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PROTEOLYTIC ACTIVITY OF *PSEUDOMONAS CHLORORAPHIS* 5N ISOLATED FROM RHIZOSPHERE SOIL

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ABSTRACT

A Gram-negative bacterial strain characterized by high proteolytic activity was isolated from rhizosphere soil under winter wheat. Morphological and biochemical testing as well as analysis of 16S rRNA gene sequence identified the strain as *Pseudomonas chlororaphis*. In this study an extracellular endopeptidase synthesized by the strain was purified and characterized. The enzyme showed highest activity at pH 11 and at temperature 42°C. The molecular weight determined by SDS-PAGE was approx. 51 kDa. The studied endopeptidase was characterized by low thermal stability in the 37-45°C range. The activity of the enzyme was strongly inhibited by specific metallopeptidase inhibitors, which allowed classifying the studied protein as a metalloendopeptidase. Ca^{2+} and Mg^{2+} ions stimulated the activity of the enzyme, whereas Mn^{2+}, Co^{2+} and Cu^{2+} ions inhibited it.

Key words: endopeptidase, metallopeptidase, *purification*

Abbreviations

EDTA – ethylenediaminetetraacetic acid,

EGTA – ethylene glycol tetraacetic acid

PMSF – phenylmethylsulfonyl fluoride

DFP- diisopropyl phosphorofluoridate

E-64 – trans-epoxy succinyl leucylamido 4-guanidinobutane

TCA – trichloroacetic acid

INTRODUCTION

Proteolytic enzymes (peptidases, proteases), being indispensable for the functioning of all organisms, are present in animals, plants as well as in microorganisms. These enzymes are of vast industrial importance and find widespread use in the chemical and food industries. The development of environment-friendly technologies favors the use of peptidases e.g. in leather tanning, recovery of silver from photo films and in bioremediation. At present proteolytic enzymes of microbial origin account for approximately 40% of the global enzyme sales market [7, 15, 18, 23].

The ability of bacteria of the genus *Pseudomonas* to synthesize proteolytic enzymes has been documented in the literature. Both exopeptidases and endopeptidases produced by pseudomonads have been described [7, 9]. Exopeptidases of *Pseudomonas* bacteria are usually intracellular enzymes, though some exceptions are known, which carry out very important functions in basic metabolic processes of the bacterial cell [3, 10]. Endopeptidases, on the other hand, are usually released outside the cell and their main role is hydrolysis of high molecular weight proteins in the outer environment in order to make these compounds accessible for the bacterial cell. However, this is not the only function of these enzymes. Proteases of *Pseudomonas* bacteria are increasingly often scrutinized in the aspect of increased pathogenicity of clinical strains [21]. As the results of many studies have shown, extracellular endopeptidases of soil bacteria may be one of the factors determining the antagonism of *Pseudomonas* strains with regard to pathogens and pests of crop plants, such as molds or nematodes [25, 28].

Most of the known endopeptidases secreted by bacteria belonging to the genus *Pseudomonas* are alkaline metalloproteases and they frequently exhibit thermal stability [1, 9]. So far there is no detailed information in the literature regarding the characteristics of extracellular proteases synthesized by the bacterium *Pseudomonas chlororaphis*. For this reason the aim of our studies was to identify and characterize one of the extracellular endopeptidases synthesized by the rhizosphere strain 5N of this bacterium. The purified enzyme was characterized with regard to the optimal temperature and pH for its activity as well as thermal stability, and its molecular weight was determined. The effect of specific inhibitors and metal ions on the activity of the enzyme was also studied.

MATERIAL AND METHODS

Isolation and identification of bacteria

The material used in the studies was a strain of bacteria isolated from the rhizosphere of winter wheat cultivated in a field in the eastern part of Poland. Rhizosphere bacteria were isolated using King B medium [12]. Identification of the bacterial strain selected for further studies was based on biochemical and morphological traits [8] and its identity was confirmed by 16S rRNA gene sequence analysis. Amplification of 16S rRNA gene was accomplished using the universal primers 27 F and 1401R [27] 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 (PAN 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 AB667904.

Culture conditions

Cultures were set up in liquid medium, pH 7.5, composed of the mineral salts: 0.03% K_2HPO_4; 0.03% KH_2PO_4; 0.05% $MgSO_4$; 0.05% NaCl; 0.0015% $FeCl_3$ and also containing 0.25% yeast extract and 1.5% tryptone. The bacterial culture was maintained for 48 h at temperature 25°C in a shaking water bath (120 rpm). The supernatant obtained after centrifuging the culture (15 min at 10000 x g, 4°C) was filtered (0.22-µm membrane, Millipore) and used as the start preparation for purification and characterization of the enzyme.

Determination of protease activity

Proteolytic activity was determined using azocasein as the substrate [22]. Briefly, 300 µl of appropriately diluted was mixed with 300 µl 1% azocasein in 100 mM Tris-HCl buffer, pH 8.8. The reaction mixture was incubated for 30 min at 42°C. The reaction was terminated by the addition of 600 µl 10% TCA. The mix was centrifuged and after adding 500 µl of supernatant to equal volume of 1 M NaOH, the absorbance at 420 nm compared to the control sample was measured.

One unit was defined as the amount of enzyme that caused an increase in absorbance of 0.1:

E_{420}=0.1x min^{-1}.

Determination of protein content

Protein content in the initial preparation as well as after all consecutive purification steps was determined according to Bradford [2] with bovine serum albumin as a standard.

Purification of the extracellular endopeptidase synthesized by *Pseudomonas chloraphis* N5

Purification of the protease was carried out using a three-stage procedure: fractionation with ammonium sulfate, HPLC ion-exchange chromatography and ion-exchange re-chromatography using narrower salt gradient.

Fractionation with ammonium sulfate: portions of ammonium sulfate were added to the supernatant obtained after spinning down two-day cultures of bacteria, followed by filtration, to obtain 30% saturation. The solution was then centrifuged for 30 min at 13000 x g and temperature 4°C. The pellet was discarded and the supernatant was salted out to 85% concentration and centrifuged as described above. The pellet was suspended in 20 mM Tris-HCl buffer, pH 8.5 and dialyzed overnight in the same buffer. The dialyzate was concentrated in an Amicon ultrafiltration chamber with PM10 membrane. The thus obtained enzyme solution was applied to anionite Protein-Pak Q 8HR (HPLC) medium previously equilibrated with 20 mM Tris-HCl buffer, pH 8.5. Proteins bound to the bed were eluted with a linear gradient of NaCl from 0 to 0.5 M. The collected fractions containing the highest proteolytic activity were dialyzed overnight against 20 mM Tris-HCl buffer, pH 8.5 at 4°C. The dialyzate was subjected to HPLC re-chromatography in narrower NaCl gradient from 0.1 to 0.3 NaCl. The final purified enzyme preparation was used for characterization of the protein and mass spectrometry analysis.

Polyacrylamide gel electrophoresis and preparation of zymograms

SDS PAGE was done according to the procedure described by Laemmli [16]. The protein bands were visualized using Coomassie Brilliant Blue R-250 or silver staining.

In order to detect endopeptidase activity after electrophoresis under semi-denaturing conditions, polyacrylamide gels with copolymerized 0.1% azocasein were washed with deionized water containing 2.5 % Triton X-100 for 30 min at 4ºC. The gels were incubated for 60 min in 0.1 M Tris HCl buffer, pH 9.0, and then stained with amide black. The stain was then removed in 7% acetic acid solution. Proteolytic activity was detected as the presence of pale (unstained) bands against blue background of the gel with incorporated substrate.

Determination of the molecular weight of the studied enzymatic protein

The molecular weight of the enzyme was estimated by SDS-PAGE.

The following molecular weight standards were employed: phosphorylase b (97kDa), bovine serum albumin (66 kDa), ovalbumin (45kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14 kDa).

Characterization of the properties of purified enzyme

The effect of temperature on the peptidase activity was determined by assaying activity at temperature ranging from 30 to 55ºC. The optimum pH was determined in a range 4.0-12.0 in 50 mM Britton-Robison's buffer. Thermal stability was determined after 30 and 60 min preincubation of the enzyme at 37°C, 40°C and 45°C.

In order to study the effect of specific inhibitors and metal ions on activity, the enzyme was pre-incubated with an inhibitor or metal ion solution for 30 minutes at 5°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.

RESULTS

Analysis of the obtained 16S rRNA gene sequence revealed the strong similarity of the studied bacterial strain to *Pseudomonas chlororaphis* strain zong1 (99%, GenBank: HM241942) as well as to *P. chlororaphis* subsp. aurantiaca strain NCIB 10068 (99%, NCBI NR_043935) and *Pseudomonas*. sp. BIHB 989 (99%, GenBank: JF766687). The identified bacterial strain that was characterized by the ability to synthesize extracellular proteases was classified as *Pseudomonas chlororaphis* and given the name N5. The studied bacterial strain grown in liquid cultures secretes several proteases. Zymograms using azocasein as the substrate enabled the detection of the two intense bands of activity (photo1).

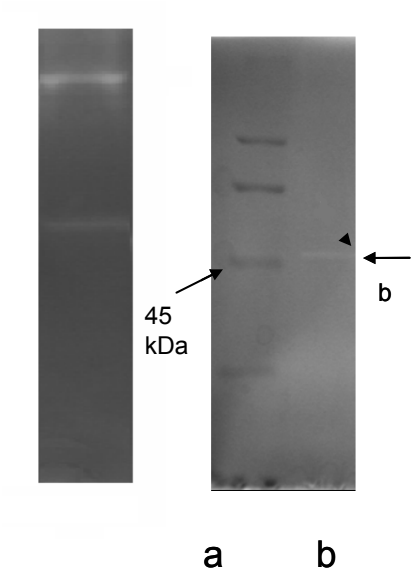

Photo 1 Zymograms: 1. the electrophoretic pattern of untreated culture supernatant, 2. The semi-denaturated electrophoretic pattern of purified endopeptidase; lane with molecular weight markers(a), purified endopeptidase (b)

One of the extracellular endopeptidases synthesized by *Pseudomonas chloraphis* N5 was purified 19.9–fold using a three stage procedure: ammonium sulfate fractionation, chromatography and ion-exchange re-chromatography (Table 1).

Table 1. Summary of the purification procedure for *P. chlororaphis* N5 protease.

Step	**Total activity [U]**	**Total protein [mg]**	**Specific activity [U/mg]**	**Yield [%]**	**Purification factor**
Culture supernatant	760.4	109.6	6.94	100.0	1.0
Ammonium sulfate precipitation (30-85%)	648.2	39.9	16.2	85.2	2.3
HPLC chromatography (Mono Q 8HR)	148.2	1.9	78	19.5	11.2
HPLC re-chromatography HPLC Q 8HR	69.2	0.5	138.4	9.1	19.9

Ammonium sulfate fractionation of the crude enzyme preparation yielded 2.3-fold purification of the studied enzyme. The next stage of purification involved ion-exchange HPLC chromatography using a NaCl concentration gradient from 0.0 to 0.5 M and resulted in over 11-fold increase in specific activity of the enzyme. After this purification step two peaks of activity were obtained (Fig. 1). Active fractions from one of them were pooled, dialyzed and re-chromatographed using a narrower NaCl gradient from 0.1 to 0.3 M. After electrophoretic separation a purified single protein band corresponding with the electrophoretic mobility of protease activity was obtained (photo 2). The molecular weight of the purified enzyme was approx. 51 kDa. The purified protease demonstrated highest activity at temperature 42°C and at pH 11. Enzymatic activity persisted at a high level over a broad pH range, from 9.0 to 12.0 (Fig. 2). The protease was characterized by high susceptibility to changes of temperature at which the reaction was carried out. At 35°C the activity of the enzyme was lower by about 50%. After exceeding the optimal temperature, the activity strongly dropped and at 55°C was only 10% of the original value (Fig. 3). Thermal stability studies demonstrated that the studied enzyme is characterized by low stability in the 37° - 45°C range. A 30-minute preincubation of the enzyme at 37° resulted in a 55% drop in activity, whereas 60-minute preincubation at the same temperature decreased its activity by 82%. Preincubation at higher temperatures caused a further loss of activity, with complete inactivation of the enzyme after 60-minute preincubation at 40°C (Fig. 4).

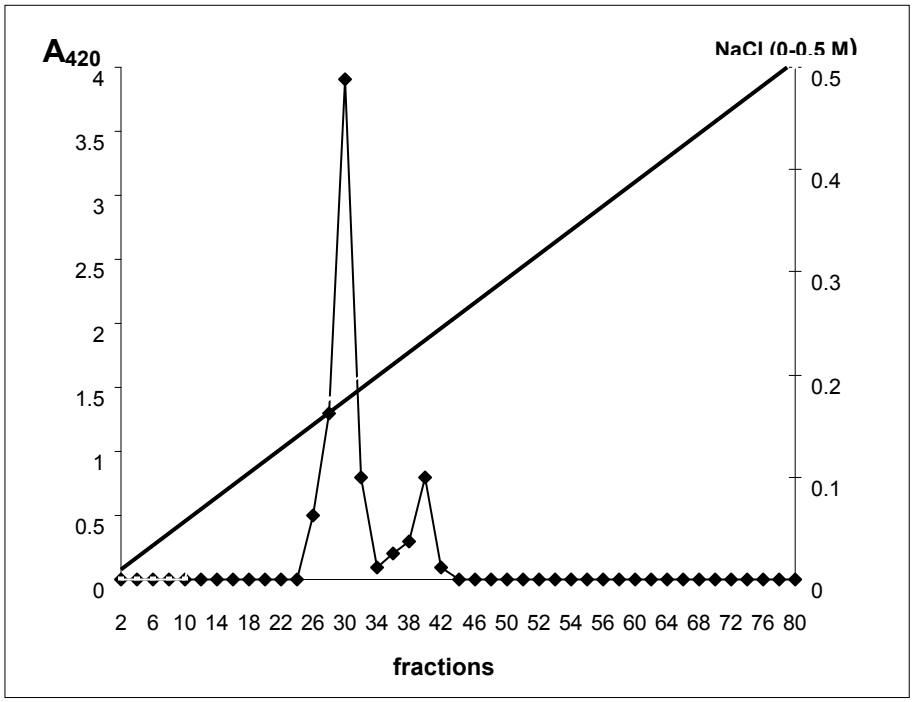

Fig. 1 Elution profile for protease *P. chlororaphis* N5 following ion exchange chromatography on Q 8HR (HPLC) column using 0 to 0.5 M NaCl gradient. Fractions with highest activity were collected at 0.18 M NaCl.

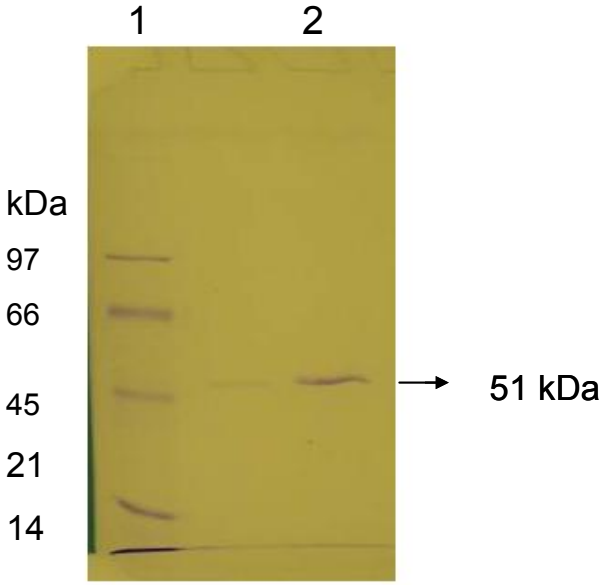

Photo 2. SDS-PAGE of the purified peptidase *of P. chlororaphis* N5. 1- molecular weight markers, 2 -enzyme preparation after second HPLC chromatography,

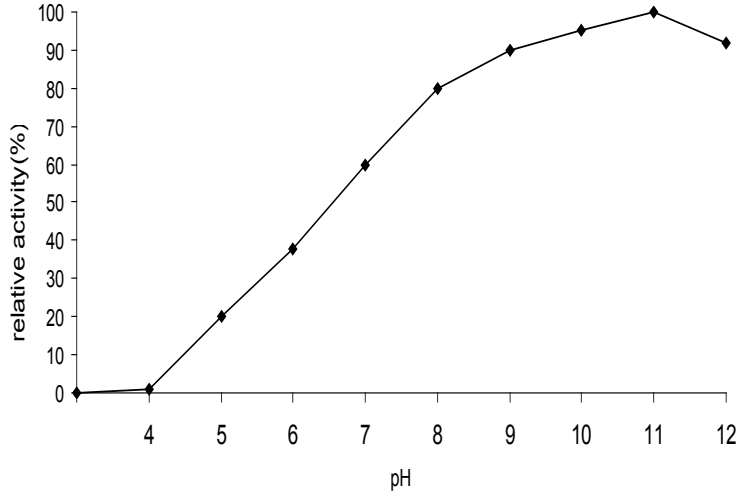

Fig. 2 Effect of pH on the activity of purified chitinase from *P. chlororaphis* strain 5N
The pH profile was determined in 50 mM Robinson's buffer by varying pH values at 42ºC.

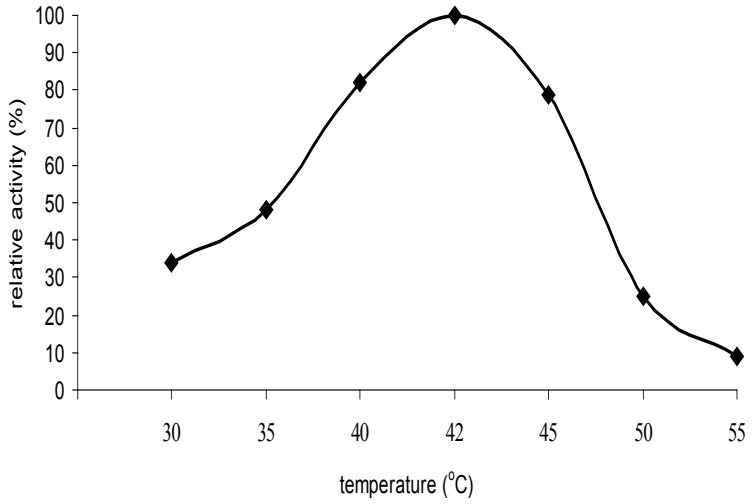

Fig. 3 Effect of temperature on the activity of purified protease from *P. chlororaphis* strain 5N

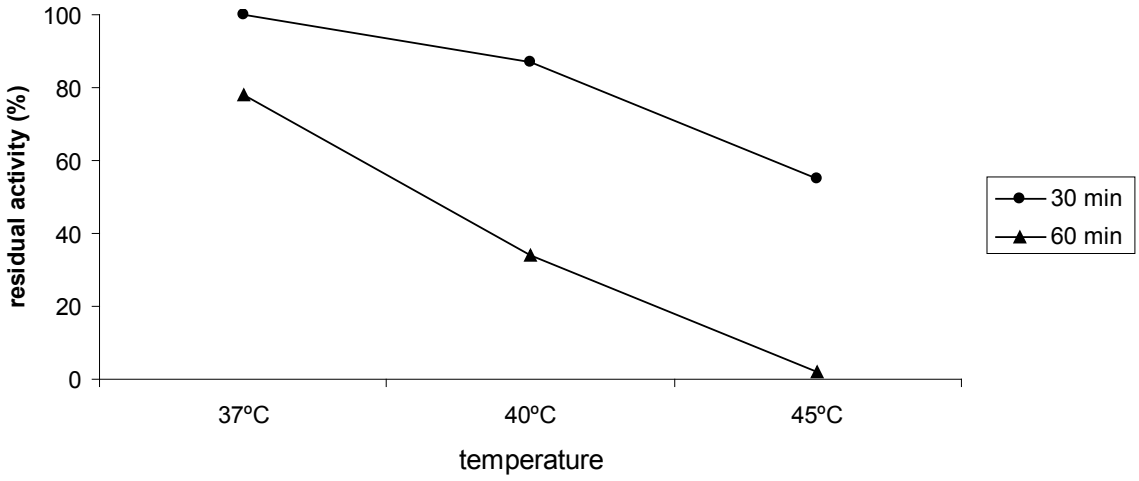

Fig. 4 Thermal stability of the purified protease

The effect of specific inhibitors on the functional groups of amino acids residues in the active center involved in enzymatic catalysis is presented in Table 2.

Table 2 Effect of inhibitors on activity of *P. chlororaphis* 5N endopeptidase

Inhibitor	Inhibitor class	Inhibitor concentration (Mm)	% activity remaining
None	-	-	100
EDTA	Metallo-	1	0
EGTA	Metallo-	1	0
1,10-phenanthroline	Metallo-	1	0
Pepstatin	Aspartic	1	100
Iodoacetamide	Cysteine	1	95
E-64	Cysteine	1	100
DFP	Serine	0.01	100
PMSF	Serine	1	96

The results unequivocally point to the characterized enzyme as a member of the metalloendopeptidases. Both EDTA, EGTA as well as 1,10-phenanthroline in concentration 1mM completely inhibited its activity.

The other specific inhibitors examined did not affect the activity of the enzyme or inhibited it to only a small degree. Table 3 presents the effect of individual metal ions on the activity of the enzyme. Ca^{2+}, Mg^{2+} and Zn^{2+} ions stabilized the activity of the endopeptidase, with Ca^{2+} ions activating it the strongest – a low concentration of the ions (0.1 mM) stimulated the activity of the enzyme, increasing it by about 50%. Partial inhibition of enzyme activity was observed in the presence of 1 mM Mn^{2+} and Co^{2+} ions. The activity of the studied protease of strain N5 was completely inhibited in the presence of Cu^{2+} ions in concentration 0.1 mM. Partial, 35% reactivation of enzyme activity after the action of EDTA was obtained in the presence of zinc ions in concentration 0. 05 mM. This effect was not observed in the presence of calcium and magnesium ions.

Table 3 Effect of metal ions on activity of *P. chlororaphis* strain 5N endopeptidase

Metal ions	Concentration (mM)	% activity remaining
None	-	100
Ca^{2+}	0.1	154
	1	163
Mg^{2+}	0.1	113
	1	118
Zn^{2+}	0.1	115
	1	100
Mn^{2+}	0.1	76
	1	76
Cu^{2+}	0.1	0
	1	0
Co^{2+}	0.1	97
	1	79

DISCUSSION

A strain of *P. chlororaphis* synthesizing extracellular endoproteases was isolated from soil. The bacterium was not found to release extracellular aminopeptidases or carboxypeptidases. No aminopeptidase or carboxypeptidase activity was found in the culture fluid when specific substrates for these enzymes were used (data not presented). Culture fluid in which endopeptidase activity was determined using azocasein as a substrate, was subjected to a purification process.

In this report we present the purification and characterization of one of the extracellular endopeptidases synthesized by strain N5 of the bacterium *P. chlororaphis*. As shown by the zymograms, strain N5 in the experimental conditions employed released to the environment at least two proteases with high activity. The purification procedure used enabled separation of these activities and obtaining a homogenous preparation of one of these enzymes. Literature data indicates that the individual species of bacteria within the genus *Pseudomonas* strongly differ from one another in the isoforms of endoproteases they release to their growth environment. The extracellular proteases synthesized by *Pseudomonas fluorescens* and *P. aeruginosa* have been particularly well characterized. Strains of the bacterium *P. fluorescens* extracellularly release a single metalloprotease with molecular weight approx. 50 kDa [11, 17]. *P. aeruginosa* strain K secretes three extracellular peptidases [22]. Interesting comparative studies on proteases synthesized by *P. fluorescens* and *P. chlororaphis* were carried out by Nicodeme at al. [20] who demonstrated that the extracellular proteases releases by these bacterial species differ in rate of migration during electrophoresis in polyacrylamide gel. This allows distinguishing these two species of bacteria from each other. Our studies have demonstrated that the optimal temperature for the activity of the enzyme is 42°C. According to Schokker and Boekel [24] the temperature optimum for peptidases synthesized by *Pseudomonas* sp. is in the interval from 30°C to 45°C. However, some bacteria of this genus produce proteases with higher temperature optimum, such as the peptidase of *Pseudomonas aeruginosa* PseA with temperature optimum 55°C [6] or that of *Pseudomonas aeruginosa* K with temperature optimum 70°C [22]. Many proteolytic enzymes synthesized by bacteria belonging to the genus *Pseudomonas*, otherwise than the studied protease N5, are characterized by high thermal stability. This, for example, is the case for peptidases synthesized by strains of bacteria responsible for the spoiling of dairy products. For example, the peptidase described by Kohlmann et al. [14] retained 36% activity after a 16-second preincubation at 72°C (conditions analogous to the pasteurization of milk). According to literature data, the temperature range, in which thermostable enzymes synthesized by *Pseudomonas* retain their activity, is from 20°C to 70°C [4; 9, 20, 26].

The studied enzyme was highly stable in a wide pH range from 6.8 to 11.2. The highest activity was noted at pH 10.9. However, in the whole range of pH values studied the enzyme retained over 80% of its activity. Many of the peptidases synthesized by *Pseudomonas* bacteria retain their activity in the pH range 6.0 to 11.0*: Pseudomonas aeruginosa* K [22], *Pseudomonas aeruginosa* MN1 [1], as well as in the pH range 5.5 to 9.0 *Pseudomonas fluorescens,* [11,13]. There are also peptidases that retain high (over 50%) activity throughout the range from 2.0 to 12.0, like the peptidase synthesized by *Pseudomonas fluorescens* 22F [24].

The majority of peptidases synthesized by *Pseudomonas fluorescens* are metallopeptidases. This was also the case for the studied *P. chlororaphis* N5 endopeptidase. Its activity was completely inhibited by compounds chelating divalent metal ions, such as EDTA, EGTA and 1,10-phenanthroline. On the other hand, such inhibitors as PMSF DFP, E-64, iodoacetamide and pepstatin did not affect the activity of the enzyme. Similar results were obtained by Kohlmann et al. [13] in their studies on the effect of different inhibitors on the activity of a peptidase synthesized by *Pseudomonas fragi* K122 as well as for *Pseudomonas aeruginosa* [4] and for *P. fluorescens*. (24).The experiments aimed at investigating the effect of different metal ions on endopeptidase activity confirmed that the studied enzyme belongs to the metallopeptidases. Many sources describe Ca^{2+} and Zn^{2+} ions as stabilizers and activators of bacterial peptidases [14, 17, 22]. The studied *P. chlororaphis* N5 enzyme, besides Ca^{2+} and Zn^{2+} ions, was also activated by Mg^{2+} ions. An essential role of zinc ions in the mechanism of catalysis by the studied protease was confirmed by the results of an experiment, in which partial reactivation of the activity of the protease previously inhibited by the chelating action of EDTA was observed. The enzyme was strongly inhibited by Cu^{2+} ions which completely abolished its activity, just like in the case of an alkaline peptidase produced by *Pseudomonas aeruginosa* MN1 [1].

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