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Encapsulation of Milk Protein with Inulin for Improved Digestibility

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Abstract:
Encapsulation is the packaging of bioactive compounds to isolate and control their release upon applying specific conditions. By using inulin as the wall material for milk protein encapsulation, it can improve the stability and reduce the rate of protein release which can be related to better digestibility. This study aims to find the best encapsulation parameters for milk protein encapsulation using inulin based on the encapsulation efficiency and evaluate the protein release by simulation of intestinal fluid *in vitro*. Protein encapsulation was prepared under various parameters including inulin concentration, stirring temperature and stirring speed. The encapsulation parameters were analysed by Response Surface Methodology to obtain the highest encapsulation efficiency, which was subsequently used for the analysis of particle size, zeta potential and protein release kinetics. The results showed that inulin concentration was the most significant in determining encapsulation efficiency, and the highest encapsulation efficiency (75.90%) was achieved at 0.47 % w/v of inulin concentration, stirring temperature of 35.17°C and stirring speed of 510.79 rpm. The inulin-encapsulated milk protein produced under the best encapsulation parameters had an average particle size value of 485.8 nm and zeta potential of -10.5 mV. Zero order kinetics model was most suited to describe the encapsulated milk protein release, indicating slow release of protein that may be associated with better digestibility.

Keywords: Inulin; Milk protein; Encapsulation; *In vitro* digestion

1. Introduction

Inulin belongs to the group of fructans that naturally occurs as plant storage carbohydrate in many members of the Asteraceae family. It is a nontoxic and degradable polysaccharide obtained from plants, such as the chicory root, and already established as a food ingredient due to its prebiotic activities [1]. Inulin is a dietary fibre that is non-digestible and only being degraded by certain colon bacteria such as the *Bifidobacterium adolescentis*, *B. angulatum*, and *B. longum* [2,3]. It is difficult to hydrolyze which qualifies inulin as a matrix molecule for capsules that have to reach the colon and to survive the upper part of the gastrointestinal tract and later degraded by bifidobacteria, which are abundantly present in the human gut [4]. Inulin also contributes to a wide array of health benefits, particularly in intestinal health and function, increasing the mineral absorption and decreasing the risk of colon cancer [1].

Encapsulation is a technique by which solids or liquids are enclosed in nano/microcapsules, protecting the functional ingredients from oxidation, degradation by enzymes and helping to mask undesirable flavor [5]. The contents can release slowly for a prolonged time until desired destination [6]. Encapsulation process can alter the physicochemical properties such as particle size and zeta potential which will subsequently affect the solubility,

stability and digestion ability of the encapsulated product [7]. In this study, milk protein was encapsulated with inulin, to improve the stability and reduce the rate of protein release in the intestine which can be related to better digestibility. People with lactose intolerance may also benefit from inulin-coated milk as encapsulation will protect the bioactive components. At the same time, encapsulation can mask undesirable odour commonly associated with milk.

So far, milk protein encapsulation has been mainly done using lipid or nanoliposome as the wall materials [8]. Although there are some studies that have used inulin as the encapsulating material for drugs and probiotic bacteria, none of them attempted on the encapsulation of milk protein itself. One study reported the effect of inulin concentration to the size and encapsulation yield of alginate-goats' milk-inulin matrix which was used to encapsulate *Bifidobacterium animalis* subsp. *lactis* BB-12 [9]. Besides the encapsulation efficiency and particle size, it is also necessary to evaluate the release rate of protein under the simulation of intestinal fluid *in vitro*. In this work, milk protein was encapsulated using inulin as the wall material and the effect of encapsulation parameters was studied by comparing the encapsulation efficiency under different parameters. The simulation of intestinal fluid *in vitro* was conducted to observe the release rate of milk protein to the surrounding and determine the kinetic release model.

2. Materials and Method

2.1 Preparation of inulin-encapsulated milk suspension

Inulin from chicory was purchased from Sigma-Aldrich. Nestle Everyday milk powder was purchased from local supermarket. Inulin-encapsulated milk suspension was prepared by protein precipitation method [10]. The milk powder contains 14.1 g of protein in 100 g of milk powder. To achieve 0.1% w/v (0.01 g) of milk protein concentration in 10 mL aqueous solution, 0.0709 g of milk powder was dissolved in 10 mL water. Next, 2 g (20 % w/v) of Na_2SO_4 (QRëC) was added into the solution. This mixture was added dropwise by a dropper into 10 mL inulin solution of varied concentration and the suspension was stirred at 300 rpm for 30 min using a magnetic hotplate stirrer at 25ºC. In this study, inulin concentration (0.1, 0.3 and 0.5 % w/v), stirring speed (150, 300, 500 and 900 rpm) and stirring temperature (25, 29, 33 and 37ºC) were varied and investigated for the purpose of optimization of the encapsulation parameters based on encapsulation efficiency, using one-factor-at-time (OFAT) method. The initial values of inulin concentration (0.1 % w/v), stirring temperature (25°C) and stirring speed (300 rpm) were chosen based on the literature [11] and preliminary experiments.

Milk protein encapsulation efficiency (EE) was calculated by the difference between the total amount added into the aqueous solution and the amount of free milk protein detected in the milk-inulin suspension. 20 µL of suspension from the milk protein-inulin mixture was taken out by a micropipette and added into a 96-well plate and mixed with 200 µL of Bradford Reagent (R&M Chemical) for Bradford's protein assay. The mixture was shaken in a vortex mixer for 5 minutes at 600 rpm. Next, the 96-well plate was transferred to a microplate reader to assay spectrophotometrically at 517 nm. A standard curve of optical density reading at 517 nm (y) vs. milk concentration, % w/v (x) was drawn at the beginning and used for the quantification of milk protein (Equation (1)).

$$y = 3.7417x + 0.3598 \quad (1)$$

The milk protein encapsulation efficiency (EE) was calculated by Equation (2):

$$\%EE = \frac{(A_{IP} - A_{FP})}{A_{IP}} \times 100 \quad (2)$$

where 'A_{IP}' is amount of the initial milk protein concentration used in the preparation of the particles and the 'A_{FP}' is the amount of free milk protein detected in the suspension [11]. All experiments were conducted in triplicates.

2.2 Response surface methodology for optimization of encapsulation parameters

The parameters (inulin concentration, stirring temperature and stirring speed) for milk protein encapsulation were analyzed by response surface methodology (RSM) using Design Expert 6.0.4 software (Stat-Ease Inc., Minneapolis, MN, USA). Historical Data Design (HDD) was used for optimization using a total of 9 combinations of experiment from the OFAT, with protein encapsulation efficiency as the response. Encapsulated milk protein produced by the best parameter combination as predicted by the RSM was selected for further characterization and *in vitro* protein release study.

2.3 Measurement of particle size distribution and zeta potential

Measurements were performed at 25°C, by Zetasizer Nano-ZS (Malvern, US) using M3-PALS technique. For particle size, refractive index of 1.45 and absorption of 0.001 were used, while for zeta potential, the viscosity, refractive index and absorption were set at 0.8872 cP, 1330 and 78.5 respectively. Analyses were performed in three different batches and the result was expressed as a mean of three measurements [11]. Non-encapsulated milk was used as the control.

2.4 *In vitro* release study by simulation of intestinal fluid

Encapsulated protein particles using inulin as coating material were passed through an *in vitro* digestion model that simulates intestine digestion, with non-encapsulated milk as the control. Simulated intestinal fluid (SIF) was prepared using phosphate buffer saline (PBS) of pH 7.4. 5 mL of encapsulated protein suspension was transferred into 15 mL centrifugal tube, added with 0.5 mL of PBS, vortexed for 2 min and let to stand. Sampling was done periodically by withdrawing 20 µL of the mixture into 96-well microplate, added with 200 µL of Bradford Reagent for Bradford's protein assay. The mixture of sample and Bradford Reagent was shaken in vortex mixer for 5 min at 600 rpm. The amount of released protein was assayed spectrophotometrically at 517 nm, and quantified using Equation (1). The *in vitro* release data of the milk protein were evaluated kinetically by zero-order and first order kinetics, Higuchi model and Hixson–Crowell model [11], represented by Equations (3) to (6) below.

Zero-order model:	$C_0-C_t = K_0t$	(3)
First-order model:	$\log C = \log C_0 - K_1t/2.303$	(4)
Hixson–Crowell model:	$C_0^{1/3} - C_t^{1/3} = K_{HC}\, t$	(5)
Higuchi model:	$Q = K_H\, t^{1/2}$	(6)

where C_t is the amount of protein released at time t, C_0 is the initial concentration of protein at time t=0, C is the remaining protein at time t, Q is the amount of protein released in time t per unit area, K_0 is the zero-order rate constant, K_1 is the first order rate constant, K_{HC} is the Hixson-Crowell constant and K_H is the Higuchi dissolution constant.

3. Results and Discussion

3.1 Encapsulation efficiency of inulin-encapsulated milk protein

Table 1 shows the effect of inulin concentration, stirring temperature and stirring speed of hot plate on the encapsulation efficiency of milk protein using inulin as the coating agent. The encapsulation efficiency of milk protein increased proportionally with the increase in inulin concentration, consistent with Sezer *et al*., 2011 [11] that observed that the particles containing the highest polymer concentration showed the highest protein encapsulation. This is due to the abundant coating material available to encapsulate protein when the concentration increased [11]. As for the effect of temperature, encapsulation efficiency decreased when the temperature was raised from 25 to 29°C, but then increased when the temperature was further raised to 33 and 37°C, although the difference between the last two temperatures was not apparent (p>0.05). Wang and Huang (2009) postulated that at higher temperatures, the structure of the coating material would become more irregular and looser to better enclose the protein and hence, the encapsulation efficiency becomes higher [13].

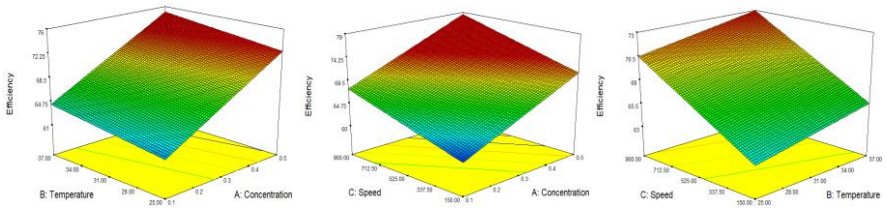
Table 1. Encapsulation efficiency of inulin-coated milk powder under selected encapsulation parameters (*different letters indicate significant differences, p<0.05)

Sample Label	Milk Protein Concentration (% w/v)	Inulin Concentration (% w/v)	Stirring Temperature (°C)	Stirring Speed (rpm)	Encapsulation Efficiency (%±SD)*
A1	0.1	0.1	25	300	61.12 ± 0.67^{a}
A2	0.1	0.3	25	300	66.76 ± 1.80^{b}
A3	0.1	0.5	25	300	72.89 ± 0.83^{c}
B1	0.1	0.1	29	300	59.49 ± 0.48^{d}
B2	0.1	0.1	33	300	64.49 ± 0.28^{be}
B3	0.1	0.1	37	300	64.76 ± 1.00^{bef}
C1	0.1	0.1	25	150	63.89 ± 0.83^{befg}
C2	0.1	0.1	25	500	61.49 ± 0.57^{a}
C3	0.1	0.1	25	900	69.48 ± 0.95^{b}

The encapsulation efficiency showed a slight decrease when the stirring speed was changed from 150 to 300 rpm (p<0.05), but no significance difference was observed between 300 and 500 rpm. However, it increased significantly at 900 rpm, indicating that higher stirring speed allows inulin and protein particles in the solution to mix well and improves encapsulation.

3.2 Response surface methodology for optimization of encapsulation parameters

Figure 1 shows the effect of encapsulation parameters (inulin concentration, stirring temperature and stirring speed) on milk protein encapsulation efficiency, using contour plots as demonstrated by the Design Expert software. Analysis of variance (ANOVA) shows that only inulin concentration (p-value = 0.0121, <0.05) was significant in determining encapsulation efficiency, while temperature (p-value = 0.4002) and speed (p-value = 0.0768) of stirring hot plate did not give significant effect to the efficiency. By using the Historical Data Design, only a linear model was given a significant p-value of 0.046 (<0.05). In order to obtain significant model in quadratic equation, adding more parameters like pH may give more prominent impact to the encapsulation efficiency.



(a) (b) (c)

Figure 1. Effect of encapsulation parameters (inulin concentration, stirring temperature and stirring speed) on protein encapsulation efficiency (a) with constant speed of 300 rpm, (b) with constant temperature of 25°C and (c) with constant inulin concentration of 0.1 % w/v

The linear equation obtained by the response surface analysis of the data showing the effect of inulin concentration, temperature and rotating speed of stirring hot plate on protein encapsulation efficiency (EE) is described as Equation (7).

$$EE = 51.05548 + 26.93080 * Concentration + 0.20592 * Temperature + 9.91047 \times 10^{-3} * Speed \quad (7)$$

Based on this model, the conditions that give the highest protein encapsulation efficiency (75.90%) was 0.47 % w/v of inulin concentration, stirring temperature of 35.17°C and stirring speed of 510.79 rpm. Although the linear model does not optimize the encapsulation parameters, their significant effect was known. Subsequently, these best conditions were used for further characterization and protein release study under simulated intestinal fluid.

3.3 Particle size distribution

The particle size of encapsulated particles was tested for the highest milk protein encapsulation efficiency estimated from RSM with the condition of 0.47 % w/v of inulin concentration, temperature of 35.17°C and stirring speed of 510.79 rpm. The highest peak was found at 485.8 nm (Table 2). This result is consistent with the previous study by (Sezer et al., 2011) who found that for the stirring speed of 500 rpm, the encapsulated particle size was around 435 nm to 537 nm whereby the nanoparticles with smaller particle size were obtained at lower amount of encapsulating material and higher stirring rates in the formulation [11]. In comparison, the particle size of non-encapsulated milk was found to be in the range of 40 to 300 nm, while for inulin particles, they formed peaks at 21.473 ±0.658, 95.919 ± 3.215 and 428.326 ±14.083 nm [12].

Table 2. Size of inulin-encapsulated milk protein and intensity of the peaks measured by particle size analyzer

Peak	Size (nm)	Intensity (%)
1	89.82 ± 19.63	13.1
2	485.8 ± 186.0	68.6
3	4256 ± 977.5	18.3

3.4 Zeta potential

Analysis of zeta potential showed an overall charge of the inulin-encapsulated protein suspension of -10.5 ± 2.0 mV, which is considered as low. It is known that the zeta potential of proteins such as the bovine serum albumin (BSA) at pH 7.0 is -20.3 ± 2.1 mV, while inulin showed zeta potential of -13.40 ± 1.77 mV. This result indicated that the outer surface of the nanoparticles consisted of inulin and caused a less negative zeta potential and smaller repulsive force among the particles. Hence, the encapsulated particles may flocculate and become less stable. The literature shows gradual decrease in zeta potential value when the inulin concentration is increasing. This is because the interaction of protein and inulin causes the coating of inulin on the surface of the protein, which shielded the surface charge of protein [14].

3.5 *In vitro* simulation of intestinal fluid and protein release kinetics

The amount of protein released following the *in vitro* treatment of simulated intestinal fluid was measured as the optical density (OD) at 517 nm using Bradford protein assay, and subsequently quantified using Equation (1). Figure 2 shows the cumulative protein release (%) of encapsulated milk protein after specific time points. The inulin-encapsulated milk protein was rapidly released initially (36% at 2 min and 73% at 4 min), thereafter the release was slow and more gradual, demonstrating the hydrolysis of inulin by phosphate buffer saline (PBS) which was used as the simulated intestinal fluid. In contrast, non-encapsulated milk was instantly released (> 90% after 2 minutes) into the PBS. Therefore, it was demonstrated that the encapsulation can extend the time of milk protein release and may allow for better absorption in the intestine.

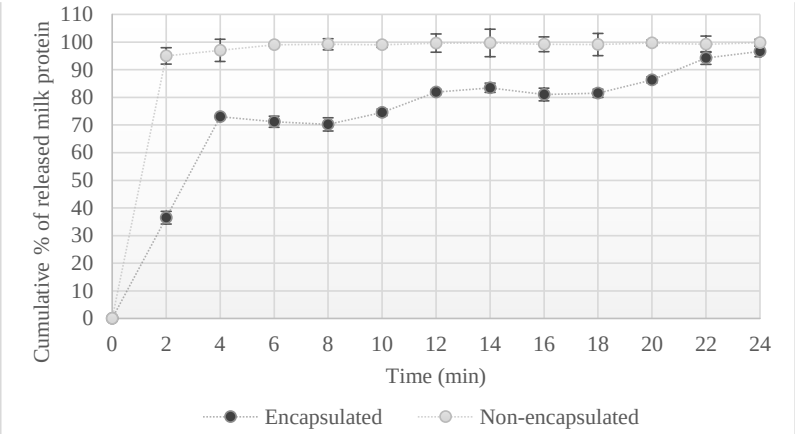


Figure 2. Amount (%) of released milk protein after *in vitro* treatment with simulated intestinal fluid

In order to propose protein release mechanism, the data obtained from *in vitro* protein release under simulated intestinal fluid condition were plotted and fitted to the following kinetic models: zero order, first order, Higuchi, and Hixson-Crowell models. According to Table 3, the release kinetic values (R^2) and the kinetic constants of the formulations were: Zero-order kinetics (R^2 = 0.8235, K_0 = 0.594) > Hixson–Crowell (R^2 = 0.8195, K_{HC} = 0.0132) > First-order kinetics (R^2 = 0.8171, K_1 = -0.0044) > Higuchi (R^2 = 0.7497, K_H = 3.7009). Thus, zero order kinetics model gives the best fit of the protein release data, similar to the findings from (Sezer et al., 2011) [11]. Zero order kinetics is for the case of initial rapid dose, followed by very slow drug or protein release, whereby a constant amount of drug is released by unit time [15,16]. This indicates that the encapsulation can be used for control or modified release of milk protein, similar to kinetics models used to describe the drug dissolution of several types of modified release pharmaceuticals such as in matrix tablets with low dosage drug in coated forms, osmotic systems, etc. [17].

Table 3. Correlation of coefficient (R^2), slope and intercept of different kinetics models based on protein release study of *in vitro* simulated intestinal fluid condition

Model type	R^2	Slope (Kinetic Constant)	Intercept
Zero order	0.8235	K_0 = 0.594	33.038
First order	0.8171	K_1 = -0.0044	1.8298
Hixson-Crowell	0.8195	K_{HC} = 0.0132	0.5747
Higuchi	0.7497	K_H = 3.7009	28.003

4. Conclusion

In this study, milk protein was encapsulated with inulin and the encapsulation parameters were optimized using Response Surface Methodology (RSM). Inulin concentration was found to be the most important parameter that affects milk protein encapsulation efficiency, while the stirring temperature and stirring speed have less significant effects. A linear model could be drawn to predict the highest encapsulation efficiency, which gave 0.47% w/v of inulin concentration, stirring temperature of 35.17°C and stirring speed of 510.79 rpm as the best parameters. The inulin-encapsulated milk protein produced under the best encapsulation parameters had an average particle size value of 485.8 nm and zeta potential of -10.5 mV. The release profile of inulin-encapsulated milk protein was studied under *in vitro* simulated intestinal fluid condition and zero order kinetics was the best model to describe the protein release behavior, indicating slow release of protein that may be associated with better digestibility. This indicates that encapsulation indeed helps in protecting the milk from rapid digestion and people with lactose intolerance may benefit from the longer time taken for the milk component to be absorbed by the body.

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