PARTIAL CHARACTERISATION AND THERAPEUTIC APPLICATION OF PROTEASE FROM A FUNGAL SPECIES

ABSTRACT

Proteases find wide application in textile, leather, food and pharmaceutical industries. Also this enzyme has considerable therapeutic importance in treating inflammation, digestive and vascular conditions. A protease obtained from a fungal isolate, *Aspergillus niger*, was tested for clinical application. The micro-organism was cultivated on a starch-casein medium. The proteolytic activity was assayed with casein substrate using Folin-Ciocalteu's method having tyrosine standard. The enzyme was precipitated using 50% ammonium sulphate. Inhibition studies on EDTA, phenyl methyl sulphonyl fluoride, dithiothreitol and pepstatin A revealed that the enzyme is an acid protease. The precipitated protease exhibited significant dehairing, destaining, declotting activites on Wistar rat model. The enzyme showed prominent anti-inflammatory activity and results were compared using diclofenac sodium.

Keywords: Acid protease, *Aspergillus niger*, Anti-inflammatory activity, De-clotting activity, De-staining, De-hairing.

INTRODUCTION

Proteases find wide applications in textile, leather, food and pharmaceutical industries [1]. Majority of these enzymes have application in textile and detergent industries [2]. Proteases are used as denture cleaners and in cleaning contact lens [3, 4]. Microbial proteases are increasingly used in treatment of various disorders viz., cancer, inflammation, cardiovascular disorders, necrotic wounds etc [5, 6]. Proteases which find applications in leather industry due to their un-hairing properties could be tried in cosmetics [7]. Proteases are also used as immune-stimulatory agents [8]. Although extensive work on proteases was reported in literature [9, 10], therapeutic application studies of protease is meagre and there are only few marketed products. These include papain, bromelain, serrapeptidase, lysostaphin, L-asparaginase and streptokinase [5]. So a study was envisaged in this area.

The experimental work was carried out using a fungal culture *Aspergillus niger* isolated from paddy soil [11]. This protease was tested for some therapeutic activities viz., de-staining, de-clotting, anti-inflammatory and de-hairing activities.

MATERIALS AND METHODS

Dehydrated media viz., peptone, yeast extract, soluble starch, potato dextrose agar were procured from Hi-media (India). Ammonium sulphate, magnesium sulphate, Folin-Ciocalteu's phenol reagent and other fine chemicals were obtained from Merck Pvt. Ltd., Mumbai.

Micro-organism and Maintenance

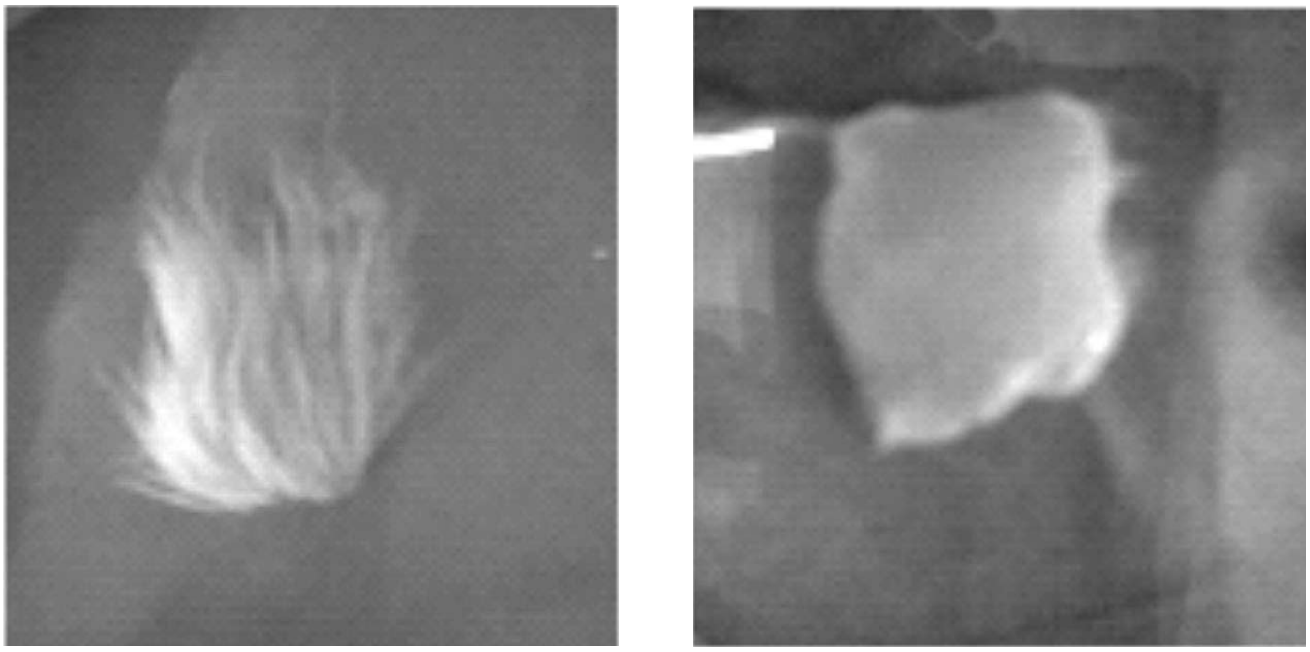
A fungal isolate from paddy field soil identified to be *Aspergillus niger* was used in the study [11]. The micro-organism was grown on potato dextrose agar (PDA) medium for 10 days at 28° and then stored at 4°.

♦ Cultivation Conditions and Extraction of the Enzyme

The fungal organism (1-10 x 10^6 cfu spore suspension) was grown on casein-peptone-potassium nitrate medium for 5 days at 28° with initial pH 7.0 and 150 rpm [11]. After incubation the proteolytic enzyme was extracted using 50% ammonium sulphate at 20° and dialysed against tris buffer (pH 5.0) for 12 h. The extracted enzyme was dissolved in tris buffer (pH 5.0) for further use.

♦ Assay of Protease

The proteolytic activity was assayed using casein as a substrate. Folin-Ciocalteau's method



(a) (b)

Fig. 1: Dehairing of rat skin (a) Control (b) After incubating with enzyme

Protease was able to remove the hair from excised rat skin after 6 h of incubation

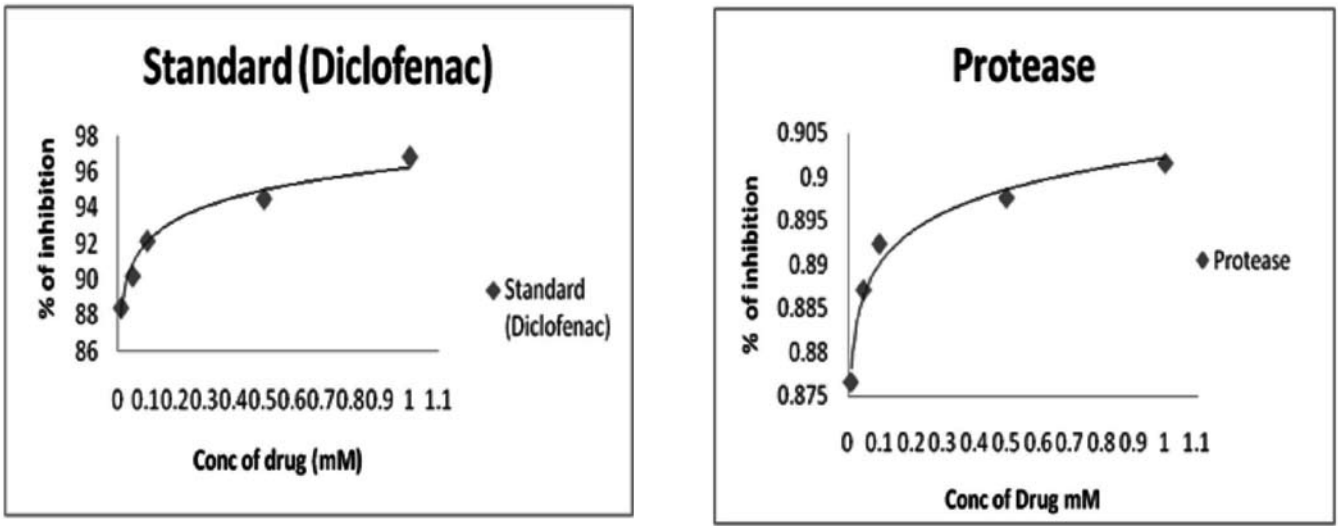


Fig. 2: Anti-inflammatory activity of standard (diclofenac) and protease extracted from *A. niger*

Percentage of inhibition of denaturation of 1 mM of protease was found to be 90% as compared with the standard

was adopted for the enzyme assay using tyrosine standard. The absorbance was measured at 660 nm (Shimadzu, Japan) [12]. One unit of protease activity (U) was defined as the amount of enzyme which releases one mcg of tyrosine per min at 37°.

♦ Characterization of Protease

In order to know the type of protease, the effect of different inhibitors viz., ethylene diamine tetra acetic acid (EDTA), phenyl methyl sulphonyl fluoride

Table I: Effect of inhibitor on protease activity

Metal ion	Concentration mM	% Relative activity
Control	-	100
PMSF	5	98.54015
DTT	5	99.27007
EDTA	5	100
Pepstatin A	1	6.934307

(PMSF), dithiothreitol (DTT) (5 mM) and pepstatin A (1 mM) on protease was studied. These inhibitors were incubated with 1 mL of the enzyme solution at 37° for 1 h. The proteolytic activity was measured under standard conditions.

♦ Application Studies

The extracted and dialysed enzyme was tested for de-hairing, de-clotting and de-staining activities using Wistar rat model. Permission was obtained from Institutional Animal Ethical Committee for conducting animal experiments. Also an *in vitro* anti-inflammatory activity study using diclofenac standard was carried out.

De-hairing of Rat Skin

Skin (2 cm x 2 cm) obtained from a Wistar rat was incubated with the 1 mL enzyme (225 U/mL) in tris-HCl buffer (pH 5). The skin was checked for loss of hair [13].

De-staining of blood:

A white cloth (2.5 cm x 2.5 cm) stained with blood from a Wistar rat was incubated with 1 mL enzyme (225 U/mL) and checked for de-staining [13].

De-clotting of Blood

Blood (2 mL) was withdrawn from retro-orbital vein of Wistar rat and was allowed to clot. The clot was incubated with 1 mL enzyme (225 U/mL) in tris-HCl buffer (pH 5) at 37° and monitored at different intervals [14].

Anti-inflammatory Activity

The anti-inflammatory effect of the protease was measured using inhibition of bovine serum albumin denaturation with diclofenac sodium as a standard [15]. Concentrations ranging from 0.01–1mM (enzyme or drug) were prepared by dissolving in dimethyl formamide (DMF) and diluted with phosphate buffer (0.2M, pH 7.4). Final concentration of DMF in all solutions was less than 2.5%. An aliquot of 1 mL of this solution was mixed with 1 mL of 1mM albumin solution in phosphate buffer (pH 7.4) and incubated at 27± 1° for 15 min. Denaturation was induced by keeping the reaction mixture at 60± 1° in a water bath for 10 min. After cooling the turbidity was measured at 660 nm. Percentage inhibition of denaturation was calculated from control where no drug was added. Each experiment was done in triplicate and average was taken. Diclofenac sodium was used as a standard for comparison.

RESULTS

The effect of various enzyme inhibitors on proteolytic activity was investigated and the results obtained are given in Table I. The activity of enzyme was inhibited by pepstatin A. This indicates presence of aspartate at the enzyme active site. PMSF (serine protease inhibitor), EDTA (metalloenzyme inhibitor) and DTT (cysteine protease inhibitor) did not affect the enzyme activity. These studies confirmed that the protease produced is an acid protease. The enzyme was then precipitated using ammonium sulphate and purified by dialysis. This preparation was studied for clinical application. The enzyme showed de-hairing activity after 5 h incubation and the hair could be removed easily (Fig. 1). The enzyme showed prominent de-clotting activity with removal of blood stain from blood stained cloth after 24 h of incubation. Extracted protease showed anti-inflammatory activity. Percentage of inhibition of denaturation was found to be 90% when compared with diclofenac potassium used as a standard drug. The results are given in Fig. 2.

DISCUSSION

De-hairing property of this acid protease can be made use of in surgical and cosmetic operations. Protease from *Pseudomonas aeruginosa* PD100 showed a similar de-hairing property [13].

Also the protease was effective in removing blood stain even without addition of detergents. This could be used as an effective blood de-stainer. Proteases isolated from *Enterobacter sp*. and *Spilosoma obliqua* showed a similar blood de-staining property [16, 17]. Organisms such as *Aspergillus* produce protease in order to utilize proteins [18]. There was significant blood de-clotting activity observed with the protease obtained from *A. niger*. The enzyme can therefore be used as an effective fibrinolytic agent. Proteases from *Aspergillus fumigates* and *Fusarium pallidoroseum* are reported to posses fibrinolytic activity[18, 19].

Proteases which break immune complexes can be used in treatment of IgA nephropathy caused mainly due to deposition of immunoglobulin IgA[20]. Proteolytic enzymes such as chymotrypsin and serratiopeptidase are known to have anti-inflammatory properties and are tested for their synergistic effect with other non steroidal anti-inflammatory agents[21]. Serrazime, a proteolytic enzyme obtained from *Aspergillus oryzae* is effective against inflammation[5]. Commercially these anti-inflammatory enzymes viz., chymotrypsin and serrapeptase are marketed under various brand names for their therapeutic properties. This protease could be further tested *in vivo* for its anti-inflammatory properties and suitability.

CONCLUSION

An acid protease extracted from *A. niger* showed significant aniti-inflammatory, de-staining and de-clotting activities. Further studies could help in asserting the usefulness of the enzyme in treatment of various vascular related disorders. The results show that the protease from this strain is comparable to other proteases discussed in literature.

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REFERENCES

1. Coral G., Arikan B., Unaldi M. N., Guvenmez H.: Thermostable alkaline protease produced by an *Aspergillus niger* strain, **Annals Microbiol.** 2003, 53(4), 491-498.
2. Charles P., Devanathan V., Anbu P., Ponnuswamy M. N., Kalaichelvan P. T., Hur B. K.: Purification, characterization and crystallization of an extracellular alkaline protease from *Aspergillus nidulans* HA-10, **J Basic Microbiol.** 2008, 48(5), 347-352.
3. Gupta R., Beg Q., Lorenz P.: Bacterial alkaline proteases: molecular approaches and industrial applications, **Applied Microbiol Biotechnol.** 2002, 59(1), 15-32.
4. Ogunbiyi L., Riedhammer T. M., Smith X.: Method for enzymatic cleaning and disinfecting contact lenses. US Patent 4, 614, 549; 1986.
5. Chanalia P., Gandhi D., Jodha D., Singh J.: Applications of microbial proteases in pharmaceutical industry: an overview, **Rev Medical Microbiol.** 2011, 22(4), 96-101.
6. Hellgren L., Mohr V., Vincent J.: Proteases of Antarctic krill-a new system for effective enzymatic debridement of necrotic ulcerations, **Cellular Molecular Life Sci.** 1986, 42(4), 403-404.
7. Loperena L., Ferrari M. D., Belobrajdic L., Weyrauch R., Varela H.: Study of *Bacillus* sp. culture conditions to promote production of unhairing proteases, **Revista Argentina de Microbiologia.** 1994, 26(3), 105-115.
8. Biziulevicius G. A.: Where do the immunostimulatory effects of oral proteolytic enzymes ('systemic enzyme therapy') come from? Microbial proteolysis as a possible starting point. **Medical Hypotheses.** 2006, 67(6), 1386-1388.
9. Singh A., Ghosh V. K., Ghosh P.: Production of thermostable acid protease by *Aspergillus niger*, **Lett Applied Microbiol.** 1994, 18(3), 177-180.
10. Machtelt B., Peter P.: *Aspergillus* as a Cell Factory for Protein Production, The Aspergilli: CRC Press; 2007, 441-455.
11. Kamath P., Subrahmanyam V. M., Rao J. V., Raj P. V.: Optimization of cultural conditions for protease production by a fungal species, **Indian J Pharm Sci.** 2010, 72(2):161-166.
12. Folin O., Ciocalteu V.: On tyrosine and tryptophane determinations in proteins, **J Biol Chem.** 1927, 73(2), 627-650.

13. Najafi M. F., Deobagkar D.: Potential application of protease isolated from *Pseudomonas aeruginosa* PD100, **Electronic J Biotechnol.** 2005, 8(2), 79-85.
14. Ramakrishna D. P. N., Reddy G., Rajagopal S. V., Kushwaha D. S., Pandey P. K., Jamal F., *et al*: Purification and Properties of an Extra Cellular Alkaline Protease Produced By *Bacillus subtilis* (MTCC N0-10110), **Int J Biotechnol Biochem (IJBB).** 2010, 6(4), 489-500.
15. Tasaka K., Meshi T., Akagi M., Kakimoto M., Saito R., Okada I. *et al*: Anti-Inflammatory Activity of a Proteolytic Enzyme, Prozime-10, **Pharmacol.** 1980, 21(1), 43-52.
16. Kanchana R., Jadhav S., Goletikar Y., Manerekar G.: Production of Alkaliphilic Protease (s) by *Enterobacter* sp for Application in Biodetergent Formulation, **Ind Biotechnol.** 2012, 8(1), 31-35.
17. Anwar A., Saleemuddin M.: Alkaline protease from *Spilosoma obliqua*: potential applications in bio-formulations, **Biotechnol Applied Biochem.** 2000, 31(2), 85-89.
18. Larcher G., Bouchara J. P., Annaix V., Symoens F., Chabasse D., Tronchin G.: Purification and characterization of a fibrinogenolytic serine proteinase from *Aspergillus fumigatus* culture filtrate, **FEBS Lett.** 1992, 308(1), 65-69.
19. El-Aassar S. A.: Production and properties of fibrinolytic enzyme in solid state cultures of *Fusarium pallidoroseum*, **Biotechnol Lett.** 1995, 17(9), 943-948.
20. Eitner F., Floege J.: Bacterial protease for the treatment of IgA nephropathy*, **Nephrology Dialysis Transplantation.** 2008, 23(7), 2173-2175.
21. Swamy A. H. M. V., Patil P. A.: Effect of some clinically used proteolytic enzymes on inflammation in rats, **Indian J Pharm Sci.** 2008, 70(1), 114-117.

Kalaskar V. V., Narayanan K., Subrahmanyam V. M.* and Rao V. J.

Dept. of Pharmaceutical Biotechnology
Manipal College of Pharmaceutical Sciences
Manipal-576 104, Karnataka.
E-mail: vm.subra@manipal.edu

*For correspondence

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