Full Paper

A cold-active extracellular metalloprotease from *Curtobacterium luteum* (MTCC 7529): Enzyme production and characterization

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A novel psychro-tolerant bacterium, *Curtobacterium luteum*, secreting an extracellular protease was isolated from the soil of Gangotri glacier, Western Himalaya. Maximum enzyme production was achieved when the strain was grown in a pH-neutral medium containing skim milk at 15°C over 120 h. The metal ions such as Zn²⁺ and Cr²⁺ enhanced enzyme production. The specific activity of purified enzyme was 8,090 units/mg after 34.1-fold purification. The 115 kDa enzyme was a metalloprotease (activity inhibited by EDTA and EGTA) and showed maximum activity at 20°C and pH 7. The enzyme was active over a broad pH range and retained 84% of its original activity between pH 6 and 8. There was no loss in enzyme activity when exposed for 3 h at 4–20°C. However, the enzyme lost 65% of activity at 30°C, and was almost inactivated at 50°C, but was resistant to repeated freezing and thawing. The enzyme activity was stimulated by manganese ions; however, it was inactivated by copper ions.

Key Words—cold-active enzymes; *Curtobacterium luteum*; Gangotri glacier; metalloprotease

Introduction

Microorganisms growing at low temperature regions are important for their metabolic contribution in the ecosphere as well as for their enzymes with potential industrial applications (Gounot, 1991; Margesin and Schinner, 1994). During the past decade it has been recognized that cold-adapted microorganisms provide a wide biotechnological potential, offering numerous economic and ecological advantages over the use of organisms and their enzymes which operate at higher temperatures (Brenchley, 1996; Gerday et al., 2000; Margesin et al., 2002; Soriano et al., 2000). Cold-active enzymes are characterized by high catalytic efficiency at low and moderate temperatures at which homo-

gous mesophilic enzymes are not active, and are thermolabile (Margesin et al., 2005). These properties of cold-active enzyme are of concern for both basic research and industrial application. The application of such enzymes enables lowering of the temperature and shortening of processing times without a loss of efficiency, which leads to saving energy consumption.

Proteases are one of the most important industrial enzymes, and they are a convenient tool whenever protein removal is needed (Rao et al., 1998). Cold-active enzymes have relatively low optimal temperatures; hence they are potentially useful in some industrial applications such as food processing, detergent additives and biotransformation of chemicals (Margesin and Schinner, 1994). Thus, it is desirable to search for new cold-active proteases with novel properties from as many different sources as possible. There are several reports on cold-active proteases produced by microorganisms from different habitats (Hamamoto et

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al., 1994; Hoffmann and Decho, 2000; Hoshino et al., 1997; Huston et al., 2000; Irwin et al., 2001; Kun-Hee et al., 1999; Zeng et al., 2003). Comparatively little information is available on enzymes from microorganisms isolated from glacier regions, which may be an ideal habitat for cold-adapted organisms because it consists predominantly of organic debris such as microorganisms, pollen, plants, animal litter and waste materials from expeditions. In the present study, we reported optimization, purification and properties of the cold-active metalloprotease produced by *Curtobacterium luteum*, a novel psychrotolerant bacterium isolated from Gangotri glacier, Western Himalaya.

Materials and Methods

Isolation of psychrotrophic bacterium producing extracellular protease. Soil samples were collected from different vicinities of Gangotri glacier, Western Himalaya. The glacier is situated between 30°44'–30°56' N and 79°04'–79°15' E, covering an area of about 75 km² and having a temperature of 5–2°C in summer and subzero in winter. The samples were serially diluted in sterile cold saline solution and plated on skim milk agar media, and incubated at 15±1°C. A clear zone of skim milk hydrolysis gave an indication of protease-producing organisms. Depending upon the diameter of the clear zone, one isolate was selected after repeated streaking for identification and detailed studies. The isolate was fully identified and characterized by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

Production of the protease. Protease production was carried out in a medium containing (g/L): glucose, 10; peptone, 5; yeast extract, 5; KH₂PO₄, 1 and MgSO₄·7H₂O, 0.2. The pH of the autoclaved broth was adjusted by adding sterilized Na₂CO₃ solution (20% w/v). The above medium was inoculated at 1.0% (v/v) with 24-h-old (OD 0.6) culture and incubated at 15±1°C in a refrigerated incubator shaker (Scigenics Biotech., Ltd., India) at 80 rpm for 48 h. The growth cultures were then centrifuged at 4°C (10,000×g, 15 min) and the supernatant was used for protease assay.

Measurement of enzyme activity. Proteolytic activity with azocasein (Sigma) as a substrate was assayed by the modified method of Secades and Guijarro (1999). The activity is expressed in units. One unit of enzyme activity was defined as the amount which yielded an increase in A₄₂₀ of 0.01 in 30 min at 30°C.

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Optimization of protease production. All the experiments were carried out in triplicate and the results are the average of these three independent findings. To determine optimum time duration for cold-active protease production, the respective broth media was inoculated with the organism and incubated at 15±1°C. The samples were withdrawn at different time intervals and the enzyme activity was assayed. To find out optimum temperature, the broth media was inoculated with the organism and incubated at different temperatures. Optimum pH condition for maximum production of protease was ascertained by inoculating the broth media having different pHs. To optimize the best condition for fermentation, the broth inoculated with the organism was incubated in static condition and in a rotary shaker (120 rpm). The effect of inducers was studied by addition of different inducers (BSA, casein, skim milk, egg albumin) at the rate of 0.5–1% to the media inoculated with the organism. To evaluate the impact of heavy metals on enzyme production, broth media was supplemented with the maximal tolerance level of different metals and incubated under optimal condition for 48 h. The heavy metals used were Co²⁺, Hg²⁺, Cd²⁺, Cu²⁺, Zn²⁺ and Cr²⁺ at the final concentration of 100, 10, 25, 100, 100 and 150 µg/ml, respectively. The enzyme production was measured as per standard method.

Purification of protease. The fermented broth media incubated at optimized conditions was centrifuged at 4°C (10,000×g, 15 min) and the supernatant was used for further purification steps. The extracellular protease in the cell free culture supernatant was obtained by ammonium sulfate precipitation to a final concentration of 80%. The amount required for percentage saturation was calculated by the method given by Deutscher (1990). All subsequent steps were carried out at 4°C. The protein precipitate was dissolved in a minimal volume of 0.05 M sodium acetate buffer and dialyzed against the same buffer for 24 h with 6–8 changes. The fraction with maximum enzyme activity obtained from ammonium sulfate precipitation was applied to DEAE-Cellulose column (Bangalore Genei, India), which was pre-equilibrated with 0.02 M sodium acetate buffer, pH 5.0. The column was eluted with a linear gradient of NaCl (0.1 to 1 M) in the same buffer. A total of 40 fractions were collected and as-

sayed for protein and enzyme activity. The fractions with high protease activities were pooled and concentrated by lyophilization (Lyophilizer MSW137, Macro Scientific Works, India) and used for characterization.

Characterization of purified protease. Determination of optimum pH and pH stability: To determine the optimum pH of purified enzyme, the activity was measured at different pHs by using the following buffers: citrate phosphate (pH 5–6), sodium phosphate (pH 7), Tris-HCl (pH 8), and glycine-NaOH (pH 9–11). Reaction mixtures were incubated at $20 \pm 2^\circ\text{C}$ for 30 min and the residual enzyme activity was measured. The effect of pH on stability of protease was determined by pre-incubating the enzyme without substrate at different pH values (5.0–11.0) for 1 h at $20 \pm 2^\circ\text{C}$. The relative activity at each exposure was measured as per standard assay procedure.

Determination of optimum temperature and thermal stability: The effect of temperature on protease activity was determined by performing the standard assay procedure within a temperature range from 4– 50°C . To determine the protease stability with changes in temperature, the enzyme was pre-incubated at different temperatures for 3 h and relative protease activity was assayed as per standard assay procedure.

Effect of metal ions and inhibitors on protease activity. Purified protease was pre-incubated with different metal ions (5 mM) such as Ca^{2+} , K^+ , Ba^{2+} , Zn^{2+} , Co^{2+} , Cd^{2+} , Hg^{2+} , Mg^{2+} , Cu^{2+} and Mn^{2+} for 30 min at 20°C and the residual activity was measured. Separate blanks with individual metal ions were prepared. The effect of protease inhibitors such as serine protease inhibitor (phenyl methyl sulphonyl fluoride, PMSF), cysteine protease inhibitors (iodoacetate, *p*-chloromercuric benzoate) metalloprotease inhibitor (ethylene diamine tetra acetic acid, EDTA; ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra-acetic acid, EGTA) and aspartic protease inhibitor (pepstatin) was investigated. After 30 min pre-incubation at $20 \pm 2^\circ\text{C}$, residual protease activity was measured by standard assay method.

SDS polyacrylamide gel electrophoresis. The SDS-PAGE of lyophilized fraction was performed, using a mini slab gel apparatus (Bangalore Genei), by the method of Laemmli (1971). The protein bands were visualized by staining the gel with Coomassie blue and the relative molecular mass of the protein was calculated using standard protein markers (Bangalore Genei) run simultaneously. The gel was analyzed with a

Gel Documentation system (GeneLine, Spectronics Corporation, New York).

Activity detection by zymogram staining. Gelatin zymography was performed in polyacrylamide slab gel containing SDS and gelatin (0.1%) as a co-polymerized substrate, as described by Sana et al. (2006) but with some modifications. The activity band was observed as a clear colorless area depleted of gelatin in the gel against the blue background.

Results and Discussion

Isolation and characterization of psychrotrophic bacteria

One hundred bacterial isolates obtained from twenty-five soil samples of Gangotri glacier were screened for secretion of extracellular protease on milk agar media at 15°C . On the basis of the largest clear zone of hydrolysis at low temperature one isolate was selected as a potent protease-producing strain, designated as GP-1, and taken for further study. The organism showed growth at 5– 42°C and a pH range of 5–12, and was a non spore-forming gram-positive irregular rod. The organism was fully characterized and identified by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology as a *Curtobacterium luteum* and given the accession number MTCC 7529.

Optimization of growth conditions for protease production

The protease production was gradually increased along with the incubation period with the highest yield at over 120 h of cultivation. However, beyond 120 h it showed a rapid decline in enzyme production that was growth independent as shown in Fig. 1. Temperature has a profound influence on protease production. In order to determine enzyme production, the cells were

Fig. 1. Effect of incubation time on growth and protease production from GP1.

incubated at 4 to 45°C in production media. The enzyme production was found to be maximal (46.4 U/ml) at 15°C over 120 h incubation at pH 7.0 (Fig. 2). According to the most widely accepted definition (Morita, 1975) the strain was psychro-tolerant. There was continuous decline in enzyme production with increase in incubation temperature and it was totally inhibited at 45°C. However, Dube and coworkers (Dube et al., 2001) reported maximal protease production at 30°C from five psychrotrophic bacteria isolated from lake sediments of Antarctica. The pH of culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. The influence of pH on the production of protease was studied at different pHs and it was found that the isolate produced maximum protease at pH 7 (Fig. 3). Kumar and coworkers (Kumar et al., 2002) reported production of neutral protease from *Bacillus* sp. S4. Therefore, it may be concluded that *C. luteum* produced neutral protease. Neutral proteases are metalloprotease, with pH optima around 7.0, which may be used in milk protein modification, nitrogen control, mash extraction and chill-haze removal in brewing, soy modification for use as flavors and in animal feeds as suggested by Schallmeyer et al. (2004).

To find out the effect of agitation on protease production, the cells were subjected to agitation at 120 rpm for 48 h incubation. More than two-fold in-

crease in protease production was noticed in shaking condition as compared to static condition. The use of cheap sources of C and N are important as these can significantly reduce the cost of production of protease. Therefore, the utilization of various sources of carbohydrates and organic nitrogen such as BSA, casein, skim milk and egg albumin were evaluated at 20°C after 48 h incubation and agitation with respect to enzyme yield. The finding suggested that the skim milk and casein were best substrates for protease production. Heavy metals present in surroundings play an important role in the growth of bacteria. The impact of heavy metals on production of cold-active protease was evaluated with maximum tolerance level of metals to the organism. The enzyme production was enhanced by Zn^{2+} (136%) and Cr^{2+} (152%) while Co^{2+} (13.5%) worsened production. The other heavy metals such as Hg^{2+} , Cd^{2+} and Cu^{2+} had no significant effect (Table 1).

Purification and characterization of protease

Protease was partially purified to homogeneity by precipitating with ammonium sulphate (60%) and using a single step ion-exchange chromatography on a DEAE-cellulose (Fig. 4). The enzyme was eluted out as unbound fractions (with 0.4 M NaCl gradient) from the DEAE-cellulose column with 34.1-fold purification and specific activity of 8,090 units/mg (Table 2).

Fig. 2. Effect of temperature on production of protease (120 h, pH 7.0).

Fig. 3. Production of extracellular protease at different pHs (20±2°C, 120 h incubation).

Table 1. Effect of heavy metals on enzyme production.

Metals	Enzyme activity (U/ml)	% activity
Control	30	100
Co^{2+}	4	13.5
Hg^{2+}	19.2	83.5
Cd^{2+}	25.6	85.5
Cu^{2+}	33	110
Zn^{2+}	40.8	136
Cr^{2+}	45.6	152

pH-optima and pH-stability profile

The enzyme was active over a broad pH range (pH 5.0–11.0) and showed optimum activity at pH 7 towards casein as a substrate (Fig. 5). About 84% of the

Fig. 4. The elution profile of protease from *C. luteum* by using DEAE cellulose column.

Fig. 5. Influence of pH on the purified protease activity ($\triangle$) and stability ($\blacksquare$).

Fig. 6. Effect of temperature on the purified protease activity ($\triangle$) and stability ($\blacksquare$).

maximum activity was manifested between pH 6 and 8. Interestingly, 52% of the maximum activity was found at pH 5. The stability of the purified protease was determined by the pre-incubation of the enzyme in various buffers with different pH for 1 h. The enzyme was relatively stable over a pH range of 6–8 and retained 68% of the original activity at 20°C, while 95% of activity was lost after 1 h at pH 11 (Fig. 5).

Temperature optima and thermal stability

The activity of purified protease was determined at different temperatures ranging from 4–50°C. The optimum temperature for activity was 20°C. Activity was significantly increased with a temperature range of 4–20°C and declined beyond 20°C with total inactivation at 60°C. High activity (33–55% of the maximum activity) was detected at 4, 10 and 30°C (Fig. 6). Most cold-active proteases display optimum activity at 30–45°C (Kulakova et al., 1999; Zeng et al., 2003). Other investigators also reported temperature optima of 20°C for proteolytic activity (Huston et al., 2000; Turkiewicz, 2003). However, some psychophilic bacteria produce proteases with optimum activity at 60°C (Irwin et al., 2001).

The studies on enzyme stability showed that the cold-active protease was stable (retained 100% of its activity) for 3 h at 4–20°C. A similar type of result was also observed by other investigators where protease retained 100% of its maximum activity at 4–20°C (Kun-Hee et al., 1999). Sixty-five percent of activity was lost at 30°C, and almost complete inactivation occurred at 50°C (Fig. 6). Thus, the enzyme was highly stable at temperatures below 30°C. Activity of the cold-active protease was not affected after repeated freezing and thawing, which may be due to adaptation of the strain to the natural habitat of Gangotri glacier where repeated freeze-thaw events are common.

Table 2. Purification steps of cold active protease from *C. luteum*.

Purification step	Total activity (U)	Total protein (mg)	Sp. activity (U/mg)	Purified yield	
				(fold)	(%)
Crude enzyme	63,080.0	266.0	237.14	—	100
(NH ₄) ₂ SO ₄ precipitation (60%) (dialyzed)	34,063.2	89.6	380.16	1.6	54
DEAE-cellulose pool (lyophilized)	3,236.0	0.4	8,090.00	34.1	5.13

Effect of metal ions and inhibitors on enzyme activity

The effect of various metal ions on the activity of purified enzyme is shown in Table 3. A stronger inhibitory effect was observed in the presence of Ca^{2+} , K^+ , Ba^{2+} , Zn^{2+} , Co^{2+} , Cd^{2+} , Hg^{2+} and Mg^{2+} . Protease activity was stimulated by Mn^{2+} . However, Cu^{2+} completely inactivated the activity at 5 mM concentration. The result suggests that manganese ion apparently protected the enzyme and played a vital role in maintaining the active conformation of the enzyme (Beg and Gupta, 2003). A similar effect of the ion was also observed by Rahman et al. (1994) and by Nascimento and Martins (2004).

Inhibition studies primarily give an insight into the nature of the active center of an enzyme. The effect of different inhibitors on the enzyme activity of the purified protease was determined (Table 3). The protease

was incubated in Tris-HCl buffer (pH 8.0) with various inhibitors at 4°C for 30 min and the residual activity was measured with casein as the substrate. The enzyme activity was almost not altered at all by 1 mM of serine, cysteine and aspartic protease inhibitor. The metalloprotease specific inhibitor such as EDTA was inhibited 85–93% of the protease activity, while EGTA exhibited 77–88% inhibition, indicating that the enzyme belongs to the group of metalloproteases. Our findings were similar to those of Marianna et al. (1999), where the protease was inhibited (92%) by EDTA.

Molecular weight of the enzyme

The purified enzyme migrated as a single band on 10% SDS-PAGE. The molecular mass of protease obtained from *C. luteum* was determined from its mobility relative to the protein standards on SDS-PAGE by us-

Table 3. Effect of various metal ions and protease inhibitors on purified protease activity.

Metal ion/ Inhibitor	Specific action	Conc.	% Residual enzyme activity
Ca^{2+} (CaCl_2)	—	5 mM	24
K^+ (KCl)	—	5 mM	23
Ba^{2+} (BaCl_2)	—	5 mM	26
Zn^{2+} (ZnCl_2)	—	5 mM	24
Co^{2+} (CoCl_2)	—	5 mM	23
Cd^{2+} (CdCl_2)	—	5 mM	12
Hg^{2+} (HgCl_2)	—	5 mM	13
Mg^{2+} (MgSO_4)	—	5 mM	19
Cu^{2+} (CuSO_4)	—	5 mM	0
Mn^{2+} (MnSO_4)	—	5 mM	135
PMSF	Serine protease inhibitor	1 mM	97
		10 mM	95
EDTA	Metalloprotease inhibitor	1 mM	15
		10 mM	7
EGTA	Metalloprotease inhibitor	1 mM	23
		10 mM	12
Iodoacetate	Cysteine protease inhibitor	1 mM	92
		10 mM	87
pCMB	Cysteine protease inhibitor	1 mM	88
		10 mM	81
Pepstatin	Aspartic protease inhibitor	1 mM	94
		10 mM	86

PMSF, phenyl methyl sulphonyl fluoride; EDTA, ethylene diamine tetra acetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra-acetic acid.

Fig. 7. SDS-PAGE of extracellular protease from *C. luteum*. Lane 1: standard molecular mass marker; Lane 2: eluted active protease.

ing a gel documentation system. By interpolation, the apparent molecular mass of the protease was estimated to be 115 kDa, assuming that the enzyme is composed of a single polypeptide chain (Fig. 7). The proteolytic activity of this SDS-PAGE protein band was confirmed by zymogram analysis (Fig. not shown).

The exploitation of biodiversity may result in the discovery of microorganisms that produce enzymes with novel properties. In this study we describe for the first time a protease produced by *C. luteum*. Although a number of protease have been reported from microbial origins, to our best knowledge this is the first report on the characterization of a protease from a psychro-tolerant *C. luteum*, which showed typical features of cold-active enzymes. The optimum temperature for protease production from *C. luteum* was 15°C. The enzyme displayed optimum activity at 20°C and high activity at 10–30°C and activity was not affected by repeated freezing and thawing. Other attractive characteristics of the cold-tolerant metalloprotease are its high activity and stability at low temperatures over a broad pH range and its resistance to various metal ions, which could be useful properties for the possible applications in different types of industries. The *C. luteum* and its enzyme could be useful for biotransformation/biodegradation at low temperature and as new tools in molecular biology. Other potential fields of application of cold-active proteases are food processing and bioremediation in cold climates (Margesin et al., 2002). The potential of *C. luteum* to utilize a wide range of organic compounds and metal ions indicates the use-

fulness of such strain as an inoculum for the energy-saving bioremediation of domestic and industrial waste at ambient temperatures in cold climates, and demonstrates the biotechnological prospective of the microbial community living in extreme environments.

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