PE4.70 Myosin Degradation Products in Dry-cured San Daniele Ham 250.00

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***Abstract*— Raw meat and San Daniele dry-cured ham samples of *Semimembranosus* muscle was extracted in denaturing and reducing condition and subjected to one-dimensional SDS-PAGE followed by immuno-detection and MALTI-TOF analyses. In raw meat the intact myosin heavy chain (MHC) (200 kDa) was present, which was identified by MALDI-TOF as skeletal myosin heavy chain 1. After the ripening period, the MHC band was markedly reduced. Simultaneously, the appearance of three new bands, two of these with apparent molecular weights of approximately 135 kDa and one of 78kDa, were clearly distinguished. Such bands were recognized by anti-MHC antibodies and two of them identified by MALDI-TOF as derived from skeletal myosin heavy chain 1. Such proteolytic products strongly suggest the involvement of cathepsin B in MHC degradation, which is known to be still active in dry-cured ham.**

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***Index Terms* — Dry-cured ham, myosin heavy chain degradation, MALDI-TOF, proteolysis.**

I. INTRODUCTION

Conversion of muscle to dry-cured ham is governed by complex interaction of biochemical processes that take place during post-mortem storage and ripening stage. Proteins and lipids constitute the major chemical components of meat and are the main subject of action of the muscle enzyme systems [13]. Proteolysis appears to be largely responsible for the post-mortem tenderising [6]. In addition, proteolysis, together with lipolysis, seems to be involved in the generation of flavour and flavour precursors in dry-cured meat products [12].

Myofibrillar proteins constitute the major components in skeletal muscle and are largely responsible for the texture of muscle tissue. Myosin and actin constitute the two major myofibrillar proteins. Myosin (about 43% of the total myofibrillar meat proteins) is the major component of thick filaments and actin (about 20% of the total) is the second most abundant. These myofibrillar proteins are not only important in muscle due to their role in contraction [7] but also for their involvement in the functional properties of meat products such as water-holding, emulsifying capacity, binding ability and gelation [1, 9], although the underlying molecular mechanisms are still debated.

Significant changes in the profiles of myosin and actin proteins have been detected by SDS-polyacrylamide electrophoresis during the processing of Serrano dry-cured ham [14]. The progressive disappearance of myosin heavy chain and light chains 1 and 2, along with actin and troponins C and I and the simultaneous appearance of several fragments with molecular mass between 150 and 16 Kda have been shown [6,15]. Lysosomal cathepsins, which are active at acidic pH values and show activity along the entire ripening process, have been proposed to be responsible for such myofibrillar protein degradation [6]. On the other hand, myosin, along with actin and α-actinin, are known to be good substrates also for the ubiquitous and muscle-specific calpain proteases, both in vitro [8] and in vivo [4].

The aim of this work was to define the myosin heavy chain degradation pattern at the end of ripening stage in dry-cured San Daniele ham obtained from three different white-breed pigs. The study has been carried out in *Semimembranosus* muscle, where lower enzymatic activity is present due to the higher NaCl content than in *Biceps Femoris* muscle [6]. One-dimensional SDS-polyacrylamide electrophoresis analyses of protein extracts followed by mass spectrometry protein identification were performed. Our results clearly show that after the long dry-curing process myosin heavy chain is markedly degraded producing a limited number of high molecular weight

fragments. Such peculiar degradation pattern can constitute the basis for identification of the endo-proteases involved in the process.

II. MATERIALS AND METHODS

A. *Samples*

The samples were obtained from crossed pigs derived from three genetic groups suitable for curing process, according to the "San Daniele" procedure [16]. Samples of raw meat and dry cured ham were from *Semimembranosus* muscle (SM) (an external type muscle). Analyses were carried out on raw meat and on dry-cured hams ripened for 15 months.

B. Protein extraction and quantification

Extraction of proteins was carried out according to Pineiro et al. [10] or Di Luccia et al. [2] method with minor modifications. Samples (100 mg) were freed of connective and adipose tissue, ground over ice and suspended using SDS- or CHAPS-containing solution under reducing conditions. Finally all extracts were subjected to desalting by ultra-filtration and stored at –30°C.

The protein concentration was determined by UV absorbance at 280nm, after sample dilution with SDS- or CHAPS-containing solution.

C. *SDS-PAGE and Western blotting*

Proteins were separated by SDS–PAGE according to Laemmli [5] in 8% or 13% polyacrylamide gels using the Mini 2D (Bio-Rad, Richmond, Calif., USA) electrophoresis equipment. High range standard proteins of known molecular weight were used (Sigma Chemical Co., Milan, Italy; myosin 200 KDa, β-galactosidase 116 KDa, phosphorylase B, 97 kDa, bovine serum albumin 66 kDa, glutamic dehydrogenase 55 KDa, ovalbumin 45 kDa, glyceraldehyde-3-phosphate dehydrogenase 36 KDa). The gels were subsequently stained in a 0.25% (w/v) solution of Comassie Brilliant Blue R and the bands were quantified by densitometry using ImageQuant software (Amersham). The apparent masses of the protein bands were estimated from a calibration curve obtained by plotting the migration distances of the standard proteins versus their known molecular masses. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using Mini Trans-Blot (Bio-Rad). Western blotting was performed with anti-myosin heavy chain antibody (anti-MHC) (1:2000) (Sigma) or anti-actin antibody (1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in phosphate buffered saline (PBS) containing 3% (w/v) non-fat dry milk. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

D. *Protein identification by mass spectrometry*

Electrophoretic bands were excised from SDS-PAGE and destained with 25 mM ammonium bicarbonate (NH_4HCO_3) and 5% (v/v) acetonitrile (ACN) solution followed by a 25 mM NH_4HCO_3 in 50% (v/v) ACN solution. After overnight in-gel trypsin digestion, peptide mixtures were extracted with 1% triflouroacetic acid (TFA), subjected to ZipTip clean-up (Millipore SPA, Milan, Italy) according to the manufacturer's instructions and directly eluted with a α-Cyano-4-hydroxycinnamic acid matrix (10 g/l α-Cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.3% TFA). MALDI mass spectra were acquired with a Voyager DE Pro mass spectrometer (Applied Biosystems), in 700-4000 Da molecular weight range, in reflector and in positive ion mode, with 150 nsec delay time and an ion acceleration voltage of 20 kV. Spectra were externally calibrated using Peptide calibration Mix 4 (Proteomix) 500-3500 Da (Laser Bio Labs). Mass spectra, obtained by collecting 1000-2000 laser shots, were processed using Data Explorer version 5.1 software (Applied Biosystems). Protein identification was performed using the MASCOT search engine (http://www.matrixscience.com), and Aldente (www.expasy.org/tools/aldente) peptide mass fingerprinting (PMF) tool.

The NCBInr and Swiss Prot were used as the protein sequence databases, to produce a standardized probabilistic measure of confidence, limiting the search to mammalian proteins, allowing for one trypsin missed cleavage and with a 150 parts per million (ppm) mass tolerance error. The variable modification selected was the methionine oxidation.

III. RESULTS AND DISCUSSION

Two different extraction techniques were carried out in raw meat and dry cured samples of *Semimembranosus* muscle. In general terms, extracts obtained with 2% SDS showed 2x higher protein concentration than CHAPS extracts in all samples. Therefore, SDS extraction procedure was chosen to detect the presence of the myofibrillar proteins and their degradation products in the different samples.

Coomassie stained SDS-PAGE profiles appeared similar in all extracts of raw meat samples, independently on breed. Furthermore, the changes in the profiles after ripening stage were similar in the extracted samples from all breeds. More precisely, immuno-detection after SDS-PAGE separation of raw meat extracts showed the presence of the intact myosin

heavy chain (MHC) at 200 kDa, which was identified by MALDI-TOF as skeletal myosin heavy chain 1 (17% coverage). After the ripening period, the MHC band was still present, but markedly reduced. Simultaneously, the appearance of three new bands, two of these with apparent molecular weights of approximately 135 and one of 78kDa, was clearly distinguished (Fig. 1B). Such bands were recognized by anti-MHC antibodies (not shown) and two of them still identified as skeletal myosin heavy chain 1 by MALDI-TOF (17 and 14% coverage for the band at 135 and the band at 78kDa, respectively). Densitometric analyses of Coomassie stained SDS-PAGEs indicated that the sum of the area of the bands at 200, 135 and 78 kDa in dry cured ham extracts almost accounted for the band area of the whole MHC present in raw meat extracts, suggesting that further proteolysis of MHC into progressively smaller fragments was limited. Conversely, the band identified as actin by immunoblotting at 45 kDa appeared to be degraded by 52%±5 in dry cured ham extracts with respect to raw meat extracts, in agreement with the results obtained in Teruel dry-cured ham [6].

The finding of only major degradation products, essentially resistant to additional proteolysis, excluded the involvement of exo-peptidases during processing of dry-cured ham, while support the action of endo-proteases on the native form of MHC. Several studies have proposed that meat protein degradation is a result of the sequential action of different classes of endo-proteases, where calpains initiate destabilization of the myofibrillar structure, allowing the proteosome and cathepsins to act on the partially degradated proteins [3,4]. The degradation pattern of MHC found in our dry-cured samples strongly resembles that found by *in vitro* incubation of native myosin with muscle specific cathepsin B, which yielded MHC fragments of similar molecular weight [11]. Conversely, incubation of MHC with cathepsin D was found to give different degradation products [11]. Therefore, although yet speculative, our results support the involvement of cathepsin B, that is known to remain active until the last stage of ripening [6], in MHC proteolysis during dry-curing process.

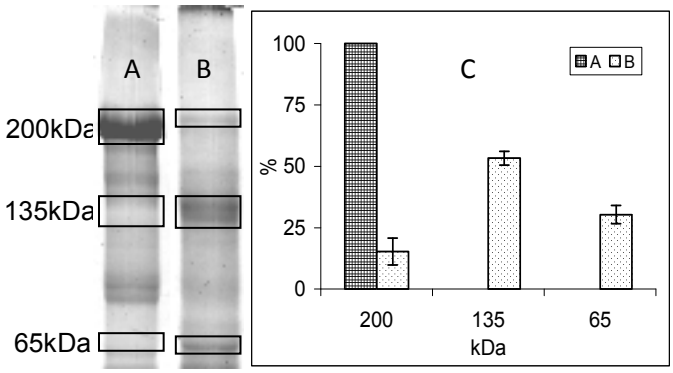

Figure 1. Myosin heavy chain and its major degradation products. SDS-PAGE on gel of 8% acrylamide of raw meat (A) and dry-cured ham after 15 months of ripening (B); Densitometric analysis: mean values ± SD of 9 samples are reported (C)

IV. Conclusion

Previous studies have proposed an essential role of cathepsin B, L and D in the degradation of myofibrillar proteins during conversion of muscle to dry-cured ham [6]. Our finding of limited number of MHC high molecular weight fragments supports this statement and proposes cathepsin B as the major candidate involved in MHC degradation. In fact, SDS-PAGE followed by immunodection and MALDI-TOF analyses showed that dry-cured ham processing produced a very characteristic MHC degradation pattern which resembles that previously produced by *in vitro* incubation of cathepsin B with native myosin.

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