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High pressure effects on the activity of cathepsins B and D of mackerel and horse mackerel muscle

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Abstract:

This work sought to determine high pressure processing (HPP) effects on the activity of cathepsins B and D in the muscle of mackerel (*Scomber scombrus*) and horse mackerel (*Trachurus trachurus*). In mackerel, the cathepsin B activity decrease reached 40% at 450 MPa while in horse mackerel, low and intermediate pressures (150 and 300 MPa) caused an activity increase (30%) but a decrease of up to 60% at 450 MPa. In both species, cathepsin D activity increased after a 300 MPa treatment (up to 2-fold for mackerel and 60% for horse mackerel) while decreasing after a 450 MPa treatment. The activity increase is probably due to HPP damage of lysosome releasing enzymes into the fish muscle. Based on the HPP effects on the activity of cathepsins B and D, 450 MPa may be used to reduce the proteolytic activity of cathepsin B prior to chilled or frozen storage of these fish species.

Keywords: High pressure processing, fish, *Scomber scombrus*, *Trachurus trachurus*, proteolytic enzymes, cathepsins B and D.

INTRODUCTION

Small pelagic fish species, such as Atlantic mackerel (*Scomber scombrus*) and Atlantic horse mackerel (*Trachurus trachurus*), are important fishery resources in many coastal areas of South European countries (MBARKI *et al.* 2009). Horse mackerel has been attracting great attention because of its moderate price and large quantities captured in West-European countries (Holland, Ireland, Spain, France, Germany and Portugal) (FAO 2003).

Loss of fish freshness is caused by a combination of physical, biochemical, and microbiological reactions. Enzymatic degradations cause the *postmortem* softening of fish muscle and facilitate the proliferation of bacterial flora (CHÉRET *et al.* 2006; HERNÁNDEZ-ANDRÉS *et al.* 2008). Endogenous protease activity is responsible for the proteolysis of fish myofibrillar proteins (CHÉRET *et al.* 2007). Cathepsins, found widely distributed in muscles and organs, are one of the proteolytic systems known to hydrolyze myofibrillar proteins during *postmortem* storage of fish muscle (JIANG 2000). During muscle storage, cathepsins B and D may be released from the lysosomal matrix into the cytoplasm and intracellular spaces as a consequence of lysosomes breakdown (BECHET *et al.* 2005).

Cathepsin B, a cysteine protease, shows an optimal pH for activity around 6.0 (NIELSEN *et al.* 2006), hydrolyzes a wide range of proteins and has an important role in the hydrolysis of tissue proteins (AOKI *et al.* 2002). Cathepsin D, an aspartic acidic protease, is considered the most important enzyme in the *postmortem* degradation of muscle because of its optimum pH and the absence of a specific inhibitor in the muscle (CHÉRET *et al.* 2005). Cathepsin D can be found in several isoenzyme forms with an activity pH range in muscle tissue between pH 3.0 and 6.0 (ASHIE *et al.* 1997).

High pressure processing (HPP) is now a commercially well-established new food preservation technology and an effective alternative to thermal treatments or the use of chemical preservatives (CHÉRET *et al.* 2006; RAMIREZ *et al.* 2009). HPP applications in food processing are of great interest because of the ability to inactivate food borne microorganisms and endogenous enzymes (CASTRO *et al.* 2008; CASTRO *et al.* 2011) while preserving the nutritional and sensory attributes of foods (WIMALARATNE *et al.* 2008). However, HPP can affect also proteins, particularly myofibrillar proteins, resulting in structural modifications and texture changes in muscle foods (ANGSUPANICH *et al.* 1999). In addition, HPP can disrupt lysosomal membranes causing

the release into the fish muscle of enzymes, such as cathepsins, leading to possible alterations of myofibrillar proteins mediated by these enzymes (OHMORI *et al.* 1992). Depending on several factors, HPP treatment effects on fish muscle can cause activation or inactivation of muscle enzymes. For instance, studies have shown that HPP treatment causes an activity increase of cathepsin B at 500 MPa, while cathepsin D activity would increase after a pressure treatments below 300 MPa and decrease at higher pressures (CHÉRET *et al.* 2005). However in cold-smoked salmon, cathepsin B activity was reduced by treatments at pressures up to 300 MPa (LAKSHMANAN *et al.* 2005).

The successful applications of HPP in other food systems suggest this technology as a potential treatment to minimize changes that occur during the storage of fish. However, the effect of HPP treatment on fish muscle is still poorly studied, particularly enzymes with proteolytic activity. Thus, the aim of this work was to determine the effect of HPP treatments on the activity of cathepsins B and D in mackerel and horse mackerel muscles which would improve frozen fish quality by reducing their proteolytic activity before freezing and subsequent frozen storage.

MATERIALS AND METHODS

Preparation of samples

Atlantic mackerel (*Scomber scombrus*) and Atlantic horse mackerel (*Trachurus trachurus*) 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 mackerel (28-33 cm and 230-280 g range) and whole horse mackerel (25-30 cm and 200-250 g range) individuals were placed in flexible polyethylene bags (three individuals per bag) and vacuum sealed at 400 mbar.

HPP Treatments

Whole fish were HPP-treated in a 55-L high pressure unit (WAVE 6000/55HT; NC Hyperbaric, Burgos, Spain) at 150, 300 and 450 MPa with 0.0, 2.5 and 5 min holding time (the 0.0 min holding time samples were carried out to study the effect of just the pressure come-up and depressurizing time). Non-pressure treated samples (untreated controls) were also studied. The pressurizing medium was water applied at 3 $MPa.s^{-1}$, 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.

Enzymatic activity

Preparation of enzyme extract

The preparation of enzymatic extract was performed by the methodology used by LAKSHMANAN *et al.* (2005). Samples (10 g) of pooled fish muscle from each 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 and after 30 min, it was centrifuged for 20 min at 14,600*g* and 4 ºC. The supernatant (used as cathepsins extract) was filtered through a Whatman nº1 filter and stored at -20 ºC prior to enzymatic activity quantifications.

Cathepsins activity

Cathepsin B activity was assayed as described by LAKSHMANAN *et al.* (2005). Enzyme extract (0.1 mL) and the 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 during 5 min. The reaction was stopped by the addition of 1 mL 3% SDS (w/v) in 50 mM Bis-Tris (pH 7.0) and the 7-amino-4-methylcoumarin (AMC) liberated was measured (excitation: 360 nm, emission: 460 nm). Cathepsin B activity was expressed as $FU.min^{-1}.g^{-1}$. Three replicates were performed for each treatment.

Cathepsin D activity assay was based on the procedure described by BUCKOW *et al.* (2010), with small modifications. Enzyme extract (0.2 mL) was mixed with 0.6 mL of a substrate solution, 2% denatured hemoglobin (w/v, #H-2625 Sigma – Aldrich Corp, Steinheim - Germany) in 200 mM citrate buffer (pH 3.7), and incubated 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 (13,000 rpm, 15 min) and the soluble peptides measured at 280 nm. Cathepsin D activity was expressed as µg $tyrosine.min^{-1}.g^{-1}$. Three replicates were performed for each treatment.

Statistical analysis

The differences between control and treated samples were tested with one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) Test. Two-way ANOVA followed by Fisher's LSD Test was used to identify differences between treatments. Differences between treatments were considered significant when $p < 0.05$. Results are reported as mean values ± standard deviation.

RESULTS AND DISCUSSION

Cathepsin B

Cathepsin B activity in untreated samples (controls) was about 16-fold higher for mackerel than for horse mackerel (Table 1). Fig. 1 and 2 show the effect of the HPP treatments on cathepsin B activity on mackerel and horse mackerel, respectively. In mackerel, HPP treated samples were significantly lower ($p < 0.05$) than in untreated samples with the exception of samples treated at 150 MPa/2.5 min and 300 MPa/5 min, for which no statistical difference was observed. A gradual decrease in the activity of cathepsin B to around 40% at 300 and 450 MPa was generally observed when the pressure level was increased (Fig. 1A). Globally, a higher effect of pressure level as compared to pressure holding time was observed, except for 300 MPa, for which the activity increased significantly with pressure holding time.

In horse mackerel, low and intermediate pressures (150 and 300 MPa for 0 min) caused an enzyme activity increase of up to 30% (Fig. 2), with a decrease being observed for 2.5 and 5 min reaching an activity value lower than the control for 150 MPa/5 min and equal for the 300 MPa for 2.5 and 5 min treatments (Fig. 2B). The activity of samples treated at 450 MPa was lower than the control, regardless of the holding time.

Comparing the results for the two species, the main conclusions could be expressed as follows. The intermediate pressure levels (150 and 300 MPa) showed a lower effect on activity of cathepsin B in horse mackerel as compared to mackerel. Pressure holding time increased the activity in mackerel and decreased it in horse mackerel. Pressure-treated mackerel showed an activity lower and up to equal to that of the control, while for horse mackerel intermediate pressure levels caused an increase in activity. For both species, the higher pressure level studied (450 MPa) caused a significant decrease in cathepsin B activity, compared to shorter holding times (0 min).

At 300 MPa, the observed effect of pressure on cathepsin B of Atlantic horse mackerel was in agreement with previous report. For instance, CHÉRET *et al.* (2005) observed that

in sea bass muscle pressure-treated at 300 MPa for 5 min, cathepsin B activity increased slightly, being this explained by the possible release of the enzyme from the lysosomes.

Cathepsin D

The Cathepsin D activity in horse mackerel is 50% higher than in mackerel, while the reverse was observed for cathepsin B (Table 1). In mackerel HPP-treatments caused a significant cathepsin D activity increase ($p < 0.05$), with the exception of 150 MPa/2.5 min and 450 MPa/5 min treatments, for which no effects were observed (Fig. 3). The activity increase was higher for 300 MPa treatments, reaching more than 2-fold for 5 minwhen compared to the control. Pressure holding time for 150 MPa treatments showed small effects on activity ; however, a 300 MPa treatment led to a significant increase, while a 450 MPa treatment provoked an activity decrease.

Similar results than for mackerel were observed for horse mackerel, (Fig. 4). In general, activity increased with pressure except for 150 MPa/5 min, 300 MPa/0 min, and 450 MPa/0 and 2.5 min treatments. Similar results to those obtained in this work for cathepsin D of mackerel and horse mackerel were obtained for the muscle of sardine (HERNÁNDEZ-ANDRÉS *et al.* 2008) and sea bass (CHÉRET *et al.* 2005). For instance, CHÉRET *et al.* (2005) observed that pressure treatments at 300 MPa increased the activity of cathepsin D, while for higher pressure levels the activity decreased to values similar to those obtained at 100 MPa. These results can be hypothetically explained by the liberation of the enzyme from the lysosomes, which appears to be the dominant effect at lower pressures and shorter holding times (JUNG *et al.* 2000), while at higher pressures enzyme inactivation would prevail. Results obtained point out the occurrence of two effects of pressure treatments on the activity of cathepsin D. At lower pressures (150 MPa and 300 MPa), cathepsin D activity increases, possibly because of enzyme release from the lysosomes into the fish muscle. At higher pressure (450 MPa) the activity decreases, possibly because the pressure inactivation effectbecomes more important than the release of cathepsin D from lysosomes.

CONCLUSION

With the exception of cathepsin B for Atlantic mackerel, lower pressures and shorter pressure holding times caused cathepsin B and D activity increases, while higher pressures/longer pressure holding times had the opposite effect. The former effect is generally attributed to enzyme release from lysosomes while the latter may reflect

pressure inactivation. HPP reduced cathepsin B activity in mackerel and horse mackerel, in all cases for the former and at 450 MPa for the latter. For cathepsin D, all pressure treatments studied resulted in an equal or higher enzyme activity as compared to the control. In conclusion, pressure treatments studied in this work (150, 300 and 450 MPa, with holding times of 0, 2.5 and 5 min) can reduce the endogenous cathepsin B activity of mackerel and horse mackerel and thus opening the possibility for a better quality retention during subsequent chilled or frozen storage preservation; however such activity inhibition was not observed for cathepsin D. These results indicate that the effect of HPP on cathepsins B and D, depend on pressure level and time intensity, but also on the fish species under study.

Acknowledgements

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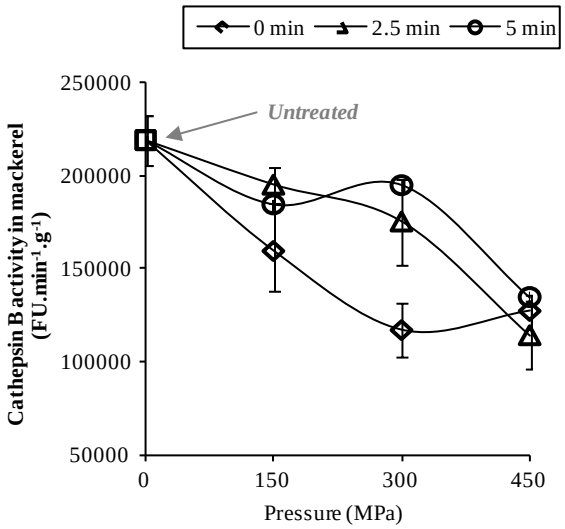
Tables

Table 1 – Cathepsin B and D activities in untreated mackerel and horse mackerel

Fish species	Cathepsin B activity ($FU.min^{-1}.g^{-1}$)	Cathepsin D activity ($\mu g\ tyrosine.min^{-1}.g^{-1}$)
Mackerel	218839 ± 13147	2.044 ± 0.138
Horse mackerel	13303 ± 361	3.31 ± 0.263

Figures

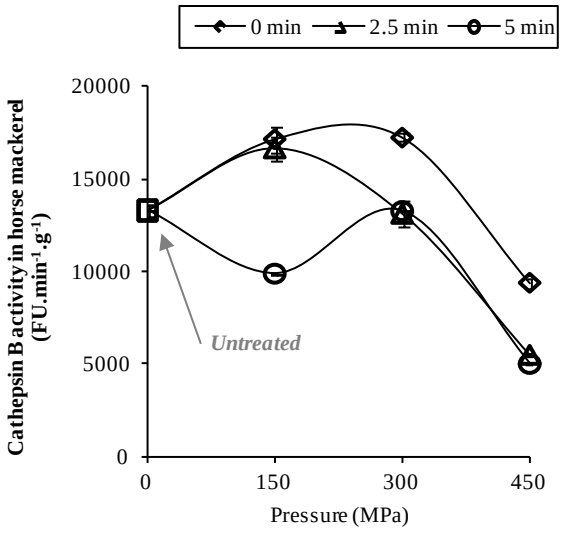


Statistical analysis[#]

Holding time	150 MPa	300 MPa	450 MPa
0 min	b*x**	b*y**	a*y**
2.5 min	a*x*	a*x**	a*y**
5 min	ab*x**	a*x*	a*y**

Different letters denote significant differences ($p < 0.05$) between pressure (x-y) or pressure holding time (a-b) values.
* Denotes significant differences with untreated samples (0.1 MPa).

Fig. 1. Pressure level and holding time effects on cathepsin B activity in mackerel.



1

Statistical analysis[#]

Holding time	150 MPa	300 MPa	450 MPa
0 min	a*x**	a*x**	a*y**
2.5 min	a*x**	b*y*	b*z**
5 min	b*y**	b*x*	b*z**

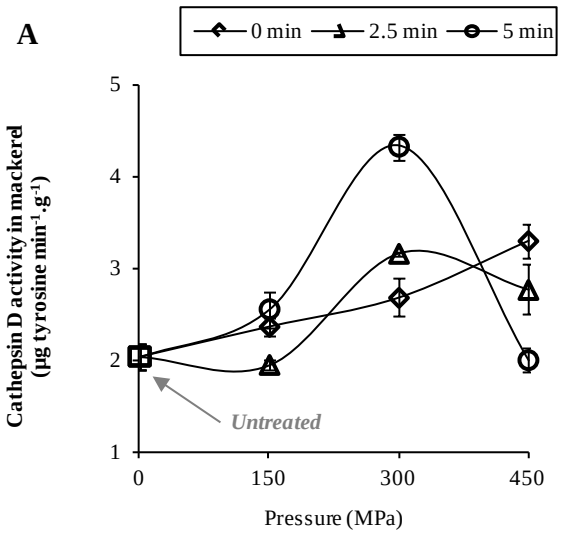
Different letters denote significant differences ($p < 0.05$) between pressure (x-z) or pressure holding time (a-b) values.
* Denotes significant differences with untreated samples (0.1 MPa).

2

3 **Fig. 2.** Pressure level and holding time effects on cathepsin B activity in horse
4 mackerel.

5

Figure 3



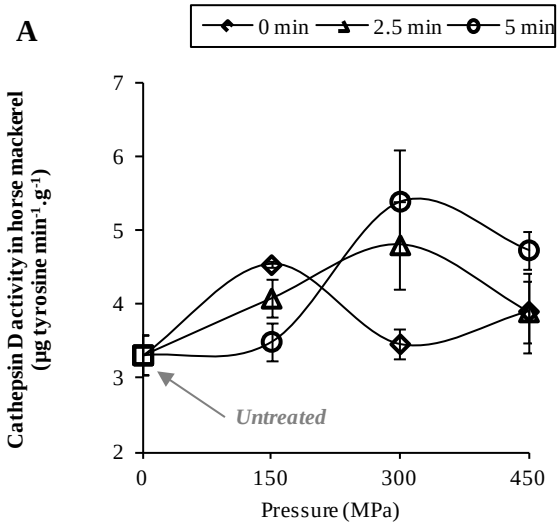
Statistical analysis[#]

Holding time	150 MPa	300 MPa	450 MPa
0 min	a*z**	c*y**	a*x**
2.5 min	b*z*	b*x**	b*y**
5 min	a*y**	a*x**	c*z*

Different letters denote significant differences ($p < 0.05$) between pressure (x-z) or pressure holding time (a-b) values.
* Denotes significant differences with untreated samples (0.1 MPa).

Fig. 3. Pressure level and holding time effects on cathepsin D activity in mackerel.

Figure 4



Statistical analysis[#]

Holding time	150 MPa	300 MPa	450 MPa
0 min	a*x**	b*xy*	b*y*
2.5 min	ab*y**	a*x**	b*y*
5 min	b*y*	a*x**	a*x**

Different letters denote significant differences ($p < 0.05$) between pressure (x-y) or pressure holding time (a-b) values.
* Denotes significant differences with untreated samples (0.1 MPa).

Fig. 4. Pressure level and holding time effects on cathepsin D activity in horse mackerel.