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Protease Inhibitor Associated Antimicrobial Activity of Pea *Pisum sativum* L. cv. Arkel

Huma Habib[1]*, Khalid Majid Fazili[2], Mohammad Afzal Zargar[3] and Bashir Ahmed Ganie[4]

[1]Department of Biochemistry, Islamia College of Science and Commerce, Srinagar 190003, India

[2]Department of Biotechnology, University of Kashmir, Srinagar 190006, India

[3]Registrar, Central University, Srinagar, 190015, India

[4]Director CORD, University of Kashmir, Srinagar 190006, India

*Corresponding Author E-mail: huma99@gmail.com

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ABSTRACT

Aqueous and organic extracts were prepared from fresh pea seeds by standard methods. The extracts were analyzed for protease inhibitory activity using Dot,blot assay. The aqueous as well as the organic extracts obtained with ethanol showed significant protease inhibitory activity. The aqueous extracts were treated with ammonium sulfate to cause differential protein precipitation. 30%, 60% and 90% ammonium sulfate saturations were used to prepare these different protein fractions. 60% and 90% fractions were positive for protease inhibitor (PI) activity on dot blot assay, the 30% fraction did not show any inhibitory activity. The assay of antibacterial activity by disc diffusion method using E.coli (ATCC 25922) strain revealed that the crude aqueous extract, 90% fraction and ethanolic extract were highly active in inhibiting the bacterial growth, the 60% fraction showed moderate antibacterial activity and the 30% fraction was not significant.

Keywords: *antibacterial activity, protease inhibitor, pisum sativum*

INTRODUCTION

Proteases are the enzymes that catalyze the hydrolytic cleavage of peptide bonds in proteins. Proteolytic enzymes, proteinases or peptidases are other names for these enzymes. These enzymes assist in processing of the cellular information, and act as molecular mediators of many vital processes, from embryonic development to immune response. As digestive enzymes, proteases cause breakdown of proteins into their individual components[1]. Proteases, on the basis of the specific catalytic residue present in their active site have been classified into serine, cysteine, aspartate and metalloproteases. During the course of evolution, each of these groups of proteins have got specialized to perform certain functions. Coagulation of blood is the responsibility of serine proteases, apoptosis is brought about by cysteine proteases, protein digestion essentially requires aspartyl proteases and metalloproteases are required for breakdown and rebuilding of the extracellular matrix[2].

Proteolysis is the key process in all living organisms. The different proteolytic events serve as intermediates in many of the signaling pathways and are of importance in major cellular events like inflammation, apoptosis, blood coagulation and hormone processing.

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Being essentially indispensible, these proteins may be potentially damaging when overexpressed or present in high concentration and need to be correctly regulated[3].

Protease inhibitors (PIs) are proteins that form stoichiometric high affinity complexes with proteases and inhibit their hydrolytic activity[4]. PIs are of very common occurrence. They have been isolated and characterized from a large number of organisms, including plants, animals and microorganisms[5-9]. Naturally occurring PIs are essential for regulating the activity of their corresponding proteases and play key regulatory roles in many biological processes. PIs act as defense proteins against insects and microorganisms and possess antibiotic activity against fungi and some viruses[10]. Most PIs interact with target proteases by binding to the active site of the protease resulting in the formation of protease-inhibitor complex and is incapable of enzyme activity[11]. Proteases regulate activation, synthesis and turnover of all proteins. Nature has developed checkpoints called protease inhibitors as one of the strategies to control them. Uncontrolled proteolysis is implicated in diseases like emphysema, systemic inflammatory response syndrome, arthiritis, pancreatitis, hepatitis etc[12].

PIs are of common occurrence in the plant kingdom. Plant PIs (PPIs) are generally small proteins that have mainly been described in storage tissues, such as tubers and seeds, but have also been found in the aerial parts of plants[13]. They are also induced in plants in response to injury or attack by insects or pathogens[14]. In plants, these PIs act as anti,metabolic proteins, which interfere with the digestive process of insects. They are in particular effective against phytophagous insects and microorganisms. The defensive capabilities of PPIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development[15].

Protease inhibitor genes have been identified and characterized in a large number of organisms. In higher organisms, nearly 2% of the genes[16] code for the proteolytic enzymes whose activity is regulated by these inhibitors. Protease inhibitor genes unlike the genes encoding complex pathways can be transferred separately from one plant species to another and expressed from the wound inducible or constitutive promoters of the host plant. This has been demonstrated that trypsin inhibitor gene from Vigna unguiculata when expressed in tobacco conferred resistance to wide range of insect pests.

A number of inherited diseases are caused due to abnormalities in protease inhibitors. These include different forms of emphysema, epilepsy, hereditary angio, neurotic oedema and Netherton syndrome[17-20]. Some of these diseases may be susceptible to treatment with the inhibitors administered as drugs, with synthetic inhibitors that take over their function, or with natural inhibitors made available by gene therapy[21]. Excessive proteolytic activities may well contribute to a number of diseased conditions, and gene therapy to introduce inhibitors to counter the effect of excessive proteases is under consideration[22,23].

In view of the tremendous biological significance associated with these inhibitors, efforts are underway for a full scale exploitation of natural resources in this context. As a part of the programme, this study aimed at analyzing the antimicobial potential of the protease inhibitory activity associated with Pea *(Pisum sativum*).

MATERIALS AND METHODS

Preparation of aqueous extract

15 g of fresh pea seeds were taken and flash frozen in liquid nitrogen. The frozen material was ground with a pestle and thawed on ice. 15 ml protein extraction buffer was added, the contents were thoroughly mixed on a vortex and centrifuged at 10,000 rpm for 20 min. The supernatant was taken in a clean tube and stored at 4^{o}C.

Preparation of Organic extracts

10 g of flash frozen pea seeds were crushed and thoroughly mixed in 90 ml of 80% ethanol. The slurries were stirred continuously for 2 hrs at 4^{o}C and centrifuged at 10,000 rpm for 10 mins. The supernatant was carefully collected, filtered and stored at 4^{o}C. The extracts with methanol, ethyl acetate and acetone were prepared in a similar manner.

Determination of protein concentration

Protein concentration in seed extract was determined by measuring UV absorbance at 280 nm or alternatively by dye binding method.

Dot blot analysis

2 µl of trypsin solution (4µg/100µl in 0.1 M Tris HCl buffer, pH 7.6) was mixed with 2 µl of extract containing inhibitor and incubated at 4°C for 5 min. This solution was spotted on a x,ray film. A clear zone is observed in the control as trypsin causes degradation of the gelatin coating on the x,ray film. In the test solution, the presence of inhibitor causes inactivation of the trypsin and thus reduces the clear zone[24].

Ammonium sulfate precipitation

Aqueous extract of pea was saturated to 30%, 60% and 90% saturation with ammonium sulfate by adding the desired amount of the salt to cause differential protein precipitations or alternatively by using the following formula.

The salt was added to the aqueous extract and stirred until completely dissolved. The solution was centrifuged at 10,000 rpm for 20 min. The pellet was dissolved in a minimum volume of the protein extraction buffer which consisted of 0.1M Tris, HCl, pH 7.6 and 10 mM calcium chloride and dialyzed overnight at room temperature. The contents were centrifuged 10,000 rpm for 10 min to get a clear supernatant.

Screening for antibacterial activity

Analysis of antibacterial activity

The antibacterial activity of aqueous and organic extracts was studied by Disc Diffusion Method[25]. 0.8 g of agar was dissolved in 20 ml of distilled water, sterilized in an autoclave and poured in petriplate. The plates were streaked with a loop using E.coli strain (ATCC 25922). 5 µl of the extract was loaded on disc and applied on the agar plate. Water or alcohol was used in a similar manner to serve as control. The plates were incubated at 37°C for 24 hrs to allow the bacterial growth. The inhibitory activity of the extract prevented the growth of bacteria around the disc and formed a clear zone. This represented the zone of inhibition. The zone of inhibition was calculated by measuring the minimum dimension of the zone around the disc.

RESULTS AND DISCUSSION

Antibacterial Activity

The antibacterial activity of the aqueous and organic extracts was assessed by Disc diffusion method using E.coli strain (ATCC 25922). Figures 1, 2, 3 and 4 represent the results of Disc diffusion method respectively for crude, 30%, 60% and 90% aqueous extracts and for ethanolic extract. The equivalent volume of water or the organic solvent was used as a control and applied simultaneously in each plate in a separate well. The control in all the cases did not produce any clear zone around but was as opaque as the rest of the plate. The clear zones represented inhibition of the bacterial growth and the zone area is a measure of the antibacterial activity. The zone of inhibition was calculated with the help of geometrical scale by measuring the minimum dimension of the zone around the disc in terms of mm (Table 1).

Protease inhibitory activity

Protease inhibitory activity of the ethanolic extract was assayed by Dot Blot method. The inhibitory activity of ethanolic extract is shown in Fig.5 in which ‘a’ represents extract and trypsin, ‘b’ and ‘e’ represents trypsin, ‘c’ represents the ethanolic extract, and ‘d’ represents buffer. ‘b’ and ‘e’ show clear zones because of the gelatin hydrolyzing activity of trypsin. ‘c’ and ‘d’ are used as controls. ‘a’ that contains trypsin preincubated with ethanolic extract does not show any clear zone around the area of application. The disappearance caused due to the ethanolic extract indicates that the trypsin inhibitory activity of the extract inactivated the trypsin and had no effect on the x-ray film.

While the battle between man and microbes continues, starting with the defeat suffered by penicillin, Methicillin, Vancomycin and other antibiotics especially ESBL, antibiotics. It is important and valuable to find compounds that potentiate antimicrobial activity against extended spectrum beta lactamase organisms such as *E. coli.*

Medicinal plants play a central role not only as traditional medicines but also as commercial commodities meeting the demand of distant markets. To compete with the growing market, there is need to expeditiously utilize and scientifically validate more medicinally useful plants. Because of the appearance

of drug resistance to antimicrobial agents, more effort is being made to find alternative antimicrobial components. It had been suggested that natural products are a preferable option to synthetic ones. Literature indicates that medicinal plants are the backbone of traditional medicine and the antibacterial activity of plant extract is due to different chemical agent in the extract with antimicrobial compounds[26]. In plants, these secondary metabolites function to attract beneficial and repel harmful organisms, serve as phytoprotectants and respond to environmental changes. In humans, however the compounds have beneficial effects including antioxidant, anti,inflammatory effects, modulation of detoxification enzymes, stimulation of the immune system, modulation of steroid metabolism and antibacterial and antiviral effects[27]. Results from the current study indicate that aqueous and organic extracts of the pea plant contained varied types of pharmacologically active compounds with antimicrobial activity. The extracts of the pea plant demonstrated varied antimicrobial activity to the test organism which was species and concentration dependent. The currents results support findings of[28] who demonstrated the presence of antimicrobial activity in seed extracts of A. caudatus.

The results obtained showed that extracts of Pea seeds exhibited inhibitory activities against E. coli at varying degrees of concentration as demonstrated by the diameters of the zones of inhibitions.Indeed herbs that have tannins as their main component are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery[29]. This is the basis for the antimicrobial use of such plants in the treatment of intestinal bleeding, diarrhea and excessive menstruation[30,31].

The increase of antibiotic resistance of microorganisms to conventional drugs has necessitated the search for new, efficient and cost effective ways for the control of infectious diseases. The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new antibacterial agents[32,33]. The use of plants is part of the Indian tradition. Many local regions all over India have a great variety of vegetation used by the local population to treat and prevent diseases. From this study we can concluded that, the seeds of pea plant has a wide range of antibacterial activity and supports the traditional use of this plants as medicines. This study demonstrated that herbal medicine can be as effective as modern medicine to combat pathogenic microorganisms.

Protease inhibitors act as antimetabolic proteins which interfere with digestive process of insects and micro,organisms. Defensive capabilities of protease inhibitors rely on inhibition of protease present in insect guts or secreted by micro,organisms causing reduction in availability of amino acids necessary for their growth and development[34].

Protease activities are regulated at the transcriptional level by differential expression and at the protein level by activation of inactive zymogens and by the binding of inhibitors and cofactors. Activation can be either autocatalytic or catalyzed by other proteases. Alternatively, proteases are sometimes activated with the assistance of activation. Protease activity is also regulated by cofactors, proteins that reversibly bind to proteases and/or inhibitors and affect their final activity, often in an allosteric manner. The blood coagulation cascade provides the most well,known examples, with tissue factor protein regulation of Factor VIIa activity[35] being the best,known cofactor to be explored by the pharmaceutical industry[36].

Table 1: Antibacterial activity of aqueous and ethanolic extract of Pisum sativum against *E. coli* (ATCC 25922) strain

Extracts of *Pisum sativum*	***E.coli* strain ATCC 25922**
Extract	Zone of inhibition (mm)
Crude extract	13.5
90% sample	10
60% sample	8
30% sample	4
Ethanolic extract	15

Fig. 1: Antibacterial activity of 30% and crude aqueous extracts using *E. coli* (ATCC 25922) strain. a) 30% aqueous extract, b) crude aqueous extract and c) control

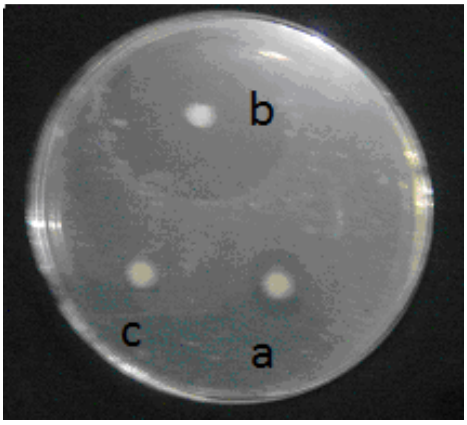


Fig. 2: Antibacterial activity of 60% aqueous extract using *E. coli* (ATCC 25922) strain a) 60% aqueous extract and b) control

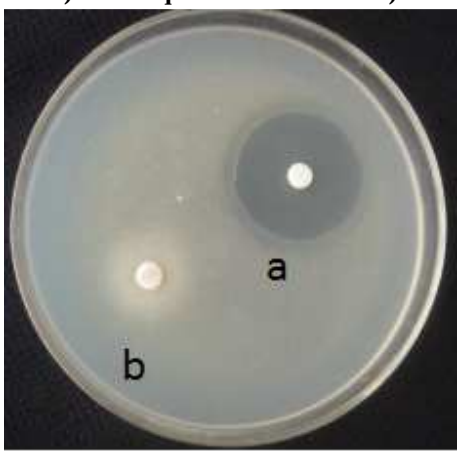


Fig 3: Antibacterial activity of 90% aqueous extract using *E. coli* (ATCC 25922) strain a) 90% aqueous extract and b) control

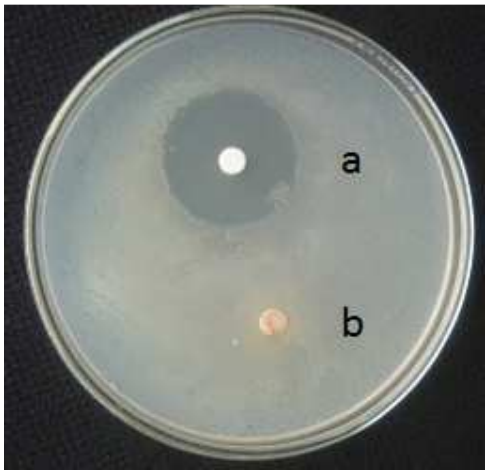


Fig. 4: Antibacterial activity of ethanolic extract using *E. coli* (ATCC 25922) strain a) ethanolic extract and b) control

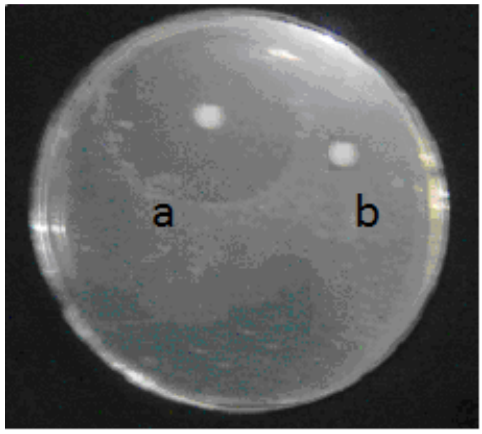
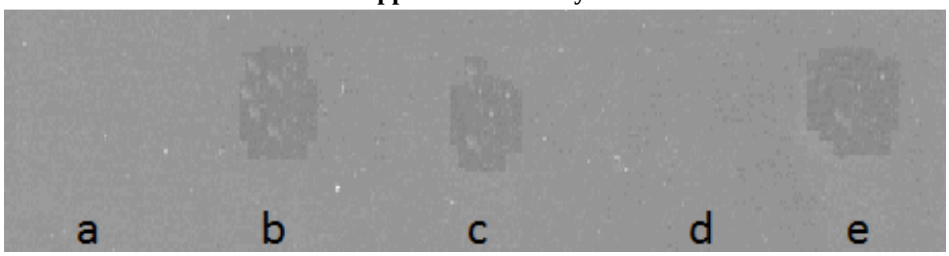


Fig 5: Dot blot assay for qualitative analysis of protease inhibitor activity. 50µl of each sample were applied on the x-ray film



a) Ethanolic extract plus trypsin, b) buffer plus trypsin, c) ethanolic extract, d) buffer and e) trypsin

CONCLUSION

Plant protease inhibitors (PPIs) are small proteins generally induced in plants in response to injury or attack by insects or pathogens. These anti,metabolic proteins interfere with the digestive process of insects and are in particular effective against phytophagous insects and microorganisms. The antimicobial potential of these inhibitirs gains importance in view of some of the inhibitors being administered as drugs in diseases like emphysema, epilepsy, hereditary angio, neurotic oedema and Netherton syndrome. This study establishes that the inhibitors derived from natural sources and in particular from pisum sativum possess highly significant anti bacterial activity that adds to their therapeutic importance.

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