Optimization of wet heat conjugation method for heat stability improvement of whey protein

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Abstract. Whey protein is known for its excellent emulsifying capacities. However, its industrial application is still limited due to the instability of heating treatment. Dry heat conjugation of whey protein with carbohydrate improved its heat stability. Nevertheless, the method required hours to days of incubation. Hence, it is considered less efficient industrially. Wet heating generally boosts the conjugation of protein and carbohydrates by providing more water to allow protein-sugar interaction. This study aims to investigate the effectiveness of the wet heat conjugation technique to enhance the heat stability of whey protein with shorter incubation time than the dry method. To this end, whey protein concentrate was wet heat incubated with its innate lactose at different temperatures and durations. The incubated WPC was then applied in oil-water-emulsion, followed by heating the emulsions to evaluate their heat stability. The results indicated that the wet heat conjugation method was only capable of increasing the heat stability of whey protein to a limited extent. The particle size of the heated emulsions was smaller than that of control (native WPC), although it was still higher than the unheated emulsions. The wet conjugation technique used in this study was more effective at inducing protein aggregation compared to protein-lactose conjugation.

1 Introduction

Whey proteins were previously only considered as a by-product of the cheese industry. Decades later, it is known for its high nutritional content. Furthermore, studies demonstrated the excellent capacity of whey proteins as natural emulsifiers [1]. Nevertheless, whey proteins are susceptible towards heating temperature. Meanwhile, heating is almost inevitable in food production, as it is required to ensure product safety. As a consequence, the application of whey proteins in the food industry is still limited.

Previous studies [2] have shown that dry heat incubation of whey proteins enables the enhancement of whey proteins' heat stability. However, the dry heat conjugation process typically requires one to eight hours to obtain the required degree of conjugation. A shorter conjugation duration would undoubtedly be highly beneficial to encourage its industrial application.

Wet heating is another alternative approach to initiate protein conjugation. It generally requires a shorter incubation time than dry heating and is easier to operate. Xi et al. (2020) [3] discovered an improved emulsifying properties of WPI upon wet heat glycation with glucose. However, the difficulty in regulating the reaction to maintain the native structure of the proteins is a major concern of this technique [4].

This study aimed to optimize the wet heat conjugation technique to facilitate the conjugation between whey protein with lactose with the goal to increase its heat stability. The obtained heat-treated proteins were then applied in oil-in-water emulsions, which were further evaluated for their stability against heat treatment.

2 Materials and methods

2.1 Materials

A commercial whey protein concentrate (WPC80; Organic whey protein®, Frenchtop Natural Care Product BV, The Netherlands) was used as the starting material. It contained 80% protein, 12% lactose, 4.4% fat, and 2.8% ash. WPC35 (Carbelac 35 (HF)®,

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Carbery, Ireland) contained 34% protein, 46% lactose, 10% fat, and 6.5% ash.

Lactose monohydrate (95% dry matter, Merck®, Germany) increased lactose content during the spray-drying conjugation. Imidazole buffer as the dissolving solution consisted of 20 mM imidazole, 50 mM NaCl, and 5 mM $CaCl_2$. Commercial sunflower oil (bought from a local shop) was used to prepare 10% (w/w) oil-in-water (o/w) emulsions. 1 N NaOH was used to adjust the pH of protein suspension. All chemicals used in this experiment were analytical grade.

2.2 Sample preparation

WPC80 or WPC35 powder was dissolved in water containing 0.015% NaN_3 to reach 10% of protein concentration (w/v). The solution was then kept overnight at 4 °C followed by pH adjustment to 7.0 by using 1N NaOH. 10 ml of the adjusted solution was then transferred to a 15 ml glass tube and incubated in a water bath at the defined temperature (i.e., 55 and 60 °C) and time (0 to 24 hours). The wet heat conjugation of WPC35 was conducted at 55 °C to avoid a too rapid Maillard reaction.

2.3 Emulsion preparation

Emulsion was prepared according to A'yun et al. (2020) [2]. The aqueous phase of all emulsions contained 0.5% of whey protein (protein content in each WPC80 and WPC35 was taken into account) in imidazole buffer. Sunflower oil was added to produce 10% (w/w) O/W emulsions. About 50 ml of this mixture was prehomogenized using an Ultra Turrax (Janke-Kunkel, Germany) at 24000 rpm for 2.5 minutes, followed by microfluidization of a subsample of 25 ml at a driving air pressure of 4 bar, corresponding to 560 bar of liquid pressure, at 30 °C for 2 minutes in a Microfluidizer M110S (Microfluidics, USA).

The heat stability test was conducted by placing 8 ml emulsion sample in a 10 ml glass tube. Subsequently, the emulsion was heated in a water bath at 60 °C for 30 minutes, followed by the particle size measurement.

2.4 Particle size measurement

Particle size and its distribution of the whey protein-stabilized emulsions, both before and after heating test, were determined using a Mastersizer 3000 (Malvern Instrument Ltd, Malvern, UK). The measurement was conducted as according to A'yun et al. (2020) [2].

2.5 Conjugation degree

The degree of protein conjugation was determined through the OPA (o-phthalaldehyde) method as adapted from Saatchi, Kiani, & Labbafi (2019)[5] with slight modifications. The method measures the number of free amino groups (-NH2) in the sample. During conjugation, the number of free $-NH_2$ groups is reduced since they react with the aldehyde group of a reducing sugar. Thus, the reduction of the free –NH2 content is an indicator of protein conjugation. Conjugation degree was calculated using Equation 1.

$$\text{Conjugation degree (\%)} = \frac{(Ao - A1)}{Ao} x\ 100 \quad (1)$$

Hereby, A_0 and A_1 are the absorbance of the native and incubated WPC, respectively.

2.6 Whey protein nitrogen index (WPNI) analysis

The amount of denatured protein after the wet heating treatment was determined using whey protein nitrogen index (WPNI) method as adapted from Leighton (1962) [6]. The denatured whey proteins were precipitated using a saturated NaCl solution followed by ultracentrifugation. The content of non-denatured protein in the supernatant was then measured using the simplified Lowry method [7].

2.7 Statistical analysis

Statistical analysis was performed on conjugation degree as well as denaturation degree data using IBM SPSS Statistics 25 software. The statistical difference were determine using One-way ANNOVA with Duncan post hoc test. A difference was regarded significant when $p < 0.05$.

3 Results and discussion

3.1 Emulsifying and heat stabilizing properties

Wet heating is commonly used to induce protein conjugation, which might also act as a pretreatment prior to spray drying. As this study mainly aimed to improve the emulsifying and heat stabilizing capacity of whey proteins, the effectivity of wet heating was first evaluated by applying the conjugates in emulsions. Meanwhile, a shorter incubation time was applied at 60 °C than at 55 °C since a higher temperature might promote faster Maillard reaction as well as (undesired) protein denaturation.

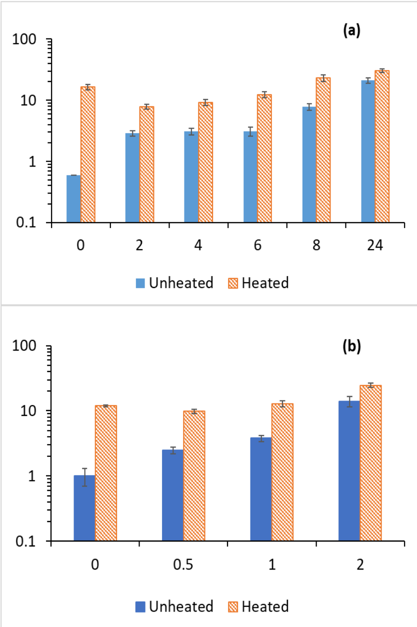

Fig. 1. Average particle size (d_{43}) of emulsions stabilized by WPC80 that was wet heat conjugated at different temperature and duration, both before and after heating at 60 °C for 30 min.

The wet heated WPC80 tended to reduce the emulsifying capacity as shown by the higher particle size of the unheated emulsions, along with the incubation time, both at 55 and 60 °C (Fig. 1). The highest particle size was shown by emulsions stabilized by WPC80 wet heat conjugated for 24 h at 55 °C (i.e. 21.0 ± 2.1 μm) and 2 h at 60 °C (i.e. 14.0 ± 2.6 μm). The average size increment was due to the increased proportion of the larger sizes (above 1 μm) as depicted by the particle size distribution (Fig. 2). A'yun et al. (2020)[2] and Wu et al. (2021)[4] suggested that this larger particle size was due to droplet aggregation. This was shown by the decreased particle size upon dilution with SDS. Prolonged incubation may promote protein polymerization due to the production of the advanced Maillard reaction products as well as inter-protein cross-linking.

Upon heat treatment of the emulsions at 60 °C for 30 min, the wet heat conjugated WPC80 at both temperatures and all incubation times could not stabilize the emulsions. This was shown by the increased particle size after heating. Nevertheless, as compared to the native WPC, the heated emulsions stabilized by incubated WPC80 for 2 and 4 hours at 55 °C and 0.5 h at 60 °C showed smaller droplet size than the native-stabilized emulsion. Meanwhile, prolonged incubations produced more droplet aggregation than the native WPC. This indicated that the applied wet heating method could not effectively improve the heat stabilizing capacity of whey proteins.

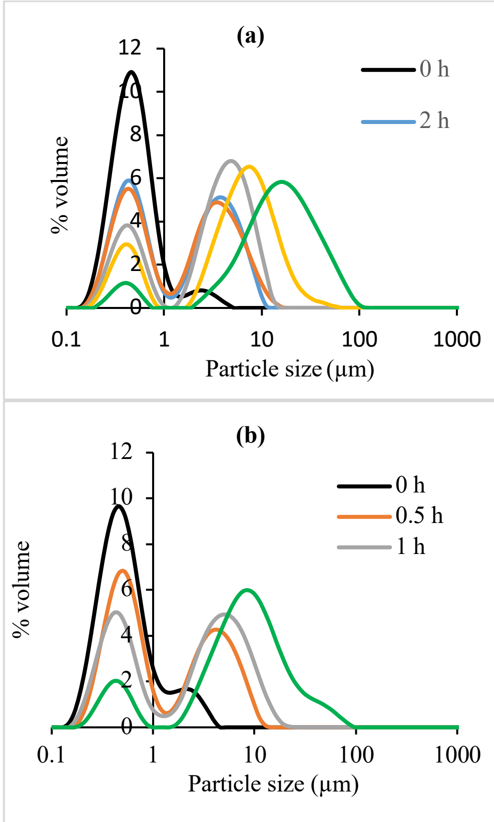

Fig 2. Particle size distribution of unheated emulsions stabilized by WPC80 that was wet heat conjugated at (a) 55 or (b) 60 °C at different incubation times (up to 24 hours).

The fact that the wet heat incubation of WPC80 could not effectively improved heat stabilizing capacity of WPC80 was then presumed due to the insufficient amount of the innate lactose. As such, wet heat conjugation was applied on whey protein with higher lactose content, that is, WPC35. The use of WPC containing a higher amount of lactose (WPC35, containing 34% protein and 46% lactose) is presumed to undergo a faster Maillard reaction as more reactant (lactose) was available for the reaction.

Figure 3 depicted the particle size of the emulsions stabilized by native and incubated WPC35. The particle size of the unheated emulsions stabilized by both native and incubated WPC35 were above 1 μm. The excess amount of lactose in WPC35, as compared to WPC80, disrupts the contact in between protein molecules in the aqueous phase. Thus reducing protein ability to form a viscoelastic layer to cover the oil droplets. Hence producing bigger droplets. Prolonged incubation to 8 h has a negative impact, as shown by the higher particle size of the unheated emulsions.

The heat stability slightly increases upon longer wet heat preconditioning of WPC35, but the effect is limited, even after 8 hours (Fig. 3). Despite the presence of high amount lactose, the innate lactose in

WPC35 might not be readily accessible for conjugation with whey proteins.

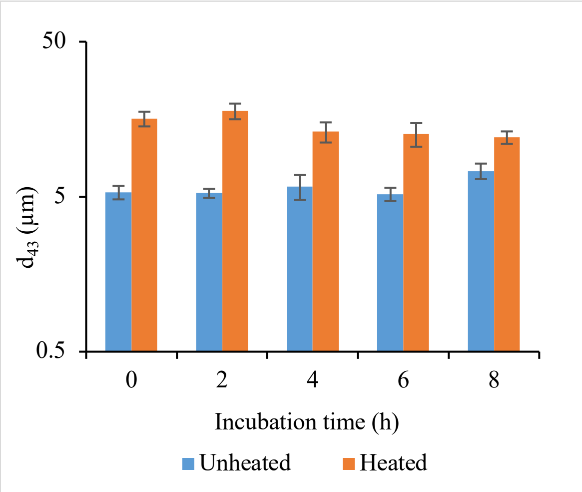

Fig. 3. Volume-weighted average particle size (d_{43}) of 10% o/w emulsions stabilized by WPC35 wet heat preconditioned at 55 °C and 10% w/v whey protein concentration in an aqueous solution at pH 7.0, before and after heating of the emulsion at 60 °C for 30 min.

3.2 Degree of conjugation and aggregation

Given that the wet heating method indicated limited improvement in the heat stabilizing properties of WPC80 and WPC35, it is intriguing to investigate if the protein conjugation occurred. Furthermore, the reduced emulsifying capacity of WPCs may have implied that the incubation tended to induce protein polymerization (e.g., due to protein crosslinking). A recent study by Nielsen et al. (2022)[8] observed a potential competition between Maillard reaction and sugar-independent protein crosslinking in whey proteins. Therefore, the incubated WPC80 was then evaluated for its conjugation and denaturation degree.

WPC80 that was wet heat conjugated at 55 °C indicated nearly no protein conjugation during the first 2 hours of incubation (Fig. 4). A significant conjugation (i.e. 9.4% conjugation degree) occurred after 8 hours of incubation. Meanwhile, prolonged incubation to 24 h was shown to lower the conjugation degree. In contrast, the degree of protein denaturation steadily increased in line with the incubation time.

A significant denaturation was observed after 2 h of incubation (i.e. 11%) and steadily increased to around 37% after 24 h. Moreover, whey protein denaturation appeared to be more dominant than protein conjugation with lactose. During heating of the whey protein solution, β-lg as the main protein constituent most likely unfolds its native structure, exposing hydrophobic residues and the free –SH group. As such, this may promote inter-protein attractive interaction to generate protein polymerization. The high degree of protein denaturation was proposed to be the cause of the reduced emulsifying capacity of the wet heat conjugated WPC. Furthermore, it may counteract the enhancement effect of protein conjugation. As a result, no heat stabilizing capacity of the wet heat conjugated WPC was observed.

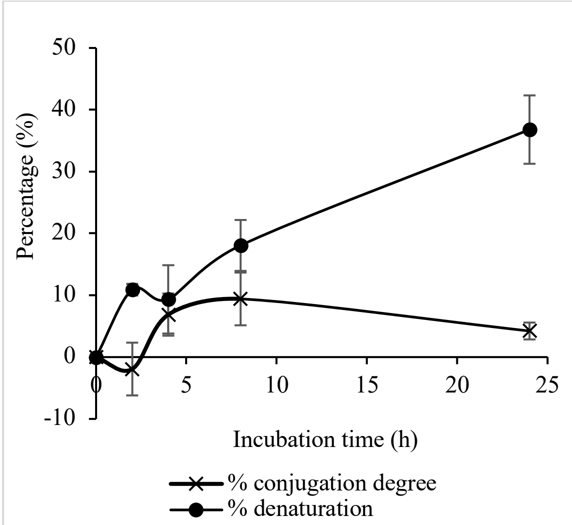

Fig. 4. Degree of conjugation (as determined by OPA method) and denaturation (determined by WPNI) of WPC80 that was wet heat conjugated at 55 °C for up to 24 hours. The standard deviations were calculated from 3 technical repetitions

4 Conclusions

The wet heat conjugation method tended to lower the emulsifying capacity of whey proteins and gave limited positive effect on their heat stabilizing capacity. These were shown by the increased particle size of the unheated emulsions stabilized by the incubated WPCs (WPC80 and WPC35).

Using WPC with higher lactose content (WPC35) did not result in better techno-functional properties of the wet heated whey proteins. The applied wet heating was found to more dominantly result in protein denaturation than protein conjugation. The findings in this research may be beneficial for future work that considers the effectivity of wet heating in protein-sugar conjugation.

Qurrotul A'yun: Writing original draft, visualization, investigation, methodology, data curation.

Chusnul Hidayat: supervision.

Paul Van der Meeren: supevision, methodology, reviewing, resources.

Data will be made available on request.

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