

Proteolysis in Serbian Traditional Dry Fermented Sausage *Petrovska klobasa* as Influenced by Different Ripening Processes

P. M. Ikonić, T. A. Tasić, L. S. Petrović, S. B. Škaljac, M. R. Jokanović, V. M. Tomović, B. V. Šojić, N. R. Džinić, A. M. Torbica, B. B. Ikonić

Abstract—The aim of the study was to determine how different ripening processes (traditional vs. industrial) influenced the proteolysis in traditional Serbian dry-fermented sausage *Petrovska klobasa*. The obtained results indicated more intensive pH decline (0.7 units after 9 days) in industrially ripened products (I), what had a positive impact on drying process and proteolytic changes in these samples. Thus, moisture content in I sausages was lower at each sampling time, amounting 24.7% at the end of production period (90 days). Likewise, the process of proteolysis was more pronounced in I samples, resulting in higher contents of non-protein nitrogen (NPN) and free amino acids nitrogen (FAAN), as well as in faster and more intensive degradation of myosin (≈ 220 kDa), actin (≈ 45 kDa) and other polypeptides during processing. Consequently, the appearance and accumulation of several protein fragments were registered.

Keywords—Dry-fermented sausage, *Petrovska klobasa*, proteolysis, ripening process.

I. INTRODUCTION

PROCESS of fermentation and drying is believed to be one of the oldest techniques for preserving meat. Hence, natural dry-fermented sausages have 2000 years long tradition of originating in China and the Mediterranean area [1]. Nowadays, many fermented sausages are commonly produced in industrial plants, but there still are regions in Europe where these products are obtained through traditional practice [2].

Petrovska klobasa is traditional dry-fermented sausage manufactured in north-western Serbia (Municipality of Bački Petrovac, Province of Vojvodina). It is a part of Slovaks' heritage, who inhabited Vojvodina in 18th century. Presently, they are producing it according to the original recipe in small household enterprises during winter, when temperatures are around 0°C or lower. Thus, it undergoes slow drying and ripening processes (90 days at least) [3], [4].

Predrag M. Ikonić, Tatjana A. Tasić, and Aleksandra M. Torbica are with University of Novi Sad, Institute of Food Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia (e-mail: predrag.ikonic@fins.uns.ac.rs, tatjana.tasic@fins.uns.ac.rs, aleksandra.torbica@fins.uns.ac.rs).

Ljiljana S. Petrović, Snežana B. Škaljac, Marija R. Jokanović, Vladimir M. Tomović, Branislav V. Šojić, and Natalija R. Džinić are with the University of Novi Sad, Faculty of Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia (e-mail: ljiljapet@uns.ac.rs, snezanasavatic@gmail.com, marijaj@tf.uns.ac.rs, tomovic@uns.ac.rs, bsojic@gmail.com, natadzin@uns.ac.rs).

This study was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. TR31032.

Proteolysis occurring during the ripening of fermented sausages is extremely important biochemical phenomena which determine the final aroma and texture characteristics of the product. This phenomenon is influenced by both muscle and microbial enzymes, and it results in formation of several low molecular weight components, i.e. peptides, amino acids, aldehydes, organic acids and amines [5]-[7].

The objective of this study was to investigate the proteolytic changes in *Petrovska klobasa* sausages during different ripening processes (traditional vs. industrial).

II. MATERIAL AND METHODS

A. Preparation of Sausages

Petrovska klobasa sausages were manufactured from lean minced pork (80% w/w) and pig fat (20% w/w), minced to 10 mm particle size and mixed with red hot paprika powder, salt, raw garlic paste, caraway and sugar. Raw materials were mixed with seasonings and the seasoned batter was immediately stuffed in collagen casings (55 mm in diameter). Raw sausages were divided into two batches which were ripened in different conditions, a traditional smoking/drying room (T) and a controlled industrial ripening room (I). The environmental conditions in traditional room were characterised by low air temperature (average $7.11 \pm 3.20^\circ\text{C}$) and high relative humidity (RH) (average $82.6 \pm 5.98\%$) (Fig. 1 (a)). On the other side, in industrial room the sausages were ripened at somewhat higher temperature (average $11.1 \pm 4.58^\circ\text{C}$), especially in first 10 days (smoking phase), and lower relative humidity (average $76.1 \pm 8.48\%$) (Fig. 1 (b)).

B. Samples

For sampling, the seasoned butter prior to stuffing (0) and three randomly selected sausages from each batch were taken after 2, 6, 9, 15, 30, 60 and 90 days of drying and ripening. Physicochemical analysis were carried out at the day of sampling, and the rest of the sausages were homogenized and stored at -20°C pending further analysis. Analyses for all samples were carried out in duplicate.

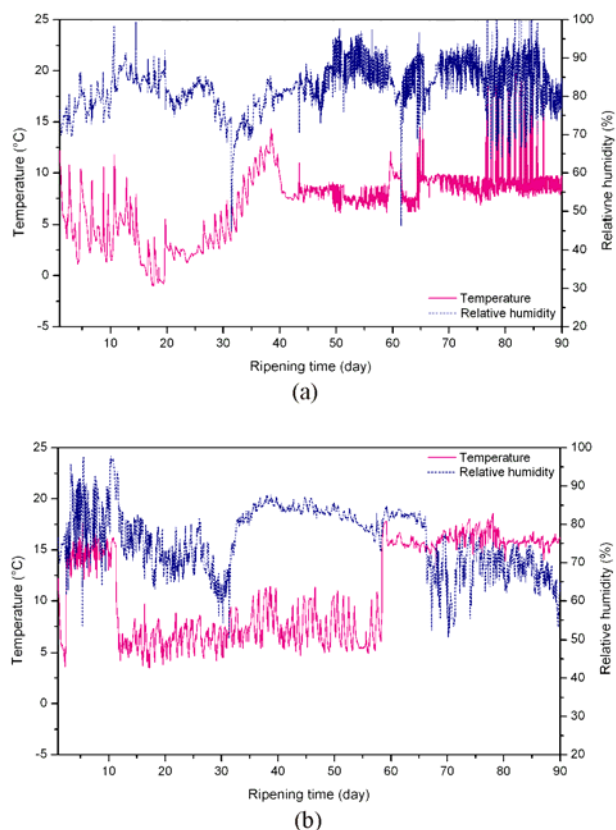


Fig. 1 Environmental temperature and relative humidity recorded during ripening of *Petrovská klobása* in traditional (a) and industrial (b) conditions

C. Physicochemical Analysis

The pH of samples was measured using the portable pH meter Testo 205 (Testo AG, USA) equipped with a combined penetration tip with temperature probe [8]. Moisture content of sausages was determined by drying the samples at $103 \pm 2^\circ\text{C}$ to constant weight [9]. The non-protein nitrogen (NPN) and free amino acids nitrogen (FAAN) were determined according to the methods described in [4]. The nitrogen fractions contents were expressed as g/100 g dry matter (dm) of sample.

D. Electrophoretic Separation of Myofibrillar Proteins-LoaC Method

Myofibrillar proteins were extracted according to the method described by [10]. The concentrations of the obtained protein extracts were determined by method of [11], using bovine serum albumin as standard protein, and adjusted with deionised water to give a final concentration of 9 mg/mL. The chip-based separations were carried out using Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) in combination with the Protein 230 Plus LabChip Kit and the dedicated 2100 expert software. The chips were prepared according to Agilent Protein 230 Kit Guide (assay protocol) and successively placed into the bioanalyzer. The complete analysis of 10 protein samples takes 25 minutes (including the start-up phase of the instrument).

E. Statistical Analysis

One way (ANOVA), Post-hoc (Duncan test) was performed using the software package Statistica 12.0 for Windows, Stat Soft, Tulsa, Oklahoma, USA. Differences were considered significant at $P < 0.05$.

III. RESULTS AND DISCUSSION

The changes in moisture content and pH along the ripening period showed the usual trends observed in this type of products (Fig. 2). However, different ripening processes (traditional vs. industrial) resulted in different drying intensity of sausages. Consequently, moisture content in industrially dried samples was lower throughout the entire processing period (Fig. 2). Moreover, due to higher initial temperature ($\approx 15^\circ\text{C}$) in industrial ripening room (Fig. 1 (b)) the fermentation process in I sausages was considerably faster, causing intensive pH decline toward the isoelectric point (pI) of actomyosin (≈ 5.0) (Fig. 2). Therefore, when the pI of myofibrillar proteins was reached the drying process of these sausages was facilitated, owing to low water holding capacity of meat [12], [13].

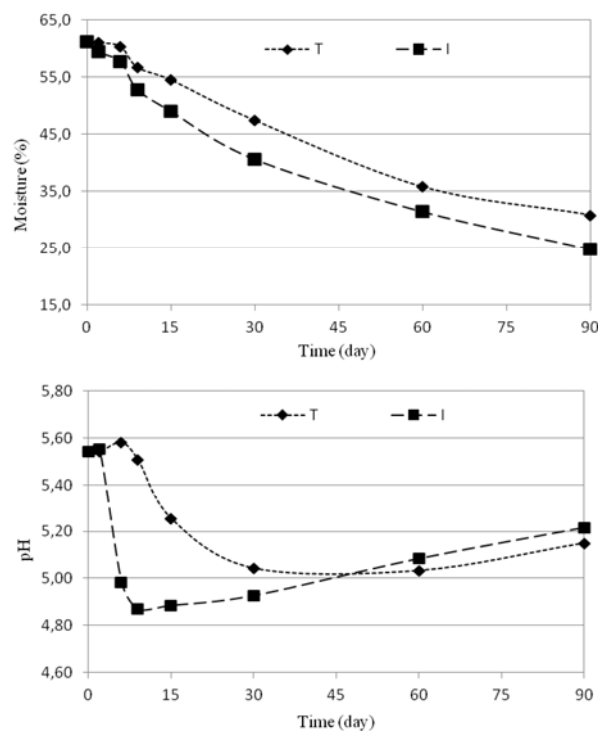


Fig. 2 The changes of moisture content and pH in *Petrovská klobása* sausages during different ripening processes

Furthermore, after only 9 days the pH value in industrially ripened samples had fallen below 5.0 (4.87). Additionally, the pH drop in I sausages was higher than in T (0.7 vs. 0.5 units). After reaching the minimal value pH started gradual increase in all sample sausages, due to degradation of proteins and liberation of peptides, amino acids, ammonia and amines, as it was proposed by a number of authors [7], [14].

The content of NPN increased progressively ($P < 0.05$) during processing in both analysed samples (Table I), being approximately 80% higher in finished products compared to raw sausage butter. In general, the obtained results are in agreement with other studies indicating that proteolysis during ripening of fermented sausages is characterised by an increase in NPN content [6], [14], [15]. However, the level of this nitrogen fraction was higher in I sausages at each sampling stage except at the end of processing period. This phenomenon is probably caused by higher temperature in the industrial room during the smoking process (Fig. 1 (b)), which favoured the growth of LAB. Consequently, due to intensive fermentation rapid pH decline occurred, positively affecting the activation of the muscle proteinases (cathepsin D-like enzymes) and degradation of myofibrillar proteins [16]-[18].

TABLE I

EVOLUTION OF NPN AND FAAN CONTENT IN *PETROVSKÁ KLOBÁSA* (EXPRESSED AS G/100 G DM) DURING DIFFERENT RIPENING PROCESSES

Batch	Time (day)								
	0	2	6	9	15	30	60	90	
NPN	T	0.63a ± 0.01	0.64a ± 0.02	0.80a ± 0.02	0.76a ± 0.02	0.76a ± 0.05	0.86a ± 0.02	1.06a ± 0.01	1.17b ± 0.01
	I	0.63a ± 0.01	0.69b ± 0.01	0.81a ± 0.02	0.81b ± 0.01	0.92b ± 0.02	0.98b ± 0.01	1.13b ± 0.00	1.10a ± 0.01
FAAN	T	0.25a ± 0.01	0.26a ± 0.01	0.28a ± 0.01	0.31a ± 0.00	0.31a ± 0.01	0.36a ± 0.00	0.47b ± 0.01	0.48a ± 0.00
	I	0.25a ± 0.01	0.27a ± 0.01	0.36b ± 0.01	0.36b ± 0.00	0.38b ± 0.01	0.39b ± 0.01	0.46a ± 0.00	0.49b ± 0.00

^{ab} indicates significant difference within column at $P < 0.05$

NPN = non-protein nitrogen;

FAAN = free amino acids nitrogen;

Regarding the FAAN, similar considerations can be applied. Namely, during first 30 days its content slightly increased in traditionally ripened sausages, when it reached the value of 0.36 g/100g dm (Table I). Unlike, the same level of this nitrogen fraction is reached after only 6 days of ripening in industrial conditions. The difference in speed and intensity of proteolysis process between T and I batches was evident until day 30. During that period, the content of FAAN fraction in I sausages gradually increased, being significantly higher ($P < 0.05$) compared to T. After 30 days of manufacturing the air temperature in traditional room was few degrees elevated (Fig. 1 (a)) and the pH had fallen, resulting in more intensive proteolysis in T sausages. Hence, after 60 days of ripening similar ($P > 0.05$) content of FAAN fraction was observed in both sample sausage (Table I). Throughout last month of processing the level of this nitrogen fraction additionally increased in both batches, being approximately 0.49 g/100 g dm.

LoaC gel-like electrophoretograms of myofibrillar proteins are given in Fig. 3, for traditional and industrial ripening processes. As it can be seen, during the processing of both examined groups of sausages notable changes in qualitative and quantitative composition of myofibrillar proteins extracts occurred. Nevertheless, some differences in the intensity of certain protein fractions between variously ripened sausages

were evident.

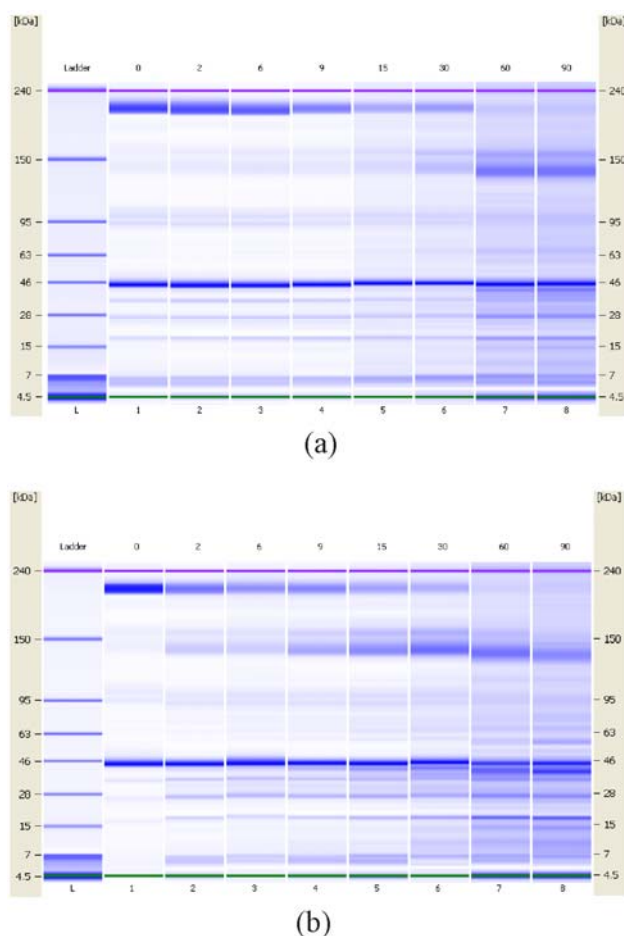


Fig. 3 LoaC gel-like image of myofibrillar proteins during ripening of *Petrovská klobása* (lanes 1-8): (a) traditional conditions, (b) industrial conditions. Lane L (Ladder), molecular weight standards ranging from 4.5 to 240 kDa

Contents of basic myofibrillar proteins with molecular mass ≈ 220 kDa (myosin) and ≈ 45 kDa (actin) were significantly reduced at the end of the manufacturing of each batch of sausages, compared to the initial ones. Thus, after three months, severe degradation of myosin resulted in almost complete disappearance of this protein fraction in both samples. However, drying process in industrial conditions, at higher initial temperatures (Fig. 1 (b)), contributed faster degradation of myosin in sausages I. This finding is in accordance with the results of several authors [2], [5], [7], who previously reported an intensive degradation of myosin during the ripening of naturally fermented sausages. On the other hand, actin has shown greater stability during ripening even it was notably degraded as well. Others [5]-[7], [19] also reported actin hydrolysis during the ripening fermented sausages. Therefore, at the end of processing (90. day) the intensity of protein band at ≈ 45 kDa was decreased, particularly in sausages ripened in industrial room (I). As it was previously mentioned, higher initial temperatures in this room caused rapid and more intensive pH decline in I

sausages (Fig. 3), positively affecting the activation of the muscle proteinases (cathepsin D-like enzymes) and certain microbial enzymes, as it was proposed in previously published work of a number of authors [2], [18], [19].

Due to co-migration of myosin (≈ 220 kDa) degradation products the intensity of protein bands at ≈ 140 and 160 kDa markedly increased during the ripening. Myosin degradation into the polypeptides with molecular weights in the range of 120 - 150 kDa was previously indicated in numerous studies [7], [15]. Furthermore, the appearance and accumulation of several polypeptides with molecular weights in the range from ≈ 15 to ≈ 42 kDa, as well as the increase in intensity of protein bands of ≈ 70 and 75 kD, was registered during the ripening of both sample sausage. Several studies reported the formation and increase of protein fragments in the ranges 50 - 100 kDa and 14 - 45 kDa [6], [7], [15], [18]. Nevertheless, more newly formed fragments appeared in sausages ripened in controlled conditions at higher initial temperatures (I). Also, total degradation and disappearance of numerous polypeptides (≈ 50 , 90 , 100 , 105 , 195 kDa) were registered in this sample, confirming the positive effect of higher temperature on proteolysis intensity [2], [18], [19].

IV. CONCLUSION

Higher initial temperature in industrial ripening room ($\approx 15^\circ\text{C}$) affected faster fermentation process, i.e. more intensive pH decline (0.7 units after 9 days) in I sausages. This phenomenon had a positive influence on drying and proteolysis process in these samples. Consequently, moisture content in I sausages was lower throughout the entire processing period. Likewise, the proteolytic changes were more significant in I samples, resulting in higher contents of nitrogen fractions (NPN and FAAN) and more intensive degradation of myofibrillar proteins. Consequently, the appearance and accumulation of several protein fragments was registered.

REFERENCES

- [1] E. Petäjä-Kanninen and E. Puolanne, "Principles of Meat Fermentation", In *Handbook of Fermented Meat and Poultry*, Toldrá F, Hui YH, Astiasarán I, Nip WK, Sebranek JG, Silveira ETF, Stahnke LH, Talon R, Eds. Ames, Iowa, Blackwell Publishing, 2007, pp. 31-36.
- [2] A. Casaburi, M. C. Aristoy, S. Cavella, R. Di Monaco, D. Ercolini, F. Toldrá, F. Villani, "Biochemical and sensory characteristics of traditional fermented sausages of Vallo di Diano (Southern Italy) as affected by the use of starter cultures", *Meat Sci.* vol. 76(2), pp. 295-307, 2007.
- [3] T. Tasic, P. Ikonić, A. Mandić, M. Jokanović, V. Tomović, S. Savatić, Lj. Petrović, "Biogenic amines content in traditional dry fermented sausage *Petrovska klobasa* as possible indicator of good manufacturing practice", *Food Control* vol. 23(1), pp. 107-112, 2012.
- [4] P. Ikonić, T. Tasic, Lj. Petrović, S. Škaljac, M. Jokanović, A. Mandić, B. Ikonić, "Proteolysis and biogenic amines formation during the ripening of *Petrovska klobasa*, traditional dry-fermented sausage from Northern Serbia", *Food Control* vol. 30, pp. 69-75, 2013.
- [5] L. C. Roseiro, C. Santos, M. Sol, M. J. Borges, M. Anjos, H. Gonçalves, A. S. Carvalho, "Proteolysis in *Painho de Portalegre* dry fermented sausage in relation to ripening time and salt content", *Meat Sci.* vol. 79(4), 784-794, 2008.
- [6] Ü. Dalmiş and A. Soyer, "Effect of processing methods and starter culture (*Staphylococcus xylosum* and *Pediococcus pentosaceus*) on proteolytic changes in Turkish sausages (sucuk) during ripening and storage", *Meat Sci.* vol. 80(2), pp. 345-354, 2008.
- [7] M. Spaziani, M. Del Torre, L. Stecchini, "Changes of physicochemical, microbiological, and textural properties during ripening of Italian low-acid sausages. Proteolysis, sensory and volatile profiles", *Meat Sci.* vol. 81, pp. 77-85, 2009.
- [8] ISO 2917, "Meat and meat products. Measurement of pH (Reference method)," Switzerland: International Organisation for Standardisation, 1999.
- [9] ISO 1442, "Meat and meat products. Determination of moisture content (Reference method)," Switzerland: International Organisation for Standardisation, 1997.
- [10] F. Toldrá, E. Rico, J. Flores, "Cathepsin B, D, H and L activities in the processing of dry-cured ham", *J. Sci. Food. Agr.* vol. 62(2), pp. 157-161, 1993.
- [11] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, "Protein measurement with the Foline-phenol reagent", *J. Biol. Chem.* vol. 193, pp. 265-275, 1951.
- [12] R. Hamm, "Kolloidchemie des fleisches", Berlin: Paul Parey, 1972.
- [13] E. Huff-Lonergan, "Chemistry and biochemistry of meat", In *Handbook of meat processing*, Toldrá F, Ed. Ames, Iowa: Blackwell Publishing, 2010, pp. 5-24.
- [14] G. Kaban and M. Kaya, "Effects of *Lactobacillus plantarum* and *Staphylococcus xylosum* on the quality characteristics of dry fermented sausage "Sucuk"", *J. Food Sci.* vol. 74, 58-63, 2009.
- [15] K. Candogan, F. B. Wardlaw, J. C. Acton, "Effect of starter culture on proteolytic changes during processing of fermented beef sausages", *Food Chem.* vol. 116(3), 731-737, 2009.
- [16] K. Molly, D. Demeyer, G. Johansson, M. Raemaekers, M. Ghistelinck, I. Geenen, "The importance of meat enzymes in ripening and flavour generation in dry fermented sausages. First results of a European project", *Food Chem.* vol. 59(4), 539-545, 1997.
- [17] F. Toldrá and M. Flores, "The use of muscle enzymes as predictors of pork meat quality", *Food Chem.* vol. 69(4), 387-395, 2000.
- [18] F. Toldrá, "Dry-cured meat products", Trumbull, Connecticut: Food & Nutrition, press, inc, 2002.
- [19] J. M. Aro Aro, P. Nyam-Osor, K. Tsuji, K.-i. Shimada, M. Fukushima, M. Sekikawa, "The effect of starter cultures on proteolytic changes and amino acid content in fermented sausages", *Food Chem.* vol. 119(1), 279-285, 2010.