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Purification and characterization of a trypsin inhibitor from *Senna tora* active against midgut protease of podborer

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ABSTRACT

Proteinaceous protease inhibitors have potential application in medicines, agriculture and biotechnology. Present study was undertaken to purify and characterize a proteinaceous protease inhibitor from a medicinal plant, *Senna tora* syn. *Cassia tora*. The inhibitor was purified by ammonium sulphate precipitation, anion exchange (Q-sepharose), affinity (trypsin-sepharose) and molecular exclusion (sephadex G-75) chromatography. Zymography and denaturing polyacrylamide gel electrophoresis revealed a single band of ~20 kDa trypsin inhibitor. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) and Matrix-assisted laser desorption ionization (MALDI) analyses revealed the presence of 19,725 Da (pI 4.60) and ~19,900 Da (pI 4.57) isoform proteins in purified inhibitor. Protein identification by MALDI-peptide mass fingerprinting did not reveal high MASCOT (Matrix science) scores matching with previously known inhibitors. N-terminal amino acid sequence suggested this protein as a previously unreported inhibitor. Its dissociation constant (0.23×10^{-9} M) was indicative of a high affinity trypsin inhibitor. The inhibitor was stable over a broad range of pH (4–10) and temperature (30–60 °C). The purified inhibitor effectively inhibited total protease and trypsin-like activities of podborer (*Helicoverpa armigera*) midgut preparation. Hence, the inhibitor and its gene(s) can find application in combating against pest and protease dependent pathogens.

1. Introduction

Plant seeds have a vast repository of proteins displaying remarkable functional and structural diversity. These include lectins, ribosome inactivating proteins, enzyme inhibitors, chitinases, etc. [1]. Among enzyme inhibitors, protease inhibitors (PI) are abundantly present in seeds. Naturally, the protease inhibitors act as storage proteins, regulate the level of endogenous proteins and are inducible upon pest and pathogen attack [2]. Furthermore, they are supposedly involved in modulating apoptotic events in plant cells [3]. Recently, plant protease inhibitor has also shown promise as antiproliferative agent [4] and efficacy against pathophysiological enzymes involved in blood clotting, hemorrhage, inflammation and cancer [5,6]. Plant protease inhibitors are classified on the basis of four classes of proteases, i.e., serine-, cysteine-, aspartic- and metallo-protease inhibitors [7]. Serine protease inhibitors are the most widely studied protease inhibitors [2,5,6], and further categorized in families like Kunitz, Bowman-Birk, Potato I and II, squash and cereal superfamily. Kunitz and Bowman-Birk inhibitors are most commonly found in members of Leguminosae family. These inhibitors are distinguished on the basis of their cysteine content and number of reactive sites. Kunitz inhibitors (Mr ~18–24 kDa) have either one or two polypeptide chain(s) with four cysteine residues forming two disulphide bridges, and have a single reactive site [8]. Bowman-Birk inhibitors are small proteins (Mr ~4–8 kDa) with 14 cysteine residues forming seven intra-molecular disulphide bridges [8]. Previous studies have also established distributional and evolutionary relationships among inhibitors of leguminous subfamilies [9].

Helicoverpa armigera is a pest having wide host-range of cotton, legumes, vegetables and forage [10]. The diverse proteases (mainly serine) present in alkaline midgut are responsible for adaptive polyphagous behavior of pest [10]. Thus, PIs may help in plant protection by inhibiting pest gut protease(s) leading to physiological stress to the pest [2]. However, the pest has shown remarkable resistance against inhibitors of host plants [11]. Hence, screening of protease inhibitors from non-host plants may provide a sustainable and effective strategy for plant protection [10,11].

Senna tora (Caesalpinoideae subfamily of Leguminosae) is found throughout India, and its seeds and leaves are recommended as medicine in the traditional Indian medicine system [12]. Despite

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the presence of diverse pest varieties, *S. tora* flourishes luxuriantly, indicating its strong intrinsic resistance. Previously, we reported a proteinaceous protease inhibitor in its seeds, active against *Bacillus* sp. and *Aspergillus flavus* proteases [12]. In the present study, an unreported trypsin inhibitor (TI) from *S. tora* seeds has been purified and characterized. Its activity against *H. armigera* gut proteases (HGP) is also investigated.

2. Materials and methods

2.1. Estimation of inhibitory potential

The trypsin inhibitory activities of crude extract and protein fractions obtained after each purification step were estimated using a chromogenic substrate Nα-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) as described previously by Erlanger et al. [13]. The chymotrypsin inhibitory activity of inhibitor was assessed as reported previously by Mueller and Wedder [14] using n-glutaryl 1-phenylalanine *p*-nitroaniline (GLUPHEPA) substrate.

2.2. Purification of protease inhibitor

S. tora seeds were collected locally from the fields of Faizabad (UP, India) region. Protease inhibitor fraction was prepared as using 25 g of dried *S. tora* seeds. It was powdered, treated with chilled acetone and allowed to air dry. Subsequent washing was carried out with chilled hexane followed by extraction of crude seed extract in 100 mM sodium phosphate buffer (pH 7.1). The seed extract was subjected to ammonium sulphate saturation (0–30, 30–60 and 60–90%) and dialysis against water using 12 kDa membrane [12]. Most active ammonium sulphate fraction (30–60%) was subjected to further purification steps using anion exchange, affinity and molecular exchange chromatography. This fraction was loaded on a mono Q-sepharose ion exchange column (2 cm × 10 cm) (Amersham Pharmacia), pre-equilibrated with 50 mM Tris–HCl buffer of pH 8.0. Proteins were eluted in 2.0 ml fractions using NaCl gradient (0.1–0.6 M) at a flow rate of 0.2 ml min^{-1}. Fractions having trypsin inhibitory activity were pooled and concentrated using centricon filter (MWCO 10 kDa, Milipore). An aliquot of concentrated protein was applied on 10 ml of Trypsin-Sepharose 4B affinity column, pre-equilibrated with Tris–HCl buffer (pH 8.0, 50 mM). Bound proteins were eluted with Glycine-HCl buffer (5.0 mM, pH 2.0) and different fractions (4 ml each) were collected in different tubes containing 250 μl of 1.5 M Tris–HCl (pH 8.0). The fractions having inhibitory activity were pooled, dialyzed overnight against 50 mM Tris–HCl (pH 8.0) and concentrated using centricon filter. The purified protein from affinity column was finally subjected to molecular exclusion chromatography using Sephadex G-75 column (1.5 cm × 50 cm). The column was pre-equilibrated with 50 mM Tris–HCl (pH 8.0) and proteins were eluted in 2.0 ml fractions at a flow rate of 10 ml min^{-1}. The fractions having trypsin inhibitory activity were pooled, concentrated by centricon filter and stored (at −20 °C) in different aliquots for further analyses.

Protein concentration of different active fractions obtained after each purification step was analyzed by measuring absorbance at 280 nm against bovine serum albumin (BSA) standard.

2.3. Polyacrylamide, gelatin-PAGE activity gel and 2D gel electrophoresis

The protein fractions, obtained at each purification step, having trypsin inhibitory activity were subjected to SDS-PAGE analysis using 15% resolving gel as described by Laemmli [15]. Zymography was also performed in SDS-PAGE, copolymerized with gelatin (0.1%, w/v), to assess trypsin inhibitory activity of purified protein as described previously by Hanspal et al. [16]. Two dimensional PAGE was performed to confirm the number of active protein fractions. First dimension isoelectric focusing was performed using immobilized pH gradient strip (17 cm) (Biorad) of linear pH range (4.0–7.0) as per manufacturer's instruction. Second dimension of electrophoresis was carried out using 12% resolving gel. Molecular weight of pure protein was determined by MALDI analysis.

2.4. Protein identification [MALDI-peptide mass fingerprinting (PMF)]

The protein spots of inhibitor appeared in 2D gel electrophoresis was excised, and destained using 15 mM potassium ferricyanide and 50 mM sodium thiosulphate. After destaining, the gel pieces were dehydrated by incubating with acetonitrile for 5 min, followed by reduction and subsequent alkylation using 10 mM DTT and 50 mM iodoacetamide, respectively. Further, protein digestion with trypsin (20 ng μl^{-1}) was effected overnight at 37 °C. The spots were subjected to Matrix-assisted laser desorption ionization-Time of flight (MALDI-TOF) and peptide mass fingerprinting (PMF) analyses.

2.5. Dissociation constant (K_i) estimation

Kinetic studies were performed using BA*p*NA as a substrate employing Dixon plot [17].

Two different substrate concentrations (0.2 and 0.05 mM) and various inhibitor concentrations (0.25, 0.50, 1.25, 1.5 nM) were used. Trypsin concentration was kept constant at 0.004 nM. The reactions were performed as described earlier [13]. The reciprocal velocity $1/V_o$ (OD_{410} h^{-1} ml^{-1}) versus inhibitor concentration for each substrate concentration was plotted. A regression line for each substrate was drawn, and intersect of extrapolated lines represented the K_i value [17].

2.6. Stability studies [temperature, pH and dithiothreitol (DTT)]

The effect of temperature on stability was studied by incubating the inhibitor over a temperature range of 30–100 °C for 30 min. After incubation, the pH was adjusted using Tris–HCl buffer (pH 8.2). The pH stability was determined in pH range of 2.0–12 by employing various buffers, viz., glycine-HCl (pH 2.0–3.0), acetate (pH 4.0–5.0), phosphate (pH 6.0), Tris–HCl (pH 7.0–9.0) and glycine-NaOH (pH 10–12) [18]. After temperature/pH treatment, the inhibitor was incubated with trypsin at 37 °C for 60 min, and residual inhibitory activity was determined using BA*p*NA [13]. The effect of DTT on inhibitor's stability was performed as described previously by Yoshizaki et al. [19]. The purified inhibitor in 20 mM Tris–HCl buffer (pH 8.2) was incubated with 1, 10 and 100 mM DTT for 60 min and reaction was terminated using iodoacetamide. The residual trypsin activity was estimated as described previously by Erlanger et al. [13].

2.7. Inhibition of H. armigera gut protease (HGP) and feeding assay

H. armigera larvae were collected from chickpea fields and reared on artificial diet as suggested by Harshulkar et al. [10]. The midgut of fifth instar larvae, reared on artificial diet, was isolated. The gut tissue was homogenized in 0.2 M glycine-NaOH buffer (pH 10.0) and kept for 2 h at 10 °C. The homogenate was centrifuged at 10,000 rpm for 20 min and supernatant was used as Helicoverpa gut protease (HGP) [10,20]. The total proteolytic activity of HGP was estimated using casein as substrate [21], while trypsin-like activity was estimated against BA*p*NA [13]. For HGP inhibition, purified inhibitor (4–32 μg ml^{-1}) was incubated with HGP for 30 min and then residual protease activity was estimated. The experiment was performed thrice using 15 fifth instar larvae in each set.

Feeding assay was conducted to assess the effect of dietary host (chickpea) and *S. tora* protease inhibitor on growth and development of the podborer. The experiment was carried out in three different diets, viz., a control artificial diet, natural diet of host plant (chickpea) and diet containing protease inhibitor (0.3%, w/v). The natural diet consisted of chickpea seeds and leaves. This was replaced by fresh leaves every day in the feeding chamber. The *S. tora* inhibitor was incorporated in the artificial diet. The basic diet was supplemented appropriately with the inhibitor in a way that each larva got similar quantity of trypsin inhibitor in diet cubes (2.5 g). The larvae (24 no.) of early second instar were reared on diets and weight gain with morphological alterations was recorded every second day.

2.8. Statistical analysis

Each experiment was performed twice in triplicate and standard deviation for each value was calculated using Microsoft Excel.

3. Results and discussion

3.1. Purification and electrophoretic analysis of S. tora inhibitor

The ammonium sulphate fraction (30–60%) from crude seed protein of *S. tora* exhibited maximum inhibitory activity [12]. This is in consonance with the findings of other researchers [22]. Anion exchange (Q-sepharose) chromatography of this fraction resulted in three peaks corresponding to 0.2, 0.3 and 0.4 M NaCl elutes (Fig. 1A). The last two peaks (0.3 and 0.4 M elutes) exhibited maximum trypsin inhibitory activity. Further, affinity chromatography of these fractions on Trypsin-Sepharose matrix resulted in one peak representing purified trypsin inhibitor (Fig. 1B). Since protease-inhibitor complex under normal conditions dissociates at pH below 3.0 [23], the inhibitor was eluted at pH 2.0 in Trypsin-Sepharose column. The pure protein still retained strong inhibitory activity despite possible denaturation of inhibitor due to elution in low pH buffer (Table 1). The ion exchange and affinity chromatography processes were effective in purification of inhibitor (17.72 folds) with ~35% yield (Table 1). The yield is considered significant as trypsin might have caused some digestion of inhibitor during elution [22,23]. Molecular exclusion chromatography ensured removal of high molecular weight complexes (revealed as dimeric protein in MALDI analysis) from the Trypsin-Sepharose column