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THE INFLUENCE OF A FREE α-AMMONIUM GROUP IN THE SUBSTRATE UPON TRYPSIN-CATALYZED TRANSESTERIFICATION

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SUMMARY

1. By comparison of rates, measured by using titration, of trypsin-catalyzed hydrolysis of either L-arginine methyl ester (AME) or L-lysine methyl ester (LME) with those determined by paper chromatography analysis, it was possible to ascertain that pK_a of the α-ammonium group of both esters differs by about -1.8 units from that of the respective free amino acids.

2. In aqueous alcohol solutions, the logarithm of the ratio of concentrations of products of alcoholysis to hydrolysis at a given time shows a linear relationship with the logarithm of the ratio of ionized to un-ionized species of substrate α-ammonium group.

3. The ratio L-lysine ethyl ester (LEE):lysine also decreases with temperature, an effect which at least partially might be accounted for in terms of a decreased ionization of substrate α-ammonium group.

4. The conclusion can be reached that $k_4 > k_3$ (the deacylation rate constant in alcoholysis is larger than the corresponding one in hydrolysis). Hence, the addition of alcohol exerts a driving effect on the reaction. Besides this effect, alcohols also accelerate the reaction by a dielectric constant effect similar to that produced by acetone on the trypsin-catalyzed hydrolysis of LME or to those observed with various solvents in the trypsin-catalyzed hydrolyses of α-N-substituted substrates.

INTRODUCTION

In a previous investigation[1], it was observed that the behavior of trypsin-catalyzed hydrolysis of L-lysine methyl ester (LME) differed from that of tryptic hydrolyses of α-N-substituted esters in two ways: (1) the maximum of the activity–pH

Abbreviations: AME or AEE, L-arginine methyl or ethyl ester; LME, LEE or LPE, L-lysine methyl, ethyl or *n*-propyl ester; BAEE, benzoyl-L-arginine ethyl ester; TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

profile was at 6–6.5 instead of about 8 and (2) in aqueous ethanol solutions and at pH values below 7, the rate of liberation of titratable acid was slower than in pure aqueous media. The effect of alcohols on trypsin-catalyzed hydrolyses of the α-N-acylated substrates, benzoyl-L-arginine ethyl ester (BAEE) and *p*-toluenesulfonyl-L-arginine methyl ester (TAME), results in an increased rate of liberation of titratable acid[2–4]. For these reactions, within a given range of alcohol concentration, the plot of the rate logarithm *vs.* reciprocal of the medium dielectric constant yields a straight line having a positive slope[2–4]. The trend of the effect of alcohols with these substrates remains unchanged from pH 5.5 to 8.5 (refs. 3, 4), while in the tryptic hydrolysis of LME it does vary with pH. It is retardatory in the acid region and enhancing at pH 8 or higher[1]. It was inferred that the presence of a free α-ammonium group in LME had some bearing on the effect of alcohols and that the positive charge in α position was propitious to the transesterification reaction occurring between substrate and alcohol.

Since the titration method measures only the rate of formation of lysine, in order to ascertain the rate of transesterification, a procedure was required to determine the changes of concentration of substrate and both products. In this paper we report the results of a quantitative study of transesterification reactions catalyzed by trypsin under various conditions.

METHODS

Trypsin was a twice-crystallized salt-free preparation which, as well as LME, was obtained from Nutritional Biochemicals Corp. Substrates and standards for chromatography, L-lysine ethyl ester (LEE), L-lysine and L-arginine, were from Mann Research Laboratories. The source of L-arginine methyl and ethyl ester (AME and AEE) was Sigma Chemical Co. Ethanol and *n*-propanol were of the best grade available and were redistilled prior to use. The dielectric constant (*D*) of the various solutions was calculated from data of ÅKERLÖF[5].

The rates of hydrolysis were measured by titration to a constant pH (ref. 1) and by a method involving the chromatographic separation of substrates from free amino acids and products of transesterification. This was carried out as follows. 0.5-ml samples of the reaction mixture placed in a thermostatted vessel of the pH-stat were taken at zero time and after 5 min of enzyme action. To these samples 0.1 ml 0.2 M HCl was added in order to change pH to a value below 3 and stop the reaction. The samples were frozen immediately and kept frozen until applied to paper for chromatographic analysis. This was accomplished by one-dimensional descending paper chromatography in a solvent composed of *n*-butanol, pyridine, acetic acid and water[6]. The color was developed with copper ninhydrin reagent[7]; the spots were then cut out and eluted with 4 ml of absolute methanol. The concentration of each component was determined from readings made in a Beckman DU spectrophotometer. The solutions containing the colored derivatives formed in the presence of lysine or lysine esters were read at 530 mμ (ref. 7). It was found that compounds formed in solutions of arginine or arginine-esters absorbed light with a maximum around 506 mμ, and they were read at this wavelength. The calibration curves for each compound, except L-lysine *n*-propyl ester (LPE), had been determined previously. Since in the breakdown of both LME and AME in aqueous ethanol the amount of transesterification product equaled within variation the difference between the quantity of substrate

decomposed and that of free amino acid released, the amount of LPE formed in *n*-propanol was calculated by difference. The rates of disappearance of substrate and liberation of products were calculated by substracting the final from the initial concentration of each compound (both substrates contained a small proportion of free amino acid, namely less than 1%). Each result reported here is the average of 8–12 determinations. The standard error of the method was found to be 4.5%.

RESULTS AND DISCUSSION

Comparison of the titration and chromatographic methods

On account of pK_a shifts of amino groups produced upon esterification of amino acids[8], the rates of hydrolysis of LME (or AME) obtained by titration are lower than the actual ones[1] because part of the protons set free are used to compensate for the increased basicity of product as compared to that of substrate. The fraction of the true value which can be titrated varies with pH according to the following expression:

$$\text{Fraction titrated} = 1 + \frac{1}{1 + \frac{K_a'}{H^+}} - \frac{1}{1 + \frac{K_a}{H^+}} \qquad (1)$$

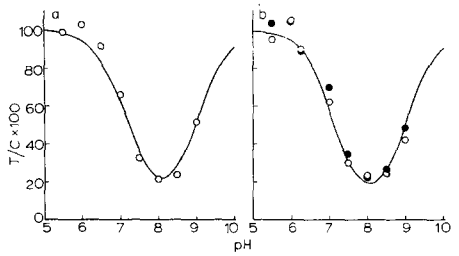
in which K_a and K_a' are the dissociation constants of α-ammonium group in the free amino acid and ester, respectively. The plot of fraction titrated *vs.* pH is an inverted bell-shaped curve with a minimum at

$$pH = \tfrac{1}{2}(pK_a + pK_a') \qquad (2)$$

Thus in an earlier work[1], it was necessary to apply a correction to the titration method in both aqueous solution and mixed solvents of a given dielectric constant, which was deduced partly on theoretical basis and partly on extrapolation of data in literature. With the aid of the chromatographic procedure, it was possible to ascertain that the values assigned previously to the pK_a of α-ammonium group in LME under the various conditions of the experiments were close to the real ones. Figs. 1a and b show the plots of the ratio of titration to chromatography value $\times 100$ (T/C $\times$ 100) obtained for the trypsin-catalyzed hydrolysis of LME at pH values ranging from 5.5 to 9 either in aqueous solution (a) or in ethanol– or *n*-propanol–water at the proportions required to yield a D value of 75.5 at 25°. The theoretical curve which best fitted (within error) the observed data in aqueous solution was that calculated ascribing pK_a values 7.2 and 9 to the α-ammonium groups of LME and lysine[9], respectively. In alcohol solutions the pK_a values were computed by adding to those in water the ΔpK_a calculated formerly[1], resulting in 9.02 for lysine, 7.2 for LME in acid media and 7.08 for the same compound in alkaline solution. Upon esterification, the pK_a of the amino group in α-amino-*n*-caproic acid changes from 10.75 to 10.37 (ref. 8). Assuming a similar shift of pK_a for the α-ammonium of lysine, it can be calculated that the error of titration caused by it would be negligible up to pH 9, which is in accordance with the results obtained.

Hydrolysis and alcoholysis catalyzed by trypsin

Fig. 2a shows the rates of breakdown of LME and formation of lysine in aqueous solution determined chromatographically as a function of pH from 5.5 to 9 at 25°.



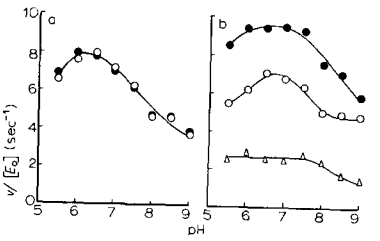


Fig. 1. Fraction titrated of the lysine set free by the action of trypsin on LME at various pH values and 25° in aqueous solution (D = 78.5) (a), or in water-alcohol solution (D = 75.5) (b). The points represent experimental values: ● in (b), ethanol–water solutions, ○, n-propanol–water. The solid lines were calculated assuming the following pK_a values for the α-ammonium group: lysine 9 at D = 78.5 and 9.02 at D = 75.5; LME 7.2 either at D = 78.5 or at D = 75.5 and pH values up to 7, and 7.08 at D = 75.5 and pH values from 7 upward. The reaction mixture contained initially 0.0125 M LME and $3.12 \cdot 10^{-6}$ M trypsin.

Fig. 2. Profiles pH–rates of formation of products and substrate decomposition measured chromatographically in the trypsin-catalyzed breakdown of LME at 25° in water (D = 78.5) (a), and in aqueous n-propanol solutions (D = 75.5) (b). ○, d[Lys]/dt; ●, $-$d[LME]/dt; △, d[LPE]/dt. Conditions as in Fig. 1.

As expected these are equal within error. In aqueous n-propanol (Fig. 2b), both rates are distinct because of the appearance of LPE. In agreement with results published earlier[1], the rate of liberation of lysine is slower in alcohol solutions than in water at pH values below 8, about the same at this pH and higher in more alkaline media. However, the reaction rate measured from substrate decomposition is always faster in alcohol solution than in water. The rate of transesterification is sensibly constant from pH 5.5 to 7.5 and then decreases progressively as pH increases. The qualitative behavior of the reaction trypsin–LME in aqueous ethanol (Fig. 3) is very similar to that observed in n-propanol solutions, but the relative proportion of alcoholysis product is greater with ethanol than with n-propanol. This result is not consistent with the conclusions reached by SEYDOUX *et al.*[10,11] concerning the efficacy of primary aliphatic alcohols for competing with water in the deacylation of L-lysyltrypsin; the longer the apolar chain the more effective the alcohol, transesterification with n-propanol being thus faster than with ethanol. Actually these authors did not measure concentrations of LEE or LPE; they calculated the deacylation constants from the rates of lysine liberation by an indirect kinetic method in the presence of various concentrations of alcohol. Again, the experimental conditions under which SEYDOUX *et al.*[10,11] worked are distinct from those of the experiments reported here, namely pH 4.8 and with 25 mM $CaCl_2$ in the medium. $CaCl_2$ modifies the esterolytic activity of trypsin through a general effect of ionic strength[12]. In addition to this, the effect of this salt on the stability of trypsin has been related to a possible change of conformation[13]. Hence, it is conceivable that interactions of lysyltrypsin and alcohols are not entirely the same either in the absence or presence of calcium or at various concentrations of H^+. Another interpretation of the apparent discrepancy could be that LPE is formed faster than LEE but further hydrolysis of the former ester occurs to a greater extent than that of LEE.

The conclusions reached previously[1] concerning the influence of a free α-ammonium group in the substrate were reached through the comparison of α-N-acylated

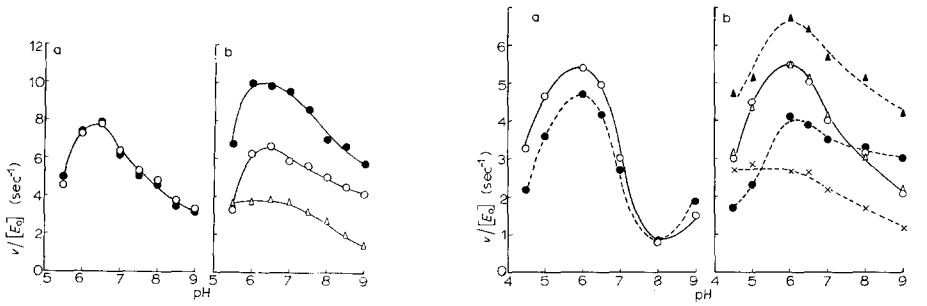


Fig. 3. Profiles pH–rate of substrate decomposition and product formation determined chromatographically in the trypsin-catalyzed breakdown of LME in aqueous ethanol solutions (b). (a) Water controls. ○ and ●, d[Lys]/dt and $-$d[LME]/dt respectively; △, d[LEE]/dt. Conditions as in Fig. 1.

Fig. 4. Profiles pH–rate of the trypsin-catalyzed hydrolysis and alcoholysis of AME at 25°. Curves in (a): data obtained by titration (without correction for pK_a shift): ○—○, in aqueous solution D 78.5; ●— — —●, in aqueous ethanol D 75.5. Curves in (b): chromatographic values: ○—○ and △—△, in aqueous solution; ●— — —● and ▲— — —▲, in aqueous ethanol. ○ and ●, d[Arg]/dt; △ and ▲, $-$d[AME]/dt; ×, d[AEE]/dt. The reaction mixture contained initially 0.0125 M AME and $3.12 \cdot 10^{-6}$ M trypsin.

esters of arginine with an unsubstituted lysine ester. The question arose as to whether the change of a guanidinium group to an ε-ammonium had some bearing on the observed effects. In order to elucidate this problem, the reaction rates of the trypsin-catalyzed hydrolysis and alcoholysis of AME were determined within a pH range of 4.5 to 9 at 25° in aqueous solution (D = 78.5) and ethanol–water solution (D = 75.5) under the same conditions of the previous experiments with LME. Fig. 4a shows the rates measured by titration without any correction for pK_a shifts. The pK_a of the α-ammonium group of arginine, like that of lysine, is close to 9 (ref. 9), and presumably upon esterification it undergoes a shift of the same order as that of lysine. Fig. 4b shows the rates of substrate decomposition, arginine liberation and formation of AEE, measured chromatographically. It can be seen that the behavior of the system trypsin–AME is quite similar to that of trypsin–LME in so far as the effects of both pH and alcohol are concerned.

Relationship between degree of ionization and transesterification

It has already been suggested[1] that the predominance of the transesterification effect of alcohols over the dielectric one (when measured by titration) in the acid region might have some bearing on the degree of ionization of the α-ammonium group of substrate. If so, there must be a correlation between the rate of transesterification relative to that of hydrolysis and the ratio of ionized to uncharged α-amino group in substrate (R–α-NH^+_3:R–α-NH_2). The ratios AEE: arginine and LEE:lysine at 5 min were calculated from the previous experimental data, and the proportion of R–NH_3^+ to R–NH_2 computed ascribing to pK_a of the α-amino group of AME and LEE the value 7.2 which is consistent with the results presented in this paper. As can be seen in Fig. 5, there is a linear relationship between the two ratios provided that they are plotted on a logarithmic scale. The correlation coefficient is 0.96.

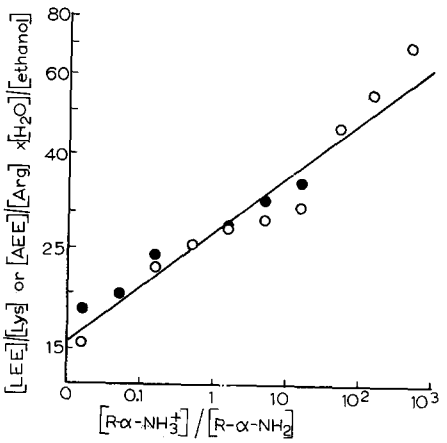


Fig. 5. Ratio of products of alcoholysis: hydrolysis as a function of the relative proportion of ionized to uncharged α-ammonium group in the substrate: LME (○) or AME (●). Conditions as in Figs. 1 and 4.

According to current views[14], the reaction of proteolytic enzymes with their substrates proceeds in three steps: (1) formation of Michaelis complex *ES*, (2) an intermediate compound acyl-enzyme *ES'* (lysyl- or arginyltrypsin in the present case) is formed at the same time that the first product P_1 (methanol) is released, and (3) the acyl-enzyme is deacylated in the presence of any nucleophile such as water, alcohols, hydroxylamine, *etc.* In solvents composed of two nucleophilic compounds, *e.g.* water and ethanol, there is a partition of acyl-enzyme between the nucleophiles and two more products P_2 (arginine or lysine) and P_3 (AEE or LEE) are formed:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \begin{cases} \xrightarrow{k_3[H_2O]} P_2 \\ \xrightarrow{k_4[\text{ethanol}]} P_3 \end{cases} \qquad (3)$$
$$\searrow P_1$$

It may be inferred from Scheme 3 that the relative proportions of LEE and lysine or AME and arginine would depend upon the values of k_4 and k_3, on the one hand, and upon the ratio of concentrations of alcohol to water, on the other. Again, if there were no effect of alcohol other than that due to alcoholysis, the ratio LEE:lysine would vary with alcohol concentration according to the relation:

$$\frac{[\text{LEE}]}{[\text{lysine}]} = \frac{k_4\,[\text{ethanol}]}{k_3\,[H_2O]} \qquad (4)$$

If k_4 and k_3 remained constant when alcohol concentration was varied, the product $[\text{LEE}]/[\text{lysine}] \times [H_2O]/[\text{ethanol}]$ would be constant too. However, alcohol like acetone decreases the medium dielectric constant, and it has been shown that the latter compound, without competing with water for the acyl group, does accelerate the trypsin-catalyzed hydrolysis of LME[1]. Consequently, if alcohol exerts the same effect as acetone, it must modify the rate of the reaction we are dealing with by two mechanisms: alcoholysis and dielectric effect. If the latter resulted in any modification

of the ratio $k_4:k_3$, the product [LEE]/[lysine] × [H_2O]/[ethanol] would no longer remain constant as alcohol concentration is increased. In order to investigate whether this occurs, the following experiment was carried out.

Effect of temperature and ethanol concentration on the quantity [LEE]/[lysine] × [H_2O]/[ethanol]

The ratio of the products of alcoholysis to hydrolysis was determined at 5°, 15°, 25° and 35° (pH 6.5) in solutions containing 6.6 and 13.2% (by vol.) ethanol. The results multiplied by the ratio of molarities of water : ethanol were plotted in logarithmic scale against reciprocal of absolute temperature. Fig. 6 shows the two lines obtained;

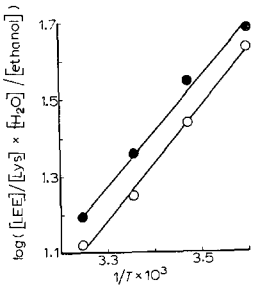


Fig. 6. Plot of log [LEE]/[Lys] × [H_2O]/[ethanol] *vs.* $1/T$ for the trypsin-catalyzed alcoholysis and hydrolysis of LME at two di-electric constant values: 75.5 (●) and 72.5 (○). Conditions as in Fig. 1.

the values at each temperature differed significantly (maximum P = 2%) for the two ethanol concentrations, suggesting that alcohol influences the reaction by two distinct mechanisms. The rate of transesterification relative to that of hydrolysis decreases as temperature is increased. Even though this effect may involve various parameters, it is of interest to point out that the ionization of amino groups diminishes as temperature is elevated. The apparent heat of ionization of α-ammonium in the lysyl residue of lysyllysine is 11 350 cal/mole, and a very close value has been given for this group in arginine[8]. Taking 7.2 as pK_a value of α-ammonium in AME or LME at 25°, it can be calculated that this would vary from approx. 7.8 at 5° to 6.9 at 35°. It seems that, at least in part, the effect of temperature like that of pH may be accounted for in terms of a modified degree of ionization of substrate.

Conclusions

Even though the situation becomes complicated because the transesterification product is also a substrate for trypsin and

$$\frac{[\text{LEE}]}{[\text{lysine}]} \times \frac{[H_2O]}{[\text{ethanol}]} \neq \frac{k_4}{k_3}$$

the results indicate that the rate constant of deacylation by alcohol k_4 is faster than the corresponding rate constant k_3 when water functions as acceptor of the acyl group. Similar observations have been made by BALLS AND WOOD[15] and BENDER AND GLASSON[16] in α-chymotrypsin-catalyzed reactions. Therefore, the addition of alcohol

increases the overall rate of substrate decomposition. Besides this, alcohol also exerts a dielectric constant effect similar to that which has been observed in the trypsin-catalyzed hydrolysis of BAEE or TAME[1-4]. The results presented here support the previous supposition that the rate of transesterification relative to that of hydrolysis depends upon the proportion of charged α-ammonium group in the substrate. However, the mechanism by which a positively charged group near the solvolyzable bond favors alcoholysis remains to be elucidated.

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