High-Pressure Effects on Lysosome Integrity and Lysosomal Enzyme Activity in Bovine Muscle

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This study was conducted to determine whether the application of high hydrostatic pressure could modify the enzymatic activity and membrane integrity of lysosomes in muscle. Several combinations of pressure (0−600 MPa) and time (0−300 s) were applied to two types of samples: purified enzymes (cathepsin D and acid phosphatase) in buffer solution and intact muscle (biceps femoris). The enzymes studied showed varying degrees of susceptibility depending on the level of pressure, holding time, and environment. Acid phosphatase activity was minimally affected by pressure in buffer solution, whereas cathepsin D was modulated significantly by the pressure and time applied. The activities of the enzymes extracted from meat increased with pressure. The cytochemical observations showed the presence of primary and secondary lysosomes in muscles. After pressurization, the membrane integrity of the lysosomes was modified. A correlation could be established between lysosomal enzymatic activities and the lysosome membrane breakdown.

Keywords: *High pressure; meat; bovine muscle; lysosome; cathepsin*

INTRODUCTION

Aging of bovine meat requires a refrigerated storage period of several days. During this period physicochemical and enzymatic phenomenon occur and the structural integrity of myofibrils is altered (Koohmaraie, 1996). Two proteolytic enzymatic systems are implicated in these modifications: the calpains and the cathepsins. However, the cathepsins are entrapped in lysosomal bodies and their participation in meat aging requires liberation into the cytosol (Goll, 1983). This release occurs during meat storage with the pH fall (Etherington, 1992).

To reduce the aging period and improve ultimate tenderness, several processes have been studied including electrical stimulation (Dutson et al., 1980), infusion of Ca^{2+} (Koohmaraie et al., 1988), acid marination (Ertbjerg et al., 1999), and ultrasonication (Got et al., 1999). High hydrostatic pressurization has also been proposed as a new technology for tenderization (Suzuki et al., 1992). This treatment is expected to accelerate lysosomal enzyme liberation. Several authors have suggested that the earlier release of lysosomal enzymes observed after pressure treatment is due to the breakdown of the lysosomal membrane (Homma et al., 1994; Elgasim et al., 1983). This breakdown correlates with increased activity of enzymes such as acid phosphatase and β-glucuronidase (Ohsumi et al., 1983). Elgasim et al. (1983) reported an increase of β-glucuronidase activity with pressure increase. However, this increase is not linear. These authors explained this profile as due to the inactivation of a fraction of this enzyme. Ohmori et al. (1992) also suggested the destruction of the membrane of the lysosomes to explain the increase in the enzymatic activity of bovine liver observed after high-pressure treatment.

Numerous parameters must be taken into account to establish an efficient process based on high-pressure technology. The main parameters seem to be the pressure, holding time, and medium composition. Ohmori et al. (1992) showed that the activity of carboxypeptidase extracted from bovine liver was unchanged at 200 MPa but decreased at 300 MPa. Horgan and Kuypers (1983) mentioned that the phosphorylase activity was increased 4 times after 1 min at 150 MPa, but that lengthening the treatment to 10 min provoked a drastic decrease in activity. Weemaes et al. (1998) reported that the minimal pressure required to inactivate polyphenoloxidases (PPO) was dependent on the medium composition.

It appears difficult to draw a conclusion on the effects of high pressure on lysosomal enzyme activities. There are no data showing clearly either the lysosomal membrane breakdown or the possible correlation between pressure levels, duration and medium composition, and lysosomal enzyme release. Therefore, the objective of this work is to study the effect of high pressure on the integrity of lysosomes and to show a correlation between lysosomal breakdown and the evolution of enzymatic activity. The effects of pressure level and holding time on cathepsin D and acid phosphatase activities are studied on purified enzymes in buffer solution and in intact muscle. In parallel, a cytochemical method using transmission electron microscopy was employed to determine the effects of pressurization on the integrity of lysosomes. This technique used acid phosphatase as the lysosomal marker and cerium as the capture agent (Robinson and Karnovsky, 1983).

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10.1021/jf9908648 CCC: $19.00
Published on Web 05/12/2000

Table 1. Independent Variables

pressure, MPa	holding time, s	pressure, MPa	holding time, s
50	20	520	260
130	60	600	300
325	160		

MATERIALS AND METHODS

Animals. Animals (dairy cows, 6 years old, 300−400-kg weight) were slaughtered in a local abattoir (Ets Levesque, Blain, France). The muscles (biceps femoris) were vacuum packed at 24 h post-mortem and stored at 4 °C until pressurization (48 h post-mortem).

Sample Preparation. Extraction of enzymes from pressurized beef was done as described by Homma et al. (1994). Commercial cathepsin D from bovine spleen (E. C. 3.4.23.5, C-3138), denatured hemoglobin (H-2625), *p*-nitrophenyl phosphate (N22002), and acid phosphatase from bovine prostate (P-6409) were purchased from Sigma (StQuentin Fallavier, France). Cathepsin D was dissolved in 0.2 M sodium acetate/acetic acid buffer (pH 3.7) and acid phosphatase was suspended in 0.1 mM sodium acetate buffer (pH 5.5).

Sample Pressurization. Commercial solutions and meat samples were vacuum packed in polyethylene bags and pressurized at 10 °C using a 3-L reactor (diameter 12 cm, height 30 cm) from Alstom Fluides and Mecanique (Nantes, France). The pilot is equipped with a thermoregulated system and the medium of pressurization is water. The conditions of treatment (pressure and holding time) were determined by the experimental design. Pressure was increased and released at 3 and 5 MPa/s, respectively. After pressure treatment, samples were kept at 4 °C and analyzed.

Biochemical Analysis. Determination of the total protein of each extract was performed by the bicinchoninic acid protein method (Sigma, Procedure N°TPRO-562) with bovine serum albumin used as the standard. Cathepsin D activity was determined with hemoglobin as the substrate according to Anson's method (1938). Acid phosphatase activity was evaluated according to Ohmori et al. (1992). The activities of cathepsin D and acid phosphatase were determined as absorbance per gram of protein. The relative activity was the ratio between treated and untreated samples. The untreated sample had 100% activity.

Experimental Design. A two-level factorial central composite rotatable design with three replicates of the center points was chosen to study the effects of the independent variables (Cochran and Cox, 1957). The variables studied were pressure and holding time. The levels of each variable are summarized in Table 1. The domain of pressure and time were explored in 11 experiments. These experiments constituted one block. The design was replicate twice.

Commercial preparations and pressurized meat extract activities of cathepsin D and acid phosphatase were the response values. Cytochemical observations constituted a qualitative response.

Statistical analysis was performed by using Statgraphics plus 2.1 software (Statistical Graphics Corp, Rockville, MD). Surface response methodology was used to study the simultaneous effect of the two variables.

Cytochemistry of Acid Phosphatase. The identification of lysosomes was based on an electron microscopy cytochemical procedure and was replicated on three different animals. This technique employed β-glycerophosphate as a substrate which the enzyme (acid phosphatase) alters into products that can be precipitated as visible deposits by a trapping agent (cerium) included in the incubation media (Robinson and Karnovsky, 1983).

Biceps femoris muscle samples of approximately 2 × 5 × 4 cm were treated under the conditions described in the experimental design. All solutions used were stored at 4 °C. After pressure treatment the muscle tissue was quickly cut into smaller pieces of approximately 2 × 2 × 10 mm and transferred to 0.1 M cacodylate buffer (pH 7.3) with 0.1 M sucrose

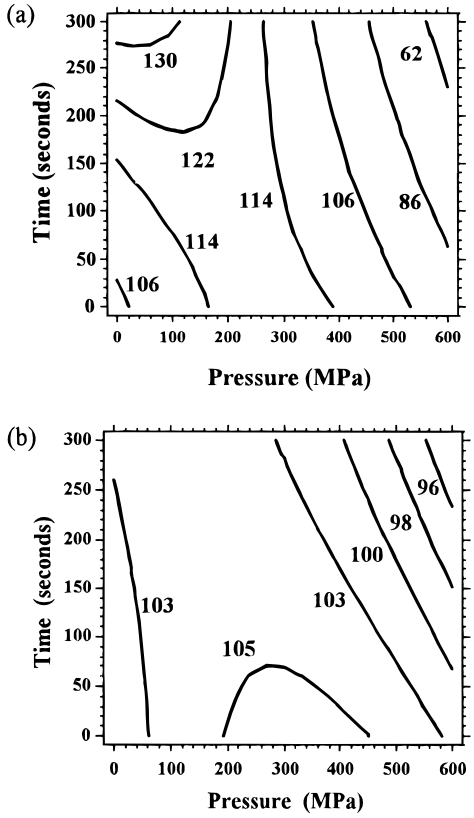

Figure 1. Contour plot of relative activity of (a) cathepsin D and (b) acid phosphatase in buffer solution as a function of pressure and time.

contained 1% glutaraldehyde or 1% paraformaldehyde. Prefixation was carried out for 1 h and samples were cut into final dimensions of 1 mm thick by 1 cm long. After several washings with cacodylate buffer, the pH was lowered with acetate buffer to the desired pH for the reaction, pH 5.0.

The reaction mixture consisted of 0.1 M acetate buffer (pH 5.0), 4 mM $CeCl_3$, and 1 mM β-glycerophosphate. The reaction was carried out at 37 °C for 3 h. The medium was replaced with fresh medium after 1 h. Controls omitted β-glycerophosphate. The tissue blocks were washed successively with acetate then cacodylate buffer. Following the cytochemical reaction, the samples were refixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4 °C.

Electron Microscopy. The samples were washed with cacodylate buffer and postfixed with 1% osmium tetroxide for 2 h. After successive dehydration with ethanol solutions (from 25% to 100%), the tissues were incubated first with different solutions of embedding media/ethanol (50/50, 75/25) and then in Spurr's embedding mixture (14300, Euromedex, Souffelweyersheim, France). The samples were embedded in silicone rubber molds and polymerized at 60 °C for 3 days. Sections of 90 nm were cut on an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. Specimens were examined in a JEOL Jem-200 CX (Tokyo, Japan) operated at 120 kV.

RESULTS AND DISCUSSION

Effect of High Pressure on Purified Enzymes in Buffer Solutions. Activity of purified cathepsin D activity in buffer solutions at different pressures and times are summarized in Figure 1a. Both time and pressure caused significant changes in activity. As shown in this figure, up to 400 MPa and 150 s, the activity of cathepsin D was higher than that of the control. The highest values (130%) were obtained at 40 MPa and 280 s. At 280 s, a pressure increase led to a

decrease of cathepsin D activity. For moderate pressures the activation of cathepsin D was similar to the results of Anese et al. (1995) for peroxidase. Whatever the pressure, the activity of the enzyme remained constant for 1 week of storage at 4 °C in buffer. Therefore, contrary to Ashie and Simpson (1996) who noticed a reactivation of cathepsin C, the treatment did not induce reversible effects at pressure below 300 MPa.

For acid phosphatase (Figure 1b) only pressure had a significant effect on the activity. For the highest time and pressure applied (600 MPa/300s), the decrease of acid phosphatase activity was only 4%.

These results show that in these conditions of treatment, the denaturation of proteins occurred only for pressures higher than 500 MPa. It appears from this study that the acid phosphatase was less pressure-sensitive than cathepsin D in solution. The lower pressure-sensitivity of acid phosphatase was in accord with the results of Seyderhelm et al. (1996) who reported a reduction of enzyme activity of 10% at 600 MPa and 30 min in Tris buffer. The difference between the two enzymes could be due to structural differences and/or to their conformation after treatment (Balny and Masson, 1993). Cathepsin D has a molecular weight between 42 000 and 53 000 Da (Zeece et al., 1986) whereas that of acid phosphatase is around 100 000−110 000 Da (Barrett and Heath, 1977).

The acid phosphatase was barostable within the conditions tested and consequently can be used as a good index of lysosomal disruption in meat extracts. In fact whatever the experimental conditions, the possible modulation of its enzymatic activity is negligible. An increase of acid phosphatase activity could only be correlated with lysosomal membrane breakdown.

Effect of High Pressure on Meat Cathepsin D and Acid Phosphatase Activities. Activities of cathepsin D and acid phosphatase extracted from pressurized meat are shown in Figure 2. It appears that meat cathepsin D and acid phosphatase activities do not show the same trend as obtained in buffer solutions. For cathepsin D, only pressure had a significant effect. This enzyme had two behaviors depending on the range of pressure and time applied. For points less than 170 MPa and lower durations of treatment, activities of cathepsin D rose progressively but remained less than the control. For higher pressure, a sharp increase of cathepsin D activity was observed. The highest values occurred at a pressure of 500−600 MPa for short times (less than 200 s).

The activities of acid phosphatase of meat at high pressure (Figure 2b) were always higher than the control and increased with all combinations of pressure and time investigated. As previously indicated, acid phosphatase is an indicator of lysosomal membrane breakdown (Ohsumi et al., 1983). Consequently, higher activity of this enzyme compared to the control could indicate lysosomal membrane breakdown in pressurized meat. This result was in agreement with those obtained by Homma et al. (1994). The increase of both cathepsin D and acid phosphatase activities with the pressure supports the breakdown of the lysosomal membrane.

The results show that the effects on enzyme activities depends on the type of enzyme and the environmental conditions of treatment. The medium composition differences between cathepsin D and acid phosphatase activities could be explained by protein−protein interactions (Galazka et al., 1996) or changes in the micro-

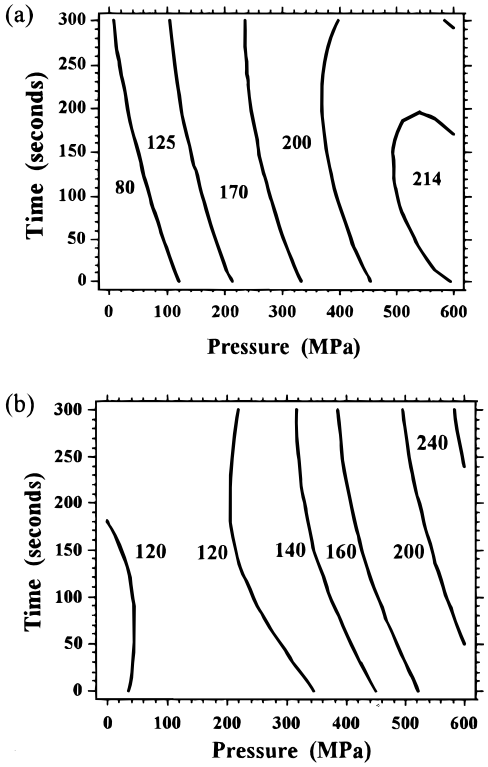

Figure 2. Contour plot of relative activity of (a) cathepsin D and (b) acid phosphatase in a complex medium as a function of pressure and time.

environment of the enzymes such as pH, ionic strength, and physicochemical equilibrium. High pressure could also affect the activity of the inhibitors of the enzymes. Homma et al. (1995) have shown the decrease in calpastatin levels, an inhibitor of the calpains, after pressurization. Etherington (1992) reported that cathepsin D does not have either an activator or an inhibitor.

The results obtained in buffer solution and meat extract suggest that the integrity of lysosomal membranes are altered by the treatment. However, the increase of activity could be the consequence of enzyme activation. So to test this hypothesis, studies of the ultrastructure of the lysosomes were carried out.

Cytochemical Observations of the Lysosomes after High-Pressure Treatment. The same experimental design as used previously was applied to bovine muscles from three animals for cytochemical observations. In the muscle tissue of the control, primary and secondary lysosomes were observed. These observations were in agreement with those obtained by Etherington (1992) and Taylor et al. (1995). The results were reproducible from one animal to another.

The primary lysosomes have an intense staining and a diameter of approximately 100 nm (Figure 3A). The secondary lysosomes were characterized by a larger diameter (around 400 nm) and a heterogeneity of structure (Figure 3B). These lysosomes result from the fusion between a primary lysosome and other cellular organelles such as a lipid droplet (Figure 3C) and/or by transport of material into lysosomes (Schellens et al., 1977; Berg et al., 1995).

For treated samples, whatever the pressure values, three populations of lysosomes were observed: unmodified, partially modified, and altered lysosomes (Figure 3D−F). Some modified lysosomes were 3 times larger than the primary lysosomes of the control and showed

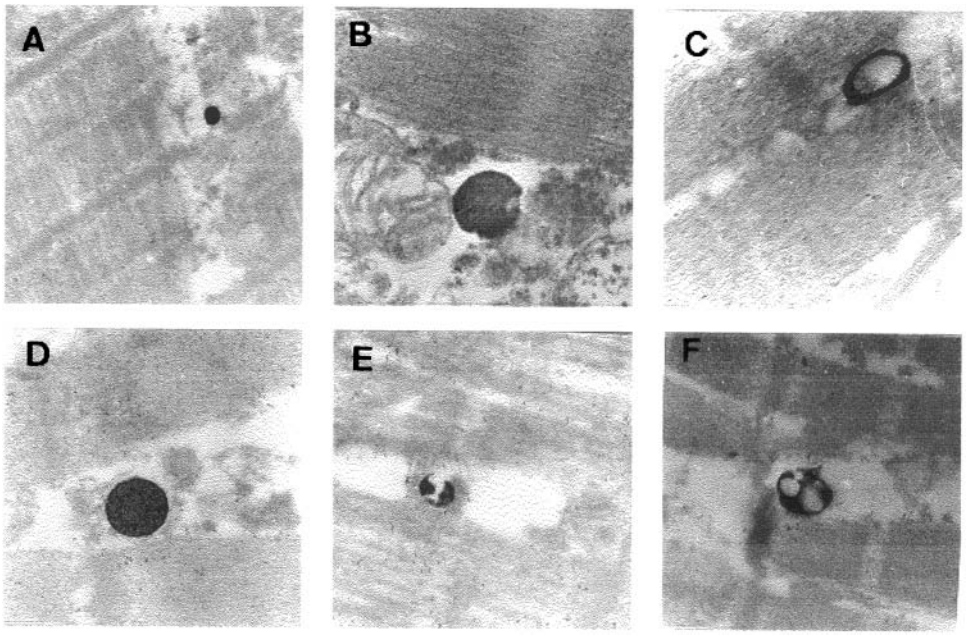

Figure 3. Ultrastructural localization of lysosomes in bovine meat. The stained reaction product represents acid phosphatase activity. Stained with uranyl acetate and lead citrate. (A) Primary lysosome with an intact membrane (15000×); (B) secondary lysosome with an heterogeneous structure (42000×); (C) secondary lysosome associated with a lipid droplet (45000×); (D) high-pressure modified lysosomes. Note the diffuse staining (43000×) (E) and (F) disrupted lysosomes. Note the breakdown of the membrane (43200× and 28000× respectively).

a more diffuse staining (Figure 3D). This could be explained by the dilution of the acid phosphatase due to the volume increase of the lysosome. This volume variation can be attributed to the modification of membrane permeability by pressure as reported by MacDonald (1992).

The altered lysosomes (Figure 3E,F) differ from the primary and secondary lysosomes by having a discontinuity in the structure (white spots). This breakdown could be the consequence of an excessive volume increase or of a direct effect of high pressure since they are not found in controls.

The microscopic observations clearly established that high pressure modified the membrane integrity of the lysosomes. All lysosomes were not altered in the same way and the effects were not linked to the pressure level. The results from the cytochemical work confirm those obtained for the enzymatic activities: the increase of acid phosphatase activity after the high-pressure treatment is a consequence of the breakdown of some lysosomes. The proportion of pressure damaged lysosomes was not quantified by the cytochemistry, but it is highly likely that this proportion increased with rising pressure, as the biochemical results suggest.

We have established a correlation between the lysosomal enzymatic activities and the lysosome breakdown. The increase of lysosomal enzyme activity might result in tenderization of meat. However, detailed studies of high-pressure treatment on meat textural modifications must be done in order to correlate these two parameters.

ACKNOWLEDGMENT

Gratitude is expressed to Mrs. S. Delepine for her technical assistance.

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Received for review August 5, 1999. Revised manuscript received March 1, 2000. Accepted March 13, 2000. This study was financed by Région Pays de la Loire of France.

JF9908648