On the Specificity and pH Dependence of Ficin-Catalyzed Hydrolyses. Some Comparisons with Bromelain Specificity†

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ABSTRACT: The specificity of ficin has been investigated using a number of ester and peptide substrates. Three families of esters were used: (i) a series of hippuric acid esters, for which the identity of the k_{cat} values suggests the rate-determining deacylation of a hippuryl–ficin intermediate; (ii) a series of *N*-acylglycine *p*-nitrophenyl esters, for which k_{cat}/K_m was found to be markedly dependent on the size of the nonpolar acyl group, increasing by a factor of 200 in going from the formyl to the *trans*-cinnamoyl derivative; and (iii) a series of *N*-benzyloxycarbonylamino acid *p*-nitrophenyl esters, in which the L-alanine and L-lysine derivatives were the best substrates examined. The effect of pH on k_{cat} has been determined for the ficin-catalyzed hydrolyses of α-*N*-benzoyl-L-arginine methyl ester and *N*-benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester. The effects of ficin and bromelain on a number of dipeptides, tripeptides, and polypeptides (bradykinin, angiotensin, and oxidized insulin A and B chains) have been studied. The results indicate that ficin and bromelain should prove generally useful in peptide sequencing, since they catalyze the hydrolysis of glycyl, alanyl, and leucyl bonds, as well as valyl, phenylalanyl, tyrosyl, and other bonds under more vigorous conditions.

Ficin, papain, and bromelain form a group of plant proteinases all of which have an essential active-site sulfhydryl group (Bender and Kézdy, 1965; Glazer and Smith, 1971). Of these enzymes, papain has been studied far more than the others, and ficin and bromelain have been too frequently dismissed as being similar to papain.

Some direct evidence is available that ficin-catalyzed hydrolyses proceed *via* acyl-enzyme intermediates. Lowe and Williams (1965) studied the ficin-catalyzed hydrolysis of methyl thionohippurate, and demonstrated spectrophotometrically the formation of a dithioester intermediate (thionohippuryl–ficin). Hollaway *et al.* (1969, 1971) have used stopped-flow techniques to study the individual steps in the ficin-catalyzed hydrolysis of *p*-nitrophenyl hippurate, with $[E]_0 \gg [S]_0$ and $[S]_0 \gg [E]_0$. The kinetic results were consistent with the minimal three-step scheme (eq 1), and permitted the

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} ES' \xrightarrow{k_{+3}} E + P_2 \quad (1)$$
$$+P_1$$

evaluation of K_s ($= k_{-1}/k_{+1}$), k_{+2}, and k_{+3}. Bernhard and Gutfreund (1956) found that the maximum velocities for ficin-catalyzed hydrolyses of the ethyl ester and amide of α-*N*-benzoyl-L-arginine were identical. This was taken as evidence that for these substrates deacylation of the acyl-enzyme intermediate is the rate-limiting step. However, a number of experiments indicates that for BzArgOEt deacylation is not the rate-limiting step (Hollaway, 1968; Whitaker, 1969; Whitaker and Lee, 1972).

Recently Hollaway *et al.* (1971) have determined the pH dependencies of K_s, k_{+2}, and k_{+3} for the ficin-catalyzed hydrolysis of *p*-nitrophenyl hippurate. In addition, Hollaway and Hardman (1973) have reported data purporting to provide evidence for a rate-limiting conformation change in the catalytic steps of the ficin- and papain-catalyzed hydrolyses of Z-LysONph.

This study was undertaken to provide some of the missing information on the mechanism and specificity of ficin and bromelain catalysis. In particular this paper reports in some detail on the ficin-catalyzed hydrolysis of a variety of ester substrates, and compares our results with the recent work of Hollaway *et al.* (1971, 1973). As an extension of this work, specificity studies have been undertaken on peptide and protein substrates, and at appropriate points comparative data obtained with the enzyme bromelain are introduced. The present work leads to a consistent picture of the specificity of both ficin and bromelain and suggests that while they have been neglected enzymes, they may be very useful for particular proteolytic digestions.

Experimental Section

Materials. *o*-, *m*-, *p*-Nitrophenyl hippurates, 2,4- and 2,5-dinitrophenyl hippurates, *p*-nitrophenyl thiohippurate, and the *p*-nitrophenyl esters of *N*-*trans*-cinnamoylglycine, *N*-acetylglycine, *N*-formylglycine, *N*-benzoylsarcosine, and *N*-benzyloxycarbonyl-γ-aminobutyric acid were synthesized by the dicyclohexylcarbodiimide method (Bodanszky and du Vigneaud, 1962). Phenyl hippurate was synthesized as described by Weiss (1893). The properties of these esters have been described previously (de Jersey *et al.*, 1969). *N*-Benzyloxycarbonyl-γ-aminobutyric acid *p*-nitrophenyl ester was recrystallized from acetone–hexane (mp 82–84°). The other *N*-benzyloxycarbonylamino acid *p*-nitrophenyl esters were obtained from Cyclo Chemical Corporation, and recrystallized from chloroform–hexane. Z-L-LysONph was prepared by the removal (Schwyzer and Rittel, 1961) of the *tert*-butyloxycarbonyl group from α-*N*-benzyloxycarbonyl-ε-*N*-*tert*-butyloxycarbonyl-L-lysine *p*-nitrophenyl ester. *p*-Nitrophenyl acetate was obtained from Aldrich Chemical Co. and recrystallized from chloroform–hexane. The *p*-nitrophenyl esters of γ-aminobutyric acid and glycine were prepared by the removal of the benzyloxycarbonyl group from the corresponding

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derivatives with HBr in glacial acetic acid. The melting points were in good agreement with previously published values. The purity of all of these esters was established at 99 ± 1%, based on the amount of nitrophenol or phenol released (measured spectrophotometrically) on complete hydrolysis of a known amount of ester.

2-Phenyloxazolin-5-one and 2-phenyl-4,4-dimethyloxazolin-5-one were prepared as described previously (de Jersey *et al.*, 1969). BzArgOEt,[1] BzArgOMe, and α-*N*-benzoyl-D-arginine ethyl ester (Mann Research Laboratories, chromatographically pure) were used without further purification. The peptides in Table V were purchased from Cyclo Chemical Corporation. Angiotensin (Ciba), bradykinin (Sigma), the oxidized A and B chains of bovine pancreatic insulin (Mann Research Laboratories), and acetonitrile (Eastman, Spectro Grade) were used without further purification. The buffer components 2-(*N*-morpholino)ethanesulfonic acid and Hepes were obtained from Calbiochem. Ficins II and III were prepared and characterized as described in the preceding paper (Kortt *et al.*, 1974). Ficin solutions of appropriate concentrations were prepared by dilution of stock enzyme solutions with freshly prepared 0.01 M acetate buffer (pH 4.5), 5 mM in cysteine and 1 mM in EDTA (buffer A). Cysteine-free ficins were prepared by gel filtration on a Sephadex G-25 column, equilibrated with 0.01 M acetate buffer (pH 4.5), 1 mM in EDTA, saturated with oxygen-free nitrogen. Enzyme concentrations were calculated assuming a molecular weight (and equivalent weight) of 25,000 (Englund *et al.*, 1968) for ficins II and III, and the $A_{1\,cm}^{1\%}$ values at 280 nm of 21.50 and 24.04, respectively. The concentrations so obtained were multiplied by the ratio of the specific activity of the enzyme sample used to the maximum specific activity of the enzyme in buffer A. These maximum specific activities were 66.8 and 86.0 for ficins II and III, respectively. It should be noted, however, that higher specific activities have subsequently been obtained: 101.8 for ficin II, and 107.5 for ficin III (Kortt *et al.*, 1974). This increase in maximum specific activity results from the removal of a ficin of lower specific activity, rather than the removal of inactive protein, since the equivalent weight (determined by thiol titration) did not show any change. Hence, the enzyme concentrations have not been adjusted to take into account these higher maximum specific activities.

Stem bromelain[2] was prepared from a buffered extract of pineapple stems as described in the Methods section.

All other reagents were analytical reagent grade.

Methods. Preparation of Bromelain. Stem bromelain was prepared from mature pineapple plants collected locally. The stems were freed of suckers, leaves and bark, cut into small splinters, and passed through a rotary-jaw crusher at room temperature to yield fine shavings. All subsequent operations were carried out at 5°. Shavings (3 kg) were homogenized for 2 min with 7.5 l. of 0.05 M acetate buffer (pH 4.6). The homogenate was filtered through cheesecloth on a Buchner funnel. The filtrate was centrifuged at 5° for 30 min at 3000*g*. The clear supernatant (buffered stem extract) was applied equally to each of two CM-cellulose columns (4.5 × 85 cm), equilibrated with 0.05 M acetate buffer (pH 4.6). The columns were washed with this buffer until the effluent had an absorbance (280 nm) less than 0.01. This usually required about 25 l. of buffer for each column. A single protein peak (fruit bromelains A and C) was eluted with 0.05 M phosphate buffer (pH 7.1), while a slowly eluting component (fruit bromelain D) was observed over about 15 l. of washing with this buffer. Stem bromelain was eluted with 0.05 M phosphate buffer (pH 7.1), 0.5 M in sodium chloride. Peak fractions of stem bromelain were pooled and precipitated by 0.3–0.7 saturation with ammonium sulfate.

Activity was measured using Z-GlyONph as the assay substrate. The assay mixture consisted of 3 ml of 0.05 M phosphate buffer (pH 6.1), 5 mM in cysteine, 1 mM in EDTA, and 100 μl of enzyme (~0.1 mg/ml) and was equilibrated at 25.0 ± 0.1° in a cuvet in the sample compartment of a Cary 14 spectrophotometer. The substrate (100 μl of a 5 × 10^{-3} M solution in acetonitrile) was added and the initial rate of hydrolysis was measured by observing the release of *p*-nitrophenol at 317 nm (Δε on hydrolysis, 5900). All rates were corrected for nonenzymic hydrolysis, determined using 100 μl of the enzyme buffer in place of enzyme. One katal (kat) is defined as the amount of enzyme which catalyzes the hydrolysis of one mol of substrate per second under the assay conditions (Kortt *et al.*, 1974). The term microkatal (μkat) follows the usual convention. Specific activity is defined as $(\mu kat/l.)/A_{280}$. Protein was estimated spectrophotometrically by measuring the absorbance at 280 nm and using the value of $A_{1\,cm}^{1\%}$ at 280 nm of 21.77 determined in this work (Kortt *et al.*, 1974).

Ester Substrates. Measurements of pH were made using a Radiometer pH meter 4, standardized acccording to Bates (1964), and are accurate to 0.01 pH unit. Stock solutions of most ester substrates were made up in acetonitrile. The exceptions were Z-LysONph which was made up in acetonitrile with 5% (v/v) water; glycine and γ-aminobutyric acid *p*-nitrophenyl esters which were prepared in *N*,*N*-dimethylformamide; and the α-*N*-benzoylarginine esters which were made up in water. Rate measurements for the hydrolysis of all substrates except the α-*N*-benzoylarginine esters were made at 25.0 ± 0.1° using a Cary 14 recording spectrophotometer, equipped with 0–0.1 and 0–1.0 absorbance slide wires and a thermostated cell compartment. Reactions were performed as follows. An aliquot of enzyme (usually 50 μl) was added to 3 ml of buffer equilibrated in a 1-cm cell in the cell compartment. Measured aliquots of substrate (20–100 μl) were added to initiate the reaction, together with aliquots of acetonitrile to maintain a constant concentration of organic solvent in each determination. The rate was measured by observing the release of the phenol at the appropriate wavelength. Molar absorption coefficients for the various phenols were determined under the conditions of each assay. The hydrolysis of the oxazolin-5-ones was followed by observing the decrease in absorbance at 250 nm (de Jersey and Zerner, 1969). In some kinetic experiments, the substrate was added first and the enzyme last. The same results were obtained irrespective of the order of addition, showing that the formation of oxazolin-5-one from the activated esters (de Jersey *et al.*, 1969) had a negligible effect. Kinetic constants K_m and V were evaluated from the initial velocity data by the method of least squares. Since $[E]_0$ was known, k_{cat} could be calculated, since $V = k_{cat}[E]_0$. The concentrations of acetonitrile used in the determinations were decided on the basis of the solubilities of the substrates.

[1] Abbreviations used: Z, benzyloxycarbonyl; BzArgOMe, α-*N*-benzoyl-L-arginine methyl ester; BzArgOEt, α-*N*-benzoyl-L-arginine ethyl ester; Z-LysONph, α-*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Z-GlyONph, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester; Z-AlaONph, *N*-benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester; BAW, 1-butanol–acetic acid–water (40:6:15 by volume); BPAW, 1-butanol–pyridine–acetic acid–water (15:10:3:12) by volume).

[2] The nomenclature used for the various proteolytic activities present in a buffered extract of pineapple stems is consistent with previous reports (Murachi *et al.*, 1964; Ota *et al.*, 1961; Ota, 1966).

The hydrolyses of BzArgOMe and BzArgOEt were measured by the pH-Stat method using a Radiometer TTTlc and SBR2c. Reaction mixtures were made up of 9.0 ml of 0.1 M NaCl, 1 mM in EDTA in CO_2-free distilled water, and 1.0 ml of substrate in water, equilibrated at 25.0 ± 0.1°. Reaction was initiated by the addition of an aliquot of enzyme (usually 50 μl), and the acid released titrated with 0.01 M NaOH. The pK_a' for α-*N*-benzoyl-L-arginine of 3.40 at 25° (Schwert and Takenaka, 1955) was used to correct initial rates determined below pH 5.5 for the incomplete ionization of the product. Values of pK_a' were determined from experimental profiles of k_{cat} and k_{cat}/K_m *vs.* pH by published procedures (Bender *et al.*, 1964).

Peptide and Protein Substrates. Peptides 1–6 (Table V) were prepared as 5 mM aqueous solutions. Peptides 7–19 were prepared as 2.5 mM solutions in ethanol–0.05 M phosphate buffer (pH 6.1) (1:1 v/v). Peptides 20–26 were prepared as 50 mM solutions in *N*,*N*-dimethylformamide. Angiotensin, bradykinin, and the oxidized A and B chains of insulin were prepared as 1 mM solutions in 0.05 M phosphate buffer (pH 7.1). Stem bromelain- and ficin-catalyzed hydrolyses of peptides were carried out at 30° in 0.05 M phosphate buffer (pH 7.1), 0.1 M in β-mercaptoethanol. Ficin II of specific activity ~67 and bromelain of specific activity 120–170 were used. The final enzyme and peptide concentrations were ~0.4 mg/ml and 1 mM, respectively (~50 mol of substrate/mol of enzyme). Hydrolysis was detected by sampling the digestion mixture for paper chromatography in BAW (100–200 nmol of peptide used) and dansylation (10–20 nmol of peptide used). For each digestion control mixtures without substrate or without enzyme were analyzed.

The oxidized A and B chains of insulin (5 ml, 1 mM in 0.05 M phosphate buffer (pH 7.1), 1 mM in EDTA, 50 mM in β-mercaptoethanol) were digested at 30° for 3 hr with stem bromelain and ficin (200 μl, ~35 mg/ml, ~15 mol of substrate/mol of enzyme). The reaction was stopped by addition of 125 μl of 6 N hydrochloric acid. Precipitated enzyme was removed by centrifugation at 0° for 15 min at 35,000*g*. Samples (75 μl) were taken for peptide mapping. Samples (20 μl) were taken also for amino-terminal analysis. The remaining supernatant was dried under vacuum and the residue taken up in 3 ml of pyridine–water (1:1 v/v). Peptides present in this solution were purified by paper chromatography and electrophoresis.

Peptide maps were run on 46 × 57 cm Whatman 3 MM paper. High-voltage electrophoresis at pH 3.6 at 50 V/cm for 90 min was carried out in the first direction. Chromatography in BPAW was carried out for 12 hr in the second direction.

Paper chromatography and electrophoresis were carried out on Whatman 3 MM paper. Chromatography was carried out in tanks equilibrated with solvent at room temperature. The solvents used were: 1-butanol–acetic acid–water (40:6:15 by volume; BAW); 1-butanol–pyridine–acetic acid–water (15:10:3:12 by volume; BPAW). High-voltage electrophoresis was carried out at pH 3.6 (pyridine–acetic acid–water; 1:10:189 by volume) and pH 5.6 (pyridine–acetic acid–water; 25:7:1968 by volume). Ninhydrin, Sakaguchi, and Pauly sprays were used to detect compounds after paper chromatography or electrophoresis. For separations on a preparative scale, samples were applied as streaks. Guide strips were used for the location of peptides which were eluted with 10% pyridine for 12 hr at room temperature.

Amino acid analyses were carried out on a Technicon amino acid analyzer. Dry samples were hydrolyzed in 6 N hydrochloric acid in evacuated, sealed tubes at 110° for 20 hr. Mild acid hydrolysis was carried out in 0.1 N hydrochloric acid in sealed tubes at 110° for 9 hr.

N-Terminal amino acids were determined by the dansyl chloride procedure (Gray and Hartley, 1963). Dansyl amino acids were identified by thin-layer chromatography on 20 × 30 cm glass plates in a sealed cabinet equilibrated with solvent. Plates were prepared with SilicAR TLC-7GF (Mallinckrodt) and activated at 110° for 1 hr just prior to use. The solvent used (benzene–pyridine–acetic acid; 80:20:1 by volume) was a modification of that used by Morse and Horecker (1966). Plates were developed for ~4 hr at room temperature.

Results

Ficin-Catalyzed Hydrolysis of Hippuric Acid Derivatives. The steady-state kinetic constants for the ficin II and ficin III catalyzed hydrolyses of several hippuric acid esters are given in Table I. Since activated esters of hippuric acid are hydrolyzed in alkaline solution *via* 2-phenyloxazolin-5-one (de Jersey *et al.*, 1969), the alkaline rate constants for the hippuric acid esters do not indicate the reactivity of the esters toward nucleophilic attack at the carbonyl carbon atom. Hence, the alkaline rate constants of the Z-Gly esters are included to give a better estimate of this reactivity (Table I). The 2,4- and 2,5-dinitrophenyl hippurates proved to be too reactive to be used as substrates. The 2,5 isomer had a k_{OH^-} of $\sim 3.4 \times 10^5$ M^{-1} sec^{-1}, while the 2,4 isomer was still more reactive.

The hydrolysis of 2-phenyloxazolin-5-one was found to be catalyzed by ficin III. k_{cat} and K_m were determined from initial velocity data; k_{cat}/K_m was also determined directly from the observed first-order rate constant (k_{obsd}) for the enzymatic hydrolysis of 2-phenyloxazolin-5-one with $[S]_0 \ll K_m$, since $k_{obsd}/[E]_0 = k_{cat}/K_m$. With $[E]_0 = 1.47 \times 10^{-6}$ M and $[S]_0 = (1.67 - 3.34) \times 10^{-4}$ M, $k_{cat}/K_m = 3.11 \times 10^3$ M^{-1} sec^{-1}, compared with a value of 3.88×10^3 M^{-1} sec^{-1} obtained from initial rate data. Because it had been shown that the more hindered oxazolin-5-one, 2-phenyl-4,4-dimethyloxazolin-5-one, could be used to titrate the active sites of papain (de Jersey and Zerner, 1969), this compound was tested as a titrant for ficin. However, the acylation of ficin by the oxazolin-5-one was slow compared with the acylation of papain, and suitable conditions for a titration of the active sites ($[S]_0 \gg K_m$) could not be achieved. However, when ficin III was incubated with 2-phenyl-4,4-dimethyloxazolin-5-one ($[E]_0 = 2.0 \times 10^{-5}$ M; $[S]_0 = 1.54 \times 10^{-4}$ M; 0.01 M phosphate buffer (pH 6.10)), progressive inhibition occurred, as adjudged by rate assay of aliquots of the reaction mixture against *p*-nitrophenyl hippurate. When spontaneous hydrolysis of residual oxazolin-5-one was complete (*ca.* 100 min), a first-order return of the enzyme to full activity was observed, with $k_{obsd} \simeq 3 \times 10^{-4}$ sec^{-1}. Hence, this experiment provided evidence for the formation of a covalent intermediate, and allowed the calculation of the deacylation rate constant for the presumed intermediate, *N*-benzoylaminoisobutyryl–ficin III.

Specificity of Ficin-Catalyzed Hydrolysis of Esters. Kinetic constants for the ficin II catalyzed hydrolysis of a series of *N*-benzyloxycarbonyl-L-amino acid *p*-nitrophenyl esters are given in Table II, and the constants for several of the corresponding D-amino acid derivatives are given in Table III.

When ficin III was reacted with *N*-benzyloxycarbonyl-D-alanine *p*-nitrophenyl ester (0.1 M phosphate buffer (pH 6.0), 8.7% v/v CH_3CN, $[S]_0 = 2.43 \times 10^{-4}$ M, $[E]_0 = 1.81 \times 10^{-5}$ M), a "burst" of *p*-nitrophenol was observed, which was

TABLE I: Kinetic Constants for the Ficin-Catalyzed Hydrolyses of Hippuric Acid Esters at 25°.

Ester	$10^4[S]_0$ (M)	k_{cat} (sec^{-1})	10^5K_m (M)	$10^{-5}k_{cat}/K_m$ (M^{-1} sec^{-1})	k_{OH^-}[a] (M^{-1} sec^{-1})	k_{OH^-}[b] (M^{-1} sec^{-1})
		Ficin II				
p-Nitrophenyl[c]	0.356–1.78	5.0 ± 0.20[d]	1.81	2.76	7,040	115
m-Nitrophenyl[c]	0.289–1.45	5.15 ± 0.20[e]	3.67	1.40	1,640	67.5
o-Nitrophenyl[c]	0.509–2.55	4.90 ± 0.40[e]	71.4	0.069	10,200	61.3
Phenyl[c]	0.997–4.99	5.10 ± 0.20[f]	26.1	0.195	34	12.1
		Ficin III				
p-Nitrophenyl[c]	0.356–1.78	7.47 ± 0.1[g]	1.97	3.79		
m-Nitrophenyl[c]	0.289–1.45	7.46 ± 0.1[h]	1.71	4.36		
o-Nitrophenyl[c]	0.509–2.55	7.30 ± 0.3[h]	62.5	0.117		
p-Nitrophenyl[i]	0.289–1.78	10.2[j]	10.0	1.02		
p-Nitrophenyl thio-[i]	0.331–1.65	11.0[j]	12.7	0.87	29,000	
2-Phenyloxazolin-5-one[i]	0.662–3.31	10.3[k]	265	0.039		

[a] Reported by de Jersey *et al.* (1969). [b] Alkaline rate constants reported by Kirsch and Igelström (1966) for the corresponding Z-Gly esters. [c] Aliquots of ficin II and ficin III, in 0.01 M acetate buffer (pH 4.5), 5 mM in cysteine, 1 mM in EDTA (specific activities 41.0 and 48.5, respectively), were added to 0.1 M phosphate buffer (pH 6.0), 3.17% (v/v) CH_3CN, to give final concentrations of cysteine and EDTA of 7.94×10^{-5} and 1.59×10^{-5} M, respectively. [d] $[E]_0 = 2.33 \times 10^{-8}$ M. [e] $[E]_0 = 2.32 \times 10^{-7}$ M. [f] $[E]_0 = 2.42 \times 10^{-7}$ M. [g] $[E]_0 = 5.11 \times 10^{-8}$ M. [h] $[E]_0 = 1.95 \times 10^{-7}$ M. [i] Determined using cysteine-free ficin III, specific activity 54.3, in 0.1 M phosphate buffer (pH 6.36), 23.2% (v/v) acetonitrile. [j] $[E]_0 = 3.24 \times 10^{-8}$ M. [k] $[E]_0 = 1.47 \times 10^{-6}$ M.

followed by rapid turnover of the substrate. Extrapolation to zero time gave a "burst" of 0.0716 absorbance unit compared with a calculated "burst" of 0.1203 for stoichiometric formation of the acyl-enzyme. With this substrate, it is not possible to achieve conditions where $[S]_0 \gg K_m$. This problem, together with the relatively rapid turnover of the acyl-enzyme, precluded the use of this ester for the titration of ficin. However, the observation of the "burst" of *p*-nitrophenol does provide further support for the acyl-enzyme intermediate.

Although not a substrate, α-*N*-Bz-D-ArgOEt was shown to be a competitive inhibitor of the hydrolysis of the L isomer, catalyzed by ficin III. At pH 6.0, $K_i = 2.5 \pm 0.3 \times 10^{-2}$ M (0.08 M NaCl–0.8 mM EDTA–4 mM cysteine; [I] = 3.22 and 5.04 mM).

Table IV shows the effect of variation of the *N*-acyl substituent on the kinetic constants for the ficin-catalyzed hydrolysis of a series of *p*-nitrophenyl *N*-acylglycinates.

Effect of pH on Ficin-Catalyzed Hydrolyses. The effect of pH on k_{cat} for the ficin-catalyzed hydrolyses of BzArgOMe and Z-AlaONph is shown in Figure 1. Steady-state kinetic constants were determined for both methyl and ethyl esters of α-*N*-benzoyl-L-arginine in the pH range from 3 to 9, and for the *p*-nitrophenyl esters of Z-Gly and Z-Ala from pH 3 to 8. Plots of k_{cat}/K_m *vs.* pH were bell shaped for each of these

TABLE II: Kinetic Constants for Ficin II Catalyzed Hydrolyses of α-*N*-Benzyloxycarbonyl-L-amino Acid *p*-Nitrophenyl Esters at 25°.[a]

Amino Acid	$10^5[S]_0$ (M)	$10^8[E]_0$ (M)	k_{cat} (sec^{-1})	10^5K_m (M)	$10^{-5}k_{cat}/K_m$ (M^{-1} sec^{-1})	k_{OH^-} (M^{-1} sec^{-1})
	0.1 M Phosphate Buffer (pH 6.0), 3.17% (v/v) CH_3CN					
Glycine	1.45–7.24	3.48	9.25 ± 0.5	1.34	6.90	134[b]
Alanine	2.37–11.84	0.58	31.0 ± 1.0	0.59	52.5	68[b]
Lysine	2.19–10.99	0.58	35.0 ± 1.2	0.87	40.2	
	0.1 M Phosphate Buffer (pH 6.36), 23.2% (v/v) CH_3CN					
Glycine	1.52–7.59	3.92	12.40	5.98	2.07	
Glycylglycine	4.13–20.65	3.92	7.45	1.72	4.33	
Alanine	2.37–11.84	0.62	29.80	2.58	11.55	
Valine	2.48–12.39	27.4	1.02	3.27	0.312	9[b]
Leucine	1.98–9.88	6.74	4.50	1.22	3.69	24[b]
Phenylalanine[c]	1.06–5.30	13.2	2.83	3.77	0.751	81[b]
Tyrosine[d]	0.69–3.47	7.42	2.27	0.34	6.68	22[b]
Lysine	2.19–10.99	0.58	55.0	5.20	10.58	31,000[e]

[a] Aliquots (50 μl) of ficin II, in 0.01 M acetate buffer, 5 mM in cysteine, 1 mM in EDTA, were added to buffer, giving final concentrations of cysteine and EDTA of 7.94×10^{-5} and 1.59×10^{-5} M, respectively. [b] Alkaline rate constants were determined in 8.94×10^{-4} M NaOH at 25°, 21.6% (v/v) acetonitrile. The alkaline rate constant for *p*-nitrophenyl acetate was determined under the same conditions, and observed rate constants were corrected using a value of 12.6 M^{-1} sec^{-1} for k_{OH^-} of *p*-nitrophenyl acetate (K. A. Connors (1961); quoted by de Jersey *et al.*, 1969). [c] 25% (v/v) acetonitrile. [d] 13.2% (v/v) acetonitrile. [e] pH 7.5–8.5.

TABLE III: Kinetic Constants for the Ficin II Catalyzed Hydrolyses of *N*-Benzyloxycarbonyl-D-amino Acid *p*-Nitrophenyl Esters at 25°.[a]

Amino Acid	$10^4[S]_0$ (M)	k_{cat} (sec^{-1})	10^4K_m (M)	10^{-3} k_{cat}/K_m (M^{-1} sec^{-1})
Alanine[b]	0.35–1.75	0.45 ± 0.1[c]	2.0	2.25
		0.40[d,e]	1.86	2.15
Leucine[b,e]		1.85[d,f]	0.24	77.1
Phenylalanine[g]	0.106–0.508	1.20	1.28	9.4
Tyrosine[h]	0.077–0.383	0.92	0.12	76.7

[a] Determined in 0.1 M phosphate buffer (pH 6.36). [b] 23.2% (v/v) acetonitrile. [c] $[E]_0 = 5.19 \times 10^{-7}$ M. [d] Determined using Henri plots (Dixon and Webb, 1964). [e] $[E]_0 = 2.13 \times 10^{-7}$ M. [f] $[E]_0 = 1.32 \times 10^{-8}$ M. [g] 25% (v/v) acetonitrile; $[E]_0 = 2.64 \times 10^{-7}$ M. [h] 13.2% (v/v) acetonitrile; $[E]_0 = 1.48 \times 10^{-7}$ M.

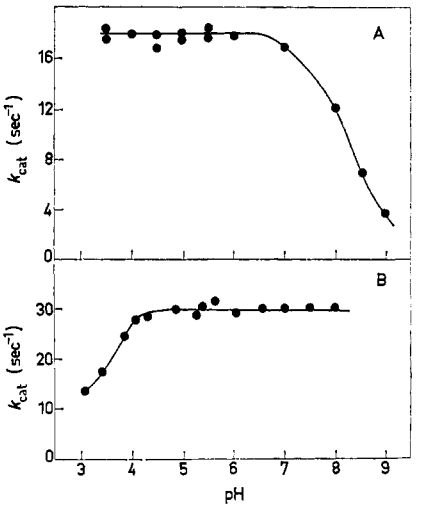


FIGURE 1: Effect of pH on k_{cat} for the ficin III catalyzed hydrolysis of BzArgOMe (A) and for the ficin II catalyzed hydrolysis of Z-AlaONph (B), at 25°. Ficin III had a specific activity of 57.3, and ficin II a specific activity of 37.5. Conditions: (A) $[E]_0 = (0.171 - 1.22) \times 10^{-6}$ M; $[S]_0 = (0.55 - 3.16) \times 10^{-2}$ M; (B) $[E]_0 = 7.76 \times 10^{-9}$ M; $[S]_0 = (0.24 - 1.18) \times 10^{-4}$ M; 23.2% (v/v) CH_3CN; 0.05 M formate, acetate, 2-(*N*-morpholino)ethanesulfonic acid, and Hepes buffers.

four substrates, and were similar to those obtained previously for ficin-catalyzed hydrolyses (Hollaway *et al.*, 1971). BzArgOEt gave a similar bell-shaped plot of k_{cat} *vs.* pH to that reported by Kramer and Whitaker (1969). Z-GlyONph gave a similar pH–k_{cat} profile to that shown for the corresponding derivative of L-alanine in Figure 1.

Specificity of Ficin and Bromelain toward Peptides and Proteins. Ficin II catalyzed the hydrolysis of peptides as shown in Table V. Stem bromelain catalyzed the complete hydrolysis of Z-Gly-~-Ala-NH_2 in 33 hr and partial hydrolysis of Z-Gly-Gly-~-Ala-NH_2 in 70 hr. Amino-terminal analysis and paper chromatography of the products of ficin- and bromelain-catalyzed hydrolyses of these peptides indicated that hydrolysis had occurred at the bonds shown.

Stem bromelain and ficin II catalyzed the hydrolysis of bradykinin as indicated: Arg-Pro-Pro-Gly-~-Phe-~-Ser-Pro-Phe-Arg. Phenylalanine and serine appeared as new amino-terminal residues after a 27-hr digestion with stem bromelain and with ficin II. Chromatography in BAW showed that hydrolysis was complete in 60 hr in the case of ficin II, but was incomplete with stem bromelain. On the basis of Sakaguchi and ninhydrin staining of the chromatograms of the two digests, the following identifications were made: Phe (R_F 0.53), Arg-Pro-Pro-Gly (R_F 0.42), Arg-Pro-Pro-Gly-Phe (R_F 0.33), Ser-Pro-Phe-Arg (R_F 0.30), and Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (R_F 0.28). The peptide Ser-Pro-Phe-Arg gave a yellow color with ninhydrin, as has been reported previously for Ser-Arg (Dreyer and Neurath, 1955). Thus in the sequence -Gly-Phe-Ser- in bradykinin the phenylalanyl bond was cleaved by stem bromelain (and probably ficin II) before the glycyl bond.

For angiotensin, stem bromelain and ficin II in 29 hr catalyzed the hydrolysis of a single bond in the sequence: Asp-Arg-Val-Tyr-~-Val-His-Pro-Phe. Valine was produced as a new amino-terminal amino acid on hydrolysis. Further, Sakaguchi and Pauly reactions of the products of hydrolysis separated by chromatography in BAW unequivocally identified the bond hydrolyzed.

Peptide maps of the ficin II and stem bromelain digests of the A and B chains of oxidized insulin are shown in Figures 2 and 3. New amino-terminal residues produced in the A chain digests were Cys(O_3H) or Asp, Ala or Tyr, Glu or Ser,

TABLE IV: Kinetic Constants for Ficin-Catalyzed Hydrolyses of *p*-Nitrophenyl *N*-Acylglycinates at 25°.[a,f]

N-Acyl Group	Ficin II[b] k_{cat} (sec^{-1})	Ficin II[b] 10^5K_m (M)	Ficin II[b] $10^{-5}k_{cat}/K_m$ (M^{-1} sec^{-1})	Ficin III[c] k_{cat} (sec^{-1})	Ficin III[c] 10^5K_m (M)	Ficin III[c] $10^{-5}k_{cat}/K_m$ (M^{-1} sec^{-1})
trans-Cinnamoyl	8.64[d]	0.76	11.4	12.28	0.67	18.3
Benzyloxycarbonyl	9.57[d]	1.94	4.93	13.34	1.83	7.29
Benzoyl	5.36[d]	2.50	2.14	7.27	2.14	3.40
Acetyl	2.30[e]	32.8	0.070	2.84	30.9	0.092
Formyl	2.50[e]	110	0.023			

[a] Determined in 0.1 M phosphate buffer (pH 6.0), 3.17% (v/v) acetonitrile. [b] Ficin II had a specific activity of 51.0. [c] Ficin III had a specific activity of 68.2, $[E]_0 = 5.11 \times 10^{-8}$ M. [d] $[E]_0 = 6.30 \times 10^{-8}$ M. [e] $[E]_0 = 4.60 \times 10^{-7}$ M. [f] No enzymatic hydrolysis of glycine *p*-nitrophenyl ester could be detected in 0.1 M acetate buffer (pH 4.5), with $[E]_0 = 8.6 \times 10^{-7}$ M. The lower pH was used because of the rapid spontaneous hydrolysis of this ester at pH 6. Similarly, no ficin-catalyzed hydrolysis of the following esters could be detected (0.1 M phosphate buffer (pH 6.0), $[E]_0 \sim 10^{-5}$ M): *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *N*-Z-γ-aminobutyric acid *p*-nitrophenyl ester, γ-aminobutyric acid *p*-nitrophenyl ester, *N*-benzoylsarcosine *p*-nitrophenyl ester.

TABLE V: Ficin-Catalyzed Hydrolysis of Peptides.

Peptide	Extent of Hydrolysis[a]
Gly-Ala (1), Gly-Gly (2), Gly-Leu (3), Gly-Val (4), Leu-Gly (5), Tyr-Gly (6), Ala-Asp (7), Ala-Asn (8), Z-Ala-Asn (9), Ala-Glu (10), Z-Ala-Glu (11), Z-Ala-Gly (12), Z-Leu-Ala (13), Z-Leu-Ala-NH_2 (14), Arg-Ala (15), Arg-Arg (16), Lys-Ala-NH_2 (17), Lys-Gly-NH_2 (18), Z-Ala-Glu-$(OBzl)_2$ (19), Glu-Ala-Ala (20)	None
Z-Ala-~-Ala-NH_2 (21), Z-Gly-~-Ala-NH_2 (22), Z-Gly-Gly-~-Ala-NH_2 (23)	Complete[b]
Z-Ala-~-Gly-NH_2 (24), Z-Gly-~-Gly-NH_2 (25), Z-Gly-~-Gly-~-Gly-NH_2 (26)	Partial (~10%)[c]

[a] Hydrolysis was carried out at 30° in 0.05 M phosphate buffer (pH 7.1), 0.1 M β-mercaptoethanol. The final enzyme and peptide concentrations were ~0.4 mg/ml and 1 mM, respectively. [b] After 33 hr. [c] After 70 hr.

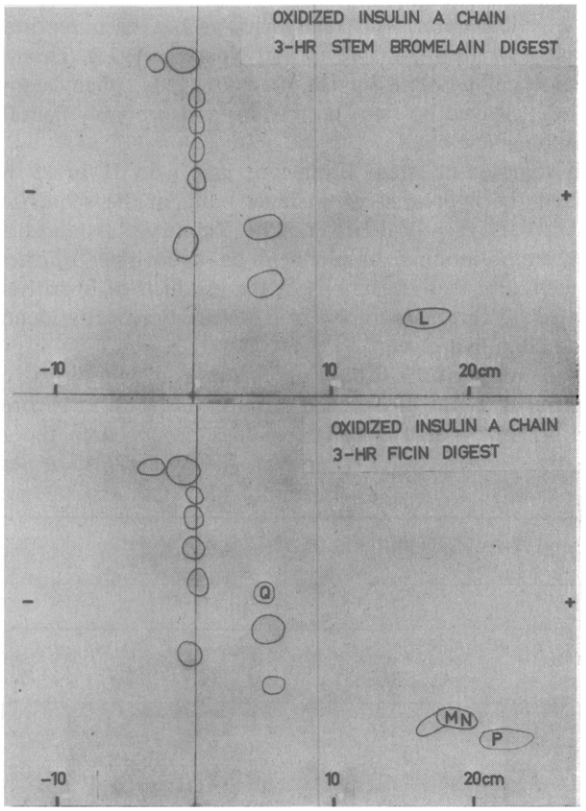


FIGURE 2: Peptide maps of stem bromelain and ficin II digests of the oxidized insulin A chain. The oxidized insulin A chain (5 ml, 1 mM in 0.05 M phosphate buffer (pH 7.1), 1 mM in EDTA, 50 mM in β-mercaptoethanol) was incubated with enzyme (200 μl, ~35 mg/ml; ficin II specific activity, ~67; bromelain specific activity, ~140) for 3 hr at 30°. Samples (75 μl) of the digests were subjected to high-voltage electrophoresis at pH 3.6 at 50 V/cm for 90 min in the first direction then chromatography in BPAW for 12 hr in the second direction. Peptides were visualized with ninhydrin.

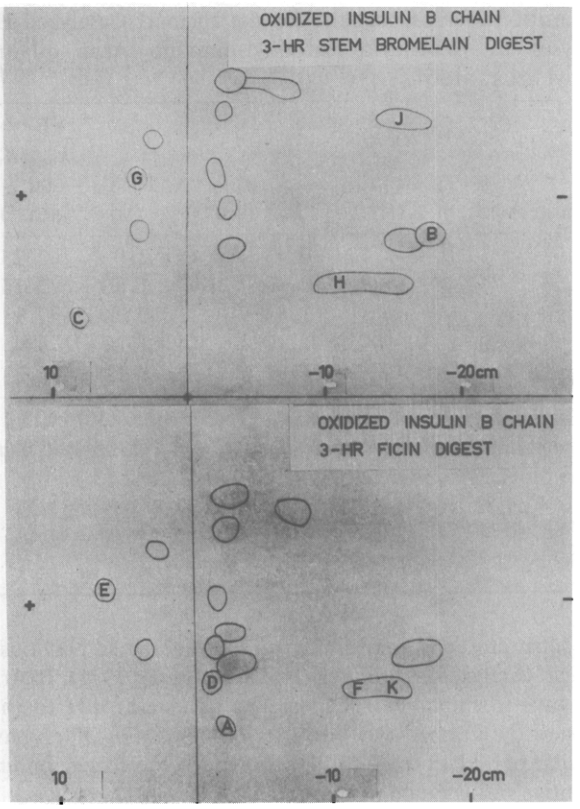


FIGURE 3: Peptide maps of stem bromelain and ficin II digests of the oxidized insulin B chain. The digest conditions and mapping procedure are as described in Figure 2.

and Leu or Ile, and in B chain were Cys(O_3H) or Asp, Glu or Ser, Thr, Gly, Ala, Val, and Leu. The amino acid compositions of some of the peptides isolated from stem bromelain digests of the insulin chains are given in Tables VI and VII. The cleavages responsible for the production of these peptides are shown in Figure 4, together with recent data of Englund *et al.* (1968). Tentative identification of peptides A–Q (Figures 2 and 3), which represent differences in the ficin and bromelain digests, were made on the basis of chromatographic and electrophoretic mobilities, and by comparison with the data of Jones and Glazer (1970) (Table VIII).

Discussion

All of the hippuric acid derivatives examined in this work had identical k_{cat} values within experimental error (Table I). This constitutes good *prima facie* evidence that deacylation of the common intermediate hippuryl–ficin is the rate-determining step in each case. The large effect of the leaving group on K_m should be noted. Hollaway *et al.* (1969) studied the ficin-catalyzed hydrolysis of *p*-nitrophenyl hippurate with $[E]_0 \gg [S]_0$, and were able to determine k_{+2} and K_s at pH 5.90 and pH 3.90. From the steady-state constants, k_{cat} and K_m, k_{+3} could be calculated. At pH 5.90, $k_{+2} = 44$ sec^{-1}, $k_{+3} = 8.5$ sec^{-1}, and $K_s = 2.8 \times 10^{-4}$ M. Hence, deacylation is the rate-limiting step at this pH. *A priori*, the large variation in k_{cat}/K_m ($= k_{+2}/K_s$) shown in Table I could be due to changes in k_{+2}, in K_s, or in both. However, since k_{+2} must remain much greater than k_{+3} for the same k_{cat} values to be obtained, and considering that even for *p*-nitrophenyl hippurate, k_{+2}/k_{+3} is only 5 at pH 5.9, it may be seen that the variation in k_{cat}/K_m must be due largely to variation in K_s. Thus, moving the nitro group from the para to the ortho position increases K_s by a

TABLE VI: Analyses of Some Peptides from the Stem Bromelain Digest of the Oxidized A Chain of Insulin.

	Amino Acid Composition[a]								
Amino Acid	A chain	ABI'1A	ABIIa1A	ABI'1'F	ABI'1'G	ABIIa2	ABI'1'E	ABI'3B	ABI'2B
Cys (O_3H)	3.46 (4)		2.15 (2)			2.00 (2)		2.02 (2)	
Asp	1.91 (2)							1.63 (2)	1.05 (1)
Ser	1.36 (2)		0.68 (1)	0.91 (1)	0.95 (1)			1.42 (2)	
Glu	3.91 (4)	2.00 (2)	0.98 (1)	1.08 (1)	1.10 (1)	0.84 (1)	0.93 (1)	2.37 (2)	1.10 (1)
Gly	1.03 (1)	2.11 (1)							
Ala	1.06 (1)		1.00 (1)					0.94 (1)	
Val	1.88 (2)	1.13 (1)	1.00 (1)					1.06 (1)	
Leu	1.90 (2)			2.03 (2)	1.53 (2)		1.07 (1)	2.02 (2)	0.86 (1)
Ile	0.84 (1)	0.81 (1)							
Tyr	1.09 (2)			0.71 (1)	0.50 (1)			0.84 (2)	
Residues[b]	1–21	1–5[c]	5–10	12–16[d]	12–16[d]	5–7	15–16[e]	8–21	16–18

[a] Uncorrected for decomposition during hydrolysis. [b] Residues as shown in Figure 4. [c] Assigned on the basis of high-voltage electrophoresis at pH 3.6 at 60 V/cm for 30 min after mild acid hydrolysis. [d] Assigned on the basis of electrophoretic mobility at pH 5.6. [e] Assigned on the basis of electrophoretic mobility at pH 3.6 and pH 5.6.

factor of about 40, assuming k_{+2} is little changed. Since k_{+2} could only increase, the factor of 40 is a minimal estimate. Similarly, the absence of a conventional leaving group, as in 2-phenyloxazolin-5-one, causes a 100-fold increase in K_s, compared with that of *p*-nitrophenyl hippurate. The difference between phenyl hippurate and *p*-nitrophenyl hippurate seems difficult to understand in these terms, barring the proposal that the nitro group interacts with the enzyme active site, causing a decrease in K_s. The more usual proposal, *viz.*, that k_{+2} for the *p*-nitrophenyl ester should be much larger because the *p*-nitrophenyl group is a much better leaving group than the phenyl group, seems untenable on the basis of the data of Hollaway *et al.* (1969) listed above.

Further evidence that small changes in the leaving group produce large changes in kinetic constants comes from a comparison of the ficin-catalyzed hydrolyses of the methyl and ethyl esters of α-*N*-benzoyl-L-arginine. The limiting value of k_{cat} for BzArgOMe (17.9 sec^{-1}) is more than four times that for the ethyl ester (4.1 sec^{-1}). Since for the ethyl ester the acylation step is rate limiting (Hollaway, 1968; Whitaker and Lee, 1972), k_{+2} for the methyl ester must be at least four times that for the ethyl ester. Since k_{cat}/K_m values differ by a factor of about 6, the major effect must be on k_{+2} rather than on K_s.

It should be noted that the above discussion is in terms of the minimal three-step scheme (eq 1). In a recent paper, Hollaway and Hardman (1973) studied the reaction of ficin with Z-LysONph, and obtained stopped-flow data with $[E]_0 \gg [S]_0$ which was difficult to reconcile with data obtained with $[S]_0 \gg [E]_0$. They have proposed that acylation of the enzyme is a more complex process than indicated by eq 1. In particular, they propose that there is a rate-limiting conformation change involved in the hydrolysis of this ester by both ficin and papain. Since the implications of the present results for the work of Hollaway *et al.* (1971, 1973) remain un-

TABLE VII: Analyses of Some Peptides from the Stem Bromelain Digest of the Oxidized B Chain of Insulin.

	Amino Acid Composition[a]							
Amino Acid	B Chain	BBI'I'A	BBI'I'C(i) and (ii)[b]		BBI'I'D	BBI'III'A	BBI'III'B	BBII'aA
Cys(O_3H)	1.96 (2)	0.98 (1)						
Asp	1.05 (1)					0.77 (1)		
Ser	0.81 (1)							
Thr	0.79 (1)							
Glu	3.18 (3)	1.11 (1)	0.94 (1)	0.95 (1)	1.35 (1)			0.75 (1)
Pro	1.02 (1)							
Gly	2.94 (3)	2.17 (2)						0.97 (1)
Ala	1.90 (2)			1.07 (1)				
Val	2.85 (3)	1.01 (1)	1.09 (1)		0.89 (1)	1.36 (1)		
Leu	4.03 (4)				1.31 (1)		1.00 (1)	
Tyr	1.30 (2)							
Phe	2.67 (3)							
Lys	0.98 (1)							
His	1.61 (2)							
Arg	1.01 (1)	0.92 (1)						1.28 (1)
Residues[c]	1–30	18–23	12–13	13–14	11–13	2–3	11	20–22[d] or 21–23

[a] Uncorrected for decomposition during hydrolysis. [b] Analyzed as a mixture of BBI'I'C(i) and BBI'I'C(ii). [c] Residues as shown in Figure 4. [d] Probably residues 20–22 on the basis of N-terminal analysis of the total digest.

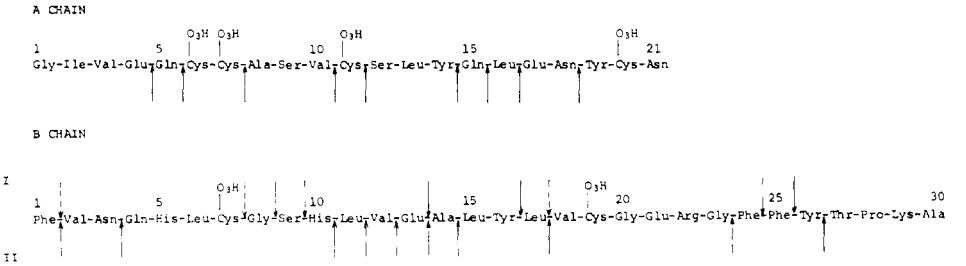


FIGURE 4: Stem bromelain catalyzed hydrolysis of the oxidized insulin chains under the conditions described in the text: (I) hydrolysis of oxidized insulin B chain by ficin (Englund *et al.*, 1968), (↓) bonds partially hydrolyzed; (II) data from this work. Bonds hydrolyzed were assigned on the basis of the data in Tables VI and VII, except the Tyr–Thr bond (26–27) which was assigned on the basis of N-terminal analysis.

TABLE VIII: Tentative Identification of Some Peptides from the Ficin and Bromelain Insulin Digests.

Peptide[a]	Residues	Identification[b]
A	1–9	Phe-Val-Asn-Gln-His-Leu-Cys(O_3H)-Gly-(Ser)[c,d]
B	1–6	Phe-Val-Asn-Gln-His-Leu
C	7–9	Cys(O_3H)-Gly-(Ser)[d]
D	18–23	Val-Cys(O_3H)-Gly-Glu-Arg-Gly
E	18–21	Val-Cys(O_3H)-Gly-Glu
F	22–23	Arg-Gly
G	18–20	Val-Cys(O_3H)-Gly
H	21–23	(Glu)-Arg-Gly[d]
J	24–30	Phe-Phe-Tyr-Thr-Pro-Lys-Ala
K	27–30	Thr-Pro-Lys-Ala[c]
L	5–10	Gln-Cys(O_3H)-Cys(O_3H)-Ala-Ser-Val
M	5–8	Gln-Cys(O_3H)-Cys(O_3H)-Ala
N	5–7	Gln-Cys(O_3H)-Cys(O_3H)
P	6–7	Cys(O_3H)-Cys(O_3H)
Q	20–21	Cys(O_3H)-Asn

[a] See Figures 2 and 3. [b] Identified on the basis of electrophoretic mobility at pH 3.6 and chromatographic mobility in BPAW under the conditions described in Figure 2. [c] Identified by comparison with data of Jones and Glazer (1970). [d] Residue in brackets may not be present.

certain, the reactions of ficin with several nitrophenyl ester substrates are currently under investigation in this laboratory.[3]

The attempts to use *N*-benzyloxycarbonyl-D-alanine *p*-nitrophenyl ester and 2-phenyl-4,4-dimethyloxazolin-5-one as titrants for ficin were unsuccessful, although evidence for the formation of a covalent intermediate was obtained with each substrate. Williams and Lucas (1970) have reported the use of *N*-Z-D-norleucine *p*-nitrophenyl ester as a titrant for papain. However, in view of the convenience and reliability of titration with 5,5′-dithiobis(2-nitrobenzoic acid) as a means of determining the concentrations of solutions of ficins (Kortt *et al.*, 1974) further search for a suitable titrant seems unnecessary at this time.

The effect of pH on the steady-state kinetic constants for the ficin-catalyzed hydrolysis of BzArgOEt and amide has been determined by a number of groups, most recently by Kramer and Whitaker (1969). Both k_{cat} and k_{cat}/K_m profiles are bell shaped, and indicate the involvement of groups with $pK_a' \sim 4.1$ and ~ 8.5 in the catalytic process. At alkaline pH values, the pH–k_{cat} profiles obtained here suggest that for BzArgOMe, acylation is the rate-limiting step, but that for Z-AlaONph, deacylation is rate limiting (Figure 1). Hollaway *et al.* (1971) have shown that k_{+3} is rate limiting for the ficin-catalyzed hydrolysis of *p*-nitrophenyl hippurate at pH 6.6, and is unchanged from pH 3.9–6.6. The acid side of the pH–rate profile for *N*-Z-L-alanine *p*-nitrophenyl ester (Figure 1) shows a pK_a' of ~3.3, compared with the literature values of 4.1–4.3 for arginine derivatives. It is possible that the lower pK_a' may be related to a change in rate-limiting step (Hollaway *et al.*, 1971). The k_{cat} for BzArgOMe is unchanged from pH 3.5 to 7 (Figure 1), whereas, for the ethyl ester, k_{cat} at pH 3.5 is only about 0.35 of the maximum value.

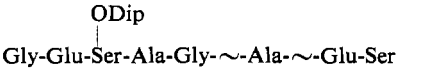
The data listed in Tables II, III, and IV represent the results of the first reasonably comprehensive study of the specificity of ficin toward synthetic substrates.[4] The *p*-nitrophenyl esters of Z-L-alanine and Z-L-lysine were hydrolyzed more rapidly than the corresponding esters of Z-glycine, Z-L-tyrosine, Z-L-leucine, Z-L-phenylalanine, and Z-L-valine (Table II). The specificity of ficin for L-alanine derivatives was further demonstrated using the α-*N*-benzyloxycarbonyl *p*-nitrophenyl esters of D-alanine and othe D-amino acids. The value of k_{cat}/K_m decreased by a factor of ~500 in going from L-alanine to D-alanine, but decreased by a factor of only ~7 for the same substitution in leucine, phenylalanine, and tyrosine substrates (Tables II and III). The major contribution to this factor in the case of the specific substrate was *via* k_{cat}. The effect of the *N*-acyl group on k_{cat}/K_m for the ficin-catalyzed hydrolysis of *p*-nitrophenyl *N*-acylglycinates is shown in Table IV. In going from the formyl to the *trans*-cinnamoyl derivative, k_{cat} increased by a factor of ~4, while K_m decreased by a factor of ~140. These results show the importance of hydrophobic bonding of the *N*-acyl group at the active site of ficin. The requirement for an α-acylamino group is shown by the fact that ficin did not catalyze the hydrolysis of glycine *p*-nitrophenyl ester, *N*-benzoylsarcosine *p*-nitrophenyl ester, or other esters tested which lacked this group. Papain shows a similar requirement for the presence of an α-acylamino group (de Jersey, 1970). Dipeptides with a free α-amino or α-carboxyl group were not hydrolyzed by ficin II. Bromelain, on the other hand, has been shown to catalyze the hydrolysis of glycine ethyl ester about half as effectively as *N*-benzoylglycine ethyl ester (Inagami and Murachi, 1963).

Ficin and bromelain catalyze the hydrolysis of alanyl and glycyl bonds in protected dipeptides and tripeptides. Pre-

[3] Preliminary experiments on the ficin-catalyzed hydrolysis of Z-LysONph under conditions closely related to those purported to exist in Hollaway's work (Hollaway and Hardman, 1973), provide no support for a rate-limiting conformational change in this system (J. de Jersey, S. E. Hamilton, and B. Zerner, unpublished results).

[4] The specific activities of ficins used in this study were lower than has subsequently been obtained (see Experimental Section). The data should be interpreted with this in mind.

viously, specificity toward these bonds had been demonstrated in the ficin-catalyzed hydrolysis of a peptide from ox liver carboxylesterase (Augusteyn *et al.*, 1969). The bonds hydrolyzed in this peptide are indicated



The glycyl and alanyl bonds indicated in Z-Gly-~-Ala-NH_2 and Z-Ala-~-Ala-NH_2 were hydrolyzed more readily than those indicated in Z-Gly-~-Gly-NH_2 and Z-Ala-~-Gly-NH_2. This specificity for alanyl and glycyl bonds is in agreement with the kinetic data presented in Table II for the ficin-catalyzed hydrolysis of *N*-benzyloxycarbonyl *p*-nitrophenyl esters of amino acids. Moreover, fruit bromelain has been reported to catalyze the hydrolysis of glycyl bonds in dipeptides and tripeptides (Bergmann *et al.*, 1937), and collagen and elastin, which are known to have high levels of glycine and alanine residues, are degraded extensively by ficin and bromelain (Tsen and Tappell, 1959; Thomas and Partridge, 1960).

Phenylalanyl and tyrosyl bonds in bradykinin and angiotensin are cleaved by ficin and bromelain. Similar results for bromelain-, ficin- and papain-catalyzed hydrolyses of bradykinin have been reported previously (Murachi and Miyake, 1970).

Studies of bradykinin, angiotensin, dipeptides, and tripeptides as substrates for ficin and bromelain indicate that these enzymes have very similar specificities. This is further emphasized by the insulin studies. The peptide maps of bromelain and ficin digests of oxidized insulin chains are identical except for a few peptides, and it is likely that these differences represent differences in degree of hydrolysis (Figures 2 and 3; Table VIII). Bromelain, unlike ficin, appears to have catalyzed the total hydrolysis of the Leu–Cys bond (residues 6–7) and the extensive hydrolysis of Gly–Glu bond (20–21) in the oxidized insulin B chain, but has produced less extensive hydrolysis of the Tyr–Thr bond (26–27) than has ficin. Ficin also partially hydrolyzes the Glu–Arg bond (20–21). In the A chain, ficin, but not bromelain, catalyzes the hydrolysis of the Ala–Ser bond (7–8) and the Tyr–Cys bond (19–20). The latter result is consistent with the relative extents of cleavage by ficin and bromelain of the Tyr–Thr bond in the oxidized insulin B chain.

Murachi and Neurath (1960) showed in a limited study that bromelain (~40 mol of substrate/mol of enzyme) catalyzed the hydrolysis of bonds before glycine and glutamine or glutamic acid in the oxidized insulin B chain. Also, in a bromelain digest of glucagon, the arginyl and alanyl bonds in the sequence -Arg-Ala-Gln- were hydrolyzed. Bromelain catalyzed the hydrolysis of the glutamyl, aspartyl, and alanyl bonds indicated in ovalbumin: -Glu-~-Ala-Gly-Val-Asp-~-Ala-Ala-~-Ser- (Satake *et al.*, 1965).

Englund *et al.* (1968) showed that ficin at low concentration (~660 mol of substrate/mol of enzyme) catalyzed the hydrolysis of glycyl, glutamyl, tyrosyl, and phenylalanyl bonds in the oxidized insulin B chain. Partial hydrolysis at cysteic acid, seryl and leucyl residues was indicated by their data. Broadly, our results are consistent with those of Englund *et al.* (1968), although more extensive cleavage is observed (Figure 4).

The present information suggests that ficin and bromelain can be used for specific hydrolysis of proteins at glycyl, alanyl, and leucyl bonds. Secondary cleavage at valyl, phenylalanyl, tyrosyl, and other bonds occurs under more vigorous conditions.

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