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Non-native states of cardosin A induced by acetonitrile: Activity modulation via polypeptide chains rearrangements

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ABSTRACT

Cardosin A (EC: 3.4.23) is an enzyme containing two polypeptide chains, purified from pistils of *Cynara cardunculus* L., a cardoon, used for milk clotting in cheese making. It is a member of the aspartic proteinases (APs), like pepsin and HIV-proteinase that are composed by two symmetric units comprising the active site. Cardosin A is thought to be involved in many cellular events such as in pollen–pistil interaction and adhesion dependent recognition mechanisms. In the present study, the structural and activity effects of different amounts of acetonitrile (ACN) in cardosin A are presented. The results indicate that low ACN concentrations (up to 10% ACN) reversibly stimulate the enzyme activity accompanied by slight secondary structure induction. In light of the structural and stability studies performed so far, cardosin A can adopt conformational alterations that can result in activity modulation via polypeptide chains rearrangements.

1. Introduction

Non-native states of proteins exist in living cells and can be involved in physiological processes and in neurodegenerative and metabolic diseases [1]. Non-native states consist in distinct protein conformational states induced by changes in the protein's environment that may reflect different or altered molecular roles. There have been several studies on protein structure–function relationships but so far there is no general answer of how protein function follows structure [2,3].

Aspartic proteinases (APs) are involved in different pathological and physiological processes [4]. Despite their function diversity they have homologous N- and C-terminal domains, arisen by ancient internal gene duplication and fusions [5]. Most structure–function relations have been investigated for pepsin, an archetypal, monomeric AP with two homologous lobes enclosing the active site [6,7]. Defining the structural–activity relations of APs should shed light in what modulates protein function both in physiological and in pathological states.

Cardosin A is an abundant AP from *Cynara cardunculus* L. that has been well characterised [8,9]. High yields of pure protein can be easily obtained making it an attractive model protein [10]. The structural stability of cardosin A at different pH values, temperatures and in 10% (v/v) ACN has been studied: it remains active in the pH range 2.5–7.5, has maximum structural stability at pH 5.0 and undergoes non-cooperative, partially reversible, thermal denaturation caused by independent unfolding of its polypeptide chains. Here, the 15 kDa polypeptide chain unfolds first, followed by the heavier and more stable chain (31 kDa) [11]. In 10% (v/v) ACN, cardosin A is less stable than in water with the small chain also unfolding first, while displaying remarkable activity enhancement (up to 70%). All this is accompanied by transition to an α/β ($\alpha+\beta$) protein [12]. Also in a recent report [13] the inactivation in 10% (v/v) ACN was shown to be biphasic, with the stabilization of unfolding intermediaries. The first inactivation phase was shown to affect the active site cleft and global protein mobility, probably due to weakening of hydrophobic interactions, with little alteration of global protein conformation. Finally the second inactivation phase was reported to affect global conformation resulting in loss of activity.

The physiological role of cardosin A is yet to be completely clarified. It was shown to accumulate in protein storage vacuoles of the stigmatic papillae and epidermic cells of the style [14,15]. Also cardosin A associates with the C_2 protein domain from phos-

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pholipase D_α (PLD_α), a putative cardosin A-binding protein from pollen, suggesting concerted and/or synergistic actions in degenerative processes during stress responses, plant senescence and/or pollen–pistil interactions [16]. However, how this can be accomplished *in vivo* is still to be elucidated.

In this investigation the biophysical characterisation of cardosin A in the presence of increasing amounts of ACN is presented as an endeavour of understanding the structure–function relationship and the folding–unfolding mechanism. The results obtained are discussed according to the different polypeptide chains conformational flexibilities and their involvement in non-native states.

2. Materials and methods

2.1. Enzyme, substrates, chemicals and solvents

Cardosin A was purified from fresh flowers collected from wild plants, identified as *C. cardunculus* L. ACN (HPLC grade) was from Romil, trifluoroacetic acid (TFA) was from Merck and N-acetyl-L-tryptophan ethyl ester (ATrEE) was from Sigma. All other chemicals were of analytical grade and obtained from Sigma or Amresco. Synthetic peptide was kindly provided by Dr. Arthur Moir (Sheffield University, UK).

2.2. Enzyme purification

Cardosin A was purified by an optimised two-step procedure described previously [10]. Purity of cardosin A was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [17] after staining with Coomassie Brilliant Blue. Cardosin A solutions were concentrated by lyophilization on a Flexy-Dry lyophilizator.

2.3. Protein concentration determination

For activity and fluorescence assays, cardosin A concentration was determined by the BCA™ protein assay (Pierce) according to the manufacturer's instructions and using bovine serum albumin for preparation of the calibration curve. For circular dichroism (CD) experiments, concentration was determined spectrophotometrically at 280 nm, using a molar absorption coefficient of $43.8 \times 10^3\ M\ cm^{-1}$ [18] for cardosin A and of $1.48\ M\ cm^{-1}$ at 300 nm for the substrate [19].

2.4. Catalytic activity

Assays (triplicates) were performed in a reaction mixture containing the synthetic peptide Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu in 50 mM sodium acetate buffer with 200 mM sodium chloride and 4% dimethyl sulfoxide (v/v), pH 4.7 with different ACN concentrations, at 25 °C. Cardosin A solutions were incubated for 1 h in reaction buffer without dimethyl sulfoxide with the appropriate ACN concentrations. Rate of hydrolysis was followed by RP-HPLC as previously reported [13]. For reactivation studies cardosin A was diluted (200-fold) in aqueous buffer (1 h at 25 °C) and assayed for activity.

2.5. Fluorescence studies

Steady-state fluorescence of cardosin A was recorded in a Jasco Spectrofluorometer FP 770 fluorimeter as described before [11]. To study the reversible nature of ACN induced changes in secondary structure, cardosin A was diluted (200-fold) in aqueous buffer (1 h at 25 °C) and spectra taken [20].

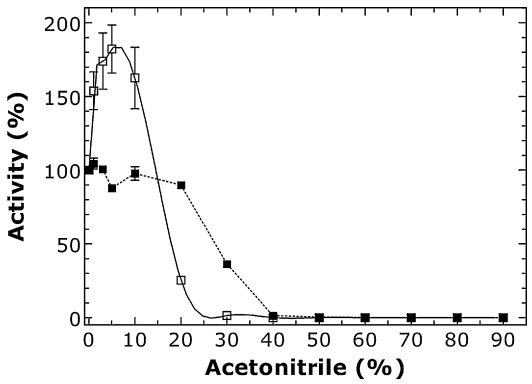

Fig. 1. Activity of cardosin A in the presence of increasing ACN concentrations. Residual activities were measured after 1 h incubation at 25 °C in the presence of the solvent (open squares) and after 200-fold dilution of ACN in aqueous buffer (closed squares).

2.6. Circular dichroism studies

Circular dichroism (CD) experiments were performed either on a F-4010 Hitachi or a Jasco J-720 Spectropolarimeter under constant nitrogen flow. Cardosin A (in the concentration range 2.38×10^{-4} to 47.6×10^{-4} mM) was incubated in 10 mM sodium phosphate buffer, pH 5, with increasing ACN concentrations for 1 h at 25 °C before spectra were taken. For the far-UV region, spectra were recorded using a 0.1 cm cylindrical path length cell, at a scan speed of $50\ nm\ min^{-1}$, with a 2 nm bandwidth and 1 s integration time. Spectra were measured four times, averaged and corrected by subtraction of the solvent spectrum obtained under similar conditions. Results are expressed in molar ellipticity, $[\Theta] = 10 M_{res} \Theta_{obs}\ l^{-1}\ p^{-1}$, where M_{res} is the mean residue molar mass; Θ_{obs} is the ellipticity (°) measured at wavelength λ; l is the optical path-length of the cell (dm) and p is the protein concentration ($mg\ mL^{-1}$).

2.7. Hydrodynamics

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 with the required ACN concentrations. All measurements were made at room temperature. The protein elution (at a flow rate of $1\ mL\ min^{-1}$) was monitored at 280 nm on an AKTA Basic system (GE Healthcare). All solvents were degassed with Helium (g) prior to use. The apparent molecular weights of cardosin A were calculated using a gel filtration LMW calibration kit (GE Healthcare) according to the manufacturer's instructions.

3. Results and discussion

The effects of increasing concentrations of ACN on cardosin A activity were investigated (Fig. 1). A bell shaped increase of the catalytic activity was observed in the concentration range of 0–10% (v/v) ACN. Above this value, the catalytic activity decreased drastically followed by complete inactivation at around 30% (v/v) ACN.

Usually, addition of small amounts of a water-miscible solvent has little effect on the biocatalyst activity and stability. However, under certain conditions enzyme activity can be induced as it is seen in this work with ACN concentrations up to 10% (v/v), although no general explanation is accepted [21–24].

Concerning reversibility, in Fig. 1 it is shown that after incubation with up to 20% ACN, cardosin A activity can be recovered after dilution in solvent free medium. This shows that in the 0–10% (v/v) ACN range the activity enhancement is caused by a reversible change of the protein. Above 20% (v/v) ACN, activity is

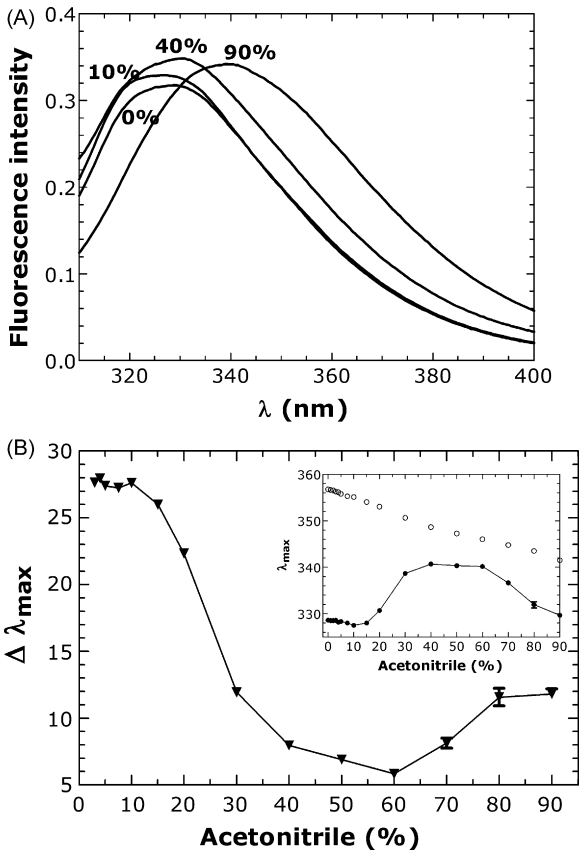

Fig. 2. Cardosin A ACN induced effects monitored by intrinsic fluorescence. (A) Fluorescence intensity spectra at 0, 10, 40 and 90% (v/v) ACN. (B) Normalized $\Delta\lambda_{max}$. $\Delta\lambda_{max} = \lambda ATEE_{max} - \lambda E_{max}$, where $\lambda ATEE_{max}$ and λE_{max} represent maximum wavelength of ATrEE (inset, open circles) and of cardosin A (inset, closed circles), respectively.

not fully recovered progressively leading to complete irreversibility at 40% (v/v), showing that cardosin A activity was lost by irreversible denaturation at this solvent concentration. In between the reversible and irreversible cardosin A denaturation, around 20% ACN, lies the transition midpoint of ACN induced effects as seen by residual activity measurements.

In order to correlate solvent-induced alterations in enzymatic activity with alterations in cardosin A structure, fluorescence and CD analyses were carried out after 1 h incubation with increasing solvent concentrations. Intrinsic fluorescence of proteins is a very sensitive indicator of the microenvironment of tryptophan residues. Cardosin A has five tryptophan residues, four belonging to the heavy chain and one to the light polypeptide chain [9]. To rule out tryptophan fluorescence dependence on solvent polarity, the fluorescence of the enzyme and of the model tryptophan derivative ATrEE [25] was compared (Fig. 2). In the inset of Fig. 2B it can be seen that the λ_{max} for ATrEE decreases steadily with increasing ACN content. After data normalization results showed that the addition of ACN did not change continuously the emission maximum wavelength. For mild concentrations of the organic solvent, up to 10% (v/v), no change of the tryptophan environment in cardosin A was seen. On the other hand, above 15% and up to 40% (v/v) ACN, a pronounced change in λ_{max} occurred, representing an exposure of cardosin A tryptophan residues towards a more polar environment, indicative of a progressive unfolding of the molecule. Further, between 40 and 60% (v/v) ACN the red shift of the emission of tryptophan fluorescence spectrum maxima progressively reaches a minimum even though complete tryptophan exposure was not observed. This can clearly be observed in Fig. 2B, where at 60% (v/v) ACN $\Delta\lambda_{max}$ is low, but still far from zero. A $\Delta\lambda_{max}$ close to zero reveals a tryptophan environment similar to that of free ATrEE, indicating that the tryptophan residues should be fully in contact with the solvent as a result of unfolding of the protein. Above 60% (v/v) ACN, the λ_{max} decreases to values closer to that of native enzyme, as indicated by the increase in $\Delta\lambda_{max}$. This reveals a rearrangement of tryptophan residues in cardosin A expressive of some packing of the protein chains.

Further examination of the effects of ACN was carried out by CD that, in the far-UV region, is particularly sensitive to protein secondary structure. The far-UV CD spectrum of native cardosin A at pH 5 [11] shows a broad minimum at 217 nm characteristic of β-structure-rich proteins [26]. In this work, far-UV CD measurements of cardosin A in 0–70% (v/v) ACN were carried out. Above 70% (v/v), no reliable spectroscopic measurements were possible due to protein aggregation or precipitation (data not shown). Analysis of spectra (Fig. 3A) revealed two distinct ACN induced effects. At higher ACN concentrations, a gradual decrease of the positive band (at ca. 195 nm) and a decrease of the negative band (at ca. 216 nm) were seen, indicating progressive loss of secondary structures. On the other hand, the spectrum of cardosin A in 5% (v/v) ACN shows an increase, even though small, of the negative band of 216 nm. Furthermore, it shows a positive band at 195 nm similar to spectra of the native enzyme, indicating a slight change in secondary structures. This change in secondary structures can be observed in the range 1–10% (v/v) ACN (Fig. 3B) where catalytic activity enhancement was detected. These observations taken together with the results obtained for cardosin A in 10% ACN, by Shnyrova et al., that suggested an increase in protein helicity [12], indicate that a rearrangement of cardosin A structure is associated with the activity enhancement. Concerning solvation phenomena in systems containing organic solvents, substrate solvation has seen to induce activity alterations [27] However, in the work presented here such

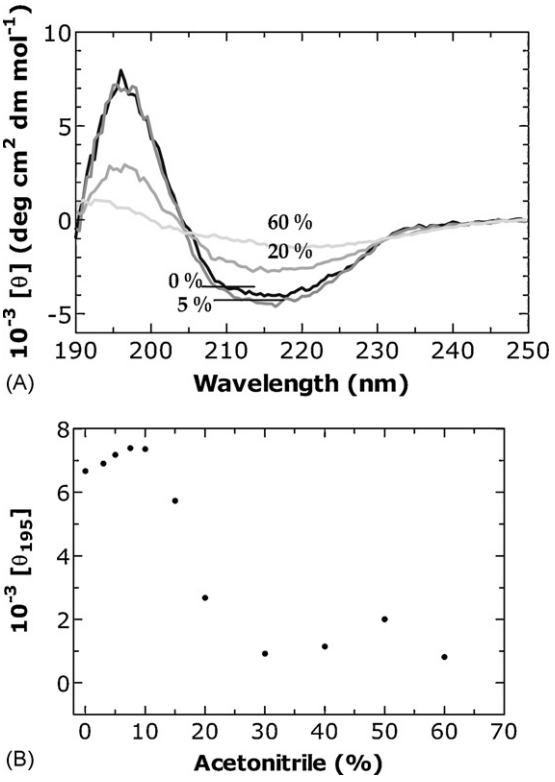

Fig. 3. Cardosin A ACN induced effects monitored by CD. (A) Cardosin A far-UV CD spectra after 1 h incubation at 25 °C. (B) CD signal intensity observed at 195 nm upon change of the % (v/v) ACN.

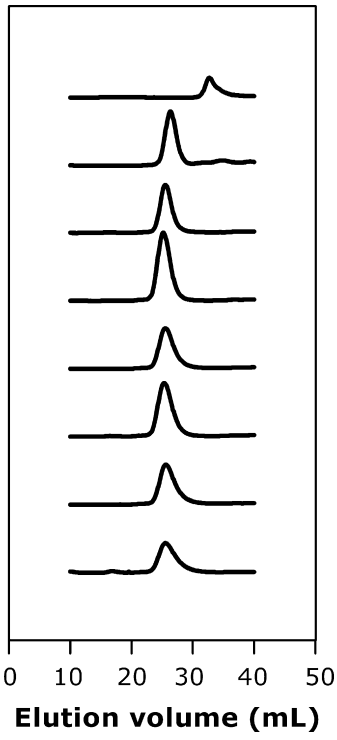

Fig. 4. Size-exclusion chromatographic elution profiles of cardosin A at different ACN concentrations. (A) Elution profiles of cardosin A. From bottom to top, ACN concentrations are 0, 2, 4, 5, 7, 10, 20 and 50% (v/v). (B) Elution volumes of cardosin A as a function of ACN concentration.

effect is not expected due to the hydrophobicity of the reaction media that contains 4% dimethyl sulfoxide (v/v), allowing full substrate solvation.

Since cardosin A polypeptide chains are held together by hydrogen bonds and hydrophobic interactions [9], with no covalent bonds involved, it seemed relevant to investigate if the increase of medium hydrophobicity (caused by solvent addition) could induce chain separation and thereby hinder activity at higher ACN concentrations. Hence, the apparent hydrodynamic molecular volume of cardosin A was analysed by SEC. Native cardosin A, with its two polypeptide chains intertwining (Fig. 4), elutes as a single peak with an elution volume (V_e) of 25.5 mL, corresponding to a 38.8 kDa molecule as already determined by Sarmento et al. [13]. When the ACN concentration was increased up to 20% (v/v), no shift in the V_e was observed. Therefore the changes in tertiary and secondary structure detected by fluorescence and CD induced by ACN did not change cardosin A hydrodynamic volume. This means that the rearrangement of tryptophan residues in cardosin A and the changes in secondary structure content occur without a transition to a more compact or expanded intermediate, suggesting a differently packed structural core. At 50% (v/v) ACN, where cardosin A is irreversibly inactivated, the peak is shifted to a higher V_e of 37.2 mL. Above 50%, data are not reliable, due to the lack of peak symmetry and to an increase of interactions of cardosin A with the matrix. Results obtained suggest two different ACN effects in cardosin A, at lower (0–20%) and at higher (30–60%) ACN concentrations. Cardosin A at 30–60% (v/v) of ACN remains inactive after solvent removal, suggesting permanent alterations in the molecule that compromise the catalytic function. This decrease in activity was accompanied by important changes in the spectroscopic properties of the enzyme. Fluorescence studies pointed to gradual dislocation of tryptophan residues towards more polar environments. The coincidence in activity loss accompanied by the spectral perturbation indicates protein denaturation as the cause of threshold inactivation of cardosin A. A pronounced reduction in hydrophobic interactions due to the addition of a water-miscible solvent must be involved with the non-cooperative protein unfolding. In the absence of such important interactions, the stability of a molecule is expected to be reduced. Similar cases have been reported, for lysozyme [28] and for ervatamin C in the presence of ACN [3]. Moreover, cardosin A SEC experiments showed that ACN addition does not affect notably cardosin A chain interactions, hydrophobic and hydrogen bonding. At lower ACN concentrations (0–20%) the effects on activity induced by the solvent are reversible, after washing out the organic solvent, showing cooperative unfolding. The activity enhancement reported was accompanied by a change in secondary structures, but not by important changes in the tertiary structure (Fig. 2). Furthermore, no differences in hydrodynamic volume were found in this range of solvent concentrations (Fig. 4) indicative of subtle molecular rearrangements in cardosin A. This indicates that cardosin A is able to adopt distinct and thermodynamically active conformations that can be explained by solvent contact. In fact, it has been reported for cardosin A [13] and thermolysin [29] that binding of ACN molecules to the enzyme active site and other regions of the molecule can take place, inducing slight changes in secondary structure with an increase in flexibility. These time dependent activity fluctuations were concomitant with a gradual increase in protein helicity, accompanied by changes in the tertiary structure.

4. Conclusions

The independent unfolding of cardosin A chains can be compared with the available data of some APs unfolding processes. Regarding pepsin, a single chain enzyme considered the archetypal AP, alkaline inactivation is due to a selective denaturation of its N-terminal lobe, with evidence for higher C-terminal stability [30,31]. This independent unfolding has already shown to play a physiological role in pepsin. It was hypothesized that taking advantage of the pH gradients inside the crypts of the stomach and of the transit of pepsin from the parietal cells, pH induced intermediate sates in pepsin participate in transportation to the stomach lumen and allow for pepsin activity regulation [7]. The physiological role of cardosin A is not well known [14]. However, the protein interacts with pollen phospholipase (PLD_α) C_2 domain via RGD and KGE domains [16]. Its association with PLD_α could facilitate disintegration of the vacuoles in the dismantling phase of a vacuolar-type cell death, but the way this is accomplished *in vivo* remains to be elucidated. Having in mind data obtained previously [11,12] and data presented here, the different chain stabilities and the pH tolerance range (pH 2.5–7.5) can be the result of an adaptation to multiple physiological functions in specific environments.

Acknowledgments

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