i	060416 (1:3)	Tap water	13.3	2.95	8
j	130416 (1:3)	Tap water	13.3	2.99	9.5
k	240516 (1:3)	Tap water	13.4	2.98	9

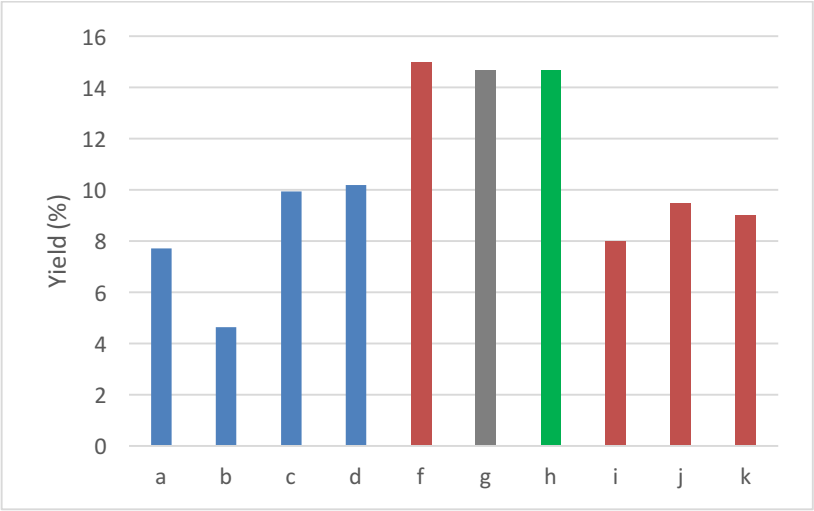


Figure 21. Yields for collagen isolated from cod skin (wet weight). Values from table 10. Method e) is not in the graph because no yield was calculated for it (missing data).

When the collagen solution had been freeze-dried it was a white substance similar to styrofoam (Figure 22). The collagen from 1:10 method was much softer than collagen from 1:3 method while the collagen from the 1:3 method was denser.

Figure 22. Freeze-dried collagen isolated from cod skin.

4.1.2 Collagen extraction with 0.7% Citric acid, 0.1% NaOH and 0.1% H_2SO_4

This method was performed once and did not work properly as the Table 11 shows the yields are only 0.5% which is not similar to previous studies (Gudmundsson & Hafsteinsson, 1997). The skin was soaked in three various solutions as described in the method chapter and washed with water until the pH was 7 and then repeated three times with each one. The problem was that the pH never managed to reach 7 in the washing between acid and alkali steps and that could have effect the results. Running water was used to wash the skin but that did not work well enough. It was mainly because of how much time the washing steps were taking. According to the protocol the skin was supposed to be washed with water 9 times in order to get the pH to 7. In the last washing step the skin was washed for hour and the pH of the water/sample solution in the last step of the process was 6.52 overnight.

It was decided to perform this method only ones due to the difficulties in the washing step and because of the low yields.

Table 11. Type of water, pH and yields of cod skin processed with Citric acid, NaOH and H_2SO_4.

Sample	Water	pH NaOH	pH sulfuric acid	pH citric acid	Yield (%)
190615	Tap water	12.8	1.74	1.75	0.5

4.1.3 Collagen extraction with 0.1 M NaOH, butyl alcohol 10% and 0.5 M acetic acid

This method did not work well, no collagen was isolated and some problems occurred during variuos steps. After the cod skin had been soaked in 0.1 M NaOH for 6 hours it was washed with water and the goal was to get pH 7, but after 1 hour of washing with water the pH was 10.88.

Butyl alcohol 10% was used instead of 1% detergent and that could probably have effect the results. The major problem was with the salting-out step when NaCl and tris (hydroxymethyl) aminomethane was used. The goal was to mix tris to the solutions until the pH was 7. The extraction solutions that was used for salting-out weight 234.74 g and when 2 l of the tris was in the solution the pH had not reach 7. To be able to continue with the method a small part of the solution was used and mixed with stronger solution in hope to get better results. 0.5 M tris was used until the pH had reach 7 and then NaCl was mixed in the solutions until it was 2.5 M. After the solution was centrifuged it appeared that nothing had precipitated. It was then decided to stop performing this method because it was obviously not working.

4.1.4 Summary of previous studies

Calculations have shown that the maximum yield of gelatin from cod skin is 17% based on the weight of wet skin (Gudmundsson & Hafsteinsson, 1997). Concentration of acid, alkali and water is important and can give different yield results. Isolation of collagen was made with different concentration of acids and the yield varied from 11-14.3% (Gudmundsson & Hafsteinsson, 1997).

Many studies represent their results as the yields of dry weight. The dry weight results in this project was 16.1-52.2%. Previous studies have shown similar yields. Collagen both ASC and PSC was isolated from largefin longbarbel catfish and the yield was 16.8% and 28% of dry weight (M. Zhang *et al.*, 2009), the yield of collagen from the skin of deep-sea redfish was 47.5% of dry weight (Wang *et al*., 2008), the yield of ASC and PSC from the skin of a balloon fish was 4% and 19.5% of the dry weight (Y. R. Huang *et al.*, 2011) and the yield of collagen isolated from the skin of brown backed toadfish was 54.3% of the dry weight (Senaratne *et al.*, 2006).

4.2 Hydrolysis of collagen

In the hydrolysis process the collagens were broken down using different enzymes to know the relationship between enzymes, amount of enzymes and degree of hydrolysis. The resulting collagen hydrolysates were white powders with hint of fish smell (Figure 23).

Figure 23. Freeze dried collagen isolated from cod skin and hydrolysed for 2 hours at 45°C with Flavourzyme+Protamex with the E/S ratio 1/50.

4.2.1 Degree of hydrolysis

First run of hydrolysis was made using the enzymes Alcalase, Flavourzyme, Neutrase and Protamex. The DH values were ranging from 5.9-13.6%. Sample hydrolysed with Alcalase had the highest degree of hydrolysis 13.6% and the sample hydrolysed with Neutrase had the lowest degree of hydrolysis 5.9% (Figure 24).

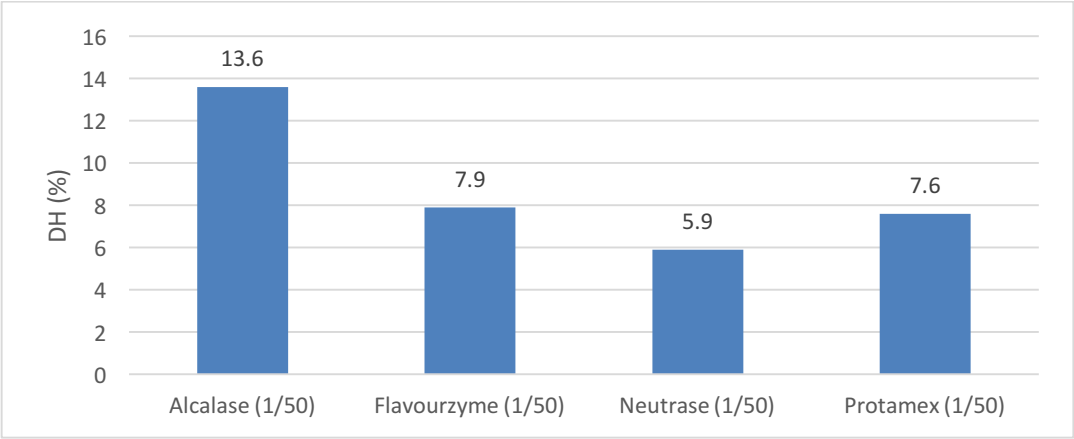


Figure 24. Degree of hydrolysis for collagen isolated from cod skin and hydrolysed for 2 hours at 45°C with Alcalase, Flavourzyme, Neutrase and Protamex with the E/S ratio 1/50.

SDS-PAGE were performed on samples shown in Figure 24 and two commercial samples from Ankra and Codland. The commercial samples had bigger peptides than samples hydrolysed in this project. Samples hydrolysed with Alcalase had the smallest peptides and as stated above it had the highest value of DH. Sample hydrolysed with Neutrase has the lowest value of DH as Figure 25 shows. There was not a big difference between samples hydrolysed with Flavourzyme, Neutrase and Protamex but sample hydrolysed with Alcalase had smaller peptides. It was also of interest to see that in Protamex and Neutrase samples a clear band can be seen around 17.5 kDa and 10 kDa.

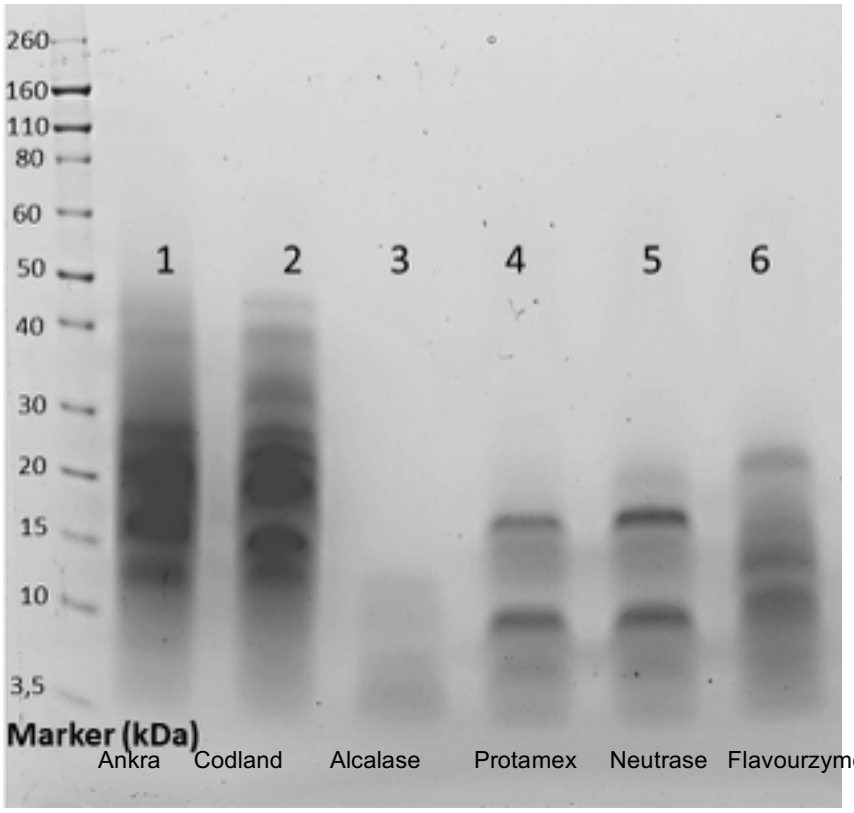


Figure 25. SDS-PAGE for samples from Ankra, Codland and samples hydrolysed with Alcalase, Protamex, Neutrase and Flavourzyme.

It was tested to use two enzymes at the same time. One sample was hydrolysed with only one enzyme, Collagenase, but the other four samples were hydrolysed with two enzymes, Flavourzyme was used in all those samples. In two of the samples it was put in the solution at the same time as the other enzymes (active for 2 hours) but in the other two it was put in hour later so it was only active for 1 hour.

The DH value was highest when Protamex and Flavourzyme were put in the collagen solution at the same time or 20.5% (Figure 26). The two samples containing two enzymes put in the solution at the same time showed higher DH than the other two where Flavourzyme was put in the solution hour later. That means the proteins are broken down more when both enzymes are active for 2 hours. Collagenase was tested and did not give higher degree of hydrolysis that the other enzymes like was anticipated whereas this enzyme is specific for collagen.

Figure 27 shows the peptide size of the samples and collagenase had broken the collagen down into very small peptides, smaller than the other samples tested.

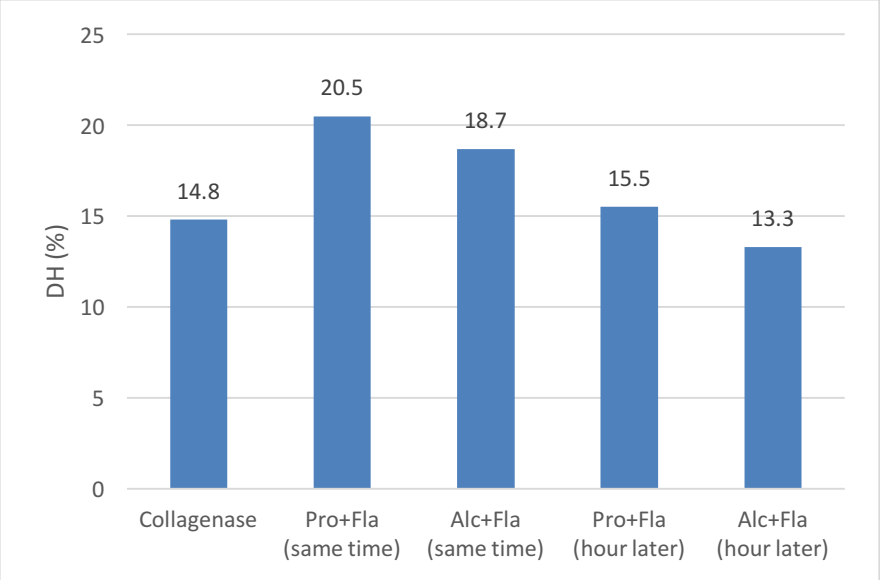


Figure 26. Degree of Hydrolysis of collagen isolated from cod skin and hydrolysed at 45°C with Collagenase, Protamex+Flavourzyme and Alcalase+Flavourzyme where the enzymes were put in at the same time (active time 2 hours) and Protamex+Flavourzyme and Alcalase+Flavourzyme where Flavourzyme were put in hour later (active time for Protamex and Alcalase: 2 hours, active time for Flavourzyme: 1 hour).

Sample	113	114	115	116	117	113	118	119	L
mg/mL	10	10	10	10	10	5	5	5	

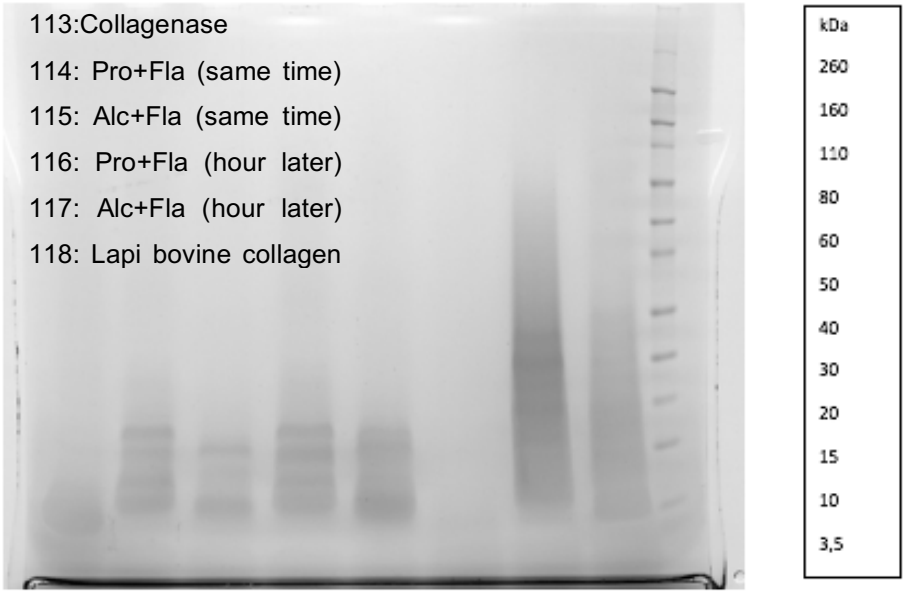


Figure 27. SDS-PAGE for samples hydrolysed with Collagenase, Pro+Fla (same time), Alc+Fla (same time), Pro+Fla (hour later), Alc+Fla (hour later) and two commercial samples: Lapi bovine collagen and Lapi fish collagen.

Various values of degree of hydrolysis was obtained when using different enzymes (Table 12).

Table 12. Degree of hydrolysis of collagen isolated from cod skin and hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme, Protamex, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L with the E/S ratio 1/25, 1/50 and 1/100.

Enzymes	Enzyme/substrate ratio	DH
1.Protamex+Flavourzyme	1/25	20.7
2.Protamex+Flavourzyme	1/50	15.8
3.Protamex+Flavourzyme	1/100	12.7
4.Protamex	1/25	11.0
5.Protamex	1/50	7.2
6.Protamex	1/100	6.4
7.Alcalase	1/25	13.5
8.Alcalase	1/50	11.7
9.Alcalase	1/100	9.3
10.Alcalase+Flavourzyme	1/25	23.3
11.Alcalase+Flavourzyme	1/50	19.7
12.Alcalase+Flavourzyme	1/100	12.6
13.Tail 37	1/25	14.8
14.Tail 37	1/50	11.8
15.Tail 37	1/100	11.5
16.TZ-02-L	1/25	16.1
17.TZ-02-L	1/50	16.3
18.TZ-02-L	1/100	9.2
19.Protamex	1/25	13.4
20.Protamex	1/50	11.7
21.Protamex	1/100	8.3
22.Protamex+Flavourzyme	1/25	15.9
23.Protamex+Flavourzyme	1/50	16.1
24.Protamex+Flavourzyme	1/100	8.1
25.Alcalase	1/25	13.0
26.Alcalase	1/50	10.0
27.Alcalase	1/100	7.0
28.Alcalase+Flavourzyme	1/25	19.4
29.Alcalase+Flavourzyme	1/50	17.1
30.Alcalase+Flavourzyme	1/100	12.0
31.Tail 37	1/25	15.3
32.Tail 37	1/50	12.8
33.Tail 37	1/100	10.8
34.TZ-02-L	1/25	11.8
35.TZ-02-L	1/50	10.5
36.TZ-02-L	1/100	7.2

The values of DH for all the samples are shown in Figure 28. The colums are average of both rounds of the OPA method. In all samples the degree of hydrolysis (DH) was highest in the samples with the E/S ratio 1/25 and the samples with E/S ratio 1/100 had the lowest DH values as expected. Alcalase+Flavourzyme with the ratio 1/25 had the highest DH of all the samples 21.3%. Alcalase is a endoprotease and is often used for the hydrolysation of collagen and that is because it can accomplish a high DH in a short time under moderate conditions (A/S, 2002; Gomez-Guillen *et al*., 2011). Flavourzyme has both exopeptidase and endoprotease activity and has a higher ability to release more free amino acids then Alcalase (Hrckova, 2002). Those two enzyme together can break the protein down to small peptides. Protamex with the ratio 1/100 had the lowest DH of all the samples 7.35%.

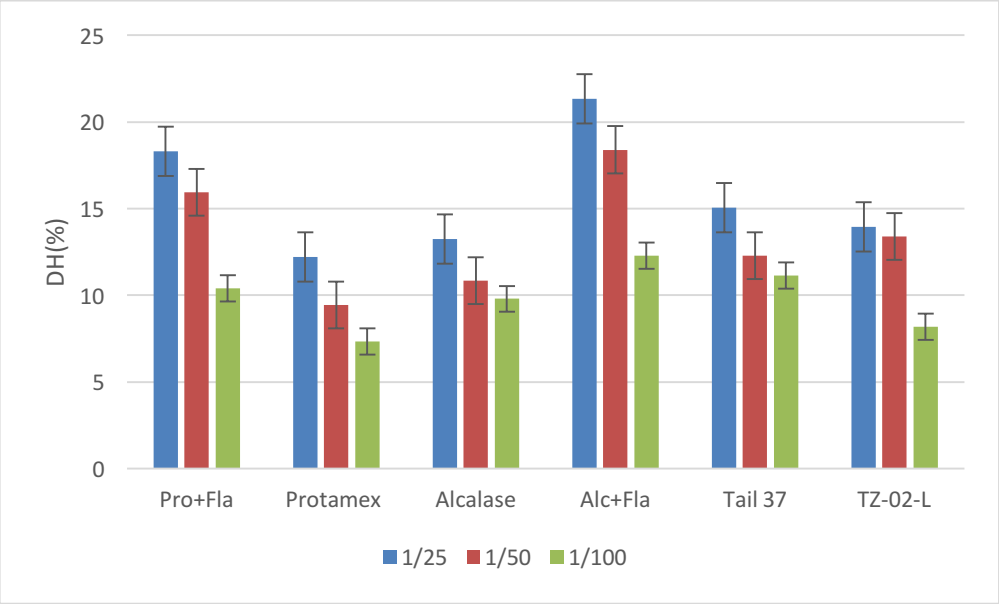


Figure 28. Degree of hydrolysis, average with standard deviation (n=2). Collagen isolated from cod skin and hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme, Protamex, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L with the E/S ratio 1/25, 1/50 and 1/100.

4.3 SDS-PAGE

The results from the SDS-PAGE method show the molecular weight of the peptides hydrolysed and filtrated in this project. Figure 29 shows the results for the samples from the pre-trial and the filtration. The peptides from the pre-trial were all hydrolysed for 2 hours with the E/S ratio 1/50. The results show that the sample hydrolsyed with Flavourzyme had the biggest peptides and it seems that the sample Hydrolysed with Protamex and Flavourzyme had the smallest peptides.

The size of filtrated samples are shown in Figure 29 and Figure 30. The peptides used in the filtration step were hydrolysed with Protamex+Flavourzyme with E/S ratio 1/50 which had high DH values and broke the collagen down to small peptides. Figure 29 shows the unfiltered samples and they are all 10 kDa or lower.

The filtration step was performed before the SDS-PAGE results were obtained. The 30 kDa filter would not have been chosen if those results had been revealed.

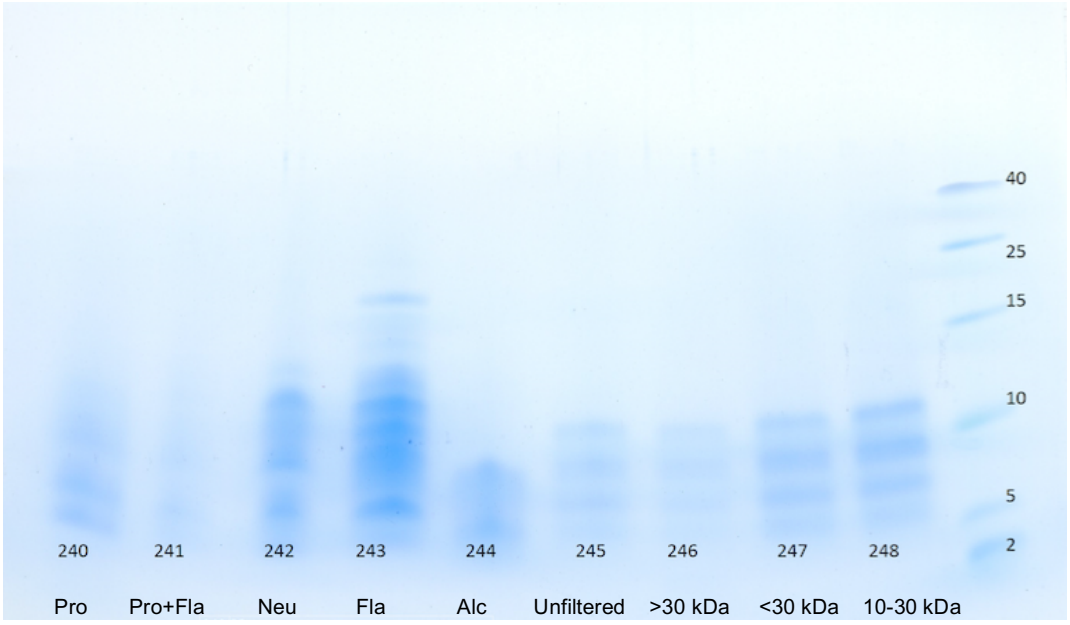


Figure 29. SDS-PAGE showing peptides from pre-trial and filtration. Samples from pre-trial: hydrolysed with Protamex (Pro), Protamex+Flavourzyme (Pro+Fla), Neutrase (Neu), Flavourzyme (Fla) and Alcalase (Alc) for 2 hours at 45°C. Samples from filtration: hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme with E/S ratio 1/50 and filtered with 30 kDa and 10 kDa filters.

Figure 30 shows the collagen peptides that were filtered through 10 kDa and 5 kDa filters. The biggest peptides were approximately 10 kDa and sample 5-10 kDa seems to contain most of them. Sample <5 kDa had some peptides over 5 kDa and that is because of there is always some range with the filters but it had less amount of bigger peptides comparing to the other samples.

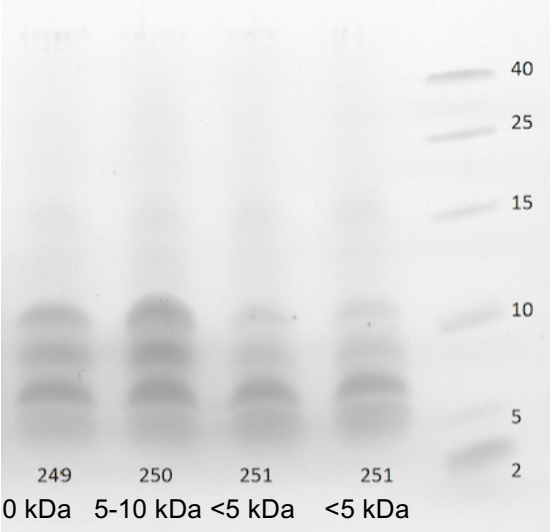


Figure 30. SDS-PAGE showing collagen hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme with E/S ratio 1/50 and filtered with 10 kDa and 5 kDa filters.

SDS-PAGE for the trial with 36 samples showed that the biggest peptides from the hydrolysis were approximately 13-14 kDa and they were in samples hydrolysed with Protamex and also with TZ-02-L with E/S ratio 1:100. Those SDS-PAGE results can be found in Appendix VIII.

4.4 Antioxidant measurements

The antioxidant activity of the peptides was measured with three methods: ORAC, Metal chelating and Reducing power. The results will be listed in the following chapters.

4.4.1 ORAC

The samples that were hydrolysed in this project had ORAC values ranging from 146-164 µmol of TE/g (Figure 31). The sample hydrolysed with Flavourzyme had the highest ORAC value 164 µmol of TE/g and the sample hydrolysed with Alcalase had the lowest ORAC or 146 µmol of TE/g of the samples hydrolysed in this project.

The commercial samples from Codland and Ankra had a lower ORAC activity. The sample from Codland had the lowest ORAC value of all the samples 91 µmol of TE/g and Ankra had 103 µmol of TE/g and they were significantly different ($p<0.05$) from samples hydrolysed in this project. SDS-PAGE results showed that they have bigger peptides than the sample processed in the project (Figure 25).

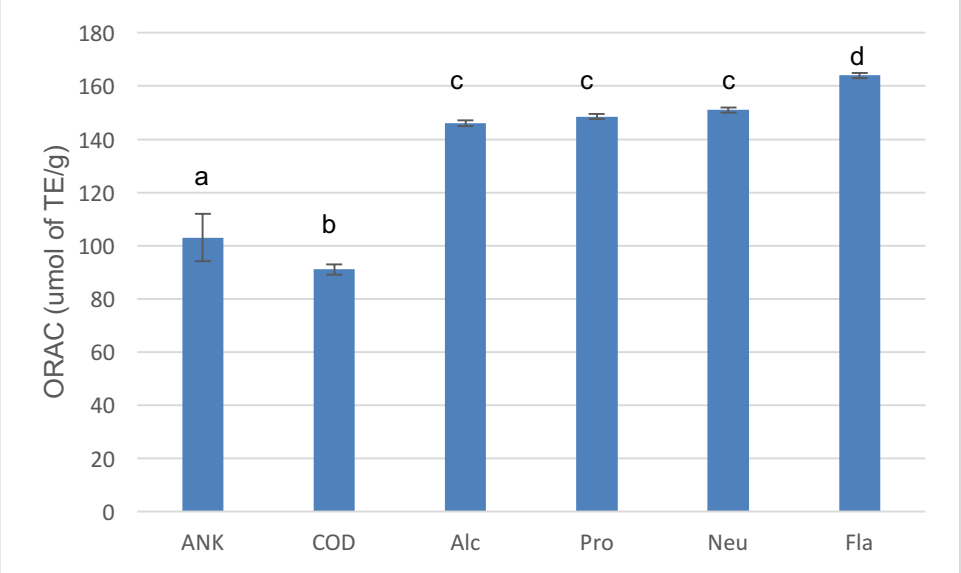


Figure 31. Oxygen Radical Absorbance Capacity (ORAC) values of hydrolysed collagen isolated from cod skin: samples from Ankra (ANK) and Codland (COD) and samples hydrolysed for 2 hours at 45°C with Alcalase (Alc),Protamex (Pro), Neutrase (Neu) and Flavourzyme (Fla) with the E/S ratio 1/50. Columns marked with the same letter are not significantly different ($p<0.05$).

ORAC values from the trial with 36 samples are shown in Figure 32. These samples were made using five enzymes and three different E/S ratios. There was significant difference between the highest and lowest values ($p<0.05$) but no significant difference was found between other samples. Protamex 1/100 had the highest ORAC activity 206 µmol of TE/g and Protamex+Flavourzyme 1/25 had the lowest value 138 µmol of TE/g. Protamex+Flavourzyme had the lowest value in all the ratios compared to other samples.

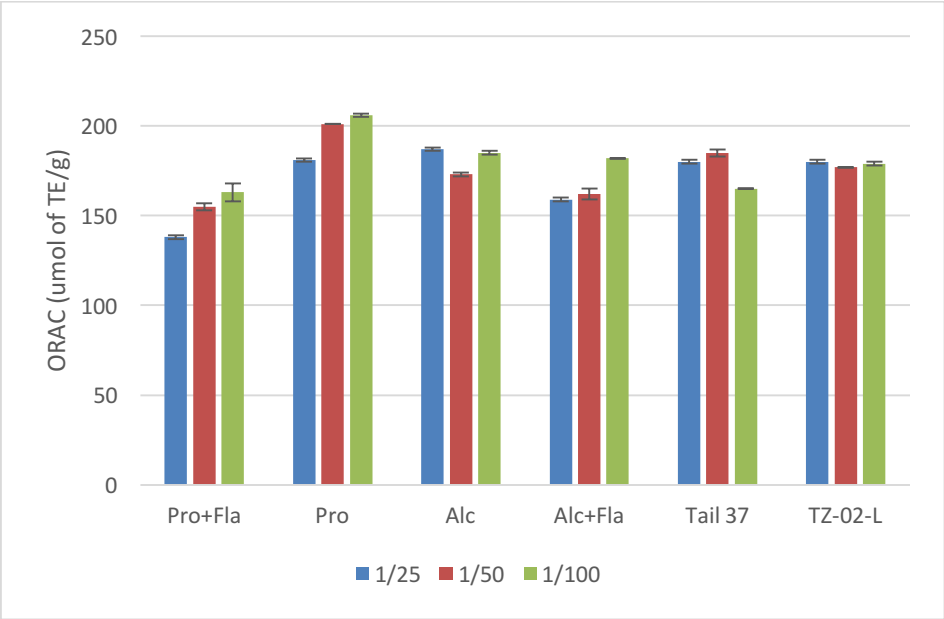


Figure 32. The ORAC value of collagen isolated from cod skin (n=2). Hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme (Pro+Fla), Protamex (Pro), Alcalase (Alc), Alcalase+Flavourzyme (Alc+Fla), Tail 37 and TZ-02-L with the E/S ratio 1/25, 1/50 and 1/100. Significant difference ($p<0.05$) between Pro+Fla 1/25 and Pro 1/100.

The correlation between ORAC values and DH of collagen peptides showed in Figure 32 were calculated. There was not much correlation (R^2 = 0.48) but it indicates that smaller collagen peptides had lower ORAC activity (Figure 33).

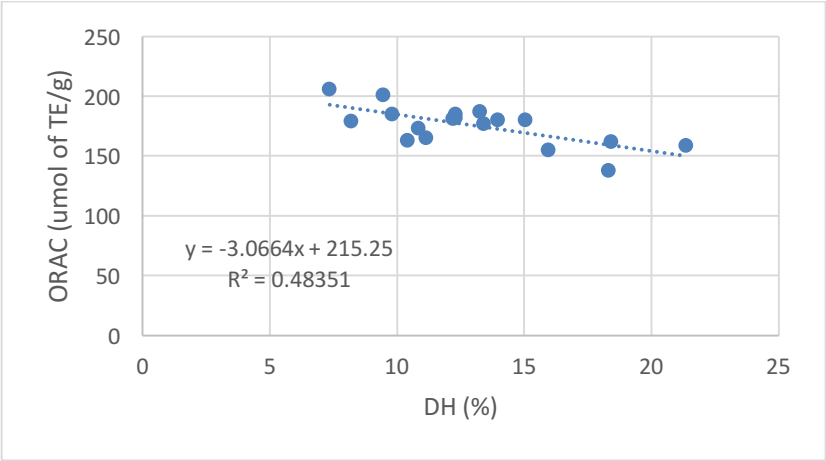


Figure 33. Correlation between ORAC values and DH of collagen isolated from cod skin. Hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme, Protamex, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L with the E/S ratio 1/25, 1/50 and 1/100.

The correlation was strongest with samples hydrolysed with Protamex, when higher amount of Protamex was used the ORAC value was lower. Samples hydrolysed with Protamex+Flavourzyme and only Protamex showed a correlation with DH (R^2= 0.82) (Figure 34).

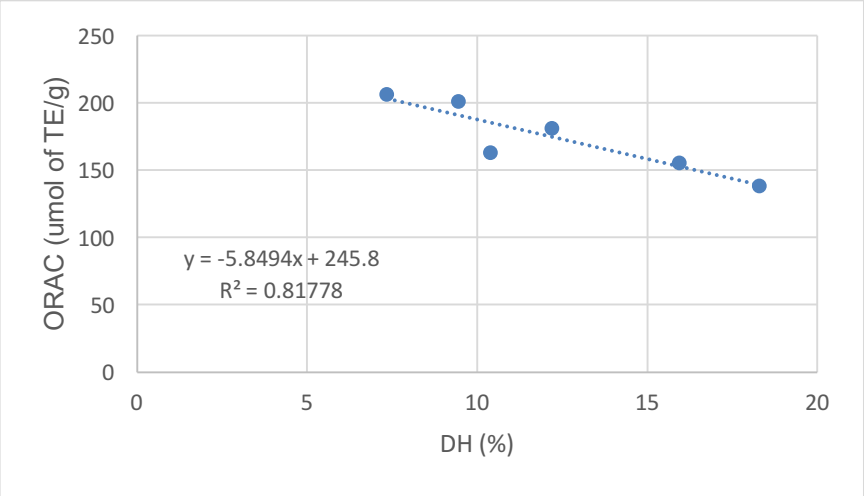


Figure 34. Correlation between ORAC values and DH of collagen isolated from cod skin. Hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme and Protamex with the E/S ratio 1/25, 1/50 and 1/100.

The ORAC values of the filtrated collagen samples are shown in Figure 35. The highest value was in the sample <5 kDa 156 µmol of TE/g. It was significantly ($p<0.05$) different from the unfractionated sample and <10 kDa samples. The unfractionated sample had the lowest ORAC activity 123 µmol of TE/g.

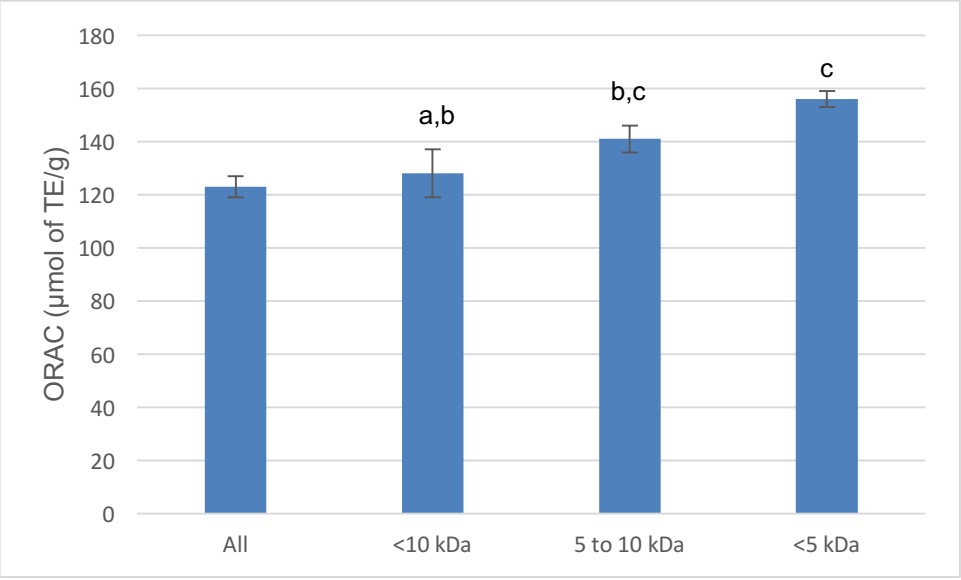


Figure 35. ORAC values of collagen isolated from cod skin, hydrolysed for 2 hours at 45°C with Protamex and Flavourzyme and filtrated with 5 kDa and 10 kDa filters. Columns marked with the same letter are not significantly different ($p<0.05$).

The results from the filtered samples are counter to the results shown in the trial with 36 samples (Figure 32). That could possibly be because of different enzymes used and how they cut the protein differently. For further research it would be good to use HPLC, FPLC or mass spectrometry to see the amino acid sequence of the peptides and see if different amino acids row are causing this difference.

Not many studies are to be found on antioxidant activity of collagen peptides for comparison. More studies are found on fish protein hydrolysates.

The results from the filtrated samples showed that smaller peptides have greater ORAC activity and that was similar to what has been showed in previous studies that higher degree of hydrolysis gives higher ORAC values for fish protein hydrolysates (Theodore, Raghavan, & Kristinsson, 2008).

Previous studies that have been performed on fish protein hydrolysates have showed higher results than found in this project. Study that was measuring ORAC activity in FPH got ORAC value approximately 500 μmol of TE/g (Halldorsdottir, Sveinsdottir, Gudmundsdottir, Thorkelsson, & Kristinsson, 2014). Other study that was researching FPH got ORAC value 670 μmol of TE/g (Halldorsdottir, Sveinsdottir, Freysdottir, & Kristinsson, 2014). FPH from pacific hake had slightly greater ORAC activity than collagen peptides hydrolysed in this project, it had 225 μmol of TE/g (Samaranayaka & Li-Chan, 2008). FPH from echinoderm byproducts that have been hydrolysed with Alcalase had ORAC values ranging from 267-426 μmol of TE/g (Mamelona, Saint‐Louis, & Pelletier, 2010) and that are higher values than obtained from Collagen peptides in this project.

4.4.2 Reducing power

The commercial sample from Ankra had the highest reducing power value 46 mg AA/protein and the sample from Codland had the second highest value 44 mg AA/protein. Sample hydrolysed with Alcalase had the lowest value of reducing power 32 mg AA/protein (Figure 36) and it had also the highest DH value. There was a little difference between all the samples measured with values ranging from 32-46 mg AA/protein.

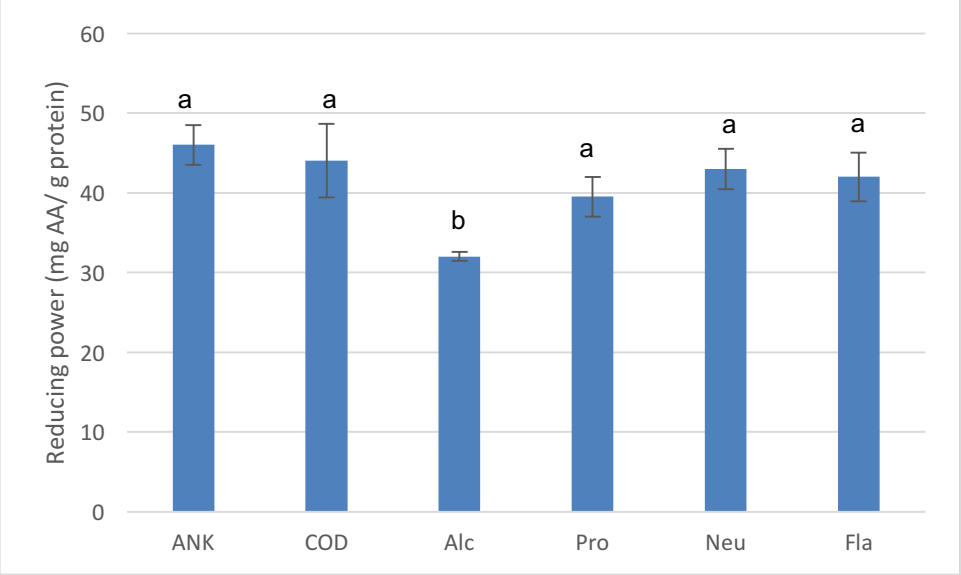


Figure 36. Reducing power of collagen isolated from cod skin, samples from Ankra and Codland and samples hydrolysed for 2 hours at 45°C with Alcalase (Alc), Protamex (Pro), Neutrase (Neu) and Flavourzyme (Fla) with the E/S ratio 1/50 and concentration 10 mg/ml. Columns marked with the same letter are not significantly different ($p<0.05$).

Reducing power was measured for collagen peptides samples hydrolysed with Protamex, Protamex+Flavourzyme, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L and with E/S ratio 1/25, 1/50 and 1/100. Reducing power of collagen hydrolysed with the enzymes mentioned above are shown in Figure 37. The values were ranging from 9-16 mg AA/g sample. It was different between samples which ratio had the highest reducing power value of the sample. But in all the samples it was either 1/50 or 1/100 that had the highest value.

The sample with the highest value was Protamex with E/S ratio 1/50 16 mg AA/g sample and the sample with the lowest value was Alcalase+Flavourzyme with E/S ratio 1/25 9 mg AA/g sample.

There was not much correlation between reducing power and DH (R^2= 0.23) (data not shown) but it indicated that smaller peptides had lower reducing power.

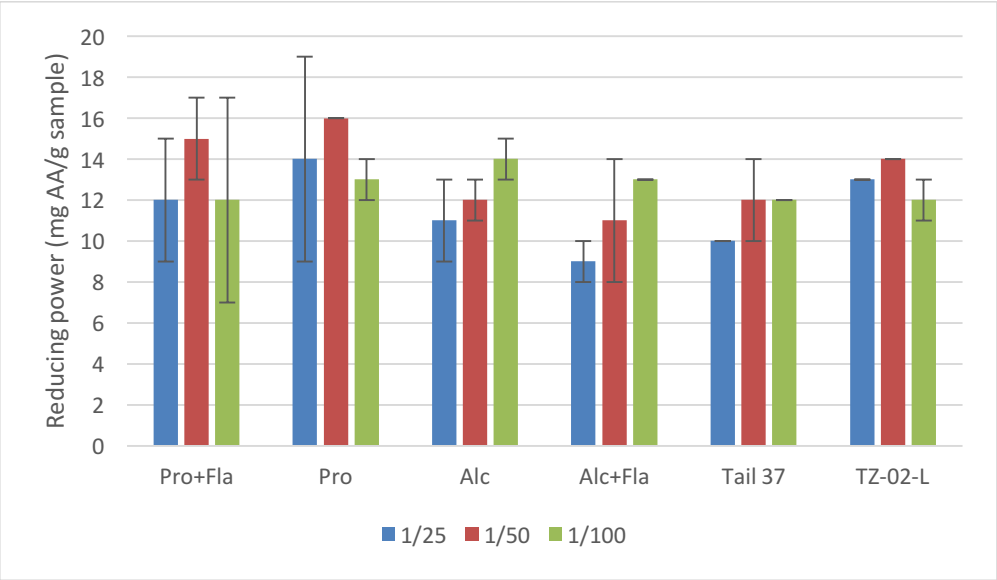


Figure 37. Reducing power of collagen isolated from cod skin (n=2) hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme, Protamex, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L with the E/S ratio 1/25, 1/50 and 1/100 and concentration 1 mg/ml. No significant difference was found between samples ($p<0.05$).

Reducing power of filtrated collagen is shown in Figure 38. There was not much difference between the samples, the values were ranging from 9-17 mg AA/sample. Collagen peptides that were <5 kDa had the lowest reducing power value 9 mg AA/g sample and collagen peptides <10 kDa had the highest reducing power value 17 mg AA/g sample.

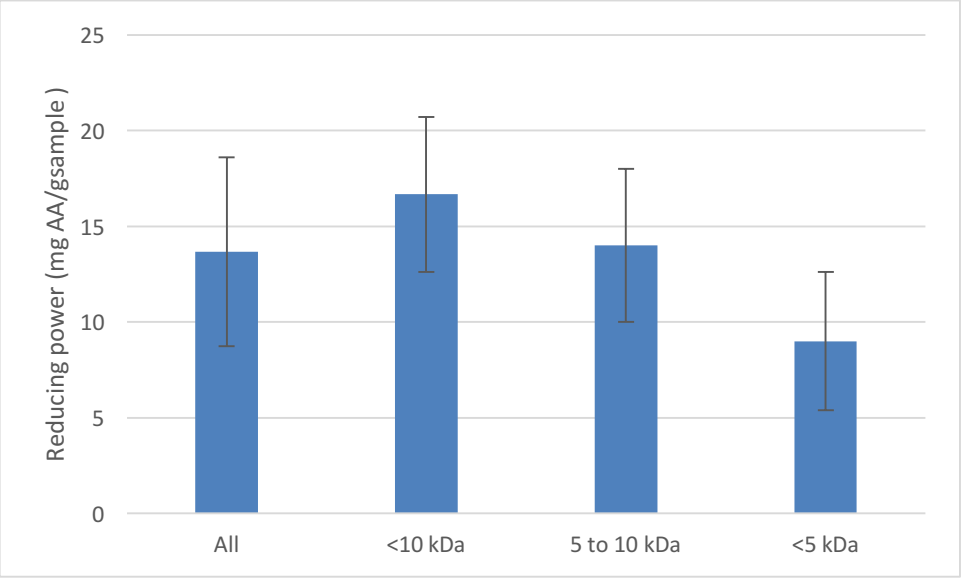


Figure 38. Reducing power of collagen isolated from cod skin, hydrolysed for 2 hours at 45°C with Protamex and Flavourzyme and filtrated with 5 kDa and 10 kDa filters and concentration 10 mg/ml. No significant difference was found between samples ($p<0.05$).

The reducing power was measured for all the samples. It was measured to estimate the ability of an antioxidant to donate an electron or hydrogen. It has been shown that peptides with greater ability of reducing power have a better capability to donate electrons (Ktari *et al*., 2013).

When all the results from reducing power measurements were reviewed, from: pre-trial, trial and filtration, it seems to be in common in them all that peptides with higher DH value and smaller peptides had lower reducing power. Previous studies that have been performed on FPH have shown similar results.

Study that was performed on FPH from grass carp showed that the reducing power was decreased when the DH value was increased. The fish proteins were hydrolysed with Alcalase and Papain and the reducing power values were higher for samples hydrolysed with Papain (X. Li, Luo, Shen, & You, 2012).

Another study found reducing power in Yellow stripe trevally FPH. Samples hydrolysed with Alcalase showed the same results as in this project, it had lower reducing power values when the DH was higher, but samples hydrolysed with Flavourzyme had similar values of reducing power regardless of the DH value (Klompong *et al*., 2007).

FPH from cod had greater reducing power than the samples hydrolysed in this project it had 7.64 mg ASE/g protein (Halldorsdottir, Sveinsdottir, Freysdottir, *et al.*, 2014). Study that was performed on Louch, which is a fresh water fish, got different results than in this project with samples hydrolysed with Protamex. They found out that the reducing power was correlated to the degree of hydrolysis for samples hydrolysed with Protamex, but samples hydrolysed with Papain had the highest value of reducing power at the second lowest DH value (You, Zhao, Cui, Zhao, & Yang, 2009).

The results from You *(*You *et al.*, 2009*).* were different from the results in this project for sample hydrolysed with Protamex, the results in this project showed that the samples with E/S ratio 1/25 which had the highest DH never had the highest reducing power. It was always samples with E/S ratio 1/50 or 1/100. The difference between those studies could be because of different raw material, as they were using fresh water fish, and also because they are using FPH and in this study only collagen was used.

4.4.3 Metal chelating

The Metal chelating values are shown in Figure 39 and the values were ranging from 27-44%. Collagen peptide hydrolysed with Alcalase had the highest value 44% and the sample from Codland had the lowest metal chelating activity 27%.

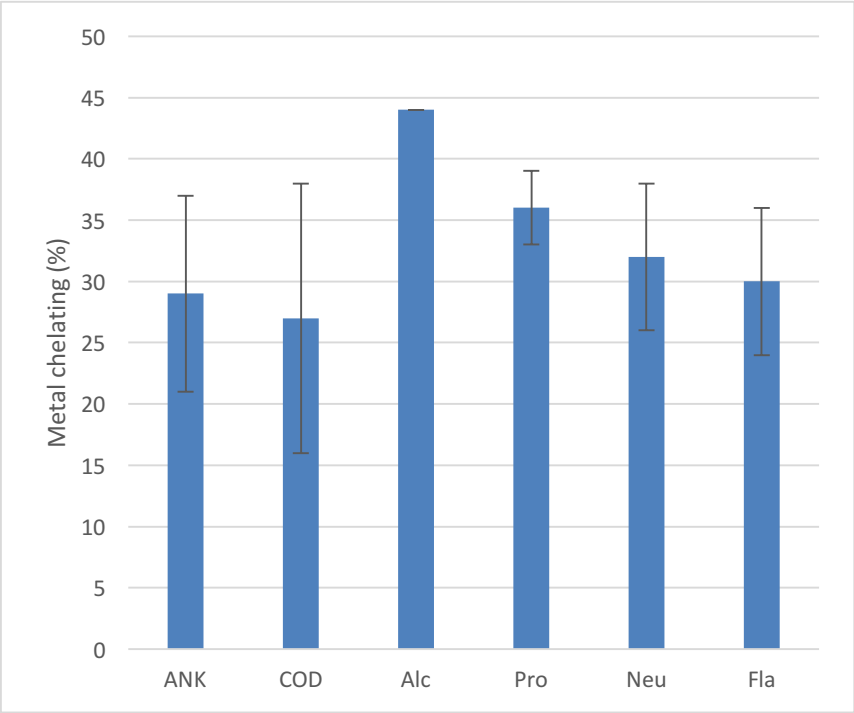


Figure 39. The metal chelating ability of collagen isolated from cod skin, samples from Ankra (ANK) and Codland (COD) and samples hydrolysed for 2 hours at 45°C with Alcalase (Alc), Protamex (Pro), Neutrase (Neu) and Flavourzyme (Fla) with the E/S ratio 1/50. No significant difference was found between samples ($p<0.05$).

The correlation between metal chelating and DH was calculated (R^2 = 0.79) for samples in Figure 39, and there was some correlation between those two variables (data not shown). That indicates that smaller peptides have higher metal chelating activity.

The metal chelating was measured for collagen peptides samples hydrolysed with Protamex, Protamex+Flavourzyme, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L and with E/S ratio 1/25, 1/50 and 1/100 (Figure 40). The values were ranging from 10-37%. Sample hydrolysed with Protamex+Flavourzyme with E/S ratio 1/100 had the greatest metal chelating ability. Sample hydrolysed with Tail 37 with E/S ratio 1/50 had the lowest value 10%.

When the metal chelating values are compared with the DH values in Table 12 it seems that the DH value has no effect on the metal chelating ability.

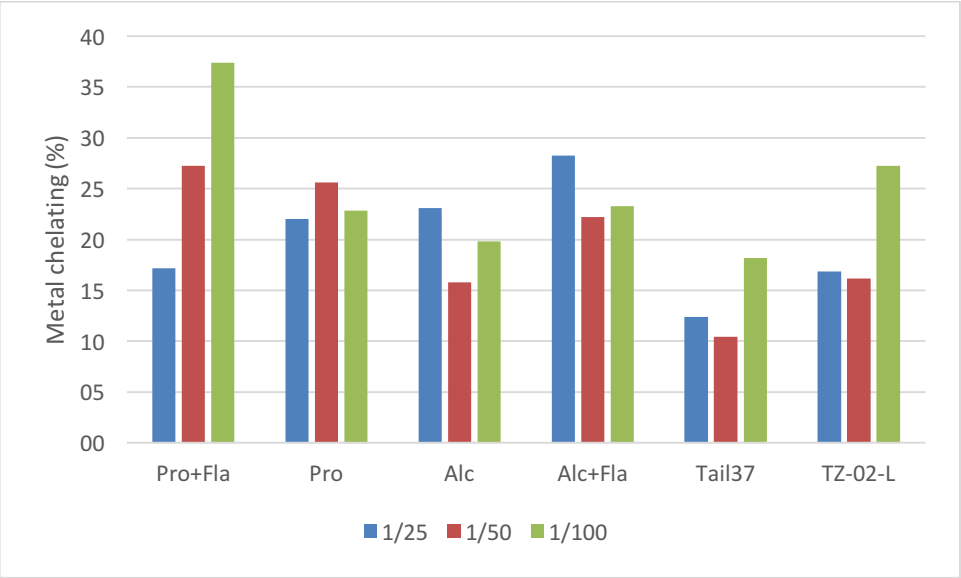


Figure 40. The metal chelating ability of collagen isolated from cod skin (n=2). Hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme, Protamex, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L with the E/S ratio 1/25, 1/50 and 1/100. No significant difference was found between samples ($p<0.05$).

There were no duplicates or triplicates performed on the samples in Figure 40. No correlation was between metal chelating and DH values in the trial with 36 samples.

The metal chelating was measured in the filtrated samples. All the samples had low values, it was ranging from 11-22%. Collagen peptides that were <5 kDa had the highest metal chelating value or 22% and the collagen peptides that were 5-10 kDa had the lowest value or 11%.

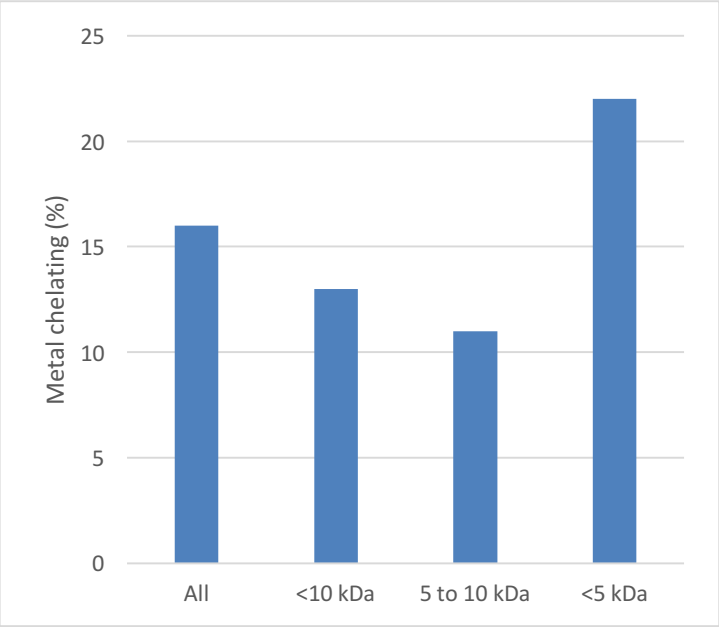


Figure 41. The metal chelating ability of collagen isolated from cod skin, hydrolysed for 2 hours at 45°C with Protamex and Flavourzyme and filtrated with 5 kDa and 10 kDa filters.

The metal chelating values in Figure 41 were under detection limits and therefor no duplicates or triplicates were performed.

Metal chelating values that are under 50% are consider low and IC_{50} could not be calculated. All the samples analyzed in this project had values under 50% so they can be considered with low ability of metal chelating.

The metal chelating ability has also been measured for fish protein hydrolysates (FPH). Previous studies have shown that FPH from cod had metal chelating 91.7% (Halldorsdottir, Sveinsdottir, Freysdottir, *et al*., 2014) and approximately 70% (Halldorsdottir, Kristinsson, Sveinsdottir, Thorkelsson, & Hamaguchi, 2013). Those samples have higher metal chelating ability than the collagen peptides in this project. Those analysis were performed with the same method as used in this project and the difference could be because of those studies had fish protein hydrolysates but in this project collagen peptides were used.

The metal chelating ability of collagen from porcine skin hydrolysed with pepsin, papain, protease from bovine pancreas and mixture of enzymes was 9.5-37.4% depending on which enzyme used and the results showed that samples with higher degree of hydrolysis had greater metal chelating ability (B. Li, Chen, Wang, Ji, & Wu, 2007). Collagen peptides from Alaska Pollock had metal chelating ability 28% when hydrolysed for 2 hours with Trypsin and 17% when hydrolysed with Flavourzyme (Guo *et al.*, 2013). The metal chelating ability was also calculated after 4 hours and 6 hours hydrolysis and the chelating activity was greater with longer hydrolysis (Guo *et al*., 2013).

Study that was performed on yellow stripe trevally showed some metal chelating activity of samples hydrolysed with Alcalase and Flavourzyme. The metal chelating activity increased when the DH was increased. Samples hydrolysed with Flavourzyme had higher MC ability than Alcalase in all DH values that were tested (Klompong *et al*., 2007). It should be kept in mind that different methods were used in analyzing the metal chelating ability of those samples and comparison therefore difficult. But those results indicate that smaller peptides have a greater metal chelating ability

4.4.4 Summary of antioxidant measurements

Like shown in this project and other studies have confirmed as well, the degree of hydrolysis and peptide size can affect the antioxidant activity. Samples with higher degree of hydrolysis and smaller peptides had lower reducing power and ORAC values but it had no correlation with Metal chelating in this project.

4.5 ACE

The ACE-inhibiting activity of collagen samples hydrolysed with Alcalase, Protamex, Neutrase and Flavourzyme (2 hours) E/S ratio 1/50 are shown in Figure 42. Samples hydrolysed with Alcalase, Protamex and Neutrase had very similar IC_{50} values 2.5-3.4 mg sample/ml but Flavourzyme had the lowest activity of all the samples hydrolysed in this project 16.9 mg powder/ml.

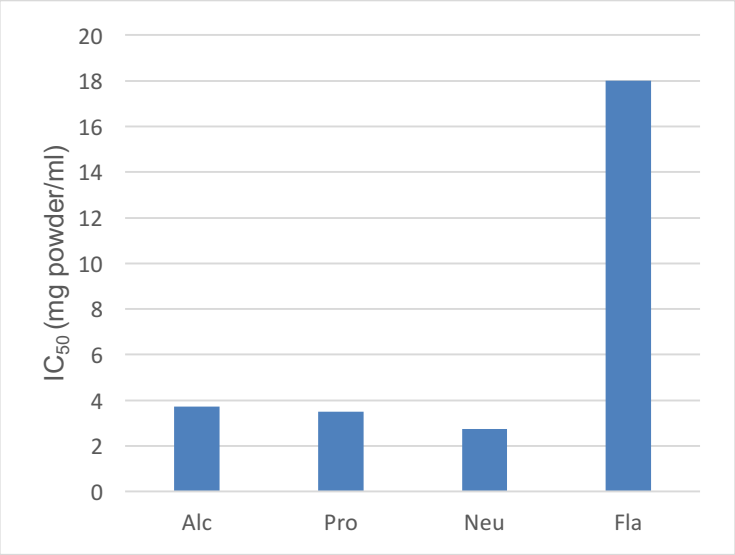


Figure 42. The ACE-inhibiting activity of collagen isolated from cod skin, hydrolysed for 2 hours at 45°C with Alcalase (Alc), Protamex (Pro), Neutrase (Neu) and Flavourzyme (Fla) with the E/S ratio 1/50.

There were no duplicates or triplicates performed on samples in Figure 42 due to time limitation. ACE inhibitory was also measured for collagen peptides from Codland and Ankra. They were clearly different from the samples produced in this project but there were too few measurements to perform statistical analysis. The Ankra sample was measured 968 mg powder/ml and the sample from Codland 116 mg powder/ml. There was no correlation between ACE-inhibiting activity and DH.

The ACE-inhibiting activity was measured for collagen peptides samples hydrolysed with Protamex, Protamex+Flavourzyme, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L and with E/S ratio 1/25 and 1/100 (Figure 43). ACE-inhibiting activity was found in samples: Protamex+Flavourzyme, Alcalase+Flavourzyme and Tail37 with E/S ratio 1/25 and the values were ranging from 1.3-1.6 mg powder/ml. The sample hydrolysed with Alcalase+Flavourzyme had the greatest ACE-inhibiting activity 1.3 mg powder/ml.

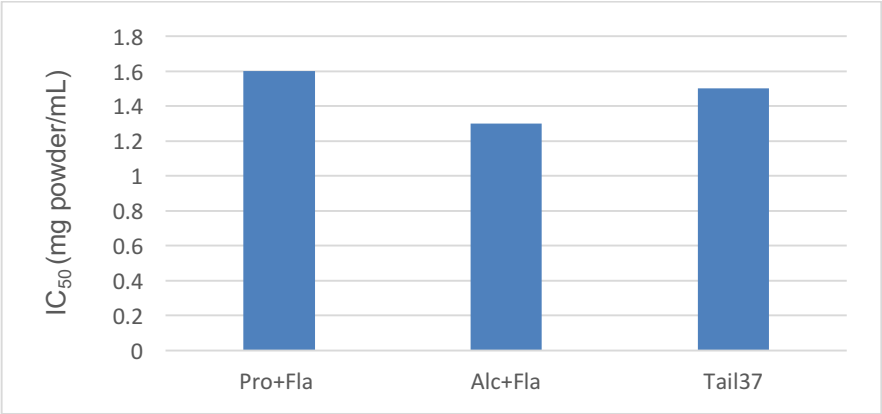


Figure 43. The ACE-inhibiting activity of collagen isolated from cod skin. Hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme, Protamex, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L with the E/S ratio 1/50.

There were no duplicates or triplicates performed on samples in Figure 43 due to time limitation.

All the samples mentioned above were ran in the ACE-inhibiting activity measurements but those three samples were the only one that showed activity. Since that were only samples with E/S ratio 1/25 that showed activity it can be assumed that higher values of DH can increase ACE-inhibiting activity.

Both of the samples hydrolysed with Flavourzyme with the E/S ratio 1/25 had ACE-inhibiting activity but as shown in Figure 42 the sample hydrolysed with Flavourzyme had the lowest ACE-inhibiting activity of the four samples hydrolysed in the pre-trial. It seems that samples hydrolysed with only Flavourzyme have low activity but when Flavourzyme is combined with another enzyme they break the protein in certain way and the collagen peptide gets higher degree of hydrolysis and greater ACE-inhibiting activity.

Filtrated collagen peptides were also measured. The results from the ACE measurements are shown in Figure 44. The peptides that are <5 kDa had the highest ACE inhibitory activity 1.4 IC_{50} (mg powder/ml).

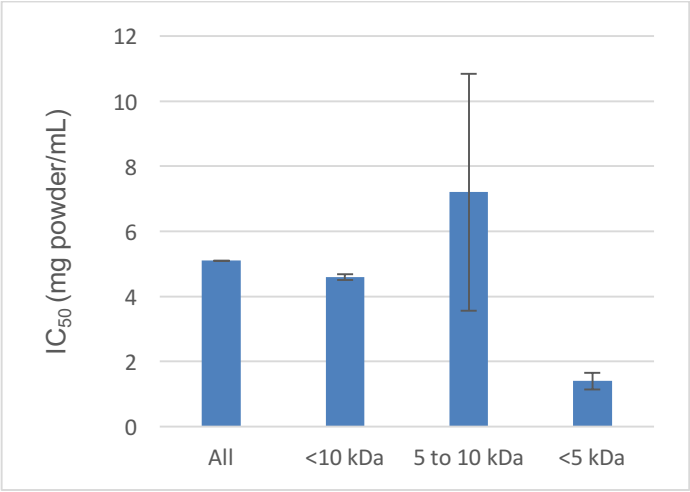


Figure 44. The ACE-inhibiting activity of collagen isolated from cod skin, hydrolysed for 2 hours at 45°C with Protamex and Flavourzyme with E/S ratio 1/50 and filtrated with 5 kDa- and 10 kDa filters and unfiltered sample (All).

Samples in Figure 44 were performed in duplicate except the unfiltered sample (All) that was only measured once. The sample filtered with <5 kDa clearly had higher activity than the other samples but there were too few measurements to be able to perform statistical analysis.

Previous studies have showed higher ACE-inhibiting activity than found in this project. ACE-inhibiting activity values for collagen peptides from skate skin were ranging from 0.68-6.19 IC_{50} (mg/ml) depending on which enzyme used. Collagen hydrolysed with Alcalase had ACE-inhibiting activity value 1,89 IC_{50} (mg/ml) so it has more activity than the samples hydrolysed with Alcalase in this project (Lee *et al*., 2011).

Gelatin from Alaska Pollock that was hydrolysed for 2 hours had ACE-inhibitory values ranging from 0.662-1.199 IC_{50} (mg/ml) depending on the enzyme used. The sample hydrolysed with Alcalase had ACE-inhibiting activity 0.840 IC_{50} (mg/ml) and sample hydrolysed with Neutrase had ACE-inhibiting activity 0.822 IC_{50} (mg/ml). Those values are lower than obtained in this project, which means they have greater ACE-inhibiting activity (Byun & Kim, 2001). It should be kept in mind that different methods were used in analyzing the ACE inhibition activity of those samples and comparison therefore difficult.

ACE values for fish protein hydrolysates of Blue Whiting were ranging from 1.34-2.41 IC_{50} (mg/ml) depending on DH of the samples. When the sample were 0% DH the IC_{50} could not be measured. The ACE-inhibiting activity was greater for samples with higher DH (Geirsdottir *et al*., 2011). Those analysis were performed with the same method as used in this project and therefore it can be concluded that collagen hydrolysated had higher ACE inhibition activity than FPH from Blue whiting.

4.6 Elastase

The elastase-inhibiting activity was measured for collagen peptides samples hydrolysed with Protamex, Protamex+Flavourzyme, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L and with E/S ratio 1/25 and 1/100. There was no activity in samples hydrolysed with Protamex+Flavourzyme, Protamex, Alcalase and Alcalase+Flavourzyme with E/S ratio 1/100. Samples hydrolysed with Tail 37 and TZ-02-L with E/S ratio 1/100 showed elastase inhibiting activity, Tail 37 had greater activity it had 8.5 IC_{50} mg/mL and TZ-02-L had 32.9 IC_{50} mg/mL (Figure 45).

Elastase-inhibiting activity was found in all samples hydrolysed with the E/S ratio 1/25, the values were ranging from 3.3-79.7 IC_{50} mg/mL. Alcalase had lower elastase-inhibiting activity than all the other samples. Collagen sample hydrolysed with Tail 37 with E/S ratio 1/25 had the highest elastase-inhibiting activity 3.3 IC_{50} mg/mL. No correlation was found between elastase-inhibiting activity and DH. The difference in inhibitions activity is therefore more likely linked to the amino acid row in the peptides.

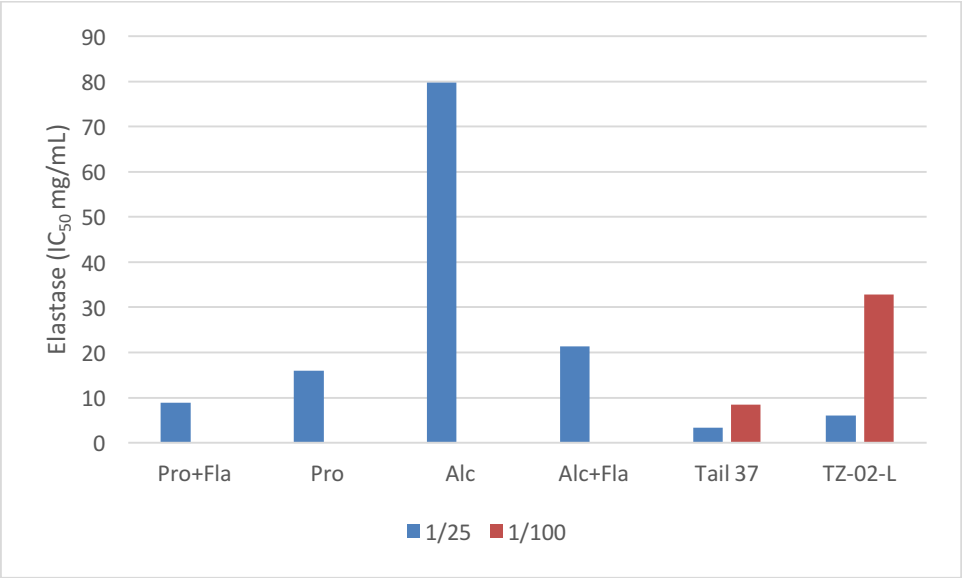


Figure 45. The elastase-inhibiting activity of collagen isolated from cod skin (n=2). Hydrolysed for 2 hours at 45°C with Protamex+Flavourzyme, Protamex, Alcalase, Alcalase+Flavourzyme, Tail 37 and TZ-02-L with the E/S ratio 1/25 and 1/100.

The highest elastase-inhibiting activity was in samples hydrolysed with Tail37 and TZ-02-L and the activity seemed to be related to the amount of enzyme used. Those enzymes were obtained from Tailorzyme while the other enzymes were from Novozyme. There might be some substance in the enzymatic preparation contributing to this elastase-inhibiting activity. That is a factor that is worthy to examine as an extension of this study by measuring the activity of the enzyme alone. Sellers of collagen hydrolysates claim that they have a positive effect on skin. The results here indicate that the hydrolysates do have an inhibiting effect in elastase and are therefore highly valuable for further research.

Not many studies are to be found on elastase-inhibiting activity of collagen but elastase-inhibiting activity was found in collagen isolated from squid skin. The collagen had been hydrolysed with Alcalase and fractionated into three groups, all the groups showed elastase-inhibiting activity (Nam, You, & Kim, 2008) but different methods were used in analyzing the elastase inhibition activity and comparison therefore difficult.

5 Conclusion

Three different isolation methods were tried in the project. One of them showed the highest yield and was used to isolate collagen for the next steps. The isolation method that gave the highest yield consisted of NaOH, acetic acid and tap water.

Samples that were hydrolysed with two enzymes at the same time had higher DH values than samples hydrolysed with only one enzyme. The trial consisted of 18 different combinations of enzymes and the results gave us range of DH values and that information can be used for further studies of collagen peptides.

The antioxidant activity was low for collagen peptides measured. Samples with high DH values seems to have lower values of ORAC. The correlation was strongest with samples hydrolysed with Protamex, when higher amount of Protamex was used the ORAC value was lower.

There was not much correlation between DH and reducing power but the results indicated that peptides with higher DH value and smaller peptides had lower reducing power.

There was no correlation between metal chelating and DH values. The peptide size had different effect on the antioxidant activity of the samples based on the different antioxidant test used. Peptide size is only one of many factors that can affect the antioxidant activity of the samples. In order to get a better understanding on the activity it is necessary to analyze the amino acid composition with mass spectrometry to get information about amino acid sequence.

Some samples showed ACE- and elastase-inhibiting activity. In both ACE- and elastase-inhibiting activity measurements it seemed that peptides with higher DH value had greater activity. Further research is needed in order to confirm some of those bioactivity measurements.

Those results show that the skin of Icelandic cod is a valuable product and instead of being thrown away as waste or sold to animal feed manufacturers at low price, it could be used to isolate collagen which is a valuable and popular product.

6 Future perspectives

It has been indicated that various factors can affect the antioxidant activity of peptides such as structural formation, position of specific amino acids and enzyme specificity and therefore further research is suggested on the construction of the collagen peptides. In order to get a better understanding on the activity it is necessary to analyze the amino acid sequence with mass spectrometry.

It would also be of interest to measure the elastase inhibiting activity of the enzymes from Tailorzyme, Tail37 and TZ-02-L, to see if they are responsible for the elastase inhibiting activity or if it is the collagen peptides. It would also be interesting to try other bioactive measurements for example cell lines and *in vivo*.

The filtration step should be repeated using smaller filters to get smaller peptides and thus be able to see if they have higher bioactivity.

References

A/S, N. (2002). Alcalase Food Grade - Product sheet.

Ahn, C. B., Lee, K. H., & Je, J. Y. (2010). Enzymatic production of bioactive protein hydrolysates from tuna liver: effects of enzymes and molecular weight on bioactivity. *International Journal of Food Science & Technology, 45*(3), 562-568.

Akoh, C. C., & Min, D. B. (2008). *Food lipids: chemistry, nutrition, and biotechnology*: CRC press.

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal, 21*(2), 143-152.

Alberts, B., Johnson, A., Lewis, J., et.al. (2002). *Molecular biology of the cell* (4th ed.). New York, USA: Garland Science.

Aleman, A., Gimenez, B., Perez-Santin, E., Gomez-Guillen, M. C., & Montero, P. (2011). Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate. *Food Chemistry, 125*(2), 334-341. doi:10.1016/j.foodchem.2010.08.058

Alemán, A., & Martínez-Alvarez, O. (2013). Marine Collagen as a Source of Bioactive Molecules: A Review. *The Natural Products Journal, 3*(2), 105-114.

AOAC-976.18. (2000). Measurement of salt in fish meal w/titrino. 17th.

AOCS-Ba-3-38. (1997). Method for analysing total fat in fish and fish meal.

Arason, S. (2012). *Hvaða aukahráefni er tæknilega framkvæmanlegt að nýta um borð í fiskiskipum*. Retrieved from Reykjavik, Iceland:

Belitz, H. D., Grosch, W., Schieberle, P. (2009). *Food chemistry* (4th ed.). Berlin, Germany: Springer.

Berisio, R., Vitagliano, L., Mazzarella, L., & Zagari, A. (2002). Crystal structure of the collagen triple helix model [(Pro-Pro-Gly) 10] 3. *Protein Science, 11*(2), 262-270.

Bieth, J. G. (2000). [The elastases]. *Journal de la Société de biologie, 195*(2), 173-179.

Boyer, R. F., Grabill, T. W., & Petrovich, R. M. (1988). Reductive release of ferritin iron: a kinetic assay. *Analytical biochemistry, 174*(1), 17-22.

Bray, A., Johnson, H., Raff, L., Walter, R. (2010). *Essential cell biology* (3rd ed.). New York, USA: Garland Science.

Buxbaum, E. (2015). *Fundamental of protein structure and function* (2nd ed.). USA: Springer.

Byun, H.-G., & Kim, S.-K. (2001). Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (Theragra chalcogramma) skin. *Process Biochemistry, 36*(12), 1155-1162.

Cao, G., & Prior, R. L. (1999). [5] Measurement of oxygen radical absorbance capacity in biological samples. *Methods in enzymology, 299*, 50-62.

Chalamaiah, M., 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.

Chary, N. S., Kamala, C., & Raj, D. S. S. (2008). Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. *Ecotoxicology and environmental safety, 69*(3), 513-524.

Chi, C.-F., Wang, B., Wang, Y.-M., Zhang, B., & Deng, S.-G. (2015). Isolation and characterization of three antioxidant peptides from protein hydrolysate of bluefin leatherjacket (Navodon septentrionalis) heads. *Journal of Functional Foods, 12*, 1-10.

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science, 66*(6), 1219-1227.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *Journal of Biological Chemistry, 252*(3), 1102-1106.

Cohen, D. M., Inada, T., Iwamoto, T., Scialabba, N. (1990). FAO Species Catalogue Volume 10. Gadiform Fishes of the World. *Food and agriculture organization of the united nations*.

Damodaran, S. (1997). *Food proteins and their applications* (Vol. 80): CRC Press.

Duan, R., Zhang, J. J., Du, X. Q., Yao, X. C., & Konno, K. (2009). Properties of collagen from skin, scale and bone of carp (Cyprinus carpio). *Food Chemistry, 112*(3), 702-706. doi:10.1016/j.foodchem.2008.06.020

Frankel, E. N. (2007). *Antioxidants in food and biology: facts and fiction*: oily Press Bridgwater, UK.
Ganske, F., Dell, E.J. (2006). *ORAC assay on the FLUOstar OPTIMA to determine antioxidant capacity*. Retrieved from USA:
Garfin, D. E. (2003). Gel electrophoresis of proteins. *Essential cell biology, 1*, 197-268.
GE. (2014). Cross flow filtration method handbook. Retrieved from
Geirsdottir, M., Sigurgisladottir, S., Hamaguchi, P. Y., Thorkelsson, G., Johannsson, R., Kristinsson, H. G., & Kristjansson, M. M. (2011). Enzymatic Hydrolysis of Blue Whiting (Micromesistius poutassou); Functional and Bioactive Properties. *Journal of Food Science, 76*(1), C14-C20. doi:10.1111/j.1750-3841.2010.01877.x
Giménez, B., Alemán, A., Montero, P., & Gómez-Guillén, M. (2009). Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. *Food Chemistry, 114*(3), 976-983.
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. doi:10.1016/j.foodhyd.2011.02.007
Gu, R.-Z., Li, C.-Y., Liu, W.-Y., Yi, W.-X., & Cai, M.-Y. (2011). Angiotensin I-converting enzyme inhibitory activity of low-molecular-weight peptides from Atlantic salmon (Salmo salar L.) skin. *Food Research International, 44*(5), 1536-1540.
Gudmundsson, M., & Hafsteinsson, H. (1997). Gelatin from cod skins as affected by chemical treatments. *Journal of Food Science, 62*(1), 37-&. doi:10.1111/j.1365-2621.1997.tb04363.x
Guo, L., Hou, H., Li, B., Zhang, Z., Wang, S., & Zhao, X. (2013). Preparation, isolation and identification of iron-chelating peptides derived from Alaska pollock skin. *Process Biochemistry, 48*(5), 988-993.
HagstofaIslands. (2015). Afli eftir fisktegundum og londunarhofnum januar 2000 - november 2015. Retrieved from http://px.hagstofa.is/pxis/pxweb/is/Atvinnuvegir/Atvinnuvegir__sjavarutvegur__aflatolur__afli_manudir/SJA01101.px/table/tableViewLayout1/?rxid=3d5eef07-4ac5-4f93-99ac-8deda3ca21a9
Halldorsdottir, S. M., Kristinsson, H. G., Sveinsdottir, H., Thorkelsson, G., & Hamaguchi, P. Y. (2013). The effect of natural antioxidants on haemoglobin-mediated lipid oxidation during enzymatic hydrolysis of cod protein. *Food Chemistry, 141*(2), 914-919.
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.
Halldorsdottir, S. M., Sveinsdottir, H., Gudmundsdottir, A., Thorkelsson, G., & Kristinsson, H. G. (2014). High quality fish protein hydrolysates prepared from by-product material with Fucus vesiculosus extract. *Journal of Functional Foods, 9*, 10-17.
Hamada, J. S. (2000). Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. *Journal of Food Science, 65*(2), 305-310. doi:10.1111/j.1365-2621.2000.tb15998.x
Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods, 4*(1), 6-24. doi:10.1016/j.jff.2011.09.001
Harper, E. (1980). Collagenases. *Annual review of biochemistry, 49*(1), 1063-1078.
Harvey, R. A., Ferrier, D. (2011). *Biochemistry* (5th ed.). Baltimore, USA: Wolters Kluwer.
Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 53*(3), 588-593.
Himaya, S., Ngo, D.-H., Ryu, B., & Kim, S.-K. (2012). An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. *Food Chemistry, 132*(4), 1872-1882.
Hlíðberg, J. B., Pálsson, J., Jónsson, G. (2006). *Íslenskir fiskar*. Ísland: Vaka-Helgafell.
Hoa, N. T., Huong, T. T. X., Minh, N. P., Dao, D. T. (2014). Investigation of enzymatic optimization by flavourzyme and celluclast for soy protein hydrolysate powder. *International Journal of Advances in Pharmacy, Biology and Chemistry, 3*(3), 550-562.
Hrckova, M., Rusñakova, M., Zemanovic, J. (2002). Enzymatic hydrolysis of defatted soy flour by three differentproteases and their effect on the functional properties of resulting protein hydrolysates. *Czech Journal of Food Sciences, 20*, 7-14.

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*(16), 4437-4444.

Huang, Y. R., Shiau, C. Y., Chen, H. H., & Huang, B. C. (2011). Isolation and characterization of acid and pepsin-solubilized collagens from the skin of balloon fish (Diodon holocanthus). *Food Hydrocolloids, 25*(6), 1507-1513. doi:10.1016/j.foodhyd.2011.02.011

Hudson, B. J. (2012). *Food antioxidants*: Springer Science & Business Media.

Insel, P., Ross, D., McMahon, K, Bernstein, M. (2011). *Nutrition* (4 ed.). USA: Jones and Bartlett Publishers.

ISO-5983-2. (2005). Animal feeding stuffs – Determination of nitrogen content and calculation of crude protein content – Part 2: Block digestion/steam distillation method.

ISO-5984. (2002). Animal feeding stuffs – Determination of crude ash.

ISO-6496. (1999). Animal feeding stuffs - Determination of moisture and other volatile matter content.

ISO-16634-1. (2008). Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content — Part 1: Oilseeds and animal feeding stuffs.

Je, J.-Y., Qian, Z.-J., Byun, H.-G., & Kim, S.-K. (2007). Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry, 42*(5), 840-846.

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*(5), 471-478.

Kim, S.-K., Kim, Y.-T., Byun, H.-G., Nam, K.-S., Joo, D.-S., & Shahidi, F. (2001). Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska pollack skin. *Journal of Agricultural and Food Chemistry, 49*(4), 1984-1989.

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.

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry, 102*(4), 1317-1327.

Ktari, N., Fakhfakh, N., Balti, R., Ben Khaled, H., Nasri, M., & Bougatef, A. (2013). Effect of degree of hydrolysis and protease type on the antioxidant activity of protein hydrolysates from cuttlefish (Sepia officinalis) by-products. *Journal of Aquatic Food Product Technology, 22*(5), 436-448.

Kumar, N. S., Nazeer, R., & Jaiganesh, R. (2012). Purification and identification of antioxidant peptides from the skin protein hydrolysate of two marine fishes, horse mackerel (Magalaspis cordyla) and croaker (Otolithes ruber). *Amino Acids, 42*(5), 1641-1649.

Lee, J. K., Jeon, J.-K., & Byun, H.-G. (2011). Effect of angiotensin I converting enzyme inhibitory peptide purified from skate skin hydrolysate. *Food Chemistry, 125*(2), 495-499.

Lehninger, N., L.D., Cox, M. (2008). *Principles of biochemistry* (5th ed.). New York, USA: W.H. Freeman and company.

Li, B., Chen, F., Wang, X., Ji, B., & Wu, Y. (2007). Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. *Food Chemistry, 102*(4), 1135-1143.

Li, X., Luo, Y., Shen, H., & You, J. (2012). Antioxidant activities and functional properties of grass carp (Ctenopharyngodon idellus) protein hydrolysates. *Journal of the Science of Food and Agriculture, 92*(2), 292-298.

Liaset, B., Nortvedt, R. Lied, E., Espe M. (2002). Studies on the nitrogen recovery in enzymic hydrolysis of Atlantic salmon (Salmo salar, L.) frames by Protamex ™ protease. *Process Biochemistry, 37*, 1263-1269.

Liceaga-Gesualdo, A., & Li-Chan, E. (1999). Functional properties of fish protein hydrolysate from herring (Clupea harengus). *Journal of Food Science, 64*(6), 1000-1004.

Liu, F., Liu, C., Lorena, D., Zhang, X., Fu, Z. (2012). Evaluation of the antioxidant activity of collagen peptide additive extracted from cod skin. *Journal of enviromental protection and ecology*, 1836-1841.

Lodish, H., Berk, A., Zipursky,S.L., Matsudaira, P., Baltimore, D., Darnell, J. (2000). *Molecular Cell Biology* (Vol. 4). New York: W.H. Freeman.

Maeda, H., Hosomi, R., Chiba, U., & Fukunaga, K. (2013). Chemical Composition of Salmon Ovary Outer Membrane and Its Protein Increases Fecal Mucins Content in C57BL/6J and Type 2 Diabetic/Obese KK-Ay Mice. *Foods, 2*(3), 415-429.

Mamelona, J., Saint-Louis, R., & Pelletier, É. (2010). Nutritional composition and antioxidant properties of protein hydrolysates prepared from echinoderm byproducts. *International Journal of Food Science & Technology, 45*(1), 147-154.
Matís. (2015). Fiskbókin. Retrieved from http://fiskbokin.is/botnfiskar/thorskur/
Matmaroh, K., Benjakul, S., Prodpran, T., Encarnacion, A. B., & Kishimura, H. (2011). Characteristics of acid soluble collagen and pepsin soluble collagen from scale of spotted golden goatfish (Parupeneus heptacanthus). *Food Chemistry, 129*(3), 1179-1186.
Mendis, E., Rajapakse, N., Byun, H.-G., & Kim, S.-K. (2005). Investigation of jumbo squid (Dosidicus gigas) skin gelatin peptides for their in vitro antioxidant effects. *Life Sciences, 77*(17), 2166-2178.
Millipore. (2003). Protein concentration and diafiltration by tangential flow filtration.
Mohammad, A. W., Suhimi, N.M., Aziz, A.G.K.A., Jahim, M.J. (2014). Process for production of hydrolysed collagen from agriculture resources: pontential for further development. *Journal of applied sciences, 14*(12), 1319-1323. doi:10.3923/jas2014.1319.1323
Molla, A. E. (2011). Processing of visceral waste proteins of beluga (huso huso) using protamex. *Biological journal of armenia, 4*(63), 39-45.
Möller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: indication for health effects. *European journal of nutrition, 47*(4), 171-182.
Nagai, T., & Suzuki, N. (2000). Isolation of collagen from fish waste material - skin, bone and fins. *Food Chemistry, 68*(3), 277-281. doi:10.1016/s0308-8146(99)00188-0
Nam, K., You, S., & Kim, S. (2008). Molecular and physical characteristics of squid (Todarodes pacificus) skin collagens and biological properties of their enzymatic hydrolysates. *Journal of Food Science, 73*(4), C249-C255.
Nazeer, R., & Anila Kulandai, K. (2012). Evaluation of antioxidant activity of muscle and skin protein hydrolysates from giant kingfish, Caranx ignobilis (Forsskål, 1775). *International Journal of Food Science & Technology, 47*(2), 274-281.
Neklyudov, A., Ivankin, A., & Berdutina, A. (2000). Properties and uses of protein hydrolysates (Review). *Applied Biochemistry and Microbiology, 36*(5), 452-459.
Ngo, D.-H., Qian, Z.-J., Ryu, B., Park, J. W., & Kim, S.-K. (2010). In vitro antioxidant activity of a peptide isolated from Nile tilapia (Oreochromis niloticus) scale gelatin in free radical-mediated oxidative systems. *Journal of Functional Foods, 2*(2), 107-117.
Nielsen, P., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science, 66*(5), 642-646.
Nollet, L. M., & Toldrá, F. (2009). *Handbook of seafood and seafood products analysis*: CRC Press.
Novozymes. (no date). Protamex.
Novozymes(a). (no date). Proteases. Retrieved from http://www.novozymes.com/en/solutions/biopharma/Brochures/Documents/2014-12715-01_Biocatalysis-Product-Sheet-Protease-3.pdf
Opheim, M., Šližytė, R., Sterten, H., Provan, F., Larssen, E., & Kjos, N. P. (2015). Hydrolysis of Atlantic salmon (Salmo salar) rest raw materials—Effect of raw material and processing on composition, nutritional value, and potential bioactive peptides in the hydrolysates. *Process Biochemistry, 50*(8), 1247-1257.
Oyaizu, M. (1986). Studies on products of browning reaction--antioxidative activities of products of browning reaction prepared from glucosamine. *Eiyogaku zasshi= Japanese journal of nutrition*.
Petsko, G. A., Ringe, D. (2004). *Protein structure and function*. London, UK: Blac well publisher.
Phanturat, P., Benjakul, S., Visessanguan, W., & Roytrakul, S. (2010). Use of pyloric caeca extract from bigeye snapper (Priacanthus macracanthus) for the production of gelatin hydrolysate with antioxidative activity. *Lwt-Food Science and Technology, 43*(1), 86-97. doi:10.1016/j.lwt.2009.06.010
Pocock, G., & Richards, C. D. (2009). *The human body: an introduction for the biomedical and health sciences*: Oxford University Press.
Pokorny, J., Yanishlieva, N., & Gordon, M. H. (2001). *Antioxidants in food: practical applications*: CRC press.
Raghavan, S., & Kristinsson, H. G. (2009). ACE-inhibitory activity of tilapia protein hydrolysates. *Food Chemistry, 117*(4), 582-588.
Ramachandran, G. N. (1976). *Biochemistry of collagen*. New York, USA: Springer.
Roslan, J., Yunos, K. F. M., Abdullah, N., & Kamal, S. M. M. (2014). Characterization of fish protein hydrolysate from tilapia (Oreochromis niloticus) by-product. *Agriculture and Agricultural Science Procedia, 2*, 312-319.

Rutherfurd, S. M. (2010). Methodology for determining degree of hydrolysis of proteins in hydrolysates: a review. *Journal of AOAC International, 93*(5), 1515-1522.
Sadowska, M., Kolodziejska, I., & Niecikowska, C. (2003). Isolation of collagen from the skins of Baltic cod (Gadus morhua). *Food Chemistry, 81*(2), 257-262. doi:10.1016/s0308-8146(02)00420-x
Samaranayaka, A. G., & Li-Chan, E. C. (2008). Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (Merluccius productus). *Food Chemistry, 107*(2), 768-776.
Scharffetter–Kochanek, K., Brenneisen, P., Wenk, J., Herrmann, G., Ma, W., Kuhr, L., . . . Wlaschek, M. (2000). Photoaging of the skin from phenotype to mechanisms. *Experimental Gerontology, 35*(3), 307-316.
Senaratne, L. S., Park, P. J., & Kim, S. K. (2006). Isolation and characterization of collagen from brown backed toadfish (Lagocephalus gloveri) skin. *Bioresource Technology, 97*(2), 191-197. doi:10.1016/j.biortech.2005.02.024
Sentandreu, M. Á., & Toldrá, F. (2006). A fluorescence-based protocol for quantifying angiotensin-converting enzyme activity. *Nature Protocols, 1*(5), 2423-2427.
Shamamian, P., Schwartz, J. D., Pocock, B. J., Monea, S., Whiting, D., Marcus, S. G., & Mignatti, P. (2001). Activation of progelatinase A (MMP-2) by neutrophil elastase, cathepsin G, and proteinase-3: A role for inflammatory cells in tumor invasion and angiogenesis. *Journal of cellular physiology, 189*(2), 197-206.
Shoulders, M. D., & Raines, R. T. (2009). Collagen Structure and Stability *Annual review of biochemistry* (Vol. 78, pp. 929-958). Palo Alto: Annual Reviews.
Sigma-Aldrich. (e.d.). Collagenase guide. General information about Sigma-Aldrich Collagenase product. Retrieved from http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/collagenase-guide.html
Sikorski, Z. E., Pan, B.S., Shahidi. (1994). *Seafood Proteins*. New York, USA.
Srinivasan Damodaran, K. L. P., Owen R. Fennema. (2008). *Fennema's Food Chemistry* (Vol. 4). USA: CRC press.
Srivastava, Y. (2013). *Advances in food science and nutrition*. Nigeria: Science and education developement institute.
Takeuchi, H., Gomi, T., Shishido, M., Watanabe, H., & Suenobu, N. (2010). Neutrophil elastase contributes to extracellular matrix damage induced by chronic low-dose UV irradiation in a hairless mouse photoaging model. *Journal of dermatological science, 60*(3), 151-158.
Theodore, A. E., Raghavan, S., & Kristinsson, H. G. (2008). Antioxidative activity of protein hydrolysates prepared from alkaline-aided channel catfish protein isolates. *Journal of Agricultural and Food Chemistry, 56*(16), 7459-7466.
Thring, T. S., Hili, P., & Naughton, D. P. (2009). Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. *BMC Complementary and Alternative Medicine, 9*(1), 1.
Venugopal, V. (2009). *Marine products for healthcare. Functional and bioactive nutraceutical compounds from the ocean.* USA: CRC Press.
Wang, L., An, X. X., Yang, F. M., Xin, Z. H., Zhao, L. Y., & Hu, Q. H. (2008). Isolation and characterisation of collagens from the skin, scale and bone of deep-sea redfish (Sebastes mentella). *Food Chemistry, 108*(2), 616-623. doi:10.1016/j.foodchem.2007.11.017
Wittenauer, J., Maeckle, S., Sussmann, D., Schweiggert-Weisz, U., & Carle, R. (2015). Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity. *Fitoterapia, 101*, 179-187. doi:10.1016/j.fitote.2015.01.005
Yang, J.-I., Liang, W.-S., Chow, C.-J., & Siebert, K. J. (2009). Process for the production of tilapia retorted skin gelatin hydrolysates with optimized antioxidative properties. *Process Biochemistry, 44*(10), 1152-1157.
You, L., Zhao, M., Cui, C., Zhao, H., & Yang, B. (2009). Effect of degree of hydrolysis on the antioxidant activity of loach (Misgurnus anguillicaudatus) protein hydrolysates. *Innovative Food Science & Emerging Technologies, 10*(2), 235-240.
Zhang, M., Liu, W. T., & Li, G. Y. (2009). Isolation and characterisation of collagens from the skin of largefin longbarbel catfish (Mystus macropterus). *Food Chemistry, 115*(3), 826-831. doi:10.1016/j.foodchem.2009.01.006
Zhang, Y., Olsen, K., Grossi, A., & Otte, J. (2013). Effect of pretreatment on enzymatic hydrolysis of bovine collagen and formation of ACE-inhibitory peptides. *Food Chemistry, 141*(3), 2343-2354.
Zhong, S., Ma, C., Lin, Y. C., & Luo, Y. (2011). Antioxidant properties of peptide fractions from silver carp (Hypophthalmichthys molitrix) processing by-product protein hydrolysates evaluated by electron spin resonance spectrometry. *Food Chemistry, 126*(4), 1636-1642.

Appendix I

Tables that summaries the isolation methods.

	Duan, Zhang, Du *et al.*	Phanturat, Benjuakul *et al.*	Guðmundsson, Hafsteinsson
Preparation	Skin washed with tap water, put in polyethylene bags and stored at -25°C.		Skin washed and treated with sodium hydroxide solution for 40 min with water at pH 7.5. Repeated 3x.
Remove non-collagenous protein	0.1 M NaOH 1:8 (w/v). Stirred for 6h. Washed with cold dH2O, until neutral pH.	0.1 M NaOH with 1:10 (w/v) at 25°C with gentle stirring, for 30 min x3. Wash with tap water until neutral pH.	Skin treated with sulfuric acid solution, washed with water. Repeated 3x.
Remove fat	Soaked in 1% detergent 1:10 (w/v) over night, wash with dH2O	Soaked in 0.05 M acetic acid 1:10 (w/v) for 3 h, with stirring. Wash with tap water until neutral pH.	Skin treated with citric acid, washed, repeated 3x. 1 kg skin / 7l acid or alcali solution.
Remove pigments			Skin extracted with distilled water over night at 45°C, without stirring.
Extraction	Treated skin was cut into small pieces and extracted with 0.5M acetic acid for 3 days with stirring	Swelled fish skin was soaked in distilled water with skin/water ratio 1:10 (w/v) at 45°C for 12 h with stirring to extract gelatin from the skin.	The mixture with the remains of the skin was then filtered in a Buchner funnel with a Whatman no. 4 filterpaper.
Centrifuge	The extract was centrifuged at 20000xg for 1 hour	Solution filtered using two layers of cheesecloth.	The clear extract was then evaporated under vacuum at 43–45 C until 85–90% of the water had been removed.
Supernatant	The supernatants were salted out by adding NaCl to a final concentration of 2.5 M in 0.05 M tris (hydroxymethyl) aminomethane, pH7.	The resultant filtrate was freeze-dried.	The concentrate containing the gelatin was then air dried in a fume hood at 45 C to remove remaining water.
Precipitate	The precipitate was obtained by centrifugation at 20000 xg for 30 min	The dry sample was blended until the fine power was obtained using a blender	One portion of gelatin liquid was freeze dried.
Dialysis	Dissolved in 0.5 M acetic acid, dialysed agains 0.1 acetic acid and disstilled water and freeze-dried to get ASC		

	Nagai and Suzuki	Senaratne, Park, Kim	Wang, An, Yang *et al.*
Preparation	Performed at 4°C	Performed at 4°C with continuous stirring	Preparation and isolation performed at 4°C
Remove non-collagenous protein			Skin washed with dH2O, cut, put in polyethylene bags and frozen at -20°C
Remove fat	0.1 N NaOH, wash with dH2O, freeze-dried	0.1 N NaOH 1:10 (w/v) in 3 days. Wash with dH2O	20 volumes of 1.0 M NaCl (0.05 M Tris–HCl, pH 7.5) for 24 h after homogenising for 2 min, wash with dH2O. Freeze-dried.
Remove pigments	Extracted with 10% butyl alcohol	10% butyl alcohol solution with ratio 1:10 (w/v) for 24 h. Washed, freeze-dried.	
Extraction	The insoluble matter was extracted with 0.5 M acetic acid for 3 days.	The lyophilized matter was extracted with 0.5 M acetic acid for 3 days.	Collagens was extracted with 0.5 M acetic acid at a sample/ solution ratio of 1:100 (w/v) for 24 h with stirring
Centrifuge	Centrifuged at 20000xg for 1 hour, the residue was re-extracted with the same solution for 2 days		Centrifuged at 20000g for 1 h and the residue was re-extracted and centrifuged under the same conditions.
Supernatant	The viscous solution was mixed and salted out by adding NaCl to a final concentration of 0.9M	The viscous solution was centrifuged, supernatant was collected and salted out, adding NaCl to a final concentration of 0.7 M and added NaCl to a final concentration of 2.3 M in 0.05 M Tris–HCl (pH 7.5).	The supernatants were combined, and precipitated by adding NaCl to a final concentration of 0.9 M.
Precipitate	Precipitate obtained by centrifugation at 20000xg for 1 h, dissolved in 0.5 M acetic acid	Precipitate separated in centrifugation at 12000xg for 1 h at 4 °C.	Precipitate obtained by centrifugation at 2500g for 0.5 h, dissolved in 0.5 M acetic acid
Dialysis	Dialyzed against 0.1 M acetic acid	Precipitate was dissolved in 0.5 M acetic acid, dialysed against 0.1 M acetic acid.	Dialysed for 48 h against 10 volumes of 0.1 M acetic acid and distilled water, changed every 8 h, lyophilized.
Pepsin		10% pepsin used for hydrolysis (48h).	

	Zhang, Liu and Li	Huang, Shiau, Chen
Preparation	Performed at 4°C with continuous stirring	Performed at 4°C with continuous stirring
Remove non-collagenous protein	Skin washed with dH2O, cut, frozen at -20°C	Skin washed with dH2O, cut, put in polyethylene bags and frozen at -20°C
Remove fat	0.1 M NaOH 1:20 (w/v), with 0.5% nonionic detergent for 24 h. Washed with dH2O until pH is neutral.	0.1 M NaOH at ratio of 1:10 (w/v) for 3 days, washed with dH2O until neutral.
Remove pigments	The fat was removed with 15% (v/v) butyl alcohol with ratio 1:24 for 24 h, changing the solution every 12 h, defatted skin washed with distilled water	10% butyl alcohol sample/solution ratio of 1:10 (w/v) for 1 day and washed with dH2O. Extracted with 0.5 M acetic acid with ratio of 1:10 (w/v) for 1 day. Centrifuged at 10,000g for 30min at 4 C.
Extraction	Defatted skins were bleached with 3% H2O2 solution for 24h, solution changed once.	
Centrifuge	The suspensions were centrifuged at 9,000g for 15 min at 4 °C, it was re-extracted in 0.5M acetic acid with a sample/ solution ratio of 1:10 for 12 h with same conditions.	
Supernatant	The supernatants were combined and salted-out by adding NaCl to a final concentration of 0.7M.	The supernatants were collected and kept at 4°C.
Precipitate	The resultant precipitate was collected by centrifugation at 9,000g for 15 min at 4 °C and then dissolved in 0.5 M acetic acid.	The precipitate was re-extracted in 0.5 M acetic acid with a sample/ solution ratio of 1:10 (w/v) for 12 h, centrifuged at 10,000g for 30 min at 4 C.
Second supernatant		The supernatants were combined. The combined extracts were salted out by adding NaCl to final concentration of 0.7 M.
Second precipitate		The resultant precipitate was collected by centrifugation at 10,000g for 15 min at 4°C and then dissolved in 0.5 M acetic acid.
Dialysis	The solution obtained was dialysed against 0.1 M acetic acid for 3 days	The solution obtained was dialysed against 0.1 M acetic acid for 3 days
Pepsin	Undissolved residue (after acid extraction) suspended in 2 volumes of 0.5M acetic acid with 1.5% (w/w) pepsin for 30 h at 4 °C with stirring.	Undissolved residue (after acid extraction) was suspended in 2 volumes of 0.5M acetic acid with 1.5% (w/w) pepsin for 30 h at 4 °C with stirring.
PSC	PSC was obtained with same method as ASC	PSC was obtained with same method as ASC
Yield	The extraction was performed 3 times and the yield value was the average of that	The extraction was performed 3 times and the yield value was the average of that

Appendix II

Protocol for freeze-dryer

Computer freeze-dryer

1. The first step is to cool down the shelves to at least -20°C.
    a. Press: semi-autofunction and put the freezing on
    b. Press: synoptic
    c. Close door
2. When the shelves are cold enough, put the samples in and place sensors under samples (between tray and sample)
3. Close the top door tightly
4. Close the bottom door, take rubber off and close tap valve
5. Start freeze-drying cycle
    a. Go to freeze-dry screen, press start cycle and confirm (y)
    b. Go to synoptic – check the pressure
    c. Check Rough pressure – it should go down
        i. It should go down – if not the door is not properly closed (put the rubber closure towards the door to close it properly)
        ii. Rough pressure goes down to ~400 mbar first and after that all pressure indicators goes down to ~0 mbar.
6. When ending the freeze-dryer it is important to check the temperature on the probe.
    a. The temperature should be ~25°C
7. Go to freeze-dry screen
    a. Press: End Cycle and confirm (y)
    b. Press: Release vacuum
8. Open the tap and release vacuum
9. Open both doors and let the ice in condenser melt

Appendix III

Protocol for isolation method used in this project

Isolation method – Phaturant et.al. (0.1M NaOH and 0.05 acetic acid)

Day 1:

1. Mix solutions for the method
    a. 0.05 M acetic acid
        i. 60.05 g/m x 0.05 m/l = 3 g/l
    b. 0,1 M NaOH
        i. 40 g/m x 0.1 m/l = 4 g/l
2. Obtain fish skin
    a. Or next morning

Day 2:

1. Get scissors, cutting board, scale, magnet stirrer and magnet, beaker or bucket (depends on the amount of cod skin used)
2. Cut the fish skin into small pieces
3. Put some extra skin into a plastic bag and in the freezer (-18°C)
    a. To use for experiments later on
4. Weigh the amount of cod skin needed
    a. In beaker or bucket
    b. If the skin+solution fits in beaker then the magnet stirrer is used
    c. If the skin+solution only fits in bucket a big spoon is used to stir the solution
5. Pour the 0.1 M NaOH solution on the cod skin with the ratio 1:10. The skins are soaked in this solution for 30 minutes and then the solution is changed.
    a. **Step 5** repeated 3 times
6. Strain the solution from the cod skin and wash it with water.
7. Cheesecloth is used to close the container used for the fish skin and rubber band is used to hold the cheesecloth in place. It is placed in the sink under the water flush to get the water circulating to wash the skin and get the pH lower.
    a. pH is measured with a portable pH meter (Portamess 913, Knick, Germany)
    b. Wash until neutral pH
8. Strain the water from the cod skin
9. Pour the 0.05 M acetic acid solution on the cod skin with the ratio 1:10. The skins are soaked in this solution for 3 hours.
10. Strain the solution from the cod skin and wash it with water.
11. Repeat **step 7.**
12. Put the cod skin in a bottle with tap water with the ratio 1:3. Put the bottle in a incubator shaker (Innova 4430, New Brunswick, Canada) at 45°C and 120 rpm overnight.

Day 3

1. Take the bottle out of the incubator shaker (Innova 4430, New Brunswick, Canada)
2. Filter the solution through two layers of cheesecloth
3. Weigh the fish skin in aluminum tray
4. Weigh the solution in aluminum trays
    a. Will be freeze-dried
5. Put the trays in the -18°C freezer

Appendix IV

Protocol for hydrolysis

Hydrolysis

1. Calculate: amount of water, protein and enzyme needed
2. Turn the incubator shaker on (Innova 4430, New Brunswick, Canada)
3. Heat water to the optimal condition of the enzyme used (sometimes over night - if it is available)
    a. Erlenmeyer (250 ml) often used
4. Adjust pH to the optimal condition of the enzyme used
    a. Use 1 M NaOH or 1 M HCl
5. Prepare the enzymes and weigh them
6. **Hydrolysis:**
    a. Put the enzymes in the water
    b. Put in the incubator shaker (Innova 4430, New Brunswick, Canada)
        i. For 2 hours
7. Turn on the hot water bath (90°C) (takes 30-60 minutes)
8. **Inactivation:**
    a. Pour the solution in plastic bags
        i. Put them in the hot water bath
        ii. For 30 minutes (depends on sample size)
9. The bags were cooled down on ice.
    a. Until the hydrolysates are 4°C.
10. Take samples:
    a. OPA
    b. SDS-PAGE
    c. More
        i. Samples stored at -18°C
11. Centrifugation: for 20 minutes at 8000 x g.
12. Taka sample:
    a. SDS-PAGE
13. The hydrolysates were then filtered through cheese-cloth, weighted.
    a. If hydrolysates are going to freeze dryer
        i. Weigh in almunium trays
    b. If not
        i. Weigh in plastic bags or boxes
14. Put them into the freezer (18°C)

Appendix V

Protocol for filtration

Labscale ™ Benchtop TFF System with Pellicon XL module

Start filtration:

1. Disconnect the tube from “Perm 1” and “Perm 2”
2. Release cap from “Ret” and “Fet”
3. Connect the Pellicon XL filter and make sure it fits.
    a. “Ret” and “Fet” – must fit
    b. “Perm 1” and “Perm 2” – must fit
4. Disconnect the tube that is used for the retentate “ret in” from the tank and put into a waste container.
5. Wash the system with 500 ml of dH20.
    a. The filters are stored in NaOH.
    b. This step is to get the NaOH out of the system.
6. When all the water has passed through, connect the tube for retentate back to the tank in “ret in”.
7. Connect a tube in “perm 2”
8. Put the other end of the tube in a container.
    a. The container is supposed to be closed (cover it as possible)
    b. Keep container on ice
9. Put the sample in the tank
10. Start the magnet
11. Start the filtration
12. Check the pressure
    a. 2 indicators
        i. Lower indicator: pressure 20-40
        ii. Upper indicator: pressure 10
13. Filter until ~100 ml of solutions is left
14. Pump the solution left in the tank out using “feed in”.

End filtration:

1. Pour 500 ml of 0.1 M NaOH in the tank
2. Wait until 250 ml of the solution is left in the tank
3. Taka the perm-tube from the container and put in the tank and circulate there for 30-60 minutes
    a. The filters needs to be stored with NaOH solution
4. Pump the NaOH left in the tank out using “feed in”.
5. Disconnect the tube from “perm 2” and close it.
    a. Wash the tube with water after use
6. Disconnect the filters and close them

Appendix VI

Protocol for OPA method

OPA according to Nielsen *et al.* 2001.

Chemical	IFL Number - delete
di-Na-tetraborate decahydrate	A1302
SDS (Na-Dodecyl-sulfate)	G4205
OPA (o-phthaldialdehyde 97%)	G3605F
Ethanol	H1040
DTT (Dithiothreitol 99%)	G1620R
Serine (Art. 7769 Merck)	D2026/D2082F

Apparatus

Erlenmeyer flasks: 100ml, 200 ml and 500 ml

Test tubes: 10 ml

A 4-decimal analytical balance

Pipettes: 400 µl, 3 mL and 4 mL

Magnetic stirrer

Whirl mixer

Spectrophotometer at 340 nm

Reagents

The OPA reagent was prepared as follows:

1. 7.620 g di-Na-tetraborate decahydrate and 200 mg SDS were dissolved in 150 ml deionized water – use stirring and heating (approx. 30°C)
2. The reagents were completely dissolved before continuing
3. 160 mg OPA was dissolved in 4 mL ethanol
4. OPA solution was transferred quantitatively to the above mentioned solution by rinsing with deionized water
5. 176 mg DTT was added to the solution by rinsing with deionized water
6. The solution was made up to 200 ml with deionized water

The serine standard was prepared as follows:

7. 50 mg serine was diluted in 500 ml deionized water (0.9516 meqv/L).

The sample solution was prepared as follows:

8. X g sample was dissolved in 100ml deionized water
9. X is 0.1 to 1.0 g sample containing 8% to 80% protein
10. The DH of the sample also influences the amount required.

Procedure

1. All spectrophotometer readings were performed at 340 nm using deionized water as the control
2. Three ml OPA reagents were added to all test tubes
3. Test tubes used for analyzing 1 sample (double determinations were) Standard 4 tubes; Blank 4 tubes; Sample 4 tubes
4. As absorbance changes somewhat with time it is important the samples stand for exactly the same time (2 min) before measuring.
5. The assay was carried out at room temperature.

Standard measuring

6. 400 µl serine standard was added to a test tube (time 0) containing 3 ml OPA reagents and mixed for 6 s.
7. The mixture stood for exactly 2 min before being read at 340 nm in the spectrophotometer.
8. Two standards were measured before the blanks along with sample values.
9. The last 2 standard were measured after having determined all blanks and sample values.
10. The mean of these standards was used for calculations
11. The typical value of the standards was OD about 0.8.

Blank measuring

12. Blanks were prepared from 400 µl deionized water and treated as described above. The typical value of a blank was OD about 0.07.

Sample measuring

13. Samples were prepared from 400 µl sample

Obs. If OD is higher that 1.0 – dilute the samples.

Calculation Determination of h

$$hh\ Serine\text{-}NH_2 = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} * 0.9516 \frac{meqv}{L} * 0.1 * \frac{100}{X} * P$$

where
Serine-NH_2: meqv serine NH_2 / g protein
X: g sample
P: protein % in sample
0.1: Sample volume in liter (L)

h is then:

$$hh\ hhhhhhhh = \frac{Serine\text{-}NH_2 - \square\beta}{\alpha\ meqv/g\ protein}$$

Where ✔ and 🚲 are shown in table 1 for specific raw materials

Table 1 – Value of constants ✔ , 🚲 and h_{tot} for different protein raw materials (Adler-Nissen 1986)

Protein	✔	🚲	h_{tot}
Soy	0.970	0.342	7.8
Gluten*	1.00	0.40	8.3
Casein	1.039	0.383	8.2
Whey*	1.00	0.40	8.8
Gelatin	0.796	0.457	11.1
Meat*	7.00	0.40	7.6
Fish*	1.00	0.40	8.6

*When raw material has not been examined, then ✔ and 🚲 are estimated to be 1.00 and 0.40 respectively

Determination of DH

$$DH = \frac{h}{h_{tot}} * 100\%$$

Where h_{tot} for specific raw materials is found in table 1.
The methods should be used with caution at low DH

2.8 OPA method for the quantification of released amino groups

Released proteinous components in each sample during digestion were determined by analyzing the degree of protein hydrolysis based on the reaction of primary amino groups with o-phthaldialdehyde (OPA). The degree of hydrolysis (DH) is defined as the percentage of peptide bonds cleaved. The OPA method was carried out as described by Church *et al.* (1983), including the recommended modifications suggested by Nielsen *et al.* (2001).

The OPA reagent was made by completely dissolving $B_4Na_2O_7$ (0.133 M) and SDS (4.6 mM) in milliQ-water (solution 1). Separately, OPA was dissolved in ethanol to a concentration of 0.3 M and transferred to solution 1. Finally, 7.33 mM DTT was added to the solution before milliQ-water was added to make the final concentration of $B_4Na_2O_7$, SDS, OPA and DTT 99.9 mM, 3.46 mM, 5.96 mM and 5.7 mM respectively.

The samples were suitably diluted according to protein content and expected DH. For the analysis procedure, a tube was loaded with 3 ml OPA reagent before 400 µl sample solution, milliQ-water (blank) or serine standard (0.95 mM $C_3H_7NO_3$) was added (time 0) and mixed on a vortex mixer.

After exactly 2 minutes the mixture was measured spectrophotometrically at 340 nm using a Genesys 20 (Thermo Fisher Scientific Inc. USA).

Calculation
Determination of h:
Serine NH = (OD−OD)/(OD− OD)× 0.9516 meqv/l × (0.1 – 100 X) × p l/g protein

Where serine-NH2 = meqv serine NH2/g protein.
X = µl sample.
P = protein percent in sample.
0.1 is the sample volume in liter.

h is then:
h = serine NH2 – 🚲/✔ meqv/g protein

Calculation of DH:
DH = h/htot× 100%

Where 🚲, ✔ and h_{tot} are constants. In the case of fish ✔, 🚲 and h_{tot} are 1.00, 0.40, and 8.6 respectively (Adler-Nissen, 1986). The DH was expressed in percent of total hydrolysis of fish proteins. For each specific sample, triplicate measurements were performed

Appendix VI

Protocol for SDS-PAGE analysis

SDS-PAGE

Materials

1. NuPAGE LDS Sample Buffer (4X) (Cat no. NP0007)(NP0008)
2. NuPAGE®Reducing Agent (10X) (Cat no.NP0004)(Cat no.NP0009)
3. NuPAGE® MES SDS Running Buffer (20X) (Cat.no NP0002)
4. NuPAGE®Antioxidant (Cat no. NP0005)
5. Protein standard (Cat no. LC5800 Novex)
6. NuPAGE 4-12% Bis-Tris Gel (Cat.no. NP0327BOX)

Instrument

1. XCell SureLock™ Mini-Cell (Cat.no. EI0001;Invitrogen, CA, USA)

Preperation

1. **Samples**
   a. 26 µL sample in eppendorf
   b. Add 4 µL NuPAGE®Reducing Agent (10X)
   c. Add 10 µL NuPAGE LDS Sample Buffer (4X)
   d. Vortex, centrifuged at 14000 rpm for 5 min
   e. Heat samples at 95 °C for 10 minutes

2. **1X MES SDS Running Buffer**

   Add 40 ml of NuPAGE® MES SDS Running Buffer(20X) (Cat.no NP0002) to 760 ml of deionized water. Put into fridge.

   For reduced samples - 200 ml of the 1X running buffer containing **500 µl NuPAGE®Antioxidant** (Cat no. NP0005) –

3. **Gel casette**

   Open the gel casette, drain away the gel packaging buffer and rinse with deionized water. Peel of the tape and pull the comb out. Rinse with deionized water.

4. **Assembling the XCell *SureLock*™ Mini-Cell.**
   a. Rinse various parts included with the Mini-Cell with deionized water before assembling it.
   b. Lower the Buffer Core into the Lower Buffer Chamber so that the - electrode fits into the opening in the gold plate.
   c. Insert the stirrer in the circle formed frame down in the bottom of the Lower Buffer Chamber.
   d. Insert the Gel Tension Wedge into the XCell *SureLock*™ unit behind the the buffer core. Make sure the Gel Tension Wedge is in its unlocked position.
   e. Insert gel cassettes into the Lower Buffer Chamber. Place one cassette behind the core and one cassette in front of the core. For each cassette, the **shorter** „well“ side of the cassette faces in towards the Buffer Core.
   f. IF you are running ONLY ONE GEL, replace the rear gel cassette with the Buffer Dam.
   g. Fill the Upper Buffer Chamber with 200 ml of the 1X running buffer containing 500 µl NuPAGE®Antioxidant Be sure to completely cover the sample well and that the Upper buffer Chamber is not leaking. Be sure to displace all air bubbles from the wells

h. Fill the Lower Buffer Chamber (anode) with 600 ml of 1X MES SDS running buffer through the gap between the Gel Tension Wedge and the back of the Lower Buffer Chamber.

Sample loading

1. Load 12 µL of the appropriate concentration of your protein sample into each well.
2. Load 5 µl of protein marker.
3. Load 2.5 µl of sample buffer into empty wells to ensure an uniform running of the stacking front.

Running gels

1. Align the lid on the Buffer Core.
2. Place the XCell *SureLock*™ Mini-Cell in a plastic box and put ice around it**.** This will cool down the system during the run.
3. Power must be off before connecting the XCell *SureLock*™ Mini-Cell to the power supply.
4. Turn on the power. Set the appropriate running conditions:

Run Conditions

1. Voltage (constant): 200 V –
2. Expected current: 100-125 mA/gel (start); 60-80 mA/gel (end) –
3. Run time: 35 min (dependent on gel percentage).

Dissembling the XCell SureLock™ Mini-Cell

1. At the end of the run, turn of the power and disconnect the cables from the power supply.
2. Remove the lid and unlock the Gel Tension Wedge. – There is no need to remove it.
3. Remove the gel cassette from the mini-cell and lay it (longer well side up) on a flat surface. Allow one edge to hang ~1 cm over the side of the benchtop.
4. **Carefully** insert the Gel Knife‘s beveled edge into the narrow gap between the two plastic of the cassette.
5. Push up and down gently on the knife‘s handle to separate the plates. You will hear a cracking sound which means you have broken the bonds which hold the plates together. Repeat until you have broken the bonds on one side, then rotate the cassette until the two plates are completely separated.
6. Upon opening the cassette, the gel may adhere to either side.
7. Immedialey fix, stain or transfer the gel as desired.

If **Coomassie blue staining** (SimplyBlue™ SafeStain, Invitrogen, Cat.no. LC6060) then rinse the gel with 100 ml deionized water. See further SimplyBlue™ SafeStain protocol – **Microwave procedure**

The microwave procedure

Takes just 12 minutes, with sensitivity as low as 5 ng

The procedure is for 1.0 mm mini-gels.

Caution: Use caution while using the stain in a microwave oven. Do not overheat the staining solutions.

1. After electrophoresis, place the gel in 100 ml of ultrapure water in a loosely covered container and microwave on High (950 to 1,100 watts) for 50 seconds until the solution almost boils.

2. Shake the gel on an orbital shaker for 1 minute. Discard the water.
3. Repeat Steps 1 and 2 two more times.
4. After the last wash, add 40 mL of Page Blue /SimplyBlue™ SafeStain and microwave on High for 45 seconds until the solution almost boils.
5. Shake the gel on an orbital shaker for 15 min. Detection limit: 20 ng BSA.
6. Wash the gel in 100 ml of ultrapure water for 10 minutes on a shaker. Detection limit: 10 ng BSA.
7. Add 100 ml of ultrapure water over night. Detection limit: 5 ng BSA.
8. Gel can be stored for several weeks in the salt solution 20% NaCl .

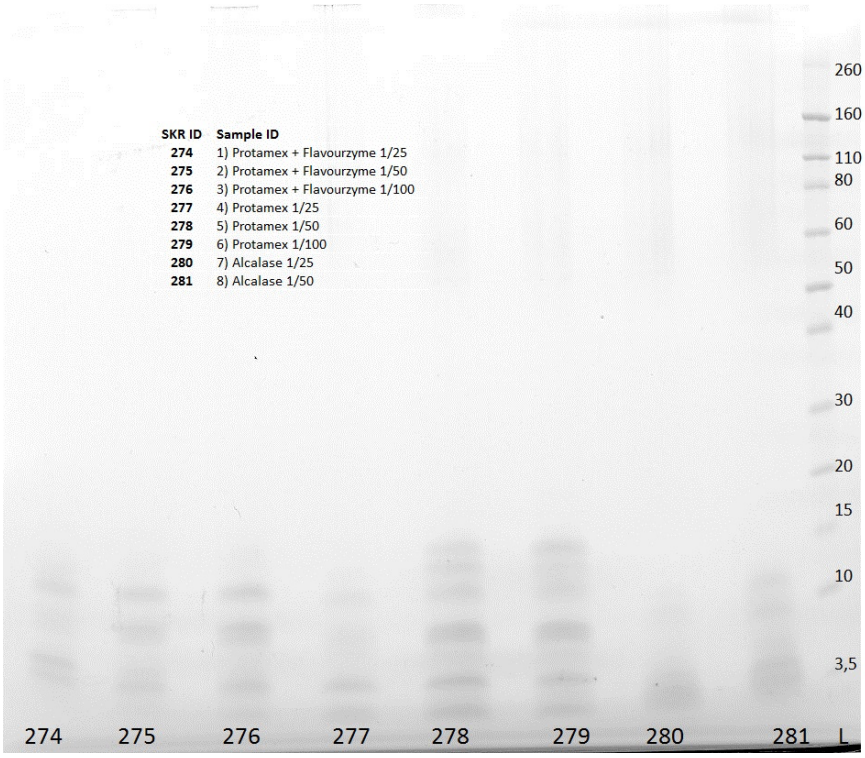
If **silver staining** take the cell apart and put the gel into a fixing solution overnight. See further Silver stain protocol.

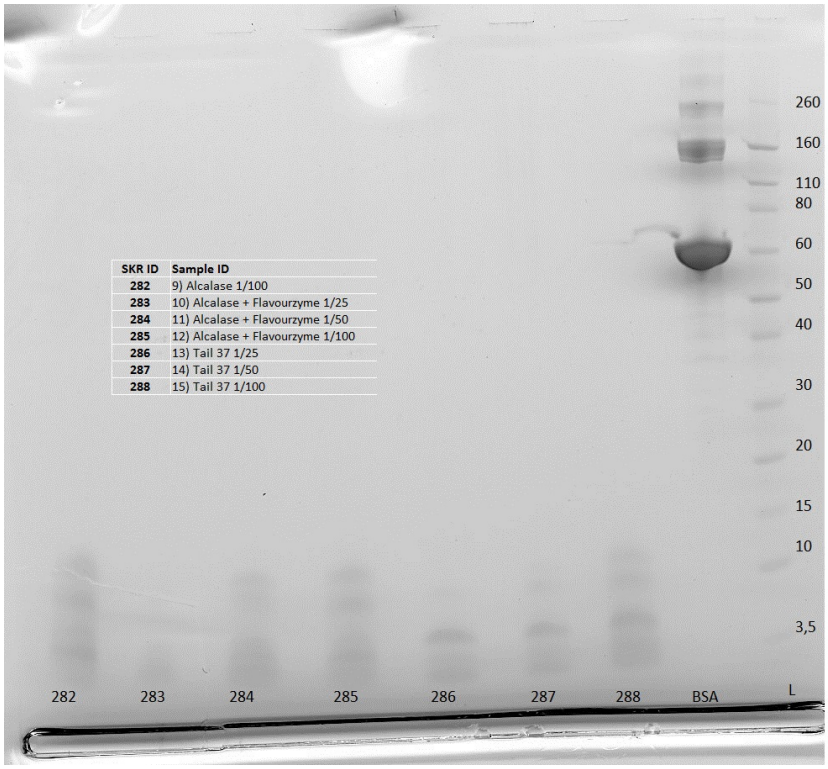
If blotting, proceed to the Western Transfer Protocol **without** removing the gel from the plate

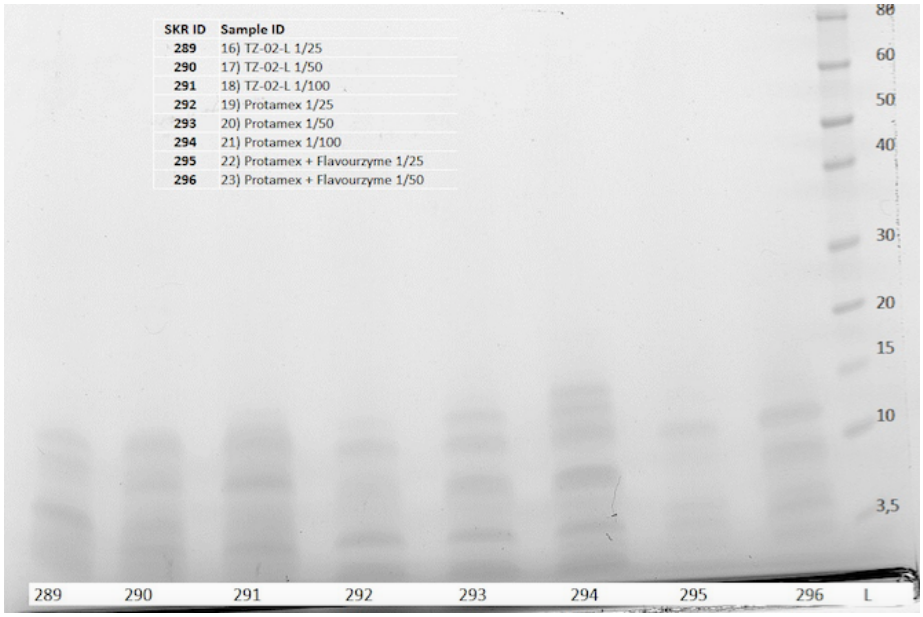
If staining, carefully remove the gel from the cassette plate – you may want to loosen one corner with the knife and allow the gel to peel away from the plate into a container with the gel facing downward.

Appendix VII

SDS-PAGE results for 36 samples in the trial






SKR ID Sample ID
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290 17) TZ-02-L 1/50
291 18) TZ-02-L 1/100
292 19) Protamex 1/25
293 20) Protamex 1/50
294 21) Protamex 1/100
295 22) Protamex + Flavourzyme 1/25
296 23) Protamex + Flavourzyme 1/50
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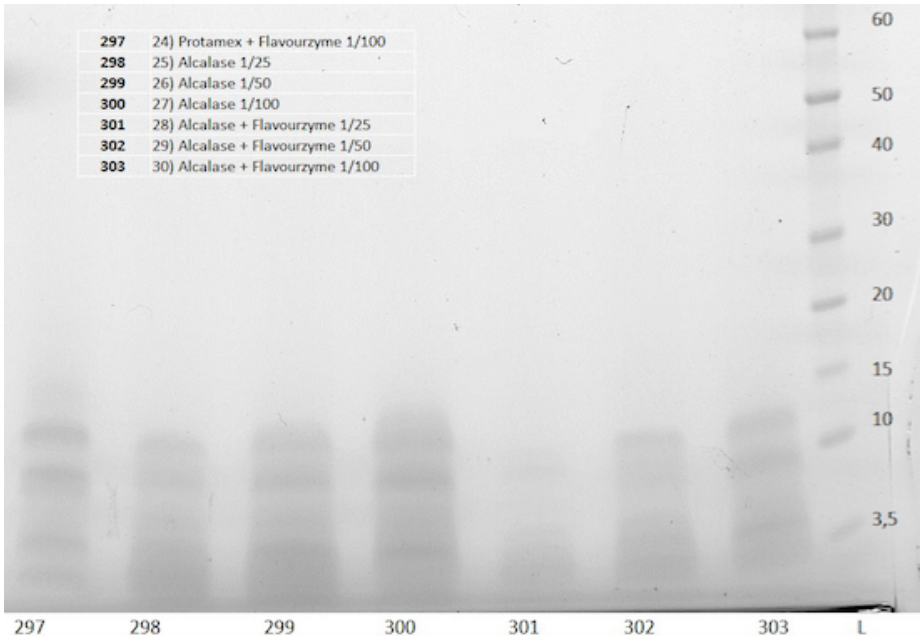
289 290 291 292 293 294 295 296 L


297 24) Protamex + Flavourzyme 1/100
298 25) Alcalase 1/25
299 26) Alcalase 1/50
300 27) Alcalase 1/100
301 28) Alcalase + Flavourzyme 1/25
302 29) Alcalase + Flavourzyme 1/50
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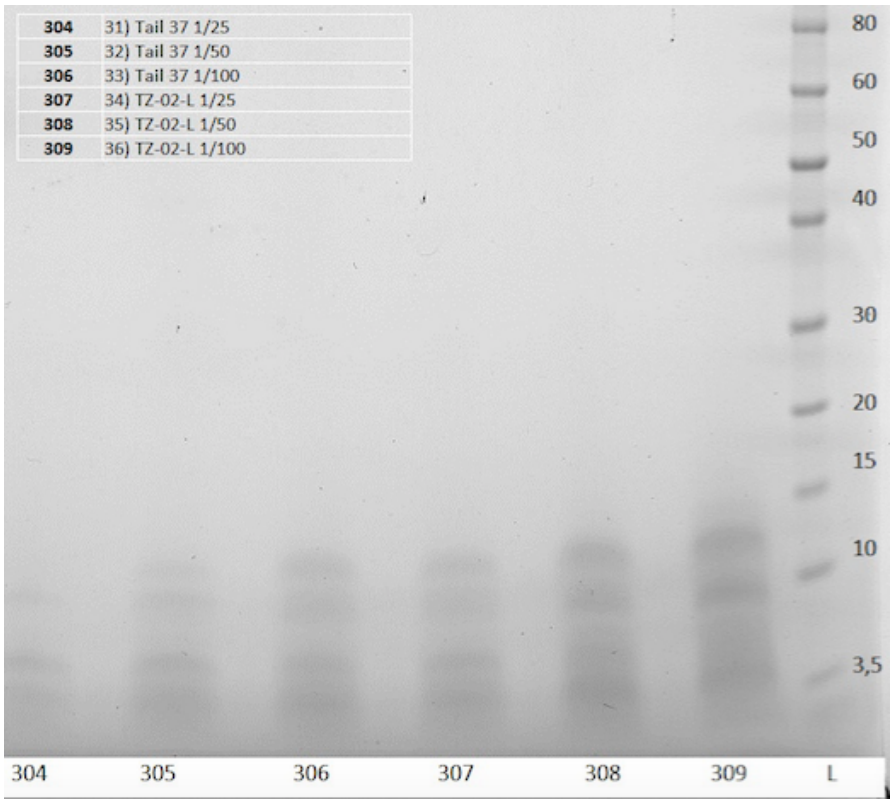
297 298 299 300 301 302 303 L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