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A novel thermostable neutral proteinase from *Saccharomonospora canescens*

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Abstract

A novel thermostable neutral proteinase, called NPS, was purified to electrophoretic homogeneity from the culture broth of *Saccharomonospora canescens* sp. novus, strain 5. The molecular mass was determined by SDS-polyacrylamide gel electrophoresis to be 35 000 Da. The enzyme exhibits a sharp pH optimum of proteolytic activity at pH 6.7. NPS was completely inactivated with inhibitors, typical for metalloendopeptidases, EDTA and 1,10-phenantroline, whereas the serine proteinase inhibitor PMSF had no effect. Atomic absorption measurements showed that the proteinase binds a single zinc and four calcium ions. The enzyme thermostability was characterized in the absence and presence of added calcium. Melting temperature, $T_m = 77°C$ and an activation energy, E_a, for the thermal deactivation of the excited protein fluorophores of 72.13 kJ mol^{-1} were calculated in the presence of 100 mM $CaCl_2$. The E_a-value is considerably higher than those obtained for a number of proteinases from microorganisms and was explained by the thermostable structure of the enzyme. Effective radiationless energy transfer from phenol groups to indole rings was observed. 68% of the light absorbed by tyrosyl residues is transfered to tryptophyl side chains. No homology was found after comparison of the NPS N-terminal sequence, including the first 26 residues, with those of other neutral proteinases from microorganisms. In contrast to the well-known bacterial neutral proteinase thermolysin and related enzymes from microorganisms, NPS possesses arylamidase and esterase activities. Further crystallographic studies will reveal the structural reasons for this specificity. Epoxy and epithio pyranosides are inhibitors of the proteinase arylamidase activity. © 1998 Elsevier Science B.V.

Keywords: Arylamidase activity; Fluorescence; Neutral proteinase; Thermostability; (*Saccharomonospora*)

1. Introduction

Zinc-containing metalloendopeptidases play an important role in the biosynthesis and metabolism of bioactive peptides [1]. They participate in the pro-

Abbreviations: NPS, neutral metalloendopeptidase from *Saccharomonospora canescens*; pNA, *p*-nitroaniline; pNP, *p*-nitrophenol; Suc, succinyl; PMSF, phenylmethanesulfonyl fluoride

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cesses of digestion, blood-pressure regulation [2], sporulation [3] and cell wall turnover regulation [4]. It was also shown that a neutral metalloendopeptidase from kidney membrane fraction degrades many neuropeptides [5]. Inhibitors of these proteinases are of considerable therapeutic value and are used for treatment of congestive heart failure, hypertension and rheumatoid arthritis [1,6,7].

The interest in neutral metalloendopeptidases from microorganisms (EC 3.4.24) is due to their important biological function and the possibilities for practical application. The amino acid sequences of neutral proteinases from *Bacillus thermoproteolyticus* [8], *Bacillus stearothermophilus* [9], *Bacillus cereus* [10], *Bacillus amyloliquefaciens* [11] and *Bacillus mesentericus / subtilis* [12] have been determined. The crystallographic structures of several thermolysin-like metalloproteinases from *B. thermoproteolyticus*, [13], *B. cereus* [14], *Streptomyces caespitosus* [15] and *Serratia marcescens* [16] are available. Thermolysin from *B. thermoproteolyticus* is the leading representative of the neutral metalloendopeptidases and serves as a model for the members of this important class of proteolytic enzymes. Substrate and inhibitor studies have been performed [1,17,18] and the structures of proteinase complexes with a variety of inhibitors have been elucidated [19–21]. These studies allowed better understanding of the hydrolytic mechanism of thermolysin and related endopeptidases.As part of our interest in the structure and function of proteinases from microorganisms, we began investigations on a proteolytic enzyme synthesized by *Saccharomonospora canescens* sp. novus, strain 5, from Bulgarian salt soils. No information is available about the structure and function of proteinases from this microorganism. Here, we describe a procedure for isolation of a chromatographically and electrophoretically pure thermostable neutral metalloendopeptidase, called NPS, from the culture broth of *S. canescens*. Our investigations on this enzyme were directed towards its physico-chemical properties, functional characteristics and substrate specificity. In contrast to the intensively studied neutral proteinase thermolysin and related enzymes, the *S. canescens* proteinase possesses arylamidase and esterase activities. The N-terminal sequence of the novel metalloendopeptidase is completely different from those of the other known neutral proteinases from microorganisms.

2. Materials and methods

2.1. Chemicals

The synthetic substrate Suc–Ala_2–Phe–pNA, 1,10-phenantroline, EDTA and DEAE 52-Cellulose SERVACEL were obtained from Serva (Heidelberg, Germany). Suc–Phe–Ala_2–Phe–pNA was purchased from BACHEM (Heidelberg, Germany). Thermolysin was a product of Sigma (St Louis, MO, USA) and Z-L-Tyr-4-nitrophenyl ester was from Fluka AG (Basel, Switzerland). Sephadex G-25 and Sephadex G-75 were obtained from Pharmacia (Uppsala, Sweden).

2.2. Purification of the neutral metalloendopeptidase from Saccharomonospora canescens (NPS)

Saccharomonospora canescens sp. novus, strain 5, was isolated from Bulgarian salt soils. Cells were grown in a medium containing 0.5% peptone, 0.5% corn steeped in liquor, 1% starch and inorganic salts in the temperature interval from 30 to 65°C. The culture broth was centrifuged at 4500 rpm for 30 min to remove the cells and the supernatant was precipitated by addition of ethanol to 70% saturation. The resulting precipitate was collected by centrifugation at 7000 rpm for 30 min and dissolved in 10 mM phosphate buffer, pH 7.0. The mixture was centrifuged at 3000 rpm for 30 min and the supernatant was loaded on a Sephadex G-75 column (3.5×100 cm) equilibrated and eluted with the buffer mentioned above. Further purification was achieved by ion-exchange chromatography on DEAE 52-cellulose column (4×30 cm) equilibrated with 10 mM phosphate buffer pH 7.0 and eluted under a linear gradient of 0–0.5 M NaCl. The final purification was performed by ion-exchange FPLC on a Mono - Q HR 10/10 column (Pharmacia, Uppsala, Sweden) using the same gradient (0–0.5 M) of NaCl. Samples were desalted by gel-chromatography on Sephadex G-25. Protein solutions were concentrated by membrane ultrafiltration (Amicon, Oosterhout, The Netherlands).

2.3. Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed as described in [22]. The gels were stained in

0.125% Coomassie brilliant blue in 45% methanol-10% acetic acid in water.

2.4. Metal ion analyses

Metal ion analyses were carried out using a Perkin Elmer model 400 atomic absorption spectrometer.

2.5. Amino acid analyses

Amino acid analyses were carried out with an automatic amino acid analyzer BIOTRONIK model LC 3000. The protein samples were hydrolyzed with 5.7 M hydrochloric acid at 110°C in evacuated sealed tubes for 24, 48 and 72 h, respectively. The contents of serine, threonine, methionine and tyrosine were obtained by linear extrapolation to zero time of hydrolysis. The amounts of valine, isoleucine and leucine were calculated from 72 h samples. For tryptophan determination a protein sample was hydrolyzed with 5% thioglycolic acid. Cysteine was determined after oxidation of the proteinase with performic acid.

2.6. Amino acid sequence analysis

N-terminal amino acid sequence analysis was performed using an Applied Biosystems sequencer, model 473 A (Weiterstadt, Germany).

2.7. Assay of the enzyme activity and kinetic studies with synthetic substrates and inhibitors

Proteolytic activity was estimated by the method of Kunitz [23], using casein as substrate. Protein content was determined by the method of Lowry et al. [24].

Kinetic measurements with the neutral proteinase from *S. canescens* and synthetic substrates were carried out in a 50 mM phosphate buffer, pH 6.7, at 25°C, using a Shimadzu recording spectrophotometer, model 3000, equipped with a thermostated compartment. Arylamidase activity was determined with Suc–Ala–Ala–Phe–pNA and Suc–Phe–Ala–Ala–Phe–pNA as substrates. Esterase activity was measured towards Z-Tyr-OpNP. The absorbance of the liberated *p*-nitroaniline and *p*-nitrophenol was monitored spectrophotometrically. The K_m and V_{max} values of the hydrolytic reactions were determined from the respective Hanes plots with five or six substrate concentrations. The enzyme concentration was determined by the absorbance at 280 nm using a molar absorptivity of $3.9 \times 10^4\ M^{-1}\,cm^{-1}$ and varied between 1.3×10^{-7} and 6.3×10^{-7} mol l^{-1}. The concentration range of the substrates was from 1×10^{-4} to 6×10^{-4} mol l^{-1}. The concentration of dimethylformamide, used as a solubilizer of the substrates, was 4% in the reaction mixture containing Suc–Ala–Ala–Phe–pNA, 10% for Suc–Phe–Ala–Ala–Phe–pNA and 40% for Z-Tyr-OpNP. Non-linear curve fitting is used for calculations with 0the kinetic data.

2.8. Proteinase inhibitors

The following proteinase inhibitors were assessed for their ability to inactivate the proteinase from *S. canescens*: ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF) and 1,10-phenantroline. 0.1 M stock solutions of EDTA in 0.1 M phosphate buffer, pH 6.7, PMSF in 100% ethanol and 1,10-phenantroline in the same solvent were prepared and stored at 4°C. Inhibitors were added to the assay mixture, containing the purified neutral proteinase, to a final concentration of 10^{-2} M. The effect of these inhibitors on the proteolytic activity of NPS is expressed as a percentage of the control activity.

Epoxy and epithio pyranosides like benzyl 2,3-anhydro-α-D-ribopyranoside, benzyl 3,4-dideoxy-3,4-epithio-α-D-arabinopyranoside and benzyl 3,4-dideoxy-3,4-epithio-β-L-arabinopyranoside were checked as potential inhibitors of the neutral proteinase. The inhibitors were synthesized as described in [25,26]. The kinetic experiments were performed using the procedures given in [27].

2.9. Effect of pH on the enzyme activity

The effect of pH on the enzyme activity was determined at a constant ionic strength by running the caseinolytic assay at various pH values.

2.10. Fluorescence measurements

Fluorescence measurements were performed with a Perkin Elmer model LS5 spectrofluorimeter, equipped

with a thermostatically controlled assembly and a Data station model 3600. The optical absorbance of the protein solutions was lower than 0.05 at the excitation wavelength to avoid inner filter effects. Tryptophyl side chains were excited at 295 nm. The temperature dependence of the tryptophyl quantum yield was determined at pH 7.0 in 0.05 M phosphate buffer in the absence of added calcium and in the presence of 100 mM $CaCl_2$. *N*-acetyl-L-tryptophanamide with a quantum yield of 0.13 (λ_{ex} = 295 nm) at 25°C [28] was used as a standard. The experimental data were analyzed according to the equation [29]:

$$Q^{-1} - 1 = k\mathrm{e}^{-E_a/RT}$$

where Q is the fluorescence quantum yield, E_a is the activation energy of the thermal deactivation of the excited singlet state, T is the absolute temperature and R is the gas constant. E_a was calculated from the slope of the Arrhenius plot $\ln(Q^{-1}\text{-}1)$ versus $1/T$.

The efficiency e of the tyrosine-to-tryptophan energy transfer was calculated using the relationship:

$$Q = Q_{Trp}\left[\mathrm{f}_{Trp}(\lambda) + e\mathrm{f}_{Tyr}(\lambda)\right]$$

where Q is the fluorescence quantum yield of the protein sample at the respective excitation wavelength λ, Q_{Trp} is the fluorescence quantum yield of the tryptophyl residues in the protein molecule after excitation at 300 nm, and $\mathrm{f}_{Trp}(\lambda)$ and $\mathrm{f}_{Tyr}(\lambda)$ are the fractional absorptions of tryptophan and tyrosine, respectively, at the excitation wavelength λ, calculated from their molar ratio in the protein.

3. Results and discussion

The crude enzyme preparation from the culture broth of *Saccharomonospora canescens* was gel-filtrated on a Sephadex G-75 column (Fig. 1). During this step of purification a considerable part of the contaminating proteins and coloured compounds were removed. Further purification was achieved by ion-exchange chromatography on a DEAE 52-cellulose column (Fig. 2). Only the fractions incorporated in the first peak exhibited a proteolytic activity. They were concentrated and the protein mixture was separated by FPL chromatography on a Mono Q column (Fig. 3). The second peak contained the *S. canescens* proteinase which was electrophoretically pure. The enzyme purification was followed by SDS-gel elec-

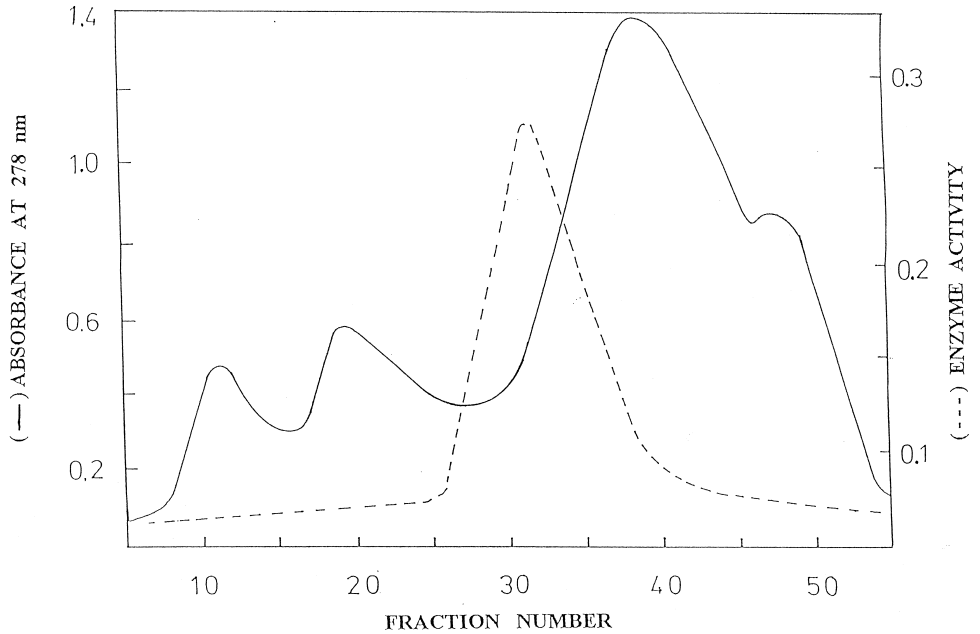

Fig. 1. Sephadex G-75 chromatography of an ethanol precipitate of a *Saccharomonospora canescens* supernatant. The column (100 × 3.5 cm) was equilibrated and eluted with 0.05 M Tris/HCl buffer, pH 7.0. (————) absorbance at 278 nm; (---) proteolytic activity.

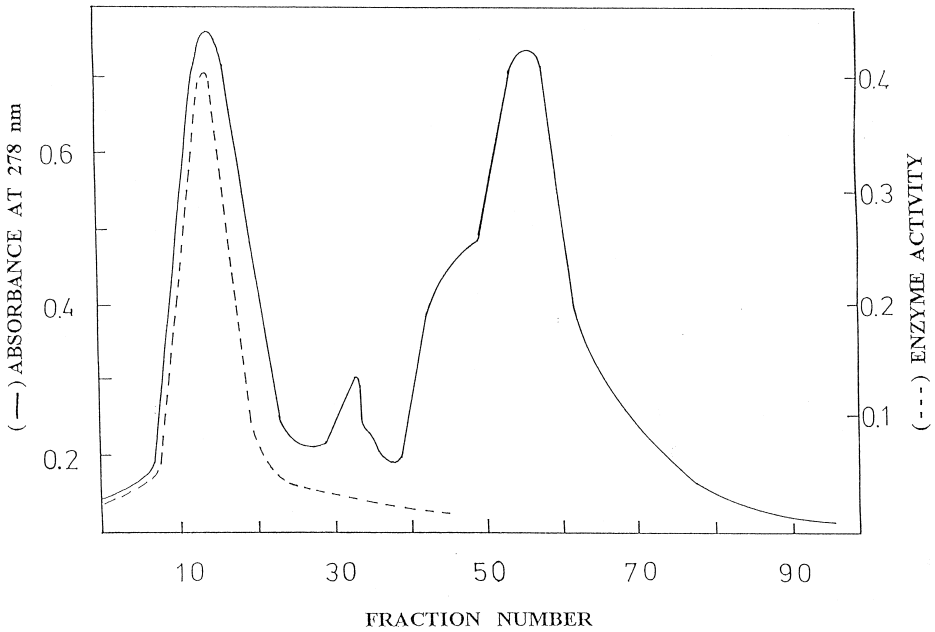

Fig. 2. Ion-exchange chromatography on a DEAE 52-cellulose SERVACEL column (30 × 4 cm) of the crude enzyme preparation, obtained after gel filtration of the precipitate from the culture supernatant. (————) absorbance at 278 nm; (---) proteolytic activity.

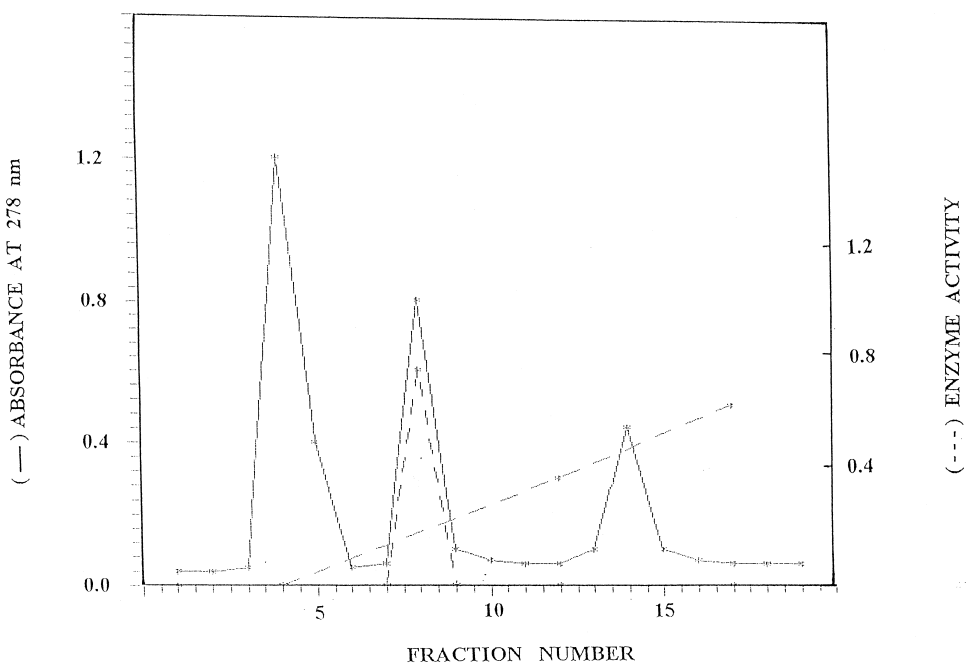

Fig. 3. Final purification of the *Saccharomonospora canescens* neutral proteinase by fast protein liquid chromatography (FPLC) on a Mono Q HR 10/10 column equilibrated with 10 mM phosphate buffer, pH 7.0. Proteins were eluted with a linear gradient of NaCl (0 – 0.5 M). (————) absorbance at 278 nm; (---) proteolytic activity.

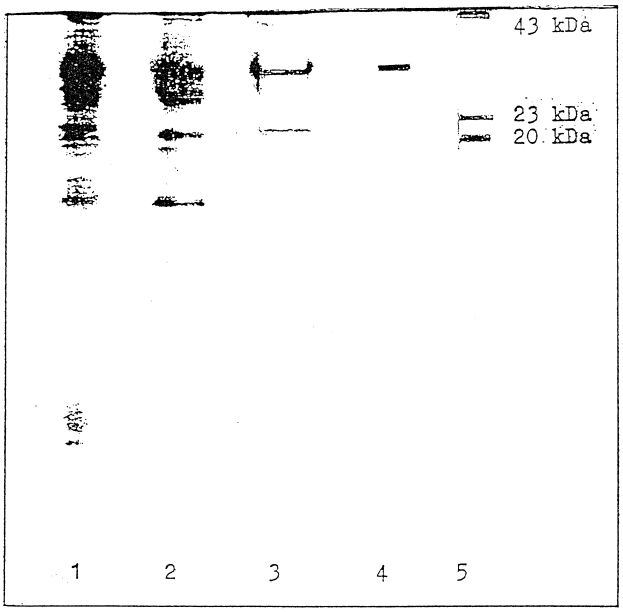

Fig. 4. SDS-polyacrylamide gel electrophoresis of the metalloproteinase preparations, obtained after the four purification steps: Lane 1, ethanol precipitate of a *Saccharomonospora canescens* supernatant; lane 2, protein composition of the ''crude'' enzyme preparation after gel filtration on a Sephadex G-75 column; lane 3, protein composition of the enzyme preparation, obtained by ion-exchange chromatography on a DEAE 52-cellulose SERVACEL column; lane 4, the neutral proteinase purified by FPLC technique and lane 5, positions of the M_r standard proteins: soybean trypsin inhibitor (20 000), trypsin (23 000) and ovalbumin (43 000).

trophoresis as it is shown in Fig. 4. In this figure the protein composition of the crude enzyme from the culture broth (lane 1) is compared with those of the enzyme preparations isolated by gel-chromatography (lane 2), ion-exchange chromatography on DEAE 52-cellulose (lane 3) and by the FPLC-technique (lane 4). The results indicate that after the last step NPS is isolated as a homogeneous enzyme. A single band was observed after gel electrophoresis of the respective sample. This conclusion was confirmed by N-terminal sequence analysis. The molecular mass of NPS, determined by SDS-PAGE, was $35\,000 \pm 1000$ Da. Soyben trypsin inhibitor (20 000), trypsin (23 000) and ovalbumin (43 000) were used as standards. M_r of 35 690 Da was calculated for the *S. canescens* proteinase from the amino acid composition. The degree of purification at each step is shown in Table 1. After the FPL chromatography the enzyme was 400-fold purified.

The pH optimum of proteolytic activity was measured with casein as a substrate using a 50 mM sodium phosphate buffer in the pH interval 5.5–8.0. NPS showed a maximum activity at pH 6.7 (Fig. 5) which indicated that it is a neutral proteinase.

The thermostability of the purified enzyme in the absence of added calcium and in the presence of 100 mM $CaCl_2$ was examined by fluorescence spectroscopy which is one of the most sensitive methods for studying protein conformation in solution and changes in conformation. The tryptophan emission maximum position, a parameter very sensitive to the fluorophore environment, was determined at different temperatures (Fig. 6). After excitation at 295 nm, NPS showed tryptophan fluorescence with a peak at 336 ± 1 nm, typical for indole groups ''buried'' in hydrophobic environment. The increase of the temperature above 42°C caused a batochromic shift of the emission maximum position of NPS in the absence of added calcium (EDTA treated enzyme) to a final value of 343.5 ± 1 nm, characteristic for ''exposed'' tryptophyl side chains. The melting temperature, $T_m = 56$°C, was determined as a midpoint of the transition curve. Addition of Ca^{2+} up to a concentration of 100 mM drastically changes the thermostabil-

Table 1
Purification of the neutral proteinase from *Saccharamonospora canescens*

Purification step	Protein (mg)	Activity (units)	Specific activity (units.mg^{-1})	Purification (fold)
Precipitation with ethanol	624.00	2650	4.3	1.0
Gel-filtration on Sephadex G-75	35.00	1010	28.9	6.7
Chromatography on DEAE-Cellulose 52	21.00	975	46.4	10.8
Gel-filtration on Sephadex G-25	4.10	700	170.7	39.7
F. p. l. c. on Mono Q 10/10	0.06	103	1716.7	399.2

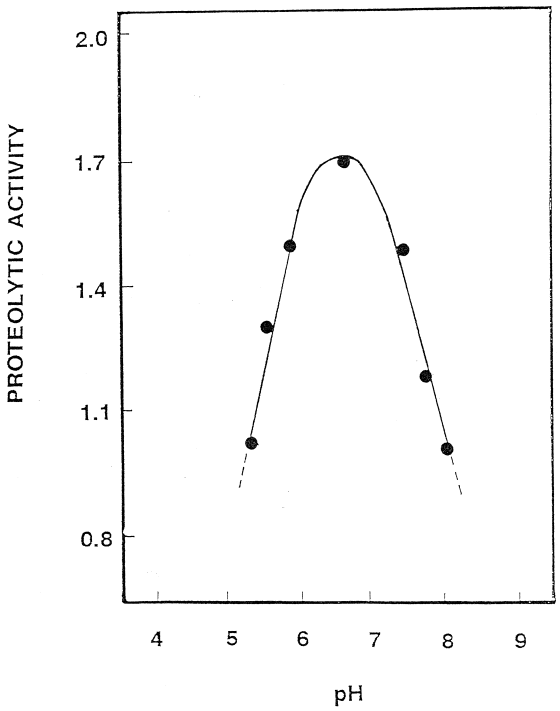

Fig. 5. pH-dependence of the proteolytic activity of the neutral metalloproteinase from *Saccharomonospora canescens*. The following buffers were used: sodium acetate (pH 5.4 – 6.0), sodium phosphate (pH 5.5 – 8.0) and Tris/HCl (pH 7.5 – 8.0).

ity and the melting temperature considerably increased to values of 60°C (2 mM Ca^{2+}), 69°C (50 mM Ca^{2+}) and 77°C (100 mM Ca^{2+}). The stability of NPS towards thermal denaturation in the presence of 100 mM Ca^{2+} was also investigated using the Arrhenius equation $Q^{-1} - 1 = ke^{-E_a/RT}$ where Q is the protein fluorescence quantum yield. Fig. 7 shows the radiationless thermal deactivation of the excited singlet state of indole chromophores. The activation energy of this process, E_a, was calculated from the plot of $\ln(Q^{-1} - 1)$ vs. $1/T$ to be 72.13 kJ mol^{-1}. This value is considerably higher than those obtained for a number of proteinases from microorganisms [30] and can be explained with the thermostable structure of NPS.

Under excitation at 280 nm, where both, phenol and indole groups absorb, the fluorescence spectrum of NPS (not shown) is dominated by tryptophyl emission. This can be explained by an effective radiationless energy transfer from phenol groups (donors) to indole rings (acceptors). The efficiency e of this process was calculated to be 0.68, i.e. 68% of the light absorbed by tyrosyl residues is transfered to indole groups. The transfer of electronic energy between chromophores depends largely on the mutual orientation in the space of their dipoles [31]. The high value of e suggests that the orientation of the tyrosyl and tryptophyl side chains in the three-dimensional structure of NPS is suitable for a very effective energy transfer at a singlet-singlet level.

The nature of the proteinase active site was checked by performing activity measurements in the presence of the specific serine proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF) or neutral metalloendopeptidase inhibitors EDTA and 1,10-phenantroline, at a final concentration of 10^{-2} M. PMSF had no measurable effect on the proteolytic activity of NPS. On the other hand, the proteinase from *Saccharomonospora canescens* was completely inactivated by the chelating agents EDTA and 1,10-phenantroline. This suggests a direct participation of a metal ion in the catalytic process.

The zinc and calcium content of NPS was determined by atomic absorption spectroscopy. The

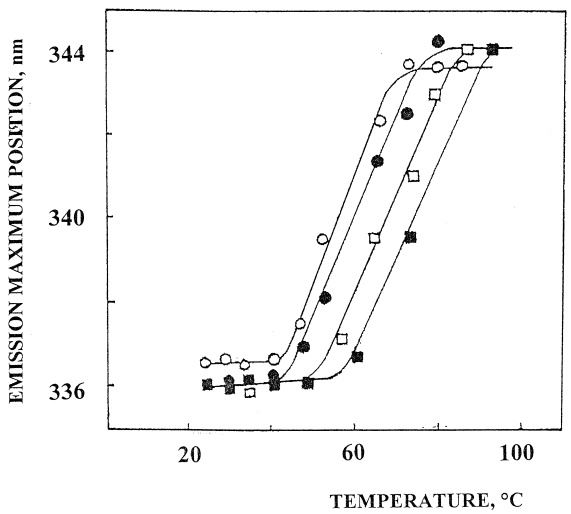

Fig. 6. Temperature dependence of the tryptophyl emission maximum position of the neutral metalloproteinase from *Saccharomonospora canescens* in the absence (○ ——— ○) and presence of 2 mM (● ——— ●), 50 mM (□ ——— □) and 100 mM (■ ——— ■) $CaCl_2$. Fluorescence spectra were recorded after excitation at 295 nm and thermal equilibration of the protein sample at the respective temperature.

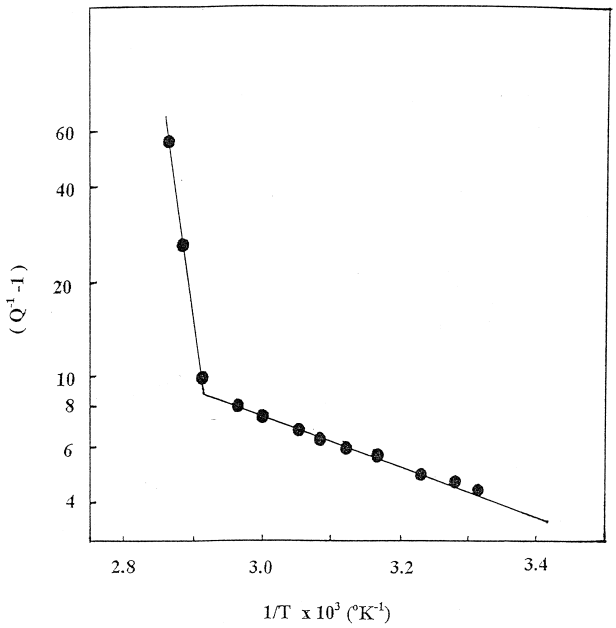

Fig. 7. Arrhenius plot for the radiationless thermal deactivation of the excited (295 nm) tryptophyl fluorophores of the neutral metalloproteinase from *Saccharomonospora canescens*. The protein sample was dissolved in 50 mM phosphate buffer, pH 7.0. The fluorescence quantum yields at various temperatures were determined relative to the fluorescence quantum yield at 25°C.

amounts of metal ions in the protein samples were calculated from calibration curves. The results showed the presence of 1.1 g-atom zinc and 3.8 g-atoms calcium per mole of enzyme. This means that each proteinase molecule contains one zinc-binding site and four calcium-binding sites. In this respect NPS is similar to thermolysine, the neutral Zn-endoproteinase from *Bacillus thermoproteolyticus*, which has the same number of metal-binding sites [13]. Most probably, the zinc ion, bound to NPS, is involved in the catalytic mechanism. Calcium ions are important for the stability of proteolytic enzymes: they protect proteinases against thermal denaturation and proteolytic (auto)degradation [32,33]. Four Ca^{2+}-binding sites are typical for the thermostable neutral proteinases; the mesophilic members of this family have less metal-binding sites [12].

On the basis of the results described here, NPS should be classified as a thermostable neutral zinc-containing proteinase.

The amino acid composition of NPS revealed that the enzyme polypeptide chain consists of 317 residues. This is characteristic for the thermophilic neutral metalloproteinases; these enzymes contain 315–319 residues per protein molecule while the polypeptide chains of their mesophilic counterparts consist of 300 residues [8–12,34].

The first 26 amino acid residues of NPS were determined by sequence analysis and compared for homologies with other neutral zinc endopeptidases:

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                10                  20          26
AVVNVYLYEH  INYGGRYIYA  AVTYTK
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This comparison did not reveal significant similarities. The N-terminal sequence of the novel proteinase from *S. canescens* is completely different from those of the known neutral proteinases from microorganisms.

Thermolysin is a leading representative of neutral metalloendopeptidases from microorganisms and serves as a model for the members of this important family of proteolytic enzymes. In order to establish similarities and differences in the specificity of this proteinase and NPS, we have performed experiments with protein and synthetic substrates. Both enzymes showed the same degree of hydrolysis with casein as a substrate. To examine whether NPS possesses arylamidase activity, the enzyme-catalyzed hydrolysis of tri- and tetrapeptide substrates, containing a *p*-nitroanilide leaving group, was analyzed. The esterase activity was investigated with a *p*-nitrophenyl ester of benzyloxycarbonyl-L-tyrosine. The initial rate of hydrolysis was examined as a function of substrate concentration to determine k_{cat}, K_m and the specificity ratio k_{cat}/K_m. The release of *p*-nitroaniline or *p*-nitrophenol was measured spectrophotometrically. The kinetic parameters for the hydrolysis of Suc–

Table 2
Kinetic parameters for the hydrolysis of synthetic substrates by the neutral proteinase from *Saccharamonospora canescens*. All experiments were carried out in a 50 mM phosphate buffer, pH 6.7, at 25°C

Substrate	K_m (M)$\times 10^5$	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}.s^{-1}$)
Suc–Ala–Ala–Phe–pNA	9.80 ± 0.80	1.10 ± 0.05	11224 ± 70
Suc–Phe–Ala–Ala–Phe–pNA	1.45 ± 0.13	1.20 ± 0.08	82758 ± 645
Z-L-Tyr-OpNP	0.54 ± 0.04	0.26 ± 0.02	482 ± 5

Ala_2–Phe–pNA, Suc–Phe–Ala_2–Phe–pNA and Z-Tyr-OpNP by the *S. canescens* proteinase are summarized in Table 2. NPS hydrolyzed the *p*-nitroanilide bond in Suc–Phe–Ala_2–Phe–pNA with a specificity ratio 7.4 times higher and a K_m value 6.8 times lower than those obtained with Suc–Ala_2–Phe–pNA as a substrate. At the same time, the catalytic constant was almost not changed. Evidently, the enhancement of the catalytic efficiency of NPS, as a result of the elongation of the substrate peptide chain, is mainly due to the influence of the Michaelis constant, i.e. to the enhanced enzyme affinity to the substrate. The data suggest that NPS has an extended substrate binding site, accomodating at least four amino acid residues at the S-side of the hydrolyzed bond. The enzyme hydrolyzed Z-Tyr-OpNP with $k_{cat} = 0.26 \pm 0.02\,s^{-1}$, $K_m = 5.4 \times 10^{-4} \pm 0.04\,M$ and $k_{cat}/K_m = 482 \pm 5\,M^{-1}\,s^{-1}$ (Table 2).

Experiments with thermolysin, using the same conditions and equipment, revealed that this proteinase is not capable of hydrolyzing the *p*-nitroanilide or *p*-nitrophenyl bonds in the three synthetic substrates, i.e. it is devoid of arylamidase and esterase activity. It was shown that thermolysin and the related metalloproteinase from rabbit kidney do not release 2-naphtylamine (2NA) when hydrolyzing peptide bonds in synthetic tripeptide substrates containing 2NA attached by amide linkage to the C-terminus [18]. Also, the *B. thermoproteolyticus* proteinase does not hydrolyze ''simple'' esters such as Ac-Tyr-OEt, Ac-Phe-OEt [35], Bz-Arg-OEt, Bz-Tyr-OpNP and alkyl esters [36 and citations therein] or amides as Bz-Tyr-NH_2, Z-Phe-NH_2 and Z-Leu-NH_2 [37]. Comparative studies with six neutral proteinases from various species of microorganisms led to the conclusion that the lack of esterase and amidase activity towards ''simple'' esters or amides of N-blocked amino acids is a common property of this group of proteolytic enzymes [37]. However, thermolysin catalyzes the hydrolysis of ester-peptide compounds such as Bz-Gly-OPhe-Ala and Bz-Gly-OLeu-Ala [36]. The results presented here demonstrate considerable differences in the substrate specificities of the neutral proteinase from *S. canescence*, on one hand and the *B. thermoproteolyticus* proteinase, on the other.

As regards to the kinetic properties, the *S. canescens* neutral proteinase reveals a noticeable similarity to the microbial serine alkaline proteinases. As it was mentioned, the neutral proteinases from microorganisms are inactive against synthetic amide and ester substrates. NPS hydrolyzed the para-nitroanilide bond in Suc–Ala_2–Phe–pNA with K_m and k_{cat} values approximately one order of magnitude lower than those for the same reaction catalyzed by serine alkaline proteinases from microorganisms. The specificity constant k_{cat}/K_m for the neutral proteinase is 2.5–15.9 times lower than those for the subtilases [27]. It can be concluded that NPS possesses a higher affinity but lower catalytic efficiency towards the substrate mentioned above in comparison to the subtilisin-type serine proteinases. This means that the substrate amino acid residues are better accepted by the neutral proteinase, but the *p*-nitroanilide bond hydrolysis, catalyzed by this enzyme, is not so effective.

The general reaction scheme for the substrate hydrolysis by serine proteinases [38] can not be applied for the neutral metalloproteinases. The thermolysin-catalyzed hydrolysis proceeds via the attack of the zinc-bound water molecule, with enhanced nucleophilicity, on the carbonyl carbon of the scissile bond [19]. In general, this mechanism should be operative for the related neutral zinc-containing proteinases. Probably, the peptide *p*-nitroanilides bind to the substrate binding site of NPS in such a way that the bond between the *p*-nitroanilide group and the phenylalanyl residue is suitably oriented for an attack of the ''activated'' water molecule. The catalytic site should be similar to that of the other neutral zinc-proteinases.

It was shown [39] that compounds containing an epoxy group like benzyl-3,4-epoxybutanoic acid (BEBA) are effective inhibitors of zinc-containing neutral proteinases. In the Michaelis complex, formed upon the interaction of such compounds with the proteinase, the epoxy group makes a complex with the active site Zn^{2+}. We have checked a series of epoxy and epithio pyranosides as possible inhibitors of NPS. These species have structural features for complexing with the zinc ion at the proteinase active site. The kinetic data definitely showed that benzyl 3,4-dideoxy-3,4-epithio-α-D-arabinopyranoside, benzyl 3,4-dideoxy-3,4-epithio-β-L-arabinopyranoside and benzyl 2,3-anhydro-α-D-ribopyranoside are inhibitors of the neutral proteinase from *Saccharomonospora canescens* with K_i values of 1.2×10^{-4}, 1.0×10^{-4} and 15×10^{-4}, respectively. These

results are in agreement with the conclusion that NPS is a neutral zinc-containing proteinase.

In conclusion, the results described here classify NPS as a novel thermostable neutral Zn-endopeptidase, synthesized by the microorganism *Saccharomonospora canescens*. In contrast to thermolysin and related enzymes, it possesses arylamidase and esterase activities. This can be explained with differences in the manner of binding of arylamide peptide substrates and ''simple'' esters of N-blocked amino acids to the respective binding sites. We plan further crystallographic investigations which can reveal the reasons for the observed kinetic behaviour of NPS.

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