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Reverse hydrolysis by cardosin A: specificity considerations

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Abstract

Cardosin A, a plant aspartic proteinase, capable of synthesising peptides, was investigated through synthesis of five methyl esters amino acid substrates as amino donors and nine benzyloxycarbonyl amino acid and peptide carboxyl donors. It was found that cardosin A is able to catalyse the synthesis of several peptide bonds, being the preference order for the carboxyl components the following: CBz.Phe > CBz.Trp. Unpredictably, Tyr could not be accepted in P_1. Results were compared and discussed according to the known specificity of pepsin, the most studied aspartic proteinase.
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1. Introduction

The concept of peptide synthesis by reversal of mass action in protease-catalysed reactions dates back to 1898 when J.H. van't Hoff suggested that the proteinase trypsin might possess the inherent capacity to catalyse the synthesis of proteins from degradation products originally generated by its own proteolytic action [1]. However, synthesis is thermodynamically unfavourable in aqueous buffers due to the presence of water as a product. To overcome this, two-phase systems have been used [2]. In fact, the denaturing and inhibiting effects induced by a solvent poorly soluble in water on enzymes are very limited (if properly selected). In addition, if the product is soluble in the organic phase, it is continuously removed from the aqueous phase thus promoting its synthesis [3].

The selection of proteinases is based on their specificity, the synthetic process being dependent on the hydrolytic mechanism of the enzyme. For this reason, and specially when using aspartic proteinases, that possess a large active cleft [4], the clear definition of the 'secondary' specificity of the enzyme (meaning the preferences of a given enzyme for the amino acid sequences surrounding the primary specificity site [4]) is of major importance in the selection of the catalyst that should be used. Cardosin A, an aspartic proteinase like pepsin, has been deeply characterised in terms of activity, specificity, structure and stability [5–7] in aqueous medium and in terms of activity in the presence of organic solvents [8,9]. It is well known that enzyme specificity may be changed profoundly on switching from one solvent to another [10]. This paper intends to investigate the potential of this enzyme in terms of its ability to synthesise peptide bonds and subsequently to gain insights into its synthetic specificity.

2. Experimental

2.1. Materials

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 and ethyl acetate) and substrates

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were purchased from Sigma. Acetonitrile was purchased from Romil.

2.2. Enzyme purification

Cardosin A was purified as described previously [5] with modifications that allowed the purification of higher amounts of protein.

Stigmas from fresh flowers were homogenised in a mortar and pestle with sodium citrate 100 mM, pH 3.5, and centrifuged. The supernatant was applied to a Hiload Superdex 75 semi prep (Amersham Pharmacia Biotech) equilibrated and eluted with 25 mM Tris–HCl pH 7.6 at a flow rate of 3 ml/min. The active fraction was further purified on a Q-Sepharose column (Amersham Pharmacia Biotech), equilibrated with 25 mM Tris–HCl pH 7.6. The proteins were eluted with a gradient of NaCl (0.2–1 M) at a flow rate of 3 ml/min. Elimination of salts from the enzyme solution was achieved by gel filtration on a G25 column (HiPrep 26/10 Desalting, Amersham Pharmacia Biotech) equilibrated with ultra-pure water at a flow rate of 10 ml/min.

All chromatographic procedures were performed at room temperature. Additionally, all solvents were degassed with Helium prior to use. Purity of cardosin A was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [11] after staining with Comassie Brilliant Blue. Densitometry analysis allowed determining purity of the samples to be 100%. Cardosin A solutions were concentrated by lyophilization. Dried cardosin A powders were either used immediately or stored at −20 °C.

Cardosin A activity was determined, as described before [7], with its specific activity being 7.81 ± 0.89 U/mg. One enzyme unit is defined as the quantity of cardosin A needed to hydrolyse 1 μmol of Lys–Pro–Ala–Glu–Phe–Phe(NO_2)–Ala–Leu per minute.

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

Enzymatic synthesis of peptides was performed in biphasic systems, constituted by an aqueous (100 μl) and an organic phase (400 μl). The aqueous phase consisted in 200 mM sodium phosphate, pH 5.0. The organic phase was constituted by a mixture of *n*-hexane/ethyl acetate (3:1 v/v) previously water saturated. Cardosin A (3 mg/ml) and methyl ester amino acids (100 mM) were solubilised in the aqueous phase. CBz amino acids (or peptides), 25 mM were solubilised in the organic phase. Reactions occurred at 30 °C under agitation (200 rpm). At regular times, samples of the organic phase were dried, solubilised in Acn 80%, TFA 0.1% (v/v), and analysed by high pressure liquid chromatography (RP-HPLC) on a C18 reverse phase column (250 mm × 4.6 mm LiChroCART™ 100 RP-18, 5 μm, Merck) on an automated chromatography system Äkta Basic 10 (Amersham Pharmacia Biotech). Peptide identification and quantification was routinely made by mass spectrometry and by amino acid analysis. Initial rates were calculated from the linear parts of product formation curves.

For Boc- blocked and Aib (dimethylglicine) containing peptides, reactions were carried out as described above but no initial rates were determined. Products were identified in the organic phase by mass spectrometry.

2.4.1. Amino acid analysis

Amino acid analysis was carried according to the method developed by Heinrikson and Meredith [12].

2.4.2. Peptide mass spectrometry

Electrospray and tandem mass spectra were acquired with a Q-TOF 2 (Micromass, Manchester). Data acquisition was carried out with a Micromass MassLynkx 3.4 data system.

3. Results and discussion

Substrates used were chosen according to the known preference of this proteinase for residues with large and hydrophobic side-chains. Cardosin A catalysed peptide synthesis was performed in biphasic systems, and the equilibrium shift favourable for the synthesis was provided by the product extraction to the organic phase. Hence, at least two main factors controlled the success of the synthesis: (i) the conformity of the reactant structure to cardosin A specificity that should determine predominantly the rate of product formation; (ii) the influence of the product structure on its solubility in both phases and, consequently, on the equilibrium position. Both these factors should determine the reaction yield. On the other hand, substrate solvation in the organic phase can affect enzyme specificity [13] and solubility and partition of substrates and products between the aqueous and the organic phases may affect reaction rates [14]. Nevertheless, it is not our goal to achieve significant yields since the characterisation of cardosin A ability to synthesise peptides is a subject of interest.

The solvent system used to accomplish such goal was chosen according to preliminary work carried out in our laboratory, where the best compromise between synthesis performance and enzyme stability was achieved [8,15]. Such system has the added advantage of allowing the catalysis of the reaction between single amino acids in order to obtain dipeptides, since dipeptides are very difficult to hydrolyse by aspartic proteinases due to the stringent secondary specificity requirements [16].

The results obtained, and summarised in Table 1 show that, in general, cardosin A acted as a useful catalyst of peptide synthesis. Like pepsin, cardosin A primary specificity is found to be markedly influenced by the nature of

Table 1
Effect of the nature of the carboxyl and amine components on coupling catalysed by cardosin A

CBz.Y	X.OMe				
	Phe (μM h^{-1})	Tyr (μM h^{-1})	Met (μM h^{-1})	Val (μM h^{-1})	Leu (μM h^{-1})
Phe	19.5	94.8	1.5	0.2	0
Tyr	0	0	0	0	0
Trp	2.0	–	0	0	0
Val.Phe	11.5	8.7	5.1	0	0
Val.Leu	–	1.5	–	–	–
Gly.Leu	16.2	8.3	0	0	0
Gly.Phe	69.8	29.4	20.8	1.2	0.7
Gly.Tyr	0	0	0	0	0
Gly.Gly.Phe	0	0	0.2	0.2	0.6

–: Low amounts of products were detected only after 5 days of reaction and no initial rates could be calculated.

the carboxyl group donor. In accordance to these expectations the preference order for the carboxyl components is the following: CBz.Phe > CBz.Trp, in agreement to what has been described for pepsin [17].

The effect of the size of the carboxyl donor component on coupling was examined as shown in Table 1. A general increase of the synthesis velocity was detected when the substrate size increased from CBz.Phe to Cbz.Gly.Phe, but not to CBz.Gly.Gly.Phe. A similar behaviour was already described for pepsin [18]. In this case it was ascribed to an increase on the synthesis product hydrolysis rate. Likewise, it seems that in the case of cardosin A the same effect could be the cause of lack of synthesis products in the condensation of CBz.Gly.Gly.Phe with the most hydrophobic substrates tested, Phe.OMe and Tyr.OMe.

The effect of valine in P_2 position (notation of Schechter and Berger [19]) in the reaction of peptide synthesis catalysed by cardosin A was investigated studying the kinetics of tripeptide production. Characteristically, the introduction of valine instead of glycine residue in P_2 position resulted in a decrease in the synthesis reaction initial rates, which confirms the enzyme's sensitivity to the amino acid residues flanking the bond to be formed according to what has been described for aspartic proteinases [4]. Isowa et al. described a similar effect of valine in P_2 position in pepsin specificity. Although the authors could couple the dipeptide CBz.Phe.Tyr.OH with H.Phe.ODPM achieving good yields, they failed in their attempt to condensate CBz.Val.Tyr.OH with the above amine component, H.Phe.ODPM [20]. These results demonstrate the extreme importance of secondary specificity of aspartic proteinases and the need of investigating it.

Even though aspartic proteinases have a known preference for hydrophobic residues, which is explained by the hydrophobic character of the S_1 and S'_1 subsites of the enzyme [21], unpredictably, Tyr was not accepted as a carboxyl donor by cardosin A. The ineffectiveness of CBz.Tyr.OH can only be explained by the subsite specificity of cardosin A at P_1. A possible explanation is that the hydroxyl group of the substrate may be so located in the enzyme–substrate complex that it could be able to form an hydrogen bond with a catalytically important carboxylate group of the enzyme [16]. Furthermore, increasing the length of the substrate (from Cbz.Tyr to Cbz.Gly.Tyr) did not overcome this effect. In fact, this result is consistent with the literature on cardosin A hydrolytic specificity since no bond of the type Tyr-X has, until the moment, been reported as hydrolysed by this enzyme [22,23]. The relatively narrower specificity of cardosin A described in the literature, and confirmed with this investigation, when compared to other aspartic proteinases, like pepsin, may be related to specific determinants of the S_1 subsite since is thought that this subsite is the primary determinant of the aspartic proteinases specificity. The S_1 subsite of pepsin features a flexible loop, Leu_{71}–Gly_{82} [21]. The equivalent loop of cardosin A differs in several amino acid residues [6] that most likely accounts to the differences noticed in their specificity.

Concerning leucine residue, it was rarely accepted as an amino donor by cardosin A (the only peptides synthesised were CBz.Gly.Phe–Leu.OMe and CBz.Gly.Gly.Phe–Leu.OMe with comparable low rates). A similar result was obtained in a study performed by Morihara and Oka with pepsin [18], in the condensation of several carboxylic donors with leucine. Similarly, in another investigation it was revealed that pepsin cleaved slowly all substrates with an aliphatic side-chain in P'_1 position [16]. This similarity of cardosin A to pepsin suggests a similar constitution of the S'_1 pocket of cardosin A that has not been resolved until the present moment [6].

The search for new blocking agents for enzymatic peptide synthesis is a growing field on chemistry, since they can be used to achieve higher yields of product formation. Butoxycarbonyl (Boc) is widely used in synthetic chemistry being less hydrophobic than CBz. Cardosin A showed to be able to catalyse the synthesis of Boc.Gly.Phe–Phe.OMe, Boc.Gly.Phe–Phe.Aib.NH_2 as well as CBz.Gly.Phe–Phe.Aib.NH_2 confirming that cardosin A is able to accommodate a long branched side-chain in its S_3 subsite (Table 2). Nevertheless, it was not possible to determine synthesis initial rates since low amounts of products were obtained even after 5 days of reaction.

Table 2
Effect of the nature of the carboxyl or amine component on coupling catalysed by cardosin A

Carboxylic donor	Amine donor	
	Phe.Aib.NH_2	Phe.OMe
Boc.Gly.Phe	–	–
Cbz.Gly.Phe	–	–

–: Low amounts of products were detected only after 5 days of reaction and no initial rates could be calculated.

Table 3
By-products synthesised by cardosin A

Carboxyl donor	Amine donor	Synthesised side-products
Cbz.Phe	Phe.OMe	Cbz.Phe.Phe.Phe.OMe
Cbz.Val.Phe	Phe.OMe	Cbz.Val.Phe.Phe.Phe.OMe
Cbz.Gly.Phe	Phe.OMe	Cbz.Gly.Phe.Phe.Phe.OMe
Cbz.Gly.Leu	Phe.OMe	Cbz.Gly.Leu.Phe.Phe.OMe
Cbz.Val.Leu	Tyr.OMe	Cbz.Val.Val.Leu.Tyr.OMe

Only reactions where side-products were detected are represented.

Transesterification reactions are normally observed only when the mechanism involves an acyl enzyme intermediate, as with lipases or serine proteases [24]. This activity is not known for aspartic proteinases like cardosin A. Nevertheless, the exact mechanism of aspartic proteinases is still under discussion and the possibility of the nucleophilic mechanism (with the formation of intermediates that contain a covalent bond) cannot be completely excluded [25]. The coupling of some peptides, as shown in Table 3, resulted in a mixture of several by-products such as Cbz.Phe.Phe.Phe.OMe, which might have been originated by enzymatic hydrolysis of the methyl ester bond on the recently produced peptide (Cbz.Phe.Phe.OMe), followed by immediate coupling of Cbz.Phe.Phe with Phe.OMe. No free ester peptide (produced either by enzymatic or non-enzymatic hydrolysis) could be detected in our experiments, although a reference to non-enzymatic degradation of Phe.OMe has been reported [26]. Our results encourage to further investigating the mechanism of aspartic proteinases.

Additionally, the coupling of Cbz.Val.Leu with Tyr.OMe originated not only Cbz.Val.Leu.Tyr.OMe product but also the Cbz.Val.Val.Leu.Tyr.OMe peptide. The amino acid composition of this peptide was determined by amino acid analysis and by molecular weight determination by mass spectrometry. Additionally, its primary structure was determined by MS–MS. We could not detect enzymatic removal of Cbz from the initial substrate. Additionally, purity of the Cbz.Val.Leu substrate was investigated by mass spectrometry and no traces of Val.Leu or Val were detected. To our knowledge, no spontaneous release of Cbz from peptides has been reported, which may indicate enzymatic degradation of this blocking agent by cardosin A.

Furthermore, coupling of Cbz.Val.Leu with Phe.OMe, Met.OMe, Val.OMe and Leu.OMe originated low amounts of products but no side-products were detected. Additionally, synthesis of Cbz.Val.Phe with Tyr.OMe originated only the expected Cbz.Val.Phe.Tyr.OMe. These findings, taken together, lead us to conclude that Cbz.Val.Val.Leu.Tyr.OMe peptide may have been formed by hydrolysis of the Cbz–Val bond and by hydrolysis of Val–Leu bond from Cbz.Val.Leu.Tyr.OMe initial product. Specific interactions of Cbz.Val.Leu.Tyr.OMe peptide with the enzyme active cleft along with the adequate solubility of this peptide in the aqueous phase (with the corresponding low solubility of Cbz.Val.Val.Leu.Tyr.OMe in the aqueous phase and subsequently extraction to the organic phase) made possible the synthesis of this side-product. Such activity could imply an application of cardosin in de-blocking processes, which would be important for chemical synthesis purposes.

4. Conclusions

In conclusion, cardosin A acted as a useful catalyst in the synthesis of peptide bonds. Additionally, the reversion of hydrolysis has shown to be useful to gain further insights into aspartic proteinase specificity, specifically in what concerns secondary specificity. Namely, in the case of cardosin A specificity it allowed to put in evidence particular characteristics of this enzyme specificity. Furthermore, the action of this catalyst in the removal of the Cbz group from blocked substrates may reveal to be a promising area of investigation.

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