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Effect of high pressure pre-treatments on enzymatic activity of Atlantic mackerel (*Scomber scombrus*) during frozen storage

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ABSTRACT

The effect of high pressure processing (HPP) pre-treatments at three pressure levels (150, 300 and 450 MPa) and holding times (0, 2.5 and 5 min) on the activity of key enzymes in Atlantic mackerel (*Scomber scombrus*) muscle was studied after HPP treatments and during frozen storage. Acid phosphatase, cathepsins (B and D) and lipase activity was determined during 3 months of accelerated storage at -10 ºC. An empirical model was applied to determine the effect of pressure level, holding time, and frozen storage time on enzyme activity. Except for a minor decrease at 450 MPa, no substantial effect on acid phosphatase activity was observed. On the other hand, lipase and cathepsins were affected by HPP and by frozen storage time. Increasing the pressure level decreased cathepsin B and lipase activity. Increasing the holding time at 150 MPa increased cathepsin B and lipase activity, while at 300 MPa lipase activity decreased and no effect was observed at 450 MPa. Cathepsin D activity increased at 150 and 300 MPa and decreased at 450 MPa, while increasing with frozen storage time. This work provides novel information on the application of HPP pre-treatments lowering enzyme activity during frozen storage of Atlantic mackerel.

Keywords: High pressure processing, frozen storage, *Scomber scombrus*, enzymatic activity, acid phosphatase, cathepsins B and D, lipase.

1. INTRODUCTION

Small pelagic fish species constitute food products of great economic importance in many European countries (Huidobro, Montero, Tejada, Colmenero, & Borderías, 1990). Although recognized as a healthy food, mackerel (*Scomber scombrus*), a species abundant in the northeast Atlantic, remains underutilized because of its short chilled shelf life (up to 9-10 days) (Sanjuas-Rey, Gallardo, Barros-Velazquez, & Aubourg, 2012). Mackerel and other fresh fish are extremely perishable when compared to other food commodities and thus freezing and frozen storage is most often used for their preservation. However, quality is lost during frozen storage due to texture, flavour and colour deterioration (Matsumoto, 1979). Deterioration of fish during frozen storage depends on many factors including fish species, storage temperature, time and endogenous enzymatic activity. According to (Burgaard & Jørgensen, 2011), frozen storage temperature did not seem to affect cathepsin D activity in trout. However, (Nilsson & Ekstrand, 1995), observed that frozen storage temperature affects lysosomal membrane integrity resulting in increased lysosomal enzymes leakage and thereby increased β-N-acetylglucosaminidase activity in trout samples stored at -18 ºC as compared to -40 ºC. The release of lysosomal lipases in trout decreased during frozen storage (Geromel & Montgomery, 1980). However, lipase activity is the principal cause of hydrolysis and formation of free fatty acids (FFA) during frozen fish storage (Gallardo, Aubourg, & Perezmartin, 1989).

HPP is a non-thermal technique for food preservation that efficiently inactivates vegetative microorganisms while retaining high quality levels (Mújica-Paz, Valdez-Fragoso, Tonello Samson, Welti-Chanes, & Torres, 2011). HPP is applied commercially in the 100-700 MPa range allowing most foods to be preserved with minimal effect on taste, texture or nutritional characteristics (Balasubramaniam, Farkas, & Turek, 2008; Mota, Lopes, Delgadillo, & Saraiva, 2013; Ramirez, Saraiva, Lamela, & Torres, 2009; Yordanov & Angelova, 2010). HPP was shown to inactivate enzymes by disrupting the bonds that determine the secondary, tertiary, and quaternary conformations without affecting the covalent bonds in the primary structure. HPP at 100–300 MPa (up to 30 min), applied on enzyme extracts from bluefish and sheephead, decreased the enzyme activity, especially of cathepsin C, collagenase, chymotrypsin, and trypsin-like enzymes (Ashie & Simpson, 1996). However, pressures up to 500 MPa enhanced the cathepsins B, H, and L activity in sea bass muscle (Cheret, Delbarre-Ladrat, De Lamballerie-

Anton, & Verrez-Bagnis, 2005), and decreased the calpain activity while evolving differently during subsequent refrigerated storage (Cheret, Delbarre-Ladrat, Verrez-Bagnis, & De Lamballerie, 2007; Cheret, Hernandez-Andres, Delbarre-Ladrat, de Lamballerie, & Verrez-Bagnis, 2006). Teixeira et al. (2013) obtained similar results, with higher activity reduction observed at about 400 MPa for acid phosphatase, cathepsin D, and calpain.

Recent previous work demonstrated an inhibition of lipid hydrolysis (Vázquez, Torres, Gallardo, Saraiva, & Aubourg, 2012) and improved functional and sensory properties (Aubourg, Torres, Saraiva, Guerra-Rodríguez, & Vázquez, 2013) in Atlantic mackerel (*Scomber scombrus*) samples subjected to HPP pre-treatments before freezing and subsequent frozen storage. However, there is limited information on the effect of HPP pre-treatments on the activity of endogenous enzymes during frozen storage of fish. The aim of this work was to study the effect of HPP pre-treatments on the activity of several quality degrading enzymes (acid phosphatase, cathepsins B and D, and lipase) during frozen storage of Atlantic mackerel.

2. MATERIALS AND METHODS

2.1. Preparation, processing and storage of sample

Atlantic mackerel (*Scomber scombrus*) caught near the Bask coast in Northern Spain (Ondarroa harbor, Bizkaia, Spain) were transported under refrigeration to the AZTI Tecnalia (Derio, Spain) pilot plant for HPP treatment within 6 hours after catch. Whole Atlantic mackerel (28-33 cm and 230-280 g range) individuals were placed in flexible polyethylene bags (three individuals per bag) and vacuum sealed at 400 mbar.

Whole fish were HPP-treated in a 55 L high pressure unit (WAVE 6000/55HT; NC Hyperbaric, Burgos, Spain) according to the following experimental design: T1 (450 MPa, 0 min), T2 (450 MPa, 2.5 min), T3 (450 MPa, 5 min), T4 (300 MPa, 2.5 min), T5 (300 MPa, 2.5 min), T6 (300 MPa, 2.5 min), T7 (300 MPa, 2.5 min), T8 (300 MPa, 5 min), T9 (150 MPa, 0 min), T10 (150 MPa, 2.5 min), T11 (150 MPa, 2.5 min), and T12 (150 MPa, 5 min). The 0 min holding time samples were carried out to study the effect of just the pressure come-up and depressurizing time. Non-pressure treated samples (T0, untreated controls) were also studied. The pressurizing medium was water applied at 3 MPa/s, yielding come up times of 50, 100, and 150 s for treatments at 150,

300, and 450 MPa, respectively; while decompression time took less than 3 s. Pressurizing water was cooled down to maintain room temperature (20 ºC) conditions during HPP treatment. HPP-treated samples were kept frozen at -20 ºC for 48 h before storage at -10 ºC and sampling after 0, 1, and 3 months. A storage temperature (-10 ºC) higher than that employed commercially (-18 ºC) was chosen to accelerate the effect of storage time.

2.2. Enzymatic activity

2.2.1. Preparation of enzymatic extract

The enzymatic extract was prepared as described by Lakshmanan et al. (2005). Fish samples (10 g) of pooled fish muscle from each of three individuals (control or HPP-treated samples) were homogenized with 50 mL ice cold distilled water for 2 min. The homogenate was kept in ice for 30 min with occasional stirring. After 30 min, it was centrifuged at 4 ºC for 20 min at 14,600×*g*. The supernatant was filtered through a Whatman nº 1 filter and stored at -20 ºC prior to enzymatic activity quantifications.

2.2.2. Acid phosphatase activity

Acid phosphatase activity was assayed with *p*-nitrophenylphosphate (*p*-NPP) as substrate following the methodology described by Ohmori et al. (1992), with minor modifications. Extracts (0.250 mL) and substrate solution (0.225 mL) of 4 mM *p*-NPP in 0.1 mM acetate buffer and 1 mM ethylenediamine tetraacetic acid (EDTA), at pH 5.5, were incubated at 37 ºC for 15 min. The reaction was stopped by adding 1 mL 100 mM potassium hydroxide (KOH) and the *p*-nitrophenol (*p*-NP) released was measured at 400 nm. Acid phosphatase activity was expressed as nmol *p*-NP/min/g of fresh fish. Three replicates were performed for each treatment.

2.2.3. Cathepsins activity

Cathepsin B

Cathepsin B activity was assayed by the methodology described by Lakshmanan et al. (2005). Enzyme extract (0.1 mL) and substrate solution (0.1 mL), containing 0.0625 mM of Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (#C5429, Sigma Aldrich Corp., Steinheim, Germany) in 100 mM Bis-Tris buffer with 20 mM EDTA and 4 mM dithiothreitol, at pH 6.5, were incubated at 37 ºC for 5 min. The reaction was stopped by adding 1 mL 3% sodium dodecyl sulphate (w/v) in 50 mM Bis-Tris (pH 7.0)

and the 7-amino-4-methylcoumarin (AMC) liberated was measured by fluorescence (excitation: 360 nm, emission: 460 nm). Cathepsin B activity was expressed as fluorescence units (FU)/min/g of fresh fish. Three replicates were performed for each treatment.

Cathepsin D

Cathepsin D activity was assayed as described by Buckow et al. (2010) with small modifications. Enzyme extract (0.2 mL) was mixed with 0.6 mL of substrate solution, 2% denatured hemoglobin (w/v) in 200 mM citrate buffer (pH 3.7), and incubated for 3 h at 37 ºC. The reaction was stopped by the addition of 0.6 mL 10% trichloroacetic acid (w/v). After vigorous stirring, the precipitate was removed by centrifugation (18,000×*g* for 15 min) and the soluble peptides measured at 280 nm. Cathepsin D activity was expressed as µg tyrosine/min/g of fresh fish. Three replicates were performed for each treatment.

2.2.4. Lipase

Lipase activity was assayed with olive oil as substrate following procedures recommended by the titrimetric enzymatic assay supplier (Sigma-Aldrich, 1999). Enzyme extract (1 mL) was mixed with the substrate solution (1.50 mL of olive oil, 1.25 mL of water, and 0.50 mL of 200 mM Tris-HCl buffer, pH 7.7) and incubated at 37 ºC for 24 h. The reaction was stopped by adding 2 mL 95% ethanol (v/v) and the liberated free fatty acids (FFA) were titrated against 25 mM sodium hydroxide (NaOH) using thymolphtalein as indicator. Lipase activity was expressed as µmol FFA/min/g of fresh fish. Three replicates were performed for each treatment.

2.3. Statistical analysis

Fish samples corresponding to each treatment were analysed after 0, 1 and 3 months of frozen storage time. The effect of pressure level and holding time were tested with a two-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey HSD) to identify differences between treatments. For each storage time, the differences between control and treated samples were tested with one-way ANOVA followed by Tukey HSD test. The level of significance was established at $p < 0.05$. Subsequent to this analysis, the data were fitted to a model to assess conditions of maximum/minimal enzyme inactivation. For this, statistical experiment design was

formulated using the Design Expert® 7.1.1 software (Stat-Ease, Inc., Minneapolis, MN, USA). The model was validated through a multifactor ANOVA test. The set of experiments follow three-level factorial design for the two factors: pressure level and pressure holding time (Box & Behnken, 1960). Error assessment was based on a replication of the central point (three times – the treatments T4, T5, and T6) and a lateral point (two times – the treatments T10 and T11). Analyses were repeated for each frozen storage time and the complete dataset obtained for each enzyme studied was fitted to the following second order polynomial model as a first approach to experimental data analysis:

$$y^i = b_0^i + b_1^i x_1 + b_2^i x_2 + b_3^i x_3 + b_4^i x_1 x_2 + b_5^i x_1 x_3 + b_6^i x_2 x_3 + b_7^i x_1^2 + b_8^i x_2^2 + b_9^i x_3^2$$

Where x_i $(i = 1 - 3)$ are the code variables for pressure level, pressure holding time, and storage time; y_i $(i = 1 - 4)$ are the dependent variables (acid phosphatase, cathepsins B and D, and lipase); and, $b_0^i \ldots b_9^i$ are regression coefficients estimated from the experimental data by multiple linear regression. This strategy allowed determining the effect of the pressure level, holding time, and frozen storage time on the enzyme activity, and to further assess conditions of maximal/minimal enzymes inactivation.

3. RESULTS AND DISCUSSION

3.1. Acid phosphatase activity

Phosphatases are very important enzymes in the regulation of various metabolic processes that occur by phosphorylation and dephosphorylation (Sparks & Brautigan, 1986). Among other degradative mechanisms, phosphatases are involved in the ATP degradation resulting in different molecules (ADP, AMP, IMP, etc.) lowering the fish freshness K value (Gill, 1992). The initial phosphatase activity (month 0) of untreated frozen Atlantic mackerel muscle (control) was 232.4±2.3 nmol *p*-NP/min/g (Table 1), which is a value similar to those reported in other studies for other fresh fish species (Kuda, Matsumoto, & Yano, 2002; Teixeira, et al., 2013). HPP-treated samples showed significant decreases ($p < 0.01$) of activity after 0 and 5 min treatments at at 450 MPa. However, when compared to frozen controls at the same storage time, the HPP effect was small, even though statistically significant in some cases, particulary at the highest

pressure level studied (450 MPa). When considering the longest time studied (month 3), a slight activity increase ($p < 0.05$) was observed in samples treated at 300 MPa.

Since pressure level, holding time and frozen storage time showed a marked effect on acid phosphatase activity, a multifactor ANOVA analysis was necessary to assess their relative influences. A significant ($p < 0.0001$) model with an F-value of 9.00 was used to confirm the significant effect of each independent variable. Acid phosphatase activity was strongly affected by frozen storage time (F value = 29.02; p-value probability > F was $p \leq 0.0001$), although an important effect of pressure could also be concluded, according to their F-value scores (18.35; p-value probability > F was $p \leq 0.0003$). The correlation value of the model was $r^2 = 0.79$ with adjusted and predicted r^2 values of 0.70 and 0.63, respectively, in addition to a signal/noise ratio of 10.86. All these statistical parameters confirmed that the empirical equation applied could be used to model the effect of HPP pre-treatment and frozen storage on the acid phosphatase activity. The model prediction for the effect of the two variables that exerted the most influence on acid phosphatase activity (frozen storage time and pressure level) is shown in Figure 1.

Approximately 40-60% of acid phosphatase was reported to be bound to lysosomes membranes with low pressures suggested to cause disruption of lysosomes and leakage of the enzyme (Cheret, Delbarre-Ladrat, De Lamballerie-Anton, & Verrez-Bagnis, 2005; Ohmori, Shigehisa, Taji, & Hayashi, 1992). According to Ohmori et al. (1992), pressures of about 200-300 MPa caused an increase of acid phosphatase activity in the cytosolic fraction and a decrease in the lysosomal fraction. The same authors conclude that higher pressure levels caused inactivation of acid phosphatase activity, which is consistent with the results obtained for acid phosphatase activity in the present work. This way, the effect of HPP on acid phosphatase activity varies, from possible increase for lower pressure caused by a release of the enzyme from the lysossomes, to a decrease for higher pressure due to a higher enzyme inactivation effect.

3.2. Cathepsin B activity

Table 2 shows the HPP effect on cathepsin B, a cysteine protease involved in the hydrolysis of myofibrillar protein during the post-mortem storage of fish muscle (Yamashita & Konagaya, 1991). At months 0 and 1 of frozen storage, the activity decreased ($p < 0.05$) with the HPP treatment, with significant differences ($p < 0.05$) at 300 and 450 MPa while increasing ($p < 0.01$) after 1 month of frozen storage of samples

treated at 150 MPa for 5 min. Except at the longest storage time (3 months), no significant differences ($p > 0.05$) were observed when comparing control and samples treated at 450 MPa for 5 min. Increasing the pressure level caused a significant decrease ($p < 0.05$) in cathepsin B activity. However, when the pressure holding time was increased, cathepsin B activity tended to increase ($p < 0.05$) to values similar to the control, being this effect more pronounced after 3 months storage.

The multifactor ANOVA analysis to assess the relative influence of the three variables yielded an F-value of 8.16 implying that the model was significant with a p-value probability > F of 0.0001. The effect of pressure level (F-value = 22.80; p-value probability > F of 0.0001) was higher than the one observed for the frozen storage (F-value = 5.96; p-value probability > F of 0.0224) and pressure holding time (F-value = 5.0311; p-value probability > F of 0.0344). The correlation value of the model was r^2 = 0.75 and the adjusted and predicted r^2 values were 0.66 and 0.46, respectively, while the signal/noise ratio was 10.82. The prediction of the model obtained for the effect of the two variables exerting the most influence on cathepsin B activity (pressure level and frozen storage time) is shown in Figure 2.

Cheret et al. (2005) observed that increasing the pressure level from 0.1 to 500 MPa (5 min), cathepsin B activity in sea bass muscle increased almost 5-fold. However, Teixeira et al. (2013) observed for the same fish that 100 to 450 MPa treatments did not affect cathepsin B activity. In this work on Atlantic mackerel muscle, 450 MPa treatments decreased the cathepsin B activity. During frozen storage, cathepsin B activity increased, possibly due to the low temperature disruption of lysosomes and the consequent release of enzymes (Nilsson & Ekstrand, 1995). It is also possible that enzyme renaturation may have occured during frozen storage.

3.3. Cathepsin D activity

Cathepsin D is an aspartic acid protease considered to be the most important enzyme in the post-mortem degradation of muscle because of its optimum pH and the absence of a specific inhibitor in the muscle (Cheret, Delbarre-Ladrat, De Lamballerie-Anton, & Verrez-Bagnis, 2005). **Error! Reference source not found.** shows the evolution of cathepsin D activity after HPP and frozen storage. The activity increased significantly ($p < 0.05$) in samples treated by HPP as compared to control samples. At month 3, the activity increased ($p < 0.05$) in all treated samples, except for 150 MPa for

5 min. Pressure holding times of 0 and 2.5 min caused an increase of cathepsin D activity when the pressure level applied increased. On the other hand, a 5 min pressure holding time increased the activity significantly ($p < 0.001$) at pressure levels until 300 MPa and decreased it ($p < 0.05$) at 450 MPa with s stronger effect shown at month 1 than in month 0. After 3 months frozen storage, cathepsin D activity increased ($p < 0.05$) slightly when increasing the pressure holding time. At month 0, increase the holding time caused an activity increase ($p < 0.05$) at 150 and 300 MPa, and a decrease ($p < 0.05$) of cathepsin D activity at 450 MPa. After 3 months storage time and at the three pressure levels studied, increasing the pressure holding time caused an activity increase ($p < 0.05$).

A multifactor ANOVA analysis was carried out to take into account the comparative effect of the frozen storage time, pressure and pressure holding time on the cathepsin D activity. The F-value obtained (15.98) implied that the model was significant (p-value probability > F of 0.0001). The analysis of the F-values obtained shows that the effect of the quadratic term of pressure level and pressure level (42.79 and 17.09, respectively) were more important that the effect of the frozen storage time and its quadratic term (4.46 and 26,06, respectively). The correlation value of the model was $r^2 = 0.81$ with adjusted and predicted r^2 values of 0.76 and 0.68, respectively, while the signal/noise ratio was 14.97. An interaction between pressure and frozen storage time was also detected (F-value of 16.25). The model prediction for the effect of the two variables with the higher influence on cathepsin D activity (pressure level and frozen storage time) is shown in Figure 3.

The application of HPP on fish muscle has been shown to have two effects on cathepsin D activity. Up to 300 MPa, activity increases, probably due to a release from the lysosomes. In some reports, this activity decreased progressively with tretaments above 300 MPa, which has been attributed to a progressive higher enzyme inactivation (Cheret, Delbarre-Ladrat, De Lamballerie-Anton, & Verrez-Bagnis, 2005).

3.4. Lipase activity

Lipase enzymes catalyze the hydrolysis of triglycerides to glycerol and free fatty acids (Kuo & Harold, 2005). Lipolysis occurs extensively in post-mortem fish muscle and has been associated with quality deterioration of the frozen tissue (Shewfelt, 1981). Lipase activity in frozen Atlantic mackerel after HPP and during frozen storage is

presented in **Error! Reference source not found.**. After HPP and freezing, lipase activity decreased ($p < 0.01$) when compared to control samples, except for 150 MPa samples treated for 5 min. HPP affected the lipase activity during frozen storage. Pressure level decreased ($p < 0.05$) the enzyme activity for all holding times applied. Increasing the pressure holding time increased ($p < 0.05$) lipase activity in 150 MPa treated samples stored for 3 months. At 300 MPa, the activity decreased ($p < 0.01$) when increasing the holding time. At 450 MPa no significant differences ($p > 0.05$) were observed.

The multifactor ANOVA analysis yielded a low F-value (4.44) implying that the model was not significant (p-value probability > F of 0.0113). Previous works showed a correlation effect of HPP treatments with FFA formation (Vázquez, Torres, Gallardo, Saraiva, & Aubourg, 2012). Increasing the pressure holding time reduced the formation of FFA at 300 and 450 MPa, while at 150 MPa the reduced lipase activity effect was observed only after 3 months storage. In the present work, lipase activity was found to be more affected, being reduced at 300 and 450 MPa, which correlated with the lower FFA formation observed in the work previously reported using the same fish samples in the scope of a collaborative project. It can be concluded that these pressure levels caused beneficial reductions in lipase activity during the storage of frozen fish.

CONCLUSIONS

The application of HPP pre-treatments before freezing and storage at -10 ºC of Atlantic mackerel changed the activity of acid phosphatase and cathepsins (B and D) involved in textural deterioration of fish muscle, indicating that HPP can be a useful pre-treatment for frozen fish preservation. HPP pre-treatments did not affect considerably the activity of acid phosphatase, but the activity of this enzyme decreased during frozen storage. Cathepsin B activity was inactivated by pressure and a slight activity recovery with the frozen storage time was observed. In contrast, cathepsin D activity was activated at intermediate pressure levels and inactivated at high pressure levels. Nevertheless, additional research is required to examine the activity of enzymes studied in frozen Atlantic mackerel kept under commercial frozen conditions (-18 °C).

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Tables

Table 1 – Enzymatic activity of acid phosphatase (nmol *p*-NP/min/g) of Atlantic mackerel muscle. Values are presented as average ± standard deviation. Different letters denote significant differences ($p < 0.05$) among pressure levels (A-C) or among pressure holding times (a-b). The symbol * denotes significant differences with control unteated samples.

Month 0	**Untreated**		232.4 ± 2.3 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	231.5 ± 8.2 abA	247.1 ± 1.9 aA	210.9 ± 5.9 aB *
	2.5	248.6 ± 6.6 aA	241.0 ± 7.6 aAB	224.4 ± 5.3 aB
	5	222.6 ± 0.4 bB	249.5 ± 3.8 aA	208.3 ± 11.8 aB *
Month 1	**Non Processed**		249.2 ± 5.4 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	251.5 ± 15.5 bAB	268.3 ± 10.2 aA	221.2 ± 3.1 aB
	2.5	262.5 ± 26.6 bA	265.8 ± 3.9 aA	218.8 ± 7.8 aB *
	5	296.1 ± 16.8 aA *	247.0 ± 3.2 aB	235.7 ± 3.4 aB
Month 3	**Non Processed**		260.2 ± 9.5 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	244.2 ± 15.0 bC *	285.7 ± 7.1 aA *	254.6 ± 5.4 aB
	2.5	261.0 ± 3.3 aB	282.1 ± 1.0 aA *	235.7 ± 8.5 abC *
	5	265.3 ± 1.4 aA	226.9 ± 4.2 bB *	231.5 ± 4.3 bB *

Table 2 – Enzymatic activity of cathepsin B (x 10^5 FU/min/g) of Atlantic mackerel muscle. Values are presented as average ± standard deviation. Different letters denote significant differences ($p < 0.05$) among pressure levels (A-C) or among pressure holding times (a-c). The symbol * denotes significant differences with control unteated samples.

Month 0	**Untreated**		2.19 ± 0.13 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	1.59 ± 0.22 aA *	1.17 ± 0.14 bB *	1.27 ± 0.09 aAB *
	2.5	1.90 ± 0.02 aA	1.75 ± 0.06 aA *	1.14 ± 0.18 aB *
	5	1.85 ± 0.21 aA	1.95 ± 0.06 aA	1.35 ± 0.15 aB *
Month 1	**Non Processed**		1.93 ± 0.12 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	1.93 ± 0.17 bA	1.23 ± 0.19 aB *	1.37 ± 0.10 aB *
	2.5	1.77 ± 0.11 bA	1.09 ± 0.14 aB *	0.60 ± 0.15 bC *
	5	2.49 ± 0.21 aA *	1.37 ± 0.20 aB *	0.83 ± 0.04 bC *
Month 3	**Non Processed**		2.08 ± 0.14 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	2.01 ± 0.01 aA	1.76 ± 0.02 bB *	1.06 ± 0.06 cC *
	2.5	1.81 ± 0.08 bA *	1.73 ± 0.06 bA *	1.72 ± 0.07 bA *
	5	2.15 ± 0.05 aA	2.23 ± 0.07 aA	2.16 ± 0.09 aA

Table 3 - Enzymatic activity of cathepsin D (µg tyrosine/min/g) of Atlantic mackerel muscle. Values are presented as average ± standard deviation. Different letters denote significant differences ($p < 0.05$) among pressure levels (A-C) or among pressure holding times (a-c). The symbol * denotes significant differences control unteated samples.

Month 0	**Untreated**		2.04 ± 0.14 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	2.37 ± 0.09 aB	2.6831 ± 0.2085 cB *	3.3000 ± 0.1862 aA *
	2.5	1.84 ± 0.03 bB	3.1950 ± 0.1108 bA *	2.7688 ± 0.2713 bA *
	5	2.56 ± 0.19 aB *	4.3274 ± 0.1403 aA *	2.0057 ± 0.1341 cC
Month 1	**Non Processed**		3.78 ± 0.21 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	2.99 ± 0.02 aA	3.75 ± 0.29 cA	3.20 ± 0.46 aA
	2.5	2.98 ± 0.15 aB	4.83 ± 0.28 bA *	3.13 ± 0.45 aB
	5	3.07 ± 0.27 aB	5.93 ± 0.13 aA *	3.07 ± 0.23 aB
Month 3	**Non Processed**		2.55 ± 0.09 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	1.08 ± 0.27 bB *	3.32 ± 0.22 bA *	3.05 ± 0.30 cA
	2.5	1.90 ± 0.21 aC *	3.52 ± 0.08 abB *	4.41 ± 0.10 aA *
	5	2.23 ± 0.23 aB	4.08 ± 0.20 aA *	3.71 ± 0.24 bA *

Table 4 - Enzymatic activity of lipase (x10^5 µmol FFA/min/g) of Atlantic mackerel muscle. Values are presented as average ± standard deviation. Different letters denote significant differences ($p < 0.05$) among pressure levels (A-C) or among pressure holding times (a-c). The symbol * denotes significant differences with control unteated samples.

Month 0	**Untreated**		6.45 ± 0.33 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	3.37 ± 0.33 cB *	4.92 ± 0.00 aA *	2.31 ± 0.58 aB *
	2.5	4.63 ± 0.29 bA *	3.73 ± 0.62 abA *	2.41 ± 0.67 aB *
	5	7.04 ± 0.33 aA	2.51 ± 0.33 bB *	2.22 ± 0.33 aB *
Month 1	**Non Processed**		5.78 ± 0.58 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	2.34 ± 0.26 aB	6.94 ± 0.58 aA	5.97 ± 0.33 aAB
	2.5	1.88 ± 0.01 bB *	3.41 ± 0.49 cA *	4.14 ± 0.67 bA *
	5	4.15 ± 0.33 aA *	5.29 ± 0.33 bA	5.49 ± 0.58 abA
Month 3	**Non Processed**		3.95 ± 0.88 *	
	Holding time (min)	**150 MPa**	**Pressure 300 MPa**	**450 MPa**
	0	3.47 ± 0.58 bB	12.14 ± 0.58 aA *	4.43 ± 0.33 aB
	2.5	8.53 ± 0.58 aA *	4.21 ± 0.68 bC	6.15 ± 0.33 aB *
	5	7.81 ± 1.16 aA *	1.06 ± 0.33 cC *	4.43 ± 0.33 aB

Figure legends

Figure 1 - Model prediction for the effect of frozen storage time (months) and pressure (MPa) on the acid phosphatase activity (nmol *p*-NP/min/g). Pressure holding time was fixed at 2.5 min.

Figure 2 - Model prediction for the effect of frozen storage time (months) and pressure (MPa) on the cathepsin B activity (FU/min/g). Pressure holding time was fixed at 5 min.

Figure 3 - Model prediction for the effect of pressure (MPa) and frozen storage time (months) on the cathepsin D activity (µg tyrosine/min/g). Pressure holding time was fixed at 0 min.

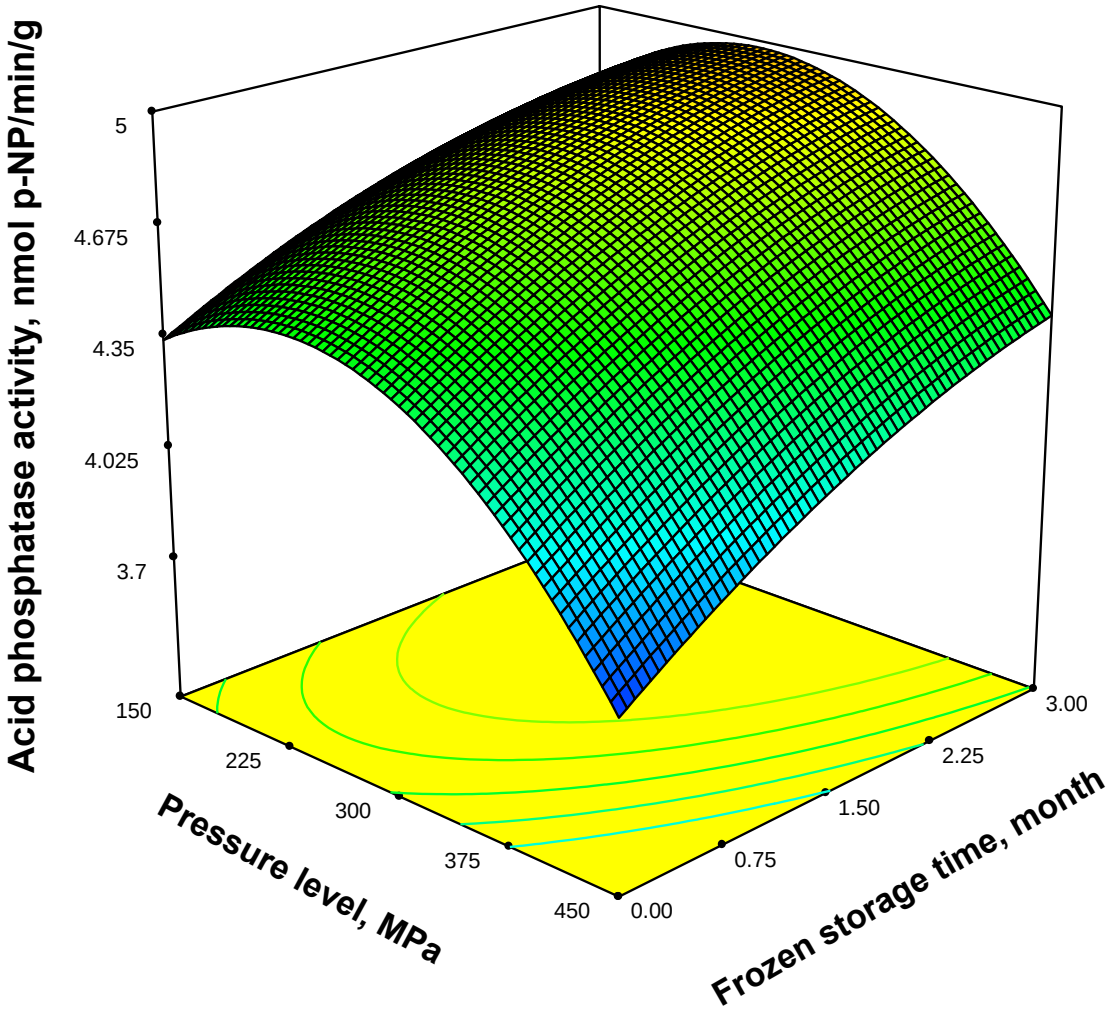
Acid phosphatase activity, nmol p-NP/min/g
5
4.675
4.35
4.025
3.7
150
225
300
375
450
Pressure level, MPa
0.00
0.75
1.50
2.25
3.00
Frozen storage time, month

Figure 1

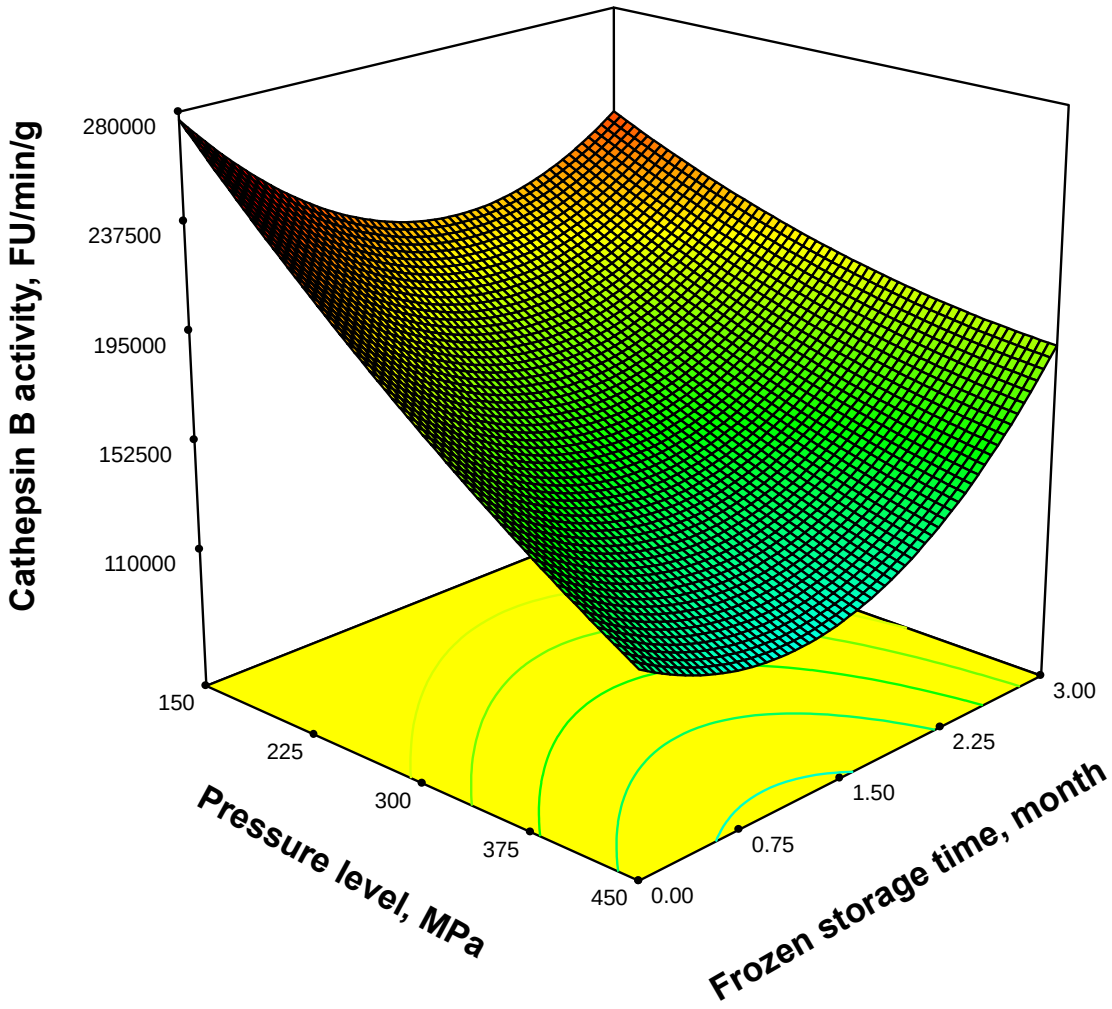
Cathepsin B activity, FU/min/g
280000
237500
195000
152500
110000
150
225
300
375
450
Pressure level, MPa
0.00
0.75
1.50
2.25
3.00
Frozen storage time, month

Figure 2

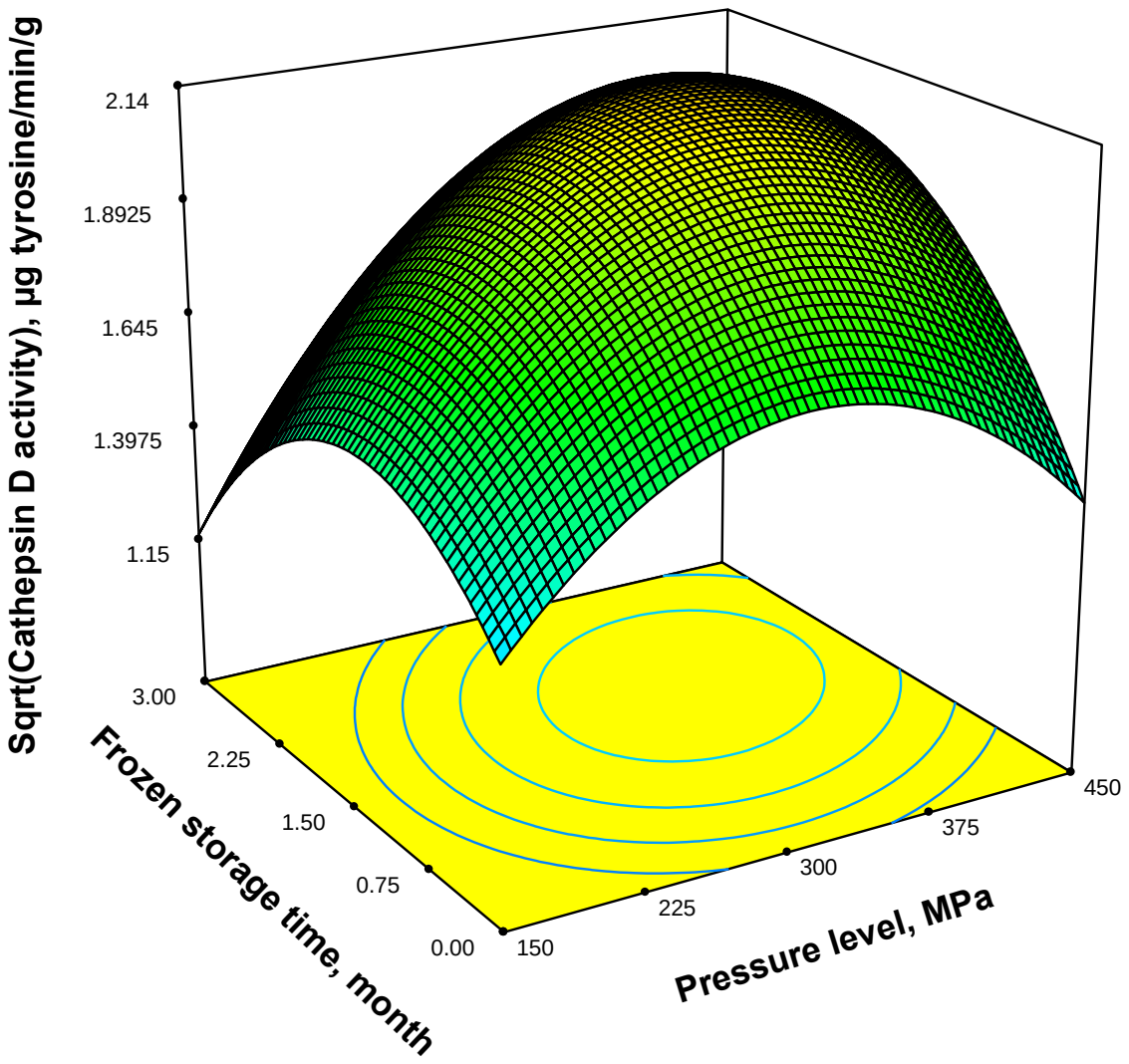
Sqrt(Cathepsin D activity), μg tyrosine/min/g
2.14
1.8925
1.645
1.3975
1.15
3.00
2.25
1.50
0.75
0.00
Frozen storage time, month
150
225
300
375
450
Pressure level, MPa

Figure 3