Glutamyl endopeptidase of *Bacillus intermedius*, strain 3-19

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Abstract A homogeneous glutamyl endopeptidase splitting peptide bonds of glutamic, rarely of aspartic acid residues in peptides and proteins, was isolated from *Bacillus intermedius* 3-19 culture filtrate using chromatography on CM cellulose and Mono S. The enzyme molecular mass is equal to 29 kDa, pI 8.4. The protease is inhibited by diisopropylfluorophosphate. The enzyme, like other glutamyl endopeptidases, reveals two pH optima at pH 7.5 and 9.0 for casein and one at pH 8.0 for Z-Glu-pNA hydrolysis. A 6 mM K_m is found for hydrolysis of the latter substrate. The enzyme activity optimum lies at 55°C, and it is stable at pH 6.5–11.0. Its N-terminal sequence shows 56% coinciding residues when compared with that of *Bacillus licheniformis* glutamyl endopeptidase.

Key words: Protease; Glutamyl endopeptidase; *Bacillus intermedius*; Isolation; Specificity

1. Introduction

Glu,Asp-specific proteases (glutamyl endopeptidases) that split specifically the peptide bonds formed by α-carboxyl groups of glutamic and — to a lesser extent — of aspartic acid belong to a subfamily of proteolytic enzymes. Initially, a protease endowed with this specificity pattern was isolated from *Staphylococcus aureus* V8 [1,2], then from *Staphylococcus aureus* 92gn [3], *Actinomyces* sp. [4], *Streptomyces thermovulgaris* [5,6], *Streptomyces griseus* [7], *Streptomyces fradiae* [8], two strains of *Bacillus licheniformis* [9,10].

The enzymes have been referred to serine proteases, but only scarce information has been collected on their structural organization. According to X-ray crystallography data, their tertiary structure seems to be related to that common for chymotrypsin family [11]. It was suggested that an unusual array of three histidine residues in *Streptomyces griseus* protease is responsible for its specific interaction with γ-carboxylate of the substrate glutamyl residue [12]; albeit, no homologous, His residues were detected in other glutamyl endopeptidases.

Thus, further research is necessary to characterize this subfamily of proteolytic enzymes. The present paper reports the isolation and characterization of glutamyl endopeptidase from *Bacillus intermedius* strain 3-19.

2. Materials and methods

2.1. Bacterial strain

Streptomycin-resistant strain *Bacillus intermedius* 3-19 was obtained from culture collection of Kazan State University microbiological department. The cells were grown as described earlier [13].

2.2. Reagents

CM cellulose was purchased from Reanal (Hungary), and Mono S HR 5/5 FPLC and Sephadex G-25 from Pharmacia (Sweden). Servalyte and glucagon were from Serva (Germany).

2.3. Proteolytic activity assessment

Casein solution (2%) in 0.1 M Tris-HCl buffer (pH 9.0), or Z-Glu-pNA [14], were used as substrates. The activity unit was defined as the amount of the enzyme capable to produce 1 μmol of *p*-nitroanaline (pNA) per min from Z-Glu-pNA under the specific conditions [3]. The value of K_m was determined using 0.2–3.3 mM Z-Glu-pNA.

2.4. Isolation of the enzyme

Sodium acetate buffers (pH 6.3) were used throughout the procedure. The protease was purified from 2 l of the culture filtrate by ion-exchange chromatography on CM cellulose column (4.5×3 cm), equilibrated and washed with 0.02 M buffer. The protease was eluted with 0.2 M buffer, then rechromatographed on CM cellulose column (1×4 cm). The enzyme was eluted with 0.15 M buffer, the eluate diluted 10 times and applied to Mono S HR 5/5 FPLC column, equilibrated with

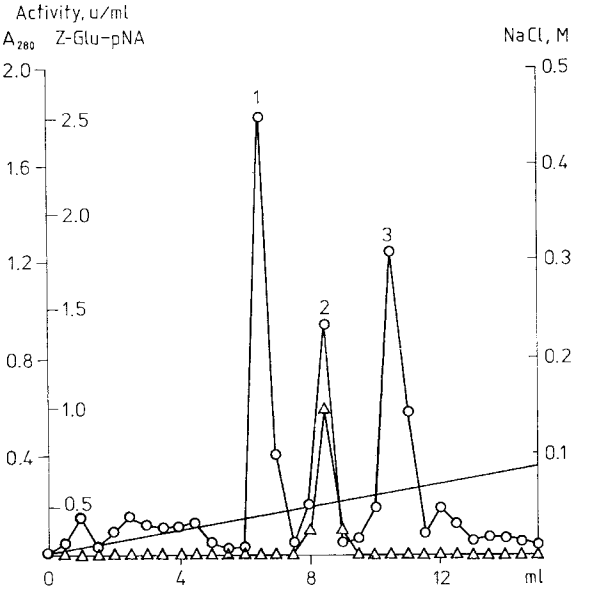

Fig. 1. Chromatography of *B. intermedius* glutamyl endopeptidase on Mono S. The mixture of enzymes obtained by CM cellulose chromatography was applied on Mono S HR 5/5 column. The enzymes were eluted with NaCl (0–0.5 M) concentration gradient in 15 mM acetate buffer (pH 6.3) containing 0.5 mM $CaCl_2$. ○, A_{280}; △, activity with Z-Glu-pNA. Fraction 2 contained pure glutamyl endopeptidase; fractions 1 and 3 contained subtilisin-like proteases to be described elsewhere.

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Abbreviations: DFP, diisopropylfluorophosphate; pNA, *p*-nitroanilide; Z, benzyloxycarbomyl; All amino acids are of the L configuration

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15 mM buffer, containing 5 mM $CaCl_2$. The protease was eluted with NaCl concentration gradient (0–0.5 M) in the same buffer (10 ml). Fraction 2 (Fig. 1) that showed proteolytic activity against Z-Glu-pNA was desalted on Sephadex G-25 and lyophilized.

2.5. Physico-chemical properties

The enzyme purity and its molecular mass were estimated by electrophoresis in 12.5% polyacrylamide gel in the presence of 0.1% of SDS. To determine pI, PBE-94 column was washed with 25 mM ethanolamine-HCl buffer (pH 9.4); then 2 mg of the protease in 2 ml of the buffer was applied on the column, which was eluted with Servalyte solution (pH 7.7). The amino acid composition of the protease and peptide fractions was determined after hydrolysis of samples with 5.7 N HCl for 48 h at 105°C with a Hitachi 835 amino acid analyzer (Japan). Half-cysteine and methionine residues were assayed after the protein oxidation with performic acid. The protease N-terminal sequence was determined after its additional purification by chromatography on 4.6×100 mm Aquapore column (Applied Biosystems, USA) using 15–60% acetonitrile gradient in the presence of 0.1% TFA. The protein was then immobilized on Immobilon P membrane and the N-terminal sequence was determined using Knauer 816 sequenator, connected to PTH On Line Analyser 120 A (Applied Biosystems, USA).

2.6. Substrate specificity

The protease specificity towards peptide substrates was tested as follows. Solutions (1 mg/ml) of Arg–Lys–Glu–Val–Tyr, Arg–Lys–Asp–Val–Tyr, oxydized insulin A-chain, B-chain or glucagon in 25 mM Tris-HCl buffer (pH 8.0), containing 5 mM $CaCl_2$, were incubated with 30 μg of the enzyme for 4 h at 37°C. Evaporated hydrolyzates were separated by HPLC on Ultrasphere Octyl column 4.6×250 mm, using a linear gradient from water to 70% CH_3CN in the presence of 0.1% CF_3COOH. The eluate was monitored at 215 and 280 nm then the amino acid composition of the fractions was assessed.

2.7. pH optima of the protease

pH optima of the protease were assessed against casein and Z-Glu-pNA as the substrates in the following buffers: 0.1 M Na-phosphate (pH 7.0–8.0), Tris-HCl (pH 7.5–9.0) and Na-glycine (pH 9.0–11.0). The same buffers were used to study pH effect on the protease stability. The activity was measured using Z-Glu-pNA as the substrate immediately after the mixing (= 100%), then after 3 and 24 h.

3. Results and discussion

3.1. Isolation of glutamyl endopeptidase

An extracellular glutamyl endopeptidase that splits specifically the peptide bonds of glutamic acid residues in peptides and proteins was isolated from *Bacillus intermedius* 3-19 culture filtrate using ion exchange chromatography on CM-cellulose followed by FPLC on Mono S that resulted in 770-fold purification of the enzyme with the yield of 12% (Table 1). The elution profile, observed in the course of chromatography on Mono S column, revealed three peaks which corresponded to proteases hydrolysing casein (Fig. 1). This paper describes a protease contained in the peak 2, active towards a chromogenic substrate for glutamyl endopeptidases Z-Glu-pNA. Homogeneity of this protease was confirmed by SDS-PAAG electrophoresis and chromatofocusing as well as by detection of a single N-terminal amino acid sequence.

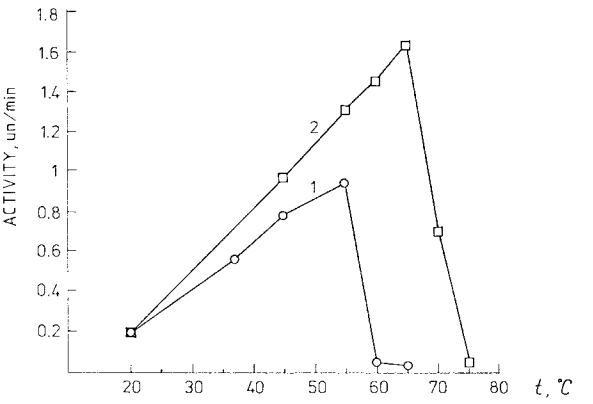

Fig. 2. Temperature dependence of glutamyl endopeptidase activity with Z-Glu-pNA was measured in: (1) 0.5 mM Tris-HCl buffer (pH 8.0) and (2) the same buffer containing 5 mM $CaCl_2$.

3.2. Enzyme characteristic

Molecular mass of 29 kDa estimated for the protease by SDS-PAAG electrophoresis is comparable with, although somewhat higher than, the values found for other glutamyl endopeptidases (Table 2). Its N-terminal sequence traced for 30 amino acid residues revealed 56% of identical residues when compared with the N-terminal sequence of *Bacillus licheniformis* glutamyl endopeptidase [9,10]:

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                  *** * ** * **  **    *   **
B. intermedius    VVIGDDGRTKVANTRVAPYNXXXXITFG-GS-
B. licheniformis  SVIGSDDRTRVTNTTAYPYRAIVHISSSIGS-
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Thus, glutamyl endopeptidases of these two *Bacillus* species are homologous and should possess the same fold of their polypeptide chains. Only limited similarity was observed between the enzyme N-terminal sequence and that of glutamyl endopeptidases from *Streptomycetes* and *Staphylococcus aureus* V8. Enzymatic properties of *Bacillus intermedius* protease and those of Glu,Asp-specific proteases from other sources are compared in the Table 3. *Bacillus intermedius* enzyme, like other glutamyl endopeptidases, has two pH optima of casein digestion at pH 7.5 and 9.0, whereas only one pH optimum at pH 8.0 was observed for hydrolysis of the chromogenic peptide substrate Z-Glu-pNA in 0.1 M Tris-HCl. K_m of the enzyme for Z-Glu-pNA hydrolysis at pH 8.0 is equal to 6 mM — the value within the range characteristic for other Glu,Asp-specific proteases – from 1.1 mM for *Actinomyces* sp. [4] to 10 mM for *Staphylococcus aureus 92 gn* [3].

The presence of Ca^{2+} ions enhances the enzyme activity and stability. Its specific activity was doubled in the presence of

Table 1
Isolation of *Bacillus intermedius* Glu,Asp-specific protease

Purification stage	Volume (ml)	Total protein (A_{280})	Activity		Yield (%)
			Total, units	Specific, units/A_{280}	
Culture filtrate	2.000	32.000	50	0.0016	100
Chromatography on CM cellulose	71	71	17	0.24	34
Rechromatography on CM cellulose	9.5	32.3	8.5	0.26	17
Chromatography on Mono S column	7.6	4.8	5.9	1.23	11.8

Table 2
Characteristics of bacterial glutamyl endopeptidases

Protease	Mol. mass Da	pI	pH Optimum of activity against		Stable at pH	Upper stability limit, °C
			Z-Glu-pNA	protein		
B. intermedius	29000	8.4	8.0	7.5; 9.0	6.5–11	55; 65 (Ca^{2+})
Str. thermovulgaris [5]	26000	6.7	6.5	6.5; 8.0	6.0–10	55
Actinomyces sp. [4]	25000	5.7	8.5	6.0; 9.0	6.0–10	55
S. aureus 92 gn [3]	25000	4.5	8.2	4.6; 8.0	5.0–10	40–45
S. aureus V8 [1]	26000	4.5	–	4.0; 7.8	3.5–9.5	45
B. licheniformis [9]	23567	4.8	8.0	–	4.0–10	51 (Ca^{2+})
B. licheniformis ATCC 14580 [10]	25000	9.0	8.0	–	4.0–10	–
Str. griseus [7]	18336	–	8.8	–	5.0–8.0	–
Str. fradiae [8]	18702	–	8.2	–	4.5–9.0	–

5 mM Ca^{2+} and remained constant after further increase of Ca^{2+} ions concentration to 30 mM. The enzyme activity in the absence of Ca^{2+} ions at pH 8.0 reveals an optimum at 55°C but drops sharply at 60°C. Temperature optimum displaces to 65°C in the presence of Ca^{2+} ions, due to the enzyme stabilization (Fig. 2). Analogous activity dependence on Ca^{2+} demonstrated Glu-specific endopeptidase from *Bacillus licheniformis* [10]. *Bacillus intermedius* protease was stable at 22°C for 3 h within 6.5–11.0 pH interval. The enzyme remains stable at pH 8.0 for 24 h in the presence of 5 mM Ca^{2+} up to 50°C, then it becomes inactivated. Similar properties were registered for *Actinomyces* sp. [4] and *Streptomyces thermovulgaris* [5] glutamyl endopeptidases.

The enzyme was completely inhibited by serine protease-specific inhibitor diisopropylfluorophosphate (DFP), but not by another commonly used inhibitor phenylmethylsulphonyl fluoride. It was not sensitive to EDTA, as well as to $Hg(CH_3COO)_2$. Several specific inhibitors of serine proteases, i.e. soybean trypsin inhibitor, ovomucoid, subtilisin inhibitor from *Streptomyces jantinus* [15] and inhibitor from sea anemone [16] failed to affect the enzyme activity.

3.3. Enzyme specificity

The enzyme splits preferably Glu–Xaa peptide bonds. Thus, the protease completely splitted Glu–Val bond in H–Arg–Lys–Glu–Val–Tyr–OH after 30 min, whereas only 5.6% of H–Arg–Lys–Asp–Val–Tyr–OH was hydrolyzed at Asp–Val bond. The enzyme splitted oxydized insulin A and B chains and glucagon completely after 4 h, cleaving the bonds as indicated by the arrows:

Insulin A-chain GIVE↓QCCASVC↓SLYQLE↓NYCN
Insulin B-chain FVNQHLC↓GSHLVE↓ALYVC↓GE↓RGFFYTPKA
Glucagon HSGGTFTSD↓YSKYLD↓SRAQD↓FVQWLMNT

Thus, *Bacillus intermedius* protease split preferably the peptide bonds formed by α-carboxyl of glutamic acid residue but, at least in a substrate devoided of glutamic acid residues, glucagon the peptide bonds of aspartic acid were also hydrolyzed. Cleavage of the bonds formed by cysteic acid, not present in proteins, confirmed that a negatively charged group in β- or γ-position of a substrate is of crucial importance for its specific hydrolysis by the glutamyl endopeptidase. Thus, the specificity of *Bacillus intermedius* glutamyl endopeptidase corresponds well to the pattern characteristic for other enzymes of the same subfamily. However, it should be indicated although that certain differences might exist between these enzymes, e.g. in more or less pronounced selectivity towards the peptide bonds formed by glutamic, rather than aspartic acid residues. It might be concluded that *Bacillus intermedius* glutamyl endopeptidase, like other Glu,Asp-specific bacterial proteases, belongs to a subfamily within chymotrypsin family of serine proteases.

Table 3
Amino acid composition of Glu,Asp-specific proteases

Amino acid	*B. intermedius*	*St. aureus* V8 [17]	*Str. griseus* [7]	*B. licheniformis* [18]	*Str. fradiae* [8]
Asx	35	35	15	18	18
Thr	28	19	19	28	21
Ser	24	9	25	32	23
Glx	16	23	4	11	5
Gly	32	21	34	29	31
Pro	8	12	4	10	3
Ala	25	15	22	12	19
1/2 Cys	2	0	4	4	4
Met	5	3	1	2	1
Val	18	19	20	13	20
Ile	20	15	7	13	7
Leu	5	8	5	5	5
Tyr	16	7	9	17	10
Phe	7	9	4	5	4
Lys	11	13	5	9	7
His	2	8	4	4	4
Arg	8	2	5	9	7
Trp	1	2	1	4	1
Total	261	220	188	222	187

References

[1] Drapeau, G.R., Boily, Y. and Houmard, J. (1972) J. Biol. Chem. 247, 6720–6726.
[2] Drapeau, G.R. (1978) Can. J. Biochem. 56, 534–544.
[3] Belyaeva, E.V., Rudenskaya, G.N., Stepanov, V.M. and Degteva, G.K. (1984) Prikl. Biokhim. Mikrobiol. (Appl. Biochem. Microbiol. Moscow) 20, 363–368.
[4] Mosolova, O.V., Rudenskaya, G.N., Stepanov, V.M., Khodova, O.M. and Tsaplina, I.A. (1987) Biokhimiya (Biochem. Moscow) 52, 414–422.
[5] Khaidarova, N.V., Rudenskaya, G.N., Revina, L.P., Stepanov, V.M., Egorov, N.S. (1989) Biokhimiya (Biochem. Moscow) 54, 46–52.
[6] Revina, L.P., Khaidarova, N.V., Rudenskaya, G.N., Grebenshchikov, N.I., Baratova, L.A. and Stepanov, V.M. (1989) Biokhimiya (Biochem. Moscow) 54, 846–850.
[7] Yochida, N., Tsuruyama, S., Nagata, K., Nirayama, K., Noda, K. and Makisumi, S. (1988) J. Biochem. 104, 451–456.
[8] Kitadokoro, K., Makamura, E., Tamaki, M., Horii, T., Okamoto, H., Shin, M., Sato, T., Fujiwara, T., Tsuzuki, H., Yoshida, N. and Teraoka, H. (1993) Biochim. Biophys. Acta 1163, 149–157.
[9] Svedsen, I. and Breddam, K. (1992) Eur. J. Biochem. 204, 165–171.
[10] Kakudo, S., Kikuchi, K., Kitadokoro, T., Fujiwara, E., Nakamura, H., Okamoto, H., Shin, M., Tamaki, M., Teraoka, H., Tsuzuki, H. and Yoshida, N. (1992) J. Biol. Chem. 267, 23782–23788.
[11] Kitadokoro, K., Tsuzuki, H., Okamoto, H. and Sato, T. (1994) Eur. J. Biochem. 224, 735–742.
[12] Nienaber, V.L., Breddam, K. and Birktoft, J.J. (1993) Biochemistry 32, 11469–11475.
[13] Itskovich, E.L., Znamenskaya, L.V., Balaban, N.P., Ershova, T.A. and Leshchinskaya, I.B. (1995) Mikrobiologiya (Microbiol. Moscow) 64, 623–629.
[14] Houmard, J. (1967) Eur. J. Biochem. 68, 621–628.
[15] Andreeva, N.A., Chermensky, D.N. and Bezborodov, A.M. (1978) Prikl. Biokhim. Mikrobiol. (Appl. Biochem. Microbiol. Moscow) 14, 25–31.
[16] Delphin, H., Dias, H., Chaves, M., Larionova, N.I., Kazanskaya, N.F., Rudenskaya, G.N. and Filippova, I.Yu. (1990) Vestn. Mosk. Univ. Ser. II, Chem. 31, 300–305.
[17] Carmona, C. and Cray, G.L. (1987) Nucl. Acids Res. 15, 6757.
[18] Svendsen, I., Jensen, M.R. and Breddam, K. (1991) FEBS Lett. 292, 165–167.