# ORGANIC ACID EFFECTS OF PROCESSING TIME ON PROTEOLYTIC CHANGES IN SEMI AND DRY SAUSAGES

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***Abstract* - Proteolytic changes in semi and dry sausages produced by two methods were determined during processing and storage for 60 days. The sausages were produced with or without organic acids in both methods. Lactic acid was used as organic acids for their acidic and proteolytic characteristics. The major changes in proteolytic characteristics of semi and dry sausage took place during the processing stage. A remarkable accumulation of free amino acids was detected in dry sausage. Higher level was in the sample with lactic acid of dry sausage, but with differences being significant for long ripened products. Proteolytic activity was observed in both with and without organic acids sausages during processing. Moreover, slight increases in proteolytic activity was detected during storage in both of organic acids added and control dry sausages, and also in semi dry sausages due to some heat-resistant proteolytic enzymes. Sarcoplasmic and myofibrillar proteins were also affected by addition of organic acids, fermentation, drying and heat processing. During fermentation, organic acid and control sausages showed intense proteolysis in both the dry and semi dry processing methods. After heating, intensive degradation of both sarcoplasmic and myofibrillar proteins due to denaturation was observed in semi dry processed samples. Bacterial proteinases contribute to the degradation of these proteins and which resulted the development of semi and dry sausage characteristics. Thus, use of organic acid in dry sausages resulting a safer product.**

**Key words: Lactic Acid, Proteolysis, Amino acid, Semi dry sausage.**

## INTRODUCTION

The meats products are traditional products have long history and an inestimable gastronomic value. Preservation is usually achieved by a combination of fermentation using water activity-lowering techniques including dehydration and addition of salt. These techniques have been the basis of traditional technologies used before the scientific techniques were understood (Campbell-Platt, 1995). All these transformations are influenced by ripening conditions, raw meat and ingredients and have a considerable effect on the organoleptic quality of fermented meat products. Breakdown products of lipolysis and proteolysis, i.e. peptides, amino acids, carbonyls and volatile flavor compounds contribute to the characteristic flavor and texture of fermented meats (Diaz, Fernández, García de Fernando, De la Hoz, & Ordóñez, 1997). Several biochemical and physical changes occur during the ripening of fermented sausages that determine the flavour and odour of the end product. These changes are mainly acidification as a result of fermentation, pH decrease, and changes in initial microflora, reduction of nitrates to nitrites and formation of nitrosomyoglobin, solubilisation and gelification of myofibrillar and sarcoplasmic proteins, proteolytic, lipolytic and oxidative phenomena, and dehydration (Casaburi, Aristoy, Cavella, Di Monaco, Ercolini & Toldra, 2007). Proteolysis of dry cured meat products has been attributed mainly either to endogenous enzymes (Toldra, 1998) or to exogenous enzymes originating from microorganisms (Mauriello, Casaburi & Villani, 2002). Nowadays, the consumer pays a lot of attention to the relationship between food and health. Therefore, Organic acids have a long history of application as food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredients. The aim of this study was to determine the proteolytic changes during processing and storage in sausages produced with or without organic acid. Two different production methods were compared, semi dry sausage and dry sausage.

## II. MATERIALS AND METHODS

### *A.* Preparation of Sausages

The sausage formulation included lean pork (85%) and pork back fat (15%) and the following ingredients (in g/kg of meat mixtures) were added: sodium chloride (15), glucose (5), pepper (3.5), coriander (1.5), all spice (1.5), cardamom (0.5), bay leaves (0.5) and sodium nitrite (0.1). The meat and fat were minced using a cutter and chopped for 2 min and mixed with the ingredients. After mixing, the sausage mixture was divided into four batches. Two of these were without organic acid, two of these with 0.30% (v/w) of lactic acid by (Kanto Chemical Co. INC Japan). The mixtures were stuffed into fibrous casing, at approximately 190 g each. The condition of semi dry sausage processing include drying 60°C, 60 min, smoke 60°C, 70 min, cooking 60°C, 70 min after that drying during 15°C, 7 days. Total processing time was 7 days for semi dry method; and the condition of dry sausage were ripened at 80% relative humidity (RH) and 27ºC, for 2 days , followed for 3 days at 80% RH and 20ºC, then for 4 days at 75% RH and 15ºC and finally for 12 days at 65% RH and 15ºC. Total processing time was 21days for the dry sausage method. The both sausages were vacuum packaged and stored at 4 °C for 60 days. Samples were taken at 0, 7 and 60 days of semi dry sausage; at 0, 21 and 60 days of dry sausage during ripening and storage.

### *B.* Microbiological analysis

Ten grams from each sample aseptically transferred into a sterile plastic bag and homogenized with 90 ml of sterilized saline (0.85% NaCl). The homogenate was prepared using a Stomacher (Exnizer 400, Organo Co., Tokyo, Japan), prior

to the preparation of 1/10 serial dilutions for microbiological analysis. The Total viable Count (TVC) determined using Plate Count Agar (Eiken Chemical Co Ltd., Japan) and incubating at 37°C for 48 h; Lactic Acid Bacteria Count (LAB) determined by Man Rogosa Sharpe agar (Oxoid Ltd., UK) and incubating at 37°C for 72 h. Tests were carried out in duplicate and the results were expressed as log cfu/g.

***C.*** **pH and moisture analysis**

pH was determined in slurries made from 10 g samples in 90 ml distilled water homogenized in an Stomacher (Exnizer 400, Organo Co., Japan) using a pH meter model HM-5S (DKK-TOA Co., Japan). Moisture contents were determined by Moisture analyzer (MX-50, A&D Co., Japan).

***D.*** **Amino acid analysis**

Samples (10 g) were homogenized (Physcotron NS 51, Microtec Co., Ltd., Japan) for 1min with 90 ml of distilled water. The homogenate was centrifuged for 20 min at 10,000 *g* at 0°C. The supernatant was filtered through Toyo filter No. 5C (ADVANTEC MFS, Inc., USA) and the samples were diluted 1:1 with 4% trichloroacetic acid (TCA) to give a final concentration of 2%. They were then incubated at 37°C for 30 min. The supernatant was filtered again with Toyo filter No. 5C. Thereafter, the solution was ultrafiltered through a Millipore filter having a pore diameter of 0.45 µm (W-13-5, Tosoh Co., Japan). Twenty microlitres of each sample were analyzed using a fully automated amino acid analyzer (L-8800A, Hitachi Ltd., Japan).

***E.*** **Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)**

Sarcoplasmic protein extracts were prepared according to the method described previously (Aro Aro *et al* 2010). Four grams of sausage samples were homogenized (Physcotron NS 51, Microtec Co., Ltd., Japan) with 40 ml of 0.03 M potassium phosphate buffer (pH 7.4) for 2 min at 13,500 rpm. The homogenate was centrifuged for 20 min at 10,000*g* at 4 °C. The supernatant contained the sarcoplasmic proteins. Myofibrillar proteins were extracted from the resultant pellet by homogenizing (Physcotron NS 51, Microtec Co., Ltd., Japan) with a solution containing 8 M Urea and 1% (w/v) β-mercaptoethanol for 2 min. The homogenate was re-centrifuged under the same conditions and the supernatant contained the myofibrillar proteins. The protein concentrations of the sarcoplasmic and myofibrillar fraction were determined by Bradford (1976) procedure. The protein concentration was adjusted with deionised water to give a final concentration of 6 mg/ml. Samples were diluted 1:1 with SDS–PAGE sample buffer to give a final concentration of 3 mg/ml and heated at 100°C for 5 min prior to electrophoresis. SDS-PAGE was done using a vertical gel electrophoresis unit (Mini-Protean 3 Cell., Bio-Rad, USA). According the method previously described, Laemmli (1970) a 12.5% separating gel with 6% stacking gel was used for sarcoplasmic and myofibrillar proteins. Ten microlitres of the sample were injected in each well including standard markers. Electrophoresis was done at 120 - 150 V. After electrophoresis was completed, the gels were stained with 0.1% (w/v) Comassie Brilliant Blue R-250, 30% (v/v) methanol and 10% (v/v) acetic acid. The gels were destained using 5% (v/v) methanol and 7.5% (v/v) acetic acid. The molecular weights of the proteins were estimated by running standard proteins of known weight in the gel. Commercial molecular weight standards (MP-0120, Sigma CO., Japan) were including soybean trypsin inhibitor A (25.7 kDa), carbonic anhydrase II (32.7 kDa), ovalbumin (47.8 kDa), bovine serum albumin (84.2 kDa) and phosphorylase B (110 kDa). Quantification of the bands was carried out first by scanning and then by taking densitometry reading using the ImageQuant TL (GE Healthcare UK Ltd., England) program.

***F.*** **Statistical analysis**

General linear model (GLM) was performed for pH, moisture content, microbial count, amino acids profiles as a function of ripening time to determine whether there were significant ($p<0.05$) differences using SPSS v16 (SPSS Inc., Chicago, USA). Tukey's test was also carried out during the ripening period.

## III. RESULTS AND DISCUSSION

### Microbiological results

The microbial changes in semi and dry sausage during ripening and storage are shown in Table 1. The initial total viable counts were 3.8 and 3.9 log cfu/g for control and samples with lactic acid, respectively. These decreased to levels in semi dry sausage on the day 7 of processing time at 15°C and then a slight decrease was observed to day 60 in control and sausages with lactic acid produced using the semi dry sausage process. The initial total viable counts were 6.3 to 6.8 log cfu/g for control and samples with lactic acid, respectively, an increased to levels in dry sausage on the day 21 of processing time and then a slight decrease was observed to day 60 in control and sausages with lactic acid produced using the dry sausage process. Lactic acid bacteria counts were 3.8 and 5.5 log cfu/g at day 0, after 7 days of process in semi dry sausage with lactic acid decrease to 2 log cfu/g, then dry sausage was increased to 9.3 log cfu/g and finally at 60 day of storage were 1.5 to 9.4 log cfu/g in both of sausages. The changes in total viable count and lactic acid bacteria counts were similar to those reported in other studies on this type of sausage. However, increased lactic acid production by lactic acid bacteria, in the presence of glucose, has been noticed in spite of an unchanged specific growth rate, suggesting that the additional energy obtained from direct fermentation of glucose is used for functions other than growth. As reported by Bover-Cid, Izquierdo-Pulido, & Vidal-Carou (2001) in

Table 1. Microbial profile during the processing and storage of semi and dry sausage. Lactic acid (LA); *(log cfu/g)*.

| Methods | Days | Total Viable count | | Lactic acid bacteria | |
|---|---|---|---|---|---|
| | | Control | LA | Control | LA |
| Semi dry | 0 | 3.82± 0.03c | 3.92± 0.02c | 3.85± 0.08b | 3.81± 0.05b |
| | 7 | 2.16± 0.08d | 2.38± 0.04c | 2.08± 0.08c | 2.09± 0.06c |
| | 60 | 1.82± 0.10c | 1.69± 0.09c | 1.69± 0.09c | 1.46± 0.15c |
| Dry | 0 | 6.31± 0.23b | 6.75± 0.11a | 2.98± 0.18c | 5.51± 0.05a |
| | 21 | 8.36± 0.09b | 9.29± 0.04a | 8.52± 0.07b | 9.31± 0.11a |
| | 60 | 8.21± 0.06b | 8.47± 0.12a | 8.46± 0.04b | 9.36± 0.04a |

some ripened meat products processed at low temperatures, fermentation is limited and thus the pH does not decrease by more than 0.2 - 0.4 units. Indeed, during the drying stage, the pH may return to similar values to those of the ripened meat due to the liberation of peptides, amino acids and ammonia from proteolytic reactions. Traditional dry sausages produced in the Massif Central (France) are dried at low temperatures and do not undergo a fermentation period (Rason, Martin, Dufour, & Lebeque, 2007).

**pH and Moisture**

The results of pH and moisture values show in the Fig 1. The initial pH values in control and samples with organic acids sausages were 5.5 to 5.9, respectively, with the semi dry sausage, The slight pH drop was observed during the first 7 days processing time and 60 day of storage no significant changes were observed in the pH values of control and samples with lactic acid on the semi dry sausage and 5.60 to 5.85, respectively, with the dry sausage. The fastest pH drop was observed during the 21 days of processing time and 5 to 5.4 respectively end of process and storage during 60 day no significant changes were observed in the pH values of control and samples with lactic acid on the dry sausage. In comparison, the final pH of semi dry sausage had higher values. However, the increase in pH after semi dry process treatment did not reach a critical value, since the sausages were exposed to ripening for 7 days of processing, which are considered safe. The reduction of enterobacteriaceae by decreasing pH is in agreement with the results previously demonstrated (Castaño, Fontan, Fresno, Tornadijo, & Carballo, 2002).

The moisture contents of control, samples with lactic acid sausages at the end of processing ranged between 31 to 35% in semi dry sausages samples (at day 7) and 40% in dry sausages ones (at day 21). No significant differences were observed between control and samples with organic acids the semi dry sausage processing method, while the decrease in moisture was significantly different between control and samples with organic acids sausages after 60 days dry sausage processing method. Both of sausage processing method and processing time significantly affected the moisture content.

**Free amino acids contents of dry sausages**

The changes in the contents of free amino acids were observed in semi and dry sausage at day 60 of storage shown in the Table 2. The total free amino acid contents of semi dry sausages in this study were ranged from 533 to 581 mg/100g and 1030 to 1136 mg/100g in dry sausage at day 60 of storage. The highest total free amino acid concentration was in the sample used lactic acid with 1136 mg/100g in dry sausage, whereas, the lowest was in the sample with lactic acid 533 mg/100g in semi dry sausage. The hydrolysis of meat proteins generates polypeptides that can be further degraded to smaller peptides and free amino acids. This degradation can be produced by endogenous and microbial enzymes as reported by different authors (Hughes *et al*., 2002). The increase in the total free amino acid concentration was detected in all batches as also reported by Hughes *et al*., (2002). The main differences in the content of total free amino acids among batches were detected at the end of the processing on day 60. The differences in total free amino acid were primarily responsible for the increment of Glu (glutamic acid) and Lys (lysine) between the sample control and other batches. Mateo *et al*., (1996) reported an increase in the total free amino acid content during the ripening of chorizo which similar to the results reported in the present work. The change occurred during fermentation and ripening process indicating that the highest enzymatic activity took place in these stages. Several authors have reported major release of free amino acids at the beginning of the process in coincidence with the fermentation stage (Diaz *et al*., 1997).

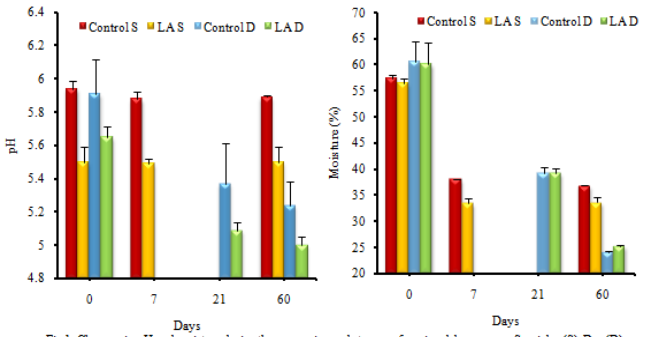

Fig 1. Changes in pH and moisture during the processing and storage of semi and dry sausage. Semi dry (S); Dry (D).

Table 2. Amino acids profiles of semi and fry sausages at 60 days. Results are expressed as mg / 100 g of sausage.

| Aas | 0 day | 60 days | | | |
|---|---|---|---|---|---|
| | | semi | | dry | |
| | | Control | LA | Control | LA |
| Asp | 5.1a | 8.72c | 2.42d | 32.45a | 23.19b |
| Thr | 7.6a | 42.29c | 40.42c | 95.38b | 101.33a |
| Ser | 7.8a | 39.17c | 36.41c | 55.03b | 71.35a |
| Glu | 23.8ab | 72.36c | 65.25c | 124.74b | 193.35a |
| Gly | 14.7ab | 31.98b | 29.24b | 84.25a | 78.94a |
| Ala | 32.4a | 89.38b | 85.45b | 168.69a | 167.82a |
| Cys | 1.0a | 1.82b | 1.91ab | 2.25a | 2.25a |
| Val | 7.3a | 32.66c | 28.79c | 73.93a | 66.82b |
| Met | 3.1a | 18.82b | 18.47b | 33.01a | 34.46a |
| Ile | 4.1a | 25.30b | 23.10b | 51.05a | 46.49a |
| Leu | 8.8a | 47.89b | 44.45b | 102.44a | 103.49a |
| Tyr | 6.7a | 4.54b | 5.01b | 8.20a | 8.15a |
| Phe | 5.9a | 28.36c | 28.16c | 50.62b | 57.10a |
| Lys | 10.1a | 57.71b | 53.12b | 36.85c | 111.09a |
| His | 6.6a | 20.02c | 18.19c | 43.42a | 36.41b |
| Arg | 10.3a | 39.13a | 36.61a | 6.90b | 7.02b |
| Pro | 5.2a | 20.87c | 16.28d | 61.29a | 27.17b |
| Total | 160.6a | 580.94c | 533.24c | 1030.45b | 1136.38a |

**Sarcoplasmic and myofibrillar proteins**

The electrophoretic patterns of sarcoplasmic and myofibrillar proteins (at 0 and 60 days of process and storage) in semi and dry sausages of two controls and organic acids added were shown in Fig. 2 respectively. Sarcoplasmic proteins with molecular weights of 78.6 kDa disappeared within the 60 days of ripening on the samples with lactic acidin dry sausage. Control in the 60 days appeared one typical band 35kDa coinciding previous findings (Aro Aro *et al*., 2010 and Candogan *et al*., 2009), while the 60 days samples with organic acid had no this typical band. The organic acid sample showed dramatically decrement of the intensity of 33-36kDa at 60 day.

In the myofibrillar fraction, the intensity of myosin heavy chains bands (around 200-220 kDa) and actin band (around 39 kDa) was decreased in

all the samples from day 0 to day 60 except the control of dry sausage. It has been suggested that sarcoplasmic and myofibrillar proteins were affected by the endogenous and microbiological enzymes, indicating that endogenous proteases and starter culture play a significant role in proteolysis during ripening of fermented sausages (Diaz *et al*., 1997). On the other hand, sausages without starter cultures showed a weaker activity of endogenous proteases compared with exogenous proteases reported by Casaburi *et al*., (2007). Several authors have reported a decrease in myosin heavy chain concentration during the ripening of dry sausages and even its complete degradation (Casaburi *et al*., 2007). The potential contribution of *lactobacilli* and *staphylococcus* species to the degradation of myofibrillar proteins has been reported by (Mauriello *et al*., 2002). Actin degradation is due to endogenous proteinases. Thus, part of the proteolytic activity on actin could be explained by the action of microbial enzymes. The results of myofibrillar protein degradation confirm that most of the proteolytic activity in traditional low-acid sausages was due to endogenous enzymes, although bacterial proteinases contributed to the degradation of these proteins.

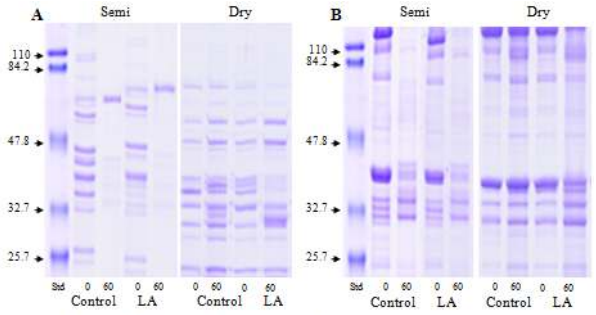

Fig 2. SDS-PAGE profile; Sarcoplasmic (A), Myofibrillar (B) of semi and dry sausages

**IV. CONCLUSION**

The organic acid as lactic acid sausages had lower pH and moisture content than control sausages. Therefore with both of production methods the use of organic acid resulted in a safer product. The proteolytic changes was followed by a degradation of sarcoplasmic and myofibrillar proteins during the processing, drying and storage was detected on the samples were brought about by both endogenous and bacterial enzymes are responsible for the degradation of sarcoplasmic and myofibrillar proteins, although bacterial proteinases contribute to the degradation of these proteins.

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**REFERENCES**

Aro Aro, J. M., Nyam-Osor, P., Tsuji, K., Shimada, K., Fukushima, M. and Sekikawa, M. (2010). The effect of starter cultures on proteolytic changes and amino acid content in fermented sausages. *Food Chemistry,* 119, 279-285.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72, 248-254.

Bover-Cid, S., Miguélez-Arrizado, M. J. & Vidal-Carou, M. C. (2001). Biogenic amine accumulation in ripened sausages affected by the addition of sodium sulphite. *Meat Science. 59***,** 391 - 396.

Campbell-Platt, G. (1995). Fermented meats - a world perspective. In: G. Cambell-Platt and P. E. Cook (ed) Fermented Meats. (pp. 39-51). Blackie Academic & Professional, UK.

Candogan, K., Wardlaw, F. B. and Acton, J. C. (2009). Effect of starter culture on proteolytic changes during processing of fermented beef sausages. *Food Chemistry,* 116, 731-737.

Casaburi, A., Aristoy, M. C., Cavella, S., Di Monaco, R., Ercolini D. and Toldra, F. (2007). Biochemical and sensory characteristics of traditional fermented sausages of Vallo di Diano (Southern Italy) as affected by the use of starter cultures. *Meat Science,* 76, 295-307.

Castaño, A., Fontan, M. C. G., Fresno, J. M., Tornadijo, M. E. and Carballo, J. (2002). Survival of *Enterobacteriaceae* during processing of chorizo de cebolla, Spanish fermented sausage. *Food Control,* 13, 107-115.

Diaz, O., Fernández, M., García de Fernando, G. D., De la Hoz, L. and Ordóñez, J.A. (1997). Proteolysis in dry fermented sausages: The effect of selected exogenous proteases. *Meat Science,* 46, 115-128.

Hughes, M. C., Kerry, J. P., Arendt, E. K., Kenneally, P. M., McSweeney, P. L. H. and O'Neill, E. E. (2002). Characterization of proteolysis during the ripening of semi-dry fermented sausages. *Meat Science,* 62, 205-216.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227, 680-685.

Mateo, J., Dominguez, M. C., Aguirrezabal, M. M. and Zumalacarregui, J. M. (1996). Taste compounds in chorizo and their changes during ripening. *Meat Science,* 44, 245-254.

Mauriello, G., Casaburi, A. and Villani, F. (2002). Proteolytic activity of *Staphylococcus xylosus* strains on pork myofibrillar and sarcoplasmic proteins and use of selected strains in the production of Naples type salami. *Journal of Applied Microbiology,* 92, 482-490.

Rason, J., Martin, J. F., Dufour, E. and Lebecque, A. (2007) Diversity of the sensory characteristics of traditional dry sausages from the centre of France: relation with regional manufacturing practice. *Food Quality and Preference,* 18, 517-530.

Toldrá, F. (1998) Proteolysis and lipolysis in flavour development of dry-cured meat products, *Meat Science 49* (Suppl. 1) pp. S101–S110.