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

LILIANA G. FIDALGO¹, JORGE A. SARAIVA¹, SANTIAGO P. AUBOURG², MANUEL VÁZQUEZ³ and J. ANTONIO TORRES⁴

¹Research Unit of Organic Chemistry, Natural and Agro-food Products (QOPNA), Chemistry Department, Aveiro University, Campus Universitário de Santiago, Aveiro, Portugal; ²Department of Food Technology, Instituto de Investigaciones Marinas (CSIC), Vigo, Spain; ³Department of Analytical Chemistry, Faculty of Veterinary Science, University of Santiago de Compostela, Lugo, Spain; ⁴Food Processing Engineering Group, Department of Food Science and Technology, Oregon State University, Corvallis, USA

Abstract


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 mackerel; 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).

Supported by the Xunta de Galicia, Spain Project 10TAL402001PR, 2010-2012, and FCT (Portugal), European Union, QREN, FEDER and COMPETE thorough QOPNA research unit, Project PEst-C/QUI/UI0062/2013; FCOMP-01-0124-FEDER-037296. Also supported by he USDA National Institute of Food and Agriculture, Grants No. 2011-31200-06041 and 2012-31200-06041.
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 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 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 at 4ºC for 20 minutes. The supernatant (used as cathepsins extract) was filtered through a Whatman Nº1 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 0.0625 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°C during 5 minutes. 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 fluorescence.
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

![Figure 1](image-url)  
![Figure 2](image-url)

*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)

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

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Cathepsin B (FU/min/g)</th>
<th>Cathepsin D (µg tyrosine/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mackerel</td>
<td>218 839 ± 13 147</td>
<td>2.044 ± 0.138</td>
</tr>
<tr>
<td>Horse mackerel</td>
<td>13 303 ± 361</td>
<td>3.31 ± 0.263</td>
</tr>
</tbody>
</table>

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

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

Statistical analysis

<table>
<thead>
<tr>
<th>Holding time (min)</th>
<th>150 MPa</th>
<th>300 MPa</th>
<th>450 MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>az*</td>
<td>cy*</td>
<td>ax*</td>
</tr>
<tr>
<td>2.5</td>
<td>bx</td>
<td>bx*</td>
<td>by*</td>
</tr>
<tr>
<td>5</td>
<td>ay*</td>
<td>ax*</td>
<td>cz</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Holding time (min)</th>
<th>150 MPa</th>
<th>300 MPa</th>
<th>450 MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ax*</td>
<td>by</td>
<td>by</td>
</tr>
<tr>
<td>2.5</td>
<td>aby*</td>
<td>ax*</td>
<td>by</td>
</tr>
<tr>
<td>5</td>
<td>by</td>
<td>ax*</td>
<td>ax*</td>
</tr>
</tbody>
</table>

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.
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.

References


Mbarki R., Sadok S., Barkallah I. (2009): Quality changes of the Mediterranean horse mackerel (Trachurus medi-


Received for publication December 1, 2012
Accepted after corrections June 27, 2013

Corresponding author:
Dr JORGE ALEXANDRE SARAIVA, PhD, Universidade de Aveiro, Departamento de Química, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal; E-mail: jorgesaraiva@ua.pt