4-P20

Localisation of Lysosomal Cathepsin B During the Post-Mortem Storage of Bovine Muscle at 4°C and 25°C using Enzyme Fluorescence Histochemistry.

Makarim Mobarak[(1)], Sean Connell[(1)], William Reville[(1)], and Michael Zeece[(2)].
(1) Biochemistry Department, University College, Cork, Ireland.
(2) Department of Food Science and Technology, University of Nebraska, Lincoln, Nebraska, USA.

Background:

Tenderness is the primary quality attribute of beef for the consumer. Bovine muscle toughens with the onset of rigor mortis, but this is gradually reversed during conditioning when the carcass is hung at 4°C for 14 days. Limited degradation of myofibrillar and associated cytoskeletal proteins contributes to the increase in tenderness during conditioning. The endogenous muscle proteases obviously suggest themselves as agents responsible for this limited degradation and there is much evidence that calpain is involved (Koohmaraie, 1996). However, there is also evidence that elevated levels of free calcium post-mortem directly cause myofibrillar degradation (Takahashi, 1996).

For many years the lysosomal cathepsins were regarded as the primary cause of post-mortem myofibrillar proteolysis. This hypothesis fell out of favour with the failure to find evidence of post-mortem degradation of actin and myosin during conditioning of meat at 4°C – actin and myosin are readily degraded by several cathepsins in *in-vitro* assay (Goll et al., 1983; Zeece et al., 1992). However, breakdown of actin and myosin is evident when conditioning is carried out at 25°C or higher (e.g. Penny and Ferguson-Pryce, 1979; Arakawa et al., 1976) and this effect is ascribed to proteolysis by cathepsins.

When assessing the potential of cathepsins to carry out post-mortem myofibrillar proteolysis, a further consideration arises – are these enzymes released from lysosomes post-mortem? Unless they are released they cannot act on the myofibrils which are present outside the lysosomes.

Moeller et al., (1976, 1977), Wu et al, (1981), Dutson (1983), O'Halloran et al., (1997), reported biochemical evidence of leakage of lysosomal enzymes into the soluble fraction in post-mortem bovine muscle. This leakage is enhanced by higher temperature post-mortem storage or by electrical stimulation of the muscle. In a more quantitative biochemical study, Chambers et al., (1994) found evidence only of a limited release of lysosomal enzymes into the soluble fraction during post-mortem storage of bovine muscle at 4°C. However, biochemical evidence of post-mortem leakage of cathepsins from lysosomes is ambiguous because of the relative insensitivity of the standard technique of assaying sub-cellular fractions prepared from muscle homogenates.

Kas et al., (1983) reported histochemical evidence of leakage of lysosomal enzymes post-mortem. On the other hand, Lacourt et al., (1986), using fluorescent localisation techniques, reported that the at-death pattern of cathepsin B localisation in bovine muscle remained unchanged over a 30-day post-mortem storage period at 4°C.

In addition to myofibrillar degradation, there is also evidence of proteolytic alteration of connective tissue during conditioning of meat (reviewed by Zeece et al., 1992). In view of the broad range of specifities found amongst the cathepsins against myofibrillar proteins, collagen, proteoglycans, etc., (Zeece et al., 1992), in view of evidence to-date regarding post-mortem leakage of cathepsins, and in view of studies correlating cathepsin activities with objective measures of tenderness, (e.g. Morgan et al., 1989), it continues to be important to know the extent to which lysosomes leak their enzymes post-mortem.

Objectives:

The present study attempts to assess the extent of leakage of cathepsin B from lysosomes during the post-mortem storage of bovine skeletal muscle, held either at 4°C or at 25°C, using a fluorescent enzyme histochemical technique.

Methods:

Cathepsin B activity was detected using the fluorescent assay described by Stauber and Ong, 1981. Skeletal muscle (*Sternomandibularis*), heart and liver samples were taken from ten 18 month old Friesian heifers at slaughter. Half of the skeletal muscle samples were stored at 4°C and the other half at 25°C; the cardiac and liver samples were stored at 4°C. The fluorescent enzyme histochemical procedure was carried out on the samples at-death and again after 1 day, 7 days and 14 days sample storage. The reaction mixture contained 1mM substrate (CBZ-Arg-Arg-4-methoxy-β-naphltylamide) dissolved in 1mM 5-nitrosalicylaldehyde, 0.2mM EDTA, 0.25mM DTT, in 0.2M ammonium acetate buffer, pH 6.0. Various controls were employed:- (a) incorporation of the protease inhibitor leupeptin (5 μM) in the reaction mixture. (b) pre-heating sections at 80°C for 10 mins., (c) omitting substrate, (d) omitting DTT or EDTA from the reaction mixture. No activity was seen in any controls.

Results and Discussion:

Cathepsin B is visualised by this technique as shining foci of activity and, as seen in the at-death samples in Figure 1, it is clear that enzyme activity is greatest in liver, intermediate in cardiac tissue, and lowest in skeletal muscle. This result conforms with well established biochemical evidence that general lysosomal activity in these three tissues is expressed in that same order.

In skeletal muscle the cathepsin B activity is primarily located near the edges of the fibres, and, as conditioning proceeds at 4°C, from day 1 through day 14 post-mortem, the shining foci gradually become smaller, dimmer, and fewer in number. Very little localised activity is seen at day-14. These changes are much more pronounced in skeletal muscle conditioned at 25°C, where almost no shining foci are seen by 7 days post-mortem – Figure 1. The tendency for cathepsin B in skeletal muscle to be localised near fibre edges has been noted in previous studies, e.g. Stauber and Ong (1981).

The same general picture showing shining foci of cathepsin B activity becoming smaller, dimmer and fewer in number is seen during post-mortem storage of bovine liver and cardiac muscle at 4°C. In both of these tissues, localised cathepsin B activity remains visible after 14 days conditioning but is greatly reduced in extent compared to at-death samples – Figure 1.

The results of the present study bear the interpretation that cathepsin B leaks from the lysosomes in post-mortem tissue and, in skeletal muscle, does so earlier and more completely at 25°C than at 4°C. No activity is detected outside the lysosomes as a result of leakage, which probably means either that the dispersed activity is too diffuse to be detected by the technique, or that the leaked cathepsin B activity is masked by protease inhibitors (cystatins). Finally, the preferential location of lysosomes close to the fibre edge in skeletal muscle places cathepsins with collagenolytic activity (e.g. B and L) close to connective tissue proteins. Cathepsins leaked post-mortem would have but a short distance to diffuse to effect connective tissue proteolysis.

Conclusions:

The enzyme histochemical evidence bolsters the existing ambiguous biochemical evidence that lysosomal enzymes leak from bovine skeletal muscle lysosomes post-mortem and are, consequently, available to digest myofibrils and other substrates. The earlier and complete release seen during conditioning at 25°C harmonises with other biochemical evidence that cathepsins effect noticeable degradation of actin and myosin during higher temperature conditioning.

References:

Arakawa, N., Inagaki, C., Kitamura, T., Fujiki, S. and Fujimaki, M. *Agr. Biol. Chem.*, **40**, 1445-1447, (1976).
Chambers, J.J., Reville, W.J. and Zeece, M.G. *Sciences Des Aliments*, **14**, 441-457, (1994).
Dutson, T.R. *J. Food Biochem.*, **7**, 223-245, (1983).
Goll, D.E., Otsuka, Y., Nagainis, P.A., Shannon, J.D., Sathe, S.K. and Muguruma, M. *J. Food Biochem.*, **7**, 137-177, (1983).
Kas, J., Vana, V., Kopecny, J. and Rauch, P. *Z. Lebensmitt-Unters. Forsch.*, **177**, 461-467, (1983).
Koohmaraie, M. *Meat Sci.*, **43**, S193-S201, (1996).
Lacourt, A., Obled, A., Deval, C., Ouali, A. and Valin, C. In: Turk, V. (ed) *Cysteine proteinases and their inhibitors*, 239-248, Walter de Gruyter and Co., New York, (1986).
Moeller, P.W., Fields, P.A., Dutson, T.R., Landmann, W.A. and Carpenter, Z.L. *J. Food Sci.*, **41**, 216-217, (1976).
Moeller, P.W., Fields, P.A., Dutson, T.R., Landmann, W.A. and Carpenter, Z.L. *J. Food Sci.*, **42**, 510-512, (1977).
Morgan, J.B., Jones, S.J. and Calkins, C.R. *J. Anim. Sci.*, **67**, 2646-2654, (1989).
O'Halloran, G.R., Troy, D.J., Buckley, D.J. and Reville, W.J. *Meat Sci.*, **47**, 187-210, (1997).
Penny, I.F. and Fegruson-Pryce, R. *Meat Sci.*, **3**, 121-134, (1979).
Stauber, W.T. and Ong Shu-Hui. *J. Histochem. Cytochem.*, **29**(7), 866-869, (1981).
Takahashi, K. *Meat Sci.*, **43**, S67-S80, (1996).
Wu., J.J., Dutson, T.R. and Carpenter, Z.L. *J. Food Sci.*, **46**, 1132-1142 (1981).
Zeece, M.G., Woods, T.L., Keen, M.A. and Reville, W.J. *Recip. Meat Conf. Proc.*, **45**, 51-61, (1992).

FIG. 1. **LOCALISATION OF CATHEPSIN B BY ENZYME FLUORESCENCE HISTOCHEMISTRY**