Thesis of Ph.D. dissertation

APPLICATION OF PROTEOMIC TOOLS IN LIVESTOCK SCIENCE

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Debrecen, 2014.

1. Background and objectives of the doctoral thesis

In the last decades, molecular biology research became highlighted in animal science, in particularly study of the macromolecules, such as DNA, RNA, proteins those are the steps of genetic information flow.

In a relatively short period, we had a great progress in the knowledge of genetic information. With the increment of knowledge formed many new disciplines those are in the era of postgenomic research. Often they are reflected as omics, such are metabolomics, transcriptomics, glycomics and proteomics as well. The omics can change essentially the practice of animal production, because they take the regulation networks and pathways into consideration those are in the background of desirable phenotypes.

The proteome means a complete set of proteins of a cell, tissue, organ or organism at a certain time. To understand its function it is necessary to identify the proteins and recognize their interactions with other molecules. Proteomics deal mainly with problems those can not be solved by DNA analysis. The application of proteomics has already widespread in the fields of human medicine and pharmacological sciences.

For the time being, proteomics methods are used in the field of animal husbandry, physiology, immunology, reproduction biology, meat and milk production experiments. The expression of cells, cell compartments, tissues and body fluids can be detected within the different treatments, making possible to understand the processes that are the results of the interactions of hundreds of proteins. Proteome analysis of different tissues of different livestock species is a huge challenge, because of variability of protein content that makes difficult choice between the fractionation and separation methods.

During research activity, I planned to perform three proteomics studies. The aims of these experiments were as listed below:

1st experiment: Effect of selenium supplement on the proteome of chicken liver

- optimizing a two-dimensional polyacrylamide gel electrophoresis method to liver tissue of chicken
- detection of changes in chicken liver proteome induced by selenium
- search for correlations between the function of protein with altered expression and the physiological effects of selenium.

2nd experiment: Proteome analysis of musculus longissimus dorsi of Merino and Tsigai sheep breeds

- optimizing a two-dimensional polyacrylamide gel electrophoresis method to the longissimus dorsi muscle of sheep
- detection of differences of two genotypes (Tsigai and Merino) in protein composition
- search for correlations between the function of protein with changed expression and differences between two breeds

3rd experiment: Proteomic analysis of skeletal muscle at different live weights in Charolais bulls

- optimizing a two-dimensional difference in gel electrophoresis method to the longissimus dorsi muscle of cattle
- investigation of differences in the proteome patterns of musculus longissimus dorsi between Charolais bulls slaughtered at 500 kg and 700 kg live weight
- search for correlations between the function of identified proteins and the investigated trait.

2. Effect of selenium supplement on the proteome of chicken liver

2. 1. Materials and methods

Twelve Cobb 500 broiler chicken were used in this experiment. The selenium concentration of feed was 0.2 mg/kg in the control group (6 chicken) and 4.25 mg/kg in the experimental group (6 chicken). Samples were harvested into cryotubes as an amount of 0.5 g per sample, 20 min after slaughter and immediately frozen in liquid nitrogen, and then were kept at -80 ˚C until subsequent analysis.

We used two-dimensional polyacrylamide gelelectrophoresis (2D-PAGE) to separate proteins of liver. Some factors influencing effectiveness of separation were optimized: extraction of proteins, pH range, amount of proteins, rehydration buffer, conditions of isoelectric focusing, concentration of polyacrylamide gel, staining method (*Table 1*).

As a result of method optimization, parameters were chosen those are appropriate to detect the largest number of spots. We performed two-dimensional polyacrylamide gel electrophorese of the two groups (control and treated), three technical replicates per sample were performed.

Gel images were analyzed and compared with Delta2D (Decodon ^{TM} GmbH, Germany) software, spots which showed significant differences between groups, were identified with liquid chromatography coupled mass spectrometry (LC-MS/MS).

Table 1. Optimization of 2D-PAGE parameters for chicken liver analysis

Protein extraction (lysis buffers)	- 8 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% 100X Bio-Lyte ampholyte - 8.5 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 0.2% 100X Bio-Lyte ampholyte - 8.5 M urea, 2 M thiourea 4% CHAPS, 60 mM DTT, 0.2% 100X Bio-Lyte ampholyte + Cleanup Kit
pH range	pH 5-8 and pH 3-10
Quantity of proteins	20, 50, 100, 150, 300 µg
Rehydration buffers	- general rehydration buffer: 8 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% 100X Bio-Lyte ampholyte, 0.002% bromphenol blue - DeStreak rehydration buffer: 8 M urea, 2 M thiourea, 2% CHAPS, 15 mg/ml DeStreak reagent, 0.2% 100X Bio-Lyte ampholyte, 0.002% bromphenol blue
Isoelectric focusing	In one step: 40000 V 20000 Vh In three steps: 1. step: 250 V 15 min 2. step: 4000 V 2.5 hr 3. step: 4000 V 20000 Vh
Concentration of the polyacrylamide gels	10%, 13%, 15%
Staining method	Coomassie G-250 and silver staining

2. 2. Major conclusion of the thesis

Based on the results of our optimization experiment, we determined the optimal parameters to performing the two-dimensional polyacrylamide gelelectrophoresis of chicken liver samples (*Table 2*).

Table 2. Ideal parameters of 2D-PAGE for chicken liver

Protein extraction (lysis buffers)	8,5 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 0.2% 100X Bio-Lyte ampholyte
pH range	pH 5-8
Quantity of proteins	150 µg
Rehydration buffers	DeStreak rehydration buffer: 8 M urea, 2 M thiourea, 2% CHAPS, 15 mg/ml DeStreak reagens, 0.2% 100X Bio-Lyte ampholyte, 0.002% bromphenol blue
Isoelectric focusing	In three steps
Concentration of the polyacrylamide gels	13%
Staining method	Coomassie G-250

Approximately 708 spots were detected on each polyacrylamide gel (gels of control and treated group as well). 18 protein spots showed significant difference in the intensity ($p<0.05$) between two groups, 14 proteins of these were identified successfully by mass spectrometry (*Figure 1*). The intensity of 8 spots were higher in the treated group while other 6 spots were detected with higher expression level in the control group (*Table 3*).

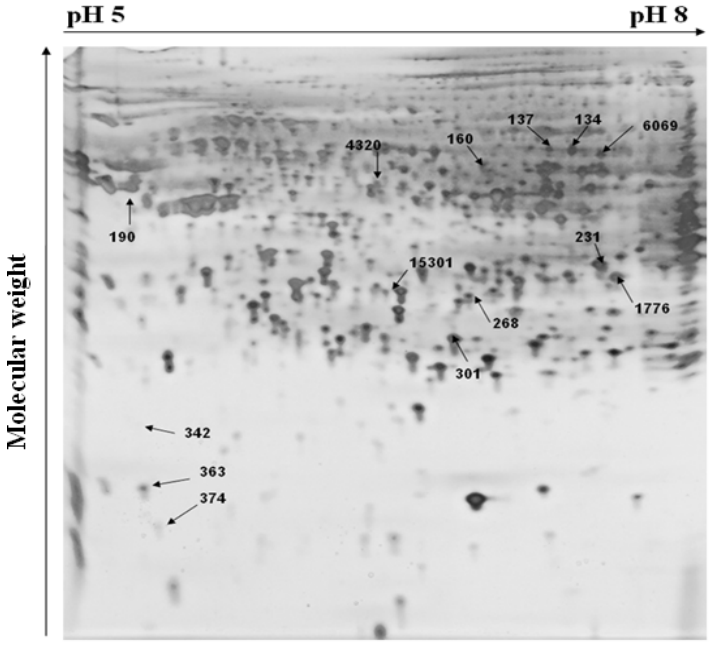

Figure 1. Representative 2-D PAGE image of chicken liver, proteins those were identified by LC-MS/MS are marked with numbers

Table 3. Differentially expressed proteins (p<0.05) between control and experimental groups in chicken liver identified by LC-MS.

Spot	Identified protein	Accession number	Homolog protein or protein family	Matched peptides/ % sequence coverage[1]	Theoretical pI/Mw (Da)[2]	Ratio[3]
134	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	P21642 (Gallus gallus)		13/26	6.6/67,311	0.5
137	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	P21642 (Gallus gallus)		22/49	6.6/67,311	0.6
160	Uncharacterized protein	E1C4W4 (Gallus gallus)	Alpha-aminoadipic semialdehyde dehydrogenase (Q2KJC9, Bos taurus)	15/38	7.0/58,053	2.3
190	Actin, cytoplasmic 1, N-terminally processed (Fragment)	F1N835 (Gallus gallus)		8/23	5.3/23,845	2.4
231	Uncharacterized protein (Fragment)	F1NFY2 (Gallus gallus)	Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic (ROLM66, Anas platyrhynchos)	19/68	6.3/38,463	0.7
268	Proteasome subunit alpha type	F1NFI8 (Gallus gallus)		10/43	6.1/29,322	1.4
301	Peroxiredoxin-6	F1NBV0 (Gallus gallus)		14/66	6.0/25,076	1.2
342	RNA-binding protein 8A	Q9CWZ3 (Mus musculus)		1/7	5.8/19,888	0.5
363	Eukaryotic translation initiation factor 5A-1	Q09121 (Gallus gallus)		5/22	5.3/15,889	0.4
374	ADF actin binding protein	C7G537 (Gallus gallus)		6/51	5.3/16,106	4.8
1776	Malate dehydrogenase, cytoplasmic	Q5ZME2 (Gallus gallus)		14/55	6.9/36,543	1.3
4320	Alpha-enolase	F1NZ78 (Gallus gallus)		16/49	6.4/47,333	1.7
6069	Uncharacterized protein (Fragment)	F1N8C3 (Gallus gallus)	acyl-CoA dehydrogenase family	22/35	-	0.6
15301	3-mercaptopyruvate sulfurtransferase	E1C8D8 (Gallus gallus)		9/47	5.8/33,221	1.3

[1] number of identified peptides and the percentage of the database protein sequence covered by matching peptides
[2] isoelectric point and molecular weight from Swissprot database
[3] ratio value of the treated group to control group

Based on our results can we conclude that the applied dose of selenium reduced the intensity of gluconeogenesis, because the expression of phosphoenolpyruvate carboxykinase in the treated group reduced by almost half, this enzyme controls the speed of gluconeogenesis (YANG et al., 2009). The reduction in gluconeogenesis can be connected with the increase of alpha-enolase level in the treated group. Alpha-

enolase catalyses the phosphoenolpyruvate synthesis in glycolysis, the phosphoenolpyruvate is the terminal product of reaction catalizated by phosphoenolpyruvate carboxykinase (PESHAVARIA and DAY, 1991). The reduction of phosphoenolpyruvate carboxykinase expression caused by selenium reduced the amount of phosphoenolpyruvate as well, and compensation of this reduction could increase the level of alpha-enolase.

The applied treatment could affect the metabolism of fatty acids, because the expression level of glycerol-3-phosphate dehydrogenase enzyme has reduced in the treated group. The terminal product of the reaction catalysed by glycerol-3-phosphate dehydrogenase is glycerol-3-phosphate, to which the activated fatty acid is connected in one pathway of triglyceride synthesis (HARDING et al., 1975). The reduction in the expression of enzyme reduces the amount of terminal product that can reduce the intensity of fatty acid production. The reduction in fatty acid level could affect the catabolism of fatty acid, this could be justified by reduced level of acetyl-coenzyme A dehydrogenase. Acyl CoA dehydrogenase catalyses the first step of beta-oxidation of fatty acids, and beta-oxidation is one of the first steps of fatty acid catabolism (THORPE and KIM, 1995).

We detected the highest difference in expression of cytoskeletal actin and ADF actin binding protein, these proteins showed 2.4 and 4.8 times higher expression level in the treated group. By these facts we can conclude that selenium causes an increase in amount of cytoskeletal actin and parallel with increase of actin, the expression of the actin regulatory protein increases as well. Because of the high dose of selenium added to the feed, liver cells were exposed to more stress. More stress could cause an increase of intensity of transport and signalling pathways in the liver cells, as the cytoskeletal actin has a key importance in performing transport and signalling pathways (SMALL et al., 1999) so it could be the reason of increasing in cytoskeletal actin level.

Three enzymes had higher expression in the treated group which play a role in antioxidant processes, however none of them are among the well-known selenium dependent enzymes. The alpha-aminoadipic semialdehyde dehydrogenase metabolises the aldehydes which produced in lipid peroxidation (FONG et al. 2006). The peroxiredoxin-6 reduces the hydrogen peroxide, the short-chain fatty acids and the

phospholipid-hydroperoxide (CHAE et al., 1994). The 3-mercaptopyruvate sulfurtransferase protein transforms the harmful cyanide and capable of binding and transport of selenium (OGASAWARA et al., 2005). The biological effect of selenium is ambivalent, lack of selenium can develop diseases, and nevertheless the overdose can cause poisoning. We did not detect symptoms of poisoning in our experimental group, but the high selenium dose could have harmful effects (4.25 mg/kg selenium in the compound feed of treated group, due to the EU regulation the maximum concentration as 0.5 mg/kg in compound feed). The overdose of selenium can damage the antioxidant system, activity of gluthatione-peroxidase reduces, the lipid peroxidation increases that could cause necrosis (damage of cell membranes, inhibition of enzyme activity) (GAÁL, 1998; MÉZES and MATKOVICS, 1986). We assume that in our experiment this could be the situation as well, because we did not detect increased glutathion-peroxidase expression, but the expression of enzymes that catalyse catabolism of the products of lipid peroxidation became higher. The increase of proteasome alpha type subunit level in the treated group can be in connection with lipid peroxidation process. The function of proteasome complex breakdown of degraded proteins (MURATA et al., 2009), probably parallel to damage of antioxidant system many proteins degraded.

3. Proteome analysis of musculus longissimus dorsi of Merino and Tsigai sheep breeds

3. 1. Materials and methods

10 rams were involved in the study, four Merino and six Tsigai ones. Animals were born in January and slaughtered at the weight of 40 kg and age of 4.5 months in average. Housing and feeding conditions were the same for each animal. They were fed ad libitum with concentrate and hay, rearing in a stable, kept is small groups. *Musculus longissimus dorsi* samples were harvested between the 12th and 13th ribs, within 20 min after slaughter into cryotubes. The samples immediately frozen in liquid nitrogen, and then kept at -80°C until subsequent analysis.

We used two-dimensional polyacrylamide gelelectrophoresis (2D-PAGE) to separate proteins of skeletal muscle. Some parameters of separation were optimized: extraction of proteins, pH range, rehydration buffer, conditions of isoelectric focusing (*Table 4*).

Table 4. Optimization of 2D-PAGE parameters for sheep muscle

Protein extraction (lysis buffers)	- 8 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2 100X Bio-Lyte ampholyte - 8.5 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 40 mM Tris, 1 mM EDTA, 0.8% 100X Bio-Lyte ampholyte
pH range	pH 5-8 and pH 3-10
Rehydration buffer	- general rehydration buffer: 8 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% 100X Bio-Lyte ampholyte, 0.002% bromphenol blue - DeStreak rehydration buffer: 8 M urea, 2 M thiourea, 2% CHAPS, 15 mg/ml DeStreak reagens, 0.2% 100X Bio-Lyte ampholyte, 0.002% bromphenol blue
Isoelectric focusing	In one step: 10000 V 50000 Vh In three steps: 1. step: 250 V 20 min 2. step: 10000 V 2.5 hr 3. step: 10000 V 50000 Vh

As a result of method optimization, parameters were chosen those are appropriate to detect the largest number of spots. We performed the two-dimensional polyacrylamide gel electrophoresis of Tsigai and Merino rams, three technical replicates per sample were performed.

Gel images were analyzed and compared with Delta2D software, spots which showed significant differences between groups, were identified with liquid chromatography coupled mass spectrometry (LC-MS/MS).

3. 2. Major conclusion of the thesis

Based on the results of our optimization experiment, we determined the ideal parameters to performing the two-dimensional polyacrylamide gel electrophoresis of sheep muscle samples (*Table 5*).

Table 5. Ideal parameters of 2D- PAGE for sheep muscle

Protein extraction (lysis buffers)	8 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% 100X Bio-Lyte ampholyte
pH range	pH 5-8
Rehydration buffer	general rehydration buffer: 8 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% 100X Bio-Lyte ampholyte, 0.002% bromphenol blue
Isoelectric focusing	In three steps

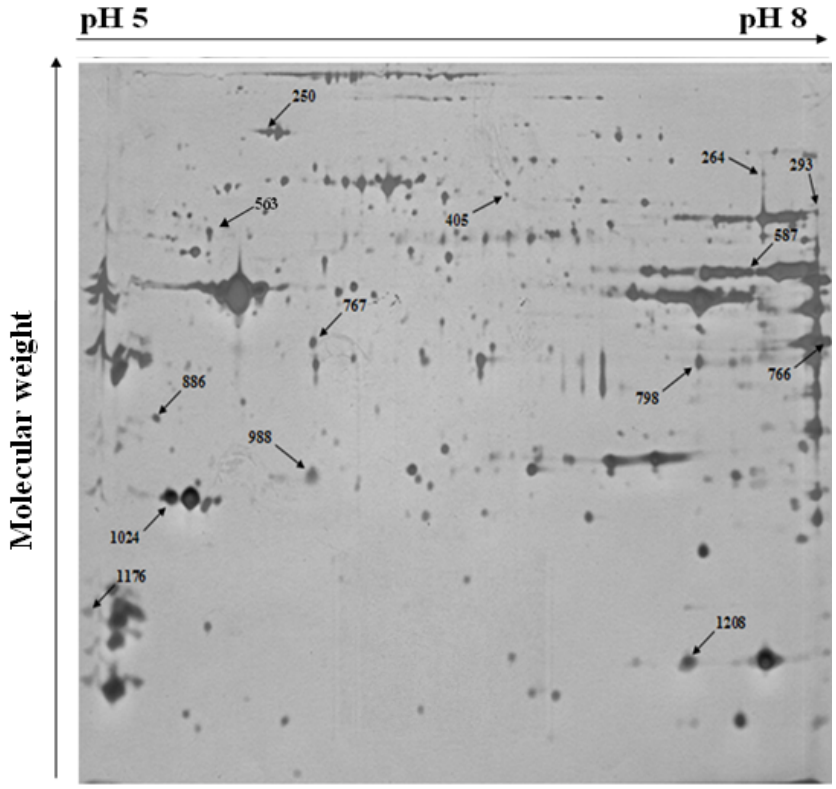

Figure 2. Representative 2-D PAGE image of sheep muscle. Proteins those were identified by LC-MS/MS are marked with numbers

Approximately 315 spots were detected on each polyacrylamide gel (on gels of Tsigai and Merino groups). 27 protein spots showed significant difference in the intensity ($p<0.05$) between two groups, 14 proteins of these, were identified successfully by mass spectrometry (*Figure 2*). The intensity of 9 spots were higher in Merino group while other 5 spots were detected with higher expression level in the Tsigai group (*Table 6*). We have made three groups, differentiated by the functions of the identified proteins, these are structural proteins, carbohydrate metabolism-related proteins, and miscellaneous proteins.

Table 6. Differentially expressed proteins ($p<0.05$) between Merino and Tsigai groups identified by LC-MS.

Spot no.	Identified protein	Accession number	Matched peptides/% sequence coverage	Theoretical pI/Mw (Da)	Ratio[1]
250	Alpha-actinin-3	Q0III9 (Bos taurus)	28/41	5.3/103,150	2.0
264	Aconitate hydratase, mitochondrial	P20004 (Bos taurus)	7/24	7.2/82,401	0.6
293	6-phosphofructokinase, muscle type	Q0IIG5 (Bos taurus)	3/16	8.6/85,162	0.4
405	Stress-induced-phosphoprotein	Q3ZBZ8 (Bos taurus)	3/15	6.1/62,481	0.4
587	Beta-enolase	Q3ZC09 (Bos taurus)	7/52	7.7/46,964	1.6
766	Glyceraldehyde-3-phosphate dehydrogenase	Q28554 (Ovis aries)	8/67	7.8/34,731	0.5
767	Actin, alpha cardiac muscle	Q3ZC07 (Bos taurus)	8/45	5.2/41,784	1.4
798	Fructose-1,6-bisphosphatase	P09199 (Ovis aries)	7/44	6.1/36,544	0.7
886	Actin, cytoplasmic (beta-actin)	P60713 (Ovis aries)	3/12	5.3/41,736	2.6
988	Myosin light chain 1/3, skeletal muscle isoform	A0JNJ5 (Bos taurus)	3/49	5.0/20,800	2.3
1024	Myosin light chain 1/3, skeletal muscle isoform	A0JNJ5 (Bos taurus)	8/66	5.0/20,800	1.3
1176	Fast skeletal myosin light chain 2	B9VGZ8 (Ovis aries)	3/27	4.9/19,102	2.3
1208	Myoglobin	P02190 (Ovis aries)	5/45	6.9/16,923	0.5

[1] number of identified peptides and the percentage of the database protein sequence covered by matching peptides
[2] isoelectric point and molecular weight from Swissprot database
[3] ratio value of the Merino group to Tsigai group

Differences in protein expression between the breeds might be caused by the difference in the intensity and direction of their selection. Tsigai can be considered as an autochthonous breed which was not exposed to high selection pressure in contrast to Merino which was strongly selected first for wool and then for meat production traits. The Merino type included in the study is a new variation among Merinos, from the cross of Merino ewes and booroola Merino rams. In addition, Tsigai breed was selected

for milk production in a period, this opposite direction of selection may cause some differences in protein expression as well.

All of the structural proteins have shown higher expression in Merino breed. The expression of both contractile (myosin light chain 1/3 protein, myosin light chain 2) and cytoskeletal proteins (alpha actinin, beta actin) showed higher expression in Merino. High expression of structural proteins can be in connection with intensive protein anabolism.

In contrary of structural proteins, the higher expression level of carbohydrate metabolism related proteins could not be connected to one or to the other breeds. The expression level of 6-phosphofructokinase, beta-enolase, glyceraldehid 3-phosphate dehydrogenase and fructose-1,6-bisphosphatase enzymes were higher in Tsigai group, while the expression level of aconitate hydratase and ATP synthase subunit beta were higher in Merino group.

In Tsigai breed, 3 among the higher expression level proteins catalyse steps of glycolysis, while the fourth protein plays a role in gluconeogenesis and all of them are located in the cytoplasm. The different expression level of enzymes, which connected to glycolysis in muscles and meat have been the object of disputes for a long time. It is mainly due to the naturally high amount in muscle. Usually, a connection was found between meat quality parameters and the expressional changes of glycolytic enzymes (SCHEFFLER et al., 2007; LAVILLE et al., 2007).

Two proteins showed overexpression in Merino which can be in connection with carbohydrate metabolism and they are located in the mitochondrion. These proteins do not take part in glycolysis, but in citric acid cycle and terminal oxidation as well.

After comparison protein markers in the literature and proteins identified by us which can be in connection with carbohydrate metabolism and other roles, we could conclude that Tsigai breed could have better meat quality parameters, because of overexpression of glycolytic enzymes and myoglobin, and lower expression of stress-induced-protein. However, we can not conclude this statement without laboratory analysis of meat quality parameters. In the future, it would be worth performing the lab

analysis of meat quality parameters of Merino and Tsigai breeds and compare the results to our conclusions that were made from identified proteins, and then these proteins can be markers of meat quality of sheep.

4. Proteomic analysis of skeletal muscle at different live weights in Charolais bulls

4. 1. Materials and methods

In a commercial beef cattle farm 10 finishing Charolais bulls were involved in the study, 5 of them were slaughtered at the live weight of 500 kg and at the age of 440 days old and five at 700 kg live weight, at 540 days old. Animals were slaughtered at a commercial slaughterhouse according to EU regulations. *Musculus longissimus dorsi* samples were harvested between the 12th and 13th ribs, within 30 min after slaughter into cryotubes as an amount of 2 g per sample.

We used two-dimensional difference in gel electrophoresis (2D-DIGE) to separate proteins of skeletal muscle. Some of the parameters of separation were optimized: extraction of proteins, pH range, conditions of isoelectric focusing (*Table 7*).

Table 7. Optimization of 2D-DIGE parameters for cattle muscle

Protein extraction (lysis buffers)	- 7 M urea, 2M thiourea, 4% CHAPS, 30 mM Tris - 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, 10mM DTT - 8 M urea, 4% CHAPS, 30 mM Tris
pH range	pH 5-8 and pH 3-10
Isoelectric focusing	In one step: 10000V 50000Vh In three steps: 1. step: 250V 20 min 2. step: 10000V 2.5 hr 3. step: 10000V 50000 Vh

As a result of method optimization, parameters were chosen those are appropriate to detect the highest number of spots. We performed the two-dimensional difference in gel electrophorese of the two groups (500 kg and 700 kg), three technical replicates per sample were performed.

Gel images were analyzed and compared with Delta2D software, spots which showed significant differences between groups, were identified with liquid chromatography coupled mass spectrometry (LC-MS/MS).

4. 2. Major conclusion of the thesis

Based on the results of our optimization experiment, we determined the optimal parameters to performing the two-dimensionale difference in gel electrophoresis of cattle muscle samples (*Table 8*).

Table 8. Ideal parameters of 2D- DIGE for cattle muscle

Protein extraction (lysis buffers)	7 M urea, 2M thiourea, 4% CHAPS, 30 mM Tris
pH range	pH 5-8
Isoelectric focusing	In three steps

Musculus longissimus dorsi proteins in the pI range between 5 and 8 were included in the comparative analysis. Approximately 350 spots were detected on each gel. Ten protein spots showed differential expression levels based on normalized spot volumes between the 500 and 700 kg weight groups (*Figure 3*). Nine spots showed a higher expression in the 700 kg weight group, while one protein spot intensity was higher in the 500 kg weight group (*Table 9*).

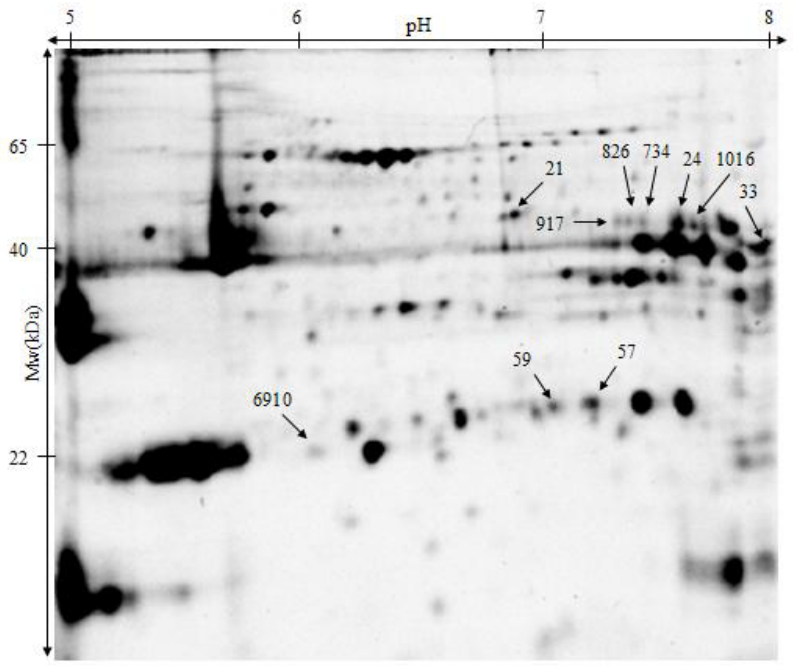

Figure 3. Representative 2D-DIGE image of m. longissimus dorsi. Proteins those are significantly different according to slaughter weight are marked with numbers

Table 9. Differentially expressed proteins (p<0.05) in Charolais bulls according to slaughter weight identified by LC-MS

Spot no.	Identified protein	Accession number	Matched peptides/ % sequence coverage[1]	Theoretical pI/Mw (Da)[2]	Ratio [3]
33	Actin, alpha skeletal muscle	P68138 (Bos taurus)	15/57	5.2/42,051	1.6
1016	Beta-enolase	Q3ZC09 (Bos taurus)	3/13	7.6/47,096	1.8
24	Beta-enolase	Q3ZC09 (Bos taurus)	5/20	7.6/47,096	1.9
734	Beta-enolase	Q3ZC09 (Bos taurus)	10/23	7.6/47,096	2.2
826	Beta-enolase	Q3ZC09 (Bos taurus)	10/31	7.6/47,096	2.3
917	Beta-enolase	Q3ZC09 (Bos taurus)	15/51	7.6/47,096	2.2
21	Alpha-enolase	Q9XSJ4 (Bos taurus)	24/64	6.4/47,326	1.4
57	Triosephosphate isomerase	Q5E956 (Bos taurus)	9/49	6.3/26,690	1.7
59	Triosephosphate isomerase	Q5E956 (Bos taurus)	16/80	6.3/26,690	1.8
6910	Heat shock protein beta-1	Q3T149 (Bos taurus)	9/70	6.0/22,393	0.4

[1] number of identified peptides and the percentage of the database protein sequence covered by matching peptides
[2] isoelectric point and molecular weight from Swissprot database
[3] ratio value of the 700- to 500 kg weight group

Spots 24, 734, 826, 917 and 1016 were all identified as beta-enolase (ENO3), in spite of being found at 5 different pI positions on the polyacrylamide gel. This heterogeneity might be the consequence of post-translational modifications that may influence the charge of the proteins. The modifications of beta-enolase have not been reported yet in cattle protein databases. Beta-enolase is the muscle-specific enolase, expressed in striated muscle. This enzyme catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis (PESHAVARIA and DAY, 1991). Its expression greatly increased during postnatal muscle growth because enolases involved in energy metabolism, specifically in pyruvate production (PICARD et al., 2010). This confirms our findings that 1.8-2.2-fold higher beta-enolase expressions were observed in the 700 kg weight group. In the literature were found similar results, ENO3 was more abundant in older pigs, indicating higher glycolytic activity in older animals.

The enolase has three major isoforms, beside beta-enolase, alpha-enolase (ENO1) has also showed an increased expression in 700 kg live weight animals. ENO1 is expressed mainly in the early stage of embryonic development but it is present in most adult tissues (ZOMZELY-NEURATH, 1983).

An additional protein was identified which is included the group of metabolic proteins as well. Triosephosphate isomerase (TPI1) was observed at two different pI positions (57, 59). Two types of posttranslational modifications of TPI1 were reported until now, both of phosphorylation and acetylation decreases the pI of proteins. In our study we have not investigated which modifications might be responsible for the differences observed in pI. Protein spots 57 and 59 had 1.7-fold and 1.8-fold higher expression in the 700 kg weight bulls compared to the 500 kg weight bulls. TPI1 plays an important role in energy production, being an enzyme that catalyzes the reversible interconversion of the dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate in glycolysis (ALBERY and KNOWLES, 1976). Several studies have found positive correlation between the triosephosphate isomerase level and the age in different species (HOLLUNG et al., 2009; KIM et al., 2009; TELTATHUM and MEKCHAY 2009). This may indicate that the different expression levels of this protein are related to muscle growth.

One of the 10 identified proteins is a structural protein. Alpha actin is a major constituent of the contractile apparatus in skeletal muscle. In Charolais bulls, the expression level of alpha actin was 1.6 fold higher in the 700 kg weight group compared to the 500 kg weight group. A significant increase of muscle fibre size was observed between 14 and 18 months old animals (TE PAS et al., 2004). This change may due to increasing number of contractile proteins within the muscle fibre.

Up-regulation of heat shock protein beta-1 (Hsp27) was observed in 500 kg weight group. Hsp27 is a member of small heat shock proteins, main cellular functions are chaperone activity, regulation of the actin cytoskeleton and control of apoptosis (GUSEV et al., 2002). Previous papers have found correlation between Hsp27 level and tenderness. There are many isoforms of Hsp27, the majority of isoforms are in negative correlation with tenderness and some of them are in positive correlation (BERNARD et al., 2007; KIM et al., 2008; MORZEL et al., 2008). We can not conclude correlation between isoforms of Hsp27 and tenderness without instrumental examination of meat quality parameters of two groups.

Based on our 2D-DIGE proteomic analysis of m. longissimus dorsi in Charolais bulls, it was concluded that proteins function in responsible for glycolytic activity and muscle growth showed higher expression in 700 kg group. Future work will examine dynamics of expression profiles in a wider range of live weight and age.

In conclusion, proteomic methods are appropriate to investigate complex traits. However their application in the animal production is limited, but in parallel with increasing knowledge and development of laboratory methods, there is a higher demand for to better understanding of production, physiological and pathophysiological traits.

5. NEW SCIENTIFIC RESULTS OF THE THESIS

1. Based on the results of method development the optimal lysis and rehydration buffer of two-dimensional polyacrylamide gelelectrophoresis for chicken liver: 8.5 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 0.2% 100X Bio-Lyte ampholyte and 8 M urea, 2 M thiourea, 2% CHAPS, 15 mg/ml DeStreak reagent, 0.2% 100X Bio-Lyte ampholyte, 0.002% bromphenol blue. The optimized lysis buffer for *m. longissimus dorsi* of sheep contains 8 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% 100X Bio-Lyte ampholyte and the composition of ideal rehydration buffer is the same. Optimal lysis buffer of two-dimensional difference in gel electrophoresis for *m. longissimus dorsi* of cattle contains 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris. For the isoelectric focusing, pH range 5-8 and three step focusing condition were optimal all of the three sample type.

2. We detected average 708 spots (SD: 44) on each polyacrylamide gel of chicken liver, average 315 spots (SD: 32) on gels of *musculus longissimus dorsi* of sheep and average 362 spots (SD: 33) on each gel of *musculus longissimus dorsi* of cattle with application of optimal parameters in pH 5-8 range.

3. The expression of alfa-aminoadipic semialdehyde dehydrogenase, cytoplasmic actin, proteasome subunit alpha type, ADF actin binding protein, cytoplasmic malate dehydrogenase, alpha-enolase and 3-mercaptopyruvate sulfurtransferase increased after high selenium induction. While the expression of phosphoenolpyruvate carboxykinase, glycerol-3-phosphate dehydrogenase, RNA-binding protein 8A, eukaryotic translation initiation factor 5A-1 and the acetyl-coenzyme A dehydrogenase proteins decreased as a result of selenium treatment. Based on our results can we conclude that the applied dose of selenium reduced the intensity of gluconeogenesis, increased the quantity of cytoskeletal actin and the expression of actin regulatory protein as well. The selenium treatment could affect the metabolism of fatty acids and the antioxidant system.

4. As a conclusion of musculus longissimus dorsi of Merino and Tsigai breeds that expression level of myosin light chain 1/3, myosin light chain 2, alpha-actinin,

beta-actin, aconitate hydratase, stress-induced-phosphoprotein and ATP synthase subunit beta were higher in Merino breed. While the expression of 6-phosphofructokinase, beta-enolase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase and myoglobin were higher in Tsigai breed. All of the structure proteins have shown higher expression in the Merino breed, this can be in connection with intensive protein anabolism. Overexpression of glycolytic enzymes and myoglobin, and lower expression of stress-induced-protein can be in connection with meat quality parameters.

5. As a result of proteomic analysis of skeletal muscle tissues of Charolais bulls with live weight of 500 and 700 kg, we concluded that alpha-enolase, beta-enolase, skeletal muscle actin, and triosephosphate isomerase proteins showed the higher expression level in the group of 700 kg bulls, those can indicate higher glycolytic activity and muscle development. While the expression of heat shock protein beta-1 was higher in the group of 500kg bulls.

6. PRACTICAL UTILIZATION OF THE RESULTS

The optimization of protein isolation, two-dimensional polyacrylamide gelelectrophoresis and two-dimensional difference in gel electrophoresis methods contribute to simple, time saver proteomic studies of chicken liver and *m. longissimus dorsi* of sheep and cattle in the future. These optimized methods may contribute to successful protein biomarker discoveries.

Our results on protein expression of chicken liver show that selenium responsive proteins, those are part of biochemical pathways, participate and help to understand the biological processes in liver.

Several identified proteins in the comparative study of sheep breeds and study of bulls with different live weights are in connection with meat quality characteristics. These proteins could be assumed as biomarker molecules, which could be used in practice to predict a meat quality parameter based on their changes in expression.

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7. LIST OF PUBLICATIONS

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