ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 9, pp. 989-993. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © V. I. Surovtsev, V. M. Borzenkov, T. V. Fedorov, O. I. Smotrov, 2007, published in Biokhimiya, 2007, Vol. 72, No. 9, pp. 1214-1219.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-144, August 19, 2007.

Ionogenic Groups in the Active Site of Lysostaphin. Kinetic and Thermodynamic Data Compared with X-Ray Crystallographic Data

V. I. Surovtsev, V. M. Borzenkov, T. V. Fedorov*, and O. I. Smotrov

State Research Center of Applied Microbiology and Biotechnology, Russian Federation Ministry of Health, 142279 Obolensk, Moscow Region, Russia; fax: (496) 736-010; E-mail: tfedorov@yandex.ru

Received May 15, 2007

Abstract—The active site of lysostaphin is shown to contain a residue of glutamic acid. As judged by a p*K* value of 9.2 (with pentaglycine bridges in peptidoglycan of staphylococci as a substrate), another ionogenic residue could be the ε-amino group of a lysine. However, the pH value near a negatively charged cell is supposed to be strongly shifted to acidity as compared to the pH of the solution volume. This shifts the enzyme pH dependence curve in solution to alkalinity. Therefore, the other group might be histidine, which is consistent with the X-ray crystallographic data. A similar shift is likely to occur for lysozyme in the case of *Micrococcus lysodeikticus* cells. Determination of p*K* of ionogenic groups in the active sites of alkaline enzymes responsible for lysis of negatively charged bacterial cells gives their apparent values because the "pericellular" and "voluminous" values of pH are not coincident.

DOI: 10.1134/S0006297907090106

Key words: lysostaphin, lysozyme, staphylococcus, active site, enzymatic catalysis

Lysostaphin (EC 3.4.99.17) is a Zn^{2+}-containing glycylglycine peptidase from the culture fluid of *Staphylococcus simulans* biovar *staphylolyticus*, which can hydrolyze pentaglycine bridges in the cell wall of various *Staphylococcus* strains, including those resistant to antibiotics. Moreover, this enzyme is a metalloelastase [1] and also catalyzes transpeptidation of glycine peptides. Unlike the overwhelming majority of cases, an imino- and not acyl-enzyme is produced as an intermediate compound, i.e., the amine moiety of the glycine peptide substrate is transferred to the carboxyl glycine acceptor [2]:

$$(Gly)_3 + E \leftrightarrow (Gly)_2 + E\text{-}NH\text{-}Gly,$$

$$RCONH\text{-}(Gly)_2 + E\text{-}NH\text{-}Gly \leftrightarrow RCONH\text{-}(Gly)_3 + E.$$

Lysostaphin-type enzymes belong to so-called **LAS**-enzymes (**L**ysostaphin, D-**A**la-D-Ala carboxypeptidase, **S**onic hedgehog enzyme). X-Ray crystallographic data have been recently obtained for lysostaphin. It is supposed that glutamic acid and histidine residues function in the active site of lysostaphin and mediate general acid/general basic catalysis [3].

The purpose of this work was to identify ionogenic groups in the active site of lysostaphin by kinetic and thermodynamic methods and compare the findings with the X-ray crystallographic data.

MATERIALS AND METHODS

Electrophoretically pure lysostaphin was isolated from the culture fluid of *S. simulans* biovar *staphylolyticus*, the strain GNTs PM No. 1030 (State Research Center of Applied Microbiology and Biotechnology) [4], and its activity was determined as described in [5]. The specific activity of the enzyme was 1520 U/mg protein. Kinetics of the lysostaphin-induced bacteriolysis of living *S. aureus* FDA209P cells were studied in the pH range of 4.5-10.3 using acetate buffer at pH range from 4.5 to 6.8, phosphate buffer at pH from 7.0 to 8.3, and acetate-ammonium buffer in the pH range of 8.8-10.3. The ionic strength was adjusted to 0.05 with NaCl.

The kinetic experiment was performed as follows: suspension of bacterial cells in 3.3 ml of buffer solution was maintained for 5 min at the temperatures under study in a thermostatic cuvette of an Ultrospec 2000 spectrophotometer (Amersham-Pharmacia, Sweden). Lyso-

* To whom correspondence should be addressed.

staphin solution (0.2 ml) was introduced into the cuvette and mixed for 3 sec. In all cases, kinetic experiments were repeated thrice. The enzyme-caused decrease in the optical density of the suspension was no more than 20%. The initial rate of the lysis was found from the slope angle of the tangent to the kinetic curve at the initial time period and expressed as the optical density (A_{525}) change per minute. The kinetic experiments were performed in the region, which followed the Lambert–Beer law (for $A_{525} \leq 0.5$) [4]. The spontaneous lysis of the *S. aureus* cells, which was negligibly low at all pH values, was taken into account.

The heat of ionization of the amino acid residue in the active site of lysostaphin was determined at 295 and 310 K from the van't Hoff equation:

$$\Delta H_{ion} = 2.303\ R\Delta pK_a T_1 T_2/(T_2 - T_1) =$$

$$= 116.7\ \Delta pK_a, \qquad (1)$$

where ΔH_{ion} (kJ/mol) is the heat of ionization of the group, R is the universal gas constant, T_2 and T_1 are absolute temperatures during the experiments, ΔpK_a is the difference of negative logarithms of the ionization constants of the group at temperatures T_2 and T_1.

Values of pK were determined from tangents to the pH-dependence curve branches with the angle coefficients +1.0 and –1.0. Projections of the intersection points of the tangents onto the abscissa axis give values of $pK_{a(app)}$ and $pK_{b(app)}$ [6]. Values of pK at 22 and 37°C (Scheme) were determined from the pH dependence of $V_{m(app)}/(K_{m(app)}[E]_0)$ by plotting experimental points and comparing deviations of the theoretical curves from these points at different pH values. The curves were described by the equation [2, 6]:

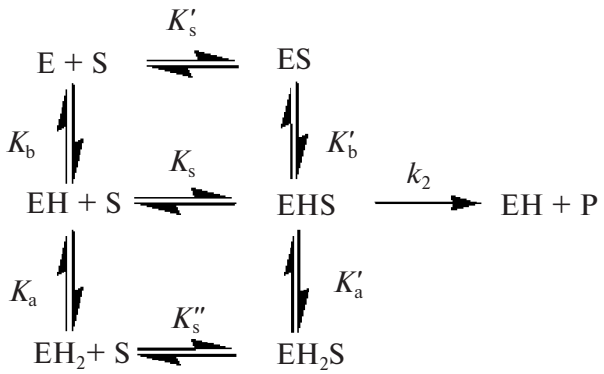
$$V_{m(app)}/K_{m(app)}[E]_0 = (k_2/K_s)(1 + [H^+]/K_a)^{-1}, \qquad (2)$$

where

$$\frac{k_2}{K_s} = \frac{\left(\dfrac{V_{m(app)} \cdot 10^{-5}}{K_{m(app)}[E]_0}\right)_{opt}}{1 + \dfrac{[H^+]_{opt}}{K_{a(app)}} + \dfrac{K_{b(app)}}{[H^+]_{opt}}}.$$

RESULTS

The pK values of ionogenic groups can be found from the pH dependence of $\log(k_{cat}/K_m)$ [6]. The concentration of the substrate from the cell wall (pentaglycine bridges) immobilized on the staphylococcus cell is unknown, and experimentally the rate of cell lysis and not the rate of substrate hydrolysis can be determined. At the principal condition of $[E]_0 << [S]_0$, this rate under the influence of lysostaphin on the *S. aureus* cells and lysozyme on the *M. lysodeikticus* is described by an equation which appears similarly to the Michaelis–Menten equation [4, 7]. Linearization of this equation gives values of $V_{m(app)}$, $K_{m(app)}$, and also $k_{cat(app)}$ equal to $V_{m(app)}/[E]_0$, where $[E]_0$ is the enzyme concentration. Data on the cell lysis can be used for determination of ionogenic groups in the active site of the enzyme capable of hydrolyzing bonds in the cell wall, if $k_{cat(app)}$ and $K_{m(app)}$ are equal or proportional to molecular constants k_{cat} and K_m. Then profiles of pH dependence of $\log(k_{cat}/K_m)$ and $\log(k_2/K_S)$ [6] are identical to $\log(k_{cat(app)}/K_{m(app)})$, and p$K$ values of ionogenic groups in the active site can be found. The proportionality of the catalytic constant $k_{cat(app)}$ and k_2 is a consequence of the coincident appearance of this rate equation with the Michaelis–Menten equation: the definite strain cell undergoes lysis on hydrolysis of a critical constant number of bonds, and as a result, $k_{cat(app)}$ is proportional to k_2. It was shown for lysozyme that $k_{cat(app)} = (n/m) \cdot k_2$, where n/m equals 10-15, n is the number of reactive bonds on the cell surface, m is the number of bonds hydrolysis of which results in the cell lysis. Consequently, the *M. lysodeikticus* cell is destroyed when 6-10% of the general number of these bonds on the surface are hydrolyzed [7]. The equality of $K_{m(app)}$ and K_S is discussed further (Eqs. (3)-(5) of the enzymatic hydrolysis of pentaglycine bridges).



Scheme of a two-stage reaction controlled by two ionogenic groups of the active site

Instead of $\log(k_{cat(app)}/K_{m(app)})$, we used the pH dependence of $\log(V_{m(app)}/(K_{m(app)}[E]_0))$. From the double reciprocal plot, $V_{m(app)}$ and $K_{m(app)}$ values were found (Fig. 1). The use of the $V_{m(app)}/K_{m(app)}$ ratio leads to the transition from relative rates and concentrations expressed in $\Delta A_{525}\ min^{-1}$ and A_{525} to an absolute unit with the dimension of $[min^{-1}]$. Respectively, if the enzyme concentration is expressed in mol/liter, the $V_{m(app)}/(K_{m(app)}[E]_0)$, similarly to k_{cat}/K_m and k_2/K_S, has the dimension of the second order rate constant $[M^{-1} \cdot min^{-1}]$.

Data on the kinetics of the bacteriolytic effect of lysostaphin at different pH values and 22°C are presented in the table and Fig. 2. In the pH range of 4.5-9.3, the $K_{m(app)}$ value is constant in the limits of experimental error and equal (0.5 ± 0.07) A_{525}. The dependence of $\log(V_{m(app)}/(K_{m(app)}[E]_0))$ on pH is symmetric and bell-shaped with the optimum at 7.55 (Fig. 2).

Point out to a possible cause of constancy of the $K_{m(app)}$ over a wide range of pH. The *S. aureus* cell surface has a strong negative charge at pH higher than 4.0 [8]. The lysostaphin isoelectric point is ~9.5 [9], and the electrostatic interaction of the enzyme with the cell wall has

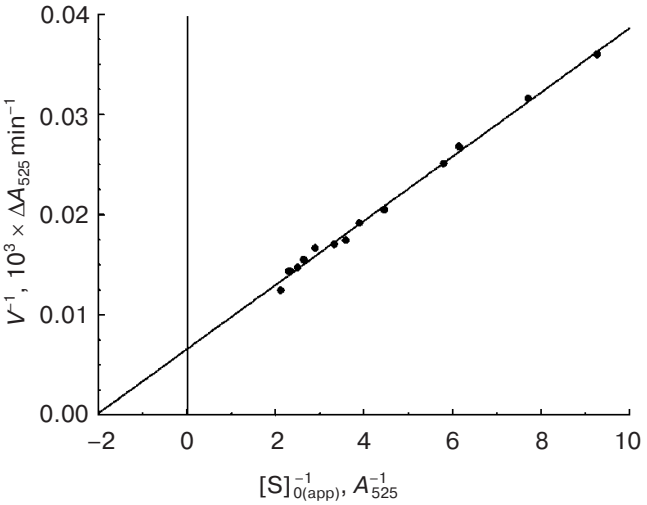


Fig. 1. Double reciprocal plot for lysostaphin-induced lysis of staphylococci (pH 4.5, 22°C).

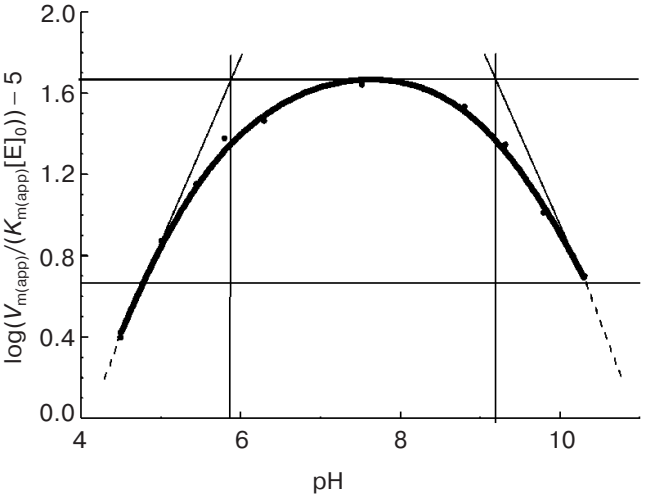


Fig. 2. The pH dependence of the logarithm of the second order rate constant of lysostaphin-induced lysis of staphylococci.

Effect of pH on kinetic parameters of lysostaphin-induced lysis of *S. aureus* cells

pH	$K_{m(app)}$, A_{525}	$\frac{V_{m(app)}}{K_{m(app)}[E]_0}\times 10^{-5}$, $M^{-1}\cdot min^{-1}$	$\log\frac{V_{m(app)}}{K_{m(app)}[E]_0} - 5$
$t = 22°C$, $[E]_0 = 1.4 \cdot 10^{-8}$ M			
4.50	0.48 ± 0.05	2.53	0.40
5.00	0.50 ± 0.06	7.43	0.87
5.45	0.48 ± 0.05	14.29	1.15
5.80	0.45 ± 0.04	23.80	1.38
6.30	0.57 ± 0.07	28.82	1.46
6.80	0.50 ± 0.05	40.0	1.60
7.55	0.47 ± 0.06	43.53	1.64
8.30	0.49 ± 0.05	42.11	1.62
8.80	0.56 ± 0.04	34.08	1.53
9.33	0.51 ± 0.05	22.26	1.35
9.80	0.83 ± 0.10	10.33	1.01
10.30	1.7 ± 0.20	5.00	0.70
$t = 37°C$, $[E]_0 = 0.36 \cdot 10^{-8}$ M			
5.0	0.64 ± 0.08	85.94	1.93
5.55	0.70 ± 0.10	148.98	2.17
6.0	0.60 ± 0.08	226.86	2.35
6.5	0.61 ± 0.08	311.50	2.50
7.0	0.62 ± 0.07	414.71	2.62
7.55	0.68 ± 0.10	485.30	2.68

to be virtually the same over the pH range 4.5-9.0. If electrostatic forces considerably contribute to the adsorption interaction of lysostaphin with the cell wall, the sorption constant and, possibly, $K_{m(app)}$ are expected to be independent of pH. At pH ≥ 9.5 the binding becomes worse, and $K_{m(app)}$ significantly increases.

The results are in agreement with the Scheme of an enzymatic reaction controlled by two ionogenic groups in the active site with K_a and K_b constants of acid dissociation in the free enzyme and K_a' and K_b' in the Michaelis complex.

Of the three forms of the enzyme presented in the Scheme, only EH can give a product. In the case of enzymatic hydrolysis of cell wall, expressions for $k_{cat(app)}$, $K_{m(app)}$, and the second order rate constant are described as Eqs. (3)-(5):

$$k_{cat(app)} = \frac{k_2}{1 + \frac{[H^+]}{K_a'} + \frac{K_b'}{[H^+]}}, \quad (3)$$

$$K_{m(app)} = K_s \frac{1 + \frac{[H^+]}{K_a} + \frac{K_b}{[H^+]}}{1 + \frac{[H^+]}{K'_a} + \frac{K'_b}{[H^+]}}, \quad (4)$$

$$\frac{k_{cat(app)}}{K_{m(app)}} = \frac{k_2}{K_s} \frac{1}{1 + \frac{[H^+]}{K_a} + \frac{K_b}{[H^+]}}. \quad (5)$$

Because the $K_{m(app)}$ value is virtually independent of pH, the dissociation constants of ionogenic groups of the free enzyme and the enzyme–substrate complex are equal ($K_a = K'_a$; $K_b = K'_b$) and $K_{m(app)} = K_S$ [6].

Thus, it is supposed that the active site of lysostaphin in the free state possesses two ionogenic groups with pK 5.9 and 9.2 which control the enzyme activity toward the staphylococcus cells and are determined from the plot of Fig. 2.

Values of pK of protein groups can be used for their identification if there are additional data, e.g. on the heat of ionization of the group or on chemical modification. These values themselves do not, as a rule, coincide with the pK of free amino acids, because they are under the influence of electrostatic and inductive effects of the adjacent groups. Therefore, the most probable values of pK ranges are indicated. Thus, these values are in the range 2.8-4.9 for β- and γ-carboxyl groups, 5.5-7.4 for the imidazole group, and 9.2-10.4 for the ε-amino group or phenol group of tyrosine residue. The probability of finding in a protein of carboxyl group with pK ~ 6 or phenol group of tyrosine with pK ~ 8 is extremely low [10]. Such a carboxyl group could exist in the case of a strictly hydrophobic environment. Data on the heat of ionization are especially significant when the active site contains a residue of aspartic or glutamic acid, because its value (~6 kJ/mol) is much lower than other heats of ionization, e.g. of imidazole of histidine residue (~29 kJ/mol) [10]. The corrected values of pK of the active site ionogenic group with pK_a of 5.9 at the temperatures of 22 and 37°C (table and Fig. 3) (considering deviations of the theoretical curve from the experimental points) are, respectively, 5.85 and 5.80, and differ by 0.05 pH unit. Consequently, using the van't Hoff equation (1) we obtain ΔH_{ion} ~ 6 kJ/mol that indicates the presence of glutamic or aspartic acid residue in the active site of lysostaphin.

The presence of a glutamic acid residue is supported by the following data. The supposed catalytic groups of the active sites of Zn^{2+}-containing proteases are presented in review [11]. Glutamic acid was present in the active site in 30 of 31 enzymes isolated from gram-positive and gram-negative microorganisms. Lysostaphin was shown to possess elastase activity [1]. The glutamic acid residue in the sequence of Ala-Ala-Thr-His-Glu is essential for enzymatic activity of metalloelastases. Lysostaphin has only one such sequence starting with Ala1 [9]. If in the case of lysostaphin the same groups of the active site are involved in catalysis on cleavage of elastin and pentaglycine bridges, Glu5 seems to be the residue involved in catalysis of hydrolysis of both substrates. Note that X-ray crystallography does not reveal a strictly hydrophobic region in the N-terminal part of lysostaphin [3].

The presence of a glutamic acid residue in the active site of lysostaphin is consistent with X-ray crystallographic data [3]. The value pK_b ~ 9.2 does not agree with these data, but corresponds to an ε-amino group of lysine or phenol group of tyrosine. Tyrosine is not an ionogenic group (the pK_a of free tyrosine for generation of phenolate ion is 10.1), and, as a rule, is a component of the sub-

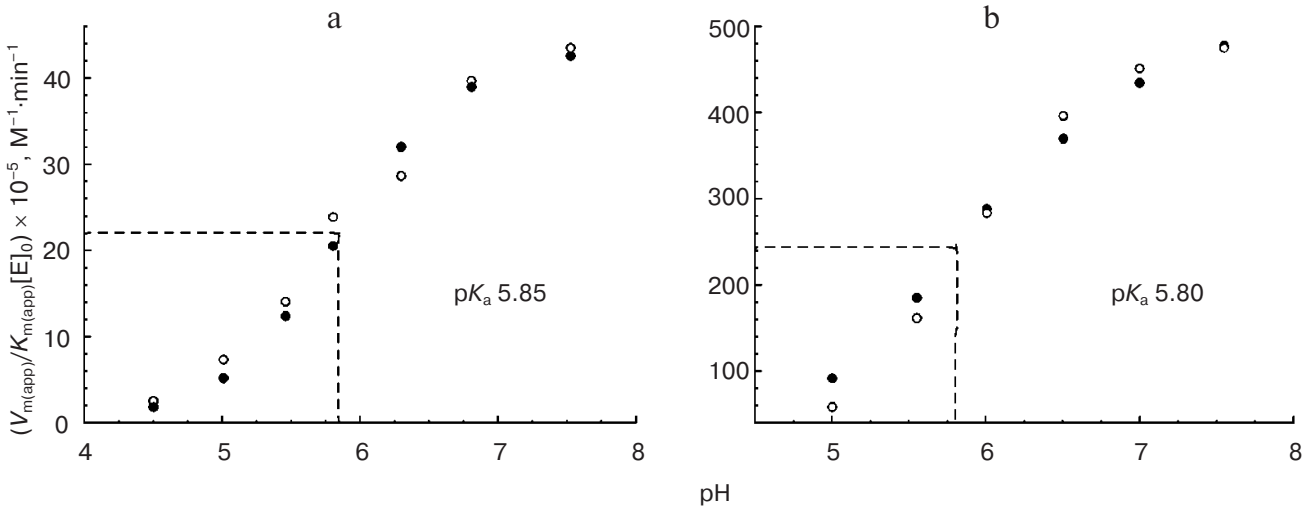


Fig. 3. Determination of p$K_{a(app)}$ at 22°C (a) and 37°C (b) by formula (4). Light circles are experimental points; dark circles are points of the theoretical curve.

strate-binding site in "tyrosine" enzymes. On modification of N-acetylhexaglycine, which is a soluble substrate of lysostaphin, with trinitrobenzolsulfonic acid (a specific reagent for amino group) the activity is completely retained [12]. This seems to exclude the presence of a lysine residue in the active site of the enzyme.

The presence of glutamic acid residue with pK_a 5.85 in a region with moderate hydrophobicity and the absence in the active site of the most probable residues with pK_a of 9.2 can be explained by the suggestion that strong negative charge of the cell influences pH value during the enzymatic reaction. The substrate (pentaglycine bridges) is located in peptidoglycan, and the value of the "pericellular" pH has to be considerably lower than the "voluminous" pH, which is determined by a pH meter. Thus, when the microbial cell, and respectively, the substrate is not charged, the true profile of pH dependence has to be shifted to acidity, which makes the pK_a of the glutamic acid residue closer to its average value in proteins. Owing to symmetry of the pH-dependence curve, the pH value of another ionized residue is shifted to histidine. Thus, it is concluded that the ionogenic groups in the active site of lysostaphin are residues of glutamic acid (possibly Glu5) and histidine (the lysostaphin molecule has no sulfhydryl groups with pH range close to that of histidine).

The pH dependence of the lysostaphin enzymatic activity is known (from this work) only for the cell substrate (pentaglycine bridges). For comparison, another enzyme capable of lysing the cells, lysozyme, can be mentioned. The pH dependence of hydrolysis of oligosaccharide (uncharged) substrates under the influence of lysozyme has been studied [13]. The results suggest that two ionogenic groups of the active site with pK value in the range of 3.0-5.0 and 6.0-6.5 are involved in the enzymatic reaction. Similar data have been also obtained for an uncharged high-molecular-weight substrate, chitin [14]. The only exception is presented by the pK values (5.0 and 9.2) of ionogenic groups, which control the catalytic activity of lysozyme on hydrolysis of the *M. lysodeikticus* cell wall [15]. Although lysostaphin and lysozyme catalyze quite different reactions leading to cell lysis, they have some similarities as follows: both enzymes are alkaline, with high values of isoelectric points (~11.0 for lysozyme); throughout the whole range of pH values studied, the cell substrates have a strong negative charge; the K_m value for hydrolysis of the cell substrate is constant in both cases (for lysozyme it has been established in [15]), and the enzymatic hydrolysis of the cells follows an equation similar to that of Michaelis–Menten. In both cases, the enzymatic hydrolysis is described by the same scheme. Thus, the pH dependence curve of lysozyme in the case of *M. lysodeikticus* is shifted to alkalinity as compared to uncharged substrates, possibly because of the lack of coincidence of the "pericellular" and "voluminous" pH values. Therefore, to obtain "true" values of pK for enzymes cleaving the cells, it is necessary to take uncharged substrates, while in the case of charged cell substrates apparent values of pK are obtained for ionogenic groups of the active site.

REFERENCES

1. Park, P. W., Senior, R. M., Griffin, G. L., Broekelmann, T. J., Mudd, M. S., and Mecham, R. P. (1995) *Int. J. Biochem. Cell Biol.*, **27**, 139-146.
2. Sloan, G. L., Smith, E. C., and Lankaster, J. H. (1977) *Biochem. J.*, **167**, 293-296.
3. Bochtler, M., Odintsov, S. G., Marcyjaniak, M., and Sabala, I. (2006) *Protein Sci.*, **13**, 854-861.
4. Fedorov, T. V., Surovtsev, V. I., Pletnev, V. Z., Borozdina, M. A., and Gusev, V. V. (2003) *Biochemistry (Moscow)*, **68**, 50-53.
5. Schindler, C. A., and Schuchardt, V. T. (1965) *Biochim. Biophys. Acta*, **97**, 242-250.
6. Berezin, I. V., and Klesov, A. A. (1976) *Practical Course of Chemical and Enzymatic Kinetics* [in Russian], MGU Publishers, Moscow, pp. 219-221.
7. Rabinovich, M. L., Klesov, A. A., and Berezin, I. V. (1976) *Bioorg. Khim.*, **2**, 689-699.
8. Akatov, A. K., and Zueva, V. S. (1983) *Staphylococci* [in Russian], Meditsina, Moscow, pp. 10-12.
9. Thumm, G., and Gotz, F. (1997) *Mol. Microbiol.*, **23**, 1251-1265.
10. Westley, J. (1972) *Enzymatic Catalysis* [Russian translation], Mir, Moscow, pp. 213-214.
11. Hase, C. C., and Finkelstein, R. A. (1993) *Microbiol. Rev.*, **3**, 823-837.
12. Kline, S. A., de la Harpe, J., and Blackburn, P. (1993) *Analyt. Biochem.*, **217**, 329-331.
13. Maksimov, V. I. (1973) *Usp. Khim.*, **42**, 2073-2094.
14. Stryer, L. (1984) *Biochemistry* [Russian translation], Mir, Moscow, p. 143.
15. Klesov, A. A., Rabinovich, M. L., and Berezin, I. V. (1976) *Bioorg. Khim.*, **2**, 795-801.