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Slovak Journal of Food Sciences

Potravinarstvo Slovak Journal of Food Sciences
vol. 12, 2018, no. 1, p. 667-672
doi: https://doi.org/10.5219/976
Received: 23 September 2018. Accepted: 3 October 2018.
Available online: 29 October 2018 at www.potravinarstvo.com

ISSN 1337-0960 (online)

BOVINE MUCOUS MEMBRANES AS A SOURCE OF ANTIMICROBIAL COMPOUNDS

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ABSTRACT

Loss of food quality, deterioration of organoleptic properties and accumulation of anti-alimentary compounds are in focus of modern food science. Nowadays, such traditional methods as processing, physical and chemical treatment are used for improving of shelf life. An alternative ways of shelf life increasing are quite a sharp problem. Antimicrobial peptides (AMPs) could be an actual alternative. According to Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php), 2884 antimicrobial peptides from six kingdoms were found and identified. Mucous membranes of farm animals due to their border position and intensive contact with different pathogens could be a capacious source of such substances. Objects of the study were bovine oral cavity mucosa, nasal cavity mucosa, tracheal cavity mucosa, rectal mucosa, tongue mucosa, saliva gland and submandibular lymph nodes. Two-dimensional electrophoresis (2DE) was performed according to the method of O'Farrell, 35 protein fractions were identified by MALDI-TOF MS and MS/MS mass spectrometry. A number of qualitative and quantitative differences were revealed. A large number of histones (H2bd-like, H2BC, HIST1H2BD, HIST2H2AC, HIST1H2AH, histone H3.3 and H2bl-like, HIST2H2AC and histone H3.3, mixture HIST1H2AJ, HIST2H2BE and histone H2A type 2-C) were found in all mucous membranes as well as several tissue-specific proteins (proteins S100-A12 and AGR2, isoforms of ribosomal proteins, myelin P2, odorant-binding protein, secretoglobin), which could be a precusors of bioactive peptides.

Keywords: AMPs; storage; shelf-life; mucous membranes; proteins

INTRODUCTION

Progressive technologies are actively implemented in the food industry. Significant part of researches is aimed on reduction of losses, stabilization of quality and increase of shelf life. Loss of food quality, deterioration of organoleptic properties and accumulation of anti-alimentary compounds directly correlate with initial quality of raw materials, storage conditions and final shelf life. One of the main reasons is microbiological contamination **(Kameník, 2013; Dikeman and Devine, 2014; Popelka et al., 2016)**.

Nowadays, the following traditional methods are used for improving of shelf life: processing (sterilization, smoking, freezing, refrigeration, salting - wet and dry), physical (low frequency and ionizing treatment, gas-modified package, cryo-treatment, etc.), chemical treatment (sulphur dioxide, benzoic acid, sorbic acid, etc.) **(Zolotokopova and Palagina, 2007; Syasin, 2011; Nesterenko and Kayatskaya, 2012; Tuniyeva, 2013, 2015; Zaitseva et al., 2014; Bilek et al., 2016)**. These methods are very effective, but, nevertheless, its implementation may lead to quality lowering, accumulation of anti-alimentary factors, activity reduction of introduced or native bioactive substances, etc.

Modern concept of foods development is primarily focused on creation of products with high quality and nutritional value. A special attention is paid in specialized and functional products with limited shelf life. In this regard, an alternative ways of shelf life increasing are quite a sharp problem. Antimicrobial peptides (AMPs), whose existence has been known for more than 60 years could be an actual alternative. According to Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php), 2884 antimicrobial peptides from six kingdoms (333 bacteriocins/peptide antibiotics from bacteria, 4 from archaea, 8 from protists, 13 from fungi, 342 from plants, and 2184 from animals) were found and identified **(Wang, 2010)**.

A wide range of AMPs were found in tissues of mammals and are classified into histatins, cathelicidins and defensins. Mainly, antimicrobial compounds were identified in blood cells (leukocytes, neutrophils, platelets) **(Tecle et al., 2010; Wang, 2010; Wang, 2014; Bahar and Ren, 2013; Jarczak et al., 2013; Zhao and Lu, 2014; Shamova et al., 2014)**. But mucous membranes of

farm animals due to their border position and intensive contact with different pathogens could be also a capacious source of such substances.

Scientific hypothesis

Despite of high availability and low cost of farm animal's by-products, the question of its use as a source of substances with antimicrobial action is not enough in focus. In this regard, the study of antimicrobial proteins and peptides contained in the mucous membranes is highly relevant due to their border position and, as a result, intensive contact with a wide range of biological agents (pathogenic and opportunistic microorganisms, viruses, fungi). It is known that the reaction of nonspecific protection is formed including signaling, regulatory and primary active substances. In this case, border tissues of animals are rich source both antimicrobial constitutive sequences and variable compounds accumulated and induced by pathogens. It is important to note that significant genomic resources of epithelium as a "first line of defense" is able to form a unique combination of proteomic profiles and capacious peptide pools both as the result of acute inflammation and a component of a booster effect.

MATERIAL AND METHODOLOGY

Objects of the study were bovine oral cavity mucosa, nasal cavity mucosa, tracheal cavity mucosa, rectal mucosa, tongue mucosa, saliva gland and submandibular lymph nodes.

Proteomic study

Two-dimensional electrophoresis (2DE) was performed according to the method of O'Farrell with isoelectric focusing in ampholine pH gradient (IEF-PAGE). The subsequent detection of the proteins was carried out by staining with Coomassie R-250 (Applichem, USA) and silver nitrate (Panreac, Spain) as described previously **(Kovalyov et al., 2006)**. The resulting digital images were edited in a graphic editor and the quantitative protein content was calculated using ImageMaster 2D Platinum version 7 ("GE Healthcare", Switzerland).

Protein fractions were excised from the gel, grinded and undergone trypsinolysis (Sigma, Germany) **(Zvereva et al., 2015)**. Obtained peptides were investigated by MALDI-TOF MS and MS/MS mass spectrometry on Ultraflex MALDI-TOF mass spectrometer (Bruker, Germany) with UV laser (336 nm) in the positive ion mode in molecular weight range of 500 – 8000 Da with calibration according to known peaks of trypsin autolysis.

Bioinformatics analysis

Analysis of obtained tryptic peptides mass spectra was performed using Peptide Fingerprint option in Mascot software (Matrix Science, USA) with MH+ mass determination accuracy of 0.01%; search was performed in databases of the National Center for Biotechnology Information, USA (NCBI).

Comparative analysis of obtained proteomic profiles was carried out with use of information module "Proteins of skeletal muscle of cows (Bos Taurus)" of the Database "Proteomics of muscle organs" (http://mp.inbi.ras.ru).

RESULTS AND DISCUSSION

A number of identified protein fractions were qualitatively or quantitatively different between the studied mucous membranes (Figure 1 and Table 1).

It was found that at least 10% muscle tissue was also presented in samples. Thus, troponin I, fast skeletal muscle (5 and 24), myosin regulatory light chain 2, skeletal muscle isoform (10), myoglobin (1) were identified. A number of major proteins, which was not detected in muscle tissues, were detected in mucous membranes. Presumably, these proteins could be a sourse a bioactive peptides.

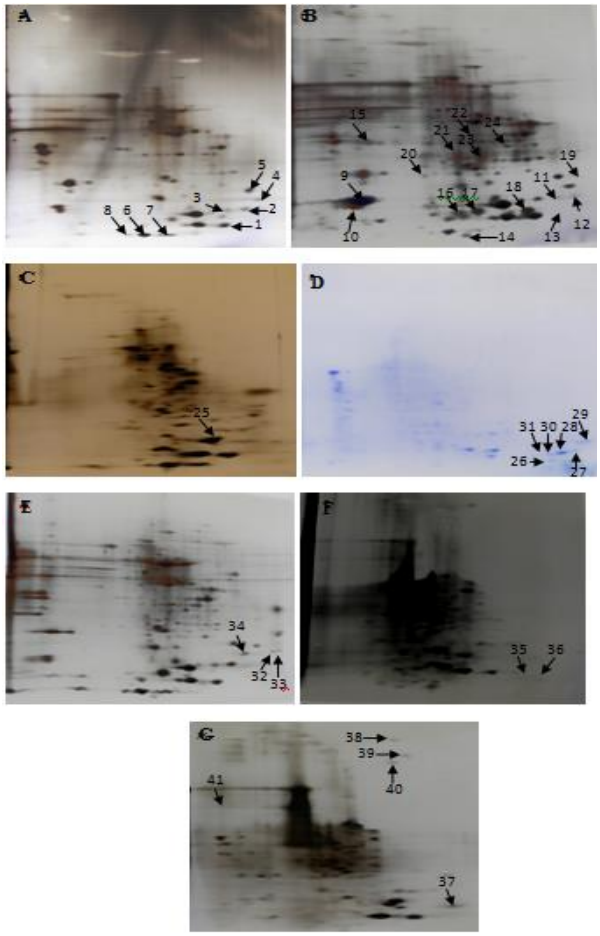

Figure 1 2DE proteins of bovine mucous membranes. Note: A – oral cavity mucosa, B – nasal cavity mucosa, C – tracheal cavity mucosa, D – rectal mucosa, E – tongue mucosa, F – submandibular lymph nodes, G – saliva gland.

Histones formed one of these groups. It's known that these proteins possessed an antimicrobial activity and may decompose into peptides with the same action **(Tagai et al., 2011)**. Some fractions were coincided in different objects. H2bd-like were identified in submandibular lymph nodes (27) and nasal cavity mucosa (11), but MW was different. MW of H2bd-like (11) was higher, presumably, due to its glycosylation in nasal cavity. The same phenomenon was revealed in relation to ubiquitin (14), which MW was arisen due to accession of hexoses.

Table 1 The results of mass spectrometric identification (MALDI-TOF MS и MS / MS) of protein fractions.

№	Protein name; (Gene symbol)	Number in Protein NCBI	S / M/ C *	Мм/pI (exp.)**	Мм/pI (calc.)**
	Oral cavity mucosa				
1	Mixture of 50s ribosomal protein l13 (RPLM)****(2) [Pseudomonas] and fragment 1-140 myoglobin [Bos taurus](MB)***(1)	WP 047273761.1 133/9/	187/4/52 133/9/51	15,0/9,60	15,8/9,79 17,1/6,90
2	Mixture of fragment 40 -166 anterior gradient protein 2 homolog isoform X1 (AGR2 and 50S ribosomal protein L13 (RPLM)****(2) [Pseudomonas]	XP 005205231.1 WP 047273761.1	159/13/62 131/13/33	18,5/9,90	20,0/8,82 15,8/9,79
3	peptidyl-prolyl cis-trans isomerase A (PPIA)***(2)	XP 006051354.1	173/21/63	18,2/9,75	17,9/8,34
4	Mixture of fragment 75 – 191 protein HP-20 homolog (MGC137014) and fragment 38 – 207 peptidyl-prolyl cis-trans isomerase B (PPIB)	NP 001040049.1 DAA25310.1	107/9/48 102/11/37	20,0/10,10	20,6/8,85 22,7/9,23
5	troponin I, fast skeletal muscle (TNNI2)	NP 001179023.1	162/21/55	22,0/10,00	21,4/8,88
6	protein S100-A12 (S100A12)***(2)	NP 777076.1	144/20/77	10,5/6,00	10,7/5,92
7	protein S100-A12 (S100A12)***(2)	NP 777076.1	210/15/63	10,0/6,50	10,7/5,92
8	protein S100-A12 (S100A12)***(2)	NP 777076.1	217/17/76	10,0/5,90	10,7/5,92
	Nasal cavity mucosa				
9	odorant-binding protein (OBP)	XP 001253219.3	118/9/53	20,0/5,20	20,0/5,20
10	myosin regulatory light chain 2, skeletal muscle isoform (MYLPF)	NP 001069115.1	173/20/78	19,0/4,90	19,0/4,91
11	Mixture histone cluster 1, H2bd-like (LOC100138359)***(2) and anterior gradient protein 2 homolog isoform X1 (AGR2)	DAA21814.1 XP 005205231.1	150/5/34 116/10/48	18,5/9,90	12,4/9,74 20,0/8,82
12	Mixture of fragment 36-93 calponin-1 (CNN1)***(2) and histone cluster 1, H2bc-like (LOC520044)***(3)	NP 001039844.1 DAA21889.1	137/3/13 146/5/24	18,5/10,20	33,3/9,05 13,8/10,11
13	myelin P2 protein (PMP2)	NP 001068707.1	214/19/77	17,0/9,90	15,0/9,67
14	Ubiquitin (LOC101902760)***(1) with signs of glycosylation (hexose)	XP 005195085.1	76/12/90	10,5/6,60	8,70/6,56
15	immunoglobulin gamma 1 heavy chain constant region	ABE68619.1	112/12/42	34,0/5,40	35,5/6,49
16	Mixture of histidine triad nucleotide-binding protein 1 (HINT1)***(1), secretoglobin family 1D member (SCGB1D)***(5)и cytochrome c oxidase subunit 5B, mitochondrial ()***(2)	NP 787006.1 NP 001071275.1 NP 001029218.1	99/5/53 199/15/63 154/12/70	16,5/6,55	13,9/6,03 11,3/8,98 13,8/8,80
17	Mixture fatty acid-binding protein, epidermal (FABP5)***(1)и secretoglobin family 1D member (SCGB1D)***(3)	DAA22652.1 NP 001071275.1	34/22/71 140/5/40	17,0/6,80	11,9/6,58 11,3/8,98
18	secretoglobin family 1D member (SCGB1D) ***(4)+ the hexose-to-peptide 2912	NP 001071275.1	201/14/65	16,5/9,50	11,3/8,98
19	troponin I, fast skeletal muscle (TNNI2)	XP 005903574.1	109/13/40	22,0/10,40	22,1/9,30
20	Mixture of immunoglobulin light chain variable region***(2) with modification Gln->pyro-Glu (Pyro-glu from Q) and immunoglobulin lambda light chain variable region (VlAMBDA1B)***(2)	AAB66578.1 AAC48559.1	247/6/60 178/15/92	29,0/6,80	11,3/6,23 13,3/4,93

№	Protein name; (Gene symbol)	Number in Protein NCBI	S / M/ C *	Мм/pI (exp.)**	Мм/pI (calc.)**
21	Mixture of alcohol dehydrogenase [NADP(+)](AKR1A1), fragment keratin, type II cytoskeletal 68 kDa, component IA (KRT76) and fragment aflatoxin B1 aldehyde reductase member 2 (AKR7A2)***(1).	NP 001069981.1 XP 002687308.1 NP 001095419.1	221/34/86 113/28/54 101/7/22	34,0/7,00	36,6/6,80 64,7/7,59 40,5/8,57
22	Mixture of immunoglobulin light chain variable region with midification + Gln->pyro-Glu (N-term Q)***(2), immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3a ***(1), C-end fragment creatine kinase M-type (CKM)***(1) and calcyclin-binding protein (CACYBP)***(1)	AAB66568.1 AEMO5841.1 NP 777198.2 NP 00102998.1	194/2/42 105/4/66 84/8/30 50/1/10	28,0/7,10	11,3/6,23 11,3/8,49 43,0/6,63 26,3/8,29
23	ATP synthase subunit d, mitochondrial (ATP5PD)	NP 777149.1	150/13/79	24,0/6,20	18,7/5,99
24	Mixture of malate dehydrogenase, mitochondrial isoform X1 (MDH2)***(2), voltage-dependent anion-selective channel protein 1 (VDAC1)и keratin, type II cytoskeletal 59 kDa, component IV (KRT6B).	XP 005225065.1 NP 776910.2 NP 001244333.1	128/2/10 218/23/77 112/18/33	31,0/9,00	35,6/8,82 30,8/8,82 60,8/8,58
	Tracheal cavity mucosa				
25	anterior gradient protein 2 homolog isoform X1 (AGR2)	XP 005205231.1	144/9/46	19,5/8,90	20,0/8,82
	Rectal mucosa				
26	Fragment 48-117 histone H2B type 1-D (HIST1H2BD)***(3)	NP 001039711.1	191/15/55	8,0/10,30	14,0/10,31
27	histone H2A type 2-C (HIST2H2AC)***(4)	DAA31670.1	545/12/49	12,0/10,50	12,90/11,02
28	Fragment 22 -119 histone H2A type 1-H (HIST1H2AH)***(3)	XP 010823702.1	381/13/61	12,5/10,70	13,90/10,88
29	Mixture of fragment 17 – 163 desmin (DES)***(1), histone H3.3 (LOC100297725)***(1) and histone cluster 1, H2bl-like (LOC100299996)***(2)	NP 001075044.1 XP 002684244.1 DAA19208.1	120/6/15 147/8/34 145/6/29	14,0/10,90	53,50/5,21 15,20/11,14 14,00/10,22
30	Mixture of histone H2A type 2-C (HIST2H2AC)****(1), histone H3.3 (LOC100297725)****(1) and cytochrome c oxidase subunit NDUFA4 (NDUFA4)***(1)	DAA31670.1 XP 002684244.1 NP 787014.1	251/10/43 147/7/30 161/778/	11,5/9,90	12,90/11,02 15,20/11,14 9,30/9,57
31	Mixture of histone cluster 1, H2aj (HIST1H2AJ)****(1), histone H2B type 2-E (HIST2H2BE)****(3) and histone H2A type 2-C ()***(4)	XP 010823687.1 NP 001092854.1 DAA31670.1	233/9/41 256/12/62 545/16/68	11,5/10,40	14,0/10,88 13,9/10,31 12,9/11,02
	Tongue mucosa				
32	60S ribosomal protein L11 isoform X1 (RPL11)***(2)	XP 005203175.1	167/13/52	20,0/10,30	21,3/9,72
33	40S ribosomal protein S10 (RPS10)***(2)	NP 001029888.1	185/5/29	20,0/10,40	18,9/10,15
34	troponin I, fast skeletal muscle (TNNI2)	NP 001179023.1	113/12/25	21,0/10,20	21,4/8,88
	Saliva gland				
35	Mixture of myelin P2 protein (PMP2)и histone H2A type 2-C (HIST2H2AC)***(2)	NP 001068707.1 DAA31670.1	162/11/50 204/3/29	15,0/10,00	15,0/9,67 12,8/11,02

№	Protein name; (Gene symbol)	Number in Protein NCBI	S / M/ C *	Мм/pI (exp.)**	Мм/pI (calc.)**
36	Aggregate hemoglobin subunit beta (HBB)	NP 776342.1	171/11/68	120,0/9,50	16,0/7,01
37	Aggregate anterior gradient protein 2 homolog isoform X1 (AGR2)	XP 005205231.1	103/6/46	100,0/7,70	19,9/8,82
38	Aggregate hemoglobin subunit beta (HBB)	NP 776342.1	195/13/82	85,0/9,30	16,0/7,01
39	protein disulfide-isomerase (P4HB)***(1)	NP 776560.1	56/39/74	55,0/4,80	57,0/4,80
	Submandibular lymph nodes				
40	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 (NDUFA2)***(1)	NP 787009.1	119/2/25	14,0/10,20	11,1/9,93
41	histone cluster 1, H2bd-like (LOC100138359)***(2)	DAA21814.1	149/4/21	12,0/10,30	12,4/9,74

Note: * S/M/C: Score – indicator of conformity or «scorecard»; Match peptides – the number of matched peptides; Coverage – % coverage of the entire amino acid sequence of the protein by identified peptides.
**mM/pI (experiment) – scores obtained as a result of electrophoretic mobility on the DE and mM/pI (calculation) – estimates made based on amino acid sequence data with consideration of signal peptide removal, but with no consideration of other post-synthetic modifications using the ExPASy Compute pI/Mw tool software.
***msms – indication of identification by tandem mass spectrometry, the number of sequenced tryptic peptides in parentheses.

Histones of other types, identified in mucous membranes in different combinations, can also make a great contribution to the formation of bioactive peptides. The following histones were identified: №12 (H2BC), №16 (HIST1H2BD), №17 (HIST2H2AC), 18 (HIST1H2AH), №19 histone H3.3 и H2bl-like, №20 (HIST2H2AC) and histone H3.3, №21 mixture (HIST1H2AJ), (HIST2H2BE) and histone H2A type 2-C (Table 1).

Protein S100-A12 (№6-8) is localized in cell membranes and cytoplasm, and is represented in the mucous membranes in large quantities in the form of three electrophoretic fractions. Its activity is associated with the production of chemo- and cytokines. He is involved in antimicrobial humoral immune response mediated by antimicrobial peptides **(Cole et al., 2001)**.

Protein fractions AGR2 were identified in oral, nasal and tracheal mucous membranes (2, 11 and 15, respectively). This protein is most fully studied in humans (O95994 in UniProt Database), and performs various biological functions. It is required for MUC2 posttranscription synthesis and secretion, plays a role in mucus production, in migration, differentiation and cell growth, and promotes their adhesion. It is usually presented in small quantities. But in mucous tissues it is presented in significant quantities, and can be an important source of active biopeptides.

There were found two another groups of proteins. Isoforms of ribosomal proteins (1, 2 and 22, 23) were identified in the oral and tongue mucosa. Moreover, the fractions № 1 and 2 belonged to the *Pseudomonas* bacteria. Myelin P2 protein (PMP2) was detected in nasal mucosa (13) and salivary gland (25). Odorant-binding protein (9) was identified in nasal mucosa, as well as 3 fraction of secretoglobin (16,17 and 18) and immunoglobulin (15,20 and 22). Presumably, all identified tissue-specific proteins can be a source of bioactive peptides

CONCLUSION

More than 35 protein fractions were identified in investigated samples. A number of qualitative and quantitative differences were revealed. A large number of histones were found in all mucous membranes as well as several tissue-specific proteins, which would be a precusors of bioactive peptides.

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Acknowledgments:

This work was supported by the Russian Science Foundation (project No. 17-76-10033).

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