Enzymatic Activity During Frozen Storage of Atlantic Horse Mackerel (Trachurus trachurus) Pre-treated by High-Pressure Processing

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Abstract
The assessment of enzymatic activity on Atlantic horse mackerel (Trachurus trachurus) during frozen storage was carried out in samples pre-treated by high-pressure processing (HPP) combinations of 150, 300 and 450 MPa with 0-, 2.5- and 5-min holding time (untreated samples were used as controls). The activities of four enzymes (acid phosphatase, cathepsins B and D, and lipase) in fish muscle were quantified during accelerated storage conditions (up to 3 months at -10 °C). The experimental data were fitted to second-order polynomial models to determine the effect of pressure level, holding time and frozen storage time on these enzyme activities and to identify conditions of maximum/minimal enzyme inactivation. Acid phosphatase and cathepsin (B and D) activities were significantly ($p < 0.05$) influenced by HPP, showing behaviours during frozen storage different from control samples. Acid phosphatase and cathepsin B activities de- creased ($p < 0.05$) with HPP treatments, being this effect more intense for cathepsin B, particularly at 450 MPa. Regarding cathepsin D, the activity increased ($p < 0.05$) at intermediate pressure (300 MPa) and decreased ($p < 0.05$) at higher pressure (450 MPa). During frozen storage, cathepsin D enzymatic activity tended to increase over time indicating activity recovery of these enzymes. Although a predictive model for its activity was not acceptable, the increase in lipase activity during storage was the most pronounced trend observed.

Keywords High-pressure processing · Frozen storage · Trachurus trachurus · Acid phosphatase · Cathepsins · Lipase

Introduction
Freezing and frozen storage are widely employed to retain the properties of fish before consumption. However, quality is lost during frozen storage due to texture, flavour and colour deterioration (Matsumoto 1979) caused by several factors including the activity of endogenous enzymes. In fresh fish muscle, lysosomal enzymes, such as cathepsins and acid phosphatase, play an important role in myofibrillar and connective tissue degradation (Chéret et al. 2007; Hultmann et al. 2012; Ladrat et al. 2003). For instance, Yamashita and Konagaya (1991) observed that cathepsin B hydrolyses important myofibrillar proteins, including connetin, nebulin and myosin, causing a drastic degradation of the muscle structure and consequently quality loss. These muscle enzymes could be used as final quality indicators (Toldrá and Flores 2000). During frozen storage, several hydrolytic enzymes are released and can cause fish muscle quality loss. Burgaard and J0rgensen (2011) showed that frozen storage temperature did not seem to affect rainbow trout (Oncorhynchus mykiss) cathepsin D activity. On the other hand, Nilsson and Ekstrand (1995) observed that in the same fish species, frozen storage temper- ature affected the integrity of lysosomal membranes, resulting in an increase of lysosomal enzyme leakage and thus in- creased |3-N-acetylglucosaminidase activity when stored at -18 °C instead of -40 °C.

Lipolysis occurs extensively in fish muscle post-mortem and is associated with quality deterioration in the frozen tissue (Shewfelt 1981). Lipase activity was reported to be the principal cause for the formation of free fatty acids during albacore (Thunnus alalunga) frozen storage (Gallardo et al. 1989) and was reported by Geromel and Montgomery (1980)to be released from the lysosomes during frozen storage of trout (Salmo gairdneri). Also, it has been shown that free fatty acids interact with proteins leading to texture deterioration during frozen storage (Mackie 1993).

High-pressure processing (HPP) has been shown to inactivate microorganism and enzymes extending the shelf-life of many food products, while retaining high levels of quality (Yordanov and Angelova 2010; Múјica-Paz et al. 2011). HPP can inactivate enzymes by disrupting the bonds that determine their secondary, tertiary and quaternary conformations, with- out affecting the covalent bonds in the primary structure. According to Ashie and Simpson (1996), pressures up to 300 MPa decreased cathepsin C, collagenase, chymotrypsin and trypsin-like enzyme activities in extracts from fresh blue- fish (Pomatomus saltatrix) and sheephead (Semicossyphus pulcher). Chéret et al. (2005b) observed that pressures up to 500 MPa increased the cathepsins B, H and L activities in fresh sea bass (Dicentrarchus labrax L.) fillets. However, Teixeira et al. (2013) obtained different results for acid phosphatase, cathepsin D and calpain from the same fresh fish species (sea bass), having observed that activity reduction was maximal at 400 MPa.

Atlantic horse mackerel (Trachurus trachurus) is a medium-fat species abundant in the Atlantic Northeast that has recently drawn a great deal of commercial interest (Aubourg et al. 2004). In a recent previous work, valuable information was obtained concerning HPP application in this fish species to inhibit lipid damage development during sub- sequent frozen storage. HPP pre-treatments (150-450 MPa for 0-5 min) led to a

marked inhibition of lipid hydrolysis on frozen Atlantic horse mackerel (Torres et al. 2013); at the same time, the sensory analysis and the assessment of water holding capacity, colour and texture after cooking revealed no identifiable significant differences between fresh and HPP- treated fish (Torres et al. 2014). Concerning the activity of endogenous enzymes, only few reports on the effect of HPP pre-treatments followed by refrigeration storage have been published, while a published work on the effect of HPP pre- treatments during subsequent frozen fish storage is practically non-existent. The effect of these pre-treatments on enzymatic activities during the frozen storage of another species, Atlantic mackerel (Scomber scombrus), was recently published by Fidalgo et al. (2014). Thus, the aim of this study was to investigate the effect of HPP pre-treatments on the activity of acid phosphatase, cathepsins (B and D) and lipase in frozen Atlantic horse mackerel during 3 months of frozen storage under accelerated storage conditions (-10 °C).

Materials and Methods

Chemicals

Sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), acetic acid, trizma hydrochloride (Tris-HCl), dithiothreitol (DTT), 2-bis-(2-hydroxyethyl)-amino-2-(hydroxymethyl)- 1,3-propanediol (Bis-Tris), ethylenediaminetetraacetic acid (EDTA), p-nitrophenol, thymolphthalein, sodium hydroxide (NaOH), citric acid, trisodium citrate and L-tyrosine were obtained from Sigma-Aldrich (Steinheim, Germany). Other chemicals, such as potassium hydroxide (KOH) and sodium acetate, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain). Substrates used in the determination of enzymatic activity, e.g. p-nitrophenyl phosphate disodium salt hexahydrate (p-NPP, #N22002), Z-arginine-arginine-7-ami- do-4-methylcoumarin hydrochloride (Z-Arg-Arg-7-AMC HCl, #C5429), haemoglobin from bovine blood (#H2625) and olive oil (#O1514), were also purchased from Sigma- Aldrich.

Raw Material, Processing and Storage Conditions

Fresh Atlantic horse mackerel (T. trachurus), caught near the Bask coast in Northern Spain (Ondarroa Harbour, Bizkaia, Spain), were transported under refrigeration to the AZTI Tecnalia (Derio, Spain) pilot plant for HPP treatment. Whole fish individuals (25-30 cm and 200-250-g range) were placed in flexible polyethylene bags (three individuals per bag) and vacuum sealed at 400 mbar.

Whole fish were treated by HPP in a 55-l high-pressure unit (WAVE 6000/55HT; NC Hyperbaric, Burgos, Spain) at 150, 300 and 450 MPa for 0-, 2.5- and 5-min holding times following the experimental design presented in Table 1. Experiments with 0-min pressure holding time were carried

Table 1 Experimental design

Treatment code	Pressure level, holding time
T0	Control
T1	150 MPa, 5 min
T2	150 MPa, 2.5 min
T3	150 MPa, 0 min
T4	150 MPa, 2.5 min
T5	300 MPa, 5 min
T6	300 MPa, 2.5 min
T7	300 MPa, 2.5 min
T8	300 MPa, 2.5 min
T9	300 MPa, 0 min
T10	450 MPa, 5 min
T11	450 MPa, 2.5 min
T12	450 MPa, 0 min

out to study the effect of the pressure come-up and depressurising time. Non-pressure-treated samples (T0, un- treated control) were also studied. The pressurising 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. Pressurising water was cooled down to maintain room temperature (20 °C) conditions during HPP treatment. HPP- treated and control samples were frozen in static air at -20 °C for 48 h before storage at -10 °C for sampling after 0,1and3months.Ahighertemperature(-10 °C) than the one employed commercially for frozen storage (-18 °C) was chosen to accelerate time effects and thus reduce the duration of experiments. After 0, 1 and 3 months, fish was partially thawed to allow skin removal and cutting of the white muscle which was packaged again in polyethylene bags and immediately frozen at -20 °C. Each sample was thawed during 2-4h at 4 °C before preparing each enzymatic extract.

Enzymatic Activity

Preparation of Enzymatic Extract

The preparation of enzymatic extracts was performed follow- ing the methodology used by Lakshmanan et al. (2005). Ten grams of fish samples were homogenised with 50 ml of ice cold distilled water for 2 min (8000 rpm, IKA Ultra-Turrax T25 homogeniser, IKA®-Werke GmbH & Co., Staufen, Germany). The homogenate was kept in ice with occasional stirring. After 30 min, the homogenate was centrifuged at 14,600xg and 4 °C for 20 min (Laboratory Centrifuge 3K30, Sigma, Osterode, Germany). The supernatant was filtered (Whatman n°1) and stored at -20 °C prior to enzymatic activity quantification.

Acid Phosphatase Activity

Acid phosphatase activity was assayed with p-NPP as substrate following with only minor modifications the methodology described by Ohmori et al. (1992). Enzymatic extract (250 (il) was mixed with 4 mM pNPP (225 (il) in 0.1 mM acetate buffer and 1 mM EDTA (pH 5.5). After incubation at 37 °C for 15min, the reaction was stopped by adding 1000 (il of100 mM KOH. The p-NP released was measured at400 nm (LAMBDA 35 UV/Vis spectrometer, PerkinElmer Instruments Inc., MA, USA). Acid phosphatase activity was expressed as nanomole p-NP/minute/gram of muscle fish. Three replicates were performed for each treatment.

Cathepsins B and D Activity

The activity of cathepsin B was assayed by the methodology described by Lakshmanan et al. (2005). Enzyme extract (100 (il) and substrate solution (100 (il) containing 0.0625 mM Z-Arg-Arg-7-AMC HCl in 100 mM Bis-Tris buffer, 20 mM EDTA and 4 mM DTT (pH 6.5) were incubatedat37 °C for5 min. The reaction was stoppedby adding 3 % SDS (w/v, 1000 (il) in 50 mM Bis-Tris (pH 7.0). The free aminomethylcoumarin (AMC) liberated was determined by fluorescence (excitation 360 nm, emission 460 nm; FluoroMax 3 spectrofluorometer, Horiba Scientific, NJ, USA). Cathepsin B activity was expressed as fluorescence units (FU)/minute/gram of muscle fish. Three replicates were performed for each treatment.

Cathepsin D activity assay was based on the procedure described by Buckow et al. (2010) followed with small modifications. The enzyme extract (200 (il) was mixed with the substrate solution (600 (il) containing 2 % denatured haemoglobin (w/v) in 200 mM citrate buffer (pH 3.7). After incubation at 37 °C for3 h, the reaction was stopped byadding 600 (il of 10 % TCA (w/v). After vigorous stirring, the precipitate was removed by centrifugation (18,000xg for 15 min; Elmi Micro Centrifuge CM-50, Porvoo, Finland). Soluble peptides were measured at 280 nm (LAMBDA 35 UV/Vis spectrometer, PerkinElmer Instruments, Inc.). Cathepsin D activity was expressed as microgram tyrosine/ minute/gram of muscle fish. Three replicates were performed for each treatment.

Lipase

Lipase activity was determined following the titrimetric enzymatic assay described by Sigma-Aldrich (1999). The enzymatic reaction consisted of the enzyme extract (1000 (il) and substrate solution, including 1500 (il of olive oil, 1250 (il of distilled water, and 500 (il of 200 mM Tris-HCl buffer (pH 7.7). After incubation at 37 °C for 24 h, the reaction was stopped by adding 2000 (il of 95 % ethanol (v/v). The free fatty acids (FFA) liberated were titrated using 25 mM NaOH and thymolphthalein as indicator. Lipase activity was expressed as micromole FFA/minute/gram of muscle fish. Three replicates were performed for each treatment.

Statistical Analysis

For each treatment, fish samples were analysed after 0, 1 and 3 months of frozen storage time. The effect of pressure level and pressure holding time were tested with a two-way analysis of variance (ANOVA) followed by a multiple comparisons test (Tukey's honestly significant difference, HSD) to identify differences between treatments. At each storage time, the differences between control and HPP samples were tested with one-way ANOVA followed by Tukey's HSD test. The level of significance was established at $p<0.05$. Subsequent to this analysis, the data were fitted to a model to determine

Table 2 Enzymatic activity of acid phosphatase (nmol *p*-NP/min/g) of Atlantic horse mackerel muscle

Frozen storage time (month)	Pressure (MPa)	Control	Pressure holding time (min)		
			0	2.5	5
0	0.1	251.6±3.6			
	150		256.1±4.8 (aA)	*230.8±13.2 (bA)*	*219.9±32.7 (cA)*
	300		*193.3±12.3 (aC)*	*188.2±3.9 (aC)*	*187.2±4.6 (aA)*
	450		*216.8±4.5 (aB)*	*214.1±1.2 (aB)*	*202.7±3.9 (aA)*
1	0.1	195.1±5.5			
	150		*253.9±0.6 (aA)*	*253.9±3.1 (aA)*	*228.7±6.1 (bA)*
	300		*236.9±6.1 (aB)*	*216.1±1.0 (bB)*	*215.2±4.4 (bB)*
	450		*223.2±2.3 (aC)*	*208.1±4.9 (bB)*	189.9±2.5 (cC)
3	0.1	280.7±3.9			
	150		*198.3±2.0 (cC)*	*264.7±0.3 (aA)*	*243.8±2.6 (bA)*
	300		273.2±3.7 (aA)	*240.7±3.1 (bB)*	*217.6±4.0 (cB)*
	450		*219.7±2.3 (aB)*	*225.6±1.9 (aC)*	*220.1±12.0 (aB)*

Values are presented as average± standard deviation with different letters used to denote significant differences ($p<0.05$) between pressure levels (A–C) or holding times (a–c). Values in italic represent significant differences with untreated control samples (0.1 MPa) for each storage month

conditions of maximum/minimal enzyme inactivation. For this, the experiment design was formulated using Design Expert® (Version 7.1.1, Stat-Ease, Inc., MN, USA). The mod- el was validated through a multifactor ANOVA test. The set of experiments followed a three-level factorial design for the two factors: pressure level and holding time (Box and Behnken 1960). Error assessment was based on a triplication of the central point (T6, T7 and T8 treatments) and duplicated lateral point (T2 and T4 treatments) as shown in Table 1. 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:

$$y^i = b^i_o + b^i_1 x_1 + b^i_2 x_2 + b^i_3 x_3 + b^i_4 x_1 x_2 + b^i_5 x_1 x_3 + b^i_6 x_2 x_3 + b^i_7 x_1^2 + b^i_7 x_1^2 + b^i_8 x_2^2 + b^i_9 x_3^2$$

where x_l (l =1-3) are the code variables for pressure level, holding time and storage time; y_l (l =1-4) are the dependent variables (activity of acid phosphatase, cathepsins B and D and lipase); andb'_o... b'_9 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 determining conditions of maximal/minimal enzyme inactivation.

Results and Discussion

Acid Phosphatase Activity

Phosphatases participate in degrading adenosine triphosphate (ATP) in fish muscle yielding adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inosine monophosphate (IMP). Quantification of these nucleotides is used to calculate the fish freshness K value (Gill 1992). At the beginning of frozen storage (month 0), the acid phosphatase activity of unpressurised Atlantic horse mackerel muscle was

Table 3 Analysis of variance (ANOVA) for the enzymatic activity of acid phosphatase (nmol *p*-NP/min/g) of Atlantic horse mackerel muscle

Source	Sum of squares	*df*	Mean square	*F* value	*p* value prob>*F*
	14362.95	3	4787.65	16.55	<0.0001
x_1-Pressure	5971.74	1	5971.74	20.65	<0.0001
x_2-Time	2871.73	1	2871.73	9.93	0.0036
x_3-Frozen storage time	6999.49	1	6999.49	24.20	<0.0001
Residual	8966.95	31	289.26		
Lack of fit	5789.91	22	263.18	0.75	0.7273
Pure error	3177.04	9	353.00		
Cor total	23329.90	34			

Table 4 Enzymatic activity of cathepsin B (×10^5 FU/min/g) of Atlantic horse mackerel muscle

Frozen storage time (month)	Pressure (MPa)	Control	Pressure holding time (min)		
			0	2.5	5
0	0.1	13.30±0.36			
	150		*17.14±0.71 (aA)*	*16.28±.33 (aA)*	*9.88±0.10 (bB)*
	300		*17.22±0.21 (aA)*	13.58±0.23 (bB)	13.26±0.99 (bA)
	450		*9.39±0.27 (aB)*	*5.50±0.09 (bC)*	*5.01±0.47 (bC)*
1	0.1	22.95±0.14			
	150		*18.22±0.66 (bB)*	*17.60±0.26 (bA)*	22.19±0.31 (aA)
	300		22.34±1.38 (aA)	*19.40±0.44 (bA)*	22.22±1.21 (aA)
	450		*8.06±0.23 (aC)*	*5.57±0.17 (bB)*	*6.00±0.18 (bB)*
3	0.1	23.39±1.67			
	150		22.07±0.23 (aA)	*16.21±0.81 (bB)*	*13.98±0.85 (cB)*
	300		23.15±0.21 (aA)	*20.21±0.45 (bA)*	*18.02±0.72 (cA)*
	450		*13.03±0.25 (aB)*	*10.66±0.42 (bC)*	*9.76±0.37 (bC)*

Values are presented as average± standard deviation with different letters used to denote significant differences (p<0.05) between pressure levels (A–C) or holding times (a–c). Values in italic represent significant differences with untreated control samples (0.1 MPa) for each storage month

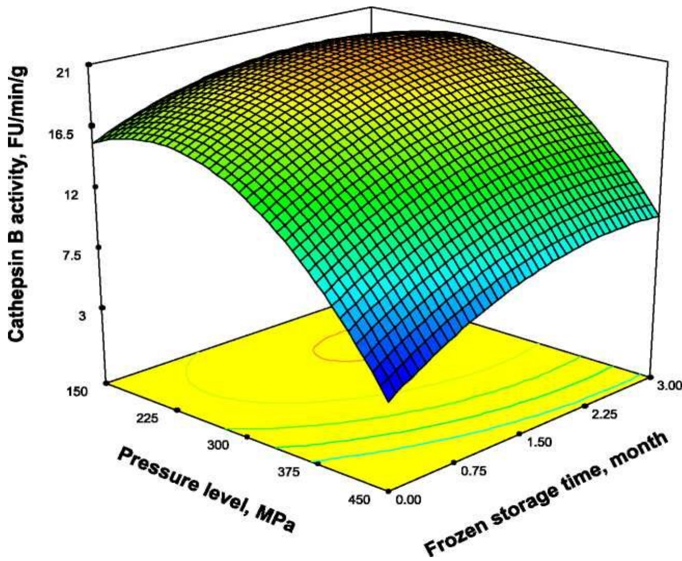
251.6± 3.6 nmol p-NP/min/g (Table 2), a value similar to those obtained for other fresh fish species (Kuda et al. 2002; Teixeira et al. 2013). The activity of untreated samples de- creased after 1 month of frozen storage and increased at month 3. While activity decrease can be attributed to protein denaturation due to freezing as reported by Matsumoto (1979), further activity increase might be related to rupture of lysosomes and release of the enzyme during frozen storage as reported by Nilsson and Ekstrand (1995). At 0-month frozen storage time, the acid phosphatase activity of treated samples remained the same only for the 150-MPa and 0-min holding time condition, while an activity decrease (p <0.05) was observed for HPP pre-treatments at all other pressure levels and holding times. After 1 month, acid phosphatase activity of pressurised samples was higher than in the control samples, except for samples processed at 450 MPa for 5 min. However,
after 3 months, the activity was lower than in the control samples, except for the 300-MPa and 0-min holding time pre-treatment. In general, increasing the pressure level in- creased the acid phosphatase activity reduction independent of the HPP pre-treatment holding time. A similar behaviour has been previously observed by other authors during refrigerated storage. For instance, Ohmori et al. (1992) showed that acid phosphatase was inactivated by increasing pressure up to 500 MPa, while Teixeira et al. (2013) reported similar results in sea bass processed up to 400 MPa, having observed a slight decrease in activity.

A multifactor ANOVA analysis was performed to assess the relative influence of pressure level, holding time and storage time (Table 3). A significant (p < 0.0001) model with an F value of 16.55 was obtained, but the model correlation value was r^2 = 0.62 with adjusted and predicted r^2 values of

Table 5 Analysis of variance (ANOVA) for the enzymatic activity of cathepsin B (×10^5 FU/min/g) of Atlantic horse mackerel muscle

Source	Sum of squares	*df*	Mean square	*F* value	*p* value prob>*F*
	888.47	9	98.72	15.83	<0.0001
x_1-Pressure	486.10	1	486.10	77.93	<0.0001
x_2-Time	15,.2	1	15.02	2,41	0.1332
x_3-Frozen storage time	108.99	1	108.99	17.47	0.0003
x_1–x_2	11.09	1	11.09	1.78	0.1945
x_1–x_3	11.35	1	11.35	1.82	0.1894
x_2–x_3	2.03	1	2.03	0.33	0.5731
x_1^2	262.11	1	262.11	42.02	<0.0001
x_2^2	32.54	1	32.54	5.22	0.0311
x_3^2	25.60	1	25.60	4.10	0.0536
Residual	155.94	25	6.24		
Lack of fit	81.78	16	5.11	0.62	0.8062
Pure error	74.16	9	8.24		
Cor total	1044.41	34			

Fig. 1 Model prediction for the effect of frozen storage time (month) and pressure (MPa) on the cathepsin B activity (*10^5 FU/ min/g) of Atlantic horse mackerel muscle. Pressure holding time was fixed at 2.5 min



0.58 and 0.51, respectively, in addition to a signal/noise ratio of 14.32. This indicates that the proposed model for acid phosphatase activity cannot be used for prediction purposes.

Published research of HPP effects on acid phosphatase activity during frozen fish storage species is scarce. On the other hand, approximately 40-60 % of acid phosphatase has been reported to be bound to lysosome membranes. Low pressure levels have been suggested to cause disruption of lysosomes and leakage of the enzyme, thus increasing the quantifiable acid phosphatase activity (Ohmori et al. 1992; Chéret et al. 2005a), while Ohmori et al. (1992) concluded that higher pressure levels caused its inactivation. These observations are consistent with the results obtained in the present work.

Cathepsins B and D Activity Cathepsin B

Table 4 shows the HPP effect on cathepsin B activity during frozen storage. This enzyme is a cysteine protease involved in the hydrolysis of myofibrillar proteins during the post-mortem storage of fish muscle (Yamashita and Konagaya 1991). The

Table 6 Enzymatic activity of cathepsin D (µg tyrosine/min/g) of Atlantic horse mackerel muscle

Frozen storage time (month)	Pressure (MPa)	Control	Pressure holding time (min)		
			0	2.5	5
0	0.1	3.32±0.26			
	150		*4.54±0.04 (aA)*	3.93±0.36 (aA)	3.50±0.25 (aB)
	300		3.47±0.20 (bA)	*4.80±0.32 (aA)*	*5.40±0.70 (aA)*
	450		3.91±0.42 (aA)	3.90±0.54 (aA)	*4.74±0.25 (aA)*
1	0.1	2.64±0.11			
	150		2.29±0.22 (aB)	3.40±0.36 (aA)	3.06±0.50 (aA)
	300		*5.04±0.61 (aA)*	*4.54±0.42 (aA)*	*5.14±0.64 (aB)*
	450		*4.11±0.41 (aA)*	*4.07±0.21 (aA)*	3.80±0.44 (aA)
3	0.1	0.84±0.15			
	150		1.52±0.25 (aB)	*1.63±0.05 (aB)*	1.55±0.08 (aA)
	300		*3.16±0.09 (aA)*	*2.69±0.20 (aA)*	1.51±0.19 (bA)
	450		0.99±0.14 (bB)	1.42±0.35 (abB)	*2.11±0.61 (aA)*

Values are presented as average± standard deviation with different letters used to denote significant differences ($p<0.05$) between pressure levels (A–C) or holding times (a–c). Values in italic represent significant differences with untreated control samples (0.1 MPa) for each storage month

Table 7 Analysis of variance (ANOVA) for the enzymatic activity of cathepsin D (μg tyrosine/min/g) of Atlantic horse mackerel muscle

Source	Sum of squares	*df*	Mean square	*F* value	*p* value prob>*F*
	40.75	9	4.53	18.85	<0.0001
x_1-Pressure	0.95	1	0.95	3.94	0.0592
x_2-Time	0.01	1	0.01	0.04	0.8487
x_3-Frozen storage time	22.70	1	22.70	94.50	<0.0001
x_1–x_2	0.50	1	0.50	2.08	0.1629
x_1–x_3	0.49	1	0.49	2.05	0.1654
x_2–x_3	2.52	1	2.52	10.50	0.0036
x_1^2	6.40	1	6.40	26.64	<0.0001
x_2^2	0.29	1	0.29	1.21	0.2820
x_3^2	1.72	1	1.72	7.14	0.0136
Residual	5.52	23	0.24		
Lack of fit	3.48	14	0.25	1.10	0.4573
Pure error	2.04	9	0.23		
Cor total	46.28	32			

initial activity observed in untreated Atlantic horse mackerel was 13.30±0.36x10^5 FU/min/g. The activity of the untreated samples increased almost twofold after 1 and 3 months of frozen storage. In general, increasing pressure and holding time caused a higher activity reduction pattern in cathepsin B activity. During storage, pressure-treated samples showed an increased activity ($p<0.05$) suggesting a possible reversible pressure inactivation that was more substantial after 3 months of storage at the highest pressure level studied (450 MPa).

A multifactor ANOVA analysis was completed to assess the relative influence of the three variables, yielding an F value of 15.83 and implying that the model was significant with a p value probability>F of0.0001 (Table 5). The effect of pressure level was higher than the one observed for the frozen storage and holding time. The model also showed an important effect of the quadratic term of pressure level implying a strong pressure level effect on enzyme activity. The correlation value of the model was r^2=0.85 and the adjusted and predicted r^2 values were 0.80 and 0.72, respectively, while the signal/noise ratio was 14.54. The prediction of the model obtained for the effect of the two variables exerting the highest influence on cathepsin B activity (pressure level and frozen storage time) showed that increasing the pressure level caused higher activity decreases, while activity recovery occurred during storage time (Fig. 1). A

Fig. 2 Model prediction for the effect of pressure (MPa) and frozen storage time (month) on the cathepsin D activity (pg tyrosine/min/g) of Atlantic horse mackerel muscle. Pressure holding time was fixed at 2.5 min

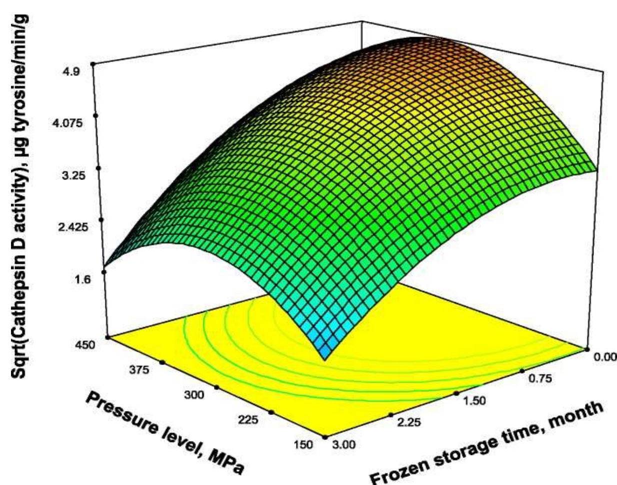


Table 8 Enzymatic activity of lipase ($\times 10^5$ μmol FFA/min/g) of Atlantic horse mackerel muscle

Frozen storage time (month)	Pressure (MPa)	Control	Pressure holding time (min)		
			0	2.5	5
0	0.1	4.63±1.16			
	150		4.05±0.58 (abB)	5.63±0.58 (aA)	3.37±1.46 (bA)
	300		2.79±0.73 (aB)	5.07±0.11 (aA)	4.34±1.00 (aA)
	450		6.36±1.16 (aA)	4.63±0.01 (abA)	3.08±0.33 (bA)
1	0.1	10.51±0.67			
	150		*2.50±0.33 (bC)*	*4.81±0.93 (aB)*	*5.21±1.00 (aB)*
	300		*5.96±0.33 (bB)*	*5.82±0.29 (bAB)*	8.66±0.58 (aA)
	450		*12.53±0.33 (aA)*	*7.52±1.00 (bA)*	*6.84±0.67 (bAB)*
3	0.1	8.66±0.58			
	150		10.30±0.33 (aA)	6.94±0.50 (bB)	*4.82±0.33 (cB)*
	300		7.79±0.01 (bB)	9.91±0.51 (aA)	*5.98±0.33 (bB)*
	450		7.21±1.00 (aB)	8.48±0.88 (aAB)	8.08±1.15 (aA)

Values are presented as average± standard deviation with different letters used to denote significant differences ($p<0.05$) between pressure levels (A–C) or holding times (a–c). Values in italic represent significant differences with untreated control samples (0.1 MPa) for each storage month

similar effect was observed during frozen storage in work with a different fish species, Atlantic mackerel (Fidalgo et al. 2014), in which it was observed that cathepsin B activity decreased with pressure level increments, with activity recovery being observed during frozen storage. In fresh fish, 20-30 % of cathepsin B was inactivated by pressures between 400 and 500 MPa (Ohmori et al. 1992).

Cathepsin D

This aspartic acid protease is considered to be the most important enzyme in the degradation of muscle because there is no specific inhibitor of cathepsin D activity and the pH of post-mortem muscle is in the optimum pH range for its activity (Chéret et al. 2005b). Table 6 shows a gradual decrease of the cathepsin D activity during storage in the untreated control samples, from an initial value of 3.32±0.26 to 0.84±0.15 pg tyrosine/min/g in month 3. Overall, processed samples showed a similar pattern, with higher activity observed in treated samples when compared to controls, indicating that pressure treatments caused an increment of activity. This behaviour can be caused by a release of enzyme from lysosomes as proposed by Chéret et al. (2005b) to explain activity increase in fresh fish for the same enzyme after 300-MPa treatments. In the present study, 300-MPa pre-treatment showed an increase ($p<0.05$) of activity at months 1 and 3. Minor effects were observed when the holding time was increased. The activity increased ($p<0.05$) in samples treated at 300 MPa (month 0) and 450 MPa (month 3) and decreased ($p<0.05$) for a pressure level of300 MPa at month 3.

A multifactor ANOVA analysis was carried out taking into account the comparative effect of the frozen storage time, pressure and holding time on the cathepsin D activity (Table 7). The F value obtained (18.85) implied that the model was significant (p value probability>F of 0.0001). The analysis of the F values obtained shows that the frozen storage time effect (F value=94. 50; $p<0.0001$) was more important than the pressure level effect (F value =3.94; $p<0.0592$) and its quadratic term (F value=26.64; $p<0.0001$). The correlation value of the model was $r^2=0.88$ with adjusted and predicted r^2 values of 0.83 and 0.74, respectively, while the signal/noise ratio was 15.03. An interaction between holding time and frozen storage time was also detected (F value= 10.50; $p<0.0036$). The model prediction for the two variables effect with the higher influence on cathepsin D activity (pressure level and frozen storage time) is shown in Fig. 2.

Table 9 Analysis of variance (ANOVA) for the enzymatic activity of lipase ($\times 10^5$ μmol FFA/min/g) of Atlantic horse mackerel muscle

Source	Sum of squares	*df*	Mean square	*F* value	*p* value prob>*F*
	81.37	3	27.12	10.04	< 0.0001
x_1-Pressure	5.69	1	5.69	2.11	0.1565
x_2-Time	1.20	1	1.20	0.45	0.5096
x_3-Frozen storage time	74.07	1	74.07	27.43	< 0.0001
Residual	83.71	31	2.70		
Lack of fit	63.93	22	2.91	1.32	0.3442
Pure error	19.79	9	2.20		
Cor total	165.08	34			

Previous research concerning the effect of HPP treatment on cathepsin D activity in frozen fish and during frozen storage is limited, but in a recent published work, Fidalgo et al. (2014) presented results similar to those reported in the present work, namely that an intermediate-level pressure (300 MPa) caused an increase of cathepsin D activity during frozen storage of Atlantic mackerel. For fresh fish, the few publications available on the effect of HPP on fish during chilled storage similar results were observed. For instance, Chéret et al. (2005b) observed that cathepsin D activity in- creased at 300 MPa in fresh sea bass, which was attributed to a release of enzymes from lysosomes.

Lipase Activity

The hydrolysis of triglycerides to glycerol and FFA is catalysed by the action of lipases (Kuo and Harold 2005). During post-mortem, lipolysis occurs extensively in fish muscle, and this has been associated with quality deterioration of the frozen tissue (Shewfelt 1981). Table 8 shows the effect of the HPP pre-treatment on frozen horse mackerel and during frozen storage. The lipase activity in untreated control samples was 4.63±1.16x 10^5 |imol FFA/min/g inmonth 0. This activity increased after 1 and 3 months of frozen storage by about 2.5 and twofold, respectively. No significant differences ($p>0.05$) were observed between untreated and HPP samples for month 0. A different behaviour was observed after 1 month of frozen storage when the lipase activity decreased ($p<0.05$) at 150 MPa (all holding times), 300 MPa (0 and 2.5 min) and 450 MPa (2.5 and 5 min) and increased ($p<0.05$) at 450 MPa (0 min). After 3 months, enzyme activity changes were observed only for samples treated for 5 min at 150 and 300 MPa. After 0 and 1 months, 300- and 450-MPa treatments caused a progressive activity increase ($p<0.05$), being more significant for 0 and 2.5-min holding time samples. In general, the main effect observed was lipase activity recovery with storage time. Regarding pressure level effect, globally, a significantly activity increase ($p<0.05$) was observed with increasing pressure level, being main exceptions to this behaviour observed occurred after 1 (2.5 and 5 min) and 3 months of storage (0 min). Concerning holding time, different effects were observed, depending of the frozen storage month studied. For instance, after 0 month, the activity decreased ($p<0.05$) with the increment of holding time for 150 and 450 MPa and showed no changes ($p>0.05$) for 300 MPa; however, at the last storage time (month 3), the effect of holding time was distinct, de- creasing ($p<0.05$) at 150 and 300 MPa and no change ($p<0.05$) at 450 MPa, with the increment of holding time.

The multifactor ANOVA analysis (Table 9) yielded a low F value (10.04) implying that the model was significant (p value probability y>F of 0.0001), but the correlation value of the model was $r^2=0.49$ with adjusted and predicted r^2 values of 0.44 and 0.35, respectively, while the signal/noise ratio was 9.12. This implies that the model cannot be used for prediction purposes for lipase activity. Previous work showed an inhibitory effect of FFA formation in frozen fish as a result of aprevious HPP treatment (Torres et al. 2013). These authors also observed that in untreated control samples, the FFA content increased progressively during frozen storage, which correlates well with the lipase activity increase observed in the present study for the untreated samples.

Conclusions

Increasing the pressure level resulted in a progressively higher decrease of acid phosphatase and cathepsin B activities during
frozen storage, reaching 20 and 60 %, respectively, when
compared to their initial activity. For cathepsin D, an increase of its activity with pressure was observed, particularly at 300 MPa, being associated to a possible release of the en- zymes from lysosomes. For lipase, the main observation was that the activity increased with storage time. The effect of holding time on the enzymes studied was significantly lower than pressure level and frozen storage effect. Although, the findings here presented can lead to improvements of frozen fish quality, additional research is needed to examine the activity of the same enzymes in frozen horse mackerel kept at the frozen storage temperature used commercially (-18 °C).

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