Biotechnology Letters (2005) 27: 1195–1198
DOI 10.1007/s10529-005-0016-x

Purification and characterization of an extracellular cold-active serine protease from the psychrophilic bacterium *Colwellia* sp. NJ341

Quan-Fu Wang[1,2], Jin-Lai Miao[2,*], Yan-Hua Hou[3], Yu Ding[2], Guo-Dong Wang[2] & Guang-You Li[2]
[1]*College of life Sciences, Ocean University of China, 266003, Qingdao, P.R. China*
[2]*Key Laboratory of Marine Bio-active Substances, First Institute of Oceanorgarphy, State Oceanic Administration, 266061, Qingdao, P.R. China*
[3]*Harbin Institute of Technology, 264209, Weihai, P.R. China*
**Author for correspondence (Fax: +86-532-88967430; E-mail: miaojinlai@163.com)*

Received 7 April 2005; Revisions requested 4 May 2005; Revisions received 7 June 2005; Accepted 10 June 2005

Key words: Antarctic, cold-active serine protease, *Colwellia*, psychrophilic bacteria

Abstract

Colwellia sp. NJ341, isolated from Antarctic sea ice, secreted a cold-active serine protease. The purified protease had an apparent Mr of 60 kDa by SDS-PAGE and MALDI-TOF MS. It was active from pH 5–12 with maximum activity at 35 °C (assayed over 10 min). Activity at 0 °C was nearly 30% of the maximum activity. It was completely inhibited by phenylmethylsulfonyl fluoride.

Introduction

Cold-active or cold-adapted enzymes are produced by organisms existing in permanently cold habitats. Cold-active metalloproteases and cellulases have been used as cleaning detergents, in leather processing, food processing and molecular biology (Cavicchioli *et al.* 2002). Bacteria living within Antarctic sea ice have a high potential for biotechnological applications (Russell *et al.* 1997) as they produce a variety of cold-active enzymes (Nichols *et al.* 1999). However, we believe that this is the first report of the purification and characterization of an extracellular cold-active serine protease from a psychrophilic bacterium, *Colwellia* sp. NJ341.

Materials and methods

Bacteria and their cultivation

Two hundred sixty strains were isolated from the sea ice in Antarctica (68°30′E, 65°00′S) during 2001–2002. Strains were conserved in the Key Laboratory of Marine Bio-active Substances, Qingdao, China. Strains were inoculated in the sea water medium (peptone 0.5% and yeast extract 0.1%, pH 7.5) at 8 °C with shaking at 100 rpm.

Assay of protease activity

Protease activity was determined by a modified method of Folin & Ciocalteau (1927). Briefly, 50 μl purified protease was added to the reaction mixture containing 2% (w/v) casein in 50 μl 50 mM Tris/HCl (pH 8). The mixture was incubated at 35 °C for 10 min, the reaction stopped by adding 100 μl 10% (w/v) trichloroacetic acid, and centrifuged at $9000\times g$ for 15 min. The protein remaining in the supernatant was determined by the Folin-phenol reagent. One unit of protease activity was defined as the amount of enzyme that liberated 1 μg tyrosine per min. A blank was run in the same manner except the enzyme was added after the addition of 10% (w/v) TCA. All experiments were done in duplicate.

Protein determination and zymogram

Protein was assayed according to Lowry using bovine serum albumin (BSA) as a standard. A zymogram was performed as described by Liu *et al.* (1997).

Results and discussion

Characterization of Colwellia *sp. NJ341*

Protease activity of the cultures was screened from 260 strains of Antarctic bacteria taken from sea ice, and strain NJ341 showed the highest protease activity (126 U ml^{-1}). Strain NJ341 was identified as the genus *Colwellia* from its biochemical characteristics and 16S rDNA sequence. Phylogenetic tree analysis by neighbor-joining method also exhibited that strain NJ341 was more closely related to the genus *Colwellia* (levels of similarity, 89.8–94.8%), and its closest relative was *C. piezophila*. It was named *Colwellia* sp. NJ341.

Purification of an extracellular cold-active protease

The purification of the protease was summarized in Table 1. A purification of approx. 31-fold with a yield of 9% was achieved. The SDS-PAGE analysis of the purified enzyme revealed a single band and the concentrated protein with protease activity was reexamined by zymogram and exhibited a single active protein band (Figure 1). MALDI-TOF MS of the purified protease gave a single peak with an approx. Mr of 60 kDa (Figure 2a). With the peptide masses obtained from MALDI-TOF MS of tryptic peptides (Figure 2b), extensive searches from protein databases in NCBI did not yield any other protease sequences to this protease.

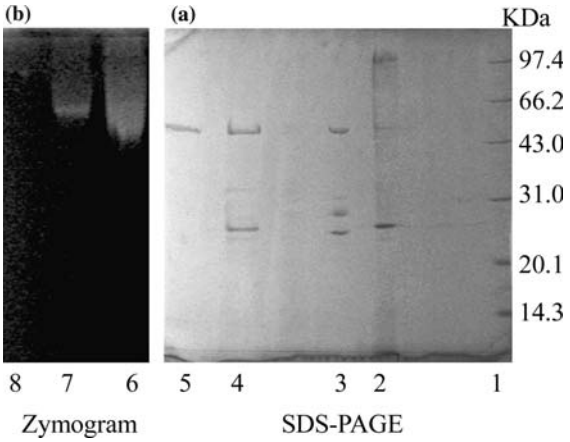

Fig. 1. (a) Protein profile of purification step on 12.5% SDS-PAGE. lane 1, protein molecular mass markers (expressed in kDa); lane 2, Ammonium sulphate precipitated; lane 3, DEAE-Sephadex A50; lane 4, Sephadex G-75; lane 5, DEAE-Sepharose Fast Flow (purified protease). (b) Zymogram. lane 6, partial purified protease; lane 7, purified protease; lane 8, PMSF (10 mM) + purified protease.

Effect of pH and temperature on the protease activity and its stability

The purified protease from *C.* sp. NJ341 exhibited the maximum activity at pH 8–9 (Figure 3a). The maximum activity was about 35 °C and its protease activity under 0 °C exhibited nearly 30%

Table 1. Purification process of protease from *Colwellia* sp.NJ341.

Purification steps	Total activity (Units)	Total protein (mg)	Specific activity (U mg^{-1})	Purification fold	Enzyme yield (%)
Crude extract	61838	140	441	1	100
Ammonium sulphate precipitated	33442	38	889	2	54
DEAE-Sephadex A50 column chromatography	14872	4	3541	8	24
Sephadex G-75 column chromatography	12601	2	5727	13	20
DEAE-Sepharose Fast Flow column chromatography	5530	0.4	13826	31	9

Cells were removed by centrifugation (12000 × *g* for 30 min at 4 °C) and the supernatant was collected and precipitated with 75% saturated $(NH_4)_2SO_4$ solution (3 h, 4 °C). The protease was applied to DEAE-Sephadex A50 column (1.6 × 40 cm), pre-equilibrated with 50 mM Tris/HCl (pH 7.5) buffer, and eluted with 500 mM NaCl at 18 ml h^{-1}. The protease eluate was applied to a Sephadex G-75 column (1.6 × 100 cm), and eluted with 50 mM Tris/HCl (pH 7.5) buffer at 12 ml h^{-1}. The active fraction was applied to a DEAE-Sepharose Fast Flow (1.0 × 10 cm), pre-equilibrated with 50 mM Tris/HCl (pH 7.5) buffer, and eluted with 300 mM NaCl at 24 ml h^{-1}.

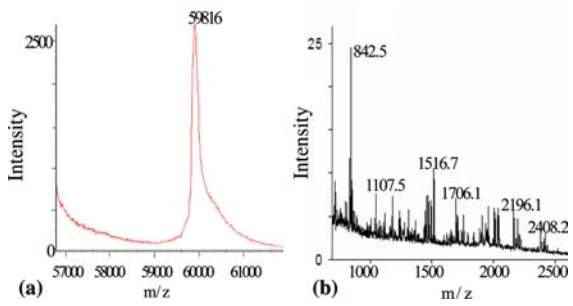

Fig. 2. (a) MALDI-TOF MS spectrum of the purified protease. (b) MALDI-TOF MS spectrum of tryptic peptides from the purified protease. The peptide masses of the purified protease were matched with protein sequences from protein databases in NCBI using the MASCOT search program (www.matrixscience.com).

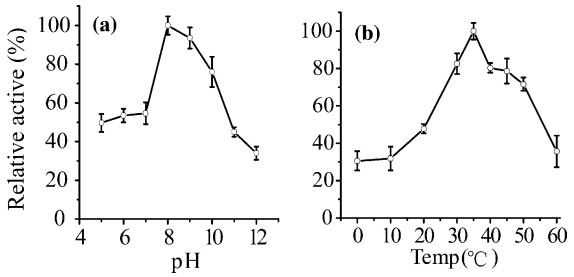

Fig. 3. (a) Effect of pH on the activity of the purified protease. The reaction buffer used were the following (50 mM each): sodium acetate/acetic acid (pH 5); KH_2PO_4/Na_2HPO_4 (pH 6–7); Tris/-HCl (pH 8–9) and $Na_2HPO_4/NaOH$ (pH 10–12). (b) Effect of temperature on the activity of the purified protease. Purified protease was incubated in 50 mM Tris–HCl (pH 8) at 0–60 °C for 10 min.

of the maximum activity (Figure 3b). But the protease activity retained about 50% of its maximum activity for 50 min at 40 °C (date not shown). These results indicated that this protease was cold-active protease.

Effect of inhibitors and metal ions on protease activity

The purified protease was completely inhibited by PMSF (Figure 1b), suggesting that this protease was a serine protease. Treatment with EDTA and EGTA was slightly inhibitory, and the cysteine protease inhibitors E-64 had no effect. It was completely inhibited by Fe^{2+}, while only partially inhibited by other metals such as Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, Pb^{2+}, Mn^{2+} and Al^{3+}. It was sensitive to urea, SDS and thiourea, indicating that hydrogen bonds played an important role in maintaining enzyme activity (date not shown).

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). In this paper, we describe, for the first time, the characteristics and purification of a cold-active protease from the genus *Colwellia*. It was active over a wide range of pH (5–12) with optimum activity at around 35 °C but, most importantly, its activity at 0 °C was nearly 30% of the maximum. These properties made it potentially useful for industrial applications particularly in processes where there may be a risk of microbial contamination or a temperature instability of reactants or products. This present protease had a better thermostability than other cold-active proteas that have been reported (Marianna *et al.* 1999, 2003, Secades *et al.* 2003) although it was inactivated at 50 °C over 30 min.

Acknowledgements

This work was supported by Program of National Natural Science Foundation of China. (No. 40406003)

References

Cavicchioli R, Siddiqui KS, Andrews D, Sowers KR (2002) Low-temperature extremophiles and their applications. *Curr. Opin. Biotechnol.* **13**: 253–261.

Folin O, Ciocalteau V (1927) On tyrosine and tryptophane determinations in proteins. *J. Biol. Chem.* **73**: 627–650.

Gerday C, Aittaleb M, Arpigny JL, Baise E, Chessa JP, Garsoux G, Petrescu I, Feller G (1997) Psychrophilic enzymes : a thermodynamic challenge. *Biochim. Biophys. Acta* **1342**: 119–131.

Liu PC, Lee KK, Tu CC, Chen SN (1997) Purification and characterization of a cysteine protease produced by pathogenic luminous Vibrio harveyi. *Curr. Microbiol.* **35**: 32–39.

Marianna T, Ewa G, Halina K, Maria Z (1999) Biosynthesis and properties of an extracellular metalloprotease from the Antarctic marine bacterium *Sphingomonas paucimobilis*. *J. Biotechnol.* **70**: 53–60.

Marianna T, Marzena P, Halina K, Stanisław B (2003) A cold-adapted extracellular serine proteinase of the yeast *Leucosporidium antarcticum*. *Extremophiles* **7**: 435–442.

Nichols DS, Sanderson K, Bowman J, Lewis T, Mancuso CA, McMeekin TA, Nichols PD (1999) Developments with Antarctic microorganisms: PUFA, culture collections,

bioactivity screening and cold adapted enzymes. *Curr. Opin. Biotechnol.* **10**: 240–246.

Russell NJ (1997) Psychrophphilic bacteria – molecular adaptations of membrane lipids. *Comp. Biochem. Physiol.* **118**: 489–493.

Secades P, Alvarez B, Guijarro JA (2003) Purification and properties of a new psychrophilic metalloprotease (Fpp2) in the fish pathogen *Flavobacterium psychrophilum*. *FEMS Microbiol. Lett.* **226**: 273–279.