

The Characterisation of the Collagenolytic Activity of Cardosin A Demonstrates its Potential Application for Extracellular Matrix Degradative Processes

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Abstract: Type I collagen is the major fibrous protein of mammals being needed to strengthen and organise the extracellular matrix (ECM). Connective tissue components are modulated by matrix metalloproteinases, which are critical for disintegration and remodelling of ECM under physiological and pathological conditions.

Cardosin A is an abundant aspartic proteinase (AP) from *Cynara cardunculus* L. that has been shown to be able to hydrolyse fibrillar collagen within the α -chains. The aim of this work is the characterisation of collagen degradation by cardosin A, since in the native state fibrillar collagen is resistant to most proteolytic enzymes. The pattern of type I collagen hydrolysis by cardosin A is defined and maintained for at least 24 hours of digestion, suggesting that cardosin A can hydrolyse collagen at a small number of specific peptide bonds. N-terminal sequencing of hydrolysis products identified one cleavage site as being Phe⁴⁶⁴-Gln⁴⁶⁵ in the α 2 chain of collagen I. Two peptides were synthesised correspondent to collagen I specific sequences, in order to produce two polyclonal antibodies, that allowed the identification of three collagen fragments following cardosin A cleavage. Defining the mechanism of collagen cleavage by collagenases and other enzymes, like cardosin A, is important for the comprehension of physiological and pathological processes affecting the ECM. To our knowledge, this is the first study of *in vitro* collagenolytic activity of a plant AP. Therefore, in view of the cardosin A restricted specificity towards collagen this enzyme may be proposed for an eventual medical or technical procedures assisting ECM remodelling.

Keywords: Type I collagen; cardosin A; aspartic proteinase; fibrosis; adhesion.

INTRODUCTION

Type I collagen is the major protein component of the extracellular matrix (ECM) in all higher vertebrates, corresponding to 30 % of total protein. It belongs to the fibrillar group of collagens and constitutes more than 95 % of total collagen in many tissues, such as skin, tendons and bone [1,2]. Collagen is synthesised as a soluble procollagen, containing N- and C- propeptides. These are cleaved by specific N- and C- proteinases to generate monomers that compose collagen fibrils [3,4]. Collagen molecules are composed of three helical chains: two α 1 with a molecular weight of 100 kDa and one α 2 with a molecular weight of 95 kDa. Each chain is characterized by the presence of two telopeptides, in both terminals and by a repeated sequence of 338 Gly-X-Y triplets [2,5].

The mechanism of fibrillar collagen degradation has been the subject of considerable interest mainly due to its subjacent medical implications. Disturbance in the balance between ECM production and degradation leads to formation

of chronic ulcers with excessive ECM degradation, or to fibrosis, for example hypertrophic scars or keloids characterized by excessive accumulation of ECM components. Postsurgical fibrosis and consequent adhesion formation is an everyday problem in clinical practice, a major cause of morbidity and expense, and an occasional cause of mortality [6].

Although knowledge of fibrosis pathogenesis has improved in recent years, but a great deal about these phenomena remains unclear [7]. In the native state collagen is resistant to most endogenous and exogenous proteolytic enzymes. Even bacterial proteinases have weak degradative activity against collagen [8,9]. The only mammalian proteinases that have been shown to attack the native triple helical region of type I collagen are the interstitial collagenases of the matrix metalloproteinases (MMPs) family, MMP-1, MMP-8 and MMP-13. These collagenases cleave the type I collagen triple helix across all three chains at Gly⁷⁷⁵-Leu/Ile⁷⁷⁶, generating the characteristic TC^A and TC^B fragments [10-13]. These fragments can be further degraded either by these collagenases or by non-specific proteinases from the ECM [14]. Proteinases with broad specificity, such as cysteine proteinases, attack only the extra

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helical regions (representing only 4 % of the molecule) that are located at both ends of native collagen (telopeptides) [15]. Because the telopeptides are involved in intra- and intermolecular links, this attack may separate individual molecules. More recently it was demonstrated that cathepsin K, a mammalian cysteine proteinase, degrades type I collagen by using a mechanism reminiscent of bacterial collagenases [16,37].

None of these enzymes appear suitable for post-surgical applications such as the reduction in the amount of fibrotic tissue that is formed as a consequence of surgical intervention; such treatment requires a collagenolytic activity that produces a small number of specific fragments by digestion within the triple helix, combined with minimal damage to the surrounding healthy tissue. This is particularly relevant in view of the work of Gardi *et al.* [17], which demonstrates very clearly that both *in vivo* & *in vitro* the generation of a range of low molecular weight fragments of collagen will increase rather than diminish the fibroproduction.

Cardosin A and cardosin B are dimeric aspartic proteinases (EC 3.4.23) from pistils of *Cynara cardunculus* L. whose milk-clotting activity has traditionally been used in Portugal for cheese making [18]. Although the function of these proteinases present in the flowers of *Cynara cardunculus* L. is still unclear, several studies have been developed concerning proteolytic processing, physical-chemical characterisation and its specificity aspects such as a strong preference to cleave bonds between residues with large hydrophobic side-chains [19-23]. Our studies *in vitro* have demonstrated that cardosin A is suitable for limited proteolysis, while cardosin B has a more general proteolytic activity [20,22,23]. Furthermore, cardosin A has been characterized regarding catalytic and specificity aspects in non conventional environments [20,21,24] and concerning its thermal stability using biophysical approaches [25].

We have therefore, instigated two parallel research studies to evaluate the usefulness of the cardosins for the treatment of surgical adhesions. The present study demonstrates that cardosin A cleaves collagen within the triple helix; parallel studies, that are currently in progress, using an animal model of intestinal adhesion produced by normal surgical procedures within the abdominal cavity show that chitosan sponges with immobilised cardosin A cause a significant reduction in the amount of fibrotic tissue that is formed after intervention [26-28]. To our knowledge, this is the first study of *in vitro* collagenolytic activity of a plant aspartic proteinase. Thus, the present work intends to characterize *in vitro* cardosin A activity with the aim to determine whether cardosin A cleaves native type I collagen in its triple helix and identify the cleavage sites. Since, proline-rich proteins are unusually difficult to sequence, namely by mass spectrometry [29], two peptides (corresponding to the two sequences flanking the identified cleavage bond) were synthesised and two polyclonal antibodies were produced in order to identify collagen fragments following proteolytic cleavage. The results allowed us to propose models to explain the interaction between the enzyme and the substrate during hydrolysis.

EXPERIMENTAL PROCEDURES

Enzyme Purification – Fresh flowers of *Cynara cardunculus* L. were collected from wild plants and cardosin A was purified as described previously [19]. After salt removal from enzyme solution by size exclusion chromatography (*Hiprep 26/10 Desalting*, Amersham Pharmacia Biotech), cardosin A solution was concentrated by lyophilisation (*Flexy-Dry* lyophilisator) for 24 hours at 100 mtorr and -50°C . Purity of cardosin A was measured by SDS-polyacrylamide gel electrophoresis (BioRad) according to Laemmli [30].

Preparation of Acid-soluble Type I Collagen - Native human type I collagen (Sigma) was dissolved in 0.5 M acetic acid overnight at 4°C and then dialysed against 0.2 M phosphate-citrate buffer, pH 5.0 for cardosin A assays, or 50 mM Tris-HCl buffer, pH 7.4 for MMP-1 digestion (for comparative purposes). Type I collagen was used at a final concentration of 8 mg/ml (Micro BCATM, Pierce).

Enzymatic Assay – The cardosin A was added to type I collagen at a weight ratio of active enzyme:substrate of 1:50 and the reaction was carried out at 37°C or at 25°C for 24 hours. Cardosin A activity was previously verified according to Veríssimo *et al.* [23] with slight modifications. Although cardosin A had demonstrated activity at physiological conditions [27] the reactions were carried out at pH 5.0, optimal conditions for cardosin A activity, in order to achieve higher yields for sequence purposes.

Peptide Sequence Analysis of Collagen Fragments – Type I collagen fragments generated by cardosin A were separated by SDS-PAGE on a 10 % gel (*Mini-Protein II*, BioRad), electroblotted to a polyvinylidene difluoride membrane (BioRad) using 3-cyclohexylamino-1-propanesulfonic acid buffer (Sigma) for 16 hours at 400 mA. After transfer, the membrane was stained with 0.1 % Coomassie Brilliant Blue (Sigma) containing 50 % methanol and 10 % acetic acid (Merck) and destained with 50 % methanol (Merck). The type I collagen fragment bands were excised and N-terminal sequencing was performed on a 476A protein sequencer (Applied Biosystems). The first 10-14 residues were determined and compared with known sequences of type I collagen (SWISS-PROTTM Protein Data Bank).

Peptide Synthesis - Criteria for selection of suitable sequences for synthesis were: a) the greatest homology with human type I collagen (and not with other collagen types or other ECM proteins); b) the presence of rare amino acids in native collagen chain; c) the absence of modified prolines. Two peptides corresponding to the sequences 447-457 and 482-492 of the 2(I)-chain, namely -Gln-Gly-Val-Gln-Gly-Gly-Lys-Gly-Glu-Gln- (peptide 1) and -Glu-Arg-Gly-Leu-His-Gly-Glu-Phe-Gly-Leu- (peptide 2), were synthesised using a Fmoc chemistry (The Krebs Institute, University of Sheffield, U.K.). The synthesised peptides were purified by reversed-phase HPLC on a C₁₈ column (Vydac) and correct assembly was verified by sequence analysis and by mass spectroscopy. Peptide 1 was synthesised with a C-terminal polylysine tree to produce an immunogen that did not require coupling to any carrier protein, while peptide 2 was coupled

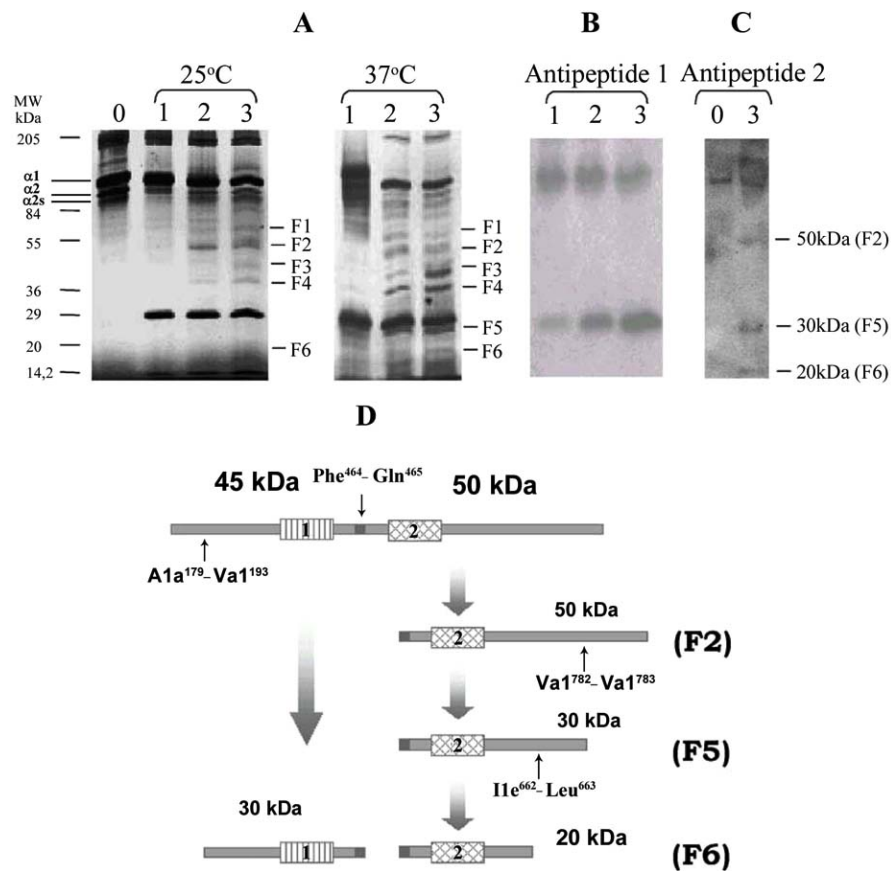


Fig. (1). (A) SDS-PAGE time-course of the digestion of human type I collagen by cardosin A. Collagen was digested at 25 or 37 °C in 200 mM phosphate-citrate buffer (pH 5.0). At selected times the reactions were stopped with equal volume of SDS-PAGE denaturing solution and the samples loaded on a 4-15 % gradient gel (*PhastSystem* – Amersham Pharmacia Biotech). The gel was silver stained. Lanes represent: 0 – intact type I collagen, with 1 and 2 chains; 1-3 – time-course of digestion of type I collagen by cardosin A, 1, 8, and 24 hours. (B) Identification of human type I collagen products of cardosin A cleavage using anti-peptide 1 antibody. The lanes represent the time-course of the reactions (1-3: 1, 8 and 24 hours) and one fragment of 30 kDa was detected. (C) Identification of human type I collagen products of cardosin A cleavage using anti-peptide 2 antibody. Lane 0 represents intact collagen and lane 3 represents collagen digested by cardosin A for 24 hours. (D) Proposed mechanism for the action of cardosin A on human type I collagen. Sequencing data has shown that cleavage occurs at residues 464/465. F1-F6 correspond to further analysed collagen fragments.

to thyroglobulin *via* the NH₂ group using glutaraldehyde [31].

Preparation of Anti-peptide Antibodies for Type I Collagen Detection - New Zealand white rabbits were immunised five times at fortnightly intervals by subcutaneous injection of 250 µg of peptide (sequence 1) or conjugated to thyroglobulin (sequence 2) emulsified with complete (first immunisation) or incomplete (booster immunisations) Freund's adjuvant (Sigma). Serum samples from rabbits were then tested by ECL detection method (Amersham Pharmacia Biotech) for reactivity with both immunising peptides and human type I collagen.

SDS-PAGE and Immunoblotting - Collagen fragments were analysed on a 4-15 % gradient polyacrylamide gel (*PhastSystem*, Amersham Pharmacia Biotech) under reducing conditions. The gels were silver stained using an established protocol [32]. Gels were scanned on a Gel Image Analyzer (FX-710 scanner, Quantity ONE software - Bio-Rad), in order to estimate molecular masses and protein band optical densities. The electrophoresed samples were

transferred to nitrocellulose membranes and blocked for 1 hour at room temperature in 5 % milk (w/v) in TBS-T (Sigma) before immunoblotting. Blocked membranes were incubated overnight at 4 °C under agitation with a rabbit anti-peptide antibody or control serum, diluted 1:100 in TBS-T. After three washes with TBS-T, the membranes were incubated for 2 hours at ambient temperature with the HRP labelled second antibody, diluted 1:1000 in TBS-T. The membranes were then washed three times with TBS-T and detection was carried out with ECL reagents (Amersham Pharmacia Biotech) following the manufactures instructions.

RESULTS

Enzymatic Assay - To investigate the mechanism of collagen fragmentation by cardosin A, human type I collagen was incubated at 25 or 37 °C with the enzyme (enzyme/substrate mass ratio of 1/50) for 1, 8 and 24 hours and the reactions were stopped by adding an equal volume of SDS-PAGE denaturing solution. Blank assays were also performed by incubating type I collagen in the same conditions without proteinase. Fig. (1A) shows intact human

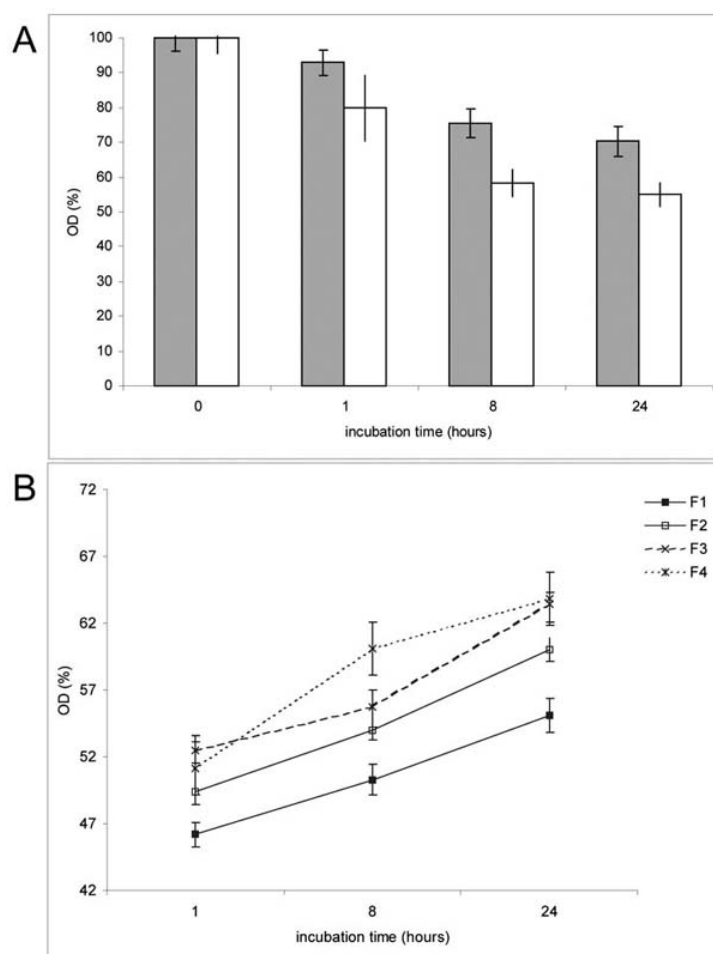


Fig. (2). Optical density analysis of human type I collagen digestion by cardosin A: **A-** The graph represents digestion of $\alpha 1$ (■) and $\alpha 2$ (□) chains of type I collagen during incubation time with enzyme; **B-** Graphic representation of F1-F4 collagen fragments production during reaction. Hydrolysis reaction was carried out at 37°C. 100% was set to optical density of intact collagen chains. The optical density was determined by densitometry of product bands (n=5). On the plot are represented deviation bars.

type I collagen (commercially obtained, lane 0) with its characteristic $\alpha 1$ and $\alpha 2$ chains. It is also possible to visualise $\alpha 2$ s that has been described as being a result of collagen commercial extraction from tissues with pepsin [33]. The pattern of type I collagen hydrolysis by cardosin A (25°C and 37°C) was found to be well defined and maintained until 24 hours of digestion, suggesting that cardosin A can hydrolyse collagen molecules at a small number of specific peptide bonds. With the anti-peptide1 antibody one fragment of 30 kDa was revealed and the anti-peptide 2 antibody allowed the detection of F2, F5 and F6 collagen fragments (Fig. 1B-C). At last, Fig. (1D) represents a proposed mechanism for the action of cardosin A on human type I collagen.

Fig. (2) shows that some of the products generated after 8 hours of digestion (F1, F2, F3 and F4) are not further digested, since their concentration increases during the reaction time. $\alpha 1$ and $\alpha 2$ collagen chains digestion is also verified.

In order to analyse the resultant products of collagen degradation by cardosin A, similar experiments with MMP-1 were performed. Fig. (3) shows $\alpha 1$ and $\alpha 2$ collagen chains

and it is possible to visualise $\alpha 2$ s. Among the generated products by cardosin A, one has an apparent molecular weight of 25 kDa, equivalent to the characteristic TC^B product of MMP-1 activity [10-13], which may suggest a cleavage site at approximately one-quarter of the distance from the C-terminal, namely at Gly⁷⁷⁵-Ile/Leu⁷⁷⁶ peptide bond. The collagen chains are simultaneously digested by both enzymes.

Identification of Type I Collagen Cleavage Sites by N-Terminal Sequencing – With the aim of identifying the cleavage sites of type I collagen by cardosin A, N-terminal amino acid sequencing was performed. Evidence for a cleavage site inside the triple helix corresponding to the Phe⁴⁶⁴-Gln⁴⁶⁵ of $\alpha 2$ -chain of type I collagen was found. Sequencing studies on other peptides were less useful since the high content of proline and hydroxyproline residues in collagen molecules reduces the efficiency of both the sequencing process and the identification of the specific point of cleavage within the highly repetitive protein sequence.

Immunological Detection of Cardosin A Hydrolysis Products of Human Type I Collagen – In view of the

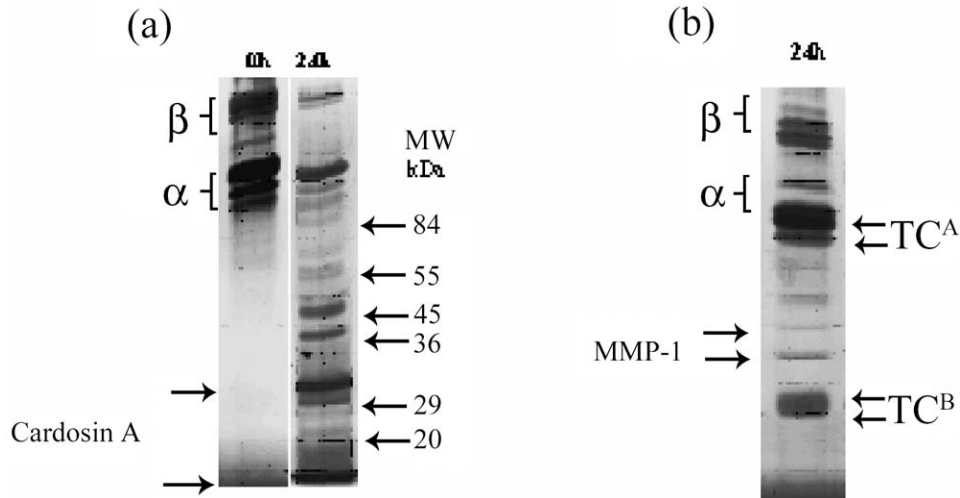


Fig. (3). SDS-PAGE of human type I collagen digestion with cardosin A (a) and MMP-1 (b). Collagen was digested for 24 hours at 37 °C in 200 mM phosphate-citrate buffer (pH 5.0) for cardosin A assay and in 50 mM Tris-HCl buffer (pH 7.4) for MMP-1. The molecular weights of the main fragments of cardosin A collagenolytic action are indicated in the figure (a), as well as for the characteristic TC^A (75 kDa) and TC^B (25 kDa) fragments of MMP-1 (b). Both enzymes are identified by arrows: dimeric cardosin A (30 kDa and 15 kDa subunits) and MMP-1 (latent and active forms respectively, 55 and 45 kDa).

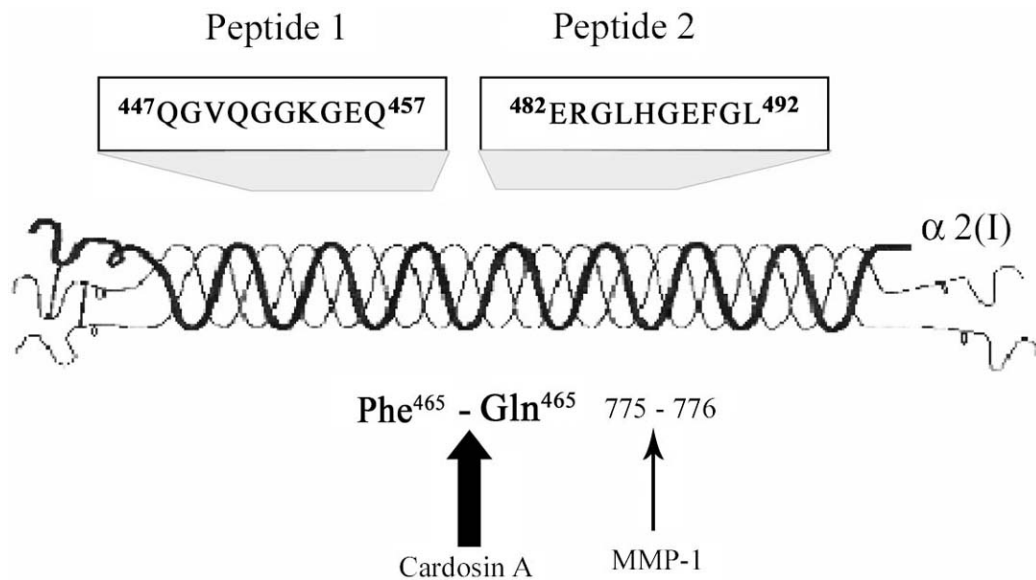


Fig. (4). Schematic representation of the sequences of 2-chain of human type I collagen selected for peptide synthesis. These peptides were used to produce specific antibodies towards adjacent regions of the cleavage site (Phe⁴⁶⁴-Gln⁴⁶⁵) identified by N-terminal sequencing. MMP-1 cleavage site is represented and corresponds to Gly⁷⁷⁵-Leu⁷⁷⁶ of $\alpha 2(I)$.

difficulty of using protein sequencing to characterise the resultant fragments, two peptides were synthesised and used to produce specific antibodies towards two adjacent regions of the identified cleavage site (Phe⁴⁶⁴-Gln⁴⁶⁵). The two specific anti-peptide antibodies produced, were found to be reactive to type I collagen fragments following proteolytic cleavage. Their positions are shown in the Fig. (4).

Two different temperatures, 37 and 25 °C, were used for anti-peptide antibody detection procedures. At 37 °C (physiological temperature), fragments with molecular masses of 50 and 30 kDa were found to be immunopositive to the anti-peptide 2 antibody. Therefore both fragments must contain residues 482-492 (peptide 2 sequence), corresponding to this region of $\alpha 2$ -chain (Fig. 1C). Since a

more slower digestion rate was achieved at a lower temperature, a second digestion was carried out at 25 °C and the pattern of digestion analysed using anti peptide 1 antibody. The antibody reacted with a 30 kDa fragment, the concentration of which increased with time (Fig. 1B).

DISCUSSION

Several investigators have reported that collagen denaturation occurs over 35-37°C [34, 35]. However, Sato *et al.* [36] reported contrasting results, based on CD studies, showing that approximately 90% of helicity of native type I collagen is maintained at 37 °C. Our work corroborate these studies since cardosin A has shown to produce the same hydrolysis pattern of type I collagen at 25 and 37 °C.

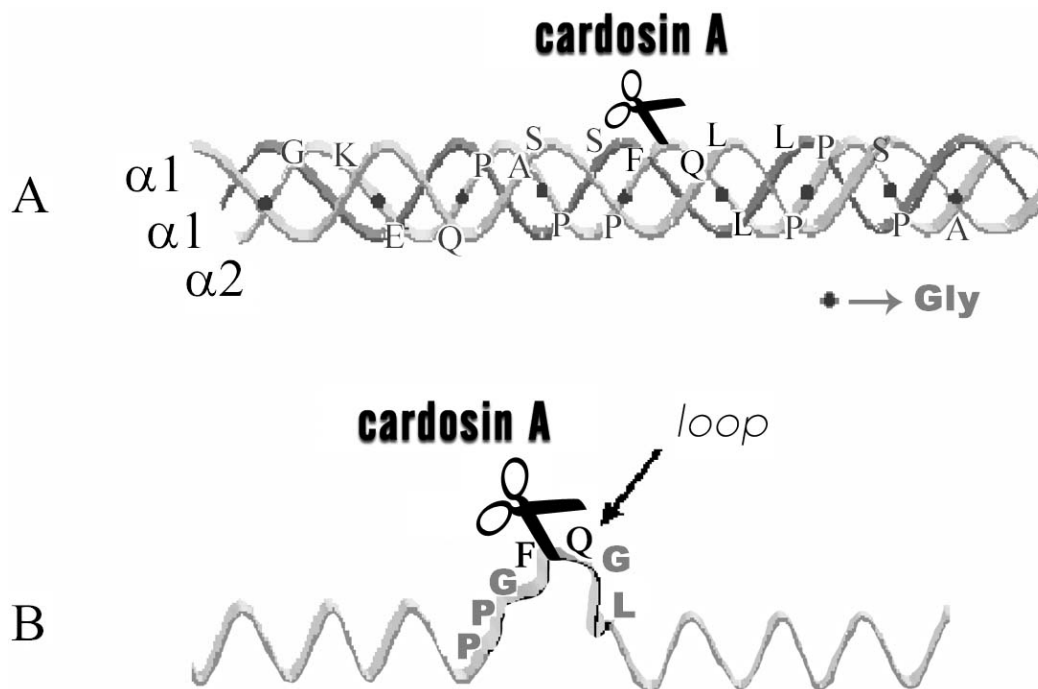


Fig. (5). Proposed mechanism for collagenolytic degradation of human type I collagen by cardosin A. Type I collagen molecules consist of two $\alpha 1$ -chains and one $\alpha 2$ -chain associated in triple helix conformation. These models take into account the known secondary specificity of cardosin A, towards hydrophobic residues. (A) In this model hydrophobic residues are located on the surface of collagen molecule involving the three α -chains for cleavage within native triple helix at Phe⁴⁶⁴-Gln⁴⁶⁵ peptide bond. (B) Cleavage within triple helix at Phe⁴⁶⁴-Gln⁴⁶⁵ peptide bond by cardosin A is sustained by loop formation involving hydrophobic residues of $\alpha 2$ -chain of collagen.

Results of type I collagen degradation by MMP-1 were compared with cardosin A collagenolytic activity (Fig. 3). Among the generated products by cardosin A, one has an apparent molecular weight of 25 kDa, equivalent to the characteristic TC^B product of MMP-1 activity, which may suggest a cleavage site at approximately one-quarter of the distance from the C-terminal, namely at Gly⁷⁷⁵-Ile/Leu⁷⁷⁶.

The sequencing data and immunodetection results, suggested the following mechanism for cardosin A digestion of collagen (Fig. 1D). Sequencing has shown that cleavage occurs at residues Phe⁴⁶⁴-Gln⁴⁶⁵. This would produce 2 fragments with molecular weights of 45 and 50 kDa, exactly as it has been demonstrated by this study. The 50 kDa fragment must contain the peptide 2 sequence as it cross reacts with the antibody raised against this region, which is again consistent with the proposed model. Additional cleavage points must also exist, since additional fragments of 30 and 20 kDa were produced. N-terminal analysis showed that the 20 kDa fragment begins at residue 465 and must therefore extend, approximately, to residue 660-680. The 30 kDa fragment is immunopositive with anti-peptide 2 antibody and it must therefore extend to a residue position between 760-780. Examination of the collagen sequence suggests that the 20 kDa fragment arises from cleavage at Ile⁶⁶² and the 30 kDa fragment by cleavage at Val⁷⁸², consistent with the known specificity of cardosin A. An additional fragment of 30 kDa that cross reacted with the anti-peptide 1 antibody and therefore suggests an additional cleavage as occurred at Ala¹⁷⁹. Graph A in Fig. (2) shows that $\alpha 1$ collagen chain is also digested. Furthermore, in Fig. (1A) it is possible to visualise two fragments, F1 and a weak

band between F2 and F3 (37°C), suggesting the occurrence of cleavage in the same region on $\alpha 1$ collagen chain, having in mind the proposed mechanism for cardosin A collagenolytic activity.

Type I collagen is one of the most abundant molecules in mammals and its degradation is an important physiological and pathological issue, since in the native state fibrillar collagen is resistant to most proteolytic enzymes.

Only three collagenolytic mechanisms have been described until the present. One is used by interstitial collagenases that cleave the stable triple helices of collagen. The second is used by broad specific proteinases, such as cysteine proteinases, which cleave the nonhelical telopeptides of native collagen molecules. A third mechanism, reminiscent to bacterial collagenases, was recently proposed for cathepsin K, a cysteine proteinase involved in osteoclastic bone resorption [37]. This enzyme shows the peculiarity of cleaving native collagen at multiple sites of the triple helices.

Cardosin A has been shown to cleave preferentially between two hydrophobic residues [23,24]. Since the Phe-Gln peptide bond does not have the precise characteristic hydrophobic motif required for cardosin A hydrolysis, two models for proteolytic activity may be proposed (Fig. 5). The first model is based on the amino acid composition that is close to the hydrolysed bond, involving the surface residues of the triple helix of collagen molecule. We can notice that all amino acids exposed are large and hydrophobic (Fig. 5A). The second model is based on modelling studies of protein with helical structure [38]. These studies revealed that slight

structural modifications, such as loop formation, may occur without protein denaturation allowing the adaptation of the substrate to the enzyme [39]. Once again hydrophobic residues are involved (Fig. 5B).

All these approaches taken together allow us to conclude that cardosin A shows collagenolytic activity, directed within the helical region, hitherto believed to be resistant to all non-mammalian proteinases.

The results also show that cardosin A uses a mechanism of collagenolytic activity by acting within the triple helix of collagen molecules and probably at both telopeptide ends, similar to the mammalian cathepsin K collagenolytic action [37,40]. To our knowledge, this is the first report of a plant proteolytic enzyme with collagenolytic activity. In fact, cardosin A uses a more specific hydrolysis mechanism when compared with bacterial collagenases, behaving, therefore, more closely as animal collagenases on ECM remodelling.

Since fibrotic states result from the excessive synthesis and deposition of collagen and since collagen type I degradation plays a central role in the resolution of this pathology, our data may lead to the improvement of key areas given that it has been demonstrated that cardosin A hydrolyses collagen within the helices. This collagenolytic activity of cardosin A could be used in surgery to assist on the ECM remodelling or in other medical or technical processes where an increase of ECM degradation is required.

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ABBREVIATIONS USED

TIMPs	=	Tissue Inhibitors of Metalloproteinases
MMPs	=	Matrix Metalloproteinases
ECM	=	Extracellular Matrix
TC	=	Tropocollagen
ECL	=	Enhanced Chemiluminescence
HRP	=	Horseradish Peroxidase
TBS-T	=	Tris-Buffered Saline-Tween

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