Title: Superchilled, chilled and frozen storage of Atlantic mackerel (*Scomber scombrus*) fillets – changes in texture, drip loss, protein solubility and oxidation

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Abstract

Changes in quality characteristics in relation to protease activity and protein oxidation in chilled, superchilled and frozen mackerel fillets during storage were studied. The solubility of sarcoplasmic proteins was quite stable in mackerel samples for all storage experiments, whereas the solubility of myofibrillar proteins decreased in both superchilled and frozen samples. A significant correlation ($r=0.983$, $p<0.05$) between the increased activity of cathepsin B+L in chilled fillets and softening of the fish flesh during storage was revealed. Contrary to chilled samples, the texture of superchilled mackerel fillets became tougher along the storage period, which can be explained by a higher rate of myofibrillar oxidation ($r=0.940$, $p<0.05$). The hardness and drip loss decreased slightly at the end of frozen storage. Superchilling preserved the quality of mackerel fillets with the least side effects in relation to protein solubility, drip loss and softening of the fish tissue as compared to chilled and frozen storage.

Keywords: superchilling, chilling, Atlantic mackerel, frozen storage, protease activity, quality parameters

Introduction

Pelagic fish is a valuable food source due to its high nutritive value and essential nutrients such as omega-3 fatty acids, fat-soluble vitamins (E and D) and easily digestible proteins (Venugopal, 2009). However, it is highly perishable due to endogenous enzymes boosting proteolysis of muscle proteins, oxidation of lipids and metabolic activities of microorganisms (Puolanne and Halonen, 2010, Standal et al., 2018).The high perishability constrains long-distance delivery of fresh fish. Proper handling and preservation methods are therefore needed to increase the shelf life and ensure safety while retaining sensory- and nutritional quality. Chilling of fish is a commonly used practice to increase its shelf-life, while improving quality and safety during storage by reducing the rate of enzymatic proteolysis, lipid oxidation, and microbial degradation (Lauzon et al., 2010). A large amount of fresh seafood is currently transported on chilled ice over several hundreds of kilometres, which makes up about one-third of the overall transport weight. This drastically increases the transportation costs and leads to higher CO_2 emissions, resulting in less efficiency as more transport units have to be used (Thordarson et al., 2017).

Frozen storage at -18°C to -40°C helps to ensure fish safety for a long time of storage and distribution, while hindering chemical and microbial changes in the product (Lauzon et al., 2010). However, it may impair the sensory quality (Montero and Borderias, 1990) and requires much higher energy consumption in comparison with chilling (Thordarson et al., 2017).

Nevertheless, other preservation methods based on control of temperature can be used to reduce biochemical degradation and microbial spoilage occurring during storage and distribution of fish. One of them is superchilling, which may act as an attractive compromise between conventional chilling and freezing (Duun and Rustad, 2008). Superchilling is defined as a method of preserving food by partial freezing (Duun and Rustad, 2007), e.g. by applying low temperatures (< - 30 °C) for a short time so that the outer layer of the product is frozen, and further storage of the products at temperatures just below its initial freezing point (1-2 °C) leading to an even distribution of ice within the product. This enables delivery of higher amounts of valuable products with lower energy consumptionand in less transport units, because

the ice shell inside the product acts as a thermal inertia (Gallart-Jornet et al. 2007). Superchilling has been used for several decades in the fish processing as one of the most efficient refrigeration preservation techniques helping to significantly increase the shelf life of fish products compared to commonly used chilling technology (Duun and Rustad 2008; Magnussen et al. 2008).Up to now, superchilling preservation technology has been successfully applied in preservation of cod, salmon, sea bass, tilapia (Duun and Rustad 2007; Kaale, et al., 2014; Liu et al. 2010; Cyprian et al. 2013), as well as other seafood products. However, despite the successful application of superchilling in extending the shelf-life of the seafood products, this method may result in thermal denaturation of proteins decreasing protein solubility and leading to increased drip loss and changes in the fish muscle hardness due to cell damage and protein aggregation (Bahuaud et al., 2008; Duun and Rustad, 2008; Liu et al., 2013). Also, there is very little information on the influence of superchilling on quality parameters of small pelagic fish, with no studies performed on Atlantic mackerel, which has recently received greater attention due to its increasing capture production and economic importance (FAO, 2015).

According to the European Market Observatory for Fisheries and Aquaculture Products (EUMOFA), Atlantic mackerel ranked among the top small pelagic commodity groups both in volume and value in 2017 in Europe (EUMOFA, 2018). Therefore, the development of effective preservation technologies to prolong shelf life and preserve quality of pelagic fish products, while minimizing production, delivery and storage costs, become increasingly important. In respect to this concern, the aim of the present study was to find the best preservation technology by assessing the potential of chilling, superchilling and freezing to maintain the quality of Atlantic mackerel (*Scomber scombrus*) during storage.

2. Materials and Methods

2.1 Sample preparation

Atlantic mackerel (*Scomber scombrus*) was used as raw material in the present study. The fish were caught on the 21st of January 2017, transported in RSW-tanks (Refrigerated Sea Water) and landed at Pelagia A.S. processing plant (Selje, Norway) on the 23rd of January, where it was mechanically filleted. Average weight of the resulting skin-on fillets was 89 ± 9.6 g, with an average length of 17 ± 1.4 cm. The mackerel fillets were packed in 15 kg vacuum packs, placed on ice and transported by boat to SINTEF (Trondheim, Norway). Upon arrival to SINTEF on the 25th of January, the fish was divided into three parts (for chilled, superchilled and frozen storage experiments) and vacuum-packed. Sample packages of four mackerel fillets each were placed in BST-090 type bags from "Three Seal Bags" series (Rolf Bayer Vacuumverpackung GmbH) having the following parameters: thickness of 90 μm, gas permeability (O_2, N_2 and CO_2) of up to 60 $cm^3/m^2*d*bar$ and water vapor permeability of up to 4 g/m^2*d. The vacuum-bags with the fish were further heat-sealed using a vacuum sealing machine (Webomatic Vacuum packaging system, Super max, 3000 sensor). Temperature data loggers type SL52T (Signatrol, UK) were manually inserted into vacuum bags with the fish fillets to monitor fluctuations of temperature during chilled, superchilled and frozen storage experiments.

Superchilling was conducted in an Impingement Lab Freezer (JBT-Frigoscandia, Sweden) at -37°C (air temperature) for 1.5 min at Energy's laboratory of Norwegian University of Science and Technology – NTNU (Trondheim, Norway). The fish was further subjected to chilled and superchilled storage experiments (on ice at +4ºC and -1.7ºC, respectively) performed at NTNU, as well as frozen storage (i.e. vacuum packed fish fillets were put at -27ºC storage facilities at SINTEF. A storage temperature of −25°C to −30°C is recommended for frozen storage of fishery products in Europe – and is commonly used in pelagic industry in Norway. Sample collections and analyses for chilled and superchilled fish were performed at four and six different times, respectively. For chilled storage: on day 1 (directly upon arrival to the laboratory, corresponding to day 4 after catch) and after 2-, 5- and 7-day storage. For superchilled

experiment: on day 1 (the same as for chilled fish) and after 2-, 5-, 9-, 14- and 19-day storage. The frozen mackerel samples were analyzed on 1-, 9- and 12-month storage. Prior to analysis, the frozen mackerel samples were first thawed overnight at +4°C. The number of replicates varied between n = 3 to n = 5 for the different analyses.

2.2 Chemical and physicochemical assays

Specific proteolytic activity

The activity of cathepsin B+L was determined in centrifuged tissue fluid (CTF) of chilled and superchilled mackerel samples as previously described by (Hultmann & Rustad, 2004). The CTF preparation was performed as described by Nilsson (1994). Briefly, about 10-15 g of muscle was weighted out in a centrifuge tube and further centrifuged for 30 minutes at 8000 x g. The supernatant was used as the CTF for determination of cathepsin B+L activity. Prior to measurements, the amount of proteins in the extracts was determined by BioRad protein assay, using bovine serum albumin as a standard (Bradford, 1976). The analyses were run in triplicate.

Drip loss

For determination of drip loss, mackerel fillets were removed from the vacuum bags and blotted dry with a tissue paper before weighing. The remaining liquid in the vacuum bag was also weighed and the drip loss was calculated as the percentage of fish weight loss after removing the liquid (Kaale et al., 2014).

Protein solubility

Water- and salt soluble proteins were extracted from mackerel muscle by a modification of the method of Licciardello et al. (1982), as previously described by Hultmann & Rustad (2002). The extraction procedure was performed once on each fish fillet.

148 Protein content in the extracts was determined by using the method of Bradford (1976), with bovine serum
149 albumin (BSA) as a standard. The analyses were run in four replicates and the mean value ±SD was
150 calculated.
151
152 ***Protein oxidation***
153 Protein carbonyl groups were determined by DNPH-based Enzyme-Linked Immunosorbent Assay (ELISA)
154 in a 96-well polystyrene plate as a measure of protein oxidation (Buss et al., 1997). The indirect ELISA kit,
155 STA-310 OxiSelectTM, was purchased from Cell Biolabs, Inc. (San Diego, CA, USA).
156 Briefly, extracts of water- and salt-soluble proteins were used to determine total carbonyls in sarcoplasmic
157 and myofibrillar proteins. Before the analysis, all samples and BSA-standards were diluted with 1X PBS
158 to obtain solutions with protein concentration 10 µg/ml, and then 100 µl of each sample were introduced
159 in a 96-well protein binding plate for incubation overnight at 4°C. Then, each well was washed three times
160 with 250 µl 1X PBS, and 100 µl DNPH working solution were added followed by incubation for 45 minutes
161 at room temperature under dark.
162 After this, the wells were washed five times with 250 µl 1X PBS/ethanol (1:1, v/v) with a 5-minute
163 incubation on an orbital shaker with 5 minutes between washes. Further, 200 µl ELISA blocking solution
164 were added in each well followed by a 2-hour incubation at room temperature on an orbital shaker. After
165 the incubation, the wells were washed three times with 250 µl ELISA wash buffer and 100 µl diluted anti-
166 DNP antibody were added followed by one-hour incubation at room temperature on an orbital shaker and
167 subsequent washing with ELISA wash buffer. Further, 100 µl diluted HRP-conjugated secondary antibody
168 were added to the wells, and the same washing procedure was performed for five times. Then, 100 µl ELISA
169 substrate solution were added in each well and incubated at room temperature on an orbital shaker for
170 approximately 15 minutes. The enzyme reaction was ended by adding 100 µl ELISA stopping solution in
171 all wells, and the absorbance was read immediately on a plate reader (Tecan, Austria) at 450 nm.
172 Carbonyl groups were determined in the four parallels for each protein extract (n = 4), and the average
173 value with standard deviation were calculated. The results were expressed in nmol carbonyls/mg protein.

174

175 ***Texture parameters***

176 Hardness and cohesiveness of chilled, superchilled and frozen mackerel samples were measured along the
177 whole internal dorsal part of the fillets using a TA.XT2 Texture Analyzer (SMS Stable Micro Systems,
178 Ltd., Surrey, UK) equipped with a 1 kg load cell according to the method described by Hultmann & Rustad
179 (2002). The fish fillets were kept on ice before the analysis. Texture Profile Analysis (TPA) as a double-
180 compression test was used for determination of the flesh hardness and cohesiveness. Mackerel fillets were
181 compressed twice by a flat-ended cylinder of 12 mm in diameter at a constant speed of 1 mm s^{-1} until it had
182 reached 60 % of its height, carefully avoiding myocommata. The holding time between the compressions
183 was 5 s. The maximum resistance force was recorded in Newton (N) and expressed as fillets' hardness.
184 Cohesiveness was calculated as the area of work during the second compression divided by the area of
185 work during the first compression. From four to five measurements were run on each fillet and the average
186 was calculated.

187

188 **2.3 Statistical analysis**

189 Statistical analysis and data processing were conducted using Statgraphics Centurion XVI. Statistical
190 significance of the experimental data was verified by using Student's t-test and Analysis of Variance
191 (ANOVA). To establish a relationship between certain parameters, Pearson correlations were calculated.
192 Differences were considered significant at $p<0.05$.

193

194 **3. Results and discussion**

195

196 ***Specific proteolytic activity***

197 For the chilled-stored group, cathepsin B+L activity increased insignificantly ($p<0.05$) on the 7th day
198 storage, peaking at 1.24-fold of the initial value (Figure 1). For the superchilled mackerel samples, this

trend was slightly accelerated and cathepsin B+L activity reached the maximum level of chilled-stored samples on the 5thday of superchilled storage. This phenomena can be explained by a greater release of enzymes from lysosomes due to a mechanical damage of muscle cells by small ice crystals formed during superchilling of fish (Kaale et al., 2014), compared to chilled fish. Although cathepsin B+L activity in CTF of superchilled samples had no significant changes along the storage period, it increased significantly in comparison with chilled samples. These results are in agreement with a study published by Bahuaud et al. (2008) who reported an accelerated breakage of lysosomes by ice crystals and an increased release of cathepsin B+L in Atlantic salmon fillets subjected to superchilling before ice storage, when compared with chilled-stored salmon. Meanwhile, cathepsin B+L activity of superchilled mackerel samples increased moderately within the first nine days of storage (Figure 1), it further stabilized, peaking at 1.09-fold of the initial value on the 19th day of storage. This may be explained by a greater release of cathepsins B and L from the cell damaged by small ice crystals formed during superchilling in the beginning of storage (Bahuaud et al., 2008).

Figure 1

Drip loss

There was a significant ($p<0.05$) increase in drip loss for both the chilled and superchilled mackerel samples during storage (Figure 2). This is in agreement with the results reported by Kaale et al. (2014), who found significant differences in water loss during chilled and superchilled storage of salmon. However, superchilled samples had lower values of drip loss compared to chilled samples during the whole storage period. A significant increase in drip loss in both chilled and superchilled mackerel fillets during storage can be partially explained by the effect of cathepsin B+L activity (Figure 2), resulting in faster breakdown of proteins, which decrease the ability of muscle proteins to bind and hold water (Puolanne and Halonen, 2010).

Drip loss of frozen mackerel samples increased gradually during the first 9 months of storage and then dropped, reaching the value of 5.54% on 12th month. Moreover, it was significantly lower after 12 months of frozen storage compared to the last day of chilled and superchilled storage of mackerel samples.

Figure 2

Protein solubility

Muscle proteins undergo denaturation during storage of fish, leading to a decrease in amount of soluble proteins (Duun and Rustad, 2008). Therefore, changes during chilled, superchilled and frozen storage in sarcoplasmic (water-soluble) and myofibrillar proteins (salt-soluble) of mackerel samples were determined in the study (Figure 3A-B). There were no significant changes between methods of storage with respect to water-soluble proteins, because they are generally more stable during storage than salt-soluble ones (Duun and Rustad, 2008). This is in accordance with the results reported by Standal et al (2018). The content of water-soluble proteins remained unchanged after a 12-month frozen storage, which is in contrast with the studies of Leelapongwattana et al. (2005) and Saeed and Howell (2002) revealing a reduction in protein extractability during frozen storage of fish. The reason for the small differences in content of water-soluble proteins can be explained by the lower content of sarcoplasmic proteins compared to myofibrillar proteins – leading to less significant changes during storage compared to myofibrillar proteins (Tejade, 2001).

Solubility of myofibrillar proteins decreased significantly during superchilled storage of mackerel, which can be explained by a combined effect of denaturation of cells by small ice crystals and detrimental activity of proteases (Duun and Rustad, 2008) released from lysosomes, as shown by the negative correlation of content of myofibrillar proteins and cathepsin B+L activity($p<0.05$, $R=-0.645$). Freezing also decreased the solubility of myofibrillar proteins (Figure 3B), more notably from 9 to 12 months of storage, which is in agreement with the results of Medina et al. (2009) and Standal et al (2018). The decrease in extractability of myofibrillar proteins observed in this study is in agreement with the results of earlier investigations

(Rodriguez et al., 2006; Duun and Rustad, 2007; Medina et al. 2009). The amount of extracted salt-soluble proteins in superchilled mackerel samples was significantly lower than in chilled fillets, which is in agreement with the study on cod by Duun and Rustad (2007). Similarly to the latter study, the extractability of myofibrillar proteins in chilled samples increased slightly at the end of storage in the present study. The lower extractability of myofibrillar proteins in both superchilled and frozen mackerel samples during storage was also observed by Duun and Rustad (2007; 2008), Medina et al. (2009) and Standal et al (2018) working with cod, salmon, horse mackerel and Atlantic mackerel, respectively.

Figure 3

Protein oxidation

During chilled storage, the protein oxidation expressed as carbonyl content was significantly reduced in the sarcoplasmic, but remained table in the myofibrillar proteins (Figure 4A-B). This is in agreement with previous research of Standal et al. (2018) revealing no significant changes in carbonyl content of myofibrillar proteins during chilled storage of Atlantic mackerel fillets for 9 days at 4ºC.

Moreover, no significant variation in water-soluble proteins of mackerel samples were observed during a 14-day superchilled storage. Nevertheless, salt-soluble proteins of superchilled fillets suffered from a significant increase in carbonyl content from 9thday of storage (Figure 4B). Similarly, the oxidation rate in both sarcoplasmic and myofibrillar proteins varied significantly along 1 year of frozen storage, with a pronounced increase in amount of protein carbonyls after month 9 (Figure 4 A and B).

A significant increase in protein carbonyls during storage of frozen and superchilled mackerel samples can be explained by the cell disruption by ice crystals during freezing and cell puncture by small ice crystals during superchilling, liberating various pro-oxidants (H_2O_2, iron, myoglobin, etc.) which further increase the oxidative status of unfrozen phase of the fish (Standal et al., 2018).This phenomenon is ascribed to

cryo-concentration of pro-oxidant solutes around protein molecules in the unfrozen portion of water of the product, leading to increased oxidation (Standal et al., 2018).

Myofibrillar proteins were characterized by a higher carbonyl content than sarcoplasmic ones for all storage times (4-5 times higher at the end of storage time for chilled, superchilled and frozen samples). Similar results were obtained for chilled and frozen storage of mackerel samples (Standal et al., 2018), as well as thin-lipped mullet (Tokur and Polat, 2010). This tendency can be explained by a higher perceptibility of salt-soluble proteins to denature both during processing and storage (Duun and Rustad, 2007).

Texture parameters

There was an insignificant decrease of flesh hardness (Figure 5A) and breaking strength (Figure 5B) in chilled mackerel fillets in comparison with superchilled samples during storage. The texture softening in chilled mackerel samples can be associated with increased proteolytic activity leading to myocyte apoptosis (Ge et al., 2015). In support of this hypothesis, a significant correlation ($r=0.983$, $p<0.05$) between the activities of cathepsin B+L in mackerel samples and softening of the fish flesh during chilled storage was found. This tendency can be explained by a detriment effect of chilled storage on the integrity of lysosomes, leading to their breakage and the resulting release of cathepsins (Ge et al., 2015). The post-mortem tenderization of fish muscle during chilled storage has been largely studied for several decades and has been mainly attributed to activities of endogenous proteases on myofibrilar proteins (Ashie and Simpson, 1996). The progressive post-mortem softening of fish flesh primarily relates to weakening of Z-discs of myofibrils (Seki and Tsuchiya, 1991) and myosin-actin junctions (Yamanoue and Takahashi, 1988), as well as alterations in the pericellular connective tissue (Ando et al., 1991).

However, contrary to the increased rate of proteolytic activity in superchilled mackerel fillets (Figure 1) in comparison to chilled-stored fillets, the texture of superchilled samples became tougher during storage (Figure 5A). This could be explained by a higher rate of myofibrillar oxidation in the superchilled fillets (Figure 4A), leading to an increase in fish tissue hardness during storage. Moreover, a significant correlation ($r=0.940$, $p<0.05$) between the formation of carbonyl groups in myofibrillar proteins and the increase in

hardness of superchilled mackerel fillets during storage confirmed this hypothesis. It is generally agreed that oxidative modifications of proteins often lead to a decreased tenderness of both fish and meat (Lund et al., 2008; Kim et al., 2012; Listrat et al., 2016). According to these studies, the toughening effect is often accompanied by formation of protein crosslinks, including collagen crosslinks (Montero and Borderias, 1990; Kim et al., 2012; Listrat et al., 2016), as well as formaldehyde production in the muscle and the aggregation of myofibrillar protein and stroma (Montero and Borderias,1990).

The hardness of frozen mackerel samples was gradually decreasing during storage, reaching the value of 7.33±1.38 N on month 12. However, the softening of the muscle tissue varied insignificantly along the storage period, being probably associated with gradually reduced drip loss ($r=0.825$, $p<0.05$) preventing drying-induced toughening of the fish flesh during frozen storage.

Figure 5

4. Conclusion

The study has revealed that compared to chilled and frozen storage, superchilling may successfully preserve the fish muscle integrity from structural breakdown, resulting in lower values of drip loss and less fish tissue softening. No significant differences ($p<0.05$) were found between solubility of sarcoplasmic proteins in chilled, superchilled and frozen samples along the whole storage period. However, the extractability of myofibrillar proteins decreased significantly during superchilled and frozen storage. Protein oxidation increased significantly ($p<0.05$) in myofibrillar proteins of superchilled samples in comparison with chilled samples, partially contributing to a slight increase in the muscle tissue hardness ($r=0.940$, $p<0.05$). However, frozen mackerel samples had much higher content of protein carbonyls in myofibrillar proteins at the end of storage period compared to both chilled and superchilled samples. Therefore, we can conclude

that preservation of mackerel fillets by superchilling could be an alternative to chilled storage in regard to detrimental changes in protein characteristics.

5. Acknowledgments

The study displayed in the paper is based upon work supported by the JPI project ProHealth “Innovative processing to preserve positive health effects in pelagic fish products”, RCN 259582/E50. Pelagia Selje is acknowledged for providing fish for the experiments.

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Legends to figures

Figure 1. Activity of cathepsins B+L of chilled and superchilled fillets reported as increase in FI/g wet weight/min.

Figure 2. Drip loss in chilled, superchilled and frozen samples during storage. Day 1 shows drip loss in fish arrived in the lab.

Figure 3. Amount of sarcoplasmic (A) myofibrillar proteins (B) in chilled, superchilled and frozen samples during storage.

Figure 4. Carbonyl content in sarcoplasmic (A) and myofibrillar proteins (B) in chilled, superchilled and frozen samples.

Figure 5. Hardness (A) and cohesiveness (B) of chilled, superchilled and frozen samples during storage.