A DEMONSTRATION OF THE INVOLVEMENT OF CATHEPSINS IN POSTMORTEM MYOFIBRILLAR PROTEIN DEGRADATION AT HIGH TEMPERATURE.

SANCHO R., JAIME I., BELTRAN J.A. and RONCALES P.

Dept. of Food Technology. Faculty of Veterinary Science. University of Zaragoza, SPAIN.

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SUMMARY

The degradation of actin throughout 48 hours of postmortem storage of muscle fibres at 4 and 20C was studied by means of SDS-gel electrophoresis and immunoblotting using a commercial monoclonal antibody.

Electrophoretic patterns showed an enhanced proteolysis of meat held at 20C, as revealed by the increased number and intensity of degradation peptides in the region of 30 kDa, as well as by the appearance of a new peptide of a molecular weight between those of actin and troponin T. Western blotting of actin demonstrated by the first time the degradation of this protein at 20C, while not at 4C, at the same time that at least a peptide proceeding from actin proteolysis was clearly evident.

When muscle fibres were incubated at 20C in the presence of cathepsin inhibitors E-64 and pepstatin, which do not inhibit calpains, immunoblots showed no degradation of actin. This clearly demonstrated that cathepsins are effectively involved in actin degradation in meat stored postmortem at a temperature of 20C.

INTRODUCTION

Tenderness is the most important organoleptic characteristic of meat, and it is of prime importance for the consumers. It is known that meat becomes more tender during ageing, so that postmortem storage of meat is the method currently used for achieving tenderisation.

The mechanisms responsible for postmortem tenderising are still unclear but it is generally accepted that at least two proteolytic systems could be responsible for these changes, namely calpains and cathepsins.

Meat ageing is a highly variable process, depending on a variety of biological factors (age, sex, muscle type) and processing conditions. A number of methods have been so far investigated to accelerate the tenderising of meat, such as electrical stimulation of carcasses (Uytterhaegen et al., 1992), high hydrostatic pressure (Koohmaraie et al., 1984), infusion of Ca^{2+} (Koohmaraie, 1992), addition of endogenous and exogenous proteases (Etherington, 1992), ultrasonication (Roncalés et al, 1993), rapid chilling (Jaime et al., 1992, 1993), delayed chilling (Whipple et al., 1990) and high temperature conditioning (Smulders et al., 1990). Most of these methods have been tried out to destabilise the lysosomes and therefore increase lysosomal protease activity in muscles.

Many studies have shown that delayed chilling or HTC in the postmortem period speed up the ageing process and result ultimately in increased tenderness. Yu and Lee (1986) reported that delayed chilling at 25C for 8 h increased Longissimus mucle tenderness. Whipple et al. (1990) found that at 1 day postmortem, the difference between delayed chilling and control was significant, while at 6 or 14 days postmortem the difference in tenderness was negligible.

Two mechanisms have been proposed to explain the tenderising effect of delayed chilling: the prevention of cold shortening and the activation of proteolysis (Dutson et al., 1977). High temperature conditioning enhances the disruption of the lysosomal membranes, causing the release of lysosomal enzymes (Moeller et al., 1977) and a more extensive degradation of myofibrillar proteins (Yates et al., 1983). Futhermore, Yates et al. (1983) reported that incubation of bovine muscle at 37C promoted a more intense proteolytic change than did incubation at 4C, with degradation of myosin and troponin at 37C. On the contrary, Whipple and Koohmaraie (1991) reported that incubation of calpains (at 25C) with myofibrils did not result in breakdown of myosin. This suggests that calpains are not involved in this proccess.

Bandman and Zdanis (1988) already reported that cathepsins degraded myosin heavy chain at 37C while not at 0-4C. We have carried out a study on the effect of cathepsins on actin degradation at moderately high temperatures (20C).

MATERIALS AND METHODS

Samples were obtained from *Longissimus dorsi* muscle from beef of a carcass weight about 250 kg. They were obtained within 1-2 h, after slaughter.

Monoclonal anti-a-sarcomeric actin, Anti-mouse IgG peroxidase conjugate, Pepstatin A and Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) were obtained from Sigma Chemical Co. (UK).

Incubation of myofibrils

Myofibrils were immersed in 100 mmol KCl, 20 mmol KH_2PO_4, 0,1 mmol EDTA, 1 mmol $MgCl_2$ and 1 mmol NaN_3 buffer (ph-7), with a catheptic inhibitor (E-64 and pepstatin A at 1 mmol concentration) or without inhibitor (control). Samples were incubated at 4C or 20C for 48 h.

Myofibrillar proteins isolation

Myofibrils were prepared following the procedure described by Olson et al. (1976), using 100 mmol KCl, 20 mmol KH_2PO_4, 0,1 mmol EDTA, 1 mmol $MgCl_2$ and 1 mmol NaN_3 as isolating medium. Protein concentration was determined by the Nessler method. Samples were stored at -20C.

SDS-Polyacrylamide gel electrophoresis

We used a modification of the Laemmli procedure, according to Greaser et al. (1983). This system uses a 1,5 mm-thick slab gel consisting of a 15% polyacrylamide resolving gel and a 3% polyacrylamide stacking gel. The acrylamide:bysacrylamide crosslinker ratio was 200:1 in the resolving gel and 20:1 in the stacking gel. Electrophoresis was performed at a constant voltage of 120 V at the stacking gel and then at 250 V at resolving gel for about 5 h. Gels were stained using a solution of Coomassie blue R-250 and destained until the background was clear.

Myofibrillar protein suspensions were dissolved in 0,05 M Tris-HCl buffer (pH 6,8) and boiled for 5 min in the presence of 6% SDS. After heating, 40% sucrose was added in a ratio of 1:3 (v/v) and final protein concentration was adjusted to 10 mg/ml. Ten ml of these myofibril solutions were applied to the gels.

Electroblotting of polyacrylamide gels

Protein from SDS-polyacrylamide were transferred from the gel to nitrocelullose paper according to the procedure used by Towbin et al. (1979). Transfers were run at 17 min at 135 mA.

Immunoblotting procedure

The Western blots (nitrocelullose transfer) were incubated with 5% ovoalbumin in phosphate buffered saline (PBS), pH 7,4 for 4 h to inhibit non-specific binding. The transfers were then incubated with monoclonal anti-a-sarcomeric actin at dilution 1:200 for 2 h. The dilutions were done in 5% ovoalbumin in PBS, pH 7,4. The transfers were washed in PBS for 30 min. Bound antibody was detected by incubation for 2 h with 1:200 dilution of anti-mouse IgG peroxidase conjugate in 3% ovoalbumin in PBS pH 7,4.

Blots were developed with 0,6 mg/ml 4-chloro-1-naphthol, 2 ml methanol, 8 ml PBS and 10 ml H_2O_2 during 15 min.

RESULTS AND DISCUSSION

The myofibrils were incubated for 48 h at two temperatures: 4C and 20C. Electrophoretic patterns (Figure 1) showed an enhanced proteolysis of meat held at 20C, as revealed by the increased number and intensity of degradation peptides in the region of 30 kDa, as well as by the appearance of two new peptides, one of 33 kDa and another one of a molecular weight between those of actin and troponin T. An increased degradation of myofibrillar proteins known to be sensitive to proteases action such as troponin T, myosin, desmin, a-actinin and tropomyosin was not observed.

It has been described that high temperature conditioning may disrupt the lysosomes, thus releasing cathepsins (Moeller et al., 1976). These proteases would degrade myosin (Bandman and Zdanis, 1989), actin (Schwartz and Bird, 1977) and a-actinin (Matsukura, 1984) at 37C. In vitro studies in which cathepsins were added to actin extracts and incubated, demonstrated that actin proteolysis occurred (Schwartz and Bird, 1977; Okitani et al., 1981; Ouali et al., 1987). On the contrary, actin degradation has not been demonstrated under conditions of refrigeration (Goll et al., 1983).

In order to check if these new peptides observed after incubation at 20C proceeded from actin degradation, an immunological study using a commercial monoclonal anti-actin antibody was performed. As shown in Figure 2, Western blotting of actin demonstrated the degradation of this protein at 20C, as opposed to 4C storage. At the same time, at least a degradation peptide proceeding from actin proteolysis was clearly evident.

It is well known that actin is not hydrolysed by calpains (Goll et al., 1983). Several studies, on the contrary, have demonstrated that it is degraded by cathepsin B (Schwartz and Bird, 1977; Ouali et al., 1987), cathepsin L (Okitani et al., 1981) and cathepsin D (Schwartz and Bird, 1977; Okitani et al., 1981; Ouali et al., 1987). Cathepsins B, L and D are located within the lysosomes, but as a result of high temperature and acidic pH, lysosomal membranes may rupture (Weisman, 1964; Stagni and Bernard, 1968), and therefore degrade actin.

Muscle fibres were then incubated at 20C in the presence of cathepsin inhibitors E-64 (inhibitor of cathepsin B and L) and pepstatin (inhibitor of cathepsin D), which do not inhibit calpains. Immunoblots shown in Figure 3 demonstrated that no degradation of actin was evident when inhibitors were present. These results effectively prove that cathepsins are involved in actin degradation in meat stored postmortem at a temperature of 20C.

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FIGURES

1.- Schematic representation of a 15% SDS-PAGE of myofibrilar proteins obtained from beef *Longissimus thoracis* after 48 h of storage: A) control, B) samples incubated at 4 C, C) samples incubated at 20 C.
2.- Western blotting of myofibrillar proteins reacted with monoclonal anti-actin antibody after 48 h of storage, and separated on 15% SDS polyacrylamide gels: a) samples incubated at 4 C, b) samples incubated at 20 C.
3.- Western blotting of myofibrillar proteins reacted with monoclonal anti-actin antibody after 48 h of storage at 20 C, and separated on 15% SDS polyacrylamide gels: 1, 2) control, incubated without cathepsin inhibitors; 3, 4) incubated with cathepsin inhibitors (E-64 and pepstatin).