

Purification and Biochemical Characterization of a Novel Alkaline Protease Produced by *Penicillium nalgioense*

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Received: 29 September 2013 / Accepted: 19 February 2014 /

Published online: 2 March 2014

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Abstract *Penicillium nalgioense* PNA9 produces an extracellular protease during fermentation with characteristics of growth-associated product. Enzyme purification involved ammonium sulfate precipitation, dialysis, and ultrafiltration, resulting in 12.1-fold increase of specific activity (19.5 U/mg). The protein was isolated through a series of BN-PAGE and native PAGE runs. ESI-MS analysis confirmed the molecular mass of 45.2 kDa. N-Terminal sequencing (MGFLKLLKGSLATLAVVNAGKLLTANDGDE) revealed 93 % similarity to a *Penicillium chrysogenum* protease, identified as major allergen. The protease exhibits simple Michaelis-Menten kinetics and K_m (1.152 mg/ml), V_{max} (0.827 mg/ml/min), and k_{cat} (3.2×10^2) (1/s) values against azocasein show that it possesses high substrate affinity and catalytic efficiency. The protease is active within 10–45 °C, pH 4.0–10.0, and 0–3 M NaCl, while maximum activity was observed at 35 °C, pH 8.0, and 0.25 M NaCl. It is active against the muscle proteins actin and myosin and inactive against myoglobin. It is highly stable in the presence of non-ionic surfactants, hydrogen peroxide, BTNB, and EDTA. Activity was inhibited by SDS, Mn^{2+} and Zn^{2+} , and by the serine protease inhibitor PMSF, indicating the serine protease nature of the enzyme. These properties make the novel protease a suitable candidate enzyme in meat ripening and other biotechnological applications.

Keywords *Penicillium nalgioense* · Extracellular enzyme · Alkaline protease · Purification · Characterization

Introduction

Alkaline proteases are industrially important enzymes with diverse applications ranging from the food and feed to the detergents industry. Identification of new sources of these enzymes with application-relevant characteristics is of great interest. Fungi of the genus *Penicillium* and other acid-tolerant fungi produce mainly acid proteases [1]; however, a number of them have been shown to produce alkaline proteases often thermotolerant and very stable in the presence of surfactants and oxidizing agents. Neutral and alkaline serine proteases were isolated from the culture filtrates of *Penicillium waksmanii* [2], *Penicillium chrysogenum* [3, 4], *Penicillium*

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charlesii [5], *Penicillium citrinum* [6], *Penicillium cyaneo-fulvum* [7], and *Penicillium cyclopium* [8].

A rich natural habitat for a large number of members of the genus *Penicillium* is the surface of fermented or cured meat products. We have reported earlier a study on the mycoflora of traditional Greek sausages which demonstrated the dominance of *Penicillium* spp. over other isolated fungi: *Penicillia* were isolated from 90.8 % of visibly moldy sausages [9]. Among them, *Penicillium nalgiovense* made a significant contribution in the total number of isolates. *P. nalgiovense* is used industrially as a starter culture for certain types of European sausages, and an earlier study with a large number of *P. nalgiovense* isolates from mold-fermented sausages showed that the majority of the examined cultures had proteolytic and/or lipolytic activity [10]. Although this activity contributes greatly to the development of the final properties of the fermented product (texture, flavor, and aroma) [3, 11, 12], no particular protease or lipase enzyme from *P. nalgiovense* has so far been purified and characterized.

Preliminary studies with *P. nalgiovense* isolates from our earlier work [9] showed that one particular strain (PNA9) produced increased alkaline proteolytic activity at low temperatures. In this view, we carried out bioreactor cultivations of *P. nalgiovense* PNA9 to study the characteristics of production and proceeded on the purification and characterization of the enzyme.

Materials and Methods

Microorganism and Culture Conditions

The sausage isolate *Penicillium* PNA9 used in this study was identified phylogenetically as *P. nalgiovense* by comparison of a 575-bp fragment of genomic DNA containing the partial sequence of the 18S rRNA gene using BLAST (GenBank Accession Number: KF515707, BankIt1652703).

P. nalgiovense PNA9 was plated in 9-cm diameter petri dishes on malt extract agar (MEA) and incubated for 7 days [13]. Cultures were maintained at 4 °C, while spores maintained freeze dried. For submerged cultivations, the following chemically defined medium was used (per liter): glucose, 10 g; KNO₃, 9 g; KH₂PO₄, 0.5 g; FeSO₄·7H₂O, 1 mg; L-cysteine, 0.1 mg; biotin, 0.005 mg; and thiamine, 0.005 mg. Cultivations were also carried out using the following complex medium (per liter): soybean meal, 10 g; glucose, 10 g; polypeptone, 5 g; yeast extract, 1 g; KH₂PO₄, 1 g; and NaCl, 1 g (pH 6.5).

Erlenmeyer flasks (250 ml) containing 50 ml of sterile media were inoculated with 1 ml spores' suspension containing 10⁷ spores/ml—the spores collected from 7-day-old plate cultures. Following inoculation, the flasks were incubated in a shaker incubator at 200 rpm and 27 °C for 48 h and the contents of 3 flasks were used as inoculum for bioreactor cultivations.

Bioreactor cultivations were carried out in a BioFlo 110 New Brunswick Scientific (USA) stirred tank bioreactor (STR) with a working volume of 2 l. The agitation system of the reactor was consisted of two 6-bladed Rushton-type impellers (52 mm), operated at the stirrer speed of 200 rpm. The temperature was maintained at 27 °C. The air flow rate was 1 vvm. The oxygen sensor was calibrated by sparging the medium with air (dissolved oxygen tension, DOT 100 %) and N₂ (DOT 0 %). Following sterilization (120 °C/15 min), the pH of the medium was adjusted with titrants (1 M NaOH and HCl solutions) at the chosen level of pH 8.0 and remained under control during fermentation by automatic addition of titrants. Experiments were carried out in triplicate and mean values are presented.

Assay of Proteolytic Activity

Proteolytic activity was determined using azocasein as the substrate [14]; 120 μ l of diluted enzyme solution were added to 480 μ l Tris buffer containing 5 mM $MgCl_2$ and 1 %w/v azocasein (Sigma), and the mixture was incubated at 35 °C for 30 min. The reaction was terminated by adding 600 μ l of 10 %v/v trichloroacetic acid. The mixture was left for 30 min on ice and then was centrifuged at 15,000 $\times$ g, at 4 °C for 10 min. The supernatant (800 μ l) was neutralized by the addition of 200 μ l 1.8 N NaOH solution, and the absorbance was read on a spectrophotometer at 420 nm.

Protease Purification

Protease purification was done in broth filtrates from fermentations carried out with the chemically defined medium described above, which did not contain any protein or peptide sources. Preliminary studies in broth filtrates collected over the fermentation time-courses were carried out with tricine-SDS-12 % (w/v) PAGE at pH 8.5 [15] and band intensities at around 45 kDa were found to increase up to the 78-h sample. To purify the enzyme in active form, broth filtrates were treated in a number of steps, as follows:

1. Ammonium sulfate precipitation. Ammonium sulfate (258 g) was added to 500 ml broth filtrate (4 °C) to achieve 80 % of saturation. Following centrifugation at 9,000 $\times$ g for 25 min, the precipitate was dissolved in 100-ml potassium phosphate buffer (pH 7.5) and dialyzed thoroughly into it overnight.

2. The dialyzed preparation was concentrated to 6.8 ml by ultrafiltration (4 °C) using a centrifugal filter unit with a membrane for 50 kDa (Amicon, Millipore) (crude enzyme concentrate).

3. Crude enzyme concentrate was subjected to a series of BN-PAGE and native PAGE runs according to Wittig et al. [16]. Isolation of the protein of interest was achieved by carrying out native electrophoresis at pH 7.5 in the absence of urea, using 15 % (w/v) acrylamide and 0.4 % bisacrylamide gels and SERVA Blue G 1 % for preparing the blue-stained cathode running buffer.

4. A post-electrophoretic in-gel activity assay was carried out with cut-off lanes being placed in tubes with azocasein containing reaction buffer (as described above).

5. Elution of protein from native gels. The lanes of interest were placed in 1.5-ml microcentrifuge tubes with 200 μ l of elution buffer (Ornstein-Davis buffer) and eluted for 6 h. Eluates containing the purified enzyme were stored at –20 °C until used.

The procedure of protease purification was repeated three times and showed very good reproducibility.

Protein concentration of the purified enzyme was determined by the method of Bradford [17] using bovine serum albumin (Sigma) as the standard.

Molecular Mass Determination and N-Terminal Sequencing

The recovered enzyme from native PAGE gels was subjected to tricine-SDS-12 % (w/v) PAGE at pH 8.5 according to Schägger [15] for a rough estimation of its molecular weight. For mass determination by mass spectrometry, gel excising and destaining was done from 15 % polyacrylamide tricine-SDS-PAGE gels containing 0.1 % bromophenol blue. This method of extraction elutes the protein directly into a solution of formic acid/water/2-propanol (1:3:2 v/v/v). Molecular mass determination of the purified enzyme was done by electrospray ionization mass spectrometric (ESI-MS) analysis. Following elution of the protein into the above solution, the sample was processed according to Cohen and Chait [18] and the protein was finally eluted into the electrospray source with a solution consisting of 70 % acetonitrile/2.5 %

acetic acid at 6 ml/min. ESI-MS was performed on a TSQ-700 triple-quadrupole mass spectrometer (Finnigan MAT Corp., San Jose, CA) using the standard conditions for protein analysis by ESI-MS according to Mirza et al. [19].

For amino acid sequencing, the enzyme sample was transferred from SDS-PAGE gel onto a polyvinylidene (PVDF) membrane and the protein was subjected to N-terminal sequencing [20]. Edman degradation sequencing of the protein was done using an Applied Biosystems Procise Sequencer (ABI 494 protein sequencer). Homology searches were performed with BLAST (Basic Local Alignment Search Tool, NCBI-algorithm blastp) in the UniProtKB database.

Enzyme Kinetic Analyses

Determination of K_m and V_{max} was done using azocasein as substrate. The enzyme was incubated with different concentrations of azocasein (0.25–4.0 mg) and activity was assayed using the standard method. The kinetic parameters K_m , V_{max} , and k_{cat} were calculated through the Lineweaver-Burk double reciprocal plots.

Effects of Temperature, pH, and NaCl Concentration on Purified Enzyme Activity

Protease activity of the purified enzyme (1 $\mu\text{g}/\mu\text{l}$) against azocasein (pH 8.0) was determined at different temperatures by performing the assay at 10, 15, 20, 30, 35, 40, and 45 °C. Thermal inactivation was examined by incubating the purified enzyme at 30, 40, 50, and 60 °C for 60 min. Aliquots were withdrawn to test for remaining activity at pH 8.0 and 50 °C. The non-heated enzyme served as the control (100 %).

Next, the optimum temperature was fixed and the effect of pH value ranging from 4.5 to 10 was determined. pH stability was tested by keeping the purified enzyme in different buffers (100 mM) at 4 °C for 1 h, and the residual activity was determined under standard assay conditions. The following buffer systems were used: citric acid- Na_2HPO_4 buffer, pH 6.0; phosphate buffer, pH 7.0; Tris-HCl buffer, pH 8.0; and glycine-NaOH buffer, pH 9.0–10.0. The effect of the NaCl concentration was determined under the optimum conditions of pH and temperature while the following concentrations were tested: 0, 0.25, 0.5, 1, 1.5, 2, 2.5, and 3 M.

Effects of Metal Ions, Surfactants, Oxidizing Agents, and Enzyme Inhibitors

The effect of various metal ions (5 mM) on enzyme activity was tested using CaCl_2 , MnSO_4 , MgCl_2 , and ZnSO_4 . The effects of the surfactants SDS, Tween 80 and Triton X-100 and the oxidizing agent H_2O_2 on enzyme stability were also studied. The effects of the enzyme inhibitors phenylmethylsulfonyl fluoride (PMSF, 5 mM), dithio-bisnitrobenzoic acid (DTNB, 1 mM), and the metal complexing agent ethylenediaminetetraacetic acid (EDTA, 5 mM) on the activity of the purified enzyme were studied. The purified enzyme was preincubated with each one of these factors at 4 °C for 1 h, and the residual activity was measured against azocasein at pH 8.0 and 35 °C. The activity of the enzyme without any additions was considered as 100 %.

Evaluation of Purified Enzyme Activity Against Myosin, Actin, Myoglobin, and Elastin

Myosin (220 kDa, Sigma), actin (43 kDa, PROGEN), and myoglobin (14 kDa, Sigma) were used as substrates (2 mg/ml), and the proteolytic activity of the purified enzyme (1 $\mu\text{g}/\mu\text{l}$) was evaluated after 1, 2, 3, 4, 6, 7, and 8 h of incubation by SDS-5 % (w/v) PAGE against the same standard proteins. Tests were carried out at pH 6.0. The intensity of the produced bands was

measured on an image analysis system (Olympus, Germany) using the software Gel-Pro Analyzer (version 6.3, Media Cybernetics).

Elastinolytic activity was assessed according to Reichard et al. [21] using elastin congo red (Sigma) as substrate (1 g/l in 0.2-M sodium borate buffer, pH 8). The activities of the purified protease and porcine elastase (Sigma) were compared by monitoring the time required to liberate 50 % of the bound dye from the substrate.

All enzyme assays were carried out in triplicate and mean values are presented here.

Results

Protease Production in *P. nalgiovense* Fermentation

The profile of extracellular proteolytic activity (alkaline) of *P. nalgiovense* PNA9 was monitored in fermentations carried out with a complex medium containing the protein/peptide sources soybean meal, polypeptone, and yeast extract, and a minimal, chemically defined medium, without any proteins/peptides in its composition (Fig. 1). The second medium was used for protein purification purposes. Proteolytic activity was 1.8-fold higher in the case of the complex medium, while almost similar production patterns were obtained with a maximum at 72 h in the complex medium and 78 h in the chemically defined medium. Proteolytic activities reduced sharply beyond these time-points, and this was in parallel with the low growth rates of the fungus during the second half of fermentation. Biomass levels reached 6.5 g/l at 100 h and 5.2 g/l at 78 h and slightly declined until 120 h when runs were stopped (not shown). According to Fig. 1, protease is produced by *P. nalgiovense* as a growth-associated product.

Purification, Molecular Mass Determination, and N-Terminal Sequencing of the Protease

P. nalgiovense PNA9 protease was purified from fermentation broth filtrates as described in the “Materials and Methods” section. The partial purification results are shown in Table 1. Ammonium sulfate precipitation followed by dialysis resulted in 1.24-fold increase of specific activity, while ultrafiltration resulted in an overall 12.1-fold increase of specific activity. The

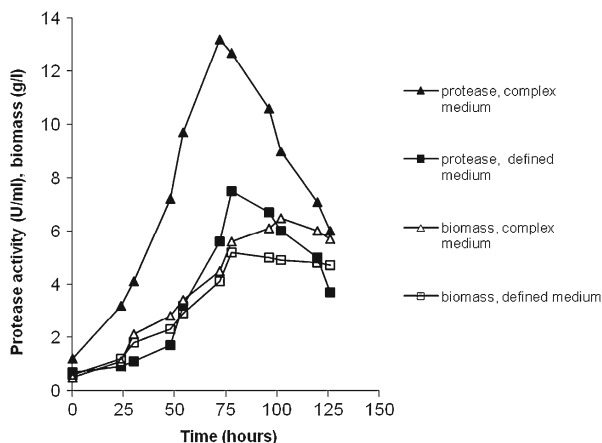


Fig. 1 Time-courses of extracellular proteolytic activity and biomass of *P. nalgiovense* PNA9 in fermentations carried out with a complex and a minimal, chemically defined medium

protein was then isolated in BN-PAGE as described (Fig. 2) and in-gel activity was assayed protease positive in cut-off lanes placed in tubes with azocasein containing reaction buffer. The recovered enzyme from BN-PAGE gels was subjected to tricine-SDS-12 % (w/v) PAGE at pH 8.5, and its molecular mass was estimated to be approximately 45 kDa. Molecular mass determination of the purified protease by ESI-MS analysis confirmed 45.2 kDa. N-Terminal sequencing was carried out for the first 30 amino acids and their sequence was found to be the following: MGFLKLLKGSLATLAVVNAGKLLTANDGDE.

Kinetic Analysis

The values for K_m and V_{max} , as determined through the Lineweaver-Burk plot of Fig. 3, were 1.152 mg/ml and 0.827 mg/ml/min, respectively. The k_{cat} value toward azocasein was determined to be 3.2×10^2 (1/s).

Effect of Temperature, pH, and NaCl Concentration on Protease Activity

The purified protease was found to be active within the temperature range of 10–45 °C with maximum proteolytic activity at 35 °C (Fig. 4). The thermal stability profile of the purified enzyme showed that it is completely stable at 30 °C. At 40 °C, the protease retained 80 % of its initial activity after 1 h incubation. Incubation at 50 °C and 60 °C, resulted in significant losses of activity and the residual activity was 40 and 25 %, respectively, within 15 min (Fig. 5).

The effect of pH on protease activity was studied by using azocasein as substrate at various pH values at the optimum temperature of 35 °C. The pH-activity profile of the purified protease is shown in Fig. 6. The protease is active in the pH range of 4.0 to 10.0, with an optimum at pH 8.0 (100 % relative activity). The relative activities at pH 7.5 and 9.0 were 85 and 82 %, respectively, of that at pH 8.0. The pH stability profile showed that the protease is highly stable between pH 8.0 and 9.0 (Fig. 7). A 57 % of its initial activity was retained at pH 10.0 while 72 % at pH 7.0.

Protease activity of the purified enzyme against azocasein was determined in the presence of a wide range of NaCl concentrations (0–3 M). Enzyme activity was retained over this range of concentration while the maximum activity was observed at 0.25 M (results not shown).

Effects of Metal Ions, Surfactants, Oxidizing Agents, and Enzyme Inhibitors

The effects of these factors on the protease are detailed in Table 2. Addition of CaCl_2 and MgCl_2 (5 mM) enhanced protease activity by 132 and 112 % of the control. In contrast, addition of MnSO_4 and ZnSO_4 (5 mM) reduced the activity by 57 and 25 %, respectively.

The enzyme was found to be highly stable in the presence of the non-ionic surfactants Tween 80 and Triton X-100 (5 %, v/v), but was inhibited (33 % residual

Table 1 Partial purification of the protease from *P. nalgiovense* PNA9

Enzyme fraction	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Broth filtrate	500	6,600	4.12	1.6	–	100
(NH_4) $_2\text{SO}_4$ precipitation followed by dialysis	40.9	6,570	3.30	1.99	1.24	99.54
Ultrafiltration (50 kDa)	6.8	5,475	0.28	19.5	12.1	82.9

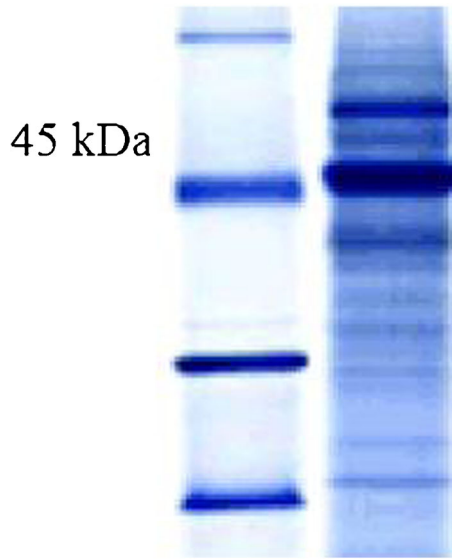


Fig. 2 Isolation of the alkaline protease in BN-PAGE gels

activity) by the strong anionic surfactant SDS used at the concentration of 0.5 %, w/v. The protease retained 72 % of its activity following treatment with H_2O_2 at the concentration of 5 %, v/v.

Enzyme activity was also determined in the presence of various enzyme inhibitors. Complete inhibition was caused by the serine protease inhibitor PMSF, indicating the serine protease nature of the enzyme. Most of the activity was retained (90 %) in the presence of the thiol agent BTNB, while EDTA caused only a slight reduction in activity (13 % of initial activity was lost).

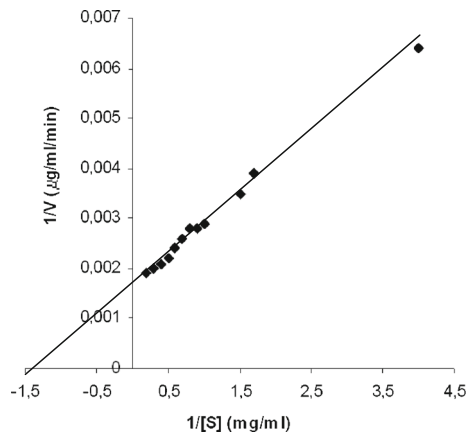


Fig. 3 Lineweaver-Burke double reciprocal plot for determination of K_m and $V_{\max}$ of the purified protease using azocasein as substrate

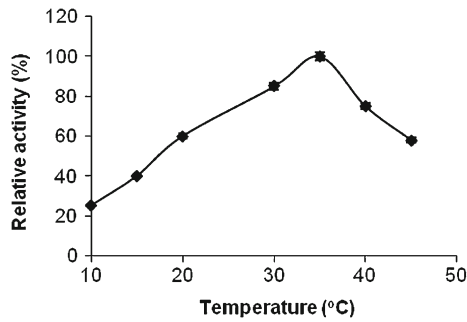


Fig. 4 Effect of temperature on protease activity. Standard deviations were ± 2 % (based on three replications)

Proteolytic Activity Against Myosin, Myoglobin, and Elastin

Proteolytic activity of the purified enzyme against myosin, actin, and myoglobin was tested over a time period of 1–8 h at pH 6.0. As shown in Table 3, the protease was highly active against actin, moderately active against myosin, while inactive against myoglobin. Elastinolytic activity was not detected.

Discussion

Preliminary research (not shown here) with the sausage isolated *Penicillium* strain revealed the presence of extracellular alkaline proteolytic activity in culture broths during the growth phase. The strain was then identified as *P. nalgiovense*, and the data presented here shows that the alkaline proteolytic activity is due to a single protease that is produced as a growth-associated extracellular product. Production was increased when a complex medium rich in peptides and peptones was used, while production characteristics (e.g., onset of production, time of maximum production, decrease of production) remained of the same trend when a chemically defined medium was used for protease purification purposes (Fig. 1). Proteolytic activities in both media were decreased during the second half and towards the end of fermentations. This can be explained by the elimination of carbon and nitrogen sources in the substrate and the progress of an autolysis process, common in filamentous fermentations [22, 23] that lead to

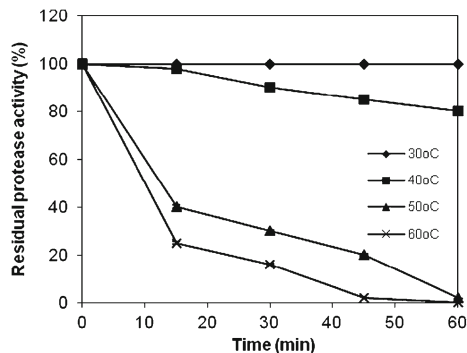


Fig. 5 Effect of temperature on the stability of protease. Standard deviations were ± 2 % (based on three replications)

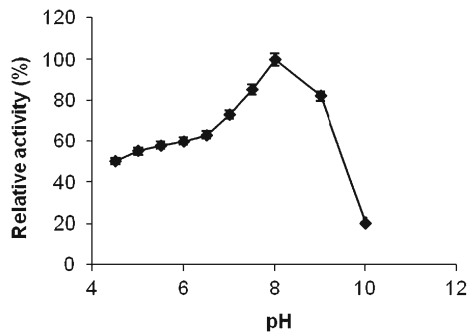


Fig. 6 Effect of pH on protease activity. Standard deviations were ± 2 % (based on three replications)

liberation of intracellular proteases and degradation of protein products. It was beyond the aim of the present study to study the effects of the composition of culture medium on protease production; however, the presence of intact peptides may have induced protease production as have been observed in many cases with different microorganisms [24, 25].

Ammonium sulfate precipitation followed by dialysis and ultrafiltration was an effective procedure in increasing the specific activity of the enzyme by more than 12-fold, corresponding to 19.5 U/mg (Table 1). BN-PAGE was effective in isolating and purifying the active protease. The procedure yielded a pure, homogeneous protein as evidenced by detection of a single band in polyacrylamide gel electrophoresis under non- and denaturing conditions, e.g., BN-PAGE (Fig. 2) and SDS-PAGE, respectively. The molecular mass of the protease was found to be 45.2 kDa. Since it was completely inhibited by PMSF (Table 2), the isolated enzyme is a serine protease. Serine proteases are widely distributed in filamentous fungi, and a number of them have been isolated and characterized [26–30]. Neutral and alkaline serine proteases were isolated from culture filtrates of several *Penicillium* species as mentioned in the “Introduction” section [2–8]. In general, the molecular masses of microbial proteases are rarely more than 50 kDa [31]. Alkaline serine proteases isolated from *Penicillium* spp. have masses in the range of 32–45 kDa, while the respective enzymes from *Aspergillus* spp. have a wider range of molecular masses [32].

Alignment studies for the 30 amino acids identified in the N-terminal of the protein chain were carried out within the UniProtKB protein database [33]. Comparing N-terminal regions of submitted proteins (Table 4), it appeared that the highest sequence identity (93 %) exists between the protease under investigation and an alkaline serine protease produced by a *P. chrysogenum* strain [34], followed by an alkaline serine protease produced by *Penicillium*

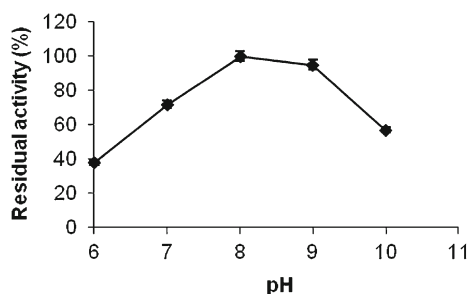


Fig. 7 Effect of pH on protease stability. Standard deviations were ± 2 % (based on three replications)

Table 2 Effect of various metal ions, surfactants, oxidizing agents, and enzyme inhibitors on alkaline protease activity. The activity is expressed as a percentage of the activity in the absence of the factor under investigation

Metal ions	Residual activity (%)
None	100
CaCl ₂ (5 mM)	132
MnSO ₄ (5 mM)	57
MgCl ₂ (5 mM)	112
ZnSO ₄ (5 mM)	27
Surfactants	
None	100
SDS (0.5 %, w/v)	35
Tween 80 (5 %, v/v)	100
Triton X-100 (5 %, v/v)	100
Oxidizing agent	
None	100
H ₂ O ₂ (5 %, v/v)	72
Enzyme inhibitors	
None	100
PMSF (5 mM)	0
DTNB (1 mM)	90
EDTA (5 mM)	87

nordicum (85 %) [35], and finally the protease produced by *P. chrysogenum* Pg222 (81 %) [3, 26]. The protease with the highest similarity in its N-terminal region has been identified to be a major allergen [34]. *P. nalgiovense* strains are known to produce penicillin [9] whose presence in meat products is implicated in food allergies. Several proteases also produced by *Penicillium* spp. are known to act as allergens [36]. Our present results, however, offer only an indication that the isolated protease may have such a role and further research is required to establish it.

Based on the Michaelis-Menten equation and the Lineweaver-Burk plot, the estimated values of K_m , V_{max} , and k_{cat} against azocasein show that the protease exhibits simple Michaelis-Menten kinetics and possesses high substrate affinity for azocasein, as well as catalytic efficiency (Fig. 3).

Table 3 Protease activity against the muscle proteins myosin, actin, and myoglobin

Incubation time (h)	% of SDS-PAGE band intensity against the controls		
	Myosin	Actin	Myoglobin
1	98.1	61.2	100
2	65.2	34.5	100
3	48.7	12.5	100
4	39.1	8.9	100
6	21.3	3.5	100
7	15.4	2.7	100
8	11.7	1.2	100

Table 4 Comparison of N-terminal amino acid sequence of the purified protease of *P. nalgiovense* PNA9 with other proteases produced by *Penicillium* spp.

N-Terminal amino acid sequence (30 aa)	Protease
MGFLKLLKGSLATLAVVNAGKLLTANDGDE	<i>P. nalgiovense</i> . This work
MGFLKLLSTSLATLAVVNAGKLLTANDGDE	<i>P. chrysogenum</i> . Chu et al., 2002
MGFLKLTFTSLATLAVVNAGKLLTASDGDE	<i>P. nordicum</i> . Karolewicz and Geisen, 2005
MGFLKVLATSLATLAVVNAGKLLTGSDGDE	<i>P. chrysogenum</i> . Benito et al., 2006

Experiments with the purified extracellular alkaline protease of *P. nalgiovense* PNA9 showed that thermal stability and proteolytic activity are higher at temperatures around 30 °C. This characteristic could be of interest for meat technology applications, e.g., dry-curing of meat products, where most of the ripening process takes place at temperatures around 30 °C. Also, the preservation of enzymatic activity over a wide range of NaCl concentrations (0–3 M) is another characteristic that applies to the above mentioned area of meat technology. The NaCl content of dry-cured meat products is in the range of 1–2 M, and its role as an inhibitor of the endogenous (muscle) proteolytic enzymes is well known [37]. Therefore, upon application, the alkaline protease of *P. nalgiovense* PNA9 should be expected to replace to some extent lost endogenous enzymatic activity and contribute itself to protein hydrolysis during the ripening process. Indeed, proteolytic activity of the purified enzyme against selected muscle proteins revealed that it is highly active against actin and moderately active against myosin. This is in contrast with the properties of protease EPg222, produced the dry-cured ham-isolated *P. chrysogenum* Pg222, which is very active against myosin and moderately active against actin [3]. Myoglobin is not affected by both proteases. The purified enzyme showed no proteolytic activity against the connective tissue protein elastin, while collagenolytic activity was not assessed.

The biochemical characterization of the isolated protease shows that it is active over a wide range of pH values (4.0–10.0); however, maximum activity and stability were obtained in the pH area of 8.0–9.0. Similarities exist between the protease under investigation and the protease produced by *P. charlesii* [5]. The protease produced by *P. charlesii* has a similar molecular mass (44 kDa); it is a serine protease and it is active over a wide pH range of 3.0 to 10.0 with optimum pH in the area of 7.0–9.0. Similarities also exist with the alkaline serine proteases produced by *P. waksmanii* [2] and *P. chrysogenum* [4], both having optimal activity at pH 8.0 and 35 °C, but lower molecular masses (32 and 41 kDa, respectively).

Among the metal ions tested, Ca^{2+} and Mg^{2+} enhanced enzymatic activity, a characteristic also observed with the proteases produced by *P. waksmanii* [2] and *P. chrysogenum* [4], *A. clavatus* [28], and several bacterial alkaline serine proteases [38, 39]. Mn^{2+} and Zn^{2+} had an inhibitory effect on enzymatic activity. Similarly, Mn^{2+} was found to reduce proteolytic activity of the CP-1 alkaline serine protease by *Serratia rubidaea* [39], while Zn^{2+} had a slight stimulatory effect.

The alkaline serine protease of *P. nalgiovense* PNA9 was highly stable in the presence of the non-ionic surfactants Tween 80 and Triton X-100 but lost most of its activity in the presence of the anionic detergent SDS. Enzymatic activity was also highly preserved in the presence of 5 % (v/v) H_2O_2 . The presence of the metal complexing agent EDTA had only a slight inhibitory effect on the proteolytic activity of the enzyme, while the thiol reagent DTNB had practically no effect. Therefore, the protease under investigation shows an extremely good profile in terms of stability and high activity in the presence of surfactants, oxidizing agents, and enzyme inhibitors. Proteases with high stability and activity characteristics in the high

alkaline range have a high industrial demand for various biotechnological applications. The major application of such proteases is in the detergent industry because the pH of laundry detergents is in the range of 9.0–12.0. The low temperature optimum however of the protease of *P. nalgiovense* PNA9 is a characteristic that could be particularly useful and suited to the current trend of washing at low-normal temperatures.

Conclusion

A novel alkaline serine protease with technologically important properties was purified and characterized. The enzyme is active from 10–45 °C, pH 4.0–10.0, and 0–3 M NaOH. It is active against muscle proteins and highly stable in the presence of non-ionic surfactants, hydrogen peroxide, BTNB, and EDTA. Therefore, the isolated protease as a cold-active alkaline protease with the above-described properties may be further researched in terms of production conditions and protein studies in view of specific applications.

Acknowledgments The authors would like to thank Prof. E.M. Papamichael from the Department of Chemistry, University of Ioannina for his helpful suggestions regarding the methodologies followed in this work.

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