

ANALYSIS

ANALYTICAL STUDY AND ANTIMICROBIAL ACTIVITY OF ALPHA-DEFENSIN 2 DISSOLVED IN PHARMACOPEIA BUFFERS WITH DIFFERENT pH

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Abstract: Human neutrophil peptides (HNPs, alpha-defensins) are a group of six defensins being considered as new antimicrobial drugs because of their multifunctional efficiency against bacteria, viruses and fungi. Regardless of the unique biological properties, alpha-defensins are unstable compounds and their activity depends on many physical and chemical factors as well as on the kind of the used cultivation media. This leads to research difficulties and obstructions in their therapeutic application. The purpose of this study was to determine the antibacterial activity of alpha-defensin 2 dissolved in pharmacopeia buffers and in parallel to develop selective and accurate analytical tests for identification and assay of alpha-defensin 2 in the course of study. The antibacterial effect of alpha-defensin 2 was determined against the strain *Escherichia coli* (ATCC 25922). It was found that 10 mg/L of Human neutrophil peptide-2 (HNP-2, alpha-defensin 2) dissolved in pH 9.0 buffer caused 90% inhibition of the bacterial respiratory activity. This buffer was considered as a suitable environment for deploying the antibacterial activity of the alpha-defensin. A selective MS analysis method for the identification of alpha-defensin 2 in sample mixtures was developed. Also, the HPLC method with alternative selectivity was elaborated for identification and assay of mixtures containing alpha-defensin 2 and aminoacids in Mueller Hinton broth. The procedure includes development of system suitability test determination.

Keywords: pH, antimicrobial activity, analytical study, alpha-defensin 2, buffers

Human neutrophil peptides (HNPs, alpha-defensins) are a group of six defensins being considered as new antimicrobial drugs because of their multifunctional efficiency against bacteria, viruses and fungi. Regardless of the unique biological properties, alpha-defensins are unstable compounds and their activity depends on many physical and chemical factors as well as on the kind of the used cultivation media. This leads to research difficulties and obstructions in their therapeutic application. The purpose of this study was to determine antibacterial activity of alpha-defensin 2 dissolved in pharmacopeia buffers and in parallel to develop selective and accurate analytical tests for identification and assay of alpha-defensin 2 in the course of study.

The antibacterial effect of alpha-defensin 2 was determined against the strain *Escherichia coli* (ATCC 25922). It was found that 10 mg/L of Human neutrophil peptide-2 (HNP-2, alpha-defensin 2) dissolved in pH 9.0 buffer caused 90% inhibition of the bacterial respiratory activity. This buffer was considered as a suitable environment for deploying the antibacterial activity of the alpha-defensin. A selective MS analysis method for the identification of alpha-defensin 2 in sample mixtures was developed. Also, the HPLC method with alternative selectivity was elaborated for identification and assay of mixtures containing alpha-defensin 2 and aminoacids in Mueller Hinton broth. The procedure includes development of system suitability test determination.

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HNPs are a group of six defensins, representing antimicrobial peptides (AMPs) with cationic moiety. They include in their molecules from 29 to 35 amino acids and three disulfide bonds between Cys1–Cys6, Cys2–Cys4, and Cys3–Cys5 residues in the secondary structure (1-3). Multifunctional efficiency against bacteria, viruses and fungi determines them as new antimicrobial drugs compared with antibiotics with large growing bacterial resistance (4, 5). HNP-2 is secreted in mucosal surface including eye in concentration between 0.2 µg/mL and 1.0 µg/mL (6). The mechanism of action is based on destroying the cell membrane by "carpet" and "barrel stave" models and also on targeting some intracellular structures (7). For HNP-2 there was reported antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter aerogenes*, *Bacillus cereus*, *Mycobacterium avium* and *M. intracellulare* (8, 9). HNP-2 is effective in vitro against *M. avium-intracellulare* (10). Antiviral activity of HNP-2 is studied against HIV-1, HSV-1/HSV-2, IAV, AAV, and HPV. The mechanism of antiviral activity involved blocking of the receptor binding, receptor down-modulation, extracellular aggregation, block fusion and cell signaling modulation (11, 12). Other function of HNPs includes chemo attractions for different types of immune cells including monocytes, CD4+ T-cells. The expression of HNPs is a mark of cancer progression and metastatic dissemination. High levels of HNPs are reported in patients with Crohn's disease, ulcerative colitis, Wegener's granulomatosis or lupus nephritis (13-15). In serum and cerebrospinal fluids of patients with Alzheimer disease are found high levels of HNP1-2 and hBD-2 human beta-defensin-2 (hBD-2) (16).

Regardless of the unique biological properties, alpha-defensins are unstable compounds and the evaluation of their antibacterial activity depends on many physical and chemical factors as well as on the kind of the pH and culture media used. This leads not only to research difficulties but also to obstructions of their application in the medicinal practice. Therefore, the purpose of this study was to determine the antibacterial activity of alpha-defensin 2 in buffered media and in parallel to develop selective and accurate analytical tests for identification and assay in the course of the study.

EXPERIMENTAL

Reagents

Acetonitrile HPLC grade, methanol HPLC grade, deionized water (DI), glutamic acid RS,

L-arginine aspartate RS, buffer solutions with pH = 2.0, 4.0, 7.4 and 9.0 pH units prepared according to Eur. Ph. 9, culture medium Brain Heart Infusion Broth (BHI, #M210, HiMedia, India, ingredients: calf brain infusion, beef heart infusion, proteose peptone, dextrose, sodium chloride, disodium phosphate with final pH = 7.4 ± 0.2), culture medium Mueller Hinton Broth (MHB, #M0405B, Thermo Scientific-Oxoid, UK, ingredients: beef infusion, casein acid hydrolysate, starch with final pH = 7.3 ± 0.1), lyophilized amorphous powder of Human Neutrophil Peptide-2 alpha-defensin 2 (M.W. 3371.0, C₁₄₇H₂₁₇N₄₃O₃₇S₆), Peptide Institute, Japan, provided by Pepta Nova GmbH.

MS analysis of HNP-2

Instruments

Mass analysis was performed by using Waters Micromass ZQ, Waters Reagent Manager pump with flow rate 20 µL/min of rate solution from Methanol – Water (70:30 v/v) throughout the system. Used gas is N₂ with debit 150 µL/min. Temperature of the instrument's analyzer is 120°C and the temperature of ionization camera – 250°C.

Preparation of stock solutions

The stock solution of alpha-defensin 2 was prepared by dissolving 0.12 mg lyophilized amorphous powder in DI water to obtain a solution with a concentration of 240 mg/L

Preparation of test solution for mass analysis

25 µL stock solution of alpha-defensin 2 was diluted to 250 µL with borate buffer (pH = 9.0).

Preparation of solution for additional ionization

The solution for additional ionization was prepared by mixing 0.1% solution of formic acid in water and 0.1% solution of formic acid in methanol in ratio 30 : 70 v/v. The measured pH for the first solution was 2.60 pH units and for the second – 3.60 pH units.

HPLC determination of alpha-defensin 2 – model mixture with glutamic acid and L-arginine aspartate

Instruments

HPLC analysis was performed by isocratic elution with a flow rate of 1.0 mL/min. A high performance liquid chromatographic system (SHIMADZU Corporation, LC-20 AD quaternary pump), Shimadzu DGU-20A5 vacuum degasser and a Shimadzu SPD-20A UV/VIS detector were used for analysis. The data were recorded using Lab Solutions

Software. The separation was carried out at 25°C, using column Unisol, Adela Technologies, C18 ODS, 250 × 4.6, 100 Å, 5 µm with mobile phase – acetonitrile : DI water – 50 : 50 v/v, previously filtered and degassed. The analytical wavelength was 210 nm.

Preparation of solutions

Preparation of stock solution of alpha-defensin 2

0.12 mg lyophilized amorphous powder of alpha-defensin 2 was dissolved in DI water to obtain solution with a concentration of 240 mg/L.

Preparation of stock solution of glutamic acid

10.0 mg crystal form of glutamic acid was dissolved in DI water to obtain solution with a concentration of 0.1 mg/mL.

Preparation of stock solution of L-arginine aspartate

10.0 mg L-arginine aspartate was dissolved in DI water to obtain solution with a concentration of 0.1 mg/mL.

Preparation of mixture of glutamic acid and L-arginine aspartate

1.0 mL stock solution of glutamic acid was diluted with 1 mL DI water and mixed with 2.0 mL stock solution of L-arginine. 20 µL of the obtained mixture was injected in the HPLC apparatus.

Preparation of mixture of glutamic acid, L-arginine aspartate and alpha-defensin 2

The test solution of alpha-defensin-2 was prepared by diluting 50 µL stock solution of alpha-defensin 2 to 1.0 mL with DI water. 0.5 mL stock solution of glutamic acid was diluted to 1.0 mL with DI water and mixed with 1.0 mL stock solution of L-arginine. 1.0 mL of this solution was mixed with 50 µL test solution of alpha-defensin 2. 20 µL of the obtained mixture was injected in the HPLC apparatus.

HPLC determination of alpha-defensin 2 in culture media

Preparation of BHI solution

0.37 g of granulated culture medium was dissolved in 10.0 mL DI water and the obtained solution was filtrated through a membrane filter with pore size 0.45 µm. 20.0 µL of this solution was injected in the chromatograph.

Preparation of MHB solution

0.22 g granulated culture medium was dissolved in 10.0 mL DI water. After filtration, 0.1 mL

of this solution was diluted to 100.0 mL with DI water. 20 µL of this solution was injected in the chromatograph.

Preparation of model mixture containing alpha-defensin 2 and MHB:

25 µL stock solution of alpha-defensin 2 was diluted with 475 µL borate buffer with pH = 9. 40 µL of this solution was mixed with 160 µL solution 1 : 1000 MHB. The chemical stability of this solution was examined by injecting in the HPLC apparatus in every 30 minutes.

Bacterial strains and growth conditions

The assayed bacterial strains included: *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 3865), both from the American Type Culture Collection (USA). The strains were maintained in Trypticase Soy Agar/Broth (TSA/TSB, #M1968/#LQ508, HiMedia, India) at 37°C under aerobic conditions. All experiments were performed using MHB and agar (MHA, #CM0337B, Thermo Scientific-Oxoid, UK). Before each experiment, the bacterial strains were cultured on MHA overnight; thereafter, one colony was re-suspended in 10 mL MHB and incubated for other 18 h at 37°C. This liquid culture served for preparation of a bacterial suspension in 0.9% NaCl with cell density of 1×10^8 colony-forming units (CFU/mL) (0.5 McFarland) which was further diluted to 5×10^5 CFU/mL in the broth microdilution method.

Determination of minimal inhibitory and bactericidal concentrations

The antimicrobial activity was estimated in 96-well microplates by broth microdilution method test according to ISO 0776-1 : 2006 (E). The standardized bacterial inoculum was prepared as described in the previous section "Bacterial strains and growth conditions". The inoculum's turbidity was adjusted to McFarland 0.5 (1×10^8 CFU/mL) after spectrophotometric determination ($\lambda = 600$ nm) of the bacterial density and 50 µL were re-suspended in 10 mL MHB. 50 µL from this final bacterial suspension was added to each assayed sample in 96-well microtitre plates containing equivalent volume of MHB loaded with HNP-2 in serial dilution from 10 to 0.156 mg/L. Four different stock solutions of HNP-2 (buffers with pH 2.0, 4.0, 7.4 or 9.0) were tested for antimicrobial activity in parallel to the buffers. Untreated samples served as the negative control, whereas the growth media was used to control the sterile conditions. The antibiotic gentamycin was applied as a positive control in serial dilution

from 4 mg/L to 0.0031 mg/L. The EUCAST clinical breakpoints (1 mg/L for *S. aureus* and 2 mg/L for *E. coli*) were used for the susceptibility assessment of the strains tested. The samples were incubated overnight at 37°C. The lowest concentrations without visible growth were determined as minimal inhibitory concentrations (MICs). The experiments were performed in triplicate. The minimal bactericidal concentrations (MBCs) were determined after MIC readings of 100 µL from each sample on MHA at 37°C overnight. MBCs were read as concentrations inhibiting the growth of bacterial colonies up to 100%.

Determination of bacterial redox activity

For determination of the bacterial redox (respiratory) activity the samples used for the BMD assay were further incubated (37°C) with the dye 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, #M2128, Sigma-Aldrich) at final concentration 0.05 mg/mL for 180 min. The resulting violet formazan crystals were dissolved by an equivalent volume of organic solvent (5% HCOOH in isopropanol) and the absorbance was measured at 550 nm (reference 690 nm) on an ELISA reader (BioTek Elx800, USA) against a blank solution (MHB). High intensity of the color correlates to the high bacterial respiratory activity and lower antimicrobial effect of alpha-defensin 2 in various buffers. The MTT test is based on the reduction of the tetrazole3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its respective formazan. It was used in our study to evaluate quanti-

tatively the population of the dead bacteria in parallel to the CFU enumeration, as far as dead bacteria are unable to reduce the MTT dye. For example, the use of MTT for testing the bactericidal activity of functionalized textiles has already been reported several times (20).

RESULTS AND DISCUSSION

HNP-2 (alpha-defensin 2) was identified in the sample mixtures using selectively MS analysis to determine mass changes in the peptide macromolecule of alpha-defensin 2 corresponding to the formation of multiple charges ions trough of protonation ($M+zH$)^{z+}. In Figures 1, 2 and 3 were shown mass spectra of M^{4+} , M^{3+} and M^{2+} ions corresponding to average molecular masses – 846.05, 1124.84, 1688.47 *m/z*.

The formula used for analysis is:

$$M = z_1(m_1 + mp),$$

where: M is mass of the molecular ion; z – number of charges; mp – mass of the proton; m_1 - mass to charge ratio.

HPLC method with alternative selectivity was applied (17) for assay of alpha-defensin 2 in mixture with amino-acids that are part of peptide composition – glutamic acid and L-arginine aspartate in MHB. The procedure includes development of system suitability test determination. Some chromatographic parameters such as *retention time*, *resolution*, *relative retention*, *symmetry factor*, *LOD* and *LOQ* were appointed for optimization of conditions in respect of time. The results are shown in Table 1.

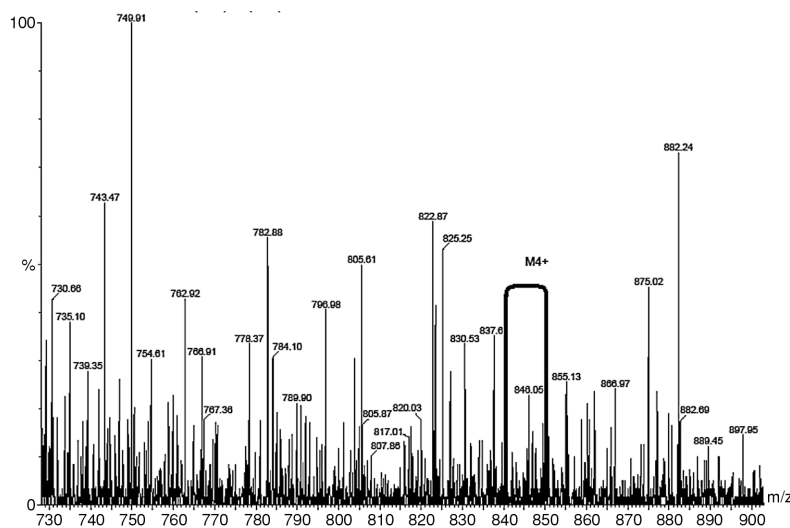
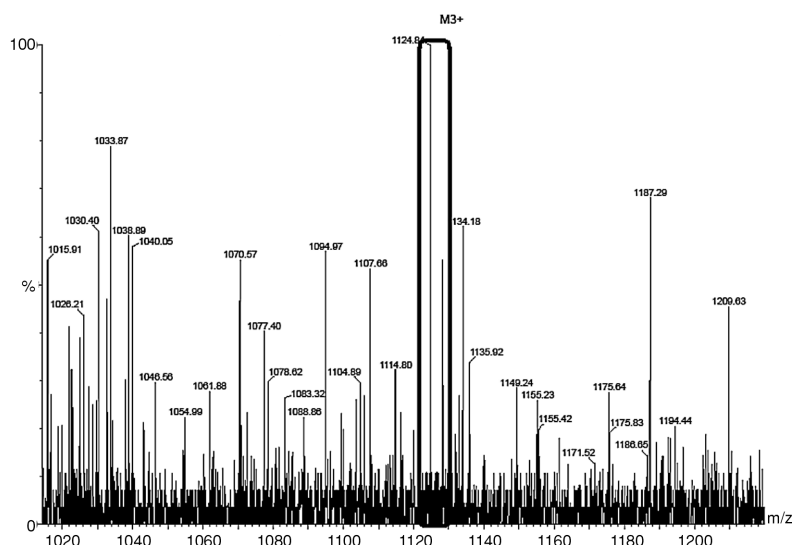
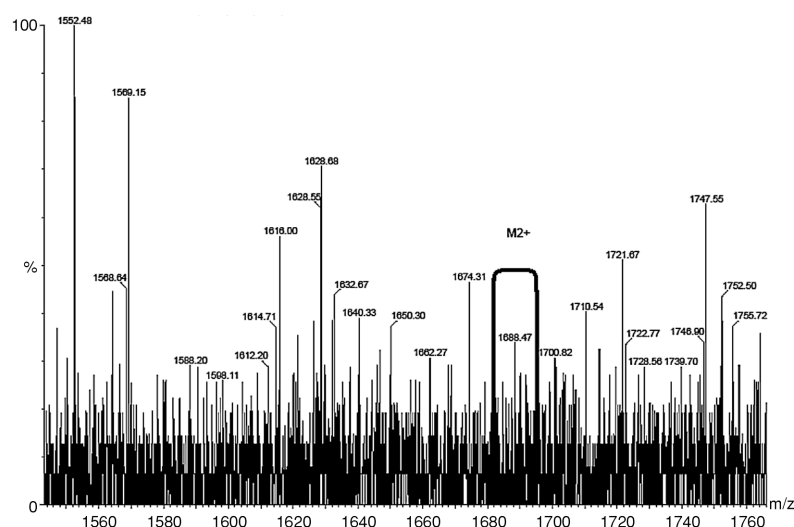


Figure 1. Mass spectrum of M^{4+} of HNP-2.

Figure 2. Mass spectrum of M^{3+} of HNP-2.Figure 3. Mass spectrum of M^{2+} of HNP-2.

The *resolution* of the mixture alpha-defensin 2/amino acids in the used mobile phase acetonitrile: DI water – 50 : 50 v/v (Fig. 4) is suitable for pharmacokinetic investigations and assay tests. For the time interval of 240 min, the retention time of alpha-defensin 2 is in the method tolerance – under $\pm 2\%$ (Table 2). The peptide's chromatographic profile is not changed in the presence of culture media in a buffer solution with pH = 9.0 in contrast to solutions of different pH, where the peptide disrupts. For identification of mixtures more acceptable is the flow rate of about 1.0 mL/min for the used mobile phase.

Antimicrobial activity of HNP-2

The antimicrobial activity of HNP-2 was tested on one *S. aureus* strain (Gram-positive) and one *E. coli* strain (Gram-negative) by using four different pharmacopoeia buffers with pH 2.0, 4.0, 7.4 or 9.0 for dissolving the protein. The maximal tested concentration for both strains (10 mg/L) was chosen according to previously published data (18, 19). HNP-2 inhibited effectively the growth of the Gram-negative strain *E. coli* at a minimal inhibitory concentration of 10 mg/L when it was dissolved in buffer with pH 9.0. This effect was bacteriostatic as

far as HND-2 did not inhibit the colony formation of *E. coli* after cultivation of the treated bacteria on MHA for 24 h (37°C). Concentrations of HNP-2 up to 50 mg/L did not exert a bactericidal effect, as well. However, the number of colony forming units at concentration of 10 mg/L did not exceed 1×10^4 CFU/mL which is by one logarithm less than the density of the initial bacterial inoculum (5×10^5 CFU/mL) and is an evidence for inhibition of the bacterial growth. The inhibition of MTT reduction after treatment with alpha defensin paralleled the

reduction in the number of CFU. The *S. aureus* strain turned out to be not sensitive to the alpha-defensin 2 exposure in all four buffers tested.

The redox activity of *E. coli* was determined after treatment with HNP-2 with the MTT-dye assay. HNP-2 dissolved in buffer with pH 9.0 inhibited the bacterial respiratory metabolism of the *E. coli* strain up to $91.1 \pm 0.6\%$ as visible from Figure 5 and Table 3. The effect of the buffer itself was $36.57 \pm 1.2\%$ inhibition, i. e. with 54.5% less than that of the protein dissolved in the same buffer. In

Table 1. Chromatographic parameters from system suitability test.

Parameter	Alpha - defensin 2	Glutamic acid	L-arginine aspartate
Retention time (min) \pm SD	1.82 \pm 0.0194	2.15 \pm 0.0180	3.81 \pm 0.0745
Resolution	-	1.348	4.266
LOD (mg/mL)	0.00024	-	-
LOQ (mg/mL)	0.00012	-	-
Relative retention	-	1.281	2.271
Symmetry factor	1.01	0.66	0.14

Table 2. Content in % of alpha-defensin 2 at pH = 9 in MHB.

Time (min)	t_r (min)	Area (AU)	Height (AU)	Area (%)	Height (%)
0	1.779	1024482	25394	98.684	98.690
30	1.819	145512	7168	16.238	23.313
60	1.847	84552	4469	14.778	18.991
90	1.840	87595	4593	15.774	19.925
120	1.831	90706	4819	16.555	20.751
150	1.825	96166	5123	17.479	21.810
180	1.844	62455	3621	14.506	19.603
210	1.813	93692	5160	18.077	23.079
240	1.822	74167	4270	16.657	22.111

Legend: t_r - retention time of analyte in min; Area - area under the peaks in absorption units (AU) and % Height - height of the peaks in absorption units (AU) and %.

Table 3. Comparison between the antibacterial effect of borate buffer with and without HNP-2 on the redox activity of *Escherichia coli* (ATCC 25922) after 24 h of exposure.

Sample	Absorbance ($\lambda = 550$ nm) \pm SD*	[%] of untreated control \pm SD
Control	3.08 \pm 0.087	100 \pm 9.8
10 mg/L HNP-2 in 50 : 50 buffer (pH 9) and bacterial suspension in MHB	0.729 \pm 0.016	8.905 \pm 0.62
50 : 50 buffer (pH 9) and MHB	1.954 \pm 0.065	63.43 \pm 2.1

Legend: *SD - standard deviation.

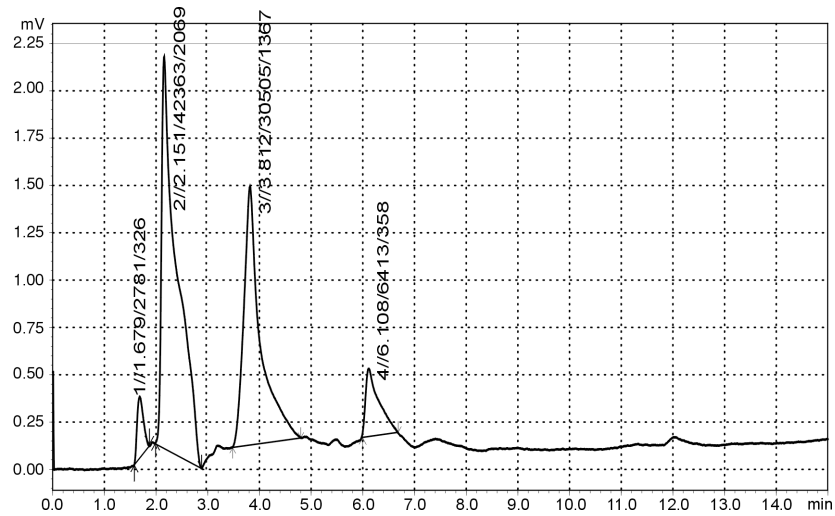


Figure 4. Chromatogram of mixture alpha-defensin 2, glutamic acid and L-arginine aspartate.

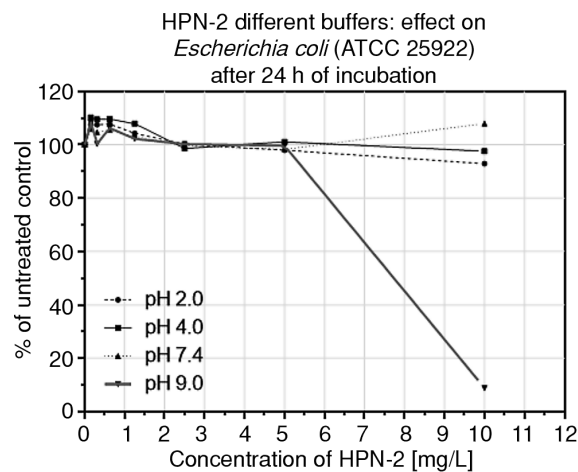


Figure 5. Effect of HNP-2 on the redox activity of *Escherichia coli* (ATCC 25922).

contrast, the other three buffers (2.0, 4.0 and 7.4 pH) used for dissolving of HNP-2 did not provide a stable milieu for revealing its antibacterial properties, most probably due to the low stability of the protein. The highest activity of HNP-2 was observed at a concentration of 10 mg/L, whereas all other concentrations were found to be ineffective. Our result correlated with the data published by Ericksen et al. (19) about the antibacterial effect of HNP-2 against the same strain *E. coli* used in our study (ATCC 25922). The authors found that HNP-2 inhibited the growth of the treated bacteria with 90% at a concentration of 8.4 ± 1.3 mg/L. This effect was calculated after measurement of the optical density of the bac-

terial culture (OD_{650}) whereas in our experiment we used the MTT dye to determine the respiratory activity of the survived bacteria.

The effect of HNP-2 on *Escherichia coli* (ATCC 25922) in buffer with pH 9.0 was compared to the effect of the buffer itself. The effect of the buffer was sevenfold weaker than this of the defensin. The results are presented in Table 3.

CONCLUSION

The antibacterial effect of alpha-defensin 2 against the *Escherichia coli* strain ATCC 25922 was demonstrated after stabilization of the peptide in a

buffer with pH 9.0. Under these conditions, the minimal inhibitory concentration of HNP-2 was determined at 10 mg/L causing 90% inhibition of the bacterial metabolic activity. In order to determine mass changes in the peptide macromolecule, a selective MS analysis method for the identification of alpha-defensin 2 in sample mixtures was developed. Also, the HPLC method with alternative selectivity was elaborated for the identification of mixtures and assay of alpha-defensin 2 in MHB. The procedure includes development of system suitability test determination.

In conclusion, the pH 9.0 buffer efficiently stabilizes the peptide macromolecule of alpha-defensin 2, thereby providing a suitable environment for deploying its antibacterial activity.

Conflict of interest

The authors declare no conflicts of interest.

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