

Changes in Enzymatic Activity of Fish and Slaughter Animals Meat after High Pressure Treatment at Subzero Temperatures

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The aim of this study was to determine changes in the activity of proteolytic enzymes and transglutaminase of fish and mammal meat after pressurization at subzero temperatures. The activity was measured at the optimal pHs determined for enzymes from particular types of tested meat. It was found that increasing the pressure in the range of 60–193 MPa, did not change significantly the activity of acidic proteases of cod flesh, while the activity of neutral and alkaline proteases decreased drastically. Proteolytic enzymes from salmon flesh were more resistant than those from cod flesh. They maintained or increased (neutral protease) activity after pressurization. The activity of the endogenous enzymes of bovine meat increased with pressure increase, except for acidic proteases, the activity of which was reduced after treatment at 193 MPa to the level similar to unpressurized meat. Endogenous proteases of porcine meat were activated by high-pressure treatment. It has been shown that activity of TGase in unpressurized flesh from cod was 5 times higher than that from unpressurized salmon. Depending on the type of meat, these enzymes were also significantly different in their sensitivity to pressure. The pressure of 60 and 193 MPa led to a complete inactivation of the TGase in cod flesh, while the activity of salmon flesh TGase was decreased only by 15 and 21%, respectively.

INTRODUCTION

High-pressure technique is one of the most widely used non-thermal methods of food preservation, especially in meat, vegetable, and fruit industries. However, the practical use of this technique should take into account that pressurization causes not only inactivation of spoilage and pathogenic microorganisms in food but also affects most components, which are responsible for product quality [Truong *et al.*, 2015]. It is known that compounds with a low molecular weight, including aromatic substances, dyes or biologically-active molecules such as vitamins, remain unchanged [Tokuşoğlu 2016; Martysiak-Zurowska *et al.*, 2017]. However, pressure induces changes in other food components, such as proteins, including the enzymes, therefore in some cases may limit the usefulness of this method for the preservation or mild food processing. Depending on pressure and temperature, certain enzymes may be activated, while others are inactivated [Truong *et al.*, 2015]. These effects are determined by enzyme properties and origin and by the environmental conditions. Endogenous enzymes are important components of food products affecting their quality [Klomkloa *et al.*, 2012]. Some of them cause unfavorable effects, therefore, they should be inactivated during processing and storage of foods. On the other hand, some

enzymes lead to such changes in food which enhance its acceptability by the consumers.

Meat is a source of numerous proteolytic enzymes, which play an important role in tenderization of mammals meat. Limited and selective enzymatic proteolysis during aging leads to changes in the structure of meat, in connective tissue, and some of the myofibrillar proteins. Fragmentation of the myofibrils in mammals meat is positively correlated with its tenderness after heat treatment [Toldra & Reig, 2015]. In the case of fish flesh, enzymatic degradation of myofibrillar proteins leads to deterioration of fish texture, decreases the shelf-life, and promotes spoilage [Stoknes *et al.*, 2005; Ahmed *et al.*, 2015; Toldra & Reig, 2015].

Proteolytic enzymes are present in lysosomes and other organelles, in the sarcoplasm and intercellular liquid. They exhibit different sensitivity to high pressure. These enzymes derived from seafood meat are more sensitive to high pressures than their mammalian counterparts [Ashie & Simpson, 1996]. Pressure in the range of 100–200 MPa, generally increases the activity of cathepsins in mammals meat. This results from the release of enzymes from lysosomal into sarcoplasmic fluids caused by pressure-induced damage in lysosome membrane [Ohmori *et al.*, 1992]. The acid protease (including cathepsin D) of beef meat does not lose its activity after pressurization at 500 MPa, while neutral proteases are inactivated already under the pressure of 400 MPa [Ohmori *et al.*, 1991; Jung *et al.*, 2000]. Besides cathepsins also calpains are involved in tenderization of meat. The effect of high pressure on calpain activity depends on sev-

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eral factors: the pressure range, calpastatin activity in these conditions and changes in muscle proteins, *i.a.* fragmentation of myofibrils. At moderate pressures (100–150 MPa), the activity of calpain in the meat of rabbit and sea bass remains practically unchanged [Cheret *et al.*, 2005; Homma *et al.*, 1995]. It has been found, however, that under these conditions the denaturation of myofibrillar proteins takes place, which increases their susceptibility to enzymatic hydrolysis. At a higher pressure – above 300 MPa – the activity of calpain in sea bass, salmon, and rabbit meat drastically increases [Cheret *et al.*, 2007a; Homma *et al.*, 1995; Lakshmanan *et al.*, 2005].

Another enzyme, which affects the quality of food products, is TGase – glutamine gamma-glutamyltransferase. It is the calcium-dependent enzyme occurring in plants [Serafini-Fracassini & Del Duca, 2008], fish [Klomkiao *et al.*, 2012] and mammals meat [Esposito & Caputo, 2005] that catalyzes inter- and intra-molecular cross-links between proteins, peptides, and various primary amines [Toldra & Reig, 2015]. This property of TGase has been proven to be useful in improving functionalities such as gelation and emulsification [DeJong & Koppelman, 2002]. Endogenous TGase in fish flesh can catalyze the formation of cross-linked myosin heavy chains. This setting reaction of the fish *surimi* is thought to be important with respect to the rheological properties of fish flesh paste. The sensitivity of TGase to pressure treatment depended on its origin. The activity of TGase from horse mackerel (*Trachurus trachurus*) decreased by about 35% after pressurization at 300 MPa and 25°C for 15 min [Montero *et al.*, 2005], while Alaska Pollock TGase was completely resistant to the pressure in the range of 100–300 MPa [Ashie & Lanier, 1999]. There is no information about the influence of high pressure at subzero temperature on endogenous TGase activity in fish flesh. Our earlier work has shown that moderate pressure at subzero temperature causes denaturation of cod and salmon proteins and induces gelation of washed flesh from these fish [Malinowska-Pańczyk *et al.*, 2014]. Information about the activity of TGase in fish flesh treated by high pressure at subzero temperature is important for applying this technique to design new products from fish flesh.

Therefore, the effect of high pressure on the endogenous enzymes of meat should also be taken into account during determining the high-pressure process parameters. A number of studies have been conducted regarding the high-pressure effect on muscle enzymes but only fragmentary data are reported concerning pressure influence on protease and TGase of fish flesh and mammal meat at subzero temperature without freezing of water.

MATERIALS AND METHODS

Materials

Post rigor cod (*Gadus morhua*) and salmon (*Salmo salar*) fillets, pork *Longissimus dorsi* and bovine *Biceps femoris* muscle were purchased at a local market.

Pressure treatment

Samples of mammal meat and skinned fish flesh were minced in a meat grinder (mesh diameter $\phi=3$ mm), vacuum packed in polyethylene bags and pressurized at 60,

111 and 193 MPa at -5, -10 and -20°C, respectively. Pressurization step lasted 40 min while decompression was performed for 10 min. Under these conditions, the samples were pressure-treated in the unfrozen state. The procedure has been previously described in detail by Malinowska-Pańczyk *et al.* [2014].

General proteolytic activity

General proteolytic activity (GPA) in fish flesh and animal meat was estimated according to Erickson *et al.* [1983] with slight modification. Fifty grams of minced meat were centrifuged at 5000×g and 4°C for 30 min (MPW 350R centrifuge, MPW Med. Instruments, Poland). The supernatant was collected and used for enzyme assay at pH 2.5–9.5. Two mL of hemoglobin (2.5% solution dissolved in 0.01 mol/L HCl), 1 mL of McIlvain buffer (pH 2.5, 3.0, 3.2, 4.0, 4.2, 4.6, 5.4, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.4, 7.6, and 7.8) or Tris-HCl buffer (pH 8.5, 9.0, and 9.5), and 0.5 mL of the supernatant enzyme solution were added to a test tube. Mixtures with cod and salmon samples were incubated at 55°C for 3 h. Additional reactions with the supernatant of salmon sample were carried out at 65°C and pH 6.6, 6.8, 7.0, 7.4, 7.8, 8.5, 9.0, and 9.5. These conditions were selected according to Stoknes & Rustad [1995], as those that ensure the maximum activity of the proteolytic enzymes. In the case of pork and beef meat, the GPA was estimated at 37°C for 5 min and 3 h, respectively. At the end of the incubation period, 6 mL of 12% TCA (w/v) were added to each test tube. For the blank sample, the TCA was added to the supernatant solution immediately at the beginning of incubation. Thirty min after TCA addition, the solution was filtered through the filter paper (Filtrak 65 g/m², Munktel & Filtrak GmbH, Barenstein, Germany) and the absorbance was recorded at 280 nm (Spectrophotometer Spectroquant PHARO300, Merck Millipore, Germany). Proteolytic activity was expressed as an increase in absorbance at 280 nm (AbU) per h (or min in the case of pork meat) and mg protein.

The effect of high pressure on the proteolytic activity of the tested meat was estimated at pH in which the enzyme showed the maximum activity, *i.e.* 3.0, 7.0 and 8.5 for cod and salmon flesh, 4.2, 6.2 and 7.8 for beef meat, and 4.0, 6.0 and 7.0 for pork meat.

Transglutaminase activity

The activity of TGase was measured according to Montero *et al.* [2005] and Worratao *et al.* [2005]. A solution containing: 20 mmol/L Tris (2-amino-2-(hydroxymethyl)propane-1,3-diol), 5 mmol/L EDTA (disodium ethylenediaminetetraacetate), 2 mmol/L DTT (DL-dithiothreitol), and 10 mmol/L NaCl (pH 7.5) was added to minced cod and salmon flesh (1:2 w/v) and the sample was homogenized at 10,000 rpm for 2 min (Heidolph Silent Crusher, Heidolph Instruments GmbH, Germany). The homogenate was centrifuged at 9000×g and 4°C for 20 min. Five hundred μ L of the supernatant were mixed with 200 μ L of 1 mol/L TAE buffer (pH 6.0), 300 μ L of 0.1 mol/L carbobenzyloxy L-glutaminyglycine, 50 μ L of CaCl₂, and 50 μ L of 2 mol/L hydroxylamine. The samples were incubated at 37°C for 10 min and 1 mL of 12% TCA was then added. The mixture was centrifuged at 4000×g for 15 min at room temperature and absorbance of the supernatant was



measured at 525 nm. TGase activity was calculated based on the calibration curve with L-5-N-hydroxyglutamine (Sigma-Aldrich, cat. no. G2253) used as a standard. The amount of enzyme catalyzing the formation of 1 $\mu\text{mol/L}$ L-5-N-hydroxyglutamine within 1 min was assumed as a unit of activity.

Protein assay

The Biuret method was used to determinate protein concentration in meat juices and the supernatant obtained during TGase activity determination. Bovine serum albumin was used as a standard.

Statistical analysis

The data presented in figures and table are average values of at least three replications with standard deviation. The analysis of variance (one-way procedure) was performed to evaluate differences between treatments using the Statistica ver. 8.0 software [StatSoft Inc., USA].

RESULTS AND DISCUSSION

Effect of pH on the activity of proteolytic enzymes of meat

Optimal pH at which the endogenous proteolytic meat enzymes show the maximum activity may vary between species and even within one species [Wasson, 1992]. Thus at the first

stage of the study, the pH values at which endogenous enzymes have the maximum activity were determined. The results showed that acid, neutral and alkaline endogenous proteases of cod flesh had the optimal activity at pH 3.0, 7.4, and 8.5, respectively (Figure 1). Different values were demonstrated by Angsupanich & Ledward [1998], who showed that the activity of endogenous proteases of cod (*Gadus morhua*) after 3-h incubation at 55°C was the highest at pH 3.3, 6.6, and 9.0. In contrast, proteolytic enzymes of the flesh of Pacific cod (*Gadus macrocephalus*) after incubation at 50°C for 30 min were the most active at pH 3.2–3.6 and 7.8–8.0 [Erickson *et al.*, 1983].

The general proteolytic activity (GPA) of salmon flesh after 3-h incubation at 55°C was the highest in the acidic environment at pH 3.0 (Figure 2). At this temperature and higher pH values, the enzymes demonstrated minimal activity. Stoknes & Rustad [1995] showed that neutral and alkaline proteases of salmon flesh were more active at 65°C than at 55°C. In this work, it has also been found that at the temperature of 65°C the neutral proteases exhibited the maximum activity at pH 7.0 whereas the alkaline ones at pH 8.5 (Figure 2).

Acidic proteases of beef meat, in contrast to the fish flesh enzymes, had the maximum activity at higher pH values (pH 4.2). In turn, neutral and alkaline proteases were the most active at pH 6.2 and 7.8, respectively (Figure 3). For comparison, Ohmori *et al.* [1991] demonstrated that

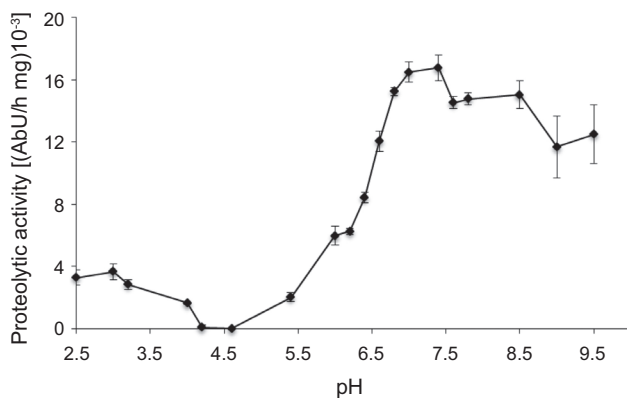


FIGURE 1. Effect of pH on the general proteolytic activity of cod flesh.

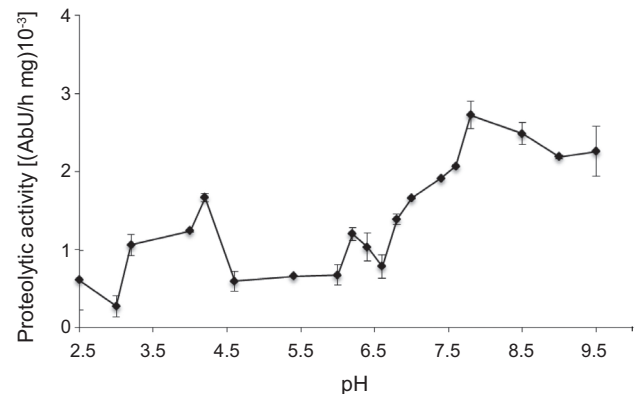


FIGURE 3. Effect of pH on the general proteolytic activity of beef meat.

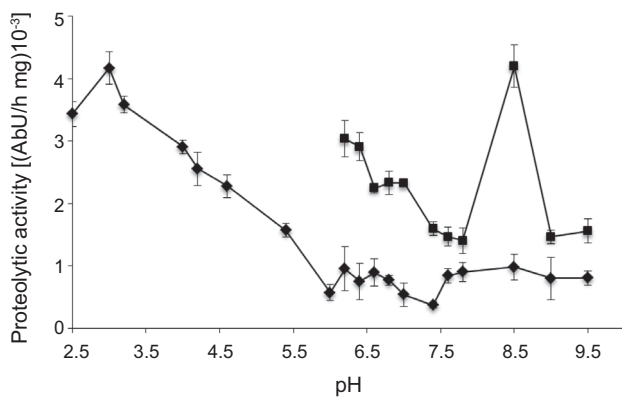


FIGURE 2. Effect of pH on the general proteolytic activity of salmon flesh; (◆) at 55°C and (■) at 65°C.

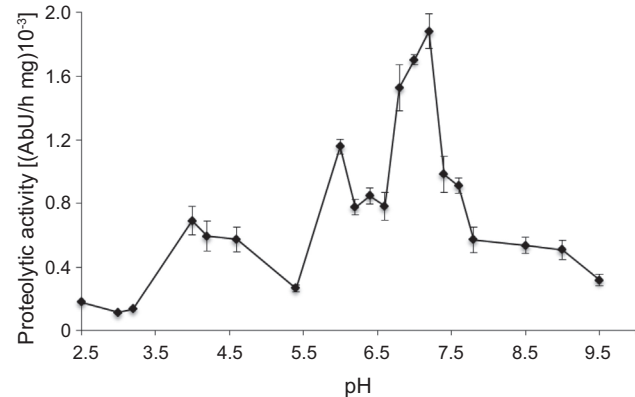


FIGURE 4. Effect of pH on the general proteolytic activity of pork meat.

beef enzymes were the most active at pH 3.0, 7.0, and 8.0. The values obtained by these authors are similar to those presented in our study, and a small discrepancy may be due to differences in the parameters of the assay: 24-h incubation at 30°C and 3-h incubation at 37°C, respectively. In contrast to the beef proteolytic enzymes, the proteases of pork meat were active at pH 4.0, 6.0, and 7.0 (Figure 4). These data are consistent with the findings reported by García-Barrientos *et al.* [2006].

Effect of high pressure treatment on the activity of proteolytic enzymes

Changes in the activity of endogenous proteolytic enzymes after pressurization were measured at the optimal pHs that were determined for enzymes from particular types of meat.

Fish flesh

It was stated that proteases from fish muscle are thought to be more pressure sensitive than those from mammals meat [Ashie & Simpson, 1996] but Jantakoson *et al.* [2012] report-

ed no change in protease activity in shrimp muscle after high pressure treatment up to 800 MPa at 28°C for 30 min. In our study, with increasing pressure in the range of 60–193 MPa at -5 to -20°C, respectively, the activity of acidic proteases of cod flesh did not change significantly, while the activity of neutral and alkaline proteases decreased 3 times (Figure 5A). Angsupanich & Ledward [1998] also reported the lowering of the activity of neutral cod proteases after pressurization at 200 MPa for 20 min at room temperature. However, the activity of acidic and alkaline proteases increased slightly under these conditions, but decreased at higher pressures of 400–800 MPa. In the case of enzymes of the flesh of sea bass (*Dicentrarchus labrax* L.), calpains (neutral and alkaline proteases) completely retained their activity after pressurization at 100 MPa, but at 300 MPa they were inactivated. The loss of calpains activity at high pressure was due to structural modifications and dissociation of the heterodimeric form of the protein [Cheret *et al.*, 2007b], while Homma *et al.* [1995] suggested the possibility of autolysis. The decreased calpain activity possibly prevents degradation of myofibrillar

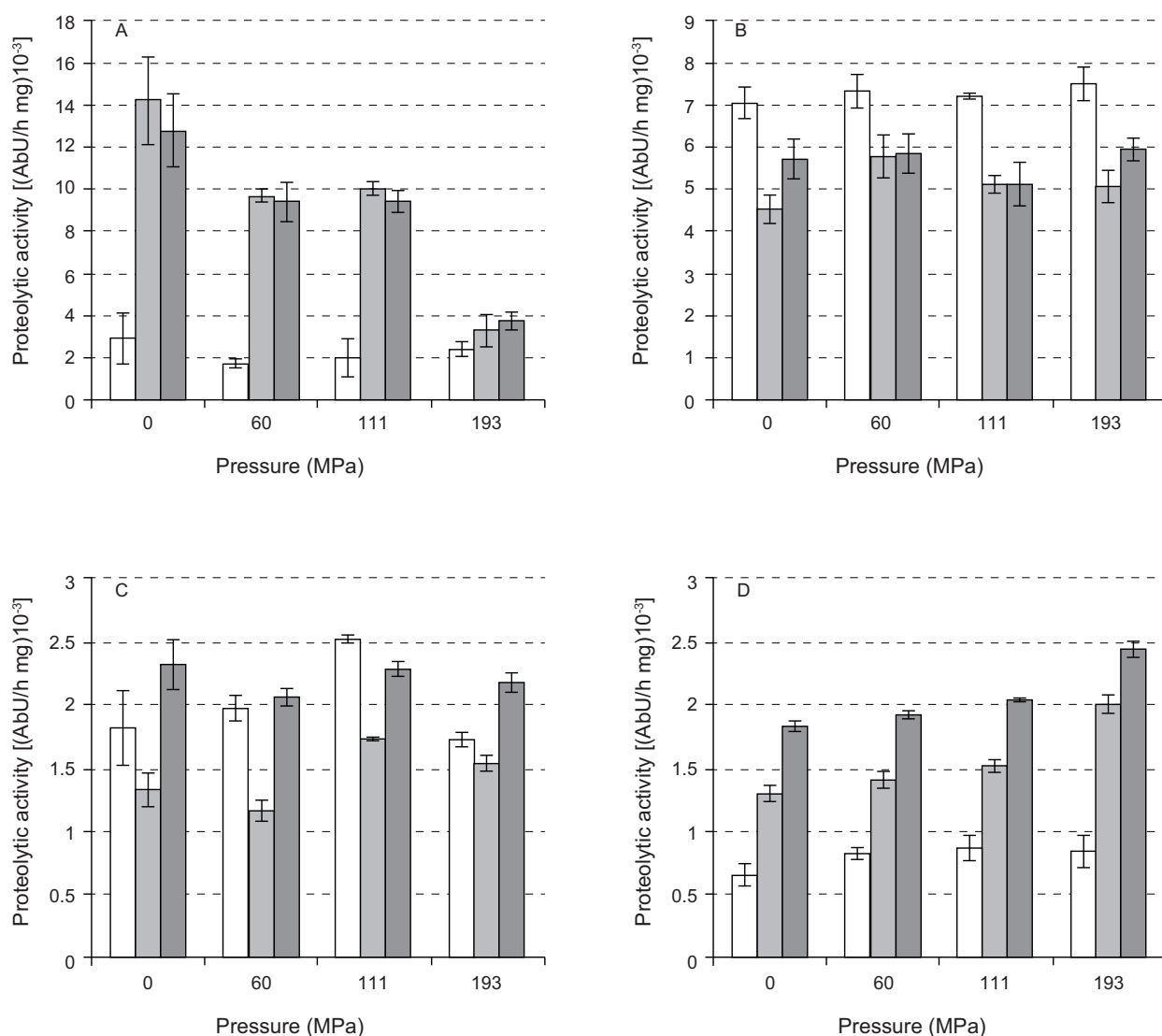


FIGURE 5. The activity of endogenous enzymes of A) cod: pH 3.0 (□), 7.0 (■), 8.5 (▣); B) salmon: pH 3.0 (□), 7.0 (■), 8.5 (▣); C) beef: pH 4.2 (□), 6.2 (■), 7.8 (▣); D) pork: pH 4.0 (□), 6.0 (■), 7.0 (▣) meat after pressure treatment at subzero temperature.

fish proteins under high pressure. Such a possibility was highlighted in the review article of Campus [2010] and in a work by Duranton *et al.* [2014].

Proteolytic enzymes of salmon flesh were more resistant to pressures in the range from 60 to 193 MPa at the temperature from -5 to -20°C than cod enzymes (Figure 5B). The activity of acidic and alkaline proteases did not change after pressure treatment at 60–193 MPa, whereas the activity of neutral proteases increased after pressurization of 60 MPa. At the higher pressure (above 60 MPa) these enzymes activity decreased to the value noted in the untreated control samples. Higher pressure-resistance of proteolytic salmon enzymes compared to cod enzymes may be due to the protective effect of lipids. Salmon flesh contains about 7%, and cod flesh only 0.5–1% of lipids [Łuczyńska *et al.*, 2014; Zeng *et al.*, 2010]. In the available literature, there is no data on the activity of proteases in salmon flesh as affected by pressure treatment. Only Lakshmanan *et al.* [2005] have shown that the activity of the endogenous protease of cold-smoked salmon flesh at pH 6.0 decreased after pressure treatment at 150 MPa and 9°C, and then increased at 200 and 300 MPa to the level as in the unpressurized sample. In this range of pressure, the activity of proteolytic enzymes at pH 6.5 did not change significantly. A similar phenomenon was observed in the case of cathepsin D in Atlantic horse mackerel, the activity of which increased about 2-fold after pressurization at 300 MPa for 5 min, while 450 MPa treatment provoked a decrease in its activity to the same value as in the unpressurized sample [Fidalgo *et al.*, 2014].

Mammals meat

The activity of the endogenous enzymes of bovine meat, except at 7.8 pH, increased by about 25% after pressurization at 111 MPa and -10°C (Figure 4C). However, after the treatment at 193 MPa their activity decreased to the level similar to that in the unpressurized meat. Ohmori *et al.* [1991] have shown that the activity of the acidic, neutral, and alkaline proteases of bovine meat did not change even after pressure treatment of 400 MPa at room temperature. The increase of cathepsins B, D and L activity after pressurization up to 400 MPa was also observed in beef meat [Homma *et al.*, 1994]. However, Koohmaraie *et al.* [1984] and Homma *et al.* [1995] observed the decreased calpain activity in pressurized beef (100 MPa, 37°C, 2 min) and rabbit meat (200 MPa, 2°C, 5 min), respectively. Almost complete inactivation was observed at 400 MPa [Homma *et al.* 1995], which was related to the lower distribution of calpains in pressurized beef muscle, as observed with the immunogold electron microscopy [Borjigin *et al.*, 2006].

Next, endogenous acidic and neutral proteases of porcine meat (with optimal activities at pH 6.0 and 7.0, respectively) were activated by high-pressure treatment (Figure 4D). Their activity increased by 66 and 36%, respectively, with pressure increase up to 193 MPa. Some authors have pointed out that the activation of neutral and alkaline proteases (calpains) in pressurized meat may be induced by an increasing concentration of calcium in the cytosol as a result of disruption of the endoplasmic reticulum and mitochondria [Cheret *et al.*, 2007a]. In contrast, Grossi *et al.* [2012]

TABLE 1. Activity of endogenous TGase of cod and salmon flesh after pressurization at 60 and 193 MPa.

Pressure (MPa)	Activity of endogenous TGase (U/mg proteins)	
	Salmon flesh	Cod flesh
0.0	0.417±0.028 ^a	2.136±0.094 ^a
60	0.353±0.015 ^b	Nd. ^b
193	0.328±0.078 ^b	Nd. ^b

Nd. – not detected; ^{a,b} – values for a particular column followed by different letters differ significantly ($p < 0.05$) (mean ± SD, $n = 6$).

showed that the activity of μ -calpain in fresh porcine *longissimus* muscle decreased by 15 and 80% after pressurization at 50 and 100 MPa, respectively. However, m-calpains activity was almost unaffected under these conditions. Complete inactivation of both m- and μ -calpain was achieved at 200 MPa [Grossi *et al.*, 2012].

In the case of acidic proteases (pH 4.0) of pork meat, the increase of pressure caused only 28% increase in their activity. This may be due to the release of acidic proteases to the cytosol as a result of disintegration/modification of the lysosomal membranes [Jung *et al.*, 2000; Kubo *et al.*, 2002; Duranton *et al.*, 2014]. In the available literature there is lack of data on the effect of pressure treatment on the endogenous enzymes of raw porcine meat. However, Campus *et al.* [2008] showed that activities of cathepsin B and B+L of dry-cured pork loins were not affected significantly at 300 and 350 MPa while at 400 MPa there was a decrease of around 20% of their initial activity. In contrast, increasing activity of cathepsins B and L in brine-enhanced *semitendinosus* has been noticed after pressurization at 600 MPa [Grossi *et al.*, 2012].

Effect of high pressure treatment on transglutaminase activity

TGase isolated from various organisms differs in the optimum pH at which it exhibits the maximum activity. In oysters meat (*Crassostrea gigas*), this enzyme shows the maximum activity at pH 8.0 [Kumazawa *et al.*, 1997], while in tilapia flesh (*Oreochromis niloticus*) at pH 7.0–7.5 [Worratao *et al.*, 2005]. The optimal temperature ensuring the maximum activity of the enzyme also depends on its origin. TGase from carp and bream flesh is the most active at 50°C, whereas that from the flesh of threadfin breams (*Nemipterus virgatus*) and white croaker (*Genyonemus lineatus*) – at 40 and 30°C, respectively [Tsukamasa *et al.*, 2002]. These authors showed also that TGase from different sources retained at least 80% of its maximum activity at 37°C. Therefore, in our study TGase activity in cod and salmon flesh was measured at this temperature. It has been shown that the activity of this enzyme in cod was 5 times higher than in salmon (Table 1).

Depending on the type of meat, these enzymes are also significantly different in sensitivity to pressure. The pressure treatment at 60 and 193 MPa led to complete inactivation of TGase in cod flesh, whereas the activity of salmon flesh TGase was decreased by only 15 and 21%, respectively (Table 1). Montero *et al.* [2005] have also shown that the activity of TGase from horse meat (*Trachurus trachurus*) subjected

to a pressure treatment at 300 MPa and 25°C for 15 min decreased by 35%. Zhou *et al.* [2013] demonstrated inactivation of threadfin bream (*Nemipterus bleekeri*) TGase as a result of the decrease of ϵ -(γ -glutamyl) lysine linkage formation. In contrast, endogenous TGase of Alaska pollock (*Theragra chalcogramma*) flesh and microbial TGase added to surimi were resistant to 15-min pressure treatment in the range 100–300 MPa at 4°C [Ashie & Lanier, 1999], which indicates that this enzyme has different pressure sensitivity depending on its origin.

CONCLUSIONS

The high pressure treatment at subzero temperature causes a number of changes in the components of the muscle tissue of fish and mammals [Malinowska-Pańczyk *et al.*, 2014]. Some of them, *e.g.* inducing gelation of proteins, may be used in the food industry for designing new products with improved functional and sensory properties, while others limit the use of this technique. The present study has demonstrated high pressure effects on the activity of proteolytic enzymes of fish flesh and mammals meat. Pressurization can be useful in reducing the softening of fish flesh because it decreases the activity of neutral and alkaline proteases of cod flesh. By contrast, the exposure of mammals meat to high pressure of 193 MPa at -20°C may enhance the endogenous proteases participating in meat tenderization. Moreover, such phenomena may coincide with the inactivation of microorganisms, since the pressure of 193 MPa leads to a reduction in the number of meat spoilage microflora [Malinowska-Pańczyk & Kołodziejaska, 2013].

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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