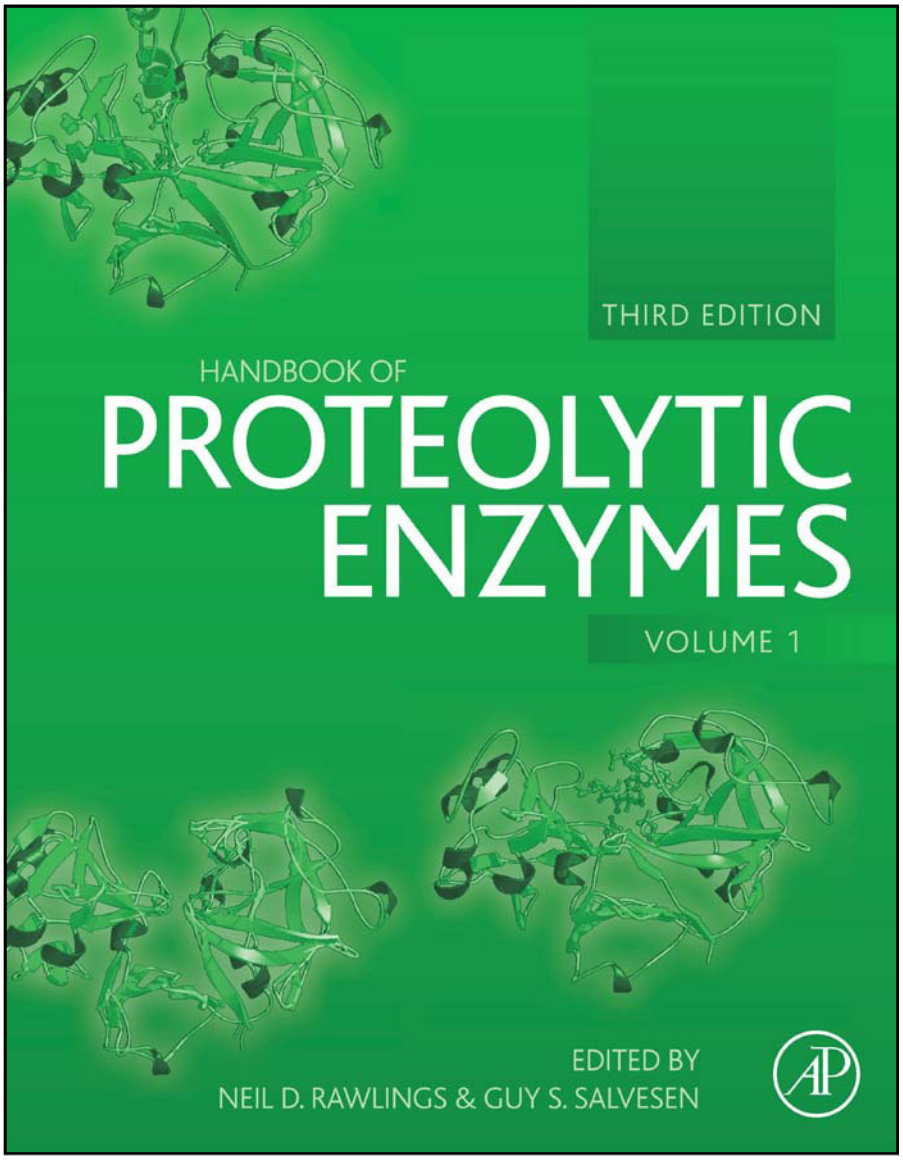

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Chapter 23

Phytepsin

Databanks

MEROPS name: phytepsin
MEROPS classification: clan AA, family A1, subfamily A1A, peptidase A01.020
IUBMB: EC 3.4.23.40 (BRENDA)
Tertiary structure: Available
Species distribution: subkingdom Viridiplantae
Reference sequence from: *Hordeum vulgare* (UniProt: P42210)

Name and History

In the plant kingdom, aspartic proteinases (APs) are ubiquitously expressed. All plants appear to contain the enzyme since its presence is observed in some tissues of every plant so far tested. In 1997, the name ***phytepsin*** (*phyto* (Lat.) = plant) was introduced to denote all related plant APs and was adopted by NC-IUBMB. However, in the literature, no universal name is commonly used to represent all plant APs. Instead, the names tend to indicate the plant species or tissue from which the specific enzyme is purified. In this review, the name phytepsin is used to denote plant APs in general and specific names are given where applicable. A related group of secreted plant APs from *Nepenthes* and other carnivorous pitcher plants is presented in Chapter 24.

Some of the first phytepsin purifications described in the literature were from rice [1], buckwheat [2], squash and cucumber seeds [3], and wheat [4]. The enzymes were obtained in sufficient quantity and purity to enable basic kinetic analyses. Flowers of the cardoon plant (*Cynara cardunculus* L.) contain a milk-clotting activity that has been exploited for centuries in traditional cheese making in Portugal and Spain. This activity was eventually found to be due to a family of APs [5,6]. The X-ray crystal structure of the active form of one of the enzymes, cardosin A, has been reported [7]. Barley (*Hordeum vulgare* L.) grains also contain a prominent phytepsin activity [8]. The corresponding enzyme was purified from resting barley grains [9] and the X-ray crystal structure of the recombinantly produced zymogen form was subsequently solved [10]. In the 1990s, several other phytepsins were also identified and purified from monocotyledonous and dicotyledonous plants which have led to a general understanding of the kinetic and structural features of phytepsins [11,12].

Interestingly, a plant-specific insert (PSI), a domain containing an extra protein sequence of about 100 amino acids, was first observed in barley phytepsin [13] and later in numerous other phytepsins [12]. The region has no sequence homology to mammalian or microbial APs and thus the PSI region was quickly characterized as a unique structural feature of phytepsins. However, it soon became apparent that naming the insertion as a 'plant-specific' domain was not quite appropriate for all phytepsins, as several phytepsins lacking PSI have been detected since the early reports, including a phytepsin-like AP from the tobacco chloroplast nucleoids [14] and nucellin from barley ovaries [15]. Moreover, recent genome-wide studies on the phytepsin genes in *Arabidopsis* [16–18], cardoon [19], and rice [20] have revealed that phytepsins form a diverse set of APs with several subgroups, numerous distinct structural features, diverse subcellular localization, and multiple functions.

Activity and Specificity

The partially purified squash seed phytepsin cleaves the oxidized insulin B chain after polar (Tyr) and hydrophobic (Phe, Leu) residues [3]. The activity of barley grain phytepsin has been measured using hemoglobin as a substrate, showing pepstatin-sensitive protein cleavage at pH 3.7 [9,21]. Enzymatic activity has also been shown to be active using a native gel electrophoretic method with immobilized edestin [22]. Purified phytepsin hydrolyzes hemoglobin and a chromophoric substrate, Pro-Thr-Glu-Phe↓Nph-Arg-Leu (NovaBiochem), optimally at pH 3.5–4.1 [9,23]. Insulin B chain, glucagon and melittin have also been used to characterize the hydrolytic specificity of barley phytepsin. The cleavage of insulin B chain by barley phytepsin proceeds as follows:

FVNQHLCGSHL↓VEA↓L↓YLVCGERGF↓F↓YTPKA

The cleavage typically occurs either between two residues with hydrophobic side chains (Leu, Ile, Val, Phe) or next to one hydrophobic residue. In glucagon, the Asp↓Tyr bond was also readily cleaved [23]:

HSQGTFTSD↓YSKY↓L↓DS↓RR↓AQDF↓VQW↓L↓MNT

A similar type of hydrolytic specificity against insulin B chain has also been observed for several other phytepsins, with only slight variation [24]. Besides insulin B chain and hemoglobin, other *in vitro* substrates tested as potential phytepsin substrates include albumin, gliadin, chromophoric peptides, casein [12] and firefly luciferase [25].

Most phytepsins are inhibited by pepstatin [12]. In addition, several substrate-analog inhibitors which are effective against cathepsin D, a mammalian lysosomal AP, have also been tested against barley phytepsin [9]. A systematic series of synthetic inhibitors developed against mammalian and retroviral APs have also been tested against the recombinantly produced cardoon enzyme (cyprosin); several inhibited cyprosin with measured K_i values in the nanomolar range [26]. Endogenous AP inhibitors have been detected or purified from several plant species including potato [27,28], tomato [29,30], squash [31], and *Anchusa strigosa* [32]. AP inhibitor has also been purified and characterized from the seeds of *Vigna radiata* where it is suggested to regulate phytepsin activity during germination [33]. AP inhibitor from *Lupinus bogotensis* efficiently inhibits an AP from the guts of coffee berry borer, an insect pest, suggesting a defensive role against plant pathogens of some endogenous plant AP inhibitors [34]. Solution structure obtained using NMR of the squash aspartic proteinase inhibitor has recently been published [35].

Structural Chemistry

Primary Structure

The overall primary translation product of most phytepsins is similar to their mammalian and microbial AP counterparts, consisting of an endoplasmic reticulum (ER) signal sequence followed by a self-inhibition peptide of around 40 residues (propeptide) preceding the mature enzyme sequence [12,13,36]. In many phytepsins, the conserved catalytic active site residues are Asp-Thr-Gly and Asp-Ser-Gly in the N-terminal and C-terminal regions, respectively, although most mammalian and microbial enzymes contain the Asp-Thr-Gly sequence on both sides of the active site. Whether the Asp-Thr/Ser-Gly variation confers any biological significance remains to be determined. Due to the presence of the PSI sequence, the primary translation product of phytepsins containing this area is significantly larger (~500 amino acids) than those of most mammalian enzymes. A seven-member family of glycosylphosphatidylinositol (GPI)-anchored APs has been identified from the *Arabidopsis thaliana* genome [37]. A putative viral-type AP is encoded by part of the *BARE*-1 retroelement in the barley genome [38].

The cardoon floral AP group (including cardosins and cyprosins) has been extensively studied and several members have been sequenced [19,39,40]. In addition, genome-wide identification and analyses of phytepsin genes have been reported for *Arabidopsis* [16–18] and rice [20]. Thus, at least in these plants, phytepsins form a diverse set of enzymes with several subfamilies and diverse expression patterns. The *Arabidopsis* genome contains 51–69 phytepsin genes that have been classified into three subgroups (typical plant APs, nucellin-like APs, and atypical plant APs), depending on their domain organization and their active site sequence motifs [17]. In rice, 96 putative phytepsin genes have been identified and their chromosomal location, phylogenetic relationships,

and genetic structure have been detailed [20]. The results from *Arabidopsis* and rice point to the multiple functions of phytepsins in different developmental stages and plant tissues. Numerous examples of tandem duplications as well as evidence of restricted expression pattern for phytepsins suggest that a high degree of specialization exists among phytepsins.

Tertiary Structure

X-Ray structural analyses of cardoon [7] and barley [10] phytepsins have provided most of the structural information about plant phytepsins. The crystal structure of a zymogen form of barley phytepsin, solved at 2.3 Å resolution, is presented in Figure 23.1. The enzyme structure consists of two similar ß-barrel domains with the two catalytic active site residues, Asp36 and Asp223 (illustrated in ball-and-stick in Figure 23.1), located in the interdomain cleft. The phytepsin propeptide wraps around the mature protein and, together with the first 13 residues of the N-terminus of the mature enzyme, fully blocks it. The PSI domain is attached to the C-terminal domain of the enzyme by two flexible polypeptide linkages, permitting some freedom in the positioning of PSI relative to the main body of the enzyme. The PSI domain is comprised of five amphipathic helices forming a helical bundle with a large internal hydrophobic core. The structure of PSI is similar to saposins and saposin-like proteins in animal cells (see below). The 1.7 Å resolution crystal structure of cardoon phytepsin (cardosin A) illustrates the structure of a fully processed active phytepsin with unique plant complex type glycans [7]. The distinctive feature of cardosin A among phytepsins is the presence of the Arg-Gly-Asp (RGD) cell-attachment motif. The crystal structure shows that the RGD-motif is located at the base of the molecule opposite the active site and projects itself outward from the molecular surface, possibly indicating a protein-protein interaction motif for this area. Comparison of the zymogen form of barley phytepsin to the mature cardosin A structure suggests that only minor conformational changes occur during activation of plant phytepsins [10].

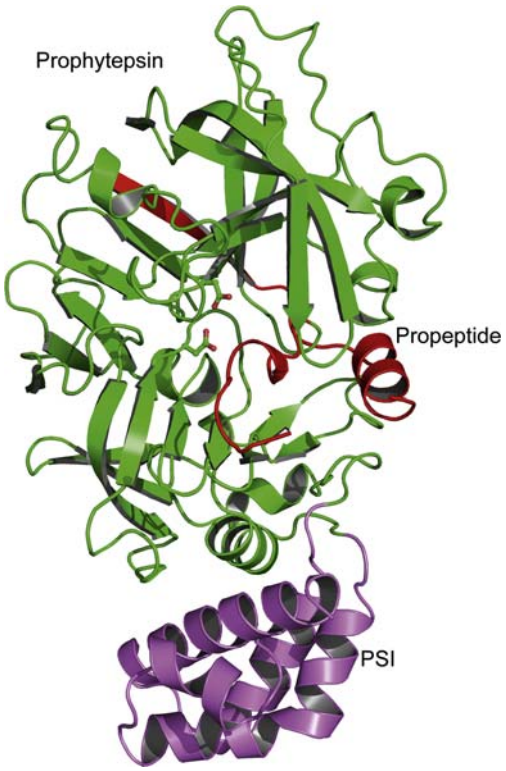

FIGURE 23.1 The crystal structure of the zymogen form of barley phytepsin. Propeptide (6–26 out of the 41 residue sequence) and the first 13 residues of the mature enzyme are shown in red, the PSI region in magenta, and the mature enzyme in green. Active site residues Asp36 and Asp223 are shown in ball-and-stick representation.

Crystallographic analysis of wheat (*Triticum aestivum* L.) xylanase inhibitor-I (TAXI-1) revealed its surprisingly close structural homology to phytepsins [41,42]. Crystal structure of TAXI-1 in complex with *Aspergillus niger* xylanase shows an interesting example of the divergence of protein function within the context of a common structural framework. TAXI-1 belongs to a group of wheat defense system proteins that are active against plant cell wall-degrading xylanases secreted by plant pathogenic microorganisms. The structure shows that the C-terminal lobe of TAXI-1 forms a tight complex, with the active site region of xylanase sterically blocking its action. Thus, this protein is an inhibitor in spite of its closely related AP fold. TAXI-1 does not contain a PSI domain and it is apparently proteolytically non-functional due to critical residue changes in the active site cleft. Multiple TAXI-1 homologs are present in soybean, rice, and *Arabidopsis* genomes [43]. It is an intriguing possibility that some TAXI-1 genes found in various plant species may be identical to the numerous inactive mutant APs found by researchers investigating APs in the same species.

Preparation

Phytepsins have mostly been purified from crude extracts by affinity chromatography using immobilized pepstatin as an affinity matrix [11,12]. The expression level of phytepsins in plant tissues is generally low and most purification procedures have yielded only milligram amounts of pure protein. In addition, the preparations often contain several enzyme forms or processing intermediates, further hindering detailed analysis. Bacterial and eukaryotic cell expression systems have been utilized for some phytepsins. For example, the proform of the rice enzyme, oryzasin 1, has been expressed in *E. coli* as a fusion protein linked to glutathione-S-transferase (GST). The resulting purified enzyme underwent autocatalytic activation and exhibited

proteolytic activity using hemoglobin as a substrate [44]. Atypical phytepsins (CDR1) from *Arabidopsis* [45] and rice [46] have also been successfully expressed in *Escherichia coli*. However, a general problem with bacterial expression methods has been very low yield of the correctly folded product. In contrast, eukaryotic cell expression systems have been successfully used for the production of phytepsins. The baculovirus-infected insect cell expression method has been used to obtain barley prophytepsin for crystallographic and other purposes [10,47]. The cDNA encoding the cyprosin precursor from cardoon flowers was expressed in *Pichia pastoris* cells and an active enzyme was purified from the culture media [26]. *Pichia* expression system has also been used for the expression of one of the *Arabidopsis* phytepsins (AtAP A1) [24]. These examples demonstrate the usefulness of eukaryotic expression systems for obtaining reasonable quantities of correctly folded and fully functional phytepsins.

Biological Aspects

Proenzyme Activation

Phytepsins are predominantly vacuolar proteases although these enzymes have also been detected in other cell organelles and in the extracellular matrix (see below). Phytepsins are expressed in their zymogen form, undergo N-terminal processing during activation, and then some of them undergo further internal processing to obtain the mature enzyme. The PSI region is often removed during processing resulting in the formation of a two-chain enzyme [47–52]. The reason some phytepsins exist as monomers and the others as two-chain forms is not known. Both autocatalytic and heterocatalytic steps are needed for the activation of phytepsins. These mechanisms presumably depend both on the prevailing pH as well as on the presence of processing proteases within the particular intracellular compartment traversed by the phytepsin precursor.

Intracellular Transport

Several sequence-specific regions may play a role as targeting signals to direct phytepsin family members to vacuoles or to exit the cell. For barley phytepsin, the suggested targeting region includes the NPLR-peptide in the self-inhibitory peptide immediately prior to the N-terminus of the mature enzyme and also some areas in the PSI domain [10,53–55]. The C-terminal peptide region has also been suggested to function as an intracellular targeting signal sequence for phytepsins [51]. The moss *Physcomitrella patens*, a seedless plant, has been used in the visualization of the phytepsin-GFP (green fluorescent protein) fusion during its route from the Golgi complex into a vacuole [56]. APs have also been localized to the cell wall of maize pollen [57] and other diverse cellular locations include photosystem II membranes of spinach plastids [58], intercellular fluids of rice and *Arabidopsis* [59], and the latex of *Ficus racemosa* [60].

Since its discovery as part of phytepsin sequence in the early 1990s, the PSI domain has evoked a vigorous discussion about its function. Several theories for its function have been suggested, including a defensive role against invading pathogens [61,62], disruption of cell membranes during programmed cell death [61], and as a mediator in the vacuolar targeting of phytepsin precursors [10]. Phytepsins destined to vacuoles typically contain PSI in their primary structure [48,56,63] whereas phytepsins destined to other parts of the cell do not [14,15,45,64]. PSI is structurally curiously similar to saposins, sphingolipid-activating proteins in mammalian cells [10]. Saposins interact with lipids and promote sphingolipid degradation in animal cells among other functions [65]. Prosaposin is a lysosomal protein and saposin-type domains have also been found in other proteins such as acid sphingomyelinase and acyloxyacyl hydrolase in animal cells. Recent discoveries that the intracellular trafficking of prosaposin [66] and acid sphingomyelinase [67,68] is mediated by sortilin, a type I transmembrane receptor protein, support the hypothesis that the PSI region may not only bring prophytepsin into contact with membranes but may also cause it to interact with a membrane-bound sortilin-type receptor in the Golgi apparatus during its route into vacuoles. However, there is no direct evidence for sortilin-type proteins in plant cells, although plant cells are known to contain numerous membrane-bound proteins functionally linked to intracellular trafficking [69]. Whether some of these proteins show structural similarity to sortilins is an interesting question for future studies. These studies may also specifically elucidate the role of the PSI in the intracellular transport of phytepsins.

Distribution in Tissues and Function

Phytepsins have been detected or purified from a diverse array of common and exotic plant species [11,12,17,20]. These enzymes have been observed in numerous tissue types in many plants and thus it is likely that they play an important role in a variety of proteolytic processes within cells and in the extracellular space. In seeds, they probably take part in the modification of storage proteins and regulation of seed development and germination [21,63]. In cardoon, cynarases as well as cardosins A and B are present in floral organs where they regulate floral development, reproduction and postembryonic seed development [70]. In addition, *Arabidopsis* phytepsin apparently functions as an anti-cell-death component in reproduction and embryogenesis [64,71]. Phytepsins are also known to be elevated in a number of environmental stress situations, suggesting a role

in the cellular response to stress [72,73]. Several reports also suggest that phytepsins play an important defensive role against pathogens [46,59,62,74]. In rice, phytepsins are part of a hybrid sterility mechanism [75]. Adding to the functional, structural, and locational diversity of the phytepsins is the recent discovery of unique plant APs with multiple transmembrane domains [76,77]. This is the first example of plant APs apparently acting as intramembrane signal peptide peptidases. These unique APs have been detected and linked to pollen function in *Arabidopsis* [76] as well as to development of the vegetative shoot apex of rice [77]. The recent investigations related to phytepsins continue to illustrate the large diversity within this family related to both structure and function. The phytepsin family represents an efficient protein design from which incredible diversity of form and function has evolved.

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Further Reading

Phytepsins have been reviewed in disease resistance [78], incompatibility mechanisms in plants [79] and industrial applications [80].

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