# Kinetics of the Bromelin-catalysed Hydrolyses of α-*N*-Benzoyl-L-arginine Ethyl Ester and α-*N*-Benzoyl-L-argininamide

By K. BROCKLEHURST,* E. M. CROOK, and C. W. WHARTON

(*Department of Biochemistry and Chemistry, St. Bartholomew's Hospital Medical College, Charterhouse Square, London, E.C.*1)

THE acyl-enzyme mechanism represented by (1) has been suggested repeatedly to describe the catalysis of the hydrolysis of ester and amide substrates by the plant proteolytic enzymes papain, ficin, and bromelin.[1–7]

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_2} ES^1 \xrightarrow{k_3} E + P_2 \quad (1)$$
$$+ P_1$$

In scheme (1), ES is the Michaelis complex, $K_S$ its dissociation constant, $P_2$ the carboxylic acid product, $P_1$ the alcohol or ammonia product of an ester or amide substrate S, and $ES^1$ is the acyl-enzyme which is usually considered to be an intermediate common to related ester and amide substrates.

Although recently the existence of an acyl-enzyme intermediate has been demonstrated for papain and for ficin by spectrophotometric observation of the thionohippuryl enzyme[6]† and of *trans*-cinnamoylpapain,[7] the original suggestion of the acyl-enzyme hypothesis stemmed from kinetic data.[3,4] The values of the turnover rate constant, $k_{cat}$, for the catalysis of the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester (BAEE) and the corresponding amide (BAA) by papain and by ficin were found to be very similar. Since the rates of non-enzymic hydrolyses of ethyl esters are usually considerably greater than those of the corresponding amides, the similarities in the values of $k_{cat}$ for the enzymic hydrolyses were interpreted in terms of a rate-limiting deacylation of a common acyl-enzyme intermediate. Similarly the unique feature of bromelin—namely the 140-fold difference in the values of $k_{cat}$ for the bromelin-catalysed hydrolyses of BAEE and BAA, recognised by Inagami and Murachi,[5] has been interpreted by these authors in terms of the common acyl-enzyme hypothesis by assuming that deacylation ($k_3$) is rate-limiting for the BAEE hydrolysis whereas acylation ($k_2$) is rate-limiting for the BAA hydrolysis. More recently[8,9] the kinetic data for the papain-catalysed hydrolyses have been analysed more fully, making certain assumptions, and this analysis indicates that $k_{cat}$ does *not* reflect the common deacylation step but rather that for both BAEE and BAA, $k_{cat}$ is determined by both $k_2$ (acylation) and $k_3$ (deacylation) and that for the ester the predominantly rate-limiting step is deacylation whereas for the amide the predominantly rate-limiting step is acylation.

We now report for the first time a similar analysis of the data of Inagami and Murachi[5] for the bromelin-catalysed hydrolysis of BAEE and BAA. This analysis yields the surprising result that the supposedly common deacylation constant, $k_3$, is *ca.* 190 times greater for the bromelin-catalysed hydrolysis of BAEE than for the bromelin-catalysed hydrolysis of BAA.

The constants of equation (1) are related to those of the usual Michaelis–Menten equation (2) by equations (3) and (4).

$$E + S \overset{K_m(app)}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \quad (2)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (3)$$

$$K_m(app) = (k_{-1} + k_2)k_3/k_1(k_2 + k_3) \quad (4)$$

If it is assumed that $k_{-1} \gg k_2$, equation (4) becomes (5) and (6) follows from equations (3) and (5).

$$K_m(app) = [k_3/(k_2 + k_3)]K_S \quad (5)$$

$$k_{cat}/K_m(app) = k_2/K_S \quad (6)$$

To explain the similarity in the values of $k_{cat}/K_m(app)$ for the bromelin-catalysed hydrolyses of BAEE and BAA, Inagami and Murachi[5] suggested that $k_{-1} \ll k_2$ in which case $k_{cat}/K_m(app)$, which from equations (3) and (4) equates to $k_1k_2/(k_{-1}+k_2)$ reduces to $k_1$, the second order constant for the formation of the Michaelis complex. Consideration of the value[5] of $k_{cat}/K_m(app)$ (lim) (2·9 M.$^{-1}$ sec.$^{-1}$) for the bromelin-catalysed reactions, however, suggests that this ratio cannot represent $k_1$ since the formation of a Michaelis complex is usually considered[10] to be diffusion controlled with a rate constant of *ca.* $10^8$ M$^{-1}$ sec.$^{-1}$. It is unlikely, therefore that $k_{-1} \ll k_2$.

† Preliminary investigations in this laboratory indicate the formation of thionohippurylbromelin ($\lambda_{max}$ 316 mμ) on admixture of methyl thionohippurate and the enzyme.

If $k_2$ is eliminated from equations (5) and (6), equation (7) results.

$$k_{cat} = k_3 - [k_3 K_m(app)/K_S] \quad (7)$$

If $k_3$ and $K_S$ are independent of pH, a plot of $k_{cat}$ against $K_m$(app) should be linear with intercepts of $k_3$ and $K_S$ on the $k_{cat}$ and $K_m$ (app) axes, respectively, and slope of $-k_3/K_S$. The Figure

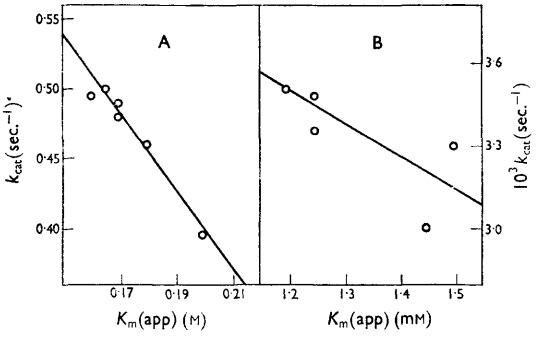


FIGURE. *Plots of* $k_{cat}$ *against* $K_m$(app) *for the bromelin-catalysed hydrolyses of* (A) BAEE *and* (B) BAA *in the pH range* ca. 5—8.

[The points are taken from Inagami and Murachi[5] and the lines are the least-squares regression lines for equation (7)].

shows the plots for BAEE and BAA for the data in the pH range *ca.* 5—8 where it is assumed that $k_3$ and $K_S$ are essentially independent of pH (see ref.8 for a discussion of this assumption in the case of the analogous papain-catalysed hydrolyses). Thus regression of $k_{cat}$ on $K_m$(app) permits the computation of $k_3$ and $K_S$. It is possible to calculate $k_2$ (lim) in two ways, [8,9] firstly by making use of equation (6) and the known value of $k_{cat}/K_m$(app) (lim) and secondly from equation (8) which is obtained by rearrangement of equation (3) for the data in the pH-independent region.

$$1/k_2 \text{ (lim)} = 1/k_{cat} \text{ (lim)} - 1/k_3 \text{ (lim)} \quad (8)$$

The constants obtained from this analysis of the bromelin-catalysed hydrolyses together with those obtained by Whitaker and Bender[8] for the analogous papain-catalysed hydrolyses are presented in the Table.

If the assumptions implicit in this analysis are correct, the very large difference found in the values of $k_3$(lim) for the bromelin-catalysed hydrolysis of BAEE and BAA compels the view that either these two substrates are not bound in the same way by the same sites on the enzyme or that in the case of BAA, the ammonia released consequent upon the acylation of bromelin by BAA is bound strongly to the enzyme in such a way that it inhibits the subsequent deacylation step. Whilst the binding of BAEE and BAA to entirely different sites on the enzyme would not be expected in view of the similarity in structure of the two substrates, a suitably aligned, highly electrophilic centre in the enzyme could introduce a strong antiproductive component into the binding of the amide, which would be reflected in a low value of $K_S$, a low value of $k_2$ and possibly a low value of $k_3$. The low value of $k_3$ for the amide hydrolysis compared with that for the ester hydrolysis could arise in at least two ways: firstly, if a different nucleophilic centre in the enzyme is acylated by the amide as a result of the antiproductive binding and secondly if the same centre is acylated by the amide as by the ester but a conformational change is required to effect the acylation by the amide and $k_3$ reflects a

TABLE

*Kinetic constants of bromelin- and papain-catalysed hydrolyses of* α-N-*benzoyl*-L-*arginine ethyl ester and* α-N-*benzoyl*-L-*argininamide at* 25·0°

| Enzyme | Substrate | $K_m$(app)(lim) | $10^2 k_{cat}$(lim) | $10^2 k_2$(lim) | $10^2 k_3$(lim) | $K_S$ |
|---|---|---|---|---|---|---|
| | | mM | sec.$^{-1}$ | sec.$^{-1}$ | sec.$^{-1}$ | mM |
| Bromelin[a] .. .. | BAEE | 170 | 50 | 101[b]<br>106[c] | 94±5[d] | 349±32[d] |
| Bromelin[a] .. .. | BAA | 1·2 | 0·35 | 1·2[b]<br>1·3[c] | 0·49±0·06[d] | 4·25±1·63[d] |
| Papain[e] .. .. | BAEE | 15 | 1600 | 6490±1390 | 2020±170 | 54·5±11·7 |
| Papain[e] .. .. | BAA | 30 | 800 | 970±209 | 2870±2510 | 36·2±7·8 |

[a] Experimental data from Inagami and Murachi (ref. 5); [b] calculated from $k_{cat}/K_m$(app)(lim) and $K_S$ using equation (6); [c] calculated from $k_{cat}$(lim) and $k_3$(lim) using equation (8); [d] the standard errors refer to the least-squares regression constants of equation (7); no statistics for the individual points of the regression are available; [e] data from Whitaker and Bender (ref. 8).

subsequent conformational change of the acyl-enzyme required to permit deacylation. These possibilities are being investigated.

(*Received, August 2nd*, 1967; *Com.* 818.)

[1] H. Gutfreund, *Discuss. Faraday Soc.*, 1955, **20**, 167.
[2] A. Stockwell and E. L. Smith, *J. Biol. Chem.*, 1957, **227**, 1.
[3] E. L. Smith, *J. Biol. Chem.*, 1958, **233**, 1392.
[4] B. R. Hammond and H. Gutfreund, *Biochem. J.*, 1959, **72**, 349.
[5] T. Inagami and T. Murachi, *Biochemistry*, 1963, **2**, 1439.
[6] G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 189.
[7] M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, 1964, **86**, 5333.
[8] J. R. Whitaker and M. L. Bender, *J. Amer. Chem. Soc.*, 1965, **87**, 2728.
[9] M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, 1966, **88**, 5880.
[10] H. Gutfreund, "An Introduction to the Study of Enzymes", Blackwell Scientific Publications, Oxford, 1965, p. 70.