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Purification and Characterization of Serine Proteinase 2 from *Bacillus intermedius* 3-19

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Abstract—A proteinase secreted in the late stationary phase was isolated from the culture fluid of *Bacillus intermedius* 3-19 by ion-exchange chromatography on CM-cellulose followed by FPLC on a Mono S column. The enzyme was completely inhibited by the serine proteinase inhibitors diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride. The maximum proteolytic activity against the synthetic chromogenic substrate Z-Ala-Ala-Leu-pNA was observed at pH 9.0. The molecular weight of the enzyme is 28 kD and its isoelectric point is 9.2. We have also determined pH- and thermostability and K_m and k_{cat} of this proteinase. The enzyme has been classified as a thiol-dependent serine proteinase. N-Terminal amino acid sequence (10 residues) and amino acid composition of the protein were also determined. By the mode of hydrolysis of peptide bonds in the oxidized B-chain of insulin, this enzyme is similar to the thiol-dependent serine proteinase 1 from *B. intermedius* 3-19 secreted during vegetative growth.

Key words: proteinase, thiol-dependent serine proteinase (*Bacillus intermedius*), purification, properties

Gram-positive bacteria can quickly respond to changes in the environment and initiate sporulation in critical situations. The relation between spore formation and the synthesis of serine proteinases in bacilli and the role of these enzymes in maternal cell lysis and processing of spore envelope-forming proteins are discussed in the literature [1].

During the early stationary growth stage, *B. intermedius* cells secrete a serine proteinase into the surrounding medium; it is a member of the thiol-dependent serine proteinase subfamily of subtilisins. The enzyme has been purified to homogeneity, and its properties have been studied [2, 3]. In the late stationary growth stage, serine proteinases are also found in the culture medium of *B. intermedius*; one of these has been identified as a subtilisin-like proteinase [4]. The index 2 was assigned to this enzyme, which is synthesized in the late stationary growth stage, to distinguish it from the thiol-dependent serine proteinase 1 synthesized during the vegetative growth stage. Studies of physical, chemical, and enzymatic properties of this protein are necessary to determine the functional role of "late" proteins and particularly subtilisin-like proteinase 2, which is secreted just before the lysis of maternal cells and release of endospores into the medium [5].

The goal of the present study was to isolate the subtilisin-like proteinase 2 from the culture medium of *B. intermedius* at the late stationary growth stage and to determine the properties of this enzyme.

MATERIALS AND METHODS

Bacterial strain. The streptomycin-resistant strain *B. intermedius* 3-19 from the collection of the Department of Microbiology, Kazan State University, was used in this study. The culture medium was the same as described previously [5]. Bacteria were grown at 30°C on a shaker at 200 rpm for 46 h. The medium-to-flask volume ratio was 1 : 5. Cell-free culture fluid was obtained by centrifugation for 50 min at 4500*g*.

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

Proteolytic activity was determined using casein (2%) in 0.05 M Tris-HCl buffer (Serva, Germany),

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pH 8.5 [6], and the synthetic chromogenic substrates Z-Ala-Ala-Leu-pNA and Z-Glu-pNA [7] synthesized in the Faculty of Chemistry, Lomonosov Moscow State University, by the method of Houmard [8]. The enzyme amount providing the hydrolysis of 1 μmol substrate per 1 min (under the given experimental conditions) was taken as 1 unit of activity (U).

Protein was determined using a spectrophotometer at 280 nm assuming the optical density of 1 mg/ml protein solution to be 1 in a cuvette with path length of 1 cm.

Isolation of the enzyme. The enzyme was isolated from 2 liters of culture fluid of *B. intermedius* as described previously [9]. Re-chromatography was conducted on a Mono S FPLC column in 0.015 M sodium acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$. Proteins were eluted with 0-0.5 M NaCl gradient in the same buffer. Protein fractions hydrolyzing the substrate Z-Ala-Ala-Leu-pNA were desalted on Sephadex G-25 and then lyophilized.

Physical and chemical properties. The purity degree and molecular weight of the subtilisin-like proteinase 2 were determined by electrophoresis in 12.5% polyacrylamide gel with 0.1% SDS. The amino acid composition of the enzyme was determined on a Hitachi 835 amino acid analyzer (Japan) after hydrolysis with 5.7 M HCl for 48 h at 105°C. Semi-cystine and methionine residues were assayed after oxidation with performic acid. The N-terminal sequence was determined by the Edman method in samples additionally purified by chromatography on an Aquapore column, 4.6 × 100 mm (Applied Biosystems, USA), with 15-60% acetonitrile gradient in the presence of 0.1% trifluoroacetic acid for 40 min (HPLC mode). The protein was then immobilized on Immobilon P membranes and sequenced on a Knauer-816 sequencer (Applied Biosystems).

The Michaelis constant K_m was measured using the synthetic substrate Z-Ala-Ala-Leu-pNA dissolved in 20% dimethyl formamide (DMFA). The K_m value was determined from a plot as described previously [10], and the catalytic constant (k_{cat}) was calculated using the ENZFITTER software.

Isoelectric point of the enzyme was determined by the isoelectrofocusing in 5% polyacrylamide gel in the presence of 2% Biolyte 3/10 ampholines on the IEF Cell mini-column (Bio-Rad, USA). The column was calibrated with a protein calibration kit (Serva) [10].

Effect of inhibitors. Diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), tosyllysine chloromethyl ketone (TLCK), ethylenediamine tetraacetic acid (EDTA), *o*-phenanthroline (Sigma, USA), and $HgCl_2$ were used in the enzyme/inhibitor molar ratio of 1 : 100 and *p*-chloromercuribenzoate (*p*-CMB) was taken in the molar ratio of 1 : 130. The inhibitory proteins duck ovomucoid, soybean trypsin inhibitor (Sigma), and inhibitor from a sea anemone [11] were taken in the molar ratio of 1 : 10. The inhibition was conducted for 1 h at 22°C, and the residual hydrolytic activity against Z-Ala-Ala-Leu-pNA was determined under standard conditions.

Substrate specificity. Activity of the enzyme against natural substrates was determined using the oxidized B-chain of insulin as described previously [3]. The enzyme solution (10 μl, 1 mg/ml) in 0.02 M $NaHCO_3$, pH 8.5, was added to the substrate dissolved in the same buffer at the concentration of 1 mg/ml and incubated for 4 h at 37°C. Dried hydrolyzates were separated by HPLC on an Ultrasphere Octyl column, 4.6 × 250 mm, in a linear H_2O–70% acetonitrile gradient in the presence of 0.1% CF_3COOH. The optical density at 215 and 280 nm was measured in eluates, and the fractions were lyophilized, hydrolyzed with 5.7 M HCl at 105°C, and concentrated followed by analysis on a Hitachi 835 amino acid analyzer (Japan).

Enzymatic characteristics. The pH optimum of the protease was determined using casein and Z-Ala-Ala-Leu-pNA in 0.05 M Tris-HCl, pH 7.5-10.0, as substrates. The pH stability of the enzyme was determined using 0.05 M phosphate buffers, pH 6.0-8.0, and Tris-HCl buffers, pH 7.5-10.0, from the hydrolysis of Z-Ala-Ala-Leu-pNA after enzyme preincubation in a buffer for 2 and 22 h at room temperature. Temperature optimum was determined from the hydrolysis of Z-Ala-Ala-Leu-pNA in a reaction mixture containing 0.5 mM Ca^{2+} at temperatures ranging from 22 to 65°C. To determine the enzyme thermostability, the enzyme solution was pre-incubated for 1 h at 22, 37, 45, 50, and 55°C in presence or absence of 0.5 mM $CaCl_2$ with following measurement of the hydrolytic activity against Z-Ala-Ala-Leu-pNA. The enzyme activity without pre-warming was taken as the control.

Mathematical data processing was performed in the Microsoft Excel environment by the calculation of root-mean-square deviation (σ). The data were assumed significant at $\sigma \le 15\%$. Significance of differences was evaluated from the Student's *t*-test, and $p \le 0.05$ was taken as the criterion of significance.

RESULTS AND DISCUSSION

The subtilisin-like proteinase 2 synthesized by *B. intermedius* in the late growth stage was purified by ion-exchange chromatography on CM-cellulose followed by FPLC on a Mono S column (Fig. 1). Three protein fractions (*1*, *2*, and *3*) were collected, two of which (*1* and *3*) were active against the specific subtilisin substrate Z-Ala-Ala-Leu-pNA. We studied properties of the predominant protein (fraction *3*) named subtilisin-like proteinase 2. Re-chromatography of fraction *3* on the Mono S column was performed to prepare the chromatographically homogeneous protein (Fig. 2). This purification step resulted in twofold increase in proteinase purity in comparison to the previous purification step (Table 1). The

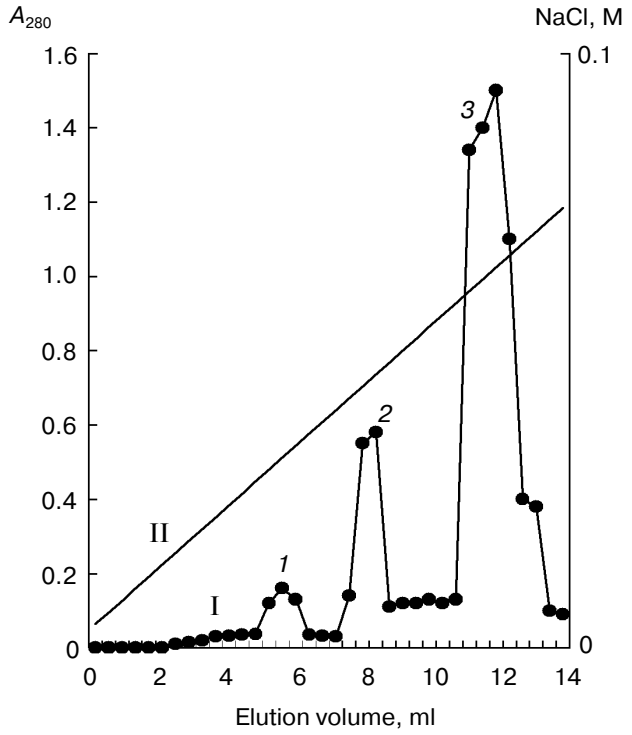

Fig. 1. FPLC of *Bacillus intermedius* proteinases on a Mono S column. I) A_{280}; II) NaCl gradient (0-0.5 M) in 15 mM sodium acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$; *1*, *3*) fractions that hydrolyze Z-Ala-Ala-Leu-pNA.

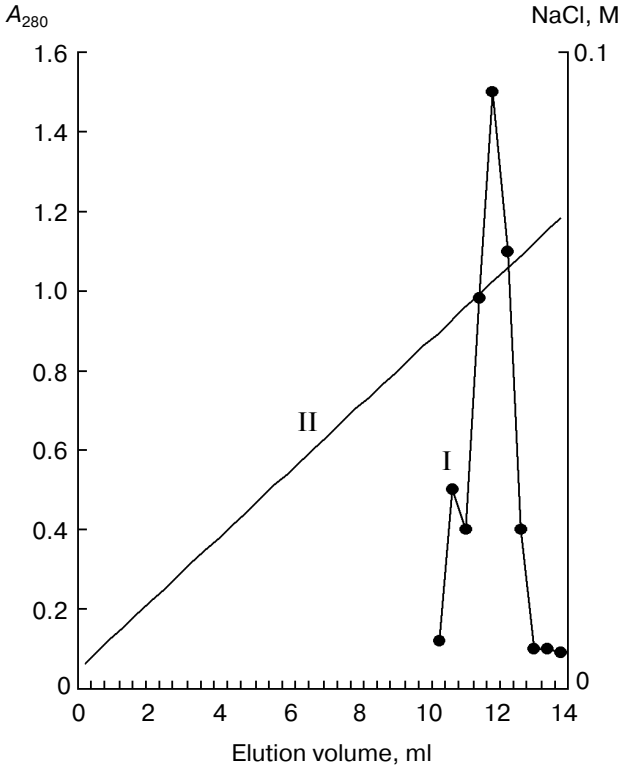

Fig. 2. Re-chromatography of *Bacillus intermedius* subtilisin-like proteinase 2 on a Mono S column. I) A_{280}; II) NaCl gradient (0-0.5 M) in 15 mM sodium acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$.

four-step purification procedure yielded the enzyme with the purity degree of 160 and yield of 11%.

The enzyme purity was confirmed by SDS-PAGE, which demonstrated a single polypeptide with molecular weight of 28 kD (Fig. 3). The K_m and k_{cat} values are 5.4 μM and 16,545 sec^{-1}, respectively. Interestingly, the Michaelis constant of this enzyme differs substantially from that of known subtilisins from microorganisms, such as subtilisin BPN′ (K_m = 1.86 mM) [12], *T. vulgaris* proteinase (K_m = 1.9 mM) [13], and *Str. thermovulgaris* proteinase (K_m = 0.3-0.4 mM) [14]. Significant differences in values of the constants are also observed between the *B. intermedius* 3-19 proteinases 1 and 2 (for proteinase 1, K_m = 1.25 mM and k_{cat} = 0.15 sec^{-1}) [3]. Thus, the subtilisin-like proteinase 2 secreted by the *B. intermedius* cells in the late stationary growth stage far more actively binds and cleaves the substrate in comparison to both the subtilisin-like proteinase 1 produced by *B. intermedius* at the early stationary growth stage and subtilisins from some other microorganisms.

The p*I* value (9.2) of subtilisin-like proteinase 2 is the same as that of subtilisin-like proteinase 1 [2].

The studies on the effect of enzyme inhibitors on the subtilisin-like proteinase 2 activity showed that the specific serine proteinase inhibitors PMSF and DFP com-

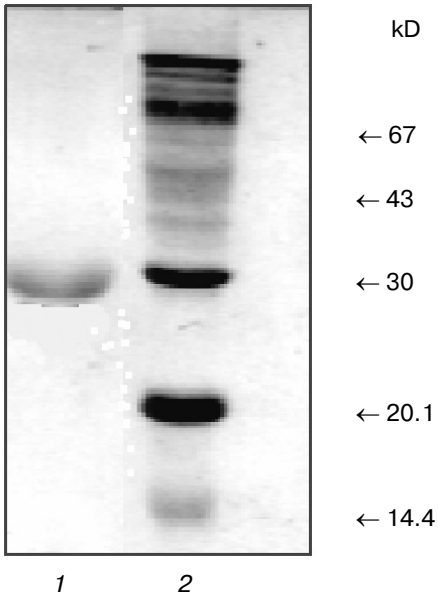

Fig. 3. SDS-PAGE of subtilisin-like proteinase 2 (*1*). *2*) Protein standards: bovine serum albumin (67 kD), ovalbumin (43 kD), carboanhydrase (30 kD), trypsin inhibitor (20.1 kD), and lysozyme (14.4 kD).

Table 1. Isolation of subtilisin-like proteinase 2 from culture fluid of *Bacillus intermedius* 3-19

Purification step	Volume, ml	Protein, A_{280}	Activity		Purity degree	Yield, %
			total, U	specific, U/A_{280}		
Culture fluid	1900	30400	4921	0.162	1	100
Chromatography on CM-cellulose	42	111.3	1218	10.9	67	24.8
Chromatography on Mono S (FPLC)	40	48	612	12.8	79	12.74
Re-chromatography on Mono S	30	21	543	25.9	160	11.0

pletely inhibit the activity of the enzyme (Table 2). The metalloproteinase inhibitors EDTA and *o*-phenanthroline and the trypsin-type proteinase inhibitor TLCK do not inhibit activity of the enzyme. Subtilisin-like proteinase 2 is inhibited by thiol-specific reagents, namely mercuric salts (by 70%) and *p*-CMB; the latter inhibits the enzyme activity by only 25%, possibly because of the large size of its molecule. Natural inhibitory proteins differ in their effect on the subtilisin-like proteinase 2 activity; duck ovomucoid inhibits the activity by 70%, soybean trypsin inhibitor by 25%, whereas the inhibitor from a sea anemone has no effect on the enzyme activity (Table 2).

Thus, the subtilisin-like proteinase 2 from *B. intermedius* resembles both thiol-dependent subtilisins from *Str. thermovulgaris* [14] and *T. vulgaris* [13, 15] and thiol-dependent serine proteinase 1 from *B. intermedius* [16] in its inhibition spectrum and appears to be a member of the thiol-dependent serine proteinase subfamily of subtilisins.

We determined the substrate specificity of thiol-dependent serine proteinase 2 against natural oligopeptide substrates from the cleavage of oxidized B-chain of insulin (Fig. 4). Multiple peptide fragments produced in the course of hydrolysis are indicative of broad substrate

Table 2. Effect of inhibitors on the activity of *Bacillus intermedius* 3-19 subtilisin-like proteinase 2

Inhibitor	Enzyme to inhibitor molar ratio	Residual activity, %
DFP	1 : 100	0
PMSF	1 : 100	0
TLCK	1 : 100	100
EDTA	1 : 100	100
o-Phenanthroline	1 : 100	100
p-CMB	1 : 130	75
$HgCl_2$	1 : 100	32
Duck ovomucoid	1 : 10	30
Inhibitor from sea anemone	1 : 10	100
Soybean trypsin inhibitor	1 : 10	75

Table 3. Properties of *Bacillus intermedius* 3-19 subtilisin-like proteinases

Property	Subtilisin-like proteinase 1 [2, 3, 16]	Subtilisin-like proteinase 2
Molecular weight	32.5 kD	28 kD
K_m	1.25 mM	0.0054 mM
k_{cat}	0.15 sec^{-1}	16545 sec^{-1}
pH optimum:		
casein	10.0	8.5
Z-Ala-Ala-Leu-pNA	8.5	9.0
pH stability	6.3-11.0	6.3-9.5
Temperature optimum (Ca^{2+})	50°C	55°C
Thermostability (Ca^{2+})	22-45°C	22-45°C
p*I*	9.2	9.2

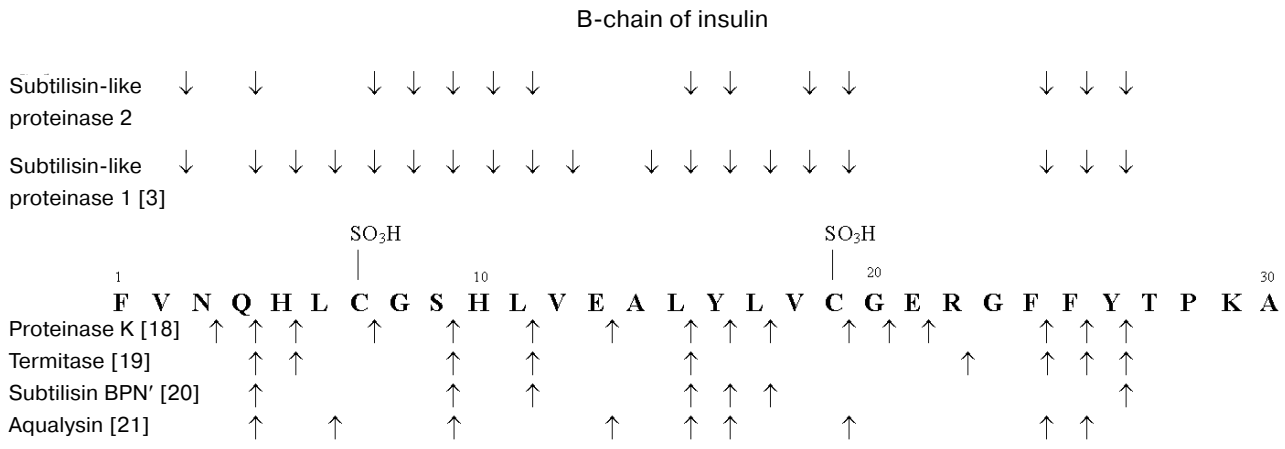

Fig. 4. Hydrolysis of the oxidized B-chain of insulin by bacterial subtilisins.

specificity of the enzyme, which is characteristic of typical subtilisins [17]. Thiol-dependent serine proteinase 2 actively hydrolyzes the bonds formed by carboxylic groups of hydrophobic residues of leucine, phenylalanine, and tyrosine (Leu11–Val12, Leu15–Tyr16, Phe24–Phe25, etc.) and hydrophilic serine, cysteine, and glutamine (Ser9–His10, Cys7–Gly8, Gln4–His5, etc.). Proteinase 2 resembles thiol-dependent proteinase 1 from *B. intermedius* [3] in the mode in which it hydrolyzes peptide bonds in oxidized B-chain of insulin. When com-

Table 4. Amino acid composition of microbial subtilisins

Amino acid	*B. intermedius* subtilisin-like proteinase 2	*B. intermedius* subtilisin-like proteinase 1 [2]	Subtilisin Carlsberg [23]	Subtilisin BPN′ [22]	Proteinase K [25]	Termitase [24]	*Str. thermovulgaris* proteinase [14]
Asx	32	43	28	28	31	35	37
Thr	19	21	19	13	21	21	26
Ser	29-30	31	32	37	36	29	31
Glx	14	20	12	15	12	14	19
Gly	31	38	35	33	34	33	38
Pro	13	17	9	14	9	13	14
Ala	38	48	41	37	33	44	48
½ Cys	7	1-3	0	0	5	1	2
Met	5	1	5	5	5	1	1
Val	31	33	31	30	19	23	23
Ile	14	15	10	13	10	14	15
Leu	17	20	16	15	10	9	10
Tyr	6	6	13	10	17	15	16
Phe	3	4	4	3	6	3	5
Lys	8	10	9	11	8	10	10
His	7-8	5	5	6	4	4	4
Arg	3	6	4	2	12	5	6
Trp	—	–	1	3	5	3	3
Total	279-280	319-321	274	275	277	277	308

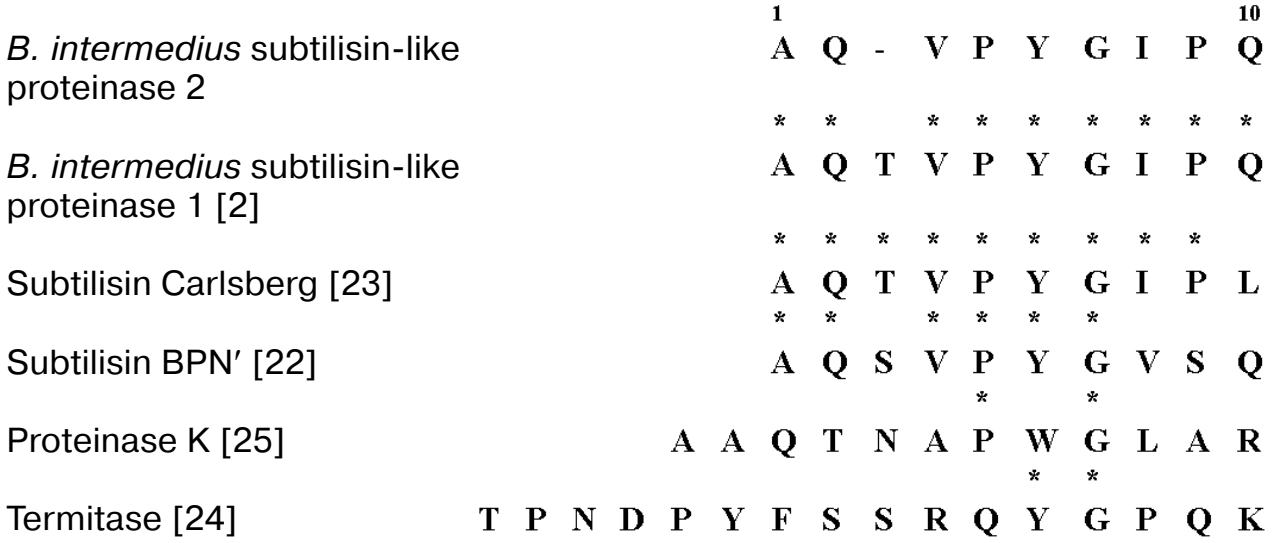

Fig. 5. N-Terminal amino acid sequences of bacterial subtilisins.

pared to other known proteinases, such as proteinase K, termitase, subtilisin BPN′, and aqualysin [18-21], the thiol-dependent serine proteinase 2 from *B. intermedius* resembles proteinase K from the fungus *Tritirachium album* in substrate specificity.

Maximum hydrolytic activity of thiol-dependent serine proteinase 2 was found at pH 9.0 against the synthetic substrate Z-Ala-Ala-Leu-pNA and at pH 8.5 against casein (Table 3). The enzyme is stable at pH values from 6.3 to 9.5 for 2 h, but significant loss of activity is observed at higher pH values and when preincubation is extended to 22 h.

Temperature optimum of the enzyme is 55°C. The enzymatic activity increases by 20% in presence of Ca^{2+}. The enzyme is stable at temperatures from 22 to 45°C in the presence of Ca^{2+}. At 50°C, the enzyme loses up to 35% of its activity in presence and up to 80% in absence of Ca^{2+}. Further increase of pre-incubation temperature leads to a profound activity drop.

The thiol-dependent serine proteinase 2 molecule consists of 280 amino acid residues, 40-41 amino acid residues shorter than subtilisin-like serine proteinase 1, and is distinguished from typical subtilisins by enhanced content of Asp residues (Table 4). Since the isoelectric point of the enzyme is higher than 9, one can assume that aspartic acid (and probably glutamic acid as well) are present in the form of amide. The thiol-dependent serine proteinase 2 molecule is enriched also with cysteine, methionine, and histidine compared to molecules of thiol-dependent serine proteinase 1 [2], subtilisins BPN′ and Carlsberg, and known thiol-dependent proteinases [22-24]. The N-terminal sequences of thiol-dependent serine proteinases 1 and 2 from *B. intermedius* were compared and found to be the same over a distance of 10 amino acids (Fig. 5). It is worth noting that subtilisin 2 more closely resembles classic subtilisins than thiol-dependent proteinases in N-terminal sequence of 10 amino acid residues. Its N-terminal sequence matches that of subtilisin Carlsberg by 90% and subtilisin BPN′ by 70%; far less homology (20%) is observed with termitase and proteinase K [22-25].

Thus, the subtilisin-like proteinase 2 from *B. intermedius*, according to its physical and chemical properties, sensitivity to inhibitors, and amino acid composition, can be classified as a member of the thiol-dependent serine proteinase subfamily of subtilisins.

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