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Review Article

Functionalization of whey proteins by reactive supercritical fluid extrusion

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Abstract

Whey protein, a by-product from cheese-making, is often used in a variety of food formulations due to its unsurpassed nutritional quality and inherent functional properties. However, the possibilities for the improvement and upgrading of whey protein utilization still need to be explored. Reactive supercritical fluid extrusion (SCFX) is a novel technique that has been recently reported to successfully functionalize commercially available whey proteins into a product with enhanced functional properties. The specific goal of this review is to provide fundamental understanding of the reinforcement mechanism and processing of protein functionalization by reactive SCFX process. The superimposed extrusion variables and their interaction mechanism affect the physico-chemical properties of whey proteins. By understanding the structure, functional properties and processing relationships of such materials, the rational design criteria for novel functionalized proteins could be developed and effectively utilized in food systems.

Key words: reactive supercritical fluid extrusion, whey proteins, modification, transformation, functionalization

1. Introduction

The utility of food proteins is determined by its nutritional and functional properties. In the case where the protein is used as ingredient in a food system, the functional properties become more important than nutritional properties. Nowadays, consumers are interested in wellness and food. The market for protein-based functional ingredients has surged, and the food industry is constantly looking for new functional proteins that allow it to produce a lower cost product with the same quality or a superior quality product at the same cost. Intensive research efforts on food proteins are aimed at modifying inexpensive, available proteins to enhance their functionalities so that more costly proteins in the formulation could be spared. Whey is a by-product obtained during cheese manufacture. Whey proteins (WPs) are inexpensive, available and often used in a variety of food formulations and constitute a significant share of the dairy ingredients market. Their abilities to form gels, films, foams, emulsions and sols are important in food applications and product development (Foegeding & Davis, 2011). However, the possibilities for the improvement and upgrading of WP utilization still need to be explored.

The importance of correlating protein functionality to structure as a first step in a more systematic approach to the understanding and designing of processes to improve, as well as to predict, functionality has been recognized and constitutes one of the eternal objectives of numerous studies over the past decades (Kinsella, 1976; Morr,1990; Van Vliet *et al.*, 2002; Walstra, 1993; Zheng *et al.*, 2008). Although many researchers have established that properties like solubility, hydrophobicity and flexibility have a direct effect on protein functionality, yet, the exact magnitude to which the structures of macromolecules like proteins play a role in the expression of a given functional property is hard to predict.

Many successful protein functionality augmentations have been achieved through structure modification by chemical, enzymatic or physical techniques. Physical treatments of WPs have been extensively studied to improve their functionalities (Iordache & Jelen, 2003; Liu *et al.*, 2005;

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Lopez-Fandino, 2006). Among the physical methods, protein functionality modification by extrusion technology has received considerable attention as a means for texturization of proteins and the use of twin-screw extrusion has proved to be instrumental in the development of many new products. The combination of shear, temperature and pressure during extrusion processing creates conformational changes and chemical reactions in proteins (Manoi & Rizvi, 2009a). Texturization of protein by conventional cooking extrusion is a currently practiced industrial technology. It is known that the product's texture results from a complete restructuring of the polymeric material into an oriented pattern followed by cross-linking at the die end of the extruder (Martinez-Serna & Villota, 1992). Supercritical fluid extrusion (SCFX) is an innovative food processing technology that offers sub-100ºC expansion using direct supercritical fluid carbon dioxide (SC-CO_2) injection, providing several advantages over conventional extrusion methods (Manoi & Rizvi, 2008). Until now, only a few investigators have addressed the mechanisms of protein functionalization and its reactivity during extrusion process and the effects of SC-CO_2 treatments on WP functionalities. This review provides an overview of functional properties of WPs and their modification via reactive SCFX process. The transformation and reactivity of WPs during the extrusion process and the possibilities of utilizing modified WPs as dairy-based ingredients in food products were also established.

2. Functional properties and modifications of whey proteins

WP products are generally used as food ingredients because of their versatile functional and nutritional properties. Their desirable functional properties such as solubility, foaming, emulsification, heat-induced gelation and coagulation, water binding capacity and retention, dispersability, viscosity and turbidity have been primarily revealed and utilized in food systems (Foegeding & Davis, 2011; Firebaugh & Daubert, 2005). The properties of whey based protein products are mainly dependent on the processing technology. Several different treatments including heat treatments and membrane fractionation techniques significantly influence their properties and consequently their possible uses (Almécija *et al.*, 2007). The functional behavior of WPs during food processing, however, is much more complicated. The native proteins reflect a number of functional properties in aqueous solutions which are modified during processing to affect the protein functionality. Therefore, the functional properties of protein ingredients are the result of intrinsic properties of WPs and a number of extrinsic factors. Intrinsic factors include amino acid composition and sequence, conformation, molecular size, net charge, inter- and intra- cross-links, hydrophilic/hydrophobic ratio, and rigidity/flexibility of the protein in response to external conditions. The relationship between intrinsic properties of WPs and extrinsic factors such as temperature, pH, salts, and protein concentration are critically important for elucidating and controlling the functional properties of WPs (de Wit, 1998).

Protein functionality is often associated with secondary and tertiary structural changes (unfolding and heat denaturation) (Foegeding & Davis, 2011). Proteins in whey have relatively low molecular weight and are able to expose hydrophobic groups when partially unfolded. Therefore, WPs rapidly migrate and adsorb onto an air-water interface, reducing surface tension, and allowing them to form stable foams (Philips & Kinsella, 1990). This property is usually identified with the foaming ability of WP products in an aqueous solution (de Wit, 1998). In contrast, WP aggregates impair foaming properties. An important aspect of WPs is their success as emulsifiers in food systems. Important factors determining their emulsification properties are protein concentration, pH, ionic strength, concentration of calcium and lactose, the processing history, and the storage conditions (Mccrae *et al.*, 1999). The good emulsifying properties of WPs allow the introduction of fat globules as structural elements of heat-induced WP gels. Moreover, the well-known heat-induced interactions between WPs and casein micelles make milk an interesting base for all kinds of textured products with high nutritional values (de Wit, 1998).

Modification of WPs to enhance or alter their functional properties can be accomplished by chemical, enzymatic, or physical techniques (Augustin & Udabage, 2007). Chemical modification alters the non-covalent forces (van der Waals forces, electrostatic interactions, hydrophobic interactions and hydrogen bonds) determining protein conformation in a manner that results in desired structural and functional changes. Enzymatic modification generally involves proteolytic hydrolysis of the protein to yield a mixture of peptides (Buchert *et al.*, 2010). Enzymes can be used to introduce intramolecular or intermolecular crosslinks into a protein structure (Eissa & Khan, 2006). Such cross-links between protein chains by enzymatic or chemical reactions make protein polymers with specific structure that brings favorable textural and rheological properties (Buchert *et al.*, 2010; Dickinson, 1997). Physical protein modification method may involve heat treatment, complex formation with biopolymers, or a texturization process (Onwulata *et al.*, 2011). Thermal treatment resulting in partial protein denaturation may elicit desired improvements of functional behavior. Texturization of protein involves physical treatments such as fiber spinning or thermoplastic extrusion for meat extender products (Walsh & Carpenter, 2003; Walsh *et al.*, 2008). These processes impart structural integrity to proteins. Recently, novel food processing technologies have been used to modify functionalities of food biopolymers particularly proteins (López-Fandiño, 2006). Several studies on the effects of high pressure treatments on protein functionalities have been reported (Hossei-Nia *et al.*, 1999; Iordache & Jelen, 2003; Liu *et al.*, 2005; Xu *et al.*, 2011; Zhong & Jin, 2008). Krešić *et al.* (2008) utilized high pressure, ultrasound and tribomechanical activation techniques to modify rheological and thermophysical properties of WPI and WPC powders and dispersions. Among these techniques, high pressure treatment has the most potential to enhance the rheological properties of WPs that could be appropriate for utilization in many food formulations such as bakery, dairy and sausage products. In addition, Dissanayake and Vasiljevic (2009) studied heat treatment and microfluidization process in combination, followed by spray drying process to stabilize WPs against heat by producing microparticulated species that enhanced surface and colloidal properties of WPs. Their results have implications for the use of WPs as an additive in heat-processed foods.

3. Modification of whey proteins by reactive extrusion

Reactive extrusion has been defined as an extruder-conducted process that involves the concurrent reactions of the feed polymers (Brown & Orlando, 1988). The reactive extrusion process provides the environment with adequate residence time at the proper temperature for melting, mixing, and reaction of the polymer and additives. Subsequently, the feed polymer is modified by changing the molecular weight of feed polymer, grafting or adding a functional monomer to the polymer. It reactively combines one polymer with another and induces chemical changes that improve the properties of the modified material such as enhanced thermal stability, mechanical strength, elongation, adhesive strength and other mechanical properties. Reactive extrusion was developed in 1980s, and has been generally used for the production and modification of a wide variety of synthetic polymers and blends (Xie *et al.*, 2006).

The most commonly used technique to reactively texturize WPs is thermoplastic extrusion (Martinez-Serna & Villota, 1992). Extrusion cooking has been applied to dairy ingredients to improve functional properties, replaces the traditional by continuous process, and develops foods with new texture characteristics (Onwulata *et al.*, 2011). Extrusion with their shearing screws operating at varying speed and heating can alter the conformational structure of globular proteins changing the molecular structure of proteins known as texturization and/or forming new functionalities (Qi & Onwulata, 2011). These changes further impart unique functional properties to dairy proteins, resulting in new protein-based food ingredients. The new functional behavior depends on the extent of modification and the degree of structural change imparted and is controlled by adjusting parameters such as extrusion temperature and moisture level. Such texturized proteins can be used to produce puffed high-protein snacks (Onwulata, 2010). Softer gels and expanded structures can be made using supercritical fluid extrusion and cold extrusion techniques that avoid elevated temperatures and minimize possible damage to the nutritive components and functionality of the texturized dairy proteins (Manoi, 2009). The early reason for texturizing protein is to develop a physical structure which will provide, when eaten, a sensation of eating meat. Textured proteins can broaden the range of food applications to include use as meat analogues or meat extender (Walsh & Carpenter, 2003; Walsh *et al.*, 2008). In these cases, the structure of the protein must be made to resemble that of muscle in order to attain the proper texture. WPs have been considered as the new ingredient for meat alternative market and snack because it is readily available, inexpensive, and a high protein source (Onwulata *et al.*, 2003; Walsh & Carpenter, 2003).

Hale *et al.* (2002) developed meat extender for beef patties by extruding 2 parts of WPC and 1 part of corn starch using water, 0.1 N HCL, or 0.2 M NaOH as the liquid. The sensory results showed that consumers identified no difference in taste or texture between burgers made using 40% base texturized WPs and 100% beef. Walsh *et al.* (2008) investigated the range of WP in a WP/starch mixture needed to produce an extrusion-textured whey product that contained a fibrous texture and found that the consumer acceptability of beef patties extended by 50% texturized WPs containing 48% protein was acceptable to consumers. Onwulata *et al.* (2003) studied the functionality of texturized WPs by extruding three different types of WP including WPC, WPI, and whey albumin at 38% moisture content at different cooking temperatures. They found that varying temperature in the extruder demonstrated different degrees of WP denaturation, which might be useful for different products. Walsh and Carpenter (2003) developed a new snack product by extruding a dry mix comprising 2 parts of WPC80 and 1 part of corn starch at a rate of 25 g/min and adding 0.1 M NaOH solution at a rate of 11 g/min at 145-147ºC. The resulting product was expanded, crunchy and with small even cell size. Onwulata *et al.* (2006) extruded WPI pastes (60% solids) in a twin screw extruder at 100ºC with four different pH adjusted water streams; acidic solutions (pH 2.0 and 2.5), and alkaline solutions (pH 11.5 and 12.4). The results indicated that alkaline treatment increased insolubility and pasting properties (viscosity). This condition also produced rod-like microstructures and formed fine-stranded fiber-like structures in texturized products. Acidic conditions increased solubility and decreased WPI pasting properties. Recently, Onwulata *et al.* (2010) reported the possibilities of using the pretexturized dairy proteins obtained by extrusion process of various dairy protein sources including non-fat dried milk (NDM), WPC, and WPI to boost the protein content in puffed snacks based on corn meal. The extrusion temperature and water level adjustment led to differences in texture, moisture and solubility of extruded milk proteins due to protein structure alteration during extrusion process. The authors concluded that the extrusion temperature ranging from 50 to 100ºC was sufficient to alter the structure of the dairy proteins suitable for use in snack products.

4. Transformations of proteins during reactive extrusion process

The extrusion process has been utilized to alter WP conformational structures and functionalities for providing extruded proteins with a wide variety of textures (Tunick & Onwulata, 2006). In general, proteins are susceptible to both conformational changes and chemical reactions during extrusion. The combination of shear, temperature and pressure during extrusion processing creates opportunities for protein molecular transformations and denaturation (Ledward & Tester, 1994). The increase in pressure and temperature as a result of both transfer of heat from the heated barrel and the conversion of mechanical energy into heat energy accompanied with the shearing and mixing of the extruder screw causes protein denaturation, which exposes the reactive free sulfhydryl (SH) groups, non-polar amino acids, and peptides that are normally concealed in the native proteins (Onwulata *et al.*, 2003; 2006). The behavior of WPs during food processing is very complex and is governed by their heat sensitivity and depends not only on their intrinsic properties but also on their susceptibility to denaturation. Denaturation is defined as a major change of the protein native structure, without alteration of the amino acid sequence and is a consequence of an altered balance between the different forces, such as electrostatic interactions, hydrogen bonds, disulfide bonds, dipole-dipole interactions and hydrophobic interactions that maintain a protein in its native state (Alting, 2003). Therefore, denaturation of globular proteins is a prerequisite to “activate” the new functionality of proteins such as texturing, gelling, foaming and emulsifying properties.

Ledward and Mitchell (1988) proposed that during the extrusion process proteins possibly (1) form randomly aggregated or oriented spherical molecules, or (2) aggregate as strands, either randomly or oriented in the direction of flow. However, the effects of extrusion on the molecular changes of WPs are still difficult to isolate because high protein concentrations are exposed to several processes simultaneously. Only few investigators have addressed the mechanisms of WPs reactivity during extrusion, especially at high levels of protein concentrations with limited water content. However, Areas (1992), Ledward and Tester (1994), and Yuryev *et al.* (1990) concluded that S-S bonds, non-specific hydrophobic and electrostatic interactions are all responsible for protein texturization by extrusion. The structure formation of protein extrudates is believed to result from a complete restructuring of the polymeric material in an oriented pattern. The forces which stabilize the tertiary and quaternary structures of the proteins are weakened by a combination of increased temperature and shear within the extruder (Camire, 1991). During extrusion, the proteins completely disaggregate through mechanical mixing to form a homogeneous suspension. Consequently, the proteins are denatured, dissociated and unraveled, allowing alignment of the denatured protein molecules in the direction of the flow (Li & Lee, 1996). The reaction sequence is depicted in Figure 1. The proteins then cross-link at the die end of the extruder to impart a network to the extrudates. However, the way that protein cross-links with protein in the extrusion process is still unclear, and no unified model or mechanism for protein–protein interactions during extrusion processing has been proposed to date.

5. Functionalization of whey proteins by reactive supercritical fluid extrusion (SCFX)

Reactive supercritical fluid extrusion (SCFX), a novel extrusion technology for production of highly expanded starch foam, was patented by Rizvi and Mulvaney (1992). Instead of steam this process uses supercritical carbon dioxide (SC-CO_2) as a blowing agent, a nutrient carrier, and as an in-line process modifier (Alavi *et al.*, 1999). SC-CO_2 is an environmentally friendly solvent, chemically inert, physiologically safe and easily recycled, which is ideal for food processing.

Thermodynamically, SC-CO_2 has a liquid-like density and gas-like diffusivity and viscosity which lead to rapid wetting and allow penetration of complex structure (Rizvi *et al.*, 1995). The supercritical conditions of CO_2 are relatively easy to achieve (critical temperature = 31°C, critical pressure = 7.38 MPa) as demonstrated in Figure 2. SCFX is conducted at high pressure and at temperatures below 100°C with lower shear which offers major advantage over to steam-based extrusion processing. The potential of using SCFX for producing a range of puffed food products such as ready-to-eat cereals, pasta, and confectionery products has been reviewed (Mulvaney & Rizvi, 1993; Rizvi *et al.*, 1995). Its distinct low-

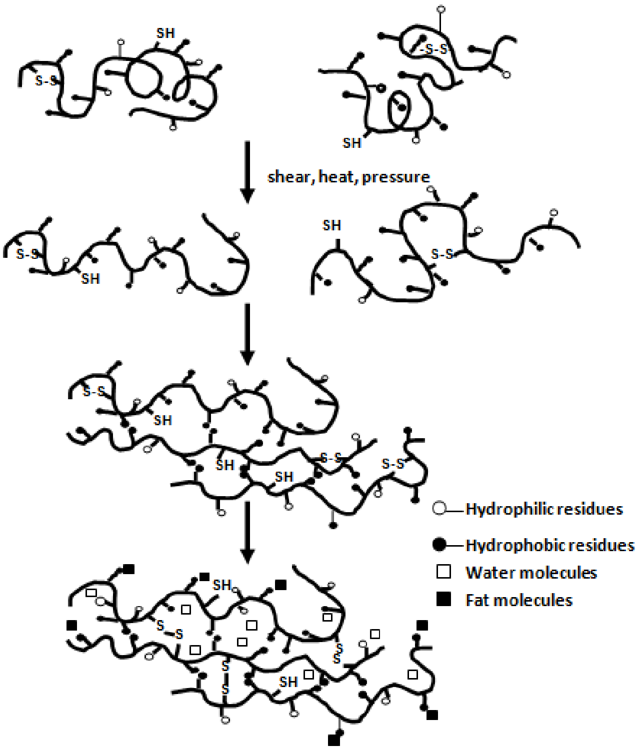

Figure 1. Schematic diagram of a protein molecule denaturing, aligning in the direction of flow and cross-linking through hydrophobic interactions and disulfide bond formations with another protein during extrusion processing (modified from the protein transformation proposed by Li & Lee, 1996).

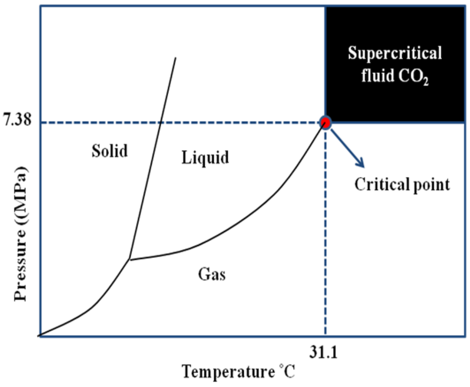

Figure 2. Supercritical fluid phase diagram

temperature and low-shear conditions due to high moisture allow for the retention of heat sensitive ingredients. The delicate balance of temperature, pressure, shear and internal environment created by introduction of SC-CO_2 during SCFX processing creates opportunities for chemical reactions and conformational changes in proteins.

SCFX process dynamics can be divided into two stages- I) flow of protein melts containing SC-CO_2 through the nozzle and extruder die, II) exit of extrudate from the die. At a macroscopic level, the predominant phenomena include pressure drop experienced by protein melt in stage I, bulk diffusion of CO_2 and heat transfer in stage II. At the microscopic level, the predominant phenomena include nucleation of bubbles as the protein melt saturated with SC-CO_2 undergoes a pressure drop in stage I, expansion of the individual bubble induced by a net driving force acting upon the surrounding protein matrix and diffusion of CO_2 from protein matrix into the bubble in stage II. Therefore, the expansion process consists of three steps- a) dissolving SC-CO_2 in the polymer melt to form a polymer and SC-CO_2 solution, b) cell nucleation caused by rapid pressure drop, and c) cell growth and extrudate expansion at the die exit as the pressure quenches to atmospheric level (Alavi *et al.*, 1999; Rizvi *et al.*, 1995).

The SCFX process is more versatile and controllable. In this process, the pressure drop can be manipulated by adjusting the operating conditions; therefore the cell size, cell density, and product expansion can be varied to produce a wide range of products having desired mechanical properties. The combination of shear, temperature and pressure during high-pressure reactive extrusion processing can create opportunities for both conformational changes and chemical reactions in proteins and form new functionalities. The high pressure extrusion process of proteins could be achieved by introduction of dense carbon dioxide. Utilization of dense carbon dioxide in the extruder can decouple the two roles of water in the conventional extrusion cooking processes, i.e., where it plays the role of both a plasticizer and a blowing agent in making expanded extrudates. Expanded extrudates can thus be made at sub 100°C temperatures, which obviates the need for high temperature treatment of heat sensitive proteins and also provides a precise control of the extent of denaturation and reactivity achieved. The nature of the interactions among the various other processing parameters like the ionic strength, the pH and pressure obtained via injected carbon dioxide, shear rate and temperature are not known.

The effects of SC-CO_2 treatments on the functionalities of commercial WP products including WPI and WPC powders and dispersions were investigated by Zhong and Jin (2008). The WPI dispersion was treated with SC-CO_2 at 40°C and 10 MPa for 1 h, whereas WPI and WPC powders were treated with at 65°C and 10 or 30 MPa for 1 h. The authors indicated that the gelling properties were apparently enhanced by SC-CO_2-treatments in all samples. According to the surface hydrophobicity and rheological results, both compositional and structural changes may have contributed to enhanced WP functionalities. In addition, Xu *et al.* (2011) studied the effect of SC-CO_2 treatments on the structure and conformation of WPI using intrinsic fluorescence spectroscopy and fourier transform infrared (FT-IR) spectroscopy techniques. The SC-CO_2 treatment at 60°C leads to partial denaturation of its fractions and exposure of more hydrophobic regions of proteins. It induced the secondary structure change as indicated by a decrease in α-helix content, hydrogen bonds and an increase in the amount of β-sheet. Their results confirmed that the structure and conformation of proteins were modified through SC-CO_2 treatment.

The texturization process of WPs by reactive SCFX was originally proposed by Manoi and Rizvi (2008) as shown in Figure 3. In this research, WP functionalities were modified using a novel reactive SCFX process. A twin-screw extruder cooperated with SC-CO_2 generation unit was used as a continuous bioreactor to generate microcellular extrudates by precisely controlling the above variables. High pressure extrusion of WPs under different pH conditions and in the presence of mineral salts, combined with a delicate control of heat, shear, and internal environments created by introduc-

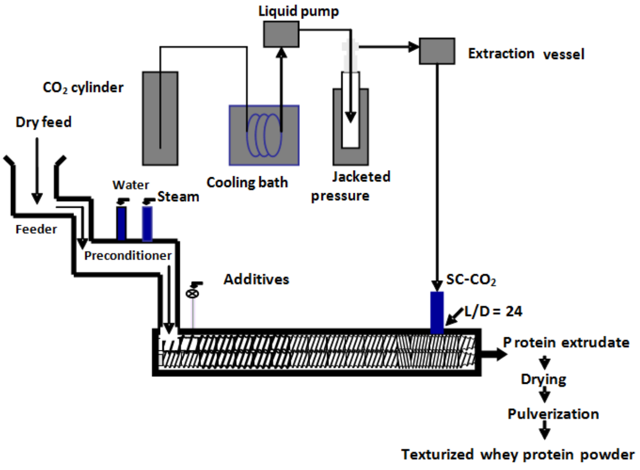

Figure 3. Schematic for production of texturized whey proteins by reactive supercritical fluid extrusion (RSCFX) (modified from Manoi, 2009: Manoi & Rizvi, 2008).

tion of SC-CO_2, was used to texturize and develop unique functional properties in commercially available WPC. High pressure treatment can alter structure and functional properties of proteins by modifying their secondary and tertiary structure (Galazka *et al.*, 2000; López-Fandiño, 2006). The cold-gelling property of modified WPs by SCFX was reported by Manoi and Rizvi (2008). The rheological behavior of modified WPs was found to be strongly dependent on the pH and SC-CO_2 levels used during extrusion. The highest apparent viscosity (η = 2.06 Pa·s) and elastic modulus (G' = 10 kPa) values were observed in the modified WPs produced at extremely acidic condition (pH 2.89) with SC-CO_2 injection and were significantly higher than those exhibited by the unextruded control (η = 0.008 Pa·s, and G' = 0.04 Pa). A 20% (w/w) modified WP dispersion exhibited a highly viscous and creamy texture with particle size in the micron-range (mean diameter ~ 5 μm) which could serve as a thickening/gelling agent or as a fat replacer in food formulations over a wide range of temperatures. In addition, the cold, gel-like emulsions prepared with texturized WPs by reactive SCFX process could be beneficial for controlling the texture of emulsion-filled gel products and their derivatives. Manoi and Rizvi (2009b) reported that homogeneous gel-like emulsion of creamy consistency has been successfully produced by incorporation of corn oil with modified WP dispersion in water. Their results indicated that only 4% (w/w) modified WP was needed to emulsify 80% corn oil and it showed higher thermal stability upon heating to 85°C. Modified WPs also yielded excellent emulsifying properties (emulsion activity index, EAI, = 431 m^{-2} g^{-1}, emulsion stability index, ESI, = 13,500 h) compared to the commercial WPC-80 (EAI = 112 m^{-2} g^{-1}, ESI = 32 h) (Manoi & Rizvi, 2009b). Emulsions prepared with small amounts of modified WPs showed an enhanced adsorption of proteins at the oil-water interface, which prevented flocculation and coalescence of the oil droplets, and an increase in the viscosity of the continuous phase, which prevented creaming by trapping the oil droplets within the gel matrix. These attributes helped generate very stable oil-in-water emulsions of important utility in food formulations and should be useful in new product development (Manoi & Rizvi, 2009b). It is possible that structural changes in modified WPs due to denaturation and polymerization induced by reactive SCFX process lead to an increased surface hydrophobicity and molecular flexibility, allowing an effective adsorption of protein molecules at the oil-water interface.

7. Conclusions

Reactive extrusion, a multi-functional and thermal-mechanical process, has allowed a larger number of food protein modification and functionalization. Effects of reactive SCFX processes on the modification of proteins are usually susceptible to both conformational changes and chemical reactions during extrusion under controlled process conditions such as temperature, screw speed, flow rate, pressure, and environment conditions such as pH, moisture content, and ionic strength. The increase in pressure and temperature as a result of both heat transfer from the heated barrel and the conversion of mechanical energy into heat energy accompanied with the shearing and mixing of extruder screw cause protein denaturation and melting within the extruder barrel subsequently alter the functionalities of proteins. The technological approaches and prototypes to allow the development of unique WP products having a wide range of improved functional properties using a novel reactive process have been established. Appropriate modifications could produce desired functional ingredients in the food industry.

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