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EXPERIMENTAL ARTICLES

Effect of Nutrients on the Accumulation of Glutamyl Endopeptidase in the Culture Liquid of *Bacillus intermedius* 3-19

E. V. Shakirov*, L. A. Gabdrakhmanova*[1], N. P. Balaban*, M. R. Sharipova*, G. N. Rudenskaya, and I. B. Leshchinskaya***

**Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Russia*

***Moscow State University, Vorob'evy gory, Moscow, 119899 Russia*

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Abstract—The effect of nutrients and growth conditions on the accumulation of glutamyl endopeptidase in the culture liquid of *Bacillus intermedius* 3-19 was studied. Glucose and other readily metabolizable carbon sources were found to suppress the production of the enzyme, whereas inorganic phosphate and ammonium cations enhanced it. Protein substrates, such as casein, gelatin, and hemoglobin, did not affect enzyme production. Some bivalent cations (Ca^{2+}, Mg^{2+}, Co^{2+}) increased the production of glutamyl endopeptidase, but others (Zn^{2+}, Fe^{2+}, Cu^{2+}) acted in the opposite way. The rate of enzyme accumulation in the culture liquid increased as the growth rate of the bacterium decreased, so that the maximum enzyme activity was observed in the stationary growth phase. Based on the results of this investigation, an optimal medium for the maximum production of glutamyl endopeptidase by *B. intermedius* 3-19 was elaborated.

Key words: proteinase, glutamyl endopeptidase, biosynthesis, catabolite repression, growth conditions.

Proteolytic enzymes are widely used in molecular biology to study the primary structure of proteins and peptides and the structural and functional organization of proteins.

Three groups of proteolytic enzymes differing in substrate specificity are presently known: (1) trypsin and trypsin-like proteases; (2) chymotrypsin, its analogues, and subtilisin-like proteinases; and (3) elastase-like enzymes.

In 1972, an enzyme cleaving bonds between the α-carboxylic groups of glutamic and aspartic amino acids was isolated from the culture liquid of *Staphylococcus aureus* and characterized [1]. This initiated the study of a large new group of serine proteinases, also called glutamyl endopeptidases, whose strict substrate specificity is determined by the presence of negatively charged side glutamyl and aspartyl residues in substrate molecules [2]. Investigations with the use of the chromogenic substrate Z-Glu-pNa showed that these proteases are widely spread among microorganisms, actinomycetes and bacilli in particular [3–5]. Being dominant in staphylococci, glutamyl endopeptidases are synthesized by sporogenous bacteria in relatively small amounts. The regulation of the biosynthesis of these enzymes in microorganisms is as yet poorly understood.

[1]Corresponding author. E-mail: leila.gabdrakhmanova@ksu.ru

Earlier, we showed that the streptomycin-resistant strain *Bacillus intermedius* 3-19 produces an extracellular thiol-dependent proteinase [6]. Later, this proteinase (which turned out to be glutamyl endopeptidase) was isolated and characterized [7, 8]; however, the conditions promoting its biosynthesis have not yet been investigated.

The aim of the present work was to elaborate a nutrient medium and determine the growth conditions that would be optimal for the maximum accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius* 3-19.

MATERIALS AND METHODS

The streptomycin-resistant strain *Bacillus intermedius* 3-19 (B-3833) was obtained from the All-Russia Collection of Industrial Microorganisms.

The basal medium for the cultivation of this strain contained (%) peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; and $MnSO_4$, 0.01 (pH 8.5). The medium was sterilized at 1 atm. Some components of the cultivation medium were sterilized separately and were added to the medium immediately before inoculation. These are solutions of inorganic phosphate (Na_2HPO_4), NH_4Cl, and other salts sterilized at 1 atm, solutions of carbon sources, casein, and gelatin sterilized at 0.5 atm, and a hemoglobin solution sterilized by boiling for 15 min.

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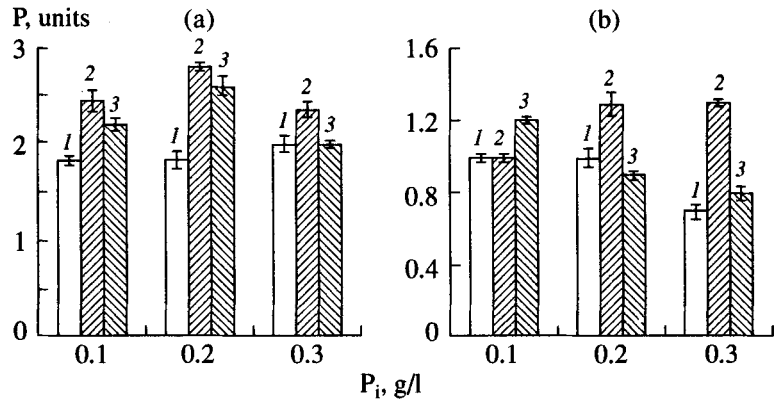

Fig. 1. Effect of peptone and inorganic phosphate on the accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius* 3-19: (a) 20 g/l peptone; (b) 30 g/l peptone; (*1*) peptone 1, (2) peptone 2, (*3*) peptone 3. P is the productivity of enzyme biosynthesis expressed in arbitrary units. P_i is inorganic phosphate in the medium.

Hemoglobin and gelatin were from Sigma (United States), casein Hammarsten was from Serva (Germany). Peptone 1 and peptone 2 of animal origin were purchased from the Semipalatinsk and Vinnitsa meat processing plants, and the peptone 3 of plant origin was from a factory in Tbilisi. These peptones differed in their inorganic phosphate content.

Cultivation was carried in flasks 20% full of the growth medium on a shaker (200 rpm) at 30°C. The medium was inoculated with 1 vol % of a 12- to 16-h-old culture grown in the presence of 500 μg/ml streptomycin. Culture growth was monitored nephelometrically.

Proteolytic activity was determined with *N*-carbobenzoxy-L-glutamic acid *p*-nitroanilide (Z-Glu-pNa). One unit of proteolytic activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per 1 min.

The efficiency of production of glutamyl endopeptidase was defined as the ratio of the proteolytic activity of the culture liquid to biomass and was expressed in arbitrary units. The specific growth rate was calculated by the formula $\mu = d(\ln D)/dt$. The specific rate of accumulation of glutamyl endopeptidase was calculated by the formula $\varepsilon = d(\ln A)/dt$.

Data were statistically processed as described in the handbook [9].

RESULTS AND DISCUSSION

The first stage involved a search for the type of peptone and the concentration of inorganic phosphate optimal for the maximum accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius*. As can be seen from data presented in Fig. 1, both peptone 2 and peptone 3 appeared to be suitable for the efficient production of glutamyl endopeptidase. Optimal concentrations of peptone and inorganic phosphate in the growth medium providing for the maximum accumulation of glutamyl endopeptidase were found to be 20 and 0.2 g/l, respectively. The production of glutamyl endopeptidase was almost independent of the origin of peptone (animal or plant) but depended on the total content of inorganic phosphate in the medium. The deficiency of inorganic phosphate in peptones (this is typical of some peptones of animal origin) could be compensated for by the addition of increased amounts of phosphate salts to the growth medium.

Based on data in the literature indicating that glucose suppresses the biosynthesis of many microbial proteases [10], we investigated the effect of this and some other sugars on the production of glutamyl endopeptidase by *B. intermedius*. It is evident from Fig. 2 that the presence of 0.5% glucose or maltose in the cultivation medium led to a twofold decrease in the production of glutamyl endopeptidase. Glucose and maltose at a concentration of 1% diminished the production of glutamyl endopeptidase by 75 and 55%, respectively, whereas higher concentrations of these sugars did not affect enzyme biosynthesis. Various concentrations of galactose, lactose, and sucrose in the cultivation medium also inhibited the production of glutamyl endopeptidase by *B. intermedius*, albeit to a lesser degree than glucose or maltose.

Therefore, like many other microbial proteases, the glutamyl endopeptidase of *B. intermedius* is subject to catabolite repression by easily metabolizable carbon sources. In connection with this, to improve the production of glutamyl endopeptidase, *B. intermedius* should be cultivated in media containing only peptone as the source of carbon and energy.

Using such media, we investigated the dynamics of growth and the accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius* (Fig. 3). According to a two-phase model proposed by Coleman *et al.* [11], the microbial synthesis of enzymes subject to catabolite repression primarily occurs in the phase of retardation or even complete cessation of growth. In accordance with this model, the glutamyl endopeptidase of *B. intermedius* appeared in the culture liquid in the phase of growth retardation, and its level peaked in the stationary phase. The specific rate of enzyme accu-

mulation (Fig. 3, curve *4*) was at a maximum when the specific growth rate of *B. intermedius* (Fig. 3, curve *3*) had already decreased to almost zero.

There is substantial evidence indicating that spore formation and the synthesis of alkaline proteinases are coordinately controlled [12]. Taking into account that *B. intermedius* 3-19 cells grown in a medium without glucose begin to produce spores on the 14th h of growth [6], we may state that the period of active accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius* 3-19 coincides with the period of spore formation.

Growth and metabolism of aerobic and facultatively anaerobic bacteria depend on the oxygen concentration in the cultivation media. Figure 4 shows the effect of aeration (the lower the ratio of the medium volume to the flask volume, the higher the degree of aeration) on the level of glutamyl endopeptidase in the culture liquid of *B. intermedius*. It is evident that the medium-to-flask volume ratio 1 : 4 is optimal for the maximum production of glutamyl endopeptidase.

We also investigated the effect of ammonium ions, as a supplementary source of nitrogen, on the production of glutamyl endopeptidase by *B. intermedius*. Figure 5 shows that the addition of NH_4Cl to the growth medium brought about a 15% stimulation of enzyme production, indicating that a combined addition of organic and inorganic nitrogen was beneficial for culture growth and enzyme accumulation.

It is known that bivalent cations are essential for the catalytic activity of enzymes. In particular, glutamyl endopeptidases can be considered Ca^{2+}-dependent enzymes [2, 3]. We have previously shown that Ca^{2+} cations exert a stabilizing and activating effect on the glutamyl endopeptidase of *B. intermedius* [7]. On the other hand, the effect of Ca^{2+} and other bivalent cations on the biosynthesis of this enzyme has not yet been investigated.

Figure 6 shows that the production of glutamyl endopeptidase was maximum at a concentration of Ca^{2+} cations in the medium equal to 5 mM (in the cultivation medium containing 1 mM Ca^{2+}, enzyme activity was 15% lower) and a Mg^{2+} concentration equal to 2 mM. At the same time, the presence of Mn^{2+} cations in the medium did not influence the production of glutamyl endopeptidase, while Fe^{2+}, Zn^{2+}, and Cu^{2+} at concentrations of 1–5 mM considerably suppressed it. Of much interest is the fact that Co^{2+} cations at a concentration of 3 mM increased the production of glutamyl endopeptidase by 2.5 times (Fig. 6, curve *6*) but inhibited culture growth.

The slight beneficial effect of Ca^{2+} and Mg^{2+} on the glutamyl endopeptidase activity of the culture liquid of *B. intermedius* was probably related to the stabilizing effect of these cations on the enzyme, whereas Co^{2+} cations evidently stimulated its production. It should be noted that other authors observed either an inhibitory action of Co^{2+} cations on the activity of proteinases or

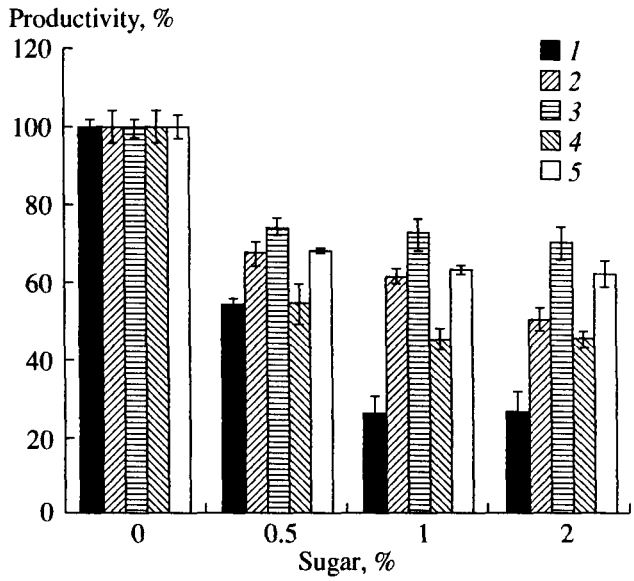

Fig. 2. Effect of sugars on the accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius* 3-19: (*1*) glucose; (*2*) galactose; (*3*) lactose; (*4*) maltose; and (*5*) sucrose. Productivity of enzyme biosynthesis in sugar-free medium was taken to be 100%.

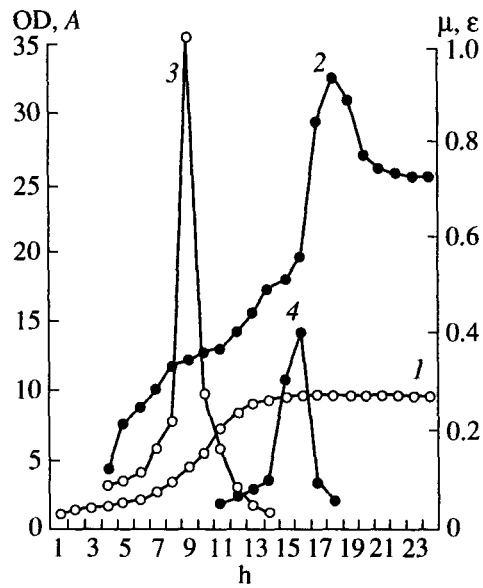

Fig. 3. Growth of *B. intermedius* 3-19 and the accumulation of glutamyl endopeptidase: (*1*) optical culture density OD_{590} expressed in units; (*2*) glutamyl endopeptidase activity *A* expressed in units/ml; (*3*) specific growth rate μ expressed in h^{-1}; (*4*) specific rate of glutamyl endopeptidase accumulation ε expressed in h^{-1}.

the absence of any effect [13, 14], and some reported a stabilizing effect on glutamyl endopeptidases [3, 15].

It is known that the addition of protein substrates to growth media can stimulate the biosynthesis of proteinases [6, 10]. However, we failed to reveal any stimulatory effect of casein, gelatin, and hemoglobin added to the medium at concentrations of 0.5 to 2% on the production of glutamyl endopeptidase by *B. intermedius*.

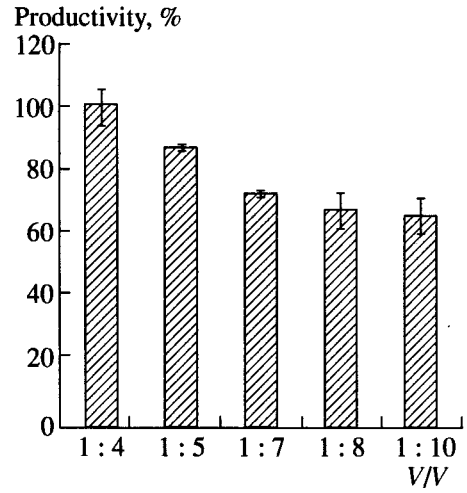

Fig. 4. Effect of aeration on the production of glutamyl endopeptidase by *B. intermedius* 3-19. The volume of flasks was 100 ml. The productivity of enzyme biosynthesis at the medium-to-flask volume ratio *V/V* = 1 : 4 was taken to be 100%.

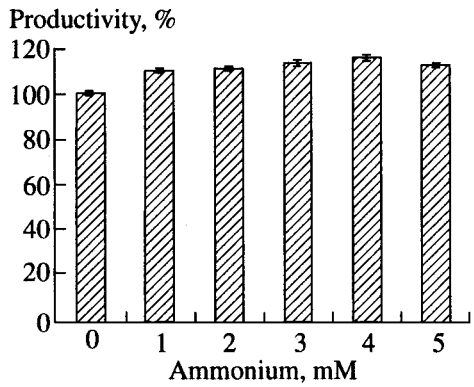

Fig. 5. Effect of ammonium on the accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius* 3-19. The productivity of enzyme biosynthesis without the addition of ammonium was taken tio be 100%.

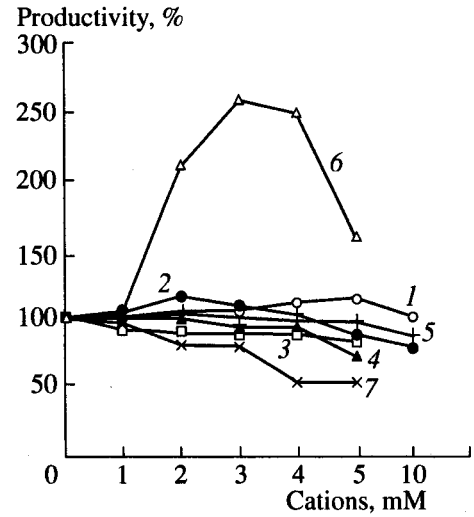

Fig. 6. Effect of bivalent cations on the accumulation of glutamyl endopeptidase in the culture liquid of *B. intermedius* 3-19: (*1*) Ca^{2+}, (*2*) Mg^{2+}, (*3*) Cu^{2+}, (*4*) Fe^{2+}, (*5*) Mn^{2+}, (*6*) Co^{2+}, and (*7*) Zn^{2+}.

To summarize, *B. intermedius* efficiently produces glutamyl endopeptidase in the medium containing peptone as the sole source of carbon and the following salts (%): $CaCl_2 \cdot 2H_2O$, 0.06; $MgSO_4 \cdot 7H_2O$, 0.05; NaCl, 0.3; $MnSO_4$, 0.01; Na_2HPO_4, 0.02; and NH_4Cl, 0.02 (pH 8.5).

The production of glutamyl endopeptidase by *B. intermedius* 3-19 and the biosynthesis of other serine proteinases have much in common, but greatly differ in the effect of Co^{2+} cations.

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