Proteolytic Enzymes: Electrophilic Participation in the Acylation Step

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Acylation of α-trypsin and purified fractions of stem-bromelain by substituted phenyl ester substrates has a low sensitivity to Hammett σ consistent with the involvement of an electrophilic interaction with the ether oxygen. General-base assisted thiol attack is shown to be a minor route in acylation of thiol proteases. Stem bromelain's heterogeneity is confirmed and a fractionation procedure is reported for isolation of macro-quantities of the constituent enzymes.

ELECTROPHILIC assistance in the acylation of proteases by their substrates is an attractive hypothesis which is distinct from catalysis involving proton transfer to the leaving group from a tetrahedral intermediate. Recent *X*-ray crystallographic work has provided evidence for an electrophilic component at the active centre of the alkaline protease subtilisin BPN′ where a sulphonyl oxygen of the tosylated enzyme, an analogue of a tetrahedral intermediate, resides in a space surrounded by NH groups of amide links. This component is thought [1] to accept and stabilise the oxyanion of the tetrahedral intermediate during catalysis. There is also evidence that trypsin possesses a similar electrophilic interaction because *X*-ray crystallographic studies of the trypsin–trypsin-inhibitor complex indicate that the oxygen derived from the carbonyl of the lysine-15 peptide link of the inhibitor

[1] J. D. Robertus, J. Kraut, R. A. Alden, and J. J. Birktoft, *Biochemistry*, 1972, **11**, 4293.

is surrounded by peptide NH groups from the enzyme backbone; it is thought that these stabilise the anionic oxygen of a tetrahedral adduct formed between the carbonyl of the peptide of lysine-15 and the hydroxy of serine-195 of the trypsin.[2] The adduct does not proceed to acyl enzyme.

It has been argued that a low Hammett or Brønsted sensitivity for acylation of an enzyme by substituted phenyl ester substrates is consistent with electrophilic participation. This low sensitivity has been observed for three proteolytic enzymes of widely differing genetic origins [3-6] suggesting a convergence in natural selection although the primary structure of the enzymes, papain, chymotrypsin, and alcalase is markedly different.

We decided to study the acylation of α-trypsin and bromelain, two enzymes with similar characteristics to the above three, by substituted phenyl ester substrates to see if the low selectivity was general and to provide possible evidence for an overall theory of electrophilic participation in the acylation step of protease catalysis. We report a simple and efficient fractionation procedure for the enzymes from the juice of the stem of the pineapple (from the Bromeliaceae family, a monocotyledon) a plant considerably different from the source of papain (*Carica papaya*, a dicotyledon).

EXPERIMENTAL

Materials.—Ion exchange celluloses were purchased from Whatman and were pre-cycled and de-fined according to instructions in the Whatman Handbook. Substrates and inhibitors were from previous investigations; [4-6] materials such as reagents and buffer components were obtained from B.D.H. and were either of analytical reagent grade or were recrystallised or redistilled prior to use. Analytical reagent grade dioxan was purged of peroxides by allowing it to percolate through a column of active alumina (an aqueous solution of KI was used to check the absence of peroxides). Acetonitrile was distilled several times from P_2O_5, then from CaH_2, and stored over molecular sieves (4 A). Twice distilled water was used throughout. Crude enzyme in the form of an acetone-precipitated powder, as described by Heinicke and Gortner,[7] was obtained from the Dole Pineapple Corporation.

Methods.—Fractionation of stem bromelain. A difficulty in the study of the proteolytic enzymes from the juice of the stem of the Bromeliaceae is that no satisfactory fractionation procedure has been reported for macro-quantities of the enzyme. Considerable heterogeneity was discovered by El-Gharbawi and Whitaker [8a] but the conditions appear to be quite delicately balanced because although the method has been employed by Silverstein and Kézdy [8b] both Ota *et al.*[8c] and ourselves were able to isolate only one active peak using Amberlite IRC-50 columns under the same conditions. Two reports [9] have described the use of sulphoethyl-Sephadex as a column support, but, although they confirm the heterogeneity of stem bromelain they do not provide a fast and convenient fractionation for gram quantities of active protein. Kinetic work requires that the enzyme be homogeneous unless exceptional circumstances prevail; active-site studies do not necessarily require homogeneity and the sequence studies of Husain and Lowe [10] have a built-in control to allow for this. We describe here a fast (less than a week) and simple fractionation of stem bromelain capable of operating at room temperature.

Crude acetone-dried powder (10 g) was stirred with 0.2M-Tris buffer (100 ml) containing thioglycolic acid (0.1 ml) and adjusted to pH 8.50 (prior to addition of the powder). Stirring was continued for 1 h at room temperature, the suspension was centrifuged (at maximum speed on an MSE-50 instrument with the 6 × 100 ml head) and the supernatant solution dialysed *versus* distilled water (5 l) overnight at 4°. Recentrifugation of the dialysate, which deposited solid matter overnight, gave a total volume usually of *ca.* 170 ml with pH 8.50.

De-fined, pre-cycled Whatman DE 52 ion-exchange cellulose was equilibrated in 0.2M-Tris buffer (pH 8.50) and a 1.5 × 1.05 cm^2 column packed and equilibrated with 0.005M-Tris buffer at the same pH. The dialysate was applied to the column and eluted with 0.005M-Tris; a sharp protein peak was eluted almost at the ' break-through ' volume. The eluted fraction was ' water-white ' whilst the brown pigment of the crude powder and presumably most of the acidic protein was retained on the column, the former as a sharp band at the top. Some pigment was retained by the IR 45 resin as used in the procedure of Murachi *et al.*[11] but we find the DE 52 cellulose to be the most efficient decolouriser.

A Whatman CM 52 or CM 32 column (2.5 × 3.5 cm^2) was packed under the same conditions as before and then equilibrated with 0.005M-Tris buffer at the same pH. The eluate from the DE 52 column (up to 20 ml) was applied and a ' disc ' of physically altered material appeared at the top of the CM column where the protein adsorbed. The ' disc ' was only noticeable when large quantities of protein were applied. Elution with 0.005M-Tris at pH 8.50 gave a ' break-through ' peak with inactivateable protein.* The column was subjected to gradient elution (90 ml h^{-1}) with salt concentration increasing linearly from 0.005 to 0.4M using two reservoirs, one containing buffer at 0.005M (400 ml) and the other containing buffer at 0.005M and NaCl at 0.4M (total 400 ml). Fractions (20 ml) were collected using a Beaumaris fraction collector, and the absorption of the effluent was monitored continuously at 253 nm with an LKB Uvicord type 4701A optical unit equipped with a Uvicord type 4701A control unit output to a Record continuous recording ammeter. The resultant fractionation pattern had four major peaks A—D which were proteinaceous as judged from spectra run on individual fractions

* The protein in all the fractions was assayed *after* prior ' activation ' with 4-thiocresol and *all* the protein peaks were inactive towards substrate without this treatment.

[2] R. Huber, 6th Harden Conference, Wye, 1974.
[3] R. E. Williams and M. L. Bender, *Canad. J. Chem.*, 1971, **49**, 210.
[4] A. Williams, *Biochemistry*, 1970, **9**, 3383.
[5] A. Williams and G. Woolford, *J.C.S. Perkin II*, 1972, 272.
[6] A. Williams, E. C. Lucas, and A. R. Rimmer, *J.C.S. Perkin II*, 1972, 621.
[7] R. M. Heinicke and W. A. Gortner, *Economic Botany*, 1957, **11**, 225.
[8] (*a*) M. El-Gharbawi and J. R. Whitaker, *Biochemistry*, 1963, **2**, 376; (*b*) R. M. Silverstein and F. J. Kézdy, *Fed. Proc.*, 1970, **29**, abstract 3893; (*c*) S. Ota, S. Moore, and W. H. Stein, *Biochemistry*, 1964, **3**, 180; (*d*) G. Feinstein and J. R. Whitaker, *ibid.*, p. 1050.
[9] (*a*) S. Ota, K. Horie, and F. Hagino, *J. Biochem.* (*Tokyo*), 1969, **66**, 413; (*b*) S. Scocca and Y. C. Lee, *J. Biol. Chem.*, 1969, **244**, 4852.
[10] S. S. Husain and G. Lowe, *Biochem. J.*, 1968, **110**, 53.
[11] J. Murachi, M. Yasui, and Y. Ysuda, *Biochemistry*, 1964, **3**, 48.

using a Unicam SP 800 spectrophotometer. Peaks B—D were separated and rechromatographed on CM 32 columns after being desalted by passage through Sephadex G25 (Pharmacia) and excellent gaussian-type peaks were observed at the eluate volumes expected from the initial chromatography. Gel electrophoresis at pH 9.5 indicated that the peaks B—D were homogeneous and enabled the discs from gel electrophoresis of the crude material to be identified. The discs moved towards the cathode indicating that the protein was positively charged at this pH; the relative mobility of the constituent enzymes was B > C > D.

Crude stem bromelain from Sigma, Koch–Light, and B.D.H. gave elution patterns for displacement chromatography on CM 32 cellulose similar to the material from the Dole Co. The yield of protein from 1 g of acetone powder as judged from the absorbance at 280 nm for a typical fractionation was: B, 48 mg; C, 55 mg; D, 29 mg and the fraction of carbohydrate: B, 3.0%; C, 1.1%; D, 1.3%.

Disc electrophoresis was carried out using polyacrylamide gels at room temperature with a Shandon apparatus. Each sample tube contained 7.5% acrylamide small pore gel and the upper large pore gel was omitted. Each enzyme sample containing 80—180 μg protein was diluted in 10% sucrose and 0.1 ml was layered on top of the gel. A steady current of 5 mA/tube was applied for *ca.* 2 h with the anode in the upper compartment; gels were stained overnight with Naphthalene Black and destained in 7% acetic acid using a current of 5mA/tube for 3—5 h with cathode in the upper compartment.

Specific activity was measured using the catalysed hydrolysis of methyl hippurate (0.025M) in a total of 10 ml made up with 0.1M-NaCl containing 0.001M-EDTA at 35°. The pH was kept at pH 6.00 using 0.01M-NaOH titrant in a Radiometer pH-stat assembly (Titratigraph SBR2c; Titrator 11; pH meter 25; syringe burette SBU1c). The specific activity is defined as the number of micromoles of substrate hydrolysed per min per mg protein. Active site titration was carried out for bromelain using Ellman's reagent and since release of thiol was slow the reaction was followed to completion which usually took up to 1 h. Up to 0.65 SH groups per molecule were found for the bromelain fractions assuming a molecular weight of 20 000.[8d]

Carbohydrate content was measured using the orcinol–sulphuric acid method, reading the absorbance of the reaction mixture at 540 nm after incubation at 80° for 15 min.

Protein content of enzyme samples was estimated from the absorbance at 280 nm assuming 1 absorbance unit is equivalent to 1 mg ml^{-1} for a 1 cm path length cell.

Fractionation of trypsin. The method of Schroeder and Shaw [12a] was followed on a sample of bovine trypsin obtained from the Boehringer Co. The trypsin was assayed by active site titration with 4-nitrophenyl 4-guanidinobenzoate as in ref. 12*b*.

Kinetics.—Substrates releasing acid on hydrolysis were studied using the pH-stat as described for the assay; those esters undergoing a u.v.–visible spectral change were studied using either a Unicam SP 800 or a Beckman DBG instrument. In a typical spectrophotometric experiment the activated enzyme (50 λ of stock) was added to 2.45 ml buffer solution at pH 6.00 (0.1M ionic strength; phosphate buffer) containing substrate (25 λ) from a stock solution in acetonitrile. Michaelis–Menten parameters were derived from initial rates and the concentration of substrate *via* fitting to the hyperbolic Henri equation.[13] Values of the enzyme concentration required to calculate k_0 were derived from the active site titrations described above. In the cases where the substrate concentrations never exceeded the value of K_m, pseudo-first-order rates were observed (usually with substrates measured spectrophotometrically) and the rate constants were derived from logarithmic plots using experimental infinity values.

TABLE 1

Hydrolysis of substituted phenyl mesylglycinate, hippurate, and other esters by bromelain (fraction B) [a]

Substituent	λ/nm	k_0/K_m [b] (mesylglycinate)	k_0/K_m [b] (hippurate)
4-Nitro	325	153	4 540
4-Methoxy	290	28.5	648
4-Formyl	330	101	
4-Chloro	281	37.4	1 250
4-Acetyl	295	126	2 460
3-Nitro	355	55.9	2 390
Parent	270	17.7	513
4-Fluoro	280	13.8	344
4-Hydroxy	290	14.2	
3-Chloro	280		1 180
4-Amino	310		262
4-Methyl	285		892
Methyl hippurate		1.69 (K_m > 0.29M)	
4-Nitrophenyl acetate	350	0.64	

[a] 35°, ionic strength made up to 0.1M with NaCl, phosphate buffer, pH 6.00, 0.001M-EDTA. [b] Units are 1 mol^{-1} s^{-1}.

TABLE 2

Hydrolysis of phenyl hippurate and other esters by bromelain (fraction D) [a]

Substituent	$k_0^{-1}K_m$/l mol^{-1} s^{-1}	K_m/l mol^{-1}
4-Nitro	1 130	
3-Nitro	352	
4-Acetyl	1 090	
4-Chloro	409	
4-Methyl	260	
4-Methoxy	260	
Parent	205	
4-Fluoro	172	
3-Chloro	593	
4-Amino	107	
Methyl hippurate	7.0	0.044
Methyl *N*-acetylglycinate	0.0829	0.24
Methyl *N*-benzoylphenylalaninate	7.36	>0.01 (3% CH_3CN)
Benzyl acetylglycinate	0.957	
Ethyl *N*-benzoylargininate (BAEE)	2.21	>0.3
Methyl *N*-benzyloxycarbonyl-L-lysinate	1.18	>0.2

[a] 35°, 0.1M ionic strength made up with NaCl, phosphate buffer for the phenyl ester substrates, pH 6.00.

RESULTS

The hydrolysis of the substituted phenyl esters by both trypsin and bromelain are first order in enzyme and substrate concentrations. A slight amount of non-linearity as required by the Henri equation is observed where the higher substrate concentrations approach the value of K_m. The bimolecular rate constants (k_0/K_m) obtained by division of the pseudo-first-order rate constants by the enzyme concentration are summarised in Tables 1—4. The dependence of k_0/K_m on σ

[12] (*a*) D. D. Schroeder and E. Shaw, *J. Biol. Chem.*, 1968, **243**, 2943; (*b*) T. Chase and E. Shaw, *Biochem. Biophys. Res. Comm.*, 1967, **29**, 508.

[13] A. Williams, ' Introduction to the Chemistry of Enzymes,' McGraw-Hill, 1969, p. 119.

TABLE 3

Hydrolysis of substituted phenyl hippurates by α-trypsin [a]

Substituent	$k_0K_m^{-1}$/l mol^{-1} s^{-1}
4-Nitro	1 320
3-Nitro	205
4-Chloro	84.2
Parent	38.7
4-Methoxy	42.1
4-Acetyl	366
4-Fluoro	38.8
4-Methyl	42.6
2-Nitro	174
3-Chloro	82.9

[a] 25°, ionic strength made up to 0.1M with NaCl, phosphate buffer, pH 7.00.

TABLE 4

Hydrolysis of esters with bromelain (fraction C) [a]

Ester	$K_0K_m^{-1}$/l mol^{-1} s^{-1}
Methyl hippurate	2.46
Methyl *N*-nicotinoylglycinate	0.99

[a] 35°, ionic strength made up to 0.1M with NaCl, pH 6.00.

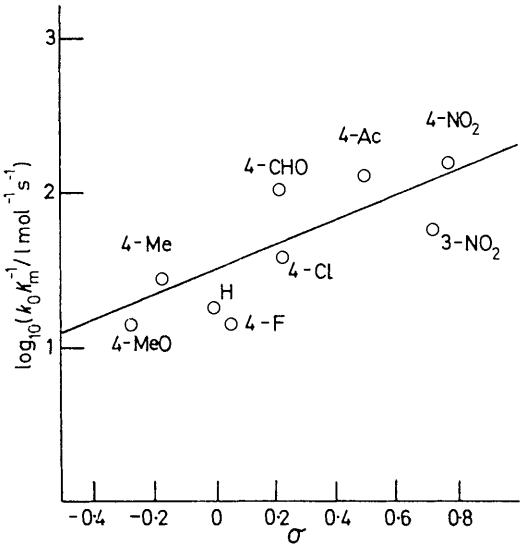


FIGURE 1 Dependence of k_0/K_m on σ for substituted phenyl mesylglycinate substrates of bromelain (fraction B). Conditions as in Table 1, line is arbitrary of slope 0.8

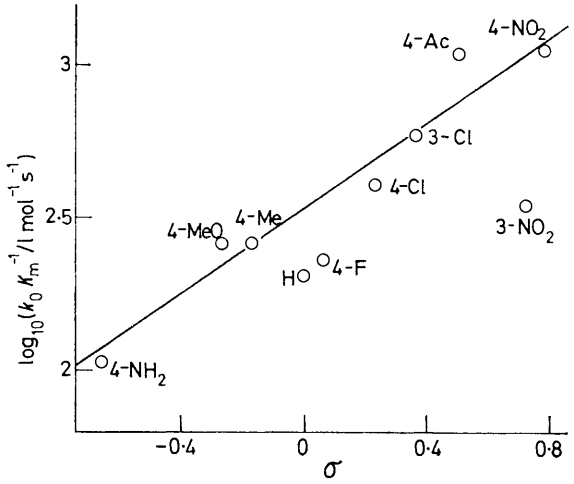


FIGURE 2 Dependence of k_0/K_m on σ for substituted phenyl hippurates and bromelain (fraction B). Conditions as in Table 1, line is arbitrary of slope 0.7

and σ$^-$ for the various esters and enzymes are illustrated (Figures 1—4).*

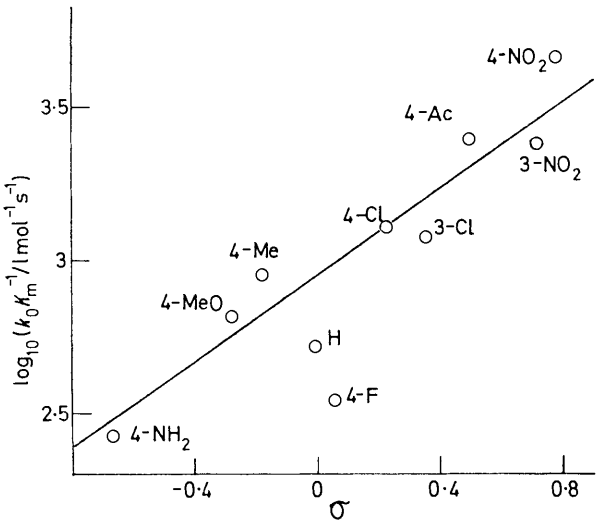


FIGURE 3 Dependence of k_0/K_m on σ for substituted phenyl hippurate substrates of bromelain (fraction D). Conditions as in Table 2, line is arbitrary of slope 0.7

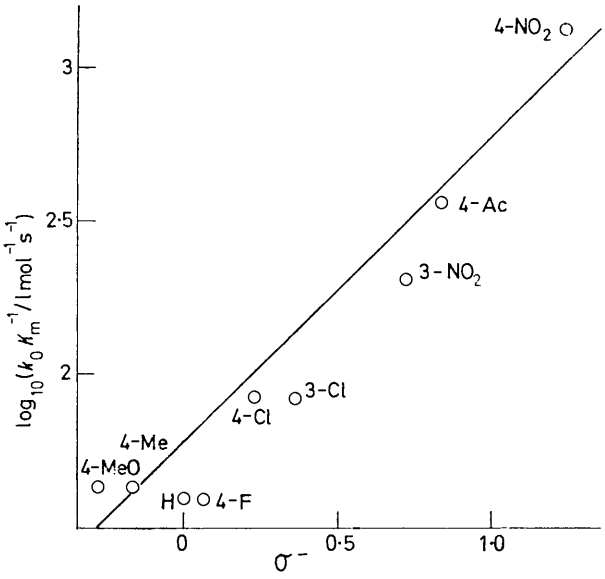


FIGURE 4 Dependence of k_0/K_m on σ$^-$ for substituted phenyl hippurate substrates of α-trypsin. Conditions as in Table 3, line is arbitrary of slope 1.0

In many cases (see Tables) the K_m value is too high for accurate measurement because of solubility problems and where possible a lower limit on this parameter is given.

Solvent composition has a marked effect on bromelain kinetic parameters and details of this are given in Table 5 for the enzyme from fraction B. With the methanol solvent some of the observed inhibition is due to the methanolysis reaction.

Since kinetic data have not been reported for homogeneous bromelains it is difficult to compare the results of this work with literature values. However the parameters for brome-

* Error limits on the parameters are <10%; the deviations exhibited in the Figures are not due to error but presumably arise from variations in the microscopic medium which are impossible to assign at present.

lain from fraction D for BAEE seem to have similar values to those obtained by Inagami and Murachi [14a] for a presumably heterogeneous enzyme.

TABLE 5

Variation of k_0/K_m with solvent composition [a]

Ionic strength/M	0.1	0.2	0.4	0.8	1.2
$k_0K_m^{-1}$/l mol^{-1} s^{-1}	1.69	1.77	2.02	2.31	2.83
Ionic strength/M	1.6	2.0	2.4		
$k_0K_m^{-1}$/l mol^{-1} s^{-1}	3.19	3.5	4.03		
CH_3CN (%)	0	5	10	15	25
$k_0K_m^{-1}$/l mol^{-1} s^{-1}	1.69	1.09	0.825	0.586	0.389
Dioxan (%)	0	0.5	1	1.5	2.0
$k_0K_m^{-1}$/l mol^{-1} s^{-1}	1.69	1.32	1.01	0.713	0.437
Methanol (%)	0	5	10	15	20
$k_0K_m^{-1}$/l mol^{-1} s^{-1}	1.69	1.05	0.673	0.573	0.406
Methanol (%)	25				
$k_0K_m^{-1}$/l mol^{-1} s^{-1}	0.280				

[a] 35°, except in the ionic strength experiment the ionic strength was adjusted to 0.1M with NaCl, pH 6.00.

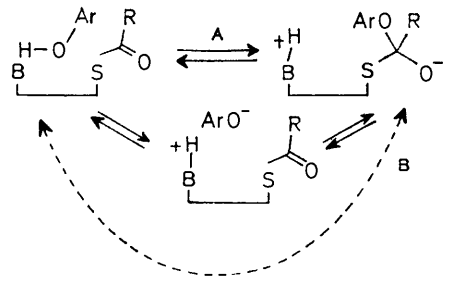
DISCUSSION

There is ample evidence consistent with an acyl-enzyme intermediate in bromelain-catalysed hydrolyses implying at least a three step pathway (1).[4] The

$$RCOX + EH \underset{}{\overset{K_s}{\rightleftharpoons}} RCOX{\cdot}EH \xrightarrow[-HX]{k_2} RCOE \xrightarrow[+H_2O]{k_3} RCO_2H + EH \quad (1)$$

function k_0/K_m is equivalent to k_2/K_s (see ref. 14, p. 38) and is useful because it does not involve complications due to non-productive binding [15] and is essentially equivalent to a bimolecular rate constant for reaction between free enzyme and free substrate to give acyl-enzyme.

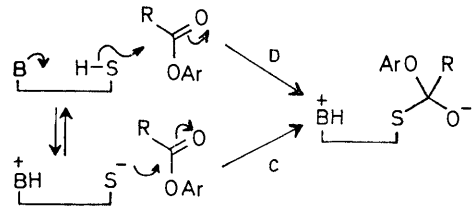
The use of phenyl ester substrates ensures that any manifestation of electrophilic character in the acylation is not due to general acid facilitating departure of the phenolate leaving group (presumably from a tetrahedral intermediate). Guthrie [16] estimates that the departure of methoxide ion from the tetrahedral adduct $CH_3OC(CH_3)(OH)O^-$ has the rate constant *ca.* 10^7 s^{-1}; it is reasonable that departure of a phenolate ion from a similar adduct would have a rate constant close to the diffusion controlled limit and therefore not be subject to catalysis. Since phenolate ions are more nucleophilic than the un-ionised species and present in considerable proportion at the pH values employed path B [reaction (2)] for deacylation by reaction with phenol is unlikely to involve general base catalysis (path A); the microscopic reverse of deacylation, namely decomposition of the tetrahedral intermediate, will therefore take the stepwise path involving no general acid catalysis. These arguments are equally valid for the corresponding serine proteases.



(2)

Zwitterion Mechanism for Thiol Proteases.—There is no experimental evidence which clearly distinguishes a zwitterionic mechanism (C) from a general base catalysed removal of a proton from the thiol (D) as in equation (3) although the former has been favoured for papain.[17] Since the thiol group has pK_a *ca.* 8 it is unlikely that general base catalysis is necessary for its action as a nucleophile because a significant fraction will exist as an anion and the ionisation be essentially diffusion controlled. This type of argument cannot be used in the serine type protease because the serine has a relatively high pK_a and is not present as its anion in significant concentrations at neutral pH and there is an advantage to be gained by a concerted general base assisted nucleophilic attack.

Electrophilic Component of Acylation.—Bromelain. The Hammett sensitivity (Figures 1—3) for acylation of bromelains by phenyl ester substrates is considerably lower than that normally expected for thioanion attack on substituted phenyl esters (ρ *ca.* 2 for mercaptoethanol



(3)

thioanion with aryl *N*-benzyloxycarbonylglycinates; [18a] ρ *ca.* 2 for mercaptoacetate dianion with aryl acetates; [18b] ρ *ca.* 1.7 for cysteine thioanion with aryl acetates; [18c] ρ *ca.* 1.7 for cysteine thioanion with aryl *N*-mesylglycinates [18b]). This lower selectivity results from a smaller negative charge in the transition state of the rate-limiting step (or the *equivalent* of a smaller negative charge) presumably from some form of electrophilic assistance.

The low Hammett ρ is consistent also with an earlier transition state than in the above model reactions and

[14] (*a*) T. Inagami and T. Murachi, *Biochemistry*, 1963, **2**, 1439; (*b*) K. Brocklehurst, E. M. Crook, and C. W. Wharton, *Chem. Comm.*, 1967, 1185; (*c*) T. Murachi and N. Takahashi, in 'Structure-Function Relationships of Proteolytic Enzymes,' eds. P. Desnuelle, H. Neurath, and M. Ottesen, Munksgaard, Copenhagen, 1970, p. 298; (*d*) C. W. Wharton, *Biochem. J.*, 1974, **143**, 575.

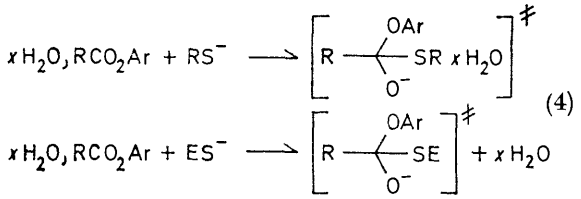
[15] (*a*) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 1965, **34**, 49; (*b*) F. E. Brot and M. L. Bender, *J. Amer. Chem. Soc.*, 1969, **91**, 7187.

[16] J. P. Guthrie, *J. Amer. Chem. Soc.*, 1973, **95**, 6999; 1974, **96**, 3608.

[17] (*a*) L. Polgár, *Eur. J. Biochem.*, 1973, **33**, 104; (*b*) L. Polgár, *FEBS Letters*, 1974, **47**, 15.

[18] (*a*) J. F. Kirsch and M. Igelström, *Biochemistry*, 1966, **5**, 783; (*b*) A. Williams, E. C. Lucas, and K. T. Douglas, *J.C.S. Perkin II*, 1972, 1493; (*c*) G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 199; (*d*) W. P. Jencks and M. Gilchrist, *J. Amer. Chem. Soc.*, 1968, **90**, 2622; (*e*) A. R. Fersht and W. P. Jencks, *ibid.*, 1970, **92**, 5442.

this could arise from an extra powerful nucleophile and/or a very good leaving group.[18d,e] It is inconceivable that the reactivity of the cysteine thioanion *per se* is enhanced because an anion is presumably at maximal reactivity compared with the neutral form with or without general base catalysis. Extra leaving ability of the phenol leaving groups could only be from electrophilic catalysis at the ether oxygen of the ester. It is not practicable to discuss electrophilic assistance in terms of the component k_2 and K_s parameters as these are only separable under exceptional conditions. Since substituent effects on ground-states for model and enzyme reactions are identical [see equation (4)] differences in k_2/K_s come only from substituent effects on the transition state of the rate-limiting step which is the intra-complex addition of thioanion to ester to give tetrahedral intermediate. This energy level is the most unstable in the potential energy reaction co-ordinate surface. Energy levels corresponding to the intermediates and transition states between ground state and the maximal transition state are not relevant to a discussion of substituent effects on k_2/K_s. If we were able to dissect k_2/K_s then these levels would be relevant to a discussion of the intermediate steps.



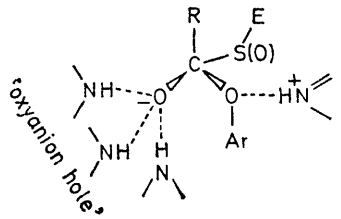
(4)

It is impossible to assign the interaction of the transition state with groups at the active site for bromelain since the tertiary structure is unknown and we must rely on the papain structure [19b] and the assumption that bromelain's active site is closely similar. Perusal of a model (built using co-ordinates, supplied by Dr. J. Drenth, and Kendrew models) of the complex between an ester substrate and papain as described by Lowe and Yuthavong [19b] indicates that the -NH-CO- interactions with aspartate-158 and glycine-66 must be altered (probably weakened by distortion) for formation of the tetrahedral intermediate to occur with the thiol of cysteine-25. When the oxygen of the ester derived from the ether link is within hydrogen bonding distance of the imidazolium NH^+ (of histidine-159) the oxyanion from the carbonyl oxygen resides in a region surrounded by peptide NH groups of serine-24 and cysteine-25 and the amide NH of glutamine-19. This interaction which is the same sort as proposed earlier,[1,4,5] aids formation of the tetrahedral intermediate by a form of chelate effect and the advantage over solvation by water molecules in the model reactions [equation (4)] is the entropic effect of the loss of solvating waters in the enzyme case.[20]

[19](*a*) J. Drenth, J. N. Jansonius, R. Koekoek, H. M. Swen, and B. G. Wolthers, *Nature*, 1968, **218**, 929; (*b*) G. Lowe and Y. Yuthavong, *Biochem. J.*, 1971, **124**, 107.

[20] W. P. Jencks and M. I. Page, Abstracts 8th FEBS Meeting, Amsterdam, 1972.

The action of the glutamine amide NH acting as an electrophile has been mentioned before [21] for papain.



Possible electrophilic participation in acylation of bromelain or trypsin

We do not believe that any interactions of amide NH groups with the oxyanion can give rise to a lower selectivity because amide is not expected to be a better hydrogen bond donor than water owing to its higher pK_a. We are therefore led to the conclusion that it is the electrophilic interaction of the imidazolium ion with the ether oxygen which causes the lower selectivity. This is quite reasonable since this acid would certainly be a better donor than water and is conveniently located to hydrogen bond in papain at least.[21] Some arguments for the close similarity of the active sites of bromelain and papain are given later.

Trypsin. The low value of the Hammett sensitivity of k_0/K_m for this serine protease compared with that expected for reaction of alcohols with phenyl esters [4,5] is consistent in view of the similarity of trypsin to the closely related α-chymotrypsin which also exhibits a low sensitivity.[3,4] Perusal of the structure of di-isopropylphosphoryl-trypsin [22] indicates that one of the phosphoryl oxygens (the phosphoryl system is in effect an analogue of a tetrahedral intermediate and hence virtually of a transition state for acylation) lies in a pocket ringed by peptide NH groups of residues serine-195, aspartic acid-194, and glycine-193. This arrangement has been discussed previously for the stabilised adduct between trypsin and trypsin inhibitor.[2] For reasons given for the bromelain case, namely that solvation by water hydrogen bonding is unlikely to be less effective than by amide, we believe that this electrophilic interaction is not the cause of the low sensitivity; the latter is most likely due to interaction of the ether oxygen with the imidazolium NH^+ of histidine-57. Of course, as described for the bromelain case, the electrophilic interaction with the oxyanion contributes considerably to the stabilisation of the tetrahedral intermediate by an entropy effect.

Generality of Electrophilic Assistance.—Kinetic evidence exists for an electrophilic component in the acylation step for bromelain, papain,[6] trypsin, α-chymotrypsin,[3,4] and alcalase; [5] structural work on papain,[21] trypsin,[2,22] α-chymotrypsin,[23] subtilisin BPN',[1] and interpretation of structural work on tosyl-α-chymotryp-

[21] J. Drenth, H. M. Swen, W. Hoogenstraaten, and A. L. Æ. Sluyterman, *Proc. k. ned. Akad. Wetenschap.*, 1975, **C78**, 104.

[22] (*a*) R. M. Stroud, L. M. Kay, and R. E. Dickerson, *J. Mol. Biol.*, 1974, **83**, 185; (*b*) M. Krieger, L. M. Kay, and R. M. Stroud, *ibid.*, p. 209.

sin [24] as a transition state or tetrahedral intermediate analogue of acylation or deacylation in the α-chymotrypsin reaction provides data which can be interpreted as evidence for an electrophilic participation. This electrophilic assistance involves stabilisation of an incipient oxyanion of a tetrahedral-like transition-state by amide NH groups where the advantage over bulk water is entropic. There is further structural evidence discussed by Polgár [25] for hydrogen bonding of the NH^+ of the imidazolium ion with the ether oxygen of the ester (or with the heteroatom involved in the substrate link). We believe this latter interaction gives rise to the low sensitivity of k_0/K_m to the phenyl substituent in the phenyl ester substrates.

Little work on model systems has been reported to try and see whether electrophilic participation at ether oxygen (or heteroatom) or carbonyl oxygen has an activating effect. The reason for this is that it is difficult to design a molecule where the interaction is specific for either the ether or carbonyl oxygen and with good acids whether the interaction is *via* electrophilic participation (*i.e.* hydrogen bonding) or *via* some other electronic mechanism. Cases where the latter difficulty is overcome do indicate an effect due to electrophilic assistance but the site of the assistance is usually ambiguous.[26]

Comparison of Bromelain and Papain.—Although the bromelains are glycoproteins, in all other respects they closely resemble papain having approximately one active SH residue per molecule, a histidine imidazolyl residue within 5 Å of a reactive cysteine,[10] similar active site and other sequences,[14c] and a similar mechanism.[14] Although the absolute kinetic parameters are somewhat different the relative magnitude of k_0/K_m for a series of substrates (Table 6) suggests that the structure at the active site of bromelain is close to that of papain. Moreover the pattern of points on the Hammett plots (Figures 1—3) are similar to the pattern for the corresponding papain reactions.[6]

TABLE 6

Comparison of k_0/K_m for bromelain- and papain-catalysed hydrolyses [a]

Substrate	Bromelain (fraction D)	Bromelain (fraction B)	Papain [b]
4-Nitrophenyl hippurate		4 540	250 000
4-Nitrophenyl mesylglycinate		153	5 750
4-Nitrophenyl acetate		0.64	6.74
Methyl hippurate	7.0		246
Methyl acetylglycinate	0.083		2.06
Benzyl acetylglycinate	0.957		207
4-Nitrophenyl hippurate	1 130		250 000

[a] 35°, ionic strength made up to 0.1M with NaCl, pH 6.00. [b] Data from ref. 6, A. Williams, E. C. Lucas, and H. C. Hawkins, *J.C.S. Perkin II*, 1972, 627, and E. C. Lucas and A. Williams, *Biochemistry*, 1969, **8**, 5125, are for the pH of maximal activity.

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[24] A. Williams and G. Salvadori, *J. Chem. Soc.* (*B*), 1971, 2401.

[25] (*a*) L. Polgár and B. Asbóth, *J. Theor. Biol.*, 1974, **46**, 543; (*b*) L. Polgár, *ibid.*, 1971, **31**, 165; (*c*) L. Polgár, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 1972, **7**, 29, 319.

[26] A. Williams and G. Salvadori, *J.C.S. Perkin II*, 1972, 883 and references quoted therein.