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The Adsorption of Lysozyme on Living *Escherichia Coli* Bacterial Cells in the Presence of Charged Amino Acids and Glycine

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Abstract. The effect of glycine, glutamate, aspartate, lysine, histidine, and arginine on the adsorption of lysozyme on living Escherichia coli bacterial cells under conditions close to those for the functioning of the enzyme in saliva was studied. It was shown that the addition of the listed amino acids at concentrations of 1.5 mM for glycine and 5 mM for charged amino acids reduced the desorption constant of lysozyme by 1.4–2.0 times, while the rate of cell lysis (lysozyme activity) increased by 1.4–1.9 times. The maximum amount of lysozyme that can bind to bacteria (adsorption capacity) did not change. An increase in the efficiency of cell lysis in the presence of glycine and charged amino acids can be explained by an increase in the productive adsorption of the enzyme on the surface of bacterial cells.

1 Introduction

Because of the problem of antibiotic resistance of bacteria increasing in urgency, an active search for alternative antimicrobial agents is being carried out all over the world. Great interest is devoted to bacteriolytic enzymes, in particular lysozyme [1]. The most studied and commercially available lysozyme is chicken egg lysozyme, which is similar in structure and properties to human lysozyme [2]. Currently, lysozyme is widely used in medicine and biotechnology [1, 3–6]. However, the ability of microorganisms to evolve the mechanisms of resistance against the action of bacteriolytic enzymes [7, 8] requires the search for new approaches to enhance the antibacterial activity of the enzyme. One of the mechanisms for acquiring bacterial resistance to the action of lysozyme is unproductive sorption of the enzyme, which means binding of the enzyme on the surface of the bacterial cell, leading to its deactivation and, accordingly, to a slowdown in cell lysis up to its complete stop [9]. It is already known that a number of bacteria possess specialized complexes of biopolymers that bind lysozyme [10]. Glycine and charged amino acid supplements have recently been found to increase the rate of lysis of Gram-negative bacterial cells [11, 12], but the mechanism of this activation is currently unclear.

To study the thermodynamic parameters of enzyme sorption directly on living bacterial cells, a special measurement technique was developed [13, 14], which we use in this work to investigate the effect of amino acids on lysozyme sorption. As model cells, we chose

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Escherichia coli, which is closely related to various pathogenic bacteria of the Enterobacteriaceae family [15]. The study was carried out on chicken egg lysozyme as a model enzyme, which is used in drug preparations and biologically active additives used in the oral cavity [16–18], under conditions close to those of saliva, namely, osmolarity equal to 70 mM and pH 7.0 [19]. Understanding the mechanisms of the effect of amino acids on the enzymatic lysis of bacterial cells will expand our understanding of the regulation of lysozyme activity (both salivary lysozyme itself and lysozyme in drugs) and help in the development of new highly effective antibacterial drugs based on lysozyme with activating additives.

2 Materials and methods

Materials: chicken egg lysozyme, MES, Tris ("Amresco", USA), sodium hydroxide, dibasic sodium carbonate, sodium chloride, magnesium sulfate ("Panreac", Spain), hydrochloric acid, acetic acid ("Component-Reaktiv", Russia), L-histidine, L-lysine (Serva, Germany), L-arginine, L-aspartic acid (Merck, Germany), monosodium L-glutamate ("MeiHua" (梅花), China), glycine ("Roth", Germany), glucose ("Rokett Frer", France), yeast extract ("Biospringer", France), peptone, tryptone, bacto agar ("BD Difco", USA), preparation of lyophilized *Micrococcus luteus* bacterial cells ("Sigma", USA)

Escherichia coli (museum strain MH-01) was generously provided by Anatoly A. Belogurov (National Medical Research Center of Cardiology, Moscow). Bacteria were grown according to the standard procedure [20]. Freshly grown E. coli cells in the form of a suspension with a concentration of 10^9 CFU/ml in a buffer solution of 10 mM Tris-MES-CH_3COOH were frozen by immersing the tubes in liquid nitrogen in portions of 500 µl. For each experiment, the suspension of *E. coli* bacteria was defrosted, centrifuged at 6000 rpm (2073 g) for 7 minutes. Next, the supernatant was removed, and 400 µL of buffer solution was added to the precipitate. All measurements in the work were carried out in 10 mM Tris-MES-CH_3COOH-Na_2CO_3 buffer solution with pH 7.0 (at 37 °C). For all solutions of free amino acids, the pH was adjusted to value of 7.0 by adding NaOH or HCl solutions.

The rate of lysis of *E. coli* cells (lysozyme activity) was measured by turbidimetry by the rate of change in absorption of A_{650} ($-dA_{650}/dt$) [21, 22]. The rate of change in absorbance ($v = -dA_{650}/dt$) is proportional to the change in the number of cells over time (-dCFU/dt) [21, 22]. A UV-1800 spectrophotometer (Shimadzu, Japan) was used in the work. The measurements were carried out at a wavelength of 650 nm and a temperature of 37°C. When measuring, the initial concentration of bacterial cells in the suspension was $3 \cdot 10^8$ CFU/ml ($A_{650} = 0.45$). After adding bacterial cells and the required amount of the effector solution to the reaction mixture before measuring the rate of enzymatic lysis, the background change in optical absorption was recorded for 3-4 minutes to be taken into account in the correction for background lysis without enzyme or cell sedimentation. The rate of the background change in A_{650} uptake in all experiments did not exceed the error in measuring the rate of lysis of bacterial cells by lysozyme. During measurements, lysozyme was added to a final concentration in the cuvette of 0.1 µg/mL. The rate of enzymatic cell lysis was measured for 5 minutes.

The adsorption of lysozyme on bacterial cells was measured according to the method previously presented in the literature [13, 14]. A suspension of *E. coli* cells was prepared in a buffer solution with a final concentration of $3 \cdot 10^8$ CFU/mL (optical absorption A_{650} = 0.45). A solution of lysozyme (to final concentrations of 50–250 µg/mL) and effectors (to a final concentration of 1.5 mM for glycine and 5 mM for other amino acids) were added to the suspension. The time for establishing adsorption equilibrium was 3-5 minutes at a temperature of 37 °C [13, 14], so the suspension was incubated for 7 minutes in a dry-air thermostat TSO-1/80 model 1005 (SKTB-SPU, Russia) at 37 °C on a Multi Bio RS-24

(Biosan, Latvia) with a rotation speed of 15 rpm. Then the samples were centrifuged in a Minispin centrifuge (Eppendorf, Germany) for 7 minutes at a rotation speed of 6000 rpm (2073 g). The supernatant obtained after centrifugation was used to further measure the concentration of unbound lysozyme by its activity on the auxiliary substrate *M. luteus* [13, 14]. The free enzyme concentration was calculated using a calibration curve of *M. luteus* cell lysis rate versus lysozyme concentration. Using the obtained concentrations of free lysozyme and knowing the initially added amount of lysozyme, the amounts of lysozyme bound by cells were calculated and sorption isotherms were constructed.

Each experimental value was obtained from the measurement results of at least 3 independent experiments. Experimental errors were calculated using the Student's t-distribution for the confidence interval P = 0.95.

3 Results and discussion

As was shown in previous works, the dependences of the rate of *E. coli* cell lysis by lysozyme on the concentration of free amino acids (up to 5 mM) belong to two main types: 1) dependence with a maximum activity at a 1.0–1.5 mM of glycine concentration, 2) monotonically increasing activity with increasing concentration of charged amino acids [13, 14]. To compare the parameters of lysozyme adsorption on bacteria and lysozyme activity, we chose concentrations of 1.5 mM for glycine and 5 mM for other amino acids. Figure 1 shows lysozyme adsorption isotherms in the absence and presence of effectors. All dependences obtained are satisfactorily described by the Langmuir adsorption isotherm equation. The values of the calculated adsorption parameters included in the Langmuir adsorption equation are presented in Table 1. The table also presents bacterial cell lysis rates (enzyme activity) in the presence and absence of effectors. As it can be seen from the table, the B_{max} values (maximum adsorption capacity) do not change within the experimental error in the presence of the selected effectors. This suggests that the added free amino acids do not change the number of available enzyme binding sites on the bacterial surface. However, amino acids at these concentrations reduce desorption constants values up to 2 times, for example, in the presence of aspartate. A simultaneous increase in the rate of cell lysis indicates an improvement in the productive adsorption of the enzyme, which precedes a successful catalytic act. This effect can be caused both by the influence on the conformation of the enzyme itself and by a change in the medium at the phase boundary near the surface of the bacterium. The effect of simultaneously enhancing the binding of lysozyme by bacteria and increasing bacteriolytic activity in the presence of free amino acids is fundamentally different from the effect of changing the parameters of enzyme adsorption and its bacteriolytic activity with a change in the ionic strength of the solution, which was previously reported in the literature for lysozyme and *E. coli*, when an increase in the proportion of adsorbed enzyme at low ionic strength by 3–5 times led to a corresponding decrease in the activity of lysozyme, which is explained by an increase in the proportion of unproductively adsorbed inactive enzyme [13]. According to the previous data, an increase in the productive sorption of lysozyme was observed on *Lactobacillus plantarum* cells at a pH value corresponding to the maximum activity; however, on lactobacilli, an increase in productive sorption was not associated with a change in the desorption constant, but was accompanied by a change only in the maximum adsorption capacity of the cell surface [14].

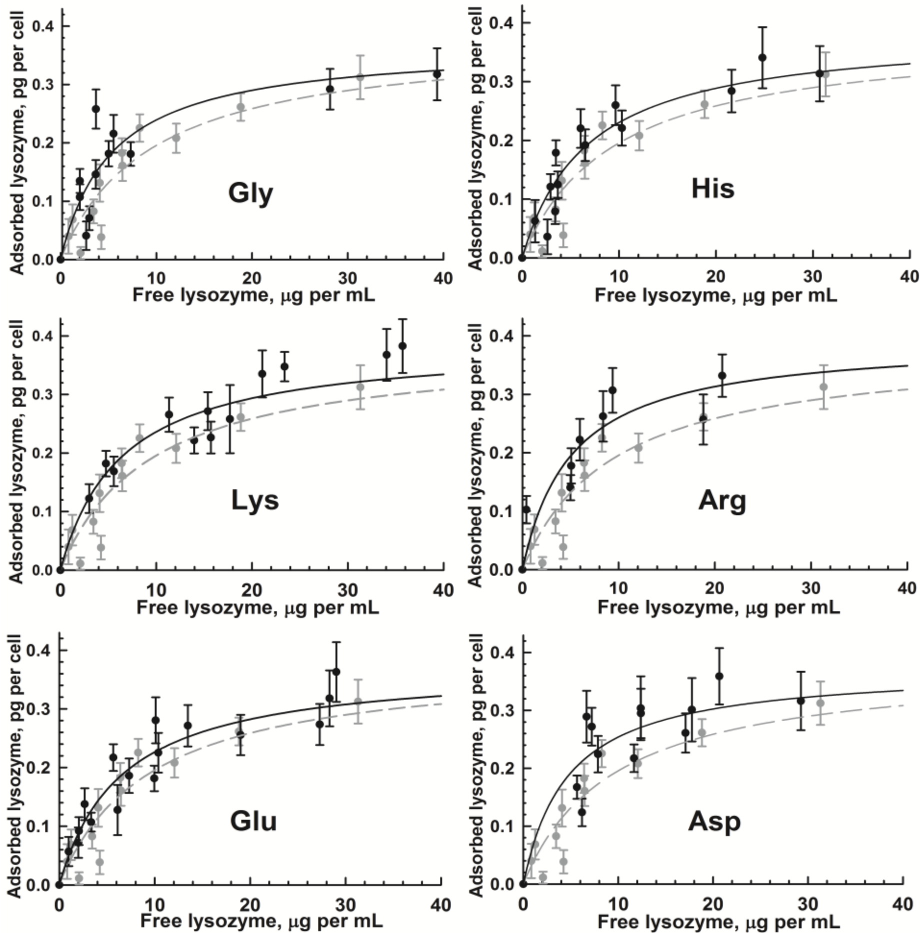

Fig. 1. Adsorption isotherms of lysozyme on *E. coli* bacterial cells in the absence of an effector (dashed gray curve and gray experimental points) and in the presence of an effector (solid black curve and black experimental points). The concentration of glycine is 1.5 mM, the concentration of other free amino acids is 5 mM.

Table 1. Relative rates of enzymatic lysis of live E. coli cells and adsorption parameters of lysozyme on the surface of bacteria in the presence of glycine and charged amino acids.

Added effector	**-**	**Gly**	**His**	**Lys**	**Arg**	**Glu**	**Asp**
Relative rate of *E. coli* cell lysis by lysozyme in the presence of an effector (increase in rate compared to rate without effector)	1	1.8 ±0.2	1.5 ±0.2	1.4 ±0.2	1.9 ±0.2	1.5 ±0.2	1.9 ±0.3
Desorption constant of the enzyme on the surface of bacteria (K_d), 10^{-7} M	6.6 ±1.3	3.8 ±0.9	4.8 ±0.8	4.5 ±1.0	3.6 ±0.8	4.5 ±0.8	3.3 ±0.8
Maximum adsorption capacity (B_{max}), pg per bacterial cell	0.38 ±0.03	0.36 ±0.03	0.38 ±0.03	0.39 ±0.02	0.39 ±0.02	0.37 ±0.02	0.37 ±0.02
The ratio of the desorption constant in the absence of an effector to the desorption constant in the presence of an effector ($K_d^{without\ effector}/K_d^{with\ effector}$)	1	1.8 ±0.8	1.4 ±0.5	1.5 ±0.6	1.9 ±0.8	1.5 ±0.8	2.0 ±0.9

The concentration of glycine is 1.5 mM, the concentration of other free amino acids is 5 mM.

4 Conclusion

Thus, the set of obtained experimental data suggests that the facilitation of enzyme sorption on cells by increasing productive sorption makes a significant contribution to increasing the efficiency of cell lysis by lysozyme in the presence of charged amino acids and glycine. The study of the effect of additional low molecular weight substances on cell lysis and sorption of lysozyme on the substrate (bacteria) is of great practical importance in the development of antibacterial drugs, hygienic and antiseptic agents based on lysozyme.

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