1   **Changes on enzymatic activity and on**
2   **sarcoplasmic and myofibrillar proteins during**
3   **frozen storage of hake (*Merluccius merluccius*)**
4   **pre-treated by high-pressure**

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ABSTRACT

The effect of high pressure processing on enzymatic activity and muscle proteins of European hake (*Merluccius merluccius*) was evaluated in an accelerated frozen storage study (−10 °C for 5 months). A central composite design with five pressure levels (range 150–450 MPa) and frozen storage time (range 0–5 months) was used, were tested.

Results showed that increasing the pressure level and frozen storage time causes an increase of acid phosphatase activity. Otherwise, increasing these two variables resulted in a decrease of cathepsin B and calpains activities. Furthermore, frozen storage and high-pressure pre-treatment did not affect significantly the cathepsin D activity, verifying causing only a slight increase using lower pressure levels caused during storage. Concerning sarcoplasmic and myofibrillar proteins, a definite effect of pressure on myofibrillar proteins content could not be implied. However, sarcoplasmic content was highly affected by the pressure level and at the same time, an important effect of the frozen storage time could be observed, resulting in a slightly decrease of sarcoplasmic proteins content using higher pressure levels (430.7 and 450 MPa).

This work allowed to verify that the application of high pressure processing as a pre-treatment before frozen storage could be used to lowering the enzymatic activity of frozen European hake and then reduce the deleterious effect of enzymatic activity during frozen storage.

Keywords: High-pressure processing, frozen storage, *Merluccius merluccius*, acid phosphatase, cathepsins B and D, calpains.

Highlights:

- High-pressure processing (HPP) pre-treatments caused changes on enzyme activities during frozen storage of European hake;
- Acid phosphatase activity increased with HPP pre-treatment and during frozen storage;
- HPP (≥ 300 MPa) caused a decrease of cathepsin B and calpains activities and sarcoplasmic proteins content;
- No effect of pressure and frozen storage on cathepsin D activity was observed.

1. Introduction

European hake (*Merluccius merluccius*) is an abundant Gadiform species of great market value because of its excellent organoleptic features. Although freezing and frozen storage retain the properties of fish before consumption, the quality is lost during frozen storage due to texture, flavour and colour deterioration (Matsumoto, 1979)⁽¹⁾; being this deterioration can be caused by endogenous enzymatic activity. Burgaard & Jørgensen (2011) showed that frozen storage temperature of trout did not seem to affect cathepsin D activity. On the other hand, Nilsson & Ekstrand (1995) observed that in the same fish species, there is an increase in β -N-acetylglucosaminidase activity, which is a result of the increased lysosomal enzymes leakage due to the effect of frozen storage temperature on the lysosomal membrane integrity. Additionally, Geromel & Montgomery (1980) noted that the release of lysosomal lipases in trout decreased during frozen storage, being the hydrolysis, and formation of free fatty acids during frozen storage, caused by lipase activity (Gallardo, Aubourg, & Perez-Martin, 1989)⁽⁵⁾.

High pressure (HP) technology is a non-thermal technique for food preservation that efficiently inactivates vegetative microorganisms and endogenous enzymes while retaining high quality levels (Mújica-Paz, Valdez-Fragoso, Samson, Welti-Chanes, & Torres, 2011)⁽⁶⁾. This technology 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. The enzymatic activity can be inhibited by HP to a certain extent depending on the enzyme type, the treatment condition and fish species (Chéret, Delbarre-Ladrat, de Lamballerie-Anton, & Verrez-Bagnis, 2005; Chéret, Delbarre-Ladrat, Verrez-Bagnis, & de Lamballerie, 2007; Teixeira et al., 2013, 2014)⁽⁷⁻¹⁰⁾. In addition, the inhibition of proteases during the refrigerated storage time could contribute to the delay of fish texture degradation. However, the effect of HP pre-treatments on enzymatic activities during the frozen storage was recently published by several authors using Atlantic mackerel (*Scomber scombrus*) (Fidalgo, Saraiva, Aubourg, Vázquez, & Torres, 2014a; Pazos et al., 2015)^(11,12), horse mackerel (*Trachurus trachurus*) (Fidalgo, Saraiva, Aubourg, Vázquez, & Torres, 2014b)⁽¹³⁾, sardine (*Sardina pilchardus*) (Méndez et al., 2016)⁽¹⁴⁾ and barramundi (*Lates calcarifer*) (Truong, Buckow, Nguyen, & Stathopoulos, 2016)⁽¹⁵⁾. Nevertheless, research concerning HP pre-treatment of European hake before freezing step and frozen storage is inexistent. Therefore, the aim of this work was to study the

effect of HP pre-treatments (from 150 to 450 MPa for 2 minutes) on the activity of several quality degrading enzymes (acid phosphatase, cathepsins B and D, and calpains) and muscle proteins (sarcoplasmic and myofibrillar) during 5 months of ~~accelarated~~accelerated frozen storage at -10 °C of European hake, to reduce the time needed to study the effect of storage:

2. Materials and methods

2.1. Samples preparation, storage and sampling

European hake (102 individuals) were caught near the Galician Atlantic coast (North-Western Spain) and transported under ice to the laboratory. Whole hake individuals were placed in flexible polyethylene bags (three individuals per bag) and vacuum sealed at 400 mbar. The length and weight of the specimens ranged 27.5-29.5 cm and 180-205 g, respectively.

HP treatments at 150, 169.27, 300, 430.73 and 450 MPa were performed in a 55-L high pressure unit (WAVE 6000/55HT; NC Hyperbaric, Burgos, Spain) according to the experiment design that will be described in the **Table 1** Error: No se encuentra la fuente de referencia; in all cases, a pressure holding time of 2 min was employed. Water applied as the pressurising medium at 3 MPa s⁻¹ yielded 50, 56.42, 100, 143.58 and 150 s as the come-up times for the 150, 169.27, 300, 430.73 and 450 MPa treatments, respectively, while decompression time was less than 3 s. Cold pressurising water was used to maintain temperature conditions during HHP treatment at room temperature (20 °C).

After HP treatments, hake individuals were kept at -20 °C for 48 hours and then stored at -10 °C with samples analysed after 0.00, 0.32, 2.50, 4.68 and 5.00 months according to the following design of pressure-level/frozen-storage-time treatments described in the **Table 1** Error: No se encuentra la fuente de referencia-

Three replicates (n=3) for each treatment (T-0 to T-16) were analysed independently. Each analysis was based on the corresponding extract from the fish white muscle pooled from two individual fish. A frozen storage temperature (-10 °C) higher than commercial practice (-18 °C) was chosen as an accelerated test condition to estimate the extent of the pressure level and frozen storage effects on the chemical quality indices of frozen hake.

2.2. Enzymatic activity determination

2.2.1. Preparation of enzymatic extract

The enzymatic extract was prepared as described by (Lakshmanan, Patterson, & Piggott, (2005)(Lakshmanan, Patterson, & Piggott, (2005). Fish samples (10 g) of pooled fish muscle from each of three individuals (control or HP-treated samples) were homogenized with 50 mL ice cold distilled water for 2 min, using an IKA Ultra-Turrax T25 homogenizer (2 min, 8000 rpm; Janke & Kunkel, Deutschland, Germany). The homogenate was kept in ice for 30 min with occasional stirring. After 30 min, it was centrifuged at 4 °C for 20 min at 14,600×g (Laboratory Centrifuge 3K30, Sigma, Osterode, Germany). The supernatant was filtered through a Whatman n° 1 filter and stored at -80 °C 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, Shigehisa, Taji, & Hayashi, (1992) (Ohmori et al., 1992), with minor modifications. The reaction solution was composed by 0.250 mL of extracts and 0.225 mL of substrate solution, containing 4 mM *p*-NPP in 0.1 mM acetate buffer (pH 5.5) and 1 mM ethylenediaminetetraacetic acid (EDTA), being followed with incubation taking place at 37 °C for 15 min. The reaction was stopped by adding 1 mL of 100 mM potassium hydroxide and the *p*-nitrophenol (*p*-NP) released was measured at 400 nm (Multiskan Go microplate spectrophotometer, Thermo Scientific, Waltham, EUA). Acid phosphatase activity was expressed as nmol *p*-NP per minute per gram of muscle. Three replicates were performed for each treatment.

2.2.3. Cathepsins activityies

Cathepsin B activity was assayed by the methodology described by (Lakshmanan et al., (2005). A mixture of 0.1 mL of enzyme extract and 0.1 mL of substrate solution, containing 0.0625 mM of Z-arginine-arginine-7-amido-4-methylcoumarin hydrochloride (Z-Arg-Arg-7-AMC HCl, #C5429) in 100 mM Bis-Tris buffer (pH 6.5) with 20 mM EDTA and 4 mM dithiothreitol, was incubated at 37 °C for 5 min. The reaction was stopped by adding 1 mL 3% sodium dodecyl sulfate (w/v) in 50 mM Bis-Tris (pH 7.0) and the AMC liberated was measured by fluorescence (excitation: 360 nm, emission: 460 nm; Hitachi F2000 fluorescence spectrophotometer, Tokyo, Japan).

Cathepsin B activity was expressed as fluorescence units (FU) variation per minute per gram of muscle. Three replicates were performed for each treatment.

Cathepsin D activity was assayed as described by (Buckow, Truong, & Versteeg, (2010), with some adaptations. A volume of 0.2 mL of enzyme extract was mixed with 0.6 mL of substrate solution (2% denatured haemoglobin, w/v, in 200 mM citrate buffer, pH 3.7), and incubated for 3 h at 37 °C. The reaction was stopped by the addition of 0.6 mL of 10% trichloroacetic acid (w/v). After vigorous stirring, the precipitate was removed by centrifugation (18,000×g for 15 min; Centrifuge 3 K30, Sigma, Osterode, Germany) and the soluble peptides measured at 280 nm (Multiskan Go microplate spectrophotometer, Thermo Scientific, Waltham, EUA). Cathepsin D activity was expressed as µg tyrosine per minute per gram of muscle. Three replicates were performed for each treatment.

2.2.4. Calpains activity

Activity of calpains was measured using the method of (Sasaki, Kikuchi, Yumoto, Yoshimura, & Muraichi, (1984)(Sasaki, Kikuchi, Yumoto, Yoshimura, & Muraichi, (1984). Enzyme extract (0.05 mL) and substrate solution (0.05 mL, 0.125 mmol/L L-methionine-7-amido-4-methylcoumarin trifluoroacetic salt in 100 mmol/L Bis-Tris, 5 mmol/L calcium chloride, pH 6.5) were mixed and incubated at 37 °C during 2 min. The reaction was stopped by adding a solution composed by 30 mmol/L monoethanoic acid, 21 mmol/L acetic acid, and 9 mmol/L sodium acetate (1.5 mL, pH 4.3). The fluorescence of AMC liberated was measured (Ex = 360 nm; Em = 460 nm) using a spectrofluorometer (Hitachi F2000 fluorescence spectrophotometer, Tokyo, Japan). Calpains activity was expressed as FU variation per minute per gram of muscle. Three replicates were performed for each treatment.

2.3. Extraction and quantification of sarcoplasmic and myofibrillar proteins

Sarcoplasmic (low-salt-soluble) and myofibril (high-salt-soluble) proteins extraction from the sardine hake muscle was performed by modification of a previously published protocol (Pazos, da Rocha, Roepstorff, & Rogowska-Wrzesinska, 2011) (Pazos et al. 2011). Briefly, 0.5 g of fish muscle were homogenized using an Ultra-Turrax high-performance disperser in ten volumes of Tris buffer (10 mM Tris-HCl, pH 7.2), containing 5 mM protease inhibitor (phenylmethylsulphonyl fluoride, PMFS). The sarcoplasmic protein fraction was obtained from the supernatant after centrifugation of

the homogenate at 40,000×g (4 °C, 20 min). The pellet was then homogenized with a saline solution (0.6 M sodium chloride, 10 mM Tris buffer, 5 mM protease inhibitor phenylmethylsulphonyl fluoride – PMFS, pH 7.2), and myofibril proteins were isolated from the supernatant after centrifugation at 4,500×g (4 °C, 20 min). Sarcoplasmic and myofibril proteins were stored at –80 °C until use. Protein concentrations were determined in both fractions by bicinchoninic acid assay (Smith et al., 1985)(Smith et al. 1985). Contents were calculated as g/100g muscle.

2.4. Statistical analysis

The effect of pressure level was tested with a one-way analysis of variance (ANOVA) followed by a multiple comparisons test (Tukey's honestly significant difference, HSD) to identify differences between treatments. The level of significance was established at $p < 0.05$.

The experimental design was statistically analyzed using the Design Expert® 7.1.1 (Stat-Ease, Inc., MN, USA). The set of experiments followed a central composite design (CCD). This design has three groups of points: a) two-level factorial design points; b) axial points; c) centre points. This design has 5 levels of the independent variables with desirable statistical properties. In this case, to study the range 150–450 MPa, the design gives the following levels: 150, 169.27, 300, 430.73 and 450 MPa. The models used, and the statistical approach are described previously (Pita-Calvo, Guerra-Rodríguez, Saraiva, Aubourg, & Vázquez, 2018) (Pita-Calvo, Guerra-Rodríguez, Saraiva, Aubourg, & Vázquez, 2017).

3. Results and discussion

3.1. Enzymatic activity

3.1.1. Acid phosphatase activity

Acid phosphatase was used as an indicator of the level of lysosome disruption according to (Ohsumi, Ishikawa, & Kato, (1983)Ohsumi, Ishikawa, & Kato, (1983). Error: No se encuentra la fuente de referencia shows the evolution of acid phosphatase activities after the different pressure conditions and during frozen storage. The acid phosphatase activity on fresh fish was $2.64 \pm 0.56 \mu\text{mol p-NP/min/g}$. The activity of this enzyme was affected by the high-pressure pre-treatment and the frozen storage time. Storage for 24–48 hours at –20 °C (T12) led to a decrease of the activity in approximately 60% and statistically unchanged ($p < 0.05$) after pre-treatment at 300

MPa. Decrease of the acid phosphatase activity can be attributed to protein denaturation due to freezing, as reported by (Matsumoto, 1979)Matsumoto, (1979). In contrast, on frozen samples untreated by high pressure, storage caused a decrease of the activity when compared to the fresh one, obtaining with a reduction in about 85% after 4.68 months (T15), but when compared to the untreated control at the month 0 (T12), this activity reduction was about 55%. In the case of high pressure-treated samples, lower values of acid phosphatase activity were also obtained at the end of frozen storage using pressure levels of 169.27 MPa (T3) and 300.00 MPa (T7), showing a reduction of activity ($p < 0.05$) on of approximately 85% and 55%, compared to enzymatic activity of fresh fish and frozen control (T12), respectively.

A multifactor ANOVA analysis was completed to assess the relative influence of the two variables (pressure level and storage time), yielding an F value of 10.59 and implying that the model was significant with a p-value probability $> F$ value of 0.011. The effect of frozen storage time was higher than the one observed for pressure level. The model also showed an effect of the quadratic term of frozen storage. The correlation value of the model was $r^2 = 0.91$ and the adjusted and predicted r^2 values were 0.83 and 0.60, respectively, while the signal/noise ratio was 8.83. The prediction model is shown in **Figure 1**, where. It can be see that increasing the pressure level and frozen storage time causes an increase of acid phosphatase activity.

There are a few publications of the effect of HPP on acid phosphatase activity during frozen fish storage, using different species, such as Atlantic mackerel (Fidalgo et al., 2014a; Pazos et al., 2015)), horse mackerel (Fidalgo et al., 2014b) and sardine (Méndez et al., 2016) (Méndez et al. (2016). In general, increasing the pressure level higher than 300 or 450 MPa caused a significant reduction of the initial acid phosphatase activity (Fidalgo et al., 2014b, 2014a). However, the prediction model indicated that with higher pressure levels and longer frozen storage times, acid phosphatase activity increased, which could be related to the possible effect on lysosomes, since this enzyme (40-60%) has been reported to be bound to lysosome membranes. So, the combined effect of high pressure and frozen storage could cause disruption of lysosomes and leakage of the enzyme, increasing the quantifiable acid phosphatase activity (Chéret et al., 2005; Ohmori et al., 1992) (Chéret et al., 2005; Ohmori et al., 1992).

3.1.2. **Cathepsin B activity**

Cathepsin B is a cysteine protease involved in the hydrolysis of myofibrillar protein during the post-mortem storage of fish muscle (Yamashita & Konagaya, 1991). High pressure pre-treatment effect on cathepsin B activity during frozen storage is shown in the Error: No se encuentra la fuente de referencia. Fresh fish showed a cathepsin B activity of $42.49 \pm 15.18 (x10^4)$ FU/min/g. After the freezing step and high-pressure pre-treatment, there is was no significant effect ($p < 0.05$) on the cathepsin B activity. Along frozen storage, cathepsin B activity decreased ($p < 0.05$) to residual values of about 1-3% after 4.68 months, compared to fresh fish. High-pressure pre-treatment caused a significant effect ($p < 0.05$) on cathepsin B activity, registering resulting in residual values lower than 4% using pressure levels of 430.73 and 450 MPa as early as 0.32 and 2.50 months, respectively. A similar effect was verified in previous work done in Atlantic mackerel (Fidalgo et al., 2014a) and horse mackerel (Fidalgo et al., 2014b), in which increasing the pressure level caused a reduction of the cathepsin B activity, being more pronounced at 450 MPa. This effect of high pressure it was already verified using fresh fish, wherein 20-30 % of cathepsin B was inactivated by pressures between 400 and 500 MPa (Ohmori et al., 1992).

The ANOVA was completed to assess the relative influence of the two variables (pressure level and storage time) yielding an F value of 8.38, and implying that the model was significant with a p-value probability $> F$ value of 0.011. In this case, the effect of pressure level was higher than that observed for frozen storage time. The correlation value of the model was $r^2 = 0.86$ and the adjusted and predicted r^2 values were 0.82 and 0.65, respectively, while the signal/noise ratio was 12.27. **Figure 2** shows the prediction for this enzyme activity. It can be see that the increasing of the pressure level and frozen storage time causes a decrease of cathepsin B activity.

3.1.3. Cathepsin D activity

Cathepsin D is an aspartic acid protease considered to be the most important enzyme in the post-mortem degradation of fish or meat muscle because of its optimum pH and the absence of a specific inhibitor in the muscle (Chéret et al., 2005). Error: No se encuentra la fuente de referencia shows the evolution of cathepsin D activity after high-pressure pre-treatment and frozen storage. Frozen storage and high-pressure pre-treatment did not affect significantly the enzymatic activity ($p > 0.05$). Along frozen storage, samples pre-treated with lower pressure levels caused showed a slight increase ($p < 0.05$) of cathepsin D activity during storage, being more pronounce at 300 MPa,

with an increase of 7-fold after 5 months. In previous work, this a similar effect was also observed using Atlantic mackerel (Fidalgo et al., 2014b). The application of high pressure on Atlantic mackerel muscle showed to have two effects on cathepsin D activity: (a) <300 MPa, activity increased, probably due to a release from lysosomes; (b) >300 MPa, activity decreased, which has been attributed to a progressive higher enzyme inactivation (Chéret et al., 2005). In our study, a similar behaviour was observed using lower pressures lower than 300 MPa.

The ANOVA was not significant. The correlation value of the model was $r^2=0.20$ and the adjusted and predicted r^2 values were 0.0 and -0.69, respectively, while the signal/noise ratio was 2.39. Neither pressure level nor storage time showed a significant effect on the cathepsin D activity.

3.1.4. Calpains activity

Cytoplasmic calpains are cysteine proteases which need calcium ions for enzyme activation (Sriket, 2014). These enzymes are thought to cause the weakening and disintegration of the Z-line of fish myofibrils (Lakshmanan et al., 2005), and therefore cause limited proteolysis of myofibrillar proteins, making them susceptible to further degradation by other proteases (Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004; Hultmann & Rustad, 2007) (Delbarre-Ladrat et al., 2004; Hultmann & Rustad, 2007).

Results of calpains activity during frozen storage are shown on the Error: No se encuentra la fuente de referencia, in which fresh fish showed an initial calpain activity of 83.56 ± 8.08 ($\times 10^3$) FU/min/g. After freezing (month 0), the calpains activity of control (T12) and high pressure (T9) samples significantly decreased ($p<0.05$) of about 57% and 64% of the initial activity, respectively. Along frozen storage there was a slower decrease of activity, obtaining lower values of activity (60-90% of reduction) observed on samples pre-treated at higher pressure levels (300.00, 430.73 and 450.00 MPa). The decrease in calpains activity at higher pressure levels was already observed by other authors using fresh sea bass (Chéret et al., 2005; Teixeira et al., 2013) and it was is probably caused by the dissociation of both subunits of calpains (μ - and m-calpain) (Chéret et al., 2007).

— The model for calpains activity was significant (**Figure 3**). In this case pressure level, frozen storage time, the interaction between them, and the quadratic term for pressure level were significant model terms. The correlation value of the model was

$r^2=0.99$ and the adjusted and predicted r^2 values were 0.98 and 0.97, respectively, while the signal/noise ratio was 24.19. The prediction of the model obtained showed that increasing the pressure level and along frozen storage time caused higher activity decreases.

3.2. Assessment of sarcoplasmic and myofibrillar proteins content

A marked effect of pressure level could be observed on the sarcoplasmic protein content (Error: No se encuentra la fuente de referencia). Thus, a lower mean content on such proteins group was detected in fish corresponding to the highest pressure tested at all storage times; differences were found significant ($p < 0.05$) in most cases. Contrary/Differently, scarce differences could be observed as a result of the pressure applied when the myofibrillar proteins are encountered/quantified. Thus, lower mean values were detected in fish corresponding to the highest pressure checked/tested when frozen storage times of 0, 0.32 and 4.68 are considered; by the contrary, high levels in such proteins content are implied when frozen storage times of 2.50 and 5.00 are taken into account. As a result, a definite effect of pressure on myofibrillar proteins content could not be implied/concluded.

——— Most previous research has shown a greater effect of pressure on sarcoplasmic proteins than on the myofibrillar ones. This conclusion was observed for chilled Coho salmon previously submitted to HP processing (135-200 MPa for 30 s) (Ortea et al., 2010) and for frozen mackerel previous treated under HP conditions (125-200 MPa for 0 min) (Pazos et al., 2015). Contrary, when frozen sardine previously submitted to HP processing (125-200 MPa for 0 min) was analysed, a higher effect of pressure was implied on the sarcoplasmic proteins fraction (Méndez et al., 2017).

——— Concerning the sarcoplasmic proteins, the multifactor ANOVA analysis (pressure level and frozen storage time effects) yielded a significant ($p < 0.001$) model with an F value of 32.77. The evaluation of the F-values for the two independent variables confirmed their individual effects. Thus, sarcoplasmic proteins content was highly affected by the pressure level (F-value = 132.00; p-value probability $> F$ was $p < 0.00001$); meantime, an important effect of the frozen storage time could also be observed (F-value of 4.29; p-value probability $> F$ was $p < 0.01$). The correlation value of the model was $r^2 = 0.9704$ with adjusted and predicted r^2 values of 0.9408 and 0.9109, respectively, and a signal/noise ratio of 15.22. These statistical parameters

confirmed that an empirical coded equation could be used to model the effect of previous pressure applied and frozen storage time on the sarcoplasmic proteins content:-

where Y is the sarcoplasmic protein content (g/100 fish muscle), A is the pressure level applied (MPa) and B is the frozen storage time (months). Concerning the myofibrillar proteins content, the multifactor ANOVA analysis (pressure level and frozen storage time effect) did not show a significant effect ($p > 0.05$).

4. Conclusion

The application of HPP pre-treatments before freezing and storage at accelerated -10°C for 5 months of European hake (*Merluccius merluccius*) was studied by evaluation of the enzymatic activity and on sarcoplasmic and myofibrillar proteins content. Acid phosphatase activity increased with the increase of pressure level and frozen storage time, contrarily, a decrease of cathepsin B and calpains activities was observed. Moreover, no effect of pressure level and frozen storage time was verified on cathepsin D activity. Sarcoplasmic content was highly affected by the pressure level and frozen storage time, resulting in a slightly decrease of sarcoplasmic proteins content using higher pressure levels (430.7 and 450 MPa).

The application of high pressure processing as a pre-treatment before frozen storage caused changes on the activities of acid phosphatase, cathepsins (B and D) and calpains involved in textural deterioration of fish muscle, indicating that HPP can be a useful pre-treatment for frozen fish preservation.

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Highlights:

- High-pressure processing (HPP) pre-treatments caused changes on enzyme activities during frozen storage of European hake;
- Acid phosphatase activity increased with HPP pre-treatment and during frozen storage;
- HPP (≥ 300 MPa) caused a decrease of cathepsin B and calpains activities and sarcoplasmic proteins content;
- No effect of pressure and frozen storage on cathepsin D activity was observed.
- HPP pre-treatment can be used reduce deleterious enzymatic activity during frozen storage.

Table 1 – Experimental design.

Code	HHP treatment (MPa)	Storage time (Months)
T-0	<i>Fish without HHP processing and freezing process - fresh fish</i>	
T-1	300.00	2.50
T-2	169.27	0.32
T-3	169.27	4.68
T-4	430.73	4.68
T-5	300.00	2.50
T-6	300.00	2.50
T-7	300.00	5.00
T-8	150.00	2.50
T-9	300.00	0.00
T-10	430.73	0.32
T-11	450.00	2.50
T-12	0.10	0.00
T-13	0.10	0.32
T-14	0.10	2.50
T-15	0.10	4.68
T-16	0.10	5.00

Table 2 – Enzymatic activity of Acid Phosphatase ($\mu\text{mol p-NP/min/g}$), Cathepsin B ($\times 10^4 \text{ FU/min/g}$), Cathepsin D ($\mu\text{g tyrosine/min/g}$) and Calpains ($\times 10^3 \text{ FU/min/g}$) of hake samples pre-treated by high pressure and during frozen storage.

Code	Pressure (MPa)	Storage time (Months)	Enzymatic activity			
			Acid phosphatase	Cathepsin B	Cathepsin D	Calpains
T0	Fresh control*		2.64 ± 0.56 <i>a</i>	42.49 ± 15.18 <i>a</i>	1.105 ± 0.357 <i>d</i>	83.56 ± 8.08 <i>a</i>
T1	300.00	2.50	0.78 ± 0.04 <i>bcd</i>	28.64 ± 18.45 <i>ab</i>	2.104 ± 1.198 <i>cd</i>	31.64 ± 6.09 <i>cde</i>
T2	169.27	0.32	0.89 ± 0.04 <i>bcd</i>	46.31 ± 20.82 <i>a</i>	3.630 ± 1.021 <i>bc</i>	70.83 ± 4.83 <i>ab</i>
T3	169.27	4.68	0.47 ± 0.11 <i>cd</i>	1.19 ± 0.80 <i>b</i>	4.935 ± 1.331 <i>b</i>	31.64 ± 5.84 <i>cde</i>
T4	430.73	4.68	0.55 ± 0.11 <i>bcd</i>	0.38 ± 0.27 <i>b</i>	1.974 ± 0.683 <i>cd</i>	8.76 ± 3.22 <i>g</i>
T5	300.00	2.50	0.82 ± 0.04 <i>bcd</i>	22.86 ± 13.92 <i>ab</i>	2.711 ± 0.155 <i>bcd</i>	22.69 ± 6.54 <i>def</i>
T6	300.00	2.50	0.68 ± 0.10 <i>bcd</i>	33.74 ± 01.40 <i>ab</i>	2.386 ± 0.608 <i>cd</i>	28.92 ± 5.58 <i>cdef</i>
T7	300.00	5.00	0.42 ± 0.03 <i>d</i>	0.69 ± 0.54 <i>b</i>	7.972 ± 1.425 <i>a</i>	15.94 ± 0.61 <i>fg</i>
T8	150.00	2.50	0.76 ± 0.07 <i>bcd</i>	54.23 ± 19.00 <i>a</i>	1.654 ± 0.043 <i>cd</i>	72.21 ± 3.96 <i>ab</i>
T9	300.00	0.00	0.61 ± 0.06 <i>bcd</i>	35.46 ± 15.18 <i>ab</i>	1.952 ± 0.698 <i>cd</i>	30.27 ± 2.58 <i>cde</i>
T10	430.73	0.32	0.53 ± 0.04 <i>bcd</i>	1.69 ± 0.95 <i>b</i>	1.887 ± 0.883 <i>cd</i>	18.28 ± 0.72 <i>efg</i>
T11	450.00	2.50	0.62 ± 0.16 <i>bcd</i>	1.47 ± 0.38 <i>b</i>	3.430 ± 0.907 <i>bcd</i>	19.71 ± 0.87 <i>efg</i>
T12	0.10	0.00	0.97 ± 0.12 <i>bc</i>	30.83 ± 13.66 <i>ab</i>	1.707 ± 0.822 <i>cd</i>	35.93 ± 3.80 <i>cd</i>
T13	0.10	0.32	1.01 ± 0.20 <i>b</i>	18.36 ± 7.31 <i>ab</i>	1.535 ± 0.474 <i>cd</i>	39.71 ± 6.12 <i>c</i>
T14	0.10	2.50	0.86 ± 0.08 <i>bcd</i>	39.56 ± 21.97 <i>a</i>	2.064 ± 0.361 <i>cd</i>	58.38 ± 0.85 <i>b</i>
T15	0.10	4.68	0.43 ± 0.01 <i>d</i>	0.90 ± 0.06 <i>b</i>	1.955 ± 0.279 <i>cd</i>	36.88 ± 4.43 <i>c</i>
T16	0.10	5.00	0.41 ± 0.06 <i>d</i>	0.72 ± 0.17 <i>b</i>	2.479 ± 0.982 <i>bcd</i>	22.37 ± 3.84 <i>defg</i>

* Fish without HHP processing and freezing process

Table 3 - Assessment of sarcoplasmic and myofibrillar protein content (g/100g muscle)* in frozen hake muscle previously processed under various pressure levels. Average values of three independent determinations (n=3); results are shown as average $\pm$ standard deviation. For each protein group and for each frozen storage time, average values followed by different letters (a, b) denote significant ($p<0.05$) differences as a result of the pressure applied. Symbol * means that this sample correspond to fish without HHP processing and freezing process.

Code	Pressure (MPa)	Storage time (Months)	Total sarcoplasmics	Total myofibrillars
T0	Fresh fish		2.34 ± 0.23 <i>ab</i>	9.11 ± 0.61 <i>a</i>
T12	0.1	0.00	2.44 ± 0.10 <i>b</i>	8.41 ± 0.48 <i>a</i>
T9	300.0	0.00	2.03 ± 0.10 <i>a</i>	8.24 ± 0.93 <i>a</i>
T13	0.1	0.32	2.34 ± 0.25 <i>b</i>	8.92 ± 1.26 <i>a</i>
T2	169.3	0.32	2.20 ± 0.25 <i>b</i>	8.90 ± 0.87 <i>a</i>
T10	430.7	0.32	1.25 ± 0.16 <i>a</i>	7.60 ± 2.93 <i>a</i>
T14	0.1	2.50	2.15 ± 0.22 <i>b</i>	8.81 ± 0.98 <i>a</i>
T8	150.0	2.50	2.16 ± 0.24 <i>b</i>	7.66 ± 1.34 <i>a</i>
T1	300.0	2.50	2.36 ± 0.48 <i>b</i>	7.42 ± 0.45 <i>a</i>
T5	300.0	2.50	1.79 ± 0.28 <i>b</i>	7.45 ± 0.58 <i>a</i>
T6	300.0	2.50	2.05 ± 0.08 <i>b</i>	7.31 ± 0.49 <i>a</i>
T11	450.0	2.50	1.12 ± 0.09 <i>a</i>	11.50 ± 0.76 <i>b</i>
T15	0.1	4.68	2.28 ± 0.26 <i>b</i>	8.53 ± 0.23 <i>a</i>
T3	169.3	4.68	1.94 ± 0.51 <i>b</i>	8.52 ± 0.45 <i>a</i>
T4	430.7	4.68	1.14 ± 0.09 <i>a</i>	7.83 ± 0.48 <i>a</i>
T16	0.1	5.00	2.09 ± 0.31 <i>a</i>	7.88 ± 0.66 <i>a</i>
T7	300.0	5.00	1.87 ± 0.35 <i>a</i>	8.22 ± 0.85 <i>a</i>

Figures

Figure 1. Model prediction for the effect of pressure level (MPa) and frozen storage time (months) on acid phosphatase activity.

Figure 2. Model prediction for the effect of pressure level (MPa) and frozen storage time (months) on cathepsin B activity.

Figure 3. Model prediction for the effect of pressure level (MPa) and frozen storage time (months) on calpains activity.

Figure 1

Design-Expert® Software
Factor Coding: Actual
Acid phosphatase ((10⁻²) nmol p-NP/min/g fish)
● Design points above predicted value
○ Design points below predicted value
0.894375
0.422015
X1 = A: Pressure
X2 = B: Frozen Storage

Figure 2

Design-Expert® Software

Factor Coding: Actual

Cathepsin B (fluorescence/min/g fish)

● Design points above predicted value

○ Design points below predicted value

54231.5

378.737

X1 = A: Pressure

X2 = B: Frozen Storage

Figure 3

Design-Expert® Software
Factor Coding: Actual
Calpains (fluorescence/min/g fish)
● Design points above predicted value
○ Design points below predicted value
72207.5
8763.46
X1 = A: Pressure
X2 = B: Frozen Storage