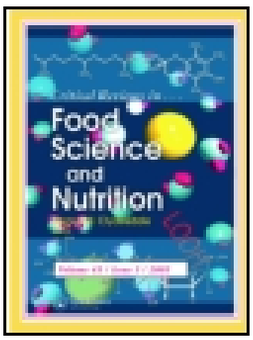

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Plant proteases for bioactive peptides release: A review

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ABSTRACT

Proteins are a potential source of health promoting biomolecules with medical, nutraceutical and food applications. Nowadays, bioactive peptides production, its isolation, characterization and strategies for its delivery to target sites are a matter of intensive research. *In vitro* and *in vivo* studies regarding the bioactivity of peptides has generated strong evidence of their health benefits. Dairy proteins are considered the richest source of bioactive peptides, however proteins from animal and vegetable origin also have been shown to be important sources. Enzymatic hydrolysis has been the process most commonly used for bioactive peptide production. Most commercial enzymatic preparations frequently used are from animal (e.g., trypsin and pepsin) and microbial (e.g., Alcalase® and Neutrase®) sources. Although the use of plant proteases is still relatively limited to papain and bromelain from papaya and pineapple, respectively, the application of new plant proteases is increasing. This review presents the latest knowledge in the use and diversity of plant proteases for bioactive peptides release from food-proteins including

both available commercial plant proteases as well as new potential plant sources. Furthermore, the properties of peptides released by plant proteases and health benefits associated in the control of disorders such as hypertension, diabetes, obesity, and cancer are reviewed.

Keywords

phytoproteases, hydrolysates, papain, bromelain, functional foods, bioactivity

ACCEPTED MANUSCRIPT

1. INTRODUCTION

Proteins are essential constituents of foods and contribute to a wide range of nutritional, functional and biological properties. Food proteins are an important source of energy and essential amino acids for normal growth, life maintenance, and reproduction. Their functional properties contribute to the physicochemical and sensory properties of foods. Furthermore, their partial digestion by proteases may produce peptide sequences with specific biological properties (bioactive peptides) which make them potential ingredients for functional foods (Udenigwe and Aluko, 2012). Biologically active peptides, encrypted within food protein sequences, can be released during digestion, food processing (e.g., milk fermentation and cheese ripening) and protein hydrolysate production. Proteolytic enzymes have the ability to modify proteins through limited or extensive cleavage, releasing free amino acids, peptides or polypeptides with physicochemical properties different from the original protein (Pacheco-Aguilar et al., 2008). Proteolytic enzymes from proteolytic system of starters, proteases endogenous to food, or added enzymes (e.g., rennet) differ in their specificity and therefore in their capacity to release bioactive sequences (Korhonen and Pihlanto 2006; Sabbione et al., 2016).

Studies *in vitro* and *in vivo* have demonstrated that some peptide sequences released from food proteins would prevent metabolic disorders and lifestyle-related diseases. Bioactive peptides can act on the cardiovascular, digestive, endocrine, nervous and immune systems, potentially helping to prevent hypertension, diabetes, obesity, cancer and cardiovascular diseases (Ichimura et al., 2009; Jakala et al., 2009; Li-Chan et al., 2012; Chakrabarti et al., 2014). Peptides that have the capacity to inhibit the angiotensin I-converting enzyme (ACE) may have an anti-hypertensive effect (Iwaniak et al., 2014). Peptides that induce cytosine secretion may stimulate satiety and

reduce obesity and those that stimulate the release of insulin or inhibit the activity of the enzyme dipeptidyl peptidase IV (DPP-IV) would have a positive effect in patients with diabetes mellitus (Jakubowicz and Froy, 2013; Liu et al., 2013). In addition, some peptides with antioxidant activity may prevent DNA damage and lipid peroxidation and thereby lessen cancer development and oxidative stress (Chakrabarti et al., 2014; Stevenson 2012).

ACE inhibitory and antioxidant activity are the two properties most claimed for bioactive peptides found in protein hydrolysates and functional foods. Antihypertensive properties in milk-fermented products such Calpis® and Evolus® have been associated with the presence of the casein-derived peptides VPP and IPP which possess high ACE inhibitory activity (IC_{50} of 9 and 5 μM, respectively) (Nakamura et al., 1995; Erdman et al., 2008). Similarly, protein hydrolysates obtained from bonito fish, commercialized as Vasotensin® (metagenics.com) and peptACE® (naturalfactors.com) display antihypertensive activity, attributed to the presence of the peptide LKPNM and a mixture of nine bioactive peptides, respectively (Chalamaiah et al., 2012). Bioactive peptides in protein hydrolysates from milk (Medeiros et al., 2014; Lacroix and Li-Chan 2012a), egg (Memarpoor-Yazdi et al., 2012a;2012b; Chen et al., 2009), meat (Udenigwe and Howard 2013: Di Bernardini et al., 2012), fish (Najafian et al., 2013; Huang et al., 2012), soy bean (Margatan et al., 2013; Kong et al., 2008), chickpeas (Medina-Godoy et al., 2012), amaranth (Velarde-Salcedo et al., 2013), other food-proteins (Udenigwe and Aluko 2012; Chen and Chi 2012; Tavares et al., 2012a; Iwaniak and Dziuba 2009) and non-conventional protein sources (Kang et al., 2012; Fan et al. 2010; McDonagh & FitzGerald, 1998) have also been identified. The fact that bioactive peptides have been derived from many different protein sources has increased interest in the production, commercialization and the design of novel

functional foods containing these peptides. Peptide-rich functional protein hydrolysates have a range of beneficial effects on physicochemical and sensory properties of food and, the presence of bioactive peptide sequences can have additional beneficial health effects on diverse pathological conditions (Ha et al., 2012). Protein hydrolysate production with functional and bioactive peptides is an important emerging area in nutrition, medicine and foods. However, the lack of suitable technologies for their efficient release and process optimization has hampered their production (Tavares et al., 2011a; Liu et al., 2013).

Hydrolysis efficiency depends of factors such as protein substrate, type and specificity of proteases, degree of hydrolysis (DH), as well as stability of peptides through the entire hydrolytic process (Zarei et al., 2012; 2016; Tavares et al., 2011a; Santos et al., 2011). Numerous methods for monitoring DH during a hydrolytic process have been developed using different basic principles: a) trichloroacetic acid soluble nitrogen determination (TCA-SN), b) determination of free amino groups produced during hydrolysis (2,4,6- trinitro benzene sulfonic acid or TNBS, O-phthaldialdehyde or OPA, and formol titration) and c) the titration of protons released during peptide bond hydrolysis (pH-STAT) using a base. The pH-STAT has the advantage to make continuous measurements of the hydrolytic process; however, and depending of sample, its use can affect the sensorial property of hydrolysate by adding a high proportion of cations (Aspevik et al., 2016).

Most hydrolytic processes use one protease at a time; however, a combination of two enzymes with different specificity has also been explored (Zeng et al., 2018). Sometimes, proteins partially-hydrolyzed are further treated with pepsin and trypsin to simulate gastrointestinal digestion (Tavares et al., 2011a; Papillo et al., 2014). Other proteases widely used for bioactive

peptides production include commercial preparations such as Alcalase, Neutrase, Flavourzyme, and Thermolysin derived from bacteria and fungi (Rui et al., 2012). The use of plant proteases in the production of bioactive peptides is still scarce. Cysteine proteases such papain, ficin and bromelain, obtained naturally from latex, fruits, leaves or stem of papaya, fig, and pineapple respectively are currently the most used plant proteases. However, serine proteases such as zingibain, cucumisin and actinidin obtained from ginger rhizome, melon and kiwifruit, respectively, are three new emerging plant proteases which have been considered recently (Ha et al., 2012; Nafi et al., 2013 Mazorra-Manzano et al., 2013).

The search for novel specialized proteolytic enzymes with preference for specific peptide bonds for the selective release of bioactive peptides requires further study in order to improve process efficiency. This review summarizes the recent studies regarding bioactive peptide production using traditional and novel plant proteases. The various plant proteases, the protein substrates used, and the bioactive properties of peptides produced and their potential to prevent or treat disorders such as hypertension, diabetes, obesity, and cancer, are reviewed.

2. DISTRIBUTION, CLASSIFICATION AND PROPERTIES OF PLANT PROTEASES

Proteases, proteinases or peptidases are a group of hydrolytic enzymes that carry out the hydrolysis of peptide bonds in proteins and peptides. Proteases mainly involve two groups of enzymes known as exo- and endo-peptidases. Exopeptidases act at the amino- or carboxyl ends, while endopeptidases act on the interior of the protein sequence used as substrates. Their properties and functions have attracted the attention of the scientific community and industry in an attempt to exploit their physiological and biotechnological properties. In many cases,

proteases are central to many biotechnological processes in the food industry, research, and medicine, as well as being critical in the development of therapeutic agents against diseases such as cancer, Alzheimer and AIDS (Cooper, 2002).

They are widely distributed in nature from microorganisms to animals and plants. The recent genomic sequencing of several organisms has revealed a high number of the proteases from different classes, structures and important physiological roles. According to the MEROPS database (http://merops.sanger.ac.uk/index.htm) peptidases are classified by their structural and evolutionary criteria and actually represent 244 families and 55 clans (Rawlings et al., 2014). The serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic (EC 3.4.23) and metallo (EC 3.4.24) peptidases, that possess the catalytic residues Ser, Cys, Asp or metal-ion cofactor (e.g., Zn^{+2}, Co^{+2} and Mn^{+2}) in their active center, respectively, are the most abundant.

Proteases are involved in most plant functions, such as protein processing, digestion, growth, reproduction, defense, apoptosis, senescence, etc. (Feijoo-Siota and Villa, 2011; Van der Hoorn and Jones et al., 2004). The complete genome of the model plant *Arabidopsis thaliana* encodes for close to 900 sequences of known and putative peptidases, however the number in others plants is still unknown and only a limited number, mainly those found in abundance, has been identified (**Table 1**).Cysteine proteases (CPs) account for approximately 16% of the total sequences deposited in Merops database. Papain (EC 3.4.22.2), bromelain (EC 3.4.22.32) and ficin (EC 3.4.22.3) from papaya, pineapple and fig respectively, are the most well-known plant proteases used in food processing, pharmaceutics and other industrial processes. These three enzymes represent 5% of the global sales of proteases (Illanez, 2008). Other well-known CPs includes zingipain (EC 3.4.22.67) from ginger rhizome and actinidin (kiwillin) from Kiwi fruit

(Choi et al., Ha et al., 2012 1999; Teh et al., 2016). Cysteine proteases are more abundant in plants even though the number of serine proteases (SPs) is higher in plant genome (Schaller, 2004). However, some *Cucurbitaceae* species also contain significant serine protease activity and could represent up to 50% of the total protein extracted from fruits and latex (Sharma et al., 2009: Antao y Malcata 2005). Cucumisin (EC 3.4.21.25) from melon *Cucumis melo* fruit, is the best-known plant SPs (Arima et al., 2013; Antao y Malcata 2005). Other SPs identified in plants include pomiferin (o 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), dobiumin from *Solanum dobium (Ahmed et al., 2009),* hordolisin from barley *Hordeum vulgare* (Terp et al., 2000), crinumina from *Crinum asiaticum* (Singh *et al., 2010),* Nerifolin from *Euphorbia neriifolia* L. (Yadav et al., 2012), and others (Antao y Malcata 2005; Domsalla and Melzig, 2008). According to Merops classification they have been grouped into the same S8 family of alkaline protease subtilisin (also known as Alcalase® from *Bacillus licheniformis*), subfamily A, and clan SB (Rawlings et al., 2014).

Pepsin is the most representative enzyme of the aspartic protease (APs) family. This animal gastric enzyme has been widely studied and characterized, and is considered a model enzyme in the structure-function relationship of APs (Sinkovits et al., 2007). Most members of the APs belong to the family A1 (Pepsin-like), also known as "acid proteinases", which are active at acidic pH and are inhibited by Pepstatin A. However, some members, such as chymosin, are also active at neutral pH (Chitpinityol and Crabbe, 1998). Typical plant APs are structurally distinctive of the family A1 since they contain an internal region of about 100 amino acids (plant specific insert) not generally present in the family. Actually, only two typical plant APs have

been characterized and its tridimensional structure known: phytepsin (PDB 1qdm) and cardosin A (PDB 1b5f) from barley *Hordeum vulgare* L. and *Cynara cardunculus*, respectively, from which their zymogen and mature enzyme (without prosegment) 3D structure, respectively, have been resolved (Kervinen et al., 1999; Frazao et al., 1999). These enzymes are heterodimeric with two conserved catalytic motifs: Asp-Thr-Gly (DTG) and Asp-Ser-Gly (DSG) at substrate binding cleft, differing from the characteristic animal and microbial catalytic motifs DTG/DTG counterpart (Faro and Gal, 2005; Rawlings et al., 2014). The general name "phytepsins" (EC 3.4.23.40) has been adopted by the Enzyme Commission of the IUBMB for all typical APs. However, the name of the specie or tissue from which each enzyme has been purified, is used most commonly to give them a common name (Vairo-Cavalli et al., 2013). The phytepsins (family A1), include all the typical APs such as A1, A2 and A3 from *Arabidopsis thaliana* (Chen et al., 2002), cardosins and cyprosins from *Cynara cardunculus* (Sarmento et al., 2009; Pissarra et al., 2007), cenprosins from *Centauria calcitrapa* (Domingos et al., 2000), cirsin from *Cirsium vulgare* (Lufrano et al., 2012), oryzasin from rice *Oryza sativa* (Asakura et al., 1995), soyAP1 and soyAP2 from soy *Glycine max* (Terauchi et al., 2006) and others. Plant APs grouped in subfamily A1B are atypical. Examples in this family includes the constitutive disease resistance CDR1 and PCS1, which promotes cell survival, proteases from Arabidopsis, as well as the nucellin-like proteinases such as nephentensin from the insectivorous plant Nephenthes, and nucellin from *Oryza sativa*, respectively (Faro and Gal, 2005; Ge et al., 2005; Xia et al., 2004).

Among all plant proteases, metalloproteases (MPs) are the least characterized and most of the functional and structural information has been obtained from vertebrate sources and comparison of gene sequences (Sun et al., 2007). However, plant MPs activity has been detected in several

sources such as pea seeds, germinated maize, sugarcane, buckwheat seed, sorghum, soybean leaves, *Arabidopsis thaliana* and wheat (Marino et al., 2014; Ramakrishna et al., 2010; Ramos and Selistre-de-Araujo, 2001) which are part of the metalloproteases matrix (MMP) family belonging to the metzincins clan and have dependence on metal ions cofactors, generally zinc (Zn^{2+}) (Marino et al., 2014; Marino and Funk, 2012). Microbial metalloproteases (e.g., thermolysin, collagenases and keratinases) have been used in therapeutic applications and for biotechnological processes such as protein hydrolysates production, dehairing and bating of leather, peptide synthesis of precursors for the sweetener aspartame production, etc (Mansfeld, 2007), but the use of plant MPs is still limited due to lack of their characterization. However, the abundance and catalytic properties of cotinifolin from *Euphorbia cotinifolia* open possibilities to explore its utilization in biotechnology and industrial applications in a near future (Kumar et al., 2011).

3. APPLICATION OF TRADITIONAL AND EMERGING PLANT PROTEASES FOR THE RELEASE OF BIOACTIVE PEPTIDES

Proteases are the most commercialized enzymes due to their multiple biotechnological uses which include such applications as: their use as biological detergents, meat tenderizing, leather treatment, digestive agents, cheese making and functional protein hydrolysates production (Li et al., 2013; Shah et al., 2014). Protein hydrolysates production with nutritional and functional properties are commonly used in the formulation of microbial media, food supplements, animal feed and food ingredients (Benjakul et al., 2014, McCarthy et al., 2013). Recently, the production of protein hydrolysates with bioactive peptide sequences represents a new research area with high impact in health, medicine and food industry. The wide diversity of commercial

proteases with special catalytic characteristics of specificity and stability opens new possibilities to improve and optimize hydrolytic processes.

A survey in the scientific literature indicates that the most cited proteases for production of bioactive peptides includes the use of Alcalase®, a commercial preparation obtained from the microbial fermentation of *Bacillus licheniformis*, followed by porcine pepsin, trypsin, Flavourzyme®, papain, Neutrase® chymotrypsin and Protamex® (**Figure 1**). Interestingly, three enzymes from animal source (pepsin, trypsin and chymotrypsin) and three from microbial origin (Alcalase, Flavourzyme and Neutrase), were cited in approximately 75% of the papers reviewed. However, the references to plant proteases for bioactive peptide production was only around 15%. Although citations to papain and bromelain represented close to 75% of those references.

Table 2 shows the use of traditional and emerging plant proteases for bioactive peptides production from different protein sources. The cysteine proteases papain and bromelain have been the plant proteases most explored for this purpose. Other plant proteases obtained from cardoon, fig, ginger, kiwi and Asian pumpkin, also have been considered for biotechnological processes; however, at the present, their potential use for bioactive peptides production has been limited (Babij et al., 2014; Vairo-Cavalli et al., 2013). Bioactive peptides production represents a key area of research due to their high potential use as nutraceuticals and in functional foods to enhance health and reduce disease risk where they can have an effect on the immune, cardiovascular, nervous and intestinal systems (Udenigwe and Aluko, 2012). Diverse functions, including anti-microbial, anti-oxidative (Arruda et al., 2012; Dabrowska et al., 2013; Memarpoor-Yazdi et al. 2012b), anti-hypertensive, anti-thrombotic, cholesterol lowering effect, immunomodulatory (Hosomi et al., 2012; Shimizu et al., 2009; Memarpoor-Yazdi et al. 2012a;),

anti-cariogenic or properties related to emotional state (Pihlanto-Leppala 2001), have been associated to some peptide sequences. An efficient release of an encrypted peptide (within the protein sequence) depends mainly on the protease used and extend of the hydrolytic process. Therefore, a better understanding of the catalytic properties of proteases is necessary in order to manipulate and optimize the hydrolytic process to obtain products with specific characteristics. Protease specificity and protein substrate are some of the most important factors that define the bioactivity of the released peptide sequence. Proteases with broad substrate specificity generally yield non-predictable degradation products during the hydrolytic process, but their use is still attractive in producing hydrolysates with high degree of hydrolysis (DH) where small fragments may possess activity (Barbana and Boye 2011). On the contrary, a limited hydrolysis using proteases with high specificity would lead to an efficient release of peptides with expected sizes and properties. Total protein hydrolysis by the action of a single protease is not always possible and generally requires a combination of different enzymes/conditions for an efficient release of small fragments and free amino acids. Therefore, a complementary or sequential use of a mixture of endo- and/or exo-proteases with diverse specificity is required for a complete release of bioactive fragments (Barbana and Boye 2011; Memarpoor-Yazdi et al., 2012a; 2012b). Several strategies based on natural digestion process have been explored using proteases with different specificity (e.g., pepsin, trypsin and chymotrypsin) and different pH conditions. The wide diversity of plant proteases with unique catalytic properties, diverse specificity and stability, has made their future commercial use attractive. However, further research on the characterization of these new emerging plant proteases, in order to select an appropriate enzyme and the design of an efficient reasonable hydrolytic process is needed. Their abundance in various natural sources,

their catalytic properties and some recent successful applications make them scientific and commercially attractive. In addition, the limited/restricted use of non-plant proteases (e.g., animal and recombinant sources) due to ethical, religious reasons or regulatory restrictions in some countries provides new opportunities for the use of new plant proteases.

4. FOOD PROTEINS AS SOURCE OF BIOACTIVE PEPTIDES

For a long-time, the food industry has used enzymatic processes for the production of hydrolysates to improve the functional or nutritional properties of the protein used. Enzymatic hydrolysis has been also widely applied to improve digestibility, solubility, reduce allergenic properties from some proteins and to release bioactive peptides. Bioactive peptides production has received considerable attention due to the beneficial action that some peptide sequences have over the immune, cardiovascular, nervous and intestinal systems (Udenigwe and Aluko, 2012).

The screening of potential bioactive sequences released from food proteins by enzymatic process has been the focus of several recent studies. Milk proteins, mainly caseins (α- β- and κ-casein) and whey proteins (α-albumin, β-lactoglobulin, bovine serum albumin and lactoferrin), are considered the richest sources of bioactive peptides. Antihypertensive, antioxidant, opioid, immunostimulant, antibacterial, mineral carrier, etc., are properties commonly explored (Haque et al., 2009). Other proteins such as lysozyme and albumin from hen egg-white, duck and ostrich also contain encrypted peptide sequences with antioxidant, antihypertensive and immunomodulant properties (Jimenez-Saiz et al., 2013; Chen et al., 2009; Miguel and Aleixandre 2006). Muscle proteins from beef, pork, chicken, fish, crustaceans, and mollusks have also been reported as potential sources of bioactive peptides, mainly with antioxidant properties. Storage proteins from grains and cereals such as soy, beans and flaxseed also contain

peptide sequences with anticancer, antioxidant, antithrombotic, antidiabetic and antihypertensive activity (Cavazos and Gonzalez de Mejia, 2013; Velarde-Salcedo et al., 2013). In addition, non-conventional protein sources including worm silk and microalgae have also been considered as a source of bioactive peptides (Fan et al., 2010 Kang et al 2012; Fitzgerald et al., 2012; Samarakoon et al., 2013). In order to be an effective bioactive peptide, it must be of small molecular size and be resistant to digestive peptidases (Tavares et al., 2011; Fujita and Yoshikawa et al., 1999), or even enhance its activity through its digestion and absorption process during its transit to the target site (Tavares et al., 2011b; Fujita and Yoshikawa et al., 1999).

5. BIOACTIVE PROPERTIES OF PEPTIDES RELEASED BY PLANT PROTEASES

Milk proteins are the best known source of bioactive peptides. However, several studies and analyses *in silico* of other protein sequences have revealed that proteins from plants, grains, meat, fish and seafood also contain peptide sequences with bioactive properties (Iwaniak and Dziuba 2009a; 2009b). In fact, most proteins could be considered precursors of bioactive peptides sequences (Dziuba et al., 2004). Bioinformatics analysis has revealed that by using trypsin, ficin and papain specificity, several bioactive peptides from different protein sources can be released. The number, sequence and properties of these *in silico* predicted peptides depend on the protein and protease used; however, only few studies have examined further both their release via proteolytic digestion and their *in vitro* or *in vivo* properties (Thewissen et al., 2011; Majumder and Wu, 2010).

5.1 Peptides with Antihypertensive Properties

Hypertension (HP) is a chronic medical condition consisting in the increase of the arterial blood pressure. It is the major risk factor for cardiovascular morbidity and mortality since it can lead to such diseases as coronary heart disease, stroke, heart and kidney failure, etc., and therefore is one of the major causes of premature death (WHO, 2016). HP is a significant health problem worldwide where, only in the US, around 80 million people (1 of each 3 adults) have high blood pressure (AHA statistical update, 2016).

An enzymatic cascade of reactions, known as the renin-angiotensin system, regulates blood pressure. Briefly, the aspartic protease renin catalyzes the hydrolysis of the angiotensinogen (produced in the liver) to yield the decapeptide angiotensin I which subsequently, by the action of the angiontensin-converting enzyme (ACE) at the C-terminal, release a dipeptide to yield angiotensin II. This octapeptide stimulates the release of aldosterone, a hormone that increases blood volume due to its action on the kidney. ACE plays a dual role in arterial blood pressure regulation where it catalyzes the production of the vasoconstrictor angiotensin II and inactivates the vasodilator bradykinin. Therefore, the inhibition of this enzymatic cascade (ACE or renin inhibition) has been the target for the design of therapeutic drugs and the discovery of new natural bioactive compounds (Cooper, 2002). ACE inhibition has been the preferred target since its inhibitors have an antihypertensive effect. ACE inhibitory peptides have received special attention. This property has been highly evaluated due to the peptide potential for use in functional foods, investigate by several in vivo and in vitro studies (Rodriguez-Figueroa et al., 2013; Tavares et al., 2011b; 2012b).

Although milk proteins, specifically caseins, are an important source of antihypertensive peptides, recent research supports that all food proteins contain peptides with ACE-inhibitory activity (Martinez-Maqueda et al., 2012; Iwaniak et al 2014). Animal (i.e., digestives enzymes), microbial and vegetable proteases are commonly used for bioactive peptides production. For example, pepsin is used for antihypertensive peptide production from egg white where their beneficial effect on spontaneously hypertensive rats SHRs has been shown (Miguel and Aleixandre 2006). The plant protease papain, alone or in combination with trypsin, over the same protein substrate, has produced peptides with antihypertensive activity *in vitro* (Memarpoor-Yazdi et al 2012a; 2012b). Flaxseed protein hydrolysates produced with papain or ficin inhibited the activity of the two blood pressure regulating enzymes, renin and ACE (Udenigwe et al 2009). *Microalgae palmaria* hydrolysate, produced with papain, was also able to inhibit renin (Fitzgerald et al 2012). Bovine serum albumin (BSA) hydrolysate produced with papain showed the ability to inhibit ACE, renin, and dipeptidyl peptidase-IV (DPP-IV) indicating the potential use for the treatment of hypertension and type-2 diabetes. SLR, YY, ER, and FR sequences inhibited ACE showing an IC_{50} of 0.17, 0.18, 027, and 0.42 mM, respectively. In addition, a fraction obtained (1 kDa) showed antihypertensive activity in SHRs (Lafarga et al., 2016a). Cottonseed protein hydrolysate produced with papain showed higher ACE inhibitory activity than those produced by alcalase, flavourzyme, trypsin, neutrase and pepsin (*Gao et al., 2010*). Its fractions, one lower than 5 KDa (prepared by ultrafiltration UF) and another of 0.82-2.43-kDa (obtained by gel filtration G-25), yielded IC_{50} values of 0.792 and 0.159 mg/mL, respectively. Soy protein isolate (SPI) and β-conglycinin-rich fraction hydrolysates, both prepared with papain, showed more than double ACE inhibitory activity of that obtained with pepsin, had IC_{50}

values of 0.177 and 0.170 *vs* 0.361 and 0.588 mg protein/mL, respectively (Margatan et al., 2013). Lentil protein hydrolysate, produced under simulated gastrointestinal digestion (GIS) condition using papain, exhibited the highest ACE inhibition activity (IC_{50} values range of 0.053-0.190 mg/mL) in comparison with those produced with bromelain and a mixture of alcalase/flavorzyme (Barbana and Boye 2011).

During gastrointestinal digestion, enzymes may break up proteins and peptides, increasing or decreasing the bioavailability of ACE inhibitory peptides. Predigested proteins (hydrolysates) with ACE inhibitory activity can resist further gastrointestinal hydrolysis, thereby exerting an *in vivo* ACE inhibitory activity. A recent study reported that after GIS, the overall ACE-inhibitory activity of whey peptides was not severely affected, demonstrating resistance and the release of new short bioactive fragments (*Tavares et al., 2011a*). The presence of residual rennet, endogenous milk proteases and microorganisms in raw milk used for cheese making seem to be responsible for casein hydrolysis, leading to the presence of bioactive peptides found in ripened cheeses (Settanni and Moschetti, 2010). Bioactive peptides found in cheese-like model systems manufactured with sheep's and goats's milk, renneted with plant proteases from *C. cardunculus* (cardosins) suggests the potential use of phytoproteases for the production of functional cheese with ACE-inhibitory peptides (Silva et al 2006). Furthermore, whey protein hydrolysates prepared with the same plant extract showed antihypertensive effects in SHR, upon administration by gastric intubation, suggesting that the use of whey protein hydrolysates produced with cardosins, can be an effective nutraceutical ingredient in functional food formulation for hypertension control (Tavares et al., 2012a). Bioactive fragments found include the KGYGGVSLPEW (f16–26), DKVGINY (f97–103), KVGINYW (f98–104), DKVGINYW

(f97–104) sequences from α-lactalbumin, and the decapeptide DAQSAPLRVY (f33–42) from β-lactoglobulin (Tavares et al., 2011a, 2011b). In addition, ECA-inhibitory peptides have been generated by endogenous proteases from potato tuber (Makinen et al., 2016).

In some cases, combination of enzymes during protein hydrolysis can be more efficient than used separately. Lysozyme hydrolysates obtained with a mixture of trypsin and papain exhibited higher ACE inhibitory and antioxidant activities than those obtained when used alone (Memarpoor-Yazdi et al. 2012a; 2012b; Asoodeh et al. 2012). Gelatin hydrolysates from blacktip shark prepared with papain, exhibited ACE inhibitory activity, property that increased with DH (10-40%) from an IC_{50} value of 2.45 g/L to values ranging from 0.94 to 1.77 g/L (Kittiphattanabawon et al. 2013). Gelatin hydrolysates (40% DH) also exhibited capacity to inhibit the oxidation of LDL cholesterol, protect DNA scission from hydroxyl and peroxyl radical *in vitro* and inhibit lipid oxidation in both b-carotene linoleate and cooked comminuted pork model systems (Kittiphattanabawon et al. 2012; 2013). Usually, the strength of antihypertensive peptides is compared with that of antihypertensive drugs such as captopril, which possesses an ACE inhibition IC_{50} value as low as 0.022 μM (Fujita and Yoshikawa, 1999). Most bioactive peptides from food proteins have moderate ACE inhibitory potency within the IC_{50} range values from 100 to 500 μM; however, these are preferred due to their natural origin and their decreased side-effects. The most common antihypertensive peptides DPP and IPP, isolated from hydrolysates of milk proteins (β-casein), have IC_{50} values around of 9 and 5 μM, respectively. However, LKP and IPW peptides, found in fish and chicken meat hydrolysates, showed low IC_{50} values of 0.32 and 0.22 μM, respectively (Erdman et al., 2008).

Studies related with the effect of food processing over the bioactivity of peptides are scarce. However, a recent study showed that hydrolysates with antihypertensive activity prepared from bovine albumin proteins using papain, preserved its activity *in vitro* and *in vivo* (using SHR) after its inclusion in bread production, indicating that hydrolysates bioactivity was stable to the baking process and offers high potential for use as a functional antihypertensive ingredient (Lafarga et al., 2016b).

5.2 Antioxidant Properties

A diversity of complex oxidative reactions naturally occurring in biological systems can lead to *in vivo* oxidative stress due to excessive reactive oxygen species (ROS) which can induce damage to nucleic acids, proteins, and unsaturated fatty acids provoking cellular damage that contributes to carcinogenesis, cirrhosis, Alzheimers, and coronary artery diseases (Chakrabarti et al., 2014; Fan et al. 2010). On the other hand, quality deterioration and/or possibly creation of toxic/carcinogenic compounds caused by oxidative reactions is a concern for the food industry, therefore a diversity of strategies to increase its shelf life are commonly used. The consumers´ preference for natural preservatives has led to the replacement of synthetic antioxidants by natural compounds; however, they differ in their ability to inhibit oxidation as well as in their mechanism of action (Xiong 2010). *In vitro* evaluation of antioxidant properties include methods to determine radical-scavenging activity such as the DPPH (2,2-diphenyl-1-picrylhydrazyl), DMPO (5,5-dimethyl-1-pyrroline-N-oxide) and ABTS (2,2-azino-bis(3ethylbenzothiazoline-6-sulphonic acid), or evaluate their protective action to oxidation of the low density lipoprotein (LDL cholesterol) and supercoiled plasmid DNA strand (Kittiphattanabawon et al. 2013; Je et al, 2007). Their capacity to reduce metal ions by FRAP (ferric ion reducing antioxidant power) and

CUPRAC (cupric ion reducing antioxidant capacity) assays, or the protective action on target molecules by inhibiting its consumption such as the ORAC (oxygen radical absorbance capacity) and TRAP (total radical-trapping antioxidant potential) assays, are commonly used (Wang et al., 2012; Hsu et al., 2011; Kang et al., 2012; Kim et al., 2013b). Authors refers reader to Tan and Lim (2015) for better description of principles, strengths and weaknesses of the different methods.

Nutraceutical properties of bioactive peptides, next to their health beneficial effects, have increased their use as valuable ingredients in food, medicine and cosmetology. Animal proteins, especially fishery products and their sub-products, are the preferred sources for production of peptides with antioxidant properties (Chalamaiah et al., 2012). Several proteolytic enzymes such as bromelain, papain, flavorzyme, protamex, neutrase, and alcalase have been commonly used for this purpose (Je et al 2007; Kang et al., 2012; Chalamaiah et al., 2012; Elavarasan et al., 2014). Papain and bromelain are amongst the most commonly plant proteases used with excellent results. Hydrolysates from freshwater carp (*Catla catla*), produced with bromelain, had higher radical scavenging activity (DPPH assay) than those obtained with alcalase, flavorzyme and protamex (Elavarasan et al. 2014). Hydrolysates from fish *Sphyrna lewini* muscle prepared using papain also showed DPPH and ABTS radical scavenging activity (Wang, et al 2012). A protein hydrolysate from tuna backbone produced with pepsin and papain exhibited a higher DPPH and hydroxyl radical scavenging activity (DMPO assay) than those obtained with alcalase, chymotrypsin, neutrase, and trypsin. However, papain hydrolysates presented the lowest superoxide radical scavenging activity using a riboflavin/EDTA solution UV-irradiated (Je et al., 2007). Papain-hydrolyzed gelatin (from blacktip shark) inhibited lipid oxidation in both

β-carotene linoleate and cooked comminuted pork model systems, showing the capacity to inhibit human LDL cholesterol and DNA oxidation even after GIS conditions. Even though a clear relationship between bioactive properties and specific peptide sequence has not been established, it is expected that the specific bioactivity of peptides is related to its sequence and structure (Aleman et al., 2011; Xiong 2010). Hydrolysates from marine microalgae *Navicula incerta* obtained with papain showed higher antioxidant activity than those obtained with alcalase, chymotrypsin, neutrase, pepsin, pronase-E and trypsin. Two bioactive peptide sequences (PGWNQWFL and VEVLPPAEL) with capacity to protect HepG2/CYP2E1 cells ethanol-induced cytotoxicity were identified (Kang et al., 2012).

It is well known that milk proteins are an excellent source of ACE-inhibitory peptides; however, studies on their antioxidant properties are scarce. Cynarasas or phytepsins (EC 3.4.23.40) from cardoon (genus Cynara) were able to release peptides from milk proteins with antioxidant ACE-inhibitory activity *in vitro*. Antihypertensive properties *in vivo* using SHR were also confirmed (Cavalli et al., 2013; Tavares, et al 2011; 2012a; 2012b). Some active fragments showed similar sequences, such as YQEP (f191–194) and YQEPVLGP- (f191-) from β-casein and RPKHPIKH- (f16-) and RPK (f16–18) from α-casein (**Table 3**). In addition, some peptides with similar sequences (or part of it) such as VPKVK $_{f\beta 95\text{-}99}$, TQEP $_{f103\text{-}106}$ and TQEPVLGP $_{f191\text{-}198}$ fragments from β-casein, in addition to ACE inhibitory activity also had antioxidant activity (Silva et al., 2006). A casein hydrolysate obtained at the highest DH (76%) with a serine proteinase from Asian pumpkin seeds had antioxidant activity of 2.15 µM Trolox/mg, 96.15 µg Fe (III)/mg and 814.97 µg Fe (II)/mg using FRAP, DPPH and Fe ion chelating activity methods, respectively (Dabrowska et al., 2013).

White egg protein hydrolysate prepared by papain digestion had high antioxidant and ACE inhibitory activity (Chen and Chi, 2012). However, ostrich egg white protein hydrolysates obtained with trypsin presented higher antioxidant activity than those obtained with papain (Tanzadehpanah et al. 2012).

Use of plant proteases for antioxidant peptides production from plant proteins has also been explored. Hydrolysates from soy protein isolate (SPI) produced with bromelain and papain showed higher antioxidant (superoxide dismutase-like activity and lipid peroxidation inhibition) than those produced with flavourzyme or neutrase; their activities increasing with DH (Lee et al, 2008). A protein hydrolysate from palm kernel cake produced with papain showed a higher DPPH radical scavenging activity (around 73 %) than those made with pepsin, alcalase, chymotrypsin, bromelain, flavourzyme and trypsin (with 50, 28, 22, 12, 6 and 6%, respectively) (Zarei et al., 2012). Antithrombotic and antioxidant activities in amaranth hydrolysates, prepared by activation of endogenous acid proteases, have also been reported (Sabbione et al., 2016). The use of non-conventional protein for antioxidant peptides production has also been considered. Hydrolysates from silk sericin have been produced using alcalase, papain and bromelain; however, higher antioxidant activity (evaluated by ABTS and DPPH, peroxidation inhibition, reducing power and ferrous ion chelating ability) was shown by the ones produced with alcalase (Fan et al., 2010).

5.3 Peptides with DPP-IV Inhibitory Activity for Diabetes Treatment

Diabetes is a major contributor to global mortality and morbidity. In 2014, the number of adults with this disease was estimated of 422 million (NCD RiskC, 2016). Several synthetic drugs (e.g,

acarbose) and natural compounds (e.g., catechins and plant extracts) have been associated with antidiabetic properties due to their capacity to inhibit key enzymes in blood sugar regulation, decrease blood glucose, increase glucose uptake and stimulate insulin secretion (Liu et al., 2013; Lee et al., 2011; Guasch et al, 2012). Another attractive option in pharmacological interventions includes the use of bioactive peptides with capacity to inhibit key enzymes (e.g., Dipeptidyl-aminopeptidase IV and α-glucosidase) that regulate blood sugar preventing or delaying the complications related to this disease. Dipeptidyl-aminopeptidase IV (DPP-IV, EC 3.4.14.5) is a key enzyme in intestinal digestion and absorption mechanisms. Its activity has been associated with the degradation of the incretins GLP-1 (glucagon-like peptide-1) and GIP (gastric inhibitory polypeptide) in the brush border membrane of mammalian small intestine, kidney and other tissues (Jacubowicz and Froy., 2013; Lacroix and Li-Chan 2012b). GLP-1 and GIP have important roles in glucose homeostasis regulation in the gastrointestinal tract within the first minutes of nutrient ingestion. They have a glucose-dependent stimulation role in insulin secretion, glucagon suppression and enhancement of β-cell mass. Incretins have a short biological half-life due to its rapid degradation by DPP-IV and neutral endopeptidases, along with their elimination by the kidney (Jacubowicz and Froy, 2013). Therefore, it is assumed that peptides with capacity to modulate GLP1 release, possess antidiabetic properties. Incretin-based therapies and DPP-IV inhibitors have proven to be useful for the treatment of type-2 diabetes (Tulipano et al., 2011; Anagnostis et al., 2011; Riedel et al., 2009). Diabetic treatment involves frequent injections of DPP-IV inhibitors to maintain therapeutic efficacy. The presence of bioactive peptides in protein hydrolysates with capacity to inhibit DPP-IV *In vitro* and reduce blood glucose level *in vivo* has promoted the production of hydrolysates with antidiabetic

properties (Lacroix and Li-Chan 2012b). The tripeptides IPl (diprotin A) and VPL (diprotin B) were the first potent DPP-IV inhibitors available, with IC_{50} in the µM range (Lacroix and Li-Chan 2012a). However, more potent inhibitors (e.g., linagliptin and alogliptin with IC_{50} in the nM range) are commercially available (Juillerat-Jeanneret, 2014). *In silico* analysis of food protein sequences indicate that caseins from cow's milk and collagen from bovine meat and salmon are rich potential sources of peptides with DPP-IV inhibitory activity (Lacroix and Li Chan 2012a ; Nongonierma and FitzGerald 2014). The dipeptides GA, GP and PG are the predominant sequences found in food proteins (Lacroix and Li Chan 2012a) which have been found to be effective. Under full gastrointestinal simulated digestion (GIS) of β-lactogobulin and α-casein, the peptides released, IPA and VAPFPEV, respectively, showed DPP-IV inhibitory activity (Kopf-Bolanz et al., 2014). Milk protein hydrolysates with DPP-IV inhibitory activity were also obtained using proteases from microbial (i.e., Thermolysin, Protease N "Amano" K, Protease A "Amano" 2, Umamizyme K, Alcalase, Protin SD-NY10, Flavorzyme and Protamex), animal (i.e., pepsin and trypsin) and plant (i.e., bromelain) sources (Lacroix and Li-Chan 2012b). Whey protein pepsin-hydrolyzed showed the highest DPP-IV inhibitory activity, while a whey β-lactoglobulin-rich fraction trypsin-hydrolyzed presented several peptides with capacity to inhibit DPP-IV with two potent sequences LKPTPEGLD and LKPTPEGLDLEIL (with IC_{50} of 45 and 57 µM, respectively) (Lacroix and Li Chan 2014). Reports using plant proteases for the production of bioactive peptides with DPP-IV inhibitory activity are scarce with only the use of bromelain or papain being reported (Boots et al., 2009; Lacroix and Li-chan 2012b; Li-Chan et al., 2012;Nongonierma et al., 2015). In addition, no one DPP-IV inhibitory peptide sequence released by plant proteases has yet to be identified. The skin gelatin hydrolysate from Atlantic

salmon using bromelain showed lower DPP-IV inhibitory activity than that obtained with flavorzyme or alcalase (*Li-Chan et al. 2012a*). However, when sodium caseinate was the substrate, the hydrolysates prepared with bromelain presented higher DPP-IV inhibitory activity than the enzymes described above, including pepsin (Lacroix and Li-Chan 2012b). Recently, a quinoa protein hydrolysate prepared with papain presented an IC_{50} DPP-IV inhibitory activity of 0.88 mg/mL. Again, bioactivity of peptides depend of substrate, DH, and protease used. In addition, it is not surprising that some peptides hold bi-functionality (Haque et al., 2009; Lacroix et al., 2016). For example, the IQKVAGTW, VLDTDY, LKALPMH (from β-lactoglobulin) and WLAHKAL (from α-lactalbumin) peptides obtained by the hydrolysis with pepsin can inhibit DPP-IV and ACE activity (Lacroix et al., 2016; Lacroix and Li-chan, 2014), while quinoa protein papain-hydrolyzed showed antioxidant and DPP-IV inhibition activities (Nongonierma et al., 2015). Only two *in vivo* studies on the glycemic regulatory effect of hydrolysates produced with plant proteases have been reported (Lacroix and Li-Chan 2016). The oral and ileal feeding of Male Sprague Dawley rats with a papain-hydrolyzed rice endosperm and bran protein had a positive effect in glycemia reduction under intraperitoneal glucose tolerance test, reducing the serum DPP-IV activity, stimulating the cell line GLUTag for incretin GLP-1 secretion (Ishikawa et al., 2015). In addition to the observed increase in insulin secretion and decrease in blood glucose level, a similar effect with DPP-IV and GLP-1 was observed when the same animal models were ileal administered with papain-hydrolyzed zein protein (Mochida et al, 2010). The use of plant proteases for the production of bioactive peptides with DPP-IV inhibitory activity for the treatment of type-2 diabetes, offers a new and promising strategy that requires to be explored in the evaluation and process optimization for protein hydrolysates production.

5.4 Hypocholesterolemic and Hypolipidemic Peptides

Globally, obesity is a major public health concern related with the increase of risk to hypertension, heart disease and diabetes. The increase of cholesterol and/or triglycerides (TGs) in plasma or the reduction of high-density lipoprotein levels (dyslipidemia or hyperlipidemia) contributes to the development of atherosclerosis. Strategies to modulate these levels are associated with a reduction in the risk of cardiovascular diseases (CVD). Protein hydrolysates containing certain peptide sequences that have shown promising hypolipidemic effect *in vitro*, *ex-vivo* (e.g., cultured mammalian cells) and *in vivo* (i.e., animal models) (Howard and Udenigwe, 2013; Liu et al., 2013). Hypolipidemic peptides act in hepatocytes, adipocytes and gastroinstestinal tract by binding bile acids, cholesterol micelles disruption and by inhibiting their absorption. Besides, they can act affecting the activity of hepatic and adipocyte enzymes and by activating gene expression of lipogenic proteins such as the low density lipoprotein (LDL), its receptor (LDL-R) and the enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR). This last one is the major regulatory enzyme for cholesterol biosynthesis and its inhibition has been the preferred target aimed to reduce the rate of cholesterol biosynthesis (Udenigwe and Rouvinen-Watt 2015; Howard and Udenigwe, 2013). LPYP and IAVPGEVA peptides from soy protein hydrolysates have been reported to inhibit HMGCoAR. Based on these peptide sequences, a potent competitive inhibitory peptide with SFGYVAE sequence (IC_{50} = 0.03 μM) was recently designed. Other effective hypolipidemic peptides include soystatin (VAWWMY) from soy glycinin, and lactostatin (IIAEK) from β-lactoglobulin (Howard and Udenigwe, 2013). With the exception of lunasin (peptide found naturally in soy, wheat and rye), most hypolipidemic peptides with the capacity to inhibit the expression of HMGCoAR or

cholesterol absorption has been released from food proteins by the use of pepsin, trypsin, alcalase and microbial proteases (from *Bacillus* sp.) (Howard and Udenigwe, 2013; Pak et al., 2012; Nagaoka et al., 2010). To the best of our knowledge, papain is the only plant protease used to produce hydrolysates with hypocholesterolemic properties. A low molecular weight peptide fraction from pork meat papain-hydrolyzed was capable to reduce the cholesterol levels (VLDL and LDL) in plasma and liver of rats by interfering cholesterol absorption, while those hydrolysates produced with pepsin or trypsin did not show hypocholesterolemic effect (Moritmatsu et al., 1996). Fish hydrolysates from Alaska Pollock also reduced the cholesterol level but by increasing the expression level of cholesterol 7 α-hydroxylase (CYP7A1) in liver (Hosimi et al., 2012). However, no hypocholesterolemic active peptide sequence has yet been identified.

5.5 Antimicrobial Properties

The rise of bacterial resistance against synthetic antimicrobial agents and their use limitations as preservatives in food systems have greatly increased the interest for natural antimicrobial compounds. Antimicrobial peptides possess a great potential to replace synthetic antimicrobial agents due to their low toxicity and less environmental risk. Aquatic organisms have been the preferred source for the isolation of natural antimicrobial peptides resulting in an increased interest for a natural product-based drug. Antimicrobial peptides production by hydrolysis of food proteins catalyzed by plant proteases has gained relevance recently. Bromelain was efficient in releasing antimicrobial peptides from Leatherjacket (*Meuschenia sp*.) protein. This hydrolysate was effective against *Staphylococcus aureus* and *Bacillus cereus* (Salampessy et al. 2010). In other study, a casein hydrolysate at high DH produced using serine proteinases from

Asian pumpkin inhibited the growth of *B. cereus* and *Pseudomonas fluorescens* but not *Escherichia coli* (Dabrowska et al., 2013). Cysteine proteases from *Jacaratia corumbensis* latex was also used for casein hydrolysis. After 2 h hydrolysis, the product was active against *Enterococcus faecalis, B. subtilis, E. coli, P. aeruginosa, Klebsiella pneumoniae* and *S. aureus* (Arruda et al., 2012). Efficiency among proteases from different sources (i.e., animal, microbial and plants) to release antimicrobial peptides have been compared. Ovoalbumin hydrolysates produced with pepsin presented higher antimicrobial activity than those obtained with papain (Tang et al., 2013). However, hydrolysates from sea cucumber *Actinopyga lecanora* produced with plant proteases (i.e., papain and bromelain) showed maximum antibacterial activity as compared to those hydrolyzed by other proteases (i.e., pepsin, trypsin, alcalase, and flavourzyme). The hydrolysate produced with papain showed activity against the pathogen *S. aureus*, while bromelain-hydrolyzed showed the highest activity against *Escherichia coli, Pseudomonas aeruginosa,* and *Pseudomonas sp*. (Ghanbari et al., 2012). On the other hand, the hydrolysis of oyster (*Crassostrea gigas*) protein with alcalase and bromelain, released a cysteine-rich antimicrobial peptide (CgPep33) that showed activity against *E. coli, B. subtilis, P. aeruginosa* and fungi (Liu et al., 2008). In addition, using the same combination of enzymes and substrate, a peptide fraction of 5–10 kDa showed inhibitory activity against herpes virus (Zeng et al., 2008).

5.6 Other Bioactive Properties

Antihypertensive, antidiabetic, antioxidant, antimicrobial, anticholesterolemic and antilypidemic activity are among the most studied bioactive properties. However, to a lesser degree, the study of peptides with immunomodulating, antiproliferative, antitrombotic, appetite suppressant and

calcium-binding capacity have been also considered. However, it is likely that some bioactivities remain to be discovered. Soy hydrolysates prepared with alcalase and papain showed a higher immunomodulating effect on the proliferation of murine spleen lymphocytes *in vitro* as compared to those prepared with flavourzyme, trypsin, Protease A and Peptidase R. Those peptides were of low molecular weight and contained a high content of positive charges characteristics (Kong et al., 2008). The digestion of soybean β-conglycinin with bromelain and others enzymes yielded peptides with capacity to inhibit appetite (appetite suppressors). Peptides released with bromelain produced the highest capacity to suppress appetite in rats under meal-feeding conditions. The mechanism was through cholecystokinin (CCK) secretion by the enteroendocrine cells (Sufian et al., 2011). In another study, wheat germ protein papain-hydrolyzed, presented peptide fragments with high calcium-binding capacity. However, those obtained with alcalase possessed a higher capacity to bind calcium due to their high content in acidic and hydrophobic amino acids (McDonagh and FitzGerald, 1998).

Other bioactive properties of peptides include acetylcholinesterase (AChE) inhibitory activity, which opens possibilities for their use as a therapeutic agent in Alzheimer's diseases (AD). The inhibition of the hydrolysis of acetylcholine activates the central cholinergic system and alleviates cognitive deficits. Tuna liver protein hydrolysate produced with alcalase, neutrase, protamex and flavourzyme released peptides with AChE inhibitory activity as well as excellent antioxidant activities against DPPH, hydrogen peroxide and hydroxyl radical scavenging, reducing power and protection effects on hydroxyl radical-induced DNA damage *in vitro* (Ahn et al., 2010). Although no plant proteases were considered in that study, the production of antioxidant peptides with high efficiency as described above, resemble their potential. The use of

plant proteases to produce peptides with AChE inhibitory activity has been recently demonstrated with the red seaweed *Palmaria palmate* hydrlysate produced with papain. The two peptides IRLIIVLMPILMA and LMAASWAIY sequences showed AChE inhibitory activity with an IC_{50} of 0.432 mg/mL (288.77 µM) and 0.348 mg/mL (339 µM), respectively (Hayes et al., 2015). In another study, antiproliferative activity was reported for the LPHVLTPEAGAT (1206 Da) and PTAEGGVYMVT (1124 Da) peptides sequences found in tuna hydrolysate produced with papain and protease XXIII, respectively. These peptides showed a dose dependent inhibition effect on MCF-7 cells with IC_{50} values of 8.1 and 8.8 µM, respectively (Hsu et al., 2011). Production of peptides with antithrombotic activity constitutes another biological function with potential biomedical application. Pork meat hydrolysates prepared with papain presented a peptide fraction with antithrombotic activity, which was just as effective as aspirin (50 mg/kg body weight) (Shimizu et al., 2009). In addition, hydrolysis of bovine caseinomacropeptide (CMP) by *C. cardunculus* yielded bioactive peptides with antithrombotic activity. The identified peptide sequence, TVQVTSTAV (f161–169), was equivalent to those obtained by hydrolysis of ovine CMP with trypsin ($f_{163–171}$ TAQVTSTEV and $f_{165–171}$ QVTSTEV) (Tavares et al., 2011b).

6. FUTURE TRENDS AND CONCLUSIONS

Plants represent an important and promising source of proteases for biotechnological applications, such as the production of bioactive peptides and functional ingredients. Plant proteases are diverse and offer a wide range of temperature and pH activity profiles. The diversity of substrate specificities among different plant proteases supports their potential use for the production of bioactive peptides through the digestion of proteins from various sources. The type and source of enzyme, as well as the strategy for its use (e.g., pure form, mixed or the

sequential action) will depend on enzyme specificity, protein substrate used, and extent of hydrolysis and the expected bioactivity of the peptides released.

Hydrolysates produced by the action of plant proteases have, so far, been evaluated for their antihypertensive and antioxidant properties towards treating diseases such as hypertension, cancer and diabetes. Antimicrobial, antidiabetics, antiobesity, anti-hypercholesterolemia, and immunostimulating properties have also been considered. Most studies have evaluated the bioactivity of these hydrolysates *in vitro*; hence, studies *in vivo* are still relatively scarce. In addition, the peptide sequences responsible for bioactivity of hydrolysates have not been identified in most cases.

Plant proteases such as bromelain and papain have been used to great success as biotechnological processing aids, yet these few examples represent a tiny fraction of what might be available. Therefore, more research regarding identification and the use of emerging new plant proteases is highly recommended. The use of bioinformatics-aided prediction studies represents a new area of opportunity in order to select an appropriate combination of protease/substrate to optimize proteolysis. However, the *in silico* design of proteases with high specificity towards a specific protein sequence still requires *in vitro* experiments for confirmation.

Plant proteases represent a growing market for industrial enzymes due to their ability to increase functional properties such as solubility, foaming, and emulsifying properties in foods. Their use as aids in the production of functional/bioactive hydrolysates from food proteins offers unique opportunities in the pharmaceutical and food industries for the production of functional ingredients, nutraceuticals and functional foods.

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Table 1. Abundance and diversity of proteolytic enzymes in some plant sources[1]

Plant specie	**Known and putative peptidases**
Arabidopsis *Arabidopsis thaliana****	306 SP, 232 AP, 142 CP, 115 MP, 101 PP
Papaya Carica papaya	7 **CP**, 3 SP , 3 PP
Kiwi *Actinidia deliciosa*	7 **CP**
Pinaple *Ananas comosus*	6 **CP**, 2 PP, 1 AP, 1 SP
Fig *Ficus glabrata/Ficus carica*	3 **CP**/ 3 **AP**
Ginger *Zingiber officinale*	3 **CP**
Sodom apple *Calotropis procera*	1 **CP**
Melon *Cucumis melo*	6 **SP**, 5 CP, 3 PP, 2 AP
Osage orange *Maclura pomifera*	1 **SP**
Cardoon Cynara cardunculus	12 **AP**

[1]Numbers of peptidases correspond to sequences deposited in MEROPS database.

[*]Numbers in Arabidopsis corresponds to the total DNA sequences of peptidases identified in its complete genome.

Letters in bold indicate the type of peptidases most abundant reported in plant sources which genome has not been sequenced.

Abbreviations: AP, Aspartic peptidase; CP, Cystein peptidase; CP, Serine peptidase; MP Metallo peptidase, PP, Mixed mechanism.

Table 2. ***Type of plant proteases, source and food proteins used for bioactive/functional peptides production***

Type	Given name	Source	Protein Substrate	Bioactivity	References
Cysteine					
	Papain	*Carica papaya* latex or fruit	Hen egg white lysozyme[1]	Antioxidant, antimicrobial and ACE-inhibitory peptides	Memarpoor-Yazdi et al 2012a, 2012b; Asoodech et al., 2012
			Duck egg white proteins	Antioxidant hydrolysates	Chen et al., 2009
			Hen egg white proteins	Antioxidant and ACE-inhibitory peptides	Chen and Chi 2012
			Red blood cell fraction from deer, sheep, pig and cattle	Antioxidant hydrolysate	Bah et al., 2016
			Ostrich egg white	Antioxidant peptides	Tanzadehpanah et al., 2012
			Chicken muscle from Black-bone silky fowl *Gallus gallus domesticus Brisson;*	Antioxidant and ACE-inhibitory peptides	Gu et al., 2012;

			Bovine (Boss taurus) brisket sarcoplasmic proteins	Antihypertensive activities	Di Bernardini et al., 2012
			Bovine globulins	Hydrolysates with antihypertensive activity *in vitro* and *in vivo*	Lafarga et al., 2016a
			Chicken breast proteins	Antioxidant peptides	Sun et al., 2012
			Pork meat proteins	Hipocholesterolemic; Antitrombotic peptides	Morimatsu et al., 1996; Shimizu et al., 2009
			Microalgae *Navicula incerta*	Antihepatotoxic and antioxidative peptides	Kang et al 2012
			Macroalgae *Palmaria palmata*	Acetylcholinesterase (AChE) inhibitory peptides	Fitzgerald et al., 2012
			Microalgae *Nannochloropsis oculata*	ACE-inhibitory peptides	Samarakoon et al., 2013
			Mussels *Rudtapes philippinarum* and *Mytilus coruscus*, silk sericin	Antioxidant peptides	Kim et al., 2013a, 2013b; Fan et al. 2010

			Soy protein isolated, beta-conglycinin- and glycinin-rich fractions, bean proteins, cottonseed protein, lentil protein concentrate	ACE-inhibitory peptides	Margatan et al., 2013;Rui et al., 2012; Gao et al., 2010; Barbana et al., 2011
			Soy protein	Immunomodulating peptides	Kong et al., 2008
			Soy protein isolate	Antioxidant peptides and ACE-inhibitory peptides	Lee et al., 2008
			Chickpeas protein concentrate	Antioxidant peptides and ACE-inhibitory peptides	Medina-Godoy et al., 2012
			Palm kernel cake	Antioxidant peptides; Iron-Chelating	Zarei et al., 2012, 2016
			Quinoa protein	Antioxidant and DPP-IV inhibitory activity hydrolysates	Nongonierma et al., 2015

			Flaxseed protein	Renin and ACE-Inhibitory peptides	Udenigwe et al., 2009
			Rice protein	Antioxidant hydrolysates	Guo et al., 2013; Zhang et al., 2010
			Ziziphus jujube seed protein	Antioxidant hydrolysates	Kanbargi et al., 2016
			Zein protein	Hydrolysates with capacity to induce GLP-1 secretion and reduction of DPP-IV activity *in vivo*	Mochida et al., 2010
			Gelatin from blacktip shark skin	Antioxidant and ACE-inhibitory peptides	Kittiphattanabawon et al., 2012, 2013
			Fish skin gelatin and protein muscle from loach *Misgurnus anguillicaudatus,*	Antioxidant hydrolysates	You et al., 2010; You et al., 2009;

			Fish muscle from patin *(Pangasius sutchi)*, Shark *Sphyrna lewini* Stone fish *Actinopyga lecanora*, Triakidae fish *Sphyrna lewini*, tuna backbone and silver carp processing by-products	Antioxidant peptides	Najafian et al., 2013; Wang et al., 2012; Bordbar et al., 2013, Luo et al., 2013; Je et al., 2007; Zhong et al., 2011
			Fish (Cirrhinus mrigala)	ACE-inhibitory peptides	Elavarasan et al., 2016
			Tuna dark muscle	Cancer cell antiproliferative activity	Hsu et al., 2011
			Alaska pollock muscle	Hypocholesterolemic activity	Hosomi et al., 2012
			Boarfish (Capros aper Linnaeus)	ACE-inhibitory hydrolysates	Hayes et al., 2016
			Threadfin bream (N. Japonicus) frames	Antioxidant and ACE-inhibitory hydrolysates	Gajanan et al., 2016

			Sea cucumber *Actinopyga lecanora*	Antibacterial peptides	Ghanbari et al. 2012
			Bullfrog skin, *Rana catesbeiana Shaw*	Antioxidant peptides	Qian et al., 2008
			Caseinate	DPP-IV inhibitory peptides; Antioxidant peptides and ACE-inhibitory peptides	Boots 2009; Luo et al. 2014
			Whey proteins	Hydrolysates with antioxidant and DPP-IV inhibitory activity	Le Maux et al., 2016
			Buffalo milk proteins	Antioxidant and ACE-inhibitory peptide fractions	Abdel-Hamid et al., 2017
	Bromelain	Pineaple fruit or stem	Fish muscle from Shark *Sphyrna lewini*, rock fish *Actinopyga lecanora* and Carp *(Catla catla)*	Antioxidant peptides	Wang et al., 2012; Bordbar et al., 2013; Elavarasan et al., 2014
			Threadfin bream (*N. Japonicus*) frames	Antioxidant and ACE-inhibitory peptides	Gajanan et al., 2016

			Red blood cell fraction from deer, sheep, pig and cattle	Antioxidant hydrolysates	Bah et al., 2016
			Sea cucumber *Actinopyga lecanora;* oyster (Crassostrea gigas)*;* Insoluble muscle protein from Fish leatherjacket (*Meuchenia sp.*)	Antibacterial peptides	Ghanbari et al. 2012; Liu et al., 2008; Salampessy et al., 2010
			Sodium caseinate	Calcium binding caseinophosphopeptides	McDonagh & FitzGerald, 1998
			Silk sericin	Antioxidant peptides	Fan et al. 2010
			Rice protein	Antioxidant/solubility and emulsifying properties	Guo et al., 2013
			Palm kernel cake	Antioxidant and Iron-Chelating peptides	Zarei et al., 2012, 2016
			Lentil protein concentrate	ACE-inhibitory peptides	Barbana et al., 2011

			Gelatin from Atlantic salmon skin, caseinate, whey proteins	Dipeptidyl-peptidase IV-inhibitory activity	Li-Chan et al., 2012; Boots 2009; Lacroix and Li-Chan 2012b
			Protein isolates from Pinto Durango and Negro 8025 beans	Antioxidant, α-amylase, α-glycosidase, DPP-IV and ACE-inhibitory activity peptides, and induced insulin secretion in NS-1E cells.	Oseguera-Toledo et al., 2015
			Milk proteins	Peptide fractions with ACE-inhibitory properties	Medeiros et al., 2014
			Soy protein isolate	Antioxidant and ACE-inhibitory peptides	Lee et al., 2008
			Soy β-Conglycinin	Appetite suppressor (stimulate cholesystokinin secretion)	Sufian et al., 2011
	Ficin	*Ficus sp* latex tree	Flaxseed protein	Renin- and ACE-inhibitory peptides	Udenigwe et al., 2009

			Caseins	Antioxidant peptides	Di Pierro et al., 2014
			Egg-white proteins	Antioxidant hydrolysates	Cho et al., 2014
	Actinidin	Kiwifruit	Hemp (Canabis sativa L) protein isolate	Hydrolysates with antioxidant (DPPH radical scavenging and ORAC activity) and ACE-inhibitory activity	Teh et al., 2016
	Zingibain	Ginger rhizoma			
	Jacaratia Cistein Protease	*Jacaratia corumbensis* O. Kuntze latex	Casein	Antimicrobial peptides	Arruda et al., 2012
Serine	Pomiferin	*Maclura pomifera* latex fruit	Caseins and whey proteins	Antioxidant and ACE-inhibitory activity peptides	Corrons et al., 2012;2017, Bertucci et al., 2015
	Asian Pumpkin Serin Proteinasa	*Cucurbita ficifolia* Asian pumpkin seeds and pulp	Caseins	Peptide fractions with antioxidant and antimicrobial activity	Dabrowska et al., 2013
			β-lactoglobulin and whey protein concentrate	DPP-IV, ACE and α-glucosidase inhibitory activity	Babij et al., 2014

			Egg-White by-product protein	ACE-Inhibitory peptides	Pokora et al., 2014
Aspartic	Cyprosin, Cardosin (A-H), Phytepsins, Cynarases	*Cynara cardunculos*	Whey proteins from bovine milk	Peptides with Antioxidant and ACE-Inhibitory activity and peptide concentrates with Antinociceptive, Anti-inflamatory and Antiulcerogenic activity	Tavares et al., 2011a, 2011b, 2011c, 2012a, 2012b;2013 Tavares and Malcata 2012
	Onopordosin	*Onopordum acanthium* L. (Asteraceae)	Ovine and Caprine milk	Antioxidant and Anti-hypertensive peptide	Silva et al., 2006
	Arctiumisin	*Arctium minus* (Hill) Bernh flowers	Whey proteins	Antioxidant hydrolysates	Cimino et al., 2016

[1]A higher ACE-inhibitory activity of peptides was obtained by hydrolyzing in combination with trypsin.

[2]The bioactive properties are assigned to the crude protein hydrolysates when bioactive sequences were not identified or peptide fractionation was no accomplished.

Table 3. Peptides sequences with bioactive activity released by plant proteases from food-proteins

Protein/peptide sequence	Plant protease	Biological Property		Reference
α-lactalbumin		***ACE Inhibitory activity (IC_{50})***	***Antioxidant activity***	
KGYGGVSLPEW $_{f16–26}$	*Cardosin from C. cardunculus*	300.9 (μM)		Tavares et al., 2011b
KGYGGVSL $_{f16-23}$		0.70 (μM)		
DKVGINY $_{f97–103}$		99.9 (μM)		
KVGINYW $_{f98–104}$		25.4 (μM)		
DKVGINYW $_{f97–104}$		12.2 (μM)		
β-lactoglobulin				
DAQSAPLRVY $_{f33–42}$		13 (μg/mL)		

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β-casein			TROLOX Eq. (µM)	Silva et al., 2006
VPKVK $_{f95-99}$		93.75 (µg/mL) 169-689 (µg/mL)	0.079	
YQEP $_{f103-106}$		499.99 (µg/mL)	0.072- 0.102	
YQEPVLGP $_{f191-198}$			0.124	
α_{s1}-casein		36.68 (µg/mL)		
RPK		892.83 (µg/mL)	--	
RPLHPIKH		95.46 (µg/mL)	--	
YQKFPQY$_{f90-96}$			0.068	
	SP from Asian pumpkin *Cucurbita ficifolia*			
Ovalbumin		33.9 (µg)		
SWVE $_{f148-151}$		73.44 (µg)		Pokora et al., 2014

DILN $_{f86–89}$				
Caseinomacropeptide			**Antithrombotic activity** (claimed in Qian et al., 1995)	
TVQVTSTAV $_{f161–169}$				Tavares et al., 2011b
Bovine serum albmin	*Papain from Carica papaya*			
SLR, YY,ER, & FR		0.17, 0.04, 0.27 & 0.42 (mM)		Lafarga et al., 2016a
Barley protein concentrate				
FQLPKF		28.2 (µM)		Gangopadhyay et al., 2016

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GFPTLKI		41.2 (μM)		
Palm kernel cake protein				
GGIF & YLLLK		Iron-chelating activity (IC50 1.4 and 0.2 μM)		Zarei et al., 2016
***Sphyrna lewini* muscle**		Scavenging activity (EC_{50} mg/mL) on hydroxyl (0.15 and 0.24), ABTS (0.34 and 0.12), superoxide anion (0.09 and 0.11) and DPPH (3.63 and 4.11) radical		Wang et al., 2012
WDR and				
PYFNK				

		Antiproliferative activity against MC7-cell (IC_{50} 8.1 µM)	
Tuna fish protein			
LPHVLTPEAGAT		Antioxidant and antihepatotoxic activity (protection of HepG2/CYP2E1 cells of alcohol-induced cytotoxicity)	Hsu et al., 2011
Microalgae *Navicula incerta*			Kang et al., 2012

PGWNQWFL		Scavenging activity for hydroxyl, DPPH, superoxide and alkyl radicals (IC_{50} 0.118, 0.154,0.316 and0.243 mg/mL, respectively) in vitro, inhibited MDA level, and regulated antioxidative enzymes in vivo	
VEVLPPAEL			
Mussel Mytilus coruscus			Kim et al., 2013b
SLPIGLMIAM			

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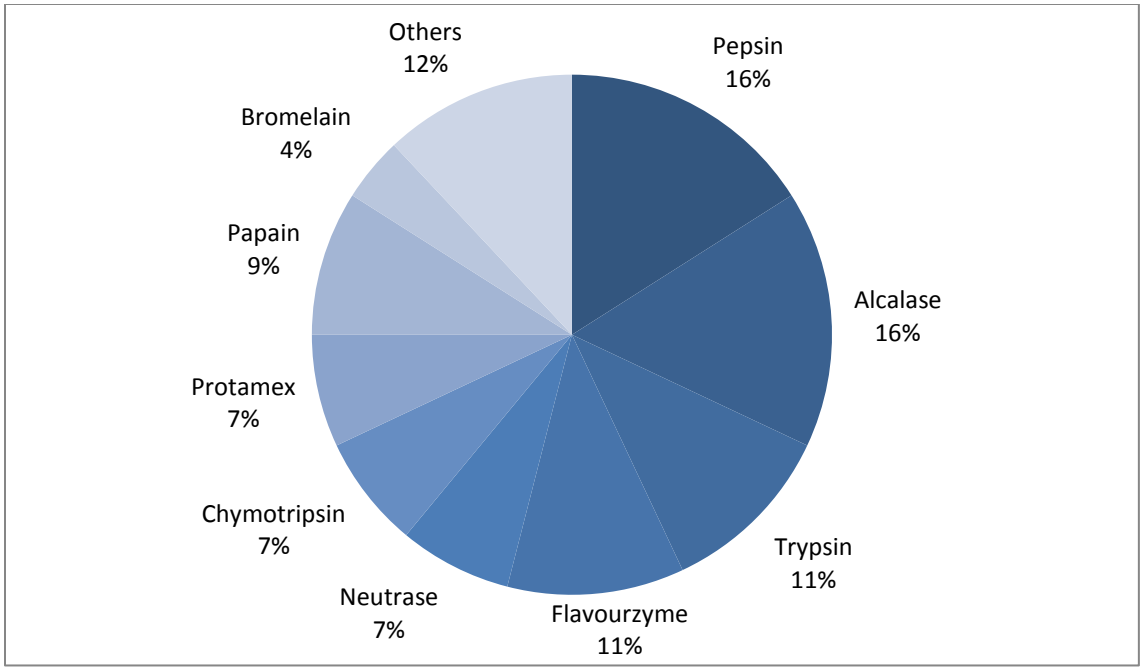

Figure 1. Cite frequency for proteases used in bioactive peptides production*, *Data obtained from 150 research articles from Scopus, Web of Knowledge and Pubmed. Enzyme inclusion criteria were at least one bioactivity evaluated from their hydrolysates, independently from the number of enzymes evaluated*

