SERINE PROTEASE INHIBITOR FROM *GUIZOTIA ABYSSINICA*

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**ABSTRACT**

Proteinase inhibitors are important for the regulation of proteinases involved in numerous biological processes. Protein inhibitors of proteinases are widely distributed in plants, being particularly abundant in storage tissues such as seeds and tubers. In seeds, proteinase inhibitor plays a role in defense in protecting the seeds from infestation against insects, nematodes and phytopathogens. Protease inhibitors from plant origin are shown to be effective in combating various diseases. Niger (*Guizotia abyssinica*) is an oil yielding plant cultivated for its oil rich seeds. The seed of niger contains ~18% of protein. Serine protease inhibitors were isolated and partially purified from niger seeds. The isolated protein inhibited the trypsin and chymotrypsin activity. The inhibition of serine proteases by the isolated protein was found to be concentration dependent. The trypsin and chymotrypsin inhibitors isolated from the oil seed of niger seeds is thermostable upto 70° C. The study highlights the abundance of protease inhibitors in oil rich seed.

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INTRODUCTION

Proteinaceous inhibitors against proteinases are widely distributed in plants. These are particularly abundant in storage tissues such as seeds and tubers [1,2]. The inhibitors of the Bowman-Birk (BBI) family [3] are small serine proteinase inhibitors found in seeds of legumes and in many other plants. Characteristically, their molecular masses range between 6000-9000 daltons and they contain seven disulfides bonds that help stabilize their active conformation. All the BBIs exhibit two regions of tandem homology, each with an independent reactive site loop of nine residues formed by two very well conserved cysteine residues. Using the convention described by Scheter and Berger [4], it is the P1 residue of the reactive site that confers inhibitory specificity, Lys/Arg for trypsin and Tyr/Phe/Leu for chymotrypsin. The consensus sequence for various BBI reactive sites of monocots and dicots is CTXSSPPQC [5].

Much evidence shows that many different protease inhibitors in their pure forms suppress carcinogenesis [6]. BBI has been extensively studied as an anticarcinogenic protease inhibitor. The soybean derived BBI when given to animals by several different routes of administration or in the diet, is effective in suppressing carcinogenesis in different species of mice, rats and hamsters, in several organ systems and tissue types and in cells of hematopoietic, epithelial and connective tissue origin. Studies by Kennedy et al [7] show that BBI suppresses carcinogenesis in animals known to have a genetic susceptibility to cancer. Animal carcinogenesis studies have shown that dietary amounts as low as 0.01% BBI can suppress liver carcinogenesis in mice [8]. It is thought that the strength of the BBI as a cancer preventive agent lies in its ability to reverse the initiation cell division. It is hypothesized that BBI suppresses carcinogenesis by its ability to inhibit serine proteases or the expression of certain proto-oncogenes [9].

Only about half of the BBI administered orally is taken up into the blood stream via the intestinal epithelial cells or crosses the intestinal lumen and is distributed throughout the body [10]. BBI possesses all the important properties of an ideal proteinase inhibitor, to be used as a cancer preventive agent viz chemical and thermal stability, water solubility, and ability to inhibit transformation \textit{in vitro}. However the relatively high molecular mass of BBI is a limitation, as sufficient amounts of the BBI do not reach organs outside the GI tract when administered orally leading to a low bioavailability. The poor bioavailability can be alleviated if the size of the BBI is reduced such that it is capable of reaching the target tissue when administered orally. Cyclization of small peptides is a routine method of improving efficacy by reducing degradation and enhancing binding affinity \textit{via} the minimization of entropic losses. Smaller peptides containing the protease inhibitory loops are therefore attractive alternates to the BBI. A novel cyclic peptide of 14 amino acid residues isolated from sunflower seeds, homologous to the BBI class of trypsin inhibitors across a nine residue loop region is far more effective as a proteinase inhibitor than BBI [11]. The exceedingly high potency (K\textsubscript{i}=100 pM for trypsin) is attributed to the considerable structural rigidity obtained through its cyclic nature, which is also stabilised by a single internal disulfide residue similar to the active site loop of BBIs. Furthermore the recent discovery of 34 amino acid residue cyclic trypsin inhibitors from squash seeds suggest that naturally occurring cyclic proteinase inhibitors may be more prevalent. Apart from these and the plant cyclotides information on other such ‘natural cyclic peptides’ in the plant kingdom is sparse. Taking into account the need for potent small peptides for wide and varied applications the preliminary work is carried to isolate, and characterize from niger (\textit{Guizotia abyssinica}) seeds.

MATERIALS AND METHODS

Tris-HCl, Sodium dodecyl sulphate, acrylamide, N,N, methylenebisacrylamide, ammonium persulphate (APS), N N N’ N’\textsubscript{4} tetraethylenemethylenediamine (TEMED), β-mercaptoethanol, trypsin, chymotrypsin, BAPNA, BTPNA were procured from Sigma – Aldrich.

Defatting Niger seed flour

Niger seeds were purchased from local market. The seeds were cleaned manually to remove immature and infected seeds. The adhered dust particles were removed by washing the seeds under running tap water and air dried on a filter paper at 25 ± 2 °C. The cleaned seeds were ground to a fine powder and defatted with hexane (1:5 w/v) for 14-16 h at 25 ± 2 °C with occasional stirring. The slurry was filtered and residual hexane was removed by air drying at 25 ± 2 °C. The defatted fine powder obtained was stored at 4 °C until used.

Isolation of protein by Ammonium sulphate precipitation

The dried defatted Niger seed powder was extracted for 16 h at 4 °C with 5 volumes of sodium phosphate buffer, pH 7.0. The extract was centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant served as the crude extract. To the supernatant (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was added to 35 % saturation. The precipitated protein was removed by centrifuging at 15000 rpm for 45 min at 4 °C. (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was added slowly to the supernatant at 4 °C to 70 % saturation and allowed to stand at 4 °C overnight and centrifuged at 12000 rpm for 10 min at 4 °C. The precipitate thus obtained was dissolved in 0.1 M phosphate buffer pH 7.0. Dialysis was done against 0.01M phosphate buffer of pH7.0. The dialysed sample was used as the source of protein inhibitor.

Protein estimation

Protein concentration was determined by the Folin Ciocalteau method. Bovine serum albumin (BSA) was used as the standard.
Assay methods

The trypsin and chymotrypsin activity and their inhibitory activity were spectrophotometrically measured by assaying amidolytic activity for trypsin and chymotrypsin in the absence and presence of a known quantity of inhibitor using the chromogenic substrates BAPNA and BTPNA respectively. All the spectrophotometric measurements were performed in ELICO UV-Visible recording spectrophotometer.

Assay of trypsin and trypsin inhibitor activity

Trypsin was assayed according to the modified photometric method of Kakade et al., [12] using the substrate BAPNA. Forty mg of BAPNA was dissolved in 2 mL dimethylsulfoxide (DMSO) and then diluted (1:100) in 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM CaCl₂ prior to enzyme assay. The assay reaction consisted of 0.5 mL of trypsin solution (40-50 μg of trypsin in 1 mM HCl), 0.5 mL of water and 1.25 mL of the substrate. The reaction was carried out at 37 °C for 10 min and the reaction arrested by adding 0.25 mL of 30 % acetic acid. The absorbance of p-nitroaniline liberated was measured at 410 nm against an appropriate blank in which the reaction was arrested by adding 30 % acetic acid prior to the addition of BAPNA.

For the inhibition studies, the trypsin solution was incubated with an aliquot of inhibitor solution for 10 min at 37 °C and the reaction started by the addition of 1.25 mL BAPNA diluted in buffer and incubated at 37 °C for 10 min. The reaction was arrested by addition of 30 % acetic acid and the residual trypsin activity was measured by recording 410 nm.

Trypsin and trypsin inhibitory unit

One trypsin (TU) unit is arbitrarily defined as an increase in absorbance of 0.01 at 410 nm under conditions of assay. The trypsin inhibitory unit (TIU) is defined as the number of trypsin units inhibited under the same conditions [12].

Assay of chymotrypsin and chymotrypsin inhibitory activity

The reaction velocity was determined by measuring the absorbance of p-nitroanilide at 410 nm resulting from the hydrolysis of BTPNA. The stock of BTPNA (20 mM) was prepared by dissolving 16.2 mg in 2 mL of DMSO and then made up to 100 mL with 80 mM Tris-HCl buffer, pH 7.8 containing 100 mM CaCl₂ and 20 % DMSO (v/v). Chymotrypsin solution (40-50 μg in 0.5 mL of 1 mM HCl) was added to 0.5 mL of distilled water and incubated with 1.25 mL of substrate at 37 °C for 10 min. The reaction was stopped by adding 0.25 mL of 30 % acetic acid and the liberated product, p-nitroaniline measured at 410 nm against an appropriate blank.

Chymotrypsin inhibitory assay was performed similar to that of trypsin inhibition assay and the residual activity of chymotrypsin was calculated by measuring the absorbance at 410 nm.

Chymotrypsin and chymotrypsin inhibitory unit

One chymotrypsin (CU) unit is arbitrarily defined as an increase in absorbance of 0.01 at 410 nm under assay conditions. The chymotrypsin inhibitory unit (CIU) is defined as the number of chymotrypsin units inhibited under the same conditions.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE at alkaline pH (8.3) was carried out according to the method of Laemmli [13] in a discontinuous buffer system.

Zymogram

(a) Gelatin embedded assay

Gelatin–PAGE [14] was performed by adding gelatin (1 %, w/v final concentration) to the acrylamide gel. Following electrophoresis, the gel was washed with distilled water three times and then incubated at 37 °C in 0.1 M Tris-HCl buffer (pH 8.0 for trypsin and pH 7.8 for chymotrypsin) containing either trypsin or chymotrypsin (40 μg/mL) for 1 h for gelatin hydrolysis. The gel was washed with distilled water and stained with CBB R-250 and destained. The presence of the proteinase inhibitor was detected as a dark blue band in a clear background due to the complex of the unhydrolyzed gelatin with the stain.

(b) APNE Stain assay

Following electrophoresis, the gel was washed with distilled water and immersed in 0.1 M Tris-HCl, pH 8.2 containing trypsin (40 μg/mL) or chymotrypsin (pH 7.8) for 60 min at 25 °C. The gel was rinsed thrice in distilled water. APNE (N-acetyl-DL-phenylalanine β-naphthylester) reagent was prepared as mentioned below. (a) Seven mg of tetra azotized o-diamisidine in 10 mL of 0.1 M phosphate buffer pH 7.4 was prepared. (b) APNE 2 mg was dissolved in 0.2 mL DMSO. (a) and (b) were mixed just before use and the gel was stained using the above buffer. Clear zone was visualized in the gel against a pink background after staining the gel. The clear bands indicated the presence of inhibitor.

Thermal stability studies

Aliquots of the inhibitor solution (0.5mL) containing 500μg of the inhibitor were incubated at 100 ± 3 °C in water bath. Aliquots were removed at different intervals of time and immediately cooled on ice. The trypsin/chymotrypsin inhibitor activity was assayed as described earlier.

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RESULTS AND DISCUSSION

Fractionation of niger proteinase inhibitor

The niger seed flour was de-fatted and extracted with 0.02M sodium phosphate buffer in cold condition. The extract was subjected to 30-75% ammonium sulphate fractionation. The precipitate was dialysed against sodium phosphate buffer using a cut off of 3 kDa membrane. Lowry’s method of protein estimation was carried out. Amount of protein present in crude extract and dialysed ammonium sulphate precipitate was ~1.5 mg/mL and 0.6 mg/mL respectively. Trypsin and chymotrypsin inhibitory activity in the extract and ammonium sulphate fraction was carried using BAPNA and BTPNA as the substrate. The fractions showed ~6000 TIU/mg protein and 4500 CIU/mg protein respectively.

SDS-PAGE and gelatin embedded native PAGE

The crude extract and the partially fractionated sample were electrophoresed on PAGE under denaturing and reducing conditions. Gel visualization was carried out by staining with CBB R-250. Niger seeds possess ~12 to 18% of proteins and the major proteins in the seed are the storage proteins. 11S and 2S globulin proteins occur in several isoforms and could be visualized (Fig. 1). The dialysed protein was electrophoresed on 10% native gel with 10% gelatin substrate to detect trypsin inhibitory activity and detected by staining with CBB R-250.

![Figure 1: Protein profile of the crude extract and ammonium sulphate precipitate on SDS-PAGE. 40µg of sample was treated with β-marcaptoethanol and 10% SDS in boiling temperature for 10 minutes. The gels were stained with CBB R-250. Lane 1: Crude sample, Lane 2: 30-75% ammonium sulfate fractionated sample, MWM: molecular weight marker](image)

Native PAGE specific staining for trypsin inhibitory activity (Fig. 2 A) and chymotrypsin inhibitory activity (Fig. 2 B) was carried out. The bands appeared on the gel corresponds to the various forms of inhibitors, present in the seed extract.

![Figure 2: Native-PAGE (10% T, 2.7% C) profile of inhibitors showing trypsin and chymotrypsin inhibitory activity. Gelatin-embedded native-PAGE showing protease inhibition. The gels were stained for (A) trypsin inhibitory activity and (B) chymotrypsin inhibitory activity with APNE](image)
To assess the type of inhibition present in the seed extract, porcine trypsin was taken as the enzyme source. 50 µg of trypsin was incubated with various concentration of extract and checked for the inhibition. With increasing concentration of inhibitor the trypsin was inhibited. The IC$_{50}$ for trypsin and chymotrypsin by the protein fraction of the seed extract was 69 and 96 µg/mL of protein respectively (Fig 3).

**Temperature stability of protease inhibitor**

There was slight reduction in inhibitory activity when the niger protein fraction was subjected up to 70 $^\circ$C and retained 50 % of inhibitory activity up to 80 $^\circ$C (Fig 4). However, there was loss in the inhibitory activity of both trypsin and chymotrypsin when incubated at 100 $^\circ$C. The possible occurrence of many cysteine residues forming disulfide bonds may count for this striking stability in structural conformation and inhibitory properties of protein [15].

**CONCLUSION**

Niger, an oil rich seed contains upto 18 % of protein. The studies show the presence of thermostable serine protease inhibitors viz., trypsin and chymotrypsin inhibitory activity. The inhibition of serine protease was dose dependent. The works provide several avenues in search of novel serine protease inhibitor for pharmacological use.

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