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Production, characterization, gene cloning, and nematocidal activity of the extracellular protease from *Stenotrophomonas maltophilia* N4

Urszula Jankiewicz,[1,*] Ewa Larkowska,[1] and Maria Swiontek Brzezinska[2]

Department of Biochemistry, Warsaw University of Life Sciences, SGGW, Nowoursynowska 159, 02-776 Warsaw, Poland[1] *and Department of Environmental Microbiology and Biotechnology, Nicolaus Copernicus University, Lwowska 1, 87-100 Torun, Poland*[2]

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A rhizosphere strain of the bacterium *Stenotrophomonas maltophilia* N4 secretes the serine protease PN4, whose molecular mass is approximately 42 kDa. The optimal temperature for the enzyme activity of the 11-fold purified protein was 50°C and the optimal pH was 10.5. The activity of the enzyme was strongly inhibited by specific serine protease inhibitors, which allowed for its classification as an alkaline serine protease family. Ca^{2+} ions stimulated the activity of the protease PN4, while Mg^{2+} ions stabilized its activity, and Zn^{2+} and Cd^{2+} ions strongly inhibited its activity. The enzyme has broad substrate specificity. For example, it is able to hydrolyse casein, keratin, albumin, haemoglobin, and gelatin, as well as the insoluble modified substrates azure keratin and azocoll. The gene that encodes the 1740 bp precursor form of the enzyme (accession number: LC031815) was cloned. We then deduced that its amino acid sequence includes the region of the conserved domain of the S8 family of peptidases as well as the catalytic triad Asp/His/Ser. The bacterial culture fluid as well as the purified protease PN4 demonstrated biocidal activity with regard to the nematodes *Caenorhabditis elegans* and *Panagrellus* spp.

[**Key words:** Serine protease; *Stenotrophomonas maltophilia*; Nematocidal activity; Biocontrol]

Presently, there has been an increasing focus in the agricultural sciences on microsphere organisms that favourably affect the growth of plants. The importance of rhizosphere bacteria in the ecosystem is a result of their enzymatic activity, including the activity of chitinases and proteases. Chitinases are enzymes that hydrolyse the β-1,4-glycosidic bonds in chitin, which is one of the most common natural polymers in nature. Proteases (peptidases, proteinases) are hydrolases [EC 3.4. 21-24] that degrade peptide bonds in proteins and peptides. Interest in microorganisms that produce proteases has grown due to the versatile use of these enzymes in medicine, biotechnology, protection of the environment and numerous branches of the chemical and food industries (1–3). Extracellular proteases produced by microorganisms have an important role, particularly as bionematocides (4,5), in biological plant protection. Most of the described proteases with nematocidal activity are fungal enzymes that are able to degrade the cuticle of nematodes. While studying bacterial virulence factors that are involved in infection, researchers have focused on enzymes with specific activity against keratin and collagen (6,7). Their effectiveness in biocontrol is determined by the structure of the cuticle of adult nematodes. The cuticle is a non-cellular layer that is produced by a subcutaneous layer and is composed mainly of proteins, including keratins and collagen fibres. The structure of collagen also depends on the developmental stage of the nematode (8,9). A serine protease that is synthesized by *Bacillus* sp. has been described as an enzyme that contributes to the breakdown of the nematode cuticle (10). The metallopeptidase that is produced by *Pseudomonas fluorescens* is also nematocidal (11); however, the majority of nematocidal bacterial proteases are classified as belonging to the serine proteases of the subtilisin family with a conserved amino acid triad, Asp-His-Ser, in the active centre (12–14).

The rhizosphere bacterium *Stenotrophomonas maltophilia* is also involved in promoting the growth of plants, partly through the secretion of chitinases and proteases with fungistatic and nematocidal activities (15–17). The main objective of the present study was to determine the role of the extracellular keratinolytic and collagenolytic peptidases of *S. maltophilia* N4 in the antagonism of these bacteria towards free-living soil nematodes. To do so, the studied enzyme was purified and characterized, and the gene that encodes the precursor protein of the active enzyme was cloned.

MATERIALS AND METHODS

Experimental material and culture conditions The source of the studied protease was strain *Stenotrophomonas maltophilia* N4 (accession number: AB667906). The bacteria were grown in mineral media (g/l): KH_2PO_4 3; K_2HPO_4 3; $MgSO_4$ 0.5; NaCl, 2; $FeCl_3$ 0.005 that was supplemented with 10 g shredded feathers from the helmeted guinea fowl (*Numida meleagris*). The cultures were maintained for 90 h with shaking at 120 rpm, at 28°C. *Caenorhabditis elegans* N2 nematodes were obtained from the collection of The Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN, USA). They were cultivated on Petri dishes containing Nematode Growth Medium (NGM) at room temperature. *C. elegans* were fed *Escherichia coli* strain OP50 (18). *Panagrellus* spp. nematodes were purchased at an aquarium supply store and cultivated in a sterile 1-L bottle. The bottom of the bottle contained an approximately 2 cm layer of shredded sterilized oatmeal in

* Corresponding author. Tel.: +48 22 5932560; fax: +48 22 5932562.
E-mail address: urszula_jankiewicz@sggw.pl (U. Jankiewicz).

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deionized water. Microworms were transferred into the above-described media in the bottle, and the bottle was stoppered with cotton wool. Six to eight days after initiating culture, nematodes were seen moving along the walls of the bottle, and they were removed aseptically manner for further studies.

Determination of enzymatic activity We then pelleted bacterial cultures at day four, and the supernatant was used for the detection and determination of proteolytic activity as well as for purification of the enzyme.

The activity of the peptidase in both the crude extract and at the individual stages of the study was determined using 1% azocasein in 100 mM Tris–HCl buffer at pH 8.5 as the substrate. The enzymatic reaction was maintained for 30 min at 40°C. Absorbance was measured at 420 nm and compared with control samples. Enzymatic activity was given in units that were defined as an increase in absorbance: $E_{420} = 0.1 \times ml^{-1} \times h^{-1}$.

Purification of the protease PN4 The clear culture supernatant was used as the crude material for enzyme purification. The initial stage of purification consisted of salting out the proteins with ammonium sulphate to 90% saturation. The protein pellet was suspended in a minimal volume of Tris–HCl buffer at pH 8.5 and dialysed at 4°C for 12 h with two exchanges of the buffer. The obtained protein solution was then separated using ion exchange chromatography on a DEAE Sepharose CL-6B column (Sigma–Aldrich). Proteins bound to the column were eluted with an increasing gradient from 0 to 0.5 M NaCl in 20 mM Tris–HCl buffer, pH 8.5. The protein fractions with highest activity were concentrated (Vivaspin 20, Sartorius AG) and subjected to gel chromatography on Superdex 200 (Sigma–Aldrich) in 50 mM Tris–HCl buffer at pH 8.5.

The purified enzyme was used for the identification of proteins by mass spectrometry and for further studies of the biochemical characteristics of the enzyme and evaluation of its biocidal activity against nematodes.

Determination of protein content Protein concentration was determined with bovine serum albumin as a standard (19).

Electrophoretic separation Electrophoretic separations were performed using 12% polyacrylamide gel containing SDS (20). Zymograms were obtained after electrophoretic separation of the samples in polyacrylamide gel containing 0.1% haemoglobin. On completion of electrophoresis, the gels were incubated for 1 h in 0.5% Triton X-100 solution and then transferred to 100 mM Tris–HCl buffer at pH 8.5 and stained with 0.1 % amide black solution. Proteolytic activity was observed as bright bands where the substrate was digested, against the dark blue background of the gel.

Proteomic analysis of the purified protein fraction A protein sample that was previously digested with trypsin was separated on a nanoAcquity UPLC (Ultra Performance LC) system and analysed with an Orbitrap-based mass spectrometer (IBB, PAN). The results were analysed using BLAST (NCBI).

Biochemical properties of purified protease PN4 The optimum pH was determined by testing proteolytic activity across the range 5.5–12.0 in 50 mM Britton-Robison's buffer. To determine the optimal temperature, we conducted reactions between 30°C and 60°C. To evaluate the thermal stability, the enzyme solution was preincubated for 30, 60 or 90 min at 40°C, 50°C or 60°C, after which the incubation was continued at 40°C. The non-preincubated enzyme was used as a control (100%).

Effect of metal ions, inhibitors, and other chemical compounds on the activity of purified PN4 protease To study the effect of specific inhibitors on enzyme activity, the enzyme was preincubated with an inhibitor solution for 30 min at 4°C, after which the remaining activity was tested. The effect of metal ions on activity was determined following preincubation of the enzyme for 30 min at 4°C in the presence of metal ions and other chemicals at a final concentration of 1.0 and 5.0 mM, after which the substrate was added and the residual activity was tested.

Substrate specificity of protease PN4 Substrate specificity was studied by the method of Anson (21) in the presence of 1% solutions of the following soluble substrates: casein, albumin, haemoglobin and gelatin or keratin in 100 mM Tris–HCl buffer at pH 8.5. One unit (1 U) of proteolytic activity was defined as the amount of enzyme required to liberate 1 μmole of tyrosine within 1 min of the reaction under the specified conditions.

Keratinolytic activity was measured with keratin azure as the substrate in 50 mM Tris–HCl buffer at pH 8.5 (22). Collagenolytic activity was determined using azocoll as a substrate (23).

In both cases the reactions were conducted for 3 h at 40°C using a rotary shaker at 200 rpm. One unit of enzyme activity was the amount of enzyme that caused a change in absorbance of 0.1 at 595 nm under the specified conditions.

Cloning of the structural gene of the protease Genomic DNA was isolated from *S. maltophilia* N4 cells using a Genomic DNA Purification Kit (Fermentas). Primers for PCR were designed using the sequence of gene pr2, which encoding the serine protease of *S. maltophilia* strain PS5 and available in GenBank (accession number JF317278). The gene was amplified using the primers F: 5′ ATGTCCCAGGTAACGCAACCG and R: 5′GCTTTCAGTACTGGGCGTTGAGGG, and the expected length of the product was 1740 bp. The PCR product, after elution from an agarose gel (kit Gel-out, A&A Biotechnology), was ligated into plasmid pJET 1.2 using the CloneJet PCR kit (Thermo Scientific). For transformation procedures, competent *E. coli* DH5α cells were used. To obtain the nucleotide sequence of the cloned protease gene, plasmids that were isolated from the transformed bacteria were sent to the Laboratory of Mass Spectrometry of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. The obtained nucleotide sequences were analysed using BLAST (NCBI).

Determination of nematocidal activity To evaluate the effect of cultured supernatant from *S. maltophilia* N4 bacteria and of isolated protease PN4 on nematodes, a kill tests were performed.

Twenty-five *C. elegans* nematodes were placed in a sterile manner on the surface of a Petri dish (6 cm diameter) with NGM media. Then, drops of either culture supernatant or solution of protease PN4 were placed at four points on the media. The supernatant and protease solution were sterilized by passing them through membrane filters (pore size 0.22 μm). Control cultures were made in the presence of sterile Tris–HCl buffer. Observations were made every 8 h for two days, recording the number of live and dead nematodes in the observation field of a light microscope. To study the survival of *Panagrellus* spp., 100 μl of culture supernatant or of purified enzyme were added to a 1.5 ml Eppendorf tubes that contained 25 nematodes. The control sample was prepared either with sterile buffer or with thermally inactivated culture supernatant (10). The samples were incubated for 5 or 10 h at room temperature, after which dead nematodes in the observation field of a light microscope were recorded.

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

RESULTS

The obtained supernatant served as a source of proteases to determine proteolytic activity and to perform protein purification. Analysis of zymograms revealed the presence of at least three extracellular proteases that were synthesized by *S. maltophilia* N4 and that differed in their molecular mass (Fig. 1a,b). For further studies, the protease PN4, whose activity is visible on the zymogram in the form of a bright band with a molecular mass of approximately 42 kDa, was selected. Table S1 presents the consecutive stages of purification of the peptidase PN4. The three-stage procedure yielded more than an 11-fold increase in concentration of purified enzymatic preparation. The purification process resulted in the extraction of one major protein band with a weight of approximately 42 kDa (Fig. 1c).

Biochemical properties of protease PN4 The studied enzyme showed significant activity between the temperature of 40°C and 55°C, with its temperature being optimum at 50°C (Fig. S1). The protease PN4 demonstrated nearly total thermal stability during the 90 min preincubation at 40°C; however, after the same period of incubation at 50°C, the activity of the enzyme dropped to only 30% of the initial value (Fig. 2). The optimal pH for the protease PN4 was 10.5, but it retained 90% of its activity in the pH range between 9.5 and 11.5 (Fig. S2).

The results indicate that the specific serine proteases inhibitors, Pefablock and DFP (diisopropylfluorophosphate) inhibit the activity of the enzyme at the 80–90% level. Activity was also inhibited in the presence of metal-dependent protease inhibitors. The remaining protease inhibitors, including iodoacetamide or pepstatin, did not affect the activity of the enzyme. A similar effect was observed in the presence of Aprotini, a typical trypsin inhibitor (Table 1).

Table 2 presents the effect of the individual metal ions on the activity of the enzyme. The enzyme was found to be activated only by Ca^{2+} ions, whereas Mg^{2+} ions were able to stabilize the activity. The activity of the peptidase was most greatly reduced by the divalent Zn and Cd ions. The ionic detergents, which are presented in Table 2, were observed to have a modest inhibitory effect on the activity of the enzyme. The protease PN4 utilized a broad spectrum of substrates, and its activity was particularly elevated in the presence of casein, albumin, keratin and haemoglobin and somewhat lower against gelatin. The enzyme was also active against modified insoluble substrates, such as azocoll and keratin azure (Table 3).

Proteomic analysis and protease gene sequence analysis The peptide sequences obtained after trypsin digestion of the protein and MS analysis were used to identify the