Journal Pre-proof

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PII: S0023-6438(19)31097-7

DOI: https://doi.org/10.1016/j.lwt.2019.108755

Reference: YFSTL 108755

To appear in: *LWT - Food Science and Technology*

Received Date: 13 June 2019

Revised Date: 16 October 2019

Accepted Date: 18 October 2019

Please cite this article as: Fidalgo, L.G., Delgadillo, I., Saraiva, J.A., Autolytic changes involving proteolytic enzymes on Atlantic salmon (*Salmo salar*) preserved by hyperbaric storage, *LWT - Food Science and Technology* (2019), doi: https://doi.org/10.1016/j.lwt.2019.108755.

Autolytic changes involving proteolytic enzymes on Atlantic salmon (*Salmo salar*) preserved by hyperbaric storage

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ABSTRACT

The effect of hyperbaric storage (HS, 50-75 MPa at 10-37 ºC) on proteolytic enzymes and muscle proteins of Atlantic salmon (*Salmo salar*) was assessed and compared to atmospheric pressure (AP, 0.1 MPa) at the same storage temperature and ~~conventional~~ refrigeration (AP, 5 ºC). Generally, activities of acid phosphatase, cathepsin B and D, and calpains decreased when compared to fresh salmon, with a more' pronounced effect of storage temperature of 37 ºC in HS/AP samples. However, activity recovery was observed for some enzymes, as the case of cathepsins B and D, and calpains, whose showed an increase of residual activity for samples stored at 60 MPa/10 ºC and 75 MPa/25 ºC after 50 and 25 d, respectively. A pronounced increase of myofibrillar fragmentation index (MFI) was observed at 75 MPa (25/37 ºC) after 10 d (3.2-/4.3-fold, respectively). Otherwise, at 60 MPa/10 ºC, a decrease of MFI values was observed after 50 d of storage. For sarcoplasmic proteins, no effect was observed at 60 MPa/10 ºC during 30 d of storage, with a slight increase after 50 d. At 75 MPa/25 ºC, a

decrease of sarcoplasmic proteins content (46%) was obtained after 10 d with no further changes during the 25 d of storage.

Keywords: Hyperbaric storage; *Salmo salar*; lysosomal enzymes; calpains; myofibrillar fragmentation index.

1. Introduction

During fish spoilage, there is a breakdown of various components and the formation of new compounds responsible for the changes in odour, flavour and texture, which are mainly caused by the metabolic activity of microorganisms, endogenous enzymatic activity (autolysis) and by oxidation of lipids **(Gram & Huss, 1996)**. Higher autolytic activity of major muscle endogenous proteases induces hydrolysis of key myofibrillar proteins, and thus contributes to weakening of the myofibrilar structure during *post-mortem* storage. The main proteolytic systems are the cytoplasmic calpains and the lysosomal cathepsins, such as cathepsins B, L, H and D **(Stagg, Amato, Giesbrecht, & Lanier, 2012)**.

The concept of hyperbaric storage (HS) at low (LT) and room (RT) temperatures has been studied lately as a possibility to improved fish preservation by some authors **(Fidalgo, Lemos, Delgadillo, & Saraiva, 2018; Ko, Jao, Hwang, & Hsu, 2006; Otero, Pérez-Mateos, Holgado, Márquez-Ruiz, & López-Caballero, 2019; Otero, Pérez-Mateos, & López-Caballero, 2017)**, with results showing the efficiency of HS in extending the shelf-life of fresh fish. The effect of HS/RT on enzymes activity from fresh fish is very scarce. However, the effect of high pressure (100-500 MPa for few min) in fish enzymes activity was extensively studied. It was suggested that the changes of enzyme activity in this case can be attributed to proteolytic enzymes release due to

perturbation of protein structure and the rupture of cell membrane by moderated pressures, contributing to enzyme activity increase, while higher pressure levels promote denaturation **(Chéret, Delbarre-Ladrat, de Lamballerie-Anton, & Verrez-Bagnis, 2005)**. Moreover, the difference in enzyme structure also affects its behaviour under pressure, since some proteases can have maximum activity and stability at a particular pressure-temperature combination due to structural modifications **(Chéret, Hernández-Andrés, Delbarre-Ladrat, de Lamballerie, & Verrez-Bagnis, 2006)**, as well as fish muscles can induce a protective effect on specific enzymes against high pressure, as observed in myofibril-bound serine proteinases of silver carp **(Qiu, Xia, & Jiang, 2013)**.

The effect of HS in enzymatic activity was already studied for a reduced number of food products. The activity of a pectin methylesterase extract from strawberry juice was not affected by HS during 15 d (pressures up to 200 MPa at 20 ºC), and this was suggested to explain the viscosity losses observed under HS, due to a possible enzymatic effect during longer periods under pressure **(Bermejo-Prada, Segovia-Bravo, Guignon, & Otero, 2015)**. **Pinto et al. (2017)** observed a decrement of peroxidase activity (to a residual activity of about 16.8%) of watermelon juice after 10 d at HS (at variable room temperature), compared to 49.2% of conventional refrigeration (5 ºC), while both polyphenol oxidase and pectin methylesterase showed enzymatic activity reductions similar to 5 ºC (atmospheric pressure).

So, the aim of this work was to study the effect of HS on the main proteolytic enzymes of Atlantic salmon, ~~compared to refrigeration.~~ as a first insight of this preservation method applied on fresh fish. For this, an initial screening during 10 d to evaluate the microbial evolution to define storage time frames within which salmon would still be considered accepted for consumption was carried out. The

pressure/temperature conditions of storage were selected according to previous work: 60 MPa at 10 ºC; 50, 60 and 75 MPa at 25 ºC, and 75 MPa at 37 ºC that revealed reduction/inhibition of spoilage microbial growth during the 10 d of storage time, except for 50 MPa/25 ºC, for which was observed a similar microbial behaviour to refrigeration (atmospheric pressure and 5 ºC). Afterwards, according to the microbial stability, the two best conditions studied in these previous work**,** 60 MPa/10 ºC and 75 MPa/25 ºC, were also evaluated during a longer storage time (50 and 25 d, respectively). The results were compared to control samples stored at atmospheric pressure (AP, 0.1 MPa) at the same storage temperatures (10, 25 and 37 ºC) and under refrigeration (RF, 5 ºC) during the same time. Enzymatic activities (phosphatase acid, cathepsins B and D, and calpains) and myofibrillar fragmentation index and sarcoplasmic protein content were evaluated, as an attempt to correlated proteolytic activity with proteins changes.

2. Materials and methods

2.1. Samples preparation and storage experiments

Farmed Atlantic salmon (*Salmon salar*) was acquired from a local market next to the University (5 min driving distance) before each experiment storage (the fish was caught between 24-48 h). Portions of dorsal muscle (5-10 g) were cut in aseptic conditions, removing the skin, and were packaged in low-oxygen permeable barrier bags (PA/PE-90; Plásticos Macar – Indústria de Plásticos Lda., Palmeira, Portugal). To avoid deterioration, samples were always kept on ice, and storage assays were initiated as soon as possible that all samples were prepared (within maximum of 2 h).

Storage experiments were carried out between January and March 2018 and were divided in two parts: (1) Different combinations of pressure/low temperatures over 10 d

of storage were carried out (HS/LT: 60 MPa at 10 ºC; HS/RT: 50, 60 and 75 MPa at 25 ºC; 75 MPa at 37 ºC); (2) Longer storage conditions at HS/LT (60 MPa/10 ºC) and HS/RT (75 MPa/25 ºC) were further studied, during 50 and 25 d, respectively. Control samples were always kept at the same temperatures and at 5 ºC under atmospheric pressure conditions, in exactly the same conditions (in the dark and immersed in the same fluid used for compression), except for pressure. Samples were evaluated for enzymatic activity (phosphatase acid, cathepsins B and D, and calpains), myofibrillar fragmentation index and sarcoplasmic protein content.

The first set of HS experiments (1) was performed using a 100-mL high-pressure equipment (pressure vessel: 35-mm inner diameter × 100-mm height; High-pressure system U33, Institute of High Pressure Physics, Warsaw, Poland). The second set (2) was carried out using a different high-pressure equipment for HS/LT (60 MPa/10 ºC): 200-mL high-pressure equipment (pressure vessel: 35-mm inner diameter × 250-mm height; SFP FPG13900, Stansted Fluid Power, Stansted, United Kingdom); and for HS/RT (75 MPa/25 ºC): 2-L high-pressure equipment (pressure vessel: 100-mm diameter × 250-mm height; FPG7100, Stansted Fluid Power, Stansted, United Kingdom). Both high-pressure equipment use a mixture of propylene glycol and water (40:60, v/v) as pressurization fluid.

2.2. Enzymatic activity

The enzymatic extract was prepared using ice-cold distilled water as described by **Lakshmanan, Patterson, & Piggott (2005).** The enzymatic extracts were stored at -80 ºC prior to enzymatic activity quantifications.

Acid phosphatase activity was assayed with *p*-nitrophenylphosphatate (*p*-NPP) as substrate following the methodology described by **Fidalgo, Saraiva, Aubourg,**

Vázquez, & Torres, (2014). Cathepsin B and D activities were assayed by the methodology described also by **Fidalgo et al. (2014)**, using Z-Arg-Arg-7-AMC and denatured haemoglobin as substrates, respectively.

Activity of calpains was measured using the method described by **Sasaki, Kikuchi, Yumoto, Yoshimura, & Murachi, (1984)**, with some adaptations. Enzyme extract (50 µL) and substrate solution (50 µL, 0.125 mmol/L L-met-AMC TFA in 100 mmol/L Bis-Tris, 5 mmol/L calcium chloride, pH 6.5) were mixed and incubated at 37 °C for 2 min. The reaction was stopped (1.5 mL; 30 mmol/L monochloroacetic acid, 21 mmol/L acetic acid, and 9 mmol/L sodium acetate, pH 4.3) and fluorescence of liberated AMC was measured (excitation: 360 nm, emission: 460 nm; Hitachi F2000 fluorescence spectrophotometer, Tokyo, Japan).

Three replicates of enzymatic activity were performed for each condition. From these data, residual activity was calculated as:

$$Residual\ activity\ (\%) = \frac{A}{A_0} \times 100$$

where A is the enzymatic activity of the salmon sample after storage and A_0 is the enzymatic activity of the initial sample (0 d).

2.3. Myofibril fragmentation index

Myofibril fragmentation index (MFI) was determined by the method of **Zhang et al. (2013)**. Muscle tissue was pulverized in liquid nitrogen (0.5 g) and homogenized with 25 mmol/L phosphate buffer (30 mL; 0.1 mol/L potassium chloride, 1 mmol/L EDTA acid, pH 7.0) for 1 min (10000 rpm; MICCRA D-9 Homogenizer, MICCRA GmbH, Müllheim, Deutschland). The suspension was filtered to remove connective tissue and the residue was washed with the same phosphate buffer (10 mL). Then, filtrate was centrifuged (1000×g, 15 min, 4 ºC; Heraeus Biofuge Stratos, Thermo,

Electron Corporation, Massachusetts, EUA), the precipitate was resuspended in phosphate buffer (10 mL) and centrifuged again. This step was repeated twice and the pellet was resuspended in buffer solution (10 mL). Protein concentrations were determined and after adjustment to a concentration of 0.5 mg/mL, using the same buffer, absorbance measurements at 540 nm were done (Multiskan Go microplate spectrophotometer, Thermo Scientific, Waltham, EUA). Protein concentrations were determined by the Bradford assay modified by **Zor & Selinger (1996)** and using bovine serum albumin (BSA) as standard (0.1-0.6 mg BSA/mL of phosphate buffer). MFI was calculated by multiplying measurements with 150.

2.4.Sarcoplasmic protein content

Sarcoplasmic protein was extracted according to the method of **Wang, Hang, Luo, & Shen (2013)**. Salmon samples were minced (5 g) and homogenised with cold deionised water (1:5, w/v) for 1 min (10000 rpm; MICCRA D-9 Homogenizer, MICCRA GmbH). The homogenate was kept in at 4 ºC for 30 min to extract sarcoplasmic protein and centrifuged for 20 min (14,000 ×g, 4 ºC; Heraeus Biofuge Stratos, Thermo, Electron Corporation). The supernatant was collected, and the protein concentration determined by the Bradford assay method **(Zor & Selinger, 1996)**.

2.5.Statistical analysis

The results of the first set of experiments (1) of effect of storage condition and storage time were tested with a two-way analysis of variance (ANOVA) and the second set of experiments (2) with a one-way ANOVA, followed both by a multiple comparison test (Tukey HSD) to identify the differences. The significance level was set at 5%.

175

176 ## 3. Results and discussion

177 ### 3.1. Effect of hyperbaric storage on the lysosomal proteases

178 #### 3.1.1. Acid phosphatase

179 **Fig. 1a** shows the acid phosphatase activities in salmon muscle for 10 d of storage
180 time. At AP/5 ºC, residual activity decreased (35%; $p < 0.05$) after 3 d of storage with
181 no further changes ($p > 0.05$) during the 10 d. At AP/10 ºC, a higher residual activity
182 was obtained (~85%) but at AP/25 and 37 ºC, residual activity was lower (22 and 23%,
183 respectively; $p < 0.05$). **Rode & Hovda (2016)** observed that acid phosphate also
184 slightly decreased (about 10%) during 11 d at 0.5 ºC.

185 After 10 d, 60 MPa (10 and 25 ºC) caused an increase of 1.5- and 1.2-fold,
186 respectively; $p < 0.05$) of the initial activity. Contrarily, at 50 MPa/25 ºC and 75
187 MPa/25 and 37 ºC a decrease ($p < 0.05$) of activity was observed, to values similar to
188 AP samples. In these HS samples, the observed residual activity was low (20-34%),
189 being not statistically different ($p > 0.05$) to control samples at AP/5, 25 or 37 ºC. The
190 storage assay during longer times corroborate these results for both storage conditions,
191 60 MPa/10 ºC and 75 MPa/25 ºC **(Table 1)**, being observed an increase and decrease,
192 respectively, of residual activity during storage time (50 and 25 d, respectively).

193 Acid phosphatase can be used as an indicator of lysosome disruption, according to
194 **Ohsumi, Ishikawa, & Kato (1983)**, since 40-60% is bound to lysosomes membranes
195 **(Nilsson & Ekstrand, 1993)**. So, as lysosomes are very sensitive to pressure, during
196 storage time acid phosphatase could be released from the organelles, for example,
197 comparing storage assays at 25 ºC, higher values were observed at 60 MPa, when
198 compared to 50 and 75 MPa. However, at a higher pressure of 75 MPa/25 ºC, a protein
199 denaturation effect caused by pressure and storage temperature could explain the

observed lower residual activities. Differently, at 50 MPa/25 ºC, the observed low residual activity could be related to the higher microbial activity observed in these samples **(Fidalgo et al., 2018)**.

3.1.2. Cathepsin B

Cathepsin B activity in Atlantic salmon muscle was evaluated after 10 d of HS and AP conditions, and the results are shown in the **Fig. 1b**. Cathepsin B activity was not affected by AP/5 ºC after 10 d, which is in agreement with results presented by **Hultmann & Rustad (2004)** and **Duun & Rustad (2008)** also in Atlantic salmon. However, cathepsin B activity decreased when the storage temperature was increased to 25 or 37 ºC (at AP; 42 and 3%, respectively). Compared to AP/5 ºC, HS at 60 MPa/10 ºC caused a reduction of residual activity ($p < 0.05$) after 10 d of storage (48%), being statistically ($p < 0.05$) similar to control samples at AP/10 ºC after 3 d. At 25 ºC, there were no differences on cathepsin B activity between HS and AP samples, except for an increase for 60 MPa after 3 d, but activity was reduced ($p < 0.05$) when compared to fresh and AP/5 ºC samples. Furthermore, at 37 ºC, the activity was reduced to values lower than 6% in all samples (AP and HS) already after 3 d. Temperature storage seemed to clearly affect cathepsin B activity, reducing it significantly at 37 ºC to values below the detection limit. Moreover, pressure storage seemed to have no effect on cathepsin B activity, being observed a reduction of activity similar to the respective control samples at AP. Generally, this reduction can result from the enzyme denaturation caused mainly by the storage time under pressure. When cathepsin B activity was compared to AP/5 ºC, lower values were observed under HS after 10 d, as for instance, residual activities of about 29%, 37% and 35% at 50, 60 and 75 MPa (25 ºC) were obtained when compared to 89% at AP/5 ºC.

For the longer storage assay (**Table 1**) was observed that at 60 MPa/10 ºC residual activities decreased between the 6th and 10th d (but in this case without significant differences; $p > 0.05$), but then increased (~2.2-fold) after 50 d of storage. For 75 MPa/25 ºC was observed that cathepsin B activity also decreased ($p < 0.05$) when compared to fresh fish and AP samples after 25 d of storage (13%).

Cathepsin B is a lysosomal cysteine protease that hydrolyses a wide range of proteins and has an important role in the hydrolysis of tissue proteins **(Barrett & Kirschke, 1981)**. Cathepsin B activity increase might be due to disruption of lysosomes and consequent release of the enzyme, thus favouring contact with substrate **(Chéret et al., 2005)**, being this a possible explanation of the results observed after longer storage time (50 d) at 60 MPa/10 ºC. On the other hand, in the present work, using a high storage pressure/temperature of 75 MPa/25 ºC, an opposite behaviour was obtained, with a strong effect of pressure on the denaturation of this enzyme.

3.1.3. Cathepsin D

Cathepsin D is an aspartic protease and its activity evolution in the conditions studied in this work are shown in **Fig. 1c**. AP/5 ºC showed a significant reduction ($p < 0.05$) of cathepsin D activity only after 10 d of storage (48%). For AP/10 and 25 ºC, similar ($p > 0.05$) residual activities were obtained (71% and 69%, respectively) after 3 d, when compared to fresh fish samples. However, at AP/37 ºC, a lower residual activity (21%) was observed.

Generally, HS seemed to not affect cathepsin D activity during storage, since residual activities were similar to AP/5 ºC after 30 d of storage and the respective control samples at AP. However, at 60 MPa/25 ºC, there was an increase of residual activity during storage, increasing from 50% (6 d) to about 100% (at the dy 6 and 10 d).

At 37 ºC were observed the lowest residual activities (7% and 21%). Cathepsin D is a lysosomal enzyme and the verified activity increase at 60 MPa/25 ºC could be caused by the disruption of the lysosomes and consequent enzyme release **(Chéret et al., 2005)**.

For the storage assay during longer time were also observed no significant effects ($p > 0.05$) of HS on cathepsin D activity (**Table 1**), with only an increase of residual activity ($p < 0.05$) from the 30^{th} to 50^{th} d (from 80% to 130%, respectively) for samples stored at 60 MPa/10 ºC.

3.2.Effect of hyperbaric storage on the cytosolic enzymes

3.2.1. Calpains

Fig. 1d shows calpains (cysteine proteases) activity during the different storage conditions. Under AP/5 ºC conditions, it was verified that the residual activity remained unchanged ($p > 0.05$) after 6 d and decreased (71%; $p < 0.05$) after 10 d. At AP/10 ºC after 3 d, there was a decrease ($p< 0.05$) of 87%. Increasing storage temperature caused the residual activity to progressively decrease to values of about 51% (AP/25) and 2.5% (AP/37 ºC).

Similar to AP samples, HS caused a decrease of the residual activity during storage. At 60 MPa/10 ºC, residual activity was lower (60%, $p < 0.05$) than AP/5 and 10 ºC after 3 d and decreased (9%, $p < 0.05$) after 10 d. A similar effect was observed at 25 ºC immediately after 3 d, with pressure levels of 50, 60 and 75 MPa showing reductions ($p < 0.05$) to residual activities of about 12%, 7% and 31%, respectively, being significant different ($p < 0.05$) to AP/10 ºC (51%). However, at 37 ºC, it was not verified a pressure-effect on calpains activity, since HS samples showed similar values to AP/37 ºC (< 2.5%). Moreover, these samples showed the lowest residual activities,

indicating a significant effect of storage time, regardless the pressure level. **Bessiere, Cottin, Balny, Ducastaing, & Bancel, (1999)** observed that pressure treatments induced a dissociation of the two subunits of calpains (μ- and m-calpains) with this causing activity decrease **(Saido, Sorimachi, & Suzuki, 1994)**. Besides that, it was observed an effect of storage temperature on calpains activity, since lower residual activities were verified for samples stored at 37 ºC.

For longer storage times (**Table 1**) was verified a similar behaviour. However, a possible activity recovery was observed for samples stored at 60 MPa/10 ºC and 75 MPa/25 ºC, showing a slight increase of residual activity from 30th d (2%) and 18th d (8%), respectively, to 50th d (29%) and 25th d (15%), respectively.

3.3.Effect of hyperbaric storage on the proteins muscle

3.3.1. Myofibrillar fragmentation index

Myofibrillar fragmentation index (MFI) results during the different storage conditions are shown in **Fig. 2a**. Storage at AP/5 ºC did not affect MFI of salmon samples during 10 d of storage, as well as at AP/37 ºC after 3 d, since they did not show significant differences ($p > 0.05$) when compared to initial salmon samples. However, at AP/10 and 25 ºC, there was a decrease (34%) or increase (143%), respectively, of MFI values.

After 10 d, HS at 60 MPa/10 ºC showed a decrease of MFI values (45%), being however not significant different ($p > 0.05$) to samples stored at AP/5 ºC. Similar results ($p < 0.05$) were observed for 60 MPa/25 ºC (71%). On the other hand, the major influence on MFI was for 75 MPa. This pressure level clearly caused a pronounced increase of MFI values, increasing immediately 1.5- and 2.7-fold at 75 MPa/25 ºC and 75 MPa/37 ºC, respectively, after 3 d. Moreover, MFI values at 75 MPa increased

progressively until the 10th d (3.2- and 4.3-fold, respectively). Under these conditions, a linear correlation was observed between the MFI values and storage time (75 MPa/25 ºC: % relative MFI = 24 × storage d + 102, r^2 = 0.84; 75 MPa/37 ºC: % relative MFI = 23 × storage d + 210, r^2 = 0.95).

For the storage assay using longer times, similar results were observed (**Table 1**) . There was a decrease for 60 MPa/10 ºC until the 50th d of storage (from 27.11 ± 1.64 to 17.44 ± 0.86, respectively). Contrary, and similar to previously stated, at 75 MPa/25 ºC, MFI values increased 4.8-fold at the 25th d of storage, from 21.35 ± 2.57 to 101.46 ± 1.90, respectively.

Muscle is composed mainly of myofibrillar proteins, which are strongly degraded by proteolysis during fish *postmortem*. MFI could reflect the extent of muscle myofibrillar protein degradation, being an useful indicator of I-band (composed by actin) rupture state and breakage of intermyofibrils linkages **(Volpelli, Failla, Sepulcri, & Piasentier, 2005)**. High MFI values indicate higher damage in the myofibrils proteins (D. Wang et al., 2016), being more significant using a pressure storage of 75 MPa, which was visually confirmed immediately after 3 d of storage, mainly at 37 ºC. According to **Zhou, He, Su, & Huang (2016)**, after a high pressure treatment (200-400 MPa, 2 min), muscle sarcomeres became shorter and the number of suspended particles of myofibrillar extracts increased. Differently, in the present work, lower pressure levels (60-75 MPa) were used and during longer storage time (10 d), but still, the combined effect of 75 MPa and longer storage time was enough to cause damage on myofibrillar proteins.

Otero et al. (2019) stated that HS at low temperature (50 MPa/5 ºC) caused differences on the electrophoretic pattern of the myofibrillar fraction of Atlantic mackerel what could be due to a direct effect of pressure on myofibrillar proteins but

also to a pressure-induced effect on the autolytic capacity of endogenous proteases. In the present work, there was no effect on MFI of AP/5 ºC samples during the 10 d of storage, but a pronounced effect of storage at 25 ºC was verified, mainly at 75 MPa/25 and 37 ºC, with a higher increase of MFI values when compared to AP/25 ºC.

3.3.2. Sarcoplasmic proteins content

The sarcoplasmic proteins content of samples stored at different conditions is shown in the **Fig. 2b**. The solubility of sarcoplasmic proteins of salmon was affected by storage time and temperature. At AP/5 ºC, there was a decrease ($p < 0.05$) after 10 d (35%). The decrease of sarcoplasmic protein during refrigerated storage time at 4 ºC is in accordance with the results obtained by other authors (Aubourg, Piñeiro, Gallardo, & Barros-Velazquez, 2005) using turbot (*Psetta maxima*). Increasing storage temperature at AP, showed no changes ($p > 0.05$) on sarcoplasmic proteins content at AP/10 and 37 ºC, but at AP/25 ºC was obtained a reduction to 70%.

At 60 MPa/10 ºC, there were no significant differences ($p > 0.05$) on sarcoplasmic proteins content, neither at 60 MPa/25 ºC, even though a low value was obtained after 6 d (67%) in the latter samples. However, at 75 MPa/25 ºC, sarcoplasmic proteins decreased ($p < 0.05$) to a similar value to AP/5 ºC (39%) after 10 d. A 75 MPa/37 ºC, the pressure effect was more immediately detected, with a reduction ($p < 0.05$) to 58% after 3 d and maintained not statistically different ($p > 0.05$) during the 10 d (67%), being similar to AP/37 ºC ($p > 0.05$).

For longer storage times (**Table 1**) was observed that at 60 MPa/10 ºC the sarcoplasmic proteins content decreased ($p < 0.05$) progressively after the 15^{th} d, from an initial value of 19.77 ± 2.06 to 11.62 ± 2.23 mg BSA/g fish, reaching a value of 5.65 ± 0.63 mg BSA/g fish (29%) after 30 d. However, at the 50^{th} d, sarcoplasmic proteins

increased ($p < 0.05$) again (3.2-fold) to a similar value to those obtained in the initial fresh fish (18.34 ± 1.80 mg BSA/g fish). At 75 MPa/25 ºC, the longer storage assay for 25 d (**Table 1**) confirmed the behaviour obtained previously during 10 d (**Fig. 2b**), verifying a decrease (54%; $p < 0.05$) of sarcoplasmic proteins content values during storage time (from 14.28 ± 1.46 to 7.75 ± 1.11 mg BSA/g fish).

Sarcoplasmic proteins are mainly composed of enzymes associated with energy-producing metabolism (e.g. glycolysis and citrate cycle) **(Nakagawa, Watabe, & Hashimoto, 1988)**. The effect of high pressure processing on sarcoplasmic proteins was studied by several authors, with results indicating that increasing pressure level caused a decrease of sarcoplasmic proteins content **(Marcos, Kerry, & Mullen, 2010)**. According to **Marcos, Kerry, & Mullen (2010)**, changes in muscle protein solubility could indicate a protein denaturation, being the solubility decrease due to the formation of insoluble protein aggregates that can no longer be extracted. However, the decreased protein solubility observed suggests certain denaturation of sarcoplasmic proteins induced mainly by storage time, since values were not different from the respective control samples at AP (including AP/5 ºC). However, at 60 MPa/10 ºC, no effects ($p < 0.05$) were observed on sarcoplasmic proteins during 30 d of storage, but an increase was verified after 50 d, indicating a storage effect on sarcoplasmic protein extractability.

4. Conclusion

A stronger effect on proteolytic activity and proteins muscle of salmon was observed during HS (RT and LT, during 10 days or 25 and 50 days, respectively), being this more considerable with increasing temperature storage, while at low temperatures/pressure, muscles proteins seemed to be less affected. In this work was also observed that HS caused changes on the activities of several deleterious enzymes,

which are involved in textural deterioration of fish muscle. Thus, these results indicated that HS could be a useful methodology to improve fish preservation. However, additional research is of interest to try correlate the activity of these enzymes and the changes observed in the muscle protein profile.

Conflicts of interest

The authors hereby declare there is no conflict of interests.

Acknowledgments

Thanks are due to the University of Aveiro and FCT/MCT for the financial support for the QOPNA research Unit (FCT UID/QUI/00062/2019) through national founds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement and for supporting the PhD grant of Liliana G. Fidalgo (SFRH/BD/96984/2013).

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Table 1 – Enzymatic activities (acid phosphatase, cathepsin B, cathepsin D, and calpains), myofibrillar fragmentation index, and sarcoplasmic proteins content of Atlantic salmon (*Salmo salar*) stored under hyperbaric storage: 60 MPa/10 ºC and 75 MPa/25 ºC during 50 and 25 days, respectively; and under atmospheric pressure (0.1 MPa) at same temperature (10 and 25 ºC) and refrigeration (4 ºC). Different letters along each column denote significant differences ($p < 0.05$) between storage conditions and days (a-f).

Conditions		**Enzymes activity** [(1)]				**Protein stability**	
		Acid phosphatase	**Cathepsin B**	**Cathepsin D**	**Calpains**	**MFI** [(2)]	**Sarcoplasmic proteins** [(3)]
Fresh fish	*0 days*	100 ^{c}	100 ^{c}	100 ^{ab}	100 ^{a}	27.11 ± 1.64 ^{b}	19.77 ± 2.06 ^{ab}
60 MPa/10 ºC	*6 days*	162.8 ± 11.2 ^{b}	109.8 ± 3.6 ^{bc}	67.6 ± 9.8 ^{b}	27.4 ± 1.1 ^{de}	23.88 ± 0.73 ^{c}	15.63 ± 2.36 ^{bcd}
	15 days	146.5 ± 10.0 ^{b}	94.7 ± 5.4 ^{c}	99.8 ± 17.6 ^{ab}	15.0 ± 1.8 ^{ef}	16.36 ± 0.42 ^{de}	11.62 ± 2.23 ^{d}
	30 days	111.4 ± 4.4 ^{c}	69.6 ± 5.9 ^{d}	80.0 ± 20.6 ^{b}	2.3 ± 0.4 ^{f}	13.93 ± 0.24 ^{e}	5.65 ± 0.63 ^{e}
	50 days	221.1 ± 5.5 ^{a}	154.5 ± 8.8 ^{a}	129.5 ± 14.1 ^{a}	28.9 ± 2.3 ^{d}	17.44 ± 0.86 ^{d}	18.34 ± 1.80 ^{bc}
AP/5 ºC	*6 days*	42.2 ± 1.5 ^{d}	101.4 ± 16.0 ^{c}	77.7 ± 21.2 ^{b}	66.7 ± 0.5 ^{c}	30.37 ± 1.58 ^{a}	24.87 ± 0.82 ^{a}
AP/10 ºC	*6 days*	46.1 ± 3.4 ^{d}	129.6 ± 1.2 ^{b}	70.5 ± 10.5 ^{b}	83.9 ± 11.9 ^{b}	8.28 ± 1.40 ^{f}	14.07 ± 2.65 ^{cd}
Fresh fish	*0 days*	100 ^{a}	100 ^{a}	100	100 ^{b}	21.35 ± 2.57 ^{h}	14.28 ± 1.46 ^{a}
75 MPa/25 ºC	*6 days*	37.0 ± 2.3 ^{bc}	49.9 ± 9.1 ^{b}	99.6 ± 13.7	15.3 ± 1.3 ^{d}	56.37 ± 1.29 ^{d}	9.35 ± 1.51 ^{b}
	10 days	40.0 ± 2.0 ^{b}	6.7 ± 6.0 ^{d}	71.1 ± 13.3	13.6 ± 2.2 ^{d}	69.55 ± 0.39 ^{c}	9.63 ± 1.65 ^{b}
	18 days	28.5 ± 3.3 ^{de}	25.3 ± 2.6 ^{cd}	71.1 ± 7.6	8.4 ± 1.0 ^{e}	85.19 ± 0.51 ^{b}	7.27 ± 0.97 ^{b}
	25 days	22.3 ± 2.9 ^{e}	12.5 ± 3.9 ^{d}	63.8 ± 10.1	14.9 ± 0.4 ^{d}	101.46 ± 1.90 ^{a}	7.75 ± 1.11 ^{b}
AP/5 ºC	*6 days*	42.2 ± 1.5 ^{b}	99.1 ± 15.3 ^{a}	77.9 ± 21.3	66.6 ± 0.5 ^{c}	23.82 ± 1.12 ^{f}	18.03 ± 2.03 ^{a}
AP/25 ºC	*6 days*	32.3 ± 3.2 ^{cd}	42.2 ± 3.0 ^{bc}	69.4 ± 17.5	126.7 ± 2.0 ^{a}	30.61 ± 3.55 ^{e}	10.05 ± 0.74 ^{b}

[(1)] Enzymatic activities are shown as residual activities compared to the initial values;

[(2)] MFI: Myofibrillar fragmentation index;

[(3)] Sarcoplasmic proteins values are shown in mg Bovine Serum Albumin (BSA)/g fish muscle.

Figures

Figure 1. Residual activities (%) of acid phosphatase (a), cathepsin B (b), cathepsin D (c) and calpains (d) of fresh Atlantic salmon (full grey bar) and after 3 d (diagonal listed bars), 6 d (horizontal listed bars) and 10 d (vertical listed bars) of storage under: hyperbaric storage (60 MPa at 10 ºC; 50, 60 and 75 MPa at 25 ºC; 75 MPa at 37 ºC) and under atmospheric pressure (AP, 0.1 MPa) at the same temperature (10, 25 and 37 ºC) and under refrigeration (AP/5 ºC). Different letters denote significant differences ($p < 0.05$) between storage days for each storage condition (a-f) and between storage conditions for each storage day (A-C).

Figure 2. Relatives values of myofibrillar fragmentation index (a) and sarcoplasmic proteins (b) of fresh Atlantic salmon (full grey bar) and after 3 d (diagonal listed bars), 6 d (horizontal listed bars) and 10 d (vertical listed bars) of storage under: hyperbaric storage (60 MPa at 10 ºC; 60 and 75 MPa at 25 ºC; 75 MPa at 37 ºC) and under atmospheric pressure (AP, 0.1 MPa) at the same temperature (10, 25 and 37 ºC) and under refrigeration (AP/5 ºC). Different letters denote significant differences ($p < 0.05$) between storage days for each storage condition (a-d) and between storage conditions for each storage day (A-C).

Figure 1

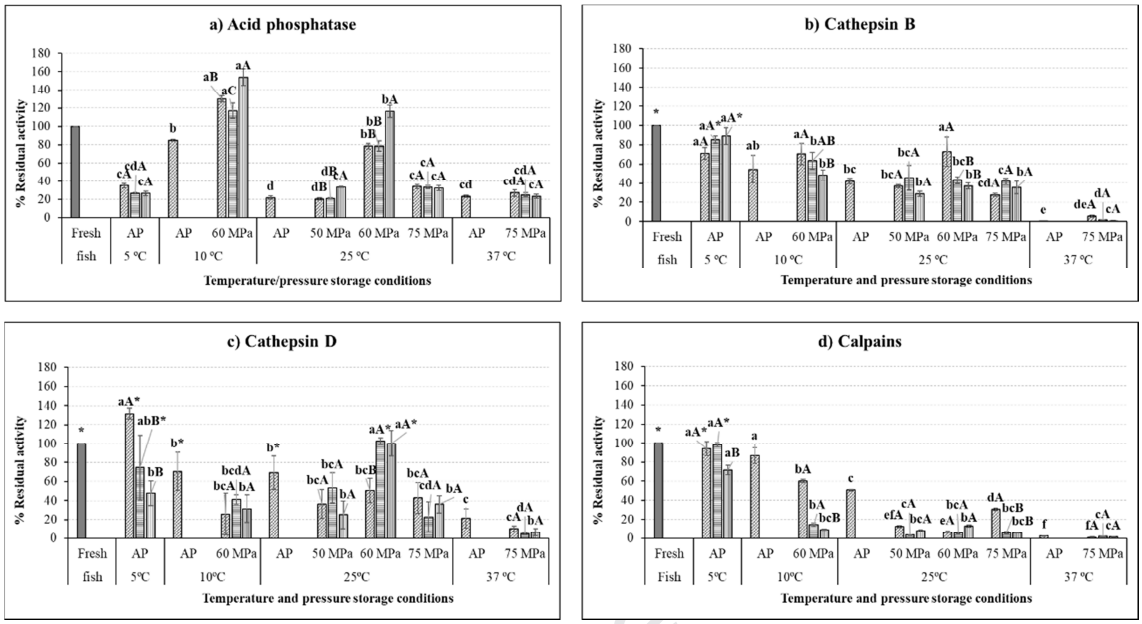
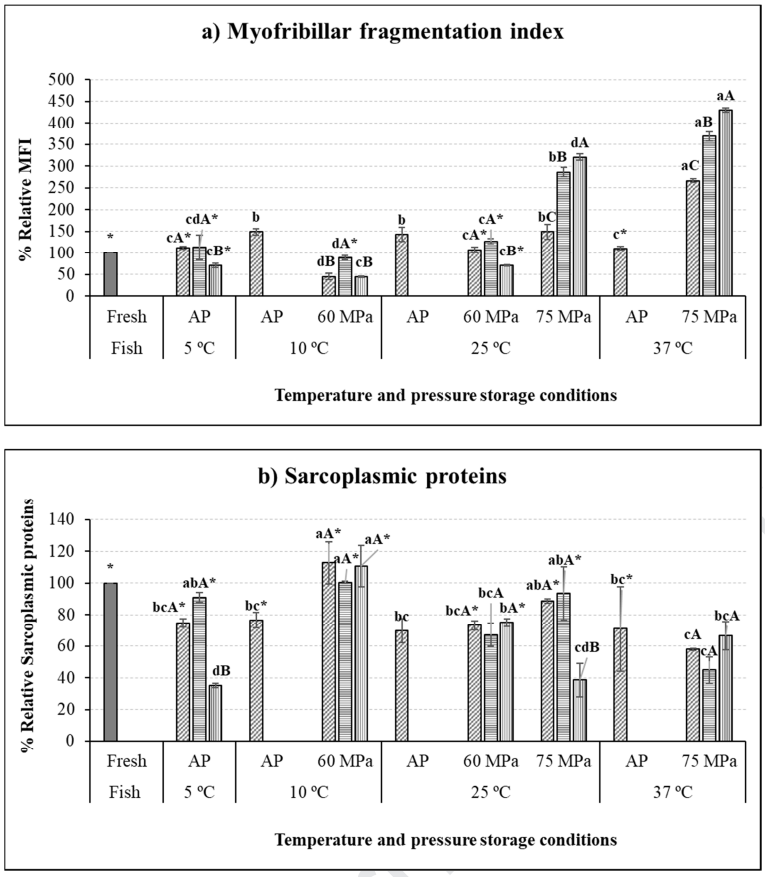


Figure 2


a) Myofribillar fragmentation index
% Relative MFI
500
450
400
350
300
250
200
150
100
50
0
*
cA*
cdA*
cB*
b
dB
dA*
cB
b
cA*
cA*
cB*
bC
bB
dA
c*
aC
aB
aA
Fresh
AP
AP
60 MPa
AP
60 MPa
75 MPa
AP
75 MPa
Fish
5 ºC
10 ºC
25 ºC
37 ºC
Temperature and pressure storage conditions

b) Sarcoplasmic proteins
% Relative Sarcoplasmic proteins
140
120
100
80
60
40
20
0
*
bcA*
abA*
dB
bc*
aA*
aA*
aA*
bc
bcA*
bcA
bA*
abA*
abA*
cdB
bc*
cA
cA
bcA
Fresh
AP
AP
60 MPa
AP
60 MPa
75 MPa
AP
75 MPa
Fish
5 ºC
10 ºC
25 ºC
37 ºC
Temperature and pressure storage conditions

Highlights

- ~~Hyperbaric storage (HS) affected proteolytic activity and muscle proteins;~~
- Proteolytic activity decreased under HS, mainly at higher storage temperature;
- Activity recovery after longer times under HS;
- Atmospheric pressure (AP) storage affected muscle proteins;
- Lowest myofibrillar fragmentation index ~~for salmon samples stored~~ at 60 MPa/10 ºC;
- HS at 60 MPa/10 ºC did not affect sarcoplasmic proteins content.

Conflict of interest

The authors have no conflict of interest to disclose.