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GENERATION OF BIOACTIVE PEPTIDES IN MEAT RAW MATERIALS EXPOSED TO PROTEASES OF DIFFERENT ORIGIN

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Abstract

There are plenty of methods nowadays used for the technological correction of meat raw materials and increasing the functionality of meat products. Among them, the enzymatic hydrolyses using proteases of microbial, plant or animal origin causes considerable interest. Sarcoplasmic and myofibrillar proteins of meat products are subjected to proteolysis during enzymatic treatment, resulting in the peptides generation, including those with high physiological activity and a pronounced therapeutic and preventive effect. Usually they are low molecular weight compounds consisting of several amino acid residues. It should be noted that, unlike drugs, such peptides are able to rapidly penetrate the gastrointestinal tract membranes and further into the bloodstream and the rest of the organism. The aim of this work was to study the bioactive peptides generation in various meat raw materials due to enzymatic hydrolyses by enzymes of animal (pepsin, trypsin) and plant (papain, bromelain) origin. *Bos taurus* and *Sus scrofa* skeletal muscle samples were injected with 5 ml of proteases in 50 g of raw meat. The samples were kept for 40 min at 30 °C for exposure to trypsin and pepsin solutions, 30 min at 30 °C for papain and bromelain. The optimum pH value was not established for the enzymes in the samples, the fermentation process was carried out at the native pH of the raw meat. Humidity in the room was 50-55 %. As a control, the corresponding muscle samples without enzymatic treatment were used. According to the results of a one-dimensional gel electrophoresis under denaturing conditions using sodium dodecyl sulfate (SDS-PAGE), obvious quantitative differences were found in the protein profiles of the studied raw materials, and different profiles were obtained for proteases of different origin. A subsequent two-dimensional isoelectric focusing electrophoresis in an ampholine pH gradient (IEF-PAGE) confirmed the obtained results and revealed the used proteases features. Thus, animal proteases, possessing high specificity, affect only a certain part of muscle proteins, but almost entirely destruct them to small peptides (including low molecular weight) and free amino acids. In addition, a greater proteolytic activity of trypsin compared to pepsin was noted. Plant origin proteases affect the majority of muscle proteins with a sufficiently low specificity and destruct them to many fragments, as evidenced by the presence of numerous new protein fractions on 2D-electrophoregram. Using mass spectrometry (MALDI-TOF MS and MS/MS) some short peptides were detected and identified in samples treated with animal proteases. It was practically not possible to detect short peptides after plant proteases treatment due to the insufficient suitability of mass spectrometry to determine the very low molecular weight of generated peptides. Thus, plant origin proteases generate intermediate fractions of some muscle proteins and bioactive peptides more actively and efficiently. The raw materials processing by proteolytic enzymes, in our opinion, can be regarded as the most effective way to obtain biologically active peptides.

Keywords: proteolytic enzymes, enzymatic hydrolyses, biologically active peptides, one-dimensional gel electrophoresis, SDS-PAGE, two-dimensional electrophoresis, IEF-PAGE, MALDI-TOF, mass spectrometry identification

The increase in meat produced in large modern commercial livestock complexes leads to more meat raw with non-traditional technological characteristics. This actualizes the problem of effective meat processing. E.g., intensive pig exploitation and selection for meatiness, keeping without walking and in large groups, early weaning of piglets, significant fluctuations of the microclimate, etc., exceed the adaptive capabilities of animals, reduce their resistance to technological stress and adversely affect meat quality [1]. Hypodynamia, intensive fattening during commercial growing and breeding for meatiness which leads to disturbances of glycogen metabolism are generally accepted causes of exudativeness and dark sticky meat [2]. Targeted enzymatic modification improves nutritional and biological value of such meat providing its more effective use [3-5].

In the last decade, special attention has been paid to the substances of a protein nature, the biologically active peptides [6]. Their presence in raw materials and finished meat products contributes to better digestion of proteins of animal origin [7-9]. Myofibrillar proteins disintegrate into polypeptides under the action of endogenous muscle enzymes, primarily cathepsin D (at low pH), and further into peptides and free amino acids. Peptides are decomposed by endogenous and microbial enzymes into free amino acids, and the degradation is primarily determined by pH values [10].

The proteolytic activity of lactic acid bacteria strains was studied on meat raw materials, including sarcoplasmic and myofibrillar proteins [11-13]. Thus, under the influence of cells and cell extracts of the strain *Lactobacillus plantarum* CRL 681, originally isolated from meat products, the proteolysis of both sarcoplasmic and myofibrillar proteins occurred with the formation of various peptides of hydrophobic nature. During the proteolysis of myofibrillar proteins, the amount of lysine, arginine, and leucine increased to the maximal level, whereas sarcoplasmic proteins mainly released alanine [11]. Similar study was carried out for *L. curvatus* CECT 904 and *L. sakei* CECT 4808. Both strains expressed proteolytic activity towards sarcoplasmic proteins. Adding cells resulted in peptide degradation, whereas adding cell extracts provided formation of hydrophilic and hydrophobic peptides. In addition, the level of produced free amino acids was higher for *L. sakei* strain [12]. The same authors evaluated activity of *L. casei* CRL 705-derived proteinase and aminopeptidase towards muscle proteins. Proteinases of whole cells cleaved sarcoplasmic proteins to a wide range of peptides; partial hydrolysis was also connected with cell extracts. In mixing cells and cell extracts with sarcoplasmic protein extracts, the peptide profiles changed significantly and production generation of free amino acids was higher [13].

Native amino acid sequences, as well as those formed during the autolysis, enzymolysis of peptides, during thermal and other technological treatments, can be functionally active. Bioactive peptides with hypotensive, opioid, antioxidant, antithrombotic, antimicrobial, immunomodulatory and other biological activities, which have a therapeutic or prophylactic effect on the pathogenesis of a number of diseases, are found and studied [14, 15]. Since most of the currently known bioactive peptides do not penetrate from the digestive tract into the blood, their action is probably mediated through the receptors of the intestinal epithelium or is carried out directly in the intestinal lumen [15, 16]. Such peptides are released and activated during digestion in the gastrointestinal tract or during meat processing [17]. In particular, proteolysis (drying, fermentation) in the course of industrial processing releases ACE-I inhibitors and peptides with

antioxidant activity [18]. The activity of peptides depends on the amino acid composition, molecular weight, chain length, type and charge of the amino acid at the N- and C-terminus, hydrophobic and hydrophilic properties, spatial structure, etc. For example, peptides with higher ability to inhibit the angiotensin-converting enzyme (ACE) usually have aromatic or alkaline amino acids at the N-terminus, a greater amount of hydrophobic and positively charged amino acids at the C-terminus [19]. The relationship between activity and peptide structure is currently under study. Many bioactive peptides of natural origin are structurally different from the peptides resulting from the post-translational modification of proteins. They contain non-protein amino acids (β-alanine, γ-aminobutyric acids), D-amino acids, and alkylated amino acids. H-peptide bonds and ring structures are characteristic of peptides with low molecular weight. Together with pyroglutamic acid residues, they provide protection against the proteases with substrate specificity to peptides containing α-amino acids with normal bonds, which makes it possible to preserve the functionality of the peptide until it is digested [3, 14]. Many peptides containing 2-9, less often up to 25 amino acid residues in a strictly determinate sequence [20], are extracted from meat raw materials, and many of them are obtained by proteolysis with enzymes of various origins.

Pepsin, a member of the peptidase family A1, is the predominant digestive protease in the gastric juice of the vertebrates. Unlike some other endopeptidases, it hydrolyzes only peptide bonds, but not non-peptide amide or ester bonds. When an unpurified myosin light chain was treated with pepsin, an ACE inhibitory octapeptide was detected, which was evaluated as a temporarily effective hypotensive substance [21]. Another peptide, obtained by hydrolysis with pepsin of porcine myosin, retained ACE inhibitory activity after heating myosin B at 98 °C for 10 min [22]. It was proven that bioactive peptides can be obtained not only from myofibrillar proteins but also from regulatory proteins, such as troponin and tropomyosin. In 2003, the ACE-inhibitory peptide was isolated from porcine troponin C, hydrolyzed with pepsin. This peptide showed relatively high resistance to digestive proteases, and it can be expected that it will function in vivo as an antihypertensive agent [23]. In another experiment, after the treatment with pepsin, two new ACE-I inhibitory peptides from porcine skeletal troponin were identified. One of them showed the strongest ACE inhibitory activity among the previously detected peptides obtained from troponin [24]. Trypsin belongs to serine proteinases and is synthesized by the pancreas in the form of an inactive precursor (proenzyme) of trypsinogen. Trypsin hydrolyzes peptide bonds formed by the carboxyl groups of L-arginine and L-lysine. Papain is a non-specific thiol protease and the main component of the milk sap protein of the tropical plant *Carica papaya*. Due to its proteolytic properties, it is used in the food industry to soften meat [25, 26]. Papain (300 U/kg) was used to increase the amount of free amino acids in dry fermented sausages [27]. The antithrombotic activity of papain hydrolysate from defatted pork meat (crude fragments and peptides purified by cation exchange chromatography) was estimated in vivo [28]. After the administration to mice per or, the initial peptide fraction with an average molecular weight of 2500 Da showed antithrombotic activity at a dose of 210 mg/kg of body weight, and the fraction 2517 Da purified by cation-exchange chromatography was active at a dose of 70 mg/kg (its activity was equivalent to the activity of 50 mg/kg aspirin). The plant enzyme bromelain is present in large quantities in fruits, leaves, and stems of plants of the *Bromeliaceae* family, of which pineapple is most known (*Ananas comosus*). Like other proteases, it degrades myofibrillar proteins and collagen, which often leads to the softening of meat [29]. The effect of bromelain, papain and collagenolytic enzyme MCP-01 on beef meat at low temperatures (4 °C) was studied. Using

scanning electron microscopy, the differences were found in the destruction of muscle fibers [30].

In this paper, we established for the first time that in processing beef and pork with enzymes of animal (pepsin, trypsin) and plant (bromelain, papain) origin, the formation of bioactive peptides is most likely when using plant enzymes.

The aim of the work was to study peptide profiles of meat raw, presumably having biological activity, under the action of enzymes of animal and plant origin.

Techniques. Bovine cattle (*Bos taurus*) meat (coxal part) and pig (*Sus scrofa*) meat (carbonate) stored after slaughter for 48 hours at 2±2 °C were used. The effects of the following enzymes were studied: pepsin from the gastric mucosa of pigs (10000 U/g) and trypsin from pancreas of a bull (2000 U/g) (HIMEDIA, India); papain from the milk sap of papaya (1100 U/g) and bromelain from the pineapple stem (1310 U/g) (Sigma, USA). In one-dimensional electrophoresis, the enzymes were used at concentrations of 0.5; 1.5; 2.5 %. The samples in which concentrations were effective were further fractionated by two-dimensional electrophoresis.

The solutions of proteases (5 ml per 50 g of meat raw) were injected into the sample of whole muscle (500 g), then allowed for 40 min at 30 °C for trypsin and pepsin preparations, and for 30 min at 30 °C for papain and bromelain. The fermentation was conducted at the native meat pH under 50-55 % room humidity.

The fractional composition of proteins was analyzed by one-dimensional electrophoresis in polyacrylamide gel (12.5 % SDS-PAGE) in a VE-10 chamber (Helicon, Russia) at a constant voltage of 160 V. When the front reached the dividing gel, the voltage was increased to 180 V and the separation was continued for 4-5 hours. The amount of protein introduced is 20 μg per sample. The samples of animal tissue (1 g) were homogenized; proteins were extracted with 3 ml of distilled water. The resulting mass was transferred into a test Eppendorf tube. The slurry was centrifuged for 8 min at 14000 g. One milliliter of the supernatant was placed into a clean Eppendorf tube and 1 ml of the buffer of preparing samples (with dye) was added. The samples were put in a boiling water bath and heated for 2-3 min at 95-100 °C. Then 10-20 μl mixture was added to the gel slots. The marker was a mixture of 11 recombinant proteins (250, 150, 100, 70, 50, 40, 30, 20, 15, 10, and 5 kDa) (Thermo, USA). Coomassie R-250 (Fisher Bioreagents, England) was used for staining. The protein composition was analyzed using the UniProt Protein Database (http://www.uniprot.org/) [31].

The two-dimensional (2D) electrophoresis was performed according to O'Farrell with isoelectric focusing in an ampholine pH gradient (IEF-PAGE); the subsequent detection of proteins was performed by staining with silver nitrate [32]. When making the preparations for 2D electrophoresis, 100 mg of a crushed sample was homogenized in 2 ml of lysing solution (9 M urea, 5 % mercaptoethanol, 2 % Triton X-100, 2 % ampholines, pH 3.5-10, teflon-glass system). The homogenate was clarified by centrifugation (5 min, 800 g); the supernatant fraction containing the protein extract was used for fractionation in equal volumes (50-75 μl).

After the trypsinolysis, the protein fractions were identified by MALDI-TOF (matrix-assisted time-of-flight laser desorption/ionization) and MS/MS (tandem) mass spectrometry (Ultraflex mass spectrometer, Bruker, Germany) with a UV laser (λ = 336 nm) in the mode of detection of positive ions in the mass range of 500-8000 Da with their calibration by the known peaks of autolysis. To study the spectrum of short peptides (with m/z 1500-5000), 100 mg of the preparation was homogenized in 2 ml buffer (5.8 mg KH_2PO_4; 232 mg $Na_2HPO_4 \cdot 2H_2O$; 2.2 g NaCl; 0.5 ml 10 % Triton X-100; 1.87 g KCl) and fur-

ther diluted with water to 50 ml. The homogenate was clarified by centrifugation (5 min, 800 g). In the supernatant fraction, the spectra of the peptides were determined. The analysis of the mass spectra of tryptic peptides was performed using Mascot software, the Peptide Fingerprint option (MatrixScience, USA) at the 0.01 % accuracy of MH^+ mass determining with a search in the NCBI databases [33, 34].

Results. In bovine meat (Fig. 1, A), enzymes of animal origin slightly increased the amount of proteins of a molecular weight of more than 70 kDa (presumably myosin and α-actinin), indicating proteolytic changes in the actomyosin complex. The proteins of 25-70 kDa did not change significantly. In the zone below 15 kDa, new protein bands occurred, which indicates the accumulation of fragments with low molecular weight via cleaving off from major proteins. The effect of pepsin and trypsin on bovine proteins practically did not differ.

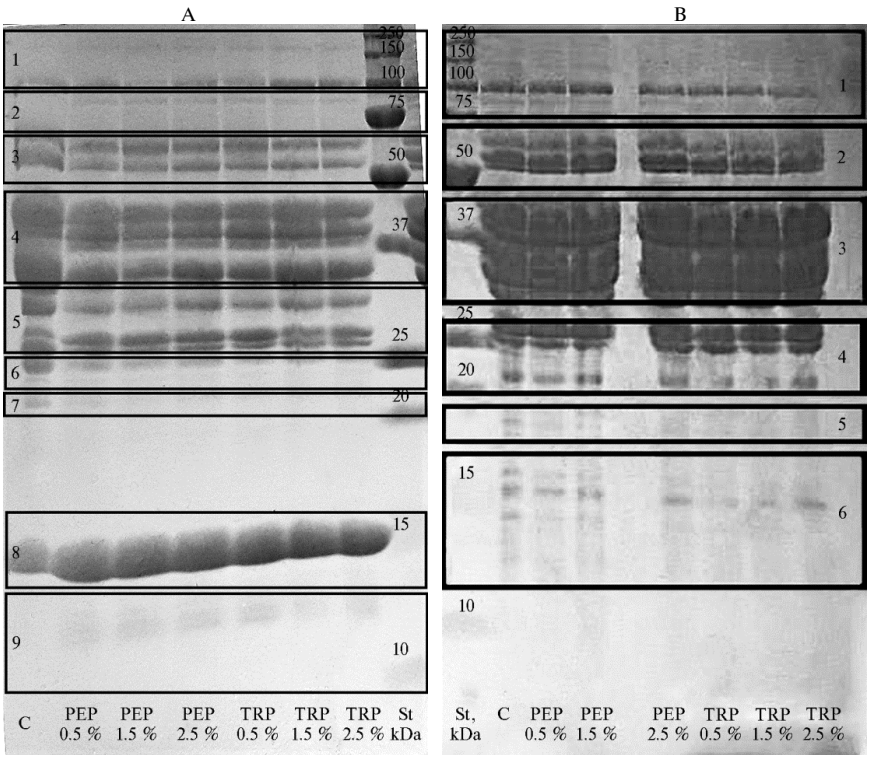

Fig. 1. One-dimensional electrophoresis of proteins derived upon processing extracts of bovine cattle *Bos taurus* (coxal part) (A) **and pig *Sus scrofa* (carbonate)** (B) **skeletal muscles with enzymes of animal origin:** St — protein marker, C — control (without enzymatic processing), PEP — pepsin, TRY — trypsin; 0.5 %, 1.5 %, 2.5 % — the concentration of enzymes.

A: 1 — myosin-10 (222.9-229 kDa); 2 — α-actinin (102-105 kDa); 3 — elastin (64-72 kDa), desmin (53-55 kDa); 4 — α- and β-tubulin (47-52 and 35-52 kDa), α-actin (41.5-42 kDa); 5 — musculoskeletal troponin-T of fast/slow type (30-32 kDa), α/β-tropo-myosin (32.5-32.7 kDa); 6 — musculoskeletal troponin-T of fast type (25-33 kDa), troponin-1 (23-25 kDa); 7 — cofilin 2 (21-22.5 kDa); 8 — hemoglobin (15 kDa); 9 — fragments of high-molecular proteins.

B: 1 — heavy chains of myosin (205-210 kDa), α-actinin (100 kDa), muscle creatine kinase (80 kDa); 2 — elastin (64-66 kDa), α- and β-tubulin (53 and 55 kDa); 3 — G-actin (42 kDa), tropomyosin-1 (39 kDa), troponin-T (35-38 kDa), tropomyosin-2 (32 kDa); 4 — light chains of myosin (16-27 kDa), troponin-1 (23-25 kDa), light chains of myosin-A1 (20.7 kDa); 5 — troponin C (20 kDa), light chains of myosin-2 (18 kDa); 6 — myoglobin (17 kDa), skelemin (15 kDa), fatty-acid-binding protein (14-15 kDa), fragments of myoglobin (8-12 kDa).

In the porcine meat (Fig. 1, B) the content of heavy chains of myosin decreased. A protein of about 18 kDa was significantly degraded (presumably, this corresponds to the light chains of myosin 2). In the samples with pepsin, the intensity of the bands of 14 kDa (presumably ribonuclease) and 8-12 kDa (pre-

sumably α- and β-chemokines), significantly decreased, with trypsin, these bands were absent. It is noted that trypsin has a more pronounced effect on pig meat than pepsin.

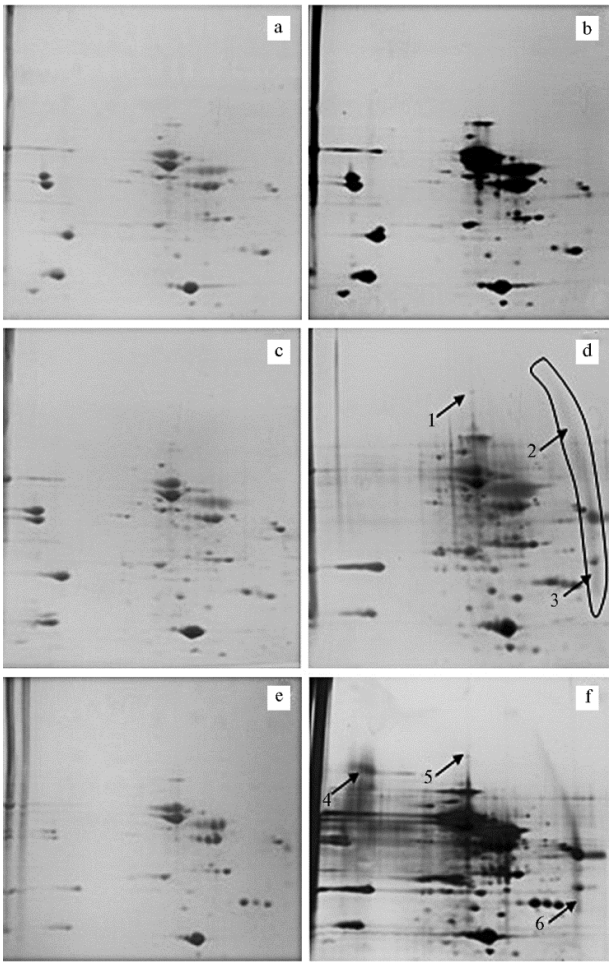

Fig. 2. Two-dimensional electrophoresis of proteins derived upon processing of bovine cattle *Bos taurus* skeletal muscle (coxal part) extract with enzymes of animal origin: a, b — control; c, d — treatment with 1.5 % pepsin; d, e — treatment with 1.5 % trypsin; staining with Coomassie R-250 (left) and silver nitrate (right). The arrows indicate zones of atypical fragments and aggregates of muscle proteins: 1, 5 — track of mitochondrial aconitase 2 fragments (*ACO2* gene product), 2, 3, 6 — aggregate of fragments (60-278 amino acid residues) of isoform 2 of protein 1, containing 4.5 LIM domains (*FHL1*), 4 — fragment (530-1912 amino acid residues) of myosin-1 (*MYH1*).

The computer densitometry upon 2D electrophoresis with Coomassie R-250 staining showed a 15-37 % decrease in the total proteins. A more sensitive staining with silver nitrate made it possible to identify a number of intermediate fragments as bands of degraded tracks of major proteins, e.g. muscle creatine phosphokinase, aldolase A, mitochondrial aconitase, and β-enolase. Moreover, by 2D electrophoresis (Fig. 2), the fragments of the C-terminus of the heavy chain of myosin were detected, normally aggregating at the start of the gel at IEF, which indicates proteolytic changes in the actomyosin complex.

The presence of protein track in the alkaline zone (in the oval, see Fig. 2), which covers the weight range from 400 to 5 kDa, attracts the particular attention. To identify its nature, the fragments of this track were examined in the upper and lower zones. In all cases, it was identified as protein 1 isoform 2, containing 4.5 LIM domains, with a weight of not more than 32 kDa. However, the observed distribution of fragments was significantly wider. That is, during proteolytic processing, this protein apparently retained the core part (positions 60-278) with the formation of aggregates that form a non-ordinary electrophoretic track.

Since the 2D electrophoresis revealed a decrease in the amount of protein material after the exposure to proteases, we studied the changes in the spectra of short peptides (of 8 to 40 amino acid residues) in such samples (Fig. 3). After treatment with proteases, the spectra changed significantly (see Fig. 3). In the control and upon pepsinization, the resultant peptides consisted of 12-40 amino acid residues, and exposition to trypsin led to a pool of peaks, corresponding to lower weights, mainly of 1500-3000 m/z (12-24 amino acid residues) (Table 1).

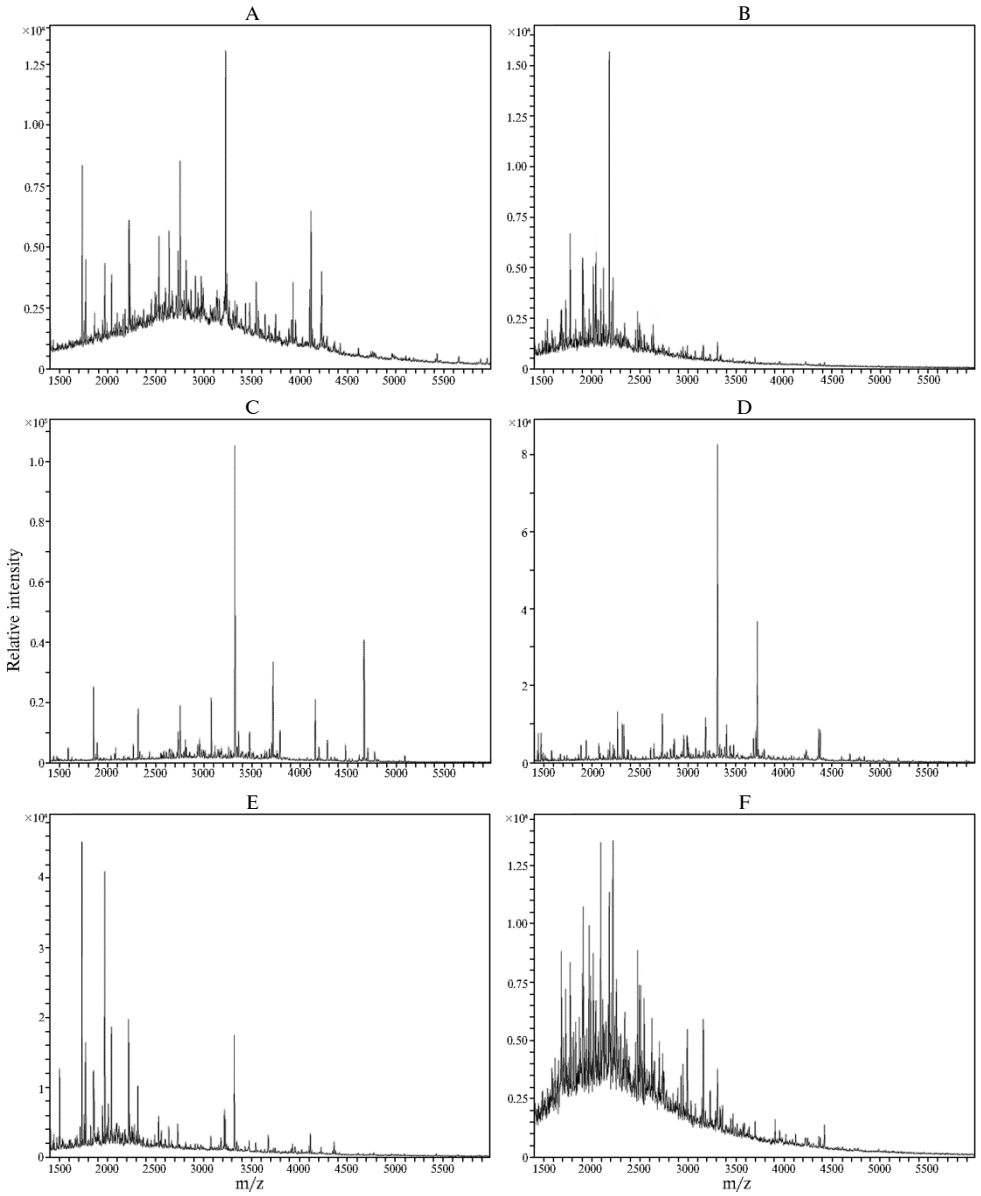

Fig. 3. Tryptic peptide profiles of bovine cattle *Bos taurus* (coxal part) (left) **and pig *Sus scrofa* (carbonate)** (right) **skeletal muscle extracts in the m/z 1500-5000 range:** A, B — control, C, D — 1.5 % pepsin, E, F — 1.5 % trypsin. For description of the identified peptides with m/z 1853.8, 3077.4, 4157.8, 4665.5, 3322.8, 1940.9 and 1952.9 see Table 1.

1. Mass-spectrometric identification (MALDI-TOF MS and MS/MS) of short peptides (m/z 1500-5000) **derived upon processing meat raw extracts with proteases of animal origin**

No. in the Protein Database NCBI	S/M/C	m/z (position in the AA sequence)	Amino acid sequence of identified peptides/proteins
		Beef (pepsin)	
		Myoglobin peptides (*gene MB*)	
NP 776306.1	144/7/100	1853.8 (139-154)	FRNDMAAQYKVLGFHG
		3077.4 (2-30)	GLSDGEWQLVLNAWGKVEADVAGHGQEVL
		4157.8 (71-107)	TALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEF
		Fructose bisphosphate aldolase peptides A (*gen ALDOA*)	
NP 001095385.1	92/4/26	4665.5 (31-70)	IRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVL
		3322.8 (2-2)1	PHQYPALTPEQKKELCDIAH

Continued Table 1

Beef (trypsin)			
Fructose bisphosphate aldolase peptides A (gene ALDOA)			
NP 001095385.1	86/6/30	3322.8 (2-21)	PHQYPALTPEQKKELCDIAH
Pork (pepsin)			
Peptides of chain A L-lactate dehydrogenase (LDHA)			
NP 001165834.1	124/7/25	1940.9 (92-109)	VVITAGARQQEGESRLNL
Pork trypsin			
Calsequestrin peptide-1 (CASQ1)			
Q05JF3	58/2/51	1952.9 (35-47)	EEGLDFPEYDGVD

Note. AA — amino acids. S/M/C: **S**core (measure of compliance), **M**atch peptides (number of coincident peptides), **C**overage, % (covering the complete amino acid sequence of the protein with the identified peptides).

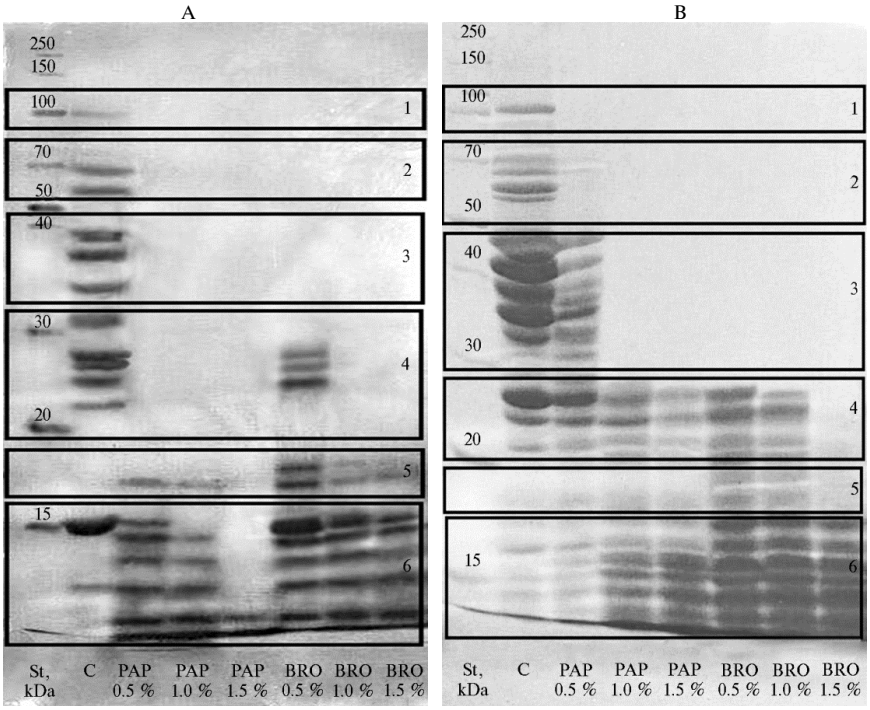

Fig. 4. One-dimensional electrophoresis of proteins derived upon processing extracts of bovine cattle *Bos taurus* (coxal part) (A) **and pig *Sus scrofa* (carbonate)** (B) **skeletal muscles with enzymes of plant origin:** St — protein marker, C — control, PAP — papain, BRO — bromelain; 0.5 %, 1.5 %, 2.5 % — the concentration of enzymes.

A: 1 — α-actinin (103-104 kDa); 2 — elastin (64-66 kDa), desmin (53-55 kDa); 3 — aldolase A (39.5 kDa), musculoskeletal troponin-T of fast type (35-38 kDa), β-tropomyosin (32.5-32.7 kDa); 4 — musculoskeletal troponin-T of slow type (28.6-32 kDa), troponin-1 (23-25 kDa), cofilin (21-22.5 kDa), light chains of myosin-A1 (20.7 kDa); 5 — troponin C (20 kDa), light chains of myosin-2 (18 kDa), myoglobin (17 kDa); 6 — skelemin (15 kDa), protein binding fatty acids (14-15 kDa), myoglobin fragments (8-12 kDa).

B: 1 — α-actinin (100 kDa), muscular creatine kinase (80 kDa); 2 — myoalbumin (70 kDa), elastin (64-66 kDa), catalase (58 kDa), β-tubulin (55 kDa); 3 — G-actin (42 kDa), muscular aldolase (40 kDa), tropomyosin-1 (39 kDa), troponin-T (35-38 kDa), tropomyosin-2 (32 kDa); 4 — light chains of myosin-1 (16-27 kDa), troponin-1 (23-25 kDa), light chains of myosin-A1 (20.7 kDa); 5 — troponin C (20 kDa), light chains of myosin-2 (18 kDa), myoglobin (17 kDa), skelemin (15 kDa); 6 — protein binding fatty acids (14-15 kDa), myoglobin fragments (8-12 kDa).

In the pork, under the influence of plant enzymes (Fig. 4, B), there was significant destruction of proteins with molecular weights above 50 kDa (with papain) and above 30 kDa (with bromelain). The protein fragments of less than 20 kDa accumulated intensively, especially in the samples with bromelain. According to the effect, the plant proteases differed noticeably from the animal ones, probably because of their much lower specificity to potentially attacked proteins.

The protein profile of bovine cattle (Fig. 5) underwent significant destruction: almost all proteins with a weight of more than 20 kDa were destroyed.

The protein fragments significantly accumulated in the range below 20 kDa. At the same time, papain worked more efficiently.

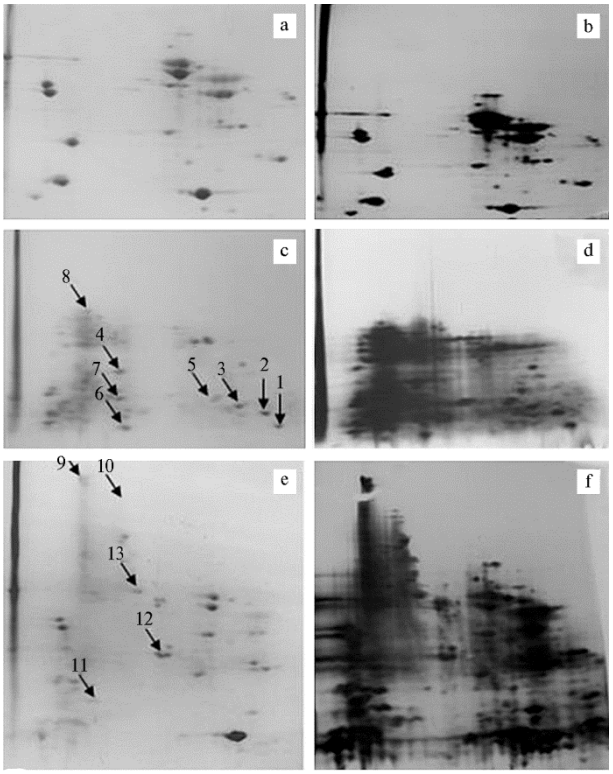

Fig. 5. Two-dimensional electrophoresis of proteins derived upon processing extract of bovine cattle *Bos taurus* skeletal muscle (coxal part) with enzymes of plant origin: a, b — control; c, d — treatment with 0.5% papain; d, e — treatment with 0.5 % bromelain; staining with Coomassie R-250 (left) and silver nitrate (right). The arrows indicate proteins: 1, 2, 3 — fragments of myoglobin (product of the *MB* gene), 4 — a mixture of fragments of actin (*ACTG2*) and myosin 1 (*MYH1*), 5 — canonical myoglobin (*MB*), 6 — fragment of actin (*ACTA1*), 7 — fragment of myosin 7 (*MYH7*), 8, 9, 10 — fragment of myosin 2 (*MYH2*), 11 — musculoskeletal light chain of myosin 1/3 (*MYL1*), 12 — fragment of myosin 1 (*MYH1*), 13 — mixture of fragments of myosin 2 (*MYH2*) and myosin 1 (*MYH1*).

When treating beef with bromelain, the fractions of the α- and β-tropomyosins were detected in a residual amount and myoglobin remained practically intact. Under the influence of papain, the total gel staining remained high due to the appearance of a heterogeneous mixture of fragments of high molecular weight (15-60 kDa). When treated with papain, the fragments of different types of heavy chains (MYH1, MYH2, and MYH7) of myosin were detected, localized in the muscle fibers of the fast and slow types. The action of bromelain turned out to be more specific for fast-type fibers (fractions of MYH7 fragments were not found). The number of bands in one-dimensional gel electrophoresis changed significantly during the processing of pork and beef with plant enzymes (Table 2).

In the extracts treated with plant proteins, spectrum of short peptides (Fig. 6) showed the appearance of additional peaks. When treated with papain, the peptides detected were of m/z up to 4500, with bromelain these were no more than of 3000 (mostly up to 2500). We have constructed the amino acid sequence of the peptide of *Bos taurus* actin family (without additional detail and reference to a gene) resulted from the treatment of beef with bromelain.

2. The number of protein bands on the one-dimensional electrophoregrams derived upon processing cattle *Bos taurus* (coxal part) and pig *Sus scrofa* (carbonate) skeletal muscle extracts with different concentrations of plant enzymes

Molecular weight, kDa	Control 1	Pepsin, %			Trypsin, %			Control 2	Bromelain, %			Papain, %		
		0.5	1.5	2.5	0.5	1.5	2.5		0.5	1	1.5	0.5	1	1.5
						Beef								
> 250	–	–	–	–	–	–	–	–	–	–	–	–	–	–
150-250	1	1	1	1	1	1	1	1	–	–	–	–	–	–
100-149	1	1	1	1	1	1	1	1	–	–	–	–	–	–
70-99	–	1	1	1	1	1	1	4	–	–	–	–	–	–
50-69	2	2	2	2	2	2	2	3	–	–	–	2	–	–
37-49	3	3	3	3	3	3	3	3	–	–	–	5	–	–

Continued Table 2

25-37	6	6	6	6	6	6	6	4	1	1	–	4	2	1
20-24	2	2	2	2	2	2	2	2	2	2	2	3	2	2
15-20	–	–	–	–	–	–	–	4	6	6	4	5	5	5
10-15	2	4	4	4	4	4	4	2	2	2	2	2	2	2
					Pork									
> 250	1	–	–	–	–	–	–	1	–	–	–	–	–	–
150-250	1	1	1	1	1	1	1	–	–	–	–	–	–	–
100-149	1	1	1	1	1	1	1	–	–	–	–	–	–	–
70-99	2	2	2	2	2	2	2	2	–	–	–	–	–	–
50-69	3	3	3	3	3	3	3	1	–	–	–	–	–	–
37-49	3	3	3	3	3	3	3	2	–	–	–	–	–	–
25-37	6	6	6	6	6	6	6	5	3	–	–	–	–	–
20-24	2	2	2	2	2	2	2	2	–	–	–	–	–	–
15-20	3	2	2	1	1	–	–	1	3	3	3	2	1	–
10-15	4	4	4	4	3	3	3	1	4	4	4	4	4	1

Note. Controls 1 and 2 are non-fermented raw materials (for the variant with enzymes of animal and plant origin, respectively). Dashes indicate that there are no protein fractions in the specified molecular weight range, in which obvious changes have occurred.

The peptide was conservative for actins and corresponded to the positions 244-257 of amino acid residues in the canonical musculoskeletal actin of *Bos taurus* (*ACTA1* gene), the m/z position 2066.0 (I/LPDGQVI/LTI/LNERF). Obviously, short peptides in this case should be identified using multidimensional chromatography, which is more adapted to work with short peptides.

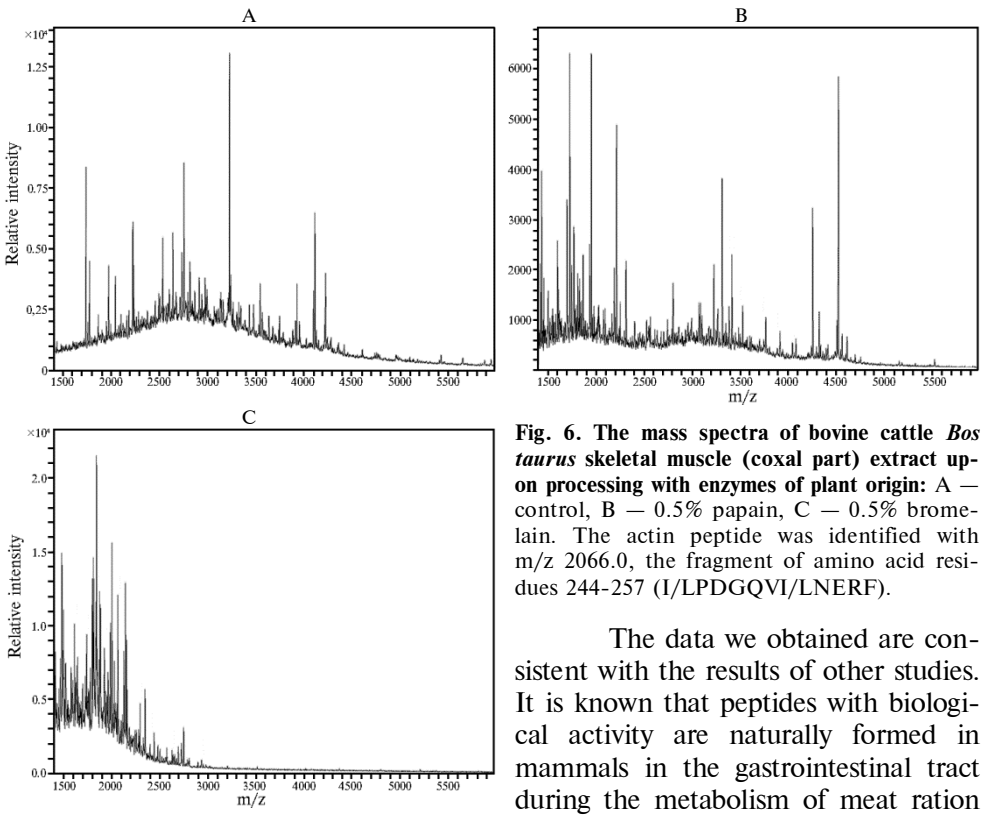

Fig. 6. The mass spectra of bovine cattle *Bos taurus* skeletal muscle (coxal part) extract upon processing with enzymes of plant origin: A — control, B — 0.5% papain, C — 0.5% bromelain. The actin peptide was identified with m/z 2066.0, the fragment of amino acid residues 244-257 (I/LPDGQVI/LNERF).

The data we obtained are consistent with the results of other studies. It is known that peptides with biological activity are naturally formed in mammals in the gastrointestinal tract during the metabolism of meat ration proteins under the influence of digestive enzymes secreted in the small intestine [35-38]. To generate such potentially functional peptides, commercial exogenous proteinases derived from animal tissues (pepsin and trypsin), plants (papain, ficin and bromelain) and microbial sources (Alcalase®, Flavourzyme®, Neutrase®, collagenase or proteinase K) are commonly used to mimic the gastrointestinal digestion [37, 39, 40]. For the production of some biologically active peptides, enzymatic hydrolysis of collagen from meat or by-products of slaughter (cuttings, organs, hemoglobin), in addi-

tion to meat raw materials, is carried out [41]. T. Lafarga et al. [42] studied the release of potential biologically active peptides angiotensin-I-converting enzyme (ACE-I, EC 3.4.15.1), renin (EC 3.4.23.15) and dipeptidyl-peptidase IV (DPP-IV, EC 3.4.14.5) from bovine and porcine proteins, including hemoglobin, collagen and serum albumin. These proteins commonly found in meat by-products (bones, blood, and meat cuttings) play the key roles in controlling hypertension and the development of type 2 diabetes and other diseases associated with metabolic syndrome. The new peptides included the ACE-I inhibitory tripeptide Ile-Ile-Tyr and the inhibitory DPP-IV tripeptide Pro-Pro-Leu, corresponding to the sequences of positions 182-184 and positions 326-328 of porcine and bovine serum albumin, which can be released after hydrolysis, respectively, by papain and pepsin. In other studies [43], the inhibitory and antioxidant activity of angiotensin-I-converting enzyme (ACE-I) of sarcoplasmic proteins isolated from the pectoral muscle (Pectoralis profundus) of bovine cattle (*Bos taurus*) and hydrolyzed by papain was determined. Sarcoplasmic protein hydrolysates were subjected to membrane ultrafiltration and 10 kDa and 3 kDa filtrates were obtained. As a result, 11 peptides were characterized from the total hydrolysates fraction, 15 from the fractions of filtrate 10 kDa, 9 peptides from the fractions of filtrate 3 kDa. The authors identified the similarities between the amino acid sequences of the peptides identified by them and the known antioxidant and inhibitory ACE-I peptides described in the BIOPEP database. Pork myofibrillar proteins were studied as promising sources of biologically active peptides [44]. After simulating gastrointestinal digestion of certain porcine myofibrillar proteins with the use of pepsin, trypsin and chymotrypsin, peptide sequences inhibiting dipeptidyl peptidase IV were most frequently found among intact proteins. All in all, a total of 399 peptides were detected with various activities (enzyme inhibition, antioxidant, hypotensive, stimulating, antiamnesic activity, regulation of body functions).

Thus, we identified a quantitative decrease in the components of beef and pork protein profiles under the action of proteases of various origins. The proteases of animal origin act on the raw materials more evenly and more specifically than the plant ones. For the plant proteases, the formation of intermediate fragments is characteristic, which was not observed in the samples with proteases of animal origin. In all cases, new protein fragments are found, including the ones with low molecular weight, as confirmed by mass spectrometry. By tandem mass spectrometry, we identified some candidate fragments which may have biologically active properties. The greatest changes in the protein profile of meat raw materials are in treating with proteases of plant origin, therefore, in this case, the formation of biologically active peptides can be anticipated.

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