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Proteomic analysis of water soluble and myofibrillar protein changes occurring in dry-cured hams

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Abstract

The myofibrillar fraction of raw ham muscles and dry-cured hams with different ripening times was extracted in denaturing and reducing conditions and subjected to two-dimensional gel electrophoresis. The two-dimensional maps gave overall pictures of the already noted progressive disappearance of actin, tropomyosin and myosin light chains during ripening. In addition, two fragments from Myosin Heavy Chain proteolysis, marked as myosin chain fragments MCF1 and MCF2, were identified by immunodetection and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Furthermore, a new form of actin on two-dimensional gel was identified by MALDI-TOF peptide mapping. In 12-month-old dry-cured ham, most myofibrillar proteins were completely hydrolyzed. At this stage of ripening, in fact, in some Parma and S. Daniele dry-cured ham samples, myosin heavy chain fragments and other unidentified neo-formed spots were found. Some of the sarcoplasmic proteins in water extracts from pork meat markedly decreased in amount or disappeared totally, during ripening. Surprisingly, two-dimensional gel electrophoresis maps of the water soluble protein fraction from dry-cured ham showed the presence of two spots identified as tropomyosin α- and β-chain. This result suggests that some of the saline soluble myofibrillar proteins can disappear from this fraction because of salt solubilization and not due to complete enzyme action. Two-dimensional gel electrophoresis (2-DGE) has proved a powerful tool to evaluate the enzymatic susceptibility of meat proteins and the evolution of protein map fragmentation throughout ripening process as well as a means of obtaining a standard fingerprinting map characterizing the final product.
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1. Introduction

The palatable characteristics of dry-cured ham, mainly texture and flavor, are due to intense proteolytic phenomena occurring during the ripening process (Aristoy & Toldrà, 1995; Belletti, Dazzi, Chizzolini, Palmia, & Parolai, 1983; Hansen-Moller, Hinrichsen, & Jacobsen, 1997; MacCain, Blumer, Craig, & Steel, 1968; Toldrà, Rico, & Flores, 1993).

The origin of proteolysis is attributed to the activity of endogenous enzymes, since the effective activity of microorganisms on myofibrillar and sarcoplasmic proteins in dry-cured ham has not yet been well defined (Cordero & Zumalacarregui, 2000; Molina, Silla, Flores, & Monzò, 1989a; Molina, Silla, Flores, & Monzò, 1989b; Molina & Toldrà, 1992; Rodriguez, Nunez, Cordoba, Bermudez, & Asensio, 1988). Proteolytic activity

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on muscle proteins has been essentially attributed to cytosolic enzymes, calpains and lysosomal proteinases, and cathepsins (Goll et al., 1983; Huff-Lonergan et al., 1996; Jiang, 1998; Koohmaraie, 1992; Olson, Parrish, Dayton, & Goll, 1997; Penny, 1974; Taylor, Geesink, Thompson, Koohmararie, & Goll, 1995; Taylor, Tassy et al., 1995; Whipple & Koohmaraie, 1992), which act for a longer time (Toldrà & Flores, 1998; Toldrà & Etherington, 1988). The evolution of myofibrillar protein hydrolysis during the ripening process has been studied by mono-dimensional gel electrophoresis (Toldrà, Flores, & Sanz, 1997; Toldrà et al., 1993). The principal changes were observed in myosin heavy chains (MHC), myosin light chains (MLC1 and MLC2) and troponin C and I with the appearance of numerous fragments in the 50–100 and 20–45 kDa regions. However, there is little information as to how the single components of sarcoplasmic and myofibrillar proteins change during the curing process (Monin et al., 1997).

In the last few years, proteomic studies have improved two-dimensional gel electrophoresis (2-DGE), making it possible to resolve more complex protein mixtures. This technique provides for isoelectric focusing (IEF) in immobilized pH gradient (IPG) in the first dimension and an orthogonal second dimension in sodium dodecyl sulphate–polyacrylamide gradient gel electrophoresis (SDS–PAGE). The protein changes are revealed by imaging analysis and the spots are identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) peptide mass mapping after tryptic "in gel" digestion of excised protein spots. The 2-DGE and MALDI-TOF MS analysis represent a powerful combination of technologies with which high resolution separation of proteins and their rapid identification is possible. Proteomics has emerged as a new experimental approach, in part because mass spectrometry has simplified protein analysis and characterization, and several important and recent innovations have extended the capability of mass spectrometry. The proteomic approach has already succeeded in identifying modifications of meat protein components during *post-mortem* storage, revealing a more complex electrophoretic pattern than that obtained by mono-dimensional electrophoresis (Lametsch & Bendixen, 2001; Lametsch, Roepstorff, & Bendixen, 2002).

In this work, a proteomic study of water soluble and myofibrillar protein fractions was performed to obtain a better understanding of enzymatic action throughout the ripening process of hams.

2. Materials and methods

2.1. Samples

Analyses were carried out on raw meat from hams, 72 h after slaughtering, and dry-cured hams ripened for 6, 10 and 14 months, as reported on the labels. Five samples of dry-cured ham were examined from each different factory to verify the validity of the results.

Sequencing grade Trypsin (TPCK treated) was purchased from Boehringer (Mannheim, Germany). Solvents were HPLC-grade from Carlo Erba (Milan, Italy). The electrophoretic reagents were analytical grade, carrier ampholytes and IPG DryStrip were from Amersham-Pharmacia.

2.2. Extraction of water soluble and myofibrillar proteins

Samples (50 g) were freed of connective and adipose tissue and homogenized at 2 °C with 200 ml of water milliQ (Millipore). The homogenate was centrifuged under refrigeration at 2 °C and 5500*g* (Labofuge 400R, Heraeus Instruments) for 20 min to obtain a pellet and a clear supernatant. The latter contained the sarcoplasmic fraction and the pellet contained myofibrillar and connective tissue proteins. The pellet was washed three times with distilled water, centrifuged and freeze dried. All dried samples (10 g) were re-suspended overnight at 4 °C in 50 ml of denaturing solution [cholamidopropyldimethylhydroxypropanesulfonate (CHAPS) 4%, 8 M Urea and 65 mM dithiothreitol (DTT)] to extract myofibrillar proteins. These proteins were recovered in the supernatant after centrifugation at 14,500*g* for 5 min (Biofuge, Heraeus Instruments) and used for 2-DGE.

2.2.1. High-resolution 2-DGE: first dimension

IEF was carried out using the Multiphor II system (Pharmacia Biotech, Uppsala, Sweden) and Immobiline DryStrips gel (pH 4–7, 18 cm) was re-hydrated overnight directly with the sample, in an Immobiline DryStrip Reswelling Tray (Amersham Pharmacia). One hundred microliters of sample was dissolved in 300 μl of a solution containing 8 M Urea, 2% (w/v) CHAPS, 10 mM DTT, 2% (v/v) IPG Buffer, pH 4–7, and a trace of bromophenol blue (Bjellquist et al., 1993). The program run was 1 mA of current and 500 V for 1 h and then 3500 V for 16 h.

After IEF, IPG gel strips were equilibrated for 12 min in equilibration buffer (50 mM Tris–HCl, pH 6.8, 6 M Urea, 30% glycerol and 2% SDS) plus 2% DTT to re-solubilize the proteins and to reduce –S–S– bonds, and for 5 min in equilibration buffer plus 2.5% iodoacetamide and a trace of bromophenol blue to block –SH groups (Bjellquist, 1993).

2.2.2. High-resolution 2-DGE: second dimension

The second dimension was performed as a modification of the Laemmli system (Laemmli, 1970) using the Investigator 2-D Electrophoresis System (Millipore). The IPG strips were laid on top of the polymerized linear gradient (9–16%) and overlaid with 0.5% agarose in

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