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Biochemical Properties and Potential Applications of a Solvent-Stable Protease from the High-Yield Protease Producer *Pseudomonas aeruginosa* PT121

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Abstract An organic solvent-stable protease from *Pseudomonas aeruginosa* PT121 was purified in a single step with 55% recovery by hydrophobic interaction chromatography on a Phenyl Sepharose High Performance matrix. The purified protease was homogenous on SDS-PAGE and had an estimated molecular mass of 33 kDa. The optimal pH and temperature conditions for enzyme activity were 8.0 and 60°C, respectively. The enzyme was classified as a metalloprotease based on its strong inhibition by EDTA and 1,10-phenanthroline and exhibited good stability across a broad pH range (6.0–11.0). The protease was quite stable in the presence of various water-miscible organic solvents. This is a unique property of the protease which makes it an ideal choice for application in aqueous-organic phase organic synthesis including peptides synthesis. The synthetic activity of the protease was tested using *N*-carbobenzoxy-L-asparagine (Z-Asp) and L-phenylalaninamide (Phe-NH_2) as substrate in the presence of various water-miscible organic solvents for aspartame precursor synthesis. The highest yield was obtained in the presence of 50% DMSO (91%). The synthesis rate in the presence of DMSO was also much higher than the rates in the other tested organic solvents, and the initial rates of Z-Asp-Phe-NH_2 synthesis in mixtures of various water-miscible organic solvents, with the exception of ethanol, correlated with the yields of Z-Asp-Phe-NH_2. Furthermore, the PT121 protease was able to use various carboxyl components (Z-AA) and Phe-NH_2 as substrates to catalyze the syntheses of the dipeptides, indicating that this protease has a broad specificity for carboxylic acid residue.

Keywords Purification · Characterization · Organic solvent-stable protease · Enzymatic synthesis

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Introduction

Proteases are among the most important hydrolytic enzymes and account for approximately 40% of the total enzyme sales in various industrial market sectors such as detergent, food, pharmaceutical, leather, diagnostics, waste management, and silver recovery [1]. In recent years, proteases have attracted a great deal of attention from organic chemists [2]. Under normal aqueous conditions, proteases catalyze the hydrolysis of peptide bonds, but the direction of the reaction can be reversed in the presence of organic solvents. This extends the potential applications of these enzymes [2, 3]. Unfortunately, enzymes are generally very labile catalysts and easily lose their activity in the presence of organic solvents. Although some physical and chemical methods have been employed to improve the activity and stability of proteases in the presence of organic solvents, there is an increasing need for natural organic solvent-stable proteases that can be used in organic syntheses [4, 5].

Organic solvent-stable enzymes are naturally stable and exhibit high activity in the presence of organic solvents. They are usually secreted by a relatively novel group of extremophilic microorganisms [5, 6]. Recently, some solvent-stable protease producers have been successfully isolated from nature, and most of these belong to the *Bacillus* genus [7–10] and *Pseudomonas* genus [11–13]. Among the reported proteases from solvent-tolerant microbes, the levels of production are less than 2,000 U/ml. From the viewpoint of industrial applications, the production levels of the organic solvent-stable proteases reported so far are inadequate. Moreover, there are only a few reports on the application of these proteases in organic synthesis. The first reported solvent-stable protease from the PST-01 strain was used to catalyze the synthesis of various dipeptides in the presence of organic solvents [14]. A mutant solvent-stable protease from *Bacillus licheniformis* RSP-09-37 was used to synthesize a kyotorphin precursor and to catalyze the transesterification reaction in the presence of organic solvents [15, 16].

Previously, an organic solvent-stable producer *Pseudomonas aeruginosa* PT121 was isolated from oil-contaminated soil samples, and its protease showed an extraordinarily high production yield. The crude protease was quite stable in the presence of 50% solutions of various organic solvents [17]. In this study, we report the purification and characterization of a protease from *P. aeruginosa* PT121. We studied the stability of the protease and its ability to catalyze the synthesis of the dipeptides in the presence of various organic solvents.

Materials and Methods

Materials

Dimethyl sulfoxide (DMSO), ethylene glycol, dimethylformamide (DMF), 1,4-butanediol, 1,2-propanediol, methanol, ethanol, and isopropanol (all HPLC grade) were purchased from Sinopharm (Shanghai, China). Casein was purchased from Sigma (St. Louis, MO, USA), and tryptone was obtained from Oxoid (Basingstoke, UK). *N*-Carbobenzoxy-L-aspartate (Z-Asp), *N*-carbobenzoxy-L-leucine (Z-Leu), *N*-carbobenzoxy-L-methionine (Z-Met), *N*-carbobenzoxy-L-alanine (Z-Ala), *N*-carbobenzoxy-L-valine (Z-Val), *N*-carbobenzoxy-glycine (Z-Gly), and L-phenylaninamide (Phe-NH_2) were purchased from GL Biochem (Shanghai, China). All other chemicals were of analytical grade and were purchased from Sunshine (Nanjing, China).

The *P. aeruginosa* PT121 strain was used in this study. It was stored in our laboratory, and a sample was also deposited in the China Center for Type Culture Collection (Wuhan, China) with the accession number CCTCCM208029.

Culture Conditions for Protease Production

Inocula were prepared by transferring loopfuls of the fresh cultured strain in Luria–Bertani (LB) liquid medium followed by incubation at 37°C on a shaker at 180 rpm. The cultures were grown overnight and used to inoculate 50 ml of the optimized protease production medium in 250-ml Erlenmeyer flasks. The composition of the medium was as follows (in g/l): tryptone, 10.0; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.5; $MgSO_4$, 0.3; $CaCl_2$, 1.0; NaCl, 1.0; and glycerol, 6.3. The pH was adjusted to 7.0 with 1 M NaOH. The flasks were incubated at 37°C on a shaker at 180 rpm. After 72 h of growth, the cells or cell debris were removed by centrifugation at 10,000×*g* and 4°C for 10 min. The supernatants were harvested and used as the crude protease solution.

Protease Activity Assay

The protease activity was assayed as described by Shimogaki et al. [18] with slight modifications. Two milliliters of 50 mM Tris–HCl buffer (pH 8.0) containing 2% (*w*/*w*) casein and 2 ml diluted protease solution were mixed (both preincubated at 40°C for 10 min). The reaction mixture was then incubated at 40°C for 10 min, and the reaction was terminated by adding 4.0 ml TCA mixture (containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). The mixture was further incubated at 4°C for 20 min, followed by centrifugation at 15,000×*g* for 15 min. The absorbance of the supernatant was measured at 280 nm. One unit (U) of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the conditions described above. The amount of tyrosine was determined from the tyrosine standard curve. The protease activity was calculated by the following formula: $PA=(144A_{280}-0.493)\times D/T$, where PA is protease activity (U), D is dilution times, and T is reaction time (min).

Protein Estimation

The protein concentration was determined by the method of Bradford [19] using the Bradford Protein Assay Kit (Tiangen, Nanjing, China) with bovine albumin as the standard.

Protease Purification

Hydrophobic interaction chromatography was performed on a Phenyl Sepharose High Performance column (1×5 ml; Amersham Biosciences, Sweden) that was preequilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 1.2 M NaCl. The crude protease solution (containing 1.2 M NaCl, pH adjusted to 8.0) was applied to the column at a flow rate of 1.0 ml/min using the ÄKTA prime plus system (Amersham Biosciences, Sweden), and the effluent was monitored at 280 nm. The column was washed with equilibration buffer until the absorbance of the effluent reached the baseline level. The bound protein was then eluted with 50 mM Tris–HCl (pH 8.0) buffer at a flow rate of 1.0 ml/min. The fractions showing absorbance at 280 nm were collected and analyzed for protease activity. The active fractions were pooled and used as the purified enzyme for further analysis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

SDS-PAGE was carried out according to the method of Laemmli [20] using a 15% cross-linked polyacrylamide gel and an Amersham gel electrophoresis unit. The protein bands were stained with Coomassie brilliant blue R-250 (Sigma). The molecular weight of the protease was estimated by comparing the relative mobility of proteins of different molecular sizes using standard molecular weight markers in the range 116.0–14.4 kDa (Fermentas, Germany)

Determination of the Optimum pH for Enzyme Activity and Stability

The optimum pH for the activity of the purified protease was determined in various buffer systems in the pH range 6.0–11.0 using casein as the substrate. The pH stability of the enzyme was studied by preincubating the enzyme in buffers of different pH values (pH 4.0–12.0) at 30°C for 1 h. In all cases, the residual activity was determined under standard assay conditions.

Determination of the Optimum Temperature for Enzyme Activity and Stability

The effect of temperature on the purified enzyme was studied by assaying the enzyme at different temperatures using casein as the substrate. The thermal stability was assessed by incubating the purified protease at various temperatures. After incubation for 1 h, the samples were removed and rapidly cooled in an ice bath. The residual activity levels were determined at the assay temperature, as described above.

Effect of Metal Ions, Inhibitors, and Surfactants on Protease Activity

The effect of divalent cations, including Co^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Ba^{2+}, and Ni^{2+} (in the form of their chlorides), was tested by preincubating the protease in 5 mM solutions of these ions at 40°C. After incubation for 30 min, the residual activity of the protease was determined, as described above. The effect of inhibitors and surfactants was studied by incubating the protease solution with different concentrations of these at 40°C for 10 min. The residual activity was assayed using the standard method.

Measurement of K_m and V_{max}

The K_m and V_{max} values of the purified protease were determined under the optimal reaction conditions (60°C, pH 8.0) by measuring the proteolytic activity using various concentrations of the casein substrate with 5 min incubation. All experiments were performed in triplicate. A double-reciprocal plot was constructed, and Sigma Plot software was used to calculate the kinetic parameters.

Effect of Organic Solvents on Protease Stability

Two milliliters of the purified PT121 protease solution (0.25 mg/ml) was added to 2 ml of various organic solvents in a universal bottle with a screw cap and incubated at 30°C with constant shaking at 140 rpm. The residual activity was investigated after 0, 1, 3, 5, 7, 10, and 15 days. Samples were carefully removed from the aqueous phase in the case of water-immiscible solvents.

Reaction Conditions for Z-Asp-Phe-NH_2 Synthesis

The purified PT121 protease was precipitated with 70% acetonitrile at 4°C for 30 min. The precipitate was redissolved in Tris–HCl buffer (100 mM, pH 8.0) to obtain the desired concentration (approximately 10,000 U/ml, 0.5 mg/ml). The acyl donor Z-Asp (100 mM) and nucleophile L-Phe-NH_2 (200 mM) were dissolved in the Tris–HCl buffer in the presence of 50% solutions of various organic solvents. Small amounts of the sample solution were removed from the reaction mixture after 24 h of incubation in order to measure the product yields. The samples were analyzed by high performance liquid chromatography (Dionex P680 HPLC) using a Kromasil 100-5C18 column (4.6 mm× 250 mm, Kromasil, Sweden) and UVD170U detector at 254 nm. Aliquots of the sample solutions were diluted 1/10 with the eluent (acetonitrile/H_2O (40/60, *v*/*v*) containing 0.05% trifluoroacetic acid).The flow rate of the carrier was 1.0 ml/min and the oven temperature was 37°C. The retention times of Z-Asp and Z-Asp-Phe-NH_2 were 4.6 and 6.4 min, respectively. The product yield and synthesis rates were calculated based on the amount of Z-Asp (carboxyl component) and Z-Asp-Phe-NH_2 (product), respectively. Reactions in which the protease was not included served as the controls.

Rates of Z-Asp-Phe-NH_2 Synthesis in the Presence of Various Organic Solvents

The rates of Z-Asp-Phe-NH_2 synthesis were measured before the product precipitated, under initial reaction conditions, i.e., when less than 10% of the substrate had disappeared. The reactions were performed under the conditions described above. Small amounts of the sample were removed from the reaction mixtures at reasonable intervals in order to measure the reaction rates. All experiments were performed in triplicate.

Synthesis of Z-AA-Phe-NH_2 in the Presence of Organic Solvents

Various carboxyl components of the substrates (Z-Asp, Z-Leu, Z-Met, Z-Ala, Z-Val, and Z-Gly) were used for synthesis the dipeptides with the amine component of Phe-NH_2. The reactions were performed with 100 mM Z-AA, and 200 mM Phe-NH_2 in the presence of 50% solutions of various water-miscible organic solvents at 30°C for 24 h incubation. Small amounts of the sample solution were removed from the reaction mixture in order to measure the product yields. The product yield was calculated based on the amount of Z-AA (carboxyl component) and Z-AA-Phe-NH_2 (product).

Results and Discussion

Purification of the Organic Solvent-Stable Protease

The extracellular protease produced by *P. aeruginosa* PT121 was purified by hydrophobic interaction chromatography on a Phenyl Sepharose High Performance column. Figure 1 shows the elution of the desired protein. The bound protein was eluted with 50 mM Tris–HCl (pH 8.0) buffer without NaCl at a flow rate of 1.0 ml/min, and line 1 showed the natural negative gradient of the NaCl concentration in the process of washing the column. Two protein peaks eluted from the Phenyl Sepharose High Performance column, but only the eluate fractions with NaCl concentration between 0.6 and 0.15 M contained the protease activity (the first peak). This was a symmetrical peak that corresponded to the desired

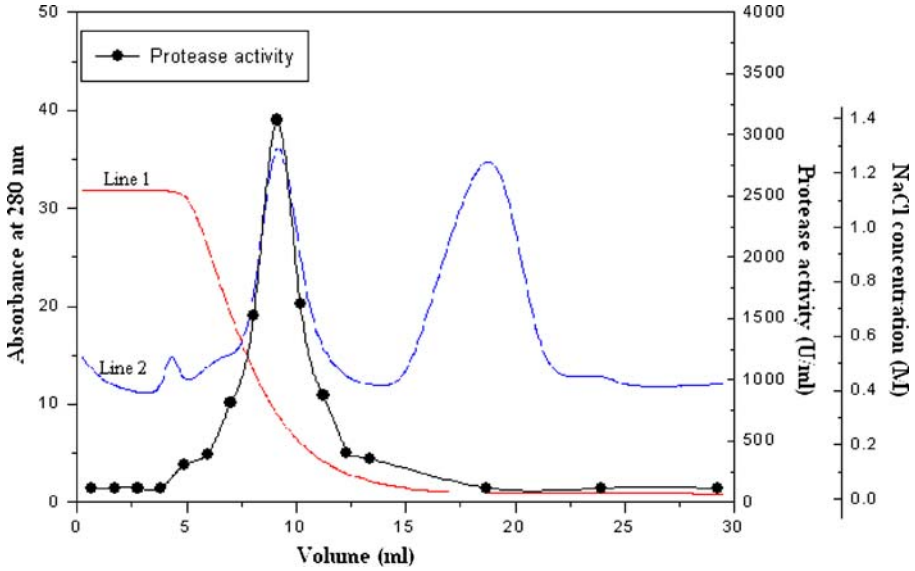

Fig. 1 Purification of the PT121 protease on the Phenyl Sepharose High Performance matrix. The details of the experiment are described in the text. The symbol (●) represents protease activity, and *Line 1* represents the concentration of NaCl in the process of washing the column with 50 mM Tris–HCl buffer (pH 8.0). The protein content of the eluate was monitored online by measuring the absorbance at 280 nm (*Line 2*)

product. Table 1 shows that 55% of the total activity could be recovered with 2.2-fold purification. Figure 2 shows the results from SDS-PAGE analysis of the crude protease solution and purified enzyme preparation. The purified enzyme was homogeneous with a molecular mass of 33 kDa. A similar one-step purification method was also reported for the alkaline protease from haloalkaliphilic *Bacillus* sp. [21], which was purified by hydrophobic interaction chromatography on a Phenyl Sepharose 6 Fast Flow column. Although the purification of some solvent-stable proteases has been reported, the recovery of most of these has been low. This can be attributed to the more complex purification procedures applied in these cases, which result in low production yields. The first reported solvent-stable protease PST-01 was purified by ammonium sulfate precipitation and two successive hydrophobic interaction chromatography steps. The purified protein was obtained in 25.6% yield and with 102-fold purification [12]. Five steps, including ion exchange (DEAE-Sepharose CL-6B and CM-Sepharose) and gel filtration chromatography (Sephacryl S-100 matrix), were used to isolate the solvent-stable protease from *Bacillus subtilis* TKU007 with 58-fold purification and 23% yield [22]. *P. aeruginosa* PT121 was reported to be a high-yield protease producer. SDS-PAGE analysis of the crude protease solution and purified enzyme preparation confirmed that the high amount of the solvent-stable protease in the crude protease solution facilitated the use of a simple purification procedure.

Table 1 Purification of a protease from *Pseudomonas aeruginosa* PT121.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude	19,572	2.0	9,786	100	1
Phenyl Sepharose chromatography	10,765	0.5	21,530	55	2.2

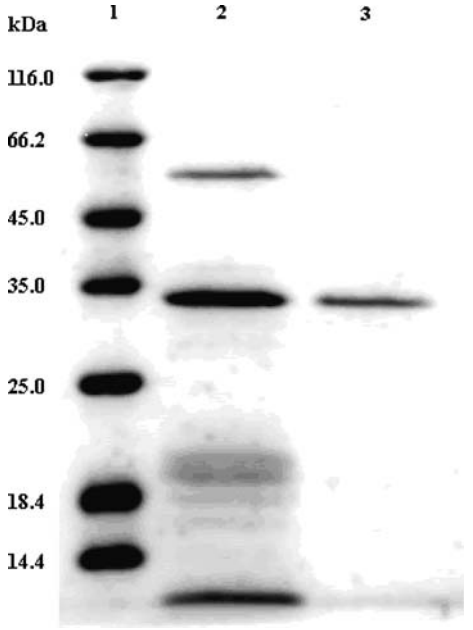

Fig. 2 SDS-PAGE analysis of the purified protease from *P. aeruginosa* PT121. *Lane 1*, molecular weight marker; *lane 2*, crude protease solution (5 μg protein); *lane 3*, purified protease from *P. aeruginosa* PT121 (1 μg protein)

Optimum pH for Enzyme Activity and Stability

The purified protease was found to be highly active (in terms of casein hydrolysis) in the pH range 7.0–9.0 at 40°C. The maximum activity was recorded at pH 8.0. This was consistent with the behavior of other proteases from *P. aeruginosa* [12, 23]. However, only 40% of the optimum activity was retained at pH 6.0, and 13% was retained at pH 11.0 (Fig. 3), but no activity of the protease was detected at pH 4.0. Regarding the pH stability

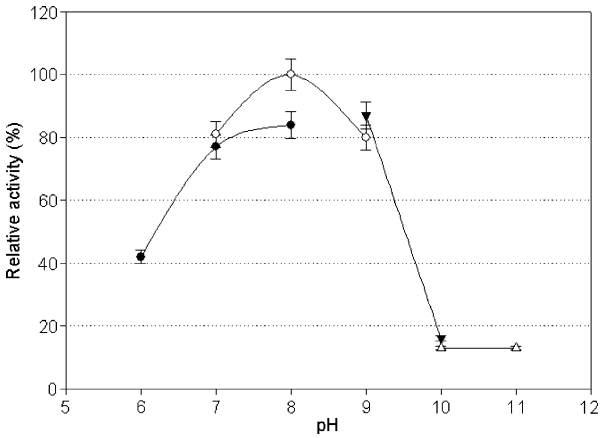

Fig. 3 Effect of pH on protease activity. The activity of the PT121 protease was measured at different pH values at 40°C using casein as the substrate. The buffers used were 50 mM NaH_2PO_4–NaOH (●) (pH 6.0–8.0); 50 mM Tris–HCl (○) (pH 7.0–9.0); 50 mM Gly–NaOH (△) (pH 9.0–10.0); and Na_2HPO_4–NaOH (▲) (pH 10.0–11.0). Each experiment was done in triplicate and the difference between three sets of experiments was less than 5%

of the PT121 protease, the enzyme was found to be very stable over a broad pH range (6.0–11.0), and all activity was conserved at these pH values.

Optimum Temperature for Enzyme Activity and Stability

The maximum activity of protease PT121 was observed at 60°C, though considerable activity was also observed in the temperature range 40–70°C (Fig. 4). The purified protease showed good stability and retained more than 90% of its initial activity after incubation for 1 h from 30 to 50°C (Fig. 5). However, the activity decreased to 75% after incubation for 1 h at 60°C. The protease activity rapidly decreased after incubation at temperatures higher than 60°C (only 3% activity remained after incubation at 70°C). The temperature optimum and thermostability of the protease were similar to those of many other reported proteases from *P. aeruginosa* [24, 25]. The protease was further characterized for its K_m and V_{max} towards casein as a substrate. The K_m was 3.97 mg/ml, while the V_{max} was 7.58 μmol/min. Lower K_m values on casein were found in those of *P. aeruginosa* PseA protease (2.69 mg/ml) [23], *P. fluorescens* 22F protease (2.44 mg/ml) [26], *Bacillus* sp. TKU004 protease (2.98 mg/ml) [27], and *B. subtilis* TKU007 protease (0.13 mg/ml) [22]. As regard to V_{max} of PT121 protease (7.58 μmol/min), which is far more than 0.14 of *Bacillus* sp. TKU004 [27], 0.86 of *Bacillus subtilis* TKU007 [22], 1.26 of *Bacillus cereus* [28], and 3.03 of *P. aeruginosa* PseA [23].

Effect of Metal Ions on Protease Activity

The effect of various metal ions on the hydrolytic activity of the protease was tested by adding 5 mM solutions of the divalent ions to the purified PT121 protease solution. The protease was markedly activated by Co^{2+} with a 124% increase in the specific activity in comparison to that of the control. A slight inactivation (5%) was observed in the presence of 5 mM Zn^{2+}, while Ca^{2+} and Mn^{2+} at the same concentration increased the activity by 6% and 5%, respectively. In contrast, Mg^{2+}, Ba^{2+}, and Ni^{2+} had moderately inhibitory effects, leading to decreases in the activity by 25%, 28%, and 48%, respectively.

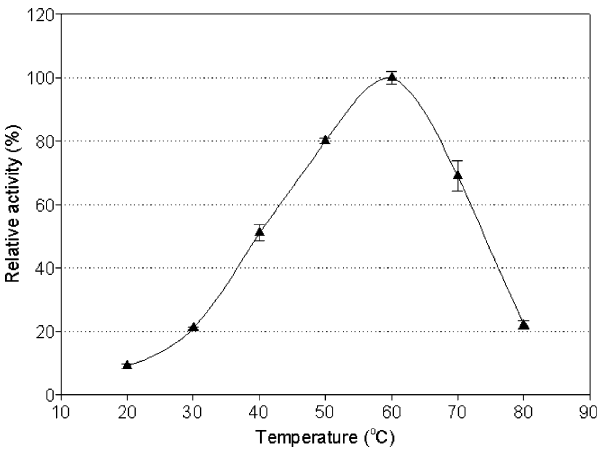

Fig. 4 Optimum temperature for protease activity. The effect of temperature on protease activity was studied by performing the enzymatic reaction at different temperatures in the range 20–80°C at pH 8.0 using 50 mM Tris–HCl buffer. The activity measured at 60°C was regarded as the 100% value. Each experiment was performed in triplicate, and the difference between the three sets of experiments was less than 7%

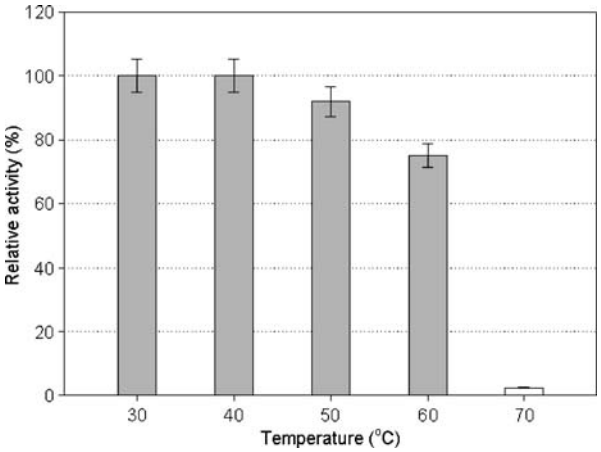

Fig. 5 Thermal stability of the protease. The purified solution was incubated at 30, 40, 50, 60, and 70°C for 1 h. The residual activity of the incubated protease was assayed under standard assay conditions. The activity of the protease that had not been incubated was regarded as the 100% value. Each experiment was done in triplicate and the difference between three sets of experiments was less than 5%

Influence of Different Inhibitors and Surfactants on Protease Activity

The effect of various inhibitors and surfactants on the protease is summarized in Table 2. The purified protease was strongly inhibited by 5 mM solutions of EDTA and 1,10-pheanthroline (95% and 78% inhibition, respectively), indicating that this protease is a metalloprotease. Serine protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) did not inhibit this protease, while sulfhydryl reagents such as DDT, glutathione, and β-mercaptoethanol inhibited its activity (31%, 43%, and 67% inhibition, respectively).

Table 2 Effect of inhibitors and surfactants on protease activity.

Reagent	Concentration (mM)	Relative activity
None	–	100
EDTA	5 mM	5
1,10-Pheanthroline	5 mM	22
Phenylmethylsulfonyl fluoride (PMSF)	5 mM	100
Urea	5 mM	96
1,4-Dithio-DL-threitol (DTT)	5 mM	69
Glutathione	5 mM	57
β-Mercaptoethanol	5 mM	33
SDS	5%	0
Cetyltrimethylammonium bromide (CTAB)	5%	69
Triton X-100	5%	113
Tween 80	5%	125

The purified protease was preincubated with various reagents at 40°C for 10 min, and the residual protease activity was determined. The activity of the protease in the absence of reagent was regarded as the 100% value. Each experiment was performed in triplicate, and the difference between the three sets of experiments was less than 5%

Enzymes are usually inactivated by the addition of surfactants to the reaction solution. The activity of the protease increased by 13% and 25% in the presence of 5% (*v*/*v*) Triton X-100 and Tween 80 (nonionic surfactant), respectively; however, the protease activity reduced by 31% and 100% in the presence of 5% cetyltrimethylammonium bromide (CTAB) and 5% SDS, respectively. Similar behavior has been reported for the surfactant-stable protease from *Bacillus cereus* in the presence of 0.5% Triton X-100; this enzyme showed a 2-fold increase in proteolytic activity [8]. Another surfactant-stable protease has been reported to be very stable in the presence of 2% solutions of nonionic surfactants such as Tween 20, Tween 40, and Triton X-100, and its activity decreased to 0% in 1 mM SDS [27]. The protease for application in detergent industry usually with some special properties, including stable in a wide of pH and various surfactants, relatively low substrate specificity, high-yield production, and simple separation [29]. One of the important detergent enzyme subtilisin showed maximum activity at pH values of 8.0–10.0 and at a temperature of 60°C is similar with the PT121 protease [8]. Considering the stability of the PT121 protease in the presence of surfactants and with high-yield production, this protease may find potential application in laundry detergents.

Half-Life of the Protease in the Presence of Organic Solvents

The effect of various organic solvents on the stability of the purified PT121 protease was investigated. Table 3 shows the half-life of the PT121 protease in the presence and absence of various organic solvents. The half-life of the PT121 protease in the presence of 50%(v/v) water-insoluble solvents such as chloroform, cyclohexane, hexane, octane, decane, and

Table 3 Effect of organic solvents on protease stability.

Organic solvent	Half-life (days)
DMSO	>100
Glycerol	>100
1,4-Butanediol	>100
1,2-Propanediol	>100
Ethylene glycol	>100
Butanol	12.0
Isoamyl alcohol	11.5
Hexanol	15.9
DMF	8.9
Methanol	7.1
Ethanol	5.5
Isopropanol	5.0
Acetone	3.5
Chloroform	2.6
Cyclohexane	2.9
Hexane	3.8
Octane	4.5
Decane	5.7
Tetradecane	5.2
None	4.7

The purified PT121 protease (2 ml) was incubated at pH 8.0 and 30°C with constant shaking at 140 rpm in the presence of 2 ml organic solvent for 15 days. The residual activity was measured after 0, 1, 3, 5, 7, 10, and 15 days. The half-life was calculated from the exponential regression curve

tetradecane was 2.6, 2.9, 3.8, 4.5, 5.7, and 5.2 days, respectively. The half-life of the PT121 protease in the absence of any organic solvent was 4.7 days. The protease was quite stable in the presence of 50%(v/v) glycerol, 1,4-butanediol, propylene glycol, ethylene glycol, DMSO, butanol, isoamyl alcohol, and hexanol. The half-life in these solvents was much longer than that in the absence of organic solvent. In general, the half-life of the protease in the presence of water-miscible organic solvents was higher than that in the absence of organic solvent, with the exception of acetone. Generally, most enzymes are unstable and are easily denatured in such hostile environments because organic solvents (particularly hydrophilic solvents) have a tendency to strip essential water from enzyme molecules. Water is very important for enzymes since multiple hydrogen bonds are formed between water and protein molecules, and these promote the conformational mobility of protein molecules [30]. The PT121 protease was very stable in a range of organic solvents. Thus, this protease is a naturally solvent-stable protease.

A curious phenomenon was also observed in the presence of various alcohols such as glycerol, 1, 4-butanediol, propylene glycol, ethylene glycol, butanol, isoamyl alcohol, and hexanol. The protease was found to be more stable in these solvents than in the absence of organic solvent. Similar results were reported by Ogino [31] and Karbalaei-Heidari [32]. We speculate that the enhanced stability of these proteases in the presence of these alcohols was due to the decrease in the water content (some deleterious reactions such as deamidation of Asn/Gln residues and hydrolysis of peptide bonds are caused by water). At the same time, the type of the organic solvents such as glycerol and ethylene glycol also facilitate the formation of multiple hydrogen bonds that enhance the conformational mobility of protein molecules [33]. Recently, the solvent stability of some solvent-stable proteases was investigated, but the relationship between enzyme stability and the nature of the organic solvent (especially in terms of solvent polarity) could not be established. The PST-01 protease was very stable in various water-miscible solvents such as ethylene glycol, DMSO, triethylene glycol, and methanol [12]. Rahman et al. [11] observed that the protease from *P. aeruginosa* strain K was quite stable in the presence of 25% solutions of various hydrophobic solvents such as hexadecane, dodecane, decane, and isooctane, while the half-life of the PseA protease in the presence of 25% solutions of various organic solvents (both hydrophobic and hydrophilic solvents) was very similar to that in the absence of organic solvent [34]. The mutant protease from *Bacillus licheniformis* RSP-09-37 is capable of tolerating a high acetonitrile content, which is otherwise quite harmful to other reported solvent-stable proteases [15]. This suggests that the stability of enzymes in the presence of organic solvents depends on both the enzyme and the type of organic solvent used.

Effect of Various Organic Solvents on Z-Asp-Phe-NH_2 Synthesis

Z-Asp-Phe-NH_2 was synthesized by the protease in the presence of various water-miscible organic solvents. Table 4 shows the Z-Asp-Phe-NH_2 yields in the presence of 50% (*v*/*v*) solutions of various organic solvents. The yields in the presence of DMSO, DMF, methanol, ethylene glycol, 1,2-propanediol, 1,4-butanediol, ethanol, and isopropanol were 91%, 75%, 52%, 51%, 44%, 42%, 30%, and 22%, respectively. No byproduct was detected in these reaction mixtures. The product of these reactions separated out in the form of crystals, and the amount depended on the product yield. The crystal was easily recovered and was positively identified as the desired product by reverse-phase HPLC and mass spectroscopy (Fig. 6d).

The effect of organic solvents on the rate of dipeptide synthesis was investigated (Table 4). The synthesis rate in the presence of DMSO was much higher than the rates in

Table 4 Effect of organic solvents on the synthesis rate and yield of Cbz-Asp-Phe-NH_2 synthesized using the PT121 protease.

Organic solvent	Synthesis rate (μmol/min/mg protein)	Product yield (%)
DMSO	804±20	91
DMF	400±8	75
Methanol	228±12	52
Ethylene glycol	168±4	51
1,2-Propanediol	140±4	44
1,4-Butanediol	120±8	42
Ethanol	156±4	30
Isopropanol	92±4	22

The reactions were performed with 100 mM Z-Asp, 200 mM Phe-NH_2, and 0.25 mg/ml of the purified protease in the presence of 50% solutions of various water-miscible organic solvents

the other tested organic solvents, and the initial rates of Z-Asp-Phe-NH_2 synthesis in mixtures of various water-miscible organic solvents, with the exception of ethanol, correlated with the yields of Z-Asp-Phe-NH_2. Further, the synthesis rates in the tested alcohols (ethanol, ethylene glycol, 1,2-propanediol, and 1,4-butanediol) were very similar. Therefore, the type of organic solvent influences the catalytic specificity of the enzyme. The PST-01 protease has also been used in Z-Asp-Phe-NH_2 synthesis in the presence of 50% DMF, but the yield was less than 15% [14]. However, these reactions required a large excess of nucleophiles such as Phe-NH_2 (the nucleophile should be present in 50-fold excess of the acyl donor). In contrast, the PT121 protease showed high catalytic activity in Z-Asp-Phe-NH_2 synthesis in the presence of 50% DMSO, DMF, ethylene glycol, and Methanol (>50%), at a low nucleophile to acyl donor ratio (2/1, 200 mM and 100 mM, respectively). Since the highest catalytic activity was observed in the presence of 50% DMSO, the product was easily recovered, and several crystals were separated out during the reaction (Fig. 6). Thus, the solvent-stable protease from *P. aeruginosa* PT121 is a promising biocatalyst for application in non-aqueous media.

Proteases have been proved as useful catalysts for organic synthesis, especially in the field of peptides synthesis. Recent examples are the synthesis of various short peptides by commercial proteases like trypsin, thermolysin, subtilisin, papain, and pepsin [2]. The Bz-

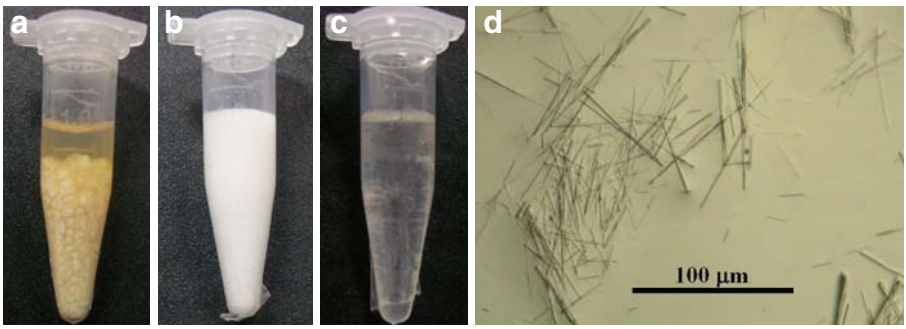

Fig. 6 Synthesis of Z-Asp-Phe-NH_2 in the presence of 50% DMSO using the crude and purified protease preparations as the catalysts. **a** Crude protease; **b** purified protease; **c** control without protease; and **d** Z-Asp-Phe-NH_2 crystal

Arg-Gly-Asp-OEt (RGD precursor) was synthesized by trypsin starting from Bz-Arg-OEt and Gly-Asp-$(OEt)_2$ in the presence of 70% ethanol [35], and thermolysin has been used in synthesis of F-Asp-Phe-OMe (aspartame precursor) in aqueous-organic biphasic system [36]. On the other hand, a number of researchers turned their attention to finding new synthetic applications, such as peptide isosteres synthesis [37] and esterification/trans-esterification [16], and selectively trigger the self-assembly of peptide hydrogels (macroscopic biomaterials) [38]. The reasons for this success arise from the commercial availability of these enzymes at mostly reasonable cheap prices. However, most natural bacteria secrete protease in low yield is a limiting factor for application. The solvent-stable protease from *P. aeruginosa* PT121 with high-yield production and simply purification, it would be more suitable for synthetic applications.

Carboxyl Component Specificity of the PT121 Protease for the Dipeptide Synthesis

We are presently using various carboxyl components (Z-AA) and Phe-NH_2 as substrates to investigate the carboxyl components substrate synthetic specificity of the PT121 protease. Table 5 shows the Z-AA-Phe-NH_2 yields in the presence of 50% (*v*/*v*) solutions of organic solvents. The result proved that the protease is able to select phenylalanine (Phe) on the amine side of the peptide bond with broad specificity for carboxylic acid residue. This property is similar with the well-known commercial protease—thermolysin, which is the preference for hydrophobic/aromatic residues on the amine side of the peptide bond and non-specific for carboxylic acid residue. Therefore, thermolysin was selected as a suitable model enzyme to selectively trigger the self-assembly of peptide hydrogels (macroscopic biomaterials) via reversed hydrolysis [38]. In addition, the primarily results also indicated that organic solvent is an important factor affected the substrate specificity of the enzyme: Z-Leu-Phe-NH_2 was synthesized in DMSO, methanol, and ethanol but not in DMF and isopropanol, for instance. Now we are preparing to deeply investigate the substrate specificity and catalytic mechanism for promoting the application of the protease more widely.

Conclusions

In this study, a solvent-stable protease from *P. aeruginosa* PT121 was purified in a single step. In this case, the purification procedure was simple and resulted in high yields of the

Table 5 The carboxyl components substrate specificity of the PT121 protease for the dipeptide synthesis in the presence of organic solvents.

Organic solvent	Product yield (%)					
	Z-Asp-Phe-NH_2	Z-Leu-Phe-NH_2	Z-Met-Phe-NH_2	Z-Ala-Phe-NH_2	Z-Gly-Phe-NH_2	Z-Val-Phe-NH_2
DMSO	91	53	90	55	22	0
DMF	75	0	68	29	21	0
Methanol	52	44	90	58	18	0
Ethanol	30	14	78	49	22	0
Isopropanol	22	0	54	32	26	0

solvent-stable protease from the crude protease solution. The high stability and activity of the PT121 protease in the presence of different organic solvents proved that this enzyme is an excellent candidate for use as a biocatalyst in organic synthesis in the presence of organic solvents.

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