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Isolation and partial characterization of protease from *Pseudomonas aeruginosa* ATCC 27853

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Abstract: Enzymatic characteristics of a protease from a medically important, referent strain of *Pseudomonas aeruginosa* ATCC 27853 were determined. According to sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS--PAGE, and gel filtration, it was estimated that the molecular mass of the purified enzyme was about 15 kDa. Other enzymatic properties were found to be: pH optimum 7.1, pH stability between 6.5 and 10; temperature optimum around 60 °C while the enzyme was stable at 60 °C for 30 min. Inhibition of the enzyme was observed with metal chelators, such as EDTA and 1,10-phenanthroline, suggesting that the protease is a metalloenzyme. Furthermore, the enzyme contains one mole of zinc ion per mole of enzyme. The protease was stable in the presence of different organic solvents, which enables its potential use for the synthesis of peptides.

Keywords: protease; *Pseudomonas aeruginosa*; purification; characterization.

INTRODUCTION

A wide range of investigations on *Pseudomonas aeruginosa* and its exoenzymes were performed: from targeted treatment of infections to decomposition of natural materials and bioremediation.[1–6] Since this strain has the property of forming biofilms (specific communities of cells encased in an extracellular matrix composed of proteins, nucleic acids, and cell debris), *P. aeruginosa* has advantages in the invasion of a host and survival under different environments, in comparison to other strains. The ability to form biofilms and synthesize numerous exoproducts, such as lipase, phospholipase, alkaline phosphatase, exotoxin and proteases, is regulated by cell to cell communication, quorum sensing

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(QS).[6–9] *P. aeruginosa* produces several extracellular proteases, including LasB elastase, LasA elastase, and alkaline protease.[10] Proteases are assumed to play an important role during acute *P. aeruginosa* infection, however details of their action are sometimes unclear.[11,12]

The prototype strain, *P. aeruginosa* ATCC 27853 has been used as a reference control strain in different kinds of experiments, including: testing of antimicrobial activity of new compounds or combination therapy against *P. aeruginosa*, identification of virulence factors, particularly extracellular enzymes, quailty control testing, drug carrier testing *etc*.[13–16] It has become clear that numerous extracellular enzymes acting as virulence factors, controlled by QS, are important in the development of *P. aeruginosa* biofilms.[17] However, a detailed characterization of extracellular enzymes from microorganisms of medical importance, such as *P. aeruginosa* ATCC 27853, has not yet been reported. In this study, extracellular protease from the prototype strain *P. aeruginosa* ATCC 27853 was isolated and its enzymatic properties were characterized, considering the protease as a potential, new target for the design of antibacterial therapy at the level of exoproduct formation and interaction.[18]

EXPERIMENTAL

Materials

Phenyl-Toyopearl 650 was purchased from Tosoh Bioscience (Montgomeryville, PA, USA). Hammersten casein was purchased from Merck (Darmstadt, Germany). Sephadex G-75 was supplied by Pharmacia (Uppsala, Sweden). Molecular mass standards were supplied by Serva (Heidelberg, Germany). The chemicals used for electrophoresis were purchased from Sigma Chemicals (St. Louis, MO, USA). The equipment employed for chromatography and electrophoresis was purchased from Hoefer Scientific Instruments (San Francisco, CA, USA).

Microorganism

P. aeruginosa ATCC 27853 was provided by American Type Culture Collection (USA).

Culture conditions

P. aeruginosa ATCC 27853 was cultured at 30 °C for 20 h in Luria-Bertani (LB) medium (0.5 % NaCl, 0.5 % yeast extract and 1 % tryptone) agitated at 100 cycles min^{-1} with a horizontal shaker model LT-W (Küchner, Birsfelden, Switzerland). An actively growing culture was dispensed into an Erlenmeyer flask (1 %), and fermentation was realized in LB medium at 30 °C for 120 h. A culture filtrate was then collected after 96 h and used for protease isolation and purification.

Proteolytic activity assays

Proteolytic activity was determined using 0.6 % Hammersten casein solution (50 mM Tris–HCl, pH 7.6) as a substrate. The enzyme solution (1 ml) was mixed with the substrate solution (5 ml) and incubated at 30 °C. After 10 min, 5 ml of trichloroacetic acid (TCA) solution (0.11 mol L^{-1} TCA, 0.22 mol L^{-1} sodium acetate and 0.33 mol L^{-1} acetic acid) was added to the reaction mixture, which was followed by additional 20 min incubation. The precipitate was removed by filtration or centrifugation. The absorbance (A) of the filtrate was measured at 275 nm using a UV–Visible light spectrophotometer (Gilford, Gilford Instruments, Oberlin, OH, USA). One unit of protease activity is defined as the amount of the enzyme that gives an absorbance value equivalent to 1 μg of Tyr per min at 30 °C.[18]

Protease activity inhibition and stability in organic solvents was determined according to Morihara method.[19] In short, the activity was determined by incubating 1 ml of 2 % casein solution (pH 7.6) with 1 ml of enzyme solution at 40 °C for 10 min. The reaction was stopped by the addition of 2 ml of TCA solution followed by 20 min incubation at 40 °C. After filtration, the amount of liberated Tyr was determined spectrophotometrically at 660 nm (Gilford, Gilford Instruments, Oberlin, OH, USA) using Folin-Ciocalteu reagent (dilution 1:4) to develop color. One unit of enzyme activity is defined as the amount of enzyme that results in an ΔA_{660} of one.[18]

Purification of protease

All procedures were performed at 4 °C. The culture filtrate, obtained by centrifugation at 7500 rpm for 15 min (Sorvall, rotor SS-1, New Town, Conn., USA) was lyophilized and resolved in 50 mmol L^{-1} Tris–HCl buffer (pH 7.60), supplemented with 20 % ammonium sulfate, with final concentration of 1 mg ml^{-1} of proteins. This crude sample was purified by hydrophobic chromatography using Phenyl Toyopearl 650 resin previously equilibrated (20 % ammonium sulfate solution in 50 mmol L^{-1} Tris–HCl buffer, pH 7.6). The protease was collected as the flow-through, while all the contaminating proteins were bound to the resin.

Protein concentrations were determined by the Bradford method using crystalline bovine serum albumin (BSA) as the standard.[20] In some cases, the concentration of proteins was determined spectrophotometrically using the equation c (mg mL^{-1}) $=1.55A_{280} - 0.76A_{260}$.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS PAGE, was performed using 12.5 % polyacrylamide gels,[21] under reducing conditions and a standard protein mixture containing: lysozyme (14.4 kDa), trypsin inhibitor (21 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (BSA) (67 kDa), phosphorylase B (97 kDa), and myosin (220 kDa).

Gel filtration

The molecular mass of the proteolytic enzymes was determined by gel filtration[22] on a Sephadex G-75 column (2.5 cm×75 cm), previously equilibrated with 50 mmol L^{-1} Tris–HCl buffer (pH 7.6) containing 0.5 mol L^{-1} NaCl. The column was calibrated using cytochrome *c* (12.5 kDa), chymotrypsin (25 kDa), ovalbumin (45 kDa), and BSA (67 kDa) as a molecular mass protein standard mix. The molecular masses were determined by plotting the log of molecular masses against the elution volumes.

Optimum pH

The pH optimum was determined using casein as substrate for the protease. 50 mmol L^{-1} buffers of different pH values were used to assay enzymatic activity: sodium citrate buffer (2.79–5.78), Sorensen's phosphate buffer (5.57–7.80), Tris–HCl buffer (7.62–9.39) and borate–NaOH buffer (9.50–11.41). Other conditions were as for the standard assay method.

pH Stability

The buffers used to test pH stability were: sodium citrate buffer (2.79–5.78), Sorensen's phosphate buffer (5.57–7.80), Tris-HCl buffer (7.62–9.39) and borate–NaOH buffer (9.50––11.41). Reaction mixtures (5 mg of enzyme in 1.2 ml buffer solution) were incubated at 30 °C for 3 h. The remaining enzymatic activity was measured under standard test conditions.

Optimum temperature

Using the standard reaction mixture, the proteolytic activity was monitored in 50 mmol L^{-1} Sorensen's phosphate buffer (pH 7.6) at different temperatures (from 25–90 °C) for 10 min.

Thermal stability

The samples in 50 mmol L^{-1} Tris-HCl buffer (pH 7.6) were incubated at different temperatures for various periods of time and then quickly cooled. Standard enzyme assays were then used to determine the enzyme activity.

Effects of inhibitors

The effects of different agents, such as: phenymethylsulfonyl fluoride (PMSF), *p*-chloromercuribenzoic acid (*p*CMB), 1,10-phenanthroline, ethylenediaminetetraacetic acid disodium salt (EDTA), dithiothreitol (DTT), were investigated. Enzyme solution in 50 mmol L^{-1} Tris–-HCl buffer (pH 7.6) was incubated after the addition of 5 mmol L^{-1} of inhibitor at 30 °C for 30 min, and remaining activity was determined by standard methods using casein as described by Morihara.[19]

Proteolytic apoenzyme reactivation

The enzyme activity was completely deactivated by treatment with 1 mmol L^{-1} 1,10-phenanthroline at 30 °C for 15 min. Following deactivation, solutions of different metal ions were added (Cu^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+}) to a final concentration of 1.2 mmol L^{-1}, and then after incubation at 30 °C for 30 min, the remaining activity was determined under standard conditions.

Metal analysis

Elemental metal analysis (Zn, Cu, Co, Fe and Mn) was performed by means of flame atomic absorption using a Perkin Elmer SAS7500A atomic absorption spectrophotometer (Norwalk, MA, USA).

Substrate specificity

Activities against *N*-hippuryl-L-Lys, *N*-hippuryl-L-Phe, elastin, collagen model, heat-killed *S. aureus*, against synthetic esters, such as *N*-benzoyl-L-Arg-ethyl ester (BAEE), *N*-benzoyl-L-Tyr-ethyl ester (BTEE), and *N*-acetyl-L-Tyr-ethyl ester (ATEE), as well against synthetic oligopeptide-*p*-nitroanilide (*p*-NA) substrates, such as *N*-succinyl-Ala-Ala-Pro-Phe-*p*-NA, *N*-succinyl-Ala-Ala-Pro-Leu-*p*-NA, and *N*-benzoyl-Arg-*p*-NA were determined according to the methods recommended by Biochemica Merck (Darmstadt, Germany).

Organic solvent stability

The effects of various organic solvents (methanol, ethanol, acetone, 1-butanol, 2-propanol, chloroform, *n*-hexane and *N*,*N*-dimethylformamide (DMF)) on the crude protease were investigated. The culture supernatant was incubated in the presence of 30 % (v/v) of organic solvent for a fixed period of time (from 24 to 240 h). The experiments were performed at 30 °C on a rotary shaker at 160 strokes min^{-1}, according to the Ogino method.[18] A crude sample without organic solvent was assayed under the same experimental conditions and was used as a control.

RESULTS AND DISCUSSION

Purification of protease

The fermentation broth was concentrated by lyophilization and the proteolytic enzyme produced by the *P. aeruginosa* ATCC 27853 was purified from the lyophilisate by hydrophobic chromatography and gel filtration chromatography. A summary of the purification procedure is given in Table I. The purification of

protease was accomplished relatively easily, since the protease did not bind to the Phenyl-Toyopearl gel, while almost all the other contaminating proteins from the crude sample were bound to the column. After hydrophobic chromatography, the enzyme had been purified 5-fold with 57.5 % recovery. After gel filtration chromatography, the protease had been purified 30 times with a yield of 25.3 %. This was the final purification step prior to SDS PAGE, which was used to assess the protein homogeneity. The SDS PAGE of the purified protease is shown in Fig. 1.

TABLE I. Purification of the protease from *P. aeruginosa* ATCC 27853

Purification step	Total protein mg	Total activity mU	Specific activity mU mg^{-1}	Yield %	Purification fold
Crude preparation (lyophylisate)	100	1660	16.6	100	1
Phenyl-Toyopearl 650	10.84	954.5	88	57.5	5.3
Sephadex G-75	0.84	420	498	25.3	30

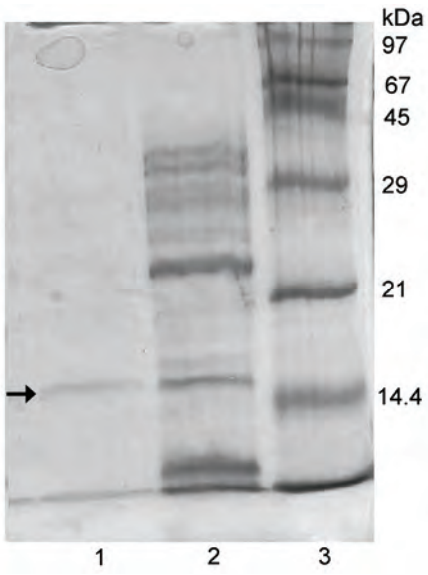

Fig. 1. Molecular mass determination of protease by SDS PAGE (12.5 %) under reducing conditions. Lane 1 – purified protease, lane 2 – crude protease preparation and lane 3 – markers: lysozyme, 14.4 kDa; trypsin inhibitor, 21 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; BSA, 67 kDa; phosphorylase B, 97 kDa (myosin as a 220 kDa protein is too large to migrate under these gel conditions and it remained at the top of the gel lane).

Molecular mass determination of the protease

The molecular mass of the protease from *P. aeruginosa* ATCC 27853 determined by gel filtration on Sephadex G-75 was 14 kDa and by SDS PAGE, 15 kDa (Fig. 1). The group of proteolytic enzymes produced by *P. aeruginosa* strains includes at least four endopeptidases with molecular masses ranging from 20 to 50 kDa.[18] Using the ExPASy UniProt data base, 22 extracellular proteases from *P. aeruginosa* were found. As is given in Table II, among the identified proteases, six belong to the group of alkaline metalloproteinase, with a length of

479 amino acids (50 kDa), and five to the group of elastase LasB, with a length of 498 amino acids (54 kDa). With the exception of putative uncharacterized protease (also known as staphylolytic protease LasA), all the proteases as pre-proenzymes have a length of more than 400 amino acids (45 kDa), and according to available data, all of them have molecular mass ranging from 20 to 50 kDa, when they are in the form of mature extracellular protease. Molecular mass determination of proteases is difficult because of the presence of a protease-related processing intermediary protein, from which the mature enzyme is formed.[28] The molecular mass of the protease from *P. aeruginosa* ATCC 27853, obtained by SDS PAGE, suggests it is a small protease, different from any other hitherto characterized protease from *P. aeruginosa*. *P. aeruginosa* ATCC 27853 was declared as a strain producing elastase and alkaline protease, both having a molecular mass of about 30 kDa.[29]

TABLE II. *P. aeruginosa* proteases (search was performed using the network service of ExPASy)

Protein name (EC 3.4.24.-)	Accession number	Length of pre-proenzyme (AA)/calculated mass of pre-proenzyme (kDa)	Mass of extracellular protein, kDa
Putative uncharac-terized protein	P72166	263/28	20[18]
Protease lasA	P14789	418/46	20[23]
Alkaline proteinase	Q6SQM7	459/50	–
Alkaline proteinase	P72120	476/50	–
Alkaline metallo-proteinase	Q03023, Q4Z8K9, Q02J90, B7UWT0, A3LKRI7, A6V8W2	479/50	–
Organic solvent tolerant protease	Q6UL02	479/50	33[24]
Pseudolysin	P14756	498/54	33[25]
PseA protease	Q3Y6H8		
Elastase LasB	A3KXZ5, A3LEH5, A6V146, B7UZP0, Q02RJ6		35[26]
Elastase	A9QUN1		34[27]
Organic solvent tolerant elastase	A7LI11		33[24]

Effects of pH on the activity and stability of the protease

The effect of pH on the protease activity toward casein was examined at various pH values at 30 °C. The enzyme from ATCC strain was the most active in the pH range 7–8, as shown in Fig. 2. This pH optimum is similar to that of the protease isolated from *P. aeruginosa* ME4[30] and lower than those of san-ai protease and aeruginolysin protease (pH 9).[18,19]

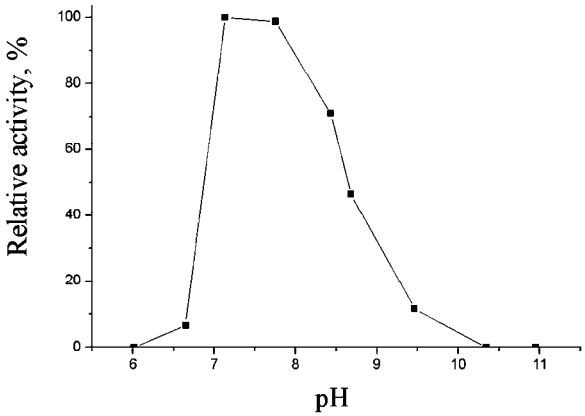

Fig. 2. Effect of pH on protease activity. The pH optimum was determined using casein as the substrate for protease in 50 mmol L^{-1} buffers of various pH values.

The stability of the ATCC enzyme was examined under various pH conditions. The enzyme was stable between pH 6.5 and pH 10, when the incubation was performed at 30 °C for 3 h, as shown in Fig. 3. This is similar to the pH stability of the protease from *P. aeruginosa* ME4.[30] On the other hand, the protease from the *P. aeruginosa* san ai strain and the alkaline protease aeruginolysin have different pH stability ranges (pH 5.5–11.5 and pH 5–9, respectively).[18,19]

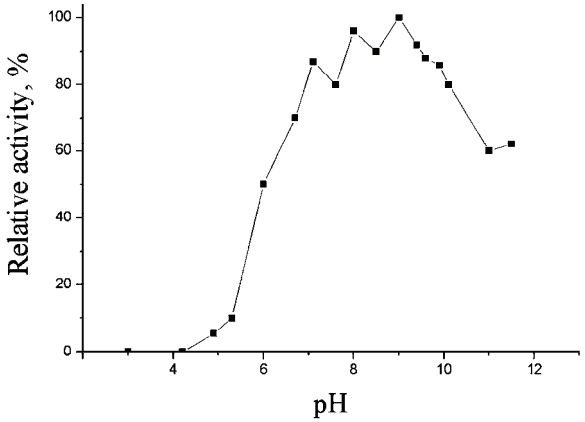

Fig. 3. The pH stability of the protease. Reaction mixtures (enzyme in buffer solutions) were incubated at 30 °C for 3 h. The remaining activity was measured under standard enzyme test conditions.

Effects of temperature on the activity and stability of the protease

The effect of temperature on the protease activity towards casein was examined at various temperatures for 10 min at pH 7.6 (50 mmol L^{-1} Sorensen's phosphate buffer). The optimum temperature of the ATCC protease was found to be around 60 °C, as shown in Fig. 4. This temperature optimum is the same as those reported for the proteases from the san ai strain and aeruginolysin,[18,19] and higher than that of protease from *P. aeruginosa* ME4, which is 50 °C.[30]

The thermostability of the enzyme was examined by measuring the residual activity after incubation at various temperatures for different periods of time. As shown in Fig. 5, the residual activity of the ATCC enzyme at pH 7.6 (50 mmol L^{-1} Tris–HCl buffer) was more than 50 % after incubation for 30 min at 60 °C, and about 43 % after incubation for 15 min at 70 °C. Thus, the ATCC protease is more stable than aeruginolysin (10 min at 60 °C), but less stable than the san-ai protease (90 min at 60 °C).[18,19]

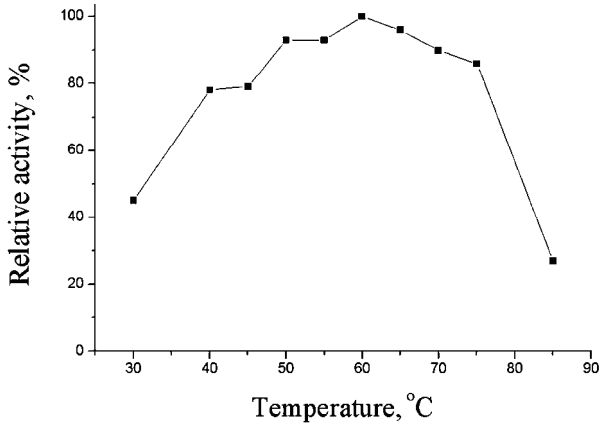

Fig. 4. Effect of temperature on protease activity. Using the standard reaction mixture, the proteolytic activity was monitored in 50 mmol L^{-1} Sorensen's phosphate buffer (pH 7.6) at different temperatures (from 25–90 °C) for 10 min.

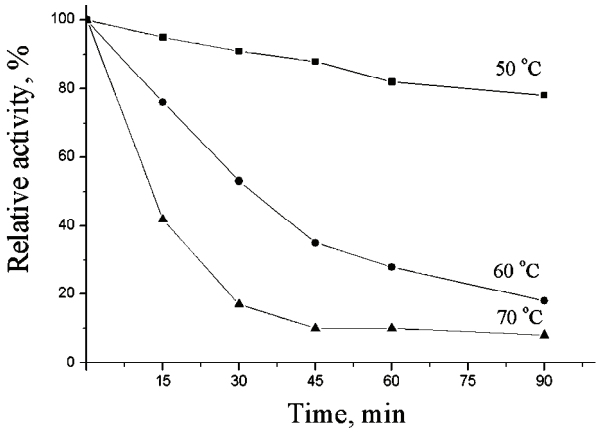

Fig. 5. Thermal stability of the enzyme. The enzyme solutions in 50 mmol L^{-1} Tris–HCl buffer (pH 7.6) were incubated at different temperatures for various periods and then quickly cooled. Standard enzyme assays were then used to determine the enzyme activity.

Effect of inhibitors

The protease activity was inhibited by EDTA and 1,10-phenanthroline, 96 % and 100 % respectively. The inhibition observed with the metal chelators EDTA and 1,10-phenanthroline suggests that the protease is a metalloenzyme. Additionally, the protease activity was significantly inhibited by DTT (54 %). This signifies that the enzyme contains a disulfide bond as part of its monomeric structure and that the activity of the enzyme is disulfide bond-dependent. This

effect is consistent with its aforementioned thermal stability, which was shown to be primarily the result of disulfide bond formation.[18,31] The serine protease inhibitor PMSF had no significant effect on the enzyme activity (inhibition of 5 %). *p*CMB had no effect on the protease activity, which suggests that the enzyme activity does not depend on sulfhydryl groups. Inhibition by metal chelators such as EDTA and 1,10-phenanthroline is a common property of almost all *P. aeruginosa* endopeptidases,[18,23,25,30] suggesting that the protease from *P. aeruginosa* ATCC 27853 is similar to other *P. aeruginosa* proteases, with the exception of serine protease Ps-1, which is not a metalloendopeptidase.[32] However, it should be noted that, with the exception of LasA,[33] *P. aeruginosa* metalloendopeptidases are not inhibited by reducing agents such as DTT or mercaptoethanol, suggesting that the activity of the enzyme is not disulfide bonds dependent. A similar inhibition pattern was reported for san ai and ME4 proteases.[18,30]

Enzyme reactivation

To determine the metal ion in *P. aeruginosa* ATCC 27853 metalloenzyme, the enzyme was treated with 1,10-phenanthroline and the obtained apoenzyme was reactivated by addition of different metal ions: Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+} and Mg^{2+}. Only Zn^{2+} efficiently restored the activity of the apoenzyme to 73 % of its original level, indicating that Zn^{2+} is essential for the protease. Reactivation of protease with the other ions was less than 50 % (Mn^{2+} restored the activity to 45 %; the other ions restored less than 10 % of the activity). This result was verified by atomic absorption spectrometry, which demonstrated one mol of Zn^{2+} per mol of enzyme. It was reported previously that Zn^{2+} is also present in other *P. aeruginosa* proteases, including aeruginolysin,[19] elastase,[34] LasA,[33] san ai[18] and ME4 protease.[30]

Substrate specificity

The protease acts on the protein substrate casein but not on elastin–orcein or on heat-killed *Staphylococcus aureus*. Thus, the enzyme is not an elastase and has no staphylolytic activity. No activity was found against chromogenic oligopeptide-*p*-NA substrates, *i.e.*, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-NA, *N*-succinyl-Ala-Ala-Pro-Leu-
-*p*-NA and *N*-benzoyl-Arg-*p*-NA, the dipeptides hippuryl-L-Lys and hippuryl-L-
-Phe, and the pentapeptide $(Gly\text{-}Pro)_5$. Activity against synthetic substrates, such as ethyl esters: *N*-benzoyl-L-Arg-ethyl ester (BAEE) and *N*-benzoyl-L-Tyr-ethyl ester (BTEE), was very low but detectable, including activity against *N*-acetyl-L-
-Tyr-ethyl ester (ATEE). The enzyme was not active against Leu-*p*-NA.

Although the rules governing the substrate specificity of the protease from *P. aeruginosa* ATCC 27853 remain unclear, it should be emphasized that aeruginolysin[35] isolated from various strains of *P. aeruginosa* (IFO 3080, IFO 3455,

and T 30; stock cultures at the Institute of Fermentation of Osaka) and serralysin proteases have quite similar substrate specificity with a preference for small- to medium-sized substrates with hydrophobic residues at their P1' positions.[18,35]

Organic solvent stability

The effects of various organic solvents (such as methanol, ethanol, acetone, 1-butanol, 2-propanol, chloroform, *n*-hexane and *N*,*N*-dimethylformamide (DMF)) on the crude extracellular enzyme were investigated. The stability of the protease in organic solvents is shown in Fig. 6. The enzyme was stable in selected organic solvents, concentration 30 %, for 24 h, with the exception of 2-propanol, chloroform and ethanol. The protease activity remained unaltered in *n*-hexane, acetone, 1-butanol and methanol, even after a 240-h long exposure to these organic solvents. The stability of the protease in organic solvents may allow its employment in organic solvents for the synthesis of peptides.

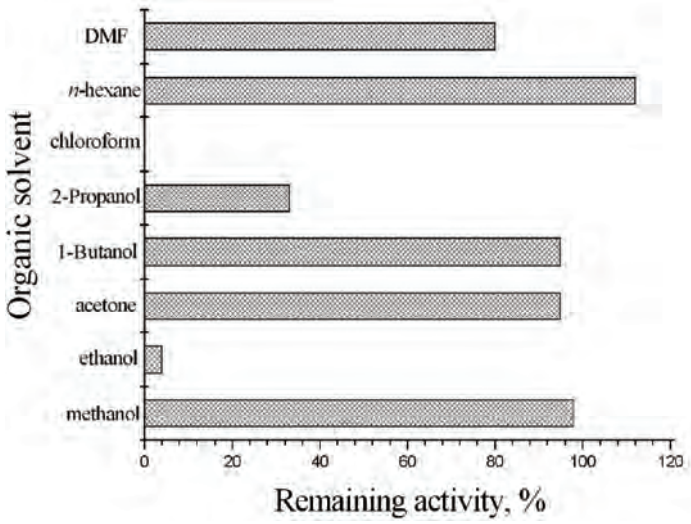

Fig. 6. Organic solvent stability of the enzyme. The effects of various organic solvents (30 % (v/v) of organic solvent) on the crude protease were investigated. A crude preparation without organic solvent was assayed under the same experimental conditions and was used as a control with 100 % activity.

CONCLUSIONS

Extracellular protease from the referent *P. aeruginosa* ATCC 27853 strain was purified, characterized, and its stability in water and different organic solvents determined. The protease molecular mass, pH optimum and substrate specificity indicate that a new protease has been identified. Enzymatic characterization of the protease yielded important information about its optimal catalytic conditions and organic solvent stability.

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ИЗВОД

ИЗОЛОВАЊЕ И ДЕЛИМИЧНО КАРАКТЕРИСАЊЕ ПРОТЕАЗЕ ИЗ *Pseudomonas aeruginosa* ATCC 27853

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У овом раду је окарактерисана екстрацелуларна протеаза медицински значајног, референтног соја *Pseudomonas aeruginosa* ATCC 27853. Молекулска маса пречишћеног ензима одређена SDS PAGE и гел филтрацијом износи око 15 kDa. Одређени су следећи ензимски параметри: pH оптимум 7,1; pH стабилност у опсегу 6,5–10; температурни оптимум 60 °C, а ензим је стабилан на 60 °C 30 min. На основу инхибиције ензима помоћу EDTA и 1,10-фенантролина, утврђено је да протеаза представља металоензим. Показано да протеаза садржи 1 мол јона цинка по молу ензима. Протеаза је стабилна у присуству различитих органских растварача, што омогућава употребу за синтезу пептида.

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