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Review

Properties and applications of phytepsins from thistle flowers

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ABSTRACT

Aqueous extracts of thistle flowers from the genus *Cynara*—*Cardueae* tribe Cass. (*Cynareae* Less.), Asteraceae Dumortier—are traditionally used in the Mediterranean region for production of artisanal cheeses. This is because of the presence of aspartic proteases (APs) with the ability to coagulate milk. Plant APs, collectively known as phytepsins (EC 3.4.23.40), are bilobed endopeptidases present in an ample variety of plant species with activity mainly at acidic pHs, and have two aspartic residues located on each side of a catalytic cleft that are responsible for catalysis. The cleavage of the scissile peptide-bond occurs primarily between residues with large hydrophobic side-chains. Even when aspartylendopeptidase activity in plants is normally present at relatively low levels overall, the flowers of several species of the *Cardueae* tribe possess APs with extremely high specific activities in certain tissues. For this reason, in the last two decades, APs present in thistle flowers have been the subject of intensive study. Present here is a compilation of work that summarizes the known chemical and biological properties of these proteases, as well as their biomedical and biotechnological applications.

Contents

1. Introduction

Aspartic proteases (APs) are widely distributed among vertebrates, plants, yeasts, nematodes, parasites, fungi, and viruses, thus playing key roles in a variety of physiological and pathological contexts (Davies, 1990; Simões and Faro, 2004).

According to the MEROPS database (Rawlings et al., 2012) created by Rawlings & Barrett, plant APs are distributed among the families A1, A2, A3, A11, A28, and A33 of clan AA; family A8 of the clan AC and families A22, and A24 of clan AD. Along with pepsin-like enzymes from various sources, most plant APs belong to the A1 family (the pepsin family; Rawlings et al., 2012). These phytoAPs are present in an ample variety of plant species and have

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been characterized and purified from different tissues such as seeds, flowers, and leaves (Feijoo-Siota and Villa, 2011; González-Rábade et al., 2011; Lufrano et al., 2012). Because of their diversity plant APs have been grouped into 3 classes—i.e., typical plant-aspartic, atypical plant-aspartic, and nucellin-like proteinases—depending on their putative domain organizations and their active-site–sequence motifs (Faro and Gal, 2005). Typical plant APs belong to the subfamily A1A, while the other two classes are grouped almost exclusively in subfamily A1B. The Nomenclature Committee of the IUBMB has adopted the general name phytepsins (EC 3.4.23.40) for all typical plant APs (NC-IUBMB, 1997). In the literature, however, a universal name to refer to these peptidases is not commonly found, and the names used tend to indicate a particular species or tissue from which each enzyme has been purified.

All the peptidases in family A1 are endopeptidases, mainly active at acidic pH, and with two aspartic residues responsible for catalytic activity. The two catalytic Asp residues, like the protein lobes in which they are contained, are homologous to each other. The two domain-structure was suggested as having arisen from an ancestral-gene duplication since retroviral, retrotransposon, and badnavirus proteases (*Pfam PF00077*) are much smaller and appear to be homologous to either of the two lobes of the eukaryotic APs (Tang, 2004). APs are synthesized in a precursor form containing an N-terminal signal peptide and a propeptide. APs display specificity for residues with large hydrophobic side-chains on either side of the scissile bond—e.g., Phe, Val, Ile, Leu—at P1 and P1′ (Dunn and Hung, 2000; Rawlings et al., 2012). Pepstatin A—a tightly binding, reversible peptidase inhibitor—is the one most widely used in the family A1 (Rawlings et al., 2012).

Plant APs that have been characterized from the A1 family are mostly targeted to lytic vacuoles or to the vacuolar-protein storage-body compartment, even though some of the APs are either directed at the endoplasmatic reticulum or else secreted into the cell wall or towards the apoplast (Almeida et al., 2012; González-Rábade et al., 2011; van der Hoorn, 2008). *In-silico* analysis of targeting signals for most putative APs of the *Arabidopsis thaliana* genome is in accordance with the expected localization in the secretory system, but also predicts other sites, such as the chloroplast or mitochondria, with several APs indicated as being bound to membranes (Faro and Gal, 2005). Inactive proenzymes are in general activated autocatalytically at acidic pH *in vitro*, but the process is not always complete under these conditions, suggesting that *in vivo* completion of maturation might require action of other protease(s) and/or exopeptidase(s) (Castanheira et al., 2005; Rawlings et al., 2008).

This review focusses on characterization of the APs present in the flowers of several species of the tribe *Cardueae* Cass. (*Cynareae* Less.), family Asteraceae Dumortier, intensively studied as a result of the occurrence of these peptidases. Here the known chemical and biological properties of these proteases are summarized as well as their biomedical and biotechnological applications.

2. Thistle flowers as producers of phytepsins

Because of the presence of APs with the ability to coagulate milk, thistle flowers from the genus *Cynara* are traditionally used in the Mediterranean region for production of artisanal cheeses (Roseiro et al., 2003; Silva and Malcata, 2005a). Moreover, consumer constraints on the use of rennets for religious reasons, diet (e.g., vegetarianism) or opposition to genetically engineered foods have prompted study of these enzymes as rennet substitutes (Roseiro et al., 2003). *Cynara cardunculus* L. (cardoon) produces two identified groups of typical plant APs—cardosins and cyprosins—in mature flowers. Even when overall aspartylendopeptidase activity in plants is normally present at relatively low levels, these flowers possess extremely high AP activity in certain tissues (Cordeiro et al., 1994a; Duarte et al., 2006; Figueiredo et al., 2006; Ramalho-Santos et al., 1996, 1997).

From *C. cardunculus*-dried-flower extracts, obtained at alkaline pH (8.3), three glycosylated peptidases with milk-clotting activity were isolated and named cynarases 1, 2, and 3 (Cordeiro et al., 1994a; Heimgartner et al., 1990). The name cynarase was later replaced by cyprosin according to the AP-naming conventions (Cordeiro et al., 1994a). The purification process included fractionation by ammonium-sulfate precipitation (30–80% saturation) and anion-exchange chromatography on DEAE-Sepharose and MonoQ columns (Heimgartner et al., 1990). Each purified peptidase consists of two subunits, one large (32.5–35.5 kDa) and one small (13.5–16.5 kDa), that show pI microheterogeneity (at *ca.* 4.0) upon analysis by two-dimensional electrophoresis (Cordeiro et al., 1994a). Among these three peptidases, cyprosin 3 is the one most similar to chymosin—the enzyme used to coagulate milk of different mammals in the manufacture of cheese (Cordeiro et al., 1992).

In contrast, cardosins have been purified and characterized from fresh stigmas of *C. cardunculus* (Veríssimo et al., 1995). Contrary to expectation, cardosins and cyprosins have never been co-purified (Pimentel et al., 2007). The purification process used for cardosins involved extraction at acid pH followed by size exclusion on Superdex 200 and anion-exchange chromatography on MonoQ. At acidic pH between 75% and 90% of total extracted enzyme activity correspond to cardosin A, which enzyme in terms of specificity and kinetic parameters proved to be similar to chymosin, whereas cardosin B, the second most active, was similar to pepsin (Faro et al., 1995; Ramalho-Santos et al., 1996, 1997; Veríssimo et al., 1995, 1996). Both cardosins are glycosylated enzymes (Costa et al., 1997).

In later studies, Sarmento et al. (2009) purified and characterized four new APs from the pistils of *C. cardunculus*, thus elevating the number of APs that had been isolated, purified, and biochemically characterized from this species to nine. These four enzymes—cardosins E, F, G, and H—are dimeric (*ca.* 27 kDa and 11 kDa for the heavy and light chains, respectively), glycosylated, and with maximum activity at around pH 4.3. The primary structures of the three were partially determined by N- and C-terminal sequencing and by peptide mass-fingerprinting through both MALDI-TOF/TOF and LC-MS/MS analyses. These enzymes resemble cardosin A more than they do cardosin B or cyprosin. The genes for cardosin A, cardosin B, and two APs not yet isolated (the cardosins C and D) were characterized and their expression in *C. cardunculus* analyzed by real-time polymerase-chain reactions (Pimentel et al., 2007).

Another member of genus *Cynara* that has been investigated is *C. humilis*, from which species Esteves (1995) isolated only a cardosin-A-like enzyme from its fresh flowers by acid extraction. Two groups of investigators have compared this enzyme with chymosin and the *C. cardunculus* peptidases (Esteves et al., 2002, 2003; Vioque et al., 2000).

The presence of APs has also been reported in different anatomical parts and at various developmental stages in the inflorescence of *Cynara scolymus* L., commonly known as artichoke (Llorente et al., 1997). In subsequent studies, Llorente et al. (2004) obtained, from fresh or frozen artichoke flowers, homogenates at pH 6.0 with maximal proteolytic activity at pH 5.0 and a pI of around 4.0. Adsorption with activated carbon, together with anion-exchange and affinity chromatography, led to isolation of a heterodimeric milk-clotting proteinase consisting of a 30-plus a 15-kDa subunit. The amino-terminal sequence of the heavy chain proved to be identical to cardosin-A larger subunit. Furthermore, three APs were acid-extracted from the stigmas of dried artichoke flowers at pH 3.0. This step was followed by a purification process consisting of ultrafiltration and ion-exchange chromatography on a Q-Sepharose column. These purified enzymes were named cynarases A, B, and C (Sidrach et al., 2005).

Table 1
APs present in several species of the tribe *Cardueae* Cass. (Cynareae Less.). These are members of the A1 family (MEROPS database). In the second column, sequence-access numbers of the databases UniProtKB/TrEMBL or of GenBank are indicated when possible and the name assigned by authors cited.

Species	Enzyme	Reference
Centaurea calcitrapa	Aspartic proteinase Q96383	Domingos et al. (1998)
Cynara cardunculus	Cyprosin A X69193	Cordeiro et al. (1994a)
	Cyprosin B (aka cyprosin 3) Q39476/X81984	White et al. (1999)
	Cardosin A Q9XFX3/AJ132884	Faro et al. (1995)
	Cardosin B Q9XFX4/AJ237674	Faro et al. (1995)
	Cardosin C	Pimentel et al. (2007)
	Cardosin D	Pimentel et al. (2007)
	Cardosin E P85136	Sarmento et al. (2009)
	Cardosin F P85137	Sarmento et al. (2009)
	Cardosin G P85138	Sarmento et al. (2009)
	Cardosin H P85139	Sarmento et al. (2009)
Cynara humilis	Cardosin A	Esteves (1995)
Cynara scolymus	Cynarase A	Llorente et al. (2004)
	Cynarase B	Sidrach et al. (2004)
	Cynarase C	
Cirsium vulgare	Cirsin JN703462	Lufrano et al. (2012)
Onopordum turcicum	Aspartic proteinase	Tamer (1993)
Onopordum acanthium	Onopordosin	Brutti et al. (2012)

A heterodimeric (30.0 + 16.0 kDa) glycosylated peptidase, designated cenprosin, was isolated from dried flowers of *Centaurea calcitrapa* L. with a maximum activity between pH 4.0 and 5.0 (Domingos et al., 1998). The enzyme was purified in its precursor form (of 50 kDa) by means of ammonium-sulfate precipitation followed by ion-exchange and hydrophobic-interaction chromatography. The primary sequence of the zymogen was deduced from the corresponding cDNA (Domingos et al., 2000).

APs with milk-clotting activity were obtained at pH 3.0 from fresh flowers of *Silybum marianum* (L.) Gaertn., commonly referred to as the *blessed milk thistle*. A depigmented-enzyme preparation gave maximal activity at pH 3.8 on haemoglobin as the substrate (Vairo Cavalli et al., 2005). An AP named silpepsin was obtained by size exclusion on Superdex 200 and anion-exchange chromatography on a MonoQ-Sepharose column (Vairo Cavalli et al., 2009).

At least three AP DNA precursors from flowers of *Cirsium vulgare* (Savi) Ten. have been cloned, and one of them, in its zymogenic form, (procirsin) has been characterized and expressed. The recombinant procirsin displays typical proteolytic features of APs—e.g., acidic pH optimum, inhibition by pepstatin, and a cleavage preference between hydrophobic amino acids. This recombinant protein also displays a high specificity towards κ-casein and has milk-clotting activity (Lufrano et al., 2012).

Other sources of APs with milk-clotting activity are flowers of the thistle genera *Onopordum turcicum* Danin, *Carduus acanthoides* L., *Carduus thoermeri* Weinm., *Arctium minus* (Hill) Bernh., and *Onopordum acanthium* L. (Brutti et al., 2012; Cimino et al., 2010; Tamer, 1993). The enzymes from *O. turcicum* have been extracted at pH 5.0 and were partially purified by ammonium-sulfate precipitation, size exclusion, and ion-exchange chromatography. They have a pI around 3.3–3.7 and a maximal activity at pH 5.0 (Tamer, 1993). The nature of the catalytic type of the proteases from *O. turcicum* involved in milk clotting have not yet, however, been elucidated. APs with milk-clotting activity were obtained by extraction at pH 6.0 and pH 3.0 from *C. acanthoides* and *C. thoermeri*, respectively (Lufrano et al., 2007). The crude extracts with proteolytic and milk-clotting activity were prepared from fresh flowers of *A. minus* at pH 7.0, partially purified (Sephadex G-25), and characterized. An analysis of the crude extract by isoelectric focussing and zymogram showed only one active band (of pI 5.0), with an activity inhibited by pepstatin (Cimino et al., 2010). Milk-clotting activity has also been reported for extracts obtained at pH 3.0 from fresh flowers of *O. acanthium*. The partially purified enzyme preparation, because of the presence of APs with a pI of 4.4, showed maximal activity at pH 2.5 on haemoglobin as the substrate (Brutti et al., 2012).

Table 1 summarizes names given to APs of different organisms of the tribe *Cardue* that have been studied and sequence-accession numbers when available. The amino-acid sequences of the thistle-APs (Fig. 1), when compared with those of phytepsin or pepsin, form a separate group from either of the other two—*cf*. the phylogenetic tree shown in Fig. 2. Pimentel et al. (2007) have proposed that an AP ancestral gene duplicated and gave rise to cyprosins and cardosins during evolution of *C. cardunculus* and that thereafter duplications occurred within both groups.

3. Molecular structure of typical plant APs from thistle flowers

Up to now, the APs purified, cloned, and characterized from flowers of species of the *Cardueae* tribe belong to the group of typical peptidases from the family A1. These peptidases are initially translated as inactive single-chain precursors, the zymogens, and subsequently processed to the mature form of the enzymes. The primary structure of the precursors is characterized by the presence of a signal sequence that directs the zymogen to the endoplasmic reticulum, followed by a propeptide of approximately 40 amino acids (a prosegment), an N-terminal domain, the plant-specific insert (PSI), and a C-terminal domain (Fig. 3). The primary structure of the precursors is well-conserved among typical plant APs (Dunn, 2002; Simões and Faro, 2004). Mature forms of known thistle-flower APs, as described in Section 3, have two chains: a heavy one of about 30 kDa—the N-terminal domain—and a light one of about 15 kDa—the C-terminal domain (Cordeiro et al., 1994b; Domingos et al., 2000; Ramalho-Santos et al., 1998; Vieira et al., 2001).

With respect to tertiary folding of the thistle-flower APs, the crystallographic structure at high resolution (1.7 Å) has been determined only for mature cardosin A from *C. cardunculus*. By this technique, the molecule (PDB code: 1B5F; Fig. 4) is seen to be composed of two glycosylated polypeptide chains of 31 and 15 kDa in the form of two distinct lobes that are homologous in their polypeptide-chain folding. The secondary structure follows the pepsin-like single-chain–folding topology. Essentially it consists of a double-Ψ formed by two interlocked motifs, each comprising a loop and a strand that together resemble the Greek letter Ψ (Castillo et al., 1999; Frazão et al., 1999). The active site is located between those two domains at the bottom of a large cleft. The two halves of the enzyme act as independent folding structures that move relative to one other and contribute a single catalytic aspartate each (Asp35 and Asp218) in a co-planar arrangement of the two carboxylate residues (Frazão et al., 1999). This three-dimensional ordering is also found in almost all the PDB structures of APs, even though—and in striking contrast to the abundant previous structural findings—a refinement of the crystalline structure of apoplasmepsin II has suggested that the two carboxylate groups in the catalytic dyad are non-coplanar. Furthermore, the

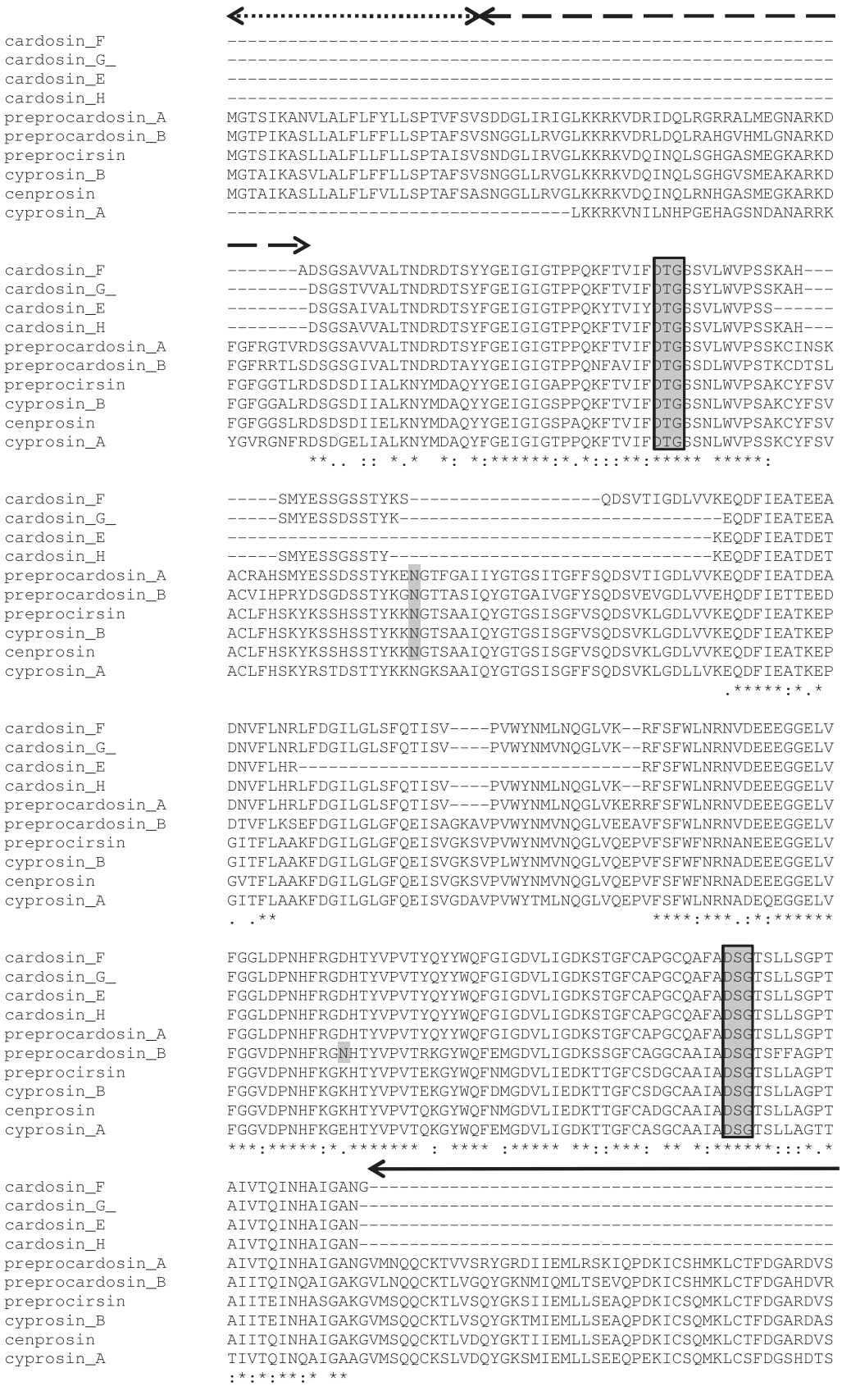

Fig. 1. Amino acid sequence alignment of thistle APs. The regions corresponding to the N-terminal signal peptide (⟵⟶), the prosegment (⟵⟶) and the plant-specific insert (⟷) are indicated by arrows over the corresponding sequences. Potential *N*-glycosylation sites are grayed out and the catalytic triads are boxed. Sequence of the enzymes used for the alignment are cenprosin, CAA70340.1; cyprosin A, CAA48939.1; cyprosin B (aka cyprosin 3), CAA57510.1; cardosin A, CAB40134.1; cardosin B, CAB40349.1; cardosin E, P85136; cardosin F, P85137; cardosin G, P85138; cardosin H, P85139; and cirsin, AFB73927.2. The available sequences of cyprosin A and cardosins E, F, G, and H are not complete.

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cardosin_F          ------------------------------------------------------------
cardosin_G_         ------------------------------------------------------------
cardosin_E          ------------------------------------------------------------
cardosin_H          ------------------------------------------------------------
preprocardosin_A    SIIESVVDKNNDKSSGGIHDEMCTFCEMAVVWMQNEIKQSETEDNIINYANELCEHLSTS 416
preprocardosin_B    SMIESVVDKNNDKSSGG---EICTFCEMALVRMQNEIKRNETEDNIINHVNEVCDQLPTS 417
preprocirsin        SIIESVVDKNNGKSSGGANDEMCTFCEMAVVWMQNQIKRNETEDNIINYVNELCDRLPSP 420
cyprosin_B          SIIESVVDENNGKSSSGVHDEMCTFCEMAVVWMQNQIKRNETEDNIINYVNELCDRLPSP 420
cenprosin           SIIESVVDKNNGKSSGGVHDEMCTFCEMAVVWMQNQIKRNQTEDNIINYVNELCDRLPSP 420
cyprosin_A          MIIESVVDKSKGKSS-GLHDEMCTMCQMAVVWMQNQIRQNETEENIINYVDKLCERLPSP 385


cardosin_F          SEELNVK------------------FGLTPEQYIL---KGEATQCISGFTAMDAT-LLGP 252
cardosin_G_         SEELNVK------------------FGLTPEQYIL---KGEATQCISGFTAMDAT-LLGP 237
cardosin_E          SEELNVK------------------FGLTPEQYIL---KGEATQCISGFTAMDAT-LLGP 195
cardosin_H          SEELNV-------------------FGLTPEQYIL---KGEATQCISGFTAMDAT-LLGP 236
preprocardosin_A    SEELQVDCNTLSSMPNVSFTIGGKKFGLTPEQYILKVGKGEATQCISGFTAMDAT-LLGP 475
preprocardosin_B    SAESIVDCNGISSMPNIAFTIGSKLFEVTPEQYIYKVGEGEAATCISGFTALDIMSPQGP 477
preprocirsin        MGESAVDCNSLSSMPNIAFTIGGKVFELCPEQYILKIGEGEAAQCISGFTAMDVAPPRGP 480
cyprosin_B          MGESAVDCNSLSSMPNIAFTIGGKVFELCPEQYILKIGEGEAAQCISGFTAMDVAPPRGP 480
cenprosin           MGESAVDCNDLSSMPNIAFTIGGKVFELCPEQYILKIGEGEAAQCISGFTAMDVAPPRGP 480
cyprosin_A          MGESAVDCSSLSSMPNIAFTVGGKTFNLSPEQYVLKVGEGATAQCISGFTAMDVAPPHGP 445
                      *  *                  * : ****:    :* :: *******:*     **


cardosin_F          LWILGDVFMRPYHTVFDYGNLLVGFAEAA 281
cardosin_G_         LWILGDVFMRPYHTVFDYGNLLVGFAEAA 266
cardosin_E          LWILGDVFMRPYHTVFDYGNLLVGFAEAA 224
cardosin_H          LWILGDVFMRPYHTVFDYGNLLVGFAEAA 265
preprocardosin_A    LWILGDVFMRPYHTVFDYGNLLVGFAEAA 504
preprocardosin_B    IWILGDMFMGPYHTVFDYGKLRVGFAEAV 506
preprocirsin        LWILGDVFMGRYHTVFDYGKSRVGFAEAA 509
cyprosin_B          LWILGDVFMGRYHTVFDYGKLRVGFAEAA 509
cenprosin           LWILGDVFMGQYHTVFDYGKLRVGFAEAA 509
cyprosin_A          LWILGDVFMGQYHTVFDYGNLRVGFAEAA 474
                    :*****:**  ********:  ******.
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Fig. 1. (*continued*)

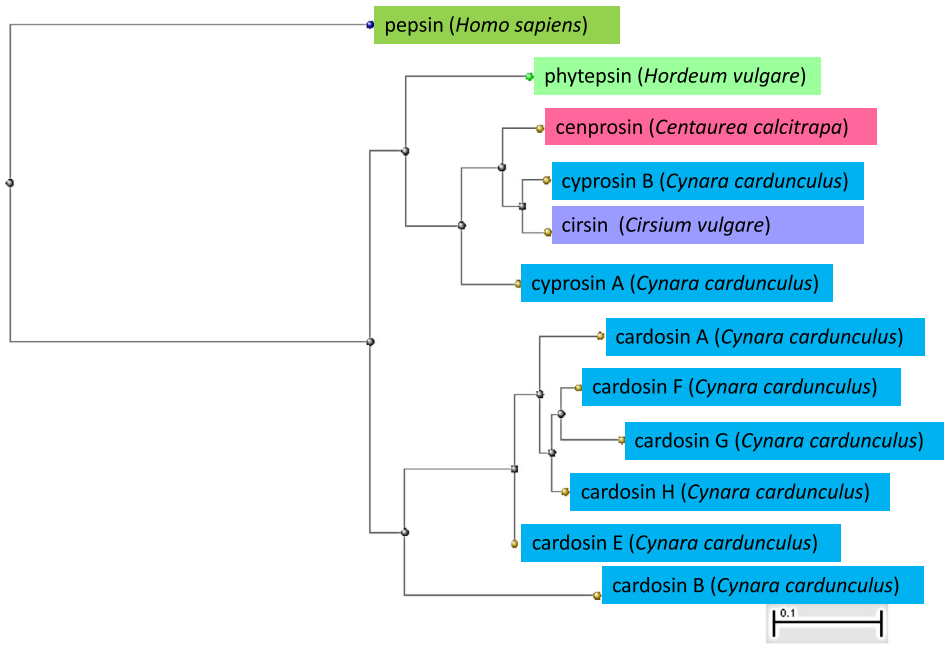


Fig. 2. Phylogenetic relationship among several thistle-flower AP. Phylogenetic analysis was carried out by the neighbor-joining method, with the distance indicated according to Grishin and the (protein), phytepsin precursor and pepsinogen are also included. On the basis of the thistle-AP family tree, the APs can be divided into two groups. Sequence of the enzymes used for construction of the tree were pepsinogen, AAI71897.1; phytepsin, P42210; cenprosin, CAA70340.1; cyprosin A, CAA48939.1; cyprosin B (aka cyprosin 3), CAA57510.1; cardosin A, CAB40134.1; cardosin B, CAB40349.1; cardosin E, P85136; cardosin F, P85137; cardosin G, P85138; cardosin H, P85139; cirsin, AFB73927.2.

non-coplanar arrangement may be necessary for initiating the catalytic mechanism—before formation of the tetrahedral intermediate—as suggested from results obtained by crystallographic studies, molecular-dynamics simulations of the HIV protease, and quantum–mechanical and molecular-mechanics simulations of β-secretase (Friedman and Caflisch, 2010). Accordingly, the co-planar

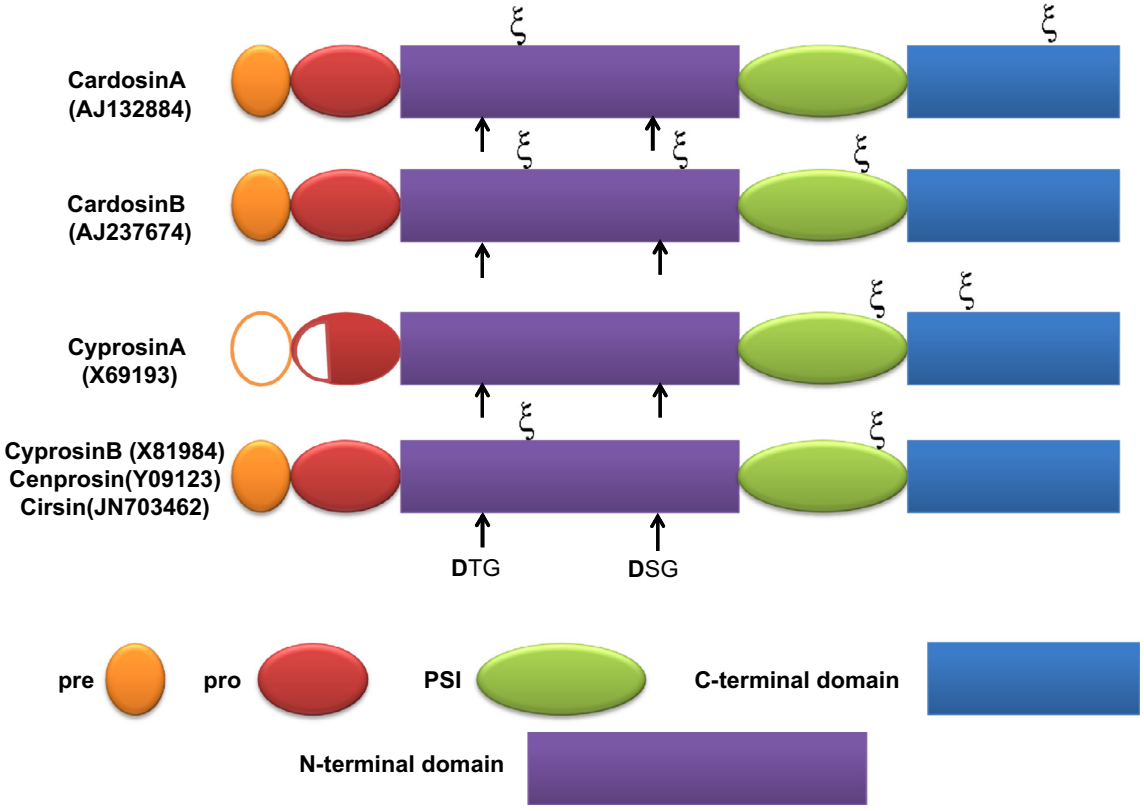


Fig. 3. Schematic representation of the domain organization of thistle-AP precursors from *Cynara cardunculus* (cardosin A and B and cyprosin A and B), *Centaurea calcitrapa*, and *Cirsium vulgare* (cirsin). Accession numbers for each sequence are indicated in brackets and complete sequences colored. All the enzymes include a signal peptide (pre), a propeptide (pro), and a plant-specific insertion (PSI domain) separating the two chains of the mature form (N- and C-terminal domains). Uncolored segments of cyprosin A represent sequences not available on databases consulted. Positions of conserved catalytic motifs, DTG and DSG, and *N*-glycosylation sites (ξ) are indicated. Adapted from Pissarra et al. (2007).

conformation of the APs observed in crystalline structures would likely be a result of the low temperatures required in crystallographic preparations. For plant APs, however, a more in-depth analysis is still needed for elucidating the three-dimensional arrangement of the catalytic site. The aspartates in plant-AP sequences are contained in two conserved catalytic motifs: Asp-Thr-Gly (DTG) and Asp-Ser-Gly (DSG)—those differing from the characteristic counterpart animal and microbial catalytic motifs (DTG/DTG, Fig 3B). A water molecule bound to both aspartate carboxyls by hydrogen bonding is the putative nucleophile in the hydrolytic mechanism (Frazão et al., 1999).

The crystal structure of cardosin A has an extra β-hairpin of 20 residues in the N-terminal domain that is folded down onto the active-site cleft and encloses substrates and inhibitors there (Frazão et al., 1999). This flexible loop is known as the *flap* (Fig. 4B), and constitutes an asymmetric feature of the structure of pepsin-like peptidases. The flap carries residues essential for specificity, that can interact with substrates as part of the S1 subsite contributing to hydrolytic specificity (Andreeva and Rumsh, 2001; Hong and Tang, 2004; Rawlings and Bateman, 2009). During the catalytic cycle, however, the flap must open to allow entrance of the substrates into the cleft and release of the hydrolytic products (Hong and Tang, 2004).

Three disulfide bridges have been found in mature cardosin A at conserved positions within the A1 family. Two of those secondary-structure linkages are within the heavy chain (Cys45-Cys56 and Cys206-Cys210) and the third within the light chain (Cys249-Cys282). In addition to these secondary structures within each of the chains, the heterodimer is held together by hydrophobic interactions and hydrogen bonds. An additional conserved feature of the APs is a *cis* peptide bond between Thr22 and Pro23, with this latter *cis*-Pro being located at the tip of the VIb β-turn (Frazão et al., 1999). The *cis* peptide bonds in which proline (Xaa-Pro) is involved are preferentially located near the protein's surface, primarily in bends and turns, and would play a key role in the mechanism of protein folding (Pal and Chakrabarti, 1999; Stewart et al., 1990).

All phytepsins from cardoon flowers, cardosins, and cyprosins are glycosylated enzymes. Cardosin A has two N-linked glycosylation sites (at Asn67 and Asn257) occupied by oligosaccharides resistant to the removal by endoglycosidase H, the resistance of which is indicative of prior Golgi processing (Costa et al., 1997; Duarte et al., 2008). In both subunits of cardosin A, the oligosaccharides identified are of the plant-modified type with a proximal fucose and xylose (Costa et al., 1997). These glycans are located on the enzymatic surface away from the active-site cleft (*cf.* Fig. 4), and therefore the possibility that the oligosaccharide chains play a significant role in enzymatic activity and specificity is unlikely. Rather, those sugar residues may enhance conformational stabilization and/or correct protein processing (Frazão et al., 1999). The cardosins E, F, G, and H isolated from *C. cardunculus* flowers are glycosylated heterodimers, but the nature of those glycans remains unknown (Sarmento et al., 2009). Cyprosins, also from cardoon flowers, are likewise heterodimeric enzymes containing high mannose-type glycosylations. One of those enzymes has glycosylation on the Asn398 and Asn445 residues (Cordeiro et al., 1994a), while in another cyprosin isoform amino-acid sequence the Asn139 and Asn400 were identified as putative glycosylation sites (White et al., 1999). In both of these examples, one of those glycosylated sites was localized in the PSI domain. Finally, two putative *N*-glycosylation sites in procirsin (of *C. vulgare*) were also predicted at these same residues Asn139 and Asn400 (Lufrano et al., 2012).

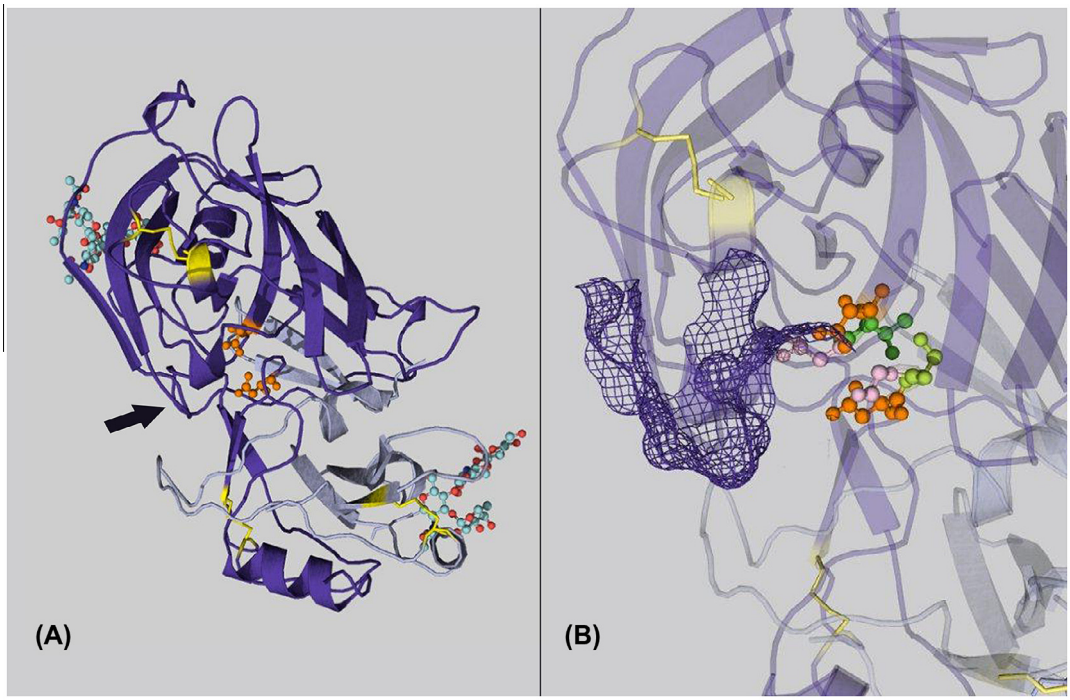


Fig. 4. Cartoon representation of a cardosin-A molecule (PDB 1B5F; Frazão et al., 1999). A. The molecule contains two glycosylated peptide chains (31 kDa in deep purple and 15 kDa in light gray). The folding of cardosin A is typical of the A1 family. The protein is bilobed with two domains separated by the large cleft where the active site is located (indicated by an arrow); the Asp35 and Asp218, within the active site, are shown in orange and in ball-and-stick representation. The two glycans, represented also as ball and sticks, are N-linked to Asn67 and Asn178, cyan, red and blue balls represent C, O, and N atoms, respectively. Disulfide bonds, as well as Cys involved in the bridges, are shown in yellow. B. The cardosin A active site. The residues of the catalytic triads are shown in ball-and-stick representation; Asp35 and Asp218 are in orange, Thr33 in green, Gly34 and Gly217 in pink and Ser216 in lyme. The electron density of the flap is shown as a deep purple mesh. (For visualization of these indications of color in the figure, the reader is referred to the web version of this article.)

The motif Arg-Gly-Asp (RGD, residues 246–248)—this, in mammalian proteins, being typically associated with a functional integrin cell-attachment ligand—was found in the C-terminal portion of the 31-kDa chain of cardosin A. This sequence is located in a loop that connects two strands on the enzyme surface opposite to the active-site groove (Faro et al., 1999; Frazão et al., 1999). Another amino-acid motif that has been identified from the crystalline structure of cardosin A, Lys-Gly-Glu (KGE, residues 455–457) mimics the RGD motif in terms of charge distribution and position relative to the protein core (Simões et al., 2005). The cardosins E, F, G, and H share the RGD motif—and presumably the KGE motif as were (Sarmento et al., 2009)—with cardosin A.

4. Activities and specificities

APs tend to bind 6–10 peptide residues of the polypeptide substrates in their active site and, as already stated, cleave those peptides at two aspartic-acid–containing catalytic sites. None of the peptidases in family A1 require cofactors, and all are inhibited by pepstatin A. Table 2 lists the K_i values of this inhibitor for several *Asteraceae* APs. The AP-specificity typically favors bonds with residues containing large hydrophobic side-chains on each side of the scissile bond, but several AP members have more restricted specificities that enable the protein-processing functions (Rawlings et al., 2012).

Like the majority of other milk-clotting enzymes, thistle APs cleave κ-casein at the bond Phe105-Met106 (Lufrano et al., 2012; Ramalho-Santos et al., 1996; Sampaio et al., 2008). Some of these enzymes cleaved α_s- and β-caseins in different peptide bonds according to the APs' specificity (Table 3). Cardosin B also degraded ovine α_s- and β-caseins, but not to the same extent. In unfractionated sodium-caseinate preparations, α_s-caseins were more susceptible to proteolysis by cardosin B than β-casein, whereas with purified caseins, the opposite was observed (Silva and Malcata, 1999).

Cyprosin 3 has shown higher clotting activity with ewe's milk than chymosin, as well as a more specific hydrolysis of proteins in that type of milk (Cordeiro et al., 1992). The three cyprosins have a pH optimum around 5.1 with bovine casein as substrate. Through the use of a fluorometric protease-activity assay, based on release of trichloroacetic-acid-soluble fluorescent peptides from fluorescein-isothiocyanate-labelled casein, cyprosin 3 was found to have the highest specific activity and cyprosin 1 the lowest (Heimgartner et al., 1990). The use of a library of 46 synthetic chromophoric octapeptides with systematic variation in the amino-acid residues confirmed that three isoforms of cyprosin 3 preferentially cleaved peptide bonds between two hydrophobic amino acids. From a set of four selected peptides, Lys-Pro-Ile-Val-Phe-Nph-Arg-Leu gave the lowest K_m values (15–25 mM), while Lys-Pro-Ile-Leu-Phe-Nph-Arg-Leu showed the highest k_{cat} values (34–85 s^{-1}). The reader is referred to Table 4 for more detailed comparison of the substrates and kinetic parameters. The K_i values

Table 2
Interaction of pepstatin A with natural and recombinant APs from *Cynara cardunculus* flowers.

Endopeptidase	Pepstatin K_i (nM)
Cyprosin 3[a]	<0.1
Cyprosin B_R[*,b]	0.5
Cardosin A[c]	3
Cardosin A_R[d]	1.5
Cardosin B[c]	1

$_R$ Recombinant.
[*] aka cyprosin 3.
Data are taken from: [a]Cordeiro et al. (1998); [b]White et al. (1999); [c]Veríssimo et al. (1996), [d]Castanheira et al. (2005).

Table 3
Specificity of action of cardosin A and cardosin B from *Cynara cardunculus* upon isolated bovine, caprine and ovine α_{s1}-, β-, and κ-caseins. To our knowledge, the independent action of cardosin A on ovine caseins has not been made available to date.

Endopeptidase	Bovine caseins		
	α_{s1}-Casein	β-Casein	κ-Casein
Cardosin A	Phe23-Phe24 Phe153-Tyr154 Trp164-Tyr165 Tyr165-Tyr166[a]	Leu127-Thr128 Leu165-Ser166 Leu192-Tyr193[b]	Phe105-Met106 [b]
Cardosin B	Phe23-Phe24 Phe150-Arg151 Phe153-Tyr154 Trp164-Tyr165[a]	Leu165-Ser166 Leu192-Tyr193[c]	Phe105-Met106[c]
	Caprine caseins		
	α_{s1}-Casein	β-Casein	κ-Casein
Cardosin A and cardosin B	Phe153-Tyr154[d]	Leu127-Thr128 Leu190-Tyr191[d]	Lys116-Thr117[e]
	Ovine caseins		
	α_{s1}-Casein	β-Casein	κ-Casein
Cardosin B	Leu156-Asp157 Trp164-Tyr165[f]	Leu127-Thr128 Leu165-Ser166 Leu90-Tyr191[f]	Phe105-Met106[f]

Data are taken from: [a]Ramalho-Santos et al. (1996); [b]Pires (1998a); [c]Pires (1998b); [d]Silva and Malcata (2000b); [e]Silva and Malcata (2005a); [f]Silva and Malcata (1999).

for pepstatin A were below 0.1 nM for the three isoforms. The pH optimum for cyprosin 3 proved to be around 4.1 with the synthetic peptide Lys-Pro-Leu-Gln-Leu-Nph-Arg-Leu as substrate (Cordeiro et al., 1998). The kinetic parameters for the hydrolysis of Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu by a purified preparation of recombinant wild-type cyprosin from *Pichia pastoris*, when compared with those of the natural cyprosin 3, were consistent with a pH optimum between 4.5 and 5.0. The k_{cat} and K_m values determined for the naturally occurring isoform ($29 \pm 3\ s^{-1}$ and 25 ± 5 μM, respectively) were 3-fold higher and 2-fold lower than the corresponding respective values derived at pH 5.0 for the recombinant enzyme. The temperature dependence of the specificity constant (k_{cat}/K_m) for this chromogenic-substrate hydrolysis by recombinant cyprosin increased progressively in magnitude, reaching a maximum value at 55 °C. Recombinant cyprosin displays a remarkable stability at temperatures up to 55 °C and at pH values as high as 6.0 (White et al., 1999). These researchers also studied the primary specificity of recombinant cyprosin using an inhibitor-peptide library—in which statine was retained as the centerpiece, occupying the P1–P1′ positions—and systematically examined the effect of substitutions in other positions. Some of those inhibitors were found to have subnanomolar potencies against the plant enzyme.

Table 4
Kinetic parameters for hydrolysis of synthetic peptides by naturally occurring and recombinant APs from flowers of the Asteraceae family.

Endopeptidase	Substrate	K_m (mM)	k_{cat} (s^{-1})	Optimun pH
Cyprosin 3α[a]	Lys-Ser-Ala-Lys-Phe∗Nph-Arg-Leu	221	16	
	Lys-Pro-Ile-Gln-Phe∗Nph-Arg-Leu	38	21	
	Lys-Pro-Ile-Val-Phe∗Nph-Arg-Leu	15	18	
	Lys-Pro-Ile-Leu-Phe∗Nph-Arg-Leu	98	35	
Cyprosin 3β[a]	Lys-Ser-Ala-Lys-Phe∗Nph-Arg-Leu	267	15	
	Lys-Pro-Ile-Gln-Phe∗Nph-Arg-Leu	30	13	
	Lys-Pro-Ile-Val-Phe∗Nph-Arg-Leu	19	15	
	Lys-Pro-Ile-Leu-Phe∗Nph-Arg-Leu	58	54	
Cyprosin 3γ[a]	Lys-Ser-Ala-Lys-Phe∗Nph-Arg-Leu	279	37	
	Lys-Pro-Ile-Gln-Phe∗Nph-Arg-Leu	38	70	
	Lys-Pro-Ile-Val-Phe∗Nph-Arg-Leu	26	64	
	Lys-Pro-Ile-Leu-Phe∗Nph-Arg-Leu	36	86	
Cyprosin B_R[‡b]	Lys-Pro-Ile-Val-Phe∗Nph-Arg-Leu	25×10^{-3}	29	4.5–5.0
Cardosin A[c]	Lys-Pro-Ala-Glu-Phe∗Nph-Ala-Leu	0.11	55	4.5
	Leu-Ser-Nph∗Ahx-Ala-Leu-OMe	0.64	13	
Cardosin A_R[d]	Lys-Pro-Ala-Glu-Phe∗Nph-Ala-Leu	0.22	43	4.0–4.5
Cardosin B[c]	Lys-Pro-Ala-Glu-Phe∗Nph-Ala-Leu	0.11	89	
	Leu-Ser-Nph∗Ahx-Ala-Leu-OMe	0.08	86	5.0
Cardosin E[e]	Lys-Pro-Ala-Glu-Phe∗Nph-Ala-Leu	0.64	25	
Cardosin G[e]	Lys-Pro-Ala-Glu-Phe∗Nph-Ala-Leu	0.37	35	
Procirsin$_r$[f]	(MCA)Lys-Lys-Pro-Ala-Glu-Phe∗Phe-Ala-Leu-Lys(DNP)	7.1	4	4.0

α, β, and γ are isoforms of cyprosin 3.
$_R$ recombinant.
[‡] aka cyprosin 3.
∗Indicates scissile bond.
Nph is *p*-NO_2-Phe; MCA is 7-amino-4-methylcoumarin amide; DNP is 2,4-dinitrophenyl.
Data are taken from: [a]Cordeiro et al. (1998); [b]White et al. (1999); [c]Veríssimo et al. (1996); [d]Castanheira et al. (2005); [e]Sarmento et al. (2009), [f]Lufrano et al. (2012).

In the example of cardosin A, cleavage of the β-chain of oxidized insulin (Table 5) occurred at the bonds Leu15-Tyr16, Leu17-Val18, and Phe24-Phe25 (Veríssimo et al., 1995). The optimum pH for hydrolysis of the synthetic peptide Lys-Pro-Ala-Glu-PheNph-Ala-Leu was 4.5, and values determined for the apparent active-site ionization constants pK_{e1} and pK_{e2} of the free enzyme were 2.5 ± 0.2 and 5.3 ± 0.2, respectively. Proteolytic activity was inhibited by pepstatin A (K_i = 3 nM) and diazo-acetyl-*nor*-leucine methyl ester (aka DAN; Veríssimo et al., 1996). The primary specificity of both recombinant and natural cardosin A was investigated through use of two peptide libraries. While both enzymes had a preference for tyrosine and phenylalanine in the P1′ position, at P1, phenylalanine was the only amino acid accepted in the S1 pocket readily, though leucine was as well but only moderately (Castanheira et al., 2005).

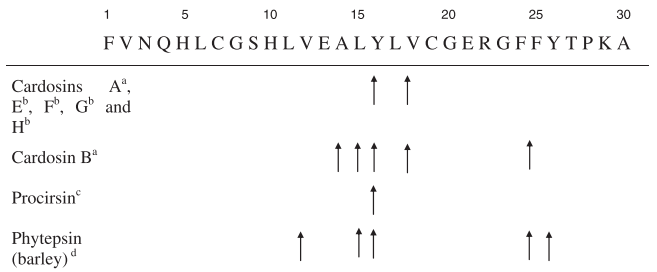
Cardosin B has a slightly broader specificity than cardosin A. Cleavage of the oxidized insulin β-chain by cardosin B occurred between the following bonds: Glu13-Ala14, Ala14-Leu15, Leu15-Tyr16, Leu17-Val18, Phe24-Phe25, and Phe25-Tyr26. The pH dependence of the kinetic parameters K_m, k_{cat}, and k_{cat}/K_m for cardosin B was investigated with the peptide substrate Leu-Ser-Nph-Ahx-Ala-Leu-OMe, and the following respective values for the apparent active-site ionization constants pK_{e1} and pK_{e2} of the free enzyme were determined at 3.73 ± 0.09 and 6.7 ± 0.1 (Veríssimo et al., 1996).

With respect to activity, cardosins E, F, G, and H resemble cardosin A more than they do cardosin B or cyprosin, with cardosins E and G being more active than cardosin A towards the synthetic peptide Lys-Pro-Ala-Glu-Phe-Nph-Ala-Leu. When the specificity of cardosins E, F, G, and H was assessed by cleavage of the β-chain of oxidized insulin, the four enzymes were able to cleave that substrate at the same peptide bonds as cardosin A (Table 5). Nevertheless, the selectivity of these enzymes varied in terms of the velocity exhibited with each scissile peptide bond (Sarmento et al., 2009).

Cenprosin showed maximal activity with fluorescein–isothiocyanate–haemoglobin at pH 3.5 (Domingos et al., 2000).

The activity of recombinant procirsin (the cirsin zymogen) was tested with a series of fluorogenic substrates. Only the typical AP fluorogenic substrate (MCA)Lys-Lys-Pro-Ala-Glu-Phe-Phe-Ala-Leu-Lys(DNP) was significantly cleaved, with a maximum activity at pH 4.0. Kinetic parameters were determined at this pH (K_m: 7.13 ± 1.30 μM and k_{cat}: 4.24 ± 0.05 s^{-1}, with a catalytic efficiency of 594 mM^{-1} s^{-1}). Substrates designed for proteases described as atypical APs, (MCA)Lys-Leu-His-Pro-Glu-Val-Leu-Phe-Val-Leu-Glu-Lys(DNP) and (MCA)Lys-Lys-Leu-Ala-Asp-Val-Val-Asn-Ala-Leu-Glu-Lys-Lys(DNP) were poorly cleaved and not cleaved at all, respectively. No hydrolysis was observed with the fluorogenic BACE-1 substrate (MCA)Lys-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys(DNP), the renin substrate 1-Arg-Glu(EDANS)Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(DABCYL)Arg, or the HIV-protease substrate 1-Arg-Glu(EDANS)Ser-Gln-Asn-Tyr-Pro-Ile-Val-Asn-Lys(DABCYL)Arg. The most susceptible cleavage site was at Leu15-Tyr16 when oxidized insulin β-chain was hydrolyzed with recombinant procirsin (Lufrano et al., 2012).

Table 5
Thistle-AP specificity towards insulin β-chain compared to phytepsin from *Hordeum vulgare*. Arrows indicate the cleavege sites.



Data taken from: [a]Veríssimo et al. (1995); [b]Sarmento et al. (2009); [c]Lufrano et al. (2012); [d]Kervinen et al. (1993).

5. Tissue localization

In-situ localization studies have shown that APs are not randomly distributed within plants. Rather, they are located in different specific tissues depending on the species (Simões and Faro, 2004). Moreover, in each part of the cell, proteolytic activity is performed by a separate protease specifically targeted to particular subcellular compartments (Pesquet, 2012).

This cellular anatomical specificity of plant APs has suggested a huge number and wide diversity of these enzymes, where characteristic may account for the finding that genes encoding the cyprosins and the cardosins are organized as a multigene family expressed during early stages in the development of the *C. cardunculus* flowers (Pimentel et al., 2007). Cordeiro et al. (1994b) have demonstrated by Northern blotting that synthesis of the mRNA encoding cyprosin starts during the early stages of floral development and switches off upon flower maturation. In addition, cardosin mRNA was detected in young inflorescences, but not in pistils of fully opened ones, thus indicating that the expression of the structural gene is developmentally regulated (Faro et al., 1999; Vieira et al., 2001). Furthermore, mRNA and protein analyses of both cardosins suggested that the two enzymes accumulate during seed maturation and that cardosin A is later synthesized *de novo* when the radicle emerges (Pereira et al., 2008).

Cyprosins accumulate in flowers only and in particular in mature flowers. Using immunostained Western blots with polyclonal antibodies against the large subunit of cyprosin 3; Cordeiro et al. (1994a) detected the presence of cyprosin, in low amounts, in very young flowers. These authors demonstrated that the amount of enzyme increased towards the later stages of development and was mostly present in the violet-colored parts of the styles and corollas, they also localized cyprosins in the epidermal cell layer of the styles by immunogold labelling.

The expression of cardosin A was found to occur from the beginning of flower development and was highly restricted to the pistils. Fig. 5 shows a schematic representation of the thistle gynoecium. Cardosin A accumulated mainly in the protein-storage vacuoles of stigmatic papillae (Fig. 5A), but was also located in subepidermal parenchymal layers of the stigma. At a much lower proportion or abundance, cardosin A was present in the large central vacuole of style epidermal cells, but was not detected in cytoplasm, cell wall, or organelles (Duarte et al., 2006; Pissarra et al., 2007; Ramalho-Santos et al., 1997). In the embryo (*C. cardunculus*), the cardosin-A precursor form was seen to accumulate in protein bodies and cell walls, a different localization from that described in cardoon flowers, thus suggesting a tissue-dependent pattern for accumulation of the protein (Pereira et al., 2008; Pissarra et al., 2007).

Localization of cardosin B is clearly different from that of cardosin A, since the former was reported as accumulating in the inner region of pistils until the later stages of floral development. Moreover, the presence of this cardosin in the transmitting tissue increased from the upper region of the stigma to the lower style (Fig. 5A and B), with the same accumulation occurring also on the ventral surface of the ovary wall and in the nucellus (Duarte et al., 2006; Figueiredo et al., 2006; Vieira et al., 2001). This localization led Pissarra et al. (2007) to suggest that cardosin B seemed to establish an inner route from the upper stigma to the embryo sac in the ovule (*c.f.* Fig. 5). Immunocytochemistry, with a monospecific antibody, localized cardosin B on the cell wall and in the

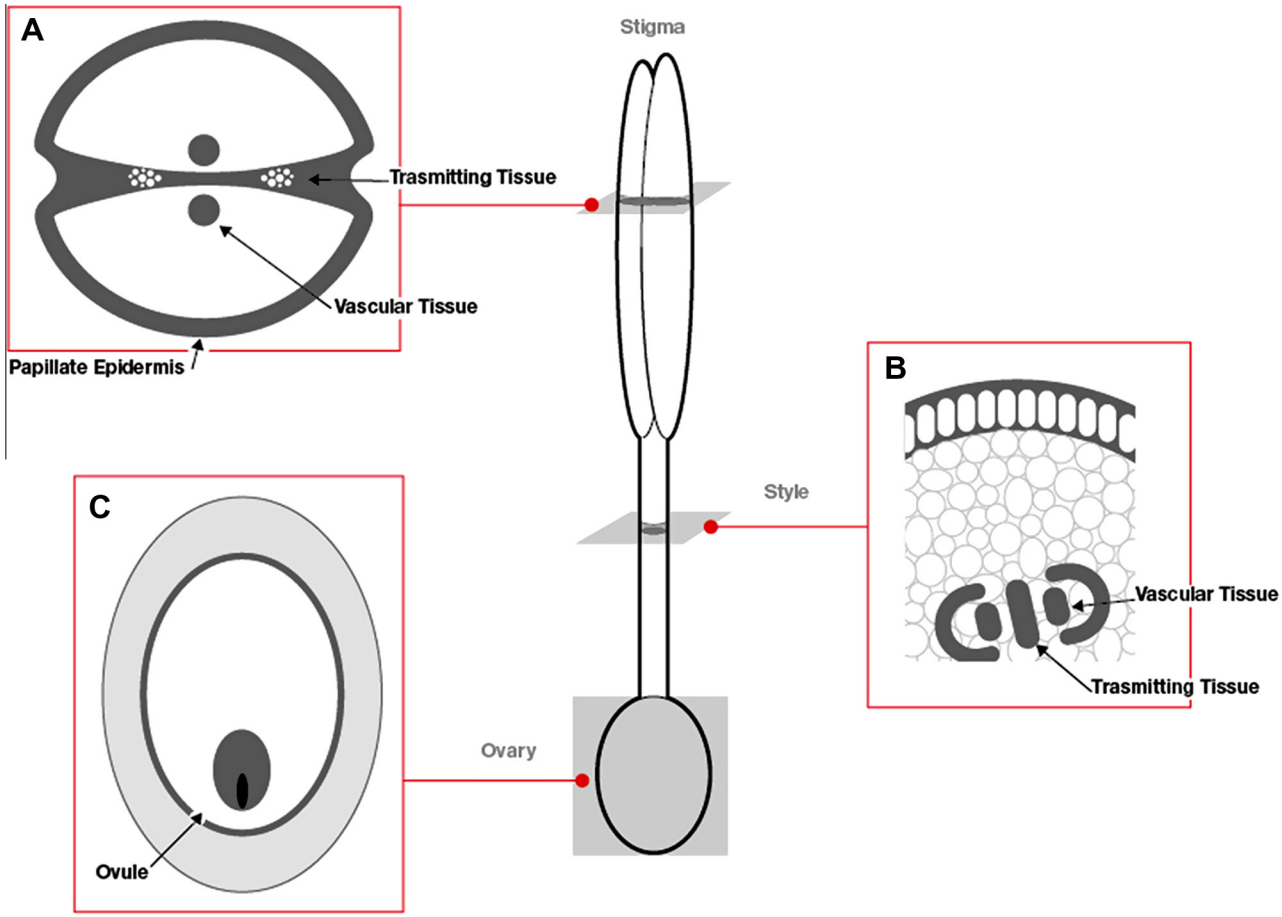


Fig. 5. Schematic diagram of a thistle gynoecium. (A) Transverse section of the stigma. (B) Transverse section of the style. (C) Longitudinal section of the ovary. The gynoecium consists of a single compound pistil of two carpels, a single two-cleft style, and an inferior ovary with one locule and one basal ovule. The stigma is bifid and covered by the stigmatic papillae (Faro et al., 1998; Singh, 2004).

extracellular matrix of the floral transmitting tissue (Vieira et al., 2001). When cardosin B was expressed in two different heterologous systems (e.g., the dexamethasone-inducible expression in transgenic *Arabidopsis* and the *Agrobacterium*-mediated transient expression in *Nicotiana tabacum* abaxial leaf epidermis), the protease was found mainly in the vacuole, but was also detected on the cell wall (Soares da Costa et al., 2010).

6. From proenzymes to mature peptidases

6.1. Zymogen inactivation

In the prophytepsin crystal structure, anchorage of the prosegment and part of the N-terminus in the active-site cleft is achieved by ionic interactions between the Lys11/Tyr13 of the mature-enzyme sequence and catalytic aspartic acids at the bottom of the cleft (Kervinen et al., 1999). The Lys/Tyr sequence is also found in mammalian-AP-zymogen propeptides and on well established evidence appears to be responsible for the ionic interactions with Asp residues of the active site (Richter et al., 1998). Many plant APs, such as cyprosin, contain a Lys/Tyr sequence in a position equivalent to the Lys11/Tyr13 of prophytepsin (*Hordeum vulgare* L.), and an inactivation mechanism similar to the one proposed for this propeptidase has been suggested there as well (Simões and Faro, 2004; White et al., 1999). Even when procirsin has this sequence in position Lys10/Tyr12, a recombinant procirsin remains active is indeed notable (Lufrano et al., 2012) and highlights the need for conducting further studies on the AP-precursors activation/inactivation mechanisms.

Castanheira et al. (2005) proposed that the procardosin-A propeptide—as prosegments of many other AP zymogens—has a role in its own inactivation. Cardosin A and cardosin B, however, do not contain the aforementioned sequence, neither in the prosegment nor in the N-terminus of the mature enzyme (Simões and Faro, 2004).

6.2. Processing

Cardosins, cyprosins (*C. cardunculus*), cenprosins (*C. calcitrapa*), and cirsin (*C. vulgare*), like typical plant APs, are synthesized as zymogens that require enzymatic processing for maturation (Brodelius et al., 2005; Domingos et al., 2000; Lufrano et al., 2012; Ramalho-Santos et al., 1998).

The *in vitro* processing of native procardosin A was likewise found to be sequential, starting with a cleavage between the 31-kDa fragment and a PSI with the consequent accumulation of an intermediate form that was only partially processed. Next cleavage occurred at the C-terminal of the PSI before final removal of the prosegment (Ramalho-Santos et al., 1998). The *in vitro* processing of the recombinant procardosin A was also found to be a multistep process, but in this instance the first step was excision of the propeptide to generate an active intermediate with the PSI domain still present in the structure. Removal of the PSI was subsequently effected by sequential cuts out of the precursor. *In vitro* activation

of this recombinant procardosin led ultimately to generation of an active form, where the two polypeptide chains still remained associated by a disulfide linkage (Castanheira et al., 2005). On the basis of their findings, Castanheira et al. (2005) proposed that completion of *in vivo* maturation might require action of another protease or exopeptidase(s). Duarte and colleagues (2008) followed processing of an isoform of cardosin A by heterologous expression in two systems (a transient form in tobacco, and an inducible one in *Arabidopsis thaliana*). The resulting data suggested that cleavage at the N-terminus of the PSI preceded prosegment removal, so as to produce an intermediate form of 35 kDa beforehand and a mature form of 31 kDa afterwards. As the prior intermediate form of 50 kDa predicted by the initial cleavage of procardosin A at the C-terminus of the PSI was rarely seen, the cut in the N-terminal PSI should either immediately precede or else occur very shortly after excision of the C-terminus. Thus, removal of the PSI took place before the loss of the prosegment, in contrast to the model for the maturation of plant APs, the well characterized AP phytepsin from barley (Duarte et al., 2008). These *in vivo* results differed, however, from those *in vitro* described by Castanheira et al. (2005), again reinforcing the idea that other enzymes, and not simply a drop in the pH, are necessary for activation. Processing of the prophytepsin from barley was also shown to be incomplete *in vitro*, and markedly different from the maturation pattern observed *in vivo* (Duarte et al., 2008; Glathe et al., 1998).

When cardosin B was transcribed by dexamethasone-inducible expression in *Arabidopsis* and by *Agrobacterium*-mediated transient expression in tobacco epidermal cells, processing of the enzyme was sequential. The signal peptide was first removed upon primary translation product's entrance into the endoplasmic reticulum, and an initial cleavage occurred between the heavy chain and the PSI. The latter was next eliminated by scission between this region and the light chain, and finally the prosegment was deleted to produce cardosin B's fully processed form, in which heavy and light chains are held together by hydrophobic interactions and hydrogen bonds (Soares da Costa et al., 2010).

When cyprosin 3 (aka cyprosin B) was expressed in *Pichia pastoris*, processing of the initial protein translation product started with elimination of the prosegment. In contrast to the naturally occurring cyprosin, the light chains remained attached by disulfide bonding to the heavy chains in this system. Removal of the PSI in plants must therefore take place at a slightly different location from those sites observed in *P. pastoris*. The processing steps were only marginally affected by inclusion of the aspartic proteinase inhibitor, pepstatin, in the culture medium of the *Pichia* cells. In this system, processing likely takes place through action of host-cell peptidases at exposed sites that are susceptible to heterocatalytic attack (White et al., 1999).

The cenprosin proform (65 kDa) isolated from fresh flowers of *C. calcitrapa* was reported to undergo *in vitro* autoproteolytic processing at pH 3.0 and pH 5.0. Although cleavage sites were not determined, the molecular masses of the intermediate forms estimated by sodium-dodecylsulfide–polyacrylamide-gel electrophoresis—50, 43, 30, 21, and 16 kDa—were in good agreement with those found for cardosin A (Domingos et al., 2000).

The cirsin zymogen expressed in *E. coli* has been shown to be active without autocatalytic cleavage of its proenzyme-specific domain. This feature contrasts with the autoactivation through acid-triggered prosegment removal described for recombinant cardosin and cenprosin (Lufrano et al., 2012).

6.3. Targeting

As mentioned in Section 3, thistle APs enter the secretory pathway because of the presence of a signal peptide responsible for their translocation into the ER. Ramalho-Santos and coworkers (1998) suggested that the C-terminal sequence VGFAEAA, found in cardosin A and in other plant APs, may represent a putative vacuolar targeting signal in APs for this ultimate localization. This sequence is highly similar to the VFAEAIA array found in barley lectin that was proven to be sufficient for the vacuolar targeting of the protein. As will be discussed in Section 6.4, the PSI was also proposed as a vacuolar-sorting signal.

In the cardoon, cardosin A is synthesized as a preproenzyme of 66 kDa that is translocated into the ER, where the signal peptide is then excised and the protein becomes glycosylated at its two predicted N-glycosylation sites. As mentioned briefly in Section 3, both sites, Asn 70 and Asn 363, are occupied by oligosaccharides predominantly resistant to removal by endoglycosidase H, thus indicating prior processing in the Golgi (Costa et al., 1997). When cardosin A was expressed in tobacco-leaf epidermis and in *Arabidopsis* seedlings, the enzyme was correctly sorted to the vacuole, but in both those heterologous systems the N-glycan derivatives were complex and acquired partial endoglycosidase-H resistance during transport. Inhibition of ER-Golgi traffic by coexpression of either dominant inhibitory form of the Sar1 and Rab-D2 GTPases—which hydrolases block ER-to-Golgi traffic via different mechanisms—led to an accumulation of procardosin A, whose buildup meant that transport of this proenzyme to the Golgi was taking place in COPII-coated vesicles. Moreover, brefeldin A—a drug that causes Golgi fusion with the ER, thus inhibiting anterograde transport of secreted products and vacuolar markers—inhibited processing of cardosin A in tobacco protoplasts. A 35-kDa intermediate of mature cardosin was found in the vacuole fraction, suggesting that the final step in the post-translational processing occurred in that compartment. Once cardosin A reached the vacuole—in contrast to phytepsin, whose protease was degraded in vacuoles of tobacco protoplasts—the cardosin was found to be stable for several days in tobacco leaves and protoplasts, and also in *Arabidopsis*-seedling vacuoles (Duarte et al., 2008).

Like cardosin A, cardosin B has two putative *N*-glycosylation sites, Asn138 and Asn252; but, in contrast to the glycosylation pattern of cardosin A, the glycans bound to cardosin B—both in naturally occurring or recombinant cardosin B (expressed in *Arabidopsis* and *Nicotiana tabacum*)—are sensitive to endoglycosidase-H digestion. This glycosylation pattern implies that glycans are of the high-mannose-type and suggests that either those sugars are inaccessible to the Golgi processing enzymes. Soares da Costa and coworkers (2010, 2011) suggested that this is a result of the cardosin B conformation, or the protein leaves the Golgi at an early step before the Golgi modifying enzymes are able to derivatize them. In the Golgi, the enzyme is transported through the organelle in a RAB-D2a-dependent route and is delivered to the vacuole via the prevacuolar compartment in a RAB-F2b-dependent pathway (Soares da Costa et al., 2010, 2011).

Since cardosin B was secreted in cardoon pistils, localization of the enzyme in vacuoles of the cardoon-ovary cells, as well as in heterologous systems, suggested that the differential targeting of cardosins A and B in cardoon pistils resulted principally from differences in cells in which those two proteins were expressed (Soares da Costa et al., 2010, 2011). These authors deduced that complete processing of cardosin B was occurring in the endoplasmatic reticulum before transport to the Golgi. Based on observations obtained thus far on previously published results with cardosins, the mechanisms of protease assembly and transport through the endomembrane system may be tissue-dependent as a result of the differentiation of trafficking pathways according to environmental stimuli and signals received from the adjacent cells (Oliveira et al., 2010; Soares da Costa et al., 2011).

6.4. Plant-specific inserts (PSIs)

A unique feature of typical plant AP precursors is the presence of an internal domain, known as the plant-specific insert (PSI), whose function is as yet not completely understood. According to the three-dimensional structure of the phytepsin precursor, this additional sequence of approximately 100 residues is thought to form an external loop on the surface of the protein (Kervinen et al., 1999). The PSI structure resembles that of saposin-like proteins (aka the SAPLIPs), a group of lipid-interacting species that have various cellular functions and feature a characteristic location of six cysteines and several hydrophobic residues (Munford et al., 1995). PSIs have been called swaposins, because of a reverse homology of the N- and C-termini of the PSI, in which two respective termini of a given PSI are "swapped" with opposite termini of two different saposins. As a consequence, protein primary structures result with different connectivities, but with similar overall three-dimensional shapes (Brodelius et al., 2005; Bryksa et al., 2011; Egas et al., 2000; Törmäkangas et al., 2001). In fact, a rearranged form of cyprosin recombinant PSI—in which the N- and C-terminal sequences were swapped to resemble the structural arrangement observed in saposins—functioned as an efficient activator of a human glycohydrolase *in vitro* with comparable activity to that of saposin C (Brodelius et al., 2005). The PSI domain of crystalline barley prophytepsin contained a "closed" tertiary structure similar to that of NK-lysin and saposin B, but the crystalline recombinant potato PSI exhibited a tertiary structure similar to open V-shaped configuration of saposin C (Bryksa et al., 2011).

The functions of the PSIs are still unclear. These domains, however, have been proposed to function as vacuolar-targeting signals in a way similar to involvement of saposins in the cotransport of procathepsin D to the lysosome (Domingos et al., 2000; Simões and Faro, 2004; Soares da Costa et al., 2010). When procyprosin was expressed in *P. pastoris* cells, the mature enzyme was secreted by the yeast into the medium, therefore suggesting the enzyme's PSI did not contain the appropriate intracellular targeting signals functional for *Pichia* (White et al., 1999).

Plant APs are found as either monomeric or heterodimeric forms. Whereas the monomeric APs retain the PSI, the heterodimers lose their PSIs during proteolytic maturation, and as mentioned in Section 3, all known thistle APs are heterodimeric. Ramalho-Santos et al. (1998) have proposed that the mechanism by which the PSI is removed from procardosin A involves a specific conformation of the precursor. Since the PSI domain contains five amphipathic helices linked to each other by three disulfide bridges appropriately positioned to give helical hydrophobic domains, the PSI is able to interact with a membrane during procardosin-A intracellular transport so as to achieve the proper conformation for processing at the PSI-cleavage sites. In fact, a recombinant PSI from procardosin A was found to interact with phospholipid membranes to promote release of the aqueous contents of phospholipid vesicles in a pH-dependent manner, with higher leakage activity occurring at acidic pHs. A similar dependence has also been found for saposins C and D with respect to their ability to bind and thereafter induce leakage in synthetic vesicles (Egas et al., 2000).

The sequence encoding the entire PSI was deleted from procardosin A and procirsin and the mutants expressed in *E. coli* in order to evaluate the significance of the PSI domain in the proteolytic processing and activity of the wild-type proenzymes. The mutant procardosin A without the PSI domain was easily auto-activated at acid pH and behaved like the natural enzyme in terms of primary specificity (Castanheira et al., 2005), whereas expression of a PSI-deleted procirsin led to an inactive protein, thus suggesting a critical role for this internal segment in the folding of the mature cirsin (Lufrano et al., 2012). Both mutants were expressed by the same procedure.

In a similar approach, White and coworkers (1999), deleted nucleotides encoding the PSI of procyprosin 3 and replaced five residues (Lys238-Gly-Val-Met-Ser242) immediately preceding the PSI with the corresponding residues (Val238-Pro-Leu-Ile-Gln242) from human cathepsin D, a sequence known to form a surface loop in this region of the human enzyme. The expression of this insert-deleted construct in *Pichia* resulted in production of lower amounts of an inactive recombinant protein. These researchers claimed that in *Pichia* the PSI appeared to be essential for insuring that the nascent polypeptide was folded properly into a form such that subsequent processing would produce mature enzyme (White et al., 1999). Nonetheless, findings from the expression of this inactive mutant of procyprosin 3 were in agreement with data reported for expression of the PSI-deleted procirsin, and that correspondence was consistent with the high degree of similarity between the amino-acid sequences and the structures of both enzymes (Lufrano et al., 2012), thus suggesting that the PSIs of these enzymes might be involved in the correct folding of the respective proteins.

As observed by Cordeiro et al. (1994b), the PSI sequences exhibit a high diversity (*cf.* Fig. 1), one greater than the sequence diversity of the parent APs themselves, and this variation might have resulted from adaptations necessary during evolution. The PSIs in plant APs may, therefore, have different functions depending on the enzyme in question (Törmäkangas et al., 2001; Brodelius et al., 2005; Terauchi et al., 2006; Duarte et al., 2008) .

7. Physiological roles

The great diversity of APs within either the same plant or tissue suggests existence of different roles for each AP that may be related to specificity, catalytic efficiency, and/or subcellular localization (Mazorra-Manzano et al., 2010). The APs of *C cardunculus* are encoded by a multigene family composed of several members, and in this species—with the cardosins and cyprosins coexisting within the same plant—both types of APs are expressed in early stages of floral development (Pimentel et al., 2007; Ramalho-Santos et al., 1997; Vieira et al., 2001). Even though the biological roles of the cardosins and the cyprosins in the cardoon flowers are not completely understood, the pistil-specific localization of both enzymes suggests a possible participation in several flower-associated phenomena—such as flower senescence, defense mechanisms against insects and/or pathogens, and reproduction (Sarmento et al., 2009; Soares da Costa et al., 2010). Therefore, Cordeiro et al. (1994b) have proposed that cyprosins in flowers have a role in senescence, since these enzymes specifically accumulate and have maximum activity in mature organs. The specific localizations of cardosin B in the cell wall and the extracellular matrix of the transmitting tissue of the style have led researchers to think that this enzyme may be involved in expansion or loosening of transmitting-tract extracellular matrix to accommodate the growing pollen tube. However, because of the cardosin's relatively high abundance within the extracellular matrix of this tissue, its broad specificity, and its high specific activity, a defensive function has also been proposed, (Vieira et al., 2001). Cardosin B has also been implicated in development of the embryo sac where that cardosin is also localized, along with being associated as well with events of programmed cell death in the nucellus (Figueiredo et al., 2006).

As mentioned earlier in Section 3, cardosins A, C, E, F, G, and H possess RGD and KGE motifs; with the first of these being a well-known cell-attachment motif characteristic of integrin-binding proteins and the second one mimicking the RGD in both structure and charge. A possible function of RGD-containing cardosins has been suggested related to the adhesion-mediated proteolytic

mechanisms associated with pollen-tube extension. These mechanisms function in a similar way to the proteases that act together with integrin during cell proliferation and invasion (Faro et al., 1999; Pimentel et al., 2007). Phospholipase Dα has been identified as a cardosin-A–binding protein. Interaction of both these enzymes is mediated by the phospholipase-Dα C2 domain, a microenvironment that has been suggested to be facilitating protein–protein interactions. Nevertheless, cardosin B—it harboring RGN and EGE instead of RGD and KGE motifs—does not bind phospholipase Dα. Simões and coworkers (2005) suggested that formation of a complex between cardosin A and phospholipase Dα indicated possible actions (concerted and/or synergistic) in degenerative processes, such as those observed during stress responses, plant senescence, and/or pollen–pistil interactions.

Besides the proposed physiological functions related to protease activity *per se*, and those associated with the cell-attachment motifs, the PSI domain of plant APs could have roles based on its membrane-interacting properties that are independent of the remainder of the proenzyme (Bryksa et al., 2011). Finally, the leakage produced by PSI in synthetic vesicles may simulate an analogous function *in vivo* in the defense against pathogens or in programmed cell death (Egas et al., 2000).

Brodelius and coworkers (2005) suggested that the PSI may be involved in regulation of sphingolipid metabolism, because of PSI's tissue localization and saposin-C-like activity *in vitro* by recombinant cyprosin PSI. In addition, they proposed, as a possible function of the PSI, a role in the biogenesis of membranes in tissues undergoing high growth rates, such as flowers under development after opening and the primordial root in the embryo, where the turnover of lipids is high. Furthermore, procardosin A seemed to be acting as a bifunctional enzyme that could participate in the processing and/or degradation of seed-storage proteins, as well as in membrane-lipid conversion during water uptake and solute leakage in actively growing tissues since the proenzyme was found to accumulate in the protein bodies and cell walls of embryos (Pereira et al., 2008; Pissarra et al., 2007).

8. Biotechnological and biomedical applications

The several biotechnological and biomedical applications of thistle-flower APs reviewed in this section include manufacture of cheeses—a process which dates back to the period when the Romans occupied the Iberian Peninsula—along with other absolutely contemporary uses that are considered either potential or at present under evaluation.

In view of the specific action of the cardosin A from *C cardunculus* on collagen, this enzyme has been proposed for possible assistance in technical medical procedures or for remodelling the extracellular matrix. In addition, it has been evaluated for dissociating the embryonic cortex in order to establish primary neuronal cultures (Duarte et al., 2005, 2007). Neurons treated with cardosins exhibit normal morphology and function when compared to cultures prepared with the widely used trypsin-dissociation method (Duarte et al., 2007). Furthermore, use of cardosins resulted in an improvement of neuronal regeneration at an early stages of culture after cell dissociation (Duarte et al., 2009).

Immobilization of cardosin A in chitosan sponges by entrapment did not induce alterations in the specificity of the enzyme, thus maintaining the ability to hydrolyze type-I collagen. The biocompatibility of that form of immobilized cardosin A was tested in rats submitted to abdominal surgery (Pereira et al., 2005). According to the outcome observed, the authors proposed that these chitosan sponges would be promising vehicles for introduction of cardosin A into the abdominal cavity for prevention and reduction of adhesion formations.

In addition, because of their stability in aqueous-organic biphasic systems, cardosins A and B were able to catalyze the enzymatic synthesis of dipeptides and tripeptides in such media (Sarmento et al., 1998, 2004; Shnyrova et al., 2006).

8.1. Cheese preparation

Cardoon-flower aqueous extracts, as a rich source of milk-clotting peptidases, have been widely used for manufacture of traditional artisanal Portuguese, Spanish, and Italian cheeses (Aquilanti et al., 2011; Cordeiro et al., 1992; Veríssimo et al., 1995). Because of their unique texture, flavor, and aroma, these cheeses are marketed under the Registry of the Protected Geographical Indications and Designation of Origin.

Farmers collect cardoon flowers during the months of June and July and store them in dry places until use during autumn and winter. Aqueous extracts of the flowers of *C. cardunculus*, *C. humilis*, and/or *C. scolymus* have proven to be successful catalytic substitutes for animal rennet, and have thus been used since antiquity in the manufacture of goat and ewe cheeses in several rural areas of the Iberian Peninsula—*viz* Serra da Estrela, Serpa, Azeitão, Los Pedroches, La Serena, Flor de Guía, and Torta del Casar (Delgado et al., 2010; Reis and Malcata, 2011; Reis et al., 2000; Silva and Malcata, 2000a; Sousa and Malcata, 1997).

In general, the enzymatic coagulation of milk is a two-stage process. The predominant step in the early phases of the renneting reaction is specific proteolysis of the κ-casein molecules at the Phe105-Met106 bond in bovine and ovine κ-casein and at the Lys116-Thr117 bond in caprine κ-casein, with both substrates splitting the protein into two molecules: the hydrophobic *para*-κ-casein and the hydrophilic caseinomacropeptide. The second phase comprises coagulation of casein micelles after destabilization by proteolytic attack (Dalgleish, 1987; Silva and Malcata, 2005a). The vegetable rennet, in addition to its clotting capacity, has a strong proteolytic activity that eventually leads to an extensive breakdown of the caseins in the cheese matrix, thus contributing to the special features of the final product (Macedo and Malcata, 1997). The ripening time of calf-rennet–produced cheeses made from ewe's milk can be accelerated by adding different amounts of cardoon enzymes without losing any organoleptic properties of the control cheeses (Galán et al., 2008).

With an aim at achieving a standardization of the vegetable rennet along with a preservation of the organoleptic characteristics of those Mediterranean varieties, these cheeses have been the subject of intense research efforts (Aquilanti et al., 2011; Macedo and Malcata, 1997; Ordiales et al., 2012; Pereira et al., 2011; Pinho et al., 2001; Sousa and Malcata, 1997). The current state of Portuguese ovine and caprine cheeses has been reviewed comprenhensively by Reis and Malcata (2011).

The presence of a cardosin A-like enzyme in *C. humilis* is responsible for its milk-clotting activity (Esteves et al., 2002). The action of cardosin A from C. *cardunculus* and *C. humilis* on ovine and caprine caseins has been well documented (Ramalho-Santos et al., 1996; Silva and Malcata, 2000a, 2000b; Sousa and Malcata, 1998). For more detailed information, the reader is referred to Table 3. The use of either of those species as vegetable rennets has no significant effect on the resulting cheeses' chemical components, flavor, or aroma, or on the water activity of the cheeses from ewes' milk, although higher lactic-acid contents were observed with the enzymes from the former source (Vioque et al., 2000).

Other researchers (Raposo and Domingos, 2008; Reis et al., 2000) have demonstrated the existence of milk-clotting activity in the flowers of *C. calcitrapa* (Asteraceae). The greatest limitation to the widespread use of APs of this species in the preparation of cheeses is the low yield of the final product, along with heterogeneity of the proteinase profile from the flowers. The use of plant-

cell culture for the production of biomass as an alternative for the intensive production of these proteinases without dependence on seasonal flowering has been proposed (Lourenço et al., 2002; Raposo and Domingos, 2008). Extracts of purple starthistle flowers have also been tested as potential proteolytic additives in the ripening of commercial bovine cheeses, since those preparations produced significant changes in the composition of the cheeses and increases in degradation of both the α_s- and the β-caseins. The cheeses obtained from that rennet, however, had a slightly more bitter taste than control cheeses (Reis et al., 2000).

As mentioned in Section 2, the presence of APs with milk-clotting activity has also been detected in crude aqueous extracts of flowers from several species of the Asteraceae (Brutti et al., 2012; Cimino et al., 2010; Lufrano et al., 2007; Tamer, 1993; Vairo Cavalli et al., 2005). Aqueous extracts of the flowers of O. acantium were found to be a suitable vegetal curd when used to obtain semihard cheeses from bovine milk (Brutti et al., 2012). The flowers of *S. marianum* were proposed as an alternative source of vegetable rennet. Different peptide profiles were observed in goat's and ewe's caseinates through a hydrolysis brought about by blessed-milk-thistle extracts, with less extensive degradation occurring than with bovine caseins and the same enzyme extract (Vairo Cavalli et al., 2005, 2008).

8.2. Production of bioactive peptides

Bioactive peptides present in the amino-acid sequence of food proteins are of particular interest in nutrition and food science, because those protein fragments have been shown to play physiological roles (Meisel, 1997; Silva and Malcata, 2005b). Current research encompassing bioactive peptides is the subject of intensive scientific efforts worldwide.

Bioactive peptides have been found in cheese-like model systems mimicking the real cheeses manufactured with sheep's and goats's milk that has been renneted with *C. cardunculus* proteases. Ripened cheeses contain numerous peptides originating mainly from breakdown of caseins during ripening. Antioxidant capabilities and inhibition of the angiotensin-converting enzyme (ACE) are among bioactivities attributed to these peptides. The YQEP (f191–194), VPKVK (f95–99), and YQEPVLGP-∗ (f206-∗) peptides from β-casein along with the RPK (f16–18) and RPKHPIKH-∗(f16-∗) from α_{s1}-casein have ACE-inhibitory activity, while peptides released upon cleavage of the peptide bond Leu190-Tyr191 (present in ovine or caprine β-casein), and corresponding to the sequence YQEP-∗ (f191-∗) of β-casein, have antioxidant activity (Silva et al., 2006).

Bovine whey-protein concentrates, as well as purified proteins from whey after hydrolysis by the proteases from cardoon flowers, also released highly potent ACE-inhibitory peptides. Among all the peptides obtained in this manner, the bioactive fragments generated from α-lactalbumin—*viz* KGYGGVSLPEW (f16–26), DKVGINY (f97–103), KVGINYW (f98–104), DKVGINYW (f97–104), as well as the decapeptide DAQSAPLRVY (f33–42) from β-lactoglobulin—were different from previously reported peptides obtained by hydrolysis of the same substrate with other proteases (Tavares et al., 2011a,b). Even when the peptides were partially hydrolyzed after simulated gastrointestinal digestion, the overall ACE-inhibitory activity was not severely affected (Tavares et al., 2011a). Furthermore, whey-protein concentrates hydrolyzed with *C. cardunculus* extracts—as well as the ultrafiltration fraction of molecular weight below 3 kDa, fractions 16–26 and 97–104, from α-lactalbumin and the fraction 33–42 from β-lactoglobulin—had antihypertensive effects upon adminitration by gastric intubation to hypertensive rats. Moreover, the hydrolyzed concentrate and fraction 16–26 from α-lactalbumin (KGYGGVSLPEW) showed ACE-inhibitory activity *in vivo*: the pressor effect of angiotensin I was significantly lower and the response to bradykinin increased when hypertensive rats were pretreated with either product. In view of these results, Tavares et al. (2012) proposed that whey-protein concentrates hydrolyzed by *C. cardunculus* extracts would be an effective nutraceutical ingredient for the formulation of functional foods aimed at hypertension control.

Two ovine caseinomacropeptide-derived peptides—TAQVTSTEV (f163–171) and QVTSTEV (f165–171)—obtained by tryptic hydrolysis, have been reported to have antithrombotic activity. Since a peptide with an equivalent sequence—TVQVTSTAV (f161–169)—has been identified upon the hydrolysis of bovine caseinomacropeptide by aqueous extracts of *C. cardunculus* (Tavares et al., 2011b), the production of peptides with antithrombotic activity constitutes another potential biomedical application to be explored for these extracts.

9. Conclusions

The known chemical and biological properties of typical APs present in the flowers of the Cardueae tribe are summarized as well as their biomedical and biotechnological applications. Even when aspartylendopeptidase activity in plants is normally present at relatively low levels overall, the flowers of the thistle possess extremely high AP activities in certain tissues. In spite of all the data gathered in the last two decades, certain nagging questions still remain regarding: regulation of catalysis; the biologically relevant target substrates; possibility of different roles for the proenzymes and the mature enzymes; significance of the heterogeneity of these enzymes in each thistle species; purpose of the independent domain PSI in the precursor form, and whether or not PSI still has a function when exscinded from the enzymatic precursor. Nevertheless, the recent rapid progress in the study of enzyme function and structure–function relationships would lead us to believe that these pressing questions will be resolved within the foreseeable future.

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