In silico determination of substrate spectrum of lactonases, hydrolyzing various N-acyl homoserine lactones

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Abstract. The rapid growth in the number of resistant pathogenic bacteria has led to a decrease in the effectiveness of the existing antimicrobial agents. N-acyl homoserine lactones (N-AHLs) are the key molecules responsible for the formation of antibiotic resistance of gram-negative bacteria. The combination of various lactonases, capable of hydrolyzing a wide range of N-AHLs, with antibiotics, is one of the most appropriate ways to solve the problem of maintaining the effectiveness of the latter. The most interesting is the combination of lactonases with different substrate spectrum of action. In this study, using the molecular docking method, we investigated the substrate range of various lactonases in order to select enzymes suitable to combine with hexahistidine-tagged organophosphorus hydrolase (His6-OPH), for which high lactonase activity against a number of N-AHLs and the possibility of complex formation with antibiotics have been shown previously. It was found that all the studied lactonases hydrolyze predominantly long chain N-AHLs, while, among all studied lactonases, the SsoPox enzyme from the class of phosphotriesterases-like lactonases was the best candidate for the development of combined enzyme preparations.

1. Introduction
Most of the bacteria (both gram-positive and gram-negative) are known to be resistant to various antibiotics. In recent years, the number of such resistant strains of pathogenic bacteria has increased significantly. This, in turn, led to a rapid loss in the effectiveness of the majority of antibiotics that are currently used in practice, and resulted in the need to increase their effective doses [1]. The study of the resistance mechanisms in pathogenic bacteria showed that in both gram-positive and gram-negative cells, one of the main mechanisms for the development of resistance is the Quorum Sensing (QS) formation. QS is the ability of pathogenic bacterial cells to interact with each other through signaling molecules within the stable highly concentrated populations [2]. Most gram-negative bacteria use various N-acyl homoserine lactones (N-AHLs) as inducers of QS development. Different bacteria are characterized by the synthesis of various N-AHLs, and the N-acyl chain length of N-AHLs can vary from C4 to C18. For example, such common bacterial pathogens as Pseudomonas aeruginosa, Burkholderia cepacia, Yersinia enterocolitica use C12-homoserine lactone (C12-HSL), C8-HSL and C6-HSL, respectively, as signaling molecules of QS [2,3].

To date, the most appropriate way to inhibit the QS mechanism is the destruction of the inducer molecules themselves. The destruction of these molecules leads to a loss of the ability of cells to transition into the QS state. This, in turn, increases their susceptibility to the action of a wide range of antimicrobial agents and eliminates the need to increase the applied dose of antibiotics [4]. Of greatest
interest is the enzymatic hydrolysis of N-AHLs by lactonases - enzymes that break down the ester bond of lactones, thereby opening the lactone ring [4,5]. Currently, however, there are no known available and produced in significant quantities enzymes with lactonase activity that are effective against a wide range of N-AHLs, responsible for the formation of resistance of pathogenic bacterial cells. In this regard, there are no effective methods for the combined use of such enzymes with the antimicrobial agents in order to increase the effectiveness of existing antibiotics.

One of the enzymes which are exhibit lactonase activity is the hexahistidine-tagged organophosphorus hydrolase (His\textsubscript{6}-OPH). It has been previously shown that, in addition to the ability to hydrolyze a wide range of organophosphorus compounds, His\textsubscript{6}-OPH effectively catalyzes the hydrolysis of the lactone ring in molecules of various N-AHLs [6]. In addition, it was found that the enzyme is able to maintain a sufficiently high enzymatic activity and stability in the presence of a number of β-lactam antibiotics, as well as antimicrobial agents of different chemical structure [6,7,8]. However, the substrate spectrum of action of His\textsubscript{6}-OPH is not as wide as the range of N-AHLs synthesized by gram-negative bacteria. Therefore, it is advisable to combine various enzymes capable of catalyzing the hydrolysis of a number of N-AHLs and other lactonases for their subsequent combination with antibiotics should help to create new drugs with different focuses and a wide substrate spectrum of action.

So the purpose of this study was to select enzymes capable of hydrolyzing various N-AHLs, to study their substrate spectrum of action and the possibility of their combination with His\textsubscript{6}-OPH, in order to obtain catalytically active drugs that provide antibiotics with increased effectiveness against resistant cells of gram-negative bacteria. Initially, an analysis of the literature data was carried out, and enzymes exhibiting lactonase activity were selected, and based on the use of computer simulation methods, their substrate spectrum of action was determined \textit{in silico} for various N-AHLs with or without 3-oxo substitution.

2. Results and discussion

Based on an analysis of published data, 6 enzymes capable of hydrolyzing a range of N-AHLs were selected (Table 1). The choice of enzymes was based on their belonging to a different classes of lactonases: 4 enzymes (AaL, AidC, AiiA and AiiB) from the class of Metallo-β-lactamases, and 2 (GKL and SsoPox) from the class of Phosphotriesterases-like lactonases (PLLs), as well as on the differences in the structure of their active site.

| Enzyme | Origin | Me in Active site | k\textsubscript{eff}, (10\textsuperscript{3}s\textsuperscript{-1}M\textsuperscript{-1}) | Substrate |
|--------|--------|-------------------|-------------------|-----------|
| AaL [9] | Alicyclobacillus acidoterrestres | Co\textsuperscript{2+} | 1300 | C4-HSL |
| AidC [10] | Chryseobacterium sp. | Zn\textsuperscript{2+} | 1700 | C7-HSL |
| AiiA [11] | Bacillus thuringiensis | Zn\textsuperscript{2+} | 16 | C6-HSL |
| AiiB [12] | Agrobacterium tumefaciens | Zn\textsuperscript{2+} | 16 | C6-HSL |
| GKL [13] | Geobacillus kaustophilus | Zn\textsuperscript{2+}, Fe\textsuperscript{2+} | 0.430 | C8-HSL |
| SsoPox [14] | Sulfolobus solfataricus | Co\textsuperscript{2+}, Fe\textsuperscript{2+} | 36 | 3OC10-HSL |

Using the molecular docking method the most probable interactions of various N-AHLs with or without 3-oxo substitution with the active sites of the selected enzymes were simulated at pH 7.5 (the
closest pH to physiological conditions) and 10.5 (corresponding to the maximum enzymatic activity of His$_6$-OPH [15]) (Figures 1,2).

Figure 1. Possible interactions of N-AHLs with the active site of (A) Aal, (B) AidC, (C) AiiA, (D) AiiB, (E) GKL and (F) SsoPox enzymes at pH 7.5. N-AHLs are shown as green sticks. Ions in enzymes active site are colored differently: Co$^{2+}$, Zn$^{2+}$ and Fe$^{3+}$ ions are colored violet, blue and brown respectively. Interactions between lactone ring of N-AHLs and ions in the enzyme active site are indicated by yellow dashed lines.
Figure 2. Possible interactions of N-AHLs with the active site of (A) Aal, (B) AidC, (C) AiiA, (D) AiiB, (E) GKL and (F) SsoPox enzymes at pH 10.5. N-AHLs are shown as green sticks. Ions in enzymes active site are colored differently: Co$^{2+}$, Zn$^{2+}$ and Fe$^{3+}$ ions are colored violet, blue and brown respectively. Interactions between lactone ring of N-AHLs and ions in the enzyme active site are indicated by yellow dashed lines.

The models of enzyme-substrate interaction, obtained from molecular docking, demonstrated that, regardless of the N-acyl chain length, both 3-oxo substituted and unsubstituted N-AHLs are located in the active site of the selected lactonases at a distance sufficient for efficient hydrolysis of their lactone ring. This suggests that the studied enzymes must be able to hydrolyze N-AHLs with different N-acyl chain length. The exception was the GKL enzyme, for which a significant remoteness of the molecules of the investigated substrates from his active sites was established and, therefore, the probability of effective hydrolysis was absent.

To more accurately assess the enzyme-substrate interactions, as well as to determine the effect of the substrate N-acyl chain length, in the obtained models, the affinity values of N-AHLs to the surface of the analyzed lactonases were calculated at both pH values (Table 2). The affinity was estimated as
minimum of potential energy during electrostatic and hydrophobic interactions, and hydrogen bonding between ligand (N-AHL) and receptor (enzyme).

Table 2. Affinity of N-AHLs to the surfaces of various enzymes at different pH values

| #     | AMP | pH | Affinity (kJ·mol⁻¹) | p value |
|-------|-----|----|---------------------|---------|
|       |     |    | Mean               | Median  | Upper (Lower) Bounds |         |
|       |     |    |                    |         |                      |         |
| AaL   | C4  | 7.5| -20.9              | -20.1±2.6 | -18.9(-22.5)         | 0.296   |
|       |     | 10.5| -21.6              | -21.3±2.1 | -19.7(-22.9)         |         |
|       | C6  | 7.5| -22.8              | -22.6±2.1 | -20.9(-24.7)         | 0.400   |
|       |     | 10.5| -22.0              | -21.3±1.9 | -20.5(-23.4)         |         |
|       | 3OC6| 7.5| -20.8              | -19.7±2.7 | -19.2(-21.2)         | 0.006   |
|       |     | 10.5| -22.9              | -22.8±1.6 | -21.3(-23.7)         |         |
|       | C7  | 7.5| -22.9              | -22.8±2.2 | -20.9(-24.2)         | 0.873   |
|       |     | 10.5| -22.8              | -22.6±2.0 | -21.0(-23.3)         |         |
|       | C8  | 7.5| -22.9              | -22.8±2.2 | -21.0(-24.9)         | 0.714   |
|       |     | 10.5| -22.6              | -22.0±1.9 | -21.3(-23.8)         |         |
|       | 3OC8| 7.5| -23.5              | -23.8±2.3 | -21.0(-24.6)         | 0.751   |
|       |     | 10.5| -23.7              | -23.6±1.3 | -22.6(-24.6)         |         |
|       | 3OC12| 7.5| -24.9              | -24.7±1.3 | -23.9(-25.4)         | 0.815   |
|       |     | 10.5| -24.9              | -24.7±1.6 | -23.8(-25.4)         |         |
| AidC  | C4  | 7.5| -21.0              | -21.1±0.9 | -20.2(-21.3)         | 0.435   |
|       |     | 10.5| -21.3              | -21.1±0.8 | -20.9(-21.6)         |         |
|       | C6  | 7.5| -23.6              | -23.4±1.3 | -22.3(-24.2)         | 0.047   |
|       |     | 10.5| -22.7              | -22.4±1.3 | -21.9(-23.0)         |         |
|       | 3OC6| 7.5| -23.4              | -23.0±1.1 | -22.6(-24.0)         | 0.148   |
|       |     | 10.5| -22.8              | -22.6±0.9 | -22.2(-23.3)         |         |
|       | C7  | 7.5| -23.3              | -23.4±1.3 | -22.2(-23.8)         | 0.792   |
|       |     | 10.5| -23.3              | -23.2±1.4 | -22.2(-24.4)         |         |
|       | C8  | 7.5| -23.5              | -23.0±1.2 | -23.0(-23.4)         | 0.144   |
|       |     | 10.5| -22.9              | -22.4±1.5 | -21.7(-24.0)         |         |
|       | 3OC8| 7.5| -24.4              | -24.5±1.0 | -23.5(-25.1)         | 0.054   |
|       |     | 10.5| -23.6              | -23.4±0.5 | -23.1(-24.2)         |         |
|       | 3OC12| 7.5| -25.2              | -25.1±1.2 | -24.3(-25.7)         | 0.004   |
|       |     | 10.5| -23.9              | -23.6±1.0 | -23.4(-24.3)         |         |
| AiiA  | C4  | 7.5| -21.2              | -20.7±1.6 | -20.1(-22.8)         | 0.977   |
|       |     | 10.5| -21.1              | -20.5±1.6 | -20.1(-22.5)         |         |
|       | C6  | 7.5| -22.8              | -22.4±1.9 | -23.1(-24.0)         | 0.071   |
|       |     | 10.5| -23.5              | -23.2±0.8 | -23.0(-24.2)         |         |
|       | 3OC6| 7.5| -23.9              | -23.6±1.6 | -22.7(-24.6)         | 0.977   |
|       |     | 10.5| -24.0              | -23.8±1.5 | -22.7(-24.8)         |         |
|       | C7  | 7.5| -23.9              | -22.8±2.4 | -21.9(-25.9)         | 0.156   |
|       |     | 10.5| -22.8              | -22.0±1.8 | -21.0(-24.9)         |         |
|       | C8  | 7.5| -23.5              | -23.0±1.8 | -21.9(-25.0)         | 0.721   |
|       |     | 10.5| -23.2              | -23.4±1.5 | -21.7(-24.6)         |         |
|       | 3OC8| 7.5| -24.0              | -23.8±1.0 | -23.4(-24.7)         | 0.863   |
|       |     | 10.5| -24.0              | -23.8±1.0 | -23.1(-24.7)         |         |
|       | 3OC12| 7.5| -24.4              | -23.8±2.2 | -22.4(-26.3)         | 0.281   |
As a result, it was found that with an increase in the N-acyl chain length of N-AHLS, their strength of interaction with the surface of enzymes increases (i.e., C6-HSL interacts more strongly than C4-HSL, etc. etc.). Moreover, for different lactonases, the affinity values in enzyme-substrate complexes were comparable. The strongest interaction was revealed in the case of the SsoPox enzyme, where the maximum value of the analyzed parameter was noted in the “SsoPox – 3OC12” model at pH 10.5 (−28.9 $\text{kJ mol}^{-1}$). Comparison of the data obtained for N-AHLS’ pairs with or without 3-oxo substitution (C6-HSL vs 3OC6-HSL; C8-HSL vs 3OC8-HSL) showed that in most cases the presence

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| Enzyme | substrate | $K_{d}$ (M) | $K_{d}$ (M) | $K_{d}$ (M) | $K_{d}$ (M) | $K_{d}$ (M) | $K_{d}$ (M) | $K_{d}$ (M) | $K_{d}$ (M) | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **AiiB** | C4 | 7.5 | -20.8 | -21.1±1.7 | -19.2(-22.1) | 0.532 |
| | 10.5 | -20.5 | -20.5±1.5 | -19.7(-21.7) | |
| | 7.5 | -21.3 | -21.3±1.8 | -19.4(-22.6) | 0.622 |
| | 10.5 | -21.7 | -21.5±1.2 | -20.9(-22.1) | |
| | 7.5 | -22.6 | -22.8±1.3 | -21.4(-23.8) | 0.724 |
| | 10.5 | -22.8 | -22.8±1.0 | -21.9(-23.3) | |
| | 7.5 | -22.0 | -22.0±0.9 | -21.7(-22.9) | 0.717 |
| | 10.5 | -22.2 | -22.2±1.5 | -21.0(-23.6) | 0.014 |
| | 7.5 | -23.4 | -23.4±1.1 | -22.7(-23.8) | |
| | 10.5 | -22.0 | -22.0±1.6 | -21.0(-22.9) | |
| | 7.5 | -24.6 | -24.7±1.0 | -23.8(-25.4) | 0.022 |
| | 10.5 | -23.1 | -22.6±1.6 | -21.7(-24.7) | |
| | 7.5 | -21.5 | -21.5±2.0 | -19.4(-23.3) | 0.020 |
| | 10.5 | -23.9 | -23.4±2.0 | -22.2(-25.9) | |
| **GKL** | C4 | 7.5 | -20.4 | -19.9±1.7 | -19.4(-20.8) | 0.025 |
| | 10.5 | -21.8 | -22.0±1.7 | -20.2(-22.9) | |
| | 7.5 | -21.5 | -21.5±0.6 | -21.0(-21.7) | 0.157 |
| | 10.5 | -22.7 | -21.7±2.2 | -21.3(-22.9) | |
| | 7.5 | -21.0 | -20.7±1.1 | -20.5(-21.6) | 0.412 |
| | 10.5 | -21.3 | -21.3±0.7 | -20.9(-21.7) | |
| | 7.5 | -23.5 | -22.6±3.0 | -21.0(-25.3) | 0.200 |
| | 10.5 | -21.8 | -21.7±0.8 | -21.0(-22.2) | |
| | 7.5 | -21.7 | -21.3±1.2 | -20.9(-22.5) | 0.011 |
| | 10.5 | -22.9 | -22.8±0.8 | -22.2(-23.4) | |
| | 7.5 | -23.6 | -23.4±1.5 | -22.6(-24.2) | 0.431 |
| | 10.5 | -23.1 | -22.8±1.4 | -21.9(-24.2) | |
| | 7.5 | -26.3 | -24.9±3.0 | -23.9(-28.0) | 0.485 |
| | 10.5 | -26.5 | -25.7±2.2 | -24.7(-28.6) | |
| | 10.5 | -22.0 | -21.5±2.6 | -19.8(-23.0) | |
| **SsoPox** | C4 | 7.5 | -22.7 | -22.2±1.4 | -21.7(-23.4) | 0.075 |
| | 10.5 | -23.4 | -23.2±1.2 | -22.7(-23.7) | |
| | 7.5 | -24.7 | -24.7±1.5 | -23.4(-25.4) | 0.306 |
| | 10.5 | -25.1 | -25.1±1.4 | -23.9(-25.5) | |
| | 7.5 | -26.6 | -26.6±1.5 | -25.2(-28.2) | 0.158 |
| | 10.5 | -25.5 | -25.3±2.1 | -23.8(-27.6) | |
| | 7.5 | -25.5 | -25.5±0.7 | -25.1(-25.9) | 0.103 |
| | 10.5 | -24.6 | -24.5±1.3 | -23.5(-25.9) | |
| | 7.5 | -26.8 | -26.4±1.8 | -25.6(-27.9) | 0.005 |
| | 10.5 | -24.7 | -24.7±1.9 | -23.1(-25.8) | |
| | 7.5 | -23.2 | -22.6±1.5 | -22.3(-23.6) | <0.001 |
| | 10.5 | -26.1 | -25.9±1.3 | -25.1(-27.2) | |
| | 7.5 | -28.0 | -27.8±2.4 | -25.9(-30.3) | 0.308 |
of a substitution in the N-AHL molecule increased its strength of interaction with the surface of the enzyme.

Based on the results obtained from one-way analysis of variance (ANOVA) (Table 2, p value), it was found that, in almost all cases, a change in the pH of the medium did not have a statistically significant \((p > 0.05)\) effect on the molecular affinity of N-AHLs to the surface of the enzyme. Only in 10 cases the pH variation turned out to be statistically significant for the enzyme-substrate interaction: \(\text{AaL-3OC6} (p = 0.006); \text{AidC-C6} (p = 0.047); \text{AidC-3OC12} (p = 0.004); \text{AiiB-3OC8} (p = 0.022); \text{AiiB-3OC12} (p = 0.020); \text{GKL-C4} (p = 0.025); \text{GKL-C8} (p = 0.011); \text{SsoPox-C8} (p = 0.005)\) and \(\text{SsoPox-3OC8} \ (p < 0.001)).\) It should be noted that the interaction of the AiiA enzyme with N-AHLs was pH independent.

Thus, it has been shown that all lactonases studied in this work, with the exception of GKL, are able to hydrolyze various N-AHLs, mainly long chain ones. Moreover, the best results (both in terms of accessibility of the active site of the enzyme and in terms of the affinity of N-AHLs to its surface) were obtained with the SsoPox enzyme belonging to the class of PLLs. Therefore, the combination of SsoPox with His\(_6\)-OPH is useful to obtain the most effective antimicrobial drugs with lactonase activity and a broader spectrum of action than each of the enzymes, introduced into this combination for its subsequent complexation with antibiotics, has.

3. Conclusions
This research enabled conclusion that among the studied lactonases, SsoPox from the PLLs class is the best candidate for the development of combined enzyme preparations with His\(_6\)-OPH. The use of molecular docking methods in further studies for the search and selection of enzymes capable of selectively hydrolyzing short-chain or long-chain N-AHLs with or without 3-oxo substitution molecules may allow the creation of combined antimicrobial agents with high efficiency and specificity of action.

4. Computational methods
The structures of N-AHLs were created using ChemBioDraw software (ver. 12.0, CambridgeSoft). After that, energy minimization using ChemBio3D with force field MM2 was applied. Finally, the structures in protein data bank (.PDB) format were converted to the PDBQT format (PDB format with partial charges and atom types) using AutoDockTools (as part of MGLTools ver. 1.5.6, available at http://mgltools.scripps.edu/) [16]. Atomic charges were calculated with the Gasteiger-Marsili method.

To calculate the surface charge distribution of enzymes at a certain pH, adaptive Poisson-Boltzmann solver (APBS) and PDB2PQR servers (ver. 1.4.2.1 and 2.1.1, respectively, available at http://www.poissonboltzmann.org/) with PARSE forcefield and default settings were used [17,18]. After that, structures in PQR format were converted to the PDBQT format using AutoDockTools [16].

The dockings of ligands to the enzyme surface were realized using AutoDockVina (ver. 1.1.2, available at http://vina.scripps.edu/) [19] on a desktop computer equipped with Intel Pentium Dual-Core CPU E5400 2.7 GHz and 3 GB of available memory. Briefly, the grid box was approximately centered on the center of mass of the enzyme. The size of the grid box was chosen so that any enzyme surface was within the box with an additional margin. Calculations were performed with default program options.

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