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Cardosin A as a model aspartic proteinase for the study of organic solvent effects An overview on catalytic and structural aspects

A.C. Sarmento [a,*], C. Oliveira [a], E. Pires [b], P. Halling [c], M. Barros [a]

[a] *Cell Biology Centre, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal*
[b] *Center for Neurosciences of Coimbra, University of Coimbra, 3500 Coimbra, Portugal*
[c] *Department of Chemistry, University of Strathclyde, Glasgow G1 1XL, Scotland, UK*

1. Introduction

Water has proven to be one of the several limitations for broadening the scope of applications of enzymes in biocatalysis, especially when the reactants involved are poorly water-soluble. The introduction of an organic solvent into the reaction system has numerous advantages. These include a direct action of the solvents on the reactants (improving their solubility) and on the reaction products (improving or diminishing their solubility), thereby increasing the productivity of the reaction system. Nevertheless, it has been documented that the introduction of an organic solvent into the reaction mixture may induce alterations on enzyme activity, enzyme stability and thermostability, and enzyme specificity [1] thereby imprinting new properties to old enzymes.

The knowledge about the factors affecting activity, stability, structure and selectivity of enzymes in media with organic solvents has been improved using thermodynamic, kinetic, spectroscopic and physical approaches; this understanding is now being developed to a level almost comparable to that of biocatalysts in aqueous media [1].

Cardosin A is a fine example of a small number of aspartic proteinases that have been isolated and purified from plants [2]. These proteinases share a number of common characteristics within the group: all have acid pH optimum, are heterodymeril, inhibited by pepstatin and preferentially cleave bonds between hydrophobic amino acids. Along with Cardosin A, there are examples of very well known enzymes within this class: pepsin, renin, human immunodeficiency virus type 1 protease or chymosin among others.

Cardosin A has been successfully used for peptide synthesis in biphasic systems, revealing good yields when compared with pepsin [3,4]. When exposed to small concentrations of organic solvents, in a biphasic system, Cardosin A has shown a decrease in stability with only some organic solvents, for example, ethyl acetate [5,6]. Cardosin A has proven to be an excellent catalyst. Firstly, it is very stable, loosing only around 20% of it's initial activity after 4 weeks at room temperature in aqueous buffer [7]. Nevertheless, in order to achieve better yields, a deeper knowledge of enzyme specificity is necessary, as well as knowing how the addition of organic solvents to the reaction media can alter it. Cardosin A may also become an attractive model to study the hydrolytic behaviour of aspartic

* Corresponding author. Tel.: +351-34-370970; fax: +351-34-426408.
E-mail addresses: cristina@bio.ua.pt (A.C. Sarmento), mbarros@bio.ua.pt (M. Barros).

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proteinases in media containing organic solvents: secondary specificity is an important factor for catalysis and it's tertiary structure has already been solved [8].

Thus, in this work we study the effect of organic solvents on Cardosin A hydrolytic specificity and test the overall possibilities of the enzyme as a catalyst in the presence of ethyl acetate and *n*-hexane, which have different hydrophobicities. On the other hand, any hydrolytic activity and specificity alterations will be correlated with solvent induced effects on structure. This will be achieved by probing the dynamic conformation of Cardosin A in organic solvents using a proteolytic enzyme, endoproteinase Glu-C, as a probe. To our knowledge, there is only one study where the authors applied limited proteolysis (LP) (using thermolysin) as a probe to assess protein structure in trifluoroethanol, an organic solvent that promotes and stabilises α-helix formation [9].

2. Materials and methods

Cardosin A was purified by a two-step procedure, performed as previously described [2] with slight modifications.

All reaction media were prepared as follows: aqueous phase was prepared by adding an excess of the selected organic solvent (*n*-hexane and ethyl acetate) to equal volume of aqueous buffer and vigorously agitated. The organic phase was removed after saturation of the aqueous phase, and used immediately.

The k-, α- and β-caseins (Sigma) were incubated with Cardosin A (E/S 1/500 w/w) at 30 °C for 60 min. Aqueous buffer was sodium phosphate 100 mM, pH 6.2. At selected times aliquots were taken and reaction stopped by addition of equal volumes of denaturing solution and heated at 100 °C, for 5 min. Samples were analysed by SDS-PAGE according to the method of Laemmli [10]. β-chain of oxidised insulin (1 mg/ml, from Sigma) was incubated with the enzyme (0.25 mg/ml) at 37 °C, in 100 mM sodium formate buffer, pH 3.0. Aliquots were taken and reaction stopped with equal volume of 12 ml/l TFA. Samples were analysed by HPLC, on an Äkta Basic system (Amersham Pharmacia Biotech) equipped with a C18 reverse-phase column. The mobile phase consisted of 0.1% (v/v) TFA. Elution was achieved by a gradient of acetonitrile (0–100% v/v) at a flow rate of 0.8 ml/min. Detection was at 215 nm.

Limited proteolysis assays were performed at 25 °C. Cardosin A was incubated with endoproteinase Glu-C (Sigma) with a substrate/protease ratio of 200/1 (w/w). Aliquots of reaction media were taken and analysed by SDS-PAGE. Digestion fragments were transferred to PVDF membranes and sequenced in an automated sequencer (Applied Biosystems), by Edman degradation.

Activity measurements were made by hydrolysis of a synthetic peptide (Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu) specifically delineated for Cardosin A specificity studies [2], with modifications.

3. Results and discussion

Having in consideration all parameters that may alter stability and activity of Cardosin A in low water systems, we used systems with high water content. In this way, interpretation will be facilitated. In fact, in these high water content systems, the enzyme is solubilised, with its ionisation state controlled by the buffer and with water activity of the system being approximately 1, in all solvents tested. So only the effect of protein-solvent contacts will be evaluated.

Fig. 1 shows hydrolysis of α-, β- and k-caseins with Cardosin A in the systems tested: aqueous buffer, aqueous buffer with *n*-hexane and ethyl acetate. These

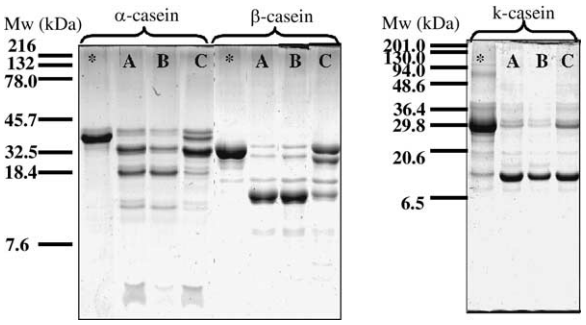

Fig. 1. SDS-PAGE of α-, β- and k-caseins hydrolysis by Cardosin A at 30 °C. Each lane (A, B and C) represents 60 min incubation with Cardosin A: (A) control reaction in 100 mM sodium phosphate buffer, pH6.2; (B) aqueous buffer with *n*-hexane; (C) aqueous buffer with ethyl acetate. The (❋) represents substrates prior to incubation.

substrates were chosen since the hydrolytic profile of Cardosin A action in aqueous medium had already been established [11]. These substrates are very complex possessing several susceptible bonds to Cardosin A action. Results show that the hydrolytic action is maintained, suggesting that there are no new peptide bonds cleaved by Cardosin A in systems B and C (at least not detectable by this technique). Nevertheless, an alteration of the SDS-PAGE pattern can be seen (lane C of α- and β-caseins), assumingly by a reduction on the velocity rate of Cardosin A in the presence of ethyl acetate. Time courses of hydrolysis of these substrates (data not shown) confirmed this hypothesis showing that Cardosin A is less active in this system.

Since it has been proved that organic solvents may alter the tertiary structure of proteins, thus exposing new peptide bonds to the solvent, any new hydrolysis product detected could be assigned to the organic solvents effect on the enzyme (specificity alterations). Nevertheless, this effect could be in reality be caused by a 3D structure alteration of the substrate (in this case, caseins). In order to avoid this effect, we have also used a simpler substrate than casein. A substrate with no tertiary structure and with only some elements of secondary structure.

As in the case of caseins the specificity of Cardosin A towards β-chain of oxidised insulin has already been studied in aqueous medium (Fig. 2A).

As we can see in Fig. 2B the presence of ethyl acetate in the reaction medium induced the appearance of two new peptides (α and β) that were identified by amino acids analysis (Table 1).

The presence of the peptides alpha and beta, suggested the possibility of identifying the first cleaved bond promoted by Cardosin A on β-chain of oxidised insulin. Also, it was possible to localise the peptide bond that was hydrolysed, giving origin to these new products alpha and beta (Table 1). In this way, it seemed that organic solvents rather than promoting specificity alterations (in terms of hydrolysis of new peptide bonds), influenced hydrolysis rates. Reaction velocities were deeply investigated (Table 2) and the results show, that although there is a strong dependence of hydrolysis rates on the media composition, the preference order is followed, being the velocity of hydrolysis of Leu_{15}-Tyr_{16} approximately four times higher than hydrolysis of Phe_{25}-Tyr_{26}. The only exception happens when the medium has *n*-hexane. In this case (Table 2), formation velocity of peak D is

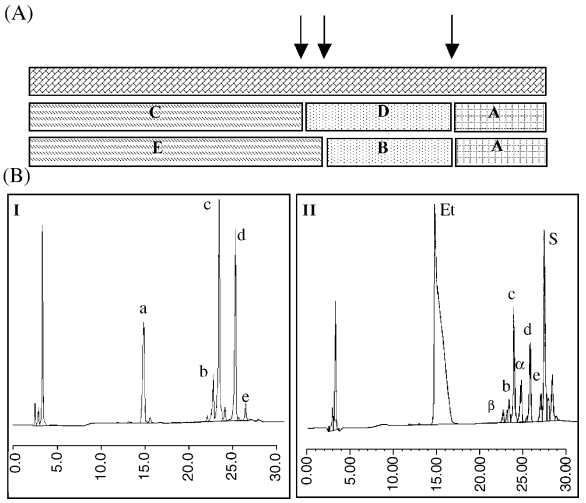

Fig. 2. β-chain of oxidised insulin hydrolysis, by Cardosin A in 100 mM sodium formate buffer, pH 3.0, at 37 °C: (A) schematic representation of bovine β-chain of oxidised insulin hydrolysis by Cardosin A in aqueous medium; arrows indicate hydrolysed peptide bonds, by Cardosin A; (B) RP-HPLC chromatograms (at 215 nm) of β-chain of oxidised insulin 10 min digestion in: I—aqueous buffer; II—aqueous buffer with ethyl acetate (Et). a–e are fragments originated by Cardosin A hydrolysis in aqueous medium. The α and β represent new fragments only found in the system with ethyl acetate.

Table 1
Characterisation of α and β peptides originated from hydrolysis of β-chain of oxidised insulin, by Cardosin A

Peptide	Cleavage site	Peptide sequence
α	Leu_{15}-Tyr_{16}	Tyr_{16}-Ala_{30}
β	Leu_{17}-Val_{18}	Val_{18}-Ala_{30}

Table 2
Initial rates of peptide production (C and D corresponding to hydrolysis of Leu_{15}-Tyr_{16} and Phe_{25}-Tyr_{26}, respectively), produced by hydrolysis of β-chain of oxidised insulin, by Cardosin A, as a function of media

	C peak	D peak
Aqueous	100	100
n-Hexane	78	153
Ethyl acetate	6	5

100% was set to the highest amount of each product in aqueous medium.

Table 3
Kinetics parameters of hydrolysis of Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu, by Cardosin A, at 25 °C in 50 mM sodium acetate, 4% DMSO, pH 4.7 (**A**), aqueous buffer with *n*-hexane (**B**) and aqueous buffer with ethyl acetate (**C**)

	A	B	C
K_m (mM)	0.04	0.04	0.138
K_{cat} (min^{-1})	198	202	150
K_{cat}/K_m ($min^{-1}\,mM^{-1}$)	4950	5050	1086

K_m and K_{cat} were calculated according to the Lineweaver–Burk equation.

increased and is even higher than in the aqueous buffer, suggesting a facilitation, by the solvent, of Phe_{25}-Tyr_{26} peptide bond hydrolysis.

Considering the results obtained with caseins and β-chain of oxidised insulin hydrolysis by Cardosin A, there is either an effect of the organic solvents on reaction velocities (caseins, Fig. 1) and a facilitation of hydrolysis of one peptide bond (β-chain of oxidised insulin, Table 2). In order to elucidate these results, kinetic parameters (K_m and K_{cat}) were calculated. A synthetic peptide (Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu) was used. This peptide is suitable for this study, since Cardosin A cleaves only one peptide bond, Phe–Phe(NO_2).

Results are shown in Table 3 and it can be seen that *n*-hexane (B) doesn't induce any significant alterations on the calculated parameters, being very similar to the ones in aqueous medium (A). This result is in agreement to caseins hydrolysis study, where *n*-hexane had little effect on the reaction rate. The increased rate obtained in β-chain of oxidised insulin hydrolysis (Table 2), in the presence of *n*-hexane, can not be explained by the calculated kinetic parameters. This may be explained since the hydrolysed amino acid sequence, by Cardosin A, in β-chain of oxidised insulin (Arg·Gly·Phe·Phe-Tyr·Thr·Pro) is quite different from the one of the synthetic peptide (Lys·Pro·Ala·Glu·Phe-Phe(NO_2)·Ala·Leu) used for kinetic parameter determination. In this way, the driving forces promoting interaction between substrate and the active cleft of Cardosin A are necessarily different. The exact mechanism exerted by *n*-hexane is still unknown, but it seems that its effect depends on the substrate tested.

On the other hand, ethyl acetate changes the overall catalytic performance of Cardosin A. As it can be seen (Table 3), Cardosin A in ethyl acetate, has reduced affinity towards the synthetic peptide than in any other media tested, which correlates well with the caseins and β-chain of oxidised insulin hydrolysis studies.

LP has been reported to be an excellent technique to attain structural information in proteins, at the molecular level. So, LP of Cardosin A in systems with organic solvents was tested, as a new approach, to probe solvent induced alterations on Cardosin A. Fig. 3 shows the results obtained using endoproteinase Glu-C as a probe. Hydrolysis pattern in aqueous medium shows one major fragment of 16.5 kDa (✻) produced from the 31 kDa chain cleavage. In *n*-hexane, the hydrolytic pattern is quite similar to the aqueous one, revealing, with this approach, no solvent induced structural effects.

This correlates well with the results obtained with specificity studies of Cardosin A, in the presence of *n*-hexane, shown in this work. Concerning LP results, with Cardosin A in the presence of ethyl acetate, there is a different hydrolysis pattern. The same fragment of 16.5 kDa, produced in aqueous conditions is observed, but another fragment (✻) of 22.6 kDa is detected. Destabilisation of Cardosin A structure induced by ethyl acetate (demonstrated by LP results) maybe the cause of the low catalytic performance of Cardosin

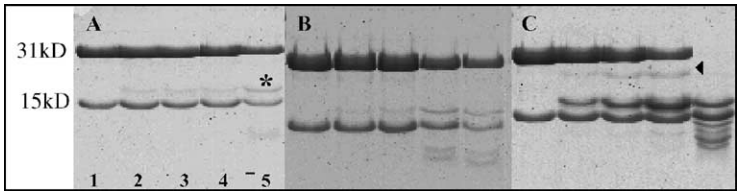

Fig. 3. SDS-PAGE of Cardosin A incubated with endoproteinase Glu-C, at 25 °C in: (A) 50 mM ammonium bicarbonate; (B) aqueous buffer with *n*-hexane and (C) aqueous buffer with ethyl acetate. Reaction times were: **1**: 0 h, **2**: 30 min, **3**: 60 min, **4**: 3 h and **5**: 24 h. The (✻) and (**3**) represent sequenced hydrolysis fragments of Cardosin A.

A in this medium. Amino acid sequencing of both fragments confirmed that there was no specificity alterations induced by the solvent on endoproteinase Glu-C, being the new fragment a result only of Cardosin A conformational alteration.

4. Conclusions

Caseins hydrolysis by Cardosin A in the presence of *n*-hexane and ethyl acetate has revealed the same products. In this reaction, *n*-hexane has shown to produce little effect on the hydrolysis rate, while ethyl acetate decreased the rate considerably. A similar effect was detected using β-chain of oxidised insulin. In this way, it was possible, with this substrate, to discover the first hydrolysed bond by this catalyst. However, *n*-hexane has shown to increase the hydrolysis rate of one specific peptide bond of β-chain of oxidised insulin, by a mechanism still unknown.

In order to investigate if these effects were only associated with the Cardosin A active site, or if a global structural alteration was involved, LP studies were carried out. The results confirmed that there is a structural effect induced by ethyl acetate witch may explain the reduced activity shown.

Aspartic proteinases can be used as model proteins in the study of the effect of organic solvents on enzymatic catalysis, since they possess a large active cleft with several subsites and where specificity alterations, even subtle ones, are related to alterations in each subsite. This is only possible in this case, and not with other proteinases classes where the specificity is only dependent on the primary specificity (P1-P′1).

Cardosin A, being a very stable enzyme when exposed to organic solvents, and when its subsite residues become completely clarified, may, then, be used as a model protein for the understanding of the effects of organic solvents on such complex active sites, allowing a deeper investigation of molecular aspects of these effects.

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