Identification of species- and tissue-specific proteins using proteomic strategy

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Abstract. Proteomic technologies have proven to be very effective for detecting biochemical changes in meat products, such as changes in tissue- and species-specific proteins. In the tissues of cattle, pig, horse and camel M. longissimus dorsi both tissue- and species specific proteins were detected using two dimensional electrophoresis. Species-specific isoforms of several muscle proteins were also identified. The identified and described proteins of cattle, pig, horse and camel skeletal muscles (including mass spectra of the tryptic peptides) were added to the national free access database “Muscle organ proteomics”. This research has enabled the development of new highly sensitive technologies for meat product quality control against food fraud.

1. Introduction
The aim of proteomics is identification of all proteins, their biological activity, post-translation modifications and interactions in a cell, as well as identification of changes in the proteome as a response to changed biological conditions. The typical workflow in proteomics includes protein extraction and separation, protein and peptide identification and data analysis. The most common method used for detection of proteins or peptides in proteomics is mass-spectrometry. This strategy has multiple applications including for meat science research; however, it is limited by huge biochemical heterogeneity of proteins and inability to precisely detect low-abundance proteins.

In recent decades, the scientific community witnessed the rapid development and improvement of “-omic” methods with high throughput. The development of these methods has also changed the experimental approaches in food science [1,2].

In life sciences, including agriculture, food and animal sciences, the use of proteomics is a great step forward both for safe and high quality food production and improvement of animal husbandry and sustainability. The meat composition, sensory characteristics and nutritional value are important characteristics to determine meat quality and consumer acceptability. Meat quality is closely linked with biological peculiarities of an animal. Obviously meat quality characteristics (like tenderness, water binding capacity, nutrient composition, autolytic changes etc.) are complex and multicomponent systems. Their detailed description would provide the next step toward understanding processes that cause changes in their characteristics and subsequent meat quality management [3,4,5]. Proteomics is a prospective approach to studying mechanisms that are the basis for various meat quality traits.
At present, several methods based on identification of species-specific DNA (different variations of polymerase chain reaction) as well as the alternative method of enzyme-linked immunosorbent assay (ELISA) based on the specific reaction antigen-antibody are used in the laboratory analysis of meat and meat-based products. Despite all their advantages, these methods have several significant drawbacks. A large part of Brucellosis, one of the most widespread zoonosis, is a contagious chained disease affecting a great number of animals and, in a smaller proportion, people [10, 22]. In the animals, this disease is usually manifested as either chronic or latent infection. Causal agents of the infection are bacteria from Brucella genus which can have different virulence and host affinities.

The appearance of brucellosis in human population is closely related to its incidence in animal populations, and this correlation is the only way that the disease can be observed, studied and controlled. Humans are most commonly affected by consuming either meat or dairy such as milk or young cheese produced from uncooked goat or sheep milk. Transmission of brucellosis from animal to human occurs through the air or via skin wounds. People working in the higher risk professions, such as the farming or meat industry as well as the veterinary or lab professionals, show higher incidence rate [8, 18]. An estimate of a half of a million people per year seeking medical attention due to brucellosis has been given by World Health Organisation (WHO), although it is believed that the number of affected people is up to 25 times greater.

Brucellosis, although in many countries controlled or completely eradicated, remains great health and economic problem. This is especially true in the regions in which highly contagious *Brucella melitensis*, causing disease in sheep, goats, and humans.

studies in proteomics is performed using two-dimensional electrophoresis (2DE). The Brucellosis, one of the most widespread zoonosis, is a contagious chained disease affecting a great number of animals and, in a smaller proportion, people [10, 22]. In the animals, this disease is usually manifested as either chronic or latent infection. Causal agents of the infection are bacteria from Brucella genus which can have different virulence and host affinities.

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Brucellosis, although in many countries controlled or completely eradicated, remains great health and economic problem. This is especially true in the regions in which highly contagious *Brucella melitensis*, causing disease in sheep, goats, and humans. Combination of high-performance liquid chromatography (HPLC) and tandem mass-spectrometry is also a very promising approach to animal protein analysis.

The aim of this research was to identify potential biomarkers of meat (beef, pork, horse and camel meat) species-and tissue- specificity based on the proteomic profiles of their skeletal muscles.

2. Materials and methods

The following muscle types and animal species were used in the research:
- cattle (*Bos taurus*) m. Longissimus dorsi (OOO KRROS, Moscow region);
- pig (*Sus scrofa*) m. Longissimus dorsi (OOO Velcom, Moscow region);
- horse (*Equus caballus*) m. Longissimus dorsi (MPZSafa, Moscow region);
- camel (*Camelus bactrianus*) m. Longissimus dorsi (Almaty Technological University).
The 2DE by O’Farrell with isoelectrofocusing in ampholine (IEF-PAGE) or immobiline (IPG-PAGE) pH gradients was used as the main proteomic technology; the following protein detection was carried out by staining with Coomassie R-250.

For 2DE, 100 mg of a mixed muscle was homogenized in the Teflon-glass system in 2 ml of the lysing solution: 9 M urea, 5% mercaptoethanol, 2% triton X-100, 2% ampholines with pH 3.5-10 (IEF-PAGE). Homogenate was then clarified by centrifugation at 800 g for 5 min and the supernatant with solubilized proteins (the extract) was used for separation. In the immobiline pH gradient (IPG-PAGE), the lysing solution with 9 M urea, 4% CHAPS, 2% ampholines with pH 3.5-10 and 0.6% dithiothreitol was used.

Protein identification was performed after tryptic proteolysis by MALDI-TOF MS and MS/MS mass-spectrometry using a MALDI-TOF mass-spectrometer Ultraflex (Bruker, Germany) with UV-laser (336 nm) in the positive ion mode and a mass range of 500-8000 Da with their calibration according to the known trypsin autolysis peaks.

The mass-spectra of tryptic peptides were analyzed by the Mascot software, Peptide Fingerprint option (Matrix Science, USA) with accuracy of MH+ mass detection of 0.01%, with the use of the database of the National Center for Biotechnology Information (NCBI).

3. Results and discussion
Up to 170 protein fractions were obtained and over 120 proteins were identified (table 1 and figure 1).

| Detected proteins | Identified proteins |
|-------------------|---------------------|
| cattle            | 115                 | 51                  |
| pig               | 145                 | 108                 |
| horse             | 130                 | 61                  |
| camel             | 170                 | 114                 |

Table 1. Number of identified proteins extracted from cattle, pig, horse and camel m.Longissimus dorsi.

A. Pig Sus scrofa

B. Cattle Bos taurus
Figure 1. Proteomic maps of m. Longissimus dorsi of pigs (A), cattle (B), horses (C) and camels (D). Staining with Coomassie R-250. Arrows and numbers (1-61; 1-55;1-51) indicate fractions identified by MALDI-TOF MS (see the information module “Skeletal muscle proteins” in the free-access database “Muscle organ proteomics”, http://mp.inbi.ras.ru). ТПМ – tropomyosin; МЛЦ – myosin light chains; ТнТ – troponin T; Α/Д – actin- desmin.

Among 108 totally identified protein fractions on the two-dimensional electrophoregrams of porcine skeletal muscles, 18 were isoforms of different troponin proteins known as strictly specific for the mammalian muscle tissues [6]. Among these proteins, eight were characterized as products of TNNT1 gene expression, seven as products of TNNT3 gene, and three as products of TNNI2 gene.

Information on proteomic studies of pig troponins is still very limited. In the pig (Sus scrofa) genome, eight genes encoding different troponins have been found to date.

In the analyzed muscles, three protein products of genes encoding troponins I (No. 84, 94 and 95) were identified in addition to troponins T. Two of them (No. 101 and 102) corresponded to transcript 73853890 / Q4JH15 (gene TNNI2), and the third one to transcript 47522664 / B3VFA9 (gene TNNI1).

As a result, this research established that there are three genes encoding troponins T (TNNT1, TNNT2, TNNT3), three genes for troponins I (TNNI1, TNNI2, TNNI3) and two genes for troponins C (TNNC1, TNNC2) in the pig genome.

Two protein fractions (No. 68 and 79) identified as protein products of the porcine TNNT3 gene corresponded to transcript 55741811 / Q75NG9, and no special information in the NCBI and UniProt databases was found. Taking into consideration the experimentally determined pI value, these fractions were named astroponin T fast skeletal muscle acidic (fTnt-a) and troponin T fast skeletal muscle neutral (fTnt-n). Ozgur Ogut et al., while examining chicken (Gallus domesticus) muscle, assumed that both acidic and basic TnT isoforms would modulate the Ca\(^{2+}\) sensitivity of muscle contraction [7].

Proteins specific to muscle type were also found. For example, pig muscle contained pig Troponin I, fast skeletal muscle, (TNNT2) TNNI2 with Mm/pI 21.1/9.00 and pig Troponin I, slow skeletal muscle, isoform TnI-S4 (TNNI1) TNNI1 with Mm/pI 23.0/9.50 (positions 101 and 102 in figure 1A, respectively).

Protein No 13 is of special interest to us. It was identified by Mascot program as a hypothetical protein containing a crystalline domain – a product of a gene from locus LOC494560 (protein sequence coverage 77%, score – 371, taking into account the MS/MS results). Therefore, this is first
real evidence of a protein product that corresponds to the predicted hypothetical protein. We regard this significant result as a certain contribution to the full annotation of the *Sus scrofa* genome.

Among the identified major bovine muscle proteins (figure 1B), nine (No. 6-10, 15, 16, 18 and 48) were the products of the TNNT3 gene. At the same time, the multiplicity of fast Troponin T isoforms was revealed, which was also noted by other authors [8,9].

This research into the horse meat proteome led, in general, to identification of 61 protein fractions (figure 1C). New direct data was obtained regarding equine proteins, the existence of which was earlier only predicted [10]. Of particular interest among the identified horse meat proteins is protein Dj-1 (Figure 1C No 54). This protein is known to protect human cells from oxidative stress and prevents the development of apoptosis, while mutations in the gene encoding Dj-1 are a cause of several forms of Parkinsonism (see Q99497 UniProt). The obtained data can be considered an indirect confirmation of the hypoallergenic properties of horse meat.

Although among the identified proteins were, for example, myosin light chains, which are important components of the main muscle engine – the actomyosin complex – direct studies of even major horse skeletal muscle proteins appears to be extremely limited. For instance in the PubMed database only one study was found that reported the myosin light chain 3 detected in the biopsies of the horse muscle using proteomic technique [11]. Thus, the results obtained in our study can be considered novel.

When studying the camel proteome (figure 1D), in 16 identified protein fractions, different types of post-translational modifications were found. Among them were acetylation of N-terminal amino acids [+Acetyl (Protein N-term)], phosphorylation of serine and threonine residues [+Phospho (ST)] and others not discussed herein. Y. Zahedi et al. identified two full-length slow TnT and one fragment of fast TnT in camel [12]. As a result of our research, 18 camel mitochondrial proteins were identified. This result is of a significant interest, as direct data on these proteins are unavailable.

In addition to protein polymorphism analysis and other basic research in the field of muscular biochemistry, the proteomic strategies are challenging new applications in applied science, from protein biomarkers and pharmacological targets to developing food quality and composition control methods [13,14].

As a result of identification in the current study, typical mammalian muscle tissue proteins were revealed. These proteins are not species-specific, for example, tropomyosins (see table 2) with regard to tropomyosin β-chain (TPM2) with the following sequence of amino acids:

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001MDAIKKKMQMLKLIDKENAIQRAEQADEAKKQAEIDRCKQLEEEQQLQKLMGTEDEVEKYSVSDAQEKLEQAEEKKTADEADVASLRQIQVEEELD010IRAQERLATALQKLEEAERKAKDESERGLKVENRAMKDEEMELQEMQEQM0151KEAKHIAEDSRKYEEVARKLVILEGEARSEERAEVESRARQLEEL0201RTMDQALKSLMASEEYSTKEDKYYEIIKLLLEELKIAETRAEFAERSVAM0251KLEKTIDDEETLASAKEENVIHQITLDQTTLELNNL
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**Table 2.** Comparative data on identification of tissue-specific β-Tropomyosin (TPM2) of cattle, pig, camel and horse skeletal muscles.

| Animal species     | Number of amino acid residues | Mm | pI | Match, % |
|--------------------|--------------------------------|----|----|----------|
| *Sus scrofa*       | 287                            | 33.5 | 4.80 | 29.0     |
| *Bos Taurus*       | 284                            | 32.8 | 4.66 | 89.0     |
| *Equus caballus*   | 284                            | 32.8 | 4.66 | 64.0     |
| *Camelus bactrianus* | 284                          | 33.0 | 4.66 | 85.0     |

By analyzing the proteomic profiles, we revealed several proteins that could be suitable as markers of mammalian muscle tissue. Among them are β-enolase, myosin light chain, troponin I, 5-
triosephosphate isomerase. A search for other protein biomarkers is being carried out. A necessary prerequisite for determination of these markers is protein thermal stability. It can be of scientific interest to study fast myosin behavior during thermal and other types of meat processing, since according to Guerrero, M. et al., fast myosin is an exceptional marker for skeletal muscles [15]. The high sensitivity of the protein separation by 2DE makes it possible to identify the animal species origin of these marker proteins (figure 2).

![Figure 2. Two-dimensional electrophoregrams with species-specific proteins, Coomassie 250 stain.](image)

Species-specific differences in the electrophoretic mobility of myoglobins as well as troponins, β-enolase isoforms and myosin light chain isoforms was evident when using 2DE to examine beef and pork muscle, as shown in figure 2.

The results of some species-specific muscle protein identification are presented in table 3.

**Table 3.** Skeletal muscle proteins identified by mass-spectrometry (MALDI-TOF MS and MS/MS) methods.

| Protein (name in NCBI) / gene symbol | Species             | Mm/pI  |
|-------------------------------------|---------------------|--------|
| Troponin T fast skeletal muscle type |                     |        |
| (TNNT3)/ fTnT3/17                   | *Bos taurus*        | 32.0/8.60 |
|                                     | *Sus scrofa*        | 34.0/8.65 |
|                                     | *Equus caballus*    | 32.0/9.10 |
|                                     | *Camelus bactrianus*| 31.5/10.20 |
| Myosin light chain 1/3, skeletal    | *Bos taurus*        | 21.5/5.10 |
|                                     | *Sus scrofa*        | 21.0/4.90 |
4. Conclusion
The results of the presented proteomic analysis have opened the way to the development of new highly sensitive technologies for meat product quality control, based on the analysis of species-specific isoforms of several muscle proteins.

Proteins that can be considered as markers of the presence of mammalian muscle tissue in meat products were identified. The information obtained will aid in quantifying the proportion of muscle tissue in meat products, including emulsified and heat-treated products.

Proteins of the contractile actomyosin complex (myosin light chains, tropomyosins, troponins) and enzymes participating in numerous interdependent carbohydrate metabolic pathways (glyceraldehyde 3-phosphate dehydrogenase, β-enolase) were chosen as the most informative proteins in terms of species specificity. In addition, α- and β-hemoglobins were added to the list as highly species-specific proteins for horse and camel meat.

The identified and described proteins of cattle, pig, horse and camel skeletal muscles (including mass spectra of the tryptic peptides) were added to the national free access database “Muscle organ proteomics” (http://mp.inbi.ras.ru). Information modules: Proteins of porcine skeletal muscle (Sus scrofa), Proteins of bovine skeletal muscle (Bos taurus), Proteins of horse skeletal muscle (Equus caballus) and Proteins of camel skeletal muscles (Camelus bactrianus) within the database were constructed.

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