# Biotechnological applications of proteases

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**Abstract**

This review presents some of the hottest topics in biotechnological applications which is proteases in biocatalysis. Obviously, one of the most relevant areas of application is in the hydrolysis of proteins in food technology, and that has led to a massive use on proteomics. Their aim is to identify the different proteins via peptide maps obtained after a specific protease hydrolysis. However, concepts like degradomics are taking a more relevant importance in the use and study of proteases and will be also properly discussed. Other protease applications like in cleaning (detergents development), pharmaceutical or fine chemistry will be analyzed. This review goes from basic areas such as protease classification to a discussion of the preparation of protease immobilized biocatalysts, considering the different problems raised by the use of immobilized proteases due to the peculiar features of the substrates, large macromolecules. Production of bioactive peptides via limited hydrolysis of proteins will occupy an important place in this review.

**Key words:** Protease; bio-functional peptides; degradomics; protease immobilization; steric hindrances; controlled proteolysis.

## 1. Introduction

Food biotechnology, considered as applications of biotechnological processes, started before 6000 B.C. Fermenting grapes or brewing beer are examples of this initial food biotechnology, even though they are crude ones at that (Mishra et al., 2016). In fact, the application of enzymes in food technology was already established in these processes. Another example of this “primitive” food biotechnology (Mishra et al., 2016) is the use of rennet. This shows the successful use of a protease mixture from the stomach of calves in cheesemaking for centuries (Moschopoulos, 2016). Actually, proteases may be remarked among hydrolases for their industrial uses, being a collection of enzymes of the outmost relevance that is still applicable in food industry, followed by transferases (Vermelho et al., 2016).

Enzymes are well inside the concept of *Biotechnology* (*"any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use"* (Food and Agriculture Organization, 2017). As previously presented, proteases are among the first enzymes utilized for this kind of application and their utility still stands strong. However, the ways of utilization of these enzymes have changed over time. Initially, they were mainly used as extracts of animal or vegetable tissues, or complete microorganism cells. The advances in enzyme isolation and production techniques have facilitated the direct application of purified enzymes (Schmid et al., 2001; Kirk et al., 2002; Kaul and Asano, 2012; Li et al., 2013). These technical advances permitted to extend the range of known enzymes and improve the characterization of their conformations and catalytic mechanisms, making their application simpler (Pogson et al., 2009; Li et al., 2013; Felici et al., 2017; Strack, 2017). At this point, both academic and industrial interests meet (Neurath, 1999), or it could be said that both basic and applied sciences focus on

proteases. They offered a wider range of likely applications, and also permitted to understand many life cycles of living beings (Huang et al., 2017). Proteases, thanks to their variants and different activity specificities, show their intimate relation with biological cycles of the diverse living beings (Perez-Silva et al., 2016; López-Otín and Overall, 2002; Huang et al., 2017). These life-cycles, in turn, mirror adaptive and evolutionary courses of living beings, and can even alter as an answer to pathological processes (Huang et al., 2017). In this sense, degradomics, a strong “omic” approach (Perez-Silva et al., 2016; López-Otín and Overall, 2002) has permitted a wide look at proteases biodiversity. A degradome can be defined as the whole diversity of proteases in an organism, tissue or cell at a determined time. Degradomics is focused on elucidating the proteases that are presented at a defined time in a particular medium (which may be cross-referenced with the knowledge on the physiological state of that specific specimen) including information on the enzyme-substrate and/or enzyme-inhibitor also presented (Perez-Silva et al., 2016; López-Otín and Overall, 2002). Degradomics enriches this "catalogue" of protease diversity (Fig. 1).

This complexity of vital cycles and their changes among different organisms provides a huge range of proteases having different functions and, consequently, a wide variety of specificities and structures (Krem et al., 2000; Puente et al., 2003). This large library of proteases may be used in different processes, and it is constructed mainly through this huge diversity of performances towards enzyme specificities and reactions media (Li et al., 2013; Sanman and Bogyo, 2014). The protease specificity mirrors how this biocatalyst interacts with the substrate to produce its hydrolysis, the core of protease utilizations (Fig. 2), and this noticeably will be imitated on the properties of the final product (McDonald, 2015). Understanding how proteases perform its function and

how to control these functions is of relevance in the search for new biocatalysts (Castro et al., 2011). This concept will be further discussed below.

In this context, this review intends to highlight subjects related to the features and types of processes in this wide universe that includes the utilization of proteases.

## 2. Particularities of protease activities

Although the folding and the primary structure of a protease are of the utmost significance in determining the interaction between the substrate and the enzyme, theories stress that a second way of interaction from a post-binding stereo-adjustment would also be very important (Michel, 2016). This way, the substrate-enzyme connection would be determined by both preexisting conformations (*Conformational Selection*) and the own changes induced by the substrate on the enzyme conformation (*Induced Fit*) (Michel, 2016; Johnson, 2008). The importance of the *induced fit* in enzyme specificity has been debated (Johnson, 2008), but the influence of the flexibility of the protein chain and their transition states for protease performance have been fully accepted. The interaction of the protease molecule with its substrate, or its structural characteristics have been used to divide proteases into several useful classifications. For example, if the peptide substrate runs through the whole extent of the protease active site framework and is cleaved somewhere in its middle point, the enzyme is called *endopeptidase* (McDonald, 1985). In a similar way, we call *exopeptidades* those proteases that act next to the end of the polypeptide chains, They can be named *amino*peptidases if they attack at the n-terminus or *carboxypeptidases* if they hydrolyze peptide bonds at the c-terminus. The exopeptidases are categorized as dipeptidyl- or tripeptidyl- peptidases if they eliminate peptides of two or three terminal amino acids (McDonald, 1985).

Proteases are affected in their activity by the pH value in the reaction medium as all enzymes. This optimal pH may condition the usefulness of a protease for a specific industrial application. Thus, proteases are also classified by their optimum pH, as alkaline (pH > 7.0) (or even high alkaline proteases (pH > 10.0)), neutral (around pH 7.0) or acidic proteases (pH < 7.0), (Sumantha et al., 2006, Gupta and Ayyachamy, 2012).

In the international system for the nomenclature and classification of enzymes (EC number) developed in the 1950s (Webb, 1993), proteases are located in class 3, as Hydrolases, subclass 3.4., hydrolysis of peptide bonds, which is divided between 13 sub-subclasses depending on their catalytic mechanism (McDonald, 1985). The better understanding of protease structures, their substrates and inhibitors, have permitted other classifications of protease considering their chemical structures, such as MEROPS peptidase database, that was founded in 1996 (Rawling and Barrett 1993; Rawling et al. 2008; Rawling et al., 2012; Rawlings et al., 2016), and since then it has gathered a large library of peptidases, that in 2016 reached a total of 2457 entries (Rawlings, 2016). Other classification is based on the catalytic sites of the protein and their tertiary structure, taking into account the iconic amino acid or metal present in the active site they are divided into: Cysteine peptidases (C), Aspartic peptidases (A), Serine peptidases (S), Metallo peptidases (M), Mixed catalytic type (P) and Unknown type (U) (Rawling and Barret, 1993; Polgár, 2005; Rawling et al., 2008). Serine proteases are known for their very well described catalytic triad including a catalytic Ser, and they are a very easily recognized type of proteases (Rawlings and Barrett, 1993; Barrett and Rawlings, 1995), of which subtilisin-like and trypsin-like proteases are the most interesting family of enzymes considering their multiple applications.

It is also possible to highlight the difference in the grades of protease specificity (McDonald, 1985). There are proteases able to attack peptides and proteins in very different positions, while other proteases are much more selective, attacking only a very determined amino acid sequence. Obviously, these diverse specificities determine their possible uses. For some applications, a high specificity or selectivity may be a disadvantage, while in other cases it may positive and even become the key for the successes in the application, as we will discuss below.

*2.1. Narrow specificity or selectivity as an advantage*

In some cases, a severe control over the hydrolysis of a protein by a protease is desired. Proteases with a very high specificity, that is, those that recognize only a few sequences, have significance in some cases. Trypsin is a good example of this. The enzyme hydrolyzes protein molecules where they exhibit arginine or lysine as P1 (Olsen et al., 2004). It may seem unfavorable to utilize a protease that produces so low a degree of hydrolysis in the substrate, but in some circumstances we may contemplate that “less is more”.

Two examples show the advantage of a narrow specificity of proteases: cheese manufacture, specifically aiming at the making of the milk clot and analysis of mass spectrometry for proteins of the hydrolyzed proteins. Trypsin, either pancreatic porcine or bovine, is the family of proteases most broadly utilized in the hydrolysis of protein samples for proteoma analysis, useful due to its high specificity. This way, the treatment can release specific and “standard” peptide fragments that enable to achieve “peptide maps” (Giansanti et al., 2016; Trevisol et al., 2016). This allows the identification of the samples under study. Trypsin hydrolysis of proteins also has the advantage of producing not very long peptides with basic C-terminus, which are suitable for collision

induced dissociation (CID) tandem mass spectrometric analysis (Stosova et al., 2008; Kim et al., 2010; Tsiatsiani et al., 2015). Other sources of trypsin-like or even other enzymes have been considered to be used in sample preparation for proteomic studies (Wang et al., 2009; Trevisol et al., 2016). Kiser et al. (2009) suggested trypsin from *Streptomyces erythraeus* as a candidate for its utilization in proteomic analysis because it is more resistant to urea action, significantly more active, and much more resistant to autolysis than bovine trypsin. A previous chymotrypsin digestion of the protein, followed by trypsin treatment has been shown to be more effective in the hydrolysis of membrane proteomes, where arginine and lysine are less frequent and decrease trypsin activity (Fischer and Poetsch, 2006; Fischer et al., 2002). Amongst non-trypsin proteases, Lys-C has been a remarkable protease, followed by chymotrypsin, Glu-C, pepsin, LysN, AspN, and ArgC (Tsiatsiani et al., 2015; Giansanti et al., 2016).

This way, trypsin is finding many uses when moderately large fragments of protein chains are desirable, and this is based on the possibility of producing a reproducible, controlled, and low degree of hydrolysis of the protein substrates.

It should be considered that protease autolysis can be a drawback during the preparation of samples in proteomic analysis (Gobom et al., 1997). This is a particular and especially undesirable feature of proteases, when a protease molecule becomes the substrate for another protease molecule, producing contamination of the samples with these protease chain fragments. To prevent this, low concentrations of proteases are utilized, reducing the autolysis process but also decreasing the target reaction rate. Techniques such as immobilization, as will be discussed later, can solve this cause of sample contamination (Gobom et al., 1997).

Another example of an application that benefits from a high specificity of the enzyme is cheese production. Cheesemaking is traditionally initialized by milk

coagulation after protease modification to destabilize the casein micelles, followed by the precipitation of these unstructured proteins. In the first step, calf rennet (having pepsin and chymosin activities as major components) is the “protease” most used in cheesemaking (Machopoulou, 2016). Bovine chymosin is an aspartyl protease obtained from the abomasum of suckling calves. This is the main component of calf rennet and has a very narrow specificity that enables the specific breakage of the Phe 105-Met 106 bond of the κ-casein (Machopoulou, 2016). In this way, cheese manufacture usually starts via this controlled κ-casein hydrolysis producing the milk protein coagulation. Any appropriate rennet substitute must fulfill both this high specific caseinolytic activity and small-generalized proteolytic activity (Mohanty et al., 1999; Kumar et al., 2005), keeping the possibility of protein precipitation and targeted clot generation. Later, it will be discussed how an excessive grade of proteolysis enhances the protein hydrolysate solubility, harming the generation of the initial clot. Diverse proteases (plant, microbial or animal sources) have been evaluated as rennet substitutes (Ghorai et al., 2009). We can highlight the use of an aspartyl protease from *Rhizomucor miehei* in cheese production (Mohanty et al., 1999; Kumar et al., 2005). Actually, several strains of *Rhizomucor miehei* have been evaluated as milk-clotting enzymes producers, together with a commercial enzyme from *A. niger* var. *awamori* strain (GCAAP4) or a recombinant strain of *Aspergillus niger* var *awamori* (Mohanty et al., 1999; Kumar et al., 2005). These new proteases can hydrolyze some undesired peptide bonds of k-casein, but their specificities are close enough to that of calf chymosin (Moschopoulou, 2017).

### *2.2. Narrow specificities as a disadvantage*

Analysis of amino acid composition is a standard protein determination protocol which is broadly utilized in physiology, pharmacology, food chemistry, proteomics and

nutrition studies. The amino acid profile of the hydrolysate shows the dietary protein quality in protein assessment (Fountoulakis and Lahm, 1998; Weiss et al., 1998; Masuda and Dohmae, 2010). The total amino acid analysis technique comprises hydrolysis of the problem protein followed by separation and detection of all the amino acids. However, this hydrolysis phase is still today a key point not fully resolved. The analysis needs that the target peptides or proteins are fully hydrolyzed to release all the amino acid residues. It must be considered that a full enzymatic hydrolysis of proteins to amino acids is still not possible, as a “super enzyme” able to hydrolyze each peptides bond in each existing protein is unavailable. One answer to this problem is the combined utilization of several proteases with dissimilar specificity. Thus, some researches have suggested enzymatic alternatives to the classic chemical acid hydrolysis, alternatives that utilize sequential treatments with different proteases to achieve a high percentage of hydrolysis, but the “boiling acid” treatment is still the process of choice even with the problems that this method has. The enzymatic strategy remains restricted to specific utilizations, like the identification of some amino acids that are unstable in hot acidic medium, such as tryptophan. Acid heat treatments, mainly in the presence of oxygen, produce the degradation of tryptophan (Cuq et al., 1983). Thus, enzymatic hydrolysis by pronase from *Streptomyces griseus* has been applied to determine the amount of tryptophan in a sample, but the process did not achieve the full release of this amino acid. This has caused alkaline hydrolysis to remain as the most utilized alternative protocol (Fountoulakis and Lahm, 1998; Weiss et al., 1998; Masuda and Dohmae, 2010; Yamskov et al., 1986; Delhave, 1992).

## 3. Protease applications

As recently reviewed, protein hydrolysis finds a broad variety of applications on diverse biotechnology processes (Tavano, 2013). A small summary was sketched in Fig. 3. New protease industrial processes are continually being presented. The increase in hydrolysis specificity and product purity while reducing environmental impact, and especially when it comes to food production (Tavano, 2013) compared positively proteases with chemical processes. Some other protease applications will be discussed below.

*3.1. Food Biotechnology*

Diverse biotechnological techniques may be used in food manufacture in a very wide sense, from enhancement protocols related to planting or breeding, to modifications of specific stages of processing, either to improve food quality or to replace old processes with a high negative environmental impact (Stover and Mehta, 2017).

Food products are very complex and involve diverse matrices presenting different compositions that include diverse kinds of constituents. All components are interacting among them, resulting in the specific features of the final food. Among these constituents, peptides and proteins contained in the food medium take an outstanding position (Lacou et al., 2015). Proteins play crucial roles in those aspects involved in the bio- and techno-functional features and/or nutritional value of foods (Lacou et al., 2015; Wouters et al., 2016). The hydrolysis of these proteins affects the food matrix properties, which may produce positive effects such as modifications of sensory quality (such as texture or flavor), enhanced digestibility, decreased allergenicity or liberation of bioactive peptides (Chen, 2008; Henzel and Watanabe, 2003; Lacou et al., 2015). Changes of chemical processes by enzymatic hydrolysis processes is an attractive

option as enzymatic treatments fully maintain the chemical species existing in the food sample. The acid protein hydrolysis onto the food will affect most constituents of the food matrix and/or will generate compounds that may be harmful for human wellbeing (Castro et al., 2011).

However, a critical item when studying enzyme samples directed to food processes is the security assessment of the enzyme producer strain. That way, to find a protease that may be applicable from the point of view of their specificity and/or stability, is not enough. It is required that both the enzyme and the enzyme-producing microorganism are accepted as health-safe. This certificate is granted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). This is an international expert scientific agency that is administered by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). It has been holding meetings since 1956, firstly to assess the safety of food additives. As described in "General Specifications and Considerations for Enzyme Preparations used in Food Processing": "*Enzyme preparations consist of biologically active proteins, at times combined with metals, carbohydrates and/or lipids. They are obtained from animal, plant or microbial sources and may consist of whole cells, parts of cells, or cell free extracts of the source used. They may contain one or more active components as well as carriers, solvents, preservatives, antioxidants and other substances consistent with good manufacturing practice. They may be liquid, semi liquid, dry or in an immobilized form (immobilized enzyme preparations are preparations which have been made insoluble in their intended food matrix by physical and/or chemical means)*" (FAO, 2017). And later, regarding to microbial sources "*Microbial sources used in the production of enzyme preparations may be native strains or variants of microorganisms, or be derived from native strains or variants by the processes of selective serial culture*

*or genetic modification. Production strains for food enzyme preparations must be non-pathogenic and non-toxigenic…*". Protease samples may be used as "food additives" if they are approved by the FDA for specific uses and considered GRAS (generally recognized as safe) substances. A substance may be GRAS only if its general recognition on safety is founded on the opinions of experts considered to be qualified to evaluate the safety of the substance (FDA, 2017).

*3.1.1. Health-related properties of proteins*

Diverse special diets can be calculated to offer some specific protein nutritional requirements or even supplement nutritional necessities or generate health benefits. In particular instances, the protein resource may come from combinations of free amino acids or even a blend of both small peptides and amino acids (Clemente, 2000). This might be alternatively performed by the use of unmodified protein supplements. Both free amino acids and peptides can be absorbed, however short-chain peptides are frequently favored due their enhanced absorption kinetics (Jahan-Mihan et al., 2011). Moreover, as described by Clemente (2000), protein hydrolysates achieved by enzymatic treatments and composed by peptides with specific features and molecular size are demanded for specific preparations. These features of the hydrolysates resulted from the protease choice and the procedure design. Thus, in some instances, when the patient needs a general supply of amino acids via protein hydrolysates, a high hydrolysis percentage of the protein hydrolysis can be required (Clemente, 2000). This may be achieved employing proteases with a broad specificity, using a mix of diverse enzymes or carrying out a step by step hydrolysis of the protein utilizing a consecutive proteolysis employing different proteases in each hydrolysis step (Boutrou et al., 2013). In other cases, a specific peptide is the goal of the hydrolysate preparation (a bioactive

peptide, for example) or even a specific breakage of a single peptide bond (such as hypoallergenic formulas). These instances usually need to select the most suitable protease with the proper narrow specificity.

Nowadays, the employment of dietary complements based on protein hydrolysates has already gone beyond the bounds of protein intake to patients who could not assimilate native proteins because of illness, such as Crohn's disease or pancreatitis (Posovszky, 2016). For instance, the utilization of protein hydrolysates in sports nutrition has improved its importance, with uncountable researches showing their efficacy in helping in the recovery of the muscle connective tissue matrix after exercise (Thomson et al., 2015; Holm et al., 2017). Moreover, these supplements present an insulinotrophic effect, which causes muscle anabolic result (Yuan et al., 2017), specially on those hydrolysates with high concentration of Leu (Rittiga et al., 2017). However, researchers remark the difficulty in defining or isolating the actual cause for some benefits produced by the consumption of protein hydrolysates. The combination of several effects would be the most accepted justification, since these peptides would couple their nutritional effects to other "bioactivities", an area of study which has received increasing attention nowadays (Thomson et al., 2015; Holm et al., 2017).

*3.1.1.1. Reduction of food protein allergy*

Food allergy is defined as ‘‘an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food’’ (Sicherer and Sampson, 2014), including both IgE-mediated and non–IgE mediated allergenic reactions (Berin, 2015). When food protein illnesses are intermediated by IgE, the percentage of the protein recognized by IgE is named the epitope, which can be “sequential” or “linear”, depending on the tridimensional or the primary structures of the

allergenic protein, respectively. The conformational epitopes are more easily broken by food processes, e.g., thermal treatment, but for the linear epitopes these treatments may not be sufficient. The hydrolysis of the protein chain may be the only way to prevent the identification of the epitope by the immunological response system (Martins and Galeazzi, 1996; Sicherer, 2002; Sathe and Sharma, 2009). A proper selection of a protease with the desired specificity and the hydrolysis protocol can radically decrease protein allergenicity. This will depend on the percentage of protein hydrolysis and even on the filtration protocols used in the final purification to discard remaining unmodified proteins. The ideal process should imply a minimum amount of downstream steps (Burton et al., 2002). Thus, well-performed proteolyses have been utilized to decrease the allergenicity of milk proteins or gluten in foods (Rizzello et al., 2007; Osborn et al., 2017). The selection of the enzyme and the percentage of hydrolysis that is necessary to get the desired effect require deep attention. A poor design of the process can produce the opposite effect. If a low percentage of hydrolysis is achieved and only a superficial hydrolysis is attained, this can increase the allergenic reactivity of the protein by revealing epitopes previously hidden in the core of the protein structure. A study performed by Panda et al. (2015) confirmed that the hydrolysis of soybean protein isolate (SPI) by chymotrypsin or bromelain amplified the food allergenicity measured by IgE immunoblots. Cabanillas et al. (2012) showed a 65% reduction in IgE reactivity for roasted peanut protein when hydrolyzing for 300 min using Flavourzyme, while a 30 min treatment produced an augmentation in IgE reactivity when measured using an ELISA. Using Alcalase as catalyst, a vanishing of IgE reactivity was appreciated after that treatments times.

Depending on the percentage of protein hydrolysis, the products may be named as extensively hydrolyzed formulas (small oligopeptides) and partially hydrolyzed

formulas (where about 90% of the peptides present a molecular weight <3 kD) with clear alterations in the allergenic features (Vandenplas et al., 2014; Berin, 2015) and even acceptance responses, since a very high hydrolysis degree can alter the product taste, as discussed below. For example, Meinlschmidt et al. (2016) showed, also with hydrolysis of soy protein isolate, that the Pepsin, Alcalase and Papain hydrolyses were suitable in the destruction of the main soybean allergens. However this treatment amplified the bitterness of the hydrolysates. Although Alcalase showed the highest efficiency in the hydrolysis (13% hydrolysis), Papain and Flavourzyme were more useful because of the production of a less marked bitter taste (Meinlschmidt et al., 2016).

Soy protein extract exhibits at least 16 IgE-binding proteins with molecular weights ranging from 7.5 to 97 kDa, which may be implicated in allergy of soy derivatives (Cordle, 2004). Therefore, substantial research efforts are intended at modulating soy allergenicity and checking enzymatic protocols to diminish soy allergenic reactivity.

Pepsin and trypsin were utilized to alter other legume proteins allergicities (Pinto et al., 2009). The allergenic effects were significantly different when employing different protein extracts, like chickpea, lentil or sweet lupin proteins. Lentil and chickpea proteins lost their immunogenic effects after some few minutes of enzyme treatment, while the diminution of the antigenic activity of the major globulin protein from sweet lupin was slowly decreased and needed longer hydrolysis treatment. The antigenic epitopes existing in the hydrolysate were fully degraded after 30 min by trypsin hydrolysis, while if pepsin was used, it still exhibited about 23 % of the initial antigenicity (Pinto et al., 2009). In many examples, a large variety of different allergen peptides can be found in a single food.

Many studies show the effectiveness of well-performed hydrolysis in the production of protein hydrolysates with a potential utilization in the diet of human beings, (especially children) suffering food allergy (Osborn et al., 2017). Cow, peanuts, eggs, soy, wheat and especially milk proteins are the most important food allergens for young children. Milk proteins can produce allergic reactions versus the three main proteins presented: α-lactalbumin, β-lactoglobulin and caseins (Sharma et al., 2001). A recent study of The Cochrane Library (Osborn et al., 2017), shows that among children at high danger of allergy who cannot be only breast fed, the continued supplementation with a hydrolyzed protein formula decreases the danger of infant cow milk allergy and development of infant allergy compared with the utilization of a cow milk formula.

Von Berg et al. (2016) gave indications that the utilization of some hydrolysate formulas as breastmilk replacements in the first four months of life of high-risk children is coupled with an effect that avoids the allergic reaction in the moment of consumption. This researcher showed that this treatment also presented eczema development until the children reached 15 years. Children who were frequent users of casein hydrolysate presented a decreased probability of becoming asthmatic between the age of 11 and 15 years. The same study showed the problems for establishing precise explanations on these hydrolysates positive effects and strengthened the hypothesis that the hydrolysis of allergenic epitopes is not the only positive effect from consuming a protein hydrolysate, because each kind of hydrolysate (depending on the protein source and enzyme treatment used) may produce diverse side-effects. It is likely that the exact kind and percentage of peptides, now known already with possible bioactivity, can also produce extra profits (see *3.1.1.2.*).

Consumption of gluten from any *Triticum* species or similar proteins of rye or barley and their crossbred varieties, can severely distress genetically predisposed

individuals to inflammatory illness of the small intestine (Rizzello et al., 2007). Celiac and non-celiac gluten sensitive patients show a small intestinal enteropathy presenting villous atrophy, a chronic inflammation of intestinal lamina propria, a compromised epithelial barrier and augmented epithelial permeability (Rizzello et al., 2007). In celiac patients, dietary gluten activates production of anti-gliadin antibodies and anti-transglutaminase 2. Only the anti-gliadin antibodies are produced in non-celiac gluten sensitives. Gluten proteins exhibit two main portions: soluble (gliadins) and insoluble (glutenins) aqueous alcohols portions (Matysiak–Budnik et al., 2005; Stepniak et al., 2006; Sestak and Fortgang, 2013). Nowadays, the only actual treatment for celiac disease is a gluten-free diet (Rizzello et al., 2007). The high amount of proline residues in gluten (12-17% of all amino acids) produces that this protein is resistant to full proteolytic degradation by human pancreatic and gastric proteases, which rose interest among the researchers who intended to use nondigestive proteases for gluten hydrolysis (Matysiak–Budnik et al., 2005; Stepniak et al., 2006; Sestak and Fortgang, 2013). Ehren et al. (2009) evaluated the gluten detoxification features of two food-grade enzymes, dipeptidyl peptidase IV (DPPIV) from *Aspergillus oryzae* and aspergillopepsin (ASP) from *Aspergillus niger*. Used individually, neither ASP nor DPPIV efficiently broken immunotoxic gluten epitopes. However, the joint utilization of DPPIV and ASP permitted the detoxification of moderate quantities of gluten, even when an excess of casein was presented or in whole-wheat bread. Rizzello et al. (2007) used a combination of sourdough lactobacilli and fungal proteases to remove the toxicity of wheat flour during long-time fermentation and suggested that this process was an efficient approach to eliminate gluten toxicity. Other studies analyzed the utilization of prolyl endoproteases from *Aspergillus niger* for this goal (Matysiak–Budnik et al., 2005; Stepniak et al. 2006; Sestak and Fortgang, 2013). It has been previously stated that in

some instances a single protease cannot produce the required degree of hydrolysis. Li et al. (2016) employed a sequential protein hydrolysis, which enabled allergenicity decrease. Sequential-hydrolysis of wheat flour by alcalase and papain was found more efficient in decreasing the amount of detectable gliadin than every individual enzyme hydrolysis. Under optimal conditions of sequential enzymatic hydrolysis, gliadin was almost completely destroyed. This gave a flour extract showing decrased allergenic effects than the hydrolyzate obtained with individual hydrolysis using chymotrypsin flavourzyme, trypsin or pepsin.

*3.1.1.2. Bio-functional properties*

Peptides are capable of exerting a positive effect on body functions beyond those traditionally recognized nutritional functions, giving extra health effects (Biesalski et al., 2009; Kitts and Weiler, 2003; Kris-Etherton et al., 2002) such as beneficial impacts on the digestive, immune, cardiovascular or nervous systems (Fig. 4). These helpful effects of peptides are influenced by their sequence and amino acid composition (Sirtori et al., 2009; Hernández-Ledesma et al., 2011; Arroume et al., 2016). Together to the features that render these bioactivities, food peptides (i.e. those ingested through diet) must also exhibit some extra-features such as the capacity of being absorbed (possibility of its transport through intestinal cells) and resistance to digestive proteolysis (Vij et al., 2016). Many peptides fulfill these preconditions as bioactive compounds and therefore they may present likely utilities as nutraceuticals. Together with improving animal or human health, these peptides are potential natural additives for food. This is because some of their action mechanisms as bioactive compounds can have positive effects in the food matrix itself (Li-Chan, 2015). Antioxidant or antimicrobial peptides, for example, may apply their function in food, with direct positive effects on its preservation.

Although the connection between the peptide structures and their activities is not completely understood, some researches detected some features that favor the bioactive properties of peptides. These include amino acid sequence, size, presence or absence of specific amino acids in particular locations, etc. Some of these peptide properties can be achieved by the right selection of the enzymatic protocol that is employed to produce these peptides. That is, a proper selection of the protease and an adequate control of the hydrolysis process is required (Ambroggio et al., 2016). Although the most usual way to manufacture bioactive peptides is via protein hydrolysis using microbial fermentation or natural digestive procedures, many *in vitro* enzymatic methods have been suggested (Korhonen and Pihlanto, 2006; Agyei and Danquah, 2011; Hernandez-Ledesma et al., 2011). Actually, bearing in mind that diverse enzymes can originate peptides with a broad range of effects, non-digestive proteases, such as Thermolysin or Alcalase, have been utilized for the production of peptides that differs from those released by stander human-digestion, studying their possible bioactivity activities.

The features of the amino acids that form the peptides present great relevance to determine the potential bioactivity of the peptide, together with using a protease with the required specificity. Therefore, the proper selection of the substrate-protein will also be critical on the final features of the produced peptides (Amarowicz, 2008; Tavano, 2013). Therefore, the selection of the protein substrate source, will determine the characteristics of the final peptide hydrolysate (after their controlled proteolysis). As it is evident, even though the same enzyme is used in the process of production of these peptides, they will be different depending on the composition of the substrate protein (Tavano, 2013).

Another relevant consideration is that the produced hydrolysate will be composed of a mixture of amino acids and many different peptides. This library of peptides often exhibits many bioactivities. Even a single peptide may have several potential bioactivities. Neves et al. (2017) showed that the hydrolysates produced by hydrolysis of salmon trimmings protein catalyzed by Corolase PP have both DPP-IV and ACE antioxidant capacity and inhibition activities. Vij et al. (2016) presented that an isolated peptide derived from casein hydrolysates (Val-Leu-Pro-Val-Pro-Gln-Lys) presented both ACE inhibition and antioxidant capacities, together with a demonstrated effect in transepithelial transport that enable its assimilation.

*3.1.1.2.1. Antihypertensive peptides*

Some peptides present antihypertensive activity, in many instances by inhibiting angiotensin-I-converting enzyme (ACE). This is a peptidyldipeptide hydrolase coupled to the renin-angiotensin system which is very relevant in the regulation of the cardiovascular function and the blood pressure. The ACE inhibition produces the decreasing of blood pressure (Izzo and Weir, 2011). Several researches have shown the ACE-inhibitory properties of diverse peptides (ranging from 2 to 12 amino acids) constituted by many hydrophobic or cationic (such as Lys and Arg) amino acids at the C-terminal positions (Schmelzer et al. 2007; Hernández-Ledesma et al. 2011). Considering that pepsin possesses preferential specificity towards hydrophobic residues and trypsin preferential activity in Arg- and Lys- at P1, both enzymes have been successfully utilized in the production of peptides with antihypertensive properties using milk protein as substrate (Mullally et al., 1997). Ferreira et al. (2007) demonstrated that Ala-Leu-Pro-Met-His-Ile-Arg peptide achieved after β-lactoglobulin hydrolysis catalyzed by trypsin owned a strong ACE-inhibition power. Ko et al. (2017) determined the

sequence of the peptides achieved after hydrolyzing the marine sponge *Stylotella aurantium* which presented ACE inhibitory activity. Two dipeptides were purified and identified: Tyr-Arg (337.2 Da) and Ile-Arg (287.2 Da). Wang et al. (2017) established the ACE inhibition potential of Tyr-Ser-Lys obtained after trypsin hydrolysis of rice bran protein. However, peptides presenting very diverse sequences behave as ACE inhibitors. Abdelhedia et al. (2017) showed that Pro-Thr-Val-Pro-Lys-Arg-Pro-Ser-Pro-Thr, Pro-Leu-Pro-Lys-Arg-Glu, Val-Val-Pro-Phe-Glu-Gly-Ala-Val and Ile-Ala-Gly-Pro-Pro-Gly-Ser-Ala-Gly-Pro-Ala-Gly, peptides achieved by Esperase® catalyzed hydrolysis of smooth-hound viscera proteins, were capable of inhibiting ACE through a complex net of interactions involving hydrogen bonds, hydrophobic, electrostatic and van der Waals. Other researches also suggested ACE-inhibitory activities of peptides with a proline element at the carboxyl terminal. However, as proline is resistant to digestive proteases (Li et al., 2004; Korhonen and Pihlanto, 2006), the release of these peptides requires to be catalyzed using alternative proteases as biocatalysts in the protein hydrolysis.

*3.1.1.2.2. Antioxidant peptides*

Free radicals can be produced *in vivo* through standard reactions or by ingestion/absorption of substances, presented in the environment or diet, which may become oxidizing reagents. The excess of these radicals can produce negative effects on the human health, for example damage in proteins, the oxidation of low density lipoproteins in membranes or DNA mutations (Sarmadi and Ismail, 2010)

Several reports show that peptides can present antioxidant power, even though the exact mechanism is still not properly understood. It has been speculated to be mainly because of to inhibition of singlet oxygen quenching potential, harmful lipid

oxidation, free radical scavenging or chelation of metal ions (Erdmann et al., 2008; Hernandez et al., 2012). It is assumed that the peptide antioxidant effects may be related to a sum of different peptide features, like the presence of electron-donating amino acid moieties in the peptide sequence or the existence of several hydrogen atoms (Samaranayaka and Li-Chan, 2011). Because of diverse physicochemical features of amino acids, the antioxidant abilities of peptides are associated with the occurrence of certain groups in the peptide sequence, such as methionine, histidine, tyrosine, proline or tryptophan. Peptides containing hydrophobic amino acids (e.g., Leu or Val at the N-terminus) seems to be more suitable to have significant antioxidant activity *in vivo* owing to enhanced accessibility to hydrophobic substances, like fatty acids (Amarowicz, 2008). Thus, it is obvious that again in the instance of the antioxidant effects of protein hydrolysates depend on the utilized protease, substrate protein and process design (Amarowicz, 2008).

Guo et al. (2017) presented that sea horse proteins hydrolyzed by papain gave a peptides mixture that presented enhanced antioxidant power compared to those produced using flavorzyme or trypsin. Moreover, they showed that the hydrolysis grade is a significant parameter to regulate the antioxidant potential of the peptides. This antioxidant activity was higher after 40 min of papain hydrolysis than after 120 min. Therefore, for some uses, producing large peptide fragments through controlled hydrolysis may be a key factor.

As discussed before, some peptides may present activities that make them interesting as food additives. This is the case of antioxidant peptides. Antioxidants are usually added in foods to delay lipid peroxidation and also to prevent the production of compounds resulting from food oxidation. This can drive to unwanted alterations in texture, color or flavor of the foods (Samaranayaka and Li-Chan, 2011). The addition of

these compounds to the food is necessary. However, not only should their antioxidant activity be taken into account, but also their effects on the fabrication process. Moreover, the sensorial features should be considered of the final product, since some peptides may have bitter taste, as we will discuss in depth later. For example, in a study presented by Yarnpakdee et al. (2015), where *Nile tilapia* protein was hydrolyzed by Protamex, Flavourzyme, Alcalase or papain, the final product presented an augmented antioxidant activity as the grade of hydrolysis increased. Peptides released by Alcalase or papain possessed the strongest metal chelating and ABTS radical scavenging activities. Both peptide extracts presented a high amount of hydrophobic amino acids and had lysine, glutamic acid/glutamine and aspartic acid/asparagine as the dominant amino acids. Although the Alcalase hydrolysate was the highest antioxidant activity, the papain hydrolysate was the one with the best organoleptic acceptance. This was because the Alcalase product was much more bitter. This showed the requirement for cautious selection of the enzyme.

*3.1.1.2.3. Other bioactive peptides*

Many other possible “bioactivities” may be found in the literature. Guo et al. (2017) established the anti-fatigue activity of peptides obtained after papain hydrolysis of sea horse protein extract. Sabbione et al. (2016) showed the antithrombotic activity of amaranth protein produced after imitation of gastrointestinal digestion, and also detected that various peptides are capable to cross the Caco2-TC7 cell monolayer. Alamdari and Ehsani (2017) underlined the antimicrobial peptides obtained by hydrolysis of milk proteins using digestive proteases or by lactic acid bacteria fermentation.

Opioid peptides presented their biological activity by coupling to opioid receptors, presenting an opiate-like effect. A ‘‘typical’’ opioid peptide from milk proteins is the peptide Tyr-Gly-Gly-Phe produced via cheese fermentation. The presence of the tyrosine residue at the N-terminus and another aromatic amino acid at the fourth position are critical aspects for this effect, as they enable the peptide coupling with the opioid receptor (Silva and Malcata, 2005).

The enzyme dipeptidyl peptidase IV (DPP-IV) is involved in the regulation of serum glucose in humans. Its inhibitors can be utilized in type 2 diabetes treatment as antidiabetic drugs (Lacroix et al., 2017). Many peptides present potential as inhibitors of DPP-IV. Nongonierma et al. (2017) produced inhibitor peptides by bovine milk protein hydrolysis catalyzed by trypsin. Many researches have shown the bioactive potential of milk proteins and their proteolyzed peptides, but it is essential to measure the actual activity of the peptides *in vivo* and not only *in vitro*. For example, Lacroix et al. (2017) showed that milk protein derived DPP-IV inhibitors may be hydrolyzed by intestinal brush border membrane peptidases and have low permeability. These facts decrease their potential for action *in vivo*, becoming a nice example of the necessity of considering the peptides stability and bioavailability when evaluating their bioactivities.

### *3.1.2. Modulating the techno-functional properties of foods*

Proteins can fulfill diverse non-nutritional functions in food products because of their physicochemical features which determine the performance of proteins in a food media, determining relevant “techno-functional” features of the foods such as protein capacity for gelation, foam formation or emulsifying, as well as solubility, and fat or water taking (Wouters et al., 2016; Foegeding and Davis, 2011; Panyam and Kilara, 1996; Kinsella and Melachouris, 1976). Functional features of proteins came from a

equilibrium between the native three-dimensional structure of a protein and the continuous structural conformational transitions. This last is clearly modulated by forces such as hydrophobic or ionic interactions. These can alter these different protein conformational forms. This conformational equilibrium is frequently coupled to alterations in the secondary or tertiary structure of proteins, and with its surface-activity relationship (Wouters et al., 2016; Foegeding and Davis, 2011). Proteolysis of proteins is a potent instrument in the alteration of these techno-functional features of proteins. This can alter three relevant issues in this balance: it reduces the protein molecular weight; it augmented the global number of ionizable groups in the medium (by the release of a greater number of protein chain terminal groups proportional to the number of broken peptide bonds); and can produce the exposure of hydrophobic groups from the core of the protein structure. This ultimately produces different alterations in these proteins-environmental interactions. These effects will be clearly determined by the degree of protein hydrolysis. However, although the alterations in the protein techno-functional properties induced by proteolysis are the consequence of these main parameters, they also depend on the environmental conditions and the hydrolysis conditions (especially the protease used and extent of the hydrolysis performed). The proteins are transformed into a peptide extract containing different percentages of peptides of diverse dimensions, free amino acids and even intact native protein molecules. In this new condition, depending on the initial medium pH, for example, the released carboxyl and amino terminal groups may be in their dissociated or protonated forms, which alters the protein interactions with the medium and may even change the global pH value of the food. Hydrolysis conditions need to be properly determined to prevent the consequences of a bad protocol design that can damage the protein functionality and even produce disapproving sensorial effects, such as accumulation of

bitter-flavored peptides (Jung et al., 2005). It is a requirement to avoid that the intended technological effects can harm the sensory features of the food, which is an important challenge in food processing. Many food constituents, such as amino acids and peptides, can contribute to the final sensory-specific foods features, such as the texture, shape, color or taste (Rolls, 1982).

Protein solubility is determined by electrostatic and hydrophilic interactions, and the enhancement of protein solubility is the most remarkable property on protein techno-functional features after the hydrolysis process (Panyam and Kilara, 1996). It is usually proportional to the growth in the new ionizable amino and carboxyl terminus groups produced after the protein hydrolysis of peptide bonds (Wouters et al., 2016). However, the hydrolysis step requires to be controlled, because this treatment also exposes hydrophobic moieties initially hidden inside the protein core. This increases peptide attractions/aggregation interactions producing a decrease in solubility. A high grade of hydrophobicity and sulfhydryl disulfide interactions increases protein insolubility, even after a high percentage of hydrolysis (Creusot et al., 2006; Paraman et al., 2007; Creusot and Gruppen, 2008). However, the optimal grade of hydrolyses is determined by the final goal. For instance, a high hydrolysis grade may have negative effects on protein solubility. However, the exposition of hydrophobic groups augments the hydrophobic surface, which can be favorable if the peptide extract is going to be used as an emulsifier (Panyam and Kilara, 1996). In some examples, the highest emulsification capability has been achieved with a low hydrolysis percentage of the protein, and an augmentation in the accessibility of large hydrophobic peptide units at the oil–water interface, promoting larger emulsion formation (Panyam and Kilara, 1996).

Although proteolysis can decrease gelling properties because of the small size of the products, low grades of hydrolysis can also enhance the protein gelling properties.

Enhanced gelation features and a higher foaming capacity of protein extracts were described when the protein hydrolysis was performed during a short time period (Sanchez and Burgos, 1996; Totosaus et al., 2002). Protein gelation requires coupling between protein molecules; in many instances after the unfolding of the native protein structure, and in this way gelation needs large peptides.

Wouters et al. (2016) described that the impact of the flavor and smell of the protein constituents cannot be ignored when these are utilized in food technology looking for their technological features. This fact should be further reinforced when hydrolysates are utilized. Peptides and amino acids, along with other molecules like sugars or salts, determine the taste sensation of foods. Human gustatory system can detect five basic flavors: salty, sour, bitter, sweet and umami. The last three tastes are the major ones related to peptides influence (Iwaniak et al., 2016).

Bitter taste warrants special care because this can be a cause for product rejection because of the distaste produced by this disgusting flavor. This may be an adaptive evolution of mammals for avoiding foods that are potentially toxic, which have a bitter taste in many instances (Glendinning, 1994). The flavor of peptides can change depending on the amino acid sequence of their constituents. Researches show an association between bitter flavor of the peptides and their chain length. The bitterness of peptides augments if they are formed by up to eight amino acid groups. Moreover this is influenced by its overall hydrophobicity and the specific amino acid located in N- and C-terminus. For example, if tyrosine or phenylalanine are in any terminus position, they can determine bitter taste (Saha and Hayashi, 2001; Maehashia and Huang, 2009; *Iwaniak et al.,* 2016). Hence, the right protease selection to perform the protein extract hydrolysis can decrease disagreeable flavor of the final product and even produce peptides with wanted tastes. For example, during manufacture and ripening in cheese-

making, a gradual proteolysis is usually considered to be a requirement for the development of the right flavor of some cheeses (e.g., Brie or Camembert) (Engel et al., 2001; Singh et al., 2003). In fact, proteolysis is present during the main biochemical alterations in cheese-making, bearing in mind that in most cases, the first step in chasee production is an enzymatic coagulation. Later, it will suffer a sequential transformation of flavor and texture, to some extent determined by the hydrolysis ratio during the ripening process (Fox, 1989). For many cheeses, proteolysis is not limited to the action of external added enzymes, but also to microorganism enzymes. It occurs in the blue-veined cheeses, where besides giving their typical look; blue molds produce a typical smell and flavor via the transformations catalyzed by their enzymes. The same occurs for the mold surface in cheeses such as Camembert and Brie (Souza et al., 2001; Seratlic et al., 2011).

Umami taste (as the one induced by monosodium L-glutamate) has been broadly accepted as the fifth basic kind of savor and named broth-like, meaty or savory taste. It is coupled to the taste characteristics and the chemical structure of L-glutamyl oligopeptides (Zhang et al., 2017). The presence of glutamic acid in di- or tripeptides was strongly associated with umami taste (Iwaniak et al., 2016) since it was described that the anionic L-glutamyl oligopeptides might present a umami taste (Zhang et al., 2017). Soy sauce is characterized by its strong and distinct umami taste, which is produced via fermentation hydrolysis of soy proteins. Zhuang et al. (2016) showed the relevant role of peptides in the umami taste of soy sauce and that the amino acid sequences of the peptides responsible for the umami taste in this product were Glu-Gln-Gln-Gln-Gln, Ala-Gln-Ala-Leu-Gln-Ala-Gln-Ala, Leu-Pro-Glu-Glu-Val, Ala-Leu-Pro-Glu-Glu-Val, and Glu-Ala-Gly-Ile-Gln, being the presence of glutamic acid/glutamine

persistent. Yu et al. (2017) recognized the umami hexapeptides from *Takifugu obscurus* as Lys-Gly-Arg-Tyr-Glu-Arg.

*3.2. Feed Biotechnology*

The relevance of feed proteases is increasing and these enzymes can supplement animal feedstocks to enhance nutrient bioavailability of the food constituents or as an element in animal diet formulation to help protein digestion in the gastrointestinal tract (Pariza and Cook, 2010). Sucu et al., (2014) studied the influence of a supplemental dietary mixture of different proteases in granular form (a cysteine protease and a serine endopeptidase of the subtilisin family - 3.4.22.2 and EC 3.4.21.62 respectively) on the productivity variables in dairy cattle, using as model lactating Holstein cows. The outcome of the experiment showed that supplemental protease enhances milk and meat production effectiveness and enhances parameters of nitrogen status. The utilization of a novel serine protease expressed in *Bacillus licheniformis* (RONOZYME ProAct) as feed protease, permitted a significant augmentation in the degree of the hydrolysis, solubilization and digestibility of proteins (Fru-Nji et al., 2011), improving broiler performance by enhancing protein and energy digestibility. The use of a mixture of enzymes containing lipases and proteases during the feather hydrolysis process may enhance the energetic properties of the feather meal when added in diets for adult dogs (Pacheco et al., 2016)

Proteolysis during the natural ensiling processes happened because of microbial and plant proteases and it can enhance the nutritional features of the final product, augmenting the availability of many nutrients. Proteases are not only adequate to enhance the use of protein in the foods, but they also enhance starch digestion (Young et al., 2012). The destruction of the food matrix may benefit the digestion of other

“digestive enzyme-dependent” constituents. Windle et al. (2014) studied the effects of the addition of a protease from *Aspergillus niger* with a low pH optimum activity (around pH 3.0) to corn plants gathered at diverse silage fermentation degrees, using 20 mg or 2,000 mg of protease/kg of wet forage. The results showed that the concentration of soluble proteins augmented with time of ensiling on protease presence. The treatment of corn plants with the higher protease amount increases the proteolysis during ensiling, yielding in vitro starch digestibility results after 45 days of ensiling. Similar results required 150 days of ensiling in untreated corn silages (Windle et al., 2014).

In all these uses of proteases, they must have features that make it resilient to the medium to which they will be exposed, such as the presence of proteases in the cow rumen or drastic pH values (Morgavi et al., 2001). The environment of the silage is commonly acidic, the pH ranging between 3.8 and 4.8 (Young et al., 2012).

### *3.3. Non-Food Applications*

Cleaning solutions with a broad range of uses may also incorporate proteases. They are utilized in cleaners to eliminate silver from X-ray and photographic films and also to clean surgical instruments or contact lens, laundry or detergent formulations for industrial uses. They are particularly utilized in the cleaning of material employed in food industrial processes (such as meat or milk industries), where the equipment will directly contact the product. In these instances, a classical chemical cleaning protocol becomes risky if the chemical compound is incorporated into the food (O’Donnell et al., 2010). Some features are required for a protease to be used as detergent additive, such as stability against surfactants, activity and stability in alkaline pH, high water solubility, and a very wide specificity that enables to digest diverse proteins (Ma et al., 2011). Thus, alkaline proteases are the most suitable for this application.

Proteases are also utilized in pharmaceutical uses. For example, they may be used in the digestion of keratinized skin. The thick layer of dead skin and the keratin that constitutes the higher percentage of corns may be degraded by proteases, augmenting its solubility and making regenerating epithelia and the elimination of the scar simpler, and helping in the healing processes. Keratinase-based cosmetic products are efficient in the reduction or elimination of corns and calluses (Gupta et al., 2013; Singh et al., 2016).

## 4. Improving proteases for biotechnology applications

As previously discussed, the utility of proteases depends on diverse points that comprise the selection of the most suitable enzyme, bearing in mind its specificity, activity and stability in the food media (pH, process temperature). Thus, in some instances the search for new enzymes that work under extreme conditions is required, even the genetic modification of the enzyme to get specific features may become necessary (Tavano, 2013). Some other strategies are being evaluated to get an even better behavior of enzymes, including the exploration for less known functions. These include the knowledge of the parameters that influence the behavior of the enzyme in a specific process. Among them, we can remark the protein structural limitations or performance under the conditions of the medium, or even know the details intrinsic to the process that can be overcome, such as the solubility of the enzyme in the medium that makes its separation and/or recovery and reuse very complex.

Like any other enzyme or protein, protease chain integrity is necessary to maintain their functions (McGeagh et al. 2011; Wang et al. 2009; Talbert and Goddard, 2012). Many parameters simultaneously determine the protein stability: helix dipole interactions, loop tension, ionic interactions, the entropy of water, salt bridges, planarity

of conjugated systems, pi–pi stacking, torsion potentials, hydrogen bonds, bond stretching, Van der Waals forces and disulfide bridges (Eijsink et al. 2004). Drastic conditions of handling or temperature or pH value changes during processing can lead to alterations in the enzyme conformation and produce important functional alterations (McGeagh et al. 2011; Wang et al. 2009; Talbert and Goddard, 2012). Moreover, since the protein chain of a protease molecule may be the substrate of other protease molecule, proteases can present autolysis as an additional and particular problem to be solved in the biocatalysts design. This may reach special relevance during protease storage or when the sample cannot be contaminated by chain protein fragments from the protease, for example in samples utilized for mass spectrometry (Gobom et al., 1997).

The performance of proteases may be enhanced by either physical procedures or chemical modifications. The use of ultrasounds in reactions catalyzed by proteases has presented some positive effects in some instances (Trieu-Cuot and Gripon, 1982; Seratlić et al., 2011). Chemical methods comprise alterations in amino-acid side chains with reactive functional moieties which can react with chemicals by intra- or/and intermolecular cross-linking, or covalent coupling (Fágáin, 1995). This aims to enhance protein c stability and/or enzyme activity via reduction of enzyme surface flexibility and decreasing protein unfolding or subunit dissociation (in oligomeric enzymes). In some instances, the alterations may improve the resistance versus chemical reagents or inhibitors, or reduce the autolysis. Different compounds have been utilized for chemical modification of diverse enzymes, such as succinic anhydride, which reacts specifically with the ε-amino groups of the side chain of the lysine residues or the terminus $NH_2$ and alter the ionic nature of the group from cationic to anionic. Glutaraldehyde crosslinking has been utilized to stabilize enzymes by introducing intermolecular bonds. The

covalent immobilization of caboxymethylcellulose to the protein surface via reductive alkylation with $NaBH_4$ enhanced enzyme pH and thermal stabilities (Villalonga et al., 2000). In addition to these chemical modifications of the enzyme chains, protease immobilization techniques may add some advantages. We will discuss in more detail this aspect in the following point.

*4.1. Immobilization of proteases: objectives, problems and alternatives*

The immobilization of enzymes is a general requisite for their industrial implementation as biocatalysts to simplify the reuse of these relatively expensive biomacromolecules (Dicosimo et al., 2013; Cantone et al., 2013; Sheldon and Van Pelt, 2013; Liese and Hilterhaus, 2013). Most of the considerations that must be taken into account when immobilizing an enzyme are similar for proteases or any other enzyme, we will remark in each separate case where the immobilization of proteases can make a difference.

The necessity for enzyme immobilization to facilitate enzyme reuse has promoted an intense research trying to combine immobilization to the solution of other enzyme limitations (Sheldon and Van Pelt, 2013). Thus, enzyme operational stability may be improved just by immobilizing the enzymes inside porous supports, because this will prevent enzyme aggregation, interaction with external interfaces, and, very relevant in the case of proteases, ultimately avoid autolysis (Garcia-Galan et al., 2011).

Enzyme structural rigidity may be improved via immobilization if many enzyme-support stable bonds are formed, if the spacer arms are short enough and the support is rigid (Garcia-Galan et al., 2011; Mateo et al., 2007). The enzyme groups implied in this multipoint covalent attachment should maintain their relative positions (the movement will just reduce to the length of the spacer arm) under any inactivating condition. To

maximize this structural rigidification, several aspects need to be considered. It requires the use of a proper immobilization system, which involves diverse parameters not always considered. First, the use of a proper support (rigid, having large internal surfaces that permit a good enzyme-support geometric congruence and many active groups) (Garcia-Galan et al., 2011) is necessary. However, this alone is not enough to maximize the multipoint covalent attachment; a proper active group needs to be selected. This group should be very reactive with nucleophiles located in the enzyme surface and stable enough under immobilization conditions. For example, epoxyde (Mateo et al., 2007), glutaraldehyde (Barbosa et al., 2014), glyoxyl (Mateo et al., 2006) or vinylsulfone (Dos Santos et al., 2015) have been reported to be very efficient for this objective. Finally, the optimal results will be only reached if an immobilization protocol that maximizes the enzyme/support interactions: moderately high temperatures, alkaline pH values, and one very important variable, time to permit the enzyme support multi-interaction (Pedroche et al., 2007). The final surface of the support should preferably be as inert as possible to prevent undesired enzyme/support interactions during biocatalyst operation or storage (Santos et al., 2015). Immobilization may also permit the prevention of enzyme subunit dissociation of multimeric enzymes if all enzyme subunits are involved in the immobilization. This should produce an increase in multimeric enzymes stabilities (Fernandez-Lafuente, 2009; Poltorak et al., 1998; Lencki et al., 1992). In some instances, immobilization may be coupled to purification (Barbosa et al., 2015). For example, using heterofunctional supports a final multipoint covalent attachment immobilization may be achieved, while the first enzyme immobilization via physical interaction with adsorbent groups permits its purification (Barbosa et al., 2013).

Moreover, immobilization may also improve some other enzyme properties. For instance, immobilized enzymes may have a lower inhibition (Garcia-Galan et al., 2011).

One of the first examples of inhibition reduction after immobilization was showed using a protease from an extreme thermophile by Cowan and Daniel (1982), and later it has been showed by other researchers in other enzymes (Mateo et al., 2004). Enzyme activity may increase after immobilization by an actual positive change in enzyme conformation that produces a more active enzyme conformation (Secundo, 2013), by an improved stability if the activity is determined under drastic conditions, or by many other reasons which have been recently reviewed (Rodrigues et al., 2013). The conformational changes (Secundo, 2013) may also alter enzyme specificity or selectivity. Immobilization has become a very useful technique to tune enzyme catalytic properties, although this modulation is nowadays performed just in an empiric way (Garcia-Galan et al., 2011; Rodrigues et al., 2013).

Immobilization may be performed just by physical adsorption on the support (Jesionowski et al., 2014). This may be simple and efficient, and usually may permit the reuse of the support after enzyme inactivation (Jesionowski et al., 2014). However, it has some problems (Fig. 5). First, it is not as mild as many authors consider, for example ion exchange requires the promotion of many enzyme-support ion bridges that may produce enzyme inactivation even though each individual bond is weak (Santos et al., 2015). Second, the support will be never fully inert, making the promotion of new enzyme-support bonds during operation possible, perhaps fixing incorrect structures of partially inactivated enzymes (Santos et al., 2015). In fact, it has been recently shown how the enzymes immobilized on cation exchangers may form very strong support/unfolded enzyme composites when inactivated (Virgen-Ortíz et al., 2017; Virgen-Ortíz et al., 2016). Finally, the low energy of each ion bond does not fix the relative positions of the involved groups. That is, a strong rigidification may not be expected by these techniques (Garcia-Galan et al., 2011). However, it may be very

useful to prevent enzyme subunit dissociation of multimeric enzymes (Fernandez-Lafuente, 2009). Entrapment of enzymes is a simple technique of enzyme immobilization (Katiyar and Ali, 2015; Bibi et al., 2015; Biró et al., 2016; Reetz et al., 1996). However, it may hardly improve enzyme properties (exception made on multimeric enzymes, preventing enzyme dissociation, or generation of enzyme favorable environments), and tends to be not very simple to be performed at large scale. However, it has been used in many instances to design biosensors (Gupta and Chaudhury, 2007; Cosnier, 1999). Moreover, enzyme leakage is a problem of this immobilization strategy, that has been usually solved increasing the size of the enzyme molecule (e.g. attaching the enzyme to a polymer, or making an enzyme aggregate) (Nguyen et al., 2016; Wilson et al., 2004; Cui et al., 2013; Matto and Husain, 2006). Covalent immobilization may produce a high rigidification if an intense multipoint covalent attachment is achieved but after enzyme inactivation, both enzyme and support will be discarded and the immobilization protocols are more sophisticated (Garcia-Galan et al., 2011). Thus, covalent immobilization is only recommended if the immobilization really provides a significant improvement on the enzyme properties (Mateo et al., 2007).

Regarding the supports, the cheapest one is the immobilization without supports (Cao et al., 2003; Sheldon, 2007). Crosslinked enzymes were the first proposal, but the reproducibility was not simple, and enzyme activity losses by chemical modification were significant (Cao et al., 2003; Sheldon, 2007; Ghafourifar et al., 2013). The crosslinked enzyme crystals (CLECs) (Khalaf et al., 1996; St. Clair and Navia, 1992; Häring and Schreier, 1999; Yan et al., 2015) yielded better results, and later the strategy was simplified through the concept of crosslinked enzyme aggregates (CLEAs). CLEAs are an immobilization alternative with good acceptance in academia (Cao et al.,

2003; Sheldon, 2007; Sheldon et al., 2005; Sheldon, 2011) (Fig. 6). The crystals are expensive and complex to be produced, but enzyme aggregates production is easy. The enzymes immobilized following these protocols may be stabilized (Sheldon et al., 2005; Sheldon, 2011) and may be a good solution to stabilize multimeric enzymes of complex structures (Fernandez-Lafuente, 2009) (Fig. 6). In the case of proteases, this may be a valid method of immobilization if they are going to be used in some organic chemistry reaction (synthesis of small peptides, resolution of racemic mixtures of esters or amides, or similar). However, if they are going to be used in the hydrolysis of proteins, it should be considered that only the external proteases (if properly oriented) may be active, as by steric limitations a substrate larger or similar in size to the protease may not access the core of the CLEA particle, and even less of the CLEC particle (Garcia-Galan et al., 2011) (Fig. 7). Together with steric hindrances and diffusional limitations, CLEAs have the problem of a low mechanical resistance in aqueous media (Garcia-Galan et al., 2011).

Porous pre-existing supports are the most utilized materials to immobilize enzymes: their mechanical resistance may be selected according to the reactor, loading may be very high (Garcia-Galan et al., 2011) (e.g., 100 mg/packed ml of wet support or 1g of enzyme per g of solid support using agarose (Zucca et al., 2016)) and may produce operational stabilizations as stated above (Mateo et al., 2007).

However, using proteases, there are some applications where the use of porous supports may be problematic. The most obvious one is in the modification of solid substrates, like textile materials; here the use of porous supports may be unsuitable to immobilize a protease (Garcia-Galan et al., 2011) (Fig. 8). The hydrolysis of protein aggregates may be performed using porous biocatalyst if they are re-solubilized, e.g., using high concentrations of caotropic agents like urea or guanidine (Garcia-Galan et

al., 2011). This makes the use of highly stabilized proteases compulsory to maintain their function under these drastic conditions (e.g. very stable proteases further stabilized by multipoint covalent attachment) (Mateo et al., 2007). Very large protein-substrates are also a problem as it makes the use of large pores in the support compulsory (Fig. 9). This reduces the specific area of the support (that way, volumetric loading capacity) and the mechanical resistance of the support (Garcia-Galan et al., 2011). In any case, the protease orientation regarding the support surface will play a critical role, as only properly oriented protease molecules may be accessed by the protein-substrate (Hernandez and Fernandez-Lafuente, 2011) (Fig. 10). Steric hindrances increase with support loading. It is possible that medium loaded protease immobilized biocatalysts have a good activity versus proteins, while when this biocatalyst is fully loaded with protease, the activity may virtually disappear (Garcia-Galan et al., 2011; Rodrigues et al., 2013; Hernandez and Fernandez-Lafuente, 2011) (Fig.10).

The use of nanoparticles may be an alternative to the use of porous materials, magnetic nanoparticles may be handled even if they have a very small diameter (very small particles are required to have good enzyme loading because diameter and specific area in non-porous supports are inversely correlated) by using a magnet (Vaghari et al., 2016; Kumari and Singh, 2016; Bosio et al., 2016; Cipolatti et al., 2014; Hwang and Gu, 2013). They can permit to stabilize proteins via multipoint attachment. If properly oriented, they may even act on solid substrates (Fig. 11) (Hwang and Gu, 2013), but they are not devoid problems: the enzyme is not protected from interactions with external interfaces or even from proteolysis (not by an enzyme immobilized in the same particle, but by enzymes immobilized on other particles) (Fig. 12) (Garcia-Galan et al., 2011; Cipolatti et al., 2016; Betancor et al., 2005). The incidence of these problems may be reduced coating the protease with a polymer but this needs to be

optimized to permit the access of the substrate to the protease active center (Cipolatti et al., 2016; Betancor et al., 2005) (Fig. 12).

Another alternative to immobilize proteases in an active form versus complex substrates is the use of smart polymers. This way the protease may act almost like a free enzyme under certain circumstances, and precipitate under other conditions (Cirillo et al., 2014; Roy and Gupta, 2003; Sardar et al., 2000) (Fig. 13). These polymer-immobilized enzymes may be used versus any substrate, but also have some limitations and problems. First, proteolysis of the modified protease is possible as well as any other intermolecular or interface inactivating interaction (Fig. 13). Second, protease stabilization is limited, as the polymer will not be very rigid. Finally, they can be only used if the final product is fully soluble; otherwise precipitated protease/polymer recovery will be not possible.

Therefore, although immobilization is a potent tool to improve enzyme properties (Garcia-Galan et al., 2011; Mateo et al., 2007), the final use of the catalyst when using proteases may exclude some kind of supports and render others less suitable to improve enzyme properties.

There are many examples of the use of immobilized proteases in the literature. We will comment just some of the most recent ones. Recent studies showed that Trypsin covalently immobilized onto modified magnetite nanoparticles exhibited a higher $K_m$ (12.1 mM) than the free trypsin (5.1 mM), which suggested conformational alterations on the enzyme structure after their covalent insolubilization (Atacana et al., 2017). However, this immobilized trypsin biocatalyst could be reused several cycles and maintained around 59% of its initial activity when utilized in casein hydrolysis. The effective hydrolysis of casein employing this immobilized enzyme biocatalyst was confirmed via liquid chromatography–mass spectrometry, showing that were similar to

the results achieved using free trypsin and demonstrating the prevention of autolysis (Atacana et al., 2017).

Chymotrypsin was immobilized in glyoxyl agarose (Bahamondes et al., 2017), finding diffusional problems on the activity recovery. This was modulated by the textural properties of the support and the enzyme loading: the diffusional problems increased with the particle size and enzyme loading.

Ficin was immobilized on glyoxyl-agarose, observing that 30% of the activity was retained under mild conditions (Siar et al., 2017). However, the biocatalyst stability greatly improved, this permitted the catalyst to retain double activity at pH 10, 3 folds more activity at 80ºC and it became 3 times more active in the presence of 2 M urea than the free enzyme. Moreover, the biocatalyst could be reused for 5 cycles at 55ºC in casein hydrolysis maintaining the initial activity (Siar et al., 2017).

Trypsin was immobilized in a lignocellulosic support (corn cob powder—CCP) activated with glyoxyl groups, glutaraldehyde and IDA-glyoxyl (Bassan et al., 2016). The retention of catalytic activity in the optimal biocatalyst was next to 75%. These biocatalysts were very stable at 65ºC, which were appropriate for the synthesis of some bioactive peptides.

### *4.2. Bioreactors*

Martin et al. (2004) stated “Bioreactors are generally defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, pressure, nutrient supply and waste removal). The high degree of reproducibility, control and automation introduced by bioreactors for specific experimental bioprocesses has been

key for their transfer to large-scale applications." Different types of bioreactors have been found to utilize proteases.

Enzyme membrane bioreactors make enzyme immobilization to reuse the enzyme unnecessary (Pietro et al., 2008) (although the positive effects of a proper immobilization are also skipped). In this bioreactor, a semi-permeable membrane module is used in the reactor. The membrane must permit to the pass of peptides of the desired size and amino acids from the reaction mixture, while the enzyme, whole substrate proteins and large peptides remain into the reaction tank. Membrane bioreactors combine selective elimination of products from the reaction media with controlled biochemical reactions (Giorno and Drioli, 2000). They present some advantages compared to other bioreactors. For example, the use and reutilization of soluble enzymes is permitted, inhibitory effects of the products may be prevented by continuous removal of the reaction products, and the maximum molecular size of the product-peptides may be selected by the molecular weight cut-off of the membrane. Compared to the use of free enzyme in a standard bioreactor, the advantages are clear. For example, the inactivation of the protease is not necessary to halt the process, and enzyme reuse is possible, as the enzyme is confined inside the bioreactor. However, the use of free enzymes in membrane reactors presents the same problems of the use of actual free enzymes, such as lower stability or autolysis.

### *4.3. Proteases in non-conventional media for synthetic purposes*

There is extensive literature on the application of hydrolases enzymes in organic synthesis carried out in non-aqueous media such as organic solvents, solvents-free reaction medium or ionic liquids (Wang et al., 2016; Milner and Maguire, 2012; Carrea and Riva, 2000). Proteases have been also applied for synthetic purposes. In fact,

based on the principle of reversibility, proteases can catalyze the hydrolysis of ester and amide bonds and the reverse reaction (e.g., ester and peptide bond formation). In particular, proteases have been applied in biocatalysis especially for peptide synthesis either by a kinetically controlled or an equilibrium-controlled approach (Jakubke, 1994; Deschrevel et al., 2003; Yazawa and Numata, 2014).

For kinetically controlled synthesis, serine and cysteine proteases form reactive acyl enzyme intermediates with an activated substrate (usually an ester, although it may also be an amide). In a second step, the acyl group should be preferentially transferred to the amino groups of amino acids or peptides (aminolysis). However, in the presence of water the hydrolysis of the acyl enzyme or the hydrolysis of the synthesized peptide (a water molecule attacks the acyl enzyme intermediate) also occur. Then, the activated acyl donor substrate decreases gradually during the course of the reaction, and the effect of hydrolysis increases (Kasche, 1986).

Thus, in a kinetically controlled synthesis, the aminolysis/hydrolysis ratio has to be increased for obtaining a high peptide yield (Fig. 14). This can be obtained by means of the use of efficient nucleophiles and/or working in low water systems (Klein et al., 2000), but it is necessary to stop the reaction before the rate of hydrolysis exceeds the rate of aminolysis and maximum yields may start to decrease. Another possibility to reduce hydrolysis is by favoring the precipitation of the products, as shown by Ungaro et al. (2015) for the synthesis of Z-Ala-Phe-OMe starting from Z-Ala-OH and HCl•Phe-OMe. The reaction was catalyzed by thermolysin at 50°C in acetate buffer and calcium acetate to enhance the thermal stability of the enzyme, and in the presence of ammonium sulfate for promoting precipitation of the peptide product. Z-Ala-Phe-OMe is the precursor of L-Ala-L-Phe, an interesting dipeptide for food applications because of its bitterness. As the reaction maximum yield is determined by the kinetic properties of

the catalysts, the search of enzymes with better features or the modification of the enzymes (via genetic, physical or chemical ways, including immobilization) may also improve the results obtained using these processes (Kasche, 1986; Rodrigues et al., 2013).

In equilibrium-controlled synthesis using proteases, the acyl donor is usually a free carboxyl group of an amino acid or of a peptide (Kasche, 1986). Proton transfer occurs during the first ionization step, followed by condensation, as shown in Fig. 14. The yields in this kind of reaction are determined only by the thermodynamics of the process, and the change in the enzyme can only increase the reaction rate or avoid enzyme inactivation or inhibition under the usually drastic utilized conditions. The major drawbacks of the equilibrium-controlled synthesis are usually the lower yield and a slower reaction rate with respect to the kinetically controlled reactions. Consequently, higher amounts of biocatalyst are necessary. Moreover, optimal reaction conditions are required to shift the equilibrium toward peptide formation and the use of an appropriate organic solvent as reaction medium might be beneficial for this purpose, provided that the amino acids are soluble in it (Kasche, 1986).

Thus, it appears clear that when proteases are used for synthetic purposes, either in a kinetically controlled or in an equilibrium controlled process (Fig. 14), the use of organic solvents as reaction media is useful to reduce the hydrolysis of the protease-acyl complex, owing to the lower quantity of water present in the reaction.

The extreme of this situation is when proteases are used in neat organic solvents. In this condition, the enzyme (e.g., in the form of a lyophilized powder or immobilized onto a support) can be suspended (or, even dissolved) in the reaction medium with a low (less than 5% v/v) amount of aqueous buffer to improve enzymatic activity (Secundo and Carrea, 2003). Nevertheless, the amount of water present in the

reaction medium (better expressed as water activity, “aw”) may have a strong influence on the catalytic properties of the enzyme and on the reaction yield (Bell et al., 1995). The use of enzymes in neat organic solvents is a conceptually different situation compared to the employment of the enzyme in water-organic solvents mixtures. In fact, in this latter case, the organic solvent is added to favor the dissolution of insoluble reactants, but in equilibrium the hydrolytic reaction may prevail on the synthetic one, depending on the difference in the pKs of the amino and carboxyl groups involved, the excess of one of the substrates, and the concentration of solvent that may be used. In this regard, one very important feature of an organic solvent for a proper medium for this kind of reactions is its capacity to increase the pK of the carboxylic acid (Fernandez-Lafuente et al., 1991; Rosell et al., 1998).

A similar situation is also observed in the case of enzymes in water/solvent biphasic systems or in reverse-micelle systems where the enzyme is dissolved in water, even if the organic solvent is the most abundant part (Carrea and Riva, 2000; Zaks and Klibanov, 1988). There are different advantages using enzymes in dry organic solvents. In fact, it is possible to transform substrates that are unstable or poorly soluble in water avoiding water dependent side-reactions, including the denaturation of enzymes that, in anhydrous organic media, tend to have higher thermal stability. Furthermore, in an organic solvent it is possible to prevent microbial contamination and to facilitate enzyme recovery that will be in aggregated form. Furthermore, it is possible to modulate the regio- and enantioselectivity of a given enzyme by changing the organic solvent.

Among the families of proteases, subtilisins are considered to be the most efficient enzymes in organic media (Klein et al., 2000). Subtilisins are bacterial serine proteases, classified today as subtilisin BPN’ or subtilisin Carlsberg, and are commercialized by several companies.

An interesting example on the use of proteases for the synthesis of food related compounds is the modification of sugars such as lactose, glucose, maltose, sucrose or maltotriose (Riva et al., 1988; Carrea et al., 1989). Sucrose esters constitute an interesting class of biosurfactants used in the preparation of microemulsions suitable as delivery systems of food-derived bioactive compounds (Flanagan and Singh, 2006). Thanks to the capabilities of *Bacillus subtilis* subtilisin, (crystalline enzyme, type VIII) and of protease N (a less purified form of subtilisin), it was possible to regioselectively acylate, to a level of gram scale, sucrose in 1-O'-position, obtaining 1'-O-mono-butyrylsucrose (57% yield). The reaction was performed in anhydrous dimethylformamide with 2,2,2-trichloroethyl butyrate as acyl donor. Likewise, 6'-O-mono-butyryl cellobiose, 6'-O-mono-butyryllactose, 6'-O-mono-butyrylmaltose, 4'-O-mono-butyryllactose and 3'-O-mono-butyryllactose were also synthetized. Before their use as biocatalyst of these reactions, both enzyme preparations were dissolved in 0.1 M aqueous phosphate, adjusted to pH 7.8, and lyophilized. In fact, it is known that the optimal ionization state of the enzyme before drying, markedly influence the catalytic activity of subtilisin in organic solvent (Zaks and Klibanov, 1988). A more detailed study on the preferential position acylated by subtilisin of sugars was conducted investigating the esterification of several enantiomeric benzyl and naphthyl glycopyranosides (Danieli et al., 1999).

Another interesting application of subtilisin in non-aqueous media, related to the food field, is the acylation of starch, which is possible thanks to the reaction of hydroxyl groups of the anhydroglucose unit monomer with substrates containing an acyl group. Acetylated starch derivatives with low degree of substitution of mol of acyl per mol of anhydroglucose are utilized as additives in the food industry to control and adjust the rheological behavior of pastes. Starch succinates strengthened starch swelling

capability at lower temperatures, while alkenyl succinate starch derivatives give to the starch emulsifying capabilities (Alissandratos and Halling, 2012). Dordick and coworkers (Bruno et al., 1995) showed the possibility of acylating a thin film of amylose (an organic solvent-insoluble polysaccharide consisting of α-1,4-linked glucose moieties), by catalysis in organic solvents, using an organic-soluble enzyme preparation of subtilisin Carlsberg (from *Bacillus licheniformis*). The peculiarity of this process is that acylation of the insoluble polymer occurs only in the presence of a soluble enzyme form.

The high stability and versatility of subtilisin was also proved by suspending it in protic ionic liquids for the transesterification reaction of N-acetyl-L-phenylalanine ethyl ester with 1-propanol. Among the different ionic liquids tested, subtilisin was only active in diethanolammonium chloride, while chymotrypsin was not active in the same ionic liquids (Falcioni et al., 2010).

The above examples of application of subtilisin indicate that using proteases in non-aqueous media can be a useful methodology for the production of various compounds and even macromolecules useful for the food industry. Nevertheless, for a more diffuse use of this methodology, it is important to find other stable and versatile proteases able to catalyze the synthetic reactions in non-aqueous media. This may allow enlarging the possibility to develop biocatalytic methods, both in water and in non-aqueous media, for the preparation of interesting industrial products.

## 5. Proteases sources

Considering that proteases are very related to the vital cycles of all living beings, the numerous tissues of animals and plants or whole microorganism cells (even the most primitive ones) may be considered as proteases sources (Davesena, 2010). Moreover, the features of these proteases will mirror their living circumstances. Thus,

there are many changes among proteases from different species and a long history of changes rising from the long natural evolution. However, most proteases have features that show mild life conditions, mainly considering animal and plant sources (Castro et al., 2011). However, among microorganisms this could differ. It is possible to search those microorganisms that survive under extreme conditions of pressure, ionic strength, temperature or pH (the so-called extremophiles) (Ratyanarayana et al., 2005; Elleuche, et al., 2014; Banciu and Muntyan, 2015), expecting that their proteases may be adjusted to these drastic conditions. This vision of microorganisms as a new library of naturally-differentiated enzymes (Tavano, 2016; Mishra et al., 2016), together with other benefits of using microorganisms on a large scale, shows the relevance of microbial proteases. Since some industrial procedures needs using proteases under conditions distant from the physiological ones (e.g., their applications in detergents with alkaline characteristics) or even in the diverse media that constitute food matrices, including a variety of pH values, concentrations and nature of salts, etc., the amplification of the collection of protease availability is a permanent goal (Tavano, 2016; Mishra et al., 2016; Sandhya et al., 2005; Cobb et al., 2012). Frequent alterations from novel market demands make industries to search improved enzymes, and the exploration for new enzyme sources may be a good begin.

### *5.1. Microbial proteases*

Microorganisms as a source of enzymes has a handful of advantages like their natural features and the possibility of creating new features in these microorganism and their enzymes with simplicity (Vermelho et al., 2016; Mishra et al., 2016). Both cases show the notable capability of microorganisms to acclimate to altered environmental conditions, such as high temperature, drastic pH, concentrations of media constituents,

or presence of chelating agents (Souza et al., 2015; Mishra et al., 2016; Sandhya et al., 2005; Cobb et al., 2012). That is, there is interest in evaluating enzymes in microorganisms, that propose a huge amount and metabolic diversity (Li et al., 2013), enabling to search the most suitable enzyme, even employing the traditional "screening" culture.

Many interesting proteases have been found by screening extremophiles, which naturally synthetize enzymes that retains their function under extreme conditions, such as high pressure, high radiation exposition, high salinity, drastic pH or extreme temperatures, finding proteases whose stability may be very interesting (Van den Burg, 2003 Eijsink et al., 2005). Thermostable enzymes have been isolated from thermophilic microorganisms which live at 60-80°C (hyperthermophiles grow at temperatures over 80ºC). They present proteases with rigid enough structures to resist thermal denaturation or to the presence of organic solvents (Fontana et al., 1998; Mishra et al., 2016). In this way, the use of proteases in protocols performed at high temperatures is an actual benefit, enhancing the velocity of the reaction not only by the effect of the enzyme kinetics, but also by permitting the joint action on the substrate (proteins), that will be partially unfolded at high temperature. Folded proteins may resist better the hydrolysis catalyzed by proteases, but once they are submitted to high temperatures, proteins may become partially denatured and this increase the accessibility of the proteases to their target places. For example, the protease catalyzed hydrolysis of hard-to-degrade animal proteins generated in the meat industry, was improved by using thermostable proteases at high temperature. The high temperature produced the protein-substrate thermal unfolding and yielded higher proteolysis vulnerability of the proteins (Suzuki et al., 2006). Moreover, high temperatures may decrease

contamination of the product by microorganisms, that could contaminate the reaction medium or the substrate.

Psychrophilic, the cold-adapted microorganisms, can grow under 0◦C, and even at −20◦C, this should be related to enzymes adapted to these specific thermal condition, and include the production of cold-adapted proteases (Siddiqui and Cavicchioli, 2006). The main protease characteristic that justifies their adaption at these cold temperatures comprises a very flexible structure, which equilibrates the low energy existing in these very cold environments. This suggests that these proteases exhibited a decreased activation enthalpy and more negative activation entropy when compared with standard proteases. This provides economic profits, because utilizing lower temperatures may produce energy savings during operation. Moreover, the utilization of these cold-adapted proteases possesses other advantages, since the processes performed at low temperatures can prevent chemical degradation of the food product (e.g., of vitamins). This chemical degradation will produce a decrease of the nutritional power of the final product. The use of high temperatures may also favor losses of volatile compounds, altering the final flavor of the product, Besides, cold-adapted proteases may be inactivated by small augmentations of temperature, that facilitate stopping of the proteolytic process with minor increments of the temperature (Lylloff et al., 2016; Cavicchioli et al., 2011). A cold-adapted protease produced by a deep-sea cold-adapted bacterium, *Pseudoaltermonas* sp. SM9913, was used to hydrolyze marine fish, pork and shrimp meat samples treated at 0ºC. The treatment liberated more essential amino acids and free taste when utilizing cold-adapted protease than when treated with mesophilic proteases (He et al., 2004).

Halophilic microorganisms are other kind of interesting extremophiles. They are capable to grow under hypersaline conditions (DasSarma and DasSarma, 2015). They

have been also sources of interesting proteases, providing specifc advantages for some food processes. Hypersaline conditions may permit performing food proteolysis processes without strictly sterile conditions, thanks to the microorganisms growth inhibitory effects in other microorganisms due to the low water activity of these media. Low water activity conditions are the situation of aqueous-organic solvent mixtures. This makes that halophilic enzymes usually keep high activity in organic media. This converts the halophilic proteases in suitable industrial biocatalysts in synthetic processes (DasSarma and DasSarma, 2015).

Microbial proteases are relevant examples in the families of acidic or alkaline proteases. Microbial rennin-like proteases constitutes a good example of acid proteases (Moschopoulos, 2016). Neutral proteases, mainly fungal neutral proteases, are relevant constituents of commercial enzymes preparations, which have uses in protein modification, food processing and baking and also in pharmaceutical, animal feeds, leather industries (Sumantha et al., 2006, Gupta and Ayyachamy, 2012). Its frequent high affinity for hydrophobic amino acids gives an advantage as a de-bittering reagent. Nowadays, *Aspergillus oryzae* is the most important fungal source of neutral proteases (Sumantha *et al*., 2006). Their main uses comprise meat protein recovery, casein and whey protein hydrolysis, fish protein hydrolysis, gelating hydrolysis, soy sauce production, soy protein hydrolysis and meat tenderization (Sumantha *et al*., 2006). The thermostable protease *Thermolysin*, produced by *Bacillus stearothermophilus* which can act at 80ºC (Mattheus, 1988) is attracting the interest of the researchers.

Alkaline proteases are very relevant, because of their stability and activity at alkaline pH values. Subtilisin Carlsberg produced by *Bacillus licheniformis*, Subtilisin Novo and Subtilisin BPN are some popular serine alkaline proteases. These enzymes can be used in flavor and color development in cookies, improvement of dough texture,

treatment of flour in the manufacture of baked goods, cheese flavor development, meat tenderizing, bating and dehairing of skins, improving digestibility of animal feeds, etc. (Guntelberg and Otteson, 1952, Smih et al., 1968; Kumar and Takagi, 1999; Sumantha et al., 2006).

Thus, the diverse natural conditions where microorganisms may live and, therefore, their innate enzyme variants, can have a broad range of microbial proteases to be studied and evaluated. This agrees with the industrial requirements, because the constant progresses in industrial procedures often need an increasing variety of catalytic features of the proteases. In this context, the prospects of utilizing techniques like site-directed mutagenesis or directed-evolution, again present benefits to the utilization of microorganisms as sources of enzymes (Eijsink et al., 2005; Li et al., 2013). Creating proteases with enhanced functions to reach the necessities of particular commercial uses may be achieved with these techniques (Li et al., 2013). Enzymes from microorganisms can be improved applying site-directed mutagenesis techniques (including deletions, insertions, or/and recombination), permitting the achievement of a “rationally designed” protease (Eijsink et al., 2005; Li et al., 2013). Alternatively, directed evolution uses selective pressure to a collection of variants of a desired biological entity to identify those variants having properties next to the desired ones. It is based on the production of a large genetic variety followed by selection/screening. This "laboratory evolution” increases the rate and mimics natural evolution, it has an enormous potential to enhance enzyme features. However, this strategy is time-consuming (Coob et al., 2012). The fact is that approximately 90% of industrial enzymes are recombinant forms (Adrio and Demain, 2014).

Microbial proteases are frequently synthetized as extracellular proteins *in nature*; and this is another relevant advantage of using microorganisms as proteases

producers. These enzymes are directly secreted into the fermentation broth and ease downstream processing, preventing some retrieval and purification steps of the enzyme during its manufacture, steps that cannot be prevented using proteases directly obtained from animals or plants (Savitha et al., 2011; Souza et al., 2015). One problem of extracellular enzymes is that the enzymes are diluted in the whole culture medium, and this may become a problem in the industrial scale of protease production.

Fungi are often utilized in proteases production, because it has advantages depending on the kind of medium utilized in their growing. Fungi can grow on cheap materials and secrete high quantities of enzymes into culture medium which could facilitate their downstream processing (Anitha and Palanivelu, 2013, Souza et al., 2015). Different ways of cultivation can be used depending on the convenience and features of the microorganism utilized. Both solid state and submerged fermentation are frequently utilized in the production of proteases by fungi (Devasena, 2010). Some species of filamentous fungi, like *Aspergillus, Penicillium* and *Paecylomices* have showed to be great producers of extracellular protease in submerged fermentation. This production strategy has advantages in control of process and can present an easy recovery of extracellular enzymes. Other advantages comprise low production costs, low wastewater output, lower mechanical energy expenditures (due to being a static process), simplicity and the low moisture content can greatly decrease bacterial contamination during fermentation. The main drawbacks of this fermentation are the lack of control of temperature and pH (Sandhya et al., 2005).

### *5.1.1. Some outstanding microbial proteases*

Alcalase is a commercial protease cocktail; it was initially obtained from *Bacillus subtilis*, a microorganism that is capable to produce diverse alkaline extracellular

proteases. The first of these proteases was determined by Linderstrom-Lang and Ottesen and purified by Gtintelberg and Ottesen, and now is named subtilisin Carlsberg (DeLange and Smith, 1968), but it has also been called subtilisin A, subtilopeptidase A, and then Alcalase. Initially if was used in the detergent industry, due its alkalophilic features, but many researches show a great range of applications in the modification of foods. Cabanillas et al. (2012) described that roasted peanut proteins decreased a 65% its IgE reactivity after 300 min of hydrolysis using Flavourzyme as a catalyst and a 100% decrease in this reactivity using Alcalase after only 30 min of hydrolysis. Sweet sorghum grain proteins and salmon hydrolysates exhibited the highest ACE inhibitory activity when hydrolyzed using Alcalase (Ahn et al., 2012; Wu et al., 2016). The enhancement of the antioxidant activity of the chickpea protein was also showed after Alcalase hydrolysis (Ghribi et al., 2015).

Flavourzyme is another commercial peptidase preparation (supplied by Novozymes) containing different endo- and exopeptidases from *Aspergillus oryzae* such as: alkaline protease 1, neutral proteases 1 and 2, dipeptidyl peptidases 4 and 5, leucine aminopeptidases 2 and A, (Merz et al., 2015a; Merz et al., 2015b). They have many applications in academy and the industry because of the synergy among endo and exopeptidases, that has been determined critical for an effective hydrolysis of proteins. But this mixture proteases presents some disadvantages such as: some changes of the blend composition from batch to batch reduce the reproducibility of the hydrolysis process; lower control over the exact modification on the substrate; each enzyme can be altered by changes in the medium in a different way and even be affected in different ways during storage (Merz et al., 2015a; Merz et al., 2015b). Novozymes uses the exopeptidase activity to defined the activity of Flavourzyme® 1000L preparation utilizing the synthetic substrate H-Leucine-para-nitroanilide (Leu-

pNA), i.e. this preparation contains a minimum of 1000 leucine aminopeptidase units per gram preparation, but the other proteases are not comprised in the supplier information. When defatted soy flour was hydrolyzed using Flavourzyme 1000 L, Alcalase 2.4 L FG and Novozym FM 2.0 L, enhanced gelling and foaming features were achieved, mainly when Flavourzyme was utilized (Hrcková et al., 2013).

Thermolysin, as suggested by itsname, is a thermostable zinc endopeptidase obtained from *Bacillus thermoproteolyticus* (Mattheus, 1988). Yokoyama et al. (1992) described a potent ACE inhibitory activity of the tuna muscle after thermolysin hydrolysis. This was explained by thermolysin specificity, that produces peptides with Leu, Phe, Ile or Ala at the amino terminus. When α-lactalbumin and β-casein was hydrolyzed by this protease, a high ACE inhibitory activity was found on the final product. Diverse peptides of the proteolyzate presented this functionality (Otte et al., 2007). Sequential hydrolysis of a commercial preparation of casein hydrolysate from bovine milk with immobilized trypsin and thermolysin released peptides with biological activities as antithrombotic, opioid agonists, antihypertensives, bifidogenic, antioxidant, mineral carriers, immunostimulants, as well as peptides that show gastrointestinal mucosal protection activity (Rocha-Martin et al., 2017).

## 6. Conclusion

Proteases are and will very likely remain one of the most utilized enzymes at both academic and applied levels. The relatively recent advances in microbiology (metagenomics) and genetics (directed evolution) may provide researchers with proteases whose properties are near the industry requirements. Moreover, production of enzymes is also experiencing an impressive development in recent times. Furthermore, progress in material science, nanotechnology, solids chemistry, and protein chemistries

to mention a few examples may permit achieving immobilized protease biocatalysts with significantly improved properties overcoming the current drawbacks of the immobilization processes. New bioreactors may also increase the range of processes where enzymes may be utilized.

Therefore, proteases may have a future full of successful new applications, not only in food and cleaning technologies, but also in pharmaceutical and fine chemistry industries. Thus, proteases should be expected to maintain their prominent position in enzyme utilization in the medium and even long term.

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**Figure legends**

Fig. 1. Schematic representation of the relationship between degradomic and protease repertories for biotechnological applications.

Fig. 2. Schematic representation of how proteases interact with the substrate or under certain environmental conditions to promote the characteristic of the process and final product.

Fig. 3. Schematic representation of protease potential applications.

Fig. 4. Schematic representation of different pathways for bioactive peptides release.

Fig. 5. Enzyme immobilization via ion exchange. It requires multipoint immobilization and the ion exchange capacity of the support during operation may fix altered conformations of the enzyme.

Fig. 6. Stabilization of multimeric enzymes structure during the preparation of CLEAs.

Fig. 7. Usefulness of proteases CLEA versus different substrates. They can attack neither very large nor solid substrates, being mainly useful for very small substrates (relative to the protein size).

Fig. 8. Utility of proteases immobilized on porous supports versus different substrates. They may be useful for substrates as large as the protease, but may be inadequate for much larger substrates .

Fig. 9. Advantages and drawbacks of using supports with very large pores. While this biocatalyst may be used versus large substrates, its loading capacity and mechanical resistance will be quite compromised.

Fig. 10. Effect of orientation and loading on the activity versus large substrates of enzyme immobilized on supports having flat surfaces. Only properly oriented proteases

will be active versus large substrates, and the requirements will increase when the loading of the support does.

Fig. 11. Advantages of using nanoparticles to immobilize proteases: the immobilized enzymes may be used to hydrolyze even solid substrates.

Fig. 12. Drawbacks of the immobilization of proteases in nanoparticles: interactions with macromolecular structures are not prevented. Coating with polymers may be a simple solution.

Fig. 13. Proteases modified with smart polymers: advantages and drawbacks.

Fig. 14. Thermodynamically controlled synthesis of amide bonds using proteases. Yields are determined by the thermodynamic constant of the process and they are independent from the catalyst.