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# The Mechanism of Trypsin-catalyzed Hydrolyses. The Cinnamoyl-trypsin Intermediate[1-3]

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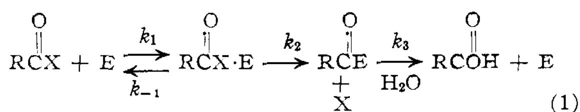
The trypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole proceeds through a preliminary adsorption of the substrate on the enzyme, followed by two catalytic steps: first acylation of the enzyme forming the acyl-enzyme intermediate, *trans*-cinnamoyl-trypsin, and then deacylation of this compound producing cinnamic acid and regenerating the enzyme. The kinetics of the acylation and deacylation steps have been determined, including the effect of *p*H and of urea on the deacylation of *trans*-cinnamoyl-trypsin. The difference spectrum of *trans*-cinnamoyl-trypsin *vs.* trypsin has been determined. The trypsin- and α-chymotrypsin-catalyzed hydrolyses of N-*trans*-cinnamoylimidazole show similarities with respect to the stepwise catalytic sequence, *p*H dependence of the deacylation reaction and similarities in the spectral and kinetic behavior of the acyl-enzyme intermediate. These similarities lead to the conclusion that these two serine proteinases share common active sites from the catalytic point of view and that the mechanisms of their reactions are similar, in particular with respect to acyl-enzyme formation.

## Introduction

The mechanism of α-chymotrypsin-catalyzed reactions proceeds in three steps; a preliminary adsorption of substrate on the enzyme is followed by two catalytic steps in which the substrate is first converted to an acyl-chymotrypsin intermediate followed by hydrolysis of the acyl-chymotrypsin. In previous papers in this series direct spectrophotometric evidence has been presented for the formation and decomposition of the cinnamoyl-α-chymotrypsin intermediate.[2,6] It is the purpose of the present paper to give evidence, primarily kinetic and spectrophotometric, for the formation and reactions of the analogous cinnamoyl-trypsin intermediate formed in a trypsin-catalyzed hydrolysis.

Trypsin and chymotrypsin are proteolytic enzymes of considerable similarity. Their precursors, trypsinogen and chymotrypsinogen are both important constituents of bovine pancreatic juice.[7] Trypsin and chymotrypsin have been classified as serine proteinases; that is, they are proteolytic enzymes which contain a unique serine residue that reacts with organophosphorus compounds leading to an inactive derivative.[8] The molecular weights of these enzymes are approximately equal and both are known to possess one active site per molecule. The active site of both enzymes, as defined by the reactive serine residue, contains additional common components: both enzymes contain the sequence Gly-Asp-Ser-Gly, although the two sequences then diverge.[9,10] Both enzymes catalyze the hydrolysis of esters as well as amides, and in addition probably all other carboxylic acid derivatives. Both enzymes are protein in nature, containing no coenzyme or prosthetic group and therefore the catalytic activity of these enzymes must be associated directly with the constituents of the protein.[11]

There are several indications in the literature that trypsin-catalyzed hydrolyses, like those of chymotrypsin, proceed through an acyl-enzyme intermediate. The trypsin-catalyzed hydrolyses of a series of benzoyl-L-arginine esters proceed with identical maximal velocities.[12] These esters include methyl, ethyl, isopropyl, benzyl and cyclohexyl esters whose alkaline rate constants, reflecting nucleophilic attack, would be expected to differ by at least a factor of ten.[13] The identical enzymatic rates cannot, therefore, be explained on the basis of a single nucleophilic enzymatic catalytic step[14] but can easily be explained by a two-step catalytic process in which each of these esters is converted in a first fast step to the intermediate benzoyl-L-arginyl-trypsin which then decomposes in a second (rate-determining) step, to products. The trypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate has been investigated recently using the stopped-flow technique which allows the observation of both the pre-steady state and steady state portions of the reaction.[15] This reaction can be straightforwardly interpreted in terms of two catalytic steps, an acylation and a deacylation reaction, as the chymotrypsin reaction has been analyzed.[16] These two pieces of experimental evidence, one of which involves a specific substrate, constitute reasonable, although indirect, evidence for the sequence



$$\mathrm{R\overset{O}{\overset{\|}{C}}X} + \mathrm{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{R\overset{O}{\overset{\|}{C}}X{\cdot}E} \xrightarrow{k_2} \mathrm{R\overset{O}{\overset{\|}{C}}E} + \mathrm{X} \xrightarrow[\mathrm{H_2O}]{k_3} \mathrm{R\overset{O}{\overset{\|}{C}}OH} + \mathrm{E} \quad (1)$$

(1) This research was supported by grants from the National Institutes of Health.

(2) Paper XII in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, M. L. Bender and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2550 (1962).

(3) Some of the results of this paper have been presented in preliminary form: M. L. Bender, E. T. Kaiser and B. Zerner, *ibid.*, **83**, 4656 (1961).

(4) Alfred P. Sloan Foundation Research Fellow.

(5) National Institutes of Health Postdoctoral Research Fellow.

(6) M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).

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(11) M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).

(12) G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, **179**, 665 (1949).

(13) M. L. Bender and K. A. Connors, unpublished observations. L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 211.

(14) A study of the effect of structure on reactivity indicates that the acylation of chymotrypsin exhibits the characteristics of a nucleophilic reaction; M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962).

(15) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, **37**, 751 (1959).

(16) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

for catalysis by trypsin, completely analogous to that for chymotrypsin given earlier.

In this paper the spectrophotometric techniques developed for chymotrypsin are applied to the trypsin reaction. Since cinnamoyl-α-chymotrypsin had been characterized earlier,[6] it was decided to prepare the corresponding acyl-enzyme intermediate, cinnamoyl-trypsin and to subject these two compounds to as extensive a comparison as possible. For this purpose the reaction of N-*trans*-cinnamoylimidazo'e with trypsin was investigated.

## Experimental

**Materials.**-N-*trans*-Cinnamoylimidazole was prepared by the reaction of cinnamoyl chloride with imidazole in benzene solution.[17] N-α-Benzoyl-L-arginine ethyl ester hydrochloride was obtained from California Biochemical Research (A grade). Trypsin used in these experiments was a twice-crystallized salt-free preparation from Mann Biochemicals Corp. (Lot No. E.2081). *trans*-Cinnamic acid (Eastman Kodak Co.) was twice recrystallized from ethanol-water (m.p. 134.5-135.5°). Acetonitrile (Eastman Kodak Co.) was purified by double distillation from phosphorus pentoxide (b.p. 80.5°). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was obtained from Matheson, Coleman and Bell (m.p. 170-171°).

The buffer systems used in these experiments were required to have the attributes of reasonable buffering action in the region of *p*H 5 to 9 and negligible precipitation and complexation reactions with calcium ion. To meet these requirements Tris-acetic acid buffers were chosen. One solution consisted of 30 ml. of 0.16 *M* acetic acid, 4.5 ml. of 1.0 *M* Tris, 10 ml. of acetonitrile, 10 ml. of 0.5 *M* calcium chloride and a sufficient volume of doubly distilled water to make up a total volume of 100 ml. This buffer, which was 0.048 *M* in acetic acid, 0.045 *M* in Tris, 0.05 *M* in calcium chloride and 10% in acetonitrile, had a *p*H of 5.35.[18,19] Acetonitrile was omitted in the preparation of the other buffer, and a correspondingly greater amount of water was used. The acetonitrile-free buffer had a *p*H of 5.25.

**Trypsin Solutions.**—In contrast to the preparation of chymotrypsin solutions, the preparation of stable, spectrophotometrically clear, kinetically reproducible solutions of trypsin requires special care and attention. For this reason their preparation is discussed here in detail. Trypsin (0.2195 g.) was dissolved as completely as possible in 25 ml. of the 10% acetonitrile buffer described above. The resulting solution was centrifuged, and the supernatant was allowed to stand overnight in the refrigerator. Some precipitate formed on standing, but ultramicrofiltration produced a stable, spectrophotometrically clear solution of trypsin. A Schleicher and Schuell Co. ultramicrofiltration apparatus equipped with an S and S membrane filter (type A, medium size, 50 mm.) supported by a Teflon disk and No. 604 S and S filter paper was used. Comparison of solutions which were centrifuged but not ultramicrofiltered with these solutions indicates that in general clearer trypsin solutions of higher, more reproducible activity were obtained after ultramicrofiltration. Aliquots from the above stock solution were further diluted with the Tris-acetic acid buffer to give solutions of varying trypsin concentration for kinetic studies.

The concentrations of trypsin in these solutions were determined by the measurement of their ultraviolet absorbances at 280 mμ since there is, at the present time, no titration procedure applicable to trypsin like the one for chymotrypsin.[17] The molarities of the trypsin solutions were calculated from eq. 2

$$\text{trypsin molarity} = \frac{\text{absorbance at 280 m}\mu \times 0.694}{24{,}000} \quad (2)$$

(17) G. R. Schonbaum, B. Zerner and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(18) All *p*H measurements were determined on a Radiometer model 4C *p*H meter. The *p*H meter was standardized against *p*H 4.01 potassium hydrogen phthalate solution or against *p*H 9.18 borax solution.

(19) For an example of the use of a Tris-acetic acid buffer system, see D. K. Myers and E. C. Slater, *Biochem. J.*, **67**, 558 (1957).

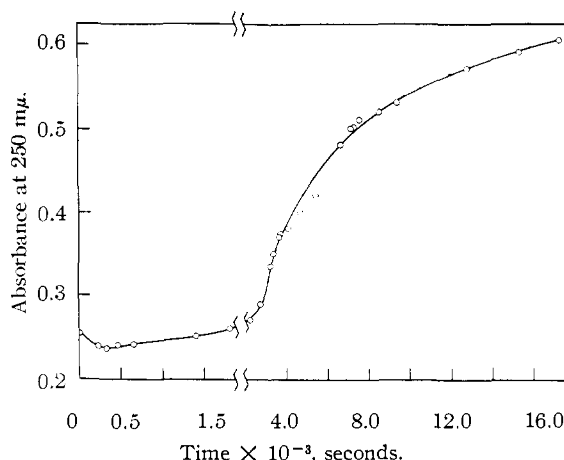


Fig. 1.—The reaction of N-*trans*-cinnamoylimidazole with trypsin at 25.0° in 10% acetonitrile-water at *p*H 5.2. $[E_0] = 1.23 \times 10^{-4}$ *M*, $[S_0] = 8.85 \times 10^{-6}$ *M*.

where 0.694 is the optical factor for conversion from aborbance to mg./ml.[20] and 24,000 is the molecular weight of trypsin.[21] Determination of the molarity of the stock solution whose preparation is described above by the absorbance method indicated that only about 60% of the trypsin originally weighed out dissolved in the solution. As has been noted before, the presence of calcium ion is required to maintain the activity of trypsin solutions.[22]

Trypsin activities were determined spectrophotometrically by following the rate of hydrolysis of N-α-benzoyl-L-arginine ethyl ester.[23] The activity has been defined as

$$\text{activity} = \frac{\text{absorbance change}}{\text{time in sec.} \times \text{mg. of trypsin/ml.}} \quad (3)$$

The average activity of four separate solutions reported in Table I was 0.87 ± 0.05 absorbance unit/sec./mg. of trypsin/ml. (*p*H 8.0, 25°, 0.05 *M* Tris buffer). This agreement indicates the excellent reproducibility of the trypsin solutions used. In a recent report on the chromatographic purification of trypsin, the maximum activity of trypsin preparations at 37° was 0.445 absorbance units/sec./mg. of trypsin/ml.[24] This value measured at 37° is considerably less than that of the present solutions measured at 25°. One reason for this discrepancy might be the fact that the sources of the trypsin used were different. It should be noted that trypsin preparations which were ultramicrofiltered exhibited greater activity, and solutions which were only centrifuged had activities similar to those reported by Liener.[24] An obvious need in this area is a titration procedure for the active sites of trypsin, as has been developed for chymotrypsin.[17]

**Kinetics.**—The kinetics of the acylation of trypsin by N-*trans*-cinnamoylimidazole were followed at 335 mμ using a Cary model 14 recording spectrophotometer equipped with 0.0-0.1 and 0.0-1.0 slide wires and a thermostated cell compartment at 25.0 ± 0.1°. In a typical experiment 3.0 ml. of the enzyme solution was equilibrated in the cell compartment for 15-20 minutes. The reaction was initiated by the introduction of 25 μl. of a solution of N-*trans*-cinnamoylimidazole in acetonitrile with stirring. The *p*H of each reaction was measured at the conclusion of the experiment. A typical kinetic plot is shown in Fig. 2.

The kinetics of the formation of cinnamate ion from *trans*-cinnamoyl-trypsin, that is, the deacylation of *trans*-cinnamoyltrypsin, was measured spectrophotometrically at

(20) E. W. Davie and H. Neurath, *J. Biol. Chem.*, **212**, 507 (1955).

(21) L. W. Cunningham, Jr., F. Tietze, N. M. Green and H. Neurath, *Disc. Faraday Soc.*, **13**, 58 (1953); C. M. Kay, L. B. Smillie and F. A. Hilderman, *J. Biol. Chem.*, **236**, 118 (1961).

(22) N. M. Green and H. Neurath, *ibid.*, **204**, 379 (1953).

(23) G. W. Schwert and Y. Takenaka, *Biochim. et Biophys. Acta*, **16**, 570 (1955).

(24) I. E. Liener, *Arch. Biochem. Biophys.*, **88**, 216 (1960). The maximum activity obtained here is also higher than that reported by R. D. Cole and J. M. Kinkade, Jr., *J. Biol. Chem.*, **236**, 2443 (1961).

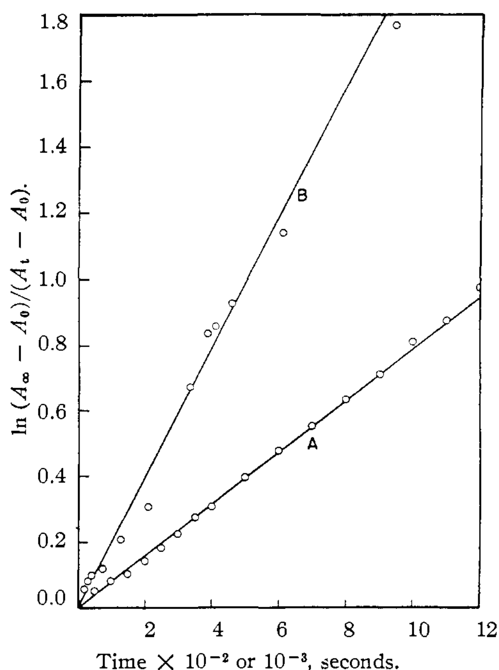


Fig. 2.—First-order plots of acylation and deacylation reactions of N-*trans*-cinnamoylimidazole with trypsin; *p*H 5.2, 10% acetonitrile-water, 25.0°: A, acylation, $[E_0] = 2.54 \times 10^{-5}$ *M*, $[S]_0 = 4.5 \times 10^{-6}$ *M*, time scale in hundreds of seconds; B, deacylation, $[E_0] = 1.23 \times 10^{-4}$ *M*, $[S]_0 = 4.5 \times 10^{-6}$ *M*, time scale in thousands of seconds.

250 mμ, since at this wave length a minimum in the enzyme absorption spectrum occurs. In a typical experiment 3.0 ml. of enzyme solution was placed in both the sample and reference cells so that the high absorbance (between 2 and 3 absorbance units) of the enzyme solution would be blanked out. The run was initiated as before by the addition of a microliter quantity of N-*trans*-cinnamoylimidazole in acetonitrile solution to the trypsin solution in the sample cell. At 250 mμ there occurs initially a slow, slight decrease in absorbance due to the acylation reaction, followed by a gradual increase in absorbance which obeys first-order kinetics precisely after several thousand seconds; this is the deacylation reaction. A typical curve is given in Fig. 1, similar in form to many α-chymotrypsin-catalyzed hydrolyses of cinnamate substrates. A typical kinetic plot is shown in Fig. 2.

**Measurement of the Difference Spectrum of *trans*-Cinnamoyl-trypsin.**—Enzyme solutions were introduced in both the sample and reference cells, care being exercised to balance the two solutions. Acetonitrile (25 μl.) was introduced into the reference cell and an acetonitrile solution of N-*trans*-cinnamoylimidazole (25 μl.) was added to the sample cell, initiating the reaction. The absorption spectrum was recorded from 340 to 240 mμ at least every 200 seconds. The spectrum was recorded at the rate of 1 mμ per second.

## Results

**Acylation of Trypsin with N-*trans*-Cinnamoylimidazole.**—Although the reaction of N-*trans*-cinnamoylimidazole with α-chymotrypsin is a facile reaction, its reaction with trypsin is slow indeed. For this reason it was decided to study the acylation of trypsin with this substrate under conditions in which the enzyme concentration considerably exceeds that of the substrate, leading to pseudo first-order kinetics. This procedure is the same as that used in the reaction of methyl cinnamate with chymotrypsin; justification is given in that paper[2] for the validity of these kinetics. Table I summarizes a number of acylation reactions of trypsin carried out at *p*H 5.2, in which the enzyme concentration is varied from $2.54 \times 10^{-5}$ *M* ([enzyme]/[substrate] = 5.8) to $2.03 \times 10^{-4}$ *M* ([enzyme]/[substrate] = 46.2). *p*H 5.2 was selected for most reactions because at lower *p*H's the spontaneous hydrolysis becomes important while at higher *p*H's deacylation becomes important.

TABLE I

THE ACYLATION OF TRYPSIN WITH N-*trans*-CINNAMOYLIMIDAZOLE

| $[E]_0 \times 10^5$,[e] *M* | $[E]_0/[S]_0$[e] | $k_{obs} \times 10^3$, sec.$^{-1}$ | $k_{acylation}$,[e] $M^{-1}$ sec.$^{-1}$ |
|---|---|---|---|
| 2.54[a] | 5.8 | 0.75[d] | 29.6 |
| 5.00[a] | 11.4 | 1.60[d] | 32.3 |
| 9.88[a] | 22.5 | 3.20[d] | 32.4 |
| 10.0[a] | 22.7 | 3.50[d] | 35.0 |
| 12.7[a] | 28.9 | 4.10[d] | 32.3 |
| 15.0[a] | 34.0 | 5.35[d] | 35.7 |
| 20.3[a] | 46.2 | 6.85[d] | 33.7 |
| | | | 33.0[f] |
| 10.4[b] | 23.7 | 6.80 | 65.3 |
| 10.4[b] | 23.7 | 6.40 | 61.6 |
| | | | 63.5[f] |

[a] 25.0°, *p*H 5.19–5.29, Tris-acetic acid buffer, 10% acetonitrile–water (see Experimental section). [b] 25.0°, *p*H 5.23, Tris-acetic acid buffer, 1.6% acetonitrile–water. [c] N-*trans*-Cinnamoylimidazole = $4.4 \times 10^{-6}$ *M* for all runs. [d] These values have been corrected for the spontaneous hydrolysis of the substrate under these conditions ($k = 4.7 \times 10^{-5}$ sec.$^{-1}$). The correction is less than 1% at the highest trypsin concentration, but about 6% at the lowest. [e] The trypsin concentrations and therefore the second-order acylation constants which are derived from them are internally consistent but depend on the use of an optical factor for determination of concentration[20] which is not wholly satisfactory on an absolute basis. [f] Average.

TABLE II

DEACYLATION OF *trans*-CINNAMOYL-TRYPSIN

| $[E]_0 \times 10^5$, *M* | $[S]_0 \times 10^6$, *M* | *p*H[d] | $k_3' \times 10^4$, sec.$^{-1}$ |
|---|---|---|---|
| 6.45 | 7.91 | 5.27 | 1.96[a] |
| 12.3 | 8.85 | 5.21 | 1.96[a] |
| 16.2 | 15.8 | 5.21 | 1.68[a] |
| 7.5 | 17.1 | 5.21 | 1.74[b] |
| 15.2 | 8.61 | 5.15 | 1.64[b] |
| 11.5 | 8.68 | 5.43 | 1.89[c] |
| 2.9 | 8.68 | 6.38 | 12.8[c] |
| 20.0 | 8.68 | 6.68 | 18.3[c] |
| 11.4 | 8.68 | 7.10 | 51.0[c] |
| 12.4 | 8.68 | 7.48 | 68.0[c] |
| 11.2 | 8.68 | 7.69 | 117[c] |
| 9.67 | 8.68 | 8.62 | 134[c] |
| 8.65 | 8.68 | 9.18 | 131[c,e] |
| 8.65 | 8.68 | 9.18 | 153[c] |

[a] 10% acetonitrile-water. [b] 3.2% acetonitrile-water. [c] 1.6% acetonitrile-water. [d] At *p*H's higher than 5.2, the acylation reaction was first carried out at *p*H 5.2 and then Tris buffer was added to the cuvette in sufficient amount to bring the reaction solution to the desired *p*H value. [e] At *p*H 9.18 a definite end-point in the absorbance change at 250 mμ was not reached, and a continual upward drift was observed. The cause of this drift is not known, but it could result from gradual changes in the enzyme since trypsin may not be stable in the basic solution even in the presence of calcium ion. In order to minimize the error due to the side reaction, the rate constant for deacylation was calculated by the Guggenheim method.

At all trypsin concentrations of a given activity, the reactions follow pseudo-first-order kinetics pre-

cisely. Since this kinetic behavior requires the presence of a constant excess amount of enzyme, the kinetic result indicates that the reaction being observed is one involving trypsin, and not one involving the small amount of chymotrypsin that may be present in the trypsin preparation.[26] It is evident from Table I that the reaction is first order in trypsin.

**Deacylation of *trans*-Cinnamoyl-trypsin.**—The deacylation of *trans*-cinnamoyl-trypsin, like that of *trans*-cinnamoyl-α-chymotrypsin, is a first-order kinetic process. Since the deacylation is in general slower than the acylation reaction, after sufficiently long times it is possible to determine its rate constant directly. Table II summarizes the rate data for the deacylation of *trans*-cinnamoyl-trypsin at a number of *p*H's. A graph of the variation of the deacylation rate constants with *p*H is shown in Fig. 3.

At pH 5.2, where most of the acylation kinetics were carried out, the rate constant for the acylation reaction was always larger than that for the deacylation reaction. For example, in 10% acetonitrile–water at the lowest concentration of trypsin used for acylation, the ratio $k_a/k_d$ is about 4 while it is nearly 37 at the highest trypsin concentration. In 1.6% acetonitrile–water the ratio $k_a/k_d$ has even higher values at corresponding trypsin concentrations since acylation (but not deacylation) is more rapid in the more aqueous solvent. These relative rate constants indicate that the deacylation reaction does not complicate the acylation kinetics by regeneration of extra enzyme. This of course is not a great factor here since large enzyme/substrate ratios were used.

**Hydrolysis of *trans*-Cinnamoyl-trypsin in 7.74 *M* Urea.**—The alkaline rate of deacylation of *trans*-cinnamoyl-trypsin in 7.74 *M* urea was followed spectrophotometrically in the same manner as the corresponding reaction for *trans*-cinnamoyl-α-chymotrypsin.[6] Since the preparation of *trans*-cinnamoyl-trypsin from *trans*-cinnamoylimidazo'e at *p*H 5 does not lead to a quantitative yield of product (*vide infra*) some N-*trans*-cinnamoylimidazole will be present. For this reason some samples were prepared with an initial excess of substrate to show that the presence of the starting material was not affecting the observed kinetics. At the high *p*H values at which these runs were carried out (*p*H 12.8–13.5), any starting material is rapidly consumed and cannot account for the slow, observed reaction. Reproducibility of the data was checked at 5 *p*H values, with 5 acyl-enzyme preparations, and with freshly prepared buffers on three different occasions (13 runs).

**The Difference Spectrum of *trans*-Cinnamoyl-trypsin *vs.* Trypsin.**—Difference spectra of *trans*-cinnamoyl-trypsin *vs.* trypsin were obtained under conditions in which the [enzyme]/[substrate] ratio was much greater than 1 (see Table I). Under these conditions the acylation reaction follows pseudo-first-order kinetics, and the acylation and deacylation reactions can then be considered to be two consecutive first-order reactions. In all cases the acylation rate constant was considerably greater than the deacylation rate constant, so that

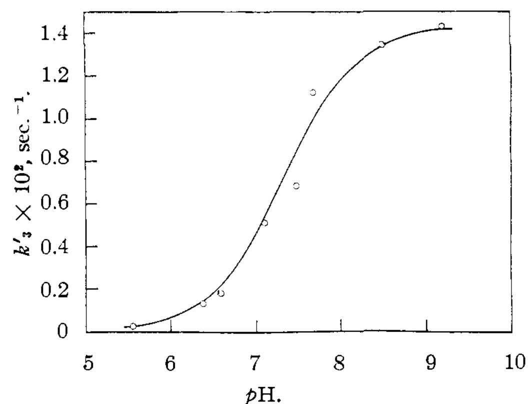


Fig. 3.—The deacylation of *trans*-cinnamoyl-trypsin at 25.0° in 1.6% acetonitrile–water.

the concentration of the unstable intermediate *trans*-cinnamoyl-trypsin formed transiently was as high as 90% of the initial substrate concentration. At the time of the maximal amount of acyl-enzyme it was possible to calculate from the known rate constants not only the concentration of the intermediate but also the concentrations of the reactant and product. The absorbance values of the *trans*-cinnamoyl-trypsin difference spectrum were of course corrected for the small absorbances due to the reactant and product. A summary of the difference spectra of *trans*-cinnamoyl-trypsin obtained in two solvents is given in Table III.

TABLE III

THE DIFFERENCE SPECTRUM OF *trans*-CINNAMOYL-TRYPSIN *vs.* TRYPSIN[c]

| Wave length mμ | Molar Absorptivities[c] 1.6% acetonitrile–water[a] | 10% acetonitrile–water[b] |
|---|---|---|
| 250 | 3,100 | 1,615 |
| 260 | 5,340 | 4,290 |
| 270 | 9,430 | 9,165 |
| 280 | 14,350 | 14,000 |
| 290 | 18,200 | 18,000 |
| 300 | 19,130 | 19,250 |
| 310 | 15,020 | 15,600 |
| 320 | 8,540 | 8,350 |
| 330 | 2,980 | 2,825 |

[a] These are average values of two runs. In the first run the concentrations of acyl-enzyme, reactant and product were 90.0, 2.3 and 7.7% of the initial substrate concentration, respectively. In the second run the concentrations were 77.7, 20.6 and 1.7%, respectively. The agreement at all wave lengths but 280 and 320 mμ was better than 2%. [b] These are average values of two runs. In the first run the concentrations of acyl-enzyme, reactant and product were 84.0, 4.8 and 11.2% of the initial substrate concentration, respectively. In the second run the concentrations were 79, 7 and 14%, respectively. The agreement at all wave lengths but 250 and 330 mμ was better than 5%. [c] The absolute value of the molar absorptivity is based on N-*trans*-cinnamoylimidazole as the primary standard. The concentration of *N*-trans-cinnamoylimidazole was 4.38 $\times 10^{-6}M$. The concentration of trypsin varied from 5.5 $\times 10^{-5}M$ to 1.04 $\times 10^{-4}M$ so that the background absorbance due to trypsin at 280 mμ varied from 1.9 to 3.6 absorbance units.

The difference spectrum of *trans*-cinnamoyl-trypsin *vs.* trypsin is reasonably reproducible, even though the transient nature of this intermediate does not present optimum conditions for the observation of a spectrum. The agreement among the

various spectra is quite good, particularly at the maxima. It should be noted that no major effect on the absorption maximum of the acyl-enzyme due to variation in the solvent was observed.

## Discussion

As pointed out in the Introduction a number of criteria exist for concluding that trypsin and $\alpha$-chymotrypsin are similar to one another. On the other hand, the view may be taken that trypsin requires the specificity provided by lysine and arginine derivatives whereas $\alpha$-chymotrypsin is specific for tyrosine, phenylalanine and tryptophan derivatives.[25] However, the specificities of the two enzymes may not be widely different, on the basis of a study of the relative rates of reaction of trypsin and $\alpha$-chymotrypsin with N-$\alpha$-benzoyl-L-arginine ethyl ester, a specific substrate for trypsin, and acetyl-L-tryrosine ethyl ester, a specific substrate for $\alpha$-chymotrypsin. The catalytic rate constant of the trypsin-catalyzed hydrolysis of the latter compound, the non-specific substrate, is 13% of that of the former compound, the specific substrate. Furthermore the catalytic rate constant of the $\alpha$-chymotrypsin-catalyzed hydrolysis of the former compound, the non-specific substrate, is 20% of that of the latter compound, the specific substrate.[26] Apparently the specificities of the two enzymes do not differ from one another very markedly.

It is the thesis of this paper that even though the specificities of these enzymes might differ from one another, the mechanisms of their catalytic action are the same. Mechanism, of course, is the subject of this series of papers and the mechanistic similarity of trypsin and $\alpha$-chymotrypsin is the important point of this paper.

As pointed out in the Introduction, trypsin and chymotrypsin are similar with respect to biological origin, molecular weight, types of substrates on which they act, the presence of a single active site per molecule, the presence of a DFP-inhibitible serine hydroxyl group in this active site, and a portion of the peptide sequence surrounding this active site. To these similarities can be added similarities with respect to the stepwise catalytic sequence, $p$H dependence of the catalytic action and, finally, similarities in the spectral and kinetic behavior of the acyl-enzyme.

The results presented in this paper for the trypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole give direct experimental confirmation of the two-step catalytic mechanism of eq. 1, supporting the earlier work of Schwert and Eisenberg[12] and of Stewart and Ouellet.[15] The acylation of trypsin by N-*trans*-cinnamoylimidazole is considerably slower than that of $\alpha$-chymotrypsin. Unfortunately, the kinetic data available, shown in Table IV, involve only second-order acylation rate constants which include both the equilibrium constant[27] of the adsorptive step and the first-order rate constant of acylation itself. It may be surmised, although it is not proved, that the difference in the second-order acylation constants is due to a difference in the adsorptive equilibrium constants and not a difference in the rate constants of acylation themselves. That this may be so may be seen in a comparison of the first-order deacylation rate constants for *trans*-cinnamoyl-$\alpha$-chymotrypsin and -trypsin. The rate constants for the deacylation of these two compounds, as seen in Table IV, are practically identical. Furthermore the effects of $p$H on the deacylation rate constants are also similar (Table IV). Previously it had been shown that the acylation rate constants of the trypsin and $\alpha$-chymotrypsin-catalyzed hydrolyses of $p$-nitrophenyl acetate (as well as the deacylation rate constants) were remarkably similar to one another differing by only a factor or two.[15] Furthermore the $p$H dependence of the deacylation of acetyl-trypsin and acetyl-$\alpha$-chymotrypsin were reported to be similar, depending on groups with apparent $pK_a$'s of 7.0 and 7.3, respectively.[15,28] The kinetic and $p$H similarities found in the present and previous investigations must mean that the differences between trypsin and $\alpha$-chymotrypsin in specificity, which are evident in the binding and rate constants of specific substrates (and which are evident in the present second-order acylation rate constants) disappear when one observes the individual catalytic steps with relatively non-specific substrates. This result implies that the catalytic entities operating in the deacylation must be identical for the two enzymes.

TABLE IV

A COMPARISON OF THE ACYLATION AND DEACYLATION REACTIONS OF TRYPSIN AND $\alpha$-CHYMOTRYPSIN USING N-*trans*-CINNAMOYLIMIDAZOLE AS SUBSTRATE

| Parameter | Trypsin | $\alpha$-Chymotrypsin[b] |
|---|---|---|
| $k_{acylation}$, $p$H 5.2, $M^{-1}$ sec.$^{-1}$ | 33,[a,d] 63.4[c,d] | $2.9 \times 10^3$ [a]<br>$12 \times 10^3$ |
| $k_{deacylation}$, $p$H 5.2, sec.$^{-1}$ | $1.87 \times 10^{-4}$ [a]<br>$1.69 \times 10^{-4}$ [c] | $1.4 \times 10^{-4}$ |
| $\lambda_{max}$ of cinnamoyl-enzyme, m$\mu$ | 296[c] | 292 |
| $\epsilon_{max}$ of cinnamoyl-enzyme | 19,300[c] | 17,700 |
| $k_{OH}$ of cinnamoyl-enzyme in 7.74 $M$ urea, $M^{-1}$sec.$^{-1}$ | $4.5 \pm 0.5 \times 10^{-2}$ | $4.1 \pm 0.3 \times 10^{-2}$ |
| $pK_a$ of deacylation of cinnamoyl-enzyme | 7.3[c] | 7.15 |

[a] 10% acetonitrile–water; Tris–acetic acid buffer. [b] 1.6% acetonitrile–water; acetate buffer. [c] 1.6% acetonitrile–water; Tris–acetic acid buffer. [d] Assuming the molecular weight of trypsin is 24,000 and using protein absorbance as a measure of concentration.

The $p$H dependence found in the deacylation of *trans*-cinnamoyl-trypsin may be compared with $p$H dependencies found for the over-all catalytic constants of specific substrates. In the trypsin-catalyzed hydrolysis of N-$\alpha$-benzoyl-L-arginine ethyl ester it was found that the catalytic activity may be represented by dependence on a group with an apparent $pK_a$ of 6.25[30] or 6.02[31] in water at 25°.

(25) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(26) T. Inagami and J. M. Sturtevant, *J. Biol. Chem.*, **235**, 1019 (1960).

(27) It is assumed that the adsorption is a pre-equilibrium, although this point has been proved by no means. The assumption rests on analogy with other reactions of trypsin.

(28) A discrepancy exists between the $p$H dependence of acylation by trypsin[15] and by $\alpha$-chymotrypsin[29] as determined by stopped-flow techniques. In the former case it is found that $K_m$ is $p$H independent while in the latter case $k_{acylation}$ ($k_2$) is $p$H dependent. No apparent explanation exists at present to reconcile these data.

(29) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci.*, **42**, 719 (1956).

The differences presumably were due to difference in ionic strength of the media used in the two investigations. While the apparent $pK_a$ found in the present work does not agree quantitatively with these results, the general findings are similar and indicate that the process being observed here is related to those observed with specific substrates.[32]

Two further indications of the similarity of *trans*-cinnamoyl-trypsin and *trans*-cinnamoyl-$\alpha$-chymotrypsin are seen in a comparison of the spectra of these two compounds given in Fig. 4 and summarized in Table IV, and a comparison of the alkaline hydrolytic rate constants of *trans*-cinnamoyl-trypsin and *trans*-cinnamoyl-$\alpha$-chymotrypsin in 7.74 *M* urea (Table IV). Like *trans*-cinnamoyl-$\alpha$-chymotrypsin, the spectrum of *trans*-cinnamoyl-trypsin cannot be analyzed from a structural point of view because of our ignorance of the effect of the enzyme environment on the spectrum. However, it appears superficially that the two intermediate compounds are quite similar.[33] Likewise when the two intermediate compounds are converted to ordinary esters by the denaturing solvent 7.74 *M* urea, they act kinetically similar to one another and to the model compound O-cinnamoyl-N-acetylserinamide. Thus it may be said that *trans*-cinnamoyl-trypsin, like *trans*-cinnamoyl-$\alpha$-chymotrypsin, is an ester of a serine moiety of the enzyme.[6]

(30) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955).

(31) T. Inagami and J. M. Sturtevant, *Biochim. et Biophys. Acta* **38**, 64 (1960).

(32) The reaction of $p$-nitrophenyl acetate with trypsin has been found to depend on groups with $pK$a's of 7.00, 6.02 and 6.26 in phosphate, acetate or cacodylate buffers, respectively. Variation with buffer may explain part of these differences; G. H. Dixon and H. Neurath, *Federation Proc.*, **16**, 173 (1957).

(33) The maxima of the difference spectra of *trans*-cinnamoyl-$\alpha$-chymotrypsin and *trans*-cinnamoyl-trypsin differ by approximately 4 m$\mu$. This is a significant difference, but is considerably smaller than the shift between O-*trans*-cinnamoyl-N-acetylserinamide and *trans*-cinnamoyl-$\alpha$-chymotrypsin caused by the latter's enzymatic environment. The spectral difference observed between the $\alpha$-chymotrypsin and trypsin derivatives indicates that there is a small difference in the environments of the two cinnamoyl groups.

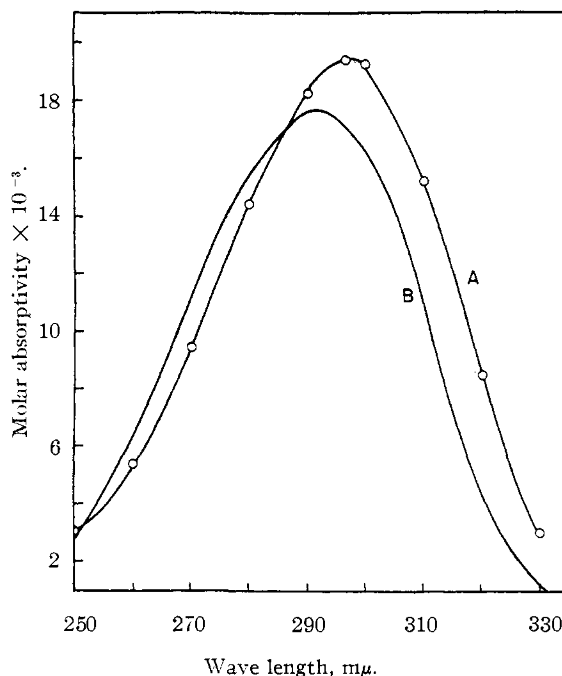


Fig. 4.—Difference spectra of *trans*-cinnamoyl-trypsin (A), and *trans*-cinnamoyl-$\alpha$-chymotrypsin (B); see text for details.

In conclusion it appears that in all important mechanistic aspects catalyses by trypsin and by $\alpha$-chymotrypsin are similar. The formation of an acyl-enzyme intermediate has now been demonstrated for two related serine proteinases[8]; it is not unreasonable to extrapolate this mechanism to all enzymes of this family.

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