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EXPERIMENTAL ARTICLES

The Cellular Location of Proteolytic Enzymes of *Bacillus intermedius*

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Abstract—The activities of proteinases in the culture fluid and cellular fractions of *Bacillus intermedius* 3-19 grown under various conditions were studied. Thiol-dependent serine proteinase was the prevalent enzyme in the total pool of extracellular proteinases (70%); its catalytically active form was also detected in the cell membrane and, during active enzyme production, in the cell wall. Another enzyme, glutamyl endopeptidase (10% of the total pool), was detected in the cell membrane; it was also found in the cell wall and cytoplasm during active enzyme secretion into the growth medium. The production of these enzymes was maximal on medium containing inorganic phosphate and gelatin and decreased 2- to 4-fold on medium with glucose and lactate. The level of activity of extracellular enzymes correlated with that of corresponding membrane-bound proteins. The addition of $CoCl_2$ (2 mM) into the medium caused an essential increase in extracellular glutamyl endopeptidase activity and promoted the release of the membrane-bound enzyme into the culture fluid. Proteolytic activity towards casein was also detected in the cytoplasm. The proteinases localized in the cytoplasm were shown to differ in their properties from those secreted.

Key words: thiol-dependent serine proteinase, glutamyl endopeptidase, location, *Bacillus intermedius*

Representatives of the genus *Bacillus* secrete into the external medium a wide variety of neutral and alkaline proteinases, of which many have been thoroughly studied [1–4]. The amino acid sequences for some of these enzymes are known. Their genes have been cloned and their nucleotide sequences have been determined [4–7]. The expression of the genes coding for proteinases is known to proceed in parallel with the process of spore formation [8] and to be controlled by the *hpr*, *sac Q* (*Hy*), *sac U* (*Hy*), *prtR*, and *iep* genes [9, 10]. However, the regulation mechanisms of protease biosynthesis and secretion are still poorly understood [6, 7, 11].

Bacillus intermedius 3-19 secretes into the medium serine proteinases. The thiol-dependent proteinase is prevalent among them; it has been classified as subtilisin-like proteinase [1, 12]. This microorganism also secretes glutamyl endopeptidase (up to 10% of the total pool of extracellular proteinases). The latter splits specifically the peptide bonds formed by the carboxyl groups of glutamic acid and, to a lesser extent, of aspartic acid and, according to its properties, is referred to serine proteinases [2, 13]. The third proteinase (less than 5% of the total pool of extracellular proteinases) is active toward Glp-Ala-Ala-Leu-pNA, like common subtilisin. Both thiol-dependent proteinase and glutamyl endopeptidase were characterized, their kinetic and catalytic properties were studied, and their amino acid sequences were determined [12, 13]. The gene encoding glutamyl endopeptidase has been cloned and sequenced [14]. The synthesis of these enzymes are inhibited by glucose [15, 16]. Inorganic phosphate in the medium increases the yield of the enzymes. The activity of thiol-dependent proteinase was shown to be threefold higher upon the addition of gelatin to the medium [15]. The productivity of glutamyl endopeptidase increased twofold after the addition of $CoCl_2$ (2 mM) to the medium [16].

The present paper reports on the distribution of proteinases between the cellular fractions and the culture fluid of *B. intermedius* under various conditions of growth. These data may be helpful for the understanding of the processes of formation and secretion of extracellular enzymes.

MATERIALS AND METHODS

The streptomycin-resistant strain *Bacillus intermedius* 3-19 (Str 500) from the culture collection of the Microbiology Department, Kazan State University, was used in this work.

The basal medium for the cultivation of *B. intermedius* contained (%) peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.01, $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; $MnSO_4$, 0.01; pH 8.5. To the experimental variants of the medium, solutions

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Table 1. Extraction of glutamyl endopeptidase and thiol-dependent serine proteinase (U/g of cells) from intact cells and protoplasts of *B. intermedius* with the solutions of divalent metal salts and detergents

Solution	Z-Glu-pNA		Z-Ala-Ala-Leu-pNA	
	Cells	Protoplasts	Cells	Protoplasts
Tris–HCl buffer, pH 8.0, 0.5 M	12*	30*	100*	160*
$CaCl_2$, 1 M	10*	15**	70*	140*
$CaCl_2$, 2 M	8*	30*	80*	150*
$CaCl_2$, 3 M	8.5*	27*	76**	135*
$MgCl_2$, 1 M	7.5*	25*	80*	160*
$MgCl_2$, 2 M	8.3**	35**	100*	180**
$MgCl_2$, 3 M	10*	33*	110*	168**
NaCl, 1 M	6*	28*	42*	80*
NaCl, 2 M	6.4*	25*	60*	110*
NaCl, 3 M	8*	20*	50*	90*
EDTA, 0.1 M	7	0	20*	70*
Tween 20, 1%	5*	7**	40*	100*
Triton X100, 1%	5*	10*	40*	100*
Triton X305, 1%	8*	10*	60*	100*

* $\sigma \leq 15\%$; ** $\sigma \leq 20\%$.

of $CaCl_2$ (2 mM), Na_2HPO_4 (0.01%), glucose (1%), gelatin (1%), and sodium lactate (2%) were added before inoculation. Cultures were incubated on a shaker (200 rpm) in 1-l flasks containing 250 ml of the medium at 30°C. The cells of a 24-h culture grown on antibiotic-containing medium were used as the inoculum (1 vol %). Streptomycin sulfate (500 μg/ml) was added aseptically before cultivation. The cells were harvested by centrifugation and washed with 10 mM Tris–HCl buffer (pH 7.5) containing 10% sucrose, until the assay for residual activity in supernatant gave a negative result.

Cell lysate was obtained by treating cells with lysozyme (1 mg/ml, Serva, Germany) for 30 min at 37°C in 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA and 10 mM $MgSO_4$. Nucleic acids were precipitated with streptomycin sulfate: Cell lysate was incubated for 1 h at 4°C and centrifuged at 15000 *g* for 30 min. The supernatant was assayed for proteinase activity.

B. intermedius protoplasts and their lysates were prepared as described earlier [17]. Proteolytic activity was determined in the culture fluid, in the cell wall after separating it from protoplasts by centrifugation at 10000 *g* for 10 min, and in the cytoplasm obtained after the lysis of protoplasts followed by precipitation of nucleic acids with streptomycin sulfate.

Solubilization of the enzymes was achieved by treating intact cells and protoplasts with solutions of inorganic salts, EDTA, and detergents in 1 mM Tris–HCl buffer (pH 8.0). In the case of protoplasts, the buffer contained 20% sucrose. After incubation at room temperature for 20 min, the suspension was centrifuged at 10000 *g* for 30 min. The supernatant was assayed for proteinase activity.

Casein solution (2%, Serva, Germany) in 0.1 M Tris–HCl (pH 8.5) and the peptides Z-Glu-pNA and Z-Ala-Ala-Leu-pNA were used as substrates, and proteolytic activity was measured as described in [12, 13, 18]. The unit of caseinolytic activity was defined as the amount of enzyme releasing 1 μmol of tyrosine in 1 min. The unit of proteolytic activity toward peptide substrates was defined as the amount of the enzyme producing 1 μmol of *p*-nitroaniline (pNA) in 1 min under specific conditions. The results were multiplied by 1000. Specific proteinase activity was expressed in corresponding units per 1 g of cells (U/g).

The content of thiol-dependent proteinase and glutamyl endopeptidase in cell fractions was calculated from the specific activity of homogeneous proteinases toward the respective substrates [12, 13].

Statistical analysis of the data obtained was performed with Microsoft Excel for PC, using the standard deviation calculation and taking 20% to be an insignificant difference in the results.

RESULTS AND DISCUSSION

In order to study the location of proteolytic enzymes and the distribution of their activity between the culture fluid and the cellular fractions, stationary-phase (20-h) *B. intermedius* cultures undergoing sporulation were used. Bacteria were grown under conditions allowing activation or repression of extracellular proteinases by various factors.

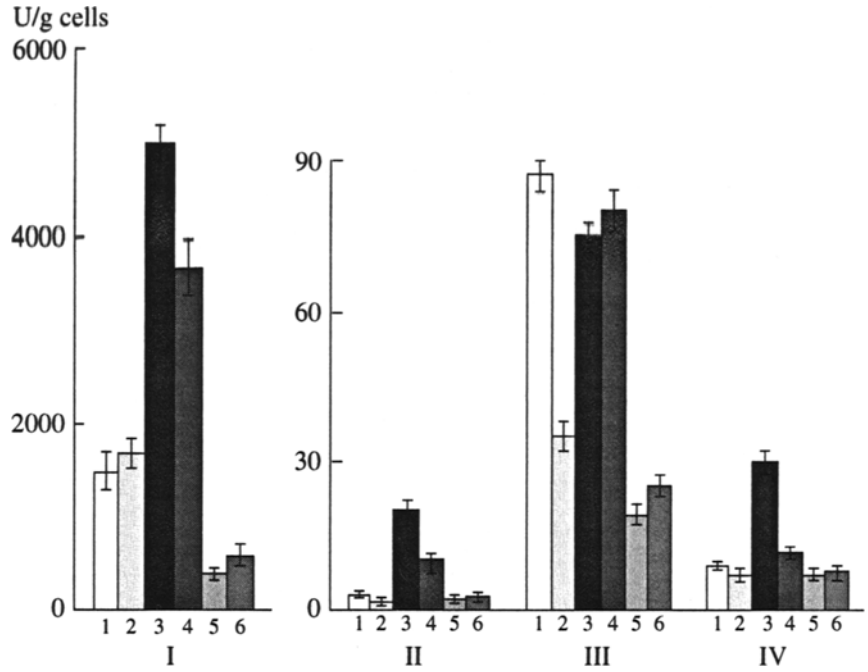

Fig. 1. Specific activity (U/g cells) toward casein in the (I) culture fluid, (II) cell wall, (III) membrane, and (IV) cytoplasm of *B. intermedius* 3-19 cells cultivated on (1) basal medium and media containing (2) 0.1 mM $CoCl_2$, (3) 0.01% Na_2HPO_4, (4) 1% gelatin, (5) 1% glucose, and (6) 2% lactate.

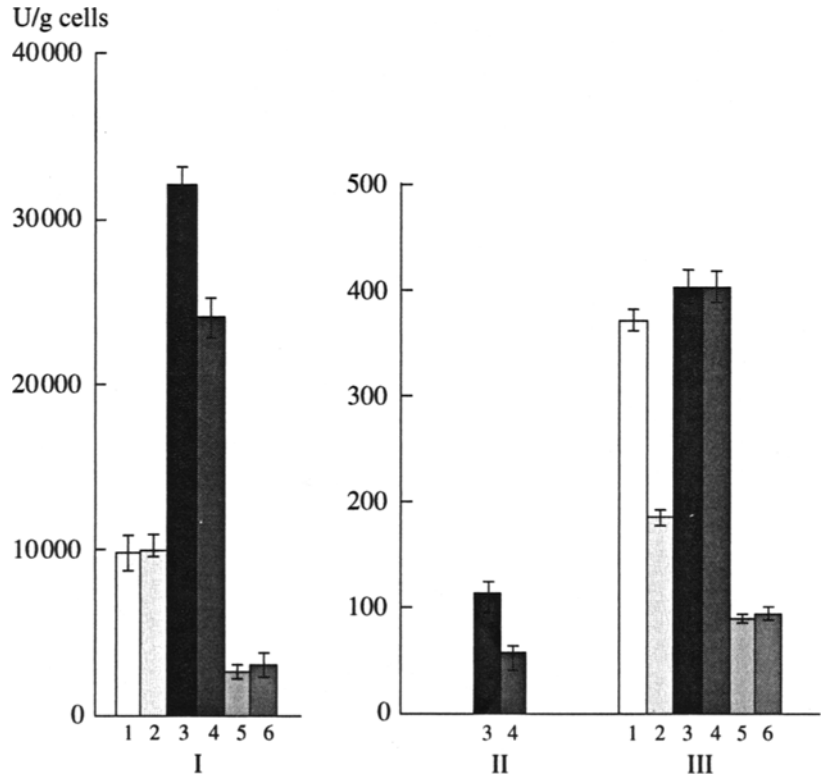

Fig. 2. Specific activity (U/g cells) toward Z-Ala-Ala-Leu-pNA in the (I) culture fluid, (II) cell wall, (III) membrane, and cytoplasm of *B. intermedius* 3-19 cells cultivated on (1) basal medium and media containing (2) 0.1 mM $CoCl_2$, (3) 0.01% Na_2HPO_4, (4) 1% gelatin, (5) 1% glucose, and (6) 2% lactate.

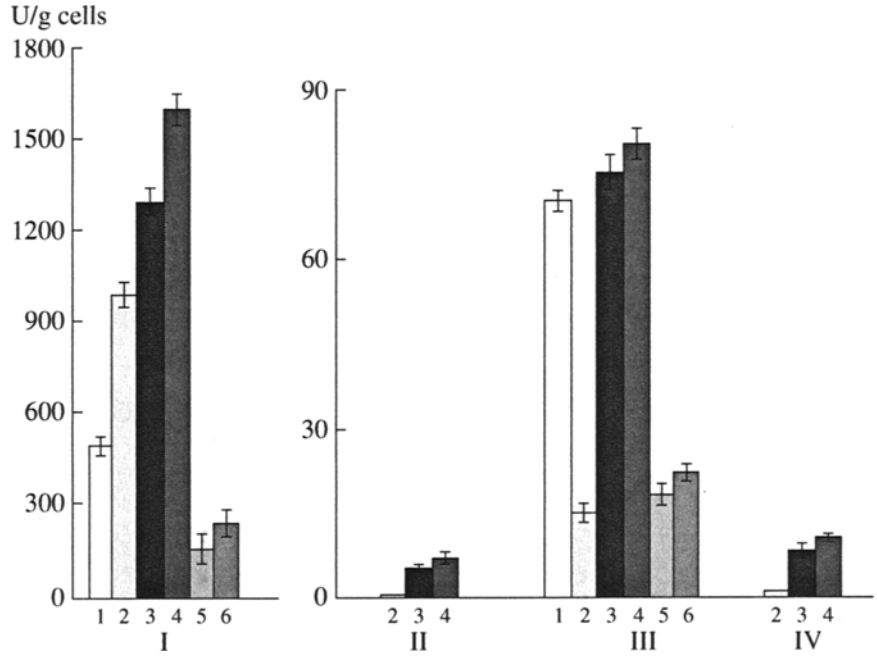

Fig. 3. Specific activity (U/g cells) toward Z-Glu-pNA in the (I) culture fluid, (II) cell wall, (III) membrane, and (IV) cytoplasm of *B. intermedius* 3-19 cells cultivated on (1) basal medium and media containing (2) 0.1 mM $CoCl_2$, (3) 0.01% Na_2HPO_4, (4) 1% gelatin, (5) 1% glucose, and (6) 2% lactate.

When cells were grown in the basal medium, caseinolytic activity was detected in total cell lysates, but activity toward peptide substrates was absent. The treatment of intact cells and protoplasts of *B. intermedius* with concentrated solutions of inorganic salts (1–3 M) and detergent solutions (1%) caused the release of proteinases (Table 1). Maximal release of proteinases from the cell surface was observed after treatment with solutions of metal salts ($MgCl_2$, $CaCl_2$, and NaCl in a concentration of 2 M). When the cells and protoplasts were treated with solutions of Tween 20 (1%), Triton X100 (1%) and Triton X305 (1%), the enzyme was solubilized less efficiently; a further increase in the detergent concentration to 5% did not promote the process. The release of proteinases from protoplasts was found to be twice more effective than that from intact cells.

The allocation of proteinases in the cells and distribution of their activity are reported in Figs. 1–3. To determine the total activity of the pool of proteinases, casein was used as the substrate. The activity of the dominant proteinases was measured using the chromogenic peptide substrates Z-Glu-pNA (for glutamyl endopeptidase) and Z-Ala-Ala-Leu-pNA (for thiol-dependent proteinase). 86–98% of the proteolytic activity toward all three substrates was found in the culture fluid. Inorganic phosphate (P_i) and gelatin enhanced the extracellular proteinase activity 3- to 4-fold, while glucose and Na-lactate decreased it 2- to 4-fold. The addition of $CoCl_2$ to the medium resulted in a 2-fold increase in the culture productivity with respect to extracellular glutamyl endopeptidase and had no effect on the enzymes active against casein and Z-Ala-Ala-Leu-pNA.

In contrast to the activity toward peptide substrates, caseinolytic activity was observed in all cell fractions: membrane, cytoplasm, cell wall (Figs. 1–3). The bulk of the activity toward peptide substrates could be solubilized from the protoplast surface (regardless of the growth conditions).

The activity of the membrane-bound enzymes was maximal on media containing P_i and gelatin and decreased 4-fold on media containing lactate and glucose, when the corresponding extracellular enzymes were repressed. In the presence of $CoCl_2$, the culture productivity with respect to membrane-bound glutamyl endopeptidase decreased 5-fold. It is probable that $CoCl_2$ promotes the release of the membrane-bound enzyme and formation of extracellular protein. Thus, the level of activity of extracellular enzymes correlated with that of corresponding membrane-bound proteins.

In the cell wall, glutamyl endopeptidase and thiol-dependent proteinase were detected when the cells were grown on media containing P_i and gelatin and when the activity of the extracellular enzyme was maximal. Caseinolytic activity in the cell wall was detected in all variants, supporting the idea that, in the pool of *B. intermedius* proteinases, other secreted enzymes exist.

No catalytically active form of thiol-dependent proteinase was found in the cytoplasm regardless of the cultivation conditions. Glutamyl endopeptidase was present in active form in the cytoplasm of maximally induced cells.

Table 2. Relative contents of glutamyl endopeptidase and thiol-dependent proteinase (% of the caseinolytic activity) in the cell fractions and culture fluid of *B. intermedius* 3-19

Fraction	Control medium	$CoCl_2$, 0.1 mM	Na_2HPO_4, 0.01%	Gelatin, 1%	Glucose, 1%	Lactate, 2%
Culture fluid						
Proteinase pool	100	100	100	100	100	100
GE*	11	40	17	28	20	15
TP**	70	59	72	70	60	60
Cell wall						
Proteinase pool	100	100	100	100	100	100
GE*	0	0	20	50	0	0
TP**	0	0	30	50	0	0
Membrane						
Proteinase pool	100	100	100	100	100	100
GE*	58	25	60	60	50	55
TP**	40	52	38	40	48	43
Cytoplasm						
Proteinase pool	100	100	100	100	100	100
GE*	0	0	>1	>1	0	0
TP**	0	0	0	0	0	0

Note. Caseinolytic activity was taken as 100% in each fraction.
* GE, glutamyl endopeptidase.
** TP, thiol-dependent proteinase.

Table 3. Effect of various reagents, temperature, and pH on the proteolytic activities (% of the control) in the cell fractions of *B. intermedius*

Inhibitors, temperature, and pH	Cell wall	Membrane			Cytoplasm
	Casein	Casein	Z-Glu-pNA	Z-Ala-Ala-Leu-pNA	Casein
37°C	100	100	100	100	100
50°C	147**	123*	200*	150*	128*
pH 7.5	100*	90*	95*	70*	100*
pH 8.0	100*	100*	100*	100*	90*
pH 10	70**	100*	20**	100*	40*
PMSF	50*	15**	100*	10**	105*
EDTA	88*	100*	105*	98*	0
o-Phenanthroline	80*	93*	106**	100*	0
p-CMB	44*	74*	100*	70*	28**
N-Ethylmaleimide	38*	30*	100*	59*	0
Dithioerythritol	40**	53*	100*	53*	0
Mercaptoethanol	50**	78*	100*	–	–
Urea, 2 M	64*	80**	100*	–	14**
Urea, 4 M	34**	20*	100*	–	0

Note: Proteolytic activity in the control was taken as 100%. "–" means that the determination was not carried out.
* $\sigma \leq 10\%$, ** $\sigma \leq 15\%$.

It is worth noting that the distribution of the enzyme activities varied depending on the conditions of cultivation. The proportion of extracellular enzymes increased up to 98% when the cultivation medium contained P_i and gelatin. On the contrary, when glucose and lactate were present in the medium, the proportion of enzymes located in the membrane increased to 5–11%. The proportion of glutamyl endopeptidase activity in the culture fluid was less (87–92%) than that of thiol-dependent proteinase (95–98%). In the membrane, the content of catalytically active glutamyl endopeptidase was higher (55–60%) than that of thiol-dependent proteinase (38–50%).

Our results show that extracellular and membrane-bound proteinases are repressed by certain easy assimilated carbon sources (glucose and lactate). It is well known that extracellular alkaline proteinases are involved in the initiation of bacterial sporulation [3, 8]. On the other hand, endogenous glucose inhibits the initial stages of sporulation [8]. It is possible that both proteinase biosynthesis and sporogenesis are regulated by catabolite repression [3].

The release of the bulk of proteinases into the growth medium is apparently related to the role of these enzymes during sporulation: they maintain a high rate of protein turnover and thus promote spore formation from endogenous sources. The fact that the content of membrane-bound proteinases is maximum in induced cells indicates that they, along with the extracellular enzymes, may play a role in the peripheral utilization of proteins. As follows from Figs. 1–3, the activity of membrane-bound enzymes did not exceed 80–90 U/g toward casein, 370–400 U/g toward Z-Ala-Ala-Leu-pNA, and 70–80 U/g toward Z-Glu-pNA.

From the values of specific activity of homogeneous proteinases toward respective substrates [12, 13], the amounts of glutamyl endopeptidase and thiol-dependent proteinase in the membrane and in the culture fluid (Table 2) were estimated. The obtained data show that thiol-dependent proteinase dominated the culture fluid (up to 72%); it was maximally active on medium containing P_i. The largest amount of extracellular glutamyl endopeptidase was observed on medium with gelatin (25%). In the cell wall, both enzymes were found only when bacteria were grown on media containing Pi and gelatin; their ratio was about 1 : 1. In the cell membrane, glutamyl endopeptidase was shown to be the dominant enzyme; its proportion in the pool of proteinases was 60%, with the exception of media containing $CoCl_2$, which caused the release of the membrane-bound enzyme into the medium. As follows from data in Table 2, the content of minor proteases in the membrane fraction was about 2%.

Thus, the value of the ratio of the total amount of glutamyl endopeptidase and thiol-dependent proteinase to the caseinolytic activity suggests that, in addition to these enzymes, *B. intermedius* cells secrete other proteinases (<5%). The medium containing P_i and gelatin is optimal for the synthesis of the major extracellular proteinases.

The effect of various inhibitors on the proteolytic activity in the cell fractions was studied (Table 3). Caseinolytic activity in the cell wall fraction decreased in the presence of phenylmethylsulfonyl fluoride (PMSF), EDTA, and *o*-phenanthroline. Probably, in addition to serine proteinase, a metalloproteinase also occurs in the pool of proteinases. When reagents against the thiol group were used, partial inhibition (up to 62%) took place. This may be evidence of the presence of a subtilisin-like thiol-dependent proteinase. The fact that the caseinolytic activity decreased at pH 10 suggests the presence of a neutral protease in the pool of proteolytic enzymes.

In the membrane fraction, PMSF but not EDTA or *o*-phenanthroline caused a decrease in the caseinolytic activity, indicating the dominant role of serine proteinases in this fraction. Inhibitory analysis of the proteolytic activity toward Z-Glu-pNA showed the enzyme in the membrane to be a glutamyl endopeptidase: it was resistant to PMSF, EDTA, and thiol reagents (Table 2). The inhibitory effect of thiol reagents on the activity toward Z-Ala-Ala-Leu-pNA and suppression of this activity in the presence of PMSF may indicate the presence of a subtilisin-like thiol-dependent proteinase. It is possible that the precursors of extracellular enzymes were detected in a catalytically active form in the membrane.

In the cytoplasm, PMSF did not affect caseinolytic activity; EDTA and *o*-phenanthroline completely inhibited the intracellular enzyme, and thiol reagents inhibited the enzyme by about 80%. The addition of 2–4 M urea caused denaturation of proteinases located in the membrane and in the cell wall. Thus, a proteinase activity dependent on the presence of metal ions and inhibited by reagents against sulfhydryl group was found in the cytoplasm. The results of inhibitory analysis of the proteolytic activity in the cell fractions allow us to suggest that secreted proteinases are located in the cell wall and membrane. Cytoplasmic proteinases differed from the membrane-bound and extracellular enzymes in their properties. Intracellular enzymes are usually enzymes with a narrow substrate specificity and low activity toward native proteins [1].

Thus, proteolytic enzymes of *B. intermedius* were shown to be secreted by cells into the culture fluid and were detected in the cell fractions. Thiol-dependent proteinase and glutamyl endopeptidase were mainly located in the surface cell structures: the membrane and the cell wall. Such a location is typical of exoenzymes. A common regulation of the formation of extracellular enzymes and corresponding proteins located in the membrane indicates that the latter also participate in the catabolism of extracellular substrates. The changes in the relative levels of proteinases in the cell fractions, correlating with changes in the growth conditions, may be evidence of the influence of environmental factors

on the secretion of enzymes into the medium. Proteolytic enzymes located in the cytoplasm differed in their properties and substrate specificity from those secreted.

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