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BIOPHARMACY

ANTIMICROBIAL PRESERVATIVE EFFECTIVENESS OF NATURAL PEPTIDE ANTIBIOTICS

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Abstract: The constantly growing resistance of microbes to drugs and other substances which fight microbial infections leads to search for new antimicrobial substances. Among substances which attract the scientists attention are antimicrobial peptides. Such compounds are quite common in nature and belong to the most important elements of the innate immune system of all living organisms. Numerous antimicrobial peptides have been isolated from insects, amphibians, mammals, plants and bacterial species. In this study we investigated the *in vitro* activity of two animal peptides, citropin 1.1 and protegrin 1 alone and in combination against microbial strains proposed for the evaluation of preservatives: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Aspergillus niger* ATCC 16404. The results of the antimicrobial preservative effectiveness were compared to the values received for benzalkonium chloride, popular preservative of medicines and cosmetics.

Keywords: peptides, preservative effectiveness test, antimicrobial activity

One of the primary requirements posed with respect to many drugs and cosmetics is their sterility and resistance to infections during application and storage. Hence, the presence of preservatives also prevents the synthesis of the products of metabolism of microorganisms, which may be the cause of skin and mucosa irritation. The hitherto applied preservatives are mostly organic derivatives of organic acids, aldehydes, alcohols and phenols, derivatives of guanidine, organic derivatives of mercury, and ammonia salts. Inorganic preservatives are above all boric acid, sodium sulphite and iodate. Following the pro-ecological tendency, the world cosmetic industry tries to diverge from the synthetic preservatives in favour of natural compounds. In particular, this pertains to biocosmetics, which by definition should base on natural ingredients of plant or animal origin. It seems it is worth considering the application of new compounds of peptide structure. Only few peptides (from the group of lantibiotics – compounds of peptide structure, of bacterial origin) have been used as preservatives so far [1].

However, modern chemotherapy places highest hopes on endogenous peptide antibiotics [2]. Endogenous peptide antibiotics constitute one of the most important parts of the immune system of eukaryotic organisms [3]. They are secreted at the first moments of infection; they have non-specific activity, yet microorganisms are very effectively killed already after a few minutes. They are of greatest significance in insects, where peptides secreted directly to hemolymph constitute the first line of defence against bacteria [4]. Among mammals, rich in peptides are neutrophiles and all types of epithelium, which have a direct contact with the surroundings abundant in pathogenic microorganisms [5].

In recent years, a large number of natural antimicrobial peptides as well as several synthetic analogues have been tested with respect to their possible application as chemotherapeutic agents [6]. Due to the low toxicity of peptide antibiotics particularly ardent hopes are put on the possibility of the application of endogenous ones as biopharmaceuticals in modern chemotherapy of infectious diseases. Nevertheless, there are no reference data in literature on attempts to use them as preservatives.

In the present study, we were interested in investigating peptide antibiotics as the group of preservatives. We synthesized two antimicrobial peptides (protegrin 1, the peptide isolated from porcine leukocytes [7] – RGGRLCYCRRRFC--VCVGR-NH_2 and citropin 1.1, the peptide pro-

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duced by frog *Litoria citropa* [8] – GLFDVIKK--VASVIGGL-NH_2). The microbiological tests were conducted on pharmacopeal reference strains proposed for the evaluation of preservatives by: *European Pharmacopeia* (5th Edition, 2005) and *Farmakopea Polska VI* (Polish Pharmacopeia VI). The results of the test were compared to the values received for benzalkonium chloride, popular preservative of medicines and cosmetics.

EXPERIMENTAL

Antimicrobial agents

The peptides were synthesized by solid-phase procedures on Polystyrene AM-RAM resin (0.76 mmol/g, Rapp Polymere, Germany) using 9-fluorenylmetoxycarbonyl (Fmoc) methodology [9]. The Fmoc groups of each amino acid were deprotected by 20% piperidine in dimethylformamide (DMF) in two steps: 2 min and 20 min. The coupling reactions were carried out with 4-fold excess of Fmoc-amino acids in DMF in presence of Triton X-100 using diisopropylocarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. The peptides were cleaved from resin with trifluoroacetic acid (TFA) in the presence of 2.5% ethanedithiol (EDT), 2.5% triisopropylsilane (TIS) and 2.5% water as scavengers. The cleaved peptides were concentrated and precipitated with diethyl ether. Protegrin was cyclized by 0.1 M iodine in methanol. The crude peptides were purified by reverse-phase high performance liquid chromatography on Knauer K501 two-pump system with Kromasil C8 column 10 x 250 mm (5 µm particle diameter, 100 E pore size) employing acetonitrile-water mixtures (containing 0.1% TFA) as an eluent, flow rate 5 mL/min, absorbance at 226 nm. The fractions with purity greater than 95% were pooled and liophilized.

Benzalkonium chloride was obtained from Sigma-Aldrich (Poznań, Poland).

Organisms

The following reference strains proposed for the evaluation of the preservatives by *European Pharmacopeia* (5th Edition, 2005) and *Farmakopea Polska VI* were tested: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Aspergillus niger* ATCC 16404. All microorganisms were from Polish Collection of Microorganisms (Polish Academy of Sciences, Institute of Immunology and Experimental Therapy, Wrocław, Poland)

Antimicrobial assay

The minimal inhibitory concentration (MIC) was determined using a microbroth dilution method with Mueller-Hinton broth (Becton Dickinson) (bacteria) and Sabourauds Dextrose broth (fungi). An initial inoculum of microorganisms was 10^5 CFU/mL. Polypropylene 96-well plates (Nunc GmbH & Co. KG, Germany) were incubated for 18 h at 37°C (bacteria) and 48 h at 20-25°C (fungi) in air. The MIC was taken as the lowest drug concentration at which observable growth was inhibited. The minimal bactericidal or fungicidal concentration (MBC) was established as the lowest concentration of each drug that resulted in more than 99.9% reduction of the initial inoculum. Appropriate dilutions of broth were surface plated on Plate Count agar to determine the number of microorganisms. Experiments were performed in triplicate.

Determination of preservative effectiveness

Preservative effectiveness was determined in sterile water. The concentrations of drugs used in the preservative test were determined according to the value of MIC and MBC. The appropriate amounts of tested compounds (256 µg/mL of citropin, 128 µg/mL of protegrin, 64 µg/mL of benzalkonium chloride and citropin + protegrin – 1:2) in 5 mL of sterile, deionized water were separately inoculated

Table 1. Activity of antimicrobial peptides and benzalkonium chloride against reference strains.

Compound	Minimal inhibitory concentration and minimal bactericidal or fungicidal concentration (MIC/MBC) [µg/mL]				
	E. coli ATCC 8739	*S. aureus* ATCC 6538	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
Citropin 1.1	128/256	8/8	256/256	16/16	32/32
Protegrin 1	4/4	8/8	8/16	8/8	64/128
Benzalkonium chloride	16/16	2/2	32/32	8/8	64/64

Table 2. The results of preservative effectiveness test for citropin, protegrin, the mixture of citropin and protegrin, and banzalkonium chloride. The table presents the results of two measuring series. (-) – the increase of microbes was not observed.

Time	Compounds	Number of microorganisms per gram of test solution				
		E. coli ATCC 8739	*S. aureus* ATCC 6538	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
0 h		2×10^5	10^5	$2{,}6 \times 10^5$	$3{,}1 \times 10^5$	$1{,}4 \times 10^5$
6 h	Citropin	$4{,}2 \times 10^5$ $4{,}6 \times 10^5$	2×10^4/-	10^4 5×10^4	-/-	-/-
	Protegrin	$1{,}9 \times 10^5$ $2{,}4 \times 10^5$	10^4/-	$1{,}6 \times 10^5$ $2{,}1 \times 10^5$	-/-	-/-
	Citropin + Protegrin	$2{,}6 \times 10^5$ $3{,}2 \times 10^5$	-/-	9×10^4 $1{,}6 \times 10^5$	-/-	-/-
	Benzalkonium chloride	-/-	-/-	-/-	-/-	-/-
24 h	Citropin	5×10^4/-	-/-	-/-	-/-	-/-
	Protegrin	2×10^4 3×10^4	-/-	-/-	-/-	-/-
	Citropin + Protegrin	2×10^4 5×10^4	-/-	-/-	-/-	-/-
	Benzalkonium chloride	-/-	-/-	-/-	-/-	-/-
2 days	Citropin	10^4/-	-/-	-/-	-/-	
	Protegrin	-/-	-/-	-/-	-/-	-/-
	Citropin + Protegrin	-/-	-/-	-/-	-/-	-/-
	Benzalkonium chloride	-/-	-/-	-/-	-/-	-/-
7 days	Citropin	-/-	-/-	-/-	-/-	-/-
	Protegrin	-/-	-/-	-/-	-/-	-/-
	Citropin + Protegrin	-/-	-/-	-/-	-/-	-/-
	Benzalkonium chloride	-/-	-/-	-/-	-/-	-/-
14 days	Citropin	-/-	-/-	-/-	-/-	-/-
	Protegrin					
	Citropin + Protegrin	-/-	-/-	-/-	-/-	-/-
	Benzalkonium chloride	-/-	-/-	-/-	-/-	-/-
28 days	Citropin	-/-	-/-	-/-	-/-	-/-
	Protegrin	-/-	-/-	-/-	-/-	-/-
	Citropin + Protegrin	-/-	-/-	-/-	-/-	-/-
	Benzalkonium chloride	-/-	-/-	-/-	-/-	-/-

with proper volume of suspension bacteria or fungi to achieve an approximate population of 10^5 cells per mL of the sample. The prepared samples were stored at 20-25°C throughout the time of the study. At 0 h, 6 h, 24 h and 2, 7, 14, 28 days after inoculation the appropriate volume (0.1 mL) of inoculated samples were transferred onto Tryptic Soy Agar (TSA; bacteria) and Sabourauds Dextrose Agar (SDA; fungi). The TSA plates were incubated at 37°C for 3 days and SDA plates were stored at 20-25°C for 5 days and after that total plate counts were performed.

RESULTS

The first stage involved determining the minimal inhibitory concentration and minimal bactericidal concentration of the examined substances. The tests were determined using a microbroth dilution method using polypropylene plates. Protegrin was the most active among the examined substances. Protegrin was inhibiting the growth of the examined bacteria in concentration of 4-8 µg/mL. For protegrin inhibition of the growth of fungi *C. albicans* and *A. niger* was observed for concentrations of 8

and 64 μg/mL, respectively. The values of MBC for both peptides were not significantly different from the value of MIC. Lower activity of citropin against protegrin and benzalkonium chloride was observed in case of *E. coli* and *P. aeruginosa* strains. The values of MIC and MBC were a departure point to calculate the amounts of substances necessary to conduct a preservative effectiveness test.

In preservative effectiveness test the concentrations of antimicrobial agents were used: citropin 256 μg/mL, protegrin 128 μg/mL, and benzalkonium chloride 64 μg/mL. The compounds in 5 mL of sterile, deionized water were separately inoculated with proper volume of suspension bacteria or fungi to achieve an approximate population of 10^5 cells per mL of the sample. At 0 h, 6 h, 24 h and 2, 7, 14, 28 days after inoculation the appropriate volume (0.1 mL) of inoculated samples were transferred onto Tryptic Soy Agar (TSA; bacteria) and Sabourauds Dextrose Agar (SDA; fungi). The presence of bacteria was observed after 6 hours for peptides and their mixture. The test conducted for benzalkonium chloride did not indicate any presence of microbes under these conditions. The tests conducted after 24 hours and 2 days (only for citropin) revealed the presence of *E. coli* in the examined liquids. Throughout the time of the test there was no increase of the examined fungi both for the solutions of the examined peptides and for benzalkonium chloride. Preservative effectiveness test was conducted twice. Microbiological trials from each test were performed in triplicate.

DISCUSSION AND CONCLUSION

Microbial infections constitute a serious problem in the modern drug delivery. Despite considerable progress in the creation of new cosmetics and drugs of modern delivery systems, there are still various problems with preservation of these preparations. Extending the durability of these products and preventing the lesions caused by bacteria, fungi and physicochemical factors is one of the most important issues in modern pharmacy. The preservatives used should exhibit various properties, e.g. be nontoxic, act in low concentrations, have a wide spectrum of action and they should not cause irritations or allergies. The preservatives currently used exhibit high antimicrobial activity. However, they have an adverse influence on human body, for example cause allergic reactions. Therefore, there is a constant need for new preservatives.

The data presented above illustrate the potential of cationic antibacterial peptides in preservation of medicines and cosmetics. They were tested with the pharmacopeal method on reference strains and in conditions for conventional preservatives. The present study indicated that both peptides are effective substances against all tested microorganisms.
The main trump of the presented peptides is that they are natural compounds. They may be used in many oral deliveries without any toxic reactions. Peptides are susceptible to the activity of proteolytic enzymes of gastrointestinal tract. Both high antimicrobial activity and other risks connected with uncontrolled and undesirable activity of peptides are eliminated in gastrointestinal tract. These substances undergo enzymatic degradation in the initial part of gastrointestinal tract.

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