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Characteristics of a Novel Secreted Zinc-Dependent Endopeptidase of *Bacillus intermedius*

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Abstract—A novel zinc-dependent metalloendopeptidase of *Bacillus intermedius* (MprBi) was purified from the culture medium of a recombinant strain of *Bacillus subtilis*. The amino acid sequence of the homogeneous protein was determined using MALDI-TOF mass spectrometry. The sequence of the first ten residues from the N-terminus of the mature protein is ASTGSQKVTV. Physicochemical properties of the enzyme and its substrate specificity have been studied. The molecular weight of the metalloproteinase constitutes 19 kDa, the K_m and k_{cat} values are 0.06 mM and 1210 sec^{-1}, respectively, and the p*I* value is 5.4. The effect of different inhibitors and metal ions on the enzyme activity has been studied. Based on the analysis of the amino acid sequence of the active site motif and the Met-turn together with the enzyme characteristics, the novel bacterial metalloproteinase MprBi is identified as a metzincin clan adamalysin/reprolysin-like metalloprotease.

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Most metalloendopeptidases are zinc-dependent (zincins) and contain in the active site the highly conserved sequence HEXXH with two histidine residues as the zinc ligands. Depending on the position of the third zinc ligand, zincins are subdivided into gluzincins (HEXXH + E), aspzincins (HEXXH + D), and metzincins (HEXXHXXGXXH/D + Met-turn) [1]. A characteristic feature of metzincins is the extended motif of the active site and the presence of the conserved methionine residue in the 1,4-β-loop that is called a Met-turn. Metzincins are subdivided into the families of astacins, serralysins, matrixins, and adamalysins/reprolysins [1]. In spite of the significant differences in their sequences, these enzymes have similar tertiary structure of the catalytic domain [2]. Their active sites contain three histidine residues (zinc ligands), as well as residues of glutamate and glycine also playing a significant role in the catalysis. The tyrosine residue of the Met-turn in astacins and serralysins plays the role of "tyrosine switch", being the fifth zinc ligand and that is responsible for the substrate binding and/or stabilization of the transition state during catalysis [3]. In the representatives of matrixins and adamalysins/reprolysins, the tyrosine residue in the Met-turn is replaced by a proline residue, and these enzymes are activated by the mechanism of the "cysteine switch" [4].

Eukaryotic metzincins are usually multidomain proteins that, besides the catalytic domain, contain other domains on the C-terminus of the molecule. The exception is astacin (20.3 kDa) that was first isolated from the alimentary tract of the crawfish *Astacus astacus* L. The mature form of this protein comprises a protease domain of 200 amino acid residues (a.a.) with N-terminal alanine [5]. Astacin is synthesized as an inactive precursor with a signal peptide on the N-terminus of the catalytic domain. Only one bacterial astacin-like endopeptidase has been described: it is flavastacin of *Flavobacterium meningosepticum*, whose sequence contains a highly conserved zinc-binding motif of the active site **HE**LM**H**AI**G**FY**H** and Met-turn SV**MMY** with a tyrosine residue [6]. Bacterial serralysins are also synthesized as zymogens with a propeptide at the N-terminus, but the secretory signal is located in the C-terminal region [1].

Abbreviations: Dnp, dinitrophenyl; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; MprBi, metalloendopeptidase of *Bacillus intermedius*; *p*CMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride.

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Eukaryotic metzincins play an important role in embryonic development and tissue differentiation. They are also involved in the immune reactions and genesis of pathological states (rheumatoid arthritis, inflammation processes, atherosclerosis, oncological diseases), cell migration, organogenesis, tissue degradation, as well as division, penetration, and metastasis of cancer cells [1, 2].

The functional importance of these enzymes necessitates their investigation as potential medications. In this connection, bacterial proteins that are analogs of eukaryotic metzincins are of special interest, being not only an ideal model for fundamental investigations, but also possessing high potential for their practical application. Searching for, isolation, and investigation of bacterial metzincins have become possible with the development of postgenomic technologies, since microorganisms synthesize trace amounts of these enzymes, and thus their isolation is connected with problems of cloning and increase in expression efficiency. The goal of this study was to elaborate an effective method for isolation of a homogeneous preparation of the secreted zinc-dependent metalloproteinase of *B. intermedius* from the recombinant strain of *B. subtilis* and to investigate the properties of the homogeneous protein.

MATERIALS AND METHODS

In this work we used multicopy plasmid pSA1, a derivative of plasmid pCM4 containing the insertion of genomic DNA of *B. intermedius* (1 kb) with the gene of the secreted metalloproteinase (*mprBi*, GenBank accession number EU678894). The recipient strain, a strain of *B. subtilis* BG 2036 with deletions of the genes of extracellular proteases (Δ*apr*, Δ*npr*), was kindly provided by Prof. E. Ferrarri (Genencor Int. Inc., USA).

The *B. subtilis* cells were transformed with plasmid DNA according to the method of Anagnostopolous and Spizizen [7]. The recombinant strain was cultivated on a vibration stand (B. Braun, Germany) for 30 h at 200 rpm in a medium described earlier [8]. The volume of the medium constituted 1/5 of the flask capacity. The culture liquid was freed from cells by centrifugation (20 min, 10,000*g*).

The proteolytic activity of the metalloproteinase was determined by the cleavage of azocasein [9, 10] using a spectrophotometer (BioRad, USA) at 450 nm. The unit of activity was determined as the amount of the enzyme hydrolyzing 1 μg of the substrate per minute.

Protein concentration was determined spectrophotometrically, considering that the optical density of a solution of 1 mg/ml in a 1-cm cuvette at 280 nm constitutes 1.0. Protein concentration was also determined by Bradford's method [11].

The metalloproteinase of *B. intermedius* was isolated from 1 liter of culture liquid of the recombinant *B. subtilis* strain using fractionation with ammonium sulfate, collecting the fraction of 0.2-0.7 saturation. To remove the ammonium sulfate, the collected fraction was dialyzed against 0.05 M Tris-HCl, pH 7.3. The enzyme was then purified on a Butyl Sepharose column (HiTrap, Pharmacia). The metalloproteinase binds to Butyl Sepharose when ammonium sulfate concentration in the protein solution is close to the value at which the protein is inactivated and precipitated (35%). The column with Butyl Sepharose was equilibrated with 0.05 M Tris-HCl, pH 7.3, containing 5 mM Ca^{2+} and 35% ammonium sulfate. Before application to the column, the enzyme was dialyzed against the same buffer. To elute the enzyme, ammonium sulfate concentration in the buffer was lowered to 20-15%. Fractions containing the purified enzyme were pooled and dialyzed against 0.05 M Tris-HCl, pH 7.3, containing 5 mM Ca^{2+}.

The purity of the preparations was determined by SDS-PAGE in 12.5% polyacrylamide gel according to Laemmli [12]. The gels were stained with Coomassie Brilliant Blue G-250 (Serva) or with a solution of $ZnCl_2$. In the case of the second method (the sensitivity is 30 ng of protein [13]), the gel was preliminarily incubated 0.2 M imidazole solution for 10 min and then placed into 0.3 M solution of $ZnCl_2$. Both solutions were prepared using deionized water. The molecular weight of the enzyme was determined by electrophoresis with the use of the following protein standards: BSA (66 kDa), ovalbumin (45 kDa), papain (20.6 kDa), and lysozyme (14.4 kDa) (Sigma, USA).

To study the effect of inhibitors on the activity of the metalloproteinase, the enzyme preparation was incubated in the presence of an inhibitor for 1 h at room temperature, and then the residual activity was determined by the hydrolysis of azocasein. The residual activity was expressed as percentage relative the activity in the absence of inhibitors in the reaction mixture. The tested inhibitors were phenylmethylsulfonyl fluoride (PMSF) (a specific serine proteinase inhibitor), EDTA and 1,10-phenanthroline (metalloproteinase inhibitors), *p*-chloromercuribenzoate (*p*CMB), $HgCl_2$, and protein trypsin inhibitor. The inhibitors were added to the enzyme solution to achieve final concentrations of 0.5 and 5 mM.

The homogeneous enzyme preparation was analyzed using MALDI-TOF spectrometry. The metalloproteinase was treated with trypsin as described by Sigma-Aldrich (www.sigmaaldrich.com/etc/medialib/docs/Sigma/General_Information/maldi_tof_ms_analysis.Par.0001.File.tmp/maldi_tof_ms_analysis.pdf). The resulting peptides were analyzed using a Vision 2000 TOF mass-spectrometer (Thermo Bioanalysis, Great Britain). The data were processed using the Peptide Mass Fingerprint (www.matrixscience.com) and Peptide Mass (www.expasy.net/tools/peptide-mass.html) programs.

The N-terminal amino acid sequence of the protein was determined by Edman degradation using a Model 816

Protein Sequences device (Giessen, Germany) equipped with a 120A PTH analyzer (Applied Biosystems, USA). The location of the signal peptide was determined by the analysis of the nucleotide sequence of the gene *mprBi* using the SignalP 3.0 program (www.cbs.dtu.dk/services/SignalP).

The Michaelis constant K_m was determined by the hydrolysis of azocasein, plotting the data in the Lineweaver–Burk coordinates. The isoelectric point of the enzyme was determined based on its amino acid sequence using the ProtParam program (www.expasy.net/cgi-bin/protparam).

The substrate specificity of the metalloproteinase was determined by the hydrolysis of the synthetic substrates Dnp-Ala-Ala-Leu-Arg-NH_2, Dnp-Gly-Gly-Phe-Arg, Dnp-Gly-Gly-Ile-Arg, Dnp-Gly-Gly-Lys, Dnp-Gly-Gly-Leu-Arg, and Dnp-Ala-Ala-Val-Arg according to the method described by Lyublinskaya et al. [14]. The unit of enzyme activity was determined as the amount of the enzyme hydrolyzing 1 μmol of the substrate per minute under the conditions employed. The peptides obtained after hydrolysis of the B-chain of oxidized insulin were analyzed using MALDI-TOF mass-spectrometry assay (http:/expasy.net/tools). The activity of the metalloproteinase in the hydrolysis of casein according to Hammerstein (Sigma, USA) and ovalbumin (Sigma) was determined by the method described by Kaverzneva, using 2% solution of the corresponding substrate in 0.1 M Tris-HCl, pH 9.0 [15]. The unit of the activity was determined as the amount of the enzyme producing 1 μmol tyrosine/min per ml of the enzyme solution.

The effect of bivalent metal ions on the activity of the metalloproteinase was studied using chlorides of calcium, magnesium, cobalt, copper, and nickel at final concentrations varying from 1 to 20 mM, and zinc chloride was used at final concentration 0.01-20 mM. Solutions of the bivalent metals were added to the enzyme solution and incubated at room temperature for 15 min, and then the activity of the enzyme was determined by hydrolysis of azocasein and expressed as percentage relative the enzyme activity in the absence of the metal ion.

The dependence of the enzyme activity on pH of the reaction medium was determined by hydrolysis of azocasein in 0.05 M Tris-HCl containing 5 mM Ca^{2+} in the pH range from 7.2 to 9.5. To study the effect of pH of the medium on the enzyme stability, the enzyme was incubated in the same buffer solutions (pH 7.2-9.5) for 24 h at room temperature. Then the substrate was added and the activity was determined as described above.

The effect of temperature on the enzyme activity was determined by hydrolysis of azocasein in 0.05 M Tris-HCl, pH 8.0, containing 5 mM Ca^{2+}, at temperatures varying from 22 to 70°C. To study the thermostability of the enzyme, the enzyme was first incubated in the indicated buffer for 40 min at temperatures from 22 to 70°C, and then the substrate was added and the activity was measured at 37°C as described above.

Statistical analysis of the experimental data was done using Microsoft Excel. To describe and compare enzyme parameters, 95% confidence limits for mean values were calculated.

RESULTS AND DISCUSSION

Accumulation of proteolytic activity in the culture liquid of the recombinant strain *B. subtilis* bearing the plasmid with the gene of the metalloproteinase of *B. intermedius* was studied. The maximal activity was observed after 30 h of cultivation, while in the culture liquid of the wild-type strain only traces of activity were observed during the whole time of cultivation.

The enzyme was isolated from the culture liquid of 30-h culture of the recombinant strain using ammonium sulfate fractionation (0.2-0.7 saturation). In the dialyzate of the ammonium sulfate fraction, the specific activity of the enzyme increased 20-fold compared with the culture liquid, the yield of the protein being 54% (Table 1). To obtain a homogeneous preparation, the enzyme was purified on a column with the hydrophobic matrix Butyl Sepharose. The enzyme was eluted from the column with 20-15% ammonium sulfate (Fig. 1). After the chromatography the specific activity of the enzyme increased almost 350-fold, and the yield constituted 11.3% (Table 1). SDS-PAGE demonstrated that the chromatography on Butyl Sepharose yielded a homogeneous protein (Fig. 2, lane *3*), while the dialyzate of the sulfate ammonium fraction

Table 1. Isolation and purification of metalloproteinase MprBi from culture liquid of the recombinant *B. subtilis* strain

Purification step	Volume, ml	Protein, mg	Specific activity, U/mg	Yield, %
Culture liquid	760	11 400	0.05	100
Dialysis of ammonium sulfate fraction	24	312	1.03	54
Butyl Sepharose chromatography	70	3.8	17.4	11.3

contained several protein bands (Fig. 2, lane *2*). The molecular weight of the metalloproteinase by SDS-PAGE is 19 kDa.

Thus, we obtain a homogeneous preparation of the proteinase MprBi using two steps of purification, while isolation of extracellular proteinases often requires from three to five steps including ammonium sulfate fractionation, ultrafiltration, and classical ion-exchange and affinity chromatographies. Increase in a number of purification steps decreases the yield of the final product. The developed approach allowed us to decrease the time of purification and to obtain the homogeneous protein with the yield exceeding 10%.

Investigation of the effect of different inhibitors on the activity of the metalloproteinase showed that the enzyme is not inhibited by PMSF and protein trypsin inhibitor, but 1,10-phenanthroline and EDTA almost completely suppress its activity, which confirms that the enzyme belongs to the class of metalloproteinases (Table 2). High concentrations of *p*CMB almost completely inhibit its activity, suggesting the presence of a cysteine residue in the enzyme molecule.

The amino acid sequence of the homogeneous protein MprBi was determined using MALDI-TOF spectrometry (Fig. 3). The N-terminal sequence of the protein determined by Edman degradation contains 10 amino acid residues ASTGSQKVTV with N-terminal alanine. The amino acid sequence of the metalloproteinase is identical to the amino acid sequence derived from the nucleotide sequence of the *mprBi* gene (GenBank accession number EU678894). Analysis of the nucleotide sequence of the gene indicated that the signal peptide is located at the N-terminus of the protein. In the amino acid sequence of MprBi a fragment with conserved amino acid residues was found (shown bold in the sequence **HE**VG**H**NF**G**LP**H)**, this indicating that enzyme MprBi belongs to the class of zinc-dependent metalloproteinases (Fig. 4). This fragment contains three histidine residues His126, His130, and His136, glutamate residue Glu127 that is located next to the first histidine

Table 2. Effect of enzyme inhibitors on activity of metalloproteinase of *B. intermedius*

Inhibitor	Residual activity, %	
	0.5 mM inhibitor	5 mM inhibitor
PMSF	94	91
EDTA	96	5.7
1,10-Phenanthroline	5.8	0
pCMB	94	1.9
$HgCl_2$	51	40
Protein trypsin inhibitor	97	100

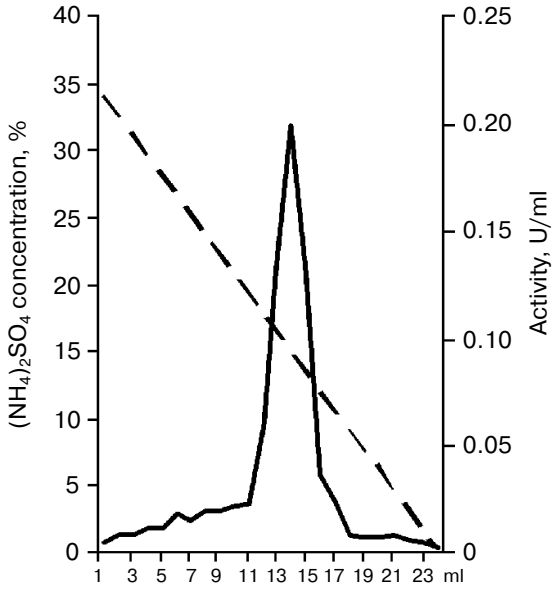

Fig. 1. Chromatography on Butyl Sepharose. Solid line, the activity of the enzyme towards azocasein; dashed line, ammonium sulfate concentration.

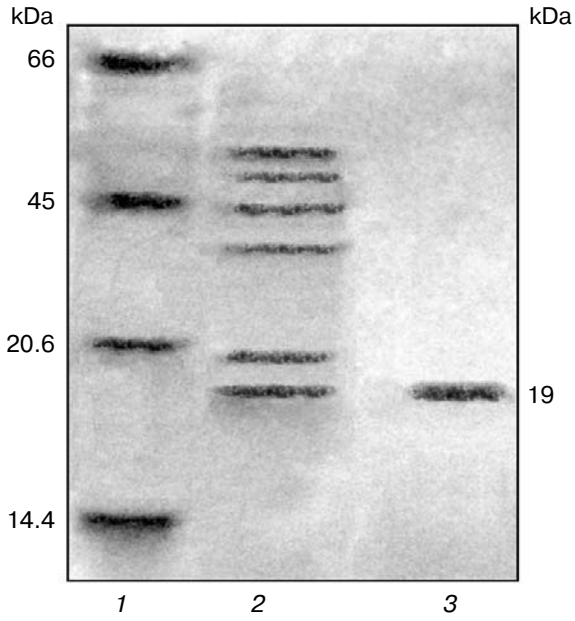

Fig. 2. SDS-PAGE of enzyme fractions: *1*) protein standards: BSA (66 kDa), ovalbumin (45 kDa), papain (20.6 kDa), lysozyme (14.4 kDa); *2*) protein fraction after precipitation with ammonium sulfate; *3*) protein fraction after Butyl Sepharose chromatography.

```
1    ASTGSQKVTV YAVADAQYRA KYSDWQTRIV SIIEQADVTF
41   NRDHDVDFVV QAVGSWTSSG SNAEQILSNL SRSFDGRGYD
81   FVTGFTANPN FDAGGIAYVY NSAPSGSAFA VNLDQGTANT
121  AKAATHEYGH NFGLPHDPQG SGIVCLMNYD YSYTVDFFDA
161  AHKNQVNRNK AWYR
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Fig. 3. Amino acid sequence of mature protein MprBi. The first ten N-terminal amino acids are shown bold (**ASTGSQKVTV**). The active site and the Met-turn motifs are shown in the frames.

Metzincin metalloproteinases	active site motif	homology with MprBi (%)	Met-turn
ASTACINS			
Astacin (crayfish)	**H E** L M **H** A I **G** F Y **H** E	42	S I **M** H Y
α-MEP (mouse)	**H E** I L **H** A L **G** F F **H** E	42	S L **M** H Y
β-MEP (rat)	**H E** F L **H** A L **G** F W **H** E	42	S V **M** H Y
BMP1/procollagen C-proteinase (human)	**H E** L G **H** V V **G** F W **H** E	50	S T **M** H Y
SPAN/BP10 (sea urchin)	**H E** I G **H** A I **G** F H **H** E	50	S I **M** H Y
Tolloid (*Dr. melanogaster*)	**H E** L G **H** T I **G** F H **H** E	50	S I **M** H Y
REPROLYSINS			
Adamalysin II (*C. adamanteus*)	**H E** L G **H** N L **G** M E **H** D	66	C I **M** R P
Atrolysin C	**H E** L G **H** N L **G** M E **H** D	66	C I **M** R P
Trimerelysin	**H E** L G **H** N L **G** M E **H** D	66	C I **M** S D
	126 127 128 129 130 131 132 133 134 135 136 137		145 146 147 148 149
MprBi (*B. intermedius*)	**H E** Y G **H** N F **G** L P **H** D		C L **M** N Y

Fig. 4. Active site and Met-turn motifs of different representatives of astacins and reprolysins [22].

residue, and glycine residue Gly133 between the second and the third histidine residues. All these residues are characteristic for the extended active site motif of seven families of metzincins [16].

The amino acid sequence of the endopeptidase MprBi contains a single methionine residue (Met147) that is located in the proximity to Tyr149. The presence of these residues in the mature molecule of MprBi suggests that the fragment CLMNY exhibits the structure of a Met-turn localized at the distance of 8 a.a. from the C-terminus of the extended active site motif. In this connection, the obtained zinc-dependent metalloendopeptidase MprBi can be referred to the metzincin clan. It is also known that the presence of a tyrosine residue in the Met-turn is characteristic only for astacins and serralysins [17-19]. In the families of matrixins and adamalysins/reprolysins, this residue is almost always replaced with a nonfunctional proline residue that is not involved in the catalysis. It should be noted that bacterial serralysins are secreted as zymogens with an N-terminal propeptide, but the secretor signal is located at the C-terminus [1]. The fact that the proteinase MprBi is synthesized with the signal peptide at the N-terminus together with the presence of the conserved Tyr149 in the Met-turn suggested that

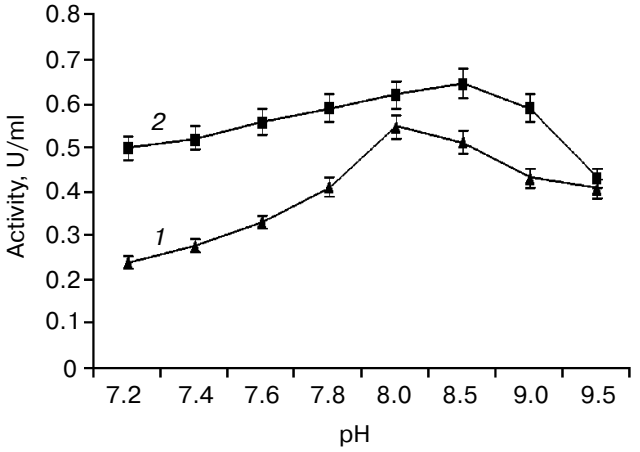

Fig. 5. The pH dependences of activity (*1*) and stability (*2*) of the metalloproteinase.

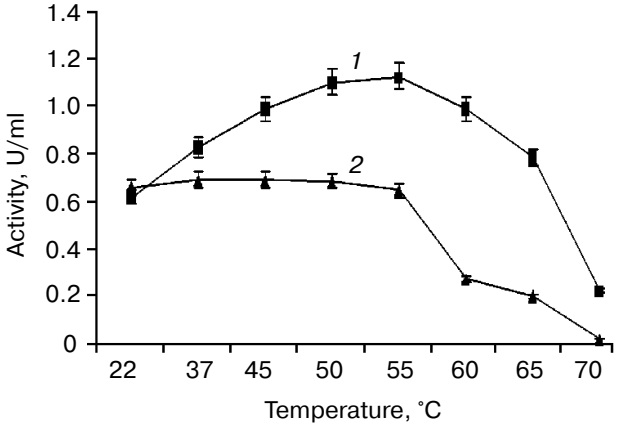

Fig. 6. Effect of temperature on activity (*1*) and stability (*2*) of the metalloproteinase.

the endopeptidase MprBi belongs to the family of astacin-like peptidases. However, it should be noted that in the extended active site motif of MprBi, the next residue to the third zinc ligand His136 is Asp137, while astacin-like peptidases contain a glutamate residue in this position (Fig. 4). In astacin-like endopeptidases, the glutamate residue Glu103 (numeration as in astacin) is oriented with the involvement of a water molecule so that that it forms a salt bridge with the N-terminal Ala1, this stabilizing the enzyme molecule. In the opinion of some researchers, the formation of a salt bridge between Glu103 and Ala1 is so important for the stabilization of the protein globule that Glu103 is often considered as the catalytic amino acid residue involved in the active site of the enzyme [20-22]. Residue Asp137 in this region of the active site is characteristic for all known representatives of the reprolysin family, i.e. the active site motif of MprBi containing 12 essential amino acid residues completely coincides with the active site motif of reprolysins. Thus, considering this fact, as well as the presence of the conserved residue Tyr149 in the Met-turn of the endopeptidase MprBi that is absent in the Met-turn of reprolysins, we suppose that the bacterial metalloendopeptidase MprBi takes an intermediate position between the families of astacins and reprolysins.

The K_m value of the enzyme is 0.06 mM, the k_{cat} value is 1210 sec^{-1}, and the isoelectric point is 5.4. The activity of the metalloproteinase is maximal at pH 8.0 (0.05 M Tris-HCl containing 5 mM Ca^{2+}), this indicating that the investigated enzyme belongs to the group of the alkaline metalloproteases (Fig. 5, curve *1*). The protein is stable in the pH range 7.2-9.0 (Fig. 5, curve *2*). The optimal temperature for the enzymatic activity is 50-55°C (Fig. 6, curve *1*). The protein is stable in the temperature range of 22-55°C (Fig. 6, curve *2*).

The specificity of the metalloproteinase MprBi towards synthetic chromogenic substrates was investigated (Table 3). MprBi hydrolyzes synthetic tetrapeptides better than tripeptides. Presumably, longer substrates bind faster to the active site of the enzyme [23, 24]. The amide of the synthetic tetrapeptide is also easily hydrolyzed by the enzyme, like tetrapeptides with the carboxyl group. Only the tetrapeptide Dnp-Gly-Gly-Phe-Arg is hydrolyzed with a low rate. The metalloproteinase does not exhibit strict substrate specificity since the spectrum of the amino acid residues forming the peptide bond that is hydrolyzed by the enzyme is rather wide.

The specificity of the enzyme towards B-chain of oxidized insulin was also studied. Investigation of the hydrolysis products by mass spectrometry confirmed that the enzyme does not exhibit a preference for certain amino acid residues of the hydrolyzed peptide bonds (Fig. 7), this indicating wide substrate specificity of proteinase MprBi. However, endopeptidase PrtA from the pathogenic insect *Photorhabdus luminescens* that belongs to the serralysin family hydrolyzes B-chain of the oxidized insulin only at the bonds formed by Val, Ala, and Leu [25].

Table 3. Specificity of the metalloproteinase towards synthetic substrates

Substrate	Activity × 10^3, U/mg
Dnp-Gly-Gly-Phe-Arg	5.15
Dnp-Gly-Gly-Leu-Arg	3.65
Dnp-Ala-Ala-Leu-Arg-NH_2	2.82
Dnp-Gly-Gly-Ile-Arg	2.64
Dnp-Ala-Ala-Val-Arg	1.45
Dnp-Gly-Gly-Lys	0.57

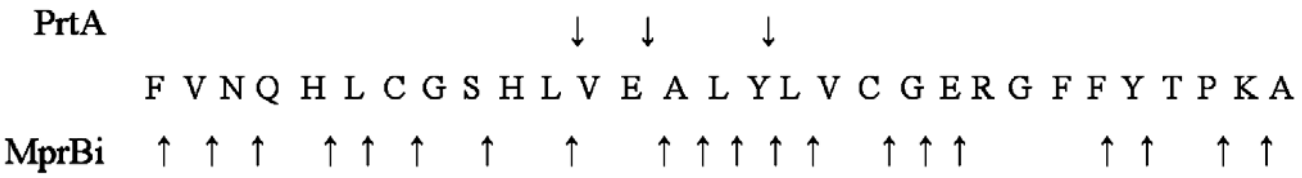

Fig. 7. Sites of hydrolysis of B-chain of insulin by metalloproteinase MprBi and endopeptidase PrtA.

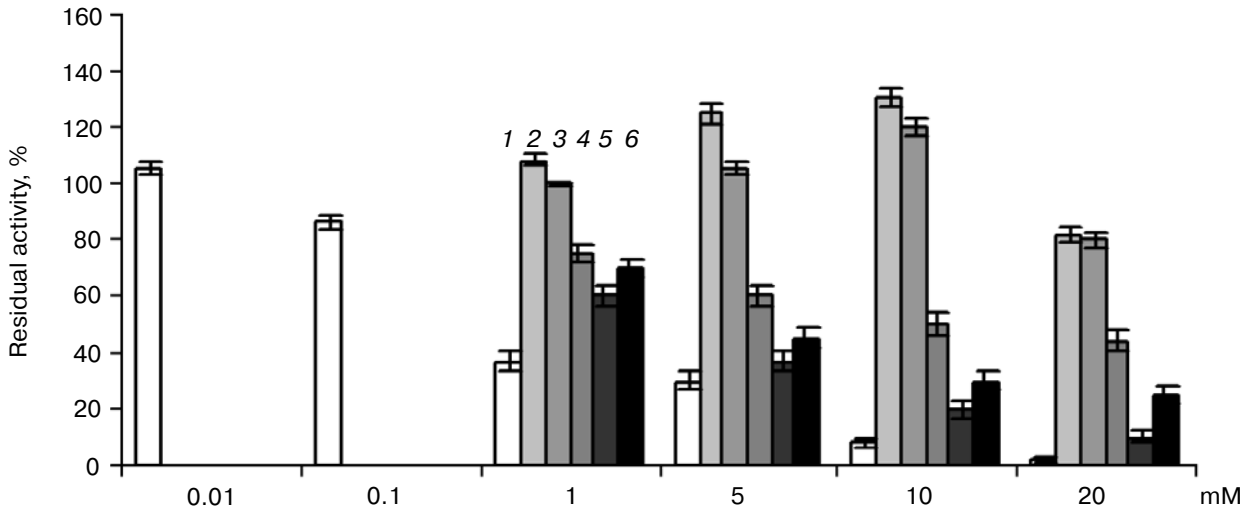

Fig. 8. Effect of bivalent metal cations on the activity of metalloproteinase MprBi: *1*) Zn^{2+}; *2*) Ca^{2+}; *3*) Mg^{2+}; *4*) Co^{2+}; *5*) Cu^{2+}; *6*) Ni^{2+}. The abscissa indicates metal ion concentration.

Among protein substrates, endopeptidase MprBi prefers casein (specific activity 32.9 U/mg) to albumin (specific activity 5.5 U/mg).

Investigation of the effect of bivalent metal cations on the enzyme activity showed that Ca^{2+} and Mg^{2+} at concentration of 10 mM increase the enzyme activity by 30 and 20%, respectively. Ions Co^{2+}, Cu^{2+}, and Ni^{2+} at concentrations from 1 to 20 mM decrease the activity of the metalloproteinase, the activity decreasing with increasing concentration of the metal ion. Low concentrations of Zn^{2+} (0.01 mM) have virtually no effect on the enzyme activity, but subsequent increase in concentration sharply decreases the enzyme activity (Fig. 8). In the case of thermolysin, the addition of Zn^{2+} at concentrations exceeding 10 μM to the enzyme solution inhibited its activity [26].

Thus, an alkaline zinc-dependent metalloproteinase of *B. intermedius* has been isolated and purified to homogeneous state. The amino acid sequence of the enzyme was determined. The substrate specificity of the enzyme, its kinetic characteristics, and enzymatic properties were investigated. A simple and efficient method for isolation of the protein from culture liquid of the recombinant strain *B. subtilis* was developed. Characteristic features of the primary structure of the protein and its biochemical properties allowed us to identify the enzyme as a metzincin clan adamalysin/reprolysin-like endopeptidase. The physiological role of the secreted proteinase MprBi in the bacterial cells and in the population in general remains unclear. This problem is of current importance since zinc metalloproteinases play a leading role both in the development of pathological processes and in treatment of many diseases [27]. In this connection, the pharmacological industry is interested in producing new highly efficient medications based on zinc-dependent metalloproteinases.

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