THE IMPACT OF CONJUGATED WPI-LACTOSE ON THE PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES AND *IN-VITRO* INFANT DIGESTION

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FACULTY OF FOOD SCIENCE AND NUTRITION
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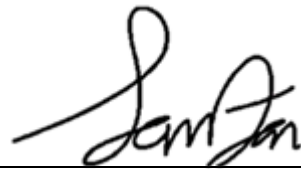

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ABSTRACT

Proteins are crucial for body metabolism, are abundant in whey proteins isolate (WPI) with a high concentration of essential amino acids. The natural form of WPI has limitations as it is susceptible to temperature, pH changes and digestibility in gastrointestinal tract of infant. The influence of protein processing is significant but is dependent upon the variety of parameters and controlled conditions. Thus, the aim of the current study to evaluate the physicochemical and functional properties of conjugates proteins of WPI-Lactose (WPI-Lac) versus native WPI under the optimized condition (temperature of reaction is 40°C, 1,3,5 and 7 days of incubation time, 0.8 of water activity (A_w) and a protein-to-disaccharide ratio of 1:0.4) of Maillard reaction (MR). This study also investigates the impact of the conjugated proteins-lactose in proteins digestibility on infant static *in-vitro* digestion model. Amongst those conjugates proteins, the WPI-Lac day 3 is showed the particularly acceptable browning colour intensity of Maillard reaction products (MRPs) (0.784±0.000, 290nm), (0.197±0.000, 420nm) and have colour index (ΔE) (90.37) and chroma value (C) (17.96) increased with the incubation time. The conjugated proteins increased in molecular weight and were elucidated in SDS-PAGE gel by slightly shifting upwards and smearing from day 1 to day 7 on incubation time (>20 kDa). 75% of the free amino group available in WPI-Lac (day 3) which indicated only 25% of leucine was conjugated with lactose and this prevent the advanced-stage reaction of MR. The conjugates protein clearly changed ($p<0.05$) in Amide I (1634.52 cm^{-1}) and Amide II (1500 cm^{-1}) from day 0 to day 7. Higher solubility of protein is shown in WPI-Lac (day 3) at 36.27%, while the $ABTS^+$ radical scavenging activity of conjugates protein significantly increased ($p<0.05$) with incubation time from 10.14% (day 0) to 61.94% (day 7), respectively. In The heating treatment of WPI-Lac (day 3) by MR and conjugated with lactose under optimized conditions of MR has enhanced the digestibility of the α-Lac which were completely disappear after 5 minutes of digestion in gastric digestion. Additionally, after 60 minutes, the α-Lac of WPI-Lac (day 3) was breakdown into smaller peptides (<10kDa) as shown in the SDS-PAGE. In *in-vitro* duodenal digestion, β-Lg of WPI-Lac (day 3) was mostly completely degraded at 100 minutes of digestion. Thus, dry MR of protein at 40 °C for three days with 0.8 water activity and conjugated with lactose may be a useful alternative for improving the digestibility of protein in infants' digestive systems. Additionally, due to the ability of β-Lg to be digested in the duodenal digestion phase, conjugated proteins have a significant potential to give strong impact on the release of the immunogenic protein.

ABSTRAK

KESAN PROTEIN ISOLAT WEI-LAKTOSA TERHADAP SIFAT FIZIKOKIMIA DAN FUNGSI SERTA PENCERNAAN BAYI IN-VITRO

Protein adalah penting untuk metabolisme badan dan terdapat dalam protein isolate wei (WPI) dengan kepekatan tinggi asid amino penting. Bentuk semula jadi WPI mempunyai had kerana ia mudah terdedah kepada suhu, perubahan pH dan kebolehcernaannya dalam saluran gastrousus bayi. Pengaruh pemprosesan protein adalah ketara tetapi bergantung kepada pelbagai parameter dan keadaan terkawal. Oleh itu, matlamat kajian ini untuk menilai sifat fizikokimia dan fungsi protein konjugat WPI-Laktosa (WPI-Lac) berbanding WPI asli di bawah keadaan optimum (suhu tindak balas ialah 40 °C, 1,3,5 dan 7 hari inkubasi, 0.8 aktiviti air (Aw) dan nisbah protein kepada disakarida 1:0.4) Kajian ini juga mengkaji kesan protein konjugasi-laktosa dalam kebolehcernaan protein pada model pencernaan in-vitro statik bayi. Di antara protein konjugat tersebut, WPI-Lac hari ke-3 menunjukkan keamatan warna peperangan yang boleh diterima untuk produk tindak balas Maillard (MRPs) (0.784±0.000, 290nm), (0.197±0.000, 420nm) dan mempunyai indeks warna (ΔE) (90.37) dan nilai kroma (C) (17.96) meningkat dengan masa inkubasi. Berat molekul protein konjugasi meningkat dan dijelaskan dalam gel SDS-PAGE dengan sedikit beralih ke atas dari hari 1 hingga hari 7 inkubasi (<20 kDa). 75% daripada kumpulan amino bebas yang terdapat dalam WPI-Lac (hari ke-3) yang menunjukkan hanya 25% leucine terkonjugasi dengan laktosa dan ini menghalang tindak balas peringkat lanjut MR. Protein konjugat jelas berubah ($p<0.05$) dalam Amida I (1634.52 cm-1) dan Amida II (1500 cm-1) dari hari 0 hingga hari 7. Keterlarutan protein yang lebih tinggi ditunjukkan dalam WPI-Lac (hari 3) pada 36.27%, manakala aktiviti penghapusan radikal ABTS+ protein konjugat meningkat dengan ketara ($p<0.05$) dengan masa pengeraman masing-masing daripada 10.14% (hari 0) kepada 61.94% (hari ke-7). Dalam Rawatan pemanasan WPI-Lac (hari ke-3) oleh MR dan konjugasi dengan laktosa di bawah keadaan optimum MR telah meningkatkan kebolehcernaan α-Lac yang hilang sepenuhnya selepas 5 minit pencernaan dalam pencernaan gastrik. Selain itu, selepas 60 minit, α-Lac WPI-Lac (hari ke-3) dipecahkan kepada peptida yang lebih kecil (<10kDa) seperti yang ditunjukkan dalam SDS-PAGE. Pencernaan duodenal in-vitro, β-Lg WPI-Lac (hari ke-3) kebanyakannya terdegradasi sepenuhnya pada 100 minit pencernaan. Oleh itu, MR kering protein pada suhu 40 °C selama tiga hari dengan aktiviti air 0.8 dan berkonjugasi dengan laktosa mungkin merupakan alternatif yang berguna untuk meningkatkan kebolehcernaan protein dalam sistem pencernaan bayi. Di samping itu, disebabkan oleh keupayaan β-Lg untuk dicerna dalam fasa pencernaan duodenal, protein terkonjugasi mempunyai potensi yang besar untuk memberi kesan terhadap pembebasan protein imunogenik.

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LIST OF ABBREVIATIONS

ABTS	-	[2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]
FTIR	-	Fourier-Transform Infrared Spectroscopy
kDA	-	Kilodalton
MR	-	Maillard reaction
MRPs	-	Maillard reaction products
MW	-	Molecular weight
SDS-PAGE	-	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
UV-VIS	-	Ultraviolet-Visible Spectrophotometry
WPI	-	Whey Protein Isolate
WPs	-	Whey proteins
α-Lac	-	Alpha-lactalbumin
β-Lg	-	Beta-lactoglobulin
PC	-	Phosphatidylcholine

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CHAPTER 1

INTRODUCTION

1.1 Background

The nutrients found in milk, including lactose, proteins, lipids, vitamins, and minerals, are remnants of whey. In the dairy industry, whey is a by-product of cheese making that consists of the water-soluble remnants that are left over once the casein fraction has been removed (Sedaghat Doost *et al.*, 2019). Whey is widely used as an ingredient in value-added products. In the food industry, whey proteins (WPs) show diverse functionality such as emulsifying, foaming formation, thermal stability, texturizing and gelling (Nooshkam *et al.*, 2020; Setiowati *et al.*, 2020; Sedaghat Doost *et al.*, 2019). Essential amino acids, which are crucial for body metabolism, are abundant in WPs. The WPs are highly valuable hydrocolloids due to their versatile properties that can be utilized to produce various food products. Whey, notably protein, has significant nutritional and technological functionalities that make it useful for food applications (Zhao, Chen & Ashaolu, 2022). High-quality globular proteins extracted by microfiltration, concentration and spray drying from the whey fraction of milk are known as whey protein isolates (WPI) (Meng & Li, 2021). WPI is highly bioavailable with a high concentration of essential amino acids with a protein content higher than 90%, meanwhile 0.5-1.0% is lactose and fat, 2.0-3.0% is ash and 4.5% is water content (Khan *et al.,* 2019; Khaire & Gogate, 2019; Guo & Wang, 2019). WPI is an essential functional ingredient utilized for various applications in the food industry and used as ingredient in infant formula (Zhang *et al.,* 2021; Hong *et al.,* 2022).

However, the natural form of WPI has limitations as it is susceptible to temperature and pH changes, which makes meeting industrial demands challenging (Jia *et al.,* 2020; Ping-Ping *et al.,* 2020; Zhang *et al.,* 2021). The structure of

proteins has a direct impact on their functional performance. WPI is a type of protein that is made up of structured globular proteins. These proteins have primary, secondary, tertiary, and quaternary structures. Under normal pH, ionic strength, and temperature conditions, the WPI are in a specific conformation known as the native state. Protein denaturation that modifies the native state of protein or unfolding and aggregation of unfolded molecules can occur under chemical, enzymatic or physical treatment that affects the WPI in conformation, physiochemical and functional properties (Guo & Wang, 2019). Consequently, finding effective modification techniques to enhance the physiochemical and functional properties of WPI is crucial. As to improve the physiochemical and functional properties of milk protein in WPI, heat treatment is widely used in all type of dairy products. Nevertheless, WPI is heat susceptible protein. The destruction of some forces that stabilize the native conformation of protein such as hydrogen bonds and hydrophobic interactions is due to the heat treatment. Protein molecules unfold due to protein conformational rearrangements, exposing reactive amino acids to the aggregation of protein molecules at certain pH values. As the main colloidal aggregates in WPI are formed by heating, the pH and calcium in WPI may trigger subsequent nonspecific aggregation (Guo & Wang, 2019). Heat processing imparted many desirable features to food, but it may also have carcinogenic and mutagenic impacts on human health as mentioned in Hamzalıoğlu & Gökmen, (2020). Added to that, Bogahawaththa *et al.* (2018) said that heating milk protein can affect immunogenic epitopes and their accessibility, resulting in altered immunogenicity. As a result, the use of the chemical modification known as Maillard reaction (MR) to form conjugates between protein and reducing sugar has been extensively researched in order to improve protein heat stability in industrial processing.

MR is a non-enzymatic reaction (Gupta *et al.,* 2018; Fay *et al.,* 2005; Teodorowicz *et al.,* 2017; Toda *et al.,* 2019; Zhang *et al.,* 2020; Wang *et al.,* 2020). There are three stages to this reaction: early, advanced, and final. Because the chemical processes at each stage are linked, they can occur simultaneously, and the MR can be influenced by a range of factors (de Oliveira *et al.,* 2016). The factors that can influence the MR include the pH, time, temperature, water activity (A_w), the type of carbohydrate present, the physical state of the matrix and the concentration of reactants (Leiva *et al.,* 2017; Garcia-Amezquita *et al.,* 2014; Morales García *et*

al., 2021). When the MR takes place, the proteins and carbonyl group in reducing sugar become cross-linked, changing the structure of the original protein, and creating new compounds that have different properties from the original protein. Since different reactions can occur during the MR, the type of reaction can affect the protein's physicochemical and functionality and the resultant products of the reaction. In a study conducted in 2019, Chen *et al.* stated that the MR initiates the covalent binding interaction between WPI and gum acacia (GA) by condensation between the reducing-end group of GA and an amino acid group in the protein. They found that the decreased WPI's surface hydrophobicity and fluorescence intensity because of the protein-polysaccharide chain bond's protective shielding effect. In addition, the MR products (MRPs) displayed noticeably higher levels of solubility, stability against heat-induced insolubility, and emulsifying properties than the WPI. Specific functional properties, such as emulsifying capabilities, thermal stability, and antioxidant effects, are significantly improved by MR with polysaccharides (Li *et al.,* 2013; Setiowati *et al.,* 2020; Wefers *et al.,* 2018; Sedaghat Doost *et al.,* 2020). In 2020, Wang *et al.* presented highly functional WPI-Inulin conjugates is lowered alpha-helix (α-helix) and beta-sheet (β-sheet) levels after MR with different hours, respectively. The conjugated protein exhibited a looser and more porous surface structure, hence enhancing emulsion activity (EA), emulsion stability (ES), 2,2'-azino-bis 3-ethylbenzothiazole-6-sulfonic acid (ABTS), and oxygen radical scavenging activity. Meanwhile, in the case of camel WPI conjugated with gum arabic under wet heating conditions, the MR led to the formation of cross-links between proteins and carbohydrates, which is proved to increase the thermal stability and solubility of the protein (Sanayei *et al.,* 2021). However, the formation of advanced MRPs (AMRPs) can occur at advanced stage of MR or advanced glycated end products (AGEs) such as occurrence of brown and harmful products called melanoidins if the reaction conditions are not controlled (Taheri-Kafrani *et al.,* 2009; Zhang *et al.,* 2019; A'yun *et al.,* 2020). According to Sedaghat Doost *et al.* 2019, AMRPs and AGEs are particularly responsible for protein nutritional and functional loss, toxicity, and off-flavour. Given the complexity of the MR, further understanding of those parameters is required to control this reaction more thoroughly.

The reaction of dietary proteins and their digested products with the regulatory functions of the gastrointestinal (GI) tract is crucial in defining protein physiological characteristics. The variables that determine the interaction of the protein with the GI tract are the physicochemical qualities, amino acid content and sequence, bioactive peptides, digestion kinetics, and non-protein bioactive components conjugated with proteins (Jahan-Mihan *et al.,* 2011). The number of studies in investigating of digestibility of conjugated protein has received as much attention. Beta-lactoglobulin (β-Lg) in WP-dextran is digested in *in-vitro* digestion model (Böttger *et al.,* 2013), while Tu and the colleagues stated that the conjugated protein demonstrated the protein pattern had a significant effect on digestibility ($p < 0.05$) and proven that increased solubility exhibited higher digestibility (Tu *et al.,* 2014; Rudloff & Lönnerdal, 1992). The presence of reducing sugars in the intensity of protein heating affects protein digestibility in intestinal phase (Tang *et al.,* 2023), meanwhile, the advanced phases of the MR masks protein epitopes, overcoming the negative effect of glycated protein's decreased digestibility on allergenicity (Corzo-Martínez *et al.,* 2010). Most *in-vitro* investigations revealed an increase in protein digestibility following milk protein heating processing, probably due to heat-induced unfolding of the globular WP (van Lieshout *et al.,* 2019).This is accordance to Zenker *et al.* (2020) that mentioned heating WP in the presence of reducing sugar caused structural changes in the protein, affecting both solubility and digestion.

There is no doubt that proteins are essential to humans since they provide as a supply of amino acids. For infant, the mother's milk is the primary source of protein. Mother's milk is universally acknowledged as the optimal nutrition for newborns. It has immunomodulatory substances that promote the development of tolerance to maternal dietary proteins absorbed through breast milk and supplemental foods for the infant. (Jensen *et al.,* 2022). When breastfeeding is not readily available, more infants are obliged to consume infant formula based on cow's milk (Fenelon *et al.,* 2018; Nguyen, 2017; Vandenplas *et al.,* 2021). Our body cannot synthesize essential amino acids. Thus, proteins in infant formula play a critical role in ensuring that infants get the building blocks they need to grow. Infant formula consists of approximately 60% WPs and half of the dry matter is lactose (Lund *et al.,* 2022). Meanwhile, WPs represent approximately 20% of milk protein (Smithers, 2008; de

Castro *et al.,* 2017). The Food and Agriculture Organization (FAO) advises evaluating protein quality based on real protein digestibility in the small intestine, however, the infant digestion investigations have clinical and ethical limitations (Maathuis *et al.,* 2017). The effectiveness of different methods for improving protein digestibility in the infant's digestive tract is dependent on the availability of digestion models that effectively reflect the complicated physicochemical and physiological events that occur in the human GI tract (Hur *et al.,* 2011). The *in-vivo* method refers to experiments conducted on animals or humans. This procedure produces the most precise outcomes, but it is expensive, time-consuming, jeopardized by ethical concerns, financial constraints, and a scarcity of resources (Nguyen *et al.,* 2015; Minekus *et al.,* 2014; Hur *et al.,* 2011; Li *et al.,* 2020). Hence, as an alternative to *in-vivo* research, simple *in-vitro* digestive models simulating the GI tract have been presented. Minekus *et al.* 2014 established and published a fundamental static model of *in-vitro*. In general, the simplest *in-vitro* procedures are those best suited to typical laboratory applications. This is due in part to the fact that, by definition, they may be completed in any ordinary laboratory without the requirement for specific equipment or supplies, as well as particularly qualified personnel (Coles *et al.,* 2005). *In-vitro* digestion models that are often used are static and dynamic models, which are discussed in the following chapter. Despite *in-vitro* models cannot completely replicate the entire complex digestive process in the human GI tract, they do provide considerable advantages over *in-vivo* models in terms of no ethical problems, low cost, and convenient sampling accessibility.

Overall, the protein digestion process in an infant's GI tract gives various outcomes. It is depending on how the protein processing occurs before the feeding infant which is related to the condition or parameter applied in the modified protein processing. It is also, depends on the method used in the digestion process which is depending on the enzyme digestion concentration, the pH of GI tract, the composition and subsequent digestive secretion, digestion and absorption, and the interaction in the digestive system between the host, the food, and micro-bacteria (Coles *et al.,* 2005). To fill in the gaps in previous studies, further research may be required to investigate the critical optimization condition in protein processing and the reliable technique of protein digestion in the GI tract of infants.

1.2 Problem statement

The biggest challenge in milk protein processing is heat-induced because of the heat instability of milk proteins, particularly the WP. During the heat processing, WPs denature and unfold and form aggregation. As a result, the WPs aggregation produce undesirable decrease product quality (Wu *et al.,* 2021). Hence, to overcome the heat instability of WPs, the conjugation of milk protein and carbohydrate using MR that has been shown to be a successful method for enhancing proteins' functional characteristics, particularly the heat stability (Wu *et al., 2021;* Setiowati *et al.,* 2020; Verhoeckx *et al*., 2019; Zhang *et al.,* 2019; Meyer *et al.,* 2018; Moimaz *et al.,* 2018 and Teodorowicz *et al.,* 2017). However, during the process of MR, it has been observed that the reactivity of proteins that have been modified with polysaccharides is significantly lower than that of monosaccharides and disaccharides. This lower reactivity leads to an early cessation of the reaction during the initial phase of the MR pathway, which ultimately results in a high concentration of Schiff base (Fabíola Cristina *et al.,* 2016; Zhu *et al.,* 2008). In a dry, non-buffered reaction, the MR of alpha-lactalbumin (α-Lac) decreases with an increase in saccharide size (Ter Haar, Schols, & Gruppen, 2011). Meanwhile, the reaction between WP and beta-casein (β-casein) decreased with increased saccharide size. Liu & Zhong, (2013) has been described the number and the length of saccharides linked to proteins and the aggregation of conjugated WPI during heating depended on their structure. Since different structures might differently influence the initial nucleophilic attack due to differences in reducing end reactivity, incubation with different saccharide isomers can also lead to differences in MR (Cardoso *et al.,* 2018). The MR can be subject to two separate temperature-related effects. During the initial phase of the MR, a higher temperature can result in a greater degree of conjugation being produced (Cardoso *et al.,* 2018; Chen *et al.,* 2012), however, the increases temperatures can alter the chemical route and produce melanoidin structures at final stage of MR, which might be affect the consumer acceptance of product (Chen *et al.,* 2019). A prolonged reaction time may not only result in a significant degree of browning, but also significantly diminish the conjugate's functionality. Therefore, the optimal reaction time for producing conjugates with superior functional properties did not necessarily correspond to the maximum conjugation degree that could be achieved (Chen *et al.,* 2019).

Conjugation of proteins with carbohydrate via MR impairs amino acid availability, particularly for lysine, and reduces the digestibility of proteins (Van Lieshout *et al.,* 2019). In addition, a decrease in the rate of protein breakdown by digestive enzymes has been associated with excessive protein aggregation resulting in by the unfolding of proteins during the heat processing of foods (Bhat *et al*., 2021). Meanwhile, Kaur *et al.* (2022) and Zhang & Vardhanabhuti, (2014) mentioned the rate of protein digestion and absorption is influenced by the chemical and physical properties of the food proteins. Additionally, the variety of the parameters that had been applied in MR, may also account for the discrepancy frequently seen in the previous studies regarding the impact of MR on protein digestibility (Teodorowicz *et al.,* 2017). The use of a wide variety of models to imitate human digestion has resulted in a demand for a better understanding of the mechanisms involved in dietary protein digestion in the human GI tract. *In-vitro* simulations of human digestion are now often employed due to their decreased labour requirements, lower costs, faster processing times, and lack of the ethical limitations associated with *in-vivo* experiments. However, *in-vitro* investigations frequently only yield data on release into the gut lumen, but human research frequently evaluate bioactive quantities in blood rather than in the GI tract. This is the challenge in contrasting bioaccessibility produced *in-vitro* with bioavailability produced *in-vivo* (Mackie *et al.,* 2020). It is impossible to compare studies since there are so many static *in-vitro* protein digestion models available in the literature that are based on different experimental conditions. On the other hand, the complex but physiologically correct dynamic *in-vitro* digestion models offer an excellent option for studying how food is digested. Nonetheless, for commercial applications, dynamic *in-vitro* models are highly expensive and challenging to administer and maintain.

The major problem with the MR in allergy research is the potential effect of conjugation on the allergenicity of food proteins because many allergenic foods are heated before ingestion. According to Toda and the co-workers in 2019, the MRPs have an impact on the gut microbiota, which may have an impact on the inflammatory status in chronic diseases like allergies. Previous research has shown that MR has a range of impacts. It is dependent upon the variety of parameters and controlled conditions that have been applied during the MR. The influence of protein