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The P_1 Reactive Site Methionine Residue of Ecotin Is Not Crucial for Its Specificity on Target Proteases

A POTENT INHIBITOR OF PANCREATIC SERINE PROTEASES FROM *ESCHERICHIA COLI**

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The importance of the P_1 reactive site for the specificity of ecotin on target proteases was examined by site-directed mutagenesis. The replacement of Met at the P_1 site with Ile, Arg, Glu, or Tyr showed little or no effect on the ability of ecotin to inhibit trypsin. Similar results were obtained for chymotrypsin, except that its replacement with Glu caused about 40% reduction of the inhibitory activity of ecotin. On the other hand, the replacement of the Met residue with Arg, Tyr, or Glu dramatically reduced its ability to inhibit elastase, while that with Ile showed little or no effect. Nevertheless, elastase could be completely inhibited upon incubation with excess amounts of the mutant ecotin containing Arg, Glu, or Tyr. Moreover, all the mutant forms of ecotin could be cleaved at the mutated P_1 site upon incubation with trypsin at pH 3.75. In addition, the replacement of a Cys residue in the disulfide bridge with Ser showed little or no effect on the ability of ecotin to inhibit trypsin, chymotrypsin, or elastase. However, the mutant ecotin containing Ser was more sensitive to inactivation by heating at 100 °C than the wild-type inhibitor. Furthermore, the wild-type ecotin whose disulfide bond had been reduced and alkylated was also more easily inactivated by heat treatment than the untreated control. These results strongly suggest that the P_1 site of ecotin is not crucial for its specificity on target proteases and that the disulfide bridge in ecotin appears to play an important role in maintenance of its structural stability.

Ecotin is a periplasmic protein in *Escherichia coli* that is capable of inhibiting trypsin and other pancreatic serine proteases, including chymotrypsin and elastase (1). The nucleotide sequence of the ecotin gene has been determined, and the deduced amino acid sequence of ecotin has revealed that the mature protein has a molecular mass of 16.1 kDa (2, 3). The inhibitor does not contain any consensus reactive site sequences of known inhibitors, suggesting that ecotin is a novel inhibitor. In addition, ecotin and its complex with trypsin have been crystallized (4, 5).

The reactive site residue, P_1, of serine protease inhibitors generally corresponds to the specificity of the cognate proteases and is encompassed by a disulfide bridge (6, 7). Thus, inhibitors with P_1 Lys and Arg tend to inhibit trypsin, those with P_1 Tyr, Phe, Leu, and Met inhibit chymotrypsin, and those with P_1 Ala and Ser inhibit elastase. The reactive site residue of ecotin has been determined to be Met^{84} and lies within a Cys^{50}-Cys^{87} disulfide bridge (3). In order to clarify further the specificity of ecotin on target proteases, mutagenesis was directed at the Met^{84} residue. We also replaced Cys^{87} with Ser to determine the role of the disulfide bond in the ecotin molecule.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The recombinant Bluescript plasmid (pBS2.2) containing the ecotin gene was constructed previously (2). Single-stranded, uracil-containing phagemids were prepared by infecting *E. coli* strain CJ236 (*dut⁻ ung⁻*) with helper phage R408 (8). Mutagenic oligonucleotides were designed to generate new restriction sites for facilitating mutant isolation (Fig. 1). The primers were annealed to the template and extended (9). The double-stranded mismatch plasmids were then transformed, and the mutated vectors were subsequently purified. The mutations were confirmed by restriction and DNA sequence analyses (data not shown).

Assays—The cleavage of fluorogenic peptides was assayed as described (10) using *N*-benzyloxycarbonyl-Ala-Arg-Arg-4-methoxy-β-naphthylamide, *N*-succinyl (Suc)[1]-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (AMC) and Suc-Ala-Ala-Ala-AMC as the substrates for trypsin, chymotrypsin, and elastase, respectively. The peptides were obtained from Peptide Institute Inc., Osaka, Japan. Reaction mixtures (0.1 ml) containing appropriate amounts of ecotin and 10 ng of trypsin, 2 ng of chymotrypsin, or 30 ng of elastase in 100 mM Tris-HCl buffer (pH 8) were incubated for 30 min at room temperature prior to the addition of the peptide substrates (0.1 mM). When assaying trypsin and chymotrypsin, 20 mM $CaCl_2$ was also included. The samples were then incubated for the next 20 min at 37 °C. After the incubation, the samples were added with 0.9 ml of 0.1 M sodium borate (pH 9.1) and then heated for 5 min in a boiling water bath. Fluorescence was measured at 310 nm (excitation) and 410 nm (emission) for 4-methoxy-β-naphthylamide and at 380 nm and 440 nm for AMC. Proteins were assayed as described by Bradford (11) using bovine serum albumin as a standard or by the absorbance at 280 nm for those proteins whose extinction coefficients are known.

Purification—E. coli cells were grown in Luria broth to mid-log phase, and their periplasmic proteins (osmotic shock fluid) were prepared by the method of Nossal and Heppel (12). The samples were adjusted to pH 3.5 by adding 1 M HCl, incubated for 10 min at 4 °C, readjusted to pH 7.8 by adding 1 M Tris base, and heated to 100 °C for 10 min. Insoluble materials were removed by centrifugation at 12,000 × *g* for 10 min, and solid ammonium sulfate was added to the supernatants to 80% (w/v) saturation. The precipitated proteins were resuspended in 1.1 M ammonium sulfate solution and loaded onto a phenyl-Sepharose column (1 × 5 cm). Proteins were eluted by linearly decreasing the salt concentration to 0.5 M. Active fractions were collected, dialyzed against 10 mM Tris-HCl (pH 7.8) containing 5 mM $MgCl_2$, and loaded onto a DEAE-cellulose column (1 × 5 cm) equilibrated with the same buffer. Proteins that did not bind to the column were collected and stored at 4 °C for further use.

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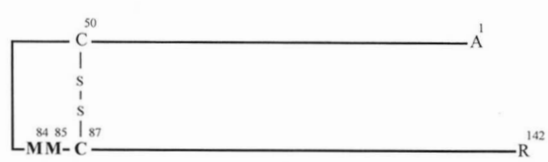
[1] The abbreviations used are: Suc, *N*-succinyl; AMC, 7-amido-4-methylcoumarin; Tricine, *N*-tris(hydroxymethyl)methylglycine; HPLC, high pressure liquid chromatography; DTT, dithiothreitol.

A



B

Changes	Primers	Restriction Site
	Met^{84} Met^{85} Cys^{87}	
None	CCCGGTTTCAACGATGATGGCCTGCCCG	
Met^{84} → Ile	5′ CCCGGTTTCAACGATCATGGCCTGCCCG	*MboI*
Met^{84} → Arg	5′ CCCGGTTTCAACTCGAATGGCCTGCCCG	*TaqI*
Met^{84} → Glu	5′ CCCGGTTTCAACTGAGATGGCCTGCCCG	*DdeI*
Met^{84} → Tyr	5′ CCCGGTTTCAACGTACATGGCCTGCCCG	*RsaI*
Cys^{87} → Ser	5′ CAACGATGATGGCCAGCCCGGATGGCAA	*BalI*

FIG. 1. **Abbreviated primary structure of ecotin (*A*) and the primers used for mutagenesis (*B*).** The amino acids represented by *single letters* are based on the nucleotide sequence of the ecotin gene (2). The mutated nucleotides are *shaded*, and the restriction sites newly created by the mutations are *underlined*.

Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed using two different methods. For determination of the purity of ecotins, electrophoresis was carried out using 10–20% (w/v) polyacrylamide gradient slab gels containing 0.1% (w/v) sodium dodecyl sulfate (SDS) as described by Laemmli (13). For separation of the cleavage products of mutant ecotins (see below), electrophoresis was performed using 4, 10, and 16% discontinuous slab gels containing SDS as described by Schagger and von Jagow (14). Anode buffer (pH 8.9) contained 0.2 M Tris-HCl, and cathode buffer (pH 8.25) had 0.1 M Tris, 0.1 M Tricine, and 0.1% SDS. The sample buffer (pH 6.8) contained 100 mM Tris-HCl, 1.5% SDS, 2% (v/v) 2-mercaptoethanol, 0.02% (w/v) bromphenol blue, and 7% (v/v) glycerol.

Protein Sequencing—Peptide fragments of ecotin were degraded with an automated gas-phase protein sequencer (Applied Biosystems, model 470A) according to the operation program 02RPTH provided for the sequencer. Phenylthiohydantoin-derivatives liberated were identified by HPLC using an Ultrasphere ODS column (2 × 250 mm, Beckman) at 49 °C. Solvents used for the elution of phenylthiohydantoin-derivatives were composed of acetonitrile, 0.1% trifluoroacetic acid adjusted to pH 4.9 at a ratio of 1/10 or 1/1 (v/v). The eluates were monitored simultaneously at 269 and 322 nm.

RESULTS

Site-directed Mutagenesis and Purification—In order to replace Met^{84} at the P_1 site of ecotin with Ile, Arg, Glu, and Tyr, site-directed mutagenesis was performed using mutagenic oligonucleotide primers (Fig. 1). We also replaced Cys^{87} with Ser to test whether the disulfide bridge in ecotin is necessary for its activity or stability. *E. coli* cells were transformed with plasmids containing the mutated ecotin genes, and its gene products were then isolated from the periplasmic space of the cells as described under "Experimental Procedures." Fig. 2 shows that the mutated inhibitors were purified to apparent homogeneity upon analysis by polyacrylamide gel electrophoresis in the presence of SDS. It is interesting to note that a single replacement of Met^{84} with Glu results in a slight change of mobility of ecotin in the gel, even in the presence of SDS.

Inhibition of Target Proteases by Mutant Ecotins—To determine whether the mutations at the P_1 reactive site alter the specificity of ecotin or affect its ability to inhibit target proteases, the inhibitory activity of the mutant forms of ecotin was compared to that of the wild-type inhibitor against trypsin, chymotrypsin, and elastase. Fig. 3*A* shows that all the mutant ecotins, in which Met^{84} was replaced with Ile and Arg and even with Tyr and Glu, are capable of inhibiting trypsin to nearly the same extents. Similar data were obtained for their ability to inhibit chymotrypsin, except that the replacement with Glu

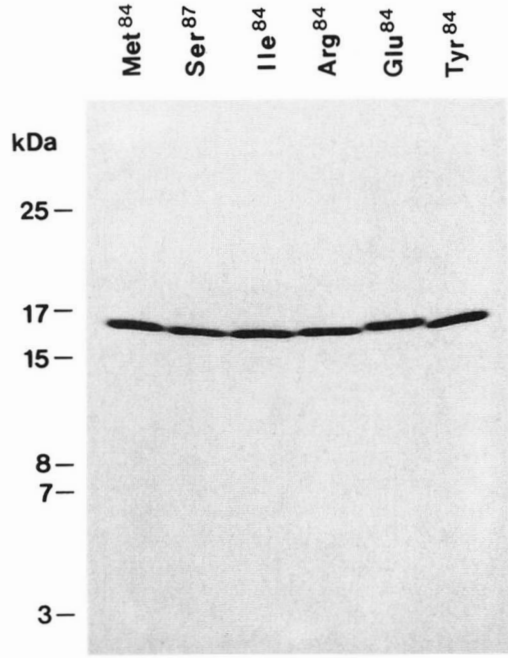


FIG. 2. **Polyacrylamide gel electrophoresis of the purified mutant forms of ecotin.** The wild-type and mutant forms of ecotin were purified as described under "Experimental Procedures." They (5 μg each) were then electrophoresed on 10–20% gradient gels in the presence of SDS (13). After electrophoresis, the gel was stained with Coomassie R-250.

resulted in about 40% decrease in the inhibitory activity of ecotin against the protease (Fig. 3*B*). On the other hand, the replacement of Met^{84} with Arg, Tyr, and Glu dramatically reduced the ability of ecotin to inhibit elastase, while that with Ile showed little or no effect (Fig. 3*C*). Nevertheless, elastase could be inhibited completely when incubated with an ~70-fold molar excess of the mutant ecotin containing Arg, Tyr, or Glu (data not shown). These results suggest that the changes of the P_1 residue do not alter the specificity of ecotin on target proteases but rather reduce its affinity to them.

The replacement of Cys^{87} with Ser showed little or no effect on the ability of ecotin to inhibit trypsin, chymotrypsin, or elastase. However, the mutant ecotin containing Ser^{87} was more sensitive to inactivation by heating at 100 °C than the wild-type inhibitor (Table I). Moreover, the wild-type ecotin whose disulfide bond had been reduced and alkylated was also more easily inactivated by heat treatment than the untreated control. In addition, prolonged storage at 4 °C or repeated freezing-and-thawing of the Cys^{87} mutant ecotin caused a significant decrease in its ability to inhibit target proteases, unlike the wild-type and other mutant ecotins (data not shown). Thus, the disulfide bridge in the ecotin molecule appears to play an important role in maintenance of its structural stability.

Cleavage of Mutant Ecotins at the P_1 Site by Trypsin—The P_1 site of ecotin has been determined previously by incubation of the inhibitor with trypsin at pH 3.75 and then by identification of the N-terminal amino acid residue at the cleavage site (3). Since replacements of Met^{84} even with Glu and Tyr were without any effect on the inhibitory activity of ecotin against trypsin, we wondered whether or not the mutant forms of ecotin are cleaved at the mutated P_1 site under the same incubation condition. Fig. 4 shows that at least two common fragments with sizes of 7.5 and 6 kDa (henceforth referred to as F7.5 and F6, respectively) are generated upon incubation at pH 3.75. However, the sum of the molecular masses of the two fragments did not exactly match with the size of intact ecotin. This result suggest that an additional cleavage may occur in the ecotin

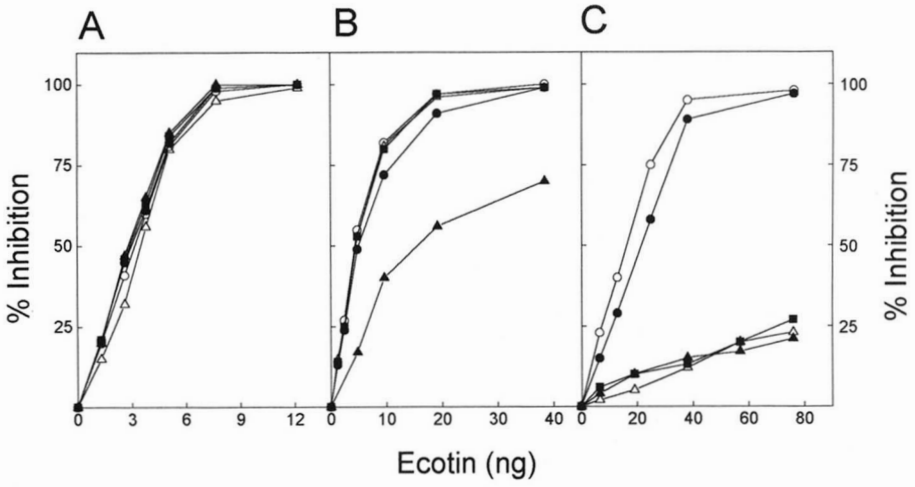


FIG. 3. **Effects of increasing concentrations of the mutant forms of ecotin on the activities of trypsin (*A*), chymotrypsin (*B*), and elastase (*C*).** The proteases were assayed in the presence of increasing amounts of the wild-type ecotin (○) and the mutant inhibitor in which the Met^{84} was replaced with Ile (●), Arg (△), Glu (▲), or Tyr (■) as described under "Experimental Procedures." The peptide substrates used for the assays were *N*-benzyloxycarbonyl-Ala-Arg-Arg-4-methoxy-β-naphthylamide for trypsin, Suc-Leu-Leu-Val-Tyr-AMC for chymotrypsin, and Suc-Ala-Ala-Ala-AMC for elastase.

TABLE I

Effect of heat treatment on the inhibitory activity of ecotin in which the disulfide bridge is eliminated

To prepare alkylated ecotin, the wild-type inhibitor (10 μg) was incubated with 1 mM DTT for 30 min and then with 10 mM iodoacetamide for 2 h at room temperature. The incubation mixture was then dialyzed against 100 mM Tris-HCl (pH 8). Aliquots (4 ng each) of the wild-type, mutant, and alkylated ecotins were heated in a boiling water-bath for the indicated periods and assayed for their inhibitory activities against trypsin (10 ng) as described in Fig. 3. The inhibitory activity of the wild-type ecotin seen against trypsin without heat treatment was expressed as 100% and the others as its relative values. Similar data were obtained from three independent experiments.

Ecotin	Relative activity of ecotin after heating for		
	0 min	30 min	60 min
		%	
Wild-type	100	98	94
$Cys^{87} \rightarrow Ser$	89	41	21
Alkylated	84	42	24

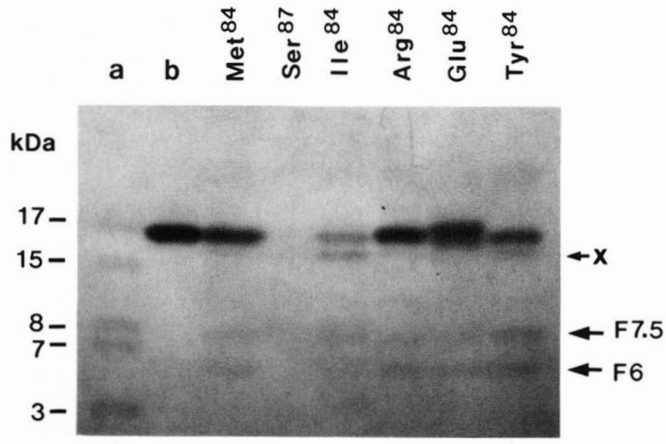


FIG. 4. **Hydrolysis of the wild-type and mutant forms of ecotin by trypsin under acidic conditions.** Hydrolysis of the inhibitors by trypsin at pH 3.75 was performed as described by Ozawa and Laskowski (14). Each of the inhibitors (5 μg) was incubated with 1 μg of trypsin in the presence of 40 mM $CaCl_2$ in a rotator for 4 days at room temperature. After incubation, the samples were electrophoresed on discontinuous polyacrylamide gels containing SDS as described under "Experimental Procedures." The gels were then stained with Coomassie R-250. *Lane a* indicates the size markers, and *lane b* shows the wild-type ecotin incubated without trypsin.

molecule. In fact, an additional band (marked with *X* in Fig. 4) was evident, particularly in the incubation mixture with the Ile^{84} and Tyr^{84} mutant ecotins. Noteworthy was the finding that the Ser^{87} mutant ecotin was hydrolyzed to near completion upon incubation with trypsin under the same conditions. This result further supports the implication that the disulfide bridge in ecotin plays a role in maintaining its structural stability, particularly under stressful conditions.

In order to confirm whether the cleavage of the mutant ecotins by trypsin indeed occurred at the P_1 site, we first needed to obtain sufficient quantities of the cleavage products. Therefore, the wild-type ecotin and the mutant inhibitors, in which Met^{84} was replaced with Glu and Tyr, were incubated at pH 3.75 with equimolar amounts of trypsin that had been covalently conjugated to Sepharose. The incubation mixtures were then adjusted to pH 1 and centrifuged to remove trypsin. When the cleavage products of the wild-type ecotin were directly subjected to separation by HPLC on a C18 column, two peptide peaks were evident (Fig. 5*B*). Of these, the second peak (marked *E*) eluted at the same retention time as intact ecotin (Fig. 5*A*). In contrast, when the same cleavage products were incubated with dithiothreitol (DTT) prior to separation by the column, three peaks were generated in addition to the DTT peak itself (*D*) (Fig. 5*C*). Upon analysis of each peak on polyacrylamide gels containing SDS, the three peaks were found to correspond to F6, intact ecotin, and F7.5 in order of elution from the column (data not shown). These results indicate that the first peak (*E** in Fig. 5*B*) consists of F6 and F7.5, which are linked by a disulfide bridge. Nearly identical data were obtained with the cleavage products of the Glu^{84} and Tyr^{84} mutant ecotins (data not shown). Because the size of F6 is similar to that of the C-terminal half of ecotin beginning from the P_1' Met^{85}, the F6 peptides obtained from wild-type ecotin and the Glu^{84} and Tyr^{84} mutant inhibitors were subjected to Edman degradation for determination of the amino acid residues at their N termini. The N-terminal 12-amino acid sequence MACPDGKKEKKF for the mutant ecotins was identical to that of the wild-type inhibitor, indicating that the wild-type ecotin and mutant inhibitors are cleaved at the same P_1 location. Thus, it is clear that Met in the P_1 site of ecotin is not crucial for its specificity on target proteases.

DISCUSSION

The specificity of most inhibitors of serine proteases is determined by interaction of the reactive site residue with the specificity pockets of target proteases (6, 7). Therefore, changes in the P_1 residues can lead to dramatic alterations in the specificity of the inhibitors. For example, upon mutation of the P_1 Met of α_1-antitrypsin to Arg, the inhibitor loses its ability to inhibit neutrophil elastase and becomes an effective thrombin inhibitor (15). However, ecotin seems to be markedly different from these inhibitors of serine proteases, because the results obtained in the present studies indicate that the changes in the

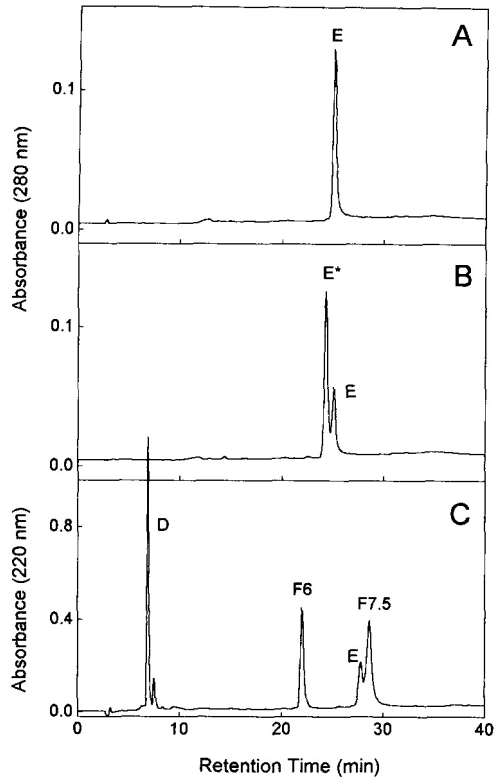


FIG. 5. **Separation of the peptide fragments of ecotin generated by trypsin treatment using HPLC.** Ecotin (20 μg) was incubated as in Fig. 4 but with trypsin (15 μg) that had been covalently conjugated to Sepharose beads. After incubation, the samples were adjusted to 0.1 M HCl, vortexed, and centrifuged. The resulting supernatant was injected directly onto a μBondapak C18 column (Millipore) (*B*) or after incubation with 10 mM DTT for 30 min at 37 °C (*C*). The peptides bound to the column were then eluted using a linear gradient of 20–60% acetonitrile containing 0.05% trifluoroacetic acid. Intact ecotin without treatment with DTT was also chromatographed as above (*A*).

P_1 reactive site do not alter the specificity of ecotin on target proteases. For example, the replacement of Met^{84} even with Glu or Tyr showed little or no effect on the ability of ecotin to inhibit trypsin. Although a marked reduction in the inhibitory activity of ecotin against elastase was observed when the P_1 residue was replaced with Arg, Glu, or Tyr, the protease could still be completely inhibited upon incubation with excess of the mutant ecotins. Thus, the changes in the P_1 residue seem to affect the affinity of ecotin for elastase but not its specificity on the protease. Similarly, the affinity of ecotin for trypsin or chymotrypsin may also be altered upon the replacement of Met^{84} as with Glu or Tyr, but to a small extent, and hence the alterations in the ability of the mutant ecotins to inhibit the enzyme may not have been easily detected under the typical assay conditions used in the present study.

Many inhibitors of serine proteases are often cleaved at the reactive site by their cognate proteases (6, 16). Ecotin has also been shown to be specifically cleaved at the P_1 Met upon incubation with target proteases at pH 3.75 (3). In the present studies, we demonstrated that the mutant ecotin in which Met^{84} was replaced with Glu or Tyr is also cleaved by trypsin at the same P_1 location under the same acidic condition. Therefore, Glu^{84} and Tyr^{84} of the mutant ecotins must somehow interact specifically with the catalytic site of trypsin, although such an interaction is very unusual for trypsin having specificity for basic amino acid residues. Noteworthy, however, is the finding that the sum of the molecular masses of F7.5 and F6 generated from these mutant ecotins and wild-type inhibitor does not exactly match with the size of intact ecotin (16.1 kDa). Moreover, an additional band (marked with *X* in Fig. 4) was evident, particularly in the incubation mixture with Ile^{84} and Tyr^{84} mutant ecotins. These results suggest that additional cleavages may occur in the ecotin molecule. In contrast, when the mutant ecotins were incubated with equimolar amounts of trypsin under the same condition, no other cleavage product except F7.5 and F6 could be seen in the eluates from a C18 column (see Fig. 5). Thus, it is possible that peptide X may be an intermediate cleavage product during generation of F7.5 and F6. However, the significance of cleavage to generate peptide X remains unclear and requires further study, such as determination of the amino acid residues at the N and C termini of F7.5, as well as the C terminus of F6.

For purification of ecotin, we also have used an alternative method involving trypsin-Sepharose affinity chromatography (1). During the study, we noticed that all the mutant forms of ecotin could bind tightly to the affinity column. Furthermore, the inhibitors were also capable of binding to the column that had been treated with diisopropyl fluorophosphate (data not shown). Therefore, it appears likely that the specific interaction between the P_1 reactive site of the inhibitor and the serine active site of the protease is not essential for tight complex formation by ecotin with trypsin and perhaps also with other target proteases.

In a number of respects, ecotin in *E. coli* is very similar to hirudin, a thrombin-specific inhibitor from the leech *Hirudo medicinalis* (17). It has been reported that replacement of the putative P_1 Lys with Glu causes a small increase in the dissociation constant of the hirudin-thrombin complex without any effect on the specificity of hirudin (18), although it is unknown whether the P_1 site in hirudin can be cleaved by thrombin under acidic conditions. It has also been shown that thrombin with its active site blocked with diisopropyl fluorophosphate is still capable of forming a complex with hirudin (19). In addition, a number of other residues aside from the Lys residue in hirudin have been demonstrated to be important for complex formation with thrombin (18–21). Therefore, it appears likely that amino acid residues other than the P_1 Met of ecotin, similar to hirudin, are involved in binding with its target proteases to form tight complexes. Mutational and structural analyses in search of the major interaction sites of ecotin with target proteases are under investigation.

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