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ORIGINAL PAPER

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IDENTIFICATION OF MARE COLOSTRUM PROTEINS

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ABSTRACT

Colostrum is an essential feed of foals. It is a source of nutrients and functional proteins significant for foals' growth and development. In the presented research using two-dimensional electrophoresis coupled via spectrometry mass MALDI-TOF in the mares' colostrum (whey proteins fraction) were identified 24 proteins representing 15 different gene products. The identified proteins were involved in supporting foals' immature immune systems and in the transport of various compounds. Further research of mares' colostrum will allow determining more gene products. An in-depth analysis of mares' milk will provide information about biochemical processes occurring in the mammary gland of the mare during the lactation period.

Key words: proteomics, colostrum, whey, mare, foals

INTRODUCTION

Colostrum is a body fluid with high biological value, rich in nutrients and regulatory components, which underlies proper growth and development of newborns. The most important compounds of milk are proteins that are not the only the source of crucial amino acids, but are also, as functional factors, involved in various metabolic pathways and proteins which helps newborns to adapt to the new extra-uterus life environment.

Proteomics allows to study and determining the protein profile of body fluids. Highly specialized proteomics tools enable protein identification which we cannot detect using traditional biochemical methods.

Due to the significance of ruminants milk in the human diet, there is a lot of research on the proteome of this body fluid [Le et al. 2011, Zhang et al. 2015a, 2015b, Tacoma et al. 2016, Delosière et al. 2019].

The proteomics approach have allowed identification of thousands of proteins of cow's milk [Delosière et al. 2019]. These included proteins associated with mammary gland development, milk components synthesis, and calf growth [Zhang et al. 2015a]. Research shows differences in the protein profile between colostrum and milk [Zhang et al. 2015b]. Furthermore, proteomic studies demonstrated protein composition of milk fat globule membrane [Lu et al. 2016, Wang et al. 2017].

Compared to cow milk, mare milk has a higher content of whey protein fraction, which is albumin-type milk [Potočnik et al. 2011]. It is widely known that whey proteins have a significant impact on infant development.

No research has been reported so far on colostrum whey low-content proteins in mares. Accordingly, this research was undertaken to study mare colostrum proteins, especially those involved in the adaptation of newborn foals to new environmental conditions. To archive this aim, we used two-dimensional electrophoresis with spectrometry mass MALDI-TOF.

MATERIAL AND METHODS

Research material and sample preparation

The experiment was carried out on colostrum collected from six mares, Polish noble half-breed in 12 hours after parturition. The animals came from horse stables

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in Nowielice and were kept in a stable-pasture system. Colostrum samples were centrifuged at 4°C (4500 g, 30 minutes), and the lipid-rich cream layer was removed to receive skim milk (fat-free precipitant). The next step was to precipitate the casein with 30% acetic acid to adjust the pH to 4.6 and centrifuged at 4°C (3380 g, 15 minutes). The obtained supernatant was collected and placed in new Eppendorf tubes. To each tube added acetone (–20°C) to extract proteins. The precipitant was diluted in lysis buffer containing: 5 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 0.2% ampholytes pH 3–10, and 2 mM TBP. Protein concentration was measured using the Protein-Assay (Bio-Rad).

Two-dimensional electrophoresis and identification of the proteins

Colostrum samples containing 800 µg of proteins were applied on 24 cm, pH 4–7 linear IPG strips, and rehydrated. Rehydration was performed in two steps: passive rehydration (6 h, 0 V) and active rehydration (12 h, 50 V). First-dimensional isoelectric focusing performed using the following procedure: 50 V for 100 Vh, 250 V for 250 Vh, 500 V for 500 Vh, 1000 V for 1000 Vh, 2h in linearly increasing voltage from 1000 V to 5000 V, and subsequently 5000 V for 90000 Vh. After the focusing process, IPG strips were equilibrated according to Lepczyński et al. [2018]. The second step of the 2d electrophoresis was to separate proteins according to their molecular weight. Equilibrated IPG strips were placed on the top of 12% polyacrylamide gels. The migration of proteins was run at 40 V for 3.5 hours and 90 V for 14 hours. After migration, the mass spectrometry identification was performed according to Dratwa-Chałupnik et al. [2016] procedure.

RESULTS

The main aim of the study was the identification of mare's colostrum proteins. To achieve this goal, we applied two-dimensional electrophoresis coupled via mass spectrometry MALDI-TOF. Among 250 spots, we identified 24 proteins, representing 15 different gene products (Table 1). Furthermore, 20 proteins were characteristic for equines (*Equus caballus*, *Equus przewalskii*, and *Equus sinus*). Proteins were separated in the 4–7 range, and molecular mass between 250–10 kDa (Fig. 1). For all identified proteins were determined theoretical and experimental molecular weight (Table 1). According to the Uniprot database, identified proteins were assigned their cell lo-

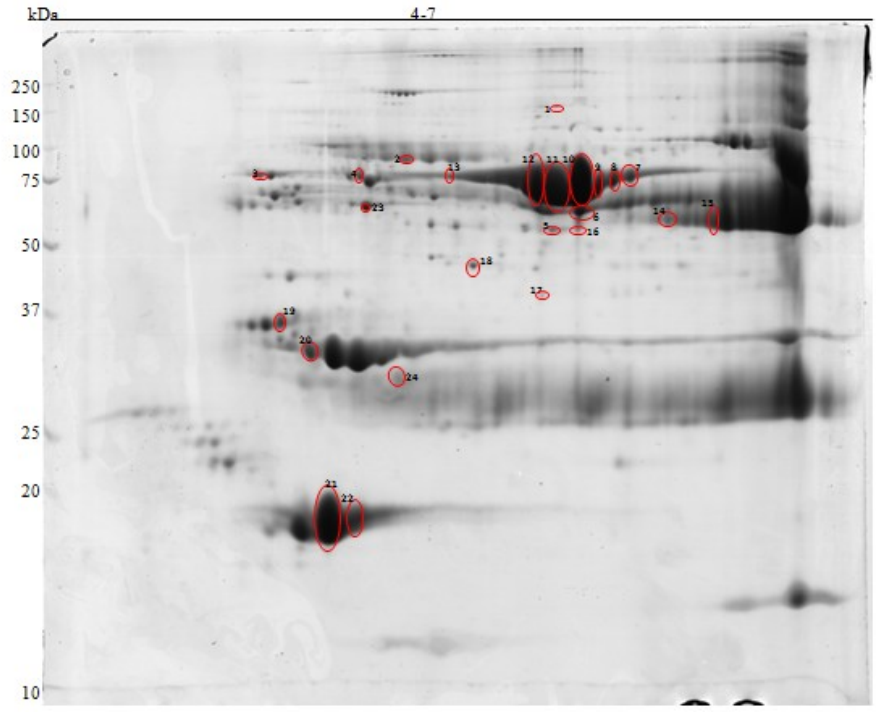

Fig. 1. Picture of mare's colostrum protein map after two dimensional electrophoresis (circles mark proteins spots as shown in Table 1)

Rys. 1. Zdjęcie mapy białkowej siary klaczy po elektroforezie dwukierunkowej (w kółkach zaznaczono spoty białkowe, zgodnie z tabelą 1)

Table 1. List of proteins identified in mare colostrum using MALDI-TOF

Tabela 1. Lista białek zidentyfikowanych w siarze klaczy z wykorzystaniem MALDI-TOF

Spot no.	Protein name	Gene name	Accession number	Number of matched peptides	Sequence coverage/ mascot score	Theoretical pI/MW (pH/kDa)	Experimental pI/MW (pH/kDa)	Cellular localization	Function	Organism
1	Inter-alpha-trypsin inhibitor heavy chain H4 isoform X3	ITIH4	XP_023476060.1	7	11/82	5.91/ 88	7.43/100	Plasma membrane	Protease inhibitor	*Equus caballus*
2	Immunoglobulin mu heavy chain constant chain secreted form	IGHM	AAU09792.1	10	31/120	5.52/50	6.35/50	Extracellular region Secreted	Antigen binding	*Equus caballus*
3	Alpha-1-antitrypsin	Spi2-8	BAG69588.1	7	22/81	5.23/47	5.23/47	Extracellular region Secreted	Protease inhibitor	*Equus caballus*
4	rho GTPase-activating protein 39	ARHG-AP39	XP_018888231.1	9	15/79	8.57/102	7.30/121	Nucleus	GTPase activator, Migration, vesicular transport activator	*Gorilla gorilla gorilla*
5	Fetuin-B	FETUB	XP_008505086.1	9	31/91	6.20/41	6.20/41	Extracellular region Secreted	Protease inhibitor	*Equus przewalskii*
6	Immunoglobulin gamma 5 heavy chain constant region	IGHG5	CAC86340.1	7	33/72	5.95/36	–	Extracellular region Secreted	Antigen binding	*Equus caballus*
7	Serum albumin	ALB	XP_008524663.1	16	35/139	5.78/70	5.95/69			*Equus przewalskii*
8	Serum albumin	ALB	XP_008524663.1	12	30/94	5.78/70	5.95/69			*Equus przewalskii*
9	Serum albumin	ALB	XP_008524663.1	12	28/130	5.78/70	5.95/69			*Equus przewalskii*
10	Serum albumin	ALB	XP_008524663.1	15	35/125	5.78/70	5.95/69	Extracellular region Secreted	Binding of various components such as fatty acids, hormones, ions	*Equus przewalskii*
11	Serum albumin	ALB	XP_008524663.1	14	33/112	5.78/70	5.95/69			*Equus przewalskii*
12	Serum albumin	ALB	XP_008524663.1	12	28/103	5.78/70	5.95/69			*Equus przewalskii*
13	Serum albumin	ALB	ALBU_HORSE	9	19/125	5.95/71	5.95/69			*Equus caballus*
14	Nuclear antigen Sp-100-like	LOC1105 - 61688	XP_021513875.1	8	31/82	9.87/39	–	Nucleus	–	*Meriones unguiculatus*
15	Immunoglobulin gamma 4 heavy chain	IGHG4	AAS18415.1	8	26/105	7.71/36	7.18/36	Extracellular region Secreted	Antigen binding	*Equus caballus*
16	Serum albumin	ALB	XP_008524663.1	8	18/83	5.78/70	5.95/69		Binding of various components such as fatty acids, hormones, ions	*Equus przewalskii*
17	Serum albumin precursor	ALB	NP_001310707.1	8	15/81	5.89/70	5.95/69	Extracellular region Secreted		*Equus asinus*
18	Complement C3 alpha chain-like	LOC103-544686	XP_008509716.1	8	32/89	4.88/31	6.41/19	Secreted	Endopeptidase activity, Component of complement system	*Equus przewalskii*
19	Proline-rich and Gla domain 4 (transmembrane) isoform 3	PRRG4	ALQ34226.1	8	68/85	6.43/18	7.08/25	Extracellular region Secreted	Calcium ion binding	*Homo sapiens*
20	Cila and flagella associated protein 206 isoform X1	Cfap206	XP_021498365.1	11	23/81	7.16/ 71	6.38/71	Cytoskeleton	Axoneme assembly Cillium movement	Meriones unguiculatus

Table 1. List of proteins identified in mare colostrum using MALDI-TOF – continued

Tabela 1. Lista białek zidentyfikowanych w siarze klaczy z wykorzystaniem MALDI-TOF – ciąg dalszy

Spot no.	Protein name	Gene name	Accession number	Number of matched peptides	Sequence coverage/ mascot score	Theoretical pI/MW (pH/kDa)	Experimental pI/MW (pH/kDa)	Cellular localization	Function	Organism
21	Beta-lactoglobulin-1 precursor	LGB1	NP_001075962.1	10	52/107	4.95/21	4.95/20	Secreted	Retinol and fatty acids binding, Stimulating cell proliferation and growth	*Eqqus caballus*
22	Beta-lactoglobulin-1	LGB1	LACB1_HORSE	9	52/77	4.95/21	4.95/20	Secreted	Retinol and fatty acids binding, Stimulating cell proliferation and growth	*Eqqus caballus*
23	Vitamin D-binding protein	GC	XP_001489400.1	9	106/32	5.46/55.9	5.32/53	Secreted	Vitamin D and albumin binding	*Eqqus caballus*
24	Interleukin-24 isoform x1	IL24	XP_008508951.1	5	73/25	9.35/23.5	9.65/23.6	Secreted	Immunomodulating cytokine	*Equus przewalskii*

calization and function, as shown in Table 1. Most of the proteins were located in the extracellular region (secreted).

DISCUSSION

Proteomics tools are successfully used in the analysis of farm animals colostrum and milk proteome: cow [Zhang et al. 2015a, Zhang et al. 2015b, Tacoma et al. 2016, Delosière et al. 2019], sheep [Cunsolo et al. 2017], and goat [Cunsolo et al. 2015, Cunsolo et al. 2017]. So far, proteomic approaches have allowed knowing the ruminant's colostrum and milk protein profile and capturing changes in protein profile during cow lactation [Zhang et al. 2015b].

Whey is a source of many regulating components, including bioactive peptides, antioxidants, and immunomodulating factors [Tai et al. 2016]. Beta-lactoglobulin (LGB1) is the main whey protein of mare milk. Presented research showed the presence of beta-lactoglobulin in mare colostrum collected in 12 h after birth (Table 1). The percentage content of LGB1 in equine milk is about 36%, whereas in ruminants between 20% in cow milk and 77% of all whey proteins in ovine [Potočnik et al. 2011]. LGB1 is a multifunctional protein. LGB1 can bind fatty acids and vitamins [Le Maux et al. 2014]. It is a retinol carrier [Król et al. 2008]. Research by Tai et al. [2016] showed that LGB1 stimulates cell proliferation and growth. Moreover, β-lactoglobulin affects the secretion of proinflammatory cytokines and regulates the Th1/Th2 ratio [Tai et al. 2016].

After birth, the foal immune system is not fully mature and requires delivery of immunomodulating proteins. Presented research showed the presence of immunoregulating proteins such as immunoglobulin mu heavy chain (IGHM) and immunoglobulin gamma 5 heavy chain (IGHG5), immunoglobulin gamma 4 heavy chain (IGHG4), nuclear antigen Sp-100-like, complement C3 alpha chain-like, the interleukin-24 precursor in mare colostrum (Table 1).

Intake of colostrum by the infant influences the correct development of innate immunity. Newborn foals are born with trace amounts of antibodies, because the epitheliochorial placenta prevents the passage of immunoglobulin from mother to fetus. In colostrum collected 12 hours after parturition, the following proteins have been identified: immunoglobulin mu heavy chain (IGHM); immunoglobulin gamma 5 heavy chain (IGHG5), and immunoglobulin gamma 4 heavy chain (IGHG4) (Table 1). The immunoglobulins delivered with colostrum protect the newborn foal against environmental pathogens. Results obtained by Markiewicz-Kęszycka et al. [2013] indicate that mare milk contains high amounts of immunoglobulins, 15.8% of the total whey proteins. Furthermore, in equine colostrum nuclear antigen Sp-100-like protein has been identified. This protein was located in the nucleus which is an antigen-stimulated by interferon.

The complement system is a primary line of protection against pathogens, a significant element of innate immunity [Alcorlo et al. 2013]. Among the identified mare colostrum proteins was the protein participating in the activation of the complement system (all three pathways)

complement C3 alpha chain-like (Table 1). The main role of the complement system is to create a membrane attack complex, which allows lysis of bacteria cells [Alcorlo et al. 2013].

Another identified protein in mare colostrum was the interleukin-24 precursor (Table 1). This protein belongs to the IL-10 cytokine family. IL-24 is produced by immune cells, including myeloid cells and lymphoid cells. Interleukin-24 is the immunoregulating cytokine [Persaud et al. 2016].

All the above-mentioned proteins were connected with regulating foals' immune response. After birth, foals are exposed to environmental pathogens. Except for immunomodulating proteins, along with colostrum are deliver protease inhibitors which have to protect proteins against degradation in the newborn digestive tract.

The presence of protease inhibitors in the study mare colostrum has been demonstrated, including alpha-1-antitrypsin, inter-alpha-trypsin inhibitor (ITIH4), and fetuin-b (FETUB) (Table 1). Research carried out by Zhang et al. [2015a] showed the presence of alpha-1-antitrypsin and ITIH4 in cow's milk. According to the authors, the observed decrease of trypsin and immunoglobulin inhibitor concentration with subsequent lactation days indicates the protective role of this inhibitor against proteolytic degradation of immunoglobulin. Fetuin-B has an endopeptidase inhibitor activity and metalloendopeptidase inhibitor activity. Nissen et al. [2012] using two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS) showed fetuin-B in cow milk.

The presence of protease inhibitors in mare colostrum indicates their protective role against protein degradation, especially the immunomodulating proteins. Regulatory proteins transfer along with colostrum is possible owing to the "leaky intestinal barrier". The physiological transfer of antibodies is possible up to 24–36 hours after birth, subsequently the enterocyte cell membrane is sealed. The Inhibiting of protein proteolysis allows their absorption in unchanged form.

Moreover, in the presented research identified in mare colostrum proteins were involved in binding and transport of compounds, such as serum albumin and vitamin D-binding protein, proline-rich and Gla domain 4 (transmembrane) isoform 3 (Table 2). Vitamin D-binding protein (GC) is a vitamin D transporting protein. GC has the availability of binding albumin [Chun 2012]. The concentration of vitamin D in mare colostrum is 4.93 $\mu g \cdot L^{-1}$ [Pieszka et al. 2016]. Delivery with colostrum vitamin D is essential for the correct growth and development of foals.

Proline-rich and Gla domain 4 (transmembrane) isoform 3 (PRRG4) is a protein belonging to the PRRG family [Yazicioglu et al. 2013]. According to the Uniprot database, the PRRG4 can bind calcium ions. Calcium is involved in many processes, including activation of some enzymes, and is a primary building block of bone mass. Calcium with vitamin D shows synergetic action, vitamin D increases the bioavailability of calcium. The content of calcium in mare milk is 93 $mg \cdot ml^{-1}$ [Claeys et al. 2014].

Among identified proteins in mares' colostrum was rho GTPase-activating protein 39, which has GTPase activity properties (Table 1). This protein belongs to the Rho GTPases family. Rho GTPases family participate in many cellular processes, including in migration, vesicular transport and cytokinesis [Hodge and Ridley 2016]. This protein is associated with mammary gland maturation.

Mammary gland duct morphogenesis is a complex process, during which cell has to proliferate and migrate to the fat pads where are differentiate into luminal and myoepithelial cell compartments. Studies showed that over-expression of Rho GTPases proteins in mammary gland tissue in the postnatal period results in higher branching and delay elongating of the milk ducts and disorganization of ductal trees [Vargo-Gogola et al. 2006]. Vesicle transport is a pathway of main milk components, among other proteins and lactose. Participating Rho GTPases proteins in vesicle transport may indicate their role in compounds secretion into milk.

CONCLUSION

To sum up, using two-dimensional electrophoresis with mass spectrometry MALDI-TOF allowed the identification of mare colostrum whey proteins, essential for biochemical process regulation. Most of the identified proteins were associated with supporting an immature foals' immunological system. Further research of the mare colostrum proteome will enable determining important proteins that respond to the correct development of foals and milk components synthesis. Identification of low-abundant mare colostrum proteins and indicate in which metabolic pathways are involved may expand knowledge about biochemical processes occurring in the mammary gland and indicate proteins involved in foal growth and development.

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IDENTYFIKACJA BIAŁEK SIARY KLACZY – BADANIA WSTĘPNE

STRESZCZENIE

Siara jest podstawowym pokarmem źrebiąt. Jest ona źródłem składników odżywczych oraz funkcjonalnych białek istotnych w procesie wzrostu i dojrzewania źrebiąt. W prezentowanych badaniach, wykorzystując elektroforezę dwukierunkową sprzężoną ze spektrometrią mas MALDI-TOF w siarze klaczy (frakcja białek serwatkowych), zidentyfikowano 24 białka, które stanowiły 15 różnych produktów genowych. Zidentyfikowane białka zaangażowane były w wspieranie niedojrzałego układu immunologicznego źrebiąt oraz w transport różnych związków. Dalsze badania nad siarą klaczy pozwolą na zidentyfikowanie większej ilości produktów genowych. Dogłębna analiza mleka klaczy dostarczy informacji o procesach biochemicznych zachodzących w gruczole mlekowym klaczy w trakcie laktacji.

Słowa kluczowe: proteomika, siara, serwatka, klacze, źrebięta

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