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Impact of High-intensity Ultrasound on Protein Structure and Functionality during Food Processing

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16.1 Introduction

Proteins have traditionally been considered to be key nutritional components in foods by providing essential amino acids. In spite of the potential of food proteins as functional ingredients (Zayas, 1997; Rutherfurd-Markwick, 2012), their use can be limited in some cases due to their low biological value, undesirable organoleptic

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properties or impaired functional properties (Matheis and Whitaker, 1984). Thus, the production of modified proteins with new or enhanced functional and biological properties will contribute to improving traditional processes for manufacturing end-products and favouring more profitable uses from an economic point of view.

Knowledge of the relationship between protein structural and functional properties is becoming increasingly important for extending their applications in food products. The elucidation of the specific mechanisms driving protein functionality would enable determination of their usefulness in the food industry, discovering which particular structures are desirable and how food proteins may be modified to improve functional properties. Numerous studies have been done to understand the relationship between structure and functionality in food proteins, but the information correlating the structure of proteins with specific functions in foods is still limited, probably due to the diversity of composition, structure and conformation of food proteins, as well as to different interactions between proteins and other components that can occur in a food system (Kinsella, 1981).

It is largely accepted that the functional properties of proteins are defined by a series of intrinsic factors, including the composition and disposition of amino acids, as well as important physicochemical properties such as flexibility, molecular size, three-dimensional conformation, active sulfhydryl and/or disulphide groups, hydrophobicity, net charge and protein/protein interactions (Kinsella, 1981; de Wit, 1998). Nevertheless, protein functionality not only depends on their physicochemical and structural features, but also on a number of extrinsic factors such as temperature, pH, salts (ionic strength) and protein concentration, as illustrated in Figure 16.1. Moreover, protein functionality can be altered during food processing due to modifications of the protein structure. The most frequent structural modifications that take place during processing are denaturalization and subsequent aggregation, although the intensity of these changes will largely depend on the severity of the processing. These modifications normally have an important impact on organoleptic properties and could also reduce the content and/or bioavailability of food proteins or their fragments derived from gastrointestinal digestion. Finally, the complex composition

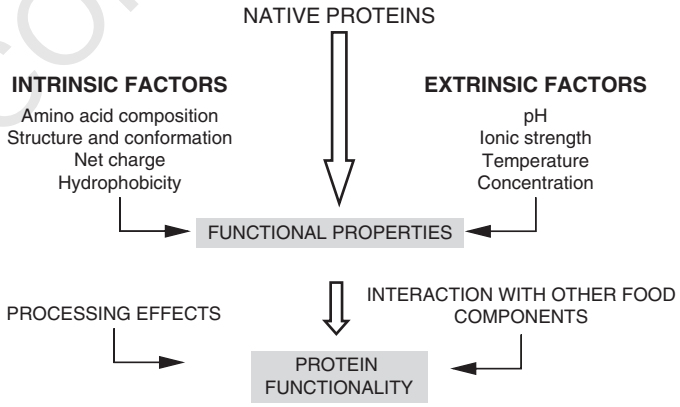

Figure 16.1 Driving factors involved in achieving functional properties and food protein functionality. Modified from de Wit *et al.* (1998) with permission from Elsevier.

of foods, which involves a variety of ingredients, as well as the type of food matrix, may also allow the interaction of proteins with other ingredients during food processing, thereby affecting structural properties and, subsequently, protein functionality (Figure 16.1).

The elucidation of the relationship between intrinsic protein properties and extrinsic factors, and the knowledge and control of the effects of food processing and interaction with other ingredients on the structural proteins in food proteins are critically important for elucidating and managing the functional properties of food proteins (de Wit, 1998). This comprehensive knowledge could allow the safe manipulation and design of food proteins with new or improved functional properties and their further inclusion as functional ingredients in a variety of foodstuffs. Food proteins can be deliberately modified to enhance their functionality and/or broaden their applications as functional ingredients and, more concretely, processing can be an effective tool to produce controlled structural changes allowing specific changes in protein functionality. In this context, food technologists in conjunction with industry are constantly searching for emergent mild processing and environmental friendly technologies, such as high-pressure processing, pulsed electric fields, etc, not only to obtain high-quality food with "fresh-like" characteristics, but also food with improved or even novel functionalities (Ashokkumar *et al*., 2008; Soria and Villamiel, 2010). As is known, treatments with high-intensity ultrasound (HIU) are an emerging technology for the preservation of food alone or in combination with other novel processing technologies, moreover they can be employed to deliberately modify the structure of food components in order to obtain improved functionality (Soria and Villamiel, 2010; Awad *et al.*, 2012; Chandrapala *et al*., ~~2012~~; Pringet *et al*., 2013).

This chapter explores the capability of HIU technology to induce structural changes in food proteins and further consequences in regard to their technological and biological properties.

16.2 Effect of High-intensity Ultrasound on Protein Structure and the Physicochemical Properties of Food Proteins

The impact of HIU on the structural and conformational properties of food proteins has been mainly attributed to physical forces generated through the cavitation phenomenon. During cavitation, micro bubbles are formed and distributed throughout the solution, which grow until a maximal critical size is reached, when they violently collapse, as observed in Figure 16.2 (Mason, 1998; Barbosa-Cánovas and Rodríguez, 2002; Soria and Villamiel, 2010; Zisu *et al*., 2010). The implosion of cavitation bubbles leads to energy accumulation in hot spots, generating extreme temperatures (5000 K) and pressures of several thousand bar, which produce, in turn, very high shear energy waves and turbulence in the cavitation zone. It is thought that the combination of pressure, heat and turbulence in the surrounding area of a collapsing bubble leads to partial protein denaturation. In this regard, Villamiel and de Jong (2000) quantified protein denaturation during pasteurization of milk by HIU. Authors described the effect of HIU (20 kHz, 150 W, 40.2–102.3 s, 55–75.5 °C) in continuous flow on whey and caseins, among other milk components. Ultrasound (US) treatment was able to denature α-lactalbumin and β-lactoglobulin, and this effect increased with temperature

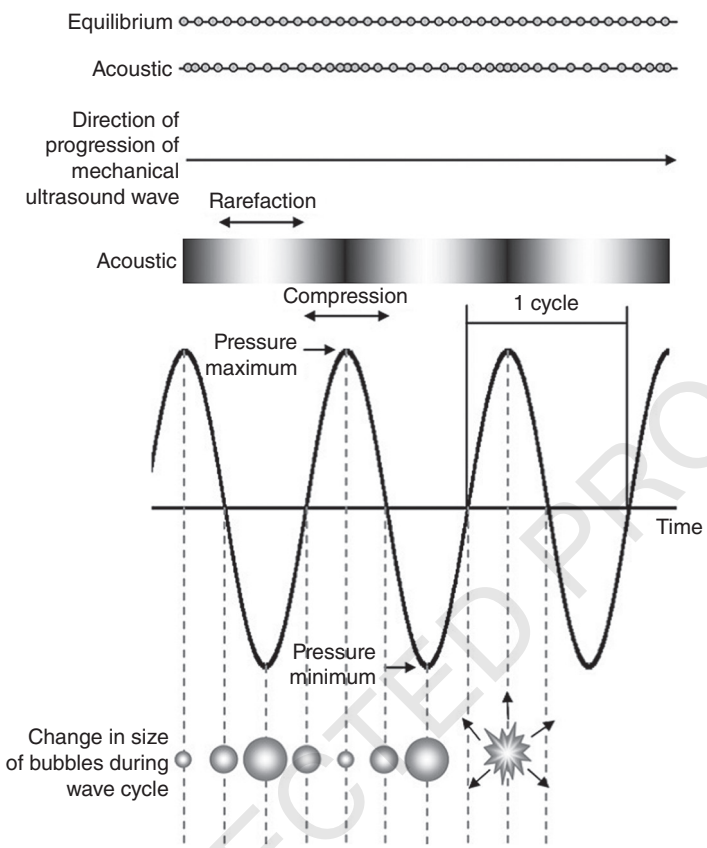

Figure 16.2 Ultrasonic cavitation. Reprinted from *Trends in Food Science and Technology* 21, 323–331. Effect of ultrasound on the technological properties and bioactivity of food: a review, by Soria and Villamiel (2010); with permission from Elsevier).

and was higher in whole milk than in skim milk. This result was ascribed to the higher solids and lower protein concentrations in the former than in the latter and/or binding locations of whey proteins on fat globule membranes. No change in caseins was detected under the assayed conditions. Likewise, the combined effect of US and heat on human milk was found to inactivate *Escherichia coli* and *Staphylococcus epidermidis* with a greater retention of IgA, lysozyme, lactoferrin and bile-salt-stimulated lipase (Czank *et al*., 2010). Christen *et al.* (2012) designed a holding US (20 kHz, 123 μm) system at different power values (3.62–25.64 W), exposure times (19.2–115.2 s) and temperatures (lower than 72 °C) to pasteurize human milk, and found optimal conditions for the inactivation of pathogens without protein denaturation. Recently, Barukcic *et al*. (2015) found a combination of US (20 kHz, 480 W, 6.5–10 min) and 55 °C to be optimal conditions for bacterial inactivation with little protein denaturation.

At the time of cavitation, sufficient shear forces are created to disrupt intermolecular bonds, such as disulphide-thiol and hydrophobic interactions, reducing the mean protein particle size and particle size distribution (Price and Smith, 1993). This has

been observed by several authors studying proteins of different types, including bovine serum albumin (BSA) (Gülseren *et al.*, 2007), whey protein concentrate (WPC) and isolate (WPI) (Krešić *et al.*, 2006; Arzeni *et al.*, 2011, 2012a; Jambrak *et al.*, 2010, 2014), milk protein concentrate (MPC) (Yanjun *et al.*, 2014), soybean proteins (Kohyama *et al.*, 1995; Jambrak *et al.*, 2009; Arzeni *et al.*, 2012a; Chen *et al.*, 2011; Hu *et al.* 2012, 2013a, 2015), egg white proteins (EWP) (Arzeni *et al.* 2012a,b) and peanut protein isolate (PPI) (Zhang *et al.*, 2014b).

These authors, among others, also described how partial molecular unfolding of proteins on sonication increases their surface hydrophobicity by unmasking previously hidden hydrophobic groups and regions inside the molecules and exposing them to a more polar surrounding environment (Nakai *et al.*, 1983). In contrast, limited changes in the free thiol groups of proteins present in WPC solutions have been observed or, if any change occurred, it was reversible, as indicated by the minimal modifications in the surface charge of proteins (Chandrapala *et al.*, 2011~~a~~; Arzeni *et al.*, 2012a).

Likewise, studies showing reduction of surface hydrophobicity and increase in particle size due to HIU-induced protein aggregation, such as those carried out by ~~Chandralapa~~, *et al.* (2011~~a~~) and Gülseren *et al.* (2007), showed that the secondary structure of β-lactoglobulin and BSA can become more ordered for long sonication treatments than in the case of proteins treated by conventional heating. However, dimerization was not detected, suggesting that sonication of food protein solutions causes predominantly physical effects. On the contrary, Zisu *et al.* (2010), observed an increase in the surface hydrophobicity and a reduction in the particle size of WPC after sonication, indicating, by native PAGE and SDS-PAGE, that HIU treatment under the studied conditions did not change protein molecular weight (primary structure), in agreement with results found for sonicated sodium caseinate, WPI and milk protein isolate (MPI) (O'Sullivan *et al.*, 2014), PPI (Zhao *et al.*, 2011; Zhang *et al.*, 2014b) and soybean proteins (Karki *et al.*, 2009; Chen *et al.*, 2011; Hu *et al.*, 2012). Moreover, it has been described that cavitation shearing might disrupt tertiary structure but leave most of the secondary structural elements intact (Stathopulos *et al.*, 2004; Hu *et al.*, 2015).

It should be noted that the effect of HIU on protein structure may vary as a function of the nature of the protein as well as the intensity and duration of the treatment applied and even the lack of temperature control. Arzeni *et al.* (2011, 2012a) pointed out the importance of the protein source as they observed that the same sonication conditions (20 kHz for 20 min) promoted either an increase or a reduction in the particle size of EWP or WPC and soybean protein isolate (SPI) dispersions, respectively.

Regarding sonication time, Krise (2011) observed no significant changes in molecular weight for EWP treated with US at 55 kHz for 12 min. Hu *et al.* (2013b,c) found that the volume-mean diameter of SPI dispersions decreased significantly during the first 20 min ($p < 0.05$) while remaining unchanged ($p > 0.05$) from 20 to 40 min. However, sonication for more than 40 min significantly increased SPI particle size according to the results obtained by Gülseren *et al.* (2007) for BSA sonicated at 20 kHz for more than 45 min. This was attributed to the increase in temperature in the surrounding area of collapsing bubbles enhancing heat-induced protein aggregation in prolonged sonication treatments, which leads to a reduction in the surface hydrophobicity, thereby increasing the particle size. SDS-PAGE results suggested that protein aggregation was due to the formation of non-covalent interactions, such as electrostatic and hydrophobic interactions, rather than covalent bonds, such as

disulphide bridges. Likewise, ~~Chandralapa~~ *et al.* (2011~~a~~) and Jambrak *et al.* (2011) observed protein aggregation for reconstituted WPC and WPI after 5 min of sonication with a 20 kHz probe and US power of 31 and 73–78 W, respectively. The contradictory results from these studies as compared to those obtained by Arzeni *et al.* (2012a), indicated above for WPC treated with 20 kHz probe and a US power of 4.27 W, might be attributed to the differences in the US power used, this being much higher in the former studies than in the latter.

In this respect, Jiang *et al.* (2014) compared the effects of low-frequency (20 kHz) ultrasonication applied at different US powers, including 150, 300 and 450 W, for 12 or 24 min at temperatures below 2 °C to black-bean protein isolate dispersions. They concluded that low-power US increased the destruction of internal hydrophobic interactions of protein molecules and accelerated protein molecular motion, resulting in protein aggregation; unstable aggregates were drastically disaggregated by cavitation at medium-power US and, finally, re-polymerization of aggregates by non-covalent linkages was observed when high-power US was used. Different protein structures can therefore be obtained by modulating the power used in each treatment.

Moreover, the effect of increasing frequency on structural changes of proteins has also been studied. Thus, Liu *et al.* (2014a) observed that at a specific energy input of 286 kJ kg^{-1}, and increasing frequency from 20 to 1600 kHz, the effects of shear on casein micelle dissociation are decreased. They attributed these results to the fact that, at high frequencies, US gives rise to a larger number of pressure antinodes and, consequently, a higher number of smaller active bubbles. The force resulting from the implosion of cavitation bubble is therefore decreased.

16.3 Effect of High-intensity Ultrasound on the Technological Properties of Food Proteins

The technological functionality of proteins is defined by rheological, emulsifying and foaming properties as well as solubility (Damodaran 2005). Changes occurring during HIU treatment in protein conformation and structure, particularly denaturation/aggregation resulting in surface hydrophobicity and particle size variation, are believed to modify the technological functionality of proteins and, hence, their degree of industrial applicability, since the functional properties of a system determine the design of unit operations, for example pumping systems, and the organoleptic characteristics perceived by consumers (Gabriele *et al.*, 2009). As indicated above, the degree of modification of the protein structure in response to the applied HIU treatment will depend on the protein nature as well as the sonication intensity and duration. Thus, the successful and effective industrial application of HIU for processing of protein-based food products or ingredients relies on the knowledge of its effect on the functionality of proteins from different sources and under different operating conditions. Over the past few years a number of studies have been carried out for this purpose using different model systems of proteins. In general, the most studied proteins have been those from milk, soybean and egg white, as they are commonly used by food manufacturers as ingredients of many food products.

Several authors have reported an improvement in solubility of whey proteins (Jambrak *et al.*, 2008; Krešić *et al.*, 2008; Zisu *et al.*, 2010, 2011), MPC (Yanjun *et al.*, 2014), α-lactalbumin (Jambrak *et al.*, 2010) and soybean proteins (Jambrak *et al.*,

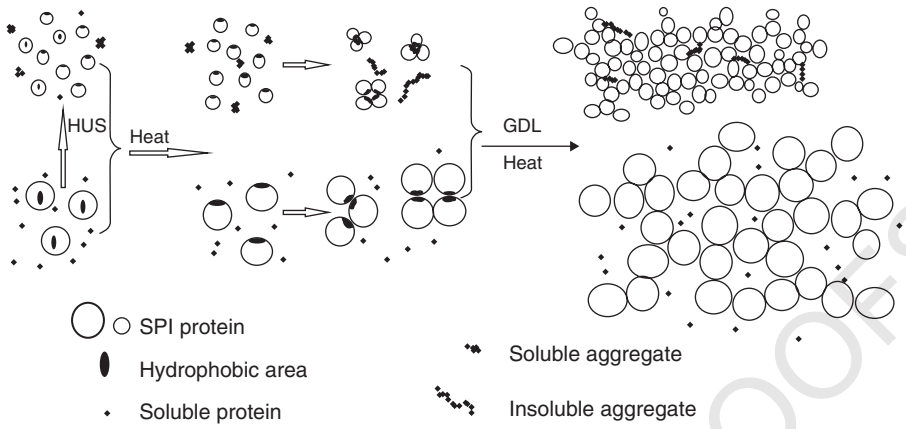

Figure 16.3 Proposed mechanism for the effect of HIU on the acid-induced gelation of soybean protein isolate (SPI). Reprinted from *Ultrasonics Sonochemistry* 20(1), 187–195. Acid-induced gelation behavior of soybean protein isolate with high intensity ultrasonic pre- treatments by Hu *et al.* (2013b); with permission from Elsevier).

2009; Karki *et al.*, 2009; Chen *et al.*, 2011; Arzeni *et al.*, 2012a; Hu *et al.*, 2013a) after HIU treatment. Although these studies were carried out at different sonication conditions, they all resulted in (1) protein denaturation on sonication, so that hydrophilic parts of amino acids from the interior are opened toward surrounding water, and (2) a decrease in particle size. Both US effects lead to an increased number of charged groups (NH_4^+, COO^-) on the protein surface (Herceg *et al.*, 2002; Krešić *et al.*, 2008) and, consequently, higher electrostatic forces, which enhance protein–water interactions by hydrogen bonds and, hence, solubility (Moulton and Wang, 1982; Morel *et al.*, 2000).

Measurement of solubility predicts the protein/solvent, protein/protein and solvent/protein interactions and hence is a good index of protein behaviour as an emulsifying, foaming and/or gelling agent (Webb *et al.*, 2002; Krešić *et al.*, 2008). In addition to solubility, surface hydrophobicity is also a determinant factor of protein functionality. Thus, increased solubility and surface hydrophobicity could provide the protein with a faster migration to, better potential adsorption at and easier rearrangement at the oil/air–water interface. This latter fact promotes intermolecular interactions at the interface, giving rise to a stable film and stabilizing the oil droplets and air bubbles from coalescence (Nakai *et al.*, 1983; Jambrak *et al.*, 2009; Hu *et al.*, 2015). Furthermore, both factors have also been related to protein gelation, as observed in Figure 16.3. The reduced particle size and subsequent formation of soluble protein aggregates on sonication (Wu *et al.*, 2011) has been shown to lead to the formation of a fine homogeneous gel network, more compact and firmer and with higher water-holding capacity (WHC) (reduced syneresis) than non-sonicated protein networks. In addition, increase in protein surface hydrophobicity during HIU treatment might facilitate intermolecular hydrophobic interactions, leading to protein re-aggregation during the heating process and the formation of heat-induced gel networks with better WHC (Kohyama *et al.*, 1995; Gülseren *et al.,* 2007; Arzeni *et al.*, 2011, 2012a; Hu *et al.*, 2013b; Yanjun *et al.*, 2014).

Thus, several authors have described that, under sonication conditions promoting reduction of particle size and increased surface hydrophobicity, the ability of several proteins, including soybean proteins (as aggregated β-conglycinin (7S) and glycinin (11S), SPI and SPC) (Jambrak *et al.*, 2009; Chen *et al.*, 2011; Hu *et al.*, 2015), PPI (Zhang *et al.*, 2014b), MPC (Yanjun *et al.*, 2014), α-lactalbumin (Jambrak *et al.*, 2010) and WPC and WPI (Jambrak *et al.*, 2008), to form and stabilize emulsions and foams is significantly improved. Reduced gelling times and formation of heat-induced gels with higher strength and elastic character has also been observed for fresh whey solutions and WPC (Zisu *et al.*, 2010, 2011; Arzeni *et al.* 2012a) and SPI (Arzeni *et al.*, 2012a; Hu *et al.*, 2013b,c) dispersions. On the contrary, as observed by Arzeni *et al.* (2012a,b), EWP showed a small but significant decrease in solubility after sonication due to the formation of small aggregates under similar sonication conditions to WPC and SPI. Likewise, no significant changes on gelation time and gel strength after HIU treatment were observed. These results highlight the importance of the nature of the protein.

Regarding the viscosity of protein dispersions, a strong correlation between the HIU-induced decrease in protein particle size and the decrease in viscosity and a flow behaviour converting to a more Newtonian one for dairy proteins (Zisu *et al.*, 2010; Arzeni *et al.*, 2012a) has been reported. However, there are controversial results since other authors have observed increased viscosity on sonication under conditions leading to a drop in particle size and increased solubility. Thus, for instance, Krešić *et al.* (2008) observed that HIU treatment for 15 min at 20 kHz and 600 W caused a significant increase in the apparent viscosity of WPI and WPC dispersions (10%, w/w), in agreement with the results observed by Jambrak *et al.* (2009) for SPI and soybean protein concentrate (SPC) dispersions (10%, w/w) (20 kHz for 30 min). The authors ascribed the increased viscosity to the higher water binding capacity of proteins upon US treatment, according to Phillips and Williams (1995).

Likewise, Zisu *et al.* (2010) reported an improvement in the heat stability of WPC and whey protein retentate used in the commercial manufacture of WPC when sonication was combined with a pre-heat treatment of 80 °C for 1 min or 85 °C for 30 s. They observed that the HIU-induced reduction in particle size and viscosity of pre-heated protein dispersions remained at levels comparable to unheated solutions after post heating at 80 °C for 20 or 30 min, suggesting that protein modifications resulting from sonication are irreversible. According to this, ~~Chandralapa~~, *et al.* (~~2011b~~, 2014) reported that the HIU-induced disruption of protein aggregates (whey/whey and whey/casein) in calcium-fortified milks does not revert on subsequent post-heating, which can be tapped in the elaboration of calcium-fortified dairy products with increased heat stability.

Similarly to protein structural properties, comparative studies on the effect of US power and frequency of technological properties of proteins have been carried out. Jambrak *et al.* (2008) studied the impact of low-intensity (500 kHz bath, <0.5 W cm^{-2}) and high-intensity (20 kHz probe, 43–48 W cm^{-2}; 40 kHz bath, 1 Wcm^{-2}) US for 15 and 30 min on the functionality of protein model suspensions (10%, w/w) of WPI, WPC and whey protein hydrolysate (HWP). The major effect on functional properties of whey protein was observed after HIU treatments for 15 min, particularly at 20 kHz using probe, which significantly increased protein solubility and foaming ability and stability. HIU for 30 min has less effect and US of 500 kHz did not impact functional properties. Jambrak and colleagues obtained similar results with α-lactalbumin

(Jambrak *et al*., 2010), SPI and SPC (Jambrak *et al.* 2009), the latter showing better functionality after HIU for 30 min with a 20 kHz probe.

Likewise, for the effect of HIU treatment duration, studies developed with proteins of a different nature, including WPC and WPI (Jambrak *et al.*, 2011), BSA (Gülseren *et al.*, 2007), milk proteins (Sfakianakis *et al.*, 2015) and SPI (Hu *et al.*, 2013b,c), have reported an impairment of protein functionality on sonication under conditions promoting an increase in particle size and, hence, a drop in solubility.

16.4 Effect of High-intensity Ultrasound on Protein Glycation by the Maillard Reaction

A special mention of protein interaction with other food components such as carbohydrates during food processing is considered in this chapter due to the relevance of this reaction to their quality and functionality. As is well known, the Maillard reaction (MR) is one of the most important and complex reactions that may spontaneously occur during food processing. The first step in this reaction is the condensation of reducing sugars with the free amino group of amino acids, peptides and/or proteins to form a Schiff base, which reorganizes to form Amadori or Heyns products. Subsequently, a cascade of consecutive reactions leads to the advanced stages in which nutritional changes, reduction of protein digestibility and browning can be produced (Corzo-Martínez *et al.*, 2012). However, during the past few years it has been well established that a safe and efficient method to generate new modified proteins with great technological and biological interest is the MR at the initial steps (Oliver *et al.*, 2006). The most frequent option is to carry out the reaction in a dry state at gentle temperatures and controlled water activity for long periods of storage, but from a practical point of view these conditions are not adequate in an industrial scale-up. The main drawbacks of this reaction are related to the long time needed, the excessive browning development and the irregular reactant contact, which might result in inefficient glycation, mainly in the case of compact or rigid proteins (Zhuo *et al.*, 2013). The MR in liquid systems at higher temperatures and shorter times can also be used for this purpose; in general, better control of the reaction is achieved, although drastic structural changes can give rise to protein aggregation and lower glycation extent (Zhu *et al.*, 2008). In this case, alternative technologies such as microwaves, high pressure, irradiation, electric fields, dynamic high-pressure microfluidization and HIU have been demonstrated to be useful for improving the MR. Sonication allows short treatment times at low processing temperatures and low energy consumption; HIU is non-contaminating, while retaining nutritive and functional properties (Stanic-Vucinic *et al.*, 2013).

The acceleration effect of US on MR has been studied in several model systems and most of the assays have focused on improving the glycation extent by US on the mixture of the amino acid or protein and carbohydrates. Thus, Guan *et al.* (2010) treated a model system of glycin-maltose (pH 10) by US (25 kHz, 1600 W, intermittent 5 s on/5 s off, <60 °C, 0–50 min) and found promotion of the intermediate (formation of hydroxymethyl furfural, A_{294}) and final stages (A_{420}) of MR. Subsequently, Guan *et al.* (2011) subjected a model system of glycin-glucose (pH 11) to similar US treatments and, according to the levels of 2,5-dimethyl-pyrazine, trimethyl-pyrazine, (Z)-9-octadecenamide, 1,2-benzenedicarboxylic acid and mono(2-ethylhexyl) ester, the major aromatic compounds of MR, significant increases in the intermediate

Table 16.1 Contents of 2-furoylmethyl lysine (mg/g lysine ± SD) obtained after acid hydrolysis of lysine–glucose model systems subjected to US (UST) and conventional (CT) heating treatments at pH 7.0.

Treatment			Time (min)			
	T (°C)	Amplitude (%)	0	15	30	60
CT	25		0.00 ± 0^{a}	120.69 ± 3.98^{a}	127.22 ± 5.18^{a}	127.99 ± 7.06^{a}
CT	40		49.67 ± 2.11^{b}	138.32 ± 12.76^{b}	$137.08 \pm 5.96^{a,b}$	142.68 ± 4.92^{b}
UST	25	50	0.00 ± 0^{a}	$125.39 \pm 9.61^{a,b}$	129.69 ± 7.82^{a}	$135.23 \pm 6.52^{a,b}$
UST	25	70	0.00 ± 0^{a}	$134.81 \pm 2.56^{a,b}$	145.07 ± 3.82^{b}	128.82 ± 7.97^{a}
UST	40	50	49.67 ± 2.11^{b}	$138.09 \pm 1.12^{a,b}$	142.94 ± 12.78^{b}	159.59 ± 9.64^{c}
UST	40	70	49.67 ± 2.11^{b}	165.18 ± 15.73^{c}	126.69 ± 10.72^{a}	$134.72 \pm 13.38^{a,b}$

[a–c] Different case letters indicate statistically significant ($p < 0.01$) differences at the same period of time. Data correspond to the sum of α- and ε-2-furoylmethyl-lysine.
Reprinted from *Food Chemistry*, 157, 186–192. Impact of high-intensity ultrasound on the formation of lactulose and Maillard reaction glycoconjugates by Corzo-Martínez *et al*. (2014); with permission from Elsevier)

products of MR and browning intensity were originated. In both studies, an increase in the antioxidant activity accompanied the increase in MR by US.

The impact of HIU (20 kHz, 400 full power, 120 μm, 50 and 70% amplitude, 25 and 60 °C, 15–60 min) on the production of lysine-glucose glycoconjugates during MR was investigated by Corzo-Martínez *et al.* (2014) in medium at neutral pH. These authors confirmed the effect of US on the intermediate and final steps of MR and moreover observed an acceleration of the reaction in the initial steps. Thus, the levels of 2-furoylmethyl-lysine (indicator of initial steps of MR) were higher in samples treated by US as compared to their counterpart heated samples (Table 16.1) and they ascribed these results to the physical effects of mixing, efficient heat/mass transfer and removal of oxygen. The latter could have avoided the oxidative cleavage of the enediols formed at the initial stages of MR.

Concerning the effect of US on protein glycation, dairy proteins have been one of those most studied. Thus, glycoconjugates of bovine serum albumin (BSA) with glucose have been formed at 60 °C, pH 10, for 30 min by US (25 kHz, 1600 full power, 10.19–17.83 W cm^{-2}, intermittent 5 s on/5 s off) (Shi *et al.*, 2010). In this study, an increase in the degree of MR was found, in agreement with the US intensity. Significant increases were found in the intermediate products content, browning, antioxidant activity and changes in the second structure of the protein. When the intensity of ultrasonic treatment of BSA alone increased, according to the far-UV CD spectra, the α-helix structure of the protein did not change, whereas in the presence of glucose, the decrease in two negative peaks at 209 and 222 nm suggested that the content of α-helix was reduced. Under neutral conditions (pH 6.5), Stanic-Vucinic *et al.* (2013) obtained β-lactoglobulin-ribose/arabinose/glucose/galactose/fructose/lactose complexes by US (20 kHz, 135 W cm^{-2}) at 10–15 °C. A significant increase was found in the early and intermediate MR products content, fluorescence, browning intensity and antioxidant activity of solutions of protein–carbohydrate complexes. The proposed treatments had the additional advantage that the low sample temperature did not considerably increase the rate of the later stages of the MR and did not change the protein to a significant level.

Recently, Perusko *et al.* (2015) proposed sonication (20 kHz, 135 W cm^{-2}, pH 8, 5–10 °C) under crowding conditions as a valuable method for modification of labile proteins, since this strategy increases the rate of the reaction at low temperatures, obtaining proteins with improved functionality. Thus, they demonstrated that the extent of whey protein (WP) glycation by arabinose under macromolecular crowding conditions using polyethylene glycol (PEG) 6000 was significantly higher than without crowding. An increase in glycation efficiency gave rise to slight modifications of WP structure. Solubility at different pH values, thermal stability and antioxidative capacity of glycated WP were augmented, mainly in the presence of PEG, as compared to sonicated non-glycated proteins.

In agreement with the above-mentioned studies, US treatment could also significantly increase the reaction rate of glycation of protein with polysaccharides to obtain complexes with improved functionality. Thus, Mu *et al.* (2010) observed an acceleration of the graft reaction between soy protein isolate (SPI) and gum acacia (GA) by US treatment (15 kHz, 5–60 min, pH 7.5, 100–400 W, 40–90 °C). The grafted SPI presented significantly higher levels of emulsifying parameters and surface hydrophobicity than native SPI. Moreover, when SPI was treated without the polysaccharide by US treatment the amount of free amino groups considerably increased, probably due to the hydrolysis of peptide bonds of SPI by US. Similar results were subsequently obtained by Zhang *et al.* (2014a), who studied the structural characteristics, surface hydrophobicity and emulsifying properties of conjugates of β-conglycinin and maltodextrins prepared by US (20 kHz, pH 7.0, 90 °C, 5–60 min). These results could be due to the fact that the bubbles originated by cavitation increase the local temperature and pressure in their surrounding area, leading to unfolding of protein and breaking of peptide linkages.

In an attempt to gain more insight onto the effect of US on protein structure and subsequent glycation, Zhang *et al.* (2014c) evaluated by MALDI-TOF and Orbitrap mass spectrometry the glycation degree of BSA before and after ultrasonication (20 kHz, 150 W cm^{-2}, intermittent 5 s on/5 s off, 15 °C, 10 min) and they found that prior to US pre-treatment of protein, only 12 sites were glycated (11 lysines, 1 arginine), whereas the number of glycation sites was increased to 42 (39 lysines, 3 arginines) after US treatment. It seems that protein could undergo a prevalent structural modification in response to ultrasonication throughout all three domains, leading to greatly improved glycation.

16.5 Effect of High-intensity Ultrasound on the Biological Properties of Food Proteins

The determination of the impact of US technology on the biological properties of food proteins is still in its infancy, since only scarce and fragmented studies, mainly focused on the effect of HIU on food allergenicity and/or on protein susceptibility to hydrolysis, have been carried out so far.

A series of studies have addressed the use of US as a tool to reduce the allergenicity of some specific proteins with dissimilar results. The reasoning underlying the use of food processing to reduce the allergenicity (IgE-reactivity) of proteins is that, on the one hand, processing could diminish the allergen load of a final processed food and, on the other hand, it could also have the ability to cause considerable changes in the structure of food allergens, in particular destroying conformational IgE epitopes.

These structural modifications could further alter food behaviour in the gastrointestinal tract during digestion and/or subsequent absorption and therefore affect the way in which food allergens are presented to the immune system. However, food processing can both reduce and increase food protein allergenicity, depending on the type of food (Mills *et al.*, 2004, 2006).

In this context, Tammineedi *et al.* (2013) investigated the effect of HIU, among other alternative non-thermal food processing techniques such as atmospheric plasma and UV-C light treatments, in reducing the allergenicity of isolated major milk bovine proteins such as α-casein, β-lactoglobulin and α-lactalbumin. For HIU treatments, a processor operating at a net power output of 500 W and 20 kHz frequency with a titanium probe of 13 mm tip diameter and a maximum sonication time of 30 min were employed. Under these treatment conditions HIU was ineffective in reducing the content and the *in vitro* allergenicity of major milk proteins as no significant differences ($p > 0.05$) in IgE binding values for control and treated samples were found by competitive indirect ELISA. Similarly, the IgG/IgE-binding properties of the major allergen in octopus, identified as tropomyosin, remained unaffected after HIU treatments performed at 37 °C and 200 W for 60 min (Shen *et al.*, 2014). Similar equipment and treatment conditions were used for the study of the effect of HIU on the reactivity of the major allergen in the fish roe of large yellow croaker, whose IgE-binding activity determined by inhibition ELISA was slightly decreased after HIU treatment (Liu *et al.*, 2014b). These results are in sharp contrast to those obtained by Li *et al.* (2006), who indicated that HIU treatment had the capacity to largely reduce the *in vitro* allergenicity of a shrimp allergen (tropomyosin) after 30–180 min of treatment at 30 Hz frequency and 800 W power, as determined by ELISA and immunoblotting analysis. In addition, these authors observed a linear relationship between the decrease in allergenicity and treatment time. By comparing these studies, we could speculate that not only longer treatment times but also higher intensities could have a more noteworthy impact on the modification of the IgE reactivity. Likewise, a complex of factors such as specific requirements for allergen structure, processing effects on tissue integrity and embedding of the allergens in the tissue matrix could also determine the potential effectiveness of novel processing methods (Mills *et al.*, 2004).

A different approach for reducing the content and, subsequently, the IgE-binding activity of two major peanut allergens, namely 7S globulin and 2S albumin proteins, in roasted peanut kernels was carried out based on a pre-treatment consisting of US-assisted enzymatic treatment (Li *et al.*, 2013). These authors demonstrated that the combination of US treatment and sequential enzymatic treatment of roasted peanut kernels was very effective in almost completely removing the two major allergens and significantly reduced IgE binding of peanut extracts. This behaviour was explained by the fact that US technology may have the potential to disrupt the peanut structure or even break peptide chains, and to facilitate the simultaneous action of sequential digestive enzymes for, subsequently, reducing the IgE-binding capacity of peanut allergens. Likewise, these authors also indicated that US-assisted enzymatic treatment could preserve more flavouring compounds than blanching-enzyme treatment due to a lower temperature.

Linking to the previous article, US technology has been also used to change the susceptibility of food proteins to simultaneous or posterior enzymatic hydrolysis with different purposes. As an example, Jia *et al.* (2010) observed that an ultrasonic pre-treatment (20 kHz frequency and 40 W power for 210 min with pulse durations of on-time 2 s and off-time 4 s) resulted in an increase in the surface hydrophobicity of

defatted wheat germ protein and loosening of the protein tissue, which could facilitate enzymatic hydrolysis and, more specifically, the release of hydrophobic amino acids and proline during subsequent enzymatic hydrolysis. These types of amino acids play important roles in the activities of the angiotensin-converting enzyme (ACE)-inhibitory peptide, thus explaining the increase of 21–40% in ACE-inhibitory activity of the hydrolysate subjected to the ultrasonic pre-treatment. However, the application of US treatment during proteolysis also enhanced the enzymatic hydrolysis of defatted wheat germ protein but had less effect on the ACE-inhibitory activity of the hydrolysate. Wheat germ protein was also pre-treated with US ranging from 200 to 1800 W prior to enzymatic hydrolysis to optimize the production of bioactive peptides with ACE inhibitory properties (Huang *et al.*, 2014). Thus, following a US pre-treatment at 600 W for 10 min, the ACE inhibitory activity of the hydrolysate was increased by 32% over the control. In good agreement with these studies, Uluko *et al.* (2013) also improved the ACE-inhibitory activity of a hydrolysate generated from the action of neutrase on milk protein concentrate with a pretreatment of US at 800 W for 5 min. Moreover, US pre-treatment increased the degree of hydrolysis and stabilized the solubility of the milk protein concentrate hydrolysates.

The application of a 70 W US pre-treatment for 30 and 45 min combined with a subsequent conventional hydrolysis with Flavourzyme yielded tilapia protein hydrolysates with the strongest nitric oxide inhibitory and antioxidative (in macrophage cell lines) activities, respectively. In addition, the 70 W US-assisted hydrolysis of untreated tilapia homogenate muscle protein, i.e. the US treatment and enzymatic hydrolysis were simultaneously carried out, gave rise to hydrolysates with high *in vitro* antioxidant properties (Kangsanant *et al.*, 2014).

Finally, it should be also mentioned that there are a considerable number of studies that have evaluated the effect of US treatments on the catalytic activity of food enzymes of industrial importance. These studies have been primarily focused on the inactivation of enzymes involved in the deterioration of fruit and vegetables, namely pectinmethylesterases, polyphenoloxidases and peroxidases, as well as on the quality and shelf-life of heat-treated milk and other dairy products. Since this topic goes beyond the main scope of this chapter, which is focused on intentionally modifying functionality of food proteins *per se* through US applications, it will not be described in detail here. However, the reader can find relevant information on this subject in a number of excellent reviews, such as those published by O'Donnell *et al.* (2010) or by Chemat *et al.* (2011).

16.6 Conclusions and Future Trends

The use of HIU in the field of food proteins has immense potential due to its ability to modify their structure and, consequently, their technological and bioactive properties. Up to now, most articles have focused on the denaturation of proteins and some hypotheses on the possible mechanisms have been stated. The impact of HIU on the structural and conformational properties of food proteins has been mainly attributed to physical forces generated through the cavitation phenomenon. This can be seen in the disruption of intermolecular bonds and hydrophobic interactions, and changes in protein particle size. Although it is noteworthy that the effect of HIU may change depending on protein nature, as well as the intensity, duration and temperature of the applied treatment, the structural modifications may improve the solubility and

gelation ability, stabilize emulsions and foams, modify the viscosity and improve the heat stability of proteins. Similarly, the bioactivity of proteins can be also modified due to HIU, although limited studies with controversial results have been carried out on this topic. Recently, it has been pointed out that HIU could be a complementary method to decrease the allergenic characteristics of proteins and improve the combined enzymatic hydrolysis of proteins, although more research is needed. A special mention of HIU applications is the use of HIU to promote the MR, as a medium to deliberately promote the structural and functional changes of proteins. Studies reported to date have pointed out the acceleration not only of the intermediate and final steps of this reaction but also the initial stages, in which no drastic impairment of functionality is caused. Thus, the glycoconjugates obtained can exhibit higher antioxidant activity as compared to their counterparts without US. Studies on the establishment of optimal processing conditions depending on the protein nature of HIU treatment are mandatory to broaden knowledge on the involved mechanisms of protein structural modifications and, consequently, to gain more insight into the potential applications of this technique in the processing of protein. All of this should be focused on obtaining proteins and derivatives with premium functionality with the aim of using them in the food industry as ingredients with added value by means of sustainable, economic and practical processing treatments.

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