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# Proteomic profile of dry cured ham adapted to low salt content

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**Abstract.** Proteomic profiles of Bayonne dry-cured ham with reduced salt content (20% approximately) was established and compared to normal ones. For each animal, one ham followed the traditional salting procedure (11 to 13 days) while the controlateral was salted for a shorter period (8 to 11 days) according to the weight of hams. The non soluble protein fraction from *Biceps femoris* muscles was studied after 12 months processing. Proteomic analysis (two-dimensional electrophoresis, image analysis and mass spectrometry identification) were performed on the 120 hams. 32 spots were significantly affected by salt; among them, 12 proteins and fragments were identified by MALDI TOF. Eight proteins or fragments belongs to the sarcoplasmic proteins (creatine kinase, glycogen phosphorylase, piroxiredoxine,  $\alpha$  crystalline,..) and the 4 remaining were 3 isoforms of actine and one actine fragment. Specific pattern was associated to salt content in Bayonne dry cured ham and we demonstrated that salt modified the solubility properties of the sarcoplasmic proteins during processing.

**Keywords.** Dry-cured ham – Proteolysis – Proteomic profile – Salt.

## **Carte protéomique du jambon de Bayonne à teneur réduite en sel**

**Résumé.** Nous avons établi des cartes protéomiques à partir du jambon de Bayonne fabriqué avec une teneur réduite en chlorure de sodium. Pour ce faire, pour chacun des porcs (n=60), un jambon a été salé normalement, à raison de 11 à 13 jours de salage selon son poids, tandis que son controlatéral était salé pendant un moindre temps (de 8 à 11 jours selon le poids). Nous avons étudié la fraction protéique myofibrillaire des muscles *Biceps femoris* des jambons de Bayonne ayant 12 mois de séchage. Les analyses protéomiques (électrophorèse bidimensionnelle, analyse d'image et identification des spots d'intérêt par spectrométrie de masse) ont été réalisées sur les 120 jambons. Au total 32 spots varient significativement en fonction de la teneur en sel. Parmi eux, 12 protéines et fragments protéiques ont pu être identifiés par MALDI TOF, 8 correspondent à des protéines ou fragments protéiques sarcoplasmiques (créatine kinase, glycogène phosphorylase, peroxyrédoxine,  $\alpha$ crystalline, etc.) et les 4 autres sont des molécules d'actine et un fragment d'actine. Nous avons montré que la teneur en sel dans le jambon de Bayonne est associée à un profil protéomique particulier et qu'elle modifie sensiblement les propriétés de solubilité des protéines sarcoplasmiques.

**Mots-clés.** Jambon sec – Protéolyse – Carte protéomique – Sel.

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## **I – Introduction**

Bayonne ham enjoys EU Protected Geographical Indication (PGI) status. This certification requires professional processors to comply with specifications that provide the consumer with a finished product of optimal quality. The curing technology is based on the addition of salt on the ham internal surface, which acts as a preserving agent but is also responsible for causing physico-chemical and biochemical phenomena that contribute to development of the textural properties. Salt affects muscle proteins by inducing denaturation (Adamsen *et al.*, 2006; Graiver *et al.*, 2006) in which the extent depends on salt concentration (Thorarinsdottir *et al.*, 2002), and

processing yields (Santé-Lhoutellier *et al.*, 2009). Proteolysis in dry-cured ham occurs throughout processing, but at different rates and to varying extents depending on salt penetration and water migration. This difference implies greater proteolytic activity in the *biceps femoris* muscle compared to *semi membranosus* muscle, which will affect its texture (Parolari *et al.*, 1994; Virgili *et al.*, 1995; Rosell and Toldra, 1998; Virgili *et al.*, 1998). Proteolytic activity on dry-cured ham proteins is essentially attributed to cathepsins, which act for a longer time (Toldrà and Etherington, 1988; Toldrà and Flores, 1998).

However, in the first stage of processing, classical muscle ageing occurs, when calpains can also act. Skrlep *et al.* (2010) demonstrated the importance of pH of green ham on proteomic profile, lower pH favouring cathepsin activities. The time course of myofibrillar protein hydrolysis during the ripening process has been studied by one-dimensional gel electrophoresis (Toldrà *et al.*, 1997; Toldrà *et al.*, 1993; Monin *et al.*, 1997) and recently using protein labchip (Theron *et al.*, 2009) and by two-dimensional gel electrophoresis (Di Luccia *et al.*, 2005). Myosin heavy chains (MHC), myosin light chains (MLC1 and MLC2) and troponin C and I are targets of proteolysis. In the soluble fraction, the presence of tropomyosin shows that solubility properties have changed, possibly due to environmental conditions such as salt. In their study on the insoluble protein fraction of *semi membranosus* and *biceps femoris* muscles, Theron *et al.* (2011) noticed numerous of soluble proteins in the proteomic pattern of *semi membranosus*, indicating changes in solubility due to denaturing caused by the salt. We aimed to determine the proteomic pattern of insoluble protein fraction from *biceps femoris* muscle undergoing two salting conditions.

## II – Material and methods

The study was based on a total of 60 pigs fed a cereal-based diet (60-80%), slaughtered at the Lahontan abattoir, and selected to meet the processing specifications of PGI Bayonne ham. The processing of Bayonne hams, which lasts 12 months in this experiment, was carried out at the Pyragena experimental station using the following sequence: salting, settling, oven drying, air drying, grease covering and ripening (Robert *et al.*, 2005). Sampling was carried out at 12 months, at the end of ripening. Proteomic analysis was performed on *biceps femoris*.

### 1. Insoluble protein extraction and electrophoresis

The method was adapted from Sayd *et al.* (2006). Frozen muscle was homogenized, using a glass bead agitator MM2 (Retsch, Haan, Germany), in 40 mM Tris HCl (pH 8) at 4°C in a ratio of 1:8 (w/v). The homogenate was centrifuged at 4°C for 10 min at 10,000 g. The supernatant was removed. The pellet was washed five times with this buffer to obtain only insoluble protein in low ionic strength buffer. After the last centrifugation, the supernatant was removed and the pellet was homogenized in 7 M urea, 2 M thiourea, 4% CHAPS (w/v), 1% DTT (w/v), at 4°C in the same ratio as the first step. The homogenate was centrifuged at 4°C for 10 min at 10,000 g. The supernatant, forming the insoluble protein fraction, was stored at -80°C. The protein concentration was determined by the RC-DC assay (Bio-Rad). First 1 mg of proteins was incorporated in a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.4% carrier ampholyte (v/v), 1% DTT (w/v), and bromophenol blue.

Samples were loaded onto immobilized pH gradient strips (pH 3-10 NL, 17 cm, Bio-Rad), and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad). Gels were passively rehydrated for 16 h. Rapid voltage ramping was subsequently applied to reach a total of 86 kVh. In the second dimension, proteins were resolved on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels using a Protean II XL system (Bio-Rad). Gels were stained with Coomassie Blue (colloidal blue). Three gels were produced per sample, giving 180 gels in all.

## 2. Image analysis and statistical treatment of data

Gels were visualized and analysed using the two-dimensional electrophoresis (2DE) image analysis software Samespots (non-linear dynamics). Aligned spots were normalized by expressing the relative quantity of each spot as the ratio of individual spot quantity to the total quantity of valid spots. Relative quantities were expressed in ppm. For one sample and one spot, the mean of three values (corresponding to the gels in triplicate) was calculated. The resulting set of average spot quantities underwent a one-way analysis of variance (ANOVA) using XLSTAT. A spot was considered significant when associated with  $p < 0.05$  in ANOVA.

## 3. Protein identification by mass spectrometry

Was performed according to the method described by Theron *et al.* (2011). Coomassie stained spots of interest were manually excised using pipette tips. The spots were then destained, dehydrated and digested by trypsin. Peptide Mass Fingerprint (PMF) of trypsin digested spots were determined in positive-ion reflector mode using a Voyager DE Pro MALDI-TOF-MS (Applied Biosystems, Courtaboeuf, France ). PMFs were compared to SwissProt (01/2008, 290 484 seq) protein sequence databases ([ftp://ftp.ebi.ac.uk/pub/databases/uniprot/knowledgebase/uniprot\\_sprot.fasta.gz](ftp://ftp.ebi.ac.uk/pub/databases/uniprot/knowledgebase/uniprot_sprot.fasta.gz)) using MASCOT 2.2 software [<http://www.matrixscience.com>]. When identification by MALDI-TOF proved unsuccessful, identification was also attempted using nano LC-ion trap MS/MS analysis. Identification of peptides was performed with Mascot 2.2, restricting the taxonomy to *mammalia* (20080417, 1177111 sequences) in the protein NCBI database.

## III – Results and discussion

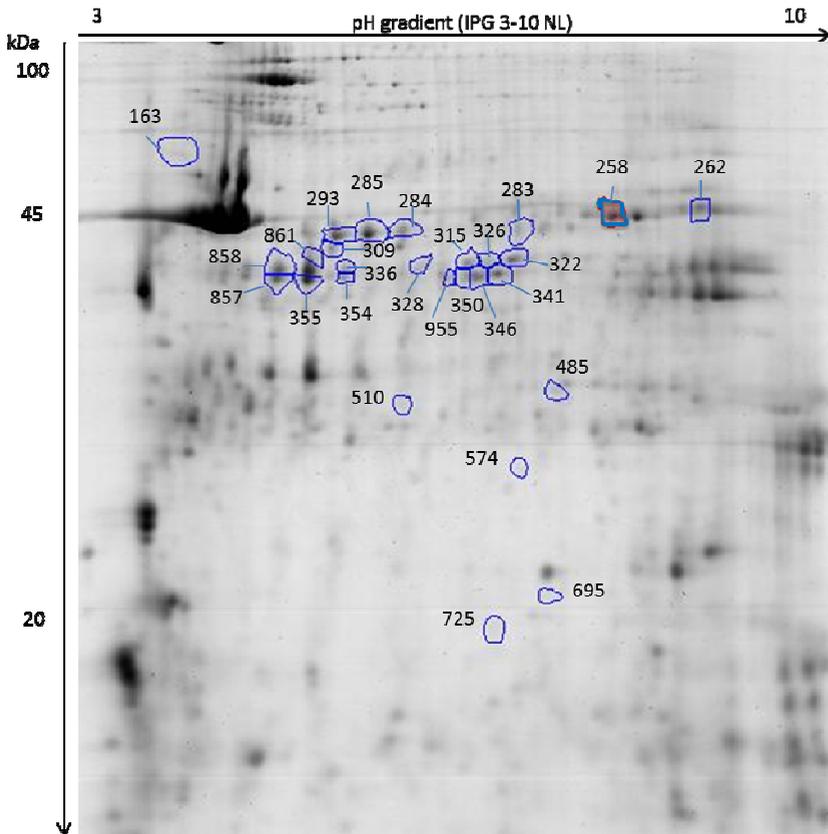
Table 1 presented the protein identified by mass spectrometry. Proteins can be grouped according their biological function and some of presented different isoforms. 32 spots were significantly affected by salt; among them, 12 were entire proteins or protein fragments. Eight proteins or fragments belongs to the sarcoplamic proteins (creatine kinase, glycogen phosphorylase, peroxiredoxin,  $\alpha$  crystalline, etc.).

**Table 1: List of the spots identified by mass spectrometry (MALDI-TOF or LC/MS/MS)**

Spot #	NCBI n°	Protein identity	Mascot score	%SC†	Matched peptides	Theoretical M/pi	Estimated M/pi	Conclusion
355	gij790202	Skeletal alpha actin	113	38%	15	42024/5.23	37500/4.8	fragment
284	gij790202	Skeletal alpha actin	85	33%	11	42024/5.23	37500/6.6	entire
285	gij790202	Skeletal alpha actin	76	32%	12	42024/5.23	40000/6.0	entire
309	gij790202	Skeletal alpha actin	91	24%	8	42024/5.23	40000/5.0	entire
258	gij194018722	Muscle creatine kinase	162	45%	19	43032/6.61	42000/8.5	entire
283	gij194018722	Muscle creatine kinase	146	44%	17	43032/6.61	39000/7.1	fragment
315	gij194018722	Muscle creatine kinase	71	17%	6	43032/6.61	38000/6.8	fragment
322	gij113205498	Enolase 3	79	39%	13	47100/8.05	38000/7.0	fragment
574	gij106073338	Muscle glycogen phosphorylase	60	18%	14	83983/6.10	30000/7.0	fragment
695	gij75063982	Alpha-crystallin B chain	65	38%	6	20116/6.76	20000/7.2	entire
510	gij194044822	Similar to peroxiredoxin 4	88	46%	10	30536/6.01	30000/6.4	entire

†%SC corresponds to the percentage of sequence coverage.

Figure 1 presented the gel and the localisation of proteins associated to salt content. Some are involved in energy metabolism. Creatine kinase (spots 258, 283, 315), participates in energy transduction. Enolase is a glycolytic enzyme, responsible for the catalysis of the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), the penultimate step of glycolysis (spot 322). Glycogen phosphorylase (spots 574), is involved in carbohydrate metabolism. Peroxiredoxin is an antioxidant enzyme (spot 510) and  $\alpha$  crystallin has chaperone-like properties including the ability to prevent the precipitation of denatured proteins. Two soluble proteins ( $\alpha$ -crystalline and peroxiredoxine) presented higher quantity in low salt ham compared to the contralateral which had normal salt.



**Fig. 1. Master gel and spots varying according to salt content.**

The 4 remaining identified proteins were 3 isoforms of actin (spots 285, 284, 309) and one actin fragment (355). Numerous spots remained not identified. The actin spots revealed that the protein was not hydrolysed and exhibited higher quantity in normal salt ham. This could be due to a possible protection of the denatured sarcoplasmic proteins of the complex actomyosin as it has been demonstrated in fresh meat. The insoluble fraction of protein revealed the presence of soluble proteins which may be explained by rapid denaturing due to salt, leading to precipitation onto the myofibrillar proteins and a jointed extraction. However In Iberian hams, Cordoba *et al.*, (1994) reported that the loss of extractibility still occurred during ripening. The dry cured ham having normal salt content (6.1%) showed higher entire actin and less

antioxidant/chaperone proteins than the low salt content dry cured ham (5.3%). The fact that non hydrolysed proteins are still present after 12 months suggested that salt or co localization of soluble with myofibrillar proteins would bring a protection from the action of proteases.

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