Non-Thermal Processing on Proteins

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Proteins represent one of the major food components that contribute to a wide range of biophysical functions and dictate the nutritional, sensorial, and shelf-life of food products. Different non-thermal processing technologies (e.g., irradiation, ultrasound, cold plasma, pulsed electric field, and high-pressure treatments) can affect the structure of proteins, and thus their solubility as well as their functional properties.

Keywords: Irradiation ; ultrasound ; cold plasma ; pulsed electric fields ; high-pressure processing ; proteins and amino acids

1. Introduction

Food proteins may undergo several chemical modifications during their processing [1]. Numerous physical, chemical and microbiological changes may occur due to the diversity of food-processed products, and the knowledge of their detailed mechanisms still needs greater research efforts to maximize the quality and stability during production and storage. The main difficulty in elucidating these mechanisms is related to the complexity of the changes occurring and the limitations of current analytical methods that are faced dealing with intractable food matrices most of the time. Technological processes that are used for food preservation and production may affect the functional, nutritional, and biological properties of food proteins. While some changes may impart health-related benefits, such as the generation of biologically active peptides [2], others may affect amino acids and generate toxic derivatives, such as lysinoalanine, D-amino acids, and biogenic amines [3]. Several non-thermal technologies have been proposed in order to reduce the impact of conventional thermal processes on food matrices such as Maillard reactions, changes in color, flavor, physicochemical composition, and so on. Ultrasound, UV irradiation, cold plasma, high-pressure processing, and pulsed electric fields are among the most investigated/applied non-thermal food processing technologies, and some of them are already have been industrialized [4] [5]. Knowledge regarding their impact on proteins and amino acids is scarce and not well explained in the literature, despite the numerous benefits of these emerging technologies over conventional food processes, such as time and energy saving, reducing solvent quantity, which matches the “green” processing concept [6].

2. Proteins and Amino Acids

Proteins are macro biopolymers that play an important role in the cells of living organisms. The structure and components of the animal and vegetal cells (e.g., lipoproteins, enzymes, hormones, antibodies, globulins, etc.) have a protein base that plays a critical role in the viability and functioning of these cells. Animal and plant proteins provide 35% and 65% of the world’s protein requirement based on the report of Young and Pellet [7], respectively.

Amino acids are the structural units of proteins and peptides [8] and they contain carbon, hydrogen, nitrogen, and oxygen as the main atoms of their structure. Among all of the known amino acids, only 20 (standard amino acids) are proteinogenic, meaning that they have a triple codon in the DNA. There are two non-standard amino acids, selenocysteine, which is abundant in eukaryotes and non-eukaryotes, and pyrrolysine, which is found in bacteria and archea [9]. In comparison to carbohydrates and lipids, proteins are rich in nitrogen (≈15–25%). Each amino acid contains a carboxyl group (-COOH), an amine group ($-NH_3$), and a side chain (R group) bonded to a core carbon atom. The side chain of each amino acid may contain other atoms, including sulfur and phosphorus. Amino acids could be categorized into alpha (α), beta (β), gamma (γ), and delta (δ) based on the location of the functional groups on the core carbon. Polarity (polar and non-polar), pH (acidic and alkaline), and side chain (aliphatic, acyclic, aromatic, containing hydroxyl or sulfur, etc.) can also be used as classification tools.

Amino acids (both proteinogenic and non-proteinogenic) play an important role in biosynthesis and neurotransmitter transport. For instance, γ-amino butyric acid (GABA, non-standard amino acid) and glutamate (standard glutamic acid) are among the most important inhibitory and excitatory neurotransmitters in the human brain, respectively. On the other

hand, glycine (standard amino acid) is necessary in the formation of porphyrins, which are used in red blood cells, whereas proline is used to synthesize hydroxyproline, which is the main component in collagen [10].

Four different structures of proteins have been identified, including primary, secondary, tertiary, and quaternary structures. In the primary structure, two amino acids are bound to each other by a peptide bond, which is an amide bond between the carboxylic acid group (-COOH) of one amino acid and an amine group ($-NH_2$) of another amino acid. When the number of bonded amino acids in a chain is less than 50, it is called peptide, and when the number of residues is more than 50, it is called polypeptide or protein. The secondary structure of proteins is based on inter-strand or intra-strand hydrogen binding. The two main kinds of secondary structure are the α-helix and β-sheet. The former is a right-handed coiled strand in which hydrogen bonds are formed between the oxygen of C=O group of each peptide bond and the hydrogen bond of N-H group of another peptide bond that was located in the fourth amino acid below in the same chain. In the β-sheet structure, hydrogen bonds are formed between two different strands, which can be paralleled or anti-paralleled. The tertiary structure is the three-dimensional shape of a polypeptide/protein. Hydrogen, hydrophobic, di-sulfide bonds, and/or electrostatic interactions between the side chains of amino acids induce protein folding to reach the maximum stability. Hydrophobic groups are located inside the protein molecules, while the hydrophilic ones are exposed outside. The interaction (e.g., di-sulfide and salt bridges, hydrophobic interactions, and hydrogen bonds) between some polypeptide chains forms their quaternary structure as a complex aggregate [10].

Amino acid composition, sequence, and structure (primary, secondary, tertiary, and quaternary) determine the molecular weight, net charge, physicochemical, and functional properties of proteins. Some of these properties that are directly related to food quality include hydrophobicity, solubility, thermal stability, gel forming, emulsifying ability, swelling, water holding capacity (WHC), and association/dissociation behaviors that dictate aspects, such as color, flavor, and texture of foods [10].

Human cells cannot synthesize essential amino acids (phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine), and thus humans rely on diet to obtain them. Animal and vegetal sources of proteins have different nutritional values and protein qualities. Animal proteins include all of the essential amino acids and they have large similarity with human ones. However, good sources of high animal proteins, such as meat, have high cholesterol and fat contents, as well as high sodium content in some cases, which can limit the consumption of meat [11]. Overall, vegetal proteins have lower nutritional quality than the animal ones due to the limitation of the essential amino acids, with the exception of some, which have been found to be suitable substitutes for meat, also that there is a difference in the protein digestibility between the animal and vegetal proteins.

3. Food Processing

3.1. Conventional Thermal Processing

Food processing is usually a required set of steps applied to food before its consumption. The main reasons for food processing include: imparting a desirable modification in the food composition, maintaining the food quality, sustain the availability of products at various times and places (products provision in out of season), food diversity (creating diverse food products to different consumers), increasing shelf life, and preparation of ready-to-eat products [12][13]. Changes occurring during processing can be beneficial, such as the inactivation of microorganisms and destruction of toxins, increasing the bioavailability of some nutrients, development of desirable flavor and texture attributes, and extending shelf life, or it may be detrimental, such as the destructive effect of heat on nutritional value of food (e.g., loss of vitamins and bioactive compounds) and the formation of harmful components (e.g., acrylamide, trans fatty acids) [14], Moreover, a loss of amino acids can occur, depending on the severity of the treatment.

The most usual traditional processes in food industries include heating, cooking, baking, freezing, milling, canning, fermentation, drying, salting (pickling, curing, or brining), extrusion, and smoking [15]. Among these processes, thermal processing (e.g., cooking, roasting, grilling, frying, boiling, pasteurizing, and sterilizing) are considered to be the most efficient in destroying pathogens, but they also have the most drastic effect food on composition, characteristics, and properties [16]. For example, proteins could be denatured during heating, depending on the temperature and the protein in question, causing the loss of their quartnary and tertiary structures and forming unfolded random shapes. Additionally, thermal treatment (90 °C, 2 h) of proteins gives rise to the formation of isopeptides, lysinoalanine, and racemization [17], alter proteins' allergenicity and stretching of some amino acids along with their peptide bonds in the primary structure [18]. Proteins during heat processing are stimulated to interact with other components in the food system. Maillard reactions are one of the most important, which involve proteins and contribute greatly to the nutritional and sensory properties of foods. The reactions are initiated by interactions between reducing sugars and amino acids and they continue with a large

set of chain reactions. These reactions may affect the color, flavor, and aroma of the food product, cause the formation of toxic compounds (e.g., acrylamide, furans, and hydroxyl propyl furfural), and decreased the digestibility and nutritional value [19][20]. Figure 1 shows a scheme of the Maillard reaction [21].

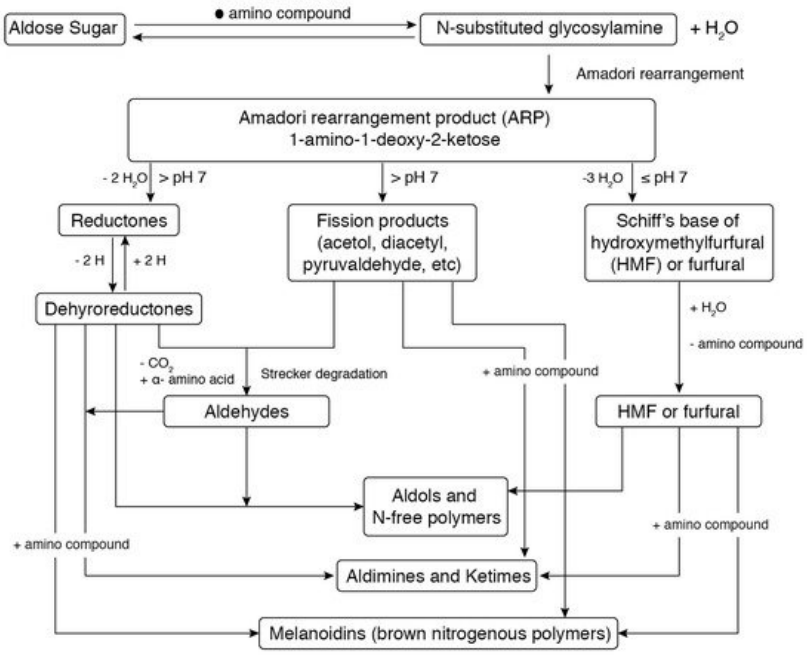

Figure 1. Scheme of Maillard reaction. Adapted from Hodge [21].

3.2. Emerging Non-Thermal Processing

Although thermal processing can contribute and assure the safety of foods, it can reduce the nutritional and sensory properties [6][22]. When considering the consumers' demand for high quality (high nutritional value, fresh taste, and desirable sensory properties, such as color and texture), strategies for minimally processed foods and alternative non-thermal technologies to conventional processing have been developed to produce microbiologically safe, fresh, and nutritious foods. These innovative food processing technologies include mainly ultrasound (US), UV irradiation, cold plasma (CP), high pressure processing (HPP), and pulsed electric fields (PEFs) [23][24]. Low energy and water requirements, as well as a higher efficiency and environmentally friendly nature, make these non-thermal techniques more preferred than the traditional thermal ones [25]. The effect of the non-thermal processing technologies on protein structures, functionalities, and allergenicity will be described in this review.

4. Impact of Non-Thermal Processing on Proteins

4.1. Surface Hydrophobicity

Table 1 shows a summary of the effects of non-thermal properties on proteins and amino acids. The number of hydrophobic groups on the surface of proteins determines their hydrophobicity [26]. Some functional properties of proteins (emulsifying, foaming and gel-forming), as well as their stability and conformation, are dependent on the hydrophobic interactions.

Table 1. Effect of non-thermal processing on proteins and amino acids.

Treatment	Substrate	Condition	Results	Reference
Ultrasound	Corn gluten meal	40 kHz, pulsed on- 10 s and off 3 s, 40 min and 20 °C.	- Molecular unfolding and exposure of hydrophobic groups - Decrease in α-helix and increase in random coil contents after heat/ultrasound and ultrasound/heat treatments	[27]
	Soy protein	20 kHz, power 65 W, 0.5, 1, 5 & 15 min	Protein extraction yield enhanced due to increasing in the solubility	[28]
	Beef proteins	2.39, 6.23, 11.32 and 20.96 Wcm^{-2}, 30, 60, 90 and 120 min	- Increase in S0 and decrease in -SH groups - Myosin aggregation and formation of higher molecular weight polymers - Decrease in α-helix and increase in β-sheet contents	[26]
	Myofibrillar proteins	200, 400, 600, 800 and 1000 W, 88, 117, 150, 173 and 193 Wcm^{-2}	Increase in S0, decrease in particle size	[29]
	Squid (*Dosidicus gigas*) mantle proteins	20 kHz, 0, 20, and 40%), 0, 30, 60, and 90 s	- Hydrophobicity was increased - The content of reactive sulfhydryl didn't change - Better emulsifying ability	[30]
	Chicken myofibrillar protein	240 w, 0, 3, 6, 9, 12 and 15 min)	- Increase in -SH groups - No changes in primary structure - Increase in β-turn and decrease in α-helix and β-sheet structures - Decrease in particle size, narrow size distribution	[31]
	Duck liver protein isolate	24 kHz, 266 W by a pulsed on-time of 2 s and off-time of 3 s for 42 min	- Increase in S0 - No changes in -SH content, primary structure and peptide bonds - Decrease in α-helix and random coil and increase in β-sheet and turn structures - Decrease in particle size	[32]
	B-Lg In Cow Milk	9.5 W, 135 W/cm^2	No significant alteration in allergenicity	[33]

Treatment	Substrate	Condition	Results	Reference
High pressure processing	Tropomyosin from shrimp	30 Hz, 800 W for 30–180 min	Allergenicity was reduced	[34]
	Hongqu Rice wines	200 and 550 MPa, 25 °C, 30 min	Free amino acids content was decreased after 6 months storage	[35]
	Brown rice	0.1–500 MPa,10 min	Free amino acids especially essential ones were increased	[36]
	Tropomyosin from shrimp	200, 400 and 600 MPa at 20 °C for 20 min	- Conversion of α-helix structure into β-sheet and random coil - Free sulfhydryl content was decreased - Surface hydrophobicity was increased by increasing the pressure from 200–400 MPa and decreased at the range of 400–600 MPa - Allergenicity was decreased	[37]
	Soy allergen (Glycinin)	100, 200 and 300 MPa for 15 min	- Polyelectrolysis was increased	[38]
	Brussels sprouts	200 and 800 MPa for 3 min, 5 °C	- The total free amino acids content was constant - The concentration of glutamin and asparagine were increased	[39]
Cold plasma	Whey protein isolate	70 kV, 1, 5, 10, 15, 30, and 60 min	- Unfolding and exposure of hydrophobic amino acids - Particle size and PDI after 15 and 30 min of treatment were increased - Free -SH groups were decreased - Oxidation of cysteine	[40]
	Grain rice flour	-	- No changes in protein bands were observed - Total aromatic acid concentrations were increased, and acidic and basic amino acid contents were decreased - The most affected amino acids were glutamic acid, serine and glutamate	[41]

Treatment	Substrate	Condition	Results	Reference
Pulsed ultraviolet light	Soy protein isolate (SPI)	1, 2, 4 and 6 min Three pulses per second with a width of 300 µs	Vanishing Gly m5 & Gly m6 bands after few minutes and decrease in allergenicity	[42]
Cold atmospheric pressure plasma		1, 2.5, 5, 7.5 and 10 min without stirring	Reduction in immunoreactivity of SPI	
Gamma-irradiation		Target doses were 3, 5, 10, 25, 50, and 100 kGy	Decrease in SPI allergenicity (Gly m5 & Gly m6) was dependent on the irradiation dose	
Pulsed electric field	Grape juice	4 µs width and with a field strength of 35 kV/cm, 1000 Hz and the total time 1 ms	- Increase in the concentration of phenylalanine, histidine, asparagine, tryptophan and ornithine - The total concentration of amino acids did not change	[43]
Radiation	B-Lg in cow milk	3, 5, and 10 kGy	Protein aggregation and alteration of IgE binding epitopes	[44]

Gly: Glycinin, SPI: Soy protein isolate, kGy: Kilo gray

4.2. Structural Changes and Aggregation

Changes in the primary structure of proteins could be detected by evaluating the variations of the amount and type of amino acids that were present. The effect of heat and ultrasound pre-treatments on the fluorescence spectra, Fourier Transform Infrared (FTIR) spectra, scanning electron microscopy (SEM), atomic force microscopy (AFM), and three-dimensional (3D) images of corn gluten meal are shown in Figure 2 [27]. The SDS-PAGE analysis is usually used to examine the protein profile and determine protein hydrolysis or protein-protein interactions. Ultrasound treatment at a frequency of 20 kHz for 30, 60, 90, and 120 min was performed on fresh beef [26]. The results showed that when the process was carried out for more than 120 min, higher molecular weight polymers were formed, and the myosin heavy chain was reduced under non-reducing conditions (i.e., in the absence of β-mercaptoethanol) at all tested ultrasound intensities (2.39, 6.23, 11.32, and 20.96 $W.cm^{-2}$). This led the authors to suggest the formation of cross-links by disulfide bonds as the mechanism responsible for myosin aggregation. Disulfide bonds may involve free radicals that formed due to cavitation during ultrasound treatment [26]. Other authors have reported that the covalent and non-covalent bonds were the main interactions that caused the aggregation of muscle proteins that were treated by ultrasounds [45][46]. However, it should be noted that some researchers have reported no effect for ultrasound treatment on protein–protein interaction. For instance, it has been shown that the banding pattern of squid mantle proteins did not change after shorter ultrasound treatment for 30, 60, and 90 s at a frequency of 20 kHz [47].

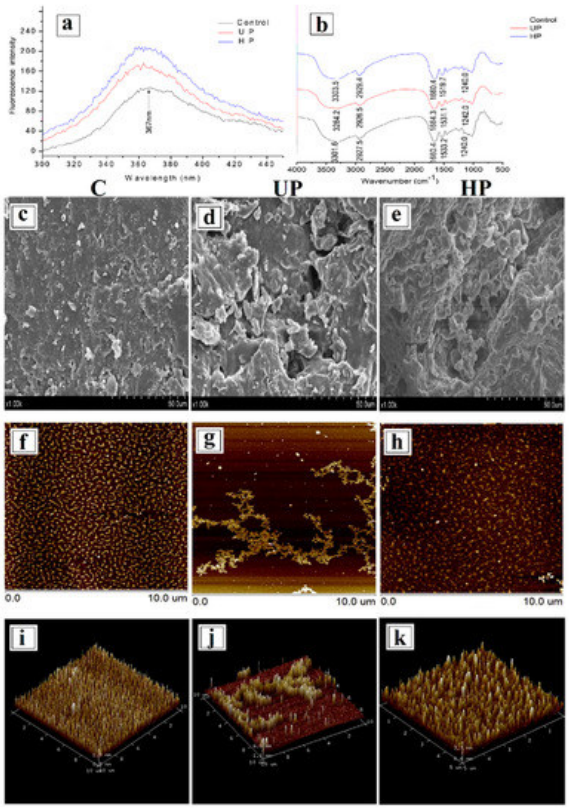

Figure 2. Effect of heat and ultrasound pretreatments on the fluorescence spectra (**a**), Fourier Transform Infrared (FTIR) spectra (**b**), scanning electron microscopy (SEM) (**c**–**e**), atomic force microscopy (AFM) microstructure (**f**–**h**), and three-dimensional (3D) images (**i**–**k**) of corn gluten meal. C, Control; UP, Ultrasound pretreatment and HP, Heat treatment, respectively. Reproduced with permission from [27].

4.3. Particle Size/Molecular Weight Distribution and Zeta Potential

Studies on the effect of ultrasound treatment on myofibrillar proteins (240 W), chicken myofibrillar protein, duck liver protein isolate (24 kHz, 266 W, 2s on, 3 s off for 42 min), and milk showed a decrease in particle size after sonication [29][31][32]. Turbulent flow and micro-streaming, caused by cavitation, agitated protein aggregates vigorously, dissociated them, and reduced the particle sizes. More homogeneous chicken myofibrillar proteins and a narrow distribution of duck liver protein isolate were obtained after ultrasound treatment, which is likely to have significant impact on the texture and mouthfeel properties of the products. After 3 min of ultasound exposure, chicken myofibrillar protein particles with irregular form were changed into a filamentous form. The smallest and more distinct particles as a result of more unfolding and exposure of hydrophobic groups appeared after 6 min The size of the particles at 15th min of treatment was larger than that at the 3rd min This was explained by the more hydrophobic interactions between proteins as the treatment time increased [31]. Gülseren et al. [48] reported an increase in the BSA particle size after sonication, which was the result of the formation of small aggregates.

4.4. Solubility and Gel-Forming/Stability

Ovalbumin shows a relative stability to pressures below 400 MPa and it does not form a gel at this pressure due to the presence of strong non-covalent bonds and four disulfide bonds that stabilize its structure. A pressure-induced gel could be formed at pressures higher than 400 MPa [49]. In biopolymers where hydrogen bonds are dominant, such as gelatin, a higher pressure (≅600 MPa) is required for stable gel [50].

Pressure-induced gels from vegetable proteins can be formed at 300 MPa for 10–30 min treatment [51]. Elastic moduli of these soft gels are significantly lower than the heat-induced ones [52]. Solid behavior of wheat proteins after high-pressure treatment was reported to be due to the weakening effect of pressure on hydrogen bonds and hydrophobic interactions ([52]. Higher pressures resulted in more solid-like and dense gel due to the formation of disulfide bonds.

The interaction between pressure and temperature is an important factor in determining the effect of high-pressure treatment on sarcoplasmic proteins. Marcos et al. [53] reported that increasing pressure that was caused the reduction of solubility and extractability of sarcoplasmic proteins [54][55]. Protein extractability is a direct measure of the quality of sarcoplasmic proteins [56][57]. The denaturation of these proteins and formation of aggregates accompanied by intermolecular disulfide bonds as a result of pressure treatment are the reasons for solubility and extractability reduction.

In the range of 200–400 MPa, no effect of temperature was observed, while, at 600 MPa, lower solubility was observed for the samples that were treated at 30 °C as compared to those that were treated at 10 °C [58].

4.5. Emulsifying Properties

The size of droplets of oil-in-water (*o*/*w*) emulsion stabilized by ovalbumin before and after the pressure pre-treatment of β-lactoglobulin below 600 MPa did not change significantly, however larger droplets were found after applying pressure pre-treatments above 600 MPa [59]. Pressure causes emulsion droplets to flocculate and the more the protein concentration and pressure level, the more flocculation was observed. A similar result was observed about 11s globulin protein of soy, when it was used as an emulsifier [60]. The stabilizing and emulsifying properties of 11s globulin were reduced after pressure pre-treatment due to the formation of intermolecular disulfide bonds that led to aggregation. When compared to the high pressure treatment (800 MPa, 30 min), a mild heating process (80 °C, 5 min) on ovalbumin, and 11s globulin (as emulsifier) caused more flocculation and consequently larger droplet size [49][59]. Emulsions containing pressurized (up to 800 MPa) whey proteins or β-lactoglobulin as emulsifier had bigger droplets than the unpressurized one. Despite the increase in hydrophobicity after high-pressure treatment, structural changes in protein decrease its emulsifying properties, however the gel-like structure of protein that is induced by pressure increases the viscosity of the system and thus stabilizes the emulsion. High-pressure treatment of emulsion stabilized by low β-lactoglobulin content did not affect the size of droplets or creaminess. A higher level of flocculation occurred after pressure treatment in the presence of a high amount of free (non-absorbed) proteins in the emulsion, due to the unfolding, aggregation, and free proteins acting as a bridge between the dispersed droplets by cross-linking them [61].

4.6. Reduction in Allergens

A reduction in allergenicity can occur due to the structural changes, including denaturation (loss of the secondary, tertiary, and/or quaternary structures), inter-molecular and intra-molecular (covalent or non-covalent) interactions, breaking disulfide bonds, and aggregation [18].

Meinlschmidt et al. (2016) investigated the effect of remote and direct cold atmospheric pressure plasma (CAPP) on the immunoreactivity of Gly m5 in soy protein isolate (SPI). The electrophoresis pattern of SPI was changed after 2.5 min treatment by remote and direct CAPP at 9 kV voltage. The disappearance of some protein bands in the SDS-PAGE of CAPP treated SPI was attributed to the formation of the insoluble proteins. Plasma offers reactive forms of nitrogen (N_2, NO, NO_2), oxygen (O_2, O_3, OH), and also UV-A and UV-B, which seriously affect proteins and promote protein-protein interactions or cross-linkage of free amino acids to proteins. As a consequence, protein solubility is simultaneously decreased by the formation of insoluble aggregates. According to the results of sandwich ELISA with mouse monoclonal anti Gly m5 bodies, the direct and remote CAPP treatment of soy protein caused a 100% and 89% reduction in immunoreactivity of Gly m5, respectively.

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