Effect of Metal Ions, Solvents and Surfactants on the Activity of Protease from *Aspergillus niger* KIBGE-IB36

Hafsa Sattar[1], Afsheen Aman[1] and Shah Ali Ul Qader[2,*]

[1]*The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi-75270, Pakistan*

[2]*Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan*

Abstract: Metal ions greatly impact on the enzymatic activity, they may form strong interaction by forming coordinate bond with enzyme-substrate at the catalytic site which may activate, inhibit or stabilized the enzyme molecules. In this study, extracellular protease from *Aspergillus niger* KIBGE-IB36 was precipitated with 40% ammonium sulfate. It was revealed that K^+, Ba^{2+}, Na^+, Mg^{2+} Zn^{2+}, Ca^{2+} boosted the protease activity whereas, Cs^+, Mn^{2+}, Cu^{2+}, Ni^{2+}, V^{2+}, Co^{2+}, Hg^{2+} and Al^{3+} showed to be inhibitor of protease. Dimethyl sulfoxide (5.0 mM) and methanol (5.0 mM) showed catalytic activity while ethanol at same concentration exhibited inhibitory effect. Protease activity augmented with Tween 80, while SDS, Triton X-100, EDTA and PMSF exhibited inhibitory effect.

Keywords: Metals, Inhibition, Activation, Protease, *Aspergillus niger*, Organic Solvents.

INTRODUCTION

Proteases or peptidases belongs from hydrolase family of enzyme and that catabolized the peptide bonds of the protein molecule. Proteases accounts for approximately 60% sale of enzyme [1]. Due to diversified catalytic properties proteases possess wide applications in food processing and leather tannery industries. Extensively utilized in meat tenderization and recovery process of silver. Considering its huge demand, microbial proteases are being studied broadly that have potential to produce enzyme in bulk capacity with better catalytic activity [2, 3]. Nevertheless, enzymes from fungal sources have attracted biotechnologist as compared to bacterial. Fungus can cultivate on low cost effective substrate and produced bulk quantity of enzyme in the fermented media. More stable enzyme also produced from them. The extracellular enzyme obtained can also be recovered by simple downstream process [4]. Among various fungal sources, *Aspergillus species* are considered as safe (GRAS) and approved by Food and Drug Administration (FDA). It is also reported that *Aspergillus niger* can synthesize protease, amylase cellulose and pectinase [5]. Currently, in 2013 *Aspergillus niger* has been utilized in animal feeds to upgrade the nutritional content of food in China [6]. The biocatalytic activity of these enzymes is mainly influenced by metal ions which as activator or inhibitor. Generally metal ions act as electrophiles that holds the functional groups in three dimensional orientations and it also form enzyme – substrate interaction by coordinate bond formation apart from this, metal ions also stabilize the catalytic active site of enzymes [7]. Therefore, metal ions in this regards plays essential part in the activation and stabilization of enzymes [8]. In several cases, solvents and surfactants also put positive impact altering hydrophobicity of enzyme molecule or by increase the solubility of substrates to recover product [9]. Therefore, in this contemporary study extracellular protease was produced from *Aspergillus niger* KIBGE-IB36 and purified by salt precipitation. Different metal ions, solvents and surfactants effect was also determined to study the proteolytic activity.

MATERIALS AND METHODS

Enzyme Production and Purification

The pure fungal strain of *Aspergillus niger* KIBGE-IB36 was isolated previously and selected for protease production [10]. The selected strain was fermented at 30ºC for 120 hours in medium containing (%): Casein, 0.25; Peptone, 2; Yeast Extract, 0.05; Glucose, 0.25; Dipotassium hydrogen phosphate, 0.3; Magnesium chloride, 0.01 and Calcium chloride, 0.01 at pH: 6.0. The thick pallet of mycelium was separated by filter paper and allowed for centrifugation at 10000 rpm for 15.0 minutes at 4ºC. The spore free filtrate was subjected to ammonium sulfate precipitation. The precipitates were obtained by centrifugation at 10,000 rpm at 0ºC for 20 minutes. The precipitates were dissolve in Tris-HCl buffer (pH: 5) and stored at 4ºC. After which the activity of protease was estimated by modified procedure of Anson using casein as the

*Address correspondence to this author at the Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan; Tel: 923212160109;
E-mail: saqader@uok.edu.pk

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substrate [11]. Partially purified protease (0.5ml) was added to 1.0 ml casein (0.5 % casein in 50mM Tris-HCl Buffer, pH 5). The enzyme and substrate was allowed to be incubated for 15 mins at 50ºC. 10% TCA (Trichloro acetic acid) was added to terminate the reaction. The reaction tubes were incubated at 37ºC for 30 mins. After incubation of 30 mins the precipitates of unhydrolyzed casein was removed from filter paper, 2ml of filtrate was mixed with 5 ml of 0.5M of Na2CO3, followed by addition of Folin and Ciocaltea reagent. The released tyrosine was measured spectrophotometrically at 660nm. "One unit of enzyme defined as the amount of protease required to hydrolysed casein to produce colour equivalent to 0.1 micro Mole of tyrosine under standard assay condition "The total protein content of cell free filtrate was estimated through Lowry *et al*. method using bovine serum albumin as standard [12].

Effect of Metal Ions

Metal ions exert pronounced effect on enzyme activity, they may speed up the activity or inhibit the enzyme-substrate reaction. The purified protease was then kept with different concentration (1mM, 5mM and 10mM) of salt solutions for 60.0 and 120 minutes at 37ºC. After every 60 minutes the samples were collected to estimate protease activity. The influence of metal ions were observed. These salts were used in recent study: BaCl2, $CoCl_2$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$, $CaCl_2$, $CsCl_2$, NaCl, KCl, $NiCl_2$, $HgCl_2$, $FeCl_3$, VoSO4 and $AlCl_3$.

Effect of Solvents

Different concentration (1mM and 5mM) of DMSO, isopropanol, methanol and ethanol were observed by incubating protease enzyme in 1:1 ratio of these solvents. Enzyme activity of protease was detected after every 30 minutes.

Effect of Surfactants, Metal Chelators and Inhibitors on Protease Activity

Effect of Tween 80, Triton X-100, SDS, EDTA and PMSF were also investigated at 1mM and 5mM concentration by incubating purified protease with above mention solvents for 30 minutes at 37ºC in 1:1 ratio.

RESULTS AND DISCUSSION

Protease from *Aspergillus niger* KIBGE-IB36 was partially purified up to 7.32 folds with maximum specific activity 161.25 U/mg (Table **1**).

Influence of Metal Ions

Various concentrations ranging from 1.0 mM, 5.0 mM and 10mM of metal ions were investigated under optimized assay conditions. It was observed that monovalent cation Na+ improved the protease activity from *A. niger* KIBGE-IB36. The increment in activity was observed around 1.5 folds (1mM) and 1.3 folds (5mM) when incubated for 120 minutes with enzyme (Table **2**). Divalent cations such as Ca^{2+}, Mn^{2+}, Zn^{2+} and Mg^{2+} with1.0 mM and 5.0mM concentration, protease from A.nigerKIBGE-IB36 retained more than 100% activity. The increment in activity was up to 123%, 136% and 145% at 1mM, 5mM and 10mM respectively when incubated with calcium ions. Similarly, metalloprotease from *Candida kefyr* 41 PSB showed 106.0% activity at 5.0 mM of Ca^{++}, Mg^{++}, Mn^{++} [15]. Previously, it has been reported that *Bacillus pumilus* serine protease improved enzymatic activity with calcium ions[16].A noticeable inhibition was observed with Cu2+, Fe2+, and Hg2+. Parallel results were also found from metalloprotease from Candida kefyr 41 PSB [15]. The complete inhibition of protease activity was observed by Hg^{++} and Cu^{++} which may be due to the interaction of sulphur groups of proteins which changes the conformational of enzyme [17]. Whereas, Cu^{++} ions attack cysteine in the amino acid sequence to inhibit the protease [18]. The interaction of Cu^{++} to His inhibits the imidazol group and reduced the catalytic performance of protease [19]. The inhibitory effect of Al^{3+} was observed from *A. niger* KIBGE-IB36 up to some extent which showed 45% and 23% relative activity at 1.0 mM and 5.0 mM concentrations, respectively.

Table 1: Partial Purification of Crude Protease from *Aspergillus niger* KIBGE-IB36

Purification steps	Enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude enzyme	24064	2336	101.96	1	100
Precipitates	7630	12	635	6.2	31.0
Dialysis	5160	6	860	8.5	67

Table 2: Effect of Different Metal Ions on the Catalytic Activity of Protease from *Aspergillus nigers* KIBGE-IB36

Metal Ions	Relative Activity (%)					
	60 minutes			120 minutes		
	1mM	5mM	10mM	1mM	5mM	10mM
Control	100			100		
K^{+}	90	78	67	87	67	45
Na^{+}	136	122	100	157	135	119
Cs^{+}	67	54	34	56	34	15
Ba^{+2}	100	100	100	100	100	100
Ca^{+2}	123	130	130	123	136	145
Co^{+2}	48	25	19	42	21	15
Cu^{+2}	43	21	12	34	19	10
Fe^{+2}	85	70	60	78	61	40
Hg^{+2}	92	84	76	80	71	60
Mg^{+2}	100	120	129	100	110	100
Mn^{+2}	110	125	124	112	121	90
Ni^{+2}	67	34	21	56	23	0
V^{+2}	0	0	0	0	0	0
Zn^{+2}	128	110	105	125	110	100
Al^{+3}	45	23	0	39	20	0

Influence of Organic Solvents

Various organic and inorganic solvents were observed 1.0 mM and 5.0 mM concentrations. From the experimental observation it was found that ethanol, methanol, isopropanol and DMSO improved activity that displayed 108%, 112%, 100% and 121% relative activity at 1.0 mM concentration of these organic solvents, respectively. At 5.0mM concentration of methanol and DMSO showed stable enzymatic activity (Table **3**). Previously, it has been reported that the protease used in non-aquous media can increase the solubility of substrates and products which eventually facilitate the recovery of product [9]. However, 5.0 mM concentration of ethanol and Isopropanol displayed reduction of activity by 23% and 44% respectively. Weak interactions between the protein structures may alter by solvents.

Influence of Surfactant, Chelators and Inhibitors

The influence of surfactants, chelator and inhibitors on protease activity is concised in Table **4**. It was noticed that catalytic activity was considerably improved in case of Tween 80 and Triton X-100 (1.0 mM) and up to 32% and 20% respectively.

Further increased in concentration (5.0 mM) of Tween 80 and Triton X-100 stabilized the protease activity. Previously it has been reported that non-ionic detergents such as Tween-X 100 and Tween-20 improved the catalytic performance of protease which was isolated from *Bacillus amylolique faciens* SP1 [14]. The partially purified protease from *A. niger* KIBGE-IB36 showed decreased in catalytic activity when protease was kept with 1.0 mM SDS. By further increased in concentration of SDS, activity was dropped. Parallel results were found from alkaline

Table 3: Influence of Organic Solvents on the Activity of Protease

Solvents	Relative activity (%)	
	1.0 mM	5.0 mM
DMSO	121	100
Isopropanol	100	56
Methanol	112	100
Ethanol	108	86

Table 4: Effect of Different Surfactants, Metal Ions Chelator and Inhibitors on the Activity of Protease

Surfactants, chelator and inhibitor	Relative activity (%)	
	1.0 mM	5.0 mM
Control	100	100
Tween 80	132	105
Triton X-100	120	100
SDS	89	36
EDTA	25	10
PMSF	20	0

protease mixed with SDS (1%) and incubated for 4.0 hours [14]. SDS has been reported to showed inhibitory effect of xylanase from *Paenibacillus campinasensis* G1-1 [20].

Protease activity was also evaluated for group classification of protease by investigating inhibitory effect. It was found that protease activity was completely inhibited when purified protease mixed with metal chelator EDTA and PMSF (serine inhibitor). The results suggested that protease belong from serine family of metalloprotease. Similar findings were detected in case of halo-alkaline protease from *Geobacillus toebii* strain LBT 77 revealed inhibitory effect in the presence of PMSF and EDTA [21].

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