# The specificity and kinetic properties of trypsin ethylated at the binding site

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Received 26 November 1984

Treatment of trypsin with triethyloxonium tetrafluoroborate at pH 8, 25 °C, results in abolition of binding to the enzyme of specific cationic substrates and inhibitors. The binding constant of soybean trypsin inhibitor to ethylated trypsin is 10000-fold smaller than to intact trypsin. However, the intrinsic ability of trypsin to recognize and react with nonspecific neutral substrates and inhibitors is not lost, and in several cases even considerably enhanced. Thus ethylated trypsin (Tret) resembles chymotrypsin in its behavior. Trypsin-like enzymes are also affected in a similar manner.

*Trypsin* *Specificity* *Binding site* *Ethylation* *Hydrophobic ligand*

## 1. INTRODUCTION

The pancreatic serine proteases trypsin and chymotrypsin have many common properties: Their molecular masses are similar; they show 40% amino acid sequence homology; their mechanism of action is the same [1,2]. They differ, however, in specificity. Chymotrypsin recognizes hydrophobic ligands and cleaves esters and peptides specifically at the carbonyl of aromatic and hydrophobic amino acids, whereas trypsin cleaves specifically at the carbonyl of the cationic amino acids lysine and arginine. Trypsin also reacts with neutral substrates but at a reduced rate [3–5]. From X-ray studies of chymotrypsin [2] and trypsin [6] and from chemical modifications of trypsin [7,8], the nature of the substrate specificity was elucidated. In chymotrypsin the binding site is a pocket lined with hydrophobic groups, whereas in trypsin the cationic ligands are anchored to the carboxylate of Asp 177 (trypsinogen numbering) in the binding site.

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It was of interest to examine how neutralization of the carboxylate binding site of trypsin would affect the specificity of the enzyme. To this end, Nakayama et al. [9] treated trypsin with triethyloxonium salt at pH 5 and obtained a modified enzyme which lost 80% of its activity towards specific trypsin substrates, but retained most of its activity towards several nonspecific neutral substrates. Recently Grooms and Bender [10] repeated the study and showed that two carboxylic groups per enzyme molecule underwent ethylation by that method.

We decided to study the kinetic properties of ethylated trypsin more thoroughly, and to examine how modification of the binding site affects specificity.

## 2. MATERIALS AND METHODS

Trypsin, chymotrypsin and soybean trypsin inhibitor (STI) were purchased from Worthington and Sigma. Benzoyl arginine ethyl ester (BAEE), acetyl tyrosine ethyl ester (ATEE), poly-L-lysine, clupeine, tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethanesulfonyl fluoride (PMSF), diphenylcarbamoyl chloride (DPCC), carboben-

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/85/$3.30 

zoxyglycine *p*-nitrophenyl ester (ZGly-pNP), carbobenzoxy-L-alanine *p*-nitrophenyl ester (ZAla-pNP), carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (ZTry-pNP) and carbobenzoxy amino acids were from Sigma. Triethyloxonium tetrafluoroborate was an Alfa product. Proflavin hydrochloride and *m*-aminobenzamidine dihydrochloride were from Aldrich. *p*-Nitrophenyl ester of carbobenzoxy-$\alpha$-amino-*n*-butyric acid (ZAbu-pNP) was prepared by the DCC method [11].

### 2.1. *Ethylation of trypsin*

To a pH-stat vessel containing 10 ml trypsin (1 mg/ml) at pH 8 (0.01 M phosphate) were added 50 mg of triethyloxonium fluoroborate in 5 portions. As the latter reagent undergoes rapid hydrolysis in aqueous solutions accompanied by proton release, the pH of the solution was kept constant by automatic addition of 1 M NaOH by the pH-stat. After the reagent had completely reacted (about 1 h), the enzyme solution was dialyzed against 0.1 M phosphate buffer, pH 7.5, for 24 h. The modified enzyme, ethylated trypsin (Tret), was then tested for activity with a variety of substrates and inhibitors.

### 2.2. *Reaction with substrates*

The specific activity of trypsin was determined by reacting it with the chromogenic active site titrant *p*-nitrophenyl *p'*-guanidino benzoate (NPGB) [12], or with BAEE, either spectrophotometrically [13] or pH-statically. The reaction with other trypsin specific substrates, polylysine, lysine methyl ester and clupeine was carried out in the pH-stat.

Ethylated trypsin (Tret) was generally assayed with ZGly-pNP.

The reactivity of trypsin or ethylated trypsin with *p*-nitrophenyl esters (50 $\mu$M) was determined at pH 8 (phosphate buffer, 2% acetonitrile) and followed spectrophotometrically at 410 nm. The initial rates of enzymic hydrolysis were corrected for the spontaneous hydrolysis of the substrates.

### 2.3. *Irreversible inhibition*

To an enzyme solution (1 mg/ml) at pH 8, 0.1 M phosphate buffer, 25°C, was added molar equivalent of DPCC [14] or 3 molar fold of PMSF or 1 mM of TLCK. At timed intervals an aliquot of the solution was assayed for activity. BAEE was used to determine the activity of trypsin and ZAla-pNP was the substrate used to estimate the activity of ethylated trypsin (Tret).

### 2.4. *Inhibition with STI*

To cuvettes containing 2 ml of 4 $\mu$M trypsin or ethylated trypsin (Tret), pH 8, 25°C, were added varied amounts of a solution of STI (10 mg/ml). After an incubation time of 5 min, the substrate solution was added to the cuvette and the residual activity of the enzyme was determined.

### 2.5. *Activation of chymotrypsinogen*

To the zymogen solution (1 mg/ml, 40 $\mu$M), pH 8, 0.1 M Tris buffer, 0.05 M $CaCl_2$ at 25°C, was added trypsin or Tret to a final concentration of 2 $\mu$M enzyme. At different times aliquots were assayed for chymotrypsin activity by following the hydrolysis of ATEE in the pH stat.

### 2.6. *Enzyme stability*

Enzyme solution, 0.1 mg/ml in phosphate buffer, pH 8, was incubated at 25°C for several days, and the residual activity was determined from time to time.

## 3. RESULTS AND DISCUSSION

### 3.1. *Cationic ligands*

The activities of trypsin and Tret towards a variety of natural and synthetic specific trypsin ligands were compared. NPGB, a specific active site titrant of trypsin reacted with Tret to the extent of 0.7–3% that of trypsin. BAEE, poly-L-lysine, clupeine, lysine methyl ester, were not susceptible to hydrolysis by Tret, and reacted with rate which was about 3% that of the intact enzyme. TLCK, a cationic specific trypsin inactivator that alkylates the active site histidine [15], failed to inactivate Tret.

Trypsin is the key enzyme activator of the pancreatic zymogens, converting them to active enzymes by cleaving a crucial peptide bond belonging to specific lysyl or arginyl residues. When Tret was used to activate chymotrypsinogen, practically no activation was observed under conditions which led to full activation by trypsin (fig.1). Trypsinogen was activated by Tret at a very slow rate compared with that obtained by the same concentration of trypsin.

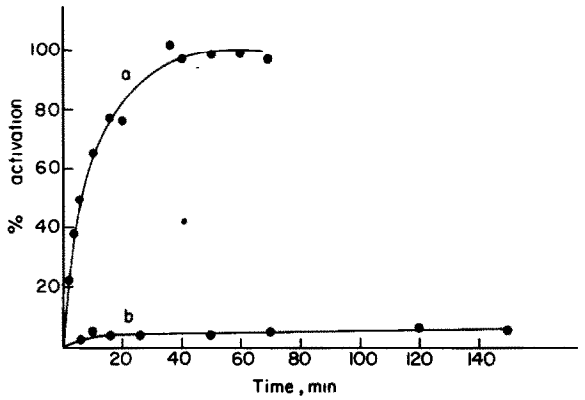


Fig.1. Activation of chymotrypsinogen by (a) trypsin and (b) Tret as a function of time. [Chymotrypsinogen], 40 $\mu$M; [enzyme], 2 $\mu$M, pH 8, 0.1 M Tris in presence of 0.05 M $CaCl_2$, 25°C.

Trypsin is competitively inhibited by cationic ligands like proflavin or *m*-aminobenzamidine. When these were added to a solution containing Tret and good nonspecific substrates, ZGly-pNP or ZAla-pNP, no inhibition of activity was observed, indicating that the modified enzyme is no longer sensitive to inhibition by cationic ligands.

Soybean trypsin inhibitor (STI) is a very potent inhibitor of trypsin, $K_d = 2 \times 10^{-10}$ M at neutral pH [16], which corresponds to standard free energy of binding $\Delta G° = -13$ kcal/mol. Trypsin can be practically titrated with this inhibitor under suitable conditions (fig.2). Fig.2 also shows that

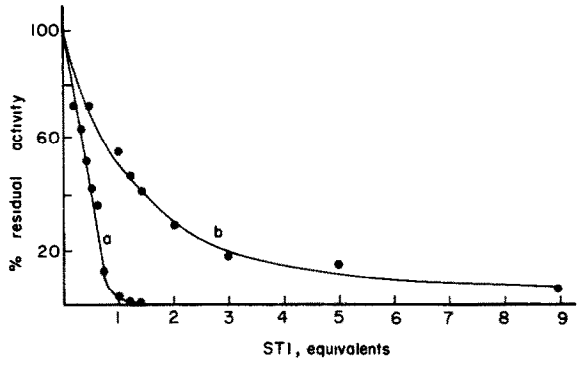


Fig.2. Inhibition of (a) trypsin and (b) Tret as a function of the concentration of soybean trypsin inhibitor. [Enzyme], 4 $\mu$M, pH 8, 25°C.

binding of STI to Tret is much poorer compared with that of intact trypsin, and $K_d$ of Tret-STI can be estimated to be $2 \times 10^{-6}$ M, which corresponds to $\Delta G° = -7.5$ kcal/mol. The difference between the standard free energies of binding of STI to trypsin and to Tret is $\Delta\Delta G° = -5.5$ kcal/mol, and if this value is due only to the effect of the ethylation of the enzyme binding site, it shows that the specificity site carboxylic group, Asp-177, contributes 40% of the free energy of trypsin and STI binding. Similar experiments with other protein inhibitors, lima bean and Bowman-Birk trypsin inhibitors, showed that they bind very weakly to Tret compared with the binding to trypsin.

### 3.2. *Nonspecific neutral ligands*

When a stoichiometric amount of STI was added to trypsin solution, the specific as well as the nonspecific activities were abolished, whereas the activity of chymotrypsin was almost unaffected under similar conditions. This proves that the nonspecific activity of trypsin is an intrinsic property of the enzyme and not due to chymotrypsin contamination.

Trypsin and Tret were reacted with several neutral substrates and inhibitors. Generally, Tret preserved trypsin ability to interact with nonspecific ligands, and ZGly-pNP, ZLeu-pNP and ZMet-pNP reacted with Tret and trypsin at comparable rates. In some cases the modified enzyme showed enhanced hydrolysis rates. Thus, ZAbu-pNP and ZAla-pNP reacted with Tret 3- and 5-fold faster, respectively, than with trypsin. On the other hand, ATEE and ZTyr-pNP reacted with Tret at a reduced rate, 1/10 that of trypsin.

Neutral covalent inhibitors of chymotrypsin were reacted with Tret. It was found that DPCC inactivated Tret about 10-fold faster than trypsin. In the presence of 40 $\mu$M inhibitor, Tret was inactivated with $t_{1/2}$ equal to 4 min, whereas trypsin was inactivated with $t_{1/2}$ approximately 35 min (fig.3). Also, PMSF reacted with Tret considerably faster than with trypsin. All the above findings indicate that ethylation of the negatively charged specificity site alters dramatically the nature of trypsin. The binding site can no longer specifically bind cationic ligands. It becomes hydrophobic in character and it can barely accommodate bulky groups like those of tyrosine derivatives. The augmented hydrophobic character alters Tret

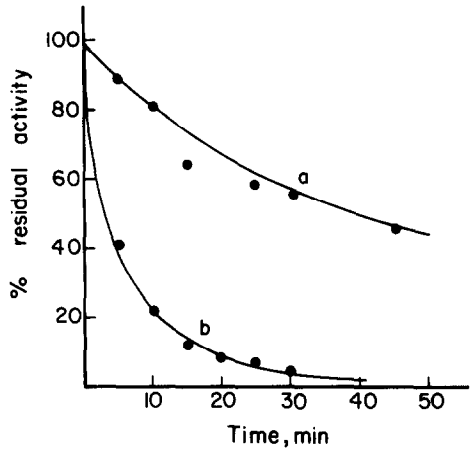


Fig.3. Inactivation of (a) trypsin and (b) Tret by DPCC as a function of time. [E] and [DPCC] 40 $\mu$M, pH 8, 25°C.

specificity, and it reacts more favorably than trypsin with relatively small neutral ligands like derivatives of alanine and $\alpha$-aminobutyric acid.

### 3.3. *Enzyme stabilization*

The loss of the ability to bind cationic ligands results in stabilization of Tret towards autolysis. Thus, when a solution of 0.1 mg/ml trypsin at pH 8 was incubated at 25°C, it lost activity with $t_{1/2}$ equal to 15 h, whereas Tret lost half of its activity in approximately 90 h. At neutral pH trypsin is known to undergo autolysis, but after ethylation the modified enzyme can hardly bind and react with other enzyme molecules, and therefore the rate of autolysis is markedly decreased.

Preliminary results show also that trypsin-like enzymes, thrombin, kallikrein and pronase, lost their specificity towards cationic substrates when treated with oxonium salts, while their ability to hydrolyze nonspecific neutral substrates was not hampered.

## ACKNOWLEDGEMENTS

The research was supported by the Technion VPR Fund–J. and A. Taub Research Fund, and by the Fund for the Promotion of Research at the Technion. We wish to thank the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, USA for providing secretarial service.

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