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Ficin-Catalyzed Reactions. Hydrolysis of α-N-Benzoyl-L-Arginine Ethyl Ester and α-N-Benzoyl-L-Argininamide[1, 2]

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Abstract. The effect of pH on the hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) by a proteolytic enzyme component purified from *Ficus carica* var. Kadota latex has been studied in detail over the pH range of 3 to 9.5. k_{cat}(lim) values for the hydrolysis of BAEE and BAA were essentially identical (5.20 and 5.01 sec^{-1}, respectively at 30°). k_{cat} values for hydrolysis of BAEE and BAA were dependent on prototropic groups with apparent pK values of 4.24 and 8.53 and 4.10 and 8.59, respectively. k_{cat}(lim) values for tht hydrolysis of BAEE and BAA were essentially identical (5.20 and groups of pK 4.33 and 8.60 and 4.55 and 8.51, respectively. Thus the pH optimum is 6.5 for both substrates. K_m(app) values for BAEE and BAA were 3.32 $\times 10^{-2}$ M and 6.03 $\times$ 10^{-2} M respectively over the pH range of 3.9 to 8.0. These data are interpreted in terms of the involvement of a carboxyl and a sulfhydryl group in the active center of the enzyme. The data do not support the concept that deacylation of the acyl-enzyme is completely the rate controlling step in the hydrolyses. Rather, it appears that the magnitude of k_2 and k_3 are not greatly different.

The 2 plant proteolytic enzymes, papain and ficin, have a number of similar properties (28). These include substrate specificity, inhibition by sulfhydryl reagents, molecular size, amino acid sequence around the essential sulfhydryl group, isoelectric point, and heat stability. Therefore, papain and ficin may be considered as homologs even though the genera from which they are obtained, *Carica* and *Ficus*, are not closely related botanically (8). *Carica* belongs to the order of Cucurbitales while *Ficus* belongs to the order of Urticales.

Most of the definitive work on these enzymes have been carried out with papain because it can be obtained readily in a crystalline state (10) and appears to be reasonably homogeneous (4, 29, 33). For papain, Smith and collaborators have determined many of the physicochemical characteristics (4, 10, 27, 29, 31, 33), have essentially elucidated the primary sequence (14) and have done extensive kinetic work on the enzyme (10, 26, 27, 30, 32). Their kinetic results have been confirmed and extended by a number of other workers (1, 3, 6, 11, 12, 18, 22-25, 35, 37).

Much less effort has been devoted to a study of the properties of ficin. The only definitive reports on the influence of pH on ficin-catalyzed hydrolyses of synthetic substrates are by Bernhard and Gutfreund (2) and Hammond and Gutfreund (5). In these cases, the ficin used was later shown to contain a minimum of 9 proteolytic enzyme components (21). This marked heterogeneity of ficin and the difficulty of purifying any one of the components to an acceptable state of homogeneity has discouraged extensive work on this enzyme.

This paper describes the effect of pH on the hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) by a homogeneous preparation of ficin from *Ficus carica* variety Kadota.

Experimental Procedures

Ficin D was purified from *F. carica* variety Kadota latex by a modification of the method described previously (13, 21). The latex was completely inactivated with *p*-chloromercuribenzoate (PCMB) and 1 $\times$ 10^{-5} M PCMB was incorporated into all eluting solutions used for chromatography. Ficin D was found to be homogeneous by a number of criteria (13, Kramer and Whitaker, unpublished data). The stock enzyme (in small aliquots) was stored frozen. Protein concentration was determined at 280 mμ in a Beckman DU spectrophotometer by use of E_{280} 32,400 (based on molecular weight of 15,500 (13, Kramer and Whitaker, unpublished data).

BAEE hydrochloride (lot no. L2690 and R1214) and BAA hydrochloride (lot no. 2766) were from

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Mann Research Laboratories. Ninhydrin, N-ethylmaleimide and L-leucine were from Nutritional Biochemical Corporation, and hydrindantin was from Sigma Chemical Company. All other chemicals were reagent grade and deionized water was used throughout.

Initial rates (less than 4%) of hydrolysis of BAEE were determined in a Radiometer pH-Stat (Titrator 11, Titragraph SBR 2c, pH meter 25, buret SUB1a). The temperature was maintained constant at 30.0° by circulating water from a thermostatically-controlled water bath through the water jacket of the reaction vessel. All reactions were carried out under nitrogen (CO_2-free and saturated with water). At pH 8.5 and above, the observed initial rates were corrected for non-enzyme-catalyzed hydrolysis. Below pH 5.5 the incomplete ionization of the product, α-N-benzoyl-L-arginine (BA), was corrected for by use of pK 3.24 for BA (35). The reaction mixture contained 0.01 M buffer, 0.001 M Versene, 0.005 M cysteine, variable amounts of substrate and 3.14×10^{-6} M enzyme in a total volume of 3 ml. Sufficient KCl was included to give a final ionic strength of 0.3 in all cases. Acetate buffer was used from pH 3.0 to 5.4, phosphate from pH 5.7 to 8.3 and borate from pH 8.6 to 9.5. After thermal and pH equilibration of the solution, the reaction was initiated by addition of a small aliquot of enzyme. The pH was maintained constant by addition of standardized 0.1 N NaOH.

Initial rates of hydrolysis of BAA were determined by the photometric ninhydrin method (19). The reaction mixture contained 0.2 M buffer, 0.005 M mercaptoethanol, 0.001 M Versene, variable amounts of substrate and 6.27×10^{-7} M enzyme in a total volume of 1.5 ml. Sufficient KCl was included to give a final ionic strength of 0.3 in all cases. Acetate, phosphate and borate buffers were used at pH 3.0 to 5.7, 5.7 to 8.3 and 8.3 to 9.5, respectively. In the case of phosphate at pH 7.0 through 8.3, the concentration of buffer was reduced appropriately to keep the ionic strength at 0.3. The reaction was initiated by addition of enzyme and the reaction was carried out in a thermostatically controlled water bath at 30.0°. At 5-min intervals, between 0 and 40 min, 0.1 ml aliquots of the reaction mixture were withdrawn and added to 1.0 ml of 0.1 M HCl to terminate the reaction. The ninhydrin assay for ammonia was carried out in the presence of 1.33×10^{-3} M N-ethylmaleimide to prevent interference of the mercaptoethanol (34). The ammonia concentration was determined from a standard curve prepared with L-leucine and use of a color factor of 0.97 for ammonia. Initial rates were determined from a tangent drawn to the initial portion of the progress curve. Correction for non-enzyme-catalyzed hydrolysis at the extremes of pH was found not to be necessary.

The data were analyzed according to the method of Lineweaver and Burk (15) by an appropriate least squares computer program. Standard deviations of the data are also included.

pK values for the pH dependence of k_{cat} and k_{cat}/K_m(app) were calculated by the method of Hammond and Gutfreund (5) by use of equation I

$$V^o/V^i = 1 + [H^+]/K_1 + K_2/[H^+] \quad (I)$$

where V^o and V^i are the limiting values at the optimum pH and values at other pHs, respectively, and K_1 and K_2 are the apparent ionization constants of the prototropic groups involved. The theoretical curves in Fig. 2 and 3 were calculated from the data of table I by use of equations II and III, respectively

$$k_{cat}/K_m(app) = k_{cat}/K_m(app))lim/(1 + [(H^+)/K^a_1] + K^a_2/(H^+)]) \quad (II)$$

$$k_{cat} = k_{cat}(lim)/(1 + [(H^+)/K^b_1] + K^b_2/(H^+)]) \quad (III)$$

where K^a_1 and K^a_2 and K^b_1 and K^b_2 are the apparent ionization constants for $k_{cat}/K_m(app)$ and k_{cat}, respectively.

Results and Discussion

The effect of pH on the rate of hydrolysis of BAEE and BAA by ficin is shown in Fig. 1 to 4 and table I.

The limiting values of k_{cat} and the effect of pH on k_{cat} are, within experimental error, identical for both BAEE and BAA. These results are in agreement with the more limited results of Hammond and Gutfreund (5) who reported that k_{cat} for BAA was 0.9 ± 0.1 of the value of k_{cat} for BAEE (which was 2.5 sec^{-1} at 25°). They have interpreted their results to mean that the rate controlling step in the hydrolysis of both BAEE and BAA is the deacyla-

Table I. *pH Dependencies, K_m(app), and Limiting Values of k_{cat} and k_{cat}/K_m (app) for the Ficin Catalyzed Hydrolyses of α-N-Benzoyl-L-Arginine Ethyl Ester (BAEE) and α-N-Benzoyl-L-Argininamide (BAA) at 30.0°.*

Substrate	Apparent pK				Limiting values		
	$k_{cat}/K_m(app)$		k_{cat}		$k_{cat}/K_m(app)$ (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	$K_m(app)$ (M × 10^2)
BAEE	4.33	8.60	4.24	8.53	160.4	5.20	3.32[1]
BAA	4.55	8.51	4.10	8.59	93.8	5.01	6.03[2]

[1] Average value over the pH range of 3.9 to 8.0.
[2] Average value over the pH range of 3.0 to 9.5.

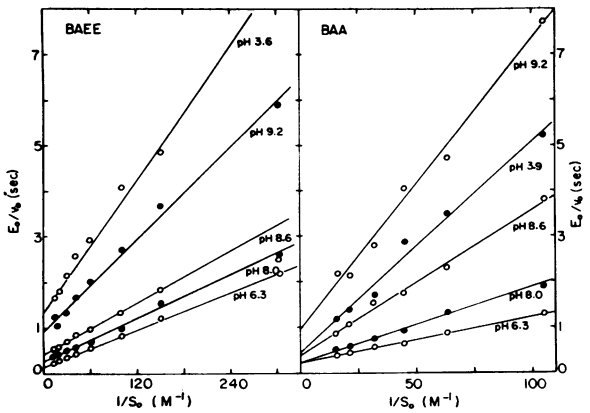

FIG. 1. Lineweaver-Burk plots showing the effect of substrate concentration and pH on the velocity of hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide by ficin at 30.0°. Reaction conditions are described in the text.

tion (k_3) of the acyl enzyme as shown in equation IV.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (IV)$$
$$+$$
$$P_1$$

E and S are the free enzyme and substrate, respectively, $E{\cdot}S$ is the Michaelis-Menten complex, ES' is the acyl-enzyme and P_1 and P_2 would be the alcohol and acid portions of an ester substrate, respectively. The postulated acyl-enzyme intermediate in ficin-catalyzed hydrolyses has been observed spectrophotometrically by Lowe and Williams (16, 17). If deacylation (k_3) were the rate controlling step k_{cat} and $k_{cat}/K_m(app)$ (which $= k_2/K_s$) would be expected to be quite different in their pH dependencies, particularly on the alkaline limb of the curve[5]. Such was found not to be the case. Also, we have found that deacylation (k_3) cannot be the complete rate limiting step in the hydrolysis of BAEE because chloroacetic acid can react readily with the essential sulfhydryl group of ficin even in the presence of saturating amounts of substrate (Whitaker, unpublished data). This would be impossible if k_3 were much smaller than k_2 (all the enzyme would be in the form of acyl-enzyme at high substrate concentrations and the essential sulfhydryl group would be acylated) but could be explained if k_2 were much less than k_3 or k_2 and k_3 were of the same order of magnitude. In view of the similarity between ficin and papain and the finding that k_2 and k_3 for hydrolysis of BAEE and BAA by papain are not greatly different (35), we suspect that k_2 and k_3 are not greatly different for ficin-catalyzed hydrolysis of BAEE and BAA. Such a situation would result in essentially identical pH $-$ k_{cat} and pH $-$ $k_{cat}/K_m(app)$ profiles, particularly in the alkaline region, which is precisely what was found (Fig. 2 and 3). The present data for k_{cat} cannot be separated into the contribution of k_2 and k_3 as was done with papain (35).

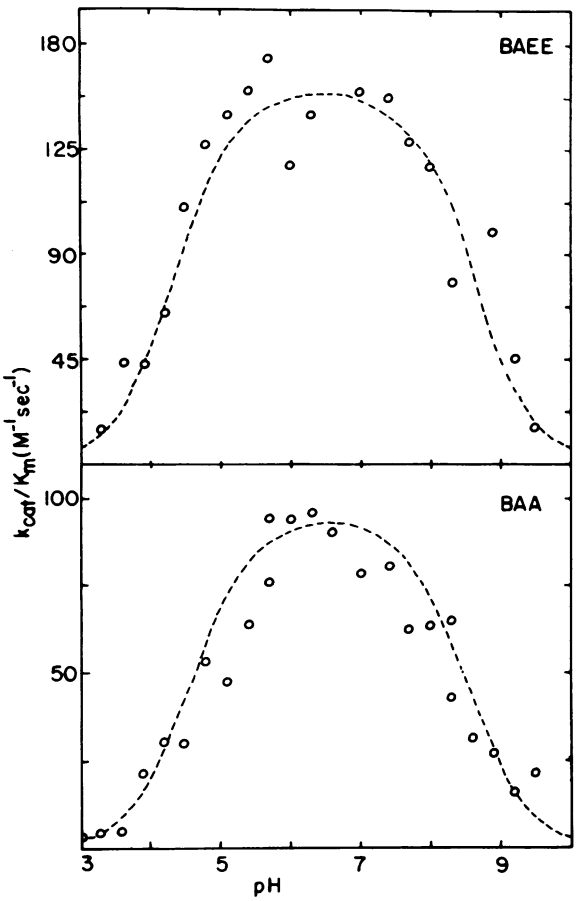

FIG. 2. k_{cat}/k_m(app)-pH profiles for the ficin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) at 30.0°. The lines are theoretical ones calculated from the data of table I by use of equation II in text.

The effect of pH on k_{cat} is identical for the hydrolysis of BAEE and BAA (Fig. 3). If deacylation (k_3) is not the complete rate limiting step for the hydrolysis of BAEE, neither can acylation (k_2) be the complete rate limiting step for the hydrolysis of BAA since $k_{cat}(lim)$ and the pH dependence of k_{cat} are identical for the 2 substrates. This presents an interesting conclusion. Since the values of $k_2(lim)$ for BAEE and BAA are of the same order of magnitude it means either that there is a substan-

[5] This assumes that the rate of acylation (k_2) is controlled by 2 prototropic groups (~4.3 and 8.5) while the rate of deacylation (k_3) is controlled by a single prototropic group (~4.3) as was found for papain (35). There are no compelling reasons to believe this is not the case for ficin.

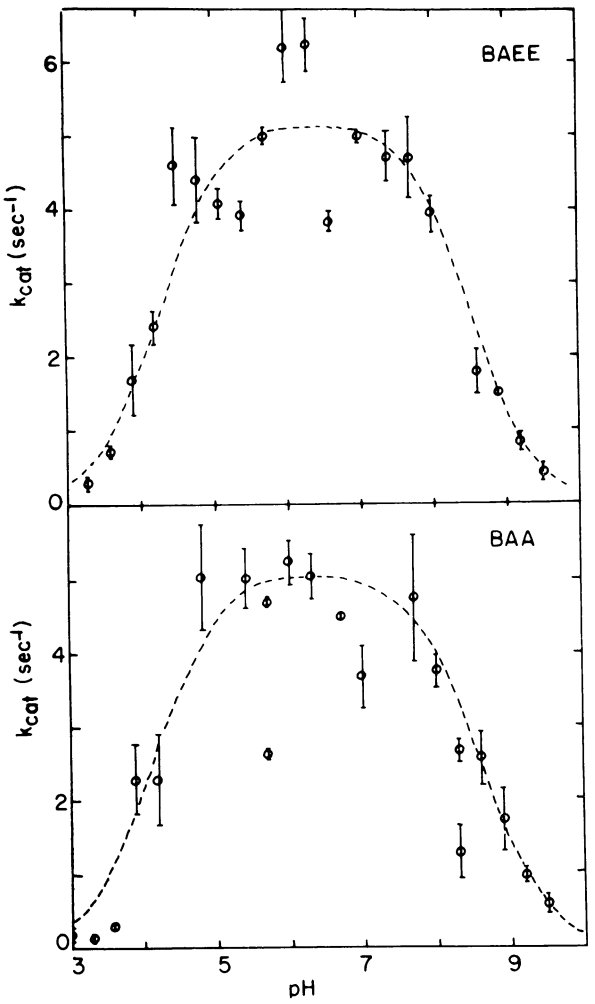

FIG. 3. k_{cat}-pH profiles for the ficin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) at 30.0°. The lines are theoretical ones calculated from the data of table I by use of equation III in text.

tial contribution from an electrophilic component in ficin-catalyzed reactions or that the reactivity of a nucleophilic sulfhydryl group toward esters and amides is essentially identical. If the latter case is true the nucleophilic attack of a sulfhydryl group on ethyl ester-amide pairs produces quite different results than the nucleophilic attack on such a pair by hydroxide ion. In the case of hydroxide ion-catalyzed hydrolysis reactivity of the ester is a thousand-fold faster than the amide. The same conclusions have been suggested in the case of papain-catalyzed hydrolyses (35).

The effect of pH on $k_{cat}/K_m(app)$ (which = k_2/K_s) for hydrolysis of BAEE and BAA by ficin reported here is essentially identical to that reported previously (5). Hammond and Gutfreund reported apparent pK values of 4.40 and 8.46 for the prototropic groups involved in the ficin-catalyzed hydrolysis of BAEE at 25°. For papain- and bromelain-catalyzed hydrolyses of BAEE the apparent pK values of the prototropic groups are 4.29 and 8.49 (35) and 4.2 and 9.0 (9), respectively at 25°. Since the effect of pH on $k_{cat}/K_m(app)$ reflects the influence of pH on the ionization of essential groups in the active center of the free enzyme (20), one must conclude that the same ionizable groups are found in the active center of all these enzymes. These groups are thought to be a carboxyl group and a sulfhydryl group on the basis of pH dependencies, heats for ionization (30), and chemical inhibition studies (7, 36). It was found that the rates of alkylation of ficin with chloroacetamide and of papain with chloromethyl ketones were dependent upon a group with pK 8.55 at 25° (7) and 8.90 at 0° (36), respectively. Further, the essential sulfhydryl group of the enzymes was alkylated in the reactions. The amino acid sequences around the essential sulfhy-

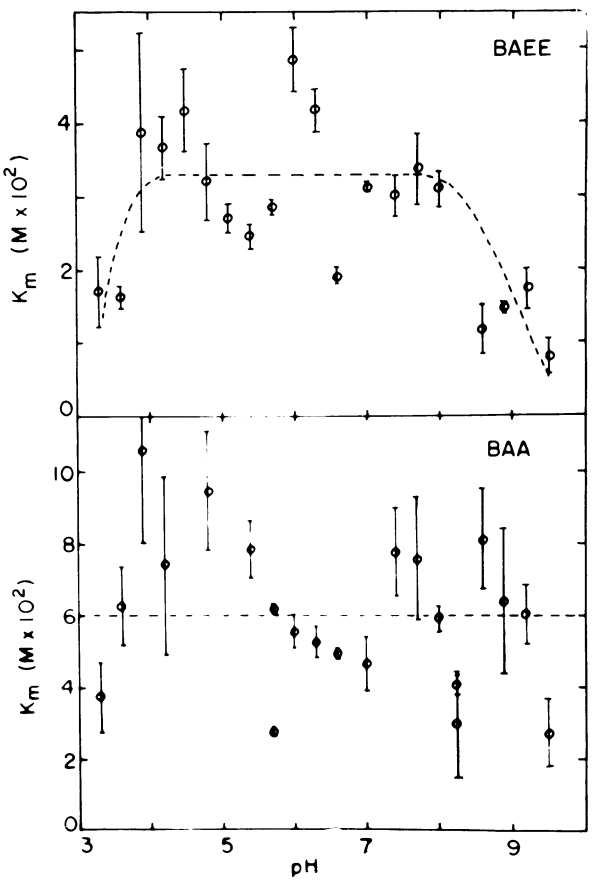

FIG. 4. K_m(app)-pH profiles for the ficin-catalyzed hydrolysis of α-N-benzoyl-L-argininamide ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) at 30.0°. For BAEE, the line is drawn through the average of the values between pH 3.9 and 8.0 and "by eye" at each extreme of pH. For BAA, the line is drawn through the average of all the values.

dryl groups of ficin and papain are very nearly the same (14, 38).

Of the 2 prototropic groups of pK ~ 4.3 and ~ 8.5 one functions in the acidic form and the other in the basic form. However, one cannot distinguish, from the kinetic data presented here, which is the base and which is the acid. With papain, it has been concluded that the base is the carboxyl group; thus the sulfhydryl group must be the acid (35). Such a conclusion would also account for the postulated substantial electrophilic nature of ficin- and papain-catalyzed hydrolyses. This would also account for the difference in pH-rate profiles for the hydrolysis of BAEE and BAA as compared with alkylation of the essential sulfhydryl group of these enzymes with chloroacetamide and chloromethyl ketones (7, 36). In the first case the rate of the reaction decreases as the pH is increased above pH 7 while in the latter case the rate is increased. If this is the correct interpretation the sulfhydryl group of ficin and papain functions as an electrophile in acylation reactions (substrate hydrolysis) and as a nucleophile in alkylation reactions.

$K_m(app)$ for the ficin-catalyzed hydrolysis of BAA appears to be independent of pH over the range of 3.0 to 9.5. On the other hand, $K_m(app)$ for the ficin-catalyzed hydrolysis of BAEE appears to decrease below pH 3.9 and above 8.0. These results are in marked contrast to those reported for the papain-catalyzed hydrolysis of BAEE and BAA (35), since $K_m(app)$ increased markedly at pH values below pH 4.5. The marked increase in $K_m(app)$ for papain-catalyzed hydrolysis of BAEE and BAA has not been satisfactorily explained (35). $K_m(app)$ values of 3.32×10^{-2} M and 6.03×10^{-2} M for BAEE and BAA, respectively at 30° are in agreement with the values of 2.5×10^{-2} M and 4.8×10^{-2} M reported previously at 25° (5). The ratio of $K_m(app)$ values for BAA to BAEE was also found to be about 2 for papain (35), pointing up once again the parallelism between the active centers of these 2 enzymes.

It is interesting and encouraging that the results reported here on a pure ficin component isolated from *Ficus carica* var. Kadota latex are essentially identical to those reported earlier (2, 5) for a preparation from *Ficus glabrata* latex which was later shown to contain at least 9 proteolytically active components (21). It is also interesting and encouraging that the results are very similar to those reported for papain. This implies that the essential features of the active center of all the multimolecular forms of ficin and of papain are identical and that amino acid changes, with concomitant changes in secondary and tertiary structure, outside the essential parts of the active center have little or no influence on the observed kinetic parameters. We have found the amino acid composition of Ficin D of *Ficus carica* var. Kadota latex to be quite different from that of 6 proteolytic enzyme components purified from *Ficus glabrata* latex (Kramer and Whitaker, Williams and Whitaker, unpublished results) which in turn are different from the amino acid composition of papain (14).

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