Kinetics of the Hydrolysis of *N*-Benzoyl-L-serine Methyl Ester Catalysed by Bromelain and by Papain

ANALYSIS OF MODIFIER MECHANISMS BY LATTICE NOMOGRAPHY,
COMPUTATIONAL METHODS OF PARAMETER EVALUATION FOR
SUBSTRATE-ACTIVATED CATALYSES AND CONSEQUENCES OF POSTULATED
NON-PRODUCTIVE BINDING IN BROMELAIN- AND PAPAIN-CATALYSED
HYDROLYSES

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1. *N*-Benzoyl-L-serine methyl ester was synthesized and evaluated as a substrate for bromelain (EC 3.4.22.4) and for papain (EC 3.4.22.2). 2. For the bromelain-catalysed hydrolysis at pH 7.0, plots of $[S_0]/v_1$ (initial substrate concn./initial velocity) versus $[S_0]$ are markedly curved, concave downwards. 3. Analysis by lattice nomography of a modifier kinetic mechanism in which the modifier is substrate reveals that concave-down $[S_0]/v_1$ versus $[S_0]$ plots can arise when the ratio of the rate constants that characterize the breakdown of the binary (ES) and ternary (SES) complexes is either less than or greater than 1. In the latter case, there are severe restrictions on the values that may be taken by the ratio of the dissociation constants of the productive and non-productive binary complexes. 4. Concave-down $[S_0]/v_1$ versus $[S_0]$ plots cannot arise from compulsory substrate activation. 5. Computational methods, based on function minimization, for determination of the apparent parameters that characterize a non-compulsory substrate-activated catalysis are described. 6. In an attempt to interpret the catalysis by bromelain of the hydrolysis of *N*-benzoyl-L-serine methyl ester in terms of substrate activation, the general substrate-activation model was simplified to one in which only one binary ES complex (that which gives rise directly to products) can form. 7. In terms of this model, the bromelain-catalysed hydrolysis of *N*-benzoyl-L-serine methyl ester at pH 7.0, $I = 0.1$ and 25°C is characterized by K_{m1} (the dissociation constant of ES) = $1.22 \pm 0.73 \text{ mM}$, k (the rate constant for the breakdown of ES to E+products, P) = $1.57 \times 10^{-2} \pm 0.32 \times 10^{-2} \text{ s}^{-1}$, K_{a2} (the dissociation constant that characterizes the breakdown of SES to ES and S) = $0.38 \pm 0.06 \text{ M}$, and k' (the rate constant for the breakdown of SES to E+P+S) = $0.45 \pm 0.04 \text{ s}^{-1}$. 8. These parameters are compared with those in the literature that characterize the bromelain-catalysed hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester and of α -*N*-benzoyl-L-arginine amide; K_{m1} and k for the serine ester hydrolysis are somewhat similar to K_m and $k_{cat.}$ for the arginine amide hydrolysis and K_{as} and k' for the serine ester hydrolysis are somewhat similar to K_m and $k_{cat.}$ for the arginine ester hydrolysis. 9. A previous interpretation of the inter-relationships of the values of $k_{cat.}$ and K_m for the bromelain-catalysed hydrolysis of the arginine ester and amide substrates is discussed critically and an alternative interpretation involving substantial non-productive binding of the arginine amide substrate to bromelain is suggested. 10. The parameters for the bromelain-catalysed hydrolysis of the serine ester substrate are tentatively interpreted in terms of non-productive binding in the binary complex and a decrease of this type of binding by ternary complex-formation. 11. The Michaelis parameters for the papain-catalysed hydrolysis of the serine ester substrate ($K_m = 52 \pm 4 \text{ mM}$, $k_{cat.} = 2.80 \pm 0.1 \text{ s}^{-1}$ at pH 7.0, $I = 0.1$, 25.0°C) are similar to those for the papain-catalysed hydrolysis of methyl hippurate. 12. Urea and guanidine hydrochloride at concentrations of 1 M have only small effects on the kinetic parameters for the hydrolysis of the serine ester substrate catalysed by bromelain and by papain.

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In connexion with the study of the matrix-perturbation of bromelain-catalysed hydrolyses caused by factors other than charge-charge interaction (see Wharton *et al.*, 1968*a,b*) it was necessary to find a substrate for bromelain which is (a) uncharged in approximately neutral media, (b) at least as good a substrate for bromelain as α -*N*-benzoyl-L-arginine ethyl ester, i.e. $k_{\text{cat.}} \geq \text{approx. } 0.5 \text{ s}^{-1}$ and $K_m \leq \text{approx. } 0.1 \text{ M}$, and (c) sufficiently water-soluble to allow determination of the Michaelis parameters without inclusion of organic solvent in the assay medium, i.e. the solubility would probably have to be greater than 0.1 M . The latter requirement, desirable in any enzyme study, is particularly important in comparative studies of free solution and matrix-supported enzyme catalyses because of the possibility that added organic solvent might modify differentially the properties of the two systems.

In our search for such a substrate for bromelain, we considered first a number of uncharged substrates that have been used by other workers in the study of hydrolytic enzymes other than bromelain. Methyl and ethyl hippurate are not sufficiently water-soluble. Cohen & Petra (1967) and Williams & Whitaker (1967) have studied the papain-catalysed hydrolysis of α -*N*-benzoyl-L-citrulline methyl ester and found this compound to be a very good substrate for papain. When it was tested as a substrate for bromelain, however, it was found that the initial velocity increased in direct proportion to the substrate concentration up to the solubility limit of the substrate (0.1 M), i.e. $K_m > 0.1 \text{ M}$. The solubility of *N*-acetyl-glycine methyl ester in water is approx. 1 M , but the bromelain-catalysed hydrolysis of this compound was barely detectable even at $[S_0] \approx 0.5 \text{ M}$ and $[E_T]$ (total enzyme concentration) $\approx 0.1 \text{ mM}$. Murachi (1970) has reported that for the bromelain-catalysed hydrolysis of acetyl-glycine ethyl ester K_m is 33 M ($k_{\text{cat.}} = 0.55 \text{ s}^{-1}$). Since the uncharged *N*-acylamino acid esters that are commonly used as substrates for other hydrolytic enzymes such as α -chymotrypsin, papain and ficin proved to be unsuitable as substrates for bromelain, we decided to synthesize *N*-benzoyl-L-serine methyl ester as a potential substrate for bromelain. This compound, which has been used as an intermediate in the synthesis of oxazolines (Fry, 1950), has not previously been used as a substrate for hydrolytic enzymes. *N*-Benzoyl-L-serine methyl ester was synthesized and found to have a solubility in water at room temperature of approx. 0.25 M . The bromelain-catalysed hydrolysis of this substrate proceeded at an easily measurable rate at substrate concentrations of 0.5 – 150 mM and a degree of saturation of the enzyme by the substrate was apparent. The present paper describes a kinetic study of the hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by bromelain and by papain.

Theoretical

Our finding that kinetic plots for the catalysis by bromelain of the hydrolysis of *N*-benzoyl-L-serine methyl ester are curved in a manner frequently taken as suggestive of substrate activation (see the Results and Discussion section) necessitated detailed consideration of the steady-state rate equation of substrate-activated catalyses and of its graphical representation. There is continuing discussion of which of the three linear plotting forms of the Michaelis-Menten equation is most useful in practice [see, e.g., Dowd & Riggs (1965) and references therein]. Our choice of the $[S_0]/v_i$ (initial substrate concentration/initial velocity) versus $[S_0]$ plot was based on the fact that it demonstrates any non-linearity that may exist much more effectively than does the $1/v_i$ versus $1/[S_0]$ plot and also still allows the fitting of a line by the method of least squares. This procedure is not applicable in a simple manner to the third linear plot, i.e. v_i versus $v_i/[S_0]$, since in this case both plotted variables are subject to error.

The objectives were (1) to define the type of modifier kinetic mechanisms which can give rise to concave-down $[S_0]/v_i$ versus $[S_0]$ plots, (2) to elucidate any simple relationships that might be required to exist between the characterizing parameters of a given modifier kinetic mechanism to permit curvature in the plot; and (3) to consider methods of determining the characterizing parameters.

Steady-state kinetics of substrate-activated enzyme catalyses: the effect of substrate activation on the shape of $[S_0]/v_i$ versus $[S_0]$ plots

In order to simplify the algebraic representation, the kinetic models in this section are described in terms of the conventional two-step mechanism, and reversible steps are regarded as unperturbed equilibria characterized by equilibrium constants rather than more complex assemblies of rate constants.

Scheme 1 is a general form of kinetic mechanism involving enzyme, substrate and modifier (M), in

Scheme 1. General kinetic mechanism involving enzyme, substrate and modifier (M) in which dead-end complexes may not form

A special case is that in which $\text{M} \equiv \text{S}$.

which dead-end complexes may not form and in which the characterizing constants are defined by eqns. (1)–(4). The interdependence of the four equilibrium constants is expressed in eqn. (5). The initial rate of formation of products (P) is given in eqn. (6) and the conservation of enzyme is expressed in eqn. (7).

$$K_{m_1} = [E][S]/[ES] \quad (1)$$

$$K_{m_2} = [ME][S]/[MES] \quad (2)$$

$$K_{a_1} = [M][E]/[ME] \quad (3)$$

$$K_{a_2} = [ES][M]/[MES] \quad (4)$$

$$K_{a_1}/K_{a_2} = K_{m_1}/K_{m_2} \quad (5)$$

$$d[P]/dt = v_i = k[ES] + k'[MES] \quad (6)$$

$$[E_T] = [E] + [ES] + [ME] + [MES] \quad (7)$$

Steady-state analysis of Scheme 1 by eqns. (1)–(7) provides eqn. (8) and differentiation with respect to $[S_0]$ gives eqn. (9).

$$[E_T][S_0]/v_i = \frac{K_{a_2}[S_0] + K_{a_2}K_{m_1} + [S_0][M] + \frac{K_{m_1}K_{a_2}}{K_{a_1}}[M]}{(kK_{a_2} + k'[M])} \quad (8)$$

$$\frac{d([S_0][E_T]/v_i)}{d[S_0]} = \frac{K_{a_2} + [M]}{kK_{a_2} + k'[M]} \quad (9)$$

If $[M]$ is maintained constant, a plot of $[E_T][S_0]/v_i$ versus $[S_0]$ (or $[S_0]/v_i$ versus $[S_0]$ when $[E_T]$ is constant) will be linear with slope $(K_{a_2} + [M])/(kK_{a_2} + k'[M])$ and intercept

$$\frac{K_{a_2}K_{m_1} + \frac{K_{m_1}K_{a_2}}{K_{a_1}}[M]}{kK_{a_2} + k'[M]}$$

In the special case where the modifier is the substrate, the rate eqn. (8) becomes eqn. (10), which on differentiation with respect to $[S_0]$ gives eqn. (11).

$$[E_T][S_0]/v_i = \frac{[S_0]^2 + \left(K_{a_2} + \frac{K_{m_1}K_{a_2}}{K_{a_1}}\right)[S_0] + K_{a_2}K_{m_1}}{kK_{a_2} + k'[S_0]} \quad (10)$$

$$\frac{d[E_T][S_0]/v_i}{d[S_0]} = \frac{kK_{a_2}^2 + 2kK_{a_2}[S_0] + \frac{kK_{m_1}K_{a_2}^2}{K_{a_1}} - k'K_{a_2}K_{m_1} + k'[S_0]^2}{(kK_{a_2} + k'[S_0])^2} \quad (11)$$

The condition that a plot of $[S_0]/v_i$ versus $[S_0]$ be concave down may be demonstrated by comparing the limiting values of eqn. (11) when $[S_0]$ tends to zero and when $[S_0]$ tends to infinity. These limits

are given in eqns. (12) and (13). Eqn. (13) is obtained by expanding the denominator in eqn. (11) and dividing numerator and denominator by $[S_0]^2$.

$$\lim_{[S_0] \rightarrow 0} \frac{d\left(\frac{[E_T][S_0]}{v_i}\right)}{d[S_0]} = \frac{1}{k} + \frac{K_{m_1}}{kK_{a_1}} - \frac{k'K_{m_1}}{k^2K_{a_2}} \quad (12)$$

$$\lim_{[S_0] \rightarrow \infty} \frac{d\left(\frac{[E_T][S_0]}{v_i}\right)}{d[S_0]} = 1/k' \quad (13)$$

The condition that a plot of $[S_0]/v_i$ versus $[S_0]$ be concave down is given in the inequality (14).

$$\frac{1}{k} + \frac{K_{m_1}}{kK_{a_1}} - \frac{k'K_{m_1}}{k^2K_{a_2}} > \frac{1}{k'} \quad (14)$$

This may be rewritten as inequality (15) in which $x = k/k'$, $a = K_{m_1}/K_{a_1}$ and $b = K_{m_1}/K_{a_2}$.

$$x^2 - (1+a)x + b < 0 \quad (15)$$

$$x^2 - (1+a)x + b = 0 \quad (16)$$

Some solutions of the equation obtained by setting the left-hand side of inequality (15) equal to zero (eqn. 16) are presented in the lattice nomogram (see Otto, 1963) (Fig. 1) as plots of $\log a$ versus $\log x$ for various values of b in the range 10^{-3} – 10^3 .

It is useful to consider the various regions and features of Fig. 1.

(I) *Solid lines (contours)*. These represent solutions to eqn. (16) and thus correspond to situations in which plots of $[S_0]/v_i$ versus $[S_0]$ will be linear.

The common boundary line (A) provides the essentially common upper root to eqn. (16). The portion of line A below the abscissa relates only to values of $b < 1$.

(II) *Region on the right of common boundary line A*. This region represents situations in which plots of $[S_0]/v_i$ versus $[S_0]$ will always be concave up, and is not considered further in the present paper.

(III) and (IV) *Regions between the common boundary line A and a given contour defined by the value of b*. These regions represent conditions (values of a , b and x) that give rise to plots of $[S_0]/v_i$ versus $[S_0]$ that are concave down. Thus the system passes from a region on the right of line A where plots are concave up, over line A into a region of downward concavity which ends when the appropriate contour defined by the particular value of b is reached. On passing over this contour the system is once more in a region of upward concavity.

The region of downward concavity corresponding to values of $b < 1$ (region III) has a 'funnel'-shaped appearance, whereas that corresponding to values of $b > 1$ (region IV) has an inverted conical shape. It is apparent from Fig. 1 that region IV is more limited in terms of the permitted variability of x and a than is region III.

Fig. 1. Lattice nomogram giving some solutions to eqn. (16), $x^2 - (1+a)x + b = 0$, and defining situations in which $x^2 - (1+a)x + b < 0$

$x = k/k'$, $a = K_{m1}/K_{a1}$ and $b = K_{m1}/K_{a1}$; k , k' , K_{m1} , and K_{a2} are the parameters that define the kinetic model given in Scheme 1 in which the modifier (M) is the substrate (S). The common boundary line (A) provides the essentially common upper root to eqn. (16). Line B is the locus that defines the limits of the constituent constants of the above parameters that characterize the hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by bromelain at pH 7.0, $I = 0.1$, 25.0°C; for the parameters see legend to Fig. 2.

It is noteworthy that the region contained within the ordinate axis and the common boundary line A corresponds to situations in which there will be downward concavity in a plot of $[S_0]/v_1$ versus $[S_0]$ even though $x > 1$, i.e. even though $k > k'$. In these circumstances b has no upper limit but a is limited to values greater than 0.1, i.e. K_{m1} must be greater than $0.1K_{a1}$. This implies that downward concavity in plots of $[S_0]/v_1$ versus $[S_0]$ can result not only from provision of a ternary SES complex whose rate constant for breakdown to products is greater than that for the breakdown of the binary ES complex, but also in another way. The alternative mechanism that can operate even when $k > k'$ involves relief at high substrate concentrations from the accumulation of a significant fraction of the enzyme at low substrate concentrations as the SE complex that cannot give rise directly to products. Thus this mechanism of substrate activation might best be described as activation by relief from non-productive binding.

When this work had been completed, Dixon & Tipton (1973) presented a discussion of substrate activation as part of a more general consideration of negatively co-operative ligand binding and pointed out that it is not necessary for x to be less than 1 to

observe kinetic plots of the form commonly interpreted as characteristic of substrate activation.

Determination of the values or limits on the values of the parameters that characterize Scheme 1

Eqn. (17), a transform of (10), is written in terms of the four parameters p , q , y and z , where $p = kK_{a2}$, $q = k'$, $y = K_{a2}[1 + (K_{m1}/K_{a1})]$ and $z = K_{m1}K_{a2}$.

$$v_1 = \frac{(p + q[S_0])[E_T][S_0]}{[S_0]^2 + y[S_0] + z} \quad (17)$$

Since the expressions used to define the four parameters involve five constants, it is not possible to isolate the constants (except for $k' = q$). Similarly, it is not possible to extract simple ratios of these constants from the parametric relationships. It is possible, however, to relate the ratios a and b of eqn. (16) to the parameters y and z . This is given in eqn. (18):

$$a = (y\sqrt{b}/\sqrt{z}) - 1 \quad (18)$$

Since a must be positive $y\sqrt{b}/\sqrt{z} > 1$. If values of y and z are known, a series of values of b may be used to calculate the corresponding values of a . These corresponding values of a and b may then be used to construct a locus to define the limits that may be placed on values of x , b and a in the particular situation characterized by the known values of y and z . The determination of the parameters y , z , p and q is discussed in the following part of the Theoretical section and the locus that defines the limits of their constituent constants for the bromelain-catalysed hydrolysis of α -*N*-benzoyl-L-serine methyl ester at pH 7.0, $I = 0.1$, 25.0°C, is presented as the dashed line (B) in Fig. 1.

Special cases of the model in Scheme 1

(i) *Compulsory substrate activation.* If $k = 0$, Scheme 1 represents compulsory substrate activation. In this case $x = 0$ and the left-hand side of the inequality (15) ($=b$) cannot be less than 0. Thus plots of downward concavity cannot be interpreted in terms of compulsory substrate activation; this phenomenon must always give rise to plots of $[S_0]/v_1$ versus $[S_0]$ of upward concavity.

(ii) *Only one type of binary complex can form and this gives rise to products.* In this situation, Scheme 1 simplifies to Scheme 2. The inequality that defines the circumstances in which plots of $[S_0]/v_1$ versus $[S_0]$ will exhibit downward concavity on the basis of the model in Scheme 2 may be obtained from inequalities (14) and (15) by letting $K_{a1} \rightarrow \infty$, i.e. $a \rightarrow 0$. Inequality (15) then becomes inequality (19) or (20):

$$x^2 - x + b < 0 \quad (19)$$

$$\left(\frac{k}{k'}\right)^2 - \frac{k}{k'} + \frac{K_{m1}}{K_{a2}} < 0 \quad (20)$$

Scheme 2. *Special case of the kinetic mechanism of Scheme 1 in which the modifier is the substrate and only one type of binary complex (that which gives rise to products) can form*

For downward concavity the left-hand side of inequality (20) must be negative. This implies that for this simplified model, $k < k'$, and further that K_{m1} must be sufficiently less than K_{a2} to prevent compensation.

Computational methods for the determination of the characterizing parameters for an enzyme catalysis subject to non-compulsory substrate activation

The four parameters that characterize the non-compulsory substrate-activation rate equation, eqn. (17), may be determined graphically if initial velocities may be determined in two ranges of substrate concentration, (i) $[\text{S}_0] < K_{m1}$ and (ii) $[\text{S}_0] > K_{a2}$ (see Trowbridge *et al.*, 1963). Preliminary plots of the data for bromelain-catalysed hydrolyses of *N*-benzoyl-L-serine methyl ester as $v_i/[\text{E}_T]$ versus $\log [\text{S}_0]$ (see Fig. 2(a) and Trowbridge *et al.*, 1963) suggested that for these catalyses the range of substrate concentration in which the rate of catalysis was readily measured is such that $K_{m1} < [\text{S}_0] < K_{a2}$, i.e. graphical determination of the parameters is not possible. Accordingly, we explored the use of computational techniques for evaluation of the parameters. It is necessary to be able to arrive at the 'best' values of the parameters of eqn. (17) starting from initial 'estimated' values, which may differ considerably from the 'best' values. Thus the object of the optimization is to refine sequentially the values of the 'estimates' in such a way as to minimize the error criterion. There are many ways of approaching this problem (Rosenbrock & Storey, 1966, p. 48).

Method 1. Eqn. (17) may be written as eqn. (21).

$$v_i - \frac{(p+q[\text{S}_0])[\text{E}_T][\text{S}_0]}{[\text{S}_0]^2 + y[\text{S}_0] + z} = 0 \quad (21)$$

In practice the left-hand side of eqn. (21) will be some non-zero value, X . It is assumed that the set of X values for a given data set will be distributed normally about zero. The best-fit values of the parameters are obtained by application of the least-squares criterion. This condition is expressed in eqn. (22):

$$\sum_{i=0}^n X^2 = \sum_{i=0}^n \left\{ \frac{v_i([\text{S}_0]^2 + y[\text{S}_0] + z) - (p+q[\text{S}_0])[\text{E}_T][\text{S}_0]}{[\text{S}_0]^2 + y[\text{S}_0] + z} \right\}^2 = \text{a minimum} \quad (22)$$

The partial differentials of eqn. (22) will be rather complicated unless the equation is simplified. This may be achieved by using an iterative technique and maintaining the denominator as a constant with respect to the dissociation constants during any one iterative cycle. It is convenient to rewrite eqn. (22) as eqn. (23), in which w_1 is defined by eqn. (24).

$$\sum_{i=0}^n X^2 = \sum_{i=0}^n w_1 \{ v_i([\text{S}_0]^2 + y[\text{S}_0] + z) - (p+q[\text{S}_0])[\text{E}_T][\text{S}_0] \}^2 \quad (23)$$

$$w_1 = 1/([\text{S}_0]^2 + y[\text{S}_0] + z)^2 \quad (24)$$

The value of w_1 is set to 1.00 for the first cycle and is readjusted for each subsequent cycle by using the values of the dissociation constants determined in the previous cycle.

Eqn. (23) may be partially differentiated with respect to p , z , q and y , a procedure facilitated by substitution of eqn. (25) and (26) into eqn. (23).

$$p[\text{E}_T] = m \quad (25)$$

$$q[\text{E}_T] = k'_E \quad (26)$$

The resulting expressions for the partial derivatives with respect to z , m , y and k'_E are set equal to zero and the set of simultaneous equations is solved for the above parameters by matrix inversion. The calculated values of z and y are used to calculate a new set of w_1 values. The iteration procedure is then repeated until the parameters achieve constant values. It is not possible, in any simple manner, to obtain standard errors for the parameters, as w_1 is defined as being constant for any one iterative cycle. In addition, the method described above places no constraints on the sign of the parameters and the method frequently produces negative values for the parameters where the data are not of the highest quality. Thus although this method was used successfully on some sets of data (see Table 3), it proved unable to produce values for the parameters in an interpretable form in a number of cases. The lack of estimates of the standard errors is also serious when fitting experimentally determined data to a relatively elaborate model of this type. A second method, capable of eliminating the above disadvantages, has been developed and is described below.

Method 2. The considerable difficulties encountered in fitting data to equations of the form of eqn. (17) arise because of extremely high correlation between the parameters. These difficulties are aggravated when, as in this study, it is possible to use substrate concentrations which cover only a small part of the range from zero to saturation. In the fitting procedure it is

Fig. 2. Kinetic plots for the hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by bromelain at pH 7.0 in 46.3 mM-potassium phosphate buffer, $I = 0.1$ at 25.0°C

This data set, which is representative of a large number of such sets, was collected on a single day. The points are experimental and the lines were calculated from the various transforms of:

$$v_i = (p + q[S_0])[E_T][S_0]/([S_0]^2 + y[S_0] + z)$$

[i.e. eqn. (17) in the Theoretical section] in which the values of the parameters ($p = 1.57 \times 10^{-2} \times 0.38 = 5.95 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$, $q = 0.45 \text{ s}^{-1}$, $y = 0.38 \text{ M}$ and $z = 1.22 \times 10^{-3} \times 0.38 = 46.36 \times 10^{-5} \text{ M}^2$) were computed by Method 2 described in the Theoretical section. (a) Plot of $v_i/[E_T]$ versus $\log [S_0]$; (b) plot of $[E_T]/v_i$ versus $1/[S_0]$; (c) plot of $[S_0][E_T]/v_i$ versus $[S_0]$; (d) plot of $v_i/[E_T]$ versus $[S_0]$. The dotted line in Fig. 2 (a) was calculated from $v_i/[E_T] = k'[S_0]/(K_{a2} + [S_0])$ in which $k' = 0.45 \text{ s}^{-1}$ and $K_{a2} = 0.38 \text{ M}$.

necessary that the parameters be constrained to be positive.

A non-linear regression procedure which was originally suggested for use in fitting data to ligand-

binding equations (Cornish-Bowden & Koshland, 1970) seemed appropriate to apply to this work. In this procedure, negative parameters are prevented by carrying out the calculations in terms of the

logarithms of the parameters. The method depends in essence on the assumption that the sum of squares of residuals can be presented approximately by a quadratic function of the logarithms of the parameters. Starting at any values of the parameters, it is possible in principle to calculate the first and second partial derivatives of the sum of squares with respect to each parameter, and use the quadratic assumption to calculate the location of the minimum. If the quadratic assumption has any validity this calculated minimum approximates to the true minimum, and the calculation is repeated as many times as are necessary to find the true minimum. When this technique was applied to the fitting of kinetic data to eqn. (17) it worked well only in the immediate vicinity of the minimum; in other words, it was necessary to have very good starting guesses. This is because eqn. (17) is rather 'overdefined', in that it contains more parameters than are actually required to produce a good fit to the data, and as a result the parameters are very highly correlated, and the sum-of-squares contours are exceedingly elongated. It was therefore necessary to complement the procedure of Cornish-Bowden & Koshland (1970) with one which was capable of proceeding efficiently from poor starting guesses when the quadratic approximation was inadequate.

Various methods of proceeding in the early iterations were tried, including various modifications of the method of steepest descent, mostly with rather unsatisfactory results. The method that was eventually used, which gave excellent results in all cases tried, was the modified simplex method of Nelder & Mead (1965). In this method, a 'simplex' is set up, consisting of $(n+1)$ sets of values for the n parameters, and the sum of squares is computed at each set of values, or 'vertex'. The worst vertex is then replaced by one obtained by reflecting the worst vertex through the centroid of the other n vertices. The amount by which this vertex is changed is then increased if the new vertex is a new best, and decreased if it is a new worst. The calculation is then repeated until the differences between the vertices become negligible. The main advantages of this method are its extreme simplicity and the fact that fewer assumptions are made about the shape of the sum-of-squares surface than in any other method. It is also particularly suitable for use in conjunction with the method described previously, because it is much more effective in the early part of the minimization, becoming very slow as the minimum is approached.

In summary, therefore, the results given were analysed by fitting to eqn. (17) by using the method of Nelder & Mead (1965) to obtain rough estimates of the kinetic parameters, which were subsequently refined by the method of Cornish-Bowden & Koshland (1970). On completion of the minimization, standard errors of the parameters were estimated as described

by Cornish-Bowden & Koshland (1970). It should be noted that since in almost all cases the calculated errors were rather large, the linear assumptions used in calculating them are unlikely to be accurate, so that the actual value must be regarded only as an index of precision.

Materials and Methods

Bromelain preparations

The starting material was food-grade bromelain [Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks., U.K. (now Miles Seravac Ltd.), batch no. E.V. 1a]. This material was purified by a method which is essentially that of Murachi *et al.* (1964) to yield what in the present paper we shall call 'bromelain'. For some experiments a preparation of the enzyme in which the chromatography on Sephadex G-100 was omitted was used. This preparation we shall call 'partially purified bromelain'. Both of these preparations are essentially the same as that which in other connexions we have called T-bromelain (Brocklehurst *et al.*, 1972).

Extraction. Crude bromelain powder (40g) was added to 0.05M-potassium phosphate buffer, pH 6.10 (200ml), and the resulting suspension stirred at room temperature (approx. 26°C) for 30min. The resulting cloudy solution was centrifuged at 10000g for 30min and the supernatant collected.

Chromatography on Amberlite CG-4B. This resin (a weakly basic anion exchanger) was chosen as a substitute for the Duolite A-2 used by Murachi *et al.* (1964) which is not obtainable in this country. In early purifications the supernatant from the extraction was applied to a column (2.5cm×45cm) of this resin which had been fully acid-base cycled. Chromatography of crude bromelain on this resin did not result in resolution of the crude enzyme into discrete components (as monitored by E_{280} measurement), but resulted in a single main peak with a long tail. Murachi *et al.* (1964) reported that chromatography on a resin very similar to Amberlite CG-4B (Duolite A-2) removes some of the yellow-coloured material from the crude enzyme. This was not the case with Amberlite CG-4B, which seems to have very little effect. As a result of this finding, this stage of the preparation was omitted from preparations of enzyme used in this work.

Chromatography on Amberlite CG-50. Murachi *et al.* (1964) applied 10g of crude bromelain in 100ml of the phosphate buffer, pH 6.1, to a 500ml column of Amberlite CG-50 resin (a weakly acidic cation exchanger).

In the present work, a column (2.5cm×100cm) of Amberlite CG-50 resin was acid-base cycled and then equilibrated overnight with 0.2M-potassium phosphate buffer, pH 6.05. A solution of crude bromelain extracted from 10g of crude powder in 100ml of

the same buffer was applied to the column. A further quantity (100ml) of this buffer was pumped through the column in 1 h and then 5 litres of 0.05M-potassium phosphate buffer, pH 6.05, was pumped through at a rate of 2 litre/h. The breakthrough fraction that resulted from this washing was discarded. The column was then eluted with 0.2M- K_2HPO_4 containing 1M-KCl at a rate of 36ml/h and 5ml fractions were collected and monitored by measurement of E_{280} . The fractions containing protein, which emerged as a single symmetrical peak after the breakthrough fraction, were pooled and then subjected to $(NH_4)_2SO_4$ fractionation. For every 100ml of collected ice cold eluate, 25.5 g of $(NH_4)_2SO_4$ was added slowly with gentle stirring. When all the $(NH_4)_2SO_4$ had been added, a moderate cloudiness was apparent in the solution. The cloudy solution was left at 4°C for 1 h and centrifuged at 10000g and 4°C for 30 min. The precipitate was discarded and the supernatant was subjected to $(NH_4)_2SO_4$ precipitation as described above, by using 6 g of $(NH_4)_2SO_4$ for each 100ml of original collected eluate. The precipitate, which was collected by centrifugation, was dissolved in the minimum volume of water at room temperature and dialysed against 3 × 5 litres of 0.01M-KCl containing 1mM-EDTA at 4°C. This enzyme, prepared by chromatography on Amberlite CG-50 and $(NH_4)_2SO_4$ fractionation, is designated 'partially purified bromelain'. This chromatographic step is the one which separates extraneous protein and most of the carbohydrate associated with the crude enzyme; these components appear in the breakthrough fraction. The composition of the breakthrough fraction varied somewhat from run to run, as did the exact elution volume of the enzyme. This was shown to be due to the exceptional pH-sensitivity of bromelain chromatography on this type of column. Variation of ± 0.1 pH unit can cause either the superimposition of the breakthrough and enzyme fractions or the emergence of the enzyme fraction in a very large volume of eluate. Crude bromelain was associated with 0.41 g of carbohydrate/g of total material, whereas after chromatography on Amberlite CG-50 the carbohydrate content was decreased to 0.025 g/g of total material.

Chromatography of 'partially purified bromelain' on Sephadex G-100. A column (2.5 cm × 100 cm) was filled with Sephadex G-100, which had been allowed to swell overnight in 0.05M-sodium acetate buffer, pH 5.2. The column was then equilibrated at 4°C by slowly passing the acetate buffer through it for 24 h. Partially purified bromelain was then applied to the column and eluted with the above-mentioned acetate buffer at a flow rate of 10 ml/h. Fractions (1 or 5 ml) were collected and protein was detected by measurement of E_{280} . The elution pattern was indicative of only a single component absorbing at 280 nm. The protein solution emerging from this

column was subjected to $(NH_4)_2SO_4$ precipitation essentially as described above in the second step of the $(NH_4)_2SO_4$ fractionation and dialysed as described above. The enzyme so obtained is designated 'bromelain'.

Chromatography on Sephadex G-100 and subsequent $(NH_4)_2SO_4$ precipitation decreases the carbohydrate content from 0.025 g/g of total material (in partially purified bromelain) to 0.02 g/g of total material (in bromelain). No circumstances have been found in which the enzyme that has been subjected to Sephadex G-100 chromatography has shown behaviour different from that of partially purified bromelain.

Other materials

Papain. This was the 2 × crystallized product of BDH Chemicals Ltd., Poole, Dorset, U.K., and was used without further purification.

Substrates. α -N-Benzoyl-L-arginine ethyl ester was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. α -N-Benzoyl-L-citrulline methyl ester was obtained from BDH Chemicals Ltd. Acetylglycine methyl ester was prepared by the acetylation of glycine methyl ester (obtained as the hydrochloride from Koch-Light Laboratories Ltd.) with acetic anhydride by the method of Wolf & Niemann (1963).

N-Benzoyl-L-serine methyl ester. This was prepared by reaction of L-serine methyl ester with benzoyl chloride. L-Serine methyl ester hydrochloride (Aldrich, Milwaukee, Wis., U.S.A.) (3.1 g) was dissolved in water (20 ml). Benzoyl chloride (2.08 g) dissolved in chloroform (40 ml) was then added and the biphasic mixture was stirred vigorously with a magnetic stirrer and $NaHCO_3$ added in small portions to maintain the pH at 8.5. After 2 h the pH of the aqueous layer was adjusted to 7.0 after prior separation from the chloroform layer. The aqueous layer was extracted with chloroform (5 × 50 ml). The chloroform extracts and the original chloroform layer were combined and dried over anhydrous $MgSO_4$. The $MgSO_4$ was then removed by filtration and the chloroform removed from the filtrate by rotary evaporation *in vacuo*. The resulting oil solidified when cooled in solid CO_2 -acetone and the solid was twice recrystallized from benzene. The final product had m.p. 85°C [Fry (1950) gives m.p. 84–86°C] and was shown to be a single component by t.l.c. in chloroform-acetone-acetic acid (79:20:1 by vol.) (Found: C, 59.0; H, 5.9; N, 6.3. Calc. for N-benzoyl-L-serine methyl ester, $C_{11}H_{13}NO_4$: C, 59.2; H, 5.8; N, 6.3%). $[\alpha]_D^{24} = 17.9 \pm 0.2^\circ$ ($c = 1$ in 95% ethanol) [Fry (1950) gives $[\alpha]_D^{25} = 17.7$ ($c = 1$ in 95% ethanol)].

Other chemicals. $(NH_4)_2SO_4$ was the BDH product 'special for enzymology.' Buffers and other solutions

were made up with AnalaR-grade reagents wherever possible. Water was distilled and subsequently deionized by passage through a Deminrolit MK.7 mixed-bed ion exchanger. In experiments in which the enzymes were used without L-cysteine or dithiothreitol in the reaction mixture, the deionized water was deoxygenated by refluxing and cooling under O₂-free N₂.

Preparation of stock solutions of activated, activator-free bromelain and papain. These were prepared by incubating the enzyme (approx. 0.1 mM) with L-cysteine or dithiothreitol (5 mM) at pH 6.5 for 15 min at room temperature, and then separating the enzyme from low-molecular-weight species on a column (1.25 cm × 30 cm) of Sephadex G-25. The column had previously been equilibrated with a deoxygenated solution of 0.01 M-KCl and 1 mM-EDTA. The enzyme was eluted with this solution, and a stream of O₂-free N₂ was passed through the eluent reservoir and over the eluting solution.

Determination of thiol contents of bromelain and papain

These were determined by using samples of activated, activator-free enzyme by titration of the enzymes' essential thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid) at pH 8.0 [see Ellman (1959) and Wharton *et al.* (1968a)]. The thiol content of bromelain was generally 0.8 mol of thiol/mol of protein, and of papain approx. 0.6 mol of thiol/mol of protein based on the following optical and molecular-weight data: bromelain, mol. wt. = 3.32×10^4 , $\epsilon_{280} = 6.33 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Murachi *et al.*, 1964); papain, mol. wt. 2.07×10^4 (Smith *et al.*, 1955), $\epsilon_{280} = 5.1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Glazer & Smith, 1961). Spectrophotometric measurements were made on Cary 15 or Cary 16K spectrophotometers. When measuring initial rates of bromelain- and papain-catalysed hydrolyses (see below) the reactions were generally studied in the presence of 5 mM-L-cysteine.

In the calculation of catalytic rate constants, the enzyme concentration was calculated from measurements of E_{280} .

Measurement of initial rates of hydrolysis of α -N-acylamino acid ester substrates catalysed by bromelain and by papain

These measurements were made in a Radiometer pH-stat. A total initial reaction volume of 5 ml was used. The reaction mixture was contained in a jacketed vessel through which water from a thermostatically controlled bath set at 25.0°C was circulated. The titrant was 0.04 or 0.1 M-NaOH, which was made up from standardized BDH concentrated volumetric reagent and O₂-free and CO₂-free deionized water. The titrant was protected from atmospheric CO₂

by guard tubes filled with self-indicating Carbosorb (BDH Chemicals Ltd.). O₂-free N₂ was passed over the surface of the reaction mixture. A typical reaction mixture is as follows: 0.5 M-KCl (1.0 ml); 0.1 M-EDTA (0.05 ml), 0.2 M-L-cysteine hydrochloride neutralized to pH 7.0 (± 0.1 ml); substrate solution (x ml); water [$5.0 - (x + y + 1.15 \text{ or } 1.05)$ ml]; enzyme solution (y ml). Any further additions to the reaction mixture (modifiers) were added in a volume which was subtracted from the water volume. The reaction was generally started by addition of the substrate. In the runs carried out in the presence of L-cysteine, the enzyme was incubated with L-cysteine-EDTA-KCl solution for 10 min before the reaction was started by addition of the substrate.

Carbohydrate determinations

These were done by the method of Winzler (1955).

Data processing

Data pairs ($[S_0]$, v_i) were processed on the University of London Atlas computer by using programs written in the EXCHLF language and on the University of Birmingham English Electric KDS9 computer by using programs written in FORTRAN IV. Copies of the FORTRAN program used to fit data to eqn. (17) may be obtained from Dr. A. Cornish-Bowden.

Results and Discussion

Kinetic plots of the data from the bromelain-catalysed hydrolysis of N-benzoyl-L-serine methyl ester

Fig. 2(b) shows a Lineweaver-Burk plot of typical data obtained from the hydrolysis of N-benzoyl-L-serine methyl ester catalysed by bromelain at pH 7.0, $I = 0.1$, and 25°C. This plot deviates somewhat from linearity giving rise to a curve of downward concavity. When the same data are plotted as $[S_0]/v_i$ versus $[S_0]$ (Fig. 2c) marked non-linearity becomes apparent, the curvature being concave down. Figs. 2(b) and 2(c) show that the catalysis of the hydrolysis of N-benzoyl-L-serine methyl ester by bromelain does not follow simple Michaelis-Menten kinetics. Downward concavity in the plot forms of Figs. 2(b) and 2(c) may arise from a number of factors: (i) the enzyme may be impure in that it may be composed of more than one species able to catalyse the hydrolysis of the substrate in non-identical ways, (ii) the substrate may be impure in that in addition to the species whose hydrolysis is catalysed by the enzyme, there is another species which may bind to the enzyme to produce a complex which catalyses the hydrolysis of the substrate more effectively than the enzyme; (iii) the enzyme may have more than one active site per molecule whose catalytic properties are non-identical

or the sites may be interactive as a result of substrate binding; (iv) the enzyme may have a single catalytic site per molecule, but be subject to non-compulsory substrate activation (see the Theoretical section).

The possible reasons for curvature in plots of enzyme kinetic data have been discussed by Trowbridge *et al.* (1963), who studied the trypsin-catalysed hydrolysis of α -N-tosyl-L- and -D-arginine methyl esters and found that these catalyses did not follow simple Michaelis-Menten kinetics. They obtained concave-up Eadie plots, which are equivalent to concave-down Lineweaver-Burk and $[S_0]/v_1$ versus $[S_0]$ plots, and interpreted their data in terms of substrate activation. It is demonstrated in the Theoretical section that concave-down $[S_0]/v_1$ versus $[S_0]$ plots may be due to non-compulsory substrate activation but may not arise from compulsory substrate activation.

Steady-state kinetic experiments do not allow the factors (i)–(iv) to be distinguished as interpretations for curved kinetic plots. Other types of experiments, however, may sometimes suggest that certain of these factors are less likely than others to be responsible for the curvature.

Factor (i). Factor (i) is very difficult to eliminate, since no absolute criteria for enzyme purity are available. The enzyme purified by the method of Murachi *et al.* (1964) is homogeneous by ultracentrifugal sedimentation, free-boundary electrophoresis and diffusion analysis (Murachi *et al.*, 1964). Chromatography on Amberlite CG-50, CM-Sephadex, DEAE-Sephadex, or Sulphoethyl-Sephadex yields a single symmetrical peak (Ota *et al.*, 1964; Murachi *et al.*, 1964). No evidence for inhomogeneity was found by disc electrophoresis on polyacrylamide gel in either the absence or the presence of 8M-urea (Chao & Liener, 1967; C. W. Wharton & I. Trayer, unpublished work). On the other hand, the chromatographically purified enzyme, which migrates as a

single band during electrophoresis on cellulose acetate (Ota *et al.*, 1964), contains small amounts of extraneous end groups in addition to the terminal valine residue (Ota *et al.*, 1964). This, together with the finding of Whitaker and his co-workers (El-Gharbawi & Whitaker, 1963; Feinstein & Whitaker, 1964) that stem bromelain can be fractionated into five proteolytically active components, each having different amino acid compositions, pH optima for casein hydrolysis and heat stabilities, casts doubt on the strict homogeneity of bromelain obtained by some methods of preparation. There is evidence, however, which, although not conclusive, suggests that such inhomogeneity as may exist in the purified bromelain preparations used in the present work is probably not responsible for the curvature in Fig. 2(b) and 2(c). First, at pH approx. 7, kinetic plots (Lineweaver-Burk, Eadie and $[S_0]/v_1$ versus $[S_0]$) for the catalysis by bromelain and by *O*-carboxymethylcellulose-bromelain of the hydrolysis of α -N-benzoyl-L-arginine ethyl ester are linear (Inagami & Murachi, 1963; Wharton *et al.*, 1968a). Secondly, the values of $v_1/[E_T]$ at fixed substrate concentrations for the catalysis of the hydrolysis of *N*-benzoyl-L-serine methyl ester by 'partially purified bromelain' and 'bromelain' are closely similar and the differences not apparently systematically distributed about zero (see the Materials and Methods section and Table 1). Thirdly, the values of $v_1/[E_T]$ at fixed substrate concentrations are closely similar when different fractions from an $(NH_4)_2SO_4$ fractionation of 'partially purified bromelain' are used for the catalysis (see Table 2). The values of $v_1/[E_T]$ in Table 2 are in good agreement with those obtained by using the normal 'partially purified' and 'fully purified' enzyme preparations (Table 1). The results given in Tables 1

Table 1. Apparent first-order rate constants at constant substrate concentrations for the hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by 'partially purified bromelain' and bromelain at pH7.0, 25.0°C and $I = 0.1$

For details see the text.

[S](M)	$v_1/[E_T] (s^{-1})$		
	Partially purified bromelain	Bromelain	$\Delta(v_1/[E_T]) (s^{-1})$
0.025	0.035	0.031	+0.004
0.050	0.057	0.056	+0.001
0.75	0.077	0.084	-0.007
0.10	0.097	0.108	-0.011
0.125	0.127	0.112	+0.015
0.150	0.133	0.132	+0.001

Table 2. Comparison of the apparent first-order rate constants for the bromelain-catalysed hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by two $(NH_4)_2SO_4$ fractions of 'partially purified bromelain' at pH7.0, 25.0°C and $I = 0.1$

Fraction 1 was obtained from a solution of partially purified bromelain at 4°C by addition of solid $(NH_4)_2SO_4$ to 42% saturation, and centrifugation. Fraction 2 was obtained by adding $(NH_4)_2SO_4$ to 50% saturation to the supernatant remaining after removal of fraction 1, followed by centrifugation. In both cases the precipitate was redissolved in water and extensively dialysed against 1.0mM-EDTA before assay.

[S](M)	$v_1/[E_T] (s^{-1})$		$\Delta(v_1/[E_T]) (s^{-1})$
	Fraction ... 1	2	
0.0125	0.023	0.018	+0.005
0.050	0.058	0.058	0.0
0.10	0.097	0.097	0.0
0.15	0.130	0.140	-0.01

and 2 were obtained from experiments carried out with an interval of 6 months between them and with different enzyme preparations. The latter two pieces of evidence indicate that if bromelain contains a kinetically significant impurity, the impurity is closely associated with it and is not separable either by chromatography on Sephadex G-100 (the step which converts 'partially purified bromelain' into 'bromelain') or by $(\text{NH}_4)_2\text{SO}_4$ fractionation. The first piece of evidence shows that any impurity in bromelain kinetically significant in *N*-benzoyl-L-serine methyl ester hydrolysis must catalyse the hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester either in a manner indistinguishable from the catalysis by bromelain or not at all.

Nakata & Ishii (1972) reported that the hydrolysis of α -*N*-benzoyl-L-arginine *p*-nitroanilide is subject to substrate activation when catalysed either by α -trypsin or by β -trypsin. This finding greatly strengthens the suggestion by Trowbridge *et al.* (1963) that the deviation of the trypsin-catalysed hydrolysis of α -*N*-tosyl-L- and -D-arginine methyl esters from simple Michaelis-Menten kinetics might be due to substrate activation.

Takahashi *et al.* (1973) have reported the resolution of bromelain prepared by the method of Murachi *et al.* (1964) into two fractions designated SB1 and SB2. These fractions differ in amino acid composition by only 2 or 3 residues of glycine, alanine and tyrosine and are essentially identical in molecular properties, active-centre sequences and catalytic properties.

In the absence of evidence to the contrary, it is tentatively suggested that impurity in the bromelain preparation is not responsible for the non-linearity of the plots in Figs. 2(b) and 2(c); fractions SB1 and SB2 appear to be so similar that their co-existence would seem unlikely to produce the 'substrate-activation' effect reported here.

Factor (ii). This factor involves the presence of impurity in the substrate. The *N*-benzoyl-L-serine methyl ester prepared as described in the Materials and Methods section provided good analytical and optical-rotation data and was pure as assessed by t.l.c. Titration with NaOH showed that there was less than 1% of *N*-benzoyl-L-serine present, and ninhydrin analysis of a stock solution of aqueous substrate that had been stored at 4°C for 2 weeks showed that the maximum possible concentration of L-serine methyl ester present was less than 0.1%. Preliminary experiments with *N*-benzoyl-DL-serine methyl ester have demonstrated that the 'substrate-activation' phenomenon characterized by the high binding constant K_a ($=0.38 \pm 0.06\text{M}$, see below) is not due to binding of low concentrations of *N*-benzoyl-D-serine methyl ester characterized by a much smaller binding constant.

Factors (iii) and (iv). These factors cannot be distinguished by the available data. In the absence of

independent evidence that bromelain catalyses exhibit co-operativity, we have chosen to interpret the rate data in terms of parameters that characterize a catalysis subject to non-compulsory substrate activation (Schemes 1 or 2). As a general view it seems reasonable that many hydrolyses of low-molecular-weight substrates catalysed by proteolytic enzymes might be found to be subject to non-compulsory substrate activation if examined over a sufficiently wide range of substrate concentration. Since the natural substrates for these enzymes are proteins, and since binding sites in such enzymes are probably composed of several sub-sites (see Berger & Schechter, 1970), each binding one amino acid residue, it seems plausible that with low-molecular-weight substrates the 'second' substrate molecule might occupy one of these sub-sites. This could result either in enhancement of the catalytic effectiveness of the enzyme acting on the substrate molecule bound at the catalytic site, by conformational changes, or by decreasing the number of non-productive binding modes of this substrate molecule.

Kinetic parameters of the bromelain-catalysed hydrolysis of N-benzoyl-L-serine methyl ester: comparison with those of the bromelain-catalysed hydrolyses of α -N-benzoyl-L-arginine ethyl ester and α -N-benzoyl-L-arginine amide

Figs. 2(a)–2(d) demonstrate the good fit of the data for the bromelain-catalysed hydrolysis of *N*-benzoyl-L-serine methyl ester to eqn. (17).

The parameters of eqn. (17) that characterize this catalysis are given in the legend of Fig. 2. The locus that defines the limits of the constituent constants of these parameters in terms of Scheme 1 is presented as line B of Fig. 1. Certain limitations must be placed on the values that may be taken by x , b and a/x . Thus, the minimum value of x is 3.3×10^{-3} and the minimum value of b is 3.21×10^{-3} . The ratio a/x has a maximum limit of 316 but has no minimum limit. If $x > 1$, a and b must be greater than approx. 300, i.e. $K_{m1} > 300 K_{a1}$ and $K_{m1} > 300 K_{a2}$. Thus although it is possible to set certain limits on the values of the ratios of the constants that characterize Scheme 1, it is not possible to define the value of any constant except that of k' .

As discussed in the Theoretical section, it is possible to simplify Scheme 1 to Scheme 2 if it is assumed that only one type of binary ES complex can form (or that all such complexes are equivalent). Scheme 2 could be interpreted in a general way analogous to the treatment of dibasic acids in terms of stages of ionization characterized by macroscopic dissociation constants (see Edsall & Wyman, 1958). There seems little advantage in this approach in the present context, as it merely hides complexity in simple parametric

formulation. Scheme 2 could also be interpreted in terms of a compulsory binding order in ternary complex-formation, i.e. the substrate molecule that undergoes transformation to products binds before the substrate molecule that acts as modifier and does not undergo transformation to products. It is interesting to adopt this interpretation of Scheme 2, because this allows a comparison of the parameters of Scheme 2 with the Michaelis parameters of other bromelain-catalysed hydrolyses. Ordered binding of ligands to enzymes is well established for multi-substrate enzymes, e.g. many NAD-linked dehydrogenases. The possibility that the substrate activation observed in the present study might involve an obligatory order of binding in what might constitute two of the sub-sites (see Berger & Schechter, 1970) for a protein or polypeptide substrate raises intriguing questions about the mechanism of binding of the amino acid residues in such substrates.

The kinetic parameters that characterize the hydrolysis of this substrate catalysed by bromelain and, for comparison, by papain, in the presence (see below) and absence of urea and guanidine hydrochloride are presented in Table 3, which also shows the kinetic parameters that characterize the bromelain-catalysed hydrolysis of α -N-benzoyl-L-arginine ethyl ester and of α -N-benzoyl-L-arginine amide. The latter parameters are discussed first because they constitute one of the most important, hitherto unresolved, problems in the kinetics of bromelain-catalysed hydrolyses and provide a background for discussion of the N-benzoyl-L-serine methyl ester hydrolysis.

Bromelain-catalysed hydrolysis of α -N-benzoyl-L-arginine ethyl ester and α -N-benzoyl-L-arginine amide

The kinetic property that most dramatically distinguishes bromelain from the somewhat similar thiol proteinases papain and ficin is the large difference in the value of $k_{\text{cat.}}$ for the hydrolyses of α -N-benzoyl-L-arginine ethyl ester and α -N-benzoyl-L-arginine amide found only for the bromelain catalyses (Inagami & Murachi, 1963). An associated feature that could be significant is that for each of these bromelain catalyses the values of the ratio $k_{\text{cat.}}/K_m$ are closely similar (see Table 3). Inagami & Murachi (1963) have interpreted the large difference in these $k_{\text{cat.}}$ values in terms of the usual common acyl-enzyme model (eqn. 27) by assuming that deacylation is rate-limiting for the ester hydrolysis (i.e. $k_{\text{cat.}} = k_{+3}$), whereas acylation is rate-limiting for the amide hydrolysis, i.e. ($k_{\text{cat.}} = k_{+2}$ for the amide system)

The constants of eqn. (27) are related to those of the simple Michaelis-Menten eqn. (28) by the well-known relationships (29–31)

$$k_{\text{cat.}} = k_{+2}k_{+3}/(k_{+2} + k_{+3}) \quad (29)$$

$$K_m = (k_{-1} + k_{+2})k_{+3}/k_{+1}(k_{+2} + k_{+3}) \quad (30)$$

$$k_{\text{cat.}}/K_m = k_{+1}k_{+2}/(k_{-1} + k_{+2}) \quad (31)$$

To explain the similarity in the values of $k_{\text{cat.}}/K_m$ for the bromelain-catalysed hydrolyses of the ester and amide substrates, Inagami & Murachi (1963) suggested that $k_{-1} \ll k_{+2}$, in which case $k_{\text{cat.}}/K_m$ (eqn. 31) reduces to k_{+1} , the second-order rate constant for the formation of the adsorptive complex. Consideration of the value (Inagami & Murachi, 1963) of $k_{\text{cat.}}/K_m$ ($2.9 \text{ M}^{-1} \cdot \text{s}^{-1}$) for the bromelain-catalysed reactions, however, suggests that this ratio cannot represent k_{+1} , since the formation of an adsorptive complex is usually considered to be diffusion-controlled with a rate constant of approx. $1 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (see Gutfreund, 1965). It is unlikely therefore that $k_{-1} \ll k_{+2}$. It could be suggested that the similarity in the values of $k_{\text{cat.}}/K_m$ for the amide and ester hydrolyses might be due to fortuitous compensation of the smaller value of k_{+2} for the amide hydrolysis by a proportionately larger value of $k_{+1}/(k_{-1} + k_{+2})$ (or k_{+1}/k_{-1} i.e. $1/K_s$, if $k_{-1} \gg k_{+2}$) than obtains for the ester hydrolysis. Such compensation, although not impossible, seems unlikely, and this casts doubt on the interpretation suggested by Inagami & Murachi (1963) of their finding that $k_{\text{cat.}}$ (ester)/ $k_{\text{cat.}}$ (amide) = 140. We would like to propose an alternative interpretation of the relationships that exist between the kinetic parameters of these bromelain-catalysed hydrolyses. We have shown previously, in another connexion (Brocklehurst *et al.*, 1968), that one phenomenon which is characterized by a decrease in the magnitude of $k_{\text{cat.}}$ without change in the magnitude of $k_{\text{cat.}}/K_m$ is non-productive binding of the substrate. When the acyl-enzyme model (eqn. 27) is extended to include binding of the substrate by the enzyme in modes that will not permit acylation (SE), it becomes eqn. (32).

The parameters that characterize eqn. (32) are related to those of the simple Michaelis-Menten model (eqn. 28) by eqn. (33) and (34) in which K_m^* and K_s' are defined by eqns. (35) and (36).

Table 3. *Kinetic parameters for some bromelain and papain-catalysed hydrolyses at pH 7.0 and 25.0°C*

For the hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by bromelain the kinetic parameters are those of Scheme 2; for the relationship of these to the parameters of Scheme 1 see the text. References: (i) this work; (ii) Inagami & Murachi (1963); (iii) Lowe & Williams (1965). Values given $\pm$ s.e.m.

Enzyme	Substrate	<i>I</i>	Modifier	K_m (mM)	k_{cat} or k (s ⁻¹)	k_{cat}/K_m or k/K_m (M ⁻¹ .s ⁻¹)	K_{s2} (M)	k' (s ⁻¹)	k'/K_{s2} (M ⁻¹ .s ⁻¹)	Reference
Bromelain	<i>N</i> -Benzoyl-L-serine methyl ester	0.1	—	1.22 $\pm$ 0.73	0.0157 $\pm$ 0.0031	12.83	0.38 $\pm$ 0.06	0.45 $\pm$ 0.04	1.18	(i)*
Bromelain	<i>N</i> -Benzoyl-L-serine methyl ester	1.15	—	1.62	0.0138	8.53	0.35	0.52	1.48	(ii)†
Bromelain	<i>N</i> -Benzoyl-L-serine methyl ester	1.15	1 M-Urea	0.35	0.012	36.33	0.17	0.33	1.93	(i)†
Bromelain	<i>N</i> -Benzoyl-L-serine methyl ester	1.15	1 M-Guanidine hydrochloride	3.23	0.035	10.83	0.20	0.38	1.90	(i)†
Bromelain	α - <i>N</i> -Benzoyl-L-arginine ethyl ester	0.1	—	170	0.5	2.93	—	—	—	(ii)‡
Bromelain	α - <i>N</i> -Benzoyl-L-arginine amide	0.1	—	1.2	0.0035	2.92	—	—	—	(ii)‡
Papain	<i>N</i> -benzoyl-L-serine methyl ester	0.1	—	52 $\pm$ 4	2.80 $\pm$ 0.10	54.00	—	—	—	(i)†
Papain	<i>N</i> -Benzoyl-L-serine methyl ester	1.0	—	24 $\pm$ 1	2.30 $\pm$ 0.03	96.17	—	—	—	(i)†
Papain	<i>N</i> -Benzoyl-L-serine methyl ester	1.0	1 M-Urea	30 $\pm$ 1	2.50 $\pm$ 0.03	83.33	—	—	—	(i)†
Papain	<i>N</i> -Benzoyl-L-serine methyl ester	1.0	1 M-Guanidine hydrochloride	30 $\pm$ 2	2.40 $\pm$ 0.03	96.83	—	—	—	(i)†
Papain	Methyl hippurate	0.3	—	20.5 $\pm$ 2	2.72 $\pm$ 0.06	133 $\pm$ 3.0	—	—	—	(iii)§

* Kinetic parameters computed by Method 2 (see the Theoretical section).

† Kinetic parameters calculated by Method 1 (see the Theoretical section).

‡ These data are from experiments at pH 6.0 but the k_{cat} -pH and K_m -pH profiles are essentially flat in the pH range 6-7.

§ Data from experiments at pH 6.0 and 35°C.

$$k_{\text{cat.}} = \frac{k_{+2}k_{+3}}{\frac{k_{+3}K_m^*}{K_s'} + k_{+2} + k_{+3}} \quad (33)$$

$$K_m = \frac{k_{+3}K_m^*}{\frac{k_{+3}K_m^*}{K_s'} + k_{+2} + k_{+3}} \quad (34)$$

$$K_m^* = (k_{-1} + k_{+2})/k_{+1} \quad (35)$$

$$K_s' = k_{-1}'/k_{+1}' \quad (36)$$

Comparison of eqns. (33) and (34) with the analogous eqns. (29) and (30) that result from the steady-state treatment of eqn. (27) demonstrates that if two catalyses are characterized by similar parameters for the formation of the productive adsorptive complexes, and for the subsequent acylation and deacylation steps, but one catalysis is more subject to non-productive binding than is the other, the former catalysis will be characterized by both a lower $k_{\text{cat.}}$ and a lower K_m but the ratio $k_{\text{cat.}}/K_m$ will be unaffected. Thus if bromelain catalyses the hydrolyses of α -*N*-benzoyl-L-arginine ethyl ester and α -*N*-benzoyl-arginine amide by similar mechanisms characterized by similar values of K_m^* (see eqn. 35) and similar values of $k_{+2}k_{+3}/(k_{+2} + k_{+3})$, the observed values of the Michaelis parameters for the two catalyses could result from a much larger non-productive component in the binding of the amide than in the binding of the ester.

One way in which this could arise would be if the susceptible primary-amide group (R_3) of the amide were bound to an appreciable extent at a site on the enzyme (ρ_1) which is occupied by the *N*-acylamino group (R_1) of the substrate when it is bound pro-

ductively (see Scheme 3). Since for *N*-acylamino acid amide substrates both R_1 and R_3 are amide groups, this type of non-productive binding might be expected to exist in most, if not all, catalyses by proteolytic enzymes of the hydrolysis of this class of substrate. Similarly, R_3 could bind non-productively in ρ_2 , the site which may exist in bromelain for the binding of the amino acid side chain, in this case the arginine side chain.

Bromelain-catalysed hydrolysis of N-benzoyl-L-serine methyl ester

The four kinetic parameters for the apparent substrate-activated bromelain-catalysed hydrolysis of *N*-benzoyl-L-serine methyl ester bear a remarkable resemblance to the two sets of kinetic parameters for the bromelain-catalysed hydrolysis of α -*N*-benzoyl-L-arginine amide and α -*N*-benzoyl-L-arginine ethyl ester (see Table 3); k and K_{m_1} for the *N*-benzoylserine methyl ester hydrolysis are somewhat similar to $k_{\text{cat.}}$ and K_m for the α -*N*-benzoyl-L-arginine amide hydrolysis and k' and K_{a_2} are somewhat similar to $k_{\text{cat.}}$ and K_m for the α -*N*-benzoyl-L-arginine ethyl ester hydrolysis. We tentatively propose the following interpretation of this similarity. For the *N*-benzoyl-L-serine methyl ester hydrolysis K_{m_1} and k are the parameters which, in terms of the model given in Scheme 2, characterize the binding of a substrate molecule to give the binary enzyme-substrate complex and the subsequent chemistry which results in its hydrolysis. The relatively low values of both K_{m_1} and k could result as suggested for the α -*N*-benzoyl-L-arginine amide hydrolysis from a large non-productive component in the binding of *N*-benzoyl-L-serine

Scheme 3. Schematic representations of the productive binding of an *N*-acylamino acid amide by a proteolytic enzyme such as bromelain

It is considered that binding of the *N*-acylamino group [R_1 in (a) and RCO-NH in (b)] to a site in the enzyme, ρ_1 , and of the side chain (R_2) to a site ρ_2 presents the group in the substrate that contains the susceptible linkage [R_3 in (a) and CO-NH_2 in (b)] to the catalytic site of the enzyme, ρ_3 .

methyl ester to give the binary complex. Since the susceptible linkage in *N*-benzoyl-L-serine methyl ester is not an amide group, the reason for strong non-productive binding cannot be the same as that proposed for α -*N*-benzoyl-L-arginine amide. It could occur, however, for another reason. Whereas α -*N*-benzoyl-L-arginine amide and α -*N*-benzoyl-L-arginine ethyl ester may be trifunctional substrates, in which the orientation of the susceptible linkage at the catalytic site of bromelain results from binding of the *N*-benzoylamino group (R_1) at a particular site (ρ_1) on the enzyme and of the arginine side chain (R_2) at another site (ρ_2) (see Scheme 3), *N*-benzoyl-L-serine methyl ester might serve only as a bifunctional substrate. This would be the case if the hydroxymethyl side chain is not bound to any significant extent in the ρ_2 site. If this is so, non-productive binding could result from binding of R_1 of *N*-benzoyl-L-serine ethyl ester in site ρ_2 . Alternatively, there may be other sub-sites in the bromelain-binding site at which *N*-benzoyl-L-serine methyl ester binds relatively strongly but non-productively.

In terms of our chosen model, the parameters K_{22} and k' characterize the binding of a second *N*-benzoyl-L-serine methyl ester molecule to give a ternary complex, SES (see Scheme 2), and the subsequent chemistry which results in the hydrolysis of one *N*-benzoyl-L-serine methyl ester molecule. It is noteworthy that the value of k' is similar to that of k_{cat} for the α -*N*-benzoyl-L-arginine ethyl ester hydrolysis. In the *N*-benzoyl-L-serine methyl ester hydrolysis the function of the substrate molecule that is causing the 'activation', and is not itself undergoing hydrolysis, is envisaged as a blocking of one or more of the binding sites which it is suggested may be involved in non-productive binding of *N*-benzoyl-L-serine methyl ester in the binary complex.

It has been common practice to use low-molecular-weight substrates to study proteolytic enzymes. Evidence is accumulating, however, that there are a large number of possible interactions between the amino acid residues in the active sites of some proteinases, notably papain (Berger & Schechter, 1970; Wolthers *et al.*, 1970), and the amino acid residues of large polypeptide substrates, and that low-molecular-weight substrates may bind in non-productive modes in such active sites. We have shown previously (Brocklehurst *et al.*, 1968) that postulated non-productive binding modes could satisfactorily resolve the contradictory conclusions reached by Whitaker & Bender (1965) and by Sluyterman (1968) concerning the rate-limiting step in papain-catalysed hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester, if this ester could bind to papain in a mode which does not permit acylation of the enzyme but which may increase the reactivity of the thiol group of cysteine-25 towards alkylating agents. This suggestion subsequently received strong support from the demon-

stration by Whitaker (1969) that the rate of alkylation of the thiol group of cysteine-25 is enhanced by the binding of the inhibitor α -*N*-benzoyl-D-arginine ethyl ester. Hinkle & Kirsch (1971) have produced evidence that the principle mode by which both papain and ficin bind *p*-nitrophenyl esters of *N*-acylamino acids is non-productive. They point out that the elimination of a non-productive binding mode for a reagent by substrate binding can produce enhancement of the reactivity of a functional group in the enzyme towards the reagent. This is analogous to our present interpretation of the substrate-activated bromelain-catalysed hydrolysis of *N*-benzoyl-L-serine methyl ester and is an alternative type of interpretation to that commonly offered for rate enhancements occasioned by substrate binding, i.e. substrate-induced conformational changes in the enzyme (Koshland, 1958). It is probable that, at least in some cases, blocking of non-productive binding modes by substrate binding will be accompanied by conformational changes in the enzyme. It remains to be discovered whether in a given case these factors support or oppose each other and to what extent.

Some of the points discussed in this section have been reported in a preliminary communication (Brocklehurst *et al.*, 1967).

Effect of urea and guanidine hydrochloride on the kinetics of the bromelain-catalysed hydrolysis of N-benzoyl-L-serine methyl ester and of α -N-benzoyl-L-arginine ethyl ester

The kinetic parameters that characterize these catalyses in the presence and absence of the potential modifiers urea and guanidine hydrochloride are given in Table 3.

The effect of these potential modifiers on the *N*-benzoyl-L-serine methyl ester hydrolysis was studied to ascertain their effectiveness in compensating this substrate for its lack of an arginine or citrulline side chain. The observed activation of the trypsin-catalysed hydrolysis of acetylglycine methyl ester by alkylguanidines and alkylamines has been interpreted in terms of pseudo-specific substrate formation in the adsorptive complex (Inagami & Murachi, 1964; Inagami, 1965; Mares-Guia & Shaw, 1965).

The effect of urea and guanidine hydrochloride at concentrations of 1M on the bromelain-catalysed hydrolysis of *N*-benzoyl-L-serine methyl ester is unremarkable. Further, the effect of these potential modifiers at concentrations of 1M on the initial rates of hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester at substrate concentrations of 0.01M and 0.1M is negligible. That a measure of competitive inhibition is not observed in the latter system is perhaps surprising. This indicates that the binding constants characterizing binding of urea and guanidine at the

Fig. 3. $[S_0]/v_1$ versus $[S_0]$ plots for the hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by papain at pH 7.0 in 46.3 mM-potassium phosphate buffer, $I = 0.1$, at 25.0°C in the presence and absence of guanidine hydrochloride

○, and —, 0.1 M-KCl; ● and ----, 1.0 M-KCl; △ and ———, 1.0 M-guanidine hydrochloride.

ρ_2 site must be greater than 0.1 M. This high apparent value of these binding constants suggests that the alkane portion of the arginine side chain may play an important part in determining the binding constant of the R_2 - ρ_2 interaction for arginine substrates of bromelain. A similar effect has been reported in trypsin-catalysed hydrolyses (Inagami & Murachi, 1964; Mares-Guia & Shaw, 1965).

Kinetics of the hydrolysis of *N*-benzoyl-L-serine methyl ester catalysed by papain

These were studied to provide a comparison with the analogous bromelain-catalysed hydrolyses. The kinetic parameters are given in Table 3. Fig. 3 shows that at $I = 0.1$ a plot of $[S_0]/v_1$ versus $[S_0]$ appears to deviate slightly from linearity at low substrate concentrations. It was considered that this deviation did not necessitate interpretation of this system in terms of substrate activation, although it is possible that the deviation may be more marked at lower substrate concentrations. The slight deviation from linearity apparent at $I = 0.1$ is even less apparent at $I = 1.0$ in either the presence or the absence of guanidine hydrochloride. Urea and guanidine hydrochloride at concentrations of 1.0 M have little effect on the Michaelis parameters for the papain-catalysed hydrolysis of *N*-benzoyl-L-serine methyl ester, which are similar to those for the papain-catalysed hydrolysis of methyl hippurate (see Table 3), as would be expected from the similarity in structure of the substrates.

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