TIC ACID TREATMENT OF PRE-RIGOR BEEF AND DISTRIBUTION OF CATHEPSIN B+L ACTIVITY.

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mary

bution of cathepsin B+L was evaluated in comminuted meat and meat-homogenates (*M. sternomandibularis* from bull) ed with lactic acid. The results showed that the relative-free activity was approximately: 76%; 73 %; 91% and 95% for acid treatments of 0 M; 0.015 M; 0.045 M and 0.060 M, respectively. A significantly larger lysosome release of enzyme been for 0.045 M and 0.060 M. Experiments using meat-homogenates revealed that cathepsins did not leak out by a e pH-dependent process.

duction

erness is a very important feature for the acceptability of meat. Meat normally achive an increased tenderness during an period, where muscle proteins are degraded by endogenous proteases (GOLL et al.,1983).
gen, the major connective tissue protein of bovine meat, which is believed to contribute to meat toughness (BAILEY, 3), has been shown to be modified by proteolysis during conditioning (STANTON & LIGHT, 1987, 1988, 1990a).
enzyme systems believed to be responsible for post-mortem tenderization are present in meat (calpains and cathepsins) NY & FERGUSON-PRYCE, 1979). It has been shown that cathepsins are capable of degrading collagen ERINGTON, 1987; STANTON & LIGHT, 1988).

activity of cathepsins are controlled in the living muscle by incapsulation in a membrane structure (lysosomes). These branes are considered to be de-stabilized post-mortem, mainly on account of a decrease in pH, and enzymes will leak nto the cytosol, causing proteolytic damage of the muscle structure. Electrical stimulation and high temperature itioning have been shown to cause an early release of cathepsins (DUTSON, 1983; WU et al., 1981,1985).

best quality meat is obtained when the carcass is chilled slowly and then aged, which for beef may be two weeks or more illing temperatures. Although conditioning improves beef quality, the cost of keeping carcasses for an extended period, es producers interested in seeking new ways of reducing conditioning time without loosing quality.

NTON & LIGHT (1990b) investigated a potential new method employing injection of lactic acid into meat to increase erization. Their results showed an increased breakdown of connective tissue in lactic acid treated meat compared to entionally produced meat.

s the aim of this work to investigate the effect of a lactic acid treatment on the release of cathepsins from bovine *M.* omandibularis .

hods and materials

ic acid treatment of meat (experiment 1): The procedure used to determine the lysosomal enzyme distribution was a ification of WU et al., (1985) and KÂS et al , (1983). *M. sternomandibularis* was removed 45 min post-mortem, from a g bull slaughtered at approximately 450 kg live weight. The meat (100 g) was comminuted with a pair of scissors in 100 of icecold isotonic sucrose solution (0.25 M sucrose and 0.02 M KCl) and filtered through a double layer of cheese-cloth. minuted meat (8 x 5 g) were placed in 8 beakers. Isotonic sucrose solution (4 x 5 ml) (reference samples) and 4 x 5 ml c-acid solution (0.045 M lactic acid; 0.205 M sucrose; 0.02 M KCl) (lactic acid samples) was added to the beakers. The ples were incubated in a waterbath (18 °C). After t=0 hr and t=24 hr, samples (2 reference and 2 lactic acid samples) were oved from the waterbath, 45 ml of icecold isotonic sucrose solution was added and the samples were placed in ice-water.

Homogenization: Directly after incubation the samples were gently homogenized with a Colora cell-homogenizer. Each sample was homogenized for 15 min at 0-4 °C. After homogenization the samples were filtered through double layer of cheese cloth and pH was recorded.
Centrifugation: The samples (45 ml) were centrifuged in a IEC M-25 High speed centrifuge at 3,000 x g (4 °C) for 10 min. The supernatant was decanted and the pellet was resuspended with a glass-homogenisator in 20 ml of icecold isotonic sucrose solution (the nuclear fraction).The supernatant was further centrifuged at 60,000 x g (4 °C) for 210 min, decanted (the soluble fraction) and the pellet was resuspended with a glass-homogenisator in 10 ml of icecold isotonic sucrose solution (the microsomal fraction). Samples were frozen in liquid nitrogen and stored at -18 °C until analysis of enzyme activity (within 4 weeks).

This procedure was repeated for muscles from two other animals but this time treated with 0.015 M and 0.060 M of lactic acid (0.015 M lactic acid; 0.235 M sucrose; 0.02 M KCl and 0.060 M lactic acid; 0.19 M sucrose; 0.02 M KCl, respectively).

pH-adjustment of meat-homogenates (experiment 2): *M. sternomandibularis* was excised, prepared and homogenized as described above (40 g of meat in 400 ml of isotonic sucrose solution). The homogenate was warmed to 18 °C in a waterbath. The meat-homogenate was placed in 8 flasks (50 ml in each) and the pH was adjusted to 6.5; 6.0; 5.5 and 5.0 (2 flasks for each pH-value) with 0.1 M isotonic lactic acid solution (0.1 M lactic acid, 0.15 M sucrose, 0.02 M KCl). The samples were kept at 18-20 °C and pH was kept constant with 0.1 M isotonic lactic acid solution or 0.1 M isotonic KOH solution (0.1 M KOH, 0.09 M sucrose). After 1 hour, the pH was raised to 7 with 0.1 M isotonic KOH solution and the samples were placed in icewater. The added volumes (lactic acid and KOH) were recorded and isotonic sucrose solution was added to a total added volume of 10 ml. Samples were centrifuged, frozen and analysed, as described above.

Enzyme activity of cathepsin B+L: The method was a modification of BARRETT (1980). Frozen samples were thawed under cold running water and 20 x diluted with assay buffer (532 mM KH_2PO_4; 48 mM Na_2HPO_4; 4 mM Na_2-EDTA; 0.1 % Brij-35; pH 6.0). Cystein buffer was freshly made before use by dissolving cystein (HCl-salt) in assay buffer (8 mM). The substrate, Cbz-Phe-Arg-NMec (Sigma), was dissolved in dimethylsulfoxid as a 0.154 mM sclution and stored at 4 °C. Before use, the substrate stock was freshly diluted to 0.02 mM with assay buffer. Tubes containing 500 µl enzyme solution and 250 µl cystein buffer were prewarmed in a waterbath at 40 °C for 2 min. The assays were started by introduction of 250 µl of 0.02 mM substrate solution into each tube with vigorous mixing. After 60 min incubation at 40 °C, the reaction was stopped by addition of 1 ml of chloroacetate buffer (100 mM Na-chloroacetate; 30 mM Na-acetate; 70 mM acetic acid, pH 4.3). Fluorescence measurements (25 °C, 10 x 10 mm acryl cell) were carried out with an SLM 48000S spectrofluorometer (450 W Xe arc lamp, SLM MC200 excitation and MC320 emission monochromator). Samples were excitated at 370 nm and the intensity of the emission at 435 nm was measured relatively to a 7-amino-4-methylcoumarin standard. Freshly prepared from 650 µl of 28.5 µM 7-amino-4-methylcoumarin (Sigma) in dimethylsulfoxid and 4.35 ml assay buffer. This solution (250 µl) was mixed with 750 µl assaybuffer and 1 ml chloroacetate buffer and used to adjust the instrument reading to 1000, corresponding to 1nmol/tube.

Calculations
Enzyme activity: One milli-unit (munit) of activity was defined as that quantity releasing 1 nmol of 7-amino-4-methylcoumarin /min. For the 60 min assay, a reading of 1000 therefore corresponded to 0.0167 munit of activity in the tube.
Relative free enzyme (%-free activity): According to WU et al., (1985) the %-free activity was calculated as: %-free activity = soluble activity in percentage of total (soluble + microsomal) activity.
Statistical analysis: Analysis of variance was used to test the effect of lactic acid treatment and incubation time on %-free activity and soluble enzyme activity.

Results and discussion

The localization of enzyme activity was examined by fractionated centrifugation. The nuclear and microsomal fraction represent the amount of cathepsinic enzymes remaining in fragmented tissue and membranous material (the nuclear fraction contains larger cell organellés, while lysosomes will be located in the microsomal fraction). The unsedimentable fraction represents the amount of cathepsins released from the lysosomes. The released cathepsins are normally considered to be the active enzymes during conditioning.

experiment 1, the nuclear fraction was ignored (no enzyme activity measured), because the main interest was ascribed to %-free activity and the amount of enzyme activity in the soluble fraction (MOELLER et al.,1977).

le 1 shows the mean of pH-values, total activity in soluble and microsomal fractions and %-free activity. pH of the samples lowered by an increasing concentration of lactic acid. The pH decrease during 24 hr incubation is not seen for the 0.060 M c acid treatment. For unknown reasons the ultimate pH of the reference samples are relatively high compared to what is ally expected for the *M. sternomandibularis* (5.70-5.75; MØLLER, 1985).

le 1: The pH-values, total activity of soluble and microsomal fractions (tot. activ. (sol./micro.)) and %-free activity in eriment 1. pH values measured at 5 °C. Each figure is the mean of two measurements.

	0.015 M lactic acid			0.045 M lactic acid			0.060 M lactic acid		
	pH	Tot. activ. (mU) (sol./micro.)	%-free activity	pH	Tot. activ. (mU) (sol./micro.)	%-free activity	pH	Tot. activ. (mU) (sol./micro.)	%-free activity
ence 0h	6.58	8.45 / 3.02	73.7	6.52	10.54 / 2.47	81.0	6.34	15.78 / 4.87	76.6
ence 24h	6.21	13.82 / 5.40	71.8	6.03	21.18 / 6.49	76.5	6.05	26.65 / 7.63	77.7
c acid 0h	6.40	8.71 / 3.69	70.3	5.91	19.42 / 1.69	92.0	4.90	20.82 / 0.62	97.1
c acid 24h	6.12	14.74 / 4.87	75.1	5.56	24.98 / 2.92	89.5	5.09	26.89 / 1.25	95.6

statistical analysis shows that 0.045 M and 0.060 M lactic acid treatment significantly ($P<0.01$ and $P<0.001$, respectively) cted %-free activity, giving higher values of %-free activity for the lactic acid treated samples compared to the reference ples. No signficant changes in %-free activity were seen during the incubation period for either of the samples.

enzyme activity of the soluble fraction increases significantly ($P<0.01$) during the incubation period for all the samples. pite this increase, %-free activity is maintained at similar levels, because of an equivalent increase in microsomal activity.

enzyme activity of the soluble fraction starts (t = 0hr) at a significantly ($P<0.05$) higher level for the 0.045 M lactic acid ment compared with the reference value (t = 0hr). The same (not significant) pattern is seen for the 0.060 M treatment. se results indicate that a lactic acid treatments de-stabilize the lysosomal membrane and that enzymes to a greater extent leak out into the cytosol compared to non-lactic acid treated samples. Lactic acid treatment should in that sense give more durable conditions for cathepsinic enzyme activity. These results are comparable with WU et al., (1981), who showed an eased amount of lysosomal enzymes in the soluble fraction after high temperature conditioning.

xperiment 1 the homogenization and centrifugation were conducted at different pH values due to the lactic acid treatment. refore, it can not be ruled out that the observed effect of lower pH on the membrane could be influenced by mechanical ss during the fractionation steps. Some previous work concerning the purification of lysosomal fractions used an stment of pH to approximately 7 prior centrifugation, but homogenization were still ducted at the respective pH's of the ples (WU et al., 1981, 1985; MOELLER et 1977).

nvestigation of this problem was intended xperiment 2, where the only difference ween samples was due to the various pH- ues during the 1 hr incubation. The results ow, that the activity of the microsomal ction is very sensitive to changes in pH (see re 1). The activity of the microsomal ction is significantly ($P<0.01$)

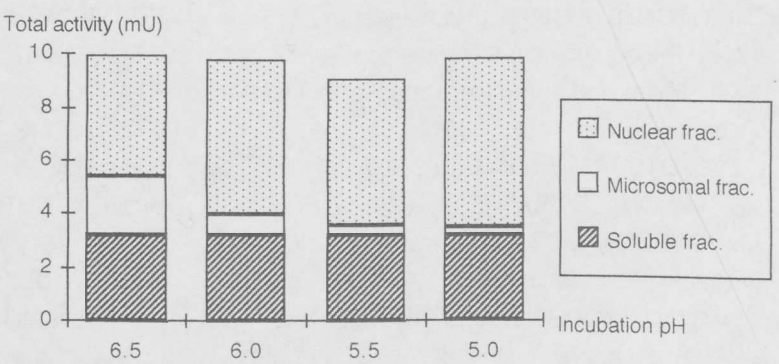

Figure 1: Activity content of nuclear, microsomal and soluble fraction in experiment 2. Each result is the mean of two measurements.

decreased with decreasing pH, similar to the results from experiment 1 (see Table 1). The amount of activity in the soluble fraction is unaffected by lowering the pH. Thus the decrease in activity of the microsomal fraction is not reflected in the soluble fraction, as seen in experiment 1, but in the nuclear fraction.
In that sense, experiment 2 shows that the de-stabilization effect of pH on the lysosomal membrane does not cause an increased release of enzymes to the cytosol as normally expected.
The effect of centrifugation on the samples at the different pH values was investigated in an experiment (not shown) similar to experiment 2, but without pH adjustment before centrifugation. This experiment gave exactly the same pattern as shown in Figure 1, i.e., the decrease in microsomal activity was not reflected in the soluble fraction, but the amount of activity in the microsomal fraction was lowered, thus giving a higher level of %-free activity. The values for %-free activity in Table 1 are therefore probably over-estimated.

The results from this work show that the lysosomal membrane is affected by a lactic acid treatment, giving higher levels of %-free enzyme activity. It is not clear to what extent lactic acid treatment actually results in a higher level of enzyme activity in the soluble fraction. Treatment of meat with 0.045 M and 0.060 M lactic acid resulted in a increase in soluble enzyme activity at 0 hr compared with the reference values, while experiment 2 showed no increase in soluble enzyme activity with decreasing pH. The increase in experiment 1 could therefore be due to the fact that homogenization is conducted at the respective pH values of the samples. Thus, an increase of soluble activity is probably affected by a combination of low pH and stress during homogenisation.
It is not yet known, whether meat-homogenates really can be used to simulate the processes in post-mortem muscle.

It is seen from the results, that the influence of pH on the lysosomal membrane is not a straight forward process. It will demand future research to determine if lactic acid treatment is capable of elevating the amount enzymes released from the lysosome in the muscle during conditioning, resulting in an improvement of tenderness.
Future work should include:
-incubation of meat-homogenates for more than one hour.
-repeating experiment 1, using a buffer-medium during homogenization and analyzing the nuclear fraction..
-enzyme distribution, collagen degradation and tenderness measurements in relation to lactic acid treatment of meat.

Conclusion

Lactic acid can induce a rapid pH-fall in pre-rigor meat and the %-free activity of cathepsins B+L will increase with increasing concentration of lactic acid, indicating de-stabilization of the lysosomal membrane. Whether this de-stabilization actually is capable of increasing the proteolytic activity of the cathepsins (increasing the enzyme activity in the cytosol) is not known and further experiments are needed.

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