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Unfolding of cardosin A in organic solvents and detection of intermediaries

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ABSTRACT

In the present study the relationship between conformational stability and enzymatic activity in the presence of organic solvents (OS) was investigated. We have found that cardosin A, the model protease investigated in this work, inactivates through a biphasic mechanism, which is incipient in aqueous buffer and becomes visible in the presence of hydrophilic OS. In fact, in OS this inactivation originates stable intermediaries that were detected in acetonitrile. This biphasic mechanism can be described in two phases: an initial one, where OS induce alterations that affect the active site cleft and global mobility, but with little interference on the global protein conformation, and, a second phase, where there is a global change in protein conformation with concomitant activity loss. It is shown that in the presence of hydrophilic OS there is a larger mobility of the enzyme, revealed by limited proteolysis, probably due to a weakening of hydrophobic interactions within the protein core.

1. Introduction

Understanding protein stability is a challenge that requires the characterisation of interactions in the wide range of conformationally heterogeneous states of proteins [1,2]. Furthermore, protein folding thermodynamics and conformational stability are central topics in biotechnology. The use of enzymes and the optimisation of their properties require detailed knowledge about their stability and unfolding.

Little information about the general folding/unfolding aspects of aspartic proteinases is available although data obtained in this subject is of vital importance due to the significant role of these enzymes in several diseases [3].

The dogma that enzymes are not active in organic solvents has been abandoned [4] and is now known that enzymes can exhibit different characteristics, such as modified and novel specificities. Nevertheless, not all non-conventional media are equivalent and properties such as solvent hydrophobicity, hydrogen-bonding capacity, miscibility in water, and others, have profound influence on the structural integrity and catalytic activity of enzymes [5,6].

Cardosin A is a model aspartic protease, and an example of a small number of plant aspartic proteinases that have been isolated, purified and characterised [7–9]. It is heterodimeric, with two chains intertwining forming two symmetric units comprising the active site [10]. We have reported the use of cardosin A for peptide synthesis in biphasic systems [8,11–13], and studied the influence of several organic solvents on its activity and specificity. These studies revealed alterations of its selectivity that were correlated to the organic solvent present [8,14].

We have demonstrated previously that 10% acetonitrile induces a higher destabilisation to the light sub-unit of cardosin A revealing its less stable nature [15]. Nevertheless, this conformational instability resulted in no activity loss, suggesting the presence of unfolding intermediaries.

This work makes a detailed description of the effect of several organic solvents on cardosin A storage stability and conformation. Additionally, stable intermediaries of cardosin A were detected and described.

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2. Materials and methods

2.1. Enzyme, substrates, chemicals and solvents

Fresh flowers were collected from wild plants, identified as *Cynara cardunculus* L. After collection stigmas were immediately frozen (−20 °C) until enzyme purification.

Organic solvents (*n*-hexane, ethyl acetate and isooctane) were purchased from Sigma (purity higher than 99%). Acetonitrile (HPLC grade) was purchased from Romil. All other chemicals were of analytical grade and were obtained from Sigma or Amresco.

2.2. Enzyme purification

Cardosin A purification was according to Sarmento [8]. Cardosin A purity was assessed by SDS-PAGE, according to Laemmli [16]. Pure cardosin A solutions were lyophilised and the dried enzyme powders were either used immediately or stored at −20 °C.

2.3. Protein concentration determination

Protein concentration was determined by the Micro BCA protein assay (Pierce) according to the manufacturer's instructions.

2.4. Peptide synthesis

Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu was synthesised using Fmoc chemistry. The synthesised peptide was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) with a C_{18} column (Vydac) and purity verified by sequence analysis and by mass spectrometry.

2.5. Assay of enzyme activities

The proteolytic activity of cardosin A was assayed using the synthetic peptide Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu as substrate.

Reaction media were prepared adding the selected organic solvent to an equal volume of aqueous buffer and vigorously agitating. The organic phase was removed and the substrate immediately dissolved in the remaining aqueous phase. Standard enzymatic assay was made in the presence of 50 mM sodium acetate, 200 mM sodium chloride and 4% DMSO (v/v), pH 4.7. Substrate was incubated at 25 °C, and reaction started by the addition of enzyme. At selected times, aliquots were taken, added to 1.5% (v/v) trifluoroacetic acid (TFA) solution. Samples were centrifuged and applied to an automated chromatography system AKTA BASIC 10 (Amersham Pharmacia Biotech), equipped with an automatic injector, and eluted with an acetonitrile gradient (30–100%, v/v) acidified with 1 ml TFA/L in a C_{18} column. Detection was performed at 257 nm. All solvents were degassed with helium (g) prior to chromatography, which was performed at room temperature.

2.6. Thermal stability

Cardosin A (36 μg/ml) was incubated in biphasic systems constituted by an aqueous phase (sodium phosphate buffer, 100 mM pH 5.0) and an organic phase (organic solvent previously water saturated). Additionally, the thermal stability and inactivation kinetics of cardosin A were investigated in the presence of 10% (v/v) acetonitrile.

Cardosin A was incubated in the organic solvents systems at 25, 37, 45, 50 and 60 °C, in 2 ml sealed glass vials (*Tracer*), under no agitation. Enzymatic activity was periodically assayed: aqueous phase aliquots (4 μl) were withdrawn, cooled to 25 °C and added to aqueous reaction media (596 μl) containing the substrate, previously equilibrated at 25 °C.

Results are expressed in percentage of retained activity, being the maximum activity (100%) correspondent to the initial activity of cardosin A in aqueous buffer.

2.7. Size exclusion chromatography experiments

Organic solvents induced unfolding of cardosin A was analysed by size exclusion chromatography (SEC) using a Superdex 75 HR 10/30 FPLC column (GE Healthcare). The mobile phase was 10 mM sodium phosphate buffer, pH 5.0. Samples were eluted at a flow rate of 1 ml/min. All measurements were made at room temperature. The protein elution profile was monitored by recording the absorbance at 280 nm on an AKTA Basic system (GE Healthcare). All solvents were degassed with Helium prior to use.

2.8. Proteolysis of cardosin A

Limited proteolysis of cardosin A with endoproteinase Glu-C was performed using an enzyme to substrate weight ratio of 1:200 (w/w) at 25 °C. A control of cardosin A was kept at 25 °C, without the addition of any proteolytic probe. Aliquots were removed at selected reaction times from the reaction mixture and analysed by SDS-PAGE, according to Laemmli [16].

2.9. SDS-PAGE and Western blotting

Cardosin A fragments (see Section 2.8) were separated by SDS-PAGE. Protein bands were Coomassie stained. After staining, gels were scanned (FX-*710*, Bio-Rad equipment) and analysed with the *Quantity 1* (Bio-Rad) software in order to estimate protein bands molecular weights.

Alternatively, after SDS-PAGE, cardosin A fragments were transferred to a poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad) using 3-cycloheylamino-1-propanesulfonic acid (CAPS) buffer (Sigma) for 1 h at 400 mA. After transfer, proteins were Coomassie stained. Cardosin A fragment bands were hand cut and N-terminal sequencing performed on a 476A protein sequencer (Applied Biosystems). The first 10–14 residues were analysed and compared with the known sequence of cardosin A [7,10].

2.10. Data analysis

When the experimental inactivation kinetic data yielded a straight line on a semi-logarithmic plot, the data were analysed considering a first-order activity decay model. For a constant temperature, Eq. (1) expresses the relation between the initial and residual activity of cardosin A, after t (days) of incubation.

$$\frac{A}{A_0} = \exp(-kt) \quad (1)$$

In this equation, A and A_0 represent, respectively, residual and initial activity of the enzyme (mM min^{-1}), k represents the observed inactivation rate constant (d^{-1}) and t the incubation time (d). A logarithmical form of Eq. (1) and non-weighted least-squares linear regression were used to estimate the inactivation rate constants.

Biphasic inactivation is the most frequent deviation from first-order kinetics that can be graphically identified by bi-exponential activity-time decay. The series model, which assumes an homogenous enzyme population inactivating in two consecutive steps, through a modified form of the enzyme (E_M) that can have equal, higher or lower activity than the initial, native enzyme (E), results in the following inactivation scheme, $E \rightarrow E_M \rightarrow D$. It is verified that a simpler double exponential equation, assuming no inactivation

mechanistic assumptions, yields the same results for the kinetic parameters than the previous model [17]:

$$\frac{A}{A_0} = (1-\alpha)\exp(-k_1 t) + \alpha\exp(-k_2 t) \quad (2)$$

where A, A_0, and t have the same meaning as in equation 1, α represents the amplitude of the second phase of the deactivation process, in terms of the relative activity of the enzyme at the onset of this phase, k_1 and k_2 are, respectively, the observed rate constants (d^{-1}) of the first and second inactivation phases. For biphasic inactivation profiles, the only general physical interpretation that can be given is that, inactivation is controlled by different reactions in each phase and the inactivation rate in the second phase is slower.

Taking into account the above considerations, the double exponential equation was used in the present study, to describe the biphasic inactivation decays observed, with the single purposes of quantification analysis, with no assumption concerning underlying deactivation mechanisms. The rate constants were estimated by least-squares non-weighted non-linear regression.

Model adequacy to describe the experimental data was established by lack of convergence or comparison of R^2 and mean sum of squares, leading to an unambiguous choice between the two kinetic models for each experiment.

2.11. Fluorescence

Steady-state fluorescence measurements were conducted in a Jasco Spectrofluorometer FP 770. Samples (20 μg ml^{-1}) were prepared as described in *thermal stability* at the appropriate temperature (25, 37, 45, 50 and 60 °C). After incubation, samples were diluted (1:10) in 10 mM sodium phosphate buffer (pH 5.0) and protein intrinsic fluorescence immediately measured, according to Pina [18]. A fluorescence excitation wavelength of 295 nm was used to avoid the contribution of the emission of residues other than tryptophan. As control, *N*-acetyl-L-tryptophanamide (NATA) fluorescence was measured in the same conditions of cardosin A.

3. Results and discussion

In a previous manuscript we have demonstrated that cardosin A shows different activity profiles in the presence of organic solvents [12] and that these induce both activity and selectivity alterations [8,14] that are dependent on the nature of the solvent. We have now extended these studies to correlate the direct effects on the active site of the enzyme with putative conformational re-arrangements of the enzyme induced by contact with organic solvents.

Time-dependent changes in structural parameters and enzymatic activity of cardosin A in the presence of water-immiscible organic solvents (hexane and isooctane), in the presence of a slightly water soluble organic solvent (ethyl acetate) and in the presence of a miscible organic solvent (acetonitrile, at 10% concentration) were investigated. As control, the enzyme was incubated in aqueous buffer, at pH 5.0 where it shows maximum storage stability [18].

The deactivation profile of cardosin A (Fig. 1) revealed that cardosin A inactivation in aqueous buffer (Fig. 1A) follows a first-order decay model at all temperatures and allows the enzyme to remain active at 25 °C after 28 days of incubation, retaining around 80% of its initial activity. In the presence of water-immiscible organic solvents, alterations of the inactivation profiles were detected (Fig. 1B–D): stability was similar, at the same temperature, in aqueous buffer, isooctane and *n*-hexane, but decreased considerably for ethyl acetate, as revealed by the higher values of the inactivation rate constants (Table 1). The reaction rate constants were about 80-fold and 180-fold higher than those in aqueous buffer, for 25 and 37 °C, respectively, with the enzyme presenting a complete and irreversible loss of activity after 2 h at 45 °C. The alterations detected are considered irreversible since activity was determined in aqueous buffer after incubation in the presence of organic solvents (OS).

Table 1
Thermal inactivation kinetic parameters of cardosin A in systems with organic solvents at different temperatures and their 95% confidence intervals

Reaction system	Temperature (°C)	k (d^{-1})	α (%)
Aqueous buffer	25	$0.641 \times 10^{-2} \pm 0.092$	>95
	37	$1.27 \times 10^{-2} \pm 0.311$	85
	45	0.368 ± 0.029	71
	50	1.78 ± 0.194	67
Aqueous buffer with isooctane	25	$0.371 \times 10^{-2} \pm 0.811$	
	37	$1.01 \times 10^{-2} \pm 0.533$	67
	45	0.591 ± 0.074	47
	50	1.94 ± 0.461	38
Aqueous buffer with *n*-hexane	25	$1.49 \times 10^{-2} \pm 0.442$	
	37	$1.14 \times 10^{-2} \pm 0.144$	73
	45	0.562 ± 0.047	47
	50	1.38 ± 0.373	10
Aqueous buffer with ethyl acetate	25	0.523 ± 0.052	49
	37	2.29 ± 0.284	42
	45	[a]	
	50	[a]	
10% acetonitrile	25	$k_1 = 0.764 \times 10^{-1}$ $k_2 = 0.598 \times 10^{-3}$	63
	37	$k_1 = 0.516$ $k_2 = 0.804 \times 10^{-1}$	22
	45	[a]	
	50	[a]	

[a] No residual activity detectable after 2 h of incubation.

Dehydration of the protein molecule, known as being responsible for affecting enzyme activity and stability in the presence of organic solvents [19,20], is not responsible for cardosin A inactivation, since in these systems, water activity (a_w) is very close to 1. In fact, inactivation does not represent hydration changes, because the lost enzyme activity was not recovered upon transfer of the enzyme back to aqueous medium.

In most cases, at the beginning of the inactivation processes, a fast, but short-lived loss of activity occurred, followed by a slower activity decay that could be fitted to a first-order model (activity measurements at $t = 0$ were not used to estimate the inactivation rate constants, when a first-order inactivation kinetics was observed). This progressive behaviour was quantified by calculating the remaining amount of relative activity after the initial fast activity loss; the results are presented in Table 1, in terms of the relative amount of activity (%) that remained after this short initial period of activity decay. This was done by extrapolating the straight line obtained in the linear regression to $t = 0$.

Nevertheless, in the presence of 10% acetonitrile the activity decay clearly followed a biphasic profile (noticeably observed, at 37 °C), which was accurately fitted by a biphasic inactivation model (Fig. 1E).

The gradual change observed for α for each reaction system studied (representing the amplitude of the second phase of the deactivation process) indicates a gradual change of the inactivation mechanism towards a biphasic one that involves the presence of a stable intermediary. Furthermore, it was verified that the experimental inactivation data for *n*-hexane and ethyl acetate could also be fitted by the biphasic model. The values estimated for k_2 and α were comparable, respectively, with the values of k obtained by the first-order model. This means that when a first-order kinetics was assumed, k corresponds to k_2, whilst the first phase could not be quantified due to its rapidity and the lower amount of activity being lost (higher α).

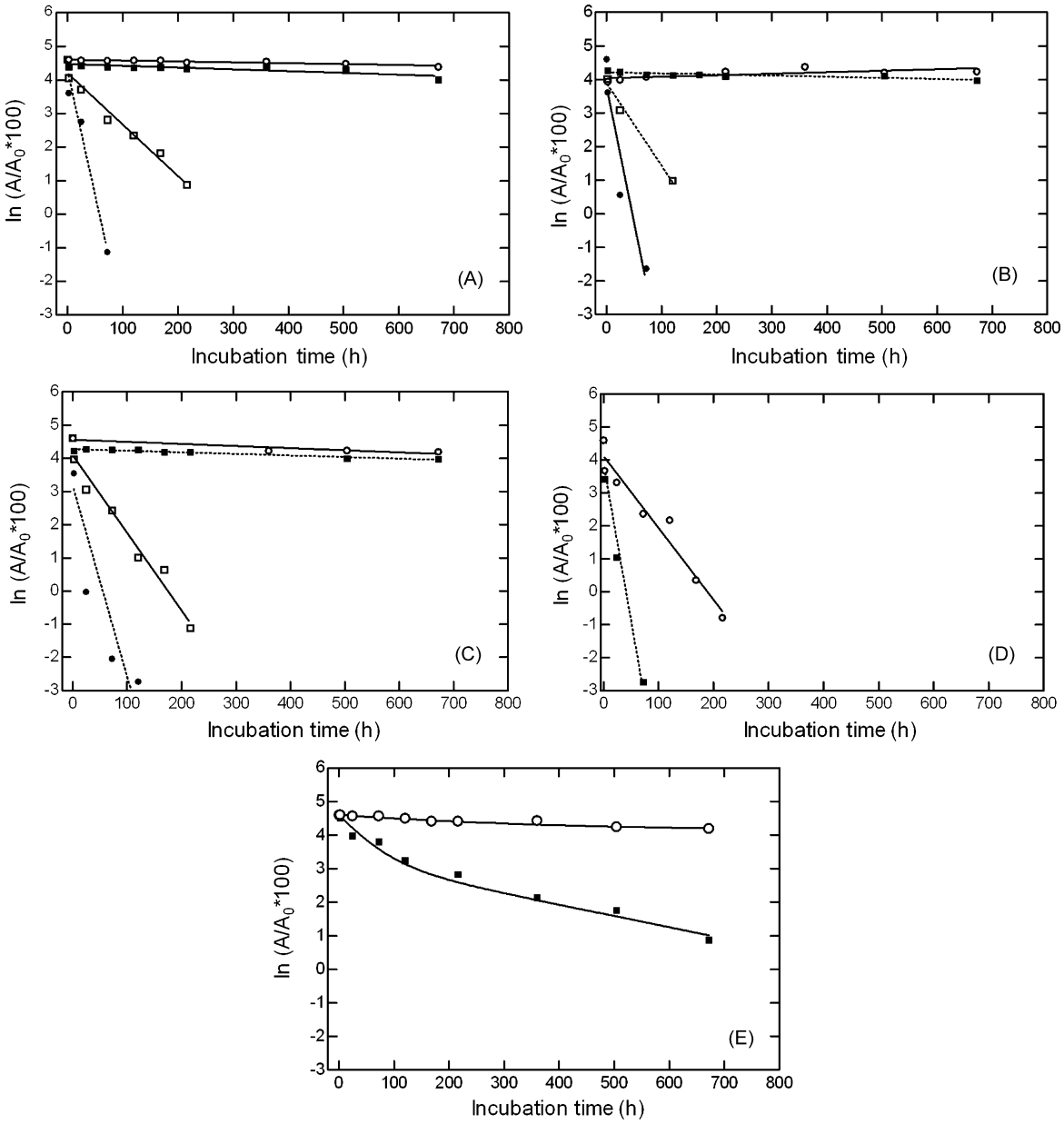

Fig. 1. Heat inactivation kinetics of cardosin A at 25 °C (○), 37 °C (■), 45 °C (□) and 50 °C (●) and model curves considering a first-order kinetics. Reaction media are: (A) aqueous buffer, (B) aqueous buffer saturated with isooctane, (C) aqueous buffer with saturated *n*-hexane and (D) aqueous buffer saturated with ethyl acetate. (E) Heat inactivation kinetics of cardosin A in 10% acetonitrile at 25 °C (○) and 37 °C (■) and model curves considering a biphasic kinetics.

A similar progressive change in inactivation kinetics, from a linear decay to a biphasic one, has been previously observed for the inactivation of horseradish peroxidase in water and phosphate buffer solutions of different ionic strength [21].

Such a change in inactivation kinetics can be explained by a series-type model of inactivation, that assumes that enzyme denaturation occurs by means of a modified enzyme, that is to say via an intermediary form of the denaturation process. In fact, it is known that inactivation proceeds through a network of reactions (that include conformational modifications, dissociation, denaturation, aggregation, coagulation and chemical decomposition) and that, frequently when apparent first-order behaviour is observed the rate of the inactivation is the rate at which the slowest, rate-limiting step of these phenomena occurs [22].

An analysis on conformation and activity changes of cardosin A during inactivation by organic solvents showed that the loss of activity occurs before any alterations of conformational changes can be detected by fluorescence measurements. In the presence of ethyl acetate inactivation is triggered by solvent contact (Fig. 1D). However, at 25 °C, changes in the intrinsic fluorescence of cardosin A were detected only after 8 h of incubation (Fig. 2). Even after approximately 70 h of incubation, the red shift of the tryptophan fluorescence spectrum maximum of cardosin A in the presence of ethyl acetate is only of 2 nm (still very far from the spectrum position of free tryptophan in water—approximately 355 nm), but the activity reduced to 11% of the initial value. At this point it should be stated that NATA fluorescence measured in the same conditions as cardosin A show no difference between aqueous and organic medium (data not shown). Therefore, the differences found correspond to conformational alterations of the protein.

At 37 °C conformational changes in the tertiary structure of cardosin A were detected, after incubation not only in ethyl acetate

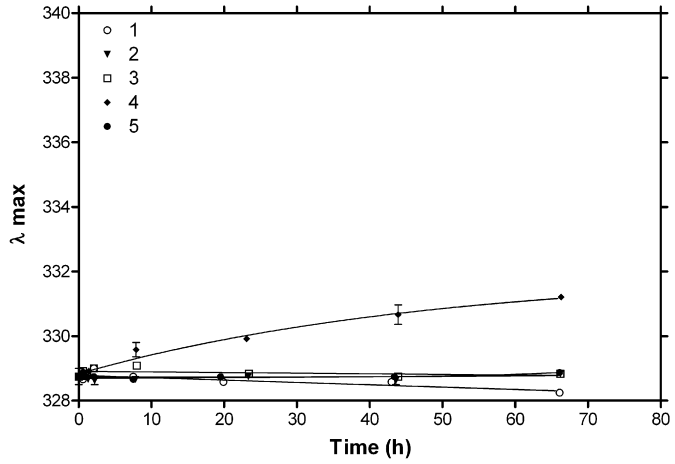

Fig. 2. Intrinsic fluorescence of cardosin A incubated in aqueous medium (1), in biphasic systems composed by an aqueous phase and isooctane (2), *n*-hexane (3), or ethyl acetate (4) and in 10% acetonitrile (5) at 25 °C. Measurements were made after dilution in aqueous buffer.

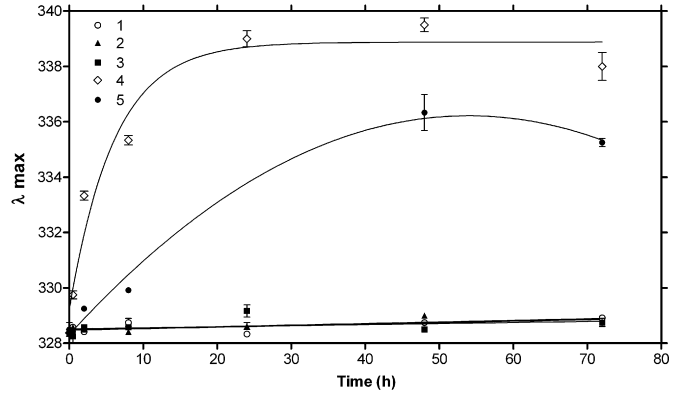

Fig. 3. Intrinsic fluoresce of cardosin A incubated in aqueous medium (1), in biphasic systems composed by an aqueous phase and an organic phase of *n*-hexane (2), isooctane (3) or ethyl acetate (4) and in 10% acetonitrile (5) at 37 °C. Fluorescence measurements were made after dilution in aqueous buffer.

but also in acetonitrile (Fig. 3). In contrast, incubation of cardosin A in aqueous buffer or in the presence of *n*-hexane or isooctane produced no tryptophan fluorescence alterations, as occurred with the inactivation profiles in these media (Fig. 1). In the presence of ethyl acetate (at 37 °C), a red shift of the tryptophan fluorescence spectrum maximum from 328 to 340 nm was detected whilst incubation in 10% acetonitrile produced a smaller red shift (from 328 to 337 nm), after 70 h of incubation. This means that ethyl acetate and acetonitrile induce different conformational alterations on the vicinity of the tryptophan residues.

The results show that apparently the initial stage of the inactivation of cardosin A is not accompanied by global conformational alterations, as shown by fluorescence. This stage corresponds to the initial fast and short decay of activity (since they occur in the same time scale), which becomes progressively more detectable for the less hydrophobic solvents. After this initial stage, where a rapid decrease of activity takes place, conformational modifications lead to further inactivation of the enzyme. Nevertheless, the intrinsic fluorescence of the protein reveals that even in this state a significant proportion of the tryptophan residues are still localised inside the protein structure. That is to say that the enzyme retains partially folded domains. This is fully consistent with our previous investigation [21] where even after thermal denaturation cardosin A retains residual structure showing an emission fluorescence of 347 nm.

Ten percent acetonitrile induces a lower change in the intrinsic fluorescence of cardosin than ethyl acetate even after 70 h of incubation (37 °C).

In acetonitrile the first phase of inactivation is slower, giving rise to a clear biphasic profile. These data are in agreement with the lower value for k_2 determined for acetonitrile.

In order to further investigate the effect of these solvents on the conformation of cardosin A, the hydrodynamic volume of the enzyme in the presence of OS was evaluated using size exclusion chromatography on a G-75 Superdex column (Fig. 4). For native cardosin A, a single peak at 25.9 ml was observed, corresponding to an estimated molecular mass of 38.8 kDa, consistent with the predicted molecular weight of native cardosin A [7]. At this point it should be stated that the apparent lack of agreement between the molecular weight of native cardosin A, as indicated by gel filtration and by SDS-PAGE, results from abnormal mobility of the protein in the gel. Such anomalous behaviour has already been reported for other glycoproteins [23].

As can be seen (Fig. 4A), the incubation of cardosin A in 10% acetonitrile, did not alter the hydrodynamic volume of the protein.

On the other hand, in the presence of ethyl acetate there is rapid loss of peptide chain integrity, as seen in Fig. 4B. After 2 h of incubation, at 37 °C, in the presence of ethyl acetate, less native cardosin A is recovered and both high and low molecular weight chromatographic protein pools are detected. The high molecular weight protein pools correspond to cardosin A aggregates. The low molecular weight protein pools are probably due to chemical degradation of peptide bonds induced by temperature [24] or due to autolysis processes (prior to inactivation) as described previously for thermolysin that inactivates in the presence of ethyl acetate mainly by autolysis [25]. In fact, incubation of cardosin A at 100 °C produces low molecular weight peptides (Fig. 4C) with the same elution volumes as those produced by inactivation of cardosin A by ethyl acetate monitored by gel filtration.

Susceptibility to proteolysis has been shown to be a sensitive method to detect subtle conformational changes of protein

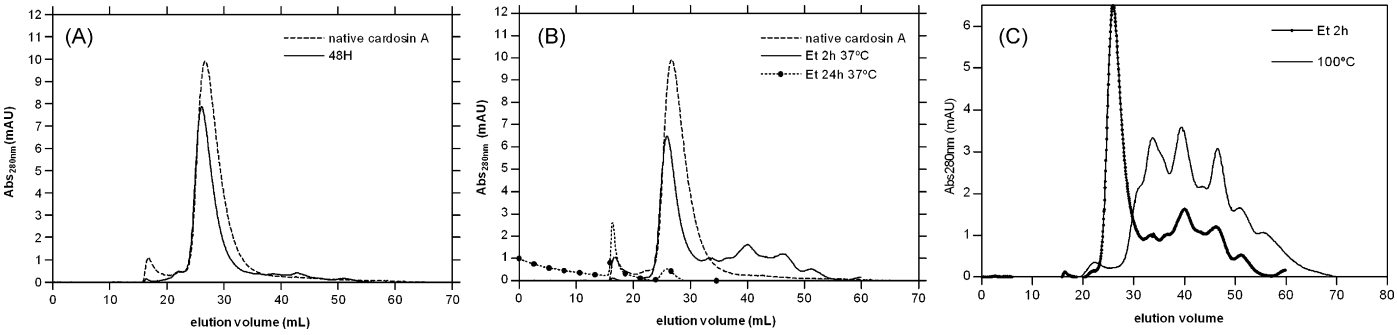

Fig. 4. Elution profiles of cardosin A incubated, at 37 °C, in the presence of 10% acetonitrile (A), ethyl acetate (B) and (C) of cardosin A thermo-inactivated (100 °C for 5 min).

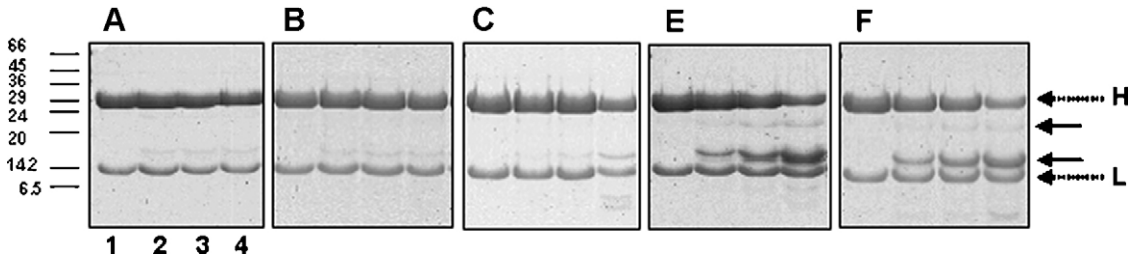

Fig. 5. SDS-PAGE of cardosin A incubated with endoproteinase Glu-C, at 25 °C in: (A) 50 mM ammonium bicarbonate, (B) aqueous buffer with isooctane, (C) aqueous buffer with *n*-hexane, (E) aqueous buffer with ethyl acetate and (F) aqueous buffer with 10% acetonitrile. Reaction times were: (1) 0 h (prior to proteolysis), (2) 0.5 h, (3) 1 h and (4) 3 h. Arrows represent sequenced hydrolysis fragments of cardosin A. H-cardosin A heavy sub-unit and L-cardosin A light sub-unit.

Table 2
N-terminal sequences of cardosin A fragments obtained by limited proteolysis

Fragment (kDa)	N-terminal sequence	Fragment sequence
22.6	SSDSSTYKEN	59–239
16.5	QDFIEATDEAD	100–239

Cardosin A was treated with V8 at 25 °C for 24 h, analysed by SDS/PAGE, blotted, and protein sequenced.

molecules [26]. Therefore, in order to obtain further insights into the events that take place during the first phase of cardosin A inactivation limited proteolysis was carried out. This technique allows assessing to subtle conformation alterations of the protein as well as to local residue mobility modifications.

Limited proteolysis of cardosin A by endoproteinase Glu-C (V8) in aqueous buffer occurs at one peptide bond giving rise to one peptide detected by SDS-PAGE with estimated weight of 16.5 kDa (Fig. 5).

The same trend of proteolytic susceptibility of cardosin A was obtained both in the presence of *n*-hexane and isooctane (Fig. 5), suggesting that, from all putative cleavage sites, this peptide bond is the only one that is accessible to the proteolytic probe. In contrast, in the presence of ethyl acetate and in the presence of 10% acn one additional fragment, of about 22.6 kDa, was found. The 16.5 kDa fragment was also detected at higher amounts, revealing higher susceptibility to proteolysis. These fragments were identified by N-terminal sequencing (Table 2). Sequencing analysis of the 16.5 kDa fragment identified the sequence QDFIEATDEAD, corresponding to the first 11 amino acids of the fragment originated by E_{99}–Q_{100} peptide bond hydrolysis which, in the crystal structure of the enzyme, is located in a N-terminal portion of a β-sheet and characterised by a certain residue mobility (Figs. 6 and 7), in cardosin A heavy chain [10]. The 22.6 kDa fragment is the result of the accumulation of the C-terminal fragment originated by E_{58}–S_{59} peptide bond hydrolysis, of the 31 kDa polypeptide chain of cardosin A. This peptide bond is located in a linkage region of a α-helix and a β-sheet and characterised by low residue mobility in cardosin A crystal structure [10]. Ethyl acetate and acetonitrile induced destabilisation of the protein, leading to an increase of the number of peptide bonds

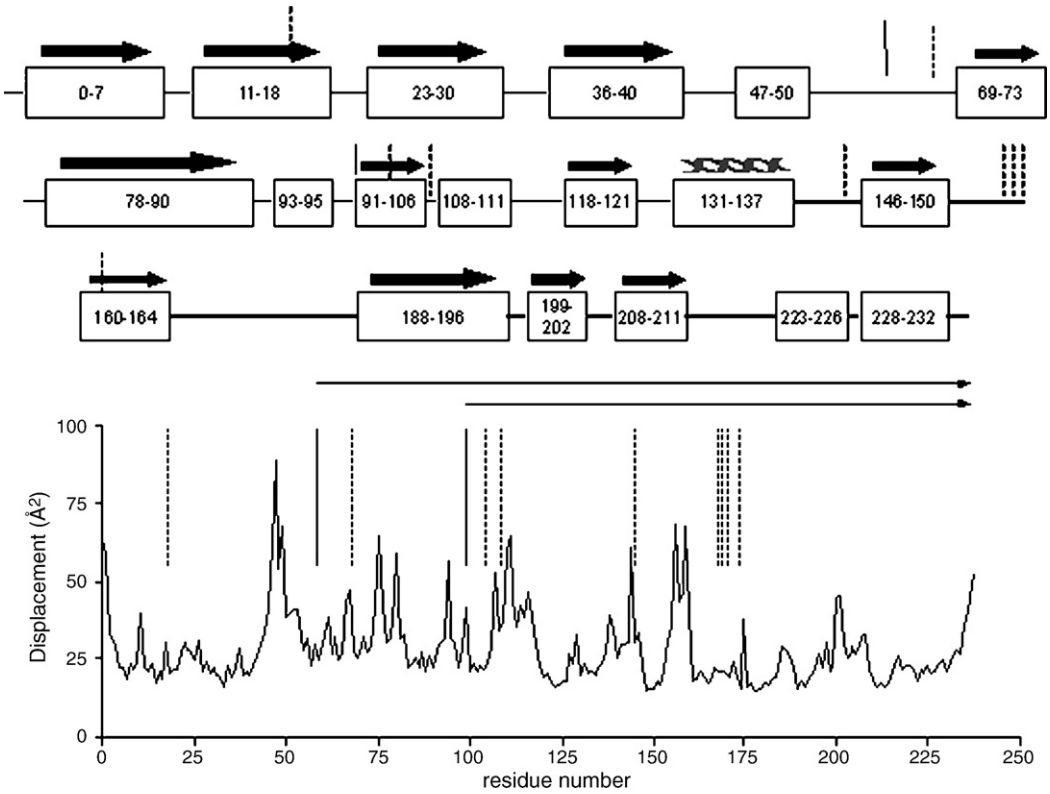

Fig. 6. Temperature factor profile for cardosin A. Mean residue factors (averaged over all backbone atoms) are plotted along the amino acid sequence of the 31 kDa polypeptide chain. Data was retrieved from crystallographic values of cardosin A (PDB: **1B5F**). Boxes at the top of the figure indicate the presence of segments of secondary structure (helices and strands). All putative V8 nick sites are indicated by dashed lines and the actual nick sites cut by the enzyme are indicated by full lines.

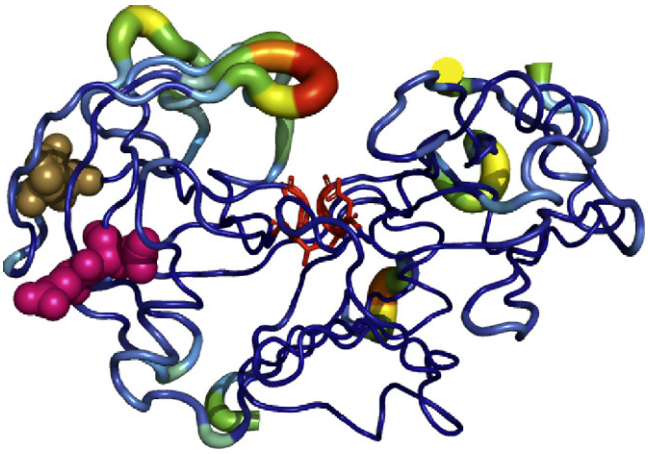

Fig. 7. Cartoon representation of cardosin A. The active site aspartates side chains (red) are depicted as ball-and-stick representation. The limited proteolysis sites are represented in pink (first cleavage site) and brown (second cleavage site). Cartoon was generated by Pymol [29]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

cleaved by V8. This effect was more pronounced for ethyl acetate than for acetonitrile, in agreement with the higher destabilisation induced by ethyl acetate as seen with intrinsic fluorescence and size exclusion chromatography.

Cardosin A conformational determinants (Fig. 6) can help to understand the reasons why some sites of limited proteolysis are preferential towards others. The fact that 16.5 kDa fragment is produced in all media is related to the fact that this region not only is exposed to the solvent but also has some mobility (Figs. 6 and 7). In the presence of ethyl acetate and acetonitrile this proteolytic susceptibility increases and faster accumulation of this fragment was observed.

On the other hand, the 22.6 kDa product (resulting from E^{58}–S^{59} peptide bond hydrolysis), is located in a region of low relative mobility (Fig. 6). The low mobility of this peptide bond justifies the absence of hydrolysis in aqueous conditions, since it is only relatively exposed to the solvent [10]. Therefore, the presence of ethyl acetate and acetonitrile molecules in the system apparently leads to an increase of residue mobility.

Our results show that there is a general trend where the most hydrophobic organic solvents induce less damage to the catalyst. This can also be viewed in terms of solubility since more hydrophobic solvents are less soluble in the aqueous phase and therefore less solvent molecules can interact with the protein. Ethyl acetate induced faster inactivation of the enzyme but in the presence of acetonitrile, cardosin A inactivation is slower and originated a clear biphasic profile, possibly, by the presence of stable intermediaries.

4. Conclusions

Knowledge on protein folding/unfolding and of denaturation is of great significance for the biotechnology industry as it has a direct effect on the overall stability of an enzyme and therefore on the commercial viability of any process. In what concerns aspartic proteinases, which are of physiological, commercial and pathological relevance, very few studies have been performed on the stability of this family. Studies on pepsin [27,28] have demonstrated the formation of inactive intermediaries during pH-induced denaturation that exhibit native-like characteristics.

Using organic solvents, we have demonstrated that the dynamic structure of cardosin A was perturbed by these molecules, resulting in small but clearly detectable local structural changes. Indeed, it is known that intermediaries can be detected by studying protein denaturation by several techniques sensitive to different distinct structural features. Increased conformational instability of the molecule, when in the presence of ethyl acetate and acetonitrile, was demonstrated by limited proteolysis. Limited proteolysis also assigned this area of enhanced mobility to a specific region (centred on residues 99–100) of the heavy sub-unit revealing that in the presence of organic solvents, this particular region of cardosin A shows a much faster, highly localised, rate of conformational change and therefore less stable.

Data presented in this work indicate that inactivation of cardosin A in the reaction systems studied proceeds through a biphasic mechanism, which is incipient in buffer and becomes progressively more detectable for the less hydrophobic solvents, reaching a clear and quantifiable biphasic profile in acetonitrile. With increasing hydrophilicity of the organic solvent, the second phase of inactivation accounts, progressively, for a lower relative amount of activity being lost (lower α), being the intermediary more stable in acetonitrile (lower k_2) than in ethyl acetate. The first inactivation phase corresponds to an increase of the enzyme mobility (as seen by limited proteolysis) and (at least in some cases) to a loss of protein integrity (involving phenomena such as aggregation and rupture of peptide bonds) as seen by size exclusion chromatography. All these events lead to the formation of a stable intermediary. On the other hand, complete inactivation occurs in the second phase and involves global conformational alterations that were detected by fluorescence.

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