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ANALYTICAL STUDIES OF PROTEASE EXTRACTED FROM *AZADIRACHTA INDICA*

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ABSTRACT

Investigations have been carried out on proteases extracted from *Azadirachta indica* using variable concentration of PBS (phosphate buffered saline, ranging from 3 to 9) buffer in order to estimate the total protein and protease content. The results showed that *Azadirachta indica* showed higher protein (PH 5 to 9 using Tris HCl and ice cold acetone) and protease (using casein and citrate buffer) content in case of pH 6 to 8. Overall, the results showed that protease activity of leaves extracted from *Azadirachta indica* may be responsible for wound healing properties.

KEYWORDS: Proteases; *Azadirachta indica*; protein; protease

1. INTRODUCTION

Proteases (also labeled as peptidases, proteinases and proteolytic enzymes) are found in all living organisms from viruses to animals including human which are capable of hydrolyzing various peptide bonds in proteins effective but these are more effective in removing or repairing the damaged or infected tissues from wounds.[1, 2, 3] Actually, the growth and development in all the unicellular as well as multicellular organisms occurs as a result of an overall balance between proteolysis and protein synthesis.[4] These proteases are extensively used in various pharmaceutical industries especially for cell culture and tissue dissociation, peptide synthesis, recombinant antibody fragments, purification of nucleic acids (unwanted proteins digestion), peptide sequencing and proteolytic digestion of proteins in proteomics etc.[5, 6]

There are number of protease inhibitors which is already reported and widely distributed in the plant and animal kingdom.[7, 8] Most of them are already purified as well as characterized from many different species i.e. unicellular as well as multicellular organisms.[9] While the physicochemical properties of these protease inhibitors have been extensively investigated, their immunobiological effects, e.g. immunostimulatory /immunosuppressive/ immunoadjuvant effect, remain relatively unexplored. In the present study, our group focused on protease extracted from the fresh leaves of medicinal plant, *Azadirachta indica* (frequently known as neem) belongs to the family, *meliaceae*. This medicinal plant already showed number of medicinal properties i.e. antihelminthic,[10, 11] antifungal, antibacterial,[12, 13] etc.

The objective of our study is to screen the proteases of different pH values extracted from medicinal plant i.e. *Azadirachta indica* for empathetic immunobiological aspects of proteolytic enzymes and summarize their applications in the field of pharmaceutical sciences. Commercially, proteolytic enzymes from *Azadirachta indica* have received special scrutiny because of their broad or immense substrate specificity as well as activity in wide range of pH and temperature. As there is no proclamation on the study of protease in Azardichta indica, an attempt was made to study proteases in medicinal plants which may play a role in wound healing property of the plant

2. MATERIALS AND METHODS

2.1 Congregation of fresh leaves of *Azadirachta indica*

Healthy looking plant leaves of *Azadirachta indica* were collected or gathered from the garden of Vidya Pratishthan's School of Biotechnology, Baramati, District Pune, Maharashtra, India.. These fresh plant leaves were macerated with liquid nitrogen (- 196 °C) to prepare fine powder using mortar and pestle. The powder was stored at 4 °C for extraction, analyzing and estimate its protein content and this content was further used for the investigation of protease enzyme assay from the leaves powder of Azardichta indica. All these immunopharmacological studies were carried out at 0 - 5°C.

2.2 Extraction of protein from *Azadirachta indica*

For protein estimation, weigh 2 g of leaves powder is added into 50 ml tube and add double the amount of extraction buffer (i.e. 20 mM tris HCl) dissolved in PBS of different pH concentration ranging from 3 to 9. The pH of the medium was calibrated as well as adjusted by 1N NaOH or 1N HCl. Afterwards, incubate the leaves powder along with extraction

buffer for 5 minutes at room temperature and then centrifuged at 6000 rpm for 10 minutes at 4°C. Collect the supernatant after centrifugation and then add equal quantity of ice cold acetone. Incubate all these samples for 8 minutes at room temperature and then centrifugation at same speed and time as mentioned above. After centrifugation, supernatant was discarded and collect the pellet and washed with ice cold acetone to remove the pigments as well as lipids.[14] Finally, the protein concentration was determined by using Nano drop method.

2.3 Crude Protease production by *Azadirachta indica* and enzyme assay

The extraction of protein (Tris HCl and ice cold acetone) from different concentration of PBS buffer (pH 3 to 9) using fresh plant leaves of *Azadirachta indica*. Finally, the protein was dissolved again in different pH values of PBS buffer ranging from 3 to 9. Centrifuged the protein content at 10000 rpm at 4^{o}C for 30 minutes and the supernatant (crude enzyme extract) was collected and used for analyzing and estimate its protease content from *Azadirachta indica*.

Protease activity was determined by calorimetric assay using casein as substrate. In this study, crude enzyme extract was assayed by using 1% casein dissolved in citrate buffer (pH 7). For these studies, add equal quantity of casein and crude enzyme extract in 50 ml tube and allowed to stand for 2h. Afterwards, TCA solution was added to stop the enzymatic reaction and then centrifuge at 10000 rpm for 20 minutes. The supernatant was collected and add equal quantity of NaOH solution in comparison with TCA solution. Incubate all these samples for 20 minutes at room temperature. Afterwards, 500 µl of Folins colins reagent was added and the intensity of blue color was measured at 700 nm within half an hour using spectrophotometer.[14]

2.4 Analytical studies (pH and temperature) of crude protease from *Azadirachta indica*

For these analytical studies, different pH from 5 to 8 was prepared for the use as PBS buffer. Serially dilution of different series of crude enzyme extract was mixed with similar quantity of BSA dissolved in PBS buffer of different pH values. BSA used as standard for these studies. After incubation of 1 h at room temperature and optical density (OD) was measured after with spectrophotometer at 595 nm.

In addition, different temperatures were selected (0°C, 4°C, 18°C, 45°C and 90°C) in order to determine the effect of protease activity of crude extract. Similar quantity of crude enzyme extract and BSA solution dissolved in PBS of different pH values. Again, performed lowry

test and OD was measured at 595 nm using spectrophotometer. For both these studies, two controls were taken i.e. enzyme control and the next one is BSA.

2.5 Statistical analysis

Data are reported as means ± standard error (S.E). The difference between the control and treated groups of proteases is determined by One way ANOVA test (Bonferroni multiple comparison test). *$*P < 0.05$; $**P < 0.01$; $***P < 0.001$*.

3 RESULTS

3.1 Estimation of protein content from *Azadirachta indica*

To determine the protein content from fresh plant leaves of *Azadirachta indica* using Tris HCl and ice cold acetone which is determined through Nanodrop. The results showed that *Azadirachta indica* showed higher range in protein content (pH 5 and 8) as compared to blank. BSA used as standard for these studies and the results showed the protein content at different dilutions of PBS (pH ranging from 3 to 9) which is determined in Nanodrop as shown in **Fig.1**.

3.2 Estimation of crude protease production

The effect of variable pH concentration of PBS buffer (3 to 9) dissolved in protein extracted from the leaves of *Azadirachta indica* for the estimation of protease which is determined through spectrophotometer as shown in **Fig. 2**. The results showed that PBS (pH 7) concentration of leaves protein of *Azadirachta indica* attained higher level of protease content followed by pH 8 as compared to control. BSA used as standard for these studies.

3.3 Effect of pH and temperature on crude protease

The effect of pH and temperature on protease concentration at different pH and temperatures (0°C, 4 °C, 18 °C, 45 °C and 90 °C) values as shown in **Fig.3**. The optimum temperature and pH of crude enzyme extract was found to be active at 37^{o}C and pH (6, 7.2 and 8), while protease was still active at 60^{o}C too.

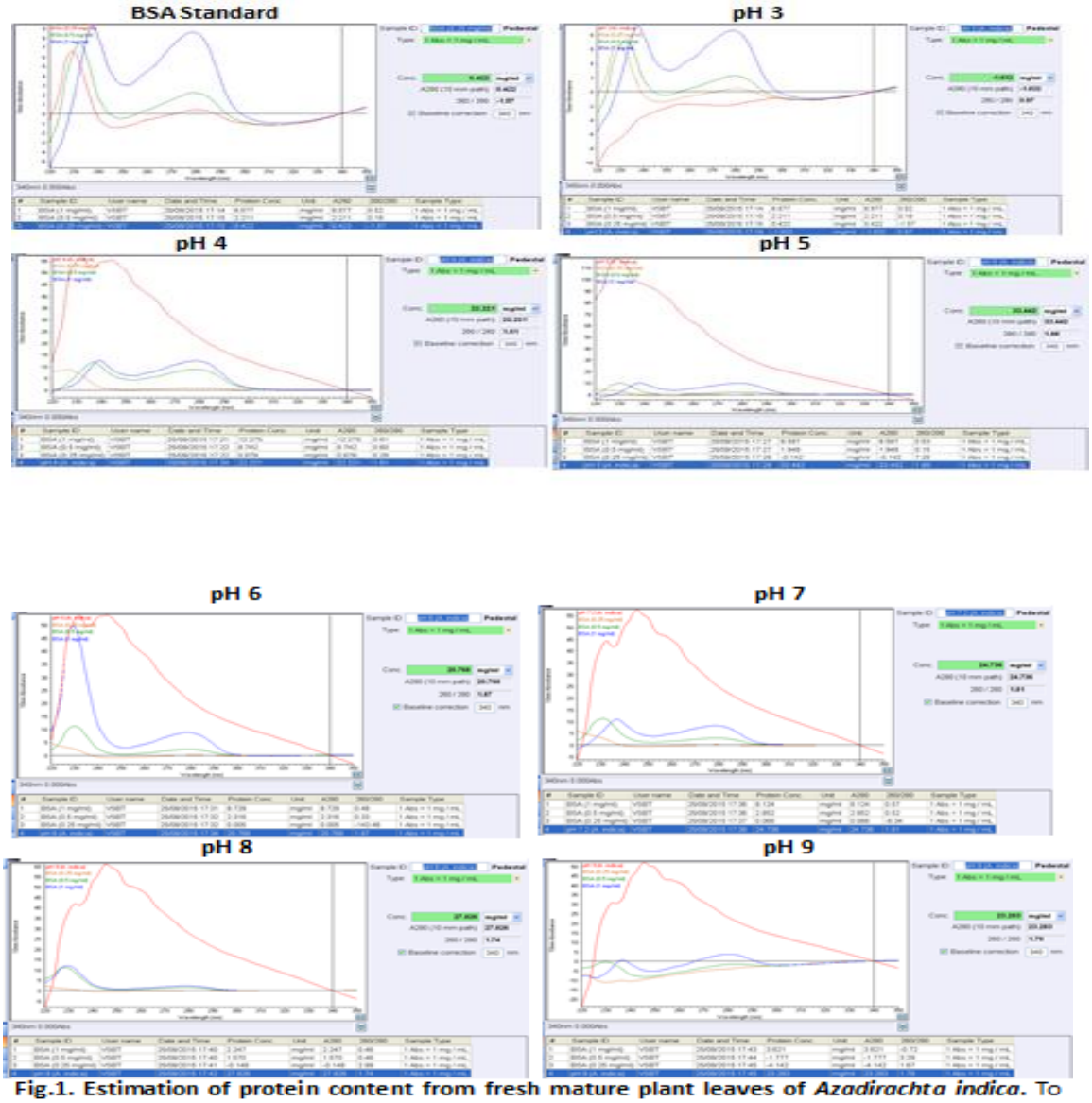

Fig.1. Estimation of protein content from fresh mature plant leaves of *Azadirachta indica*. To determine the protein content using Tris HCl and ice cold acetone from different pH values of PBS buffer (3 to 9) which is determined through Nanodrop. Results are expressed in mg/ml. BSA used as standard for these studies.

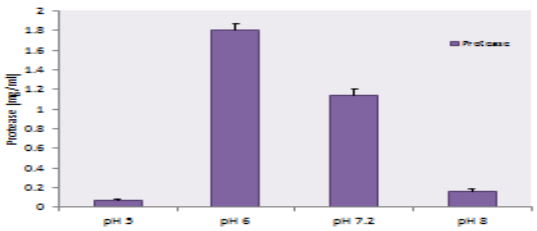

Fig.2. Estimation of protease content from fresh mature plant leaves of *Azadirachta indica*. Serially dilution of crude enzyme extract was mixed with similar quantity of BSA dissolved in PBS buffer of different pH values. BSA used as standard for these studies. After 1h incubation at room temperature and optical density (OD) was measured after with spectrophotometer at 595 nm . Results are expressed in mg/ml.

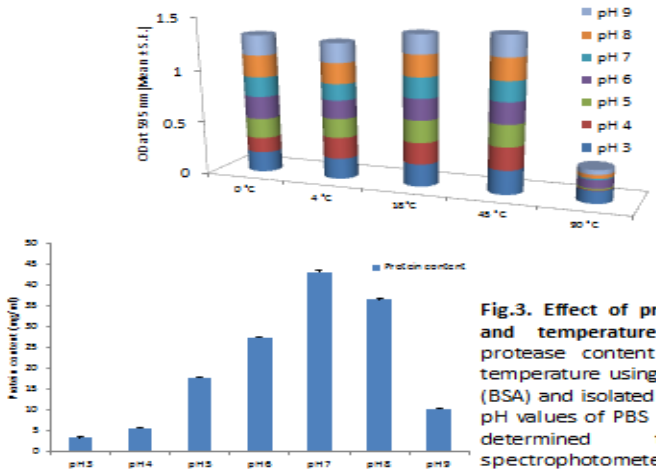

Fig.3. Effect of protease at different Ph and temperature. To determine the protease content at different ph and temperature using Bovine serum albumin (BSA) and isolated protease from different pH values of PBS buffer (3 to 9) which is determined through UV-visible spectrophotometer.

DISCUSSION

Azadirachta indica one of the traditional medicinal plants in India which is a promising source of proteases involved in various immunopharmacological activities such as wound healing etc. Generally, these proteases are generally used in various immunopathological conditions such as cardiovascular diseases and neurodegenerative disorders.[15, 16] Most of researchers focused on proteases isolated from various medicinal plants are major source for pharmaceutical industry as potential drug target for number of human diseases.[15] In addition, most of infectious or contagious pathogenic micro-organisms require proteases for replication or use proteases as virulence factors or protease target therapies for number of human diseases e.g. AIDS.[17] Finally, proteases are also play a key role in plant proteins (processing, maturation and destruction) and also played an important role in biotechnological industry because of their usefulness as biochemical reagents or in the manufacture of numerous products.[3, 4] Therefore, attempt was made to determine the protease activity of fresh plant leaves of *Azadirachta indica*. Buffers of different pH range (3 to 9) were used for extraction of the fresh leaves to identify the best buffer for extraction of protein, protease using casein as substrate and also identified its activity of protease on human whole blood for the estimation of lymphocytes, monocytes and granulocytes count and human peripheral blood mononuclear cells for the estimation of CD14 FITC monocyte surface marker by flow cytometry.

In order to estimate the total protein content from the leaves of *Azadirachta indica* showed higher variation in response to different pH (ranging from 3 to 9) of the buffer that was used for extraction using Tris HCl and ice cold acetone. The results showed that increase in protein content was observed with increase in pH of the buffer. Maximum protein content was

observed with pH 5, 7.2 and 8. Further increase in pH showed decline in total protein extraction (Fig 1). After getting the protein content from fresh leaves of *Azadirachta indica* and further dissolved in PBS of different concentration of pH. For estimation of protease, maximum activity was obtained at pH 6, 7.2 and 8 only using buffer for extraction was citrate buffer (pH 7). In addition, the optimum temperature of proteolytic enzymes of *Azadirachta indica* was measured by incubating with BSA along with different concentration of proteases stored at different pH and temperatures. The results showed that the protease was still active at 45 °C and pH (6, 7.2, 8) afterwards the protease activity still declined at higher temperature i.e. 90°C.

CONCLUSION

Protease extracted from *Azadirachta indica* using variable concentration of PBS buffer (pH ranging from 3 to 9). These proteases showed many fascinating medicinal properties (immunomodulatory, anti-inflammatory etc) and also involved in various applications especially for biotechnology. However the isolation and purification of proteases from this plant i.e. *Azadirachta indica* will assist us to recognize the mechanism of various disease models.

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AUTHORS' CONTRIBUTION

All authors are involved in conception and design, drafting the research article and approved the final manuscript.

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