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Effect of processing parameters on salting of herring.

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Summary

Herring is a fatty fish that contain Omega-3 fatty acid. Consumption of herring gives us many essential nutrients connected to their proteins, and lipids that are very rich in omega-3 fatty acids. These omega-3 fatty acids are highly susceptible to oxidation. For this good preservation technique is very important. The primary purpose of salting is preservation. The ripening process appears to be mainly proteolytic, leading to a degradation of muscle proteins resulting in an increase in soluble nitrogenous compounds such as peptides and free amino acids.

The function of proteolytic enzymes is to break down the peptide bonds of proteins resulting in smaller peptides and formation of smaller peptides and amino acids from the protein molecules. During lipid oxidation, endogenous antioxidants are consumed and several primary and secondary oxidation compounds are formed, which results in reducing the sensory quality and may damage protein functionality because of cross-reactions with proteins. Lipolytic enzymes are responsible for enzymatic hydrolysis of fat, breaking down fat into free fatty acids and glycerol.

During the ripening and salting of the herring, the raw taste of the herring gradually changes. In this case, addition of salt to the fish is an effective way to slow down the enzymatic reactions and at a very high concentration (20-25 %) of salt, the enzymatic process decreased to almost no activity level. This will increase the shelf life of the product without freezing it.

The objective of this study was to study the proteolytic and lipolytic activity in herring, active during the ripening process of herring. The enzymatic activity during salting was studied as a function of pH, temperature and salt concentration, and the activity in both muscle and intestines was studied. In this thesis, some selected proteolytic enzymes, for instance; trypsin, chymotrypsin and cathepsins have been analysed. How the biochemical parameters of herring changes by the enzymatic ripening procedure has also been studied; proteolytic and lipolytic enzyme activity and how protease activity affect the peptide content in herring.

The highest General proteolytic activity (GPA) was found in intestine at pH 6. The activity goes up to higher level in the presence of salt and buffer. The lowest activity was found in muscle tissue at pH 7. The activity of cathepsin B was higher in intestine at pH 6, whereas for the muscle very low activity was found at pH 5 also it had the maximum activity at 30 ^{0}C to

40 ^{0}C. Trypsin had shown the higher activity at pH 5 in 40 °C and in every cases intestine shown the higher activity than muscle. Chymotrypsin was found to have about 10 times higher activity than trypsin, the second highest measured enzyme in herring filet. It means that the changes in chymotrypsin activity will have higher impact on total proteolytic activity. Acid soluble peptide of herring muscle decreased gradually when salt content increased and If NaCl is exchanged with KCl also increase the activity; this shows that the proteases are affected by the type of ions.

Content

1 Introduction

The use of pelagic fish species for salting is a very ordinary practice in several European countries. Salting is a conventional preservation technique, and herring (*Clupea harengus*) is salted in many European countries for instance in Norway, Finland, Denmark, Iceland, Holland, Russia and Germany.

The primary purpose of salting is preservation, but during salting the fish are transformed into a salted, well-appreciated delicacy. The ripening process appears to be mainly proteolytic, leading to a degradation of muscle proteins resulting in an increase in soluble nitrogenous compounds such as peptides and free amino acids (Gudmundur et al., 2000; Kiesvaara, 1975).

The ripening process involves enzymatic degradation of proteins and lipids in herring muscle. Ripening takes a long time and is believed to be seasonally dependent. Seasonal changes in the herring raw material such as changes due to sexual maturity, fat content and enzyme activity, probably after the ripening process and the quality of the final product(Olsen & Skara, 1997)

During the salting various changes occur that change the chemical and physicochemical properties of fish. The changes can in general be divided into two phases, salting and ripening. Salting as such is characterized by salt penetration into the fish tissues and it ends when the concentration of salt in the tissues equals that of the surrounding brine. Changes occurring during salting stage, salt uptake, water content changes and fish weight changes, have been well documented. (Valle & Nickerson, 1967a, 1967b; Zugarramurdi & LupÍN, 1980)

Protein degradation occurring during ripening depends on the activity of proteolytic enzymes (Engvang & Nielsen, 2000; Nielsen & Borresen, 1997). Enzymes from the internal organs are of prime importance for the proteolysis (Hauch Nielsen, 1995; Luijpen, 1959). Muscle enzymes can however not be ruled out and these enzymes may also be important for the ripening process.

Ripening of herring is traditionally carried out by storing gutted or ungutted herring together with salt, sugar (and spices) in barrels for several month, in order to obtain characteristic

changes in texture and flavor commonly believed to be caused by enzymes originating from digestive tract.(Gildberg, 1994; Olsen & Skara, 1997). Some studies also indicates that eviscerated herring ripens more slowly than ungutted herring.

The influence of intestinal enzymes on the ripening process of herring, relative to the muscle proteases, was examined by processing the raw material in different ways, i.e with intestines (deheaded) and without intestines (gutted and filleted). Potential diffusion of proteolytic enzymes from the intestine.was investigated by measuring general proteolytic activity (GPA), both in muscle and in pyloric caeca during the ripening period. (Olsen & Skara, 1997)

Enzymes from muscle tissue also participate in the proteolysis (Hauch Nielsen, 1995) and may therefore be of importance for the ripening.(Gudmundur et al., 2000; G. Stefánsson, Henrik, & Gudmundsdóttir, 1995). In Scandinavia and many parts of Europe the industrial practice is to use fresh herring for salting. Early work indicates that herring which freezes during salting takes up salt more quickly than fresh salted herring (Gudmundur et al., 2000).

Ripening is a long process and during this long ripening period many biochemical reactions take place and the herrings develop a characteristic texture.(Mette Christensen, Eva Andersen, Line Christensen, Mogens L. Andersen, & Caroline P. Baron, 2011; Hauch Nielsen, 1995)

The ripening is chiefly believed to be caused by enzymes but the origin of enzymes has as yet not clearly been identified but it has been found that substantial proteolytic breakdown occurs in the fish muscle during ripening(Olsen & Skara, 1997).Most of the research on understanding the ripening process has investigated the role of proteolytic enzymes and their relation to protein breakdown (M. Christensen, E. Andersen, L. Christensen, M. L. Andersen, & C. P. Baron, 2011; Hauch Nielsen, 1995).

The aim of this study was to investigate the proteolytic and lipolytic activity in herring important for the ripening process in herring. The enzymatic activity during salting was studied as a function of pH, temperature and salt concentration, and the activity in both muscle and intestines was studied.

1.1 Atlantic Herring (*Clupea harengus*)

The scientific name for the herring found in the North Atlantic is "*Clupea harengus"*. The scientific name for the Pacific herring, a closely related species, is "*Clupea pallasii"*. The name Atlantic herring is used when it is necessary to distinguish it from the Pacific herring. The name Baltic herring is used to describe small "*Clupea harengus"* caught in the Baltic Sea. In Norwegian it named as "Sild"(Stroud, 2001).

1.1.1 Morphological features

Herring is one of the most important fish species in the North Atlantic and Baltic Sea, with an annual catch exceeding 2 million metric tonnes. Atlantic herring (*Clupea harengus*) (figure 4.1) is classified as fatty fish species. The body of the herring is deeper than it is thick, and the length of the fish is about five times the greatest depth. The upper part of the body is dark blue green, or steel blue, and the snout is blackish blue; the sides and belly are silvery. The lower jaw protrudes slightly beyond the upper. There is a single short back fin, a short anal fin near the tail, and a deeply forked tail fin. The pelvic fins are behind the start of the back fin, whereas on the sprat they are in front. The body is covered with large, thin, loosely attached scales. The mouth is large, and contains small weak teeth. The lateral line is not visible, and there is no barbell (Stroud, 2001).

Most of the herring landed in Britain are between 23 and 30 cm long; herring caught off Norway and Iceland are often larger, up to 36 cm. Occasionally a herring reaches a length of about 43 cm, but this is exceptional. The weight of a herring in relation to its length. The weight for a given length can vary considerably from season to season and from year to year (Stroud, 2001).

Figure 1.1: Atlantic Herring (Clupea harengus)

(Source:http://www.scottsbt.com/fishids/idmisc/herring.htm)

1.1.2 Geographical distribution

The herring is found on both sides of the north Atlantic. In the north east Atlantic it occurs from the Bay of Biscay in the south to Spitzbergen and Novaya Zemlya in the north, while in the north west Atlantic it occurs from the coast of Maine northwards. The most important fishing grounds are the North Sea, the Baltic Sea, and the coastal waters of Britain, Norway, Iceland and Canada (Stroud, 2001).

1.1.3 Life history

The herring is a pelagic fish, and may be found anywhere between 2 and 400 m below the surface of the sea. The female herring lays its eggs on the sea bed, usually in water 10-80 m deep, on hard ground covered with small stones, shells or seaweed to which the naturally sticky eggs can attach themselves. One female may lay 20,000-40,000 eggs. The eggs are fertilized in the water by the male herrings, which discharge their sperms at the same time as the females lay their eggs. They mature, mostly at 3 years of age. The adult herring feeds mainly on animal plankton, particularly the tiny copepod called “Calanus” near the surface of the sea (Stroud, 2001).

1.1.4 Seasonal Variation

The quality of the herring depends on the season it was caught. There are three different categories: (Pedersen, 1989)

- Winter quality: The herring has a low fat content as well as roe and milt. It can be caught during January to March. This fat content is about 14 %.

- Summer quality: Fat content is from medium to high and the texture of the fat and lard is loose.
- Quality H: Fat content is high and the texture of the fat and lard is hard. It can be caught from July to December. This fat is about to 29 %.

1.1.5 Herring processing and preservation

Preservation and processing of herring is important for the consumers because the fishermen catch this fish seasonally in a large scale. If this enormous amount of fish is not properly preserved, it will be spoiled with time. Good preservation methods are necessary to ensure the access to highly nutritious herring during the whole year.

The objectives of all preservation technique are to prevent or slow down the spoilage in herring muscle that is mainly due to bacterial growth, enzymatic activity and. Oxidation. A very well known method is salting to prevent the bacterial growth and spoilage. *Canning* and *Marinating versus salting* are also some common practices to process herring.

Although some herring is distributed and sold unprocessed, either whole or as boned herring, most of the catch is processed in some way before sale. The main food outlets are for smoked, salted, marinated and canned products; quick freezing and cold storage are used as a means of preserving some of the catch prior to making these products, and for preserving some of the finished products (Stroud, 2001).

1.1.5.1 Salting

The conventional way of salting is to place the whole herring in barrels with salt overnight in order to form blood brine and to fill the barrels with saturated brine before storage. In this method, the herring stay edible for over a year if stored at low temperature, and develop a characteristic texture due to multiple biochemical reactions which involve significant degradation of muscle myosin as well as protein oxidation and degradation (Mette Christensen et al., 2011)

This method was further developed to “sharp salting” in which the herring has been used is gutted and beheaded first and then placed in brine with up to 20% of salt that was changed at frequent time intervals. It took minimum 2-3 months of ripening in order to get the desired

taste and texture. The benefit of using the high concentration of salt is that increases the shelf life of the product up to 12 month.

There is another method in practice that used also the same principle named the sugar salting method. The benefit of adding sugar to brine is, that decreases the salt concentration to 10-15% and that results more balanced and less salty taste compared to sharp salting method. This method also decreases the storages time to 4-6 weeks to form the edible product. Pickling is also popular method to preserve herring in Nordic countries in which vinegar used to remove the salty taste.

1.1.5.2 Freezing and cold storage

Whole ungutted herring, frozen within a few hours of capture and properly cold stored at -30°C, can be kept 7 months before thawing to make good quality smoked, canned or marinated products. Ideally the herring should be frozen not later than 24 hours or, when the fat content is high, 18 hours after capture, and the herring should be kept properly chilled between catching and freezing. Herring can also be frozen after splitting or filleting if required; they are most conveniently frozen in blocks in a horizontal plate freezer (Stroud, 2001).

Freezing the herring before adding salt can therefore speed up the ripening process while developing similar taste and texture, which is very advantageous considering large scale industrial food processing (Gudmundur et al., 2000).

The possible oxidation of fatty acids in fish during frozen storage should however be taken into consideration, as long periods of freezing before further processing can cause a decrease in nutritional value and development of unwanted smell and taste (Pirestani, Sahari, & Barzegar, 2010; Undeland & Lingnert, 1999)

1.1.6 Biochemical composition

The chemical composition of fish varies greatly from one species and one individual to another depending on age, sex, environment and season. The variation in the chemical composition of fish is closely related to feed intake, migratory swimming and sexual changes in connection with spawning. (Huss, Boerresen, Dalgaard, Gram, & Jensen, 1995).

Herring is available throughout all the year round, but the size and fat content varies a lot during a year. The fat content is at its peak in June and July, where it can reach about 20%. On the other hand the fat content dramatically drops down to 3-5%, or even as low as 1% right after spawning by December/January before it starts to increase again. (Stroud, 2001).

The lipid fraction is the component showing the greatest variation. Often, the variation within a certain species will display a characteristic seasonal curve with a minimum around the time of spawning. Figure 1.2 shows the characteristic variations in the North Sea herring (1.2). (Huss et al., 1995).

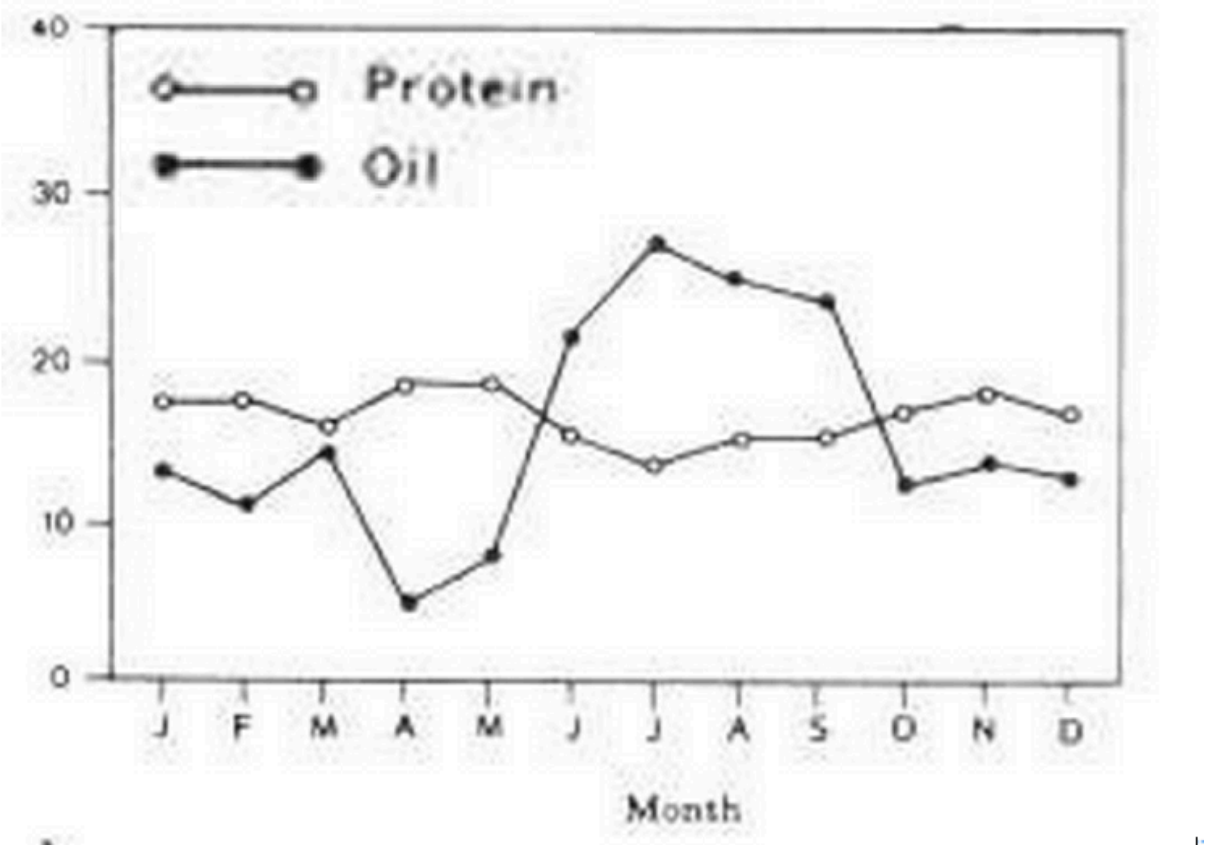

Figure 1.2: Seasonal variation in the chemical composition of herring fillets (Clupea harengus). Each point indicates the mean value of eight fillets. Adopted from (Huss et al., 1995)

There is a significant difference between lean fish and fatty fish. Lean fish; for example cod, saithe and sprat stored lipid in liver. On the contrary, fatty fish like Herring stored lipid in fac cells distributed in other body tissues for example beneath the skin, in body cavity etc (Huss et al., 1995).

The variations in water, lipid and protein contents in Herring are shown in Table 1.2.

Table 1.1: chemical compositions of the fillets of Herring (Murray & Burt, 2001).

Species	Scientific name	Water %	Lipid %	Protein %
Herring	*Clupea harengus*	60-80	0.4-22.0	16.0-19.0

The herring as a food has a high energy value because most of the fat is in the flesh; the raw flesh of a moderately fat herring, containing 11 per cent fat, has an energy value of about 7·4 kJ/g (Stroud, 2001). Herring also contain appreciable amounts of iron, calcium and iodine.

Water is the main component in fish; and the water content varies between 69 and 82g/ 100g. There is a correlation between the content of water and content of fat in the fish. Figure 4.2 shows that it is possible to estimate the fat content based on its water content (Stroud, 2001).

(Kent, Lees, & Christie, 1992) found that, the variation in the percentage of fat is reflected in the percentage of water, since fat and water normally constitute around 80 % of the fillet. As a rule of thumb, this can be used to estimate the fat content from an analysis of the amount of water in the fillet.

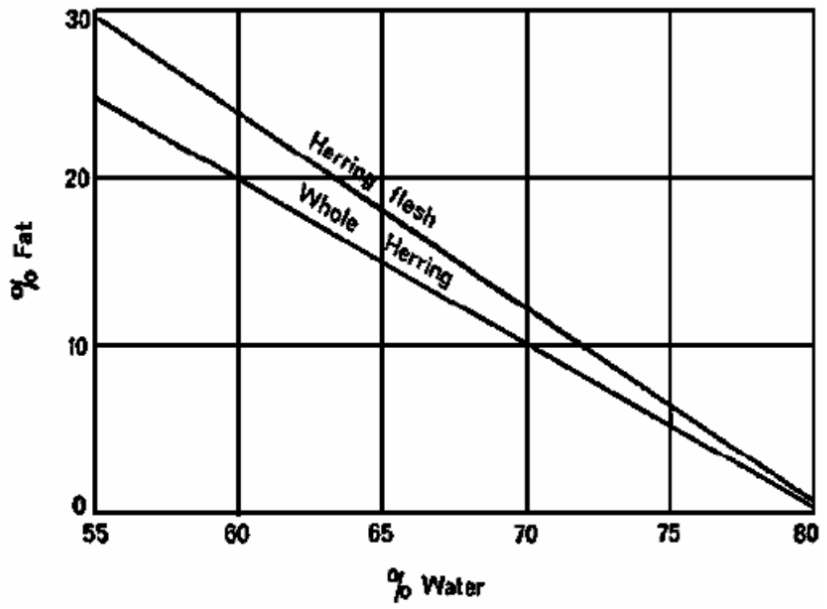

Figure 1.3: Fat content in Herring (Stroud, 2001).

The proteins in fish muscle tissue can be divided into the following three groups (Huss et al., 1995):

1. Structural proteins (actin, myosin, tropormyosin and actomyosin), which constitute 70-80 % of the total protein content (compared with 40 % in mammals). These proteins are soluble in neutral salt solutions of fairly high ionic strength (³0.5 M).

2. Sarcoplasmic proteins (myoalbumin, globulin and enzymes), which are soluble in neutral salt solutions of low ionic strength (<0.15 M). This fraction constitutes 25-30 % of the protein.

3. Connective tissue proteins (collagen), which constitute approximately 3 % of the -protein in teleostei and about 10 % in elasmobranchii (compared with 17 % in mammals).

The N-containing extractives can be defined as the water-soluble, low molecular weight, nitrogen- containing compounds of non-protein nature. This NPN-fraction (non-protein nitrogen) constitutes from 9 to 18 % of the total nitrogen in teleosts (Huss et al., 1995).

Table 1.2: Major components in the NPN-fraction of Herring (Huss et al., 1995).

Compound in mg/100 wet weight[1)]	Fish Herring
1) Total extractives	1200
2) Total free amino-acids:	300
Arginine	<10
Glycine	20
Glutamic acid	<10
Histidine	86
Proline	<1.0
3) Creatine	400
4) Betaine	0
5) Trimethylamine oxide	250
6) Anserine	0
7) Carnosine	0
8) Urea	0

The amount of vitamins and minerals is species-specific and can furthermore vary with season. In general, fish muscle is a good source of the B vitamins and, in the case of fatty species, also of the A and D vitamins. As for minerals, fish muscle is regarded as a valuable source of calcium and phosphorus in particular but also of iron, copper and selenium. Saltwater fish have a high content of iodine. In Tables 1.3 some of the vitamin contents in herring are listed. Because of the natural variation of these constituents, it is impossible to give accurate figures (Huss et al., 1995).

Table 1.3 Vitamins in herring fillet (Murray & Burt, 2001).

Fish	A (IU/g)	D (IU/g)	B_1(thiamine) (µ/g)	B_2 (riboflavin) (µ/g)	Niacin (µ/g)	Pantothenic acid (µ/g)	B_6 (µ/g)
Herring fillet	20-400	300-1000	0.4	3.0	40	10	4.5

1.1.7 Enzymatic process during in ripening of herring

Enzymatic reactions are essential in the improvement of texture and taste of food products, and enzymatic ripening is an ancient process and food where enzymes are important for ripening has been produced since long before enzymes were known. Industrial application of enzymatic processes is not very old and has only been used for the last 20 years. (Aardal, 2013). (Vilhelmsson, 1997) reported that some marine enzymes for instance chymotrypsin, trypsin, elastase, collagenase and alkaline phosphatase have cold adaptive properties compared to mammalian enzymes. Some of these enzyme from marine sources has shown higher catalytic activity compared to mammalian enzyme for example when proteolysis must occur at low temperature.(Vilhelmsson, 1997)

The raw taste of herring changes stepwise to a salty ripened flavor by ripening and salting of the herring product. This formation is a very complicated process thus an outcome of the changes in flavor is the result from the biochemical (or enzymatic) processes that took place in the herring. The chemical processes are a complex set of reactions and in this type of chemical reaction; normally some proteolytic and lipolytic enzyme breaks proteins and lipids into smaller fragments respectively. The function of proteolytic enzymes is to break down the

peptide bonds of proteins resulting in smaller peptides and formation of relatively smaller peptides and amino acids from the protein molecules. This plays a significant role for changing the taste because amino acids have their own and particular flavor. During these processes the texture is also changed because protein is responsible to keep the cell structure intact, so protein breakdown means the degradation of cellular structure.(Aardal, 2013)

The properties of the digestive enzymes can be and are often exploited when ripening fish - herring is often barreled in brine with the intestines intact, utilizing the digestive enzymes to enhance the ripening process. If the fish were gutted and rinsed before storage, the proteolytic enzymes would be dominated by the intracellular cathepsins, and the ripening process would be slowed down. (Engvang & Nielsen, 2000; Hauch Nielsen, 1995; Moe & Munksgaard, 1991).

Lipolytic enzymes are responsible for enzymatic hydrolysis of fat, breaking down fat into free fatty acids and glycerol. This plays important role in digestive function. Lipolytic enzymes are mostly found in the digestive system of the animal. While whole ungutted herring is ripening in brine in sealed barrels, enzymatic lipid hydrolysis is the most important consideration not the microbial or oxidative breakdown.

During the ripening and salting of the herring, the raw taste of the herring gradually changes. In this case, addition of salt to the fish is an effective way to slow down the enzymatic reactions and at a very high concentration (20-25 %) of salt, the enzymatic process decreased to almost no activity level. This will increase the shelf life of the product without freezing it. However, exposing the herring to salt for extended periods of time will also severly change the sensory properties. High concentration brine will over time dry out the meat and yield a firm, tough texture (G. Stefánsson & Guðmundsdóttir, 1997)

A brief summary of proteolytic and lipolytic enzymes are given below

1.1.7.1 Proteolytic enzymes

Proteolytic enzymes (alternatively called proteases) break down the protein into small peptide fragments by cleaving the peptide bonds in proteins. This is a very common and most significant enzymatic processes in living organisms. Proteolysis is a process by which many of valuable biological processes are regulated.

The digestive proteolytic enzymes from aquatic organisms that are most commonly studied include pepsin, trypsin, trypsin-like, chymotrypsin, gastricin, and elastase (DeVecchi & Coppes, 1996), while the most studied muscle proteases include different cathepsins, calcium-activated proteases, heat-activated proteases, multicatalytic proteases, and metalloproteases. Most studies of enzymes from the digestive tract aim to characterize the digestive system or parts of this to learn more about the uptake and utilization of nutrients, or they focus on utilization of extracted enzymes. Studies of enzymes from fish muscle most often focus on the quality and shelf life of the fish muscle. Proteolytic activity in the liver of fish has not been well characterized (Sovik & Rustad, 2005).

Distribution of proteases are very diverse and depends on tissue type. The highest proteolytic activities are found in the internal organs, but high activity can also be found in muscle where the enzymes are important in catalyzing protein turnover (Foegeding, Lanier, & Hultin, 1996). Proteolytic activity also depends on pH, temperature and the life cycle of the fish, and enzymatic concentration and activity differ on season and diet (German, Horn, & Gawlicka, 2004).

Endogenous fish muscle proteases are located in intracellular fluids and in the sarcoplasm, or they are associated with various cell organelles. In the live animal, the proteases function in muscle protein turnover. After death, the biological regulation of the enzymes is lost, and the enzymes hydrolyze muscle proteins and resolve the rigor mortis contraction (Foegeding et al., 1996)

During iced storage of raw fish, the quality of fish muscle will deteriorate during iced storage of raw fish. Endogenous proteases, which are able to hydrolyze different proteins in the muscle, are important early in the deterioration process (Cepeda, Chou, Bracho, & Haard, 1990; Hultmann, 2004).

Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Barrett, 1994). Proteases are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent

groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960).

The proteolytic activity depends on location in the muscle, the life cycle of the fish, pH and temperature. In addition, the activity in situ is controlled by the presence of endogenous activators (mostly reducing compounds and metal ions) or inhibitors (both broad and specific inhibitors may be found in fish muscle). The enzymes are involved in protein catabolism in the muscle, and are important for the creation of gonads during the sexual maturation process and spawning migration (when the fish is not eating, but is using its own proteins for building gonads). For farmed fish, a change in culture conditions may change the enzyme content and proteolytic enzyme activity of fish muscle (Haard, 1992).

The content of free amino acids in the muscle of aquatic animals is normally higher than in muscle of land animals. The free amino acids play an important role as taste and flavor components, and the free amino acid content in muscle from aquatic organisms normally ranges from about 0.5% (w/w) to 2% of muscle weight. (Hultmann, 2004).

General proteolytic activity is usually measured using hemoglobin as a substrate. The activity varies with origin of the enzymes, in addition to incubation temperature and pH in the reaction mixture (Stoknes & Rustad, 1995).

In this work, in addition to general proteolytic activity, some selected proteolytic enzymes have been determined, for example; cathepsins , trypsin and chymotrypsin.

1.1.7.2 Cathepsins

The name cathepsin, which is derived from the Greek word kathepsein (to digest), was proposed for the protease that was active in a slightly acidic environment. (Turk et al., 2012). The cathepsins are involved in deterioration of muscle texture, and different cathepsins may act in concert to autolyze fish muscle (Hultmann, 2004). The cathepsins are related to protein catabolism of the fish during spawning migration, as the activity in white muscle is markedly higher in fish during spawning migration compared to fish in feeding migration (Yamashita & Konagaya, 1990).

Cathepsins are found in lysosomes. Cathepsin B has plays a role in lysosomal protein breakdown. Many types of cathepsins have been identified, among them, and cathepsins B

and L are the most important for deterioration of muscle texture (Hultmann, 2004). The activities differ between muscle fractions and fish species (Yamashita & Konagaya, 1990). The optimum activity of cathepsins are about 40-50 °C, and the activity is highly associated with temperature the activity decreases with reduction of temperature and increase vice-versa. However, usually they are effective best at pH 3-4; but at pH 6.0-6.5 few of them also maintain fairly high activity. Cathepsins B and L are activated by reducing compounds.

(Yamashita & Konagaya, 1990) reported that, in the muscle any treatment that causes breakdown of lysosomes will lead to an increased autolytic activity. Because of this, the fish muscle will be more vulnerable to deterioration of muscle texture. This may be especially important after thawing of fish, where ice crystals may have disrupted the lysosomes during the freezing process or the frozen storage (Hultmann, 2004).

1.1.7.3 Trypsin

Trypsin is a serine protease with a molecular weight of 24 kDa, found in the digestive system of many vertebrates, where it hydrolyses proteins (Rawlings & Barrett, 1994). Trypsin prefers to cleavage the positively charged amino acid side chain; where chymotrypsin prefers aromatic side chains.

It synthesized in the pancreas and secreted as an inactive trypsinogen from the pancreatic acinar cells together with chymotrypsinogen and other zymogens. Trypsinogen is activated by enteropeptidase in the small intestine and by the trypsin itself. Trypsin activates chymotrypsinogen and the other pancreatic zymogens (proelastase and procarboxypeptidase), and each active proteolytic enzyme has its substrate specificity. (Torrissen & Male, 2000).

Trypsin purified from anchovy viscera has been estimated to having a maximum activity at pH 9.0 and 45°C for casein and pH 8.0 for synthetic substances (Heu, Kim, & Pyeun, 1995). Flavor testing done on ripened octopi with added bovine and fish trypsin have shown that elevated levels of amino acids such as glutamate, alanine, leucine, serine, lysine, arginine and proline adds positive flavor to the seafood product (Lee, Simpson, & Haard, 1982).

It also helps to improve the texture of fish products; to tenderize meat; in the extraction of seasonings and flavorings from vegetable or animal proteins and in the manufacture of sauces (Protease, 2010). Generally, fish proteases are less thermostable and shows higher activity at low temperatures compared to bovine proteases (Asgeirsson & Bjarnason, 1991).

1.1.7.4 Chymotrypsin

Chymotrypsin, an endopeptidase, is a digestive enzyme existing in pancreatic tissues of vertebrates and inverbrates which is secreted into the duodenum (Geiger, 1985). The three types of chymotrypsin (A, B, C) have been found in mammals but only two types of chymotrypsin (A and B) have been found in fish (Yang et al., 2009). Chymotrypsin concentration is 105 times higher in the gut than in non-gut tissue but the activity and concentration vary according to fish species and the environment in which the fish live (de la Parra, Rosas, Lazo, & Viana, 2007; von Elert et al., 2004).

Fish chymotrypsin usually has two different forms: cationic (chymotrypsin B) and anionic (chymotrypsin A) (Yang et al., 2009). The inactive form of chymotrypsin (chymotrypsinogen) can be activated by trypsin which partially cleaves it into two parts while still maintaining an S-S bond (Geiger, 1985). The pH range of chymotrypsin activity is 7.5-9.0 (Kristjansson & Nielsen, 1992). Compared with mammalian chymotrypsin, fish chymotrypsin has similar amino acid composition and a molecular weight of 25-28.8 kDa (Cohen, Gertler, & Birk, 1981)

Chymotrypsin, together with trypsin, is thought to be very important for ripening of herring products. Inhibitor studies using chymostatin as a 100% inhibitor of chymotrypsin showed relatively lower amounts of valine, leucine, isoleucine, methionine, phenylalanine and tyrosine, but an otherwise unchanged relative amount of single free amino acids (Engvang & Nielsen, 2000).

Trypsin is an important protease, which can activate chymotrypsin during the digestive process. Therefore, the factors that influence the activity of trypsin will affect the activity of chymotrypsin. Both internal factors (trypsin phenotypes and fish life stage) and external factors (water temperature and fish starvation, feeding and nutrient condition) affect the activity of chymotrypsin. (Zhou., Budge, Ghaly, Brooks, & Dave, 2011)

1.1.7.5 Lipolytic enzymes

A lipase is an enzyme that catalyzes the hydrolysis of fats (lipids) (Svendsen, 2000) Lipolytic enzymes (lipases) hydrolyse triglycerides into fatty acids and glycerol, a very important process in digestive reactions of fats. Lipases can be found in many parts of the body, including blood, gastric juices, pancreas and pancreatic tissues, intestinal juices and adipose

tissues. Lipases facilitate the movement of lipids stored intracellularly - lipases in the bloodstream hydrolyse triglycerides in the bloodstream for energy usage and in adipose tissue for reentry into the bloodstream, giving them a role in biological membrane functions (Aardal, 2013).

This enzymatic lipid metabolism is especially important for many marine organisms, as carnivores get a lot of their energy intake from triacylglyceroles and wax esters in crustaceans, copepods, worms and fish (Cowey & Sargent, 1977).

Lipolytic enzymes have the ability to hydrolyse fat, due to this they offer a broad application in research, cosmetics, medicine, therapeutic usage and industry. In 2001, about a third of the total lipase sales were detergent enzymes (Sharma, Chisti, & Banerjee, 2001). A variety of lipases have been described in fish (Søvik, 2005), and have molecular weights ranging from 54-60 kDa, pH optimum 7-8 and temperature optimum 25-37°C (Mukundan, Gopakumar, & Nair, 1985).

The aim of the study presented in this thesis is to investigate the enzymatic activity profiles of digestive enzymes involved in the ripening process of herring as a function at pH, temperature and salt.

2 Materials and Method:

2.1 Raw Materials:

2.1.1 Frozen herring

First batch of frozen herring (whole fish) were collected from SINTEF Fisheries and Aquaculture Marine laboratories (SeaLab) at Brattøra, Trondheim on 1st July 2013. This herring was caught in the Trondheim fjord. The fish was kept in -40 degree freezer and at the day 1 it was taken out and the extract was prepared for further analysis. The crude extract from this batch was used for the analysis of proteolytic activity. Figure 2.1 shows the frozen herring taken out from freezer, before making the fillet.

Figure 2.1: Frozen herring from Trondheim fjord supplied by SINTEF.

2.1.2 Fresh herring

Fresh herring was supplied by SINTEF at 26 of November 2013. The extract was made by Trude Johansen and stored at -40C. This extracts was used for the analysis of lipolytic activity measurements.

2.1.3 Marinated Herring samples

Frozen herring fillets (141±3g) with skin, vacuum packed in 20 kg cardboard boxes produced 08.01.13 at Norway Pelagic, Kabelvåg was delivered at SINTEF Fisheries and Aquaculture and stored at -28°C till 1.07.13. The herring fillets were taken out of the freezer 28.06.13 and thawed at 4°C for two days and two to three hours in room temperature. The fillets were manually deskinned and stored at 4°C over night. The fillets were cut in pieces of 3-4 cm (weight around 5g). The pieces were premarinated (11%NaCl) for 17 hours at 4°C. Ratio fish to brine was 1:1. The premarinade was then removed and 10 different brines were added to the herring pieces.

Table 2.1: Salt content at different samples

Sample	Salt (% of reference level)	% KCl	% NaCl
1	100	100	0
2	100	75	25
3	100	50	50
4	100	25	75
5	100	0	100
6	80	0	100
7	60	0	100
8	40	0	100
9	80	25	75
10	60	25	75

Each box contained 61.5% herring and 38.5% brine. The boxes were stored at 4°C for 4 weeks. Samples were then removed, frozen in plastic bags and stored at -20°C until analysed for acid soluble peptides.

2.2 Preparation of samples:

2.2.1 Preparation of crude extracts for determination of General Proteolytic Activity (GPA):

The samples were thawed at room temperature until they were soft enough to be cut. This usually took 30 min to few hours depending on the size of the sample. The filets were separated by a scalpel from backbone after that each filet was trimmed with scalpel, the intestines was separated carefully and washed with distilled water, the fillet remove to avoid visual blood and intestines. By a sharp knife, skin and fins were also removed. The muscle and the intestine have been performed the same procedure separately. Then the samples were homogenised in a food processor before further analysis. Samples were taken out for the determination of dry matter and freeze-drying.

The muscle and the intestines were homogenized separately. Samples were weighed out into centrifugation bottles, the rest of the procedure was either done in a cold room (+4°C) or on ice in the laboratory. This procedure performed on ice in laboratory. The samples were homogenized in distilled water (1:1; here 60gm of fish was mixed with 60ml of water.) using an Ultra Turrax for 20 s, stirred for 10 min, and subsequently centrifuged at 10400X for 20 min at 4°C. The Ultra Turrax was run for a few second in pure water after each sample.

The supernatants were filtered through glass wool, and pH values of the water extracts were recorded. Protein concentrations in extracts were determined by the BioRad method (Bradford, 1976) with bovine serum albumin as standard. The analyses were performed in triplicate. The crude extract were distributed into suitable plastic bottles and stored at -40°C.

2.2.2 BIORAD method for Protein determination:

Protein concentrations in extracts were determined by the BioRad method (Bradford, 1976) with bovine serum albumin as standard. The analyses were performed in triplicate. The biorad method is based on the fact that the maximum absorbance of an acid solution of coomassie brilliant blue G-250 shifts to 595 nm when the color is bound to protein. The concentration of BSA stock solution was 1mg/ml. it was taken out from the freezer and thawed and kept in ice. The dye reagent was delivered as concentrate and kept in cold room

or on ice. It was diluted as 1:4 with distilled water. Diluted dye reagent was kept in dark bottle at room temperature and it could be kept in 14 days.

Dilutions from the stock solutions of the standard BSA as 0.2; 0.4; 0.6; and 0.8 mg/ml. 100 µl of suitable diluted samples were pipet out into test tubes. Three parallels were taken for each sample. The standards and the samples and 5.0 ml of dye reagent were added to the blank and mixed by whirl mixer. The absorbance was read out at 595 nm wavelengths after 5 minutes.

2.2.3 Determination of enzymatic activity

General proteolytic (GPA) and lipolytic (GLA) activity, enzymatic activity of chymotrypsin, trypsin, and cathepsin B was measured. Analysis of single enzymes used the same method (I. Stoknes & Rustad, 1995), changing only the substituted 7-amino-4-methylcoumarin substrate specified for each enzyme. GPA was measured using a modification of the hemoglobin substrate method (Barrett, 1972) and the amount of acid soluble peptides were determined by Lowry's protein measurement method (Lowry, Rosebrough, Farr, & Randall, 1951a). GLA used a method involving a liposomal dispersion (Izquierdo & Henderson, 1998).

2.2.4 Determination of General proteolytic activity (GPA)

The procedure for the first step of the determination of proteolytic activity is incubation of the enzyme extracts with the substrate haemoglobin. Each enzyme extract was analysed in triplicate.

The proteolytic activity of the enzyme extract was determined in a two-step method according to (Barrett, 1972) and (Lowry et al., 1951a) with minor modification (Stoknes & Rustad, 1995). Haemoglobin was used as a substrate for measuring the proteolytic activity as an increase in acid soluble peptides.

The incubation mixture consisted of 1.2 mL of phosphate-citrate buffer (McIlvaine, 1921) and 0.4 mL of substrate (bovine hemoglobin Sigma H-2625 1% w/v), and to the zero sample 2 mL of 5% w/v TCA (trichloroacetic acid, Merck) was also added.

The samples were preincubated for 10 min in water baths before 0.4 mL of suitably diluted enzyme extracts were added. Incubation time was set to 1 h at 10 °C and 40 °C. The reaction was stopped by addition of 5% w/v TCA and mixed on a whirl mixer. The samples were

cooled for 30 min and then filtered, (parallel one filtered twice and one filter paper was all three parallel) before the amount of short peptides in the filtrate was determined according to Lowry method (Lowry et al., 1951a).

2.2.5 Determination of protein content, Lowry method

Reagent used in Lowry:

Alkaline copper reagent: 1 part 1% $CuS0_4$, 1 part 2% potassium sodium tartate, 100 parts 2% Na_2C0_3 in 0.1 M NaOH

Folin phenol reagent: 1 part folin phenol reagent, 2 parts distilled H_20

Bovine serum albumin (BSA): Bovine serum albumin (BSA) was used as a standard. Activities are expressed as mg hemoglobin cut per g water-soluble protein per hour and are given in arbitrary units (U) based on the mean of three measurements. Activity was determined at three different pHs 5, 6 and 7, and at two different temperatures, 10 and 40°C.

The determination of protein content in a solution after incubation of enzyme extracts with a certain substrate, indicates the amount of the proteins the enzymes have cut and the proteolytic activities of the different enzyme extracts can then be compared.

Measured OD was compared to blank samples (blanks had TCA added immediately with no incubation time) for accurate measured enzymatic activity. Absorbance was measured at 750 nm using a spectrophotometer for all samples, and activity was calculated using a measured standard curve using bovine albumin serum (sample concentrations 12.5, 25, 50, 100, 150, 200 and 300 μg/ml).

2.2.6 Acid-Soluble Peptides.

Proteins in the water soluble extract were precipitated with tricloroacetic acid (TCA) as described by (Le, Datta, & Deeth, 2006). Equal volumes of TCA and extract were mixed, left at room temperature for 30 minutes and filtered through black band filter. The final TCA concentration was 10 %. Then the protein content of the extracts were determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951b). Determination was done in triplicate.

2.2.7 General lipolytic activity (GLA) measurements

Activity of lipases was determined by spectrofluorimetry according to the method described by (Roberts, 1985) and later by (Izquierdo & Henderson, 1998) with minor modifications. The nonfluorescent substrate, 4-methylumbelliferyl heptanoate was solubilized in a liposomal dispersion of soya lecithin. This substrate is hydrolysed to heptanoic acid and the highly fluorescent compound, 4-methylumbelliferone through the action of lipases.

Mixtures of 20 μl substrate and 40 μl of enzyme solutions, suitably diluted in disodium hydrogenphosphate-citric acid buffer (McIlvaine, 1921) at pH 5 and pH 6, were incubated for 15 min in water-baths at 4, 10, 24, 30 and 40 $^{\circ}$ C.

Reactions were stopped by adding 3 ml of cold 1 mol/l Tris HCl, pH 7.5. Experiments confirmed a linear relationship between increase in fluorescence and time under these conditions. For zero time samples extracts were incubated in water-baths at 80 $^{\circ}$ C for 30 min, centrifuged at 3000rpm for 10 min, and diluted and measured as the respective sample.

The fluorescence of each sample measured immediately with a spectrofluorometer, excitation excitation wavelength 365 nm, slit 10 nm and emission wavelength 450 nm, silt 5 nm. Activities are expressed as an increase in fluorescence and given in arbitrary units (U) based on the mean of three measurements.

Liposomal dispersion: 10 mg 4-metylumbelliferyl hepatone (4-MUH) and 27.1 mg L-α-phosphatidyl choline (soya lecithin) was mixed with 34.6 ml 2:1 chloroforom:methanol and evaporated to about 1 ml on a rotary evaporator at 40^0C. The evaporated solution was transferred to a test tube before evaporating the rest of the solvent on a heat block with N_2 and completely drying before desicator (about 1 hour). 8.65 ml 0.15 M NaCl was added to the solution before mixing on the vortex mixer for 20 minutes at maximum and leaving the tube in an ultrasound bath for 30 seconds. This mixing ultrasound steps were repeated until most of the crystals on the tube wall had dissipated in the salt solution (3-4 times). The dispersion was filtered throgh a 0.45 μm and 0.22 μm filter (Milipore) and kept in a refrigerator (4^0C) or in a cold room until used (up to 4 days after preparation).

2.2.8 Specific proteolytic activity measurements

Specific proteolytic activity was determined according to the method described by (Hultmann, Bencze Rørå, Steinsland, Skåra, & Rustad, 2004). The peptidase activity was measured against a synthetic fluorogenic substrate, N_{α}-carbobenzoxy-arginine-arginine-7-amido-4-methylcoumarin (Barrett & Kirschke, 1981).

100 µl of suitably diluted enzyme extract was incubated with 100 µl substrate at 4° C. The reaction was stopped after 10 minutes by adding stopping solution. A blank was prepared by adding distilled water instead of enzyme extract to the reaction mixture. When the enzymes cut the substrate, 7-amino-4-methylcoumarin (AMC) is liberated.

Fluorescence of AMC was measured at 460 nm (5 nm slits) after excitation at 360 nm (10 nm slits) at Fluorescence Spectrometer. Activities are expressed as the increase in fluorescence (fluorescence valoe of blank subtracted from that of sample), given as increase in fluorescence per gm wet weight per minute during incubation. The analyses were run in triplicate.

Chemicals/solutions:

- Substrate stock solution: 3 mM substituted 7-amino-4-metylcoumarin (substrate) in dimethylsulfoxide (DMSO). To be stored in aliquots of 300 uL in eppendorf tubes at -20 °C.
- Substrate working solution: Dilute stock solution 1:32 (300 uL stock) with distilled water (9,300 uL).
- 150 mM mM bis-Tris, 30 mM EDTA, 6 mM DTT at pH 6.0 or 7.0 (assay buffer for cathepsin B- and B+L-like enzymes).
- 1 % SDS 50 mM bis-Tris buffer pH 7.0 (stopping solution).

Substrates for different (groups of) enzymes:

- *t*-butoxy-glutaminyl-alanyl-arginine-4-methylcoumaryl-7-amide: trypsin-like activity.
- Succinyl-leucyl-leucyl-valyl-tyrosine-4-methylcoumaryl-7-amide: chymotrypsin-like activity
- Carbobenzoxy-arginyl-arginine-4-methylcoumaryl-7-amide: cathepsin B-like activity.

- Carbobenzoxy-phenylalanyl-arginine-4-methylcoumaryl-7-amide: cathepsin B+L-like activity.

3 Result and Discussion:

3.1 Amount of soluble proteins

Biorad result shows the extractability of amount of protein from extract. From the figure 3.1 it was detected that the amount of soluble protein was higher in the intestine and this result was expected.

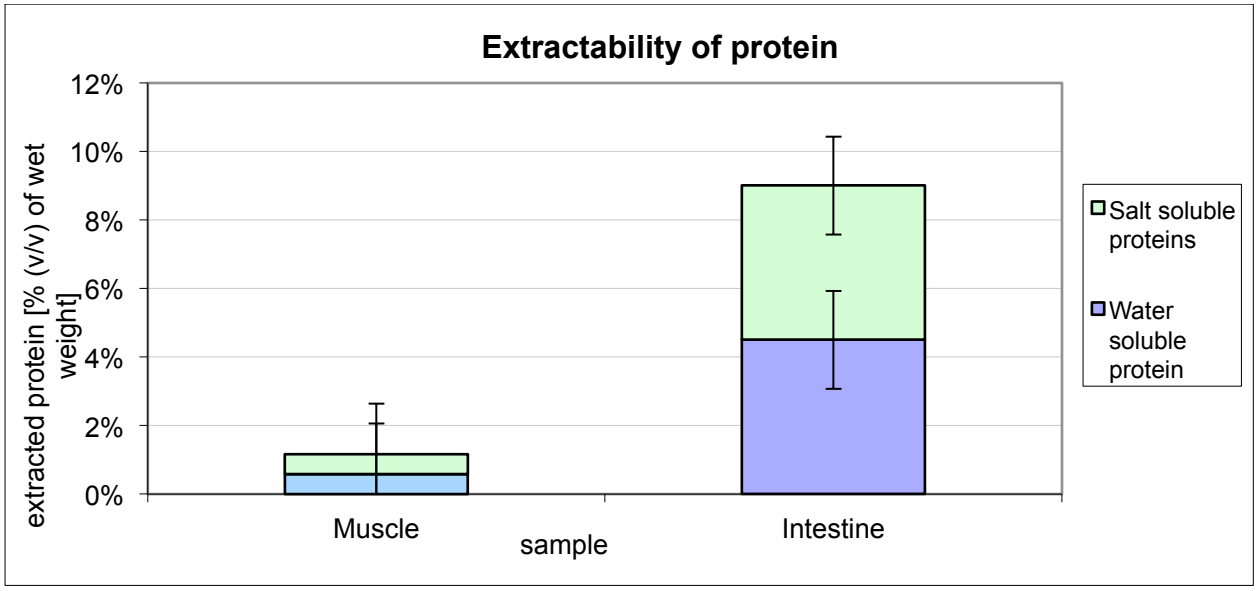

Figure 3.1: Amount of soluble protein (salt soluble and water soluble) content in extract.

3.2 Enzymatic analysis

3.2.1 General proteolytic activity (GPA)

The activity was measure by breakdown of hemoglobin and the results are shown for muscle and intestine samples in figure 3.2 and figure 3.3.

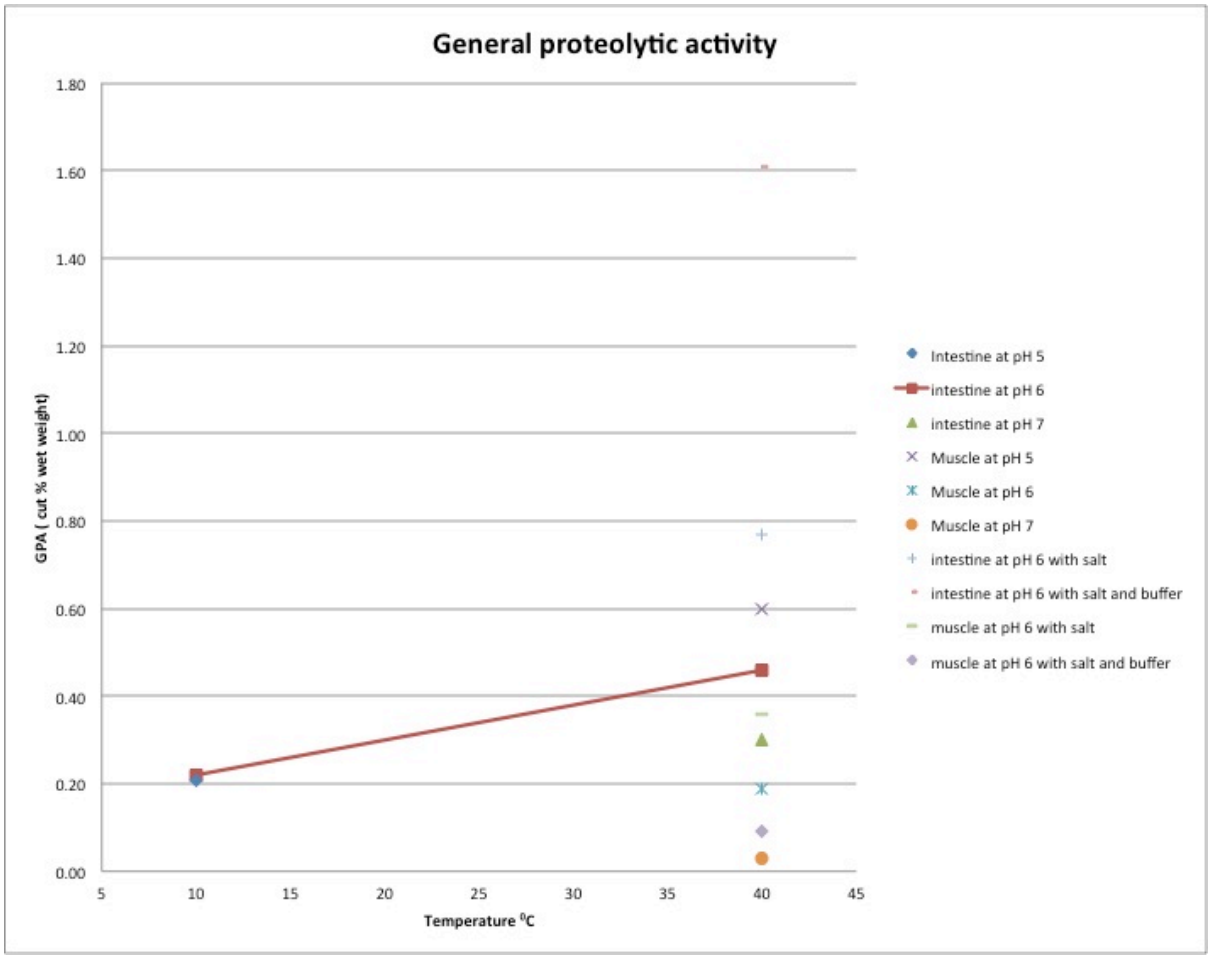

Figure 3.2: General proteolytic activity of herring intestine and muscle at different temperature conditions. Values are given as means of three parallels.

The figure 3.2 showing the gerenal proteolytic activity (cut in % of wet weight) of herring muscle and intestine at 10^0C and 40^0C at three different pH (pH 5, 6 and 7). A higher level of general proteolytic activity was detected in intestine at pH 6 (40^0C) than that of intestine at pH 6 (10^0C). The activity increased to a bit higher level when it contains salt and the activity increased more than that when it contains buffer in addition with salt. In intestine higher activity was found at pH 6.

On the other hand, the lowest activity was found in muscle tissue at pH 7 (40^0C). There is a very slight difference in activity when the temperature is low (10^0C) between muscle at pH 5 and intestine at pH 6. In case of muscle higher activity was found at pH 5 than at pH 6 and 7. When the samples contained, salt or salt and buffer, the activity was not increased for muscle even when there was buffer the activity was less than when there was only salt.

Our results are, however, in accordance with findings of (Sovik & Rustad, 2005) who found proteolytic activity in cod liver to be remarkably high at acidic pHs.

It was also found in case of muscle that, the addition of salt increases the activity but addition of buffer with salt potentially decreases the activity. So, changes in the salt significantly affect the protein degradation. Addition of salt affected the pH , pH was adjusted by adding suitable base.

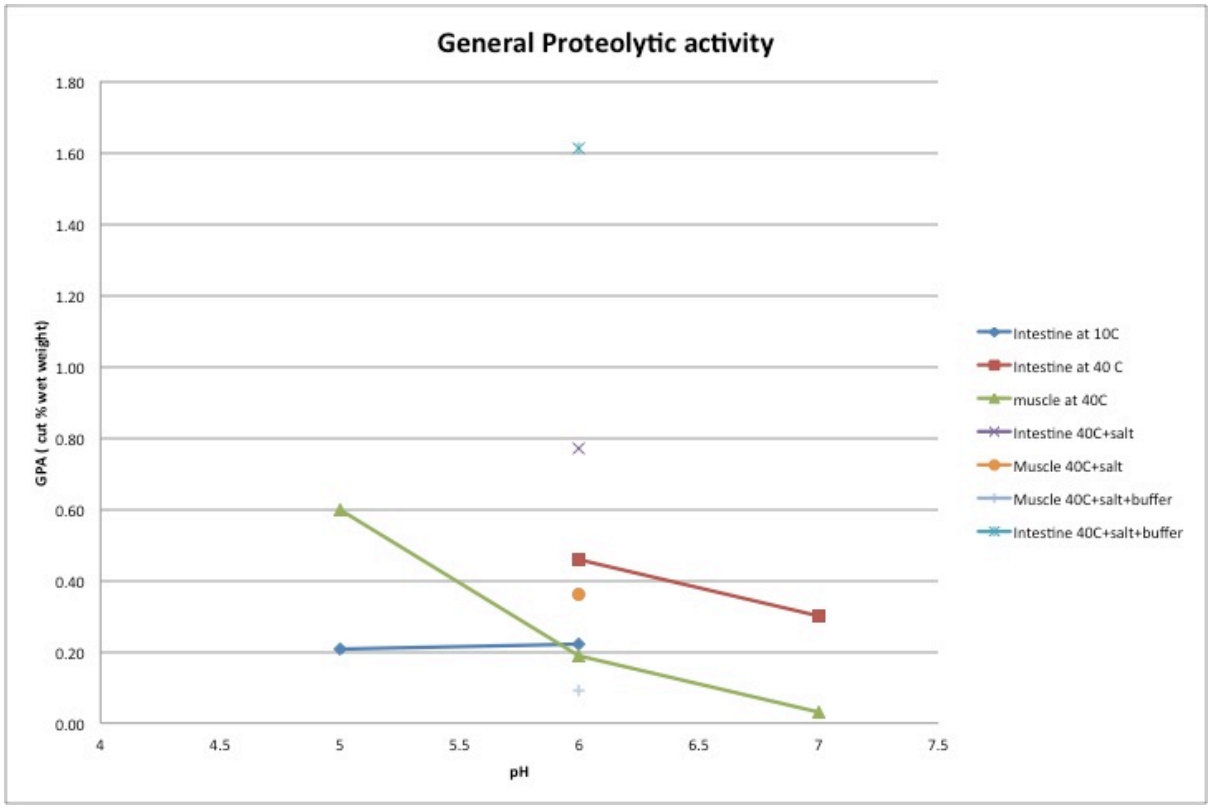

Figure 3.3: General proteolytic activity of herring intestine and muscle at different pH. The values are given as mean of three parallels.

Figure 3.3 represent the general proteolytic activity at different temperatures condition.

The general proteolytic activity measures a "sum" of different proteolytic activities. In this case, the overall maximum activity was detected at pH 6, and that was expected. For muscle the activity at 40^0C is highest at pH 5, due to cathepsins. The activities gradually decreased when the pH gradually increased and the lowest activity was found at pH 7. At pH 6 muscles had higher activity when salt was added to the samples but at the same condition the activity

decreased when buffer was added togather with the salt. The same results was found by (Sovik & Rustad, 2005).

Sovik & Rustad, (2005) found proteolytic enzymes from cold adapted fish have been found to exhibit higher activity at low temperatures. The pH and temperature relationships of the muscle proteases of herring were studied in separate experiments using hemoglobin as substrate. In the acidic range these proteases exhibited considerable activity over higher temperature (Iren Stoknes, Rustad, & Mohr, 1993).

The activity in intestine at low temperature (10 ^{0}C) had no significant change between pH 5 and pH 6 but in high temperature for instance at 40 ^{0}C the activity was detected higher at pH 6 than that of pH 7. This was very similar to the findings of (Stoknes et al., 1993). The expected higher activity in intestines is at pH 6. In this work highest activity was found when there was salt in and it was increased drastically when salt and buffer both was present. Stoknes et al., (1993) found that, in herring, the highest proteolytic activity was found in the intestines and the proteolytic activity was highest at acidic pH. At lower temperatures (around 40°C) the dominating proteolytic activity was found in the intestines (Stoknes et al., 1993).

Stoknes et al., (1993) concluded that, in herring, proteolytic activity exhibiting an acidic pH-optimum seemed to dominate completely in all tissue fractions examined at 40°C. The activity was far higher in the intestinal fraction than in the other samples analysed. In comparison, only limited activity could be observed in muscle at low pH, and in the alkaline region an even lower activity was detected. That partially supports the result we got.

3.2.2 Specific proteolytic activity

Figure 3.4, 3.5 and 3.6 show the result of the specific proteolytic activity measurements during the ripening periods.

3.2.2.1 Cathepsin B

Cathepsin B is a lysosomal cysteine protease found in lysosomes (Barrett & Kirschke, 1981). The most studied proteases in fish muscle are the cathepsins (Sovik & Rustad, 2005). Cathepsin B, C, and L have all been found to show optimum at pH values between 5 and 6.5

and could therefore be the dominating proteases measured at pH 5 (Sovik & Rustad, 2005). Activity of cathepsin B was measured against synthetic fluorogenic substrates, CBz-Arg-Arg-4-methylcoumaryl-7-amide (Barrett & Kirschke, 1981).

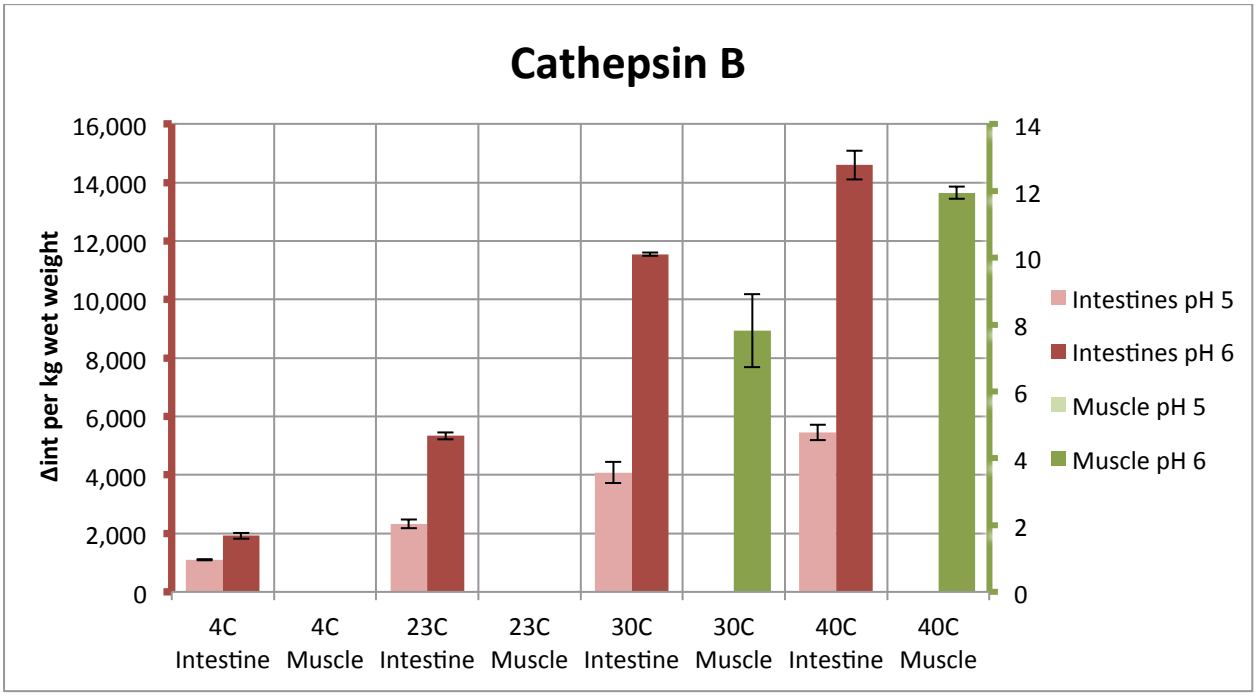

Figure 3.4: The activity of cathepsin B

The activity of cathepsin B (from figure 3.4) was higher in intestine at pH 6 than pH 5, whereas for the muscle very low activity was found at pH 5 than that of pH 6. Cathepsin B had the maximum activity at 30 ^{0}C and 40 ^{0}C at pH 6 in intestine. Inn muscle highest activity was found in 40 ^{0}C at pH 6. The activity of cathepsin B gradually increased with the increase of temperature at pH 5 in intestine. Similar result was also found at intestine at pH 6 but the activity was much higher that at pH 5. The highest activity of cathepsin B in herring muscle was found in 40 ^{0}C.

(Sovik & Rustad, 2006) found in (Aranishi, Hara, Osatomi, & Ishihara, 1997) that, Cathepsin B from cut off had maximum activity at 50 °C in cod and saithe, 35 °C in tusk and ling and 20 °C in haddock, while cathepsin B in liver had highest activity at 50 °C in saithe and tusk, and 35 °C in cod and haddock. All species showed maximum cathepsin B activity at the same

temperature in viscera (35 °C). Cathepsin B from hepatopancreas (a digestive gland) of carp (*Cyprinus carpio*) has been found to have a temperature maximum of 45 °C (pH 6) (Aranishi et al., 1997). Cathepsin B from mackerel muscle showed temperature optimum at 55 °C (pH 6.5) (Jiang, Lee, Tsao, & Lee, 1997), from *Mujil auratus* muscle at 45 °C (pH 6.0) (Bonete, Manjon, Llorca, & Iborra, 1984).

This is little higher than we found from Herring, but closely similar findings in some of the other species also noted by researchers.

3.2.2.2 Trypsin

Trypsin and chymotrypsin are synthesized as inactive zymogen precursors (trypsinogen and chymotrypsinogen) to prevent unwanted destruction of cellular proteins, and to regulate when and where enzyme activity occurs. Both are serine protease.

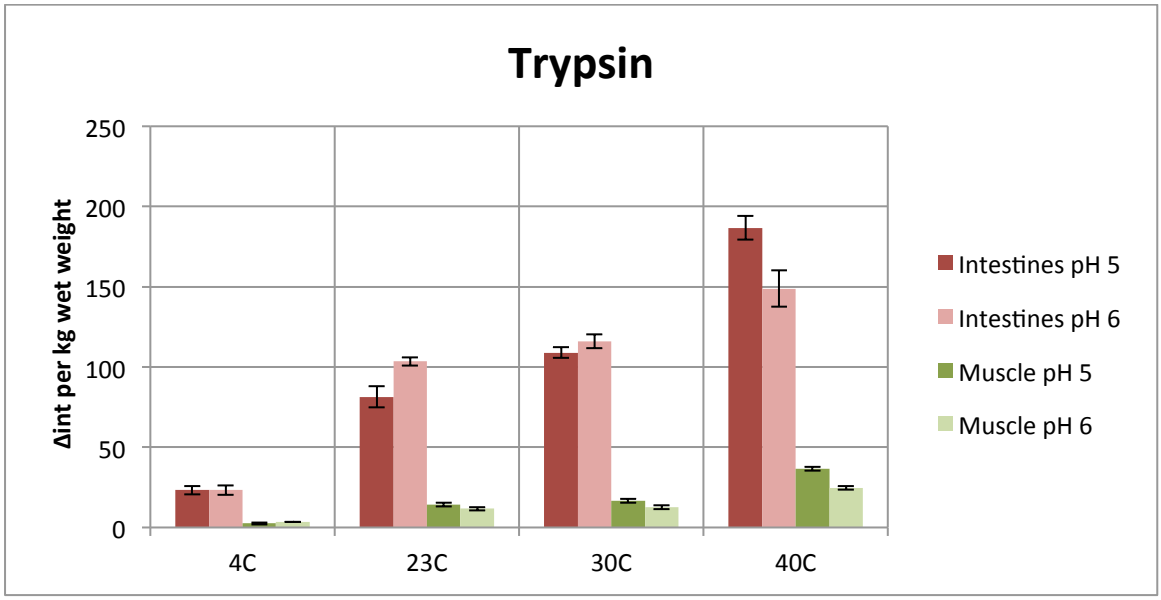

Figure 3.5: the activity Trypsin

In intestine of herring, trypsin had shown the highest activity at pH 5 in 40 °C, but when the temperature decrease then the activity increase at lower pH for instance 5. Similar pattern of result also found for herring muscle at 40 °C. in every cases intestine shown the higher activity than muscle. The lowest activity was found 4 °C for both intestine and muscle. For muscle pH always showed more activity than pH 6. When the temperature increase from 23

to 30 °C, the activity increase with temperature for both pH 5 and 6. At very low temperature i.e. 4 °C there is no significance difference in activity for both muscle and intestine at pH 5 and pH 6 accordingly.

Peptidase activity of trypsin, chymotrypsin increased to a considerably higher level in the thawed salted herring muscle in comparison with the fresh salted herring. (Gudmundur Stefánsson et al., 2000).

3.2.2.3 Chymotrypsin

Chymotrypsin has 245 amino acid residues and 5 pairs of disulfide linkages with a molecular mass around 24000. Generally, the single polypeptide molecular weight is 25-28 kDa . The inactive form of chymotrypsin (chymotrypsinogen) can be activated by trypsin which partially cleaves it into two parts while still maintaining an S-S bond. N h

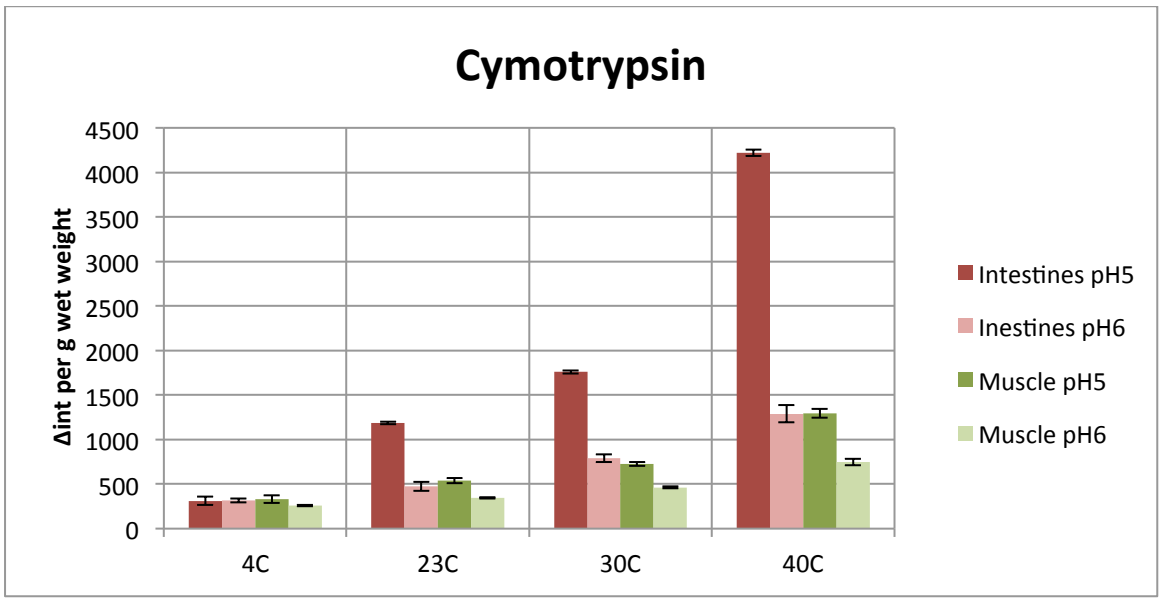

Figure 3.6: the activity of Chymotrypsin.

Studies have shown that high salt content may prevent activation of the intestinal serine proteinases by affecting either the proenzymes or the key-activating enzyme. (Gudmundur Stefánsson et al., 2000). As shown in figure 3.5 and figure 3.6, chymotrypsin measured to

have an about 10 times higher activity than trypsin, the second highest measured enzyme in herring filet. It means that the changes in chymotrypsin activity will have higher impact on total proteolytic activity.

The pH range of chymotrypsin activity is 7.5-9.0 (Kristjansson *et al*., 1992). In our results the highest activity was found at pH 5. At pH 5 the activity of chymotrypsin in intestines gradually increased with temperature and it reached its peak at 40 °C. It means that, temperature has a significant effect on the stability of chymotrypsin. Related results was found by (Sabapathy & Teo, 1995). (Sabapathy & Teo, 1995) reported an optimal temperature range of 45-55^{o}C for chymotrypsin extracted from rabbitfish and observed denaturation of the enzyme at a temperature of 60°C.

3.2.3 General lipolytic activity (GLA)

Activity of lipases was determined by spectroflurimetry according to the method describing by (Roberts, 1985) and (Izquierdo & Henderson, 1998) with a minor modification.

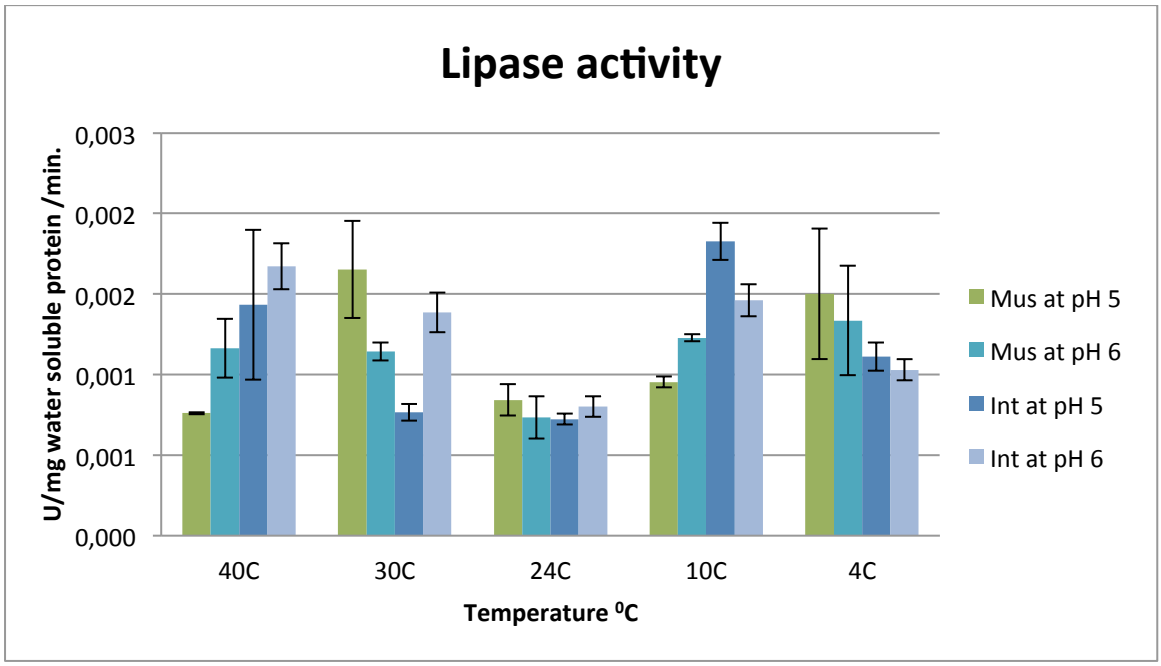

Figure 3.7: General Lipolytic avtivity

From the figure 3.7, the result we found in our study is very low. It can be said with the comparison of the other studies that there is almost no activity found. It due to may be any error, while we were preparing the liposomal dispersion solution or any experimental error.

From figure 3.7 we can assume that the highest activity was found for muscle at low temperature while intestine in higher. Lipase activity at pH 6 was higher in intestine at 40°C. Distinct differences in lipolytic activity in different tissues of fish have also been described by other researchers. Lipase activity (pH 7) was found to be highest in intestines in oil sardine, in stomach and pyloric caeca in mullet, in red muscle in mackerel and in the liver/hepatopancreas in rohu (Nayak, Viswanathan Nair, Ammu, & Mathew, 2003). Lipase activity at pH 6 was considerable higher that the activity at pH 5 for intestine.

3.2.4 Total peptide content

The amount of water soluble protein is higher at lower salt content showing that reducing the salt content in the marinade will result in increased protein solubility and might lead to more protein leaking out of the herring into the brine. If 25% of the NaCl is exchanged with KCl, then the amount of water-soluble proteins is also increased in figure 3.8. This shows that the type of ions, not only the salt concentration, influences the proteolytic activity. This should however be investigated further.

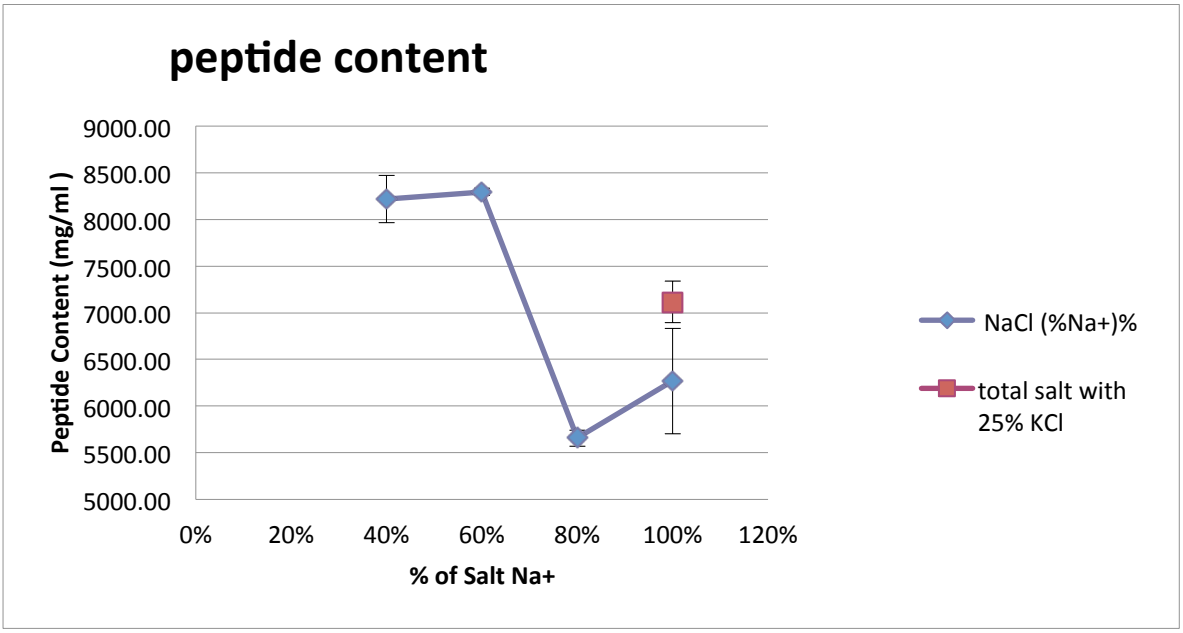

Figure 3.8: total peptide content

The peptide content slightly increase when the temperature shifted from 40 % to 60 %, but this is very little and negligible and similar things happed in the case of 80 % to 100 %.

However, there was a drastic decrease observed when the temperature decreased from 60 % to 80 %.

3.2.5 Acid soluble peptide

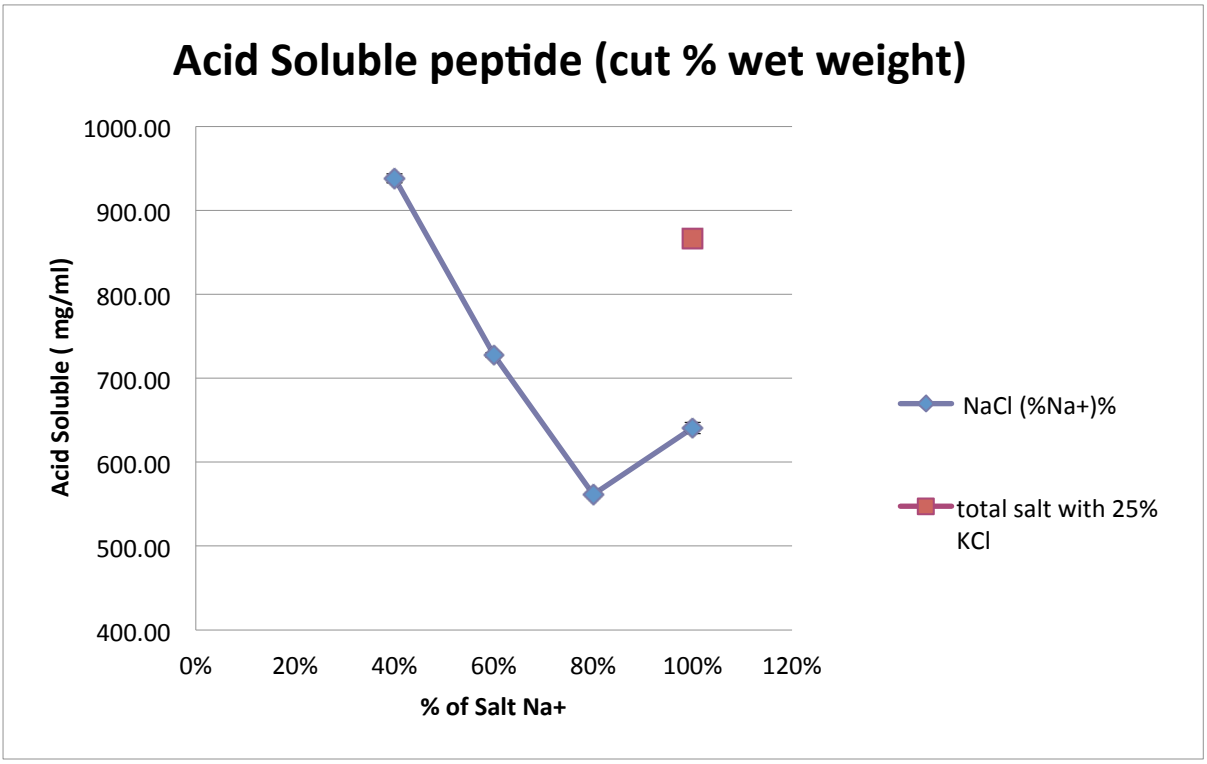

Figure 3.9: Acid Soluble peptide

Acid soluble peptide measured as cut % wet weight. Herring muscle with different % of salt. Acid soluble peptide of herring muscle decreased gradually when salt content increased, from 40 % to 80 % of the salt content in the control sample. If 25 % of NaCl is exchanged with KCl also increase the activity; this shows that the proteases are affected by the type of ions. But from 80 to 100 % the change is small. High amounts of free amino acids and acid-soluble peptides indicate that more of the proteins in the samples are degraded.(Siri Lise. Sovik & Rustad, 2005).

4 Conclusion

Preservation techniques for herring is very important in order to keep the nutritional content for example protein and fat, intact for longer period. Herring can be preserved in many ways, and some of the preservation techniques are hundred years old but the important issue is there is many ways to improve the ripening procedure. For this reason its important to know the biochemistry of such procedure. Ripening procedure can be modified in such a way so that the taste, smell and texture would be satisfactory, time of ripening process could be shorter.

This thesis is a representation of the result of, how the biochemical parameters of herring changed by the enzymatic ripening procedure for instance; proteolytic and lipolytic enzyme activity. This work also shows, how protease activity affect the peptide and amino acid content in herring.

The overall maximum general proteolytic activity was detected in intestine at pH 6. The activity increased to higher level when it contains salt and buffer. The lowest activity was found in muscle tissue at pH 7. There is a very slight difference in activity when the low temperature. Salt and buffer does not afftet the proteolytic activity in muscle but it significantly after the intestine. So, changes in the salt significantly affect the protein degradation. The activities gradually decreased when the pH gradually increased. The highest proteolytic activity was found in the intestines and the proteolytic activity was highest at acidic pH.

The activity of cathepsin B was higher in intestine at pH 6 than pH 5, whereas for the muscle very low activity was found at pH 5 than that of pH 6. Cathepsin B had the maximum activity at 30 ^{0}C and 40 ^{0}C at pH 6 in intestine. In muscle highest activity was found in 40 ^{0}C at pH 6. The activity of cathepsin B gradually increased with the increase of temperature at pH 5 in intestine.

Trypsin and chymotrypsin are serine proteases synthesized as inactive zymogen precursors. In herring, trypsin had shown the higher activity at pH 5 in 40 °C. Similar pattern of result also found for herring muscle at 40 °C. in every cases intestine shown the higher activity than muscle. At very low temperature i.e. 4 °C there is no significance difference in activity for both muscle and intestine at pH 5 and pH 6 accordingly. In this work, chymotrypsin measured to have an about 10 times higher activity than trypsin, the second highest measured enzyme

in herring filet. It means that the changes in chymotrypsin activity will have higher impact on total proteolytic activity.

The amount of water-soluble protein is higher at lower salt content showing that reducing the salt content in the marinade will result in increased protein solubility and might lead to more protein leaking out of the herring into the brine. Is salt is replaced the activity changed at a significance level, its not only concentration dependent but also ion dependent. The peptide content also affected by temperature.

Acid soluble peptide of herring muscle decreased gradually when salt content increased and If NaCl is exchanged with KCl also increase the activity; this shows that the proteases are affected by the type of ions.

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Appendix

Raw data and calculations used to obtain the results showed in this thesis can be found as several Microsoft excel worksheets on the attached CD. The files are named after their content and divided by type of experiment.