

High Pressure Effects on the Activities of Cathepsins B and D of Mackerel and Horse Mackerel Muscle

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Abstract

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We determined high pressure processing (HPP) effects on the activities of cathepsins B and D in the muscles 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 at 450 MPa a decrease of up to 60%. In both species, cathepsin D activity increased after a 300 MPa treatment (up to 2-fold for mackerel and 60% for horse mackerel) and decreased on 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 activities 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*; mackerel and horse mackere; proteolytic enzymes

Small pelagic fish species, such as the 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).

The loss of fish freshness is caused by a combination of physical, biochemical, and microbiological reactions. Enzymatic degradation causes the postmortem

softening of fish muscle and facilitates 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 hydrolyse 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).

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Cathepsin B, a cysteine protease, shows optimal activity at pH around 6.0 (NIELSEN & NIELSEN 2006), hydrolyses a wide range of proteins and plays an important role in the hydrolysis of tissue proteins (AOKI *et al.* 2002). Cathepsin D, an aspartic acid 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 3.0 and 6.0 (ASHIE & SIMPSON 1997).

High pressure processing (HPP) is now a commercially well-established new food preservation technology and an effective alternative to the thermal treatments or 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, 2011) while preserving the nutritional and sensory attributes of foods (WIMALARATNE & FARID 2008). However, HPP can affect also proteins, particularly myofibrillar proteins, which results 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 increase of cathepsin B activity at 500 MPa, while cathepsin D activity will increase after a pressure treatment 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 of 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 minimise changes that occur during the storage of fish. However, the effect of HPP treatment on fish muscle is still poorly studied, particularly regarding enzymes with proteolytic activity. Thus, the aim of this work was to determine the effects 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 during subsequent frozen storage.

MATERIAL AND METHODS

Sample preparation. 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 h after the catch. Whole mackerel (28–33 cm and 230–280 g range) and whole horse mackerel (25 to 30 cm and 200–250 g range) specimens 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 5.0 min holding time (the 0.0 min holding time samples were prepared to study the effects of just the pressure come-up and depressurising time). Non-pressure treated samples (untreated controls) were also studied. The pressurising medium was water applied at 3 MPa/s, yielding come up times of 50, 100, and 150 s for the treatments at 150, 300, and 450 MPa, respectively; while decompression took less time than 3 seconds. Pressurising water was cooled down to maintain room temperature (20°C) conditions during HPP treatment.

Enzymatic extract preparation. Preparation of the 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 homogenised with 50 ml ice cold distilled water for 2 minutes. The homogenate was kept in ice with occasional stirring and after 30 min, it was centrifuged at 14,600 g and 4°C for 20 minutes. The supernatant (used as cathepsins extract) was filtered through a Whatman N°1 filter and stored at –20°C prior to enzymatic activity quantification.

Cathepsins activity. Cathepsin B activity was assayed as described by LAKSHMANAN *et al.* (2005). The enzyme extract (0.1 ml) and the substrate solution (0.1 ml), containing 0.0625mM of Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (#C5429, Sigma-Aldrich Corp., Steinheim, Germany) in 100mM Bis-Tris buffer with 20mM EDTA and 4mM dithiothreitol at pH 6.5 were incubated at 37°C during 5 minutes. The reaction was stopped by the addition of 1 ml 3% SDS (w/v) in 50mM 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 fluorescence

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

Fish species	Cathepsin B (FU/min/g)	Cathepsin D (µg tyrosine/min/g)
Mackerel	218 839 ± 13 147	2.044 ± 0.138
Horse mackerel	13 303 ± 361	3.31 ± 0.263

units (FU)/min/g. 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. The enzyme extract (0.2 ml) was mixed with 0.6 ml of the substrate solution, 2% denatured hemoglobin (w/v, #H-2625; Sigma-Aldrich Corp, Steinheim, Germany) in 200mM citrate buffer (pH 3.7), and incubated at 37°C for 3 hours. 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 were measured at 280 nm. Cathepsin D activity was expressed as µg tyrosine per min per g. Three replicates were performed in each treatment.

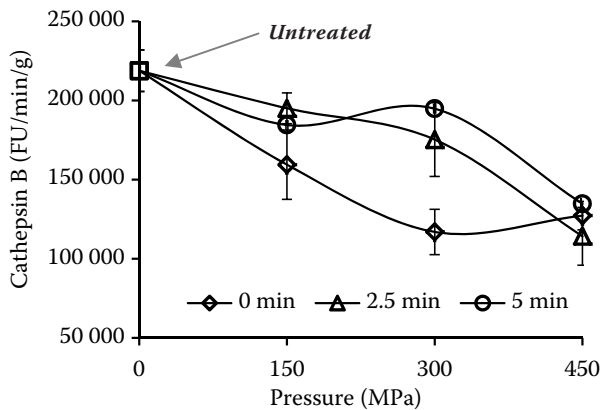
Statistical analysis. The differences between the control and treated samples were tested with one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (*LSD*) test. Two-way ANOVA followed by Fisher's *LSD* test was used to

identify the differences between treatments. The differences between the treatments were considered significant when $P < 0.05$. The results are reported as mean values ± standard deviation.

RESULTS AND DISCUSSION

Cathepsin B. Cathepsin B activity in the untreated samples (controls) was about 16-fold higher with mackerel than with horse mackerel (Table 1). Figures 1 and 2 show the effects of the HPP treatments on cathepsin B activity in mackerel and horse mackerel, respectively. In mackerel, cathepsin B activity in HPP treated samples was significantly lower ($P < 0.05$) than that in the untreated samples with the exception of the 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 (Figure 1). Globally, a higher effect of the pressure level as compared to that of the pressure holding time was observed, except for 300 MPa, for which the activity increased significantly with the pressure holding time.

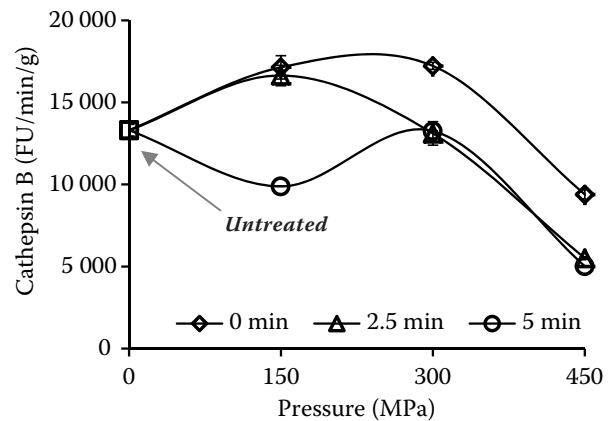
In horse mackerel, low and intermediate pressures (150 and 300 MPa for 0 min) caused an enzyme activity increase of up to 30% (Figure 2), with a decrease observed for 2.5 and 5 min reaching an activity value



Statistical analysis [#]			
Holding time (min)	150 MPa	300 MPa	450 MPa
0	bx*	by	ay*
2.5	ax	ax*	ay*
5	aby	ax	ay*

[#]Different letters denote significant differences ($P < 0.05$) between pressure (x–z) or pressure holding time (a–b) values
*significant differences with untreated samples (0.1 MPa)

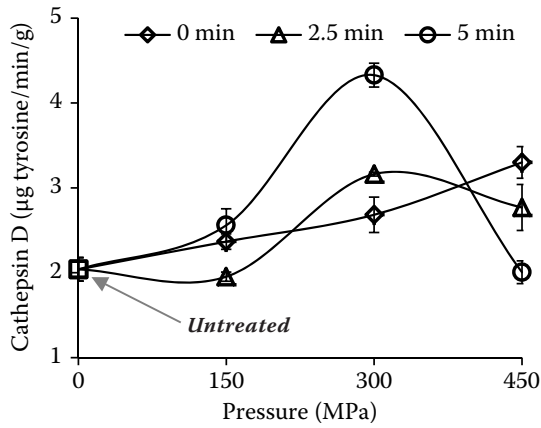
Figure 1. Pressure level and holding time effects on cathepsin B activity in mackerel



Statistical analysis [#]			
Holding time (min)	150 MPa	300 MPa	450 MPa
0	ax*	ax*	ay*
2.5	ax*	by	bz*
5	by*	bx	bz*

[#]Different letters denote significant differences ($P < 0.05$) between pressure (x–z) or pressure holding time (a–b) values
*significant differences with untreated samples (0.1 MPa)

Figure 2. Pressure level and holding time effects on cathepsin B activity in horse mackerel



Statistical analysis [#]			
Holding time (min)	150 MPa	300 MPa	450 MPa
0	az*	cy*	ax*
2.5	bz	bx*	by*
5	ay*	ax*	cz

[#]Different letters denote significant differences ($P < 0.05$) between pressure (x–y) or pressure holding time (a–b) values
*significant differences with untreated samples (0.1 MPa)

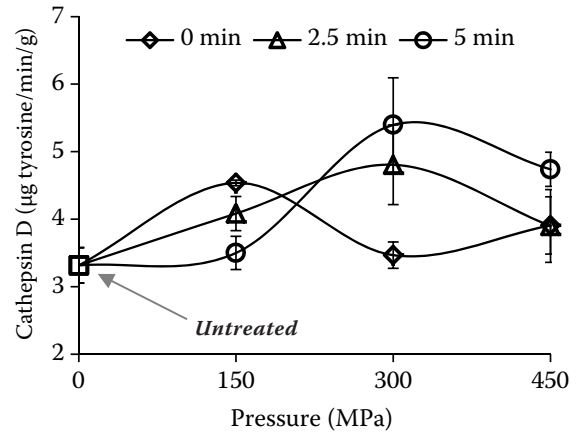
Figure 3. Pressure level and holding time effects on cathepsin D activity in mackerel

lower than the control for 150 MPa/5 min and equal for the 300 MPa for 2.5 and 5 min treatments (Figure 2). The activity of the samples treated at 450 MPa was lower than that of the control, regardless of the holding time.

Comparing the results obtained with the two species, the main conclusions can be made as follows. The intermediate pressure levels (150 and 300 MPa) showed a smaller effect on the activity of cathepsin B in horse mackerel as compared to mackerel. The pressure holding time increased the activity in mackerel and decreased it in horse mackerel. Pressure-treated mackerel showed an activity lower than 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 as compared to shorter holding times (0 min).

At 300 MPa, the observed effect of the pressure on cathepsin B of the Atlantic horse mackerel was in agreement with previous reports. 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, the being is explained by the possible release of the enzyme from the lysosomes.

Cathepsin D. The cathepsin D activity in horse mackerel was 50% higher than that in mackerel, while



Statistical analysis [#]			
Holding time (min)	150 MPa	300 MPa	450 MPa
0	ax*	bxy	by
2.5	aby*	ax*	by
5	by	ax*	ax*

[#]Different letters denote significant differences ($P < 0.05$) between pressure (x–y) or pressure holding time (a–b) values
*significant differences with untreated samples (0.1 MPa)

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

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, with which no effects were observed (Figure 3). The activity increase was higher in 300 MPa treatments, reaching more than 2-fold value for 5 min when compared to the control. The pressure holding time for 150 MPa treatments showed small effects on the activity; however, a 300 MPa treatment led to a significant increase, while a 450 MPa treatment provoked an activity decrease.

Similar results as for mackerel were observed for horse mackerel (Figure 4). In general, the 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 with the muscles of sardine (HERNÁNDEZ-ANDRÉS *et al.* 2008) and sea bass (CHÉRET *et al.* 2005). For instance, CHÉRET *et al.* (2005) observed that the pressure treatments at 300 MPa increased the activity of cathepsin D, while at 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 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. The results obtained point out the occurrence of two effects of the pressure treatments on the activity of cathepsin D. At lower pressures (150 and 300 MPa), cathepsin D activity increases, possibly because of the enzyme release from the lysosomes into the fish muscle. At higher pressure (450 MPa) the activity decreases, possibly because the pressure inactivation effect becomes more important than the release of cathepsin D from lysosomes.

CONCLUSIONS

With the exception of cathepsin B in Atlantic mackerel, lower pressures and shorter pressure holding times caused cathepsins B and D activity increases, while higher pressures/longer pressure holding times had the opposite effect. The former effect is generally attributed to the enzyme release from lysosomes while the latter one may reflect pressure inactivation. HPP reduced cathepsin B activity in mackerel and horse mackerel, with the former in all cases and with the latter at 450 MPa. For cathepsin D, all pressure treatments studied resulted in an equal or higher enzyme activity as compared to the control. In conclusion, the 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 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 effects of HPP on cathepsins B and D depend on the pressure level and time intensity, but also on the fish species under study.

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