



His₆-OPH and Its Stabilized Forms Combating Quorum Sensing Molecules of Gram-Negative Bacteria in Combination with Antibiotics

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Abstract

Background: In most species of gram-negative pathogenic and conditionally pathogenic bacteria, quorum sensing-dependent systems have been discovered, in which various N-acyl-L-homoserine lactones function as signaling molecules inducing the development of resistance towards the effects of antibiotics.

Objectives: The purpose of this study was to investigate the possibility of stabilizing lactonase activity of His₆-OPH by forming enzyme polyelectrolyte non-covalent complexes (EPNCs) containing both this enzyme and polyanionic polymer. It also targeted at studying the efficacy of these EPNCs in combination with a variety of antibiotics (ampicillin, gentamicin, kanamycin, and rifampicin) against gram-negative bacteria. Cheap non-toxic biodegradable polyamino acids (poly-L-glutamic acid and poly-L-aspartic acid) were selected as polyanionic polymers to produce EPNCs.

Methods: Recombinant *Escherichia coli* strain SG13009 [pREP4], transformed by plasmid encoding His₆-OPH, was used for His₆-OPH production. The His₆-OPH lactonase activity was determined according to known methods, using a pH-sensitive indicator (cresol red) and a colorimetric method. The minimum inhibitory concentration (MIC) of antibiotics was determined with the suspension of bacteria cells, with antibiotic added in a concentration range from 0 to 500 mg/L at 37°C during 16 hours.

Results: All the tested enzyme preparations were equally efficient in catalyzing the hydrolysis of N-acyl-homoserine lactones of various structures containing and lacking a 3-oxo group in the acyl radical. It has been established that the presence of enzyme preparations facilitates the reduction of MIC of antibiotics (ampicillin, gentamicin sulfate, kanamycin sulfate, and rifampicin) on the growth of highly concentrated (10⁶ cells/mL) cell populations of *Pseudomonas aeruginosa* B-6643 and *Escherichia coli* B-6645. Polyelectrolyte complexes of this enzyme have an increased efficiency at a lower pH (6.5) of the medium in comparison to its highly purified form. It is possible to reduce the minimum inhibitory concentration of antibiotics by 10% to 850% in combined use of antibiotics and His₆-OPH enzyme preparations.

Conclusions: The discussed approaches for the use of original biological products based on His₆-OPH opens up opportunities for creating new ways of effectively controlling bacterial diseases in animals.

Keywords: Organophosphorus Hydrolase, Lactonase, N-Acyl-Homoserine-Lactones, Polyelectrolyte Complex, Minimal Inhibitory Concentrations, Antibiotics

1. Background

Rapidly increasing resistance of pathogenic bacterial strains to existing antimicrobial drugs is a global issue that with the onset of the XXI century has been compounded by the lack of new classes of antibiotics (1).

One of the causes of bacteria being resistant to different antimicrobial agents is their ability to exist in the form of highly concentrated cell populations, with the mechanism of "Quorum Sensing" (QS) in bacteria being the prerequisite for the populations' formation. The efficient con-

trol of bacterial populations, which are resistant to antibiotics, is an urgent issue (2). An alternative is the use of antibiotics in combination with substances directly affecting certain mechanisms that ensure the stability of cell populations. An example is the combination of β -lactam antibiotics with clavulanic acid, sulbactam or ortazobactam, inhibiting β -lactamase enzymes, which are synthesized by bacteria to hydrolyze antibiotics (1).

In most species of gram-negative pathogenic and conditionally pathogenic bacteria, QS-dependent sys-

tems have been discovered, in which various N-Acyl-L-Homoserine Lactones (AHLs) function as signaling molecules, inducing the development of resistance towards the effects of antibiotics (3).

It is known that a number of enzymes (AHL-lactonases) degrade QS-signaling molecules, such as AHLs by opening the lactone ring (4). It has also been shown that hexahistidine-tagged organophosphate hydrolase (His₆-OPH) exhibit a lactonase activity against a number of AHLs (5). It turned out that His₆-OPH had a higher lactonase activity in individual substrates, and the substrate activity range of this enzyme was greater than that of natural lactonases.

It is known that the activity of various enzymes could be stabilized (6), including that of organophosphate hydrolase (7), due to the formation of enzyme polyelectrolyte non-covalent complexes (EPNC). It has previously been found that polyanions in EPNCs were the most successful “partners” for recombinant enzyme His₆-OPH to maintain a high catalytic activity in hydrolysis reactions of organophosphorus compounds (8).

2. Objectives

The purpose of this study was to investigate the possibility of stabilizing lactonase activity of His₆-OPH by forming EPNCs, containing both this enzyme and polyanionic polymer. Another aim was determining the efficacy of these EPNCs in combination with a variety of antibiotics (ampicillin, gentamicin, kanamycin, and rifampicin) against gram-negative bacteria. Cheap non-toxic biodegradable polyamino acids (poly-L-glutamic acid (PLE₅₀) and poly-L-aspartic acid (PLD₅₀)) were selected as polyanionic polymers to produce EPNCs (9). It was assumed that the use of His₆-OPH and EPNC could stabilize the lactonase activity of this enzyme allowing its use at neutral pH values typical for most living organisms, rather than at a pH of 10.5, which is optimal for the action of His₆-OPH (10).

3. Methods

3.1. Cells and Chemicals

Poly-L-glutamic acid sodium salt (PLE₅₀, MW = 7500 Da) and poly-L-aspartic acid sodium salt (PLD₅₀, MW = 6800 Da) were used to prepare polyelectrolyte complexes of the His₆-OPH enzyme (Alamanda Polymers, Huntsville, AL, USA). The study used antibiotics (ampicillin, gentamicin sulfate, kanamycin sulfate, and rifampicin), lactones L-Homoserine lactone hydrochloride (HSL), N-Butyryl-DL-homoserine lactone (C₄-AHL), N-Hexanoyl-DL-homoserine

lactone (C₆-AHL), N-Decanoyl-DL-homoserine lactone (C₁₀-AHL), N-Dodecanoyl-DL-homoserine lactone (C₁₂-AHL), N-Tetradecanoyl-DL-homoserine lactone (C₁₄-AHL), N-(β-Ketocaproyl)-L-homoserine lactone (3-oxo-C₆-AHL), N-(3-Oxooctanoyl)-L-homoserine lactone (3-oxo-C₈-AHL), N-(3-Oxodecanoyl)-L-homoserine lactone (3-oxo-C₁₀-AHL), N-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂-AHL), N-(3-Oxotetradecanoyl)-L-homoserine lactone (3-oxo-C₁₄-AHL), and other reagents purchased from Sigma (Saint Louis, MO, USA).

Gram-negative bacteria cells of *Pseudomonas aeruginosa* B-6643 and *Escherichia coli* B-6645 were purchased from All-Russian Collection of Industrial Microorganisms.

Recombinant *Escherichia coli* strain SG13009 [pREP4] (Qiagen, Hilden, Germany) transformed by plasmid encoding His₆-OPH (11) was used for His₆-OPH production.

3.2. Cell Culture

Culturing of *Pseudomonas aeruginosa* B-6643 and *Escherichia coli* B-6645 cells at pH 7.0 was carried out in an IRC-1-U temperature controlled shaker (Adolf Kuhner AG, Basel, Switzerland) at 28°C and 180 rpm in 750 mL Erlenmeyer flasks with 200 mL of LB medium. The cell concentration was monitored at 540 nm using the Agilent 8453 UV-visible spectroscopy system (Agilent technology, Waldbronn, Germany) with the use of calibration graphs showing linear dependence of the optical density of the cell suspension from known cell concentration in the sample (mg/mL).

Recombinant *Escherichia coli* strain SG13009 [pREP4] cells were cultivated, and the His₆-OPH enzyme was isolated and purified as published previously (12). The protein concentration was determined by the Bradford method. The purity of the enzyme was monitored by electrophoresis, as described previously (8).

3.3. Preparation of Enzyme Polyelectrolyte Non-Covalent Complexes

To produce an EPNC, an aliquot of a PLE₅₀ or PLD₅₀ solution prepared in distilled water at a concentration of 20 mg/mL was added to a solution of highly purified His₆-OPH in 0.1 M carbonate buffer (pH 10.5) (protein concentration of 0.16 ± 0.01 mg/mL and activity 695 ± 15 U/mL). The aliquot volume was calculated so that the enzyme/polymer molar ratio was 1:5. Next, the mixture was held for 30 minutes at +8°C. The effective hydrodynamic diameter of particles of prepared complexes was determined at 25°C by DLS using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK), and was equal to 35 ± 5 nm.

3.4. Lactonase Activity Measurement

The His₆-OPH lactonase activity was determined according to known methods, using a pH-sensitive indicator (cresol red) and a colorimetric method (13, 14). The reaction was carried out in a 2.5-mM bicine buffer (pH 8.2) containing 0.1 M of NaCl. The unit of lactonase activity was defined as enzyme concentration that brings hydrolysis of 1 μmol of substrate over 1 minute at 25°C. The values of the catalytic performance of enzyme preparations in reactions of AHLs hydrolysis (catalytic constant (k_{cat}), Michaelis constant (K_m), and the efficacy constant ($k_{eff} = k_{cat}/K_m$)) were calculated using the Michaelis-Menten equation. The hyperbolic function «Hyperbl» and the Origin 8.1 software were used to approximate the experimental data so that the catalytic constants could be determined.

3.5. Minimum Inhibitory Concentration Measurement

The value of the minimum inhibitory concentration (MIC) of antimicrobial agents with an EPNC either being absent or present (12.5 μg/mL) was used to estimate the influence of antibiotics on highly concentrated gram-negative cell populations. The MIC of antibiotics was determined with the suspension of bacteria cells exposed in physiological saline (10⁶ cells/mL) with antibiotic added at a concentration range from 0 to 500 mg/L at 37°C during 16 hours. To evaluate the residual concentration of viable cells in the samples after exposing them in the presence of the antimicrobial agent, the concentration of intracellular ATP was determined by known luciferin-luciferase method (15, 16), using a standard ATP reagent (Lyumtek Ltd., Moscow, Russia). The intensity of bioluminescence was recorded using a MicroLuminometr 3560 (New horizons diagnostics Co, MD, USA). The received data was linearized to determine the MIC (as described previously when calculating the accuracy of approximation) (17). When the experimental data obtained in at least 3 replications was processed, the mean values and standard deviation (± SD) were calculated.

4. Results

4.1. Determining the Catalytic Characteristics of His₆-OPH and its Polyelectrolyte Complexes in AHL Hydrolysis

The researchers studied the characteristics of hydrolyzing lactones (which are inducers of quorum sensing in a number of gram-negative bacteria) under the effect of His₆-OPH and its EPNC, obtained using PLE₅₀ and PLD₅₀. Hydrolysis kinetics was determined by plotting velocity versus substrate concentration. The K_m , k_{cat} , and k_{eff} values were calculated by fitting the data to the Michaelis-Menten equation (Table 1).

In general, for His₆-OPH or its EPNC, the catalytic characteristics established in the reactions with the studied AHLs differed slightly. The highest AHL catalytic hydrolysis constant was determined for the enzyme in the reaction with the lactone C₀.

It has been found that when AHLs were used as a substrate with the side chain elongated from C₄ to C₁₄ carbon atoms, there was a certain increase in the K_m values and a simultaneous decrease in the efficacy constant of the enzyme preparations (k_{eff}).

Regarding AHLs containing the 3-oxo group in the acyl radical, the k_{cat} value decreased with the chain elongated from C₆ to C₁₄ carbon atoms, wherein the minimum K_m value and simultaneously the maximum k_{eff} value were established in hydrolyzing 3-oxo-C₁₂-AHL.

In general, it could be argued that all the investigated enzyme preparations of His₆-OPH were equally able to actively catalyze the hydrolysis of structurally different AHLs containing and lacking the 3-oxo group in the acyl radical.

4.2. The Effect of Combining Various Antibiotics with His₆-OPH or Its Polyelectrolyte Complexes on the MIC of Antibiotics Against Different Gram-Negative Bacteria Cells

The effect of *E. coli* and *P. aeruginosa* bacteria in combination with various antibiotics when His₆-OPH or its EPNC was administered in highly concentrated suspensions of gram-negative bacteria on the effectiveness of the latter was examined. The MIC values were established for the 4 antibiotics with the enzyme preparations absent or present.

For all antibiotics studied and for two bacterial cultures used in the work, it was found that the presence of the enzyme preparations reduced the MIC values for all antibiotics (by 10% to 85%) (Table 2).

By exposing cells to 2 different pH values (6.5 and 7.5), a significant impact of the parameter on the effectiveness of the enzyme preparations in combination with antibiotics was found in terms of reduction in MIC for gram-negative bacteria. It was shown that by lowering the pH value to 6.5, the effectiveness of the purified enzyme drastically declined (the MIC reduction in the presence of the drug did not exceed 6%), with the EPNC efficiency decreasing less markedly (the decrease in the MIC in the presence of the drug reached 415%). As expected, this fact confirmed that the stability of His₆-OPH catalytic performance increased as part of an EPNC.

5. Discussion

The experimental data analysis allowed the identification of the following key patterns in AHLs hydrolysis re-

Table 1. The Catalyst Characteristics of His₆-OPH and Polyelectrolyte Complexes of This Enzyme in Hydrolysis Reactions of Various AHLs^a

Substrate	K _m (μM)	k _{cat} (s ⁻¹)	10 ⁻³ k _{eff} (M ⁻¹ s ⁻¹)
His₆-OPH			
HSL	307 ± 7	4.70 ± 0.08	15.31 ± 0.01
C ₄ -AHL	415 ± 5	2.61 ± 0.03	6.29 ± 0.04
C ₆ -AHL	531 ± 8	3.19 ± 0.12	6.01 ± 0.02
C ₁₀ -AHL	540 ± 8	3.12 ± 0.12	5.78 ± 0.02
C ₁₂ -AHL	545 ± 8	2.9 ± 0.12	5.32 ± 0.02
C ₁₄ -AHL	603 ± 8	2.72 ± 0.22	4.51 ± 0.03
3-oxo-C ₆ -AHL	638 ± 10	4.22 ± 0.22	6.61 ± 0.02
3-oxo-C ₈ -AHL	603 ± 8	4.61 ± 0.25	7.65 ± 0.03
3-oxo-C ₁₀ -AHL	188 ± 6	2.68 ± 0.03	14.26 ± 0.01
3-oxo-C ₁₂ -AHL	103 ± 5	1.87 ± 0.15	18.16 ± 0.03
3-oxo-C ₁₄ -AHL	153 ± 3	1.45 ± 0.22	9.48 ± 0.07
His₆-OPH-PLD₅₀			
HSL	310 ± 10	4.74 ± 0.16	15.29 ± 0.02
C ₄ -AHL	417 ± 5	2.63 ± 0.15	6.31 ± 0.03
C ₆ -AHL	538 ± 4	3.31 ± 0.13	6.15 ± 0.03
C ₁₀ -AHL	543 ± 5	3.24 ± 0.15	5.97 ± 0.03
C ₁₂ -AHL	552 ± 8	2.97 ± 0.12	5.38 ± 0.02
C ₁₄ -AHL	618 ± 5	2.71 ± 0.15	4.39 ± 0.03
3-oxo-C ₆ -AHL	644 ± 5	4.42 ± 0.15	6.86 ± 0.03
3-oxo-C ₈ -AHL	610 ± 4	4.70 ± 0.12	7.71 ± 0.03
3-oxo-C ₁₀ -AHL	193 ± 5	2.74 ± 0.15	14.20 ± 0.03
3-oxo-C ₁₂ -AHL	127 ± 5	1.85 ± 0.05	16.52 ± 0.10
3-oxo-C ₁₄ -AHL	167 ± 3	1.43 ± 0.04	8.56 ± 0.01
His₆-OPH/PLD₅₀			
HSL	305 ± 9	4.72 ± 0.14	15.48 ± 0.02
C ₄ -AHL	412 ± 6	2.65 ± 0.17	6.43 ± 0.03
C ₆ -AHL	525 ± 5	3.33 ± 0.15	6.34 ± 0.03
C ₁₀ -AHL	541 ± 5	3.25 ± 0.15	6.01 ± 0.03
C ₁₂ -AHL	549 ± 4	2.99 ± 0.14	5.45 ± 0.04
C ₁₄ -AHL	610 ± 5	2.69 ± 0.15	4.41 ± 0.03
3-oxo-C ₆ -AHL	635 ± 5	4.3 ± 0.15	6.77 ± 0.03
3-oxo-C ₈ -AHL	600 ± 5	4.70 ± 0.15	7.83 ± 0.03
3-oxo-C ₁₀ -AHL	190 ± 3	2.72 ± 0.09	14.32 ± 0.03
3-oxo-C ₁₂ -AHL	112 ± 5	2.07 ± 0.15	18.48 ± 0.11
3-oxo-C ₁₄ -AHL	177 ± 7	1.69 ± 0.12	9.55 ± 0.02

^aData are expressed as mean ± SD.

actions under the influence of His₆-OPH and its EPNC. Biological products on the basis of this enzyme have high

enough lactonase activity in comparison with known lactonases (18) in the C-O hydrolysis of molecules in AHLs of

Table 2. The Minimal Inhibitory Concentrations of Antibiotics for Gram-Negative Bacteria in the Presence of Enzyme Preparations on the Basis of His₆-OPH (12.5 µg/mL) or Without Them at pH 7.5 and 6.5^a

Antibiotic Additive	MIC, µg/mL			
	None	His ₆ -OPH	His ₆ -OPH/PLE ₅₀	His ₆ -OPH/PLD ₅₀
pH 7.5				
<i>E. coli</i> cells				
Ampicillin	4900 ± 50	445 ± 10	4454 ± 50	3578 ± 50
Gentamicin	3800 ± 50	87.9 ± 2	79.6 ± 3.1	94.8 ± 3.5
Kanamycin	1 ± 0.05	0.5 ± 0.01	0.4 ± 0.01	0.3 ± 0.01
Rifampicin	3 ± 0.05	1.2 ± 0.01	0.8 ± 0.03	0.5 ± 0.02
<i>P. aeruginosa</i> cells				
Ampicillin	3600 ± 50	498 ± 3	1115 ± 50	1250 ± 30
Gentamicin	5100 ± 50	61 ± 1	66.7 ± 3.1	59.7 ± 2.0
Kanamycin	120 ± 5	15 ± 0.5	8 ± 0.1	6 ± 0.2
Rifampicin	6 ± 0.2	2.1 ± 0.01	1.8 ± 0.01	1.7 ± 0.01
pH 6.5				
<i>E. coli</i> cells				
Ampicillin	4655 ± 50	4470 ± 10	4600 ± 50	3480 ± 50
Gentamicin	3610 ± 50	3430 ± 20	1334 ± 10	1194 ± 10
Kanamycin	0.95 ± 0.05	0.95 ± 0.01	0.8 ± 0.01	0.7 ± 0.02
Rifampicin	2.85 ± 0.05	2.81 ± 0.01	1.9 ± 0.01	1.8 ± 0.02
<i>P. aeruginosa</i> cells				
Ampicillin	3433 ± 50	3260 ± 3	1717 ± 50	2446 ± 32
Gentamicin	4867 ± 50	4620 ± 1	973 ± 2.8	948 ± 10
Kanamycin	115 ± 5	110 ± 0.5	40 ± 1.3	42 ± 2
Rifampicin	5.5 ± 0.2	5.2 ± 0.01	4.0 ± 0.5	4.3 ± 0.1

^aData are expressed as mean ± SD.

various structures. By varying the length of the acyl radical in the range of C₆-C₁₂, the efficacy constants of enzyme preparations reached their maximum values.

Comparison of the results to published data regarding the side chain influence on the characteristics of AHLs hydrolysis showed consistency with previously established characteristics of lactones hydrolysis with the His₆-OPH enzyme (5) and with the lactonase isolated from *Bacillus thuringiensis* cells (19). In particular, the catalytic performance of hydrolysis was improved for lactones from 3-oxo-C₆-AHL to 3-oxo-C₈-AHL. However, the hydrolysis of AHLs not containing the 3-oxo-group demonstrated differences in the efficacy of His₆-OPH and the lactonase isolated from *Bacillus thuringiensis*. The difference was that in the first case, the maximum value of k_{eff} was observed for C₄-AHL, and with the chain elongated to C₁₄-AHL this parameter decreased, while for *Bacillus thuringiensis* lactonase the oppo-

site trend was observed up to C₁₀-AHL, and the maximum effectiveness of the enzyme was observed for the latter substrate. These trends seem to be associated with structural features of the enzyme molecules that exhibit lactonase activity.

The differences shown in Table 2 in the values of minimum inhibitory concentrations of antibiotics in the presence of enzyme preparations on the basis of His₆-OPH are probably due to antibiotics affecting the enzyme activity.

The trends of reducing the MIC of antibiotics combined with His₆-OPH enzyme preparations appear to have been caused by the destruction of AHLs molecules that induce quorum sensing in gram-negative bacteria cells and by the resulting increase in the efficacy of antibiotics. Thus, due to the use of His₆-OPH preparations, bacterial cells were directly deprived of a possible transition to a stable state. Consequently, the more effective impact of anti-

crobial agents was made on the cells, which are part of highly concentrated populations. This result is significant because it demonstrates the possibility of reducing the amounts of single effective doses of applied antibiotics, while the development of bacteria cells resistance to antibiotics is reduced due to “quorum sensing.”

The discussed approaches to the use of original biological products based on His₆-OPH open up opportunities for creating new ways of effectively controlling bacterial diseases in animals. The biologics presented, which ensure the hydrolysis of lactone inductors forming stable populations of gram-negative bacterial cells (*Escherichia sp.* and *Pseudomonas sp.* et al.) that cause various diseases, present a competitive alternative to expensive lactonases secreted from gram-positive cells of the *Bacillus* genus.

Taking into account the already known properties of His₆-OPH enabling its usage in the form of EPNCs, purified and non-purified enzyme for deep detoxification of organophosphorus compounds in vitro (8) and in vivo (20), it is possible to suppose that the enzymatic preparations in combination with antibiotics being introduced into body will possess double activity against bacterial infections and pesticides entering the body with dirty foods and water samples.

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Footnotes

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