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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.

Keywords: Myofibrillar proteins; Dry-cured ham; Two-dimensional gel electrophoresis; Immunoblotting; MALDI-TOF mass spectrometry

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

running buffer (25 mM Tris, 198 mM glycine and 0.1% SDS). SDS–PAGE was run at 16 W/gel and 500 V_{max}. The gel was stained using Coomasie Blue R-250 as described above.

2.3. Immunoblot analysis

After 2-DGE, the proteins were transferred from the polyacrylamide gel on polyvinylidene difluoride (PVDF) membrane (Millipore) by using a MILLIBLOT^{TM} system (Millipore), following the electroblotting procedures described in the manufacturer's instructions. Electrophoretic transfer was accomplished in 3 h at a constant 1250 mA/cm^2 of gel.

Immunodetection was carried out as described by Molina, Fernandez-Fournier, De Frutos, and Ramos (1996). The first incubation was made with a human antibody monoclonal Anti-Skeletal Myosin developed in rabbit by Sigma while a peroxidase conjugated anti-rabbit (Bio-Rad) was used as a secondary antibody.

2.4. In-gel digestion of 2-DGE separated proteins

Sample preparation was essentially the same as previously described by Shevchenko, Wilm, Vorm, and Mann (1996). Briefly, proteins from 2-DGE were excised from the gel, crushed and repeatedly washed with acetonitrile and 25 mM NH_4HCO_3 solution 50% v/v (Sigma) until destaining was achieved. The proteins were in-gel reduced with 10 mM dithiothreitol in 25 mM NH_4HCO_3 (45 min, at 55 °C) and *S*-alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 (30 min, at room temperature and in the dark). After extensive washing with 25 mM NH_4HCO_3, gel particles were dried and re-hydrated with a digestion solution of 12.5 ng/μl of trypsin in 25 mM NH_4HCO_3. After 1 h incubation in an ice bath, the excess trypsin was removed and the spots were recovered from the 25 mM NH_4HCO_3 solution. The samples were finally incubated at 37 °C overnight. The total digest was removed and the gel pieces were treated in an ultrasonic bath with 50% acetonitrile in formic acid 5% (twice). The solutions recovered were mixed together and lyophilized.

2.5. MALDI-TOF-mass spectrometry analysis

Mass spectrometry experiments were carried out on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N_2 laser ($\lambda = 337$ nm, 3-ns pulse width, 20-Hz repetition rate). The instrument operated with an accelerating voltage of 20 kV. Samples were mixed with the matrix, α-cyano-4-hydroxy-cinnamic acid prepared by dissolving 10 mg in 1 ml of aqueous 50% acetonitrile containing 0.1% TFA, on the MALDI target and air-dried. External mass calibration was performed with low-mass peptide standards and mass measurement accuracy was ±0.3 Da. The mass spectra were acquired in the reflector mode using Delay Extraction (DE) technology.

Raw data were analyzed using a software program furnished by the manufacturer and are reported as average masses.

2.6. Protein identification

Two software packages, both available on-line, PROWL-ProFound (web site: http://prowl.rockefeller.edu) and Protein Prospector MS-Fit (web site: http://prospector.ucsf.edu), maintained and updated daily at the Rockefeller University (NY) and University of San Francisco (CA), respectively, were used to identify protein spots from independent non-redundant protein sequence databases. These electronic tools were used for selection of protein molecular mass filter, adjustable incomplete cleavage, taxonomic choice of the organism under investigation, peptide mass error, mass changes due to protein modifications (e.g., cysteine alkylation) isomorphs or post-translational modifications. Up to one missed tryptic cleavage was considered and a conservative mass accuracy of ±0.5 Da was used for all tryptic-mass searches. A number of top candidates with high scores from the peptide matching analysis were further evaluated by comparing their calculated pI and MW using the experimental values obtained from 2-DGE. These two parameters were used with large tolerances ($\Delta pI = 1$ and $\Delta MW = \pm 15\%$ Mw) as filters to exclude false positive candidates from the output lists.

3. Results

3.1. Two-dimensional gel electrophoresis map of fresh raw ham

3.1.1. Water soluble proteins

Fig. 1 shows the 2-DGE map of water soluble meat proteins from pork skeletal muscles of fresh raw ham.

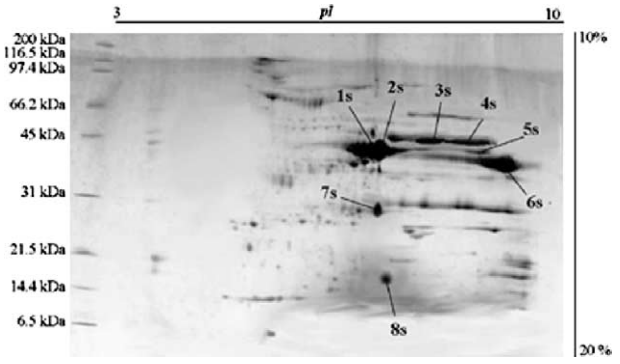

Fig. 1. Two-dimensional gel electrophoresis map of the sarcoplasmic protein fraction from pork skeletal muscles of fresh raw ham.

Water soluble protein extracts allowed us to localize the main sarcoplasmic protein spots, in the area whose approximate pI ranges between about 6.0 and 9.0 and estimated MWs ranges between 116.5 and 14.4 kDa. Yan et al. (2001) identified rat skeletal muscle protein using 2-DGE and mass spectrometry. They performed a first dimension in the 3–10 pH range, where the main myofibrillar and sarcoplasmic proteins were well separated, the former ranging between pH 3.5 and 7.0 and the latter between pH 6.0 and 10.0, as also occurs for pork sarcoplasmic proteins. The most represented were identified as enolase B (theoretical MW 52.3 kDa; estimated MW 46.8 kDa), creatine kinase (41 kDa), and glyceraldehyde 3-phosphate dehydrogenase (36 kDa). On the basis of these results, we could identify the spots labeled 4s and 5s, with an estimated MW of 46 kDa, as pig enolase B, 1s and 2s, with an estimated MW of 43.2 kDa, as pork creatine kinase and 6s, with an estimated MW of 36.5 kDa, as pig glyceraldehyde 3-phosphate dehydrogenase. For an unequivocal identification of sarcoplasmic proteins, we carried out peptide mass mapping of the spots labeled in Fig. 1 (see below).

3.1.2. Myofibrillar proteins

The 2-DGE map of the myofibrillar proteins from pork skeletal muscles of raw ham is shown in Fig. 2. The spots were initially identified on the basis of their relative position (p*I* and MW) as indicated in the literature (Di Luccia et al., 1992; Giometti, Danon, & Anderson, 1983; Pernelle, Righetti, & Wahrmann, 1988). The main protein spots were identified as myosin light chains (MLC1 fast and slow, MLC2 fast and slow and MLC3 fast) and tropomyosins (TPMs), α and β chains. In particular, it was possible to detect the heterogeneity of α and β TPM, spots 4m of Fig. 2, according to 2-DGE by Pernelle et al. (1986) and isoforms of MLC1 and MLC2, spots 2 and 3, according to 2-DGE by Pernelle et al. (1986) and Pernelle, Righetti, Wahrmann, and Herve (1990).

The spots which have not yet been identified include three major ones, 7m, 8m and 9m (Fig. 2), which range between an estimated molecular weight of 46.5 and 40 kDa. The actin spot, which has a MW of 42 kDa as reported by Swiss Prot data bank, should fall between these two values. In a recent work, Lametsch et al. (2002) identified a spot of 40 kDa as actin by using 2-DGE and MALDI/TOF mass spectrometry. To ascertain the exact position of actin on our two-dimensional map, identification was carried out by MALDI-TOF MS. Furthermore, immunoblotting detection and MALDI-TOF MS were used to localize MHC and its fragments.

3.2. Immunoblotting detection of MHC on 2-DGE map of raw ham meat

Western blot was performed on the 2-DGE map of myofibrillar protein fraction to detect the localization of myosin heavy chain and its enzymatic hydrolysis products.

The human antibody monoclonal Anti-Skeletal Myosin, which labels the A bands of human and animal skeletal muscle, allowed recognition only of some of the components localized at the right side of the 2-DGE. The molecular weights of these components range between 100 and 10 kDa (Fig. 3). Considering that monoclonal antibodies cross-react with a specific epitope localized on the proteins, that the examined samples were myofibrillar proteins and that only some of the spots on the right side of the 2-DGE map and no other myofibrillar proteins were recognized, strongly suggests that these spots derived from MHC (223 kDa) proteolysis.

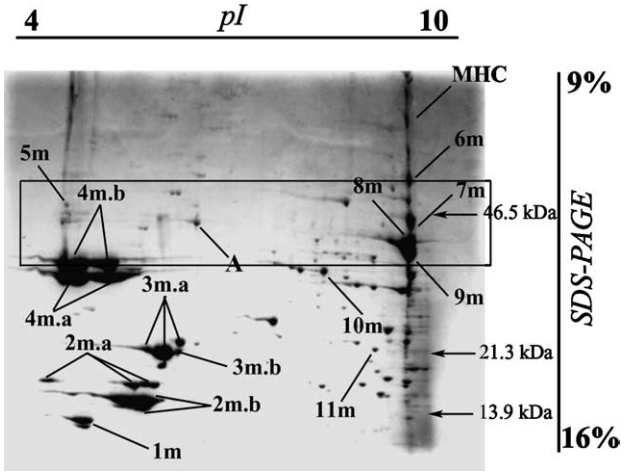

Fig. 2. Two-dimensional gel electrophoresis map of the myofibrillar protein fraction from pork skeletal muscles of fresh raw ham.

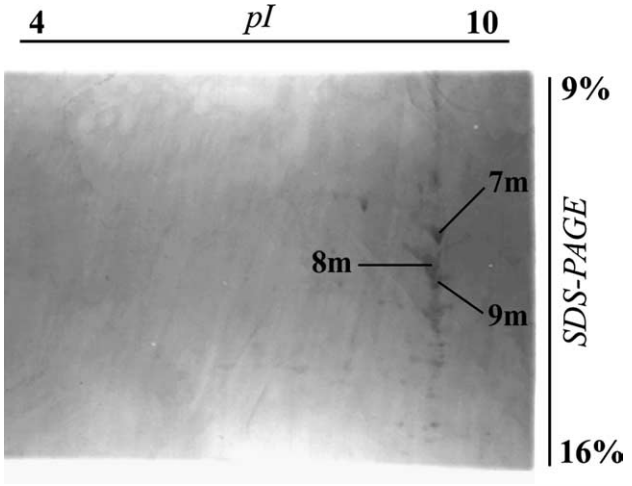

Fig. 3. Western immunoblotting detection of MHC on a two-dimensional gel electrophoresis map of the myofibrillar protein fraction from raw ham.

Therefore, the major spots named 7m, 8m and 9m fragments with MW of about 46, 43 and 40 kDa, respectively, were renamed myosin chain fragments (MCFs), MCF1, MCF2 and MCF3, respectively (Fig. 2). Lametsch et al. (2002) found only one spot derived from MHC with 56 kDa; this was probably due to a greater number of spots in the 2-DGE map because of the concomitant presence of sarcoplasmic and myofibrillar fractions.

3.3. Identification of spots by MALDI-TOF MS

3.3.1. Water soluble protein fraction

The spots of sarcoplasmic proteins, extracted in water from raw meat, were spread at the side of the 2-DGE map corresponding to a 6.0–10 pH range. Most were previously identified on the basis of known p*I* and MW. However, to ensure an exact identification an "in-gel digestion" of manually excised spots was carried out using trypsin with high specificity, and the peptide mixtures deriving from tryptic hydrolysis were analyzed by MALDI-TOF MS. By measuring the molecular masses of peptides from digestion, with the aid of PROWL ProFound and Protein Prospector MS-Fit software, 8 spots were identified, these being the most represented, as reported in Table 1. Six spots are glycolytic enzymes: enolase B, spots 3s and 4s, glyceraldehyde 3-phosphate dehydrogenase, spot 5s, aldolase A spot 6s, triosephosphate isomerase, spot 7s; spots 1s and 2s are creatine kinase, involved in high ATP turnover of muscle and nerve cells, and spot 8s is myoglobin.

For pork proteins not present either in protein or genomic databases, the search was referred to human, supposing a high degree of alignment as verified for myofibrillar proteins.

3.3.2. Myofibrillar protein fraction

To remove any doubt as to the nature of spots recognized by the monoclonal antibody a MALDI-TOF-MS analysis was performed following excision from the gel and in-situ trypsin digestion as above. Table 2 summarizes the peptide map of spots (labeled by consecutive numbers in Fig. 2) identified by using software packages, PROWL ProFound and Protein Prospector MS-Fit. In the case of spots whose peptide mass fingerprint matched with that of human skeletal MHC, primary structures were aligned and compared with those deduced from nucleotide sequences of pig skeletal MHC genes (in Nucleotide NCBI, GeneBank). In this fashion, human and pig skeletal MHC showed a homology of 94% while pig cardiac and skeletal MHC showed 84%. These homologies were evaluated by ExPasy MBS online software.

The peptide signals from spots 1m and 3m matched MLC3 and MLC1, respectively. MCL3 is present only in the fast twitch fiber and derived from the same gene of MLC1 by a process of alternative promoter utilization and differential RNA splicing of the two primary transcripts (Nabeshima, Fujii-Kuriyama, Muramatsu, & Ogata, 1984; Periasamy et al., 1984). This RNA splicing determines a deletion of 41 amino acids at N-terminal in MCL3, generating the so-called "difference peptide" (Frank & Weeds, 1974; Matsuda, Maita, Umegane, & Katoi, 1980). In fact, the difference between the two proteins is a peptide of 44 amino acids, since the peptide 1–52 is specific for MLC1 whereas the peptide 1–8 is specific for MLC3 (Seidel & Arnold, 1989), the sequence common to MLC1 and MLC3 is a peptide of 141 amino acids, 53–193 for MLC1 and 9–149 for MLC3 (Seidel et al., 1987). Thus the sequence of the peptides 12–19, 48–61 and 93–108 of pig MLC3 correspond to the peptides 56–63, 92–105 and 137–152 of human MLC1. This is a further confirmation of the great similarity of human and pig muscle proteins.

The signals derived from spot 2m matched human regulatory MLC2 skeletal muscle.

The MALDI spectrum observed for spots 4m.a and 4m.b led to identification of these components as isoforms of human TPM α and β, and the spot 12m as a fragment of TPM β. The protein spot marked A matched with pig actin, whereas, surprisingly, the spot 5m, on the acid side of 2D gel and with a estimated molecular weight of 50 kDa, also matched pig actin (Fig. 2). All the remaining spots produced peptide signals matching pig MHC cardiac muscle composed of 1935 amino acids, and their MW ranged between 70 and 10 kDa, except for spot 7m which matches with MHC smooth human muscle isoform. The peptide mass of spot 6m corresponds to a first portion of N-terminal side including amino acid residues from 148 to 657 while spots 7m, 8m, 9m, 10m and 11m correspond to complementary portions including amino acid residues from 1110 to 1697. Therefore, *post-mortem* proteolysis due to endogenous muscle enzymes generated at least 6fragments from the MHC parent protein.

3.4. Two-dimensional gel electrophoresis maps of dry-cured hams

3.4.1. Water soluble proteins

Fig. 4 shows the 2-DGE sarcoplasmic map of dry-cured ham. Comparison with the 2-Dge map of raw ham highlights remarkable differences caused by the ripening process. In the 2-DGE map of dry-cured ham, there can be seen: (i) a diminution of spot intensities of proteins with estimated MW between 66.2 and 28 kDa; (ii) the disappearance of creatine kinase; (iii) a partial disappearance of protein with estimated MW ranging between 28 and 14.4 kDa; (iv) the appearance of spots between 150 and 66.2 kDa, two more acidic spots with estimated MW around 38 kDa, whose pI range between 4.5 and 4.8, and two spots with

Table 1
Identification of sarcoplasmic proteins in fresh meat and in dry-cured hams, separated by 2-D gel electrophoresis (see Figs. 1 and 4)

Spot	Identification	Theoretical p*I*	Theoretical MW (Da)	Peptide mass $M - H^+$ (Da)	ΔMass (compared with theoretical) (Da)	Position	Sequence
1s–2s	Creatine kinase, B chain (P12277) human	5.34	42644.3	759.0	−0.3	210–215	DWPDAR
				1232.6	−0.1	87–96	DLFDPIIEDR
				1511.1	−3.8	1–13	MPFSSHNALKLR
				1644.6	−1.7	293–307	AGVHIKLPNLGKHEK
				2467.7	−0.4	87–107	DLFDPIIEDRHGGYKPSDEHK
3s–4s	*Enolase B* (P13929) human	7.73	46855.8	506.3	0.0	5–8	IFAR
				661.4	0.0	427–431	FRNPK
				704.4	0.0	126–131	GVPLYR
				1542.7	1.0	358–371	LAQSNGWGVMVSHR
				1805.0	0.1	32–49	AAVPSGASTGIYEALELR
				2355.3	0.9	162–182	LAMQEFMILPVGASSFKEAMR
				2673.4	1.1	228–252	TAIQAAGYPDKVVIGMDVAASEFYR
				2743.8	0.5	202–227	DATNVGDEGGFAPNILENNEALELLK
5s	Glyceraldehyde 3-phosphate dehydrogenase pig (P00355)	8.52	35704.85	795.5	0.1	225–231	LTGMAFR
				805.6	0.2	3–10	VGVNGFGR
				909.4	−0.1	105–114	AGAHLKGGAK
				1358.9	0.2	321–332	VVDLMVHMASKE
				1558.1	0.3	232–245	VPTPNVSVVDLTCR
				1615.5	−0.3	70–83	AITIFQERDPANIK
				1763.4	−0.4	307–320	LISWYDNEFGYSNR
				2248.6	−0.4	84–104	WGDAGATYVVESTGVFTTMEK
				2887.5	0.2	78–104	DPANIKWGDAGATYVVESTGVFTTMEK
				4089.2	0.4	269–306	GILGYTEDQVVSCDFNSDTHSSTFDA GAGIAL NDHFVK
6s	Fructose biphosphate Aldolase A human (P04075)	8.39	39288.8	1342.7	0.0	87–98	ADDGRPFPQVIK
				1647.1	0.3	42–55	RLQSIGTENTzEENR
				1802.8	−0.1	42–56	RLQSIGTENTEENRR
				2273.1	0.9	111–133	GVVPLAGTNGETTTQGLDGLSER
				3178.6	1.1	173–200	YASICQQNGIVPIVEPEILPDGDHDLKR
7s	Triosephosphate isomerase human (P00938)	6.51	26538.3	532.3	0.0	190–193	GWLK
				954.5	0.0	6–13	FFVGGNWK
				1458.8	0.1	110–112	HVFGESDELIGQK
				1466.8	0.1	175–187	TATPQQAQEVHEK
				1614.9	0.1	99–112	RHVFGESDELIGQK
				2322.2	0.0	113–134	VAHALAEGLGVIACIGEKLDER
				3029.8	0.3	219–247	ELASQPDVDGFLVGGASLKPEFVDIINAK
8s	*Myoglobin* Pig (P02189)	6.83	16953.4	748.6	0.2	134–139	ALELFR
				941.7	0.3	146–153	YKELGFQG
				1593.2	0.4	17–31	VEADVAGHGQEVLIR
				1827.3	−0.4	80–96	GHHEAELTPLAQSHATK
				1881.5	0.5	103–118	YLEFISEAIIQVLQSK
				1955.5	0.5	79–96	KGHHEAELTPLAQSHATK
				2585.1	1.6	97–118	HKIPVKYLEFISEAIIQVLQSK
				3375.5	0.8	1–31	GLSDGEWQLVLNVWGKVEADVA-GHGQEVLIR

estimated MW below 14.4 kDa. Therefore the action of endogenous enzymes on sarcoplasmic proteins throughout the ripening period involves a partial proteolysis of proteins ranging between 66.2 and 28 kDa and a stronger hydrolysis of proteins between 21.5 and 14.4 kDa, as well as the formation of components with MW below 14.4 kDa. The unexpected presence of the most acidic spots prompted us to identify them by MALDI-TOF mass spectrometry.

3.4.2. Myofibrillar proteins

The 2-D patterns of the 6- and 10-month-old dry-cured hams are shown in Fig. 5(a) and (b) while three 12-month-old Parma dry-cured hams are shown in Fig. 6(a)–(c), and three 12-month-old S. Daniele dry-cured hams in Fig. 7(a)–(c).

The 2D-map of 6-month-old ham (Fig. 5(a)) shows the absence of MLC3 spots, a weak A spot and single sub-forms of MLC1and MLC2 which are clearly visible

Table 2
Identification of myofibrillar proteins in fresh meat and in dry-cured hams, separated by 2-D gel electrophoresis (see Figs. 2–4)

Spot	Identification	Theoretical p*I*	Theoretical MW (Da)	Peptide mass $M - H^+$ (Da)	ΔMass (compared with theoretical) (Da)	Position	Sequence
1m	Myosin light chain 3 skeletal Muscle (MLC3) pig Q29069	4.63	16729.96	1010.9	0.4	13–20	EAFLLFDR
				1200.8	0.2	26–36	ITLSQVGDVLR
				1514.7	0.0	49–62	KVLGNPSNEELNAK
				1794.7	−0.2	94–109	VFDKEGNGTVMVTELR
2m.a	Myosin light chain 2 skeletal	4.91	19085.65	1173.9	0.3	43–52	DGIIDKEDLR
2m.b	Muscle (MLC2) human GI: 220346			1192.9	0.3	33–42	EAFTVIDQNR
				1319.4	−0.2	94–106	GADPEDVITGAFK
				1560.4	−0.4	92–106	LKGADPEDVITGAFK
				1988.3	0.4	74–91	EASGPINFTVFLTMFGEK
3m.a	Myosin light chain 1 skeletal			1010.3	−0.2	56–63	EAFLLFDR
3m.b	Muscle (MLC1) human P05976	4.97	21013.91	1200.9	0.3	69–79	ITLSQVGDVLR
				1513.4	0.6	92–105	KVLGNPSNEELNAK
				1722.7	−0.1	137–152	VFDKEGNGTVMGAELR
				1915.0	0.1	49–63	EQQDEFKEAFLLFDR
				2519.6	0.4	44–63	IEFSKEQQDEFKEAFLLFDR
				3646.6	0.9	106–136	KIEFEQFLPMLQAISNNKDQGSYEDFVEGLR
4m.a	α-Tropomyosin (P09493) human	4.69	32708.5	1308.11	0.1	234–244	EAETRAEFAER
				1727.2	−0.6	92–105	IQLVEEELDRAQER
				1801.3	−0.5	153–167	HIAEDADRKYEEVAR
				1883.4	−0.5	190–205	CAELEEELKTVTNNLK
				2202.3	0.2	106–125	LATALQKLEEAEKAADESER
				2386.3	0.1	169–189	LVIIESDLERAEERAELSEGK
4m.b	β-Tropomyosin (P07951) human	4.66	32850.7	721.9	−0.4	239–244	AEFAER
				893.9	−0.5	162–168	YEEVARK
				845.9	−0.5	232–238	LKEAETR
				941.8	−0.6	153–160	HIAEDSDR
				1169.9	−0.7	169–178	LVILEGELER
				1297.9	0.3	60–70	YSESVKEAQEK
				1726.8	−1.0	92–105	IQLVEEELDRAQER
				1882.8	−1.1	91–105	RIQLVEEELDRAQER
				2043.8	−2.3	162–178	YEEVARKLVILEGELER
				2201.8	−0.3	106–125	LATALQKEEAEKAADESER
				2385.8	−0.4	169–189	LVILEGELERSEERAEVAESK
5m	Actin pig P02568	5.23	41816.70	1198.4	−0.1	51–61	DSYVGDEAQSK
				1515.3	0.4	85–95	IWHHPFYNELR
				1790.8	−0.1	239–254	MQKEITALAPSTMKIK
				1955.9	0.1	96–113	QEYDEAGPSIVRRKCF
				2246.2	0.2	292–312	DLYANNVMSGGTTMYPGIADR
				2308.1	0.8	316–336	EITALAPSTMKIKIIAPPERK
6m	Myosin heavy chain cardiac muscle (MYH7_P) pig P79293	/	Fragment	1319.5	−0.1	352–363	LTGAIMHFGNMK
				1887.4	−0.5	641–657	GSSFQTVSALHRENLNK
				2541.8	0.6	148–169	SDAPPHIFSISDNAYQYMLTDR

(*continued on next page*)

Table 2 (*continued*)

Spot	Identification	Theoretical p*I*	Theoretical MW (Da)	Peptide mass $M - H^+$ (Da)	ΔMass (compared with theoretical) (Da)	Position	Sequence
7m	Myosin heavy chain smooth muscle (MYST) human P35749	/	Fragment	1912.7	−0.2	1425–1440	LQQELDDLVVDLDNQR
				1926.8	−0.1	1397–1411	FQKEIENLTQQYEEK
				2685.7	1.2	1814–1837	VRKATQQAEQLSNELATERSTAQK
				3153.2	0.7	1536–1562	ALETQMEEMKTQLEELEDELQATEDAK
8m	Myosin heavy chain cardiac muscle (MYH7_P) pig P79293	/	Fragment	1185.5	0.0	1772–1781	EQDTSAHLER
				1229.5	−0.2	1317–1326	RQLEEEVKAK
				1508.6	0.8	1306–1317	LTYTQQLEDLKR
				2927.3	0.9	1532–1557	QLEAEKLELQSALEESEASLEHEEGK
9m	Myosin heavy chain cardiac muscle (MYH7_P) pig P79293	/	Fragment	1344.1	0.4	1435–1447	SNAAAAALDKKQR
				1646.9	0.1	1290–1303	QLDEKEALISQLTR
				1692.9	0.0	1304–1317	GKLTYTQQLEDLKR
				2086.2	0.2	1110–1126	ELQARIEELEEELEAER
				2122.3	0.2	1196–1214	HADSVAELGEQIDNLQRVK
				2211.1	0.9	1588–1606	RNHLRVVDSLQTSLDAETR
				2615.9	0.6	1421–1444	LQNEIEDLMVDVERSNAAAAALDK
10m	Myosin heavy chain cardiac muscle (MYH7_P) pig P79293	/	Fragment	1343.9	0.2	1435–1447	SNAAAAALDKKQR
				2086.8	0.7	1110–1126	ELQARIEELEEELEAER
				2122.2	0.1	1196–1214	HADSVAELGEQIDNLQRYK
				2615.7	0.4	1421–1444	LQNEIEDLMVDVERSNAAAAALDK
11m	Myosin heavy chain cardiac muscle (MYH7_P) pig P79293	/	Fragment	1598.1	0.2	1677–1689	RNNLLQAELEELR
				1947.7	−0.2	1504–1521	NLQEEISDLTEQLGSSGK
				1986.4	−0.5	1618–1634	MEGDLNEMEIQLSHANR
				2510.4	0.1	1677–1697	RNNLLQAELEELRAVVEQTER
12m	β-Tropomyosin (P07951) human	/	Fragment	1182.1	0.4	218–226	EDKYEEEIK
				1339.9	0.2	206–217	SLEAQADKYSTK
				1430.6	0.1	66–77	EAQEKLEQAEKK
				1461.8	−0.1	129–140	VIENRAMKDEEK

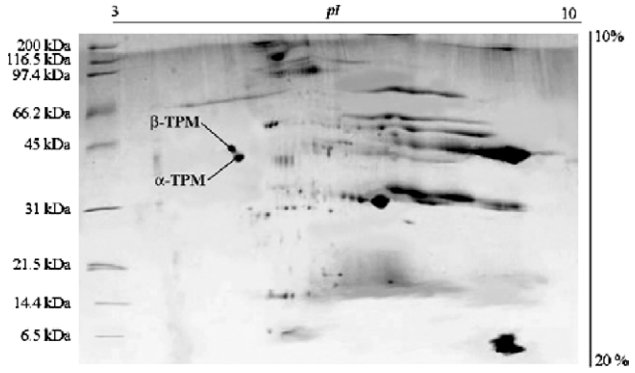

Fig. 4. Two-dimensional gel electrophoresis map of a sarcoplasmic protein fraction from pork skeletal muscles of dry-cured ham.

in addition to the spots relative to TPM, α and β chains, MCF1 and MCF2, and other spots from MHC fragmentation.

In the case of 10-month-old ham, the 2-D pattern (Fig. 5(b)) shows traces of the MLC fraction and further disappearance of spots from MHC fragmentation, on the right side of the gel, when compared with the 6-month-old product. At 10 months of ripening, the TPMs are completely absent.

Lastly, the effect of the intense proteolysis which takes place at complete ripening (labeled 12–14 months) shows 2D-patterns (Figs. 6 and 7) and lacks spots which correspond to A, TPM/s and MLC/s components. In particular, Figs. 6(a) and 7(a) show a 2D map lacking any protein fraction indicating the occurrence of the most intense proteolytic activity. Figs. 6(b) and 7(b) how a notable number of spots in the MHC fragmentation area for S. Daniele ham and a few spots of tropomyosins as well as a few spots on the left side of the gel for Parma ham. Figs. 6(c) and 7(c) show a 2D map with a smear distributed on the gel where the myofibrillar proteins should be present instead. Furthermore, TPMs were also observed in the pattern of Fig. 7(c). It is worth noting that the disappearance of actin (spot A)

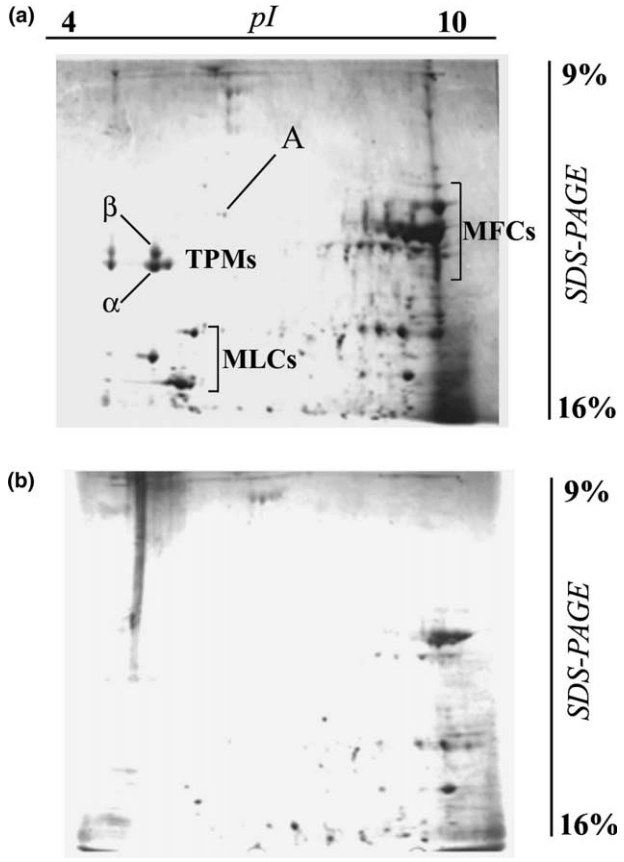

Fig. 5. Two-dimensional gel electrophoresis maps of a myofibrillar protein fraction from dry-cured hams at different ripening stages: (a) 6 months; (b) 10 months.

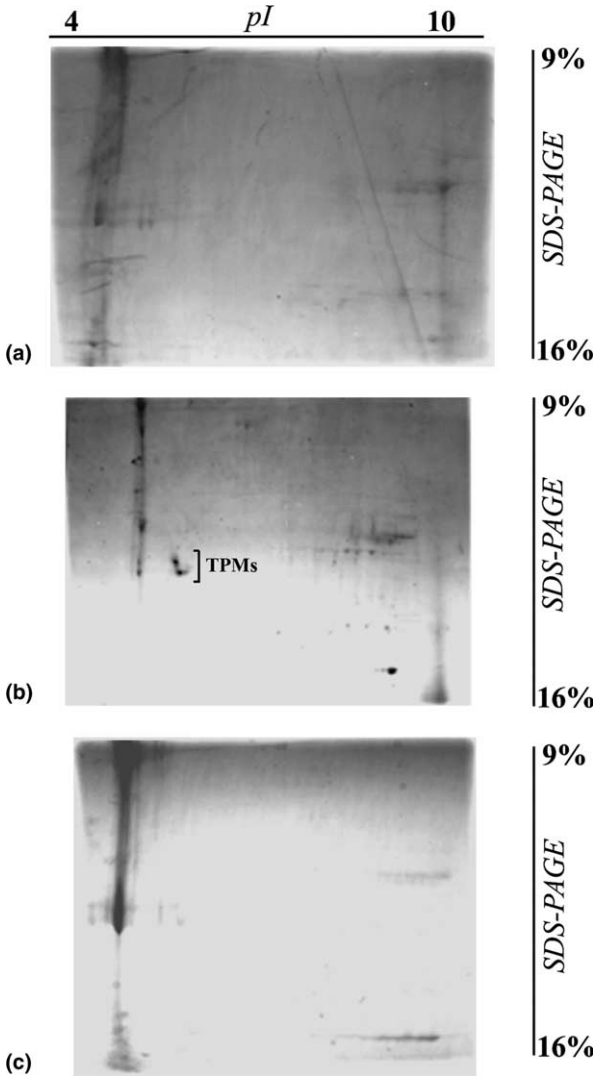

Fig. 6. Two-dimensional gel electrophoresis maps of myofibrillar protein fraction from three different Parma dry-cured hams at 12 months of ripening.

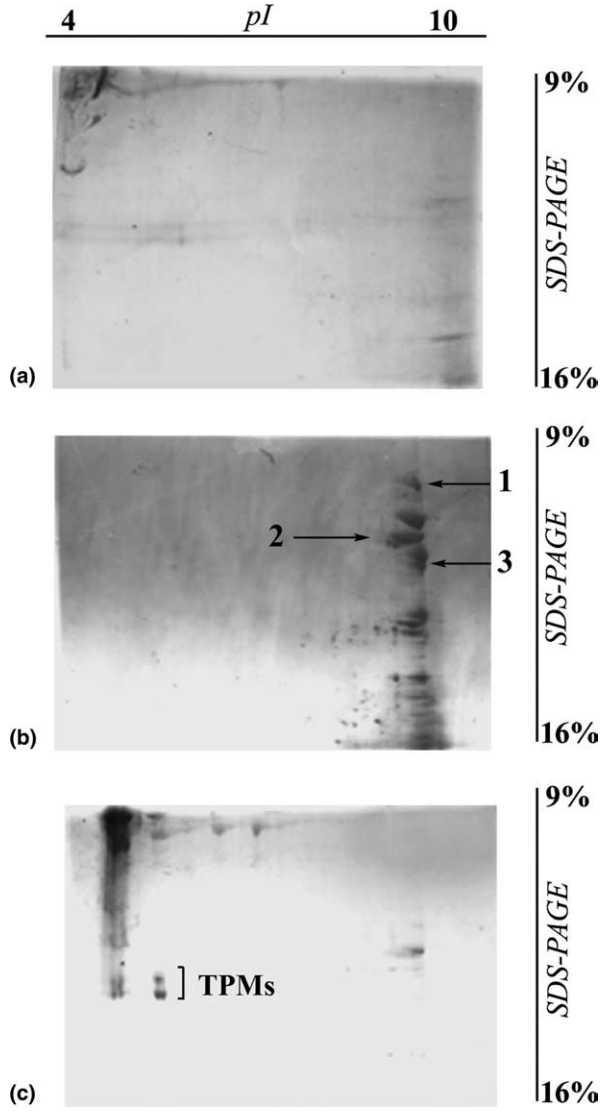

Fig. 7. Two-dimensional gel electrophoresis maps of myofibrillar protein fraction from three different S. Daniele dry-cured hams at 12 months of ripening.

could indicate two different ripening times; actin is present up to 6 months and absent at 10 months, while at 12–14 months in two out of six cases TPMs were present (Figs. 6(b) and 7(c)).

The more or less extensive proteolysis observed in the maps of Parma and S. Daniele final products shows how endogenous enzyme activities of genotype and technological processing can influence Italian dry-cured ham characteristics with the same Protected Designation of Origin.

3.5. Identification of protein spots in dry-cured ham 2D maps by MALDI-TOF mass-spectrometry

3.5.1. Water soluble proteins

The 2-DGE of water soluble proteins extracted from dry-cured ham showed a strong reduction of some protein spots, which were identified as enolase B, creatine kinase, and glyceraldehyde 3-phosphate dehydrogenase (Fig. 4).

On the 2-DGE of sarcoplasmic extracts from dry-cured hams, two spots also appeared at an estimated p*I*'s of about 4.80 and 4.50 and estimated MW of 34 and 33 kDa, respectively, i.e., on the acid side of the gel, contrary to the expectations for sarcoplasmic proteins. By MALDI-TOF MS analysis of the in-gel triptic digests from these two spots we could identify them unequivocally as Tropomyosin α-chain and Tropomyosin β-chain, respectively.

The incomplete hydrolysis of sarcoplasmic proteins during the ripening process suggests a slower action of the endogenous enzymes on the water soluble proteins; this hypothesis seems to be confirmed by the spots of triosephosphate isomerase and aldolase A and the presence of TPMs which remained intact in the water extract of final products.

3.5.2. Myofibrillar proteins

The six 2-DGE maps of myofibrillar proteins from fully matured Parma and S. Daniele dry-cured hams show different 2D patterns (Figs. 6 and 7). Only two of these maps, Figs. 6(b) and 7(c), exhibit small spots of tropomyosins which are not present in the others. These results are consistent with the finding of tropomyosin in the sarcoplasmic fraction.

The presence of numerous spots localized only on the alkaline side of Fig. 7(b) prompted identification of the main spots by MALDI-TOF/MS. Following the procedure reported above, the spots indicated as 1, 2 and 3 in Fig. 7(b) were excised from the gel and analyzed. All peptide masses matched with MHC, confirming that the spots on the right-hand side of the gel represent MHC fragments as previously obtained from raw meat. It is noteworthy that although the ham was labeled 14 months, the MHC fragments were still present. On the other hand the lack of the A and MLC fractions is proof of a ripening period of at least 12 months.

4. Discussion

In this work, we showed the sarcoplasmic and myofibrillar protein changes, occurring in the ham muscle during dry-curing, in addition to identifying myofibrillar fragments and showing how sarcoplamic proteins disappeared by proteolysis. A further goal was to perform an in-depth analysis by 2-DGE of the separated protein fractions with respect to total meat protein analysis. This objective was achieved by fractionating the meat proteins and dissolving the sarcoplasmic fraction in water to ensure complete insolubilization of the myofibrillar fraction.

Lametsch and Bendixen (2001) resolved 1000 individual protein spots, from 5–200 kDa, by using two-dimensional electrophoresis during post-mortem aging in pork muscle, and detected the changes of the 15 most represented proteins by image analyses. More recently, Lametsch et al. (2002) have determined the most susceptible proteins to endogenous enzyme action in the first 48 h post-mortem; they identified fragments derived from the sarcoplasmic fraction (glycogen phosphorylase, pyruvate kinase, dihydrolipoamidesuccinyl transferase, phosphopyruvate hydratase and creatine kinase) and the myofibrillar fraction (myosin heavy chain, actin and troponin). The MHC (as a fragment of 56 kDa) and the actin spot of 40, as well as a 32 kDa actin fragment appeared among these latter compounds.

Our 2-DGE maps of fresh ham are less complex, because the sarcoplasmic and myofibrillar fractions were analysed separately. At 72 h post-mortem identification was carried out of the most abundant myofibrillar protein fragments, MCFs, and of a number of minor ones derived from MHC; moreover, a relevant heterogeneity of tropomyosin and MLCs was observed (Lametsch et al., 2002). We also identified the 5m spot as an actin form, although its isoelectric point and molecular weight were very different to expectation.

The presence of MHC fragments in the first 48 and 72 h (our results) suggests that there exists a synergistic contribution of calpains and lysosomal proteinases to the post-mortem tenderization of pork. It is generally accepted that in the first 72 h the calpains specifically attack certain proteins of the Z-line, such as desmin, filamin, nebulin, and, to a lesser extent, connectin (Davies et al., 1978; King, 1984; Lusby, Ridpath, Parrish, & Robson, 1983; Penny, Etherington, Reeves, & Taylor, 1984), and that cathepsins preferentially attack myosin and actin (Gault, 1992; Jiang, Lee, & Chen, 1992; Jiang, Wang, & Chen, 1992; Koohmaraie, Whipple, Kretchmar, Crouse, & Mersmann, 1991) as the pH falls from 6.5 to 5.5. Koohmaraie et al. (1991), using SDS–PAGE to determine the effective myofibril fragmentation index in longissimus muscle steaks, observed that desmin was extensively hydrolyzed in pork muscle from 1 day post-mortem, whereas in beef and lamb the effect was less. At 14 days they observed the absence of the desmin band in all three species. These authors also observed that the pH fell to 5.5 more rapidly in pork muscle than in beef and lamb. These results indicate that desmin and 30 kDa-derived fragments, arising from myofibril disruption, and MHC and actin fragments, deriving from myofibrillar protein hydrolysis, could be considered molecular markers for proteolysis.

Longer aging of meat, as in dry-cured ham production, is characterized by the intensive activity of cathepsins which produce peptides and amino acids. Their combination is responsible for flavor development during ripening (Toldrà & Flores, 1998). The 2-DGE maps of meat proteins represent the fragmentation of meat proteins which generates peptides throughout ripening. Comparison of the 2-DGE maps provides a detailed picture of the protein changes during proteolysis. At 6 months neo-formed components with MW of 46.5 and 23.4 kDa were observed, which disappear at 10 months, whereas the MCF2 fragment with a molecular weight of 46.5 kDa and minor components with lower molecular weight remain. It is also evident that some of these components are difficult to detect by mono-dimensional electrophoresis techniques (IEF and SDS–PAGE) because of their co-migration.

Lastly, at 12–14 months of ripening we observed six different 2-DGE maps of myofibrillar proteins from two typical Italian dry-cured hams, four of them lacking main spots identified as A, TPMs and MLCs. Although small spots of α and β TPMs appear in two of the six 2-DGE maps of myofibrillar proteins, all the 2-DGE maps of sarcoplasmic proteins show the appearance of two acidic spots identified as α and β tropomyosin. Therefore, the complete absence of TPMs in the 2-DGE maps of the myofibrillar proteins is not only due to enzyme action but to their solubilization in the water-soluble protein fraction (sarcoplasmic proteins). This phenomenon must be ascribed to solubilization of myofibrillar saline soluble proteins caused by adding salt and its diffusion in the ham during ripening. The other components which change during the ripening process in the sarcoplasmic fraction were identified as enolase B, creatine kinase, and glyceraldehyde 3-phosphate dehydrogenase; two unidentified spots with a MW less than 14.4 kDa also appeared. It is noteworthy that several authors (Fadda et al., 1999a, 1999b; Martin, Cordoba, Rodriguez, Nunez, & Asensio, 2001; Santos et al., 2001; Sanz et al., 1999) who studied the effect of lactic acid bacteria on sarcoplasmic proteins observed complete hydrolysis of enolase B, creatine kinase, and glyceraldehyde 3-phosphate dehydrogenase in a few days, whereas endogenous muscle enzyme activity produces a reduction in these proteins only after 12 months of dry-cured ham ripening.

The modifications of sarcoplasmic protein patterns and the presence of intact TPMs in water extracts led us to hypothesize a less intensive action of endogenous enzymes on water-soluble proteins, mainly cathepsins, during ripening. In other words, the presence of intact TPMs in the water-soluble fraction could make it less susceptible to lysosomal enzymes (cathepsins), since the other myofibrillar proteins, mainly MHC, MLCs and A, were completely hydrolyzed. This result confirms that the myofibrillar proteins are an elective substrate of lysosomal enzymes and suggests a minor susceptibility of water-soluble proteins to endogenous enzyme action.

The separate analyses of meat proteins, unlike 2-DGE analyses of whole meat proteins, have highlighted the solubilization of the tropomyosins and their

presence at the final ripening stage in the sarcoplasmic fraction. At the same time, simpler maps have been obtained and possible overlapping of fragment spots of the two fractions have been avoided.

The application of proteomic study to proteolysis occurring in dry-cured ham production has allowed an evaluation of the changes in muscular proteins during ripening. An aspect shown by 2-DGE maps is that protein heterogeneity and some neo-formed components exhibiting the same estimated MW or approximate pI are not detectable by mono-dimensional gel electrophoresis studies. A proteomic study, therefore, is a useful tool in finding molecular markers as indicators of the technological process in meat aging and ripening.

Finally, it would be interesting to relate sensory textural analyses to the different dry-cured ham 2-DGE maps to obtain a better understanding of the relationship between product quality and proteolysis, and, use this information to select the breed or modify the environmental conditions in the ripening rooms (Parolari, Virgili, & Schivazappa, 1994; Russo et al., 2002; Toldrà & Flores, 1998). Such a study could also contribute to identification of objective parameters to define quality and traceability of typical dry-cured hams based on a standard fingerprint obtained with the 2-DGE maps.

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