UNIVERSITI PUTRA MALAYSIA

FORMATION, CHARACTERIZATION AND APPLICATION OF THIOLMODIFIED BETA-LACTOGLOBULIN FIBRILS COMPLEX WITH CHITOSAN

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By

CHANG HON WENG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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December 2017

Chair : Professor Tan Chin Ping, PhD
Faculty : Food Science and Technology

β-lactoglobulin (β-LG) fibrils have diverse functionalities that make them good emulsifiers and promising foaming agents. However, fabrication of β-LG fibrils under highly acidic conditions (pH 2) has limited their application in food matrixes for food consumption. In addition, β-LG fibrils become unstable when the pH changes and are prone to aggregation. Hence, this study examined the effect of the thiol-modification on β-LG fibrils to improve their functionalities and provide stability against pH changes. Thiol-modified β-LG fibrils were further incorporated into fish oil emulsions by complexing them with chitosan, followed by microencapsulation.

In the first stage, the effect of thiol-modification on β-LG fibrils was examined by modifying the carboxyl side groups. The results showed that the highest esterification was obtained at molar ratios of 4:1 (propanethiol:carboxyl groups) under pH 9. Thiol-modification significantly ($p < 0.05$) enhanced the foaming capacity (550.0 ± 16.7% to 727.8 ± 9.6%), foam stability and emulsifying stability index of the β-LG fibrils. In the second stage, complexation of thiol-modified β-LG fibrils with chitosan was carried out and incorporated into a fish oil emulsion. The results showed that the emulsion droplet size increased with smaller polydispersity indexes upon increasing the chitosan concentrations (0.1% - 0.5%, w/w). The addition of chitosan improved the emulsion stability and decreased the extent of creaming and turbidity loss rate, which improved the oxidative stability of the emulsion significantly ($p < 0.05$). Moreover, chitosan-coated emulsion conferred higher heat stability under thermal treatments (63 °C and 100 °C) as indicated by the consistent droplet sizes.

In the third stage, the fish oil emulsion stabilized with thiol-modified β-LG fibril-chitosan complexes was microencapsulated via spray drying using different inlet temperatures (160 °C, 170 °C and 180 °C). The results showed that the fish oil emulsion microencapsulated at 160 °C exhibited significantly higher microencapsulation efficiency ($p < 0.05$) with enhanced reconstitution properties. Fish oil microcapsules stabilized by thiol-modified β-LG fibril/0.5% chitosan complexes

exhibited slightly higher glass transition temperature with a smooth-surfaced as observed via scanning electron microscopy. In the fourth stage, the storage stability and *in-vitro* digestibility of the microencapsulated fish oil emulsion stabilized with thiol-modified β-LG fibril-chitosan complexes was studied, revealing minimal changes in terms of surface colour and peroxide and p-anisidine values coupled with higher oil retention over 4-weeks of storage period. It was also stable against different ionic strengths. This is related to the thicker wall materials formed by thiol-modified β-LG fibril/0.5% chitosan complexes. It was found that chitosan coating slightly hinder the enzymatic digestion process.

The findings of this study suggest that thiol-modification improved the functionalities of β-LG fibrils, including greater tolerance to pH and environmental changes. In addition, the complexation of thiol-modified β-LG fibrils with chitosan contributed to the stability and improved microencapsulation efficiency (> 89%) of a fish oil emulsion.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBENTUKAN, PENCIRIAN DAN APLIKASI PENGUBAHSUAIAN THIOL KE ATAS FIBRIL BETA-LAKTOGLOBULIN SERTA GABUNGANNYA DENGAN KITOSAN

Oleh

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Fibril β-laktoglobulin (β-LG) mempunyai pelbagai fungsi yang menjadikan mereka sebagai pengemulsi yang baik dan agen berbuih yang berkesan. Walau bagaimanapun, fabrikasi fibril β-LG pada pH 2 yang sangat berasid menyebabkan aplikasinya terhad dalam matriks makanan. Selain itu, fibril β-LG adalah tidak stabil terhadap perubahan pH dan terdedah kepada pengagregatan. Oleh itu, kajian ini mengkaji kesan pengubahsuaian fibril β-LG dengan tiol untuk memperbaiki fungsinya dan kestabilannya terhadap perubahan pH. Fibril β-LG yang telah diubahsuai dengan thiol dimasukkan ke dalam emulsi minyak ikan dan bercampur dengan kitosan, dan kemudiannya melalui proses mikroenkapsulasi.

Di peringkat pertama, kesan tiol terhadap fibril β-LG dengan mengubahsuai kumpulan sampingan karboksil β-LG telah dikaji. Keputusan kajian menunjukkan bahawa esterifikasi tertinggi didapati pada nisbah molar 4:1 (propanethiol:kumpulan karboksil) pada pH 9. Pengubahsuaian fibril β-LG dengan tiol berjaya mempertingatkan kapasiti buih (550.0 ± 16.7% to 727.8 ± 9.6%), kestabilan buih dan indeks kestabilan emulsi fibril β-LG dengan ketara ($p < 0.05$). Di peringkat kedua, fibril β-LG yang diubahsuai dengan tiol dan ditambah dengan kitosan, kemudiannya dimasukkan ke dalam emulsi minyak ikan. Keputusan kajian menunjukkan peningkatan kepekatan kitosan (0.1% - 0.5%, w/w) telah memyebabkan saiz titisan emulsi menjadi besar, dan indeks polydispersity yang lebih kecil. Penambahan kitosan meningkatkan kestabilan emulsi dengan kadar krim dan kadar kekeruhan yang lebih rendah dan meningkatkan kestabilan oksidatif emulsi dengan ketara ($p < 0.05$). Emulsi bersalut kitosan mempunyai kestabilan haba yang lebih tinggi di bawah rawatan haba (63 °C dan 100 °C) seperti yang ditunjukkan oleh saiz titisan yang konsisten.

Di peringkat ketiga, emulsi minyak ikan terbentuk dengan gabungan fibril β-LG yang diubahsuai dengan tiol dan kitosan telah melalui pengeringan semburan dengan menggunakan suhu salur masuk yang berbeza (160 °C, 170 °C dan 180 °C). Keputusan

kajian menunjukkan emulsi minyak ikan yang telah dimikroenkapsulasi mempamerkan kecekapan mikroenkapsulasi yang lebih tinggi dengan ketara ($p < 0.05$) dan sifat penyesuaian semula telah dipertingkatkan. Mikrokapsul minyak ikan yang dibentuk dengan gabungan fibril β-LG yang diubahsuai dengan tiol/ 0.5% kitosan mempamerkan suhu peralihan kaca yang lebih tinggi dan permukaan kapsul yang licin melalui pengimbasan mikroskopi elektron. Di peringkat keempat, kestabilan penyimpanan dan kecerahan *in-vitro* emulsi minyak ikan terbentuk dengan gabungan fibril β-LG yang diubahsuai dengan tiol dan kitosan yang dimikroenkapsulasi telah dikaji, menunjukkan perubahan warna, nilai peroksida dan nilai p-anisidin yang minimum dan pengekalan minyak yang lebih tinggi dalam tempoh penyimpanan selama 4 minggu. Tambahan pula, sampel tersebut adalah stabil dalam keadaan kekuatan ionik yang berlainan. Ini disebabkan oleh permukaan dinding tebal sampel yang terbentuk dengan gabungan fibril β-LG yang diubahsuai dengan tiol/ 0.5% kitosan. Lapisan kitosan didapati telah menghalang proses pencernaan enzimatik serba sedikit.

Penemuan kajian ini menunjukkan bahawa pengubahsuaian fibril β-LG dengan tiol akan meningkatkan fungsinya dengan toleransi yang lebih cekap terhadap pH dan perubahan persekitaran. Selain itu, gabungan fibril β-LG yang diubahsuai dengan tiol dan kitosan dapat meningkatkan kestabilan dan kecekapan mikroenkapsulasi emulsi minyak ikan.

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I certify that a Thesis Examination Committee has met on 7 December 2017 to conduct the final examination of Chang Hon Weng on his thesis entitled "Formation, Characterization and Application of Thiol-Modified Beta-Lactoglobulin Fibrils Complex with Chitosan" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AOR	Angle of repose
ANOVA	Analysis of variance
A.u	Arbitrary units
BSA	Bovine serum albumin
CS	Chitosan
DHA	Docosahexaenoic acid
EAI	Emulsifying activity index
EPA	Eicosapentaenoic acid
ESI	Emulsifying stability index
GRAS	Generally recognized as safe
O/W	Oil-in-water
Pa.s	Pascal per second
p-Ani	p-Anisidine value
PDI	Polydispersity index
pI	Isoelectric point
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
SDS	Sodium dodecyl sulfate
TEM	Transmission electron microscopy
Tg	Glass transition temperature
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
ThT	Thioflavin T

WPI	Whey protein isolate
α-la	αlpha-lactalbumin
β-LG	Beta-lactoglublin

CHAPTER 1

INTRODUCTION

Self-assembly of dairy globular protein into amyloid fibrillar structures has been studied extensively due to its great potential in food applications. Fibrils are often characterized by a high ratio of length versus width. If the fibrils are composed of a cross β-sheet conformation structured perpendicular to the fibril axis, they are described as "amyloid". Various food proteins, such as bovine serum albumin (Veerman, Sagis, Heck, & van der Linden, 2003), soy glycinin (Akkermans, et al., 2007) and ovalbumin (Veerman, de Schiffart, Sagis, & van der Linden, 2003), have been reported to form fibrils under specific condition. Beta-lactoglobulin (β-LG) is a major whey fraction found in bovine milk and is the only fibril-forming protein of whey isolate (WPI). It forms fibrils when it is subjected to high thermal treatment (> 80 °C) at pH 2 for 20 hours. β-LG is suitable to be used as a model protein to investigate the formation of fibrils due to its availability, dynamic properties and structurally well-defined arrangement (Bolder, Vasbinder, Sagis, & van der Linden, 2007).

The idea of β-LG fibrillation is initiated by previous studies which found that the formation of gel by β-LG at pH less than 4 is characterized by a structural "thin filament" (Langton & Hermansson, 1992). A subsequent study found that heat-induced β-LG at pH 2 and low ionic strength produced semi-flexible, fine and long protein aggregates (Aymard, Nicolai, Durand, & Clark, 1999). The formed protein aggregates are typically long in the order of microns, with diameters in the nanometer range. It is widely accepted that the fibril formation of β-LG is often induced at high temperature (> 80 °C) and under acidic condition (pH 2) (Bolder, Hendrickx, Sagis, & van der Linden, 2006). Heat-induced fibrillation of β-LG is often associated with an increment of β-sheet content resulting from the transformation of α-helical to β-sheet conformation. The unique dimension of β-LG fibrils with a high aspect ratio results in a high excluded volume (Philipse, 1996), making them a promising food additive for colloidal dispersions. Previous experimental studies also highlighted the diverse functionalities of β-LG fibrils as foaming (Oboroceanu, Wang, Magner, & Auty, 2014), gelling (Graveland-Bikker & de Kruif, 2006), and encapsulating agents (Serfert, et al., 2014).

There are a total of 4 working chapters as shown in Figure 1.1 in which the scope of the study will be basically focusing on the thiol-modification of β-LG fibrils and the application of thiol-modified β-LG fibrils-chitosan complex in fish oil emulsion and encapsulated fish oil. In the first stage, the effects of thiol-modification on the characteristics of β-LG fibrils were firstly evaluated by modifying the carboxyl side groups of the β-LG fibrils. Thiol-modification was based on the molar ratios of thiol reagents:carboxyl groups. Fabrication of β-LG fibrils is usually performed under extremely acidic condition (pH 2), which make the resulting fibrils inappropriate for food applications. The pH for most food products is typically in the range of pH 4 to 7 (Ardy Kroes-Nijboer, et al., 2012). Thus, it is essential to look attentively at the stability of the fabricated fibrils against pH changes. When the pH is close to the isoelectric point of β-LG (~ pH 5.1), the β-LG fibrils is vulnerable to aggregation, with

partial β-sheet disruption often observed. In an effort to enhance the fibrils stability against pH changes, researchers have investigated the possibility of electrostatic interaction between β-LG fibrils and sodium dodecyl sulphate (SDS) (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008) or soybean lecithin (Mantovani, Fattori, Michelon, & Cunha, 2016). In fact, protein modification provides an alternative way to better address the shortcomings of β-LG fibrils by conferring the fibrils with enhanced functionalities and higher stability against pH changes. However, fibril modification is often ignored, with past studies mostly focusing on the modification of relatively pure globular protein solutions (Jones, Adamcik, Handschin, Bolisetty, & Mezzenga, 2010). The significance study of the first stage included exploring the possibilities to enhance functionalities of β-LG fibrils via grafting of functional groups (esterification). Esterified milk proteins (Sitohy, Chobert, & Haertlé, 2001) and methyl esters of β-LG (Halpin & Richardson, 1985) have shown improved functional properties as well. The hydrophobic core of β-LG was unravelled with more exposing functional groups upon fibrillation. After denaturation and acid hydrolysis, fibrillar protein has more exposed functional groups. This allows modification via cross-linking to become more effective. Hence, it was proposed that more impact can be achieved by thiol-modification using β-LG fibrils as opposed to globular native β-LG attributable to the higher excluded volume of fibrils (Munialo, de Jongh, Broersen, van der Linden, & Martin, 2013). Thiol-modified β-LG fibrils were produced at this stage and the thiol-modified β-LG fibrils with the best physical properties were chosen and used in the second stage of this study.

In the second stage, the complexation of thiol-modified β-LG fibrils with different concentrations of chitosan was performed. Then, the fabricated thiol-modified β-LG fibrils-chitosan complex was utilized as a stabilizer to produce fish oil-in-water emulsions via high pressure homogenization. The resulting emulsions were characterized in terms of their physical properties and stability. The growing demand for fish oil is credited to its beneficial health effects. Fish oil contains substantial amount of omega-3 polyunsaturated fatty acids which have been reported to enhance cardiovascular activity, strengthen the immune system and reduce cholesterol level. This makes them a valuable ingredient in food fortification. However, omega-3 fatty acids are prone to oxidative degradation due to its unsaturated carbon-carbon bonds, thus limiting its application in the food industry (Kagami, et al., 2003). Further degradation may also release unhealthy secondary oxidation products (Fetterman Jr & Zdanowicz, 2009). Hence, it is important to apply an efficient encapsulation system such as emulsion to protect the fish oil against oxidative degradation. Chitosan is a commonly used stabilizer in oil-in-water emulsions. It is a natural cationic polysaccharide. However, chitosan cannot be applied alone in emulsion system as it is a non-surface active stabilizer. The significance study of the second stage included more beneficial impact could be achieved by complexing polysaccharides with protein in order to produce polysaccharides-protein complexes with enhanced functionalities. Complexation of chitosan with an effective emulsifier such as thiol-modified β-LG fibrils can bring out the advantageous properties of chitosan in producing a stable emulsion. Thiol-modified β-LG fibrils could adsorb onto the oppositely-charged chitosan prior to their incorporation into fish oil-in-water emulsions. In this case, thiol-modified β-LG fibrils act as an emulsifier by reducing the interfacial tension between the oil and aqueous phases. Similarly, chitosan acts as a stabilizer to provide the fish oil emulsions with higher stability via viscosity enhancement (Dickinson, 2003). The complexation between protein and polysaccharides would form a protective layer

around the fish oil droplets that protects against environmental stresses such as high temperature, ionic strength and extreme pH conditions. The resulting fish oil emulsion with better physical properties and highest stability was chosen prior to fish oil microencapsulation in the third stage.

Subsequently, the third stage of this study involved the microencapsulation of fish oil-in-water emulsion stabilized using the best thiol-modified β-LG fibrils-chitosan complex at three different inlet temperatures (160 °C, 170 °C and 180 °C). The most stable emulsion with enhanced physical properties from the second stage was subjected to spray drying. The resulting spray-dried fish oil microcapsules were then characterized in terms of their physical properties which include encapsulation efficiency (oil retention), reconstitution and morphological properties. Fish oil has a strong odour and vulnerable to oxidative degradation which was attributed to the presence of polyunsaturated fatty acids. Besides, previous research has implied the limitation of β-LG fibrils in spray-dried products due to its heat instability. The significance of the study for the third stage included masking off the undesirable fishy odour and inhibiting the fish oil from oxidative deterioration by encapsulation. Moreover, chitosan which employed in the study conferred higher heat stability and its complexation with thiol-modified β-LG fibrils combined the advantageous properties of both oppositely-charged biopolymers for enhanced encapsulation properties. The fish oil microcapsules produced with the best encapsulation properties were used in the fourth stage.

Finally, in the fourth stage, the spray-dried fish oil microcapsules were evaluated in terms of their stability against environmental stresses (ionic strength and pH) and when subjected to a 4-week storage period. In addition, the *in vitro* release behaviour of fish oil from the microcapsules under simulated gastrointestinal condition was also examined. Omega-3 fatty acids are vulnerable to oxidative deterioration and the gastrointestinal stability and bioavailability of orally-consumed fish oil are a matter of concern as well. In other words, the targeted bioactive compound such as fish oil may undergo degradation en route to the intestines. The significance of study for the fourth stage included of providing protection and high gastrointestinal stability to the fish oil against gastric environment. The fabrication of emulsion is an intermediate processing step prior to microencapsulation. Microencapsulation of functional ingredient such as fish oil is a promising approach to protect omega-3 fatty acids against oxidative deterioration and to produce microcapsules with desirable attributes such as controlled release and enhanced bioavailability (Drusch, Serfert, Van Den Heuvel, & Schwarz, 2006). Wall materials such as chitosan, whey proteins and plant gums are commonly incorporated into fish oil emulsion prior to fish oil microencapsulation (Pourashouri, et al., 2014). The combination between chitosan and thiol-modified β-LG fibrils is expected to confer enhanced stability to fish oil microcapsules and protect the sensitive core (fish oil) from deterioration. Besides, chitosan-coated emulsion was found to be highly stable against oxidation and less susceptible to enzymatic digestion, making it an ideal delivery system for lipophilic ingredients (Klinkesorn & McClements, 2009). Previous studies highlighted the importance of the mucoadhesive property of chitosan which allow for longer adsorption residence time in the gastrointestinal passage (Shin, Chung, Kim, Joung, & Park, 2013).

The objectives of this study were:

1. To examine the effect of thiol-modification on the physical properties and functionalities of β-LG fibrils.
2. To evaluate the stability and physical properties of fish oil-in-water emulsions stabilized using thiol-modified β-LG fibril-chitosan complex.
3. To investigate the application of thiol-modified β-LG fibril-chitosan complex as wall material on the characteristics of spray-dried fish oil microcapsules.
4. To determine the effect of the thiol-modified β-LG fibril-chitosan complex on the storage stability and *in vitro* release of the spray-dried fish oil microcapsules.

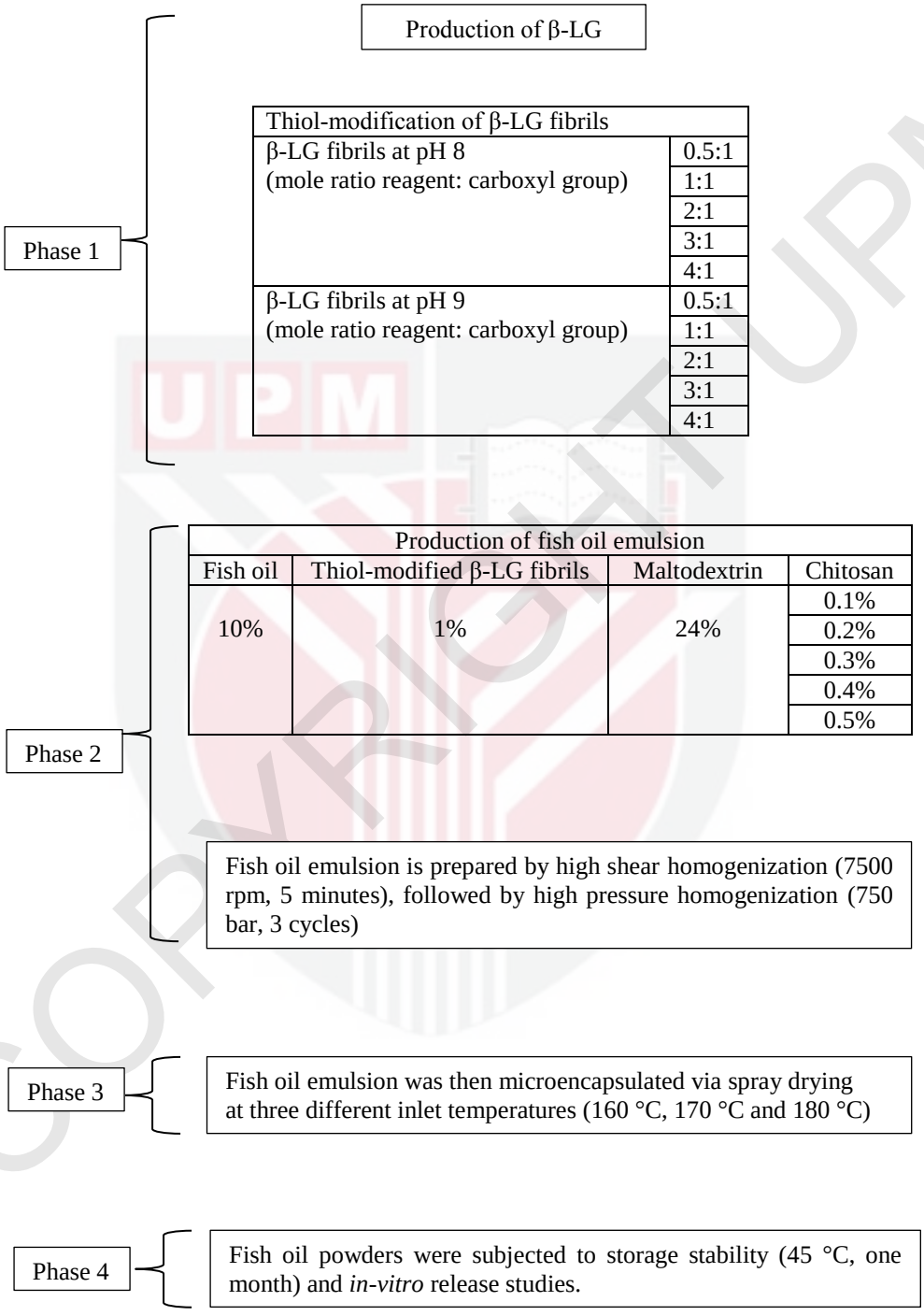

Figure 1.1: Schematic flow diagram of the working chapters.

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