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International Journal of Current Research
Vol. 6, Issue, 01, pp.4393-4397, January, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PARTIAL PURIFICATION AND CHARACTERIZATION OF *PSEUDOALTEROMONAS PISCICIDA* JCR 18 PROTEASE

***Jaquline Chinna Rani, I. and Jayasurya Kingsley**

Department of Plant Biology and Biotechnology, Loyola College, Chennai – 600 034

ARTICLE INFO

Article History:
Received 20th September, 2013
Received in revised form
16th October, 2013
Accepted 01st December, 2013
Published online 26th January, 2014

Key words:
Protease,
Pseudoalteromonas,
Casein,
Marine,
SDS-PAGE

ABSTRACT

Proteases able to function in a broad range of alkaline pH, temperature, salts, solvents and detergents are the most preferred from an industrial point of view. An extracellular alkaline protease with thermos table properties was isolated from a marine bacterium *Pseudoalteromonas piscicida* strain JCR 18 obtained from seawater, off the coast of Marina, Chennai. The enzyme was isolated and partially purified using gel chromatography and SDS-PAGE and was seen to be a monomer having a molecular weight of 30kDa. The specific activity was seen to be 172.4 U/mg at 27-fold purification and with an yield of 3%. It exhibited optimum activity at a temperature of 50°C and pH 9. It was inhibited by PMSF and DFP showing it to be a serine protease. The activity was only slightly enhanced by metal ions like Ca^{2+}, Mg^{2+}, and Mn^{2+}showing that it is cofactor-independent. Increased activity was seen with casein as substrate. This protease with its high specific activity, alkaline pH range, thermo stability and reduced cofactor requirement would be best suited for detergents, gelatinization of x-rays and dehairing applications in leather industries.

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INTRODUCTION

Proteases are one of the three groups of industrially most important enzymes accounting for nearly 60% of the total enzyme market (Amoozegar *et al.,* 2007) and find varied applications in a number of industries viz., detergent, tanning, dairy, baking, brewing and protein hydrolysis, peptide synthesis, infant formula preparations and isolation of DNA (Kaur *et al.,* 1998; Tari *et al.,* 2006; Bhaskar *et al.,* 2007; Dodia *et al.,* 2008; Shumi *et al.,* 2004; Najafi *et al.,* 2005). More than 300 tons of enzymes, the majority of which are proteases are annually produced from bacteria alone, namely *Bacillus sp*. (Shumi *et al.,* 2004; Venugopal and Saramma, 2008). Proteases make up more than half ofthe total worldwide sale of enzymes (Rao *et al.,* 1998; Singh *et al.,* 2001) of which 25% comprises the alkaline proteases alone (Mala *et al.,* 1998). The total world-wide sale of proteases was estimated to touch $ 6 billion in 2011 (Yossan *et al.,* 2006; Gupta *et al.,* 2002; Krishna *et al.,* 2009) and the market demand is on the rise due to its diverse applications. The marine environment which constitutes 71% of the earth's surface houses millions of microorganisms with potential enzymes. It is to be noted that there is a higher population of proteolytic bacteria in marine habitats than in freshwater or soil (Atlas and Bartha, 1981). The production of copious amounts of extracellular enzymes is seen as the fastest means to search for food in this complex and vast environment.

****Corresponding author: Jaquline Chinna Rani, I.***
Department of Plant Biology and Biotechnology, Loyola College, Chennai – 600 034

Bacteria secrete proteases in an attempt to acquire organic compounds enriched with nitrogen from marine environments. Most of these are seen to produce more than one kind of proteases (Sanchez-Porro *et al.,* 2003). These salt-loving bacteria called "halophiles" have numerous advantages namely high cell yield at challenging salt concentrations, low risk of contamination by other bacterial strains, enzymes active at high salt concentrations, higher resistance to chelating agents and enzymes that are active at extremes of pH and temperature.

Recently, there is great interest in enzymes isolated from moderate halophiles over those obtained from non – or extreme – halophiles as these can perform in the presence and absence of salt (Ventosa *et al.,* 1998). Fluctuations in salt levels in industrial processes is a common phenomenon and therefore biocatalysts that continue to function under these conditions are preferred (Karbalaei-Heidari *et al.,* 2009). Halotolerant proteases offer acceleration advantage in proteolytic hydrolysis which decrease production time involved in preparation of oriental salt-fermented foods like soy, shrimp, lance and anchovy sauces which has intensified efforts for a thermostable and saline tolerant protease (Vijayaraghavan *et al.,* 2012). Thermostable enzymes can function at high temperatures which aids faster reaction rates, increased solubility of nongaseous reactants and products and lower incidence of contamination by mesophilic organisms (Gomes and Steiner, 2004; Usharani and Muthuraj, 2010). *Pseudoalteromonas piscicida* is a marine bacterium which is lethal to fishes and hence the name (*pisces*-fish; *cidal*-kills).

They are popular "biofoulers" and seen growing attached to surfaces. They are psychrotrophs with a broad temperature range of 10-30°C and predominantly halophilic with growth at saline levels of 10-30 ppt. The genus comprises both pigmented and non – pigmented forms. They are known to produce autotoxic antibiotic compounds which befits their sub culturing once a week. The extracellular protease from the isolate *Pseudoalteromonas piscicida* JCR 18 (GenBank ID: JQ340776) is seen to be ideal in all these aspects which was the focus of this present study.

MATERIALS AND METHODS

Culture of the microorganism and enzyme production

The bacterial isolate used in this study was obtained from seawater off the Marina coast, Chennai. The enzyme was produced in Erlenmeyer flasks containing 500 ml of modified YTSS medium (pH 8) containing (g/l): Yeast extract, 0.5% ; Casein 0.5 % ; NaCl, 2.0 % ; $NaNO_3$,0.5%; Glycine, 0.1%. The flask was incubated at 37°C at 150 rpm for 48 h in a rotary shaker and then spun at 10,000 rpm for 20 min in cold to remove the bacterial cells. The supernatant was collected and used for further studies.

Protease assay

The protease assay based on Anson (1938) was used with slight modification (Sigma Aldrich protocol). 1% casein in 0.1M Tris-Cl buffer (pH 7.5) was taken to which 0.1 ml of suitably diluted cell-free supernatant was added and incubated for 10 min. To this, 110 mM TCA was added and the tubes incubated at 37°C for 30 min. The mixture was filtered through Whatman filter paper and the filtrate was used in colour development. To 1 ml of the filtrate, 2.5 ml of 500 mM sodium carbonate, and 0.5 ml of 1:1 diluted Folin-Ciocalteau reagent was added and incubated at room temperature for 30 min. The filtrate was read at 600nm using a standard curve for tyrosine. One unit of protease activity is defined as the unit that will hydrolyse casein to produce color equivalent to 1.0µmole (181µg) of tyrosine per min at pH 7.5 at 37°C.

Protein Concentration

The protein concentration was estimated by the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard.

Partial purification of crude protease

The crude protease was fractionated by precipitation with ammonium sulphate (40-80%), centrifuged at 10,000rpm for 10 min. The precipitate was resuspended in 0.1 M Tris-HCl buffer, pH 8 and then dialyzed against several volumes of 0.001 M Tris-HCl buffer (pH 8). The complete removal of salt was confirmed by addition of a pinch of barium chloride to the buffer and if the solution turned milky white (formation of barium sulphate), dialysis was continued until the buffer remained colorless on addition of salt.

Gel filtration chromatography (using Sephadex G-75)

The dialyzed sample was loaded onto a column of Sephadex G-75 (2.2 x 60cm) equilibrated with 0.1 M Tris-HCl (pH 8). The column was eluted at a flow rate of 30ml/h with the same buffer. Fractions of 2 ml were collected and absorbance at 280nm was read. Fractions showing highest protease activity were subsequently pooled together and used for further studies.

SDS-PAGE

SDS-PAGE was carried out on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of Laemmli, 1970 and subjected to silver staining.

Characterisation of enzyme

Effect of temperature on protease activity

The effect of temperature on protease activity was assayed by incubating the reaction mixture at different temperatures ranging from 30-80°C and assaying for the activity under standard assay conditions.

Effect of pH on protease activity

A solution of 1% casein was prepared in different buffers namely sodium phosphate (pH 5-7), Tris-Cl (pH 8.0) and glycine-NaOH (pH 9-12) and the activity studied under the normal assay conditions.

Effect of protease inhibitors on protease activity

The effect of various protease inhibitors (5mM) such as serine inhibitors (phenylmethylsulphonyl fluoride [PMSF] and diisopropyl fluorophosphate [DFP]), cysteine-inhibitors (p-chloromercuric benzoate [pCMB] and β-mercaptoethanol [β-ME], iodoacetate), and a chelator of divalent cations (ethylene diamine tetra acetic acid [EDTA]) were determined by pre-incubating the enzyme solution with the above separately for 30 min at 50°C before adding the substrate. The residual protease activity was measured. The level of inhibition was expressed as percent activity remaining compared to a control without inhibitor (Karbalaei-Heidari *et al.,* 2009).

Effect of various metal ions on protease activity

The purified enzyme was incubated with metal ions viz., calcium, magnesium, aluminium, cobalt, cadmium, ferric iron, zinc, mercury and copper at 5 mM at 50 °C for 60 min and the effect of the ions on the enzyme activity was measured by adding the above to separate reaction mixtures. The residual activity was determined.

Effect of protein substrates

The activity of the enzyme in different protein substrates namely casein, BSA, egg albumin and gelatin was studied by incubating the enzyme with the respective substrates (2mg/ml). The reaction mixture was incubated at 50° C for 30 min and the assay was performed as before.

RESULTS AND DISCUSSION

The results obtained in this work revealed the ability of *P. piscicida* JCR18 (Fig.1) to produce extracellular alkaline

Table 1. Summary of purification steps of alkaline protease from *P.piscicida* JCR 18

Purification step	Total Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude Extract	29200	4560	6.4	1	100
Ammonium sulphate precipitation, dialyzed	9650	620	15.6	2.4	33
Sephadex G -75 chromatography	862	5.0	172.4	27	3.0

protease. Fractionation using gel chromatography revealed protein peaks showing protease activity. The peak with maximum protease activity was taken and the partially purified protein was seen to have a specific activity of 172.4 $Umg^{-1}$and the purification of approximately 27- fold with 3% yield was obtained (Table 1). The protease was found to be a monomer with a molecular weight of 30kDaon SDS-PAGE (Fig. 2). Most of the proteases reported so far are monomers (Croocker *et al.,* 1999; Almas *et al.,* 2009; Martinez-Rosales and Castro-Sowinski, 2011; Nolasco *et al.,* 2002).The purified enzyme was seen to have maximum activity at 50°C (Fig. 3) and atpH 9 (Fig. 4) showing clearly it is an alkaline protease. The enzyme was strongly inhibited by PMSF and DFP (Fig. 5) while the metal ions Mg^{2+}, Ca^{2+} and Mn^{2+} (Fig. 6) increased protease activity to a small extent.

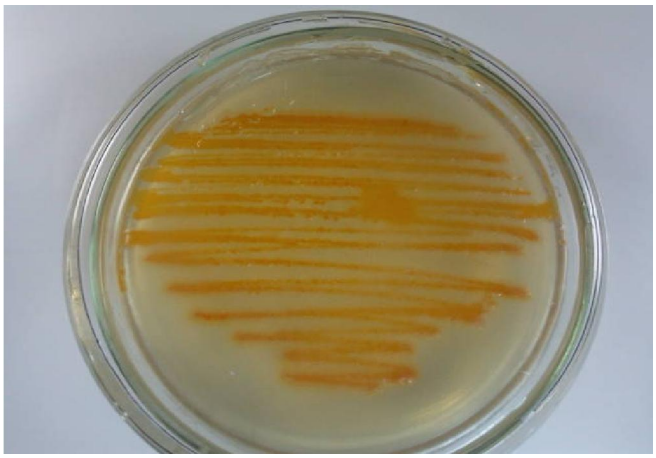
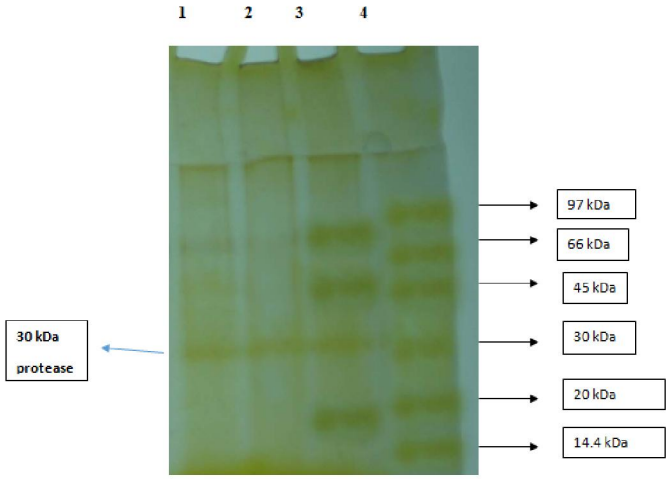


Fig. 1. *Pseudoalteromonas piscicida* JCR 18 on YTSS Agar Plate



LANE 1, 2: SEPHADEX G- 75 FRACTION
LANE 3: DIALYSED FRACTION
LANE 4: STANDARD MARKER PROTEINS

Fig. 2. Silver-stained polyacrylamide gel of purified fractions of *P.piscicida* protease

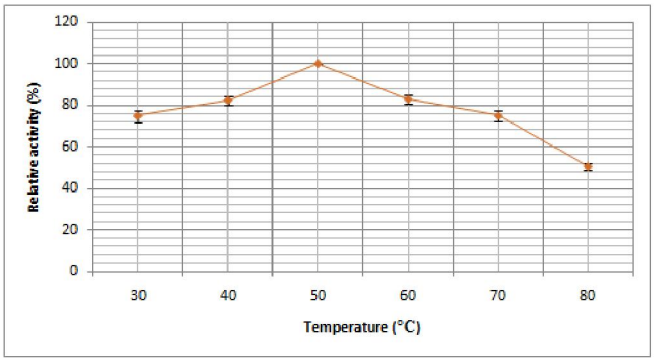


Fig. 3. Effect of temperature on protease activity of *P. piscicida*

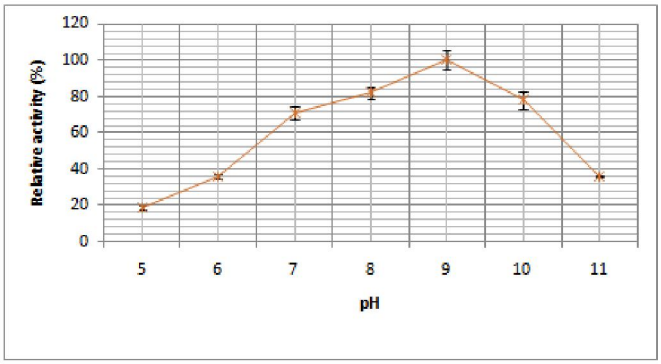


Fig. 4. Activity of protease at different pH

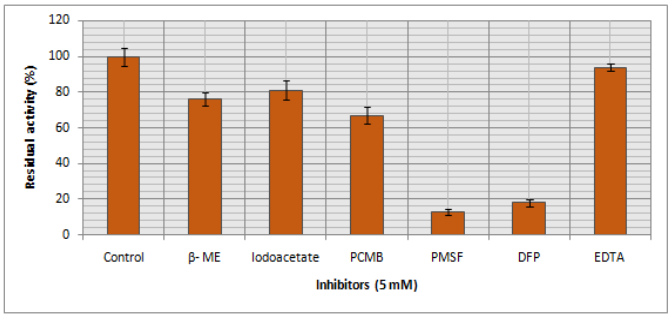


Fig. 5. Residual activity of the enzyme in the presence of inhibitors

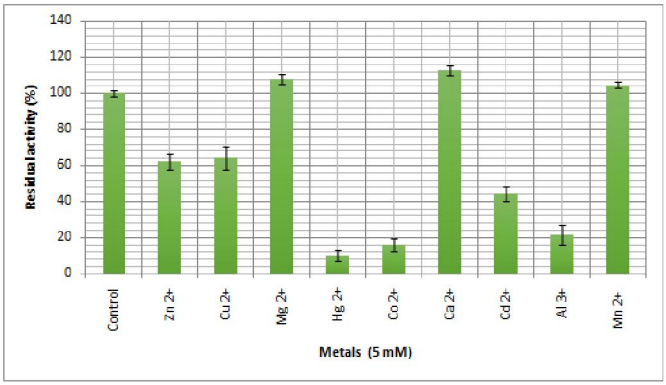


Fig. 6. Residual activity of the enzyme in the presence of metals

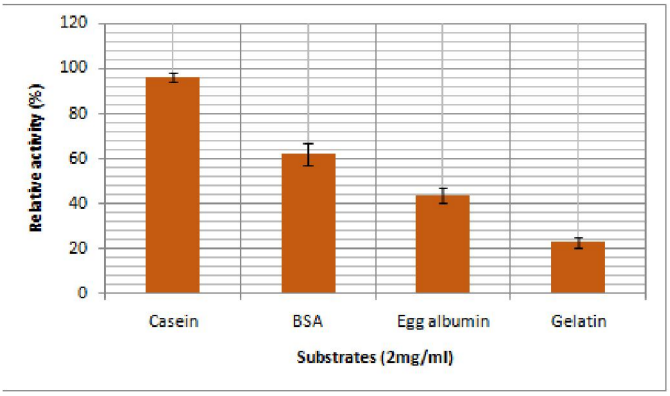


Fig. 7. Relative activity of the enzyme in the presence of different substrates

Evidently, the isolated enzyme may not be a metalloprotease, and is not Ca^{2+} dependent, though the metal ions caused a slight increase in enzyme activity. These are major advantages in the detergent industry as detergents having high amounts of chelating agents like EDTA, EGTA function as water softeners and assist in removal of stains (Walsh, 2002). Of the substrates, casein was seen to be the best in enhancing activity (Fig. 7). *P.piscicida* is a marine bacterium and non-pathogenic to humans which offers an industrial advantage. The simple medium requirements consisting of yeast extract, sea salts and peptone for growth and protease production makes it economical. The enzyme has other industrially ideal properties of activity at optimum pH of 9 and temperature 50°C which make it suitable for detergent applications. The low yield (3%) of the enzyme is compensated by the high specific activity of 172.4 Umg^{-1}. The enzyme is low molecular weight and is a monomer with no stringent cofactor requirement which is another added industrial advantage. However, the above characteristics can be greatly improved by protein engineering or by enhancing the expression by cloning the gene into a heterologous system. There is abundant scope for further research on optimization of fermentation characteristics using different bioprocess models, crystallographic structural elucidation and enzyme engineering of this protease towards development of the enzyme for a wide range of applications" (Haritha *et al.,* 2012).

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