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Influence of Acidic Exopolysaccharide of *Xanthomonas campestris* IBPM 124 on the Kinetic Parameters of Extracellular Bacteriolytic Enzymes

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Abstract—Interactions of a negatively charged exopolysaccharide of *Xanthomonas campestris* IBPM 124 with its extracellular enzymes (muramidase, endopeptidase, and neutral phosphatase) and also with egg lysozyme, lysostaphin, muramidase of *Streptomyces globisporus*, and a bacteriolytic enzyme complex of *Streptomyces albus* were studied. All these enzymes were positively charged under the conditions of their maximal activity. It was shown that interaction of the acidic exopolysaccharide from *X. campestris* with these enzymes changed their kinetic parameters. The change was either positive (increase in reaction rate) or negative (decrease in reaction rate) and depended on the enzyme and type of substrate cleaved. Due to such interactions, the acidic exopolysaccharide secreted by *X. campestris* into the environment not only retained and transported positively charged exoenzymes into the near-cellular space, but also regulated their activity.

Key words: *Xanthomonas campestris*, lysoamidase, bacteriolytic enzymes, polysaccharide, interaction

Under deficit of nutrients, the bacterium *Xanthomonas campestris* IBPM 124 secretes into the environment nearly 20 different proteins including a metalloprotease (EC 3.4.24.) [1], a neutral phosphatase (EC 3.1.3.) [2], and three bacteriolytic enzymes—a muramidase (EC 3.2.1.), an endopeptidase (EC 3.4.), and an N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28) [3-5]. The bacteriolytic enzymes destroy target bacterial cells and help their producer to occupy a specific ecological niche by supplying the bacterium with nutrients that are formed with participation of proteases and other enzymes.

Together with the enzymes, *X. campestris* IBPM 124 secretes a negatively charged exopolysaccharide with a molecular mass of 1300 kD whose structure is formed by a repeating unit that comprises N-acetylglucosamine and N-acetylmannuronic and N-acetylgalacturonic acids [6]. Enzyme secretion starts in the lag phase; the exopolysaccharide appears in the medium and is intensively accumulated in the second half of the exponential growth phase [7].

The physiological role of the exopolysaccharide is probably not less important than that of the enzymes. In particular, it was shown that under the conditions of their maximal activity, some alkaline exoenzymes of *X. campestris* are electrostatically bound to the exopolysaccharide. These enzymes are the muramidase, endopeptidase, and neutral phosphatase [8]. The electrostatic interaction of proteins with polysaccharides in aqueous solutions is rather well studied [9]. As for the influence of such interaction on the enzymatic activity of proteins, there are few articles in the literature on this subject [10]. This work was devoted to investigation of this issue.

The data reported here should help to understand better the mechanisms of interaction and functioning of substances that are secreted by microorganisms into their environment and play an essential role in vital activity of microbial associations in natural ecosystems.

MATERIALS AND METHODS

Isolation and purification of enzymes. The microbial enzyme preparation, lysoamidase, containing the exopolysaccharide and exoenzymes was obtained from culture liquid of the bacterium *X. campestris* grown at the pilot plant in the Institute of Biochemistry and

Abbreviations: Ant-Ala-Ala-Phe-pNA) anthraniloyl-alanyl-alanyl-phenylalanine-para-nitroanilide; DNPCh) 3,4-dinitrophenyl-tetra-N-acetyl-β-D-chitotetraoside; pNPP) *p*-nitrophenylphosphate.

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The bacteriolytic endopeptidase from the lysoamidase preparation [4] was purified by gel filtration on Sephacryl S-200 and ion-exchange chromatography on CM-Sephadex followed by chromatography on a Mono S column in an FPLC system.

The bacteriolytic muramidase and N-acetylmuramoyl-L-alanine amidase were purified from culture liquid of *X. campestris* by a procedure [3-5] that includes ion-exchange chromatography on DEAE-Toyopearl and gel filtration on Toyopearl HW-50F. The metalloprotease was isolated from the lysoamidase preparation [1] by ion-exchange chromatography on DEAE-Sephadex and gel filtration on Sephadex G-75. Neutral phosphatase was obtained [2] by chromatography on SP-Sephadex and recirculation gel chromatography on Sephadex G-75.

Protein was determined by absorption at $\lambda = 280$ nm and also according to the method of Bradford [11].

Purification of the exopolysaccharide. The exopolysaccharide was purified by treatment of lysoamidase with pronase (1 mg/ml) for removal of the protein. Thus obtained material was first chromatographed on a column (14 × 14 cm) with DEAE-Spheron 1000 and then on a column (14 × 14 cm) with QAE-Sephadex A-50. It was then subjected to ultrafiltration through an XM-100A membrane and dialyzed. The content of the exopolysaccharide was measured using the anthrone method [12].

Enzyme assay. Activities of the muramidase, endopeptidase, and N-acetylmuramoyl-L-alanine amidase using a natural substrate was estimated by decrease in optical density in a cell suspension of *Staphylococcus aureus* (at 400 nm) in 10 mM Tris-HCl buffer, pH 8.0, at 50°C (for the N-acetylmuramoyl-L-alanine amidase, at 55°C) on a Shimadzu UV-160A spectrophotometer. The initial reaction rate (v) was expressed in relative units (decrease in optical density by 0.001 per min per mg protein).

Activities of the endopeptidase, N-acetylmuramoyl-L-alanine amidase, and metalloprotease using a synthetic substrate was measured by cleavage of the fluorogenic tripeptide Ant-Ala-Ala-Phe-pNA and recording the fluorescence intensity at 420 nm on a Hitachi M 850 spectrofluorimeter (Japan). The reaction was carried out in 0.01 M MES-buffer, pH 6.5, at 50°C (for the N-acetylmuramoyl-L-alanine amidase, at 55°C). The initial reaction rates were expressed in relative units (increase in the fluorescence intensity (I_{420}) of the cleaved substrate by 0.01 per min per mg protein). Activity of the muramidase was determined using DNPCh as substrate in 10 mM Tris-HCl buffer, pH 8.0, at 50°C. It was recorded by the increase in the absorption at 400 nm of dinitrophenol released (ΔA_{400}). The initial reaction rates are expressed in relative units (increase in optical density by 0.001 per min per mg protein).

Activity of the metalloprotease using casein as a substrate was measured as described elsewhere [1]. The amount of enzyme that released 1 μmol of tyrosine per min at 37°C was taken for the unit of activity.

Activity of phosphatase was measured by the rate of hydrolysis of *p*-nitrophenylphosphate [2]. The amount of enzyme that released 1 μmol *p*-nitrophenol per min at 37°C was taken as the unit of activity.

Enzymatic activity of egg lysozyme and the muramidase from *S. globisporus* was measured in 10 mM Tris-HCl buffer, pH 6.6, at 40°C using a special substrate for lysozyme (*Micrococcus lysodeikticus* cells, 0.43 mg/ml); activity of lysostaphin and the enzyme complex from *S. albus* was determined in 10 mM Tris-HCl buffer, pH 8.0, at 40°C, using *S. aureus* cells as the substrate (0.15 mg/ml).

Calculation of the kinetic parameters. The initial reaction rates (v) were calculated by the slope angle of tangents to the rectilinear segments of curves representing the process of substrate cleavage in no less than three parallel experiments. The secondary kinetic parameters (Michaelis constants, K_m) and maximum reaction rates (V) were determined by constructing plots in double reciprocal coordinates according to Lineweaver–Burk (v^{-1}; S^{-1}).

The constants of inhibition (K_i) and activation (K_a) of some tested enzymes by the exopolysaccharide were calculated by equations for the respective types of reactions [13, 14].

The mean square deviation in five determinations of v was ±2.5%; for the constants K_m, V, K_a, and K_i it was ±7%.

Chemicals. The following reagents were used: DEAE-Toyopearl, Toyopearl HW-50F (Toyo Soda, Japan); DEAE-Spheron, QAE-Spheron (Chemapol, Czech Republic); Sephacryl S-200, DEAE-Sephadex, Sephadex G-75 (Pharmacia, Sweden); Ant-Ala-Ala-Phe-pNA (Diagnostikum, Russia); *p*-nitrophenylphosphate (Serva, Germany); DNPCh (Koch-Light, UK); lysozyme (Serva, Germany); lysostaphin (Sigma, USA); substrate for lysozyme (Difco Lab., USA); enzyme complex G-8709014 from *Streptomyces albus* and M-1 muramidase 870914 from *Streptomyces globisporus* (kindly provided by Dr. A. I. Severin). Other reagents were domestic products of analytical and high purity grades.

RESULTS AND DISCUSSION

Effect of the exopolysaccharide on the activities of the muramidase, endopeptidase, and phosphatase using synthetic substrates. The effect of the exopolysaccharide on the activity of muramidase towards DNPCh as a substrate is shown in Fig. 1a. According to the traditional classification, this inhibition is of mixed type [15, 16]. The exopolysaccharide decreases the catalytic activity of the muramidase ($V' < V^0$) and weakens the binding of enzyme to substrate ($K'_m > K^0_m$). Using a more complete classification, this mode of inhibition is of type I_i of biparametrically coordinated inhibition ($K'_m > K^0_m$, $V' < V^0$, $i > 0$), where K'_m is the effective Michaelis constant determined in the presence of inhibitor (i) and V' is the

maximum reaction rate determined at the same concentrations of inhibitor or activator; K_m^0 and V^0 are the analogous parameters of the enzymatic reactions (without inhibitor, $i = 0$) [13, 14].

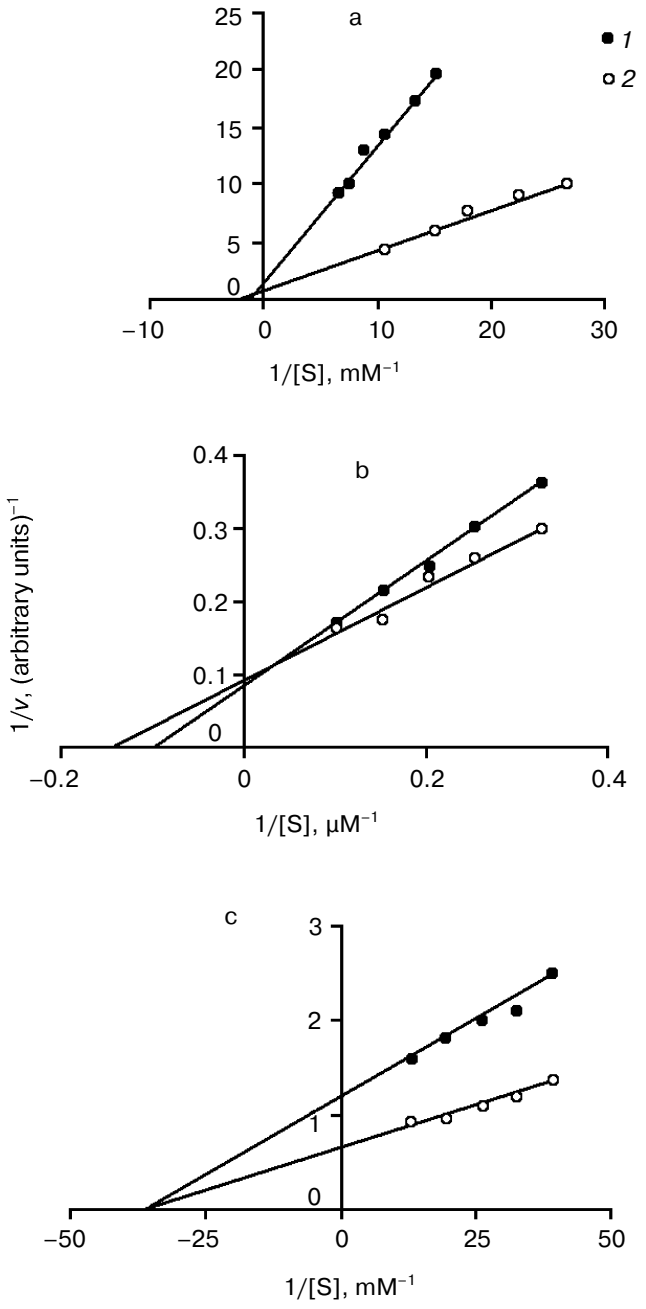

Fig. 1. Lineweaver–Burk plots for the muramidase (a), endopeptidase (b), and phosphatase (c) in the presence (*1*) and absence (*2*) of the exopolysaccharide when synthetic substrates were used. a) Muramidase, 13 μg/ml; substrate (dinitrophenyl-chitotetraoside), 0.037-0.15 mM; exopolysaccharide, 0.13 mg/ml; b) endopeptidase, 8.9 μg/ml; substrate (Ant-Ala-Ala-Phe-pNA), 3.03-9.8 μM; exopolysaccharide, 0.7 mg/ml; c) phosphatase, 1.8 μg/ml; substrate (pNPP), 0.026-0.079 mM; exopolysaccharide, 0.144 mg/ml.

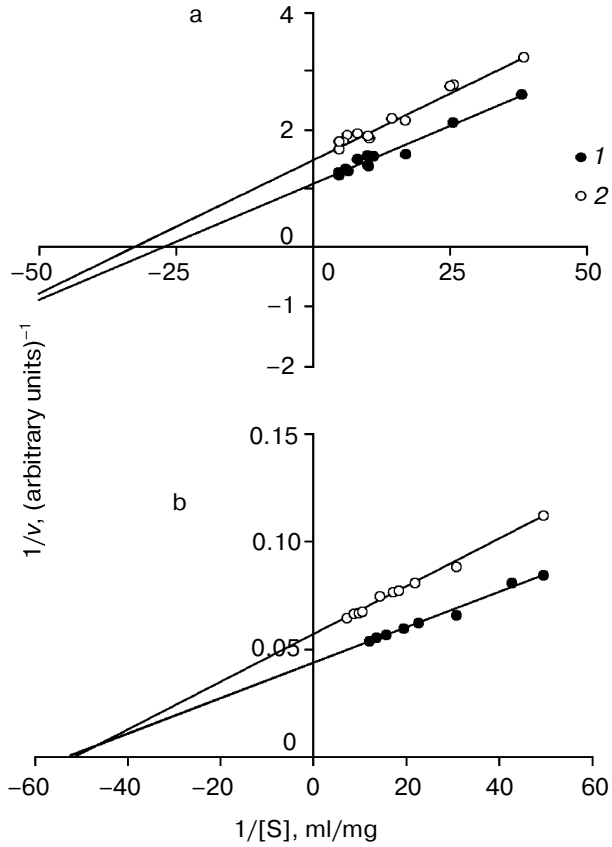

Fig. 2. Lineweaver–Burk plots for the muramidase (a) and endopeptidase (b) in the presence (*1*) and absence (*2*) of the exopolysaccharide when the natural substrate (*S. aureus* cells) was used. a) Muramidase, 9 μg/ml; substrate (*S. aureus* cells), 0.026-0.22 mg/ml; exopolysaccharide, 0.7 mg/ml; b) endopeptidase, 8.9 μg/ml; substrate (*S. aureus* cells), 0.02-0.141 mg/ml; exopolysaccharide, 0.7 mg/ml.

The interaction of the bacteriolytic endopeptidase and the exopolysaccharide was accompanied by difficulties in the binding of enzyme to the synthetic substrate Ant-Ala-Ala-Phe-pNA ($K_m' > K_m^0$) and increase of the maximum reaction rate ($V' > V^0$), which is characteristic of type V_i inhibition, i.e., pseudo-inhibition of enzymes (Fig. 1b) [13, 14]. This type of enzyme inhibition and the equations for calculating the appropriate K_{Vi} constants of enzyme inhibition are not included in the traditional classification [15, 16].

The plots depicting the effect of initial rates of pNPP cleavage by neutral phosphatase on the substrate concentration in the presence and absence of the exopolysaccharide are given in Fig. 1c. As seen, the exopolysaccharide prevents the binding of enzyme to substrate ($K_m' > K_m^0$) and decreases the maximum reaction rate ($V' < V^0$). This effect of the exopolysaccharide also follows type I_i biparametrically coordinated inhibition [13, 14].

Thus, it was revealed that electrostatic interaction of the exopolysaccharide with these enzymes leads to inhibi-

Table 1. Effect of the exopolysaccharide from culture liquid of *X. campestris* IBPM 124 on the activity of *X. campestris* exoenzymes and other bacteriolytic enzymes

Enzyme	Substrate	Activity, %		Isoelectric point
		without exopolysaccharide	with exopolysaccharide*	
Egg lysozyme	*M. lysodeicticus*	100	6	10.5-11.0
Lysostaphin of *S. staphylolyticus*	*S. aureus*	100	0	9.5-10.0
Enzyme complex of *S. albus*	*S. aureus*	100	193	9.0
Muramidase of *S. globisporus*	*M. lysodeicticus*	100	145	8.2
Muramidase of *X. campestris*	*S. aureus*	100	130	9.0-10.0
Endopeptidase of *X. campestris*	*S. aureus*	100	112	9.0-10.0
N-Acetylmuramoyl-L-alanine amidase of *X. campestris*	*S. aureus*	100	100	4.5
Phosphatase of *X. campestris*	p-NPP	100	50	8.3
Metalloprotease of *X. campestris*	casein	100	100	5.0

* The protein/exopolysaccharide ratio is 1 : 100.

tion of their activity towards low-molecular-weight synthetic substrates.

Effect of the exopolysaccharide on activities of the muramidase and endopeptidase using *S. aureus* cells as a substrate. It was especially important to study how the activity of bacteriolytic enzymes would change in the presence of the exopolysaccharide towards their natural substrate, i.e., bacterial cell walls, which are characterized by a complicated chemical structure [17]. Due to the peculiarity of natural substrates (bacterial cell walls) used in the work, the activity of the enzymes and their respective kinetic parameters were expressed in relative units.

The dependence of initial rates of lysis of *S. aureus* cells by the muramidase from *X. campestris* on the substrate concentration in the presence and absence of the exopolysaccharide is shown in Fig. 2a. The data indicate that the interaction of the muramidase with the exopolysaccharide hampers the binding of the enzyme to the substrate ($K'_m > K^0_m$) and increases the maximum rate of substrate cleavage ($V' > V^0$). This dependence is referred to as type II'''_a biparametrically discoordinated activation of enzymes [13, 14]. In the classic literature there is neither a classification of such types of enzyme activation nor an equation for calculating the respective constants [15, 16].

This suggests that the exopolysaccharide affects both the associative and catalytic sites of the enzyme active center during formation of a ternary enzyme–polysaccharide–substrate complex. This example vividly illustrates that use of only synthetic substrate as a model for determination of enzyme interaction with polysaccharide would not correctly estimate the effect of the exopolysaccharide on the activity of the muramidase towards one of its natural substrates—*S. aureus* cells (the muramidase can also hydrolyze cells of *Micrococcus lysodeikticus*, *Bacillus subtilis*, *Streptomyces chrisomallus*, and some other gram positive bacteria). Thus, the mechanism of formation of the ternary enzyme–polysaccharide–substrate complex is different and depends on the substrate used.

When *S. aureus* cells are used as a substrate, the interaction of the bacteriolytic endopeptidase with the exopolysaccharide leads to a slight decrease in the Michaelis constant value ($K'_m < K^0_m$) and the maximum reaction rate ($V' > V^0$) (Fig. 2b). This is characteristic of biparametrically coordinated activation (type I_a) of enzymes [13, 14].

The different effect of the exopolysaccharide on substrate cleavage by the endopeptidase (Figs. 1b and 2b) may be due to the presence of a hydrophobic fragment comprising two alanine residues in the molecule of the synthetic substrate Ant-Ala-Ala-Phe-pNA.

Effect of the exopolysaccharide on the activity of some other bacteriolytic enzymes. We also studied the influence of the exopolysaccharide from *X. campestris* on some bacteriolytic enzymes isolated from other microorganisms and interacting with it electrostatically (Table 1). As seen from this table, the exopolysaccharide drastically inhibits the activity of egg lysozyme, possessing high muramidase activity, and lysostaphin, a lytic complex from *Staphylococcus staphylolyticus*. Besides, the exopolysaccharide activates the enzyme complex of *Streptomyces albus* and the muramidase of *Streptomyces*

Table 2. Kinetic parameters of enzymes from *X. campestris* IBPM 124 during their interaction with the exopolysaccharide

Enzyme	Substrate	K_a, mg/ml	K_i, mg/ml
Muramidase	*S. aureus*	1.04	
	DNPCh		0.05
Endopeptidase	*S. aureus*	2.06	
	Ant-Ala-Ala-Phe-pNA		1.09
Phosphatase	p-NPP		0.17

globisporus. This again confirms our suggestion that formation of the triple complex exopolysaccharide–enzyme–substrate depends on both the enzyme itself and the substrate properties.

Effect of the exopolysaccharide on the activities of the N-acetylmuramoyl-L-alanine amidase and metalloprotease. Under the conditions of their optimal activity, the N-acetylmuramoyl-L-alanine amidase and metalloprotease of *X. campestris* are not bound to the exopolysaccharide electrostatically [8] (see p*I* values in Table 1), and so we used these two enzymes as controls. The studies revealed that the exopolysaccharide had no effect on either the activity of the metalloprotease towards Ant-Ala-Ala-Phe-pNA and casein or that of the N-acetylmuramoyl-L-alanine amidase towards Ant-Ala-Ala-Phe-pNA and *S. aureus* cells. The Michaelis constants and maximum reaction rates of these enzymes did not change in the presence of the exopolysaccharide.

These data show that the exopolysaccharide changes the properties of enzymes bound to it electrostatically. The character of these changes depends on the nature of the substrate used. Moreover, a comparative study of the K_i and K_a constants of enzyme inhibition by the exopolysaccharide (Table 2) reveals that binding of the exopolysaccharide to the endopeptidase is 20 and 6 times weaker than its binding to the muramidase and phosphatase, respectively, during cleavage of synthetic substrates and 2 times weaker than its binding to the muramidase during the cleavage of a natural substrate. This suggests that the mechanism of formation of the enzyme–polysaccharide–substrate complex varies.

The experimental data also indicate that electrostatic interaction of these enzymes with the exopolysaccharide of *X. campestris* is accompanied by changes in the kinetic parameters (K_m^0 and V^0) of reactions catalyzed by these enzymes. These changes can be positive or negative and depend on the type of enzyme and the substrate used.

In conclusion, the acidic exopolysaccharide secreted by *X. campestris* into the environment retains and moves exoenzymes in the near-cellular space and also regulates their activity. Since the exopolysaccharide has the ability to inactivate some foreign bacteriolytic enzymes, it may also protect the cells against their action.

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