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Detection of protein aggregation markers in raw meat and finished products

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Abstract: The effect of animal and plant proteases as well as starters, or starter cultures, on protein aggregates formation in raw pork and beef as well as meat products was studied. The proteomic analysis of raw meat revealed that animal proteases – pepsin and trypsin – caused the aggregation of isoform 2 of protein 1 containing 4.5 LIM domains. Vacuum packaged meat showed the same results during storage, while unpacking led to the acceleration of the aggregation process due to autolysis. In addition, mixed aggregated fragments, such as muscle creatine phosphokinase and glutathione-S-transferase, actin and perilipin, and type II keratin appeared in those samples. Starters with *Pediococcus pentosaceus* 31 from the Russian National Collection of Industrial Microorganisms (VKPM-8901) caused myoglobin and troponin I aggregation, while the formation of soy proteins aggregates (glycinin G1 and glycinin A3B4) was detected in meat products as a result of the autolysis process and the use of cholesterol-lowering starters. All in all, proteases which cause protein aggregation may be less effective for raw meat tenderization, whereas the proteins identified may be used as quality biomarkers.

Keywords: Proteins, meat, proteases, aggregates, biomarkers

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INTRODUCTION

Protein aggregation in this paper is defined as the interaction of denatured protein molecules resulting in the formation of weak or strong (for example, disulfide) molecular linkages. These linkages contribute to larger particles formation. Mostly, protein aggregation in meat occurs as a result of thermal denaturation when heating or autolysis in the rigor mortis stage.

Denatured and then aggregated proteins become less soluble and lose their biological properties. Rigor mortis also causes changes which lead to protein aggregation.

On the other hand, proteolytic enzymes are capable of weakening intermolecular interactions in proteins and provide proteolysis, which contributes to tissue tenderization [1]. Both proteolytic and meat enzymes act on protein substrate fragments and thereby influence protein components structures. The enzymes facilitate aggregates dissotiation, free SH-groups formation, and muscle tissue properties partial recovery after denaturation or rigor mortis. As a result of the enzyme impact, meat becomes tender, juicy, with pronounced taste and aroma.

The proteolytic activity of lactic-acid bacteria strains against sarcoplasmic and myofibrillar proteins was studied by different research groups [2-4].

In 2015, the hydrolysis of myofibrillar proteins during the fermentation of sausage models with starters or their mixture was studied by Lopez *et al.* [2]. The researchers used such starters as *Lactobacillus curvatus* CRL705 and *Staphylococcus vitulinus* GV318. The results had shown that the mixed culture accelerated proteolysis significantly. Thus, two-dimensional electrophoresis confirmed the hydrolysis of actin, myosin light chain 1/3, myosin light chain 2, and myosin heavy chain. 33 peptides from troponin T, myosin light chain 2, and, in particular, from actin were also identified by means of LC-MS/MS. In the matter of actin primary structure,

three of its regions were very sensitive to degradation. Although the inoculation of the mixed starter cultures accelerated proteolysis, peptides and amino-acids contents were reduced while *L. curvatus* CRL705 alone increased the abovementioned contents.

In 2018, De Almeida *et al.* [3] evaluated the ability of four species of lactic acid microorganisms to form low molecular weight peptides in a sausage model with a low sodium content. As a whole, 86 low molecular weight peptides obtained mainly from myofibrillar proteins were identified (56 of them – from actin). The inoculation of *L. curvatus* CRL705 and *Enterococcus mundtii* CRL35 led to the formation of 56 and 43 low molecular weight peptides, respectively. Larger quantity of peptides per unit time was released when using *Lactobacillus plantarum* and *L. sakei* in comparison to other models. Each strain in the models allowed creating the unique profile of small peptides and amino acids which might be used as a biochemical characteristic to differentiate certain fermented foods.

In the research conducted by Basso *et al.* [4], *Lactobacillus sakei* (DSM 6333), *Lactobacillus plantarum* (B21), and *L. farciminis* (DSM 20184) have demonstrated proteolytic activity to meat sarcoplasmic proteins. Thus, the 94kDa-band disappeared in samples with *L. farciminis* and *L. plantarum* and narrowed significantly in samples with *L. sakei.* Bands with a molecular mass from 94 kDa to 38 kDa decreased in all samples. According to capillary electrophoresis analysis, no signals corresponding to the retention time of 8.64 and 8.66 minutes were observed in all the samples. Moreover, MALDI-TOF MS method identified 94-kDa and 38-kDa bands as an isoform of muscle glycogen phosphorylase and glyceraldehyde-3-phosphate dehydrogenase.

In 2001, Italian researchers [5] obtained controversial results on the proteolytic activity of 27 *Staphylococcus xylosus* strains to sarcoplasmic and myofibrillar proteins. Neither electrophoretic nor spectrometric analysis confirmed the proteolytic activity of some strains. SDS-polyacrylamide gel electrophoresis revealed changes in pork protein profile for only 12 out of 24 strains which were able to hydrolyze muscle protein extracts on agar.

Montowska *et. al.* [6] has proved a favorable effect of trypsin along with super-high frequency treatment on rapid and efficient recovery of peptide markers in meat products. Meat was subjected to heating – considerable protein aggregation was observed – and then analyzed by means of tandem mass spectrometry to detect specific peptide markers. It was established that treatment of heat-treated product with trypsin for one hour contributes to the structure recovery of desirable peptide markers which could be easily identified using the known methods.

Ageing effects meat taste, tenderness, water-binding capacity (WBC), colour, and juiciness. The monitoring of biochemical processes occurring during meat ageing allows meat products quality biomarkers to be revealed [7]. Change in the proteome of muscle exudate from genetically similar pigs with the same meat characteristics within a regular ageing period, namely, 7 days, was studied [1]. It has been found that some quality characteristics of meat such as meat tenderness, cooking losses as well as the colour index CIE b* change considerably due to autolysis, notably, at the end of the ageing process. These data illustrated structural changes in the pork meat during the ageing process, which affected proteomic profiles. Three main groups of proteins which changed significantly during the ageing were detected: stress proteins, metabolic enzymes, and structural proteins. Undoubtedly, proteolysis plays a key role in protein fragmentation (for instance, enolase and titin). It has been also observed that the number of stress proteins increase. To monitor these changes, myofibrillar or sarcoplasmic proteomic fractions are usually used. However, using the muscle exudate as a more available substrate allowed previous research to be expanded. For example, it has been discovered that vinculin and peroxiredoxin-6 correlate to the WBC and the tenderness of meat, respectively. Such protein biomarkers are useful for monitoring the fresh meat quality and forecasting the autolysis course.

Oxygen, contained in meat tissues, also takes part in the formation of meat raw materials texture, namely, tenderness and juiciness: a high oxygen level contributes to protein intermolecular cross-linking and aggregates formation [9, 10].

The results of the research conducted by Moszkowska *et. al.* [11] have shown the formation of protein aggregates in the muscle tissue packaged in an oxygen-containing atmosphere. These aggregates, which consist of myosin heavy chains, are the result of cross-linking proteins and are able to affect the meat tenderness adversely. Aggregate formation depends not only on a type of packaging but also on a method of packaging. Thus, larger aggregates were observed in *biceps femoris* muscles in comparison to *longissimus lumborum* muscles and in meat packaged in the modified gas atmosphere (MGA) rather than in vacuum. It may be explained by the metabolism of muscles analyzed due to differences in postmortem glycolysis extent, the characteristics of muscle fibers and their glycolytic and oxidative properties.

Both SDS-gel electrophoresis and diagonal-PAGE, as well as immunoblotting, demonstrated the oxidative cross-linking of myosin heavy chains in meat packaged in MGA with high oxygen content. Moreover, aggregates of myosin heavy chains (MHC) were also detected in other packages containing high oxygen concentration (MGA and vacuum + MGA); apparently, protein oxidation occurred which led to myosin binding due to its polymerization [12]. Such an aggregation of MHC, occurred under severe temperature conditions (190°C), and took place in both raw meat and finished meat products [6].

The aggregate formation can be related not only to oxidation but to proteolysis as well. In this work, we identified a number of protein aggregates of different origin formed as a consequence of proteolytic and oxidative processes.

STUDY OBJECTS AND METHODS

In this research, we studied *m. longissimus dorsi* samples from Bos Taurus before and after treatment

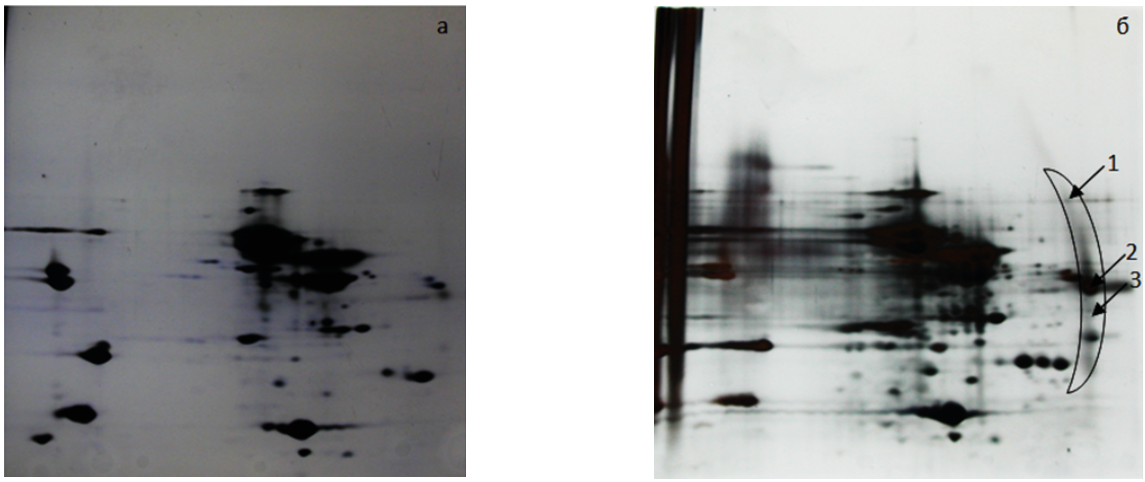

Fig. 1. Two-dimensional electrophoregrams of bovine skeletal muscle tissue samples: (a) control; (b) treated with trypsin. Silver nitrate staining. Arrows denote monomeric form, ellipse denotes track of protein 1, isoform 2 containing 4,5 LIM domains.

with proteolytic enzyme of animal and plant origin and a number of starters. Whole muscle of 500 g was injected with proteases solutions in the amount of 5 ml per 50 g raw meat and kept at 30°C for 40 min for trypsin and pepsin, and for 30 min for papain and bromeline treatment. In case of starter cultures, a sample was kept in vacuum at 11°C for 9 days.

We used 1.5% pig pepsin and bovine trypsin solutions (Himedia, India, 10, 000 NFU/mg and 2, 000 U/g, respectively) and 0.5% papain and bromeline solutions (Sigma, USA, 1.1 U/mg solid and 2, 370 U/g prot., respectively). The starter suspensions concentration was determined as 10^9 CFU/ml using McFarland standard. The following starters were used: *Pediococcus pentosaceus* 31 (VKPM-8901), *Pediococcus acidilactici* 38 (VKPM-8902), *Lactobacillus sakei* 105 (VKPM-8905), and *Lactobacillus curvatus* 2 (VKPM-8906).

Another set of experiments was carried out using uncooked smoked sausage samples. The control sample did not include starters. The experimental sample no. 1 had starters from the Microorganisms Collection of Moscow State University of Food Production. The experimental sample no. 2 was prepared using starters from Bactoferm SM 194 Chr. Hansen, Denmark, such as *Pediococcus pentosaceus*, *Staphylococcus carnosus*, *Staphylococcus xylosus*, *Lactobacillus sake*, and *Debaryomyce shansenii*. Uncooked smoked sausage for the experiment was manufactured by a halal meat production factory in the Moscow region. It included such ingredients as beef of best quality, beef and horse meat of good quality, soy granules, beef external fat, vegetable fiber with a coloring agent, nitrite salt, and spices.

In the third set of experiments, skeletal muscles of Bos Taurus (*m. longissimus dorsi*) were separated from a carcass in 30 min after slaughter and vacuum-packaged into individual packages. The samples were kept at ± 2°C for 4 and 7 days until analyzed.

Protein fractionation, colouring, computer densitometry, and mass-spectrometric identification were carried out as described in previous research [13, 14]. Protein identification was financially supported by the Ministry of Education and Science of the Russian Federation and performed in Shared Knowledge Center 'Industrial biotechniques' (RFMEFI62114X0002).

RESULTS AND DISCUSSION

The enzyme preparations listed are widely used in the meat industry. For instance, the American Food and Drug Administration of the Department of Health Protection and Human Services generally recognized five exogenous enzymes as safe (GRAS) to use in the meat industry. These are papain, bromeline, ficin, and *Aspergillus oryzae* and *Bacillus subtilis* proteases [15]. Therefore, a potential formation of complexes limiting the exoprotease usage due to their reduced effectiveness in meat is of great interest.

The silver nitrate staining of a bovine skeletal muscle treated with trypsin and pepsin revealed a protein track with molecular mass of 15–100 kDa in the alkaline zone of pH gradient (Fig. 1). The track was not detectable in staining with CBB R-250. The former staining technique is 100 times more sensitive than the latter one, which means a small quantity of the protein involved. The analysis of gel fragments from upper and lower zones of this track revealed isoform 2 of protein 1 containing 4.5 LIM domains, *FHLI* gene. The monomeric form of the protein corresponded completely to its physical and chemical parameters (molecular mass and pH) and contained more than 90% of protein substances in the track.

The amino acid sequence of the protein does not exceed 32 kDa in mass, whereas that in the track was considerably wider. It implies that the proteolytic treatment causes aggregates formation that gives an unusual electrophoretic track. The analysis of vacuum-packaged skeleton muscles of Bos Taurus also detected an identical track of the protein by day 4 of storage that decreased by day 7 as a consequence of the autolytic process. After unpacking, the trace formation process tended to accelerate during 3 days, which confirms that both proteolytic enzymes and free oxygen take part in this process.

The track formation can be explained by the fact that animal proteases act in injection sites, and the stage of

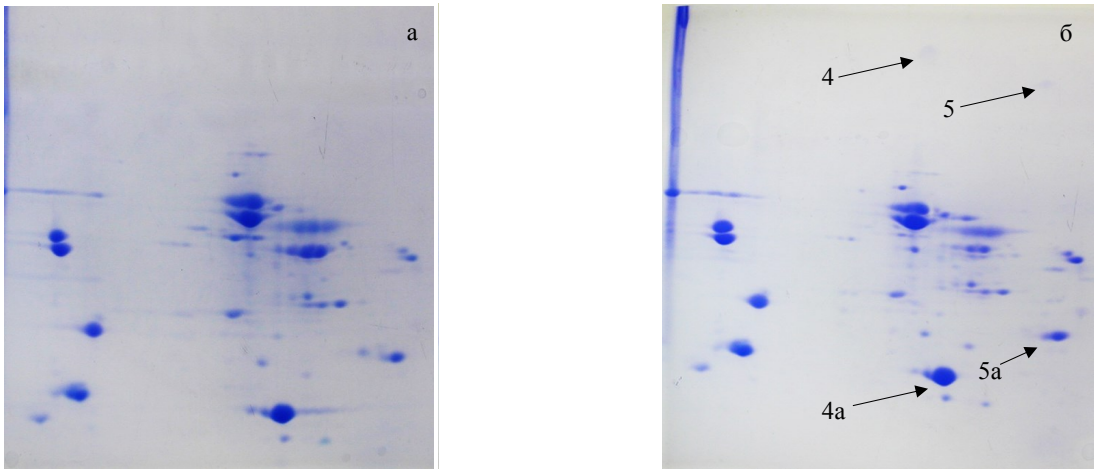

Fig. 2. Two-dimensional electrophoregrams of bovine skeletal muscle tissue samples: (*a*) control; (*b*) treated with *Pediococcus pentosaceus* 31 VKPM-8901. CBB R-250 staining. Arrows denote protein aggregates, arrows with letters denote their monomeric forms.

The track formation can be explained by the fact that animal proteases act in injection sites, and the stage of aggregates formation is impossible to record. However, one of the proteolysis stages is the removing of similar molecules. This implies that a track with reduced mass is formed which can be detected only at low protease concentrations, i.e. at some distance from injected sites. Plant proteases do not have such an effect, probably, because their pH optimums are more adapted to raw meat conditions.

Thus, bromeline resulted in the formation of five high molecular fractions of myosin heavy chains fragments with molecular weights of 170, 150, 60, and 44 kDa, respectively. Consequently, polypeptides with a high molecular weight, a few short peptides, and a small number of free amino acids are formed as a result of bromeline action. Bromeline has a low actomyosin affinity. The data resulted coincide with those of Istrati *et. al*. [8].

Another case of protein aggregates formation was detected when using *Pediococcus pentosaceus* 31 VKPM-8901as a starter. Fig. 2 demonstrates weight high molecular protein aggregates (160–200 kDa) that were identified as myoglobin and troponin (Table 1). Their subunits molecular weights did not exceed 21.5 kDa, and their monomers were represented in large quantities on the two-dimensional electrophoregram. In addition, fractions with much higher weight (up to 3% of monomers) were also recorded.

In vacuum-packaged skeletal muscle from Bos Taurus, aggregates of other proteins were also revealed (Fig. 3). Aggregates of mixed fragments, such as muscle creatine phosphokinase and glutathione-S-transferase, actin and perilipin, and type II keratin, appeared. A cause of their formation might be in a low oxygen concentration and naturally occurring autolysis. These factors slow down the aggregates formation process through the removal of structurally similar molecules complex from submolecular structures, which can be seen on the electrophoregrams.

Aggregation was also observed in uncooked smoked sausage samples with starters from the MSUFP collection (Fig. 4). Two of the samples contained soy protein aggregates (soy was used in the sausage formulation). The control sample (without starters) included two aggregates of glycin G1 fragments. The sample with starters had glycin G1 fragments and a glycinin A3B4 aggregate, and the third sample did not contain aggregates. These results demonstrate that the presence of

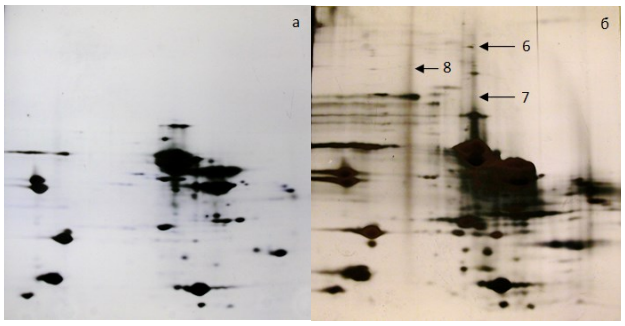

Fig. 3. Two-dimensional electrophoregrams of vacuum-packaged bovine skeleton muscle: (*a*) control; (*b*) day 7 of storage. Silver nitrate staining.

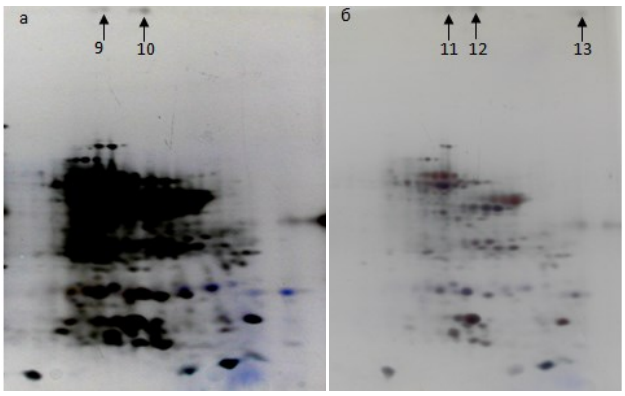

Fig. 4. Two-dimensional electrophoregrams of protein fragments of uncooked smoked sausages samples with aggregates detected: (*a*) control; (*b*) experiment. Silver nitrate staining.

Table 1. Results of mass-spectrometric identification (MALDI-TOF MS and MS/MS) of protein aggregates fractions

№	Protein name (gene symbol)	Numbers in Protein NCBI	S/M/C *	Мм/pI (exp.)**	Мм/pI (calc.)**
1	Aggregate of fragments (60–278 in amino acid sequence) of isoform 2 of protein 1 containing 4.5 LIM domains (FHL1)***(2)	NP 001106730.1	138 / 4 / 15	60.0 / 8.20	31.9 / 8.76
2	Isoform 2 of protein 1 containing 4.5 LIM domains (FHL1)	NP 001106730.1	120 / 26 / 63	31.0 / 40	31.9 / 8.76
3	Aggregate of fragments (60–278 in amino acid sequence) of isoform 2 of protein 1 containing 4.5 LIM domains (FHL1)***(1)	NP 001106730.1	95 / 5 / 20	24.0 / 8.50	31.9 / 8.76
4	Aggregate of myoglobin (MB)***(2)	NP 776306.1	159 / 14 / 75	200.0 / 7.30	17.1 / 6.90
5	Aggregate of fast skeletal muscle troponin I (TNNI2)	XP 00503574.1	129 / 17 / 48	160.0 / 8.20	21.4 / 8.88
6	Aggregate of muscle creatine phosphokinase and glutathione-S-transferase P (GSTP1)	AAD30974.1 NP 803482.1	150 /14 / 43 88 / 7 / 60	180.0 / 6.70	43.0 / 6.63 23.6 / 6.89
7	Aggregate of skeletal muscle alpha-actin (ACTA1) и S-terminal of perilipin 4 fragments (LOC510990)***(1)	AAI34666.1 XP 015327565.1	189 / 17 / 54 177 / 9 / 14	67.0 / 6.80	42.0 / 5.23 102.1 / 5.97
8	Aggregate of type II keratin fragments	OO220327.3	51 / 6 / 11	160.0 / 5.40	66.0 / 8.16
9	Aggregate of glycinin G1 fragment 324–493 [Glycine soy] (GY1)	KHN10744.1	159 / 14 / 37	300.0 / 6.70	55.8 / 5.95
10	Aggregate of glycinin G1 fragment 324–493 [Glycine soy] (GY1)	KHN10744.1	159 / 13 / 34	300.0 / 7.30	55.8 / 5.95
11	Aggregate of glycenin G1 fragment 322–492 [Glycine soy (GY1)	KHN10744.1	153 / 12 / 29	300.0 / 6.70	55.8 / 5.95
12	Aggregate of glycenin G1 fragment 324-436 [Glycine soy] (GY1)***(1)	KHN10744.1	129 / 5 / 15	300.0 / 7.30	55.8 / 5.95
13	Aggregate of glycenin G1 fragment 375–503 [Glycine max] (Gly A3B4)***(1)	BAA19059.1	135 / 6 / 17	280.0 / 9.20	58.2 / 5.46

*S/M/C is characteristics widely used for mass-spectrometric analysis: Score is suitability mark or 'score record'; Match peptides is number of coincided peptides; Coverage is percentage of amino acid sequence covered by identified peptides.
**Mm/pl (exp.) is experimental values obtained on the basis of electrophoretic mobility on DE, and Mm/pI (calc.) is values calculated from amino acid sequence data, taking into account the signal peptide removal but with no consideration for other postsynthetic modifications, using 'Compute pI/Mm tool' software (ExPASy).
***msms is the reference to identification confirmed by means of mass-spectrometry (brackets include number of sequenced tryptic peptides).

oxygen and the use of several types of proteases make aggregates formation with plant proteins possible.

CONCLUSION

According to the data obtained, only certain proteases provoked protein aggregation, and the presence of free oxygen accelerated the process. The proteases affected several types of raw meat proteins and also plant proteins in meat products (in particular, soy ones). Additionally, some proteins were also involved but one-dimensional electrophoresis was not able to detect them because of small quantities. Those proteins had alkali pI, therefore the use of immobilized pH gradients was useless to detect them as opposed to ampholines non-equilibrium pH gradient electrophoresis. The results obtained, along with those of previous research [12, 14], allowed us to assume that cross linkages formation and the aggregation of muscle proteins, primarily myosin, tended to reduce meat water-binding capacity, juiciness, and tenderness and affected meat flavor adversely. The negative impact of protein polymerization on the calpain activity also might retard the tenderization process in early proteolysis in the presence of free oxygen [12].

Apparently, those proteases which led to protein aggregate formation were less effective for meat tenderization and formation of consumer characteristics of finished meat products.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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