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Michaelis–Menten Kinetics for Determining Enzymatic Activity of Lysostaphin

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Abstract—The rate of lysostaphin-catalyzed lysis of staphylococci follows the Michaelis–Menten equation at $[E]_0 << [S]_0$, i.e., the activity of the enzyme is proportional to its concentration. This equation is proposed for determining the specific activity of lysostaphin. The apparent activation energy of hydrolysis of pentaglycine bridges in *Staphylococcus* peptidoglycan is 77.9 kJ/mol.

Key words: lysostaphin, lysis, immobilized substrate, Michaelis–Menten equation

Bacteriolytic enzymes are clearly interesting for both theory and practice. Lysostaphin (EC 3.4.99.17) is a Zn^{2+}-containing glycylglycine endopeptidase from culture liquid of *Staphylococcus simulans* biovar *staphylolyticus* that hydrolyzes pentaglycine bridges in the cell wall of various *Staphylococcus* strains, including those resistant to antibiotics. The enzyme is of the endopeptidase family, for which amino acid residues in the active site and the catalytic mechanism are unknown [1].

To determine the activity of lysostaphin, two groups of approaches using immobilized substrates have been proposed. Colorimetric approaches are based on determination of products resulting from lysis of target cells during a certain time interval, and turbidimetric methods are based on degree of lysis of the enzyme-sensitive cells [2]. By turbidimetry, the activity can be determined uninterruptedly using the unique feature of lysostaphin. However, approaches of both groups fail to reveal proportionality between the rate of lysis and the enzyme concentration.

Kline et al. [1] reported the colorimetric evaluation of activity of lysostaphin with N-acetylhexaglycine as a soluble substrate. The method reveals a linear dependence between the enzymatic activity and absorbance of a trinitrophenylated product of hydrolysis at 405 nm. However, different mechanisms of the enzymatic effect of lysozyme were earlier shown on using either the soluble substrate or *Micrococcus lysodeicticus* cells [3]. Thus, to study the catalytic mechanism of the bacteriolytic enzyme both soluble and insoluble (immobilized on target cells) substrates should be used.

The initial rate of lysis by lysozyme of *M. lysodeicticus* cells was shown [4] to be proportional to the initial concentration of the enzyme and to be hyperbolic relative to the initial concentration of the substrate, i.e., to follow the Michaelis–Menten equation:

$$v_0 = \frac{k_{cat}\,[E]_0[S]_0}{K_m + [S]_0}$$

(the main condition is $[E]_0 << [S]_0$), where k_{cat} is the turnover number of the enzyme at saturating concentration of the substrate, $[E]_0$ and $[S]_0$ are initial concentrations of the enzyme and substrate, and K_m is the Michaelis constant, which is numerically equal to the concentration of the substrate at the half-maximum rate. The latter is $V_{max} = k_{cat}[E]_0$. This equation unambiguously relates the maximal rate of the reaction with the initial concentration of enzyme. Therefore, V_{max} can be also used for determination of the specific activity of the enzyme in addition to the initial rate of the reaction. In the case of immobilized substrates, effective (apparent) values of kinetic constants are obtained.

Thus, if in the case of cell-immobilized substrate the initial and maximal rates of lysis are proportional to concentration of the enzyme, the correct biochemical determination of activity of the enzyme should be based on the Michaelis–Menten equation.

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The purpose of this work was to apply the Michaelis–Menten equation for determination of the specific activity of lysostaphin under conditions of the maximal reaction rate with staphylococci as target cells.

MATERIALS AND METHODS

Lysostaphin was isolated from culture liquid of *S. simulans* biovar *staphylolyticus* (State Research Center of Applied Microbiology) No. 1030. Purification of the enzyme was described earlier [5]. Activity of the preparation at different purification stages was from 300 to 1500 units per mg protein. Protein was determined by the method of Bradford using electrophoretically pure lysostaphin ($A^{1\%}_{280}$ 24.7 [5]) as the standard. According to [6], the activity unit was taken as the quantity of enzyme that within 10 min decreased twofold the initial optical density (0.250 at 620 nm) of *S. aureus* cell suspension in a cuvette with optical pathlength of 1 cm in 6 ml of 0.01 M phosphate buffer (pH 7.5) containing 0.15 M NaCl at 37°C.

For kinetic experiments, the unit of enzyme activity was taken as the quantity of the enzyme in 3-ml volume in a 1-cm pathlength cuvette at 22°C that decreased the initial absorption of *S. aureus* culture (strain ATCC 538P, $A_{525(\text{initial})} \leq 0.500$) in the same buffer by 0.01 per 1 min at 525 nm.

Cultures of *S. aureus* were grown on enzymatic fish and casein hydrolyzates. In experiments, live cells and acetone powder fraction suspension not precipitated in 30 min were used. Culture supplemented with the dye Procion Blue F3GA (0.1 mg/ml) resulting in formation of large clusters of cells was also used [7].

The value of $V_{\max(\text{app})}$ was determined from the Lineweaver–Burk and Eadie–Hofstee equations [8]. The degree of lysis in the kinetic curves was not higher than 20% of the initial absorption. The value of $V_{\max(\text{app})}$ was used for determination of specific activity of lysostaphin (units per mg protein) and the apparent activation energy which was evaluated from the Arrhenius plot $\ln V_{\max(\text{app})} = f(1/T)$.

RESULTS

Bacteriolytic enzymes perform catalysis under heterogeneous conditions. Because the concentration of the cell-immobilized substrate is uncertain, the reaction rate is evaluated by degree of lysis of target cells in a definite time interval. Cell lysis seems to require that a certain crucial number of bonds are cleaved, and its degree is proportional to the decrease in concentration of the substrate [4]. The change in absorption of the cell suspension should follow the Lambert–Beer law, because only in this case the rate of lysis in optical units per 1 min will be proportional to decrease in the concentration of the cells and, consequently, in the concentration of the true substrate (pentaglycine bridges). In the case of suspensions of staphylococci, these conditions were met at the absorption of about 0.5 and wavelength of 525 nm.

Relative rate of lysostaphin-catalyzed lysis of target cells under various culture growth parameters

Growth phases of *S. aureus* culture	Relative rate of enzymatic splitting of target cells
Linear	6
Stationary	3
Stationary + + Procion Blue F3GA	2
Type of nutritional medium: enzymatic fish hydrolyzate	4
enzymatic casein hydrolyzate	1

The decrease in concentration of the immobilized substrate in the course of reaction catalyzed by a bacteriolytic enzyme is determined by lysis of cells with varied surface features. We have shown that for *S. aureus* culture the degree of enzymatic lysis was different for target cells of the same strain grown under different conditions (table).

According to the literature, concurrently with pentaglycine bridges there are bridges that include Ser residues that are responsible for resistance of such bridges to lysostaphin. The Ser/Gly ratio varies in *Staphylococcus* strains, and this results in different rates of lysis [9]. Based on these data, it is reasonable to standardize target cells using suspension of acetone powder of a particular strain, e.g., of *S. aureus* FDA309P specified by low virulence and inability to cluster during growth.

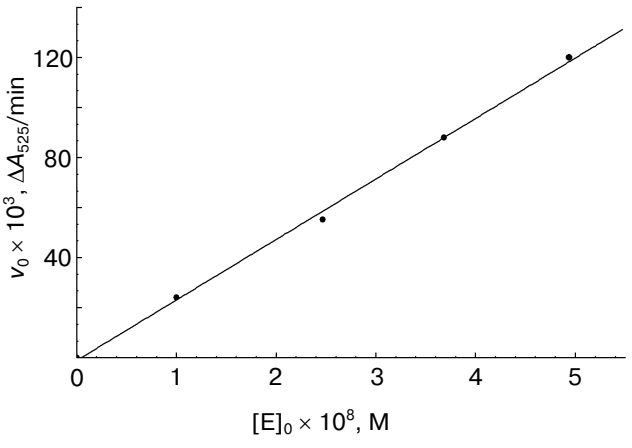

Fig. 1. Dependence of the initial rate of lysis of living *Staphylococcus* cells in stationary growth phase on the initial concentration of lysostaphin.

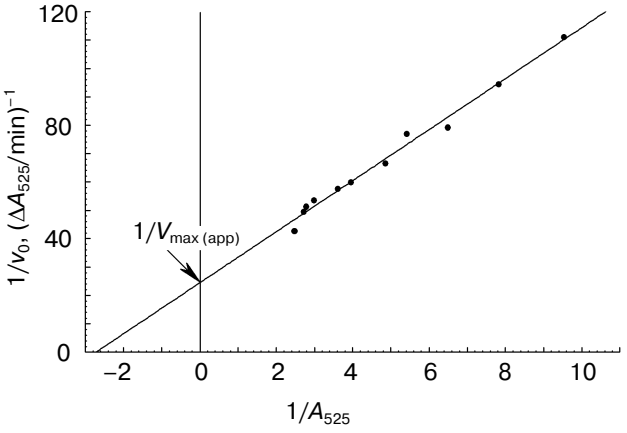

Fig. 2. Lineweaver–Burk plot for living *Staphylococcus* cells.

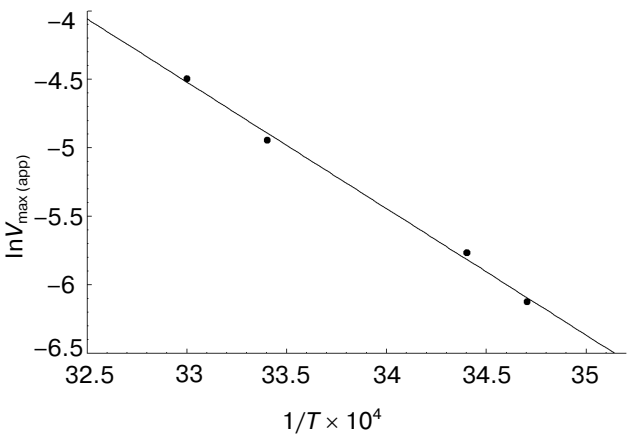

Fig. 4. Arrhenius plot for lysis of acetone powder suspension from *Staphylococcus* cells. $E_{(app)} = -R\tan\alpha$; $E_{(app)}$ is an apparent activation energy of enzymatic reaction, R is a universal gas constant.

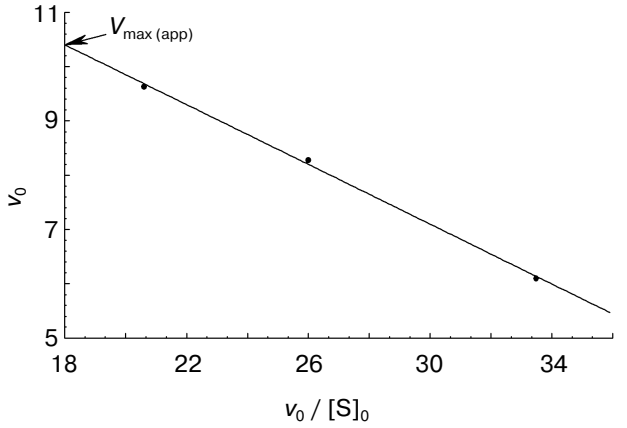

Fig. 3. Eadie–Hofstee plot for acetone powder from *Staphylococcus* cells (the dependence of $10^3 \cdot \Delta A_{525} \cdot \text{min}^{-1}$ on $10^3 \cdot \Delta A_{525} \cdot \text{min}^{-1} / \Delta A_{525}$). Each point on the plot is the mean of at least three determinations.

Figure 1 shows the linear dependence between the concentration of lysostaphin and the rate of lysis of target cell suspension, and this suggests that it follows the Michaelis–Menten equation. The value of $V_{max(app)}$ obtained from linear anamorphoses determines the concentration of the native enzyme in the sample and can be used to determine the specific activity of lysostaphin (Figs. 2 and 3). The latter for electrophoretically pure preparation of lysostaphin was 10,130 and 2570 units/mg for suspensions of *S. aureus* living cells and acetone powder, respectively. Moreover, values of $V_{max(app)}$ at different temperatures allow us to determine the apparent activation energy of the enzymatic process from the Arrhenius plot (Fig. 4). The activation energy of hydrolysis of pentaglycine bridges of target cells by lysostaphin is 77.9 kJ/mol, which is rather a high value for enzymatic reactions [10].

Using of plots of linear anamorphoses (Figs. 2 and 3) for calibration simplifies the determination of the specific activity of lysostaphin if the initial rates of lysis of target cells are obtained at constant concentration of the substrate. For samples with different enzyme content, the ratio of the initial rates is equal to the ratio of the enzyme concentrations (Fig. 1). It follows from the Michaelis–Menten equation that at the constant concentration of the substrate $v_1/v_2 = V_{max\ 1}/V_{max\ 2}$ and the specific activity is expressed in V_{max} units per 1 mg protein.

Thus, the Michaelis–Menten equation can be used to determine the specific activity of lysostaphin in the presence of the cell-immobilized substrate.

REFERENCES

1. Kline, S. A., de la Harp, J., and Blackburn, P. A. (1994) *Analyt. Biochem.*, **217**, 329-331.
2. Zhow, R., Chen, S., and Rescei, P. (1988) *Analyt. Biochem.*, **171**, 141-144.
3. Saint-Blancard, J., and Jolles, P. (1972) *Biochimie*, **54**, 7-15.
4. Berezin, I. V., Klesov, A. A., and Rabinovich, M. L. (1976) *Bioorg. Khim.*, **2**, 681-688.
5. Fedorov, T. V., Surovtsev, V. I., Pletnev, V. Z., Borozdina, M. A., and Gusev, V. V. (2003) *Biochemistry (Moscow)*, **68**, 50-53.
6. Schindler, C. A., and Schuchardt, V. T. (1965) *Biochim. Biophys. Acta*, **97**, 242-250.
7. Sugai, M., Ooku, K., Takata, T., Miyake, Y., and Suginaka, H. (1990) *FEMS Microbiol. Lett.*, **67**, 175-178.
8. Cornish-Bowden, E. (1979) *Principles of Enzyme Kinetics* [Russian translation], Mir, Moscow, p. 47.
9. Thumm, G., and Gotz, F. (1997) *Mol. Microbiol.*, **23**, 1251-1265.
10. Laidler, K. (1966) in *Kinetics of Organic Reactions* [Russian translation], Mir, Moscow, p. 291.