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The Action of α-Chymotrypsin on Tyrosine Ethyl Ester

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The splitting of tyrosine ethyl ester by α-chymotrypsin was studied by means of continuous titration in a pH-stat. The reaction was followed to about 99 % hydrolysis, and to explain the obtained results it is necessary to assume the simultaneous or consecutive uptake of 3 molecules of substrate before reaction takes place. This supports the view that transpeptidation is the first step in the splitting of this substrate.

Numerous investigations have been carried out on the action of α-chymotrypsin [1–3], and a good deal of these are made with phenylalanine derivatives as substrates, *e.g.* tyrosine ethyl ester (TEE). However, these investigators usually study the initial velocities of the reaction in order to analyse the results according to Michaelis and Menten [4]. If, however, the suggestion made by Kuk-Meiri and Lichtenstein [5] that the splitting of TEE should start with a transpeptidation is correct, a Michaelis-Menten analysis could very easily lead to misinterpretations. In order to distinguish between this and other suggested theories for the mechanism, the reaction was studied carefully and the results analysed according to the "steady state" method in the way suggested by Christiansen [6,7]; it is known that this method has given new information about the behaviour of the pancreatic enzymes on other substrates [8].

EXPERIMENTAL

Crystalline α-chymotrypsin was kindly placed at our disposal by *Novo Terapeutisk Laboratorium*, Copenhagen, Denmark.

TEE was a commercial product (L-tyrosine ethyl ester from Nutritional Biochemical Corporation, U.S.A.).

The experiments were carried out in 30 ml 0.2 M KCl, in which the desired amount of substrate was dissolved and adjusted to the desired pH by means of KOH. Substrate concentrations varied from 0.678 mmole/l to 9.50 mmole/l. The temperature, unless stated otherwise, was 27.09°C and the pH was 6.838. The hydrolysis was followed by continuous automatic titration with 0.10 M KOH + 0.2 M KCl in a selfrecording pH-stat. The degrees of reaction were calculated as the proportion between the titration value at the desired time and the titration value after complete hydrolysis.

The potassium hydroxide is added from a syringe in quanta corresponding to 1/100 mm movement of the piston. It should be noticed that at the highest degrees of reaction

Table 1. E = enzyme concentration in arbitrary units. t = times necessary to obtain the degrees of reaction, a, given in the first line. In order to confirm the $E \cdot t$ law the figures in the vertical columns $E \cdot t_n$ should be identical for each value of n, but different for different values of n. For further explanation see text.

a = 0.143			0.286		0.429		0.571		0.714		0.857	
E	t_1	$E \cdot t_1$	t_2	$E \cdot t_2$	t_3	$E \cdot t_3$	t_4	$E \cdot t_4$	t_5	$E \cdot t_5$	t_6	$E \cdot t_6$
8	0.95	7.6	2.1	16.8	3.4	27.2	4.0	32.0	6.0	48.0	8.1	64.8
6	1.2	7.2	2.8	16.8	4.2	25.2	5.0	30.0	7.7	46.2	10.5	63.0
4	2.0	8.0	4.1	16.4	6.4	25.6	7.6	30.4	12.5	50.0	18.6	74.4
2	3.8	7.6	8.0	16.0	12.7	25.4	15.4	30.8	26.0	52.0	38.7	77.4
1	8.3	8.3	17.3	17.3	27.2	27.2	32.8	32.8	56.5	56.5	87.0	87.0

the velocity was considerably lower than 1 quantum/min so that errors on calculated times of one or two minutes are to be expected in this area.

RESULTS

From Table 1 is seen that the chronometric integral, with acceptable accuracy, can be written in the form $E \cdot t = f(x_1 x_2 ... x_n)$ where E is enzyme concentration, t is time and x_1, x_2, ...x_n are the variables which determine the reaction rate, *e.g.* degree of reaction, pH and concentration of TEE. In a single experiment the only variable on the right side usually is the degree of reaction. In a series of experiments, where only the enzyme concentration is varied, the validity of writing the chronometric integral, as mentioned above, can be determined by comparing the times necessary to reach a given degree of reaction with different enzyme concentrations. Table 1 records the times necessary to obtain some different degrees of reaction for different enzyme concentrations and the products of these times and enzyme concentrations.

The dependence of activity on temperature and pH, as well as the pH optimum dependence on temperature, was investigated. The results are shown

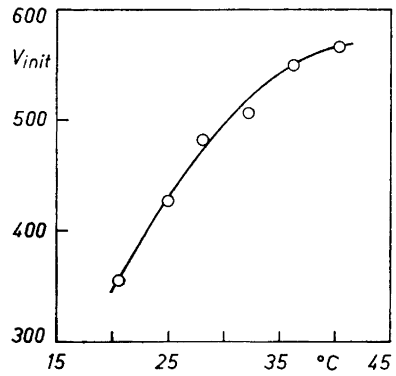

Fig. 1. Initial velocity as function of temperature. pH = 6.7.

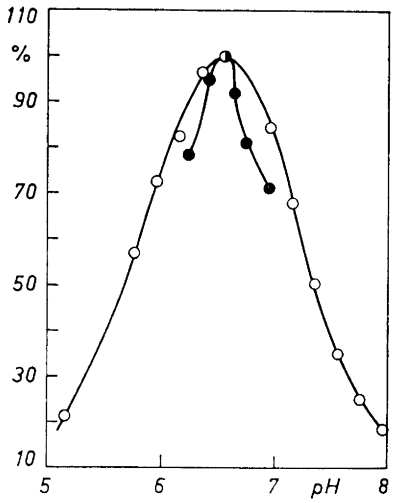

Fig. 2. A graph of initial velocity *versus* pH at two temperatures. ○, $t = 28.10°C$; ●, $t = 41.45°C$. Abscissa gives pH and ordinate gives the slope of the titration curve in per cent of the maximal slope at the same temperature. Substrate and enzyme concentrations are equal in all experiments at the same temperature. The dissociation of the amino group gives rise to a change in the fraction of the liberated acid that is titrated, this fraction being diminished in the pH range 6—8. The true optimum of the reaction is at pH 6.85.

in Figs. 1 and 2. It is interesting to compare these two figures with the same results obtained with subtilisin splitting the same substrate [9]. In the latter case, the changes of pH optimum with temperature clearly demonstrated that the enzyme attacked only the positively charged substrate mlecules; but the curves presented here in no way indicate that the same mechanism is the

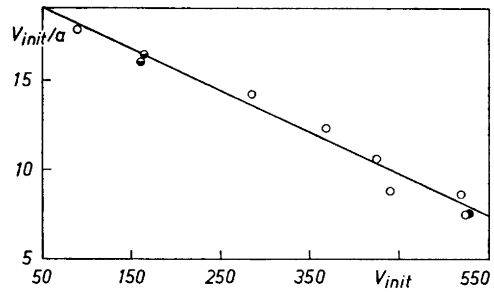

Fig. 3. The figure gives initial velocity divided by initial substrate concentration as ordinate and initial velocity as abscissa. Initial velocity is determined as the slope (in arbitrary units) at the origin of the lower concentrations and as the slope when d^2c/dt^2 is zero at the highest concentrations. (See: Discussion and Fig. 8 for further explanation). The two points marked ◒ are twin determinations at 10 mg/30 ml; ● is 70 mg/30 ml recrystallized from ethyl acetoacetate; the other points represent 5, 20, 30, 40, 50, 60, and 70 mg in 30 ml.

case with chymotrypsin, which suggests a fundamental difference in the action of the two enzymes on this substrate.

The reaction has often been described by a Michaelis-Menten function. From Fig. 3 it is seen that our results too satisfy the Michaelis-Menten equation. Using the method for graphical representation, suggested by Eadie [10], in which (initial velocity)/(substrate concentration) is plotted *versus* initial velocity, a reasonably straight line is obtained as seen in the figure. If, however, the reaction in its entire course should have a reaction scheme as the one suggested by Michaelis and Menten, the chronometric integral should consist of a zero order term and a first order term:

$$E \cdot t = A \cdot a - B \cdot \log(1-a) \qquad (1)$$

where a is the degree of reaction and A and B are constants. Indeed, this is not true for this reaction, which can easily be seen, for instance by plotting t/a *versus* $\log(1-a)/a$. If the reaction could be described by eqn. (1), this graph should be a straight line with the intercept on the ordinate axis being A and the slope B. With different initial substrate concentrations, A should be proportional to the initial substrate concentration and B should depend linearly on the same or be independent of this concentration.

In this way it can be clearly demonstrated that the experimental results recorded in Tables 2—7 in no way fit eqn. (1). It is further seen from such a

Tables 2—7. These tables give measured and calculated values of t together with corresponding values of a. The 6 tables represent 6 different concentrations of TEE and for every table is given a, A', B', C' and D'. (The B' values are based on calculations with $\log_{10}$.) The deviations between t_m and t_c at the highest degrees of reaction is understandable as the number of hydroxyl quanta from the titration outfit was below 1 quantum/min. The number of quanta for complete hydrolysis are given for every experiment as s_∞.

Table 2. a = 5 mg/30 ml; A' = 2.70; B' = 44.0; C' = −0.395; D' = 0.00170 and s_∞ = 224.

t_m	t_c	a
0	0.00	0.0000
5	4.61	0.1964
10	9.76	0.3750
15	15.28	0.5267
20	20.48	0.6383
25	25.78	0.7276
30	30.90	0.7946
35	36.18	0.8482
40	41.28	0.8883
45	45.58	0.9151
50	50.02	0.9375
55	54.37	0.9553
60	59.81	0.9732
65	63.16	0.9821
70	70.18	0.9910

Table 3. a = 10 mg/30 ml; A' = 5.50; B' = 46.6; C' = −0.189; D' = 0.00110 and s_∞ = 466.

t_m	t_c	a
0	0.00	0.0000
5	4.84	0.1759
10	10.07	0.3369
15	15.17	0.4678
20	20.35	0.5772
25	25.72	0.6795
30	30.67	0.7382
35	35.64	0.7940
40	40.37	0.8369
45	45.72	0.8755
50	50.21	0.9013
55	55.35	0.9249
60	59.42	0.9399
65	64.50	0.9549
70	69.18	0.9657
75	73.89	0.9742
80	78.63	0.9807
85	82.89	0.9850
90	89.40	0.9893
95	105.23	0.9936

graph that a negative term at the right side should be added to (1) and that this term should be relatively more important at low substrate concentrations than at high substrate concentrations, where it should be important only at the higher degrees of reaction. It is then reasonable to suggest that a second order term should be added to (1) making it:

$$E \cdot t = A'a - B'\log(1-a) + C'\left(\frac{1}{1-a} - 1\right) \qquad (2)$$

This equation is able to describe the obtained results with excellent accuracy except at the very highest degrees of reaction where calculated times will be too low as compared with those measured. It will be seen from the calculations below that the term which becomes significant at the highest degrees of reaction should be positive. Therefore a fourth term is added to the right side of (2) giving as chronometric integral:

$$E \cdot t = A'a - B'\log(1-a) + C'\left(\frac{1}{1-a} - 1\right) + D'\left(\frac{1}{(1-a)^2} - 1\right) \qquad (3)$$

We determined the best fitting values of A', B', C' and D' for six experiments with different TEE concentrations and the same enzyme concentration. These values are in Tables 2—7.

Table 4. a = 20 mg/30 ml; A' = 11.0; B' = 44.2; C' = — 0.080; D' = 0.00045 and s_∞ = 939.

t_m	t_c	a
0	0.00	0.0000
5	4.80	0.1512
10	10.00	0.2971
15	15.05	0.4207
20	20.14	0.5282
25	25.19	0.6187
30	30.29	0.6954
35	35.26	0.7572
40	40.40	0.8094
45	45.17	0.8488
50	50.12	0.8818
55	55.14	0.9084
60	60.22	0.9297
65	64.71	0.9446
70	69.56	0.9574
75	74.74	0.9680
80	78.72	0.9744
85	83.80	0.9808
90	88.35	0.9851
95	94.52	0.9893
100	102.25	0.9925
105	112.54	0.9947

Table 5. a = 30 mg/30 ml; A' = 16.2; B' = 46.5; C' = — 0.072; D' = 0.00027 and s_∞ = 1390.

t_m	t_c	a
0	0.00	0.0000
5	4.92	0.1302
10	10.10	0.2561
15	15.28	0.3698
20	20.28	0.4683
25	25.41	0.5576
30	30.54	0.6353
35	35.61	0.7014
40	40.53	0.7561
45	45.68	0.8043
50	50.49	0.8417
55	55.53	0.8741
60	60.46	0.9000
65	65.73	0.9223
70	68.84	0.9388
75	75.35	0.9518
80	79.94	0.9619
85	84.82	0.9705
90	89.46	0.9770
95	93.94	0.9820
100	99.96	0.9871
105	105.77	0.9906
110	111.00	0.9928
115	115.79	0.9942

Table 6. a = 40 mg/30 ml; A' = 20.2; B' = 46.5; C' = − 0.071; D' = 0.00022 and s_∞ = 1849.

t_m	t_c	a
0	0.00	0.0000
5	4.79	0.1152
10	9.87	0.2293
15	14.93	0.3342
20	19.98	0.4300
25	24.80	0.5127
30	29.87	0.5906
35	34.93	0.6593
40	40.05	0.7198
45	45.01	0.7701
50	49.97	0.8129
55	55.03	0.8496
60	60.18	0.8805
65	64.88	0.9037
70	70.27	0.9254
75	75.30	0.9416
80	80.08	0.9540
85	85.03	0.9643
90	89.93	0.9724
95	94.89	0.9789
100	100.22	0.9843
105	104.38	0.9876
110	108.71	0.9903
115	113.13	0.9924
120	120.00	0.9946

Table 7. a = 50 mg/30 ml; A' = 28.2; B' = 47.0; C' = − 0.045; D' = 0.00017 and s_∞ = 2298.

t_m	t_c	a
0	0.00	0.0000
5	4.31	0.0870
10	9.47	0.1867
15	14.70	0.2820
20	19.85	0.3703
25	24.77	0.4487
30	29.94	0.5248
35	35.01	0.5931
40	40.02	0.6540
45	45.15	0.7097
50	50.30	0.7597
55	55.44	0.8003
60	60.24	0.8359
65	65.17	0.8660
70	70.14	0.8916
75	74.91	0.9121
80	80.07	0.9304
85	84.86	0.9443
90	89.64	0.9556
95	94.66	0.9652
100	99.48	0.9726
105	104.47	0.9787
110	109.45	0.9835
115	113.92	0.9869
120	117.48	0.9891
125	122.96	0.9927
130	127.96	0.9935

REACTION MECHANISM

Assuming a steady state, we suggest the following reaction mechanism, based upon eqn. (3):

$$X_1 + 2\ TEE \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 + TEE \rightleftharpoons X_3 + P_1 \qquad (\pm 2)$$
$$X_3 \rightarrow X_1 + P_2 \qquad (+ 3)$$

In this scheme X_n means a form of the enzyme or a combination of enzyme and one or several substrate or product molecules. P_n is the reaction products; P_2 may represent either one or more molecules and nothing is assumed about their configuration.

Treating this reaction scheme according to the steady state method, in the way described by Christiansen [6,7], and using the following symbols: x_n = concentration of X_n; w_n = reaction probability for the n'th reaction; k_n = velocity constant for the same reaction; c = concentration of TEE; a = initial concentration of TEE; $s = -\mathrm{d}c/\mathrm{d}t$; E = enzyme concentration and t = time, we have:

$$s = x_1w_1 - x_2w_{-1}$$
$$s = x_2w_2 - x_3w_{-2}$$
$$s = x_3w_3$$

Remembering that $E = x_1 + x_2 + x_3$, we get by rearrangement and addition:

$$E/s = \frac{1}{w_1} + \frac{1}{w_2} + \frac{1}{w_3} + \frac{w_{-1}}{w_1 w_2} + \frac{w_{-2}}{w_2 w_3} + \frac{w_{-1} w_{-2}}{w_1 w_2 w_3}$$

Looking at the reaction scheme we see that we can introduce:

$$w_1 = k_1 c^2;\ w_{-1} = k_{-1};\ w_2 = k_2 c;\ w_{-2} = k_{-2}(a-c) \text{ and } w_3 = k_3,$$

if assuming that the concentration of $P_1 = (a-c)$. This changes the equation to:

$$E/s = \left(\frac{k_{-1}}{k_1 k_2} + a\frac{k_{-1}k_{-2}}{k_1 k_2 k_3}\right)\frac{1}{c^3} + \left(\frac{1}{k_1} - \frac{k_{-1}k_{-2}}{k_1 k_2 k_3}\right)\frac{1}{c^2} + \left(\frac{1}{k_2} + a\frac{k_{-2}}{k_2 k_3}\right)\frac{1}{c} + \left(\frac{1}{k_3} - \frac{k_{-2}}{k_2 k_3}\right)$$

Integrating this gives:

$$E \cdot t = Aaa - (B_1 + B_2 a)(\ln 10)\log(1-a) + C\frac{1}{a}\left(\frac{1}{1-a} - 1\right) + \tfrac{1}{2}(D_1 + D_2 a)\frac{1}{a^2}\left(\frac{1}{(1-a)^2} - 1\right)$$

a is the degree of reaction $= (a-c)/a$; $A = \frac{1}{k_3} - \frac{k_{-2}}{k_2 k_3}$; $B_1 = \frac{1}{k_2}$; $B_2 = \frac{k_{-2}}{k_2 k_3}$; $C = \frac{1}{k_1} - \frac{k_{-1}k_{-2}}{k_1 k_2 k_3}$; $D_1 = \frac{k_{-1}}{k_1 k_2}$ and $D_2 = \frac{k_{-1}k_{-2}}{k_1 k_2 k_3}$.

This calculated chronometric integral is identical with (3) if: $A' = A \cdot a$; $B' = B_1 + B_2 \cdot a$; $C' = C \cdot \frac{1}{a}$ and $D' \cdot 2a^2 = D_1 + D_2 \cdot a$.

From Figs. 4—7 it is seen that the dependence of A', B', C', and D' on a is in agreement with the calculated dependence.

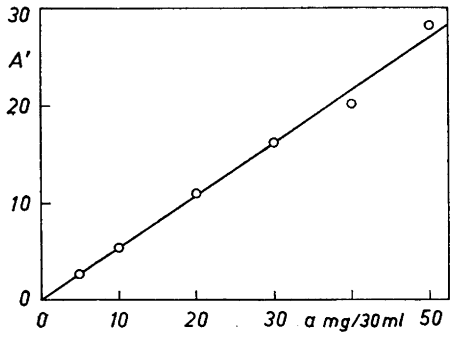

Fig. 4. A' from Tables 2—7 *versus* a.

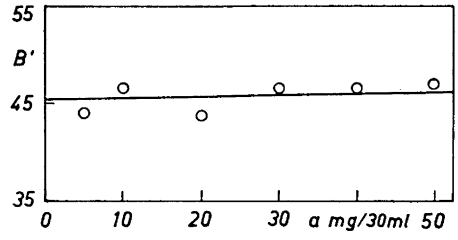

Fig. 5. B' from Tables 2—7 *versus* a.

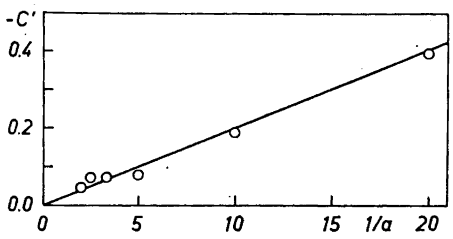

Fig. 6. C' from Tables 2—7 *versus* $1/a$.

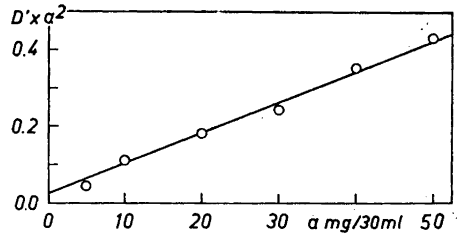

Fig. 7. D' from Tables 2—7 multiplied by a^2 as ordinate and as abscissa a.

DISCUSSION

The reaction mechanism suggested here differs from mechanisms, previously proposed, for the investigated reaction. A mechanism involving three steps has, however, been suggested before [2,3] and it has also been suggested that a crucial step should involve a transpeptidation [5]. Based on kinetical facts alone, it is not possible to conclude that the step involving two molecules of TEE is involving a transpeptidation; but it seems necessary to assume the participation of three molecules of TEE in order to explain the obtained results. Referring to the investigations by Kuk-Meiri and Lichtenstein [5], it seems reasonable to assume that the first two molecules form a dipeptide, and that the reaction thereafter takes place by substituting one of the molecules in the resulting tyrosyl-tyrosine ethyl ester (TTEE) with a third molecule of TEE. It seems reasonable to explain the kinetical data by assuming that the dipeptide synthesis is made directly from TEE so it can be written schematically:

$$2\ \text{TEE} \rightarrow \text{TTEE} + \text{EtOH}.$$

Another possibility, a reaction between tyrosine (T) and TEE:

$$\text{T} + \text{TEE} \rightarrow \text{TTEE}$$

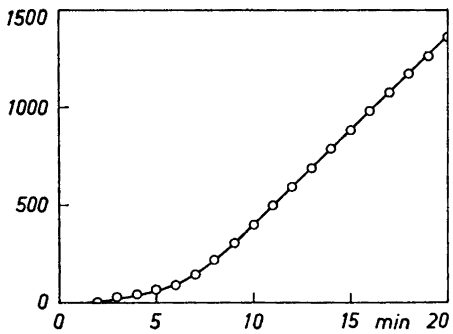

Fig. 8. Experiments with saturated TEE solutions. Note the obvious increase in reaction velocity in the first minutes.

as was suggested by Tauber [11] for the formation of phenylalanyl-phenylalanine ethyl ester does not seem to be supported by the kinetical data.

It should be noticed that the fourth term in the chronometric integral is needed for a second reason: It appears so that it is impossible to construct any reaction mechanism that gives a negative coefficient to the term corresponding to the highest reaction order. The necessity for this negative term is seen by plotting t/a *versus* $\log (1-a)/a$, especially at the lowest substrate concentrations.

The chronometric integral, suggested here, conforms with the experimental results to a very high extent, and it seems that the proposed reaction mechanism is one of the simplest that can explain the experimental facts. However, there is evidence which indicates that the reaction mechanism is in fact more complicated than the one proposed here. The deviation from the $E{\cdot}t$ law may not be large enough to disprove its validity but it might suggest the need for corrections. Secondly, a positive value of $\mathrm{d}s/\mathrm{d}t$ $(= -\mathrm{d}^2c/\mathrm{d}t^2)$ is seen in the first minutes for higher substrate concentrations and in solutions saturated with TEE, which indicates that under these circumstances the reaction does not attain a steady state immediately; that some reaction not identical with the overall reaction takes place in the first minutes (Fig. 8).

Further information on where hydrogen and hydroxyl ions enter in the reaction scheme, as well as whether several protolytic forms of substrate enter in the reaction, can probably be obtained by studying the four constants at different pH values. Experiments of that kind are in progress in this laboratory.

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