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Identification and proteolytic processing of procardosin A

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Plant aspartic proteinases contain a plant-specific insert (PSI) of about 100 amino acids of unknown function with no similarity with the other aspartic proteinases but with significant similarity with saposins, animal sphingolipid activator proteins. PSI has remained elusive at the protein level, suggesting that it may be removed during processing. To understand the molecular relevance of PSI, the proteolytic processing of cardosin A, the major aspartic proteinase from the flowers of cardoon (*Cynara cardunculus* L.) was studied. Procardosin A, a 64-kDa cardosin A precursor containing PSI and the prosegment was identified by immunoblotting using monospecific antibodies against PSI and the prosegment. Procardosin A undergoes proteolytic processing as the flower matures. PSI was found to be removed before the prosegment, indicating that during processing the enzyme acquires a structure typical of mammalian or microbial aspartic proteinase proforms. *In vitro* studies showed that processing of PSI occurs at pH 3.0 and is inhibited by pepstatin A and at pH 7.0. Sequence analysis allowed the identification of the cleavage sites, revealing that PSI is removed entirely, probably by an aspartic proteinase. Cleavage of the PSI scissile bonds requires, however, a conformation specific to the precursor since isolated cardosins and pistil extracts were unable to hydrolyse synthetic peptides corresponding to the cleavage sites. In view of these results, a model for the proteolytic processing of cardosin A is proposed and the molecular and physiological relevance of PSI in plant aspartic proteinase is discussed.

Keywords: aspartic protease; milk-clotting enzyme; cardosin; proteolytic processing; saposin.

Aspartic proteinases (AP) are a widely distributed class of endoproteases that share significant similarities at the amino-acid-sequence level and at the structural level [1, 2]. The typical characteristics of the family are an acidic pH optimum, inhibition by pepstatin A, preference for bonds between hydrophobic amino acids, and sequence similarities, in particular the conservation of the catalytic triads Asp-Thr-Gly or Asp-Ser-Gly [1–4]. AP have been implicated in diverse physiological processes, such as digestion or blood-pressure regulation, and in some pathological conditions, including infection by fungi and retroviruses or cancer [5, 6].

Like many other proteases, AP are synthesised as inactive zymogens and undergo proteolytic processing leading to the activation of the enzyme [1]. Most AP contain an N-terminal prosegment of 46–50 amino acids that is removed during processing [7]. In pepsinogen, the prosegment is located along the active-site cleft, stabilised by salt bridges that are disrupted at low pH [8]. In cathepsin D, in addition to the N-terminal prosegment and a C-terminal dipeptide, there is another sequence that is removed [9]. Removal of this 2–7-amino-acid sequence gives rise to two-chain active cathepsin D.

Plant AP have been much less studied, although they are known to be consistently present throughout the whole plant kingdom [5]. In the majority of plants AP are located in seeds, whether quiescent or germinating. They are believed to participate in storage-protein cleavage, which is necessary for germination [5, 10]. AP have also been found in leaves of some plants. In leaves they have been implicated in mechanisms of defense against pathogens [11, 12]. Species of the genus *Cynara* contain considerable amounts of AP in flowers. Recent data indicate that the major AP from cardoon (*Cynara cardunculus* L.), cardosin A, may be involved in the sexual reproduction of the plant [6] and may have a defensive role as well [13].

A few primary structures deduced from the cDNAs of AP from different plant families have been determined [5, 6, 12, 14]. They share about 66% identity with each other and 40% identity with cathepsin D, the closest AP of non-plant origin. However, the similarity with cathepsin D is higher in the N-terminal and C-terminal domains, since all plant AP studied contain an insertion of about 104 amino acids, with no similarity to AP from other origins, called plant-specific insert (PSI). PSI has remained elusive at the protein level, suggesting that it may be at least partially removed during processing [15]. The molecular and physiological relevance of PSI remains unknown. However, a significant similarity (33% identity) with saposins, animal sphingolipid-activator proteins [16], has been noted, including the conservation of six cysteine residues [17].

We have studied two AP from cardoon, cardosins A and B [15]. Both AP are two-chain glycosylated enzymes, and they are thought to have arisen by gene duplication [5]. The structure of the glycans is of the plant-modified type [18]. Both enzymes preferably cleave bonds between residues with bulky hydrophobic side chains, but cardosin B displays a broader specificity and

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Abbreviations. AP, aspartic proteinase; PSI, plant-specific insert.

Enzymes. Cathepsin D (EC 3.4.23.5); pepsin (EC 3.4.23.1); renin (EC 3.4.23.15).

a higher proteolytic activity [19, 20]. The localisation of cardosin A has been studied in detail [13]. The enzyme accumulates in protein-storage vacuoles of the papillar epidermis of the stigma, the pollen-receptive surface of the pistil [13]. In the present work, the proteolytic processing of cardosin A and the relevance of PSI are investigated.

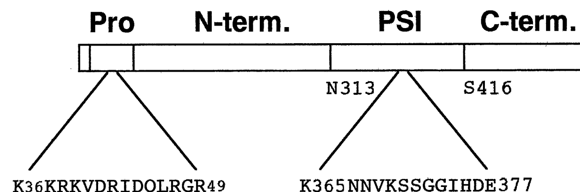

Fig. 1. Design of the monospecific antibodies against PSI and the prosegment. Numbering corresponds to the complete sequence of cardosin A, including the presegment and prosegment. The first and the last amino acids belonging to the PSI are shown. Peptides with the sequences indicated were synthesised and used in the production of monospecific antibodies. A polyclonal antibody against mature cardosin A was also produced. Pro, prosegment; N-term., N terminus; C-term., C terminus.

EXPERIMENTAL PROCEDURES

Plant material. Pistils of flowers of *C. cardunculus* were collected from plants grown from seeds supplied by the Botanical Gardens of the University of Coimbra, Portugal. Pistils were collected at two stages of development, closed (immature) capitula and open (mature) capitula, and were stored at −80°C until usage.

Antibody production. Peptides corresponding to sequences of PSI and the prosegment of cardosin A (KNNVKSSGGIHDE and KKRKVDRIDQLRGR, respectively) were synthesised chemically (Bio-Synthesis). These peptides were used for the production of monospecific antibodies in New Zealand rabbits, using thyroglobulin as carrier, essentially as described [13, 21]. The sera obtained was purified in an ECH Sepharose 4B matrix (Pharmacia) conjugated with the peptide used for immunisation according to the manufacturer's instructions. The presence of anti-peptide Ig was assessed by dot-blot analysis using the peptide linked to ovalbumin as antigen. A polyclonal antibody against cardosin A, purified as described previously [15], was produced in a New Zealand rabbit following the method of Sambrook et al. [21].

Protein extraction. Pistils at different stages of development were ground in a mortar and pestle under liquid nitrogen. For the identification of procardosin A the ground tissues were homogenised at 20% (mass/vol.) in 100 mM Tris, 100 mM Bicine, 2% (mass/vol.) SDS, 8 M urea, and the homogenates incubated for 2 h at 37°C under agitation. Extracts obtained were centrifuged at 12000 *g* for 15 min at 4°C, and the supernatants were kept at −20°C. For the extractions at pH 7.0 NaCl/P_i (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.0) was used. For the extractions at pH 3.0 200 mM sodium citrate pH 3.0 was used, with or without 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 100 μM iodoacetamide or 1 mM EDTA (Sigma). Extracts obtained were centrifuged at 12000 *g* for 15 min at 4°C. The supernatants were collected and divided into two fractions of equal volume: one was immediately mixed 1:1 with 200 mM Tris, 200 mM Bicine, 4% SDS, 8 M urea; the other was left for 1 h at room temperature before being mixed with the same solution. All samples thus obtained were kept at −20°C. Protein concentration in the samples was determined using the bicinchoninic acid protein assay reagent kit (Pierce) according to the manufacturer's instructions.

Immunoblotting analysis. Protein samples (10−20 μg) were loaded onto 15% polyacrylamide gels for SDS/PAGE, and electrophoresis was performed in a Mini Protean apparatus (Bio-Rad) according to the method of Laemmli [22]. Alternatively, 1-μg protein samples were loaded onto 12.5% PhastGels, and SDS/PAGE was performed in PhastSystem (Pharmacia) according to the manufacturer's instructions. Proteins separated in the Mini Protean apparatus were transferred onto nitrocellulose membranes by electroblotting in 10 mM Caps, 10% methanol, pH 11 at 500 mA for 1 h. Proteins separated in PhastSystem were transferred onto nitrocellulose membranes using the same apparatus according to the manufacturer's instructions. The membranes were incubated in 5% (mass/vol.) skimmed milk in 0.1% (by vol.) Tween 20 (Merck) in NaCl/P_i, for 45 min at room temperature, then incubated overnight with 1:200 or 1:400 dilutions of the primary antibody in NaCl/P_i/0.1% Tween 20. The membranes were washed 3 times in NaCl/P_i/0.1% Tween 20 for 10 min and incubated with a 1:1000 dilution of swine anti-rabbit IgG conjugated to horseradish peroxidase (Dako) for 1 h. The membranes were washed three times in NaCl/P_i/0.1% Tween 20 for 10 min, and peroxidase activity was measured by luminol chemiluminescence using the enhanced chemiluminescence method (Amersham) according to the manufacturer's instructions. Stripping of antibodies and reprobing of membranes with different antibodies were performed according to the manufacturer's instructions.

Sequence analysis. Proteins to be sequenced were transferred to poly(vinylidene difluoride) membranes using the method described above and stained with Coomassie Blue (Sigma). Automated N-terminal sequencing was performed on an Applied Biosystems 473-A Protein Sequencer (Perkin Elmer, Applied Biosystems Division). Prior to C-terminal-sequence analysis, electroblotted cardosin A chains were treated with phenylisocyanate under basic conditions to modify the lysine side chains [23]. C-terminal-sequence analysis was performed on a Procise 494C Sequencer (Perkin Elmer, Applied Biosystems Division) using a chemical protocol modified slightly from that described in [24]. The alkylated thiohydantoins were identified on-line by reverse-phase analysis on a 140 C microgradient system from the same firm.

Hydrolysis of synthetic peptides with the cleavage sites. The peptides IGANGVMNQQ, EHLSTSSEEL and RGTVRDSGSA, corresponding to sequences around the cleavage sites between the prosegment and the 31-kDa fragment, the 31-kDa fragment and PSI, and PSI and the 15-kDa fragment, respectively, were synthesised chemically by Dr Arthur Moir (University of Sheffield, UK). Each peptide was incubated with isolated cardosins (enzyme:substrate mass ratios of 1/200, 1/100 and 1/40) in 0.1 M sodium formate pH 3.1 or in 0.1 M sodium citrate pH 6.0, and the reaction was allowed to proceed at 30°C. At selected times aliquots were taken and applied to a Biobrene activated filter (Perkin Elmer, Applied Biosystems Division) for automated amino acid sequencing.

RESULTS

Identification of a cardosin A precursor containing PSI. Antibodies intended to be specific for PSI and the prosegment of cardosin A were produced against synthetic peptides bearing the sequences KNNVKSSGGIHDE and KKRKVDRIDQLRGR, respectively (Fig. 1). These regions are predicted to be exposed, based on their amino acid content and on the model of the secondary structure of PSI [17]. In immunoblots of SDS/polyacrylamide gels the anti-PSI Ig revealed a strong reaction with a polypeptide with an apparent molecular mass of 64 kDa present in

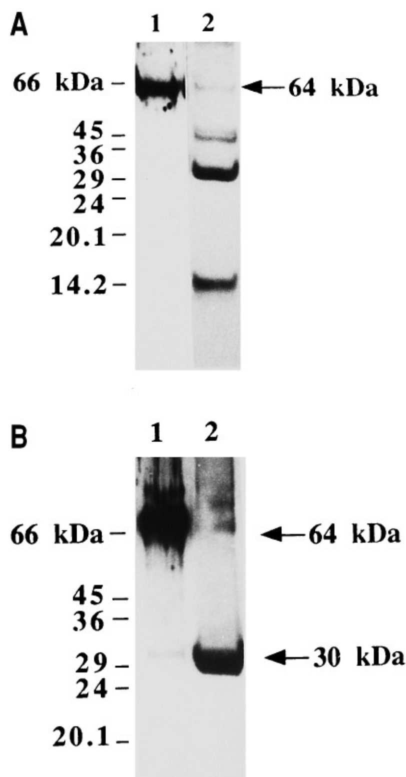

Fig. 2. Identification of a cardosin A precursor containing PSI (A) and its evolution with the maturation of the flowers (B). (A) 10 μg protein from immature pistils were loaded onto 15% SDS/polyacrylamide gels and analysed by immunoblotting with the antibodies anti-PSI and anti-(cardosin A) Ig. The anti-PSI Ig reacted specifically with a 64-kDa band (lane 1), also detected by the anti-(cardosin A) Ig (lane 2). The 31-kDa and 15-kDa bands correspond to the mature chains of cardosin A. A 45-kDa band detected by the anti-(cardosin A) Ig possibly corresponds to the non-dissociated chains of the enzyme. (B) 20 μg protein from immature (lane 1) and mature (lane 2) pistils were analysed as in (A) using the anti-PSI Ig. In mature pistils the PSI was detected predominantly in a 30-kDa band (lane 2), suggesting that the precursor undergoes processing as the flowers mature.

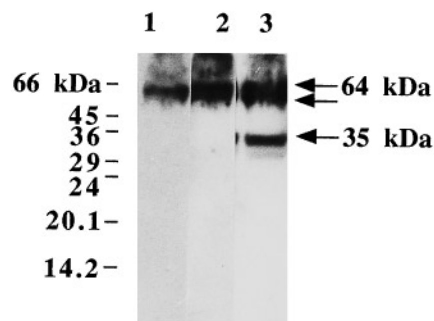

Fig. 3. The identified precursor contains the prosegment, and the PSI is processed before the prosegment. 10 μg protein from immature (lanes 1 and 2) or mature (lane 3) pistils were analysed as in Fig. 2 using the anti-pro and anti-PSI Ig. The anti-pro Ig reacted with the 64-kDa band (lane 2), which was confirmed by stripping the antibodies and reprobing the same membrane with the anti-PSI Ig (lane 1). The 60-kDa band detected by the anti-pro Ig possibly corresponds to procardosin B. In mature pistils the prosegment was detected predominantly in a 35-kDa band. The results indicate that PSI is processed before the prosegment.

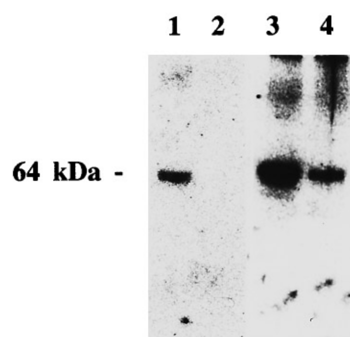

Fig. 4. Conditions for *in vitro* processing of PSI. 1 μg protein from immature pistils mixed with denaturing solution immediately following extraction or after 1 h at room temperature was loaded onto 12.5% SDS/polyacrylamide gels and analysed by immunoblotting on PhastSystem using the anti-PSI Ig. Extracts were made at pH 3.0 (lanes 1 and 2) and pH 7.0 (lanes 3 and 4), before (lanes 1 and 3) or after (lanes 2 and 4) the 1-h incubation.

extracts of immature pistils, and no reaction with other pistil proteins (Fig. 2A). This 64-kDa protein was also detected by the anti-pro Ig (Fig. 3) and the polyclonal anti-(cardosin A) Ig (Fig. 2A), indicating that it is a cardosin A precursor containing both PSI and the prosegment. The apparent molecular mass of 64 kDa on SDS/PAGE would be expected for the full-length native procardosin A. Thus, this precursor is called procardosin A. The absence of reaction of the anti-PSI Ig with any of the chains of mature cardosin A (31 kDa and 15 kDa) suggests that PSI is at least partially removed during proteolytic processing of procardosin A.

With the maturation of the flower the PSI is detected predominantly in a protein with an apparent molecular mass of 30 kDa (Fig. 2B). This result suggests that processing of procardosin A follows the maturation of the pistil, the precursor existing in mature pistils already mainly in an intermediate form.

PSI is processed before the prosegment. When the anti-pro Ig was tested on immunoblots of extracts of immature pistils, it reacted with two bands with apparent molecular masses of 60 kDa and 64 kDa (Fig. 3). Stripping of the antibodies and reprobing of the membrane with the anti-PSI Ig were carried out to confirm that both antibodies were reacting with the same band, namely that with an apparent molecular mass of 64 kDa (Fig. 3). The 60-kDa band may correspond to the precursor of cardosin B, the full amino acid sequence of which is not available. That the anti-PSI Ig is specific for PSI of cardosin A, while the anti-pro Ig is not specific for PSI, is probably due to the lower similarity between the plant AP in PSI, compared with the rest of the protein [25]. When the anti-pro Ig was tested on immunoblots of extracts of mature flowers, the strongest reaction occurred with a band with an apparent molecular mass of 35 kDa (Fig. 3). The evolution of procardosin A along the maturation of the pistil is clear.

If the prosegment were processed before the PSI, the anti-pro Ig should react with procardosin A and no other band. However, the anti-pro Ig reacted clearly with a 35-kDa band. The value of 35 kDa would be expected for a polypeptide with the prosegment preceding the 31-kDa subunit of cardosin A. In that same hypothesis, one would expect the anti-PSI Ig to react with two bands in the 60-kDa region (the precursor with and without the prosegment). This result was not observed. These results strongly support the hypothesis that the PSI is processed before the prosegment.

Conditions of *in vitro* processing of the PSI. *In vitro* studies were carried out to gain insights into the way the PSI is processed *in vivo*. However, these studies were performed using

Table 1. N-terminal and C-terminal sequences of the 31-kDa and 15-kDa chains of cardosin A. Heterogeneity was detected in the C terminus of the 31-kDa chain and the N terminus of the 15-kDa chain. In these cases, the sequences are indicated by order of occurrence. The C terminus of the 15-kDa chain is not processed, because the identified sequence precedes a stop codon in the cDNA [6]. Numbering corresponds to the complete sequence of cardosin A, including the presegment and prosegment.

Chain	N terminus	Cleavage site	C-terminus	Cleavage site
31-kDa chain	DSGSA...	Arg68−Asp69	...GAN	Asn309−Gly310
			...ANGV	Val311−Met312
			...NGVM	Met312−Asn313
15 kDa chain	TSSEE...	Ser414−Thr415	...VGFAEAA	no cleavage
	SSEE...	Thr415−Ser416		
	SEE...	Ser416−Ser417		

total protein extracts and not a purified precursor, since attempts to purify procardosin A were not satisfactory. Namely, extracts were made at neutral and acidic pH. When an extract of immature pistils made at pH 3.0 was left for 1 h at room temperature, the reaction of the anti-PSI Ig with procardosin A was completely lost (Fig. 4), which suggests that the PSI was removed and possibly degraded. On the contrary, if the same experiment was carried out using an extract made at pH 7.0, the disappearance of the band corresponding to procardosin A was partially inhibited (Fig. 4). These results suggest that removal of the PSI occurs at an acidic pH. The effect of the presence of various inhibitors for the different classes of proteinases in the pH 3.0 buffer was studied. The presence of pepstatin A, a specific inhibitor of AP, at 1 µM in the pH 3.0 buffer was sufficient to completely inhibit the processing of the PSI. No other proteinase inhibitor used had this effect (data not shown). Since AP, including cardosin A, have acidic pH optima, the results implicate AP activity in the removal of the PSI from procardosin A. Considering the great abundance of the enzyme in the pistils [13], it is probable that cardosin A is the AP involved in the removal of the PSI.

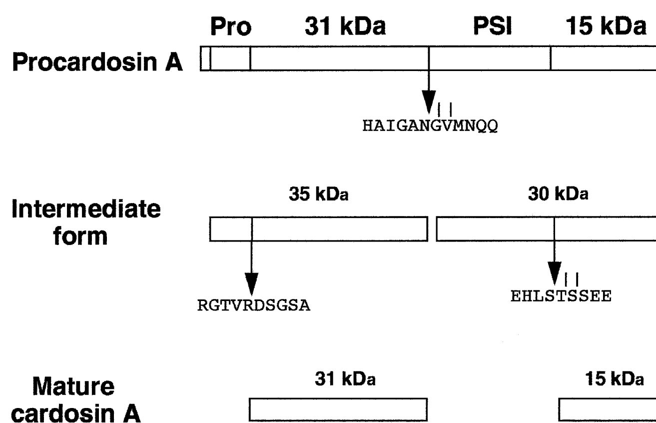

Fig. 5. Proposed model for the proteolytic processing of procardosin A. The cleavage sites between the prosegment (Pro) and the 31-kDa fragment, the 31-kDa fragment and PSI, and PSI and the 15-kDa fragment are indicated by arrows. Vertical lines indicate secondary processing sites. Removal of PSI appears to occur in the sequential order indicated, with the existence of an intermediate form.

Identification of the sites of cleavage. N-terminal [15] and C-terminal sequencing of the mature chains of cardosin A allowed the identification of the sites where cleavage occurs during the processing of procardosin A (Table 1), by comparison with the amino acid sequence deduced from the cDNA [6]. Arg68−Asp69 is the cleavage site between the prosegment and the 31-kDa fragment. The main cleavage site that gives rise to the C-terminal of the 31-kDa chain is Ans309−Gly310. Val311−Met312 and Met312−Ans313 are secondary sites of cleavage also identified in this region. The main cleavage site that yields the N terminus of the 15-kDa chain is Ser414−Thr415. Secondary sites of cleavage are Thr415−Ser416 and Ser 416−Ser417. The C terminus of the 15-kDa chain does not undergo processing, since the sequence that was identified is located immediately before the first stop codon in the cDNA [6]. Despite the heterogeneity in both cleavages, these results indicate that PSI is completely and precisely removed during processing, since the cleavage sites are located at the borders between the 31-kDa fragment and PSI and between PSI and the 15-kDa fragment (Fig. 1). PSI in cardosin A comprises amino acids Asn313−Ser416. Thus, procardosin A is converted during processing to a 'typical' AP, with the precise removal of PSI.

Hydrolysis of synthetic peptides corresponding to the cleavage sites. Isolated cardosins and synthetic peptides corresponding to the 31-kDa fragment/PSI (IGANGVMNQQ) and the PSI/15-kDa fragment (EHLSTSSEEL) cleavage sites were used to identify the aspartic proteinase responsible for processing of the PSI. Hydrolysis of the peptide substrates was monitored by automated amino acid sequencing, and reactions were carried out at pH 3.1 and 6.0 for short (15 min and 30 min) and long (up to 48 h) periods. Both cardosins were unable to cleave the two peptides under these experimental conditions. The same result was observed when the peptides were incubated with pistil extracts (total protein:substrate mass ratio of 1/100), suggesting that cleavage of the scissile bonds requires a conformation specific to the precursor.

In contrast, cardosin A cleaved consistently the synthetic peptide containing the prosegment/31-kDa fragment cleavage site (RGTVRDSGSA) at the Arg−Asp bond. However, only about 5% of the peptide was hydrolysed after 16 h incubation with isolated cardosin A (data not shown). This result indicates a slow rate of hydrolysis, since longer incubation times yielded proportionally higher amounts of product. Cleavage of the peptide bonds was completely inhibited by 1 µM pepstatin A but not by a cocktail of inhibitors specific for other classes of proteinases when the peptide was incubated with pistil extracts, suggesting that cardosin A may also be involved in the processing of the cleavage site between the prosegment and the 31-kDa fragment.

DISCUSSION

Based on the results obtained it is possible to propose a scheme for the proteolytic processing of procardosin A (Fig. 5). The reaction of the anti-PSI Ig with a 30-kDa band (Fig. 2B) and that of the anti-pro Ig with a 35-kDa band (Fig. 3) suggest

Barley lectin	V-FAEAIA
Cardosin A	VGFAEA-A
Cyprosin	VGFAEA-A
Tomato AP	VGFAEA-A
Brassica AP	VGFAEA-A
Arabidopsis AP	VGFAEA-A
Barley AP	IGFAKA-A
Orizasin	VGFAKS-A

Fig. 6. Alignment of C-terminal sequences from plant AP with the vacuolar targeting signal from barley lectin. The underlined sequence, belonging to barley lectin C-terminal propeptide, has been shown to be sufficient for the vacuolar targeting of the protein [43]. C-terminal sequences deduced from the cDNAs of cardosin A [6], cyprosin [25], tomato AP [12], *Brassica napus* and *Arabidopsis thaliana* AP [14], barley AP [44] and orizasin [45] are shown. Cardosin A and barley AP are vacuolar proteins [13, 46].

that the first cleavage site to be processed is that between the 31-kDa fragment and PSI. In addition, a monospecific antibody against the 15-kDa chain of cardosin A also reacts with a 30-kDa band [13]. These results suggest that processing is sequential, with the accumulation of an intermediate form being only partially processed (Fig. 5). In the removal of PSI, cleavage seems to occur first between the 31-kDa fragment and PSI and afterwards at the border of PSI and the 15-kDa fragment. Processing is completed when the prosegment is removed. In the processing of procathepsin D or pepsinogen, for example, pseudoforms have been identified that have partially removed prosegments [26, 27]. Occurence of similar intermediate cleavages in the removal of the prosegment of cardosin A cannot be excluded.

Three types of processing have been observed in the activation of AP: complete self-processing (intra or intermolecular); self-processing partially assisted by a different proteinase; and fully assisted processing [7]. Pepsinogen is included in the first category, procathepsin D in the second, and prorenin in the third. Processing of cathepsin D requires the action of lysosomal cysteine proteinases [28]. Even though cardosin A was unable to cleave synthetic peptides with the PSI cleavage sites, the enzyme is probably involved in the processing of the precursor. However, we cannot exclude the possibility that a vacuolar processing enzyme, similar to those existing in protein-storage vacuoles of castor bean [29] and in immature seeds of soybean [30], is involved in the cleavage of the Asn309−Gly310 bond. Vacuolar processing enzymes cleave seed and vacuolar proproteins precursors at the C-terminal side of asparagine residues [31] and together with an AP are involved in the post-translational processing of storage proteins in castor bean [32].

The mechanism by which PSI is removed from procardosin A is not clear, but it involves a specific conformation of the precursor. The PSI domain contains potential amphipathic helices with cysteine residues properly positioned to give helical hydrophobic domains [17, 33]. Proteins with such domains are known to interact with the lipid bilayer [34, 35]. It is possible that procardosin A during its intracellular transport interacts directly with the membrane via the PSI domain, thus rendering the proper conformation for processing at the PSI cleavage sites.

Upon removal of the PSI, procardosin A acquires a structure typical of mammalian or microbial AP proforms. We do not know whether this intermediate form is active, but processing into the mature form requires the removal of the prosegment. Taking into account the primary specificity of plant AP [4] and of cardosin A [19, 20], it would be expected that the cleavage site between the prosegment and the 31-kDa fragment (Arg68−Asp69) was probably processed by a serine or cysteine proteinase. Cardosin A, but not cardosin B, was able to hydrolyse specifically the Arg−Asp bond of a synthetic peptide corresponding to this site, although at a very slow rate.

The physiological role of the proteolytic processing of vacuolar plant AP has not been elucidated entirely. The results obtained in the present work suggest that processing may be a safety mechanism to prevent proteolytic activity of newly synthesised proteinase. Due to its location [17] PSI may function as a barrier to the access of the substrate into the active-site cleft. As mentioned above, the nature of PSI may also allow procardosin A to be transported intracellularly to the vacuole as a membrane-associated protein. This would be a very straightforward strategy to overcome prevacuolar activation of plant AP, and to prevent unwanted proteolytic activity of dislocated proteinase due to overexpression or defects in the vacuolar sorting machinery. Cardosin A is expressed by the epidermal cells of the pistils in very abundant quantities [13]. Once PSI is removed, activation could be controlled by the prosegment itself, which probably occurs at a slow rate in the vacuole.

Although the question of the vacuolar sorting determinant of cardosin A is not addressed specifically in the present work, some evidence is available to discuss the hypothesis first put forward by Guruprasad et al. [17], suggesting that PSI is involved in the vacuolar targeting of plant AP. This hypothesis was raised based on the observed similarity to saposins, which have been reported to interact in human cells with a fraction of cathepsin D in the endoplasmic reticulum [36], then to be cotransported to the lysosome [37, 38]. In plants, proteins are targeted to the vacuole by short amino acid sequences located in N-terminal or C-terminal propeptides or in the mature protein [39−41]. In barley lectin [42, 43], the C-terminal propeptide (VFAEAIA) is highly similar to the sequence identified at the C-terminus of cardosin A (Fig. 6). Similar C-terminal sequences are also present in other plant AP (Fig. 6). These observations suggest that the VGFAEAA sequence may be the vacuolar targeting signal in vacuolar plant AP. It is possible, however, that PSI in some way cooperates with the putative targeting signal here proposed. Mutagenesis studies in a plant heterologous system should be used to test this hypothesis.

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