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Biochimica. — *Catalytic properties of stem bromelain, ficin and papain in the acidic pH region. A comparative pre-steady-state and steady-state study.* Nota di PAOLO ASCENZI (*), PATRIZIA ADUCCI (**), GINO AMICONI (*), ENEA MENEGATTI (***), MARIO GUARNERI (***) e ALESSANDRO BALLIO (*), presentata (****) dal Corrisp. D. CAVALLINI.

ABSTRACT. — Pre-steady-state and steady-state kinetics for the hydrolysis of p-nitrophenyl esters of N-α-carbobenzoxy (-L-) amino acids catalyzed by stem bromelain (from *Ananas sativus*; EC 3.4.22.4), ficin (from *Ficus glabrata*; EC 3.4.22.3) and papain (from *Carica papaya*; EC 3.4.22.2) have been determined between pH 2.5 and 6.0 ($I = 0.1$ M) at 21 ± 0.5 °C. The different kinetic behaviour of these cysteine proteinases, especially in the pH dependence of the deacylation step, has been related to the nature of the amino acid residue present at position 158. In fact, differences in the intensity of the electrostatic field contributed by the 158 side chain may affect the acid-base equilibrium of the Cys25-His159 catalytic diad, and thus the enzyme activity.

KEY WORDS: Stem bromelain; Ficin; Papain; Cysteine Plant Proteinases; Enzyme kinetics; pH effects.

RIASSUNTO. — *Proprietà catalitiche della bromelaina da fusto, della ficina e della papaina in condizioni di pH acido. Studio comparativo in condizioni di stato-pre-stazionario e di stato-stazionario.* Le proprietà cinetiche di stato-pre-stazionario e di stato-stazionario relative all'idrolisi di p-nitrofenil esteri di *N*-α-carbobenzossi (-L-) aminoacidi catalizzata dalla bromelaina da fusto (da *Ananas sativus*; EC 3.4.22.4), dalla ficina (da *Ficus glabrata*; EC 3.4.22.3) e dalla papaina (da *Carica papaya*; EC 3.4.22.2) sono state determinate fra pH 2,5 e 6,0 ($I = 0,1$ M) a 21 ± 0.5 °C. Il diverso comportamento cinetico di queste proteinasi a cisteina, in particolar modo nella dipendenza dal pH dello stato di deacilazione, è stato correlato alla natura del residuo aminoacidico presente in posizione 158. Infatti, differenze nell'intensità del campo elettrostatico indotte dal residuo 158 possono influenzare l'equilibrio acido-base della diade catalitica Cys25-His159, e quindi l'attività enzimatica.

INTRODUCTION

Extensive detailed investigations on cysteine proteinase action indicate a number of common mechanistic features; among them, the transient existence of an acyl-intermediate, involving the active centre thiol group (*i.e.*, Cys25) and the participation of the neighbouring histidyl residue (*i.e.*, His159), has been clearly established [1-7]. Nevertheless, changes in position, mobility, conformation and/or nature of amino acid residues in the neighbourhood of the Cys25-His159 catalytic diad may account for differences in the pH dependence of kinetics of cysteine proteinase catalyzed reactions [3-20].

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In order to gain a better insight on the modulation of cysteine proteinase action by pH, detailed pre-steady-state and steady-state kinetics for the hydrolysis of p-nitrophenyl esters of *N*-α-carbobenzoxy-glycine (ZGLyONp), -L-alanine (ZAlaONp), -L-valine (ZValONp) and -L-tyrosine (ZTyrONp) catalyzed by stem bromelain (from *Ananas sativus*; EC 3.4.22.4), ficin (from *Ficus glabrata*; EC 3.4.22.3) and papain (from *Carica papaya*; EC 3.4.22.2) have been determined between pH 2.5 and 6.0 ($I = 0.1$ M) at 21 ± 0.5 °C. These results have been analyzed taking into account the molecular properties of cysteine proteinases considered [21-25] as well as their catalytic behaviour towards specific active site probes [5, 7-20].

Materials and methods

Stem bromelain was purified from the lyophilized powder of pinapple stem (from Sigma Chemical Co., St. Louis, U.S.A.) as detailed [8, 9]. Ficin was isolated from the dried latex (from Kock-Light Laboratories Ltd, Colnbrook, U.K.) as reported [10]. Papain was purified from commercial preparations (from Sigma Chemical Co., St. Louis, U.S.A.) as detailed [13].

ZGlyONp, ZAlaONp, ZValONp and ZTyrONp were obtained from Sigma Chemical Co., (St. Louis, U.S.A.).

All other products were from Merck AG (Wuppertal, F.R.G.).

All chemicals were of analytical grade and used without further purification.

The stem bromelain, ficin and papain catalyzed hydrolysis of ZGlyONp, ZAlaONp, ZValONp and ZTyrONp was monitored spectrophotometrically at 360 nm ($\Delta\varepsilon_{360} = 4.5\ mM^{-1}\ cm^{-1}$ [2]), $T = 21 \pm 0.5$ °C, between pH 2.5 and 6.0 (in formate buffer, pH 2.5 to 3.5, and acetate buffer, pH 3.5 to 6.0, sodium salts; $I = 0.1$ M), with a Varian double-beam spectrophotometer (Cary 219) and a Durrum-Gibson rapid-mixing stopped-flow apparatus [2, 3].

Values of kinetic parameters for stem bromelain, ficin and papain catalyzed hydrolysis of ZGlyONp, ZAlaONp, ZValONp and ZTyrONp were obtained from the experimental data according to the standard treatment of the catalytic mechanism of cysteine proteinases (Scheme I) [2-7]:

(I)
$$E + S \overset{K_s}{\longleftrightarrow} E \cdot S \overset{k_{+2}}{\longrightarrow} \underset{\underset{P_1}{+}}{E \cdot P} \overset{k_{+3}}{\longrightarrow} E + P_2$$

where E is the enzyme, S is the substrate, $E \cdot S$ is the reversible rapidly-formed enzyme · substrate complex, $E \cdot P$ is the acyl · intermediate, and P_1 and P_2 are the hydrolysis products (*i.e.*, p-nitrophenol and the *N*-α-carbobenzoxy(-L-)amino acid, respectively).

Values of k_{cat}, K_m and k_{cat}/K_m were determined from the intercepts on the ordinate and abscissa as well as the slope, respectively, of plots of $1/v$ *vs* $1/[S_0]$, with $[S_0] \geq 5x[E_0]$ [2, 3]. Values of k_{+2}, K_s and k_{+2}/K_s were obtained from the intercepts on the ordinate and abscissa as well as the slope, respectively, of plots of $1/k^{app}$ *vs* $1/[E_0]$ with $[E_0] \geq 5x[S_0]$ [2, 3]. Values of k_{+3} calculated by substitution

of the experimentally determined values of k_{cat}, K_m, k_{+2} and K_s into eqs. 1 and 2 [2, 3]:

$$k_{+3} = (k_{+2} \cdot k_{cat})/(k_{+2} - k_{cat}) \quad (1)$$

$$k_{+3} = (k_{+2} \cdot K_m)/(K_s - K_m) \quad (2)$$

were the same within the errors.

An average error value of ± 8% was evaluated for kinetic parameters as reported [2, 3].

For further experimental details as well as biochemical procedures see Ascenzi *et al.* [2, 3].

RESULTS AND DISCUSSION

Over the whole pH range explored (pH 2.5 to 6.0), the stem bromelain, ficin and papain catalyzed hydrolysis of ZGlyONp, ZAlaONp, ZValONp and ZTyrONp conforms to simple Michaelis-Menten kinetics, and pre-steady-state and steady-state data may be consistently fitted to the minimum three-step mechanism of cysteine proteinases (Scheme I) [2-7], as indicated from the excellent agreement between values of k_{cat}/K_m (obtained where $[S_0] \geq 5x[E_0]$ and those of k_{+2}/K_s (determined where $[E_0] \geq 5x[S_0]$ (see fig. 1 as well as table I):

Data shown in fig. 1 and table II indicate that the stem bromelain, ficin and papain [3] catalyzed hydrolysis of ZGlyONp, can be described in terms of only one apparent ionizing group, dissociating in the pH range explored (pH 2.5 to 6.0). This model (*i.e.*, the minimum device for describing the data) is supported by values of the Hill coefficient for pH titrations being equal to 1 within the experimental error (0.98 to 1.02). However, since this is a phenomenological viewpoint, it does not exclude *a priori* that two (or more) amino acid residues may physically partecipate in modulating kinetic parameters below pH 6.0 as suggested from literature [3-7, 14, 18, 26, 27].

As far as the reversible pH effects on pre-steady-state and steady-state parameters for stem bromelain, ficin and papain catalyzed hydrolysis of ZGlyONp, ZAlaONp, ZValONp and ZTyrONp are concerned, significant differences in the pH dependence of the deacylation step (*i.e.*, k_{+3}) are observed (see fig. 1 as well as tables I and II). In fact, values of k_{+3} for stem bromelain and ficin catalyzed hydrolysis of ZGlyONp, ZAlaONp, ZValONp and ZTyrONp are essentially pH independent; on the other hand, values of the deacylation rate constant for papain action show a five-fold change over the pH range explored (2.5 to 6.0) (see fig. 1 as well as tables I and II). Such findings indicate that the restoration of free stem bromelain and ficin is preceded or accompanied by the disappearance, from the functional viewpoint, of the catalytically relevant group (see fig. 1 as well as table II), possibly its pK_a being forced below pH 2.5 during the dissociation of the E·P adduct (see Scheme I), or rather because it does not contribute at all to the deacylation step. On the other hand, the restoration of free papain is accompanied by protonation of the functionally relevant amino acid residue (see fig. 1 as well as table II). These data may be taken as indicative of different conformational transitions, occurring in stem bromelain, ficin and papain

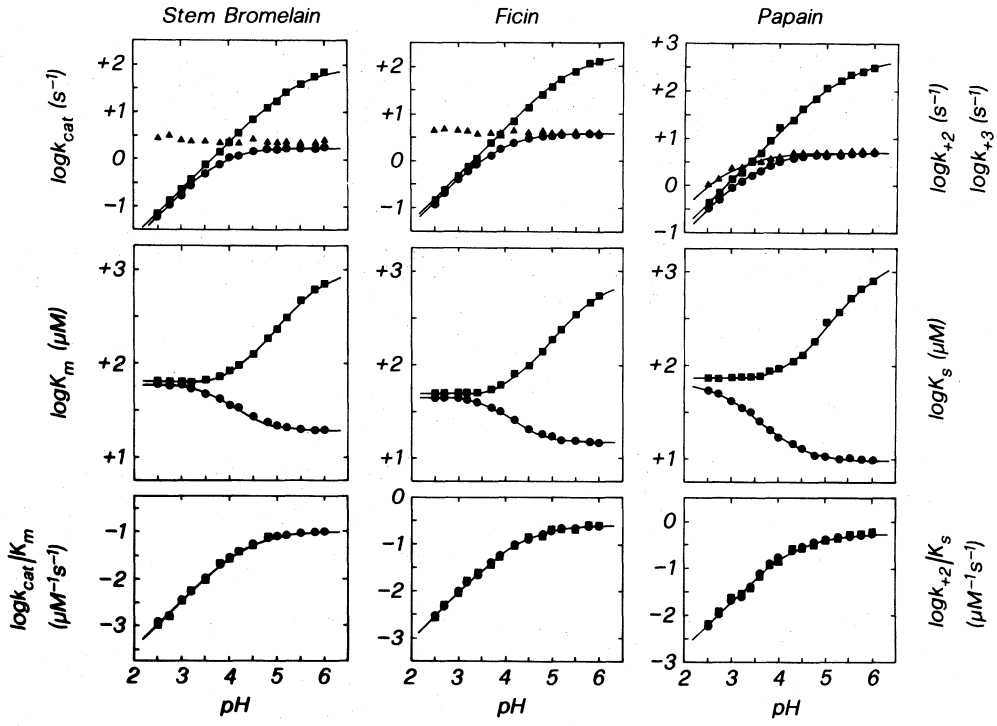

Fig. 1. – pH dependence of $\log k_{cat}$ (●), $\log k_{+2}$ (■), $\log k_{+3}$ (▲), $\log K_m$ (●), $\log K_s$ (■), $\log k_{cat}/K_m$ (●) and $\log k_{+2}/K_s$ (■) for the hydrolysis of ZGlyONp catalyzed by stem bromelin, ficin and papain at $T = 21 \pm 0.5$ °C and $I = 0.1$ M. Values of pre-steady-state and steady-state parameters for the papain catalyzed hydrolysis of ZGlyONp, obtained between pH 3.0 and 6.0 at $T = 21 \pm 0.5$ °C and $I = 0.1$ M, were taken from Ascenzi *et al.*, [3]. Continuous lines, calculated with sets of parameters given in table II and according to Ascenzi *et al.*, [3], are theoretical curves for a simple system (*i.e.*, involving one ionizing group). Over the whole pH range explored (pH 2.5 to 6.0), values of k_{+3} for stem bromelain and ficin catalyzed hydrolysis of ZGlyONp (▲) are essentially pH independent with average values of $2.6 \pm 0.3\ s^{-1}$ and $4.1 \pm 0.3\ s^{-1}$ respectively. For further details see text.

during catalysis. This view, based on the assumption that the acid-base equilibrium of the Cys25-His159 catalytic diad affects the pH profiles of $k_{+2}/K_s\ (= k_{cat}/K_m)$, k_{+2} and k_{+3} [2-4, 6, 7, 28] (reflecting the ionization(s) in the free enzyme (E) as well as in the $E \cdot S$ and $E \cdot P$ adducts, respectively [28]), agrees with previous results reported for related systems [1, 3-6, 29, 30].

The limited molecular information available for stem bromelain [21, 23] and ficin [22, 24] does not allow a circumstantial comparison with the detailed structure-function relationship of papain [3-7, 25]. However, a large body of evidence, based on comparative kinetic studies with specific active site probes [5, 7-20], suggests that differences in the modulating effects of pH on catalytic properties of stem bromelain, ficin and papain can be ascribed to changes in the disposition of cationic site(s) and/or hydrophobic area(s) in the neighbourhood of the Cys25-His159 catalytic diad [7, 9-14, 16-20]. Thus, it has been proposed [7, 9-14, 16-20] that if stem bromelain and ficin contain an aspartic residue analogous to Asp158 in papain, the pK_a of its carboxy group is probably significantly lower ($pK_a \leq 2$) than that of the corresponding

TABLE I. – *Values of Pre-Steady-State and Steady-State Parameters for Stem Bromelain, Ficin and Papain Catalyzed Hydrolysis of* ZGlyONp, ZAlaONp, ZValONp and ZTyrONp (*).

Proteinase	Substrate	pH	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	k_{+2} (s^{-1})	K_s (μM)	k_{+2}/K_s ($\mu M^{-1}s^{-1}$)	k_{+3} (s^{-1})
Stem bromelain	ZGlyONp	2.5	7.8×10^{-2}	70	1.1×10^{-3}	8.0×10^{-2}	72	1.1×10^{-3}	2.9
		6.0	2.2	23	9.6×10^{-2}	67	6.8×10^{2}	9.9×10^{-2}	2.3
	ZAlaONp	2.5	0.21	55	3.8×10^{-3}	0.22	57	3.9×10^{-3}	5.3
		6.0	5.2	16	0.32	1.3×10^{2}	4.4×10^{2}	0.30	5.2
	ZValONp	2.5	1.3×10^{-3}	40	3.3×10^{-5}	1.4×10^{-3}	42	3.3×10^{-5}	2.3×10^{-2}
		6.0	2.0×10^{-2}	10	2.0×10^{-3}	0.90	4.8×10^{2}	1.9×10^{-3}	2.0×10^{-2}
	ZTyrONp	2.5	2.0×10^{-3}	20	1.0×10^{-4}	2.1×10^{-3}	21	1.0×10^{-4}	4.2×10^{-2}
		6.0	3.0×10^{-2}	5.0	6.0×10^{-4}	1.5	2.4×10^{2}	6.2×10^{-4}	3.2×10^{-2}
Ficin	ZGlyONp	2.5	0.18	48	3.8×10^{-3}	0.19	50	3.8×10^{-3}	4.0
		6.0	3.7	15	0.25	1.3×10^{2}	5.5×10^{2}	0.24	3.7
	ZAlaONp	2.5	0.40	25	1.6×10^{-2}	0.42	27	1.6×10^{-2}	6.8
		6.0	6.8	16	0.43	3.1×10^{2}	7.0×10^{2}	0.44	7.1
	ZValONp	2.5	2.0×10^{-3}	20	1.0×10^{-3}	2.1×10^{-2}	21	1.0×10^{-3}	0.42
		6.0	0.30	5.0	6.0×10^{-2}	20	3.2×10^{2}	6.3×10^{-2}	0.31
	ZTyrONp	2.5	1.6×10^{-3}	10	1.6×10^{-3}	1.7×10^{-2}	11	1.5×10^{-3}	0.22
		6.0	0.15	2.0	7.5×10^{-2}	1.4	1.9×10^{2}	7.4×10^{-2}	0.16
Papain	ZGlyONp	2.5	0.20	56	3.6×10^{-3}	0.30	71	4.2×10^{-3}	0.86
		6.0 (**)	4.0	9.5	0.42	3.0×10^{2}	7.5×10^{2}	0.40	4.0
	ZAlaONp	2.5	0.70	48	1.5×10^{-2}	1.1	80	1.4×10^{-2}	1.8
		6.0	9.2	9.1	1.0	5.0×10^{2}	5.0×10^{2}	1.0	9.3
	ZValONp	2.5	3.0×10^{-2}	40	7.5×10^{-4}	4.0×10^{-2}	58	6.9×10^{-4}	0.10
		6.0	0.60	7.0	8.6×10^{-2}	40	4.6×10^{2}	8.7×10^{-2}	0.61
	ZTyrONp	2.5	9.0×10^{-2}	5.0	1.8×10^{-2}	0.12	6.5	1.8×10^{-2}	0.38
		6.0	1.9	1.0	1.9	75	40	1.9	1.9

(*) Data were obtained at $T = 21 \pm 0.5$ °C and $I = 0.1$ M.
(**) From Ascenzi *et al.* [3].

TABLE II. – pK_a *and Midpoint Values Fitting the* pH *Titrations of Kinetic Parameters for Stem Bromelain, Ficin and Papain Catalyzed Hydrolysis of* ZGlyONP (*).

Kinetic Parameters	Stem Bromelain	Ficin	Papain (**)
k_{cat}	$pK_a = 4.0 \pm 0.1$	$pK_a = 3.9 \pm 0.1$	$pK_a = 3.8 \pm 0.10$
k_{+2}	$pK_a = 5.60 \pm 0.15$	$pK_a = 5.60 \pm 0.15$	$pK_a = 5.80 \pm 0.15$
k_{+3}	— (***)	— (***)	$pK_a = 3.10 \pm 0.15$
K_m	midpoint = 4.1 ± 0.1	midpoint = 4.15 ± 0.10	midpoint = 3.65 ± 0.10
K_s	midpoint = 5.05 ± 0.15	midpoint = 5.00 ± 0.15	midpoint = 5.15 ± 0.10
$k_{+2}/K_s (= k_{cat}/K_m)$	$pK_a = 4.5 \pm 0.1$	$pK_a = 4.4 \pm 0.1$	$pK_a = 4.5 \pm 0.1$

(*) Data were obtained at $T = 21 \pm 0.5$ °C and $I = 0.1$ M.

(**) From Ascenzi *et al.* [3].

(***) Over the whole pH range explored (pH2.5 to 6.0), values of k_{+3} for stem bromelain and ficin catalyzed hydrolysis of ZGlyONp are essentially pH independent (see fig. 1) with average values of $2.6 \pm 0.3\ s^{-1}$, and $4.1 \pm 0.3\ s^{-1}$, respectively.

residue in papain (pK_a 3.0 to 4.0). On this basis, different pH effects observed on the deacylation step (see fig. 1 as well as tables I and II) may be explained in terms of intensity changes, different for the three cysteine proteinases, of the electrostatic field, induced by charged residues (such as Asp158 in papain), modulating the acid-base equilibrium of the Cys25-His159 catalytic diad, and thus the enzyme activity.

As a whole, the reported data represent the first example of pH modulation of cysteine proteinase action exclusively controlled by the deacylation step, and support the view [31] that the enzyme act is a collective phenomenon in terms of structural changes associated to catalysis that accompany the motion along the reaction coordinate and propagate through the protein structure.

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