Functional properties of antimicrobial peptides extracted from hens' platelets

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Abstract: Analysis of studied literature showed that there were only single, sometimes conflicting data on existing birds' thrombodefensins, their antimicrobial activity. The problem of birds'TD influence on persistent properties and microorganisms' ability to form biofilms hasn't been studied yet. This determined purposes of our research – the aim is to study some functional properties of antimicrobial peptides extracted from hens' platelets. **Objective.** Antimicrobial peptides were extracted from various tissues of vertebrates and human, special significance is taken on by the antimicrobial peptides which were extracted from platelets and have antibacterial, antiviral, antimycotic and anticancer activity. **Materials and methods.** At the terminal stage of fattening acid-soluble proteins of broiler chick's platelets were got by means of the method of low-pH extraction [6]. Protein content in these low-pH extracts were determined with the method by M.M. Bradford (1979) with using of CoomassieBrilliantBlueG-250 dye (SIGMA, Germany) [7]. Antimicrobial properties of the acid-soluble proteins from platelets were estimated in vitro by means of the plate method in the case of Gram-positive microorganism *Bacillussubtilis83*. The concentration inhibiting growth of 50% of colonies compared to the control was considered as the minimal bactericidalconcentration (MBC). The preparation activity was re-counted taking in account the protein content in low-pH extract. Spectrum of antimicrobial activity of acid-soluble proteins from platelets was determined in the case of such cultures: *Bacilluscereus*, *Micrococcusluteus*, *Staphylococcusaureus, Enterococcusfaecalis*, *EscherichiacoliK12*, *Klebsiellapneumoniae 278*, *Enterobactercloacae, Candidaalbicans*. Influence of various TD concentrations on anti-lactoferrin activity (ALfA) was determined on thesecultures: *Escherichiacoli K12*, *Enterococcusfaecalis*и*Candidaalbicans*. Studying of TD persistency regulation was accomplished in two stages. In the first stage we extracted clones by plating 24 hours' culture on solid nutrient medium according instructions by Miller (1976) [4]. Then we chose a clone with the highest level of ALfA. In the second stage we studied ingluence of TD dilutions on ALfA of microorganism by co-incubation. ALfA of microorganisms was determined by means of complex of apparatuses for enzymeimmunoassay by "STAT FAX" using sets for lactoferrin determination "Lactoferrin-EIA-BEST" by "Vektor-BEST", which was evaluated in ng/ml [2]. Estimation of low-pH platelet extract influence on the ability to form a biofilm we did in vitro in the case ofcultures capable of biofilm formation: *Enterobactercloacae*(3 strains), *Enterobacteragglomerans, StaphylococcusaureusP 209, Klebsiellapneumoniae 278, Candidaalbicans*(3 strains) [40]. Activity level was counted as ratio A492test/A492control. Measure more 1,1 were considered as a positive result. **Results and Discussion.** Summing up our findings we should note: at first, there are antibiotics owning a wide spectrum of antimicrobial action in hens' platelets, at second, platelet cationic proteins are capable to change biological properties of microorganisms, these properties determine their interaction with macroorganism, which can be used in working out a new class of antimicrobial preparations in perpective.
Annotaion: In platelets of agricultural birds (broiler chicks) there were discovered antimicrobial peptides– thrombodefensins (TD), which possess a broad spectrum of action in the case of Gram-negative, Gram-positivebacteria's forms and *Candidaalbicans.*Thrombodefensins exert multidirectional influence on biological characteristic of microorganisms: they inhibitanti-lactoferrin activity (ALfA)and exert modifying influence on biofilm formation.
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1. Introduction

More and more agents get resistant to antibiotics which exert toxic action on organs and tissues of human and animal organisms, therefore, there is a burning issue upon research of new preparations having wide spectrum of antimicrobial activity and low toxicity for macroorganism's own cells[17; 18; 19; 20; 21; 22; 29; 30; 31]. A fine alternative for antibiotics is considered therapy based on using antimicrobial peptides, which are innate components of the immune system [1; 9; 11; 12; 13; 16; 34; 35; 36].

Antimicrobial peptides were extracted from various tissues of vertebrates and human, special significance is taken on by the antimicrobial peptides which were extracted from platelets and have antibacterial, antiviral, antimycotic and anticancer activity [5; 10; 14; 15; 23; 25; 26; 28; 33].

Analysis of studied literature showed that there were only single, sometimes conflicting data on existing birds' thrombodefensins, their antimicrobial activity. The problem of birds'TD influence on persistent properties and microorganisms' ability to form biofilms hasn't been studied yet. This determined purposes of our research – the aim is to study some functional properties of antimicrobial peptides extracted from hens' platelets.

2. Materials and methods.

At the terminal stage of fattening acid-soluble proteins of broiler chick's platelets were got by means of the method of low-pH extraction [6]. Protein content in these low-pH extracts were determined with the method by M.M. Bradford (1979) with using of CoomassieBrilliantBlueG-250 dye (SIGMA, Germany) [7].

Antimicrobial properties of the acid-soluble proteins from platelets were estimated in vitro by means of the plate method in the case of Gram-positive microorganism *Bacillussubtilis83*. The concentration inhibiting growth of 50% of colonies compared to the control was considered as the minimal bactericidalconcentration (MBC). The preparation activity was re-counted taking in account the protein content in low-pH extract.

Spectrum of antimicrobial activity of acid-soluble proteins from platelets was determined in the case of such cultures: *Bacilluscereus*, *Micrococcusluteus*, *Staphylococcusaureus, Enterococcusfaecalis*, *EscherichiacoliK12*, *Klebsiellapneumoniae 278*, *Enterobactercloacae, Candidaalbicans.*

Influence of various TD concentrations on anti-lactoferrin activity (ALfA) was determined on thesecultures: *Escherichiacoli K12*, *Enterococcusfaecalis*и*Candidaalbicans*. Studying of TD persistency regulation was accomplished in two stages. In the first stage we extracted clones by plating 24 hours' culture on solid nutrient medium according instructions by Miller (1976) [4]. Then we chose a clone with the highest level of ALfA.

In the second stage we studied ingluence of TD dilutions on ALfA of microorganism by co-incubation. ALfA of microorganisms was determined by means of complex of apparatuses for enzymeimmunoassay by "STAT FAX" using sets for lactoferrin determination "Lactoferrin-EIA-BEST" by "Vektor-BEST", which was evaluated in ng/ml [2].

Estimation of low-pH platelet extract influence on the ability to form a biofilm we did in vitro in the case ofcultures capable of biofilm formation: *Enterobactercloacae* (3 strains), *Enterobacteragglomerans, StaphylococcusaureusP 209, Klebsiellapneumoniae 278, Candidaalbicans*(3 strains) [40]. Activity level was counted as ratio A492test/A492control. Measure more 1,1 were considered as a positive result.

Calculated number materials were worked up statistically with finding averages, standart deviations and mean errors. Validity of differences of compared measures was estimated by Student t-criterion.

3. Results

In the process of the experiment we studied antimicrobial properties of acid-soluble proteins from hens' platelets in vitro in the case of Gram-positive microorganism *Bacillussubtilis 83*as the most sensitive culture [Suleymanov K.G., 1998].

The native preparation of platelet cationic protein (PCP) of hens suppressed growth of *B. subtilis*entirely. Incubating PCP with *B. subtilis*in dilution 1:2 there was recorded growth of 33 CFU, after incubation with the protein in dilution 1:4 there was registered growth of 99 CFU of *B. subtilis*. The low-pH extract of hens' platelets slowed down growth of 58,6% colonies of the studied microorganism, and the PCP dilution 1:16 turned out the minimal bactericidal concentration suppressing growth of 50% of colonies *B. subtilis*hence the antimicrobial PCP hens' activity made up 16 units.

Having found presence of antimicrobial activity of hens' PCP we decided to estimate antimicrobial spectrum of TD action in the case of various specimens of coccal, rod-like Gram-positive and Gram-negative forms of bacteria, and also of fingi.

Our experiments resulted in revealing high level of antimicrobial activity of acid-soluble hens' platelet proteins in the case of spore-forming Gram-positive bacterium *B. cereus*. Activity of the low-pH TD extract was 32 units, as that the minimal bactericidal concentration was 0,10 mg/ml.

Treating Gram-positive culture *M. luteus*with low-pH hens' PCP extract in dilution 1:8 we registered growth of 226 CFU, which was almost 50% less than in the control experiment (546 CFU). Therefore, hen PCP activity in the case of this microorganism was 8 units (MBC 0,38 mg/ml).

The activity of the hen platelet antimicrobial protein in the case of microorganism *S. aureus* was high enough. In PCP dilution 1:8 we registered growth of 1320 CFU, which was almost 50% less than in the control experiment ($p<0,05$), therefore, its activity was estimated as 8 units and the MBC as 0,38 mg/ml.

Antimicrobial activity of acid-soluble proteins from hen platelets in the case of Gram-negative

microorganism was a bit lower. TD activity was 6 units, the MBC in the case of *E. coli* was 0,51 mg/ml.

There was PCP having sufficiently high activity in the case of microorganisms*E. faecalis,K.pneumonia* and *E. cloacae.* Hen PCP activity in the case of *E. faecalis*was 16 units and the MBC was 0,23 mg/ml. Also PCP dilution 1:16 turned out the MBC suppressing growth of 50% of *K.pneumonia* (16 units), and the MBC in the case of this microorganism was 0,23 mg/ml. A dilution 1:32 appeared to be the MBC suppressing growth of 50% of *E. cloacae*, activity of the preparation was 32 units and the MBC was 0,12 mg/ml.

TD activity in the case of the yeast-like fungus *C.albicans* was 4 units, as that the MBC was 0,76 mg/ml.

Estimating the antimicrobial spectrum of the low-pH hen platelet extract we can conclude that TD activity varies depending on a microorganism species. So, antimicrobial hen platelet protein has high antimicrobial activity in the case of spore-forming Gram-positive bacteria and enterobacteria, and a bit less activity in the case of the studied specimens of the coccail group and the fungi *Candidaalbicans.*

Lactoferrin is one of the immune system components in a mammal organism, it takes part in the system of nonspecific humoral immunity exerting bacteriostatic and bacteriocidal action against many bacteria, fungi, viruses and protozoa. Microorganisms are capable of suppressing a lot of factors of natural defense of a host, including lactoferrin, however, PCPs has the property to inhibit some factors of microorganism persistency [8; 24; 27; 32; 39].

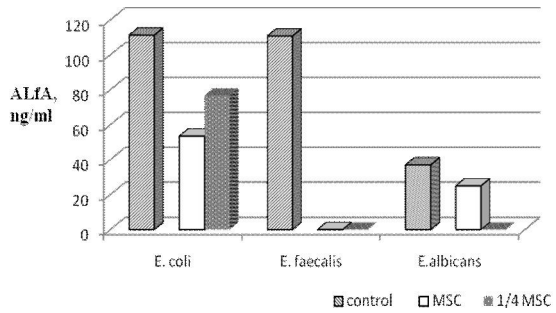
Analyzing our findings we discovered high initial heterogeneity of populations of *Escherichiacoli K12*, *Enterococcusfaecalis* and*Candidaalbicans*in ALfA, the average numbers of the index were 181,3±13,75,88,8±14,31 and 177,9±21,59ng/ml thereafter.

Cultivation *E. coli* in the hen PCP presence at theminimal suppressing concentration (MSC)went with 2,08 times ALfA decrease compared to the control experiment ($p<0,05$). The examined index changed a bit less cultivating bacteria with the TD dilution 1:4 MSC, *E. coli*'s ALfA decreased in 31,2% ($p<0,05$) in this dilution.

We noted that as TD concentration decreases as *E. coli*'s ability to inhibit lactoferrin rises.

Cultivating bacteria *E. faecalis* with hen platelet low-pH extract in MSCand ¼ MSCdilutions ALfA of this microorganism got indefinite ($p<0,001$).

The bird platelet antimicrobial protein in the experimented dilutions also exerted inhibiting influence on *C. albicans*' ALfA.



Note: ** — validity of differences of ALfA measures in the control experiment and after co-incubation with platelet low-pH extract ($p<0,01$); *** — ($p<0,001$)

Pic. 1 – Microorganisms' ALfA change under effect of platelet low-pH extract dilutions

Thus, our findings prove suppressing influence of hen thrombodefensins on antilactoferrin activity of microorganisms, inhibiting ability was noted in all dilutions and against all cultures being under study.

The ability of microorganisms to form biofilms is an important factor of pathogenicity, it is one of the basic strategy raising survival of bacteria in environment including in host. Bacteria's ability to exist in biofilms makes big difficulties as it greatly increases resistance of bacteria to antibacterial and disinfectant substances, to negative environmental influence such as low and high pH values, big osmotic force et al., and also to host immune defense action[9; 37; 38].

Our experiments resulted in revealing stimulative influence of hen TD on microorganisms' ability to form biofilms. Co-cultivation hen PCP with studied microorganisms intensified the ability to form biofilms in 100% of cases at that *E. cloacae*'sfilm formation (FF) coefficient credibly increased on average in 12,2%, *Eagglomerans* – in 13% ($p<0,01$),*K. Pneumonia* – in 15,6% ($p<0,01$) and*S. aureus* – in 5,8% compared to the starting level of the index.

Table 1.**Influence of hen platelet antimicrobial proteins on the ability of microorganisms to form biofilms**

Micro-organisms	*Film formation index $S_x \pm x$*		
	control	*MSC*	*1/4MSC*
E. cloacae1247	1,10±0,01	1,31±0,018***	1,28±0,014***
E. cloacae1236	1,10±0,016	1,23±0,009**	1,19±0,014*
E. cloacae1479	1,25±0,012	1,33±0,012	1,35±0,019
E. agglomerans	1,39±0,051	1,54±0,052**	1,57±0,062**
S. aureus	1,04±0,026	1,12±0,044	1,1±0,017
K. pneumoniae	1,22±0,026	1,21±0,028	1,41±0,03**
C. albicans 446	1,19±0,042	1,14±0,079	1,20±0,059
C. albicans 447	1,33±0,044	0,83±0,07**	1,01±0,110
C. albicans 275	1,03±0,021	0,71±0,054**	0,83±0,40*

Note: * — validity of differences of the film formation index in the control experiment and after co-incubation with platelet low-pH extract ($p<0,05$); ** ($p<0,01$); ***($p<0,001$).

Data upon dose-depended effect of influence of the PCP on the ability of microorganisms to form a biofilm is a deal of great interest. As our study showed the most stimulative effect in the case of the biofilm formation ability was registered in co-incubation bacteria with at concentration equal ¼ of the MSC, in other words as the PCP concentration decreases as the ability of bacteria to form biofilms grows.

Perhaps, under antimicrobialplatelet peptide action in vitro the most part of microbial population perishes, and keeping viability cells-persisters being present in a bacterial population before its contact with TD provide for more active biofilm formation.

As our experiments showed TD exert mostly inhibiting influence on the ability of *C. albicans* to form biofilms. Biofilm formation decrease after co-incubation the fungi with hen platelet antimicrobial proteins happened in 83,3% of cases. So, under influence of the MSC and ¼ of the MSC of hen TD average values of FFof*C. albicans 447* decreased in 37,5% ($p<0,01$) and 24% thereafter, and of *C. albicans 275*in 31,1% ($p<0,01$) and 19,4% ($p<0,05$) compared to the starting level of the index.

As you can see from our findings antimicrobial peptides from bird platelets exert modifying influence on biofilm formation by microorganisms stimulating biofilm formation by bacteria and inhibiting this ability of fungi.

Summing up our findings we should note: at first, there are antibiotics owning a wide spectrum of antimicrobial action in hens' platelets, at second, platelet cationic proteins are capable to change biological properties of microorganisms, these properties determine their interaction with macroorganism, which can be used in working out a new class of antimicrobial preparations in perpective.

List of sources

1. Bukharin O.V., Suleymanov K.G. (1997) Nature and biological function of platelet cationic protein. Progress of modern biology, №3, 10-15.
2. Valysheva I.V., Valyshev A.V., Kartashova O.L. (2003) A new method of evaluation of microorganism antilactoferrin activity. Microbiology journal, №4, 64-67.
3. Lakin G.F. (1990) Biometrics, Moscow: The Higher School, 288 p.
4. Miller J. (1976) Experiments in molecular genetics (translation from English), Moscow: World, 350 p.
5. Sipaylova O.Y., Nesterov D.V. (2013) Antimicrobial low-molecular peptides: factors of nonspecialdefence of animal organism. Bulletin of OSU, №12, 169-171.
6. Sycheva M.V., Sheida E.V., Kartashova O.L., Zhukov A.P. (2009) Antimicrobial activity of thrombodefensins of various animal species. Proceedings of Orenburg SAU, №4(24), 177-179.
7. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochev., 72, P.248-254.
8. Brock, J. Lactoferrin: a multifunctional immunoregulatory protein? / J. Brock //Immunol. Today. – 1995. - №16(9). – P. 417-419.
9. Brown, K.L. Cationic host defense (antimicrobial) peptides / K.L. Brown, R.E. Hancock // Curr. Opin. Immunol. – 2006. – Vol. 18. – P. 24-30.
10. Brumfitt, W. Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci / W. Brumfitt, M.R. Salton, J.M. Hamilton-Miller // J. Antimicrob. Chemother. – 2002. – Vol. 50. – P. 731-734.
11. Bulet, P. Anti-microbial peptides: from invertebrates to vertebrates / P. Bulet, R. Stocklin, L. Menin // Immunol. Rev. – 2004. – Vol. 198. – P. 169-184.
12. Elsbach, P. What is the real role of antimicrobial polypeptides that can mediate several other inflammatory responses? / P. Elsbach // J. Clin. Investig. – 2003. – Vol. 111. – P. 1643-1645.
13. Ganz, T. Defensins: antimicrobial peptides of innate immunity / T. Ganz // Nat. Rev. Immunol. – 2003. – Vol. 3. – P. 710-720.
14. Gennaro, R. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides / R. Gennaro, M. Zanetti // Biopolimers. – 2000. - Vol.55. - P. 31-49.
15. Hallock, K.J. MSI-78, an analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain / K.J. Hallock, D.K. Lee, A. Ramamoorthy // Biophys. J. – 2003. – Vol. 84. – P. 3052-3060.
16. Hancock, R.E.W. Cationic peptides: effectors in innate immunity and novel antimicrobials / R.E.W. Hancock // Lancet Infect. Dis. – 2001. – Vol. 1. – P 156-164.
17. Hancock, R.E.W., Chapple D. S. Peptide antibiotics / R.E.W. Hancock // Antimicrob. Agents Chemother. – 1999. – Vol. 43. – P. 1317-1323.
18. Hancock, R.E.W. The role of cationic antimicrobial peptides in innate host defences / R.E.W. Hancock, G. Diamond // Trends Microbiol. – 2000. – Vol. 8. – P. 402-410.
19. Hancock, R.E.W. Cationic peptides: a new source of antibiotics / R.E.W. Hancock, R. Lehrer // Trends Biotechnol. – 1998. – Vol. 16. – P. 82-88.

20. Hancock, R.E.W. Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics / R.E.W. Hancock, A. Patrzykat // Curr. Drug Targets Infect. Disord. – 2002. – Vol. 2. – P. 79-83.
21. Hancock, R.E.W. Role of membranes in the activities of antimicrobial cationic peptides / R.E.W. Hancock, A. Rozek // FEMS Microbiol. Lett. – 2002. – Vol. 206. – P. 143-149.
22. Hancock, R.E.W. The role of antimicrobial peptides in animal defenses / R.E.W. Hancock, M.G. Scott // PNAS. – 2000. - Vol. 97. - P. – 8856-8861.
23. Hirsch, J.W. Comparative bactericidal activities of blood serum and plasma serum / J.W. Hirsch // J. Exper. Med. – 1960. – Vol. 112. – P. 15 – 22.
24. Human lactoferrin and peptides derived from its N terminus are highly effective against infectionswith antibiotic-resistant bacteria / P. H. Nibbering, E. Ravensbergen, M. M. Welling et al. // Infect. Immun. - 2001. - No. 69. - P. 1469–1476.
25. In vitro antibacterial properties of pexiganan, an analog of magainin / Y. Ge, D. L. MacDonald, K. J. Holroyd et al. // Antimicrob. Agents Chemother. – 1999. – Vol. 43. – P. 782-788.
26. In vitro antiviral activity of dermaseptins against herpes simplex virus type 1 / A. Belaid, M. Aouni, R. Khelifa et al. // J. Med. Virol. – 2002. – Vol. 66. – P. 229-234.
27. Jenssen, H. Modelling of anti-HSV activity of lactoferricin analogues using amino acid descriptors / H. Jenssen, T.J. Gutteberg, T. Lejon // J. Pept. Sci. – 2005. – Vol. 11. – P. 97-103.
28. Jenssen, H. Peptide antimicrobial agents / H. Jenssen, P. Hamill, R.E. Hancock // Clinical Microbiology Reviews. – 2006. - Vol. 19. - No 3. - P. 491-511.
29. Johnstone, S.A. In vitro characterization of the anticancer activity of membrane-active cationic peptides / S.A. Johnstone // Anticancer drug des. – 2000. – Vol. 15. – P. 151.
30. Kavanagh, K. Histatins: antimicrobial peptides with therapeutic potential / K. Kavanagh, S. Dowd // J. Pharm. Pharmacol. – 2004. – Vol. 56. – P. 285-289.
31. Klaenhammer, T.R. Bacteriocins of lactic acid bacteria / T.R. Klaenhammer // Biochimie. – 1988. – Vol. 70. – P. 337-349.
32. Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts / J. H. Andersen, S. A. Osbakk, L. H. Vorland et al. // Antiviral Res. – 2001. – Vol. 51. – P. 141-149.
33. Lee, D.G. Structure and fungicidal activity of a synthetic antimicrobial peptide, P18, and its truncated peptides / D.G. Lee, K.S. Hahm, S.Y. Shin // Biotechnol. Lett. – 2004. – Vol. 26. – P. 337-341.
34. Lehrer, R. Defensins: Endogenous Antibitic Peptides of Animal Cell / R. Lehrer, T. Ganz, M. Selsted // Cell. – 1991. – V. 64. – P. 229 – 230.
35. Lehrer, R.I. Antimicrobial peptides in mammalian and insect host defence / R.I. Lehrer, T. Ganz // Curr. Opin. Immunol. – 1999. - №11 (1). - P. 23-27.
36. Lehrer, R. Defensins: Antimicrobial and Cytotoxic Peptides of Mammalian Cell / / R. Lehrer, T. Ganz, A. Lichtenstein // Ann. Rev. Immunol. – 1993. - №1. – P. 105 – 128.
37. Persister Cells and the Mechanism of Multidrug Tolerance in Escherichia coli /I. Keren, D. Shah, A. Spoering et.al. // Specialized J. Bacteriol. – 2004. - Vol. 186. – P. 8172-8180.
38. Lyte, M. Stimulation of Staphylococcus epidermidis growth and biofilm formation by catecholamine inotropes / M. Lyte, P.P.E. Freestone, C.P. Neal // Lancet. - 2003. – Vol. 361. – P. 130-135.
39. McKeown, S.T. The cytotoxic effect of human peptid-1 (HNP1) and lacto-ferrin on oral squamous cell carcinoma (OSCC) in vitro / S.T. McKeown // Oral oncol. – 2006. – Vol. 42 (7). – P. 685.
40. Merritt, J.H., Kadouri D.E., O'Toole G.A. (2005) Growing and Analyzing Static Biofilms, Curr. Protoc. Microbiol., 1B.1.1 – 1B.1.17.

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