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Isolation and characterization of a metal ion-dependent alkaline protease from a halotolerant *Bacillus aquimaris* VITP4

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A halotolerant bacterium *Bacillus acquimaris* VITP4 was used for the production of extracellular protease. Fractional precipitation using ammonium chloride was used to obtain the enzyme. The protease exhibited optimum activity at pH 8.0 and 40°C and retained 50% of its optimal proteolytic activity even in the presence of 4 M NaCl, suggesting that it is halotolerant. The molecular mass of protease, as revealed by SDS-PAGE was found to be 34 kDa and the homogeneity of the enzyme was confirmed by gelatin zymography and reverse-phase HPLC. Upon purification, the specific activity of th enzyme increased from 533 U/mg to 1719 U/mg. Protease inhibitors like phenyl methane sulphonyl fluoride and 2-mercaptoethanol did not affect the activity of the enzyme, but EDTA inhibited the activity, indicating the requirement of metal ions for activity. Cu^{2+}, Ni^{2+} and Mn^{2+} enhanced the enzyme activity, but Zn^{2+}, Hg^{2+} and Fe^{2+} decreased the activity, while Mg^{2+}, Ca^{2+} and K^{+} had no effect on the enzyme activity. The protease was quite stable in the presence of cationic (CTAB), anionic (SDS) and neutral detergents (Triton X-100 and Tween-20) and exhibited antimicrobial activity against selected bacterial and fungal strains. The stability characteristics and broad spectrum antimicrobial activity indicated the potential use of this protease in industrial applications.

Keywords: Alkaline protease, Antimicrobial activity, *Bacillus aquimaris* VITP4, Gelatin zymography, Halostability, Metal ion binding

Proteases find widespread application in food processing, pharmaceutical, meat tenderization, organic synthesis, detergent and leather industries[1]. Extracellular proteases that can be purified easily have been commercially exploited to assist protein degradation in various industrial processes[2]. Recent awareness of environmental pollution caused by chemical-based industries has necessitated the development of enzyme-based processes as alternatives to currently employed chemical processes. Proteases have potential applications in the industries for partial or total replacement of currently employed toxic chemical processes[3].

Proteases from marine microorganisms are currently receiving increased attention due to their inherent stability at different values of pH, temperature and salinity[4]. Proteases to be used as detergent additives should be stable and active in the presence of detergents. They are used as an active ingredient in the development of biopharmaceutical products like contact lens cleaners and in cleaning a wide variety of polymeric protein substrates[5]. They find extensive application in different industries viz. food, pharmaceutical, silk and for recovery of silver from used X-ray films[6]. The halophilic proteases are less suitable in saline fermentation processes, because they need at least 12.5% (w/v) NaCl for the expression of high activity[7]. Some marine bacteria belonging to the genera *Bacillus, Pseudomonas* and *Vibrio* have been reported to produce proteases with novel attributes like thermostability, salt tolerance and solvent stability[8].

In the present study, we have purified and characterized a protease from a halotolerant bacterium *Bacillus aquimaris* VITP4 and also investigated the antimicrobial activity of the enzyme against specific human pathogens.

Materials and Methods

Chemicals

All chemicals used were of analytical grade. All microbiological media used were dehydrated media (Hi-Media, Mumbai).

Microorganism and inoculum preparation

Isolation of the moderately halotolerant bacterium *Bacillus aquimaris* VITP4, producing substantial

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amounts of extracellular protease has been previously reported[9]. The isolate was cultured and maintained on Zobell agar slants and the culture was regenerated every 2-3 weeks on a fresh plate from the frozen stock culture. For protease production, 0.5 M salt medium containing peptone (10 g/l) and yeast extract (10 g/l) was used. The optimum conditions[9] for cell growth and protease secretion were 37°C, 48 h, agitation speed of 150 rpm and medium pH 7.5.

Protease assay and total protein determination

After cultivation for 48 h, cultures were centrifuged at 9168 *g* for 10 min at 4 °C. The cell-free supernatant was used as crude preparation to measure protease activity using casein as the substrate. Protease assay was carried out using tyrosine as standard as previously described[9]. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of tyrosine/min under the defined assay conditions (over a range of pH and temperature). The amount of protein content of the sample was estimated by the method of Lowry *et al*.[10] using bovine serum albumin (BSA) as the standard. All the experiments were carried out independently in triplicates and repeated twice.

Purification of protease

The crude protease extract was subjected to ultra-filtration through nitrocellulose membrane filter (Amicon, MWC: 10 kDa). The activity of the concentrate was assayed as mentioned above. The concentrate was further saturated to 70% with ammonium sulphate and allowed to stand at overnight 4°C. The resulting precipitate was collected by centrifugation at 10,000 *g* for 20 min at 4°C. The precipitate was dissolved in 50 mM potassium phosphate buffer (pH 8.0) and kept for dialysis at 4°C against the potassium phosphate buffer.

Determination of molecular weight and homogeneity by electrophoresis and HPLC

The dialyzed fraction was subjected to sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) to estimate the molecular mass of the protein and also for ascertaining its homogeneity. SDS-PAGE was performed on a slab gel containing 12% polyacrylamide[11]. To 25 µl of protein sample, 25 µl of sample buffer[11] was added, boiled in boiling water bath (90°C) for 7-8 min and was loaded into the gel. Protein was detected by staining with Coomassie brilliant blue. Molecular mass was calculated using the standard graph by plotting the relative mobility of each standard protein (Genei, Bangalore) against the log of its molecular mass.

Homogeneity and authenticity of the protease was examined by gelatin zymography using gelatin (20 mg/ml) as copolymerized substrate in the resolving gel. The purity of the sample was authenticated by HPLC (C_{18}) analysis at 280 nm for the dialysed fraction.

Effect of pH, temperature and NaCl

The pH for optimal activity of protease was determined in the following buffer systems: potassium phosphate buffer (pH 6.0-8.0) and glycine-NaOH buffer (pH 9.0-12.0). The effect of temperature on the catalytic activity was studied under the standard assay conditions at different temperatures (10-70°C). For halostability, the enzyme was pre-incubated with NaCl (0-4 M) at 37°C for 1 h. Caesin was used as the substrate for these studies.

Effect of protease inhibitors, metal ions and detergents

The effect of different additives on proteolytic activity of protease was investigated by incubating the enzyme in the presence of different concentrations at 37°C for 30 min. Protease inhibitors (1-10 mM) such as PMSF, EDTA and β-mercaptoethanol and metal ions (2.5-10 mM) such as Cu^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+} and K^{+} were used for the study.

In order to investigate the effect of detergents on the enzyme activity, aliquots were pre-incubated with cationic (CTAB), anionic (SDS) and neutral detergents (Triton X-100, Tween 20 and Tween 80), each at a final concentration of 10 mM at 37°C for 30 min.

The residual enzyme activity was calculated in percentage with reference to the activity of the enzyme without the above-mentioned additives (metal ions, inhibitors and detergents).

Determination of antimicrobial activity

The initial screening of pathogens which are susceptible to the antimicrobial activity of protease was carried out by disc-diffusion method[12]. Based on the zone of clearance around the paper discs containing protease, organisms were selected for further quantification of antimicrobial activity. The indicator organisms were grown individually in nutrient broth for 24 h at 37°C. The culture broth at 1% level was added into warm, but unset plate count agar, mixed uniformly and poured into sterile Petri

plates. Appropriate number of wells each of 5 mm diameter was made in the solidified agar using a sterile borer. Aliquots (5 µl, 10 µl, 15 µl) of protease were added into the wells. The plates were pre-incubated at 4°C for 3 h to allow the test material to diffuse into the agar and then the plates were incubated at 37°C for 24 h. Subsequently, the plates were examined for zone of clearance around the individual wells. The diameter of zone of clearance was measured and expressed as arbitrary units per ml (AU/ml) as per the calculation below

$$\text{AU/ml} = \frac{\text{Diameter of the zone of clearance (mm)} \times 1000}{\text{Volume taken in the well } (\mu\text{l})}$$

Results and Discussion

Purification of extracellular protease

The culture supernatant containing the protease was concentrated by membrane filtration using a 10 kDa nitrocellulose membrane, followed by fractional ammonium sulphate precipitation. A summary of the purification of the VITP4 protease is shown in Table 1. The extra-cellular protease exhibited increased specific activity values at each level of purification, leading to a purification fold of 3.2. The pure protein exhibited the specific activity of 1719 U/mg.

Determination of homogeneity by electrophoresis and HPLC

Electrophoretic pattern of the dialysed sample revealed a prominent single band (Fig. 1a). Using simple regression analysis, the relationship between relative mobility and molecular weight data was expressed as y = -1.0271x + 5.193 with an R^2 value of 0.9. The molecular mass of protease was calculated to be 34 kDa. The molecular mass of alkaline proteases produced by several bacteria including *Bacillus* ranges from 15 to 30 kDa[13]. However, the molecular mass of halophilic alkaline proteases has been reported to range from 40 to 130 kDa[14-16].

Gelatin zymography carried out under native conditions revealed a clear hydrolytic zone of proteolytic activity against a dark blue background (Fig. 1b), confirming the presence of protease in the fraction. The zymogram suggested that gelatin was markedly susceptible to hydrolysis by VITP4 protease. Since gelatin is a denatured form of collagen[17], the protease may find use in the preparation of hydrolyzates from collagen, which find applications in the pharmaceutical, cosmetic and food industries.

The purity of protease was further checked by using reverse phase HPLC. Elution of protease with different ratios of mobile phase (water/acetonitrile) revealed only one sharp peak (as detected at 280 nm), confirming the purity of protein sample.

Effect of pH and temperature on enzyme activity and NaCl stability

The pH profile of VITP4 protease was determined at 37°C. The extra-cellular protease was active in the pH range of 7.0-10.0, with an optimum at pH 8.0 (Fig. 2). There was a decline in the activity at pH lower than 8.0, suggesting the alkaline nature of the enzyme. However, recent reports on alkaline proteases have reported the pH optimum in the range of pH 9.0-12.0[14,15,18]. The enzyme exhibited

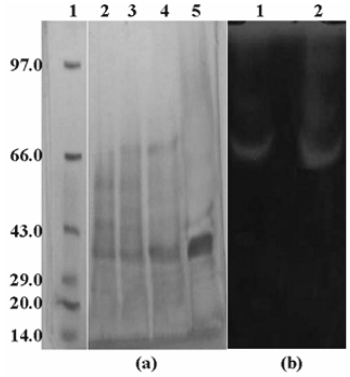

Fig. 1—(a): SDS-PAGE of VITP4 protease using 12% acrylamide gel [Electrophoretic pattern of the dialyzed sample revealed a single prominent band Lanes: 1, protein marker; 2, crude supernatant; 3, membrane concentrate; 4, ammonium sulfate precipitated sample; and 5, dialysed sample; and (b): Gelatin zymogram of VITP4 protease Lanes: 1, membrane concentrate; and 2, ammonium sulfate precipitated sample]

Table 1—Purification of VITP4 protease

Purification step	Volume (ml)	Activity (U/ml)	Protein conc (mg/ml)	Specific activity (U/mg)	Purification fold
Culture supernatant	200	800	15	533	1
Membrane filtration	40	1,910	19	1,005	19
Ammonium sulphate precipitation	2	1,100	09	1,222	23
Dialysis	1	1,375	08	1,719	32

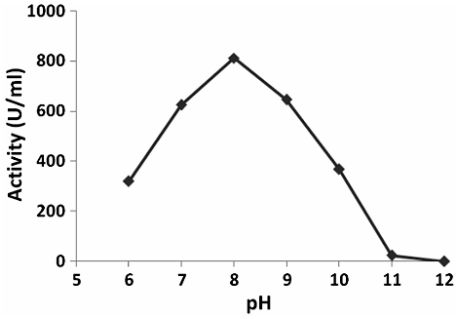

Fig. 2—Effect of pH on protease activity [The optimum pH was determined with casein as substrate dissolved in the following buffer systems: potassium phosphate buffer (pH 60-80) and glycine-NaOH buffer (pH 90-120)]

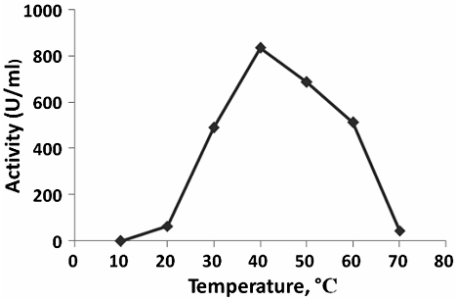

Fig. 3—Effect of temperature on protease activity [The optimum temperature was determined for the protease at pH 80 and the temperature range of (10-70°C)]

proteolytic activity in the range of 30-60°C with an optimum at 40°C (Fig. 3), indicating its mesophilic nature with respect to temperature.

Salt tolerant proteases are significant from industrial perspective[19]. The VITP4 protease maintained a significant activity up to a concentration of 2 M NaCl, although it exhibited the highest activity in the absence of NaCl (Fig. 4). Even in the presence of 4 M NaCl, the protease retained about 40% of its activity, indicating the halotolerant behaviour of the enzyme. The NaCl requirement of VITP4 protease at 40°C was quite less in comparison to other halophilic proteases. However, the halotolerant VITP4 protease showed stability over a broad range of NaCl concentrations as compared to halophilic proteases[16-18]. In contrast, the catalytic activity of most proteases from extremely halophilic microorganisms those normally survive in a 4.0 M salt environment decreases dramatically and irreversibly, when exposed to lower salt concentrations[20]. For example, an extra-cellular protease from the haloalkaliphilic archaeon *Natrialba magadii* does not have any activity at a concentration less than 5.8% (w/v)

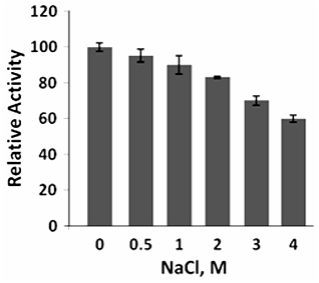

Fig. 4—Halostability of VITP4 protease at pH 80 and 37°C [The enzyme was incubated with NaCl (0-4 M) for 1 h prior to the assay. Activity at 0 M NaCl was taken as 100%]

NaCl[15]. Thus, the bacterium exhibited a broad salt tolerance and produced optimum levels of extracellular protease in the presence of salt concentration at approximately the same proportion as found in sea water.

Effect of protease inhibitors

Small alterations of the macromolecule structure may have profound effects on protein behavior, as the biological function and native structure of a protein are closely interconnected. The inhibition study provides an insight into the nature of enzyme, its cofactor requirements and nature of the active centre[21,22]. In order to understand the nature of the alkaline protease, the proteolytic activity was assayed in the presence of different protease inhibitors. Alkaline proteases, in general, could belong to either serine, cysteine or metalloproteases. The purified enzyme was not inhibited by β-mercaptoethanol (10 mM) and PMSF (10 mM), indicating that it might not belong to either cysteine or serine protease family. However, the activity decreased when treated with EDTA. The enzyme lost 40% of its initial activity (in the absence of EDTA), indicating the requirement of metal ions for the enzyme activity (Table 2).

Effect of metal ions

The influence of metal ions is among the many external factors that affect the catalytic activity and/or stability of the enzymes. Cations are known to increase the thermal stability of other *Bacillus* alkaline proteases[23]. Some of them are known to play a vital role in maintaining the active conformation of the enzyme[24,25]. Therefore, the activity was assayed in the presence of different concentrations of divalent metal ions. The cations used in the present study showed differential effects on the enzyme activity. The activity increased in the presence of Cu^{2+}, Ni^{2+}

Table 2—Effect of protease inhibitors, metal ions and detergents on the enzyme activity

[Observed enzyme activity in the absence of any additive was taken as 100%]

Additive	Relative activity at	
	5 mM	10 mM
PMSF	98	96
EDTA	76	60
βME	98	96
$CuCl_2$	145	210
$NiCl_2$	112	135
$MnCl_2$	115	118
$ZnCl_2$	70	40
$HgCl_2$	75	65
$FeCl_2$	82	78
SDS	94	90
CTAB	93	89
Triton X-100	98	97
Tween 20	78	72

and Mn^{2+} (Table 2), of which Cu^{2+} exhibited the maximal increase (210%) in the activity. Incidentally, only a few proteases have shown enhanced activity in the presence of Cu^{2+}, such as from *Serratia rubidaea*[26], *B. mojavesnis*[27] and *B. licheniformis*[28]. In contrast, the VITP4 protease was partially inhibited by Zn^{2+}, Hg^{2+} and Fe^{2+}, whereas Mg^{2+}, Ca^{2+} and K^{+} had no effect on the enzyme activity. Zn^{2+} concentrations greater than 0.1 mM have been shown to inhibit metalloproteases[29]. The inhibition could be due to the formation of zinc monohydroxide that bridges the catalytic Zn^{2+} to the side chain of the active site of the enzyme[29].

Effect of detergents

The commercial application of the enzymes depends on their ability to remain functional in the presence of a variety of external factors. Thus, the activity of VITP4 protease was assayed in the presence of cationic, anionic and neutral detergents. The protease exhibited significant stability to SDS (anionic), CTAB (cationic) and non-ionic detergents like Triton X-100, Tween-20 and Tween-80 (Table 2). The enzyme activity was moderately inhibited in the presence of Tween-20. These attributes of salt tolerance and stability to detergents were similar to some of the marine bacteria[8,30,31].

The fact that VITP4 protease was resistant to chemical denaturants suggested that the protein might be having a well-packed structure and very rigid native conformation. It is believed that structural properties of proteins might be closely related to protein stability, and increased protein stability might be generally correlated with greater protein rigidity[32]. The VITP4 protease could hence be used as a promising additive in commercial detergents.

Table 3—Relative antimicrobial properties of VITP4 protease

Organism	Protease (µl)	Inhibition zone (mm)	Activity (AU/ml)
Antibacterial			
Escherichia coli	5	—	—
	10	8	800
	15	21	**1400**
Pediococcus sp	5	2	400
	10	24	**2400**
	15	30	2000
Pseudomonas sp	5	—	—
	10	—	—
	15	12	**800**
Yersinia enterocolytica	5	6	1200
	10	14	1400
	15	25	**1668**
Antifungal			
Candida albicans	5	8	1600
	10	24	**2400**
	15	30	2000
Candida famata	5	2	400
	10	16	**1600**
	15	21	1400
Neurospora crassa	5	—	—
	10	—	—
	15	10	**667**

Inhibition is represented in terms of arbitary units. See 'Materials & Methods' for detail

Antimicrobial activity

Among the several bacterial and fungal strains tested, the extra-cellular protease produced by *B. aquimaris* VITP4 exhibited antimicrobial property against *Candida albicans*, *C. famata*, *Escherichia coli*, *Neurospora crassa*, *Pediococcus* sp., *Pseudomonas* sp., and *Yersinia enterocolytica*. The diameters of inhibition zone of various aliquots of VITP4 protease are presented in Table 3. This broad spectrum antagonistic activity of protease may find applications in topical ointments. However, further investigation is needed to exploit the applicability range of this protease. Earlier, similar broad range antimicrobial property has been reported for the protease secreted by *B. proteolyticus* CFR3001 against human pathogens[17]. *Bacillus* spp. secrete many enzymes, including proteases that degrade slime and biofilms, thus allowing them to penetrate slime layers around Gram negative bacteria. Further *Bacillus* spp. are often used commercially in

bioremediation mixes, or as probiotic agents in the aquaculture[33-35].

Conclusion

A low molecular weight halotolerant alkaline protease with optimal activity at pH 8.0 and 40°C was isolated and purified from *Bacillus aquimaris* VITP4. The metal ions like Cu^{2+}, Ni^{2+} and Mn^{2+} enhanced the activity of the protease. Activity of the enzyme decreased in the presence of EDTA, indicating that it is a metal ion-dependent enzyme. The enzyme showed stability against detergents and antimicrobial activity against human pathogens, thus may have potential in commercial application.

Acknowledgement

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