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My parents and sisters are warmly thanked for their support and care.

SUMMARY

This thesis presents a study of proteolytic and lipolytic activities in by-products from cod species (Atlantic cod, saithe, haddock, tusk and ling) caught in three different fishing grounds and in three different seasons. The work is part of an EU-project; "Utilisation and stabilisation of by-products from cod species" (QLK1-CT2000-01017), with the aim to increase the utilisation of by-products to produce value-added food ingredients. In order to accomplish this, knowledge about the natural variations in quality and stability of by-products according to species, season and fishing ground is necessary so that appropriate handling and storage procedures can be worked out. This knowledge is also important for evaluating the suitability of the by-products for different uses, e.g. direct consumption, drying, salting and/or canning, extraction of fish oil and/or bioactive substances, processing into surimi based products, fish protein concentrates or hydrolysates, etc. Although many studies of different enzymes in fish have been published, there is a lack of knowledge about enzymes in by-product fractions. This study provides a basic characterisation of enzymes responsible for the breakdown of important macromolecules, such as proteins and lipids, in viscera, cut off and liver fractions. Each by-product sample is made up from 15 fishes in order to provide enough raw material for all analyses and to simulate industrially interesting batches.

Species affected the general proteolytic activity (pH 5 and 7), and activity of trypsin and chymotrypsin in viscera. Viscera from Atlantic cod, saithe and haddock had a higher proteolytic activity compared to tusk and ling. In cut off and liver general proteolytic activity (pH 5 and 7), activity of cathepsin B and collagenase varied according to species.

Variations according to season were found in activity of trypsin, chymotrypsin, elastase, cathepsin B, collagenase and lipase (pH 7) in viscera from Atlantic cod. The results clearly indicate that viscera samples from the Icelandic Sea had lower enzymatic activities in April-June compared to the other seasons. Cut off samples from the Icelandic Sea also had lower activity of cathepsin B and collagenase in April-June compared to Feb.- March, the lipolytic activity was,

however, higher in April-June compared to Oct.-Dec. In liver samples seasonal differences were found in activity of cathepsin B and lipase (pH 5).

Fishing ground influenced general proteolytic activity (pH 3 and 7), activity of trypsin, chymotrypsin, elastase, cathepsin B, collagenase and lipase (pH 7) in viscera from Atlantic cod. Generally, the enzymatic activities in viscera were highest in samples from the Icelandic Sea, except for pepsin which was highest in samples from the south coast of Ireland. Activity of cathepsin B was higher and activity of collagenase lower in cut off samples from the south coast of Ireland compared to the Icelandic Sea. Lipolytic activity was higher in cut off samples from the Barents Sea compared to the Icelandic Sea. Cathepsin B and collagenase in liver was also influenced by fishing ground.

Trypsin, chymotrypsin and cathepsin B in crude extracts of viscera from fish of the cod species lost 50% of initial activity after 10 min incubation at 60°C. Elastase, collagenase and lipase (pH 5 and 7) lost 50% of initial activity after 10 min at 50°C. In crude extracts of liver, cathepsin B and collagenase lost 50% of initial activity after 10 min at 50°C, and lipase (pH 5 and 7) after 10 min at 60°C. Cathepsin B and lipase (pH 5 and 7) in crude extracts of cut off required 10 min incubation at 50°C in order to lose 50% of initial activity, and collagenase required 10 min at 40°C.

Significant variations in quality parameters and enzymatic activities in by-products from cod fish according to species, seasons and fishing grounds have been demonstrated. Generally, the enzymatic activities in by-products are high and it is important that they are rapidly and continuously stored at cold temperatures. It is also important that the by-products are treated as valuable raw material in the same way as the main product, already from the time the fish is caught. Separating the different by-product fractions, and fractions from the lotidae family from the gadidae family, will ensure that the fractions with low enzymatic activities are not contaminated by fractions with higher enzymatic activities. Viscera from Atlantic cod caught in the Icelandic Sea and from fish of the gadidae family may be better raw materials for protein hydrolysates, especially if autolytic or semi autolytic processes are used, or for extraction of proteolytic enzymes. Viscera samples from Atlantic cod caught in

the Barents Sea have lower lipase activity and may therefore be a better raw material for extraction of marine oils compared to samples from the Icelandic Sea. Due to the high lipase activities in both viscera and liver, cold extraction of lipids would probably be the most favourable method for producing fish oil. However, if heating is needed it should be rapid up to temperatures above 60°C.

Cut off from ling and saithe, Atlantic cod caught in the Barents Sea (April-June and Oct.-Dec.) and Icelandic Sea (April-June) may be used for minced products or surimi based products. Cut off from haddock, Atlantic cod caught at the south coast of Ireland and in the Icelandic Sea (Feb.-March) is a poor raw material for these kinds of products due to the high activity of cathepsins B.

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III Sovik, S. L., & Rustad, T. (2005). Effect of season and fishing ground on the activity of lipases in byproducts from cod (*Gadus morhua*). Lebensmittel Wissenschaft und Technologie. In press.

IV Sovik, S. L., & Rustad, T. (2005) Effect of season and fishing ground on the activity of cathepsin B and collagenase in by-products from cod species. Lebensmittel Wissenschaft und Technologie. In press.

V Sovik, S. L., & Rustad, T. Effect of species, season and fishing ground on the activity of elastase in viscera from cod species. Submitted to Journal of Aquatic Food Product Technology.

1. INTRODUCTION

1.1. Objectives

During the initial period of industrialised fisheries, fish resources appeared to be inexhaustible, and by-products were looked upon as worthless. However, it was soon acknowledged that this "waste" was a problem when it started to accumulate in every fishing harbour. In Norway this was first recognised as a serious problem in the early 1970s (Gildberg, 2004a). Since then a lot of research regarding fishery by-products have been carried out. However, a systematic survey of variations in enzymatic activities in by-products according to species, seasons and fishing grounds have not been published. The overall aim of this thesis has been to characterise enzymes responsible for the breakdown of major macromolecules, such as protein and fat, in the by-product fractions and compare samples from different cod species, seasons and fishing grounds. The thesis focuses on enzymatic activities in liver, viscera and cut off fractions from cod species.

The quality of by-products limits the possibilities for utilisation of the raw material, and enzymatic activities along with microbial degradation are the most important factors determining raw material quality. Variations in enzymatic activities and thereby quality is important when finding possible uses for the different by-product fractions. In processes utilising by-products, such as production of fish protein hydrolysates (FPH), minced and surimi based products, extraction of lipids, enzymes and/or other bioactive compounds, etc. the activity of the endogenous enzymes in the raw material needs to be controlled and knowledge about how these activities change according to temperature is important.

More specifically, the following topics have been addressed in separate papers:

- Variations in general proteolytic activities in the by-products, as a function of pH and temperature, between species and fishing grounds. Paper I: Proteolytic activity in byproducts from cod species caught at three different fishing grounds.
- Variations in trypsin and chymotrypsin activity in viscera between species, season and fishing ground, and the heat stability of the enzymes in crude extracts. Paper II: Seasonal changes in trypsin and chymotrypsin activity in viscera from cod species.

- Lipolytic activities in by-products from Atlantic cod, as a function of pH and temperature, variations according to season and fishing ground, and heat stability of the enzymes in crude extracts. Paper III: Effect of season and fishing ground on the activity of lipases in byproducts from cod (*Gadus morhua*).
- Cathepsin B and collagenase activity in by-products, variations according to species, season and fishing ground, and heat stability of the enzymes in crude extracts. Paper IV: Effect of season and fishing ground on the activity of cathepsin B and collagenase in by-products from cod species.
- Variations in elastase activity in viscera between species, season and fishing ground, and heat stability of the enzyme in crude extracts. Paper V: Effect of species, season and fishing ground on the activity of elastase in viscera from cod species.

1.2. Marine by-products

The most common understanding of the term "by-products" are all the raw material, edible or inedible, left over during the preparation of the main product (Gildberg, 2002). For fish of the cod species this is skin, bones, heads, collar bone, cut off, backbones, shells, liver, roe, milt, tongues, stomachs, intestines, gall bladder and swim bladder. These fractions of the fish have traditionally not been seen as part of the main product, and have been thrown away (RUBIN, 2000; RUBIN, 2004). However, liver, roe and tongues from large cod are to some extent utilised for human consumption. In white fish fillet production a major part (about 60%) of the fish is considered as by-products (Gildberg, 2002). By-products have also been referred to as "waste", but recently the more positive concept of "rest raw material" has been used.

Whole fish are also sometimes considered as by-products, e.g. catch of unwanted species and farmed fish that has died or been slaughtered because of disease (RUBIN, 2000; RUBIN, 2004).

Figure 1.1 illustrates products and by-products from processing of cod on shore.

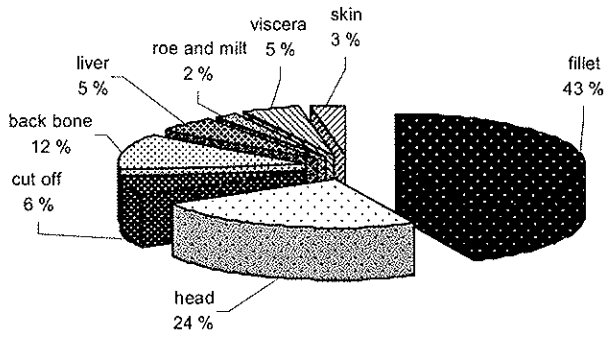

Figure 1.1: Products and by-products from on shore processing of cod (http://www.biotech.ntnu.no/fishbyprod/).

The attitude towards by-products started changing during the nineties, and the fishing industries became more aware of the potential value in by-products. The change was brought on by both economic as well as ecological concerns, and has led to increased interest in by-products both from a research and business point of view (RUBIN, 2004).

1.3. Amount and potential value of marine by-products

Although it is difficult to estimate the available quantity of by-products on a world basis, it is likely that at least 25 million ton could be recovered if every part of the fish had been brought ashore. In addition it is estimated that more than 20 million ton of by-catch fish is wasted at sea. Hence, the total estimate would be about 45 million ton (Gildberg, 2002).

There are no statistical data available on the value of by-products on a world basis, and the potential value of such an extremely inhomogeneous material is also hard to estimate. To give an indication, Gildberg (2002) used data available from the Norwegian fisheries, extrapolated, and estimated the worlds by-product quantity of 45 million ton to correspond to a value of about 12 billion US$.

In Norway about 80-90% of the by-products generated at sea are dumped, the remainder is used for fish meal. About 66% of the by-products generated on shore are utilised, mainly for fish meal, silage and feed. About 10% is processed into food or speciality products, these 10% accounted for almost half of the value from by-products (http://www.biotech.ntnu.no/fishbyprod/). In 2002 the Norwegian fisheries produced 540 000 ton of by-products, the major part came from the cod fisheries, 236 000 ton. White fish such as cod, saithe and haddock, are delivered eviscerated and decapitated from the fishermen (RUBIN, 2004). It has been estimated that the value of by-products from the fisheries in Norway may increase to about 5 billion NOK (about 720 million US$) within 2005-2010 (RUBIN, 2000). Growth in the fishing industries and in the fish farming industries is resulting in new challenges in handling, storing and utilising the increasing amount of by-products (RUBIN, 2004).

1.4. Utilisation of by-products

Fishery by-products can be categorised into four major groups: 1) material used for fertilisers, 2) for feed, 3) for food and 4) speciality products. Fertilisers are regarded as the least profitable way of utilising the by-products. Traditionally the major utilisation of by-products has been for feed production, the profitability in this production is also quite low. Processing by-products into food and food ingredients normally gives a far better profitability, and extracting and purifying high value biochemicals from special by-product fractions are generally the most profitable (Gildberg, 2002). The problem with the latter way of utilisation is that there will still be a considerable amount of unutilised mass left over. Possible products from by-products are listed in Table 1.1.

Table 1.1: Possible value added products produced from by-products (Arason, 2002).

Food ingredients	Health food ingredients	Fine chemicals
• Flavour attributes • Proteins • Collagen • Fish oils • Ingredients with functional properties -gel forming -foaming -water binding -emulsifying	• Ingredients with nutritional properties -proteins -vitamins -minerals • Nutraceuticals/ Pharmaceuticals -speciality fish oils -hormones -glucosamine -chitosan -other physiologically active extracts and compounds	• Biocompounds -enzymes -substrates -hormones -gelatine -chitosan -etc.

1.4.1 Marine oils

By-products from cod species are sources of marine oils, known to have beneficial health effects. Liver contains between 50 and 70% lipids with more than 30% n-3 fatty acids, viscera contains 2-9 %, and cut off 1% lipids. Lipid content in by-products from cod species is affected by species, season and fishing ground (Falch et al., submitted; 2003). The primary products of lipolytic enzymes are glycerol and free fatty acids. The limits for free fatty acids in crude fish oil extracts are 2-5% according to guideline specifications (Young, 1985). Knowledge about lipolytic enzymes in by-products, variation according to species, season and fishing ground, is therefore important in order to ensure that fish oil from by-products meets the specifications.

Today the industry carries out the fat extraction using high temperatures. This can lead to lipid oxidation, reduction of oil quality and reduced market value of the fish oil. Dumay et al. (2004) presented an alternative method for fat extraction from by-products of cod using broad spectrum proteases in order to disrupt tissues and cell membranes. The hydrolysis conditions used were not optimized in the study, but the general conclusion was that the use of proteases enhanced extraction of total fat, phospholipids, eicosapentaenoic acid and docosahexaenoic acid. No global tendencies were found regarding which enzymes were most efficient for extracting

lipids, the results varied according to by-products. The endogenous proteases were not commented in the study, but it is likely that they are partly responsible for these variations, and should be taken into consideration when optimizing such a process.

1.4.2 Fish mince, surimi and surimi based products

Cut offs may be a suitable raw material for the production of fish mince, surimi and surimi based products. Minced meat is less stable than intact muscle. The most important parameter of fish mince is the gel-forming ability of the proteins, this determines the textural attributes of the finished products (Venugopal & Shahidi, 1995).

Surimi is a myofibrillar protein concentrate produced by repeated washing of fish mince to remove water-soluble and odor-bearing compounds consisting of enzymes, sarcoplasmic proteins, blood, inorganic salts and some lipids. Surimi is used as the raw material for preparation of seafood analogs, such as shrimp, crab legs, scallop and lobster tail. In addition, the surimi industry has the potential to develop novel products, such as luncheon meat, sausages, pastas and protein drinks. A major problem in fish mince and surimi production is softening of the gel due to the action of endogenous proteases (Venugopal & Shahidi, 1995).

1.4.3 Fermented products

Fermentation of fish products has been used extensively in Southeast Asia for preparation of flavoured products. Fermented products from fish may be classified broadly into two types: 1) fish and salt formulations, and 2) fish, salt and carbohydrates. In the former category, fermentation results from autolytic enzymes present in the tissue while high levels of salt (> 20%) prevents microbial deterioration of the meat. Fish sauce and fish paste are produced by this method. The long fermentation time for fish sauce (5-12 months) may be reduced by addition of exogenous proteases (Venugopal & Shahidi, 1995). All kinds of fish material can be used for production of fish sauce provided that the material contains enough proteolytic enzymes to give an acceptable rate of autolysis (Gildberg, 1992). The trypsin-like enzymes play a key role in the autolytic process of fish sauce

fermentation (Orejana & Liston, 1981), and the amount of such enzymes is a major factor affecting the production time. By selecting fish material containing high concentrations of trypsin-like enzymes (the intestines), it is possible to reduce the production time from several months to a few weeks (Gildberg, 1992). Gildberg (2001) showed that male Arctic capelin and Atlantic cod intestines can be utilised as a raw material for the production of high value fish sauce for human consumption. It is likely that also other mixtures of by-products can be utilised for the production of both fish sauce and fish paste.

1.4.4 Fish protein concentrates (FPC) and hydrolysates (FPH)

The development of FPC was one of the earliest attempts to recover fish protein from processing wastes. The method of solvent extraction has frequently been employed when producing FPC. The fish proteins are not hydrolysed, but concentrated intact by the removal of water and oil from the substrate (Kristinsson & Rasco, 2000). Two types of FPC can be prepared. Type A is a colorless and odorless product having less than 1% fat. This product has poor functionality, may contain residual solvents, and suffers from high preparation costs. Type B is prepared by drying and grinding fish mince, and therefore has a fishy odor due to higher amounts of lipids (Venugopal & Shahidi, 1995). FPC was the precursor to the field of enzyme hydrolysis of fish protein (Kristinsson & Rasco, 2000).

Protein hydrolysis can be accomplished with enzymes, acids or alkali. Enzymatic hydrolysis is strongly preferred over strictly chemical methods for producing hydrolysates in nutritional applications. Enzymatic hydrolysis can produce hydrolysates with well defined peptide profiles (Lahl & Braun, 1994). This approach gives an effective recovery of proteins, in addition to upgrading the functional and nutritional properties of the by-product proteins (Shahidi et al., 1995; Liaset et al., 2000; Slizyte et al., 2005). Enzymatic hydrolysis of fish by-products may be accomplished by autolytic processes, by addition of commercial enzymes or a combination of both (semiautolytic). From an industrial point of view, the autolytic or semi autolytic processes are likely to be most interesting since a costly heating step, in order to inactivate endogenous enzymes, can be avoided. Heating of the raw

material prior to the hydrolysis has also been found to reduce the yield of the protein containing fractions (Slizyte et al., 2004) and could destroy nutritional components, result in unwanted reactions (Maillard reaction), or even create unknown components in the final product (Lahl & Braun, 1994). Knowledge about raw material composition and activity, stability and specificity of endogenous proteolytic enzymes, variation according to species, season and fishing ground, is important when industrial processes are worked out (Slizyte et al., 2004; 2005; Dauksas et al., 2005). A main challenge in the production of fish protein concentrates is quality control and ensuring that product specifications are met all year round. Controlling the endogenous enzymes and adjusting process parameters such as time, temperature, pH and amount of added commercial enzymes according to the quality of the raw material are important parameters in this respect.

Fish silage is not treated here, since it is not considered suitable for human consumption.

1.4.5 Extraction of enzymes and bioactive compounds

Marine enzymes offer a wide range of unique applications, including deskinning of fish and squid, and purification and cleaning of fish roe for caviar production. In addition descaling of fish, extraction of carotenoproteins from shellfish processing discards, use of gastric enzymes of fish as a rennet substitute in cheese manufacturing, ripening of fish and production of fish sauce, and production of FPH are among other ways of utilising marine enzymes (Shahidi & Kamil, 2001).

Perhaps the most extensively studied enzymes from the marine environment are the various enzymes, including trypsin, chymotrypsin and elastase, that have been isolated from Atlantic cod viscera (stomach, pyloric caeca and intestines). The cod enzymes have been shown to have higher catalytic efficiency than corresponding mammalian enzymes, they are active at low temperatures and are heat labile (Vilhelmsson, 1997). Digestive enzymes from cold adapted fish may be particularly useful as industrial aids in situations where it is advantageous to inactivate the added enzymes by mild heat treatment after the intended process is complete (Simpson & Haard, 1984c)

In Iceland extraction of enzymes from cod intestines has been commercialised. Preparations used to produce flavourings for food processing and innovative cooking (Primex Marine Biotechnology), and enzyme ingredients used in both pharmaceutical and cosmetic skin ointments (Zymtech Inc) are being produced.

The fish silage processing technology was developed in the 70s and revealed the possibility of recovering bioactive peptides from fish silage. The peptides may be valuable immuno stimulants. *In vitro* and *in vivo* studies have shown that certain fractions in fish protein hydrolysates may stimulate the non-specific immune defence system (Gildberg, 2004a). Fish protein hydrolysates have also been found to have a hypocholesterolemic effect (Wergedahl et al., 2004) and anti-hypertensive activities (Bordenave et al., 2002; Hwang & Ko, 2004). Other bioactive peptides such as growth factors and secretagogues (gastricin and cholecystikinin) have also been reported in FPH (Ravallec-Ple et al., 2000).

1.5. Cod species

Atlantic cod (*Gadus morhua*), saithe (*Pollachius virens*), haddock (*Melanogrammus aeglefinus*), tusk (*Brosme brosme*) and ling (*Molva molva*) are all members of the order gadiformes. All together this order has 10 families, including the gadidae and lotidae families. Atlantic cod, saithe and haddock are members of the gadidae family, while tusk and ling are members of the lotidae family. Differences between the two families include the number of fins, gadidae having three dorsal fins and two anal fins, while the lotidae have one to three dorsal fins and one anal fin (www.fishbase.org).

Table 1.2 shows the spawning periods reported in the literature for cod, saithe, haddock, tusk and ling.

Table 1.2: Reported spawning period for fish of the cod species.

Reference	*Cohen et al., 1990*	*Bergstad et al., 1987*	*Thorarinsdottir et al., 2004*	*Muus & Nielsen, 1998*
Location	*General*	*Barents Sea*	*Icelandic waters*	*General*
Atlantic cod	December-June	March- April	March- April (May)	January- April
Saithe	November-February	January-April	January-February	February- April
Haddock	February- June	March- June	April- May	March- June
Tusk	April- July		April- July	April- June
Ling	March- July		May- June	March- July

1.5.1. Atlantic cod (*Gadus morhua*)

The Atlantic cod is generally considered a demersal fish, although its habitat may become pelagic under certain hydrographical conditions, when feeding or spawning. The presence of Atlantic cod usually depends on prey distribution rather than on temperature. However, larger fish are found in colder waters in most areas (0-5°C) (Cohen et al., 1990).

Atlantic cod has a high growth rate, maximum length of the species is 2 m. It may live up to 20 years and is a voracious and omnivorous species. Juveniles feed mainly on invertebrates, and older fish on invertebrates and fish, including young cod. Deep-water cod show preference for herring throughout the summer and autumn (peak June-July), but in winter and during the spawning period, they sustain themselves on mixed food in coastal areas (Cohen et al., 1990). The qualitative variations of food are temporal and geographic, and the diversity of prey increase from northerly to southerly regions (Du Buit, 1995).

Atlantic cod is one of the most important commercial fishes, and has been called "the beef of the sea". It has been exploited ever since man began to fish in the seas of Europe. The total catch reported for this species to FAO for 1999 was 1 092 859 ton. Iceland and Norway were the countries with largest catches. Atlantic cod is marketed fresh, chilled or frozen as fillets or whole, salted, dried, in brine or smoked. Other products obtained from cod are salted cheeks, liver oil and roe (http://www.fao.org/figis/servlet/FiRefServlet).

1.5.2. Saithe (*Pollachius virens*) 

Saithe is an active, gregarious, pelagic fish occurring in inshore and offshore waters down to about 200 m. Maximum length of the species is 1.3 m, and maximum age 24 years. Small fish in inshore waters feed on small crustaceans (copepods, amphipods, and euphausiids) and small fish, while the large saithe prey predominantly upon fishes (Cohen et al., 1990).

Saithe is an important specie, similar to cod and haddock, and may replace them in some products. The total catch reported to FAO for 1999 was 339 987 ton. The countries with the largest catches were Norway and Faeroe Islands. Saithe is marketed fresh, chilled as fillets, frozen, canned, dried salted and in brine (http://www.fao.org/figis/servlet/FiRefServlet).

1.5.3. Haddock (*Melanogrammus aeglefinus*) 

Haddock is a demersal species found from 10 to 450 m depth, more common from 80 to 200 m, over rock, sand, gravel or shells, usually at temperatures between 4 and 10°C. Maximum length for the fish is 1 m and life expectancy is about 14 years. The haddock is an omnivorous fish, feeding mainly on small bottom-living organisms including crustaceans, molluscs, echinoderms, worms and fishes (Cohen et al., 1990).

Haddock is important in North Atlantic fisheries. The total catch reported for this species to FAO for 1999 was 249 317 ton. The countries with the largest catches were the United Kingdom and Norway. It is marketed fresh, chilled as fillets, frozen, smoked and canned. It is also processed into fishmeal and used for animal feeds (http://www.fao.org/figis/servlet/FiRefServlet).

1.5.4. Tusk (*Brosme brosme*) 

The tusk lives on rough, rock, gravel or pebble bottoms on both sides of the North Atlantic. It generally keeps far from the shore, near the bottom at depths from 20 to 1000 m, mostly between 150 and 450 m in the north-eastern Atlantic. Maximum length of the fish is 1.2 m and it lives a maximum of about 20 years. It

feeds mostly on crustaceans and shellfish, and also on benthic fishes (flatfishes and gurnard) and even on starfishes (Cohen et al., 1990).

The total catch of tusk reported to FAO for 1999 was 34 743 ton. The countries with the largest catches were Norway and Iceland. Utilisation is fresh or frozen as fillets, but also dried, salted and in brine (http://www.fao.org/figis/servlet/FiRefServlet).

1.5.5. Ling (*Molva molva*) 

Ling is a demersal fish that lives on rocky bottoms at depths of 15 to 600 m or more, commonly from 100 to 400 m. Maximum length of the fish is 2 m, and the maximum age is 10 years for males and 14 years for females. It feeds mostly on fish (cod, herring, and flatfish) but also on crustaceans (lobsters), cephalopods and echinoderms (starfishes) (Cohen et al., 1990).

The total catch of ling reported to FAO in 1999 was 53 870 ton. The countries with the largest catches were Norway and the United Kingdom. Ling is marketed frozen, as fresh fillets, dried, salted, in brine and also as fishmeal (http://www.fao.org/figis/servlet/FiRefServlet).

*The drawings of the fishes are from http://www.fao.org/figis/servlet/FiRefServlet.

1.6. Enzymes

1.6.1. Proteolytic enzymes

Proteolytic cleavage of peptide bonds is one of the most frequent and important enzymatic modification of proteins. Historically, enzymatic proteolysis has been associated with protein digestion. Physiologists and biochemists interested in the process of protein digestion in animals and man were among the first scientists to study enzymes. Hence the digestive proteases from pancreatic and gastric secretions are among the best characterised enzymes and much of the current knowledge of protein structure and enzyme function comes from studies of these proteases (Neurath, 1989). Proteolytic enzymes also have many other physiological functions, such as the activation of zymogens, blood coagulation and the lysis of fibrin clots,

the release of hormones and pharmacologically active peptides from precursor proteins, and the transport of secretory proteins across membranes. They are present in all forms of living organisms (Neurath, 1984).

Four classes of proteases are recognised according to their catalytic mechanism; serine, cysteine, aspartic and metallo proteinases (Neurath, 1989). This classification is based on a functional criterion, namely the nature of the most prominent functional group in the active site. Members of the same functional family are usually evolutionarily related, but there are exceptions to this rule (Branden and Tooze, 1999). A short introduction to the four classes of proteases will be given, before a more thorough description of the proteases investigated in this thesis is presented. The focus will be on proteases in fish.

1.6.1.1. Serine proteases

Two major evolutionary families are present in this class: the mammalian serine protease which includes chymotrypsin, trypsin and elastase and the bacterial serine protease which includes subtilisin. Enzymes in this family have a common catalytic triad of Ser, Asp and His (Neurath, 1989), and very similar mode of action, even though they are not otherwise detectably related (Creighton, 1993).

The mammalian serine proteases have a very similar three dimensional structure and catalyses the hydrolysis of peptide bonds in very similar ways. They do, however, differ strikingly in their preference for amino acid side chains in the substrate specificity pocket. Chymotrypsin prefers aromatic side chains, trypsin positively charged side chains and elastase small uncharged side chains (Branden & Tooze, 1999).

Chymotrypsin: Chymotrypsin is widely distributed among living species and its biological role is to catalyse the hydrolysis of proteins in the small intestine. It is selective for peptide bonds on the carboxyl side of the aromatic side chains of tyrosine, tryptophan and phenylalanine, and of large hydrophobic residues such as methionine. Chymotrypsin is synthesised as a precursor, chymotrypsinogen, in the pancreas of mammals (Stryer, 1995). Cod does not possess a well defined pancreatic organ, and the pancreatic tissue is associated with the mesentery of the pyloric ceca

(Bishop & Odense, 1966). Chymotrypsin, along with other digestive proteases such as trypsin, elastase, carboxypeptidase A and B is produced as zymogens by the endocrine cells located in the mesentery (Overnell, 1973). After secretion of chymotrypsinogen into the duodenum, it is conformed into its active form, chymotrypsin, by the action of trypsin (Stryer, 1995).

Chymotrypsin has been characterised from several fish species, including Atlantic cod- *Gadus morhua* (Raae & Walther, 1989; Asgeirsson & Bjarnason, 1991), rainbow trout- *Oncorhynchus mykiss* (Kristjansson & Nielsen, 1992), carp- *Cyprinus carpio* (Cohen et al., 1981a and b) and anchovy- *Engraulis japonica* (Heu et al., 1995). Chymotrypsin has been reported to exist as two isozymes in fish (Raae & Walther, 1989; Asgeirsson & Bjarnason, 1991; Kristjansson & Nielsen, 1992). The molecular weight of chymotrypsin from Atlantic cod is reported to be 26-27 kDa and the pH optimum 7.8-9 (Raae & Walther, 1989; Asgeirsson & Bjarnason, 1991). This is similar to a molecular weight of 25-28.8 kDa and pH optimum of 8-9 for chymotrypsin from other fishes (Cohen et al., 1981a; Kristjansson & Nielsen, 1992; Heu et al., 1995). The temperature optimum of fish chymotrypsin is 45-55°C (Kristjansson & Nielsen, 1992; Heu et al., 1995). Chymotrypsin from fish adapted to cold waters is generally more active at low temperatures and less thermo stable compared to bovine chymotrypsin, and unstable at pH values below 5 (Asgeirsson & Bjarnason, 1991).

Trypsin: Trypsin is the best studied of the serine proteases in fish, and it has been characterised in Atlantic cod (Asgeirsson et al., 1989; Simpson et al., 1989; Raae & Walther, 1989), Greenland cod- *Gadus ogac* (Simpson & Haard, 1984a and b) Antarctic fish- *Paranotothenia magellanica forster* and trout- *Salmo gairdineri* (Genicot et al., 1988), rainbow trout (Kristjansson, 1991), Atlantic salmon- *Salmo salar* (Outzen et al., 1996), chum salmon- *Oncorhynchus keta* (Sekizaki et al., 2000), carp (Cohen et al., 1981a & b), anchovy (Heu et al., 1995), menhaden- *Brevoortia spp* and mullet- *Mugil spp* (Pavlisko et al., 1999). It is secreted as a zymogen and converted to its active form by enteropeptidase, a specialised proteolytic enzyme secreted by intestinal cells. The free trypsin activates more trypsinogen along with

zymogens of other digestive proteases. Trypsin cleaves polypeptide chains on the carboxyl side of arginine and lysine residues (Lehninger et al., 1993).

The molecular weight of trypsin from Atlantic cod has been reported to be in the range 24 to 27 kDa, and the reported pH and temperature optimum ranges from 8-9 and 40 to 55°C, respectively (Asgeirsson et al., 1989; Simpson et al., 1989; Raae & Walther, 1989). Molecular weights of trypsin from other fishes are in the range 23.5-25.7 kDa, optimum pH 7.5 to10 and optimum temperatures ranging from 35 to 63°C (Kristjansson, 1991; Heu et al., 1995; Simpson et al 1989; Pavlisko et al., 1999; Genicot et al., 1988). Trypsin from Atlantic cod has been reported to exist as one (Raae & Walther, 1989; Simpson et al., 1989; 1990) or three (Asgeirsson et al., 1989) isozymes, while in other fish it has been reported to exist as one (Kristjansson, 1991; Heu et al., 1995; Pavlisko et al., 1999), two (Hjelmeland & Raa, 1982), three (Murakami & Noda, 1981) or four (Cohen et al., 1981a; Outzen et al., 1996) isozymes. Trypsin from fish adapted to cold waters is also generally more active at low temperatures, less thermo stable (Asgeirsson & Bjarnason, 1991), and have lower activation energy compared to bovine trypsin. Trypsin from Atlantic cod has been found to have activation energy of 31.8-37.2 kJ/mole compared to 51.5-53.1 kJ/mole for bovine trypsin (Asgeirsson et al., 1989; Simpson et al., 1989). Fish trypsin is unstable at pH values below 5 (Asgeirsson & Bjarnason, 1991).

Elastase: Elastase is an endopeptidase with the ability to hydrolyse elastin, a fibrous protein in connective tissue, in addition to various soluble proteins. Two types of serine elastases, named elastase I and elastase II, are produced in the mammalian pancreas. Elastase I preferably cleaves bonds between alanine and alanine or glycine, whereas elastase II has an enlarged binding pocket and therefore preferably cleaves bonds between leucine, phenylalanine or tyrosine and glycine or alanine (Gertler et al., 1977). It is synthesised initially in the pancreas as an inactive precursor, and is activated by trypsin in the intestines. Elastase has been purified from the internal organs of several fish species such as Atlantic cod (Raae & Walther, 1989; Gildberg & Overbo, 1990; Asgeirsson & Bjarnason, 1993), rainbow trout (Bassompierre et al., 1993), Atlantic salmon (Berglund & Smalas, 1998), carp (Cohen et al., 1981a), dover

sole- *Solea solea* L (Clark et al., 1985), catfish- *Parasilurus asotus* (Yoshinaka et al., 1982) and African lung fish- *Protopterus aethiopicus* (de Haen & Gertler, 1974).

The reported molecular weight of elastase in Atlantic cod ranges from 24.8-28, the pH optimum 7.6-8.5 and the temperature optimum 40°C (Raae & Walther, 1989; Gildberg & Overbo, 1990; Asgeirsson & Bjarnason, 1993). Elastase from other fishes has a molecular weight of 24-27, pH optimum of 8-9.5 and temperature optimum of 45-60°C (Yoshinaka et al., 1982; 1984; Bassompierre et al., 1993; Berglund & Smalas, 1998). Fish elastase is unstable at pH below 5 (Yoshinaka et al., 1982; Asgeirsson & Bjarnason, 1993).

Serine collagenase: Collagenases are generally defined as enzymes that are capable of degrading the polypeptide backbone of native collagen under conditions that do not denature the protein. Two types of proteases with collagenolytic activity have been reported in fish, and are thought to have different physiological functions; metallo collagenase and serine collagenase. Serine collagenase has been purified from the internal organs of cat fish (Yoshinaka et al., 1987), Atlantic cod (Kristjansson et al., 1995), filefish – *Novoden modestrus* (Kim et al., 2002), mackerel- *Scomber japonicus* (Park et al., 2002) and tuna- *Thunnus thynnus* (Byun et al., 2003) and probably has a digestive function. The molecular weights of the serine collagenases from Atlantic cod, filefish, mackerel and tuna were 24.1, 27, 14.8 and 15 kDa, while the pH optimum was between 7 and 9.5 and the temperature optimum between 45 and 55°C. The collagenolytic serine protease from catfish was most stable at pH 6-9 (Yoshinaka et al., 1987), from tuna at pH 6-7.5, while Kristjansson et al. (1995) found collagenolytic serine protease to be unstable at pH values below 7 in Atlantic cod.

The activation energies of a collagenolytic serine proteinase from Antarctic krill, *Euphausia superba* Dana, were found to be 27.5 and 34.6 kJ/mole for two synthetic substrates (Turkiewicz et al., 1991).

1.6.1.2. Cysteine proteases

This class includes several lysosomal cathepsins, the cytosolic calcium activated proteases (calpains), and the plant proteases papain and actinidin. The major catalytic amino acid is a cysteine (Neurath, 1989).

Cathepsin B: Cathepsin B is a lysosomal cysteine protease that hydrolyses a wide range of proteins and has an important role in the hydrolysis of tissue proteins (Barrett & Kirschke, 1981). With its dual action as an endopeptidase and a peptidyldipeptidase, cathepsin B is equipped to participate in both the early and late stages of lysosomal protein breakdown. It is formed from a precursor that is latent in the tissues (Mort & Buttle, 1997) and is converted to the mature form by biological reactions, such as acidification (Mach et al., 1994), action of other proteolytic enzymes (Dalet-Fumeron et al., 1993; Kawabata et al., 1993) or auto activation by cathepsin B itself (Rowan et al., 1992; Mach et al., 1993). Together with other cathepsins it is considered one of the main causes of post mortem degradation of muscle (Ashie et al., 1996; Kolodziejska & Sikorski, 1996). Enhanced activity of cathepsin B along with cathepsin D, H and L in spawning fish has been reported, pointing out an important physiological role of these enzymes in muscle catabolism (Yamashita & Konagaya, 1990a). Bonete et al. (1984a) measured cathepsin B activity in muscle, liver, heart, spleen and gonads from the fishes *Mujil auratus, Sparus aurata* and *Lightonatus mormyrus*, and found that the spleen was the organ showing highest activity in units per g tissue, however, muscle extract accounted for 95% of the fish total cathepsin B activity.

Cathepsin B has been purified from muscle of several fish species, including carp (Hara et al., 1988), *Mujil auratus* (Bonete et al., 1984b), chum salmon (Yamashita & Konagaya, 1990b) and mackerel- *Scomber australasicus* (Jiang et al., 1994) - *Scomber japonicus* (Aoki et al., 1995; 2002). The molecular weight of cathepsin B or cathepsin B-like enzyme from fish muscle is in the range 23-29 kDa, the pH optimum 5.5-6.5 and the temperature optimum 45-55°C (Hara et al., 1988; Jiang et al., 1994; Aoki et al., 1995; 2002). The decrease in catalytic activity of fish cathepsin B is quite fast when pH is changed from the optimum, and instability is

reported above pH 7-7.5 and below pH 4-5 (Hara et al., 1988; Yamashita and Konagaya, 1990b; Jiang et al., 1994). The activation energy found for cathepsin B from the *Mujil auratus* muscle is 44.4 kJ/mole (Bonete et al., 1984b).

1.6.1.3. Aspartic proteases

Like the members of the other protease families, aspartic proteases perform many functions. The best known gastric enzymes, such as pepsin, gastricsin and chymosin, are involved in digestion and have only limited substrate specificities. Cathepsin D is a lysosomal enzyme that degrades proteins intracellularly. Other aspartic proteases produce very specific cleavage in single proteins and are involved in control of blood pressure and virus activation (Creighton, 1993). Gildberg (1988) has reviewed aspartic proteases in fish.

Pepsin: Pepsin is the principal proteolytic enzyme in gastric juice, it is synthesised as the zymogen pepsinogen. Pepsinogen contains an amino-terminal precursor segment that is proteolytically removed in the formation of pepsin. This activation occurs spontaneously below pH 5. The rate formation of pepsin is independent of the concentration of pepsinogen, which shows that activation is intramolecular (Stryer, 1995). The effect of temperature on activation of fish pepsinogens has been found to be less marked compared to porcine pepsinogen (Squires et al., 1986a).

The first fish pepsin crystallised was from salmon in 1940 (Norris & Elam, 1940). Today pepsin from several fish has been characterised, Atlantic cod (Brewer et al., 1984; Gildberg et al., 1990), Polar cod- *Boreogadus saida* (Arunchalam & Haard, 1985), Greenland cod (Squires et al., 1986a & b), capelin- *Mallotus villosus* (Gildberg & Raa, 1983), adult and juvenile salmon (Sanchez-Chiang et al., 1987) and palometa- *Parona signata* (Pavlisko et al., 1997).

Many fish have two significantly different pepsins, pepsin I and pepsin II, which digest haemoglobin at maximal rate in the pH range 3-4 and 2-3, respectively (Gildberg, 1988).When "crude pepsin" fractions are examined a broad pH optimum in the range 2-3 is shown (Arunchalam & Haard, 1985). Fish pepsins have higher pH optimum and are less stable in strong acidic conditions than mammalian pepsins. They are very efficient at low temperatures, but less thermo stable than mammalian

pepsins. The molecular weight of fish pepsin is similar to that of mammalian pepsin, 35 kDa (Gildberg, 1988). Cod pepsins are glycoproteins, and their amino acid composition resembles that of porcine cathepsin D more than that of porcine pepsin (Gildberg et al., 1990).

Pepsin act very slowly on small peptides and haemoglobin is the most widely used substrate for analyses of pepsin activity (Gildberg, 1988). The temperature optimum of fish pepsin on haemoglobin is in the range 35-40°C (Gildberg & Raa, 1983; Arunchalam & Haard, 1985; Pavlisko et al., 1997). The Arrhenius activation energy is lower for fish pepsins, 12.1 and 13.4 kJ/mole in Polar cod (Arunchalam & Haard, 1985) and 30.5 kJ/mole in Atlantic cod (Brewer et al., 1984) compared to 46.0-50.2 kJ/mole in warm blooded animals (Haard et al., 1982).

Cathepsin D and E: Cathepsin D is similar to pepsin in its specificity and molecular structure, but the physiological role is usually quite different. Cathepsin D is a lysosomal enzyme active in intracellular protein turnover (Gildberg, 1988). It has been isolated from a variety of tissue cells, but seems to be particularly abundant in cells from liver, spleen and muscle (Barrett, 1977). Fish muscle and liver has been reported to contain about 5-10 times more cathepsin than mammalian muscles (Gildberg, 1988).

Cathepsin D or cathepsin D-like activity has been described in among muscle of chum salmon (Yamashita & Kongaya, 1990a, 1992), carp (Goldman-Levkovitz et al., 1995), sardine- *Sardina pilchardus* (GomezGuillen & Batista, 1997), herring- *Clupea harengus* (Nielsen & Nielsen, 2001) and tilapia- *Tilapia mossambica* (Doke et al., 1980), liver of Antarctic icefish- *Chionodraco hamatus* (Capasso et al., 1999) and ovary from rainbow trout (Brooks et al., 1997) and seabream- *Sparus aurata* (Carnevali et al., 1999). Studies have shown that cathepsin D activity is elevated in spawning fish, and that female fish have higher activity compared to male fish (Yamashita & Konogaya, 1990a; GomezGuillen & Batista, 1997).

Haemoglobin is also the most commonly used substrate for cathepsin D. Studies suggest that it has a preference for bulky hydrophobic amino acids (Goldman-Levkovitz et al., 1995; Nielsen & Nielsen, 2001). Both the pH and temperature optimum differ among studies of cathepsin D in fish, ranging from 2.5 to

3.8 and 37 to 55°C, respectively (Doke et al., 1980; Goldman-Levkovitz et al., 1995; GomezGuillen & Batista, 1997; Capasso et al., 1999; Nielsen & Nielsen, 2001). Fish cathepsin D is a glycoprotein containing one or two glycosylation sites per molecule (Nielsen & Nielsen, 2001; Brooks et al., 1997; Capasso et al., 1999), and the molecular weight of cathepsin D found in fish is 36-40 kDa. Activation energy for cathepsin D from carp and *Mujil auratus* is reported to be 33.5 and 35.1 kJ/mole, respectively (Goldman-Levkovitz et al., 1995; Bonete et al., 1984b), this is somewhat lower than the activation energy for cathepsin D from mammalian fibroblasts, 45.2 kJ/mole (Schwabe & Kalnitsky, 1966).

Cathepsin E is a tissue proteinase with similar specificity and physiological role as cathepsin D. It has a molecular weight of about 100 kDa and optimum activity at pH 2.5 (Barrett, 1977).

Studies of cathepsin E from fish are scarce, but it has been described in muscle of chum salmon. The molecular weight was found to be 90 kDA and the pH optimum 2.8 (Yamashita & Kongaya, 1992).

1.6.1.4. Metallo proteases

Metallo proteases use bound metals in their active sites, usually Zn^{2+}, and include medically important enzymes such as angiotensin-converting enzyme, enkephalinase and collagenase. Carboxypeptidase A and B and thermolysin are the most thoroughly studied representatives of this class (Creighton, 1993).

Collagenase: Metallo collagenases or specific collagenases are zinc containing enzymes that require calcium for optimum activity and stability. They cleave the collagen helix at a specific locus under physiological conditions, and are involved in the remodelling of the extracellular matrix (Harris & Vater, 1982; Sellers & Murphy, 1981). Enzyme catalysed degradation of collagen and other extracellular matrix components have been implicated in quality deterioration of seafood products by several investigators (Sikorski et al., 1984; Bremner & Hallet, 1985; Ando et al., 1992). Collagenases are secreted as zymogens and the activity is controlled by factors other than the enzyme concentration (Monfort & Perez-Tamayo, 1975). A number of inhibitory mechanisms involving tissue inhibitors and blood serum

proteins have been found for these enzymes (Woessner, 1991). After death these mechanisms would gradually cease to be effective. Moreover, the collagenases are activated by both cysteine and serine proteinases (Unemori & Werb, 1988). Collagenase is bound to collagen fibres, reticulum fibres and basement membranes (Montfort & Perez-Tamayo, 1975). In fish the greatest concentrations of collagen is found in the skeleton, fins and skin. In fillets the content is higher towards the tail end (Sikorski et al., 1984).

Collagenolytic metallo protease from muscle of winter flounder- *Pseudopleuronectes americanus* (Teruel & Simpson, 1995), rainbow trout (Saito et al., 2000) and hepatopancreas of the marine crab- *Scylla serrata* (Sivakumar et al., 1999) has been characterised. The molecular weight of the collagenolytic metallo proteases from rainbow trout and marine crab was 54-55 kDa. This is somewhat higher than the molecular weight reported for collagenase from tadpole (Hori & Nagai, 1979) and rabbit (Unemori & Werb, 1988), 43 and 43-47 kDa, respectively. The molecular weight of the procollagenases was, however, reported to be 53 and 57 kDa in rabbit. Reported pH optimum for collagenase is around neutral, pH 7-7.5, while it is reported to be stable in the pH range 6-8 (Hori & Nagi, 1979; Teruel & Simpson, 1995; Sivakumar et al., 1999). Optimum temperature of partially purified collagenolytic enzyme from winter flounder muscle was reported to be 40°C (pH 7.5) (Teruel & Simpson, 1995).

1.6.2. Lipolytic enzymes

Lipases are defined as enzymes hydrolysing long chain acylglycerols at an oil–water interface (Martinelle & Hult, 1994), and are indispensable for the biological turnover of lipids. They are required as digestive enzymes in the transfer of lipid from one organism to another, that is from plant to animal and from animal to animal. Within the organism, they are instrumental in the deposition and mobilisation of the fat that is used as an energy reservoir. In addition they are involved in the metabolism of intracellular lipids and therefore in the functioning of biological membranes (Brockerhoff & Jensen, 1974). These enzymes are of particular importance in the marine environment, as lipids constitute a considerable fraction of the energy intake of marine carnivorous fish (Watanabe, 1982).

Controlling lipase activity in the by-products is necessary in order to minimise lipolysis of nutritionally valuable fat, and ensuring that fish oil extracted from by-products meets the guideline specifications. The primary products of lipolysis are free fatty acids and glycerol. Free fatty acids are easily oxidised and may impart off-flavour in the by-products and products produced from them.

Lipolytic enzymes are hormonally modulated and a variety of slow acting (thyroxin, cortisol, growth hormone, prolactin) and fast acting (adrenaline, nor-adrenaline, somatostatin) agents have been shown to stimulate lipase activity, perhaps indicating multiple mechanism of hormone action (Sheridan, 1988). Antilipolytic hormones (insulin), and metabolites (fatty acids, acyl co A, CoASH, etc.) are other factors controlling the lipolysis of storage fat (Hochachka & Somero, 1984).

Different lipases have been described in fish: triacylglycerol lipase (Gjellesvik et al., 1989; Sidell & Hazel, 2002), pancreatic lipase (Patton, 1975; Leger et al., 1977), bile salt-dependent lipase (Patton et al., 1977; Gjellesvik, 1991), phospholipase (Chawla & Ablett, 1987; Aaen et al., 1995) and lipoprotein lipase (LPL) (Liang et al., 2002). The molecular weights of the fish lipases range from 54-60 kDa, and the pH and temperature optimum ranges from 7-8 and 25-37°C, respectively (Mukundan et al., 1985; Gjellesvik et al., 1992).

1.6.3. Activation energy

Activation energy (E_a) is the difference between the energy levels of the ground state and the transition state. The rate of the reaction reflects this activation energy, a higher activation energy corresponds to a slower reaction. Reaction rates can be increased by raising the temperature, thereby increasing the number of molecules with sufficient energy to overcome this energy barrier (Lehninger et al., 1993). A high activation energy of a reaction means that the reaction is highly temperature dependent. Enzymes accelerate chemical reactions by decreasing the activation energy. Knowledge about the activation energies of the enzymes present in the by-product fractions is important in order to predict the breakdown of macromolecules during storage and processing at given temperatures.

The studies of Arrhenius and van`t Hoff in the 1880-ties were the starting point for all modern theories of the temperature dependence of rate constants. The Arrhenius plot provides a simple method of evaluating E_a (Cornish-Bowden, 2001).

The activation energies of similar types of enzymes are grouped around certain values. Peptidases from mammals are usually associated with activation energies of 42- 67 kJ at temperatures between 0 and 40°C, most values are close to 50 kJ (Schwabe & Kalnitsky, 1966).

1.6.4. Heat stability of enzymes

The optimum temperature for activity is understood to be due to the counteracting effects of increasing temperature on reactant mobility and "effective collision" on one hand, and denaturation on the other hand. When the temperature of an enzyme system is raised beyond the optimum, the rate of denaturation is increased several fold more than the rate of the enzyme-catalysed reaction (Ashie et al., 1996).

Thermal stability of enzymes is related both to enzyme structure and to factors in the micro environment. Factors within the enzyme structure that contribute to thermal stability include the number of salt-bridges, hydrogen and disulfide bonds, ligands (prosthetic groups and metal ions), hydrophobic interactions, and the amino acid sequence. Factors in the micro environment that may have significant effects on heat stability are the relative amounts of free and bound water, substrate binding, pH and the presence of salts (Ashie et al., 1996; Kristjansson & Kinsella, 1991; Adams, 1991).

The high catalytic efficiency of enzymes from cold adapted species at low temperatures is associated with a low thermal stability. A decrease in activation energy is achieved structurally by a decrease in the number of enthalpy-driven interactions that have to be broken to reach the transition state, though indicating lower stability (Georlette et al., 2004). It has been suggested that the lower stability of cold adapted enzymes from psychrophile organisms is due to lack of evolutionary pressure for stable enzymes in the low temperature environment (Miyazaki et al., 2000).

2. THE RAW MATERIAL

2.1 Sampling and preparation of raw material

By-products from five species of cod were analysed, Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), saithe (*Pollachius virens*), tusk (*Brosme brosme*) and ling (*Molva molva*). Sampling took place at three times during 2001: February/March, April/June and October/December. Fish by-products were sampled from fish caught at three different fishing grounds, Barents Sea, Icelandic Sea and south coast of Ireland. In order to minimize the influence of size, size requirements were included in the trial. Atlantic cod was used to study the influence of size and was therefore collected in 3 size groups, 50-60 cm, 60-70 cm and 70-80 cm. The other species had the following size requirements; haddock: 45-55 cm, saithe: 70-80 cm, tusk: 40-50 cm and ling: 70-80 cm. Figure 2.1 illustrates the handling and sampling procedure for the fish.

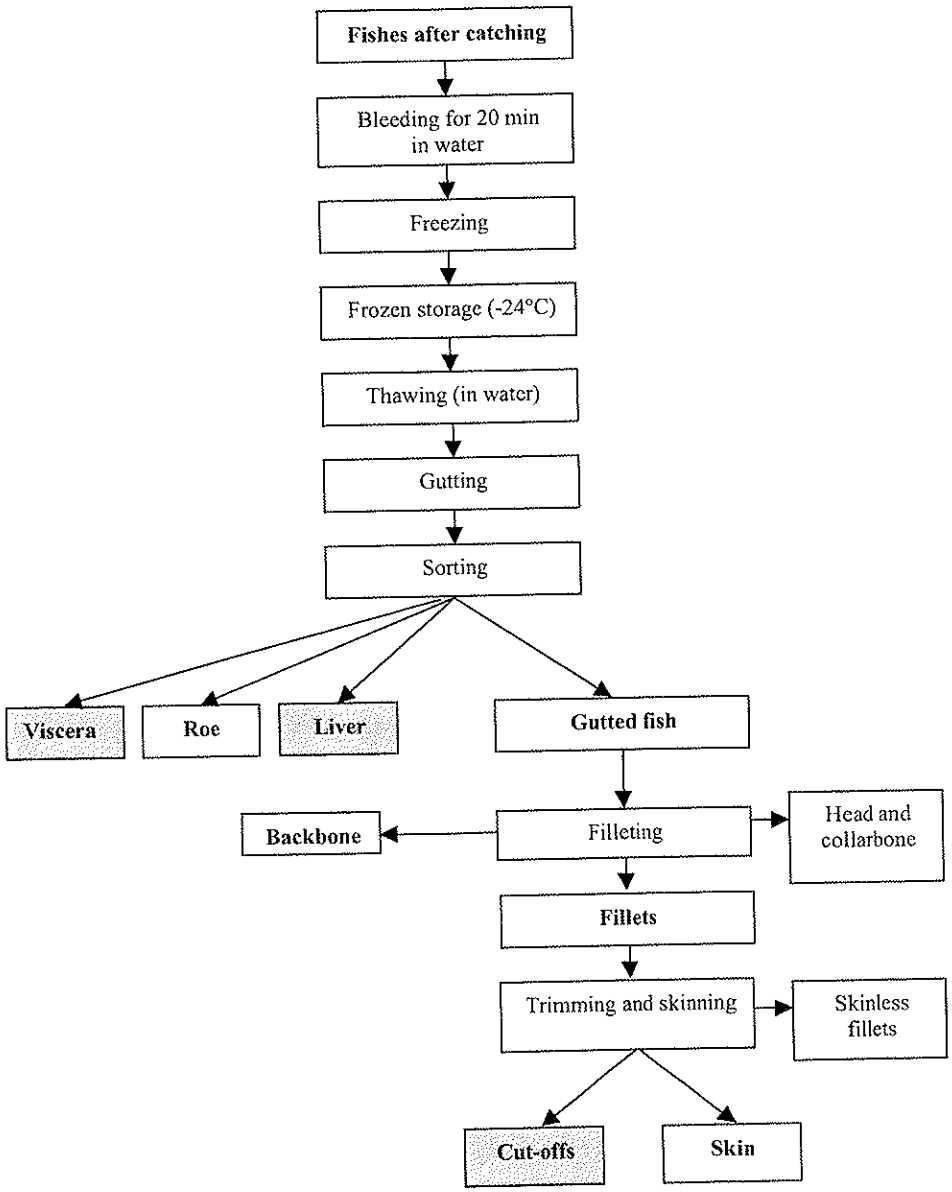

Figure 2.1: Procedures for handling the fish onboard and sampling.

SINTEF Fisheries and Aquaculture, IFL (Icelandic Fisheries Laboratories) and UCC (University College Cork) were responsible for collection and sample preparation. The fish were frozen whole after bleeding onboard vessels and stored at -24°C. Before processing the fish were thawed in water (ratio 1:4.5) for

approximately 22 hours, to a core temperature of 0 to 1°C. Weight and length of the gutted fish were recorded. The by-products collected were viscera, roe, liver, cut-offs (v-cut and belly flap) and skin. In this study only the viscera, liver and cut off were further analysed. Each sample was made up of by-products from 15 fish of the same species. The samples were crudely homogenised using a food processor or a Warring Blender. Handling, homogenisation and distribution of the samples were done within 14 days after catch. The samples were sent to the lab, where they were stored in a freezing room at a temperature of -40°C until analyses could be conducted.

2.1.1 Evaluation of the sampling procedure

Ideally all the species should have been caught at the same time in all three fishing grounds, and all species should have been caught in all three fishing grounds in all three seasons. This was, however, not the case. Seasons became wider than planned due to weather problems (the vessels could not go out), or enough fish was not caught within the range of the season. Not all species were caught in large enough amounts to make up samples, or not in large enough amounts to make up samples to send to all of the partners, therefore all species are not represented in all by-product fractions. Table 2.1 to 2.10 gives an overview of the samples collected from the different fishing grounds, seasons and species, and some sample characteristics.

Each sample was made up of by-products from 15 fish of the same species in order to provide enough raw material to send to all the partners in the EU-project, but also in order to simulate industrially interesting batches. Only one sample of each by-product fraction was collected for saithe, haddock, tusk and ling. Three samples were collected for Atlantic cod, these three samples were from fish of different size (50-60, 60-70 and 70-80 cm). No significant differences were, however, found between the three size categories, and these samples were put into one group in this study. The fact that only one sample was available for most of the species from the different fishing grounds and seasons, makes it difficult to interpret the data, and limits the value of the work done in this thesis. It may have produced information of greater value if the number of fishing grounds, seasons and/or species had been reduced, and more samples of the same fishing grounds, seasons and/or species had been studied.

Since samples have been collected at three different fishing grounds several fishing boats and different people have been involved in the sampling, this may of course be a factor contributing to the variance in raw material between fishing grounds. However, if by-products are to be industrially utilized the raw material will most likely come from several different fishing boats, and different people will be involved in the process. The variance in raw material due to differences in routines onboard fishing boats is therefore a variance the industry must be prepared to meet.

Season of catch is of major importance when enzymatic activity is evaluated in fish, due to seasonal spawning and feeding behaviour. Enhanced activity of several cathepsins has been reported in spawning fish (Yamashita & Konagaya, 1990a, GomezGuillen & Batista, 1997). The spawning season of fish of the cod species is quite long (see table 1.2) and varies according to species and fishing ground. It is therefore difficult to predict whether the fish caught in the different fishing grounds and seasons were spawning, especially since most of the species could have been spawning in more than one of the seasons. Since the samples were made up of by-products from 15 fish, it is also possible that some of the fish were spawning and others not. The ratio of spawning fish to non-spawning fish would then be of major importance with respect to the quality of the by-products. Falch et al. (submitted) found that the gonadosomatic indexes (milt and roe weight as percentage of the whole body weight) were higher in fish caught in the Barents Sea in Feb.-March, compared to the other seasons. The largest Atlantic cod (70-80 cm) did, however, also have a higher lipid content in liver in Feb.-March (Falch et al., submitted), this is in contradiction to what is expected in spawning fish. For the Icelandic samples, roe was found in all species at all sampling periods, except haddock in June. The gonadosomatic index was also in the Icelandic samples highest in the Feb.-March collection for all species. The number of fish which roe and milt were collected from was, however, low (Thorarinsdottir et al., 2004), and the influence of these fish on the parameters measured in pooled samples from 15 fish is not clear. It is likely from these results that no samples of fish were taken during the peak of spawning, and from the proximate analysis it may be reasonable to assume that spawning occurred between the Feb.- March and the April- June sampling in the

Barents Sea and the Icelandic Sea. Information about the gonadosomatic index of the fish caught at the south coast of Ireland was not available.

2.2. Raw material composition

2.2.1 Viscera

The pH in crude extracts of viscera ranged from 6.32 to 7.44. Atlantic cod viscera from the Icelandic Sea had significantly lower pH ($p < 0.05$) than Atlantic cod viscera from the other fishing grounds in all three seasons. Diet and feeding status of the fish are probably the main factors influencing pH in viscera.

Table 2.1 to 2.4 shows dry matter, total protein, water-soluble protein and free amino acids in the viscera collected from the different fishing grounds in the three seasons.

Table 2.1: Dry matter (g/ 100g wet weight) in viscera from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	21.0 ± 0.9	3	18.9 ± 0.8	3	19.9 ± 0.2
Saithe	1	20.0	1	19.7	1	27.0
Haddock	1	20.3	1	22.3	1	22.3
Tusk	1	19.5	1	21.9	1	28.4
Ling	1	15.8	1	18.5	1	18.5
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	18.2 ± 0.4	3	17.1 ± 0.2	3	21.3 ± 4.4
Saithe	1	22.8	1	20.9	1	19.1
Haddock	1	17.1	1	15.3	1	15.4
Tusk			1	16.0		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	17.5 ± 0.9	2	23.0 ± 1.5	2	19.2 ± 0.8
Saithe	1	18.2			1	19.0
Haddock	1	19.2	1	18.1	1	15.7
Ling	1	14.6	1	17.2	1	11.6

Atlantic cod and saithe from the Icelandic Sea and Atlantic cod and haddock from the Barents Sea had lowest amount of dry matter in viscera samples from April-June. Since the fish should be feeding in this period the opposite was expected. In cod and ling from the south coast of Ireland the amount of dry matter was highest in

April- June, as expected. The content of dry matter in the viscera fraction is most likely determined by the quality and quantity of feed in the stomach and intestines. The quality of stomachs and intestines for consumption are evaluated on basis of their degree of filling. Stomach and intestines that are filled are bloated and thin, and considered of poor quality. Stomachs and intestines from cod, saithe and haddock from northern Norway are found to be of good quality from the end of January to out April, and in this period these by-products may be sold for consumption (RUBIN, 2002)

Table 2.2: Total protein (g/ 100g wet weight) in viscera from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	10.5 ± 0.4	3	11.7 ± 0.4	3	10.1 ± 0.1
Saithe	1	12.6	1	11.8	1	18.5
Haddock	1	10.6	1	9.2	1	10.0
Tusk	1	3.3	1	6.9	1	16.1
Ling	1	8.7	1	10.5	1	12.3
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	11.5 ± 1.3*	3	11.6 ± 0.3	3	13.4 ± 2.9
Saithe	1	12.2	1	12.7	1	12.0
Haddock	1	10.9	1	9.4	1	10.1
Tusk			1	11.9		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	13.3 ± 0.9	2	9.0 ± 0.5	2	13.2 ± 0.7
Saithe	1	12.4			1	12.7
Haddock	1		1	9.3	1	7.4
Ling	1	11.5	1	9.9	1	8.1

Tusk viscera from the Icelandic Sea had low amounts of protein in Feb.- March and April- June. Atlantic cod viscera from the south coast of Ireland had significantly ($p < 0.05$) lower amounts of total protein in April-June compared to the other seasons. This parameter is probably also mostly determined by feeding status.

Table 2.3: Water-soluble protein (g/ 100g wet weight) in viscera from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	0.73 ± 0.07	3	0.97 ± 0.00	3	0.64 ± 0.03
Saithe	1	0.86	1	0.66	1	1.99
Haddock	1	1.02	1	0.40	1	0.85
Tusk	1	1.73	1	1.32	1	1.5
Ling	1	1.26	1	1.44	1	1.25
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	1.22 ± 0.19	3	1.69 ± 0.20	3	1.53 ± 0.16
Saithe	1	1.48	1	1.35	1	2.1
Haddock	1	1.41	1	1.24	1	1.76
Tusk			1	1.29		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	0.68 ± 0.11	2	0.75 ± 0.31	2	0.99 ± 0.41
Saithe	1	0.48			1	2.26
Haddock	1	1.31	1	0.50	1	1.66
Ling	1	2.07	1	1.61	1	1.78

Samples of Atlantic cod from the Barents Sea had significantly ($p < 0.05$) higher amount of water-soluble protein in the viscera compared to the other fishing grounds (all three seasons). Tusk and ling from the Icelandic Sea and ling from the south coast of Ireland had higher amounts of water-soluble protein in viscera compared to the other species, with exception of saithe in Oct.- Dec. Samples of Atlantic cod from the Icelandic Sea and the Barents Sea had significantly ($p < 0.05$) higher amounts of water-soluble protein in April- June compared to the other seasons. The onset of feeding occurs quite suddenly in late autumn or early summer. An enhanced production of digestive enzymes with the onset of feeding has been reported (Love, 1975), and may explain the higher values of water-soluble protein seen in some of the samples in April- June.

Table 2.4: Free amino acids (g/ 100g wet weight) in viscera from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	28.7 ± 1.5	3	19.9 ± 2.2	3	31.5 ± 2.8
Saithe	1	33.2	1	30.1	1	17.1
Haddock	1	27.4	1	26.5	1	15.1
Tusk	1	4.1	1	6.1	1	8.0
Ling	1	14.2	1	11.4	1	6.1
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	15.1 ± 3.9	3	7.9 ± 4.1	3	19.2 ± 8.9*
Saithe	1	7.9	1	26.8	1	14.8
Haddock	1	1.8	1	19.0	1	7.6
Tusk			1	7.0		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	29.5 ± 0.5	2	30.7 ± 7.0	2	34.5 ± 0.9
Saithe	1	33.4			1	17.0
Haddock					1	22.7
Ling	1	16.4	1	27.4	1	11.5

Amount of free amino acids was lower in tusk and ling than in the other species.

Figure 2.2 shows the relationship between water-soluble protein and free amino acids. Amount of water-soluble protein was significantly ($p < 0.05$) negatively correlated to amount of free amino acids.

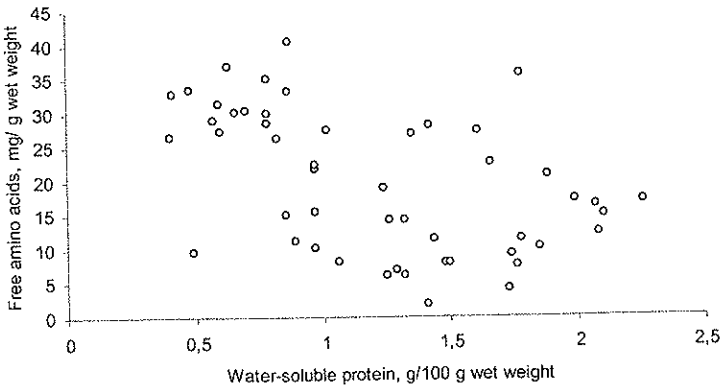

Figure 2.2: Relationship between amount of water-soluble protein (g/100g wet weight) and free amino acids (mg/g wet weight) in viscera from cod species (Pearson correlation coefficient = -0.53).

2.2.2 Cut off

The pH in crude extracts of cut off ranged from 6.74 to 7.37. Cut off samples of Atlantic cod from the Barents Sea and the Icelandic Sea, had lowest pH in Feb.- March and highest in Nov.- Dec., while samples from the south coast of Ireland had lowest pH in April- June. Saithe, haddock, tusk and ling from the Icelandic Sea also showed lowest pH in April- June. Lower pH in muscle of cod fish during heavy feeding in the summer months have been reported (Love, 1975; Love, 1979; Rustad, 1992). The amount of muscle glycogen at the instant of death is one of the most important determinants of the ultimate pH in fish meat. Fish in poor "biological condition" have muscles with comparatively low glycogen reserves, and flesh with comparatively high pH (Haard, 1992). The muscle pH of wild cod (post rigor) has been reported in the range 6.3- 7.1 (Rustad et al. 1991; Rustad, 1992; Hultmann & Rustad, 2002).

Table 2.5 to 2.8 shows the content of dry matter, total protein, water-soluble protein and free amino acids in cut off samples collected from the different fishing grounds in the three seasons.

Table 2.5: Dry matter (g/100g wet weight) in cut off from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	17.7 ± 0.8	3	15.0 ± 0.8	3	17.9 ± 0.5
Saithe	1	19.1	1	16.6	1	20.6
Haddock	1	19.1	1	18.4	1	19.5
Tusk	1	19.4	1	19.4	1	19.0
Ling	1	18.6	1	18.0	1	21.5
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	17.6 ± 0.4	3	18.3 ± 0.6	3	17.6 ± 0.4
Saithe	1	19.9	1	20.4	1	20.2
Haddock	1	18.5	1	19.4	1	18.6
Tusk			1	31.1		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	19.9 ± 0.2	2	19.4 ± 0.3	2	25.9 ± 3.4
Saithe	1	21.9			1	21.9
Haddock			1	19.7		
Ling	1	19.3				

Comparing dry matter in cut off from different species shows that dry matter tended to be lower in Atlantic cod than in the other species. Cut off samples also showed seasonal variation with respect to dry matter. Cut off from Atlantic cod, saithe, haddock and ling caught in the Icelandic Sea and Atlantic cod caught at the south coast of Ireland had the lowest content of dry matter in April- June and the highest in Nov.- Dec. Samples from the Barents Sea had highest content of dry matter in April- June. Samples from the south coast of Ireland had the higher amount of dry matter (in all seasons) compared to the other fishing grounds.

Nutritional status and sexual maturity of fish can have a marked effect on the water content and thereby dry matter of the flesh. Depletion of nutrients during sexual maturation or starvation can result in an increase in the moisture content of lean muscle (Haard, 1992). A seasonal change in amount of dry matter in the cut off is expected due to the spawning/ feeding behaviour of the fish. In fillets it has been reported to be lowest in spring and early summer after spawning (Eliassen & Vahl, 1982a; Love, 1975). The content of dry matter in cod muscle has been found to be 17- 20% (Hultmann & Rustad, 2002; Hultmann, 2003).

Table 2.6: Total protein (g/100g wet weight) in cut off from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	16.7 ± 1.0	3	13.6 ± 0.5	3	16.1 ± 0.5
Saithe	1	17.6	1	15.2	1	19.0
Haddock	1	15.5	1	17.3	1	17.9
Tusk	1	22.9	1	17.5	1	17.2
Ling	1	17.3	1	17.1	1	20.0
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	16.3 ± 0.5	3	15.3 ± 1.1	3	15.7 ± 0.5
Saithe			1	18.3	1	18.9
Haddock	1	17.7	1	17.2	1	16.7
Tusk			1	16.5		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	16.2 ± 0.2	2	16.1 ± 1.4	2	23.5 ± 3.0
Saithe	1	18.0			1	18.9
Haddock			1	17.4		
Ling	1	14.7				

The total amount of protein tended to be lower in cut off samples from Atlantic cod compared to the other species, with some exceptions. In Atlantic cod from all three fishing grounds the amount of total protein was lower in April- June. In samples of Atlantic cod from the Icelandic Sea and the Barents Sea the amount was highest in Feb.- March, while in the samples from the south coast of Ireland it was highest in Oct.- Dec. Total protein content is expected to change with season in the same way as dry matter and the highest values were expected in the Oct.-Dec. samples, when the fish had recovered from spawning (Eliassen &Vahl, 1982a; Ingolfsdottir et al., 1998). The results in our study suggest that the samples taken in Feb.- March are caught prior to spawning, and the fish caught in April- June is recovering from spawning.

The cut off samples contain somewhat less protein than fish muscle, which normally ranges from 18 to 22% of the tissue weight (Haard, 1992), but is of nutritionally good quality (See chapter 2.2.4).

Table 2.7: Water-soluble protein (g/ 100 g wet weight) in cut off from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	2.17 ± 0.30	3	2.18 ± 0.11	3	1.02 ± 0.03
Saithe	1	1.87	1	2.27	1	2.1
Haddock	1	1.84	1	1.86	1	2.24
Tusk	1	2.17	1	2.00	1	1.85
Ling	1	2.57	1	2.39	1	1.62
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	1.84 ± 0.05	3	2.03 ± 0.15	3	1.92 ± 0.02
Saithe	1	2.19	1	2.79	1	2.50
Haddock	1	1.26	1	2.42	1	2.17
Tusk			1	1.25		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	1.78 ± 0.18	2	3.00 ± 0.65	2	1.43 ± 0.15
Saithe	1	1.76			1	1.84
Haddock			1	2.23		
Ling	1	0.86				

Samples of Atlantic cod from all three fishing grounds, saithe from the Icelandic Sea and the Barents Sea and haddock from the Barents Sea had the highest amount of water-soluble protein in April- June. Hultmann (2003) reported amount of

water-soluble protein in farmed cod muscle to be 2.4 ± 0.2 g/100 g ww, while Rustad et al. (1991) reported amount of water-soluble protein in wild cod muscle to be 3.9 ± 1.0 g/100 g ww.

Table 2.8: Free amino acids (g/ 100 g wet weight) in cut off from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	10.7 ± 9.0	3	1.2 ± 0.2	3	1.3 ± 0.2
Saithe	1	0.9	1	1.2	1	0.8
Haddock	1	27.4	1	1.2	1	2.8
Tusk	1	0.8	1	0.6	1	0.3
Ling	1	1.6	1	0.8	1	0.8
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	2.8 ± 1.6	3	1.0 ± 0.1	3	1.3 ± 0.1
Saithe	1	2.2	1	1.5	1	0.9
Haddock	1	1.3	1	13.6	1	0.5
Tusk			1	0.3		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	5.9 ± 0.3	2	17.2 ± 7.0	2	3.3 ± 0.7
Saithe	1	8.9			1	8.6
Haddock						
Ling	1	0.8				

All species except for saithe from the Icelandic Sea had the highest amount of free amino acids in Feb.- March, this also holds for Atlantic cod and saithe from the Barents Sea, and may be explained by the onset of spawning. The breakdown of muscle protein may have started leading to increased amounts of free amino acids, but has not yet progressed enough to reduce the amount of protein. Samples of Atlantic cod from the Icelandic and Barents Sea also had high standard deviations in this analysis, suggesting that not all of the fish had started the breakdown of muscle protein. The content of free amino acids in the muscle of aquatic animals is normally higher than that from land animals. The free amino acid content in muscle from aquatic organisms normally ranges from about 0.5 to 2% of muscle wet weight (Haard, 1992).

Figure 2.3 shows a significant ($p < 0.05$) positive correlation between amount of total protein and dry matter in cut off. Several researchers have reported an inverse

relationship between water and protein in cod muscle, showing maximum and minimum values corresponding to the period of spawning (Love et al., 1974; Eliassen & Vahl, 1982a; Ingolfsdottir et al., 1998).

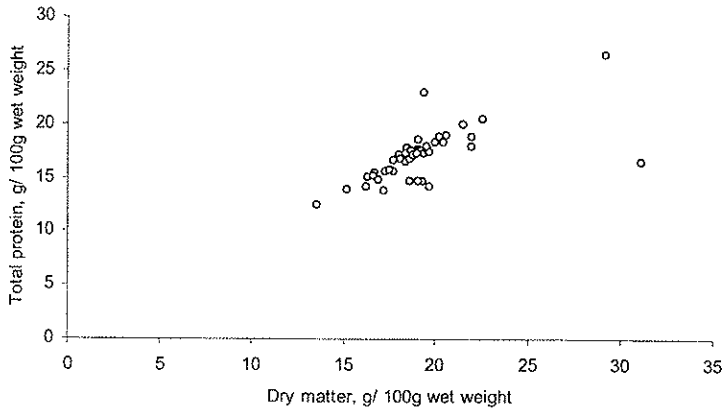

Figure 2.3: Relationship between amount of total protein (g/100g wet weight) and dry matter (g/100g wet weight) in cut off from cod species (Pearson correlation coefficient = 0.64).

2.2.3 Liver

Table 2.9 and 2.10 characterises the liver collected from the different fishing grounds in the three seasons.

The pH in liver was somewhat lower than in the other by-product fractions, 6.7 ± 0.02. No variations according to species, season or fishing ground were found.

Table 2.9: Dry matter (g/ 100g wet weight) in liver from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	62.7 ± 5.1	3	63.5 ± 4.4	3	60.4 ± 2.9
Saithe	1	72.1	1	63.1	1	86.1
Haddock	1	58.4	1	55.7	1	61.6
Tusk	1	72.8	1	61.6	1	75.1
Ling	1	58.7	1	45.9	1	78.4
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	73.5 ± 1.3	3	64.2 ± 5.1	3	60.6 ± 2.8
Saithe	1	68.5	1	70.5	1	72.7
Haddock	1	58.5	1	69.0	1	70.1
Tusk			1	56.7		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	58.5 ± 8.8	1	52.9	3	45.5 ± 6.0
Saithe	1	57.3			1	52.7
Haddock	1		1	51.0	1	61.4
Ling	1	47.0	1	45.9	1	58.6

The amount of dry matter in liver is high due to the large amounts of lipids in this fraction, and is also influenced by the nutritional status of the fish. Since liver lipids are known to decrease leading to an increased ratio of water, and reach a minimum during spawning (Jangaard et al., 1966; Eliassen & Vahl, 1982b; RUBIN, 2002), it was expected to find lowest values for dry matter in liver samples from Feb.- March and highest values in Oct.- Dec. This was also the case for saithe and haddock from the Barents Sea, however, the opposite was the case for Atlantic cod samples from all three fishing grounds. Samples of Atlantic cod from the Icelandic Sea and the south coast of Ireland had the highest amount of dry matter in Feb.- March and the lowest in Oct.- Dec. Saithe, haddock, tusk and ling from the Icelandic Sea and ling from the south coast of Ireland had lowest amount of dry matter in samples from April- June, when the fish is expected to be feeding. Fatty livers are considered to be of higher quality than less fatty livers. In cod, saithe and haddock caught in the northern parts of Norway the liver was found to be of high quality trough out the year, except during the spawning period (RUBIN, 2002). dos Santos et al. (1993) reported that cod fed low lipid diets (prawns) stored considerably less lipid in liver, and therefore had higher moisture content, than cod fed high fat diets (herring). Cod

in the Barents Sea and the Icelandic Sea is reported to feed on capelin (Dolgov, 2002; Palsson, 1983), while it is not a part of the diet of cod in southern regions, such as the south coast of Ireland (Du Buit, 1995). This may explain why the amount of dry matter in liver was lower for samples from the south coast of Ireland compared to the two other fishing grounds.

Lower amount of dry matter in liver may also indicate starvation. During starvation of cod, hepatic lipids is the first reserve to be used (Black & Love, 1986) giving a lower amount of dry matter.

Cod liver from wild fish has been reported to have a dry matter content of 60 ± 12% (Rustad et al., 1991), this is close to the values found in this study.

The amount of water-soluble protein in liver was higher than in viscera, and in the same range as in cut off, 2.1 ± 0.07. It did not vary according to species, season or fishing ground.

Table 2.10: Free amino acids (g/ 100g wet weight) in liver from fish of the cod species caught in the Icelandic Sea, the Barents Sea and at the south coast of Ireland in three seasons. Values for Atlantic cod are mean values ± standard error of the mean. N = pooled samples of 15 fishes.

Icelandic Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	3	7.34 ± 1.01	3	6.18 ± 0.267	3	8.41 ± 1.8
Saithe	1	3.22	1	5.14	1	2.65
Haddock	1	8.01	1	12.03	1	6.14
Tusk	1	1.83	1	2.05	1	3.14
Ling	1	2.53	1	2.90	1	3.76
Barents Sea						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	8	3.89 ± 0.43	3	4.28 ± 0.61	3	5.87 ± 1.67
Saithe	1	2.05	1	3.50	1	2.26
Haddock	1	0.74	1	4.98	1	3.73
Tusk			1	4.61		
South coast of Ireland						
Species	**N**	**Feb.- March**	**N**	**April- June**	**N**	**Oct.- Dec.**
Atlantic cod	2	9.76 ± 0.82	1	13.27	3	16.30 ± 3.69
Saithe	1	12.82			1	3.91
Haddock			1	15.32	1	8.86
Ling	1	5.82	1		1	5.63

Atlantic cod and haddock from the Icelandic Sea had higher values of free amino acids compared to the other species in all three seasons. Samples from the

Barents Sea had lower amounts of free amino acids compared to samples from the two other fishing grounds in all three seasons.

The values for total protein in liver are not presented here. Due to problems during freeze drying (the step before the C/N analysis) and problems in weighing out homogenous amounts for the analysis, the results were rejected. Analysing the total protein in liver using the Kjeldahl-method would possibly have given better results. Results from Iceland on the liver samples from the fish caught in the Icelandic Sea indicate that the total protein content in liver varied between 4 and 8 % (Thorarinsdottir et al., 2004).

Figure 2.4 and 2.5 show the relationship between water-soluble protein and dry matter, and water-soluble protein and free amino acids in liver samples from fish of the cod species. In liver samples there was a significant ($p < 0.05$) negative relationship between free amino acids and pH, and water-soluble protein and dry matter. A significant positive relationship was found between water-soluble protein and free amino acids.

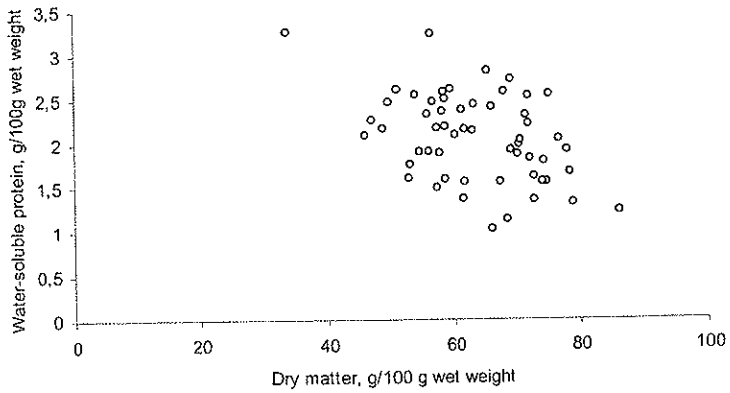

Figure 2.4: Relationship between amount of dry matter (g/100g wet weight) and water-soluble protein (g/100g wet weight) in liver from cod species (Pearson correlation coefficient = -0.44).

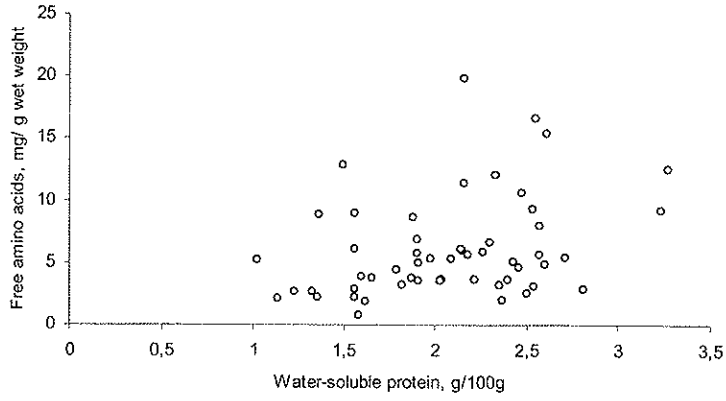

Figure 2.5: Relationship between amount of water-soluble protein (g/100g wet weight) and free amino acids (mg/g wet weight) in liver from cod species (Pearson correlation coefficient = 0.33).

2.2.4 Total amino acid composition and protein efficiency ratio (PER) in cut off and viscera

Thirteen samples of cut off and 8 samples of viscera were digested with 6M hydrochloric acid at 105°C for 22 hours (Blackburn, 1968), followed by neutralisation of the hydrolysates. Total amino acid composition was analysed using HPLC. Figure 2.6 shows the total amino acid composition of cut off and viscera, compared to egg and wheat protein. Table 2.11 shows the protein efficiency ratio of cut off and viscera compared to the protein efficiency ratio found by Shahidi et al., for Atlantic cod offal (head, viscera, liver and skeletal frame) and muscle. PER is defined as the weight in grams gained per gram protein consumed (Damodaran, 1996). The time and expense required for measuring PER directly in rats makes it impractical for quality control and regulatory control of food products (Alsmeyer et al., 1974), therefore a number of equations have been worked out for this purpose. Liver is not likely to be utilised for its proteins but rather for the content of nutritionally important lipids, and has therefore not been included in this analysis.

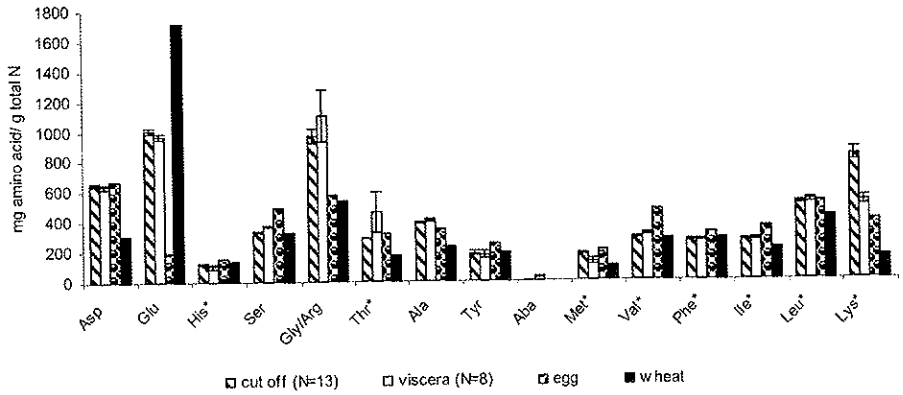

Figure 2.6: Amino acid composition (mg aa/g total N) of protein in cut off and viscera compared to egg and wheat protein (aa* = essential amino acid). Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes. (Amino acid composition of egg and beef protein are from Garrow & James, 1996).

Table 2.11: Calculated protein efficiency ratio (PER) of cut off and viscera from fish of the cod species compared to PER of Atlantic cod offal (head, viscera, liver and skeletal frame) and muscle.

	Cut off	Viscera	Cod offal[d]	Cod muscle[d]
PER[a]	2.91 ± 0.24	3.03 ± 0.3	2.09	2.87
PER[b]	3.35 ± 0.73	3.29 ± 1.22	2.36	3.24
PER[c]	3.25 ± 0.17	3.09 ± 0.54	2.31	2.99

[a] PER = - 0.468 + 0.45[LEU] – 0.105[TYR]
Equation according to Alsmeyer et al., 1974.
[b] PER = - 1.816 + 0.435[MET] + 0.780[LEU] + 0.211[HIS] – 0.944[TYR]
Equation according to Alsmeyer et al., 1974.
[c] PER = 0.08084 [$\sum AA_7$] – 0.1094, where $\sum AA_7$ = threonine + valine + methionine + isoleucine + leucine + phenylalanine + lysine
Equation according to Lee et al., 1978.
[c] Values from Shahidi et al., 1991.

Figure 2.6 shows that the fish proteins had somewhat lower content of the essential amino acids histidine, methionine, valine, phenylalanine and isoleucine compared to egg protein. The fish proteins, however, had a higher content of the essential amino acid lysine compared to egg protein. The fish protein had lower content of the essential amino acid histidine compared to wheat protein, the amount

of phenylalanine was in the same range, while threonine, methionine, valine, isoleucine, leucine and lysine were higher in the fish protein. The PER of the by-poducts are somewhat higher than both cod offal and cod muscle. This makes viscera and cut off a well suited raw material for production of protein based foods and food ingredients.

3. EXPERIMENTAL ASPECTS

A general description of materials and methods are given in the articles. This section will provide a short background for the conditions chosen in the respective enzymatic assays.

3.1. Peptidase activity

The fluorogenic substrates used to measure the different peptidase activities are listed in Table 3.1.

Table 3.1: Fluorogenic substrates used in this work.

Peptidase activity	Substrate	Sigma nr
Trypsin	N-t-BOC-Gln-Ala-Arg-AMC	B 4153
Chymotrypsin	N- Suc-Leu-Leu-Val-Tyr-AMC	S 6510
Elastase	N-methoxy-Suc-Ala-Ala-Pro-Val-AMC	M 9771
Collagenase	Suc-Gly-Pro-Leu-Gly-Pro-AMC	*
Cathepsin B/L	CBz-Arg-Arg-AMC	C 5429

* The substrate is purchased from Bachem

These substrates consist of a polypeptide chain of 2 to 5 amino acids coupled to 7-amido-4-methylcoumarin. When the peptide chain is hydrolysed adjacent to the amide group, the amide is converted to an amine, an aromatic substance which is able to fluoresce when excited with light of about 360 nm and emits light of 460 nm. The fluorescence is measured by a fluorescence spectrophotometer and gives a quantitative picture of the activities. The increase in fluorescence per minute is used

as a measure of activity (U). These arbitrary units are best suited for internal comparison.

Peptidase activity was tested in different buffers at pH 7. pH 7 was chosen because it is close to the natural pH of the by-products. Trypsin and chymotrypsin activity in viscera was tested in 0.1M Bis Tris buffer at different $CaCl_2$ concentrations, 50mM and 100mM. At higher $CaCl_2$ concentrations the liquid turned opaque with the addition of stopping solution, 1 % SDS in 50mM Bis Tris buffer, pH 7. Cathepsin B activity in cut off, liver and viscera was tested in 0.3M phosphate citric acid buffer with 2mM, 10mM and 20mM EDTA. A concentration of 2mM was chosen for further analysis. Collagenase activity was tested in 0.1M Bis Tris buffer with 50mM $CaCl_2$, 100mM $CaCl_2$ and 50mM $CaCl_2$ and 50mM NaCl. 0.1M Bis Tris with 50mM $CaCl_2$ was chosen for further analysis of collagenase activity.

The relationship between time and increase in fluorescence for the different substrates was tested, and was linear up to 30 minutes at substrate concentrations of 0.0625mM.

3.2. Lipase activity

The samples were screened for lipase activity using a fluorescent method described by Roberts (1985) and later by Izquierdo and Henderson (1998) with minor modifications. The principle of the method is making a non-fluorescent substrate, that releases a highly fluorescent compound upon hydrolysis. The non-fluorescent substrate, 4-methylumbelliferyl heptanoate (4-MUH) was solubilised in a liposomal dispersion of soya lecithin. This substrate is hydrolysed to heptanoic acid and the highly fluorescent compound, 4-methyllumbelliferone (4-MU) through the action of lipases. The increase in fluorescence per minute was used as a measure of activity (U). These arbitrary units are best suited for internal comparison.

$$\text{4MUH (non-fluorescent)} \xrightarrow[H_2O]{\text{lipase}} \text{heptanoic acid + 4-MU (fluorescent)}$$

Several digestive lipases catalyse the hydrolysis of the fatty acid ester bond of the non-fluorescent compound, 4-MUH, to yield one molecule of the highly

fluorescent compound, 4-MU, and one molecule of heptanoic acid. Each molecule of 4-MU produced therefore reflects the hydrolysis of one molecule of fatty acid by the lipase. The measurement of the lipase activity is based on detection of the fluoresce of the 4-MU produced by lipases. Although the assay may be conducted at pH 1-9, it is expected to be more sensitive and specific for acid lipase (Roberts, 1985).

The effect of buffers on the lipase measurement was tested, and phosphate citric acid buffer was chosen for further analysis for both pH 5 and 7. Six mM taurocholic acid (Sigma T-4009) and 0.5 μg/ml co-lipase (Sigma 3028) was added to the buffers and activity measured at both pH 5 and 7 in some of the samples. Taurocholic acid activates the bile salt dependent lipase, while the co-lipase counteracts the inhibitory effect of the taurocholic acid on the pancreatic lipase in the system.

The linearity of the lipase assay according to time was also checked and a linear relationship between the increase in fluorescence and time up to 15 min was found.

The lipase assay was also tested on a commercial lipase from *Candida rugosa* (Sigma L 8525) and one unit commercial lipase corresponded to an increase of 18.7 in fluorescence. A colorimetric method using olive oil emulsion as substrate and copper (II) acetate for determination of free fatty acids after lipolysis was tried out. This method was, however, rejected because it was too insensitive for the purpose of this study. Using the commercial lipase from *Candida rugosa* the fluorimetric method was approximately 1000 times more sensitive in determining lipase activity compared to the colorimetric method.

3.3 Evaluation of the enzymatic analysis

The general proteolytic activity was analysed using 2% haemoglobin as substrate at three different pH; 3, 5 and 7. The activity measured at pH 3 in viscera samples is mostly due to pepsin from the acid environment of the stomach, while the activity measured at pH 7 is most likely due to the alkaline peptidases of the intestines. In cut off and liver the activity measured at pH 3 is most likely due to the acid cathepsins (D and E), activities at pH 5 due to other cathepsins (eg. B, L and H),

the activity at pH 7 may also be due to cathepsins, calpains, collagenase and alkaline proteases.

Digestion of haemoglobin at low pHs (2-3) is the most common way of analysing pepsin activity (Brewer et al., 1984; Arunchalam & Haard, 1985; Squires et al., 1986a & b; Gildberg, 1988; Batista & Pires, 2002). General proteolytic activity, non-specific and/or total proteolytic activity is mostly analysed using haemoglobin (Anson, 1938; Stoknes et al., 1993; Chakrabarti et al., 1995) or casein (Chong et al., 2002; Castillo-Yanez, et al., 2004; Lundstedt et al., 2004) as substrates. Since casein will denature at acidic pHs, haemoglobin was chosen as substrate in this work.

For measuring peptidase activity there is a vast number of substrates available (based on flurometric, colorimetric and titrimetric methods). Sigma lists 14 substrates for assaying trypsin, 13 for chymotrypsin, 6 for elastase, 8 for cathepsins B and 8 for collagenase. Substrates containing a peptide chain coupled to *p*-nitroanilide appears to be the most commonly used substrate for assaying trypsin (Simpson & Haard, 1984a & b; Genicot et al., 1988; Asgeirsson et al., 1989; Batista & Pires, 2002), chymotrypsin (Raae, 1990; Asgeirsson & Bjarnason, 1991; Raae et al., 1995; Batista & Pires, 2002) and elastase (Raae & Walther, 1989; Gildberg & Overbo, 1990; Asgeirsson & Bjarnason, 1993; Bassompierre et al., 1993). Also substrates based on methyl/ ethyl esters are commonly used for trypsin (Overnell, 1973; Simpson & Haard, 1984a & b; Heu et al., 1995) and chymotrypsin (Overnell, 1973; Asgeirsson et al., 1989; Asgeirsson & Bjarnason, 1991; Heu et al., 1995).

For cathepsins B the by far most used substrate today is Arg-Arg-AMC (Yamashita & Konagaya, 1989a & b; An et al., 1994, Aoki et al., 1995; 2000; 2002; Porter et al., 1996; Aranishi et al., 1997). Barret & Kirschke (1981) stated that the most sensitive, safe and convenient leaving group for substrates of the cysteine proteinases so far discovered is 7-amino-4-methylcoumarin, and that the Arg-Arg-AMC is an almost ideal substrate for cathepsins B. The most commonly used substrate for assaying collagenase is its natural substrate collagen (Monfort & Perez-Tamayo, 1975; Hori & Nagai, 1979; Kim et al., 2002; Park et al., 2002). 2,4-dinitrophenyl derivates with an octapeptide based on the amino acid sequence around the cleavage site have been found to have a high degree of specificity, but the

sensitivity was not equal to that achieved using collagen as substrate (Harris & Vater, 1982). 2,4-dinitrophenyl derivates have, however, been used in some studies (Hori & Nagai, 1979; Yoshinaka et al., 1987). Other synthetic substrates used for assaying collagenolytic activity are *p*-phenylazobenzyloxycarbonyl derivates (Teruel & Simpson, 1995; Hernandez-Herrero et al., 2003) and AMC derivates (Stoknes & Rustad, 1995a & b; Hultmann & Rustad, 2002). In this work the AMC substrates were chosen for all the peptidases, because of their sensitivity and ease of use.

Several methods for assaying lipase activity are reported in the literature, eg. radioactive methods using ^{14}C-labeled substrates (Albro & Latimer, 1974; Albro et al., 1985; Ozkizilcik et al., 1996), titrimetric methods (Borlongan, 1990; Gargouri et al., 1995; Nayak et al., 2003), methods based on chromatographic (thin layer and gas-liquid) techniques to separate and measure the products of hydrolysis (Albro & Latimer, 1974; Patton et al., 1977; Lie & Lambertsen, 1985), colorimetric methods based on *p*-nitrophenyl derivates (Albro et al., 1985; Gjellesvik et al., 1992) and fluorimetric metods based on 4-methylumbelliferyl esters (Jacks & Kircher, 1967; Dooijewaard-Kloosterziel & Wouters, 1976; Izquierdo & Henderson, 1998; Hoelne-Reitan et al., 2003). Several reviews on lipase detection and assays have been published in recent years (Thomson et al., 1999; Beisson et al., 2000; Hendrickson, 1994). The method based on 4-methylumbelliferyl heptanoat was chosen in this work because it is sensitive and makes it possible to also evaluate low lipase activities, as is the case in the cut off fractions.

Since some of the enzymatic activities in this study is very high it might have been better to use less sensitive substrates in some of the assays. Less sensitive substrates might have given less standard deviations and results that were easier to interpret. However, also other researchers have reported high deviations in activities of digestive enzymes in cod (Gildberg, 2004b). The concentration of certain digestive enzymes may be highly seasonal, gender and age specific, and can vary tremendously within a species as well as between species (Kristinsson & Rasco, 2000).

3.4 Statistical treatment

Data obtained in this study was analysed using MINITAB and Unscrambler. The distribution of the data was initially checked in the Andersson-Darling Normality test. However, since the number of samples in this study is small it is difficult to interpret whether the data is normally distributed or not, this resulted in the use of both non-parametric (Kruskal-Wallis test) and parametric (One Way ANOVA) tests. In paper I the results from the non-parametric test are presented, while in paper II- V results from the parametric test are presented. All data have been tested with both the non-parametric and the parametric test, and the main conclusions hold for both the tests. Pearson correlation test was used to determine the extent to which the variables were correlated. The significance level was set at 95 % ($p < 0.05$) for One Way ANOVA and Pearson correlation test, and 90% ($p < 0.10$) for the Kruskal-Wallis test in Paper I. Specific activities, U/mg water-soluble protein, were used when comparing different species, seasons and fishing grounds and are presented in the papers. In the following total activities, U/g wet weight, will be presented in the figures.

An effect of species was found and since all species were not available from all fishing grounds and seasons, samples from Atlantic cod only have been used to study effects of fishing grounds and seasons in the following.

PCA analysis was preformed in Unscrambler on activities expressed as U/g wet weight. The samples were centered, weighed by multiplying with (1/standard deviation), and cross validated. The bi-plots of principal component one and two are presented.

4. RESULTS AND DISCUSSION

In the following the effect of species, season and fishing ground on the proteolytic and lipolytic activities in viscera, cut off and liver samples are summarised, for more details see the respective papers.

4.1. Effect of species

In Paper I some of the species were pooled because of the small number of samples. Atlantic cod and saithe were treated as separate groups, while haddock, tusk and ling were treated as one group. Since tusk and ling belong to the lotidae family it would probably have been wiser to pool these samples together, keeping the haddock samples out. In the following discussion all species have been treated separately.

4.1.1. Viscera

Figure 4.1, 4.2 and 4.3 shows the mean maximum general proteolytic activity at pH 5 and 7, mean maximum activity of trypsin, chymotrypsin, elastase, collagenase and cathepsin B pr g wet weight viscera from the five species.

A significant effect ($p < 0.05$) of species was found on the general proteolytic activity at pH 5 and 7, but not at pH 3 (Paper I). The activity measured at pH 3 is most likely caused by pepsin in the stomach, while the activity at pH 7 is due to the alkaline proteases of the intestines. Pepsin and different cathepsins may contribute to the activity measured at pH 5 in viscera. Munilla-Moran and Saborido-Rey (1996) also found the enzymatic profile of fish intestine to be more species specific than of the stomach. Also other researchers have reported differences in proteolytic activities between species (Khalil et al., 1987; Hidalgo et al, 1999). Our findings for general proteolytic activity at pH 7 were further supported by the results of trypsin and chymotrypsin, which also differed significantly ($p < 0.05$) between the five species (Paper II). Generally, at pH 7 viscera from Atlantic cod, saithe and haddock showed higher activity than tusk and ling. Atlantic cod, saithe and haddock are members of the gadidae family, while tusk and ling are members of the lotidae family. Tusk and ling also differ from the other species in that they can live at depths down to 1000m (www.fishbase.org). Both adaptability to deeper water, and also genetical differences between the two families may explain the differences in enzymatic activities between the species seen in this study.

No significant differences in specific activity (U/mg water-soluble protein) of elastase (Paper V), cathepsin B and collagenase (Paper IV) in viscera were found between species. However, figure 4.2 and 4.3 clearly show that also the mean

maximum total activity of elastase, cathepsin B and collagenase (U/ g wet weight) were lower in tusk and ling compared to the other species.

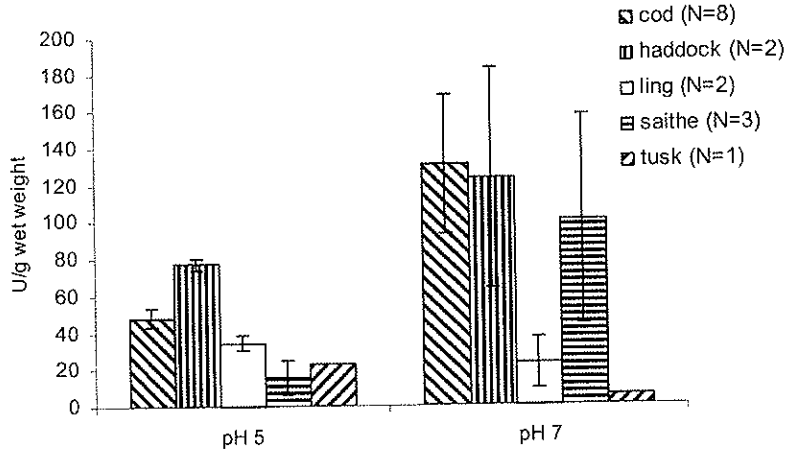

Figure 4.1: Mean maximum general proteolytic activity measured at pH 5 (50°C) and pH 7 (50°C) in viscera from Atlantic cod, haddock, ling, saithe and tusk, expressed as U/g wet weight sample (U = mg haemoglobin cut per hour). Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

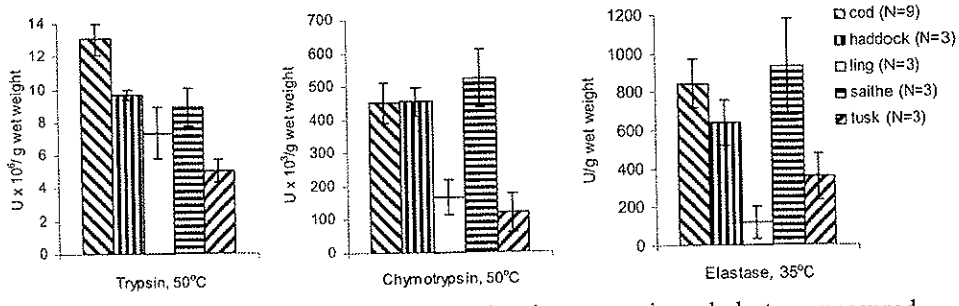

Figure 4.2: Mean maximum activity of trypsin, chymotrypsin and elastase measured at pH 7 in viscera from Atlantic cod, haddock, ling, saithe and tusk, expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

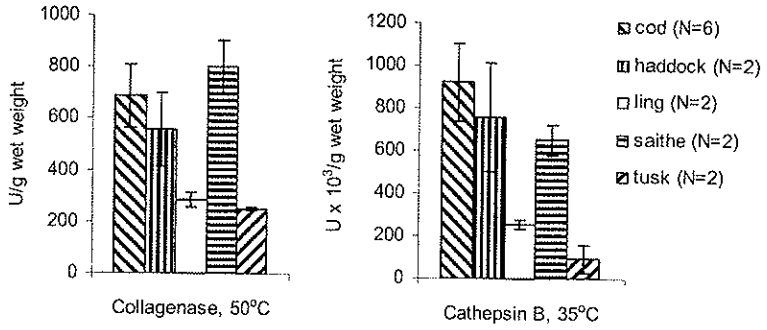

Figure 4.3: Mean maximum activity of collagenase and cathepsin B measured at pH 7 in viscera from Atlantic cod, haddock, ling, saithe and tusk, expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

Figure 4.4 shows a PCA plot of viscera samples from the Icelandic Sea, also demonstrating the differences between the species. The bi-plot shows that viscera samples from tusk and ling had lower activity of trypsin, chymotrypsin and elastase, lower amounts of free amino acids and higher amounts of water-soluble protein, compared to viscera from the other species. It also shows that activity of trypsin and amount of free amino acids were closely and positively correlated (see chapter 4.3).

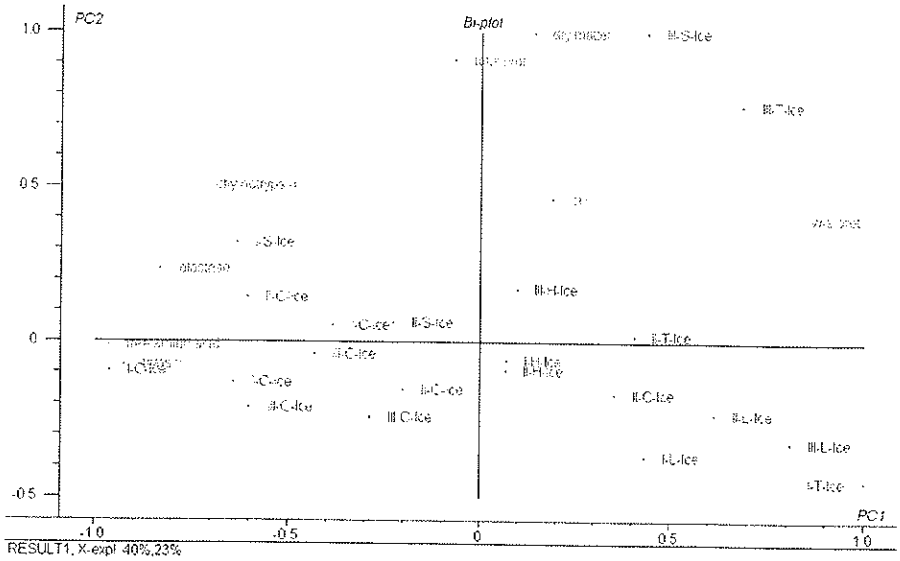

Figure 4.4: PCA-bi-plot of viscera samples from cod species caught in the Icelandic Sea in three different seasons presenting trypsin (50°C), chymotrypsin (50°C) and elastase (35°C) activity (U/ g wet weight), and some background characteristics. I, II, III- seasons, C- Atlantic cod, S- saithe, H- haddock, T tusk, L- ling, Ice- Icelandic Sea.

4.1.2. Cut off

Figure 4.5 and 4.6 shows the mean maximum general proteolytic activity, mean maximum activity of collagenase and cathepsin B pr g wet weight cut off from the five species.

A significant ($p < 0.05$) effect of species was found on general proteolytic activity (U/ mg water-soluble protein) at pH 5 and pH 7 in the cut off fraction. Also in cut off the activity was highest in Atlantic cod, saithe and haddock (Paper I). From Figure 4.5 it is clear that this also holds for the activity at pH 3 when the activity is expressed as U/ g wet weight. The general proteolytic activity at pH 3 in both cut off and liver is most likely due to acid cathepsins, mainly cathepsin D, but also cathepsin E may contribute (Gildberg, 1988). The cathepsin B activity was significantly ($p < 0.05$) higher in cut off samples from haddock, while collagenase activity was highest in ling (Paper IV). Since samples from different seasons are pooled when comparing the different species, it is possible that differences in spawning between the species contribute to the differences in enzymatic activities found in this study. A higher activity of cathepsins in muscle of spawning fish has been reported (Yamashita & Konagaya, 1990a; GomezGuillen & Batista, 1997), and it is possible that the intensity of spawning or the number of spawning fish in the haddock groups were higher compared to the other species. No observations or explanations for elevated activities of collagenase in fish tissue, as seen in the ling samples, have been found reported. Ontogeny, diet, fasting, exercise, body temperature and pressure are other factors influencing the amount of enzymes in muscle tissue (Haard, 1992).

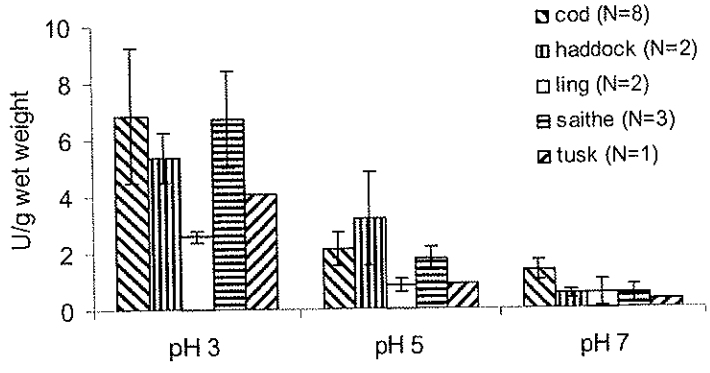

Figure 4.5: Mean maximum general proteolytic activity measured at pH 3 (35°C), pH 5 (50°C) and pH 7 (65°C) in cut off from Atlantic cod, haddock, ling, saithe and

tusk, expressed as U/g wet weight sample (U = mg haemoglobin cut per hour). Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

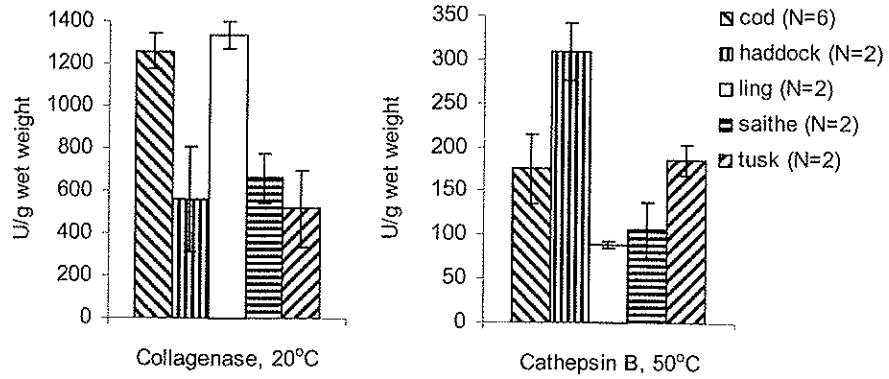

Figure 4.6: Mean maximum activity of collagenase and cathepsin B measured at pH 7 in cut off from Atlantic cod, haddock, ling, saithe and tusk, expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

4.1.3. Liver

Figure 4.7 and 4.8 shows the mean maximum general proteolytic activity, and mean maximum activity of collagenase and cathepsin B pr g wet weight liver from the five species.

Only a minor effect of species was found on general proteolytic activity in liver (Paper I). Cathepsin B activity was significantly ($p < 0.05$) higher in liver samples from haddock, while tusk and ling again showed the lowest activity (Paper IV). A higher enzymatic activity in liver just before, and during spawning, is likely. Eliassen and Vahl (1982b) suggested that most of the energy needed for gonad development in cod was derived from liver, leading to a reduction in liver size prior to spawning. Collagenase activity was highest in ling (Paper IV).

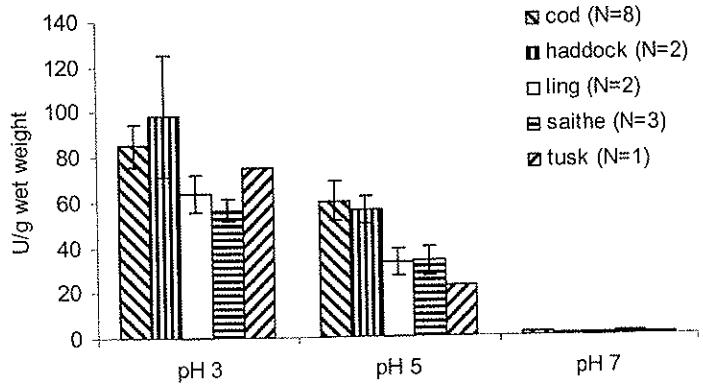

Figure 4.7: Mean maximum general proteolytic activity measured at pH 3 (50°C), pH 5 (50°C) and pH 7 (50°C) in liver from Atlantic cod, haddock, ling, saithe and tusk, expressed as U/g wet weight sample (U = mg haemoglobin cut per hour). Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

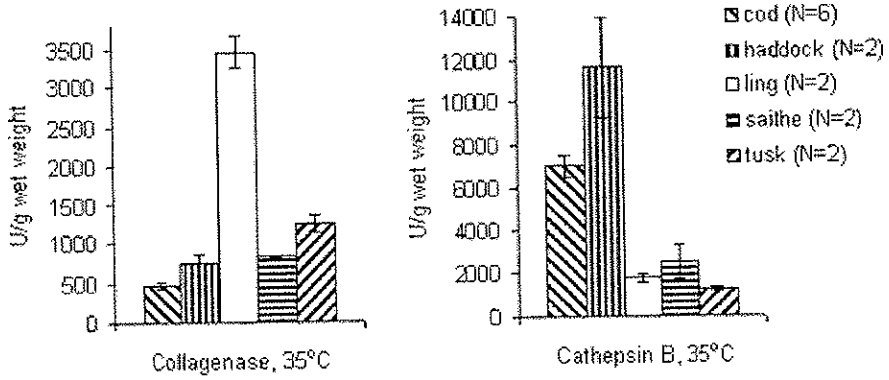

Figure 4.8: Mean maximum activity of collagenase and cathepsin B measured at pH 7 in liver from Atlantic cod, haddock, ling, saithe and tusk, expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

Figure 4.9 shows a PCA plot of liver samples from the Icelandic Sea (Feb.-March and April- June), also demonstrating the differences between the species. The bi-plot shows that liver samples from ling have higher activity of collagenase, while haddock and cod (Feb.-March) have higher activity of cathepsins B. It also shows that activity of cathepsins B and amount of free amino acids were closely and positively correlated (see chapter 4.3).

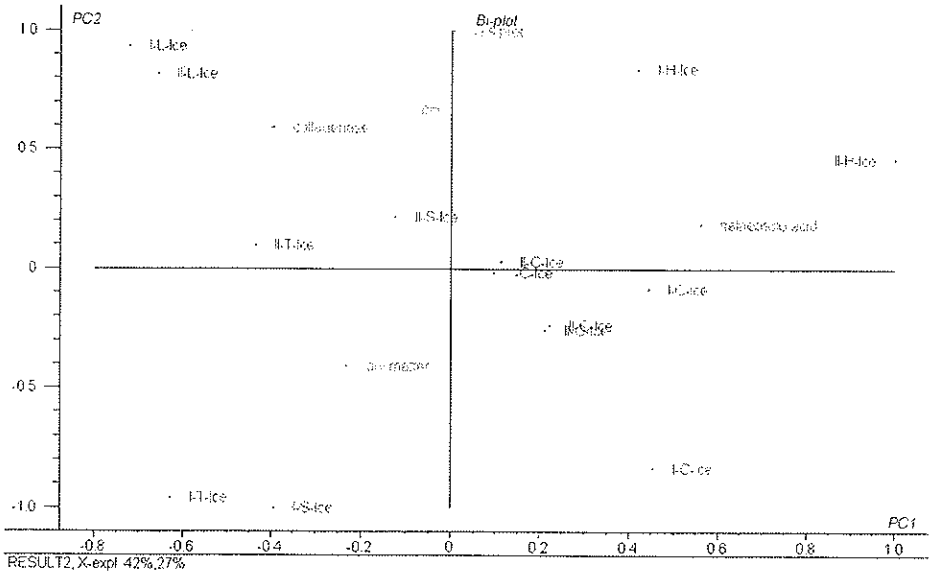

Figure 4.9: PCA-bi-plot of liver samples from cod species caught in the Icelandic Sea in three different seasons presenting cathepsins B (35°C) and collagenase (35°C) activity (U/g wet weight), and some background characteristics. The label for cathepsins B and free amino acids is on top of each other, at the left side of the plot. I, II, III- seasons, C- Atlantic cod, S- saithe, H- haddock, T tusk, L- ling, Ice- Icelandic Sea.

4.2. Effect of fishing ground and season

4.2.1. Viscera

Figure 4.10, 4.11, 4.12 and 4.13 shows the mean maximum general proteolytic activity, mean maximum activity of trypsin, chymotrypsin, elastase, collagenase, cathepsin B and lipase pr g wet weight viscera from Atlantic cod caught in the different fishing grounds in different seasons.

A minor effect of fishing ground was seen on general proteolytic activity at pH 3 and pH 7 in Feb.-March. Samples from the south coast of Ireland had the highest proteolytic activity at pH 3, while samples from the Icelandic Sea had the highest proteolytic activity at pH 7 (Paper I). Viscera from Atlantic cod caught in the Icelandic Sea in Feb.-March also had a significantly ($p < 0.05$) higher activity of trypsin (Paper II) and elastase (Paper V) compared to viscera from Atlantic cod caught in the two other fishing grounds, and activity of cathepsin B and collagenase compared to viscera samples from the south coast of Ireland (Paper IV). Also the lipase activity at pH 7 was significantly higher in viscera samples from the Icelandic

Sea compared to the Barents Sea in Feb.-March (Paper III). The viscera samples from the Barents Sea have been found to have lower amount of lipids (2.1 %) compared to samples from the Icelandic Sea (4.8 %) (Falch et al., 2003).

An effect of fishing ground was also seen on the activity of trypsin, chymotrypsin (Paper II) and elastase (Paper V) in viscera from Atlantic cod caught in the Barents Sea and the Icelandic Sea in April-June, and in the Icelandic Sea and south coast of Ireland in Oct.-Dec. The activity was generally higher in viscera samples from Atlantic cod caught in the Icelandic Sea compared to the other fishing grounds.

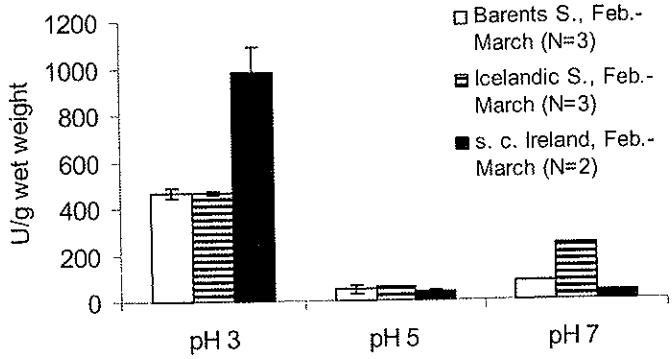

Figure 4.10: Mean maximum general proteolytic activity measured at pH 3 (35°C), pH 5 (50°C) and pH 7 (50°C) in viscera from Atlantic cod caught in the Barents Sea, Icelandic Sea and south coast of Ireland in Feb.-March, expressed as U/g wet weight sample (U = mg haemoglobin cut per hour). Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

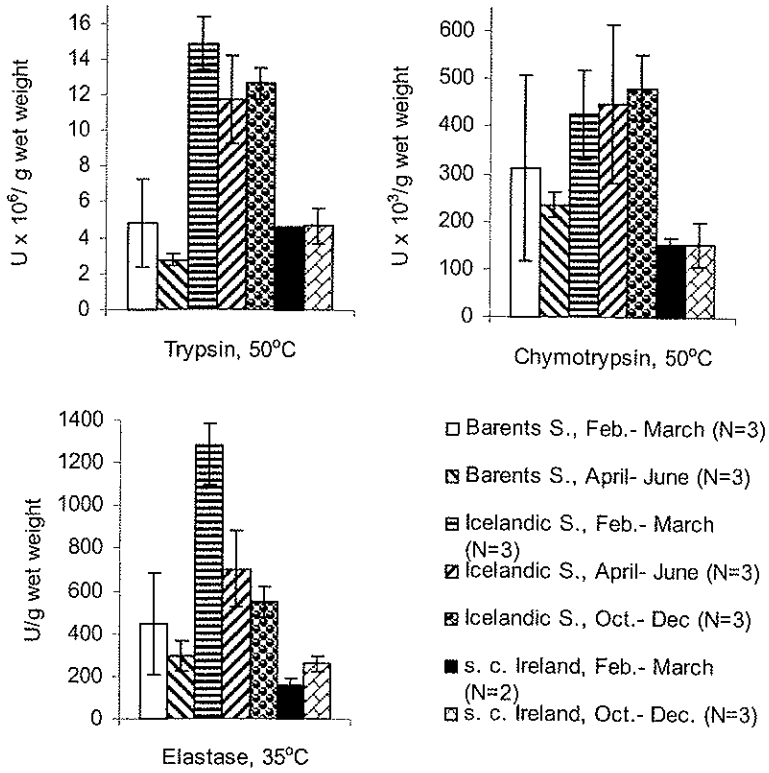

Figure 4.11: Mean maximum activity of trypsin, chymotrypsin and elastase measured at pH 7 in viscera from Atlantic cod caught in the Barents Sea (Feb.-March, April-June), Icelandic Sea (Feb.-March, April-June, Oct.-Dec.) and south coast of Ireland (Feb.-March, Oct.-Dec.), expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

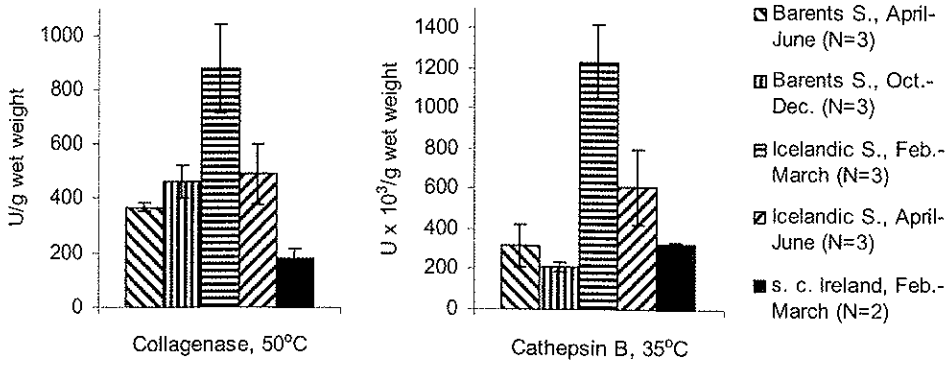

Figure 4.12: Mean maximum activity of collagenase and cathepsin B measured at pH 7 in viscera from Atlantic cod caught in the Barents Sea (April-June, Oct.-Dec.), Icelandic Sea (Feb.-March, April-June) and south coast of Ireland (Feb.-March), expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

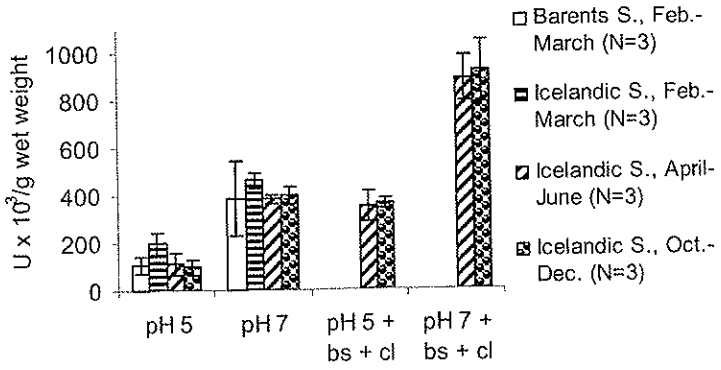

Figure 4.13: Mean maximum activity of lipase at pH 5 (20°C), pH 7 (35°C), pH 5 with addition of bile salt and co-lipase (35°C) and pH 7 with addition of bile salt and co-lipase (35°C) in viscera from Atlantic cod caught in the Barents Sea (Feb.-March) and the Icelandic Sea (Feb.-March, April-June, Oct.-Dec.), expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

Season affects viscera from Atlantic cod caught in the Icelandic Sea, Barents Sea and south coast of Ireland differently. Seasonal differences in enzymatic activities were more pronounced in viscera from Atlantic cod caught in the Icelandic Sea compared to Atlantic cod caught in the Barents Sea and south coast of Ireland. For all enzymes studied, viscera samples from the Icelandic Sea, April-June had lower specific activity compared to samples from other seasons (Paper II, III, IV and V). Viscera samples from Atlantic cod caught in April-June also had lower lipid content (2.7 %), than samples from the two other seasons (4.8 % and 3.5 %)(Falch et al., 2003).

Viscera samples from the Barents Sea also showed lower activity of trypsin, chymotrypsin and elastase in April-June compared to Feb.-March, the differences were, however, not significant (Paper II & V). Samples from Feb.-March had higher activity of cathepsin B and collagenase compared to the samples from Oct.-Dec (Paper IV).

Samples from the south coast of Ireland showed significantly lower elastase activity in Feb.-March compared to Oct.-Dec.

Water temperature, mean depth and activity of the fish, spawning and feeding are factors likely to differ between season and fishing ground, these factors are also

likely to influence enzymatic activities in fish. The samples in our study are from wild fish, and data on feeding status of the fish was not available. However, pepsin has been found to be unresponsive to protein level in the diet in several fish (Reimer, 1982, Lundstedt et al., 2004). Also chymotrypsin and trypsin has been found to change little with protein changes in the diet (Lundstedt, 2004). Several studies have, however, concluded that enzymatic activities in fish intestines and gut are affected by feed (Hofer, 1979; Fernandez et al., 2001; Garcia-Carreno et al., 2002), and studies of stomach content of cod and haddock have shown that type of feed differed significantly between both season and area (Du Buit, 1995; Adlerstein et al., 2002). Genetic differences are also important factors influencing enzymatic activities. Fish caught in the same fishing ground at different seasons may belong to different genetic subfamilies due to the vertical migration of cod, and thereby offer an explanation for the differences in enzymatic activities between both season and fishing grounds. Temperatures experienced by individual fish before catch is difficult to predict. Seasonal differences in mean depth and ambient temperature has been observed in tagged cod living in the Barents Sea. As a result of horizontal and vertical migrations, the cod experienced higher temperatures in winter and spring than in summer (Godø & Michalsen, 2000). Increased concentration of enzymes during cold acclimation has been reported and proposed as the most important response to seasonal variations in temperatures (Hazel & Prosser, 1974; Hochachka & Somero, 1984; Ekberg, 1962). We found the specific activity of all enzymes to be lower during spring/summer in cod from the Icelandic Sea. No tagging studies of cod from the Icelandic Sea have been found, but if the result from the Barents Sea is representative also for cod from the Icelandic Sea, this is when the cod experiences the coldest temperatures. Some of these differences were, however, cancelled out when total activity was used to compare seasons. The samples from summer had the highest concentration of water-soluble protein indicating higher concentrations of other enzymes. Metabolic enzymes found to have higher activities during cold acclimation of fish, are among others, succinic dehydrogenase (Hazel, 1972), citrate synthase and phosphofructokinase (Guderley & Gawlicka, 1992) from muscle, aspartate aminotransferase, citrate synthase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase and pyruvate kinase in the visceral retia mirabilia (blood

vessels) (Fudge et al., 1997) and enolase, aldolase, triphosphate isomerase and lactate dehydrogenase in heart ventricle (Pierce & Crawford, 1997). Thermally induced changes in isozymes have also been described in different tissue in a number of animals (Poly, 1997), and may explain why the optimum temperature of elastase in viscera (Paper V) changed with season and lipase (pH 5 and pH 7) in cut off changed with season and fishing ground (Paper III).

Another possible explanation for the higher amount of water-soluble protein, and lower specific activity, in samples from April- June, is the feeding status of the fish. Cod fish are known to be feeding excessively during summer and it is likely that the fish stomachs and intestines contain more partially digested material at this time compared to other seasons. This may lead to an increase in amount of water-soluble protein and therefore a decrease in specific activities. The pancreatic tissue secretes most of the digestive enzymes, and therefore the relative proportion of pancreatic tissue in the viscera will be of major importance for enzymatic activities. Stomachs from cod, saithe and haddock from the northern part of Norway were found to have highest amounts of feed from May to August, somewhat less feed from September to mid-January, and low amounts of feed from mid-January till end of April (RUBIN, 2002).

Figure 4.14 and 4.15 show PCA plots of Atlantic cod viscera, also demonstrating the differences between the seasons and fishing grounds. Figure 4.14 shows that Atlantic cod viscera from the Icelandic Sea had higher activity of trypsin, chymotrypsin and elastase compared to samples from the two other fishing grounds. Figure 4.15 shows that viscera from the Icelandic Sea Feb.- March had high activity of cathepsins B and collagenase, and that the activity of cathepsins B and collagenase activity was positively correlated in viscera.

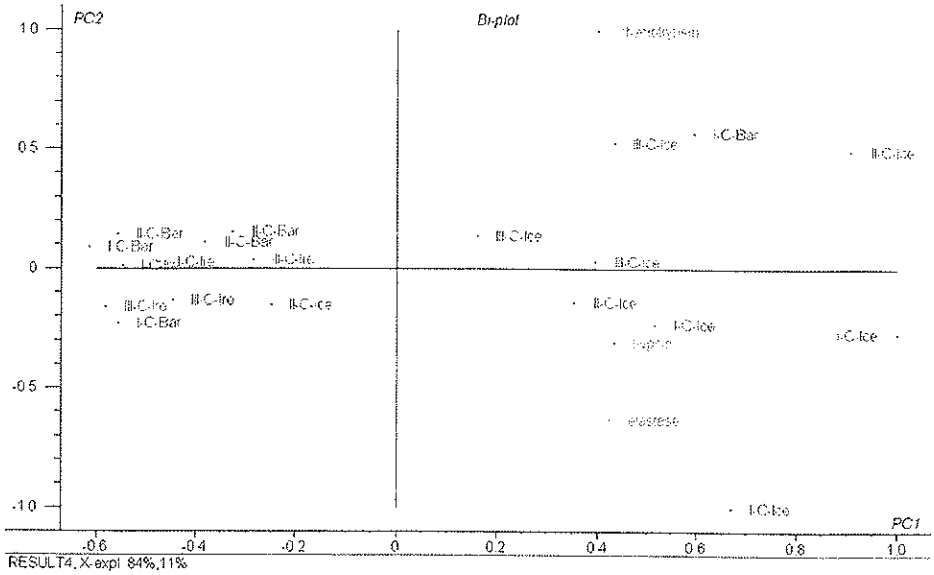

Figure 4.14: PCA-bi-plot of viscera samples from Atlantic cod caught in the Icelandic Sea, the Barents Sea and the south coast of Ireland in different seasons, presenting trypsin (50°C), chymotrypsin (50°C) and elastase (35°C) activity (U/ g wet weight). I, II, III- seasons, C- cod, Ice- Icelandic Sea, Bar- Barents Sea, Ire- south coast of Ireland.

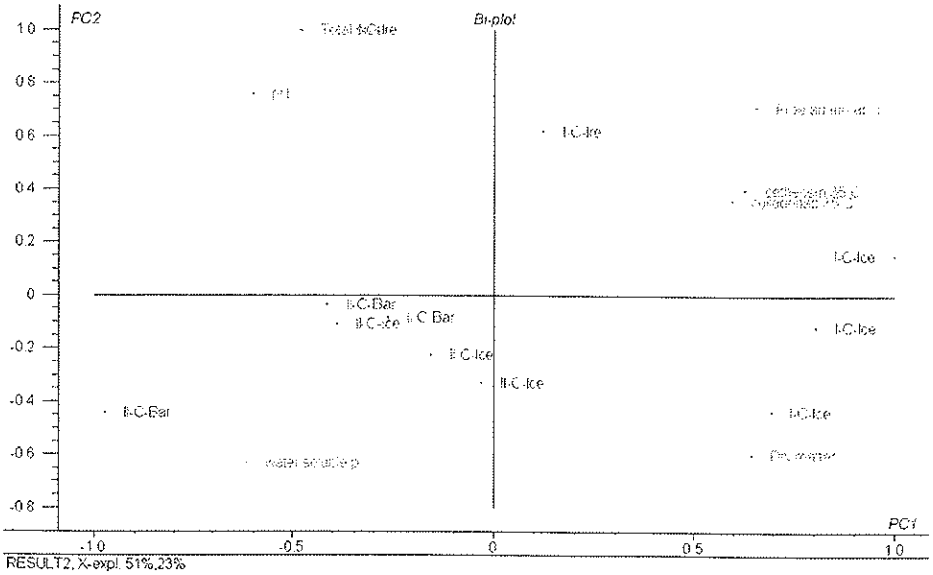

Figure 4.15: PCA-bi-plot of viscera samples from Atlantic cod caught in the Icelandic Sea the Barents Sea and the south coast of Ireland different seasons, presenting cathepsins B (35°C) and collagenase (35°C) activity (U/ g wet weight) and some background characteristics. I, II, III- seasons, C- cod, Ice- Icelandic Sea, Bar- Barents Sea, Ire- south coast of Ireland.

4.2.2. Cut off

Figure 4.16, 4.17 and 4.18 shows the mean maximum general proteolytic activity, mean maximum activity of collagenase, cathepsin B and lipase pr g wet weight cut off from cod caught in different fishing grounds in different seasons.

Cut off samples from the south coast of Ireland, Feb.-March, had significantly higher activity of cathepsin B and lower activity of collagenase compared to samples from the Icelandic Sea (Paper IV) (Table 4.3). The samples from Ireland also had higher general proteolytic activity at pH 3 indicating a high activity of cathepsin D and possibly E. The difference was, however, not significant. Yamashita and Konagaya (1990a) reported high activities of both cathepsins B and D, along with other cathepsins in muscle of chum salmon in spawning migration, while Gomez-Guillen and Batista (1997) have reported maximum cathepsin D-like activity during spawning in sardine. Whether cod stops feeding during spawning or eats intensively throughout the year does not appear to be clear, however, either way they do not absorb enough nutriments to satisfy the needs of maturation (Love, 1975). Guderley et al. (2003) reported cathepsin D activities to be twice as high in the muscle of starved cod compared to fed cod. Starvation is known to increase the proteolytic activity in several tissues such as liver, kidney, spleen, red and white muscle. White muscle experiences the largest increase, and in species like cod, muscle protein breakdown reaches a maximum 3-4 weeks into a fasting period (Navarro & Gutierrez, 1995). The activity of collagenase was lower in the samples from the south coast of Ireland, and this is in accordance with the fact that the connective tissue of fish is not depleted during starvation (Sikorski et al., 1984).

An effect of fishing ground on activity of cathepsin B and collagenase was also found in cut off samples from April- June. Samples from the Barents Sea had significantly ($p < 0.05$) higher activity compared to samples from the Icelandic Sea (Paper IV).

Cut off samples from the Barents Sea, Oct.- Dec. had higher activity of lipase at pH 5 and pH 7 compared to samples from the Icelandic Sea (Paper III). Factors such as swimming activity (Jonas & Bilinski, 1964) and fatty acid composition in the feed (Liang et al., 2002) may influence the lipase activity in fish tissue.

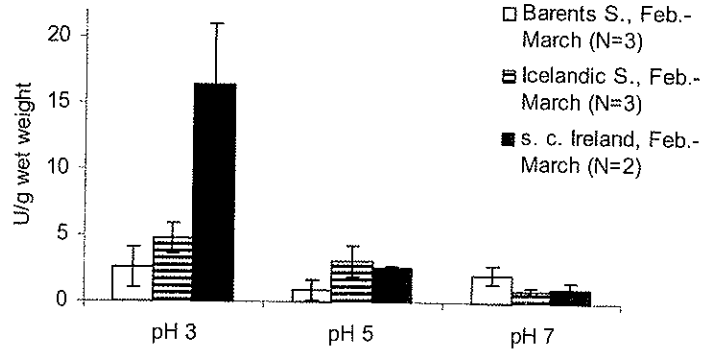

Figure 4.16: Mean maximum general proteolytic activity measured at pH 3 (35°C), pH 5 (50°C) and pH 7 (50°C) in cut off from Atlantic cod caught in the Barents Sea, Icelandic Sea and south coast of Ireland in Feb.-March, expressed as U/g wet weight sample (U = mg haemoglobin cut per hour). Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

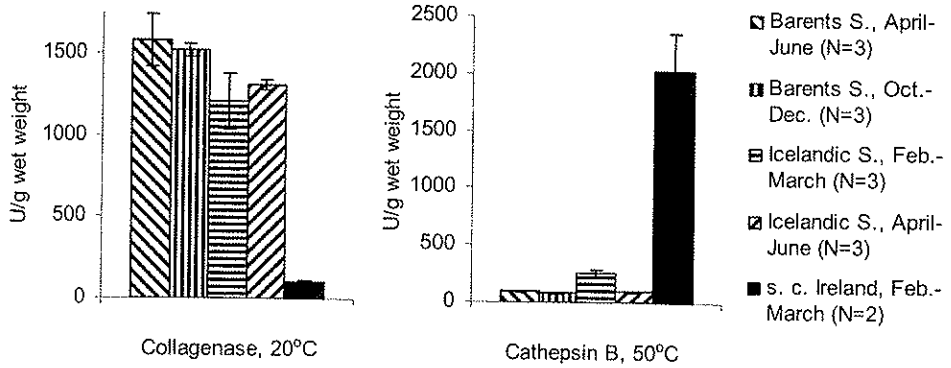

Figure 4.17: Mean maximum activity of collagenase and cathepsin B measured at pH 7 in cut off from Atlantic cod caught in the Barents Sea (April-June, Oct.-Dec.), Icelandic Sea (Feb.-March, April-June) and south coast of Ireland (Feb.-March), expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

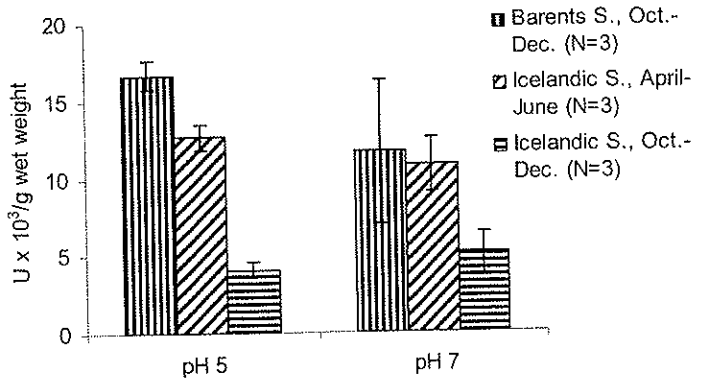

Figure 4.18: Mean maximum activity of lipase at pH 5 (35°C) and at pH 7 (35°C) in cut off from Atlantic cod caught in the Barents Sea (Oct.-Dec.) and the Icelandic Sea (April-June, Oct.-Dec.), expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

Cut off samples from Atlantic cod caught in the Icelandic Sea, April-June had lower activity of cathepsin B and collagenase compared to samples from Feb.- March (Paper IV), the lipase activity at pH 5 and 7 was, however, significantly higher compared to samples caught in Oct.-Dec. (Paper III). Atlantic cod may be spawning in Feb.-March (Cohen et al., 1990) and our results are therefore in accordance with findings by Yamashita and Konagaya (1990a), who found elevated activities of lysosomal proteases, including cathepsin B, along with a 40% reduction in activity of cysteine protease inhibitor, in the white muscle of salmon during spawning. Bonete et al. (1984a) also found variation in cathepsin B activity in muscle, liver, heart, spleen and gonads from the fishes *Mujil auratus*, *Sparus aurata* and *Lightonatus mormyrus* according to year and season. Cut off samples from the Barents Sea showed no seasonal variation in cathepsin B activity, while the results for collagenase were inconclusive.

4.2.3. Liver

Figure 4.19, 4.20 and 4.21 shows the mean maximum general proteolytic activity, mean maximum activity of collagenase, cathepsin B and lipase pr g wet weight in liver samples from Atlantic cod.

The general proteolytic activity at pH 3 and cathepsin B activity was higher in liver samples from Atlantic cod caught at the south coast of Ireland compared to the other fishing grounds. The differences were, however, not significant.

Liver samples from the Icelandic Sea, Feb.-March, showed significantly ($p < 0.05$) higher activity of collagenase compared to samples from the south coast of Ireland. Liver samples from the Barents Sea, April-June, had significantly lower activity of cathepsin B and higher activity of collagenase compared to samples from the Icelandic Sea, April- June (Paper IV).

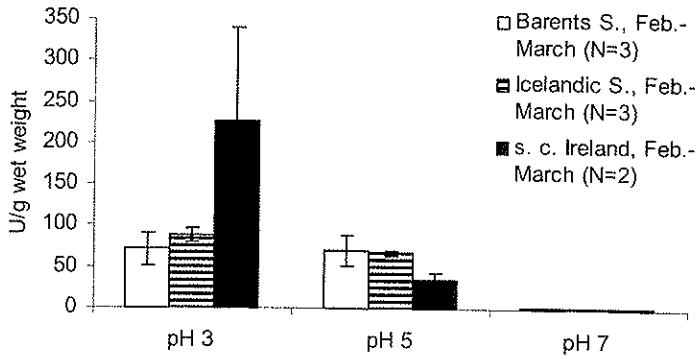

Figure 4.19: Mean maximum general proteolytic activity measured at pH 3 (50°C), pH 5 (50°C) and pH 7 (65°C) in liver from Atlantic cod caught in the Barents Sea, Icelandic Sea and south coast of Ireland in Feb.-March, expressed as U/g wet weight sample (U = mg haemoglobin cut per hour). Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

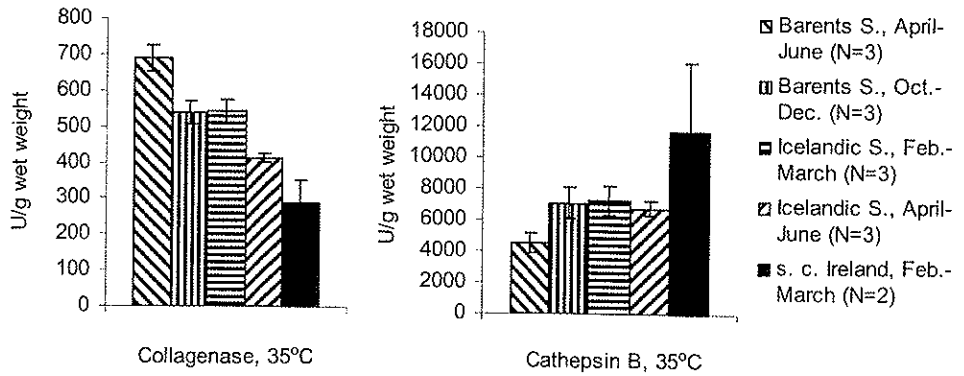

Figure 4.20: Mean maximum activity of collagenase and cathepsin B measured at pH 7 in liver from Atlantic cod caught in the Barents Sea (April-June, Oct.-Dec.), Icelandic Sea (Feb.-March, April-June) and south coast of Ireland (Feb.-March), expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

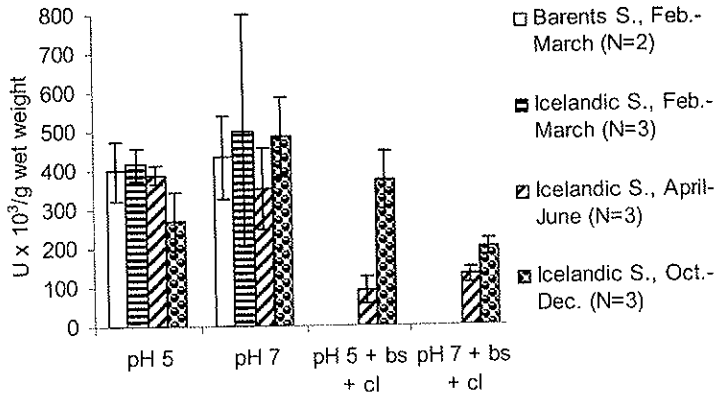

Figure 4.21: Mean maximum lipase activity at pH 5 (50°C), pH 7 (50°C), pH 5 with addition of bile salt and co-lipase (35°C) and pH 7 with addition of bile salt and co-lipase (20°C) in liver from Atlantic cod caught in the Barents Sea (Feb.-March) and the Icelandic Sea (Feb.-March, April-June, Oct.-Dec.), expressed as U/g wet weight sample. Mean values with standard error of the mean as bars are plotted. N = number of pooled samples of 15 fishes.

Activity of cathepsin B in liver samples from the Barents Sea, April-June, was significantly higher than in Oct.-Dec. (Paper IV). The lipase activity at pH 5 with addition of bile salt and co-lipase in liver samples from the Icelandic Sea was significantly lower in samples from April-June compared to Oct.-Dec. (Paper III).

4.3. Correlations

All samples analysed for the respective variables have been used in the correlation test, regardless of fishing grounds, seasons and species. The correlation test was done on specific enzymatic activities (U/ mg water-soluble protein). The correlations found for the by-products are summed up in Table 4.1.

Table 4.1: Correlations found in the by-product fractions. +/- indicates whether the correlations were positive or negative. All correlations were significant at 95% level ($p < 0.05$).

Fraction	Correlations		+/-
Viscera	Water-soluble protein	Gen. prot. act., pH 5 and 7	-
		Trypsin	-
		Chymotrypsin	-
		Elastase	-
		Collagenase	-
		Cathepsin B	-
		Lipase, pH 5 and 7	-
	Free amino acids	Gen. prot. act., pH 5 and 7	+
		Trypsin	+
		Chymotrypsin	+
		Elastase	+
		Collagenase	+
		Cathepsin B	+
		Lipase, pH 5 and 7	+
Cut off	Water-soluble prot. Dry matter	Lipase, pH 5 and 7	+
		Cathepsin B	+
		Collagenase	-
Liver	Free amino acids	Cathepsin B	+
		Collagenase	-
		Lipase, pH 5 and 7	-

In general, high enzymatic activity in viscera leads to increased amounts of free amino acids and decreased amounts of water-soluble protein. Trypsin is an activator of the other digestive peptidases in the intestines, it is therefore expected that the activity of the peptidases increases simultaneously. Trypsin, chymotrypsin, elastase, cathepsin B and metallo collagenase are all endopeptidases and therefore not expected to give an increase in free amino acids. However, activity of endopeptidases renders the proteins more susceptible to degradation by other peptidases, including exopeptidases, thereby giving a rise in amount of free amino acids. The correlation between collagenase activity and free amino acids was positive in viscera and negative in liver, this difference may be explained by the presence of different collagenases in the two fractions and differences in activity of other proteases. As mentioned above trypsin activates all the other peptidases in viscera. This is not the case in liver, and it is likely that factors activating cathepsin B represses collagenase in this fraction. Therefore the correlation between free amino

acids and collagenase in liver is most likely caused by collagenase being negatively correlated to cathepsin B. Also the lipase activity was positively correlated to free amino acids in viscera and negatively correlated in liver. This suggests that lipases is activated by the same factors as peptidases in viscera, while in liver lipases are repressed by factors activating exopeptidases or by the exopeptidases themselves. Correlation between lipase activity and water-soluble protein was negative in viscera and positive in cut off.

Proteolytic enzymes in cut off were not correlated to amount of water-soluble protein or free amino acids. Activity of cathepsin B was positively correlated, while collagenase was negatively correlated to dry matter (Paper IV). Quality deteriorating characteristics of fish that may be caused by collagenolytic activities, like gaping, has also been found to increase with decreasing water content in the muscle (Sikorski et al., 1984).

4.4. Apparent activation energies

The apparent activation energies are calculated using the Arrhenius equation in the temperature range 5°C to 35-50°C, depending on the maximum activity of the samples. Table 4.2 shows the apparent activation energies calculated for the different enzymes in this study.

The apparent activation energies for proteolytic enzymes active at pH 3 and pH 5 were significantly lower in viscera samples compared to cut off and liver samples. This indicates that proteolytic enzymes active at pH 3 and pH 5 in cut off and liver are more sensitive to temperature changes compared to those in viscera. The apparent activation energy found for general proteolytic activity at pH 3 in viscera is in good agreement with the activation energy of pepsin from Atlantic cod, 30.5 kJ/mole (Brewer et al., 1984). There were no significant differences in apparent activation energies for proteolytic enzymes active at pH 7 in cut off, viscera and liver (Paper I). Jonas et al. (1983) found the activation energies of proteolytic enzymes from the alimentary canal of silver carp, common carp and sheat fish, active at pH 7.5, to be 58.5, 64.4 and 65.6 kJ/mole, respectively.

No differences in apparent activation energy for collagenase were found between the three fractions. The activation energies ranged from 32-38 kJ/mole. This

is in the same range as the activation energies of a collagenolytic serine proteinase from Antarctic krill (*Euphausia superba* Dana) which was found to be 27.5 and 34.6 kJ/mole for two synthetic substrates (Turkiewicz et al., 1991).

The apparent activation energy of lipase at pH 5 was significantly higher in cut off than in viscera and liver indicating that lipase (pH 5) in cut off is more temperature dependent. The apparent activation energy of lipase at pH 7 with addition of bile salt and co-lipase was significantly lower in cut off compared to viscera and liver. Addition of bile salt reduces the temperature dependency of lipases active at both pH 5 and 7 in cut off, while no changes in temperature dependency are seen for lipases in liver and viscera (Paper III).

Table 4.2: Apparent activation energies for different enzymes in by-products from cod species ± standard error of the mean. N = pooled samples of 15 fishes.

Enzyme	N	Cut off	N	Viscera	N	Liver
Proteases, pH 3	16	46.4 ± 2.6	16	28.1 ± 2.2	16	45.9 ± 2.8
Proteases, pH 5	16	67.1 ± 5..3	16	44.7 ± 3.8	16	76.9 ± 3.6
Proteases, pH 7	16	50.4 ± .0	16	47.5 ± 3.5	16	40.5 ± 5.1
Trypsin			36	53.6 ± 1.2		
Chymotrypsin			35	33.2 ± 1.2		
Elastase			36	47.1 ± 2.5		
Cathepsin B	14	18.3 ± .2	15	33.8 ± 2.3	14	39.1 ± 4.5
Collagenase	14	38.6 ± 3.0	15	34.9 ± 2.2	14	32.3 ± 3.2
Lipases, pH 7	9	25.5 ± 5.5	12	25.5 ± 3.4	10	22.1 ± 1.0
Lipases, pH 5	9	37.6 ± 6.3	11	23.2 ± 4.4	11	19.8 ± 1.2
Lipases, pH 7 + bs + cl	3	9.7 ± 2.0	6	26.0 ± 1.4	6	23.1 ± 1.8
Lipases, pH 5 + bs + cl	3	24.4 ± 1.9	6	23.3 ± 3.0	6	21.8 ± 2.7

4.5. Heat stability

In order to inactivate enzymes in the by-products, high temperatures are required, heating is, however, expensive and in order to keep processing costs down knowledge about the heat stability of the endogenous enzymes is needed. The results from the heat stability studies showed that cathepsin B was the most heat stable enzyme in the viscera fractions. Cathepsin B had the highest residual activity after 10 min incubation at 60°C and after 60 min incubation at 50°C (Paper IV). Trypsin was more heat stable than chymotrypsin, and they were both more heat stable than elastase and collagenase. Lipase active at pH 5 in viscera was somewhat more heat

stable than lipase active at pH 7, judged from residual activity after 10 min incubation at 60°C and after 60 min incubation at 50°C (Paper III).

Also in the cut off fractions cathepsin B was the most heat stable enzyme, and showed the highest residual activity after 10 min incubation at 60°C and after 60 min incubation at 50°C (Paper IV). Also the lipases at both pH 5 and 7 showed considerable activity after 10 min incubation at 60°C, however, more than 95% of initial activity was lost after 60 min at 50°C (Paper III)..

In crude extracts of liver, collagenase appears to be the most heat stable, judged from 10 min incubation at 60°C. However, the lipolytic enzymes are the most heat stable judged from the residual activity after 60 min incubation at 50°C. Table 4.3 illustrates the heat stability of different enzymes in by-products.

Table 4.3: Heat stability of different enzymes in by-products. Temperatures where the measured activity was less than 50% and less than 10% of initial activity, and the residual activity after 10 minutes incubation at 40°C, 50°C and 60°C ± standard error of the mean. N = pooled samples of 15 fishes.

Enzyme	By-product	N	T where activity was		% of initial activity measured at		
			<50%	<10%	40°C	50°C	60°C
Trypsin*	Viscera	10	60	60	101.2 ± 12.5	80.9 ± 27.0	2.6 ± 2.0
Chymotrypsin*	Viscera	10	60	60	95.1 ± 3.9	57.4 ± 17.3	3.0 ± 2.2
Elastase	Viscera	6	50	60	105.9 ± 17.0	14.8 ± 8.8	3.0 ± 3.0
Cathepsin B	Cut off	5	50	> 80	65.0 ± 5.6	30.8 ± 17.7	18.8 ± 16.5
	Viscera	5	60	60	102.9 ± 9.8	92.4 ± 22.9	8.8 ± 9.4
	Liver	5	50	50	77.0 ± 18.2	6.4 ± 5.5	5.2 ± 5.7
Collagenase	Cut off	5	40	60	22.5 ± 6.1	12.3 ± 13.3	7.2 ± 5.0
	Viscera	5	50	60	80.2 ± 4.6	37.6 ± 15.9	3.7 ± 3.1
	Liver	5	50	50	85.5 ± 1.6	29.7 ± 18.9	8.6 ± 9.2
Lipases, pH 5	Cut off	2	50	70	187.5 ± 33.6	25.5 ± 0.12	17.1 ± 9.4
	Viscera	4	50	60	66.7 ± 18.0	21.6 ± 20.2	2.3 ± 1.8
	Liver	4	60	60	80.8 ± 15.1	66.4 ± 22.3	6.6 ± 3.5
Lipases, pH 7	Cut off	2	50	60	155.4 ± 65.7	26.0 ± 4.7	10.0 ± 0.7
	Viscera	4	50	60	86.8 ± 20.1	37.9 ± 41.5	1.4 ± 1.8
	Liver	4	60	60	157.6 ± 41.6	104.3 ± 24.7	1.7 ± 2.3

*The residual activity was measured at 50°C for trypsin and chymotrypsin, and at 35°C for the other enzymes.

The results show that enzymes in the different by-product fractions respond differently to temperature. The presence of different molecular species of enzymes, concentration of proenzymes, inhibitors, activators and stabilising factors offer possible explanations for this. Since endogenous enzymes have somewhat different

optimum and denaturing temperatures, changes in processing temperatures is likely to change both the degree of hydrolysis and the composition of the end product when FPH are produced.

Heat stability of several peptidases is known to be highly dependent on pH and presence of calcium. Kristjansson (1991) found rainbow trout trypsin to be stable for 30 min at a temperature up to 40°C at pH 5.4, at pH 8 the enzyme was stable up to 50°C (Kristjansson, 1991). Berglund and Smalas (1998) showed that purified elastase from the pancreas of North Atlantic salmon was most heat stable at pH 8, which was the pH optimum of the enzyme. pH dependency is difficult to interpret since several processes such as acid- and temperature- induced unfolding, autolysis and refolding can take place (Outzen et al., 1996). In this study we have incubated the crude extracts at their natural pH and then examined residual activity at pH 7. The pH of the viscera extracts varied from 6.6 to 7.1, which is probably either above or below the pH were the enzymes studied are most stable. This might be one of the explanations for the rather large variation in the results. Kristjansson (1991) found that in the absence of $CaCl_2$ trypsin was almost fully inactivated after 10 min of heating at 55°C, whereas in presence of 15mM $CaCl_2$ more than 80% of original activity remained after the same treatment. Another study found that chymotrypsin lost more than 90% of maximum activity in absence of $CaCl_2$, but less than 40% in presence of 15mM $CaCl_2$ after 20 min incubation at 50°C (Kristjansson & Nielsen, 1992). Kristjansson et al. (1995) also found that collagenolytic serine protease purified from the intestines of Atlantic cod was stabilised against thermal inactivation by calcium ions. Simpson et al. (1989) reported that addition of 0.02M $CaCl_2$ to substrate buffer elevated the temperature optima by 5°C for trypsin from Greenland cod and Atlantic cod. Enzyme activity has in the present study been measured in a buffer containing 0.1M $CaCl_2$, but the crude enzyme extracts were incubated without buffer. The crude extracts from by-products may contain varying concentrations of calcium.

Figure 4.22 shows the temperature dependence of trypsin and chymotrypsin from saithe viscera compared to the corresponding enzymes in Porcine Trypsin Novo (PTN).

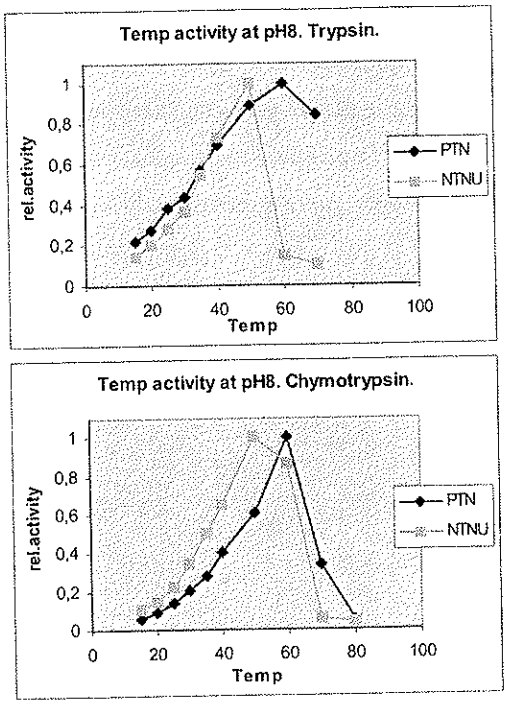

Figure 4.22: Temperature dependence of trypsin and chymotrypsin from saithe viscera (NTNU) compared to Porcine Trypsin Novo (PTN).

It is a common assumption that enzymes from cold adapted organisms are less thermostable and have relatively low temperature optima compared to the homologous enzymes from warm-blooded animals. In order to compare fish enzymes to enzymes from warm-blooded animals we sent a crude enzyme extract from saithe viscera (caught in the Icelandic Sea) to Novozyme AS where trypsin and chymotrypsin activities were compared to a product called Porcine Trypsin Novo (PTN), which is based on an extract from porcine glands. Surprisingly the temperature optima for the fish enzymes were almost as high as the temperature optima for the porcine enzymes (50-55°C versus 60°C) (Figure 4.22). Trypsin from saithe did, however, lose activity much more rapidly when temperature was increased above the optimum, compared to PTN. The PTN-trypsin also appeared to be more active than the saithe trypsin at temperatures between 15 and 30°C, this is

contradictory to the common assumption that enzymes from cold adapted organisms have higher activities at low temperature compared homologous enzymes from warm-blooded animals. Chymotrypsin from saithe viscera is, however, more active at low temperatures compared to PTN. The saithe extract was also compared to PTN in a hydrolysis experiment, and the saithe extract was found to have marginally better hydrolysing properties when tested on casein and soy isolate at 40°C compared to PTN (Søvik et al., 2004).

5. SUGGESTIONS FOR FUTURE WORK

In order to accomplish a profitable utilisation of by-products, more knowledge about the quality of each fraction is necessary. Consumer preferences for by-products sold whole (liver, dried stomach and intestines etc.) need to be linked to quality parameters and enzymatic activities. Splitting the viscera fraction into stomachs and intestines, may reveal more differences between species, season and fishing ground. Characterisation of changes in the by-products during different storage conditions needs to be studied. Further characterisation of enzymatic activities during spawning/life cycle of different species are also of interest. Differences between species found in this study needs to be confirmed in a study where spawning fish are excluded. A study of the quality and enzymatic activities in by-products over several years would be of interest in order to compare seasonal variations with the year to year variation.

Heat stability of enzymes from all three fishing grounds and seasons should be characterised. This work suggests that different isozymes dominate in samples from different seasons and fishing grounds, and this may result in differences in heat stability. It may be of interest to study the presence and amount of factors influencing the heat stability of the enzymes, such as divalent ions, inhibitors, activators, etc.

In this study the lipolytic activity was studied in by-products from cod only, it is, however, of great interest to characterise and compare these activities in the other species as well.

A study of by-products from farmed fish where the feeding history of the fish is known may possibly reveal more of the influence feeding status has on the enzymatic activities in the different by-product fractions.

6. CONCLUDING REMARKS

By-products are complex and highly perishable fractions, resulting in high standard deviations in many of the analyses. However, the present work clearly demonstrates significant variations in quality parameters and enzymatic activities in by-products from cod fish according to species, seasons and fishing grounds.

General proteolytic activity (pH 5 and 7) was more affected by species than fishing ground. General proteolytic activity (pH 3) was not affected by either.

Season significantly affected activity of trypsin, chymotrypsin and elastase in viscera from cod caught in the Icelandic Sea. Cod caught in the Barents Sea showed no significant seasonal changes. Activity of trypsin, chymotrypsin and elastase in cod viscera was affected by fishing ground. Significant differences between the five species were found for trypsin and chymotrypsin. Viscera fractions from the gadidae family and cod caught in the Icelandic Sea have the highest proteolytic capacities judged from the peptidases examined in this study, and may therefore be a suitable raw material for extraction of enzymes, autolytic and/or semiautolytic production of FPH, and production of fish sauce and/or paste.

Species, season and fishing ground significantly affected activity of cathepsin B and collagenase in cut off samples, while viscera samples were not significantly affected. Cut off from ling and saithe, cod caught in the Barents Sea (April-June and Oct.-Dec.) and Icelandic Sea (April-June) may be used for minced products or surimi based products. Cut off from haddock, cod caught at the south coast of Ireland and in the Icelandic Sea (Feb.-March) is a poor raw material for these kinds of products due to the high activity of cathepsins B.

The lipase activity was higher at pH 7 compared to pH 5 in liver and viscera, and on the same level in cut off. Lipase activity in cut off and viscera was somewhat affected by season and fishing ground, while no significant effects were found for liver lipases. Due to the high lipase activities in viscera and liver, cold extraction of lipids or procedures using partial hydrolysis would probably be the most favourable method for producing fish oil. However, if heating is needed it should be rapid to temperatures above 60°C.

The enzymatic activities in by-products are high and it is important that they are rapidly and continuously stored at low temperatures. Separating different by-

product fractions, and fractions from the lotidae family from the gadidae family, will ensure that fractions with low enzymatic activities are not contaminated by fractions with higher enzymatic capacities.

Generally, crude extracts from by-products needed heating up to 50-60°C for 10 min in order to lose 50% of initial activity of the enzymes studied.

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