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ANTI-PROTEASE ACTIVITY AND HEAT STABILITY PROPERTY OF PIPER LONGUM SEEDS EXTRACT

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ABSTRACT

Long pepper (*Piper longum*) also called as Indian long pepper (pipli) belongs to the family of Piperaceae extensively used as spice and as medicine in South Asian continent. Herein we are reporting the property of inhibiting the activities of proteases like trypsin and chymotripsin by the crude protein of *Piper longum*. It exerts protease inhibitory activity up to 68% at 25 µg crude protein dose against Serine proteases. The protein was stable up to a temperature of 70^0C, active over a wide range of pH from 2 to 12. This is the first report on protease inhibitor property of *Piper longum* crude protein.

KEYWORDS: *Piper longum*, Protease inhibitors, trypsin and chymotrypsin.

INTRODUCTION

In many developing countries pulses are the rich source of proteins with only complaint of poor digestibility. This is due to the presence of anti-nutritional factors including protease inhibitors. These reduce the nutritional potential of the legume protein leading to stomach distension, flatulence and some are toxic.[1] It is found that protease inhibitors are extremely

widespread throughout the plant kingdom. These are also involved in regulation of protein turnover, apoptosis, stress tolerance and defense against pathogens and pests.[2,3]

The legume seed protease inhibitors normally contain trypsin/chymotrypsin inhibitors.[4,5,6] These inhibit proteolytic enzymes of digestive tract and reduce the ability of body to utilize food proteins, casing defect in protein efficiency ratio. However, these leguminous seeds lots of applications in medicine, agriculture, food technology.[7]

Species of the genus Piper are important medicinal plants used as medicine to treat various diseases.[8] *Piper longum* is an Indian long pepper, is used as a spice and seasoning, whicg is close associate of *P. Nigrum.*[9] This plant seeds are readily available and inexpensive. The fruits and roots have numerous medicinal attribution to various diseases like chronic asthma, to hepatotoxicity and tumors.[10, 11,12]

Present study is to highlight the inhibitory action of crude protein of *Piper longum* seeds against proteases like trypsin and chymotrypsin.

MATERIALS AND METHODS

1.1. Chemicals

Trypsin (EC 3.4.21.4), Chymotrypsin (EC 3.4.21.1), Ammonium sulphate and all other chemicals unless otherwise mentioned were of analytical grade procured from Merck (Germany). Solvents were distilled before use.

1.2. Isolation of proteins from Piper longum

The protein was isolated from *Piper longum* by 55% ammonium sulphate precipitation. Where 10g of powder of *Piper longum* vortexed with 300 ml of double distilled water for two hours at 20^{o}C. Later the extract centrifuged at 10000 rpm for 20 min at 4^{o}C. The supernatant collected was subjected to 55% ammonium sulphate precipitation, kept for votexing at 4^{o}C overnight. Further the precipitated crude protein was separated by centrifugation. The obtained crude precipitate of protein was subjected to dialysis against double distilled was for 72 hours with an interval of six hours. The dialyzed sample was examined and confirmed is free of unwanted salts.

1.3. Protease inhibitory activity

The protease inhibitory activity was assayed according to the method of Satakee M et al 1963.[13] 50 µL aliquot of trypsin and chymotrypsin was pre incubated separately with

different concentrations of Protease inhibitor. To the above denatured casein was added as substrate of 0.4 mL (2%) in a final volume of 1 mL using 0.2 M Tris-HCl buffer of pH 8.5 for 2 h at 37^0C. After incubation, the reaction was stopped by adding 1.5 mL of 0.44 M TCA and the mixture was allowed to stand for 30 min. The reaction mixture was centrifuged at 1500g for 15 min. An aliquot (1 mL) of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of Folin–Ciocalteu reagent (1:2 v/v). The colour developed was read at 660 nm. Activity was expressed as units/hr. Protease inhibitor activity of the enzyme is finally expressed in terms of percent inhibition.

1.4 DPPH radical scavenging activity along with protease enzymes

DPPH is a stable purple coloured nitrogen-centered free radical that gets reduced to a yellow coloured diphenylpicryl hydrazine by the fractions in a concentration-dependant manner. DPPH radical scavenging activity was assessed according to the method described by Aquino *et al.*[14] Different doses of crude protein of *piper longum* and constant amount of proteases like trypsin or chymotrypsin was mixed with 1 ml of freshly prepared 0.5 mM DPPH ethanolic solution and 2 ml of 0.1 M acetate buffer. The resulting reaction mixtures were incubated at 37 °C for 30 min, and the absorbance was measured at 517 nm. The % DPPH radical scavenging activity was calculated using the following formula

$$\% \text{ Inhibition of DPPH radical scavenging activity} = \frac{(\text{Abs of control} - \text{Abs of samples})}{\text{Abs of control}} \text{ X } 100$$

1.5. Thermal and pH stability of Protease inhibitor

The effect of temperature on trypsin inhibitory activity of extracts from crude protein of *piper longum* were tested by incubating at different temperatures 37, 40, 50, 60, 70, 80, 90, 100^0C for 30 min. after cooling the samples to room temperature the residual trypsin inhibitory activity was determined as described earlier.

The effect of pH on the trypsin inhibitory activity was examined at pH ranging between 2-12 for 30 min at room temperature using the buffers: glycine-HCl (pH 2 to 3), sodium acetate-acetic acid (4 to 5), Sodium phosphate buffer (pH 6), Tris-HCl (pH 7 to 9) and glycine-NaOH (pH 10 to 12). The residual inhibitory activity was measured as described earlier and the final concentration of used buffers is of 50mM.

1.6. Salt stability

The crude protein of *piper longum* were incubated at room temperature for 30 min in the presence of NaCl ranging from 0% to 3% and were tested for inhibitory activity against trypsin and chymotrypsin, the residual inhibitory activity was measured.

1.7. Statistical analysis

All experiments were carried out in triplicate to check the reproducibility of results. The data presented here are the averages of triplicate determinations and the standard deviations for all the values were < ± 5%.

RESULTS AND DISCUSSION

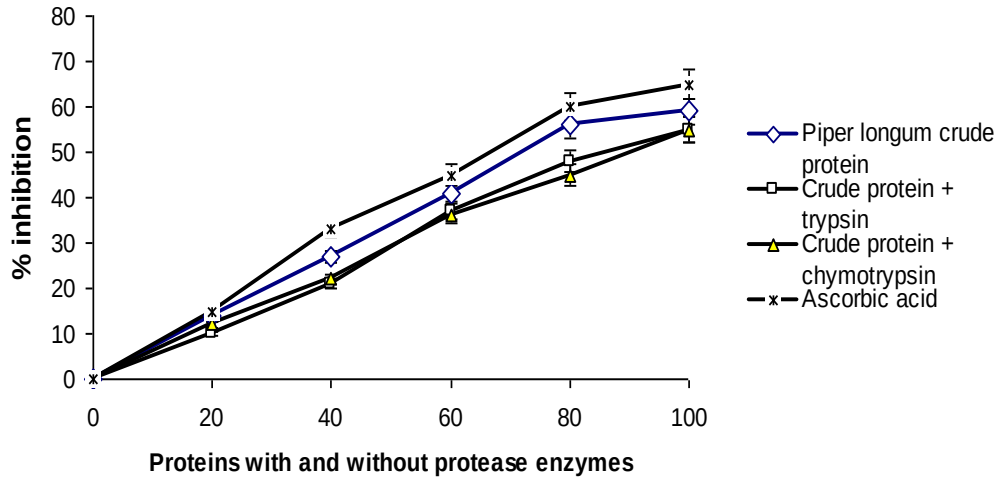

Figure-1: DPPH radical scavenging activity of crude proteins with and without protease enzymes.

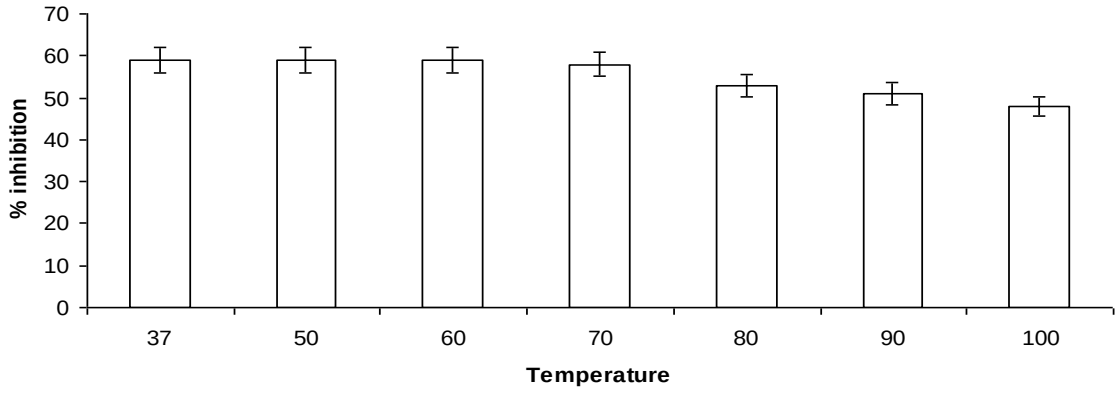

Figure-2: Effect of temperature on DPPH radical scavenging activity of Piper longum crude proteins.

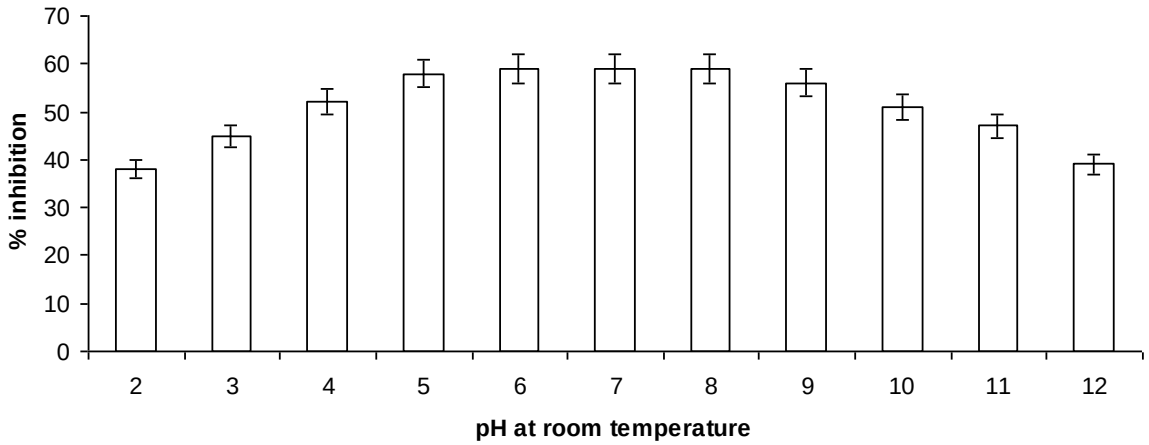

Figure-3: Effect of pH on DPPH radical scavenging activity of Piper longum crude proteins.

The aqueous extract of *Piper longum* seeds subjected to ammonium sulphate (w/v) fractionation for protein precipitation and it was found that 55% saturation was efficient for precipitating the protein. The dialyzed crude protein shows strong serine protease (Trypsin and Chymotrypsin) inhibitory activity.

To analyze the protease inhibitor activity of crude proteins a fixed interval dose dependent DPPH radical scavenging activity was done. Where different doses of protein enzymes are mixed with crude proteins of *Piper longum* incubated at room temperature and subjected to antioxidant analysis.

Figure-1 shows that, no effect of protease enzymes on crude proteins when compared to crude protein alone, bar indicates standard deviation from triplicate determination.

The protease inhibitor crude proteins of *Piper longum* were stable up to 70^0C without any loss in its activity, as the temperature increased from 75^0C to 100^0C the inhibitory activity decreased gradually (Fig. 2).

The inhibitory activity of enzymes trypsin and chymotrypsin was tested at different pH between 2.0 and 12.0 (Fig. 3), the crude protein is stable over a broad range of pH. However there was some decrease in activity at more acidic and more basic pH's, but it was generally stable at weak acidic pH to weak basic pH. The possible occurrence of cysteine residues forming disulphide bonds may responsible for this stability.

CONCLUSION

This is the first report a protease inhibitor nature of seeds of *Piper longum*, and showed a potent inhibitory activity against both trypsin and chymotrypsin. Therefore, future studies in this direction have to be performed to completely elucidate the characteristic features of Protease inhibitory activity of *Piper longum* proteins.

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