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Characterization and Mass Spectrometry Analysis of Aminopeptidase N from *Pseudomononas putida* Lup

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Abstract

An intracellular aminopeptidase N synthesized by *Pseudomonas putida* Lup was purified and characterized. The approx. 150-fold purified enzyme showed highest activity against A-β-naphthylamide at pH 7.5 and at temperature 40°C and was 100% thermostable for 240 min at 40°C. *P. putida* lup aminopeptidase N is a monomer with molecular mass approx. 99 kDa determined by SDS-PAGE and gel permeation chromatography. The enzyme has broad substrate specificity, but is the most active against protein substrates with N-terminal alanine and arginine. The activity of *P. putida* Lup aminopeptidase N is strongly inhibited in the presence of specific metallopeptidase inhibitors and is partly recovered in the presence of Zn^{2+} and Co^{2+} ions. Co^{2+}, Mg^{2+} and Ca^{2+} ions increased the activity of the enzyme. Moreover, the enzyme was inhibited by inhibitors of cysteine enzymes. Analysis of fragments of the amino acid sequence of the purified enzyme demonstrated high similarity to PepN of *Pseudomonas putida* GB-1.

K e y   w o r d s: *Pseudomonas putida*, aminopeptidase N, purification of enzymes

Introduction

Peptidases perform several significant functions in both eukaryotic and prokaryotic organisms. Intracellular peptidases are involved in processes such as splitting off signal peptides from newly synthesized proteins, activation of inactive precursors, inactivation of regulatory proteins and degradation of damaged peptides. Extracellular peptidases can be toxins and virulence factors, whereas others demonstrate low specificity and participate in the breakdown of proteins in the environment to short peptides or single amino acids, which can then be taken up and utilized by the cell (Addlagatta *et al.*, 2006; Jankiewicz and Bielawski, 2003). An important role in cellular protein metabolism in both eukaryotic and prokaryotic organisms is played by aminopeptidases N (APN, EC 3.4.11.2). APN found in living cells is a common exopeptidase with broad substrate specificity.

Mammalian aminopeptidases N are membrane enzymes that carry out diverse physiological functions, such as receptors for corona- and other human viruses. They also participate in angiogenesis and stimulation of tumor growth and regulation of blood pressure (Kumar *et al.*, 2009). The role of these exoproteases in physiological processes of the host justifies intensive studies on these enzymes and their inhibitors. In bacteria, aminopeptidases N occur in the cytosol where they participate in ATP-independent protein metabolism and play principal role in the maturation, activation and final stage of peptides hydrolysis to single amino acids (Addlagatta *et al.*, 2006; Lazdunski *et al.*, 1975). Bacterial APN in particular are the main intracellular enzymes releasing N-terminal alanine from protein substrates.

APN belong to the zinc-dependent metallopeptidases, grouped, according to the Merops classification, into family M1 included in the Gluzincin superfamily (thermolysin-like peptidases). All of these enzymes are metallopeptidases with a single zinc ion in the active center (Hooper, 1994; Rawlings and Barrett, 1993). APN have a characteristic conservative Zn-binding $HEXXH(X)_{18}E$ motif, in which a Zn ion is coordinated by two histidine residues and distal glutamic acid. The glutamate next to the first Zn^{2+}-binding histidine in the HEXXH motif is essential for water hydrolysis of

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peptide bond and subsequent release of the substrate (Kumar *et al.,* 2009; Peer, 2011). The structure of the active center has been studied in detail for the APN of the gram-negative bacterium *Escherichia coli.* It has been found that structurally it exhibits strong similarity to thermolysin. The APN of *E. coli*, which is composed of 870 amino acids, contains four domains, and the active center is located in domain II, encompasing amino acids residues from 194 to 443 (Matthews, 1988). There are also reports in the literature suggesting the presence of two active centers in APN, one responsible for aminopeptidase activity and one for endopeptidase activity (Chandu *et al.*, 2003). However, more recent data rule out this hypothesis (Addlagatta *et al.*, 2006). They are enzymes with broad substrate specificity, showing highest activity against protein substrates with the alkaline amino acid residues: R and K or hydrophobic ones, *e.g.* A, L, M, at the N-terminus (Chandu and Nandi, 2003; McCaman and Villarejo, 1982).

The objective of the described studies was to purify, identify and characterize the intracellular aminopeptidase synthesized by a strain of the bacterium *Pseudomonas putida.*

Experimental

Materials and Methods

Biological materials. A strain of bacteria synthesizing an intracellular aminopeptidase N (ApN) was isolated from the rhizosphere of winter wheat cultivated in a field in the eastern part of Poland. Bacteria were isolated using King B medium (King *et al.*, 1954). Identification of the strain selected for further studies was based on biochemical and morphological traits (Holt *et al.*, 1994). The identity of the strain was confirmed by 16S rRNA gene sequence analysis. Amplification of 16S rRNA gene was accomplished using universal primers 27 F and 1492 R (Watanabe *et al.*, 2001). The template in the reaction was genomic DNA isolated from bacterial cells in late logarithmic stage of culture, using a Genomic DNA Purification Kit (Fermentas). The purified PCR product was sequenced in the DNA Sequencing and Oligonucleotides Synthesis Laboratory at IBB PAS (Warsaw, Poland). The obtained nucleotide sequences were compared with those deposited in the available data bases GenBank/EMBL/DDBJ using the program BLAST. The obtained 16S rRNA gene sequence was deposited in the DDBJ database with access number AB667905.

Composition of culture medium, growth conditions and preparation of enzyme extract. The liquid growth medium used was a mineral medium according to Bassalik, composed of: 0.03% K_2PO_4; 0.03% KH_2PO_4; 0.05% $MgSO_4$; 0.05% NaCl and 0.0015% $FeCl_3$, supple-

mented with 1.5% tryptone, 0.25% yeast extract and 0.3% glucose. The pH of the medium was adjusted prior to autoclaving to 7.0.

Bacteria were grown for 48 h with shaking at 120 rpm and temperature 28°C. Growth of bacteria was monitored by following OD_{600}. After appropriate culture time the bacteria were spun down (12000×g, 20 min) and the bacteria in the pellet, after discarding the supernatant, were washed twice in Tris-HCl buffer, pH 8.5 and disrupted by sonication. The clear intracellular protein extract obtained after centrifugation was used as the preparation for purification of the aminopeptidase.

Enzymatic activity. Aminopeptidase activity was determined using synthetic amino acid derivatives of β-naphthylamide. The incubation mixture consisted of: enzyme preparation, 0.05 M Tris-HCl buffer, pH 7.5 and substrate in final concentration 1 mM. The reaction was conducted for 30 minutes and then terminated by the addition of 0.1% solution of diazo salt of o-aminotoluene (Fast Garnet GBC), dissolved in 1 M acetate buffer, pH 4.2 with the addition of 10% Tween 20. The absorbance of the colored product was determined photometrically at 525 nm. Absorbance readings were calculated to give μmole product formed using a standard curve prepared for five concentrations of β-naphthylamine.

One unit of activity was defined as one μmol naphthylamine formed in 1 min reaction time.

Purification of the enzyme. All purification stages (except for HPLC chromatography) were carried out at 4°C. Fractionation with ammonium sulfate was performed by salting out the enzyme preparation in the first stage of fractionation to 35%, and in the second to 85% saturation with ammonium sulfate. The protein deposit was dissolved in 20 mM Tris-HCl buffer, pH 7.8 and dialyzed overnight against the same buffer. The obtained preparation was subjected to low pressure ion-exchange chromatography on anionite cellulose DEAE 52.

Prior to chromatography, the column was equilibrated with 20 mM Tris-HCl buffer, pH 7.8. Protein was eluted with linear NaCl gradient from 0 to 0.5 M. Fractions with highest enzymatic activity were pooled and dialyzed for 12 hours against 20 mM Tris-HCl buffer, pH 7.8. The next stage of purification involved preparative electrophoresis in BIO-RAD Model 491 Prep Cell apparatus, with 7.5% separating gel and 4% concentrating gel, at constant voltage of 240 V. Separation was in 25 mM Tris – 192 mM glycine buffer, pH 8.3. Proteins were eluted from elution chamber with 25 mM Tris-HCl, pH 7.8. Fractions showing highest alanyl aminopeptidase activity were used for the next purification step, *i.e.* high pressure ion-exchange chromatography HPLC. The enzyme solution was applied to Protein-Pak Q 8HR column, which was first equilibrated with 15mM Tris-HCl buffer, pH 7.8. Elution was with linear NaCl gradient from 0.2 to 0.5 M. Fractions with highest

aminopeptidase activity were used for enzyme characterization and MS analysis experiments.

Determination of protein content. Measurements of protein concentration at all stages of the study were made using the Bradford method (1976). Absorbance values were converted to μg protein using a standard curve plotted for five concentrations of bovine serum albumin.

Electrophoretic separations and detection of aminopeptidase activity. Electrophoresis under native and denaturing conditions was according to the procedure described by Laemmli (1970). The protein bands were visualized using Coomassie Brilliant Blue R-250. Aminopeptidase activity in polyacrylamide gel following electrophoretic separation was detected after incubation of the gel in 1 mM solution of the substrate in Tris-HCl buffer, pH 7.5.

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: Alcohol dehydrogenase (150 kDa), bovine albumin (67 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). The void volume of the column (Vo) was determined using dextran blue.

Characterization of the properties of purified enzyme. The optimum pH was determined in a range of 5.0–9.0. The buffer systems used were: 50 mM Britton-Robinson buffer for the pH range of 5.0–9.0 and 50 mM Tris-HCl buffer for the pH range of 6.8–8.5. The optimal temperature was determined in the range from 25 to 55°C. Thermal stability was determined after 1, 2, 3 and 4 h preincubation of the enzyme at temperatures: 40, 45 and 50°C.

Determination of Michaelis-Menten constants. The substrate affinities were determined in the presence of those substrates with which high enzymatic activity (over 60% compared to A- β-naphthylamide activity) was observed; the final concentration of the substrates in the reaction mix was from 0.015 to 1.5 mM. The Km value was determined using the graphical Lineweaver-Burk method.

The effect of metal ions on activity was determined following preincubation of the enzyme for 30 min at 4°C in the presence of divalent metal ions (Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}) in final concentration of 0.1 and 1.0 mM, after which the substrate was added and the residual activity tested.

In order to study the effect of specific inhibitors on activity, the enzyme was preincubated with an inhibitor solution for 60 minutes at 4°C after which the remaining activity was tested

Reactivation of protease activity after inhibition by EDTA was carried out in the presence of Ca, Mg and Zn ions following dialysis of the preparation.

Analysis of fragment of the amino acid sequence of the purified enzymatic protein. Both the highly purified enzyme preparation and the protein band (approx. 99 kDa) excised from polyacrylamide gel were subjected to mass spectrometry analysis (IBB PAN). The protein concentration in the sample was 10 μg/ml. A protein sample previously digested with trypsin was separated on a nanoAcquity UPLC (Ultra Performance LC) system and analyzed with an Orbitrap-based mass spectrometer.

All results presented in this paper in the form of numerical values are means from three independent repetitions. The mean error, reflecting maximal deviation of the results of measurements from the mean, did not exceed 5%.

Abbreviations:

K_m – Michaelis-Menten constant
APN – Aminopeptidase N
β-NA – β naphtylamide
EGTA – ethylene glycol tetraacetic acid
EDTA – ethylenediaminetetraacetate

Results

The intracellular aminopeptidase synthesized by *Pseudomonas putida* Lup was purified over 150-fold in a four step procedure. The enzymatic activity of the enzyme during its purification and characterization was determined using A-β NA as a substrate (Table I).

Table I
Purification of *P. putida* Lup aminopeptidase.

Steps	Total aminopeptidase activity [U]*	Total protein [mg]	Specific activity [U/mg]	Fold	Yield purification [%]
Crude enzyme	10227.3	1605.6	6.4	1.0	100
Ammonium sulfate precipitation (35-85%)	8454.9	619.4	13.7	2.1	82.7
Ion-exchange chromatography cellulose DEAE 52	6715.4	186.6	36	5.6	65.7
Preparative electrophoresis	2312.5	7.9	292.7	45.7	20.6
Ion-exchange chromatography HPLC Mono Q 8HR	908.5	0.93	976.9	152.6	8.9
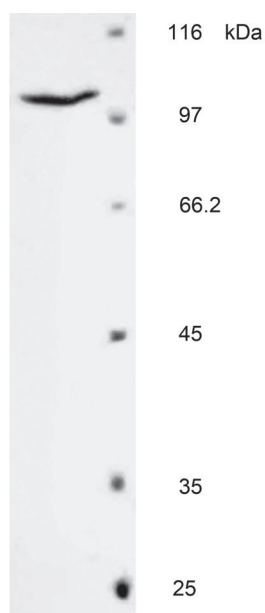
Fig. 1. SDS-PAGE of the purified aminopeptidase.

studied enzyme is a monomer. Proteomic analysis of the purified enzymatic preparation using mass spectrometry (MS) demonstrated that the amino acid sequence of the studied enzymatic protein is highly similar to the sequence of the aminopeptidase N of *Pseudomonas putida* GB-1 (GenBank accession no. YP_001667790 which is identical to that translated from the nucleotide sequence of Gene ID: 58693290). The peptides derived from the studied aminopeptidase overlap with 45% of the aa sequence of aminopeptidase N from *P. putida* GB-1 (Fig. 2). High overlap of the obtained peptide sequence within the conserved domain of APN in *Pseudomonas* bacteria between amino acid residues 212 and 421 was determined. APN in strain GB-1 is composed of 885 amino acid residues. Its calculated molecular weight was 99.549 kDa and the theoretical pI was 5.06.

The purified *P. putida* Lup aminopeptidase has broad substrate specificity (Table II). The enzyme was shown to be active against the following amino acid derivatives of β-naphthylamide: A-β-NA (100%), R-β-NA (60%), K-β-NA (25%), L, S (10%) and G, M-β-NA (5%). The substrate affinity of *P. putida* lup aminopeptidase was determined for the substrates the enzyme had the highest activity against. Km constants determined did not show great variation, even though the lowest K_m was obtained during enzymatic reaction against A-β-NA, which points to the highest affinity of the enzyme for this particular substrate. The effect of specific inhibitors on the activity of the studied enzyme was also examined (Table III). Partial inhibition of enzyme activity observed when inhibitors such as EDTA and

Salting out with ammonium sulfate and low pressure ion-exchange chromatography on DEAE cellulose 52 resulted in a sample purified almost 6-fold with 65.7% recovery of total activity. Considerable, over 8-fold purification of the enzyme was obtained after preparative electrophoresis. After the final step of purification, ion-exchange HPLC chromatography, over 150-fold purification of the enzyme was achieved. The molecular weight of the enzyme determined by SDS PAGE was approx. 99 kDa (Fig. 1). This result was confirmed using molecular sieve chromatography, which determined a molecular weight of 98 kDa. This value shows that the

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001MRTEQPQVIY LKDYQAPEYL IDETHLTFEL FEDHTLVHAQ LVMRRNPARG
051AGLPPLVLDG QQLELLRASL DDLELQAGDY QLEADSLTLH PKAERFTLDT
101SVKIHPESNT ALEGLYKSGK MFCTQCEAEG FRKITYYLDR PDVMSTFTTT
151VIAEQHRYPV LLSNGNPIGS GPAEDGRHWA TWEDPFMKPA YLFALVAGDL
201WCVEDTFTRQ SGRDVILRIY VEPENIDKCD HAMVSLKKSM RWDEEVYGRE
251YDLDIFMIVA VNDFNMGAME NKGLNIFNSS CVLARAETAT DAAHQRVEGV
301VAHEYFHNWS GNRVTCRDWF QLSLKEGFTV FRDAEFSADM NSRTVKRIED
351VAYLRTHQFA EDAGPMAHPV RPDSFIEISN FYTLTVYEKG AEVVRMVRTL
401LGAEGFRKGS DLYFERHDGQ AVTTDDFIKA MEDANGVDFT QFKRWYSQAG
451TPRLEVSEAY DAAAQAYSLT FRQSCPQTPD KAEKLPFVIP VELGLLDAAG
501NDLPLQLAGE ATAQGTSRVL SVTEAEQTFT FQRIQAKPLP SLLRGFSAPV
551KLSFPYDRDQ LMFLMQHDSD GFNRWEAGQQ LSVQVLQELI GQHQRGEALK
601LDQRLITALG TVLGNESLDP AMVAEMLSLP GEAYLTEISQ VADVDAIHAA
651REFARQQIAE QLFDALWARY QANREVSRST EYVASAEHFA RRSLQNIALS
701YLMQSGKPQV LEATLEQFEH CDNMTERLTA LAVLVNSPFE AERAKALEA
751AEHFKDNPLV MDQWFSVQAA SALPGGLARV KALMQHPAFT LKNPNKVRA
801IGAFAGQNLV NFHAADGSGY RFLADLVIEL NALNPQIASR QLAPLTRWRK
851YDEARQALMK GELERILASG ELSSDVYEVV SKSLA
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Fig. 2. Sequence of aminopeptidase N from *P. putida* GB-1.

Sequence alignment of aminopeptidase N from *P. putida* GB-1 (GenBank accession no. YP_001667790 which is identical to that translated from the nucleotide sequence of Gene ID: 58693290) and studied aminopeptidase from *P. putida* lup (letters underlined). Bold letters: Zn ion is coordinated by two histidine residues (H 303, 307) and distal glutaminic acid (326), the glutamate (304) is essential for water hydrolysis of peptide bond and subsequent release of the product.

Table II
Substrate preferences and affinity of purified *P. putida* Lup
aminopeptidase.

Substrate	activity (%)	Km value [M]
Ala-β-NA	100	6.8×10^{-5}
Arg-β-NA g	60	7.8×10^{-5}
Lys-β-NA	25	7.5×10^{-5}
Leu-β-NA	10	nd
Ser-β-NA	10	nd
Gly-β-NA	5	nd
Met	5	nd

Not-determined-nd

Table III
Effect of inhibitors on activity of *P. putida* Lup aminopeptidase.

Inhibitor	Concentration (mM)	Activity (%)
None	–	100
EDTA	1	85
	10	20
1,10-Phenantroline	1	70
	3	5
Iodoacetamide	0.5	60
	1	47
E-64	0.01	50
	0.1	40
DFP	0.1	98
	1	96
Pepstatin A	3	100
Bestatin	0.0005	10
Amastatin	0.00005	0

Table IV
Effect of metal ions and reducing agent on activity
of *P. putida* Lup aminopeptidase.

Metal ion	Concentration (mM)	Relative Activity (%)
None	–	100
Co²⁺	0.1	120
	1.0	140
Ca²⁺	0.1	120
	1.0	130
Mg²⁺	0.1	115
	1.0	130
Zn²⁺	0.1	10
	1.0	0
Pb²⁺	0.1	15
	1.0	10
Cu²⁺	0.1	65
	1.0	18
Mn²⁺	0.1	95
	1.0	85
Reduced glutathione (GSH)	0.1	120
	1.0	180
Dithioerythritol (DTE)	0.1	160
	0.5	90

1.10 phenanthroline were added to the reaction mixture, it indicates that the protein is a metallopeptidase. In this case partial reactivation of activity was obtained after adding Co²⁺ (20%) and Zn²⁺ions (50%). These results were confirmed in reaction with bestatin and amastatin, which are diagnostic inhibitors for metalloexopeptidases. However, inhibition of the enzyme was also demonstrated in the presence of cysteine protease inhibitors E64 and iodoacetamine. Moreover, after adding compounds counteracting the oxidation of -SH groups to the reaction mixture, such as reduced glutathione (GSH) or dithioerythritol (DTE), enhanced activity was observed, which is typical for cysteine enzymes (Table IV). Inhibitors specific for the remaining catalytic types of proteases (serine and aspartyl) did not cause any change in enzyme activity. The studied aminopeptidase was activated about 30–40% when Ca,
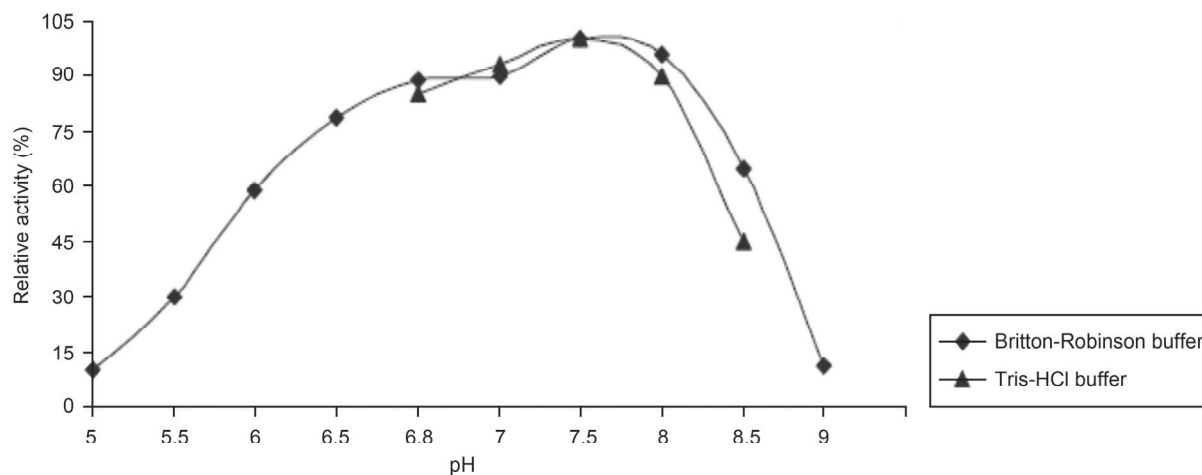

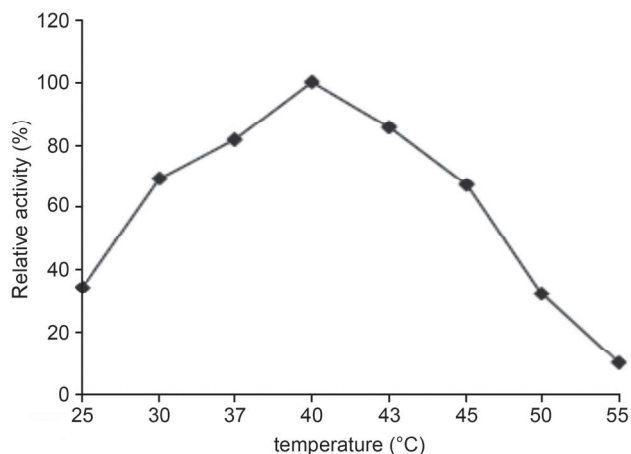Fig. 3. Effect of pH on *P. putida* Lup aminopeptidase activity.

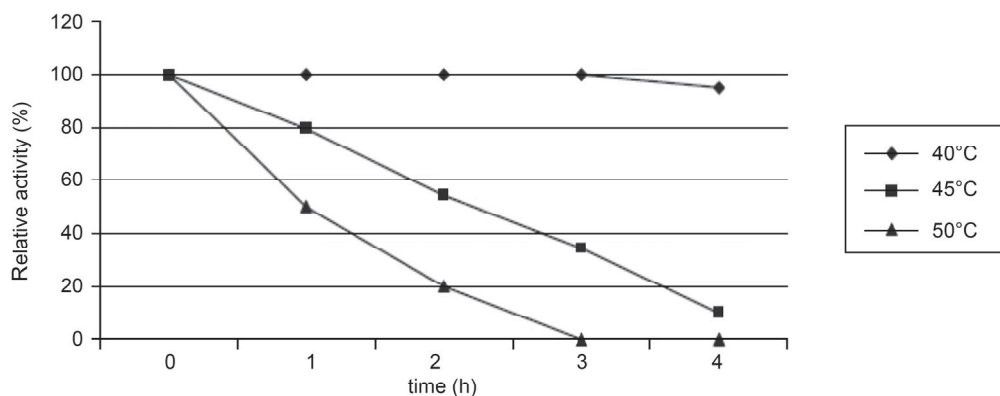Fig. 4. Effect of temperature on *P. putida* Lup aminopeptidase activity.

Fig. 5. Thermostability of *P. putida* Lup aminopeptidase at temperatures ranging from 40°C to 50°C.

are in the fragment from residue 212 to 421 (Hooper, 1994). So far the best characterized ApN of Gram-negative bacteria are those from *E. coli* (Ito *et al.*, 2006) and *Neisseria meningitidis* (Nocek *et al.*, 2008). The structure of these proteins has been investigated using crystallography methods, providing detailed information regarding their active center and the spatial structure of their functional domains. In the APN molecule from *E. coli,* four domains have been distinguished: N-terminal β-domain M^1-D^{193}, catalytic domain: F^{194}-G^{444}, middle β-domain: T^{445}-Y^{546} and C-terminal α-domain: S^{547}-A^{870} (Addlagatta *et al.*, 2006). The similarity of the amino acid sequence of aminopeptidases N of *E. coli* and *P. putida* is slightly over 50%.

There is far less information about the ApN of *Pseudomonas* sp. In case of *P. putida* ApN no detailed biochemical characterization of the enzyme has yet been conducted.

Mg and Co ions in concentration 1.0 mM were added to them in the incubation mixture. Partial inhibition of activity was observed after the addition of Pb and Zn ions in low, 0.1 mM concentration (Table IV).

The temperature and pH optima determined for A-βNA were 40°C and pH 7.5, respectively. The studied aminopeptidase demonstrated high activity in a broad pH range from 6.0 to 8.5 (Fig. 3). On the other hand, the activity of the enzyme was strongly dependent on temperature and slight deviations from the optimal temperature resulted in its significant decrease (Fig. 4). The studied aminopeptidase was characterized by high thermal stability after 240 min preincubation at 40°C (Fig. 5). Preincubation for the same length of time at 45°C resulted in almost complete loss of activity.

Discussion

APN produced by *P. putida* belong to family M1, subfamily M01 and has been given the number 005. The conserved domain for family M1 stretches from amino acid residue 15 to 384, conserved residues for aminopeptidase N of Gram-negative bacteria – M01.005

The aminopeptidase studied by our group has similar substrate specificity, except that its activity is the highest when the amino acid residue at the N-terminus of the protein substrate is alanine, and not arginine, like for the *E. coli* ApN. The substrate specificity of the *E. coli* ApN for N-terminal amino acids can be arranged as follows: A > R > K > P/G (Addlagatta *et al.*, 2008). A somewhat different substrate specificity was found for aminopeptidase N of *Streptococcus thermophilus* A. The enzyme demonstrated highest activity towards the following substrates: K-AMC: 100% > L 93% > R: 80 > M: 28% > A: 20% (Chavagnat *et al.*, 1999). Similar results were obtained also for *S. thermophilus* YRC001. For this reason the aminopeptidase N of these bacteria have been termed lysyl aminopeptidase (Motoshima *et al.*, 2003). On the other hand, the N-like aminopeptidase of *Lactobacillus curvatus* DPC2024 showed highest activity with protein substrates carrying N-terminal L (100%), K (63%), F (58%), M (26%) and A (6%) (Abdallah *et al.*, 1999). Studies on the APN of *E. coli* K12 have revealed that it is a metallopeptidase but a thiol group of a cysteine is also involved in catalysis

(Yoshimoto *et al.,* 1988). However, it has not yet been determined which of the 8 cysteine residues present in the APN sequence plays a crucial role for the activity of the enzyme (Nocek *et al.*, 2008). It cannot be excluded that the binding of the inhibitor to one of the cysteine residues located close to the active site of APN results in distortion of its structure and changes in the conformation of the protein molecule. A similar phenomenon is observed for the studied ApN from *P. putida* lup. The enzyme is strongly inhibited by specific inhibitors of cysteine enzymes and metallopeptidases. Stimulation of enzyme activity in the presence of reducing compounds confirms the importance of the -SH groups of cysteine in enzymatic catalysis or in maintaining the proper conformation of the enzyme molecule. The amino acid sequence of the *P. putida* APN contains amino acids residues involved in the binding of zinc ions: H 303, H 307 and E 326. The significant role of zinc ions in catalysis is also supported by the partial reactivation by these ions of the studied aminopeptidase previously treated with EDTA. The studied aminopeptidase was activated in the presence of Co ions and to a somewhat lesser degree by Ca and Mg. A similar dependence was observed for the PepN-like enzyme in *Lb. curvatus* DPC2024 (Abdallah *et al.*, 1999). Activation by Co ions has also been described for aminopetidase N *S. thermophilus* YRC001 aminopeptidase (Motoshima *et al.*, 2003).

The molecular mass of the studied enzyme determined by SDS PAGE is about 99 kDa. A similar molecular mass calculated based on the amino acid sequence of the genes coding the enzymes is given for other *P. putida* aminopeptidases N. Aminopeptidases N with similar molecular masses have also been found in *E. coli* K 12: 87 kDa (McCaman, and Villarejo, 1982) and S*treptococcus thermophilus* YRC001: 96.4 kDa (Motoshima *et al.*, 2003). The pH and temperature optima for the studied enzyme are 7.5 and 40°C, respectively. APN of *S. thermophilus* A shows optimal activity under similar conditions: pH 7.0 and 37°C (Chavagnat *et al.,* 1999). Similarly, the N-like aminopeptidase of *Lb. curvatus* demonstrates optimal activity at pH 7.0 and 40°C (Christensen *et al.*,1999)

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