Effect of electrohydraulic shockwave treatment on tenderness, muscle cathepsin and peptidase activities and microstructure of beef loin steaks from Holstein young bulls

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Abstract

Hydrodynamic pressure processing (HDP) or shockwave treatment improved tenderness of beef loin steaks by a reduction of 18 % in Warner-Bratzler shear force (WBSF). Endogenous muscle proteolytic activities (cathepsins and peptidases) and protein fragmentation of sarcoplasmic and myofibrillar proteins detected by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) were not influenced by HDP. However, physical effects on the microstructure using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were clearly detected, namely, a disruption of the structure at the muscle fiber bundles and an increased endomysium space was observed. The present paper supports the evidence of physical disruption of the muscle fibers as the cause behind the tenderness improvement. The paper discusses the possible mechanisms responsible for the meat tenderisation induced by HDP treatment.

Keywords: Hydrodynamic pressure processing, shockwave, meat tenderisation, cathepsin activity, peptidase activity, muscle microstructure.

1. Introduction

Meat tenderness has long been recognized as the most important quality trait for consumer acceptability of fresh meat as steaks (Mennecke, Townsend, Hayes, & Lonergan, 2007). Hence, meat tenderness greatly influences important aspects regarding the commercialization and acceptability of fresh meat such as eating satisfaction, repeated purchase and price (Grunert, Bredahl, & Brunsø, 2004). Meat tenderness is the consequence of the architecture and the integrity of the skeletal muscle cell (Lonergan, Zhang, & Lonergan, 2010). Namely, three factors determine meat tenderness: (1) the degree of shortening of muscle fibers after rigor mortis, (2) the extent of fragmentation of muscle proteins responsible for the structure as a consequence of protein breakdown or proteolysis and (3) the specific physical phenotype which is associated to particular genetic and environmental effects, composition, connective tissue content (Bolumar, Enneking, Toepfl, & Heinz, 2013). The relative contribution of these individual factors determines the final tenderness.

Taking into account the importance of meat tenderness, many investigations have tackled the problem to find solutions which can be applied in the meat industry (Thompson, 2002). Industrial meat tenderization strategies include biological methods such as control of pH and temperature at slaughter, electrical stimulation, tenderstretch and tendercut, hot boning for improving muscle chilling and stretching, traditional aging, chemical methods such as post-exsanguination vascular infusion, addition of exogenous proteases, solubilizing agents like salt, marination, addition of calcium, and mechanical methods such as grinding, blade or needle tenderization and the application of high pressure processing (HPP) pre- or post-rigor in combination or not with temperature (Bolumar et al., 2013). These methods allow certain manipulation of the levels of shortening of the sarcomere, protein hydrolysis and distension of muscle structure. However, these methods also have important drawbacks such as they are difficult to implement at the slaughterhouse, cannot cope with the high variation of the cattle, require high processing cost in terms of time and energy (14-21 days of maturation) or are encompassed with an important price reduction of the product (for instance: grinding).

69 A novel post-mortem intervention to tenderize meat is the processing by hydrodynamic pressure
70 processing (HDP) or shockwave. A shockwave is the instantaneous development of pressure waves
71 which travel through water and any material which is an acoustical match to water (for instance:
72 meat). The travelling of the pressure wave through the meat induces a tenderization effect (“rupture
73 effect”) which is likely due to dissipation of mechanical stress at the boundaries of acoustic
74 impedances. HDP applied to meat tenderization represents a low-cost and non-invasive method which
75 has no negative impact on the microbiological and chemical product stability (Solomon, Long, &
76 Eastridge, 1997; Moeller et al., 1999; Claus et al., 2001; Bowker, Callahan, & Solomon, 2010,
77 Solomon, Liu, Patel, Bowker, & Sharma, 2006; Solomon, Sharma, & Patel, 2011). So far, most of the
78 tenderization studies by HDP-treatment have been carried out in prototypes which rely on the use of
79 explosives. The use of explosives in the meat industry has associated concerns of safety for operators
80 and potential restrictions due to contact with residues. An alternative is the generation of shockwaves
81 by electrical discharges under water as it is the prototype employed in the present study. The use of
82 underwater electrical discharges allows a continuous processing as load of explosives is not required
83 and permits a better parameterisation of shockwave intensity by adjusting electrical capacity, voltage
84 and number of pulses (Bolumar et al., 2013). Therefore, the potential applicability of shockwave
85 generated by electrical discharges as a tenderizing method for meat considered as tougher by the
86 consumer like young bulls is very relevant.

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88 Two main mechanisms have been reported to be responsible for the tenderization effect by HDP: (1)
89 an instantaneous effect on the meat structure due to alterations within the sarcomeric structure of the
90 myofibrils (Zuckerman, & Solomon, 1998) and separations at the Z-line (Claus, 2002) and (2) an
91 enhanced or accelerated maturation (Solomon, Berry, Paroczay, Callahan, & Eastridge, 2002;
92 Callahan et al., 2002; Paroczay, Solomon, Berry, Eastridge, & Callahan, 2002; Heinz, Töpfl,
93 Schwägele, & Münch, 2011). The latter must be due to enzymatic activation of proteases and/or to
94 facilitated contact and breakdown of the structural muscle proteins caused by the alteration suffered in
95 the integrity of the skeletal cell. The mechanisms behind the accelerated maturation after HDP are not
96 fully understood. Therefore, it is of importance to investigate the effect of shockwave treatment on the

endogenous proteolytic activities like cathepsins and peptidases since no studies have been reported relating shockwave and protease activities so far to the best of our knowledge.

In the present study, the effect of HDP shockwave on the tenderness, cathepsin and peptidase activities, muscle protein hydrolysis and microstructure from steaks of young bulls is investigated in order to gain insights about the mechanisms leading to meat tenderisation by shockwave.

2. Materials and methods

2.1. Muscle samples

Boneless beef strip loins (*longissimus lumborum*) from Holstein young bulls (average 2.5 years) were purchased from a local slaughterhouse. The strip loins were sliced into steaks of 26 mm-thick and immediately after vacuum packed in plastic bags (total bone guard (TBG) bags, Cryovac Sealed Air Corporation, New Jersey, USA). Half of the steaks were randomly assigned for control and the other half for HDP or shockwave treatment. Traceability of the steaks from the individual animal was maintained along the whole study. Five animals were included in the study.

2.2. Experimental design

Samples were taken at days 1 and 7 after treatment. Tenderness measurements were performed after 7 days of storage under refrigerated conditions, simulating the commercial life of meat from processor to supermarket and consumer. Proteolytic enzyme activities (cathepsin and peptidases) were measured at day 1 after shockwave treatment in order to ascertain if the shockwave treatment was inducing an effect on them. Analysis of protein hydrolysis by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and microscopy analysis by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were carried out at day 1 and 7 in order to analyse the effect of shockwave on the microstructure. Samples for tenderness and microscopy analysis were delivered to the laboratory the immediate day of sampling in order to prevent from any structural change due to storage conditions. Samples for proteolytic activities and protein pattern analysis were frozen at the respective day in liquid nitrogen and kept at -80 °C till analysis.

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2.3. Hydrodynamic pressure processing (HDP) or shockwave treatment

The meat steaks were submitted to HDP in the shockwave prototype constructed by the German Institute of Food Technologies (DIL, Quakenbrueck, Germany). This prototype is based on electrical discharge under water. The system consists of a high voltage power supply, a capacitor bank as well as a high voltage/ current switch to discharge the stored electric energy across the electrodes. By variations of charging voltage and capacity, the energy per pulse can be varied from 36 to 14400 J per pulse. The treatment intensity can be further adjusted by the number of pulses applied. The following settings were used in the present study: voltage (36 kV), distance from meat to spark (20 cm) and one single pulse.

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2.4. Warner-Bratzler shear force measurement

The tenderness was measured by using the Warner-Bratzler shear force (WBSF) procedure. Briefly, the steaks were cooked on a grill at 100 °C (Elektro Bratplatte - Glatt (8 kW), 800x700x290 mm, GGM Gastro International GmbH, Ochtrup, Germany). The grilling time of the samples was standardized by preliminary tests to reach a final internal temperature of 71 °C. Subsequently, the cooked meat steaks were left overnight in a chilling chamber (4 °C) to allow cooling down and permit the temperature of the steaks to homogenise before measuring. The day after, strips of 10 mm-thick were cut by using a scalpel. At least five strips for each steak, and a minimum of two steaks per loin and treatment (i.e. control and shockwave) were measured. The tenderness was detected in a texture analyser TA.XT plus (Stable Micro Systems Ltd, Surrey, England) by using the standardised Warner-Bratzler shear force blade. The operational settings were as follows: test mode in compression strength, initial height of cell 3 cm, pre-test speed 1 mm s⁻¹, test speed 3.30 mm s⁻¹, post-test speed 10 mm s⁻¹, travel distance 40 mm and detection of sample 50 g. The maximum shear force was calculated from the graph in order to compare the tenderness of the samples.

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2.5. Preparation of enzyme extracts for cathepsin/peptidases assays

2.5 g of muscle were homogenised in 25 ml of 50 mM sodium citrate buffer containing 1 mM EDTA and 0.2% (v/v) Triton X-100 at pH 5.0 (for cathepsins) or 4 g of muscle in 20 ml of 50 mM disodium phosphate buffer, pH 7.5, containing 5 mM EGTA (for peptidases). In both cases, the extracts were homogenised (3 x 10 s at 27.000 rpm on ice) with a polytron (Kinematica, Switzerland), centrifuged at 10,000g for 20 min at 4 °C and the supernatants filtered through glass wool and used for the enzyme assays.

2.6. Assay of cathepsin and peptidase activities

Cathepsins were assayed as described by Toldrá and Etherington (1988) using N-CBZ- L-arginyl-L-arginyl-7-AMC, N-CBZ-Lphenylalanyl-L-arginine-7-AMC (Sigma, St. Louis, MO), at pH 6.0, for cathepsins B and B+L. For each assay, 50 µl of extract was diluted with 250 µl of reaction buffer (40 mM sodium phosphate at pH 6.0, containing 0.4 mM EDTA, 10 mM cysteine, and 0.05 mM substrate) and then incubated for 15 min at 37 °C. One unit (U) of proteolytic activity was defined as the amount of enzyme capable of hydrolysing 1 nmol of substrate per minute at 37 °C

Muscle aminopeptidase activities were measured by fluorometric assays using aminoacyl-7-amido-4-methyl coumarin as substrates (aa-AMC) (Sigma Chemical Co., St. Louis, MO) (Toldrá & Flores, 2000). Alanyl aminopeptidase (AAP) was assayed using 0.1 mM alanine-AMC as substrate in 100 mM phosphate buffer, pH 6.5, with 2 mM 2-mercaptoethanol. Arginyl aminopeptidase (RAP) was assayed using 0.1 mM arginine-AMC in 50 mM phosphate buffer, pH 6.5, with 0.2 M NaCl. Leucyl aminopeptidase activity (LAP) was assayed using 0.25 mM leucine-AMC in 50 mM borate-NaOH buffer, pH 9.5, with 5 mM magnesium chloride. Pyroglutamyl aminopeptidase (PGAP) was assayed with 0.1 mM pyroglutamic-AMC in 50 mM borate-HCl, pH 8.5, containing 1 mM DTT. Methionyl aminopeptidase (MAP) was determined by using 0.15 mM Ala-AMC as substrate, in 100 mM phosphate buffer, pH 7.5 containing 10 mM dithiothreitol (Flores, Marina, & Toldrá, 2000).

Dipeptidyl peptidases (DPP) I, II, III and IV were assayed as previously described by Sentandreu and Toldrá (2001), using AMC (Bachem, Switzerland or Sigma, St. Louis, MO), as fluorescent substrates.

DPP I was measured using 0.5 mM Gly-Arg-AMC in 50 mM sodium acetate/acetic acid buffer, pH 5.5, containing 5 mM DTT; DPP II: 0.5 mM Lys-Ala-AMC or Gly-Pro-AMC in 50 mM sodium acetate/acetic acid buffer, pH 5.5, containing 0.04 mM bestatin; DPP III: 0.5 mM Arg-Arg-AMC in 50 mM sodium tetraborate/potassium phosphate buffer, pH 8.0, containing 0.05 mM CoCl₂. DPP IV: 0.25 mM Gly-Pro-AMC in 50mM tris-base buffer, pH 8.0, containing 5 mM DTT. Then 50 µl of each enzyme preparation were added to 250 µl of the respective substrate solution.

In all cases, the reaction was incubated at 37 °C and the fluorescence continuously monitored at 355 nm and 460 nm as excitation and emission wavelengths, respectively for AMC derivatives, using a Fluoroskan Ascent fluorimeter (Thermo Electron Co, Finland). Three replicates were performed for each enzyme assay. One unit (U) of proteolytic activity was defined as the amount of enzyme capable of hydrolysing 1 µmol of substrate per hour at 37 °C.

2.7. Detection of protein fragments by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS–PAGE)

Muscle sarcoplasmic and myofibrillar proteins were extracted as described by Goransson et al. (2002). SDS-PAGE analysis (Laemmli, 1970) of the obtained protein extracts were performed using 10% resolving polyacrylamide gels for sarcoplasmic and myofibrillar proteins, respectively. 12 µg of protein was injected per lane. Broad range molecular weight standards; myosin (200.00 kDa), β-galactosidase (116.25 kDa), phosphatase b (97.40 kDa), serum albumin (66.20 kDa), ovalbumin (45.00 kDa), carbonic anhydrase (31.00 kDa), trypsin inhibitor (21.50 kDa), lysozyme (14.40 kDa) and aprotinin (6.50 kDa), were ran simultaneously (Bio-Rad, Hercules, CA, USA). Proteins were visualised by staining with Coomassie Brilliant Blue R-250 and subsequently unstained overnight.

2.8. Confocal laser scanning microscopy (CLSM)

Protein components were stained by fluorescein isothiocyanate (FITC) (Thermo Fisher Scientific Inc., IL, USA) (absorption 492 nm; emission 520 nm) resulting in green colour and lipids were marked by Nile Red (Sigma Aldrich, Co., St Luis, MO, USA) (absorption 554 nm; emission 606 nm) effecting

red colour in the micrographs. 2 µL FITC solution were put on a microscope slide, then a meat slice (1mm thick) was positioned on it and Nil Red was applied on samples top side. Diffusion time of fluorescing dyes was 12 h at 4 °C. CLSM Nikon ECLIPSE E 600 (Nikon Corporation, Japan) was used for structure characterization of beef muscle fibers.

2.9. Scanning electron microscopy (SEM)

Random samples (volumetric elements of edge length of 1.5 mm) were taken from the meat, frozen in super-cooled liquid nitrogen and inserted into the cryo-preparation system (Emitech K 1250, France). There the free water from the sample was removed by sublimation. Finally, the surface was sputtered with gold in deep frozen state. The prepared samples were transferred into the SEM (JEOL JSM 6460 LV, Japan) at approx.-180°C. An electron beam is generated in the scanning electron microscope (SEM) which is accelerated to 1 - 30 kV voltage. This primary electron beam is directed in an isometric pattern to the sample surface. The secondary electron signal caused by the primary beam will be changed according to the state of the sample surface and the blade angle of the beam. Therefore the image which is built up in the Braun tube arises from light spots whose brightness is changed. Since the secondary electrons can be forced by magnetic lenses to follow a curved course, it is possible to make surface profiles visible which lie outside of the direct target line of the collector. So a big depth of the field is possible and in addition a tri-dimensional effect can be produced. The generated image is recorded electronically.

2.10. Data analysis

The results were analysed by a one-way analysis of variance (ANOVA) using the software (Past online version 2.17b, Hammer & Harper) in order to discriminate the significant differences.

3. Results and discussion

The values of WBSF for control and shockwave-treated meats are shown in Table 1. Clearly, shockwave treatment reduced the maximum cutting force by 1 kg (17.8 % tenderness improvement) (Table 1). This is in agreement with several works reporting tenderness improvements around 20 %,

which have been revised by Solomon et al. (2006) and Bolumar et al. (2013). Therefore, HDP shockwave is a useful postharvest technology for reducing time and cost associated with maturation and improving the tenderness of tough primal muscles.

Muscle cathepsin and peptidase (amino- and dipeptidyl-) activities from control and shockwave-treated meat can be observed in Table 2. Cathepsin activities were not affected by shockwave treatment showing quite high p-values (Table 2). Statistical significance is normally accepted if p-value is as maximum 0.1 ($p\text{-value} < 0.1$). Nevertheless, all cathepsin activities, B, B + L and H, trended to be lower in the shockwave-treated meat than in the non-treated control meat although the differences were not statistically significant in any case. Aminopeptidase activities, AAP, RAP, LAP, PGAP and MAP, also did not show statistically significant differences between control and shockwave-treated samples (Table 2). In addition, all aminopeptidases activities apart from LAP were again lower in the shockwave-treated meat. Although the differences were not statistically significant, it can be highlighted that aminopeptidase activities, RAP and MAP, had the lowest p-values, 0.2084 and 0.1589, respectively (Table 2). Similarly, dipeptidyl peptidases, DPP I, II and IV, also showed lower activity in the case of meat treated by shockwave whereas DPPII was slightly higher though the differences were not statistically significant for any of them (Table 2). Overall, this is the first time to the best of our knowledge that muscle cathepsin and peptidase (amino- and dipeptidyl-) activities have been quantified after shockwave treatment and it can be concluded that endogenous proteolytic activities, cathepsin and peptidases, were not affected by HDP shockwave treatment.

Contrary to HDP treatment, other physical treatments like electrical stimulation (Goransson et al., 2002) and high pressure processing (HPP) (Campus, Flores, Martinez, & Toldra, 2008) have been reported to influence muscle cathepsin and peptidase activities to some extent. Electrical stimulation affected the activation of cathepsin activities (Maribo, Erthjerg, Andersson, Barton-Gade, & Moller, 1999) as well as exoprotease activities, inducing the activation of DPP I and II, and the inhibition of MAP, LAP, and PGAP while other studies have reported electrical stimulation did not influence cathepsin activities (Dransfield, Etherington, & Taylor, 1992) and aminopeptidases, AAP and RAP

(Goransson et al., 2002). HDP or shockwave is a treatment in which momentary high pressures can be reached. Pressure effect on muscle proteolytic activity has been investigated. Several scientific publications have reported an increased cathepsin activity after HPP likely due to its release from lysosomes (Homma, Ikeuchi, & Suzuki, 1994; Ohmori, Shigehisa, Taji, & Hayashi, 1992; Grossi, Gkarane, Otte, Ertbjerg, & Orlie, 2012). Whereas cathepsin activities, were not activated by HPP treatment in dry cured loin as the lysosome possibly was already broken previously during the processing (Campus et al., 2008). HPP treatment has also been reported to reduce activity of aminopeptidases and dipeptidylpeptidases likely by enzyme denaturation (Campus et al., 2008). Since time exposed to pressure is significantly reduced in shockwave treatment (fraction of seconds) compared to HPP (minutes), the effect on proteolytic activity, if so, might be negligible. It can be concluded that muscle cathepsin and peptidase activities measured were not influenced by shockwave treatment in the present study. Nevertheless, a tendency to decrease the cathepsin and peptidase activities after shockwave likely due to the pressure conditions during the treatment can be appreciated on the data. This trend is in any case statistically significant. The proteolysis due to muscle cathepsin and peptidase activities would then be expected to be in the same order for control and shockwave-treated meat what is important for the development of the typical flavour of matured meat (Moya, Flores, Aristoy, & Toldrá, 2001; Spanier, Flores, McMillin, & Bidner, 1997).

SDS-PAGE analysis of sarcoplasmic and myofibrillar proteins at day 1 and 7 are depicted in Figure 1. No marked differences can be observed in the protein bands between control and shockwave-treated meats for sarcoplasmic and myofibrillar proteins neither at day 1 nor at day 7 (Figure 1). Evident changes are observed in the sarcoplasmic fraction due to the maturation of 7 days, particularly a decreasing of protein intensity of bands between 50-60 kDa and in the region between 45-35 kDa. Proteolysis of myofibrillar proteins due to maturation during storage (7 days) is not observed (Figure 1, C and D images) being longer maturation time needed to observe noticeable changes using this methodology. This is in agreement with the results reported by Schilling et al. (2002) who did not find any difference in protein solubility, for both protein fractions, sarcoplasmic and myofibrillar, between control and HDP-treated meats. In contrast, Bowker, Fahrenholz, Paroczay, & Solomon (2008a)

reported that HDP influenced sarcoplasmic proteins corresponding to 143, 65, 44, 36 and 19 kDa after similar maturation time (8 days) and correlated these changes with WBSF suggesting that HDP and aging cause changes to sarcoplasmic proteins that may be indicators of proteolysis and tenderization. In agreement with our results, Bowker, Fahrenholz, Paroczay, Eastridge and Solomon (2008b) did not detect changes visualized by using SDS-PAGE in the protein intensity of myosin heavy chain (MHC), actin, myosin light chain-1 (MLC-1), troponin C (TnC) and myosin light chain-2 (MLC-2) neither by HDP nor ageing (8 days) whereas desmin decreased by ageing. However, in the same study HDP treatment influenced troponin T (TnT) degradation as confirmed by using a more sensitive technique like Western blot. They reported that HDP in combination with aging decreased the intensity of the TnT band and enhanced the accumulation of the 30 kD fragment arisen from the TnT degradation suggesting that HDP tenderization was caused by both physical disruption of the myofibril apparatus and enhanced post-mortem proteolysis (Bowker et al., 2008b). An accelerated maturation after shockwave treatment, which will mean a shorter maturation time, has been described in the literature (Solomon et al., 2002; Callahan et al., 2002; Paroczay et al., 2002; Heinz et al., 2011).

Changes in the microstructure of tissue can be detected by using CLSM and SEM. In CLSM, a point light source (laser) is focused on a small area within the sample, and a confocal point detector is used to collect the resulting signal. The lens acts as a condenser as well as collector. This provides an image of the in-focus plane called as “optical section”. This means that only the in-focus regions are detected and the out of focus parts remain black. The main advantage of CLSM is the minimal sample preparation and thus the sample preserves the physical structure. Procedures like embedding, fixing or sectioning are not necessary. To identify different components, it is necessary to use differential fluorescent probes. Food components such as lipids, proteins and phospholipids can be stained selectively by adsorption or chemical covalent interaction. In our CLSM analysis, proteins display green colour and lipids are red. CLSM images showed very clearly the structural integrity of control beef muscle fibers at day 0 as well as at day 7 where intact muscle cells are observed (Figure 2, images A – C) while shockwave treatment causes considerable disruptions of muscle fibers (Figure 2, images D –F). In addition, it can be observed that an ageing for 7 days has also no significant

influence on the meat structure analyzed by CLSM. Furthermore, increased endomysium space, which corresponds to the space between muscle fibers, can also clearly be observed for shockwave-treated meat in the SEM images (Figure 3). The disruption of the muscle fibers is connected with the appearance of additional protein networks likely release of intracellular content which is observed in the figure 3 as white networks (comparing images A, B, C (control) to images D, E and F (shockwave-treated)). Overall, changes in the microstructure of the meat after shockwave treatment are evident in Figures 2 and 3. Main changes were observed in the endomysium (i.e. space between muscle fibers). There is an increased endomysium space which might be presumably due to a displacement within the muscle fiber at the collagen level. Juncture myofibrillar fragmentations of the Z-lines and A-band/ I-Band using transmission electron microscopy (TEM) were reported by Zuckerman and Solomon (1998) as well as jagged edges associated with the Z-line and the thin filaments were described by Claus (2002) what suggested that physical tearing rather than proteolysis is behind the observed tenderization. In addition, Zuckerman, Bowker, Eastridge and Solomon (2013) recently reported through SEM analysis that shockwave treatment disrupted the integrity of the collagen fibril network of the endomysium in both non-aged and aged samples and that WBSF and connective tissue changes were greater in shockwave-treated samples in comparison to the control what it is in agreement with our results. These data suggest that shockwave alterations in the connective tissue network at the level of the endomysium also contribute to meat tenderization. In this sense, the amount and chemical composition of connective tissue, which is largely a function of the age and the specific muscle, can be considered as “background toughness” (Sentandreu, Coulis, & Ouali, 2002). Currently there are very few treatments that allow the contribution of connective tissue to tenderness to be manipulated (Purslow, 2005). Therefore, HDP treatment could be an alternative and promising method to influence the “background toughness”. It would be interesting to test the effect of HDP treatment on the same muscle having different degree of collagen like for instance cows and young bulls in order to confirm this fact.

HDP or shockwave treatment has been extensively investigated during the last two decades as a meat tenderization method. However, the precise mechanisms underneath the observed effect still remain

unclear. Two main effects have been empirically observed after HDP: (1) an instantaneous effect mediated by physical effects on muscle structure mainly displacement of Z-line, increased endomysium and disruption at the collagen level and (2) enhanced maturation. The enhanced maturation is not well understood. The release of cathepsin from the vacuoles due to the impact of high pressure shockwave could be a mechanism which might contribute to explain the enhanced maturation observed in HDP-treated meats. An increased cathepsin activity by application of high pressure conditions likely due to disruption of lysosome and release of cathepsin has been confirmed (Grossi et al., 2012). Analogously, meat tenderization by HPP treatment of post-mortem meat, which only occurs combined with high temperature (60 °C), is likely caused by lysosome breakdown and subsequent release to the medium of proteolytic activity (Hugas, Garriga & Monfort, 2002). In a recent study, Sikes et al. (2010) stated that meat was tenderized after treatment (200 MPa, 60 °C, 20 min) and ascribed the effect to an enzymatic occurring activity suggesting the cathepsins as responsible. The role of the cathepsin, if so, in the enhanced maturation by shockwave treatment and according to our data, may come from a facilitated contact with the substrate (muscle proteins) rather than enzyme activation. Our data support the fact regarding the effect of the shockwave on the microstructure of the meat as a tenderizing mechanism since no activation of proteolytic system or enhanced protein fragmentation was observed. One must note that myofibrillar protein fraction, which is considered as responsible for the tenderness, is not greatly influenced in ageing experiments of approximately 1 week as the present. More sensitive techniques must be applied to unveil the effect of HDP on the muscle biochemistry after shockwave treatment. It can be hypothesised that even though the endogenous proteolytic system seems not to be strongly affected by HDP, the HDP-induced changes on muscle microstructure could be facilitating the accessibility of certain proteins to the endogenous proteolytic enzymes whereby the proteolysis of key elements of the muscle structure would be favoured in this way. This will require additional investigations on the effect of specific endogenous proteases under shockwave conditions using protease inhibitors and advanced proteomic methodologies.

4. Conclusions

HDP or shockwave technology is an effective method to tenderize steaks of beef loin (*longissimus lumborum*) (18 % WBSF reduction). Enzymatic activation of endogenous proteolytic system (cathepsin and peptidases) as well as muscle protein fragmentation detected by conventional SDS-PAGE was not observed after shockwave treatment. But microstructure modifications at the muscle fibers bundles were clearly observed by CLSM and SEM. The present work provides evidence to support the physical effects of HDP on muscle microstructure but further research is needed to better understand the biochemistry behind the accelerated maturation described in the literature. Molecular and mechanistic studies are needed to describe the fundamentals of the ‘rupture effect’ observed after shockwave treatment in order to gain insight and bring about a target application of the shockwave technology.

Acknowledgements

The research leading to these results has received funding from the European Union's Seventh Framework Programme managed by REA Research Executive Agency <http://ec.europa.eu/research/rea> (FP7/2007-2013) under grant agreement number 2870340 [Development of Shock Wave Technology for Packed Meat]. Grant PROMETEO/2012/001 from Conselleria d'Educació, Formació i Ocupació of Generalitat Valenciana (Spain) is also acknowledged. Collaboration of Mrs Andrea Aleixandre in the enzymes analysis is acknowledged.

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Figure captions

Figure 1. SDS-PAGE analysis of sarcoplasmic and myofibrillar proteins from control and shockwave-treated beef *longissimus thoracis* at day 1 and 7 of storage under refrigerated conditions.

Lane C: control sample, lane SW: shockwave-treated sample, lane St: standard proteins (kDa)

A: sarcoplasmic proteins at day 1, B: sarcoplasmic proteins at day 7, C: myofibrillar proteins at day 1, D: myofibrillar proteins at day 7.

Figure 2. Confocal laser scanning microscopy (CLSM) images from control and shockwave-treated beef *longissimus thoracis* at day 1 and 7 of storage under refrigerated conditions.

A: control at day 1, B: control at day 7, C: control at day 7, D: shockwave-treated at day 1, E: shockwave-treated at day 7, F: shockwave-treated at day 7.

Figure 3. Scanning electron microscopy (SEM) images from control and shockwave-treated beef *longissimus thoracis* at day 1 and 7 of storage under refrigerated conditions.

A: control at day 1, B: control at day 7, C: control at day 7, D: shockwave-treated at day 1, E: shockwave-treated at day 7, F: shockwave-treated at day 7.

Figure 1

Figure 2

Figure 3

1 Table 1. Tenderness[#] improvement by hydrodynamic shockwave treatment of beef loin steaks (mean
2 ± standard deviation).

Control		Shockwave	
Average value		Average value	Improvement
(kg)		(kg)	(%)
5.6 ^a ± 1.5		4.6 ^b ± 0.9	17.8

3 Different superscript letter within the same row means a significant difference at p-value < 0.01.

4 [#]: The texture was measured by Warner-Bratzler shear force (WBSF) procedure after 7 days of
5 storage which simulates the commercial life of meat from processor to supermarket and consumer.

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1 Table 2. Muscle cathepsin and peptidase (amino- and dipeptidyl-) activities from control and
2 shockwave-treated beef *longissimus thoracis* (mean ± standard deviation).

	Control	Shockwave	p-value [#]
Muscle cathepsin activities (nmol/ min.g)			
Cathepsin B	31.21 ± 22.32	27.00 ± 19.79	0.6609
Cathepsin B+L	48.03 ± 17.43	40.31 ± 12.58	0.2712
Cathepsin H	5.53 ± 2.48	5.01 ± 2.17	0.6258
Muscle peptidase (amino- and dipeptidyl-) activities (µmol/ h.g)			
Alanyl aminopeptidase (AAP)	5.27 ± 0.89	5.12 ±0.63	0.6946
Arginyl aminopeptidase (RAP)	3.80 ± 0.94	3.36 ±1.49	0.2084
Leucyl aminopeptidse (LAP)	1.51 ±0.42	1.51 ±0.50	0.7617
Pyroglutamyl aminopeptidase (PGAP)	0.26 ± 0.19	0.22 ±0.20	0.6848
Methionyl aminopeptidase (MAP)	5.85 ± 2.04	4.07 ±2.07	0.1589
Dipeptidyl peptidases (DPP) I	2.71 ± 1.45	2.32 ±1.44	0.5571
DPP II	0.29 ± 0.08	0.29 ± 0.17	0.9716
DPP III	4.17 ± 1.01	4.09 ± 1.00	0.8633
DPP IV	0.20 ± 0.14	0.17± 0.12	0.6472

3 [#]: (1 – (p-value)) x 100 equals to the probability that the samples are significantly different among
4 them.