Molecular cloning and characterization of cDNA encoding cardosin B, an aspartic proteinase accumulating extracellularly in the transmitting tissue of *Cynara cardunculus* L.

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Abstract

Cardosins A and B are related aspartic proteinases from the pistils of *Cynara cardunculus* L., whose milk-clotting activity has been exploited for the manufacture of cheese. Here we report the cloning of cardosin B cDNA and its organ, tissue and cytological localization. The cDNA-derived amino acid sequence has 73% similarity with that of cardosin A and displays several distinguishing features. Cardosin B mRNA was detected in young inflorescences but not in pistils of fully opened inflorescences, indicating that its expression is developmentally regulated. The proteinase, however, accumulates in the pistil until the later stages of floral development. Immunocytochemistry with a monospecific antibody localized cardosin B to the cell wall and extracellular matrix of the floral transmitting tissue. The location of cardosin B in the pistil is therefore clearly different from that of cardosin A, which was found at protein storage vacuoles of the stigmatic papillae and has been suggested to be involved in RGD-mediated proteolytic mechanisms. In view of these results the possible functions of cardosin B in the transmitting tissue are discussed.

Introduction

Aspartic proteinases (aspartic endopeptidases, EC 3.4.23) are one of the four main classes of proteinases, the others being serine, cysteine and metallo proteinases (Barrett *et al.*, 1999). Because of their involvement in pathological conditions such as AIDS, malaria, cancer (James, 1998) and, more recently, Alzheimer's disease (Sinha *et al.*, 1999; Vassar *et al.*, 1999; Yan *et al.*, 1999; Lin *et al.*, 2000), aspartic proteinases have long been the subject of intensive research (Blundell *et al.*, 1998). Aspartic proteinases have been found in retroviruses, bacteria, fungi, parasites, animals and plants (James, 1998) and, in spite of their widespread occurrence in nature, they share common features in terms of catalytic activity, amino acid sequence and 3-dimensional (3D) structure. With the exception of a few aspartic proteinases from bacteria (Oda *et al.*, 1998) and the recently identified BACE, the Alzheimer's β-secretase (Sinha *et al.*, 1999; Vassar *et al.*, 1999; Yan *et al.*, 1999; Lin *et al.*, 2000), the majority of proteins belonging to this class are characterized by their susceptibility to pepstatin inhibition and are active at acidic pH. On sequence databases, aspartic proteinases can be identified by the presence of the conserved hydrophobic~hydrophobic~DT/SG sequence motif found once in retroviral enzymes, which are active as homodimers, and twice in all other microbial and eukaryotic homologues due to duplication and subsequent fusion to the ancestral gene (Tang *et al.*,

1978). Each DT/SG sequence contributes one catalytic aspartate and resides within the large extended active-site cleft which separates the two symmetric domains observed in the common overall fold of aspartic proteinase 3D structures (Blundell *et al.*, 1998).

In plants, aspartic proteinases have been found in seeds, leaves, flowers and petals of many species and in the pitchers of several insectivorous species (Mutlu and Gal, 1999). Most of them are secreted or accumulate in protein storage vacuoles (Rodrigo *et al.*, 1991; Runeberg-Roos *et al.*, 1994; Ramalho-Santos *et al.*, 1997; Mutlu *et al.*, 1999). In seeds, aspartic proteinases are thought to participate in storage protein processing at the initial stages of seed germination, whereas in the pitchers of insectivorous plants they are probably involved in protein degradation of trapped organisms. Extracellular aspartic proteinases from tomato and tobacco leaves degrade secreted pathogenesis-related proteins *in vitro* (Rodrigo *et al.*, 1989, 1991) and expression of the tomato proteinase gene is induced by wounding (Schaller and Ryan, 1996). Recently the aspartic proteinases from developing tracheary elements of barley root-tip (Runeberg-Roos and Saarma, 1998), daylily petals (Panavas *et al.*, 1999) and the degenerating nucellar cells of the barley ovule (Chen and Foolad, 1997) have been associated with programmed cell death of specific tissues.

We have been working on two aspartic proteinases, named cardosin A and cardosin B, from the pistils of *Cynara cardunculus* L. (family Asteraceae), whose milk-clotting activity has been exploited in Portugal for the manufacture of cheese. Cardosin A has been studied extensively (Veríssimo *et al.*, 1996; Ramalho-Santos *et al.*, 1997, 1998; Costa *et al.*, 1997; Faro *et al.*, 1999) and, very recently, its crystal structure was determined at high resolution (Frazão *et al.*, 1999). Cardosin A accumulates in protein storage vacuoles of the stigmatic epidermal papillae and in vacuoles of the epidermal cells of the style (Ramalho-Santos *et al.*, 1997). Cloning of cardosin A cDNA revealed the presence of an RGD-cell attachment motif within its sequence and led to the identification of a putative receptor in pollen which interacts with the RGD sequence of the proteinase (Faro *et al.*, 1999). These results have suggested a possible role for cardosin A in adhesion-mediated proteolytic mechanisms involved in the pollen-pistil interaction.

Much less is known about cardosin B. In order to gain more insight into the possible biological function(s) of cardosin B we have cloned its cDNA and studied its organ, tissue and cytological localization. In contrast to cardosin A, cardosin B accumulates in the cell wall and in the extracellular matrix of the transmitting tissue, suggesting that the two cardosins may have different roles in the pistil of *C. cardunculus*.

Material and methods

Plant material

Organs of *Cynara cardunculus* L. were collected in the field, in the central region of Portugal, between May and July. Inflorescences were collected at three stages of development: closed, partially opened and fully opened capitula. Seeds were stored at room temperature, while the other organs, roots, midribs, leaves, bracts and flowers were frozen in liquid nitrogen and kept at −80 °C prior to analysis.

cDNA cloning

Total RNA was isolated from inflorescence buds of *C. cardunculus* as described (Pawlowski *et al.*, 1994) and the poly(A) mRNA was isolated using a PolyATract mRNA isolation kit (Promega). Primers used in the initial RT-PCR reaction are Primer S (GTTGCACTAACGAACGATAGG) and Primer R (TTATTGGAGCTGGTAGAATGG). RACE reactions were performed using the Marathon cDNA amplification kit (Clontech) according to the manufacturer's instructions. Primers flanking the open reading frame of cardosin B cDNA are Primer #S (ATGGGTAC-CTCAATCAAAGCAA) and Primer #R (TCAAGCT-GCTTCTGCAAATCC). DNA sequencing was performed in a Vistra DNA Automatic Sequencer 725 (Amersham). Primers were labelled with Texas Red using the 5′ oligonucleotide Texas Red labelling kit (Amersham) and sequencing reactions were carried out with the Termo sequenase premixed cycle sequencing kit (Amersham) by the dideoxynucleotide chain termination method.

RT-PCR analysis

Total RNA was isolated from 100 mg of plant tissue with the RNeasy Plant mini kit (Qiagen). For analysis of cardosin B mRNA expression, the quality and relative amount of RNA was estimated by agarose gel electrophoresis. RT-PCR was performed with the Superscript RT-PCR kit (Gibco-BRL) with similar amounts of total RNA and the following

primers: Primer S (GATCTCGGCTGGGAAAGCG) and Primer R (ATACCATTGCAGTCTACTATCG). PCR products were visualized on an agarose gel as a 689 bp product.

Antibody production

A cardosin B-specific peptide bearing the sequence CVIHPRYDSGD was synthesized at the University of Sheffield (Dr A. Moir, Krebs Institute, Sheffield, UK), purified by reversed-phase HPLC on a C18 column (HPLC Technology) and used for the production of an antibody with thyroglobulin as carrier, essentially as described by Sambrook *et al.* (1989). The thyroglobulin-peptide complex, containing 500 μg of the peptide, was emulsified with Freund's complete adjuvant (Sigma) and injected subcutaneously into a New Zealand rabbit, from which pre-immune blood had been collected. A second injection was given three weeks later with the same amount of thyroglobulin-peptide solution emulsified with Freund's incomplete adjuvant and the rabbit was bled three weeks after this last injection.

Protein extraction and western blotting analysis

Each sample of seeds, roots, midribs, leaves, bracts, corollas and stamens was ground in a mortar and pestle under liquid nitrogen. The same was done to pistils at different stages of development. The tissues were then homogenized at 20% w/v in 100 mM Tris-Bicine, 2% SDS, 2% 2-mercaptoethanol, 8 M urea, and the extracts obtained were centrifuged at $12\,000 \times g$ for 15 min at 4 °C. The supernatants were collected and stored at −20 °C. The protein extracts described above were loaded onto 15% polyacrylamide gels for SDS-PAGE, according to the method of Laemmli (1970). For immunodetection, proteins were transferred onto PVDF membranes overnight in the cold room at 20 V. The membranes were then incubated in blocking solution, 0.5% w/v of skimmed milk in TBS containing 0.1% v/v Tween 20, for 45 min at room temperature, and then incubated for 1 h with 1:400 dilution of the cardosin B antibody or 1:200 of the cardosin A antibody (Ramalho-Santos *et al.*, 1998) in blocking solution. The membranes were washed five times in blocking solution for 5 min and incubated with a 1:1000 dilution of swine anti-rabbit IgG conjugated to horseradish peroxidase for 1 h. The membranes were again washed five times in blocking solution for 5 min and peroxidase activity was developed by luminol chemiluminescence using the ECL method according to the manufacturer's instructions (Amersham).

Immunohistochemistry and immunogold transmission EM

Small pieces of both stigmas and styles were fixed in 4% v/v paraformaldehyde, 0.5% v/v glutaraldehyde, 2% w/v sucrose and 0.05% w/v CaCl$_2$ in 1.25% w/v PIPES buffer pH 7.2, for 2 h at room temperature, dehydrated in a water/ethanol series and embedded in LR White Resin (London Resin, Basingstoke, UK). Semi-thin sections were collected on glass slides coated with 0.1% poly-L-lysine. The sections were first treated with sodium *m*-periodate (saturated solution) followed by 0.1 M hydrochloric acid. After etching, sections were incubated with cardosin B antiserum (1:60) diluted in TBS 500 (0.01 M Tris-HCl pH 7.5, 500 mM NaCl) containing 0.2% v/v Tween-20 and 10% non-immune goat serum (Sigma, G-9023). Immunodetection was performed with Histostain-Plus (Zymed) according to the manufacturer's instructions with the following modifications: washing steps were with TBST 500 (TBS 500 plus 0.2% v/v Tween 20) and the substrate chromogen solution was replaced with Sigma *Fast* BCIP/NBT alkaline phosphatase substrate diluted in 10% w/v polyvinyl alcohol (PVA 40–70 kDa, Sigma). Sections were mounted in double-distilled and de-ionized water.

Immunogold labelling of cardosin B was performed in ultrathin sections on uncoated nickel grids. Sections were blocked in 5% w/v non-fat dried milk in TBS 500. After blocking, sections were incubated with the cardosin B antiserum (1:100) followed by colloidal gold-labelled protein A (20 nm) (Sigma) diluted 1:65 in 0.01 M Tris-HCl pH 7.5 buffer containing 0.5% v/v fish gelatin plus 0.02% PEG. Control sections were prepared as experimental sections except that buffer or pre-immune serum was substituted for anti-cardosin B. Sections were stained in uranyl acetate and lead citrate before observation on a transmission electron microscope (EM 10C; Zeiss, Oberkochen, Germany) operating at 60 kV.

Results

Cloning and sequencing of cardosin B cDNA

Because cardosins are so abundantly expressed in pistils, several clones were randomly picked up from a closed inflorescence cDNA library and sequenced.

A)

B)

Figure 1. A. cDNA-derived amino acid sequence of cardosin B and its alignment with those of cardosin A and phytepsin. Dots denote gaps in the sequence. The signal and pro sequences are indicated. The predicted plant-specific insert sequence is underlined. The active-site aspartates are in bold and the RGD cell-attachment motif present in cardosin A is in bold italics. Putative N-glycosylation sites are marked by stars. The conserved cysteines in the saposin-like domain are shadowed and the tyrosine common to saposins and the plant-specific insert (PSI) is bold underlined. Cardosin B, *Cynara cardunculus* aspartic proteinase, GenBank accession number CAB40349; Cardosin A, *C. cardunculus* aspartic proteinase, CAB40134; Phytepsin, *Hordeum vulgare* aspartic proteinase, P42210. B. Domain organization of cardosin A and cardosin B precursors. Both structures include a signal peptide (Pre), a prosegment (Pro) and a PSI domain separating the two chains of the mature form. The cysteines in the PSI domain are highlighted and the differences in the positions of N-glycosylation sites between the two proteinases are also depicted.

One of them was a partial cDNA clone of cardosin B, by comparison with the previously determined partial amino acid sequence of the proteinase (Veríssimo *et al.*, 1996). Based on the sequence of this cDNA fragment, primers were designed and a full-length cardosin B cDNA was amplified through rapid amplification of cDNA ends (RACE) from a closed inflorescence (lower portion) cDNA Marathon library.

The cardosin B cDNA contained an open reading frame of 1521 bp whose sequence is available at the EMBL/GenBank databases with the accession number AJ237674. The cDNA-derived amino acid sequence (Figure 1) contains a signal sequence, a pro segment at the N-terminus and a downstream polypeptide sequence that includes the two chains of the mature proteinase. The alignment of the deduced amino acid sequences of cardosin A, cardosin B and phytepsin is also shown in Figure 1. Sequence similarity searches using Blastap revealed that cardosin B displays 73% similarity to cardosin A and 59%, 60% and 58% similarity to phythepsin (accession number P42210), oryzasin (Q42456) and the aspartic proteinase from *Brassica* (accession AAB03108) respectively. An important feature of all plant aspartic proteinases isolated so far is the presence of an insert that separates the two-chain domains and is not present in the mature enzyme. In the case of cardosin B, and according to the information on the primary structure of the protein, this internal segment named PSI (plant-specific insert) is 101 amino acids long. As previously shown by Guruprasad *et al.* (1994), the predicted secondary structure of this domain displays a significant similarity with those of saposins and saposin-like proteins such as NK-lysin and amoeba pore-forming peptide (Liepinsh *et al.*, 1997). In the particular case of cardosin B, the overall similarity at the amino acid sequence level between its PSI and saposins is about 30%. Six conserved cysteines, one invariant tyrosine and a putative N-glycosylation site are remarkably conserved between the sequences of cardosin B PSI and saposins. The two active-site aspartic acid residues of cardosin B are located in the 34 kDa chain, each contained within the conserved DTG and DSG motifs of plant aspartic proteinases. In contrast to cardosin A, cardosin B does not contain the RGD motif, characteristic of integrin-binding proteins. Instead, a putative N-glycosylation site is created in the same region, as the Asp is mutated into Asn. This mutation gives rise to a downstream Asn-His-Thr, a consensus sequence for N-glycosylation in
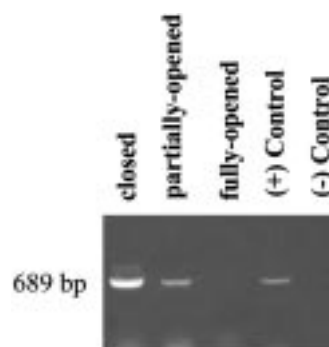

Figure 2. Expression of cardosin B mRNA during floral development. RT-PCR analysis of cardosin B mRNA expression throughout different stages of floral development: closed, partially opened and fully opened capitula. The fourth lane from the left-hand side, (+) control, contains a PCR product from a cardosin B cDNA clone using the same pair of primers, and the fifth lane, (−) control, shows the result of a PCR reaction with the specific cardosin B primers and a cardosin A cDNA clone as template.

which the Asn was shown by protein sequencing to be occupied (data not shown).

Expression of cardosin B mRNA is developmentally regulated

Because cardosin B is found in flowers, expression of its mRNA was evaluated at three different stages of floral development (closed, partially opened and fully opened capitula). Because of the high degree of similarity between the cDNA sequences of cardosins A and B, RT-PCR analysis was used for that purpose. In this assay, cardosin B mRNA was detected as a PCR-amplified fragment of about 689 bp. As shown in Figure 2, the primers used in the RT-PCR reactions did not PCR-amplify a cDNA cardosin A clone indicating therefore that they are specific for cardosin B. The highest levels of cardosin B mRNA were observed at the initial stages of floral development in the closed capitula and its amount decreased throughout floral development (Figure 2). No cardosin B mRNA was detected at the later stages of flower development (Figure 2). Taken together, these results suggest that expression of cardosin B mRNA in the pistil of *C. cardunculus* L. is likely to be developmentally regulated.

Cardosin B is specifically accumulated in pistils throughout floral development

To study the expression of cardosin B at the protein level, a polyclonal antibody was produced against a synthetic peptide bearing a cardosin B sequence
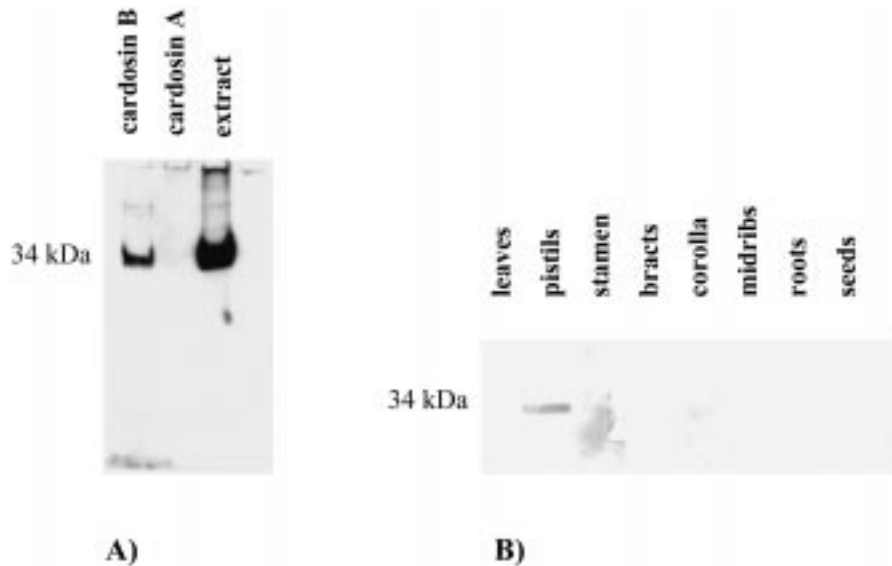
Figure 3. A. Specificity of the anti-cardosin B antibody. Equal amounts of purified cardosin B and purified cardosin A were loaded on a 15% polyacrylamide gel, transferred onto a PVDF membrane and immunodetected with the anti-cardosin B polyclonal antibody by using the ECL method from Amersham. A strong reaction was also observed with cardosin B present in extracts of pistils and no cross-reactivity was observed with any other protein of the extract. B. Organ-specific location of cardosin B. Samples of protein extracts from several organs of *C. cardunculus* were loaded on 15% polyacrylamide gels, blotted and immunodetected with the anti-cardosin B antibody and visualized by chemiluminescence (ECL from Amersham).

Figure 4. Accumulation of cardosin B during floral development and its distribution in mature pistils. A. Protein extracts from closed and partially opened flowers were separated on 15% polyacrylamide gels, blotted and immunodetected with the polyclonal anti-cardosin B antibody. Extracts of mature pistils corresponding to each of the segment depicted in C were also analysed with the anti-cardosin B antibody and with an anti-cardosin A monospecific antibody (B). The band recognized by the anti-cardosin B antibody corresponds to the 34 kDa chain of mature cardosin B whereas the one recognized by the cardosin A antibody is the 31 kDa chain.

(CVIHPRYDSGD), with low homology to cardosin A. This sequence corresponds to a region of the enzyme that is probably exposed, based on the assumed similarity to the 3D structure of cardosin A (Frazão *et al.*, 1999). The anti-cardosin B polyclonal antibody recognized the 34 kDa chain of purified cardosin B, showing no reaction at all with purified cardosin A (Figure 3A). Furthermore, the antibody did not react with any other pistil proteins as shown in Figure 3A. The antibody was subsequently used to examine the expression of cardosin B in different plant organs by immunoblot analysis. The antibody did not recognize any of the proteins from seeds, bracts, midribs, roots, leaves or stamen extracts. As expected, cardosin B was
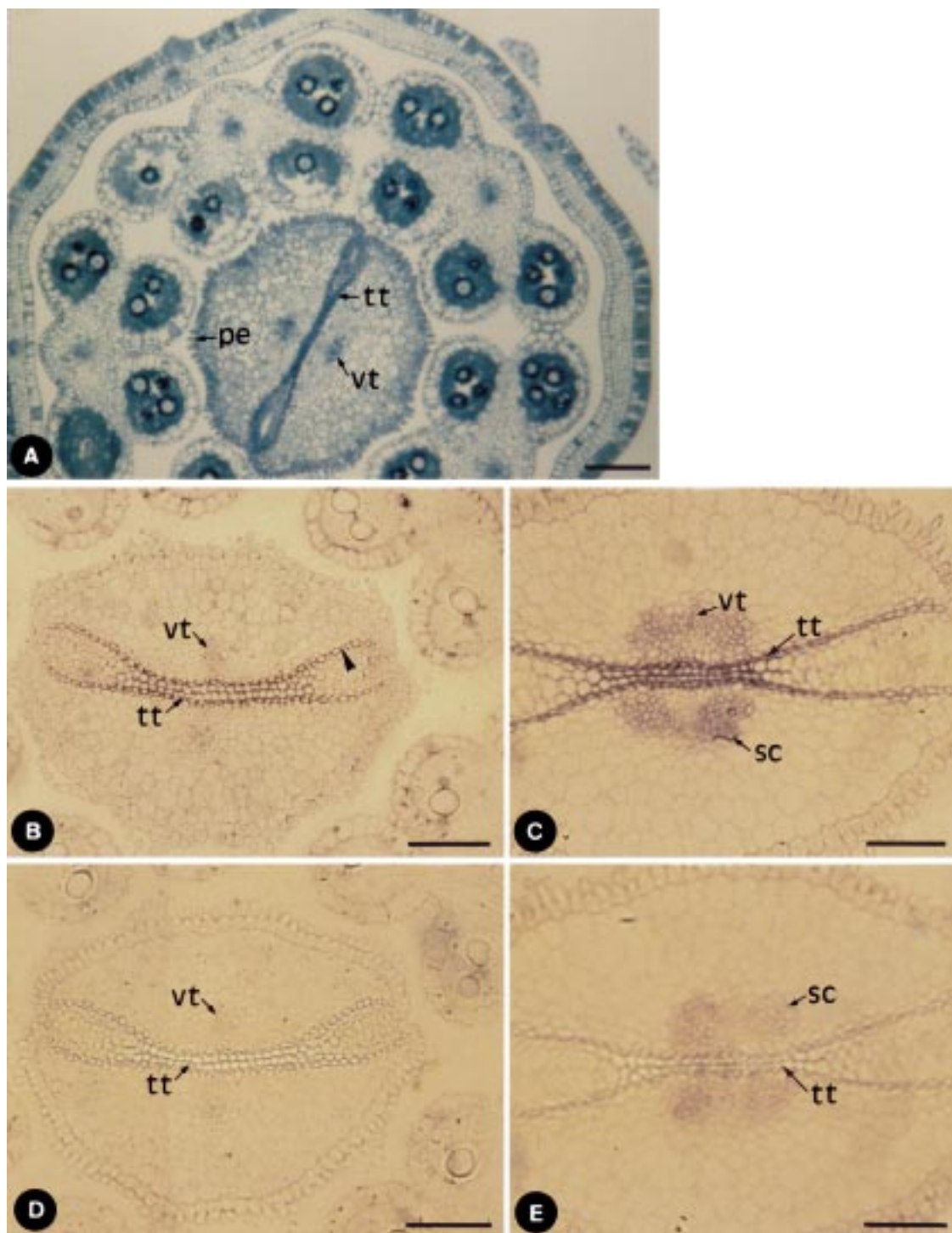
Figure 5. Histolocalization of cardosin B in flowers (florets) of *C. cardunculus*. A. Structure of a closed floret showing, from the outside, fused petals, anthers with developing spores, and stigma. The stigma, with two longitudinal grooves, has a glandular papillate epidermis (pe), parenchyma, vascular tissues (vt), and transmitting tissue (tt). Bar, 100 μm. B and C. Localization of cardosin B in cross-sections of the stigma. Intense staining occurs in the transmitting tissue (tt) of both closed (B) and opened (C) florets. The transmitting tissue occupies the central core of the structure and also extends as subepidermal layers (arrowhead) to the stigma grooves. No colour development was observed either in the papillate epidermis or in other tissues like vascular tissues (vt) or sclerenchyma (sc) developed in the stigma of opened florets. Bars, 10 μm. D and E. Control sections of stigma, of both closed (D) and opened florets (E), where buffer was substituted for primary antibody. Bar, 10 μm.
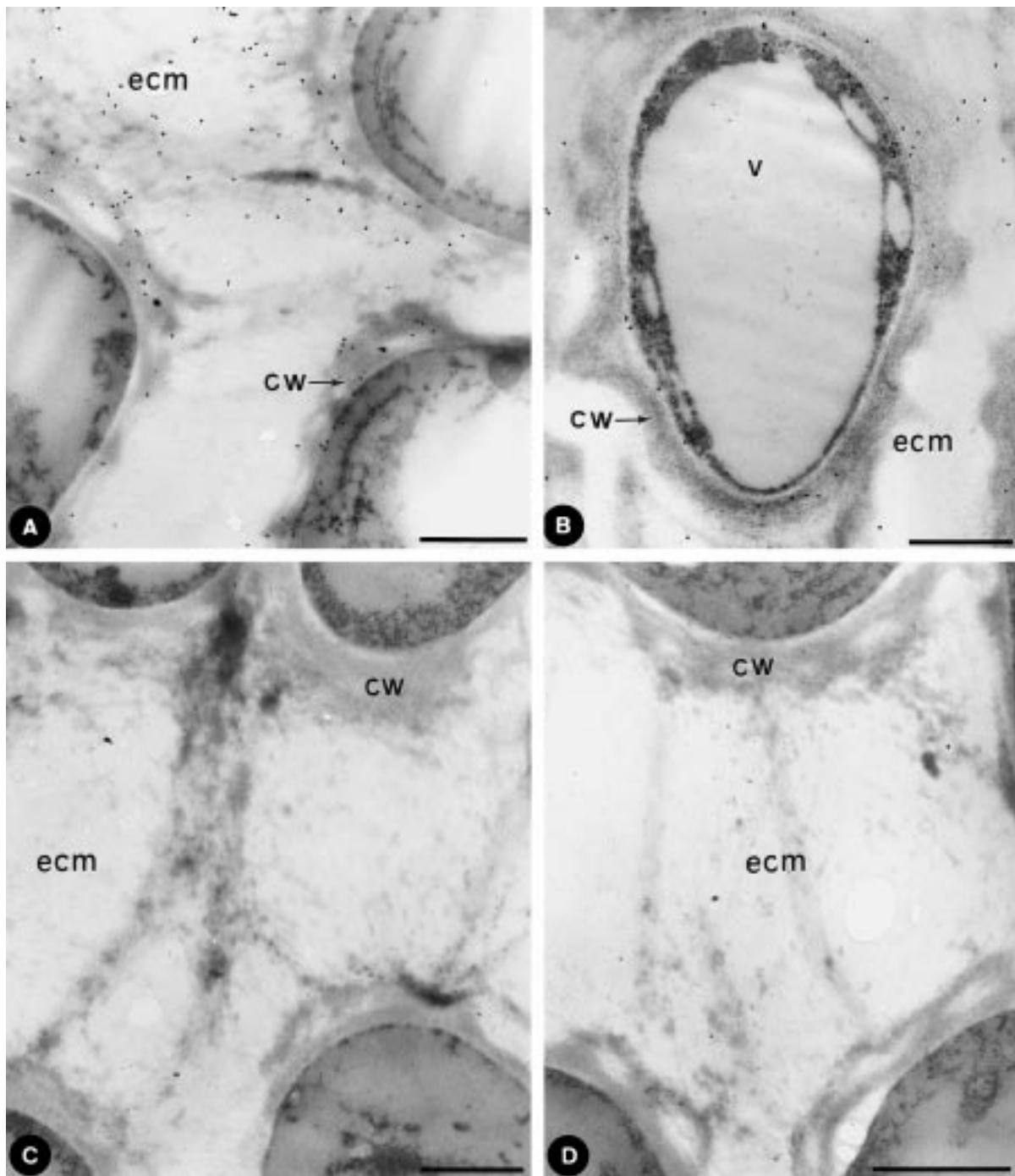
Figure 6. Immunogold-electron microscopy localization of cardosin B in the transmitting tissue. Numerous gold particles are present in the extracellular matrix (ecm) and cell walls (cw) of the transmitting tissue cells (A) and some labelling can be seen in the cytoplasm, while the vacuoles (v) are devoid of labelling (B). No appreciable labelling is present over the cells including the cell wall and extracellular matrix (ecm), either in sections without an incubation in primary antiserum (C) or in sections incubated with the preimmune rabbit serum (D) as control sections. Bars, 1 μm.

detected predominantly in the pistil of *C. cardunculus* L. (Figure 3B). Expression of cardosin B was also examined throughout floral development. In contrast to cardosin A (Ramalho-Santos *et al.*, 1997), cardosin B levels are high from the initial stages of floral development (Figure 4A). The distribution of cardosin B along the pistil is also different to that of cardosin A (Figure 4B). Immunoblotting analysis of extracts obtained from different pistil segments revealed that while cardosin A is abundantly expressed at the upper parts, cardosin B is distributed along the pistil and is the only cardosin detected by this assay in extracts of the lower part of the pistil.

Cardosin B has an extracellular location in the transmitting tissue

In order to examine the distribution of cardosin B *in situ*, in both style and stigma tissues, immunohistochemical and immunogold electron microscopy (EM) analysis were performed with monospecific antibodies raised against a cardosin B synthetic peptide. Immunolight microscopy revealed that in the stigma and the style colour development was restricted to the transmitting tissue of both closed and opened flowers (Figure 5); however, at the stigma level immunostaining for cardosin B was also very intense in contiguous subepidermal layers at the longitudinal grooves (Figure 5, B and C). No colour development was observed either in other tissues such as epidermis, cortical parenchyma and vascular bundles or in control sections (Figure 5D and E).

By immunogold-EM analysis of stigma and style sections, numerous gold particles were observed in the cell wall and extracellular matrix of the transmitting tissue (Figure 6A). Some gold particles could also be detected in the cytoplasm of a few cells in the transmitting tissue but not in the vacuoles of the same cells (Figure 6B). No appreciable labelling was seen over the cells of the vascular bundle and epidermis. Pre-treatment of grids with sodium periodate to oxidize carbohydrates and thus avoid possible non-specific glycan recognition by serum showed no difference in the distribution of the gold particles. Incubation of control sections with buffer (Figure 6C) or non-immune serum (Figure 6D) instead of primary antibody did not yield any appreciable labelling.

Discussion

Aspartic proteinases have been cloned from several plants (Runeberg-Roos *et al.*, 1991; Cordeiro *et al.*, 1994; Asakura *et al.*, 1995; Schaller and Ryan, 1996; D'Hondt *et al.*, 1997; Faro *et al.*, 1999; Panavas *et al.*, 1999). With the exception of nucellin from barley ovule nucellar cells (Chen and Foolad, 1997), the primary structures of their precursors are very similar. Like other plant aspartic proteinases, cardosin B is synthesized as a single-chain precursor and has to undergo further proteolytic processing in order to generate the two-chain mature form. Comparison between the amino acid sequences of the precursor and mature forms of cardosin B revealed that, in addition to the signal sequence, the 44 amino acid long prosegment and the PSI domain are also removed during the maturation of the protein. Studies on the activation and proteolytic mechanisms of plant aspartic proteinases have shown that the first cleavage, upon the removal of the signal sequence, occurs either inside (Glathe *et al.*, 1998; White *et al.*, 1999) or at the boundaries of the PSI domain (Ramalho-Santos *et al.*, 1998). In procardosin A, the PSI domain is removed before the prosegment, indicating that the enzyme acquires a structure typical of the gastric aspartic proteinase zymogens, before the final maturation process takes place. The prosegments of the gastric zymogens are well known for their role in keeping the precursors inactive as they get into the active-site cleft and prevent substrate binding (James, 1998). In prophytepsin, however, a variation of the mechanism of inhibition used by the gastric aspartic zymogens was unrevealed by the crystal structure (Kervinen *et al.*, 1999). The active site is locked by the interaction of Lys-11 and Tyr-13 of the N-terminal region of the mature form with the catalytic aspartates. Interestingly, these residues are not conserved in cardosin B amino acid sequence, and most likely another inactivation mechanism may operate in its case.

The hallmark feature of plant aspartic proteinases is the internal segment known as PSI domain, whose sequence has no homology with those of their mammalian, parasite or microbial counterparts. The PSI domain has been sugested to be involved in the transport of plant aspartic proteinase precursors to the vacuole because of its structural resemblance to saposins (Guruprasad *et al.*, 1994), proteins that are co-transported with cathepsin D to lysosomes (O'Brien and Kishimoto, 1991) and display membrane-binding properties (Liepinsh *et al.*, 1997). Moreover, a putative

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membrane receptor binding site involved in Golgi-mediated transport to vacuoles was found on the 3D structure of prophytepsin PSI (Kervinen *et al.*, 1999), further strengthening this hypothesis. However, the fact that cardosin B is found extracellularly, albeit it contains a PSI domain, argues against the above hypothesis and suggests that another yet unknown sorting signal may co-operate with the PSI in targeting vacuolar aspartic proteinases to this organelle. Taking into account the membrane-binding properties conferred to proteins by the saposin fold, it seems more plausible that the primary role of PSI is promoting the association to the membrane after translocation into ER, thus preventing unwanted proteolysis during the intracellular transport of the precursors. After the association to the membrane in the ER, positive sorting information is then required by vacuolar aspartic proteinases whereas extracellular ones, such as cardosin B, follow the default pathway and are secreted out. In this regard we cannot exclude, however, that cardosin B is secreted simply because the specific vacuolar sorting receptors are not expressed in the transmitting tissue cells. In any case, the accumulation of cardosins A and B in different cell compartments indicates that they can be good models to study the molecular mechanisms involved in the vacuolar targeting of plant aspartic proteinases.

Cardosin B is the first proteolytic enzyme to be found in relatively high abundance at the extracellular matrix of the transmitting tissue. Other hydrolases, such as S-RNases (McClure *et al.*, 1990) and chitinase (Wemmer *et al.*, 1994; Ficker *et al.*, 1997), have also been found in stylar transmitting tissues. The S-RNases are involved in self-incompatibility mechanisms whereas chitinases, which are highly expressed in pistils of Solanaceae, have been proposed to be involved in defensive mechanisms against pathogens (Ficker *et al.*, 1997). The same defensive function can also be proposed for cardosin B, a hypothesis that is in close agreement with the broad specificity and high specific activity of this proteinase. However, on the basis of the results described here and similarly to what has been proposed for other stylar genes (Graaf, 1999), whose expression is developmentally regulated, it is also conceivable that cardosin B has additional role(s) in pistil development and/or in pollen-pistil interactions.

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