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ORIGINAL PAPER

Native and Biotechnologically Engineered Plant Proteases with Industrial Applications

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Abstract Proteases occupy the most relevant position among industrial enzymes. Plant proteases have been used in medicine, detergent manufacturing, and food science for many years, but their production is diminishing in favor of those of microbial origin because lower production costs. Papain, bromelain, and ficin are the most frequently employed plant proteases, although new proteases with new and more appealing physicochemical properties for industry are still emerging. DNA technology and genetic engineering shall play, without a doubt, an important role for the production of these proteases at the industrial level. The present review focuses on the applications of traditional plant proteases as well as new proteases discovered during the last 20 years, some of which have already been genetically engineered either to increase production or to strengthen some of their physicochemical properties. The review also refers to the protease classification, action pattern, and main characteristics.

Keywords Plant proteases · Industrial enzymes · Protein engineering · Genetic engineering · Applications

Introduction

Proteases are enzymes that hydrolyze the peptidic linkages in proteins and, from the point of view of industry, are the most important type of enzymes because they represent ca. 60% of all commercialized enzymes in the world. The fields of application are very diverse, including food science and technology, pharmaceutical industries, and detergent manufacturing. Main producers worldwide include Novo Industries, Gist-Brocades, Genencor International, and Miles Laboratories. The wide distribution of proteases among plants, animals, and microorganisms demonstrates that they are necessary for livings organisms, playing important physiological roles in quite diverse biological processes (Rao et al. 1998). The number of industrially used proteases of plant origin is small (Aehle 2004) and some cysteine proteases (CPs) such as papain, bromelain, and ficin are still being used in a variety processes. Plant proteases were used in ancient times as illustrated in Homeric writings such as the Íliad, by indicating that the latex from the fig tree may be used for milk-clotting and the cheese-making. Also, pineapple juice and again fig tree latex have been used as antihelmintic. Because the industrial application of enzymes requires low cost in their production as well as large scale production, plant enzymes are being replaced by those of microbial origin. So, the number of plant proteases commercially available is limited (Table 1). In the last years, however, plant proteases are the object of renewed attention from the pharmaceutical industry and biotechnology not only because of their proteolytic activity on a wide variety of proteins but also because often they are active over a range of temperatures and pHs (Dubey et al. 2007). All these have stimulated the research and increased the number of works on plant proteases. Recent biotechnological developments and, particularly, protein engineering predict the appearance in the near future of plant proteases with more and improved industrial properties.

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Table 1 Commercially available plant proteases

Enzyme	CAS number	Source	Suppliers [a]	Physical form
Papain	9001-73-4	Papaya latex	Sigma, MP Biomedicals, Sisco Research Laboratories, Beta Pharma, Pierce Biotechnology, Worthington Biochemical Corporation, Enzyme Development Corporation	crystallized, papain agarose, buffered aqueous suspension, lyophilized powder, crude powder
Chymopapain	9001-09-6	Papaya latex	Sigma, 3B Scientific Corporation	lyophilized powder,soluble powder
Ficin	9001-33-6	Fig tree latex	Sigma, Pfaltz & Bauer, Enzyme Development Corporation	saline suspension, lyophilized powder, ficin agarose
Bromelain	9001-00-7	Pineapple stem	Sigma, MP Biomedicals, Pfaltz & Bauer, Beta Pharma, Enzyme Development Corporation	lyophilized powder
Actinidin	39279-27-1	Kiwi fruit	Biochem Europe	powder
Cucumisin	82062-89-3	Sarcocarp of melon fruit	Sigma	lyophilized powder

[a] Other suppliers can be found in http://www.chemicalbook.com

Classification and Protease Nomenclature

"Peptidase" is the recommended term described in the Enzyme Nomenclature of the Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) for any protein that carries out the hydrolysis of peptide bonds. Peptidases are normally known as proteases, proteinases, or proteolytic enzymes. Because of the variety in their catalytic behavior, proteases are difficult to classify following the rules established for the rest of enzymes, and instead, three criteria are used for their classification:

Catalyzed Reaction

This is the main property established by the NC-IUBMB that classifies these enzymes within group 3 (hydrolases) and subgroup 4 (hydrolases of peptide bonds). Subclass 3.4 may be in turn subdivided into endo- or exo-peptidases (amino-terminal or carboxy terminal) depending on their ability to hydrolyse internal peptide bonds or bonds located at the ends; endo-peptidases are by far more important from the industrial point of view (Barrett 1994).

Nature of the Active Site

Hartley set a classification of proteases according to their catalytic site (Hartley 1960), in which proteases were divided into six mechanistic classes: serine endopeptidases (EC 3.4.21); cysteine endopeptidases, formerly denoted as thiol proteases (EC 3.4.22); aspartic endopeptidases, first known as acid proteases; glutamic endopeptidases (EC 3.4.23); metalloendopeptidases (EC 3.4.24); and threonine endopeptidases (EC 3.4.25), with a fifth group including peptidases with unidentified mode of action (EC 3.4.99). Serine, threonine, and cysteine proteases are catalytically very different from aspartic and metallo-proteases in that the nucleophile of catalytic site of the former group is part of an amino acid, whereas it is an activated water molecule for the last two types.

Structure-Based Evolutive Relationships

Rawlings et al. (1993) classified proteases according to their amino acidic sequence and relationships in families and clans. Proteases were placed within the same family if they shared sufficient sequence homology, and families believed to have a common ancestor were placed within the same clan. The names of clans and families in the *MEROPS* database are built on the letters S, C, T, A, G, M, and U, which refer to the catalytic types. However, some of the clans are mixed type and contain families with two or more catalytic types and designated with the letter "P." This classification resulted in the creation of the MEROPS peptidase database, which is constantly revised: http://merops.sanger.ac.uk (Rawlings et al. 2010). The plant proteases most frequently used belong to the groups of cysteine, aspartic, and serine proteases (SPs).

Plant Cysteine Proteases

Native Plant Cysteine Proteases

CPs of plant origin, particularly the enzymes of the tropical plants *Carica papaya* (papain, chymopapain, caricain, glycil endopeptidase), *Ananas comosus* (fruit bromelain, stem bromelain, ananain, comosain), and *Ficus glabrata* (ficin) are of considerable commercial importance, due to their strong proteolytic activity against a broad range of protein substrates and because they are active over a broad

range of pH and temperature. According to the MEROPS database, CPs are divided into ten clans: CA, CD, CE, CF, CH, CL, CM, CN, CO,and C-, and to date, plant CPs have been described as belonging to five of these clans (CA,CD, CF,CO, and CE). Most plant CPs belong to the C1 family, also known as the papain family (clan CA). A complete list of CPs may be found the MEROPS database (Rawlings et al. 2010).

The proteases of the C1 family are synthesized with a signal peptide that leads them to specific cellular compartments and a pro-peptide at the amino end that must be cleaved in order for the enzymes to be active (Rawlings and Barrett 1994). The structure of these proteases reveals an α-helix and a β-barrel-like separated by a groove containing the active site, formed by the Cys-25 and His-159 residues, which are located at each side of the groove and are well conserved in all members of the family (Fig. 1) (Drenth et al. 1968). Other residues important for catalysis are Gln-19 (Ménard et al. 1995), which precedes the Cys-25 and is believed to help in the formation of an oxyanion hole, and Asn-175, which orients the His-159 imidazolium ring (Theodorou et al. 2007). The structures of these enzymes are very similar, and the catalytic mechanism is identical in all so far studied. They can differ, however, in the amino acids lining the substrate-binding pocket, which are responsible for binding the amino acid side chains of the substrate. They could, therefore, differ in substrate specificity, cleaving different peptide bonds of the substrate protein (Storer and Ménard 1994).

The majority of plant CPs exhibit optimum pH between 5.0 and 8.0 and molecular masses in the 25–30 kDa range, except a few in the range of 50–75 kDa (Dubey et al. 2007). The physiological roles attributed to plant CPs include their involvement in plant growth and development as well as in senescence and programmed cell death (Martínez et al. 2007). Roles in protein mobilization, cell signaling, response to biotic and abiotic stimuli, and as a defense mechanism (Van Der Hoorn and Jones 2004) have been also invoked (Grudkowska and Zagdańska 2004; Schaller 2004; Beers et al. 2004).

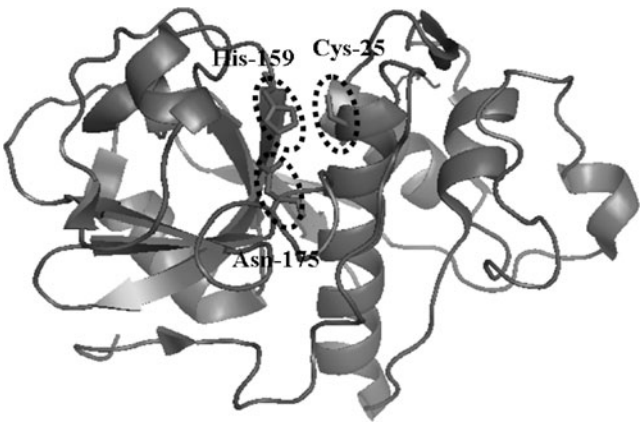

Fig. 1 Three dimensional model of papain, PDB code 1PPN (Pickersgill et al. 1992)

As for their location, plant CPs are present in most plant organs and, accordingly, have been obtained from: PsCYP15A from pea roots (Vincent and Brewin 2000); GP2 and GP3 from ginger rhizomes (Kim et al. 2007), from sweet potato (*Ipomoea batatas*) roots (Huang et al. 2005); FLCP-1 and FLCP-3 from *Phaseolus* leaves (Popovic et al. 1998); bromelain (Rowan et al. 1990), and ananain (Lee et al. 1997) from stems. The most ubiquitous group are found in fruits, i.e. balansain I, macrodontain I in *Bromeliaceae* (Pardo et al. 2000; Lopez et al. 2000), araujiain in *Asclepiadaceae* (Priolo et al. 2000); papain, chymopapain, papaya glycil endopeptidase, and caricain from *C. papaya* latex (Azarkan et al. 2003). Papain like cysteine proteases are usually lysosomal (vacuolar) or secreted proteins (Dubey et al. 2007).

Enzymes in C. papaya Latex

C. papaya is cultivated in tropical and subtropical zones of the globe. In the past, the crude latex came mainly from Kivu, East Congo and Uganda, but today, Central America, India, and Vietnam are also important producers. The latex composition varies slightly according to the country of origin, but it is composed of about 15% dry matter, 40% of which is formed by enzymes, mostly CPs including papain, chymopapain, caricain, and glycil endopeptidase (Baines and Brocklehurst 1979) (Table 2). These enzymes accumulate in the laticifers as zymogens and become activated after the latex is released from the plant (Silva et al. 1997; Moutim et al. 1999; Azarkan et al. 2003).

The process to obtain papaya enzymes consists of two main stages: latex extraction and drying by either sun-drying or spray-drying, both types commercially available. Spray-dried preparations are, however, more refined. One kilogram of this latex yields approximately 200 g of crude papain (Uhlig 1998). These preparations must be treated with reducing agents in order to protect the free thiol functions from air oxidation that would originate the loss of their proteolytic activity. Addition of low molecular mass thiol (i.e., dithiothreitol, cysteine) allows the regeneration of the free thiol function when suited. Procedures for the purification of the distinct cysteine proteases from crude latex of *C. papaya* include: chromatography on ion-exchange supports, such as SP-Sepharose Fast Flow and hydrophobic supports such as Fractogel TSK Butyl 650 (M), Fractogel EMD Propyl 650(S), or Thiophilic gels. The use of covalent or affinity gels is recommended to provide preparations of cysteine endopeptidades with a high free thiol content (Lawers and Dekeyser 1997; Azarkan et al. 2003).

Table 2 Cysteine proteases from *Carica papaya* latex

Name (EC number)	Alternative names	UniprotKB code	Molecular mass (Dalton)	Protein Sequences	Reference
Papain (EC 3.4.22.2)	Papaya proteinase I	P00784	23,000	(completely sequenced) 212 aa	(Mitchel et al. 1970) (Husain and Lowe 1970)
Chymopapain (EC 3.4.22.6)	Papaya proteinase II	P14080	27,000	(completely sequenced) 218 aa	(Jacquet et al. 1989) (Watson et al. 1990)
Caricain (EC 3.4.22.30)	Papaya proteinase Ω Papaya proteinase III Papaya peptidase II Papaya peptidase A	P10056	23,500	(completely sequenced) 216 aa	(Dubois et al. 1988) (Azarkan et al. 2003)
Glycyl endopeptidase (EC 3.4.22.25)	Chymopapain M Papaya proteinase IV Papaya peptidase B	P05994	23,313	(completely sequenced) 216 aa	(Ritonja et al. 1989a) (Lynn and Yaguchi 1979)

Papain (EC 3.4.22.2) is a minor latex component (5–8%). It was first isolated in 1879, was the first to be crystallized (Drenth et al. 1968; Kamphuis et al. 1984), and has been often used as a model in structure studies for other CPs. Besides its protease activity, Papain also exhibits amidase, esterase, transamidase, transesterase, and thiolesterase activities (Barbas and Wong 1987; Johnston 1956). The optimum pH (5.0 to 7.0) of papain varies with the nature of the substrate, being 7.0 when casein is used as the substrate. This enzyme is extremely temperature stable in comparison to other proteases. Effective activity is demonstrated over the temperature range of 10–90 °C (Whitehurst and Van Oort 2010).

Caricain (EC 3.4.22.30) (Dubois et al. 1988; Pickersgill et al. 1991) was the second protease to be purified from papain latex and is also known as papaya proteinase III, papaya proteinase A, or papaya proteinase Ω (this last name given because is one of the last proteases to be eluted from cation exchange chromatography columns due to its basic character).Two similar but distinct cDNAs have been shown to code for caricain (Revell et al. 1993).

Chymopapain (EC 3.4.22.6): the term chymopapain was coined in 1941 to distinguish a proteolytic activity remaining in papaya latex after the removal of papain (Jansen and Balls 1941). There are five similar but distinct clones coding for precursor chymopapains (Taylor et al. 1999). All of these isoforms have a free cysteine residue at position 117 (mature chymopapain numbering) not involved in disulfide bond formation or in the active site, and this property does differentiate chymopapain from the rest of the latex proteases (Azarkan et al. 2003). Chymopapain has characteristics similar to those of papain but differs in its activity towards proteins. It is less heat sensitive and more stable to pH 2 than papain and caricain (Uhlig 1998).

Glycyl endopeptidase (EC 3.4.22.25) is also known as chymopapain M, papaya proteinase IV, papaya peptidase B (Buttle et al. 1989).

Enzymes from A. comosus

Bromelain is a crude, aqueous extract from the stems and immature fruits of pineapples (*A. comosus* from the family of *Bromeliaceae)*. It contains a mixture of different proteases as well as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, and carbohydrates. The major protease present in extracts of plant stem is termed "stem bromelain" (EC 3.4.22.32) or also ananase, whereas the major enzyme fraction found in the juice of the pineapple fruit is named "fruit bromelain" (EC 3.4.22.33). Two additional CPs were detected only in the stem by a procedure involving active-site-directed affinity chromatography: ananain (EC 3.4.22.31) and comosain (Table 3) (Rowan et al. 1988, 1990; Napper et al. 1994), all commercially available bromelain being derived from the stem. Crude commercial bromelain from pineapple stem has been purified by successive use of ion exchange chromatography, gel filtration, and ammonium sulfate fractionation. From 10 g of the starting material, 0.87 g of purified bromelain is obtained (Murachi et al. 1964; Wharton 1974; Vanhoof and Cooreman 1997; Devakate et al. 2009).The optimum pH for stem bromelain activity is 6.0–8.5 for most of its substrates, and its temperature optimum range is 50 to 60 °C. Fruit bromelain has much higher proteolytic activity compared to stem bromelain and a broader specificity for peptide bonds (Polaina and MacCabe 2007).

Enzymes from Ficus sp.

Ficin (EC 3.4.22.3), also known as ficain, is isolated from dried latex of *F. glabrata* (Sgarbieri et al. 1964). It is also present in other species of *Ficus*, such as *Ficus carica* and *Ficus elastica*. Although crude ficin is of considerable commercial importance, very few studies have been carried out on the ficin isolated from the latex of others *Ficus*

Table 3 Cysteine proteases from *Ananas comosus*

Name (EC number)	Uniprot code	Molecular mass (Dalton)	Protein Sequences	Glycosylation	Reference
From pineapple stems:					
Stem bromelain (EC. 3.4.22.32)	P14518	23,800	(completely sequenced) 212 aa	glycosylated	(Harrach et al. 1995) (Ritonja et al. 1989b)
Ananain (EC 3.4.22.31)	P80884	23,464	(completely sequenced) 216 aa	not glycosylated	(Harrach et al. 1995) (Lee et al. 1997)
Comosain (Not included in IUBMB)	Q9S8M1	24,509 and 23,569 23,550 and 23,560	N-term.sequence N-term.sequence	glycosylated highly glycosylated	(Harrach et al. 1995) (Napper et al. 1994) (Harrach et al. 1998)
From pineapple fruits:					
Fruit bromelain (EC. 3.4.22.33)	O23791	23,000	N-term. sequence	not glycosylated	(Cooreman 1978)

species. A green fig weighing 10–15 g contains about 100–150 mg (Uhlig 1998). Ficin isolated from the latex of a *Ficus* tree is known to occur naturally in multiple forms (Jones and Glazer 1970) distinguishable by ion-exchange chromatography (Williams and Whitaker 1969; Kramer and Whitaker 1969; Liener and Friedenson 1970; Malthouse and Brocklehurst 1976). The optimum pH range is from 5.0 to 8.0 and the optimal temperature is from 45 to 55 °C (Polaina and MacCabe 2007). As of today, only three fragments of ficin have been studied (a fragment around the catalytic Cys, a fragment around the catalytic His, and N-terminal fragment). The amino acid sequence determined for neighboring residues of the active-site Cys was found to closely resemble the corresponding sequence in papain (Devaraj et al. 2008).

Industrial Applications of Plant Cysteine Proteases

Brewing Industry

The application of proteolytic enzymes to chillproof beer was patented by Leo Wallerstein in 1911 (De Clerck 1969). Crude papain or bromelain (Kennedy and Pike 1981; Jin and Toda 1988) and ficin (Priest and Stewart 2006) are used in the brewing industry in order to obtain good colloidal properties at low temperatures, thus eliminating cloud formation (Jones 2005). Currently, papain is not so widely used because additive-free beers prevail in some European countries.

Baking Industry

Proteases are used in the baking industries in order to hydrolyze gluten, so the baking mass may be easier prepared. In this sense, and because of their rapid rate of reaction, broad optimal pH, and temperature as well as the lack of amylase or pentosanase side activities (Polaina and MacCabe 2007), these enzymes may find a natural niche for industrial application. Gluten hydrolyzates are currently under study to generate new products with higher-added value (Wang et al. 2007a, b). Bromelain has been also used to obtain hypoallergenic wheat flour because of its ability to break the wheat glutenin IgE epitope Gln-Gln-Gln-Pro-Pro (Tanabe et al. 1996).

Food Processing

Softness and tenderness of meat have been identified as the most important factors affecting consumer satisfaction and the perception of taste. Consumers are willing to pay a premium for a guaranteed tender product with the potential to increase the value of the middle meats over $60 per carcass (Miller et al. 2001). Tenderness is a complex trait. Generally, the two primary structural features of muscle that influence tenderness are integrity of the myofibrils (termed the actomyosin effect) and the connective tissue contribution (termed background effect). Only five enzymes are considered as having a GRAS status by the US federal agencies (CFR 1999, 2009), namely papain, ficin, bromelain, *Aspergillus oryzae* protease, and *Bacillus subtilis* protease. Other two plant CPs, actinidin (EC 3.4.22.14) and zingibain (EC 3.4.22.67), are good candidates for tenderization (Naveena et al. 2004; Sullivan and Calkins 2010).

Studies carried out with GRAS enzymes revealed that papain showed the greatest ability to improve tenderness in meat, although caution must be taken as juiciness and textural changes can be negatively affected. Bromelain increased tenderness and degraded collagen more than the contractile proteins, while ficin gave the most balanced degradation of both myofibrillar and collagen proteins (Sullivan and Calkins 2010). Papain and

bromelain are also used to manufacture different sauces (Díaz et al. 1996) and dry cured ham (Scannell et al. 2004).

Fish Protein Hydrolysate

The waste materials generated by manufacturing plants processing marine species are becoming a great problem in countries where fishing is an important industry. Traditional methods of fish protein hydrolysate (FPH) production exploit the endogenous enzymes of the viscera to produce such hydrolysates. The enzymatic hydrolysis of the waste materials appears as a possibility in order to obtain products with higher added value. FPHs have potential applications either as a source of bioactive peptides or as nitrogenous substrates for microbiological media (Horn et al. 2005; Sumantha et al. 2006; Safari et al. 2009; Klompong et al. 2010). Commercial proteases of plant origin such as papain, bromelain, ficin, and alcalase (contains ananain) have been employed to generate FPH (Aspmo et al. 2005), and papain has been extensively studied and described by a number of different authors over the last 60 years (Sen et al. 1962; Sripathy et al. 1962; Hale 1969; Ritchie and Mackie 1982; Quaglia and Orban 1987; Vega and Brennan 1988; Hoyle and Merritt 1994; Gildberg 1994; Kristinsson and Rasco 2000; Gilmartin and Jervis 2002).

Use of Cysteine Proteases in Pharmacy and Medicine

Infections caused by gastrointestinal nematodes have profound and severe consequences on millions of people all over the world and cause important economic losses in farm-exploited animals. Traditionally, extracts of *C. papaya, Ficus* spp., and *A. comosus* have been employed for treating gastrointestinal nematodes since they degrade their cuticles (Berger and Asenjo 1940; Hammond et al. 1997; Mueller and Mechler 2005). At present, treatment is based on synthetic drugs, but acquired resistance against them has stimulated research on the traditional methods for fighting infestation (Behnke et al. 2008). Ongoing studies show that extracts containing bromelain (Thomson et al. 2001; Hordegen et al. 2003, 2006), ficin, papain, chymopapain, and others (Stepek et al. 2005) may be a good alternative to synthetic drugs.

Chymopapain has been used to treat herniated disks through chemonucleolysis, a medical procedure that involves the dissolving of the gelatinous cushioning material in an intervertebral disk (Simmons et al. 2001; Sagher et al. 1995). Papain, bromelain, and ficin have been used as anti-inflammatory drugs to replace glucocorticoids and nonsteroidal anti-rheumatic (Lotz-Winter 1990; Maurer 2001), and the mixture of proteases derived from pineapple stem has also been proposed for the treatment of arthritis (Brien et al. 2004).

Proteolytic enzyme mixtures (containing trypsin, chymotrypsin, and papain) and bromelain are currently being investigated in the treatment of breast, colorectal, and plasmacytoma cancer patients thus showing good results (Beuth 2008; Salas et al. 2008).

Papain and bromelain have also been used for debridement of burns (Rosenberg et al. 2004); although the Food and Drug Administration asked pharmaceutical companies to stop the use of topic papain because of the side effects (Shuren 2008). Papain is the main ingredient of Papacarie, a gel used for chemomechanical dental caries removal. This gel effectively breaks unions among the collagen fibrils in the dentine thus avoiding the use of rotary cutting tools (Lopes et al. 2007; Kotb et al. 2010).

Bromelain has been investigated to be applied in clinically important antibody identification (Rookard et al. 2009). Papain and ficin are used for the preparation of Fab fragments from IgG to be employed in assay systems where the presence of the Fc region may cause problems (Mariani et al. 1991; Boguslawski et al. 1989). Papain is also used to digest IgM (Newkirk et al. 1987) as well as in red cell serology to modify the red cell surface to enhance or destroy the reactivity of many red cell antigens as an adjunct to grouping, antibody screening or antibody identification procedures. Papain has also been shown to be useful in platelet serology (Lown and Dale 1995). Finally, this enzyme is commonly used in cell isolation procedures (Huettner and Baughman 1986; Kinoshita et al. 2003; Driska et al. 1999).

Organic Chemistry

Ficin (Tai et al. 1995; Sekizaki et al. 2008), papain and, to a lesser extent, bromelain are widely used in the synthesis of amino acids and peptides (Rai and Taneja 1998; Barbas and Wong 1987; Uyama et al. 2002; Narai-Kanayama et al. 2008; Burton et al. 2002; Stevenson and Storer 1991; Chen et al. 1998; Theppakorn et al. 2004; Lang et al. 2009). Also, papain, stem bromelain, and other plant CPs: araujiain (not yet included in IUBMB) from *Araujia hortorum*, asclepain C from *Asclepias curassavica*, and funastrain from *Funastrum clausum* have been assayed with good results for the synthesis of arginine-base surfactants (Morcelle et al. 2009).

Detergents

Detergent proteases represent the single largest volume of enzymes sold for industrial use, accounting for as much as 35% of the total industrial enzyme market (Cherry and Fidantsef 2003). Papain satisfies the requirements needed in a protease to be used in the detergent industry since these enzymes must be active over a wide range of temperatures

and partly stable at basic pH values. Subtilisins, serine proteases from *Bacillus licheniformis* and *Bacillus amyloliquifaceins*, are the enzymes normally employed in detergent formulations (Van Beckhoven et al. 1995). The uses of chemically modified alkaline proteolytic enzymes (Hamsher and Tate 1973) and of papain are patented (Kosaka 1995a, b). However, and despite all this, no published information on the use of papain in detergents is available. This may be so because it loses most of its activity at extreme pH values (Khaparde and Singhal 2001). In order to overcome this, papain has been chemically modified with different dicarboxylic anhydrides so the optimum pH changed to a more basic value (pH 9) and the optimal temperature to 80 °C. The stability of the modified papain increased by immobilization on starch gel. Modified papain retained activity comparable to the commercial enzyme detergents so it can be an inexpensive alternative to the alkaline proteases that are also used in detergents (Roy et al. 2005; Sangeetha and Abraham 2006).

Immobilization

Many applications require that proteases be immobilized on different media because in that form are more resistant to denaturation are more easily manipulated, can be reused and, what is most important, particularly for the food and pharmaceutical industries, the reaction product is not contaminated by the enzyme. New immobilization approaches are being investigated, such as the use of magnetic composite microspheres for papain immobilization (Lei et al. 2004), papain on Sepharose 6B in presence of cysteine (Homaei et al. 2010), native and modified papain on cotton fabric (Li et al. 2007; Xue et al. 2010), and bromelain oriented immobilization on metal affinity support (Gupta et al. 2007) (Table 4).

Biotechnologically Engineered Cysteine Proteases and Industrial Uses

It is perhaps in the field of enzymology that the advances of biotechnology are more evident. CPs are, however, poorly explored from a biotechnological point of view. They were first used as crude extracts and then in semi-purified or fully purified forms and their chemical modification has just began.

As pointed out before, papain is the most widely used protease of the CP group and, consequently, the most technologically developed. In this regard, a peptide nitrile hydratase activity has been engineered into papain by means of a single mutation (Gln19Glu) in the active site (Dufour et al. 1995), and the mutant was expressed in *Pichia pastoris*. Papain is stable in organic solvents (Stevenson and Storer 1991). The hydrolysis of both peptide and non-peptide nitriles requires the use of organic co-solvents because of the low water solubility. Due to the natural amidase activity, the mutated CP may be used for the hydrolysis of peptide nitriles in aqueous-organic media (Versari et al. 2002). The mutated enzyme was also used to synthesize amidrazones and showed several advantages over other nitrile-hydrolyzing enzymes (Dufour et al. 1998). The bioengineered proregion of proteinase IV (glycyl endopeptidase) (Taylor et al. 1995) produced in *Escherichia coli* was tested with good results as inhibitor of the intestinal CPs in the larvae of the herviborous pest Colorado potato beetle. Thus, plant protease proregion could prove an interesting complement to the inhibitors currently used for regulating proteinase activity in complex biological systems (Visal et al. 1998). Also, the *BAA* gene (the gene encoding fruit bromelain) was cloned and expressed in *Brassica rapa* under the control of the cauliflower mosaic virus (CaMV) 35S promoter, thus causing high resistance of the plant against the soft rot pathogen *Pectobacterium carotovorum* ssp. *carotovorum*. These results suggest that the gene could be useful to protect the plant against pathogenic bacteria (Jung et al. 2008).

Particular industrial uses require of enzymes stable at very harsh conditions such as temperature, pressure, pH, organic solvents, etc. By means of genetic engineering, it has been possible to increase the stability of papain to temperatures that do not affect its normal activity, using as template the unusually stable CP ErvataminC (not yet included in IUBMB) from the medicinal plant *Ervatamia coronaria* (Thakurta et al. 2004). The generation of three mutations in the interdominium region of papain increased the half life to 94 min at 60 °C and 45 min at 65 °C (Choudhury et al. 2010).

As far as basic research goes, plant CPs have been engineered to be used in studies related to interaction among amino acids in the active sites of papain (Ménard et al. 1990), ananain (Carter et al. 2000), and caricain (Taylor et al. 1994; Ikeuchi et al. 1998) (Table 5). Results of several of these studies are shown in Table 6.

Plant Aspartic Proteases

Native Plant Aspartic Proteases

In the MEROPS database, plant aspartic proteases (APs) have been distributed among the A1, A3, A11, and A12 families of clan AA, and the A22 family of clan AD. Majority of plant APs, together with pepsin-like enzymes from many different origins, belong to the A1 family (Rawlings et al. 2010). The name used for NC-IUBMB to denote all related plant APs is phytepsin (3.4.23.40).

Table 4 Immobilization methods of plant cysteine proteases

Immobilization matrices	Enzymes	Results (casein as substrate enzymatic assays)	Commentary
Magnetic composite microspheres (Lei et al. 2004)	Free papain (FP) Immobilized papain (IP)	Optimum temperature (at pH 7) FP: 65 °C/IP: 80 °C Optimum pH (at 40 °C) FP: 6.5/IP: 8.0 Km (mg/ml) FP: 21.64/IP: 7.08	The immobilized papain exhibited better adaptability to alkaline environment and reusability than the soluble one.
Oriented immobilization on Cu-iminodiacetic acid carrier Sepharose 6B (Gupta et al. 2007)	Free stem bromelain (FB) Immobilized stem bromelain (IB) Cross-linked stem bromelain (CB)	Optimum temperature (at pH 7.5) FB, IB and CB 60 °C. Optimum pH (at 37 °C) FB: 7.5/IB: 6.0/CB: 8.0 Km (mg/ml) FB: 1.08/IB: 0.42/CB: 1.56	The single His 158 residue of stem bromelain is used for orientation of the enzyme. Bromelain immobilized is more resistant to thermal inactivation.
Activated cotton fabric by sol–gel method (Li et al. 2007)	Free papain (FP) Immobilized papain (IP)	The thermostability of IP shows no significant change compared to the FP. Optimum pH (at 40 °C) FP: 6.0/IP: 7.0 At pH 3,0 IP retains 80% of the original activity whereas FP retains only 16%	The optimum pH for immobilized papain is shifted to alkaline side and its adaptability to environmental acidity is significantly increased.
Activated cotton fabric (Xue et al. 2010)	Immobilized papain (IP) Immobilized modified papain with: 1,2,4benzenetricarboxylic anhydre (IBP) pyromellitic anhydre (IPP)	Optimum temperature (at pH 8) IP, IBP and IPP 80 °C. IPP is more thermoestable than IP and IBP. Optimum pH (at 45 °C) IP: 6.0/IBP and IPP: 9.0 Km (mM) IP: 8.12/IBP: 7.00/IPP: 5.30	Immobilization of papain after chemical modification increases its stability and reusability in alkaline conditions. Cotton fabric immobilized modified papain has potential applications in the functional textiles field.
Sepharose 6B (Homaei et al. 2010)	Free papain (FP) Immobilized papain (IP) Papain co-immobilized with cysteine (IPC)	Optimum temperature (at pH 7.5) FP: 60 °C/IP and IPC: 80 °C. Optimum pH (room temperature) FP: 6.5/IP and IPC: 8.0 Km (μM) FP: 0.62/IP: 0.79/IPC: 0.57	Both immobilized forms of papain exhibit a marked increase in stability to temperature and extreme pH values with respect to free form. The temperature profile of papain- coimmobilized with cysteine shows a broad range of activity compared with free and immobilized form.

The primary structure of almost all plant APs includes: a signal sequence at the amino terminus, responsible for translocation into the ER, a prosegment involved in inactivation or in the correct folding, stability and intracellular sorting (Simoes and Faro 2004), and a fragment known as the plant-specific insert (PSI) located between the amino and carboxy termini, and encompassing 100 amino acids (Fig. 2) (Simoes and Faro 2004). The PSI fragment has been identified only in plants but shows structural similarity to the precursor of mammalian saposins, lysosomal sphingolipid-activating proteins. Hence, PSI is called swaposin domain (Muñoz et al. 2010). The PSI fragment exhibits a putative membrane-binding region and may play a role in vacuolar transport of APs (Egas et al. 2000), although biological function has not been completely established. PSI is present in all plant AP genes with the exception of

Table 5 Expressed recombinant plant cysteine proteases

Enzyme/EMBL-Bank	Host	Cloning	Objective	References
Papain/M15203 (Cohen et al. 1986)	*E. coli*	Propapain	yield: 400 mg/L yield: 3 mg/L	(Choudhury et al. 2009) (Taylor et al. 1992) (Vernet et al. 1989) (Cohen et al. 1990)
	E. coli	Mutant K174R Mutant K174R/V32S Mutant K174R/V32S/G36S	Improving thermostability	(Choudhury et al. 2010)
	Autographa californica/ Spdoptera frugipeda Sf9	Propapain	yield: 300 μg/L	(Vernet et al. 1991)
	Baculovirus-insect cell expression system	Propapain Mutant D158N	Role Asp 158 in the enzyme mechanism	(Ménard et al. 1990)
	Baculovirus-insect cell expression system and *S. cerevisiae*	Mutant Q19E Mutant Q19H Mutant Q19N/S21A Mutant Q19A Mutant Q19S	Effect on the catalytic properties of the papain of the residue Gln19	(Ménard et al. 1991) (Ménard et al. 1995)
	Autographacalifornica/ Spdoptera frugipeda Sf9	Propapain Mutant V133A/S205E Mutant V33A/V157G/S205E	Study S2 subsite specifity	(Khouri et al. 1991)
	Autographacalifornica/ Spdoptera frugipeda Sf9	Mutant Q19E	Engineering nitrile hydratase activity into a papain	(Dufour et al. 1995)
	P. pastoris	Propapain Mutant Q19E	Synthesis amidrazones	(Dufour et al. 1998)
	S. cerevisiae	Propapain	Amino acids which are tolerated at Asn175 yield: 110 μg/L	(Vernet et al. 1993)
	S. cerevisiae	Propapain	yield: 1.7 mg/L	(Ramjee et al. 1996)
	E. coli	Propeptide	Study thermal unfolding of papain propeptide	(Gutiérrez-González et al. 2006)
Chymopapain/X97789 (Taylor et al. 1999)	*E. coli*	Prochymopapain	Characterization yield: 8–12 mg/L	(Taylor et al. 1999)
Caricain/X66060 (Revell et al. 1993)	*E. coli*	Procaricain Mutant E50A Mutant D158E Mutant E50A/D158E	Role of Glu50 and Asp158 in the enzyme mechanism	(Ikeuchi et al. 1998)
Glycyl endopeptidase/ X78056 (Taylor et al. 1995)	*E. coli*	Propeptide	Proregion to target proteases of pests	(Visal et al. 1998)
Ananain/AJ002477 (Robertson and Goodenough 1997)	*E. coli*	Proananain Mutant E50A Mutant E35A	Role Glu50 and Glu35 in the enzyme mechanism	(Carter et al. 2000)
Fruit bromelain/D14059 (Muta et al. 1993)	Transgenic plants (binary plant expression vector pIG 121 Hm)	Preprobromelain	OverexpressBAA in transgenic plant (enhanced resistantce to bacterial soft rot)	(Jung et al. 2008)

nucellin (Chen and Foolad 1997), an AP-like protein from tobacco chloroplasts (Nakano et al. 1997) and AP encoded by the *cdr*-1 gen from *Arabidopsis* (Xia et al. 2004).

Processing of plant APs into fully active enzymes involves several proteolytic steps that include elimination of signal peptide and propeptide, thus originating heterodimeric or monomeric forms (Mutlu and Gal 1999). In heterodimeric plant APs, the PSI fragment is removed by proteolysis prior to activation (Glathe et al. 1998; Ramalho-Santos et al. 1997). The vast majority of plants APs are heterodimeric

Table 6 Kinetic constants of biotechnologically engineered plant CPs

Enzyme	Substrate	Specific[a] activity (U/mg, 37 °C)	T_{max} (°C)	$t_{1/2}$ at 60 °C (min)	Commentary
Recombinant wild-type papain (1 mg/ml)	Azocasein at pH 6.5	125.94	–	–	Production of active papain with activity comparable with commercially native papain using *E. coli* as expression host. (Choudhury et al. 2009)
Commercial papain (2 mg/ml)		221.73	–	–	
Wild type papain	Azocasein at pH 6.5	128.1	50	77	Improve the thermostability of papain by introducing three mutations at its interdomain region (half-life $t_{1/2}$ extended by 94 min at 60 °C). (Choudhury et al. 2010)
Mutant papain K174R/V32S		121.3	60	114	
Mutant papain K174R/V32S/G36S		130.2	65	171	
		K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}mM^{-1}$)	
Wild type papain	Cbz- Phe- Arg- AMC	0.086	45	520	Determination of a glycosylation site required for the high yield of propapain. (Ramjee et al. 1996)
Wild type papain	Cbz-Phe-Arg-AMC at pH 6.5	0.089	41.6	464	Change of specificity of mutant papain V133A/S205E compared to wild-type enzyme.
	Cbz-Arg-Arg-AMC at pH 6.5	2.27	1.18	0.51	
	Cbz-Cit-Arg-AMC at pH 6.5	1.48	8.14	5.50	
Mutant papain V133A/S205E	Cbz-Phe-Arg-AMC at pH 6.0	0.19	16.9	89	Change of specificity of mutant papain V33A/V157G/S205E and important decrease of his activity compared to wild-type enzyme. (Khouri et al. 1991)
	Cbz-Arg-Arg-AMC at pH 6.0	0.94	9.3	11.2	
	Cbz-Cit-Arg-AMC at pH 6.0	1.296	5.67	4.37	
Mutant papain V33A/V157G/S205E	Cbz-Phe-Arg-AMC at pH 6.0	0.43	2.78	7.4	
	Cbz-Arg-Arg-AMC at pH 6.0	5.6	0.79	0.12	
	Cbz-Cit-Arg-AMC at pH 6.0	2.03	0.42	0.20	
Papain recombinant	Cbz-Phe-Arg-AMC at pH 6.5 and 25 °C	0.0892	41.6	464	These mutations result in 65-315-fold decreases in K_{cat}/K_m, supporting the hypothesis that the side chain of Gln19 contributes to transition state stabilization in the oxyanion hole of papain. (Ménard et al. 1995)
Mutant papain Q19E		0.224	1.58	7.1	
Mutant papain Q19H		0.032	0.047	1.48	
Mutant papain Q19N/S21A		0.243	1.20	4.93	
Mutant papain Q19A		0.30	2.4	7.7	
Mutant papain Q19S		0.27	0.23	0.76	
Native chymopapain	Bz-Phe-Val-Arg-pNA at pH 7.0 and 37 °C	0.0938	9,198	98,059	The recombinant chymopapain hydrolyses a peptide substrate with similar efficiency to native chymopapain (Taylor et al. 1999)
Wild type chymopapain		0.0701	3,750	53,495	
Wild type caricain	Pyr-Glu-Phe-Leu-pNA at pH 6.75 and 25 °C	1.18	4.41	3.70	(Ikeuchi et al., 1 998)
Mutant caricain E50A		1.28	3.90	3.05	
Mutant caricain D158E/E50A		0.75	1.57	2.08	
Mutant caricain D158E		2.61	3.65	1.4	
Wild type ananain	Pyr-Glu-Phe-Leu-pNA at pH 4.0	–	–	1.65	[b] Comparisons of the kinetics of recombinant wild-type ananain with E50A and E35A mutants proves that these charged groups are not essential for catalysis. (Carter et al. 2000)
Mutant ananain E50A		–	–	1.52	
Mutant ananain E35A		–	–	1.96	
Wild type ananain	Pyr-Glu-Phe-Leu-pNA at pH 7.0	–	–	4.53	
Mutant ananain E50A		–	–	4.36	
Mutant ananain E35A		–	–	2.07	

Table 6 (continued)

Enzyme	Substrate	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}mM^{-1}$)	Commentary
		Nitrile hydratase activity			
Mutant Q19E	MeOCO-PheAla-CN at pH 5.0 and 22 °C	50	57	1.15	A peptide nitrile hydratase acitvity has been engineered into a papain by mutation Q19E, which causes a significant increase in $K_{cat.}$ (Dufour et al. 1995)
Wild type papain		5.27	<0.00015	<0.00003	
		Amidase activity			
Mutant Q19E	Cbz-Phe-Ala-amide at pH 5.0 and 22 °C	>400		0.105	
Wild type papain		>500		0.603	

[a] One enzyme unit is defined as the amount of soluble protease required to release 1 μg of soluble azopeptides/min

[b] pH values tested in this study were from pH 3 to pH 9

Cbz carboxybenzyl, *Bz* benzoyl, *pNA* *p*-nitroanilide

proteins with a large subunit of 28–35 kDa, and a small subunit of 11–16 kDa and only a reduced number are monomeric proteins with molecular mass of 36–65 kDa (Mutlu and Gal 1999). Their crystallographic structure reveals that plant APs are formed by beta strand secondary structures arranged in a biloval conformation (Simoes and Faro 2004).

The catalytic center is formed by two Asp residues that activate a water molecule, and this mediates the nucleophilic attack on the peptide bond (James 2004). The majority of APs contain a common motif in both lobules Asp-Thr-Gly (DTG) but plants APs contain Asp-Ser-Gly (DSG) in one of them (Fig. 3). APs are active at acidic pH and are specifically inhibited by pepstatin A (Chitpinitoyl and Crabbe 1998).

Most plant APs do not have particular roles, but some have been traditionally ascribed to roles such as hydrolysis of store proteins, stress response, plant senescence and programmed cell death, plant sexual reproduction (Simoes and Faro 2004), and antimicrobial functions (Mendieta et al. 2006). Plant APs are widely distributed among gymnosperms. AP activity has been detected in the seeds of pine species (Salmia 1981), whereas in angiosperms, APs have been detected in monocotyledonous plants such as barley, rice, wheat, sorghum, and maize (Asakura et al. 1995; Asakura et al. 1997; Sarkkinen et al. 1992; Belozersky et al. 1989; Radlowski et al. 1996; Garg and Virupaks 1970), and in dicotyledonous plants like sunflower, cacao, *Arabidopsis*, *Brassica*, spinach, potato, tobacco, tomato, *Cynara cardunculus*, *Centaurea calcitrapa*, and carnivorous plants such as *Nepenthes* (Park et al. 2001; Domingos et al. 2000; Heimgartner et al. 1990; D'Hondt et al. 1997; Voigt et al. 1995; Guevara et al. 2001; Rodrigo et al. 1989; 1991; Tokes et al. 1974; Kuwabara and Suzuki 1995; Guilloteau et al. 2005).

Great majority of the purified plant APs are intracellular and accumulate essentially inside protein storage vacuoles (Simoes and Faro 2004). Exception to this intracellular location are the secreted APs found in the extracellular matrix of tobacco (Rodrigo et al. 1991) and tomato leaves (Rodrigo et al. 1989), cardosin B found in the extracellular matrix of the floral transmitting tissue in *C. cardunculus* (Vieira et al. 2001), the APs from *Nepenthes* (Tokes et al. 1974), the AP encoded by the *Arabidopsis cdr*-1 gene (Xia et al. 2004), and the AP from spinach localized to the plastids (Kuwabara and Suzuki 1995).

Enzymes from Extracts of C. cardunculus

The best studied APs are those from *C. cardunculus* extracts, which are used in the dairy industry and include cardosins and cyprosins. Heimgartner and coworkers isolated three proteases that were termed as cynarases 1, 2, and 3. These are heterodimeric proteases only present in

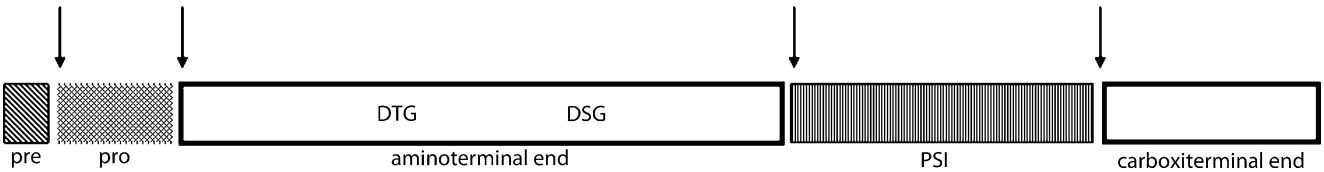

Fig. 2 Schematic representation of the primay structure cardosin A. *Pre* signal peptide; *pro* prosegment; *PSI* plant-specific insert. *Arrows* indicate processing sites

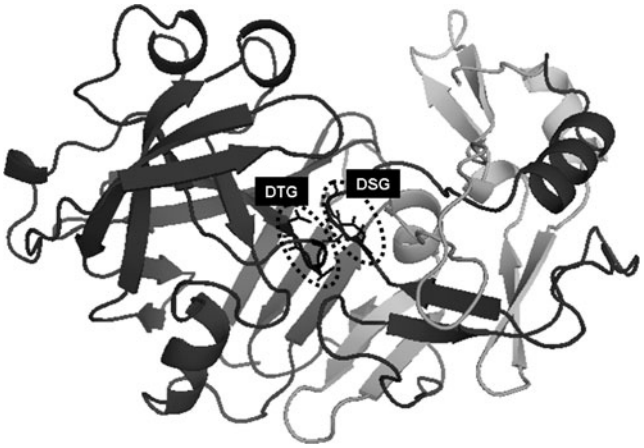

Fig. 3 Three dimensional model of cardosin A, PDB code: 1B5F (Frazão et al. 1999).The heavy chain (35 kDa) is shown in *black*, the light chain (15 kDa) in *gray*

the violet part of the flower and have molecular masses about 49–50 kDa (Heimgartner et al. 1990; Campos et al. 1990). Cardosin A (Veríssimo et al. 1995, 1996), the most abundant of the cardosins, accumulates in the protein storage vacuoles of the stigmatic epidermal papillae and in the vacuoles of the epidermal cells in the stylus (Ramalho-Santos et al. 1997). Cardosin B accumulates in the cell wall and in the extracellular matrix of the transmitting tissue (Vieira et al. 2001). Although Cardosin B is less abundant than Cardosin A, its proteolytic activity is by far higher (Faro et al. 1999). Cardosin A has an Arg-Gly-Asp (RGD) motif, which is known to function in cell surface receptor binding by extracellular proteins. Both cardosins are formed by two subunits of 31 and 15 kDa in the case of CardosinA, and 34 and 14 kDa in the case of CardosinB (Veríssimo et al. 1996). More recently have been isolated, purified, and biochemically characterized four new APs from pistils of *C. cardunculus* named cardosins E, F, G, and H, which resemble cardosin A more than they resemble cardosin B or cyprosin (Table 7). These cardosins were purified by gel filtration and anion exchange liquid chromatography (Sarmento et al. 2009).

Industrial Applications of Plant Aspartic Proteases

Dairy Industry

Several plant extracts have been used for centuries for milk clotting instead of animal rennets: *Galium verum*, *Withania coagulans*, *F. carica*, *C. cardunculus*, *C. scolymus*, *Cayduus nutans*, *Cnicus benedictus*, *Drosera peltata*, *Datura stramonium*, *Pisum sativum*, *Lupinus hirsutus*, *Ricinus hirsutus*, *Pinguicula vulgaris*, *Leucas cephalotes*, *Crotalaria burhia*, *Rhazya stricta*, and *Streblus asper* (Wallace 1922). However, the vast majority of these extracts are not suitable for the cheese industry due to their high proteolytic activity. Cheeses made with plant coagulants can be found in the Mediterranean basin, both in East African and Southern European countries. Spain and Portugal have the largest variety and production of cheeses using *Cynara* sp. as the plant coagulant and are normally produced at an artisanal scale. Among the different plant species to produce satisfactory final products, *C. cardunculus*, *C. humilis*, and *C. scolymus* have to be reported (Roseiro et al. 2003). The cheeses are elaborated by dried flower extracts acting on raw ovine and/or caprine milk and cheese and are very appreciated worldwide (Table 8). These cheeses have a characteristic soft creamy texture, an exquisite flavor, sometimes slightly bitter but piquant when more mature (Galan et al. 2008). This is due to the fact that plant APs cleave alpha, beta, and kappa caseins, while animal chymosin only cleaves kappa caseins.

Currently, there are no registered commercially available coagulants of vegetable origin, although there is a Spanish patent for the production of a dried powder from the

Table 7 Aspartic proteases from *Cynara Cardunculus*

Name (EC 3.4.23.40)	UniprotKB code	Molecular mass (Dalton)	Protein sequences	Reference
Enzymes from dried flowers:				
Cynarase 1 Cynarase 2		Natives dimeric proteins 49,000		(Heimgartner et al. 1990)
Cynarase 3 (cyprosin B)	Q0VTQ7	(32,0–34,0)–(14,0–18,0)		
Enzymes from fresh flowers:				
Cardosin A	Q9S8D5 Q9S8D4	31,000–15,000	Partial sequences (both subunits)	(Faro et al. 1995; Veríssimo et al. 1996)
Cardosin B	Q9S8D3	34,000–14,000	Partial sequences (both subunits)	
Cardosin E Cardosin F	P85136 P85137	27,669–10,691 27,356–10,778	Partial sequences (both subunits) Partial sequences (both subunits)	(Sarmento et al. 2009)
Cardosin G	P85138	27,409–10,691	Partial sequences (both subunits)	
Cardosin H	P85139	27,134–10,778	Partial sequences (both subunits)	

Table 8 Cheeses elaborated with *Cynara* L. proteases in the Iberian Peninsula (Roseiro et al. 2003)

Country	Cheese
Portugal	Serra da Estrela
	Serpa
	Castelo Branco
	Évora
Spain	Casar de Cáceres
	Torta del Casar
	La Serena
	Los Pedroches
	Los Ibores

aqueous extract of the flowers from *Cynara* sp. (Cáceres and Fernández 2000).

Biotechnologically Engineered Aspartic Proteases and Industrial Uses

Cheeses elaborated with natural *Cynara* sp. extracts have high price because all the manipulations involved in growing the plants and the subsequent enzyme preparation from the plants. Besides, these extracts most often contain several enzyme forms or processing intermediates that complicate the purification and characterization of the APs for basic research. Because of this, several APs from *Cynara* sp. have been cloned and expressed successfully in bacterial and eukaryotic expression systems. The production by yeast of the cyprosin B (cynarase 3) has been patented (Soares et al. 2000). This was the first clotting protease of plant origin produced by fermentation technology (Sampaio et al. 2008). Recombinant cyprosin produces a proteolysis similar to that obtained with natural enzymes present in the crude extract from *C. cardunculus* (Fernández-Salguero et al. 2003).

Contrary to microbial and animal APs, plant APs have received little scientific attention until now. Currently, several plant APs are under study and many have been expressed successfully and may have industrial application in the near future (Table 9).

In this regard, studies with APs from *Solanum tuberosum* have revealed their antimicrobial activity (Guevara et al. 2002) including antifungal activity (Mendieta et al. 2006). The authors cloned, expressed, and purified PSI (swaposin domain) from StAPs (StAsp-PSI) and found that the recombinant protein still maintained its cytotoxic activity (Muñoz et al. 2010). Both StAPs and StAsp-PSI were able to kill human pathogenic bacteria in a dose dependent manner but were not toxic to human red blood cells under the experimental conditions tested. Therefore, StAPs and StAsp-PSI could contribute to the generation of new tools to solve the growing problem of resistance to conventional antibiotics (Zasloff 2002)

Plant Serine Proteases

Native Plant Serine Proteases

For years, it was believed that SPs were rare in plants, but recently, SPs have been purified from a number of plant species. Most plant SPs belong to the S8 family (MEROPS database) that is divided into two subfamilies, with Subtilisin as the prototype for subfamily S8A and Kexin the typical example for subfamily S8B. Kexin-type proteins appear to be absent from plants (Schaller 2004).

The basic mechanism of action of SPs involves transfer of the acyl portion of a substrate to a functional group of the enzyme (Dunn 2001). The two basic steps of catalysis by this group of enzymes includes, first, the formation of an ester between the oxygen atom of serine and the acyl portion of the substrate, which produces a tetrahedral intermediate and releases the amino part of the substrate and, second, the attack of water on the acyl-enzyme intermediate, which breaks it down and releases the acidic product, while regenerating the original enzyme form (Antao and Malcata 2005).

Members of the subtilisin family are synthesized as preproenzymes, then translocate across cell membranes via the prepeptide (or signal peptide), and finally are activated by cleavage of the propeptide (Dunn 2001).

Members of the S8 family have a catalytic triad in the order Asp, His, and Ser in the sequence. In the S8A subfamily, the active site residues frequently occur in the motifs Asp-Thr/Ser-Gly (which is similar to the sequence motif in families of aspartic endopeptidases in clan AA), His-Gly-Thr-His, and Gly-Thr-Ser-Met-Ala-Xaa-Pro (Rawlings et al. 2010). The first crystal structure of a subtilisin-like serin protease, the SBT3 from tomato (*Solanum lycopersicum*), has been recently resolved (Ottmann et al. 2009; Rose et al. 2010) (Fig. 4).

In recent years, several plant SPs have been isolated from various plant species with distinct locations, ranging from seeds to latex or fruits. Primary methods of purification of plant serine proteases include ammonium sulfate precipitation, column chromatography, and gel filtration; secondary techniques include affinity chromatography, chromatofocusing, and hydrophobic iteration chromatography. Serine proteases have been found and extracted from the seeds of barley (*Hordeum vulgare*), soybean (*Glycine max*), and rice (*Oryza sativa*); from the latex of *Euphorbia supina*, *Wrightia tinctoria*, dandelion (*Taraxacum officinale*), African milkbush (*Synadenium grantii*), and jackfruit (*Artocarpus heterophyllus*); from the flowers, stems, leaves, and roots of *Arabidopsis thaliana*; from the storage roots of sweet potato (*I. batatas*) and corn (*Zea mays*); from the sprouts of bamboo (*Pleioblastus hindsii*); from the leaves of tobacco (Nicoti-

Table 9 Expressed recombinant plant aspartic proteases

Enzyme/EMBL-Bank	Host	Cloning	Objective	References
Cardosin A/cardA	*E. coli*	Procardosina A	Characterization	(Faro et al. 1999)
AJ132884		Procardosin A without PSI	Role PSI	(Egas et al. 2000)
(Faro et al. 1999)		Mutant D32A	Activation and proteolytic processing of procardosin A	(Castanheira et al. 2005)
Cyprosin B/CYPRO11	*P. pastoris*	Procyprosin B	Activation and proteolytic processing of procyprosin B	(White et al. 1999)
X81984				
(Cordeiro et al. 1994)		Mutant with autoactivation sequence and without PSI	Role PSI	
	S. cerevisiae	Procyprosin B	Production in a bioreactor	(Sampaio et al. 2008)
AP from *S. tuberosum*/StAPs AY672651	*E. coli*	StAPs-PSI (cloned PSI sequence)	Assays antimicrobial activity	(Muñoz et al. 2010)
(Guevara et al. 2005)				
A. thaliana/CDR1 AY243479 (Xia et al. 2004)	*E. coli*	ProCDR1 Mutant D108A	Characterization proCDR1 Study active site	(Simões et al. 2007)

ana tabacum), lettuce (*Lactuca sativa*), common bean (*Phaseolus vulgaris*), and tomato (*Lycopersicon esculentum*); and from the fruits of melon (*Cucumis melo*), *Cucurbita ficifolia*, osage orange (*Maclura pomifera*), suzumeuri (*Melothria japonica*), "Ryukyu white gourd" (*Benincasa hispida*), Japanese large snake gourd (*Tricosanthes bracteata*), and yellow snake gourd (*Tricosanthes kirilowii*) (Antao and Malcata 2005).

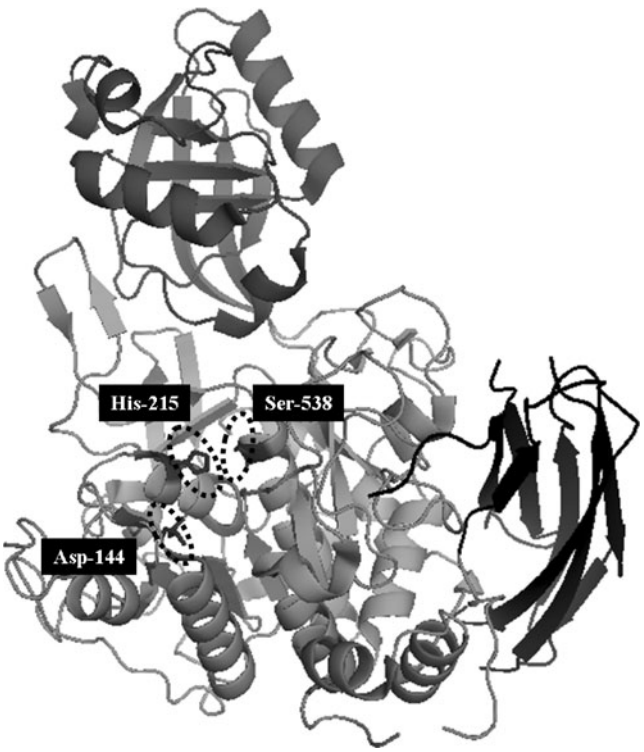

Fig. 4 Three dimensional model of SBT3 (*S. lycopersicum*), PDB code: 3I6S (Ottmann et al. 2009)

Plant SPs are involved in many physiological processes including microsporogenesis, which occurs within the polinic sacs in anthers and was the first role demonstrated for a plant subtilisin-like protease (Kobayashi et al. 1994). SPs can be also induced after a pathogen attack, thus generating a hypersensitive response that causes necrosis and tissue death (Tornero et al. 1996); signal transduction which leads to hypersensitive cell death (Coffeen and Wolpert 2004); differentiation of specialized plant tissues or organs (Berger and Altmann 2000); senescence (Wang et al. 2004; Distefano et al. 1999) and the initiation of storage protein mobilization during early germination (Muntz et al. 2001; Sutoh et al. 1999).

Plant SPs known at present vary from 19 to 110 kDa in molecular mass, but the majority lies within the 60–80 kDa range. The optimum pH for their activity is in the alkaline range (pH 7–11), with the exception of hordolisin and SEP-1 from barley with optimum pH of 6 and 6.5, respectively, as well as Ara12 from *A. thaliana*, RSIP from maize and protease C1 from soybean, which act best in the 3.5–6.5 pH range. The optimum temperature of most plant SPs is between 20 and 50 °C (Antao and Malcata 2005). Most members of the family are inhibited by general serine peptidase inhibitors such as diisopropyl-fluorophosphate and phenylmethylsulfonyl fluoride.

Cucumisin (EC 3.4.21.25)

Cucumisin from sarcocarp of melon fruit (*C. melo*) is an extracellular subtilisin-like SP that is expressed at high levels in the fruit (comprises more than 10% of the total protein content in the fruit) and accumulates in the juice. It was the first plant subtilisin type protease characterized (Kaneda and Tominaga 1975; Yamagata et al. 1994; Uchikoba et al. 1995). The amino acid sequence Gly-Thr-

Ser-Met around the reactive serine residue of cucumisin is identical to that of subtilisin, a microbial SP. Its optimun pH is around 10, and its optimal temperature is 70 °C (Kaneda and Tominaga 1975).

Other SPs with potential industrial applications include the cucumisin-like proteases that have been isolated from *Euphorbia supine*, *Cucumis trigonus* Roxburghi; Macluralisin from *M. pomifera*; taraxilisin from dandelion (*T. officinale*), and Wrightin, from the Latex of *W. tinctoria* (Arima et al. 2000; Rudenskaya et al. 1995, 1998; Terp et al. 2000; Tomar et al. 2008; Asif-Ullah et al. 2006). In the case of the latex of *E. milii*, two SPs have been isolated: eumiliin (not included in IUBMB) and milin (not included in IUBM). Although the vast majority of proteases in latex are CPs, the latex of *Moraceae* and *Euphorbiaceae* do contain SPs (Fonseca et al. 2010). Finally, letttucine has been isolated from *L. sativa* leaves (Lo Piero et al. 2002) (Table 10).

Industrial Applications of Plant Serine Proteases

Contrary to CPs which, as indicated above, have been widely used in food science and technology as well as in medicine, few plant SPs have found a place in industry. CPs have a limitation due to the fact that their proteolytic activity is readily suppressed by air-induced oxidation and metal ions. Therefore, CPs require mild reducing and chelating agents for activation and activity, whereas SPs do not require these reagents. Thus, they could be in turn useful and economical for industrial applications that would include the following:

Dairy Industry

The milk clotting activity of cucumisin suggests that it might be suitable for milk-clotting production. Cucumisin exhibited the same milk-clotting activity of CPs such as papain, but in addition, it produced much less bitter-tasting peptides than those formed by more typical plant CPs (Uchikoba and Kaneda 1996). Other SP enzyme, Lettucine (not included in IUBMB), from *L. sativa* leaves, is able to provoke a significant disorganization of the micellar structure of casein. Its proteolytic activity was analyzed under various technological parameters, such as temperature and pH, and the results were highly consistent with the milk-clotting process (Lo Piero et al. 2002).

Food Processing

Traditionally, the Kachri fruit, *C. trigonus* Roxburghi, has been used as meat tenderizer in the Indian subcontinent. Asif-Ullah demonstrated that the proteolytic activity of the fruit was in part due to a SP. This protease was stable at basic pH values and at high temperature, suggesting its potential application in the food industry (Asif-Ullah et al. 2006).

Molecular Biology

Recently the protease from *C. melo* has been used for DNA extraction from nail clippings in an efficient way. The use of nail clippings when circumstances do not allow to obtain blood samples can be an alternative source of genomic DNA that can be easily stored. Yoshida and coworkers checked the keratinolytic activity of crude proteases prepared from different plants (*C. melo*, *F. carica*, *Actinidia chinensis*, *Pyrus communis*), as well as pure papain or bromelain, and compared the results with proteinase K. They found that the keratinolytic activity in the crude preparation of *C. melo* was 1.78 times higher than that of proteinase K and the activity of the protease solution might be associated with the nature of cucumisin (Yoshida-Yamamoto et al. 2010).

Table 10 Plant serine proteases

Source	Enzyme	UniprotKB code	Molecular mass (kDa)	Protein Sequence	Glycoprotein	References
Cucumis melo	Cucumisin	Q39547	54	Partial sequence	yes	(Kaneda and Tominaga 1975) (Yamagata et al. 1994)
Maclura pomifera	Macluralisin	Q9S8G2	65	N-terminal sequence	yes	(Rudenskaya et al. 1995)
Taraxacum officinale	Taraxilisin	–	67	N-terminal sequence	yes	(Rudenskaya et al. 1998)
Wrightia tinctorica	Wrightin	–	57	–	yes	(Tomar et al. 2008)
Euphorbia milii	Eumiliin	–	30	N-terminal	yes	(Fonseca et al. 2010)
	Milin	–	51			(Yadav et al. 2006)
Lactuca sativa	Lettucine	–	40	–	nd	(Lo Piero et al. 2002) (Lo Piero and Petrone 1999)
Cucumis trigonus		–	67	N-terminal	nd	(Asif-Ullah et al. 2006)

Pharmacy and Medicine

Molluscicides have been used for the control of schistosomiasis by the reduction of the intermediate host snails of the parasite *Schistosoma mansoni* from the fresh water reservoirs. *E. milii* latex has been reported as a molluscicidal agent (Sermsart et al. 2005), with milin being likely responsible for the latex lethality on snails. So, it may be used as a molluscicide to control transmission of the endemic disease schistosomiasis (Yadav and Jagannadham 2008).

Results from experiments performed in vivo with the plant SP eumiliin showed that it is possible to provoke drastic degenerative events, including myonecrosis and inflammatory responses. Eumiliin caused formation of edema and an increase in sensitivity to pain in mice. Eumiliin presented fibrinogenolytic activity and this in turn suggested that eumiliin could have application in the future as an antithrombotic drug (Fonseca et al. 2010).

Possible Applications in the Food and Biotechnological Industries

Wrightin (not included in IUBMB) from the latex of *W. tinctoria*, might be a potential candidate for operations requiring high temperatures because it retains complete activity at 70 °C after 60 min of incubation, and 74% of activity after 30 min of incubation at 80 °C. Furthermore, it is stable over a broad range of pH (5–11.5) and remains active in the presence of various denaturants, surfactants, organic solvents, and metal ions (Tomar et al. 2008).

Milin exhibits activity and high stability over a broad range of pH, temperature, and at high concentrations of chemical denaturants (Yadav et al. 2010), and it has low susceptibility to autodigestion at room temperature. Therefore, it could be of use for the food and textile industries. The fact that Milin is not inhibited by the proteinaceous inhibitor soyabean trypsin inhibitor (SBTI) could facilitate the frequent use of plant proteins for nutritional purposes (Yadav et al. 2006).

Biotechnologycally Engineered Serine Proteases and Industrial Uses

Little information on plant SPs genes has been reported to date, with the cucumisin gene (Genbank code: AY055805) being one exception (Yamagata et al. 1994).

The expression pattern for this gene is quite specific for fruits (only during fruit development and for tissues such as the placenta, locule, and perpheral tissues around seeds in the fruit). Yamagata and coworkers studied the regulation of the promoter by using β-glucuronidase as the reporter gene. A stretch of only 20 bp of the promoter sequence that contained a regulatory enhancer is enough to mediate the fruit-specific expression pattern observed for this gene. This pattern is most likely established via action of fruit-specific transcripcional positive or negative regulators interacting with cis-acting DNA sequences within the analyzed promoter fragment. The Cucumisin promoter region may be useful for genetic engineering of fruits (Yamagata et al. 2002) and is protected by a worldwide patent (Yamagata et al. 2007).

Conclusions

Today, hundreds of different proteases are known in living organisms, and all of them carry out the same reaction: hydrolysis of the peptide bonds of proteins. All differ, however, in substrate specificity, optimal pH and temperature, and other parameters that make their properties unique. In recent years, newly identified plant proteases have been found to exhibit promising properties for their application. Up to now, papain, bromelain, and ficin are best known at the industrial level among plant proteases. Although plant proteases were first employed in raw state or partly purified at best, today, they are accessible in fairly well purified form and with new or optimized properties, thanks to the use of genetic engineering techniques. Probably the future will see new proteases with properties only hinted today.

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