Effect of prerigor lactic acid treatment on lysosomal enzyme release in bovine muscle

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Abstract: An improved method for isolating intact lysosomes from beef muscle is presented, using an unspecific bacterial protease (nagarse, 50 μg ml^{-1}) prior to homogenisation. Nagarse treatment resulted in 100% increased cathepsin B + L activities in the membrane fraction (subcellular fraction containing lysosomes). By using this method the subcellular distribution of lysosomal enzymes, as affected by parameters related to meat marination, was investigated in beef muscle. Different prerigor lactic acid treatments were performed in a muscle homogenate, in a muscle mince finely cut with scissors, and in whole muscle. The effect of lower pH on the level of free (not bound) enzyme activities was dependent on muscle integrity. Lowering pH in the muscle homogenate from 7.2 to 5.0 shifted the majority of cathepsin B + L and β-glucuronidase activities from the membrane fraction into the myofibrillar fraction. In contrast, decreased pH of the muscle mince increased enzyme activities in the soluble fraction ($P < 0.05$). Also the lactic acid injection procedure resulted in increased activities of lysosomal enzymes in the soluble fraction ($P < 0.01$). Storage for up to 4 days resulted in a gradual increase in free enzyme activity also in control samples, but to a lower final level as compared to lactic acid treated samples.

Keywords: beef; muscle; cathepsin B + L; lysosomal integrity; acid marination

INTRODUCTION

Several protease systems within the muscle could be involved in the *post mortem* tenderisation process.[1] The role of each of these systems in the ageing process is still debated but sarcoplasmic calpains have been suggested to be the main proteases.[2–4] It has also been suggested that the normal *post mortem* accumulation of lactic acid causes the lysosomal membrane to become fragile and leak enzymes[5–7] and that the released lysosomal enzymes have the potential to cause myofibrillar weakening.[8–10]

Marinading is performed to give flavour to meat and to improve tenderness and is traditionally the process of immersing meat into an acidic solution of vinegar, wine or fruit juice.[11,12] However, this can lead to an uneven distribution of the marinade in the meat. A concept similar to curing involves injection of lactic acid into pre-rigor muscle.[13] This procedure may result in an accelerated distribution of acid throughout the meat and an earlier release of proteolytic enzymes from the lysosomes. Cathepsin B and L are known to be able to cleave myofibrillar and collagenous proteins,[10,14,15] and an early activation is likely to cause an early effect on tenderness.

Lysosomal cathepsins must be released from their containment within the lysosome to participate in the tenderisation that occur during ageing and through marination. A release of lysosomal enzymes during acid marination has not been directly shown. To determine the localisation of lysosomal enzymes, most studies have used a homogenisation of the meat followed by an enzyme determination in the subcellular fractions after differential centrifugation. The enzyme activity in the sedimentable fractions containing lysosomes has been referred to as bound and the activity in the final supernatant (designated the soluble or the non-sedimentable fraction) as free activity. Increased free lysosomal enzyme activity has thus been implied with storage time.[5,7,16,17] High temperature conditioning, early *post mortem*, results in lower muscle pH and increased free lysosomal enzyme activity.[6,18,19] An accelerated pH decline by electrical stimulation has also shown increased free enzyme activity.[16,17,20]

The role of lysosomal enzymes in acid marination is not fully understood. The objectives of the present work were, within the concept of acid marination, to study the influence of an early *post mortem* lactic acid

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induced pH reduction on the release of lysosomal enzymes at three different levels of integrity of beef muscle structure. Furthermore, the effect of storage time on the release of lysosomal enzymes in beef muscle was investigated.

MATERIALS AND METHODS

Experimental design

The effect of lactic acid induced pH reduction on lysosomal enzyme release was studied in three separate experiments differing widely in the integrity of the muscle structure: (1) a muscle homogenate; (2) muscle finely minced by scissors to pieces of approximately 50 mm^3; and (3) whole muscle.

Muscles

Muscles were excised from 2–4 years old cows within 30 min of slaughter and trimmed for visible connective tissue and fat. In all experiments, lactic acid was added with 2 h of slaughter. *M sternomandibularis* was used for the muscle homogenate and minced muscle experiments while *M pectoralis profundus* was used for the whole muscle experiment. The overall conclusions from the experiment were not expected to be influenced by muscle type as the effect of pH on lysosomal integrity concerns basic subcellular properties.

Muscle homogenate experiment

M sternomandibularis was homogenised as described below. After filtration through cheesecloth samples were withdrawn, and pH was adjusted to 7.2 (no addition), 6.0, 5.5 and 5.0 by addition of 0.3 M lactic acid. Following incubation for 60 min at 0°C, pH was adjusted back to pH 7.2 with 0.3 M KOH. Samples were subcellular fractionated and enzyme activities of each fraction were determined as described below.

Muscle mince experiment

In this experiment the acid was added by immersing muscle that had been minced with scissors in 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 of 0.012 M. After addition of buffer (10 ml g^{-1} tissue) each muscle sample (1.5 g) was divided into approximately 30 small pieces by scissors. Following incubation at 15°C or 30°C for either 1 h or 24 h, excess buffer was decanted. Samples were then homogenised, subcellular fractionated and assayed for enzyme activities as described below.

Whole muscle experiment

Lactic acid (0.3 M) was added by injection using a multi—pipette with fixed needles. The muscle sample (200–300 g) was injected to a level of 11–15% (w/w) of the original weight. Injections were at three depths with approx 0.5 cm between each needle injection point. At selected times, *post mortem* pH was measured using a direct insertion probe electrode (Ingold Lot 406-M3). After 24 h at 15°C, samples (15 g) were excised and vacuum packed before storage at 4°C up to 12 days. At the *post mortem* times of 2 h, 1 day, 4 days and 12 days samples were homogenised, subcellular fractionated and assayed for enzyme activities as described below.

Homogenisation

All procedures were carried out at 0–4°C. Samples of tissue (1.5 g) were added to 3 ml of buffer A (100 mM sucrose, 100 mM KCl, 50 mM tris-HCl, 10 mM sodium pyrophosphate, 1 mM Na_2EDTA, pH 7.2) containing 50 μg ml^{-1} nagarse (Sigma, protease Type XXVII) dissolved immediately before use, and were finely minced with scissors and incubated for 5 min. Excess buffer was decanted off and buffer A (20 ml g^{-1} tissue) without nagarse was added. Homogenisation was performed using a motor-driven Potter-Elvehjem-type homogeniser (glass/Teflon, clearence 0.15 mm) by making 10 complete passes at 1500 rpm. The homogenate was filtered through cheesecloth.

Subcellular fractionation

The filtrate was centrifuged at 1100 × *g* for 10 min to obtain a myofibrillar fraction. The supernatant was then centrifuged at 3000 × *g* for 10 min to obtain a heavy mitochondrial fraction. Then the supernatant was 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). The myofibrillar fraction was resuspended in 5 ml and the other pellets in 2 ml buffer (85 mM Na acetate, 15 mM acetic acid, 1 mM Na_2EDTA, pH 5.5) and rapidly frozen in liquid nitrogen. A membrane fraction, for simplicity of presentation of results, was defined as the combined activities of the heavy mitochondrial, lysosomal and microsomal fractions.

Assays of enzyme activities

The combined cathepsin B + L activities were determined fluorimetrically according to Ref 21 using the substrate N-CBZ-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-NMec). The fluorimetric assay for β-glucuronidase was performed as described previously[18] using 4-methylumbelliferyl-β-D-glucuronide as substrate. All assays were done in triplicate. Activities of cathepsin B + L and β-glucuronidase are expressed as mU (the release of 1 nmol of product per min).

Statistical analysis

Results were examined by standard analysis of variance. Statistical calculations were carried out using SAS STAT™ software (ANOVA procedure).

Table 1. Distribution of cathepsin B + L and β-glucuronidase activity (mU g^{-1} muscle ± SD) in subcellular fractions after marination of finely cut bovine *M sternomandibularis*. Incubation conditions *post mortem* (time, temperature and pH) during marination were as indicated

	Time (*h*)	*Temp* (°*C*)	*pH*	*Myofibrillar*	*Membrane*	*Soluble*	*Total*
Cathepsin B + L	1	15	7.2	0.29 ± 0.04	1.14 ± 0.21	0.29 ± 0.03	1.71 ± 0.28
			5.5	0.26 ± 0.01	1.09 ± 0.01	0.39 ± 0.06	1.74 ± 0.05
		30	7.2	0.27 ± 0.05	1.05 ± 0.13	0.44 ± 0.02	1.76 ± 0.20
			5.5	0.22 ± 0.00	1.03 ± 0.05	0.61 ± 0.02	1.86 ± 0.04
	24	15	7.2	0.26 ± 0.00	1.08 ± 0.01	0.62 ± 0.06	1.97 ± 0.06
			5.5	0.26 ± 0.01	1.02 ± 0.03	0.66 ± 0.04	1.93 ± 0.03
		30	7.2	0.25 ± 0.02	0.77 ± 0.01	1.00 ± 0.05	2.02 ± 0.08
			5.5	0.25 ± 0.03	0.72 ± 0.00	1.18 ± 0.02	2.16 ± 0.00
β-Glucuronidase	1	15	7.2	0.75 ± 0.09	1.87 ± 0.39	0.80 ± 0.07	3.41 ± 0.55
			5.5	0.68 ± 0.03	1.88 ± 0.02	1.09 ± 0.05	3.65 ± 0.00
		30	7.2	0.62 ± 0.11	1.57 ± 0.36	1.07 ± 0.16	3.26 ± 0.64
			5.5	0.50 ± 0.04	1.44 ± 0.02	1.09 ± 0.01	3.03 ± 0.05
	24	15	7.2	0.51 ± 0.03	1.25 ± 0.03	0.77 ± 0.02	2.52 ± 0.04
			5.5	0.63 ± 0.00	1.21 ± 0.01	0.87 ± 0.04	2.71 ± 0.05
		30	7.2	0.62 ± 0.04	0.90 ± 0.02	1.06 ± 0.05	2.58 ± 0.01
			5.5	0.80 ± 0.11	0.86 ± 0.05	1.28 ± 0.01	2.94 ± 0.07

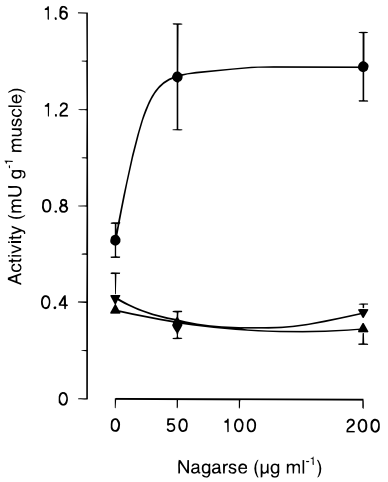

Figure 1. Effect of nagarse treatment before homogenisation on distribution of cathepsin B + L activity in subcellular fractions. Means of duplicate determinations are for each subcellular fraction shown as mU g^{-1} muscle; error bars indicate the SD values. ●, Membrane fraction; ▲, soluble fraction; ▼, myofibrillar fraction.

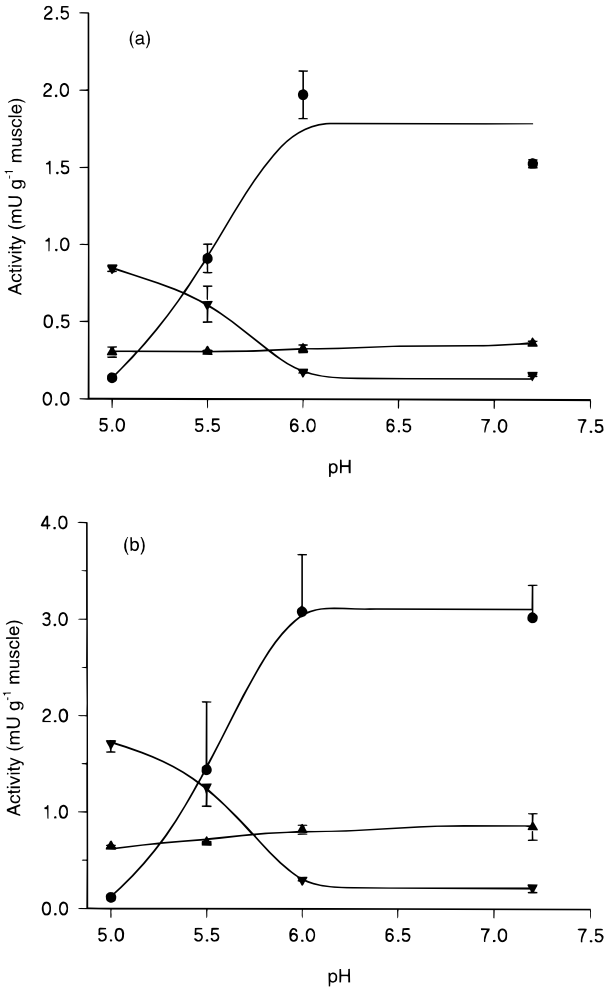

Figure 2. Effect of pH in muscle homogenate on distribution of (a) cathepsin B + L and (b) β-glucuronidase activity in subcellular fractions. Means of duplicate determinations are for each subcellular fraction shown as mU g^{-1} muscle; error bars indicate the SD values. ●, Membrane fraction; ▲, soluble fraction; ▼, myofibrillar fraction.

RESULTS

Effect of nagarse

The effect of nagarse incubation prior to homogenisation on lysosomal enzyme distribution in subcellular fractions was investigated. *M sternomandibularis* was finely cut with scissors and incubated for 5 min with either 0, 50 or 200 μg ml^{-1} nagarse in buffer A. Homogenisation and subcellular fractionation were performed as described in the Materials and methods section. Figure 1 shows that nagarse treatment (50 μg ml^{-1}) resulted in a 100% increase of the cathepsin B + L activity in the membrane fraction (from 0.66 to 1.34 mU g^{-1} muscle). Activities in the myofibrillar and soluble fractions were not affected by nagarse treatment. Effect of

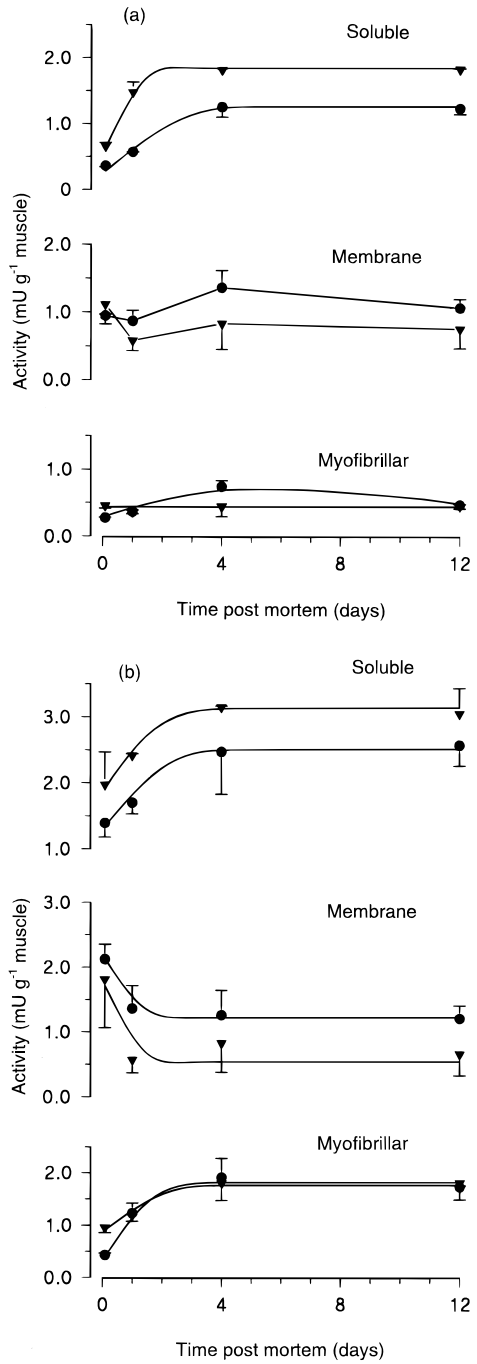

Figure 3. Effect of storage of control and lactic acid injected *M pectoralis profundus* on the distribution of (a) cathepsin B + L and (b) β-glucuronidase. Results are for each subcellular fraction shown as mU g^{-1} muscle, ●, Control; ▼, lactic acid injected to 11–15% (w/w).

incubating with 50 and 200 μg ml^{-1} nagarse were similar. For the subsequent experiments, incubation with 50 μg ml^{-1} nagarse was performed prior to the homogenisation. To obtain information on the physical effect of nagarse treatment on myofibrils, SDS-PAGE was performed on the first pellet obtained after homogenisation and centrifugation (myofibrillar fraction). Nagarse incubation resulted in pronounced degradations of major myofibrillar proteins, including myosin heavy chain and actin (results not shown).

Muscle homogenate

Figures 2(a) and (b) shows the effect of pH on the distribution of lysosomal enzymes in subcellular fractions when adding lactic acid to the muscle homogenate. Reducing pH from 7.2 to 6.0 only resulted in minor changes in enzyme distribution. A pronounced redistribution of activity occurred between pH 6.0 and 5.0. The cathepsin B + L activity in the membrane fraction decreased from 1.9 to 0.14 mU g^{-1} muscle and increased in the myofibrillar fraction from 0.18 to 0.85 mU g^{-1} muscle (Fig 2(a)). Similarly, β-glucuronidase activity decreased from 3.1 to 0.12 mU g^{-1} muscle in the membrane fraction and increased from 0.30 to 1.7 mU g^{-1} muscle in the myofibrillar fraction when lowering pH from 6.0 to 5.0 (Fig 2(b)). Cathepsin B + L and β-glucuronidase activities in the soluble fraction were not affected by pH.

Muscle mince

Small pieces of muscle were marinated under different conditions with respect to incubation time, temperature and pH of the buffer (marinade). Table 1 shows the means of cathepsin B + L and β-glucuronidase activities in the subcellular fractions. Analysis of variance for main effects of lactic acid and incubation time and temperature showed that lowering the pH in the marinade (7.2 to 5.5) increased cathepsin B + L and β-glucuronidase activities in the soluble fraction ($P < 0.05$). Increased incubation time (1–24 h) resulted in increased cathepsin B + L activities in the soluble fraction ($P < 0.001$) and decreased β-glucuronidase activities in the membrane fraction ($P < 0.01$). Increased incubation temperature (15–30°C) decreased cathepsin B + L and β-glucuronidase activities in the membrane fraction as well as increased activities in the soluble fraction ($P < 0.01$). Increasing both incubation time and temperature added further to the observed differences with respect to decreased enzyme activities in the membrane fraction and increased activities in the soluble fraction. Enzyme activities in the myofibrillar fraction were not affected ($P > 0.1$) by pH, time and temperature in this experiment. Total activity increased with incubation time for cathepsin B + L and decreased with incubation time for β-glucuronidase ($P < 0.01$).

Whole muscle

In lactic acid injected samples, the pH declined below 5.5 after less than 1 h and reached an ultimate pH at 24 h of approximately 5.1 (5.4 in non-injected controls). Figure 3 shows the distribution of cathepsin B + L (Fig 3(a)) and β-glucuronidase (Fig 3(b)) in the subcellular fractions during storage. Analysis of variance for main effects of lactic acid and storage were performed. Lactic acid injection resulted in increased cathepsin B + L and β-glucuronidase activities in the soluble fraction ($P < 0.01$) and decreased β-glucuronidase activities in the mem-

brane fraction ($P < 0.05$) while the measured enzyme activities in the myofibrillar fraction were unaffected ($P > 0.1$). Storage also increased cathepsin B + L and β-glucuronidase activities in the soluble fraction ($P < 0.01$) and decreased β-glucuronidase activities in the membrane fraction ($P < 0.05$). Furthermore, storage increased β-glucuronidase activity in the myofibrillar fraction ($P < 0.001$). For controls, the cathepsin B + L and β-glucuronidase activities in the soluble fraction gradually increased during storage from 2 h to 4 days and with no further increase up to day 12 (Fig 3(a) and (b)).

Overall, the whole muscle experiment shows that low pH induced by lactic acid injection results in an enhanced release of lysosomal enzymes as enzyme activities generally increased in the soluble and decreased in the membrane fraction, although the latter effect was only significant for β-glucuronidase.

DISCUSSION

To study the localisation and possible release of lysosomal enzymes *in situ*, the amounts of enzymes leaking from the lysosomes during homogenisation and subsequent isolation should be kept as low as possible. One problem in isolating intact lysosomes is the resistance to physical disintegration of the muscle tissue because an effective homogenisation tends to disrupt some lysosomes. Nagarse is an unspecific bacterial protease. It has previously been used to facilitate the isolation of intact mitochondria from muscle.[22,23] Nagarse incubation before homogenisation increased total cathepsin B + L activity (the combined activity in all fractions) by 35% (data from Fig 1). This suggests that nagarse incubation promoted a more efficient disintegration of muscle cells during homogenisation. This explanation is supported by the observations that nagarse induced pronounced degradation of proteins in the myofibrillar fraction (results not shown). Nagarse did not disturb the measurements of enzyme activities since nagarse, at a concentration 10 times in excess of that present after homogenisation, showed no activity in either the cathepsin B + L or the β-glucuronidase assays (data not shown). Also, to reduce the risk of nagarse interference in subsequent steps, excess nagarse-containing buffer was decanted prior to homogenisation, although any released lysosomal enzyme activity present in that solution would be lost.

The homogenisation procedure using nagarse resulted at early *post mortem* times in only approximately 20% of total cathepsin B + L activity in the soluble fraction (data from Fig 2(a) at pH 6.0 to 7.2) and approximately 25% of total β-glucuronidase activity in the soluble fraction (data from Fig 2(b) at pH 6.0 to 7.2). Our method thus compares favourably with methods from other studies measuring the activity of free (not membrane bound) lysosomal enzyme activities in muscle.[7,17,24–26] For instance, Chambers *et al*[7] reported 45% of total cathepsin D and 35% of total β-glucuronidase activity in the soluble fraction at early *post mortem* times.

For analysing the effect of lactic acid treatment on the release of lysosomal enzymes, three different structural levels of muscle were studied: (i) a muscle homogenate, (ii) muscle finely minced by scissors before marination and (iii) whole muscle injected with lactic acid. In the muscle homogenate, the cell structure is broken down before the addition of acid and a uniform acid distribution is obtained almost immediately. Small pieces of muscle were used in the muscle mince experiment to reduce the time of acid diffusion into the meat. To accelerate the acid distribution in the whole muscle experiment, the lactic acid injection was performed with a small distance between each needle injection point.

In the muscle homogenate, the activity of the membrane fraction was very sensitive to changes in pH between 6.0 and 5.0 as enzyme activities in the membrane fraction decreased and activities in the myofibrillar fraction increased (Fig 2(a) and (b)). In this experiment, pH was lowered and then pH was titrated back to 7.2 before subcellular centrifugation. It cannot be ruled out that these pH changes caused irreversible effects on the enzyme activities, as the sum of activities (total activity) decreased with decreasing pH. It can be speculated that the disrupted cell structure in the muscle homogenate experiment enabled either released enzymes or lysosomes to move freely and associate with components in the myofibrillar fraction. This could explain why lower pH resulted in increased activities in the myofibrillar rather than in the soluble fraction, as seen in the muscle mince and whole muscle experiments.

In the muscle mince experiment, the marinading buffer was decanted prior to subcellular fractionation. It is possible that some released lysosomal enzymes had sufficient time to migrate into the solution that was decanted, in which case the amount of free enzyme activity was, to some extent, underestimated in that experiment.

The effect of storage time was investigated in the muscle mince (1–24 h *post mortem*) and whole muscle experiment (up to 12 days *post mortem*). In both experiments the overall tendencies were towards increased enzyme activities in the soluble fraction and decreased activities in the membrane fraction with increasing storage time (Table 1, Fig 3(a) and (b)). This demonstrates that β-glucuronidase and cathepsins were released from lysosomes into the sarcoplasm not only after lactic acid treatment but also in non-treated or normal meat during storage. For the latter part, these results agree with other studies. Dutson and Lawrie[5] reported that the specific activity of free β-glucuronidase increased with storage up to 14 days. Pommier[16] observed an increase in free cathepsin D and β-glucuronidase from 4 to 24 h. Ageing from 1 to 14 days increased free cathepsin B + L and β-glucuronidase.[17] Chambers *et al*[7] showed increased specific activity of β-glucuronidase and cathepsin D in a soluble fraction

and decreased β-glucuronidase activity in a membrane fraction after ageing up to 14 days.

The high temperature (30°C) incubation in the muscle mince experiment also resulted in increased cathepsin B + L and β-glucuronidase activities in the soluble fraction and decreased activities in the membrane fraction (Table 1). The release of enzymes by increased incubation temperature was additionally enhanced by lower muscle pH. These observations are supported by other studies. High temperature incubation of early *post mortem* muscle results in an increased rate of pH decline[19] and has previously been shown to increase the activity of free cathepsin C and β-glucuronidase[18,19] and to increase the specific activity of free β-glucuronidase and β-galactosidase.[6]

In conclusion, muscle incubation with nagarse increased the cathepsin B + L activity in the membrane fraction, indicating that nagarse incubation promoted a more efficient disintegration of muscle cells during homogenisation. Lactic acid addition to a beef muscle homogenate resulted in decreased cathepsin B + L and β-glucuronidase activities in the membrane fraction and increased activities in the myofibrillar fraction. In a muscle mince and in whole muscle pre-rigor, lactic acid resulted in an enhanced level of enzyme activities in the soluble fraction. These results indicate that acid marination of beef can cause lysosomal enzymes to be released. Storage up to 4 days resulted in an increased amount of free enzyme activity in both lactic acid treated and control samples. Further studies are designed to examine the relationship between increased release of lysosomal enzymes, protein degradation and tenderness in beef muscle injected with lactic acid.

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