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Biotechnological Applications of Plant Proteolytic Enzymes

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Preface

Proteases (also termed as proteolytic enzymes or proteinases) refer to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. These enzymes are widely distributed in all plants, animals and microorganisms. Proteases fields of application are very diverse, including food science and technology, pharmaceutical industries and detergent manufacturing.

This book is a review about the results obtained during the last decade in biotechnological application of plant proteolytic enzymes. In the last years, plant proteolytic enzymes 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. All these have stimulated the research and increased the number of works on plant proteolytic enzymes.

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Chapter 1
An Overview of Plant Proteolytic Enzymes

D'Ipólito Sebastián, María Gabriela Guevara, Tito Florencia Rocío, and Tonón Claudia Virginia

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1.1 Introduction

Enzymes are proteins that act as highly efficient catalysts in biochemical reactions. This catalytic capability is what makes enzymes unique and they work efficiently, rapidly, and are biodegradable. The use of enzymes frequently results in many benefits that cannot be obtained with traditional chemical treatments. These often include higher product quality and lower manufacturing cost, less waste, and reduced energy consumption. Industrial enzymes represent the heart of biotechnology processes and biotechnology (Whitehurst and van Oort 2009; Sabalza et al. 2014)

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Proteases are enzymes that hydrolyze peptide bonds of 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 (Feijoo-Siota and Villa 2011). These enzymes polarize the carbonyl group of the substrate peptide bond by stabilizing the oxygen in an oxyanion hole, which makes the carbon atom more vulnerable for attack by an activated nucleophile. Proteases can do this in four major ways, which gives the names to four catalytic classes: cysteine proteases, serine proteases, metalloproteases, and aspartic proteases (Dunn 2002).

These enzymes are widely distributed in all plants, animals, and microorganisms. Proteases account for approximately 2% of the human genome and 1–5% of genomes of infectious organisms (Puente et al. 2003). In plants, the *Arabidopsis* genome encodes over 800 proteases, which are distributed over almost 60 families, which belong to 30 different clans (van der Hoorn 2008). The distribution and the family size are well conserved within the plant kingdom because poplar and rice have similar distributions (García-Lorenzo et al. 2006).

1.2 Classification of Proteases

1.2.1 Catalyzed Reaction

This is the main property established by the Enzyme Nomenclature of the Committee of the International Union of Biochemistry and Molecular Biology (ENCIUBMB) 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 exopeptidases (amino-terminal or carboxy-terminal) depending on their ability to hydrolyze internal peptide bonds or bonds located at the ends; endopeptidases are by far more important from the industrial point of view (Barrett 1994).

1.2.2 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 metalloproteases 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.

1.2.3 Structure-Based Evolutive Relationships

Rawlings and Barrett (1994) 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 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).

1.3 Plant Proteases

Plant proteases are involved in many aspects of plant physiology and development (van der Hoorn 2008). They play a pivotal role in processes such as protein turnover, degradation of misfolded proteins, senescence, and the ubiquitin/proteasome pathway (Beers et al. 2000). Proteases are also responsible for the posttranslational modification of proteins by limited proteolysis at highly specific sites (Schaller 2004). They are involved in a great diversity of cellular processes, including photoinhibition in the chloroplast, defense mechanisms, programmed cell death, and photomorphogenesis in the developing seedling (Estelle 2001). Proteases are thus involved in all aspects of the plant life cycle ranging from mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Schaller 2004).

1.3.1 Plant Cysteine Proteases

Fourthly, Cysteine proteases (CPs) family are recognized until today in which the nucleophile is the sulfhydryl group of a cysteine residue. The catalytic mechanism is similar to that of serine-type peptidases in that the nucleophile and a proton donor/general base are required, and the proton donor in all cysteine peptidases is a histidine residue as in the majority of the serine entered forms (Domsalla and Melzig 2008). Although there is evidence in some families that a third residue is required to orientate the imidazolium ring of the histidine, a role analogous to that of the essential aspartate seen in some serine peptidases. There are a number of families in which only a catalytic dyad is necessary (Barrett et al. 1998).

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 in the MEROPS database (Rawlings et al. 2010).

Plants offer an attractive alternative for the production of CPs as they occur naturally in different tissues, in some cases in excessive amount (González-Rábade et al. 2011). CPs of the tropical plants *Carica papaya* (papain, chymopapain, caricain, and glycyl endopeptidase), *Ananas comosus* (fruit bromelain, stem bromelain, ananain, and 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 (Feijoo-Siota and Villa 2011). CPs have been isolated 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 and McDonald 2009); FLCP-1 and FLCP-3 from *Phaseolus* leaves (Popovič et al. 2002); and 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; López et al. 2000), araujiain in *Asclepiadaceae* (Priolo et al. 2000); papain, chymopapain, papaya glycyl 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).

According to the review by González-Rábade et al. (2011), proteases like papain, bromelain, and ficin are employed in different industrial processes and medicines (Uhlig and Linsmaier-Bednar 1998). Some of these proteases are used in the food industry for cheese, brewing and beverage industries for the preparation of highly soluble and flavored protein hydrolysates (papain-like proteases), as a food complement (Kleef et al. 1996; La Valle et al. 2000; Losada Cosmes 1999) to soften meats and dehydrated eggs (Bailey and Light 1989; Lawrie 1985; Miller 1982), and for the production of emulsifiers, among other uses (Pardo et al. 2000). Uses in other industries include culture medium formulation (Headon and Walsh 1994), isolation of genetic material (Genelhu et al. 1998), and the use of keratinases in the leather industry for dehairing and bating of hides to substitute toxic chemicals (Foroughi et al. 2006). Also, they are used in the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems (Rao et al. 1998). Proteases also have an important application in the pharmaceutical industry. Plant extracts with a high content of proteolytic enzymes have been used in traditional medicine for a long time. They have been used for the treatment of cancer (Batkin et al. 1988; Targoni et al. 1999), as antitumorals (Guimarães-Ferreira et al. 2007; Otsuki et al. 2010), for digestion disorders (Kelly 1996; Mello et al. 2008), and swelling and immune-modulation problems (Leipner et al. 2001; Lotti et al. 1993; Melis 1990; Otsuki et al. 2010). A good example is bromelain, derived from pineapple, which has been shown to be capable of preventing edema, platelet aggregation, and metastasis due to its capacity of modifying cell surface structures by

peptide cleavage. Salas et al. (2008) reviewed the pharmacological activity of plant cysteine proteases, emphasizing their role in mammalian wound healing, immunomodulation, digestive conditions, and neoplastic alterations.

1.3.2 Plant Aspartic Proteases

Aspartic proteases (APs, EC 3.4.23) are a family of proteolytic enzymes widely distributed among living organisms and are found in vertebrates, plants, yeast, nematodes, parasites, fungi, and viruses (Rawlings and Salvesen 2013). Aspartic proteases differ from the serine and cysteine peptidases in the way that the nucleophile that attacks the scissile peptide bond is an activated water molecule rather than the nucleophilic side chain of an amino acid (Domsalla and Melzig 2008).

Plant APs have been characterized and purified from a variety of tissues such as seeds, flowers, and leaves: (1) seeds of *Arabidopsis thaliana* (Mutlu et al. 1999), rice (Asakura et al. 1997; Doi et al. 1980), barley (Kervinen et al. 1999; Runeberg-Roos et al. 1991; Sarkkinen et al. 1992), hempseed (St. Angelo et al. 1969, 1970), cucumber, and squash (Polanowski et al. 1985); (2) leaves of the tomato plant (Rodrigo et al. 1989); (3) leaves and tubers of the potato plant (Guevara et al. 2001, 2004); (4) maize pollen (Radlowski et al. 1996); and (5) flowers of thistle (Heimgartner et al. 1990; Verissimo et al. 1996), among others. Some of these APs, like the ones found in barley, resemble mammalian cathepsin D. It has been suggested that plant APs are involved in the digestion of insects in carnivorous plants (Garcia-Martinez and Moreno 1986; Takahashi et al. 2009; Tökés et al. 1974), in the degradation of plant proteins in response to pathogens (Rodrigo et al. 1989, 1991), during development processes (Asakura et al. 1997; Runeberg-Roos et al. 1994), protein-storage processing mechanisms (Doi et al. 1980; Hiraiwa et al. 1997), stress responses (de Carvalho et al. 2001; Guevara et al. 1999, 2001), and senescence (Bhalerao et al. 2003; Buchanan-Wollaston 1997; Cordeiro et al. 1994; Lindholm et al. 2000; Panavas et al. 1999). These enzymes are distributed among families A1, A3, A11, and A12 of clan AA and family 22 of clan AD (Faro and Gal 2005; Mutlu et al. 1999; Rawlings et al. 2014; Simões and Faro 2004). The majority of plant APs have common characteristics as that of AP A1 family, are active at acidic pH, are specifically inhibited by pepstatin A, and have two aspartic acid residues responsible for the catalytic activity (Simões and Faro 2004).

Plant APs are classified into three categories: typical, nucellin-like, and atypical (Faro and Gal 2005). The swaposin domain is only present in typical plant APs inserted into the C-terminal domain as an extra region of approximately 100 amino acids known as "plant-specific insert" (PSI) (Simões and Faro 2004). The PSI domain has a high structural homology with saposin-like proteins (SAPLIPs), a large protein superfamily widely distributed from primitive eukaryotes to mammalians (Bruhn 2005; Michalek and Leippe 2015). Individual SAPLIPs generally share little amino acid sequence identity. However, SAPLIP protein sequences include highly conserved cysteine residues that form disulfide bonds and give

SAPLIPs a stable structure; secondary protein structure consists mainly of α-helices joined by loops (Andreu et al. 1999; Bruhn 2005; Munford et al. 1995). The SAPLIPs or Sap domains may exist for itself independently as a functional unit or as a part of a multidomain protein; they are autonomous domains with a variety of different cellular functions, all of them associated with lipid interaction. SAPLIP activities are classified into three major groups: (1) membrane targeting by the SAPLIP domain; (2) presentation of lipids as substrate for an independent enzyme, either by extraction from the membrane or by disturbance of the well-packed lipid order; and (3) membrane permeabilization by perturbation owing to single molecules or by pore formation of oligomeric proteins (Bruhn 2005). The SAPLIP family includes saposins, which are lysosomal sphingolipid-activator proteins (O'Brien and Kishimoto 1991), NK-lysin, granulysin, surfactant protein B, amoebapores, domains of acid sphingomyelinase and acyloxyacyl hydrolase, and the PSI domain of plant APs (Munford et al. 1995; Stenger et al. 1998; Vaccaro et al. 1999).

The PSI domains of plant APs are named swaposins since they arise from the exchange (swap) of the N- and C-terminal portions of the saposin-like domain, where the C-terminal portion of one saposin is linked to the N-terminal portion of the other saposins (Simões and Faro 2004). This segment is usually removed during the proteolytic maturation of the heterodimeric typical plant APs (Davies 1990; Domingos et al. 2000; Faro and Gal 2005; Glathe et al. 1998; Mutlu et al. 1999; Ramalho-Santos et al. 1997; Törmäkangas et al. 2001; White et al. 1999). However, in monomeric typical plant APs, the PSI domain is present in the mature protein (Guevara et al. 2005; Mendieta et al. 2006). *Solanum tuberosum* APs 1 and 3 (*St*APs 1 and 3) are included into the group of monomeric typical plant APs (Guevara et al. 1999, 2001, 2005).

All enzymes employed commercially in milk coagulation are APs, with acidic optima pH, and high levels of homology between their primary structures and similarity between their catalytic mechanisms (Silva and Malcata 2005).

The most widely used AP is rennet, which has chymosin as its active component (Vioque et al. 2000). Rennet is obtained from the stomach of calves but it is costly and scarce. Most companies produce recombinant rennet of calf origin in different microbial hosts (Seker et al. 1999). Some plant APs have shown to possess similar characteristics to calf-derived rennet and hence have attracted attention in the food industry. In Portugal and some regions of Spain, the use of extracts from dried flowers of *C. cardunculus* L. has been successfully maintained since ancient times for the production of many traditional varieties of sheep and goat cheeses, further strengthening the suitability of this rennet for the production of high-quality cheeses (Reis and Malcata 2011; Roseiro et al. 2003a, b; Sousa and Malcata 2002). Therefore, over the last decades, much effort has been made in understanding the properties of this unique plant coagulant. Several authors have dedicated their research efforts to characterize the milk-clotting enzymes present in cardoon flowers, their role in the hydrolysis of caseins in vitro, and their effect in the proteolysis process during ripening (Agboola et al. 2004; Brodelius et al. 1995; Esteves et al. 2001; Esteves et al. 2003; Faro et al. 1992; Ramalho-Santos et al. 1996; Roseiro et al. 2003a, b; Silva et al. 2003; Silva and Xavier Malcata 1998; Silva and

Malcata 1999, 2000, 2005; Sousa and Malcata 1997, 1998, 2002). Due to the potential of these cardoon enzymes to serve as alternative rennets in larger-scale production processes, several strategies have also been undertaken in more recent years to either develop more standardized formulations of the native enzymes or explore their production in heterologous systems to generate synthetic versions of these proteases (Almeida and Simões 2018).

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 (Planta 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).

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 *St*APs (*St*Asp-PSI) and found that the recombinant protein still maintained its cytotoxic activity (Muñoz et al. 2010). Both *St*APs and *St*Asp-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, *St*APs and *St*Asp-PSI could contribute to the generation of new tools to solve the growing problem of resistance to conventional antibiotics (Zasloff 2002).

1.3.3 Plant Serine Proteases

Serine proteases (SPs) use the active site Ser as a nucleophile. The catalytic mechanism is very similar to that of cysteine proteases, and some serine proteases are even evolutionarily related to cysteine proteases. With more than 200 members, serine proteases are the largest class of proteolytic enzymes in plants. Plant serine proteases are divided into 14 families. These families belong to nine clans that are evolutionarily unrelated to each other. Families S8, S9, S10, and S33 are the largest serine protease families in plants, with each containing approximately 60 members. Biological functions for serine proteases have been described for some of the subtilases (SDD1 and ALE1; family S8, clan SB), carboxypeptidases (BRS1 and SNG1/2; family S10, clan SC), and plastid-localized members of the S1, S26, and S14 families (DegPs, Plsp1, and ClpPs) (van der Hoorn 2008).

Feijoo-Siota and Villa (2011) have reviewed several origins to SPs. These enzymes 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 (*Nicotiana 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 (*Trichosanthes bracteata*), and yellow snake gourd (*Trichosanthes kirilowii*) (Antão and Malcata 2005).

SPs are usually considered to act principally as degradative enzymes. Plant subtilases (subtilisin-like SPs), however, have been documented to be involved in several physiological processes including symbiosis (Takeda et al. 2007), hypersensitive response, the infection of plant cells (Laplaze et al. 2000), pathogenesis in virus infected plants (Tornero et al. 1997), germination (Sutoh et al. 1999), signaling (Déry et al. 1998), tissue differentiation (Groover and Jones 1999), xylogenesis (Ye and Varner 1996), senescence (Distefano et al. 1999; Huffaker 1990), programmed cell death (Beers et al. 2000), and protein degradation/processing (Antão and Malcata 2005).

Cucumisin, an enzyme derived from melon, remains the best plant SP characterized to date (Antão and Malcata 2005), purified from *Cucumis melo* (Kaneda and Tominaga 1975). Plant subtilisins, also referred to as cucumisin-like proteases (in recognition of the first subtilisin isolated from a plant (Yamagata et al. 1994)), have been isolated from *Cucumis melo* (Yamagata et al. 1994), *Solanum lycopersicum* (Meichtry et al. 1999), *Alnus glutinosa* (Ribeiro et al. 1995), and *Arabidopsis* (Zhao et al. 2000). Cucumisin-like SPs have also been isolated from other sources, like the latex of *E. supina* (Arima et al. 2000a; Taylor et al. 1997), the sprouts of bamboo (*Pleioblastus hindsii* Nakai) (Arima et al. 2000b), and the fruits of *Melothria japonica* (Uchikoba et al. 2001). A cucumisin-like protease from kachri fruit (*Cucumis trigonus Roxburghi*) is used as a meat tenderizer in the Indian subcontinent (Asif-Ullah et al. 2006).

Macluralisin, from the fruits of *M. pomifera* (Raf.) Schneid (Rudenskaya et al. 1995), taraxilisin, from the latex of dandelion (*T. officinale Webb s. I.*) roots (Rudenskaya et al. 1998), SP A and B, from the sarcocarp of yellow snake gourd (*Trichosanthes kirilowii*) (Uchikoba et al. 1990), an SP from the seeds of tropical squash (*C. ficifolia*) (Dryjanski et al. 1990), and several other SPs from barley (Fontanini and Jones 2002), oat (Coffeen and Wolpert 2004), soybean (Tan-Wilson et al. 1996), and common bean (Popovič et al. 2002) are other SPs isolated from plants.

Some SPs have been studied for their medicinal properties (Andallu and Varadacharyulu 2003; Andallu et al. 2001; Doi et al. 2000; Jang et al. 2002; Andallu and Varadacharyulu 2003; Andallu et al. 2001; Doi et al. 2000; Jang et al. 2002), examples of these are the a subtilisin-like SP, named as indicain, isolated from the latex of *Morus indica* by Singh et al. (2008) and milin, an SP purified from the latex of *Euphorbia milii* (Yadav et al. 2006). Many of the medicinal applications have been proved by clinical studies (Asano et al. 2001; Cheon et al. 2000; Doi et al. 2001; Nomura 1999). Milin, another SP of plant origin, isolated from *E. milii*, is a good candidate for applications in the food industry (Souza et al. 1997; Schall et al. 2001).

1.3.4 Plant Metalloproteases

Metalloproteases contain catalytic metal ions that activate water for nucleophilic attack while stabilizing the oxyanion hole. Of the different types of proteases, metalloproteases are the most diverse in terms of both structure and function. More than 50 families have been identified in 24 clans. MPs in plants include exo- and endoproteases in many different subcellular locations, with degradative or highly specific processing function (Schaller 2004).

In plants, MPs are involved in nodulation, plastid differentiation, thermotolerance, regulation of root and shoot meristem size, sensitivity to auxin conjugates, and meiosis (Casamitjana-Martínez et al. 2003; Bartel and Fink 1995; Bölter et al. 2006; Chen et al. 2000, 2005, 2006; Combier et al. 2007; Davies et al. 1999; Golldack et al. 2002; Helliwell et al. 2001; Sakamoto et al. 2002; Sanchez-Moran et al. 2004; Sjögren et al. 2006).

Leucine aminopeptidases (LAPs) are ubiquitous MPs, thought to be involved in the regulation of protein half-life in plants, which is largely influenced by the N-terminal amino acid residue (Varshavsky 1996). In addition to the common LAP, which is constitutively present in all plant species (Bartling and Nosek 1994; Chao et al. 2000), some plants of the nightshade family (*Solanaceae*) have additional LAPs expressed in the reproductive organs upregulated under several stress conditions, including osmotic stress, wounding, and pathogen infection (Chao et al. 1999; Gu et al. 1999; Hildmann et al. 1992; Pautot et al. 1993, 2001; Schaller et al. 1995).

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteases belonging to the metzincin clan of metalloendopeptidases, EC subclass 3.4.24, MA (M) clan according to the MEROPS database (Rawlings et al. 2010). This family is characterized by a highly conserved catalytic domain containing an HEXXHXX GXX(H/D) zinc-binding sequence followed by a conserved methionine that forms a tight 1,4-β turn called "Met-turn" (Rawlings et al. 2014).

Members of this family have mainly been studied in mammals but have also been found in simpler animals and plants (Massova et al. 1998). Members of the MMP family have been also identified in plants, but only few of them have been characterized. Ragster and Chrispeels (1979) described the first MMP in higher plants, and ethylenediaminetetraacetic acid (EDTA)-sensitive Azocoll-degrading enzyme in leaves of soybean (*Glycine max*). This protein was purified and characterized only in 1991 and was named soybean metalloendoproteinase-1 (SMEP-1) because of structural and biochemical similarities to vertebrate MMPs (Graham et al. 1991; McGeehan et al. 1992; Pak et al. 1997). Other MMPs were then studied in *Arabidopsis* (Maidment et al. 1999; Golldack et al. 2002), cucumber (Delorme et al. 2000), *Medicago* (Combier et al. 2007), soybean (Liu et al. 2001), tobacco (Schiermeyer et al. 2009; Mandal et al. 2010), and loblolly pine (Ratnaparkhe et al. 2009). Members of the MMP family have been identified in plants, but only few of them have been characterized.

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Chapter 2
Milk-Clotting Plant Proteases for Cheesemaking

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Contents

2.1 Introduction

Cheesemaking represents one of the most important processes in modern dairy industry and a daily practice in familiar farms. Cheese is a basic food in the diet of many cultures, highly appreciated by its sensory attributes, nutritional characteristics, and bioactive components (López-Expósito et al. 2017). Transformation of milk into cheese is more than a simple process since it involves several complex physicochemical and biochemical steps, which will define the final characteristics of the different types of cheeses. Milk coagulation is the first step in cheesemaking where the enzymatic coagulation represents the most used mechanism in cheese

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production. Rennet from animal sources contains the chymosin enzyme as the main clotting agent. Several efforts have been put in the development of new, suitable chymosin substitutes to supply the demand of the increasing cheese production, counteracting the decay in its production from its natural source (Fox et al. 2017). Microbial proteases and recombinant chymosin have supplied in part this demand; however, due to ethical, religious, and cultural reasons, natural sources are preferred. Recently, special interest has focussed in the use of plant proteases as rennet substitute. The use of some plant proteases in the manufacture of artisanal cheeses has been successfully applied and gained acceptance leading to the valorization of some cheeses produced in some world regions (Shah et al. 2014; Ben Amira et al. 2017a). This chapter describes the milk-clotting properties of some plant coagulants used for cheese production at artisanal, experimental, and commercial scale in some world regions. Some technological aspects for the formulation and standardization of coagulants from plant origin and its impact in physicochemical, technological, and sensorial properties of curds and cheeses are also considered. Cheese production is in constant growth and it is expected that in the near future the use of plant proteases in cheesemaking will increase, producing new specialty cheese favoring the cheese market.

2.2 Rennet for Cheese Production

Milk provides a variety of functional compounds (protein, fat, carbohydrates, minerals, and vitamins) essential for human nutrition. Worldwide milk production was estimated in 750 million tons in 2016, mostly from cows (85%) and other significant quantities from buffalo, sheep, goat, and camel produced in countries such as India, Pakistan, Egypt, Iran, Italy, France, and Spain (IDF 2017). A great diversity of cheeses with distinctive flavors, texture, and forms are produced daily worldwide using these sources. The exact number of cheese varieties is unknown. However, around 80% of the global cheese production (estimated in 20 million tons) is from cow's milk (IDF 2017). Cheese from buffalo, goat, and sheep milk is almost limited to little farms at artisanal scale production. Transformation of milk to cheese is an important activity in the dairy industry with a constant annual growth of 3.5% in EU and American countries. This growth has been associated to an increase in global population, per capita incomes, eating trends, and lifestyles changes, in addition to the market development for cheese-based products and ingredients.

From the technical point of view, cheese production involves the separation of milk solids (mainly casein protein and fat) from whey. In most cases, proteolytic enzymes are added to milk for clotting, where caseins are separated as a coagulum (with fat trapped in the gel network) with a consequent whey release. Historically, the earliest cheese was accidentally produced around 5000 BC during milk transportation in bags made from stomachs of animals. Since then, natural rennet from calf abomasum (main source of chymosin) is the preferred milk-clotting agent due to its high efficiency to clot milk, resulting in an appropriate curd texture and high

yield of cheese produced. However, the increase in cheese production coupled with the decrease in chymosin supply from its natural source has increased the milk-clotting enzymes demand, incentivizing the screening of microbial and plant sources as calf rennet substitutes. Fermentative processes using selective fungal strains such as *Rhizomucor miehei*, *Rhizomucor pusillus,* and *Cryphonectria parasitica* have resulted adequate to produce proteases with high similarity in structure and catalytic properties of chymosin. In addition, genetically engineered strains of *Escherichia coli*, *Aspergillus niger*, and *Kluyveromyces lactis,* carrying the calf *Bos taurus* chymosin gene, have been successfully used to produce recombinant chymosin (fermentation-produced chymosin, FPC) (Andrén 2011). Nowadays, most cheeses (around 80%) produced worldwide use standardized FPC as the milk-clotting enzyme. However, plant proteases represent an attractive option and are recently emerging as new milk-clotting agents in cheesemaking. Political, religious, or cultural restrictions for the consumption of animal-derived ingredients or genetically engineered foods in some countries have incentivized the research and use of plant proteases (Roseiro et al. 2003). Plant origin coagulants in cheesemaking represent an artisanal practice in many regions of the world, in which knowledge is crossing frontiers and expanding their use in the production of specialty cheese.

2.3 Plant Origin Coagulants in Cheesemaking

Cheese production involves the use of chymosin and chymosin-like proteases for milk coagulation. Enzymatic coagulation is an essential step in most cheese manufacture where the casein component of the milk protein system forms a gel network that entraps fat. The bovine chymosin has been the preffered enzyme used for this purpose; however, proteases from animal, microbial and plant sources have also been used successfully. In this regard, plant proteases, obtained from different tissues including fruits, flowers, stems, latex, etc., possess attractive catalytic properties with diverse optimum conditions of pH and temperature to clot milk (Table 2.1). Flowers from cardoon plant species (*C. cardunculus* and *C. scolymus*) contain high amount of milk-clotting proteases (cardosins and cynarases) with similar catalytic properties to chymosin. They belong to the same group of chymosin (aspartic proteinase, EC. 3.4.23), so far considered the most suitable proteases for cheesemaking (Yegin and Dekker 2013). However, proteases from other catalytic groups are also considered as an attractive option to diversify the characteristics of cheeses produced. Cysteine proteases (EC. 3.4.22) such as papain, bromelain, zingibain, and actinidin from papaya, pineapple, ginger, and kiwi, respectively, and serine proteases (EC. 3.4.21) from melon (i.e., cucumisin),

Cucurbita ficifolia, and *Solanum dubium* are some examples of plant proteolytic enzymes with attractive properties for cheesemaking and food processing (Pardo et al. 2010; Mazorra-Manzano et al. 2013a). In Mediterranean countries, the use of plant coagulants represents a traditional practice for the production of artisanal cheeses, where extracts from cardoon flowers represent the most successful case.

Table 2.1 Physicochemical properties of plant proteases with milk-clotting activity

Plant source	Enzyme	Type	Tissue	Optimum conditions		Reference
				Temp (°C)	pH	
Asclepias curassavica	Asclepian cI, cII	CP	Latex	50.0	8.5	Liggieri et al. (2009)
Bromelia hieronymi	Hieronymain III	CP	Fruit	40	7.3–10.7	Bruno et al. (2008)
Bromelia pinguin	Pinguinain	CP	Fruit	20–70	7.2–8.8	Payrol et al. (2008)
Calotropis procera	Procerain Procerain B	CP	Latex	55 50	7.0–9.0 6.5–8.5	Kumar and Jagannadham (2003), Singh et al. (2010a)
Citrus aurantium	Citrus proteases	AP;CP;SP	Flower	65	4–9	Mazorra-Manzano et al. (2013b)
Crinum asiaticum	Crinumin	SP	Latex	70	8.5	Singh et al. (2010b)
Euphorbia milii	Milin	SP	Lx	60	8.0	Yadav and Jagannadham (2009)
Euphorbia nivulia	Nivulian-II	CP	Latex	50	6.3	Badgujar and Mahajan (2014)
Moringa oleifera		AP	Flower	70	NR	Pontual et al. (2012)
Morus indica	Indicain	SP	Latex	80	8.5	Singh et al. (2008)
Solanum dubium	Dubiumin	SP	Seed	70	11	Mohamed et al. (2009a)
Streblus asper	Streblin	AP	Stem	55	5.5	Tripathi et al. (2011)
Withania coagulans	WcAP	AP	Fruit	60	5.5	Salehi et al. (2017)
Zingiber officinale	Zingibain	CP	Rhizobium	60	5.0	Hashim et al. (2011)

Abbreviations: *AP* aspartic proteinase, *CP* cysteine protease, and *SP* serine protease

Traditional cheeses such as Serra da Estrela, Serpa, La Serena, and Torta del Casar are some examples of cheeses elaborated with cardoon flower extract; these are highly valuated products in Portugal and Spain that some have been protected with a denomination of origin (PDO). In Sudan, the berries from *Solanum dubium* has been used for many years to make white-soft goat and sheep cheeses, while in Mexico, the ripened berries from wild "trompillo" (*Solanum elaeagnifolium*) have been used for production of pasta filata cheese type, known as "Asadero" (Chávez-Garay et al. 2016).

2.3.1 Milk-Clotting Properties of Plant Proteases

Plant extracts, with enough amount of proteolytic enzymes, have the capacity to clot milk under optimum enzymatic activity conditions. Their milk-clotting enzymatic mechanism is initiated, in most cases, similar to chymosin, this is, by the hydrolysis of the Phe_{105}-Met_{106} peptide bond in the casein micelle-protective protein, κ-casein (κ-CN). This enzymatic reaction conduces to the release of the hydrophilic portion known as glycomacropeptide ($f_{106-169}$), located at the casein micelle surface, causing a decrease in electrostatic and steric repulsion forces between micelles, conducing to casein aggregation and clot formation (nonenzymatic phase) (Horne and Lucey 2017).

Milk-clotting activity (MCA) is an important parameter for the evaluation of enzymatic preparations used as coagulants in cheesemaking. The conventional MCA protocol includes the evaluation of rennet strength, referring to the amount of enzyme able to clot a volume of milk in 40 min at a reference temperature (32–35 °C) using standardized skim milk as substrate. Differences in optimum pH/temperature for activity among plant proteases require of a comparative evaluation with standardized chymosin, employing the same protocol. To evaluate the milk-clotting activity in of a new plant extract, this must be capable to transform milk into curd in suitable times (about 40–60 min) and have a comparable specificity to chymosin (Harboe et al. 2010). Under conventional cheesemaking process conditions (32–37 °C/pH 6.3–6.8), the milk-clotting performance of rennet candidate will depend of the amount, type of protease, specificity, and optimum conditions for its activity. Cysteine protease found in raw extracts from kiwifruit (*A. deliciosa*), ginger rhizome (*Z. officinale*), and aguama fruit (*B. pinguin*) presented appropriate specific MCA (2.7, 2.3, 2.59, and 1.5 U mg^{-1}, respectively). However, the MCA of these extract was temperature-dependent obtaining maximum activity at temperatures above 40 °C (Mazorra-Manzano et al. 2013a; Moreno-Hernández et al. 2017a). Other protease sources such as cardoon extracts (*C. cardunculus* and *C. scolymus*) presented higher MCA values (61 IMCU mL^{-1}) under standard temperature/pH conditions, increasing its activity at lower pH due to the presence of acidic (aspartic) proteolytic enzymes (Chazarra et al. 2007; García et al. 2014).

Table 2.2 describes the milk-clotting characteristics of some crude enzymatic preparations and partially purified proteases from plant sources. In contrast with pure enzymes, some crude extracts are complex in proteinase composition resulting in low specificity ratio (MCA/proteolytic activity). Therefore, the presence and activity of different proteases over milk caseins can affect the milk-clotting performance of plant rennet. Hydrolysis of caseins other than k-CN affects the cheese yield and functional properties of curd. Nonspecific hydrolytic reactions could result in excessive proteolysis; however, these characteristics could result attractive for some type of cheeses due to the development of cheese flavors and/or acceleration of cheese ripening (Roa et al. 1999; Delgado et al. 2010).

Table 2.2 Milk-clotting properties of plant proteases in comparison with chymosin

Plant source	Milk-clotting activity (MCA)	MCA/PA ratio	Reference
Raw extracts			
Cardoon flowers (*C. cardunculus*)	61 IMCU mL^{-1}	NA	García et al. (2014)
Kiwifruit mesocarp (*A. deliciosa*)	2.7 U mg^{-1}	5 (104)	Mazorra-Manzano et al. (2013a)
Rhizome (*Z. officinale*)	2.3 U mg^{-1}	3.2 (162)	
Melon mesocarp (*C. melo*)	1.5 U mg^{-1}	2.5 (208)	
Fruit mesocarp (*B. pinguin*)	2.59 U mg^{-1}	1.3 (162)	Moreno-Hernández et al. (2017b)
Berries (*S. elaeagnifolium*)	818 U mg^{-1} (whole)	584 (11.7)	Chávez-Garay et al. (2016)
	1089 U mg^{-1} (seeds)	837 (8.2)	
Berries (*W. coagulans*)	5.71 U mg^{-1}	47.6 (NA)	Salehi et al. (2017)
Latex (*C. procera*)	566 U mg^{-1}	1788 (1)	Freitas et al. (2016)
Purified enzymes			
Cardosin A (*C. cardunculus*)	1160 IMCU g^{-1}	NA	Silva et al. (2003)
Cardosin B (*C. cardunculus*)	7556 IMCU g^{-1}	NA	
Cynarase A (*C. scolymus*)	4651 IMCU g^{-1}	22.91 (25)	Sidrach et al. (2005), Chazarra et al. (2007)
Cynarase B (*C. scolymus*)	30,000 IMCU g^{-1}	22.42 (3.8)	
Cynarase C (*C. scolymus*)	43,000 IMCU g^{-1}	34.87 (2.7)	
Actinidin (*A. deliciosa*)	1 RU mg^{-1}	0.46 (10.2)	Grozdanovic et al. (2013)
GP-II (*Z. officinale*)	314 U mg^{-1}	1653 (2.1)	Hashim et al. (2011)
Cucumisin (*C. melo*)	117 TMCA	109 (NA)	Uchikoba and Kaneda (1996)
Dubiumin (*S. dubium*)	3520 U mg^{-1}	2490 (2)	Mohamed et al. (2009a)

Values in parenthesis for MCA/PA ratio represent a relative factor between chymosin and plant coagulant evaluated in the same study. *NA* not available

In addition to the type of protease and complexity of the enzymatic preparations, other factors such as enzyme concentration, pH, temperature, calcium ions, and salts, among others affect the milk-clotting process. Enzymatic milk coagulation (clot formation) is observed when approximately 70–80% of the κ-CN has been hydrolyzed. Typically, the milk-clotting time (Ct) shows linearity with the inverse of rennet activity (within a range of concentration) (McMahon and Brown 1984). Since temperature and pH affect the proteolytic activity, Ct will depend of the enzymes and reaction conditions. These factors strongly affect the interaction between rennet-destabilized micelles for transition to gel in the second phase of milk coagulation. Generally, Ct decreases if the temperature increases and/or the pH decreases (Chazarra et al. 2007; Beka et al. 2014). As milk clotting progresses, calcium ions are essential for the development of gel matrix (nonenzymatic phase). Therefore, addition of calcium chloride ($CaCl_2$) to milk (10–40 g $CaCl_2$ 100 kg^{-1}) is a common practice in cheesemaking. Its effect in gel behavior is associated with the reduction of repulsive forces between rennet-altered micelles promoting hydropho-

bic interactions (increasing the speed of curd formation) (Harboe et al. 2010; Freitas et al. 2016). All factors described above affect the milk-clotting properties of plant proteases similarly to chymosin and microbial proteases (Mohamed et al. 2010; García et al. 2014).

2.3.2 *Preference and Specificity of Plant Proteases Over Milk Proteins*

The use of proteolytic enzymes for milk coagulation represents the most common process in cheesemaking. The hydrolytic events that take place define the protease properties as coagulant and its potential as chymosin substitute. The direct observation of the casein protein profile after milk coagulation (by SDS-PAGE or Urea-PAGE) allows a rapid exploration of the preference of proteases over different caseins. Most studies have evidenced that plant proteases such as those found in *Centaurea calcitrapa*, *Citrus aurantium*, and *Bromelia penguin* extracts hydrolyze preferentially κ-CN in the Phe_{105}-Met_{106} peptide bond during early proteolytic phases, producing the amino *para*-kappacasein fragment ($F_{1\text{-}105}$ κ-CN) and the glycomacropeptide ($F_{106\text{-}169}$ κ-CN) (Tavaria et al. 1997; Mazorra-Manzano et al. 2013b; Moreno-Hernández et al. 2017a). Liquid chromatography-mass spectrometry analysis has also showed that proteases have a higher preference to cleavage this site; however, other peptide bonds in α_{s1}-CN, β-CN, and κ-CN can also be cleaved during the milk-clotting process. Cardosins A and B hydrolyze with high-preference hydrophobic regions such as Phe_{23}-Phe_{24}, Phe_{153}-Tyr_{154}, and Typ_{164}-Tyr_{165} in α_{s1}-CN, and Leu_{127}-Thr_{128}, Leu_{165}-Ser_{166}, and Leu_{192}-Tyr_{193} peptide bonds in β-CN. Minor differences between casein preparations from bovine, caprine, or ovine have been observed (Silva and Malcata 1999; Vairo et al. 2013). Figure 2.1 shows the cleavage preference of some plant proteases on peptide bonds of caseins.

The specificity of milk-clotting proteases, determined by its milk-clotting/proteolytic activity ratio (MCA/PA) (Table 2.2), is an important parameter that defines its potential use as chymosin substitute in cheesemaking. Proteases showing high values of MCA/PA (i.e., chymosin) are associated with high yield of curds, and good textural and flavor characteristics of cheeses. On the contrary, a low ratio results in weak curds, low yield (increases the loss of protein in whey), present a soft texture, and often produce bitter cheeses. Most plant rennets had showed lower MCA/PA ratios than chymosin. However, some plants such as *Cynara* sp. flowers, *Solanum* sp. berries, and *C. procera* latex have MCA/PA ratio values similar to chymosin (García et al. 2014; Chávez-Garay et al. 2016; Freitas et al. 2016). Crude extracts from kiwifruit, ginger rhizome, and melon mesocarp have MCA/PA ratios lower (104, 162, and 208 times, respectively) than calf rennet; however, formed curds still present good characteristics and rheological properties comparable to those obtained with chymosin (Mazorra-Manzano et al. 2013a). This parameter (MCA/PA ratio) improves when proteases used are partially purified (Uchikoba and Kaneda 1996; Hashim et al. 2011; Grozdanovic et al. 2013).

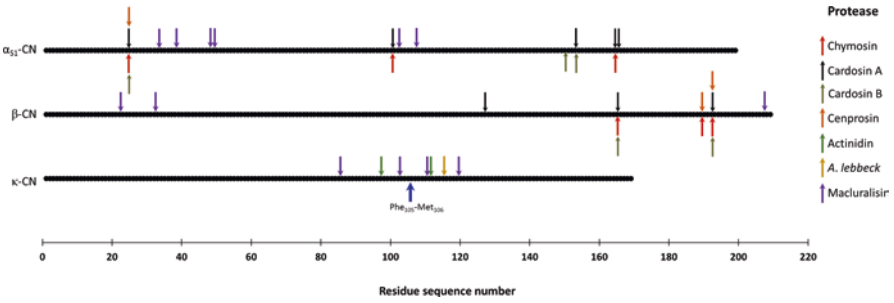

Fig. 2.1 Specificity of plant proteases toward milk caseins. Primary cleavage sites in α_{s1}-casein (α_{s1}-CN), β-caseins (β-CN), and κ-casein (κ-CN) by milk-clotting plant proteases. Most clotting proteases hydrolyze at high frequency the Phe_{105}-Met_{106} in κ-CN during early stages of coagulation. Arrows indicate susceptible bond to cleavage by chymosin on α_{s1}-CN (Phe_{24}-Phe_{25}, Arg_{100}-Leu_{101}, and Trp_{164}-Tyr_{165}), β-CN (Leu_{165}-Ser_{166}, Ala_{189}-Phe_{190}, and Leu_{192}-Tyr_{193}), and κ-CN (Phe_{105}-Met_{106}) (Moller et al. 2012); Cardosin A on α_{s1}-CN (Phe_{24}-Phe_{25}, Arg_{100}-Leu_{101}, Phe_{153}-Tyr_{154}, Trp_{164}-Tyr_{165}, and Tyr_{165}-Tyr_{166}), β-CN (Leu_{127}-Thr_{128}, Leu_{165}-Ser_{166}, and Leu_{192}-Tyr_{193}), and κ-CN (Phe_{105}-Met_{106}); Cardosin B on α_{s1}-CN (Phe_{24}-Phe_{25}, Phe_{150}-Arg_{151}, and Phe_{153}-Tyr_{154}), β-CN (Leu_{165}-Ser_{166} and Leu_{192}-Tyr_{193}), and κ-CN (Phe_{105}-Met_{106}) (Vairo et al. 2013); Cenprosin on α_{s1}-CN (Phe_{24}-Phe_{25}), β-CN (Ala_{189}-Phe_{190} and Leu_{192}-Tyr_{193}), and κ-CN (Phe_{105}-Met_{106}) (Tavaria et al. 1997); Actinidin on κ-CN (Arg_{97}-$Hist_{98}$, Phe_{105}-Met_{106}, Phe_{105}-Met_{106}, and Lys_{111}-Lys_{112}) (Lo Piero et al. 2011); *Albizia lebbeck* protease on κ-CN (Phe_{105}-Met_{106} and Asp_{115}-Lys_{116}) (Egito et al. 2007); Macluralisin on α_{s1}-CN (GLy_{33}-Lys_{34}, Asn_{38}-Glu_{39}, Ser_{48}-Thr_{49}, Thr_{49}-Glu_{50}, Lys_{102}-Lys_{103}, and Pro_{107}-Gln_{108}) β-CN (Ser_{22}-Ile_{23}, Lys_{32}-Phe_{33}, and Ile_{207}-Ile_{208}), and κ-CN (Ala_{85}-Lys_{86}, His_{102}-Leu_{103}, Phe_{105}-Met_{106}, Pro_{110}-Lys_{111}, and Ile_{119}-Pro_{120}) (Corrons et al. 2017)

2.3.3 Plant Origin Coagulants and Cheese Properties

Several systematic research on the curd properties, yield, and sensory quality of cheeses produced with plant proteases has been recently conducted (Ordiales et al. 2014; García et al. 2016; Wang et al. 2017). Plant protease activities over milk proteins have a significant effect on curd and cheese properties. A high rate of κ-casein hydrolysis results in low milk-clotting time. Long exposition to proteases can cause proteolytic degradation of the casein network, thus reducing approximately 0.3–0.7% the curd yield (Jacob et al. 2011). In addition, enzymatic preparations with high proteolytic activity can affect dramatically the yield and curd properties. Commonly, dynamic rheometry (dynamic oscillating shear deformation) is used to study the behavior of candidate "rennet substitute" during milk curd formation (Ben Amira et al. 2017a). Properties such as standard gelation time, gelation time rate, set-to-cut time, and gel hardness are determined. Temperature affects the curd formation through the regulation of enzyme activity and micelle hydrophobic interactions. Some plant coagulants show shorter gelation times in comparison with calf chymosin when clotting temperature increases. Confocal microscopy analysis has revealed that plant proteases clot milk better at low temperatures (by a mechanism where a gel network is better-interconnected and smaller pores are formed) than at high coagulation temperatures (Esteves et al. 2003). Higher clotting temperatures

(70 °C) cause over-proteolysis of the gel network, thus affecting its rheological properties and resulting in a poorly interconnected structure and curd firmness. A rheological analysis of curds elaborated with *Withania coagulans* proteases suggested that short clotting times do not necessarily correlate with good gel properties. Hence, temperature range and enzyme concentration are important parameters to consider in cheese manufacture (Beigomi et al. 2014).

After milk coagulation, the cutting time (Tc) results crucial to maximize protein and fat transfer from milk to casein curd, thus curd can have the right technological properties. Cutting the curd facilitates whey removal; however, cutting the curd too early can lead to the loss of fines (some milk solids), while late Tc affects whey removal and more water get trapped, yielding high-moisture cheeses. Besides, factors such as pH at draining, cooking temperature, and curd processing (press, salted) affect cheese yield (Jacob et al. 2010). The use of plant rennet from *Cynara* sp. showed similar gel building rates and higher curd stiffness than microbial rennet from *R. miehei* (Esteves et al. 2003; Zhao et al. 2004). However, texture profile analysis of miniature fresh curds, elaborated with proteases from kiwifruit, melon, and ginger, presented similar hardness and cohesiveness than those prepared with chymosin. However, cheese elaborated with plant coagulants presented lower curd yields (2–5% reduction) than chymosin (Mazorra-Manzano et al. 2013a). On the other side, proteases from *Dregea sinensis* stems, *Withania coagulans* berries, and kiwifruit *Actinidia chinensis* were adequate to produce high-quality mozzarella-type cheeses from buffalo milk. Although they presented similar physiochemical composition, cheese elaborated with plant coagulant presented a higher and complex presence of flavored compounds (Nawaz et al. 2011; Puglisi et al. 2014; Wang et al. 2017). Table 2.3 shows some characteristics of cheeses produced with plant proteases under commercial and experimental conditions.

2.3.4 Plant Proteases and Cheese Ripening

Enzymatic reactions occurring during cheese manufacture are complex but essential for the development of flavors in matured cheeses. Conversion of fresh curd into ripened cheese occurs by diverse biochemical reactions where proteolysis, lipolysis, and microbial fermentation are involved. Free amino acids and peptides are important in the development of cheese flavor, therefore protease action is an important factor for their release and subsequent flavor development. During cheese ripening, proteolytic events by the action of proteases from coagulants, microflora (starter culture and natural microbiota), and endogenous enzymes (e.g., plasmin and cathepsin D) take place. Residual coagulant in curd after whey drainage represents the major source of proteolytic enzymes in many cheeses. A proportion between 15 and 30% of clotting protease added to milk remains active in the casein gel. They also contribute to textural changes by breakdown of the protein network and flavor development during cheese storage (Visser 1993; Sousa et al. 2001). Besides, polypeptides and small peptides formed (depending of enzyme specificity) can

Table 2.3 Experimental and commercial cheeses made with plant coagulants

Cheese type	Country	Milk source	Plant coagulant	Production scale	Cheese properties	Reference
Asadero	Mexico	Cow	Powder or extracts from *Solanum elaeagnifolium* berries	Artisanal	Pasta filata-type, melt properties, tangy flavor, stringy texture, and minimal aged	Martínez-Ruiz et al. (2013), Chávez-Garay et al. (2016)
Caciofiore della Sibilla	Italy	Ewe	Raw extract from *Carlina acanthifolia* petioles	Artisanal	Soft texture, bitter flavor, and aromatic	Cardinali et al. (2016)
Casteloes	Portugal	Bovine	Raw extract from *Centaurea calcitrapa*	Experimental	Semisoft cheese, slightly bitter flavor, and clean taste	Reis et al. (2000)
Dangke	Indonesia	Cow	Raw latex from papaya (*Carica papaya*) tree	Artisanal	Yellowish-white, cottage-type, smooth surface, and free from cracks and mold	Rana et al. (2017)
Domiati	Egypt	Buffalo, cow and mixtures	Coagulant from Albizia *lebbeck* and Sunflower (*H. annuus*) seeds	Artisanal/ semi-industrial	Salty fresh-type, high-moisture, semisoft, and springy texture	Darwish (2016)
Fresco	Mexico	Cow	Kiwifruit (*Actinidia deliciosa*) extract	Experimental	Semisoft and creamy taste	Mazorra-Manzano et al. (2013a)
			Ginger rhizome extract	Experimental	Soft texture	
Fresco	Spain	Goat	Cardoon (*Cynara cardunculus*) extract	Experimental/ Semi-industrial	Vegetal odor, slightly hard texture, and bitter and salty with goat taste	Garcia et al. (2012)

(continued)

Table 2.3 (continued)

Cheese type	Country	Milk source	Plant coagulant	Production scale	Cheese properties	Reference
Jibna-beida	Sudan	Goat, sheep	Extract from *Solanum dobium* berries	Artisanal	White fresh-type, crumbly texture, and slightly bitter taste	Mohamed et al. (2009b), Yousif et al. (1996)
Manchego	Spain	Ewe, goat, cow, sheep, and mixtures	Coagulant powder from cardoon (*C. cardunculus*)	Experimental	Soft texture, intense bitter and creamy taste	Prados et al. (2006)
Mozzarella	Italy	Cow	Kiwifruit (*Actinidia chinensis*) extract	Experimental	Pasta filata-type, lack bitterness, and similar flavor of Mozzarella (PDO)	Puglisi et al. (2014)
Mozzarella	Pakistan	Buffalo	Raw extract from paneer booti (*Withania coagulans*) berries	Experimental	Pasta-filata type, fatty cheese, lightly bitter taste, and semihard texture	Nawaz et al. (2011)
Mozzarella	China	Buffalo	Raw extract from *Dregea sinensis* stem	Experimental	Compact, smooth, and uniform shape, semihard texture, butterfat flavor, and intense fruity-sweet aromas	Wang et al. (2017)
Nettle	Spain	Cow	Extract from fresh nettle leaves (*Urtica dioica*)	Experimental	Fresh-type, soft-spreadable and creamy texture, flavored and aromatic curd	Fiol et al. (2016)

(continued)

Table 2.3 (continued)

Cheese type	Country	Milk source	Plant coagulant	Production scale	Cheese properties	Reference
Peshawari	Pakistan	Cow, buffalo	Purified ginger rhizome protease (*Zingiber officinale*)	Experimental	Semihard texture	Hashim et al. (2011)
Serra da Estrela	Portugal	Ewe, sheep	Dried thistle flower from *Cynara cardunculus* or *C. humilis*	Artisanal/ Semi-industrial (PDO)	Yellow-white color, young age but soft-sliceable, solid at ripening, aromatic and flavored notes creamy consistency with slightly bitterness	Guiné et al. (2016)
Torta del Casar	Spain	Ewe, sheep	Dried flowers of *C. cardunculus*	Artisanal/ semi-industrial (PDO)	Soft-bodied cheese, slightly bitter and salty taste, ripened and creamy consistency	Ordiales et al. (2014)
Warankashi	Nigeria	Cow	Sodom apple leaf (*Calotropis procera*) or pawpaw (*Carica papaya*)	Artisanal	Soft-bodied cheese, sweet flavor	Hussein et al. (2016)

PDO protected by a denomination of origin

contribute to cheese flavor characteristics (although some peptides present bitter flavor). In addition, these protein fragments act as flavor precursors as they serve as substrates for microbiota metabolism during flavor formation (McSweeney 2004). Proteolytic activity of proteases is a catalytic parameter that will depend on the protein substrate used for its evaluation. The capacity of plant protease to degrade caseins determines its caseinolytic activity and represents an attractive feature to improve and accelerate cheese ripening. The susceptibility of caseins to the proteolytic action of different rennet affects the ripening process. The proteolytic action of residual cardoon (thistle) proteases on caseins during ripening of La Serena cheese has a stronger relationship with flavor development than prevailing microflora or native enzyme activity (Roa et al. 1999). Bovine and caprine caseins resulted more susceptible to the cardosin action than ovine caseins. Proteolysis and textural changes occurring during ripening of cheese elaborated with cardoon extracts results in softer and creamy cheeses than those obtained with chymosin

(O'Mahony et al. 2003; Pino et al. 2009; García et al. 2016). The high hydrolysis rate on α_{s-1} and β caseins conduces to the development of the cheese sensory characteristics and shorter ripening time. These important features of cardoon proteases had resulted essential in the development of the distinctive flavor in Torta del Casar and La Serena cheeses, a key characteristic for the Designation of their Origin Status (Sousa and Malcata 2002; Delgado et al. 2010; Ordiales et al. 2014).

2.4 Milk-Clotting Enzyme Preparations Based on Plants

The high concentration of proteases in some parts of plants has attracted the attention of the researcher community as well as the industrial and pharmaceutical sectors. The evaluation of extraction processes, purification, characterization, and stabilization of enzymatic preparations with potential use in cheesemaking has recently increased. The standardization and stabilization of enzymatic formulations in liquid or powder form is essential for their commercialization and industrial application. However, in addition to its natural abundance, other parameters have to be considered for its utilization. Generally, before the purification and standardization process, some proteases, even the animal calf rennet or recombinant chymosin, require additional steps for its activation or refolding steps to recover the enzyme in its functional form (Marston et al. 1984; Gasser et al. 2008). Most plant sources have proteases in its active form and can be easily extracted and isolated from different tissues (i.e., flowers, roots, fruits, stems, leaves, and latex). Most of the times, its direct application in cheesemaking is technologically feasible (Sun et al. 2016). However, factors such as variation in concentration, distribution in ecotypes, season, and physiological stage can affect their coagulant performance. Therefore, several strategies to standardize enzymatic preparations are considered. Figure 2.2 outlines the typical steps for analysis, purification, and characterization of milk-clotting plant preparations for cheesemaking.

2.5 Extraction and Concentration

In addition to proteases, plant tissues have diverse biological compounds like polyphenols, gums, polysaccharides, inhibitors, prooxidant metals, and non-proteolytic enzymes (i.e., phenoloxidases) that can interfere with protease activity and stability. Several strategies to improve proteolytic enzymes extraction, purification, and stabilization have been developed. The type of buffer used for extraction (e.g., chemical nature, pH, and ionic strength), temperature, and time of extraction, in addition to the biochemical properties of proteases, are some factors to be considered. Acetate or citrate buffers are commonly employed to improve activity and stability of aspartic proteinases (Mazorra-Manzano and Yada 2008; Beka et al. 2014), while Tris(hydroxymethyl)aminomethane buffer is frequently employed for the extraction

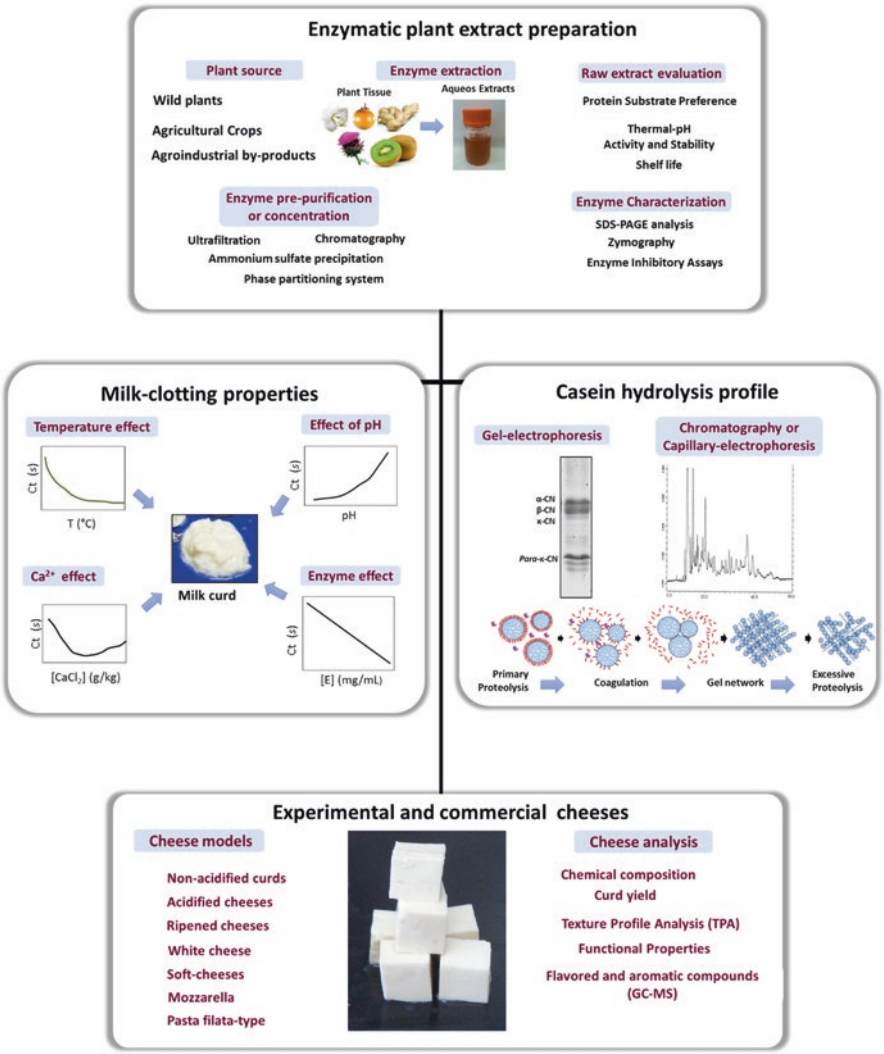

Fig. 2.2 Plant origin coagulants preparations, characterization, and application in cheesemaking process

of cysteine or serine proteases. Reducing agents like dithiothreitol (DTT) and cysteine are also important to stabilize cysteine protease activity. Other compounds like ethylendiaminetetraacetic acid (EDTA) and polyvinylpyrrolidone (PVP) as metal and polyphenol chelating agents, respectively, are used to prevent oxidation and tannin–protein aggregation during enzyme extraction (Priolo et al. 2000; Liggieri et al. 2009; Babazadeh et al. 2011). Crude cardoon coagulants prepared at different pH show significant changes in protein content and activity, producing skim milk gels with different firmness, rheological properties, and water holding capacity.

Surface response methodology indicated that grinding time and plant tissue-extraction solution ratio affected parameters such as MCA/PA ratio, coagulant efficiency, and viscoelastic properties of casein gels (Ben Amira et al. 2017b, c). Enzyme isolation from raw extracts requires the use of concentration and purification technics. In this way, ultrafiltration and ammonium sulfate precipitation for enzyme pre-concentration are commonly used. Pre-concentrated enzymes are further subjected to chromatographic methods including ion-exchange (anionic or cationic), size-exclusion, and affinity chromatography (Mahajan and Badgujar 2010; Huang et al. 2011; Tripathi et al. 2011; Kumari et al. 2012; Salehi et al. 2017).

Recently, a three-phase partitioning system (TPP) has been used for simple and quick enzyme recovery and purification. This non-chromatographic method is a versatile tool for one-step bioseparation of target proteins in complex mixtures. The procedure involves the mixing of crude plant extracts with ammonium sulfate $(NH_4)_2SO_4$) and an organic solvent, usually *tert*-butanol (*t*-BuOH). After centrifugation, enzymes or proteins are selectively partitioned as an interfacial precipitate between aqueous phase (rich in saccharides and polar compounds) and solvent layer rich in pigments, lipids, and other nonpolar compounds (Gagaoua et al. 2017). Recently, the use of a single TPP step has been employed successfully for recovery of proteolytic enzymes with milk-clotting properties, including zingibain, cucumisin, actinidin, calotropain, and ficin, with high purification factors (4 to 14-folds) and recovery yields (120–250%) (Gagaoua et al. 2014; Gagaoua et al. 2015; Gagaoua and Hafid 2016; Gagaoua et al. 2017).

2.6 Liquid and Powder Preparations

Artisanal cheese production represents an important proportion of cheeses marketed worldwide. The recent artisanal cheese valorization has motivated the development of new varieties and the standardization of processes for its industrial production. Several milk-clotting proteases extracted from some fresh or dried plant tissues are used directly for cheesemaking at artisanal level. Dried flowers of *C. cardunculus* and berries from *Solanum dubium* are some examples of milk-clotting proteases sources used to manufacture traditional ovine and bovine cheeses. On the other hand, liquid plant coagulants preparations for cheesemaking are also used. The extraction procedure from some plant sources is simple, of low cost, and suitable for its local distribution. Liquid enzymatic preparations distribute more homogeneously in milk to perform milk coagulation. However, they are more sensitive to thermal denaturalization, pH changes, proteolysis, and microbial spoilage. For this reason, several food additives and preservatives are added to extracts in order to avoid the loss of enzyme activity. Sodium chloride, pH buffering chemicals, and stabilizers (glycol, glycerol, and sorbitol) are some of the preservative substances added to extend their shelf life (Harboe et al. 2010). The knowledge of the optimum pH/temperature range and the effect of different salts and denaturant substances are key factors in formulation and stabilization of enzymatic preparations. Many

researches have documented the stability of plant proteases in high ionic strength buffers, surfactants (e.g., SDS and Tween-80), reducing agents, and organic solvents (methanol, ethanol, and acetone) (Tripathi et al. 2011; Prakash et al. 2012; Moreno-Hernández et al. 2017b).

On the other hand, coagulants in the form of powder are well suited for shipment at warm temperature and over long distances. Freeze-drying (lyophilization) of enzymatic liquid preparations improves enzyme stability and extends its shelf life. Freeze-drying causes minimal structural changes in enzymes and its activity is usually better preserved. Cardoon rennet (*C. cardunculus*) powder (by freeze-dryer) did not show significant changes in milk-clotting activity, improved its microbial quality, and was stable for one-year storage. Moreover, ewe cheeses made with powder vegetable coagulant showed similar organoleptic characteristics and textural attributes than those cheeses produced with fresh crude extracts (Fernández-Salguero et al. 2002; Tejada et al. 2007; Tejada et al. 2008). In addition, its powdered form offered a more standardized dosage for cheesemaking.

2.7 Conclusions, Trends in Future Research

The successful application of some plant proteases sources (e.g., cardoon flower, and *solanum dubium* and *S. elaeagnifolium* berries) as milk-clotting agents for cheesemaking has represented a traditional practice in some world regions such as in Mediterranean, Indian, and Latin America countries. The worldwide shortage of calf rennet and the increase in cheese production have increased the demand for new sources of coagulant enzymes, problem partially solved by the use of recombinant chymosin. Preference for natural coagulant sources will impulse the search for novel, natural, stable, and efficient coagulants. Trends in consumption of clean label (e.g., non-GMO and natural) foods warrant the attention toward the screening of efficient and stable milk-clotting enzymes from novel sources that meet industrial and market demand. The great availability and diversity of plant proteases sources with potential use in cheesemaking will lead to explore new strategies to reduce limitations associated with source availability, natural concentration variation (e.g., plant tissue culture), and stability under storage. The presence of proteases with limited specificity in some plant extracts will be resolved by its characterization and use of new purification strategies to obtain reproducible and stable preparations for successful cheese production. In addition, the screening of new plant sources by proteomics and genomic approaches will facilitate the identification of new promising sources of plant proteases with potential application in other biotechnological processes such as the production of protein hydrolysates with functional and/or bioactive properties.

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Chapter 3
Use of Plant Proteolytic Enzymes for Meat Processing

Thirawat Tantamacharik, Alan Carne, Dominic Agyei, John Birch, and Alaa El-Din Ahmed Bekhit

Contents

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3.1 Introduction

Meat scientists and industries are constantly searching for natural and environmentally friendly ways to improve the tenderness of their meat products. There is also a search for appropriate strategies for treating or valorizing protein wastes generated from meat-processing operations. A promising way to achieve both goals is by applying proteases obtained from plant sources. Plant proteases are preferred over proteases of microbial origin for many reasons such as: (1) plant proteases are functional over a broad range of temperature and pH; (2) there are less concerns surrounding possible toxicity or biohazard risk as their source of origin are often from readily consumable food materials; and (3) they may provide additional incomes for farmers. Therefore, with such specifications, plant-based proteases with properties suitable for applications in meat industries remain an interesting field of research. This chapter will provide an overview of discovered plant proteases and their applications in meat processing and meat waste utilization.

3.2 Meat Tenderization

Toughness in meat is a characteristic resulting primarily from the effect of connective tissue combined with interaction of myofibrillar proteins. The meat tenderization process involves the controlled limited hydrolysis of connective tissue proteins and the myofibrillar structure. Meat myofibrils are primarily composed of actin and myosin, and connective tissue is composed primarily of collagen and elastin (Bailey and Light 1989; Lawrie 1998). The relative proportion of these components determines the structural composition of a meat cut and therefore influences its tenderness. Consequently, disruption of these structural proteins leads to the tenderization of meat (Wheeler et al. 2000). Various methods have been evaluated for achieving the tenderization of meat. These approaches have been mainly directed to reducing the amount of intact connective tissue without causing extensive degradation of myofibrillar proteins. Treatment by proteolytic enzymes is one of the more popular methods of meat tenderization (Qihe et al. 2006). A variety of proteases that have different hydrolytic specificity are available from various plant species, as summarized in Fig. 3.1. Several of these proteases have not been fully evaluated in applications such as meat tenderizing.

Numerous studies have suggested that the most important quality feature of meat and determinant of eating satisfaction is tenderness. Achieving tenderness in tougher meat cuts is therefore the main challenge in terms of consumer acceptability (Jeremiah 1981; Miller et al. 2001). A number of reviews have suggested that the most common cause of unacceptability in beef, pork, and lamb was the toughness of the meat (Bekhit et al. 2017; Jeremiah 1982; Miller et al. 2001). In addition to that, Miller et al. (2001) found that approximately 78% of meat consumers in five cities across the USA are willing to pay a premium price for tender beefsteaks. Consumers also recognize the inherent tenderness variability in different meat cuts. Additionally, consumers tend to associate the differences in tenderness with

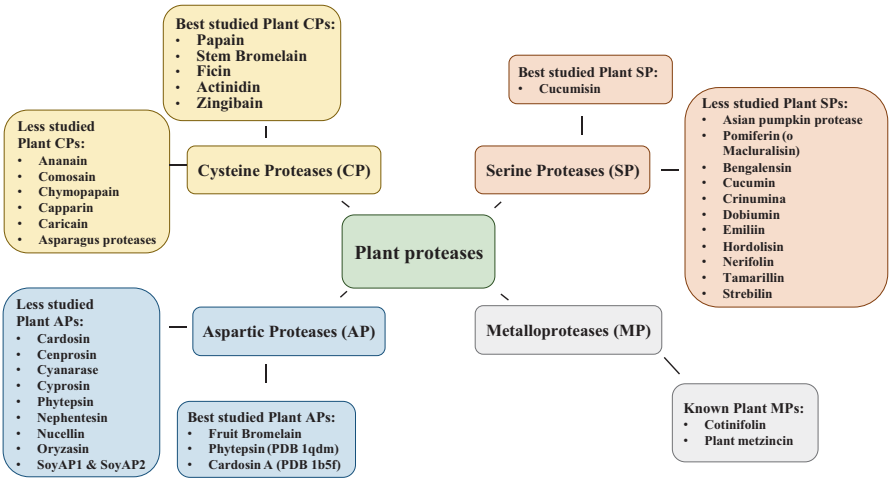

Fig. 3.1 Plant proteases, their classifications, and level of investigation in meat studies

price where the more tender the cut, the higher the price. Consumers also associate different cooking methods for different meat cuts as there is a wide variation in the texture of meat within a carcass that in part relates to the amount of connective tissue that is present. Polkinghorne et al. (2008) estimated that less than 10% of the meat of a carcass is considered to be prime grilling cuts that fetch a premium price. There is a considerable commercial difference between tender and less tender meat cuts. For example, a kilogram of beef fillet is currently sold in New Zealand for NZ$45–60/kg while a kilogram of beef silverside has a market value of NZ$10–12. Thus, increasing the tenderness of tougher meat cuts can increase the value of the remaining 90% of the carcass if the tenderness level can be improved through meat tenderizing interventions (Bekhit et al. 2017). Improving the tenderness of meat cuts and maintaining consistency in meat quality would result in attracting a larger consumer base, a higher retail price, and more frequent consumption (Feldkamp et al. 2005; Lusk et al. 2001; Platter et al. 2005; Polkinghorne et al. 2008). Therefore, the search for innovative meat tenderization techniques is of significant interest to the meat industry.

3.2.1 Factors Influencing Meat Texture

Inconsistency and variability in the texture of meat cuts are two of the major issues facing the meat industry. This is due to the wide biological diversity of skeletal muscle between animal species (Pette and Staron 1990). This difference in the structural and contractile properties of muscle tissue reflects the high degree of functional specialization that forms the basis of the structure, and functional plasticity and adaptability of muscle tissue (Sentandreu et al. 2002). Therefore, meat cuts

from different parts of an animal carcass, aside from differences relating to animal species, will respond differently to any postmortem aging process, which in part is controlled by the effect of endogenous proteases on the meat structure. Hence, any exogenous meat tenderizing strategy using proteases needs to be tailored to the characteristics of the particular meat cut.

3.2.2 The Mechanism of Meat Tenderization

The endogenous process postmortem of converting muscle into meat product is a three-step process: (1) pre-rigor phase, (2) rigor phase, and (3) tenderizing phase. The pre-rigor phase is the first stage after postmortem where the muscle remains excitable, depending on the duration of survival of the nervous system, and may last up to 30 minutes in beef (Chrystall and Devine 1985). During the rigor second phase, the cellular energy currency and energy storage compounds such as adenosine triphosphate (ATP), phosphocreatine (PC), muscle glycogen, etc., become depleted. The duration of this phase is highly variable, depending on meat storage conditions, species of animal, and muscle type. Upon rigor onset, the elasticity of the muscle is reduced and the toughness of the muscle increases. The tenderizing phase is dictated by the chilling conditions used, as well as biological variations between muscle group and animal species. The difference in the toughness of meat cuts is defined by the degree of crosslinking of collagen in the connective tissue which defines the background toughness (Sentandreu et al. 2002). Traditionally, two factors that influence the tenderness of cooked meat are the cooking method used and the humidity during the cooking process, which affects the extent of conversion of collagen to gelatin (Sentandreu et al. 2002).

Animal muscles contain endogenous proteases and protease inhibitors that have important roles in the maintenance and development of muscle tissue in the live animal. The hydrolytic capability of these endogenous proteases has been utilized during traditional aging of muscle postmortem in the process of converting muscle to meat. The levels of endogenous protease and protease inhibitors control the extent to which a meat cut is tenderized during the aging process.

The main endogenous proteases in muscle such as calpains are thought to be responsible for the tenderization of meat during postmortem (Bernard et al. 2007; Goll et al. 2003; Koohmaraie and Geesink 2006; Neath et al. 2007; Nowak 2011). Calpains are a subfamily of 14 calcium-dependent cysteine proteases, whose physiological role in muscle is to break down myofibrillar proteins (tropomyosin, troponin I, troponin T, C-protein, connectin (titin), and desmin) as part of the maintenance and remodelling of muscle tissue in the live animal. Postmortem meat tenderness is influenced by calpain I (μ) and calpain II (m) as well as the activity of calpastatin, which act as a calpain inhibitor (Goll et al. 2003; Koohmaraie and Geesink 2006; Moldoveanu et al. 2008). The majority of μ calpain resides in the Z-line (~66%), while the rest is situated in the I-band (~20%) and the A-band (~14%) (Nowak 2011). Calpain II is also mainly (~52%) situated in the Z-line, and about 27% and

21% are found in the I-band and A band regions of the sarcomere, respectively (Koohmaraie 1994; Kumamoto et al. 1992). The proteolytic activity of endogenous calpain in muscle is modulated by an inhibitor, calpastatin, which forms a complex with calpain. Calpastatin has a molecular mass of ~60–70 kDa and is comprised of four inhibitory domains (Maki et al. 1987; Nowak 2011). There are three regions (A, B, and C) within these domains. Besides the inhibitory activity of calpastatin region B, regions A and C were found to be capable of activating calpain I and calpain II (Tompa et al. 2002). Calpastatin can be inactivated by heat treatment during the cooking process. However, they are unable to inhibit the activity of other exogenous proteases such as trypsin, chymotrypsin, and papain (Nishiura et al. 1978). Apart from calpastatin, little is known about other endogenous inhibitors of other peptidases present such as the cystatins (a family of cysteine peptidase inhibitors) (Ouali and Talmant 1990; Shackelford et al. 1991), and serine peptidase inhibitors (Ouali 1999; Zamora et al. 1996) in skeletal muscle cells. As the concentration of endogenous proteases in muscle varies between animal species, this will affect the degree of tenderness achieved by these endogenous proteases during traditional aging of meat (Chéret et al. 2007; Gerelt et al. 2005).

3.3 Waste Generated from Meat Industries

Industrial processing of livestock and poultry generates a large amount of by-products such as blood, hair, wool, feather, skin, beak, horn, nail, hoof, bone (containing collagen), meat trimmings, and internal organs. These by-products are either disposed of by landfill or incinerated, or utilized in various ways mainly as animal feed and production of glue. Meat-processing waste was ranked as the second largest waste category in commercial and industrial food in New Zealand waste streams after dairy product waste, with an estimate of 17,182 tons generated in 2011 (Reynolds et al. 2016).

Some of this waste material is disposed of in landfill or by incineration which has potential damaging effects on the environment. As a result, meat industries are incurring considerable disposal costs and are missing out on potential revenue streams that could be generated by better utilization of this waste.

Some of this meat waste is being converted into relatively low-value by-products such as animal feed, organic fertilizers, and pet foods (Adel et al. 2002). There is also the possibility to utilize the protein component of this waste, including production of protein hydrolysates, that can be utilized in higher value-added products (Gbogouri et al. 2004). Protein hydrolysates have been shown to have enhanced functional properties such as increased solubility, fat absorption, foaming stabilization, and emulsifying properties that can contribute valuable characteristics to processed food products (Klompong et al. 2007).

Hydrolysates of protein containing animal by-products are reported to have potential health-enhancing effects and therefore are finding uses as nutraceutical ingredients in food and pharmaceutical formulations (Khan et al. 2011; Lasekan

et al. 2013; Toldrá et al. 2012). Antioxidant, antimicrobial, and antihypertensive (ACE inhibitor) activities are the most extensively studied functional properties of bioactive peptides from meats and meat-processing by-products.

In addition, protein hydrolysates derived from meat waste can be used as a nutritional source for patients who are not able to digest intact protein. These protein hydrolysates also have potential application in food products as flavorings, as well as presenting a considerable source of essential amino acids (Martínez-Alvarez et al. 2015; van Boekel 2006). For this reason, the flavor industry is interested in finding novel protein hydrolysates generated from cheap and abundant underutilized protein sources which may be used as a supplement to develop new flavors.

In addition, such protein hydrolysates can be used to provide an essential source of nitrogen in large-scale biotechnology cultivation of microorganisms to produce high-demand useful industrial products such as antibiotics, organic acids, and enzymes (Brandelli and Riffel 2005). A common ingredient in commercial growth media is peptone which provides the essential carbon and nitrogen source for the microorganism. All of these properties make animal protein hydrolysates of interest for manufacture of products for human consumption or for animal feed.

However, in the 1990s, the outbreak of bovine spongiform encephalopathy (BSE) in Europe coupled with the link established between consumption of BSE-infected meat and the development of Creutzfeldt–Jakob disease led to a sharp decline and/or ban in the use of slaughterhouse waste material as a food ingredient. Meat waste was considered to be a possible transmission pathway for the above mentioned disease. In addition, legislations in some countries does not allow animal waste disposal in landfill. Although BSE has been eliminated in most countries, the use of animal by-products directly as animal feed ingredients is currently legally restricted. In Europe, only third category by-products (EC Regulation No. 1069/2009, Article 33) can be utilized after appropriate technological processing to produce low value-added products such as animal feeds, silages, and fertilizers, etc., or derived products of greater added value in cosmetic, sanitary, or veterinary medicinal products. In the USA, the use of animal by-products is also restricted for various purposes such as food, medicinal, pharmaceutical, and cosmetic purpose (Liu 2002; Martínez-Alvarez et al. 2015).

Worldwide, it is estimated that approximately 60 million tonnes of animal by-products (Hamilton and Consultation 2004) are being processed by the rendering industry each year, of which ~42% are processed in North America, ~25% in the European Union, and ~17% are processed in Argentina, Australia, Brazil, and New Zealand combined (Martínez-Alvarez et al. 2015). Data on the amount of animal by-product waste derived from slaughter and processing fluctuate but are estimated at 33–43% (w/w) of the live animal weight (Hamilton and Consultation 2004). Meeker (2009) reported that the amount of by-products derived from slaughter and processing of cattle, pigs, and broilers may be up to about 49%, 44%, and 37% of the live animal weight, respectively. In the specific case of chickens, blood and feather accounts for 2–6% and up to 10% of the total bird weight, respectively (Meeker 2009).

As indicated above, protein by-products from meat industries are being generated in considerable quantity each year by meat-processing industries. These by-products present a remarkable resource of underutilized protein. By breaking

down or modifying their structure and converting them into hydrolysates using proteases, a range of biological activities and functional properties are generated that have considerable potential for utilization in a range of products. The potential of many plant proteases has not been fully evaluated for application in meat tenderizing or for the processing of meat waste. While some plant proteases have been evaluated for meat tenderization, it may be that plant proteases will have better application with processing of meat waste.

3.4 Plant Proteases: Sources and Classifications

Proteases have been isolated from a variety of plants and documented in the literature, as summarized in Fig. 3.1. The most well-studied plant proteases include papain from papaya latex, bromelain from pineapple stem, ficin from fig fruit, actinidin from kiwifruit, and zingibain from ginger rhizome and are currently the most well-studied plant proteases. Other plant sources such as asparagus (Ha et al. 2013; Yonezawa et al. 1998) and tamarillin from tamarillo (Li et al. 2018a, b) contain new emerging proteases which have not been well studied and may have potential useful applications in meat-processing industry. Plant proteases can be categorized according to their catalytic mechanism. Several of the plant proteases are thiol proteases (CP), having cysteine in the active site, and are the most studied and commonly used. In addition, serine (SP), aspartic (AP), and metallo (MP) proteases are represented among the plant proteases. Although a wide range of plant proteases have been documented in the literature, several of them have not been given an appropriate EC classification such as asparagus proteases (Ha et al. 2013).

3.4.1 Plant Cysteine and Serine Proteases

Papain (EC 3.4.22.2), bromelain (EC 3.4.22.32), and ficin (EC 3.4.22.3) are the most well-known plant cysteine proteases (CP) used in food processing, pharmaceutics, and other industrial processes. These three enzymes accounted for approximately 5% of the global sales of commercial proteases in 2008 (Illanes 2008). This is in part because these proteases are highly abundant and obtained from relatively accessible plant material sources. Two other CPs that have emerging potential are actinidin (also known as kiwillin) and zingibain (EC 3.4.22.67) (Choi et al. 1999; Ha et al. 2012; Teh et al. 2016). Cysteine proteases are more abundant in plants even though the total number of serine proteases (SPs) known is greater across all of the plant genomes (Schaller 2004). Some *Cucurbitaceae* species contain significant serine protease activity and could represent up to 50% of the total protein extracted from fruits and latex (Antão and Malcata 2005; Sharma et al. 2009). Cucumisin (EC 3.4.21.25) from melon *Cucumis melo* fruit is the best known

of the plant SPs (Antão and Malcata 2005; Arima et al. 2013). Other SPs identified in plants include pomiferin (or macluralisin) from *Maclura pomifera* latex (Corrons et al. 2012), SPs from Asian pumpkin (Babij et al. 2014), bengalensin from *Ficus benghalensis* (Sharma et al. 2009), cucumin from *Cucumis trigonus* Roxb (Asif-Ullah et al. 2006), dobiumin from *Solanum dobium* (Ahmed et al. 2009), hordolisin from barley *Hordeum vulgare* (Terp et al. 2000), crinumin from *Crinum asiaticum* (Singh et al. 2010), nerifolin from *Euphorbia neriifolia* L. (Yadav et al. 2012), tamarillin from tamarillo or tree tomato (*Cyphomandra betacea* (F)) (Li et al. 2018b), streblin from *Streblus asper* (Tripathi et al. 2011), and others (Antão and Malcata 2005; Domsalla and Melzig 2008). In comparison with cysteine proteases, serine proteases do not require the use of reductants and chelating agents for their hydrolytic reaction. Moreover, serine proteases were also found to have high stability under elevated temperature and the presence of oxidizing agents and the surfactants (Ahmed et al. 2009).

3.4.2 Plant Aspartic Proteases

The only plant APs that have been characterized and their three-dimensional structures analyzed are phytepsin (PDB 1qdm) and cardosin A (PDB 1b5f) from barley *Hordeum vulgare* L. and *Cynara cardunculus*, respectively (Frazão et al. 1999; Kervinen et al. 1999). These enzymes are heterodimeric with different conserved catalytic motifs: Asp-Thr-Gly (DTG) and Asp-Ser-Gly (DSG) at the substrate binding cleft, that differ from the characteristic animal and microbial catalytic motifs DTG/DTG counterpart (Faro and Gal 2005; Rawlings et al. 2013). The general name "phytepsins" (EC 3.4.23.40) has been adopted by the Enzyme Commission (EC) of the International Union of Biochemistry and Molecular biology (IUBMB) for all typical APs. However, the name of the species or tissue from which each enzyme has been purified is used as the basis for naming the extracted enzymes (Cavalli et al. 2013). The phytepsins (family A1), include all the typical Aps, including the A1, A2, and A3 protease isoforms from *Arabidopsis thaliana* (Chen et al. 2002), the cardosins and cyprosins from *Cynara cardunculus* (Pissarra et al. 2007; Sarmento et al. 2009), the cenprosins from *Centaurea calcitrapa* (Domingos et al. 2000), cirsin from *Cirsium vulgare* (Lufrano et al. 2012), oryzasin from rice *Oryza sativa* (Asakura et al. 1995), the AP1 and AP2 isoforms from soy *Glycine max* (Terauchi et al. 2006), and others. The plant APs grouped in subfamily A1B are atypical. Examples in this family include the constitutive disease resistance CDR1 and PCS1, which promotes cell survival, proteases from Arabidopsis, as well as the nucellin-like proteinases such as nephentesin from the insectivorous plant Nepenthes, and nucellin from *Oryza sativa*, respectively (An et al. 2002; Faro and Gal 2005; Ge et al. 2005; Xia et al. 2004).

3.4.3 Plant Metalloproteases

Among all plant proteases, the metalloproteases (MPs) are the least characterized (Sun et al. 2007). However, the proteolytic activity of plant MPs has been detected in several sources such as wheat, buckwheat seed, pea seeds, germinated maize, sugarcane, soybean leaves, sorghum, and *Arabidopsis thaliana* (Macedo et al. 1999; Marino et al. 2014; Ramakrishna et al. 2010; Ramos and Selistre-de-Araujo 2001). The matrix metalloproteases (MMP) family belonging to the metzincins clan have a dependence on metal ion cofactors, generally zinc (Zn^{2+}) (Marino and Funk 2012; Marino et al. 2014). Although many plant MPs have been discovered, the use of plant MPs is still limited due to a lack of their characterization. The potential abundance and knowledge of the catalytic properties of cotinifolin from *Euphorbia cotinifolia,* a native broadleaf red shrub from Mexico and South America, opens the possibilities to explore the utilization of MPs in biotechnology and industrial applications in the near future (Kumar et al. 2011).

3.5 Applications of Plant Proteases in Meat Tenderization

The process of meat tenderization involves essentially a limited proteolysis of higher order protein structures in meat. The endogenous proteases present in meat are responsible for the tenderization effect that is achieved during the aging of meat postmortem. There is the potential for adding exogenous proteases from different sources to achieve additional tenderization (Lantto et al. 2009). Studies have shown that connective tissues and muscle proteins can be digested by exogenous proteases (Abdel-Naeem and Mohamed 2016; Grzonka et al. 2007). Although there are a wide variety of proteases available from plants, bacteria, and fungi, the plant proteases have been the most studied. Approximately, 95% of the commercial proteases used in the USA are plant-derived. Microbial-derived tenderizers have not been used widely due to the preference for proteases sourced from readily consumable products from plants (Ionescu et al. 2008a, b). Several plant proteases have been documented in the literature to have the capability of hydrolyzing muscle proteins. Examples of plant proteases are summarized in Fig. 3.1. The most commonly used proteases for commercial meat tenderization are papain, bromelain, ficin, and actinidin (Abdel-Naeem and Mohamed 2016). Reports by Garg and Mendiratta (2006) and Naveena et al. (2004) also suggested that proteases sourced from ginger rhizome (Zingibain) and fruits of *Cucumis trigonus* Roxb (Cucumin) plant were effective in tenderizing meat products. The pH, temperature, and hydrolysis capability of well-studied plant proteases used in proteolytic degradation of myofibrillar proteins and collagen are summarized in Table 3.1.

Although, some plant proteases have been demonstrated to have relatively broad substrate specificity as well as exceptional hydrolytic capability, the extent

Table 3.1 A summary of the most commonly studied plant proteases and their properties

Plant proteases	EC	Source	Range of active temperature and pH	Optimal temperature and pH	Relative degree of hydrolysis of myofibrillar	Relative degree of hydrolysis of collagen	References
Papain	3.4.22.2	Latex of papaya also known as pawpaw (*Carica papaya*)	50–80 °C, pH 4.0–9.0	65–75 °C, pH 6.0–7.0	High	Moderate	Arshad et al. (2016), Bekhit et al. (2017), Calkins and Sullivan (2007), Kilara et al. (1977), Smith and Hong-Shum (2011)
Bromelain	3.4.22.32 (stem) 3.4.22.33 (fruit)	Fruit, leaves, and stems of the *Bromeliacea* family, of which pineapple (*Ananas comosus*) is the most commonly studied	50–80 °C, pH 5.0–8.5	50–60 °C, pH 6.0–8.5	Moderate	High	Arshad et al. (2016), Bekhit et al. (2017), Calkins and Sullivan (2007), Smith and Hong-Shum (2011)
Ficin	3.4.22.3	Latex from trees and fruit of the genus *Ficus*	45–75 °C, pH 5.0–9.0	45–55 °C, pH 5.0–8.0	Moderate	High	Arshad et al. (2016), Bekhit et al. (2017), Calkins and Sullivan (2007)
Actinidin	3.4.22.14	Kiwi fruit also known as gooseberry	40–60 °C, pH 4.5–8.5	58–62 °C, pH 7–8.5	Mild	Mild	Bekhit et al. (2017), Dufour (1988), Eshamah et al. (2014), McDowall (1970)
Zingibain	3.4.22.67	Ginger rhizome (*Zingiber officinale)*	45–70 °C, pH 4.5–8.0	60–70 °C, pH 6.0–7.0	Mild	Moderate	Adulyatham and Owusu-Apenten (2005), Bekhit et al. (2017), Ha et al. (2012), Kim et al. (2007), Pawar et al. (2007), Thompson et al. (1973)

of proteolysis can be difficult to control, and the proteases often excessively hydrolyze a protein substrate leading to an undesirable, overly proteolyzed product (Schaller 2004). The results from a study conducted by Ha et al. (2012) showed considerable differences in protease activity depending on the substrate used for assay and that protease assays with connective tissue and meat myofibril extracts provide a realistic evaluation of the potential of a protease for application in meat tenderization. The actinidin protease preparation was found to be most effective at hydrolyzing beef myofibril proteins due to mild hydrolytic capability and the zingibain protease preparation was found to the most effective at hydrolyzing connective tissue proteins. This indicates the potential uses of these plant-derived proteases for meat tenderization applications, as opposed to the challenge of trying to control the degree of meat tenderization with the more active papain and bromelain proteases. Another additional consideration with plant proteases is that the reliability of the protease source is governed by several factors such as variation in climatic conditions and the availability of land for plant growth (Rao et al. 1998).

3.5.1 Papain

Papain (EC 3.4.22.2) (molecular weight of 23.4 kDa) is a cysteine protease of the peptidase C1 family. It is sourced from the papaya plant (*Carica papaya*), particularly from papaya fruit latex. The functional role of papain in plant physiology is thought to provide protection for the plants against insects (Konno et al. 2004). The three-dimensional structure of papain was determined by Kamphuis et al. (1984). Papain has optimum activity at approximately 65 °C and pH 6.0–7.0, depending on the substrate (Kilara et al. 1977; Smith and Hong-Shum 2011). Papain has been shown to have a broad spectrum of proteolytic activity over a relatively wide range of pH (5.0–8.0) and retains activity above 65 °C (Smith and Hong-Shum 2011). A study by Berger and Schechter (1970) showed that papain has a specificity for amino acids with aromatic side chains such as Phe and Tyr at the P2 position. Synthetic peptides and inhibitors were used to map the active sites of papain. Within the active site, Cys25 and His159 are the two essential residues for the protease activity (Bekhit et al. 2014).

Papain has a high thermal and pressure stability which requires intense process conditions for adequate inactivation, as to achieve 95% inactivation of papain, 22 min processing at 900 MPa and 80 °C was required (Arshad et al. 2016; Maróstica and Pastore 2010). Papain has been used in the meat industry as a tenderizer owing to its proteolytic effect and capability to hydrolyze myofibrillar proteins. Additionally, the hydrolytic capability of papain is enhanced with heat-denatured collagen. Papain has therefore been applied to meat cuts from older animals as when the meat reaches higher temperatures during cooking, the highly cross-linked collagen characteristic of older animals shrinks and begins to convert to gelatin and is more susceptible to hydrolysis by papain (Wilson et al. 1992). For many years,

papain has been used to breakdown tough fibers. However, papain proteolysis can be challenging to control and tends to over-hydrolyze meat due to over-proteolysis of myosin which gives rise to a "mushy and/or grainy" texture in the tenderized meat. This has limited the use of papain as a commercial meat tenderizer.

3.5.2 Bromelain

Bromelain or bromelin comprises a group of endopeptidases present in the leaves, stems, and root, and in high abundance in the fruit of the *Bromeliacea* family. Of the *Bromeliacea* family, bromelain from the pineapple plant (*Ananas comosus*) is the best-known and well-studied member. Fruit and stem bromelain are immunologically different where stem bromelains (EC 3.4.22.32) are cysteine endopeptidases, whereas fruit bromelains (EC 3.4.22.33) are aspartic endopeptidases. Crude extracts of stem bromelain (EC 3.4.22.32) contain a mixture of other minor cysteine endopeptidases such as ananain and comosain. Bromelain is a glycosylated single-chain protein with a molecular weight of 24.5 kDa. Its structure consists of 212 amino acid residues and includes seven cysteines, of which one is involved in catalysis. The remaining six cysteines form three disulfide bridges. Purified bromelain is stable when stored at −20 °C; it has an optimum activity at pH 6–8.5 and within a temperature range of 50–60 °C. Although the structures of the bromelains are similar, in that they are both single-chain glycosylated proteins with similar molecular weights (fruit bromelain MW = 25 kDa), fruit bromelain has a much higher proteolytic activity and a broader specificity for peptide bonds in comparison to stem bromelain (Kim and Taub 1991). The hydrolytic specificity of bromelain is slightly less than that of papain as has been determined by proteolysis of synthetic peptides at pH 5.0–7.0 and optimal temperature of 50 °C (Smith and Hong-Shum 2011). While only two main proteases have been detected in pineapple fruit, up to four protease components have been separated by chromatography of the crude extracts of pineapple stem (Rowan et al. 1990). The catalytic activity of most of these proteases has been extensively investigated in multiple studies using various synthetic peptides (Inagami and Murachi 1963; Napper et al. 1994; Rowan et al. 1990). Results from these studies have shown that although the proteases are related, they are distinctive in terms of hydrolytic specificity and have small sequence differences (Lee et al. 1997). Bromelain is important for tenderization of meat in industries with controlled environment and is useful for assurance of the microbiological quality and purity. Like other proteases, bromelain degrades myofibrillar proteins and collagen, often resulting in over-tenderization of meat (Melendo et al. 1996). Ionescu et al. (2008a, b) investigated the use of bromelain with adult beef cuts, with the best results being achieved using 10 mg protease/100 g meat, with a tenderization time of 24 h at 4 °C, followed by thermal inactivation at a rate of 1 °C/min to 70 °C.

3.5.3 Ficin

Ficin (EC 3.4.22.3) is a class of cysteine (with thiol functional group) or sulfhydryl proteases from the genus *Ficus.* Ficin is a well-known plant protease used in meat tenderization (Maróstica and Pastore 2010) as well as an enhancer of the solubility of muscle proteins (Ramezani et al. 2003). Crude ficin extracts have been shown to contain 10 proteases (Kramer and Whitaker 1964). These proteases have different properties, including molecular weight and conditions for optimum activity. The most extensively studied ficins are the cysteine endopeptidases found in the latex of *Ficus glabrata* and *Ficus carica.* Proteases from other species sourced from the latex of *Ficus glabrata*, *Ficus anthelmintica*, and *Ficus laurifolia* are less well known. In 2008, it was shown that ficin obtained from *F. racemose* has a molecular weight of 44.5 kDa and showed a maximum activity in the optimal pH range of 4.5–6.5 at 60 °C (Arshad et al. 2016; Maróstica and Pastore 2010). Ficin protease had optimal activity over a range of pH 5–8 and temperature 45–55 °C making it suitable for fresh meat processing. Previous studies on ficin observed that the pH is dependent on the substrate concentration and has a half-life at 60 °C of 1.5 h (Kramer and Whitaker 1964). These properties make ficins a beneficial class of plant protease for use in meat tenderization.

3.5.4 Actinidin

Actinidin (EC 3.4.22.14, also known as actinidain or actinidia anionic protease) is a novel sulfhydryl or cysteine protease extracted from kiwifruit. The *Actinidia deliciosa* variety is the most commonly used plant source for actinidin. It also belongs to the papain family due to its sequence and structural similarities with papain proteases (Baker 1980; Carne and Moore 1978; Kamphuis et al. 1985). Actinidin has been fractionated by ion exchange chromatography into six isoforms of similar molecular weight of 23.5 kDa with isoelectric points (pIs) ranging from 3.9 to 9.3, and the acidic isoforms being the more prominent species (Nieuwenhuizen et al. 2007; Sugiyama et al. 1996). The specific activity of actinidin has been well characterized and shows a wide substrate specificity and pH range (4.5–6.0) (McDowall 1970) with the optimal temperature being 58–62 °C (Dufour 1988). Despite the striking similarity in structural homology, the specificity of actinidin is somewhat different to that of papain. Substrates with aromatic N-substituents had higher Km values for actinidin in comparison with papain, indicating differences in substrate–enzyme affinity between the two proteases. This variance was ascribed to amino acid differences within the active site of actinidin, especially the substitution of Ser 205 in papain with a Met in actinidin (Met 211), which makes the hydrophobic pocket at the S2 subsite notably shorter. It has a molecular weight of 32 kDa. Actinidin is used commercially in the meat industry to tenderize meat (Varughese et al. 1992) and enhance the chemical processes related to degradation of the myofibrillar proteins into peptides. It is also involved in the activation of m-calpain throughout postmortem aging (Ha et al. 2012). Actinidin has many applications in the food industry because of its advantages

over other plant proteases such as papain and ficin. Actinidin shows mild tenderizing activity, even at high concentrations, preventing surface mushiness. It has a relatively low inactivation temperature (60 °C) which makes the tenderization process easier to control (Eshamah et al. 2014; Tarté 2009). With previous application to meat, it has been found that actinidin has a milder tenderizing effect (Han et al. 2009) on collagen than other traditionally used proteases such as papain (Lewis and Luh 1988). This may limit its usefulness for underutilized cuts with high connective tissue content; however, it exhibits a more controlled tenderizing action on the myofibrillar structure, which is advantageous in terms of minimizing the mushy texture and off-flavors often experienced with the use of papain proteases (Ashie et al. 2002).

3.6 Applications of Plant Proteases on the Production of Protein Hydrolysates from Meat and Meat-processing By-products

Meat trimmings and by-products are in many countries taken out of production for the human consumption due to various reasons such as being not commonly consumed by customers (e.g., intestines, feet, and stomach), have low value (liver, and kidney) or due to hygienic and religious reasons (e.g., blood). There has been an increasing interest in utilizing these by-products with the aim of reducing waste of natural resources and adding-value to the processing of animals (Lafarga and Hayes 2014; Mora and Toldrá 2014). The production of bioactive compounds has been one of the most promising processing options as it generates compounds that have health and functional roles in food systems and could potentially have high value that justify the additional cost and efforts in processing the by-products. Bioactive peptides are short amino acid sequences (between 2 and 20 amino acid residues) that exhibit a physiological effect upon consumption in a food system. The generation of bioactive peptides is achieved by the hydrolysis of proteins due to the actions of proteases (either during the digestion of food after consumption, use of a fermentation process or use of purified or semi-purified protease preparations). Some of the biological activities that could be exerted by bioactive peptides are summarized in Fig. 3.2. Selected bioactivities of peptides obtained by plant proteases that have been reported for meat and meat industry by-products will be described below.

3.6.1 Muscle Proteins

3.6.1.1 Antioxidant Peptides

The production of highly reactive species of oxygen and nitrogen (known as ROS and RNS, respectively) in biological systems, including in food, is well known and has been reviewed by Bekhit et al. (2013) for meat. Oxidative stress caused by ROS and RNS

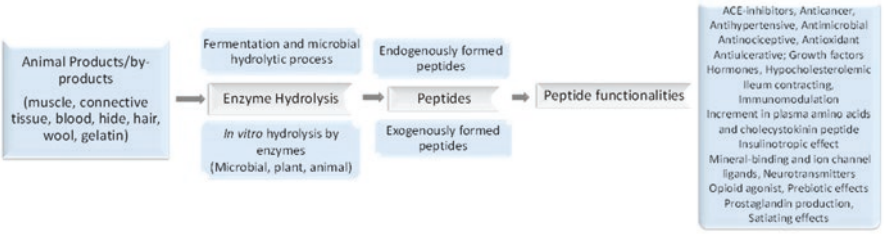

Fig. 3.2 Schematic representation of production of peptides from the meat industry and their potential bioactivities. Modified from Agyei et al. (2017)

lead to several pathological diseases and deterioration of foods. Antioxidants are capable of delaying oxidative processes and improve the healthiness and the quality of foods.

Antioxidant peptides were generated from pork myofibrillar proteins (Saiga et al. 2003) using papain (hydrolysis was carried out for 24 h at 37 °C and pH 7). The antioxidant activities of crude protein hydrolysate were better than those obtained by actinase E and were comparable to α-tocopherol in a linoleic acid peroxidation assay, but lower activities were found with the DPPH radical-scavenging activity and the metal-chelating activity assays. The hydrolysate obtained from papain treatment was purified and 5 peptides (D–S–G–V–T; I–E–A–E–G–E; D–A–Q–E–K–L–E; E–E–L–D–N–A–L–N; and V–P–S–I–D–D–Q–E–E–L–M) were identified. The D–A–Q–E–K–L–E peptide that originated from tropomyosin had the highest antioxidant activity. Under similar conditions, but lower pH (6.0), Kim et al. (2007) used papain and generated very high antioxidant activities in venison hydrolysate. The antioxidant activity (DPPH, hydroxyl, superoxide, and peroxyl radical-scavenging activity assays) of the papain hydrolysate was better than alcalase, neutrase pepsin, trypsin and chymotrypsin hydrolysates. Kim et al. (2007) reported the active peptides to be M–Q–I–F–V–K–T–L–T–G and D–L–S–D–G–E–Q–G–V–L.

Similarly, Arihara et al. (2005) reported three peptides (D–L–Y–A, S–L–Y–A, and V–W) obtained from pork actomyosin hydrolyzed with papain with high in vitro and in vivo antioxidant activity. The peptides showed anti-fatigue activity in mice subjected to treadmill exercise. The use of papain in addition to microbial proteases from *Streptomyces* and *Bacillus polymixa* generated pig collagen hydrolysates that had better antioxidant activity than the individual proteases (Li et al. 2007). Four peptide sequences (Q–G–A–R, L–Q–G–M, L–Q–G–M–Hyp, and Hyl–C) were isolated from the hydrolysate.

3.6.1.2 Other Activities

Prebiotic activity was reported by Liepke et al. (2002) and Arihara et al. (2013) reported papain hydrolyzed pork actomyosin to enhance the growth of *Bifidobacterium* strains. The peptide with E-L-M was found to be very active in

promoting the growth of 11 *Bifidobacterium* strains in skim milk. Papain hydrolyzed pork muscle exhibited cholesterol-lowering activity (Morimatsu et al. 1996) and antithrombotic activity (Shimizu et al. 2009).

3.6.2 Blood Proteins

Blood generated in abattoirs each year accounts to 4% of the live animal weight or 6–7% of the lean meat content of the carcass (Bah et al. 2013), which ends up as blood meal for low-value animal feed and fertilizer or discarded as effluent. Blood contains approximately 18% protein that can be recovered and used as a source of bioactives. Hydrolysates of blood proteins displayed antihypertensive, antioxidant, antimicrobial, and opioid activity and a comprehensive review on the topic is available (Bah et al. 2013). The aim of the following section is to provide an update for this information focussing on peptides generated using plant proteases. Lafarga et al. (2016a, b) reported bovine serum albumin hydrolysates obtained by papain treatment that demonstrated in vitro and in vivo antihypertensive activity. The peptides S-L-R, Y-Y, E-R, and F-R had ACE inhibitory activity (EC50) of 0.17, 0.04, 0.27, and 0.42 (mM), respectively (Lafarga et al. 2016a).

Plasma from deer, sheep, and pig blood was hydrolyzed using papain and bromelain as well as with microbial proteases individually. The peptide hydrolysates were obtained after 1, 2, 4, and 24 h of hydrolysis (Fig. 3.3) and were investigated for their antioxidant and antimicrobial activities. Papain resulted in more extensive hydrolysis of the proteins than bromelain (Fig. 3.4). Plasma hydrolysates generated by microbial protease exhibited higher DPPH radical-scavenging, oxygen radical-

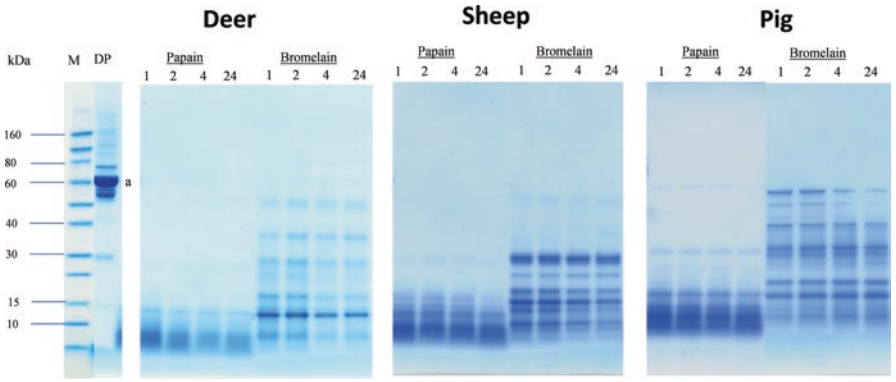

Fig. 3.3 SDS–PAGE profiles of deer plasma (DP), sheep plasma, and pig plasma treated with papain and bromelain for 1, 2, 4, and 24 h, respectively. The numbers within the figure indicate the time of hydrolysis (h). *M* standard protein markers. Modified from Bah et al. (2016a)

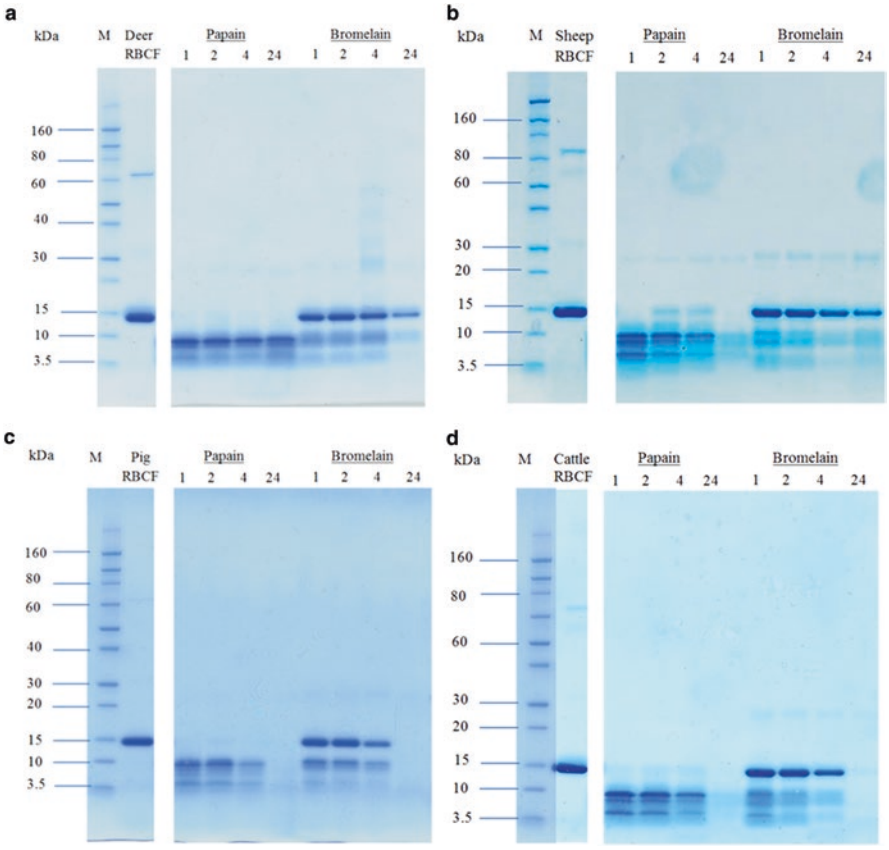

Fig. 3.4 SDS–PAGE profiles of: (**a**) deer RBCF, (**b**) sheep RBCF, (**c**) pig RBCF, and (**d**) cattle RBCF treated with papain and bromelain for 1, 2, 4, and 24 h, respectively. The numbers above the lanes indicate the time of hydrolysis (h). *M* standard protein markers. Modified from Bah et al. (2016b)

scavenging capacity (ORAC), and ferric-reducing antioxidant power (FRAP) than those generated with plant proteases for all three animal plasmas. No antimicrobial activity was found for all the hydrolysates. In a subsequent study, the red blood cell fraction (RBCF) of cattle, pig, sheep, and deer blood were hydrolyzed using the same protease to generate peptide hydrolysates (Bah et al. 2016b). Unlike plasma, red blood cell hydrolysates generated with papain had higher ferric-reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) compared to those produced with bromelain and other microbial proteases (Figs. 3.4 and 3.5). Antibacterial activity against *E. coli, S. aureus*, and *P. aeruginosa* growth was found in hydrolysates obtained with the microbial proteases only.

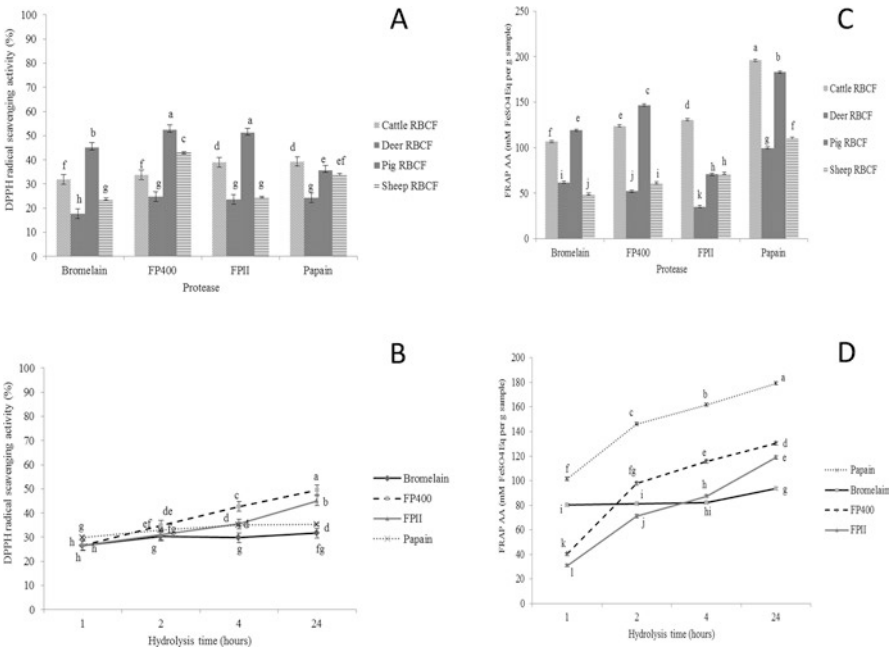

Fig. 3.5 DPPH radical-scavenging (**a**, **b**) and FRAP (**c**, **d**) antioxidant activity interaction plots. (**a**, **c**) Interaction between animal species and type of protease preparation. Means with different letters differ significantly ($p < 0.05$). (**b**, **d**) Interaction between protease preparation and time of hydrolysis. Means with different letters differ significantly ($p < 0.05$). Modified from Bah et al. (2016b)

3.7 Conclusion

The information presented in this chapter indicates that traditional plant proteases such as papain and bromelain are not really best suited as meat tenderizers due to their extensive hydrolyzing activity that can lead to mushy texture and off-flavors, whereas mild acting plant proteases such as actinidin may offer better tenderizing ability for fresh meat. On the other hand, papain seems to have better ability than many plant and microbial protease in generating peptides with antioxidant activity. As shown in Fig. 3.1, a very small fraction of plant proteases have been investigated in meat tenderization applications and even less have been investigated for the generation of bioactive peptides. More research is required for characterization and benchmarking the potential use of the lesser-known plant proteases for meat and meat by-product processing applications.

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Chapter 4
Peptide Synthesis Using Proteases as Catalyst

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4.1 Proteases: Physiological and Industrial Significances

Proteolytic enzymes (proteases) comprise a group of hydrolases (EC 3.4, NC-IUBMB) which share the common feature of acting on peptide bonds. Proteases are among the best-studied enzymes in terms of structure–function relationship (Krowarsch et al. 2005). Proteases, by catalyzing the cleavage of other proteins and even themselves, have an enormous physiological significance, their coding genes representing as much as 2% of the total human genome (Schilling and Overall 2008).

Proteases, together with lipases, represent the most important family of enzymes at industrial level, accounting for well over 50% of the enzyme market (Feijoo-Siota and Villa 2011). Proteases have been used industrially since the onset of enzyme technology in the first decades of the twentieth century; many of the early patents issued for the use of enzymes with commercial purposes were proteases, mostly from plant (papain and bromelain) and animal (trypsin and pepsin) origin. Intended uses were in brewing and in leather and rubber manufacturing (Neidelman 1991). In the decades that follow, many large-scale industrial processes were developed using now mostly microbial proteases. A common feature of them was the degradation of proteins and most relevant areas of applications were the food and beverage (Sumantha et al. 2006), detergent (Maurer 2004), leather (Foroughi et al. 2006), and pharmaceutical sectors (Monteiro de Souza et al. 2015). Acid and neutral proteases are relevant to the food industry for the production of protein hydrolyzates (Nielsen and Olsen 2002), beer chill-proofing (Monsan et al. 1978), meat tenderization (Ashie et al. 2002), and above all, for cheese production (Kim et al. 2004). Alkaline proteases are of paramount importance for the detergent industry (Sellami-Kamoun et al. 2008) and also in tannery (Varela et al. 1997; Thanikaivelan et al. 2004) and fish-meal production (Schaffeld et al. 1989; Chalamaiah et al. 2012). These conventional applications are by no means outside of continuous technological development (Monteiro de Souza et al. 2015). This is illustrated by the optimization of detergent enzyme performance under the harsh conditions of laundry at high and low temperatures, which has been a continuous challenge tackled by the construction of subtilisin (alkaline protease) variants by random and site-directed mutagenesis and by directed evolution (Kirk et al. 2002; Jares Contesini et al. 2018). It is also illustrated by the production of chymosin in microbial hosts by recombinant DNA technology and further improvement by protein engineering (Mohanty et al. 1999). Therapeutic application of proteases acting as protein hydrolases goes from conventional digestive-aids and anti-inflammatory agents to more sophisticated uses as thrombolytic drugs (i.e., urokinase and tissue plasminogen activator) and

more recently for the treatment of hemophilia. A comprehensive review on the therapeutic uses of proteases is suggested for the interested reader (Craik et al. 2011).

The potential of hydrolytic enzymes for catalyzing reverse reactions of bond formation has been known for quite some time. However, its technological potential as catalysts for organic synthesis was developed in the 1980s (Bornscheuer and Kazlauskas 1999) paralleling the outburst of biocatalysis in nonconventional (non-aqueous) media (Illanes 2016).

Proteases can not only catalyze the cleavage of peptide bonds but, in a proper reaction medium, they can also catalyze the reaction of peptide bond formation. Proteases are highly stereo- and regiospecific, active under mild reaction conditions, do not require coenzymes, and are readily available as commodity enzymes; these properties make them quite attractive catalysts for organic synthesis (Bordussa 2002; Kumar and Bhalla 2005). Such reactions will not proceed efficiently in aqueous medium where the hydrolytic potential of the enzyme will prevail, so reaction media at low, and hopefully controlled, water activity is necessary for peptide synthesis. This is a major threat since proteases, different from lipases, are not structurally conditioned to act in such environments. The use of proteases in peptide synthesis is analyzed in depth in section 3.4.

4.2 Peptides: Technological Impact

Peptides are amazing functional molecules whose technological potential has been developed vigorously in recent decades. Most current applications are related to healthcare, where bioactive peptides are increasingly being used as therapeutic agents, including peptide drugs, antimicrobial agents, vaccines, cosmetic ingredients, drug carriers, and diagnostic reagents (Albericio and Kruger 2012). Beyond that, peptides are used as taste enhancers in foods (Temussi 2012) and as nutritional supplements (Schaafsma 2009); they are also considered as valuable devices in nanotechnology (de la Rica and Matsui 2010; Seabra and Durán 2013).

4.2.1 Bioactive Peptides

In this section, the current applications of bioactive peptides as therapeutic vaccines, as cosmetic agents, and as drug delivery reagents will be reviewed.

4.2.2 Therapeutic Peptides

This is certainly the most ample and promising area for peptide research, development, and application. Peptides exhibit a remarkable specificity of action and interaction with the target, which has been attributed to their structure (Nguyen et al. 2011).

However, peptides can be readily degraded inside the human body due to the presence of multiple proteases, being this a major hurdle making peptides inferior to small-molecule drugs (Vlieghe et al. 2010); this is though a double-edged sword situation, since because of its degradability (and also by their target specificity) toxicity of peptides is quite low, which is certainly an asset for any drug (Kaspar and Reichert 2013). In fact, almost 40% of the small-molecule drug candidates are abandoned after phase I clinical trials because of toxicity (Vlieghe et al. 2010).

An interesting SWOT analysis of naturally occurring peptides as therapeutics has been raised where most of the weaknesses and resulting threats annotated point out to the need for peptide design (Fosgerau and Hoffmann 2015). In fact, therapeutic use of peptides has experienced an impressive development in recent years because of technological advances allowing the discovery of novel peptides with pharmaceutical potential, but mostly by creating artificial peptide variants using solid-phase peptide synthesis and combinatorial chemistry (Uhlig et al. 2014). Nature is an endless source of peptides with therapeutic potential and their study is on the basis of peptidomics (Dallas et al. 2015). However, natural peptides are not active enough; their physicochemical properties may be inadequate for medical use (i.e., tendency to aggregation or poor solubility) and may exhibit short circulating plasma half-life due to fast renal clearance (Werle and Bernkop-Schnürch 2006). These peptides are though excellent starting points for developing therapeutic peptides. Research and development is then mostly focused on synthetic peptides overcoming such constraints and rational design of peptides is meant to such purpose. Alanine scanning allows identifying key amino acid residues within the peptide as candidates for substitution and also allows identifying chemically labile or too reactive residues. Aggregation can be avoided by intervening hydrophobic spots, by amino acid substitution or methylation, while solubility may be modified by altering the charge distribution of the peptide. Physicochemical properties of the peptide can also be acted upon by introducing stabilizing α-helices, salt bridge formation, and other chemical modifications. Plasma half-life can be extended by protecting against proteolytic cleavage that can be done by identifying the possible cleavage sites and substituting the target amino acids, and by binding the peptide to albumin or to polyethylene glycol in order to avoid renal clearance, the latter binder being safer and more tolerable. These second-generation peptide drugs optimized for medical use by rational design are on the forefront of peptide research and development (Fosgerau and Hoffmann 2015).

β-Peptides are quite interesting candidates for medical use since they act in a way similar to common α-peptides with the advantage of lower affinity for blood cells and reluctance to proteolysis (Heck et al. 2010). The case of the dipeptide carnosine (β-alanyl-L-histidine) is illustrative, since several physiological functionalities have been ascribed to it, such as physiological buffer, metal ion chelator, immunomodulator, neurotransmitter, and antitumor agent, being considered as a prominent antioxidant and free-radical scavenger (Hipkiss and Brownson 2000; D'Arrigo et al. 2009); it has

also been claimed to prevent cell damage caused by β-amyloid protein, which is connected to Alzheimer's disease (Hipkiss 2007).

Administration route varies from case to case: most of therapeutic peptides are injectables, but oral, intranasal, and subcutaneous routes are most attractive alternatives for peptide drugs delivery. Biocompatible carrier materials, like chitosan (Prego et al. 2005), and nanoparticles of different types have also been proposed for oral delivery of peptides (Almeida and Souto 2007; Singh and Lillard 2009; Sheridan 2012). Orally administered peptides are obviously convenient to the patient, but it is challenging since degradation by their passage through the gastrointestinal tract has to be avoided. This can be also tackled by rational peptide design aiming to stabilize the secondary structure and also by the use of proper excipients and adjuvants. Anyhow, the efficacy of orally administered peptides is likely to be lower due to the difficulty in arriving to their targets, so that delivery by injection will probably continue to be the most-used route for peptide delivery.

Looking at the market, a US-FDA survey on 2012 showed that 8 out of the 76 (10.5%) new drugs approved in the period 2009–2011 were peptides intended for the treatment of lymphomas, hepatitis, type 2 diabetes, angioedema, and skin infections (Albericio and Kruger 2012). A recent survey on global peptide drug market predicted an increase from US$ 14.1 billion in 2011 to US$ 25.4 billion in 2018, which is an impressive increase of 8.5% per year in that period. This prediction is well sustained: in 2012 five new peptide drugs, namely lucinactant, pasireotide, carfilzomib, linaclotide, and teduglutide, were approved both in the USA and in the European Union (EU). Another one, peginesatide, was approved in the USA, but a year later was voluntarily recalled by the producing company because of hypersensitivity reactions in several patients under treatment (Kaspar and Reichert 2013). Administration route varied from case to case: from those five peptides, one is to be administered intravenously, two subcutaneously, one by inhalation, and one orally. By 2013, 128 peptide drugs were under clinical trial: 40 in phase I, 74 in phase II, and 14 in phase III. A significant part (about 40%) of the drug peptides under evaluation is represented by G-protein-coupled receptor (GCPR) agonists which, beyond application as agonists for type-2 diabetes, have potential applications in neurodegenerative disorders (i.e., Alzheimer's disease), cardiovascular conditions, and body weight management (Kaspar and Reichert 2013). By 2015, more than 60 peptide drugs have been approved by the US Food and Drug Administration and were in the market, 140 were under clinical trials and more than 500 were in preclinical studies; metabolic disorders and cancer were the main targets. Including also diagnostic applications, more than 100 peptides for medical use are in the market in the USA, EU, and Japan. The average number of related patents has been over 10,000/year in the last decade so a significant increase in the number of peptide drugs entering the market is to be expected in the forthcoming years; commercial success of some blockbuster peptide drugs is also a driving force for expanding the peptide market (Albericio and Kruger 2012).

Examples of synthetic therapeutic peptides that are currently in the market include the well-known hormones oxytocin (Pitocin®) and vasopressin (Pitressin®), both produced by JHP Pharmaceuticals, Leuprorelin/Leuprolide (Lupron™, Abbot Laboratories), a peptide used for the treatment of prostate cancer which generated in 2013 US$ 1.7 billion in sales worldwide (Fosgerau and Hoffmann 2015), and bivalirudin (Angiomax®, The Medicines Company), a direct thrombin inhibitor (DTI) with sales up to US$ 7.8 billion in the last quarter of 2016 (The Medicines Company Reports 2016). Other therapeutic peptides, larger in size and more complex in structure, are produced by recombinant DNA technology. Primary examples of these include insulin glargine (Lantus™, Sanofi) that generated US$ 7.9 billion in sales in 2013, and liraglutide (Victoza™, Novo Nordisk), a multifunctional glucagon-like peptide (GLP-1) and the most important member of a family of similar drugs used for the treatment of both type-2 diabetes and obesity (Fosgerau and Hoffmann 2015).

4.2.3 Peptide Vaccines

Conventional vaccines (those including whole cells or large proteins) incorporate an unnecessary antigenic load that sometimes induces over response. Peptide vaccines are an attractive option for being based on short peptide fragments inducing highly targeted immune responses. When large proteins are used, they contain many antigenic epitopes which, besides unnecessary, may become detrimental for the induction of immunity. Peptides, on the other side, may contain only epitopes causing positive immune responses (Noguchi et al. 2003). Peptides used in vaccines are small molecules, usually not exceeding 30 amino acid residues, so they are often poorly immunogenic, and proper carriers and adjuvants are required to provide chemical stability and induction of robust immune response (Purcell et al. 2007). Carriers may also act as adjuvants (Bolhassani et al. 2011). Several delivery systems for peptide vaccines have been studied including emulsions, liposomes, virosomes, polymers (i.e., polylactic acid, polyglycolic acid, and polyhydroxybutyrate), and nanoparticles of several kinds (Li et al. 2014).

Since the pioneering work of development of a synthetic peptide vaccine against malaria (Patarroyo et al. 1988), many peptide vaccines have been developed to protect against different diseases like human immunodeficiency (HIV), hepatitis C (HCV), foot and mouth disease, swine fever, influenza, and human papilloma (Li et al. 2014). However, most of them are related to cancer therapy, alone and as complements in interleukine treatment (Schwartzentruber et al. 2011; Slingluff 2011; Aranda et al. 2013; Cerezo et al. 2015).

As of 2014, 270 peptide vaccines were in Phase I stage of development, 224 were in Phase II, and only 12 have moved to Phase III being all these 12 related to cancer therapy (Li et al. 2014). At that time, none had been launched into market. Strategies for improving peptide vaccines include the use of long peptides, modification of adjuvants, incorporations of new antigens, and combination therapy with other immunologically active agents (Slingluff 2011). Developing novel safe adjuvants stimulating stronger immune response is a major challenge for peptide vaccines.

4.2.4 Cosmetic Peptides

There is a growing concern about skin deterioration (skin loss, loss of elasticity, and wrinkle appearance) as the world population is aging. Skin care is not merely a hedonistic or esthetic matter, but a matter of health for the elderly. This process involves a number of genetic, constitutional, hormonal, nutritional, and environmental factors, but extrinsic factors like UV-irradiation, smoking, and repeated facial movements are also relevant.

The increasing knowledge of skin-related aging at the molecular level is allowing the exploration of novel antiaging agents. In this scenario, peptides appear as quite relevant since they are most certainly involved in all aspects of skin homeostasis. Peptides can be readily modified by amino acid substitution and modification, building up a platform to deal with potency, solubility, toxicity, skin penetration, and cost, which are distinctive features for skin care ingredients (Fields et al. 2009). Therefore, peptides are gaining considerable attention within the cosmetic industry because of their well-documented effect as antiaging agents, reducing the onset of skin wrinkles and making skin smoother and tauter (Zhou et al. 2011).

According to their mode of action on skin, peptides (sometimes named cosmeceutical peptides) can be grouped into four categories: signal peptides, enzyme-inhibitor peptides, neurotransmitter-inhibitor peptides, and carrier peptides (Gorouhi and Maibach 2009). Signal peptides act by stimulating skin cells, mostly fibroblasts (Powers et al. 2000), with the consequent increase in collagen, and also stimulating elastin synthesis, then providing firmness and elasticity to the skin. Some reported signal peptides are the pentapeptides Val-Gly-Ala-Pro-Gly and Lys-Thr-Phe-Lys-Ser, and the tripeptide Gly-His-Lys. Enzyme-inhibitor peptides act by inhibiting metalloproteases involved in the degradation of collagen and elastin (Park et al. 2009); one salient example is the 37 amino-acid residue peptide LL-37 (Dürr et al. 2006). Neurotransmitter-inhibitor peptides inhibit acetylcholine release at the neuromuscular junction so inhibiting signal transduction pathways at that level (Lupo and Cole 2007) in a way similar to botulinum toxin (Botox), with the consequent attenuation of the repetitive contraction of the muscles involved in facial expression; one example is the hexapeptide Botox mimic Ac-Glu-Glu-Met-Gln-Arg-ArgNH_2. Carrier peptides are those delivering important substances, specifically trace elements like Cu and Mn, which are cofactors of enzymes involved in collagen and elastin formation and in wound healing processes; one example is the tripeptide Gly-Gln-Pro-Arg (Bachem Product Monographs 2017). Several peptides used in cosmetics act by other mechanisms associated with skin appearance and aging delay, such as scavenging reactive oxygen species, organizing collagen fibers, and acting as anti-inflammatory agents. For instance, carnosine is a natural dipeptide with well-documented antioxidant activity that, beyond being used in dietary supplements, is used in cosmetic applications (Babizhayev 2006). Synthetic carnosine derivatives have also been used for that purpose (Stvolinsky et al. 2010).

Beneficial effects of cosmetic peptides are mostly based on in vitro studies and the efficacy of peptides absorption in a stable form into the dermis is a matter to deal

with when using these compounds for skin care. However, soft clinical data are strongly supporting their beneficial effect, and, in fact, peptides have now become important ingredients in several cosmetic products. Commercial cosmetic products containing peptide ingredients like the acetylated hexapeptide Argireline®, the palmitoyl oligopeptide Matrixyl™, the pentapeptides Leuphasyl®, and Vialox® have already well-established markets (Fields et al. 2009). It is estimated that more than 25 peptides are currently being used in cosmetic preparations and many more are in advanced stages of development (Bachem Product Monographs 2017). Lipopeptides are particularly interesting cosmetic ingredients because they combine anti-wrinkle and surfactant properties, being key components in moisturizing and cleansing cosmetics (Kanlayavattanakul and Lourith 2010). They are amphiphilic cyclic peptides of no more than 10 amino acid residues linked to β-hydroxy acids; their structure confers them multifunctionality: detergency, emulsification, foaming, hydration, and antimicrobial activity, so they are well-appreciated as cosmetic ingredients for skin care (Varvaresou and Iakovou 2015).

4.2.5 Peptides as Drug Carriers and Diagnostic Reagents

The selective permeability of the cell membrane is a major hurdle for drug delivery to their target. Internalization is essential to many drugs so that effective transporters are required. Cell-penetrating peptides are small cationic, often amphipathic, molecules with outstanding capacity for cell membrane translocation being good candidates for drug delivery. Mechanism of cell penetration may vary according to the peptide length and the properties of the carried molecule (Mueller et al. 2008), but penetration systems can be categorized in energy-dependent endocytosis and energy-independent direct translocation across the membrane, both depending on peptide–membrane interactions (Yeung et al. 2011). As drug transporters, peptides have some drawbacks due to their poor stability, short half-life, and susceptibility to protease digestion. However, extensive research is aimed to overcome these barriers. Recent advances in elucidating the mechanisms of cell penetration and in peptide design using rational strategies are placing peptides in a prominent position for accurate and safe delivery of bioactive molecules to target cells (Copolovici et al. 2014). In addition, advances in peptide synthesis and scale-up have reduced their cost (Bray 2003; Vlieghe et al. 2010), so becoming attractive components of drugs and diagnostic reagents. An interesting application of cell-penetrating peptides is in the delivery of imaging agents and biosensors. Delivery of quantum dots across the blood–brain barrier, delivery of radio-immunoconjugates, visualization of viral infections, and delivery of intracellular biosensors are among the fascinating opportunities that peptides offer for medical and biological applications (Fonseca et al. 2009). The impressive development of nanotechnology has opened also attractive opportunities for peptides. Properties of peptides and nanoparticles make a virtuous coupling for application in cancer nanomedicine as drug carriers, targeting ligands, and protease substrates (Zhang et al. 2012a, b). Nontargeted distribution of drugs is

not only unsafe but also inefficient and peptides have shown to be quite convenient ligands for increasing the specificity of targeting systems, which may be applied advantageously for both therapy and diagnosis. Carrier peptides delivering therapeutic peptides represent another fascinating line of research. Peptides which are substrates for the proteolytic action of cancer-associated proteases (a set of proteases present in high amounts in cancer cells, but virtually absent in normal cells) if linked to a chemotherapeutic agent will deliver the drug upon protease hydrolysis of the conjugate. As an example, the peptide Pro-Val-Gly-Leu-Ile-Gly was conjugated with dextran and the methotrexate anticancer drug, and upon the action of the cancer-associated matrix metalloproteases (MMP-2 and MMP-9) released the drug to inhibit tumor growth (Chau et al. 2006).

4.2.6 Taste Peptides

Among the many relevant properties of peptides, taste is seldom highlighted. However, it is well known that short peptides, mainly derived from the hydrolysis of proteins, play an important role in the sensory appreciation of food taste and are, in fact, taste-endowed molecules comprising the whole spectrum of taste: sweet, bitter, sour, salty, and umami. Sweet taste in foods is associated with acceptance, while bitter taste is mostly linked to rejection. Umami (Japanese word for delicious) taste has been added to the four traditional categories to designate a meaty pleasant taste (Kurihara 2015). Taste of peptides is unrelated to the taste of its individual amino acid components and hard to correlate with its amino acid composition (Solms 1969). Table 4.1 provides a list of peptide sequences and their taste properties (Gill et al. 1996).

Taste of peptides acquired renown at the end of the 1960s with the discovery of the potent sweetener aspartame (Mazur et al. 1969). This serendipitous discovery was rather surprising at the time because no peptides were associated with sweet taste. Aspartame (*N*-(L-α-aspartyl)-L-phenylalanine 1-methyl ester) is certainly an icon of taste peptides with a sweetening power 200 times higher than sucrose. Impact of aspartame prompted the research in structure–taste relationships and several topological models were proposed to explain its mode of eliciting sweetness (Yamazaki et al. 1994; Morini et al. 2005). Peptides having hydrophobic amino acid residues in their chains are usually bitter and models for structure–bitterness relationship have been built mostly based on the resemblance between sweet and bitter receptors, but now it is accepted that different receptors exist for detecting the five basic tastes (Chandrashekar et al. 2006). As far as umami taste, there is no convincing evidence that short peptides bear such taste, which instead could be due to its partial hydrolysis into glutamic and aspartic acid. A thorough insight in taste receptor and interaction with peptides can be found in the review article by Temussi (2012).

Aspartame clearly stands out among taste peptides with a market volume exceeding 40,000 tons per year (more than 60% of the total market for noncaloric sweeteners). Alitame (L-α-aspartyl-*N*-(2,2,4,4-tetramethyl-3-thietanyl)-D-alaninamide) is another potent low-calorie dipeptide sweetener not producing toxic compounds

Table 4.1 Some relevant taste peptides

Peptide structure	Taste
Gly-Leu	Bitter
Lys-Pro	Bitter
Ser-Leu-Ala	Bitter
Gly-Pro-Phe-Pro-Val-Ile	Bitter
Lys-GlyHCl	Salty
Orn-GlyHCl	Salty
Glu-γ-Ala	Sour
Glu-γ-Glu	Sour
Asp-Glu-Glu	Sour
Asp-PheOMe	Sweet
L-Asp-D-AlaNH$_2$	Sweet
N-Ac-Phe-Lys	Sweet
N-Ac-Gly-Lys	Sweet
Ser-Glu-Glu	Umami
Gly-Asp-Gly	Umami
Ala-Glu-Ala	Umami
Val-Glu-Val	Umami
Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala	Umami

(Kim and Shin 2001) which has been approved for food use and is currently being marketed in several countries. The aspartyl residue is key to its sweetening power as early predicted by Mazur et al. (1969).

4.2.7 Nutritional Peptides

The use of peptides as nitrogen source in the formulation of foodstuffs for patients recovering from surgery, suffering malnourishment, and poor protein digestibility or absorption is considered a better alternative than supplying the free amino acids or large polypeptides. Peptides are less hypertonic and better absorbed in the intestine than free amino acids, and have better sensory properties and are less antigenic than larger polypeptides or proteins (Gill et al. 1996). Peptides can be used in oral, enteral, or parenteral nutrition (Kreymann et al. 2006). In the case of patients with functional gastrointestinal tract, oral or enteral are preferred routes than parenteral for the administration of peptides (Zaloga 2006). Beyond nutrition, many of these dietary peptides have shown to display other biological functions affecting the cardiovascular, endocrine, immune, and nervous systems (Korhonen and Pihlanto 2003; Zaloga and Siddiqui 2004).

An outstanding case of nutritional peptide is the dipeptide L-alanyl-L-glutamine, which has proved to be a very good vehicle for glutamine supply (Harris et al. 2012). Glutamine is the most abundant amino acid in blood plasma, but under stress conditions due to illness, trauma, or overtraining, glutamine concentrations in plasma and skeletal muscle may fall below normal levels and the requirement for

glutamine may exceed its de novo synthesis capacity, becoming then a conditionally essential amino acid (Wang et al. 2015). In such cases, exogenous glutamine intake becomes necessary. This has opened a buoyant market for this dipeptide which is marketed under the trade name Sustamine® by Kyowa Hakko Bio (Japan). Sustamine-based products aimed for athlete endurance have been launched in recent years with great success (Schultz 2013). The dipeptide carnosine (β-alanyl-L-histidine) is another striking example of nutritional peptide that has a well-developed market as a food supplement (Mahmood et al. 2007). Carnosine and β-alanine are currently used by athletes and bodybuilders (Culbertson et al. 2010); carnosine neutralizes the accumulation of lactic acid during highly intense exercise, accelerating the working capacity of muscle exhausted by preceding exercise (Murray 2016). As previously stated, carnosine is considered a powerful natural antiaging agent with many reported functionalities.

4.3 Production of Peptides: Available Technologies

Available technologies for the production of peptides are: extraction from natural sources, production by recombinant DNA technology, chemical synthesis, and enzyme biocatalysis (Guzmán et al. 2007).

4.3.1 Extraction from Natural Sources

Dietary proteins are a rich source of peptides, but their functionalities are hidden within the protein sequence. Release of peptides from their protein matrix can be obtained through hydrolysis by digestive enzymes, by proteolytic microorganisms or by proteolytic enzymes. Biologically active peptides are physiologically produced during the gastrointestinal digestion and fermentation of foods (Korhonen and Pihlanto 2006). In principle, peptides can be produced from any high-protein food but in practice most-used foodstuffs are milk (Nagpal et al. 2011), whey (Madureira et al. 2010; Welderufael and Jauregi 2010), and soy (Kong et al. 2008). Other sources, like marine organisms (Wilson et al. 2011) and microalga (Sheih et al. 2009), have been proposed as natural sources for peptide production. Enzymes used for the hydrolysis of proteins are from animal (i.e., α-chymotrypsin, trypsin, and pepsin), plant (i.e., papain and bromelain), and microbial sources (i.e., subtilisin and thermolysin) and the type of peptides produced is very much dependent on the enzyme used. Microbial enzymes are in general preferred for being cheaper, readily available, and quite diverse in their mode of action; use of these enzymes in immobilized form may contribute to a better catalyst performance because of increased stability and reuse, even though the macromolecular nature of the substrate may produce considerable mass-transfer limitations (Tavano 2013). However, many of the well-known bioactive peptides have been produced with animal enzymes.

This is the case of the angiotensin-converting enzyme-inhibitor peptides (Gobbetti et al. 2004) and metal-binding phosphopeptides (Meisel and FitzGerald 2003) that are produced by milk hydrolysis with trypsin and chymotrypsin.

The production of peptides from natural sources considers a first step of enzymatic hydrolysis of the protein-rich raw material, which can be done either by fermentation or enzymatic reaction with commercial proteases; the latter, being more specific and producing less contaminant materials to remove, is the preferred choice (Kim and Wijesekara 2010). This step will determine the type of peptide produced and the composition of the hydrolyzate. Recovery and purification of the peptide from the hydrolyzate may be the more complex and demanding production steps and several operations involving membrane and chromatographic fractionation have been tested (Agyei and Danquah 2011).

Production of peptides by extraction from natural sources is probably the less studied and less relevant strategy. Despite the enormous source of peptide functionalities treasured within the protein structures, technological development has been challenged by problems of large-scale separation, purification, and characterization of the resulting peptides, most information being referred to hardly scalable laboratory protocols (Bougatef et al. 2010; Agyei and Danquah 2011).

4.3.2 Production by Recombinant DNA Technology

Recombinant DNA technology can be regarded as a valuable alternative, but only in the case of large peptides whose production by other strategies can be cumbersome or unfeasible (Vlieghe et al. 2010). Insulin is the most demanded hormone worldwide; it is a 51 amino-acid residue polypeptide lying in the size borderline between peptides and proteins. Human insulin (Humulin™) was the first commercial pharmaceutical produced by recombinant DNA technology in the early 1980s (Keen et al. 1980; Johnson 1983), representing a striking example of peptide production by this route. The enormous commercial and social success of recombinant insulin has been a driving force for the development of drugs from recombinant DNA technology (Walsh 2005), but recombinant DNA technology has been mostly applied for the production of proteins where its technological impact is significant (Demain and Vaishnav 2009). However, some small peptides have been produced by recombinant DNA technology. The production of aspartame is an illustrative example: the 12-nucleotide sequence coding aspartyl-phenylalanine was chemically synthesized and cloned as multiple repeating units close to a tryptophan controlled promoter; the recombinant host cell produced the polypeptide that was further cleaved enzymatically to yield the dipeptide (Murata et al. 1993; Lee 2015). DNA sequences corresponding to small peptides should be cloned as repeated copies; otherwise, the expression efficiency attainable is low (Gill et al. 1996). Other drawbacks for the synthesis of small to medium size peptides by recombinant DNA technology are the complex extraction and purification of the product that drives into noncompetitive production costs; besides, peptides containing unnatural amino acids cannot be

produced by this route. AMPs have been produced by recombinant DNA technology using mostly *Escherichia coli* as host under the rationale that this is a sound technology for producing workable quantities (Ingham and Moore 2007). In this case, besides the abovementioned limitations, two additional challenges have to be faced since in this case the product may be toxic to the producing host and highly susceptible to degradation by endogenous proteases; this has been overcome by fusing the peptide to a carrier protein that can be afterwards cleaved at the peptide–protein junction either chemically or enzymatically (Li 2011). Summing up, recombinant DNA technology is likely to be competitive only in the case of large peptides where other strategies may render inapplicable.

4.3.3 Production by Chemical Synthesis

Chemical synthesis is the most mature technology for the synthesis of small to medium size peptides. It was originally conducted in solution, but later on replaced by solid-phase peptide synthesis (SPPS), which consists in the stepwise addition of the amino acids in the preestablished order to a solid matrix that holds the growing peptide chain (Merrifield 1986). SPPS can be conducted by the Fmoc/tBu or t-Boc/Bzl systems. In the former, the Fmoc (9-fluorenyl methoxycarbonyl) group is used for the protection of the Nα amino group and the tert-butyl group (tBu) for the protection of the side chains of several amino acids. In the latter, t-Boc (tert-butoxycarbonyl) and benzyl ester or cyclohexyl ester (Bzl) perform the respective functions of Fmoc and tBu in the former (Albericio 2000). In the first step, the C-terminal amino acid is linked to the solid matrix and then Nα group is removed (with trifluoroacetic acid in the t-Boc system and with piperidine in the Fmoc system). Then, the Nα protected amino acids are added according to the desired sequence and after each coupling a deprotection step follows. Upon completion of the peptide sequence, the peptide–matrix complex is cleaved and the side chain protecting groups are removed to yield the peptide (Illanes et al. 2009a, b).

The two most-used strategies for SPPS strategies are sequential and convergent synthesis. The former involves the stepwise addition of amino acids until the desired sequence is synthesized and it is used mainly for the synthesis of small to medium size peptides; in the latter, peptides are separately produced by sequential synthesis and then linked in solution or in solid phase to obtain the desired higher molecular weight peptide or protein. The advantage of convergent synthesis is that each peptide fragment is purified and characterized before being linked; however, reaction rates for the coupling of fragments are substantially lower than for the coupling of the activated amino acids in sequential synthesis and the C terminal of each peptide fragment may be racemized during coupling. Such problems can be circumvented by prolonged coupling reaction times and by using glycine or proline at the C-terminal (Lloyd-Williams et al. 1993; Lloyd-Williams and Giralt 2000). Convergent synthesis represents the best option for the chemical synthesis of large

peptides, which is illustrated by the large-scale synthesis of the antiviral peptide T-20 (Bray 2003).

SPPS has limitations inherent to the chemical procedures involved, which for a long time have made it a less desirable approach in the large-scale production of peptides over a certain number of amino acid residues, where recombinant DNA might be the preferred choice. Such problems include aspartimide formation, His and Cys racemization, low production efficiency of certain peptide sequences due to hydrophobicity or aggregation (amyloid peptides are a primary example of this problem), and poor solubility of the final product in some cases. However, these problems are constantly being studied and addressed by peptide chemists. For example, different protecting groups suitable for the Fmoc/tBu strategy have been developed for Asp to minimize aspartimide formation (Ruczyńsky et al. 2008), for His/Cys to minimize racemization and for Arg to decrease the lengthy deprotection times associated with its large side chain protecting groups, obtaining variable degrees of success (Behrendt et al. 2016). Backbone protection schemes and the use of pseudoprolines (modified Ser, Thr, and Cys residues) have improved production efficiency of lengthy sequences like ubiquitin (El Oualid et al. 2010) and the bovine pancreatic trypsin inhibitor (Burlina et al. 2014). Moreover, several building blocks are available to introduce posttranslational modifications (PTMs) to the sequence, which are possible to generate in the mild environment of Fmoc chemistry. These include phosphorylated Ser/Thr/Tyr, methylated Arg/Lys, *O*-glycosylated Ser/Thr, and *N*-glycosylated Asn, to name a few (Behrendt et al. 2016). Nonconventional amino acids, like citrulline or β-alanine, are also building blocks available for Fmoc/tBu synthesis. Impressive advances in SPPS have occurred recently with respect to peptide-coupling reactions driven by the development of new coupling reagents, which are paving the way for the facile and routine preparation of any desired peptide. This is expected to expand the use of peptides and peptidomimetics as drugs for the treatment of a broad spectrum of diseases (El-Faham and Albericio 2011).

Among the various strategies to improve key pharmacokinetic properties of synthetic peptides for their therapeutic use, especially cell-penetrating capabilities and increased structural and chemical stability in order to expand therapeutic targets to the intracellular environment, hydrocarbon stapling and β-hairpin mimetics (Tsomaia 2015) show promising results. For example, the stapled peptide ALRN-5281 successfully entered Phase I in 2013 as a novel growth-hormone releasing factor agonist, and ATSP-7041, a p53-related in vitro and in vivo tumor suppressor peptide is planned to enter Phase I trials. Both these synthetic products are being developed by Aileron Therapeutics Inc. As mentioned before, the β-hairpin antibiotic drug, POL7080 developed by Polyphor, is already in human clinical trials.

Manual and automated systems are available for single or multiple peptide synthesis. Most of them are laboratory devices for small-scale operation, but automated t-Boc/Bzl and Fmoc/tBu SPPS systems delivering peptides at the several kg scale are available from different suppliers (Chan and White 2000; Bruckdorfer et al. 2004). A didactic manual for SPPS by Fmoc/tBu system was published by Amblard et al. (2006).

Most peptides produced by SPPS are intended for pharmacological use so that degradation by endogenous proteases, hepatic clearance, interaction with different receptors, and low membrane permeability are barriers that attempt against their efficacy. Changes of single amino acids in the peptide sequence and modification of the peptide chain backbone may assist in solving the problem (Ahn et al. 2002). Peptidomimetics, in which the peptide is bound by either the C- or N-terminal amino acid to a nonprotein ligand, may also enhance the biological activity and efficacy (Radzishevsky et al. 2007).

As of 2015, more than 40 therapeutic peptides were already in the market being produced by SPPS and many more are now in different phases of approval; some of them are being produced at a very large scale (Albericio and Kruger 2012; Makowski et al. 2016).

4.3.4 Production by Enzymatic Synthesis

Peptides can be synthesized by nonribosomal synthetases and some peptide antibiotics, like gramicidin and bacitracin, are produced by fermentation with proper bacterial strains bearing such synthetases. However, synthetases are complex, labile, coenzymes requiring enzymes so their use as biocatalysts for the production of peptides is unlikely (Matteo et al. 1976; Martin and Demain 1980; Marahiel 2009). Proteolytic enzymes on the other hand may, under certain reaction conditions, act in reverse catalyzing peptide bonds formation instead of cleaving them, which is a more attractive technological option, since these enzymes are easy to produce, robust, do not require coenzymes, and are commercially available at low prices.

Peptide synthesis catalyzed by proteases can proceed by thermodynamic and kinetic control (Kumar and Bhalla 2005). The thermodynamically controlled synthesis of peptides (TCS) with proteases represents the reverse of the hydrolytic breakage of peptide bond catalyzed by those enzymes, as shown in the scheme (Jakubke et al. 1985):

$$\mathrm{R'COO^- + H_3^+NR'' \overset{Kion}{\rightleftharpoons} R'CO_2H + H_2NR'' \overset{Kcon}{\rightleftharpoons} R'CO\ \ NHR'' + H_2O}$$

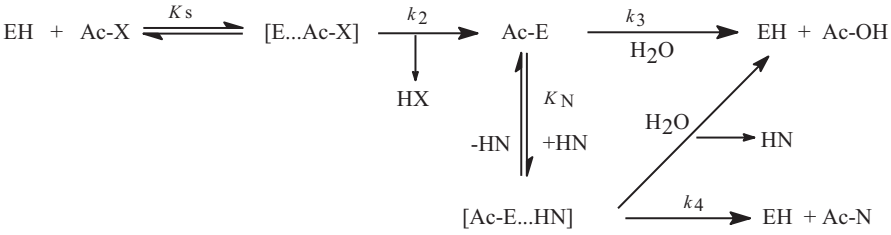
Kion and Kcon are the equilibrium constant of ionization and conversion, respectively. The synthesis and the hydrolysis of the peptide bond proceed by the same mechanism, through the acyl intermediate from a carboxylic acid which is the rate limiting step in TCS (Bordussa 2002).

This mechanism allows to use any type of protease and an acyl donor with the free carboxylic group, but the reaction rates and product yields are usually low, due to they are conditioned by the reaction equilibrium constant, and a great amount of enzyme is often required. Besides, it is necessary to carefully study the reaction conditions that lead to the displacement of the reaction equilibrium towards synthesis, i.e., by product precipitation or by modification of reaction medium

composition. The addition of organic cosolvents and the use of aqueous-organic biphasic systems are good strategies to displace the equilibrium towards synthesis, but they can produce severe compromises with enzyme activity and stability. Organic cosolvents reduce the activity of water and modify physical–chemical parameters of reaction media, but high concentrations of them are usually detrimental for enzyme activity (Halling 1994).

Aqueous-organic biphasic systems allow the peptide product partition from the aqueous phase (containing the enzyme) to the organic phase, driving the equilibrium towards synthesis and avoiding the peptide hydrolysis. However, the water–solvent interface can decrease the reaction rates because there may be denaturation of the enzymes and limitations due to the diffusion of substrates (Barberis et al. 2008). Neat hydrophobic solvents with very low water activity can be effective media for peptide synthesis by reducing product hydrolysis. However, proteases show usually low activity and stability in those media, while the most substrates (amino acids) and products (peptides) have low solubility in neat organic solvents (Quiroga et al. 2005).

The kinetically controlled synthesis of peptides (KCS) with proteases can be schematized as follows (Bordussa 2002):



EH is free enzyme; Ac-X is acyl donor substrate; [E…Ac-X] is Michaelis–Menten acyl–enzyme complex; HX is the release group; Ac-E is acyl–enzyme intermediate, HN is acceptor substrate (nucleophile), Ac-N is product of synthesis (peptide), and Ac-OH is product of hydrolysis.

The acyl donor needs to be activated as an ester or an amide in order to react with the enzyme and form a tetrahedral enzyme–substrate complex [E...Ac-X] which yields the covalent acyl–enzyme intermediate [Ac-E]. The latter can be attacked by water or other nucleophile (HN: amine, alcohol, or thiol), competing for the deacylation reaction. The success of the peptide synthesis will depend on the kinetics of those nucleophilic reactions. Generally, KCS proceeds faster and requires lower enzyme than TCS (Barberis et al. 2002, 2006). Serine or cysteine proteases can be used only as catalyst in the KCS, because they act as transferases of acyl moieties from acyl donor to nucleophile, through an acyl–enzyme intermediate (Quiroga et al. 2005, 2008).

Nevertheless, such reactions will not proceed efficiently if the reaction media is not carefully designed and evaluated. Appropriated and hopefully controlled water activities at low levels are necessary for peptide synthesis. This is the major threat

since proteases are not structurally conditioned in nature to act in such environments. Then, it was necessary to develop the media engineering and the catalyst engineering to overcome these drawbacks.

4.3.5 Media Engineering

Media engineering is related with the rational manipulation of the reaction media in order to find the best condition to the peptide synthesis (Illanes et al. 2012). Frequently, water is replaced for nonconventional media, such as organic solvents, ionic liquids, supercritical fluids, and deep eutectic mixtures.

4.3.5.1 Organic Solvents

Organic solvents, containing a controlled amount of water, can increase the solubility of substrates, especially of hydrophobic amino acids, reduce the hydrolysis of the products, improve the recovery of the products/biocatalyst, and increase the thermal stability of biocatalyst (Illanes et al. 2009a).

There are two types of biocatalytic systems using organic solvents: homogeneous systems, formed by a mixture of water and a miscible organic solvent, and heterogeneous systems in which a second phase is formed by an organic solvent immiscible with water (Barberis et al. 2008). Heterogeneous systems can be macroheterogeneous, if two immiscible liquid phases are evident, or microheterogeneous if one of the two phases (usually the aqueous phase surrounding the enzyme) is not visible to the naked eye. Both, homogeneous and heterogeneous systems, can be carried out with the free enzyme or insolubilized in the reaction medium, either because the enzyme is insoluble in the reaction medium or because it is immobilized in a solid support (carrier) (Illanes et al. 2009b). In this last case, the system will always be heterogeneous in nature.

Miscible organic solvents at moderately high concentrations are usually detrimental to enzyme activity and stability, because they tend to penetrate into aqueous microenvironment surrounding the enzyme molecules, altering the interaction patterns of the enzyme with the media and distorting its three-dimensional structure (Toth et al. 2010; Stepankova et al. 2015; Doukyu and Ogino 2010). However, polyols and glymes are notable exceptions among cosolvents (Castro 2000; Illanes and Fajardo 2001). Another way to circumscribe the problem of enzyme inactivation is the enzyme immobilization. There are several examples of proteases and other immobilized enzymes that form peptide bonds that have been successfully used for the synthesis of peptides in such media (Illanes et al. 2009b; Yazawa and Numata 2014).

Heterogeneous systems, the so-called biphasic systems, are composed of two immiscible liquids which are usually water and a hydrophobic organic solvent (Xu et al. 2013). The substrates can be dissolved in the organic phase or in the aqueous phase, but regardless of the partition of the substrate, the biocatalyst will be always in the aqueous phase. The peptide product formed can partition in the organic

phase, which is highly desirable to avoid its hydrolysis and to lead the equilibrium towards the synthesis (Bordussa 2002). Biphasic systems have been extensively used for the enzymatic synthesis of peptides and represent a good strategy because they are highly flexible and can accommodate the properties of substrates and products (Barberis et al. 2002, 2006, 2008; Trusek-Holownia 2003). The main drawback of biphasic systems is the presence of an interface that can produce diffusional restrictions of substrates, reducing the reaction rate. Although this effect can be improved with intense agitation, it can promote inactivation at the interface (Illanes et al. 2012). However, this effect has not been observed in the synthesis of peptides carried out in our laboratory using indigenous plant proteases (Morcelle del Valle et al. 2006; Quiroga et al. 2008).

The suspension of almost anhydrous enzymes in hydrophobic organic solvents can be considered as a microheterogeneous system, due to the liquid phase looks like homogeneous to the naked eye but it is a microscopically heterogeneous system since the solid enzyme is covered with a thin layer of water tightly bound to it and inside the hydrophobic organic solvent that surrounds it. The enzyme is protected from the hydrophobic organic solvent, which is very aggressive, by a layer of denatured enzyme. This system was considered as the simplest and most promising strategy for the enzymatic synthesis of peptides, since it exploits the greatest advantages of working in unconventional media, mainly high stability, easy to recover the biocatalyst, and sometimes favorable changes in substrate specificities (Klibanov 2001). The biocatalyst is simply acetonic precipitates or lyophilized of the enzyme, which are suspended in an organic medium in which it is completely insoluble (Vossenberg et al. 2012a, 2013). Immobilization is not required because the enzyme is insoluble in the reaction medium although it may be appropriated to offer a greater surface area to the substrate and provide an additional stabilization (Barberis et al. 2008). The best results were obtained with very hydrophobic solvents (log $P > 4$, where P is the partition coefficient between n-octanol and water) because of the organic solvents do not enter into the water layer and consequently, the enzyme it is more protected from direct contact with them. This strategy has serious drawbacks, such as the dramatic decrease of the enzyme activity and the low solubility of the substrate in hydrophobic solvents (Quiroga et al. 2005). However, by adding formamide or ethylene glycol (water-mimetic solvents) to the reaction medium or crown ethers during the preparation of lyophilized enzymes, better results have been achieved (van Unen et al. 2002).

Another system that can be considered as microheterogeneous are the reverse micelles, which are formed spontaneously when small amounts of water are added to the hydrophobic solvent in the presence of a surfactant under agitation (Gómez-Puyou and Gómez-Puyou 1998). However, micelles have several drawbacks: they are mechanically weak, the optimization has not been standardized and the recovery and purification of products is impaired by the surfactant (Bordussa 2002). An alternative to the classical reverse micelles are the micelles formed by water in oil (W/O), but with a high water content (95%) (Clapés et al. 2001).

Conventional organic solvents, in spite of a large number of self-evident advantages as reaction media, are generally volatile, flammable, explosive, and hazardous

to the environment and they can produce acute and chronic toxicity and carcinogenicity. To improve the human health and the environment risks associated with the use of hazardous organic solvents, tremendous efforts have been devoted for to develop alternative green reaction media (Gu 2012).

There is growing interest in developing new environmental friendly solvents with similar or even better properties than organic solvents. Thus, supercritical fluids, ionic liquids, and deep eutectic mixtures have emerged in the field of biocatalysis as new green solvents (Shanab et al. 2013).

4.3.5.2 Ionic Liquids

The ionic liquids (ILs) can be hydrophilic or hydrophobic in nature, and the main advantages that are attributed to them are ease preparation and extraction at the end of the process (Franco-Vega et al. 2014; Cao and Matsuda 2016; Hayes et al. 2015; Sprenger et al. 2015).

ILs are commonly composed of an organic cation (imidazolium, pyrrolidinium, pyridinium, and many other exotic cations) with a variety of substituents and an inorganic or organic anion, such as halides, tetrafluoroborate (BF_4^-), hexafluorophosphate (PF_6^-), bis(trifluoromethane)sulfonimide (NTf_2-), and larger anions containing sulfonyl or fluoroalkyl groups. Dialkylimidazolium cations with BF_4^- and PF_6^- anions are the most classic ILs used in biocatalysis, more specifically 1-alkyl-3-methylimidazolium salts and derivatives (Caminiti and Gontrani 2014; Sudhakar et al. 2012; Hallett and Welton 2011; Domínguez de María 2012).

ILs are classified as green solvents because they have unique physical and chemical properties such as low vapor pressure, lower volatility than organic solvents, no flammability, and high thermal and chemical stability. They are usually viscous liquids with m.p. below 100 °C. Some ILs remain liquids below 400 °C due to their strong ionic interactions (Erbeldinger et al. 2010). Besides, they can be finely tuned by means of structural changes of cation or anion, of polar or nonpolar compounds, in order to improve solvation of reagents which are sparingly soluble in water and in polar organic solvents (Sawant et al. 2011; Earle et al. 2006; Ludwig and Kragl 2007; Ahrenberg et al. 2014; Kosmulski et al. 2004; Chiappe and Rajamani 2011). The solubility of chemical compounds in ILs usually depends on the ability of them to form hydrogen bond with the anions (Novoselov et al. 2007).

Enzymes in ILs have presented higher enantioselectivity, stability, recyclability, and conversion rates than in organic solvents (Muhammad et al. 2010; Jaeger et al. 2015). Activity and operational stability of enzymes in ILs can also be increased by tuning the physicochemical properties of them (Gorke et al. 2010; van Rantwijk and Sheldon 2007).

Madeira Lau et al. (2000) discussed the catalytic activity of lipase B from *Candida antarctica* (CAL-B), free and immobilized, in anhydrous ILs. Other lipases, proteases, oxidoreductases, peroxidases, and entire cells were examined in ILs to test their activities and stabilities, and it was found that they were not denatured or deactivated. The physicochemical properties of ILs play an important role

on the stability, activity, and structure of enzymes. ILs containing anions BF_4^-, PF_6^-, or NTf_2^- allowed to express high enzyme activity, enantioselectivity, recyclability, and conversion rates (Remsing et al. 2008; Diego et al. 2009; Hussain et al. 2008; Braeutigam et al. 2007; Abe et al. 2008).

4.3.5.3 Deep Eutectic Solvents

Deep eutectic solvents (DES) are promising reaction media based on their low melting points, easy availability, biodegradability, recyclability, and low cost. They are usually formed by a quaternary ammonium or metal salt and a simple hydrogen bond donor (HDB), such as acids, amides, amines, and alcohols (Maugeri and Dominguez de Maria 2012).

DES show similar physical–chemical properties to ILs, but they are cheaper and safer solvents than ILs for synthetic biotransformation. They are mostly liquid at or below 100 °C and have higher density and viscosity than water at room temperature, poor ionic conductivity, and high polarity due to the extensive hydrogen bonds that they can form. Melting point is drastically reduced after mixing the two components, as compared to the melting points of the original ones (Khandelwal et al. 2016).

Like ILs, one of the most promising advantages of DES is their extremely low vapor pressure (low volatility) which is very attractive for using in green biocatalytic technologies (Zhang et al. 2012a, b; Abbott et al. 2011; Rub and Konig 2012). Besides, DES can be prepared from readily accessible chemicals and they have low toxicity, especially when they are derived from choline chloride (ChCl) and renewable chemicals. ChCl is a commonly used organic salt for DES since it is biocompatible with most HBD (urea, glycerol, or carboxylic acids) which are cheap and environmentally benign. Moreover, DES do not produce toxic metabolites and they are biodegradable (Smith et al. 2014; Pham et al. 2010; Angell et al. 2012).

An enzymatic process of industrial relevance is the synthesis of the protected *N*-Ac-Phe-Gly-NH2 peptide in different DES (ChCl/Gly, ChCl/urea, ChCl/isosorbide, ChCl/xylitol), catalyzed by α-chymotrypsin (Maugeri et al. 2013). Besides, immobilized papain on a magnetic material was successfully used for the synthesis of *N*-(benzyloxycarbonyl)-alanyl-glutamine (Z-Ala-Gln) in ChCl/urea (1:2) with a yield of 71.5% (Cao et al. 2015).

4.3.5.4 Supercritical Fluids

The supercritical fluids (SCF) are substances with a vapor pressure and temperature above the critical values. The special combination of the liquid and gas properties, such as viscosity and diffusivity of the gases, and density and solvating properties of the liquids, become SCF in excellent solvents for various applications. The processes involving SCF are sustainable, environmental friendly and they have low cost. Their main advantage lays in the possibility of separating and drying the product by simple expansion, while the gas can be recovered, recycled, and reused

without purification steps. The environmental benefits of using SCF in industrial processes, and the low energy consumption during operation, are the main issues for replacing the conventional organic solvents by them. Therefore, SCF are called "green solvents for the future" (Aymonier et al. 2011; Brunner 2010; Loppinet-Serani et al. 2010).

Health and safety benefits are evident in the use of CO_2 and H_2O, the most important SCF. They are noncarcinogenic, nontoxic, nonmutagenic, nonflammable, and thermodynamically stable. Another major benefit refers to the possibility of adjusting the thermophysical properties of SCF, such as diffusivity, viscosity, density, or dielectric constant, by simply varying the operating pressure and/or temperature. Moreover, SCF have excellent heat transfer properties, and they have been studied as healthy environmental heat transfer fluids (Knez et al. 2014). CO_2 is an SCF utilized as an environmentally benign solvent for enzymatic reactions. Surfactant-coated α-chymotrypsin complexes were used to synthesize dipeptides as *N*-acetyl-Phe-OEt and Gly-NH_2 in supercritical CO_2 at 308.2 °K (Mishima et al. 2003).

4.3.5.5 Aqueous Solutions

Finally, an efficient approach for hydrophobic amino acids polymerization in aqueous solution was also developed. The catalysis efficiency of papain and bromelain for oligomer peptides synthesis in aqueous solution was investigated. A set of reaction conditions (protease type, temperature, reaction time, and pH) has been tested for the polymerization reaction of L-phenylalanine methyl ester. Papain was the most efficient biocatalyst in 0.2M phosphate buffer pH 8 at 40 °C, after 3 h (Yu et al. 2016).

4.3.6 Catalyst Engineering

Proteases offer great opportunities as biocatalyst for the peptide synthesis, due to their impressive stereo- and regiospecificity, allowing for the reaction happen without protection of the amino acid lateral chains and with minimal racemization. However, proteases are usually labile enzymes and they must be converted into robust catalysts for industrial processes (Illanes 2016).

Enzyme immobilization is the best approach for increasing the enzyme stability, preventing the modification of its active site, and making possible its separation and reutilization, with considerable advantages for the bioreactor design in continuous processes (Agyei and Shanbhag 2015; Madhu and Chakraborty 2017; Kosseva 2013). However, the main drawbacks in the immobilization process are the loss of enzymatic activity and high costs (Sheldon and van Pelt 2013).

The immobilization methods can be divided into two categories: (1) immobilization in an inert matrix (carrier bound) and (2) free-support immobilization (carrier free) (Cao et al. 2003).

4.3.6.1 Immobilization in an Inert Matrix

4.3.6.1.1 Covalent Immobilization

The covalent bound is based on the activation of chemical groups of the support to react with amino acid residues of the protein. Among the 20 amino acids that make up the structure of the enzymes, the most involved in the formation of bonds with the support are Lys, Cys, Tyr, and His, and to a lesser extent Met, Trp, Arg, Asp, and Gln. The rest of the amino acids are not exposed to the outside of the protein surface due to their hydrophobic nature (Tran and Balkus 2011; Barbosa et al. 2015). This method has high operational stability and is quite flexible. That is the reason why it can be adapted to particular process characteristics (Illanes et al. 2012).

4.3.6.1.2 Immobilization by Adsorption

It is carried out by adsorption of the enzyme on a solid support through van der Waals, electrostatic, and/or hydrophobic interactions. This is a simple method, which does not involve harmful reagents for the environment and the support can be easily recovered after the enzyme has no more activity by the protein desorption. This method leads to high immobilization yields but its main drawback is that the enzyme can easily be desorbed from support, even by slight changes in the reaction medium (Stepankova et al. 2013).

4.3.6.1.3 Immobilization by Entrapment

It consists on the physical retention of the enzyme into the porous of a solid matrix generally constituted by polymers such as polyacrylamide, collagen, alginate, carrageenan, or polyurethane resins. The enzyme entrapment can be carried out inside a gel or into microcavities of a synthetic fiber by occlusion of the protein, which tends to be more resistant than gels. The entrapment is a simple method, requires little amount of enzyme and it does not undergo any alteration in its structure; but the polymerization conditions require a rigorous control in order to avoid alterations of the reactive groups of the protein (Arroyo 1998).

4.3.6.1.4 Immobilization in Nanosupports

It is based on the immobilization of enzymes in nanomaterials; which have well-established characteristics such as pore diameter (5–100 nm), defined geometry, hardness, hydrophobicity/hydrophilicity ratio, magnetic properties, and conductivity, among others; which allows the design of robust biocatalysts with greater biological activity (Illanes et al. 2012). The nanostructures for enzyme immobilization can be particles with spherical shapes, fibers, or tubes (Gutarra et al. 2016).

The main advantage is the high surface/volume ratio of the nanoparticles, allowing high load of enzyme. The lowest the size of the nanovehicle, the highest the exposure of the biocatalyst to the reaction media.

Enzyme immobilization in nanomaterials can be carried out by: enzyme adsorption on the surface, and enzyme encapsulation and entrapment in defined materials (Kim et al. 2010; Zhao et al. 2011).

Magnetic nanoparticles made of iron oxide are the most popular nanostructure for enzyme immobilization since they can be recovered easily by proximity to a magnetic field and reused for multiple cycles. Iron oxide nanoparticles have been used to immobilize a wide variety of enzymes including lipases, cellulases, pectinase, esterase, glucose oxidase, catalase, and others (Feng and Ji 2011). Stolarow et al. (2015) compared synthetic and hydrolytic activities of immobilized trypsin on magnetic microparticles, using both organic solvents and aqueous media. The immobilized enzyme showed 90% and 87% of residual activity after ten cycles in peptide synthesis reactions and in hydrolysis reactions, using 80% (v/v) ethanol and buffered aqueous solution, respectively.

Besides, nonmagnetic nanoparticles made from gold, silica, chitosan, zirconia, and other materials have also been used for enzyme immobilization. Gold nanoparticles are nontoxic and biocompatible, allowing them to be used for medical applications such as drug delivery. Chitosan is used for enzyme immobilization as membranes, fibers, and particles. Nano chitosan has shown good physical and chemical properties such as high surface area, porosity, strength, conductivity, and increased mechanical properties. Silica is largely used to produce enzyme carriers as nanoparticles, porous nanotubes, and mesoporous preparations (Ahmad and Sardar 2015). Carbon nanotubes have been frequently used to immobilize several enzymes because their preparation is relatively simple and they offer great mechanical and thermal stabilities as well as biocompatibility. The enzyme immobilization can be performed by adsorption or covalent bond into graphite tubes of cylindrical shape with diameters up to 100 nm and lengths ranging up to micrometers (Majeric et al. 2012). Cellulose nanofibers have interesting properties when compared with other nanostructures, such as reduced-mass-transfer problems due to their thinness and easy recovery (Dana et al. 2017).

4.3.6.2 Free-support Immobilization

In this method, the enzyme constitutes its own support so that concentrations close to the theoretical packaging limit are obtained (Cao and Schmid 2005). Free-support immobilized enzymes are prepared by chemical cross-linking of the protein, using glutaraldehyde as the main cross-linking agent. This strategy has been applied for the cross-linking of enzymes in solution (CLEs), of enzyme crystals (CLEC), and, more recently, of enzyme aggregates (CLEAs) (Illanes et al. 2012; Vossenberg et al. 2012b).

4.3.6.2.1 Cross-linking of Enzymes in Solution (CLEs)

CLEs are obtained by cross-linking of the dissolved enzymes, through the reaction of the surface NH2 groups with a bifunctional chemical reagent, such as glutaraldehyde (Sheldon and van Pelt 2013). Currently, they are no longer used, mainly due to their poor mechanical properties and severe mass-transfer limitations for industrial applications (Illanes et al. 2012).

4.3.6.2.2 Cross-linking Enzymatic Crystals (CLEC)

CLECs are formed by crystallization of the enzyme protein and subsequent cross-linking with a bifunctional reagent, usually glutaraldehyde (Stepankova et al. 2013; Abraham et al. 2004). They are robust catalysts, highly active, and of controllable particle size, varying from 1 to 100 μm (Sheldon and van Pelt 2013). CLECs are significantly more stable than soluble enzyme to heat denaturation, organic solvents, and proteolysis. Its high operational stability and catalytic productivity as well as its ease of recycling makes them ideal for industrial biotransformation (Amorim Fernandes et al. 2005). However, an inherent disadvantage of CLECs is the need to crystallize the enzyme previously, which translates into high costs for many applications. For this reason, CLECs are no longer available in the market and have been replaced by CLEAs (Fernandez-Lorente et al. 2011).

4.3.6.2.3 Enzymatic Cross-linked Aggregates (CLEAs)

CLEAs are produced by cross-linking enzymatic aggregates obtained by simple conventional techniques of protein precipitation, such as salts (ammonium and sodium sulfate), organic solvents (ethanol and acetone), or nonionic polymers (polyethylene glycol) (Sheldon et al. 2007). CLEAs of multimeric enzymes and combi-CLEAs were also obtained (Wilson et al. 2006a, b). They showed increased stability by prevention of the subunit dissociation and allowed multiple cascade reactions (Dalal et al. 2007). CLEAs have better mechanical properties and higher activity yields than CLEs. Its production is simpler and cheaper because purified and crystallized protein is not required as starting material (Sheldon 2011; Roessl et al. 2010).

4.3.6.3 Choice of the Best Immobilization Method

Although many immobilization techniques have been developed and applied to numerous enzymes, it is recognized that there is no universal method valid for all enzymes in all cases. However, there is a lot of information currently available and some generalizations about immobilization methods can be made, which allow to

Table 4.2 Comparison between different immobilization methods

Method	Entrapment	Cross-linking	Adsorption	Covalent bonding
Preparation	Difficult	Intermediate	Easy	Difficult
Bond strength	Medium	Weak-medium	Medium	Strong
Enzymatic activity	Low	Low	Medium	High
Support regeneration	Impossible	Impossible	Possible	Difficult
Cost of the process	Medium	Medium	Low	High
Stability	High	High	Low	High
Microbial resistance	Yes	Yes	No	No

select the most appropriate method for each specific application. They are summarized in Table 4.2. In general, the more expensive the preparation methods, the higher the stability of the biocatalyst. Simple immobilization methods such as entrapment or adsorption provide weak bonds between enzyme and support, producing catalysts that quickly loss activity and require to be replaced continuously (Arroyo 1998).

4.4 Large-Scale Production of Peptides

Most relevant technologies for peptide production are the extraction from natural sources, the production by recombinant DNA technology, the production by chemical synthesis, and the production by enzymatic synthesis. The size of the peptide determines to a great extent the technology most suitable for its production.

Extraction from natural protein sources is a plentiful reservoir for obtaining functional peptides. Problems associated to the large-scale separation, purification, and characterization of the resulting peptides have precluded a more significant impact of this strategy.

Recombinant DNA technology is quite important for the production of proteins and may be an option for the synthesis of large peptides where alternative technologies may be cumbersome and costly. In the case of small size peptides, repeated sequences of the coding nucleotides have to be cloned to be expressed and even so yields are rather low and the synthesized polypeptide has to be further processed to obtain the peptide product.

Solid-phase chemical synthesis of peptides is certainly the most mature technology, especially for producing medium size peptides (up to one hundred amino acid residues) which encompasses most of the therapeutic and cosmetic peptides. Protocols have been well established, which are amenable for automation and scale-up to match the production levels required by the market. Major drawbacks of chemical synthesis is the number of unit operations required for synthesis since protection and deprotection reactions are required in each step of amino acid addition, and the use of offensive solvents and reagents outside the context of sustainable chemistry, representing nowadays the major challenges that the commercial

production of peptides by chemical synthesis is facing. At the latter scale, costs of reagents involved in the chemical synthesis of peptides are usually high; therefore, the use of large excess, frequently used at laboratory scale and in the early stages of development for saving time and increasing yield, is inadequate for large-scale production since large amounts of reagents cannot be wasted, not only for cost but also for environmental considerations; so, reagents should be used as close to stoichiometry as possible (Bruckdorfer et al. 2004). Productivity and yield of peptide synthesis will have to be balanced with production cost, purity of the product, and security and environmental impact of the process (Guzmán et al. 2007). Process validation is another key aspect at production scale; validation implies reproducibility in terms of yields of intermediate and final products and consistency in the final composition of the product. This is a major threat in peptide synthesis because of the complexity and the number of unit operations involved in the production process. The very stringent requirements for validation, despite the high cost and long time required, stem from the unavoidable need of granting the high standards of quality and safety required by the final consumer (Andersson et al. 2000).

Enzymatic synthesis of peptides is a technological option for the synthesis of small peptides. The enzymatic synthesis, using protease as catalyst and one protected amino acid as substrate, has proven to be a scalable option for producing dipeptides, which is neatly illustrated by the commercial success of the synthesis of the sweetener aspartame using thermolysin (Yagasaki and Hashimoto 2008), and some others like the precursor of the pain reliever kyotorphin with α-chymotrypsin (Bahamondes et al. 2016). When scaling-up to production level, the advantages of enzyme processes in terms of molecular precision (implying less unit operations) and environmental sustainability (potentially low E-factor and high atomic efficiency) have to be confronted with the cost of the enzyme catalyst and the need for avoiding organic solvents as much as possible. These are major technological challenges that are being confronted by protein, catalyst, and media engineering approaches. More robust enzyme structures and immobilized enzyme catalysts are required for the efficient use of the enzyme leading to the decrease of the impact of the catalyst cost on the peptide production cost. Use of organic solvents, which are required to depress the activity of water and the hydrolytic potential of proteases (Vossenberg et al. 2013), is contradicting the principles of green chemistry which enzymatic processes are supposed to fulfill so that their replacement by neoteric solvents and other environmentally sound nonconventional reaction media is also another important challenge to be dealt with.

4.5 Concluding Remarks

Enzyme biocatalysis has evolved from reactions of molecular cleavage in aqueous medium, catalyzed mostly by hydrolases dissolved in the reaction medium, to reactions of molecular synthesis in mostly nonconventional media. The latter have a higher potential added value, so most research efforts in recent decades have been

devoted to establishing technological platforms for biocatalysis in nonaqueous media: organic solvents, ionic liquids, supercritical fluids, and deep eutectic solvents. Robust and readily available hydrolases can catalyze the reverse reactions of synthesis when performed in low water activity environments, opening up the opportunity for these well-known catalysts to be used in high added value processes for the synthesis of fine chemicals, pharmaceuticals, and other high-value bioactive molecules (Illanes 2016).

Several strategies of enzyme stabilization and activation have been developed and successfully applied to reactions in organic synthesis of potential industrial interest. A variety of enzymes produced by recombinant techniques, the development of mimetic substrates, and new reaction media have broadened the scope of enzymatic peptide synthesis (Yazawa and Numata 2014). Enzyme immobilization onto different carriers is the most usual strategy for increasing enzyme stability and reusability in biotechnological and pharmaceutical applications. However, some immobilization techniques are associated with loss of enzymatic specificity and/or activity, due probably to mass transport limitations or enzyme structural changes. For this reason, the most appropriated immobilization method must be carefully selected.

The latest scientific reports focus on the extremophile proteases as catalysts, due to their synthetic potential does not seem to have been fully appreciated to date. The structure of these proteins is somewhat different from that of commercial enzymes, making them effective at high salinity and high or low temperatures, which are often favorable to peptide synthesis. Examples of such enzymes include halophilic, thermophilic, and psychrophilic proteases (Białkowska et al. 2017).

As example, in order to explore the use of proteases from thermophiles for peptide synthesis under such conditions, putative protease genes of the subtilase class were cloned from *Thermus aquaticus* and *Deinococcus geothermalis* and expressed in *Escherichia coli*. The purified enzymes were highly thermostable and catalyzed efficiently the peptide bond synthesis at 80 °C in neat acetonitrile with excellent conversion (>90%). The enzymes keep high activity in (40–50% v/v) *N,N*-dimethylformamide, which improved substrate solubility and allowed good yields in 5+3 peptide condensation reactions. According to these results, proteases from thermophiles are promising biocatalysts to be used for peptide synthesis under harsh reaction conditions (Toplak et al. 2015).

The most relevant industrial application of peptide synthesis catalyzed by proteases is the noncaloric sweetener aspartame. A solvent-stable protease from the high-yield protease producer *Pseudomonas aeruginosa* PT121, and its mutant Y114S, enabled the pH modulation of the reaction medium to shift the thermodynamic equilibrium towards product synthesis. At lower pH, the higher solubility of the substrates (L-Phe.OMe and Z-L-Asp) was obtained, while the solubility of the product was dramatically lowered, allowing in situ product removal. The reaction-separation coupling provided the driving force for the enzymatic synthesis and resulted in high yields of 88.5%, without further purification for removing protection group of Z-aspartame (Liu et al. 2015).

The recent advances in peptide macrocycles as promising therapeutics create a need for novel methodologies for their efficient synthesis and large-scale production. Within this context, enzyme-mediated methodologies have gained great interest. Enzymes such as sortase A, butelase 1, peptiligase, and omniligase-1 represent extremely powerful and valuable enzymatic tools for peptide ligation, since they can be applied to generate complex cyclic peptides with exquisite biological activity. Therefore, the use of enzymatic strategies will effectively supplement the scope of existing chemical methodologies and will accelerate the development of future cyclic peptide therapeutics (Schmidt et al. 2017).

Lack of specificity and environmental burden associated to the chemical synthesis of peptides can in principle be overcome by enzyme biocatalysis, but strategies for enzymatic synthesis are for the most part in developmental stage and no protocols exist for validation and scale-up. In practice, this means that only small peptides (mostly dipeptides) can be efficiently synthesized in a cost-effective manner. Efforts for the enzymatic synthesis of larger peptides have been mostly unsuccessful since different enzymes and reaction protocols have to be used in each step, which is a complex task (Fité et al. 2002). This situation may evolve in the near future associated with the impressive developments in enzyme biocatalysis. Combination of chemical and enzymatic synthesis has proven to be a valuable technological option in organic synthesis since the good properties of each technology can be synergistically used in the context of one process objective (Clouthier and Pelletier 2012). This strategy is certainly applicable to the synthesis of peptides (Hou et al. 2005; Baker and Numata 2012).

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Chapter 5
Plant Proteolytic Enzymes: Their Role as Natural Pharmacophores

Carlos E. Salas, Dalton Dittz, and Maria-Jose Torres

Contents

5.1 Introduction

Proteases or proteinases are enzymes that catalyze cleavage of proteins at peptide bonds generating smaller peptides. Some of them are very specific in their choice of target site while others act rather nonspecifically and hydrolyze the protein substrate if conditions allow into short peptides. They must have appeared early in evolution along with proteins, to keep a balance between synthesis and protein degradation. Their early emergence is confirmed by their ubiquitous presence in most living forms including viruses, plants, and animals.

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Proteases involve two groups of enzymes: exoproteases, which cleave the initial or terminal peptide bonds of substrates, designated as amino- or carboxy-peptidases, respectively, and endoproteinases cleaving at inner bonds of the protein.

Classification of proteases is based upon the type of residue relevant at the active site. The hydroxyl group of serine or threonine proteinases and the cysteine group in cysteine proteinases are the nucleophiles during catalysis of these groups of endoproteinases, while activated water is the nucleophile for aspartic-, glutamic-, and metalloproteinases.

As of December 04, 2017, the database (release 12.0) lists 912,290 sequences and provides links to 1022 PDB (Protein Data Bank) (http://www.pdb.org/pdb/home/home.do) entries distributed among 268 families and 62 clans.

The MEROPS database (https://www.ebi.ac.uk/merops/), is a resource for annotation of proteases, their substrates, and their inhibitors. This database is hosted at EMBL-EBI since 2017. PANTHER (*P*rotein *A*nalysis *T*hrough *E*volutionary *R*elationships) (http://www.pantherdb.org/) is another tool that classifies proteases by families and subfamilies, their biochemical function, the biological processes, and metabolic pathways in which participate to facilitate high-throughput analysis.

Most annotated sequences belong to the serine group (36.6%), followed by metallo- (32.6%), cysteine-, (19.5%), aspartic-, (4.9%), threonine- (3.8%), plus minor groups including mixed-type proteases, and unclassified and unknown sequences.

As stated above, proteases like most proteins must have arisen early in evolution since primeval life forms required them for the digestion of food. In fact, a comparison of digestive proteases in evolved species shows similarities with those found in prokaryotic organisms arguing for a common origin.

Once proteins became constituents of cellular structure, they were required to metabolize endogenous proteins. With advances in genomic sequencing, it is now estimated that about 2% of structural genes in an organism code for these enzymes (Barrett and Fred Woessner 2013). For instance, it is estimated the presence in rice (*Oryza sativa*) of >650 genes and in *Arabidopsis thaliana* >800 genes coding for proteases (van der Hoorn 2008). A similar distribution is projected in animal organisms. Despite these impressive figures, the lack of information about their physiological function and their cognate substrates limit our knowledge of these enzymes. A further problem during dissection of a single proteolytic activity is encountered as most proteolytic enzymes act as a group of related members for a reaction(s) to take place, thus coining the concept of "protease web" (Fortelny et al. 2014).

In plants, proteolytic enzymes are found in most cell compartments participating in most stages of plant life, including growth, homeostasis, germination, breakdown of storage proteins during seed germination, organ senescence, and programmed cell death (Huffaker 1990).

An important function is their involvement in the proteasome proteolytic pathway affecting several metabolic processes, such as hormone signaling, cell cycle, embryogenesis, morphogenesis, and plant-environment interactions (van Wijk 2015).

A major source of proteolytic activities is detected in latex producing plants. Laticifers are specialized cells found in 20 plant families from angiosperm orders representing between 15 and 20,000 species (Lewinsohn 1991). The cytoplasm of laticifers stores latex containing defense metabolites which are released in response to physical damage. By analogy, the proteolytic enzymes found in some laticifers resemble the functionality of proteolytic enzymes present in mammalian circulatory system.

In this review, that focuses in the last ten years, we present evidence supporting the role of plant proteolytic enzymes as therapeutic options to treat several symptoms. Two early compilations described the advances in this field in 2008 (Domsalla and Melzig 2008; Salas et al. 2008) and recently, while we were preparing this review a revision covered this subject (Balakireva et al. 2017).

It is well established that species of Caricaceae family (*C. papaya* and *C. pubescens, syn C. candamarcensis, syn Vasconcellea pubescens,* and *syn V. cundinamarcensis*) have been used ethnopharmacologically to treat digestive disorders and skin fungal ailments by native American populations (Soplin et al. 1995). Similarly, *A. comosus* the source of bromelains was used as medicine by indigenous cultures and in 1957 Heinicke and Gortner (1957) initiated its therapeutic use. In this revision, we mainly focus on the scientific developments within this field covering the last decade. A listing of pharmacological applications discussed here is shown in Table 5.1.

5.2 Proteolytic Enzymes Inflammation and Immunomodulation

Inflammation and immunomodulation are intertwined processes in which the immune response, both innate and adaptive lead to inflammatory outcomes. The plant proteases (papain, bromelain, ficin, etc.) have been used as anti-inflammatory and especially bromelain is used as alternative and/or complementary therapy to glucocorticoids, nonsteroidal antirheumatics, and immunomodulators.

For instance, different doses of the cysteine proteinases from *Bromelia hieronymi* and bromelain used as reference on carrageenan- and serotonin-induced rat paw edema, as well as cotton pellet granuloma model, induced 40–50% inhibition of the inflammatory effect. If the enzyme fractions were treated with E-64 (cysteine proteases inhibitor), the anti-inflammatory effect disappeared, demonstrating that the effect was mediated by cysteinyl enzymes (Errasti et al. 2013). A similar result was observed using the proteolytic fraction of *V. cundinamarcensis* using the rat paw edema model. The anti-inflammatory response was equivalent to the dexamethasone positive control (unpublished data).

The anti-inflammatory action of bromelain has been linked to decreased secretion of pro-inflammatory cytokines and chemokines [granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN-γ, CCL4/macrophage inhibitory protein

Table 5.1 List of plant proteolytic enzymes with pharmacological applications

Protease	Species	Biological activity	Reference
Bromelain, CP	*Ananas comosus*	Dermatology	Ho et al. (2016)
Bromelain, CP	*Ananas comosus*	Chronic rhinosinusitis	Büttner et al. (2013)
Bromelain, CP	*Ananas comosus*	Anti-inflammatory	Shing et al. (2015)
			Amini et al. (2013)
			Secor et al. (2012)
CP	*Bromelia hieronymi*	Anti-inflammatory	Errasti et al. (2013)
Bromelain, CP	*Ananas comosus*	Digestive disorders	Zhou et al. (2017)
			Sahbaz et al. (2015)
Papain, CP	*Carica papaya*	Digestive disorders	Levecke et al. (2014)
			Mansur et al. (2014)
			Luoga et al. (2015)
Bromelain, CP	*Ananas comosus*	Wound healing/ mitogenic activity	Rosenberg et al. (2012)
			Cordts et al. (2016)
			Golezar (2016)
			Wu et al. (2012)
			Aichele et al. (2013)
			Iram et al. (2017)
SP	*Wrightia tinctoria R. Br. (Apocynaceae)*	Wound healing/ mitogenic activity	Yariswamy et al. (2013)
P1G10, CP	*Vasconcellea cundinamarcensis*	Wound healing/ mitogenic activity	Freitas et al. (2017)
			Araujo e Silva et al. (2015)
Papain, CP	*Carica papaya*	Wound healing/ mitogenic activity	Aranya et al. (2012)
			Chen et al. (2017)
			Shoba et al. (2017)
Heynein, CP	*Ervatamia heyneana latex*	Thrombolytic activity	Uday et al. (2017)
SP	*Solanum tuberosum* leaves	Thrombolytic activity	Pepe et al. (2016)
	Euphorbia cf. lactea latex		Siritapetawee et al. (2015)
	Petasites japonicus		Kim et al. (2015)
	Curcuma aromatica Salisb		Shivalingu et al. (2016)
MP-like	*Aster yomena (Kitam.) Honda*	Thrombolytic activity	Choi et al. (2014)
P1G10, CP	*Carica candamarcensis*	Thrombolytic activity	Bilheiro et al. (2013)
CP	*Cnidoscolus urens (L.)* leaves	Thrombolytic activity	de Menezes et al. (2014)

(continued)

Table 5.1 (continued)

Protease	Species	Biological activity	Reference
CP	*Pseudananas macrodontes* *Bromelia balansae* *Bromelia hieronymi*	Thrombolytic activity	Errasti et al. (2016)
Bromelain, CP	*Ananas comosus*	Antitumoral	Amini et al. (2013)
			Pillai et al. (2013)
			Romano et al. (2014)
			Miranda et al. (2017)
			Bhatnagar et al. (2015)
			Pillai et al. (2013)
P1G10, CP	*Vasconcellea cundinamarcensis*	Antitumoral	Dittz et al. (2015)
SP	*Asian pumpkin*	Bioactive peptides	Dąbrowska et al. (2013)
Papain, CP	*Carica papaya*	Bioactive peptides	Wang et al. (2012)
Papain–Bromelain, CP	*Ananas comosus*/ *Carica papaya*	Bioactive peptides	Gajanan et al. (2016)
Bromelain, CP	*Ananas comosus*	Bioactive peptides	Li-Chan et al. (2012)
CP	Latex *Jacaratia corumbensis*	Bioactive peptides	Arruda et al. (2012)
Bromelain, CP	*Ananas comosus*	Antibacterial	Pu and Tang (2017)
Papain, CP	*Carica papaya*	Antifibrotic	Sahu et al. (2017)
Papain, CP	*Carica papaya*	Drug carrier	Menzel and Bernkop-Schnürch (2018)
Bromelain, CP	*Ananas comosus*	Drug carrier	Parodi et al. (2014)
Papain, CP nanoparticles	*Carica papaya*	Antibacterial	Atacan et al. (2018)
Papain/Bromelain, CP	*Carica papaya/Ananas comosus*	Oral applications	Waleed Majid and Al-Mashhadani (2014)
			Ordesi et al. (2014)
			Divya et al. (2015)
			Mugita et al. (2017)
			Sahana et al. (2016)
			Tadikonda et al. (2017)
			Motta et al. (2014)
			Abdul Khalek et al. (2017)
			Patil et al. (2015)

(continued)

Table 5.1 (continued)

Protease	Species	Biological activity	Reference
Protease inhibitor			
Kunitz-type inhibitor	*Tamarindus indica L.* seeds	Biotechnological health-related application.	Medeiros et al. (2018)
Kunitz family of protease inhibitors	–	Antithrombogenic	Salu et al. (2018)
Trypsin and chymotrypsin inhibitors	*Erythrina velutina* seeds	Gastroprotective antielastase	Oliveira de Lima et al. (2017)
Purified protease inhibitors LC-pi I, II, III, and IV	*Lavatera cashmeriana*	Anticancer activity	Rakashanda et al. (2015)

CP cysteine protease, *SP* serine protease, *MP* metalloprotease

(MIP-1ß)], and TNF by inflamed tissue in inflammatory bowel disease using an in vitro human colon model. Bromelain also enhanced the expression of partly inflammatory cytokines IL-2 and IL-4 and IFN-γ leukocytes (Onken et al. 2008).

Fitzhugh et al. (2008) reported that bromelain decreases neutrophil migration during acute inflammation and specifically removes chemokine receptor CD128 suggesting a reduction in leukocyte binding to blood vessels that consequently impairs cell extravasation. Leukocyte migration is viewed as crucial for the inflammatory response. Furthermore, in some cases bromelain is being used to treat inflammatory symptoms in osteoarthritis or asthma (Brien et al. 2006; Secor et al. 2012).

Instead, it is not clear if papain shares the anti-inflammatory effect attributed to bromelain-like cysteine proteases, as a report shows that this protease activates human mast cells via PAR-2 cell receptors. The induced activation of mast cell is related to release of tryptase and ß-hexosaminidase (Seaf et al. 2016). Also, papain is currently used as a model substance to develop experimental osteoarthritis, lung inflammation, and rhinosinusitis, and all these conditions are associated to inflammatory processes (Patel et al. 2015; Agoro et al. 2016; Tharakan et al. 2018). Also, epicutaneous administration of papain may induce a dysfunction of the skin barrier and increase circulatory IgE and IgG. In this study, the presence of papain in serum is confirmed by identification of papain-specific IGs antibodies (Iida et al. 2014). These data suggest that papain may act as inflammatory protease departing from other cysteine proteases. Meanwhile, topical application of papain to volunteers stung by jellyfish *Chrysaora chinensis* showed pain remission and inhibition of nematocyst discharge (DeClerck et al. 2016) and another report described that papain at doses of 0.325 and 0.75 mg/kg possesses marked anti-inflammatory action against infectious arthritis, like butadion and indomethacin (Rakhimov 2001). Meanwhile, excepting for an early report describing allergic reactions and asthma during occupational exposure to bromelain (Baur and Fruhmann 1979), no additional evidence of inflammatory effect induced by bromelain was described. More recently,

Dutta and Bhattacharyya (2013) rule out the presence of toxic substances in *A. comosus* crown-leaf extracts containing bromelain. It is paradoxical that bromelain is used to treat symptoms, some of which appear to be triggered by papain. Since both papain and bromelain belong to the same group of cysteine proteases (C1A), it is conjectured that unaccounted structural differences must exist between these proteases that justify their different biological role. It is also possible that papain and/or bromelain containing fractions contain contaminating proteolytic isoforms responsible for these "unexpected" effects.

A condition known as endometriosis displaying inflammatory symptoms and affecting around 10% of females is apparently caused by estrogen in which endometrium-like tissue grows outside the uterine cavity (Eskenazi and Warner 1997). The disease is accompanied by increased level of inflammatory cytokines IL-1β, IL-6 (Harada et al. 1997), IL-8, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) (Burney and Giudice 2012). Bromelain has been used in combination with N-acetyl cysteine and α-lipoic acid as successful treatment for endometrioses both in vitro and in a rodent model (Agostinis et al. 2015).

5.3 Digestive Disorders

The efficacy of proteinases has been studied at least in three digestive disorders: ulcerative colitis, inflammatory bowel disease, and Crohn's disease. Oral bromelain was initially reported to reduce the severity of colon inflammation in a rodent model (Hale et al. 2005) and fresh pineapple juice decreases inflammation in IL-10-deficient mice with colitis (Hale et al. 2010). Colon biopsies from patients with ulcerative colitis and Crohn's disease had decreased levels of inflammatory cytokines if treated with bromelain (Onken et al. 2008). Meanwhile, bromelain effectively decreases neutrophil migration to sites of acute inflammation and support the specific removal of the CD128 chemokine receptor responsible for activation of IL-8 (Fitzhugh et al. 2008). A recent report suggests participation of TNF-α receptors in the anti-inflammatory effect of bromelain (Zhou et al. 2017). In a related study, papain applied peritoneally or oral bromelain was used to reduce or prevent intraperitoneal adhesions mainly resulting from abdominal surgery (Ochsner and Storck 1936; Sahbaz et al. 2015). However, Stevens (1968) could not confirm the protective effect of papain in an animal model.

Intestinal disorders caused by parasites have been treated with proteolytic plant enzymes. The anthelmintic efficacy of papain and bromelain against rodent cestodes *Hymenolepis diminuta* and *Hymenolepis microstoma* and *Trichuris suis* was demonstrated in vitro and in vivo (Levecke et al. 2014; Mansur et al. 2014; Luoga et al. 2015). In a study analyzing the efficacy of bromelain, actinidin, and papain against *Heligmosomoides bakeri,* papain was more efficacious than bromelain or actinidin as anthelmintic (Luoga et al. 2015).

5.4 Wound Healing and Mitogenic Activity

Initially, the ethnopharmacological properties attributed to plant proteases from the genus Caricaceae were: wound healing of fungal or viral lesions and resolution of digestive problems (Soplin et al. 1995). In most instances, the proteases bromelain and papain, alone or in combination, are nowadays applied in commercial formulations (NexoBrid™ (NXB, Debriding Gel Dressing-DGD, Debrase®) or as isolated active complex fractions in surgical wounds, experimentally induced wounds or burns, as healing enhancers (Singer et al. 2010; Rosenberg et al. 2012; Rosenberg et al. 2014; Cordts et al. 2016; Schulz et al. 2017).

Bromelain has been used to treat symptoms linked to healing: to reduce pain and manage healing after episiotomy (Golezar 2016), improving healing caused by firearm wounds (Wu et al. 2012) and healing of acute crush tendon injury (Aiyegbusi et al. 2010). For a recent review covering the surgical applications of bromelain, we refer to Muhammad and Ahmad (2017). In addition, other proteins like a serine protease from *Wrightia tinctoria* R. Br enhances healing in experimental wound incisions in mice (Yariswamy et al. 2013), and latex of rubber tree *Hevea brasiliensis* increased vascular permeability, angiogenesis, and wound healing in animal model (Mendonça et al. 2010).

Studies by our group using P1G10, the proteinase fraction from *V. cundinamarcensis* (equivalent to bromelain or papain) show that its topical application increases healing, in dermabrasion (Lemos et al. 2011), burns (Gomes et al. 2010), and incisional (Freitas et al. 2017) skin models. It also enhances protection and healing of induced gastric ulcers in animal model (Mello et al. 2008; Araujo e Silva et al. 2015). Interestingly, we observed that except for the dermabrasion model, in the other injury models the proteolytic activity is required to achieve efficacy. Also, along with the debriding effect, there is a mitogenic stimulus at the wounding site. The mitogenic property was demonstrated earlier in two of the isoforms (CMS2MS2, CMS2MS3) present in P1G10 fraction (Gomes et al. 2005). We demonstrated that the mitogenic effect found in P1G10 is independent of the proteolytic activity, as CMS2MS2 inhibited with iodoacetamide retained the mitogenic activity (Gomes et al. 2009). Therefore, we proposed that along with the debriding action induced by the proteolytic activity, a mitogenic stimulus triggered by these isoforms is contributing to the healing process (Freitas et al. 2017). Remarkably, almost 50 years before, a study anticipated the mitogenic activity of bromelain, but no further reports confirmed or rebutted this finding (Zetter et al. 1976). Meanwhile, a mitogenic action has been described in thrombin, the serine protease involved in the coagulation cascade, which acts as a mitogen in many cells, following proteolytic cleavage and activation of its cognate PAR receptor (Déry et al. 1998). Also, a cysteine protease in plerocercoid *Spirometra mansonoides* displays growth hormone-like properties (Phares and Kubik 1996). Therefore, involvement of proteases from different sources in proliferative effects is part of the function repertoire. The detailed analysis of structural features of purified proteases unrelated to the canonical proteolytic role is required to identify these novel biological actions.

5.5 Thrombolytic Activity

The thrombolytic role of plant proteases can be examined in two ways; by direct action of proteases within the circulatory network or by indirect action through cleavage of target protein substrates releasing peptides with antithrombotic or prothrombotic action. The first group encompasses two activities; procoagulant and anticoagulant, yet, there is a third group of enzymes which can act both as procoagulant and as anticoagulant depending on the concentration used in the assay.

The thrombolytic effect of plant cysteine proteases has been demonstrated in many cases; heynein—a protease from *Ervatamia heyneana* latex (Uday et al. 2017), a serine-like protease from *Solanum tuberosum* (StSBTc-3) (Pepe et al. 2016), a glycosylated serine protease from *Euphorbia cf. lactea* latex (Siritapetawee et al. 2015), a serine-like protease from *Petasites japonicas* (Kim et al. 2015), a metalloprotease-like enzyme from the edible and medicinal plant *Aster yomena* Kitamura-Honda (Choi et al. 2014), and the proteolytic fraction P1G10 from *V. cundinamarcensis* (Bilheiro et al. 2013). On the other hand, a procoagulant activity was described in a serine protease from *Curcuma aromatica* Salisb (Shivalingu et al. 2016), a cysteine protease in *Cnidoscolus urens* (L.) leaves (de Menezes et al. 2014), and in a thrombin-like activity in latex of *Asclepias curassavica* L. (Shivaprasad et al. 2009). Meanwhile, both prothrombotic and thrombolytic activity have been described in cysteine proteases from *Bromelia balansae, Pseudananas macrodontes*, and *B. hieronymi* (Errasti et al. 2016). In sum, plant proteolytic enzymes can act both as procoagulant and as anticoagulant factors.

5.6 Antitumoral

Proteases from different families and sources have been traditionally used in folk medicine for tumor treatment (Guimarães-Ferreira et al. 2007; Beuth 2008). Cysteine endoproteinases such as bromelain and papain and serine endopeptidases such as trypsin or chymotrypsin, alone or in combination, are some of the proteases with described antitumor activity (Beuth 2008). Despite the studies about their activity in cancer, the underlying mechanism of action is unclear.

Among the proteases with antitumor property, bromelain is the best studied. Its activities include modulation of cell adhesion molecules, reduction of reactive oxygen species (ROS) formation, antiproliferative effect, and induction of apoptosis. Amini et al. (2013) showed that bromelain reduced glycoprotein MUC1 in cells of gastric carcinoma. This adhesion molecule provides invasive, metastatic, and chemoresistant properties to tumor cells. When exposed to bromelain, cells with high level of MUC1 (such as gastric, pancreatic, and breast cells) displayed reduced survival as result of a cascade mediated by ER, EGFR, and PDGFR, as these receptors are stabilized by the external domain of MUC1 (Pillai et al. 2013).

In Caco-2 cells, bromelain also reduced ROS production, which is linked to gastrointestinal tumor development. This activity was observed in a concentration and proteolytically dependent manner, since inhibition of proteolytic activity by iodoacetamide did not decrease ROS production (Romano et al. 2014). Recently, the bromelain antiproliferative effect has been reported in murine melanoma as well as human gastric carcinoma and colon adenocarcinoma cells (Amini et al. 2013; Romano et al. 2014; Miranda et al. 2017).

In B16F10, a highly metastatic murine melanoma cell line, 50 and 25 μg/mL bromelain dose inhibited proliferation at 99.4% and 51.7%, respectively (Miranda et al. 2017). Bromelain also reduced the proliferation in human gastric carcinoma cell lines (KATO-III and MKN45) and in two chemoresistant subpopulations of colon adenocarcinoma (HT29-5M21 and HT29-5F12) with half-maximal inhibitory concentration at 142, 94, 34, and 29 μg/mL, respectively. In MKN45 cells, treatment with bromelain (100 and 200 μg/mL) up to 72 h interrupted Akt signaling pathway (Amini et al. 2013). Both bromelain (3 μg/mL) and iodoacetamide inactivated bromelain (1 μg/mL) reduced proliferation of human colon adenocarcinoma cells (Caco-2) suggesting that proteolytic activity is not involved in antiproliferative effect in this cell line. The downregulation of p-Akt/Akt, ERK, and total expression of p-ERK1/2/, as well as reduction of ROS production was associated to the antiproliferative effect of bromelain (Romano et al. 2014).

The proapoptotic effect of bromelain is largely described in different tumor cell lines. In MCF-7 (human breast carcinoma) cells, bromelain induced autophagy, positively regulated by p38 and JNK but, negatively regulated by ERK1/2 and ensued by apoptosis. This effect is evidenced by chromatin condensation, DNA fragmentation, and nuclear cleavage (Bhui et al. 2010). Likewise, an increase in caspase-9 and caspase-3 activity was observed when GI-101A (human breast carcinoma cells) were exposed to bromelain for 24 h in a dose-dependent manner (5, 10, and 20 μg/mL) achieving 95% of cell death at the highest concentration. The increase in caspase activity was related to an increase of cleaved cytokeratin 18 (CK18), a caspase substrate, and DNA fragmentation (Dhandayuthapani et al. 2012). At 1 and 10 μg/mL, bromelain, but not proteolytically inactive bromelain, increased caspase 3 and 7 in Caco-2 cells. In these cells, the proapoptotic effect of bromelain was not a consequence of its antiproliferative activity, since the protease at 1 μg/mL did not inhibit Caco-2 proliferation (Romano et al. 2014). In gastric cancer cells (MKN45), bromelain reduced levels of Bcl-2, activated the caspase system, and led to an overexpression of cytochrome c which, in association to a reduction in phospho-Akt, contributed to cell death (Amini et al. 2013).

Bromelain also shows a chemopreventive effect in a murine model of colon and skin cancer (Romano et al. 2014; Bhatnagar et al. 2015). In mice colon cancer induced with azoxymethane, intraperitoneal (IP) bromelain reduced crypt foci, polyps, and tumors at 1 mg/kg dose. This dose is 40-fold lower than the documented lethal IP dose of bromelain (Romano et al. 2014). Nanoparticles of bromelain (loaded with lactic-co-glycolic acid) also have chemopreventive action in anthracene-induced skin carcinogenesis murine model. In this model, topical application of formulated nanoparticles containing bromelain delayed onset of

tumorigeneses about 4 weeks, plus mortality rate as well as tumor volume were decreased by 70% and 45%, respectively (Bhatnagar et al. 2015). Compared to the chemotherapeutic effect in animals treated after tumor induction, the chemopreventive activity of bromelain nanoparticles was higher using a 10-fold lower dose than the protocol using free bromelain. Also, bromelain nanoparticles enhanced DNA protection from DMBA-induced damage, as assessed by alkaline unwinding assay and upregulating proapoptotic protein p53 and BAX and downregulating Bcl-2 antiapoptotic protein (Bhatnagar et al. 2015).

Müller et al. (2016) compared the antitumor effects of bromelain and papain in cholangiocarcinoma (CC) cell lines. Both proteases decreased proliferation, invasion, and migration of tumor cells acting downstream of NFkB/AMPK pathway, though bromelain was more effective than papain. Apoptosis was induced after bromelain and papain treatment, attaining 70% and 50% of cell death for bromelain and papain, respectively. Bromelain, but not papain, increased E-cadherin and downregulated N-cadherin in CC cell lines, in a dose- and time-dependent manner, demonstrating an inhibitory effect during the epithelial–mesenchymal transition.

In studies using the proteolytic fraction P1G10, from *V. cundinamarcensis* we confirmed the antitumoral effect. Mice bearing B16F1, a low metastasizing melanoma, subcutaneous injection of 5 mg/kg of P1G10 reduced ~70% the tumor mass and survival rate increased ~97% compared to the control. In tumor, P1G10 reduced hemoglobin and vascular endothelial growth factor (VEGF), resulting in antiangiogenic effect, and increased N-acetylglucosaminidase (NAG) which was linked to macrophage activation. P1G10 also induced around 60% of DNA fragmentation in B16F1 cells after 24 h of exposure (50 μg/mL) leading to apoptosis, as pretreatment with the pan-caspase inhibitor (ZVAD) abolishes this effect. A cell rounding and reduced ability to adhere to ECM components was initially observed after 15 min exposure to P1G10 (Dittz et al. 2015).

In clinical trials, there are evidences that proteases improve cancer treatment as a complementary systemic enzyme therapy. Stage II clinical studies demonstrate that systemic enzyme therapy (trypsin, chymotrypsin, and papain) decrease tumor—and/or side effects—therapy induced in plasmocytoma, and breast and colorectal cancer patients (Beuth 2008). These findings motivate further studies to unravel the mechanism underlying the antitumoral effect of plant proteases.

5.7 Production of Bioactive Peptides

Enzymatic hydrolysis to produce bioactive peptides with nutraceutical activity is an area of intense research. Use of plant proteases for production of bioactive peptides from food proteins is being investigated. Papain, bromelain, ficin, or pumpkin serine protease have been used separately or in combination with other proteolytic enzymes to produce antihypertensive peptides inhibiting the angiotensin-converting enzyme (Tavares et al. 2011), peptides with antithrombotic activity whose efficacy was like aspirin (Shimizu et al. 2009), peptides with antioxidant activity (Wang

et al. 2012; Gajanan et al. 2016), peptides that inhibit dipeptidyl-aminopeptidase IV and a-glucosidase during diabetes (Li-Chan et al. 2012; Lacroix and Li-Chan 2012; Nongonierma and FitzGerald 2014), peptides with hypolipidemic hypocholesterolemic activity (Morimatsu et al. 1996), antimicrobial peptides (Salampessy et al. 2010; Arruda et al. 2012; Dąbrowska et al. 2013), and immunomodulating peptides (Kong et al. 2008) are described. We refer here to a recent report reviewing the production of bioactive peptides (Mazorra-Manzano et al. 2017).

5.8 Other Applications

Without doubt, the applications of proteolytic enzymes are not restricted to the uses described above. New promising applications appear in the literature and are mentioned now.

Liposomes or nanoparticles to deliver proteolytic enzymes or products obtained by hydrolysis with plant proteases have been recently developed and described here: fabrication of core-shell nanofibers for controlled delivery of bromelain and salvianolic acid B for skin regeneration used in wound therapeutics (Shoba et al. 2017) magnetic nanoparticles containing papain as antibacterial (Atacan et al. 2018), papain-containing liposomes for treatment of skin fibrosis resulting from second degree burn (Sahu et al. 2017), a liposome for skin application of papain on hypertrophic scar (Chen et al. 2017), liposomes containing papain hydrolyzed bioactive peptides with antioxidative and ACE-inhibitory properties, from bean seeds protein (Chay et al. 2015), a bromelain hydrolyzed antibacterial liposomal peptide from rice bran protein against *Listeria monocytogenes* (Pu and Tang 2017), antihypertensive biopeptides from stone fish (*Actinopyga lecanora*) protein hydrolyzed with bromelain and stabilized by encapsulation in chitosan nanoparticles (Auwal et al. 2017), nanoparticles as well as self-emulsifying drug delivery systems displaying papain or bromelain to cleave mucin (Menzel and Bernkop-Schnürch 2018), encapsulated gold nanoparticles containing bromelain, cisplatin, and doxorubicin for treatment of osteosarcoma (Iram et al. 2017), bromelain hybrid nanoparticles on lactobionic acid conjugated to chitosan in an antitumoral study (Wei et al. 2017), nanofibers for delivery of bromelain and salvianolic acid B during skin regeneration after wounding (Shoba et al. 2017), bromelain-functionalized lipid-core nanocapsules to investigate their effect against human breast cancer cells (Oliveira et al. 2017), hyaluronic acid nanoparticles to enhance targeted delivery of bromelain in Ehrlich's ascites carcinoma (Bhatnagar et al. 2016), katira gum nanoparticles to enhance the anti-inflammatory effect of bromelain (Bernela et al. 2016), to enhance the therapeutic effect of the antibiotic levofloxacin (Bagga et al. 2016), as nanoparticles to protect against 7,12-dimethylbenz[a]anthracene-induced skin carcinogenesis (Bhatnagar et al. 2015), as oral anticancer treatment formulated as nanoparticles (Bhatnagar et al. 2014), to enhance diffusion of silica nanoparticles at the tumor

extracellular matrix (Parodi et al. 2014), and both papain and bromelain to decrease the toxicity of elastic niosomes (a microsphere used for drug delivery) and to increase the activity of metalloprotease-2 (Manosroi et al. 2012).

5.9 Protease Inhibitors

Protease inhibitors from *Tamarindus indica* L. seeds have been described with the ability to reduce levels of plasmatic leptin (Medeiros et al. 2018), two proteins from *Delonix regia* and *Acacia schweinfurthii*, of the Kunitz protease family, inhibited blood coagulation, platelet aggregation, and thrombus formation (Salu et al. 2018), two proteins fractions with gastroprotective and antielastase properties were described in *Erythrina velutina* seeds (Oliveira de Lima et al. 2017), protease inhibitors in *Lavatera cashmeriana* preventing human lung cancer cell proliferation *in vitro* (Rakashanda et al. 2015) and lupin seeds peptides inhibitors of metalloproteinases (Carrilho et al. 2009). In addition to these plant proteins, many nonprotein plant metabolites have been isolated and display inhibitory properties. Their analysis is not included here.

5.10 Oral Applications

Following an initial account describing the application of papain to remove dental decayed tissue (Bussadori et al. 2005), more than 40 reports confirm the initial finding. Several commercial preparations containing proteases, such as Papacarie® and Carie Care™: are now available and used alone or in combination, as alternatives to mechanical drilling for caries (Divya et al. 2015; Sahana et al. 2016).

It is claimed that Papacarie® does not adversely affect the microleakage of composite restorations and provides a suitable surface for bonding like conventional tooth drilling (Hafez et al. 2017), it offers a less painful alternative among children for caries removal, Carie Care™ a papain-containing formulation demonstrates antimicrobial action against *A. actinomycetemcomitans*, a major periodontal disease causing pathogen (Kush et al. 2015), local application of actinidin or papain prevents or reduces dental plaque formation and reduces oral biofilm on the tongue in elder subjects in vivo and in vitro (Mugita et al. 2017) and three randomized controlled trials; one using papain, bromelain and Miswak (teeth cleaning twig made from the *Salvadora persica*) containing dentifrice to combat dental plaque formation and gingivitis in human subjects (Tadikonda et al. 2017); the other two controlled clinical trials evaluating the long-term chemo-mechanical removal of caries and pain using Papacarie® confirming the absence of pain during the intervention and the preservation of intact dental tissue (Motta et al. 2014; Abdul Khalek et al. 2017).

5.11 Final Statement

The number of therapeutic applications involving plant proteolytic enzymes increased significantly within the last 10 years. Reports on oral applications involving Papacarie® and Carie Care™ experienced a significant increase suggesting a possible future commercial use. On the other hand, reports highlighting the use of bromelain to treat several conditions are on the rise. Treatment of digestive disorders, as anti-inflammatory and as immunomodulator, deserves attention for their link with cancer. A possible drawback is that most studies involve a proteolytic fraction composed of isoforms with diverse properties. Assessment of the biological properties of each isoform is necessary to precisely adjust formulations with the appropriate combination of isoforms to treat specific conditions.

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Chapter 6
Potential Use of Plant Proteolytic Enzymes in Hemostasis

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Contents

6.1 Introduction

Hemostasis is defined as the maintenance of the fluidity of circulating blood while at the same time protecting the organism from life-threatening bleeding at sites of vascular injury. The cell-mediated system of proteolysis of plasma coagulation proteins is essential for the survival of the organism. They propagate several enzymatic reactions that lead to the formation of a thrombus that stop bleedings. It can be divided into the processes of platelet plug formation, blood coagulation, anticoagulation, and fibrinolysis (Walsh and Ahmad 2002).

Considerable efforts have been made in recent years to unravel the suppressor mechanisms of the coagulation process. Studies with patients showing deficiency in specific coagulation inhibitors and genetically modified mice have clearly shown that extensive negative control of coagulation is essential, to prevent uncontrolled, widespread clot formation. First, circulating protease inhibitors, such as antithrombin, heparin cofactor II, TFPI, and C1 inhibitor, eliminate activated coagulation factors by attacking their active sites. The second anticoagulant modality is provided by the enzyme-based protein C/protein S pathway. Interestingly, the latter is

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implicated in endothelial-based pathways of coagulation inactivation (Versteeg et al. 2013).

Anticoagulant drugs are taken by millions of patients throughout the world. Warfarin has been the most widely prescribed anticoagulant for decades. In recent years, new oral anticoagulants have been approved for use, are being positioned as alternatives to warfarin, and represent an enormous market opportunity for pharmaceutical companies. Requests for urgent reversal of anticoagulants are not uncommon especially in the setting of critical bleeding (Dzik 2012).

Same problem occurs with the fibrinolytic therapy. There are numerous drugs, all of them associated with bleeding risk. t-PA is one of the most used drugs for the treatment of acute myocardial infarct but it is associated with brain bleeding disorders (Caldwell et al. 2006).

In this context, plant proteases and extracts are being investigated as new anticoagulant, antiplatelet, and fibrinolytic drugs. Proteases are enzymes able to hydrolyze peptide bonds. This group includes exopeptidases and endopeptidases, differentiated by where they act on the polypeptide chain. The differences between peptidases and proteases are subtle and they share the same chemical features. The term protease will be used throughout this chapter.

Proteases are divided into several subclasses: serine proteases (a Ser residue in the active site), cysteine proteases (a Cys residue in the active site), aspartic proteases (Asp is needed for catalytic activity), and metalloproteases (use a metal ion in the mechanism). Serine and cysteine proteases form covalent complexes, aspartic and metalloproteases rely on acid/base reactions (Antão and Malcata 2005).

All these types of proteases are present in plants and were linked to numerous process such as photosynthesis (Wittenbach et al. 1982), embryogenesis (Kim et al. 2009), immune response (Rodríguez-Herva et al. 2012), cell death (Lazebnik et al. 1995), and others. Not only proteases have very different activities in plants, but also have wide range of working temperature and pH levels. It makes plant proteases suitable for several uses in biotechnology and medicine (Feijoo-Siota and Villa 2010). Here we are reviewing current advances in plant proteases uses in antiplatelet, anticoagulant, and fibrinolytic applications, and also we mention some plant proteases with procoagulant activity.

6.2 Procoagulant Plant Proteases

Several procoagulant compounds have been found from a variety of species. The vast majority are proteases. For example, all the procoagulants from snake venoms characterized are proteases. It is similar to what happens in plants. Almost all procoagulants discovered up to date are proteases, although in most cases their specific mechanisms of action have not been elucidated yet (Shivaprasad et al. 2010).

Cysteine proteases from *x* latex exhibited strong and specific procoagulant action. The latex enzyme fraction exhibited strong proteolytic activity when compared to trypsin and exerted procoagulant action by reducing plasma clotting time.

Trypsin failed to induce any fibrin clot under similar conditions. The electrophoretic pattern of latex enzyme fraction-induced fibrin clot was very much similar to that of thrombin-induced fibrin clot and mimic thrombin-like action. The proteolytic activity including thrombin-like activity of *Asclepias curassavica* latex enzyme fraction was completely inhibited by iodoacetic acid (IAA) (Shivaprasad et al. 2009).

Pergularain, a cysteine protease with thrombin-like activity, was purified by ion-exchange chromatography from the latex of *Pergularia extensa*. Its homogeneity was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), native PAGE, and reverse-phase high-performance liquid chromatography (RP-HPLC). The molecular mass of pergularain by matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) was found to be 23.356 kDa. Pergularain is a glycoprotein containing 20% of carbohydrate. Proteolytic activity of the pergularain was inhibited by iodoacetic acid (IAA). Pergularain exhibited procoagulant activity with citrated plasma and fibrinogen similar to thrombin and increases the absorbance of fibrinogen solution in concentration-dependent and time-dependent manner. The authors established that thrombin-like activity of pergularain is because of the selective hydrolysis of Aα and Bβ chains of fibrinogen while γ-chain was observed to be insusceptible to hydrolysis (Shivaprasad et al. 2010).

Proteins derived from the latex (LP) of *Calotropis procera* are known for their anti-inflammatory property and they were evaluated for their efficacy in maintaining coagulation homeostasis in sepsis. In mice infected with salmonella, LP reduced the thrombocytopenia and procoagulation, while in normal mice LP showed procoagulant effect. Three latex subfractions were tested, some of them exhibited proteolytic effect on azocasein and exhibited procoagulant effect on human plasma in a concentration-dependent manner. Like trypsin and plasmin, these subfractions produced both fibrinogenolytic and fibrinolytic effects that were mediated through the hydrolysis of the Aα, Bβ, and γ chains of fibrinogen and α-polymer and γ-dimer of fibrin clot, respectively (Ramos et al. 2012).

Satish et al. characterized aqueous extracts of *Moringa oleifera* (Moringaceae) leaf and root. Caseinolytic activity of leaf extract was significantly higher than that of root extract. Similar observations were found in case of human plasma clot hydrolyzing activity, wherein leaf extract caused significantly higher plasma clot hydrolysis than root extract. Zymographic techniques were used to detect proteolytic enzymes following electrophoretic separation in gels. Further, both the extracts exhibited significant procoagulant activity as reflected by a significant decrease in recalcification time, accompanied by fibrinogenolytic and fibrinolytic activities; clotting time was decreased from 180 ± 10 s to 119 ± 8 s and 143 ± 10 s by leaf and root extract, respectively, at a concentration of 2.5 mg/mL. Fibrinogenolytic (human fibrinogen) and fibrinolytic activity (human plasma clot) was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), plate method, and colorimetric method. Zymographic profile indicated that both the extracts exerted their procoagulant activity by selectively hydrolyzing Aα and Bβ subunits of fibrinogen to form fibrin clot, thereby exhibiting fibrinogenolytic activity.

However, prolonged incubation resulted in degradation of the formed fibrin clot, suggesting fibrinolytic like activity (Satish et al. 2012).

Other plant extracts showed also procoagulant activity, *Euphorbia nivulia* latex protease has noticeable blood clotting activity followed by *Pedilanthus tithymaloides* and *Synadenium grantii*. Stem latex protease of *Pedilanthus tithymaloides* exhibits superior procoagulant activity in different mammal's blood samples. The plant latex protease could significantly reduce whole blood clotting time of human and mice blood samples. These protease fraction of lattices possesses phytoconstituents capable of arresting wound bleeding and accelerating whole blood coagulation process. It suggests good potentiality for use of latex proteases in wound management. Also, the finding of this study showed that the protease enzyme of *Pedilanthus tithymaloides* has the most potent hemostatic agent. Further characterization of these extracts is yet to be performed (Badgujar 2014).

Pharmacological properties exhibited by latex of plants are due to various biologically active compounds. As is mentioned before, this chapter is focused on proteolytic enzymes. A study conducted by Singh et al. evaluates hemostatic potential of *Tabernaemontana divaricata* and *Artocarpus altilis* from *Apocynaceae* and *Moraceae* families, respectively. The latex of these plants was initially subjected to dialysis and crude extracts were estimated for proteolytic activity using casein as the substrate. Caseinolytic activity by both the plant extracts was higher than standard proteases, papain and trypsin. However the difference was significant with papain alone. Crude enzymes (CE) from both plants exhibited coagulant activity on human platelet poor plasma by recalcification time. A significant reduction in clotting time was exhibited by *T. divaricata* compared to *A. altilis*. These results were further substantiated with fibrinogen agarose plate assay. Crude enzyme of both plants also hydrolyzed blood clot. Inhibition studies confirmed cysteine protease nature of CE. Comparative analysis revealed *T. divaricata* to be the best among the two for its hemostatic potential (Singh et al. 2015).

The procoagulant activity reviewed was due to proteolytic activity of plant lattices. In some cases proteases were isolated and resulted to be cysteine proteases (Table 6.1).

6.3 Plant Proteases with Anticoagulant Activity

Anticoagulants prolong clot formation, they are enzymes, such as serine and cysteine proteases, or nonenzymatic proteins like protease inhibitors. In this context we briefly address the difference between the most common assays used to characterize the procoagulant activity: activated partial thrombin time (APTT) and prothrombin time (PT) tests. A basic understanding of the coagulation pathway is required to interpret these tests (Fig. 6.1).

The division of coagulation in two pathways is mainly artificial, it originates from laboratory tests in which clotting times were measured after the clotting was initiated by glass (intrinsic pathway) or by thromboplastin (a mix of tissue factor

Table 6.1 Proteases and extracts with procoagulant activity

Year	First author	Plant species	Extract	Protease	Protease subfamily	Procoagulant activity	Fibrin(ogen)olytic activity
2009	Shivaprasad	*Asclepias curassavica*	Latex		Cysteine	Reduce clotting time	
2010	Shivaprasad	*Pergularia extensa*	Latex	Pergularain	Cysteine	Thrombin-like activity	Hydrolysis of Aα and Bβ chains
2012	Ramos	*Calotropis procera*	Latex			Procoagulant effect on human plasma	Hydrolysis of all chains
2012	Satish	*Moringa oleifera*	Leaf and root lattices			Decrease in recalcification time	Hydrolysis of Aα and Bβ chains
2014	Badgujar	*Euphorbia nivulia*	Latex			Reduce clotting time of whole blood	
		Pedilanthus tithymaloides	Latex			Reduce clotting time of whole blood	
		Synadenium grantii	Latex			Reduce clotting time of whole blood	
2015	Singh	*Tabernaemontana divaricata*				Reduce clotting time on poor platelet plasma	Hydrolysis of blood clot
		Artocarpus altilis				Reduce clotting time on poor platelet plasma	Hydrolysis of blood clot

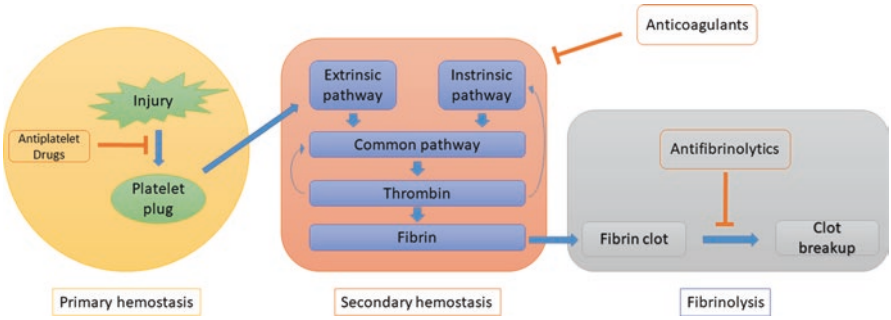

Fig. 6.1 Simplified scheme of hemostasis and the process that are conformed by formation of the platelet plug (yellow), coagulation (salmon), and fibrinolysis (grey)

and phospholipids). Thrombin is present from the very beginning, already when platelets are making the plug. Thrombin has a large array of functions, not only the conversion of fibrinogen to fibrin, but also the building block of a hemostatic plug. In addition, it is the most important platelet activator and on top of that it activates Factors VIII and V and their inhibitor protein C (in the presence of thrombomodulin), and it activates Factor XIII, which forms covalent bonds that crosslink the fibrin polymers that form from activated monomers (Pallister and Watson 2011).

The activated partial thromboplastin time (APTT) assay is used as a screening test to evaluate the overall integrity of the intrinsic/common coagulation pathway and to monitor patients on heparin therapy. This test reflects the activities of most of the coagulation factors in the intrinsic and common procoagulant pathway, but not the extrinsic procoagulant pathway that includes Factor VII and tissue factor, nor the activity of Factor XIII (fibrin stabilizing factor). The prothrombin time is a measure of the integrity of the extrinsic and final common pathways of the coagulation cascade. This consists of tissue factor and Factors VII, II (prothrombin), V, X, and fibrinogen. The test is performed by adding calcium and thromboplastin, an activator of the extrinsic pathway, to the blood sample then measuring the time (in seconds) required for fibrin clot formation (Greaves and Preston 2001).

Many recent studies showed that many plant proteases and extracts possessed anticoagulant activity. The mechanisms of action are not well understood yet, but they confirm a potential source of new therapeutic options.

A thrombolytic protease named kitamase possessing anticoagulant property was purified from edible and medicinal plant *Aster yomena* (Kitam.) Kitamase showed a molecular weight of 50 kDa by SDS-PAGE and displayed a strong fibrin zymogram lysis band corresponding to the similar molecular mass. The enzyme was active at high temperatures (50 °C). The fibrinolytic activity of kitamase was strongly inhibited by EDTA, EGTA, TPCK, and PMSF. The Km and Vmax values for substrate S-2251 were determined as 4.31 mM and 23.81 mM/mg, respectively. It dissolved fibrin clot directly and specifically cleaved the Aα and γ chains of fibrin and fibrinogen. In addition, kitamase delayed the coagulation time and increased activated partial thromboplastin time and prothrombin time. Kitamase exerted a

significant protective effect against collagen and epinephrine induced pulmonary thromboembolism in mice (Choi et al. 2014).

Gangaraju et al. characterized anticoagulant and antiplatelet activities of *Artocarpus heterophyllus* aqueous seed extracts (AqSEJ). They enhanced the clotting time of citrated human. The anticoagulant activity of AqSEJ was further strengthened by in-vivo mouse tail bleeding assay. The intravenous injection of AqSEJ significantly prolonged the bleeding time in a dose-dependent manner. Interestingly, AqSEJ specifically prolonged the clot formation process of only APTT but not PT, revealing the anticoagulation triggered by the extract could be due to its interference in an intrinsic pathway of the blood coagulation cascade. Furthermore, AqSEJ inhibited the agonists such as ADP, epinephrine, and collagen induced platelet aggregation (Gangaraju et al. 2015).

Table 6.2 summarizes the proteases and plant extracts with anticoagulant activity.

6.4 Plant Proteases with Fibrinolytic Activity

Physiologically, plasmin plays a pivotal role in fibrinolysis. Some plant proteases contribute to fibrinolysis by the activation of plasminogen to plasmin and others have fibrinolytic activity by their own (see below). During the final common pathway, thrombin promotes clot formation through fibrinogen polymerization. When fibrin forms in plasma, a small amount of plasminogen is bound (Fig. 6.1). Plasminogen activator is strongly adsorbed to the fibrin and activates bound plasminogen in situ. The plasmin released is rapidly and irreversibly neutralized by α2-antiplasmin (Peeters 1980).

Crinum species have been extensively used by traditional medical practitioners to treat various illnesses all around the world. Crinumin, a stable and active in many adverse condition serine protease from *Crinum asiaticum*, shows plasmin-like fibrinolytic activity and inhibits platelet aggregation and P-selectin exposure, as established by photography, phase contrast microscopy, whole blood optical lumi-aggregometry, and flow cytometry. Crinumin could be an efficient and inexpensive therapeutic agent for the treatment and prevention of thromboembolic diseases (Singh et al. 2011).

A protease was isolated and purified from *Artocarpus heterophyllus* (jackfruit) latex and designated as a 48-kDa antimicrobial protease (AMP48). Enzyme activity of AMP48 was strongly inhibited by phenylmethanesulfonyl fluoride and soybean trypsin inhibitor, indicating that the enzyme was a plant serine protease. AMP48 had fibrinogenolytic activity with maximal activity between 55 and 60 °C at pH 8. The enzyme efficiently hydrolyzed α followed by partially hydrolyzed β and γ subunits of human fibrinogen. In addition, the fibrinolytic activity was observed through the degradation products by SDS-PAGE and emphasized its activity by monitoring the alteration of secondary structure of fibrin clot after enzyme digestion using ATR-FTIR spectroscopy (Siritapetawee et al. 2012).

Table 6.2 Proteases and extracts with anticoagulant activity

Year	First author	Plant species	Extract	Protease	Protease subfamily	Anticoagulant activity	Fibrin(ogen)olytic activity	Other
2014	Choi	*Aster yomena*		Kitamase	Serine	Prolonged APTT and PT	Hydrolysis of Aα and γ chains	
2015	Gangaraju	*Artocarpus heterophyllus*	Aqueous seed extract			Prolonged APTT but no PT		Inhibits platelet aggregation

A 34 kDa serine protease, designated as hirtin, with fibrinolytic activity was purified to homogeneity from the latex of *Euphorbia hirta* by the combination of ion-exchange and gel filtration chromatography. Hirtin exhibited esterase and amidase activities along with azocaseinolytic, gelatinolytic, fibrinogenolytic, and fibrinolytic activities. It preferentially hydrolyzed Aα and α-chains, followed by Bβ and β, and γ and γ-γ chains of fibrinogen and fibrin clot, respectively. The optimum pH and temperature for enzyme activity were found to be pH 7.2 and 50 °C, respectively. Enzymatic activity of hirtin was significantly inhibited by PMSF and AEBSF (Patel et al. 2012).

A direct-acting chymotrypsin-like fibrinolytic serine protease was purified from *Petasites japonicus*, a medicinal herb. The molecular mass of the discovered enzyme was estimated to be 40.0 kDa as determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis, fibrin zymography, and gel filtration chromatography. The proteolytic activity of the enzyme was found to be inhibited by serine protease inhibitors, phenylmethylsulfonyl fluoride, and 4-(amidinophenyl) methanesulfonyl fluoride. An assay of enzyme activity on fibrin plates revealed that it could hydrolyze the fibrin directly. The enzyme displayed a potent fibrin(ogen) olytic activity, hydrolyzing the Aα, γ, and Bβ subunits of the human fibrinogen. The enzyme prolonged activated partial thromboplastin time and had little effect on prothrombin time (Kim et al. 2015).

*St*SBTc-3 is a potato 72 kDa subtilisin like serine protease. It degrades all chains of human fibrinogen and produces fibrin clot lysis in a dose-dependent manner. The enzyme efficiently hydrolyzes Bβ subunit followed by partially hydrolyzed Aα and γ subunits of human fibrinogen. Assays performed to determine StSBTc-3 substrate specificity using oxidized insulin β-chain as substrate show seven cleavage sites: Asn3-Gln4; Cys7-Gly8; Glu13-Ala14; Leu15-Tyr16; Tyr16-Leu17; Arg22-Gly23, and Phe25-Tyr26, all of them were previously reported for other serine proteases with fibrinogenolytic activity. The maximum *St*SBTc-3 fibrinogenolytic activity was determined at pH 8.0 and 37 °C. Additionally, *St*SBTc-3 inhibits platelet aggregation and does not exert cytotoxic activity on human erythrocytes in vitro at all concentrations assayed (Pepe et al. 2016).

A protease designated Eumiliin was isolated from the latex of *Euphorbia milii* var. hislopii by a combination of ion-exchange chromatographic steps. Eumiliin is a monomeric protein with an apparent molecular mass of 30 kDa. It has caseinolytic and fibrinogenolytic activities, but no hemorrhagic or defibrinating activities. The enzyme readily hydrolyzes the Aα-chain of fibrinogen and, more slowly, the Bβ-chain. Its fibrinogenolytic activity is inhibited by beta-mercaptoethanol and leupeptin. In contrast, EDTA and benzamidine did not affect the activity of Eumiliin. Intraplantar injection of Eumiliin caused a dose- and time-dependent hyperalgesia, which peaked 1–5 h after enzyme injection. Morphological analyses indicated that Eumiliin induced an intense myonecrosis, with visible leukocyte infiltrate and damaged muscle cells 24 h after injection (Fonseca et al. 2010).

A dimeric protease designated as EuP-82 was purified from *Euphorbia lactea* latex. Since, EuP-82 proteolytic activity was inhibited by the serine protease inhibitor (PMSF), EuP-82 was classified as a serine protease. N-glycan deglycosylation

tests revealed that EuP-82 is a glycosylated protein. MALDI-TOF MS showed that EuP-82 was a homodimer, which was its active form. The optimal conditions for fibrinogenolytic activity were at pH 11 and 35 °C. EuP-82 enzyme had broad range of pH stability from pH4 to pH12. Moreover, the enzyme was still active in the presence of reducing agent b-mercaptoethanol. EuP-82 was a proline-rich enzyme (about 20.69 mol%). Increased proline production can be found in higher plants in response to both biotic and abiotic stresses, high proline in the molecule of EuP-82 might stabilize its activity, structure, and folding. The enzyme was identified as a new serine protease. The digested products from EuP-82 cleavage of human fibrinogen were analyzed by SDS-PAGE and PMF. The results confirmed that EuP-82 could digest all subunits of human fibrinogen (Siritapetawee et al. 2015).

The proteases from turmeric species have procoagulant and fibrinogenolytic activity. Shivalingu et al. purified a protein with potent proteolytic activity named as *C. aromatica* protease-II (CAP-II). It is a monomeric protein, showing sharp peak in RP-HPLC and its relative molecular mass was found to be 12.378 kDa. The caseinolytic and fibrinolytic activity of CAP-II was completely inhibited by phenylmethylsulfonyl fluoride (PMSF). The CAP-II exhibited optimum temperature of 45 °C and optimum pH of 7.5. The CAP-II showed hydrolysis of all three subunits of fibrinogen in the order A α > Bß > γ. The CAP-II exhibited strong procoagulant activity by reducing the human plasma clotting time. It also showed fibrinolytic activity by complete hydrolysis of α-polymer and γ–γ dimer present in the fibrin (Shivalingu et al. 2016).

Proteases with fibrin(ogen)olytic activity are summarized in Table 6.3.

6.5 Discussion

Hemostasis is a complex process that includes platelet plug formation, coagulation, and fibrinolysis (Fig. 6.1). There is a broad spectrum of proteases with hemostatic activities, some of them are purified but others are extracts with proteolytic activity. The vast majority of these enzymes and extract do not act at only one level. It is important to distinguish between the fibrinolytic and fibrinogenolytic activity. The fibrinolytic activity is the ability to degrade the fibrin clot. The fibrinogenolytic activity is the ability to degrade fibrinogen. Neither both of these activities are directly related with pro or anticoagulants activities. There are fibrinolytic proteases with procoagulant (Table 6.1) or anticoagulant activities (Table 6.2). This is relevant for the decision making of what proteases are better for each therapeutic goal. The lack of a standardized test to address the biochemical characterization of these proteins makes the comparison between them very difficult. It is necessary to think about what questions the field need to address in order to characterize these novel plant proteases in such way that can contribute to the biomedical investigation of new therapies.

Table 6.3 Proteases and extract with fibrin(ogen)olytic activity

Year	First author	Plant species	Extract	Protease	Protease subfamily	Fibrin(ogen)olytic activity	Other
2011	Singh	*Crinum asiaticum*		Crinumin	Serine	Plasmin-like fibrinolytic activity	Inhibits platelet aggregation
2012	Siritapetawee	*Artocarpus heterophyllus*	Latex	AMP48	Serine	Hydrolysis of α followed by partially hydrolyzed β and γ subunits of human fibrinogen	
2012	Patel	*Euphorbia hirta*	Latex	Hirtin	Serine	Hydrolyze Aα and α-chains, followed by Bβ and β, and γ and γ-γ chains of fibrinogen and fibrin clot, respectively	
2015	Kim	*Petasites japonicus*			Serine	Hydrolyze Aα, γ, and Bβ subunits of the human fibrinogen	Prolonged APTT and little effect on PT
2010	Fonseca	*Euphorbia milii*		Eumiliin		Hydrolyze Aα followed by Bβ subunits of the human fibrinogen	Caseinolytic activity and myonecrosis
2015	Siritapetawee	*Euphorbia lactea*	Latex	Eup-82	Serine	Digest all subunits of human fibrinogen	
2016	Shivalingu	*Curcuma aromatica*		CAP-II	Serine	A α > Bß> γ	Caseinolytic activity and procoagulant activity
2016	Pepe	*Solanum tuberosum*		*St*SBTc-3	Serine	Hydrolyze Bβ followed by Aα and γ chains	Inhibits platelet aggregation

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