Evaluation of Protease Inhibitory Activity in Pea (*Pisum sativum)*

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ABSTRACT

Protease inhibitory activity was detected from aqueous extract of *Pisum sativum*. Fresh pea seeds were taken, flash frozen in liquid nitrogen, ground with pestle and mortar and then thawed on ice. The crushed sample was mixed with protein extraction buffer and centrifuged .The protein concentration of sample was determined using Bradford method .The sample was analyzed for protease inhibitory activity by standard procedures. Suitable assay was performed and an opaque zone was formed when drop of extract was put on X-ray film with respect to control (trypsin and Buffer).Salt fractionation of pea extract was performed and inhibitory activities of the salt fractionated samples were checked and results were positive. SDS-PAGE of extract was performed with respect to crude extract of pea as standard. All the samples i.e. 30%, 60% and 90% get the bands at same location.

Key words: Protease inhibitors, Pisum sativum, proteases

Abbreviations: PI: Protease inhibitors, SDS: Sodium dodecyl sulfate

INTRODUCTION

Nature has provided us with certain regulatory mechanisms which prevent over secretion and hyperactivity of proteases. The proteins that inhibit the proteases and limit their activity by competitive inhibition are called protease inhibitors. Some protease inhibitors occur in plants naturally while some are synthetic as being found in processes of blood coagulation, fibrinolysis and complement cascade of animals. Protease inhibitors help in regulation of proteolytic processes and maintain intracellular metabolism of proteins. They help in self-defense mechanisms in plants against predators, pathogens and pests (Ryan, 1990). They are important tools of crop improvement targeting plant protection and human nutrition. They are also used as antiviral agents hence can be used in therapeutics against fatal viruses e.g. Picorna, Herpes, HIV.PIs have been considered to counter a act tumor progression and metastasis (Clemente *et al.,* 2005). PI genes are currently being used to develop anti fungal, antiviral and pathogen resistant transgenic crops (Valueva *et al.,* 1999; Krattiger and Anatole, 1997). Protease inhibitor genes are also involved in regulation of Programmed Cell death in plants (Mazal *et al*., 1999). Several non – homolgous families of protease inhibitors are recognized among animal, plant and microorganism kingdoms. Protease inhibitors are abundant in storage organs and seeds of plants (Ryan, 1977). Majority of protease inhibitors studied in plant kingdom are

from Solanaceae, Leguminaceae and Graminaceae. Their synthesis is induced to high levels in response to stress, infection and wounding (Jongsma *et al.,* 1994).These inhibitor families that have been found are specific for each of the four mechanistic classes of proteolytic enzymes and are based on the active amino acid in their "reaction centre" (Kiowa *et al.,* 1997). These are Serine PI, Cysteine PI, Aspartic PI and Metallo PI.

Protease inhibitors have been worked out and isolated from many plants e.g. potato, tomato (Rancor,1968; Keilova and Tomasek,1976), black eyed pea (Louis Slade , et al., 1976), cow pea (Paulraj *et al.,* 2000), Mung beans (Maarten and Brumgartner,1978) , Medikus tubers (Zhang *et al.,* 2008), grass pea seeds, horse gram seeds, soya bean, and black gram (Maitra *et al.,* 2007). In this study an attempt is made to evaluate the protease inhibitory activity in pea (*Pisum sativum*), a frequently grown, edible, nutritious and tasty legume in Kashmir valley.

MATERIAL AND METHODS

Pea plants were collected from local seller. Seeds were washed and cleaned thoroughly.15 mg of pea fresh weight, flash frozen in liquid nitrogen were ground with a pestle and mortar and thawed on ice. 15 ml protein extraction buffer (0.1 mM Tris chloride pH 7.6 and 10mM calcium chloride) was added to powdered sample and vortexed thoroughly. Centrifugation was carried out at 10,000 rpm for 20 min. Supernatant was preserved at 4 degree Celsius.

Protein Estimation: Protein content of sample was determined by Bradford method and samples were read at 595nm (Bradford, 1976).

Dot Blot Analysis: To expedite the recognition of PIs, a method utilizing surface of an X-ray film as proteolytic substrate is employed. Positive reaction is indicated by clear zone on the film after rinsing with water (Cheung *et al.,* 1991).

Salt Fractionation: Aqueous extract of pea was treated with different concentrations of ammonium sulfate (Mw: 132.14) to precipitate different proteins. Amount of ammonium sulfate required for achieving 0-30%, 30-60%, and 60-90% saturation has been taken from nomogram or calculated. Centrifugation was carried at 10,000 rpm for 20 min. Precipitate was re suspended in protein extraction buffer and kept for dialysis for 12 hours. Supernatant obtained from each salt saturations were preserved. PI inhibitory activity of each sample was detected using Dot blot assay.

SDS -Poly Acrylamide Gel Electrophoresis: SDS - PAGE of aqueous extract of pea was carried out with mini gel apparatus in Tris glycine buffer, pH 8.8 . SDS –PAGE was performed using method of Laemmli (1970). Gel was stained overnight and destained to view the bands.

RESULTS AND DISCUSSION

Aqueous extract of pea was prepared to evaluate the protease inhibitory activity (Fig.1). The concentration of protein in aqueous pea extract was 338.75 mg%. Dot blot assays were performed in which drop of pea extract was put on X ray film with respect to buffer and trypsin as control. A clear zone was formed at zone of trypsin ,no effect was shown by buffer and an opaque zone was formed by extract .The opaque zone is formed as a consequence of the presence of inhibitors, which does not allow the proteases to digest the gelatin coated on X-ray film. Gelatin is a protein which can be degraded by proteases like trypsin. Salt fractionation of pea extract was performed and inhibitory activities of each of the salt fraction samples were checked. A drop of each of the sample was put on the X-ray surface with respect to control (Fig. 2-4). Opaque zone was formed which indicate presence of PIs in samples. 30% of salt fractionation sample used against trypsin showed little inhibitory activity because of lesser salt saturation and hence lesser purification (Fig.2). But 60% and 90% samples showed the presence of PIs which does not allow the digestion of gelatin on film (Fig. 3, 4). SDS-PAGE of salt fractionation pea extract samples was performed with respect to crude pea extract and we get four different bands over (Fig.5).It indicates presence of multi subunit PI protein. All the samples (30%, 60% and 90%) get the band at same location.

Results will be discussed in light of other family members of Leguminacea pea seeds contain a number of inhibitor proteins which have negative effects on digestibility. The researchers had shown that pea protease inhibitors can reduce the proliferation of adeno carcinoma cells in vitro and may provide benefit as dietary anti carcinogens. (Clemente *et al.,* 2005).The results also show presence of PIs in *Pisum sativum.* .The protease inhibition studies were performed on grass pea seeds, horse gram seeds, soya bean, and black gram using proteases from rohu fingerling.In case of grass pea seed, more than 50% inhibition of alkaline protease activity was recorded when the ratio of inhibitor to enzyme was 9.41 μgU^{-1}. These results also reveal that grass pea seeds also contain protease inhibitor. (Maitra *et al.,* 2007). Another study done on pea indicates presence of carboxy protease belonging to metallo or metal-activated arid serine proteases family. It strongly means that there will be definite regulatory PIs against these proteases confirming our finding. (Craigh *et al.,* 2004). Similarly pigeon pea (*Cajanus cajan* L.) extracts have been analyzed for the protease inhibitors using Gel X ray film technique for detection of electrophoretically separated protease inhibitors. (Veerapa et al., 2006). Sangeeta *et al*. (2006) confirmed marked changes in protein content of pigeon pea (*Cajanus cajan* L.) during process of germination and seed development confirming that PIs are found in these seeds for the regulatory mechanisms. So legumes are considered to be rich source of PIs. Hence it confirms our findings and thus pea (*Pisum sativum*) can be used as a source of PIs but their isolation and characterization need further studies.

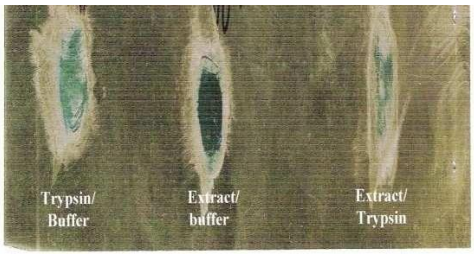

Fig.1 : Pea extract showing inhibitory activity for protease trypsin

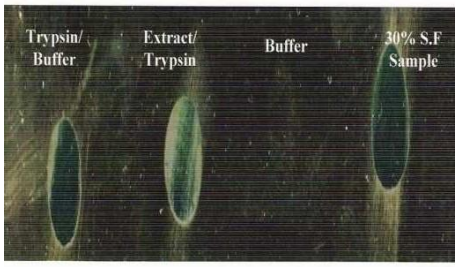

Fig.2: 30% Salt fractionation sample showing little inhibitory activity.

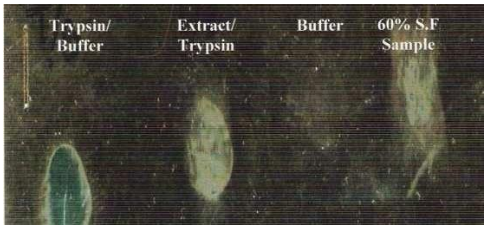

Fig.3 : 60% Salt fractionation sample showing inhibitory activity.

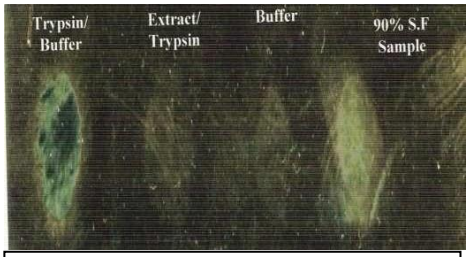

Fig. 4 : 90% Salt fractionation sample showing inhibitory activity.

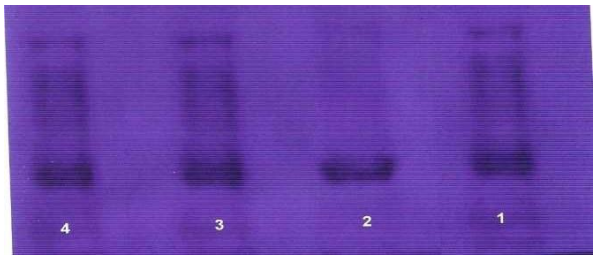

Fig.5: SDS-PAGE gel: Lane 1 to 4 represent from right to left represent crude extract, 90, 60, 30 % S.F samples

REFERENCES

Bradford, M.M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein dye binding. *Anal.Biochem*.**72:**248- 254.

Cheung, A.C., Ying, P. and Fischetti, V.A. 1991. A method to detect protease activity using unprocessed X-ray films. *Anal.Biochem*., **193**:20-23.

Clemente, A., Gee, J. M., Johnson, I. T, Mackenzie, D. A. and Domoney, C. 2005. Pea (*Pisum sativum* L.) Protease inhibitors from the Bowman-Birk class influence the growth of human colorectal adenocarcinoma HT29 cells in vitro. *J Agric Food Chem.*, **53**: 8979-86.

Craigh G. J, Grantley, W. L and Gregory A. T. 2004. Inhibitor studies and cloning of a Serine Carboxypeptidase cDNA from germinating seeds of pea (*Pisum sativum* L.). *European Journal of Biochemistry.,* **235** (3): 574 – 578

Godbole A, Sangeeta, G. Krishna, Thirumalai and R Bhatia , Chittaranjan. 2006. Changes in protease inhibitory activity from pigeon pea (*Cajanus Cajan* (L) millsp) during seed development and germination. *Journal of the Science of Food and Agriculture.* **66**(4**)**:497 - 501

Jongsma, M.A, Bakker, P.L. and Stiekema, W.J. 1994.Trypsin inhibitory activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta,* **195**:29-35.

Keilova, H., and Tomasek, V. 1976. Isolation and properties of Cathepsin D inhibitor from potatoes. *Collection of Czechoslovak Chemical Communication*., **41**:489-497.

Koiwa, H, Bressan, R.A. and Hasegawa, P.M. 1997. Regulation of protease inhibitors and plant defense. *Trends in Plant Science.*, **2**:379-384.

Krattiger,Anatole.F. 1997. Insect resistance in crops: A case study of *Bacillus Thruingenesis* and its transfer to developing countries. *ISAAA* Briefs, **2**:42. Laemmli, U.K. (1970).*Nature*.227:680.

Lawrence, Paulraj, Nirmala , Kripa, R. Koundal; 2000 .Nucleotide sequence of genomic clone encoding a cowpea trypsin inhibitor. *Electronic Journal of Biotechnology*, **4**(1).

Louis S.G. and Charles, R.C. 1976. Double headed protease inhibitors from Black Eyed pea. *The Journal of Biological Chemistry*., **251** (3).

Maarten, J. Chrispeels and Bruno Baumgartner. 1978. Trypsin inhibitor in mung bean cotyledons: purification, characteristics, sub cellular localization, and metabolism. *Plant Physiol*., **61** (4):617-623

Maitra, Sudipta; Ramachandran, Sarita and Ray, Arun Kumar. 2007. *In vitro* assay of plant protease inhibitors from four different sources on digestive proteases of rohu, *Labeo rohita* (Hamilton), fingerlings. *Aquaculture Research,* **38**(2): 156-165.

Mazal, S. Beatrice, B Massino, D . Ester, M. and Alex, L. 1999.The involvement of cysteine proteases and protease inhibitor genes in regulation of programmed Cell death in plants. *The Plant Cell*, **11**:431-443.

Mulimani. H; Kulkarni, Sudheendra and Giri, Ashok P, 2006. Detection of legume protease inhibitors by the Gel-X-ray film contact print technique. *Biochemistry and Molecular Biology Education*., **30**(1): 40 – 44.

Rancour, J.M. and Ryan,C.A. 1968 .Isolation of a carboxypeptidase B inhibitor from potatoes. *Archives of Biochemistry and Biophysics*., **125**:380-382.

Ryan, C.A. 1990. Protease inhibitors in plants: genes for improving defense against insects and pathogens. *Annu. Rev. Phytopathol*., **28**:415-449.

Ryan, C.A. 1977. Proteolytic enzymes and their inhibition in plants. *Annu. Rev. Plant Physiol.,* **24**:173-196.

Valueva, T.A and Mosolov, V.V. 1999. Protein inhibitors of proteinases in seeds, physiological functions. *Russian Journal of Plant Physiol.*, **46**:379-387.

Zhang, Kouzuma.Y, Miyaji.T. and Yonekura, M. 2008. Purification, characterization, cDNA cloning of a Bowman-Birk type trypsin inhibitor from *Apios americana* Medikus tubers. *Bioscience, Biotechnology, and Biochemistry***.,** **72**(1):171-178.