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Review

Properties and applications of phytepsins from thistle flowers

Sandra Vairo Cavalli *, Daniela Lufrano, María Laura Colombo, Nora Priolo

Laboratorio de Investigación de Proteínas Vegetales, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina

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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 . . . 16
2. Thistle flowers as producers of phytepsins. . . . 17
3. Molecular structure of typical plant APs from thistle flowers. . . . 18
4. Activities and specificities . . . 22
5. Tissue localization . . . 24
6. From proenzymes to mature peptidases. . . . 25
   6.1. Zymogen inactivation . . . 25
   6.2. Processing . . . 25
   6.3. Targeting . . . 26
   6.4. Plant-specific inserts (PSIs). . . . 27
7. Physiological roles . . . 27
8. Biotechnological and biomedical applications . . . 28
   8.1. Cheese preparation . . . 28
   8.2. Production of bioactive peptides . . . 29
9. Conclusions. . . . 29
   Acknowledgements . . . 29
   References . . . 29

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

* Corresponding author. Postal address: LIPROVE, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, C.C. 711, B1900AVW La Plata, Argentina. Tel.: +54 221 4250497x57.
E-mail address: svairo@biol.unlp.edu.ar (S. Vairo Cavalli).

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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).