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Superchilling of Salmon (*Salmo salar*)

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SUMMARY

The aim of this thesis was to study discolorations in superchilled muscle food discovered by Duun (2008) The discolorations was found in salmon, pork and cod, and was not microbiological. The hypothesis as that this is a form of drip channels formed by the degradation of the muscle, and two storage experiments were initiated to test this hypothesis with the use of biochemical tests of enzyme activity, protein solubility and microbiology.

Analyses of samples of superchilled chicken filets showed low amounts of acid soluble peptides, but with a significant increase during the last days of storage. The amount of free amino acids was low and did not change much during storage. This indicates a relatively low and stable activity of exopeptidases, while the peptidase activity rise slightly as the filets are stored and gets a lower quality.

Two storage experiments were carried out with superchilled salmon. The first experiment showed very large changes around day 1 and day 3. The temperature is equalized early on day 0 and the ice formation has stabilized on day 1. There are signs of high increase of protease activity on day 1, and the protein solubility drops with 5% on day 3, before it increase to the previous solubility on day 7. The activity of β-N-acetyl-glycosaminidase also increase in this period.

Measurements of ice crystal size was done in parallel to the biochemical analyses showed large differences between the top layer (which is frozen at the start of superchilling) and the middle.

In the second storage experiment it was found that the top layer has a larger degree of damage to the cells in the top layer, but the cells in the mid layer had signs of more damage to the organelles. This was especially visible in the higher enzyme activity of both cathepsins and glucosaminidases, which was consistently higher in the mid layer of the filets.

The original theory about the white spots that appeared late in the storage period was that they might be drip channels. This is supported by the results from the second storage experiment. It would seem that despite the outer layer being most damaged by the initial freezing, the inside of the filet has a higher damage to lysosomes and other internal organelles, which over time gives a lower quality to the inside compared to the outer parts of the filets. When thawed the inside is more degraded by enzyme activity and thus there is more liquid that need to escape and forces itself through the better quality outer layer. The paleness is probably caused by denatured proteins in the drip, but this needs more research to be confirmed.

SAMMENDRAG

Målet med denne oppgaven var å studere misfarging i superkjølt fisk oppdaget av Duun (2008). Misfargingen ble funnet i laks, svinekjøtt og torsk, i form av små hvite prikker og var ikke mikrobiologisk. Hypotesen er at dette er en form for dryppkanaler dannet ved nedbrytningen av muskelen. To lagrings eksperimenter ble satt i gang for å teste denne hypotesen ved bruk av biokjemiske tester av enzymaktivitet, proteinløselighet og mikrobiologi.

I ventetiden før det første lagringseksperiment startet ble det gjort noe arbeid på prøver av superkjølte kyllingfilet laget av en annen gruppe. Det ble funnet lave mengder syreløselige peptider, men en signifikant økning i løpet av de siste lagringsdagene. Mengden av frie aminosyrer var lav og endret seg ikke signifikant ved lagring. Dette indikerer en relativt lav og stabil aktivitet av eksopeptidaser, mens aktiviteten i endopeptidaser økte svakt når filetene lagres og får en lavere kvalitet.

Det ble gjort 2 lagringseksperimenter med superkjølt laks. Det første eksperimentet viste veldig store endringer i muskelen rundt dag 1 og 3. Temperaturen var utjevnet tidlig på dag 0 og isdannelse stabilisert på dag 1. Det var en stor økning i protease aktivitet på dag 1, og sannsynligvis var dette årsaken til at proteinløseligheten sank med 5% på dag 3, før det øke til forrige oppløselighet på dag 7 når proteaseaktiviteten var mer stabil. Aktiviteten av β-N-acetyl-glycosaminidase økte også i denne perioden.

Det ble gjort målinger av størrelsen på iskrystallene parallelt med biokjemiske analyser. De viste store forskjeller mellom det øverste laget (som er frosset ved starten av superkjøling) og midten. På to ekstra prøver fra det første eksperimentet ble det gjort noen biokjemiske eksperimenter der det øverste laget av fileten ble skilt fra resten for å se forskjellen mellom lagene.

Dette ga noen interessante resultater som førte til det andre lagringseksperimentet. Der ble alle filetene nøye lagdelt i en topp- og en midt-del. Her ble det funnet at cellene i topplaget har en større grad av cellemembranskade, mens cellene i mellomlag hadde tegn på mer skade på organeller. Dette var spesielt synlig i høyere enzymaktivitet hos de lysosomale enzymene cathepsin og glucosaminidase, som var konsekvent høyere i midtlaget i filetene.

Den opprinnelige teorien om de hvite prikkene var at de kunne være dryppkanaler. Resultatene fra det andre lagringseksperimentet støtter dette. Det virker som at til tross for at det ytre laget virker å være mest fryseskadet ved starten av lagringen så har innsiden av fileten høyere skade på lysosomer og andre interne organeller som over tid vil gi en høy grad av lekase av proteaser og andre nedbrytende enzymer. Dette gjør at over tid blir kvaliteten på innsiden lavere enn i de ytre delene av fileten. Når filetene blir tint er strukturen på innsiden mer forringet av enzymaktivitet samt de

saltløselige proteinene mer degradert. Dette gir lavere vannbindingsevne i midten, så i den tinte fileten er det dermed mer fritt vann og væske som presses ut gjennom det ytre laget som har en høyere kvalitet på dette tidspunktet. De bleke prikkene skyldes sannsynligvis denaturerte proteiner i dette drypptapet, men dette trenger mer forskning for å bli bekreftet.

CONTENTS

1 INTRODUCTION

1.1 FISH

Muscle foods are an important part of the human diet. This is both meat from cattle, sheep, poultry and fish, and they are all a good source of dietary proteins. But there are large differences in structure, amounts and properties in different types of protein, and this gives them very different properties that are important for storage, treatment and use as food.

Fish is an important source for muscle foods in Norway, and also one of the major export sectors in Norway. In 2011 2,450,000 tonnes of fish were exported, of this the largest category was salmon with 840,000 tonnes in various forms; frozen, chilled, whole fish, filets and more. The amount of exported fish has been steadily increasing since 2001, and there are no signs of the export going down in the near future(NorgesSjømatråd, 2012).

With the increasing amounts of fish export quality and transport is an increasing challenge. The customers expect high quality fish and the shelf life of the product is important for how far the export can reach, especially for fresh fish. While the differences between the individual fish can be large because of age, sex, feeding etc. the most important factor for shelf life and quality of fish is temperature. The control of the food temperature and the cold chain during transport is very important, and the prevalent way of transport and storage is in insulated boxes on ice. This is not very practical since all the ice takes up much storage capacity and needs to be refilled regularly. This has given a greater interest in different ways of storage and transport since a system without ice will give much better use of the space and with that a potential economic gain.

1.2 SUPERCHILLING

Superchilling is a method where the shelf life of many foods can be prolonged by partially freezing. This gives low temperatures, less need of external chilling, and the quality is better than in frozen foods since there is less freeze damage.

Superchilling has been studied for many years (haugland, Aune, & Hemmingsen, 2005; Sivertsvik, Jeksrud, & Rosnes, 2002), and the definition and method varies much, even the name has several versions; mainly superchilling and partial freezing, but also some other variants. But the principle is the same to a large degree: cooling the food to the initial freezing point and a few degrees under this. The initial freezing

point is the temperature at which the first ice is formed, and is between -0,5°C and -3°C (Fennema, 1975)

This will give a partial freezing of the water, initially only in the outer millimeters of the food, but if stored at superchilling temperatures the temperature will equalize in the sample and water is partially frozen out (Kaale, Eikevik, Rustad, & Kolsaker, 2011)

Other ways to increase shelf life is use of MAP and vacumpacking, and there have been done work on the effects of combining these with super chilling. MAP combined with superchilling gave promising results, but was not shown to significantly change the effects of ice formation in the muscle structure (Sivertsvik, Rosnes, & Kleiberg, 2003) Bahuaud et al. (2008) found that MAP and superchilling gave an decrease in enzyme activity, probably because of changes in CO_2 dissolution. Vacuum packing combined with superchilling gave a significant increase in shelf life and quality of muscle after superchilled storage(Kaale et al., 2011)

The importance of the age of the raw material have been investigated by Stevik, Rustad, Duun, and O'Farrell (2013) where salmon was superchilled 3 and 7 days after slaughter. They found that the age of the raw material had great effect on the quality of salmon filets. If the raw material was old the duration of superchilled storage did not have as much effect as it did for fresh raw material.

1.3 QUALITY PARAMETERS

The first thing a consumer evaluate when buying fish food is the appearance. Does the filet look fresh? And what makes a filet look fresh? Colour is one of the main parameters, and there has been done much work on the colour for farmed salmon and how to preserve it during storage. The main contributor to salmon colour is astaxanthin, and the concentration of astaxanthin is shown to decrease during frozen or chilled storage regost (Regost, Jakobsen, & Rørå, 2004). In Duun and Rustad (2008) no differences in colour were found between samples stored at different temperatures. But white spots appeared during superchilled storage, and was later also observed in cod (Duun & Rustad, 2007) and pork. The measured lightness values indicated that the colder the superchilled filets were stored the more intense the discolouration was.

Investigating these discolorations are the main aim of this thesis. Earlier work done by Duun indicate that the discolorations are not of microbial nature, and Duun also concludes that it is unlikely that they stem from leakage of common amino acids (Duun, 2008). It is suggested that the effect could be due to diffusional effects by drip channels, Taylor, Geesink, Thompson, Koohmaraie, and Goll (1995) showed such channels forming in beef when the cell membrane lifts of from the cell body, but the attachment of the cellular matrix remains unaffected. But Duun also found that

the amount of drip loss did not correspond with the groups that had the highest degree of discolouration.

As mentioned above is temperature the most important factor for prolonged shelf life. This is because a lower temperature will influence microbial and enzymatic activity and spoilage. This makes microbial counts the main way to determine the safety of foods, and a total plate count of 5 log units of colony forming units is considered the limit for whether food is fit for human consumption or not. Values higher than 5.7 log units are considered high, and 6.7 log units represents a limit for what is recommended for consumption even if total plate counts cannot be used as an absolute limit alone (ICMSF, 1986).

Drip loss is important for the industry since muscle foods are sold on weight basis. A customer is also not partial to buying meat or fish with high amounts of visible water in the package. Losing a lot of weight to drip loss is not favourable, and thus there has been a large focus on whether superchilling creates a significant increase in drip loss. Some studies have shown low drip loss in superchilled food (Sivertsvik et al., 2002; Sivertsvik et al., 2003) It has been shown that the superchilling temperature influences the amount of drip loss. Duun & Rustad (2008) found that samples that were stored at a superchilling temperature of -3.6°C had significantly lower drip loss than filets stored at -1,4 °C.

Drip loss is caused by structural changes in the muscle post mortem. Lower pH gives fiber shrinkage and myofibrillar shrinkage, and myosin is denatured (Schäfer, 2002). Cheng et al. also found that changes in the membranes during post mortem changes and storage created higher drip.

In superchilled muscle there is also a concern that the ice formation in the cells freeze out water and gives higher concentrations inside the cell, which leads to higher enzyme activity. This in turn leads to more denaturation and structural degradation giving an increase in drip loss, changes in texture and water holding capacity (Foegeding, Lanier, & Hultin, 1996)

There are many ways of measuring the water in different forms and degrees of water binding in muscle foods, both drip, liquid loss and water holding capacity is recognized as good parameters for texture and structural damage. Another interesting measurement of water in the cell is cell tissue fluid (CTF), the fluid that can be forced out of the muscle food by centrifugation at high speeds. Increase in CTF reflects loss of water holding capacity and increase with reduced ability of the tissue to hold water. This fluid contains solutes from both the extracellular and intracellular compartments. Superchilling may lead to disruption of the cell membranes because of ice crystal formation. This also includes internal membranes on organelles like lysosomes and other vesicles. CTF can therefore be seen as an indicator of degree of internal damage in the cells. This damage can also be measured by lysosomal proteins, and the amount of lysosomal enzyme activity in CTF is suggested as a measure for freezing and thawing damage. This can be

compared with lysosomal enzyme activity in extracts of water soluble proteins, which is considered to be a snapshot of the active extracellular enzymes.

1.4 PROTEIN SOLUBILITY

The properties of the proteins in fish muscle are important for many of the quality parameters; both the texture, loss and holding capacity of water are tightly knit together. The texture of fish is by nature quite soft and increased softening or gaps in the muscle are unwanted. In muscle further softening happens when proteases cleave structural proteins and thus degrades the muscle integrity. This in turn will influence the muscle's ability to retain water, and some water is lost (drip loss) or become unbound. This water forms pockets of less tightly bound water in the structure that is easier to extract (liquid loss) and is strongly related to our perception of muscle food being "dry" after cooking. As the protein structure is more degraded these pockets will gradually escape and give and increase in drip.

The extractable proteins in muscle are divided into three groups depending on how they are extracted (Foegeding et al., 1996; Haard, 1992):

- Water soluble proteins, or the sarcoplasmic proteins consists mainly of enzymes, and constitutes about 20% of the total protein in muscle.
- Salt soluble proteins, or the myofibrillar proteins consist mainly of the proteins that form the contractile network. Actin and myosin are the most prevalent group in this fraction, and constitutes about 65-70% of protein in muscle
- Insoluble proteins, which is mostly connective tissue. Collagen is the main protein in this group and constitutes about 3% of the total protein in muscle.

The amount of soluble protein differs a lot in muscle food, not only depending on which type of animal it comes from, but also by age, sex, nutrition and more.

It is known that storing fish at freezing temperatures gives a higher degree of protein denaturation (Mackie, 1993). Since superchilled storage is below the freezing point of the food it is important to monitor and get a good understanding of what happens with the proteins during superchilled storage.

Since the salt soluble proteins mainly are the contractile network, one can expect a correlation between long storage and decrease in salt soluble proteins. The long storage gives proteases longer time to cleave the proteins, leading to an increasing degree of denaturation. This affects the water holding capacity since the contractile network binds most of the water in muscle. The degree of protein denaturation can be assessed by gel electrophoresis (SDS-PAGE) by comparing the bands of myosin

and actin, if they are much cleaved the bands will be less distinct and new bands will appear.

It is vital to gather more information on the effects of different temperatures in superchilled storage. It is a balance between low temperatures giving low microbial and enzymatic activity, and high temperatures resulting in less damage to the proteins.

1.5 ENZYME ACTIVITY

As proteolytic enzymes, endogenous proteases are important for muscle protein turnover. After death the control is lost and they become important enzymes for muscle protein degrading and resolve of rigor mortis (Foegeding et al., 1996)

Calpains are generally thought to be the most important protease in muscle foods , but it would seem they are less important in fish muscle (Chéret, Delbarre-Ladrat, Lamballerie-Anton, & Verrez-Bagnis, 2007; Geesink, Morton, Kent, & Bickerstaffe, 2000; Hultmann & Rustad, 2002; Ilian, Bickerstaffe, & Greaser, 2004). Instead it is thought that various cathepsins together autolyse fish muscle. The main contributors is cathepsins B and L working in concert to deteriorate fish muscle (Aoki, Yamashita, & Ueno, 2000; Chéret et al., 2007) Cathepsin B is considered an endopeptidase

(John B. Lloyd & Mason, 1996)but there is some signs that it also can exhibit both endo- and exopetidase activity under the right conditions. Cathepsin L is an endopeptidase (John B. Lloyd & Mason, 1996) and has a very broad specificity. But unlike cathepsin B, cathepsin L is more vulnerable to inhibition than cathepsin B(Barrett & Kirschke, 1981).

Cathepsins are lysosomal enzymes and thus cathepsin activity gives an indication of the degree of damage to membranes and organelles in the cell. Another enzyme in fish lysosomes is β-N-acetyl-glycosaminidase (NAG). It has been proposed as an indicator of freeze-thawing damage by (Katarina Nilsson & Bo Ekstrand, 1993) They also propose acid phosphatase (AP) and alpha glucosidase (AG) as important enzymes (Nilsson & Ekstrand, 1995; Nilsson & Ekstrand, 1994).

It is difficult to determine the activity of these enzymes in the tissue, so free amino acids can be used as a parameter for exopeptidase activity and the accumulation of them gives a picture of the total activity over time. In the same way trichloroacetic acid (TCA) soluble peptides are used as a measurement of endopeptidases 2001 (Lund & Nielsen, 2001) However it is important to remember that TCA soluble peptides also include free amino acids.

2 METHODS

2.1 CHICKEN

2.1.1 RAW MATERIAL AND SAMPLING

Chicken breasts were received from Ytterøy kylling on the 26th of October (chicken were slaughtered on the 24th). The samples were vacuum packed and stored on ice (marked K – chilled), superchilled in an impingement freezer and stored at -1.5°C. Day of superchilling (26 th of October) is set as day 0. Samples were taken after 0, 5, 9, 16, 29 and 41 days. 4- 6 samples were taken out at each sampling point. Drip loss and protein extraction were only done on chilled samples and samples stored at -1.5°C.

Samples were removed from superchilled storage on the day before analyses and stored at +4°C overnight. Drip loss were determined by weighing the bag + sample, removing the sample, blotting it dry before weighing it, drying out the bag and weighing. Amount of drip is given I % of sample weight.

Extractions were made by homogenizing 5 g of sample in 40 ml cold distilled water with an Ultra Turrax. Homogenates were centrifuged (10000 g for 20 minutes) at 4°C. The supernatant was decanted through glass wool and made up to 50 ml with cold distilled water. Aliquots of the homogenates were frozen at -20°C and used for determination of protein content, acid soluble peptides and free amino acids.

Protein content in the extracts were analysed by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951)

2.1.2 ACID SOLUBLE PROTEINS

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

2.1.3 FREE AMINO ACIDS

Amount of free amino acids was determined in the water soluble protein extracts. Proteins were precipitated by mixing 1 ml extract and 0,25 ml 10% sulphosalisylic acid and left at 4°C for at least 45 minutes (Osnes & Mohr, 1985). The samples were centrifuged (600 g, 10 minutes, 4°C), the supernatant were diluted with double distilled water, filtered (0,2 µ pore size) and frozen at -20°C while waiting for analysis. They were analysed with reverse phase high performance liquid chromatography by the method of (Lindroth & Mopper, 1979) as modified by (Flynn, 1988).

Glycine/arginine and methionine/tryptophan were determined together, as their peaks merged. Results from individual samples were used to calculate averages for each sampling day/storage method. Total amount of free amino acids were calculated as percentage of wet weight.

2.2 SALMON 1

2.2.1 RAW MATERIAL

Filets of salmon (*Salmo salar)* were delivered from Lerøy Midnor, the age of the filets are unknown. On day -1 the filets were cut into equally sized pieces (100 ± 25 g), and vacuum packed in bags of 50 µm thickness. The next day (Day 0) the samples were chilled in an impingement freezer at −30 °C (air temperature) and 227 W/m^2 K (surface heat transfer coefficient, SHTC) for 2.1 min before storage at −1.7 ± 0.3 °C. The temperature was equalized at day 1. The chilled reference samples were stored in a cold room at 4°C. Frozen reference samples were stored at -20°C.

The ice thickness and size of the ice crystals were measured parallel to the biochemical analyses, and the results can be found in (Kaale, Eikevik, Bardal, & Kjorsvik, 2013)

2.2.2 SAMPLING

Superchilled and frozen samples were placed in the same cold room as the chilled samples the day before sampling to taw. Chilled samples were analysed on day 0, 7 and 14. Superchilled samples were analysed on day 0, 1, 3, 7, 14, 21 and 28, and frozen samples were analysed on day 30. Three filets were analysed on each sampling day.

2.2.3 MICROBIOLOGY

A sample was taken from 3 filets each sampling day up to day 14, following the NMKL method No. 91 (NMKL, 2002). Sample size was 10cm^2 and it was no more than 2mm thick, taken from the surface of the filet. Samples were taken from 2 filets each from chilled and superchilled storage on day 0, 7 and 14. Total bacteria count was done following NMKL method No.86 (NMKL, 1999). A dilution series was plated out in triplicate on commercial plate count agar (Difco); the samples were incubated for 76±4 hours at 25°C.

2.2.4 DRIP LOSS

The filet and bag was weighed and the filet was removed from the bag and weighed. Then the drip was collected with a pipette and the bag wiped of before weighing it again. Drip loss was calculated as the empty and dried bag subtracted from the total sample weight.

2.2.5 EXTRACTS

Three extracts were made from each filet; two homogenates, one with water soluble proteins and one with salt soluble proteins, and cell tissue fluid (CTF).

The homogenates were made of 4 g white muscle in 80 ml cold 0,05 M phosphate buffer (KH_2PO_4 in NaOH, pH 7). It was homogenized with an Ultra Turrax homogeniser and centrifuged at 10 000 x g for 20 min at 4°C. The supernatant was filtered through glass wool and the volume made up to 100 ml in a volumetric bottle. This is the water soluble homogenate/water soluble fraction. The sediment was resuspended with 80 ml 0,05 M phosphate buffer with salt (KH_2PO_4 in NaOH, 0,6 M KCl, pH 7) and treated the same way. This is the salt soluble fraction.

The amount of protein in the extracts was determined by BioRad protein assay (Bradford, 1976), using Bovine Serum Albumin as a standard. The analyses were run in triplicate.

Cell tissue fluid (CTF) was made with 20g white muscle cut into pieces and centrifuged (28 000 x g, 30 minutes). The liquid was then taken into eppendorf tubes of known weight using a pasteur pipette and weighed before being stored at -20°C. Measurements are given as grams of fluid per gram wet weight.

2.2.6 CATHEPSIN B+L

Cathepsin activity was done on the water soluble homogenate and CTF suitably diluted with buffer and water respectively. The activity of cathepsins B+L were measured against synthetic fluorogenic substrates, N_α-carbobenoxy-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin(Barrett & Kirschke, 1981).

Enzyme extract (100 µl, suitably diluted) was incubated with 100 µl assay buffer (150 mM bis-tris, 30 mM EDTA, 6mM DTT, pH 6, pH in reaction mixture 6,3) at 30°C for 15 minutes, before 100µl substrate (3 mM in DMSO, diluted with distilled water to 0,09375mM) was added. The reaction was stopped after 10 minutes by adding 3 ml 1% SDS in 50 mM bis-tris, pH 7. A blank was prepared using the proper dilutant for homogenate and CTF.

When the enzymes cleave the synthetic substrate 7-amino-4-methylcoumarin (AMC) is liberated. Fluorescence of AMC was measured at 440nm (5 nm slits) after excitation at 360 nm (10 nm slits) (Perkin-Elmer 3000 Fluorescence Spectrometer, Perkin-Elmer Inc., Buckinghamshire, UK). Increase in fluorescence intensity was used to calculate the activity, given as the increase in fluorescence per g wet weight * min during incubation. The analysis was run in triplicate.

2.2.7 B-N-ACETYL-GLUCOSAMINIDASE

The activity of the lysosomal enzyme β-N-acetyl-glycosaminidase was determined in the water soluble fraction by the method of (K. Nilsson & B. Ekstrand, 1993) (based on (Milanesi & Bird, 1972) and (Rehbein, Kress, & Schreiber, 1978)).

0,5 ml water soluble homogenate or CTF (suitably diluted with buffer and water respectively) was mixed with 0,3 ml reaction buffer (0,1M Na Citrate, pH 4,5), 0,2 ml 0,6M KCl and 0,2 ml substrate (1mM p-nitrophenyl-N-acetyl-β-D-glucosamide). After 30 minutes of incubation on 37°C the reaction was stopped by adding 1ml 0,3M KOH, and the absorbance was measured at 405nm. The analysis was run in triplicate.

2.2.8 Free amino acids

Measurements for free amino acids were done as in the chicken experiments, see 2.1.3.

2.3 Salmon 2

This is the second storage experiment with salmon, and it was made as similar to the first storage experiment (Salmon 1) as possible. All tests were done as in Salmon 1 expect what is mentioned in this chapter.

2.3.1 Samples

The salmon filets were cut into pieces of 200 ± 40 g of as equal thickness as possible. Chilled samples were analysed on day 0, 3, 7 and 14. Superchilled samples were analysed on day 1, 3, 7, 14, 21 and 28, and frozen samples were analysed on day 30. Three filets were analysed on each sampling day. All superchilled filets were divided in two layers, the top 3 mm and middle. The bottom layer of the filet was discarded because the muscle would be more changed by its contact with the cold metal in the impingement freezer than from the superchilled storage.

2.3.2 Microbiology

No microbial analysis was done

2.3.3 Extracts

It was made two parallels of water and salt soluble proteins from each filet.

2.3.4 Cathepsin B

The cathepsin activity was measured by cathepsin B activity instead of cathepsins B+L. The activity of cathepsin B was measured against synthetic fluorogenic substrate N_{α}-carbobenoxy-L-arginyl-L-arginine-7-amido-4-methylcoumarin. The measurements were done at 37°C and the samples were incubated with the substrate for 20 minutes.

3 RESULTS AND DISCUSSION

3.1 CHICKEN

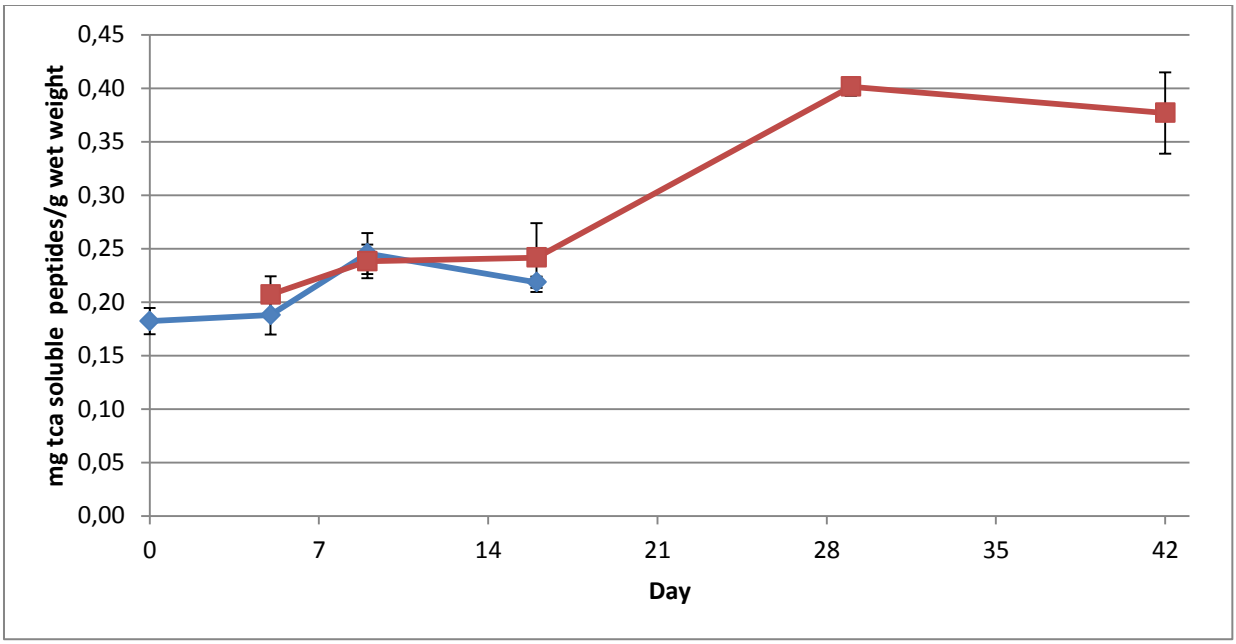

Figure 3-1 Changes in TCA soluble peptides in chicken under different storage conditions, shown as mg peptides per gram wet weight. Superchilled and chilled samples are shown as squares and diamonds respectively. Values are given as mean ± SEM (n = 6)

The amount of TCA soluble peptides was in general very low as shown in Figure 3-1. There is a significant increase from day 5 to day 29 and 42 in the superchilled samples, but the chilled samples show only very small changes. This indicates that endopeptidases are active in the superchilled samples, leading to an increase in protein breakdown and available peptides.

In contrast the amount of free amino acids (shown in figure 3-2) is stable during the entire period, with a significant decrease in the superchilled samples on day 15. Free amino acids are a parameter for activity of exopeptides, and as such it can be concluded that there is a slightly higher amount of exopeptidases in superchilled chicken compared to chilled samples, or less inhibition of them. Seeing how the chilled samples accumulate more free amino acids from day 0 to day 16 than the superchilled, the responsible exopeptidase(s) is probably more active at higher temperatures or more of it is released during storage

The dip on day 14 superchilled may be caused by an increase in microbial growth at this point, where microbes take up free amino acids to use in a period of quick growth and cell division. The amount of free amino acids in chilled samples also shows a significant decrease at the last day of storage, a time where high microbial activity can be expected. But since no microbial analysis was done this will just remain a theory.

By comparing the total amount of TCA soluble peptides and free amino acids it is probable that the main factor for protein breakdown in superchilled chicken is endopeptidases, not exopeptidases. There have not been done any measurements of enzyme activity in these samples, but from these results it could be interesting to see if the enzyme activity increases during storage for superchilled chicken filets.

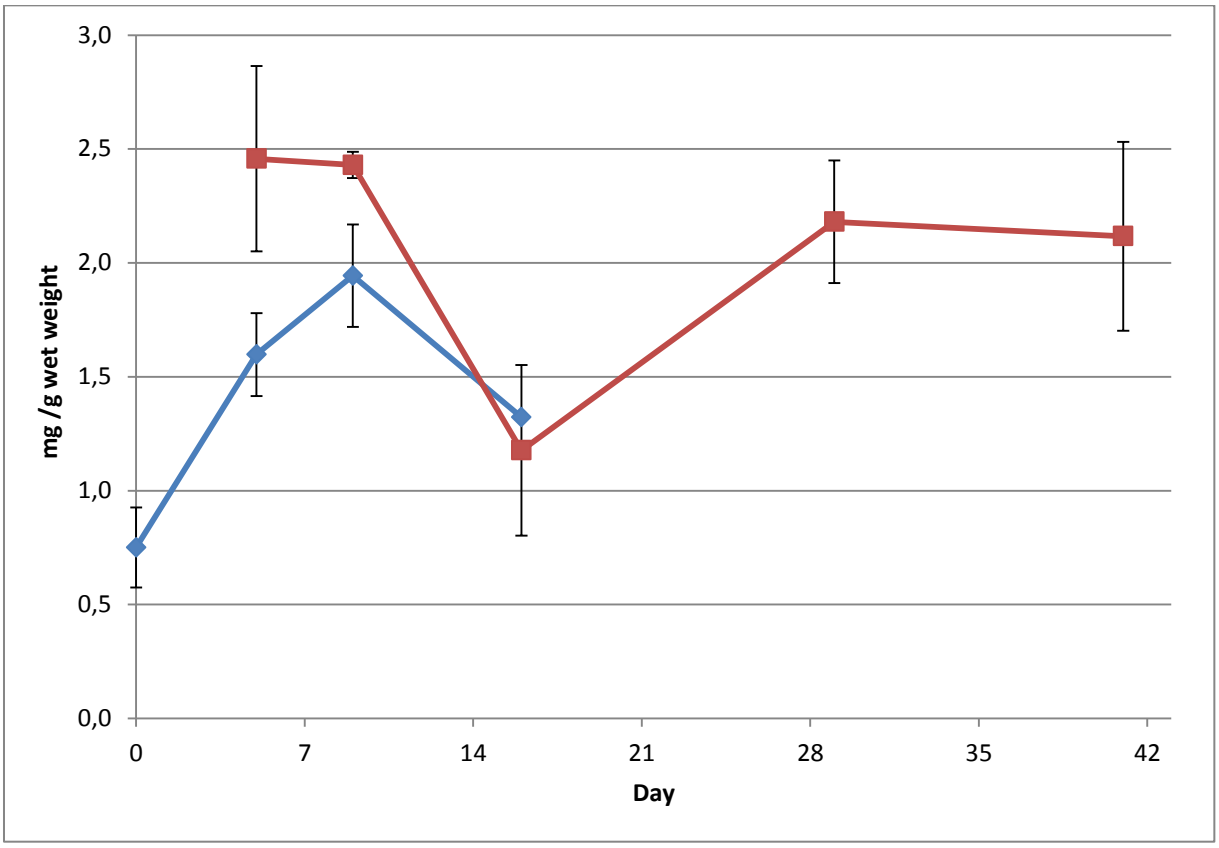

Figure 3-2: Changes in free amino acids in chicken under different storage conditions, shown as mg free amino acids per gram wet weight. Superchilled and chilled samples are shown as squares and diamonds respectively. Values are given as mean ± SEM (n = 6)

3.2 SALMON 1

3.2.1 SAMPLE QUALITY

The filets were generally in good condition until day 14 (chilled) and day 28 (superchilled). The amount of colony forming aerobic bacteria on the surface from day 0 to day 14 is shown in Figure 3-3. The superchilled filets did not change significantly between day 7 and day 14, while the chilled references quite quickly got a high microbial count. This is as expected; the shelf life of superchilled salmon is much longer than for chilled salmon, as shown in Kaale et al. 2011

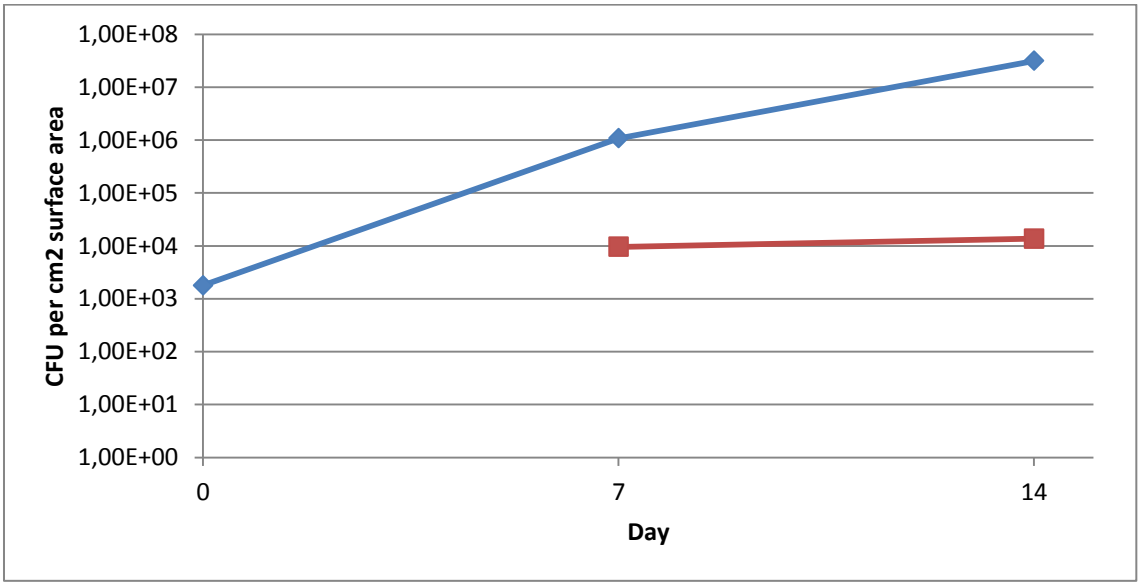

Figure 3-3: Microbiological tests on chicken under different storage conditions, shown as number of colony forming units per cm^2 surface area. Superchilled and chilled samples are shown as squares and diamonds respectively. Values are given as mean ± SEM (n = 6)

Microbial counts were not determined after day 14, but sensory evaluation of the superchilled samples by smell and texture indicate that the filets would not taste fresh and good, but was probably not unhealthy to eat. The filets differed some in texture and amount of gaping the last day, which reflects in a high variance in some measurements, especially in drip loss and amount of CTF.

There was almost no signs of white spots on day 21, only on day 28 was the discolouration clearly visible, as shown in Figure 3-4. The bottom side of the filets had large amounts of discolourations, but here it was stripes following the muscle fibres in the myocommata instead of spots. This is the side that has been in direct contact with the cold metal on the transport band of the impingement freezer, and this probably gives a higher degree of damage to the cells.

Figure 3-4: Picture of thawed salmon after 28 days of superchilled storage at -1.7°C. Top side to the right and bottom side to the left.

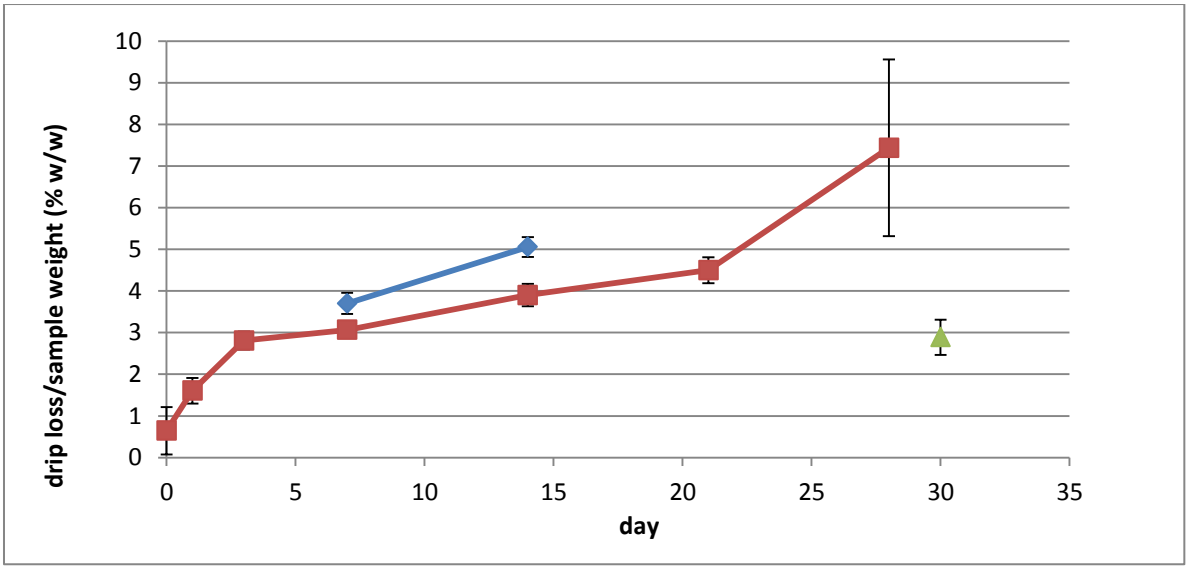

Figure 3-5: Changes drip loss in salmon under different storage conditions, shown as percentage of gram drip loss per gram wet weight. Superchilled, chilled and frozen samples are shown as squares, diamonds and triangles respectively. Values are given as mean ± SEM (n = 3)

The drip loss was higher than expected (shown in figure 3-5). Earlier experiments (Duun & Rustad, 2008) found that the drip loss was higher at high superchilling temperatures (-1.4°C 1-2% drip loss, increasing with storage), and at lower temperatures (-3.6°C) the drip loss was lost less than 0.3% of the weight during storage. This experiment was done at -1.7°C, and thus the expected drip loss was around 1-2%, but already after 3 days of storage the superchilled filets are up to 3% and rise slowly as the quality of the fish decrease and the contractile network degrade.

It is interesting that the drip loss is about 3-5% in the periods where the samples are stabilized and still of good quality (superchilled day 3 to 21, frozen day 30 and partly chilled samples day 7 and 14). This could indicate that the quality of the fish before the storage experiment started was not very good, maybe the fish was older. The filets in Duun and Rustad (2008) was stored on ice prior to and during superchilling while the samples in this experiment was kept chilled in a cold room (4°C). This will have large consequences for shelf life and general quality of the fish. Stevik et al. (2013) indicate that the quality of the raw material prior to superchilling is of major importance.

The amount of cell tissue fluid centrifuged out of the fish muscle (Figure 3-6) is steadily increasing through the storage. The superchilled samples show a change of amount of CTF per gram wet weight that is quite similar to the trends in the drip loss (figure 3-5). The chilled samples have a slightly lower amount of liquid centrifuged out, compared to their drip loss, but the tendency is that the loss of liquid and liquid forced out of the muscle are more or less unchanged during the periods where the filets are stable and still of good quality. The frozen filets have a drip loss and CTF amount that are on the same stable level, which also strengthens the thought that this is the “standard” loss/holding capacity while the filet texture still is good.

On day 28 the amount of cell tissue fluid rise a little more, probably because some of the filets at this time has low quality and the higher membrane damage release more enzymes from the lysosomes out into the cell, leading to a higher rate of further damage. The filets have a very soft texture after long storage, indicating a higher degree of breakdown of the contractile network, but also membranes; both cell membranes and organelles. This lets increasing amounts of enzymes leak out from lysosymes.

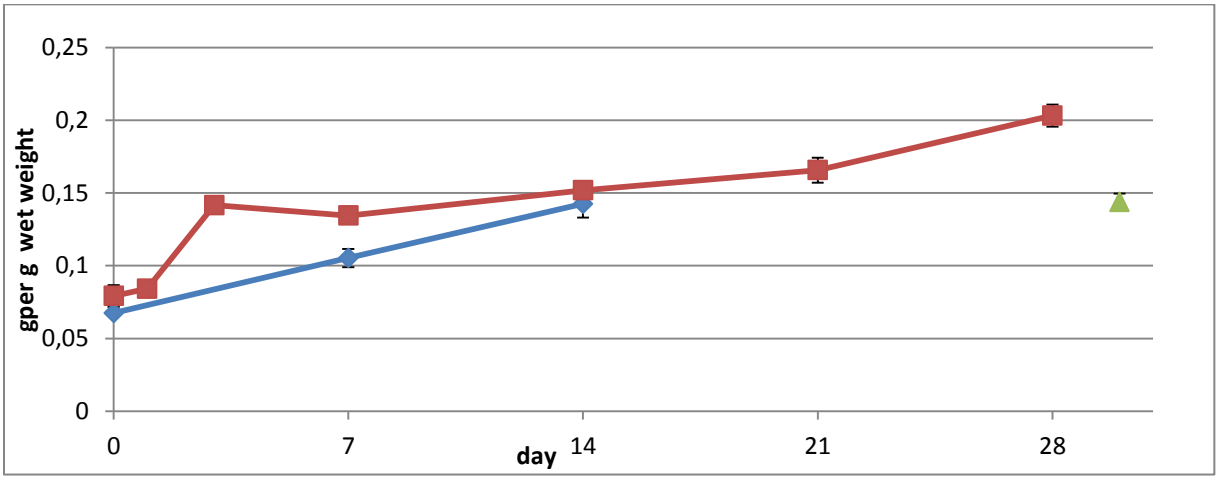

Figure 3-6: Changes in amount of cell tissue fluid per gram wet weight in salmon under different storage conditions. Superchilled, chilled and frozen samples are shown as squares, diamonds and triangles respectively. Values are given as mean ± SEM (n = 3)

Both the % of drip and cell tissue fluid are low during the first days (day 0 and 1) of superchilled storage before they increase and stabilize between day 1 and day 3. In the studies of ice crystallization done parallel to this experiment (Kaale et al., 2013) it is shown that the temperature is equalized at day 1 and the ice crystals have reached their maximum size already at day 1. The cell membranes stretch and adjust to the size of the ice crystals without showing signs of ruptured cells, but judging from the changes in CTF there is slightly higher damage to the inside of the cells. The drip loss in chilled samples is higher than in the superchilled samples, while the amount of CTF in chilled samples is lower. This demonstrates the difference between drip loss and CTF, as drip loss shows general loss of fluid as loose fluid is forced out by the structural changes, while CTF is a better measurement of internal damage in the cells and liquid over all in the system.

3.2.2 Protein solubility

Figure 3-7 shows the amount of soluble proteins. Since the extracts have been frozen before measurements the amount of salt soluble proteins are probably higher since some of them precipitate because of the freezing. Salt soluble proteins were not extracted on day 3. Day 3 also shows a very low content of water soluble proteins, but we have not found any obvious faults or errors in the extraction of proteins that day, so probably there is something happening in the muscle at this time. Previous experiments (Duun, 2008) have also seen large changes in protein solubility during the first days of storage. Several other parameters also vary this day, both the grams of cell tissue fluid per gram wet weight and NAG activity show significant increase. This all suggests that the deviant measurement is not due to an error done in the extraction process, but rather to a change in the filet.

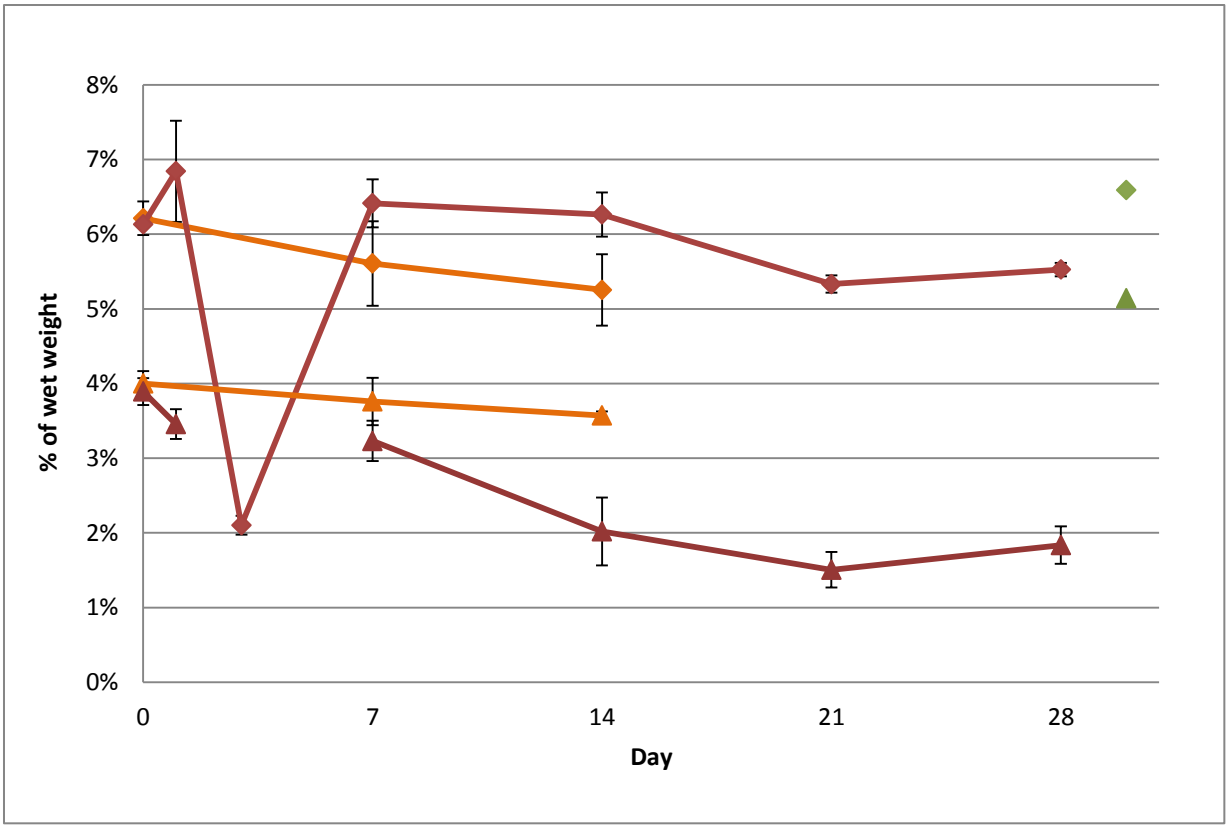

Figure 3-7: Changes in water (diamonds) and salt (triangles) soluble proteins in salmon under different storage conditions, shown as percentage of gram soluble protein per gram wet weight. Superchilled, chilled and frozen samples are shown with red, orange and green respectively. Values are given as mean ± SEM (n = 3)

The protease activity in the homogenates is decreasing, especially after long storage. This may indicate that the enzymes that are free in the cell are getting denatured. Since the chilled samples also show a slight decrease in water soluble proteins it is not probable that it comes from freeze denaturation, but rather that there are some proteolysis and proteins being washed out with the drip loss.

The salt soluble fraction is low, but the decrease between day 0 and 21 is as expected from previous experiments (Duun, 2008). This indicates a high degree of denaturation of the contractile network, but the increase in solubility between day 21 and day 28 is not significant.

The solubility in frozen samples is higher than in the chilled and superchilled samples. This could indicate that most of the denaturation in the other samples is from high enzyme activity, since the enzyme activity is thought to more or less non-existing at -20°C.

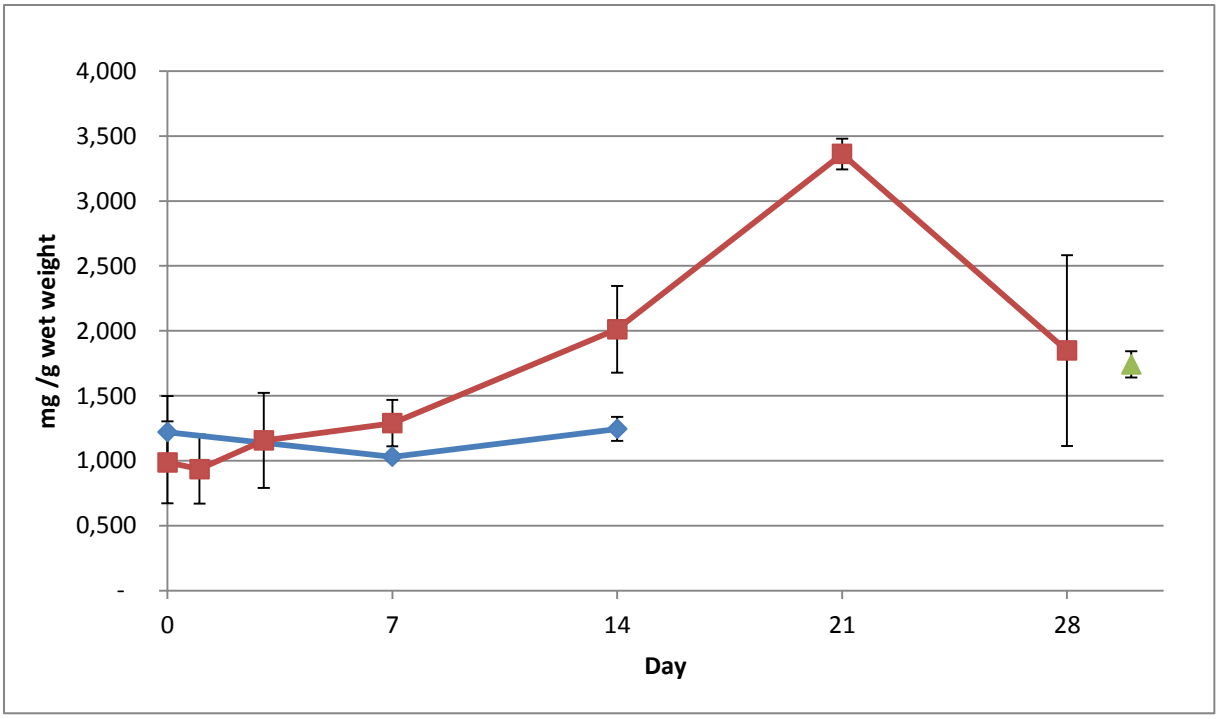

Figure 3-8: Changes in free amino acids in salmon under different storage conditions, shown as mg free amino acids per gram wet weight. Superchilled, chilled and frozen samples are shown as squares, diamonds and triangles respectively. Values are given as mean ± SEM (n = 3)

The amount of free amino acids increase slightly during storage, which indicates that there are exopeptidases active through the whole storage period. The longer the samples are stored the more free amino acids are accumulated. This might be cathepsin B activity, since there is some discussion about whether or not it is an endo- or exopeptidase. As mentioned in chapter 3.1 the decrease at the end of storage might relate to increased microbial growth using free amino acids, but because of the high uncertainty in the measurement on day 28 this is very much speculation.

The frozen sample seems to have less free amino acids, probably because there is almost no protease activity at -20°C

3.2.3 Enzyme activity

The activity of Cathepsin B+L is shown in Figure 3-9 and Figure 3-10 (based on mg protein and g wet weight*min). Since the protein concentration in superchilled samples on day 3 is so low it influences the calculations of cathepsin activity significantly when based on protein concentration. As a control an imaginary protein concentration for day 3 was made by using the average of day 1 and 7 instead of the determined value for the protein concentration. This was used as a protein concentration closer to what was expected and then used in the calculation of cathepsin activity. The increase in cathepsin activity is then just 86 int / g wet

weight*min, which makes the graph more similar to what is seen in the calculations based on wet weight where the peak is on day 1.

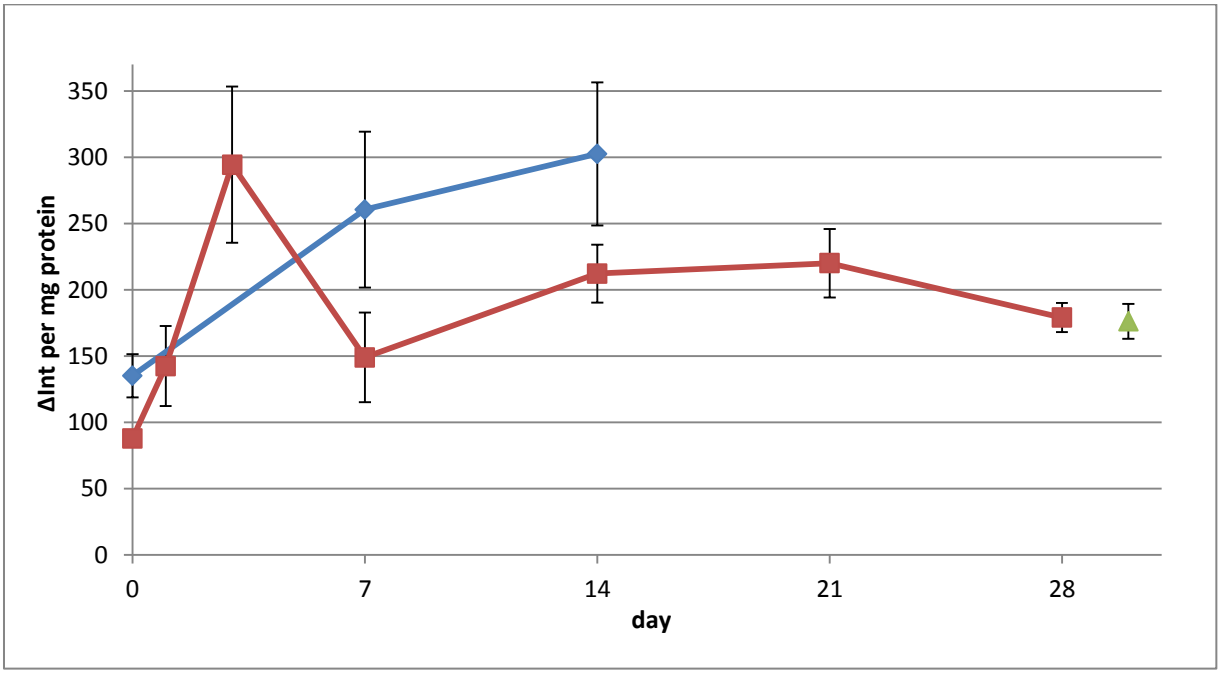

Figure 3-9: Changes in activity of cathepsin B + L in homogenates from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares, frozen storage at -20°C is represented by triangles, and chilled storage in a cold room at 4°C by diamonds. Values are given as increase in fluorescence intensity per mg protein per minute. Values are given as mean ± SEM (n = 3)

Because of the heavy influence of protein concentration on these measurements and the doubts about the reliability of those measurements some days, later discussion about cathepsin activity will be based on calculation by wet weight. The calculation by protein concentration is shown here to show the differences in the calculations and as a support of the uncertainty about the protein measurement for day 3 superchilled.

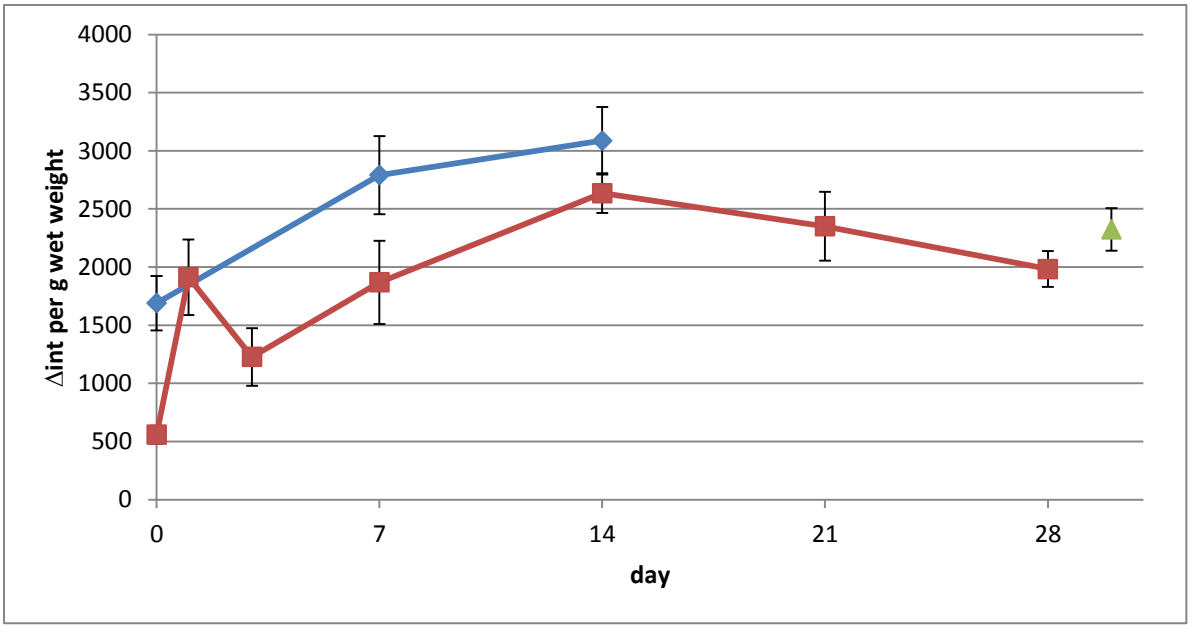

Figure 3-10: Changes in activity of cathepsin B + L in homogenates from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares, frozen storage at -20°C is represented by triangles, and chilled storage in a cold room at 4°C by diamonds. Values given as increase in fluorescence intensity per g wet weight per minute. Values are given as mean ± SEM (n = 3)

The chilled samples have higher cathepsin activity all days. This agrees with the thought that cathepsins are less active at lower temperatures and that there is more cathepsin released over time as the samples age. As more time pass the activity in the superchilled samples go down rather than rise to the same levels as in the chilled references. This can be related to the decrease in free amino acids shown in figure 3-8, but there it only is day 28 that goes down.

There is a significant increase in cathepsin activity on day 1 in superchilled samples. This is the day when the temperature is equalized in the entire filet and the ice crystals have reached their maximum size. The increase in cathepsin activity in the homogenate can be a result of damage to the lysosomes leading to an increased amount of intracellular cathepsins that also leak out through the cell membranes. It could also be that the high activity of all these proteases is what's causing the very significant decrease in protein solubility two days later.

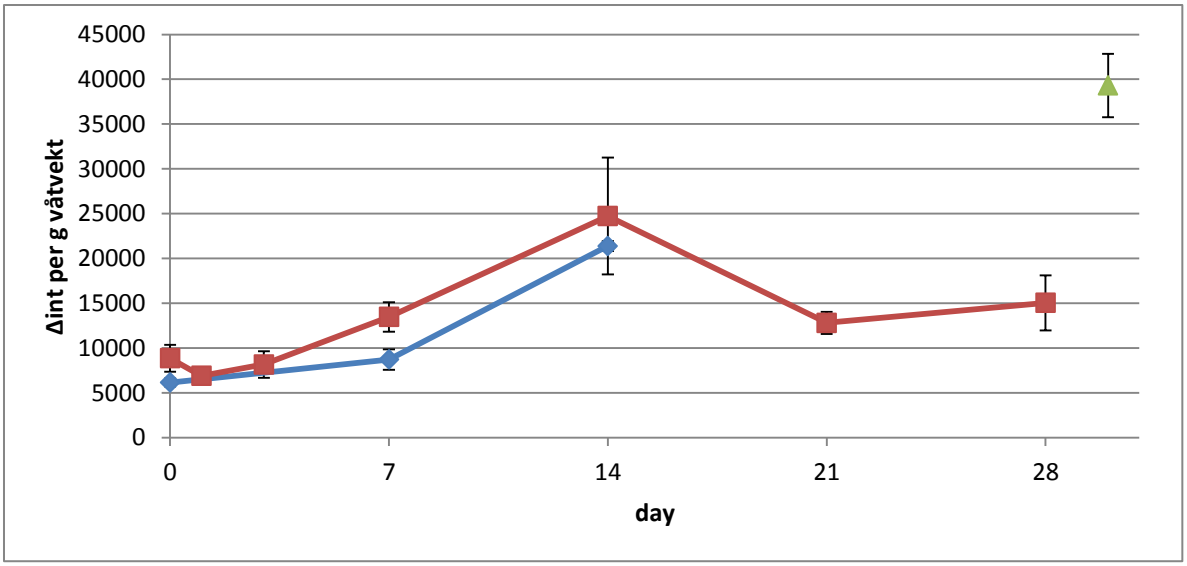

Figure 3-11: Changes in activity of cathepsin B + L in cell tissue fluid from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares, frozen storage at -20°C is represented by triangle, and chilled storage in a cold room at 4°C by diamonds. Values given as increase in fluorescence intensity per g CTF / g wet weight per. Values are given as mean ± SEM (n = 3)

Activity in superchilled and chilled samples are more or less the same, with a slight increase in superchilled samples. Also here, as in the homogenate, the activity is lower on day 21 and 28 in the superchilled samples, but there is a slight increase on day 28, which probably corresponds with the greater degradation of the filets at this time. It can be noted that the variance between the filets are high on day 14, and if adjustments are done for increased drip loss and other changing factors the difference in total cathepsin activity is not very big during the storage period.

The frozen samples have a significantly higher activity than any of the other samples. There is probably a high degree of lysosomal damage, but the low temperature has kept the proteases more or less inactive, so they have in little degree leaked out from the cell and into the homogenate. This can also relate to the relatively low amount of free amino acids and the higher protein solubility (figures 3-7 and 3-8). The frozen samples have also just been frozen and thawed once and the measurements are taken soon after complete thawing, so the proteases have had little time to react.

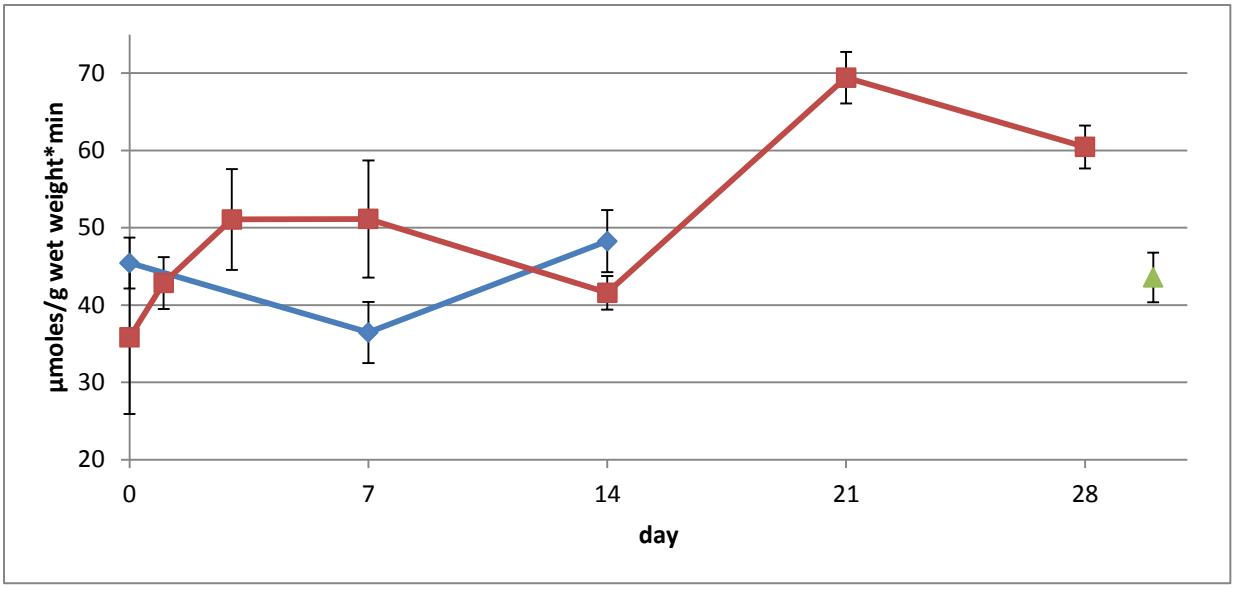

Figure 3-12: Changes in activity of β-N-acetyl-glycosaminidase in homogenates from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares, frozen storage at -20°C is represented by triangle, and chilled storage in a cold room at 4°C by diamonds. Values given as µmoles/ g wet weight*min. Values are given as mean ± SEM (n = 3)

Figures 3-13 and 3-14 show the activity of NAG in the water soluble proteins and CTF. NAG is an important lysosomal glucopeptidase and proposed as a measurement for freeze/thawing damage (Nilsson & Ekstrand, 1994) In the homogenate the activity varied greatly. This is a snapshot of the activity in the extracellular compartments of the muscle, and varies with other changes in the muscle, like loss by drip, inhibitors and proteases denaturing the enzyme.

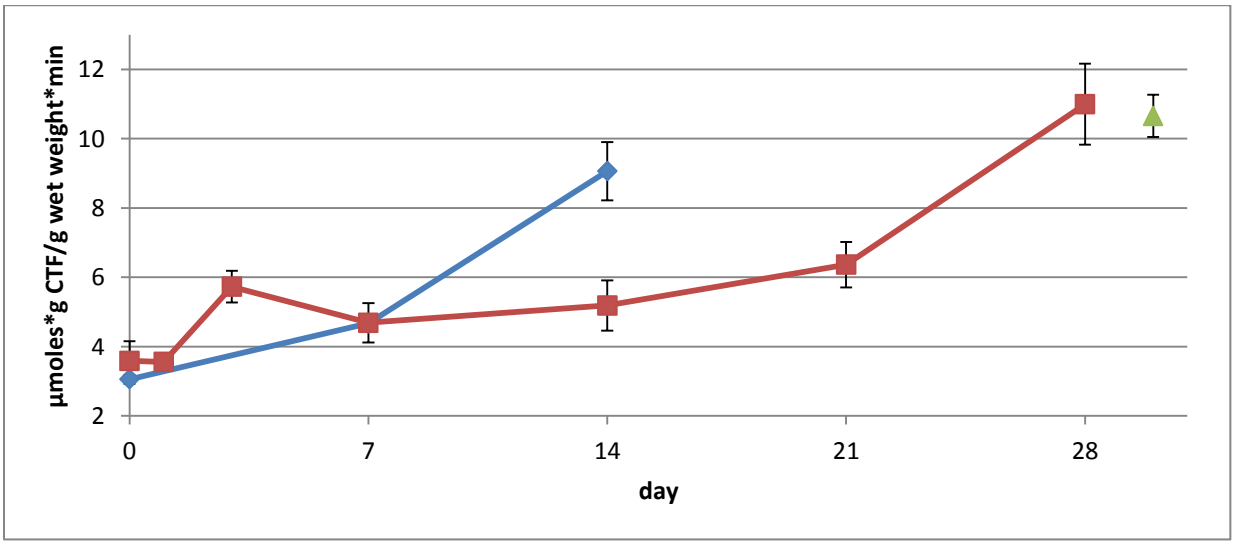

Figure 3-13: Changes in activity of β-N-acetyl-glycosaminidase in cell tissue fluid from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares, frozen storage at -20°C is represented by triangle, and chilled storage in a cold room at 4°C by diamonds. Values given as µmoles*g CTF / g wet weight*min. Values are given as mean ± SEM (n = 3)

This indicates that the total amount of NAG free in the cell (seen in figure Figure 3-13 as NAG activity in the CTF) is relatively stable once the filets have stabilised after the ice formation from superchilling (as seen in the drip loss and CTF), but the activity rise significantly when the filet show a higher degree of degradation the last day of storage (day 14 and 28 for chilled and superchilled samples respectively).

Interesting to see that here, unlike in the homogenates, the frozen reference have equal activity as the superchilled samples at day 28. In the homogenate the enzyme activity in the frozen samples is significantly lower.

Also here there is a marked difference in the measurements from day 3. Since the calculations here is not using protein solubility these measurements strengthen the theory that something is happening in the filets at this time.

3.2.4 OTHER RESULTS

There were also done some experiments on two extra superchilled filets from day 28. These results are not shown as they in many ways were just a preliminary experiment leading to the storage experiment Salmon 2 which has much more reliable results. Because it was just done on two filets the results were not statistically reliable.

The two filets was layered into a top, mid and bottom layers, one with about 2mm thick layers and the other with around 5mm thick layers. Especially the striped layer from the bottom of the filets showed much lower protein solubility and low cathepsin activity compared to the other layers. The mid layer (which was considered the “normal” cut) had higher cathepsin activity in the thin layers compared with the other layers, but almost equal activity in the mid layers. The protein solubility (both water and salt soluble)was sinking throughout the layers, with the top layers in both filets having the highest amount of soluble protein and the bottom layer having significantly ($p<0,03$) less.

3.3 SALMON 2

3.3.1 SAMPLE QUALITY

The filets were generally in good condition until day 28 (superchilled) and day 14 (chilled). No microbial analysis was done in this storage experiment, but sensory evaluation of smell and texture indicate that these filets were at the very edge of being suitable for consummation. Especially one superchilled filet was clearly not of good quality on day 28 and gave twice as high readings in all the enzyme measurements.

There was a few white spots on the filets at day 21 But on day 28 (shown in Figure 3-14) the discolouration dominated much more and was more pronounced than in Salmon 1 (Figure 3-4)

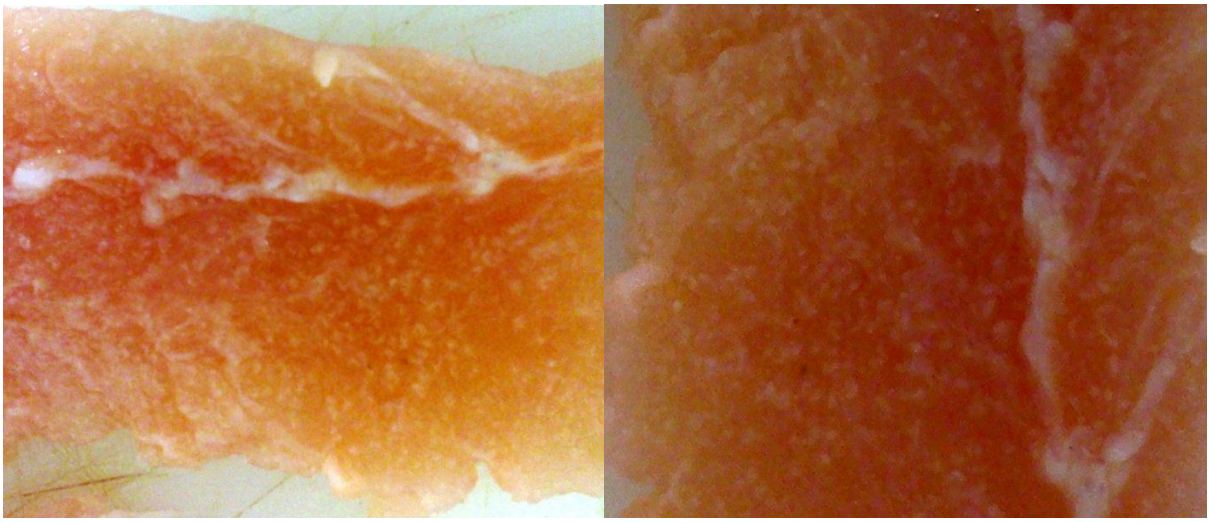

Figure 3-14: Picture of thawed salmon after 28 days of superchilled storage at -1.7°C.

Unlike in Salmon 1 (see Figure 3-5)the drip loss was more in the range of what was expected in this storage experiment. It stays under 2% during the entire period, much like what was seen in Duun & Rustad, 2008. The chilled and superchilled samples have quite similar development, which is not expected. Duun and Rustad (2008) showed little change in drip loss for chilled samples, and an increase in superchilled samples over time, while Duun and Rustad (2007)showed the opposite development.

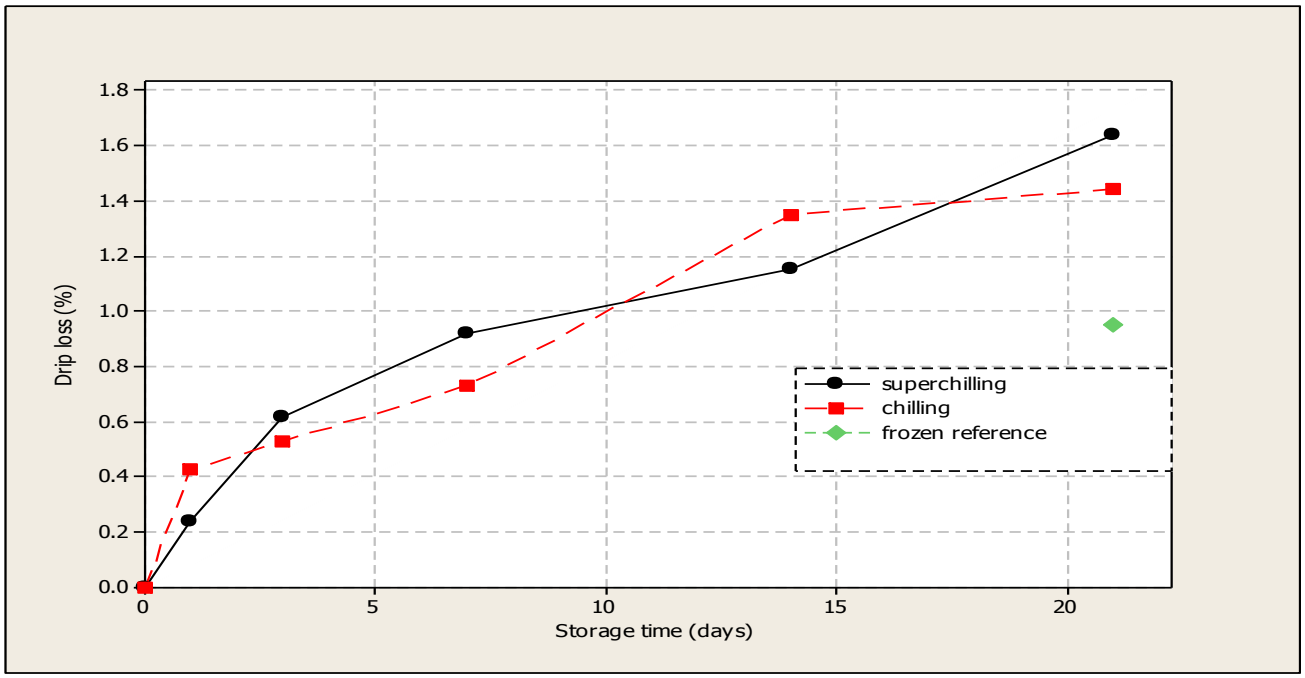

Figure 3-15: Changes drip loss in salmon under different storage conditions, shown as percentage of gram drip loss per gram wet weight. Values are given as mean (n = 2)

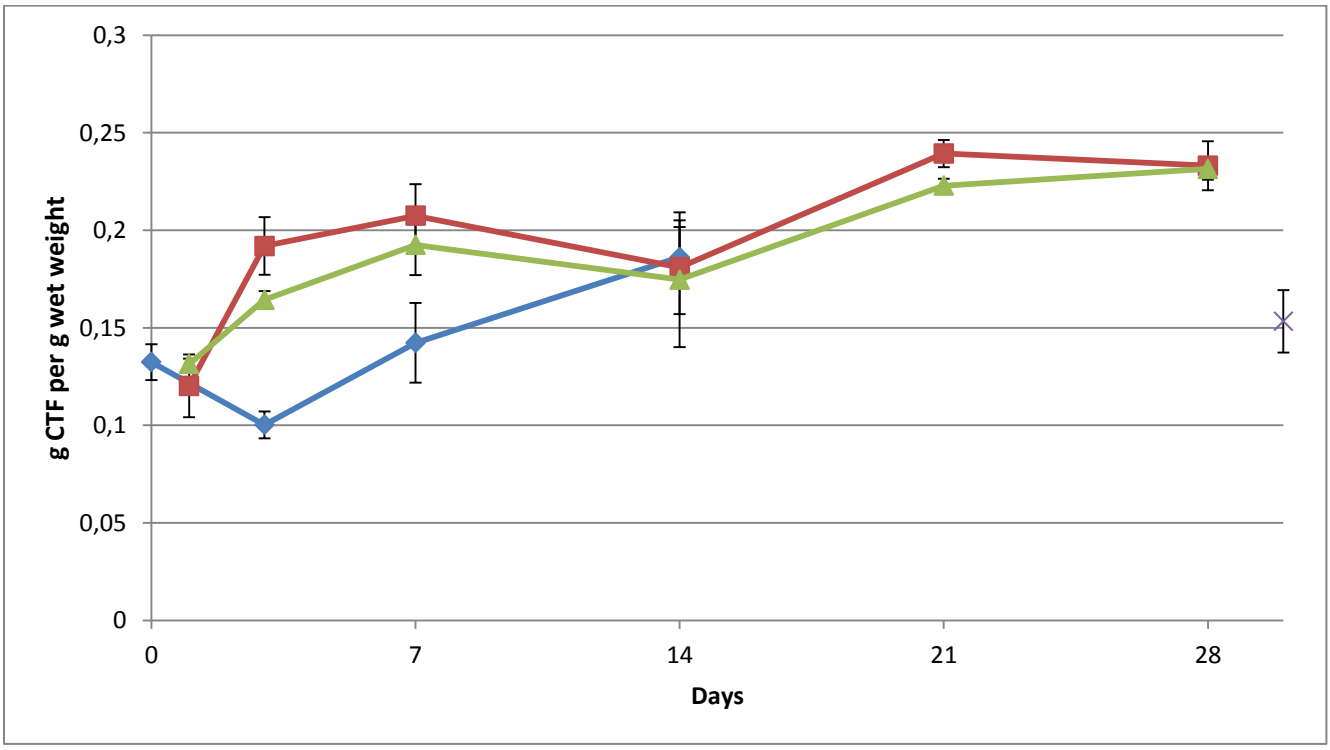

Figure 3-16: Changes in amount of cell tissue fluid per gram wet weight in salmon under different storage conditions. Superchilled storage at -1.7°C is represented by squares (top layer) and triangles (mid layer), frozen storage at -20°C is represented by a cross, and chilled storage in a cold room at 4°C by diamonds. Values are given as mean ± SEM (n = 3)

The amount of cell tissue fluid per gram wet weight (CTF) is in the same range as in Salmon 1 (Figure 3-6), both for superchilled, chilled and frozen samples. Also here there is a marked difference or stabilisation in the amount of extract after day 3 during superchilled storage.

The difference between top and mid layer is interesting. The difference is not significant at all the sampling days ($p>0,05$), but a clear trend can be seen. If CTF can be seen as a measurement of damage to the cell membranes and organelles, this shows that the top layer have a higher degree of structural damage to both cell membranes and connective tissue

3.3.2 PROTEIN SOLUBILITY

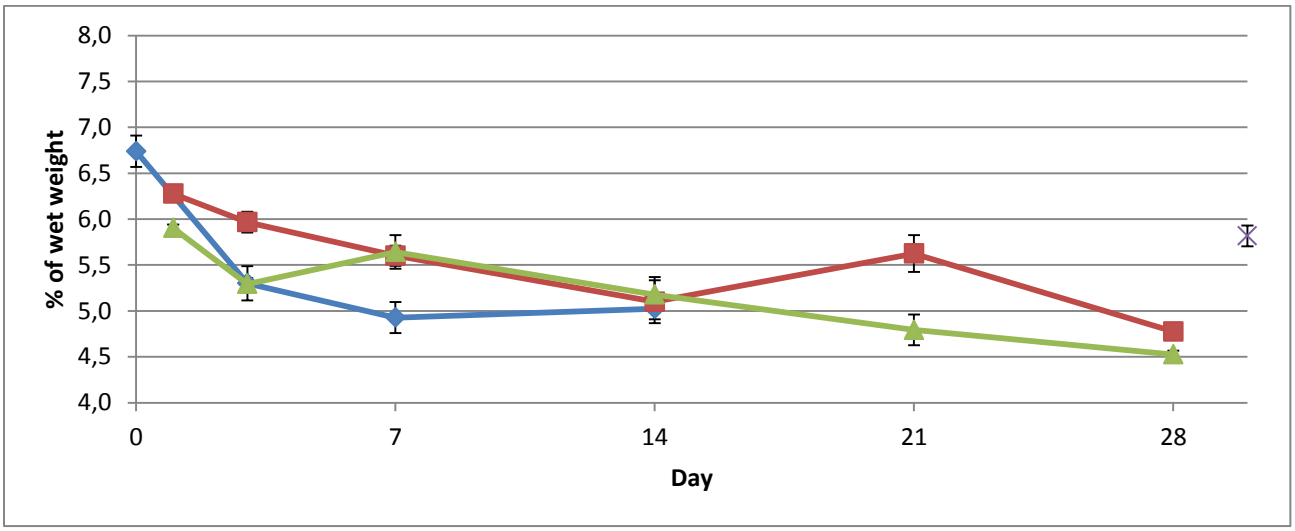

Figure 3-17: Changes in water soluble proteins in salmon under different storage conditions, shown as percentage of gram soluble protein per gram wet weight. Superchilled storage at -1.7°C is represented by squares (top layer) and triangles (mid layer), frozen storage at -20°C is represented by a cross, and chilled storage in a cold room at 4°C by diamonds. Values are given as mean ± SEM (n = 6)

Figure 4-18 shows that there is an overall decrease of water soluble proteins during storage, as seen in the first experiment (figure 3-7), but with some deviations (day 21, day 3). The frozen samples have a higher % of water soluble proteins after storage than the superchilled samples. The frozen samples also have a lower drip loss, which strengthens the theory that there is some washing out of water soluble proteins by the drip loss.

After the significant difference in protein solubility on day 3 in the first storage experiment (see Figure 3-7) there was large expectations bound to day three in the second storage experiment. As Figure 3-17 shows there is no large decrease on day 3, but the mid layer of the filets have a significantly lower amount of soluble protein compared to the top layer. Since the mid layer makes for much more of the total weight of the filet it is probable that the solubility in the mid layer is more comparable

to the measurements in Salmon 1. But the decrease in solubility on day 3 in the mid layer is still not close to the 5% decrease seen in salmon 1.

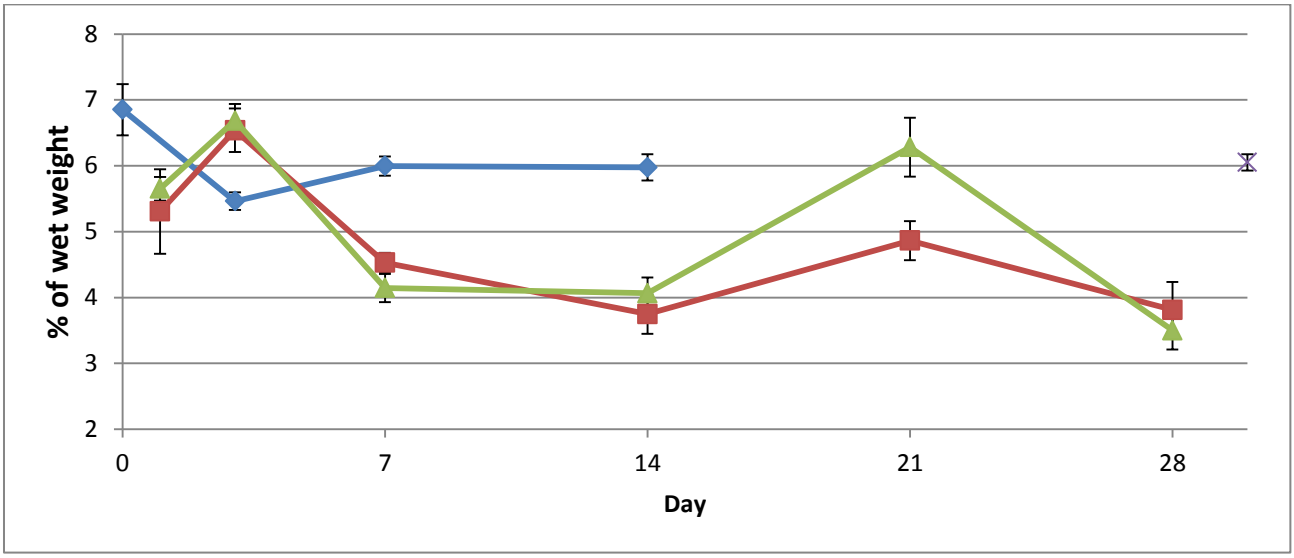

Figure 3-18: Changes in salt soluble proteins in salmon under different storage conditions, shown as percentage of gram soluble protein per gram wet weight. Superchilled storage at -1.7°C is represented by squares (top layer) and triangles (mid layer), frozen storage at -20°C is represented by a cross, and chilled storage in a cold room at 4°C by diamonds. Values are given as mean ± SEM (n = 6)

Figure 3-18 shows the amount of salt soluble proteins. This fraction is most important as a visualization of degradation and denaturation of the contractile network.

Day 3 is interesting, with a significant increase in solubility in both superchilled layers. In the first storage experiment there was no measurement of salt soluble proteins, so the results in the second experiment were highly anticipated. This is the day where most other factors seems to stabilise (drip loss, CTF and more), but it would seem that the contractile network is still going through some major changes, maybe by adjusting for the ice in the samples

As expected the solubility goes down over time, but it drops surprisingly quickly for the superchilled samples, indicating that the largest decrease in solubility happens early in the storage and then is quite stable.

The significant increase in solubility on day 21 is also a surprise. There is large differences between the individual filets this day, but the results are still significant ($p<0.05$). It might be that this is some kind of threshold where increased protease activity leads to a higher denaturation and loss of structural integrity, salt soluble proteins are the most important proteins for water holding capacity. This could give an increase in extracellular unbound water and lead to a higher extractability of the protein.

One superchilled filet had a very low percentage of salt soluble proteins on day 28, the value was only 2%. The residue in the centrifuge bottles was more than usual and more solid. Since both parallels had the same result and were made together with all the other salt soluble extracts the result should be trustworthy despite its low readings. A graph without the results from this filet was not very different; the new average was a little over 4% and didn't change the overall view of the changes in protein solubility, so the low results are included in this graph. There is no significant differences in the enzyme activity or amount of CTF from this filet, so why it acts so strangely is unknown. Hopefully SDS-PAGE will later be done on some of the samples, a high degree of denaturation in this filet is probable.

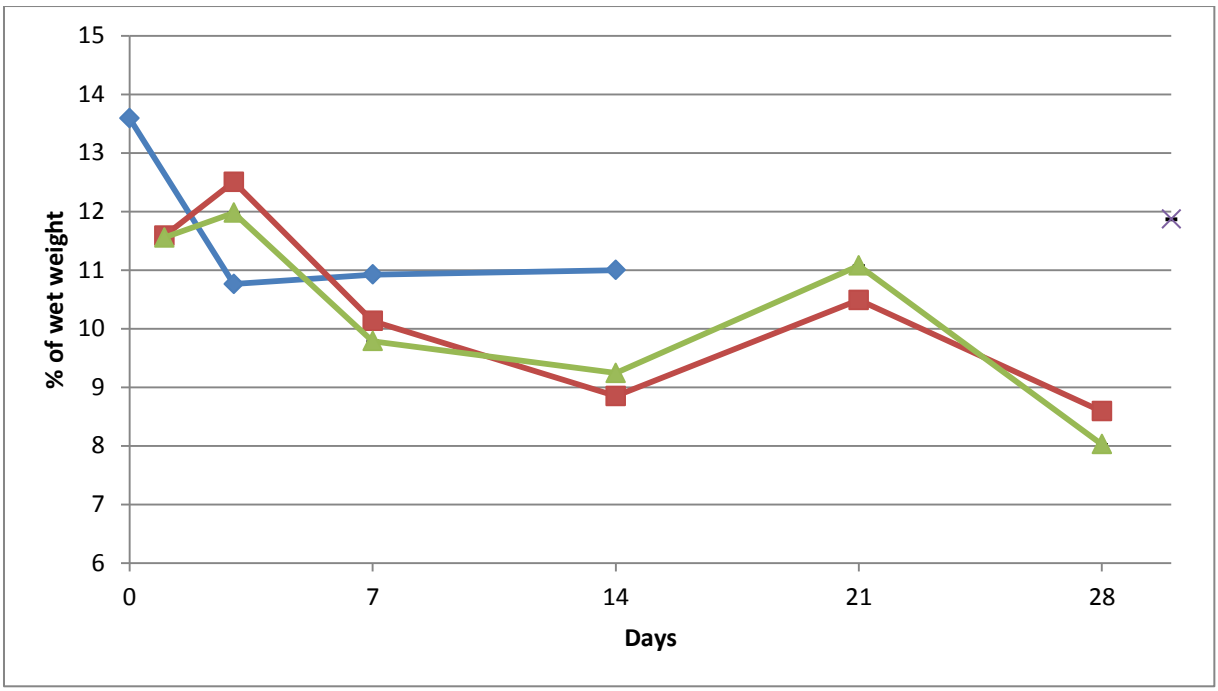

Figure 3-19: Changes in total amount of proteins in salmon under different storage conditions, shown as percentage of gram soluble protein per gram wet weight. Superchilled storage at -1.7°C is represented by squares (top layer) and triangles (mid layer), frozen storage at -20°C is represented by a cross, and chilled storage in a cold room at 4°C by diamonds. Values are given as mean ± SEM (n = 6)

The total amount of water and salt soluble proteins is shown in Figure 3-19. The chilled samples stay almost equal the entire period, with no decrease in solubility over all during storage. It is also interesting to see how the different layers from superchilled storage have a total protein solubility that is far more equal, indicating that the differences seen in water and salt soluble proteins above might just be differences from the extraction process. It could also indicate that the salt soluble proteins change properties as they are degraded.

Also here the frozen references have higher protein solubility than the superchilled samples, and this could relate to lower drip loss. But both frozen and chilled samples are higher than the superchilled samples during storage. The chilled samples are

almost stable around 6% after the first days of storage, and the frozen references have the same solubility. This is a negative difference, since low protein solubility is tightly tied to increased drip loss, loss of water holding capacity and softer texture. It could be argued that a superchilling storage temperature of -1.7°C might be too high since the damage to the contractile protein structures is so high.

3.3.3 Enzyme Activity

All results for cathepsin B activity are shown without the measurements from the filet that was of a significantly lower quality than the other samples on day 28. In all measurements it had twice as high readings or higher compared to the other samples.

The very large difference between the spoiled and the better preserved filets indicates that there is a significantly higher amount of enzymes released when the filet gets more and more spoiled. It is probable that the membranes are still largely intact during storage, despite the slightly higher measurements over time.

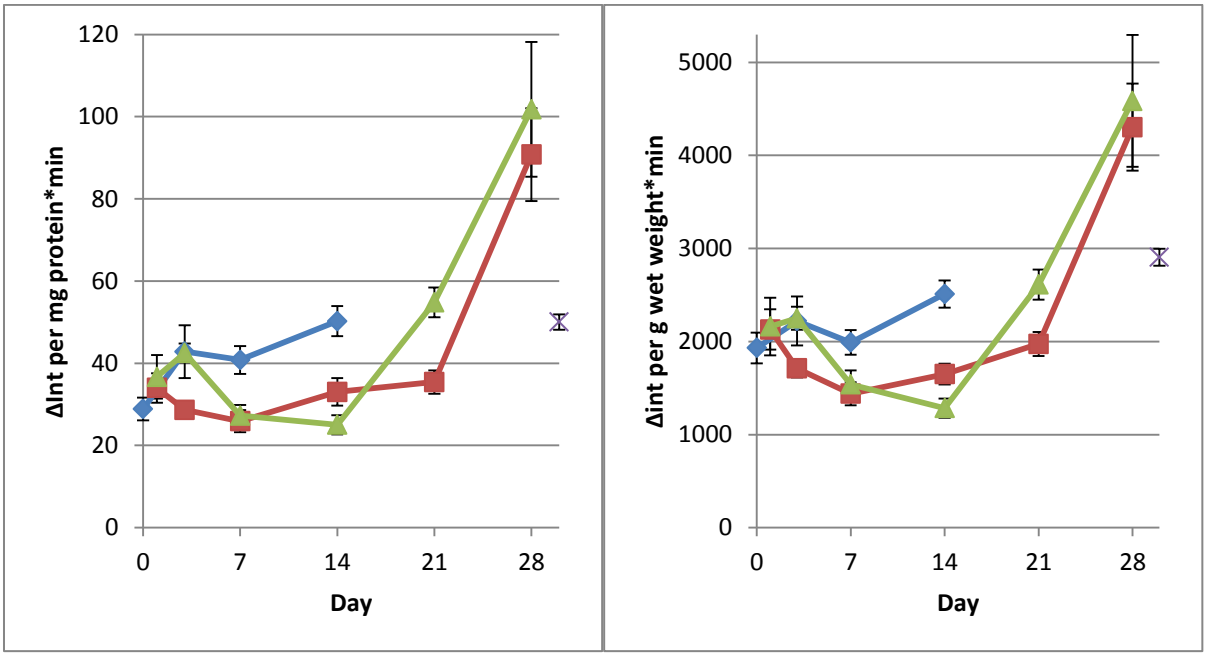

Figure 3-20: Changes in activity of cathepsin B in homogenates from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares (top layer) and triangles (mid layer), frozen storage at -20°C is represented by a cross, and chilled storage in a cold room at 4°C by diamonds. Values given as increase in fluorescence intensity per mg protein per minute (left graph) and increase in fluorescence intensity per g wet weight per minute (right). Values are given as mean ± SEM (n = 6)

The activity of cathepsin in the homogenates of the different layers (shown in figure 3-21) is largely similar to the results seen in the first storage experiment. The mid layer shows a tendency of higher measurements than the top layer almost all days,

but the difference it seldom significant. The activity in the top layer seems to be quite stable from day 3 to day 21, while the mid layer varies. The increase on day 3 in the mid layer can be related to the changes in structure mentioned earlier when discussing the changes in protein solubility and cell tissue fluid.

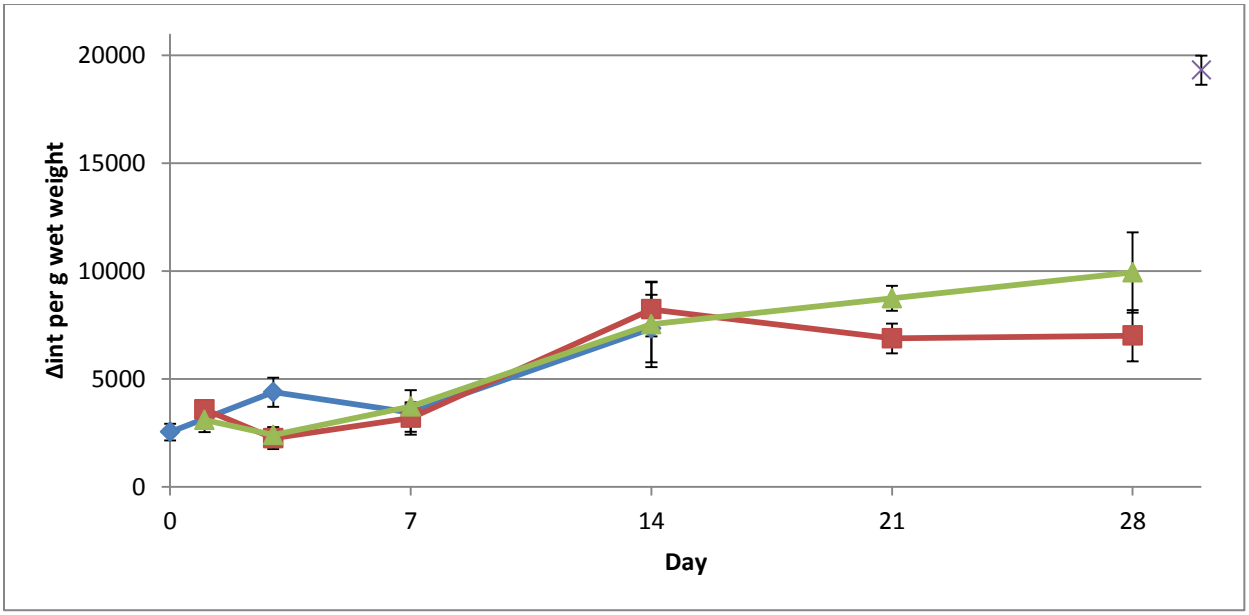

Figure 3-21: Changes in activity of cathepsin B in cell tissue fluid from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares (top layer) and triangles (mid layer), frozen storage at -20°C is represented by a cross, and chilled storage in a cold room at 4°C by diamonds. Values given as increase in fluorescence intensity per g CTF / g wet weight per minute (right). Values are given as mean ± SEM (n = 3)

The activity in the CTF is to a large degree similar to the activity in the homogenates, except that it is significantly higher, and it increases more steadily during storage. This is not surprising since we already have concluded that the CTF represents the total amount of free lysosomal enzymes and that the CTF increase while the damage on the lysosomes give a higher release of proteases which in turn cause even more damage, like a negative self-enhancing spiral.

At the last days of storage the mid layer again shows signs of higher activity than the top layer, but the difference is not significant since the variance between the filets were quite high at day 2.

The frozen samples have a significantly higher activity in the CTF than any of the other samples also in this storage experiment. There is probably a high degree of lysosomal damage, but the low temperature has kept the proteases more or less inactive, so they have in little degree leaked out from the cell and into the homogenate. The higher protein solubility in frozen samples shown earlier is probably because the filet have been stored at so cold temperatures that the enzymes are inactive.

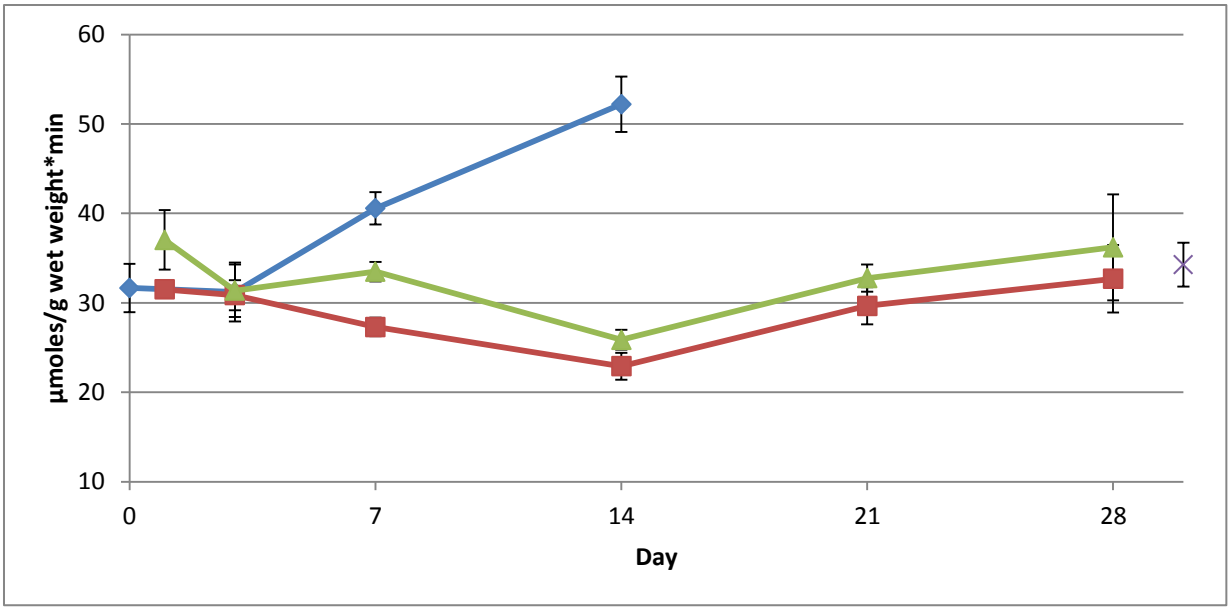

Figure 3-22: Changes in activity of β-N-acetyl-glycosaminidase in homogenates from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares (top layer) and triangles (mid layer), frozen storage at -20°C is represented by a cross, and chilled storage in a cold room at 4°C by diamonds. Values given as µmoles/ g wet weight*min. Values are given as mean ± SEM (n = 6)

The activity of NAG is a mirror image of the cathepsin activity, but the higher activity in the mid layer is clearer. Presumably the reason for this is the same as in cathepsin activity: different degrees of organelle damage. As in the first storage experiment there are fluxtations in the enzyme activity around day 1 and 3. The top layer is relatively stable, while the mid layer is quite high before it to stabilize.

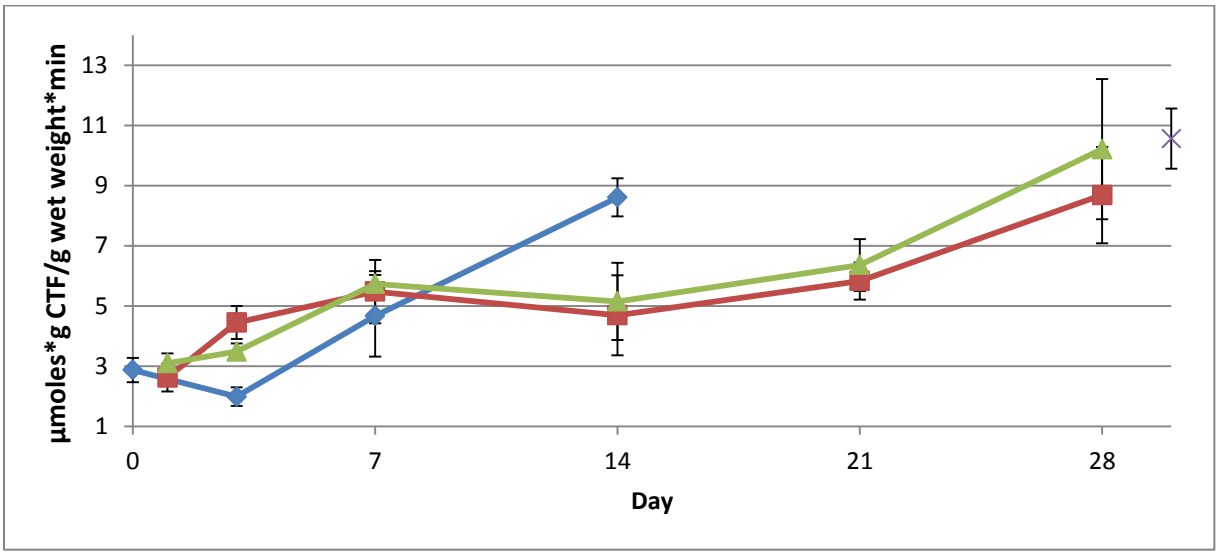

Figure 3-23: Figure 3-24: Changes in activity of β-N-acetyl-glycosaminidase in cell tissue fluid from salmon at different storage length and conditions. Superchilled storage at -1.7°C is represented by squares (top layer) and triangles (mid layer), frozen storage at -20°C is represented by a cross, and chilled storage in a cold room at 4°C by diamonds. Values given as µmoles*g CTF / g wet weight*min. Values are given as mean ± SEM (n = 3)

The NAG activity in the CTF rise the first days until it to is more or less stable from day 7 to day 21. It is interesting that all the different storage methods give more or less the same activity on their last day of storage. The activity of NAG in the CTF almost precisely mimics how one could expect a graph showing degree of membrane damage and breakdown of the contractile tissue. This is why NAG activity have been proposed as a parameter of freeze/thaw denaturation.

The samples from the mid-layer of the filets are consistently higher in almost all measurements (except day three). There is definitly a difference between the layers, and it is most certainly worth working more with, since the uncertainty here is too high to call the results significant.

4 CONCLUSION

Superchilled storage can significantly increase shelf life for fish muscle, at the same time retaining a higher quality than fully frozen fish. More knowledge on the process and effect of process parameters are still needed, but superchilling is a good alternative to traditional storage on ice.

The microbial counts clearly showed that the superchilled salmon had a longer shelf life than chilled salmon, the number of colony forming units in superchilled samples was acceptable after both 1 and 2 weeks of storage, while the chilled samples quickly became of questionable quality after 1 week and after 2 weeks of storage they were no longer suitable for human consumption.

The quality of the raw material in the first experiment can be questioned. Several of the measured parameters showed that the fish was of reduced quality and that superchilling is less effective if the raw material is old. Especially a relatively high drip loss and loss of protein solubility shows that the samples had a higher degradation than observed in some other experiments.

There were several signs that the cells in superchilled salmon experienced great internal stress and damage to organelles despite the fact that ice crystal studies showed that the cell membranes was not very damaged. This was especially visible in the measurements of lysosomal enzymes and comparison between the activity in homogenate and cell tissue fluid.

Together with the ice crystal measurements these results show that there are large changes between the inside and outside of the filet. A new storage experiment was done and all filets were carefully sectioned to give comparable biochemical measurements of the inside and outside of the cells.

The inside of the filet has much larger ice crystals that have grown over a longer time. The cell membranes are flexible and there is a low amount of ruptured cells, but the damage on the inside of the cell is greater. The top layer are frozen quickly and get a higher degree of damage to the cells the first days, but the ice crystals remain small and don’t disrupt the organelles so much.

This difference gives a higher amount of lost fluid in the top layer in the beginning, but the greater lysosome breakage in the mid layer of the filets give a higher enzyme activity inside the filet compared to outside the filet. After long storage the middle of the filet will be in a greater degree of degradation than the outside of the filet, and this is probably why the white spots appear.

When thawed, the inside is more degraded by enzyme activity and thus there is more liquid that need to escape and forces itself through the better quality top layer. The

paleness is probably caused by denatured proteins in the drip loss, but this needs more research to be confirmed.

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