Partial characterization of a crude alkaline protease from Bacillus circulans and its detergent compatibility

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Citation

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Abstract

An extracellular alkaline protease of Bacillus circulans MTCC7906 was produced using cotton deoiled meal replacing nitrogen source in the production medium at a pH of 10.5, temperature of 25°C in 96 hours of incubation. The enzyme purified by $(NH_4)_2SO_4$ precipitation and dialysis showed optimum activity at 60°C and pH 9.0 with an enzyme concentration of 3.33% of reaction mixture at a casein concentration of 14mg ml-1. SDS PAGE of the crude enzyme revealed it as 24.5 kDa protein. The enzyme was stable at room temperature for 20 days and its activity was enhanced by Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} and Ba^{2+} while inhibited by Hg^{2+}, Cu^{2+}, NH_4OH and EDTA suggesting it as a metal dependent serine protease. The alkaline protease was compatible with six local powdered detergent brands retaining more than 90% activity.

INTRODUCTION

Alkaline proteases alone account for 20% of the world enzyme market with their predominant use in detergent and leather processing industries (Kumar and Takagi, 1999; Oberoi et al., 2001). The application of such proteases requires their improved properties like usage at optimum to high temperatures, higher alkaline pH values, stability over a range of time etc. which make them detergent compatible (Gupta et al., 2002; Adinarayana et al., 2004). Furthermore, bacterial alkaline protease activity has been reported to be enhanced by various metal ions, surfactants etc. that also help to know their biochemical nature. In general, alkaline proteases have been reported to be serine centered or metallo centered (Kumar et al., 1999).

In the present study, Bacillus circulans, an isolate from vegetable waste was studied for characterization of its protease with respect to physiochemical properties and its detergent compatibility was evaluated.

MATERIAL AND METHODS

Microorganism and enzyme production- The bacterial strain selected among 32 isolates, for high protease activity was found to be a spore forming, gram positive, mobile, aerobic, halotolerant, rod shaped bacterium. It was identified as Bacillus circulans by Microbial Type Culture Collection Center and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India and deposited under accession number MTCC 7906. The enzyme was produced in Erlenmeyer flasks containing 100 ml of protease production medium, (containing gm $^{-1}$ of glucose-10, cotton deoiled meal-5, yeast extract-5, K_2HPO_4-1, $MgSO_4$-0.2 and Na_2CO_3-10, pH 10.5; Glucose and cotton deoiled meal in a CN ratio 40:1.4) incubated at 25 C with an agitation rate of 200 rpm for 96 hours on an orbital shaker. After spinning off the cells (10000rpm, 10 min, 4 C), the supernatant was partially purified.

Partial purification of crude protease- The crude alkaline protease was purified using ammonium sulphate (50-80%) and dialyzed against Tris-HCl buffer (pH 7.8) overnight with periodic change in buffer every 4 hours.

Partial characterization of alkaline protease- Alkaline protease from Bacillus circulans was characterized with respect to its optimal value of pH (7-11), temperature (30-80 C), substrate concentration (1-15 mg ml $^{-1}$) and enzyme concentration (0.05-0.5 ml per reaction mixture). The biochemical nature of the protease was characterized by studying the effect of metal ions (Ba^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+} & Co^{2+}) and inhibitors (EDTA & NH_4OH each at 10mM) on protease activity by incubating them in reaction mixture under optimized protease assay conditions.

Protease assay-The protease activity was determined in a reaction mixture of 0.1 ml enzyme, 2 ml of (0.5% w v $^{-1}$) casein solution (prepared in carbonate-bicarbonate buffer,

0.1M, pH 9.5) and 0.9 ml of distilled water and incubated at 60 C for 10 min. The proteins were precipitated by adding 3 ml of 0.5% TCA and free amino acids released by protease from casein hydrolysis were estimated by standard Lowry method. The protease activity was defined as mol of tyrosine released per minute per ml of enzyme.

Detergent compatibility studies and de-staining property- Six detergent brands (Surf excel, Ariel, Tide, Rin, Fena and Nirma) were used for studying compatibility of alkaline protease under buffered and normal conditions. Detergent solutions were prepared as per directions given on their respective sache. Casein solution (0.5% w v^{-1}) was used as substrate, was prepared either in buffer (carbonate-bicarbonate buffer, 0.1M, pH 9.5) i.e. for buffered conditions or in distilled water i.e. for normal conditions of assay. Both buffered and non-buffered solutions were used in reaction mixture comprising of 2 ml of casein solution, 0.9 ml of detergent solution and 0.1 ml of alkaline protease. The reaction mixture was incubated at optimized conditions for 10 minutes followed by protease assay. The de-staining property was studied by dipping two pieces of cloth artificially stained with blood either in detergent solution or detergent solution supplemented with enzyme followed by incubation for 10 minutes at 60 C.

RESULTS AND DISCUSSION

The alkaline protease of B. circulans was partially characterized and assessed for its compatibility with powder detergents.

Purification of crude alkaline protease- The purification of crude alkaline protease by ammonium sulphate precipitation carried out in two fractions of 0-50% and 50-80% followed by their dialysis revealed most of the activity in 0-50% fold fraction (Table 1). The crude enzyme was purified 2.45 folds. In literature, Kaur et al (1998) and Adinarayana et al (2004) reported similar results with most of the activity in 0-50% fraction and purification of 28.7 and 1.2 folds respectively.

Figure 1

Table 1: Partial purification of crude enzyme of by saturated ammonium sulphate (SAS).

Purification step	Total protein (g)	Volume (ml)	Protease activity (U ml^{-1})	Specific activity	Total activity	Recovery of enzyme (%)
Crude enzyme	1.825	250	405.8	222.3	6670.0	100
SAS						
▪ 0-50%	0.481	18	2813.2	5848.6	7237.2	87.7
▪ 50-80%	0.214	18	297.2	1388.6		12.3

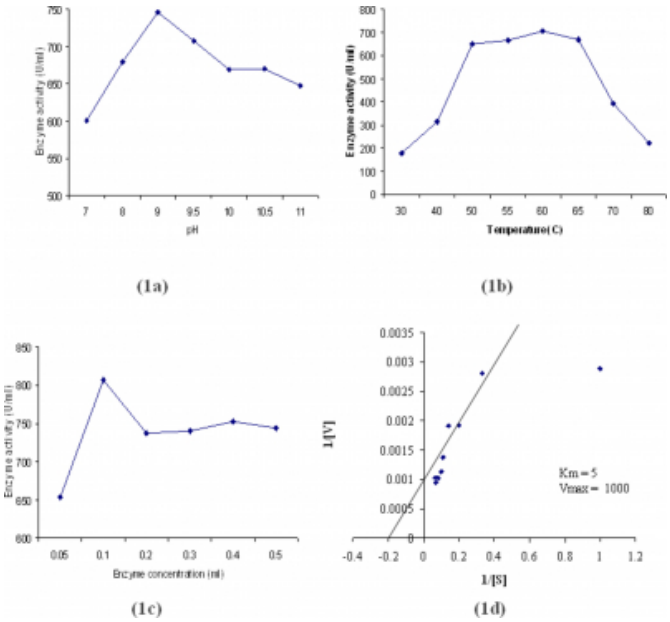
Effect of pH and temperature-The alkaline protease showed activity over broad pH (8-11) and temperature (40-65 C) ranges whereby more then 85% of enzyme activity was present (Fig.1a,1b). The pH and temperature maxima however, were 9 and 60 C respectively. In literature, both pH and temperature optima have been reported to be species specific and found to vary between 8-11 and 30-70 C respectively for alkaline protease of Bacillus sp (Wahyuntari et al. 2004, Kazan et al. 2005).

Effect of enzyme and substrate concentration- Among the different enzyme concentrations (0.05-0.5 ml) studied in a reaction mixture of 3ml, 0.1ml of enzyme that constitutes 3.33% of reaction mixture produced maximum protease activity (Fig. 1c). Kaur et al (1998) also reported 3.33% enzyme while Huang et al (2003) revealed as much as 50% of crude protease for obtaining maximum activity.

The use of different casein concentration (1-15 mg ml^{-1}) in a reaction mixture revealing a typical hyperbolic curve with 14mg ml^{-1} as the best concentration suggesting that enzyme follows Michael Menten's equation. A double reciprocal plot was prepared that showed an apparent Km of 5mg ml^{-1} and a Vmax of 1000 mol tyrosine min^{-1} ml^{-1} (Fig 1d). Similarly, Kaur et al (1998) reported Km of 3.7 mg ml^{-1} in B. polymyxa protease while Thangam and Rajkumar (2002) reported a Km and Vmax of 1.66 mg ml^{-1} , 526 U respectively for alkaline protease of Alcaligenes faecalis using casein as a substrate.

Figure 2

Figure 1: (1a) Behavior of alkaline protease of at different pH values. (1b) Behavior of alkaline protease of at different temperatures.(1c) Alkaline protease activity of enzyme of using different concentrtions of enzyme.(1d) Line weaver burke plot of alkaline protease produced by .



SDS-PAGE of partially purified protease- The purified protease was resolved on a SDS-PAGE gel showed a single band indicating a homogeneous preparation. The molecular weight of protease was determined by comparison of relative mobility of the standard marker proteins i.e. BSA (67 kDa) and Diastase (50 kDa). The comparison revealed that the protease band is 24.5 kDa (Table 2). In literature, the alkaline protease from Bacillus sp. is reported as a single band with molecular weight ranging from 16-32 kDa (Adinarayana et al. 2004; Kaur et al. 1998).

Figure 3

Table 2: Comparitive mobility of protein markers and purified alkaline protease.

Protein	Mobility (cm)	Molecular weight (kDa)
BSA	0.49	68.0
Diastase	0.44	50.0
Purified protease (0-50%)	0.844	24.5

Effect of metal ions and inhibitors- Among different metal ions studied for their influence on alkaline protease activity, the results presented in Table 3 revealed that the enzyme activity is increased by Ca^{2+} (49%), Mn^{2+} (35.6%), Zn^{2+} (30.6%), Co^{2+} (37.9%) and Ba^{2+} (26.1%), indicating that enzyme may be a metalloprotease. This fact was further strengthened by the use of a metal chelator EDTA that inhibited the enzyme activity by 73.6%.

Figure 4

Table 3: Activity of protease ( ) in the presence of different metal ions and/or inhibitors.

Metal ions / Inhibitors	Relative activity(%)
Zn^{2+}	130.6
Co^{2+}	127.9
Mn^{2+}	135.6
Ca^{2+}	149.0
Ba^{2+}	126.1
Hg^{2+}	4.6
Cu^{2+}	76.2
NH_4OH	77
EDTA	26.4

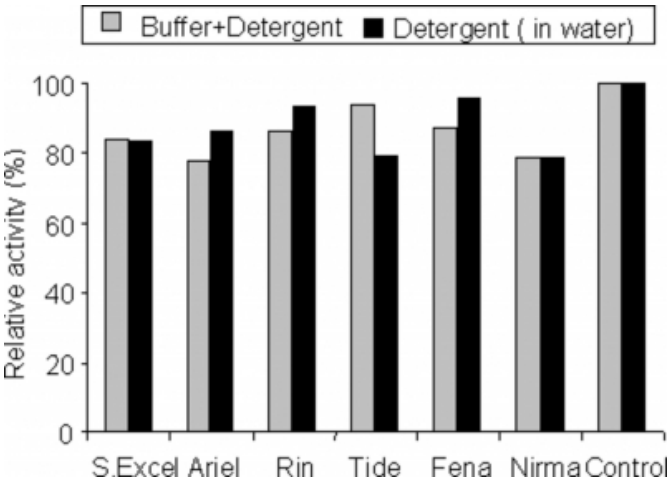
The two metal ions Hg^{2+} and Cu^{2+} inhibited the enzyme activity by 95.4% and 23.8% respectively. The liquor ammonia used at the rate of 10 mM in the incubated reaction mixture resulted in 23% reduction in alkaline protease activity, which suggested that the enzyme may be carrying serine residue at its active site also. The results thus suggest that alkaline protease of Bacillus circulans is either a mixture of proteases with metalloproteases being the major component or a metal dependent serine protease (Kaur et al 1998). Wahyuntari et al (2004) also reported metal dependent serine protease, the activity of which was decreased to 7% and 23.9% by EDTA and PMSF respectively. Elsewhere, alkaline proteases in the form of metalloproteases and/or serine proteases have been reported in literature. Whereas the former are normally activated in the presence of divalent ions like Cu^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} etc., heavy metal ions like Hg^{2+} and Cu^{2+} have been reported to be inhibitory to alkaline proteases (El-Sawh and El-Din, 2000; Singh et al. 2002). The serine proteases have been reported to be inhibited by compounds like PMSF and DFP etc (Kazan et al. 2005).

Compatibility with detergents and de-staining property- For the possible commercial exploitation of alkaline protease in

detergent industry, the alkaline protease was tested for its compatibility with six different detergents of common use. The enzyme incubated with detergent solution (either in water or in buffer) revealed that when used in water, Fena and Rin showed maximum compatibility whereas the buffered detergent solution revealed maximum compatibility of alkaline protease with Tide (Fig. 2).

Figure 5

Figure 2: Compatibility of alkaline protease of with commercial detergents.



Among the six detergents for detergents, four detergents were already containing the enzyme as quoted by their manufacturers. Therefore, their suitable controls were also run and their activities were found to be very low compared to those obtained by supplementation with enzyme of Bacillus circulans (Table 4). This revealed that the enzyme may be suitable supplement to detergents. The results of de-staining experiment showed the complete removal of stain in detergent solution supplemented with enzyme whereas blood stain was not completely removed from cloth dipped in detergent solution only (Fig 3). This suggested that enzyme may be useful to detergent industry.

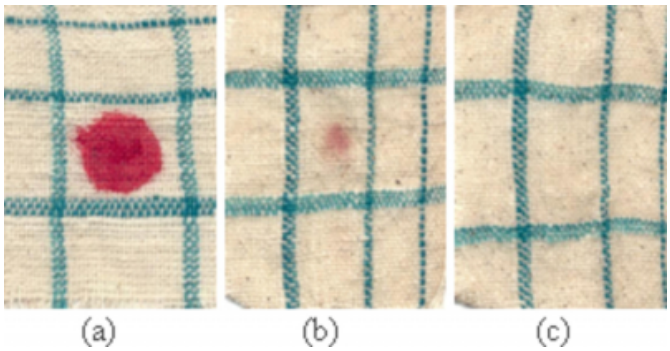
Figure 6

Table 4: Compatibility of alkaline protease of with commercial detergents.

Treatment	S.Excel	Ariel	Rin	Tide	Fena	Nirma	Control
Buffer+ Detergent	1061.69	977.07	1087.29	1188.27	1095.82	989.87	1258.67
Detergent (in water)	1053.16	1087.29	1180.44	996.98	1206.04	989.87	1258.67
Detergent without enzyme	239.64	99.56	28.44	301.51	NA	NA	NA

Figure 7

Figure 3: Blood de-staining activity of crude alkaline protease of (a, 0 minute; b, 10 minutes (detergent only); c, 10 minutes (detergent+enzyme).



Shelf life-The effect of storage of alkaline protease on its activity was determined as it is an important parameter for commercial utilization of enzyme. The enzyme stored at room temperature (30°C) for up to 30 days (Table 5) revealed that the enzyme is 91% active for 20 days and the activity is reduced to 65% in 30 days of storage suggesting that enzyme may be stored for 20 days at room temperature without much loss in its activity.

Figure 8

Table 5: Effect of storage on alkaline protease production in .

Storage Time (days)	Relative activity (%)
0	100.00
10	95.04
20	91.01
30	65.60

Storage temperature = 35°C ± 2°C

The present study thus reveals a metal dependent serine protease of Bacillus circulans MTCC 7906 which has been produced using cheap agricultural material i.e. Cotton deoiled meal. The protease is compatible with local detergents suggesting its potential as a detergent supplement for improved washing.

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