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AN ACTIVATED BY COBALT ALKALINE AMINOPEPTIDASE FROM *Bacillus mycoides*

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An intracellular arginine – specific aminopeptidase synthesized by *Bacillus mycoides* was purified and characterized. The purification procedure for studied aminopeptidase consisted of ammonium sulphate precipitation and three chromatographic steps: anion exchange chromatography and gel permeation chromatography. A molecular weight of ~50 kDa was estimated for the aminopeptidase by gel permeation chromatography and SDS-PAGE. The optimal activity of the enzyme on arginyl-β-naphthylamide as a substrate was at 37°C and pH 9.0. The enzyme showed maximum specificity for basic amino acids: such as Arg and Lys but was also able to hydrolyze aromatic amino acids: Trp, Tyr, and Phe. Co^{2+} ions activated the enzyme, while Zn^{2+}, Cu^{2+}, Hg^{2+} and Mn^{2+} inhibited it. The enzyme is a metalloaminopeptidase whose activity is inhibited by typical metalloaminopeptidase inhibitors: EDTA and 1,10-phenanthroline. Analysis of fragments of the amino acid sequence of the purified enzyme demonstrated high similarity to AmpS of *Bacillus cereus* and AP II of *B. thuringensis*.

Aminopeptidases (EC 3.4.11) belong to exopeptidases that are ubiquitous in the living world. They catalyze the hydrolysis of peptide bonds in peptide and protein substrates with the release of N-terminal amino acids. Bacterial aminopeptidases can be intracellular or extracellular enzymes. Intracellular aminopeptidases, which are the predominant type, about 90% of all aminopeptidases studied so far, carry out important functions in many physiological processes, including post-translational maturation of proteins and final stages of degradation of intracellular proteins. Their role in the regulation of gene transcription has also been proven [1]. Secreted exopeptidases, including aminopeptidases, are complemented by endopeptidases to provide the cell with nutrients that enable their growth and multiplication. In the case of pathogenic bacteria the enzymes enhance their virulence when colonizing the host organism [2, 3]. Bacterial aminopeptidases are ordered into two main groups that differ in narrow or broad substrate specificity. Enzymes with broad substrate specificity are able to cleave several different amino acid residues or groups of amino acids from the N-terminus or P1 position of a protein. On the other hand, enzymes with narrow substrate specificity are able to release one type of amino acid residues from position P1 of the peptide or protein substrate [4]. Both groups of aminopeptidases belong to stereospecific enzymes that in their majority release L-forms of amino acids. However, there are some exceptions such as aminopeptidase DmpB from *Ochrobactrum anthropi* which shows a decided preference for D-alanine as a substrate [5]. Bacterial aminopeptidases with high activity against protein substrates with N-terminal arginine are usually characterized by narrow specificity and are not a very numerous group of enzymes. So far the properties of only a few such enzymes have been described, including the aminopeptidases synthesized by *Streptococcus gordoni* and *Lactobacillus sakei* [6, 7]. Some aminopeptidases with broad specificity may also show some activity against substrates with N-terminal arginine, e.g. the alanyl aminopeptidase synthesized by *Pseudomonas* sp., or leucyl aminopeptidase of *Bacillus* sp. [8, 9].

Interest in aminopeptidases with this specificity stems from their potential participation in the intracellular metabolism of arginine. This amino acid released as a result of the direct action of aminopeptidases can not only be a product for the synthesis of new proteins but also a precursor in the synthesis of polyamines and other intermediates for many important cellular transformations. The amino acid may also be shunted into one of the catabolic arginine pathways in bacteria. Transformations of arginine in the arginine deiminase pathway (ADI) can be both a source of energy in the form of ATP or of numerous valuable metabolites. Interest in arginine metabolism is also stimulated by the demands of the biotechnology industry, mainly its pharmaceutical and food branches [7, 10].

The studies described herein focused on the intracellular aminopeptidase synthesized by a rhizosphere strain of the bacterium *Bacillus mycoides*. These bacteria belong to ubiquitous microorganisms occurring in the soil, waters and phyllosphere. The species, together with several other genetically closely related *Bacillus* species, forms the *Bacillus cereus* group [11]. *B. mycoides* strains are currently being characterized mainly from the viewpoint of their use in bioremediation and biological plant protection [12, 13]. There is no available information in the literature regarding aminopeptidases produced by *B. mycoides*. By analogy to other species from the *B. cereus* group it can be said that the best characterized aminopeptidases in this group are the leucine aminopeptidases [14, 15]. As yet

among these bacteria no aminopeptidase with high substrate preference for N-terminal arginine has been characterized.

In the current study we focused on the purification and biochemical characterization of a new arginine-specific aminopeptidase synthesized by a soil strain of *B. mycoides*. We also carried out the identification of the purified protein using MS analysis and cloned part of the gene coding for the aminopeptidase.

METHODS

Biological material and culture conditions. The source of the aminopeptidase was a soil strain of bacteria isolated from wheat rhizosphere. Based on biochemical and morphological characteristics it was classified as *Bacillus mycoides*. The bacteria were grown in liquid trtyptic soy broth (TSB) medium, pH 7.5 for 48 h with shaking (120 rpm) at 30°C. The growth of the bacteria was monitored by measuring optical density ($OD_{600\,nm}$). Bacterial cells were spun down (1000 g, 20 min), the supernatant was decanted and the cell pellet was washed twice with Tris-HCl, pH 8.8 and sonicated. The obtained intracellular protein extract was taken for aminopeptidase purification.

Enzymatic activity. Aminopeptidase activity was assayed using synthetic amino acid derivatives of β-naphthylamine (Sigma, USA) [16]. The absorbance of the colour product was determined photometrically at 525 nm. The enzyme to the control samples was added after termination of the reaction. The results of absorbance measurements were converted to μmoles product formed, using a standard curve prepared for 5 different concentrations of β-naphthylamine.

A unit of activity was taken as one μmole naphthylamine formed in one minute in the presence of 100 μl of enzyme.

Purification of enzyme. Fractionation of the enzyme preparation with ammonium sulphate was carried out by using 35% in the first stage, followed by 85% saturation in the second stage. The pellet was dissolved in 50 mM Tris-HCl buffer, pH 8.8, and dialyzed overnight against the same buffer.

Gel filtration chromatography. Prior to loading on the column the dialyzed enzyme preparation was concentrated in Amicon ultrafiltration chamber (membrane PM 10, Millipore, USA). The sample containing 1% sucrose was applied to a Sephadex G-150 column equilibrated with 50 mM Tris-HCl buffer, pH 8.8, containing 15 mM KCl. Fractions showing the studied activity in the presence of Arg-β-naphtylamid (Arg-β-NA) were pooled and taken for further purification.

Ion-exchange chromatography. Fractions with enzymatic activity were applied to a Protein-Pak Q 8HR HPLC (Waters, USA) anionite column, which was first equilibrated with 15 mM Tris-HCl buffer, pH 8.8. The enzymatic protein bound to the anionite bed was eluted with a linear gradient of KCl from 0 to 0.5 M. Collected 2.5 ml fractions showing activity were pooled and then dialyzed overnight against 15 mM Tris-HCl buffer, pH 8.8. The next step involved HPLC re-chromatography using a narrower KCl gradient, from 0.2 to 0.4 M and the same column as above equilibrated with 15 mM Tris-HCl buffer, pH 8.8.

The highly purified enzyme preparation was used as material for determining the biochemical and kinetic properties of the enzyme.

Determination of protein content. Protein concentration at all stages on the study was determined as described by Bradford [17].

Electrophoretic separations. Electrophoretic separations under native, semi-denaturing and denaturing conditions were according to the procedure described by Laemmli [18]. Protein in the gels was visualized by staining with coomassie brilliant blue R-250. After carrying out the full purification procedure, protein was staining using modified silver stain method, compatible with mass spectrometry analysis [19]. To stain polyacrylamide gels for the presence of active enzyme following native and semi-denaturing (without thermal denaturation of the enzyme solution) electrophoresis they were incubated in buffered solution of the substrate at 37°C and then stained in Fast Garnet GBC (Sigma-Aldrich, USA) solution. The location of active enzyme was indicated by red bands against a bright background.

Determination of molecular weight of the enzyme: The molecular weight of the analyzed enzyme was determined by SDS-PAGE and in the course of gel filtration on a Sephadex G-200 column. The column was calibrated using the following standard proteins: bovine albumin (67 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). The void volume of the column (V_o) was determined using dextran blue.

Characterization of the enzyme. Determination of thermal stability: the buffered enzyme preparation was subjected to 30 min incubation at four different temperatures: 30, 37, 40 and 45°C. Following the pre-incubation, the activity of the enzyme in optimal reaction temperature was assayed. The activity in a sample that was not subjected to 30 min pre-incubation was taken as 100%.

Effect of inhibitors and metal ions. In these experiments the following inhibitors were used: for metalloaminopeptidases – EDTA, 1,10-phenanthroline, for cysteine aminopeptidases – iodoacetamide, E-64, and for serine aminopeptidases – diisopropyl fluorophosphate (DFP) and for aspartyl peptidases – pepstatin A.

The enzyme was pre-incubated for 30 min in the presence of the inhibitors or metal ions at 4°C, after which substrate was added and the reaction carried out at 37°C.

Reactivation of enzymatic activity after inhibition with EDTA. After incubation with 10 mM EDTA the reaction mixture was dialyzed and the enzymatic activity was reactivated by overnight incubation with 1 mM of the metal ions Co^{2+}, Mg^{2+} or Ca^{2+}.

Determination of Michaelis–Menten constants. K_m was determined for the amino acid derivatives of β-naph-

Table 1. *B. mycoides* aminopeptidase purification procedure

Purification step	Total activity, U*	Total protein, mg	Specific activity, $U \cdot mg^{-1}$ protein	Yield, %	Purification, fold
Cell extract	407	1547	0.26	100	1
Ammonium sulfate precipitation (40–85%)	305	630	0.48	74.9	1.84
Sephadex G-150	282	206	1.36	69.3	5.18
1st Q 8HR HPLC	53.2	1.1	48.18	13.02	189.9
2nd Q 8HR HPLC	32.1	0.3	106.6	7.86	405.5

* U – μm naphtyloamine/min /100 μl of enzyme.

thylamide (β-Na) against which the enzyme was active; the final concentration of the substrates in the reaction mix was from 0.015 to 1.5 mM. The K_m value was determined using the graphical Lineweaver-Burk method.

Analysis of fragment of the amino acid sequence of the purified enzymatic protein. Both the highly purified enzyme preparation and the protein band cut out from polyacrylamide gel, corresponding with the electrophoretic mobility of a band with aminopeptidase activity, were sent to the Mass Spectrometry Laboratory at IBB PAN (Poland).

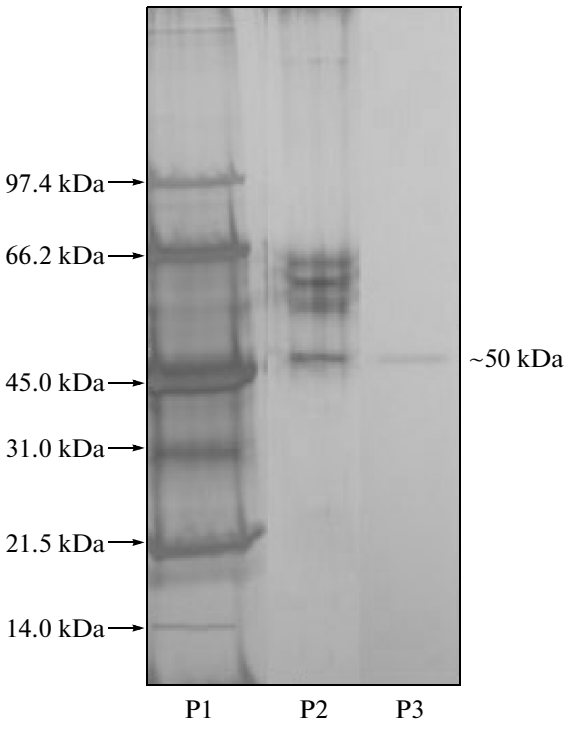

Fig. 1. SDS-PAGE of the purified aminopeptidase *of B. mycoides.*

P1–markers of molecular weight, P2 – enzyme preparation after second HPLC chromatography, silver-stained gel, P3 – enzyme activity after second HPLC chromatography; gel stained with Fast Garnet GBC (semidenaturing pattern).

Cloning of part of the aminopeptidase gene sequence. Isolation of genomic *B. mycoides* DNA was carried out with the use of Genomic DNA Purification Kit (Fermentas, Lithuania). Based on the results of MS analysis, primers for the amplification reaction (PCR) compatible with the sequence of *B. cereus* AH187 gene [Gene Bank NC_011658.1] were designed with the sequence forward: TCCCTTGCAGTCAATGTTGTTGT and rewers GACCATTGTTTTTCCGCCAACTAA. The PCR product was ligated into vector pGEM®-T (Promega, USA) and propagated in *E. coli* JM109 cells. Plasmids were isolated from white bacterial colonies in which the presence of the gene coding for aminopeptidase in the plasmid was additionally verified by PCR with the use of the above-mentioned primers. Plasmids containing a DNA insert in the form of *B. mycoides* aminopeptidase gene were sent for sequencing to the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (IBB PAN, Poland).

RESULTS

Purification of the enzyme: Intracellular *B. mycoides* aminopeptidase was purified using ammonium sulphate fractionation and a combination of low and high pressure chromatographic techniques: gel filtration and ion-exchange chromatography.

The consecutive stages of enzyme purification and the characterization of the enzyme are presented in Table 1. The procedure used resulted in over 400-fold purification of the enzymatic protein, with yield 7.9%. The most effective step was ion-exchange chromatography (HPLC) in which the enzyme was purified over 35-fold (Fig. 1, 2). In this step the studied enzymatic protein was separated from an aminopeptidase showing a substrate preference for Leu-β-NA. Activity against Leu-β-NA was demonstrated in the crude preparation and in the fraction active against Arg-β-NA after the first two purification steps. The purified enzyme preparation was taken for further studies in which the properties of the enzyme were determined.

Determination of molecular weight. The molecular weight of arginyl aminopeptidase determined by SDS-PAGE was approximately 50 kDa (Fig. 1). This result was

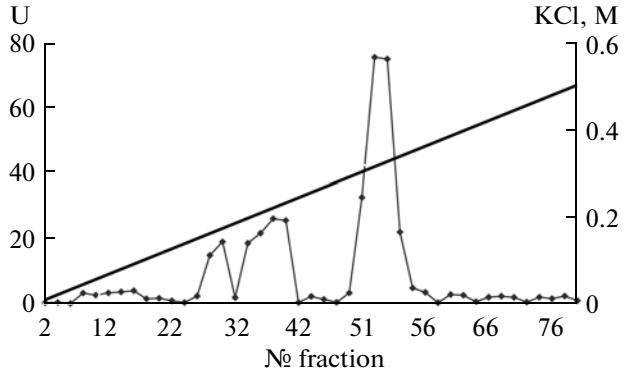

Fig. 2. Elution profile for aminopeptidase with activity against Arg-β-NA following ion exchange chromatography on Q 8HR (HPLC) column using 0 to 0.5 M KCl gradient. Fractions with highest activity were collected at 0.3 M KCl.

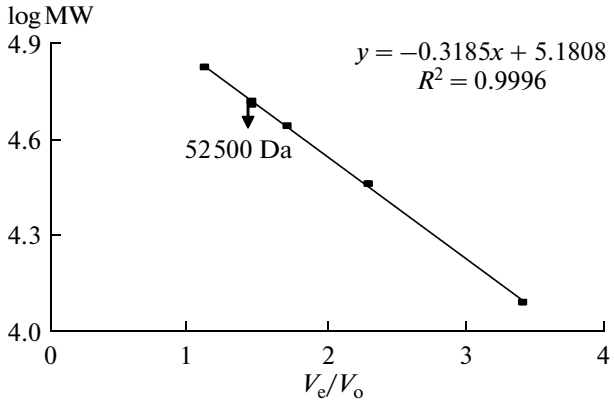

Fig. 3. Calibration curve for the V_e/V_o dependence of the standards used depending on the logarithms of their molecular weights. The black square represents the log value from which the molecular weight of the studied aminopeptidase was calculated.

confirmed by gel filtration studies in which a molecular weight for the enzyme of about 52 kDA was obtained (Fig. 3). Together these results indicate that the studied enzymatic protein does not have a subunit structure and is a monomer.

Optimal conditions for enzyme activity. The enzyme retained high activity against Arg-β-NA as a substrate only within a narrow pH and temperature range (Fig. 4). The optimal temperature for the reaction catalyzed by the studied aminopeptidase was 37°C. Small deviations from the optimal temperature resulted in a strong decrease in enzyme activity. During incubation of the reaction mix at either 30 or 39°C only 40% of the activity at the optimal temperature was determined. The pH optimum for the hydrolysis reaction catalyzed by the *B. mycoides* aminopeptidase was 9.0. The enzyme was active in a narrow pH range, from 7.0 to 9.5. Small deviations from the optimal pH value resulted in strong loss of activity by the studied enzyme. At pH 8.0 only 60% of maximal enzyme activity was observed.

Thermal stability. The thermostability of the studied enzyme was determined in the 30–45°C range, as illustrated by Fig. 5. After 30-min pre-incubation at 37°C the activity of the enzyme decreased by only 5%. However, after pre-incubation for the same time at 40°C the activity of the enzyme dropped by about 40%. After 30-min pre-incubation at 45°C the aminopeptidase *B. mycoides* retained only 12% of its activity compared to the control, not pre-incubated, sample.

Effect of specific inhibitors and metal ions. The effect of inhibitors specific for the individual catalytic groups of bacterial proteases on the activity of the purified aminopeptidase was also determined. The results of these experiments are summarized in Table 2. The activity of the studied enzyme was strongly inhibited in the presence of inhibitors specific for metallopeptidases: EDTA and 1,10-phenanthroline. The stronger inhibition was demonstrated by EDTA, which in concentration 1mM inhibited the activity of the studied aminopeptidase by 85%. Use of the other inhibitor, 1,10-phenanthroline, in the same concentration resulted in loss of about 60% activity. Inhibitors specific for serine, cysteine and aspartyl peptidases did not significantly affect the activity of purified *B. mycoides* aminopeptidase. Of all the metal ions used in the study the enzyme was activated the strongest by Co^{2+} ions which in concentration 1mM increased the activity of the ami-

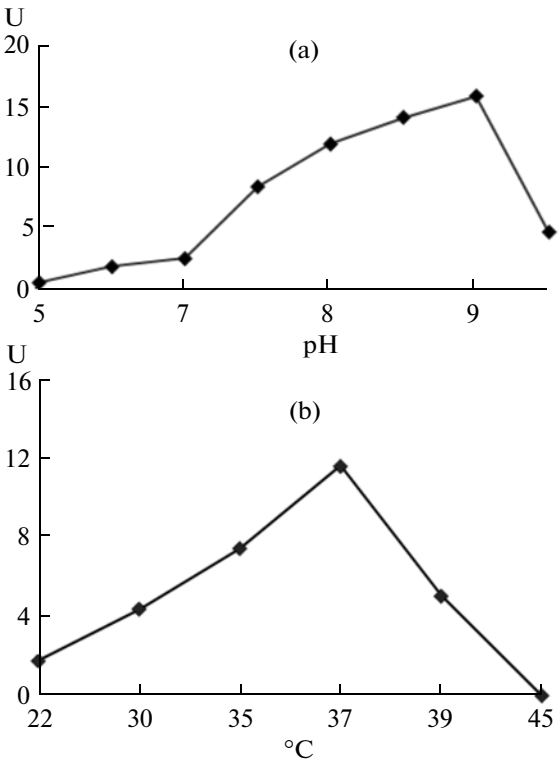

Fig. 4. Effect of pH and temperature on *B. mycoides* aminopeptidase activity.

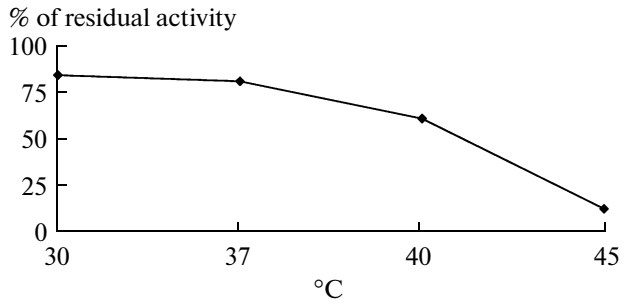

Fig. 5. Thermostability of *B. mycoides* aminopeptidase after 30 min preincubation at temperatures ranging from 30 to 45°C.

nopeptidase by 90% (Table 3). The enzyme was also activated by Ca^{2+} and Mg^{2+} ions. On the other hand, the activity of the enzyme was inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+} and Mn^{2+} ions. The strongest inhibitor was the Zn^{2+} ion which caused a loss of over 90% of aminopeptidase activity already at concentration 0.1 mM. Attempts were also made to reactivate the activity of the enzyme Co^{2+}, Ca^{2+} and Mg^{2+} ions after prior inhibition of its activity in the presence of 10 mM EDTA and dialysis of the incubation mixture. The activity of the enzyme was restored only after the addition of Co^{2+} to the incubation mix. Ions Ca^{2+} and Mg^{2+} did not affect the recovery of aminopeptidase activity.

Substrate specificity and substrate affinity. The studied enzyme showed highest activity against the substrate Arg-β-NA (Table 4). The activity of the enzyme against this particular substrate was taken as 100%. The analyzed aminopeptidase was also visibly active against naphthylamide derivatives of such amino acids Lys and of the aromatic amino acids Trp, Tyr and Phe. The purified enzyme was not active against any of the other tested substrates: Leu-, Ala-, Pro-, Gly-, His-, and Val-β-NA. The substrate affinities were determined in the presence of those substrates with which enzymatic activity was observed (Table 4). The lowest value of the constant K_m, that is the highest affinity, was obtained with Arg-β-NA. The affinity of the aminopeptidase for naphthylamide derivatives of the aromatic amino acids: Trp-β-NA and Tyr-β-NA was lower and did not show any differences between them, whereas the value of the Michaelis-Menten constant for Phe-β-NA varied from that of the others.

Table 2. Effect of inhibitors on activity of *B. mycoides* aminopeptidase

Inhibitor	Concentration, mM	Relative activity, %
None	–	100
EDTA	1	15
	10	0
1,10-Phenantroline	1	44
	3	13
Iodoacetamide	1	99
	3	98
E-64	0.01	100
	0.1	99
PMSF	1	98
	3	94
Pepstatin A	3	100

Identification of purified enzymatic protein. The purified protein was subjected to identification using the mass spectrometry (MS) technique. The peptide sequences identified using mass spectrometry showed high similarity to the sequences of aminopeptidase S (Amp S) from *B. cereus* and aminopeptidase II from *Bacillus thuringiensis*. The fragments of the amino acid sequences of the studied enzyme identified by MS demonstrated high similarity to the sequence of *B. cereus* Amp S (Fig. 6). However, only 30% overlap with the two mentioned aminopeptidases was obtained. Consequently, the additional cloning of part of the gene coding for using genomic DNA of the studied strain as PCR template, was carried out. The obtained nucleotide sequence (1065 bp) after being translated to the amino acid sequence showed full identity with the sequence of *B. cereus* AmpS (Fig. 6 line A and B).

A 354 amino acids fragment was obtained, which consists 76% of the *B. cereus* aminopeptidase sequence. The studied enzymatic protein from *B. mycoides* is built from a large amount of alanine (11.6%), glutamic acid (9.9%) and leucine (8.5%). The amino acid composition also includes a small amount of arginine (2.2%), glutamine and methionine (1.4%) as well as cysteine (0.8%). The total number of amino acids carrying a positive charge (arginine and lysine) was 34, whereas such amino acids as aspartic acid and glutamic acid were found to occur in 53 positions. The theoretical pI, calculated on the basis of the obtained sequence, was 5.12.

DISCUSSION

This study describes the complete purification procedure of an intracellular aminopeptidase synthesized by a soil strain of *B. mycoides* and the catalytic, kinetic and partly molecular characteristics of the enzyme. The purified enzymatic protein, in the form of both a solution and a band excised from polyacrylamide gel was subjected to MS analysis. Both samples yielded compatible identification of an enzymatic protein with no other protein with proteolytic activity being present.

The obtained results pointed to the similarity of the amino acid sequence of the protein to the sequence of Amp S of *B. cereus* and aminopeptidase II of *B. thuringiensis*. In spite of repeated MS analyses we were not able to achieve a larger degree of overlap with the *B. cereus* protein sequence. This can be explained in one of two ways. The first explanation could be that there were problems with the appropriate length of the analyzed peptides ob-

Table 3. Effect of metal ions on activity of *B. mycoides* aminopeptidase

Metal ion	Concentration, mM	Relative activity, %
None	–	100
Co^{2+}	1	190
	0.1	135
Ca^{2+}	1	121
	0.1	106
Mg^{2+}	1	113
	0.1	112
Zn^{2+}	1	6
	0.1	8
Hg^{2+}	1	12
	0.1	16
Cu^{2+}	1	13
	0.1	38
Mn^{2+}	1	43
	0.1	55

Table 4. Substrate preferences and affinity of purified *B. mycoides* aminopeptidase

Substrate	Activity, %	K_m, M
Arg-β-NA	100	4.3×10^{-5}
Lys-β-NA	70	1.5×10^{-5}
Trp-β-NA	55	4.4×10^{-4}
Tyr-β-NA	45	5.6×10^{-4}
Phe-β-NA	30	1.1×10^{-3}

tained after trypsin digestion, stemming from the unfortunate distribution of Arg and Lys residues in some parts of the amino acid sequence of the analyzed protein. The second possibility is that the sequence of the aminopeptidase studied by us could be similar to the *B. cereus* enzyme only to the extent observed. In order to verify the latter hypothesis cloning of part of the *B. mycoides* gene coding for the aminopeptidase was undertaken. Primers were designed for the *B. cereus* gene sequence, but in their design the results of MS analysis were also taken into account. This result allows stating that the characterized enzyme is Amp S synthesized by a soil strain of *B. mycoides*.

As yet there have been only very scant reports in the literature regarding the biochemical properties of Amp S. A problem in studies on Amp S of *Staphylococcus* was obtaining and analysis of the crystalline structure of the protein by Odintsov et al. [20]. The experience of the study group resulted in the creation in the MEROPS base of a new MQ clan of metallopeptidases within the metallopeptidase M29 family, which includes Amp S and aminopeptidase T [20, 21]. Lin et al. [15] performed studies in which they demonstrated the similarity in the structure of the active centre of *S. aureus* Amp S and aminopeptidase II from *B. sterothermophilus*. However, a comparative analysis of the complete amino acid sequences of both these aminopeptidases carried out by the mentioned authors revealed only 40% convergence.

The activity of the *B. mycoides* aminopeptidase characterized in the current study is strongly inhibited in the presence of EDTA and 1,10 phenanthroline. The active centre of the enzyme most likely contains cobalt ions, this being indicated not only by the activation of the enzyme after addition of these ions but also by reactivation of the enzyme previously treated with EDTA. The activation of bacterial metalloaminopeptidases by cobalt ions has been described in the literature many times, examples being the methionine aminopeptidase of *B. subtilis* [22], the leucine aminopeptidase of *B. kaustophilus* CCRC [23], as well as *Thermus aquaticus* aminopeptidase T [21].

The substrate preferences of the *B. mycoides* aminopeptidase studied by us differ from those described for the Amp S homologue aminopeptidase T [24]. Purified *B. mycoides* aminopeptidase demonstrated hight activity against the synthetic substrate Arg-β-NA and was not active against substrate with N-terminal leucine. This property distinguishes the studied enzyme from aminopeptidase II found in bacteria belonging to the genus *Bacillus*, which are classified as leucine aminopeptidases. There are no direct data in the literature regarding the substrate preferences of Amp S. It has only been shown that *Staphylococcus aureus* Amp S was also active against a substrate with N-terminal tyrosine [20]. The absence of activity with regard to Leu-β-NA allows to distinguish the studied aminopeptidase also from the arginyl aminopeptidase synthesized by the yeast *Debaromyces hansenii*, which was active against Arg-β-NA, Lys-β-NA, Tyr-β-NA, Phe-β-NA and Leu-β-NA [25].

The molecular weight of the *B. mycoides* aminopeptidase was determined to be about 50 kDa. Our results indicate that the enzyme is a monomer. This is shown by the results of gel filtration chromatography and by the fact that the enzyme did not lose activity after electrophoretic separation under semi-denaturing conditions. In-depth studies on the subunit structure of *S. aureus* Amp S were carried out by the above-mentioned authors [20]. The obtained crystalline form of the enzyme suggests that it is a subunit protein with homodimer structure. However, in view of the doubts stemming from the location of the active centre in a dimeric molecule the authors suggest that it may function in solution as an active monomer. Aminopeptidases T [27] and aminopeptidases II [14] are also dimers with similar molecular weight of the monomer. Aminopeptidases composed of a larger number of subunits are also known, an example of which is the trimeric arginyl aminopeptidase of *Lactobacillus sakei* [7]. A molecular weight similar to that determined in our study has been described for several monomers, including the leucyl aminopeptidase from *B. kaustophilus*, [23] and ami-

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Line 1  MSFEQTLEKY AALAVNVGVN IQPGQTLSIS APLEAVQFVR LVTEKAYKSG AKHVYVDWND  60
Line A  MSFEQTLEK- ----------------SIS APLEAVQFVR ------------HVYVDWND
Line B                              SIS APLEAVQFVR LVTEKAYKSG AKHVYVDWND
Line 1  ETLTRLKFDL APEEAFAEFP SWKAHAREEL AKEGAAFMSI YAENPDLLKG VESTRIATAH 120
Line A  ETLTR--FDL APEEAFAEFP SWK------- --EGAAFMSI YAENPDLLK- ----------
Line B  ETLTRLKFDLAPE EAFAEFP SWKAHAREEL AKEGAAFMSI YAENPDLLKGV ESTRIATAH
Line 1  KVAGEAMKVY RDYVQADKVS WCVISVPTKE WAAKVFPDVA PEEQEAKLWD AIFKATRADL 180
Line A  ---------- --------VS WCVISVPTK- ----VFPDVA PEEQEAK--- -------ADL
Line B  KVAGEAMKVYRDYVQADKVS WCVISVPTKE WAAKVFPDVA PEEQEAKLWD AIFKATRADL
Line 1  ENPVEAWKEH DKTLHTKVDY LNEKHYKALH YTGPGTDLTI ELPEKHVWAG AGSLNEKNVP 240
Line A  ENPVEAWK-- ---------- ---------- ---------- -----HVWAG AGSLNEK---
Line B  ENPVEAWKEH DETLHTKVDY LNEKHYKALH YTAPGTDLTI ELPEKHVWAG AGSLNEKNVP
Line 1  FMANIPTEEV FTMPLKTGVN GQVSSTKPLA FAGNIIDNFT LTFENGRIVD YKAEVGEEAL 300
Line A  -MANIPTEEV FTMPLK---- ---------- -AGNIIDNFT LTFENGR--- ----------
Line B  FMANIPTEEV FTMPLKTGVN GQVSSTKPLA FAGNIIDNFT TFENGRII-D YKAETGEEAL
Line 1   KHLVETDEGS HFLGEVALVP HDSPISNTNI LFYNTLFDEN ASCHLAIGNA YAFNLVGGKT360
Line A  ---------- ---------- ---------- ---------- ---------- -  AFNLVGGK-
Line B  KHLVETDEGS HFLGEVALVP HDSPISNTNI LFYNTLFDEN ASCHLAIGNA YAFNLVGGKT
Line 1   MSKEELAKNG ANASITHEDF MIGSAELDID GITADGRHEP IFRKGNWAF           409
Line A   ---------- ---------- ---------- ---------- ---------
Line B  ---------- ---------- ---------- ---------- ---------
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Fig. 6. Multiple-sequence alignment of aminopeptidase from *B. mycoides:*
Line 1 Amino acid sequence of aminopetidase *B. cereus* (GeneBank ZP_04298756); Line A – MS analysis of amino acids sequence of aminopeptidase from *B. mycoides;* Line B – amino acid sequence translated from nucleotide sequence from obtained aminopeptidase clone.

nopeptidase PepS [26]. A higher molecular weight of about 70 kDa was reported for the arginyl aminopeptidase from *Streptococcus gordonii* [6].

Since the pH optimum of the studied aminopeptidase is 9.0, the enzyme can be classified along with alkaline aminopeptidases that have been described in the literature. Examples of these are the leucyl aminopeptidase from *Streptomyces hygroscopicus*, with pH optimum 8.0 [28] the extracellular aminopeptidase from *B. subtilis* isolates, which has optimal activity in the pH 8–9 range [29] or the *B. thuringiensis* leucyl aminopeptidase with pH optimum of 10.0 [30].

It has been determined that the amino acid sequence of studied enzyme is similar to a large degree to the sequence of Amp S and aminopeptidase II of bacteria belonging to the genus *Bacillus*, grouped in clan M29 (http://merops. sanger.ac.uk) embracing thermophilic peptidases [15, 24]. However, our studies show that the *B. mycoides* aminopeptidase differs from the mentioned aminopeptidases in the lack of this characteristic thermal stability.

REFERENCE

1. Matsui, M., Fowler, J.H., and Walling, L.L., *Biol. Chem.,* 2006, vol. 387, no. 12, pp. 1535–1544.
2. Cahan, R., Axelrad, I., Safrin, M., Ohmann, D.E., and Kessler, E. *J. Biol. Chem*., 2001, vol. 276, no. 2, pp. 43645–43652.
3. Jankiewicz, U. and Bielawski, W., *Acta Microbiol. Polon*., 2003, no. 3, vol. 52, pp. 217–231.
4. Gonzales, T. and Robert-Baudouy, J., *FEMS Microbiol*., 1996, vol. 18, no. 4, pp. 319–344.
5. Fanuel, L., Goffin, C., Cheggour, A., Devreese, B., Driessche, G., Joris, B., Beeumen, J., and Frere, J.M., *J. Biochem*., 1999, vol. 341, pp. 147–155.
6. Goldstein, J.M., Nelson, D., Kordula, T., and Mayo, J.A., *Infect. Immun. J*., 2002, vol. 70, no. 2, pp. 836–843.
7. Sanz, Y., and Toldra, F., *Appl. Environ. Microbiol*., 2002, vol. 68, pp. 1980–1987.
8. Lee, G.D., Chun, S.S., Kho, Y.H., and Chun, H.K., *J. Appl. Microbiol*., 1998, vol. 84, no. 4, pp. 561–566.

9. Jankiewicz, U. and Bielawski, W., *Folia Microbiol.*, 2002, vol. 47, no. 3, pp. 230–234.
10. Lu, Ch.D., *Appl. Microbiol. Biotechnol.*, 2006, vol. 70, no. 3, pp. 261–272.
11. Hansen, B.M., Leser, T.D., and Hendriksen, N.B., *FEMS Microbiol. Lett.*, 2001, vol. 202, no. 2, pp. 209–213.
12. Bargabus, R.N., Zidack, J. Sherwood, and Jacobsen, B., *Mol. Plant-Microbe Interact.*, 2003, vol. 16, no. 12, pp. 145–1153.
13. Yudono, B., Said M., Hakstege, P., and Suryadi, F.X., *South J. Sustainable Develop.*, 2009, vol. 2, no. 1–4, pp. 64–71.
14. Kuo, L.Y., Hwang, G.Y., Lai, Y.J., Yang, S.L., and Lin, L.L., *Curr. Microbiol.* 2003, vol. 47, no. 1, pp. 40–45.
15. Lin, L.L., Chen, Y.P., Yang, J.C., Hua, Y.W., Wang, W.C., and Kuo, L.Y., *Protein J.*, 2008, vol. 27, no. 4, pp. 215–222.
16. Kolehmainen, L. and Mikola, J., *Arch. Biochem. Biophys.,* 1971, vol. 145, no. 2, pp. 633–642.
17. Bradford, M.M., *Anal. Biochem.,* 1976, vol. 72, no. 7, pp. 248–254.
18. Laemmli, U.K., *Nature,* 1970, vol. 227, no. 15, pp. 680–685.
19. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M., *Anal Chem.* vol. 68, no. 5, pp. 850–858.
20. Odintsov, S.G, Sabaa, I., Bourenkov, G., Rybin, V., and Bochtler, M., *J. Biol. Chem.*, 2005, vol. 280, no. 30, pp. 27792–27799.
21. Odintsov, S.G., Sabaa, I., Bourenkov, G., Rybin, V., and Bochtler, M.*, J. Mol. Biol.,* 2005, vol. 354, pp. 403–412.
22. You, C.H., Lu, H.Y., Sekowska, A., Fang, G., Wang, Y.P., Gilles, A.M., and Danchin, A., *BMC Microbiol.*, 2005, vol. 5, pp. 57–67.
23. Linn, L.-L., Hsu, W.-H., Wu, C.-P., Chi, M.-C., Chou, W.-M., and Hu, H.-Y., *Extremophiles,* 2004, vol. 8, no. 1, pp. 79–87.
24. Minagawa, E., Kaminogawa, S., Matsuzawa, H., Ohta, T., and Yamauchi, K., *Agr. Biol. Chem.*, 1988, vol 52, no. 7, pp. 1755–1763.
25. Bolumar, T., Sanz, Y., Aristoy, M. C., and Toldra, F., *Int. J. Food Microbiol.*, 2003, vol. 86, no. 1–2, pp. 141–151.
26. Fernandez-Espla, M.D. and Rul, F., *Eur. J. Biochem.*, 1999, vol 263, no. 2, pp. 502–510.
27. Motoshima, H., Azuma, N., Kaminogawa, S., Ono, M., Minagawa, E., Matsuzawa, H., Ohta, T., and Yamauchi, K., *Agr. Biol. Chem.*, 1990, vol. 54, no. 9, pp. 2385–2392.
28. Karadzic, I., Izrael, L., Gojgic-Cvijovic, G., and Vujcic, Z., J. *Biosci. Bioeng.*, 2002, vol. 94, no. 4, pp. 309–314.
29. Reiland, V., Fundoiano-Hershcovitz, Y., Golan, G., Gilboa, R., Shoham, Y., and Shoham, G., *Acta Cryst.*, 2004, vol. 60, pp. 2371–2376.
30. Cahan, R., Hetzroni, E., Nisnevitch, M., and Nitzan, Y., *Curr. Microbiol.,* 2007, vol. 55, no. 5, pp. 413–419.