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# Cold-active serine alkaline protease from the psychrophilic bacterium *Pseudomonas* strain DY-A: enzyme purification and characterization

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**Abstract** An extracellular protease was purified from a deep-sea psychrophilic bacterium strain DY-A which was identified as a *Pseudomonas* species. The optimal growth and protease-producing temperatures of the strain were all 10°C, and the protease was secreted only at temperatures under 20°C. The enzyme was most active at 40°C and at pH 10.0. It was inhibited by phenylmethyl sulfonylfluoride and diisopropyl fluorophosphate, indicating that it is a serine protease. Chelators such as EDTA, EGTA, 1,10-phenanthroline and 2,2′-bipyridyl produced a decrease of activity. The enzyme was sensitive to denaturing agents such as SDS, urea, and guanidine HCl and resistant to thiol-containing reducing agents such as dithiotreitol. The enzyme was active towards *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide. The native molecular mass of the enzyme determined by native PAGE and SDS-PAGE was 25 kDa.

**Keywords** Deep sea · Psychrophile · Serine protease

As one of the most important groups of industrial enzymes, cold-active protease from psychrophilic bacteria living in polar region (Vazquez et al. 1995), ice field (Margesin et al. 1991), and marine animal intestine (Hoshino et al. 1997) have been studied widely. It is not only because of their biotechnological potential for novel applications, including food processing, additives in detergents, or pharmacy, but also because that cold-active enzyme represents the lower natural limit of protein stability and is a useful tool for studies in the field of protein folding (Feller et al. 1996). However, protease isolated from deep-sea sediment is reported comparatively little. In this study we report the purification and characterization of an extracellular cold-active serine protease from deep-sea psychrophilic bacterium.

The strain DY-A, which showed strong caseinolytic activity, was isolated from deep-sea sediment collected from a depth of 5,225 m in the East Pacific (8°22′N, 145°23′W) with screening medium containing casein (1%) and yeast extract (0.2%). The maximum pH value and salinity which the strain could tolerate were pH 11.0 and 1.5 M NaCl respectively. The strain DY-A showed motility, and was Gram negative and oxidase positive. Because it was not able to grow at 37°C, we could not obtain all the data on substrate utilization by the microorganism identification system (MicroStation, BIOLOG, USA) which made it unidentifiable. As determined by 16S rDNA sequencing, strain DY-A was most closely related to the genus *Pseudomonas* (levels of similarity, 93–96.67%, Fig. 1), and thus we placed this strain in the genus *Pseudomonas*, as *Pseudomonas* sp. strain DY-A.

The growth temperature extension of *Pseudomonas* sp. strain DY-A was 4–30°C, and 10°C was the optimal temperature for growth in view of the density and stability of cell growth. Similar to the cell growth, extracellular caseinolytic activity was detected at 4–20°C, reaching the highest value (45 U/ml) at 10°C. However, no caseinolytic activity was detected at 25°C and 30°C while *Pseudomonas* sp. strain DY-A could still grow (Fig. 2). Whereas the protease could keep most activity at 30°C for more than 1 day (see below), these results indicated that the protease secretion of *Pseudomonas* sp. strain DY-A was activated by low temperature.

The purification of protease was achieved at 4°C as follows. The cell-free supernatant was subjected to a

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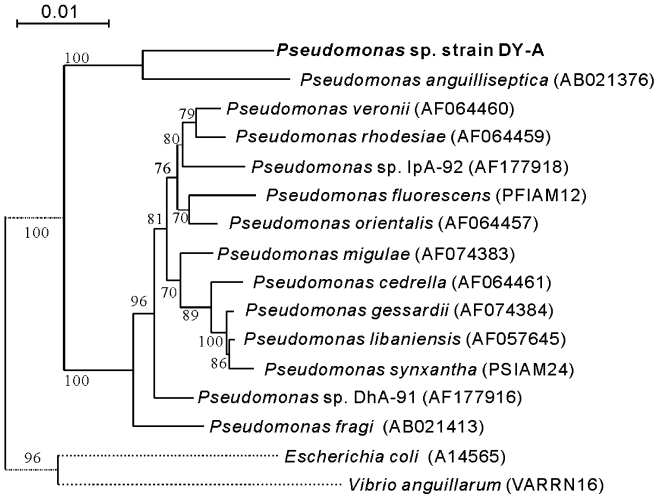


**Fig. 1** Phylogenetic relationship between the 16S rDNA sequences of *Pseudomonas sp*. strain DY-A and other *Pseudomonas* strains. *Escherichia coli* and *Vibro* were used as outgroup taxon strains. The numbers in brackets are the EMBL accession numbers of the 16S rDNA sequences of various bacteria. The tree was constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbor-joining method using the Clustal W program. The scale bar shows 0.01 substitutions per base position. Numbers refer to bootstrap values for each node out of a total of 100 replicate resamplings

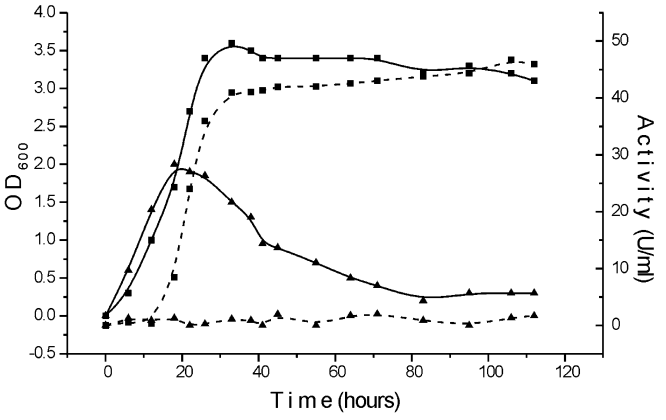


**Fig. 2** Effect of temperature on growth (*line*) and protease producing (*dash*) of *Pseudomonas* sp. strain DY-A: 10°C (■); 25°C (▲). Enzyme activity was determined by the detection of tyrosine produced from casein using Folin-Ciocalteu's Phenol reagent (Sigma). One unit of activity (U) was defined as the amount of enzyme releasing 1 $\mu$g tyrosine per 1 min at 40°C

two-step ammonium sulfate precipitation, and the protein precipitating at a salt saturation between 40% and 70% was resuspended in 10 mM Tris-HCl buffer (pH 8.0). The pre-purified protein was applied to chromatography on a DEAE Sepharose CL-6B (Pharmacia, Sweden) column, a Sephadex G-100 (Pharmacia, Sweden) column and a DEAE Sepharose CL-6B column in sequence (summarized in Table 1). The purified protein was eluted as a single peak and showed a single band equal to a molecular mass of about 25 kDa on both SDS-PAGE and native PAGE, corresponding to the active fraction detected by zymogram activity staining.

The enzyme showed a broad pH profile (pH 6.0–12.0) for casein hydrolysis and highest activity between pH 8.0 and 10.0. The highest stability of proteinase appeared at pH 10.0. Examination of the effect of temperature on the proteolytic activity of the purified protease showed that the maximal enzymatic activity was obtained at 40°C, and 60% residual activity was measured at 25°C. The protease retained 100%, 83% and 62% of initial activity after 24 h of incubation in Tris-glycine buffer (pH 10.0) at 10°, 20° and 30°C respectively. No further loss of activity was observed up to 2 weeks at 10°C. At temperatures above 30°C, the enzyme was rapidly inactivated, and no activity was detected at 60°C after 1.5 h.

The complete inhibition by 1 mM of diisopropyl fluorophosphate (DFP), phenylmethyl sulfonylfluoride (PMSF) and AEBSF (Table 2) strongly supported the hypothesis that *Pseudomonas* sp. strain DY-A protease was a serine protease. The enzyme was resistant to thiol-reducing agents such as dithiotreitol (DTT) (10 mM) and 2-mercaptoethanol (5%), suggesting that disulfide bonding was not involved in preserving proteolytic activity. On the other hand, the enzyme was sensitive to urea (4 M), SDS (1%) and guanidine-HCl (1 M), indicating that hydrogen bonds played an important role in maintaining enzyme activity. The enzyme displayed high activity towards *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitro-

**Table 1** Summary of purification of protease from *Pseudomonas* sp. strain DY-A[a]

| Preparation | Total protein (mg) | Total activity (U)[b] | Yield (%) | Specific activity (U/mg) | Purification fold |
|---|---|---|---|---|---|
| Crude enzyme | 1 331.4 | 22 500 | 100.0 | 16.9 | 1.0 |
| $(NH_4)_2SO_4$ (40%) | 416.7 | 18 399 | 81.8 | 44.2 | 2.6 |
| $(NH_4)_2SO_4$ (70%) | 91.6 | 15 163 | 67.4 | 165.5 | 9.8 |
| DEAE CL-6B[c] | 12.4 | 8 928.5 | 39.7 | 720.0 | 42.6 |
| Sephadex G-100[d] | 4.9 | 6 096.2 | 27.1 | 1 244.1 | 73.6 |
| DEAE CL-6B[e] | 3.7 | 5 265 | 23.4 | 1 423.0 | 84.2 |

[a] All steps were carried out at 4°C
[b] One unit of activity(U) was defined as the amount of the enzyme releasing 1$\mu$g of tyrosine per 1 min at 40°C
[c] Protein was eluted in a linear gradient between 0 and 0.5 M NaCl with 10 mM Tris-HCl buffer (pH8.0) containing 10 mM $CaCl_2$; desalting was carried out by dialyzing against 10 mMTris-HCl buffer (pH8.0)
[d] Protein was eluted with 10 mM Tris-HCl buffer (pH8.0)
[e] Protein was eluted in a linear gradient between 0 and 0.2 M NaCl with 10 mM Tris-HCl buffer (pH8.0) containing 10 mM $CaCl_2$

**Table 2** Effect of protease inhibitors and denaturing agents on the protease of *Pseudomonas* sp. strain DY-A

| Inhibitor and reagent | Concentration | Residual activity (%) |
|---|---|---|
| None | | 100 |
| EDTA | 1 mM | 60 |
| EGTA | 1 mM | 54 |
| 1,10-Phenanthroline | 1 mM | 67 |
| 2,2'-Bipyridyl | 1 mM | 62 |
| DFP | 1 mM | 5 |
| AEBSF | 1 mM | 7 |
| PMSF | 1 mM | 2 |
| E-64 | 10 μM | 100 |
| Pepstatin A | 1 μM | 100 |
| SDS | 1% | 46 |
| Urea | 4 M | 23 |
| HCl guanidine | 1 M | 62 |
| Dithiothreitol (DTT) | 10 mM | 89 |
| 2-Mercaptoethanol | 5% | 78 |

**Table 3** Substrate specificity of *Pseudomonas* sp. strain DY-A protease

| Substrate | Relative activity (%) [a] |
|---|---|
| *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide | 100 |
| *N*-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide | 78 |
| *N*-Succinyl-Ala-Ala-Pro-Asp-*p*-nitroanilide | 0 |
| *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide | 0 |
| *N*-Succinyl-Gly-Phe-p-nitroanilide | 0 |

[a] Proteolytic activity was determined by using synthetic oligopeptide as the substrate in 50 mM Tris-HCl (pH8.0) containing 10 mM $CaCl_2$, The *p*-nitroaniline released was determined spectrometrically at 405 nm

anilide and *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (Sigma), which are well-known substrates for chymotrypsin (Table 3).

The addition of chelator such as EDTA, EGTA, 1,10-phenanthroline and 2,2′-bipyridyl produced a decrease of activity (Table 2), suggesting that metal ions might have an important role in the preservation of the enzyme conformation. Examination of the effects of metal ions on protease producing and activity showed that the total activity recovered from medium was increased by 30% in the presence of $Ca^{2+}$ and $Mg^{2+}$ (10 mM). They enhanced the enzymatic activity slightly (8%) and had an important role in enzyme stability. Among the cations tested, $Co^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ inhibited the protease-producing and enzymatic activity, while $Fe^{3+}$, $Mn^{2+}$, $K^+$, $Li^+$, $Hg^+$, $Ag^+$ had no effect on the protease-producing and enzymatic activity.

Enzymes isolated from organisms native to cold environments generally exhibit higher catalytic efficiency at low temperatures and greater thermosensitivity than their mesophilic counterparts (Gerday et al. 1997). *Pseudomonas* sp. strain DY-A protease showed better thermostability than other cold-active protease reported (Vazquez et al. 1995; Hoshino et al. 1997; Kulakova et al. 1999; Chessa et al. 2000). Moreover, the enzyme had lower optimal enzymatic activity temperature and kept high activity at room temperature. These characteristics mean it could be applied in biotechnology such as food processing, additives in detergents and so on.

The characteristic that the protease was only secreted at low temperature may be a response to the deep-sea environment. Gene sequencing of *Pseudomonas* sp. strain DY-A is currently in progress in our laboratory. Information on its primary structure as well as the molecular characterization of the gene will contribute to elucidation of the molecular mechanisms of deep-sea microorganisms.

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