Physicochemical Properties and Heat Stability of Whey Protein Isolate-Lactose Conjugates Formed by Dry-Heating

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ABSTRACT Conjugation via MR consider as the safest and potential method in food industry. However, it is important to control the extent of conjugation via MR since the browning effect could lead to the health issues. There is great interest to understand the chemistry of MR, to improve the physicochemical properties, and to discover the potential of Maillard products with various functionalities. Whey protein isolate (WPI) has become an important source of functional ingredients in various health-promoting foods. However, WPI have problem with thermal instability that present during food processing. Therefore, this study aims to investigate the effect of dry-heating at different incubation time, then monitor the physicochemical properties and heat stability of WPI-Lactose conjugates. Conjugation of WPI with lactose was achieved by dry-heating with the ratio of lactose to WPI 1:0.4 (wt/wt). Incubation time varying from 0 to 10 days at 40°C and water activity A_w = 0.79. *o*-pthaldialdehyde (OPA) assay was used to monitor the extent of conjugation. An incubation time of 3 days was selected as the standard conjugation time based on conjugation rates and the degree of Maillard browning. The result revealed that WPI-Lactose conjugates at 3 days incubation has slightly improved heat stability of protein. Thus, a new approach of WPI-Lactose conjugates has potential to produce better heat resistance milk protein products in the future.

KEYWORDS: WPI-Lactose conjugates; Maillard Reaction (MR); Dry-Heating; Physicochemical Properties; Heat Stability.
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INTRODUCTION

Whey protein (WP) ingredients have become increasingly important in formulated foods over the past 30 years. The major WPs, beta-lactoglobulin (β-Lg) and alpha-lactalbumin (α-Lac) are responsible for the heat stability characteristics (Burrington, 2012). However, beta-lactoglobulin (β-Lg) and alpha-lactalbumin (α-Lac) proteins are adversely affected by heating. In line with the development of value-added products, researchers have improved the heat stability of whey proteins (WPs). Therefore, one of the main targets in many WPs studies is to overcome the thermal instability of the proteins. Whey protein powders should be suitably hydrated to achieve optimal performance during heat treatment.

Recent review paper published by Doost *et al.* (2019) has described both conventional (dry and wet heating) and novel (pulsed-field gradient, sonication, extrusion, high pressure, and electrospinning techniques) methods of conjugate. Although dry heating has been extensively used in many studies as a conventional method to produce WP conjugates, many others research also has been focused on new approaches to produce the conjugates on an industrial scale.

On the other hand, Visser (1988) has reported that lactose could increase the heat stability of whey proteins since lactose affects the hydrophilicity and thus, it affects the solubility behaviours of the whey proteins during heat treatment. Meanwhile, the lactose concentration can also affect the solubility of whey proteins following heat treatment. Lactose is an example of reducing sugar that is widely used in milk protein conjugation via MR. When lactose is bound to protein, conjugation has

improved the heat stability properties and emulsifying properties if comparing to the non-conjugated proteins (Nacka *et al.*, 1998).

The aim of this current study is to determine the optimal conditions, using incubation time, for WPI to conjugate most efficiently with lactose. Incubation time (e.g. from 0 to 10 days), temperature (e.g. 40°C), and humidity (or water activity, e.g. A_w = 0.79), are highly important in the food industry to control the extent of Maillard browning between WPs and sugars as they can offer functional property enhancements such as tolerance to heat treatment and can therefore offer better use as a food ingredient. In this study, the physicochemical properties and heat stability of modified WPI are also compared with unmodified WPI.

BACKGROUND THEORY

Arrhenius Equation Theory

Temperature and duration of heating were studied by Maillard (1912) himself, who reported that the rate of the reaction increases with temperature. An increase in temperature leads to an increase of the reactivity between the sugar and the amino group. The temperature dependence of a reaction rate constant *k* is often described by the well-known Arrhenius equation which is given as the following Equation (1):

$$k = A * exp\left(-\frac{E_a}{RT}\right) \quad (1)$$

where *k* is the rate constant; *A* the so-called frequency factor; *Ea* the activation energy; *R* the gas constant (8.3 J $mol^{-1}K^{-1}$) and *T* is the absolute temperature (K).

Heat Capacity Theory

DSC is a technique used for determining the quantity of heat that is either absorbed or released by a sample undergoing a physical or a chemical change. Heat capacity (*Cp*) represents the quantity of energy needed to raise the temperature of unit of mass of sample by 1°C (Durowoju *et al.*, 2017). The relationship between heat flow and heat capacity in DSC experiments can be illustrated from the following Equation (2) and Equation (3):

$$\frac{dH}{dT} = cp\frac{dT}{dt} + f(T,t) \quad (2)$$

$$C_P = \frac{dH}{dT} \quad (3)$$

where, C_p is heat capacity, d*H*/d*t* is heat flow, d*T*/d*t* is heating rate and *f(T, t)* is heat flow due to kinetic process.

METHODOLOGY

Preparation of Dry-Heating WPI-Lactose Conjugates

The WPI (BiPRO, Davisco, Foods International, Inc.) and lactose (Fonterra, Kapuni, New Zealand) mixture was prepared with final ratio of lactose to WPI is 0.4:1 (wt/wt). The solution was then freeze-dried for 24 h and divided for different lengths of time (0 to 10 days). Dried mixture incubated in water activity A_w = 0.79 at 40°C. Sample of WPI alone without lactose was used as control in this study.

Preparation of OPA Analysis

In OPA analysis, 8 mg of OPA was dissolved in 0.2 mL methanol and 0.25 mL of 20% w/v sodium dodecyl sulphate (SDS), then 10 mL of 0.1 M sodium tetraborate adjusted to pH 9.3. Then, 17.9 uL of 2-mercaptoethanol was added (Julmohammad, 2007). Absorbance was recorded at 340 nm using 96-well plate reader (EnSpire® Multimode Plate Reader, Finland). Leucine was used as standard. The solutions were pipetted into a 96-well clear plate then 200 µl of OPA reagent was added. The reaction was allowed to proceed for 5 min.

Heat Stability Analysis using Differential Scanning Calorimetry (DSC)

A Q1000 differential scanning calorimeter (DSC; TA Instruments, Newcastle, New Jersey, USA) was used for the calorimetric analysis of conjugated milk protein powder. The method used was based on that reported by Julmohammad (2017). All samples were weighed (2-3 mg) into aluminum DSC sample pans (Tzero pan), then about 6 µL (three times mass of milk protein powder) of 10 mM phosphate buffer (pH 7.4) was added slowly on to the sample inside the pan. The heating rate for the DSC scan was 5°C/min over the range 25–105°C. The DSC measurements were done in triplicate. DSC data were analysed with the Universal Analysis Software (version 3.6C) for thermal analysis, which was provided with the instrument (TA Instruments).

Statistical Analysis

Statistical analysis was carried out using IBM SPSS statistics software (Version 22, IBM Corp., 2013). The temperature values and heat capacity for DSC analysis were analysed using one-way analysis of variance (ANOVA). ANOVA was also carried out for comparison of mean values, which were further separated using Duncan significant difference test.

RESULT AND DISCUSSION

Visualization through observation of the browning effect of each conjugated milk protein powder resulted in qualitative analysis of the whey protein-sugar conjugation. The molecules causing brown pigmentation are produced during the MR, and also the formation of side products after the MR. Plus, caramelisation could occur, which means that the sugar reacted with itself and formed browning coloration, and thus did not contribute to any protein-sugar conjugation. Figure 1 shows the change in colour of WPI alone and WPI-Lactose over different incubation times.

From Figure 1, WPI alone has shown no visual colour changes in the protein powders although they were incubated for 10 days. The WPI powder is a milk protein powder comprising β-Lg and α-Lac with very trace amount (0.4 ± 0.2%) of lactose. Since there is not much lactose for sugar conjugation, the protein powder should not experience significant browning changes. Meanwhile, WPI-Lactose showed light-yellow colour after 3 to 10 days. There is a compromise between the degree of conjugation of the protein and sugar, and the subsequent MR. Lund & Ray (2017) has critically reviewed numerous different strategies for controlling Maillard reactions in foods and has reported some recent advances in strategies for controlling the Maillard reaction and subsequent downstream reaction products in food systems. In order to avoid samples becoming too brown, a possible indication of Maillard browning, incubation of samples was limited to 3 days based on the visual observation seen in Figure 1. Brown colour development is commonly applied as an indicator to detect the MR which takes place in foods, since it is easily measurable (Sun *et al.*, 2011).

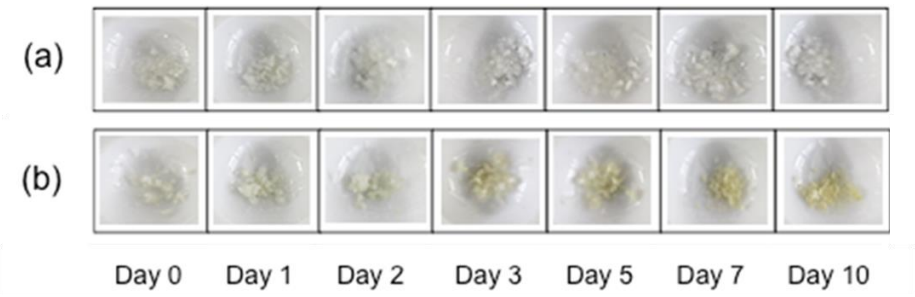

Figure 1. Conjugation from 0 to 10 days at 40°C, A_w = 0.79 for (a) WPI alone and (b) WPI-Lactose.

Protein-sugar conjugates were analysed for available primary amino groups using the OPA assay method to give an indication of the degree of conjugation. This assay has benefits in terms of its simplicity and sensitivity and is useful for routine analyses to quantify available lysine (Lys). The ε-amino group of Lys is known to be most reactive and, thus, the first that conjugates with sugars in the course of the MR (Oliver *et al.*, 2006). When sugar was conjugated to either the α-amino group of the lysine residue or the ε-amino group of the polypeptide chain, it was possible to determine the approximate the degree of conjugation by quantifying the number of lysine groups that remain at a given point during the reaction using OPA analysis (Anema *et al.*, 2005).

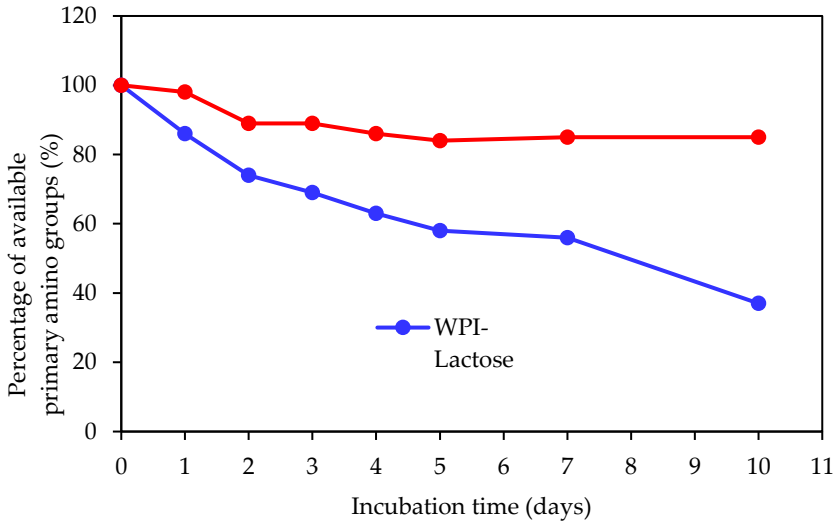

Figure 2. OPA assay expressed as percentage free amino groups of WPI alone and WPI-Lactose with as a function of incubation time (days) at 40ºC.

The number of free amino groups are expressed as a percentage of proteins of the total free amino groups from the 0-day incubation (100%). From the graph shown in Figure 2, the OPA value for WPI-Lactose decreased to 86% after 1 day of incubation and increased the OPA value declined to 69% after 3 days of incubation. After 10 days of incubation, about 63% lactose was conjugated to WPI, with only 37% OPA value remaining. This pattern of OPA value for WPI-Lactose decreased with incubation time. The current study showed that there was a significant decrease in the percentage of available amino groups after 2 days of incubation. There was 26% of amino groups available slightly higher than the 20% reported by Anema *et al.* (2005) for similar conjugation condition.

To investigate the thermodynamic and heat stability of the unmodified whey proteins and the modified whey proteins, measurements using a Differential Scanning Calorimetry (DSC) were conducted. DSC might be able to show that conjugating WPI-Lactose at 3 days could increase their heat stability compared to WPI alone. The point of T_d peak and T_d onset act as an indication for denaturation temperatures of the proteins. In this current study, the denaturation temperature T_d peak

for WPI-lactose (T_d onset = 72.74 ± 0.41 and T_d peak = 77.66 ± 0.83) has slightly increased compared to WPI alone (T_d onset = 69.61 ± 0.35 and T_d peak = 73.20 ± 0.16).

Results of WPI alone and conjugated WPI-Lactose are shown in Table 1. From the results, the heat stability of WPI has slightly improved when WPI was conjugated with lactose. Although the results not significantly change for heat stability of WPI-Lactose 3 days if compared to WPI alone, slight improvement of heat resistance shows that lactose has potential in the WPI-lactose conjugates. Chen *et al.* (2015) also reported that conjugation significantly improved β-Lg (T_d onset = 66.2°C ± 0.2 and T_d peak = 76.1°C ± 0.0) when glycated with ribose (T_d onset = 74°C ± 0.3 and T_d peak = 78°C± 0.0), glucose (T_d onset = 75.2°C ± 0.1 and T_d peak = 79.5°C ± 0.0), maltose (T_d onset = 76.3°C ± 0.0 and T_d peak = 82.2°C ± 0.2), maltotriose (T_d onset = 76.8°C ± 0.1 and T_d peak = 82.9°C ± 0.0) , fructose (T_d onset = 75.1°C ± 0.6 and T_d peak = 80.5°C ± 0.2) and galacturonic acid (T_d onset = 78.5°C ± 0.2 and T_d peak = 86.2°C ± 0.1). They suggested that this is due to the increase in in molecular size, ketose and negative charges.

Table 1. DSC parameters, heat stability range and heat capacity, for WPI alone and WPI-Lactose.

Sample (s)	T_d onset (°C)	T_d peak (°C)	Heat capacity (J/g)
WPI only	69.61 ± 0.35[a]	73.20 ± 0.16[a]	4.97 ± 0.40[a]
WPI-Lactose	72.74 ± 0.41[a]	77.66 ± 0.83[a]	5.37 ± 0.85[a]

Standard deviations represent standard errors, n=3. Data values annotated with same letters are not significantly different at $p > 0.05$, ANOVA, Duncan test.

CONCLUSION

Conjugation to form modified proteins was investigated, and optimized conditions involving 3 days heating at 40°C were chosen. Although the extent of conjugation cannot be determined accurately using OPA assay, the number of free amino groups available or percentage of conjugation, before and after conjugation can be estimated. This work has shown that sample WPI-Lactose at 3 days resulted approximately 30% of conjugation. Visual observation shows that increasing incubation times from 0 to 10 days for the conjugation of WPI to lactose at 40°C and A_w = 0.79, resulted in a browner colour, reflecting increasing Maillard browning. Finally, in this study, we have demonstrated through DSC, that conjugation can slightly improve the heat stability of WPI when conjugated to lactose compared to WPI alone.

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