Antimicrobial compounds of porcine mucosa

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Abstract. The aim of the study was to investigate porcine oral cavity mucosa (OCM), nasal cavity mucosa (NCM), rectal mucosa (RM) and tongue mucosa (TM) as sources of antimicrobial compounds. Ultrafiltrates with MW >30 kDa, MW 5-30 kDa and MW <5 kDa were obtained. All ultrafiltrates had antimicrobial activity against Escherichia coli and Proteus vulgaris. NCM ultrafiltrates revealed the highest antibacterial activity in respect to negative control: for the fraction with MW >30 kDa, the zone of microbial growth inhibition was 7.5 mm, for the MW<5 kDa fraction, it was 7 mm, and for MW 5-30 kDa fraction, it was 4.5 mm. No significant differences were found in high molecular weight proteomic profile, while qualitative and quantitative differences were observed in the medium and low molecular weight areas, especially in OCM and NCM. HPLC showed 221 tissue-specific peptides in OCM, 156 in NCM, 225 in RM, but only 5 in TM. The results observed confirmed porcine mucous tissues as a good source of antimicrobial compounds, which could be an actual alternative for reduction of microbial spoilage of foods.

1. Introduction

Analysis of scientific publications has shown there is intensive investigation of bioactive substances derived from animal tissues. The urgent need for alternative ways to combat pathogenic microorganisms that are rapidly acquiring resistance has led to comprehensive study of substances with antimicrobial action, especially in the last decade, while antimicrobial peptides have been known for more than 60 years. Thus, currently, about 2,000 antimicrobial compounds are found in tissues of different animal species.

Nowadays, a wide range of publications are dedicated to isolation and study of substances with antimicrobial action from microorganisms [1], cultured and wild plants (barley [2]), secretions from arthropods (scorpion venom [3,4]) and mollusks (oyster slime [5]), skin of amphibians, reptiles and fishes (cod [6], sturgeon [7], salamander [8], frogs [9,10]), blood cells of mammals and birds (chicken thrombocytes [11], leukocytes of goats [12], foxes [13], elk [14], cattle [15], etc.). For example, one of the largest databases supported by the University of Trieste lists more than 800 antimicrobial peptides (and some proteins) from various species such as amoeba, plants, penguins, people, etc. Various attempts have been made at classification of these compounds. Commonly, peptides are classified according to secondary structure: linear α-helical peptides (e.g., LL-37), peptides with β-strands linked by disulfide bridges (e.g., defensins), loop peptides (e.g., bactenecin), and those with a high content of specific amino acids (e.g., histatins) [16]. Antimicrobial substances are active against both gram-negative and gram-positive bacteria, and also fungi, viruses and protozoa. The mechanism of action is based on the destruction of microbial membrane due to integrity damage or interaction with certain areas of membranes. First, peptide and negatively charged membrane of the microorganism are
electrostatically attracted, then the membrane is disrupted due to pore formation, through which ions and other cell components exit the microbial cell [17].

More than 500 antimicrobial proteins and peptides were found in tissues of mammals and are classified into histatins, cathelicidins and defensins. This later category is the most widely studied, and it is subdivided into alpha-defensins (found mainly in neutrophils and Paneth cells), beta-defensins (localized in leukocytes and epithelial cells) and theta-defensins (the least studied, found in some primates) [18]. Initially, defensins in mammals were described in 1956 by Robert C. Skarnes, W. Dennis Watson as leukins and James G. Hirsch as phagocytins produced by rabbit leukocytes. H. I. Zeya and John K. Spitznagel related open substances to one molecular family, which they identified as cationic antimicrobial proteins. Only in 1985, did Michael E. Selste et al. give them their modern name – defensins [19].

Nevertheless, despite the ready availability and low cost of farm animal by-products, the question of their use as a source of substances with antimicrobial action is not enough in focus. However, the intensive development of proteomics in recent years has made it possible to study the composition of such substances. The results confirm that proteome and peptidome of barrier tissues can be significantly changed, mainly due to biosynthesis of proteins and peptides that are involved in cellular processes, metabolism, and immune protection. Moreover, overexpression of such compounds continues for some time after inflammation subsides. In addition, the particular combination of antimicrobial agents produced varies considerably depending on the type of pathogen that caused the inflammatory response. In this regard, the study of antimicrobial proteins and peptides contained in the mucous membranes of cattle and pigs 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).

In summary, it is possible that meat by-products could contain significant resources of antimicrobial proteins and peptides, and their biological activity is of primary worth for study in developing alternative approaches to food processing technology, with the aim of prolonging food shelf life.

2. Materials and Methods

The objects of study were extracts and ultrafiltrates of porcine oral cavity mucosa (OCM), nasal cavity mucosa (NCM), rectal mucosa (RM) and tongue mucosa (TM). Extraction was carried out on laboratory dispersing equipment (Labotex, Russia) with 0.9% NaCl for 24 hours, ratio 1:2, at a temperature of 4-5°C, 300 rpm. Extract was separated by centrifuging using a CM-6M (ELMI, Latvia) at 3500 rpm for 8 min, and then ultrafiltrated on PES membranes (MWCO 5 and 30kDa) by tangential filtration on a VivaFlow 200 system (Sartorius, Germany).

Antibacterial activity against *Escherichia coli* and *Proteus vulgaris* was determined by the disc-diffusion method. Cell cultures of *E. coli* and *P. vulgaris* were seeded on agar surfaces in Petri dishes, then paper discs moistened with ultrafiltrates (concentration: 0.1 g/ml 0.05 g/ml, 0.025 g/ml and 0.012 g/ml) were placed on the agar surfaces and incubated at 37°C. Zones of microbial growth inhibition were measured after 20 and 40 hours.

One-dimensional electrophoresis of extracts was carried out according to the Laemmli method in the 15% gradient SDS-PAGE with standards purchased from Fermentas (Fermentas, Lithuania).

Analysis of the peptide profile was carried out using high performance liquid chromatography (HPLC) with mass spectrometer (liquid chromatograph AGILENT 1200 C with a mass selective detector, AGILENT 6410, USA).

3. Results and Discussion

All ultrafiltrates of porcine ORM, NCM, RM and TM possessed antimicrobial activity against *E. coli* and *P. vulgaris*. NCM ultrafiltrates revealed the highest antibacterial activity: for the fraction with MW >30 kDa, the microbial growth inhibition zone was 7.5 mm, for the MW<5 kDa fraction, it was 7
mm, and for the MW 5-30 kDa fraction, it was 4.5 mm. Zones of microbial growth inhibition of ORM and TM ultrafiltrates (MW>30 kDa) were 4 mm.

Proteomic studies showed that the greatest number of low and high molecular weight proteins were detected in ORM and NCM, (about 30 bands), while in TM had 28 bands, and RM had 23 bands (figure 1).

Figure 1. One-dimensional electrophoresis of porcine extracts. St – Molecular weight marker (250, 150, 100, 70, 50, 40, 30, 20, 15, 10 and 5 kDa). Lanes: 1 – TM, 2 – OCM, 3 – NCM, 4 – RM. Dotted lines show locations of: purple – high-molecular fraction (Mm>30 kDa), green – medium molecular fraction (Mm 5-30 kDa), blue – low molecular fraction (Mm <5 kDa).

According to the UniProt Protein DataBase, we can assume that tissue-specific proteins could be present in the investigated porcine extracts but were hidden in bands of the major proteins and the intensity of bands was formed both by major and minor (tissue specific) proteins. Thus, TM and NCM both contained a band of 15-17 kDa, which may correspond to protegrin-1,2,3 (16-17 kDa) with bactericidal activity against *E. coli*, *Listeria* and *Candida albicans*, while a band in the NCM probably corresponds to an antibacterial protein PR-39 (19 kDa) with antimicrobial activity against both *E. coli* and *Bacillus megaterium*. TM and OCM may contain the antibacterial peptide AP 3910 (4 kDa), while in both OCM and NCM, in the region of 8-10 kDa, AP 2 (9 kDa) and hepcidin (9 kDa) appear as likely candidates. These compounds are also characterized by a high antibacterial activity [10].

HPLC results showed that OCM contained 221 tissue-specific peptides, NCM contained 156, RM contained 225, while in TM, only 5 such peptides occurred (figure 2).

Figure 2. Peptide profiles of extracts. A – porcine muscle tissue, B – TM, C – NCM, D – OCM, D – RM. Dotted lines show ranges of: purple – 1100-1600 Da, green – 600-100 Da, red – 100-400 Da.
It was revealed that high molecular weight ultrafiltrates of porcine NCM and TM possessed the highest antibacterial activity. Bacterial growth inhibition zones were as large as 7.0 mm in diameter. Proteomic studies did not reveal significant differences in the high molecular range, while qualitative and quantitative differences were observed in the medium and low molecular weight areas, especially in OCM and NCM. The largest number of tissue-specific peptides was observed in RM and OCM (225 and 221, respectively).

The observed results confirm the investigated porcine mucosa tissues to be a good source of antimicrobial compounds, which could be suitable as actual alternative compounds to reduce microbial spoilage of foods.

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