BBA 65966

KINETICS OF THE TRYPSIN-CATALYZED HYDROLYSIS AND ETHANOLYSIS OF L-LYSINE METHYL ESTER

M. CASTAÑEDA-AGULLÓ, GLORIA DÁVILA, CARMEN OLIVER, TERESA CRUZ AND LUZ M. DEL CASTILLO

Departamento de Biofísica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, D.F. (México)

(Received March 31st, 1969)

SUMMARY

1. The reaction of L-lysine methyl ester (LME) and trypsin in aqueous ethanol yields in the first place methanol, lysine and L-lysine ethyl ester (LEE). LEE is then acted upon by trypsin to give lysine and ethanol. Hence, kinetics are complex and involve 4 rate constants and 2 equilibrium constants.

2. An attempt was made to determine Michaelis constant (K_m) and catalytic rate constant (k_{cat}) for the first reaction, *i.e.* trypsin-catalyzed cleavage of LME, by analyzing the composition of reaction mixtures at early stages in order to have a ratio LEE:LME as small as possible.

3. At pH 6.5 the reaction follows Michaelis kinetics, but at pH 8.5 double reciprocal plots become curved suggesting activation by excess substrate. Apparently the number of substrate molecules bound per enzyme molecule increases as dielectric constant decreases.

4. The value of k_{cat} for the overall reaction increases as alcohol concentration increases. This effect may be ascribed mainly to an enhanced alcoholysis. The ratio of deacylation constants $k_4:k_3$ (alcohol:water) is also modified by a change of the medium dielectric constant. From the results presented here the suggestion is possible that the principal effect of lowering the dielectric constant is to increase the rate of acylation with respect to that of dissociation of Michaelis complex.

INTRODUCTION

In aqueous ethanol solutions, trypsin catalyzes the simultaneous hydrolysis and ethanolysis (transesterification) of L-lysine methyl ester (LME)[1,2] and L-arginine methyl ester (AME)[1]. At constant ratio ethanol:water, the relative proportion of products of the two reactions at a given time varies with pH and temperature ap-

Abbreviations: LME and LEE, L-lysine methyl or ethyl ester; AME, L-arginine methyl ester; TAME, *p*-toluenesulfonyl L-arginine methyl ester; TLME, *p*-toluene-sulfonyl L-lysine methyl ester.

parently depending upon the degree of ionization of the α-ammonium group in substrate[1]. The product of transesterification is susceptible to tryptic action so that the composition of reaction mixture is also a function of time. GLAZER[2] followed the time-course of the reaction LME-trypsin in binary mixtures of water and various alcohols. It can be seen in his results that this reaction behaves like a combination of simultaneous and consecutive reactions. At initial stages the concentration of both products increases with time, then the product of alcoholysis reaches a maximum and eventually disappears. Lysine, the product of hydrolysis, increases continuously until all LME is used up. In order to acquire a better knowledge of the complex reaction occurring between trypsin and LME in aqueous ethanol, the present investigation on the effect of two variables was undertaken. In the first place, a study was made of the time-course under the same conditions of previous experiments[1,3]. Then the effect of varying substrate concentration was analyzed. The apparent Michaelis and catalytic-rate constants (K_m and k_{cat}) were determined from data obtained at an early stage of the reaction, when the concentration of the second substrate (LEE) was small as compared to that of the first one (LME). Under these conditions, it is possible to consider simpler kinetics and express the values of K_m and k_{cat} in terms of a few rate or equilibrium constants belonging to intermediate steps.

MATERIALS AND METHODS

The trypsin sample was the same used in previous work[1]. Substrate LME was obtained from Mann Research Corp.

The concentration of lysine, L-lysine ethyl ester (LEE) and unattacked LME in hydrolysates was determined by using a paper chromatography method such as described in a former paper[1]. Data reported here are an average of 8–12 determinations.

RESULTS AND DISCUSSION

Time-course of the reaction LME-trypsin in ethanol–water

The composition of reaction mixtures was analyzed in aliquots taken at 1-min

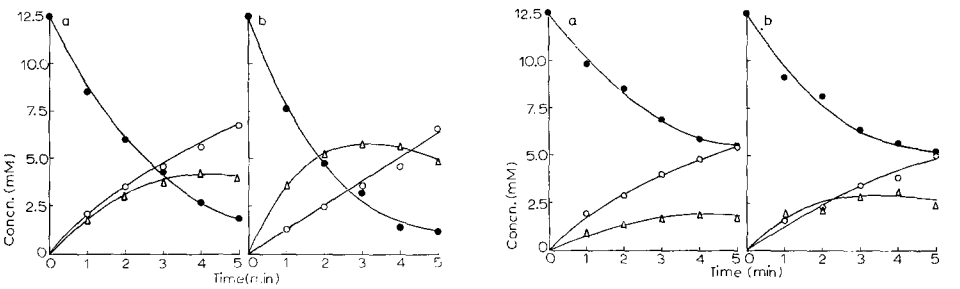


Fig. 1. Time-course of the reaction of LME and trypsin at pH 6.5 and 25° in aqueous ethanol at concentrations corresponding to dielectric constant values: 75.5 (a) and 72.5 (b). ○, lysine; ●, LME; △, LEE. Initial concn.: LME, 12.5 mM; and trypsin, 3.1 μM. No buffer was added, pH was maintained constant by continuous titration.

Fig. 2. Time-course of the reaction LME-trypsin at pH 8.5 and 25° in aqueous ethanol at two D values: 75.5 (a) and 72.5 (b). Symbols and conditions as in Fig. 1.

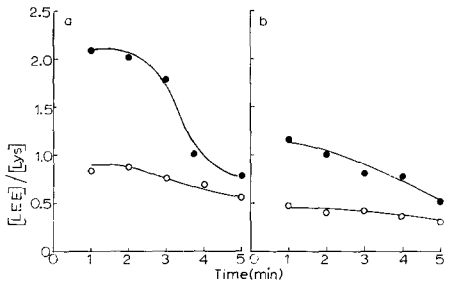
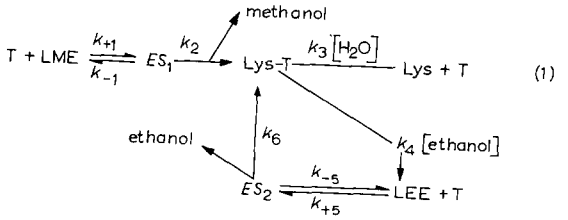


Fig. 3. Ratio of products of ethanolysis:hydrolysis as a function of time at 25° in the trypsin-catalyzed breakdown of LME in aqueous ethanol at pH 6.5 (a) and 8.5 (b) and D values 75.5 (○) and 72.5 (●). Conditions as in Figs. 1 and 2.

intervals after addition of enzyme up to 5 min. The digestion mixtures initially contained 12.5 mM LME in either 6.6 or 13.2% (by vol.) ethanol and 3.1 μM trypsin. These alcohol concentrations correspond respectively to dielectric constant values 75.5 and 72.5 at 25° (ref. 4). Figs. 1 and 2 show the results at pH's 6.5 and 8.5. The maximum concentration of LEE in all cases was reached at about 4 min. In agreement with previous results[1], the ratio LEE:lysine was higher at pH 6.5 than at pH 8.5 (Fig. 3). This ratio decreases with time because LEE, like LME, serves as substrate to trypsin.

In harmony with current ideas[5] and observed facts, the reaction path of trypsin and LME in ethanol–water can be represented as follows:

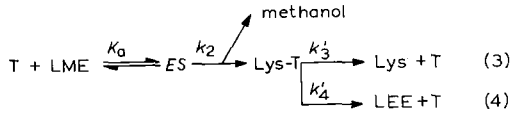


In this scheme, T stands for trypsin, ES_1 and ES_2 for the respective Michaelis complexes of trypsin and either LME or LEE, Lys-T for the intermediate compound L-lysyltrypsin. The dissociation constants of complexes ES_1 and ES_2 are $K_a = k_{-1}/k_{+1}$ and $K_b = k_{-5}/k_{+5}$. The rate constants of acylation are k_2 and k_6. Those of deacylation of lysyltrypsin with either water or alcohol accepting the lysyl group are k_3 and k_4. For the sake of convenience we will substitute k_3' and k_4' for the products $k_3[H_2O]$ and k_4[ethanol]. Making the usual suppositions of steady state and letting a_0 represent the initial concentration of LME and b or c the concentrations of LEE or lysine at time t, we could derive the following expression for the rate of formation of LEE relative to that of lysine[6]:

$$\frac{db}{dc} = \frac{k_4'}{k_3'} - \frac{k_6}{k_3'} b \left[\frac{(K_a + a_0)(k_3' + k_4') + k_2 a_0}{k_2 a_0 \left[1 + \frac{a_0}{K_a} \left(\frac{k_2 + k_3' + k_4'}{k_3' + k_4'} \right) \right] (K_b + b) + k_6 b (K_a + a_0)} \right] \qquad (2)$$

From Eqn. 2 the ratio $b:c$ must approach the value k_4'/k_3' as b approaches zero. In the same way when $b \ll a$, a condition which prevails at the beginning of the reaction,

the rate of formation of Lys-T from ES_2 must be very slow and we may consider, for simplicity, that the reaction proceeds in this manner:



Under these conditions, the values of K_m (apparent) and k_{cat} determined by the conventional procedure from the double reciprocal plot $[E_0]/v_0$ *vs.* $1/[S_0]$ must have the approximate values[5]:

$$K_m = \frac{K_a\,(k_3' + k_4')}{k_2 + k_3' + k_4'} \tag{5}$$

$$k_{cat} = \frac{k_2(k_3' + k_4')}{k_2 + k_3' + k_4'} \text{ (overall reaction)} \tag{6}$$

$$k'_{cat} = \frac{k_2 k_3'}{k_2 + k_3' + k_4'} \text{ (hydrolysis} \tag{7}$$

$$k''_{cat} = \frac{k_2 k_4'}{k_2 + k_3' + k_4'} \text{ (alcoholysis)} \tag{8}$$

The ideal values for calculation of K_m and the various k_{cat} would be the slopes of tangents to the initial portion of continuous plots of concentration of LME, lysine or LEE *vs.* time, but the method utilized does not lend itself to do this. The spread of data at short time is rather large. Compromising we chose a time of 2 min which, as can be seen in Fig. 3, is still within the zone of maximum ratio LEE:lysine. From results of composition analysis in reaction mixture after 2 min of enzyme action, the ratios $[E_0]/v_0$ (initial enzyme molarity to rate of change expressed in moles/l per sec) were calculated. Fig. 4 shows the least-square plots of reciprocal specific velocity *vs.* reciprocal of initial LME concentration for the overall reaction (disappearance of substrate) and formation of either lysine or LEE at pH 6.5 and 25° in three distinct media: aqueous solution dielectric constant ($D = 78.5$) and the two ethanol–water mixtures of D 75.5 and 72.5. The values of K_m and k_{cat} obtained from slopes and intercepts of

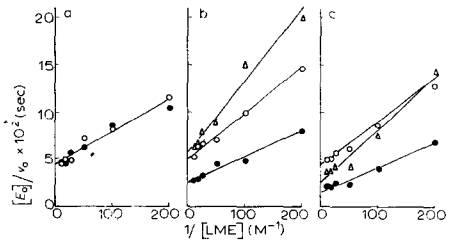


Fig. 4. Lineweaver–Burk plots of the trypsin-catalyzed cleavage of LME at pH 6.5 and 25° in aqueous solution (a) or aqueous ethanol of D values 75.5 (b) or 72.5 (c). ●, rates of substrate disappearance; ○, rates of lysine liberation; △, LEE production.

TABLE I

KINETIC CONSTANTS OF THE TRYPSIN-CATALYZED BREAKDOWN OF LME IN AQUEOUS ETHANOL

Constant	*pH 6.5*			*pH 8.5*		
	D: 78.5	*75.5*	*72.5*	*78.5*	*75.5*	*72.5*
k'_{cat} (hydrolysis) (sec^{-1})	21.7	19.6	22.0	18.2	8.2	7.0
k''_{cat} (alcoholysis) (sec^{-1})	—	17.8	37.9	—	5.1	11.6
k_{cat} (overall) (sec^{-1})	21.7	38.9	62.0	18.2	11.6	18.7
K_m (mM)	7.46	11.3	13.1	16.5	3.53	2.03
$\frac{k''_{cat}}{k'_{cat}} \times \frac{[H_2O]}{[ethanol]} \cong \frac{k_4}{k_3}$	—	41.4	38.2	—	28.3	37.0
$\frac{LEE}{Lys} \times \frac{[H_2O]}{[ethanol]} \cong \frac{k_4}{k_3}$	—	43.5	39.8	—	21.2	25.6
$\frac{k_{cat}}{K_m} \cong \frac{k_2}{K_a}$	2909	3442	4733	1106	3280	9280

these plots are given in Table I. Fig. 5 shows the results at pH 8.5 obtained under the same conditions as those at pH 6.5. In contradistinction with behavior at pH 6.5, data at pH 8.5 show a tendency to depart from a straight line. In aqueous solution only the points corresponding to 100 mM substrate fall below the line passing through the remaining points, but in alcohol all concentrations above 20 or 40 mM give rise to values which fall within a line notably curved downwards. NIEMANN and co-workers[7,8] noticed such curvature in their plots of α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl- and *N*-propionylglycine methyl ester in aqueous solution and interpreted it

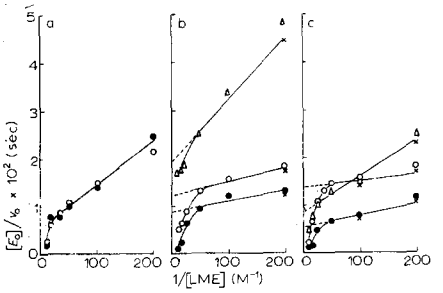


Fig. 5. Lineweaver–Burk plots of the trypsin-catalyzed breakdown of LME at pH 8.5 and 25° in aqueous solution (a) or ethanol–water at the proportions required to have *D* values 75.5 (b) or 72.5 (c). Symbols and conditions as in Fig. 4. ×, data corrected for enzymes autolysis.

on the basis of activation by excess of substrate. According to these authors, a ternary complex of enzyme and two substrate molecules would be formed which decomposes to products faster than the usual binary complex. However, in a more recent investigation, INGLES AND KNOWLES[9] reached the conclusion that the curvature of the double reciprocal plots in the α-chymotrypsin-catalyzed hydrolysis of *N*-acetylglycine esters was due to the "enzyme-blank" reaction, that is, the liberation of acidic products by the enzyme in absence of substrate. They found that the rate of this enzyme-blank'

reaction is proportional to free enzyme concentration within the range 10–100 μM. Upon correction of their data by enzyme-blank and substrate-blank reactions, the plots became linear. Even though for the present research such a high concentration of enzyme was not necessary as was with the nonspecific substrates *N*-acetylglycine esters, the possibility cannot be excluded that at pH 8.5 some enzyme autolysis occurs to an extent which would depend on the amount of free enzyme. If so, the total concentration of trypsin would be different at each point. To investigate whether our data could be corrected for enzyme loss, the following experiment was carried out. Aqueous or aqueous–ethanol solution of 5 mM Tris buffer (pH 8.5) was placed in a thermostatted vessel of the automatic titrator. Once thermal equilibrium at 25° was reached, trypsin was added to make a final concentration within the range 0.5 to 3.1 μM (the upper limit is the total trypsin concentration used throughout this work), and the recording mechanism was started. After 2 min, substrate (12.5 mM) was added, and the record continued for 5 min more. The total volume of reaction mixture was 40 ml. The resulting records of titrant (0.1 M NaOH) consumption as a function of time were compared with those obtained under identical conditions except with preincubation of enzyme at pH 8.5. No liberation of acidic products could be detected in any case prior to the addition of substrate. In aqueous solution no loss of activity could be observed either. In ethanol at $D = 75.5$, a slight decrease in rate (about 8%) was observed only with the highest concentration of enzyme. At $D = 72.5$, some diminution of activity was noted within the range 2–3.1 μM trypsin, but the maximum effect did not exceed 10%. Correction of data in Fig. 5 (indicated by $\times$) practically did not modify the situation. Since the results had been already corrected for substrate-blank reaction, the observed curvature cannot be ascribed to either substrate or enzyme autolysis. Then we have to reconsider the possibility of activation by high concentrations of substrate. TROWBRIDGE *et al.*[10] reported an important increase in the rate of tryptic hydrolysis of *p*-toluenesulfonyl L-arginine methyl ester (TAME) at pH 8 and 25° at high substrate concentrations. BECHET AND YON[11] could observe again this phenomenon, not only with TAME but also with *p*-toluenesulfonyl L-lysine methyl ester (TLME). The latter workers suggested that an allosteric effect is involved, namely that a second substrate molecule is bound by the enzyme giving rise to a change in the conformation of the catalytic site which is favorable to enzyme action. The binding could occur in an auxiliary site either before or after the formation of acyl-enzyme. In any of the two cases, the theoretical curves of BECHET AND YON[11] (when Eadie representation is used, that is v *vs.* $v/[S]$) can be decomposed into two straight lines joined by a curved portion. The slopes of these two lines, one in the region of low concentration and the other in that of high substrate concentration, are negative and equal $-K_m$ and $-K_m'$, namely Michaelis constants for the binding of the first and second substrate molecules. When our data of aqueous solution at pH 8.5 were represented by an Eadie plot (Fig. 6a), the slope of the line passing through points at high substrate concentration became positive which would mean negative values of K_m' and v'_{max}. This was already apparent in the Lineweaver–Burk plots of data in alcohol solutions. Yet, if v is plotted as a function of v divided by increasing powers of $[S]$ ($v/[S]^2$, $v/[S]^3$, *etc.*), it is possible at a certain $[S]$ power to turn the slope at high substrate concentrations into a negative one. Fig. 6 shows how this was achieved for $v/[S]^2$ in aqueous solution and for $v/[S]^3$ or $v/[S]^4$ for ethanol solutions of D 75.5 or 72.5, respectively. A feasible interpretation of the behavior of system LME-trypsin at pH 8.5 could be that a number of sites for

substrate binding exists in the enzyme[11–13], but not all of them are exposed in aqueous solution. As the medium dielectric strength decreases, a change of configuration might be elicited uncovering progressively these sites. In turn, binding additional substrate molecules would originate further conformational changes resulting in a more efficient catalytic action. Assuming that the slope values of Figs. 6b, c and d are equivalent to $-K_m'$ for the binding of 2–4 substrate molecules in addition to the one bound in the active site, the respective K_m' values would be $7.1 \cdot 10^{-3}$ $M^{1/2}$, $7.2 \cdot 10^{-4}$ $M^{1/3}$ and $9 \cdot 10^{-7}$ $M^{1/4}$.

Table I sums up the apparent values of K_m and k_{cat} as well as those of ratios k_4/k_3 and k_2/K_a at pH's 6.5 and 8.5 (the latter were computed from slope and intercept

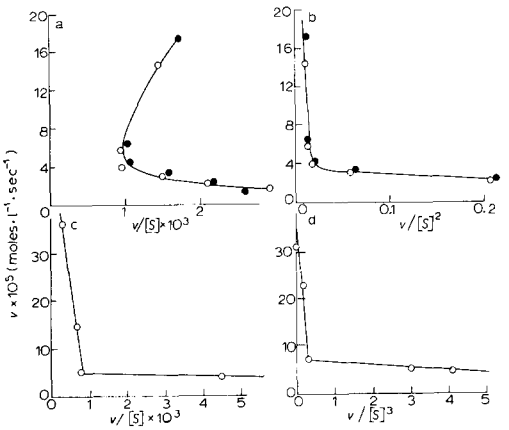


Fig. 6. Reaction velocity of the trypsin-catalyzed breakdown of LME in aqueous solution at pH 8.5 and 25° plotted as a function of $v/[S]$ (a) or $v/[S]^2$ (b). ●, $-$d[LME]/dt; ○, d[Lys]/dt. (c) and (d) plots of overall rate ($-$d[LME]/dt) in aqueous ethanol of D values 75.5 (c) and 72.5 (d) *vs.* $v/[S]^3$ and $v/[S]^4$, respectively. Conditions as in Fig. 4.

of lines through points at low $[S]$ which presumably correspond to the binding of first substrate molecule in the catalytic site). The trend of k_{cat} for the overall reaction is to increase as alcohol concentration increases especially at pH 6.5. The value of K_m increases as D decreases at pH 6.5, but the contrary occurs at pH 8.5. The apparent ratios k_4/k_3 calculated from either the catalytic rate constants or the ratio of products of alcoholysis:hydrolysis are in agreement within variation. As observed previously[1], this ratio decreases as D diminishes at pH 6.5. At pH 8.5 the reverse effect is observed. The ratio k_2/K_a increases as D decreases both at pH's 6.5 and 8.5, especially at the latter pH value. It is possible that the main dielectric effect on these trypsin-catalyzed reactions is due to an increase in the rate of acylation with respect to that of dissociation of Michaelis complex.

ACKNOWLEDGEMENT

This work was supported in part by a Frederick Gardner Cottrell grant of Research Corporation of the U.S.

REFERENCES

1 L. M. DEL CASTILLO, G. DÁVILA, L. DORANTES, C. OLIVER, R. IBARRA AND M. CASTAÑEDA-AGULLÓ, *Biochim. Biophys. Acta*, 191 (1969) 354.
2 A. N. GLAZER, *J. Biol. Chem.*, 240 (1965) 1135.
3 M. CASTAÑEDA-AGULLÓ AND L. M. DEL CASTILLO, *Biochim. Biophys. Acta*, 139 (1967) 398.
4 G. ÅKERLÖF, *J. Am. Chem. Soc.*, 54 (1932) 4125.
5 M. L. BENDER, G. E. CLEMENT, C. R. GUNTER AND F. J. KÉZDY, *J. Am. Chem. Soc.*, 86 (1964) 3697.
6 L. M. DEL CASTILLO AND M. CASTAÑEDA-AGULLÓ, in G. SOBERÓN, *Ensayos Bioquímicos*, Prensa Médica, México, D.F., 1969, p. 109.
7 J. P. WOLF AND C. NIEMANN, *J. Am. Chem. Soc.*, 81 (1959) 1012.
8 J. P. WOLF, R. A. WALLACE, R. L. PETERSON AND C. NIEMANN, *Biochemistry*, 3 (1964) 940.
9 D. W. INGLES AND J. R. KNOWLES, *Biochem. J.*, 99 (1964) 117.
10 C. G. TROWBRIDGE, A. KREHBIEL AND M. LASKOWSKI, JR., *Biochemistry*, 2 (1963) 843.
11 J. J. BECHET AND J. YON, *Biochim. Biophys. Acta*, 89 (1964) 117.
12 R. A. ALBERTY, V. MASSEY, C. FRIEDEN AND R. FUHLBRIGGE, *J. Am. Chem. Soc.*, 76 (1954) 2485.
13 R. A. ALBERTY, in D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 1, Academic Press, New York, 1959, p. 143.