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Evidence that Highly Conserved Residues of *Delonix regia* Trypsin Inhibitor Are Important for Activity

Chih-Hung Hung*, Pei-Jung Chen, and Hai-Lung Wang

Department of Medical Laboratory Science and Biotechnology, Yuanpei University, Hsinchu 300, Taiwan, ROC; fax: +8(863) 538-5353; E-mail: chihhung@mail.ypu.edu.tw

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Abstract—*Delonix regia* trypsin inhibitor (DrTI) consists of a single-polypeptide chain with a molecular mass of 22 kDa and containing two disulfide bonds (Cys44–Cys89 and Cys139–Cys149). Sequence comparison with other plant trypsin inhibitors of the Kunitz family reveals that DrTI contains a negatively charged residue (Glu68) at the reactive site rather than the conserved Arg or Lys found in other Kunitz-type trypsin inhibitors. Site-directed mutagenesis yielded five mutants containing substitutions at the reactive site and at one of the disulfide bonds. Assay of the recombinant proteins showed mutant Glu68Leu and Glu68Lys to have only 4-5% of the wild-type activity. These provide evidence that the Glu68 residue is the reactive site for DrTI and various other Kunitz-type trypsin inhibitors. The Cys139Gly mutant lost its inhibitory activity, whereas the Cys44Gly mutant did not, indicating that the second disulfide bond (Cys139–Cys149) is critical to DrTI inhibitory activity, while the first disulfide bond (Cys44–Cys89) is not required.

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Key words: *Delonix regia* trypsin inhibitor, Kunitz-type trypsin inhibitor, *Delonix regia*, reactive site, site-directed mutagenesis

Delonix regia trypsin inhibitor (DrTI), which is a member of the Kunitz family, was purified from *D. regia* (Leguminosae Caesalpinioideae) seeds. The primary structure of DrTI has been elucidated [1]. It consists of a single-polypeptide chain with a molecular mass of 22 kDa, and there are two disulfide bonds present (Cys44–Cys89 and Cys139–Cys149). DrTI is an effective inhibitor of trypsin and human plasma kallikrein, but not of chymotrypsin, plasmin, factor Xa, or tissue kallikrein. The amino acid sequence of DrTI is very similar to that of related Kunitz inhibitors, including STI (soybean trypsin inhibitor) [2], WTI (winged bean trypsin inhibitor) [3], ETI (*Erythrina caffra* trypsin inhibitor) [4], sporamin [5], and ACTI (*Acacia confusa* trypsin inhibitor) [6] (table).

The cDNA and genomic clones of DrTI have been prepared, and they share the same nucleotide sequence in the coding region; a genomic clone without intervening sequences in the coding region has been demonstrated [7]. The amino acid sequence deduced from the DrTI genomic or cDNA clones is consistent with that identified via amino acid sequencing analysis except that two amino acid residues, Ser and Lys, were present between residues Lys141 and Ser142. The DrTI cDNA clone is also present in *Escherichia coli* where its expression exhibits a strong and identical inhibitory effect on trypsin activity as seen for wild type DrTI. In plant trypsin inhibitors of the Kunitz family, the reactive site is always Arg or Lys [8, 9], indicating that DrTI, which contains a negatively charged residue (Glu68), is present at the reactive site [1].

In this work we expressed mutant proteins in *E. coli* by site-directed mutagenesis to investigate the role of the Glu68 residue in trypsin inhibitory activity of DrTI.

Abbreviations: ACTI, *Acacia confusa* trypsin inhibitor; DrTI, *Delonix regia* trypsin inhibitor; ETI, *Erythrina caffra* trypsin inhibitor; IPTG, isopropyl-1-thio-D-galactopyranoside; L-BAPNA, α-N-benzoyl-D,L-arginine-4-nitroanilide hydrochloride; PCR, polymerase chain reaction; re, recombinant; STI, soybean trypsin inhibitor; WTI, winged bean (*Psophocarpus tetragonolobus*) trypsin inhibitor.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials. Isopropyl-1-thio-D-galactopyranoside (IPTG), PCR marker, T4 DNA ligase, and thrombin were from Promega (USA); the His Trap HP column was from Amersham Pharmacia Biotech (Sweden); trypsin

Homology of DrTI [7] with other Kunitz type trypsin inhibitors: sporamin [5], STI [2], ETI [4], WTI [3], and ACTI [6]

Inhibitor	Amino acid sequence
	1 25 * 50
DrTI	SDAEKVYDIEGYPVFLGSEYYIVSAIIGAGG-GGVRPGRTRGSM-CPMSIIQEQSDLOM-GL
Sporamin	SSETPVLDINGDEVRAGGNYYMVSAIWGAGGGGALRLAHLDMMSKCASDVIVSPNDLDN-GD
STI	---DFVLDNEGNPLENGGTYYILSDITAFGG---IRAAPTGNER-CPLTVVQSRNELDK-GI
ETI	----VLLDGNGEVVQNGGTYYLLPQVWAQGGG--VQLAKTGEET-CPLTVVQSPNELSN-GK
WTI	---EPLLDSEGELVRNGGTYYLLPDRWALGGG--IEAAATGTET-CPLTVVRSPNEVSV-GE
ACTI	---KELLDADGDILRNGGAYYILPALRGKGGG--LTLAKTGDES-CPLTVVQAQSEATKRGL
	* * 75 100
DrTI	PVRF-SS----PEESQGKIYTD-TELEIEFVEKPD---CAE--SSKWVIVKDSGEARVAIGG
Sporamin	PITI-TPATADP-EST-VVMASTYQTFRF--NIATNKLCVNNVN---WGIQAHDSASGQYFL
STI	GTII-SS----PYRIR-FIAEGAHPLSLKFDSFAVIMLCVGIPTE--WSVVE--DLPEGPAV
ETI	PIRI-ES----RLRSA-FIPDDADKVRI---GFAYAPKCAPSPW---WTVLE--DEQEGLSV
WTI	PLRI-SS----QLRSG-FIPDAYSVVRI---GFANPPKCAPSPW---WTVVE--DQPQQPSV
ACTI	PAVIWTP----P-KIA-ILTPGFYLNFEF--QPRDLPACLQKYSTLPWKVEG--EASQEVKI
	125 * 150
DrTI	SEDHPQGELVRGFFKIEKLGSL---AYKLVFCPKSKSS-SGSCSDIGI---NYEGRRSLVLKS
Sporamin	KAGEFVS-DNSNQFKIELVDAN-LNSYKLTYCQ----FGSDKCYNVGRFHDHMLRTTRLALS-
STI	KIGENKD-AMDGWFRLERVSDDEFNNYKLVFCPQ-QAAEDDKCGDIGISIDHDDGTRRLVVS-
ETI	KLSEDESTQFDYPFKFEQVSDK-LHSYKLLYCE----GKHEKCASIGINRD-QKGYRRLVVT-
WTI	KLSELKSTKFDYLFKFEKVTSK-FSSYKLKYC-----AKRDTCKDIGIYRD-QKGYERLVVT-
ACTI	APKEKEQ-FLVGSFKIKPYRDD----YKLVYCEGN--SDDESCKDLGISIDADENNRRLVVK-
	175
DrTI	SDDSPFRVVFVKPRSGSETES
Sporamin	-NS-PFAVFVIKPTDV
STI	-KNKPLVVQFQKLDKESL
ETI	-EDNPLTVVLKKDESS
WTI	-DENPLVVIFKKVESS
ACTI	-DGHPLAVRFEKAHRSGA

Note: The alignment was prepared using Alscript [23]. Reactive sites are shown in the box, and gray color indicates the cysteine residues. Asterisks indicate the four residues selected for mutation in this study.

and L-BAPNA (α-N-benzoyl-D,L-arginine-4-nitroanilide hydrochloride) were from Sigma (USA); restriction enzymes and other reagents used in molecular-biological approaches were from New England Biolabs (USA); the expression vector pQE60 was from Qiagen (Germany). All other chemicals used in this work were of analytical grade.

Construction of the recombinant pQE60-DrTI plasmid for mutation. A 561 bp full-length cDNA that encoded the *DrTI* gene was cloned into pBluescript vector, yielding the plasmid pcDrTI [7]. Two primers were designed to amplify the coding region of the *DrTI* gene using the PCR method. Primer A, containing an *Nco*I site, was 5′-CATGCCATGGCGGACGCGGAGAAG-GTTTACGAC-3′, while primer B, containing a *Bam*HI site, was 5′-CGCGGATCCGGACTCCGTTTCCGAT-CCTGACCG-3′. PCR conditions were set to 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. After the resulting PCR product purification and analysis using 1.5% agarose gel electrophoresis, only one DNA band of about 580 bp was present. This fragment was cloned into pQE60 at the *Nco*I and *Bam*HI sites and the final construct, pQE60-DrTI, was used to transform *E. coli* TG1.

Site-directed mutagenesis. Five DrTI mutants were produced based on the pQE60-DrTI plasmid. The following five oligonucleotides were used to generate the indicated mutations (the bold type represents the amino acid mutation and underlining denotes the corresponding codon): Glu68**Leu**, 5′-AGTCCAGAAAAAAGCCAAGGCAAA-3′; Glu68**Lys**, 5′-AGTCCAGAACTAAGCCAAGGCAAA-3′; Lys72**Glu**, 5′-CCAGAAGAAAGCCAAGGCGAAATA-3′; Cys44**Gly**, 5′-CGGGGCTCCATGGGCCCAATGTCT-3′; Cys139**Gly**, 5′-GTGTTTGGTCCCAAAAGCAAATCC-3′. Site-directed mutagenesis was performed by PCR [10]. Mutations were verified by the DNA sequencing of the entire fragment, then verifying that the intended mutants were present. The mutant DrTI fragments were ligated into pQE60 at the *Nco*I and *Bam*HI sites to form five mutant plasmids designated as pQE60-DrTI(E68L), pQE60-DrTI(E68K), pQE60-DrTI(K72E), pQE60-DrTI(C44G), and pQE60-DrTI(C139G), respectively.

Expression and purification of recombinant DrTI and mutants. *Escherichia coli* strain M15 harboring wild-type pQE60-DrTI and various pQE60-DrTI mutant constructs were grown at 37°C in 1 liter of LB broth (1% NaCl, 1% bacto-tryptone, 0.5% bacto-yeast extract, pH 7.0) containing 100 μg/ml of ampicillin. When A_{600} reached 0.6, IPTG was added to a final concentration of 1 mM to induce the expression of fusion protein, and the culture was incubated for a further 4 h at 30°C. A maximal harvest was obtained under these conditions. Total soluble proteins were extracted in resuspension buffer (10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.5, 1 mM dithiothreitol, 0.2 mg/ml lysozyme) by repeated freeze-thawing cycles, followed by digestion with 500 ng of DNase I for 10 min at 37°C. A soluble lysate was isolated by centrifugation (10,000*g*, 10 min) at 4°C. Fifteen micrograms of crude protein extract was analyzed on 15% SDS-PAGE followed by Coomassie blue staining. Recombinant DrTI and mutants were purified by the application of the soluble lysate to a His-Trap HP affinity column containing 1 ml of Ni^{2+}-NTA-gel. His-Trap affinity columns were used in an Amersham Biosciences FPLC system and purification was carried out following the manufacturer's protocol. After the sample was applied to the column, it was eluted with 20 mM Tris-HCl at pH 8.0 containing 10 mM imidazole and then eluted with a linear gradient from 20 to 300 mM imidazole in the buffer. The eluted protein was dialyzed against two changes of 50 mM Tris-HCl, pH 8.0, buffer (1 liter each) for 16 h at 4°C. Aliquots of this dialyzed protein were stored at −80°C.

Quantitative analysis of trypsin inhibitory activity of DrTI mutant proteins. Purified reDrTI or mutant protein was reacted at 37°C for 10 min with trypsin (1 : 1 molar ratio), then L-BAPNA was added to a final concentration of 500 μM and the reaction was continued for 30 min. The optical density of the reaction mixture was then measured at 410 nm [11]. The percentage inhibition (I, %) of trypsin by DrTI was calculated using the equation: $I = [(T - T^*)/T] \times 100\%$, where T and T^* refer to the A_{410} in the absence and presence of DrTI, respectively. One inhibition unit was defined as the amount of inhibitor that was required to inhibit completely 1 μg of trypsin.

RESULTS AND DISCUSSION

Construction of expression plasmids of mutant DrTI. The expression plasmid was a pQE60 derivative and was constructed by ligating a PCR amplified fragment on pcDrTI with primer A and B, and then cloned into pQE60 at the NcoI and BamHI sites. The final construct was pQE60-DrTI. Subsequent dideoxy-sequencing showed the adduct pQE60-DrTI to contain the DrTI coding region.

DrTI is related to the Kunitz-type trypsin inhibitor family, the four Cys residues are conserved and formed two disulfide bonds, Cys44–Cys89 and Cys139–Cys149; however, at the putative reactive site a Glu residue was found in the P1 position instead of Arg or Lys as in most Kunitz-type trypsin inhibitors (table). As presented in the table, four amino acids (Glu68, Lys72, Cys44, and Cys139) were selected for mutation to determine whether the negatively charge amino acid (Glu68) was the DrTI reactive site and whether the two disulfide bonds are critical for inhibitory activity. Three mutant plasmids—pQE60-DrTI (E68L) to produce mutant Glu68Leu, pQE60-DrTI (E68K) to produce mutant Glu68Lys, and pQE60-DrTI (K72E) to produce mutant Lys72Glu—were prepared to investigate the effects of these mutations on trypsin inhibitory activity. Two other mutant plasmids—pQE60-DrTI (C44G) for the Cys44Gly mutant and pQE60-DrTI (C139G) for the Cys139Gly mutant—were constructed to examine the role of the disulfide bond on trypsin inhibition activity. Nucleotide sequence analysis of the mutant plasmids confirmed the present of the DrTI mutants formed by the site-directed mutagenesis.

Purification of recombinant proteins. The *E. coli* M15 cells were transformed using mutant plasmids. The lysate of the induced transformed cells was applied to an FPLC His-Trap HP affinity column to purify the hexahistidine-tagged mutant reDrTIs. Figure 1 presents the results after purification. The mutant reDrTIs were eluted with a linear gradient from 20 to 300 mM imidazole in 50 mM Tris-HCl, pH 8.0, buffer. The second peak fraction was reDrTI, and its yield was 3-4 mg/liter. Figure 2 shows results of 15% SDS-PAGE analysis of reDrTI and the mutants. The reDrTI and mutants had amino acid residues Met-Ala replaced by Ser at the N-terminus and 10 extra amino acid residues Gly-Ser-Arg-Ser-$(His)_6$ at the C-terminus compared to native DrTI that was purified from seeds.

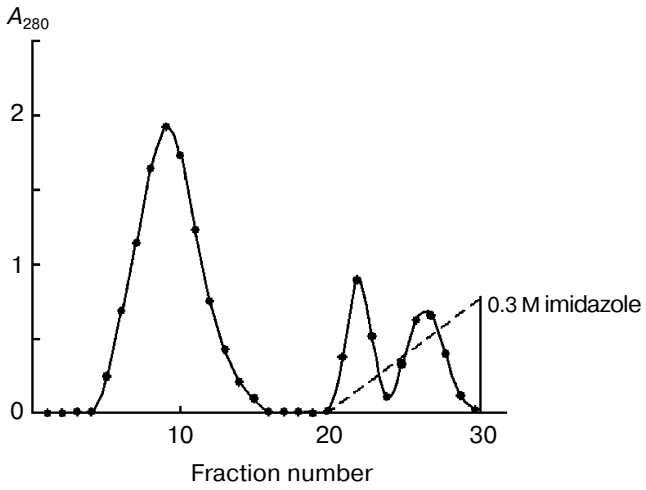

Fig. 1. Purification of reDrTI with a His-Trap HP affinity column. The sample was eluted with 20 mM Tris-HCl, pH 8.0, containing 10 mM imidazole. Elution continued through a linear gradient of 20 to 300 mM imidazole in the buffer.

Trypsin inhibitory activity of DrTI mutants. Comparing the trypsin inhibitory activity of the reDrTI showed that on a molar basis, the reDrTI exhibited the same 50% inhibition concentration as the native DrTI. Figure 3 shows the effects of various DrTI mutants on the trypsin inhibitory activity. The results indicate that the Glu68Leu and Glu68Lys mutants had significantly reduced trypsin inhibition, while Lys72Glu did not affect the trypsin inhibitory activity at all. The Cys139Gly mutant had greatly reduced trypsin inhibitory activity, while the Cys44Gly mutant showed no significant change in trypsin inhibitory activity.

The legume proteinase inhibitors are classified into two main groups according to their size and Cys content. Kunitz-type inhibitors are proteins (M_r 18,000-22,000) with one or two polypeptide chains and a low Cys content; generally there are four Cys residues arranged into two disulfide bridges, each comprising 170-180 amino acids. Bowman–Birk-type inhibitors are proteins (M_r 8,000-10,000) with high Cys content. The primary structures of various Kunitz-type trypsin inhibitors have been elucidated, and most of them have an Arg or Lys residue at the reactive site [2-4, 6].

An interesting characteristic of DrTI from *D. regia* is that a Glu68 residue is present in the expected position at the reactive site. DrTI also contains a Lys72 residue that is close to the reactive site in the reactive loop for trypsin. This may be the reactive site for trypsin for other Kunitz-type trypsin inhibitors of the Arg or Lys type. Site-directed mutagenesis provided confirmation of the DrTI reactive site. The Glu68Leu and Glu68Lys mutants resulted in the loss of almost all trypsin inhibitory activity, and Lys72Glu mutant did not experience significantly change in its inhibitory activity. Sporamin, a sweet potato tuber storage protein, also contains a Glu residue at the reactive site for trypsin [5]. Site-directed mutagenesis confirmed that the sporamin reactive site is a Glu residue for trypsin [12] suggesting that a negatively charged residue is located at the reactive site and providing evidence for a new mechanism of trypsin inhibition in the Kunitz-type trypsin inhibitors. Further biochemistry and structural studies are needed to elucidate the details of this novel DrTI trypsin-inhibiting mechanism. It should be noted that with reduced DrTI inhibitory activity against trypsin, DrTI inactivates trypsin with K_i value of 21.9 nM, while other Kunitz-type trypsin inhibitors (STI, ETI, or ACTI) have a K_i value around 1 nM [2, 4, 6].

The intramolecular disulfide bonds are presumably responsible for the functional stability of Kunitz-type inhibitors in the presence of various physical and chemical denaturants such as temperature, pH, and reducing agents [13]. Most Kunitz-type trypsin inhibitors have four conserved cysteine residues that form two disulfide

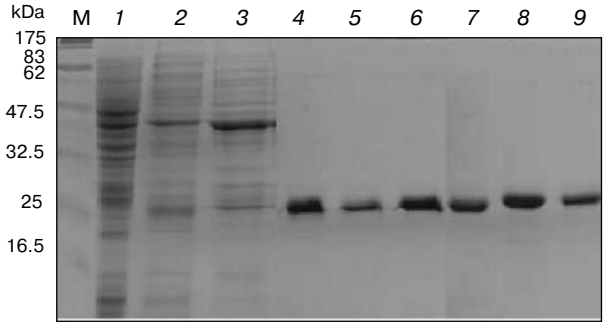

Fig. 2. SDS-PAGE analysis (15% polyacrylamide gel) of reDrTI and mutants. Lanes: M, molecular mass markers; *1*) crude extract without IPTG induction; *2*) crude extract with IPTG induction; *3*) nonspecific proteins for His-Trap HP affinity column; *4*) reDrTI; *5*) Glu68Leu; *6*) Glu68Lys; *7*) Lys72Glu; *8*) Cys44Gly; *9*) Cys139Gly.

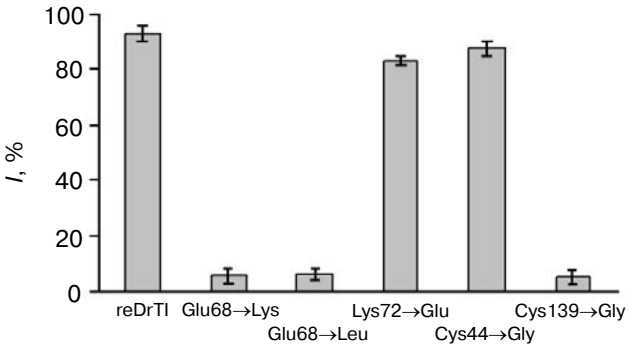

Fig. 3. Quantification of trypsin inhibitor activity of various DrTI mutants. The trypsin inhibitor activity of wild-type DrTI and the various mutants was quantified as percentage inhibition (*I*, %) as described in "Materials and Methods". Similar results were observed across three experiments, and bars represent means ± SEM with $n = 3$ per condition.

bridges, although examples with only one or no disulfide bridges are known [14-16].

Evidence from several studies suggests that the first disulfide bridge, which surrounds the reactive loop, is necessary for the inhibitory activity of most Kunitz-type trypsin inhibitors [14, 17]. However, ETI and ACTI [10, 18] reveal that the second disulfide bond is critical to the stabilization of the reactive loop. The X-ray crystallography of DrTI is similar to two Kunitz-type trypsin inhibitors from *Erythrina caffra* seeds and soybean [19-22]. The structure of Kunitz-type trypsin inhibitors has 12 antiparallel β-strands jointed by several loops. DrTI has two disulfide bonds, Cys44–Cys89 and Cys139–Cys149, which are conserved in Kunitz-type trypsin inhibitors (table). The first disulfide bond (Cys44–Cys89) in DrTI is close to the reactive site and links two loops, its location suggesting it might be a structural element stabilizing the reactive loop. However, in this study the Cys44Gly DrTI mutation did not significantly affect trypsin inhibitory activity. With the second DrTI disulfide bond Cys139–Cys149 mutation connecting two β-strands of the six-strand β-barrel, the 3D structure of the DrTI was disrupted, and the Cys139Gly DrTI mutant lost almost all trypsin inhibitory activity. This strongly suggests that the second disulfide bond (Cys139–Cys149), linking the two antiparallel β-strands, is critical to maintaining the tertiary structure of DrTI and its trypsin inhibitory activity, while the first disulfide bond (Cys44–Cys89) is not essential. However, this conflicts with the results of a study by Yao et al. [12] on sporamin where site-directed mutagenesis experiments showed that it is the first disulfide bond, and not the second, that is essential for the inhibitory activity. The reason for this disparity is not clear, although sporamin shares just under 30% of amino acid sequence identity with other trypsin inhibitors of the Kunitz family (table).

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