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Kinetic parameters analysis and pH stability of protease from a thermophilic *Bacillus* species

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Abstract: An extracellularly produced and purified protease from a thermophilic *Bacillus* strain was characterized on the basis of its stability at different pH levels to be used in various industries. Purified protease was stable over a broad pH range (5.0 to 10.0) and retained its 100% residual activity at pH-7.0 and 8.0 up to 30.0 minutes. It was also observed that 82% and 92% residual activity attained even after 120.0 minutes at pH-7.0 and 8.0, respectively. Such pH stability indicated that protease from *Bacillus* species can be a plausible candidate for various bioprocesses at commercial level. V_{max} and K_m values of purified protease were also determined by Lineweaver-Burk plot as 473 U min^{-1} and 2.3 mg ml^{-1}, respectively.

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INTRODUCTION

Proteolytic enzymes play significant role in different bioprocesses such as in synthesis of nutrients for cell development and proliferation[1]. Proteases symbolize major group of commercially available biocatalysts and account for more than 65% of the whole industrial enzyme market[2]. Different microorganisms have capability to hydrolyze the peptide bonds of protein by synthesizing protease extracellularly. Among them, *Bacillus* species comprise most of the desirable characteristics for maximum protease production that can be used in different biotechnological applications such as in dehairing process of leather industry and as an additive in detergent formulation[3]. Thermophilic organisms produce enzymes which are thermostable and have many commercial applications due to their inbuilt stability for extreme environment[4]. Currently many biotechnological and industrial processes are using more than 3000 of identified enzymes. However, these enzymes are not fulfilling the entire demand for industrial extreme conditions. A main reason for this is the fact that extreme conditions can cause enzyme denaturation that leads to loss of enzymatic activity[5]. Until now, only few proteases from thermophilic *Bacillus* species have been isolated that can withstand extreme reaction conditions including high pH and temperature. The demand for heat stable and broad pH tolerating proteases are increasing that are involved in fast reaction rate, increase the solubility of non-gaseous reactants and reduce the microbial contamination[6]. In the past few years, the catalytic properties of proteases were also enhanced by protein and genetic engineering techniques. Additionally, the use of recombinant microbes also improved the yield of extracellular proteases in the culture medium by the over expressed protease gene[7]. Alkaline proteases are required in different industries including food, textile, pharmaceutical, poultry and silk degumming processing industries[8-9].

In the current investigation, an attempt was made to characterize the purified protease on the basis of pH stability for the maximum catalytic activity of an enzyme to be used in different industrial applications. Kinetic parameters were also calculated by using lineweaver-Burk plot.

MATERIALS AND METHODS

All chemicals used were of analytical grade and casein was purchased from sigma. A high temperature tolerating *Bacillus* species was obtained from the culture bank of Department of Biotechnology, University of Karachi, Karachi. Bacterial culture was inoculated in 100.0 ml Luria Bertani's medium supplemented with 10.0 g L^{-1} casein and transferred to 900.0 ml same fermentation medium. It was incubated at 60°C for 72.0 hours at 120.0 rpm and was centrifuged at 5000 rpm for 10.0 minutes at 4°C. Cell free filtrate (CFF) was concentrated by rotary evaporator at 37ºC. The protease was purified using 80.0% ammonium sulfate followed by CM-cellulose and DEAE-cellulose column chromatography.

The protease activity[10] was determined by incubating 0.5 ml of enzyme with 0.5 ml of 10.0 mg ml^{-1} casein (prepared in 0.1M Tris-HCl buffer with pH-8.0) at 37°C and reaction was ceased by adding 1.5 ml of 0.3 M trichloroacetic acid. Centrifugation was performed at 10,000 rpm for 10.0 minutes at 4°C and enzyme assay was performed in triplicates. Absorbance was measured by spectrophotometer at 280 nm. One kunitz unit was defined as "the amount of enzyme that under test condition affects a ΔA_{280} of 0.001". Concentration of protein was determined

by dye binding method[11]. A standard curve of Bovine serum albumin (BSA) was plotted by using different concentrations ranging from 10.0 to 100.0 µg and absorbance was measured at the wavelength of 595 nm.

Enzyme stability at various pH levels (5.0 to 10.0) was investigated for 2.0 hours at 80°C and enzyme aliquots were retrieved with 30.0 time intervals of minutes. Enzyme activity was performed under standard assay conditions and percent residual activity was calculated. Sodium acetate buffers (pH-4.0 to 5.0), sodium phosphate buffer (pH-6.0), disodium hydrogen phosphate buffer (pH-7.0), Tris-HCl (pH-8.0) and Glycine-NaOH (pH-9.0 to 10.0) with same ionic strength of 50.0 mM were investigated.

Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of protease were calculated by measuring the enzyme activity at different substrate concentrations ranging from 2.50 to 25.0 mg ml^{-1} (dissolved in 0.1M Tris-HCl buffer with pH-8.0). GraphPad prism 6 software was employed to fit the initial velocity data into Michaelis-Menten constant and Lineweaver-Burk equations using its enzyme kinetics module.

RESULTS AND DISCUSSION

Purified protease from *Bacillus* species was characterized on the basis of stability at different pH levels and kinetic parameters were calculated by varying substrate concentration. The stability of protease against various pH values is a critical property for its commercial applications. Considering its utility, protease stability was determined by pre-incubating enzyme without substrate at different pH range (5.0-10.0) for 2.0 hours. It was observed that enzyme is stable over a wide pH range and retained its 100% activity at pH-7.0 and 8.0 up to 30.0 minutes (Figure 1).

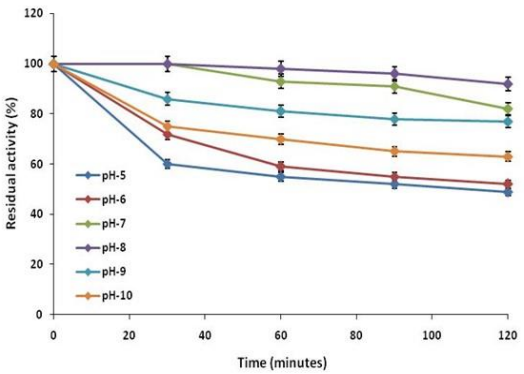

Figure 1: pH stability of protease from *Bacillus* species (Mean±S.E, n=3).

However, any alteration in pH beyond aforementioned pH values either towards the alkaline or acidic condition caused reduction in enzyme stability. The pH may create the reversible or irreversible effect on the stability of enzyme. Reversible effect appears due to protonation of the amino acids in the active site of enzyme and ultimately changes the native conformation of enzyme[12]. It has been observed that different proteases can vary in their pH stability range. The protease from *Bacillus megaterium* was observed to be 78% active in the 7.0-8.0 pH range and revealed 100% activity at pH-7.5[13]. Aspartic protease from *Rhizopus oryzae* exhibited high activity between pH values of 3.0-3.6 at 75 °C with optimum activity at pH-3.4 in Glycine-HCl buffer[14]. Current finding indicates that protease from *Bacillus* species is alkaline in nature and can be exploited in different industrial processes such as in dehairing process of leather industry[3].

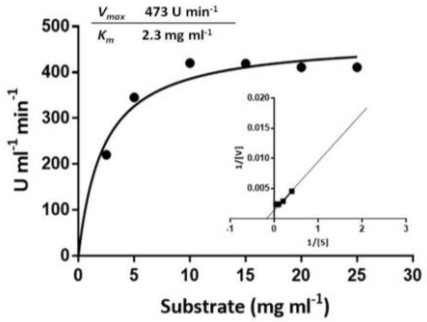

Figure 2: Kinetic parameters of protease from *Bacillus* species.

Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were also calculated by measuring catalytic activity of protease at different substrate concentration (casein) ranging from 2.5-25 mg ml^{-1}, while keeping the temperature and pH constant (Figure 2). K_m indicates the affinity of an enzyme towards its substrate. However, V_{max} represents the higher reaction rate of an enzyme at its saturation level. V_{max} and K_m values of protease were calculated by Lineweaver-Burk plot as 473 U min^{-1} and 2.3 mg ml^{-1}, respectively. Kinetic values vary among different enzymes which may always be observed due to disparity in assay procedure, reaction environment and type of substrate used[15]. It has been reported that protease from *Bacillus subtilis* showed V_{max} of 148 U ml^{-1} with K_m value of 58 µM using casein as a substrate[16]. Purified protease from *Aspergillus flavus* has V_{max} and K_m value of 60.0 U mg^{-1} and 0.6 mg ml^{-1}, respectively[17].

CONCLUSION

It can be concluded that protease from thermophilic *Bacillus* strain can be a best candidate for different biotechnological applications due to its broad pH stability range.

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