EFFECT OF POST MORTEM pH, TIME and TEMPERATURE ON THE DISTRIBUTION OF LYSOSOMAL ENZYMES IN BEEF MUSCLE

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SUMMARY

The effect of lactic acid pre rigor on the distribution of lysosomal enzymes in beef muscle was investigated in a homogenate, and in marinated and lactic acid injected samples. After homogenisation, subcellular fractions were isolated by differential centrifugation, and the activity of cathepsin B + L and of β-glucuronidase in each fraction was determined. In a meat homogenate, lowering the pH from 7.2 to 5.0 shifted the majority of cathepsin B + L activity from the membrane fractions to the myofibrillar fraction. In contrast using a marinating or an injection procedure, the activity of cathepsin B + L in the membrane fractions decreased and the activity in the soluble fraction increased with decreasing pH and with increasing incubation time. A pH reduction by lactic acid injection induced a rapid and enhanced release of proteolytic enzymes from the lysosomes.

Introduction

Several protease systems within the muscle could be involved in development of tenderness (Ouali, 1992). The normal post mortem accumulation of lactic acid causes the lysosomal membrane to become fragile and leak enzymes (Dutson and Lawrie, 1974; Wu *et al.*, 1981). Cathepsin B (Burleigh *et al.*, 1974) and Cathepsin L (Kirschke *et al.*, 1982; Mason *et al.*, 1984) have been shown to cleave native collagen by depolymerizing the cross-linked fibres.

The meat industry shows a considerable interest in novel methods resulting in decreased conditioning times and tenderness improvements in poorer quality muscles with a high collagen content. One approach is to degrade the intrinsic collagen structure to produce the desired tenderness and texture, i.e. without extensive degradation of muscle fibres. Marinating in organic acids is a traditional culinary technique used to tenderize and to improve the flavour of meat. More recently, Stanton and Light (1990) suggested a pre rigor lactic acid injection procedure for improving or upgrading lower quality muscles. This work studies the influence of post-mortem pH, time and temperature on the release of lysosomal enzymes.

Materials and methods

Muscles

Experiments were carried out using beef muscles excised within 30 min of slaughter. The muscle was cleaned for visible connective tissue and fat within 2 h after slaughter. For the studies using a meat homogenate and a marinating procedure *M. sternomandibularis* were used. Experiments with injected lactic acid were performed on *M. pectoralis profundus*.

Meat homogenate

M. sternomandibularis was homogenised as descibed below. Samples were withdrawn from the homogenate, and pH was adjusted to 7.2 (no addition), 6.0, 5.5 and 5.0 by addition of 0.3 M lactic acid. After incubation for 60 min at 0 °C, pH was adjusted back to pH 7.2 with 0.3 M KOH. Following subcellular fractionation, cathepsin B + L actvity of each fraction was determined as described below.

Marinating procedure
The marinade consisted of buffer A (pH 7.2, see below), or buffer A titrated to pH 5.5 by lactic acid (0.3 M) resulting in a final lactic acid concentration 0.012 M. After addition of marinade (10 vol,) each muscle sample (1.5 g) was divided into approximately 30 small pieces by scissors. After incubation at 15 °C or 30 °C, samples were subcellular fractionated as described below. At both temperatures incubation times of 1 hour and 24 hours were studied.

Lactic acid injection
Lactic acid was injected using a multi pipette with fixed needles to a level of 14% of the original weight of the muscle (*M. pectoralis profundus*). A square (8 x 8 cm) was injected in three depths with approx. 0.5 cm between each needle injection point. After 24 hr at 15 °C, samples (15 g) were excised and vacuum packed before conditioning at 4 °C up to 12 days. pH was measured using a probe electrode.

Homogenization and subcellular fractionation.
All procedures were carried out at 0-4 °C. The muscle was finely minced with scissors after addition of 2 vol. of buffer A (100 mM sucrose, 100 mM KCl, 50 mM tris-HCl, 10 mM pyrophosphate, 1 mM EDTA, pH 7.2) containing 50 μg/ml nagarse. After 5 min incubation, the mince was transferred to buffer A (20 ml/g tissue) and homogenised using a motor-driven Potter-Elvehjem-type homogenizer (glass/teflon, clearance 0.15 mm) by making 10 complete passes at 1500 rpm. The homogenate was filtered through cheesecloth and centrifuged at 1,100 *g* for 10 min to obtain a myofibrillar/nuclear fraction. The supernatant was then centrifuged at 3,000 *g* for 10 min to obtain a heavy mitochondrial fraction. The supernatant was then centrifuged at 27,000 *g* for 20 min to obtain a lysosomal fraction. Finally the supernatant was centrifuged at 100,000 *g* for 60 min to obtain a microsomal fraction and a soluble fraction (the final supernatant). Pellets were resuspended in a small volume of buffer (85 mM Na acetate, 15 mM acetic acid, 1 mM Na_2EDTA, pH 5.5) and rapidly frozen in liquid nitrogen.

Assays of enzyme activities
The cathepsin B + L activities were determined fluorimetrically according to Kirschke *et al.*, (1983) using the common substrate Z-Phe-Arg-NMec (where Z = benzyloxycarbonyl and NMec = 4-methyl-7-coumarylamide). The fluorimetric assay for β-glucuronidase was performed as described by Moeller *et al.*, (1976) using 4-methylumbelliferyl-β-D-glucuronide as substrate.

Results and discussion

Meat homogenate
This experiment was set up to investigate the effect of low pH on the stability of the lysosomal membrane. Lysosomes are very sensitive to changes in pH (fig. 1). For simplicity the measured activities in the lysosomal, microsomal and heavy mitochondrial fractions were combined and hereafter referred to as the membrane fraction. The activity of the membrane fraction shows a pronounced decrease with decreasing pH, i.e. from 73% of total activity at pH 6.0 to less than 8% at pH 5.0. The majority of the activity does not redistribute into the soluble fraction, but emerge in the myofibrillar fraction (10% of the total activity at pH 6.0 and 68 % at pH 5.0). It is not clear whether the lysosomal enzymes have actually been released and reappear in the myofibrillar fraction or the lysosomes have dramatically altered their sedimentation characteristics.

The distribution of β-glucuronidase activity was almost similar to the distribution of cathepsin B + L (results not shown).

Lactic acid marinade
When comparing the effect of the lactic acid marinade, pH 5.5, with the pH 7.2 marinade it can be seen that low pH consequently shifted cathepsin B + L activity from the membrane fractions into the soluble fraction (table 1). This effect is seen after 1 hour incubation at both 15 °C and 30 °C and after 24 hours incubation at both temperatures.

The effect of increasing the temperature from 15 °C to 30 °C parallels the effect of lactic acid addition (table 1). Again cathepsin B + L activity is shifted from the membrane fractions into the soluble

fraction. This is seen after 1 hour as well as after 24 hours incubation with and without lactic acid in the marinade.

The effect of increasing the incubation time from 1 hour to 24 hours can again be seen as a shift of cathepsin B + L activity from the membrane fractions into the soluble fraction. The percentage of total activity in the myofibrillar fraction is only slightly affected by pH, time and temperature in this experiment (seen as 100% minus the combined activity in the soluble and membrane fractions in table 1). The appearance of the enzyme activity in the soluble fraction is in accordance with other studies on the effect of time (Dutson and Lawrie, 1974) and high temperature conditioning (Wu *et al.*, 1981) on the distribution of lysosomal enzymes but contrasts the results from the experiment lowering pH in a meat homogenate, where the activity from the membrane fractions redistributed into the myofibrillar fraction (fig. 1). It can be speculated, that the disrupted cell structure in the meat homogenate experiment enables the lysosomes to fuse with other lysosomes/membrane systems, the enzyme activity thus sedimenting in another fraction instead of being liberated.

Lactic acid injection

Lactic acid (0.3 M) was injected in *M. pectoralis profundus* as described. After conditioning (2 h to 12 days) samples were homogenized and subcellular fractions isolated by differential centrifugation. Cathepsin B + L activity in each fraction was determined (table 2). For controls (no injected lactic acid) the enzyme activity in the soluble fraction gradually increased from 22.7% (2 h) to 44.7% (12 days) while the cathepsin B + L activity in the membrane fraction decreased during conditioning from 60.0% (2 h) to 38.5% (12 days). The results show that cathepsins are gradually released from the lysosomes during conditioning.

In lactic acid treated samples cathepsin B + L activity in the soluble fraction increased very fast (24 hours) to 60.9% at which time the activity in the membrane fraction was reduced to 24.1%. This high level of released enzyme activity was not obtained in the control even after 12 days conditioning. Prolonged conditioning time (12 days) did not further increase the activity in the soluble fraction in lactic acid treated samples. The same overall pattern was observed for β-glucuronidase (results not shown). This experiment clearly demonstrates that low pH induced by lactic acid injection releases membrane bound lysosomal enzymes. As in the marinating experiment but not in the meat homogenate, the released enzymes appear in the soluble fraction.

A high ultimate muscle pH is normally associated with tender meat. In contrast, muscles which have a normal ultimate pH, a lower pH at earlier post mortem times has been related to more tender meat (Dutson, 1983). In our lactic acid injected samples, pH declined below 5.5 in less than 1 h and reached a ultimate pH in 24 h around 5.1 (5.4 in controls). Our work indicates that a minimum concentration of 0.2 M pre rigor injected lactic acid seems necessary to obtain a lower ultimate pH as compared to controls. This is a somewhat higher concentration than used by Stanton & Light (1990). We observed that colour acceptability was negatively influenced by lactic acid concentration above 0.2-0.3 M which is in accordance with previous work (Gault, 1991; Stanton & Light, 1990).

Conclusion

Low pH in a meat homogenate by lactic acid addition destabilises the lysosomal membrane resulting in a pronounced shift of enzymatic activity from the membrane fractions into the myofibrillar fraction. In contrast using a marinating or an injection procedure shifts the activity from the membrane fractions into the soluble fraction with lower pH and with increasing incubation time. A pH reduction by lactic acid injection induces a rapid and enhanced release of proteolytic enzymes from the lysosomes.

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Table 1. Effect of temperature, time of incubation and pH in marinade on the distribution of cathepsin B + L activity in finely cut meat.

Table 2. Distribution of cathepsin B + L in bovine *M. pectoralis profundus* during ageing in (a) control and (b) lactic acid injected samples.

Fig. 1. Cathepsin B + L distribution after lactic acid addition to a meat homogenate.