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# Effect of polyelectrolytes on serine proteinase secretion by *Bacillus subtilis*

Anatoly V. Artemov and Vitaly D. Samuilov

*Department of Cell Physiology and Immunology, Moscow State University, Moscow 119899, USSR*

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Addition of polycations with molecular masses of 5–40 kDa as well as $Na^+$, stimulated serine proteinase secretion by *Bacillus subtilis* cells. Polyanions and higher-molecular-mass polycations (100–200 kDa) were inefficient. The enzyme yields in the presence of polycations or $Na^+$ were equal in magnitude. The results indicate that the cations, apparently counteracting the negative surface charge of the bacterial plasma membrane, cause the desorption of the serine (alkaline) proteinase. The synthesis of the proteinase is inferred to be stopped as the enzyme is bound to the outer surface of the plasma membrane. The desorption of the enzyme thus induces the synthesis of the new portions of proteinase.

Serine proteinase synthesis; Plasma membrane; Polyelectrolyte; (*Bacillus subtilis*)

## 1. INTRODUCTION

Extracellular enzymes of bacteria are synthesized as precursors on membrane-bound ribosomes. Transfer of the precursors across the plasma membrane is accompanied by the proteolytic removal of amino-terminal signal peptide sequences and by formation of the mature form of the enzyme [1]. $Na^+$, $K^+$ and $Mg^{2+}$, $Ca^{2+}$ and $La^{3+}$ increase the serine proteinase secretion of *Bacillus licheniformis* [2] and *B. subtilis* cells [3]. The effective concentrations of cations are increased approximately 10-fold per unit of their charge. The stimulatory effect of the cations on exoenzyme secretion is not directed to the inner surface of the plasma membrane since choline, which is a membrane-impermeant cation, was found as efficient as $Na^+$ and $K^+$ [3]. The cations tested seem to counteract the negative surface charge of the plasma membrane and of the cell wall and to induce the desorption of the cell bound enzyme.

The purpose of the present work was to study the effects of polyelectrolytes on the serine proteinase secretion by *B. subtilis*.

## 2. MATERIALS AND METHODS

*B. subtilis* strain 72 used in the industrial production of serine proteinase was grown as described previously [3]. Experiments were performed using 23–24-h cultures at the onset of the stationary growth. The cells were washed with 100 mM NaCl/0.5% glucose, suspended in 0.5% glucose/10 mM Tris-Mes (pH 7.0) and incubated in the same medium with additions of NaCl or polyelectrolytes for 30 min at 37°C with shaking (220 rpm). The optical density of the cell suspensions in a 1-cm cuvette at 650 nm was 1.0–1.5. The following polyelectrolytes were used in the experiments: polycations: poly-L-lysine hydrobromides with molecular masses of 6 and 90.3–100.5 kDa, polyethylenimine (7 and 30–40 kDa), protamine sulphate (5 kDa), poly-4-vinyl-*N*-ethylpyridinium hydrobromide (224 kDa); and polyanions: heparin (13–15 kDa), polyacrylic acid (92 kDa), dextran sulphate (100 kDa).

After incubation with electrolytes for 30 min, the proteolytic activity was assayed in the cell suspensions or supernatants obtained after sedimentation of the cells. The proteinase activity was measured as hydrolysis of *N*-acetyl-L-tyrosine ethyl ester [3] using a pH state (Radiometer, Denmark) and expressed as $\mu$g-equivalents of $H^+$/min per 1 ml; some enzyme activity in the cell suspensions incubated without electrolytes added was subtracted.

*Correspondence address:* V.D. Samuilov, Department of Cell Physiology and Immunology, Biological Faculty, Moscow State University, Moscow 119899, USSR

## 3. RESULTS

Fig.1 shows proteinase production by *B. subtilis*. The process was linear with time, considerably stimulated by NaCl and inhibited by chloramphenicol. In accordance with the data on $Na^+$, $K^+$ and choline$^+$ [3], the optimal concentration of monovalent cations was about 100 mM. The further increase of the salt concentration lowered the stimulating effect [3]. The proteolytic activity of the cell suspension and the solution obtained after the sedimentation of the cells were the same when the cells were incubated with NaCl. In the absence of the added salts, the proteinase activity of the cell suspension was higher than in the supernatant; it varied from 10 to 40% of the activity measured after incubation of the cells with NaCl.

Along with NaCl, proteinase secretion was stimulated by polycations. Table 1 shows the data obtained with polylysine, protamine and polyethylenimine. The activity of proteinase secreted

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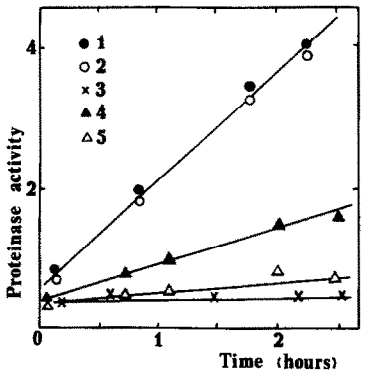


Fig.1. Effect of NaCl on serine proteinase secretion by *B. subtilis* cells. The cells incubated with NaCl: 1, cell suspension; 2, supernatant; 3, cell suspension with 25 mg/ml of chloramphenicol. The cells incubated without NaCl: 4, cell suspension; 5, supernatant.

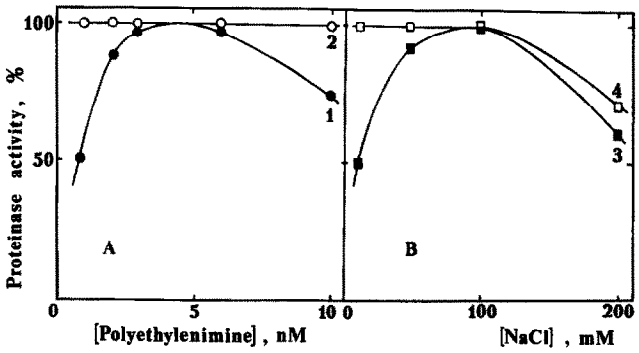


Fig.2. Effect of NaCl and 7 kDa polyethylenimine combination on serine proteinase secretion by *B. subtilis* cells. 1, polyethylenimine at different concentrations; 2, the same as 1 plus 100 mM NaCl; 3, NaCl at different concentrations; 4, the same as 3 plus 3 nM polyethylenimine. The 100% activity of proteinase in experiments A and B was 1.0 and 1.2 $\mu$g-eqv. of $H^+$/min per 1 ml, respectively.

by the cells incubated in the presence of NaCl or polycations was practically the same. The concentration optimum for polycations was variable over the range nanomoles to micromoles. As the concentrations of polycations were further increased, the stimulatory effect of polycations was hindered, and the bacterial cells were aggregated. It should be noted that the polyethylenimine concentration causing the maximum yield of proteinase was inversely correlated with the degree of polymerization: the optimal concentrations of the polymer with molecular masses of 7 and 30–40 kDa were 4 and 1 nM, respectively.

Polyanions did not stimulate proteinase secretion. The enzyme production was unaffected by heparin, polyacrylic acid and dextran sulphate, being tested at the concentrations of 0.01–100, 0.01–10 and 0.01–10 $\mu$M, respectively. The activity of serine proteinase was insensitive to NaCl and polyelectrolytes at concentrations tested (data not shown). The enzyme activity was found to be resistant to $Na^+$, $K^+$ and choline$^+$ down to 1.2 M, and to $Mg^{2+}$ and $Ca^{2+}$ to 0.3 M as has been described earlier [3].

Polyethylenimine and its combination with NaCl at the optimal concentrations demonstrated an equal level of proteinase secretion by *B. subtilis* cells (fig.2). NaCl (100 mM) prevented a decline in stimulating effect of polyethylenimine at concentrations higher than optimal (fig.2A). A drop in the proteinase yield at a NaCl concentration over 100 mM was not prevented by polyethylenimine (fig.2B). Similar results were obtained in the experiments with polylysine and NaCl.

The effect of highly polymerized polycations on proteinase secretion was also studied. Polylysine with a molecular mass of 90.3–100.5 kDa and poly-4-vinyl-*N*-ethylpyridinium with a molecular mass of 224 kDa, tested at the concentrations of 1.0 nM–1.0 $\mu$M, had no effect on secretion of the enzyme and its activity (data not shown).

Table 1

Effect of NaCl and polycations on serine proteinase secretion by *B. subtilis* cells

| Addition | Optimal concentration | Proteinase activity (%) |
|---|---|---|
| NaCl | 100 mM | 100 |
| Polylysin (6 kDa) | 1 $\mu$M | 96 |
| Protamine (5 kDa) | 10 nM | 105 |
| Polyethylenimine (7 kDa) | 4 nM | 100 |
| Polyethylenimine (30–40 kDa) | 1 nM | 100 |

The 100% activity of the enzyme was 1.9 $\mu$g-eqv. of $H^+$/min per 1 ml

## 4. DISCUSSION

Serine proteinase secretion by *B. subtilis* was found to be stimulated by polycations. The proteinase synthesized in the absence of the cations added is predominantly bound to the cells and, under these conditions, the enzyme synthesis appeared to be stopped. Along with NaCl, polycations with molecular masses of 5–40 kDa give the same yields of the enzyme. Addition of polycations in the presence of NaCl or addition of NaCl in the presence of polycations do not give an extra yield of the enzyme. Enzyme secretion is unaffected by highly polymerized polycations (100–200 kDa).

The results obtained can be interpreted as following. Serine proteinases are characterized by an alkaline isoelectric point [1] which is equal to 9.3 for *B. subtilis* [4]. Upon translocation across the plasma membrane, the enzyme is bound to the outer membrane surface by the electrostatic interaction with negatively charged sites. As these sites on the membrane surface are occupied, proteinase synthesis is terminated. The proteinase synthesis inhibition, induced by the binding of the enzyme to the plasma membrane of bacilli, resembles a repression of the synthesis of extracellular enzymes in the fungi, observed in response to an increase in the level of their content in the medium [5].

The cations added displace the enzyme into the periplasma, due to competition for the negatively charged sites of the plasma membrane. Proteinase diffuses then across the cell wall into the incubation medium. A signal of the enzyme desorption is transmitted to intracellular membrane-bound ribosomes and induces the synthesis of new portions of proteinase. Thus, the synthesis of serine proteinase is apparently regulated by the presence of the enzyme bound to the outer surface of the plasma membrane. The control mechanism of this kind can be of universal importance for synthesis of extracellular enzymes.

Stimulation of the serine proteinase synthesis by *B. subtilis* cells, observed in the presence of polycations, is similar to an immunomodulating effect of synthetic polyelectrolytes manifested as activation of immunocompetent cells by polyanions and polycations [6]. Unlike lymphocytes, the cells of Gram-positive bacteria contain cell walls composed of a peptideglycan grid involving bound teichoic acids. This grid appears to have fairly large holes, in as much as it is freely permeable to the serine proteinase with a molecular mass of 25–30 kDa [1]. As seen from fig.1, the values of the enzyme activity in the bacterial cell suspension and in the supernatant obtained by sedimentation of the cells are the same. Consequently, polyelectrolytes with medium molecular masses pass through the cell wall to the plasma membrane. The cell wall is not permeable to polyelectrolytes with high molecular masses and, because of this, the proteinase secretion is unaffected by 90.3–100.5 kDa polylysin and 224 kDa polyvinylethylpyridinium.

It should be noted that the activation of the proteinase secretion is observed at very low concentrations of polycations. This could be due to the cooperative character of their interaction with the membrane surface.

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