Kinetics of Papain Action

III. HYDROLYSIS OF BENZOYL-L-ARGININE ETHYL ESTER*

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The kinetics of hydrolysis by papain of several synthetic substrates has been previously reported (1-3). For these compounds, the Michaelis-Menten formulation was found to be applicable on the assumption that $k_0 \gg k_1$ and therefore that $K_m = k_0/k_1$ and hence $k_1 = k_0/K_m$ where

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_0} P + E \quad (1)$$

Evidence was obtained that two specific groups of the enzyme molecule participate in the $k_1$ step, namely, an ionized carboxyl group and an un-ionized thiol group.

A study has now been made of the hydrolysis of a typical ester substrate, benzoyl-l-arginine ethyl ester (BAEE) in order to compare the kinetics of hydrolysis of this compound with the kinetics of the corresponding amide. The higher precision obtainable with the ester substrate has not only confirmed the previous conclusions but has also revealed the existence of some anomalous data both with the ester and the amide caused by the presence of a minor impurity. The significance of the data and a proposed mechanism of action of the enzyme will be discussed below and in a subsequent paper (4).

EXPERIMENTAL

Materials—BAEE was prepared as previously described (5). Kinetic evidence has indicated that the compound still contained 2 to 3 per cent impurity (see below) after two recrystallizations. The substance was finally obtained pure by the following chromatographic procedure. An alumina column was prepared by suspending basic alumina (Woelm, activity Grade 1 for chromatographic analysis) in a solution of alcohol-ether (1:1). The suspension was poured into a 20 x 3-cm. glass column. To a solution of 4 gm. of BAEE in 20 ml. of ethanol were added 10 drops of bromcresol green and 10 drops of methyl red which served as indicators. The BAEE solution was passed through a second column consisting of 1:1 charcoal and Hyflo Super Cel (Johns-Mansville Company). After passing through the column the BAEE solution was colorless. The solvent was removed under reduced pressure at 40°C; recovery was of the order of 65 per cent. No ninhydrin-positive material could be detected in BAEE. On several paper chromatographic systems, only one Sakaguchi test-positive spot was observed. $[\alpha]^2_D = 17.5^\circ$ (2 per cent, water) for BAEE hydrochloride.

Crystalline mercurypapain was prepared from commercial, dried papaya latex by the method of Kimmel and Smith (6).

Methods—Hydrolysis of BAEE was studied at 25°C and 37°C over the pH range of 3.75 to 8.5. The rate of hydrolysis was estimated from the amount of base consumed while a constant pH was maintained during the reaction.

A pH-stat (Radiometer type TTT11a) was used to allow automatic addition of NaOH, this being regulated by means of a magnetic valve. A proportional band setting controlled the opening and closing of the valve, and by this mechanism, very small increments of alkali could be added as the pH setting was approached so that precision was limited only by the mixing time of the reaction mixture. The apparatus and method are fully described by Jacobsen et al. (7).

The electrodes used were a glass macroelectrode, Type G 202AT, and a calomel electrode, Type K100-4. Since no temperature compensator was used in these experiments, the electrodes were calibrated by readings with standard phthalate buffer at 25°C in a Cambridge Instrument Company, Inc., model R pH apparatus. No significant differences were observed at 25°C but a correction had to be applied at 37°C; this correction was characteristic for each glass electrode and constant over a period of at least 2 months.

The reactions were performed in a 50 ml-beaker immersed in a constant temperature water bath which was regulated to within 0.1°C. An air stirrer was used for mixing. For most of the points measured, adequate precision was obtained at five substrate concentrations: 0.05, 0.04, 0.03, 0.02, and 0.01 M. At the extremes of pH where $K_m$ values were found to increase abruptly, duplicate determinations with substrate concentrations of 0.06, 0.05, 0.04, and 0.03 M were made. Ionic strength was adjusted to 0.30 with 2.5 M KCl, a concentration previously determined to be optimal for activity (2). pH was maintained at

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1 The following abbreviations are used: BAEE, α-benzoyl-l-arginine ethyl ester; BAA, α-benzoyl-l-argininamide; BAL, 2,3-dimercaptopropanol.

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We are indebted to Dr. Philip P. Gray of the Wallerstein Company, Inc., for furnishing us with this material.

Radiometer, Copenhagen.

Glass microelectrodes were unsatisfactory. Of the three tests used, all were somewhat unstable at the temperature of hydrolysis.

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by the instrument; no buffer was necessary over the whole pH range. The pH-stat was adjusted to pH 4.01 with 0.05 M potassium hydrogen phthalate at the temperature for hydrolysis. The liquid junction of the calomel electrode was renewed between successive runs to avoid poisoning by BAL. The beaker which contained substrate and KCl was placed in position and the contents were stirred until temperature equilibrium was reached. 0.02 ml of BAL (0.0095 M) was introduced and the reaction mixture was brought to the desired pH value by addition of 1 M NaOH. The desired pH value was always exceeded by 0.1 to 1.5 pH units.

The enzyme was then added and the time of the first burette reading was recorded when the pH fell to its correct value; this allowed time for activation of the enzyme and for mixing. Burette readings were recorded at 30-second intervals for 6 minutes. The amount of enzyme added was chosen so that not more than 10 per cent hydrolysis would result in this interval, ensuring thereby a linear rate of hydrolysis with respect to time over the pH range measured. Velocities were calculated as moles per l. per second. $k_0$ and $K_m$ were determined from initial velocities as previously described utilizing a method of least squares (2). $k_1$ was calculated from the relationship $K_m = k_0/k_1$.

Enzyme concentration was estimated by the velocity of control runs at pH 5.23 and 0.06 M BAEE at the temperature of hydrolysis. This velocity was related to the initial rate of hydrolysis of BAEE at 38° under standard conditions, and the enzyme concentration was calculated from this relationship.

A factor for converting the velocity of the control at a given temperature to that at 38° was obtained by measuring the velocities at both temperatures with the same enzyme solution.

RESULTS

A plot of $K_m$ versus pH at 25° and at 37° is given in Fig. 1. The curves at both temperatures are smooth, continuous functions over the pH range measured. The form of the curves is similar to that previously obtained with BAEE (2) except in the region between pH 6.0 and 7.5. The discrepancy in this region will be discussed below. It should be noted that the values for the pH range 6.0 to 7.5 included in Fig. 1 were obtained with highly purified BAEE.

Fig. 2 shows $k_0$ to be independent of pH over most of the range at both temperatures. The values at 25° are more precise, probably because 37° is close to the upper limiting temperature of the glass electrode. Of interest are the decreasing values of $k_0$ below pH 4.5 evident at both temperatures. Unfortunately, the data in this acid pH region are not as precise because of enzyme instability. At pH 3.5 hydrolysis is linear with time only during the first 3 minutes.

If it is assumed that the curve for $k_0$ as a function of pH is that of a partial titration curve, an approximate pH' can be calculated from the pH at half of the maximal $k_0$ value. At 37° the pH' value is approximately 3.7, and at 25° it is approximately 3.5.

On the assumption that $K_m = k_0/k_1$ (2, 3), values for $k_1$ can be computed. Fig. 3 shows these calculated values of $k_1$ as a function of pH on the basis that maximal values are equal to 100 at both temperatures. In absolute terms, the maximal $k_1$ value at 37° is 1.7 times that at 25°. It is evident that both limbs of the curves resemble titration curves, and theoretical titration
FIG. 3. Calculated values of $k_1$ as a function of pH for hydrolysis of benzoyl-L-arginine ethyl ester at 25° and 37°. The curves drawn are theoretical as calculated from the mass-law equation for single, titratable groups. Values of $k_1$ are given in terms of the maxima being equal to 100 in order to compare the data at the two temperatures. The maximal average value for $k_1$ at 37° is 660 mole⁻¹ sec⁻¹; at 25°, the average $k_1$ at the maximal is 390 mole⁻¹ sec⁻¹.

curves have been drawn through the data. It is striking to find that the curves drawn are essentially identical with those previously obtained for the hydrolysis of carboxbenzoxyl-L-histidinamide and hippurylamide (3) and similar to the curve obtained with BAA (2).

On the acid side, it was found that, as with other substrates of papain, temperature has little effect on the pK' value of the group titrated, and again the conclusion that an ionized carboxyl group is involved in determining $k_1$ is warranted (2, 3).

From the shift in pK' with temperature of the alkaline limb of the $k_1$ data, the apparent heat of ionization is 5.1 kilocalories per mole at 0°, a figure in reasonable agreement with the values obtained earlier (3) and within the range for that of sulfhydryl groups (9).

Comparison of the pK' values (acidic limb) with earlier data (Table II of reference (3)) shows that these are similar for all substrates studied. The value found is 4.3 for BAEE and carboxbenzoxyl-L-histidinamide, the substrates for which the most precise data are available. Moreover, with both of these substrates pK' is independent of temperature.

The pK' values of the substrates are noticeably temperature-dependent. For comparative purposes, the only common temperature is 38°, and for BAEE and the 4 substrates studied earlier (2, 3), all of the values are in the range of 8.0 to 8.2.

Anomalous Data—It was mentioned above that BAEE which has been recrystallized twice still contains an impurity as judged in terms of anomalous kinetic data in the region of pH 6.0 to 7.5 at 37°. This is reflected in a distortion of the curves relating $K_m$, $k_0$, and $k_1$ to pH in this region. Since no similar effect was noticed in the data at 25°, it is likely that the degree of distortion is related to enzyme concentration which, relative to substrate concentration, is considerably lower at 37° than at 25°. Since the effect is limited to a narrow pH region, it is possible that the interfering impurity contains an ionizable group.

In Fig. 4 the data on the effect of pH on $k_1$, $k_0$, and $K_m$ at 37° are shown; these are the same as those given in Figs. 1, 2, and 3, except that, in the pH range from 6.0 to 7.0, only values obtained with impure BAEE as substrate are included. Repetition of determinations at points outside this critical pH region with purified substrate gave excellent reproduction of values.

A of Fig. 4 shows that maximal inhibition of $k_1$ occurs near pH 6.5, the lowest point corresponding to an apparent inhibition of the order of 25 per cent. That the points are lowered by the presence of an impurity was established by repeated recrystallization of the substrate which progressively raised the points until they approximated the theoretical curve. BAEE purified by the chromatographic method described in an earlier

![Fig. 4](https://www.jbc.org/content/378/1/1389/F4)

Fig. 4. Results obtained with impure BAEE at 37° in comparison with data previously found by Stockell and Smith (2) with BAA. (A) $k_1$ as a function of pH; the solid curve shows results with purified BAEE. (B) $k_0$ as a function of pH. (C) $K_m$ as a function of pH. The continuous line shows results with purified BAEE.
section also gave points that fell on the theoretical curve and afforded better recovery of substrate.

If all values for $k_i$ in the pH region of 5 to 7.5 are averaged, as was the procedure in plotting the results of $k_i$ versus pH for BAA (2), a flat-topped curve is obtained, as is shown in Fig. 5. It agrees very closely with the data, also given in Fig. 5, previously reported for BAA. Since the distortion apparent in the $k_i$ curve was caused by an impurity in BAEE, and since the $k_i$ curve of BAA shows exactly the same type of distortion, it can be concluded that the same or a similar impurity was present in BAA also. Such a supposition is very likely in view of the fact that the amide is prepared from the ester.

The effect of the impurity on $k_i$ is shown in B of Fig. 4. Here again the data represent points obtained with impure BAEE, and the data of Stockell and Smith (2) on BAA are included for comparison. The similarity of the curves is evident. In this case, purification of substrate resulted in a lowering of the points in the critical pH region until they approached the curve drawn.

Finally, the anomalous behavior with impure BAEE is shown by increased values of $K_n$ with a maximal at pH 6.5 (C of Fig. 4). The data are essentially the same as those obtained with BAA. Once again, purification of substrate lowered the values until they approximated the smooth curve drawn, as would be expected since $k_i$ values are obtained from the relationship, $k_i = k_o/K_n$.

Attempts to concentrate the small amount of impurity present in BAEE, after separation on alumina as described above, have indicated that the substance gives a positive ninhydrin reaction. The nature of this compound has not been established but it appears to behave like arginine ethyl ester hydrochloride. If such prove to be the case, it is likely that the anomalous kinetics is produced because of the occurrence of transfer reactions. It has been established that papain will catalyze such reactions with high efficiency in the presence of compounds containing free amino groups and will lead to formation of new derivatives including polymers (10-12). Presumably this will result in the observed effects, namely, an apparent increase in $k_o$ and a decrease in $k_i$ in the critical region where the amino compound acts as an acceptor.

**DISCUSSION**

The data for the kinetics of hydrolysis of BAEE afford additional evidence of the validity of most of the conclusions published earlier (2, 3). The fact that substrates, which differ not only with respect to the amino acid residue (arginine, histidine, glycine, and glycyl-glycine) but also with respect to the nature of the susceptible bond (amide or ester), show the same general behavior, indicates that the reaction mechanism must be identical in all cases.

In a consideration of possible mechanisms, the first feature to be noted is that positively charged substrates are hydrolysed by far the most readily and that substrates which bear a negative charge, such as carbobenzoxyglycylglycine, show inhibition of hydrolysis in pH regions where this negative charge is effective; these considerations are discussed more fully elsewhere (3, 12). It is therefore proposed that interaction occurs initially between substrate and enzyme at least in part by attraction of oppositely charged groups and that the enzyme bears a negative charge which is responsible for this effect (3, 12).

The similarity of the curves obtained for $k_i$ as a function of pH for neutral (hippuramido) or basic substrates (BAEE, BAA, and carbobenzoxyhistidinamide) is of even greater significance. Although the absolute order of magnitude for $k_i$ differs greatly with these substrates, the form of the curves, excluding the anomalies discussed above, shows the same general features; the main point is that two titratable groups, an ionized carboxyl and an un-ionized thiol group, are essential in the enzyme.

From the data presented above for the hydrolysis of BAEE, it is evident that over most of the measured range, $k_o$ is independent of pH. These findings clearly exclude the participation of hydrogen ion or hydroxyl ion as rate-limiting factors in the reaction step governed by $k_o$. Moreover, the decrease in $k_o$ below pH 4.5 indicates the participation of a titratable group in the $k_o$ step. The approximate pH$^*$ value of 3.5 to 3.7 and the apparent lack of temperature effect on this value suggest that a carboxyl group is involved and that this group must be ionized in order to obtain maximal $k_o$. The data obtained with BAA at 38° show the same characteristics of pH independence over most of the range and a decrease below pH 4.0. The peak at pH 6.5 is almost certainly the result of an artifact, as in the case of impure BAEE.

It is of interest to note, with respect to the $k_o$ data of BAA, that Stockell and Smith (2) found it necessary to postulate more than one reaction step to explain the anomalous behavior at different temperatures and changes in dielectric constant. They reported (a) a shift in pH optimum of $k_o$ at different temperatures, (b) deviations from linearity of plots in $k_o/T$ against $1/T$ at 5°, and (c) lack of a consistent effect of changes in dielectric constant on $k_o$ under various conditions. Stockell and Smith (2) assumed that two different steps were involved, steps influenced differently by pH and temperature, and that the slower one became rate-limiting under given conditions. It should be noted that certain of the earlier results with BAA can now be explained equally well by assuming the presence of an impurity of which the influence on the kinetics is affected by
both pH and temperature, as in the present studies with BAEE. In particular, it now appears that at least part of the anomalous findings is the result of a minor impurity, and that part is attributable to inactivation of the enzyme at very alkaline pH values, particularly at high temperatures. It thus appears that the effect of pH on $k_0$ and $k_1$ for the hydrolysis of BAA should be the same as for the hydrolysis of pure BAEE.

It has already been noted (3) that there is a definite relationship between the magnitude of $k_0$ and $k_1$ for different substrates. The absolute values for these constants are very similar for BAEE and BAA. Hence, the ester substrate falls into line with other compounds studied. Inasmuch as an ionized carboxyl group of the enzyme is involved in both $k_0$ and $k_1$, and the two velocity constants are clearly related, it is evident that the same carboxyl group is probably involved in both the formation and breakdown of the intermediate enzyme-substrate complex.

Clarification of the effect of pH on $k_1$ and on $k_0$ has now aided in the development of a more consistent picture of the reaction mechanism and the nature of the active site of papain. These views are developed in another paper (4).

**SUMMARY**

The kinetics of hydrolysis of benzoyl-l-arginine ethyl ester by crystalline papain has been investigated at 25° and 37°. As has been found in earlier experiments with synthetic amide substrates, a temperature-insensitive, ionized carboxyl group ($pK' = 4.3$) and an un-ionized, temperature-dependent sulfhydryl group ($pK' = 8.0$ at 37°) participate in the initial rate-limiting step governed by $k_1$. The characteristics of the $k_0$ data suggest participation of an ionized carboxyl group in this reaction step also. The fact that a relationship exists between the two velocity constants suggests that the same carboxyl group is involved in both formation and breakdown of the enzyme-substrate complex.

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