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Proteomic study of muscle sarcoplasmic proteins using AUT-PAGE/SDS-PAGE as two-dimensional gel electrophoresis☆

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Abstract

In the present study, an alternative procedure for two-dimensional (2D) electrophoretic analysis in proteomic investigation of the most represented basic muscle water-soluble proteins is suggested. Our method consists of Acetic acid-Urea-Triton polyacrylamide gel (AUT-PAGE) analysis in the first dimension and standard sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) in the second dimension. Although standard two-dimensional Immobilized pH Gradient-Sodium Dodecyl-Sulphate (2D IPG-SDS) gel electrophoresis has been successfully used to study these proteins, most of the water-soluble proteins are spread on the alkaline part of the 2D map and are poorly focused. Furthermore, the similarity in their molecular weights impairs resolution of the classical approach. The addition of Triton X-100, a non-ionic detergent, into the gel induces a differential electrophoretic mobility of proteins as a result of the formation of mixed micelles between the detergent and the hydrophobic moieties of polypeptides, separating basic proteins with a criterion similar to reversed phase chromatography based on their hydrophobicity. The acid pH induces positive net charges, increasing with the isoelectric point of proteins, thus allowing enhanced resolution in the separation. By using 2D AUT-PAGE/SDS electrophoresis approach to separate water-soluble proteins from fresh pork and from dry-cured products, we could spread proteins over a greater area, achieving a greater resolution than that obtained by IPG in the pH range 3–10 and 6–11. Sarcoplasmic proteins undergoing proteolysis during the ripening of products were identified by Matrix Assisted Laser Desorption/Ionization–Time of Flight (MALDI-ToF) mass spectrometry peptide mass fingerprinting in a easier and more effective way. Two-dimensional AUT-PAGE/SDS electrophoresis has allowed to simplify separation of sarcoplasmic protein mixtures making this technique suitable in the defining of quality of dry-cured pork products by immediate comparison of 2D maps to define the events occurring during their ripening and individuate candidate molecular markers of the characteristic proteolytic processes. Considering that, essentially, muscle endogenous enzymic activity, calpains and cathepsins, occur in the ripening process of dry-cured ham, whereas a combined action between endogenous and microbial enzymes takes place in the case of sausage ripening, these results provide deeper insight into the respective role of endogenous and microbial enzymes in performing proteolysis. Finally, image analysis and creation of data bank could be achieved to quickly identify and protect typical products.

Keywords: Two-dimensional AUT-PAGE/SDS-PAGE; MALDI-ToF fingerprinting; Sarcoplasmic proteins

1. Introduction

The conversion of muscle to meat in pig involves mainly proteolysis of myofibrillar proteins, which undergo notable changes since early stage of *rigor mortis*, even after 48 h *post mortem* [1–3]. The tenderness of meat has been thoroughly investigated to understand the biochemical mechanisms, which influence texture and flavour development as well as the technological parameters and hence meat quality [4]. Cytoplasmic proteolytic calcium dependent enzymes, named μ-and m-calpains, which act in the early stages of *rigor mortis*, significantly contribute to tenderization weakening myofibrils [5,6]. These enzymes, however, act for few days because they are specifically inhibited by calpastatin and by pH lowering. However, when pH falls to about 5.0,

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proteolytic activity on muscle proteins is continued by longer acting lysosomal proteinase, cathepsins [3,7–9]. *Post mortem* proteolysis also causes relevant changes in sarcoplamic protein fraction, which represent the water soluble fraction (quantitatively about 30–35%) of meat total protein, and the involved proteins has already been identified by proteomic-based studies [9]. Recent investigations have demonstrated that the most commonly found *Lactobacillus* species in dry fermented meats are able to hydrolyse myofibrillar and sarcoplasmic muscle proteins in vitro [10,11].

Nevertheless, as consequence of their microbiological proprieties, proteolysis occurring during dry-cured hams [4,8,12] ripening has to essentially be ascribed to endogenous enzymes, i.e. cathepsins, whereas a concomitant action of endogenous and microbial enzymes is expected in fermented dry-cured products. The respective, exact role of endogenous and microbial enzymes, however, is not well defined [13]. Bacterial peptidases seem involved principally in the degradation of peptides deriving from the previous hydrolysis of large proteins conducted by endogenous enzyme activity, thus producing amino acids and amines [7]. Basso et al. [14] established that lactic acid bacteria (LAB), usually present in meat, are able to hydrolyse preferentially selected proteins, which constitute the sarcoplasmic fraction and that bacterial hydrolytic enzymes, characterised by a high substrate specificity, probably act in LAB in the early phase of ripening. Mauriello et al. [15] selected twenty-seven *Staphylococcus xylosus* strains with proteolytic activity in "Naples-type" fermented sausages; however, they found that only twelve of those significantly changed SDS-PAGE patterns of pork proteins, and that sarcoplasmic proteins changed in a greater extent than myofibrillar proteins. Therefore, proteolytic activity and its action in the ripening phase of dry fermented sausages could depend on the strain type. Differences in myofibrillar and water-soluble protein composition between fresh meat and dry-cured hams have also been widely investigated by the 2D IPG-SDS-PAGE classic proteomic approach [16], also taking into account identification of meat proteins formerly made by Lametsch et al. [1,2]. It is noteworthy that, sarcoplasmic proteins are extensively hydrolysed and that most of these proteins have an alkaline pI. On the other hand, extensive studies about the characterization of sarcoplasmic protein fraction during ripening of pork products are lacking. By comparing our evidences with the above-mentioned studies [10,11], we could record evident changes at level of the most represented water-soluble proteins. The proteins most interested by proteolytic changes, previously identified as glycogen phosphorylase, muscle isoform, creatine kinase M chain and glyceraldehyde 3-phosphate dehydrogenase [14], are poorly focused in the IPG based electrophoresis and, moreover, they are spread in a restricted region on the alkaline side of 2D map, hence not providing an adequate resolution to completely identify protein components [1,2,16]. The aim of our study was to develop an alternative 2D electrophoresis technique, which allowed to better resolve the most represented water-soluble meat proteins, and to explore changes in their composition. This should gain advances in the understanding of the proteolytic phenomena occurring in meat ripening and obtain comparable and easy interpretable 2D maps to characterize dry-cured pork products.

This method consists of an AUT-PAGE in the first dimension and the standard SDS-PAGE in the second dimension. Two-dimensional AUT-PAGE/SDS PAGE is a technique already applied in the past for the separation of histones or their modified variants [17–19].

The most abundant sarcoplasmic proteins, as mixture of basic polypeptides with a narrow spread range of molecular masses, represented an excellent model to test our analytical technique and to delineate its capabilities.

In the present study, we compared 2D AUT-PAGE/SDS-PAGE maps of water-soluble proteins extracted from fresh meat and from dry-cured ham, a non fermented product, from "Naples-type" salami, a microbiologically fermented product, and from "Coppa", a typical semi-fermented product. Electrophoretically separated proteins have been identified by MALDI-ToF mass fingerprinting.

2. Materials and methods

2.1. Materials

Sequencing grade Trypsin (TPCK treated) was purchased from Boehringer (Mannheim, Germany). Solvents were HPLC-grade from Carlo Erba (Milan, Italy). Urea, Temed, 1-propanol, CBB R250, agarose, ACN, TFA were supplied by Bio-Rad Laboratories (Milan, Italy). SDS, ammonium bicarbonate (NH_4HCO_3), DTT, polyacrylammide solution (50% acrylammide, 0.32% bisacrylmmide) and riboflavin were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Tris–HCl and glycine were supplied by ICN Biomedicals (Costa Mesa, CA, USA). BromophenolBlue was provided by ESA Electrophoresis (Chelmsford, MA. USA). 2-Mercaptoethanol was obtained from Fluka Biochemika (Milan, Italy). Glycerol, glacial acetic acid, Triton X-100 was provided by J.T. Baker (Mallinckrodt Baker B.V., Deventer, Holland). Standard peptides for mass spectrometry external calibration were purchased in part from Applied ByoSistems (Framingham, MA, USA) and in part were purified in our lab.

2.2. Samples

Meat was provided by a local farm. Analyses were carried out on raw meat from hams, 72 h after slaughtering, and cured products ripened, respectively: "Naples-type" salami 1 month; "Coppa" 3 months; dry-cured ham 12 months.

Samples (5.0 g), kept in an ice-cold bath, were freed of connective and adipose tissue and homogenized with an Ultra-Turrax T8 homogenizer (Ika, Staufen, Germany) with 20 ml of water milli-Q (Millipore), for 10 min at 9500 rpm. The homogenate was centrifuged under refrigeration at 2 °C and 4500 × *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. Classical Bradford protein assay was used to standardise protein loadings for the first dimension. The

supernatant was then filtered on a 0.45 μm membrane (Millipore) and proteins were precipitated overnight by addition of 50 ml of −20 °C cold acetone and lyophilised. Sarcoplasmic proteins (30 μg) were finally resuspended in UAM buffer (0.5% urea, w/v, 10% acetic acid, v/v, 10% mercaptoethanol, v/v), using pironin G as tracking die, and 100 μl were used for 2D electrophoretic analysis.

2.3. Two-dimensional AUT-PAGE/SDS PAGE

Sarcoplasmic proteins were separated by AUT-PAGE in the first dimension and the standard SDS-PAGE in the second dimension.

Acid acetic/Urea/Triton X-100 gel was prepared as follows: for 20 ml of resolving gel, 4.825 ml of polyacrylammide solution (50% acrylammide, w/v, 0.32% bisacrylmmide, w/v), 0.937 ml glacial acetic acid, 14 ml Urea 8 M, 0.375 ml Triton X-100 (100%) were mixed. Polymerization was achieved by addition of 0.075 ml Temed (tetramethylendiammine) and 0.5 ml of riboflavin stock solution (10 g/5 ml deionized water) irradiating solution with a fluorescent 30-W lamp. The solution was poured into a slab gel shell, 16 cm × 18 cm, 0.75 mm thick (SE 600 Series-Vertical Slab gel unit-Hoefer Scientific Instruments, San Francisco, California). Separations were carried out in reversed conditions (electrodes were inverted): positively charged proteins migrated towards cathode, at 8 mA/gel for 16–18 h (PS 500XT DC Power supply Hoefer Scientific Instruments, San Francisco, California). Gels were stained with 0.25% Comassie Brilliant Blue R-250.

The polyacrylamide lanes were manually cut and equilibrated incubating at room temperature for 30 min, in 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 2% DTT, 50 Mm Tris–HCl pH 8.6 and a trace of bromophenol blue as tracking die. The strips were loaded at the top of 1.5 mm vertical second dimension gel (SDS-PAGE gradient 9–18%) with 0.5% (w/v) agarose in 25 mM tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3 and running conditions were 30 mA/gel until the bromophenol blue reached the bottom of the gel. Broad-range molecular weight standards (Biorad, Hercules, CA.USA) included myosin (200,000 Da), β-galactosidase (116,500 Da), phosphorylase B (97,400 Da), bovine serum albumine (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), lysozyme (14,400 Da), aprotinin (6500 Da).

Staining was performed as for first dimension.

Three gels for each sample were run to test reproducibility of analysis.

2.4. In-gel digestion of 2D gel electrophoresis separated proteins

Protein spot were manually excised and twice washed with 50 μl milli-Q water. Each gel piece was completely destained by repeated immersion into a solution 50 mM NH_4HCO_3 in 50% (v/v) aqueous acetonitrile (AcN). The destained spots were dehydrated by submersion into AcN, and dried under vacuum after AcN removal. Before digestion, 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). The dried gel piece was covered by 30 μl 50 mM NH_4HCO_3 containing 12 ng/μl trypsin maintained in tubes kept in an ice-cold bath. After 45 min action, the supernatant was removed and incubated overnight at 37 °C. The resulting tryptic digest was extracted in 40 μl of AcN/5% (v/v) formic acid solution (1/1, v/v) three-fold, the recovered solutions were then joined together and, finally, the volume was reduced to one tenth in a vacuum centrifuge for the mass spectrometric analysis.

2.5. Reversed-phase High Pressure Liquid Chromatography (RP-HPLC) of sarcoplasmic proteins

Sarcoplasmic protein mixture from fresh pork meat (100 μg) was loaded onto a 218TP52, 5 m reversed-phase C_{18}, 250 × 2.1 column (Vydac, Hesperia, CA, USA). Solvent A was water containing 0.1% (v/v) trifluoroacetic acid (TFA) and solvent B was acetonitrile containing 0.1% (v/v) TFA. A linear gradient from 20 to 60% solvent B was applied over 60 min, after 5 min of isocratic elution at 20% solvent B, at a constant flow rate of 0.2 ml/min. UV detection was carried out both at 220 and 280 nm. Protein fractions were manually collected and concentrated in speed vac (Savant) for mass spectrometric analysis.

2.6. MALDI-ToF analysis, peptide mass fingerprinting and protein identification

MALDI-ToF-mass spectrometry analysis of peptides arising from in-gel digested proteins were carried out essentially as already described by Di Luccia et al. [16]. Briefly, mass spectra were acquired on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N_2 laser (λ = 337 nm), using α-cyano-4-hydroxy-cinnamic acid (prepared by dissolving 10 mg in 1 ml of aqueous 50%, v/v, acetonitrile/0.1%, v/v, TFA). The instrument operated with an accelerating voltage of 20 kV.

External mass calibration was performed with the signal of matrix dimer at *m/z* 379.05 and with the monoisotopic masses of peptide standards including angiotensin I (*m/z* = 1296.68), bovine αs_1-casein 1–23 peptide (*m/z* = 2764.55) and bovine insulin (*m/z* = 5730.61). 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. MALDI-ToF spectra of RP-HPLC separated proteins were acquired using the same instrument described above. In the case of analysis of whole proteins, sinapinic acid was used as the matrix (Sigma), prepared by dissolving 10 mg in 1 ml of aqueous 50% (v/v) acetonitrile/0.1% (v/v) TFA, and accelerating voltage was 25 kV.

For protein identification Protein Prospector MS-Fit software was used; the entire Protein Prospector software package, maintained and updated by Mass Spectrometry facility of University of San Francisco, is available on the internet at the following web site: http://prospector.ucsf.edu.

Queries were extended to NCBI protein e-genomic databases. Up to one missed tryptic cleavage was considered and a

conservative mass accuracy of ±0.5 Da was used for all 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 2D maps. These two parameters were used with large tolerances (ΔpI = 1 and ΔMW = ±15% Mw) as filters to exclude false positive candidates from the output lists.

3. Result and discussion

Fig. 1 shows the comparison between 2D IPG (pH range 3–10)/SDS-PAGE (panel (a)) and AUT-PAGE/SDS-PAGE (panel (b)) maps of the sarcoplasmic fraction of fresh pork. In panel (a), the localization of sarcoplasmic proteins in the alkaline area of the conventional 2D IPG/SDS-PAGE map is clearly evident. In agreement with other authors, the most represented proteins are very close, having similar pI and MW [1–3,16]. In panel (b), the 2D AUT-PAGE/SDS-PAGE electrophoretic analysis shows protein spots well distributed on the entire gel area, allowing us to perform more accurate spot cutting, and then an effective protein identification by peptide mass mapping.

The addition of Triton X-100, a non-ionic detergent, into the gels causes a differential electrophoretic mobility of proteins as a result of the formation of mixed micelles between

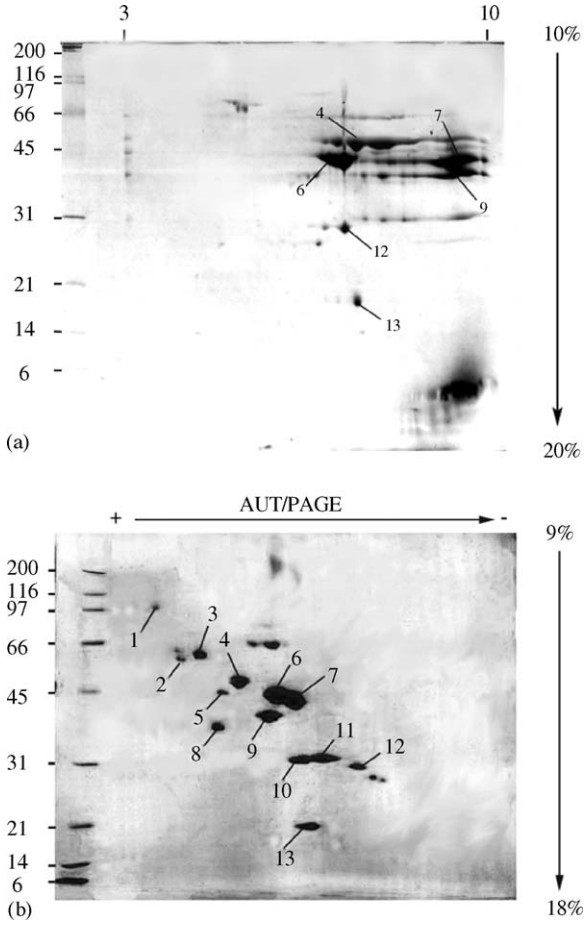

Fig. 1. Comparison of standard 2-D IPG/SDS-PAGE (panel a) and 2-D AUT-PAGE/SDS-PAGE (panel b) maps of sarcoplasmic proteins from fresh pork meat. For identification of protein spots see Table 1.

the detergent and the hydrophobic moieties of polypeptides, separating proteins with a criterion roughly similar to reversed-phase chromatography based on their hydrophobicity [17,18]. The acid pH induces a positive net charge as high as isoelectric point of proteins increases, allowing an enhanced resolution in the separation. By 2D AUT-PAGE/SDS-PAGE electrophoresis approach, applied to separation of water-soluble proteins from fresh pork and from dry-cured products, such as ham and sausages, we could spread proteins over a greater area, achieving an higher resolution degree compared to that obtained by IPG in the pH range 3–10 and 6–11.

However, by this 2D electrophoresis procedure, it was not possible to distinguish post-translationally phosphorylated protein isoforms because phosphoric groups are titrated at acid pH (about 3) as the pKs values are 2.2, 7.2 and 12.3. Even though it has not been possible to reveal protein heterogeneity due to the phosphorylation degree, the use of chromatographic techniques, such as Immobilized Metal Affinity Chromatography (IMAC) or related methods [20], coupled to mass spectrometry could overcome this drawback and permit localization of phosphate groups. It could be sometimes advantageous, however, to work with a simplified two-dimensional map, as in the present case, i.e. in the comparison of dry-cured pork products to identify candidate molecular markers to asses proteolysis. Classical 2D IPG based approach has already been successfully applied to extensive identification of all protein components of the whole sarcoplasmic and myofibrillar fractions [1,2,16] and is, without any doubt, fairly more sensitive than 2D AUT-PAGE/SDS-PAGE technique. What is new in this last approach is the capability to separate with higher resolution a restricted group of basic proteins, such as the most abundant water-soluble muscle proteins, hardly resolved by other techniques. The application of 2D AUT-PAGE/SDS-PAGE allowed us to individuate and easily compare each other 2D AUT-PAGE/SDS-PAGE maps of dry-cured pork products.

As mentioned above, 2D AUT-PAGE/SDS-PAGE electrophoresis has already been used for separation of the highly basic histone proteins for detecting their heterogeneity [17–19], but it had never been coupled to mass spectrometry for protein identification.

It might arise some controversy about MALDI-TOF identification of proteins in the presence of Triton X-100. The use of a detergent as Triton X-100 in the first dimension of electrophoresis could have prevented an adequate peptide ionization generated by tryptic digestion. In fact, even though non-ionic detergents have less effect than ionic detergents in preventing effective MALDI ionization, Triton X-100 could act as involatile solvents and its presence would result in poor matrix crystallization [21]. On the other hand, being Triton X-100 a non-ionic detergent, it could not enter at all in the SDS gel of 2D AUT-PAGE/SDS-PAGE and, in any case, it would be removed during the de-staining step of protein spots. However, the compatibility of 2D AUT-PAGE/SDS-PAGE was not a fully predictable result on the basis of these a priori considerations. Therefore, in this work we tested the effectiveness of applying MALDI-ToF mass fingerprinting to identify protein spots, reported in Table 1, thus demonstrating the compatibility of the

Table 1
Identification of sarcoplasmic proteins separated by standard 2D IPG/SGS-PAGE, 2D AUT-PAGE/SDS-PAGE or RP-HPLC

Spot	Identification	Teoretichal MW	Teorethical pI	Hydrophobicity index (HbI)[b]	Charge[c]
1	Glycogen phosphorylase, muscle form (PAN: human P11217)[a] gi [3041717]	96961	6.57	435.256	132
2	Glucose-6-phosphate isomerase (PAN: pig P08059) gi [120742]	62995	7.89	290.659	83
3	Pyruvate kinase muscle isozyme (PAN: rabbit P11974)[a] gi [2851533]	57916	7.60	274.253	82
4	Enolase B muscle isoform (PAN: human P13929)[a] gi [416950]	46856	7.73	226.704	61
5	Phosphoglycerate kinase 1 (PAN: mouse P09411)[a] gi [129903]	44405	7.52	221.639	56
6	Creatine kinase, M chain (PAN: pig Q5XLD3) gi [62286641]	43059	6.61	189.502	68
7	Fructose-bisphosphate aldolase A (PAN: rabbit P00883)[a] gi [113608]	39212	8.40	188.351	52
8	L-Lactate dehydrogenase muscle isoform (PAN: pig P00339) gi [1170740]	36487	8.22	180.265	49
9	Glyceraldehyde-3-phosphate dehydrogenase (PAN: pig P00355) gi [2506441]	35795	8.52	176.783	47
10	Phosphoglycerate mutase isozyme M (PAN: human P15259)[a] gi [130353]	28635	9.00	127.999	46
11	Carbonic anhydrase (PAN: pig Q5S1S4) gi [75042729]	29411	7.72	135.756	44
12	Triosephosphate isomerase (PAN: human P60174)[a] gi [39932641]	26538	6.51	130.476	32
13	Myoglobin (PAN: pig P02189) gi [127688]	16953	6.83	77.317	30

Protein was considered identified when percentage coverage of protein sequence was higher than 66%.
[a] Protein identified by homology.
[b] HbI: hydrofobicity index. See text for its definition.
[c] Positive charge corresponds to the sum of basic amino acids (see text).

AUT-PAGE/SDS-PAGE technique with mass spectrometry in proteomics.

Comparison of the protein migration on AUT-PAGE/SDS-PAGE with reversed-phase HPLC, where protein elution is obtained by means of a gradient of organic solvent within the chromatographic column, was also carried out. This allowed us to verify whether the migration order in AUT-PAGE/SDS-PAGE corresponded to elution in HPLC. The mixture of sarcoplasmic proteins of fresh meat was separated on a reversed-phase HPLC column (Fig. 2). Peak identification in this case involved only molecular mass measurement of intact proteins by using MALDI-ToF technique since the identity of the proteins was easily disclosed by using the information previously collected by peptide mass MALDI-ToF fingerprinting of the protein spots. The most hydrophilic proteins, at lower retention times in the column, were those showing a higher mobility in the first dimension (AUT-PAGE). However, some proteins characterised by a higher mobility in AUT-PAGE showed, unexpectedly, higher retention times in HPLC. This was the case of glucose-6-phosphate isomerase. Trying to explain this discrepancy, a hydrophobicity index (HbI) was calculated for each proteins (Table 1).

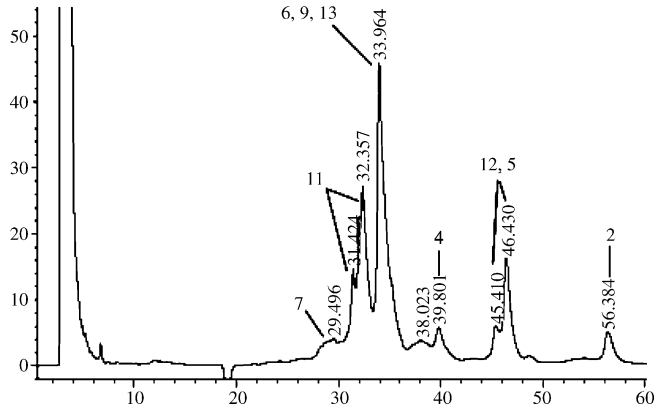

Fig. 2. RP-HPLC chromatogram of sarcoplasmic protein from fresh pork meat. For identification of protein peaks see Table 1.

Hydrofobicity index is defined as

$$\mathrm{HbI} = \sum\nolimits_n (\mathrm{AA})_i \phi_i$$

where ϕ is the hydrophobicity index of the i-esim amino acid (AA) as proposed by Black SD and Mould DR [22] in their scale of amino acid hydrophobicity and n the number of amino acids of the proteins.

The values of positive charges reported in Table 1 for each identified protein correspond to the sum of basic amino acids (arginine, lysine and histidine), assuming that at acid pH these amino acids are completely protonated, having pKa 12.0, 10.0 and 6.0, respectively.

HbIs are well correlated with the electrophoretic mobility of proteins except for spot 8 (L-lactate dehydrogenase muscle isoform), which has a HbI very close to that of spot 6 (creatine kinase, M chain). However, taking into account the differences

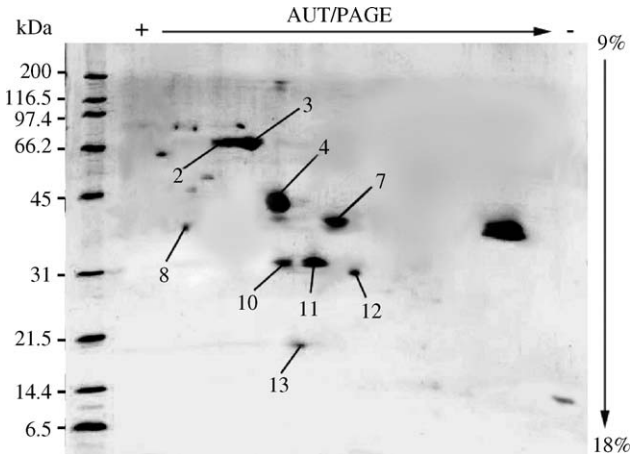

Fig. 3. Two-dimensional AUT-PAGE/SDS-PAGE analysis of sarcoplasmic proteins from 12-months aged dry-cured ham where proteolysis is accounted for muscular endogenous enzymes.

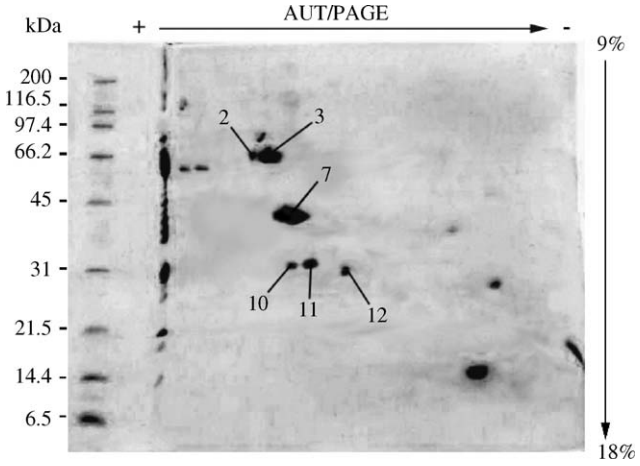

Fig. 4. Two-dimensional AUT-PAGE/SDS-PAGE analysis of sarcoplasmic proteins from 1-month aged "Naples-type" salami. In this case the proteolysis is due to a concomitant action of muscular endogenous and microbial enzymes.

in the number of positive charges calculated on the basis of the amino acid sequence (spot 8 counts 49 charges, while spot 6 counts 68 charges), we can explain the discrepancy of proteins in mobility or in R_f. Other divergences, such as the high retention time in HPLC of triosephosphate isomerase with respect to its high electrophoretic mobility, could probably be ascribed to a different kind of interaction of the protein with the aliphatic chain length of the HPLC stationary phase in the former case and with 2% Triton X-100 in the latter case.

The identification by 2D AUT-PAGE/SDS-PAGE map and MALDI-ToF of meat sarcoplasmic proteins allowed us to identify proteins most susceptible to undergo to proteolityc phenomena during seasoning. An analogous procedure of MALDI-TOF mass fingerprinting has been applied in identifying main picked protein spots of ripened products 2D maps, also comparing MALDI spectra of digest spots from fresh meat and cured products. In the case of dry-cured ham (aged up to 12 months), where only muscle proteolytic enzymes act, namely cathepsins [5,6], we observed through a long ripening time an intense hydrolysis of sarcoplasmic proteins (Fig. 3). The total disapparence of phosphoglycerate kinase 1 (44.3 kDa, spot 5), creatine kinase M (43 kDa, spot 6), L-lactate dehydrogenase muscle isoform (36.4 kDa, spot 8) and glyceraldehyde-3-phosphate dehydrogenase (35.8 kDa, spot 9) as well as glycogen phosphorylase muscle isoform (97 kDa, spot 1), and appearance of two spots with estimated molecular weight of 37 and 38 kDa, on the extreme right side of 2D map due probably to myofibrillar protein fragments, were observed. In this respect, Di Luccia et al. [16] showed the appearance in water extract from dry-cured ham of two neo-formed spots, identified by mass mapping as tropomyosin α-and β-chain. This result was attributed to the effect of salting, which can be able to solubilize in water myofibrillar proteins [16]. In our case, however, the extremely high electrophoretic mobility of these acidic proteins remains to be explained. However, the same authors also showed the presence of myosin heavy chain fragments, produced by endogenous meat enzymes action and localized in alkaline pH area of two-dimensional IPG/SDS-PAGE map. These could better explain a faster migration at acid pH. A strong decrement of enolase B (46.8 kDa, spot 4) could be expected [14], but it seems do not undergo to proteolysis. In effect, Basso et al. found that enolase B is one of proteins disappearing in the first steps of sausage ripening. Our result is consistent with the above-mentioned observation as enolase B might be a substrate of bacterial proteinase, whereas it is not hydrolysed by endogenous proteolytic enzymes acting in dry-cured hams.

A comparable proteolysis degree was observed in sarcoplasmic proteins map of dry-cured sausages "Naples-type" salami, but only 30 days of ripening is required in drying fermented sausages (Fig. 4). This could be due to inoculated or natural autochthon lactic microflora, which break down sarcoplasmic proteins faster. In this case, enolase B (spot 4), as expected, disappear completely and, as in the case of dry-cured ham, the most susceptible proteins to hydrolysis were glycogen phosphorylase (spot 1) and glyceraldehyde 3-phosphate dehydrogenase (spot 9), whereas triosephosphate isomerase (spot 12) and fructose biphosphate aldolase A (spot 7), carbonic anhydrase (spot 11) and in a less extend phosphoglycerate mutase isozyme M (spot 10) were resistant to either endogenous, was the case of dry-cured ham, or microbial enzymes activity (Fig. 4). The same results were obtained by Basso et al. [14], who in vitro assayed the rapidity of sarcoplasmic protein proteolysis by LAB, assessing complete hydrolysis of above mentioned proteins in less than 48 h of incubation, in particular of the glycogen phosphorylase. In the 2D map of sarcoplasmic fraction extracted from "Naples-type" salami polypeptide fragments formed in the course of ripening, with molecular around 37–38 kDa, were lacking.

In order to obtain indications on the kinetics of endogenous enzyme action, we investigated sarcoplasmic protein fraction of a product at intermediate ripening, that is, a sacked entire anatomic portion named "Coppa" with 3 months of seasoning. The sarcoplasmic proteins of "Coppa" ripened for 3 months, gave rise a 2D AUT-PAGE/SDS PAGE map (Fig. 5) very similar to that of fresh meat but lacking of glycogen phosphorylase (97 kDa, spot 1); furthermore, a weak spot with estimated molecular weight 14 kDa appeared. In the 2D map, all the other

Table 2
Evolution of identified sarcoplasmic proteins in ripened pork products: dry-cured ham, "Naples-type" salami and "Coppa"

Spot	Identification	Teoretichal MW	Dry-cured ham	Naples-type salami	Coppa
1	Glycogen phosphorylase, muscle form (PAN: human P11217)[a]	96961	H[a]	H	H
2	Glucose-6-phosphate isomerase (PAN: pig P08059)	62995	N.H[b]	N.H	N.H
3	Pyruvate kinase muscle isozyme (PAN: rabbit P11974)[a]	57916	N.H	N.H	N.H
4	Enolase B muscle isoform (PAN: human P13929)[a]	46856	N.H	H	N.H
5	Phosphoglycerate kinase 1 (PAN: mouse P09411)[a]	44405	H	H	N.H
6	Creatine kinase, M chain (PAN: pig Q5XLD3)	43059	H	H	N.H
7	Fructose-bisphosphate aldolase A (PAN: rabbit P00883)[a]	39212	N.H	N.H	N.H
8	L-Lactate dehydrogenase muscle isoform (PAN: pig P00339)	36487	P.H[c]	H	N.H
9	Glyceraldehyde-3-phosphate dehydrogenase (PAN: pig P00355)	35795	H	H	H
10	Phosphoglycerate mutase isozyme M (PAN: human P15259)[a]	28635	N.H	N.H	N.H
11	Carbonic anhydrase (PAN: pig Q5S1S4)	29411	N.H	N.H	N.H
12	Triosephosphate isomerase (PAN: human P60174)[a]	26538	N.H	N.H	N.H
13	Myoglobin (PAN: pig P02189)	16953	P.H	H	N.H

[a] H, hydrolysed.
[b] N.H, Not hydrolysed.
[c] P.H, Partially hydrolysed.

sarcoplasmic protein spots were less intense than the analogous in the 2D map of fresh meat, indicating that a slight hydrolysis occurred. "Coppa" is a sacked entire anatomical part, neck muscles; in the proteolysis the main role should be played by the endogenous enzymes, whereas microbial enzymes probably act only on the surface. Sarra et al. [23], in fact, demonstrated that in Culatello, a similar product of sacked entire meat portion, microflora (*Staphylococcus xylosus*) is distributed in the peripheral zone, forming a biofilm under the creasing. Therefore, the whole product underwent proteolysis from endogenous enzymes. Our result clearly demonstrated that glycogen phosphorylase is a substrate of both endogenous enzymes and bacterial proteinases, whereas the neoformed spot could be a fragment deriving from hydrolytic action on this protein. In Table 2, identified sarcoplasmic proteins undergoing to proteolysis and surviving proteins during ripening have been schematised. Taken into account differences in the microbiological properties of the examined pork products, comparison of 2D AUT-PAGE/SDS-PAGE maps, assessing proteolysis, led us to

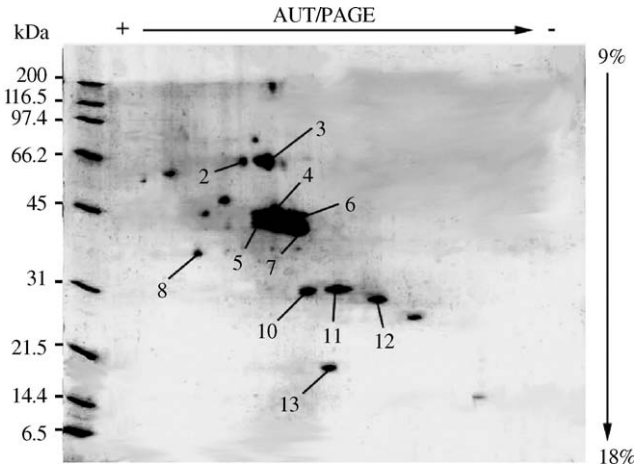

Fig. 5. Two-dimensional AUT-PAGE/SDS-PAGE analysis of sarcoplasmic proteins from 3-months aged "Coppa". Proteolysis is probably carried out by microbial enzymes on the surface, in a very limited extend, and by endogenous muscular enzymes in the internal part of he product.

infer that endogenous enzymes acted on sarcoplasmic proteins slower than microbial enzymes, and only in a secondary phase of the ripening process.

However, to confirm these preliminary results, a study on intermediate and prolonged ripening seasoning times for "Coppa" should be necessary.

4. Conclusion

A remarkable contribution to the questioned issue concerning the role played by the endogenous enzymes and the lactic bacteria enzymes in sarcoplasmic fraction of meat protein hydrolysis was reached by proteomic approach using 2D AUT-PAGE/SDS-PAGE and MALDI-ToF/MS. The achieving of a higher resolution in the separation of water-soluble proteins from fresh pork and dry-cured products and an easier interpretation of two-dimensional maps has allowed to assess that sarcoplasmic proteins are a substrate of microbial enzymes. Moreover, in each of ripened products the difference in proteolysis events were well defined and are here summarized:

- disappearance of glycogen phosphorylase and appearance of a polypeptide fragment of 14 kDa in "Coppa";
- hydrolyis of phosphoglycerate kinase 1, creatine kinase M, L-lactate dehydrogenase muscle isoform and glyceraldehyde-3-phosphate dehydrogenase as well as glycogen phosphorylase muscle form and appearance of two spots with 37 and 38 kDa as well as the spot of 14 kDa in dry-cured ham;
- hydrolyis of enolase B and of other proteins, the same as dry-cured ham but lacking of spots with molecular weight around 37–38 kDa in dry-cured sausages, in "Naples-type" salami.

These differences can be put in evidence by image analysis and creation of data bank could be important to safeguard typical products.

Finally, the 2D AUT-PAGE/SDS-PAGE technique, could be extended to achieve separations having high resolution of complex mixtures of basic proteins.

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