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Chapter 3

# Vegetable Proteins: Non-sensitizing Encapsulation Agents for Bioactive Compounds

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**Abstract**

Plant-derived proteins are remarkable macromolecules of scientific interest because they represent an alternative to the animal-derived proteins and petroleum-derived polymers. Many food proteins especially those derived from animal sources could act as antigens in humans. For instance, milk proteins extracted from cows may cause food intolerance during infancy. Further, soybean, peanuts, tree nuts, fish, crustacean shellfish and egg proteins may act as antigens in 90% of children. Since the GI tract is permeable to intact antigens the oral intake of these proteins may generate gastrointestinal (50–80%), cutaneous (20-40%) and respiratory symptoms (4–25%). Most of these allergens are water-soluble glycoproteins that are resistant to acids and enzymes. Usually, these proteins have a small molecular weight (10,000–60,000 kDa), water solubility, glycosylation residues, and a relative resistance to heat and digestion. Allergenicity is less frequent in vegetable proteins due to their less flexible and non-compact structure. Allergenicity is also related to the resistance to proteolysis, post-translational glycosylation, presence of epitopes, and enzymatic proteolysis. Moreover, proteins serve as a coating material if structural modifications in the protein, either by physical, chemical or enzymatic mechanisms are conducted. As a result, their allergenicity is reduced, and their functional properties are enhanced.

**Keywords:** encapsulation agents, allergenicity, proteins, bioactive compounds, protein derivatization

## 1. Introduction

A food allergy occurs once the body develops a specific and repetitive immune response to certain foods [1]. It can be divided into two major categories based on the mechanism involved: (i) the immunoglobulin E (IgE)-mediated, such as allergy to proteins from milk, egg, peanut

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etc. It is the most severe reaction, especially to food containing proteins, and (ii) the non-IgE-mediated allergy such as that of gluten or celiac disease, where the allergic symptom is triggered by ingestion of gluten from cereals namely wheat, rye and barley in their diets. In recent years, food allergies have caused a major health alarm affecting nearly 1% of adult population in the world and from 6 to 8% of children [2–4]. Thus, the prevalence of food allergies has increased in several regions all over the world. Further, more than 170 types of foods have been identified as potentially allergenic [5]. The animal-derived sources include eggs, milk, fish and crustacean shellfish; whereas the vegetable-derived sources include wheat, soy and nuts. The former group of proteins are responsible for causing more than 90% of allergic reactions [5, 6]. Most food allergens are stable molecules that resist the effect of food processing, cooking and the digestive process. These glycoproteins are characterized by their ability to induce a pathogenic IgE response in susceptible individuals [7]. Usually, food allergens are formed by divalent or multivalent molecules with two or more antibodies–binding sites calls epitopes, which are responsible for interacting with immune effector molecules such as the IgE antibodies [2–4]. Moreover, many food proteins especially those derived from animal sources could act as antigens in humans.

Currently, there is a worldwide search for new materials of natural origin that confers the physical, chemical and sensory characteristics to food products similar to those of synthetic additives applied on a daily basis. These synthetic compounds have been considered as potentially toxic in hypersensitive people, leading to health problems, causing allergies, hyperactivity, and cancer [8–11].

Proteins are macromolecules considered as emergents, these are versatile compounds having a good biocompatibility, biodegradability, high nutritional value, amphiphilic properties, and exhibit a strong interaction with several types of active compounds via hydrogen bonds, and electrostatic interactions [12]. Further, proteins are also able to function as emulsifiers, foaming and gelling agents [13–16]. Their chemical and structural versatility makes them suitable candidates for the delivery of bioactive hydrophobic and hydrophilic ingredients from a wide range of platforms such as particles, fibers, films and hydrogels [16].

One of the emerging and promising uses of proteins is in the microencapsulation tecnology of different compounds in the pharmaceutical, food and cosmetic fields. This technology is defined as a mechanical, chemical or physico-chemical process that isolates and protects the potentially sensitive active ingredients (i.e., liquid, solid or gas) from the damaging environment. In most cases, spherically-shaped products are obtained and the resulting particles could be classified according to their size as capsules (1–1000 μm), microcapsules (100–1000 nm) or nanocapsules (1–100 nm). In this process, the active ingredient is protected from the environment by a membrane, which in turn is named as the wall or coating material. This membrane controls the release and stability of the core material [17]. Nonetheless, to date, only a few proteins have been considered to be effective coating materials for the encapsulation of several core compounds such as vitamins, minerals, microorganisms, oils, phenolic compounds, among others. These proteins are mainly obtained from animal sources rather than plant sources. Proteins derived from milk, wheat, soy and cereals are the most widely studied for this aplication, but are considered as allergenic. For this reason, research has focused on the search for new sources of nonallergenic proteins that allows for the modification of their

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physical structures using chemical treatments such as the Maillard, hydrolysis, acylation or cationization reactions to improve the encapsulating capacity of the protein and in turn, decrease their allergenicity.

## 2. Allergenic proteins

Proteins are macromolecules derived from plants or animals that range in size from several thousand to several million Daltons [18, 19]. In general, proteins are composed of amino acids linked through peptide bonds resulting in chain lengths ranging from ~50 to >100,000 amino acids [19]. This sequence creates a characteristic three-dimensional organization (or folding) which in turn, can be organized into four structural levels.

The primary structure of proteins is formed by the linear sequence of bound amino acids. The secondary structure is due to the formation of hydrogen bonds between the carbonyl (-CO-) and amino (-NH) groups forming a folded structure. The latter describes the path that the polypeptide backbone of the protein follows in the three dimentions. The alpha and beta helices are the most important conformations of the secondary structure. Further, the tertiary structure describes the three-dimensional organization of all atoms in the polypeptide chain, including the side groups, as well as the polypeptide backbone. The quaternary structure of the protein is formed by the association between two or more proteins that exhibit a tertiary structure, resulting in a typical functionality [19, 20].

Amino acids are amphoteric in nature and thus, react with acids or bases due to the presence of alkaline ($NH_2$) and acidic (COOH) groups. Currently, there are twenty common amino acids having side chains of different size, shape, charge and chemical reactivity [19, 20]. The degree of hydrophobicity and hydrophilicity of amino acids is one of the main determinants of the three-dimensional structure of proteins. Amino acids such as glycine, alanine, valine, leucine, isoleucine, methionine and proline have non-polar aliphatic side chains; whereas phenylalanine and tryptophan have non-polar aromatic side groups. These hydrophobic amino acids are generally found within proteins, forming the so called hydrophobic core [19]. Other amino acids have ionizable side chains such as arginine, aspartic acid, glutamic acid, cysteine, histidine, lysine, and tyrosine; whereas asparagine, glutamine, serine, and threonine contain non-ionic polar groups, which are often found on the surface of the protein allowing for a strong interaction with the aqueous ambient [19].

Proteins are mainly obtained from: (i) oilseeds and protein crops such as soybeans, rapeseed, peas, lentils, broad beans and sunflowers; (ii) cereal sources such as wheat, oats, rye, barley, maize, rice, sorghum, millet and quinoa; and (iii) algae [21]. Vegetable proteins also possess several advantages such as a good biodegradability and biocompatibility, have a low cost and a high availability, and pose no health risks [22, 23].

Proteins can be classified according to their sedimentation coefficient (S), which is defined as the rate of sedimentation per unit of acceleration of the particle in a medium. This property varies according to the molecular weight, conformational space and behavior of the protein in the environment. However, there are four large families of plant proteins that are

classified according to their solubility into globulins, albumins, prolamins and glutelins. Albumin and globulins are the main constituents of plant-derived proteins [19].

Albumins are highly soluble in water and have a molecular weight ranging from 10 to 100 kDa. This group includes mostly proteins that present structural and storage functions [24]. In general, albumins contain high levels of lysine and sulfur amino acids, such as methionine and cysteine, and a high amount of disulfide bridges that favor the resistance against thermal denaturation [19, 20].

On the other hand, globulins are the main proteins obtained from vegetable sources. They are soluble in aqueous saline solutions and are mainly composed of two fractions having a sedimentation coefficient of 7S and 11S, respectively, which in turn depend on the plant source and culture conditions. Globulins are an important part of storage proteins, and have a molecular weight of more than 100 kDa. They are rich in arginine, aspartic acid, glutamic acid and their amides [19, 20].

Glutelins and prolamins are proteins soluble in basic aqueous solutions and hydroalcoholic mixtures, respectively. Their structure has been poorly studied, but these fractions represent agglomerates of globulins bound together by disulfide bonds and hydrophobic interactions, resulting in a complex morphology [21].

Currently, there are 16,712 recognized protein families in the Pfam database, but only 255 (~1.13%) of them are considered as allergens. Further, only 0.16% of the top 20 families account for ~80% of all reported cases of food allergenicity (**Table 1**) [25].

Further, it has been observed that the structural properties of proteins significantly affect their functionality, including the allergenic potential [25]. A survey conducted on common protein allergens reveals that they possess a wide range of physical characteristics, and none of them is unique to a class of protein allergens. Nevertheless, one report suggested that allergens tend to be ovoid in shape, although it is unclear why this should contribute

| Family | Source | Family | Source |
|---|---|---|---|
| Prolamin superfamily | Plant | Oleosins | Plant |
| Tropomyosin | Animal | Lipocalin | Animal: arthropod and mammalian |
| Cupin superfamily | Plant | Beta-1,3-glucanase | Plant |
| Profilin | Plant | Papain-like cysteine protease | Plant |
| EF-hand domain | Plant, animal | Thaumatin-like protein | Plant |
| PR-10 | Plant | Expansin, C-term | Plant: all grasses |
| Alpha/beta-caseins | Mammal | Trypsin-like serine proteases | Animal: arthropod and mammalian |
| Hevein-like domain | Plant | Enolase | Fungi and plants |
| Class I chitinases | Plant | Expansin, N-term | Plant: all grasses except 1 |

**Table 1.** Classification of allergenic proteins according to their family and source.

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to allergenicity [26]. Further, the fact of having repetitive motifs also contribute to allergenicity [26]. Many of the important allergens are exceptionally heat stable and retain their allergenicity after heating [26]. However, there are proteins not considered as allergens that possess any of the properties described previously [26].

Particular claims have been made regarding the contribution of intramolecular disulfide bonds to the allergenicity of proteins. Thus, proteins from house dust mites could induce allergenicity if disulfide bonding through site-directed mutagenesis occurs. Otherwise, allergenicity prevents making proteins unable to bind IgE. Further, the loss of disulfide bonds may cause allergens to lose their immunologic identities (i.e., the ability to bind pre-existing specific IgE antibody) and hence, the ability to initiate the novo IgE production [26]. Further, the induction of IgE antibody by wheat agglutinin could be lessened by reducing disulfide bonds with thioredoxin [26]. However, the ability of disulfide bonds to have a qualitative contribution to the allergenicity of wheat agglutinin is still unknown. A quantitative reduction of the antigenicity of a protein could result in a reduction of the allergic response to a protein without altering its intrinsic allergenicity, only if the vigor of the immune response is reduced. In order to determine whether the intrinsic allergenicity of a protein has been altered, it is necessary to assess independently its allergenicity and its antigenicity. This may be achieved experimentally by simultaneously measuring both the IgE (allergenic) and IgG (antigenic) response upon exposure. Further, it seems likely that disulfide bonds influence allergenicity, in unpredictable ways. Their presence can profoundly affect the processing and stability of antigens, and hence the release or destruction of T-cell epitopes [26]. The presence of multiple intramolecular disulfide bonds *per se* does not make a protein as an allergen, nor does their absence preclude allergenicity. For instance, ovalbumin is considered as an allergen despite of having only a single intramolecular disulfide bond that contributes little to its stability, whereas bovine serum albumin (BSA) having 17 intramolecular disulfide bonds is much less allergenic [26].

### 2.1. Allergenic animal proteins

Animal food allergens are classified into three general families such as tropomyosins, EF-hand proteins, and caseins [27]. However, regardless of their family the ability to act as an allergen appears to be related to relative identity with human homologues. Thus, if the protein has a sequence identity from 54 to 63% with respect to human homologues, then it is considered as allergenic. Nevertheless, a higher identity does not implies allergenicity [27, 28].

The family of tropomyosins is divided into four types of muscle proteins, where none of them causes an IgE response in humans [25]. In fact, no human IgE response to tropomyosin from birds or fish has been identified. This is expected since these sequences have an identity with human tropomyosins greater than 63%, which is translated in the absence of allergenic activity [27].

EF proteins form the second family of animal-derived food allergens, and they are composed of parvalbumin [27]. Parvalbumins are divided into $\alpha$- and $\beta$-parvalbumin. The $\alpha$-parvalbumin is considered to be non-allergenic, whereas $\beta$-parvalbumin is found in a variety of fish species, retaining the allergenic potential and is absent in human muscle [28].

Out of the caseins that have been shown to elicit an IgE response in humans, the rule of thumb is the closer the sequence to the human equivalent is, the less likely an IgE response will occur. Conversely, sensitization to BSA is the main predictive marker for the cross-reactivity to cow's milk that is present in 73–93% of patients with beef allergies, despite of the fact that BSA shares 76% identity to its human homolog [27, 29].

Cow's milk and egg allergies are some of the most common food allergies found in young children. It is estimated that ~3.8 and 2% of children younger than 5 years old have cow's milk and egg allergies, respectively. These allergens are commonly found in a variety of foods due to their technological and nutritional importance [6]. Thus, lactoglobulin is the major allergen in cow´s milk. Milk contains more than 20 protein fractions. In the curd, four caseins account for ~80% of milk proteins. The remaining 20% of the proteins, are globular proteins (e.g., lactalbumin, lactoglobulin, and bovine serum albumin), and are found in whey and egg proteins from both components (i.e., yolk and egg white) causing sensitization [30].

Parvalbumin represents the major clinical cross-reactive fish allergen. It contains heat-resistant linear epitopes that are s sensitizing by the interaction of metal-binding domains. In addition, other fish allergens are found in collagen and gelatin isolated from skin and muscle tissues. On the other hand, in shellfish, crustaceans and mollusks, tropomyosin is the major allergen that triggers allergic reactions [31].

### 2.2. Allergenic vegetable proteins

About 65% of plant food allergens belong to the group of prolamins, termites and the group of proteins related to pathogenesis (PR-10) [25]. The prolamin family include storage proteins, lipid transfer (LTPs), alpha-amylase/protein inhibitors and albumins from most cereal seeds. Their 3D structure consists of a compact structure with four alpha-helices stabilized by disulfide bridges and a central cavity used for lipid binding. However, similarity in their third-party structures does not indicate a similarity in their amino acid sequence between proteins in the same group. Further, prolamins from wheat are known to cause baker's asthma and celiac disease in humans. Alpha-amylase/trypsin inhibitors from various cereals, such as wheat, barley and rice, have also been involved in allergies. Some of the clinically reported allergenic plant proteins belongs to the family of prolamins such as Ara h 2, Ara h 6, Sin a 1, Ber e 1, Ses i 2 and Jug v 1. Non-specific LTPs are known to be the main food allergens in the fruits of the *Rosaceae* family. The presence of specific IgE in LTP is considered a significant risk factor for allergy and may serve as a diagnostic marker [25]. The most relevant termite-type allergens are the 7S and 11S globulins. 7S globulins include Arah1, Jugr2 and Sesi3, whereas the 11S globulins include the Arah3, soy glycinins, Bere2, Cora9 and Fage1 [32]. The third structure of the proteins belonging to the termite group consists of a series of antiparallel β leaves associated with an $\alpha$-helix forming a cavity [32]. This structure is also found in several lipocalin-like proteins involved in the transport of hydrophobic ligands, including milk β-lactoglobulin [32]. Further, plant prophylines share about 70% of the amino acid sequence homology [32–34].

All gliadin and glutenin protein fractions have been described as wheat grain allergens. The main properties of these proteins is to form a continuous viscoelastic network when flour is mixed with water to form a dough to be used in products such as bread, pastries, pasta,

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and crackers. However, wheat gluten exhibits a low solubility in aqueous solution. This fact limits the applications of wheat gluten in various types of food, since a good solubility is the main requirement for use in liquid foods and beverages. Furthermore, solubility is closely related to other functional properties of proteins such as the foaming, emulsifying, and gelling ability [35]. In soybean there are eight registered proteins which could trigger an allergic response. However, two of them named as β-conglycinin and glycinin represent ~70% of the soybean proteins. As a result, the incidence of soy protein allergy is much lower than other food allergens, such as milk or peanut proteins [36].

There are over 10 allergenic proteins identified in peanuts of which the Ara h 1, Ara h 2, and Ara h 3 are the most abundant peanut allergens and cumulatively represent approximately half of the total protein content [37]. Furthermore, the prevalence of allergy to tree nuts is estimated to be about half of that of peanut allergy. Tree nut allergic reactions tend to be severe and accidental exposures are common. Walnut, cashew, almond, pecan, Brazil nut, hazelnut, macadamia nut, pistachio, and pine nut are the most common tree nuts responsible for allergy cases in the USA and Europe. Moreover, from 20 to 50% of peanut allergic patients are also allergic to tree nuts [38].

## 3. Mechanism of action of allergenic proteins

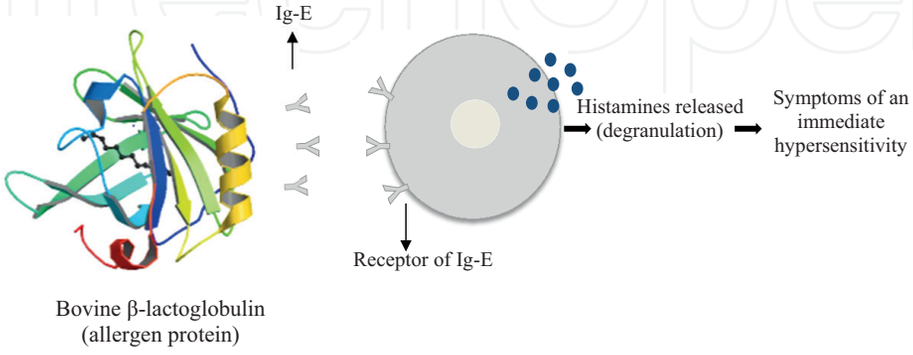
The prevalence of food allergy has been steadily increasing around the world. The relevant risk factors for food allergies are: (i) an increased use of antacids which is translated in a reduced digestion of allergens; (ii) a reduced consumption of omega-3 polyunsaturated fatty acids in the diet; (iii) a reduced consumption of antioxidants; (iv) genetics; (v) male gender; (vi) race, since it is increased among asian and black children as compared to white children; (vii) an increased hygiene; (viii) a northern climate; (ix) obesity, since this is an inflammatory state; (x) timing and route of exposure to foods (increased risk for delaying allergens with possible environmental sensitization); and (xi) vitamin D insufficiency [1, 39]. Most food allergens belong to only a limited number of proteins, and around 65% of plant food allergens belong to just four protein families, such as the prolamin, cupin, Bet v 1-like, and profiling, whilst animal food allergens can be classified into three main families named as tropomyosins, EF-hand proteins and caseins [40]. In general, children food allergies to milk, egg, wheat, and soy typically resolve during childhood, whereas allergies to peanut, tree nuts, fish, and shellfish are persistent. The prognosis also varies with disorder; for instance, food allergy related to eosinophilic esophagitis appears to have a relatively poor chance of resolution. For instance, the resolution rates are slow for allergies that have been commonly outgrown, such as those to milk, egg, wheat, and soy [39].

The human body has a series of physiological barriers for protection against foreign antigens. In the digestive system, these barriers are composed of two groups: (i) non-immunological such as the gastric acid, pancreatic enzymes, intestinal enzymes, mucus, the membrane of the microvilli, the mucosal layer and intestinal peristalsis), and (ii) immunological, such as IgA, IgE, IgM, IgG, lymphocytes, macrophages, Peyer's patches, intestinal secretory IgA and secretory IgA in breast milk [30]. Usually, when the immune system recognizes food proteins as a foreign body, immunoregulatory mechanisms are established that lead to the acquisition

of tolerance. Alterations in these regulatory mechanisms alter the induction of tolerance, resulting in food allergy [30]. An allergy reaction require a complex interaction between the protein and the immune system [26]. The National Institute of Allergy and Infectious Diseases of the United States, identified four categories of immune-mediated adverse food reactions such as IgE-mediated, non–IgE-mediated, mixed, and cell-mediated reactions. The most prevalent non-IgE-mediated reactions are eosinophilic esophagitis (EoE), the food-protein induced enterocolitis syndrome, proctocolitis, entheropathy and celiac disease [41]. The IgE-mediated reaction is by far, the most well established mechanism, where the antibodies bind to the high affinity receptors of mast cells and basophils, and to the low affinity receptors on macrophages, monocytes, lymphocytes and platelets. Thus, IgE are able to bind a specific receptor on the surface of mast cells and basophils, when two or more of these captive IgE molecules bind to their specific antigen, becoming cross-linked on the surface of the cell [26]. Once the allergens penetrate the mucosal barriers and bind to the IgE of mast cells and basophils, these cells release mediators that cause vasodilatation, smooth muscle contraction and mucosal secretion, giving rise to the typical symptoms of immediate hypersensitivity (**Annex 1**) [30].

In order to crosslinking to takes place, at least two antibody molecules must bind to the inducing allergen. An allergen must therefore contain at least two IgE binding sites, each one contains at least 15 amino acid residues. This implies a lower size limit for protein allergens of approximately 30 amino acid residues [26]. The IgE-mediated allergic immune response can be divided into three phases: (i) the sensitization phase in which B lymphocytes switch to the production of specific IgE, (ii) the effector phase consisting of an acute reaction and a facultative late-phase reaction; (iii) a chronic phase that may be the result of repetitive late phase reactions. The acute reaction causes activation of mast cells and basophils releasing histamine, leukotrienes, and other mediators known to be responsible for the wheal and flare reaction occurring in the skin and at various mucosal sites including the eye, nose, lung, and gastrointestinal tract [7]. The IgE-mediated reaction occur immediately or within 1–2 h of ingestion, whereas non-IgE–mediated reactions generally have a delayed onset beyond 2 h of ingestion [42].

A limited number of foods are responsible for the majority of reactions in IgE-mediated food allergy. For instance, allergy to cow milk, eggs, wheat, and soy are more common




**Annex 1.** IgE-mediated protein hypersensitivity.

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in infants and young children, whereas seafood, peanuts, and tree nuts are the most common causes of food allergy in adults [40]. The IgE-mediated reaction in skin includes hives and angioedema, whereas the gastrointestinal manifestations include mouth and lip pruritus, abdominal pain, vomiting, and diarrhea. On the other hand, a variety of respiratory tract symptoms that generally involve IgE-mediated responses, includes rhinorrhea and wheezing, whereas isolated asthma or rhinitis are unusual [42]. Further, the IgE-mediated food allergy may cause the dietary protein–induced syndromes such as enteropathy and enterocolitis. These in turn, cause profuse vomiting, diarrhea, dehydration and lethargy. Other syndromes include proctocolitis, gastroesophageal reflux, infantile colic, constipation and the Heiner syndrome [42].

## 4. Proteins used as coating materials: an emergent trend

There are many coating materials considered as "generally regarded as safe (GRAS)" which are used in the encapsulation process. Most of them are derived from natural sources such as natural gels (e.g., gum arabic, alginates, carrageenan and mesquite gum), modified starches, maltodextrin and proteins (e.g., whey proteins, gelatin, soy, rice, sunflower and peas) [13, 18, 43–49].

Animal-derived proteins are the most widely used coating materials, either alone or in complexes with polysaccharides applying techniques such as coacervation and spray drying. For instance, Chi and City [50] used whey protein to encapsulate rambutan oil by spray drying. They obtained an EE and yield of 69.9 and 28.5%, respectively using gelatin as a coating material; whereas whey proteins rendered an EE of 73.9% and yield of 58.6%. The best yield and EE was achieved with milk proteins instead of proteins obtained from tissues. Likewise, several researchers have evaluated the encapsulation activity of proteins such as gelatin [19, 45, 51], sodium caseinate in combination with lactose, or maltodextrin and other protein-carbohydrate complexes [19]. Remarkably, Rubio and his team [52] patented the process to obtain microcapsules and nanocapsules based on free whey proteins or complex polysaccharides employing "Blow-spinning," "blow-spraying," "electrospinning" and "electrospraying" as encapsulation techniques. In general, animal proteins tend to be more soluble, flexible and smaller in size at a broader pH range than proteins obtained from plant sources. For instance, casein proteins have a molecular mass of 20 kDa, whereas the molecular mass of soy proteins is 350 kDa. Further, animal proteins have a much faster diffusion kinetics at the interfaces and present a greater stability in emulsions, which is the key in many encapsulation processes [12, 53]. On the other hand, the large globular nature of vegetable proteins presents a greater challenge at stabilizing the interface in an emulsion and these proteins render a low EE. These emulsions are stabilized by increasing the viscosity of the continuous phase instead of acting on the interface, leading to a lower entrapment efficiency of the dispersing compound resulting in a higher susceptibility towards oxidative and degradation reactions. However, plant proteins exhibit several advantages that make them highly attractive in the pharmaceutical, cosmetic and food fields [12, 53]. For instance, plant proteins reflect the current "green" and "clean" label trends, and they are not considered allergenic as compared to the animal-derived proteins. In addition, the niche marketing restrict the use

of animal proteins in the diet and increase their cost promoting plant proteins as ideal coating materials from more abundant sources [12, 53].

In order to replace synthetic polymers and animal-derived products, there is a growing interest in the industrial use of renewable resources from natural origin having unexplored applications. Thus, natural macromolecules such as plant proteins have drawn considerable attention due to their availability, biodegradability, renewable character and various physicochemical properties that make them able to form films. The film forming ability of proteins is based on the unfolding characteristics of the protein structure in a solvent. This unfolding is favored by pH changes, addition of electrolytes, heat treatments, or solvent removal [13, 19, 20, 43, 54–56]. The solvents used to prepare the protein solutions are mostly water, ethanol, and rarely acetone. Proteins must be in an open or extended form in order to allow the molecular interactions for the formation of the film to take place. Further, these interactions depend on the protein structure (degree of extension) and the respective sequence of hydrophobic and hydrophilic amino acids in the protein. Therefore, vegetable proteins are very suitable materials for the encapsulation process of active ingredients in the food, pharmaceutical and cosmetic fields [12, 46, 53].

### 4.1. Encapsulation methods with proteins

Since the encapsulation methods are diverse and complex, they are classified according to the type of organic solvent, the energy expenditure, or application field. However, the most common classification method is related to the production process. In this case, it could be classified as physical, chemical or physicochemical processes [46, 57]. In general, the encapsulation process involves three steps: (i) the formation of a wall around the core material; (ii) complete closure of the wall, so any possible leakage of the core is avoided; (iii) generation of a capsule, either by chemical reactions or physical treatments [58]. The particle shape thus obtained depends on the physicochemical properties of the core, the coating material and the technique implemented (**Figure 1**) [21].

The morphology (shape and structure) of the microparticles is in turn, classified into two categories: capsules and spheres [18]. In the first case, the capsule is composed of a cluster of particles having a liquid or solid core surrounded by a continuous solid coating, which is generated only by chemical methods. In the second type, the sphere is formed mechanically, either by a process of atomization or milling process. Whereby, the active ingredients are finely dispersed as fine solid particles or liquid droplets within the matrix. A third category may rise comprising more complex structures, such as multilayer capsules or multilayer spheres. Further, both capsule and spherical morphologies should not have defects or pinholes to ensure a greater stability. Therefore, the presence of defects may increase the rate of oxidation or hydrolytic degradation (**Figure 1**) [18, 53]. Further, microparticles might exhibit from irregular to spherical shape. In fact, the coating material is generally adapted to the outline of the particle having a wide variety of shapes [21].

The most common encapsulation techniques include spray drying, extrusion, coacervation, liposome formation, fluid bed coating, inclusion complexes, ionic gelation, lyophilization, cocrystallization and emulsification. As mentioned previously, their morphology is classified into two major categories named as microspheres and microcapsules. Microspheres are typically

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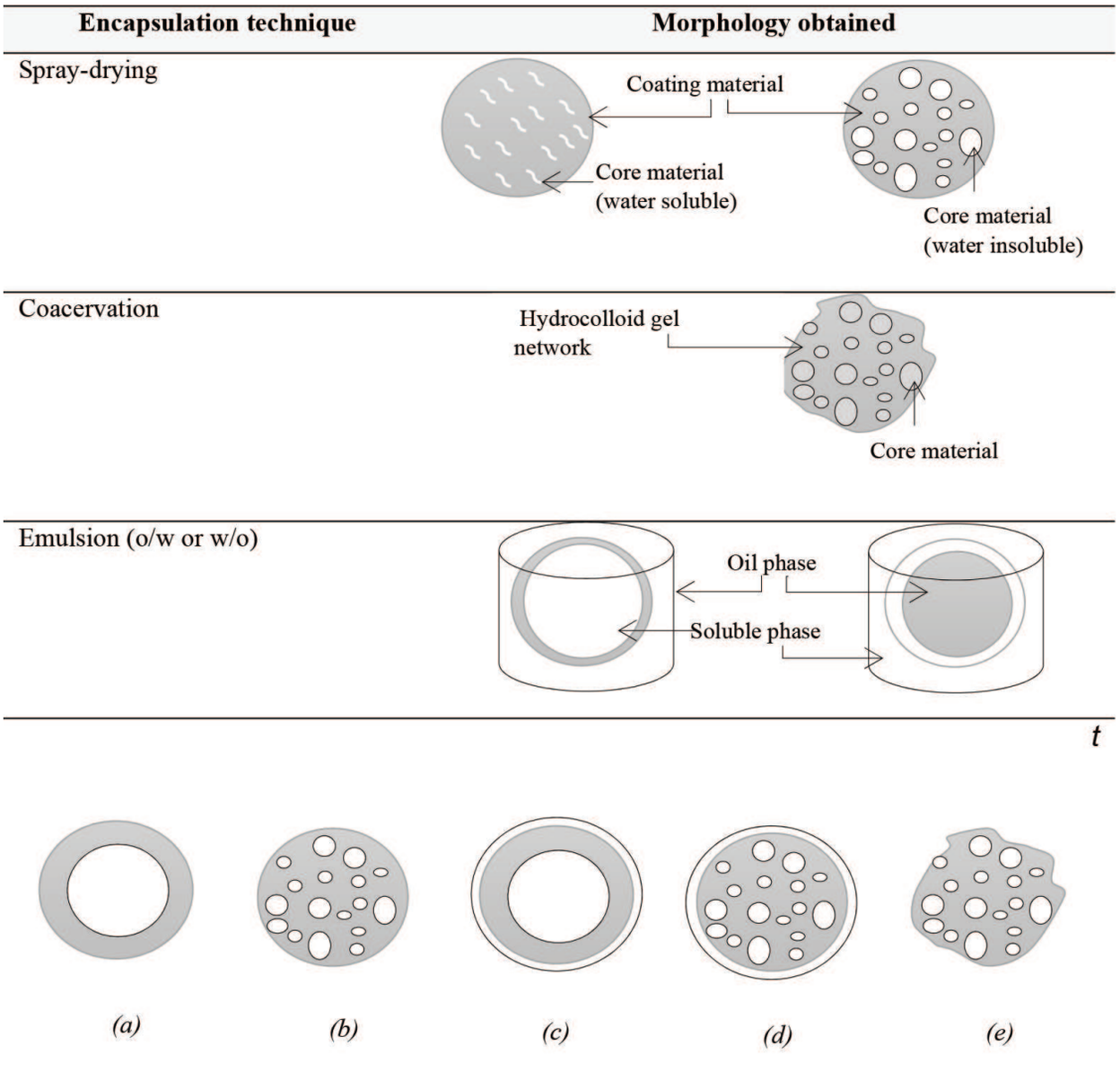



**Figure 1.** Morphology of the particles obtained by microencapsulation: (a) single microcapsule, (b) microsphere, (c) multilayer microcapsule, (d) multilayer microspheres, and (e) irregular microcapsule.

formed by a physical process such as spray drying, fluid bed coating, extrusion, and multiple emulsification [18, 59, 60]. On the other hand, the chemical process associated to the capsule formation include phase separation, ionic gelation, coacervation and liposome formation [18, 44, 57]. The different methods used for microencapsulation and the resulting main features are presented in **Table 2**.

The choice of the encapsulation technique for a particular process depends on: (i) the desirable size, biocompatibility and biodegradability of the particles; (ii) the physicochemical properties of the core and the coating materials; (iii) the intended use; (iv) the desired release mechanism from the core; and (v) the production costs. Some of the most commonly used encapsulation techniques which employ proteins as the coating material are described as follows:

| Encapsulation method | Particle size (μm) | Max. load (%) | Type of process | Reference |
|---|---|---|---|---|
| Simple coacervation | 20–200 | <60 | Chemical | [61] |
| Complex coacervation | 5–200 | 70–90 | Chemical | [61] |
| Co-current spray drying | 1–100 | <40 | Physical | [21] |
| Counter-current spray drying | 50–200 | 10–20 | Physical | [21] |

**Table 2.** Properties of the microcapsules produced by different techniques.

### *4.1.1. Emulsification*

The emulsification process involves the formation of colloidal systems formed by two immiscible liquids in which the dispersed phase is in the form of small droplets (i.e., between 0.1 and 10 μm) distributed in a continuous or dispersing phase. Emulsions are unstable if they are allowed to stand for some time. As a result, the molecules of the dispersed phase are redistributed forming a layer that can precipitate or migrate to the surface depending on the density gradient between the two phases [21]. In general, emulsions are classified according to the continuous phase when one liquid is dispersed into another. For instance, an O/W emulsion is formed when a hydrophobic liquid is dispersed in water or in a water-soluble liquid. On the contrary, a W/O emulsion is formed when a water-soluble liquid is dispersed in a hydrophobic solvent [57]. Moreover, the stabilization process of the emulsions is achieved by three mechanisms:

i. Formation of a strong emulsifier layer or film around the individual droplets of the suspended liquid;

ii. Formation of an electrostatically charged layer on the surface of the individual droplets;

iii. Viscosity increase of the dispersant medium. Thus, as the viscosity of the dispersant liquid increases, the Brownian motion slows down decreasing the probability of particles to agglomerate. As a result, the sedimentation rate of the particles or their possible flocculation rate is reduced.

One of the mostly widely used strategies to achieve the stabilization of emulsions is by the incorporation of surfactants in the system. These compounds have amphiphilic (i.e., hydrophilic and hydrophobic) regions in their structure and hence, they have the ability to reduce the interfacial tension between the phases of the emulsion system leading to a better stability [62]. Polysaccharides are the most commonly used emulsifiers and are mainly represented by native and denatured starches, phospholipids and proteins. Plant protein properties such as water solubility, amphiphilicity, the ability to self-associate and interact with a variety of substances, a high molecular weight and flexibility make them ideal for encapsulation by emulsification followed by techniques such as spray drying, coacervation, ionic gelation and solvent evaporation [21].

The absorption and emulsifying properties of the proteins at the interface of the colloidal system depend on many factors such as protein structure, state of aggregation, pH, and ionic

strength [63]. For instance, caseins, which have a random spiral conformation, tend to form an interweaved layer; whereas whey proteins that are globular in shape usually form aggregates at the interface of the colloidal system [64]. Further, bovine serum albumin, whey protein and proteins isolated from plant sources such as soybean, pumpkin seed, quinoa and peas have been used as emulsifiers in many encapsulation systems, including W/O and W1/O/W2 double emulsions [65–67]. Proteins could also be combined with other emulsifiers to stabilize emulsions. In fact, protein-polysaccharide complexes having electrostatic interactions between molecules of opposite charge at a certain pH and ionic strength range could stabilize emulsions. For instance, the sodium caseinate/soybean lecithin complex is an stable emulsion which is used to encapsulate phenolic compounds, simultaneously [68].

*4.1.2. Spray drying*

Microencapsulation by spray drying is the most widely known technique used to encapsulate food ingredients such as vitamins (C and E), fragrances, probiotic bacteria, lipids, vegetable oils, minerals (i.e., iron), anthocyanin pigments, milk, and foodstuffs [45, 47, 48, 60, 69]. The technique of microencapsulation by spray drying has been used since the 1950s and is currently applied at the industrial and academic level due to its rapid speed, economy and simplicity. It involves the dispersion of the active ingredient with the encapsulation material that is pumped into a spray chamber followed by dehydration with circulating hot air at a temperature between 150 and 200°C [45, 47, 48, 60, 69]. Spray drying provides a relatively high EE as compared to other methods. The highest EE achieved with spray drying is between 96.0 and 100.0%, which is much higher than those obtained with alternative methods [18]. Several parameters need to be controlled during the spray drying process. The inlet and outlet drying air temperatures, the feed flow of the product, the residence time and the characteristics of the raw material are the most important factors [18]. The initial mixture needs to be in a form of a dispersion, a solution, or an O/W emulsion having a low viscosity. The process is divided into different stages:

i. Atomization of the feed mixture;
ii. Interaction of the liquid phase with hot air;
iii. Evaporation of the droplets;
iv. Separation of the formed microcapsules.

The ideal materials for spray drying should have a low viscosity at high concentrations, a high solubility, have a good emulsifying and film forming ability, and hold efficient thermal properties (i.e., low effective diffusivity and low conductivity) to protect the encapsulated material during the drying process [21]. Thus, the drying process can be carried out following three different patterns: (i) co-current, if the solution is atomized in the same direction than the hot air flow (**Figure 2a**); (ii) countercurrent, if the sprayed microdroplets move in the opposite path than the flow of hot air (**Figure 2b**); and (iii) mixed, if the spray droplets move without a defined trajectory and at a high entropy. Thus, the air flow moves in opposite and/or concurrent directions (**Figure 2c**).

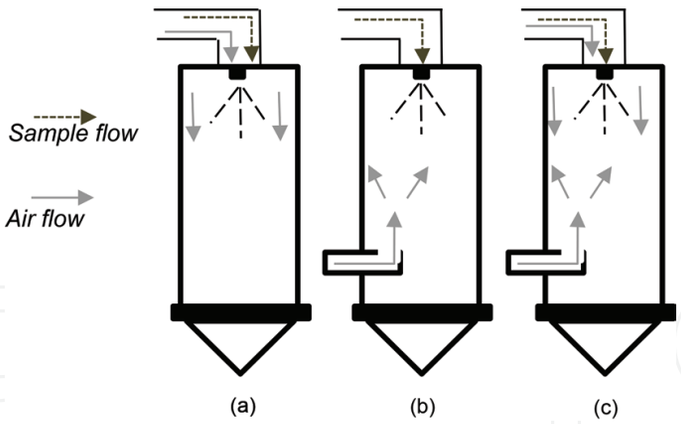



**Figure 2.** Spray-drying pattern types: (a) co-current, (b) counter-current, and (c) mixed.

The average particle size obtained by the atomization process varies between 1 and 100 μm for the co-current and between 50 and 200 μm for the counter-current drying pattern [21].

*4.1.3. Coacervation*

Coacervation is a chemical method of phase separation. The term "coacervation" was introduced in the colloid chemistry field by Bungenberg de Jong and Kruyt in 1929 to describe the spontaneous separation of the liquid/liquid phases that can occur when polyelectrolytes of opposing charges are mixed in an aqueous medium [17, 44, 70–73]. Coacervation is defined by the International Union of Pure and Applied Chemistry (IUPAC) as the separation of a colloidal system into two liquid phases. Thus, coacervation is an intermediate state between dissolution and precipitation and hence, it leads to a partial desolvation in opposition to the exhaustive solvation associated to the process of precipitation. Therefore, any factor that involves polymer desolvation causes the coacervation phenomenon [53]. The coacervation-triggering phenomena include temperature changes, pH modification and the addition of an incompatible anti-solvent, salt or another polymer [18, 53]. The coacervation procedure can be classified into two types: (i) simple or (ii) complex coacervation, depending on the number of polymers involved [18]. Complex coacervation mainly occurs by electrostatic interactions between two or more solutions of opposite-charged polymers producing two immiscible liquid phases: (i) one is the continuous phase having a low polymer concentration, whereas, the second one is composed of the polymer rich dense phase, also named as the coacervate phase, which in turn is used to coat a variety of active core ingredients. Usually, the coacervate complexes possess the combined functional properties of each polymer involved [21]. On the other hand, simple coacervation only implies one polymer, and thus, it is not very popular in the food and pharmaceutical fields [18, 43]. In general, the process of coacervation involves the following steps:

i. Dispersion: A vigorous stirring of the active ingredient (liquid or solid particles) is carried out in a solution of the polymer, or a mixture of polymers that will form the wall material;

ii. Induction: Coacervation is induced by one of the previously described phenomena. The system of the solution becomes transparent and under the microscope the coacervate droplets have an appearance similar to that of an emulsion;

iii. Deposition: The adsorption of the coacervate droplets around the core material takes place. The cloudy supernatant is clarified as the process of coacervation evolves;

iv. Coalescence: Microscopic droplets of the coacervate form a continuous layer around the core;

v. Hardening: The system is subjected to cooling and a crosslinking agent is added to render a stiff shell (optionally);

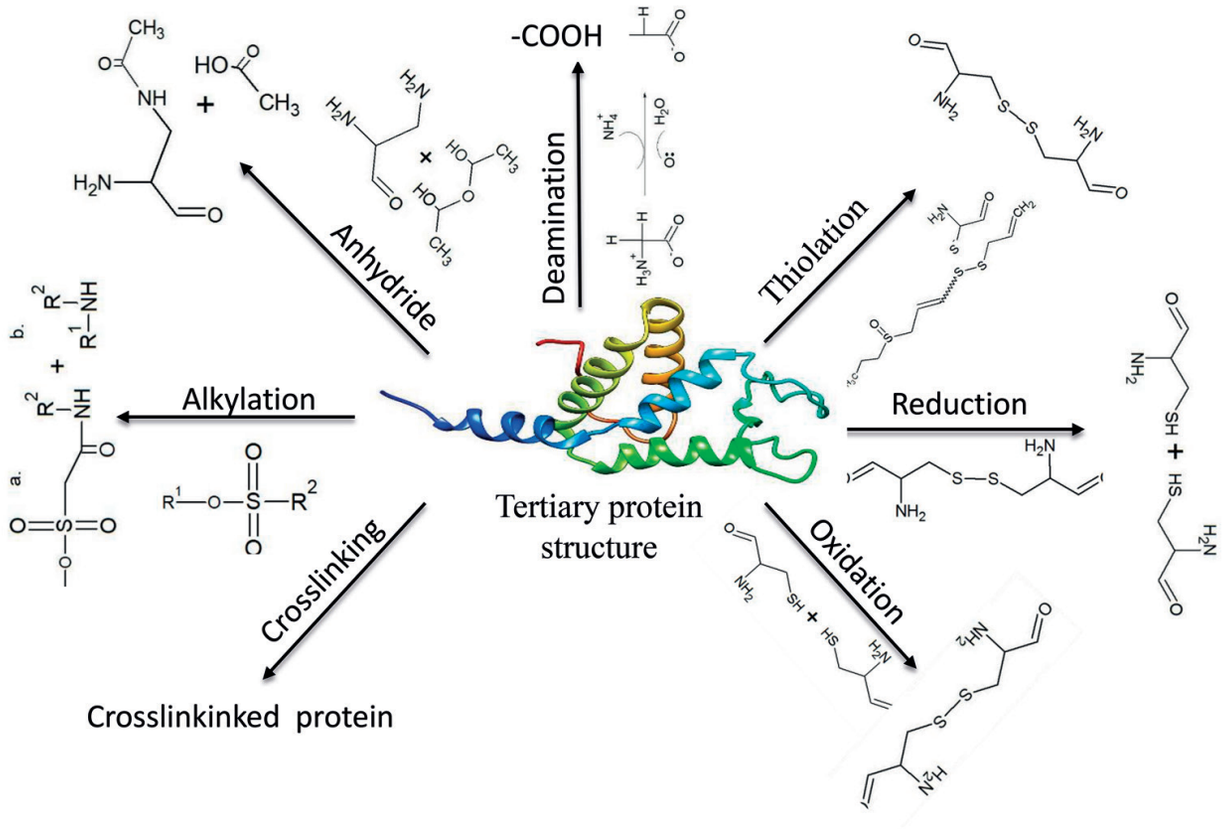
vi. Separation: Microcapsules are isolated by centrifugation or filtration. Thus, spherical microcapsules as small as 4 μm are obtained having a large loading capacity (i.e., 90.0%).

### 4.2. Structural changes in proteins: a strategy to enhance their functionality

In recent years, the demand for multifunctional products has increased, and researchers need to develop or modify techniques to improve the functionality of proteins since most of them show no variability in their desirable functional properties [12, 19, 21]. Even though proteins are versatile materials with interesting properties, it may be necessary to modify the inherent properties such as solubility, hydrophobicity, hydrophilicity, the gelling, emulsifying, foaming and allergenicity. As a result, a versatile material is obtained, with less allergenicity and a wide variety of applications in different fields [12, 19, 21]. Several modifications can be conducted in proteins since amino acids have side chains of different sizes, shapes, charges and chemical reactivity. The reactivity of a protein, in terms of its ability to be chemically modified will be largely determined by the composition and location of the amino acids on its three-dimensional structure. These modifications can be made by physical (i.e., pressure and temperature), chemical or enzymatic methods [19]. Some of the feasible modifications of proteins are illustrated in **Annex 2**.

The term "crosslinking" is commonly used to describe the intra or inter-covalent bonding of a protein. As a result, the molecular size and shape, and the functional properties may be affected by crosslinking. Crosslinking can also be used for the stabilization and subsequent modulation of the release properties of the protein-based controlled release systems. Different methods can be used for crosslinking purposes, ranging from physical to enzymatic and chemical modifications [46, 74]. Crosslinking can be controlled by the proper reaction mechanism, reactive groups of the protein, and type of crosslinking reagent. The number of reactive groups per protein chain, types of functional groups, and spacer length of the crosslinking agent determine the resulting crosslinking density. The most relevant physical modifications of proteins imply variations in temperature and pressure that lead to structural changes or their denaturation. The main disadvantage of the heat treatment as a crosslinking method is that the reaction is difficult to control. The denaturation process of proteins begins when the structure itself becomes an intermediate product. As the reaction progress, the structure is altered, but the secondary structure remains unchanged. Once the chains unfold completely, the denaturation process is concluded. The magnitude of these changes depends on the protein source and environmental conditions (e.g., pH, solvent, presence of salts, surfactants, etc.) [75]. The more reactive the reducing sugar is the stronger and darker the gel becomes [12, 43, 46, 56, 76].

The enzymatic modification of proteins is another useful method. Various enzymes have the ability to crosslink proteins. Examples thereof include transglutaminase (TG), disulphide




**Annex 2.** Schematic representation for the typical chemical modifications of proteins.

isomerase, peroxidase, lipoxygenase, and catechol oxidase. In a recent overview on enzymatic crosslinking, microbial TG, lactoperoxidase and glucose oxidase are highlighted as the enzymes available on a sufficient large scale for industrial applications [19]. TG is an acyltransferase that catalyzes the introduction of c-(y-glutamyl)-lysine crosslinks into proteins, making TG an interesting enzyme for food grade protein crosslinking. TG has been used to crosslink several proteins [19]. In general, caseins appear to be more susceptible to TG-induced crosslinking than whey-proteins, possibly due to the predominantly random structure of caseins, in contrast to the globular structure of whey proteins [19]. A less known type of crosslinking involves the peroxidase-catalyzed reaction between the side chains of two tyrosines, resulting in a C-C bond between the two carbons in an ortho position with respect to the phenol group [19]. This type of crosslinking is very strong due to the intermolecular formation of bonds. The use of co-substrates to enhance the crosslinking efficiency of peroxidases reduces the amount of enzyme needed and therefore increases the cost effectiveness. The co-substrates are phenolic compounds such as mono- and di-hydroxy benzene derivatives (e.g., catechol, ferulic acid, and *p*-hydroxybenzoic acid) and probably act as a spacer between the protein molecules [19].

On the other hand, the chemical modification of proteins has been the most widely studied method among scientists. These reactions are mainly represented by deamidation, acylation, chemical hydrolysis, and cationization reactions. There is a plethora of research conducted

on the use of chemical agents for protein crosslinking [19]. The most widely used reagents are bifunctional having two reactive groups that can be used to introduce inter- and/or intramolecular bonds into proteins [19]. These reagents in turn, can be classified as homobifunctional or heterobifuntional. Homobifunctional reagents (i.e., glutaraldehyde) have two identical functional groups, whereas heterobifunctional reagents have two different functional groups. It is difficult to control the reaction conditions (i.e., pH, ionic strength and protein:reagent ratio) of homobifunctional reagents to ensure an intra- or intermolecular crosslinking. On the other hand, heterobifunctional reagents can be used in a more discriminating way. In this case, crosslinking occur in separate sequential steps, and the formation of intermolecular crosslinks can be avoided or stimulated. Tannins are complex polyphenolic substances that can be derived from galls, but also from fruits (e.g., pomegranate) and tea. Tannic acid (TA) is capable of complexing or crosslink proteins by forming a multiple hydrogen bonds network [19]. Proteins can hereby be physically crosslinked and thus, become more resistant to enzymatic degradation. This type of crosslinking is partially reversible. Further, some metal ions can oxidize TA and proteins, whereas other metals could inhibit the formation of hydrogen bonds.

All of these reactions modify the secondary or tertiary structure of the proteins using different compounds that could form linear biopolymers, biopolymers with hydrophobic linkage aggregates outside the structure, cationic or anionic biopolymers, or biopolymers in which certain specific amino acids are exposed. All of these modifications allow for an effective interaction of protein derivatives at the interface of emulsions, or with the active compound in the core, resulting in an improved EE. On the other hand, a protein could loss its original conformation or bonding which is responsible for its allergenicity, e.g., formation of disulfide bonds, which are responsible for binding the IgE and hence, triggering allergenicity.

Currently, many researchers are searching for alternatives to improve the encapsulation properties of vegetable proteins isolated from legumes (e.g., soybeans, peas, chickpeas and lentils), sunflower seeds and cereals (e.g., oats, wheat, barley and corn) [19–21, 44, 71, 77]. The chemical and enzymatic hydrolyses, the acylation [21], cationization [21, 78] and Maillard reactions [12, 19, 77] are the best chemical treatments to improve their functionality [12, 43]. For instance, Nesterenko et al. [46] studied native and modified soybean and sunflower seed proteins to encapsulate $\alpha$-tocopherol by spray drying. Likewise, the EE increased from 82.6% for native soy protein to 94.8% for the modified product. Moreover, the EE of the native sunflower seed protein was 79.7%, whereas that of the acylated proteins was 99.5%. They concluded that the structural modification of the proteins increased the affinity between the active ingredient and the coating material, improving the encapsulation process, hydration and net protein load. Similar results were obtained by Chen Lia et al. 2015 [76], who encapsulated oleoresin obtained from tomato using soybean proteins conjugated with gum arabic as the coating material. These chemical modifications produced by Maillard reactions were conducted by heating at 60°C and at a relative humidity of 79% for 3, 6 and 9 days. They obtained EE of 69.25, 76.47, 80.91 and 84.69% for the native and modified proteins treated for 3, 6 and 9 days, respectively. The 3D change in the protein structure favors the stabilization of the emulsions, the encapsulation process and improves their biocompatibility.

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