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Isolation and Characterization of Glutamyl Endopeptidase 2 from *Bacillus intermedius* 3-19

N. P. Balaban[1]*, A. M. Mardanova[1], M. R. Sharipova[1], L. A. Gabdrakhmanova[1], E. A. Sokolova[1], A. V. Garusov[1], E. I. Milgotina[2], G. N. Rudenskaya[2], and I. B. Leshchinskaya[1]

[1]Faculty of Biology, Kazan State University, ul. Kremlevskaya 18, Kazan 420008, Russia; E-mail: Nelly.Balaban@ksu.ru
[2]Faculty of Chemistry, Lomonosov Moscow State University, Moscow 119899, Russia; fax: (7-095) 939-3181; E-mail: laboratoriahps@hotmail.com

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Abstract—The culture filtrate of *Bacillus intermedius* 3-19 was used for isolation by chromatography on CM-cellulose and Mono S columns of a proteinase that is secreted during the late stages of growth. The enzyme is irreversibly inhibited by the inhibitor of serine proteinases diisopropyl fluorophosphate, has two pH optima (7.2 and 9.5) for casein hydrolysis and one at pH 8.5 for Z-Glu-pNA hydrolysis. The molecular weight of the enzyme is 26.5 kD. The K_m for Z-Glu-pNA hydrolysis is 0.5 mM. The temperature and pH dependences of the stability of the proteinase were studied. The enzyme was identified as glutamyl endopeptidase 2. The N-terminal sequence (10 residues) and amino acid composition of the enzyme were determined. The enzyme hydrolyzes Glu4–Gln5, Glu17–Asp18, and Cys11–Ser12 bonds in the oxidized A-chain of insulin and Glu13–Ala14, Glu21–Arg22, Cys7–Gly8, and Cys19–Gly20 bonds in the oxidized B-chain of insulin.

Key words: proteinase, glutamyl endopeptidase, *Bacillus intermedius*, isolation, properties

In the stationary phase of growth bacteria secrete serine and neutral proteinases that play a role in adaptive processes, in particular in formation of spores. It is suggested that serine proteinases are involved both in initiation of sporulation and in synthesis of spore coat and germination of spores. It was demonstrated that proteinase TesA is secreted in the medium by sporulating cells of *Bacillus subtilis* and is involved in the formation of spore coat [1]. It was found that the product of the *clpP* gene is a proteinase that plays an important role in the phase of stationary growth of *B. subtilis*. This protein determinates cell growth in conditions of heat shock. In addition, synthesis of this proteinase is associated with expression of operons *spoIIA* and *spoIIB* [2]. The study of the proteolytic enzymes that are secreted by cells during the late stationary phase of growth is thought to be attractive due to the suggested interaction between proteinase synthesis and the process of spore formation.

Two serine proteinases were isolated from the culture filtrate of *Bacillus intermedius* 3-19: glutamyl endopeptidase and subtilisin (thiol-dependent serine proteinase) that are generated by the bacteria in the beginning of the stationary growth phase (early proteins). The enzymes were obtained in homogenous state and characterized [3-5].

During the late stationary phase of growth of *B. intermedius* 3-19 we detected two serine proteinases in the culture filtrate, referred to as glutamyl endopeptidase 2 and subtilisin 2 [6]. These "late" enzymes are designated with index 2 to distinguish them from the early enzymes that are synthesized in the beginning of the stationary growth phase.

The objective of this study was isolation and characterization of glutamyl endopeptidase 2 from *B. intermedius* 3-19 secreted in the late stationary growth phase.

MATERIALS AND METHODS

Bacterial strain. The streptomycin-resistant strain *B. intermedius* 3-19 from the collection of the Department of Microbiology of Kazan State University was used in this study. The bacterium was cultivated in the described earlier medium [4] at 30°C and 200 rpm for 42 h. The ratio between the flask volume and the volume of medium was 5 : 1. The cells were pelleted at 4500*g* for 50 min.

Reagents. CM-cellulose (Reanal, Hungary), Mono S HR 5/5 for FPLC, and Sephadex G-25 (Pharmacia, Sweden) were used for isolation of the enzyme.

* To whom correspondence should be addressed.

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Proteolytic activity was measured using as substrates 2% casein in 0.1 M Tris-HCl buffer (Serva, Germany), pH 8.5 [7], and synthetic chromogenic substrates Z-Glu-pNA and Z-Ala-Ala-Leu-pNA [8] that were synthesized in the laboratory of the Faculty of Chemistry of Lomonosov Moscow State University by the method of Houmard [9]. The quantity of the enzyme that hydrolyzes 1 μmol of the substrate during 1 min under experimental conditions was taken for a unit of enzymatic activity.

Protein was measured spectrophotometrically taking into consideration that concentration of protein 1 mg/ml corresponds to absorbance $A_{280} = 1$ in a 1 cm pathlength cuvette.

To determine Michaelis constant (K_m), we used the substrate Z-Glu-pNA at concentrations 0.2-3.3 mM. The value of K_m was determined graphically as described elsewhere [10]; the catalytic constant (k_{cat}) was calculated using the program ENZFITTER (Biosoft, Great Britain).

Isolation of the enzyme. The enzyme was isolated from 2 liters of culture medium as described earlier [4]. The proteins were rechromatographed on a Mono S column (FPLC) in 0.015 M sodium acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$. The proteins were eluted in a gradient of NaCl concentration (0.0-0.5 M) in the same buffer. The protein fraction that was active on the substrate Z-Glu-pNA was desalted on Sephadex G-25 and lyophilized.

Physical and chemical properties. The purity of the samples and the molecular weight of glutamyl endopeptidase was determined by electrophoresis in 12.5% polyacrylamide gel with 0.1% SDS. Amino acid composition of the enzyme was estimated after hydrolysis with 5.7 M HCl for 48 h at 105°C using a Hitachi 835 amino acid analyzer (Japan). The residues of semi-cysteine and methionine were detected after oxidation by performic acid. The N-terminal amino acid was determined by the method of Edman in the samples obtained after additional purification by chromatography on an Aquapore (Applied Biosystems, USA) column (4.6 × 100 mm) in a concentration gradient of acetonitrile (15-60%) with 0.1% trifluoroacetic acid for 40 min in conditions of HPLC. Then the protein was immobilized on an Immobilon P membrane and sequenced using a Knauer-816 instrument (Applied Biosystems) [4].

The isoelectric point of the enzyme was measured by isoelectric focusing in 5% polyacrylamide gel in the presence of 2% Biolyte 3/10 ampholytes in a mini-column IEF Cell (Bio-Rad, USA). A commercial kit of marker proteins (Serva) was used for calibration of the column [10].

Substrate specificity. The substrate specificity of the enzyme was determined as described earlier [8] using the following synthetic tetrapeptides: Z-Ala-Ala-Met-Glu-pNA, Z-Ala-Ala-Trp-Glu-pNA, Z-Ala-Ala-Phe-Glu-pNA, Z-Ala-Ala-Leu-Glu-pNA, Z-Gly-Ala-Ala-Glu-pNA, Z-Ala-Ala-Trp-Asp-pNA, Z-Ala-Ala-Leu-Asp-pNA, Z-Ala-Ala-Phe-Asp-pNA, Z-Ala-Ala-Met-Asp-pNA, Z-Gly-Ala-Ala-Asp-pNA, and Z-Glu-pNA. The quantity of the enzyme that hydrolyzes 1 mg of the substrate during 1 min under defined experimental conditions was taken as a unit of enzymatic activity.

The specificity of the enzyme on natural substrates was assessed on oxidized A- and B-chains of insulin. Solutions of substrates with concentration 1 mg/ml in 0.025 M Tris-HCl buffer with 5 mM $CaCl_2$ were incubated with enzyme solution with 100 : 1 ratio (by weight) for 4 h at 37°C. Dried hydrolyzates were separated using an Altex HPLC (Beckman, USA) on an Ultrasphere Octyl column (4.6 × 250 mm) using linear gradient H_2O–70% acetonitrile in the presence of 0.1% CF_3COOH. Absorbance of eluates was registered at 215 and 280 nm; the eluates were analyzed on a Hitachi 835 amino acid analyzer (Japan) [4].

Influence of inhibitors. The following compounds were tested: phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), ethylenediamine tetraacetic acid (EDTA), benzamidine (Sigma, USA) in molar ratio enzyme/inhibitor 1 : 100, and *p*-chloromercuribenzoate (*p*-CMB) in molar ratio 1 : 130. Protein inhibitors (duck ovomucoid, soybean trypsin inhibitor (Sigma), inhibitor from marine anemone [11]) were used in molar ratio 1 : 10. Inhibition was carried out for 1 h at 22°C, the residual activity was assessed afterwards by hydrolysis of Z-Glu-pNA under standard conditions.

Enzymatic characteristics. The pH optimum of the proteinase was measured using casein and Z-Glu-pNA in 0.1 M Tris-HCl buffer, pH 7.0-10.0, as the substrates. To estimate pH stability of the enzyme, the activity was measured by hydrolysis of Z-Glu-pNA after preliminary incubation for 2 h in 0.1 M Tris-HCl buffer, pH 7.5-10.0, in the presence and in the absence of 0.5 mM $CaCl_2$. The activity of the enzyme in the presence of Ca^{2+} without preliminary incubation was taken as 100%. The temperature optimum was measured by hydrolysis of Z-Glu-pNA incubating the reaction mixture at temperatures from 22 to 65°C. When thermostability of the enzyme was assessed, the enzyme solution was incubated beforehand for 1 h at 37, 50, and 55°C in the presence and in the absence of 0.5 mM $CaCl_2$; the activity was measured by hydrolysis of Z-Glu-pNA. The activity of the enzyme without preliminary heating was taken as the control.

Mathematical treatment of results was carried out with the Microsoft Excel program that was used for calculation of mean deviation (σ). The results were considered reliable, if $\sigma \leq 15\%$. The Student criterion was used as the criterion of reliability of difference, taking $p \leq 0.05$ as the reliable level of significance.

RESULTS AND DISCUSSION

Glutamyl endopeptidase of the late stationary phase of growth was isolated using ion-exchanging chromatography on CM-cellulose and high performance liquid

chromatography on a Mono S column using FPLC (Fig. 1). We obtained three protein fractions; the first eluted at 35 mM NaCl, the second at 55 mM NaCl, and the third at 65 mM NaCl. It was shown using chromogenic substrates Z-Glu-pNA and Z-Ala-Ala-Leu-pNA that the protein from the second fraction hydrolyses the substrate Z-Glu-pNA (this property is specific for the family of glutamyl endopeptidases), but it does not hydrolyze the substrate of subtilisin Z-Ala-Ala-Leu-pNA. Therefore, this protein is glutamyl endopeptidase 2.

Figure 1 demonstrates that the obtained protein fractions are not chromatographically homogenous. Rechromatography of glutamyl endopeptidase 2 resulted in a chromatographically homogenous protein preparation (Fig. 2). The data on purification of glutamyl endopeptidase 2 (Table 1) show that after rechromatography the specific activity of the enzyme increases 1.2-fold

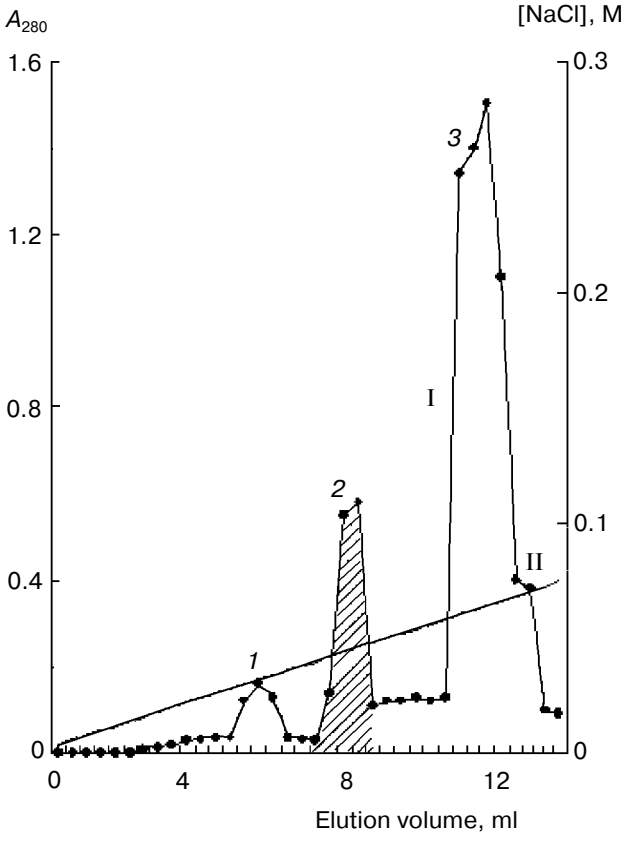

Fig. 1. Chromatography of proteinases from *B. intermedius* on the column Mono S: I) A_{280}; II) NaCl gradient (0-0.5 M) in 15 mM sodium acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$; *1*, *3*) fractions that contain enzymes that are active on the substrate for subtilisin Z-Ala-Ala-Leu-pNA; *2*) fraction that contains an enzyme that is active on the substrate Z-Glu-pNA (shaded).

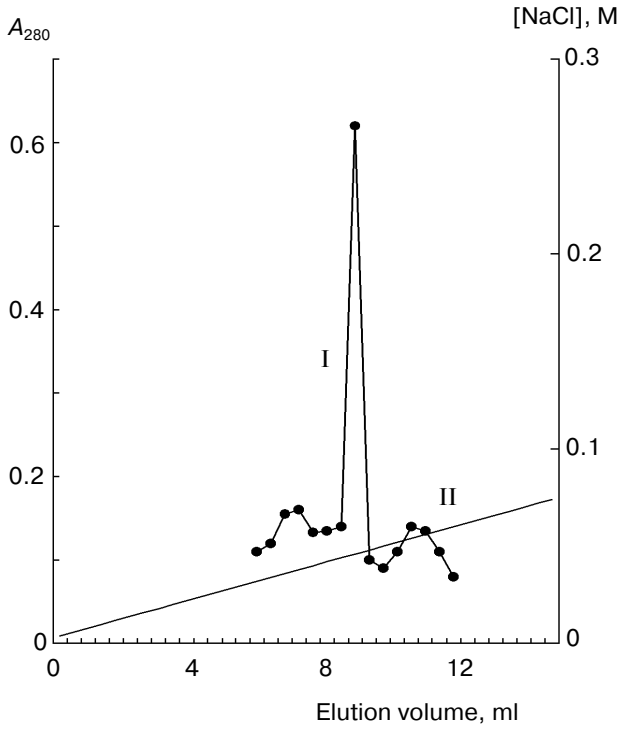

Fig. 2. Rechromatography of glutamyl endopeptidase 2 from *B. intermedius* on the Mono S column: I) A_{280}; II) NaCl gradient (0-0.5 M) in 15 mM sodium acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$. The major fraction contains the enzyme that is active on the substrate Z-Glu-pNA.

in comparison to the previous purification step. The yield of the enzyme was 19.6%.

The purity of the enzyme glutamyl endopeptidase 2 was also confirmed by electrophoresis in 12.5% polyacrylamide gel. The latter revealed the presence of a single polypeptide with molecular weight 26.5 kD (Fig. 3). The Michaelis constant (K_m) for this enzyme is 0.5 mM, k_{cat} = 81 sec^{-1}; i.e., glutamyl endopeptidase 2 binds the substrate more effectively in comparison to the enzyme of the early stationary growth phase (K_m = 6 mM) [4]. It appears that the composition or conformation of the substrate-binding region of glutamyl endopeptidase 2 differs from that of glutamyl endopeptidase 1 leading to the 12-fold increase of the binding to the substrate.

The isoelectric point of glutamyl endopeptidase 2 (8.4) is identical to that of glutamyl endopeptidase 1 from *B. intermedius* 3-19 [4].

Then we studied the effects of different inhibitors on the activity of glutamyl endopeptidase 2. The activity of the enzyme was inhibited by a specific inhibitor of serine proteinases, DFP. Another inhibitor of serine proteinases, PMSF, influences enzymatic activity insignificantly (Table 2). The enzyme is not sensitive to benzamidine,

Table 1. Isolation of glutamyl endopeptidase 2 from culture filtrate of *B. intermedius* 3-19

Purification step	V, ml	Protein, A_{280}	Activity		Purification	Yield, %
			total, units	specific, units/A_{280}		
Culture filtrate	1900	30 400	22.8	0.0008	1	100
Chromatography on CM-cellulose	42	113.3	14.04	0.126	157.5	61.6
Chromatography on Mono S column (FPLC)	17.6	5.46	4.69	0.86	1075	20.6
Rechromatography on Mono S column	7.0	4.41	4.48	1.02	1275	19.6

EDTA, and protein inhibitors; *p*-CMB decreases enzymatic activity insignificantly. Such resistance against the inhibitors was observed for many glutamyl endopeptidases, whose activities are completely inhibited only in the presence of DFP [4, 12-16]. Thus, the results indicate that the type of inhibition of glutamyl endopeptidase 2 resembles that of glutamyl endopeptidase 1 from *B. intermedius* 3-19 and of glutamyl endopeptidases from other microorganisms.

The substrate specificity of glutamyl endopeptidase 2 was studied using synthetic chromogenic tetrapeptides. The activity of glutamyl endopeptidase 2 on tetrapeptides with glutamic acid residue in position P_1 is almost 10^2-fold higher than on the tetrapeptides with aspartic acid residue (Table 3). Thus, the enzyme prefers the bonds formed by glutamic rather than by aspartic acid; this effect is typical for the enzymes from this group. For example, glutamyl endopeptidases from *B. subtilis*, *B. licheniformis*, *Staphylococcus aureus* V8, and *Streptomyces griseus* hydrolyze synthetic substrate Boc-Ala-Ala-Glu-pNA on the residues of glutamic acid by 100%, while the substrate Boc-Ala-Ala-Asp-pNA is hydrolyzed by these enzymes on the residues of aspartic acid by 0.8, 2.5, and 11%, respectively [12, 15, 17]. The amino acid in position

Table 2. Influence of inhibitors of the activity of glutamyl endopeptidase 2 from *B. intermedius* 3-19

Inhibitor	Substrate Z-Glu-pNA	
	molar ratio enzyme/inhibitor	residual activity, %
DFP	1 : 100	0
PMSF	1 : 100	84
EDTA	1 : 100	100
Benzamidine	1 : 100	100
p-CMB	1 : 130	82
Duck ovomucoid	1 : 10	100
Inhibitor from marine anemone	1 : 10	100
Soybean trypsin inhibitor	1 : 10	100

Table 3. Hydrolysis of synthetic substrates by glutamyl endopeptidase 2

No.	Substrate	Activity, units/A_{280}
1	Z-Ala-Ala-Met-Glu-pNA	1.62
2	Z-Ala-Ala-Trp-Glu-pNA	0.75
3	Z-Ala-Ala-Phe-Glu-pNA	1.86
4	Z-Ala-Ala-Leu-Glu-pNA	1.12
5	Z-Gly-Ala-Ala-Glu-pNA	1.12
6	Z-Glu-pNA	0.51
7	Z-Ala-Ala-Trp-Asp-pNA	0.03
8	Z-Ala-Ala-Leu-Asp-pNA	0.05
9	Z-Ala-Ala-Phe-Asp-pNA	0.02
10	Z-Ala-Ala-Met-Asp-pNA	0.05
11	Z-Gly-Ala-Ala-Asp-pNA	0.02

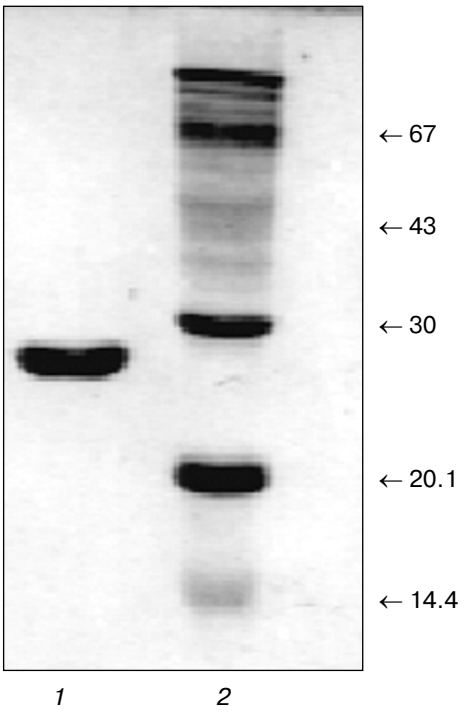

Fig. 3. Electrophoresis in polyacrylamide gel with SDS: *1*) glutamyl endopeptidase 2; *2*) markers: bovine serum albumin, ovalbumin, carboanhydrase, trypsin inhibitor, lysozyme. The molecular weights of the markers in kD are indicated on the right.

P_2 also plays an important, although not principal, role in the hydrolysis. Tetrapeptides with phenyl alanine and methionine in position P_2 were found to be preferred by glutamyl endopeptidase 2 from *B. intermedius* during hydrolysis of synthetic substrates. It was demonstrated for glutamyl endopeptidases from *B. subtilis* and *St. aureus* V8 that leucine residue in P_2 position increases enzymatic activity 10-fold, while serine, asparagine, and arginine residues in P_2 position are the strongest inhibitors of substrate hydrolysis [12, 17].

The specificity of the enzyme on the natural oligopeptide substrate was assessed by cleavage of oxidized A- and B-chains of insulin (each contains two residues of glutamic acid). In the oxidized A-chain of insulin the enzyme completely hydrolyzes during 4 h both bonds formed by glutamic acid Glu4–Gln5 and Glu17–Asp18 together with the bond Cys11–Ser12 formed by cysteic acid obtained after cysteine oxidation. It appears that the sulfo-group of cysteic acid imitates α-carboxyl groups of dicarboxylic acids allowing for hydrolysis of the bonds of cysteic acid in certain cases. The enzyme hydrolyzes the oxidized B-chain of insulin on two residues of glutamic acid Glu13–Ala14 and Glu21–Arg22, and also on two residues of cysteic acid Cys7–Gly8 and Cys19–Gly20 (Fig. 4). A similar effect was also observed for glutamyl endopeptidases from *B. subtilis* [12] and *Str. griseus* [15].

The study of the pH optimum of the enzyme using synthetic substrate Z-Glu-pNA demonstrated the pres-

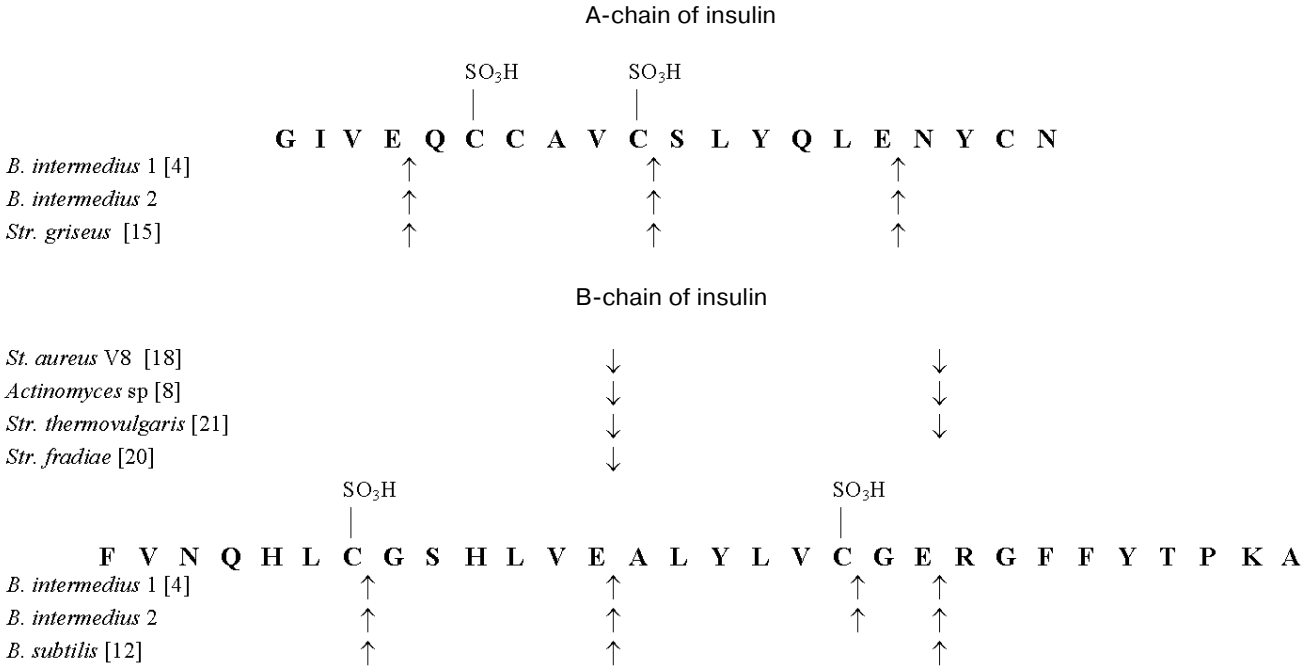

Fig. 4. Hydrolysis of oxidized A- and B-chains of insulin by bacterial glutamyl endopeptidases.

Table 4. Physical, chemical, and enzymatic properties of glutamyl endopeptidases 1 and 2 from *B. intermedius* 3-19

Properties	Glutamyl endopeptidase 1	Glutamyl endopeptidase 2
Molecular weight, kD (SDS-PAGE)	29	26.5
Amino acid composition	263	241
Michaelis constant (K_m), mM	6.0	0.5
Catalytic constant (k_{cat}), sec^{-1}	—	81
pH optimum		
on casein	7.5 and 9.0	7.2 and 9.5
on Z-Glu-pNA	8.0	8.5
pH stability		
with Ca^{2+}	7.5-9.0	7.0-10.0
without Ca^{2+}	7.5-8.0	8.0-8.5
Temperature optimum, °C		
with Ca^{2+}	65	55
without Ca^{2+}	55	50
Thermostability of the enzyme, °C		
with Ca^{2+}	22-50	37-55
without Ca^{2+}	22-37	22-37

Table 5. Amino acid composition of glutamyl endopeptidases from *B. intermedius* 3-19

Amino acid residue	Glutamyl endopeptidase	
	1	2
Asp	35	30
Thr	28	23
Ser	24	26
Glu	16	10
Pro	8	9-10
Gly	32	34
Ala	25	25
1/2 Cys	2	3
Val	18	13
Met	5	7
Ile	20	8
Leu	5	7
Tyr	16	14
Phe	7	9
Lys	11	10
His	2	2
Arg	8	7-8
Trp	1	—
Sum	263	241

ence of a single pH optimum at pH 8.5 in 0.1 M Tris-HCl buffer. Two optima were revealed for casein: at pH 7.2 and 9.5 (Table 4). These data are in accordance with the results obtained for glutamyl endopeptidases from *Staphylococcus* sp. [18, 19], proteinases from *Actinomyces* sp. and *Streptomyces thermovulgaris* [14]. In the presence of Ca^{2+}, the enzyme is stable in the pH interval 7.5-10.0, while in the absence of Ca^{2+} this interval narrows significantly to pH 8.0-9.0 and enzymatic activity decreases.

The temperature optimum of the enzyme on the synthetic substrate is 50°C in the absence of Ca^{2+}. In the presence of Ca^{2+} the temperature optimum shifts to 55°C with 30% increase in the enzymatic activity. This result confirms the stabilizing role of Ca^{2+}. Further increase in the temperature leads to strong decrease in the enzymatic activity.

Glutamyl endopeptidase 2 is highly thermostable in the presence of Ca^{2+}. Preliminary heating of the enzyme at 37, 50, and 55°C has almost no effect on its activity. In the absence of Ca^{2+} the enzyme is stable at 37°C, but its activity decreases at 50 and 55°C by 55 and 90%, respectively.

Amino acid composition of glutamyl endopeptidases 1 and 2 is presented in Table 5. The molecule of glutamyl endopeptidase 2 consists of 241 amino acid residues. The molecule of glutamyl endopeptidase 1 is 22 amino acid residues longer. Enzymes 1 and 2 contain two and three residues of semi-cysteine, respectively. In addition, these enzymes differ significantly in the content of Ile, Asp, Glu, Thr, and Val residues.

The N-terminal sequence of glutamyl endopeptidase 2 is compared with the N-terminal sequences of several known glutamyl endopeptidases in Fig. 5. The 10 amino acid-long region of the N-terminal sequence of glutamyl endopeptidase 2 completely coincides with the N-terminal sequence of glutamyl endopeptidase 1 from *Bacillus intermedius*. A high percent of identical amino acids is observed in comparison to glutamyl endopeptidases from *B. licheniformis* and *B. subtilis* (60 and 50%, respectively). This observation suggests a close relationship of these enzymes. Glutamyl endopeptidase 2 has much less homology (20-30%) with the N-terminal sequences of glutamyl endopeptidases from *Streptomyces* sp. and *Staphylococcus aureus* V8 [8, 14, 20-22].

The comparison of physical, chemical, and enzymatic properties of glutamyl endopeptidases 1 and 2 is presented in Table 4. The enzymes differ in molecular

B. intermedius 2	**V**	**V**	**I**	**G**	**D**	**D**	**G**	**R**	**T**	**K** –
	*	*	*	*	*	*	*	*	*	*	
B. intermedius 1 [4]	**V**	**V**	**I**	**G**	**D**	**D**	**G**	**R**	**T**	**K** –
		*	*	*		*		*	*	
B. licheniformis [17]	**S**	**V**	**I**	**G**	**S**	**D**	**D**	**R**	**T**	**R** –
			*	*		*		*	*	
B. subtilis [12]	**S**	**I**	**I**	**G**	**T**	**D**	**E**	**R**	**T**	**R** –
		*		*		*		*	*	
Thermoactinomyces sp.[13]	**S**	**V**	**L**	**G**	**T**	**D**	**E**	**R**	**T**	**R** –
		*	*	*		*				
Actinomyces sp.[8]	**S**	**V**	**I**	**G**	**F**	**D**	**V**	**Y**	**A**	**N** –
			*	*						
Str. thermovulgaris [21]	**S**	**D**	**I**	**G**	**T**	**N**	**T**	**G**	**W**	**M** –
		*		*						
Str. griseus [16]		**V**	**L**	**G**	**G**	**G**	**A**	**I**	**Y**	**G**	**G**
		*		*		*					
Str. fradiae [20]		**V**	**A**	**G**	**G**	**D**	**A**	**I**	**Y**	**G**	**G**
		*	*								
St. aureus V8 [22]		**V**	**I**	**L**	**P**	**N**	**N**	**D**	**R**	**K**	**Q**

Fig. 5. N-Terminal amino acid sequences of bacterial glutamyl endopeptidases.

weight (29 and 26.5 kD, respectively), Michaelis constant (6 and 0.5 mM, respectively) and in certain enzymatic properties. The data on the differences in properties and composition of glutamyl endopeptidases provide evidence to suggest the existence of isoforms of this enzyme. However, it remains obscure how these proteins are modified and how this process is regulated [23].

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