# RASONICATION OF LAMB SKELETAL MUSCLE FIBRES ENHANCES POSTMORTEM PROTEOLYSIS.

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**SUMMARY**. The ability of ultrasound treatments to disrupt membranes and to release enzymes from cellular compartments is well n. The aim of our work was to investigate if such a treatment would be able to activate endogenous proteolysis in muscle fibres. We emonstrated that ultrasonication was indeed effective in releasing lysosomal enzymes from liver cells, as shown by the presence of acid hatase activity in cytoplasm extracts. Cell membranes were damaged only to a limited extent, as revealed by turbidity measurement of rmedium. Proteolysis brought about by endogenous proteinases, after two days of fibre storage at 4°C, was assessed by means of SDS- in controls as well as in fibres treated with ultrasounds varying in intensity and time of exposition. The incipient appearance of a er of faint bands in the 30000 Da region was the only evident change of control gels. Results revealed that ultrasonic treatment of fibres ly enhanced proteolytic degradation, depending on time and output of sonication, as shown by the increased intensity of 30000 Da n bands. A distinct change featuring sonicated fibres was the degradation of a 87000 Da protein and the appearance of a 83000 Da de. It is discussed wether calpains or cathepsins are to be responsible for observer changes. Cell damage appeared to be not very tive, although it was dependent upon ultrasonication conditions. As a consequence, ultrasonication of muscle fibres may be regarded as el method for increasing postmortem proteolysis, and thus meat tenderisation, despite technical problems derived from ultrasound cation must be previously solved.

**INTRODUCTION**. It is well known that meat becomes more tender during aging, so that postmortem storage of meat is the od currently used for achieving suitable tenderisation. Proteolysis of myofibrillar proteins, mainly of high molecular weight proteins, rs to be the major contributor to the tenderising process during postmortem storage (Asghar & Bhatti, 1987). Of the endogenous ses of skeletal muscle, calcium-dependent proteases (calpains) are likely the main responsible for the increase in tenderness during rtem storage (Koohmaraie et al., 1988a). The cysteine proteinases cathepsins B, H and L and the aspartate proteinase cathepsin D, received also attention concerning their potential role in postmortem aging (Moeller et al., 1976). However, Zeece & Katoh (1989) ed that the effectiveness of cathepsins is greatly reduced under low temperature conditions and suggested that these proteases do not principal role in the tenderisation process occurring in muscle postmortem.

A number of methods have been proposed to accelerate the tenderising of meat: electrical stimulation (Davey et al., 1976), urization (Mac Farlane, 1985), infusion of $Ca^{2+}$ (Koohmaraie et al., 1988b) or addition of endogenous and exogenous proteases vett & Mc Dowell, 1987). High temperatures and low pH postmortem exerted a tenderising effect through activation of cathepsins eller et al., 1976). On the contrary, Jaime et al. (1992) reported an increased postmortem proteolysis by effect of low temperature and pH on muscle calpains. Several methods have been tried out to destabilize the lysosomes and increase lysosomal protease activity in oles: acid or alcaline treatments, triton X-100, hipertonic solutions of ClNa, pyrogens, osmotic shock and UV radiation (Park & ington, 1967). Stagni & Bernard (1968) obtained the same effect by subjecting the muscle to the action of ultrasound. Alliger (1975) ed on this new application of ultrasound, focusing on the release of enzymes and proteins from cells and subcellular particles. On this , we have carried out a study conducted to examine the effects of ultrasound on destabilization of lysosomes and other vesicles as oplasmic reticulum and mitochondria, allowing cathepsins to be released from their compartments as well as increasing free calcium centration. The main purpose was to increase postmortem myofibrillar protein degradation and thus accelerate meat tenderisation.

**MATERIALS & METHODS**. **Ultrasonic treatment of liver.** Lamb liver was obtained a day after slaugther and cut in cubes bout 5 g that were immersed in MFI buffer (20 ml) (100 mM KCl, 20 mM $KPO4H_2$, 0.1 mM EDTA , 1 mM $MgCl_2$ and $NaN_3$, pH 7) subjected then to ultrasound action for different times of application at 62 w (Branson sonifier, model 250/450). Control consisted of a ple immersed in buffer but not treated with ultrasounds. **Extraction of cell plasma fraction.** After ultrasound treatment liver samples in 15 ml of MFI buffer) were homogenized with a potter and cell plasma fraction was obtained following the method used for myofibril ction (Olson et al., 1976). Finally, supernatant was centrifuged at 30,000 g 60 min. **Acid phosphatase assay.** Acid phosphatase rity of cellular plasma fraction (20 mg of protein, approximately) was measured according to the method described by Trouet (1974) using lycerophosphate as substrate. **Muscle fibres preparation.** Muscle fibres were obtained from lamb Longissimus muscle 30 min after gther. Small groups of fibres (2 cm x 0.5 mm, approximately) were separated from a piece of muscle. The muscle bundles were included MFI buffer. **Ultrasonic treatment of fibres.** Muscle fibres immersed in MFI buffer were treated with ultrasound at 57 or 62 w for ferent times (10 s to 3 min) at atmospheric pressure. Muscle fibres were then aged at 4°C for two days. Controls not treated with trasounds were aged under the same conditions. After sonication, muscle fibres were centrifuged at 4,000 g 10 min and turbidity of rnatants was measured at 540 nm. **Myofibrillar protein isolation.** Myofibrillar proteins were isolated by the MFI method (Olson et 1976) at 3rd day postmortem, boiled in SDS samples buffer (Porzio & Pearson, 1977) and stored at -20°C. **Protein concentration.**

Protein concentration was determined by the Nessler method (Johnson, 1941). **Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).** SDS-PAGE was performed according to Greaser et al. (1983). Approximately 0.35 mg of protein were loaded on each gel. Samples were runned at 120 V and 250 V through stacking and resolving gel, respectively.

**RESULTS & DISCUSSION.** As shown in Figure 1, ultrasonication of lamb liver slices was able to destabilize lysosomes in this tissue and thus release their enzymes to cytoplasm. Acid phosphatase activity increased in a first phase (until 7 min of treatment) and decreased thereafter, probably due to ultrasound-caused inactivation. Alliger (1975) already reported that ultrasounds are able of inactivating enzymes after some time of treatment. Absorbance at 540 nm of the supernatant, used as an index of turbidity, increased with time of treatment, being three times higher at 10 min than at 0.5 min. Our results demonstrated that plasmatic membrane damage depended upon time of sonication, since increasing the time of treatment resulted in an important increase of absorbance. This fact suggest that short times should be applied to muscle samples in order to destabilize lysosomes and endoplasmic reticulum while maintaining as intact as possible the plasma membrane.

The plasma membrane of skeletal muscle was slightly affected by effect of ultrasound when a low intensity of treatment was used (Figure 2). A constant absorbance at 540 nm was obtained after one minute of application of 56 w. A more severe alteration resulted at 62 w since higher values of turbidity were obtained with small increases of treatment time (six times higher after 30 s of treatment at 62 w than at 56 w). This level of sarcolemma damage was however very little when compared to that caused to muscle samples treated with 56 w after rigor onset; supernatant absorbance was in this case even five times greater than in samples treated at prerigor with the same power. This fact should be referred to the loss of sarcolemma integrity associated to postmortem degradation (Stanley, 1991).

SDS-PAGE of ultrasound treated myofibrils (Figure 3) demonstrated that sonication brought about an activation of proteolysis since several peptides appeared at the region of 30,000 Da (30,500, 29,000, 27,500, 26,000 and 25,000 Da) as soon as after only two days of aging. Proteolytic effects were even magnified with time of treatment. However, only small differences were observed between the two ultrasonication intensities so far used. Surprisingly, no protein of a molecular weight in the range 30-45,000 Da was observed to be degraded, although appearing peptides could proceed from any protein of a high molecular weight not well defined in the gel. Moeller et al. (1976) and Troy & Tarrant (1987) reported an increase of myofibrillar fragmentation in muscle samples electrically stimulated and aged at 4°C. These authors suggested that cathepsins released after treatment could be responsible for proteolytic effects; the appearance of several peptides in the region of 30,000 Da was the most constantly found effect. Results obtained in this work have demonstrated that ultrasound treatment brought about an increase of meat aging proteolysis. These effects could be the result of either calpains activation by an increase of calcium released from sarcoplasmic reticulum or cathepsins released from lysosomes. Although we do not know thus far what proteases are most likely responsible for the changes observed, cathepsins would probably show a limited proteolytic action due to low temperature and short time of aging (Zeece & Katoh, 1989). It can be also observed in Figure 3 that the intensity of a band corresponding to a 87,000 Da protein decreased at the same time that a new band of 83,000 Da appeared in samples treated with the longest times of of exposure to ultrasounds. Some researchers have reported the appearance of peptides in this region by action of cathepsins on myosin and α-actinin (Matsukura et al., 1981). Troponin T, desmin and α-actinin have been reported to be degraded by cathepsins D and L (Matsukura et al., 1981), although their degradation was very slow at 4°C and pH 5,5. Zeece & Katoh (1989) also reported that several proteins were sensitive

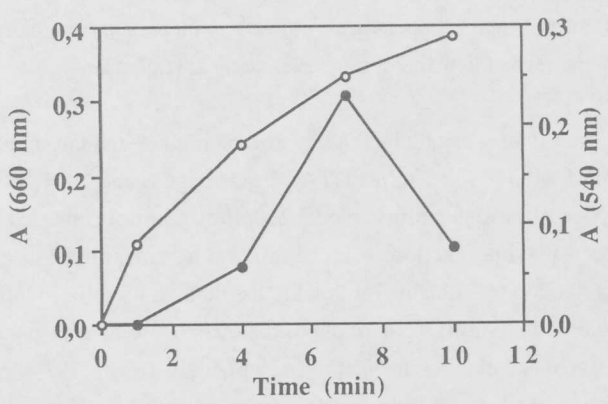


FIGURE 1. Acid phosphatase activity of lamb liver samples treated with ultrasounds for different times (○), expressed as absorbance at 660 nm, and turbidity of immersion buffer of liver samples during sonication (●), expressed as absorbance at 540 nm.

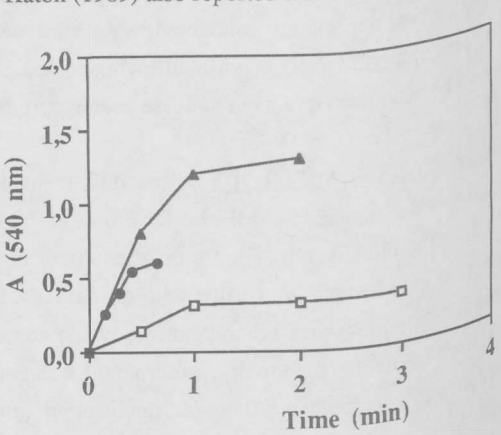


FIGURE 2. Turbidity of immersion buffer used for the sonication of lamb muscle fibres, expressed as absorbance at 540 nm. Treatments: Prerigor, 57 w (□); Prerigor, 62 w (●); Rigor, 56 w (▲).

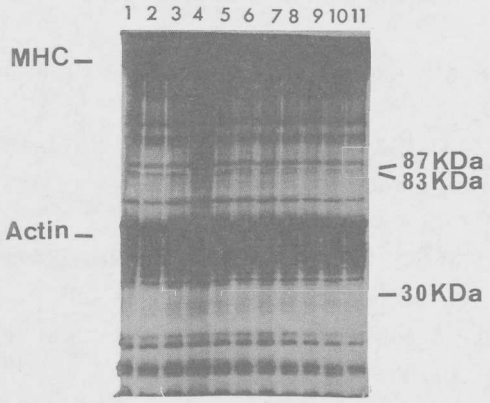


**FIGURE 3.** 15% SDS-polyacrylamide gels of myofibrils obtained from lamb Longissimus dorsi samples treated with ultrasounds ...rigor and stored at 4°C during 2 days. 1, 2, 3) controls; 4, 5, 6, 7) samples treated with ultrasounds at 57 w; 4: 30 s, 5: 60 s, 6: 2 min, ...min; 8, 9, 10, 11) samples treated with ultrasounds at 62 w; 8: 10 s, 9: 18 s, 10: 25 s, 11: 40 s.

...psins action: troponin T, myosin, α-actinin, α-tropomyosin, actin, titin, nebulin and M and C proteins, but the proteolytic effects were ...y nonexistent at 4°C. Some of this proteins have been reported, too, to be sensitive to the action of calpains: troponin T and I, ...myosin, M and C proteins, titin and nebulin (Etherington, 1984).

Ultrasounds had been shown before to be capable of releasing several enzymes from lysosomes of bovine skeletal muscle to ...plasm, such as ß-glucuronidase and cathepsins (Stagni & Bernard, 1968). Now, we have demonstrated the same effect of releasing ...rmal enzymes in lamb liver and presumably a similar effect on lamb skeletal muscle since proteolysis brought about at an early phase of ...aging was greatly increased. Among the methods proposed to increase meat tenderisation, electrical stimulation is currently being the ...used. Several authors have suggested that the proteolytic effects associated to electrical stimulation are due to cathepsins released from ...somes (Moeller et al., 1976; Troy & Tarrant, 1987), and even to calpains activation by increase of sarcoplasmic calcium levels ...hmaraie et al., 1988a). Troy & Tarrant (1987) reported the appearance of bands of 39,000 and 32,000 Da at 49-72 h postmortem in ...e muscle electrically stimulated and aged at 4 °C. According to these authors proteolytic effects were magnified if time or temperature of ...were increased. Pressurization has been reported, too, as a suitable method to increase meat tenderness by activating calpains and ...epsins. Proteolytic effects in this case were also observed at an early stage of aging (Koohmaraie et al., 1984). Results presented in this ...k suggest that the proteolytic increase observed after ultrasonication of meat is similar to that caused by electrical stimulation and ...urization at the first moment of aging. On the basis of the high correlation existing between the intensity of peptides in the 30,000 Da ...n and the tenderising of meat (Penny, 1980), our results suggest that ultrasound treatment could be envisaged as a novel method for ...ning tender meat after a short aging time. However, the results presented here are a first approach to the subject and subsequent research ...viously necessary, particularly concerning the practical application to meat cuts and carcasses.

**CONCLUSIONS**. SDS-PAGE of muscle fibres subjected to ultrasonication and stored thereafter two days at 4°C demonstrated ...this treatment significantly enhanced postmortem proteolysis, as revealed by an increased intensity of peptide bands in the region of ...00 Da, while fibre membranes were damaged only to a limited extent.

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