OMIC STUDIES ON MIOFIBRILLAR PROTEINS HYDROLYSIS DURING FERMENTATION WITH AN AUTOCHTHONOUS STARTER CULTURE

C. M. López[1], M. A. Sentandreu[2], G. M. Vignolo[1] and S. G. Fadda[1]

[1] CERELA- CONICET, San Miguel de Tucumán, Argentina

[2] IATA-CSIC, Valencia, Spain

Abstract – This study focuses on meat protein degradation during fermentation of a sausage model in order to investigate the effect of a mixed autochthonous starter culture on the hydrolysis of myofibrillar proteins. This analysis was carried out by applying proteomic and peptidomic approaches. Beaker sausage models were prepared and inoculated or not with *L. curvatus* CRL705 and *S. vitulinus* GV318. The hydrolysis of actin, myosin light chain 1/3 (MLC 1/3), myosin regulatory light chain-2 (MRLC-2) and myosin heavy chain (MHC) was elucidated by two-dimensional gel electrophoresis (2-DE). Peptides arising from troponin T, MRLC and particularly from actin were identified by Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS). These results showed that the applied starter culture exerted significant changes on these myofibrillar proteins, generating more complex peptide patterns when compared with non-inoculated samples. Results also revealed three regions of actin to be more susceptible to hydrolysis in presence of the starter culture as well as the essential role of exopeptidases during fermentation. These findings establish a solid background to further investigations about the role of starter culture in the uniqueness of fermented sausages and on the use of small peptides as quality biomarkers.

Key Words – Fermented sausages, Meat Proteins, Proteolysis, Peptidomics, Proteomics.

I. INTRODUCTION

The development of autochthonous meat starter cultures is a valuable tool to improve fermented products processing. These cultures can increase its overall quality while preserving the characteristics of the products made by artisanal technologies. Then, a starter culture comprised by two strains isolated from artisanal local products, *Lactobacillus curvatus* CRL705 and *Staphylococcus vitulinus* GV318, was designed in previous studies based on its safety and technological properties [1]. It is widely known that an extensive meat proteolysis is conducted during fermentation of these types of products, targeting both myofibrillar and sarcoplasmic fractions. However, little information is available regarding global studies on fermented meat products and the action of starter cultures on myofibrillar proteins is not clearly elucidated. Therefore, the aim of this study was to obtain the whole map of the hydrolysis of myofibrillar proteins using two-dimensional electrophoresis (2-DE) and Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) in view to determine the involvement of this promising autochthonous meat starter culture in the final product quality.

II. MATERIALS AND METHODS

Strains and culture conditions

L. curvatus CRL705 and *S. vitulinus* GV318, isolated from artisanal fermented sausage (Tucumán, Argentina) were selected based on previous results [1-2] and routinely culture in Mann Rogosa Sharpe (MRS) or Brain Heart Infusion (BHI) broth (Britania, Argentina), respectively.

Beaker sausage models (BS)

Beaker sausages (BS) models were prepared as follows: beef muscles were aseptically sampled and then, one kg of meat was thoroughly minced and mixed with the curing additives: 3% NaCl, 0.02% NaNO2, 0.75% sucrose and 0.75% glucose, previously filtered-sterilized (0.22 µm) (Millipore, USA). The batter obtained was separated into three portions: (i) BS-control that involved antibiotics: 20.000 UI/Kg penicillin, 20 mg/Kg streptomycin and 50 mg/Kg amphotericin B (Gibco, USA); (ii) BS-Lc inoculated with *L. curvatus* CRL705 (7-8 log CFU/g); and (iii) BS-Mx inoculated with both strains (7 and 6 log CFU/g for *L. curvatus* CRL705 and *S. vitulinus* GV318, respectively). Flasks containing the BS were incubated at 22 ºC. Three independent replicates were performed for each BS model. The growth and evolution of the starter culture was

followed by plating onto MRS agar and MSA and pH measuring.

Myofibrillar protein extraction and two-dimentional electrophoresis (2-DE)

Extraction of myofibrillar proteins was adapted from the method followed by Therón *et al.*[3]. For 2-DE, the samples loading, isoelectric focusing, SDS-PAGE and gel staining were performed as described by Fadda *et al*. [4]. Briefly, image analysis was carried out by using Prodigy SameSpot software (Progenesis, Non linear) submitting to a one-way analysis of variance (ANOVA) considering significant differences when associated with $p < 0.05$.

Differential spots were identified by applying a spectrometer MALDI- ToF-ToF Ultraflex II (Brunker, Germany) in the CEQUIBIEM Institute (Argentina). Results were analyzed by MS-Tagged software using Uniprot database and matches were manually verified.

These studies were carried out in two technical replicates on protein extracts from two biological replicates of each sample counting 16 gels.

Peptide extraction and sequence identification by LC-ESI-MS/MS

Ten grams from each BS sample were homogenized with 0.1N HCl (1:5 w/v). The meat slurries were centrifuged (13500 rpm at 4 ºC for 20 min) and supernatants submitted to ultra-filtration in Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-3 membrane (Millipore, USA). The obtained filtrate was freeze-dried until LC-ESI-MS/MS analyses were done.

Samples were re-dissolved, injected and separated as reported by Sentandreu *et al*. [5]. A Surveyor LC system directly coupled to a LCQ Advantage Ion trap MS instrument (Thermo Scientific, USA) and a Jupiter Proteo reverse phase column (150 x 0.5 mm; Phenomenex, USA) were used for this study. Data acquisition was done using the Xcalibur v2.0 software. Peptide identification was obtained from the MS/MS spectral data using an in-house version of the Mascot search engine v2.3 and Uniprot KB protein database. Only top ranking significant peptides were considered, taking a reference peptide score threshold of 25. Selected identifications were manually checked concerning the assignation of the identified masses to *b* and *y* ions series.

III. RESULTS AND DISCUSSION

Hydrolisis of myofibrillar proteins based on 2-DE analysis.

Changes in myofibrillar proteins during the incubation of both inoculated and non-inoculated sausage models were evaluated. BS-control at initial time (t0) as well as BS-Lc and BS-Mx at 10 days of incubation (t10) were analyzed by 2-DE. Results showed that from approximately 20 spots detected in each gel, 15 exhibited significant differences when ANOVA ($p<0.05$) was applied. These 15 spots were excised and 8 of them (spots n° 1, 2, 3, 5, 6, 11, 12, 14) were accurately identified by MALDI-Tof-Tof (Table 1).

Table 1: Identified spots by MALDI-ToF-ToF

Spots n°	MW (kDa); pI estimated	Score	Expected Mass (Da)	Protein name
1	40; 5.2	138	42340	α actin
2	17; 4.5	143	19721	MLC-1
3	116; 5.2	82	223180	MHC
5	40; 5	162	42340	α actin
6	23; 5	106	17057	MLC 1/3
11	45;5	124	42451	α actin
12	40; 4.9	170	42338	α actin
14	36; 5.5	83	42451	α actin

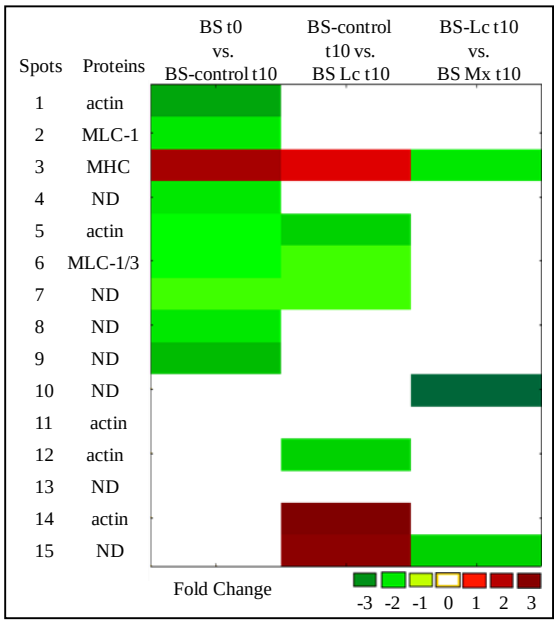
MW: molecular weight; pI: isoelectric point; MLC: myosin light chain; MHC: myosin heavy chain

These protein spots had been originated from 3 proteins: α actin, myosin heavy chain (MHC) and myosin light chain (MLC), which constitute the major fraction of myofibrillar proteins in the muscle. By comparing the theoretical and the observed molecular weights (Table 1), it could be suggested that spots n° 3 and n° 14 represented large fragments of MHC and α actin, respectively. In this case, they are considered neospots as these polypeptides were visualized only after incubation (t10) on the inoculated models (BS-Lc and BS-Mx). The other spots, with decreasing intensities during incubation, were in the range of the expected mass of their whole proteins; even when some slight degradation or post-transcriptional modification probably occurred.

In view to compare the relative abundances of the statistically significant spots, a plot was constructed (Fig. 1). The differences between two models for each spot are represented with a colour code. The red and green scales represent spot

increase or reduction between two models, respectively. The grater colour intensity for each scale, the greater change in abundance for the models considered.

Figure 1. Changes in relative abundance of the spots in the different BS models



All represented changes in relative abundance are statistically significant ($p<0.05$). ND: non-determined. Fold change: negative values (lesser abundance); 0 (no differences); positive values (higher abundance).

As can be clearly observed, α actin, MHC and MLC were hydrolyzed in the non-inoculated model (BS-control), after 10 days of incubation. However, its degradation was more remarkable when *L. curvatus* CRL705 was present. Moreover, its combination with *S. vitulinus* GV318 promoted the breakdown even of the MHC fragment, which was not registered in the other conditions.

Analysis of small peptides (<3 kDa) arising from myofibrillar proteins during incubation

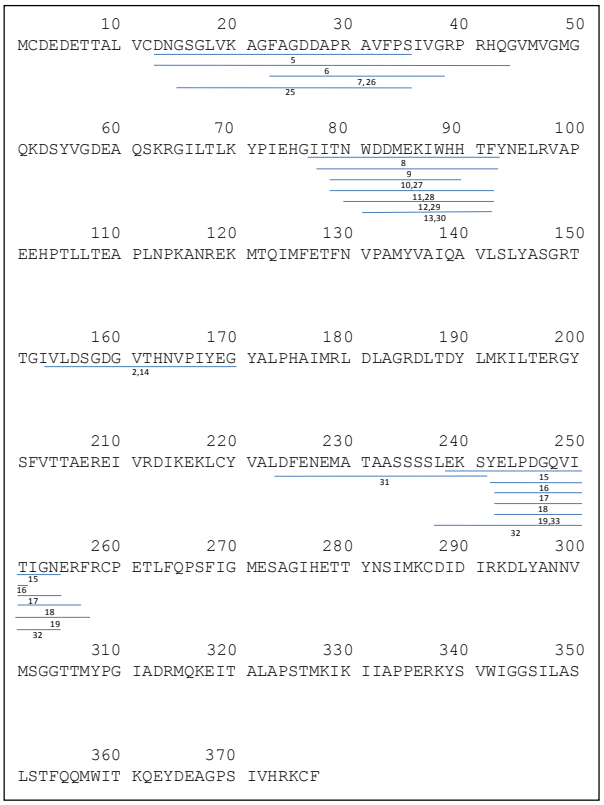
In order to identify peptides smaller than 3kDa originated from myofibrillar proteins in sausage models, LC-ESI-MS/MS was applied to BS-control, BS-Lc and BS-Mx at both initial time (t0) and 10 days of incubation (t10) by duplicate from two different batches. A total of 33 peptides from the myofibrillar fraction were identified in all the samples studied, most of them (25) originated from α actin. The rest of fragments derived from Myosin Regulatory Light Chain-2 (MRLC-2) (7) and from Troponin T (1) (Table 2). When comparing among models, BS-Lc after incubation (t10) exhibited the highest number of identified peptides. In fact, only three peptides from myofibrillar proteins were detected in BS-control t10; it might be suggested that endogenous peptidases exerted a slight hydrolysis on these proteins, as in 2-DE studies. Hence, it can be postulated that the inoculation of *L. curvatus* CRL705 notably enhances myofibrillar proteolysis, enriching the peptide composition of the meat system, although the complete mechanism of protein degradation for the different BS models remains to be elucidated.

Table 2 Sequences of identified peptides

N°	Sequence of identified peptides	Parental protein	Sample (BS)
1	APPPPAEVPEVHEEVH	Troponin T	t0
2	IVLDSGDGVTHNVPIYEG	α actin	control t10
3	TVIDQNRDGIIDKEDLRDTF	MRLC-2	control t10
4	NVKNEELDAMMKEASGPIN	MRLC-2	control t10
5	DNGSGLVKAGFAGDDAPRAVFPS	α actin	Lc t10
6	DNGSGLVKAGFAGDDAPRAVFPS IVGRPRHQG	α actin	Lc t10
7	FAGDDAPRAVFPSIVG	α actin	Lc t10
8	IITNWDDMEKIWHHTF	α actin	Lc t10
9	ITNWDDMEKIWHHTF	α actin	Lc t10
10	TNWDDMEKIWHH	α actin	Lc t10
11	TNWDDMEKIWHHTF	α actin	Lc t10
12	NWDDMEKIWHHTF	α actin	Lc t10
13	WDDMEKIWHHTF	α actin	Lc t10
14	IVLDSGDGVTHNVPIYEG	α actin	Lc t10
15	EKSYELPDGQVITIGN	α actin	Lc t10
16	YELPDGQVIT	α actin	Lc t10
17	YELPDGQVITIGN	α actin	Lc t10
18	YELPDGQVITIGNER	α actin	Lc t10
19	YELPDGQVITIGNERF	α actin	Lc t10
20	NVKNEELDAMMKEASGPIN	MRLC-2	Lc t10
21	NVKNEELDAMMKEASGPINF	MRLC-2	Lc t10
22	NEELDAMMKEASGPIN	MRLC-2	Lc t10
23	PEDVITGAFKVLD	MRLC-2	Lc t10
24	FPPDVGGNVDY	MRLC-2	Lc t10
25	GSGLVKAGFAGDDAPRAVFPS	α actin	Mx t10
26	FAGDDAPRAVFPSIVG	α actin	Mx t10
27	TNWDDMEKIWHH	α actin	Mx t10
28	TNWDDMEKIWHHTF	α actin	Mx t10
29	NWDDMEKIWHHTF	α actin	Mx t10
30	WDDMEKIWHHTF	α actin	Mx t10
31	DFENEMATAASSSSLEKS	α actin	Mx t10
32	LEKSYELPDGQVITIGN	α actin	Mx t10
33	YELPDGQVITIGNERF	α actin	Mx t10

However, when *S. vitulinus* GV318 was also present (BS-Mx t10), certain differences in peptide pattern as well as a reduction in the diversity of peptides were observed. These results, combined with 2-DE studies, might indicate that the < 3 kDa peptides were massively degraded to smaller peptides and free amino acids and eventually consumed by the microorganisms. With regards to the peptides released from α actin (Fig. 2), most of them came from three protein regions: 12- 43, 75-92 and 238-255. In addition, several peptides from these regions were common to the different models. For instance, the peptide IVLDSGDGVTHNVPIYEG was detected in both BS-control t10 (peptide n°2) and BS-Lc t10 (peptide n°14). The cleavage sites shown in Figure 2 suggested that exopeptidases might be the main responsible of this peptide diversity.

Figure 2. Peptide map of α actin as regards from the identified sequences



Complete sequence of α actin. Identified peptides are represented by lines and its numbers are in coincidence with Table 2.

Additionally, although several peptides from myofibrillar proteins such as actin, MLC-1 and 2, troponin T and titin were already reported in other meat products [6], this study constitutes the first evidence of their occurrence in fermented meat products.

IV. CONCLUSION

This study brings a global insight of the proteolysis during meat fermentation and emphasizes the role the starter cultures. Actually, the autochthonous strains had a significant effect on proteins and peptides that might contribute to the uniqueness of fermented sausages. The myofibrillar fraction was clearly cleaved, especially α actin, when *L. curvatus* CRL705 was present. Also, in the light of this data, muscle and microbial exopeptidases could be the main responsible for the higher peptide diversity observed in the inoculated models. However, further studies are necessary to determine if the peptides identified herein may be used as biomarkers of the applied technology.

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