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Evaluation of cardosin A as a proteolytic probe in the presence of organic solvents

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Abstract

This investigation showed that cardosin A not only is active in media with organic solvents, cleaving the β-chain of oxidised insulin at three susceptible peptide bonds, but also maintains its specificity in all media tested. Additionally, the presence of organic solvents in the reaction media led to modifications of enzyme selectivity, which enabled the detection of intermediate products. While solvents like ethyl acetate induced a decrease in enzymatic activity, both by reducing the amount of active enzyme and presumably due to an inhibiting effect of ethyl acetate (which might compete with the substrate for the active site of the enzyme), *n*-hexane caused an increase in the hydrolysis velocity of one peptide bond. In view of the activity and specificity of cardosin A (which shows high preference for hydrophobic residues), it is proposed as a reliable probe for limited proteolysis in the presence of organic solvents. This may become particularly useful for structural characterisation of membrane proteins.

Keywords: Enzyme catalysis; Hydrolysis intermediates; Solvent effects; Aspartic proteinases; Specificity

1. Introduction

Over the last years, we have seen a growing interest in limited proteolysis in the presence of organic solvents as a valid complementary technique in protein sequencing studies [1]. It is also a useful tool for protein surface-mapping [2,3] giving information about organic solvent-induced structural alterations. When a proteolytic enzyme is used as a probe in limited proteolysis in organic solvents, it is necessary that the enzyme has sufficient activity, stability and specificity in the presence of the solvent used [4]. The protease specificity must be clearly known, so that conclusions about substrate conformation can be drawn from the observed hydrolysis patterns. It is well known that the introduction of an organic solvent into the reaction mixture may induce alterations in enzyme activity [5,6], stability [7] and specificity [8–11]. Therefore, the probe's specificity, and any alteration in the presence of an organic solvent, needs to be precisely characterised. Cardosin A is a fine example out of a small number of plant aspartic proteinases that have been isolated, purified [12] and characterised [13–15]. This enzyme has been successfully used in the presence of several organic solvents [16–18]. Having in mind the applications of the use of organic solvents in protein sequencing and in protein surface mapping, it is of major interest to study the specificity of proteolytic enzymes that show activity in aqueous organic mixtures. Therefore, since cardosin A has proved to be active [16] and stable in the presence of organic solvents, the present work deals with the effect of organic solvents on cardosin A specificity.

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2. Experimental

2.1. Enzyme, substrates, chemicals and solvents

Cardosin A was purified from fresh flowers collected from wild plants, identified as *Cynara cardunculus* L. β-Chain of oxidised insulin, pepstatin A, *n*-hexane, ethyl acetate and iso-octane, were purchased from Sigma. Acetonitrile (HPLC grade) was from Romil and trifluoroacetic acid (TFA) was from Merck.

2.2. Enzyme purification

Cardosin A purification was based on a previously described protocol [18]. Cardosin A purity was assessed by SDS-PAGE, according to Laemmli [19]. Pure cardosin A solutions were concentrated by lyophilization. Dried cardosin 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. Hydrolysis reaction

β-Chain of oxidised insulin (1 mg mL^{-1}) was incubated with the enzyme (0.004 mg mL^{-1}) at 37 °C, in sodium formate buffer (100 mM). For the standard reaction, pH was set to 3.0. At selected times, aliquots were taken and the reaction stopped with an equal volume of TFA (12 mL L^{-1}). Reaction media were prepared by adding the selected organic solvent to an equal volume of aqueous buffer and agitating vigorously. After phase separation, the organic phase was removed, and β-chain of oxidised insulin immediately dissolved in the remaining aqueous phase. The reaction was started immediately after by enzyme addition. To avoid loss of the organic solvent, sealed vials were used.

2.5. RP-HPLC analysis

Samples were analysed by HPLC, on an Äkta Basic system (Amersham Pharmacia Biotech) equipped with a C_{18} reverse-phase column (250 mm × 4.6 mm LiChroCART 100 RP-18, 5 mm, Merck). The mobile phase consisted of ultrapure water acidified with 0.1% (v/v) of TFA. Elution was achieved by a gradient of acetonitrile (0–100%, v/v) acidified with TFA (0.1%) at a flow rate of 0.8 mL min^{-1}. Detection was at 215 nm.

2.6. Amino acid analysis

Hydrolysis products were identified and quantified by amino acid analysis, according to Heinrikson and Meredith using acid hydrolysis and pre-column derivatisation with phenylisothiocyanate (PITC) [20].

Acid hydrolysis of peptides was carried out after drying the samples in glass tubes by lyophilisation. The tubes were then inserted in an appropriate container, to which 1 mL of 6 M HCl with phenol (0.1–1%, w/v) had previously been added and hydrolysis was carried out at 150 °C for 18 h. After hydrolysis, samples were hydrated (by adding a solution of water, ethanol and triethylamine), derivatised with PITC and dried under vacuum. Samples were then dissolved in sodium phosphate buffer (12.5 mM, pH 6.4) and analysed by RP-HPLC at 254 nm. Quantification was achieved by comparing the sample composition with an amino acid standard solution (Pierce) treated in the same way as the samples.

2.7. Active-site titration

Active concentrations of cardosin A were determined according to Knight [21]. The enzyme solution was incubated with the titrant (pepstatin A) for 1 h at 25 °C and subsequently its remaining activity determined against Lys–Pro–Ala–Glu–Phe–Phe(NO_2)–Ala–Leu. A titration curve was prepared and enzyme concentration calculated by graphical estimation.

3. Results and discussion

The β-chain of oxidised insulin has been used to investigate proteinase specificity, offering several advantages such as low cost, well-defined products and no tertiary structure (Fig. 1) [22]. The cardosin A digestion profile of the β-chain of oxidised insulin in aqueous medium has been previously investigated and characterised in detail [23]. It was shown that there are three bonds susceptible to cardosin A action, giving rise to five peptides (Fig. 1a).

For all systems tested, we have verified that cardosin A was capable of hydrolysing the β-chain of oxidised insulin, with hydrolysis patterns varying according to the solvent used (Fig. 1a and b). Amino acid analysis of hydrolysis products confirmed that, with the exception of two fragments, they corresponded to the same peptides previously observed in aqueous medium [23]. Hydrolysis in the presence of ethyl acetate (Fig. 1b) led to two new well-defined products not found in the corresponding aqueous reaction. These peptides were named alpha (α) and beta (β) with their appearance being dependent on the time of reaction and on the medium used (data not shown). Identification of these products, by amino acid analysis, showed that peptide α originated by hydrolysis of the Leu_{15}–Tyr_{16} peptide bond, whereas peptide β originated by Leu_{17}–Val_{18} peptide bond hydrolysis (Fig. 1). It should be noted that, when reactions in the presence of ethyl acetate were allowed to proceed longer (24 h), peptides α and β were observed to disappear while peptides D and B increased in concentration. This indicates subsequent hydrolysis of peptides α and β by cardosin A, according to the scheme of Fig. 1. The progress of substrate hydrolysis was then investigated in the solvent-free aqueous buffer to

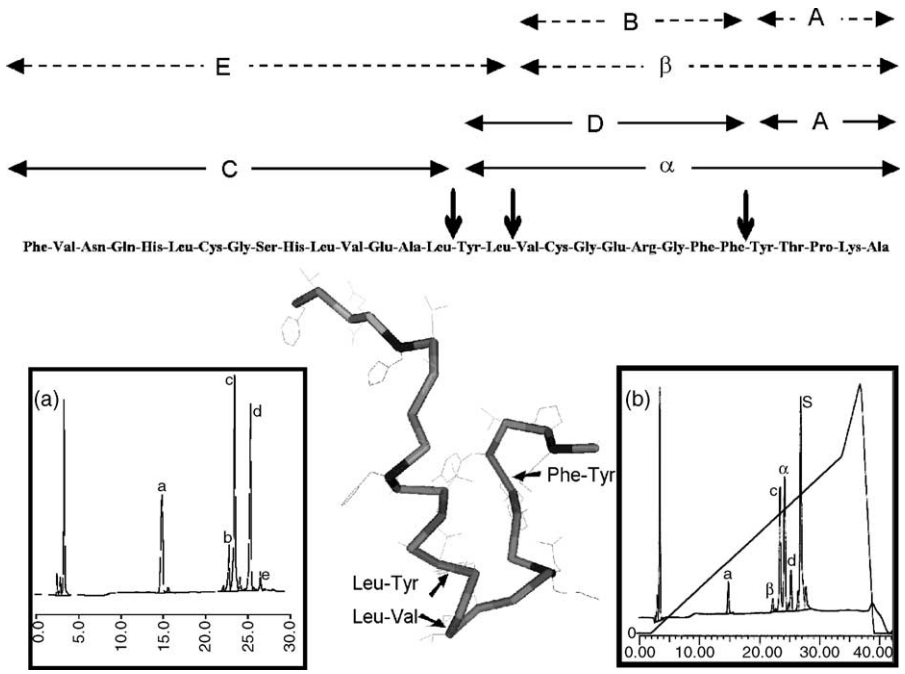

Fig. 1. Amino acid sequence, possible structure of β-chain of oxidised bovine insulin and typical hydrolysis pattern of β-chain of oxidised insulin with cardosin A. Shown is the NMR solution structure of a mutant β-chain with Cys residues replaced by Ser (PDB 1HO0). Arrows represent peptide bonds hydrolysed by cardosin A. (a) Hydrolysis reaction pattern carried out in aqueous buffer and (b) in aqueous buffer with ethyl acetate (S—substrate). Fragments produced by cardosin A hydrolysis of the substrate are shown. Dotted lines correspond to low amount products.

see the control behaviour of cardosin A, enabling the identification of the first cleavage site in the β-chain of oxidised insulin. The peptide bond Leu_{15}–Tyr_{16} is the first one to be hydrolysed giving rise to peptides C and α. Peptide C remains intact, while peptide α concentration rapidly decreases, yielding two peptides A and D (Fig. 2). Peptide α is therefore an intermediate of the reaction. Simultaneously, peptides E and β are formed (to a much smaller extent) by cleavage of the Leu_{17}–Val_{18} peptide bond. Peptide β is also a substrate for cardosin A at the Phe_{25}–Tyr_{26} peptide bond, giving rise to peptides A and B. The concentration of peptides E, B and β is very low, when compared to peptides C, D and α (Fig. 1a). After 24 h of reaction, however, an increase of peptide B and a corresponding decrease of peptide D were observed. It has to be noted that while peptides C and E are formed by single cleavages, peptide D requires two cleavages (Fig. 1). Nevertheless, quite soon peptide α reaches a steady-state level and then a linear production of peptide D is obtained.

Organic solvents can inactivate enzymatic reactions via denaturation of the catalyst (diminishing the active, soluble enzyme) [24]. Nevertheless, many agents are observed to reduce enzymatic rates, and most do not affect the dissolved enzyme concentration. Rather, they reduce the activity of the individual molecules (usually all are reduced to some extent, but sometimes a fraction is totally inactivated). For these reasons, active-site titration of cardosin A was performed in all conditions of study (Table 1). It is shown that the active enzyme concentration is medium dependent. Fig. 3 shows the relative initial velocities of peptides C, D and E formation by cardosin A, in all media tested. Data was normalised for the active enzyme concentrations. The effective velocity of peptide C formation is always higher than that of peptide D,

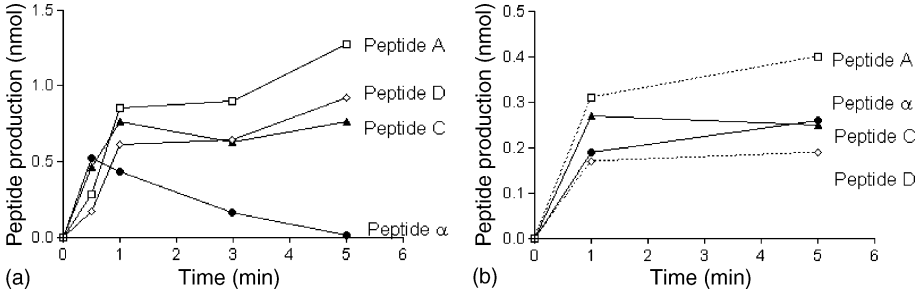

Fig. 2. Typical progress curves of hydrolysis of β-chain of oxidised insulin by cardosin A. Progress curve for the hydrolysis of β-chain of oxidised insulin in aqueous buffer (a) and in aqueous buffer saturated with ethyl acetate (b). Only major peptides were represented to facilitate reading and interpretation.

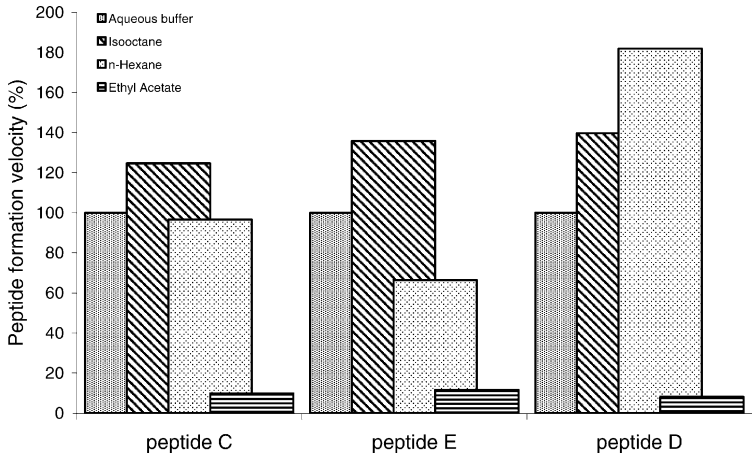

Fig. 3. Relative initial rates of peptide production as a function of media. Peptides C (Phe_1–Leu_{15}), D (Tyr_{16}–Phe_{25}) and E (Phe_1–Leu_{17}) in β-chain of oxidised insulin.

Table 1
Active-site titration of cardosin A

Reaction media	Active enzyme (nM)
AQ with iso-octane	8.66
AQ with ethyl acetate	7.39
AQ with *n*-hexane	10.31
Aqueous buffer (AQ)	12.64

which is in agreement with the Leu_{15}–Tyr_{16} peptide bond being the first hydrolysed by cardosin A, in the majority of substrate molecules (data not shown). The results show a strong dependence of the velocity of hydrolysis on the medium composition. The velocity of hydrolysis declines with decreasing hydrophobicity of the organic solvent (Fig. 3), although peptide D production is an exception. Additionally, the results show that none of the organic solvents used in this work induced alterations of cardosin A specificity (meaning that there were no new peptide bonds hydrolysed when compared to the control reaction in aqueous buffer). Nevertheless, selectivity alterations occurred, as reflected in the relative hydrolysis velocities for each peptide bond, which can be seen from the ratios of rates in Table 2. These ratios were calculated by dividing the velocity of formation of peptide C, D and E, respectively. The organic solvent does not have the same influence on the hydrolysis velocity of every peptide bond in one substrate. In fact, in the presence of *n*-hexane, there is an increase on the velocity of the Phe_{25}–Tyr_{26} peptide bond hydrolysis, which is responsible for the increase in peptide D production (Fig. 3).

Table 2
Ratios of peptide formation velocities, produced by hydrolysis of β-chain of oxidised insulin by cardosin A

Velocity ratio	1	2	3	4
Peptide C/peptide D (Leu_{15}–Tyr_{16}/Phe_{25}–Tyr_{26})	4.1	3.7	2.2	4.6
Peptide C/peptide E (Leu_{15}–Tyr_{16}/Leu_{17}–Val_{18})	4.6	4.2	6.7	3.9

Reactions were carried out in several media: 1—in aqueous buffer, and in aqueous buffer with: 2—iso-octane; 3—*n*-hexane; and 4—ethyl acetate.

In accordance with this, the influence of the solvent on enzyme selectivity can be seen from the ratio of peptide C production (hydrolysis of Leu_{15}–Tyr_{16} peptide bond) over peptide E production (hydrolysis of Leu_{17}–Val_{18}). The same applies to the relative rates of hydrolysis of the Leu_{15}–Tyr_{16} peptide bond compared with Phe_{25}–Tyr_{26} (Table 2). Since the P_1 residue is the same for the Leu_{15}–Tyr_{16} and Leu_{17}–Val_{18} peptide bonds, it is reasonable to see the ratio of the production velocities of peptide C and peptide E as a measure of cardosin A preference towards P'_1. The results seem to suggest that cardosin A has a clear preference for tyrosine in the P'_1 position (i.e. Leu_{15}–Tyr_{16}), although we should keep in mind that, for aspartic proteinases, the residues adjacent to the hydrolysed bond, i.e. secondary specificity, are of major importance for the enzyme specificity [25,26]. This effect is enhanced in the presence of *n*-hexane since the velocity ratio of Leu_{15}–Tyr_{16}/Leu_{17}–Val_{18} increases from 4.6, in aqueous buffer, to 6.7 (Table 2), mainly due to the decrease in the velocity of peptide E production (Fig. 3). Actually, only *n*-hexane significantly affects enzyme selectivity, whereas the other two solvents are more or less ineffective in terms of alteration of enzyme selectivity.

In general, it is clear that cardosin A hydrolytic behaviour is dependent on the organic solvent used in the reaction medium. In addition to the alteration of active enzyme concentration, a factor affecting cardosin A affinity for the substrate could be an alteration of its ionisation state (of the catalytic cleft or of the enzyme as a whole) induced by the solvent. In fact, an interaction of the organic solvents with the active site cleft, changing its ionisation state, would yield an alteration of the pH profile, which was not the case (Fig. 4). The results obtained indicate that there is no alteration of the pH (4.5) for optimum activity of cardosin A, although a slight broadening of the optimum was observed with iso-octane (Fig. 4).

Cardosin A has been shown to be a potentially useful proteolytic probe in limited proteolysis in the presence of a variety of organic solvents, since, not only is it active but also

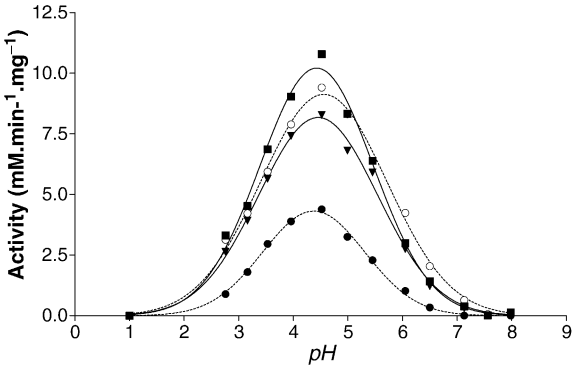

Fig. 4. Effect of the solvent on the pH dependence of cardosin A activity. Symbols correspond to: (●) aqueous buffer with ethyl acetate; (▼) aqueous buffer with *n*-hexane; (◯) aqueous buffer with iso-octane; and (■) aqueous buffer. Cardosin A activity was determined by hydrolysis of Lys–Pro–Ala–Glu–Phe–Phe(NO_2)–Ala–Leu.

its specificity is maintained. The selectivity alterations described have been shown to be helpful in the detection of reaction intermediates.

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