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Formation and characterization of thiol-modified fibrillated whey protein isolate solution with enhanced functionalities

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ABSTRACT

The effect of thiolation using propanethiol on the functionalities of fibrillated whey protein isolate (WPI) solution at different pH values was studied. Fibrillated WPI solutions were thiolated at different molar ratios of propanethiol:carboxyl group (0.5:1, 1:1, 2:1, 3:1, 4:1) and the highest esterification extent ratio was obtained at 4:1 (pH 9). We also found that the thiolation process improved the foaming capacity and foam stability. TEM micrographs evidenced aggregation of thiol-modified fibrillated WPI. A network of shortened fibrils attached to each other was formed upon thiolation, suggesting good physical interaction. This was coherent with the increment of zeta potential values, indicating a greater repulsion force to retard fibrils aggregation. Thiolation enhanced emulsifying stability index of thiol-modified fibrillated WPI solution (pH 8) and diminished its susceptibility to pH changes. This has broadened the potential application of fibrils as food ingredients.

1. Introduction

Whey protein, which is a by-product of cheese manufacturing, comprises approximately 50% of β-lactoglobulin (β-LG). As a vital protein found in the whey fraction, β-LG is a water-soluble protein in compact globular form that has been used for decades as a food additive due to its high nutritional value, easy availability and low cost. It is widely accepted that fibrillar formation of whey protein isolate (WPI) is induced under thermal treatment (>80 °C) and acidic conditions (pH 2) with low ionic strength. Strong acidic condition or proteolytic enzyme can be applied in protein hydrolysis prior to protein fibrillation, for instance WPI (Moayedzadeh et al., 2015). Two major fibril formation routes have been highlighted. The first route of fibril formation is initiated by partial denaturation of protein followed by β-sheet alignment. Second rute involves the assembly of polypeptides via β-sheet alignment into fibrillar structure resulting from denaturation and hydrolysis (Jones and Mezzenga, 2012).

Fibrillation of WPI increased β-sheets content associated with decrement of α-helix content (Mohammadian and Madadlou, 2016). Upon assembly via β-sheet stacking into long linear fibrillar aggregates after denaturation and acid hydrolysis, fibrillated WPI typically has a high aspect ratio (length/diameter), with reported lengths on the order of microns and diameter less than 10 nm (Arnaudov et al., 2003). These unique dimensions enable greater entangled network at low protein concentrations owing to its efficient structuring ability. For years, extensive research have been conducted on fibrillar structures owing to their diverse functionality as good emulsifying (Serfert et al., 2014) and foaming agents (Oboroceanu et al., 2014).

In practice, fibrillated WPI solution is fabricated under extremely acidic pH. Most food have pH values between 4 and 7 and the stability of the fibrillated WPI against pH changes when incorporated into food formulations is a matter of concern. Fibrils remain dispersed with reinforced electrostatic repulsion when the pH is further away from the isoelectric point (~5.1). However,

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fibrillated WPI is susceptible to aggregation and experience β-sheet impairment associated with persistence length decreases when the pH is increased above pH 5, resulted from decreased intrafibrillar repulsions (Jones et al., 2011). It has been proposed that disruption of β-sheets could lead to weakening of hydrogen bonds of fibril segments (Mohammadian and Madadlou, 2016). This is related to the stabilizing effect of hydrogen bonds on β-sheets (Kroes-Nijboer et al., 2012). As a result, pH adjustment led to fibril aggregation and negatively affects the viscosity, emulsifying and foaming properties, and turbidity of the fibrillated WPI solution. Recently, researchers have addressed this limitation by using soybean lecithin complexion with β-LG fibrils (Mantovani et al., 2016) or electrostatic interaction with high molecular weight poly(ethylenimine) (Gilbert et al., 2014).

Investigations into protein modification might open up additional options to better address this limitation and provide enhanced functionality. It has been reported that the modification of β-LG using *N*-ethylmaleimide led to improved foaming properties attributed to improved surface hydrophobicity as compared to native β-LG (Croguennec et al., 2006). Moreover, the glycosylation of whey protein using dextran led to greater solubility and thermal stability across a wide pH range (Wang and Ismail, 2012). However, the modification of fibrils has been largely ignored, with past investigations considering only relatively pure solutions (Jones et al., 2010). Notably, fibrils with open structure are readily modified, unlike compact globular proteins. This is because globular protein starts to unfold upon denaturation following β-sheet alignment, thus revealing the hydrophobic core and exposing functional groups. This has enabled the optimization of exposed hydrophobic cores prior to direct cross-linking with modifying agents. Recent work has employed this concept by modifying β-LG fibrils via thiolation using S-acetylmercaptosuccinic anhydride (Munialo et al., 2013), with a promising improvement of gelation efficiency on the modified β-LG fibrils. To date, protein modification is still limited to its native form.

Thus, in this study, our primary objective was to examine the effect of thiol-modification on fibrillated WPI solution, particularly the functionalities obtained by modifying the carboxyl side groups. Each β-LG monomer consists of 27 carboxyl groups that are open to esterification (Halpin and Richardson, 1985). Various studies on modifying carboxyl side groups have been conducted, with remarkable output. Esterified milk proteins showed improved emulsifying properties (Sitohy et al., 2001), whereas methyl esters of β-LG exhibit greater surface activity than ethyl and butyl esters of β-LG (Halpin and Richardson, 1985). Hence, protein modification is an alternative approach to enhance the functionalities of existing food proteins.

2. Materials and methods

2.1. Materials

Whey protein isolate (~70% β-lactoglobulin; BiPRO) was obtained from Davisco Foods International Inc., USA., and 1-propanethiol (99%) and Thioflavin T were purchased from Sigma Aldrich, Co, USA. Other chemicals were of analytical grade, and deionized water was used throughout the experiments.

2.2. Preparation of fibrillated WPI solution

A WPI solution (2.5% w/w) was made by dissolving WPI in deionized water for 2 h with magnetic stirring. After complete dissolution, the WPI solution was adjusted to pH 4.6 using 6 M HCl to precipitate the casein fraction, followed by centrifugation at 6000×*g* (Thermo Fisher Scientific, Waltham, USA) for 30 min to remove precipitated materials. The supernatant was vacuum-filtered through 0.2 μm regenerated cellulose filter paper and readjusted to pH 2 with 6 M HCl. WPI solution was then heated at 80 °C for 20 h under magnetic stirring at 300 rpm and subsequently cooled in an ice water bath for 30 min to stop the fibrillation process.

2.3. Thiolation of fibrillated WPI solution

Fibrillated WPI solution was subjected to modification with pH-adjustment to pH 8 or pH 9 using 6 M NaOH and addition of thiol reagent. 1-Propanethiol was added to the pH-adjusted fibrillated WPI solution instantly at different molar ratios of 0.5:1, 1:1, 2:1, 3:1 and 4:1 (1-propanethiol:total carboxyl residue) at a constant pH (± 0.1) under continuous magnetic stirring (350 rpm) for 24 h at room temperature. The calculation of molar ratio was based on the average molecular weight of a carboxyl residue (45 g/mol) and 27 carboxyl residues per β-LG monomer.

2.4. Quantification of carboxyl modification

Carboxyl modification was quantified based on the formation of colored hydroxymate-ferric ion chelate. In brief, 0.25 mL of sample was mixed with 0.15 mL of EDTA (0.05 M) followed by 0.25 mL of hydroxylamine hydrochloride (1 M) under vortexing. Then, 0.1 mL of sodium hydroxide (6 M) was added to the mixture. The mixture was heated at 75 °C for 5 min prior to hydrolysis and cooled to room temperature. This was followed by the addition of 0.8 mL of hydrochloric acid (1 M) to the mixture before centrifugation at 5600*g* in an Eppenforf™ MiniSpin™ microcentrifuge (Fisher Scientific, Hamburg, Germany). The supernatant fraction was collected and mixed with 80 μL of a 5% (w/v) iron (III) chloride hexahydrate solution. The absorbance of the hydroxymate-ferric ion chelate solution was read at 475 nm using a spectrophotometer (Cary 60 UV-VIS, Agilent Technologies, Santa Clara, USA) using modified fibrils as blank. A standard curve for molar adsorption of iron chelate ($R^2 = 0.98$) was prepared using ethyl thioacetate as standard.

2.5. Thioflavin T (ThT) fluorescence assay

ThT fluorescence assay was used to examine fibril formation of WPI. Upon the completion of thiolation, 4 mL of ThT solution (60 μM) in phosphate buffer (10 mM phosphate, 150 Mm NaCl at pH 7) was filtered (0.2 μm, Minisart, Sartorius) and added to 48 μL of sample. After the binding of ThT to samples for 1 min, the fluorescence intensity of samples, in arbitrary units (a.u.), was measured using a fluorescence spectrophotometer (Perkin-Elmer LS 55, USA) at excitation and emission wavelengths of 440 nm and 482 nm, respectively, and a scanning speed of 200 nm min^{-1}.

2.6. Transmission electron microscopy (TEM)

The morphology of samples was examined using a Hitachi H-7100 transmission electron microscope (Tokyo, Japan) operating at 100 kV. In summary, a drop of diluted sample (50x dilution) was deposited onto a copper grid with a carbon-coated film and left to stand for 1 min. The excess samples were drained off using a filter paper along the periphery. The grid was then placed on a drop of 2% uranyl acetate for staining, and after 1 min, excess stains were removed with filter paper. TEM micrographs were taken at 50,000× magnification.

2.7. Zeta potential

To examine the zeta potential of fibrils, all samples were diluted

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