Genomic and cDNA Cloning, Characterization of Delonix regia Trypsin Inhibitor (DrTI) Gene, and Expression of DrTI in Escherichia coli

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Degenerate primers were designed based on all possible sequences of the N-terminal and C-terminal regions of Delonix regia trypsin inhibitor (DrTI). Five hundred sixty-one bp of polymerase chain reaction (PCR) product was amplified using the above degenerate primers and genomic DNA and cDNA of Delonix regia as a template. The amplified PCR products were cloned and sequenced. DNA sequence analysis of cDNA and genomic clones of DrTI have the same nucleotide sequence in the coding region, and manifested a genomic clone without intervening sequences in the coding region. The amino acid sequence deduced from the DrTI genomic and cDNA clones agreed with that identified via amino acid sequencing analysis, except that two amino acid residues, Ser and Lys, existed between residues Lys141 and Ser142. DrTI open reading frame was then amplified and cloned in-frame with GST in pGEX4T-1 and overexpressed in Escherichia coli to yield a glutathione S-transferase (GST)-fusion protein with a calculated molecular mass of about 45 kDa. The recombinant DrTI (reDrTI) was derived by treating the GST-DrTI fusion protein with thrombin. Both the reDrTI and GST-DrTI fusion protein exhibited a strong identical inhibitory effect on trypsin activity.

Key words: Delonix regia trypsin inhibitor; Kunitz-type trypsin inhibitor; Delonix regia; molecular cloning

Seed proteins have important roles in plant survival such as maintaining seed viability, providing nutrition during early seeding, and protecting the seeds against microbes and insects.15 Protease inhibitors of seeds can inhibit trypsin while it passes through the gut of an animal, thus helping with seed dispersal, and protecting plants against pests and diseases.2–5 Protease inhibitors are present in significant quantities in Leguminosae seeds and in smaller quantities in cereals, cucurbits, potatoes and other tubers.5–8 Numerous studies have recently demonstrated the efficacy of proteinase inhibitors as defense proteins; the most direct proof comes from proteinase inhibitor overexpression in transgenic plants, which causes increases in resistance to insect pests.2–11 Besides their natural biological functions, proteinase inhibitors might also be useful in treating human pathologies such as inflammation, hemorrhage,12 and cancer.13–16

Serine proteinase inhibitors from plants are classified into families: the Kunitz trypsin, Bowman-Birk protease, potato I, potato II, barley trypsin, and squash inhibitor families.17 The legume proteinase inhibitors are further classified into two main groups according to their size and cysteine content. Kunitz-type inhibitors are proteins (Mr 18,000–22,000) with one or two polypeptide chains and low cysteine content, generally with four cysteine residues arranged into two disulfide bridges, each comprising 170–180 amino acids. Delonix regia trypsin inhibitor (DrTI) which belongs to the Kunitz family, is purified from Delonix regia (Leguminosae Caesalpinioideae) seeds. The primary structure of DrTI has been identified.18 It comprises a single-polypeptide chain with a molecular mass of 22 kDa and two disulfide bonds. The amino acid sequence of DrTI has a high similar comparative sequence of related Kunitz inhibitors, including SBTI,19 SwTI,20 PtTI,21 EcTI,22 BvTI-3c and ACTI.23,24 Finally, DrTI is an effective inhibitor of trypsin and human plasma kallikrein, but not of chymotrypsin, plasmin, factor Xa or tissue kallikrein.

Sequence comparison with other plant trypsin inhib-
itors of the Kunitz family reveals that, instead of the conserved Arg or Lys found in other Kunitz TIs, DrTI contains a negatively charged residue (Glu68) at the P1 reactive site.\(^{18}\) The present study aimed to clone the cDNA and genomic DNA of the DrTI gene and to express GST-DrTI fusion protein and reDrTI which exhibit a strong identical inhibitory effect on trypsin activity (\(E.\ coli\)). In the future, mutant proteins can perhaps be utilized by site-directed mutagenesis to investigate the role of Glu68 residue in trypsin inhibitory activity of DrTI.

### Materials and Methods

**Materials.** Isopropyl-\(-\)\(\beta\)-\(\)-\(D\)-galactopyranoside (IPTG), PCR marker, T4 DNA ligase and thrombin were purchased from Promega (Madison, WI). The expression vector, pGEX4T-1, and a HiTrap\(^{\text{TM}}\) DEAE FF column were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Trypsin, glutathione-agarose gel and L-BAPNA were from Sigma (St. Louis, MO). Restriction enzymes and other reagents in molecular-biology techniques were purchased from New England Biolabs, Boston, MA. A DNeasy Plant Maxi kit and an Oligotex mRNA Purification System were from Qiagen (Hilden, Germany). All other chemicals used were of analytical grade.

**Isolation of Delonix regia genomic DNA and mRNA, and cDNA synthesis.** Delonix regia genomic DNA was extracted from 1 g of lyophilized young leaves using a silica-gel membrane-type kit (DNeasy Plant Maxi, Qiagen, Hilden, Germany) based on a previous study.\(^{25}\) Maturing Delonix regia seeds about one month after flowering were obtained from a local source (Hsinchu, Taiwan). Total cellular RNA was isolated from the seeds by homogenizing them in 4 M guanidinium thiocyanate.\(^{26}\) Poly (A)\(^+\) was purified from total cellular RNA using the Oligotex mRNA Purification System. Poly (A)\(^+\) rich RNA from the seeds of Delonix regia was used for cDNA synthesis as described previously.\(^{27}\) The genomic DNA and cDNA samples were used for the subsequent PCR analysis.

**Amplification of Delonix regia genomic DNA and cDNA with DrTI specific primers.** On the basis of the amino acid sequence of DrTI,\(^{15}\) two degenerate PCR primers were prepared. Primer \(A\)\(_{\text{start}}\) (\(5'\)\(-\)TNGAYGNCN\(-\)GARAARGTNTAYGAYATHGA-3') encodes the first eight N-terminal amino acids of the DrTI. Primer \(A\)\(_{\text{stop}}\) (\(5'\)\(-\)NGAYTCNGTTCNGANCCNGANCNGNGYTT-3') encodes the last eight C-terminal amino acids of the DrTI. The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 50 \(\mu\)l contained 1 \(\times\) PCR buffer (20 mm Tris–HCl, pH 8.8, 10 mm KCl, 10 mm (\(\text{NH}_4\)\)\(_2\)SO\(_4\), 0.1% Triton-X-100 and 5 \(\mu\)g BSA), 500 ng of genomic DNA or 500 ng cDNA, 400 \(\mu\)M dNTP, 1.5 mm MgCl\(_2\), 50 pmole of each of paired \(A\)\(_{\text{start}}/\ A\)\(_{\text{stop}}\) primers and 2.5 units \(Pfu\) DNA polymerase (Promega, Madison, WI). The reactions were amplified in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) according to the following PCR step-cycle program: pre-incubation at 95 \(^\circ\)C for 5 min, denaturation at 95 \(^\circ\)C for 1 min, annealing at 45 \(^\circ\)C for 1 min, and extension at 72 \(^\circ\)C for 2 min. The cycle was repeated 35 times followed by a final extension at 72 \(^\circ\)C for 7 min. Reaction products were analyzed by electrophoresis on 1.5% agarose gel in Tris/acetate EDTA (40 mm Tris–HCl, pH 8.0, 5 mm sodium acetate, 1 mm EDTA). The products were detected by ethidium bromide staining.

**Construction of genomic and cDNA clones of DrTI.** DNA fragments of 0.56 kbp isolated and produced by PCR reaction by agarose gel electrophoresis were ligated to pBluescript vector (Stratagene, La Jolla, CA) which was digested Sma I and calf intestine phosphatase. Transformed \(E.\ coli\) cells (TG 1) were selected by blue-white selection. Clones with 561 bp fragments were sequenced using an ABI 310 automated sequencer. All inserts were sequenced at least twice on both strands.

**Construction of expression plasmids and overexpression of GST-DrTI fusion proteins in \(E.\ coli\).** Expression plasmid was the derivative of pGEX-4T-1 and was constructed by ligating a 561 bp BamHI/EcoRI fragment derived from pcDrTI by PCR with primer-A, \(5'\) AAGGATCCTCGGACGCGGAGAAGGTTT 3' and primer-B, \(5'\) AGGAATTC\(-\)\(T\)TA\(\text{CGACTCCCGTTCGGAT}\) 3',\(^{28}\) which contained the entire DrTI coding sequence in-frame in the BamHI/EcoRI sites of pGEX4T-1. The resulting construct, pGEX4T-1-cDrTI contained both glutathione S-transferase and the DrTI gene. All the constructs of DrTI cDNA were confirmed by sequencing the ligation products of pGEX4T-1 plasmids. To enlarge the expression of DrTI fusion protein, \(E.\ coli\) TG 1 cells harboring pGEX4T-1-cDrTI construct were grown at 37 \(^\circ\)C in 500 ml of LB broth (1% NaCl, 1% Bacto-tryptone, 0.5% Bacto-yeast extract, pH 7.0) containing 100 \(\mu\)g/ml of ampicillin. When \(OD_{\text{600}}\) reached 0.6, IPTG was added to a final concentration of 1 mm to induce fusion protein expression and the culture was incubated for a further 4 h at 30 \(^\circ\)C. A maximal harvest was obtained under these conditions. Total soluble proteins were extracted in resuspended buffer (10 mm Na\(_2\)HPO\(_4\), 1.8 mm Na\(_2\)HPO\(_4\), 140 mm NaCl, 2.7 mm KCl, pH 7.5, 1 mm DTT, 0.2 mg/ml lysozyme) by repeated freeze-thawing, followed by centrifugation (10,000 \(\times\) g, 10 min) at 4 \(^\circ\)C, the supernatant being the crude protein extract. Fifteen \(\mu\)g of crude protein extract was analyzed on 12.5% SDS–PAGE followed by Coomassie blue staining.

**Purification of GST-DrTI fusion proteins and reDrTI.** GST-DrTI fusion proteins were produced and purified to homogeneity as described previously.\(^{29}\) In brief, crude
protein extracts were loaded onto a glutathione-agarose affinity column, and then, after washing, the GST-DrTI fusion protein was eluted using 5 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. The fusion proteins were then treated with thrombin to liberate the recombinant DrTIs which were purified with a HiTrap™ DEAE FF column (1 ml). After applying the sample to the column, it was eluted with 50 mM Tris/HCl, pH 8.0, and then eluted with a linear gradient of 0 to 0.3 M NaCl in the buffer.

Trypsin Inhibitory activity of reDrTI. The trypsin inhibitory activities of GST-DrTI fusion protein, reDrTI and native DrTI were measured by incubating each DrTI with trypsin in 1 ml of 0.1M Tris–HCl buffer, pH 8.0 containing 0.01 M CaCl₂ for 5 min at 37°C. Residual trypsin activity was determined by adding 10 ml L-BAPNA (50 mg/ml in DMSO) at 37°C. After 20 min of incubation, the reaction was stopped by adding 0.5 ml of 10% acetic acid. The degree of inhibition was determined by measuring the optical density at 410 nm.30)

Results and Discussion

Amplification and analysis of DrTI cDNA and genomic fragment

The application of degenerate primers based on the amino acid sequence for DrTI was used to amplify the specific sequences of DrTI cDNA and genomic DNA, and the expected size obtained was as illustrated in (Fig. 1). The main product obtained following amplification of DrTI cDNA and genomic DNA with primers A_start/A_stop annealed at 45°C was a fragment of approximately 0.56 kbp.

The amplified DNA fragments produced by PCR reaction with cDNA and genomic DNA as template were subsequently subcloned to pBluescript vector and sequenced. Analysis of cloned DNA fragments from PCR reaction using either cDNA or genomic DNA as template revealed cloned DNA of the expected size (0.56 kbp) covering the entire DrTI. Random sampling and sequencing of clones containing the 561 bp fragment obtained detected only one type, and (Fig. 2) showed that the deduced amino acid sequence of DrTI using DNA sequence analysis of cDNA and genomic clones of DrTI had the same nucleotide sequence in the coding region, and identified the genomic clone without intervening sequences in the coding region. The amino acid sequence deduced from the cDNA clone agreed with that determined by amino acid sequencing analysis, except for two amino acid residues, Ser and Lys, between residues Lys141 and Ser142 of the amino acid sequence.18) The genomic clones isolated from soybeans encoding kunitz-type trypsin inhibitors and Bowman-Birk-type trypsin inhibitor have been reported not to contain an intron.30–32)

Construction of expression plasmids and overexpression of GST-DrTI fusion proteins in E. coli

The primers were applied to PCR on the pcDrTI to generate DrTI-encoding DNA fragments for subcloning. DNA fragments encoding 187 amino acids of DrTI flanked by EcoRI and BamHI were ligated into pGEX4T-1 using T4 DNA ligase. The desired expression vector with insert was confirmed via nucleotide sequencing. The expression plasmid was designated pGEX4T-1-cDrTI.

The GST-DrTI fusion proteins were obtained from pGEX4T-1-cDrTI expression in E. coli strain TG1 cells. The fusion proteins were purified from the E. coli lysate through affinity chromatography with a glutathione-agarose gel column. The purified fusion proteins were then treated with thrombin and purified with a HiTrap™ DEAE FF column. This study obtained a final yield of 5–6 mg purified reDrTI/liter induced E. coli at 30°C.

The homogeneity of purified reDrTIs was analyzed using SDS–PAGE and the results are shown in (Fig. 3). The N-terminal amino acid sequence of reDrTIs was determined using an automatic sequencer, and the results revealed that reDrTI has the same N-terminal amino acid sequence (about 15 amino acid residues) as native DrTI, except for two extra amino acid Gly-Ser at the N-terminus (data not shown).

Trypsin inhibitory activity of GST-DrTI fusion protein and reDrTI

The trypsin inhibitory activity of the fusion protein and reDrTI was tested and compared with that of native DrTI (Fig. 4). On a molar basis, the reDrTI and fusion protein exhibited the same 50% inhibition concentration as the native DrTI. The Ki value of reDrTI was 21.9 nM, the same as that of native DrTI.18)

General discussion

To clone the DrTI gene, PCR amplification was...
performed using Delonix regia genomic DNA isolated from young leaves or cDNA isolated from maturing seeds as a template and the same degenerate PCR primers based on all possible sequences of the N-terminal and C-terminal regions of DrTI. These amplified products had the same size as the genomic DNA and cDNA. DNA sequence analysis of cDNA and genomic clones of DrTI displayed the same nucleotide sequence in the coding region. The correct nucleotide sequence of the primer parts of DrTI was not observed in cDNA or genomic clones because the DrTI clone included degenerate primers. This result shows that the DrTI gene did not contain intervening sequences in the coding region. The amino acid sequence deduced from the cDNA clone agreed with that determined by amino acid sequencing analysis, except for the existence two amino acid residues, Ser and Lys, between residues Lys141 and Ser142 of the amino acid sequence. The difference in amino acid sequence between the cDNA and the protein of DrTI indicates that Delonix regia contains several related trypsin inhibitor genes as soya bean (Glycine max) trypsin inhibitors. 30,33)

This study obtained a final yield of 5–6 mg of purified reDrTI/liter-induced E. coli at 30°C. The reDrTI and fusion protein had the same 50% inhibition concentration as the native DrTI. The Ki value of the reDrTI was determined to be 21.9 nM, the same as that of native DrTI.
An interesting peculiarity of DrTI is that a Glu68 residue was found in the expected position for the reactive site rather than an Arg or Lys, usually present in other Kunitz-type inhibitors.\(^{18}\) Swartzia pickellii trypsin inhibitor also contains a Glu residue in the reactive site for trypsin.\(^{20}\) In the future, mutant proteins can perhaps be utilized by site-directed mutagenesis to investigate the role of Glu68 residue in trypsin inhibitory activity of DrTI.

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**References**


