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Biochemical Studies on Plant protease Inhibitors

Manjusha Dake, Sumit Singh*

Abstract:

The current work in in the manuscript involves isolation, purification of protease inhibitor from the different plant species Chick peas (Cicer arietinum), Green Tea (Camellia sinensis), Cucumber (Cucumissativus), Pumpkin (Cucurbita maxima), Ashwagandha (Withaniasomnifera) and Black eyed pea (Vigna unguiculata) and comparative analysis of protease inhibitor activities.

Methods: The protease inhibitor was extracted by homogenizing plant material in uisng 0.1 M phosphate buffer (pH-7.0). The crude inhibitor extract as well as the inhibitor purified by ammonium sulfate precipitation were analyzed for inhibitor activity against proteolytic enzymes such as Trypsin, Serratiopeptidase and papain.

Results**:** Chick Peas and Cucumber extract showed inhibitor activity for Trypsin. Cucumber extract and Pumpkin extract indicated inhibiton for Serratiopeptidase enzyme. Pumpkin extract and Aswagandha powder caused inhibition for Serratiopeptidase.

Conclusion**:** Thus the inhibitor activity shown by C. arietinum and C. sativus for Trypsin and and by C. maxima) for papain indicated their therapeutic potential.

Key words: Protease, proteolytic inhibitor, salt precipitation, enzyme activity

Introduction:

Proteolytic enzymes are essential for the survival of all kinds of organisms starting from viruses, bacteria, protozoa, metazoa, or fungi, and ending with plants and animals, and are encoded by approximately 2% of all genes (Rawlings, 2004).

Proteases have important role in many complex physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis,

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*Sumit Singh , Protein Biochemistry Laboratory, Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India,.
Dr. Manjusha dake, Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Tathawade, MS.

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activation of zymogens, release of hormones as well as pharmacologically active peptides from precursor proteins and transport of secretary proteins across membranes (Barrett, 1986). Protease inhibitors isolated from plants have important role in their defense mechanism against proteolytic enzymes released by phytophagous insects and microorganisms (Hara Saburo, 1989). These are ubiquitously distributed among plants, animals and diverse microbial flora (Joanitti et al., 2006).

They are found in seeds of plants and induced in certain plant tissues in response to herbivores or wounding (Koiwa, 1997). They act against exogenous and endogenous proteolytic enzymes from viral, fungal and mammalian origin. They are important elements of natural defense mechanism in plants against microbial pathogens and insect pests by virtue of an anti-nutritional interaction by reducing the availability of amino acids. . Thus plant protease inhibitors showing insecticidal property applied for insect pest control. Developing transgenic crops resistant to insect pests is one of the promising applications of plant protease inhibitors. Most of plant protease inhibitors are of the order of 2-20 KDa which target the cell membrane of pathogens as a major site of their action. Protease inhibitors of plan origin show extremely high affinity for proteolytic enzymes and have drawn attention against for insect defense against crops. Plant protease inhibitors are active against four classes of proteases as serine, cysteine and metallocarboxyprotease. Serine protease inhibitors form superfamily class of protease inhibitors (Gettins, 2002). Plant protease inhibitors are classified as competitive, non competitive, uncompetitive and suicide type of inhibitors (Lawrence and Koundal, 2002). Due to the direct role of protease inhibitors in regulating the activity of proteolytic enzymes, protease inhibitors play a key role in endogeneous defense system. Protease inhibitors have efficacy to prevent viral disorders and can be used as therapeutics. Inhibition of proteolytic enzymes of fungal and animal origin has generated interest for plant protease inhibitors as a protective agent that are the product of a single gene. Plant protease inhibitors are of particular interest because they are generally the product of a single gene, and inhibit proteolytic enzymes of animal and fungal origin, but rarely plant origin, and therefore are thought to act as protective agents (Baldwin and Schultz, 1983; Brattsten, 1991; Green and Ryan, 1972; Holder et al., 1987; Laskowski and Sealock, 1971).

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Microbial proteases are responsible for causing food spoilage to major extent (Chandrashekaran, 1985). Compact nature and higher content of disulfide bridges can contribute to the thermal stability of protease inhibitors (Singh and Rao, 2002). Protease inhibitors act by targeting hydrolytic enzymes of microbes (Satheeshand Murugan, 2011).

Natural plant protease inhibitors can be also applied as food preservative for seafood by increasing the shelf-life (Reppond and Babbitt 1993). Thus developing novel protease inhibitors provide an effective remedy for crop protection by management of insect pest and endogeneous pathogens. They can be employed in effective drug designing against human pathogens (Johnson and Pellecchia M, 2006), and in pharmacology (Imada C. 2005; Robert AC 2005) and in agriculture (Ahn JE, 2000). Therefore the present study is intended to involve isolation of natural inhibitors from plant source in order to find out their applications in medicine and agriculture which remained unexplored to full-fledge level. Viewing the prospects for application of plant protease inhibitors is essential for their practical proposition as therapeutics.

Materials and methods

Extraction of inhibitors from different sources

Chick peas (Cicer arietinum), Green Tea (Camellia sinensis), Cucumber (Cucumis sativus)

, Pumpkin (Cucurbita maxima), Ashwagandha (Withaniasomnifera) and Black eyed pea (Vigna unguiculata) were used as a source of protease inhibitors.

100 gm of leaves from each plant sp. collected as a plant material were washed, crushed using Mortar and pestle and blended in a grinder using phosphate buffer (0.2 M; pH 7.0) and Borate HCl buffer (0.2 M; pH 7.0). The resultant homogenate was filtered through muslin cloth and centrifuged at 10,000 rpm for 15 min at ±4°C. The supernatant obtained was used as a source of crude inhibitor to analyze the inhibition for proteolytic enzymes such as Trypsin and Papain at pH 7.0 and Serratia peptidase pH 9.0 and assayed for the enzyme inhibition activity.

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Partial purification of inhibitor by ammonium sulfate precipitation and dialysis

Supernatant fractions obtained by performing the buffer extraction of plant material as Chick peas, Green Tea, Cucumber, Pumpkin and Ashwagandha were subjected to 80 % ammonium sulfate precipitation for overnight period. The extract was then centrifuged; the resultant precipitate was dissolved in suitable buffer and dialysed against the same buffer for overnight duration. The dialysate was further utilized to check the inhibition activity against Trypsin, Papain and Serratiopeptidase.

Assay Procedure for Trypsin, Papain and Serratia Peptidase (Kunitz, 1947)

The assay procedure for proteolytic enzymes was carried out by incubating 3 ml of casein prepared in respective buffer solutions phosphate/ and Borate-HCl (0.2 M, pH 9.0) along with 1 ml of enzyme solution at 37°C for 30 min. The reaction was terminated by the addition of 3 ml TCA to remove the unreacted casein. The reaction mixture was centrifuged and the clear supernatant added with 2.5 ml sodium carbonate and 0.5 ml of Folin phenol reagent was accessed for the reaction product after incubation at 37°C for 30 min at 660 nm. A suitable control was run by inhibiting the enzyme action with TCA before incubation of the reaction mixture. Activity of Papain, Trypsin and Serratia peptidase was calculated using standard graph for tyrosine. The inhibition assay for proteolytic enzymes was carried out by following the similar assay procedure using 0.2, 0.4, 0.6, 0.8 and 1ml of inhibitor to analyze the effect of variable concentrations of inhibitor.

The inhibition assay for protease was also carried out in the presence of variable concentration of casein from 0.5 to 3.0 % (w/v).

Protein estimation:

The protein content of inhibitor were measured by Folin-Lowry method at 660 nm (Lowry et al., 1951).

Results and Discussion:

Protease inhibitors isolated from plant sources [Chick pea (Cicer arietinum), Pumpkin, (Cucurbita maxima), Cucumber (Cucumis sativus), Ashwagandha (Withania

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somnifera) Green Tea compounds (Camellia sinensis) and Black eyed peas (Vigna unguiculata)] were used to carry out inhibition studies using proteolytic enzymes-Trypsin, Papain and Serratiopeptidase.

Inhibition activity of Chick pea (Cicer arietinum**):**

The effect of variable concentrations of inhibitor from crude and semipurified chick pea extract on Trypsin indicated that increase in concentration of inhibitor lead to successive increase in the inhibition for Trypsin. From figures 1 and 2 it specifies that comparatively higher inhibition for Trypsin was observed after addition of 65µg of inhibitor isolated from crude Chick peas extract. With purified inhibitor, the inhibition observed for Trypsin was highest at 275µg of inhibitor concentration. Thus Greater inhibition was shown by Chick pea inhibitor after ammonium sulfate precipitation followed by dialysis (figure 2).

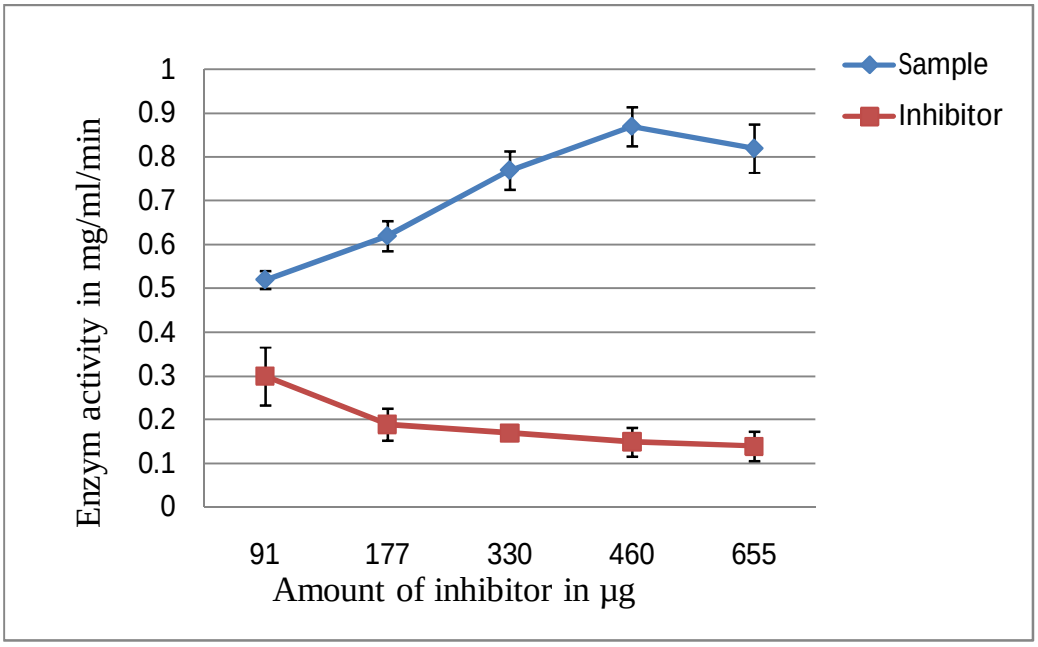

Figure 1. Effect of variable concentrations of crude inhibitor from Chick pea on the activity of Trypsin.

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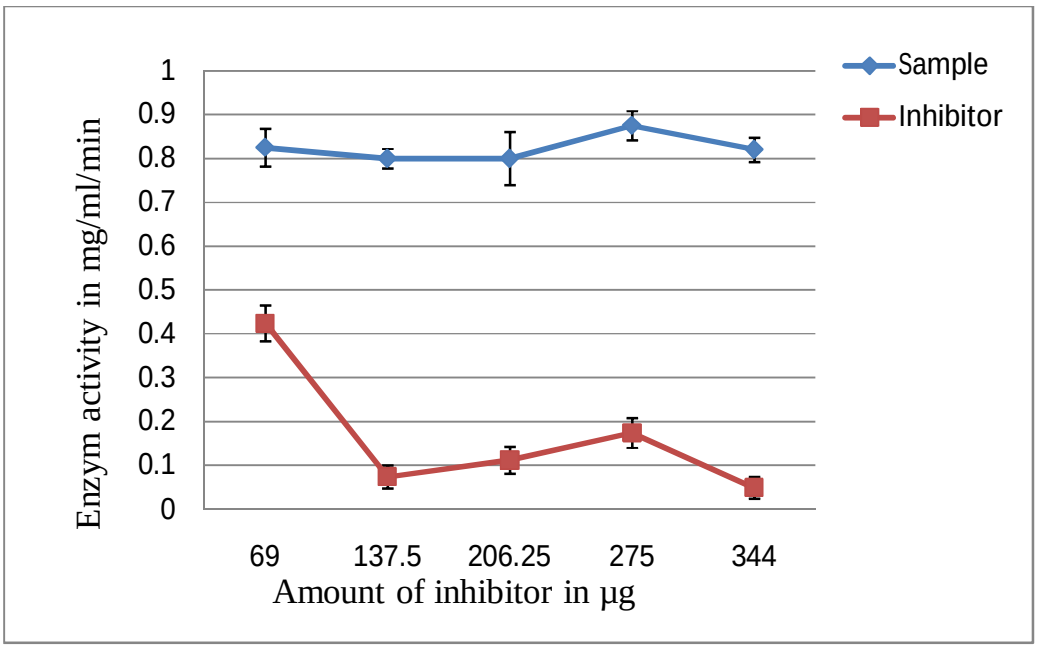

Figure 2. Effect of variable concentrations of purified inhibitor from Chick pea on the activity of Trypsin

Inhibition activity of cucumber (Cucumis sativus):

As per the experimental results observed, the crude inhibitor extract of Cucumis sativus did not show any significant inhibition of Trypsin, Serratiopeptidase and Papain. But the inhibitor purified by salt precipitation method exhibited inhibition for Serratia peptidase and Trypsin after concentration of extract by salt precipitation. The degree of inhibition was comparatively higher for Serratiopeptidase using 34µg of Cucumber inhibitor (figure 3). The inhibition observed for Trypsin was more using with 11.25µg inhibitor concentration (figure 4). But no inhibition was observed for Papain (figure 5).

Again the inhibitory effect shown by cucumber inhibitor was pronounced when the assay was carried out by initial incubation of enzyme and inhibitor followed by addition of substrate compared to when similar assay was done by simultaneous addition and incubation of enzyme, substrate as well as inhibitor. This indicates that cucumber inhibitor must be

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following the mechanism of competitive inhibition while causing the inhibition of Trypsin and Serratia peptidase.

Thus inhibitor purified from Cucumber by ammonium sulfate precipitation did not show any inhibition for papain

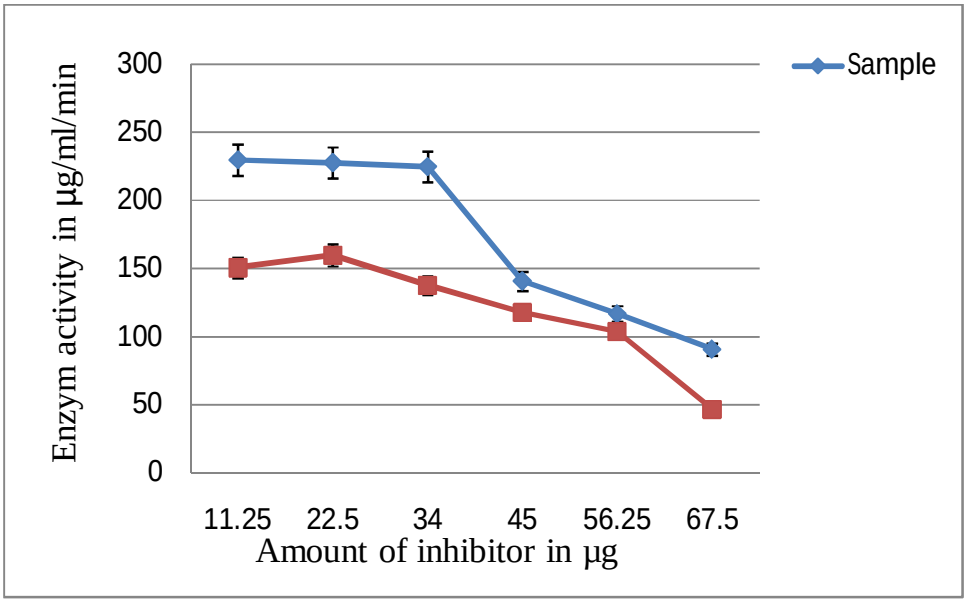

Figure 3. Effect of variable concentrations of purified inhibitor from cucumber on the activity of Serratia peptidase.

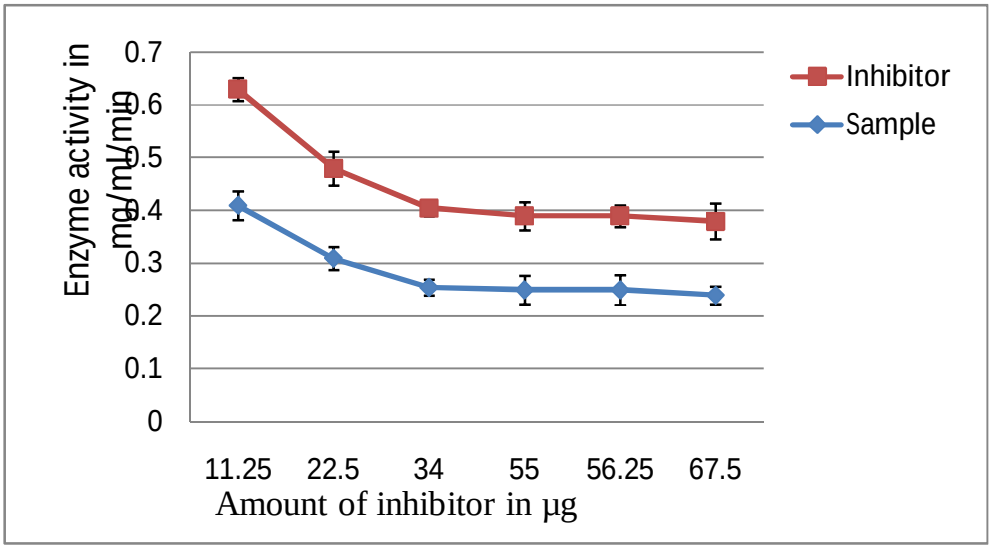

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Figure 4. Effect of variable concentrations of purified inhibitor from Cucumber on the activity of Trypsin.

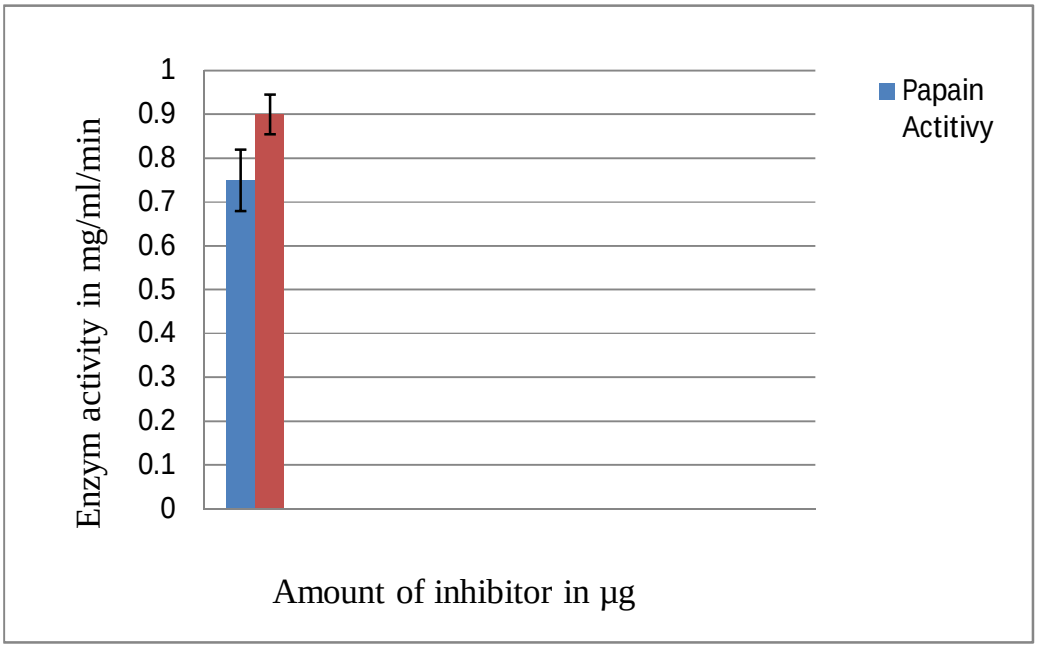

Figure 5. Effect of purified inhibitor from cucumber on the activity of Papain

Inhibition activity of pumpkin (Cucurbita maxima):

Crude inhibitor extracted from pumpkin did not show any inhibition for proteolytic enzymes Serratia peptidase, Trypsin and Papain. But the inhibitor purified from crude pumpkin extract by ammonium sulphate precipitation indicated inhibition of Serratiapeptidase, but no inhibition was observed for Papain and Trypsin.

Figure 6 indicates the effect of variable concentrations of Pumpkin inhibitor on Serratiopeptidas enzyme. Purified inhibitor from Pumpkin extract showed gradual decrease in the activity of Serratiopeptidase with corresponding increase in the concentration of proteinaceous protease inhibitor.

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The extent of inhibiton noted for Serratia peptidase was higher with the addition 27.5µg of inhibitor. But subsequent increase in the concentration of inhibitor did not show any prominent inhibitory effect on the enzyme.

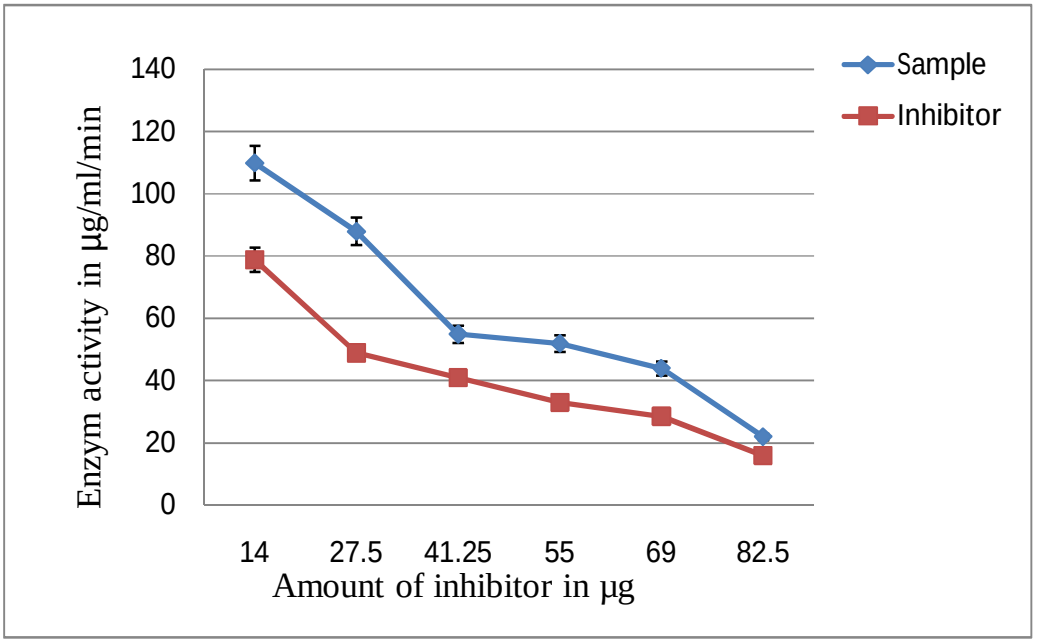

Figure 6. Effect of variable concentrations of inhibitor from Pumpkin on the activity of Serratia peptidase after dialysis.

Inhibition activity of Ashwagandha (Cucurbita maxima):

The inhibitor source used was the extract of Ashwagandha powder prepared by using phosphate buffer (0.2 M, pH 7.0). The crude inhibitor did not show any inhibition for Serratia peptidase, Trypsin and Papain. But after the purification by salt fractionation method, the inhibitory effect was noticed for Serratiopeptidase. The better inhibiton was observed using the inhibitor concentration of 11.27µg (figure 7). But Trypsin and Papain did now show decrease in enzyme activity indicating absence of inhibition or these enzymes. The inhibiton level did not altered using higher concentrations of the inhibitor.

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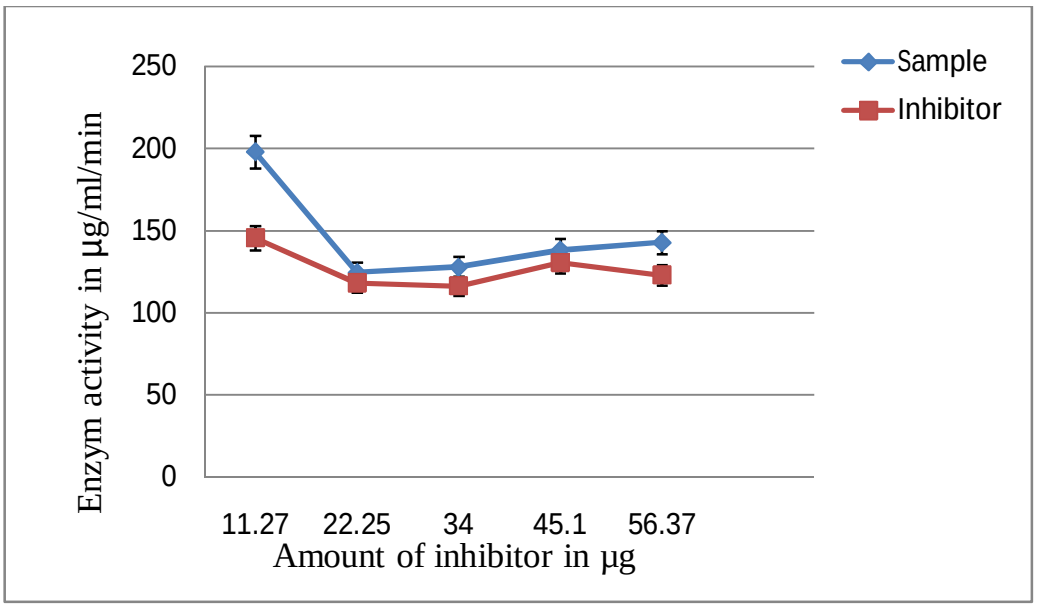

Figure 7. Effect of variable concentrations of purified inhibitor from Ashwagandha on the activity of Serratia peptidase.

Inhibition activity of Black-eyed pea (Vigna unguiculata):

The crude inhibitor extracted from black eyed peas as well as the inhibitor purified by salt precipitation method did not show any inhibition for Trypsin, Papain and Serratiopeptidase, negtive results were observed. The ammonium precipitation and the subsequent dialysis also did not show any inhibition (figures 8 and 9).

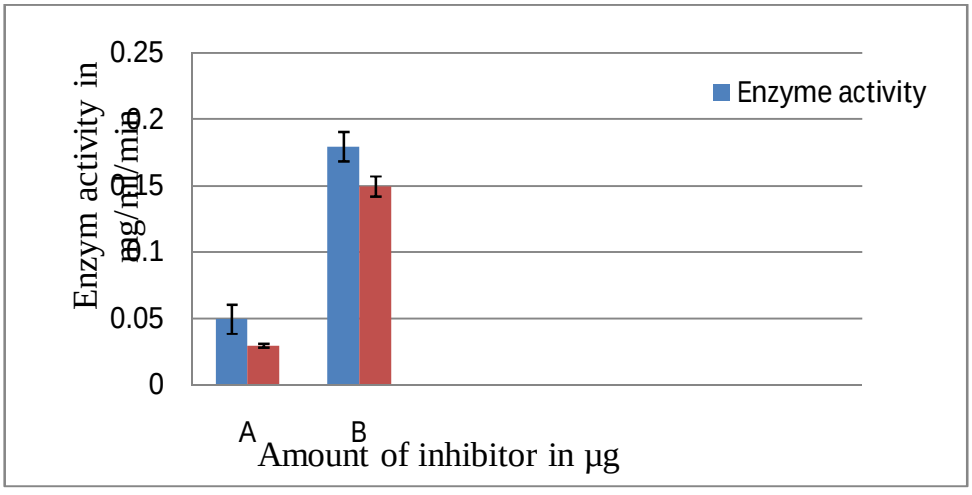

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Figure 8. Effect of the inhibitor from black eyed peas on the activity of A) Trypsin and B) Papain

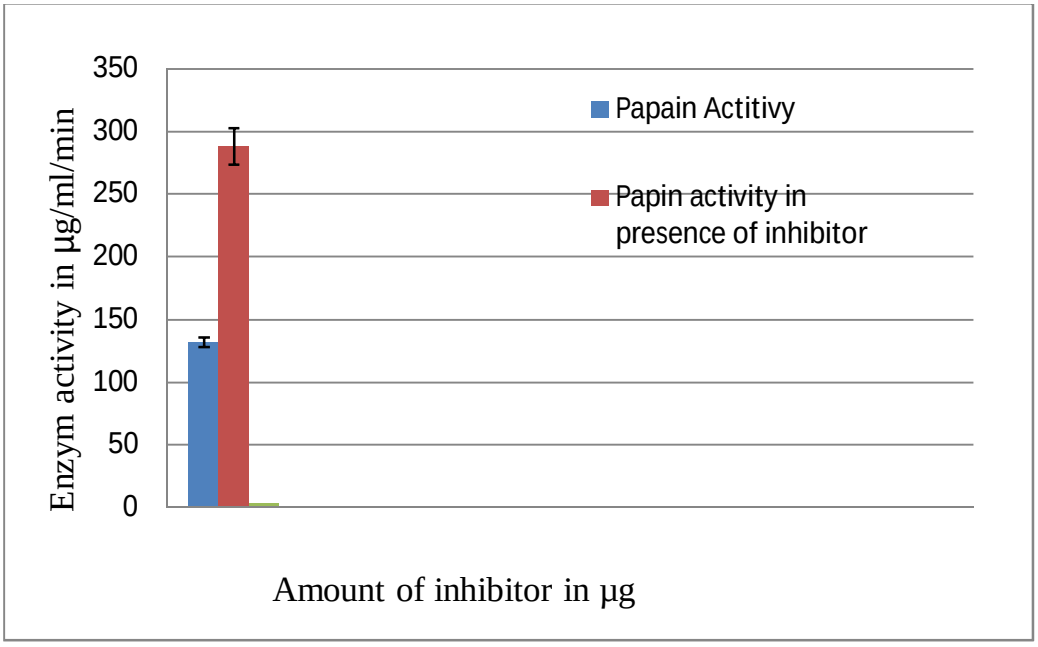

Figure 9. Activity of the Serratiopeptidase in presence of the inhibitor from Black-eyed peas.

Inhibition activity of Green Tea leaves (Vigna unguiculata):

We have investigated the inhibitory effect of polyphenols from green tea on Papain, Trypsin, and Serratiapeptidase (figure 10). Earlier studies showed that green tea exhibits the inhibitory effect on some of the proteases basically from the family of metalloproteases. But as per the experimental results observed, Green tea did not show any such inhibitiory effect on Trypsin, Papain or Serratiapeptidase even after the partial purification. Reverse results were observed with the green leaf tea extract showing increase in the activity of Trypsin, Serratiopeptidase and Papain.

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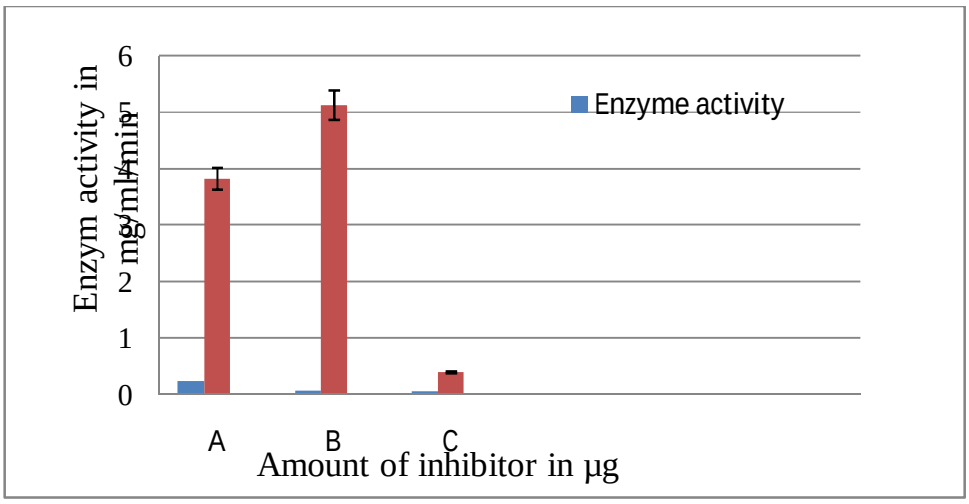

Figure 10: Activity of Trypsin in presence of green tea inhibitor extract.

A-Green tea extracts (Overnight water extraction) -3ml casein+3 ml inhibitor

B-Green tea extract (Overnight water extraction-3ml casein+1 ml inhibitor

C-Green tea extract (Buffer extraction using phosphate extraction (0.1 M, pH 7.0)

D-Green tea extract (Acetone extraction)

Conclusion:

Both Chick Peas and Cucumber extract exhibited inhibition for Trypsin where significant inhibitory effect was shown by Chick peas extract. The extract from cucumber also displayed inhibition for Serratiopeptidase after semipurification by salt precipitation method. No inhibition was observed for Serratiapeptidase and Papain by Chick pea extract even after concentration by ammonium sulfate precipitation. Similarly cucumber extract also did not show any inhibition for Papain.

Both Pumpkin extract and Aswagandha powder caused inhibition for Serratiapeptidase after semipurification by salt precipitation method. But for Pumpkin and Ashwagandha extracts, negative results were obtained indicating absence of inhibition for Trypsin and Papain. For Black-eyed peas; none of the proteases showed any inhibition. Green Tea extract prepared by using water extraction, buffer extraction and solvent extraction

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method enhanced the activity of all of the proteases i.e. Serratia peptidase, Trypsin and Papain rather than causing their inhibition.

References:

1. Rawlings, N. D., Tolle, D. P. and Barrett, A. J. (2004). MEROPS: the peptidase database, Nucleic Acids Research, 32(Database issue), D160–D164.
2. Barrett, A.V. J., & McDonald, J.K. (1986). Nomenclature: protease, proteinase, and peptidase. Biochemical Journal, 237(3), 935-935.
3. Hara, S., Makino, J., & Ikenaka, T. (1989). Amino acid sequences and disulfide bridges of serine proteinase inhibitors from bitter gourd (Momordica charantia LINN.) seeds. Journal of Biochemistry, 105, 88-92.
4. Joanitti , G. A., Freitas, S. M., & Silva, L. P. (2006). Proteinaceous protease inhibitors: structural features and multiple functional faces. Current Enzyme Inhibition, 2(3), 199–217.
5. Koiwa, H., Bressan, R.A. & Hasegawa, P. M. (1997). Regulation of protease inhibitors and plant transgenic tobacco containing genes encoding Bacillus thuringiensis delta-endotoxin or cowpea trypsin inhibitor: efficacy against Helicoverpa zea (Lepidoptera: Noctuidae). Journal of Economic Entomology, 85, 2516-2522.
6. Gettins, P. G. W. (2002). Serpin structure, mechanism, and function. Chemical Reviews, 102(12), 4751–4804.
7. Lawrence, P. K. & Koundal, K. R. (2002). Plant protease inhibitors in control of phytophagous insects. Electronic Journal of Biotechnology, 5(1), 93-109.
8. Baldwin, I. & Schultz, J. (1983). Rapid changes in tree leaf chemistry induced by damage: E'iidence for communication between plants. Science, 221, 277-279.
9. Brattsten, L. B. (1991). Bioengineering of crop plants and resistant biotype evolution in insects: counteracting coevolution. Archives of Insect Biochemistry and Physiology, 17, 253-267.

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E-Journal of Research
ISSN NO: 2395-339X

10. Green, T., & Ryan. C. (1972). Wound-induced proteinase inhibitor in plant leaves: A possible defense mechanism against insects. Science 175: 776-777.
11. Hilder, V. A., Gatehouse, A. M. R., Sherman, S. E., Barker, R. F., & Boulter, D. (1987). A novel mechanism of insect resistance engineered into tobacco. Nature , 300: 160-163.
12. Laskowski, M., Jr., Sealock, R.W. (1971). Protein proteinase inhibitors molecular aspects. In: The Enzymes, Vol. 3, pp. 375–473, Boyer, P.D., ed. New York, London: Academic Press.
13. Chandrasekaran, M. (1985). Studies on Microbial spoilage of Penaeus indicus: School of Marine Sciences, Cochin University of Science and Technology, Cochin.
14. Singh, R. R., & Rao, A. G. (2002). Reductive unfolding and oxidative refolding of a Bowman-Birk inhibitor from horse gram seeds (Doritos biflorus): Evidence for 'hyperreactive' disulfide bonds and rate-limiting nature of disulfide isomerization in folding. Biochimica et Biophysica Acta, 1597, 280-291.
15. Sateesh, L. S. & Murugan, K. (2011) Antimicrobial activity of protease inhibitor from leaves of Coccinia grandis (L.) Voigt, Indian Journal of Experimental Biology, 49(5), 366-374.
16. Reppond, K. D., Babbitt, J. K. (1993). Protease inhibitors affect physical properties of arrow tooth flounder and walleye Pollack surimi. Journal of Food Science, 58, 96-98.
17. Johnson, S., and Pellecchia, M. (2006). Structure- and fragment based approaches to protease inhibition. Current Topics in Medical Chemistry, 6, 317-329.
18. Robert, F., Nardy, L., & Thomas H. R. (2012). Serpin protease inhibitors in plant biology. Physiologia Plantarum, 145(1), 95-102.
19. Ahn, Y. B., Rhee, S. K., Fennell, D. E., Kerkhof, L. J., Hentschel, U., & Haggblom, M. M. (2003) Reductive dehalogenation of brominates phenolic compounds by microorganisms associated with the marine sponge Aplysina aerophoba. Applied and Environmental Microbiology, 69, 4159-4166.
20. Kunitz. Crystalline soyabean trypsin inhibitor III. General properties. J Gen Physiol 1947;30:291-310.

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E-Journal of Research

ISSN NO: 2395-339X

21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-75