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Chymotrypsin Inhibitor from *Erythrina variegata* Seeds: Involvement of Amino Acid Residues within the Primary Binding Loop in Potent Inhibitory Activity toward Chymotrypsin[1]

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The stoichiometry of the interaction between *Erythrina variegata* chymotrypsin inhibitor ECI and chymotrypsin was reinvestigated by analysis of their complex with ultracentrifugation and with amino acid analysis of the components separated. The amino acid analysis clearly showed that the stoichiometry of ECI and chymotrypsin was 1:1, though the apparent molecular mass of the complex was estimated to be 60 kDa. To examine the contribution of Leu64 (the P1 residue) to the inhibitory activity of ECI, a complete set of mutated inhibitors in which the amino acid at position 64 was replaced by 19 other amino acid residues was constructed by means of site-directed mutagenesis. Potent inhibitory activities (K_i, 1.3-4.6$\times10^{-8}$ M) exceeding that of the wild-type ECI (K_i, 9.8$\times10^{-8}$ M) were present in the mutant proteins L64F, L64M, L64W, and L64Y. The inhibitory activity of the mutant L64R was practically identical to that of the wild-type ECI. All other mutants exhibited slightly decreased inhibitory activities with K_i values of 1.9-4.6$\times10^{-7}$ M. These results indicate that ECI-chymotrypsin interaction involves not only the P1 site residue but also other residue(s) of ECI. A series of individual alanine mutations was then constructed in residues Gln62 (P3), Phe63 (P2), Ser65 (P1′), Thr66 (P2′), and Phe67 (P3′) in order to evaluate the contribution of each residue in the primary binding loop to the inhibitory activity. Replacement of Gln62, Phe63, and Phe67 with Ala residues decreased the inhibitory activity, the K_i values being increased by approximately 3-4-fold; but replacement of Ser65 and Thr66 had relatively little effect. This suggests that the P2, P3, and P3′ residues, together with the P1 residue, in the primary binding loop play an important role in the inhibitory activity toward chymotrypsin.

Key words: chymotrypsin inhibitor, *Erythrina variegata*, Kunitz-family protein, primary binding loop, site-directed mutagenesis.

The genus *Erythrina* is a deciduous shrub found throughout subtropical and tropical regions. Several Kunitz-family proteinase inhibitors have been isolated from various *Erythrina* species and characterized in terms of proteinase specificities (*1, 2*). We previously isolated three Kunitz-family proteinase inhibitors, ECI, ETIa, and ETIb, from the *Erythrina variegata* seeds obtained in Okinawa, and determined their amino acid sequences (*3, 4*) and inhibitory specificities (*5*). All three inhibitors exhibit inhibitory activity toward chymotrypsin, and particularly, the chymotrypsin inhibitor ECI strongly inhibits chymotrypsin; it was estimated by titration of chymotrypsin that the stoichiometry of ECI and chymotrypsin was 1:2 (*3*), whereas those of ETIa and ETIb were both 1:1. This means that ECI might be "double heads" for chymotrypsin. However, a subsequent study by limited digestion with chymotrypsin under acidic conditions unambiguously identified only Leu64 as the P1 residue for chymotrypsin. No information was obtained on a second reactive site (*4*).

In foregoing work, we showed by gel permeation chromatography on a Protein Pak 125 column that ECI forms a stable binary complex consisting of one ECI and one chymotrypsin molecule, but not a ternary complex, and that it also undergoes aggregation with chymotrypsin in a complex-forming buffer (*6*). In the present study, the stoichiometry of the ECI-chymotrypsin interaction was further corroborated by ultracentrifugation of the complex and also by quantification of each component by amino acid analysis. To understand the interaction of ECI with chymotrypsin at the molecular level, we developed an expression system for the mature ECI in *Escherichia coli* and used it to perform site-directed mutagenesis of ECI in order to generate mutant ECIs with a variety of amino acids at the P1 site in the primary binding loop. We also investigated

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Abbreviations: ECI, *Erythrina variegata* chymotrypsin inhibitor; ETIa, *E. variegata* trypsin inhibitor a; ETIb, *E. variegata* trypsin inhibitor b; HBS, HEPES-buffer saline; RP-HPLC, reverse-phase high performance liquid chromatography; RU, resonance units; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Ac-Ala-Aphe-ONP, *N*-acetyl-L-alanyl-α-azaphenylalanine *p*-nitrophenyl ester; SPR, surface plasmon resonance; tPA, tissue-type plasminogen activator.

the effects on the inhibitory activity of ECI of alanine mutations in the primary binding loop from residues 62 to 67. This allowed us to evaluate the contribution of amino acid residues around the P1 residue Leu64 to the inhibitory activity toward chymotrypsin.

MATERIALS AND METHODS

Materials—α-Chymotrypsin was obtained from Sigma Chemical, USA. The oligonucleotides used in this study were purchased from GIBCO BRL. Chameleon™ double-stranded site-directed mutagenesis kit was obtained from Stratagene. Restriction enzymes were purchased from MBI Fermentas and used as recommended by the supplier. *N*-Acetyl-L-alanyl-α-azaphenylalanine *p*-nitrophenyl ester, Ac-Ala-Aphe-ONP, for the titration of active chymotrypsin, was kindly supplied by Dr. Powers. Sephadex G-75 and Sephadex G-100 for purification of proteins, a sensor chip CM5, HEPES buffer saline (HBS), and amine-coupling kit for BIAcore were obtained from Pharmacia Biotech. All other reagent grade chemicals were purchased from Nacalai Tesque or Wako Pure Chemicals.

Stoichiometry of ECI and Chymotrypsin—The concentration of ECI was determined using the bicinchoninic acid protein assay reagent (Pierce) developed by Smith *et al.* (*7*), with bovine serum albumin as a standard protein. The chymotrypsin concentration was photometrically estimated with a coefficient of 20.4 for 1% solution with 1 cm path-length. For the complex formation, ECI was incubated chymotrypsin at chymotrypsin/ECI molar ratios of 0.5 and 2.35 in 50 mM Na-phosphate buffer, pH 6.5, containing 0.2 M NaCl for 30 min at 37°C. The ECI-chymotrypsin complex was purified by gel filtration chromatography on a Sephadex G-100 column (1.2×64 cm) in 50 mM Na-phosphate buffer, pH 6.5, containing 0.2 M NaCl. The fractions containing the ECI-chymotrypsin complex were collected and concentrated by CENTRICUT: V10 (Kurabou) at 4°C. The molecular mass of the complex was estimated by sedimentation equilibrium analysis after centrifuging an aliquot of the sample in an Optima XL-A ultracentrifuge (BECKMAN) at 10,000 rpm for 20 h at 20°C. The complex was monitored by its absorbance at 280 nm, and the partial specific volume used for the data analysis was 0.73 ml/g, calculated from the weight average of the amino acid content by the method of Cohn and Edsall (*8*). The molecular mass was evaluated from the sedimentation equilibrium curve using XLA EQ program. The remainder of the complex was subjected to RP-HPLC, and the separated components were quantified by amino acid analysis. RP-HPLC on a YMC gel C4 column (4.6×250 mm) and amino acid analysis on a Shimadzu LC6A system amino acid analyzer was performed exactly as described in the previous paper (*6*).

Expression of ECI in E. coli Cells—We previously constructed the expression plasmid for ECI as a downstream fusion to the *pel B* signal sequence, the objective being to produce in *E. coli* periplasmic space to insure correct formation of disulfide bridges and also to simplify purification (*9*). This approach was unsuccessful; the recombinant protein with the *pel B* signal peptide of 22 amino acids was produced as inclusion bodies in *E. coli* cells (*9*). Thus, the recombinant protein had to be subjected to pronase digestion to release the mature recombinant ECI. Therefore, in the present work, we wanted to express the mature ECI directly in *E. coli* cells. For this purpose, the cDNA fragment encoding mature ECI and flanked by artificial sites for *Nde*I and *Bam*HI was amplified from the cloned ECI cDNA (*9*) by PCR using two specific oligonucleotides primers, 5′-CATATGCAACCATTGCTGGATGTTGAAGG-3′ and 5′-CGCGGATCCTCATTGAGATGGTGAATTAGC-3′, as the sense and antisense primers, respectively. Since the cloned ECI cDNA contains a single *Nde*I recognition site (*9*), the internal A residue (pos. 141) was replaced with C by site-directed mutagenesis to avoid restriction enzymatic scission. The PCR product was first ligated to the plasmid pGEM T-vector (Promega); the resulting plasmid was designated pGECI. After digestion of pGECI with *Nde*I and *Bam*HI, the DNA fragments were inserted between the *Nde*I and *Bam*HI sites of pET-22b (*10*) to yield the ECI expression plasmid pETECI, and the plasmid was introduced into *E. coli* strain BL21(DE3). Overproduction in *E. coli* cells, refolding, and purification of the recombinant ECI (RECI) from the inclusion bodies were carried out, exactly as described in our previous paper (*9*). The homogeneity of the mutants was analyzed by SDS-PAGE using 15% polyacrylamide gels (*11*).

Mutagenesis—Oligonucleotide-mediated site-directed mutagenesis, using the unique site elimination method developed by Deng and Nickoloff (*12*), was used to introduce amino acid substitutions at positions 62-67 in the coding region of the recombinant ECI cDNA (pGECI), and also to eliminate the *Nde*I recognition site in the ECI cDNA, as described above. Reactions for site-directed mutagenesis were carried out using Chameleon™ double-stranded site-directed mutagenesis kits. Oligonucleotides used in this study are listed in Table I. The entire ECI coding region

TABLE I. **Oligonucleotide primers used in this study.** Unerlines (___) indicate nucleotide sequences designed for mutagenesis. Mixed bases (all intended to be equimolar): N=ACGT, K=GT, R=AG, S=CG.

Mutant	Oligonucleotide primer
L64A, L64C, L64D, L64F, L64G L64H, L64I, L64P, L64Q, L65R L64T, L64V, L64W, L64Y	5′-GCATCCCAATTCNNSTCAACGTTTATC-3′
L64E, L64N	5′-GCATCCCAATTCRSATCAACGTTTATC-3′
L64M, L64S	5′-GCATCCCAATTCAKSTCAACGTTTATC-3′
L64K	5′-GCATCCCAATTCAAATCAACGTTTATC-3′
Q62A	5′-ATTGCATCCGCTTTCCTTTCAACG-3′
F63A	5′-GCATCCCAAGCTCTTTCAACGTTT-3′
S65A	5′-CAATTCCTTGCTACGTTTATCCCT-3′
T66A	5′-CAATTCCTTTCAGCTTTTATCCCT-3′
F67A	5′-CTTTCAACGGCTATCCCTGATGGC-3′

for each candidate mutant was resequenced by the dideoxy chain termination method (*13*) to verify the presence of the intended mutation and the absence of adventitious second-site mutations. Sequencing reactions were carried out using a DNA sequencer DSQ 1000 (Shimadzu) and Takara Taq cycle sequencing kits. Each mutant plasmids was then digested with *Nde*I and *Bam*HI, inserts thus obtained were ligated with expression plasmid pET-22b previously digested with the same enzymes, and the resultant plasmids were used to transform *E. coli* BL21 (DE3).

Assay for Chymotrypsin Inhibitory Activity—Chymotrypsin inhibitory activity was assayed by the method of Kunitz using casein as a substrate (*14*). The concentration of chymotrypsin was estimated as described above, and active chymotrypsin was titrated with azapeptide, Ac-Ala-Aphe-ONP, by the method of Frank *et al.* (*15*). The concentrations of RECI and its mutant proteins were determined using the bicinchoninic acid protein assay reagent, as described above. From the inhibitory profile obtained, the inhibitory constant (K_i) was estimated by the method of Henderson (*16*).

Surface Plasmon Resonance (SPR) Measurements—Real time analysis of ECI-chymotrypsin interactions was done using BIAcore™ instrument (Pharmacia Biosensor). The principle and application of the system employing the method of surface plasmon resonance (SPR) detection was described by Karlsson *et al.* (*17*). Coupling of chymotrypsin to the sensor chip CM5 was done using the following procedure: a continuous flow of 5 μl/min of HBS [10 mM HEPES, pH 7.4, 3.4 mM EDTA, 0.15 M NaCl, and 0.005% Surfactant (Tween 20)] was maintained over the sensor surface. Carboxyl groups of the dextran matrix of the sensor chip were activated by injecting 35 μl of solution containing 0.2 M *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccinimide. Next, 35 μl of chymotrypsin solution (8 μg/ml in 10 mM acetate buffer, pH 6.0) was injected and immobilized, followed by injection of 35 μl of ethanolamine to block unreacted activated *N*-hydroxysuccinimide-ester groups. After washing with 15 μl of 10 mM HCl and excess HBS buffer, the sensor chip was ready for use. The flow rate during the coupling procedure was 5 μl/min for all steps. The SPR measurements for binding of RECI and P1 site mutants to the immobilized chymotrypsin were performed at 20 μl/min in HBS. Regeneration of the sensor chip after each analysis cycle was done by injecting 15 μl of 10 mM HCl. The concentration of all samples was determined as described above. Kinetic constants were obtained by analysis of sensorgram curves using kinetic evaluation software installed in BIAcore™.

RESULTS AND DISCUSSION

Stoichiometry of the Interaction of ECI with Chymotrypsin—In the previous study, the molecular mass of the complex consisted of ECI and chymotrypsin was estimated to be 45 kDa by gel permeation chromatography, suggesting that the stoichiometry of ECI and chymotrypsin might be 1:1 (*6*). In the present study, this was further confirmed by estimation of the molecular mass of the complex by ultracentrifugation and also by amino acid analysis of its components. To estimate its molecular mass, the ECI-chymotrypsin complex was formed at two different molar ratios of its components, as described under "MATERIALS AND METHODS," and the resulting complexes were purified by Sephadex G-100 column chromatography. These complexes were eluted at identical positions, as shown in Fig. 1. This was well consistent with the previous result of the gel permeation chromatography on a Protein Pak 125 column (*6*). The complex was then subjected to sedimentation equilibrium analysis using ultracentrifugation. Figure 2, A and B, shows the equilibration curves of the complexes formed at chymotrypsin/ECI molar ratios of 0.5 and 2.35, respectively. From this result, the molecular masses of both complexes were estimated to be approximately 60 kDa. This value was considerably higher than the value (45 kDa) estimated by gel permeation chromatography on Protein Pak 125 and lower than the calculated molecular mass (70 kDa) for a ternary complex, based on that the molecular masses of chymotrypsin and ECI are 25 and 20 kDa, respectively. However, the finding that the equilibration curves of the complexes formed at different molar ratios are essentially identical suggested that the stoichiometry of the interaction between ECI and chymotrypsin might be 1:1.

To further examine the stoichiometry, the complex was subjected to RP-HPLC equilibrated with 0.1% trifluoroacetic acid. As described in the previous paper (*6*), ECI and chymotrypsin were separated from each other by RP-HPLC, and each proteins was quantified by amino acid analysis. The results were reproducible and clearly showed that the molar ratio of chymotrypsin to ECI in both complexes was approximately 1:1. It was concluded from the analysis that one molecule of ECI interacts with one molecule of chymotrypsin. Although, at present, we have no explanation about overestimation of the molecular mass of the complex by the sedimentation equilibrium analysis, it might be due to the molecular shape of the complex.

Expression and Characterization of Recombinant ECI (RECI) in E. coli—The present result clearly demonstrates the stoichiometry of ECI-chymotrypsin interaction to be 1:1. However, the titration pattern of inhibitory activity

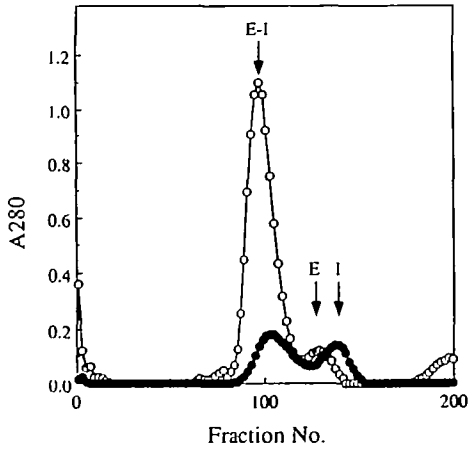

Fig. 1. **Gel filtration chromatography of the ECI-chymotrypsin complexes.** ECI and chymotrypsin were mixed at two different ratios at 37°C for 30 min, and each mixture was put on Sephadex G-100. Open and closed circles indicate the elution patterns of complexes formed at 2.35 and 0.5 chymotrypsin/ECI molar ratios, respectively. E, I, and E-I indicate chymotrypsin, ECI, and the ECI-chymotrypsin complex, respectively.

indicates that ECI might inhibit more than one chymotrypsin molecule. This observation suggests that ECI has a binding site on the primary binding loop which interacts strongly with chymotrypsin. Crystallographic studies on complexes formed by serine-proteinase inhibitors with proteinases have revealed that the P1 residue of the proteinase inhibitor interacts with the proteinase in a substrate-like manner: its side chain becomes imbedded in the S1 cavity of the proteinase (*18*). It is thus assumed that the interaction of the P1 residue Leu64 in the primary binding loop of ECI with chymotrypsin might cause a conformational change of either ECI or chymotrypsin that result in aggregation. Alternatively, residue(s) in the primary binding loop other than the P1 residue might interact with chymotrypsin in a non-substrate manner. To address this question by a genetic engineering approach, we established an expression system of ECI in *E. coli* cells and used it to construct ECI mutant proteins.

Overproduction of recombinant ECI (RECI) in *E. coli* cells was evaluated by SDS-PAGE. As in the previous study (*9*), RECI was predominantly present as inclusion bodies in the cells (data not shown). Thus, it was isolated from inclusion bodies, refolded into a native form, and purified by gel filtration on Sephadex G-75, as described previously (*9*). The direct sequencing of RECI gave the N-terminal sequence Met-Gln-Pro-Leu; this result indicated that the N-terminus of RECI has an extra methionine residue as compared with the authentic ECI. The purified RECI was found to possess a similar level of chymotrypsin inhibitory activity to authentic ECI (data not shown). Therefore, we concluded that RECI is refolded into the active conformation, which is practically the same as that of ECI. The yield of RECI was about 15 mg/liter of induced culture. Using this expression system, mutants were prepared and their inhibitory potency toward chymotrypsin was analyzed.

Preparation of the P1 Site Mutants—To investigate the contribution of the residue at position 64 in ECI to the inhibitory activity toward chymotrypsin, a complete set of mutants at position 64 of ECI was constructed by site-directed mutagenesis. All mutants were expressed in *E. coli* BL21(DE3) and purified from inclusion bodies by the procedure used for preparation of RECI, with the exception of the Asp mutant (L64D). Since mutant L64D was exclusively located in cytoplasmic fractions, the supernatant containing L64D was dialyzed against 10 mM Na-phosphate buffer, pH 7.4, and purified by gel filtration on Sephadex G-75.

The homogeneities of all purified mutants of ECI were analyzed by SDS-PAGE, as shown in Fig. 3. Each mutant migrated as a single band except for mutant L64C. Mutant L64C gave two bands with estimated molecular masses of 20 and 40 kDa in the absence of 2-mercaptoethanol, but a single band (20 kDa) in the presence of 2-mercaptoethanol (data not shown). Hence, it appeared that ECI was dimerized through covalently linkage of Cys64-Cys64 in the expression product. All mutant proteins were overproduced in an amount similar to the RECI; the yields of proteins from 1 liter of culture broth were 10–15 mg.

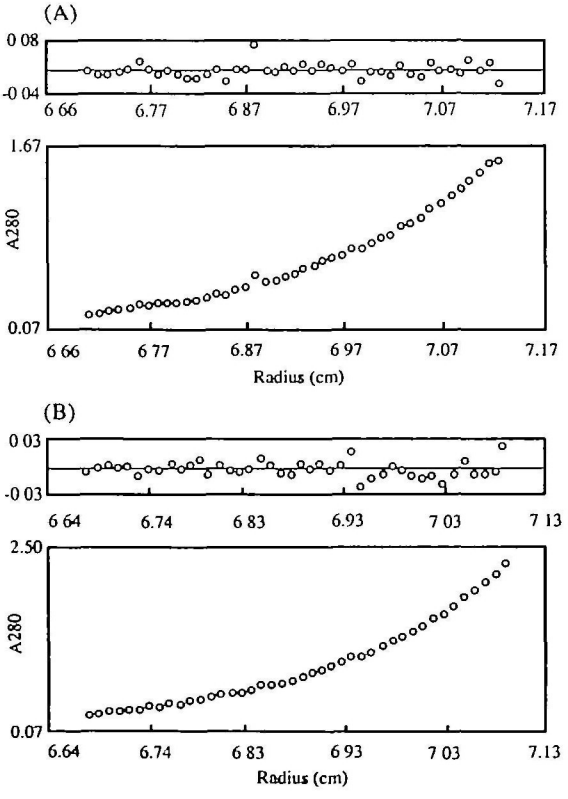

Fig. 2. **Sedimentation equilibrium analysis of the ECI-chymotrypsin complexes.** A and B show the sedimentation curves of the complexes formed at 0.5 and 2.35 chymotrypsin/ECI molar ratios, respectively. The circles represent the concentration distribution as a function of the radial position of the complex at equilibrium after 20 h at 10,000 rpm. The apparent molecular mass of the complex was calculated to be 61,259 Da from A and 57,259 Da from B.

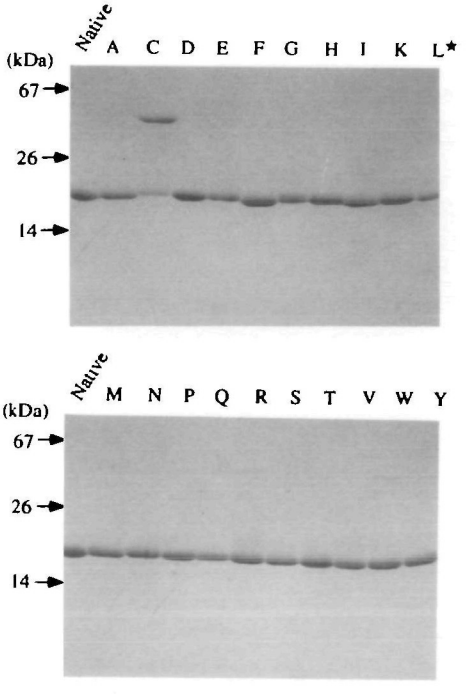

Fig. 3. **SDS-PAGE of the purified mutant proteins as well as the wild-type ECI.** SDS-PAGE was done using 15% acrylamide gel concentration, and gels were stained with Coomassie Brilliant Blue R250. Lanes are labeled with the one-letter symbol of the amino acid residue at position 64.

Characterization of the P1-Site Mutants—The P1-site mutants of ECI were characterized by two criteria: inhibitory potency toward chymotrypsin, and kinetics of interaction with chymotrypsin as estimated by SPR.

Inhibitory potencies of the mutants toward chymotrypsin were assayed using casein as a substrate. Figure 4 shows typical titration patterns of the wild-type ECI and the mutant proteins. The inhibitory constant (K_I) of each mutant was calculated from a double reciprocal plot of the data, as described by Henderson (*16*) (Table II). Among the mutants with a hydrophobic amino acid at position 64, the proteins L64F, L64M, L64W, and L64Y, in which the Leu64 residue was replaced with Phe, Met, Trp, and Tyr, respectively, possessed stronger inhibitory activities (K_I, $1.3\text{-}4.6\times10^{-8}$ M) toward chymotrypsin than did the wild-type ECI (K_I, 9.8×10^{-8} M); protein L64F proved to be the strongest inhibitor with a K_I value of 1.3×10^{-8} M. The inhibitory activity of mutant L64R was as potent as that of RECI. In contrast, the other mutants exhibited weaker inhibitory potencies than RECI, although they retained considerable inhibitory activities: their K_I values were higher about by 2 to 4-fold than that observed for RECI.

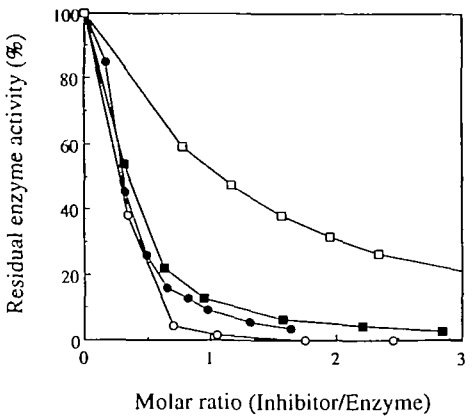

Fig. 4. **Inhibitory activity of the ECI mutants (L64X) toward chymotrypsin.** Various amounts of RECI or ECI mutants with different amino acids at the position 64 were examined for the potential to inhibit chymotrypsin. Inhibition is expressed as the remaining activity of chymotrypsin with casein as substrate. RECI (●), L64F (○), L64R (■), and L64A (□).

TABLE II. **Kinetic constants of the P1-site mutants toward chymotrypsin.** The ratios of the kinetic constants of the wild-type (wt) to those of the mutants (mt) are indicated in parentheses. The inhibitory constants (K_I) were obtained from analysis of the data of inhibitory potencies toward chymotrypsin by the method of Henderson (*16*). The apparent dissociation constants (K_d) were calculated using the relationship, $K_d = k_{off}/k_{on}$. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) were evaluated from sensorgram curves using the kinetic evaluation softwear installed in BIAcore™.

Mutant	Assay for inhibitory activity	Surface plasmon resonance measurement		
	K_I ($\times10^{-8}$ M) (mt/wt)	K_d ($\times10^{-9}$ M) (mt/wt)	k_{on} ($\times10^4$ $M^{-1}\cdot s^{-1}$)	k_{off} ($\times10^{-4}$ s^{-1})
RECI	9.8±0.9	9.0±0.5	10.7	9.7
L64F	1.3±0.3 (0.13)	3.5±0.9 (0.39)	13.1	4.6
L64M	3.9±0.1 (0.40)	3.9±1.6 (0.43)	9.4	3.7
L64W	4.5±0.9 (0.46)	6.5±0.2 (0.72)	12.7	8.3
L64Y	4.6±0.1 (0.47)	7.9±1.0 (0.88)	12.8	10.2
L64R	10.9±0.2 (1.11)	9.8±0.4 (1.09)	7.3	9.7
L64C	19.2±1.8 (1.96)	20.2±0.6 (2.24)	10.5	10.3
L64Q	22.8±1.0 (2.33)	13.3±0.1 (1.48)	4.9	9.8
L64S	23.5±0.9 (2.40)	14.6±0.6 (1.62)	7.8	8.3
L64V	23.5±1.0 (2.40)	19.1±1.0 (2.12)	5.2	9.9
L64H	29.0±3.9 (2.96)	23.9±0.6 (2.66)	6.1	14.5
L64N	29.5±1.5 (3.01)	16.8±0.3 (1.87)	8.4	14.1
L64T	32.7±1.5 (3.34)	11.7±0.0 (1.30)	11.2	13.1
L64G	32.8±0.7 (3.35)	31.4±1.0 (3.49)	4.9	15.5
L64D	33.6±1.5 (3.43)	19.4±0.8 (2.16)	6.9	13.4
L64K	34.3±0.0 (3.50)	20.7±0.6 (2.30)	6.3	13.1
L64A	36.9±1.5 (3.77)	28.4±0.5 (3.16)	4.8	13.5
L64P	39.5±0.5 (4.03)	16.2±0.1 (1.80)	8.9	14.5
L64E	41.8±0.6 (4.27)	26.3±0.5 (2.92)	4.2	11.0
L64I	45.7±2.3 (4.35)	31.6±0.2 (3.51)	5.0	15.8

Interactions of all mutants with chymotrypsin were further quantitatively analyzed by SPR with use of the BIAcore™ system (Pharmacia). Real time analysis of the interaction of each mutant with chymotrypsin was done twice using two different sensor chips of 1183.3RU and 1097.3RU, on which different amounts of chymotrypsin were immobilized. Figure 5A shows typical sensorgrams of association and dissociation using three different concentrations of RECI (62.5, 120, and 250 nM), and Fig. 5B shows sensorgrams of mutants L64F, L64R, and L64A as representatives of the stronger, less strong, and weaker, respectively, mutants proteins. The values of association rate constant (k_{on}) and dissociation rate constant (k_{off}) were calculated from curves of sensorgrams using the kinetic evaluation software installed in BIAcore™, and the appar-

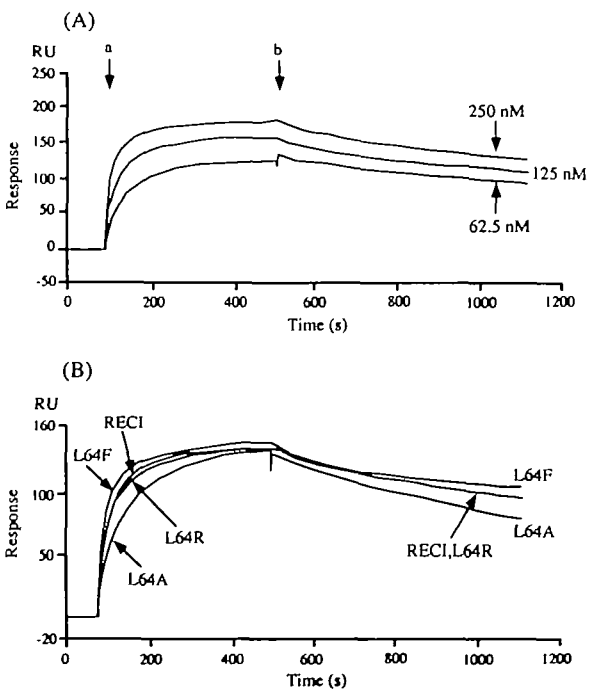

Fig. 5. **Sensorgrams of real time analyses by BIAcore of interactions of ECI or ECI mutants-chymotrypsin.** Immobilization of chymotrypsin on the sensor chip CM5 and binding analyses were done as described under "MATERIALS AND METHODS." (A) Sensorgrams illustrating binding of three different concentrations (62.5, 125, and 250 nM) of ECI with chymotrypsin immobilized on the sensor chip. (B) Sensorgrams of three ECI mutants L64F, L64R, and L64A with Phe, Arg, and Ala, respectively, at position 64, and of the RECI using 125 nM concentrations. Arrows a and b indicate the times of injection of either RECI or the mutant proteins and end of association by replacement with buffer HBS, respectively.

ent dissociation constants (K_d) were derived from the relationship between k_{on} and k_{off} ($K_d = k_{off}/k_{on}$). The kinetic constants obtained are summerized in Table II. The K_d values derived from real time analyses using the BIAcore™ were generally lower by one order of magnitude than the K_i values obtained from assays for inhibitory activities. The apparent discrepancy between these values might be attributable to the fact that the concentration of chymotrypsin immobilized on the sensor chip in BIAcore analysis was much lower than that used for the inhibitory assay. Alternatively, the results may simply indicate that ECI and its mutants could interact more strongly with the immobilized chymotrypsin than that in solution. Whatever the case, BIAcore measurements gave a similar relative binding strength to those obtained by assays for inhibitory activities. Namely, the real time analysis also showed that mutant proteins L64F and L64I are the strongest and weakest inhibitors, with K_d values of 3.5×10^{-9} and 3.2×10^{-8} M, respectively (Table II). We also found that increased affinity of mutant L64F was due to both increased association rate constant and decreased dissociation rate constant, those of mutants L64W and L64Y were mainly due to increased association rate constants, and that of mutant L64M was associated with decreased dissociation rate constant. Mutants L64Q, L64S, L64C, and L64V retained dissociation rate constants comparable to that of RECI, whereas mutants L64N, L64T, and L64P showed almost the same association rate constants as RECI.

The contribution of the P1 residue in serine-proteinase inhibitors to inhibitory activities has been extensively investigated using variants of the proteinase inhibitors prepared by semisynthetic (*19-22*) and genetic engineering methods (*23-28*). From these studies, it is generally accepted that the P1 residue of the proteinase inhibitor corresponds to specificity of its cognate proteinase. As a consequence, substitution of the P1 residue particularly influences the specificity toward proteinase. This rule is generally applicable to cases of ECI mutants. Namely, mutants with amino acids Phe, Trp, Tyr, and Met, which are good substrates for chymotrypsin, exhibit stronger inhibitory activities than do the wild-type ECI. In contrast, mutants with other amino acids at the P1 site show decreased inhibitory activities. Furthermore, since trypsin is specific for Arg and Lys, mutants L64R and L64K acquired inhibitory activity toward trypsin (data not shown). It should be noted that mutant L64R, in which Leu64 is replaced by Arg, an unfavorable substrate for chymotrypsin, retains almost the same inhibitory activity toward chymotrypsin as does the wild-type RECI, for which we have no adequate explanation.

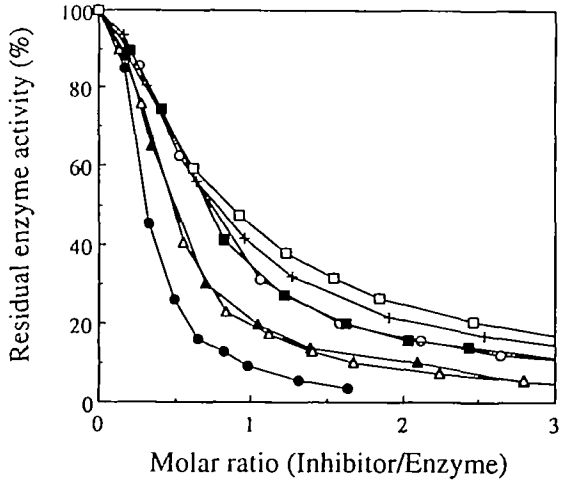

Fig. 6. **Inhibitory activity of the alanine mutations in the primary binding loop in ECI.** Various amounts of ECI (●), the mutants Q62A (○), F63A (■), L64A (□), S65A (▲), T66A (△), and F67A (+) were incubated with chymotrypsin and the remaining activity of chymotrypsin was measured using casein as the substrate, as described in Fig. 4.

The site-directed mutagenic study on the ECI mutant proteins described above indicates that even mutant proteins which have unfavorable amino acids for chymotrypsin do retain considerable inhibitory activities toward chymotrypsin. This means that ECI makes additional contacts with chymotrypsin, possibly through hydrophobic interaction, which may lead to aggregation with chymotrypsin.

Involvement of Other Residues on the Primary Binding Loop in Inhibitory Activity—The tertiary structures of Kunitz-family inhibitors have been reported for trypsin inhibitor DE3 from *E. caffra* (*29*), the proteinase K-α-amylase inhibitor from wheat (*30*), chymotrypsin inhibitor from winged bean (*31*), and trypsin inhibitor from soybean (*32*). These studies reveal that they share common tertiary structures consisting of twelve β-sheets and that the conformations of the main chains of the primary binding loops can almost be superimposed (*32*). It is thus highly likely that ECI has a common backbone folding and reactive site structure similar to those of other Kunitz-family inhibitors. Thus, some side chains of the primary binding loop in ECI might have a unique conformation that is favorable for subsites of chymotrypsin.

To investigate the contribution of each amino acid in the primary binding loop to the strong inhibitory activity of ECI, we perfomed alanine scanning mutagenesis of the residues from Gln62 (P3) to Phe67 (P3′) in the primary binding loop. As shown in Fig. 6, mutants Q62A, F63A, and F67A, in which Gln (P3), Phe (P2), and Phe (P3′) were replaced by Ala residues, exhibited lower inhibitory activity than the wild-type ECI: K_i values ($2.5\text{-}3.6\times10^{-7}$ M) of the mutants are about 3-4-fold higher than that of the wild-type. In contrast, mutants S65A and T66A exhibited almost the same inhibitory activities as the wild-type ECI (Fig. 6). The effects of alanine mutations of amino acids in the primary binding loop between Gln62 and Phe67 on inhibitory constants are shown in Table III. This result indicates that the amino acid residues Gln62 (P3), Phe63 (P2), and Phe67 (P3′) of the primary binding loop in ECI strongly interact with the amino acid residues in the subsites S3, S2, and S3′, respectively, in chymotrypsin

TABLE III. **The inhibitory constants of alanine mutants toward chymotrypsin.** The inhibitory constants (K_i) of each mutant were obtained from the data in Fig. 6.

Mutants	K_i ($\times10^{-8}$ M)
Q62A	25.1±0.8
F63A	28.6±0.5
L64A	36.9±1.5
S65A	14.4±0.5
T66A	13.5±0.8
F67A	36.2±1.9

and, together with Leu64 (P1), play a role in the strong inhibitory activity toward chymotrypsin.

Erytrina Kunitz-family proteinase inhibitors are further divided into three groups based on their abilities to inhibit trypsin, chymotrypsin, and tissue-type plasminogen activator (tPA) (*1, 2*). Group **a** includes inhibitors that are relatively specific for chymotrypsin; group **b** includes inhibitors that inhibit trypsin strongly and chymotrypsin slightly less effectively; and group **c** includes inhibitors that inhibit trypsin, chymotrypsin, and tPA. We previously isolated two Kunitz-family inhibitors designated ETIa and ETIb from *E. variegata* seeds, which are classified into group **c** and **b**, respectively, and showed that ETIa inhibits chymotrypsin less strongly than does ECI. Sequence comparison shows that the amino acid residues Leu64 (P1), Phe63 (P2), and Gln62 (P3) in ECI are replaced by Arg, Leu, and Arg, respectively, in ETIa. The present result suggests that the lower chymotrypsin inhibitory activity of ETIa may be attributable to these amino acid substitutions. This assumption can be confirmed by constructing a ETIa mutant in which Arg63 (P1), Leu62 (P2), and Arg63 (P3) are simultaneously replaced by the corresponding residues in ECI. This study is under progress in this laboratory.

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