

Structural characterization of the papaya cysteine proteinases at low pH

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Abstract

Current control of gastrointestinal nematodes relies primarily on the use of synthetic drugs and encounters serious problems of resistance. Oral administration of plant cysteine proteinases, known to be capable of damaging nematode cuticles, has recently been recommended to overcome these problems. This prompted us to examine if plant cysteine proteinases like the four papaya proteinases papain, caricain, chymopapain, and glycine endopeptidase that have been investigated here can survive acidic pH conditions and pepsin degradation. The four papaya proteinases have been found to undergo, at low pH, a conformational transition that instantaneously converts their native forms into molten globules that are quite unstable and rapidly degraded by pepsin. As shown by activity measurements, the denatured state of these proteinases which finally results from acid treatment is completely irreversible. It is concluded that cysteine proteinases from plant origin may require to be protected against both acid denaturation and proteolysis to be effective in the gut after oral administration.

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Infections with gastrointestinal nematodes have severe consequences for the health of millions of humans worldwide and cause important economic losses in livestock farming [1]. Current control of these parasitic nematodes, nowadays, relies primarily on the use of anthelmintic drugs. Three classes of synthetic anthelmintics are recognized, each having a different mode of action: the benzimidazoles act by disrupting β -tubulin, the imidazothiazoles, and/or tetrahydropyrimidines interact with nicotinic acetylcholine receptors, and the macrocyclic lactones act by opening glutamate-gated chloride channels [2]. The onset

of resistance is the most important problem arising from the increasing use of anthelmintics, suggesting that there is an urgent need for the search and development of alternative treatments against gastrointestinal nematodes of both humans and domestic animals [3].

Before the advent of synthetic anthelmintics, some plant extracts, including extracts from *Carica papaya*, *Ficus galabra*, and *Ananas comosus*, were used traditionally for several decades for the treatment of helminth infections. It has recently been shown that their effectiveness is due to the presence, in these plant extracts, of cysteine proteinases that are able to cause marked damage to the cuticles of parasitic nematodes [2,4], suggesting that these proteinases constitute strong candidates for the needed alternative strategy [2].

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The oral administration of plant cysteine proteinases, however, requires that they are able to survive both acidic pH conditions and pepsin degradation in order to be effective in the gut.

Examination of the literature reveals that several plant cysteine proteinases belonging to clan CA of this family of proteinases have been shown, in the past, to form, at low pH, partially structured states (Scheme 1) known, as the molten globule and A-states [5,6]. This is the case for papain [7] isolated from the latex of *Carica papaya* and for bromelain isolated from the stem of *Ananas comosus* [8].

At a pH of around 2, papain and bromelain but also procerain and ervatamins B, purified, respectively, from the latex of *Calotropis procera* and *Ervatamia coronaria*, all convert to an acid unfolded state exhibiting properties of a molten globule state [7–10] as shown by circular dichroism, fluorescence, and hydrophobic dye binding. These proteins indeed show substantial retention of the native like secondary structure, as evidenced by far UV-CD spectra, but lose the persistent tertiary interactions of the native state, as outlined by CD signals in the aromatic absorption region. Enhanced fluorescence of 8-anilino-naphthalene-1 sulfonic acid (ANS) by a factor of around 20–25 compared to the fluorescence in the presence of both native or completely unfolded protein indicates the presence of hydrophobic pockets, a conclusion that is supported by the observation that some of these proteinases, such as papain, tend to aggregate in the presence of salts [11].

With further decrease of pH to around 0.8, these molten globule states regain a substantial part of their lost secondary structure while maintaining an enhanced ability to bind the fluorescent probe ANS, both properties that indicate their conversion into the more structured A-states [6].

Refolding of papain, both from the A and molten globule states, to the native state has been described as fully reversible, a conclusion based on the observation that acid unfolded papain regains, upon neutralization, the spectral characteristics of the native state [7]. As generally observed for molten globule states, papain at pH 2 is also stabilized by low concentrations of urea [11]. Most other acid-denatured cysteine proteinases that have so far been investigated have also been shown, on the basis of the same criterion, to be reversibly renatured [8–10].

Thermal unfolding of papain in the pH interval 2–2.6 is also quite reversible [7,12], in marked contrast to pH 7 where aggregation is visible [13]. Thermal unfolding of papain in the molten globule state is non-cooperative and the transition curves are biphasic [7,12]. Since the

molecular structure of papain is divided essentially into two distinct lobes separated by a cleft [14], it has been suggested that the two domains unfold independently and sequentially. In the sequential unfolding of papain in the pH interval 2–2.6, the N-terminal domain has been shown to unfold first [12,15].

Not all cysteine proteinases belonging to clan CA do comply with Scheme 1. Ervatamin C, also isolated from the latex of *Ervatamia coronaria*, retains both secondary and tertiary structures along with biological activity over the wide range of pH 2–12 [16]. The thermal unfolding at low pH of both chymopapain and caricain, isolated from the papaya latex together with papain, has been described as completely irreversible [17,18], in marked contrast thus to papain [7] and despite the fact that the three papaya proteinases exhibit strong structural similarity [19]. That acid denaturation may be reversed has recently been questioned [20].

This prompted us to re-examine the structures adopted, in acidic medium, by the four cysteine proteinases that can be purified from the latex of *Carica papaya* [21].

Materials and methods

Materials. Aldrich-Chemie (Steinheim, Germany) provided methylmethanethiolsulfonate, DTT (dithiothreitol), and ANS. SP-Sepharose Fast Flow and Thiopropyl Sepharose 6B were from Amersham Biosciences AB (Uppsala, Sweden) and Fractogels EMD Propyl (S) and TSK Butyl-650 (M) were from Merck (Darmstadt, Germany). *N*-Benzoyl-D,L-arginine paranitroanilide (BAPA), and phosvitin were from Sigma Chemical (St. Louis, MO). Fluka (Buchs, Switzerland) provided bovine beta casein and pepsin from hog stomach (3834 U/mg). Boc-Ala-Ala-Gly paranitroanilide (Boc-AAG-pNA) was from Bachem AG (Bubendorf, Switzerland).

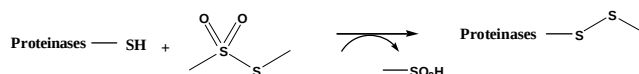
The papaya proteinases were purified from spray-dried papaya latex as described previously [21]. Purification of papain, glycine endopeptidase, and caricain up to homogeneity needed not less than three chromatographic steps on SP-Sepharose, on Fractogel EMD Propyl (S), and on Thiopropyl Sepharose 6B, respectively. Purification of chymopapain necessitated one additional step as described in [19]. At the end of their purification, the four enzymes, that contained more than 0.95 mol of essential free thiol function per mol of enzyme, were converted into their *S*-methylthioderivatives following reaction with methylmethanethiolsulfonate (Scheme 2).

The enzymes were thus protected against both air oxidation and autolysis, in a quite reversible manner, since addition of low molecular mass thiol compounds like dithiothreitol allowed the quantitative regeneration of the essential thiol functions when suited [21]. Previous studies [7–10] made use of iodoacetic acid that irreversibly converted the essential free thiol function into *S*-carboxymethylcysteine (Scheme 3).

Acid denaturation of the papaya proteinases. Acid-induced unfolding of the papaya proteinases was carried out, at different pH, in buffers containing 50 mM glycine and 157 mM KCl. pH measurements were carried out on a Multi-Calimatic pH-meter 763 with a precision of 0.01 pH unit. Unless otherwise stated, the papaya proteinases were incubated with the



Scheme 1. The reversible conversion of the native state of cysteine proteinases of the papain superfamily into partially structured states at acidic pH.



Scheme 2. Conversion of the papaya proteinases into their *S*-thiomethyl derivatives.



Scheme 3. Irreversible denaturation of the papaya proteinases at acidic pH.

buffers of specified pH and at the specified temperature and allowed to equilibrate for 30 min before taking the spectroscopic measurements. The pH was measured again after the experiment.

Fluorescence measurements. Fluorescence spectra were recorded using a Perkin-Elmer LS 55 fluorimeter and measurements were made in the concentration range where emission was linear with respect to fluorophore concentrations. In all cases, emission and excitation bandwidths were 5.0 nm each and the solution temperature in the cell was maintained, unless otherwise stated, at 10.0 ± 0.1 °C. Emission spectra were the mean of five consecutive scans.

For intrinsic fluorescence emission measurements, excitation was at 295 nm and emission spectra were collected in the range 300–520 nm. Emission spectra of ANS (excitation at 350 nm) were collected in the range 400–550 nm.

CD measurements. CD measurements were made at 10 or 25 °C on a Jasco J-710 polarimeter purged with dry nitrogen. Far-UV spectra were collected in the range 185–260 nm in a 0.2 mm circular cell while near-UV spectra were collected from 240–350 nm in a 10 mm cell. All CD spectra were an accumulation of eight scans recorded at 50 nm/min with a time constant of 2 s for a nominal resolution of 1.7 nm. The spectra were scaled to molar ellipticities [θ].

Differential scanning micro-calorimetric experiments. Calorimetric experiments were carried out in a CSC Nano DSC differential adiabatic scanning micro-calorimeter equipped with 0.3268 ml cells. A scan rate of 1 K/min and a protein concentration of 1.5 mg/ml were used.

Proteolysis of the papaya proteinases by hog pepsin and SDS-PAGE experiments. Proteolysis was performed for 2 h at 37 °C and pH 1.21, using a 2% (w/w) ratio of pepsin to papaya endopeptidases. After proteolysis and before to be loaded onto the gel, an aliquot was removed from the reaction mixture, diluted with 50 mM Tris-HCl (at pH 7.5) containing bromophenol blue and 0.8% SDS, and boiled for 5 min. The SDS-PAGE experiments were carried out on precast gels (ExcelGel, 245 × 110 × 0.5 mm, gradient 8–18%) using the Multiphore II kit from Amersham Biosciences. The running conditions were 600 V, 50 mA, and 35 W at constant temperature (15 ± 0.1) °C. Protein detection was performed using the silver staining procedure according to the manufacturers' instructions.

Other spectroscopic and analytic methods. Absorbances were measured with a Perkin-Elmer Lambda 45 spectrophotometer. Concentrations of papain, chymopapain, caricain, and glycine endopeptidase were measured spectrophotometrically using, respectively, for ϵ_{278} , values of 54981, 43908, 39200, and 40981 M⁻¹ cm⁻¹. These values were provided by Swiss-Prot Accession Nos: (P00784, P14080, P10056, and P05994). ANS concentrations were also measured by the same way using the value of 4950 M⁻¹ cm⁻¹ at 350 nm [22]. The amidase activity of papain, chymopapain, and caricain on the one hand and that of glycine endopeptidase on the other hand were measured at 37 °C and pH 6.8, using, respectively, BAPA and Boc-AAG-pNA as the substrates and the results expressed in nanomoles of substrate hydrolyzed per minute and per mg of enzyme [23].

Results

Extrinsic fluorescence emission studies

In aqueous solutions, the hydrophobic dye ANS has a low quantum yield and exhibits a λ_{max} emission at 520 nm. At pH around neutrality where the papaya proteinases are catalytically competent, these enzymes do not

bind ANS at all. Caricain, however, undergoes a transition in the pH range 2–3 resulting, at pH 2, in a blue shift in the emission peak of ANS with a maximum located at 480, a shoulder at 510 nm, and an important increase in fluorescence intensity (Fig. 1). Typically, such fluorescent characteristics are observed when a protein adopts less structured states [5,6]. As shown in Fig. 2, in the whole pH interval 3–7, the fluorescence characteristics of aqueous solutions of ANS are not affected as a result of the presence of caricain showing that this enzyme maintains its native state as low as pH 3. Further acidification, after the transition,

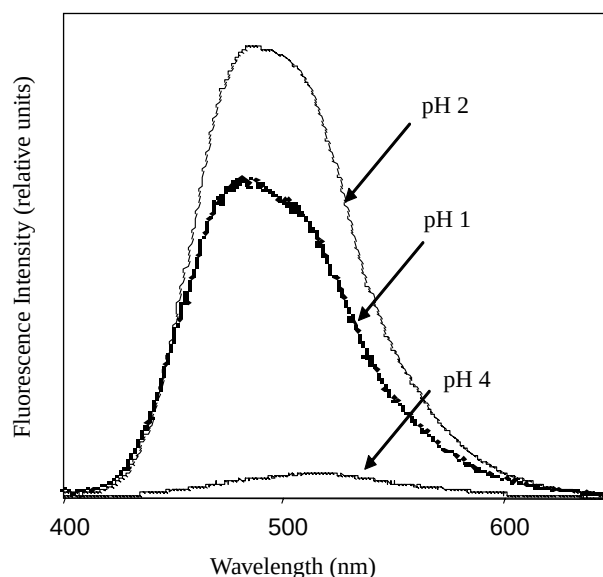


Figure 1. Emission spectra of ANS (165 μM) in the presence of caricain (3.25 μM) in buffers containing 50 mM glycine and 157 mM KCl at pH 1.0, 2.0, and 4.0 as indicated by the arrows.

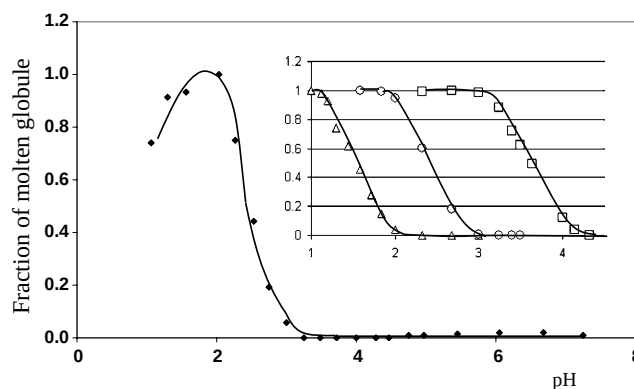


Figure 2. Spectrofluorimetric titration of the caricain-ANS complexes. Solutions were incubated at various pH values in 50 mM buffers containing 157 mM KCl for 30 min at 10 °C before fluorescence measurements. Emission spectra were collected as described in the Materials and methods section. The fraction of molten globule is the ratio of the fluorescence intensity measured at 480 nm, at each pH, relative to the maximum value observed at pH 2. Inset: Spectrofluorimetric titrations of papain-ANS (circles), chymopapain-ANS (triangles), and glycine endopeptidase-ANS (squares) complexes. See legend under Fig. 2 for further information.

results in a slight decrease in ANS fluorescence intensity indicating that a second transition occurs below pH 2. The binding of ANS to caricain thus appears to be quite similar to the binding of ANS to papain ([7] and Fig. 2) and to bromelain [8], which strongly suggests that, in acidic medium, caricain behaves as described in Scheme 1. Fluorescence studies as a function of time show that binding of ANS to caricain reaches completion within a few minutes and then slowly declines strongly, suggesting that the molten globule state of caricain might be somewhat unstable. Relatively, similar results were observed in the case of papain, glycine endopeptidase, and of chymopapain (see inset of Fig. 2). Glycine endopeptidase, however, exhibits its capacity to bind ANS in the pH range 3–4 while the chymopapain requires more acidic conditions (pH range 1–2).

Circular dichroism studies on caricain

Figs. 3A and B show the circular dichroism spectra of caricain at pH 6.8 and 3.5 (curve 1) and 2.0 (curves 2, 2a, and 2b), respectively, in the near and the far-UV regions.

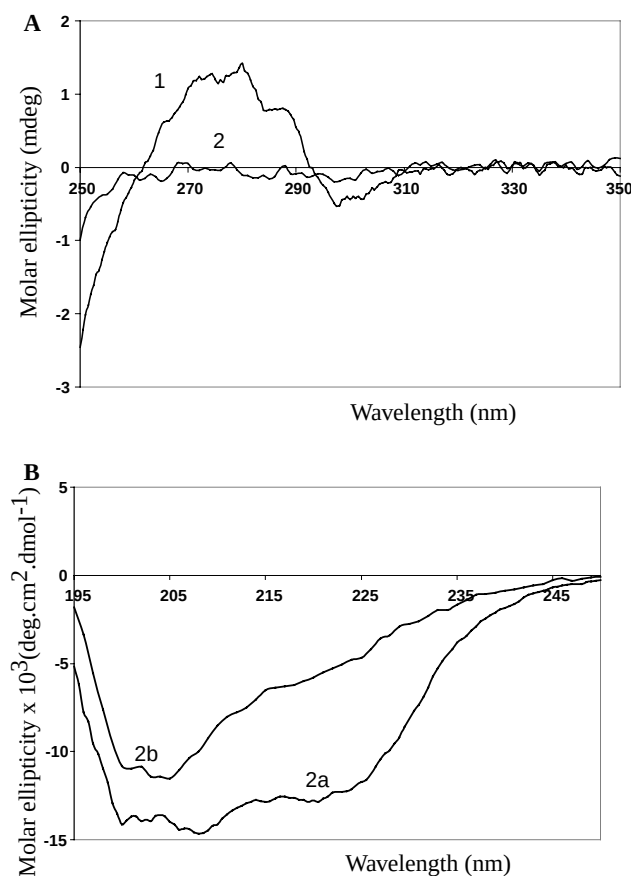


Figure 3. Circular dichroism spectra of caricain in the aromatic (A) and far-UV (B) regions. A stock aqueous solution of caricain was diluted at 25 °C in buffers containing 50 mM glycine and 157 mM KCl at pH 3.5 and 6.8 (curve 1) or 2.0 (curve 2) and the spectra in the aromatic region recorded within 30 min. Similarly, the stock solution of caricain was diluted at 10 °C in the buffer at pH 2.0 and the spectra in the far-UV region recorded after 30 (curve 2a) and 100 min (curve 2b).

In both regions, the spectra of caricain at pH 3.5 superimposed quite well to the spectra of the proteinase at pH 6.8.

Analysis of the far UV-CD spectra of caricain in the pH range 3.5–6.8 (not shown for reasons of clarity of Fig. 3B) indicates that in addition to turns (22%), both α -helices (31%) and β -sheets (16%) contribute to the secondary structure of the enzyme in its native state as expected from the X-ray structure of the enzyme [24,25].

The spectrum of native caricain in the aromatic region (250–350 nm) (Fig. 3A) results from the contribution of several strong positive signals located at 273, 280, and 286 nm, and of one small flat negative signal at 300 nm that can be assigned to tryptophan and tyrosine residues. All these signals are abolished following incubation of caricain at pH 2.0, indicating the disruption of the asymmetrical environment around the CD-active aromatic chromophores and a loss of the rigid tertiary structure. Analysis of the far UV-CD spectra (Fig. 3B) indicates that acidification of caricain, at 10 °C, up to pH 2 is not accompanied, within the first hour, by gross structural modifications which shows that at pH 2, caricain adopts for at least one hour at 10 °C a true molten globule state. However thereafter, the random coil content progressively increases (from 30% to 60%) (Fig. 3B, curves 2a and 2b, respectively), largely at the expense of the α -helix content which decreases (up to about 10%), an observation that confirms that the molten globule state of caricain is unstable and spontaneously unfolds. Further acidification up to pH 0.75 is not accompanied by regain of secondary structure (not shown).

Intrinsic fluorescence emission studies on caricain

The fluorescence emission spectrum of caricain is rather complex since not less than four tryptophan residues, located in the amino acid sequence [26] at positions 7, 26, 181, and 186, are all susceptible to contribute to this spectrum.

The side chain atoms of tryptophan-181 are well exposed at the surface of the enzyme in a polar environ-

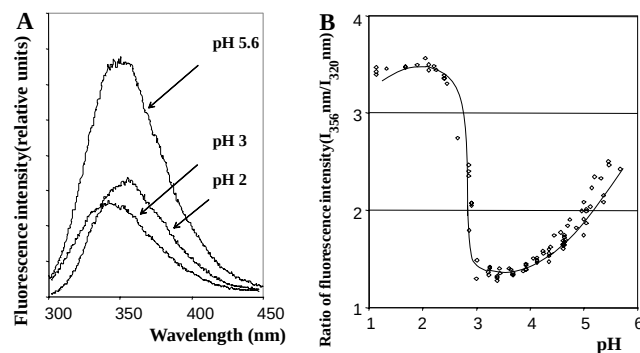


Figure 4. Emission spectra of caricain (3.25 μM) in buffers containing 50 mM glycine and 157 mM KCl at pH 5.6, 3.0, and 2.0 as indicated by the arrows (A) and spectrofluorimetric titration of caricain (B). Solutions of caricain (3.25 μM), at 25 °C, were incubated at various pH values in buffers containing 50 mM glycine and 157 mM KCl for 30 min before measurements. Fluorescence intensities were measured at 320 and 356 nm, and expressed as the ratios I_{356}/I_{320} for each pH value.

ment while the side chain atoms of the three other tryptophan residues are deeply buried in the hydrophobic core of the protein [24]. At pH around neutrality, the emission spectra of caricain exhibit maximums around 350 nm (Fig. 4A) and thus are largely dominated by tryptophan-181. Acidification of caricain up to pH 3 is accompanied by a quenching of tryptophan-181 since the global intensity decreases and the emission spectrum of caricain at pH 3 exhibits a maximum that is blue-shifted at 340 nm (Fig. 4A). Further acidification to pH 2 or lower than 2 is accompanied by the complete exposure of the tryptophan residues as shown by the emission spectra which exhibit a maximum at 356 nm. The transition between pH 2 and 3 is clearly visible in Fig. 4B wherein the ratio of the fluorescence intensities at 356 and 320 nm was plotted as a function of pH. This transition, which superimposes perfectly with that shown in Fig. 1, also suggests that acidification converts native caricain into a much less compact structure. In marked contrast, modifications of the ratio I_{356}/I_{320} in the pH interval 3–5.6 (exhibited also by papain, chymopapain, and glycine endopeptidase) are not a consequence of a conformational transition as shown by the circular dichroism spectra in both the aromatic and the far-UV regions. A close examination of the 3-D structure of caricain [24] revealed that the relative orientations of the side chains of tryptophan-181 and of histidine-159 and the distance that separates them (about 0.4 nm) are such that in its protonated form, histidine-159 makes a cation- π interaction with tryptophan-181 [27]. This leads to the quenching of the fluorescence emission of tryptophan-181 [28]. The side chain of histidine-159 is located in a hydrophobic pocket making numerous contacts with carbon atoms of cysteine-25, serine-136, aspartic acid-158, alanine-160, and tryptophan-181. An abnormal low pK_a may be assigned to the imidazole ring of histidine-159 and that conversion of the imidazole ring of histidine-159 of caricain into an imidazolium ion could proceed in the pH range 3.5–6.5 does thus not seem unlikely.

DSC studies of caricain

The endotherms of caricain, after subtraction of the buffer–buffer tracings, which were recorded at pH 7.0, 2.0, and 0.75 using a heating rate of 1 °C/min are shown in Fig. 5. The endotherms of caricain incubated at pH 0.75 or pH 2.0 for 24 h at 4 °C were flat with no visible maximum at the image of those obtained with phosvitin and β -casein, two proteins described as natively unfolded proteins [29].

For pH 7, thermal unfolding was apparently irreversible as indicated by the fact that no endotherm could be observed by rescanning the sample after having been cooled from the first scan. As a consequence, analysis of the calorimetric data was restricted to the determination of the melting temperature. The endotherm of native caricain exhibited a bell-shaped form and the maximum of the heat capacity curve occurred at 87.1 °C.

Further characterization of the molten globule state of the four papaya proteinases

In a series of experiments, the four papaya proteinases were individually incubated at 37 °C at various pH known to promote formation of their molten globule state (pH 3 and 1 in the case of glycine endopeptidase and chymopapain, respectively, and pH 2 in the case of both papain and caricain). At various time intervals, aliquots were removed and diluted 20 times in a buffer, at pH 6.8, that contains citrate, borate, and phosphate (100 mM each), and 1 mM EDTA. The percentage of the amidase activity that could be regenerated was then measured after reactivation with 2.5 mM DTT. The results of this experiment (Fig. 6) clearly show, at least in the case of caricain, that the instantaneous conversion of its native state into its molten globule state is indeed reversible. However, in their molten globule state, the four papaya proteinases rapidly and irreversibly unfold. In another series of experiments, the mixture of

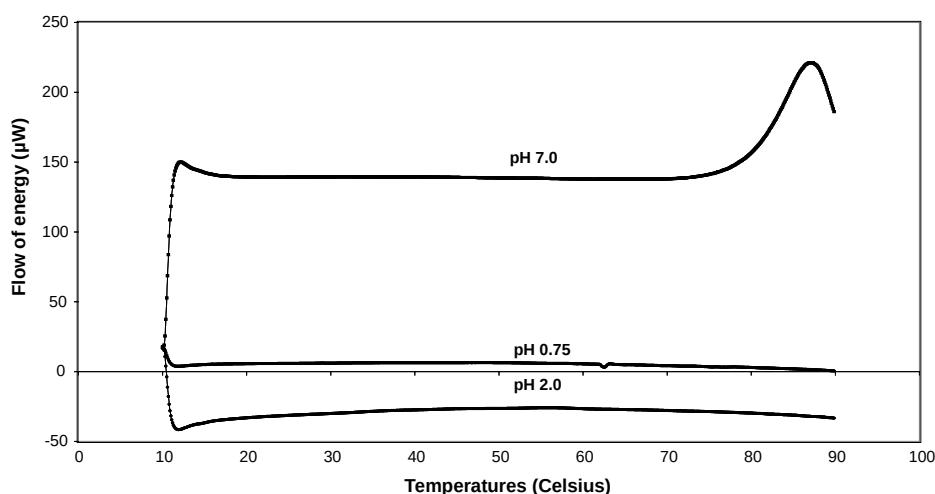


Figure 5. DSC thermograms recorded for caricain after incubation at 4 °C for 24 h at pH 6.8, 2, and 0.75.

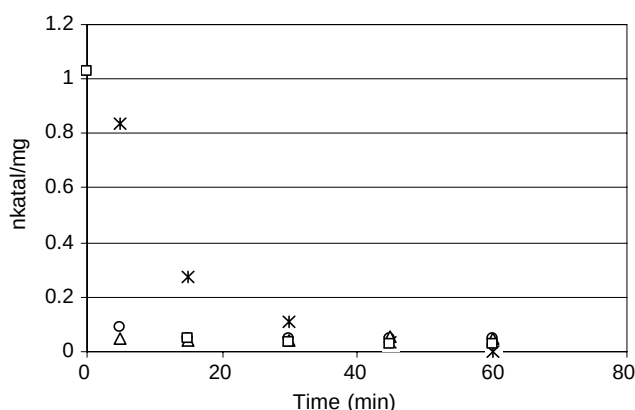


Figure 6. Regenerable enzymatic activity after incubation of papain (at pH 2, circles), caricain (at pH 2, stars), glycine endopeptidase (at pH 3, squares), and chymopapain (at pH 1, triangles) at 37 °C for various time intervals.



Figure 7. Analysis by SDS-PAGE of the proteolytic susceptibility of the papaya proteinases towards hog pepsin. Lanes 1 and 9, molecular mass standards including (from top to bottom) phosphorylase B (97,400), bovine serum albumin (66,430), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,300); lanes 2–5, papain, chymopapain, glycine endopeptidase, and caricain, respectively; lanes 6 and 7, the mixture of the papaya proteinases, before and after proteolysis, respectively; lane 8, pepsin from porcine gastric mucosa.

the four papaya proteinases were incubated with pepsin (2%, w/w) at pH 1.21 and 37 °C for 2 h and then analyzed by SDS-PAGE. The results (Fig. 7) reveal that, under these conditions, the bands corresponding to the papaya proteinases had completely disappeared after incubation with the mammalian aspartic proteinase.

Discussion

At low pH, papain, chymopapain, caricain, and glycine endopeptidase, the four cysteine proteinases that are

expressed in the papaya laticifers strongly bind the hydrophobic dye ANS.

Since both native and unfolded proteins generally do not bind ANS [5,6], this observation constitutes a strong indication that at low pH, these four papaya enzymes adopt a less structured state that is different from the native and the completely unfolded ones.

Several lines of evidence indicate that, in the case of caricain and papain, this less structured state has the typical characteristics of molten globules. For instance, at pH 2, a molecular species of caricain is characterized as a species that strongly binds ANS and at the same time is devoid of any tertiary structure while maintaining intact the secondary structure of the enzyme. Caricain is thus the sixth plant cysteine proteinase that has been shown to convert its native structure into a molten globule at acidic pH [7–10,16], suggesting that this property may be shared by other homologous enzymes including chymopapain and glycine endopeptidase.

Until now, the molten globule state of plant cysteine proteinases has generally been observed at pH 2. This is the case for caricain and also for papain ([7] and inset of Fig. 2), bromelain [8], ervatamin B [9], and procerain [10]. The cases of chymopapain (pH 1), ervatamin C (pH 0.5), [16], and glycine endopeptidase (pH 3) show that the susceptibility of the various proteinases to the proton concentration may however slightly differ.

This study has also shown the noteworthy instability of the molten globule state of the papaya proteinases. The most direct evidence of such an instability has been obtained in the case of caricain and is visible in Fig. 3B which compares the far UV-CD spectra of the proteinase after incubation, at 10 °C and pH 2, for, respectively, 30 and 100 min. Up to 30 min of incubation, the far UV-CD spectra of the molten globule were undistinguishable from the spectra of caricain in its native state. A progressive decrease of the molar ellipticities (in the 205–250 nm region) was then observed upon further incubation. Analysis of the spectrum recorded after 100 min of incubation revealed that a substantial fraction of the secondary structure characterizing native caricain had disappeared. Such an instability explains why the fluorescence intensity of the ANS–papaya proteinase complexes also declined after a time interval that was temperature dependent. It also explains the absence of endotherm exhibited by caricain after incubation at 4 °C for 24 h at pH 0.75 and 2 (Fig. 5).

After incubation at 10 °C and pH 2 for 100 min., further acidification up to 0.75 did not restore any fraction of secondary structure characterizing native caricain, strongly suggesting that the A state of this enzyme could not be observed. It is concluded that acid denaturation of the papaya proteinases leads to completely unfolded molecular species.

As a whole, the results presented in this study suggest that acid denaturation of the papaya proteinases complies with Scheme 3.

That the first step is reversible is suggested by examination of the time dependence of the regenerated activity

observed in Fig. 6 in the case of caricain. Indeed, a substantial fraction of the enzymatic activity (at least 15%) can be regenerated for incubation times up to 20 min incubation at pH 2 and 37 °C even though formation of the molten globule state of caricain requires less than 1 min. That the second step is irreversible is demonstrated by the fact that finally (after 30 min incubation at 37 °C) no enzymatic activity at all can be regenerated from any molten globule state of the papaya proteinases.

The reversible formation of the papaya proteinases molten globules, as suggested here, agrees with the conclusions drawn in previous work on papain [7] and bromelain [8]. These conclusions, based solely on spectroscopic criteria, are valid if a substantial fraction of the molten globules is not unfolded. Activity measurements, as performed in this work, yield more reliable criteria to evaluate the reversibility.

On the other hand, SDS-PAGE experiments have shown that the papaya proteinases are highly degraded by pepsin at pH 1.2 and 37 °C and converted into small peptides that could not be detected. Taking into account the high susceptibility exhibited by the four papaya proteinases towards proteolysis by pepsin at pH 1.2 and 37 °C, it is expected that only a small fraction of the proteinases will survive residence in mammal stomachs. It seems thus reasonable to recommend, in agreement with Hale [20], that plant cysteine proteinase be protected to be effective in the gut after oral administration.

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