Research Article **Biochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ENZYME KINETICS OF PROTEASE FROM *HYPSIZYGUS ULMARIUS*

SHIVASHANKAR M AND PREMKUMARI B*

Department of Life Sciences/Sericulture, Sneha Bhavana, JB Campus, Bangalore University, Bangalore- 560056, Karnataka, India.

ABSTRACT

Enzyme kinetics of protease activity from extract of edible mushroom, *Hypsizygus ulmarius* were carried out. The optimum P^H and temperature for protease activity were found to be at 10 and 40°C respectively. The P^H and temperature stability profile shows that protease enzyme is stable under alkaline conditions and up to 50°C. The extract had the greatest activity in 1% of casein as a substrate with K_m and V_{max} of 0.25mM and 0.45mM respectively. The effect of metals on activity of protease was greatly enhanced with NaCl and $NiSO_4$, However, 100% activity was seen with addition of $FeSO_4$, and decreased with $ZnSO_4$. Drastic inhibition of protease activity was seen in PMSF at 10mM.

KEYWORDS - ***Hypsizygus ulmarius,*** **Protease, Casein, Mushroom, Enzyme.**

PREMKUMARI B
Department of Life Sciences/Sericulture, Sneha Bhavana, JB Campus, Bangalore University, Bangalore- 560056, Karnataka, India.

INTRODUCTION

Protease (peptide hydrolases) constitutes a large complex group of enzyme which play an important nutritional and regulatory role in nature. These are (physiologically) necessary for living organisms as they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. They are degradative enzymes which catalyze the hydrolysis of proteins[1]. Protease accounts for about 60% of the total enzymes market in the world and 40% of the total worldwide enzyme sales[11, 6]. Alkaline proteases produced by microorganisms are of interest from a biotechnological perspective, and are investigated not only in scientific fields of protein chemistry and protein engineering but also in applied fields[14]. Among the industrial enzymes, 75% are hydrolytic in which 60% is contributed by proteases[23]. Proteases have diverse industrial applications in peptide synthesis, protein processing[20], bioprocessing of used X-ray films, leather[12], dairy and detergent industries[14, 19, 28]. So attention has been payed on proteases significance in cellular and commercial context. Mushrooms belong to the plant kingdom fungi which are fleshy, spore bearing fruiting bodies renowned for their nutritive and medicinal values. *Hypsizygus ulmarius* (Elm oyster) is relatively rare mushroom similar to oyster mushroom that belongs to phylum: Basidomycota, Class: Agaricomycetes, Order**:** Agaricales, Family**:** Lyophyllaceae, Genus: *Hypsizygus.* In nature it grows on elms and beech. This mushroom is superior in terms of yield, texture, flavor and shelf life. The niacin content is ten times higher than in any vegetables. The folic acid present in this mushroom is used to cure anemia. Since *Hypsizygus ulmarius* is a high yielding edible mushroom rich in proteins, vitamin C and B a commercial cultivation technology has been released and is gaining popularity. This mushroom is rich in antioxidants and exhibited antidiabetic activity[18]. The purification and characterization of laccase enzyme and its role in decolorization of different dyes has reported [24, 25]. Recent research has been focused on proteases and their purification, characterization for their potential applications in the industries [26, 27, 4, 21, 22, 29, 30, 32, 34, 35] and even in some clinical applications that include immunomodulatory and hypocholesterolemic actions, antitumor, anti-inflammatory, anti-allergic, anticoagulation, antithrombin activity as well as fibrino(geno)lysis stimulation [17, 31].

MATERIALS AND METHODS

Hypsizygus ulmarius mushroom were grown in association with Vinayaka mushroom cultivators, Hebbal, Bangalore Karnataka. The reagents and chemicals used were of analytical grade from Sigma Chemical Co. (St. Louis, MO).

Enzyme extraction

Crude enzyme extracts were prepared according to procedure explained [8, 13, 10]. The fresh fruiting bodies of mushroom were isolated, freeze dried and ground to a fine powder in liquid nitrogen for 15 min using a pre-chilled ceramic mortar and pestle. 80 g of cold mushroom sample were separately homogenised in 100 ml of 0.15 M NaCl using blender and left overnight on stirrer. The homogenate were centrifuged at 12,000 rpm for 45 min at 4^{o}C. The supernatant was measured and saved for further analysis.

Protein Determination

Protein content was determined using bovine serum albumin (BSA) as standard[16].

Protease Assay

The assay was carried out according to the method of [33], with slight modifications. Test sample (40 µl) of *Hypsizygus ulmarius* protease was mixed with 120 µl of 1% casein in 20 mM Tris-HCl P^{H}-9.0. The reaction mixture was incubated at 37 ^{o}C for 30 min, following incubation; 340 µl of 5% trichloroacetic acid (TCA) was added to the mixture. After centrifugation at 10,000 rpm for 10 min, the absorbance of the supernatant was read at 280 nm. A negative control (prepared by adding TCA to the enzyme to denature the enzyme prior to the addition of the substrate and thus prevent the enzymatic reaction from taking place) was run simultaneously. An increase in absorbance of

0.001 at 280nm/ml of reaction mixture/minute under standard experimental conditions is equal to one unit of protease activity.Tyrosine was used as a standard.

Enzyme kinetics

Determination of kinetic constant

Casein at different concentration (0.1%, 0.2%, 0.5%, 1%, 2%, and 5%) was used as substrate. The Km and Vmax of the enzyme was calculated based on Lineweaver-Burk plot constructed by plotting the reciprocal of substrate concentration on the X-axis and reciprocal of the enzyme reaction velocity on the Y-axis[15]. The experiment was repeated thrice and results were reproducible.

Effect of P^H and stability on enzyme activity

100 mM citrate phosphate buffer (P^H 3.0 & 4.0), 20 mM acetate buffer (P^H 5.0), 50 mM phosphate buffer (P^H 6.0, 7.0 & 8.0), Tris–HCl buffer (P^H 9.0) and 50 mM sodium phosphate-sodium hydroxide buffer (P^H10.0, 11.0 & 12.0) were used to find the optimal P^H for the proteolytic activity. The assay was performed by incubating 40 µl of enzyme in different buffers of P^H-3.0-12 for 30 min at 37^0C and assayed for protease activity using 1% casein as substrate. The same buffers were used to determine P^H stability. The enzyme and buffer were incubated for 24 h at room temperature and the standard enzyme assay described previously was performed. The unincubated enzyme is used to calculate percentage relative activity. . All the experiments were repeated thrice and the results were reproducible and statistically significant.

Effect of temperature and stability on enzyme activity

The above procedure was used to determine the optimum temperature for proteolytic activity at various temperature of 20–80^{o}C. Thermal stability were determined with enzyme in Eppendorf tube incubating at different temperatures (20-80 ^{o}C) for different intervals of time followed by rapid cooling in an ice bath and then brought to room temperature. The enzyme was then assayed and the percentage residual activities were calculated by comparison with unincubated enzyme. All the experiments were repeated thrice and the results were reproducible.

Effect of metal ions on enzyme activity

Effects of few mono and divalent metal ions (Na^+, $Zn2^+$, Li^+, $Ni2^+$, $Mg2^+$, $Cu2^+$, $Fe2^+$and $Ca2^+$) at 10 mM concentration on proteolytic activity were investigated. The percentage residual activities were determined by comparison with the standard assay mixture with absence of metal ion. All the experiments were repeated thrice and the results were reproducible.

Effect of Inhibitors on enzyme activity

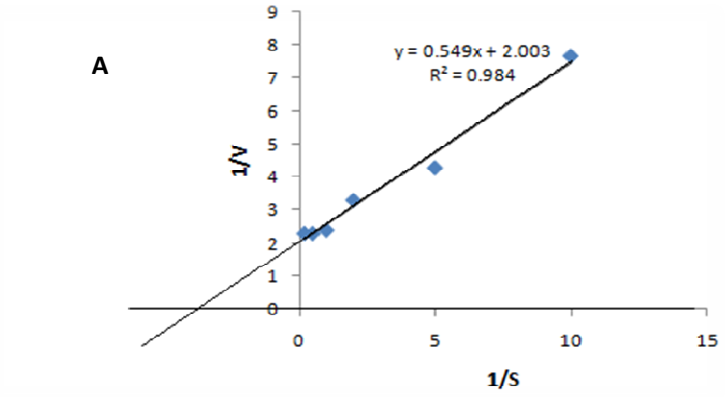
The effect of inhibitors, phenyl methane sulfonyl fluoride (PMSF) and ethylene diamine terta aceticacid (EDTA) at different concentration (0.04, 0.2, 1.0,10 mM) on the enzyme activity was determined by its pre-incubation at RT for 30 min and relative activity was measured. The enzyme activity of a control sample (without any inhibitors and other chemicals) was taken as 100. All the experiments were repeated thrice and the results were reproducible.

RESULTS

The enzyme protease tested on different concentration of substrate, casein showed the highest activity at 1% with Km and Vmax of 0.25 mM and 0.45 mM (Graph-A).

Graph– A

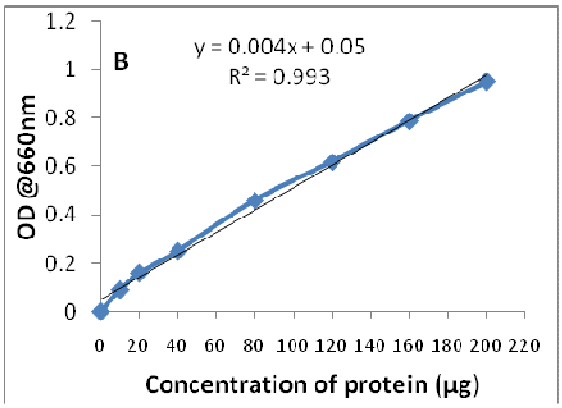
Determination of K_m and V_{max} by Line weaver-Burk plot for Hypsizygus ulmarius protease



The protein and enzyme activity were found to be 3.5mg/ml and 278µmole/ml of crude mushroom sample used (Graph-B & C).

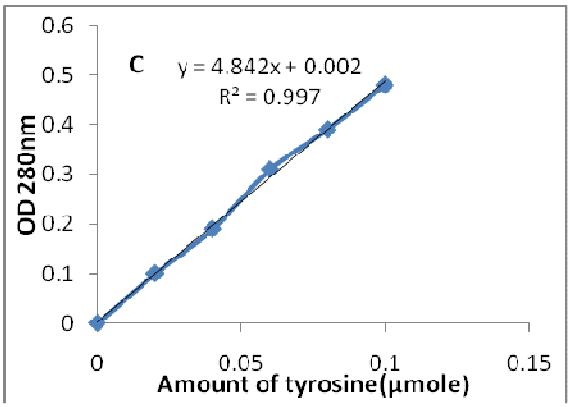
Graph –B

Standard graph to determine the total protein content µg



Graph – C

Determination of the total protease activity

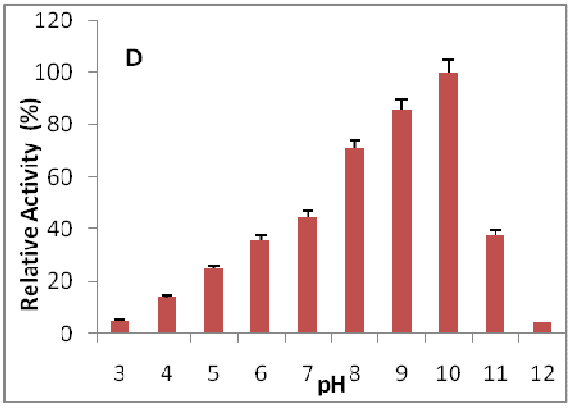


The effect of pH on protease activity and P^H stability using 1% casein is shown in Graph-D and Graph-E. The optimal P^H were found to be 10.0 with relative activity of 100%. However at P^H 8 and

9, the stability of the enzyme retained with relative activity between 70-85% when incubated for 1hr at RT. It was found that the protease enzyme is less stable at P^H below neutral.

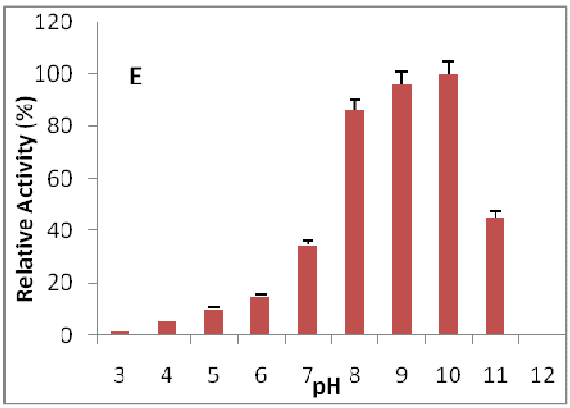
Graph – D

Effect of P^H on protease activity of Hypsizygus ulmarius.



Graph – E

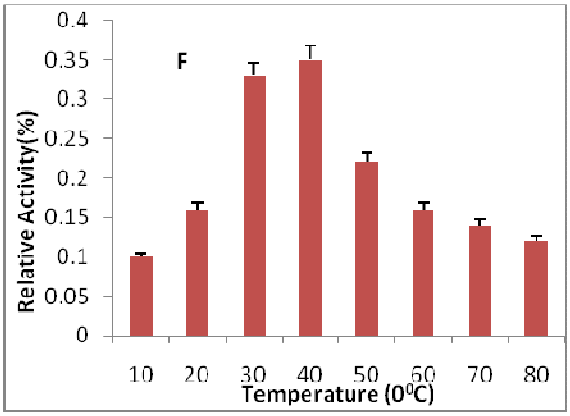
P^H stability profile on protease activity of Hypsizygus ulmarius.



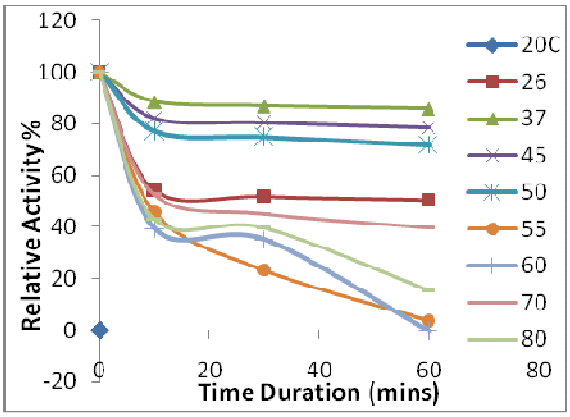
The optimum temperature for protease activity from *Hypsizygus ulmarius* was found to be between 40^0C as shown in Graph-F and was stable up to 50 ^{o}C with relative activity of 94% which is represented in Graph-G.

Graph – F

Effect of temperature on protease activity of *Hypsizygus ulmarius*

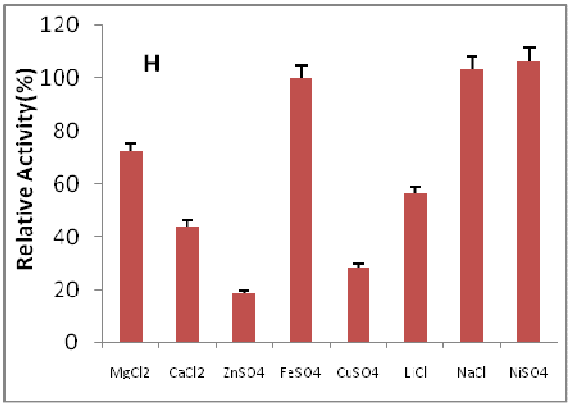


Graph – G
Thermal stability profile for protease activity of *Hypsizygus ulmarius*.



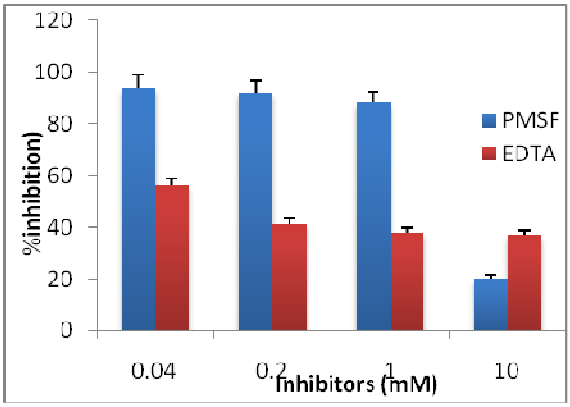
100% protease activity was observed with metal $FeSO_4$ and highly enhanced activity was seen with NaCl and $NiSO_4$. However inhibitory activity was observed for metals like $ZnSO_4$, $CuSO_4$, $CaCl_2$, LiCl and $MgCl_2$ at 10mM concentration (Graph-H).

Graph – H
Effect of metals on the activity of protease from Hypsizygus ulmarius



Among the two inhibitors tested, phenylmethanesulfonyl fluoride (PMSF) showed maximum inhibition of protease activity (20.35%), when compared to ethylene diamine tetra aceticacid(EDTA) with 36.98% at 10mM concentration (Graph-I).

Graph – I
Effect of inhibitors on the activity of protease from Hypsizygus ulmarius



DISCUSSION

In the present investigation, the enzyme kinetics of protease from *Hypsizygus ulmarius* was studied which showed highest activity at P^H-10. It is suggested that *Hypsizygus ulmarius* protease could be classified as the family of alkaline proteases since they exhibit highest activity in the alkaline conditions and they may represent serine [29, 9]. The P^H is higher when compared to the protease from *Hypsizygus marmoreus* [34] and Metalloprotease from wild basidomycete mushroom *Lepistanuda* [33]. It is known that changes in P^Hcan cause change in the activity however the enzyme is active even at P^H 9 usually this alkaline condition brings denaturation thereby resulting in the loss of catalytic activity. The stability of the enzyme in various conditions like acidic, neutral and alkaline, is important which could bring the quality in terms of industrial applications. Since temperature influences protein denaturation, enzyme inhibition and cell growth, it plays a significant role in development of the biological process. The optimal temperature for enzymatic activity of protease is obtained at 35-40 ^oC more or less to fungal proteases from *A. bisporus* (35 °C) [2, 3], but stable up 50 ^oC similar to *Hypsizygus marmoreus* (50 ^oC) [35]. The metal ions are an important factor that affects enzyme production. Enzyme enhancing or decreasing activity may be attributed to the metal ions properties towards protein ligands [7]. The enzyme activity was strongly inhibited by Zn^{2+} ions as reported by *Lepistanuda* mushroom [33] at 10 mM concentration but significantly increased by Fe^{2+} similar to serine protease in human erythrocyte membranes [5]. In this study Fe^{2+} may trigger the protease to form a compact structure or help to maintain the activity of the protease during the reaction. Among the two protease inhibitors tested PMSF at a concentration of 10 mM remarkably reduces the protease activity indicating the existence of serine residue at the active site and it has a requirement for metal ions as a cofactor [29]. In general EDTA has been known to remove some essential metal ion from the enzyme molecule resulting in the inactivation of enzyme.

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