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# The Tissue-Type Plasminogen Activator Inhibitor ETIa from *Erythrina variegata*: Structural Basis for the Inhibitory Activity by Cloning, Expression, and Mutagenesis of the cDNA Encoding ETIa

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***Erythrina variegata*** **trypsin inhibitor ETIa belongs to the Kunitz inhibitor family, but is unique in its ability to bind and inhibit tissue-type plasminogen activator (tPA). A cDNA clone encoding ETIa was isolated from the λgt11 cDNA library using specific antiserum as a probe and characterized by nucleotide sequencing. The cloned ETIa cDNA consists of 762 nucleotides and includes an open reading frame encoding a polypeptide of 198 amino acids. Comparison of the deduced protein sequence and the determined protein sequence indicated the presence of two signal peptides composed of 24 and 2 amino acids at the N- and C-termini, respectively. The cDNA encoding mature ETIa was amplified by polymerase chain reaction (PCR), ligated into the expression vector pET-22b, and expressed in *Escherichia coli* BL21(DE3). The recombinant ETIa (rETIa) was expressed in *E. coli* as inclusion bodies; it was purified to homogeneity by gel filtration on Sephadex G-75. The rETIa exhibited almost the same inhibitory activity toward trypsin and tPA as ETIa. Six mutants, in which the amino acids $Arg^{61}$, $Leu^{62}$, $Arg^{63}$, and $Ala^{65}$ were replaced by Pro, Phe, Leu/Asp, and Tyr, respectively, were constructed by site-specific mutagenesis and expressed in *E. coli*. The site-specific mutation of $Arg^{63}$ to Leu (aR63L) or Asp (aR63D) in ETIa resulted in abolition of the inhibitory activities toward both trypsin and tPA. The mutants aR61P and aL62F showed significantly reduced tPA-inhibitory activity, and furthermore the double mutant aR61P/L62F lacked tPA-inhibitory activity, despite retaining the trypsin-inhibitory activity. In contrast, the mutant aA65Y exhibited tPA-inhibitory activity to the same extent as rETIa. This result suggests that $Arg^{61}$ and $Leu^{62}$ in ETIa, in addition to $Arg^{63}$, may play an important role in the interaction with tPA.**

**Key words: cDNA cloning, *Erythrina variegata*, overexpression, tissue-type plasminogen activator inhibitor, trypsin inhibitor.**

The genus *Erythrina* is a family of deciduous leguminous trees and shrubs widely distributed in the tropics and subtropics. Several serine proteinase inhibitors from the seeds of African *Erythrina* have been isolated and characterized in terms of their inhibitory activities toward various proteinases (*1-3*). Among them, *Erythrina* trypsin inhibitor, designated ETI, belongs to the Kunitz family of trypsin inhibitors, but is unique in its ability to inhibit not only trypsin, but also to inhibit tissue-type plasminogen activator (tPA). The tPA is a serine proteinase that converts plasminogen to plasmin, which plays an important role in the dissolution of blood clots. Hence, tPA has been widely and successfully used as a therapeutic agent for the treatment of acute myocardial infarction (*4*).

ETI has been employed as an affinity ligand for purification of both one- and two-chains forms of tPA from cells (*5*), and extensive biochemical and structural studies on ETI have been carried out. The amino acid sequences of ETIs from *E. latissima* (*6*) and *E. caffra* (*7*) were determined, and the tertiary structure of ETI from *E. caffra* was analyzed at 2.5 Å resolution (*8*); this protein is the first plant-derived Kunitz family proteinase inhibitor whose tertiary structure has been determined. Furthermore, Heussen-Schemmer *et al.* (*9*) examined the reactive site of ETI for tPA by enzymatic modification and reported that the P1 residue $Arg^{63}$ for trypsin is involved in the interaction with tPA as well. Recently, Teixeira *et al.* (*10*) chemically synthesized the ETI gene on the basis of the amino acid sequence and expressed it in *Escherichia coli* cells. Subsequently, they synthesized a modified form of ETI gene encoding an extra Asp at the N-terminus to simulate soybean trypsin inhibitor (STI) and characterized its inhibitory activity; the resulting ETI mutant retained no inhibitory activity, indicating that the N-terminal Asp

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Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ECI, *Erythrina variegata* chymotrypsin inhibitor; ETIa, *E. variegata* trypsin inhibitor a; ETIb, *E. variegata* trypsin inhibitor b; IPTG, isopropyl-β-D-thiogalactopyranoside; L-BAPA, benzoyl-L-arginine *p*-nitroanilide; MCA, 4-methyl coumaryl-7-amide; PCR, polymerase chain reaction; rETIa, recombinant ETIa; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor; tPA, tissue-type plasminogen activator.

TABLE I. **Sequence around the reactive sites of ETIa and ETIb.**

| | P6 | P5 | P4 | P3 | P2 | P1 | P1′ | P2′ | P3′ | P4′ | P5′ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ETIa | Ile | Glu | Ser | Arg | Leu | Arg[63] | Ser | Ala | Phe | Ile | Pro |
| ETIb | Ile | Glu | Ser | Pro | Phe | Arg[63] | Ser | Tyr | Phe | Ile | Pro |

residue of STI prevents its binding to tPA (*11*). Despite a vast amount of structural information about ETI, the structural basis for its inhibitory activity toward tPA, however, remained to be established.

We previously isolated two trypsin inhibitors, ETIa and ETIb, and one chymotrypsin inhibitor, ECI, from the seeds of *E. variegata* obtained in Okinawa and characterized their inhibitory activities and amino acid sequences (*12, 13*). The trypsin inhibitor ETIa inhibited tPA, whereas the other trypsin inhibitor ETIb did not: ETIa was a homologue of ETI from African species, whereas ETIb was an isoinhibitor with no inhibitory activity toward tPA. Furthermore, sequence comparison of ETIa and ETIb revealed several amino acid differences at positions P3, P2, and P2′, although the P1 and P1′ residues ($Arg^{63}$-$Ser^{64}$) for trypsin were conserved in the two trypsin inhibitors (Table I). This finding suggested that the amino acid residue(s) beside the P1 and P1′ sites in ETIa might be responsible for the tPA-inhibitory activity of ETIa.

In order to understand the structural basis for the tPA-inhibitory activity of ETIa, we have isolated the ETIa cDNA clone and expressed it in *E. coli* cells, obtaining a functional inhibitor. Furthermore, six ETIa mutants, in which the amino acid residues at positions P3, P2, and P2′, as well as at the P1 site were substituted, were constructed and their inhibitory activities toward trypsin and tPA were characterized in the expectation that the results would ultimately be helpful in understanding the catalytic mechanism of tPA, a specific enzyme involved in the rate-limiting step of the endogenous fibrinolytic cascade.

## MATERIALS AND METHODS

*Materials*—Trypsin inhibitor ETIa was isolated as described previously (*12*). Restriction endonucleases and DNA-modifying enzymes were purchased from either Toyobo, MBI Fermentas, or Bethesda Research Laboratories. Plasmid pUC18 and the expression vector, pET-22b, were purchased from Bethesda Research Laboratories and Novagen, respectively. Kits for DNA ligation, random labeling, and Takara Taq Cycle sequencing were obtained from Takara Shuzo. A Chameleon™ double-stranded site-directed mutagenesis kit was purchased from Stratagene. Oligonucleotides used in this study were purchased from Greiner Japan. Human recombinant tPA, a product of Genentech, was kindly given by Dr. S. Yoshitake. Bovine trypsin was purchased from Sigma Chemicals. Substrates for trypsin and tPA, benzoyl-L-arginine *p*-nitroanilide (L-BAPA) and pyroglutamyl-glycyl-L-arginine-MCA, respectively, were purchased from Protein Research Laboratories. Nitrocellulose membrane was obtained from Schleicher & Schull. [$\alpha$-$^{32}$P]dCTP was obtained from Amersham. All other chemicals used were of analytical grade for biochemical use.

*Screening and Nucleotide Sequencing of ETIa cDNA*—A $\lambda$gt11 cDNA library containing cDNA inserts from *E. variegata* maturing seeds was previously constructed in this laboratory (*14*). Approximately $1\times10^5$ phages were screened for ETIa cDNA using the anti-ETIa serum obtained from mice according to the methods of Young and Davis (*15*). Further screenings for a full-length ETIa cDNA clone were done by plaque hybridization as described (*16*) using a partial ETIa cDNA fragment as a probe. Positive clones were isolated and phage DNA was prepared from them by the method described in Ref. *16*. The cDNA insert was excised by digestion with *Eco*RI, subcloned into plasmid pUC18, and sequenced by the dideoxy chain termination method (*17*). Sequencing reactions were carried out using a *Bca*Best sequencing kit (Takara) with [$\alpha$-$^{32}$P]dCTP. In the later part of this study, the sequencing was done with a DNA Sequencer DSQ-1000 (Shimadzu) using a Takara Taq Cycle Sequencing kit.

*Expression of ETIa*—The expression plasmid was a derivative of pET-22b (*18*) constructed by ligating a 534 bp *Nde*I-*Bam*HI fragment derived from the ETIa cDNA clone by PCR with the forward primer 5′-CATATGGTTTTGCT-CGATGGTAATGC-3′ and reverse primer 5′-CGCGGATC-CTCATGATGATTCATCTTTCTT-3′ . First, to obtain the cDNA fragment encoding mature ETIa, PCR was performed using the ETIa cDNA as a template. The amplified DNA was ligated into plasmid T-vector (Promega). After confirmation of the construction by DNA sequencing, the DNA fragment encoding mature ETIa was excised by digestion with *Nde*I and *Bam*HI, and ligated into the expression vector pET-22b previously digested with the same enzymes. The resulting construct, pET-mETIa, contains T7 lac promoter directly followed by the ETIa cDNA fragment which contained mature-form ETIa coding sequence in-frame in the *Nde*I/*Bam*HI site of pET-22b. Introduction of the resulting plasmid pET-mETIa into *E. coli* BL21(DE3) and induction with 1 mM IPTG were done according to the supplier's instruction.

*SDS-PAGE and Western Blotting*—Overproduction of ETIa in *E. coli* cells was analyzed by SDS-PAGE using 15% polyacrylamide gels (*19*) and Western blotting (*20*). Western blots were probed with the anti-ETIa serum described above, incubated with anti-mouse IgG-horseradish peroxidase conjugate and visualized by addition of the peroxidase substrate solution, 100 mM Tris-HCl (pH 7.5) containing 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride, 0.3% $H_2O_2$, and 50 mM imidazole.

*Purification of Recombinant ETIa*—After induction with 1 mM IPTG, the culture was incubated for an additional 6 h at 37°C, then the cells were harvested and lysed by sonication in 30 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl. The lysate was centrifuged at $8,000\times g$ for 15 min and the pellet was collected and washed with 1 M sucrose, followed by 2% Triton X-100/10 mM EDTA. The inclusion bodies thus obtained were dissolved in 30 mM Tris-HCl containing 30 mM NaCl, 1 mM DTT, and 8 M urea. The protein solution was dialyzed against 30 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl, 1 mM

DTT, and 4 M urea for 24 h and finally against 10 mM Na-phosphate buffer (pH 7.2). The protein was purified by gel filtration on a Sephadex G-75 column (0.8×75 cm) equilibrated with the same buffer.

*Proteinase-Inhibitory Activities*—Inhibition of trypsin by ETIa and its mutants was assayed as described by Erlanger *et al.* (*21*). Briefly, trypsin was dissolved in 1 mM HCl containing 20 mM $CaCl_2$ at the concentration of 0.02 mg/ml. One hundred microliters of the enzyme solution was incubated with 200 $\mu$l of the inhibitor solution in the appropriate buffer at 37°C for 10 min, and then 1 ml of 0.5 mM BAPA solution in 50 mM Tris-HCl buffer (pH 7.5) was added. After incubation at 37°C for 30 min, 200 $\mu$l of 30% acetic acid was added to terminate the enzyme reaction, and the absorbance at 410 nm was measured. The tPA-inhibitory activity was measured as described by Heussen *et al.* (*5*). Five micrograms of tPA in 2 ml of 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 0.1 mg/ml of BSA was incubated at 37°C for 5 min with ETIa and its mutants. Then, 5 $\mu$l of 10.6 mM Pyr-Gly-Arg-MCA in DMSO was added, and the change of fluorescence intensity with emission at 380 nm and excitation at 440 nm for 3 min, was recorded at 37°C with a fluorospectrometer RF-5000 (Shimadzu). The inhibition constant ($K_i$) was determined by the method of Bieth (*22*).

*Site-Directed Mutagenesis*—Site directed mutagenesis was performed by the unique site elimination method developed by Deng and Nickoloff (*23*) using a Chameleon™ double-stranded site-directed mutagenesis kit (Stratagene). The nomenclature of ETIa mutants and the oligonucleotide primers used for mutagenesis is shown in Table II. Mutations were introduced into the amplified ETIa cDNA fragment that had been subcloned into plasmid T-vector. After mutagenesis the ETIa cDNA fragment was sequenced to verify the presence of the desired mutation. The mutant fragment was then recovered and ligated to the expression vector, pET-22b. Expression and purification of all mutants were done by procedures identical to those described for rETIa.

## RESULTS

*Cloning and Nucleotide Sequence of the ETIa cDNA*—The cDNA library constructed from maturing *E. variegata* seeds was previously prepared using the host strain *E. coli* Y1088 (*14*). Screening the cDNA library ($1\times10^5$ plaques) with an anti-ETIa serum detected one clone (A-1). The cDNA insert (0.6 kb) thus obtained was subcloned into pUC18 and the nucleotide sequence was determined. It was apparent from the sequence analysis that the clone A-1 contained an insert of approximately 600 bp that did not include the complete reading frame of ETIa. Hence, the cDNA library was rescreened using this cDNA fragment as a probe. After tertiary screening, two clones (A-2 and 3) gave strong hybridization signals with the cDNA probe. The largest clone (A-3: 0.8 kb) was subcloned and sequenced completely. The nucleotide sequence of the ETIa cDNA and the deduced amino acid sequence are shown in Fig. 1. The ETIa cDNA insert consists of 762 nucleotides and an open reading frame encoding a polypeptide of 198 amino acid residues. Two poly(A) signals, AATAAA, were found at 64 and 103 bp downstream from the stop codon TAG. Comparison of the deduced amino acid sequence and protein sequence of ETIa indicated the presence of the signal sequence consisting of 24 amino acid residues in the N-terminal region and of the C-terminal extension peptide of 2 amino acid residues. Furthermore, one substitution ($Glu^{95}$ to $Gly^{95}$) was found in the amino acid sequence deduced from the cDNA, as compared with the reported sequence (*12*), as shown in Fig. 1.

*Expression of cDNA*—SDS-PAGE analysis of the total proteins from the induced *E. coli* and Western blotting using the anti-ETIa serum indicated that the recombinant protein gave a single band with mobility on SDS-PAGE identical to that of mature ETIa, as shown in Fig. 2. The recombinant ETIa (rETIa) was purified by gel filtration on Sephadex G-75, and the purity was confirmed by RP-HPLC (Fig. 3). A single peak was obtained and rETIa was completely purified, as judged from SDS-PAGE.

Next, the inhibitory activities toward trypsin and tPA, as well as the N-terminal sequence, were analyzed. Figure 4 shows that rETIa could inhibit trypsin to the same extent as ETIa, but is slightly less inhibitory toward tPA. The inhibition constant of rETIa was slightly increased from that of ETIa (Table III). The direct sequencing of rETIa gave the sequence Met-Val-Leu-Leu; this result indicated that the N-terminus of rETIa has an extra methionine residue. The yield of rETIa was about 30 mg/liter of induced culture.

*Construction and Characterization of ETIa Mutants*—To define the amino acid residues responsible for the inhibitory activity of ETIa toward tPA, six ETIa mutants were prepared by site-directed mutagenesis, and expressed in *E. coli* cells. The products were characterized in terms of inhibitory activities toward trypsin and tPA. The mutated amino acid residues were selected based on a comparison of the amino acid sequence around the reactive site for tPA (Table I). Namely, the P1 amino acid of ETIa ($Arg^{63}$) for trypsin and tPA was changed to a negatively charged amino acid, Asp, or a bulky amino acid, Leu, to confirm its role in inhibiting trypsin and tPA. Next, P3 ($Arg^{61}$), P2 ($Leu^{62}$), and P2′ ($Ala^{65}$) amino acids were changed to the corre-

TABLE II. **ETIa mutant and the sequences of their oligonucleotide primers.**

| Mutants | Mutation | Oligonucleotide primers* |
|---|---|---|
| (1) aR63L | Arg→Leu | 5′-TGAATCACGACTTCTTTCAGCTTTTATTCC-3′ |
| (2) aR63D | Arg→Asp | 5′-TGAATCACGACTTGATTCAGCTTTTATTCC-3′ |
| (3) aR61P | Arg→Pro | 5′-AAGAATTGAATCACCACTTCGTTCAGCTTTT-3′ |
| (4) aL62F | Leu→Phe | 5′-TGAATCACGATTTTCGTTCAGCTTTTATTCC-3′ |
| (5) aA65Y | Ala→Tyr | 5′-CACGACTTCGTTCATATTTTATTCCCGATG-3′ |
| (6) aR61P/L62F | Arg→Pro, Leu→Phe | 5′-AAGAATTGAATCACCATTTCGTTCAGCTTT-3′ |

*The mutation sites are underlined.

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1   AAT TCG CGG CCG CTA ATG AAG AGT AGC ACT TTC ATC GTC CTC GTT CTA CTT CCT TCC TTC   60
-24                 Met Lys Ser Ser Thr Phe Ile Val Leu Val Leu Leu Pro Ser Phe   -10

61  ATC TCA TAC CTA CCT TCA TCC ACT GCT GTT TTG CTC GAT GGT AAT GGT GAA GTA GTT CAA  120
-9  Ile Ser Tyr Leu Pro Ser Ser Thr Ala Val Leu Leu Asp Gly Asn Gly Glu Val Val Gln   11

121 AAT GGT GGC ACA TAC TAT CTA TTG CCA CAG GTA TGG GCA CAG GGT GGT GGA GTA CAG TTA  180
12  Asn Gly Gly Thr Tyr Tyr Leu Leu Pro Gln Val Trp Ala Gln Gly Gly Gly Val Gln Leu   31

181 GCC AAG ACT GGT GAG GAA ACT TGT CCT CTC ACT GTT GTA CAA TCT CCC AAT GAG CTC TCT  240
32  Ala Lys Thr Gly Glu Glu Thr Cys Pro Leu Thr Val Val Gln Ser Pro Asn Glu Leu Ser   51

241 AAT GGA AAA CCC ATA AGA ATT GAA TCA CGA CTT CGT TCA GCT TTT ATT CCC GAT GAC GAC  300
52  Asn Gly Lys Pro Ile Arg Ile Glu Ser Arg Leu Arg Ser Ala Phe Ile Pro Asp Asp Asp   71

301 AAG GTG AGG ATT GGG TTC GCT TAT GCT CCA AAA TGT GCA CCC TCT CCA TGG TGG ACT GTG  360
72  Lys Val Arg Ile Gly Phe Ala Tyr Ala Pro Lys Cys Ala Pro Ser Pro Trp Trp Thr Val   91

                *
361 CTT GAG GAT GGA CAA GAA GGA CTC AGC GTT AAG CTT AGT GAG GAC GAA AGC ACA CAG TTC  420
92  Leu Glu Asp Gly Gln Glu Gly Leu Ser Val Lys Leu Ser Glu Asp Glu Ser Thr Gln Phe  111

421 GAT TAT CCG TTT AAG TTT GAG CAA GTT TCT GAT AAA CTT CAC AGC TAT AAG CTT CTT TAC  480
112 Asp Tyr Pro Phe Lys Phe Glu Gln Val Ser Asp Lys Leu His Ser Tyr Lys Leu Leu Tyr  131

481 TGT GAA GGT AAA CAC GAG AAG TGT GCA AGT ATT GGG ATA AAT AGG GAT CAG AAA GGA TAT  540
132 Cys Glu Gly Lys His Glu Lys Cys Ala Ser Ile Gly Ile Asn Arg Asp Gln Lys Gly Tyr  151

541 AGG CGT TTG GTG GTG ACT GAG GAC AAT CCT CTA ACT GTT GTG TTG AAG AAA GAT GAA TCA  600
152 Arg Arg Leu Val Val Thr Glu Asp Asn Pro Leu Thr Val Val Leu Lys Lys Asp Glu Ser  171

601 TCA ACT GCA TGA AGT ACT ACT ACT GGC CAT AAA ATA TCT CGC ACC ATC TCC TGT TGA GAG  660
172 Ser Thr Ala TER                                                               174

661 ATA AAG ATA AGT CTA AAT AAA TGC TTT GAC TTT AAT GAA GTT AGT GCA AGA TCA AAT AAA  720

721 ATA ATT AAA GCT TAT GTC TTT CAA AAA AAA ACG GCC GCG AAT                         762
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Fig. 1. **Nucleotide and deduced amino acid sequences of ETIa cDNA clone.** The predicted amino acid sequence is shown below the nucleotide sequence. The two arrowheads indicate the processing sites of ETIa. Signal peptides of ETIa at the N- and C-terminal are enclosed in boxes. The putative poly(A) additional signal sequences are underlined. The asterisk indicates the amino acid replaced in the protein sequence ($Glu^{58}$ to $Gly^{58}$).

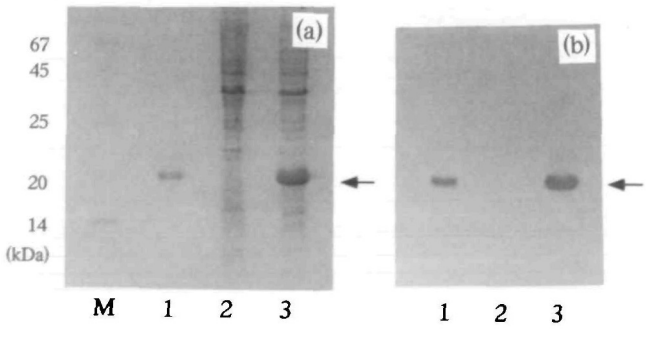


Fig. 2. **SDS-PAGE (a) and Western blotting (b) of total proteins from *E. coli* harboring pET-mETIa.** Lane 1, native ETIa; lane 2, before induction; lane 3, after induction by IPTG. M, markers: BSA (67 kDa), ovalbumin (45 kDa), $\alpha$-chymotrypsinogen (25 kDa), STI (20 kDa), and lysozyme (14 kDa).

sponding amino acid of ETIb ($Pro^{61}$, $Phe^{62}$, and $Tyr^{65}$) to investigate the importance of these amino acids for tPA-inhibitory activity.

Two mutants, designated aR63L and aR63D, in which the $Arg^{63}$ residue was changed to either Leu (aR63L) or Asp (aR63D), completely lost their inhibitory activities toward both trypsin and tPA (Fig. 5). This result agreed well with the previous report by Heussen-Schemmer *et al.* that $Arg^{63}$ is the reactive site for both trypsin and tPA (*9*).

Three mutants, aR61P, aL62F, and aA65Y, at the P3, P2, and P2′ amino acids were prepared and their inhibitory activities were analyzed. As we expected, all three mutants retained full inhibitory activity toward trypsin, although aR61P showed slightly less inhibitory activity ($K_i$: $4.8\times10^{-9}$ M) than rETIa ($K_i$: $2.1\times10^{-10}$ M). Two mutants, aR61P and aL62F, showed significantly reduced inhibitory activity toward tPA, while the mutant A65Y showed only slightly less inhibitory activity ($K_i$: $6.0\times10^{-8}$ M) than rETIa ($K_i$: $3.7\times10^{-8}$ M) (Fig. 5 and Table III). These results suggested that the $Arg^{61}$ and $Leu^{62}$ residues, in addition to the P1 site residue $Arg^{63}$, might be involved in the interaction of ETIa with tPA, while the $Ala^{65}$ residue at the P2′ site has little effect on tPA-inhibitory activity. This

was further confirmed by characterization of the double mutant aR61P/aL62F, in which $Arg^{61}$ and $Leu^{62}$ were replaced by Pro and Phe, respectively: it lacked inhibitory activity toward tPA (Fig. 5).

## DISCUSSION

*Erythrina* trypsin inhibitor, unlike the other Kunitz family inhibitors of plant origin, can bind and inhibit tPA. Hence, it should be a useful tool to investigate the catalytic mechanism of tPA. In this study, examined the structural basis for the inhibitory activity of the *E. variegata* trypsin inhibitor, ETIa, toward tPA by means of a genetic engineering approach. For this purpose, we first isolated a cDNA clone encoding ETIa. The cloned ETIa cDNA comprised 762 nucleotides and the open reading frame encoded a polypeptide of 198 amino acids, including a possible signal peptide composed of 24 amino acids and a C-terminal extension peptide of 2 amino acids. The N-terminal 24 amino acids closely resemble a classical signal sequence, with a charged residue very near the N-terminus, followed by a long stretch of hydrophobic amino acids and ending with an amino acid having a small side chain (*24*). The result suggested that ETIa may be synthesized as a preproprotein from which N-terminal and C-terminal extension peptides are cleaved during post-translational processing.

Recently, we have isolated and sequenced a cDNA clone encoding the isoinhibitor of ETIa (referred to as ETIb) from *E. variegata*. Sequencing result identified, as in the case of ETIa in this study, the presence of an identical dipeptide at the C-terminus of ETIb. Similar results were reported for plant vacuolar proteins, such as tobacco basic chitinase (*25*), tobacco $\beta$-1,3-glucanases (*26*), and barley lectin (*27*); it was reported that the C-terminal extension peptide is necessary for correct sorting of the protein to the vacuole. Since Kunitz family inhibitors are known, in general, to occur intracellularly, it is likely that the di-

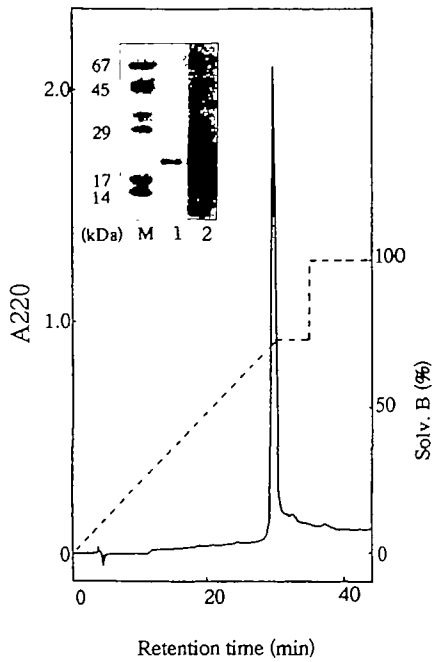


Fig. 3. **Reverse-phase HPLC and SDS-PAGE of purified recombinant ETIa.** Proteins from inclusion bodies were purified by gel filtration chromatography on a Sephadex G-75 column (0.8×75 cm). The inhibitor fraction was subjected to RP-HPLC on a YMC GEL C4 column (4.6×250 mm) equilibrated with 0.1% TFA at the flow rate of 0.6 ml/min. The protein was eluted with a linear gradient of 0 to 80% of Solvent B (80% acetonitrile in 0.1% TFA). The recombinant protein was also subjected to SDS-PAGE. Lane 1, native ETIa; lane 2, recombinant ETIa. M, markers; BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myoglobin (17 kDa), and lysozyme (14 kDa).

TABLE III. **Inhibition constants of ETIa, rETIa, and its mutants.**

| Inhibitors | $K_i$(M) | |
|---|---|---|
| | Trypsin | tPA |
| ETIa | $1.8\times10^{-10}$ | $1.4\times10^{-8}$ |
| rETIa | $2.1\times10^{-10}$ | $3.7\times10^{-8}$ |
| aR61P | $4.8\times10^{-9}$ | $8.8\times10^{-6}$ |
| aL62F | $7.7\times10^{-10}$ | $1.7\times10^{-8}$ |
| aR63L | n.d.* | n.d. |
| aR63D | n.d. | n.d. |
| aA65Y | $2.2\times10^{-10}$ | $6.0\times10^{-8}$ |
| aR61P/L62F | $4.9\times10^{-9}$ | n.d. |

*Not determined.

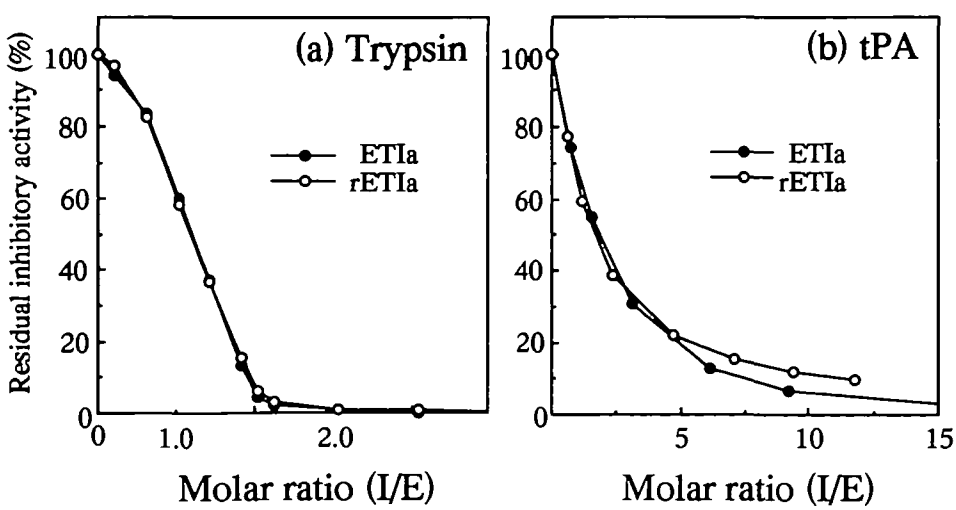


Fig. 4. **Inhibition of trypsin (a) and tPA (b) by increasing amounts of ETIa and rETIa.** A fixed amount of bovine trypsin or tPA was mixed with increasing amounts of the inhibitors, and the residual enzyme activities were assayed as described in "MATERIALS AND METHODS." The enzyme concentration in the reaction mixture was $7.2\times10^{-8}$ M for trypsin or $6.8\times10^{-8}$ M for tPA.

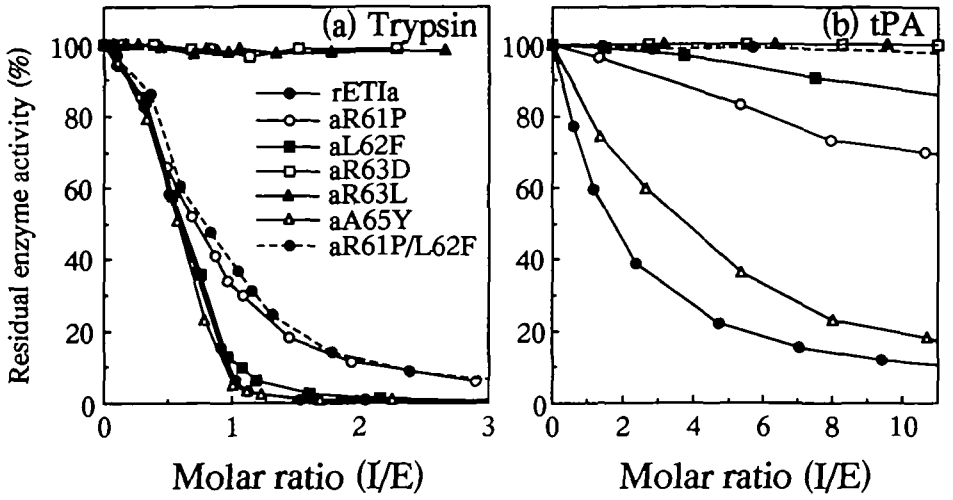


Fig. 5. **Inhibition of trypsin (a) and tPA (b) by increasing amounts of rETIa and ETIa mutants.** The conditions of the assay were the same as those described in the legend to Fig. 4.

peptides attached to the C-termini of ETIa and ETIb might function as a sorting signal, although the dipeptide signal is shorter than those found in chitinases and $\beta$-1,3-glucanases.

The deduced amino acid sequences of the ETIa cDNA clones (both A-1 and A-3) differ at position 95, where the deduced sequences have Gly instead of Glu in the determined protein sequence (*12*). This difference might be due to the difference in the *E. variegata* cultivars used for amino acid sequence determination and for cDNA library construction. Alternatively, the substitution might be attributed to polymorphism of the inhibitor genes.

The cDNA fragment encoding the matured ETIa was expressed in *E. coli* cells, and the rETIa with an extra Met at the N-terminus thus obtained could inhibit trypsin to the same extent as authentic ETIa. This result indicated that rETIa could refold into a conformation similar to that of authentic ETIa. In contrast, rETIa showed slightly less inhibitory activity toward tPA ($K_i$: $3.7\times10^{-8}$) than ETIa did ($K_i$: $1.4\times10^{-8}$ M) (Table III). Crystallographic study on the *E. caffra* trypsin inhibitor ETI showed that the N-terminal two amino acids, Val-Leu, interact with the loop structure containing the reactive site residues and play an important role in stabilizing the reactive site of ETI (*8*). The extra Met at the N-terminus of rETIa possibly causes steric hindrance to the interaction of rETIa and tPA.

Several ETIa mutants with replaced amino acid residues, either $Arg^{61}$, $Leu^{62}$, $Arg^{63}$, or $Ala^{65}$, were constructed by site-directed mutagenesis and expressed in *E. coli*. All mutant proteins could be overproduced to extents similar to that for rETIa: the yields were about 30 mg/liter of induced culture.

In the present investigation, the site-specific mutation of $Arg^{63}$ to either Asp or Leu resulted in loss of the inhibitory activities toward both trypsin and tPA. This is consistent with the result reported by Heussen-Schemmer *et al.*, that the $Arg^{63}$ residue is the reactive site for tPA, as well as for trypsin (*9*). As we expected, three ETIa mutants, aR61P, aL62F, and aA65Y, retained almost the same trypsin-inhibitory activities as rETIa. In contrast, the inhibitory activities of aR61P and aL62F toward tPA were significantly decreased, whereas that of a A65Y was only slightly decreased compared with that of rETIa. This result suggested that $Arg^{61}$ and $Leu^{62}$ may play an important role in tPA-inhibitory activity; $Leu^{62}$ appeared to be more important for interaction with tPA than $Arg^{61}$. In contrast to $Arg^{61}$ and $Leu^{62}$, the inhibitory activity of a A65Y toward tPA was only slightly decreased as compared with that of rETIa. This result suggested that $Ala^{65}$ is not critical for tPA-inhibitory activity. The characterization of the double mutants aR61P/aL62F further demonstrated an important role of $Arg^{61}$ and $Leu^{62}$ for tPA-inhibitory activity. This was further corroborated by our recent analysis of the ETIb mutant bP61R/F62L, in which $Pro^{61}$ and $Phe^{62}$ in ETIb are replaced with the corresponding amino acids Arg and Leu, respectively, of ETIa: the ETIb mutant acquired PA-inhibitory activity (Kouzuma, Y., unpublished result).

Although no crystallographic structure is at the moment available for the complex formed by tPA and ETIa, a possible role of $Arg^{61}$ and $Leu^{62}$ in ETIa for interaction with tPA can be proposed on the basis of the following information, (i) three-dimensional structural analysis of the complex formed by trypsin and STI has located the key residues involved in the trypsin-STI interaction (*28*), (ii) X-ray structural analysis of the *E. caffra* trypsin inhibitor ETI showed that the overall structure of ETI is highly similar to that of STI (*8*), and (iii) tPA is a trypsin-like serine proteinase and its amino acid sequence in the C-terminal domain is easily aligned with that of trypsin (*29*). Since $Ser^{61}$ and $Tyr^{62}$ in STI interact with two regions in trypsin: $Asn^{97}$-Thr-Leu-Asn-Asn-$Asp^{102}$ and $Ser^{214}$-Trp-$Gly^{216}$, the P3 and P2 residues ($Arg^{61}$-$Leu^{62}$), in ETIa may interact with $Asp^{366}$-Thr-Tyr-Asp-Asn-$Asp^{371}$ (equivalent to $Asn^{97}$-$Asp^{102}$ in trypsin) and $Ser^{497}$-Trp-$Gly^{499}$ (equivalent to $Ser^{214}$-$Gly^{216}$ in trypsn) in tPA. The former region, as expected, was found to contain the negatively charged residues $Asp^{366}$ and $Asp^{369}$. It is therefore likely that the $Arg^{61}$ residue is involved in an ionic interaction with the Asp residue(s). Crystallographic analysis of STI-trypsin complex shows that $Tyr^{62}$ in STI hydrophobically interacts with $Leu^{99}$ in trypsin. $Tyr^{62}$ in STI is replaced by $Leu^{62}$ in ETIa. Hence, it can be assumed that $Leu^{62}$ in ETIa might interact with $Tyr^{368}$ in tPA, by analogy with the hydrophobic interaction found in the STI-trypsin complex. The amino acids occurring at this position of the known Kunitz family proteinase inhibitors, including the ETIa isoin-

hibitor ETIb in *E. variegata*, are bulky amino acids, such as Tyr or Phe, and the proteins have no inhibitory activity for tPA. Substitution of $Leu^{62}$ in ETIa to Tyr or Phe in the known Kunitz family proteinase inhibitors might cause steric hindrance in interacting with tPA because of the side chain.

During the preparation of this paper, the 2.3 Å crystal structure of the catalytic domain of two-chain human tPA appeared (*30*). Our speculation on ETIa-tPA interaction described above is consistent with the crystal structure of the catalytic domain of tPA.

It is known that the circulating tPA in human plasma is present predominantly as a complex with a 50 kDa protein inhibitor, plasminogen activator inhibitor 1 (PAI-1), a member of the serpin family of serine proteinase inhibitors; tPA interacts with PAI-1 to form a 1 : 1 molar complex that is enzymatically inactive (*31*). It has been proposed on the basis of site-directed mutagenesis of PAI-1 that in the interaction of PAI-1 with tPA, negatively charged residues C-terminal to the reactive center of PAI-1 are involved in forming ionic bonds with positively charged residues located in a surface loop near the active site of tPA (*32*). The present study shows that the amino acid residues N-terminal to the reactive site of ETIa are crucially important for interaction with tPA. Thus, the tPA inhibitor of plant origin, ETIa, seems to inhibit tPA by a different mechanism from that of the physiological tPA inhibitor, PAI-1.

Unlike the well-studied serine proteinases such as trypsin, chymotrypsin, and elastase, tPA is a remarkably specific enzyme: a single peptide bond of plasminogen ($Arg^{560}$-$Val^{561}$) is the only known substrate of tPA *in vivo* (*33*). However, the molecular basis of the selectivity of tPA for plasminogen has not been understood. A detailed understanding of the molecular basis of the extraordinary specificity of tPA might facilitate design of proteinases with desired substrate and inhibitory specificity. The present study shows that the amino acid residues from the P1 to P3 sites in ETIa are crucially important for the interaction with tPA. A further study on the interaction of ETIa and its mutants with tPA should provide valuable information about the specificity of tPA.

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