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EXPERIMENTAL ARTICLES

Synthesis and Secretion of Proteinases by *Bacillus intermedius* in the Late Stages of Sporulation

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Abstract—In the late stages of sporulation, cells of *Bacillus intermedius* 3-19 secreted into the medium two proteinases, glutamyl endopeptidase and subtilisin, whose maximum activities were recorded in the 40th and 44th hours of growth, respectively. By estimating β-galactosidase activity as a marker of cytoplasmic membrane integrity, it was revealed that the accumulation of these proteinases in the medium was a result of their secretion and not of lysis of the cell envelope. Concentrations of peptone and inorganic phosphate ensuring the maximum production of the enzymes were established. Ammonium ions were shown to inhibit the production of proteinases by the mechanism of repression by nitrogen metabolites.

Key words: thiol-dependent proteinase, glutamyl endopeptidase, sporulation, *Bacillus intermedius.*

The ability of bacteria to react rapidly to extra- and intracellular signals reflecting changes in the environment provides for their survival under extreme conditions. Nutritional limitation can evoke in bacilli various responses such as motility, cell competence, synthesis and secretion of hydrolytic enzymes, production of antibiotics, and sporulation, of which only one is usually chosen by the cells. Sporulation in bacilli starts in the early stationary phase, which is considered to be a transitional period of preparation for sporulation [1]; sometimes, its duration exceeds the duration of the logarithmic growth phase. In this period, cells secrete large amounts of hydrolytic enzymes, including proteinases, which are excreted from the cells both before and after initiation of sporulation [2].

The study of synthesis and secretion of hydrolytic enzymes during the transition of bacilli to the dormant state provides insight into the mechanisms of cell response to extreme conditions.

Earlier, we revealed that the streptomycin-resistant strain *Bacillus intermedius* 3-19 secretes two alkaline proteinases, glutamyl endopeptidase and thiol-dependent serine proteinase, in the early stationary phase [3, 4]. The enzymes were isolated; their properties and conditions of biosynthesis were studied [5, 6]. More recently, bacilli were shown to be capable of producing alkaline proteinases in the late stationary phase corresponding to sporulation stages V and VI [7].

The aim of the present work is to study the synthesis and secretion of proteinases by *Bacillus intermedius* 3-19 in the late stationary growth phase.

MATERIALS AND METHODS

The streptomycin-resistant strain *Bacillus intermedius* 3-19 (deposited with the All-Russia Collection of Industrial Microorganisms as strain B-3833) was obtained from the wild-type strain *B. intermedius* 7P (from the Culture Collection of the Department of Microbiology, Kazan State University) by passages on medium containing streptomycin (500 μg/ml).

The strain was grown in glucose-free medium containing (g/l) peptone, 20; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.3; NaCl, 3.0; and $MnSO_4$, 0.1 (pH 8.5). The medium was sterilized at 1 atm. Solutions of inorganic phosphate (Na_2HPO_4), ammonium citrate, and ammonium chloride were sterilized separately at 1 atm and added aseptically to the medium before inoculation to give final concentrations of inorganic phosphate and ammonium salts of 0.1–0.3 g/l and 2–8 mM, respectively. Bacilli were cultivated in flasks one-fifth full of the medium on a temperature-controlled shaker (200 rpm; 30°C). The medium was inoculated with a log-phase culture grown on streptomycin-containing medium for 12–15 h. The inoculum dose was 1 vol %.

Cell growth was monitored by measuring culture turbidity with a KFK-2 photoelectrocolorimeter at 590 nm in a 1-cm cuvette. When sporulation was studied, a 50-hour culture was used as the inoculum. Spores were counted under a Carl Zeiss phase-contrast microscope (Jena, Germany) at a magnification of 1600× in 5–10 microscope fields, and the percent ratio of their number to the total number of vegetative and sporulating cells was

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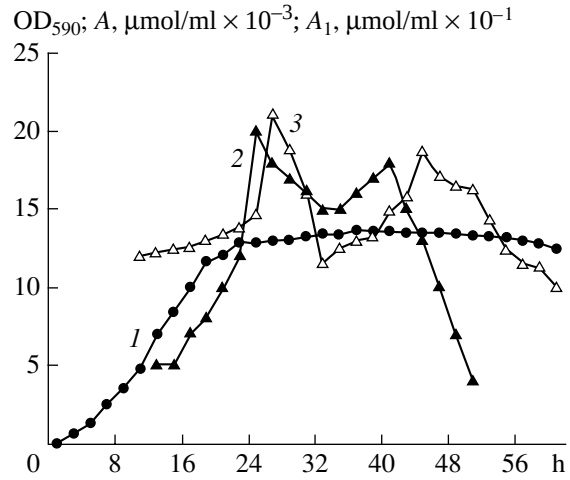

Fig. 1. Time course of (*1*) growth and synthesis of (*2*) glutamyl endopeptidase 2 (A) and (*3*) subtilisin 2 (A1) by *B. intermedius* 3-19.

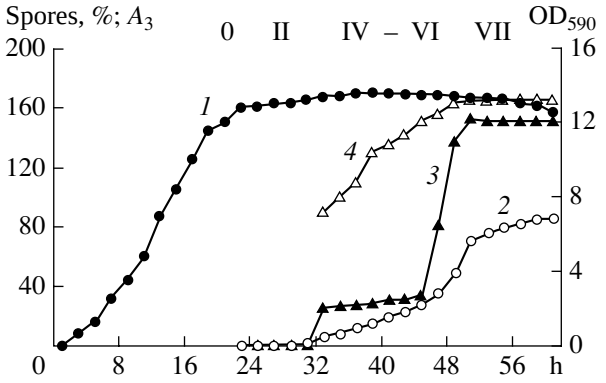

Fig. 2. Time course of (*1*) growth, (*2*) sporulation, and accumulation of β-galactosidase in (*3*) culture liquid and (*4*) cell lysates. Roman numerals indicate stages of sporulation.

calculated. Sporulation stages were determined by conventional methods.

Protein was measured spectrophotometrically, assuming a protein concentration of 1 mg/ml to correspond to an optical density (OD) of 1.0 at 280 nm in a 1-cm cuvette.

Proteolytic activity was assayed with the synthetic chromogenic substrates Z-Glu-*p*NA and Z-Ala-Ala-Leu-*p*NA. Enzyme activity was calculated as described by Lyublinskaya *et al.* [8]. One unit of proteolytic activity was defined as the amount of enzyme that hydrolyzes 1 μmol of the substrate per 1 min.

Cell productivity was calculated as the ratio of enzyme activity to the optical density of the culture.

Activity of β-galactosidase was determined by the standard method. One unit of β-galactosidase activity was defined as the amount of enzyme that increases the optical density at 420 nm by one unit per minute in 1 ml of the reaction mixture incubated at 37°C.

To obtain cell homogenates, cells were ultrasonically disrupted at 0°C on a UZDI-IV 4.2 disintegrator at 22 kHz (30 s × 15). Specific activity of extracellular and intracellular β-galactosidase was calculated per 1 g of wet biomass.

Statistical analysis (determination of standard deviation σ) was performed using the Microsoft Excel program. The results were considered confident at $\sigma \le 15\%$ and the Student criterion of $P \le 0.05$.

The results of the multifactor experiment were statistically processed using the BIOPT software package [9].

RESULTS AND DISCUSSION

The time course of *B. intermedius* growth and synthesis of proteolytic enzymes is given in Fig. 1. By using specific chromogenic substrates, Z-Glu-*p*NA and Z-Ala-Ala-Leu-*p*NA, glutamyl endopeptidase 2 and subtilisin 2 were revealed in the late phases of cell growth. Enzymes are denoted by index 2 to differentiate them from the corresponding proteinases that are secreted by bacilli in the early stationary growth phase [5, 6]. Activities of glutamyl endopeptidase 2 and subtilisin 2 reached maximum values by the 40th and 44th hours of cell growth, respectively.

To elucidate the mechanism of accumulation of glutamyl endopeptidase 2 and subtilisin 2 in the culture liquid in the late phases of *B. intermedius* growth, the time course of cell lysis was investigated. The activity of β-galactosidase, an intracellular enzyme whose release is associated with cell lysis, was chosen as a marker of the cytoplasmic membrane integrity. The activity of β-galactosidase was analyzed in the culture liquid in the course of cell growth and sporulation and, in particular, in homogenates of cells from the same growth phases. As seen from Fig. 2, the activity of β-galactosidase in the culture liquid remained relatively low (about 35 units/g biomass) up to the 44th hour of cultivation, whereas in cell homogenates, the enzyme activity gradually increased and reached 80–150 units/g biomass by the 44th–50th h of cultivation. Microscopic observations showed that cell lysis and spore release (stage VII of sporulation) began by the 48th–50th h of cultivation. In this period, the activity of β-galactosidase in the culture liquid increased sharply (to 130–150 units/g biomass). These results indicate the integrity of cell membranes in the period of intense accumulation of proteolytic enzymes, which corresponded to sporulation stages V and VI. Thus, glutamyl endopeptidase 2 and subtilisin 2 proved to be secretory enzymes, and their

Table 1. Optimization of growth medium (according to the B2 scheme) for the synthesis of subtilisin 2 by *B. intermedius* 3-19

Factor levels				Biomass, OD units	Enzyme activity, μmol/ml	Productivity, arb. units
Peptone		P_I				
*X*1	g/l	*X*2	g/l			
+	30	+	0.3	17.28	0.940	0.054
–	10	+	0.3	7.4	0.875	0.118
+	30	–	0.1	15.8	1.042	0.066
–	10	–	0.1	6.4	0.845	0.132
+	30	0	0.2	16.2	0.968	0.06
–	10	0	0.2	7.2	0.86	0.119
0	20	+	0.3	14	1.043	0.0745
0	20	–	0.1	11.6	0.974	0.08

Table 2. Optimization of growth medium (according to the B2 scheme) for the synthesis of glutamyl endopeptidase 2 by *B. intermedius* 3-19

Factor levels				Biomass, OD units	Enzyme activity, μmol/ml	Productivity, arb. units
Peptone		P_I				
*X*1	g/l	*X*2	g/l			
+	30	+	0.3	8.0	9.1	1.1
–	10	+	0.3	4.5	11.2	2.4
+	30	–	0.1	6.4	7.7	1.25
–	10	–	0.1	1.0	1.07	1.07
+	30	0	0.2	7.2	8.7	1.2
–	10	0	0.2	1.4	11.1	0.79
0	20	+	0.3	6.0	15.1	2.55
0	20	–	0.1	5.0	12.2	2.44

accumulation in the culture liquid was not related to cell lysis.

The role of extracellular proteinases in the sporulation of bacilli remains unclear. There are experimental data on the correlation in time between the synthesis of proteolytic enzymes and sporulation in bacilli. It can be assumed that these processes are subject to common regulation [2]. For instance, ScoC factor, a member of the MarR family of transcription regulators in *Bacillus subtilis*, suppresses initiation of sporulation, proteinase production, and oligopeptide transport [10]. It is assumed that certain oligopeptides regulate the transfer of orthophosphate to protein Spo0A, which then plays a key role in the initiation of sporulation in bacilli [11]. There is no information on the proteinases that are secreted in sporulation stages V and VI. We believe that these proteins play an important role in the autolysis of the mother cell at the stage of spore release into the medium. In their study of the secretion of TasA protein, which controls synthesis of proteins of the endospore coat in *B. subtilis*, Serrano *et al.* [12] were the first to consider protein secretion by the mother cell as a phenomenon necessary for spore formation. The study of suppressor mutations in strain *B. subtilis* secA12 deficient in the synthesis of SecA protein revealed that the secretion function of translocase, which is composed of Sec proteins, is a necessary condition for normal sporulation [13].

To determine concentrations of peptone (X1) and inorganic phosphate (X2) ensuring the maximum accumulation of proteinases in the culture liquid of *B. intermedius*, a two-factor experiment was carried out according to the B2 scheme [14] with a three-level variation of the factors studied. The averaged data from three replicated measurements of biomass (in terms of optial density), enzyme activities, and cell productivity are presented in Tables 1 and 2.

By using the BIOPT software package, we obtained regression equations describing, with a confidence level of 95%, the effect of concentrations of peptone and inorganic phosphate on the activities of glutamyl endopeptidase ($2Y = 13.9 - 1.2X1 + 0.78X2 - 4X1^2 - 0.25X2^2$) and subtilisin ($2Y = 1.04 + 0.03X1 + 0.02X2 - 0.13X1^2 - 0.03X2^2$). From these equations, we calculated theoretical maxima of enzyme activities (Y) in points $X1$ (peptone) and $X2$ (inorganic phosphate), which proved to be in agreement with graphical representations of the experimental data (Figs. 3, 4). It was found that the concentrations of peptone and inorganic phosphate ensuring the maximum activity of glutamyl endopeptidase 2 (Y = 15.1) were 19.0 and 0.3 g/l, respectively, whereas the maximum activity of subtilisin 2 (Y = 1.047) was achieved at concentrations of peptone and inorganic phosphate of 22.0 and 0.24 g/l, respectively. The productivity optima coincided with the optima of proteinase activities.

It is known that the presence of an additional nitrogen source in the form of inorganic or organic salts produces a considerable effect on the synthesis and secretion of proteinases in microorganisms. There are data on the repression of the production and secretion of acid proteinase in *Candida albicans* by ammonium sulfate, ammonium chloride, and ammonium acetate [15]. On the other hand, ammonium chloride was shown to stimulate synthesis of proteinases secreted by *B. intermedius* 3-19 in the early stationary growth phase: glutamyl endopeptidase and thiol-dependent proteinase (by 15 and 7%, respectively) [5, 6].

We investigated the effect of ammonium ions in the form of inorganic (ammonium chloride) or organic (ammonium citrate) salts on the synthesis of protein-

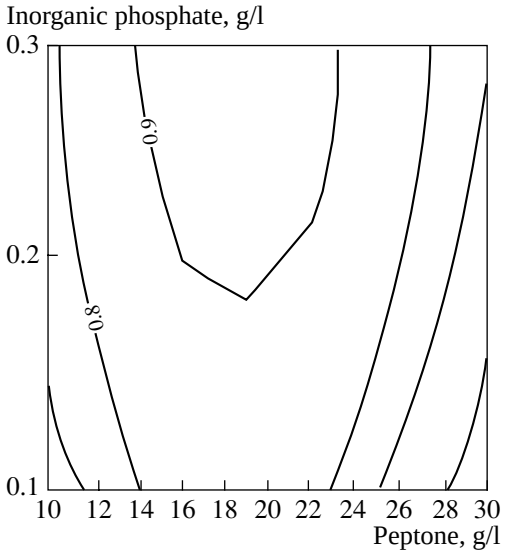

Fig. 3. Effect of peptone and inorganic phosphate on the accumulation of glutamyl endopeptidase 2 in the *B. intermedius* 3-19 culture. The maximum proteinase activity achieved in the experiment was taken as 1.0.

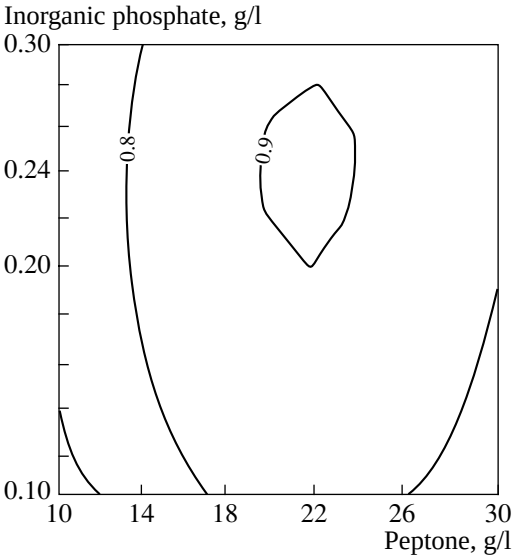

Fig. 4. Effect of peptone and inorganic phosphate on the accumulation of subtilisin 2 in the *B. intermedius* 3-19 culture. The maximum subtilisin activity achieved in the experiment was taken to be 1.0.

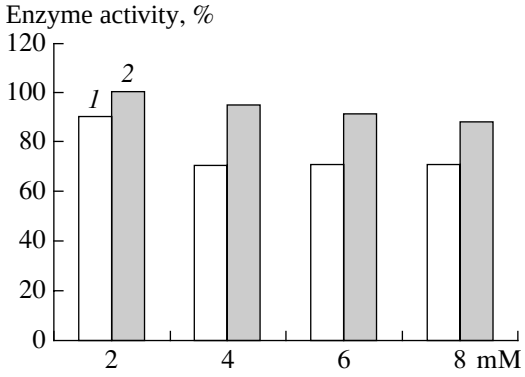

Fig. 5. Effect of ammonium ions on the synthesis of glutamyl endopeptidase 2 by *B. intermedius* 3-19. (*1*) Ammonium citrate; (*2*) ammonium chloride; $\sigma \leq 15\%$.

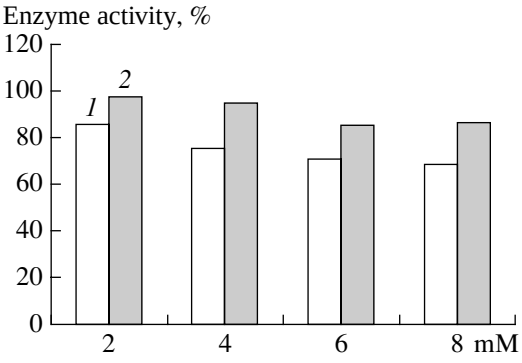

Fig. 6. Effect of ammonium ions on the synthesis of subtilisin 2 by *B. intermedius* 3-19. (*1*) Ammonium citrate; (*2*) ammonium chloride; $\sigma \leq 15\%$.

ases secreted by *B. intermedius* in the late stages of sporulation. As seen from Figs. 5 and 6, ammonium chloride in concentrations from 2 to 8 mM slightly decreased culture productivity with respect to proteinases (by 10–15%), whereas ammonium citrate markedly inhibited synthesis of both proteinases (by 30%).

Thus, the synthesis of proteinases secreted by *B. intermedius* 3-19 in the late stationary growth phase was inhibited by ammonium salts by the mechanism of end-product-controlled repression, which is typical of amino acid synthesis.

The results obtained indicate that the proteinases produced by *B. intermedius* 3-19 in the late stationary growth phase are secretory enzymes. The optimal composition of the medium ensuring the maximum enzyme production was established. Synthesis of proteinases was shown to be regulated by nitrogen-metabolite repression.

ACKNOWLEDGMENTS

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