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Research Article

IN VITRO ANTI INFLAMMATORY ACTIVITY OF PROTEASE FROM *ASPERGILLUS* SP.

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A B S T R A C T

Proteases play an important role in virulence of many human, plant and insect pathogens. The protease inhibitors of plant origin have been reported widely from many plant species. The inhibitors may potentially be used for multiple therapeutic applications in viral, bacterial, fungal diseases and physiological disorders. The *in vitro* assessment methods included protein denaturation assay, and membrane stabilization using Diclofenac sodium as standard drugs. In the present study, protease showed that the inhibition of protein denaturation at the highest concentration of 250 µ g/ml and HRBC membrane stabilization at the highest concentration of 250 µ g/ml respectively. The results indicate that the protease extracted from *Aspergillus* sp. have been possesses significantly anti-inflammatory property apart from being an anti-tumor drug.

INTRODUCTION

Proteases are abundantly and widely distributed in biological world including plant, animal and microbes (Li, 2007). A protease also called as peptidase or proteinase is group of enzyme that performs proteolysis known as hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein (Hedstrom, 2002). Proteases constitute more than 70% of industrial enzyme alone and microbial sources (bacterial and fungal) are leading supplier of these enzyme. These enzymes possess catalytic activity in broad range of temperature and pH (Murakami, 1991 & Prashant T Sanatan, 2013).

Proteases constitute one of the largest functional group of proteins involved in many normal and pathological processes. Protease inhibition of pathogenic organisms may aid in control of several diseases (Supuran *et al.,* 2002).

Several proteases are essential for propagation of disease, and hence inhibition of different proteases is emerging as a promising approach in medical applications for the treatment of cancer, obesity, hepatitis and herpes, cardiovascular, inflammatory and neurodegenerative diseases, as well as various infectious and parasitic diseases (Rao *et al.,* 1998). Aspartic proteases are a relatively small group of proteolytic enzymes. Over the last decade, they have received tremendous research interest as potential targets for pharmaceutical intervention, as many have been shown to play significant roles in physiological and pathological processes (Dash *et al.,* 2003). Study of the kinetic properties of this class of enzymes has been motivated by their pharmaceutical and commercial importance, and has evoked considerable interest regarding the role of their inhibitors.

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is a self-defence reaction in its first phase, hence regarded as the main therapeutic target and often, the best choice to treat the disease and alleviate the symptoms. It is a complex response that protects the host against tissue injury and microbial invasion. The redness, swelling, heat, pain, and loss of functions are considered as symptoms of inflammation. Prolonged inflammation contributes to the pathogenesis of many diseases such as asthma, arthritis, multiple sclerosis and even cancer (Karin and Greten, 2005; Mahesh *et al.,* 2012).

Inflammation is physiological response against microbial infection and mechanical injures which is associated with large scale of plasma accumulation along with immune cells. The available treatment to combat inflammation lies on use of Non-Steroidal (NSAID) drug in acute cases while steroidal drugs in chronic conditions. However, such management of inflammation is associated with several complications including side effects (Craik, 2011). Enzymes, especially protease emerged as better option to combat both acute and chronic inflammation in reasonable price. Serratiopepetidase is most effective protease available for use in management of inflammation (Vaisar, 2007). This enzyme is generally produced from Serratia species of bacterium but several fungal species were also explored for anti-inflammatory proteases.

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Additionally, a group of serine protease from Indian Earthworm has been studied for its anti-inflammatory potential and is underway for further molecular findings (Bhagat, 2013). Based on this background of a variety of anti-inflammatory compounds the present investigation focuses on the production of anti-inflammatory property of protease from *Aspergillus* sp. by *in vitro* methods.

MATERIAL AND METHODS

Isolation and identification of fungi

The soil samples were collected from agricultural field in Mannargudi Taluk, Thiruvarau District of Tamilnadu. The soil fungi were enumerated by the serial dilution method using Potato Dextrose Agar. The medium was sterilized at 121°C for 15 min at 15 lbs and 15 ml of the sterile medium was dispensed into sterile petridishes and kept aside for further use. One ml of microbial suspension from 10^{-3}, 10^{-4} and 10^{-5} dilutions were added into sterile Petri dishes (triplicate of each dilution) containing 15 ml of sterile Potato Dextrose Agar. 1% streptomycin solution was added to the medium before pouring into petriplates for preventing bacterial growth. All plates were incubated at 26± 2°C until mycelium grew out hyphal tips were cut and transferred to Potato Dextrose Agar (PDA). Half strength PDA was used for subculture and stock culture. Identification was based on colony and hyphal morphology of the fungal cultures, characteristics of the spores (Ellis, 1971; Barnett & Hunter, 1972).

Production of Protease

The two selected fungal (*Aspergillus oryzae* and *Aspergillus terreus*) isolates were inoculated in sterile 100 ml of protease specific fermentation broth containing (% w/v): yeast extract 1.0, $MgSO_4$ 0.02, glucose 2.0, K_2HPO_4 0.1, pH 7.0. Flasks, inoculated were incubated at 28˚C for 5-6 days in a rotary shaker. At the end of incubation, the contents of flasks were filtered through Whatmann filter paper No. 1 and then the filtrates were centrifuged at 8,000 rpm at 4˚C for 10 minutes. Pellet was discarded after centrifugation and clear supernatant was used as source of protease enzyme. The supernatant of crude enzyme was further used for anti inflammatory studies.

In-vitro anti inflammatory assay

Inhibition of protein denaturation method (Lavanya et al., 2010)

Test solution

The test solution of 0.5ml consists of 0.45ml Bovine Serum Albumin (5%w/v aqueous solution) and 0.05 ml of test solution (250 µ g/ml).

Test control solution

Test control solution of 0.5 ml consists of 0.45ml of Bovine Serum Albumin (5%w/v aqueous solution) and 0.05 ml distilled water (250 µ g/ml).

Product control solution

The Product control solution of 0.5ml consists of 0.45ml of distilled water and 0.05ml of test solution (250 µ g/ml).

Standard solution

The standard solution of 0.5ml consists of 0.45ml of Bovine Serum Albumin (5%w/v, aqueous solution) and 0.05ml of Diclofenac sodium (250 µ g/ml).

All the above solutions were adjusted to ph 6.3 using 1n HCL. The samples were incubated at 37°C for 20 minutes and the temperature was increased to keep the samples at 57°C for 3 minutes. After cooling, add 2.5ml of phosphate buffer to the above solutions. The absorbance was measured at 416nm using U V-visible spectrophotometer (Lavanya *et al.,* 2010).
Percentage inhibition= [100-(optical density of test solution-optical density of product control) ÷ (optical density of test control) ×100].

The control represents the 100% protein denaturation. The results were compared with Diclofenac sodium (250 µ g/ml).

In-vitro anti inflammatory assay by HRBC stabilization method

The principle involved here is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. Blood was collected (2 ml) from healthy volunteers and was mixed with equal volume of sterilized Alsevers solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl in distilled water) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline solution and a 10% v/v suspension was prepared with normal saline and kept at 4°C undisturbed before use. Different concentration of enzyme (50,100,150,200 and 250 µ g/ 0.5ml) in normal saline, diclofenac sodium as standard (50,100,150,200 and 250 µ g/ 0.5ml) and control (distilled water instead of hypo saline to produce 100% hemolysis) were separately mixed with 1ml of phosphate buffer, 2ml of hyposaline and 0.5ml of HRBC suspension was added to prepared. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3,000 rpm for 20 min and hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm (Kumar et al., 2011). The percentage of HRBC membrane stabilization or protection was calculated by using the following formula

$$\text{Percentage stabilization} = \frac{\text{Absorbance of control} - \text{Absorbance of Test}}{\text{Absorbance of control}} \times 100$$

Statistical Analysis

All the experimental data were carried in triplicates and expressed as means ± standard errors. The statistical analyses of the data were performed using one way ANNOVA variance SPSS version 20.0 software with advanced models (SPSS Japan, Tokyo, Japan). Differences between means were located using Tukey's test ($P<0.05$).

RESULT AND DISCUSSION

Inhibition of protein denaturation method

Denaturation of proteins is a well-documented cause of inflammation. Proteinases have been embroiled in joint responses. Neutrophils are known to be a wellspring of proteinase which conveys in their lysosomal granules numerous serine proteinases. It was beforehand detailed that leukocytes proteinase assume critical part in the improvement of tissue harm amid in fiery responses and noteworthy level of insurance was given by proteinase inhibitors (Vane & Botting 1995). Some resarchers report that denaturation of protein is one of the reason for rheumatoid joint pain because of the generation of auto-antigens in certain rheumatic sicknesses. It might be cause to in vitro denaturation of proteins (Grant *et al.,* 1970). Mechanism of denaturation is involved in alteration of electrostatic force, hydrogen, hydrophobic and disulphide bonds. Several authors have shown anti-inflammatory drugs to

show dose dependent ability to inhibit the thermally induced protein denaturation (Chou, 1997).

During inflammation leukocytes protease enzymes play an important role in tissue damage. Protease inhibitors provide significant protection against this tissue damage developed by proteases during inflammatory reactions (Das and Chatterjee 1995). These enzymes have the ability to prevent tissue damage. In the present study protease was tested for their anti inflammatory activity by protein denaturation and HRBC method. The body response to infection, injury or destruction is what we call inflammation. Inflammation is characterized by pain, swelling, redness and disturbed physical functions. It is a normal body protective function against injury and infection. One of the well documented causes of inflammation is protein denaturation. This is because, most biological proteins lose their function when denatured.

In this study , the result showed that *Aspergillus oryzae* maximum inhibition of protein denaturation of (94.23 ± 0.34) % at 250 µ g/ml and this effect was compared with the standard anti inflammatory drug diclofenac sodium of maximum inhibition (90.88 ± 1.99) % at the same concentration. The *Aspergillus terreus* recorded the maximum production of protein denaturation of (89.65 ± 0.54) % at 250 µ g/ml and it was compared with anti-inflammatory drug diclofenac sodium of maximum inhibition (86.88 ± 0.32) % at the same concentration (Table -1 & figure -1). The similar findings also reported by several workers (Williams *et al.,* 2008; Vishnu Menon and Mala Rao, 2012; Govindappa *et al.,* 2015; Mahabal & Kaliwal, 2017).

Table1 Inhibition of protein denaturation method

S. No	Concentration µ g/ ml	Protein denaturation method			
		Aspergillus oryzae	Diclofenac sodium	*Aspergillus terreus*	Diclofenac sodium
1	50	60.53 ± 0.64	56.66 ± 0.34	63.66 ± 1.36	60.44 ± 0.33
2	100	76.66 ± 0.23	70.99 ± 1.22	59.70 ± 0.39	50.44 ± 1.27
3	150	80.14 ± 1.87	78.90 ± 1.83	77.49 ± 1.88	70.66 ± 0.44
4	200	89.44 ± 0.98	80.10 ± 2.98	83.60 ± 0.53	80.32 ± 1.22
5	250	94.53 ± 0.34	90.88 ± 1.99	89.65 ± 0.54	86.88 ± 0.32

Each value represents the mean ± SD; N=3; Significance p<0.05.

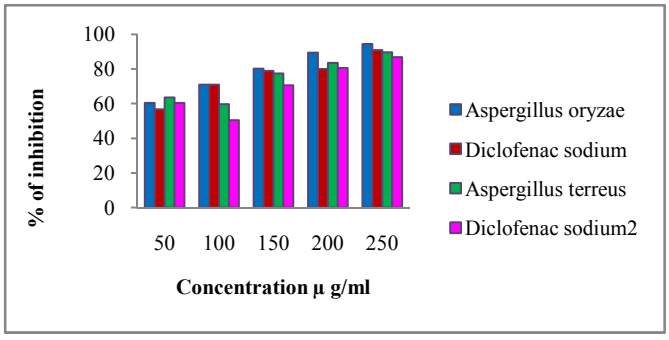

Fig 1 Inhibition of protein denaturation method

HRBC membrane stabilization assay

The HRBC membrane may be considered as a model of the lysosomal membrane which plays an important role in inflammation (Weissmann *et al.,* 1969). Lysosomal membrane stabilization is important in limiting the inflammatory responses. The mechanism is by the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases which cause further tissue inflammation and damage upon extracellular release (Murugasan *et al.,* 1981). HRBC membrane stability test is based on the finding that non-steroidal anti-inflammatory agents inhibit heat induced lysis of erythrocytes, most likely by stabilizing the membrane of the cell.

Stabilization of the RBCs membrane was studied to further establish the mechanism of anti-inflammatory action of *Aspergillus oryzae* and *Aspergillus terreus*. It was effective in inhibiting the hemolysis at different concentrations. These provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. This extract may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. *Aspergillus oryzae* at different concentration (50-250 µ g/ml) inhibited hemolysis of RBCs to varying from the concentration as shown in Table 2 & figure 2. It showed the maximum inhibition of 95.80 ± 1.66 % at 250µg/ml when compared with diclofenac as standard drug showed the maximum inhibition of 89.05 ± 2.36 % at the same concentration. *Aspergillus terreus* at different concentration (50-250 µ g/ml) inhibited hemolysis of RBCs to varying from the concentration as shown in Table 2 & figure 2. It showed the maximum inhibition 90.99 ± 0.22% at 250µg/ml when compared with diclofenac as standard drug showed the maximum inhibition, 89.42 ± 0.99% at the same concentration. The well document also confirmed by the results are reported by Garg *et al.* (2010); Valentine *et al.* (2011); Karunakaran *et al.* (2013).

Table 2 HRBC stabilization method

S. No	Concentration µ g/ ml	HRBC method			
		Aspergillus oryzae	Diclofenac sodium	*Aspergillus terreus*	Diclofenac sodium
1	50	69.01 ± 0.23	60.84 ± 1.23	72.77 ± 0.46	70.44 ± 0.44
2	100	70.53 ± 0.44	69.56 ± 0.22	78.42 ± 1.58	75.98 ± 1.36
3	150	79.89 ± 0.33	75.43 ± 0.15	80.93 ± 1.33	79.43 ± 0.68
4	200	83.88 ± 1.36	79.55 ± 1.36	88.94 ± 0.43	80.34 ± 1.55
5	250	95.80 ± 1.66	89.05 ± 2.36	90.99 ± 0.22	89.42 ± 0.99

Each value represents the mean ± SD; N=3; Significance p<0.05

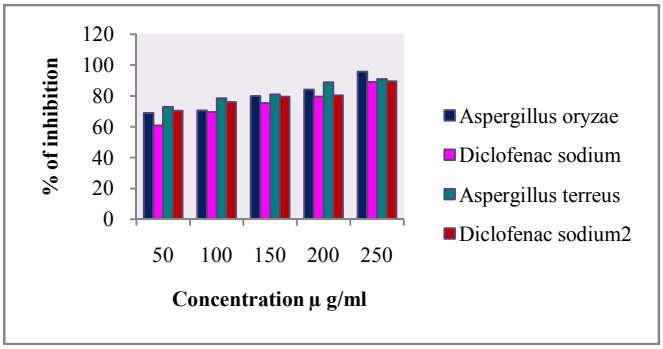

Fig 2 HRBC stabilization method

CONCLUSION

The present investigation concludes that the anti inflammatory activity, the protease showed that the inhibition of protein denaturation at the highest concentration of 250 µ g/ml and HRBC membrane stabilization at the highest concentration of 250 µ g/ml respectively. The protease extracted from *Aspergillus* sp. showed significant anti-inflammatory activity. These results appear as interesting and promising and may be effective as potential sources of novel anti-inflammatory drugs.

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