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RECOMBINANT METALLOPROTEASE AS A PERSPECTIVE ENZYME FOR MEAT TENDERIZATION

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ABSTRACT

Peptidase family M9 (MEROPS database) is true collagenases and contains bacterial collagenases from *Vibrio* and *Clostridium*. One of the producers of M9A subfamily peptidase is *Aeromonas salmonicida* (locus - ASA_3723). The aim of the study was production of recombinant metallopeptidase *Aeromonas salmonicida* by transformation *Pichia pastoris* for further meat tenderization. Laboratory amounts of recombinant peptidase were obtained and test evaluation of enzyme activity was performed. Recombinant peptidase broke the peptide bond «Pro-Leu-Gly-Met-Trp-Ser-Arg» (one of the collagen chains, (Mw = 846.06)). The concentration of the substrate (peptide) after 180 min was 2 – fold decrease as compared with control. The maximum shear force of heat-treated samples had a 1.27 – fold decrease as compared with the control. As a result of histological studies of beef shank samples, the specific effect of the supernatant on the structure of connective tissue was established. Muscle fibers have not changed. The recombinant enzyme could be used for the meat tenderization.

Keywords: meat tenderization; recombinant peptidase; M9 family peptidase

INTRODUCTION

Meat tenderness is a problem in the meat industry with important economic repercussion. The connective tissue contribution (termed a background effect) influence on tenderness. The main component of connective tissue is collagen, its content in the muscles of beef can reach 15% of dry weight **(Bailey and Light, 1989)**. Epimysium and perimysium contain type I collagen as the major component and type III collagen as a minor component; endomysium contain both type I and type III collagen as major components **(Light and Champion, 1984)**. In addition, such type of by-products can be used in the food industry **(Kotenkova and Polishchuk, 2019)**. The use of exogenous proteases to improve meat tenderness has attracted much interest recently **(Bekhit et al., 2014; Chanalia et al., 2018)**. Of particular interest for studies of collagenase activity is the M9 metallopeptidase family (MEROPS database), from microorganisms *Clostridium histolyticum* and *Vibrio alginolyticus* **(Eckhard, Schönauer and Brandstetter, 2013; Miyoshi et al., 2008; Nezafat et al., 2015)**.

Peptidase family M9 (MEROPS database) are true collagenases and contains bacterial collagenases from *Vibrio* and *Clostridium*. Active site represents of two histidine zinc ligands and the catalytic glutamate occur in the «HEXXH» motif. Based on structure and function, *Vibrio* proteases are combined into three classes. The *Vibrio* proteases in classes II and III are all members of the subfamily M9A in the MEROPS database and have collagenolytic activity. But they have significant differences in their structure and function. So, the Vibrio class II proteases have a zinc-binding motif «HEYTH», contain no C-terminal domain and cannot hydrolyse casein, while the *Vibrio* class III proteases have a zinc-binding motif «HEYVH», contain PKD-like domain and PPC domain and can hydrolyse casein. *Vibrio* collagenases hydrolyses the Pz peptide (Pz-Pro-Leu-Gly-Pro-D-Arg) by cleaving the same peptide bond Leu-Gly **(Zhang et al., 2015)**.

Peptidase M9 *Aeromonas salmonicida* is unassigned metalloprotease according to the MEROPS database (http://merops.sanger.ac.uk). It has zinc-binding motif «HEYVH» like a *Vibrio* class III peptidase, contain no C-terminal domain like *Vibrio* class II peptidase and presumable cannot hydrolyse casein. The methylotrophic yeast *Pichia pastoris* has become a fundamental tool for food biotechnology, especially for recombinant enzymes production.

Many proteases have been used in meat industry **(Angelovičová et al., 2018)**. A variety of proteases have been successfully expressed in *P. pastoris* **(Rabert et al., 2013; Queiroz Brito Cunha et al., 2018; Silva, Peres and Gattas, 2009; Macauley-Patrick et al., 2005)**, for example alkaline protease from *Aspergillus oryzae* **(Guo and Ma, 2008)**, neutral protease from *Aspergillus oryzae* **(Ke et al., 2012)**, aspartic protease from *Mucor mucedo* **(Yegin and Fernandez-Lahore, 2013)**, chymosin from *Rhizopus microsporus* **(Qian et al., 2017)**. For use in the meat industry, in particular, for meat tenderization, extracellular

aspartate protease has been expressed in *Pichia pastoris* from *Rhizomucor miehei* **(Tyagi et al., 2017)**.

The aim of the study was production of recombinant metallopeptidase *Aeromonas salmonicida* by transformation *Pichia pastoris* for further meat tenderization.

Scientific hypothesis

We are expecting the influence of the recombinant metallopeptidase *Aeromonas salmonicida* on the connective tissue of meat and meat tenderness consequently.

MATERIAL AND METHODOLOGY

The subjects were M9 peptidase gene (ASA_3723) *Aeromonas salmonicida* (laboratory collection strain, isolated from the meat surface), vector plasmid pPic9K (Invitrogen, USA), competent *E. coli* DH5α cells (Invitrogen, USA), competent *Pichia pastoris* GS115 cells (Invitrogen, USA), super-natant from recombinant clones of *Pichia pastoris*, synthetic peptide «Pro-Leu-Gly-Met-Trp-Ser-Arg» («Almabion», Voronezh, Russia), beef shank samples.

Nucleotide sequence analysis

Analysis of the nucleotide sequence encoding the peptide gene *Aeromonas salmonicida* was performed using the NCBI database (https://www.ncbi.nlm.nih.gov). For analysis and comparison of nucleotide sequences, the BLAST program was used, the search for homologous sequences was performed in the GenBank database (http://www.ncbi.nlm.nih.gov/). Bioinformatic analysis of the sequences of the *Aeromonas salmonicida* coding peptidases of genes, the design of the primers was performed using the OligoAnalyzer Tool program (https://eu.idtdna.com/calc/analyzer).

Cloning of a metalloprotease gene

PCR was performed on an ANK-32 (Sintol, Russia) in a reaction mixture containing HS-Fuzz buffer, dNTP, 5'- and 3'-terminal primers, DNA, and HS-Fuzz DNA polymerase (NEB, England). Primers used to obtain the protease gene *Aeromonas salmonicida*: AACAATCTGGGTACAAGGT-forward primer; TCAGTGGGAGGAGTCGTTG-reverse primer. Screening of recombinant clones was performed using the PCR method with standard primers for the pPic9K vector. To isolate and purify the target PCR products, a kit was used to extract DNA from an agarose gel and Cleanup Standard reaction mixtures (Evrogen, Russia). Ligase-free cloning of the obtained PCR fragments into the pPic9K vector plasmid was performed using a FusionTM CF Dry-Down PCR Cloning Kit (Clontech Laboratories Inc, USA) non-gas cloning system. Electroporation *Pichia pastoris* was carried out on an electropor with the following parameters: voltage = 1500, capacitance = 25, resistance = 200.

Electrophoresis

Analysis of recombinant *Pichia pastoris* clones for the production of target proteins. One-dimensional electrophoresis was performed according to the method of Laemmli **(Laemmli, 1970)** in 12% polyacrylamide gel with the presence of SDS.

HPLC-MS/MS

Sample preparing: the substrate «Pro-Leu-Gly-Met-Trp-Ser-Arg» synthetic peptide (one of the collagen chains, (Mw = 846.06) was treated with the supernatant from recombinant *Pichia pastoris* clones in different ratios: sample A – 0.1 mg substrate/300 μL supernatant without inhibitor Protease Inhibitor Cocktail (Roche, Switzerland); sample B – 0.1 mg substrate/300 μL supernatant with inhibitor Protease Inhibitor Cocktail; sample C – 0.02 mg substrate/300 μL supernatant with inhibitor Protease Inhibitor Cocktail; sample D – 0.02 mg substrate/150 μL supernatant with inhibitor Protease Inhibitor Cocktail.

Mass spectral analysis was performed after 1 h, 2 h, 3 h. The reaction was inhibited with EDTA and EGTA solutions – metalloproteases inhibitors. The analysis of peptidase activity was carried out on chromatograph 7890A (Agilent Technologies, USA) with a 5975C VL MSD mass spectrometer (Agilent Technologies, USA). The following parameters of mass spectrometric detection were selected for analysis: source temperature – 100 °C; desolvation gas temperature – 320 °C; desolvation gas flow rate is 8 $dm^3.min^{-1}$; nebulizer needle pressure – 30 psi. The parameters of exposure to ions in the SIM and MRM mode and the conditions of ionization by spraying in an electric field (ESI) with the registration of positive ions: range, m/z 50 – 2000; voltage on fragmenter (Frag), B 70 – 150; dissociation energy (CE), B 0 – 40.

This technique makes it possible to determine the activity of peptidases in reaction with peptides of different sequences. The extent of its degradation was determined by the area of the daughter ions of the desired peptide. The protonated ions were used to determine the molar weight of the peptide degradation products and identify their amino acid sequence.

Histological analyse

Sample preparing: beef shank samples were treated with supernatant by injection (sample E) and immersion (sample F), the exposure time was 24 hours at 18 °C. The control was treated with saline. Further, all samples were fixed in 10% buffered formalin solution for 72 hours. Sections 16 μm thick were made on a MIKROM-HM525 cryostat (Thermo Scientific, USA), mounted on Menzel-Glaser glasses (Thermo Scientific, USA) and stained with Ehrlich haematoxylin and 1% aqueous-eosin solution, then concluded glycerin-gelatin. The study of histological preparations was carried out on an AxioImaiger A1 light microscope (Carl Zeiss, Germany) using the AxioVision 4.7.1.0 computer image analysis system. Magnification on all photos 400x (lens 40x and eyepiece 10x).

Shear force analyse

Sample preparing: beef shank samples were treated with supernatant by injection (sample E) and immersion (sample F), exposure time – 24 hours at 18 °C. Control sample of meat was treated with saline. Shear force measurement of samples was performed using a Shimadzu AGS-X (Shimadzu corporation, Japan) instrument according to the standard method of Warner and Brazler **(Goodson et al.,**

2002). After exposure, some samples were cooked at 70 °C for 3 hours. The shear force measurements were performed in 4 replicates.

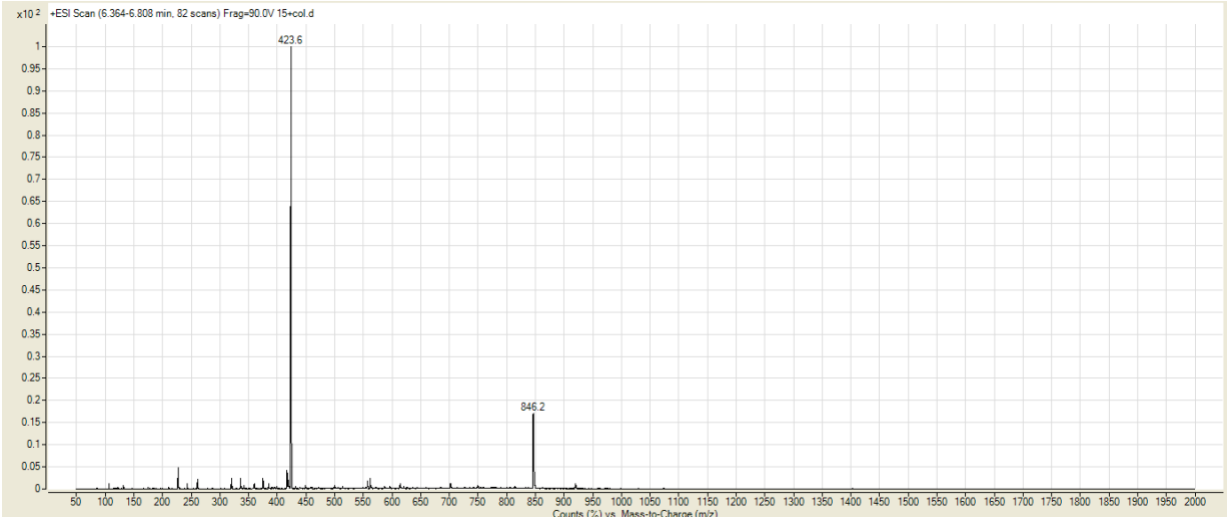

Figure 1 The mass spectrum of the peptide in the mode of selective ion monitoring (SIM) with electrospray ionization (ESI) with registration of positive measurements.

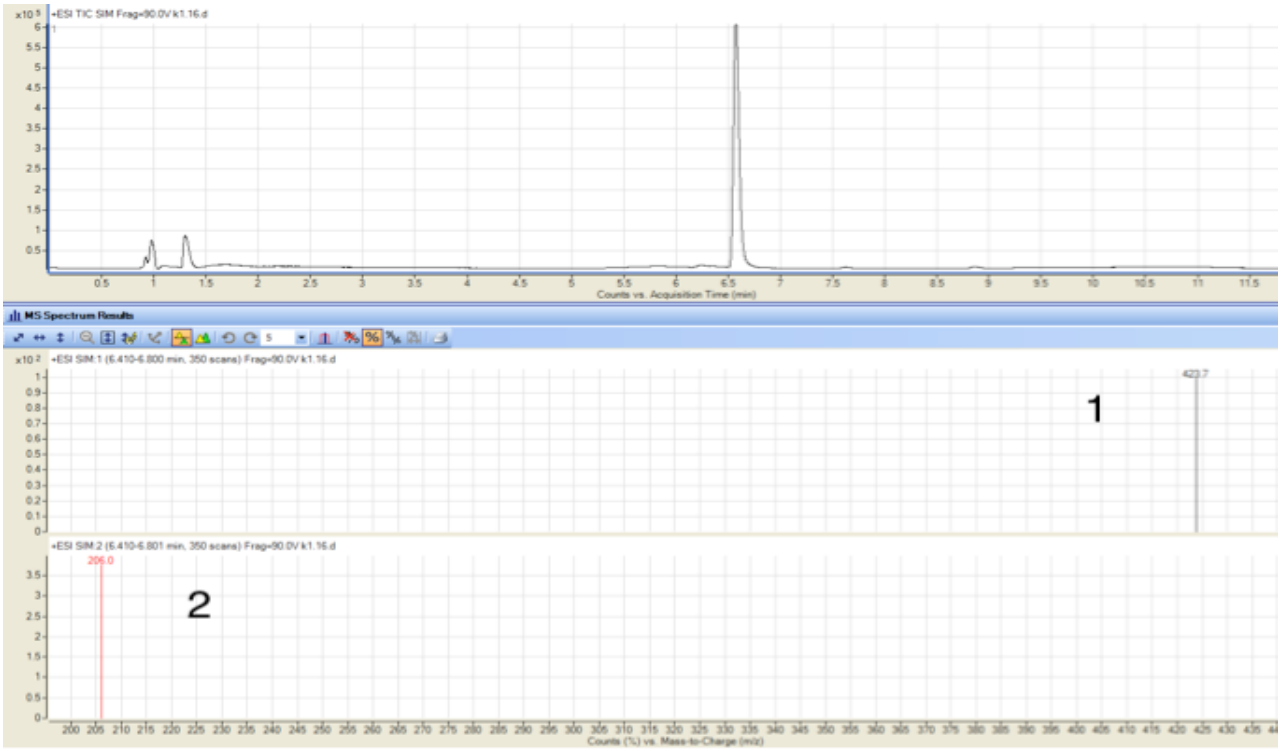

Figure 2 Chromatogram after enzymatic hydrolysis (SIM: 1 – 423.7; 2 – 206.0).

Table 1 Substrate degradation after enzymatic hydrolysis.

Time, min	Final substrate concentration, mol ±*SD*)				
	Control	sample A	sample B	sample C	sample D
60	13455.89 ±0.135*	11817.95 ±0.3531	12974.22 ±0.1641	2962.26 ±0.3602	2441.81 ±0.1302
120		8614.31 ±0.2471	9791.85 ±0.3570	2171.63 ±0.3189	2055.97 ±0.142
180	3547.54 ±0.135**	6310.73 ±0.1123	7195.99 ±0.2794	1759.57 ±0.192	1642.48 ±0.1511

Note: *- Control for A. B samples; **- Control for C. D samples.

Table 2 Result of shear force measurement of supernatant treated beef shank samples.

Cooking degree	Maximum shear force, $N.m^{-2}$ ±*SD*		
	Control	sample E	sample F
Raw beef shank	690242.6^{x} ±2.31671^{b}	413544.9 ±1.18845	639077.86 ±2.53349
Cooking beef shank	184.97 ±2.9245	149.355 ±4.6533	145.175 ±2.06103

Note: ^{X} – average value; ^{b} – standard deviation .

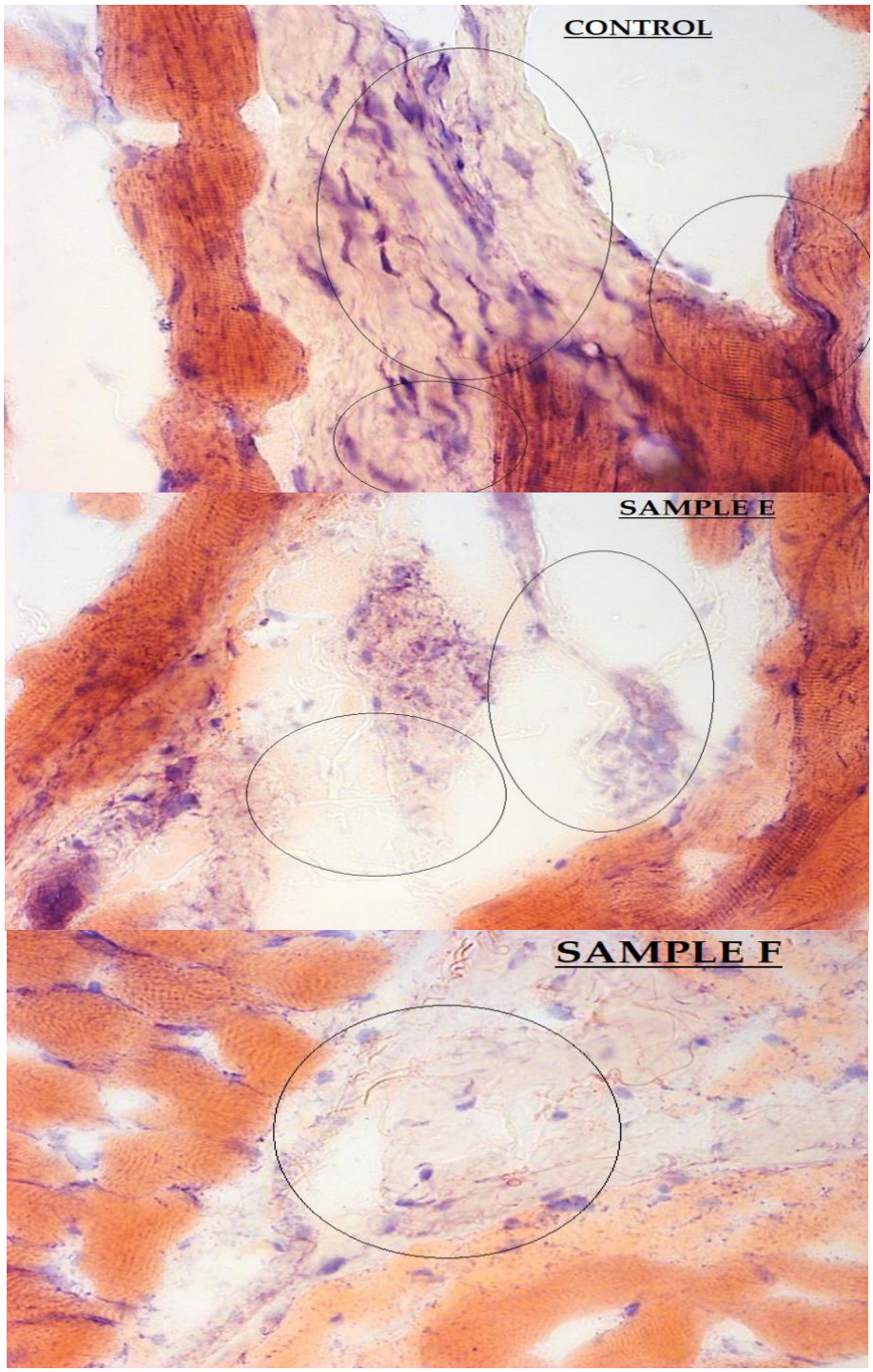

Figure 3 Result of histological analysis of supernatant treated beef shank samples. Note: *Magnification 400x.*

Statistic analysis

StatPlus 6.2.2.0 Software (AnalystSoft) was used. Average value and standard deviation (*SD*) values of shear force index and final peptide concentration was calculated. Statistical significance was determined by the examining the basic differences between values by Z-score. The differences with $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

The active domain of the M9 *Aeromonas salmonicida* peptidase gene was cloned, transformation of *Pichia pastoris* cells was carried out, and supernatant from recombinant clones was accumulated. A 40 kDa band was observed on electrophoresis. Figure 1 shows the mass spectrum of the pure peptide "Pro-Leu-Gly-Met-Trp-Ser-Arg" in the selective ion monitoring mode (SIM) with an electrospray ionization (ESI) with registration of positive reactions. To build the calibration characteristics and the quantitative content of the peptide, m/z 423.6 was chosen as the molecular ion, and m/z 846.2 as the protonated daughter ion. After enzyme hydrolysis, products were obtained from which a peak was found with m/z 206.0. This corresponds to the sum of the mass's leucine-glycine dipeptide (Leu-Gly). The chromatogram of the sample after enzymatic hydrolysis is shown in Figure 2. Such activity can be compared with *Vibrio* collagenases hydrolyses the Pz peptide (Pz-Pro-Leu-Gly-Pro-D-Arg) by cleaving the same peptide bond Leu-Gly **(Zhang et al., 2015)**. For the quantitative determination of the dipeptide (Leu-Gly), the conditions of ionization in the SIM mode were selected. The rate of hydrolysis of the peptide "Pro-Leu-Gly-Met-Trp-Ser-Arg" was studied by reducing the area of the main peak with m/z 423.7 (Table 1). Table 1 shows the comparing the final concentration (mol) of the experimental and control samples, it was found that the concentration of the substrate (peptide) after 180 min significantly decreased by 2.13 times (sample A) and by 1.87 times (sample B). Samples A and B contained the same amount of substrate and were distinguished by the presence of an inhibitor of common proteases in the sample B. A 5-fold decrease in the substrate concentration (sample C) and a 2-fold decrease in the amount of enzyme with 5-fold decrease in the substrate concentration (sample D) did not affect the degree of substrate degradation. The final concentration of the substrate in C and D experimental samples after 180 min decreased by half (Sample C) and by 2.16 times (sample D) as compared with control.

In every case the statistical differences between peptide concentration into group were $p < 0.05$. In this, Z-score was in the range of 1.8 to 2.

Results of the shear force measurement are presented in the Table 2. In all cases, sample E and sample F were softer than the control sample. Sample E in raw form was noticeably softer than the control sample and Sample F. However, after heat treatment, the average values of the shear force of the experimental samples were approximately the same (149.355 $N.m^{-2}$ and 145.175 $N.m^{-2}$). As a result of the shear force measurement, it was established that recombinant collagenase had a significant effect on meat tenderness. On average, the maximum shear force of heat-treated samples had a 1.27 – fold decrease as compared with the control. The authors **(Qian et al., 2017)** described a method for producing recombinant aspartate protease from *Rhizomucor miehei*, expressed in Pichia pastoris, able to find use in softening raw meat. The resulting enzyme had a high peptidase activity (3480.4 $U.mL^{-1}$). The effective of recombinant aspartate protease used in tenderizing pork. But pork had a low connective tissue content as compared with beef. It can be assumed that the tenderization of meat in this case is achieved by the degradation of muscle proteins, rather than connective tissue proteins. The authors **(Antipova and Glotova, 2006)** showed that the enzymes used to improve the quality of meat should have little effect on muscle tissue.

The histological analysis revealed differences between control and experimental samples. In the control the muscle fibers were characterized by a straightened shape, well-defined transverse striation and a fairly dense arrangement in the bundle. The oval-shaped nuclei were located directly under the sarcolemma of the fiber. Wavy connective tissue interimization layer tightly adhered to the bundles of muscle fibers. The nuclei in connective tissue layers were clearly detected on the preparation (Figure 3).

In the experiment sample E, marked detachment of the perimetry from the muscle bundles, its loosening. Also, disintegration of collagen fibrils, their thinning and partial fragmentation were revealed. Muscle fibers have not changed. Changes in the experiment sample F are similar to changes in the sample E, but more pronounced in the surface layers, where some homogenization of fibrillar structures was noted. In the deep layers, the loosening of connective tissue layers was mainly observed. Muscle fibers have not changed (Figure 3).

CONCLUSION

Recombinant metallopeptidase *Aeromonas salmonicida* by transformation *Pichia pastoris* was produced. Laboratory amounts of recombinant peptidase were obtained and test evaluation of enzyme activity was performed. Recombinant peptidase broke the peptide bond «Pro-Leu-Gly-Met-Trp-Ser-Arg» (one of the collagen chains, (Mw = 846.06)). The concentration of the substrate (peptide) after 180 min was 2 – fold decrease as compared with control. The maximum shear force of heat-treated samples had a 1.27 – fold decrease as compared with the control. As a result of histological studies of beef shank samples, the specific effect of the supernatant on the structure of connective tissue was established. Muscle fibers have not changed. The recombinant enzyme could be used for the meat tenderization.

REFERENCES

Angelovičová, M., Mellen, M., Zajác, P., Čapla, J., Angelovič, M. 2018. Tibia mineralization of chickens determined to meat production using a microbial phytase. *Potravinarstvo Slovak Journal of Food Sciences*, vol. 12, no. 1, p. 40-49. https://doi.org/10.5219/805

Antipova, L. V., Glotova, I. A. 2006. *The use of secondary collagen containing raw materials of the meat industry. Improvement and development of new technologies based on the enzymatic processing of collagen-containing raw materials*. SPb : GIORD, 384 p. ISBN 5-98879-007-0.

Bailey, A. J., Light, N. D. 1989. *Connective Tissue in Meat and Meat Products*. London, UK : Elsevier Applied Science, 355 p. ISBN 1851662847.

Bekhit, A. A., Hopkins, D. L., Geesink, G., Bekhit, A. A., Franks, P. 2014. Exogenous proteases for meat tenderization. *Critical reviews in food science and nutrition*, vol. 54, no. 8, p. 1012-31. https:// doi.org/10.1080/10408398.2011.623247

Eckhard, U., Schönauer, E., Brandstetter, H. 2013. Structural basis for activity regulation and substrate preference of clostridial collagenases G, H, and T. *J. Biol Chem.*, vol. 288, p. 20184-20194. https://doi.org/10.1074/jbc.M112.448548

Goodson, K. J., Morgan, W. W., Reagan, J. O., Gwartney, B. L., Courington, S. M., Wise, J. W., Savell, J. W. 2002. Beef

customer satisfaction: Factors affecting consumer evaluations of clod steaks. *Journal of Animal Science*, vol. 80, no. 2, p. 401-408.

Guo, J., Ma, Y. 2008. High-level expression, purification and characteriza-tion of recombinant Aspergillus oyrzae alkaline protease in Pichia pastoris. *Pro-tein Expression and Purification*, vol. 58, p. 301-308. https://doi.org/10.1016/j.pep.2007.12.005

Chanalia, P., Gandhi, D., Attri, P., Dhanda, S. 2018. Extraction. purification and characterization of low molecular weight Proline iminopeptidase from probiotic L. plantarum for meat tenderization. *International journal of biological macromolecules*, vol. 1, no. 109, p. 651-663. https://doi.org/10.1016/j.ijbiomac.2017.12.092

Ke, Y., Huang, W., Li, J., Xie, M., Luo, X. 2012. Enzymatic characteristics of a recombinant neutral protease I (rNpI) from Aspergillus oryzae expressed in Pichia pastoris. *Journal of Agricultural and Food Chemistry*, vol. 60, p. 12164-12169. https://doi.org/10.1021/jf303167r

Kotenkova, E., Polishchuk, E. 2019. Assessment of antimicrobial potential of substances isolated from some wastes of meat processing industry. *Potravinarstvo Slovak Journal of Food Sciences*, vol. 13, no. 1, p. 308-313. https://doi.org/10.5219/1079

Laemmli, U. K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, vol. 227, p. 680-685. https://doi.org/10.1038/227680a0

Light, N., Champion, A. E. 1984. Characterization of muscle epimysium, perimysium and endomysium collagens. *Biochem. J.*, vol. 219, no. 3, p. 1017-26. https://doi.org/10.1042/bj2191017

Macauley-Patrick, S., Fazenda, M. L., McNeil, B., Harvey, L. M. 2005. Heterologous protein production using the Pichia pastoris expression sys-tem. *Yeast*, vol. 22, p. 249-270. https://doi.org/10.1002/yea.1208

Miyoshi, S., Nitanda, Y., Fujii, K., Kawahara, K., Li, T., Maehara, Y., Ramamurthy, T., Takeda, Y., Shinoda, S. 2008. Differential gene expression and extracellular secretion of the collagenolytic enzymes by the pathogen *Vibrio parahaemolyticus*. *FEMS microbiology letters*, vol. 283, no. 2, p. 176-81. https://doi.org/10.1111/j.1574-6968.2008.01159.x

Nezafat, N., Negahdaripour, M., Gholami, A., Younes, G. 2015. Computational analysis of collagenase from different Vibrio, Clostridium and Bacillus strains to find new enzyme sources. *Trends in Pharma-ceutical Sciences*, vol. 1, no. 4, p. 213-222. https://doi.org/10.1111/tips.v1i4.52

Qian, S., Chen, F., Geng, F., Luo, Y., Gong, S., Jiang, Z. 2017. A novel aspartic protease from Rhizomucor miehei expressed in Pichia pastoris and its application on meat tenderization and preparation of turtle peptides. *Food Chemistry*, vol. 245, p. 570-577. https://doi.org/10.1016/j.foodchem.2017.10.113

Queiroz Brito Cunha, C. C., Gama, A. R., Cintra, L. C., Bataus, L. A. M., Ulhoa, C. J. 2018. Improvement of bread making quality by supplementation with a recombinant xylanase produced by Pichia pastoris. *PLoS ONE*, vol. 13, no. 2, p. e0192996. https://doi.org/10.1371/journal.pone.0192996

Rabert, C., Weinacker, D., Pessoa, A., Farías, J. G. 2013. Recombinants proteins for industrial uses: utilization of Pichia pastoris expression system. *Brazilian Journal of Microbiology*, vol. 44, no. 2, p. 351-356. https://doi.org/10.1590/S1517-83822013005000041

Silva, V. C., Peres, M. F. S., Gattas, E. A. L. 2009. Application of methylotrophic yeast Pichia pastoris in the field of food industry - A review. *Journal of Food, Agriculture and Environment*,vol. 2, p. 268-273.

Tyagi, A., Kumar, A., Mohanty, A. K., Kaushik, J. K., Grover, S., Batish, V. K. 2017. Expression of buffalo chymosin in Pichia pastoris for application in mozarella cheese. *LWT - Food Science and Technology*, vol. 84, p. 733-739. https://doi.org/10.1016/j.lwt.2017.06.033

Yegin, S., Fernandez-Lahore, M. 2013. A thermostable aspartic proteinase from Mucor mucedo DSM 809: gene identification, cloning, and functional expression in Pichia pastoris. *Molecular Biotechnology*, vol. 54, p. 661-672. https://doi.org/10.1007/s12033-012-9608-6

Zhang, Y. Z., Ran, L. Y., Li, C. Y., Chen, X. L. 2015. Diversity, structures, and collagen-degrading mechanisms of bacterial collagenolytic proteases. *Applied and Environmental Microbiology*, vol. 81, no. 18, p. 6098-6107. https://doi.org/10.1128/AEM.00883-15

Zhao, G. Y., Zhou, M. Y., Zhao, H. L., Chen, X. L., Xie, B. B., Zhang, X. Y., He, H. L., Zhou, B. C., Zhang, Y. Z. 2012. Tenderization effect of coldadapted collagenolytic protease MCP-01 on beef meat at low temperature and its mechanism. *Food Chemistry*, vol. 134, no. 4, p. 1738-1744. https://doi.org/10.1016/j.foodchem.2012.03.118

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