92. Adoption of proteomics in traditional meat products: the case of Khliaa Ezir

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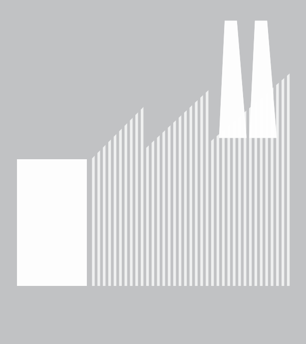
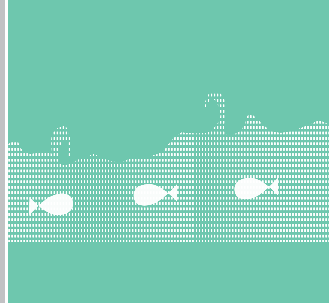
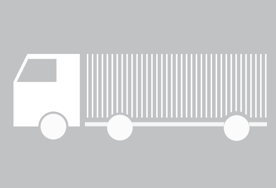

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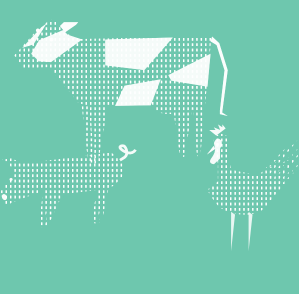
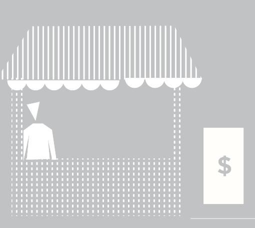

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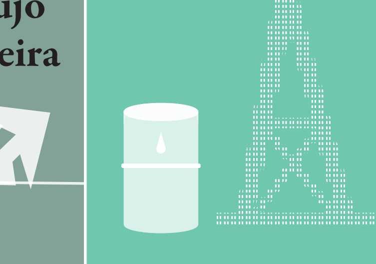
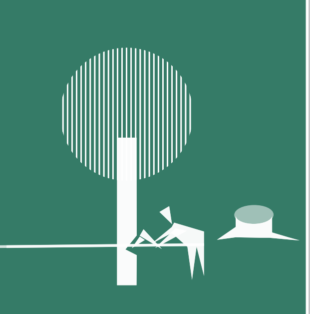

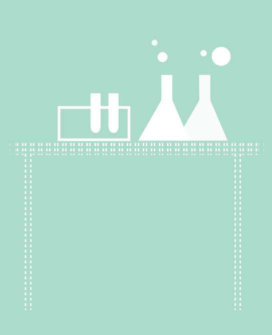

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92. Adoption of proteomics in traditional meat products: the case of *Khliaa Ezir*

H.R. Boudechicha, M. Sellama, K. Hafid, A. Boudjellal and M. Gagaoua[*]
Equipe Maquav, INATAA, Université Frères Mentouri, Constantine 1, Route de Ain El-Bey, 25000 Constantine, Algeria; gmber2001@yahoo.fr

Abstract

Khliaa Ezir is a typical Algerian meat product, prepared according to a traditional process based on empirical observations and traditional recipes, consisting of steps of trimming, marinating, cooking, ripening and ageing in earthenware jar (*Ezir*). Understanding the biological mechanisms behind the development of the sensory properties of this cultural product is of great interest for eventual product marketing. The aim of the present study was first a preliminary proteomic characterization of myofibrillar and sarcoplasmic protein extracts of the product during preparation, ripening and ageing in earthenware jar. A comparison of the proteome of the two extracts at different days and steps was carried out with 1-DE electrophoresis, showing quantitative difference in several bands. Some bands might be potential markers of the proteolysis that takes place during the preparation steps, especially during marinating and ripening. This first and preliminary proteomic data from both myofibrillar and sarcoplasmic protein extracts of *Khliaa Ezir* are consistent with a differential release of specific proteins as a function of preparation step.

Keywords: *Khliaa Ezir*, Algerian meat product, preliminary proteomic characterization

Introduction

Traditional meat products cover a wide class of food items and their characterization is of great importance. Numerous traditional ethnic meat products are commonly prepared in Algeria since the highest antiquity. They have been widely consumed for their enhanced quality, flavour, and storage properties. The inimitable colour, aroma, taste, and simulated shape of Algerian ethnic meat products enjoy a positive reputation and are regarded as the essence of human experience and wisdom. Among them, *Khliaa Ezir*, which is a traditional cured meat mostly produced in the north-east of Algeria using fresh (beef, lamb, goat or camel) meat. The particularity of its traditional process is the ripening step in an earthenware jar able to be preserved for more than one year (Boudechicha *et al.*, 2015). *Khliaa Ezir* preparation and processing, cooking habits and its organoleptic properties exhibit the deep connotation of Algerian foods of delectable, healthy, and cultural heritage.

During the processing of cured meat products which involve both (1) seasoning/curing; and (2) cooking/ripening steps, complex chemical and biochemical changes occur on the main components of raw meat (proteins and lipids) leading to a numerous modifications on colour, flavour and texture (Toldra, 2006). Cured meat products manufactured from beef are particularly a great source of proteins and free amino acids (Garrido *et al.,* 2012). Many of these free amino acids come from proteolysis, which is at the central of myriad biochemical reactions occurring in cured meat preparation, contributing to the typical flavour and texture of the final product (Lorenzo and Franco, 2012).

To understand the type and extent of proteolysis, several proteomic techniques have been successfully applied for the identification of generated peptides and their sequencing. This approach provides new insights to achieve better quality of cured meat products and reveal key biochemical mechanisms controlling the process. This may play a great role on the preservation of this meat product both by its

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cultural and historical dimension. In addition, the nutritional value of the product would be well known, hence providing a full *Khliaa Ezir* data sheet for consumers and butchers. Thus, the aim of the present study was a preliminary proteomic characterization of myofibrillar and sarcoplasmic protein extracts of *Khliaa Ezir* during preparation, ripening and ageing in earthenware jar.

Materials and methods

Preparation of Khliaa Ezir and sampling

Khliaa Ezir is prepared from fresh boneless beef meat using the traditional preparation diagram (Figure 1). Briefly, fresh selected cuts of *Semimembranosus* muscle (5-8 cm length, 4-6 cm thick) obtained from a local butcher were marinated for 7 days in a mixture of spices (salt, coriander, caraway, and smashed garlic) before cooking at an average temperature of 80 °C on water. After that, the cooked meat was immersed in a mixture of melted bovine fat and olive oil and preserved in an earthenware jar for numerous months. In this study, nine preparations of *Khiaa Ezir* were conducted in the laboratory following the traditional diagram given in Figure 1. Aliquots of each preparation were sampled at different times of the process: on fresh meat (day 0), marinade time (days 1, 3, 5 and 7), after cooking (day 8), during ripening and storage (days 2, 6, 10, 15 and 21).

Extraction of sarcoplasmic and myofibrillar muscle proteins

The sarcoplasmic and myofibrillar proteins were extracted according to the procedure described by Diaz *et al.* (1997). Briefly, each aliquot of *Khliaa Ezir* (from fresh meat to 21 day of storage on the

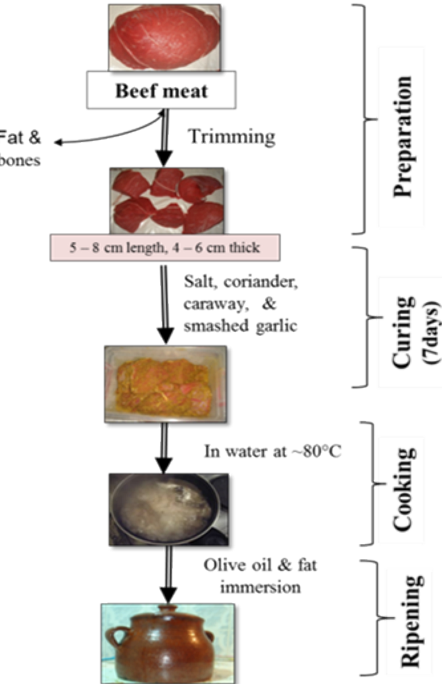

Figure 1. The traditional diagram used for the preparation of Khliaa Ezir *(Boudechicha* et al.*, 2015).*

earthenware jar) at the desired time was used for protein extraction by homogenization on 1:10 (w/v) of 40 mM sodium phosphate buffer containing 0.02% NaN_3 using a Polytron homogenizer (×20,000 rpm) (Polytron ® PT- MR 2100, Kinematica AG, Switzerland). The homogenate was then filtered through clean cheesecloth before centrifugation at 4,000 rpm for 15 min at 4 °C (Bench top centrifuges NF 400R). The supernatant was collected as the sarcoplasmic protein fraction and stored in adequate tubes at -20 °C until use. The resulting pellet was re-suspended in 10 ml of the same buffer and centrifuged twice. The final pellet was once again re-suspended in 10 ml of buffer, filtered through Whatman paper and collected as the myofibrillar protein fraction and stored at -20 °C until use. The protein concentration of both sarcoplasmic and myofibrillar protein extracts was determined using the Bradford protein assay kit obtained from Bio-Rad. A calibration curve was constructed using bovine serum albumin as the standard (Bradford, 1976).

1DE-electrophoretical analysis (glycine SDS-PAGE)

The extracts of the sarcoplasmic and myofibrillar proteins were mixed in a ratio of 1:1 (v/v) with 312.5 mM Tris buffer (pH 6.8), 0.4 mM EDTA, 7.5% (w/v) SDS, 150 mM dithiothreitol, 25% glycerol and 0.05% bromophenol blue. The mixture was then heated at 95 °C for 5 min. The protein extracts (10 µg for each well) were analysed using one dimensional SDS-PAGE electrophoresis on 3.75% stacking and 12% resolving gels according to Laemmli, (1970). A mixture of proteins with a known molecular weight ranging from 10 to 250 kDa (#161-0374) obtained from Bio-Rad Laboratories, Hercules, CA, was used. After separation, the gel was stained overnight as described by Gagaoua *et al.* (2015) with a staining solution of 4.9 mM Coomassie Brilliant Blue G-250, 50% (v/v) ethanol, and 7.5% (v/v) acetic acid. Protein patterns were then visualized after destaining the gel until a clear background was achieved. The molecular weight of the protein bands was calculated using the Un-Scan-It Gel 6.1 analysis program (Silk Scientific, Orem, UT).

Results and discussion

During the processing of *Khliaa Ezir*, the proteins undergo various modifications which depend on numerous factors. The separated sarcoplasmic and myofibrillar are depicted on Figure 2. The overall banding patterns between replicates were similar and protein bands were identified based on their molecular weights.

Myofibrillar proteins

Significant change on the protein profile of the myofibrillar proteins of *Khliaa Ezir* during preparation was observed (Figure 2a). For example, there is a progressive reduction in intensity of the two common myofibrillar and structural proteins, myosin heavy chain (MHC), and actin, appearing approximately at 200 kDa and 47 kDa, respectively. The significant decrease in abundance of these proteins seems to clearly occur after the cooking phase. In accordance, several research papers reported the effect of cooking/ ripening on meat products (Diaz *et al.,* 1997; Ruiz *et al.,* 2007). On another hand, Thorainsdottir *et al.* (2002) has shown that myosin is more sensitive to processing than actin. Since thermal treatment of meat can have an impact on the primary structure of the meat proteins, which is closely associated with both the nutritional and sensory qualities of cooked meat, characterizing cooking-induced protein modifications in meat is one of the fundamental steps to understand these effects. This work is now under investigation as a complementary work with both MS identification and characterization of the other changed proteins.

Otherwise, other high MW bands, such as the 100 kDa also decreased in intensity before its disappearance after cooking. At the same time, a progressive disappearance of other proteins such as the 61, 35 and 30

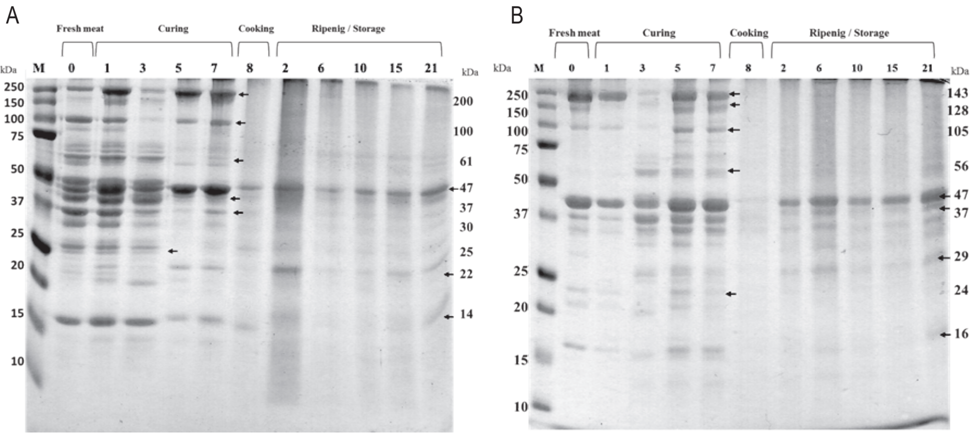

Figure 2. SDS-PAGE profile of (A) myofibrillar and (B) sarcoplasmic proteins at different preparation steps of Khliaa Ezir *(the numbers indicate days of preparation and storage).*

kDa are also observed during the last period of curing. The intensity of the band at 14 kDa decreases throughout the curing time and disappear completely after cooking. Several studies conducted on numerous traditional products showed similar changes to those described in myofibrillar proteins during meat product processing. For example, Larrea *et al.* (2006) observed that in dry cured ham the bands at 97, 22 and 14 kDa decrease progressively during the curing time, probably due to a specific sensitivity to salt. Garcia *et al.* (1997) studied the changes in proteins during the ripening of Spanish dried beef *Cecina* and also observed changes in the myofibrillar proteins: disappearance of MHC and troponin C after the smoking step (a thermal treatment).

Sarcoplasmic proteins

The electrophoretic profile of sarcoplasmic proteins is shown in Figure 2b. As for the myofibrillar protein pattern, numerous sarcoplasmic proteins were found to be changed and affected by the preparation steps of *Khliaa Ezir*. We can suppose that these proteins may undergo both truncation and aggregation after cooking as recently proposed by Wen *et al.* (2015) and Yu *et al.* (2015). Numerous proteomic studies reported that the mainly affected proteins are namely glycolytic enzymes such as lactate dehydrogenase A chain isoform 1, beta-enolase, fructose bisphosphate aldolase A isoform 1, pyruvate kinase isozymes M1/M2 isoform 4, glyceraldehyde-3-phosphate dehydrogenase and triosephosphate isomerase 1 (for a review see Picard *et al.,* 2016). In this study, the intensity of a band with a mobility very close to heavy myosin chain and an estimated MW of 143 kDa decreased throughout the preparation steps leading to its disappearance after cooking. The appearance of bands at about 105, 56 and 37 kDa has been observed during the last period of curing. The formation of compounds with similar MW has been sometimes observed during ripening of fermented sausages (Garcia de Fernando and Fox, 1991). The appearance of these proteins could result from the treatment effect leading to the formation of protein dimmers and/or aggregation of numerous proteins together. A complete mass spectrometry is under process for a better characterization of the phenomenon really occurring during *Khliaa Ezir* preparation. The intensity of band 47 kDa increased throughout the curing time and became very intense by days 5 and 7. After cooking, several bands disappeared; only this later band which seems to be highly dominant and present with a slight intensity.

According to the related literature, proteolytic processes that occur during the curing and ripening of cured meat products are mainly due to endogenous proteolytic activity (Toldra *et al.,* 1997). To this regard, Zhao *et al.* (2005) observed that the activity of these enzymes was dependent on the pH, temperature and salt content. In our study, the behaviour of the proteins is due to curing conditions (time, temperature and salt content), but this phenomenon is now under investigation with accurate techniques. The appearance of compounds of MW between 100 and 30 kDa observed throughout the marinating time could result from the breakdown of myosin heavy chain and other high molecular weight proteins. The denaturation of proteins seems to be affected by the length of curing time (unpublished data). These findings are in agreement with those reported by Martín *et al.* (1998) and Perez-Palacios *et al.* (2010) who found that salt content and the curing time affect strongly the proteolytic activity. The effect may be more pronounced on the endogenous enzymes such as cathepsins, dipeptidyl peptidases and aminopeptidases, which release large peptide fragments from proteins.

During the processing of *Khliaa Ezir*, the temperature applied during thermal treatment (80 °C) can have enhanced protein denaturation. As can be seen from Figure 2, the myofibrillar and sarcoplasmic proteins of MW greater than 47 kDa disappeared after cooking. The only exception was the band at 47 kDa which still present throughout the ripening step. Earlier studies conducted on the effects of heating on protein denaturation revealed similar changes (Di Luccia *et al.,* 2005). Tornberg, (2005) reported that between 65 and 87 °C many changes are taking place in the myofibrillar and sarcoplasmic proteins, which causes their aggregation, decreases the solubility and disappearance of the bands. Meat protein aggregation induced by cooking can lead for example to a compact structure (Promeyrat *et al.,* 2010). Furthermore, the proteins with MW less than 50 kDa still present after cooking in some ripened meat products process. This is consistent with our study suggesting that actin, the myofibrillar protein previously shown to be relatively heat stable below 100 °C (Huang *et al.,* 2011), was still detected. Finally, the identification of numerous proteins above and below their theoretical MW probably relates to *post-mortem* actomyosin complex formation and proteolysis (the work still in progress).

Conclusions

This preliminary proteomic investigation has shown the complex pattern of protein changes occurring during the traditional preparation of *Khliaa Ezir*, a cured and cooked meat product of Algeria. In summary, cooking combined with the marinating step seems to affect considerably the textural properties of the product, by significant changes on the structural proteins. Complimentary studies on the characterization and identification of the affected proteins by accurate proteomic and peptidomic techniques are now in progress for a well characterization of the mechanisms behind the sensory qualities development.

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