AN ARTIFICIAL ACYL-ENZYME AND AN ARTIFICIAL ENZYME

M. L. Bender
Departments of Chemistry and Biochemistry
Northwestern University
Evanston, IL 60201 USA

ABSTRACT. The synthesis and characterization of an artificial acyl-enzyme intermediate of chymotrypsin, and an artificial enzyme, chymotrypsin, are described. They both contain the same three catalytic groups, an imidazolyl group, a hydroxyl group, and a carboxylate ion. In the acyl-enzyme, the three groups are attached to a norbornane backbone, but in the artificial enzyme, the three catalytic groups are attached to a cycloamylose as binder. The artificial acyl-enzyme shows a rate of hydrolysis 154,000 times faster than an ordinary ester and only 18-fold slower than the real acyl-chymotrypsin. The artificial chymotrypsin is over a thousand fold slower than real chymotrypsin, presumably because of impurities in the preparation.

Chymotrypsin is a complicated enzyme. It has a molecular weight of 24,800 and consists of 245 amino acids. But it is the archetype of 20 hydrolases and is thus an important enzyme to mimic. There has been an enormous amount of research on the mechanism of action of chymotrypsin in the past[1,2] and thus a model of chymotrypsin can be soundly based. There are 3 groups known to be in the active site of chymotrypsin. These three groups and their corresponding three amino acids are shown in Table I. Identification of the first two groups has been done by

TABLE I. The Three Catalytic Groups of Chymotrypsin.

1.	The hydroxyl group of serine 195
2.	The imidazole group of histidine 57
3.	The carboxylate ion of aspartate 102

inhibition. The identification of the third group has been accomplished by a reaction of glycine ethyl ester with a carbodiimide.[1]

The most important fact about the mechanism of chymotrypsin action is that it proceeds in two kinetic steps after the initial binding, an acylation and a deacylation, with the intermediacy of a covalent acyl-

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enzyme intermediate.[2] This overall mechanism of action is shown in Figure 1: as it is also shown that the acylation may be mimicked by an

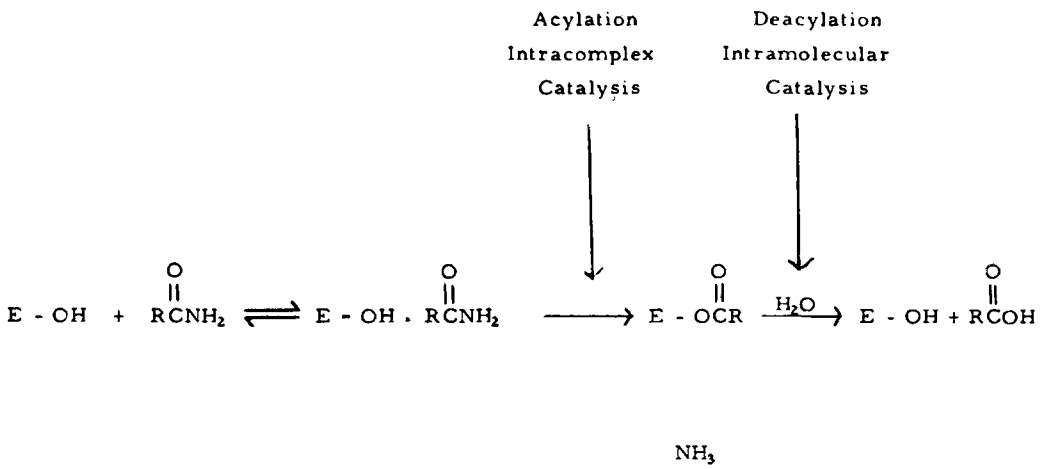


Figure 1. The overall mechanism of chymotrypsin, showing acylation, deacylation, and the acyl-enzyme intermediate.

intra-complex catalysis while the deacylation reaction may be mimicked by an intramolecular catalysis. The first evidence we had for this two step kinetic process, with the intermediacy of the acyl-enzyme, came from a spectrophotometric investigation, but the conclusive proof of the acyl-enzyme intermediate came a decade later when we carried out a stopped-flow mixing experiment involving an optimal substrate and an optimal pH (Figure 2).[3] Since a good (fast) substrate and a good (fast) pH and a bad (slow) substrate and a bad (slow) pH led to identical results of an acyl-enzyme intermediate, it has previously been postulated that every reaction catalyzed by chymotrypsin proceeds via an acyl-enzyme intermediate.[2] Therefore it is reasonable for us to attempt to model the acyl-enzyme before modeling the enzyme.

In our initial model we wanted to have a very rigid backbone where we could vary the stereochemistry with ease.[3] We therefore chose the norbornane bicyclic system on which to hang our groups. As shown in

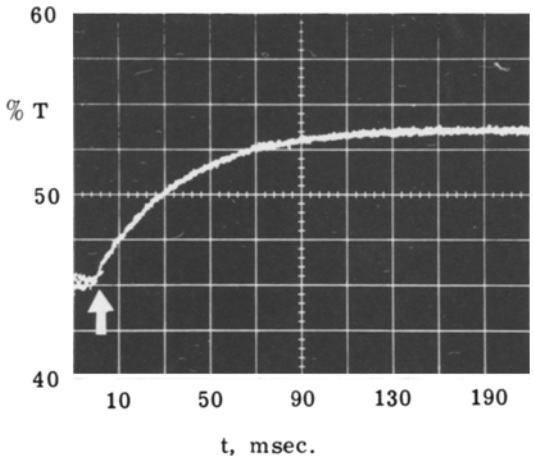
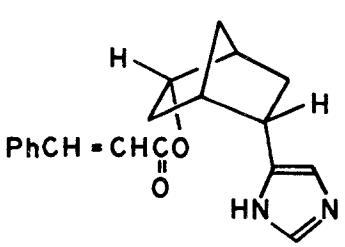


Fig. 2. Deacylation of N-(2-furyl)acryloyl-L-tryptophanyl-α-chymotrypsin. Storage oscilloscope trace. Arrow indicates where flow stops. Three consecutive runs are superimposed. After mixing: $[E_o]$ = 5.25 x 10^{-5} M, $[S_o]$ = 5.00 x 10^{-5} M, 0.05 M, Tris pH 7.90, μ = 0.15, 0.5% (v/v) acetonitrile, 25.0°, d = 2 cm.



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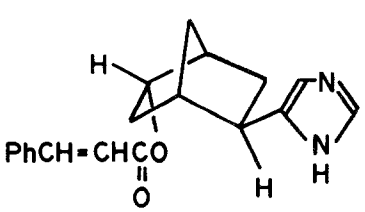

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structures 1 and 2, two structures, one with exo stereochemistry and the other with endo stereochemistry immediately reared their heads. Also seen in both 1 and 2 the ester group to be hydrolyzed is not directly across the ring from the imidazolyl group, the catalyst. If the imidazolyl group is at position 2 and the ester group is at position 6, the ester hydrolysis does not work (imidazole is not involved in the ester hydrolysis), but if the imidazolyl group is at position 2 and the ester group is at position 5, then the ester hydrolysis does work, presumably because of the positioning of a water molecule between the imidazolyl group and the ester group. This is further confirmed by the fact that both the imidazolyl and ester groups must be endo as shown in Figure 3.[4]

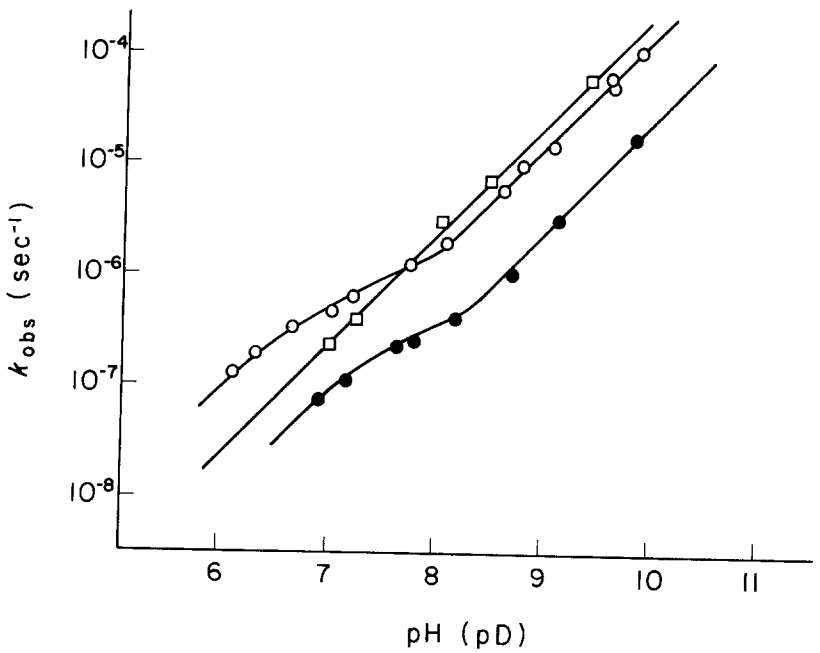


Figure 3. pH-Rate constant profiles for the hydrolyses of bicyclo[2.2.1]heptane derivatives at 60° with I = 0.1 M (KCl). (○) (endo-imidazolyl group, endo-trans-cinnamoyl group) in H_2O; (●) 1 in D_2O; (□) (exo-imidazolyl group, endo-trans-cinnamoyl group) in H_2O.

The exo-endo compound shows no involvement of the imidazolyl group, but the endo-endo compound shows involvement of the imidazolyl group in the ester hydrolysis by the fact that the endo-ester hydrolysis kinetic curve crosses over the straight line brought about by hydroxide ion at the pK of imidazole, 7. Also seen in this Figure are reactions carried out in D_2O (black dots) which indicate the presence of a water molecule and consequent proton transfers, which shows that imidazole acts as a general base, not as a nucleophile (k_{H_2O}/k_{D_2O} = 3.0). This is the first intramolecular system in which imidazole does not act as a nucleophile and is presumably due to the fact that the imidazole is attached at a distance from the ester group which is too far for direct nucleophilic attack on the ester but which is close enough for attack through a water molecule.

These results were mechanistically interesting since the k_{H_2O}/k_{D_2O} of cinnamoyl-chymotrypsin deacylation = 2.5.[6] It was so mechanistically interesting that we decided to compare rates. To our horror we found that the enzyme deacylation was still about a thousand-fold faster than our model. In analyzing our data there was obviously one group missing, a carboxylate ion. To correct this discrepancy, we added benzoate ion, 3 (the third component of the imagined active site), and produced a twenty-five hundred fold increase in the hydrolytic rate at a concentration of 0.5 M in 0.42 mole fraction dioxane-water as solvent, used to simulate the apolar nature of the chymotrypsin active site (Figure 4).[6] This was satisfying from both a mechanistic and kinetic viewpoint

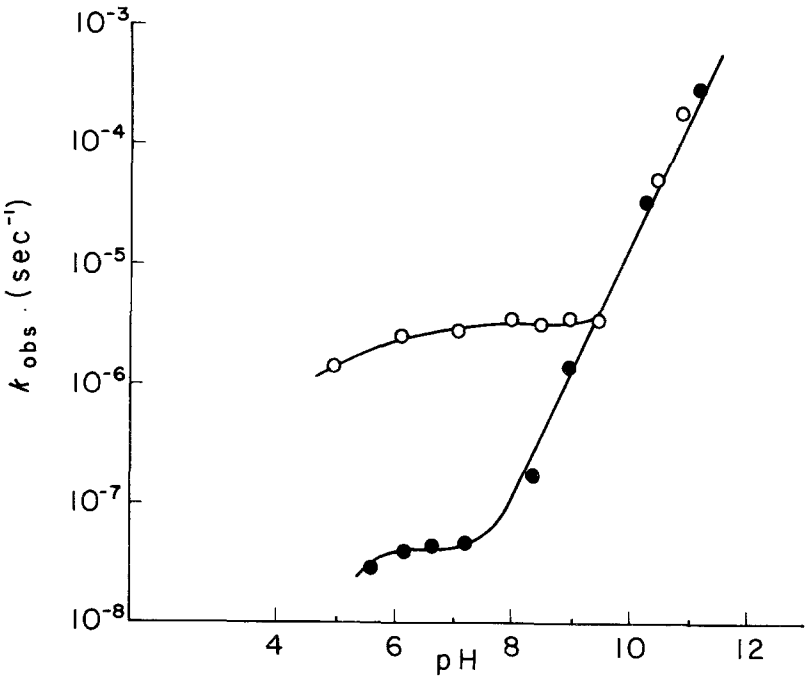
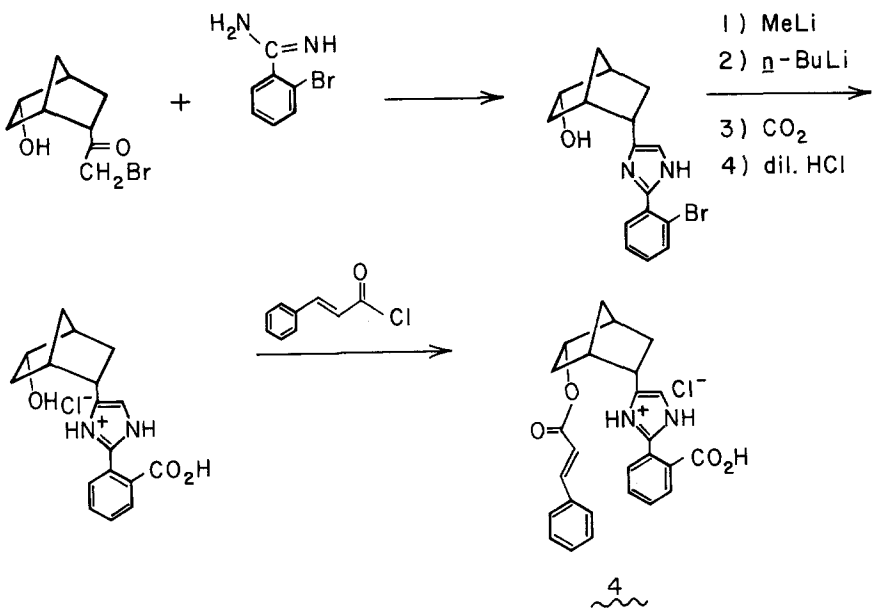


Figure 4. The effect of 0.1 M benzoate ion, ●, on the hydrolysis of 2-imidazolyl norbornane cinnamate.

since we were using the constitutents of the active site of the enzyme to produce a rate of reaction which was calculated to be within a factor of 4 of the rate of cinnamoyl-chymotrypsin deacylation. But this was accomplished using the assumption that an intramolecular catalyst (as in an enzyme) = 10 M of an intermolecular catalyst.[6]

The synthesis and reactivity of the intramolecular analog of this system, endo,endo-5-[2'-(2-carboxyphenyl)-4'(5')-imidazolyl]bicyclo-[2.2.1]-hept-2-yl trans-cinnamate, 4, have been carried out. It was synthesized from endo,endo-5-acetyl-2-hydroxy bicyclo[2.2.1]-heptane by first converting it to endo,endo-5-bromoacetyl-2-hydroxy bicyclo[2.2.1]-heptane, and then condensing it with 2-bromobenzamidine to give the corresponding imidazole derivative, carboxylating the 2-bromophenyl-imidiazole to give the corresponding carboxylate ion, and finally cinnamoylating the hydroxyl group on the norbornane ring, according to Scheme I, thereby producing 4.

Scheme I



Scheme I. The synthesis of 4. Our model of an acyl-chymotrypsin.

We have determined the rate of hydrolysis of the endo,endo compound containing intramolecular benzoate ion, 4, and shown it to be equal to 1/18 the rate of deacylation of trans-cinnamoyl-α-chymotrypsin (Table II). Furthermore, we find that an increasing dioxane concentration in the solvent increases the reaction rate as seen before.

As shown in Figure 5 and Table II there is a large increase in the rate constant for the hydrolysis of 4 over that of hydrolysis of

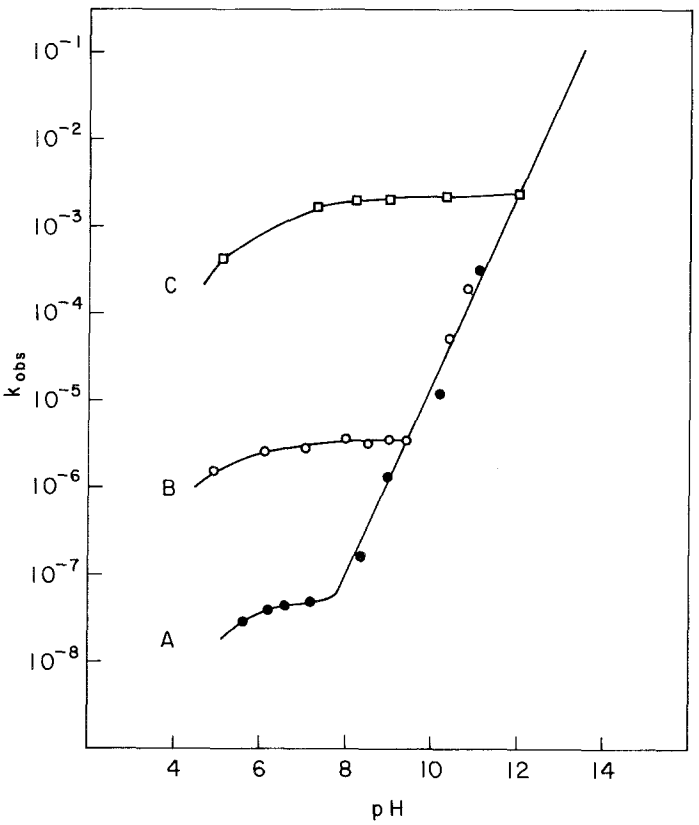


Figure 5. Hydrolysis of norbornane trans-cinnamate models. pH 7.9, 60°C.

A. endo-norbornane trans-cinnamate, (●).
B. hydrolysis of endo-norbornane trans-cinnamate + 0.5M benzoate ion, (○).
C. hydrolysis of endo,endo-5-[2'-(2-carboxyphenyl)-4'-(5')-imidazol-yl]bicyclo[2.2.1]-hept-2-yl trans-cinnamate, 4, (□).

"normal" cinnamates. Although this acceleration of 154,000, in the mixed solvent system, is 18-fold less than that of the real acyl-enzyme in water, if one suggests a differential solvating system for the active site of the artificial enzyme, as exists in the real enzyme, it is possible that both the real and artificial acyl-enzymes would have the same rate of deacylation.

Thus we have synthesized an organic chemical model of an acyl-enzyme intermediate of MW 428 whose hydrolysis rate is approximately equivalent to a real acyl-enzyme intermediate of MW 25,100 as in Table II. This

TABLE II[8]

Steps in the Development of a Model for an Artificial Acyl-Enzyme

Development	Rate Constant[a]	Hydrolysis of	Relative Rate Constant	Ref.
1. 2,5 Hydroxyl and imidazolyl group regiochemistry in the norbornane ring system confirmed	4.5×10^{-5}[a]	1	1	12
2. Endo,endo stereochemistry of the hydroxyl and imadazolyl groups	4.5×10^{-5}	1	1	12
3. The function of the imidazolyl group is a general base[b]			1,917	6,8
4. Carboxylate (benzoate) ion is necessary	8.6×10^{-5}	1 + 0.5 M benzoate		
5. Intramolecular benzoate ion is necessary	6.9×10^{-3}[c,d]	4	154,000	8

a. This rate constant corresponds to that for the rate of hydrolysis of norbornane cinnamate without an imidazolyl group. If extrapolation to 60° was required, it was done using the data of ref. 7.

b. $k_{H_2O}/k_{D_2O} = 3.0$.

c. The purity of this compound dictates that the rate constant be only good to ± 5%. The others are better.

d. The rate constant of deacylation of cinnamoyl-chymotrypsin at pH 7.9 and 60° in water is $1.3 \times 10^{-1} sec^{-1}$, which is 2,888,888 (faster than a "normal" cinnamate) or 18 fold faster than our artificial acyl-enzyme.

achievement indicates that our initial assumptions of a three amino acid active site (Table I), and the intermediacy of an acyl-eyzyme (Fig.1) are correct for the chymotrypsin structure and mechanism. Table II also indicates the many steps that the model-building has taken.

The molecular weight ratio of 58.25 for the real over the artificial acyl-enzyme which we have achieved can be explained in part by the fact that we do not utilize any amino acids in our synthesis, which immediately makes it non-utilizable for biosynthesis including transmission through the genetic chain from one generation to the next, and also reduces the water solutibility greatly. Since all life takes place in water, it reduces the utility of the artificial acyl-enzyme to non-life processes.

Now that we have successfully synthesized a model of the acyl-enzyme intermediate of chymotrypsin, we wanted to synthesize a model of the enzyme, chymotrypsin, itself. We attempted to do this by attaching the three catalytic groups, imidazolyl, hydroxyl, and carboxylate ion to the binding agent, cyclamylose as shown theoretically in Figure 6 and

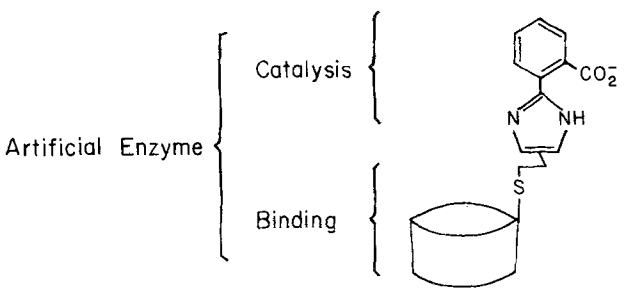


Figure 6. Theoretical synthesis of artificial chymotrypsin.

practically in Figure 7. That is to say, if the catalytic groups are

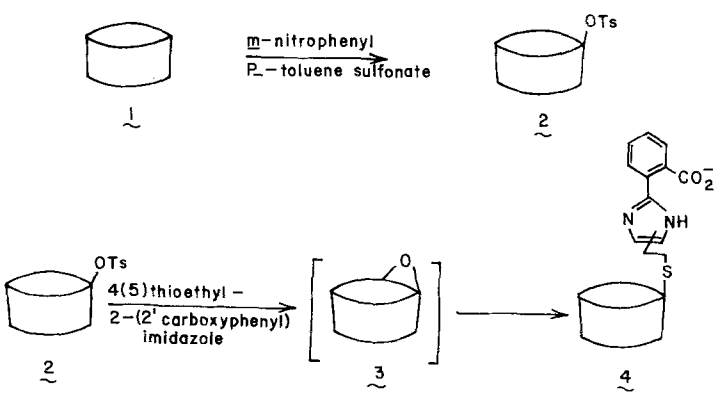


Figure 7. Practical synthesis of artificial chymotrypsin.[9]

attached to a binding agent such as cycloamylose in the same molecule, an artificial enzyme is created. The epoxide in Figure 7 is put in brackets because we have not as yet proved its intermediacy in the reaction of the cycloamylose tosylate with the good nucleophile, the sulfhydryl compound. The tosylate is a 2-compound. If the product is also a 2-compound, there is no epoxide intermediate. If the product is a 3-compound, there is an epoxide intermediate, because in it the 2 and 3 positions are equilibrated. Although Figure 6 implies that we have synthesized artificial chymotrypsin, it should be noted that so far we have managed to synthesize only an impure artificial enzyme.

However, we have some interesting data with the impure enzyme. One of the pieces of data we have is shown in Figure 8. It indicates the

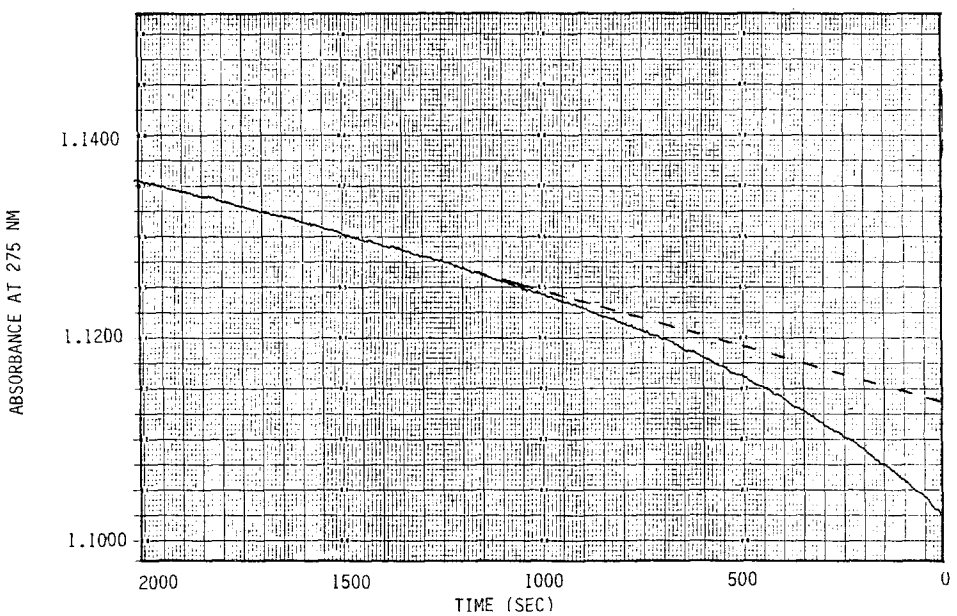


Figure 8. Artificial chymotrypsin-catalyzed hydrolysis of p-t-butylphenyl trimethylacetate at 25° in acetonitrile-water (50% v/v) at pH 10.5 borate buffer $[E]_o = 1x10^{-4}$ M; $[S]_o = 1x10^{-3}$ M.[10]

reaction of the impure enzyme with p-t-butylphenyl trimethyl acetate. This substrate was utilized for two reasons: (1) p-t-butylphenyl

acetate possesses the largest binding constant of any phenyl ester to cycloamylose; (2) p-nitrophenyl trimethyl acetate with real chymotrypsin shows a two-fold kinetic scheme, involving a curved portion and a straight portion, mirroring a presteady-state portion of the reaction, acylation, and a steady-state portion of the reaction, deacylation. As shown in Figure 8, artificial chymotrypsin shows the same phenomenon.

The two kinetic steps seen in artificial chymotrypsin catalysis imply that the mechanism of action of the artificial enzyme is as shown in Figure 9 (for the corresponding acetate ester). One obvious experiment yet to do is to prove that the acetyl group becomes attached to the cycloamylose by use of a radioactive acetate ester.

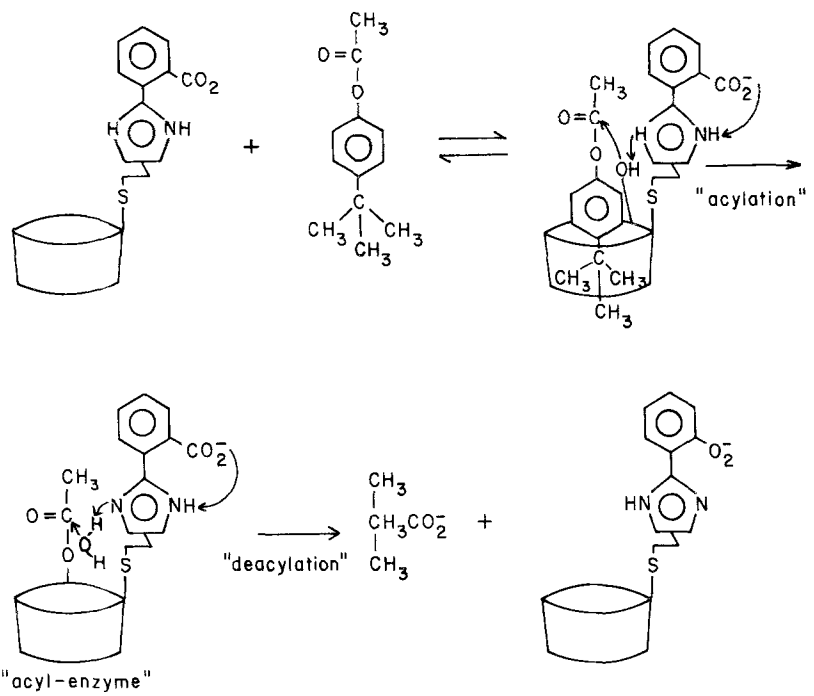


Figure 9. The mechanism of action of artificial chymotrypsin.

The impure, artificial chymotrypsin we have thus far produced is only about 1/1000 as good as real chymotrypsin as Table III will show.

TABLE III

A kinetic Comparison of Real and Artificial Chymotrypsin

Enzyme	Substrate	Rate Constant
Real Chymotrypsin	p-Nitrophenyl Acetate	$5.2 \times 10^{-2} sec^{-1}$
Artificial Chymotrypsin	p-t-Butylphenyl Acetate	$7.1 \times 10^{-5} sec^{-1}$

Thus, the impurity does not allow us to claim that we have made an artificial chymotrypsin, although we can claim that we have made artificial cinnamoyl-chymotrypsin.[11]

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