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Enzymes of SPZ7 Phage: Isolation and Properties

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Abstract—Bacteriophage enzyme preparations exolysin and endolysin were studied. Exolysin (a phage-associated enzyme) was obtained from tail fraction and endolysin from phage-free cytoplasmic fraction of disintegrated *Salmonella enteritidis* cells. A new method for purification of these enzymes was developed, and their molecular masses were determined. The main catalytic properties of the studied enzymes (pH optimum and specificity to bacterial substrates) were found to be similar. Both enzymes lyse *Escherichia coli* cells like chicken egg lysozyme, but more efficiently lyse *S. enteritidis* cells and cannot lyse *Micrococcus luteus*, a good substrate for chicken egg lysozyme. Similar properties of exolysin and endolysin suggest that these enzymes are structurally similar or even identical.

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Key words: bacteriophage enzyme, lysis of bacteria, bacteriolytic activity

Study of bacteriolytic phage enzymes is important for elucidation of mechanisms of phage integration into bacterial cells as well as for applied purposes, such as development of antibacterial preparations of new generation and high-selectivity diagnostic systems [1, 2]. *Salmonella enteritidis* belongs to the Enterobacteriaceae family; bacteria of this family are of specific interest because many of them are especially pathogenic for humans [3, 4]. Catalytic properties of bacteriolytic enzymes of SPZ7 phage were characterized for the first time.

In this work we developed convenient methods for study of enzymatic lysis on such "living substrates" as gram-negative microorganisms of the Enterobacteriaceae family and substantiated turbidimetric studies of bacterial lysis.

Abbreviations: BSA, bovine serum albumin; CFU, colony-forming units; DTT, dithiothreitol; FFH, fish-flour pancreatic hydrolyzate; GPDH, glyceraldehyde-3-phosphate dehydrogenase; PFU, plague-forming units.

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MATERIALS AND METHODS

The following reagents were used in this work: Tris-HCl buffer from ICN Biomedicals (USA); ovalbumin and myoglobin from horse heart and phosphoric acid from MP Biomedicals (USA); Coomassie G-250 from Ferak (Germany); NaOH and KOH from Ameresco (USA); HCl from Germed (Germany); NaCl from Merck (Germany); NAD^+, EDTA, Na_3AsO_4, $CuSO_4$, citric acid, CsCl, DL-dithiothreitol (DTT), polymyxin M, and lyophilized preparation of *M. lutheus* from Sigma Aldrich (USA); bovine serum albumin (BSA), chicken egg lysozyme, DOWEX 50WX8-400, and barium salt of DL-glyceraldehyde-3-phosphate diethyl acetate from Serva (Germany); Sephadex G-75 superfine, blue dextran 2000, and bovine pancreas ribonuclease A from GE Healthcare (Sweden); Durapore PVDF sterile filters from Millipore (USA); DEAE-Toyopearl 650M from ToyoSoda (Japan); Hottinger broth and fish-flour pancreatic hydrolyzate (FFH) produced in State Research Center for Applied Microbiology and Biotechnology (Russia); strains *E. coli* ATCC 25922 and *S. enteritidis* 60

from the collection of Tarasevich State Institute for Control of Medical Biological Preparations (Russia); SPZ7 phage from the collection of the State Research Center for Applied Microbiology and Biotechnology. Bidistilled water was always used.

For measuring optical density of solutions, an UV-1601 PC double-beam spectrophotometer from Shimadzu (Japan) was used. The optical pathway of the cuvettes was 1 cm. For chromatographic separation, an Econo system from BioRad (USA) was used. Proteins were detected by optical absorption at 280 nm and by measuring bacteriolytic activity of fractions. Preparations were centrifuged using OPN-8 (Dastan, Kirgiziya) and MiniSpin (Eppendorf, Germany) centrifuges and a Beckman L-5-50 (Beckman, USA) ultracentrifuge.

Phage titer in preparations was determined by a modified procedure of sterile spot counting according to Gracia [5]. Protein concentrations were measured via Coomassie G-250 binding [6] or using the biuret reaction [7, 8].

Enzyme activity measurements. In the course of this study we developed a convenient and fast method for estimation of activity of bacteriolytic enzymes using living bacterial cells. Phage host, living *S. enteritidis* cells, were used as the basic substrate. The studied enzymes were found to exhibit activity against living *E. coli* cells, which were used as an additional substrate. The latter was used during enzyme purification because there were no fractions active only against *S. enteritidis* and inactive against *E. coli* or vice versa.

Lysis of various living cells can differ, so it is important to maintain consistent growth conditions for maximal standardization of preparations. To estimate bacteriolytic activity of enzymes, *S. enteritidis* and *E. coli* cells were grown on FFH-agar for 14 h at 37°C. Cells were suspended in 0.9% NaCl, centrifuged for 5 min at 5000*g*, and then resuspended in 0.9% NaCl. Cell concentration was adjusted so that after 50-fold dilution the optical absorption at 650 nm was 0.5-0.6. Newly grown cells or cells once frozen with liquid nitrogen and stored not more than 1 month at −70°C were used for measurements. During experiment (up to 1 h), cell substrate preparations were stored at 5°C.

Bacteriolytic activity was assayed turbidimetrically via decreased optical absorption of cell suspension (A) at 650 nm. As found experimentally, for cell suspensions with optical absorption less than 1.0 the initial A value (A_o) linearly depends on the amount of added cells. After significant cell lysis, the residual optical absorption of preparations tends to a constant value (A_∞), which was conventionally taken as the optical absorption of "fully lysed disintegrated cells". The conclusion that $\Theta = (A_o - A)/(A_o - A_\infty)$ value allows evaluation of lysis degree was confirmed by independent experiments using counting of the residual colony-forming units (CFU) and by centrifugation on assay of activity of cytoplasmic enzyme glyceraldehyde-3-phosphate dehydrogenase (A_{GPDH}) in solution after separation of the cells. The data indicate that for each individual preparation the Θ value is uniquely determined by CFU and A_{GPDH} values.

We suppose that a proper assay of the level of bacteriolytic activity of enzymes is the initial rate of lysis, namely the measured values $\Delta\Theta/\Delta\tau$ or $-\Delta A/\Delta\tau$. For convenience of experiments, we took τ = 2-3 min. In the used buffer, A remained unchanged in the absence of the bacteriolytic enzyme. It can be confidently stated that significant nonenzymatic lysis or cell sedimentation did not occur.

For activity measurements, 0.01 M Tris-HCl, pH 8.5, was used. To promote osmotic cell disintegration after enzymatic disruption of cell wall, we used reaction medium with low ionic strength. When 0.02, 0.05, and 0.1 M NaCl was added to the above-mentioned buffer, the experimentally monitored $-\Delta A/\Delta\tau$ value decreased 1.9, 4.2, and 7.7 times, respectively. Activity was measured at pH 8.5, the optimal pH for the studied enzymes.

Activity of GPDH was measured via increased optical absorption at 340 nm, which corresponds with the maximal absorption of the reduced coenzyme form (NADH) [9]. The molar extinction coefficient for NADH was taken as 6220 $M^{-1}\cdot cm^{-1}$ [10]. Activity was measured in the following buffer: 50 mM glycine-KOH, pH 8.5, containing 2 mM EDTA, 0.5 mM DTT, and 3 mM Na_3AsO_4. Concentrations of NAD^+ and glyceraldehyde-3-phosphate were 1 and 4 mM, respectively. All measurements were performed at 37°C.

Determination of molecular mass of bacteriolytic enzymes. Molecular mass of the native enzymes was determined by gel-penetrating chromatography [11] using a 0.53 $cm^2 \times 27.5$ cm column with Sephadex G-75 superfine, the flow rate of eluent being 5.8 ml/h. Eluents were as follows: 0.02 M Tris-HCl, pH 7.5, containing 1 mM EDTA and NaCl at three concentrations (0.075, 0.15, and 0.3 M). Blue dextran 2000 (2000 kDa), BSA (67 kDa), ovalbumin (43 kDa), myoglobin (18 kDa), and ribonuclease A (13.7 kDa) were used as molecular mass markers. Chromatographic separation was performed at 20°C.

Isolation of enzymes. To isolate "endolysin", 30 ml of 18-h *S. enteritidis* culture at concentration 10^{10} CFU/ml was added to 3 liters of Hottinger broth; growth was continued with aeration for 2-3 h at 37°C to concentration 10^{10} CFU/ml. Purified phage lysate (300 ml) – suspension of phage particles in physiological solution – at concentration 10^{14} PFU (plague-forming units)/ml was added to the culture, which was then cultivated with aeration for 25 min. Then phage-infected cells were sedimented by centrifugation for 15 min at 6500*g*. The cells were resuspended in 100 ml of 0.05 M Tris-HCl, pH 7.0, containing 7.5 mg ribonuclease and cultivated for 60 min at 37°C. The cells were thus disintegrated, releasing the lytic enzyme. Then 5 mM EDTA was added, and the sus-

pension was centrifuged for 2.5 h at 27,000*g* and 5°C. The supernatant was separated from the pellet and filtered through filters with 0.45, 0.22, and 0.1 μm pores. The endolysin-containing supernatant was tested for bacteriolytic activity and the presence of free phage.

Phage preparation was used to obtain "exolysin". The phage was reproduced in *S. enteritidis* culture grown on dense FFH medium. The lysed bacterial lawn was washed with FFH broth. For each portion of crude enzyme, 100-500 ml of crude phage lysate with phage titer 10^{11}-10^{12} PFU/ml was obtained. The purified phage preparation was obtained by differential centrifugation in a CsCl gradient (2 h at 27,000*g*). Differential centrifugation was repeated 3-4 times. Phage in the preparations was disintegrated by multiple (25-fold) freezing to −25°C and thawing [12]. The enzyme solution was filtered using filters with 0.45, 0.22, and 0.1 μm pores. The preparations were tested for bacteriolytic activity and the presence or absence of free phage. Crude enzyme solutions were stored not more than one day at −20°C.

Enzyme solutions were thawed and proteins were precipitated by addition of $(NH_4)_2SO_4$ to 100% saturation at 0°C. If total protein concentration was less than 1 mg/ml, BSA was added to the preparation before precipitation so that total protein concentration was 1 mg/ml. This additional BSA can be almost completely separated from the active fractions during gel filtration. The protein precipitate was dissolved in 5-8 ml of 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 1 mM EDTA. The enzyme solution was applied onto a 5 cm^2 × 33 cm column with Sephadex G-75 superfine equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 1 mM EDTA. Chromatographic separation was performed at 2-3°C, the eluent flow rate being 24 ml/h.

The enzyme preparation was further purified by ion-exchange chromatography on a 2 cm^2 × 12 cm column with DEAE-Toyopearl 650 M equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. Proteins were eluted with a gradient of NaCl concentration 0.0-0.5 M (50 ml × 2). Chromatographic separation was performed at 2-3°C, the eluent flow rate being 35 ml/h.

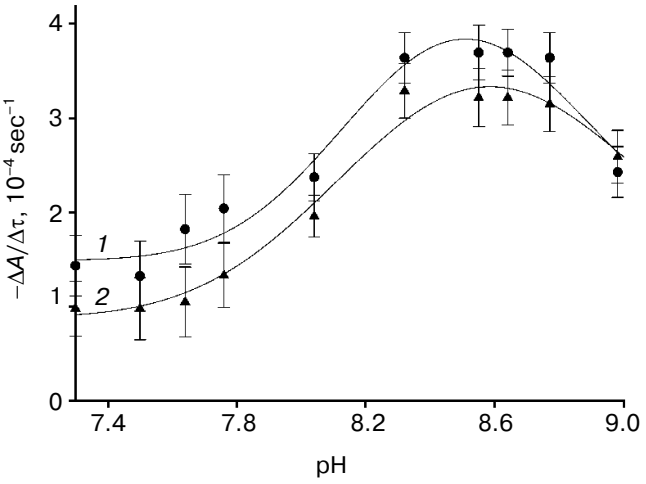

Fig. 1. pH dependence of initial rate of enzymatic lysis of *S. enteritidis* cells. Initial optical absorption 0.72. Enzyme concentration 1.2 μg/ml. Curves: *1*) exolysin; *2*) endolysin.

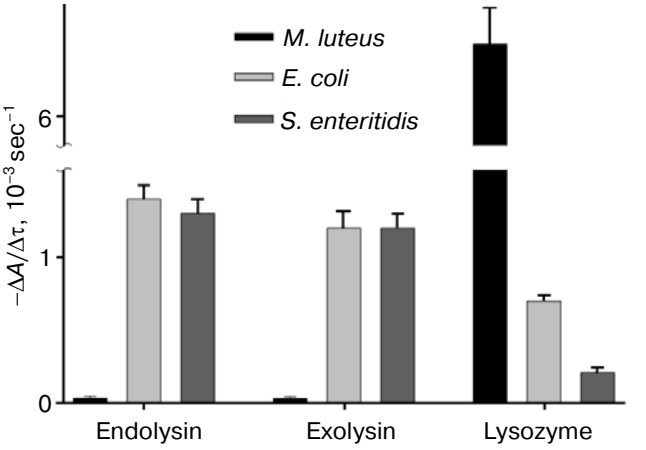

Fig. 2. Comparison of substrate specificities of endolysin, exolysin, and chicken egg lysozyme. Initial optical absorption of cell suspension 0.91-0.92. Concentrations (μg/ml): lysozyme, 5.6; endolysin and exolysin, 1.9.

RESULTS AND DISCUSSION

The measured molecular masses of endolysin and exolysin were 18 ± 2 and 18 ± 4 kDa, respectively. Thus, the molecular masses of the two enzymes are equal within the experimental error. There was no significant difference in the pH dependences of the initial rates of lysis of *S. enteritidis* cells by exolysin and endolysin (Fig. 1).

Substrate specificities of endolysin and exolysin were compared with that of chicken egg lysozyme, the best known and commercially available bacteriolytic enzyme. *Micrococcus luteus*, *S. enteritidis*, and *E. coli* were used as substrates (Fig. 2). The rates of cell lysis of the above-mentioned microorganisms were practically identical for exolysin and endolysin. It should be mentioned that substrate specificities of the studied phage enzymes significantly differ from that of lysozyme. Exolysin and endolysin lyse *S. enteritidis* more efficiently than lysozyme, but cannot lyse *M. luteus*, a good substrate for lysozyme. Both phage enzymes and lysozyme efficiently lyse *E. coli*.

Hereafter the data are presented only for "endolysin" because other characteristics of "exolysin" are also similar. Similar properties suggest that endolysin and exolysin of SPZ7 phage are structurally similar or even identical.

Linear dependence of the lysis rate on enzyme concentration is very important for detailed study of living

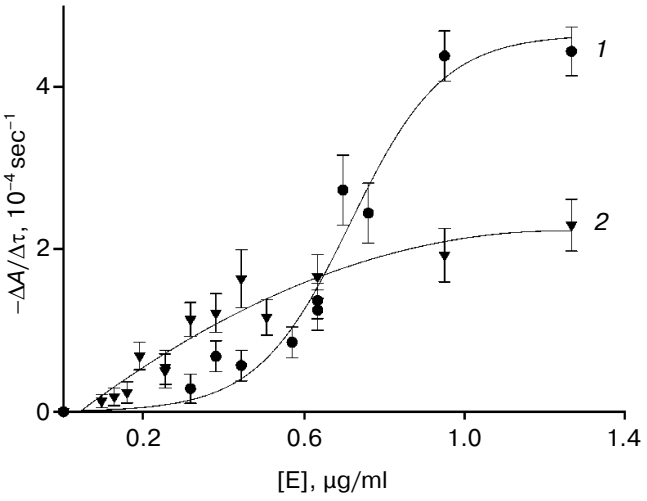

Fig. 3. Cell lysis rate versus enzyme concentration at various cell concentrations. Initial optical absorption of cell suspension was 0.94 (*1*) and 0.34 (*2*).

cells lysis by endolysin. Two dependences of the lysis rate on enzyme concentration for two different cell concentrations are presented in Fig. 3. As shown, the dependences are nonlinear and become sigmoid at high cell concentration. It should be noted that at high cell concentration the lysis rate significantly decreases at low endolysin concentration. This fact can be rationalized by sorption of some part of the endolysin on cells as "nonproductive complex" and/or more rapid poisoning and destruction of catalyst (endolysin) by components emerging from cells at higher concentration of lysed cells (e.g. enzyme proteolysis).

We studied the effect of various additives on the rate of enzymatic lysis of *S. enteritidis* cells. EDTA slightly decreases the initial rate of lysis (Fig. 4). It should be noted that addition of EDTA by itself causes a slight background cell lysis. We also studied rates of *S. enteritidis* cell lysis in the presence of Hottinger broth, DNase, and ethanol at the initial absorption of suspension of 0.9 and endolysin concentration of 1.7 μg/ml. Addition of Hottinger broth up to 5% of total volume decreased rate of lysis by 6-8%. Addition of ethanol up to 15% of total volume decreased rate of lysis by 2-4%. Addition of DNase up to concentration of 20 and 40 μg/ml increased rate of cell lysis by 6-8 and 35-43, respectively. We did not observe background cell lysis caused by Hottinger broth, DNase, and ethanol in the absence of endolysin. These data indicate that intact *Salmonella* cells as well as endolysin are relatively indifferent to these additives.

In the course of our studies we found that polymyxin M at low concentrations significantly influences the process of lysis (Fig. 5). However, the maximal effect is observed only in a narrow range of polymyxin M concentrations (2.5-3.5 μM). The effect of lower or higher concentrations was negligible. It should also be noted that the mechanism of polymyxin M action can be rather complex, because this compound is highly toxic for bacterial cells and by itself can violate cell biochemical processes.

In addition to the effect of various additives, we studied the effect of cell freezing (to −25°C) with subsequent thawing on the lysis rate. Single freezing of *S. enteritidis* suspension had practically no influence on the experimental results. However, after such treatment of *E. coli* cells the lysis rate increased more than twofold.

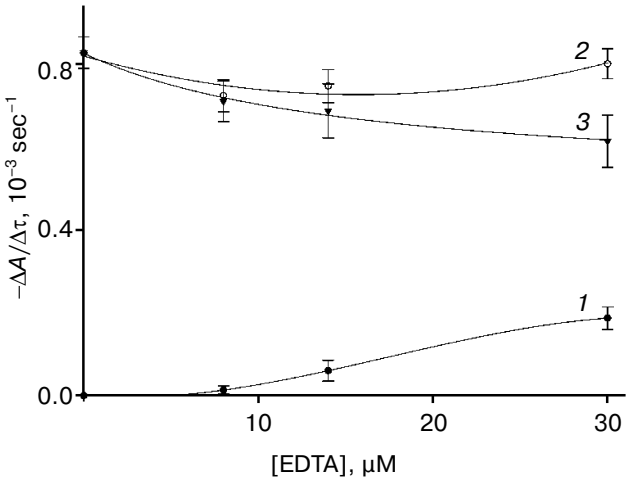

Fig. 4. Effect of EDTA on cell lysis rate in the presence of endolysin. The initial optical absorption of cell suspension was 0.91. Enzyme concentration 1.7 μg/ml. Here and in Fig. 5: *1*) background lysis in the absence of enzyme; *2*) lysis in the presence of enzyme; *3*) enzymatic lysis with subtraction of background.

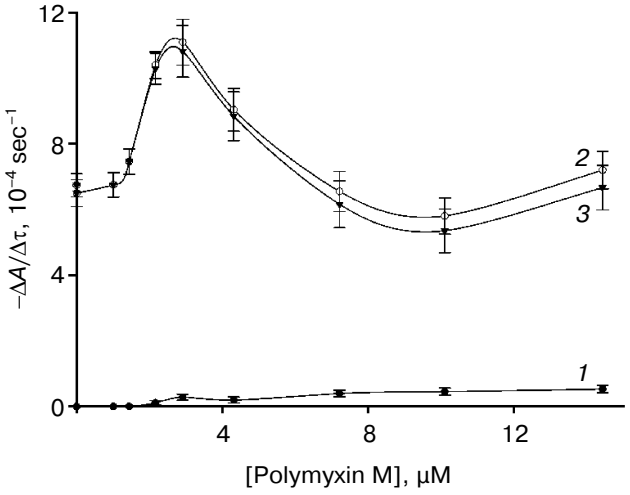

Fig. 5. Effect of polymyxin M on lysis of *S. enteritidis* cells by endolysin. Initial optical absorption of cell suspension was 0.9. Enzyme at the final concentration 1.5 μg/ml was added 5 min after incubation of cells with the other components.

The stability of the endolysin preparation was tested. The data indicate that the highly purified enzyme is very stable. Not less than 95% of the endolysin activity was retained after storage at 0.1 mg/ml concentration in 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl for more than a month at 37°C. High stability of purified enzymes can be very useful for development of new medical preparations or microbiological diagnostic systems.

In conclusion, this work lays a foundation for further detailed and thorough study of exolysin and endolysin and their action on living microorganisms of the Enterobacteriaceae family. The approaches described above might also be useful for study of catalytic properties of other bacteriolytic enzymes and their action on living cells of various microorganisms.

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