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Review

Protein hydrolysis using proteases: An important tool for food biotechnology

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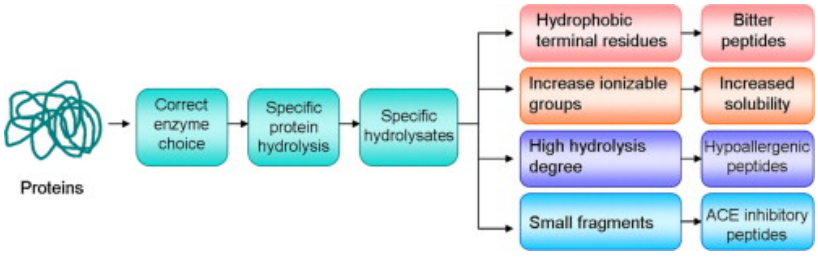
Abstract

This review intended to give a brief idea of the importance of proteases applications. Processes that involve protein hydrolysis steps find wide ranging utilizations, such as cleaning process, proteomic studies, or food biotechnology process. Many positive effects hoped for with food processing can be achieved by protein hydrolysis using specific proteases, changing nutritional, bioactive and functional properties of food proteins, which include improved digestibility, modifications of sensory quality (such as texture or taste), improvement of antioxidant capability or reduction in allergenic compounds. Protease applications in industrial processes are constantly being introduced and can be advantageous compared to chemical processes, by increasing hydrolysis specificity, product preservation and purity, and reducing environmental impact. Differences in specificity between proteases are very important to take in to consideration as a guide for the choice of protease according to the protein source to be hydrolyzed or predicted products. In this present review, some aspects of the processes that involve protein hydrolysis steps are discussed, especially considering the application of specific proteases as a tool on food biotechnology.

Graphical abstract



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Highlights

► Processes that involve protein hydrolysis steps find wide ranging applications. ► Proteases specificity is an important tool for food biotechnology process. ► Special peptides could never be achieved by chemical hydrolysis of proteins. ► The growing use of proteases is dependent on constant innovation studies. ► Enzymes stabilization continues to be a wide area for research.

Previous Next

Keywords

Proteases; Protein hydrolysis; Hydrolysates; Proteases stabilization; Food biotechnology

1. Introduction

Processes that involve protein hydrolysis steps find wide ranging applications. Such applications can be found in the detergent or leather industries, in the formulation of samples for amino acid analysis or proteomic studies, and in the development of protein hydrolysates designed for nutritional support of special patients [1], [2], [3], [4], [5], [6], [7]. For the last two examples, preservation of the properties of the generated products, peptides and amino acids, is especially desirable, thus, the hydrolysis process must be carefully controlled.

Protein hydrolysis, the cleavage of peptide bonds, can be carried out by enzymatic or chemical processes. Chemical processes, including alkaline or acid hydrolysis, tend to be difficult to control, and yield products with modified amino acids. Conventional acid hydrolysis conditions to amino acids determination, using 6M HCl at 110°C for more than 24h, can destroy tryptophan [8]. Alkaline hydrolysis can chemically reduce cystine, arginine, threonine, serine, isoleucine, and/or lysine content, and form unusual amino acid residues such as lysinoalanine or lanthionine [9]. Enzymatic hydrolysis can be performed under mild conditions, and could avoid the extreme environments required by chemical treatments. Usually, enzymatic processes avoid side reactions and do not decrease the nutritional value of the protein source [2]. Additionally, enzymes present substrate specificity which permits the development of protein hydrolysates with better defined chemical and nutritional characteristics [10].

On the other hand, enzymatic processing can present certain difficulties. Total protein hydrolysis by one protease action is still utopic. In addition, relatively small changes in protein structure can cause important damage to enzyme function, and they can present inhibitors, cofactors needs, or suffer autolysis [11], [12], [13], [14].

Thus, processes aimed at obtaining hydrolysates with specific and preserved peptides, can consider the protease application as an important tool. But achieving the objectives requires knowing perform more appropriate choice of the protease, and often also to modify this protease for best results in the desired application. In this present review, some aspects of the processes that involve protein hydrolysis steps are discussed, especially considering the application of specific proteases as a tool on food biotechnology.

2. Proteases aspects

Proteases (also termed peptidases, proteolytic enzymes and peptide bond hydrolases) are intimately associated with vital biological pathways. As a result, it is not difficult to imagine their importance, even in the case of the most primitive organisms, and their association with evolutionary processes. The range of variants and specificity reflect these evolutionary modifications. At the same time their similarities permit that common ancestry of

distinct species be established. The modifications suffered by proteins can also result in the fact that distinct proteins present similar functions – as proposed for chymotrypsin and subtilysin, which present the same catalytic mechanism for proteolysis by convergent evolution provided by unrelated proteins [8], [15], [16], [17]. The evolutionary complexity of living organisms provides an enormous range of different proteases with a large repertoire of functions and, subsequently, a wide range of structures and specificities, which arouse interest amongst researchers who seek new applications for proteases [18], [19]. The clear importance and complexity of the proteolysis in biochemical functions or pathogenical conditions in all organisms led to the development of *degradomics*, as an experimental field for the identification and characterization of proteases in an organism [19], [20], [21].

Advances in the chemical characterization of active sites and structure analysis permit that proteases can be grouped into families by common mechanism or by similar structural features [22]. Despite being relatively simple to classify enzymes, proteases do not always fit clearly into the international system for the classification and nomenclature of enzymes (EC number), which was developed in the 1950s [23]. All enzymes are divided in six classes, and proteases are classified in class 3, as Hydrolases, subclass 3.4., hydrolysis of peptide bonds. So, proteases were divided between 13 sub-classes on the basis of the catalytic reaction.

Nowadays, since the protein structure of the enzymes is better known, other forms of classification can be proposed that consider their chemical structures and thus contain information about their evolutionary families, such as MEROPS databases (peptidase database) [24], [25], [26]. Considering, principally, the tertiary structure of the protein and its catalytic sites, proteases can be classified in clans, and clans divided in families. Every clan provides information about the catalytic structure of the proteases. The names take into account the iconic amino acid or metal present in the active site: Aspartic peptidases (A), Cysteine peptidases (C), Metallo peptidases (M), Serine peptidases (S), Mixed catalytic type (P) and Unknown type (U) [24], [25], [27].

The structure around the active site of the protease determines how the substrate can bind to the sites of the protease. The surface of the protease that is able to accommodate the chain of the substrate is called the *subsite* and it can determine the substrate specificity of a given protease [28]. However, although most enzymes present a large chain/structure, only a few amino acid residues are in fact involved in the active site. Serine proteases, for example, are known for their classical catalytic triad. The geometric relationship between Asp102, His57 and Ser195 of chymotrypsin, the first structure reported for a peptidase, is very well documented [16], [27].

Although it is possible to consider that the most important characteristic of proteases is their form of action on substrates, sometimes their specificities are very complex and not clearly defined. However the mode of action can define proteases as exopeptidases or endopeptidases, which are the two main sub-classes based on substrate interaction. The peptide substrate runs through the entire length of the active site of an endopeptidase framework and is cleaved in the middle of the molecule. On the other hand, exopeptidades act near the end of polypeptide chains. Furthermore, exopeptidases are termed aminopeptidases if they act at the n-terminus, and carboxypeptidases are those acting on peptide bonds from the c-terminus. Some enzymes present both carboxy- and aminopeptidase forms, such as cathepsins, since their structure can have structural elements that provide negative charge (cathepsin H) to bind the positively charged amino terminus of the substrate, or positive charge (cathepsin X) to bind the negatively charged carboxyl terminus of the substrate [28], [29]. Finally, in describing the specificity of endopeptidases, the term oligopeptidase is used to refer to those that act optimally on substrates smaller than proteins.

The specificity of a protease determines the position at which the enzyme will catalyze peptide bond hydrolysis. The enzyme active site has a characteristic arrangement of amino acid residues which define the enzyme–substrate

interaction [17]. The knowledge of the specificity of proteases provides information that can lead to a better choice to act on a specific substrate [30], [31]. Table 1 presents a compilation of preferential cleavage of some proteases.

Table 1. Preferential cleavage of proteolytic enzymes.

Protease	**Hydrolysis reaction**
Aminopeptidase A (EC 3.4.11.1)	Release of N-terminal Leu, but also may be other amino acids, including Pro, but not Arg or Lys.
Aminopeptidase B (EC 3.4.11.6)	Release of N-terminal Arg and Lys from oligopeptides when P1′ is not Pro.
Carboxypeptidase A (EC 3.4.17.1)	Release of a C-terminal amino acid, but little or no action with -Asp, -Glu, -Arg, -Lys or – Pro.
Carboxypeptidase B (EC 3.4.17.2)	Preferential release of a C-terminal Lys or Arg
Chymotrypsin (EC 3.4.21.1)	Preferential release of N-terminal Tyr, Trp, Phe, Leu at P_1 position.
Papain (EC 3.4.22.2)	Preference for an amino acid bearing a large hydrophobic side chain at the P2 position.
Pepsin (EC 3.4.23.15)	Preferential cleavage: hydrophobic, preferably aromatic residues.
Subtilisin (EC 3.4.21.62)	Hydrolysis of proteins with broad specificity for peptide bonds, and a preference for a large uncharged residue in P1. Hydrolyses peptide amides
Thermolysin (EC 3.4.24.27)	Preferential release of C-terminal Leu and Phe at P_1 position.
Trypsin (EC 3.4.21.4)	Preferential release of N-terminal Arg and Lys at P_1 position.

Adapted from BRENDA (BRaunschweig ENzyme DAtabase) informations [32].

These differences in specificity between proteases are very important to take in to consideration as a guide for the choice of protease according to the protein source to be hydrolyzed or predicted products. A same protein chain can produce very different hydrolysates using different proteases. A potato pulp protein, for example, when hydrolysed using four different enzymes [33], endoproteases Alcalase and Novo Pro-D, exopeptidase Corolase, endo and exoproteases mix – Flavourzyme, and their combinations, resulted in distinct hydrolysates. Kamnerdpetch et al. [33] observed that the total hydrolysis degrees were 22, 8, 3 and 2% for Flavourzyme, Alcalase, Novo Pro-D and Corolase, respectively. As can be seen, the determining factor for the degree of hydrolysis was not the fact that the enzymes were endo- or exo-, but the specificity of the chain of the substrate protein. Not only the hydrolysis degree was different, the amino acid profile of the hydrolysates was also different. Novo Pro-D cleaves peptide bonds of aromatic and sulfur amino acids (His, Phe, Trp and Tyr). However, due to the combination of endo and exoprotease activities of the Flavourzyme and the broad specificity of Alcalase, which preferentially hydrolyses peptide bonds containing aromatic amino acid residues, a significant increase of methion- and aromatic amino acids in Flavourzyme and Alcalase hydrolysates was observed. Protease association can be interesting in order to increase the hydrolysis degree. According to Kamnerdpetch et al. [33], the best result (44% hydrolyses degree) was obtained from the action of a combination of 2% Alcalase+5% Flavourzyme.

Sometimes it is not enough to choose an enzyme, but it is necessary to select the source. The same enzyme, for example a trypsin from different sources, can present distinct behavior. Ovine and porcine pancreas trypsin can present very similar hydrolysis actions, as observed for β-lactoglobulin, egg white lysozyme, casein or auto-catalytic

hydrolysis production of peptide fragments. However, porcine trypsin was less susceptible to denaturation at low pH or high temperature than ovine trypsin [34], which could influence the choice of enzyme for a given application.

3. Improving proteases performance

Many hydrolytic processes meet limitations in enzyme utilization, such as enzyme chain instability, low reaction rate or even low substrate susceptibility. Whilst biological evolutionary processes have made possible the optimization of protease functions, these evolutionary modifications, obviously, do not necessarily reflect on industrial applications. However, problems regarding action or enzyme stability have been continually overcome due to advances in chemical and molecular methods [35].

Native protein states are well-packed structures, with a well defined three dimensional structure dictated by their amino acid sequence, presenting secondary, and tertiary structures. The tertiary structure gives the enzyme a core of hydrophobic amino acids (e.g. phenylalanine, tyrosine, etc.) and tends to yield acidic or basic amino acids on the surface because of their ability to form hydrogen bonds, making exposure to a hydrophilic environment favorable. Quaternary structures can also be formed by the noncovalent interactions of multiple enzyme subunits. These interactions are the basis of the catalytic cleft of the enzymatic activity. Even simple temperature variation or manipulation can lead to structural changes and cause important functional consequences [12], [36], [37]. Proteases still present a particular problem: the problem of autolysis. During storage protease enzymes can use neighboring protease chains as substrates resulting in autodigestion [13], [14]. Understanding the nature of the enzyme makes it possible to manipulate and modify the enzyme to optimize the hydrolysis process.

3.1. Managing proteases efficiency

The protein/enzyme stability can be referred to as “a protein's resistance to adverse influences such as heat or denaturants, that is, to the persistence of its molecular integrity or biological function in the face of high temperatures or other deleterious influences” [38]. According to Fagáin [38] this may arise from thermodynamic (or conformational) stability and long-term (or kinetic) stability. Thermodynamic stability concerns the resistance of the folded protein conformation to denaturation while long-term stability measures the resistance to irreversible inactivation. Irreversible thermal denaturation of a protein usually comprises an unfolding step followed by an irreversible process, such as protein aggregation or proteolysis [39]. The unfolding effect in multimeric enzymes is not easy to analyze because it is very difficult to discriminate between dissociation and unfolding destabilization effects [40]. The dissociation of the enzyme subunits is usually the first step in the inactivation of multimeric enzymes. For this reason, the prevention of enzyme dissociation and unfolding rate can be achieved by a number of chemical methods that have been described in the literature for alteration of enzyme stability and activity. These include enzyme immobilization methods, cross-linking with chemicals or chemical modification of the amino acid side-chains [41], [42], [43].

It is important to mention that a large number of factors contribute to protein stability at the same time (Van der Waals forces, hydrogen bonds, salt bridges, torsion potentials, bond stretching, planarity of conjugated systems, pi–pi stacking, the entropy of water, ionic interactions, loop tension, helix dipole interactions, and disulfide bridges) [39]. It is possible to control one factor, but it is impossible to certify which of the other processes also contributed.

Many processes that occur in foods or cleaning actions, which present a complex and variable environmental composition (presence/absence of metals, acids, detergents), can considerably damage protease utilization. Moreover, processes at high temperatures can be advantageous, since this procedure can accelerate the reaction and avoids microorganism growth. Therefore, improvement of protease stability would provide a number of advantages.

3.1.1. Chemical modifications

Many amino-acid side chains have reactive functional groups which can react with reagents by cross-linking, intra- and intermolecular or covalent coupling [38]. Chemical modification depends on enzyme characteristics to improve or keep their stability and/or activity. Modified enzymes present greater stability to environmental conditions, such as pH change or high temperature. Different dicarboxylic anhydrides have been used for chemical modification of different enzymes. Succinic anhydride reacts specifically with the ε-amino groups of the side chain of the lysine residues and changes its charges from positive to negative. Papain, for example, contains Cys–His–Asn as a catalytic triad of its active site involved in the hydrolysis of proteins and so retains its catalytic activity after succinylation of the lysine residues, which are not involved in the active site geometry. Roy et al. [44] observed that succinylated papain displays thermal and pH stability, especially in alkaline conditions, whereas activity of the native papain gradually decreases at pH values above neutral, as the negative environmental charges interact with the positive charges on the native protein chain. The same phenomenon was observed by Sangeetha and Abraham [41], where papain was chemically modified using different dicarboxylic anhydrides (citraconic, phthalic, maleic and succinic acid).

Chemical modification of lysine residues in bromelain has also been described, and was carried out using pyromellitic anhydride acid and poly maleic anhydride resulted in thermal stability and the resistance to alkali and the surfactant [43]. The modification resulted in the acylation of 8–9 of the 15 primary amino groups and altered the protein positive charge improving the stability of enzyme in extreme environmental conditions (pH and temperature) and its stability against the anionic surfactants.

Combination with molecules, such as carbohydrates, results in several changes in the structural and functional properties of the enzymes. These molecules are believed to provide additional points of hydrogen bonding with the enzyme surface, decrease dehydration and/or provide thermodynamic barriers to unfolding [37]. Covalent attachment of caboxymethylcellulose to the surface of trypsin via reductive alkylation with $NaBH_4$ improved enzyme thermostability and pH stability. On the other hand, in comparison with the native protease, the modified trypsin retained 42% of the proteolytic activity [45]. Relative movement of the catalytic domains is thought to be important for the entrance and exit of substrates and products [46], perhaps greater “stabilization” of enzyme chain can become harmful to enzyme performance.

Glutaraldehyde intramolecular crosslinking has been widely applied. In addition to being used to stabilize enzymes by introducing intermolecular crosslinking in protein chains, glutaraldehyde can also be used to modify enzymes after their immobilization, as a post-immobilization technique, or even be used to activate supports for posterior enzyme immobilization [42], [47], [48], [49], [50]. Glutaraldehyde crosslinking can increase protein stability and, moreover, avoid protease autolysis [51]. However others chemicals also can be used for this protective effect, as described by Rajput and Gupta [51], who obtained a superior effect for trypsin stabilization using dimethylsuberimidate. While the native enzyme lost 55% of its activity, the dimethylsuberimidate derivative lost only 25% of its activity owing to autolysis, and the glutaraldehyde derivative was only marginally better than the native enzyme. However, the glutaraldehyde derivative retained 90% of activity when compared to the native enzyme, while the dimethylsuberimidate derivative only 29%.

3.1.2. Immobilization of enzymes

Enzyme immobilization refers to a process that confines or localizes the enzyme in a certain defined region of space whilst retaining the catalytic activity, and which can be used repeatedly and continuously [52], [53]. The most common procedures include enzyme entrapment, protein cross-linking or covalent coupling on a solid support [54], which can be understood as a form of chemical enzyme modification [50].

To define the success of immobilization it is necessary to consider: the enzyme, the support (matrix) and the mode of attachment of the enzyme to the support [54]. The choice of immobilization procedures and support characteristics can be defined as reversible and irreversible methods. The use of irreversible methods can be very interesting for protease applications on food process. The loss of a catalyst (detachment of enzymatic chain) to the food environment can cause considerable problems, such as a hypoallergenic hydrolysate formula, for example. Methods based on the formation of covalent bonds, especially multipoint techniques (more than one covalent enzyme-support bond) can be a good choice. Multipoint enzyme immobilization can enhance the stability of enzymes via prevention of subunit dissociation and, moreover, the unfolding rate of protein chains, promoting "rigidification" of three-dimensional enzyme structures, which results in a higher resistance to the conformational changes induced by heat, organic solvents or pH [55], [56], [57], [58]. In addition, enzyme properties can be altered, including: improvement of stability, reduction of inhibition, possibility of reuse, reduction of enzyme chain aggregation and/or autolysis [55], [56], [59]. Pedroche et al. [57] demonstrated that there was no difference between the stability at 50 °C of the soluble trypsin, chymotrypsin and carboxypeptidase A and their one-point derivatives, but a significant increases in thermal stability was observed when multipoint covalent attachments were used.

Food samples may exhibit variable composition, providing different environmental pH or inhibitors concentration. Furthermore, being a good culture medium, mild temperature conditions of reactions can provide microbiological growth, making interesting use of higher temperatures of processing. In this case, the thermal stability of the immobilized enzymes will become even more attractive. Manrich et al. [60] observed that the trypsin immobilized on chitosan activated with glycidol can be 660-fold more stable than soluble enzyme and, approximately, threefold more stable than glyoxyl agarose derivative, at 70 °C.

A number of other studies have shown that immobilized proteases improving their stability to different environmental conditions. Ju et al. [61] demonstrated that chymotrypsin immobilized through covalent-bonding onto Fe_3O_4-CS nanoparticles exhibited high stability at different acid pH. Porcine pepsin immobilized on chitosan exhibited an improved resistance against thermal denaturation [62]. Additionally, Bacheva et al. [63] observed that the stability of immobilized subtilisin in an aqueous buffer containing 5–8 M urea, and in acetonitrile/60–90% dimetylsulfoxide mixtures, was substantially higher than that of the free enzyme.

Trypsin, followed by chymotrypsin, carboxypeptidase and alcalase are among the most cited in immobilization studies of proteases, specially due their wide range of applications, such as in production of food protein hydrolysates [64], [65], [66], [67], [68]. Different supports have been used and successfully applied to trypsin immobilization and stabilization, such as poly (vinyl amine) functionalized silica microparticles [69]; membrane of a cellulosic exopolysaccharide produced by Zoogloea sp. [70] and even modified spent grains [67].

But, it is not always possible to achieve 100% of recovered activity of the enzyme after the immobilization process. It is possible observe that there exists a very complex and inverse correlation between enzyme stability and activity. One explanation may be that for the enzyme to act, some chain flexibility at the active site to bind and act on substrate is required. The ideal design method provides improvements in enzyme chain rigidification, but in regions of the protein that do not affect local flexibility of the active site [39], [40], [71]. Another explanation concerning the structure of the support is that in protease immobilizations, the macromolecular nature of the substrate can be a problem, considering that the pore size of the support could became a diffusion barrier for the substrate, and enzyme activity will decrease by diffusional limitations [59]. Excessive enzymatic load on the support can also reduce the activity taking into account that a large enzymatic chain can work as a steric barrier against the substrate [72]. Trypsin immobilized on a chitosan-glutaraldehyde gels displayed 100% recovered activity for an enzymatic load of 8 mg protein/g gel, but, when 10, 12 and 14 mg protein/g gel were used, the recovered activities were 72%, 70% and 60%, respectively [60]. A proper orientation of the enzyme active center and the use of

supports containing long and flexible spacer arms, can be a important tool to keep the enzyme functionality against macromolecular substrates [73].

It is important to consider that enzyme immobilization is not only synonym of enzyme stabilization. As noted by Yust et al. [74], in addition to stabilization, the use of immobilized enzymes implies that inactivation of enzyme is not needed at the end of the process, because the catalyst can be easily removed from the reaction medium. This is especially important in food protein limited hydrolysis, where the time needed to reach thermal inactivation may accelerate the reaction rate, making difficult the control of desired hydrolysis degree [74] or even cause protein/peptide aggregation. Furthermore, even though there are many difficulties working with proteases, the reuse possibilities can result in both practical and economic advantages [75], [76].

3.1.3. Ultrasound treatment

A number of studies demonstrate the inactivation of enzymes by ultrasound treatment [77]. However, has been demonstrated that the effects of ultrasound on enzyme activity include both activation and inactivation results [78], but the activation effect is reported by only a few reports. Ma et al. [78] observed that the activity of Alcalase was increased to the maximum when treated for 4min at an ultrasonic intensity of 80W, under which Alcalase activity was increased by 5.8% over the control. But excessive treatment can decrease enzyme activity. For example, when the treatment duration exceeded 10min, Alcalase activity was lower than the control. The ultrasound effect was reported for its ability to break down molecular aggregates, leading to an increase in the number of tryptophan units on the enzyme surface, which changes its structure and causes the active center to become exposed and more accessible for the substrate. Ma et al. [78] also reported an increased number of a-helix by 5.2%, and a reduced number of random coils by 13.6% in treated alcalase.

3.1.4. Using proteases from thermophilic microorganisms

Thermostable enzymes have been isolated from thermophilic microorganisms, whose stability has attracted the interest of many investigators and a number of commercial applications have been developed as a result [79], [80], [81], [82]. Their application in processes development at high temperatures is the real advantage, considering that it has often been observed that at moderate temperatures the specific activity of a thermophilic enzyme is lower than mesophilic proteases. But, at high temperatures, close to that of optimum temperature growth of the source organism, the thermophilic proteases can acquire enhanced mobility to act, but remain rigid enough to resist denaturation, while the mesophilic enzymes can suffer unfolding [83]. For treatment of industrial wastes, such as hard-to-degrade animal proteins generated in the meat industry, proteases from thermophilic bacteria can be used at high temperatures (about 80°C), which must induce thermal denaturation of the proteins and result in greater proteolysis susceptibility [84]. Extracellular proteases from thermophilic bacteria that have been reported are serine- or metalo-proteases, while thermophilic fungi proteases are cysteine proteases. These observations are important considerations in the choice proteases for commercial application [79].

3.1.5. Tailoring enzymes

Evolution is natural tool to resolve the problem of new enzyme necessity. However, the laboratorial evolution of experimental tools, such as new technologies and computational programs, permit scientists to mimic evolutionary strategies in order to engineer new proteins that overcome enzyme limitations [39], [85]. Directed evolution has been used to improve enzyme suitability for a given application, mimicking natural evolution via two key steps: generation of genetic diversity (preparation of mutant libraries by random mutagenesis) and selection for function using aimed parameters [86], [87]. In rational protein design, mutants are projected for desired properties (usually via molecular modeling) and then generated by site-directed mutagenesis.

Yasukawa [88], generated a highly active and stable thermolysin (G8C/N60C/S65P/L144S) by combining the mutations so far revealed to be effective (Leu144 in the central α-helix located at the bottom of the active site cleft is replaced with Ser, and plus: Gly8, Asn60, and Ser65 in the N-terminal region are replaced with Cys, Cys, and Pro, respectively, to introduce a disulfide bridge between the positions 8 and 60). This mutant enzyme displayed a hydrolysis potential 10-fold higher than that of the wild-type enzyme, and its thermal inactivation at 80°C decreased by 50%.

Moreover, site-directed mutagenesis improves the catalytic properties enzyme catalytic properties, it can be used as a tool to orient protein chain immobilization [89], which can improve the activity, stability and even the selectivity of the immobilized enzyme. When Huang et al. [90] introduced a single cysteine residue into the cysteine-free subtilisin by site-directed mutagenesis, they observed that the enzyme molecules were immobilized through the side-chain sulfhydryl group of this cysteine residue and the oriented immobilized subtilisin demonstrated a higher catalytic efficiency compared to random immobilized subtilisin. There are also some examples using His in the target areas of the protein or the gene fusion of a target protein to incorporate the peptidic tag at the N- or C-terminus of the enzyme (poly-His tagged protein) permitting the immobilization of a protein via different orientations on the same support [89].

3.2. Managing substrate susceptibility

In order to increase the hydrolysis efficiency, besides making the correct choice or modification of the enzyme, it is also possible to increase the efficiency of the enzyme itself or increase the sensitivity of the enzyme to the substrate by denaturation. Although thermal treatment and destabilization of the protein enzyme chain can leave to lose protease activity, when the effect is occurs on the substrate chain it can be favorable.

Denaturation entails the unfolding of the chain and exposure of the binding sites. Changes in environmental conditions, such as temperature, pressure, pH, salt or solvent concentration can cause protein denaturation, but the destabilization of native proteins by high temperatures is a favorite tool for unfolding effect [91], [92], [93]. The known resistance of α-lactoalbumin to trypsin attack has been attributed to the presence of the covalent S—S bonds and rigid secondary structures which hide arginine and lysine and make them inaccessible [94], [95]. Thermal treatment can lead to the native state unfolding and make the protein chain more accessible [96]. Similar observations have been made for β-lactoglobulin, a dimeric protein which dissociates at high temperatures or pH values [97], [98]. This dimer-monomer transition influences its solubility and hydrolysis susceptibility and facilitates enzyme penetration. Trypsin hydrolysis of b-lactoglobulin can be temperature and pH-dependent, as demonstrated by Cheison et al. [98]. Less resistance was detected at >40°C and pH >7.5, indicating the influence of the environmental conditions on the dimer-monomer transition, which increases protein hydration and enzyme penetration and hence improves the hydrolysis process [98], [99].

Thermal treatment can change substrate accessibility for hydrolysis, improving proteolysis velocity, and it also can changes the hydrolysates profile. The proteins of chicken breast meat thermally treated showed different protein fractions after alcalase hydrolysis [100]. The chicken protein hydrolysates without thermal treatment showed fractions with a molecular weight of 10,000Da, however, after thermal treatment the size of the fractions decreased. When the effect of thermal treatment (at 50–90°C) on wheat gluten hydrolysis by papain was evaluated [101], the thermal treatment significantly increased the generation of fractions with molecular mass beyond 10,000Da of hydrolysates and greatly decreased the amounts of fractions with 10–5000Da and below 5000Da.

High-pressure and ultrasound have also been used to induce structural changes in protein chains, which can lead to substrate unfolding and improve enzyme accessibility [102]. The enthalpies for the protein denaturation are temperature- and pressure-dependent [103]. The combination of temperature and pressure treatment appears to

be more effective in causing structural changes, especially by increasing the susceptibility of disulphide bridge [93]. The effects of high-pressure treatment on Cheddar cheese ripening were investigated by O'Reilly et al. [104] indicating that pressurization increased the casein breakdown and the level of primary proteolysis in Cheddar cheese.

Ultrasound and high-pressure treatments can be applied to accelerate the diffusion of enzymes through solid matrix substrates, such as leather or meat [105], [106]. A significant loss of hardness is observed when beef is treated at 60–70°C and 200MPa, causing an acceleration of proteolysis under these conditions [107].

4. Food processes including enzymatic protein hydrolysis

Among the positive effects hoped for with food processing there are those aspects relating to the nutritional value and functional properties of foods. These include improved digestibility, modifications of sensory quality (such as texture or flavor) and health benefits, such as the improvement of antioxidant capability or reduction in allergenic compounds [108], [109]. Many of these processes can be achieved by protein hydrolysis using specific proteases. Proteolytic processes in the place of chemical processes are an attractive option as they preserve the chemical species present in the food samples. Protein hydrolysis via acid treatment directly onto the food matrix will almost certainly affect the other components and/or produce products that may be harmful.

The choice of enzymes and the hydrolysis degree must be realized taking into account taste, solubility and specific application properties of the hydrolysate product [110].

4.1. Changes in functional properties of food proteins

Functional properties of proteins connote the physicochemical properties which govern the behavior of protein in the food environment [111].

Protein hydrolysis is a powerful tool in the modification of the functional properties of proteins in food systems, including solubility, gelation, emulsifying and foaming characteristics. Proteolysis, besides decreasing the molecular weight, also increases the number of ionizable groups and can expose hydrophobic groups, which can change physical or chemical environmental interactions [112], [113], [114], [115], [116]. However, the hydrolysis conditions need to be controlled to avoid excessive protein hydrolysis that can impair functionality and cause unfavorable effects, such as production of bitter-flavored peptides [112].

Protein solubility depends on hydrophilicity and electrostatic repulsions. Apparently, the improvement of solubility is the most notable effect on protein functional properties after the hydrolysis process [108]. Increased solubility of the hydrolyzed protein is usually due to increase in the number of small peptides, and the corresponding increase in the ionizable amino and carboxyl groups. Zhao et al. [116] reported that enzymatic hydrolysis by Alcalase of an isolated peanut protein improved protein solubility and this presented a close relationship with the decrease of the surface hydrophobicity. A large number of articles in the literature present results that demonstrate the increase in protein solubility as a result of enzymatic protein hydrolysis [108], [117], [118], [119].

However, the hydrolysis processes needs to be controlled to improve solubility. Different enzymes can produce different sample responses to the hydrolysis treatment. Hydrolysates can expose hydrophobic peptides which increase peptide attractions/aggregation causing a solubility decrease [114], [120]. Paraman et al. [121] observed that highly hydrophobic and sulfhydryl disulfide interactions contributed to protein insolubility, even after a high degree of hydrolysis.

The emulsifying characteristic is an important property of food proteins which improves the utilization of dietary protein sources in food formulations [115]. Proteins can present amphiphilic properties, self-aggregate and form continuous and homogeneous membranes around oil droplets through intermolecular β-sheet interactions. So hydrophobicity, flexibility and amino acid composition, are involved in protein emulsification properties [108]. Emulsion activity and stability increased with increasing protein solubility and hydrophobicity. But, the highest emulsification capacity is obtained with a low degree of hydrolysis of the protein and increase in the availability of large peptide units at the oil–water interface, causing greater emulsion formation [108], [122]. Results obtained by Turgeon et al. [94] demonstrated that the hydrolysis of β-lactoglobulin by trypsin caused a decrease in the emulsifying power of the protein, presumably because the small peptides formed lost the capacity to interact with both aqueous and nonaqueous phases.

Protein gelation requires the unfolding of the native protein structure, followed by an association between protein strands. As a result gelation requires a solution of proteins of substantial chain size as the starting material. Usually proteolysis results in impaired gelling properties due to the small size of the products [113], [123]. But, low degrees of hydrolysis can collaborate to improve gelling properties. When Damrongsakkul et al. [124] hydrolyzed rawhide with papain and neutrase, the papain product (with longer peptide fragments) was a gelatin with gel strength and viscosity, while neutrase hydrolysis produced a collagen hydrolysate with very low viscosity.

Hrčková et al. [125] showed that the enzymatic treatment of defatted soy flour with three different proteases (Flavourzyme 1000L, Novozym FM 2.0L and Alcalase 2.4L FG) improved the foaming and gelling properties. In spite of the fact that the degrees of hydrolysis are not so different for the three enzymes, these enzymes have different specificity and produced different hydrolysates, especially when Flavourzyme was used. Flavourzyme (*Aspergillus oryzae*) is a mix of enzymes containing both endo and exopeptidases, whereas Alcalase and Novozym proteases are serine endoproteases, which are similar enzymes. Better gelation properties were reported when the protein hydrolysis was conducted over a short period. The released peptides, for example, presented better gel forming capacity than the intact protein during the first 60 min of treatment with Alcalase. However, after 60 min of hydrolysis, the hydrolysates did not form any gel.

Yust et al. [74] reported the use of immobilized alcalase to improve some functional properties of chickpea proteins by hydrolysis. All hydrolysates had better functional properties, such as solubility and foaming capacity, except emulsifying activity, than the original protein isolate.

4.2. Reduction of food protein allergy

Food allergy is defined as an adverse immunological response to food which can be mediated by IgE antibodies or not. Food allergens are generally proteins and as regards disorders mediated by IgE, the portion of the protein recognized by IgE is called the epitope. The epitope can be “linear” or “sequential”, depending on the primary structure of the protein, or the conformational, three-dimensional, form. These conformational epitopes are more easily disrupted by food preparation processes, such as thermal treatment. But, to manage linear epitopes it is necessary to reach specific amino acids sequences in the protein chain and, in these cases, cleavage of peptide bonds is one solution [126], [127], [128]. Formulas with extensive degrees of hydrolysis (peptides under 5000 Da) are termed as “semi-elemental” and partially hydrolyzed formulas (peptides between 8000 and 20,000 Da) termed hypo-allergenic [2], [129], [130].

Careful choice of enzyme and the methodology employed can drastically reduce protein allergenicity, which depends on the degree of hydrolysis and filtration techniques used posteriorly to remove residual proteins. The ideal process involves the minimum number of downstream steps [35], but ultrafiltration of the hydrolysates appears to be necessary to obtain a safe hypo-allergenic product.

Many proteins need high degrees of hydrolysis to alter their allergenicity. Hence, it is not just any protease that can accomplish this effect. Moreover, a low degree of hydrolysis, such as superficial hydrolysis, can aggravate the reactivity by exposing epitopes present in the interior of the protein chains. Cabanillas et al. [131] studied roasted peanut protein which was hydrolyzed by Alcalase and Flavourzyme. When Flavourzyme was used, a 65% decrease in IgE reactivity was noted after 300 min of hydrolysis, but at 30 min the treatment with Flavourzyme caused an increase in IgE reactivity, as detected by ELISA, whereas for Alcalase treatment a 100% reduction in IgE reactivity was observed [131]. Trypsin and pepsin were used to change other legume proteins [132], and the response was observed to be appreciably different between sweet lupin, chickpea, and lentil proteins. Chickpea and lentil lost their immunogenic capacity after a few minutes of enzyme treatment, but the antigenic activity of hydrolysates from sweet lupin major globulin was progressively reduced. The antigenic epitopes present in the hydrolysate were completely destroyed by trypsin hydrolysis after 30 min, differently from pepsin which still presented about 23% of the antigenicity.

For whey proteins, the minimal molecular mass to elicit immunogenicity and allergenicity appears to be between 3000 and 5000 Da, so the molecular weight cut-off value of the filters required must be in this range [133].

In many instances, a large range of allergen peptides can be listed. Soy protein presents at least 16 IgE-binding soy proteins with molecular masses from 7.5 to 97 kDa, which may be involved in clinical allergy. As a result, a considerable quantity of research is directed at controlling soy allergy and assessing enzymatic methods to decrease soy allergenic reactivity [134].

Although the importance of maternal milk is unquestionable, when its consumption is impossible, cow milk is usually used. However, in young children cow milk proteins, together with eggs, peanuts, soy, and wheat account for the most important food allergens [135] that can manifest reactions against the three major proteins found in milk: α-lactalbumin, β-lactoglobulin, and caseins [136]. Although cow milk protein hydrolysates are reasonable alternatives, it is not always easy to obtain an allergen-free formula. In these cases extensively hydrolyzed formulas are used, and for this purpose a mix or sequences of enzymes are used. Boza et al. [137] studied hydrolysates of casein and whey proteins, using a protease mix containing *Bacillus Licheniformis* enzymes, obtaining hydrolysates with peptides smaller than 8000 Da for whey protein and 2500 Da for the casein fraction. These hydrolysates present high nutritional values and the same amino acids as the native proteins, indicating that enzymatic hydrolysis preserves the original amino acids. Moreover, the potential antigenicity of the whey protein, measured in vitro by ELISA, was reduced 10^3 times for the whey protein hydrolysate and 10^4 for the casein fraction.

4.3. Taste preservation

Despite hydrolysate formulas produced by extreme hydrolysis being appropriate for many purposes, such as hypoallergenic formulas or high solubility proteins, when planning to use these hydrolysates for human consumption, an important factor to consider is the formation of taste. Mammals can perceive bitter taste and this can be a reason for product rejection due to aversion being caused in humans and animals. This may be an adaptive reaction for the avoidance of foods that are potentially poisonous, which usually have a bitter taste [138]. Bitter peptides can be produced during protein hydrolysis, as their properties vary depending on the type of protein and enzyme used. Studies indicate a correlation exists between bitter peptides and chain length, hydrophobicity and the amino acid present in N- and C-terminus [139], [140]. Hence, the correct enzyme choice can reduce disagreeable taste formation during protein hydrolysis.

Kodera et al. [141] obtained protein hydrolysates using D3 protease (a purified enzyme from germined soybean cotyledons) significantly less bitter than subtilisin, pepsin, trypsin, and thermolysin hydrolysates. Due to D3

substrate specificities, most hydrophobic amino acid residues in the hydrolysate are presumed not to be located at the peptide terminus, as can occur if pepsin was used.

Arai et al. [142] found that each of the several bitter peptides isolated from peptic hydrolysates of soy protein had a terminal leucine residue, but, when carboxypeptidase was used, which degrades the C-terminal structures, the bitter flavor was markedly decreased.

Wroblewska et al. [143] studied the influence of different enzymes and doses on changes in the hydrolysate's immunoreactivity, using alcalase, papain and pepsin. Differences in sensory acceptability of protein hydrolysates according their peptide composition were observed. Alcalase and then papain hydrolysis demonstrated the lowest intensity of bitter taste and the highest degree of general acceptance.

But, at the same time, bitter peptides can be desirable, as for example in cheese production. During the manufacture and ripening of cheeses, gradual proteolysis is generally considered to be a prerequisite for the development of the correct flavor of cheeses, such as Camembert or Brie. Exogenous proteases can be carefully added to accelerate ripening changes [144], [145].

4.4. Cheesemaking

In fact, proteolysis events are present amongst the principal biochemical modifications in cheese production. Taking in to account the action of proteases, including coagulation, as well as important transformations of flavor and texture, these may be significantly altered according the hydrolysis ratio during the ripening process [146], [147]. As discussed herein, small peptides generated by proteolysis can be more soluble and greater taste than intact casein or even whey proteins [148]. The liberation of amino acids, which can act as precursors for catabolic reactions, is thought to be the principal process of cheese flavor development [149].

Generally, cheese is produced via enzymatic coagulation, traditionally using calf rennet as coagulant, a two protease extract (chymosin and pepsin extract), which has a double role in cheesemaking. Chymosin is the major component and its principal role is specifically to hydrolyze the Phe105-Met106 bond of the k-casein, a micelle-stabiliser, leading to the milk protein coagulation [150]. But, rennet also contributes to proteolysis occurring during cheese ripening. Most of the coagulant activity added to the milk is lost in the whey, but, some rennet remains in the curd [150]. Although chymosin is the principal component due its specificity, during cheese ripening pepsin hydrolyzes peptide bonds of aromatic amino acids and contributes to proteolytic changes. When employing rennet from different sources, such as Lamb or kid, for example, the ratio of chymosin to pepsin in rennet is of major importance [151].

Several proteases have been investigated as likely rennet substitutes (animal, microbial or plant enzymes). Different enzymes can be used to intentionally produce new flavors or textures and are also interesting for the manufacture of “ovo-lacto-vegetarian” cheese [150], [152]. A study of the effect of aqueous extracts of flowers of *Cynara cardunculus* and animal rennet on cheese proteolysis showed marked differences between the cheeses coagulated with the two types of enzymes [153]. Casein hydrolysis was more extensive and faster and the levels of insoluble Tyr and Trp were higher using vegetable enzyme than traditional animal rennet. Pessela et al. [73] reported the casein hydrolysis in milk by rennet from *Mucor miehei*, covalently immobilized via its sugar chains previously oxidized with periodate, which act as a natural spacer arm. To prevent the coagulation of the milk in the presence of the derivative, the hydrolysis was performed at 4°C, avoiding casein precipitation, and then the hydrolyzed milk was filtered and heated to 30°C, achieving a similar aggregate to soluble rennet.

For many cheeses proteolysis is not limited to the action of added enzymes, but also to enzymes from microorganisms present. Although it is not within the scope of this review to discuss hydrolysis processes realized

by microorganisms, these cases illustrate the differences in the effect of district enzymes on the same matrix (milk proteins). A classic example is the blue-veined cheese category, where the presence of blue molds gives a characteristic appearance, and the enzymes of these molds produce typical aroma and taste. This is true for the mold surface in cheeses such as brie and Camembert [150], [154]. Proteolysis can be extremely complex. For Camembert at least five proteases are active during ripening: chymosin and bovine pepsin (in rennet), plasmin, aspartylprotease and metalloproteinases from Penicillium caseicolum, *each having a complementary and sequential role* [155].

When microorganisms are used for hydrolysis it is unlikely that only one enzyme acts. Thus, hydrolysis will be performed by a mixture of enzymes and, after liberation of the first peptides, these will be susceptible to the action of carboxypeptidases or aminopeptidases. Depending on the specificity of the enzyme that released the first peptides, distinct amino acids will be present at the extremities of the fragments and, as a result, distinct amino acid residues will be liberated over time by the exopeptidases. This will result in a wide variety of hydrolyzed species [154], [156], [157], [158], [159]. The use of specific enzymes can be interesting to produce specific changes and controlled action.

4.5. Liberation of bioactive peptides

4.5.1. Liberation of bioactive peptides

As defined by Biesalski et al. [160] “Bioactive compounds are essential and nonessential compounds (e.g., polyphenols) that occur in nature, are part of the food chain, and can be shown to have an effect on human health”. These natural constituents in food can provide health benefits beyond the basic nutritional effects, such as a “extranutritional” constituents [161]. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions and may influence health [162]. Numerous different peptides appear to have beneficial health effects on cardiovascular, digestive, immune and nervous systems, depending on their amino acid composition and sequence [163], [164].

Basically, the most common route to produce bioactive peptides is through protein hydrolysis, by digestive processes or microbial fermentation, but in vitro enzymatic processes have been widely studied [64], [164], [165]. Pepsin, chymotrypsin and especially trypsin have been the most employed enzymes and can simulate the gastrointestinal digestion effect on food peptides releases. But other enzymes, such as Alcalase or Thermolysin have been studied for peptide production. Although peptides can be liberated naturally during the digestive process, the liberation of fragments (different to those liberated by digestion) generated by different enzymes can produce peptides with a wide range of actions [164]. Bioactive peptides released via digestion or by other enzymes have been extensively studied in function of their *nutraceutical* potential, which presumes a bioactive agent from a food that can be used for the purpose of enhancing health. Such a food can be used in dosages that exceed those that could be obtained from conventional foods [166].

The bioactive mechanisms of such peptides are not entirely understood, and few studies discuss the structure–activity relationship. But some studies indicate which protease can be chosen to produce the desired fragment according to the effect required.

The Angiotensin-converting enzyme (ACE)-inhibitory peptides are usually small fragments, containing from 2 to 12 amino acids. Studies indicate that tripeptide residues play an important role in competitive binding to the active site of ACE and moreover, the most effective ACE-inhibitory peptides contain hydrophobic (aromatic or branched side chains) amino acids at C-terminal positions or are positively charged by Lys (ε-amino group) and Arg (guanidine group) as the C-terminal residue [164], [167]. This can help to explain why pepsin (preferential cleavage

between hydrophobic residues) or trypsin (preferencial cleavage in Arg- and Lys-) hydrolysis can be successfully used in antihypertensive peptide production [168]. In the whey protein, Ala–Leu–Pro–Met–His–Ile–Arg peptide, a β-lactoglobulin-derived, exercises a potent ACE-inhibition, and it can be released by a simple trypsin action [169]. Supplementary studies indicate ACE-inhibitory peptides with a proline residue at the carboxyl terminal, but proline is known to be resistant to digestive proteases [165], [170], so bacterial and fungal proteases can be used. Moreover, some sequences of the peptides obtained by *Lactobacilus* enzymes from β-casein, such as Ser–Lys–Val–Tyr–Pro–Phe–Pro–Gly–Pro–Ile or Ser–Lys–Val–Tyr–Pro, are markedly stable to digestive enzymes, as well as acidic and alkaline pH [171], which could be interesting for nutraceutical proposes, when the administrated peptides should be absorbed by intestinal mucosal without any additional cleavage.

Klompong et al. [172], reported that the antioxidative activity of protein hydrolysates from yellow stripe trevally fish species (*Selaroides leptolepis*) depends on the degree of hydrolysis, but also on the enzyme employed. At low degrees of hydrolysis (5%), Alcalase hydrolysate exhibited a better DPPH radical-scavenging activity while, at high degrees of hydrolysis (25%), Flavourzyme was more efficient. The antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities of peanut protein also were dependent on the degree of hydrolysis using Alcalase [173]. Peptide antioxidant capability can be observed to be associated with the capacity to inhibit deleterious changes during lipid oxidation and also to chelation of pro-oxidant metal ions and depends on the presence of certain amino acid residues in the peptides, such as tyrosine, histidine, methionine, and tryptophan. As a result antioxidant properties of the hydrolysates clearly depend on the enzymes employed during hydrolysis [174].

Pedroche et al. [65] observed a reduction of cholesterol, antioxidant capacity and inhibition of angiotensin-converting enzyme activity by peptides between 1800 and 1400 Da, obtained by sequential hydrolysis with immobilized trypsin, chymotrypsin, and carboxypeptidase A of the *Brassica carinata* protein.

Milk proteins are well-documented as “sources” of bioactive peptides [175]. In addition to their potential to form antihypertensive and antioxidant peptides, antithrombotic, opioid or immunomodulating peptides have been reported [176]. A “typical” opioid peptide can be obtained when the N-terminal sequence Tyr–Gly–Gly–Phe is formed. This observation leads to the conclusion that chymotrypsin can be used in this case.

4.6. Cleaning process

Protein-based residues usually represent a significant problem during cleaning procedures, especially with regard to food industrial processes (such as meat or milk industry), where the equipment can come into direct contact with the product, and in these cases chemical process can become a risk [177]. The first detergent containing protease was marketed in 1960s, using subtilisins from *Bacillus licheniformis*, but their use in the detergent industry was first proposed in 1913 [178], [179].

Alkaline proteases are the most appropriate as detergent additives as hydrolytic enzymes act at highly alkaline pH [1] (for example, subtilisin) and represent the largest volume of enzymes sold for industrial use [178], [180]. Since 1991, more than 100 new subtilases have been discovered [181] and studied for washing performance. Taking in account their resistance to chemical oxidants and washing performance at different temperatures, both important characteristics for protease use as a detergent compound [178], [179]. But subtilisins can be readily inactivated by chemical oxidants found in bleach-based detergent formulations, when the metionine catalytic residue is oxidized. Modified enzymes have been proposed, and the metionine residue is replaced with a nonoxidizable amino acid, which improves its resistance to chemical oxidants, but reduces catalytic power significantly due the change in the catalytic position [178]. M-protease from alkaliphilic *B. clausii* KSM-K16 exhibited the properties desired for a detergent additive, such as high solubility, stability against surfactants, activity and stability in alkaline pH, besides activity against various proteins, such as an unspecific enzyme [178]. Alcalase, a commercial proteolytic preparation

of different enzymes, including subtilisin, was efficient in removing casein, hemoglobin and bovine serum albumin from a model solid surface [177]. A protease from the *Bacillus* sp. Y was found to have an optimum pH of 10.0–12.5, high stability to surfactants, such as sodium dodecyl sulfate (SDS) and sodium linear alkyl benzene sulfonate [1]. In the case of use as a cleaning agent, a non-specific protease is more appropriate.

5. Conclusions

Proteases present enormous range of variants and specificity. The knowledge of the differences in specificity between proteases is very important to take in to consideration as a guide for the choice of protease according to the protein source to be hydrolyzed or predicted products. Food biotechnology processes, designed aimed at obtaining hydrolysates with specific and preserved peptides, can consider the proteases application as an important ally. Proteolysis is a powerful tool in the modification of the properties of proteins in food systems, including changes in solubility, gelation, emulsifying and foaming characteristics, reduction of protein allergy, taste transformation, or bioactive peptides liberation.

But, enzymes utilization meets limitations in some process, such as enzyme chain instability, low reaction rate or even low substrate susceptibility. Foods present a complex and variable environmental composition (presence/absence of metals, acids, inhibitors), which can considerably damage protease utilization. Moreover, processes at high temperatures can be advantageous, since this procedure can accelerate the reaction and avoids microorganism growth. Therefore, improvement of protease stability would provide a number of advantages.

Enzymes stabilization continues to be a wide area for research. Enzyme immobilization can enhance the stability of enzymes via prevention of subunit dissociation and, moreover, the unfolding rate of protein chains, promoting “rigidification” of three-dimensional enzyme structures, which results in a higher resistance to the conformational changes induced by heat or pH. In addition to stabilization, the use of immobilized enzymes implies that inactivation of enzyme is not needed at the end of the process, because the catalyst can be easily removed from the reaction medium, what is especially important in food protein limited hydrolysis.

The growing application of proteases is dependent on constant innovation studies, searching for new enzymes or proposing improved performances to the already known.

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