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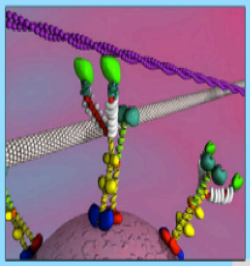
High pressure processing of fish and protein denaturation


Biological Chemistry

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by Lene E. Kramer

Universitetet i Stavanger

Nofima

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Abstract

High pressure processing (HPP) is a relative new method for inactivating microorganisms and extend the shelf life of food. This technology has gained increasing attention as an innovative and safe way to process different types of food products. Norwegian salmon is surfing an international popularity wave of sushi, which is now found in retail stores as well as restaurants. Refrigerated storage for a few days is desirable from the producers' point of view, while the consumers demand a texture, colour and eating quality equal to a fresh fish. Furthermore everyone is concerned about food safety. Can all these demands and desires be satisfied by high pressure processing?

In this thesis, a study of the protein denaturation that takes place during HPP of Atlantic salmon is presented. In addition protein denaturation was investigated for raw turbot and compared to salmon. Salmon was exposed to the pressures: 100, 150, 200, 250, 300, 350, 400, 500, 600 and 685 MPa and the pressure treated samples were compared with unprocessed control samples. To investigate the colour changes during HPP, pictures were taken for colour analysis. The denaturation of proteins in salmon during HPP was studied by differential scanning calorimetry (DSC) and polyacrylamide gel electrophoresis (PAGE). The DSC is a method to characterise the energetics of temperature induced conformational changes in proteins. PAGE separates the proteins according to size in an electric field. In addition, different gel electrophoretic techniques were used like native, combined native and denatured, and finally denatured PAGE. From these techniques more information about the proteins in their biological native form with intact complexes and subunits could be studied. Mass spectrometry (MS) was used to identify the proteins of selected bands from the gels. The result of MS analysis was fragments of peptides, which were identified by protein blasting.

Myosin and actin were the two main proteins investigated. DSC analysis determined the onset of denaturation for myosin between 200-250 MPa, while actin denatured at approximately 300 MPa. Comparing these findings with results from native gel electrophoresis, the loss of protein bands between 480-720 kDa indicated the onset of myosin denaturation starting at 200 MPa while actin was stable at all pressure loads. MS analysis of SDS-PAGE identified myosin heavy chain just below 250 kDa, while myosin light chain, troponin I and troponin C was identified around 20 kDa. Denaturation enthalpies from DSC of raw salmon and turbot

were compared, and resulting in the highest myosin denaturation for salmon, while turbot had the highest actin peak. DSC curves of turbot were uneven jagged curves; therefore HPP was not performed on turbot. Post mortem aging of turbot was also investigated by DSC and gel electrophoresis, results showed the myosin denaturation remained stable during cold storage, while actin denatured with a slow rate towards day 24.

The overall conclusions from these investigations were that DSC alone could not provide accurate information about denaturation of proteins by HPP. Native gel electrophoresis should be the preferred method for further denaturation analysis of HPP fish in combination of MS and potentially Western blotting.

1.0 Introduction

The aim of this Master thesis has been to learn about the possible effects on protein denaturation induced by high pressure processing (HPP) on Atlantic salmon (*Salmo salar*). The Atlantic salmon samples were exposed to pressures from 100-685 MPa. The different analyses used to investigate the effects of protein denaturation were differential scanning calorimeter (DSC), water holding capacity (WHC), protein gel electrophoresis (native, 2D and denatured gel), and mass spectroscopy (MS). Colour changes in the fish before and after processing were also documented by colour measurement of photography. Analysis of turbot (*Scophthalmus maximus*) to investigate protein denaturation was done using DSC analysis and gel electrophoresis.

1.1 Atlantic salmon

Norwegian salmon is found in the Atlantic Ocean. The salmon is anatropous meaning that it is born in fresh water, before it migrates to the ocean and then returns to fresh water to reproduce. Salmon is considered a healthy nutritional fish. Nutritional value of salmon is given in Table 1. Salmon is a fish with high fat content, and the fat is influenced by environmental factors such as diet, season, ranching condition, temperature and biological differences such as stage of maturity, age, sex and size. The fish flesh of salmon has a characteristic red colour due to the red carotenoid pigment astaxanthin that is also found in shrimps, krill or added to the fish feed.

1.2 Turbot

Turbot is a ray-finned flatfish. It is found close to the shore in sandy shallow waters in the Mediterranean, the Baltic Sea and the North Atlantic. Turbot has a characteristic firm flesh and is considered delicious. It has been tried cultivated in combinations to power plants with easy access to hot spill waters. Nutritional value of turbot is given in Table 1.

Table 1. The different nutritional values of salmon and turbot [1].

	Salmon	**Turbot**
Proteins (%)	18	16
Omega 3 (%)	1 - 4	1
Fat (%)	14 - 23	2
Carbohydrates (%)	>0.1	0.1

1.3 Fish muscle fibres

Fish muscles lack the tendinous system connecting muscle bundles to the skeleton as seen in mammals. Fish muscle cells run in parallel and are connected to sheaths of connective tissue (myocommata), which are anchored to the skeleton and the skin. The myomeres are shaped like a W on its side, so they fit into each other as series of cones. The most striking difference between striated fish muscle and that found in higher vertebrates, is the separation of fibre types into layers in fish [2]. The high glycolytic and anaerobic type also known as fast or white fibres constitute 90-95 % of all muscles in most fish species. The red muscle fibres are usually found as a narrow strip along the lateral line and contribute to about 10 % of the myotomal musculature. Intermediate or pink fibres are intermediate in position and by structure between red and white muscles. The red muscle fibers are also called slow fibers and are used mainly for sustained energy efficient swimming. The characteristics of this muscle type are good capillary supply, high amount of mitochondria, lipid droplets and glycogen stores. The concentration of myoglobin and cytochromes are high. The energy metabolism in red muscle is almost entirely aerobic, based mainly on lipid as fuel complemented with carbohydrates [3]. The white muscle type is used at high swimming speeds like a fast-start burst swimming for prey capture and escape response. The white muscle fibers are tightly packed with myofibrils occupying between 75 and 95 % of the fibre volume. Organelles such as mitochondria which interrupt the arrays of myofibrils, are few and both lipid droplets and myoglobin are present in very low levels in most species [4, 5]. Muscle tissue growth in fish is due to two different processes. The first process is called hypertrophy where there is growth of existing fibres; new nuclei are developed within the existing fibre. The second process is called hyperplasia where there is proliferation of new muscle stem cells to form new fibres. The growth-restricting factor for fish is the diameter of muscle fibre in order to maintain the

muscular aerobic capacity allowing diffusion of oxygen and metabolites [4, 6, 7]

1.4 Water in fish muscle

Water is the main constituent in muscle with about 66-72 % and has an important influence on quality-related parameters like appearance, texture and storage stability of muscle-based food. Not only the total water content governs the stability and quality but the cellular distribution like binding state and mobility of the water is also important [8]. Muscle tissue water can be seen in context of how tightly it is bound to subcellular structures as e.g. myofibrils. The water is kept in place and is immobilized intramuscularly by polar or nonpolar hydrophobic side chains of amino acids and capillary forces [8-10].

1.5 Protein in fish muscle

After water, protein is the most abundant with 20-22 % in contribution. Fish skeletal muscles are characterized by the precise organisation of the contractile proteins into striated myofibrils resulting from repeating units arranged in series from one Z-line to the next Z-line, called the sarcomere. Myofibril protein constitutes 50 % of the muscle protein, which is divided into about 30 % myosin and 20 % actin. Actin and myosin are the contractile apparatus of the myofibril and are shown in Figure 1 [11].

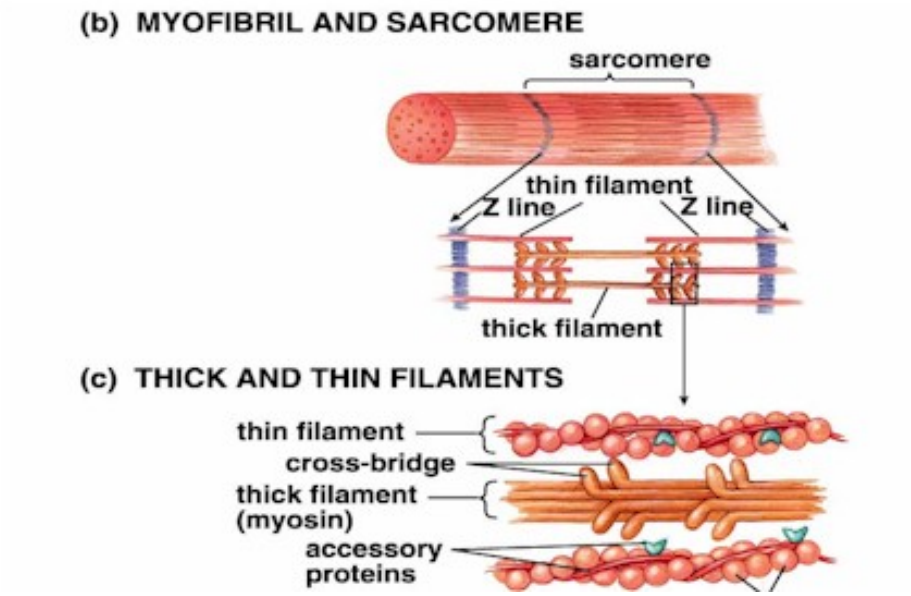

Figure 1. The sarcomere is the functional unit of a muscle. The region between the Z-lines consisting of thin and thick filaments is a sarcomere The thin filaments consist of actin, troponin and tropomyosin and the thick filaments are made up by myosin [12].

1.5.1 Structure of myosin

The thick filaments of the sarcomere contain myosin. The middle of each thick filament is called the M line. Fish skeletal myosins have complex multidomain structures containing two units of globular heads, S1, and a rod-like tail forming a coiled coil of α helices similar to mammalian myosin, as shown in Figure 2 [13]. The myosin molecule itself is composed of noncovalently bound hexameric peptides; it contains two myosin heavy chains (MHC) with an approximate molecular mass of 200 000 Dalton (Da) and four myosin light chains (MLC) with molecular mass of about 20 000 Da as seen in Figure 2A. S1 has two noncovalently bound light chains and contain an ATP binding site and a site for interaction with actin. The light chains differ one being called the essential light chain while the other is called the regulatory light chain. Each MHC consist of a globular head and a rod like tail with a molecular mass of 220 kDa. When the head of myosin is exposed to papain digestion the head domain will divide in three as shown in Figure 2B. The 25 kDa segment provides a pocket for the ATP binding site, while the 50 kDa gives the actin binding site and the 30 kDa is an α-helical rod providing the shaft for both the regulatory and the essential myosin heavy chain (MLC) [13-15].

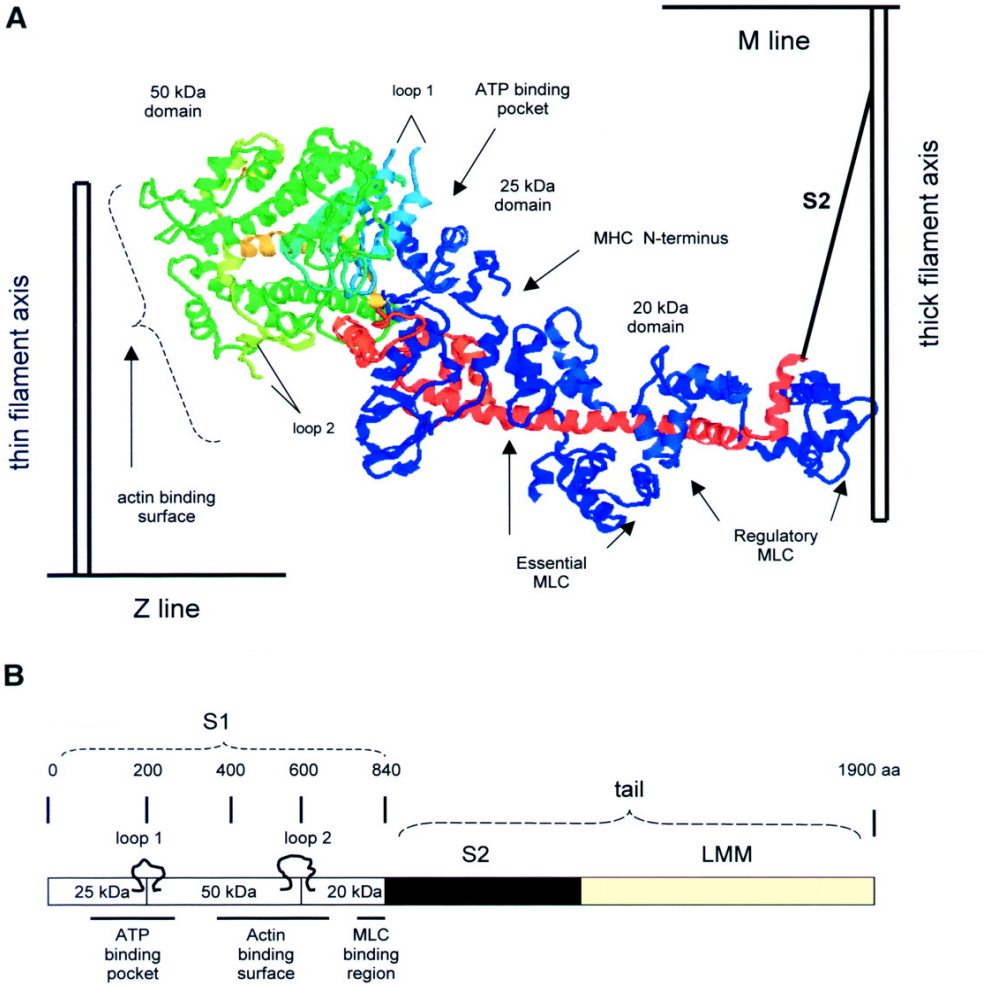

Figure 2. The structure of myosin molecule and location of clusters of molecular diversity are shown. A: The tertiary structure of S1 myosin fragment with a schematic reference to the complete molecule and the filament lattice is given. The functional regions are indicated. B: The schematic representation of the primary structure of the complete myosin heavy chain (MHC) molecule [13].

1.5.2 Actin

Actin is the main component of the thin filament found in the cytoskeleton and muscle cells. Actin is a 375 amino acid long polypeptide highly conserved and is the most abundant protein in eukaryotic cells. It participates in more protein-to-protein interaction then any other known protein. It also has the ability to switch transition between monomeric (G-actin) and filamentous (F-actin) state under the control of nucleotide hydrolysis, ions, and a large number of actin binding proteins. Actin filaments have polarity, and assemble rapidly to positive pole and slowly towards the negative pole. When the actin molecule dissembles into

a polymer they hydrolyse their tight bound ATP molecules [14]. The thin filaments consist of actin and troponin. Troponin consists of three components; the troponin C that binds the Ca^{2+}, troponin I inhibits the ATPase activity of actomyosin and troponin T provides the binding of troponin and tropomyosin [15, 16].

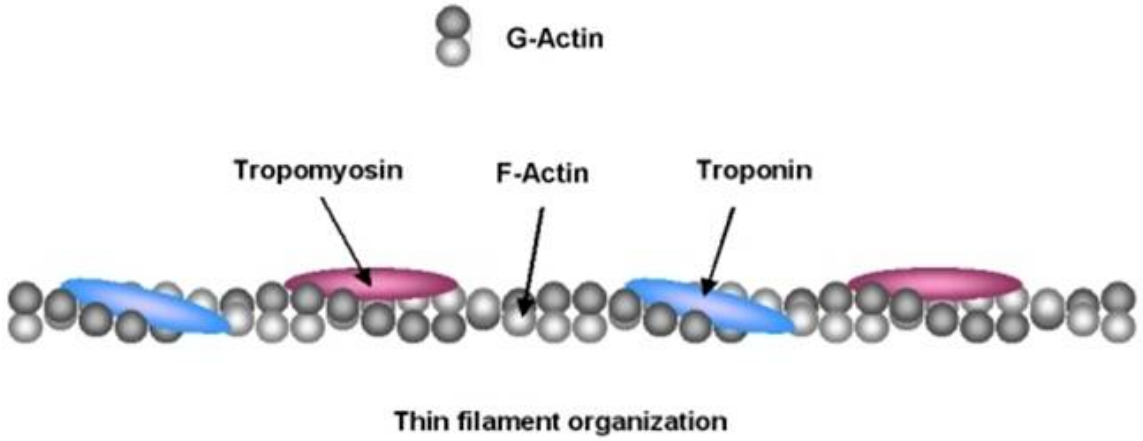

Figure 3. The schematic structure of the thin filament, with the f-actin as the structural unit, and the troponin and tropomyosin proteins that bind to the actin [17].

1.5.3 Mechanism of a muscle stroke

The contractile cycle for actin and myosin resulting in a muscle contraction can be represented into five subsequent steps shown in Figure 4.

1. The relaxation phase of the muscle begins where the S1 head of the myosin hydrolyses ATP to ADP and inorganic phosphate (Pi), while the products remain attached.
2. Ca^{2+} stimulates the contraction of the muscle, and the actin-myosin-binding site becomes accessible due to a conformational change and myosin will bind to actin. The actin-myosin-ADP+Pi complex is formed.
3. The formation of the complex promotes release of ADP and Pi resulting in a large conformational change in the head of myosin relative to its tail.
4. Another molecule of ATP binds to the S1 head.
5. Actin is released by the myosin when ATP is bound and relaxation occurs.

Rigor mortis occurs when intracellular levels of ATP drops and cannot bind the S-1 head (in step 4 in Figure 4). Actin does not dissociate and relaxation do not occur which leads to death stiffness [14, 15].

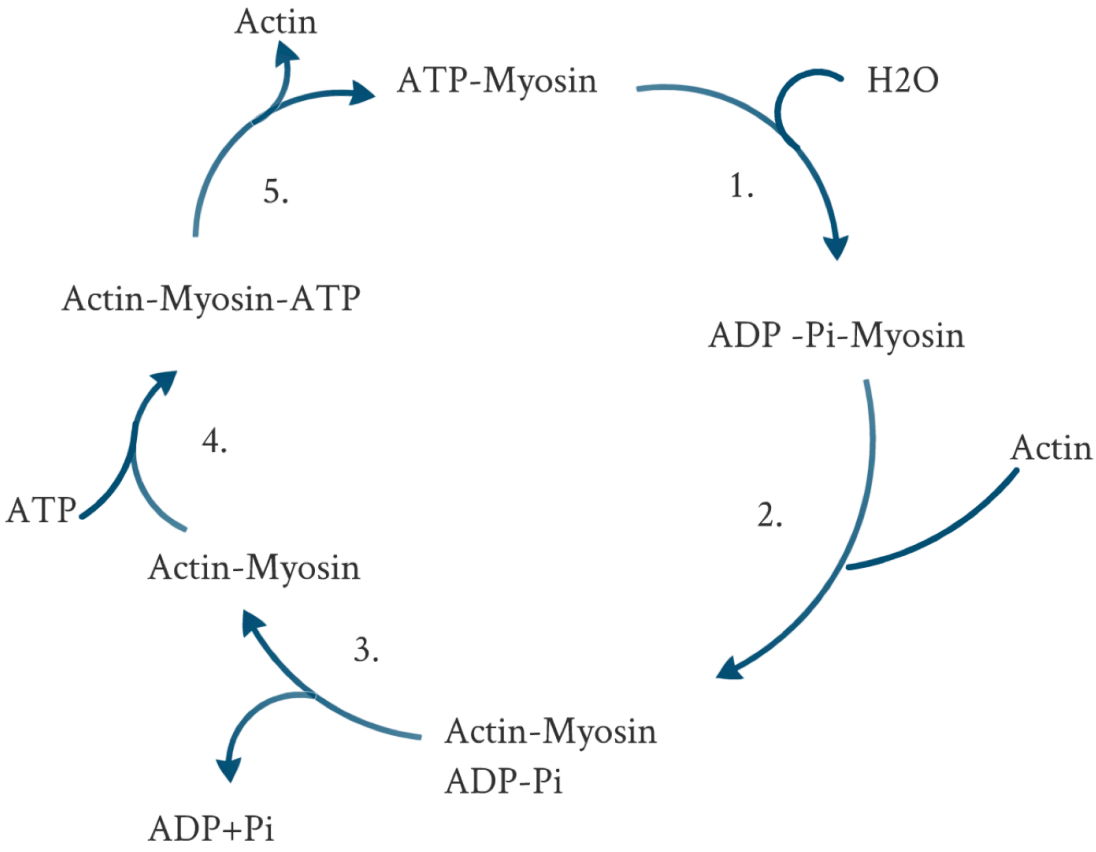

Figure 4. The mechanism of a muscle stroke where the hydrolysis of ATP drives the cyclic binding and release of the actin and myosin complex in 5 subsequent steps [16].

1.6 Post mortem aging in fish

Loss of freshness is due to complex combinations of biochemical and physical changes followed by the muscle spoilage caused by microbiological growth. As the circulation of blood ceases and oxygen supplies are depleted the anaerobic lactic acid formation takes place that leads to a drop in pH. The osmotic pressure increases, the ATP concentration decreases and lipids are oxidized. Trimethylamine N-oxide (TMAO) is degraded to trimethylamine (TMA) which is the responsible odorant for aging seafood. Due to the decreased pH of the muscle tissue, mitochondria and sarcoplasmic reticulum detoriate, and the free Ca^{2+} in cytosol increases. When fish is stored cold, two opposing mechanisms take place in fish muscle: the rigor mortis and the tenderization process. The rigor mortis is characterized by complete

exhaustion of energy rich components. ATP depletion is the onset and when ATP concentration is low enough actin and myosin forms the actomyosin complex leading to complete death stiffness (Figure 3, step 4). Many factors affect the onset and duration of the rigor mortis, especially the effect of stress before slaughtering. The tenderization process begins within hours after death and continues during post mortem storage. Tenderization is enzymatic in nature although the physio-chemical conditions as pH and osmotic pressure can affect the proteolytic activities of endogenous enzymes. The tenderization is blamed on the gradual disintegration of the extracellular matrix structure in particular. Muscle structures are modified after death and protein degradation takes place. Myofibrillar degradation is the result of proteolytic activity of the cytoskeletal components. Surprisingly the fish myofibrils are found to be structurally stable [18, 19].

1.7 Denaturation of proteins

The denaturation of proteins can be studied from two different angles, biochemically and thermodynamically. The thermodynamical investigation of high-pressure effects on protein denaturation has been performed by the DSC analysis, while the other investigation method was carried out by biochemical protein separation, the gel electrophoresis. The biochemical definition of protein denaturation is any modification in secondary, tertiary or quaternary conformation without breaking the primary structure. The proteins are built up by amino acids linked together by covalent peptide bonds. The primary structure of amino acids is the amino acid sequence. The secondary structure is characterised by coiling of the peptide chain into alpha helix and beta sheet structures, held together by hydrogen bonds. The tertiary structure refers to the three dimensional conformation of a peptide, and how these helices and sheets form domains, and is maintained by the disulphate bonds. The quaternary structure is characterized by how the different subunits are held together by non covalent interactions, driven by the hydrophobic forces. When a protein denatures and its structure is changed it looses its biological active state, and this can be reversible or irreversible. Denaturising agents can be physical like temperature and pressure, or chemical by using a strong acid or base, concentrated inorganic salts or organic solvents [14, 16].
The thermodynamically definition of protein denaturation describes the free energy change between the folded and unfolded states, and can be described as in Equation 1:

$$-RT \ln K = \Delta H - T\Delta S \quad \textbf{(Equation 1)}$$

Where *R* represents the Avogadro number, *K,* the equilibrium constant, T the temperature*, H*, the enthalpy change and *S*, the entropy change from the folded to unfolded. Equation 2 describes protein structural equilibrium.

$$N \Leftrightarrow U \quad \textbf{(Equation 2)}$$

Where *N* describes the native folded state, and *U* represents the unfolded state, although the U could represent intermediate states as well. Multi-domain proteins usually unfold stepwise, with the domains unfolding individually, either independently or with varying degrees of interactions between them. Multi-subunit proteins usually dissociate first, then the subunits unfold, unless domains are on the periphery of the aggregate where they can unfold independently [20].

The reverse of protein folding is protein denaturation, based on the energy transitions [20, 21]. When globular proteins fold, intermediate structures appear which are called the molten globular state. The molten globule is a term used for partially folded proteins that are collapsed [22]. One of the large challenges of high-performance computing has been the prediction of structure of proteins. The Levinthal paradox is a puzzle in the theory of protein folding dynamics. In 1969 Cyrus Levinthal wrote that because of the large number of degrees of freedom in the unfolded polypeptide chain, the molecule have astronomical number of possible conformations. If the protein is to attain its correct folded configuration by sequentially sampling all the possible conformations, it would require a time longer than the age of universe to arrive at its correct native conformation. The paradox is that most small proteins fold spontaneously in a millisecond or even microsecond. Proteins are often described as complex macromolecules that posses amphipathic behaviour and are dual electrolytes. The complexity of protein denaturation involves more then total energetics of denaturation of proteins groups, by biochemical investigations the proteins can be studied at a higher resolution by investigating specific protein unfolding at different levels.

Proteins in living system are found in cell membranes, in aqueous solutions and lipid transitions. When proteins denature in living systems they are referred to by different terms. Associations of protein refer to the changes occurring at molecular level like dimerization characterized by weak bonds at specific interaction sites. On the other hand the term

aggregation or polymerization, precipitation, coagulation and flocculation refers to unspecified protein-protein interactions that results in formation of larger complexes with higher molecular weights. Gelation is an orderly aggregation of native and/or partially denatured proteins, forming a three dimensional network structure in which protein-protein and protein-solvent interactions are balanced to produce a well-ordered matrix and protein-solvent interactions are balanced to produce a well-ordered matrix that is capable of holding significant amounts of water [23].

1.8 High pressure processing

High pressure processing (HPP) is a unique method for preserving the food´s natural flavour and nutrients while extending the shelf life through inactivation of microorganisms. By the public HPP is generally regarded as safe, because it is without chemical additives. HPP can retain sensory and nutritional properties of foods, and high temperature exposure can be avoided which may keep the food fresher. Today several successful food applications on the marked including dressings, sauces, salsa, packaged meats, seafood, cut fruit, chilled ready-to-eat foods, soups and juice are preserved by high pressure [24, 25].

The high pressure used in this process lies between 100-685 MPa. To describe the pressure load in these analysis 1 MPa equals 10 atmospheres that again equals 10 bar. In common terms it has been said that three elephants on top of each other balancing on a strawberry equals 500 MPa. For simplicity when 300 MPa is written it is implicated that samples were HPP at 300 MPa. The maximum pressure load has been given as both 680 and 685 MPa, this due to noise and misinterpretation between the student and the lab engineer. The packaging of HPP foods demands airtight flexible packages that can resist volume changes of about 15 %. When food is pressurised there is a reduction in volume. When the pressure drops back to normal level, the food will expand to its initial volume again. Metal or glass packages cannot be used. An advantage of HPP is that the products are processed as packaged products to avoid recontamination. Limitations to HPP are that no air pockets inside the products can be present, and the food has to contain water. Dry products will not have enough water content to make the pressurisation efficiently to destroy the microorganisms [26].

The influence of HPP directly can be to increase the quality of fresh food. The primary structure of molecules of low molecular weight such as vitamins, peptides and saccharides are though to be unaffected, while macromolecules like starch can change their structure [24].

Microorganisms are affected by pressure in different ways. Bacteria are more resistant to high pressure exposal then multicellular organisms, and spores need higher pressures to be destroyed then yeasts and bacteria. Different bacteria and different strains behave differently to pressure and when pressure level reach 400 MPa cell membranes of bacteria will be altered, and enzymes will be inactivated, which can lead to cell death. When doing inactivation studies of bacteria and spores exposed to HPP it should be done in real not in grown media. Spores are extremely pressure resistant and need more then 1200 MPa at room temperature for destruction [25]. HPP in combination with heat treatment, freezing, vacuuming or antimicrobial additives like spices can increase the safety of the products. By subsequent pressurising repeats the effect on microbial death will increase and lower pressures can be allowed [24].

The reduced shelf life of fish is seen in correlation with enzymatic activity of microorganisms on the fish. For some seafood products HPP has many benefits like higher yield, increased shelf life and elimination of food borne pathogens. HPP can reduce these effects and delay production of TMA [27, 28]. Another application of HPP is physical meat separation that vcan be used on from lobster, oysters, clams and other fresh products. HPP induce denaturation of specific proteins that holds the meat to the shell. With the knowledge about pressure intervals for gelification innovative surimi products can be produced. This again can decrease the seafood waste and increase the protein yield from the fish.

A schematic overview of the HPP mechanism can be seen in Figure 5. The vessel will be prefilled with water initially; high pressure intensifiers compress the water surrounding the sample further to achieve the given pressure. The pressure is instantly transmitted throughout the product, and treatment time is not dependent on product size or geometry
High pressure exposure can affect protein conformation and can lead to protein denaturation, aggregation or gelation depending on the protein system, the applied pressure and temperature, and the duration of the pressure treatment [23, 29].

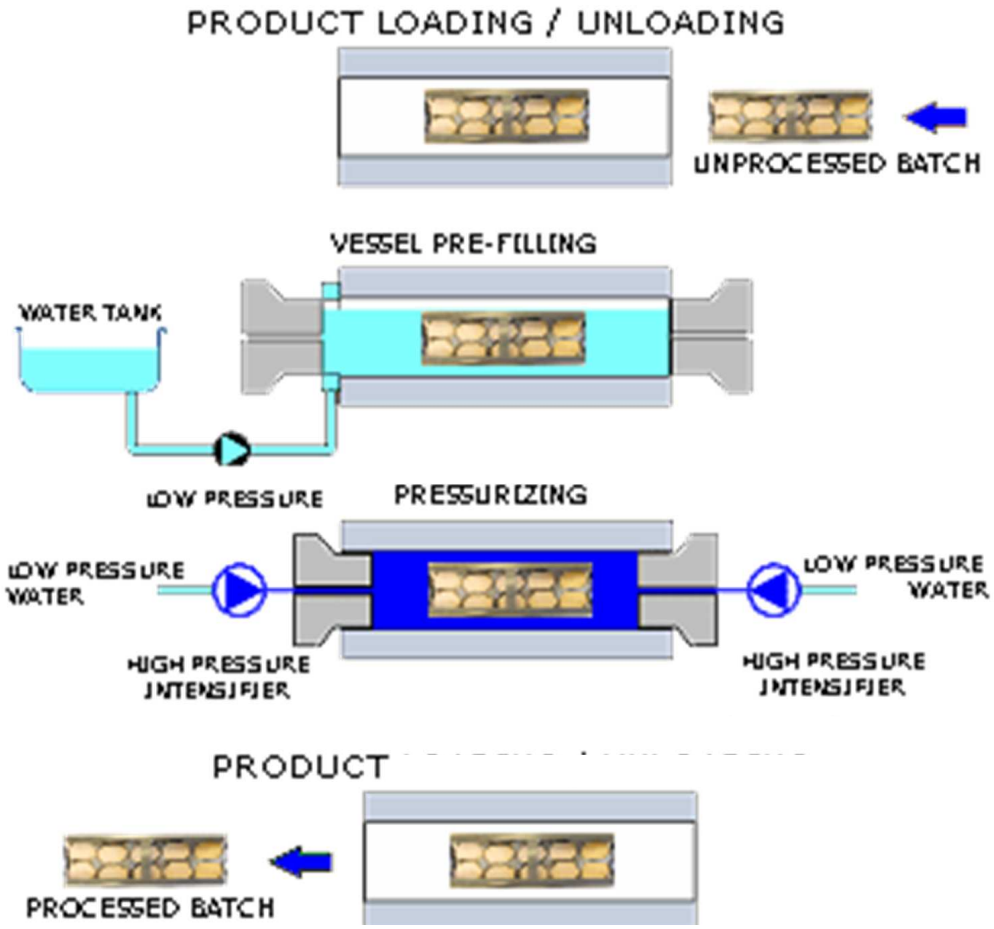

Figure 5. An illustration of the high pressure process, samples are loaded in a container with holes. The pressure chamber is prefilled with water, and by the help of high pressure intensifiers accurate pressure is applied at the given time. The process is terminated by the pressure release[26].

Denaturation of proteins by HPP depends on protein type, processing conditioning and the magnitude of the applied pressure. In order to understand the effect of pressure on a chemical reaction, le Châtelier principle states that; ¨If a thing can shrink, it will shrink if you squeeze it¨. In other words, the effect of pressure on a reaction is to move it towards the direction of smaller volumes. If products and reactants have equal contributions to the volume of the mixture, the pressure will not change the equilibrium of the system. Since proteins denature at high pressure, this implies that the partial molar volume of the unfolded protein is less than the folded protein [30]. High-pressure effects on proteins are primarily related to the rupture of non-covalent interactions within protein molecules followed by subsequent re-formation of intra- and intermolecular bonds within or between protein molecules [31].

The pressure induced denaturation will affect the secondary, tertiary and quaternary structures of proteins. The chemical bonds involved in these structures like the electrostatic interactions and the hydrophobic interactions are affected by pressure. The electrostatic interactions occur between the ionized amino acid residues: the carboxyl COO^- (e.g. Asp, Glu,) and amino NH_2 (e.g. Lys, His and Arg) groups. The hydrophobic interactions are between aliphatic (Leu, Ile and Val) or aromatic (e.g. Phe, Tyr) side chains [23].

Protein denaturation is thought to be reversible in the range 100-300 MPa and irreversible for pressures higher than 300 MPa [24]. At higher pressure than 300 MPa oligomeric proteins tend to dissociate into subunits becoming vulnerable to proteolysis, while the monomeric proteins remain unaffected [24, 32].

1.9 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a method to characterize the energetics and mechanisms of temperature induced conformational changes in biological macromolecules. It is a useful tool to study thermal properties of muscle proteins. The enthalpy is measured by the differential heat flow required to maintain a sample and an inert reference at the same temperature. The instrument is programmed to scan a temperature range by increasing linearly at a predetermined rate. The DSC thermogram is the result of thermal denaturation of muscle proteins during measurement, and further information is gained interpreting peak temperatures and corresponding enthalpy changes. According to literature denaturation of myosin is expected to occur at temperatures from 44 °C to 54 °C together with other proteins like collagen that also denatures in this region [33]. The smaller peaks number 2 at around 60 °C and peak number 3 at around 70 °C are due to sarcoplasmic denaturation. Peak number 4 is found at around 73 °C and is due to actin denaturation [33-35].

1.10 Water holding capacity

Water holding capacity of meat and fish products is the ability of a muscle to resist water loss during mechanical treatment (chopping, coarse grinding) thermal treatment, transportation and storage. WHC is related to the quality parameters of fish products like juiciness,

tenderness, taste and colour. The juiciness when tested by sensorics is described as the difference of free and bound water. However the perception has to be seen in connection with fat content, flow of saliva and softness. Hence the measurement of WHC is important value for both commercial, financial and consumer point of view.

The molecular arrangement of the myofibrillar proteins affects the state of free or bound water. The myofibrils provides a three dimensional network of filaments that provides cavities for water to be immobilised. The decrease of immobilised water is observed as a result of tightening of the space between the myofibrils as a result of protein denaturation. The WHC of muscle tissue is due to changes in the intensity of swelling of the myofibrils. As swelling occurs there is an expansion of the filament lattice that can increase the diameter of the myofibril up to 2.5 times the original value. This increase in myofibrillar volume is thought to be the reason for the water losses during muscle processing [36, 37]. The myosin, actin and tropomyosin are the water-binding components in muscular tissue. The WHC of myosin is related to the large amounts of polar amino acids like aspartic acid and glutamic acid residues [11].

1.11 Gel electrophoresis

The term electrophoresis refers to the transport of charged particles like single proteins and protein complexes along an electric field gradient. Three types of gel electrophoresis technics will be described: sodium dodecyl sulphate polyacrylamide agarose gel electrophoresis (SDS-PAGE), native (LDS) PAGE, and two dimensional gel electrophoresis (2DE-PAGE). In SDS-PAGE the proteins are completely denatured and given a negative charge by SDS [38]. They are separated according to size by the applied negative charge in the electric field. LDS-PAGE was used where the charge of a protein relies upon the intrinsic charge of the protein, and its mass. Migration to the anode occurs because most proteins carry a net negative charge at slightly basic pH. Because no denaturants are present in native LDS-PAGE, subunit interactions within a multimeric protein are generally retained and information may be gained about the quaternary structure. After native LDS-PAGE a two dimensional gel (2D-PAGE) will provide information about the different subunits that a multimeric protein contain, and prepare the proteins for mass spectrometry [39].

1.12 Mass spectrometry analysis

Mass spectrometry (MS) is a method used to analyse proteins by sequencing the amino acid sequence. The protein sample is treated with trypsin for enzymatic digestion that cleaves the protein on the carboxy-terminal side of arginine and lysine residues. This breaks the proteins into peptides. Protein samples containing formic acid are prepared and injected into a needle. The formic acid decreases the pH that helps to protonate the amino acids R-groups giving them positive charges, and subsequently the low pH inhibits trypsin activity and thereby arrests protein digestion. The needle is placed on the probe as shown in red in Figure 6. As the sample flows out of the tip of the needle, it is vaporized due to the volatile formic acid and the peptides are ionized by the action of a strong electric field. The peptide ions are guided and manipulated by electric fields into a mass analyser MS1 (Quadrupole MS). The mass analyser separates the peptides on the basis of mass divided by charge (m/z) and results are read as a mass spectrum. The isotope spectrum is given by choosing one m/z value and the quadrapole scans over the masses again and the time of flight (TOF MS) is recorded. The amino acid sequence can be determined by the masses recorded from the spectra [40].

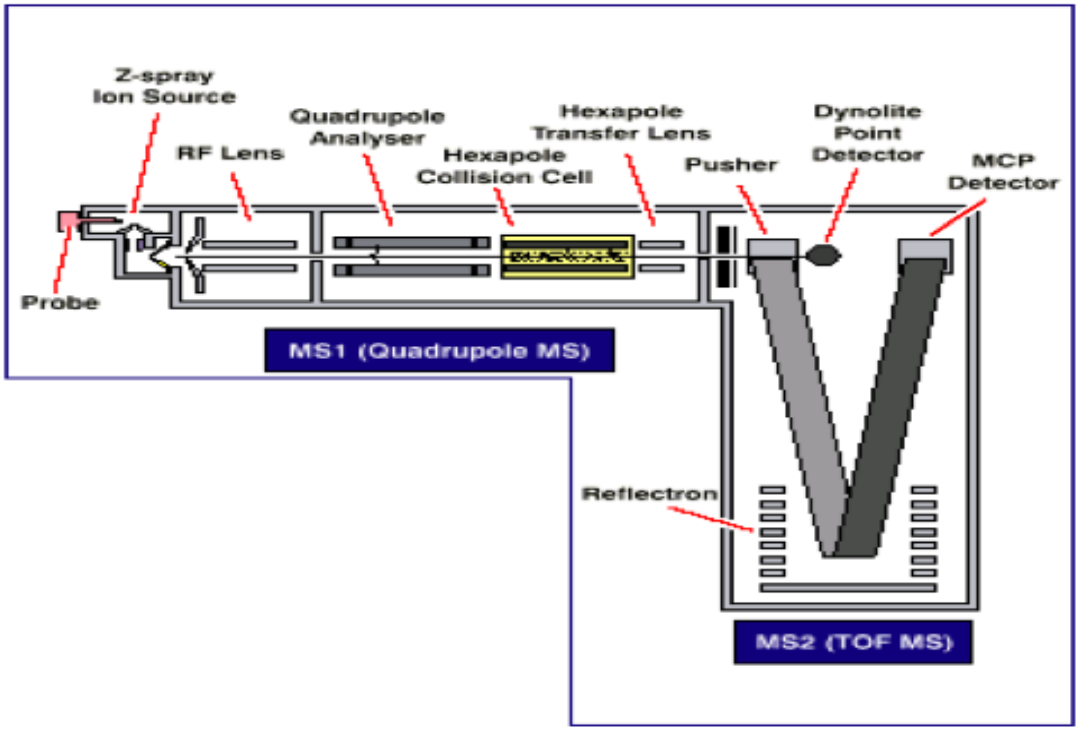

Figure 6. A schematic presentation of the different components of the mass spectrometer.

2.0 Materials and method

2.1 Experimental overview of salmon analysis

The experimental overview for salmon is given (Figure 7) where raw material of salmon back loins was analysed as raw fillets, raw minced and frozen minced samples. Unprocessed control samples were used and compared to HPP samples. The colour of the fillets was measured by DigiEye, both raw and at different pressures. The raw and pressurised samples were analysed by DSC and thermograms were given as results. The WHC was analysed from the same samples as used for DSC. The pressure treated samples were also examined by gel electrophoresis, first by native gel, then in combination with SDS-PAGE, and then as completely degenerated SDS-PAGE gel. Specific bands of the gels were cut out and further analysed by mass spectrometry for precise protein identification.

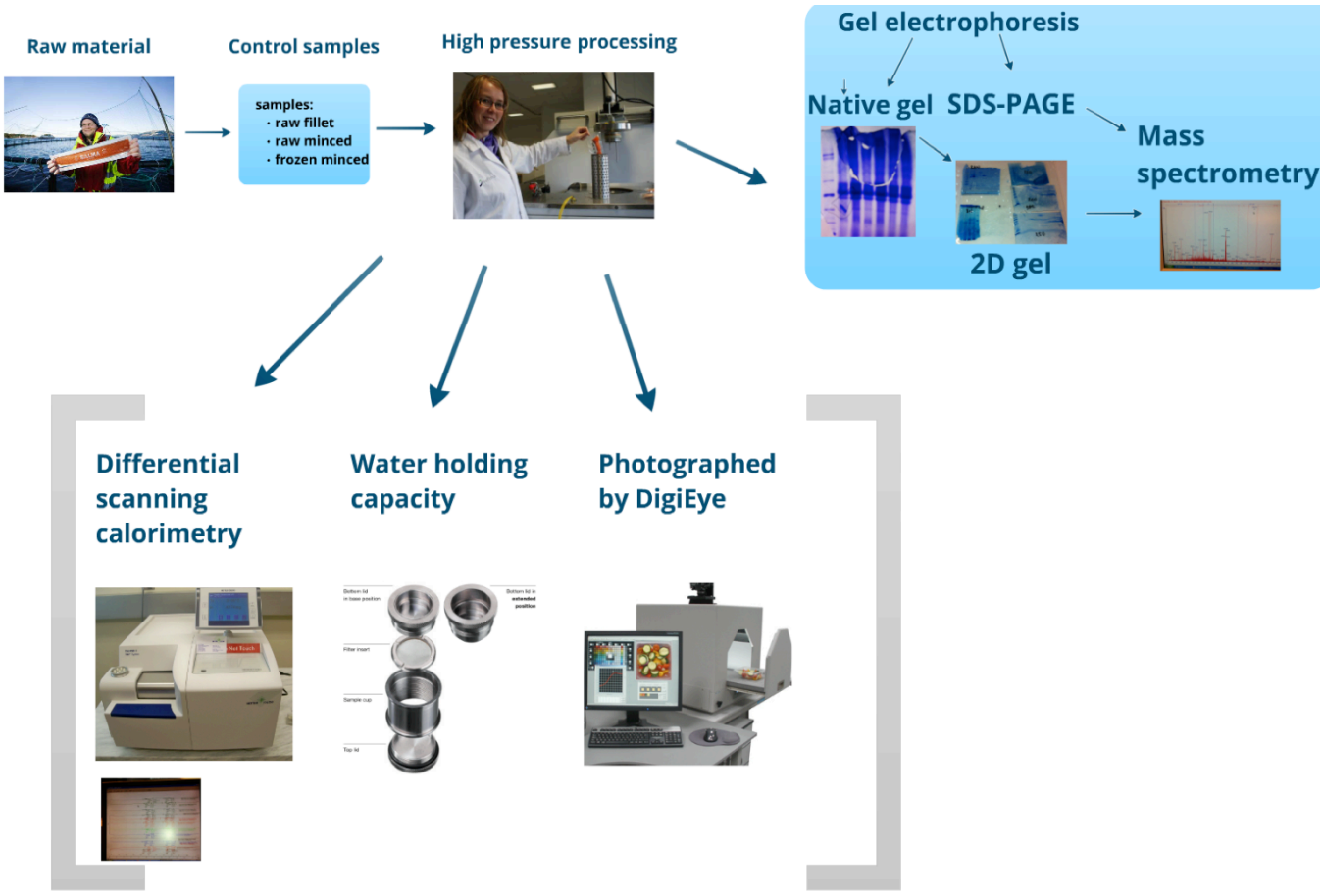

Figure 7. A flow diagram of the analyses done to investigate the effects of HPP and protein denaturation on salmon.

2.2 Experimental overview of salmon analysis

The turbot used in this work were from 1) a light exposure growth experiment and 2) a post mortem aging experiment. Differences in protein denaturation were examined by DSC and SDS-PAGE. Figure 8 gives the overall view of the analysis.

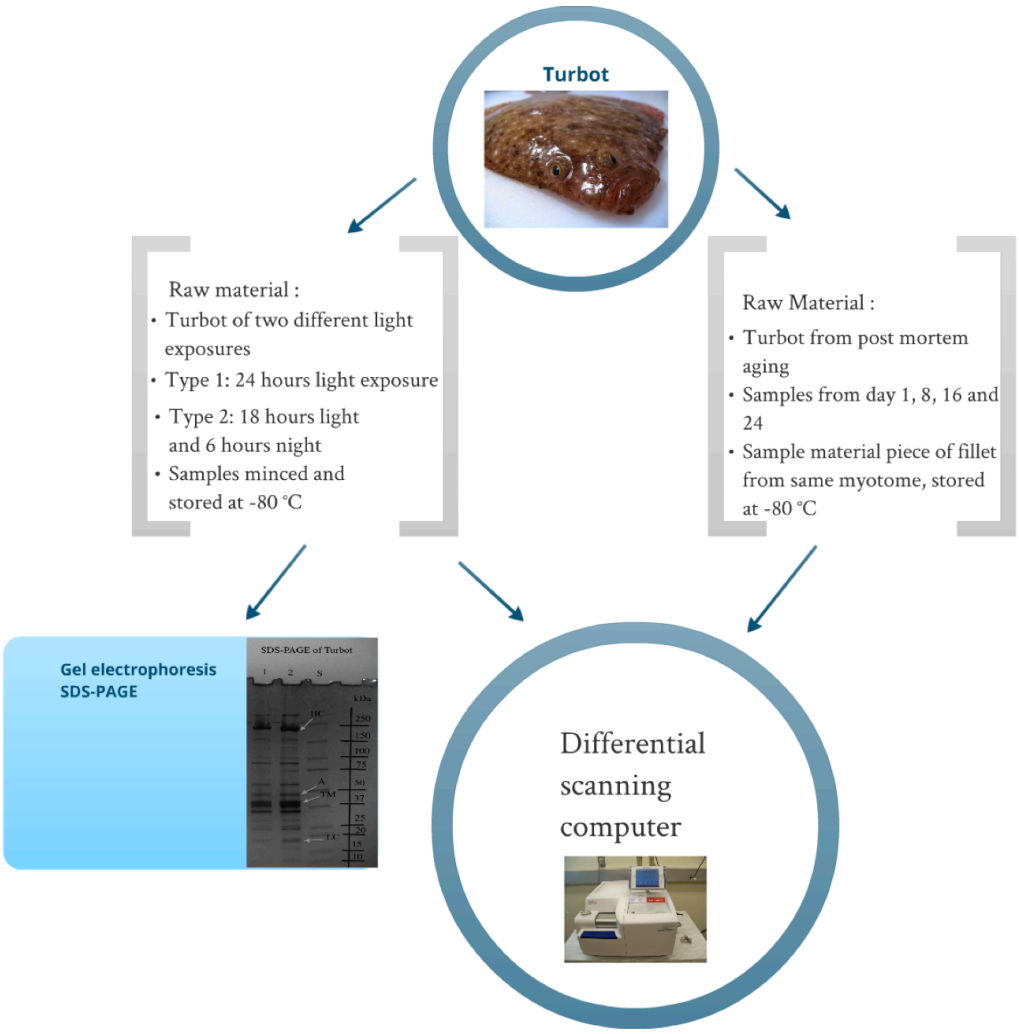

Figure 8. A flow diagram of the analyses performed to examine the turbot and protein denaturation.

2.3 Raw Material

2.3.1 Salmon

Salma (Salma Brands AS, Norway) back loins skinless and boneless fillets of farmed salmon were used. The fish was bought at the grocery store and kept at 0.5 °C. Salmon used in this experiment was 5-7 days old. On the day of experiment the fish fillets were either cut in several pieces of about 25 grams or minced. Minced fish meat was made by a grinder under cooled conditions, and aliquoted into portions of 25 grams. The minced fish was used fresh or frozen stored at -80 °C. Before the frozen samples were used for analysis they were thaw overnight in refrigerator at 0 °C.

2.3.2 Turbot

Cultivated turbot from Iceland and Norway was stored in polystyrene boxes with ice until the experimental day. Sampling from turbot was done at specific days by cutting out a piece from the same specific myotome on each fish and consequently analyse it or keep at -80 °C. First, post mortem protein denaturation was investigated by DSC at storage days of 1, 8, 16 and 24. Secondly, different light exposures of cultivated turbot of two types where type 1 was grown in light 24 hours, while type 2 had 18 hours light and 6 hours darkness. The raw material of these samples was minced frozen turbot at -80 °C, and protein denaturation was examined by DSC and SDS-PAGE.

2.4 High pressure processing

Samples were packed in double sous vide bags and vacuumed at 99.5 % (Webomatic, Bochum, Germany). Packed samples were placed in the pressure vessel of the High Pressure Laboratory Food Processing QFP pressure machine 2L (Avure Technologies Inc, Franklin, USA).

The salmon samples were pressurised in the range 100 to 685 MPa, with a holding time of 2 min. The come-up time varied with the pressure level, and it took about 3 min to reach 685 MPa. The release of pressure was instant. During pressurisation, the temperature increased due to adiabatic heating. The HPP unit was cooled to 8-9 °C by a cooling system before the experiments. After HPP, the samples were removed from the pressure chamber and kept on ice until further analyses were done.

2.5 Colour measurement of salmon

The colour of the untreated and pressurised fillets was measured by reflectance using image analysis DigiEye^{TM} (VeriVide Ltd, Leicester, UK). Each fillet was placed into an illumination chamber that ensures a uniform lightening, standard daylight (6400K) and photographed with a Nikon camera with a D80 Nikon lens. The colour of the loins was measured using the DigiPix colour measurement software. The a^*, and b^* values were obtained using white and colour standards. L^* describes the whiteness and was not be used to analyse salmon. The a^* describes the intensity of colour on the red-green axis ($a^* > 0$ = red, and $a^* < 0$ = green) and b^* is the intensity of colour on the yellow-blue axis ($b^* > 0$ = yellow, and $b^* < 0$ = blue). From these parameters the colour saturation or intensity (Chroma, C*) and the colour tone (Hue, h*) was calculated [41].

2.6 Differential scanning calorimeter

Samples from raw or HPP fillet, minced and minced frozen were weighted 60 ± 1 mg and placed in a medium pressure stainless steel DSC pan of 120 µl (Mettler Toledo, Schwerzenbach, Switzerland). From raw fillets, raw minced and frozen minced samples 8 parallels of each were examined. The pressurised samples had a minimum of four parallels of each pressure for the DSC examinations. The pan had a diameter of 7 mm and height 0.4 mm. Each pan was sealed with an inner rubber ring in the lid. A crucible sealing press (Figure 9, Mettler Toleado, Schwerzenbach, Switzerland) closed the crucible airtight. The samples were weighted using weight scale (PRX USET XS205, Mettler Toledo, Schwerzenbach, Switzerland).

DSC analysis was performed at a heating rate of 5 °C/min in the range from 0 °C to 110 °C on a Mettler Toleado DSC1/200 (Figure 9, Star System, Schwerzenbach, Switzerland). An empty pan containing approximately the same amount of distilled water as the sample was used as reference, and 2 minutes equilibration at 0 °C was done before each run. After each scan a rescan was performed with the same heating rate as the sample. The residual denaturation enthalpy (ΔH) was defined as the area under the denaturation peak using a spline baseline. Calculations of the residual denaturation enthalpy was performed by integration of the area under the graph, made by StarE software version 9.10 (Star System, Schwerzenbach, Switzerland). The specific enthalpy was found by dividing the residual enthalpy by the weight of the sample. In order to standardize the integration values, limits were given and marked at the abscissa in all the thermograms presented. The integration limits for salmon were the following: peak 1; 43-58 °C (red), peak 2; 59-61 °C (blue), peak 3; 68-73 °C (blue) and peak 4; 77-82 °C (red).

Close to the range limits there were cases where the baseline value was higher than the curve value, resulting in an apparent exothermic part. This mistake was avoided by excluding the apparent generated enthalpy from the calculation of the residual denaturation enthalpy. For turbot DSC analysis the integration limits were for peak 1; 44-58 °C (red) and for peak 4; 72-83 °C (red).

Figure 9. The figure shows a picture of the DSC machine (right side) and the crucible sealing press (left side).

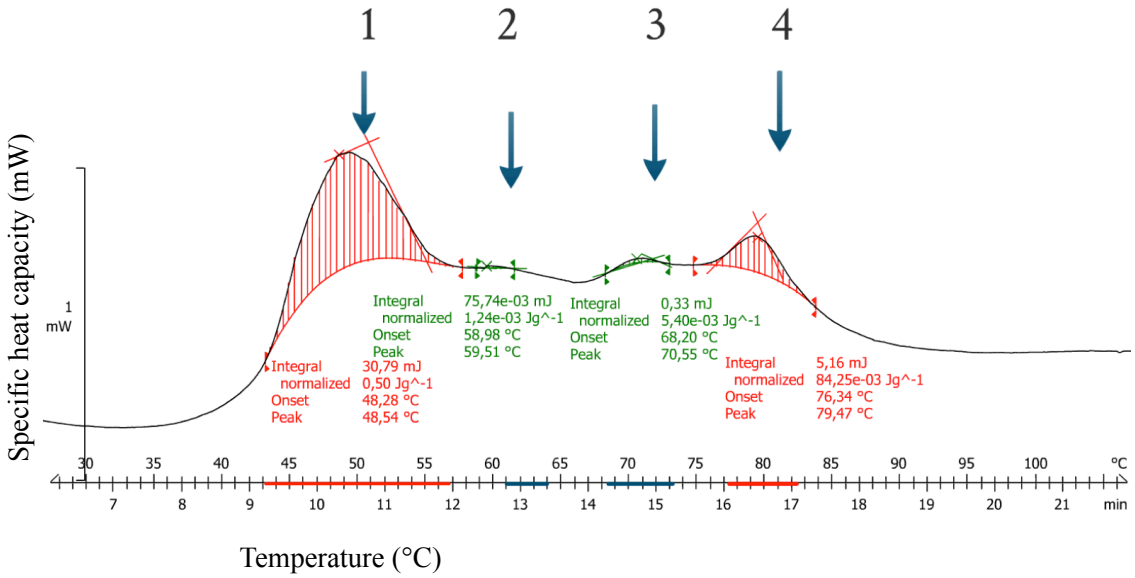

Figure 10. DSC thermogram of minced frozen Atlantic salmon muscle obtained at a scanning rate of 5 °C/min. The peaks illustrate the denaturation peaks of myosin (1), sarcoplasmic proteins (2 and 3) and actin (4). The peaks are integrated using a spline baseline. The red lines of the abscissa are indications of the temperature intervals for myosin (44-57 °C), and actin (77-81 °C), while the green lines (blue marker, 59-61 °C and 68-73 °C) show the sarcoplasmic proteins.

2.7 Water holding capacity (WHC) and drip loss

The method of holding capacity and drip loss used in these experiments was developed by Skipsnes, Østby *et al.* (2007) [35]. Sampling was done in the middle of the fillet using a scalpel to cut a circular sample. Raw fish sample was cut into appropriate pieces with a diameter of 31 mm, a height of 6 mm and a weight of 5.0 ± 1 gram (g). When minced fish samples were used, they were prepared from homogenous muscle fillet without fat tissue. Two parallels were used from each fish fillet.

2.7.1 Water content loss measurement

The samples was placed in the cooled sample cups on the filter in good contact, but without any pressure applied to it. The samples were spun in a precooled centrifuge (Rotina 420 R, Hettichlab, Tuttlingen, Germany) for 15 min at 761 g at 4 °C. Pressurized air was used to remove exudate from the bottom after the bottom was removed. The inside of bottom was

wiped with absorbent paper without touching the filter. The sample cup was weighted after the exudate was removed.

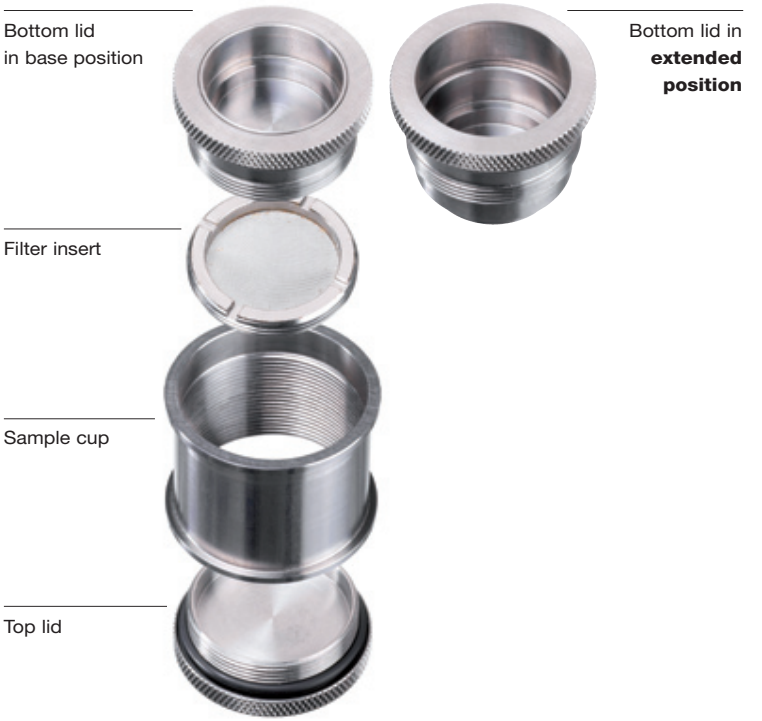

Figure 11. The sample cup used for water loss measurement.[42]

2.7.2 Dry mass measurement

The dry matter of the fish muscle was determined gravimetrically. About 5 g of fish fillet or minced fish was placed in numbered metal cups and placed in an incubator at 103-105 °C for approximately 16-18 hours [43]. The samples were cooled in an exicator before weighing, and after the weighting the dry mass was calculated according Equation 3.

$$\boldsymbol{Dry\ mass}\ \% = \frac{(V2-V0)}{V1}\ \boldsymbol{x}\ \mathbf{100}\% \qquad \text{Equation 3}$$

V0 = Weight (g) of cup
V1 = Weight (g) of sample
V2 = Weight (g) of cup and dried sample

2.7.3 Calculations of water holding capacity

The calculations of water holding capacity gives the ratio of the water remaining after centrifugation to the initial water content of the sample, using the following formula:

$$\boldsymbol{WHC} = \frac{\boldsymbol{Wo} - \boldsymbol{\Delta W}}{\boldsymbol{Wo}} \boldsymbol{x}\ \mathbf{100}\ \% \qquad \text{(Equation 4)}$$

$$\boldsymbol{Wo} = \frac{\boldsymbol{Vo}}{\boldsymbol{Vo+Do}} \times \mathbf{100}\ \% \qquad \text{(Equation 5)}$$

$$\boldsymbol{\Delta W} = \frac{\boldsymbol{\Delta Vo}}{\boldsymbol{Vo+Do}} \times \mathbf{100}\ \% \qquad \text{(Equation 6)}$$

Vo = Initial water content of the sample
ΔVo = Differences in water content of the sample, before and after centrifugation
D0 = Initial dry mass of the sample. The dry mass can be determined gravimetrically shown in Equation 3.

2.7.4 Drip loss

When fillets are minced and frozen, the water loss cannot be determined completely, because some water is left in the mincer or in the plastic bag used when processed. When fillets were pressurized a drip loss was tried to measure by weighting the bag with the fish fillet inside, and after removing the fish fillet in one piece after HPP.
The following formula was used to calculate drip loss (%):

$$\boldsymbol{Drip\ loss}\ \% = \frac{\boldsymbol{VD}}{\boldsymbol{Ws}}\ \text{x}\ 100 \qquad \text{(Equation 7)}$$

V_D = Drip volume
W_S = Initial weight of sample
The drip loss was photographed to describe the visual characteristics.

2.8 Gel electrophoresis and mass spectrometry

2.8.1 Blue native polyacrylamide gel electrophoresis

The pressurised proteins from salmon tissue were separated by native lithium dodecyl sulphate electrophoresis (LDS) in a modified blue native PAGE gel described by Reisinger and Eichacker (2008) [39] to separate proteins according to size. LDS PAGE gel of salmon tissue samples did not have any known procedure, and preliminary investigations had to be done. All solutions used were chilled to 0-4 °C to avoid further denaturation of the proteins. Raw samples of 1 mg were treated in three different ways. First, it was mixed with 20 µl 20 mM Digitonin (Sigma-Aldrich, St. Louis, MO, USA). Secondly, a dilution series of eight different concentrations (Table 2) was made from Digitonin (0-40 mM) to optimize the best detergent concentration. It was found that no detergent was needed for solubilisation. No difference in solubility was detected for the proteins separated by the native LDS-PAGE approach, most likely because the myofibrillar proteins are not membrane bound. Thirdly, it was mixed with 20 µl Tris-HCl buffer (100 mM, pH 7.2). The solubility of the tissue sample was investigated by different mixing procedures like snipping, ultrasonic mixing, and different time of incubation. No specific mixing was needed due to good solubility of the proteins. The HPP salmon samples were dissolved in 20 µl (100 mM, pH 7.2) Tris-HCl buffer. After adding the different buffer solutions, the subsequent steps were the same. Samples were spun in a precooled centrifuge at 4 °C for 10 minutes at 16100 xg. The supernatant (10 µl) was transferred to new tubes and 5 µl loading buffer was added. Subsequently samples of 10 µl were loaded onto the Native PAGE™ 3-12 % Bis-Tris Gel (Novex, Life technology, UK). The marker used was Native Mark™ unstained protein standard (Novex, Life technology, UK), and 5 µl was loaded gel. The setting for the electrophoresis was 35 Volt, 10 mA and 200 W for 24 h, and the gel was run under cold conditions in a cold room at 3 °C. The electrophoresis was stopped when the running front reached the bottom of the gel, approximately after 9 hours. The gel was cracked open by a spatula and stained with Coomassie Brilliant blue G-250 (Sigma-Aldrich, St. Louis, MO, USA), followed by consequent destaining. Pictures of the gels were taken by gel dock (InGenius, Syngene, England).

2.8.1.1 Solutions

Digitonin

20 mM 1229.34 g/mol

Table 2. Dilution series of Digitonin.

Tube	1	2	3	4	5	6	7	8	9	10
Concentration mM	0	1	2	4	5	6	10	20	30	40

Tris buffer, 100 mM, pH 7.2	30 ml
100 mM Tris	30 ml
1 mM EDTA	30 µl
dH_2O	
HCl	up to pH 7.2

Novex native buffers used for the running of the electrophoresis:

20 x cathode buffer	500 ml
50 mM Tricine	89.6 g
50 mM Bis Tris pH 7.0	104.6 g
HCl	to pH 7.0

LDS cathode buffer	1000 ml
1 x cathode buffer	50 ml
LDS	19.8 g

10 x anode buffer	1000 ml
20 x cathode buffer	50 ml
dH_2O	950 ml

Loading buffer	
750 mM ε-amino caproic acid	0.492 g
5 % Serva Blue G-250 Brilliant	0.250g

Coomassie staining

The gels were stained using Coomassie Blue Brilliant G-250 on a shaker overnight. The gels were destained by MilliQ water until the background was blank, and the protein bands were

light blue. After destaining the gels were put on a geldock (InGenius, Syngene, England) and pictures were taken.

Coomassie brilliant blue	
Serva Blue G-250 Brilliant	1g
37.5 % Acetic acid	
50 % Methanol	
dH_2O	

2.8.2 Combination of native- and SDS-PAGE

The native PAGE was combined with SDS-PAGE by the method of Schagger et al (1991)[44]. Analysis started by first running a native LDS-PAGE loaded with raw and pressurised samples. The lanes were cut out by razors and placed in falcon tubes. The protein complexes in the gel-strips were made solubilized by dissolving them in a SDS solubilisation buffer and glued to the ready made 2-D gel (NuPAGE® Novex® 4-12 % Bis-Tris gel 1.5 mm 2D Well). Air bubbles were removed between the SDS gel and the native gel by using a plastic spacer. In order to seal the gels SDS agarose overlay solution was used. The gels were run until the running font reached the bottom of the gel, approximately 2 hours at 1200 Volt, 12 mA, 24 W. The gel was stained with Coomassie Brilliant blue G-250 and photographed by gel dock (InGenius, Syngene, England).

3.8.2.1 Solutions

SDS- Solubilisation buffer	300 ml
2 % (w/v) SDS	6 g SDS
66 mM Na_2CO_3	2.12 g
0.66 % (v/v) ß-mercaptoethanol	2 ml

SDS running buffer	1000 ml
1.92 M Glycine	144.13 g
0.25 M Tris	30.3 g
1 % w/v SDS	10 g
pH adjusted to 8.4	

SDS Agarose overlay solution	100ml
0.5 % (w/v) Agarose	0.5 g

Coomassie staining

The gels were stained using Coomassie Blue Brilliant G-250 on a shaker overnight. The gels were destained by MilliQ water until the background was blank, and the protein bands were light blue. After destaining the gels were put on a geldock (InGenius, Syngene, England) and pictures were taken.

Coomassie brilliant blue

Serva Blue G-250 Brilliant	1g
37.5 % Acetic acid	
50 % Methanol	
dH_2O	

2.8.3 Sodium dodecyl sulphate gel electrophoresis

Frozen fish muscle (1 g) was added cold extraction buffer and homogenized for 1 minute using an ultra turrax T25 (Janke & Kunkel, Labortechnik, Germany) at step 5. The sample was diluted 10 times and protein determination was done by the NanoPhotometer (Implen GmbH, Germany) at A_{280} to achieve 3 mg/ml protein content. The reducing agent β-mercaptoethanol (5 µl) was added and Laemmli sample buffer (Bio-Rad, Hercules, CA, US) to obtain total protein concentration of 1.5 mg/ml. Samples were heat treated at 85 °C for 2 minutes. Subsequently samples (10 µl) were loaded onto a 4-15 % precast gel (Mini-Protean ®TGX™, Bio-Rad Laboratories, Hercules, CA, US). The marker used was Precision Plus Protein Western C™ Standard. Running buffer was added to both anode chamber and cathode chamber. The gel was running at 200 V until the running front reached the bottom of the gel after approximately 40 minutes. [40]. The gel was stained by Coomassie brilliant blue G-250 staining and pictures were taken by gel dock (InGenius, Syngene, England).

2.8.3.1 Solution

Extraction buffer, Bio-Rad	
50 mM TrisHCl	6.055 g
1 mM EDTA	2.923 g

Laemmeli sample buffer, Bio-Rad

62.5 mM Tris HCl	pH 6.8
25 % Glycerol	
2 % SDS	
0.01 % Bromphenolblue	
5 % β-mercaptoethanol	

Running buffer, Bio-Rad

25 mM Tris	pH 8.3
192 mM glycine	
0.1 % SDS	

Coomassie staining

The gels were stained using Coomassie Blue Brilliant G-250 on a shaker overnight. The gels were destained by MilliQ water until the background was blank, and the protein bands were light blue. After destaining the gels were put on a geldock (InGenius, Syngene, England) and pictures were taken.

Coomassie brilliant blue

Serva Blue G-250 Brilliant	1g
37.5 % Acetic acid	
50 % Methanol	
dH_2O	

2.8.4 Mass spectrometry of LDS-PAGE and SDS-PAGE

The type of mass spectrometer used in this experiment was a MALDI Q-TOF premier (Waters Corporation, Massachussett, USA). The mass spectrometer was used to try to identify the proteins detected by staining of the gels. The protocol of Granvogl et al. (2007) was followed [40]. MS analysis was performed on HPP salmon SDS-PAGE and the 2D-PAGE gel. A picker from OMX-S® was used to cut the different bands of proteins (Figure 12). The manufacture protocol (OMX GmbH, Germany) was followed. The gel was centrifuged (Microcentrifuge 5417R, Eppendorf, Hauppauge, NY, US) into the reactor side down of the OMX-S® tube (13000 xg for 2 min). Trypsin (20 µl) was added to the reactor and the OMX-S®

was shorty centrifuged at 3800 xg still facing reaction side down. The reaction tube was incubated in a termomixer (Thermomixer, HLC, Bovenden, Germany) for 1 hour, and stored at -80 °C. Thereafter the digestion the OMX-S® was centrifuged with the peptide sampler faced down (3 minutes, 1000 g). For desalting and subsequent eluting steps StageTip C18 (Thermo Scientific, Waltham, Massachusetts, US) pipette tips containing a carbon 18 column were used. To activate the C18-column in a StageTip an initiation solution was used. The re-equilibrium was achieved by removing the organic solvent by washing with 20 µl of 55 % formic acid and centrifugation at 150 g for 20 sec. Sample was loaded onto StageTip and peptides not bound to the matrix were removed by centrifuging at 150 g for 20 sec. Peptides bound to the C18 matrix were eluted using elution solution, and placed in new tubes and centrifuged at 1230 xg for 20 sec and ready for MS measurements [45].For electrospray ionization, the sample was loaded into a borosilicate nano ES emitter needle (Proxeon, Stockholm, Sweden). The peptide mixture was sprayed at 0.8-0.12 kV (ESI +) and a cone voltage of 40 V using a nano ESI source. MS spectra were recorded between 400-2000 m/z for at least 30 s (1 s/scan). When MSMS spectra were used the collision energies of fragmentation of the peptides in argon were set to values between 20-25 eV depending on the peptide. De novo sequence analysis was performed using MassLynx/Biolynx 4.2 software (Micromass UK Limited, England). The b- and y-ion series of spectra were interpreted manually.

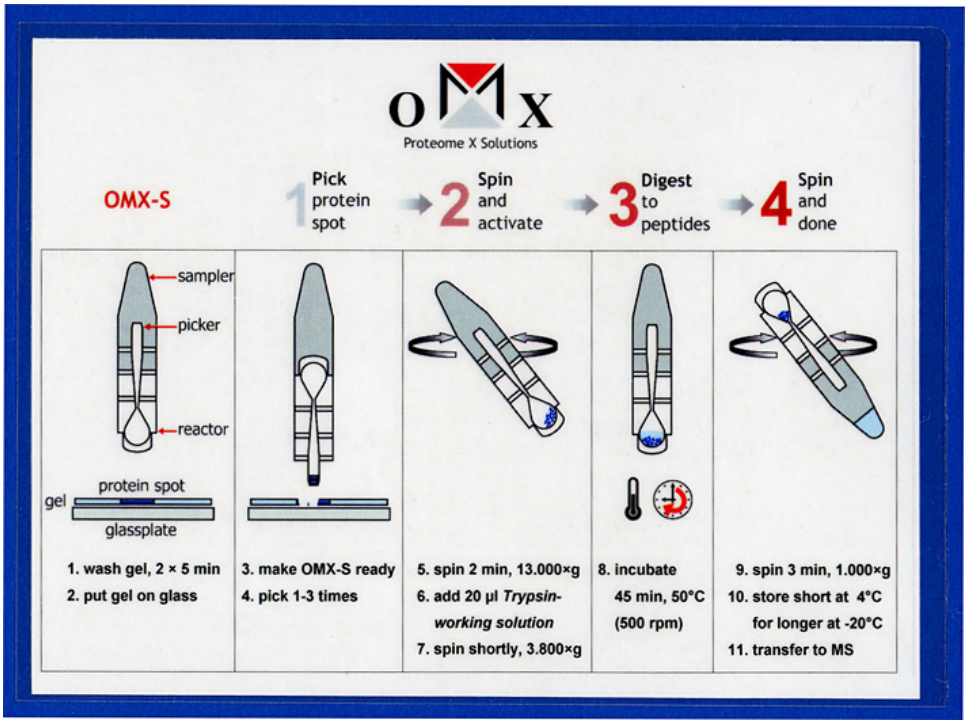

Figure 12. The figure is an illustration of the OMX-S tube that describes how the bands containing the proteins from the gel was picked, spun, digested with trypsin and made ready for mass spectrometry[45].

2.8.4 Solutions

Initiation solution	1000 µl
80 % (w/w) Acetonitrile	
5 % (w/w) Formic acid	
15 % dH_2O	

Acetonitril NH_4CH_3	10 ml
50 % (w/w) Acetonitrile	5 ml
50 % (w/w) NH_4CH_3	5 ml

Trypsin	20 µl
50 mM NH_4CH_3	18 µl
Trypsin	2 µl

Elution solution	1 ml
65 % Acetonitrile	650 µl
2 % 2-Propanol	20 µl
0.1 % Formic acid	1 µl

Formic acid	1 ml
5 % Formic acid	50 µl
95 % dH_2O	950 µl

2.9 Statistics

For the salmon variance testing ANOVA, General Linear Model, in Minitab 16 (Minitab Inc., State College Pennsylvania, USA), was used. ANOVA (general linear model) was used to determine the effect of the experimental parameters. The significant differences was according the Tukey´s multiple-comparison test when differences were $P \leq 0.05$.

3.0 Results and discussion

3.1 Appearance and colour measurement of high pressure treated salmon fillets

Raw salmon muscle can be described as having a shining pink-orange colour, a fibrous structure that is good to chew and to be odour free. When exposed to increasing pressure level the fish muscle looked more opaque, the texture felt more like rubber, and the smell changed towards cocked salmon or like cod liver oil (600 MPa). The colour changes can be seen in Figure 13. The processed salmon has been tasted, smelled and examined on appearance by the student and the supervisors. For a more accurate evaluation professional sensorics should have tested it; however this was not a part of the thesis.

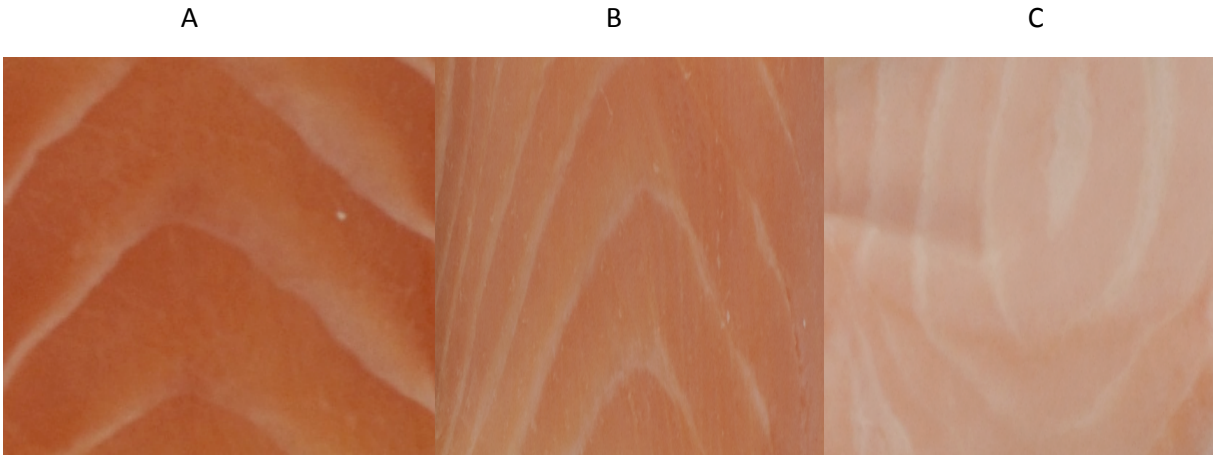

Figure 13. Photographic pictures of raw salmon (A), pressure treated salmon at 300 MPa (B), and at 680 MPa (C), taken with DigiEye gives an illustration of the colour changes induced by HPP.

The results from the colour analysis done by DigiEye are summarised in Table 3. There was no significant colour difference between 0-200 MPa in redness, yellowness or chroma. At pressure above 300 MPa the measurements gave statistically significant different results for each increase in pressure. The chroma, which describes the colour intensity, is decreasing as the pressure load increases resulting in a pale looking fish. These statistical significant colour changes are indications of protein denaturation of the salmon during HPP.

Table 3. The mean values from colourimetric measurement are based on analysis of raw fillets and pressure treated samples (100-685 MPa), where n = 2. The significance (P-values) are given were denoted by different lower-case superscript according Turkeys test.

Pressure treatment (MPa)	Redness (a*)	Yellowness (b*)	Chroma (C*)	Hue (h*)
0	32.12^{a}	31.86^{a}	45.24^{a}	44.77^{a}
100	31.75^{a}	31.87^{a}	44.99^{a}	45.09^{a}
200	32.04^{a}	31.47^{a}	44.90^{a}	44.48^{ab}
300	28.97^{b}	28.07^{b}	40.33^{b}	44.09^{ab}
400	22.94^{c}	21.30^{c}	31.31^{c}	42.77^{b}
685	18.94^{d}	18.37^{c}	26.38^{d}	44.13^{ab}
P-value	$P < 0.078$	$P < 0.035$	$P < 0.0092$	$P < 0.0091$

3.2 Differential scanning calorimetry and water holding capacity analysis of salmon

DSC analysis was performed on raw fillet, raw minced, and minced frozen salmon samples. The DSC thermograms (Figure 14) from the three different samples indicated that they appear to be comparable. By investigating the denaturation peaks enthalpies (Table 4) and denaturation temperatures (
Table 5) the significance test gave the following result: no significant differences were found between raw fillets and minced frozen material ($P < 0.05$). There was a 0.7 °C shift in denaturation temperature of myosin from for raw minced, but as long as all other parameters correlated this is a negligible finding. Based on these findings for the further investigations there were made a batch of raw minced salmon that were frozen at -80 °C, and these samples were used for further processing and analysis.

The dry mass was calculated (Equation 3), and WHC (Equation 4) was illustrated (Figure 15), giving WHC values of 89, 89 and 86 % for raw fillet, raw minced, and frozen minced

Material	Peak 1	Peak 2	Peak 3	Peak 4
Raw fillet	49.1 ± 0.5	59.6 ± 0.7	73.8 ± 5.3	79.7 ± 0.2
Raw minced	49.6 ± 0.3	60.3 ± 0.2	71.3 ± 0.3	80.2 ± 0.2
Frozen minced	48.8 ± 0.7	60.3 ± 0.6	70.8 ± 0.7	79.5 ± 0.1

respectively. There was no significant difference between WHC of raw fillet and raw minced. Although some water will be released due to mechanical processing during mincing that could not be measured because it is left in the mincer and in the plastic bag used for packaging. The minced frozen samples do loose more water then raw minced samples due to the freezing and thawing process.

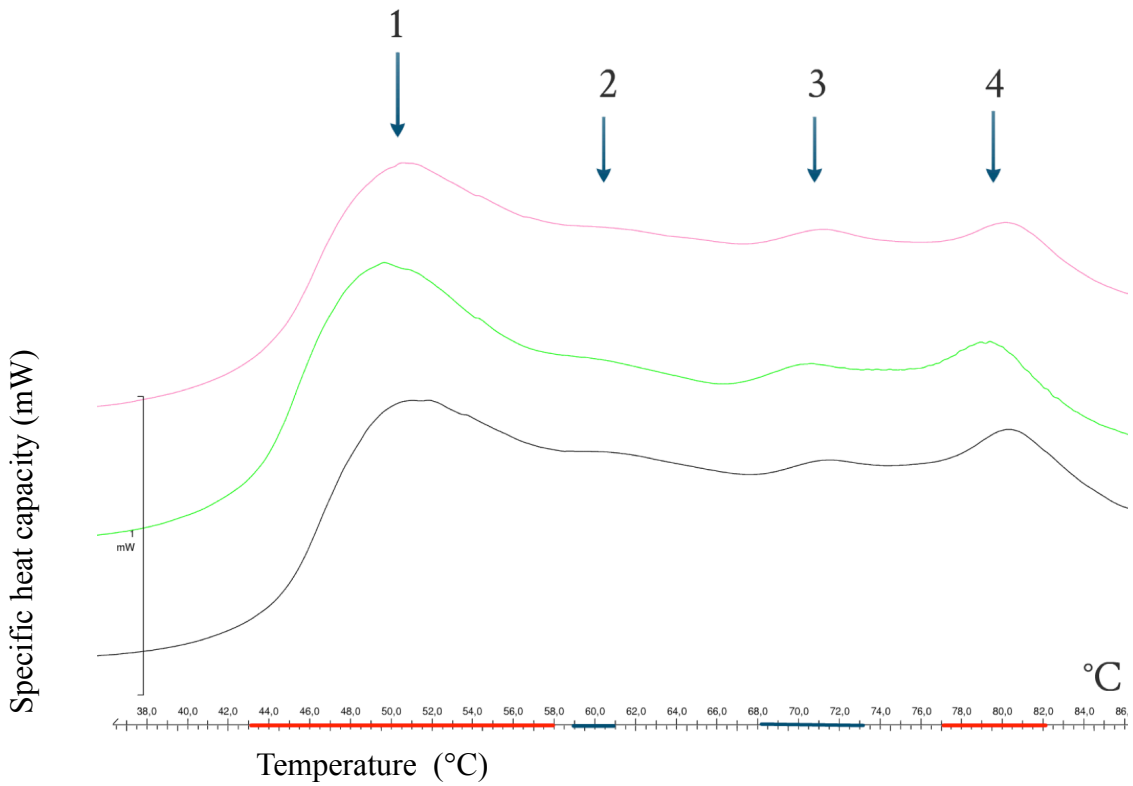

Figure 14. Thermogram of raw salmon (n = 5) in the state of raw fillet (black), raw minced (green) and frozen minced (pink). The range of the integration of peaks are shown on the abscissa for myosin (red marker, 44-57 °C), the sarcoplasmic proteins (blue marker, 59-61 °C and 68-73 °C) and actin (red marker, 77-81 °C).

Table 4. Denaturation enthalpies (J/g) for raw fillet, raw minced and frozen minced salmon samples (n = 3) .

Material	**ΔH 1**	**ΔH 2**	**ΔH 3**	**ΔH 4**
Raw fillet	0.565 ± 0.070	0.004 ± 0.000	0.013 ± 0.003	0.024 ± 0.016
Raw minced	0.406 ± 0.057	0.001 ± 0.001	0.009 ± 0.002	0.058 ± 0.003
Frozen minced	0.697 ± 0.007	Indistinguishable	0.009 ± 0.002	0.024 ± 0.003

Table 5. The denaturation peak temperatures and standard deviation are given in Celsius degrees for the different salmon samples (n = 3).

Material	**Peak 1**	**Peak 2**	**Peak 3**	**Peak 4**
Raw fillet	49.1 ± 0.5	59.6 ± 0.7	73.8 ± 5.3	79.7 ± 0.2
Raw minced	49.6 ± 0.3	60.3 ± 0.2	71.3 ± 0.3	80.2 ± 0.2
Frozen minced	48.8 ± 0.7	60.3 ± 0.6	70.8 ± 0.7	79.5 ± 0.1

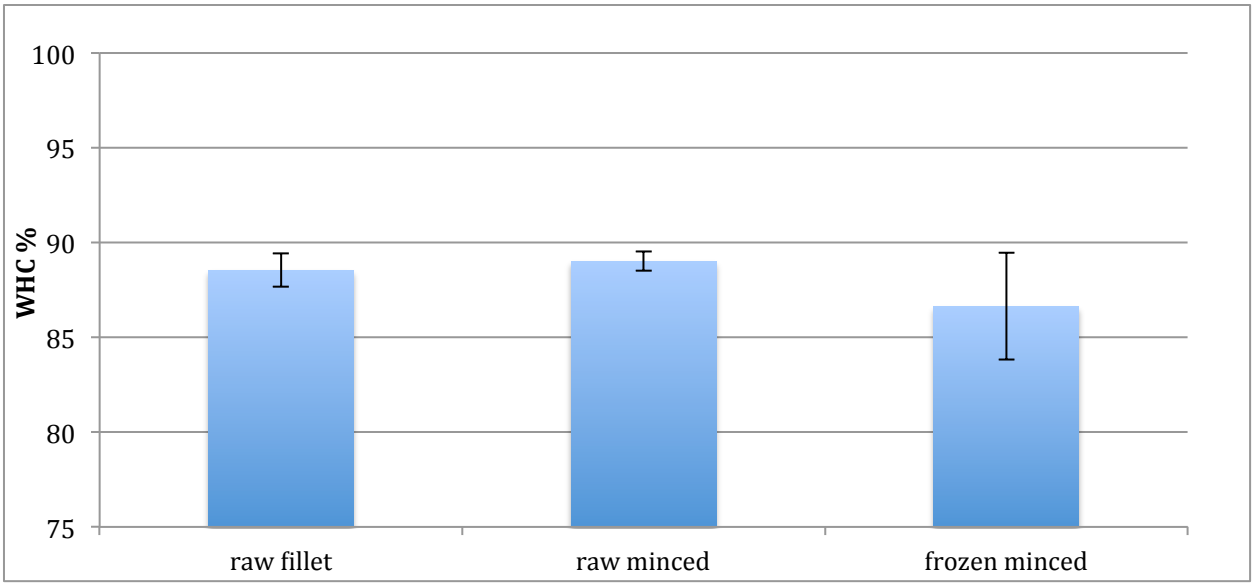

Figure 15. WHC of raw fillet, raw minced and frozen minced salmon and standard deviation (n = 8).

3.3 Differential scanning calorimetry of high pressure processed salmon

The thermograms from the DSC of salmon are given in Figure 16, 17 and 18. Figure 16 presents the average of four minced samples. The myosin peak denoted by arrow 1 show some individual differences, but peak temperatures (peak position) are even, and the actin peak (arrow 4) have low standard deviation (Table 6). By making average curves as seen in Figure 19 curve 1, the small irregularities around the myosin peak (1) even out. In the thermogram (Figure 17) of salmon at 300 MPa where arrow 1 indicates the myosin top, surprisingly they appear almost as sparks, instead of rounded curves. These 7 jagged curves shown are all parallels, and impossible to use for further interpretation of peak positions and enthalpy changes. Arrow number 3 gives indications that sarcoplasmic proteins have not been denatured by HPP at 300 MPa. The actin (arrow 4) is assumingly completely denatured because no peak or elevation can be seen.

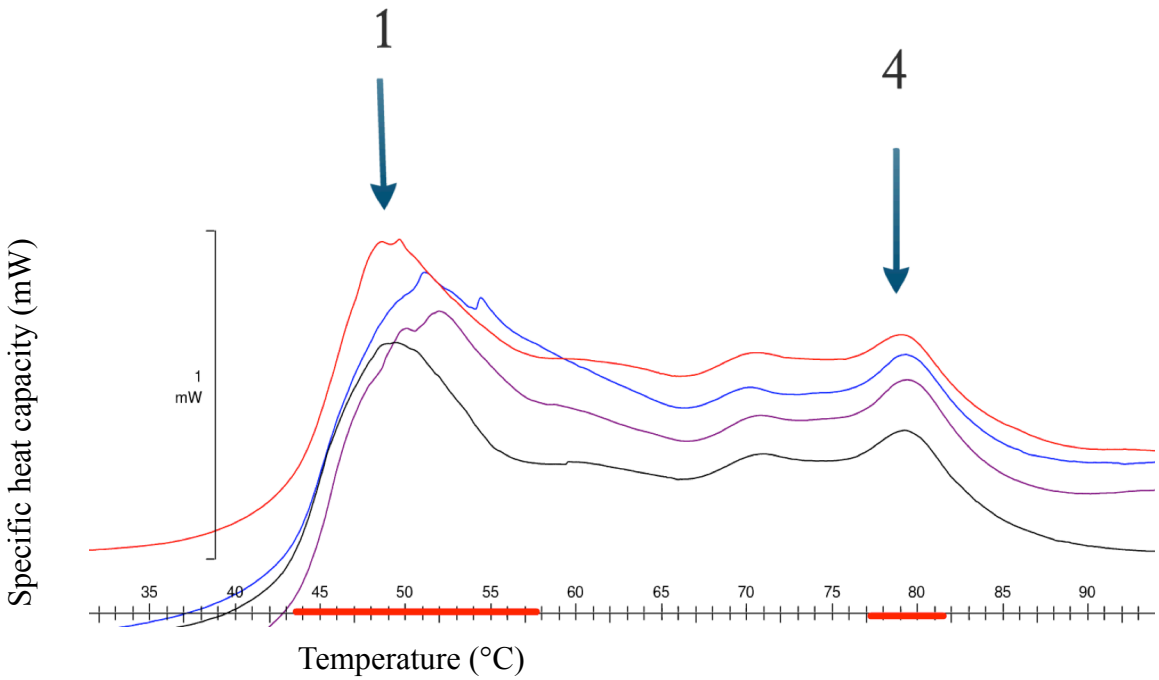

Figure 16. DSC thermogram of 4 parallel samples of frozen minced salmon obtained at a heating rate of 5 °C/min. Arrow 1 indicate the myosin peak, and arrow 4 the actin peak. The interval for integration of the peaks is shown on the abscissa for myosin (red marker, 44-57 °C) and actin (red marker, 77-81 °C).

Table 6. The denaturation enthalpy (j/g) for myosin (ΔH 1) and (ΔH 4) and peak positions in celcius degrees for raw or HPP salmon samples (MPa).

Material	ΔH 1	ΔH 4	Peak position 1 °C	Peak position 4 °C
Raw minced frozen	0.697^{a}	0.02 ± 0.003	48.8 ± 0.7	79.5 ± 0.1
HPP 100	0.396^{ab}	0.02 ± 0.002	50.2 ± 1.3	80.4 ± 1.4
HPP 150	0.233^{bc}	0.02 ± 0.006	49.4 ± 0.3	78.8 ± 0.2
HPP 200	0.087^{c}	0.02 ± 0.000	49.1 ± 2.7	79.4 ± 0.1
P-values	P < 0.001	P > 0.976	P > 0.815	P > 0.132

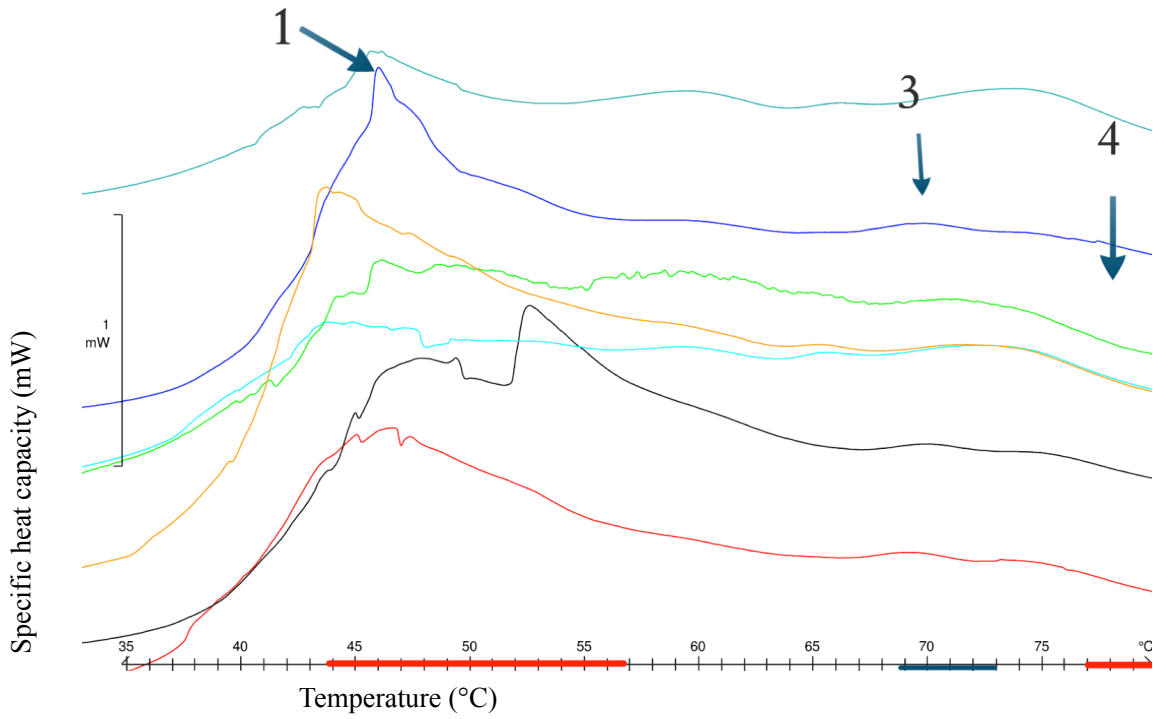

Figure 17. Thermogram of 7 frozen minced samples of salmon pressure treated at 300 MPa. The interval for integration of the peaks is shown on the abscissa for myosin (red marker, 44-57 °C), the sarcoplasmic proteins (blue marker 68-73) and actin (red marker, 77-81 °C). Arrows 1, 2, 3 indicates the myosin, sarcoplasmic and actin denaturation peaks.

In the thermogram (Figure 18) of 6 salmon samples high pressure treated at 600 MPa, the height of the myosin peaks (arrow 1) is lower, when compared to the 300 MPa (Figure 17, arrow 1). The sarcoplasmic protein seems to be denatured completely and actin curves are not present at all (arrow 4).

The average of 3 curves obtained for raw salmon and HPP samples (100-685 MPa) are shown in (Figure 19). Arrow A denotes where the myosin peaks becomes uneven at 200-250 MPa (curve 4 and 5). To combine the thermogram with denaturation enthalpies of myosin (Table 6), ΔH 1) it is clear that increased processing pressure resulted in a significantly decreased myosin denaturation enthalpy ($P < 0.001$). The myosin denaturation onset is between 200-250 MPa because the denaturation enthalpy for 200 MPa is not significantly different from 150 (Table 6), and at 250 and above indistinguishable. It is still evident that there is a myosin peak present, but the parallels are too uneven to be used for enthalpy calculations. The actin peak (arrow B) is visible up to 200 MPa, but at 250 MPa it becomes indistinguishable to calculate

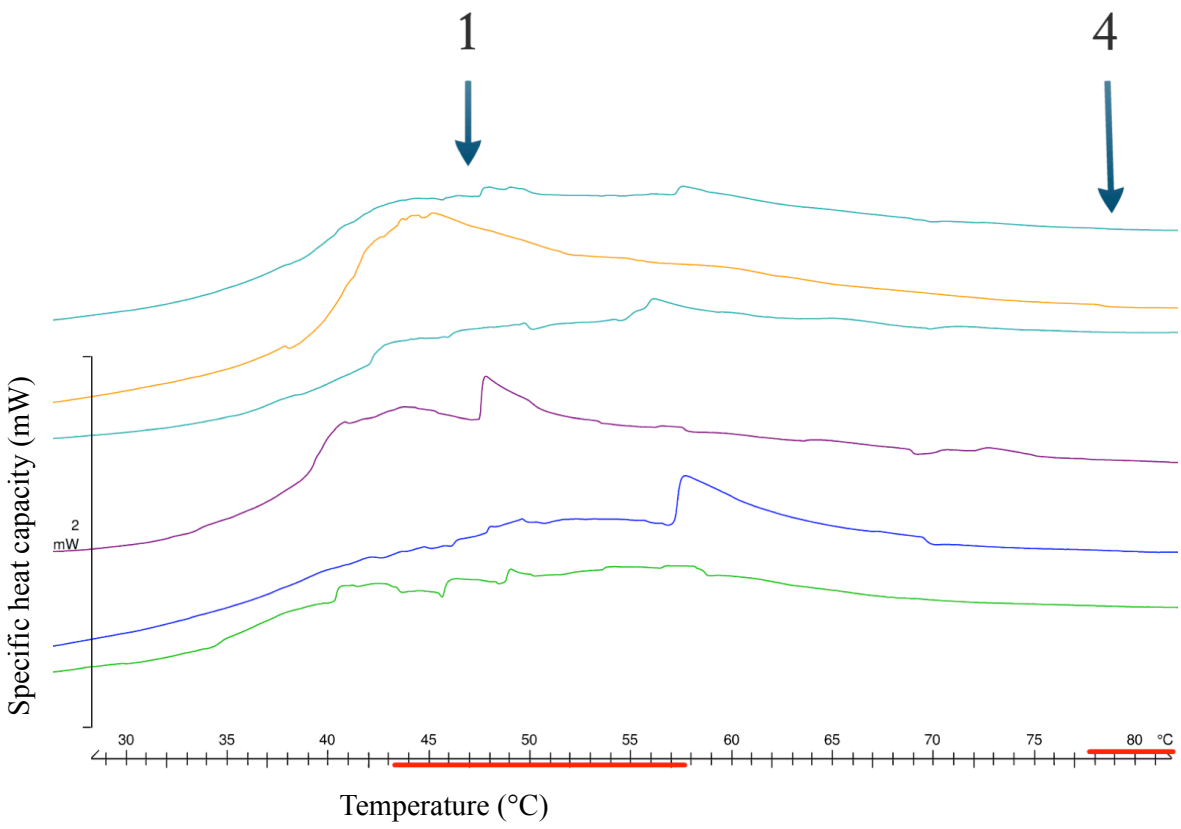

Figure 18. Thermograms of 6 frozen minced salmon samples at 600 MPa. The interval for integration of the peaks is shown on the abscissa for myosin (red marker, 44-57 °C) and actin (red marker, 77-81 °C). Arrow 1 and 4 indicates the myosin and actin peaks.

enthalpy from it. To compare these values with other DSC analysis of high pressure treated salmon sample from the literature, the following results have been previously published: Analysis of cod by Angsupanich and Ledward (1998) [46] stating: "the myosin peak has disappeared at 200 MPa, and as pressure is increased further, to 300 MPa, it is apparent that many of the sarcoplasmic proteins and actin denatured". There were differences in the pressure treatment protocol used in their experiment. While Angusipanich and Ledward (1998) made their HP treatments at ambient temperature for 20 minutes, the present study was performed with starting temperature of the water in the vessel at 9 °C.

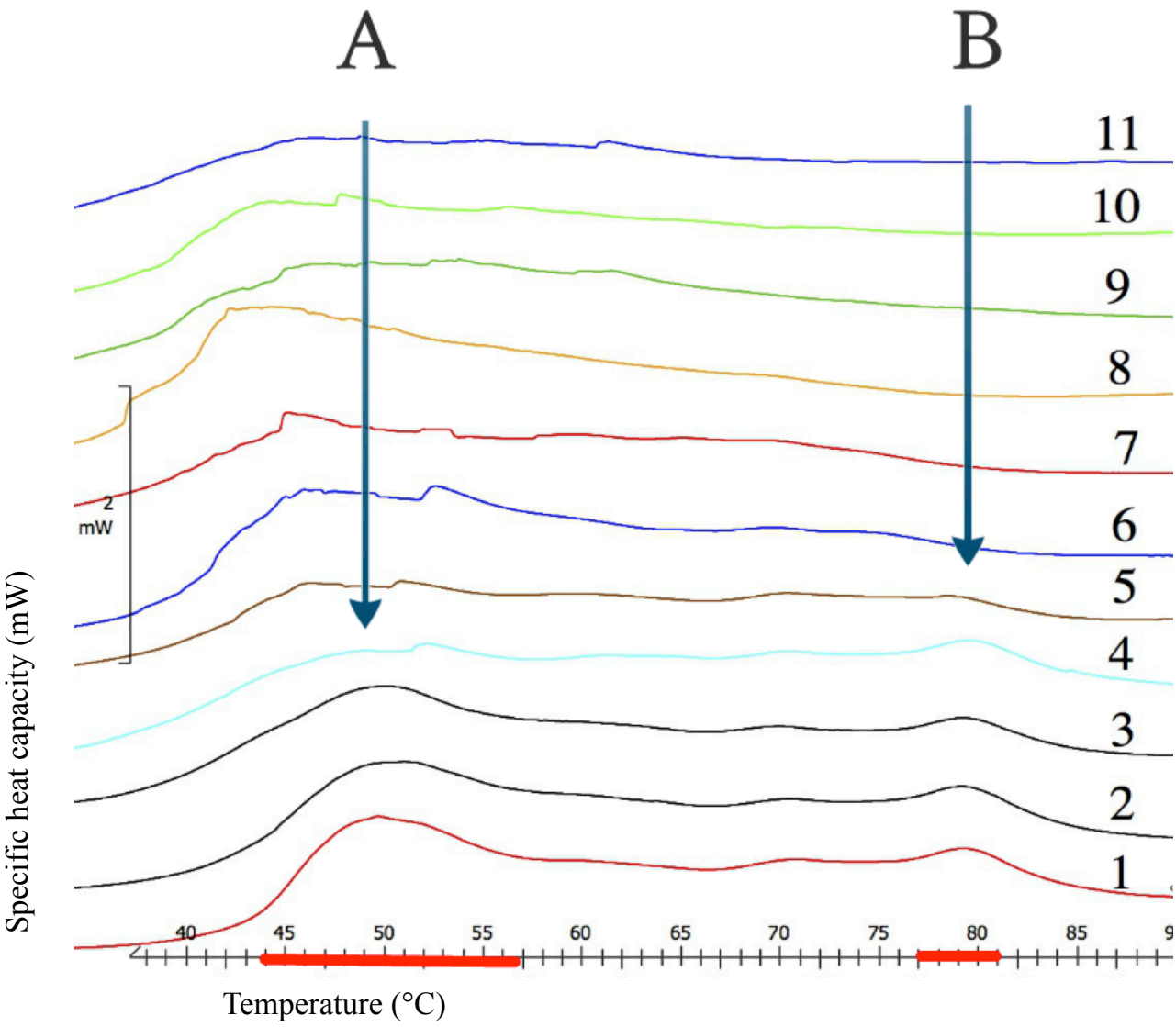

Figure 19: Thermogram of minced frozen samples (n=3) obtained at a scanning rate of 5 °C/min. Thermograms of raw (1), and HPP: 100 MPa (2), 150 MPa (3), 200 MPa (4), 250 MPa (5), 300 MPa (6), 350 MPa (7), 400 MPa (8), 500 MPa (9), 600 MPa (10) and 680 MPa (11). Arrow A is pointing at the myosin denaturation peak, and arrow B at the actin denaturation peak. The interval for integration of the peaks is shown on the abscissa for myosin (red marker, 44-57 °C) and actin (red marker, 77-81 °C).

3.4 Water holding capacity and drip loss of HPP salmon

There is a drop in WHC of pressure treated salmon from 88 to 73 %, going from 100 to 250 MPa (Figure 20). As pressure load increases above 250 MPa the changes are small, but at 685 MPa the WHC is only at 64 % and with a high standard deviation.

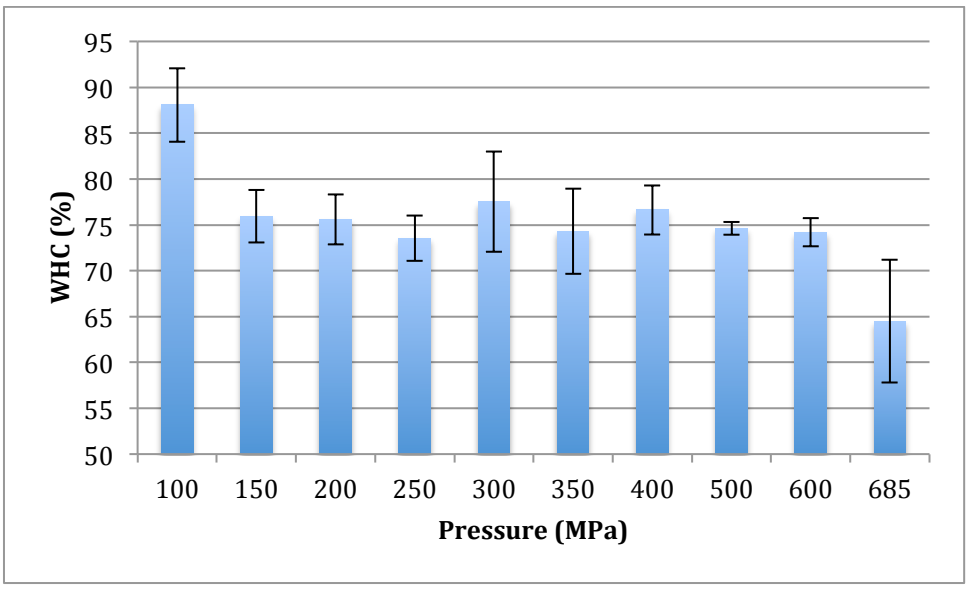

Figure 20. Water holding capacity (WHC) for raw salmon fillet (n = 4) pressurised from 100-685 MPa.

The graphical illustration (Figure 21) of the drip loss from fillets before and after processing indicates that water loss is quite high at 200, 300 and 400 MPa, but then there is a decrease at 500 MPa. Little water will leave at 500 and 600 MPa, but water loss increases again at the highest pressure 685 MPa.

In the addition to measurements of the drip loss, pictures were taken of the remaining fluid in the plastic bag where the samples were processed. This was done to compare the appearance after the different HP treatments. When the pressure load was 200 MPa (Figure 22) the drip loss was a transparent fluid with white dispersed particles. As pressure increased to 400 MPa (Figure 22) a striking difference was observed where the remaining drip loss had become more solid and with a beige colour. The white coagulated solid gave association to the coagulated egg white, and there was hardly any fluid left.

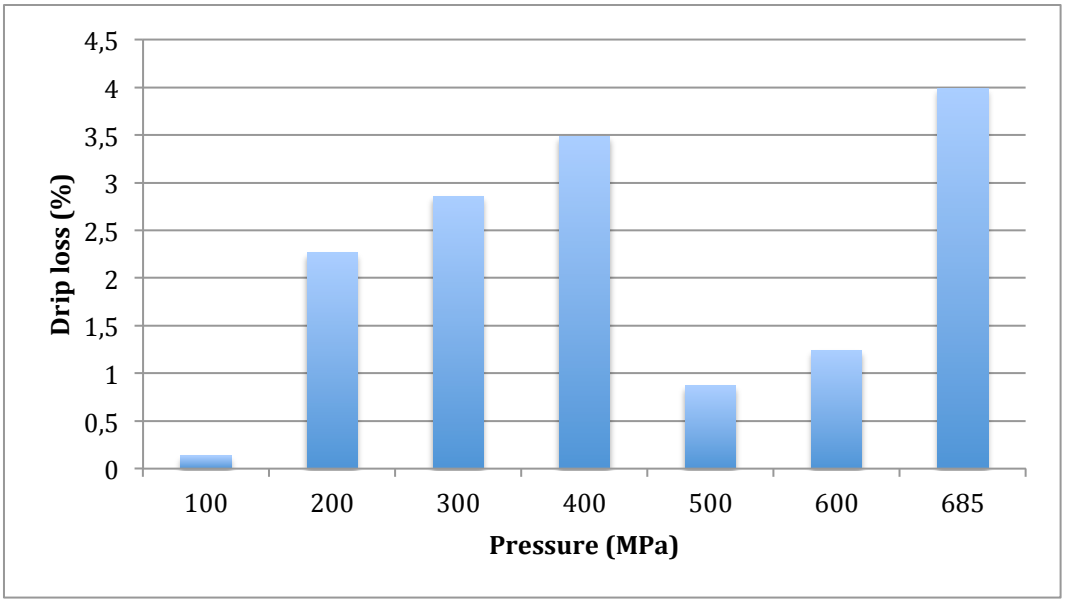

Figure 21. Percent drip loss from fillets after high pressure processing, where the pressures are given in MPa at the abscissa.

Figure 22. Pictures taken of the drip loss after removal of salmon fillet processed at 200 MPa (left) and 400 MPa (right).

3.5 Gel electrophoresis of salmon

The jagged DSC curves gave no explanation to what really happened to the myofibril during pressure treatment. Gel electrophoretic analyses were therefore performed to provide higher resolution on the molecular level for understanding the proteins under pressure. Since there is no known procedure for extraction of proteins from tissue samples without denaturation of the proteins, different extraction technics had to be tested. The best results were found when tissue samples were solubilised in Tris buffer, and no additional mixing was performed. A number of treatments were tested: 1: raw fillet of salmon, 2: 100 MPa, 3: 200 MPa, 4: 300 MPa, 5: 400 MPa, 6: 500 MPa, 7:600 MPa and 8: 680 MPa (Figure 23).

For localisation of the muscle proteins, molecular weight information from literature was used as reference [31, 38, 47-49]. Each specific band on a gel could consist of proteins with one specific size found in the sample. The denatured muscle proteins in fish are as follows:

- Myosin complex (MC) 520 kDa
- Myosin heavy chain (HC) 210 kDa
- Actin (A) 42 kDa
- Myosin light chain (LC) 15-25 kDa

Separation of the different pressure treated samples by native lithium dodecyl sulfate (LDS) - PAGE and staining with Coomassie revealed that the protein concentration of protein complexes with molecular weight of 600 kDa and higher decreased, as pressure increased above 500 MPa (Figure 23, arrow A, lane 6-8). In contrast, a pressure of 100-300 MPa appeared to increase the amount of these proteins complexes as seen relative to its raw untreated samples (Figure 23, lane 1). It was evident that a high concentration protein band at 480 kDa was lost after treatment at 200 MPa (Figure 23, arrow B). In addition, two protein bands were lost when pressure treatment increases above 200 MPa, the first with molecular weight of approximately 242 kDa (Figure 23, arrow C) and the second one at approximately 146 kDa (arrow D). It was speculated that the loss of the protein band at 480 kDa (arrow B) could indicate the loss of the myosin complex, whereas the loss of a band around 242 kDa indicate a loss of the myosin heavy chain. Since both protein bands were lost at around 200 MPa this indicated a specific sensitivity for the 200 MPa pressure increase. In addition, a protein band below 66 kDa was speculated to correspond to actin, which remained stable

throughout the increased pressure. It remained unclear whether the molecular weight of the native complexes can be calculated from information in the literature, since native proteins move differently in native LDS-PAGE gel, then SDS-PAGE [39]. In comparison to the denatured weight of actin found at 42 kDa, the literature indicated that the native weight of actin monomere was about 45 kDa. It was also described that the actin monomer binding protein, deoxyribonuclease 1(DNase 1) forms a binary complex with G-actin, giving the protein complex a molecular mass of 74 kDa [50].

The LDS-PAGE results of HP treated salmon did not explain the protein composition of the protein complexes. For separation of the protein subunits, a combination of native electrophoresis and SDS-PAGE was selected. This method allows detection of protein subunits that are assembled within the native protein complexes and it was anticipated that it should be possible to detect, whether the myosin proteins would be missing in the second dimension gel at the corresponding position of the 480 kDa band. The LDS-PAGE was run again to investigate the pressures with raw fillet (lane a) and the selected pressures of 150 (lane b), 200 (lane c), and 250 (lane d) MPa as shown in Figure 24, A. Upon staining of the gel, it became clear that the band at 480 kDa did not occur at all, in any of the samples. This was very surprising since the lost protein complexes from the first native gel (Figure 23) did not reveal a corresponding loss of a denatured protein in the second dimension gel (Figure 24 D, H and G). However, all second dimension gels revealed more or less the same protein pattern.

Identification of proteins from the complexes by mass spectrometry (Table 6) gave the following results from figure 24 A; 1: No protein, 2: No protein, 3: creatinine kinase 1,2, 4: glycogen phosphorylase, 5: Ferritin.

For second dimension PAGE, the native gel (Figure 24 A) was cut between the lanes and without staining. The formed strips were fixed on top of a denaturing SDS-gel and protein subunits were separated. The results were difficult to read because the alignment of the low and high molecular end of the cut band from the native gel (A) was not consequently aligned equal towards the standard. To facilitate a reading of the results schematic figures were drawn (C, E and G). Upon staining of the SDS-gels, all second dimension gels showed a similar pattern for separation of protein subunits, except the 200 MPa (Figure 24, E) that lost the low molecular band seen at 64 kDa seen in the other two gels (Figure 24 C, and D).

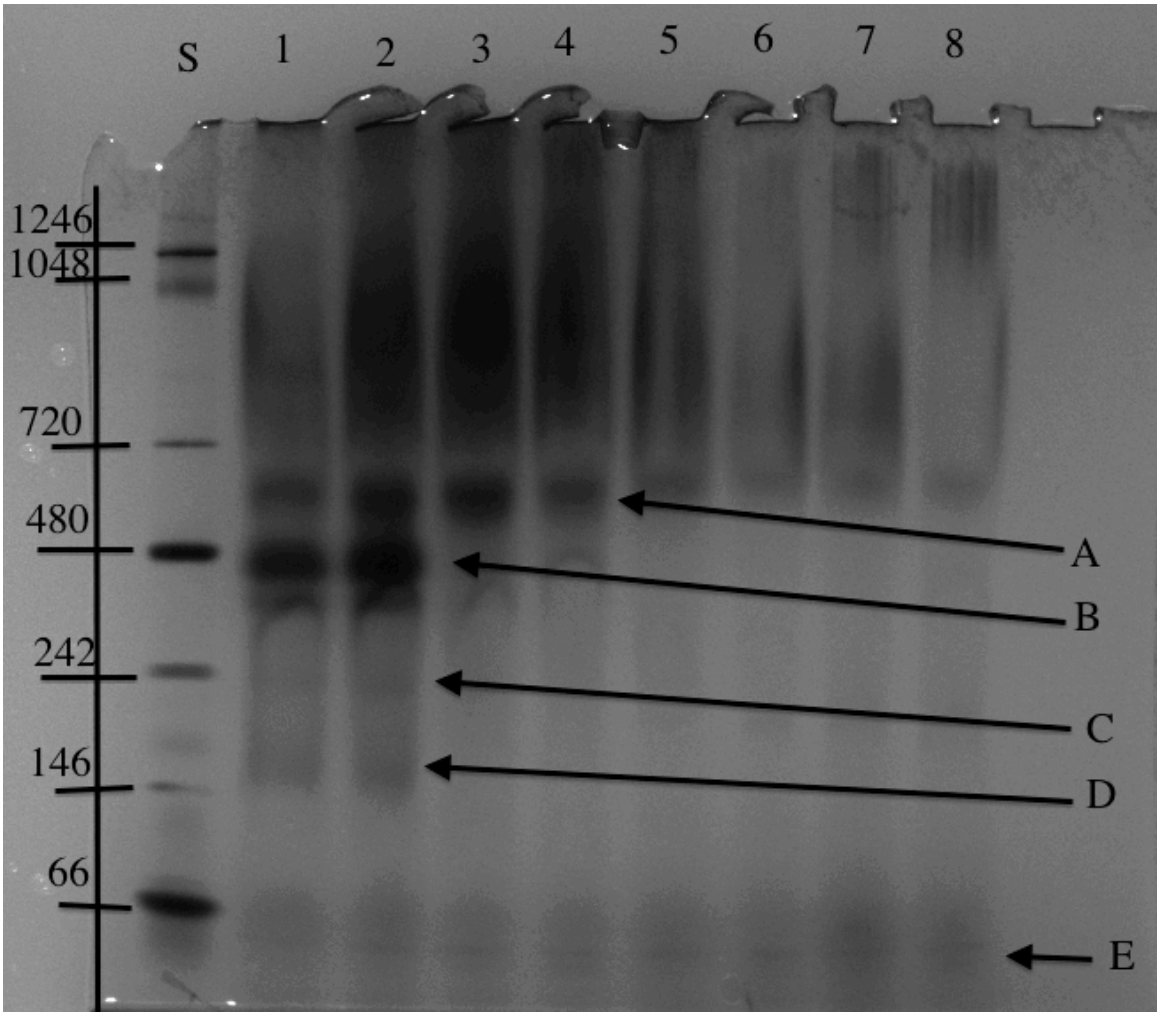

Figure 23; Native LDS-PAGE of salmon in Tris buffer. Raw fillet of salmon remained either untreated (1), or exposed to high pressure treatment of 100 MPa (2), 200 MPa (3), 300 MPa (4), 400 MPa (5), 500 MPa (6), 600 MPa (7), 680 MPa (8). Samples were loaded onto the native gel and separated together with a native molecular weight mix of standard proteins (S) with size indicated in kDa. Arrow (A-E) indicates changes of the protein bands.

Furthermore, a diffuse high molecular weight complexes separated into a number of bands with molecular weight of about 98, and 64 kDa and two bands between 50 and 64 kDa (Figure 24, D, F and H). The untreated raw sample (C) had an extra band at the high molecular weight (HW) end, at approximately 250 kDa.

The MS analysis was performed on bands cut from the gels (Figure 24). The raw salmon fillet band at 64 kDa (denoted 1, Figure 24, C) could not be identified by MS. But the band at 148 kDa found in both raw salmon and 200 MPa (Figure 24, C, E, denoted 2) was identified as Creatinine kinase 1, 2 in both. That was surprising because it was expected to find actin. The result did however correlate to the native gel (A) where the band above 146 kDa was identified (lane b, sample 3, Figure 24) as Creatinine kinase 1, 2.

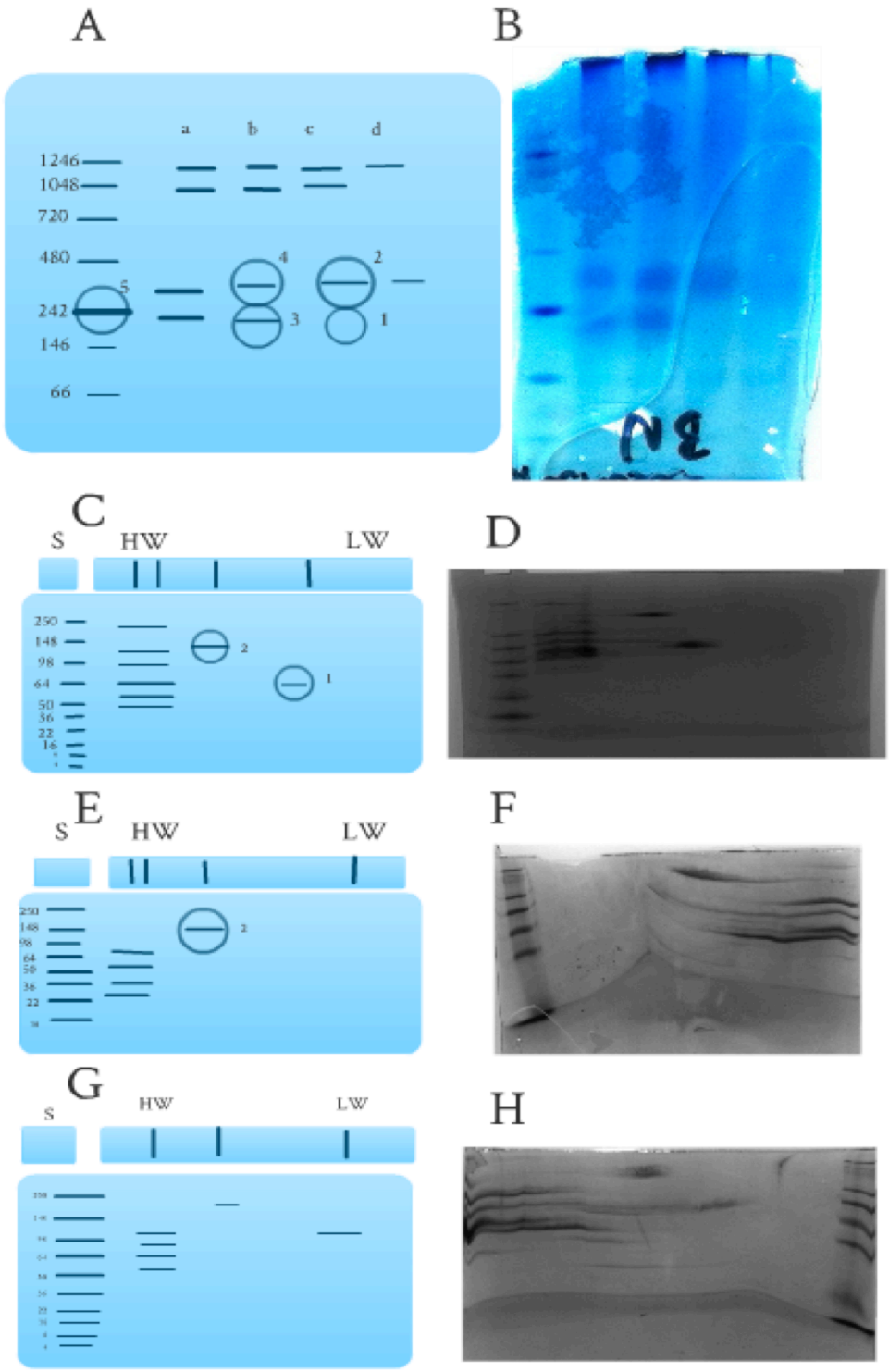

Figure 24. Separation of protein complexes and protein subunits from salmon by 2 D gel electrophoresis. The gels (A, C, E and G) are illustrations to explain the pictures of the electrophoretic gels (B, D, F and H). Illustration A is a LDS-PAGE, where the four lanes has the subsequent samples; raw fillet (a), high pressure processed (HPP) fillet at 150 MPa (b), HPP fillet at 200 MPa (c) and HPP at 250 MPa (d). Lane a, c and d were cut out and placed on top of SDS-PAGE with the following result given in D, F and H. The left lane is the standard marker with the different sized bands illustrated. The circles on the figure illstrate the samples cut out for mass spectrometry (MS). High and low molecular end of the strips are marked (HW and LW, in C, E and G). The illustrations align the same way towards the marker facilitate to the comparison.

Finally, to investigate whether high pressure treatment might generally lead to a loss of protein by aggregation in salmon, denatured SDS-PAGE analysis was performed. Raw samples, or high-pressure treated at 100, 200, 300, 400, 500, 600, and 680 MPa were solubilized and separated by SDS (Fig. 25), and visualized by Coomassie staining. Interestingly, the overall number of proteins visible after HPP appeared unchanged. The overall intensity of the protein bands subjected to 100 MPa (lane 2) and 680 MPa (lane 8) were increased. By combining the results from the previous gel electrophoresis analyses and the molecular weights from the literature background [31, 40] the following samples for MS were chosen and marked by circles on the gel (Figure 25: 1, 2 and 3). The result from the MS analysis (Table 6) identified myosin heavy chain in sample 1, enolase in sample 2, and the myosin regulatory chain in sample 3. The myosin regulatory light chain (Figure 25, arrow 3) was of great interest since the intensity of this band was highest at 100 MPa, and then decreased as pressure increased. Strangely a strong signal was found at 680 MPa again. The myosin light chain was found together with troponin C and I, and the Troponins are parts of the thin filaments of the muscle structure [51].

A possible explanation to the lack of protein changes seen on the SDS-PAGE, can be explained by previous investigations describing HPP to affect the non covalent interactions within the protein molecules [23]. By SDS-PAGE a complete protein denaturation occurs, and only the primary structure is intact. The β-mercaptoethanol added in the preparation cuts the sulphate bridges, in addition to high temperature exposure, leaving the polypeptide completely unfolded. Other SDS-PAGE of HPP analyses from fish and meat muscle samples show differences between addition of reducing agent ß-mercaptoethanol or not [31, 52, 53]. These differences are that a new band above 205 kDa is evident in the absence of ß-mercaptoethanol. As the ß-mercaptoethanol reduces the sulphate bridges, it is thought that HPP leads to the formation of sulphate bonds for myosin heavy chains [31].

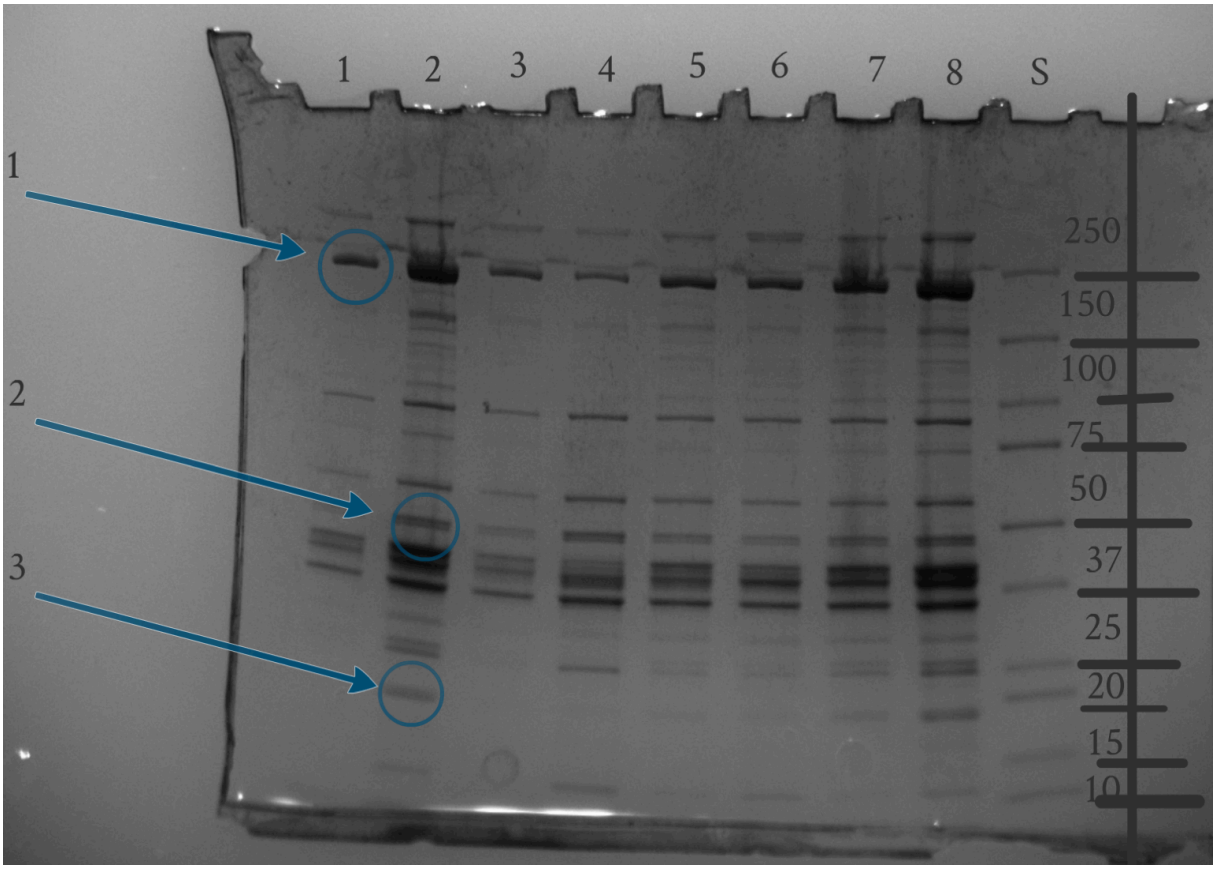

Figure 25. SDS-PAGE of salmon samples. Lanes are as given Raw (1), 100 MPa (2), 200 MPa (3), 300 MPa (4), 400 MPa (5), 500 MPa (6), 600 MPa (7), 680 MPa (8) and standard (S) with molecular weights indicated in kDa. Arrows indicate the samples used for samples for MS. Arrow 1 was identified by MS result as the myosin heavy chain (MHC), arrow 2 enolase and arrow 3 the myosin light chain (MLC).

3.6 Mass spectrometry of salmon

MS analysis was performed according to the described method in sub chapter 2.7. The protein bands marked with circles and numbers (Figure 24 and 25) were selected and cut out and prepared for MS. The raw data and MSMS spectra of myosin heavy chain can be found in the Appendix. The result from the native LDS (Figure 24) pointed out that none myofibrillar proteins were found from the native gel electrophoresis. The proteins found were all metabolic proteins, and not of great importance for these investigations. This proves the uncertainties of using only molecular weight indications for determining specific proteins found only by gel electrophoresis.

The protein bands samples from the SDS analysis (Figure 25) were investigated by MS, and with the result (Table 6) of myosin heavy chain (arrow 1), enolase (arrow 2) and myosin light chain (arrow 3). The myosin heavy chain did not come up as a sequence of salmon and the reason for this is unknown, but probably the specific peptide sequence identifying the salmon species was not one of the analysed peptide fractions.
The enolase is a metabolic enzyme for the glycolytic pathway and not of interest for these investigations.

Table 6. The protein samples analysed by mass spectrometry are summarized, and the identification of proteins are given.

Sample	**Protein**
1 (Figure 24, A)	No result
2 (Figure 24, A)	No result
3 (Figure 24, A)	Creatinine kinase 1, 2 *Salmo salar*
4 (Figure 24, A)	Glycogen phosphorylase *Salmo salar*
5 (Figure 24, A)	Ferritin
1 (Figure 24, C)	No result
2 (Figure 24, C, E)	Creatinine kinase 1, 2 *Salmo salar*
1 (Figure 25)	Myosin heavy chain *Oncorhynchus keta*
2 (Figure 25)	Enolase
3 (Figure 25)	Myosin regulatory light chain, troponin C, troponin I, *Salmo salar*

3.7 Turbot protein denaturation measured by DSC and gel electrophoresis

The DSC data for raw fillet (Figure 26), minced frozen samples (Figure 27) and post mortem aging (Figure 28) are shown. The thermogram of the raw fillet (Figure 26) gave uneven curves around the myosin peak (red marker, 50-58 °C, arrow 1), where the curves were parallels. The uneven myosin curves were also seen in the raw minced samples (Figure 27), and denaturation enthalpies could not be distinguished. The actin peak (arrow 4, Figure 26 and 27) was more stable but it was evident that the maximum peak temperatures were different as pressure load increased. The uneven curves are difficult to explain, and was the initial plan was to investigate the turbot by high pressure processing together with DSC analysis. When investigating the literature for possible explanations the work of Chevalier (2000) ([47] should be mentioned. The DSC enthalpy values described was a measure of total enthalpy denaturation of turbot muscle, without separating the enthalpy of myosin and actin as two different peaks. The thermograms in that article showed the curves as triplicates and with a rescan curve withdrawn to each. The curves look even and it is difficult to know what lies behind each presented curve. The other important finding was that even if pressure holding time is different from the salmon HPP investigations, the overall conclusions show that myosin peak is fully denatured with pressure treatment at 200 MPa, while the actin peak is particularly reduced after treatments at 180 and 200 MPa.

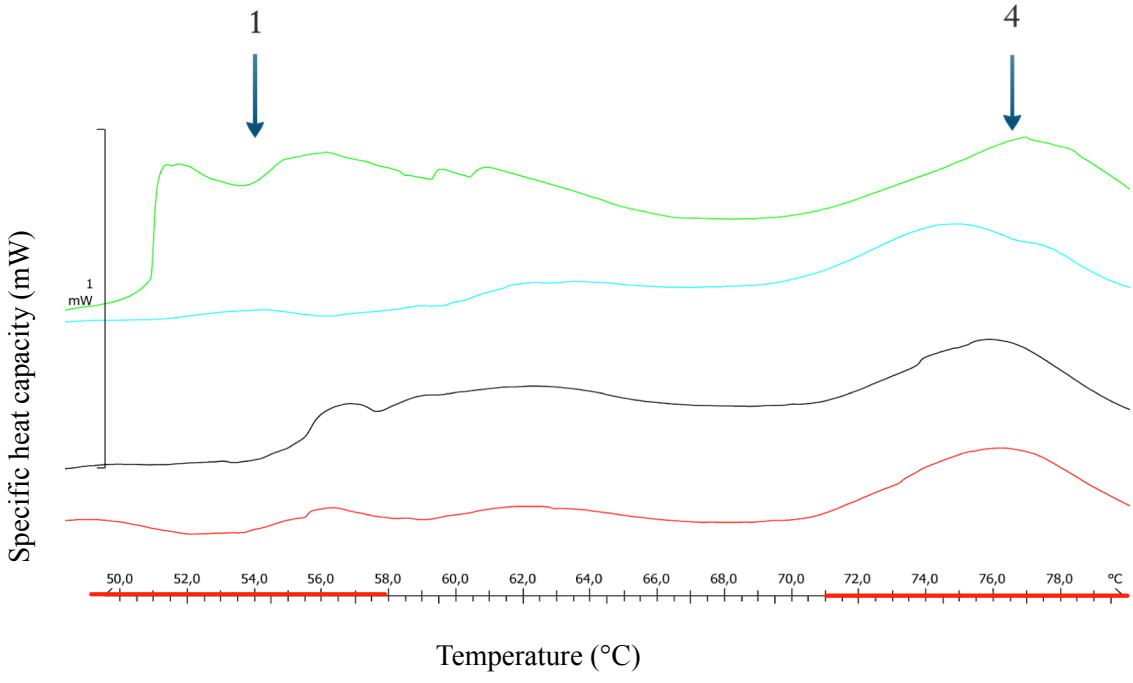

Figure 26. Thermograms of raw turbot fillet . The ranges for integration of the peaks are shown on the abscissa for myosin (red marker, 44-57 °C) and actin (red marker, 77-81 °C) denaturation.

The frozen raw turbot fillet from the post mortem storage experiment thermograph illustrates more even curves (n = 5) with a myosin peak (arrow 1) and a higher actin peak (arrow 4) (Figure 28). No significant breakdown of myosin was observed during the post mortem storage ($P > 0.75$), where the peak for denaturation remained stable ($P > 0.16$). Actin did however denature at a slow rate during post mortem storage ($P < 0.05$) (Table 8).

Raw samples of salmon and turbot filet can be compared, because interesting differences are seen among different fish species and protein denaturation by DSC analysis. When investigating the denaturation enthalpy for myosin (ΔH 1, Table 4), salmon has a higher value (ΔH 1 = 0.565) than turbot (Day 9, ΔH 1 = 0.294). When actin denaturation enthalpies (Table 4, ΔH 4) are compared from salmon (ΔH 4 = 0.024) and turbot (Day 9, ΔH 4 = 0.18), it is evident that turbot has the highest values. The salmon used in DSC experiments were 5-7 days, while turbot value was selected at day 8 to have approximately similar post mortem storage conditions. It could be tempting to assume that salmon has higher myosin and lower actin content than turbot, but that could be a very simplified assumption.

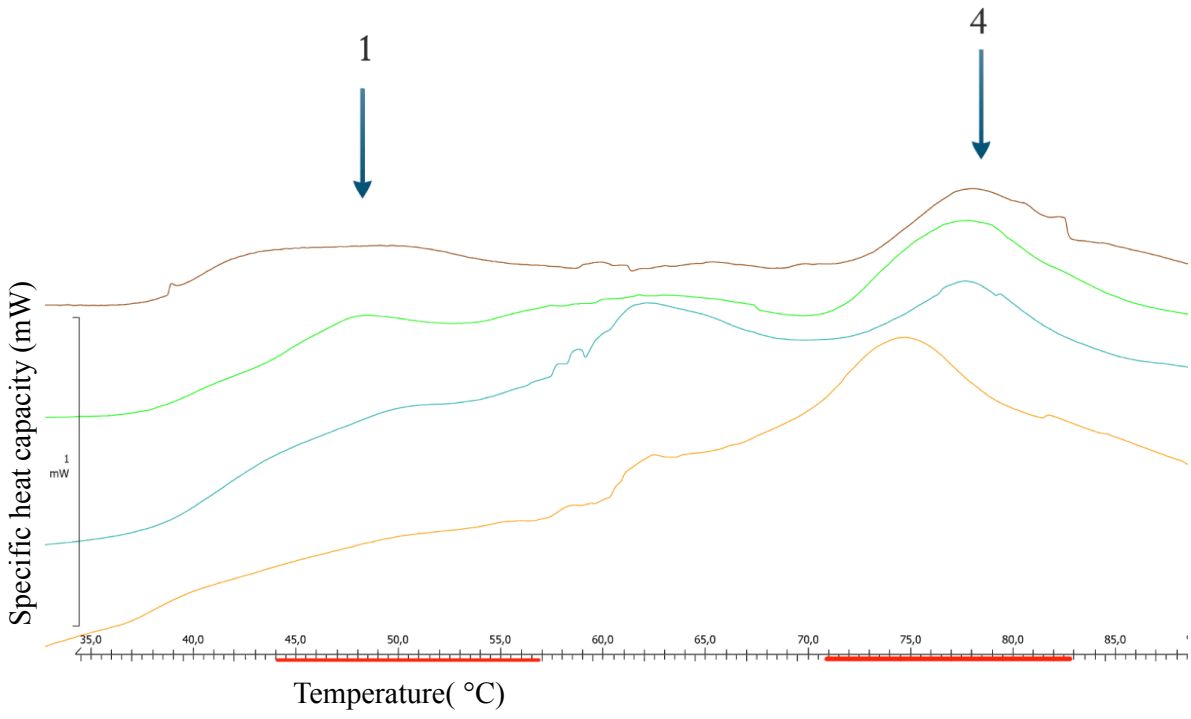

Figure 27. Thermograms of raw minced turbot . The ranges for integration of the peaks are shown on the abscissa for myosin (red marker, 44-57 °C) and actin (red marker, 77-81 °C) denaturation.

They live in different habitats and the salmon is a long distant swimmer, in comparison to the turbot that is an explosive sprinter. This fact indicates that they posses different muscle fibre composition, where turbot have more white muscle fibre types than salmon. The white muscle fibres are tightly packed with myofibrils, and very few lipid droplets are present. Turbot has a fat rim at the edge of the filet, while the salmon has fat integrated throughout the fish filet. The differences in fat content could lead to different heat conductivity, and by that means differences among the species.

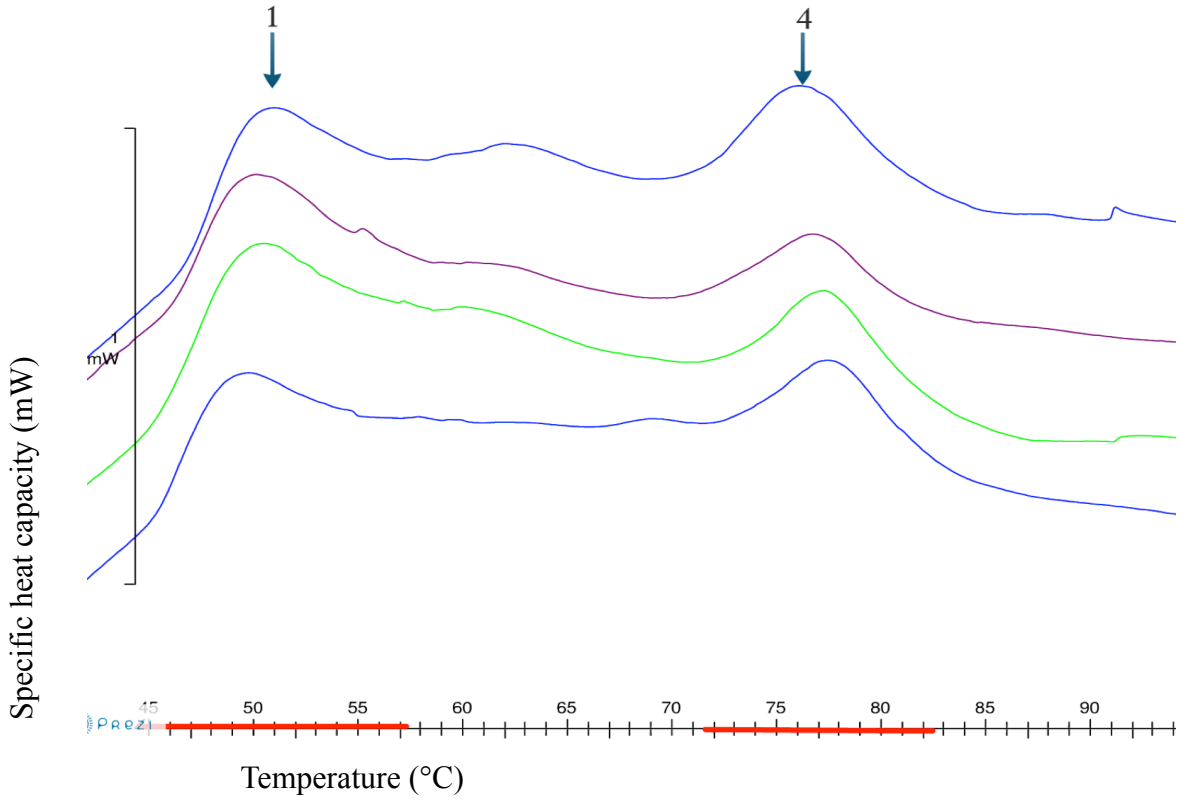

Figure 28. Thermograms of raw frozen turbot fillet from post mortem storage experiment. Lowest curve was examined at day 2 (blue), day 9 (green), day 16 (pink) and day 23, each curve representing n = 5. The ranges for integration of the peaks are shown on the abscissa for myosin (red marker, 44-57 °C) and actin (red marker, 77-81 °C) denaturation.

Table 7. The denaturation entalpies ΔH 1 and ΔH 2 (j/g), and the peak denaturation temperatures of turbot fillet (n = 6), with standard deviation values during post mortem aging at day 2, 9, 16 and 23.

Days of post mortem aging	ΔH 1	ΔH 4	Denaturation temperature 1 °C	Denaturation temperature 4 °C
2	0.292 ± 0.065	0.140 ± 0.020	48.4 ± 0.7	76.9 ± 0.2
9	0.294 ± 0.092	0.160 ± 0.040	48.2 ± 1.1	77.5 ± 2.3
16	0.286 ± 0.080	0.137 ± 0.050	48.6 ± 0.5	76.4 ± 0.4
23	0.304 ± 0.040	0.130 ± 0.030	49.4 ± 0.3	75.6 ± 0.7

The SDS-PAGE of turbot (Figure 29) is shown where the proteins were separated according to size, and the protein bands visualized by Coomassie staining. The overall number of proteins visible after the different growth experiments appeared hardly unchanged. The overall intensity of the turbot grown while exposed to light 18 h and dark 6 h (lane 2) were slightly increased. The myosin heavy chain (arrow HC), actin (arrow A), the component of thin filament troponin (TM) and the light chain (LC) of myosin were indicated according to literature [49].

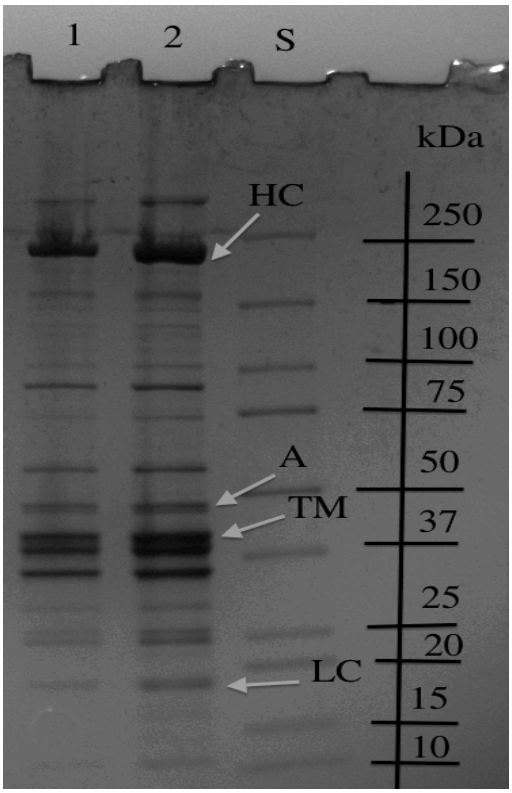

Figure 29. SDS-PAGE of minced frozen turbot with different light exposures from 24 h (1) and 18 hours light and 6 hours darkness (2). Standard (S) is used and molecular weights are given kDa).

3.8 The determination of analytic methods for analysing high pressure effects

To investigate the molecular changes of high pressure processing the appropriate analytic method should be selected. The DSC analysis of HPP salmon gave uneven curves when pressure load was at 250 MPa and above. The DSC analysis for heat treated salmon did not show similar effects like jagged curves [10]. Although pressure is applied uniformly in all directions simultaneously, pressurisation is also accompanied by adiabatic heating. The adiabatic heat occurs due to compression of the pressurizing medium, which in this case is water, and the food product. This can give an uneven temperature gradient in the food product during pressure treatment. The temperature increase in foods differs, depending on its fat level. Per 100 MPa there is an increase of 2-3 °C for water. The heat generated is continuously dissipated to the metal wall of the vessel during pressurization and pressure hold time, leading to natural convection and non-uniform temperature distribution within the vessel [54, 55].

The temperature measured in the pressure vessel was measured before the experiments and during processing. As an example, the start temperature in one experiment was 9.5 °C and when the pressure had reached 300 MPa the temperature of the water was at 14 °C. In another experiment, the starting temperature was at 8.6 °C, and the maximum temperature reached during pressurization was 21.9 °C at 600 MPa.

To return to the DSC analysis results where the uneven jagged curves as pressure increased With the question concerning the differences seen among in HPP salmon parallel samples and the adiabatic non-uniform heat described in literature [55, 56], it could be speculated if this was the explanation.

When a protein denatures the three-dimensional structure is altered, hence the biological active form is often altered. When pressure is applied to the molecule the pressure load will make the protein unfold. At the precise moment the pressure load terminates the refolding of the protein occurs [53]. Since it is believed that in a pressure treated system, the hydrophobic and electrostatic interactions are the most vulnerable, in comparison to heat treated systems where the chemical bonds affected are the H-bonds of the secondary, tertiary and quaternary structure [46]. By returning to the DSC measures the energetic transitions of the chemical bond energy release during heating, so the energy differences between H-bonds or

electrostatic interactions that are broken are probably different. To speculate further, could the uneven adiabatic temperature lead to different denaturation of the different parts of the fillet, which again lead to differences measured by the DSC, which again can be based on different bond energies released.

The native gel electrophoresis should provide more detailed information about the different protein complexes altered by the high pressure processing then the DSC. The challenge for native gel analysis is the correct identification of the myofibrillar proteins, where identification cannot be trusted by molecular weights found in the literature. Native gel electrophoresis should be combined with other accurate analysis like MS or Western blotting for accurate identifications.

Knowledge at the molecular level will give insight into the molecular changes that occur and hopefully an ability to understand what molecular changes that are responsible for the function of modified proteins.

3.9 Future experiments

- For further analysis and more specific detection of the protein changes during high pressure treatment, the plan was to combine native LDS-PAGE with Western blotting to separate and identify the proteins of interest. By gel electrophoresis, the proteins were separated according their molecular weight into bands on a gel. In Western blotting these bands containing the proteins can be transferred to a membrane and incubated with labelled antibodies to the proteins of interest. The unbound antibodies are washed away, leaving only bound antibody to the membrane, which can be detected by developing a film. As the antibodies only bind to the proteins of interest, only those would be visible. In addition the thickness of the band corresponds to the amount of protein present, and by comparison to known marker the amount of protein can be indicated. Unfortunately, there were not time to do Western blotting in this thesis however this works continues and more knowledge about high pressure effects on myosin and actin can be revealed using this method

- The differences measured between salmon and turbot on raw DSC curves are very interesting, and several other different fish species should have been included in this

type of analysis. It would be worth a try to compare salmon with mackerel on HPP and DSC analysis. Cod should have been HPP and analysed by DSC as well and compared to the turbot. Information about fish with high fat content in comparison to low fat content could maybe lead to new information for the methods

- The DSC analysis of fresh turbot fillet gave uneven curves, and the Halibut has experienced the same problems. To investigate that further the samples should have been placed in the sample cup together with a buffer solution to increase the heat conductivity during the DSC analysis

- Microbiological studies of salmon fillets exposed to the same pressures (200-250) where myosin denatures should have been performed. The HPP can also be done in successive pressure treatments, for increased inactivation of bacteria. To control the killing of the microorganisms protein denaturation is of great importance. The bacteria are usually declared dead when cell wall is destroyed, or the ribosomes are dysfunctional. Denaturation of proteins that are components of the cell wall, and the ribosomal enzyme are interesting to investigate further.

4.0 Conclusion

The aim of this Master project has been to learn about the possible effects on protein denaturation induced by high pressure processing (HPP)

- HPP do induce protein denaturation of salmon during pressurisation based on the results from DSC and gel electrophoretic analysis
- The high pressure processing of salmon fillet between 200- 250 MPa leads to significant protein denaturation of myosin, based on both the DSC results and gel electrophoretic analysis, while actin is denatured at around 300 MPa according DSC, while not at all according to LDS-PAGE.
- There is a significant colour difference in HPP salmon, where redness together with the colour intensity (Chroma) decreases as the pressure load increases

- There are differences in denaturation peaks when raw salmon and turbot are compared. Salmon has the highest denaturation peak of myosin, while turbot had the highest actin peak
- During post mortem storage of turbot myosin remained stable while actin denatured at a slow rate until day 24
- By comparing the methods used for studying the denaturation of proteins by high pressure processing, it is evident that native gel electrophoresis can provide very specific information about the proteins that denature in combination with identification using MS. While the DSC analysis of denaturation information resulted in jagged curves that could not provide specific information
- Enzymes from the metabolic pathways were identified by MS analysis, and myosin heavy chain was identified just below 250 kDa and myosin light chains were identified at approximately 20 kDa from the denatured gel electrophoresis

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6.0 Appendix

MS analysis from LDS-PAGE from Figure 24, A band marked 4:

Salmo salar creatine kinase-1 (ckm1)

MTKNCHNDYKMKFSDEEEFPDLSLHNNHMAKVLTKEMYKKLRSKSTPSGFTLDDCTQTGVDNPGHPFIMTVGCVA
GDEECYEVFKDMFDPIISDRHGGYKPTDKHKTDLNFENLKGGDDLDPAYVLSSRVRTGRSIKGYTLPPHNSRGER
RMVEKLSIEALATLDGEFKGKYYPLNGMTDAEQDQLIADHFLFDKPVSPLLLSAGMARDWPDARGIWHNDAKSFL
VWVNEEDHLRVISMEKGGNMKEVFRRFCVGLQKIEAVFKKHNHGFMWNEHLGYVLTCPSNLGTGLRGGVHVKLPK
LSTHPKFEEILTRLRLQKRGTGGVDTASVGGIFDISNADRLGSSEVQQVQMVVDGVKLMVEMEKKLEKGEAIDGM
IPAQK

454 (2+): FEEILTR

470 (2+): WHNDAK

547 (2+): TDLNFENLK

732 (2+): DLDPAYVLSSR

822 (2+): LVWVNEEDHLR

1004 (2+): VDTASVGGIFDISNADR

Salmo salar creatine kinase-2

MAKVLTKDMYAKLRDKQTPSGFTLDDVIQTGVDNPGHPFIMTVGCVAGDEESYEIF
KDLLDPIISDRHSGYKPTDKHKTDLNFENLKGGDDLDPNYVLSSRVRTGRSIKGYTLP
PHNSRGERRAVEKLSVEALNTLDGEFKGKYYPLNKMTDAEQEQLIADHFLFDKPVSP

LLLGAGMARDWPDARGIWHNDAKSFLVWVNEEDHLRVISMEKGGNMKEVFRRFC
VGLKRIEETFKKHNHGFMWNEHLGYVLTCPSNLGTGLRGGVHVKLPKLSTHPKFEEI
LTRLRLQKRGTGGVDTASVGGVFDISNADRLGSSEVDQVQMVVDGVKLMVEMEKK
LEKGEAIDGMIPAQK

768 (2+): LSVEALNTLDGEFK

895 (2+): GSSEVQQVQMVVDGVK

MS analysis from LDS-PAGE from Figure 24, A, band marked 3:

Glycogen phosphorylase, muscle form [Salmo salar]

MSKPLSDHDRKKQISVRGLAGVENVAELKVAFNRHLHFTLVKDRNVASKRDYYFAL
ANTVRDHLVGRWIRTQQYYYEKDPKRVYYISLEFYMGRTLQNTMVNLALENACDE
AIYQLGLDMEELEDMEEDAGLGNGGLGRLAACFLDSMASLGLAAYGYGIRYEFGIF
NQKIVNGWQVEEADDWLRYGNPWEKARPEYMRPVKFYGRTEHTPEGVKWVDTQV
VLALPYDTPIPGYRNNIVNTMRLWSAKAPCDFNLKDFNVGGYIQAVLDRNLCENISR
VLYPNDNFFEGKELRLKQEYFVVAATLQDIVRRFKASKFGSREIVRTDFAQLPNKVAI
QLNDTHPAMAIPELMRVLVDEEKLEWDKAWDVCVRTCAYTNHTVLPEALERWPID
LFHHLLPRHLEIIYEINRRFLQYVASKFPGDNDRLRRMSLIEEGECKKVNMAHMCIVG
SHAVNGVARIHSEILVATLFKDFYELDPHKFQNKTNGITPRRWLVMCNPGLAEVIAE
RIGEEFVRDLDQLKKLLKFIDDDAFIRDIAKVKQENKLKFAVHLEEHYKVKINPQSMF
DFQVKRIHEYKRQLLNCLHMITYYNRIKKEPNKHWTPRTIMVGGKAAPGYHTAKMII
RLITAIGEVVNHDPVIGDRLKVIFLENYRVTLAEKAIPSADLSEQISTAGTEASGTGNM
KFMLNGALTIGTMDGANVEMAEEAGEKNLFIFGMRVEEVDAMDAGKGYHASEYYN
RIPELKQAMDQISGGFFSHKQPDLFKELVDLLMHHDRFKVFADYEAYIKSQDKVNEL
YKKPKEWTKMVIHNIAGCGKFSSDRTISQYAREIWGMEPSLEKIPAPDEQLK

505.70 (2+): IPAPDEQLK
594.16 (2+): VENVAELK
667.73 (2+): WGMEPSLEK
517.19 (2+): TDFAQLPNK
556.20 (2+): FIDDD
817.93 (3+): GTEASGTGNFK

MS analysis from LDS-PAGE from Figure 24, A, band marked 5

Ferritin

SSQIRQNYSTEVEAAVNRLVNLYLRASYTYLSLGFYFDRDDVALEGVCHFFRELAEE
KREGAERLLKMQNQRGGRALFQDLQKPSQDEWGTTLDAMKAAIVLEKSLNQALLD
LHALGSAQADPHLCDFLESHFLNEQVKLIKKMGDHLTNIQRLVGSQAGLGEYLFERL
TLKHD

740.76 (2+): QNYSTEVEAAVNR

445.72 (2+): LVNLYLR

846.62 (3+): FQDLQQPSQDEWGTTLDAFK

MS analysis from LDS-PAGE from Figure 24, C band marked 2

Salmo salar creatine kinase-1 (ckm1)

MTKNCHNDYKMKFSDEEEFPDLSLHNNHMAKVLTKEMYKKLRSKSTPSGFTLDDCTQTGVDNPGHPFIMTVGCVA
GDEECYEVFKDMFDPIISDRHGGYKPTDKHKTDLNFENLKGGDDLDPAYVLSSRVRTGRSIKGYTLPPHNSRGER
RMVEKLSIEALATLDGEFKGKYYPLNGMTDAEQDQLIADHFLFDKPVSPLLLSAGMARDWPDARGIWHNDAKSFL
VWVNEEDHLRVISMEKGGNMKEVFRRFCVGLQKIEAVFKKHNHGFMWNEHLGYVLTCPSNLGTGLRGGVHVKLPK
LSTHPKFEEILTRLRLQKRGTGGVDTASVGGIFDISNADRLGSSEVQQVQMVVDGVKLMVEMEKKLEKGEAIDGM
IPAQK

454 (2+): FEEILTR

470 (2+): WHNDAK

547 (2+): TDLNFENLK

732 (2+): DLDPAYVLSSR

822 (2+): LVWVNEEDHLR

1004 (2+): VDTASVGGIFDISNADR

Salmo salar creatine kinase-2

MAKVLTKDMYAKLRDKQTPSGFTLDDVIQTGVDNPGHPFIMTVGCVAGDEESYEIF
KDLLDPIISDRHSGYKPTDKHKTDLNFENLKGGDDLDPNYVLSSRVRTGRSIKGYTLP
PHNSRGERRAVEKLSVEALNTLDGEFKGKYYPLNKMTDAEQEQLIADHFLFDKPVSP
LLLGAGMARDWPDARGIWHNDAKSFLVWVNEEDHLRVISMEKGGNMKEVFRRFC
VGLKRIEETFKKHNHGFMWNEHLGYVLTCPSNLGTGLRGGVHVKLPKLSTHPKFEEI
LTRLRLQKRGTGGVDTASVGGVFDISNADRLGSSEVDQVQMVVDGVKLMVEMEKK
LEKGEAIDGMIPAQK

768 (2+): LSVEALNTLDGEFK

895 (2+): GSSEVQQVQMVVDGVK

MS analysis of Figure 25, SDS-PAGE band 1:

Myosin heavy chain [Oncorhynchus keta]

>gi|21623523|dbj|BAC00871.1| myosin heavy chain [Oncorhynchus keta]
MSTDAEMQAYGKAAIYLRKSEKERMEAQATPFDSKNACYVTDKVELYLKGLVTARADGKCTVTVTNPDGSKEEGK
EFEEADIYEMNPPKYDKIEDMAMMTYLNEASVLYNLKERYAAWMIYTYSGLFCATVNPYKWLPVYDEEVVNAYRG
KKRMEAPPHIFSVSDNAFQFMMIDKENQSILITGESGAGKTVNTKCVIQYFATIAVSGSKKEVDPSKMQGSLEDQ
IIAANPLLESYGNAKTVRNDNSSRFGKFIRIHFQAGKLAKADIETYLLEKSRVAFQLPDERGYHIFYQLMTGHKP
ELVEMTLLTTNPYDFPMISQGHIAVPSINDKEELDATDDAITILGFTNDEKMSIYKLTGAVTHHGNLKFKQKQRE
EQAEPDGTEVADKIGYLLGLNSAELLKCLCYPRVKVGNEYVTKGQTVAQVYNAVMALAKSIYERMFLWMVIRINE
MLDTKNPRQFYIGVLDIAGFEIFDYNSMEQLCINFTNEKLQQFFNHTMFVLEQEEYKKEGIVWEFIDFGMDLAAC
IELIEKPLGIFSILEEECMFPKASDTTFKNKFYDQHLGKTKAFEKPKPAKGKPEAHFSLVHYAGTVDYNITGWLD
KNKDPLNESVILMYGKASVKLLATLYPAAPPEDKAKKGGKKKGGSMQTVSSQFRENLHKLMTNLRSTHPHFVRCL
IPNESKTPGLMENFLVIHQLRCNGVLEDLRICRKGFPSRIIYADFKQRYKVLNASVIPEGQFMDNKKASEKLLGS
IDVNHEDYKFGHTKVFFKAGLLGVLEEMRDEKLAALVGMVQALSRGFLMRREFSKMMERRESIFSIQYNIRSFMN
VKTWPWMKLYFKIKPLLQSAETEKELANMKENYEKMKTDLAKALATKKHLEEKLVALVQERADLALQVASEGQSL
NDAEERCEGLIKSKIQLEAKLKEMTERLEDEEEMNAELTAKKRKLEDECSELKKDIDDLELTLAKVEKEKHATEN
KVKNLTEEMASLDESVAKLTKEKKALQEAHQQTLDDLQAEEDKVNTLTKARTKLEQQVDDLEGSLEQEKKLRMDL
ERAKRKLEGDLKLAQESIMDLENDKQQADEKIKKKEFETSQLLSKVEDEQSLGAQLQKKIKELQARIEELEEEIE
AERAARAKVERQRADLSRELEEISERLEEAGGATSAQIDMNKKREAEFQKLRRDLEESTLQHEATAAALRKKQAD
SVAELGEQIDNLQRVKQKLEKEKSEYKMEIDDLSSNMEAVAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDI
SGQRARLLTENGEFGRQLEEKEALVSQLTRGKQAFTQQVEELKRQIEEEVKAKNALAHGVQSARHDCDLLREQFE
EEQEAKAELQRGMSKANSEVAQWRTKYETDAIQRTEELEEAKKKLAQRLQDAEETIEATNSKCSSLEKTKQRLQG
EVEDLMIDVERANAMAANLDKKQRNFDKVLAEWKQKYEEGQAELEGAQKEARSMSTELFKLKNSYEEALDHLETL
KRENKNLQQEISDLTEQIGETGKSIHELEKAKKTVETEKSEIQTALEEAEGTLEHEESKILRVQLELNQIKGEVD
RKIAEKDEEMEQIKRNSQRVVDSMQSTLDSEVRSRNDALRVKKKMEGDLNEMEIQLSHSNRQASEAQKQLRNVQG
QLKDAQLHLDDAVRVAEDMKEQAAMVERRNGLMVAEIEELRVALEQTERGRKVAETELVDASERVGLLHSQNTSL
LNTKKKLETDLVQVQGEVDDIIQEARNAEEKAKKAITDAAMMAEELKKEQDTSSHLERMKKNLEVTVKDLQHRLD
EAENLAMKGGKKQLQKLEWRVRELETEVEAEQRRGVDAVKGVRKYERRVKELTYQTEEDKKNVGRLQDLVDKLQM
KVKAYKRHAEEAEEAANQHMSKFRKVQHELEEAEERADIAETQVNKLRAKTRDSGKGKEVAE
530.69 (2+): SEVAQWR

568.21 (2+): LLTENGEFGR

618.89 (3+): DLEESTLQHEATAAALR

760.82 (+2): AALYPPPPPEDK
744.75 (2+): IEELEEELEAER

943.34 (2+): AELGEQIDNLQR

Cardiac muscle myosin heavy chain 6 alpha [Leucopsarion petersii] bony fish partial

>gi|312386772|dbj|BAJ34704.1| cardiac muscle myosin heavy chain 6 alpha [Leucopsarion petersii]
ALGNRHTKRVIQYFASIAAVGGANKKDQNKGTLEDQIIQANPALEAFGNAKTARNDNSSRFGKFIRIHFGTSGKL
SSADIETYLLEKSRVTFQLKAERNYHIFYQIMSNQKPELLEQLLITTNPYDYSYTSQGEVTVASINDADELMATD
SAFDVLGFTAEEKATVYKLTGAIMHYGNMRF

597.74(2+): ADIETYLLEK

MS analysis of Figure 25, SDS-PAGE band 2:

Enolase 3-1 [Salmo salar]

>gi|213511756|ref|NP_001135172.1| enolase 3-1 [Salmo salar]

MSIIKIHAREILDSRGNPTVEVDLYTAKGRFRAAVPSGASTGIHEALELRDGDKSRYLGKGTLKAVDHVNKDIAA
KLIEKKFSVVDQEKIDKFMLELDGTENKSKFGANAILGVSLAVCKAGAAEKGVPLFRHIADLAGHKDVILPCPAF
NVINGGSHAGNKLAMQEFMILPIGASNFHEAMRIGAEVYHNLKNVIKAKYGKDATNVGDEGGFAPNILENNEALE
LLKSAIEKAGYPDKIIIGMDVAASEFYKAGKYDLDFKSPDDPARYITGDQLGDLYKSFIKGYPVQSIEDPFDQDD
WAAWSKFTAAVDIQVVGDDLTVTNPKRIQQAVEKKACNCLLLKVNQIGSVTESIKACKLAQSNGWGVMVSHRSGE
TEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLMRIEEALGDKAKFAGKDYRHPKVN

Enolase 3-2 [Salmo salar]

>gi|213513724|ref|NP_001133193.1| enolase 3-2 [Salmo salar]
MSITKIHAREILDSRGNPTVEVDLYTAKGRFRAAVPSGASTGVHEALELRDGDKSRYLGKGTVKAVDHVNKDIAA
KLIEKKFSVVDQEKIDHFMLELDGTENKSKFGANAILGVSLAVCKAGAAEKGVPLYRHIADLAGHKDVILPCPAF
NVINGGSHAGNKLAMQEFMILPIGASNFHEAMRIGAEVYHNLKNVIKAKYGKDATNVGDEGGFAPNILENNEALE
LLKTAIEKAGYPDKIIIGMDVAASEFYKAGKYDLDFKSPDDPARYITGDQLGDLYKSFIKGYPVQSIEDPFDQDD
WAAWTKFTAAVDIQVVGDDLTVTNPKRIQQAVEKKACNCLLLKVNQIGSVTESIKACKLAQSNGWGVMVSHRSGE
TEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLMRIEEELGAKAKFAGKDYRHPKIN

693.27 (2+): YLTGDQLGDLYK

1031.07 (3+): FAPNLLENNEALELLK

1060.42 (2+): VEVDLYTAK

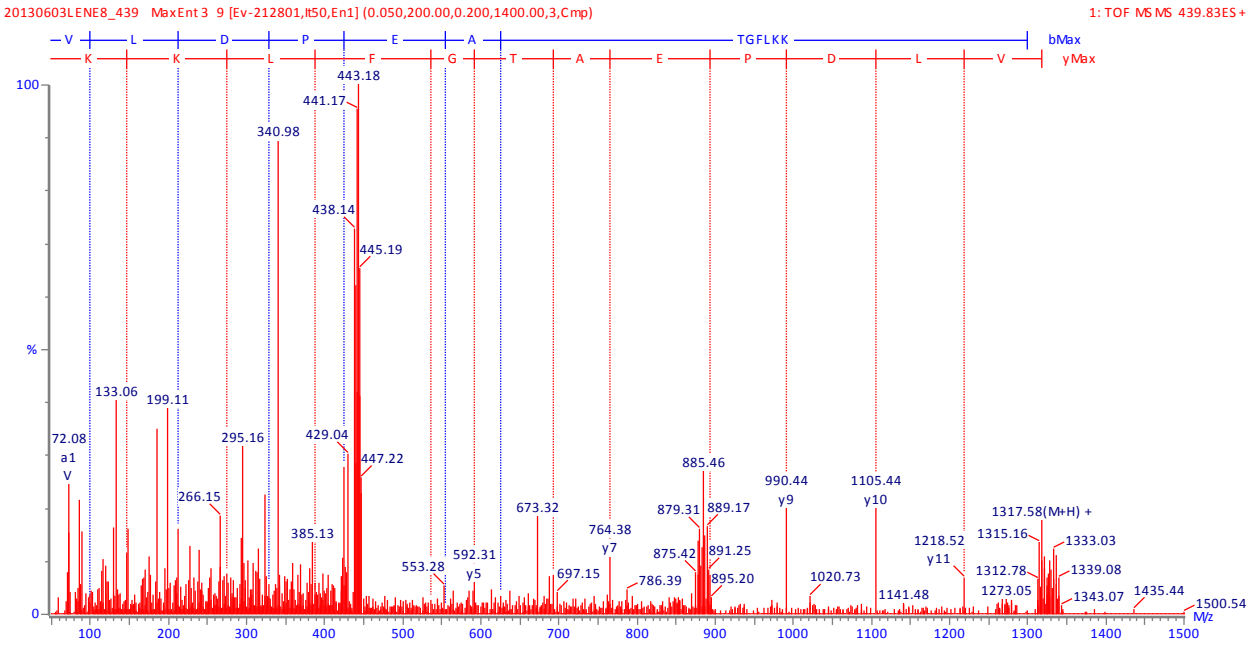
1102.42 (2+): TLLLPTGVAASEFYK

855ELGAK

MS analysis of Figure 25, SDS-PAGE band 3:

Myosin regulatory light chain 2 [Salmo salar]

>gi|30141488|emb|CAD89611.1| myosin regulatory light chain 2 [Salmo salar]
FLTMFGEKLKGADPEDVIVSAFKVLDPEATGFIKKEFLQELLTTQCDRFSAEEMKNLWAAFPPDVAGNVDYKQIC
YVITHGEEKEE
439.85 (+3): VLDPEAT



Regulatory myosin light chain [Argopecten irradians]

>gi|155651|gb|AAA27715.1| regulatory myosin light chain [Argopecten irradians]
MADKAASGVLTKLPQKQIQEMKEAFSMIDVDRDGFVSKEDIKAISEQLGRAPDDKELTAMLKEAPGPLNF
TMFLSIFSDKLSGTDSQETIRNAFAMFDEQENKKLNIEYIKDLLENMGDNFNKDEMRMTFKEAPVEGGKF
DYVKFTAMIKGSGEEEA
1002.35 (2+): GPLNFT

Troponin I, fast skeletal muscle [Salmo salar]

>gi|221221900|gb|ACM09611.1| Troponin I, fast skeletal muscle [Salmo salar]
MSEKKMTSSRRHHLKSLVLQIAFELIEVEKKEAEQVKKNFMAEIPALDLSGDQAALMEMLKKLAHTIDKVDEERY
DAEAKVNKADKEIEDLKMKVIEIQGMKKPALKKVRMSADAMLQALLGTKHKASMDFRANLKEVKKEVKEEAADAV
GDWRKNVDEQAGMDGRMKKFQG

712.76 (2+): FSADAFLAALLGTK

Troponin C, skeletal muscle [Salmo salar]

>gi|221221286|gb|ACM09304.1| Troponin C, skeletal muscle [Salmo salar]
MTDAQQEARSYLSEEMLNEFKAAFDMFDTDGGGDISTKELGQVMRMLGQNPTREELDEIIEEVDEDGSGTIDFEE
FLVMMVRLLKEDQAGKSEGELAECFRVFDKNADGYIDREEFAIIIRSSGEQISEEEIDELLKDGDKNADGMLDFD
EFLKMMENVQ
753,25 (2+): LSEEFLNEFK
879.28 (2+): FDMFDTDGGGDISTK