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Optimization of Microencapsulation of β-Lactoglobulin-Vitamin A

β-Lactoglobulin-Vitamin A의 미세캡슐화 조건 최적화

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ABSTRACT

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As one of essential nutrients, vitamin A is important for growth, development, immune and vision system. It is widely used in various types of foods as a nutritional supplement. However, the functional properties of vitamin A are not fully exhibited by its great reactivity and low stability. Microencapsulation of vitamin A could increase the stability of vitamin A,

prevent light-induced degradation and oxidation, and disperse vitamin A in water-soluble compounds. β-Lactoglobulin (β-Lg) is the major whey protein in cow's milk and has a central cavity able to bind hydrophobic ligands such as vitamin A. Furthermore, using ultra-high pressure (UHP) treatment, which changes the conformation of β-Lg, could increase the binding ability of β-Lg to vitamin A. In this study, microencapsulation condition for vitamin A using β-Lg as a wall material was optimized using response surface methodology (RSM). In order to achieve a higher microencapsulation efficiency (MEE), β-Lg was treated by UHP before encapsulating vitamin A. The UHP treatment condition of β-Lg was optimized using orthogonal array design (OAD). The microstructures of the microcapsules of β-Lg-vitamin A and microcapsules of UHP treated β-Lg-vitamin A were observed by transmission electron microscopy (TEM) and Fourier transform infrared (FT-IR) spectroscopy.

Optimal conditions for microencapsulation of β-Lg-vitamin A were 4.74:1 of the molar ratio, 1.43 h, pH 6.87, and 48.68℃ determined by RSM. The MEE under the optimized condition was calculated as 82.2% and the experimental value was 81.5%. Optimal conditions for UHP treatment of β-Lg were 300 MPa, 20 min, and 20℃ determined by OAD. The experimental MEE under the optimized condition was 94.8%.

The optimized microcapsules of β-Lg-vitamin A and microcapsules of

UHP treated β-Lg-vitamin A observed by TEM were sphere-shaped in a regular order. Vitamin A was observed by FT-IR to be inserted in the central cavity of β-Lg. These results indicate that the microencapsulation conditions for β-Lg and vitamin A were optimized by RSM, the UHP treatment conditions for β-Lg were optimized by OAD, and the microcapsules were successfully formed.

Key words: β-lactoglobulin; vitamin A; microencapsulation efficiency; ultra-high pressure; response surface methodology; orthogonal array design

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INTRODUCTION

As one of essential nutrients, vitamin A is important for growth, development, immune and vision system. Thus, vitamin A is widely used in various types of foods as a nutritional supplement (Fennema and Owen 2008; Tanumihardjo 2011; Solomons and Orozco 2003). The functional properties of vitamin A are limited by its great reactivity and low stability, resulting in a significant loss during processing and storage of foods in the presence of oxygen and light (Xie and Huang 2011). Microencapsulation is a technology proposed to entrap, protect, and deliver sensitive or bioactive components and to improve sensory properties of functional foods. Microencapsulation of vitamin A could increase the stability of vitamin A during food processing and storage, prevent light-induced degradation and oxidation (Hogan et al. 2001), and disperse vitamin A in water-soluble compounds (Gonnet et al. 2010; Loveday and Singh 2008). Microencapsulation of vitamin A has been studied using different wall materials such as cyclodextrin, gum arabic, and other polysaccharides. Compared to other wall materials, β-lactoglobulin (β-Lg) has a very similar structure with retinol-binding proteins in plasma and shows a high affinity to vitamin A (Papiz et al. 1986).

β-Lg, the major whey protein in cow's milk, garners an interest in food industry because of its nutritional and binding properties. β-Lg is a small globular protein with a molar mass of 18 kDa. The 3-dimensional tertiary structure of β-Lg displays one main α-helix, eight antiparallel β-strands arranged in a β-barrel, and a ninth β-strand involved in dimer interaction (Brownlow et al. 1997; Kontopidis et al. 2002). The β-barrel delimitates a central cavity (or calyx) able to bind hydrophobic ligands. The binding properties made β-Lg itself a natural wall material for microencapsulation of fat-soluble compounds such as vitamin A (Kontopidis et al. 2002; Liang and Subirade 2012; Perez and Calvo 1995; Sawyer et al. 1998; Sneharani et al. 2010; Wang et al. 1999). There are a few studies considering the effect of one or two combination conditions (such as temperature and pH) on vitamin A binding to β-Lg (Yaldagard et al. 2008; Grácia-Juliá et al. 2008; Blayo et al. 2014). However, there is no study about optimization of the combination condition on vitamin A binding to β-Lg.

Ultra-high pressure (UHP) treatment has been recognized as a physical tool for the modification of macromolecular compounds, such as proteins (Cheftel 1992; Hayashi 1992; Balny and Masson 1993). There are studies involving β-Lg denaturation induced by UHP to increase its binding ability to vitamin A (Considine et al. 2007; Dumay et al. 2006; Funtenberger et al.

1997). However, there is no study about optimization of UHP treatment condition on β-Lg, which can be used for encapsulation of vitamin A.

In this study, microencapsulation condition for vitamin A using β-Lg as wall material was optimized using response surface methodology (RSM). In order to achieve a higher microencapsulation efficiency (MEE), β-Lg was treated by UHP before encapsulating vitamin A. The UHP treatment condition of β-Lg was optimized using orthogonal array design (OAD). The microstructures of optimized β-Lg-vitamin A microcapsules and UHP treated β-Lg-vitamin A microcapsules were observed by transmission electron microscopy (TEM) and Fourier transform infrared (FT-IR) spectroscopy.

MATERIALS AND METHODS

1. Chemicals and reagents

Lyophilized bovine β-Lg and all-trans retinol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, methanol, potassium hydroxide, petroleum ether, and other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All of the chemicals were analytical grade.

2. Microencapsulation of β-Lg-vitamin A

β-Lg was dissolved in 10 mL distilled water at different concentrations, and 0.1 mg vitamin A was dissolved in 1 mL ethanol. The β-Lg and vitamin A solutions were mixed using an air bath oscillator (Jintan Puchen Electronics Co., Ltd., Jintan City, Jiangsu, China) at the different molar ratios of β-Lg to vitamin A (β-Lg:vitamin A = 1:1-5:1), reaction time (1-3 h), pH (5.5-7.5), and reaction temperature (30-70℃). After preparing the β-Lg-vitamin A mixture, free vitamin A that was not encapsulated was removed by dialysis. The mixture was transferred into dialysis membrane (Spectra/Por 7, Spectrum Laboratories Inc., Houston, TX,

USA) with molecular weight cut-off of 10 kDa, followed by placing the dialysis membrane in a beaker with 10% ethanol in water (replaced every 8 h) for 24 h to remove free vitamin A.

3. Determination of microencapsulated vitamin A

First, 20 mL ethanol and 10 mL 50% potassium hydroxide solution were added to the microencapsulated β-Lg-vitamin A, followed by heating in the air bath oscillator at 50℃ for 1 h to get vitamin A released from the microencapsulated β-Lg-vitamin A. The released vitamin A was extracted with 50 mL petroleum ether 3 times and concentrated using a rotary evaporator (Hei-Vap, Heidolph, Instruments GmbH & Co., Schwabach, Germany). The content of the extracted vitamin A was determined using an RP-HPLC system (Agilent Technologies Co., Ltd., Palo Alto, CA, USA), according to the ISO method (ISO 12080-2:2009) for determination of vitamin A content (International Organization for Standardization, 2009). The RP-HPLC was equipped with a DAD detector and a C18 (4.6 mm x 250 mm) column (Agilent Technologies Co., Ltd) held at 35±1℃. Mobile phase was 100% methanol. Flow rate was 1.0 mL/min and injection volume was 10 uL. Monitoring wavelength was 325 nm.

4. Determination of MEE

The microencapsulation process was monitored by MEE, which was calculated as follows:

MEE (%) = microencapsulated vitamin A/ total vitamin A × 100,

where microencapsulated vitamin A is the vitamin A content in the microencapsulated β-Lg-vitamin A and total vitamin A is the total amount of vitamin A originally added to the mixture of β-Lg and vitamin A.

5. Optimization of microencapsulation condition of β-Lg-vitamin A

5.1 Selection of independent variables on MEE for RSM

Ranges of independent variables (molar ratio of β-Lg to vitamin A, reaction time, pH, and reaction temperature) were selected for RSM design by preliminary experiments. In the first step, different molar ratios of β-Lg to vitamin A (1:1, 2:1, 3:1, 4:1, and 5:1) were compared, while other parameters were fixed (2 h, 50℃, and pH 6.5). In the second step, various reaction times (1, 1.5, 2, 2.5, and 3 h) were tested at 50℃ and pH 6.5, using the best molar ratio chosen in the previous step. In the third step, different pH of 5.5, 6.0, 6.5, 7.0, and 7.5 were tested at 50℃ using the best

molar ratio and reaction time chosen in the previous steps. Final step was to select the reaction temperature, using the best molar ratio, reaction time, and pH from the previous steps.

5.2 RSM design and statistical analysis

RSM was employed to investigate the effects of the independent variables on MEE. Based on the selected range of each of the independent variables on MEE, a central composite design (CCD) was employed to optimize microencapsulation condition of β-Lg-vitamin A. The design variables included molar ratio of β-Lg to vitamin A (A), time (B), pH (C), and temperature (D). The uncoded independent variables used in the RSM design are listed in Table 1.

Table 1. Uncoded levels for independent variables used in response surface methodology for microencapsulation of vitamin A with β-lactoglobulin

Independent variable	Unit	Symbol	$-\alpha$	-1	0	+1	$+\alpha$
Molar ratio		A	2:1	3:1	4:1	5:1	6:1
Time	h	B	0.5	1	1.5	2	2.5
pH		C	6	6.5	7	7.5	8
Temprature	℃	D	30	40	50	60	70

The experiments were designed according to the CCD using a 2^4 factorial and star design with three central points as shown in Table 2. Individual experiments were carried out in a random order. Thirty experiment settings consisting of 6 star points (star distance is 0) and 3 central points were generated with 4 factors and 3 levels by RSM. A second-order polynomial equation was:

$$MEE = a_0 + a_1A + a_2B + a_3C + a_4D + a_{11}A^2 + a_{22}B^2 + a_{33}C^2 + a_{44}D^2 + a_{12}AB + a_{13}AC + a_{14}AD + a_{23}BC + a_{24}BD + a_{34}CD,$$

where MEE is response variable, a_0, a_i, a_{ii}, and a_{ij} are constant, linear, quadratic, and interaction coefficients, respectively.

Design Expert Software (version 8.05, Stat-Ease, Inc., Minneapolis, Minnesota, USA) was used for the statistical design of the experiments and data analysis. Analysis of variance (ANOVA) was used for graphical analyses of the data to obtain the interactions between the process variables and the responses. To visualize the relationships between the responses and the independent variables, surface response and contour plots of the fitted polynomial regression equations were generated.

Table 2. Central composite design of response surface methodology for microencapsulation of vitamin A with β-lactoglobulin

Standard order	Run order	Factor 1 Molar ratio (A)	Factor 2 Time (B) h	Factor 3 pH (C)	Factor 4 Temperature (D) ℃
1	1	3:1	1	6.5	40
20	2	4:1	2.5	7	50
24	3	4:1	1.5	7	70
14	4	5:1	1	7.5	60
25	5	4:1	1.5	7	50
16	6	5:1	2	7.5	60
22	7	4:1	1.5	8	50
23	8	4:1	1.5	7	30
2	9	5:1	1	6.5	40
28	10	4:1	1.5	7	50
8	11	5:1	2	7.5	40
12	12	5:1	2	6.5	60
17	13	2:1	1.5	7	50
29	14	4:1	1.5	7	50
10	15	5:1	1	6.5	60
4	16	5:1	2	6.5	40
15	17	3:1	2	7.5	60
19	18	4:1	0.5	7	50
11	19	3:1	2	6.5	60
7	20	3:1	2	7.5	40
21	21	4:1	1.5	6	50
26	22	4:1	1.5	7	50
5	23	3:1	1	7.5	40
18	24	6:1	1.5	7	50
13	25	3:1	1	7.5	60
6	26	5:1	1	7.5	40
9	27	3:1	1	6.5	60
30	28	4:1	1.5	7	50
3	29	3:1	2	6.5	40
27	30	4:1	1.5	7	50

6. Optimization of microencapsulation of vitamin A using UHP treated β-Lg

6.1 UHP treatment

Pressure treatment was carried out using a laboratory UHP equipment (Kefa Food Equipment Co., Baotou City, Neimenggu, China). The β-Lg samples (10 mL each) prepared with optimum pH and concentration derived from RSM results were filled into polyethylene packing bags (Shenzhen San Green Industrial Co., Ltd., Shenzhen City, Guangdong, China) and put in the UHP machine. Then combinations of different pressures (100–500 MPa), times (10–50 min), and temperatures (10–50℃) were applied. After the UHP treatment, the samples were taken out of the UHP machine and mixed with vitamin A to form microcapsules using the optimum condition from the RSM results.

6.2 Selection of independent variables on MEE for OAD

Ranges of independent variables (pressure, time, and temperature) for OAD design were selected by preliminary experiments. In the first step, different pressures (100, 200, 300, 400, and 500 MPa) were compared, while other parameters were fixed (20 min and 10℃). In the second step, various times (10, 20, 30, 40, and 50 min) were tested at 10℃, using the

best pressure chosen in the previous step. In the third step, different temperatures (10, 20, 30, 40, and 50℃) were tested, using the best pressure and time chosen in the previous steps.

6.3 OAD design and statistical analysis

OAD was employed to investigate the effects of the three variables (pressure, time, and temperature) on MEE. Based on the selected range of each of the three variables on MEE, an OAD [L_9 (3^4)] matrix was employed to optimize UHP treatment condition of β-Lg. The level settings of the three variables and the experimental design were shown in Table 3 and 4, respectively. A software program for OAD (Orthogonality Experiment Assistant II, v 3.1.1, Beijing, China) was used for the statistical design of the experiments and data analysis. The results from the OAD were analyzed by range analysis and ANOVA. The average of MEE for each variable is expressed by K_i at ith (i = 1, 2, and 3) level.

Table 3. Level setting of ultra-high pressure treatment of β-lactoglobulin for orthogonal array design

Levels	Pressure (MPa)	Time (min)	Temperature (℃)
1	200	20	10
2	300	30	20
3	400	40	30

Table 4. Orthogonal array experimental design for ultra-high pressure treatment of β-lactoglobulin

Experimental No.	Pressure (MPa)	Time (min)	Temperature (℃)
1	200	20	10
2	200	30	20
3	200	40	30
4	300	20	20
5	300	30	30
6	300	40	10
7	400	20	30
8	400	30	10
9	400	40	20

7. TEM and FT-IR observations of microencapsulated β-Lg-vitamin A

Microstructures of the microencapsulated β-Lg-vitamin A prepared under the conditions optimized by RSM and OAD were observed by TEM and FT-IR spectroscopy. A Philips CM100 TEM (Philips Electronics N.V., Eindhoven, Netherlands) was operated at 60 kV. Micrographs were digitally recorded. Infrared spectra were measured with a Nicolette iS50 FT-IR spectrophotometer (Nicolet, Madison, WI, USA). The samples for FT-IR spectroscopy were freeze-dried and mixed with potassium bromide powder, followed by pressing into tablets under vacuum. For each sample, the spectrum was recorded in the 4000-400 cm^{-1} region at room temperature.

RESULTS AND DISCUSSION

1. Optimization of microencapsulation of β-Lg-vitamin A

1.1 Effects of independent variables on MEE

Figure 1 showed the effects of molar ratio (β-Lg:vitamin A), reaction time, pH, and reaction temperature on MEE. The increase of molar ratio caused an increase of the MEE. The similar effects of molar ratio on microencapsulation were reported by previous studies (Lee et al. 2002; Shpigelman et al. 2012). The highest MEE was achieved at the reaction time of 1.5 h. pH 7.0 was the most suitable for microencapsulation of β-Lg-vitamin A. Previous literatures also reported that at pH 5.5 β-Lg exists in a closed conformation where the hydrophobic cavity is not accessible to ligands to bind, while at pH 7 β-Lg has an open conformation, allowing ligands to bind at the hydrophobic cavity (Sneharani et al. 2010). The MEE decreased when the reaction temperature was higher than 50℃, suggesting that high temperature might cause vitamin A degraded during the microencapsulation process. Lešková et al. (2006) reported vitamin A rapidly loses its activity when heated at the temperature over 60℃.

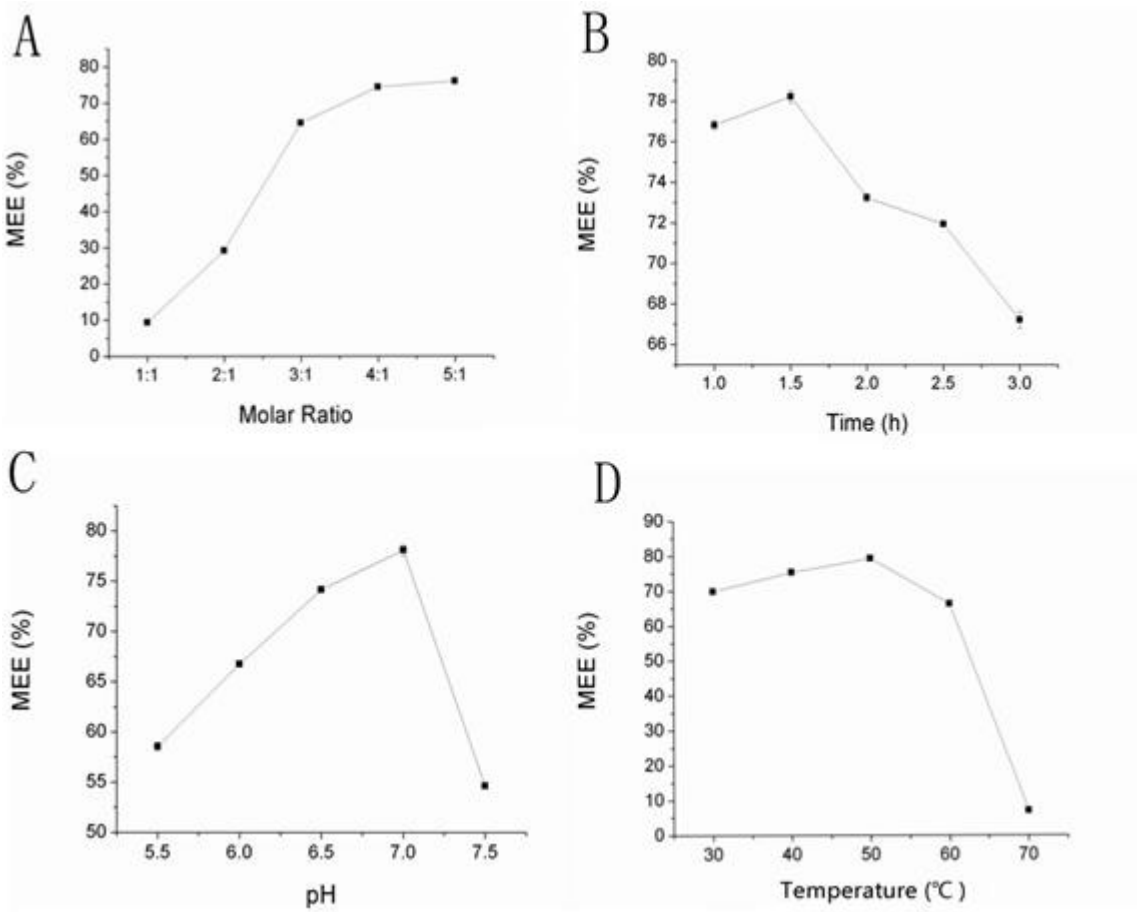

Figure 1. Effects of molar ratio of β-lactoglobulin to vitamin A (A), time (B), pH (C), and temperature (D) on the microencapsulation efficiency (MEE) of β-lactoglobulin-vitamin A.

1.2 Optimization of microencapsulation of β-Lg-vitamin A

The experimental results were shown in Table 5. Statistical analysis of ANOVA was performed to find the relationship between the independent variables and the response MEE (Table 6). The regression model had a high significance ($p < 0.0001$) and the lack of fit was not significant ($p > 0.05$), implying the current model has a high correlation between the independent variables and the responses MEE. The A, C, D, CD, A^2, C^2, and D^2 were highly significant at the level of $p < 0.01$, suggesting molar ratio (A), pH (C), and reaction temperature (D) had significant effects on the MEE. The similar effects of molar ratio, pH, and reaction temperature on the MEE were also reported by previous literatures (Lee et al. 2002; Ghosh et al. 2006; Lomas et al. 2007; Shpigelman et al. 2012). The correlation coefficient (R^2) and adjusted R^2 (R^2_{adj}) were 0.9573 and 0.9174, respectively, which indicate a high degree of correlation between the predicted and experimental data.

Table 5. Experimental microencapsulation efficiencies (MEE) of β-lactoglobulin-vitamin A resulted from response surface methodology

Standard order	Run order	Factor 1 Molar ratio (A)	Factor 2 Time (B) h	Factor 3 pH (C)	Factor 4 Temperature(D) ℃	MEE %
1	1	3:1	1	6.5	40	65.6±0.47
20	2	4:1	2.5	7	50	60.0±0.23
24	3	4:1	1.5	7	70	7.6±0.49
14	4	5:1	1	7.5	60	45.8±0.18
25	5	4:1	1.5	7	50	79.3±0.39
16	6	5:1	2	7.5	60	77.6±0.36
22	7	4:1	1.5	8	50	40.1±0.46
23	8	4:1	1.5	7	30	30.5±0.44
2	9	5:1	1	6.5	40	67.8±0.41
28	10	4:1	1.5	7	50	69.7±0.25
8	11	5:1	2	7.5	40	52.0±0.36
12	12	5:1	2	6.5	60	49.1±0.21
17	13	2:1	1.5	7	50	30.2±0.51
29	14	4:1	1.5	7	50	79.0±0.21
10	15	5:1	1	6.5	60	54.0±0.65
4	16	5:1	2	6.5	40	60.9±0.37
15	17	3:1	2	7.5	60	24.7±0.51
19	18	4:1	0.5	7	50	75.0±0.22
11	19	3:1	2	6.5	60	29.7±0.34
7	20	3:1	2	7.5	40	35.8±0.34
21	21	4:1	1.5	6	50	66.5±0.31
26	22	4:1	1.5	7	50	79.6±0.26
5	23	3:1	1	7.5	40	40.4±0.36
18	24	6:1	1.5	7	50	80.0±0.22
13	25	3:1	1	7.5	60	29.6±0.18
6	26	5:1	1	7.5	40	55.0±0.31
9	27	3:1	1	6.5	60	34.9±0.27
30	28	4:1	1.5	7	50	80.6±0.62
3	29	3:1	2	6.5	40	54.4±0.39
27	30	4:1	1.5	7	50	79.6±0.43

Table 6. Analysis of variance for the fitted quadratic polynomial model of microencapsulation efficiency of β-lactoglobulin-vitamin A

Source	Sum of squares	Degree freedom	Mean square	F value	p value
Model	11717.72	14	836.98	23.99	< 0.0001
Molar ratio (A)	2540.59	1	2540.59	72.83	< 0.0001
Time (B)	63.15	1	63.15	1.81	0.1985
pH (C)	489.58	1	489.58	14.03	0.0019
Temp (D)	725.48	1	725.48	20.8	0.0004
AB	114.91	1	114.91	3.29	0.0896
AC	171.82	1	171.82	4.93	0.0423
AD	291.15	1	291.15	8.35	0.0112
BC	141.36	1	141.36	4.05	0.0624
BD	113.36	1	113.36	3.25	0.0916
CD	355.35	1	355.35	10.19	0.0061
A^2	903.59	1	903.59	25.9	0.0001
B^2	189.67	1	189.67	5.44	0.0341
C^2	1051.12	1	1051.12	30.13	< 0.0001
D^2	5963.36	1	5963.36	170.94	< 0.0001
Residual	523.28	15	34.89		
Lack of fit	439.73	10	43.97	2.63	0.1486
Pure error	83.55	5	16.71		

R^2 = 0.9573; R^2_{adj} = 0.9174

The response surface graphs could be used to visualize the relationship between response MEE and interaction of two variables. Since reaction time had an insignificant effect on MEE, the response surface graphs used for analysis were generated from the molar ratio, pH, and reaction temperature while keeping the reaction time at 1.5 h (Figure 2). Combining the response surface graphs and statistical analysis data, molar ratio has a bigger effect on the MEE compared to pH and reaction temperature. pH and reaction temperature had similar effects on the MEE, but the effect of reaction temperature was slightly bigger than that of pH. From the three response surface graphs, the maximum MEE could be predicted to locate in the area where molar ratio was higher than 4:1, pH between 6.0 and 7.0, and reaction temperature between 45 and 55℃.

The polynomial regression equation of the response MEE could be summarized as:

$$MEE\ (\%) = 77.94 + 10.29A - 1.62B - 4.52C - 5.50D + 2.68AB + 3.28AC + 4.27AD + 2.97BC + 2.66BD + 4.71CD - 5.74A^2 - 2.63B^2 - 6.19C^2 - 14.74D^2,$$

where A is the molar ratio of β-Lg to vitamin A, B is the reaction time, C is the pH, and D is the reaction temperature.

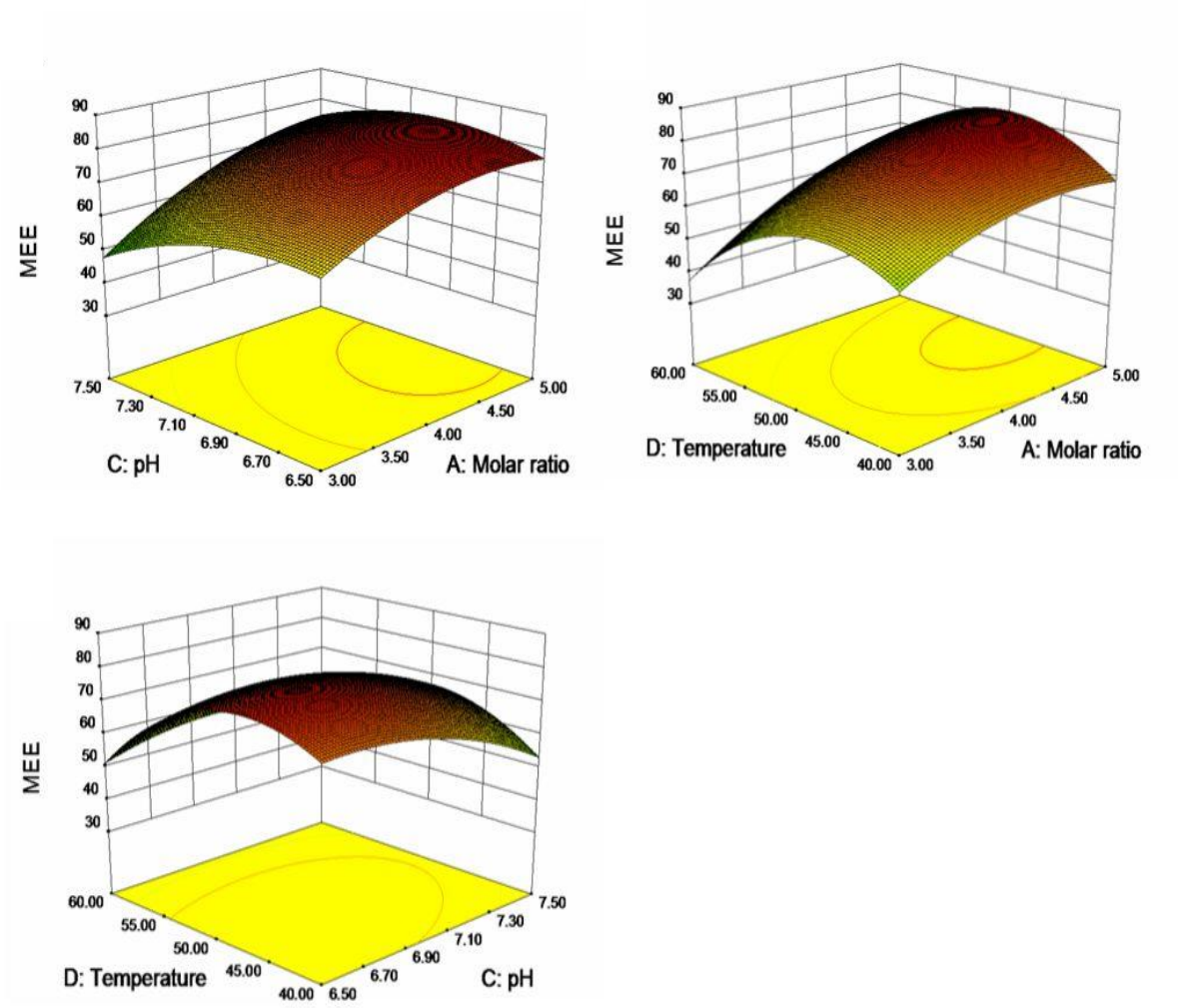

Figure 2. Response surface graphs showing effect of molar ratio, pH, and temperature on microencapsulation efficiency (MEE) of β-lactoglobulin-vitamin A.

The optimized microencapsulation condition for response MEE was obtained at molar ratio of 4.74:1, 1.43 h, pH 6.87, and 48.68℃. The MEE under the optimized conditions was calculated as 82.2% and the experimental value was 81.5±0.35%. β-Lg has been used to microencapsulate retinol, DHA, epigallocatechin-3-gallate (EGCG), and vitamin D, where the maximum MEE were all below 70% (Zimet and Livney 2008; Shpigelman et al. 2012; Wang et al. 1997; Forrest et al. 2005; Blayo et al. 2014). In this study the MEE was above 80%, suggesting microencapsulation condition was successfully optimized.

2. Optimization of microencapsulation of vitamin A using UHP treated β-Lg

2.1 Effect of UHP treatment conditions against β-Lg on the MEE of β-Lg-vitamin A

β-Lg was treated by UHP from 100 MPa to 500 MPa before encapsulating vitamin A. The MEE of microencapsulation of β-Lg-vitamin A reached the highest value when β-Lg was treated by 300 MPa, and then decreased when β-Lg was treated more than 300 MPa (Figure 3A). When β-Lg was treated under the pressure lower than 300 MPa, the pressure-induced denaturation of β-Lg could increase its binding ability to vitamin A,

meaning more vitamin A could be encapsulated to β-Lg. However, the higher pressure than 300 MPa may cause the other structural change in β-Lg, leading to decrease in its binding ability to vitamin A (Huppertz et al. 2006; Aouzelleg et al. 2004; Belloque et al. 2000). Similar results have been reported by previous literatures (Sheng et al. 2011; Claire et al. 2014). The highest MEE of the microencapsulated β-Lg-vitamin A was observed when β-Lg was treated by 300 MPa for 30 min (Figure 3B). When β-Lg was treated at 300 MPa for 30 min at 10℃-50℃. The MEE of the microencapsulated β-Lg-vitamin A reached the highest when vitamin A was encapsulated with the β-Lg which was pretreated at 300 MPa for 30 min at 20℃ (Figure 3C).

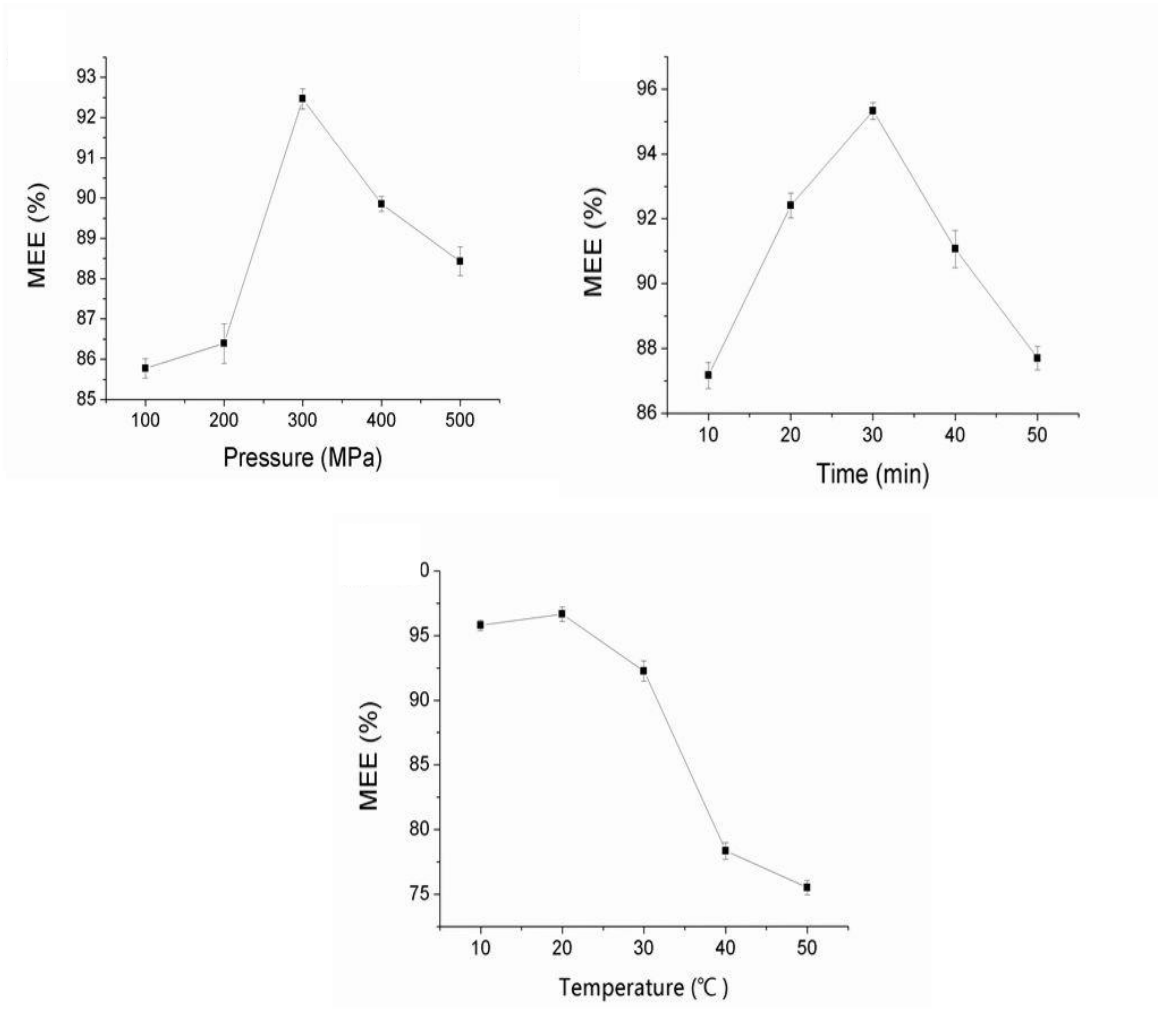

Figure 3. Effect of ultra-high pressure treatment conditions of β-lactoglobulin on the microencapsulation efficiency (MEE) of β-lactoglobulin-vitamin A

2.2 Optimization of microencapsulation of vitamin A using UHP treated β-Lg

The experimental MEE of microencapsulation of vitamin A using the UHP treated β-Lg by orthogonal array design were shown in Table 7. The variance analysis and range analysis for the MEE of the microencapsulation of vitamin A using the UHP treated β-Lg were shown in Table 8 and 9, respectively. The significance of each variable was evaluated by calculating F value. The significant variables were found to be pressure and temperature ($p < 0.05$), while the time had no significant effect on the MEE ($p > 0.05$). The range analysis indicates that the influence of the three variables on the MEE decreased in the order of pressure > temperature > time. The optimum UHP treatment conditions were determined to be 300 MPa, 20 min, and 20℃. The experimental MEE under the optimized condition was 94.8±0.48%, about 13% higher than the experimental MEE of microencapsulation of vitamin A using the β-Lg which was not treated under UHP.

Table 7. Experimental microencapsulation efficiencies (MEE) of microencapsulation of vitamin A using ultra-high pressure treated β-lactoglobulin resulted from orthogonal array design

Experimental No.	Pressure (MPa)	Time (min)	Temperature (℃)	MEE (%)
1	200	20	10	88.7±0.46
2	200	30	20	90.1±0.42
3	200	40	30	86.7±0.35
4	300	20	20	94.7±0.55
5	300	30	30	90.1±0.37
6	300	40	10	91.5±0.43
7	400	20	30	89.0±0.62
8	400	30	10	89.3±0.38
9	400	40	20	91.0±0.49

Table 8. Variance analysis for microencapsulation efficiencies of microencapsulation of vitamin A using ultra-high pressure treated β-lactoglobulin

	Sum of square	Degree freedom	F value	Significance ($p < 0.05$)
Pressure	20.065	2	130.292	*
Time	1.957	2	12.708	
Temperature	16.878	2	109.597	*
Error	0.15	2		

$F_{0.05} = 19.00$
*$p < 0.05$

Table 9. Range analysis for microencapsulation efficiencies of microencapsulation of vitamin A using ultra-high pressure treated β-lactoglobulin

	Pressure	Time	Temperature
K_1	88.477	90.770	89.810
K_2	92.087	89.823	91.920
K_3	89.773	89.743	88.607
Range	3.610	1.027	3.313

3. Microstructures observed by TEM and FT-IR spectroscopy

The microstructures were investigated by TEM at high magnification (10,000×). β-Lg was observed to be sphere-shaped in a regular order and its particle sizes were about 150-250 nm (Figure 4A). The morphology of β-Lg-vitamin A microcapsule was similar to that of β-Lg, although the encapsulated β-Lg-vitamin A was slightly bigger than β-Lg alone (Figure 4B). The UHP treated β-Lg alone and its microcapsule with vitamin A, whose particle sizes were less than 100 nm, were smaller than the untreated β-Lg alone (Figure 4C and 4D).

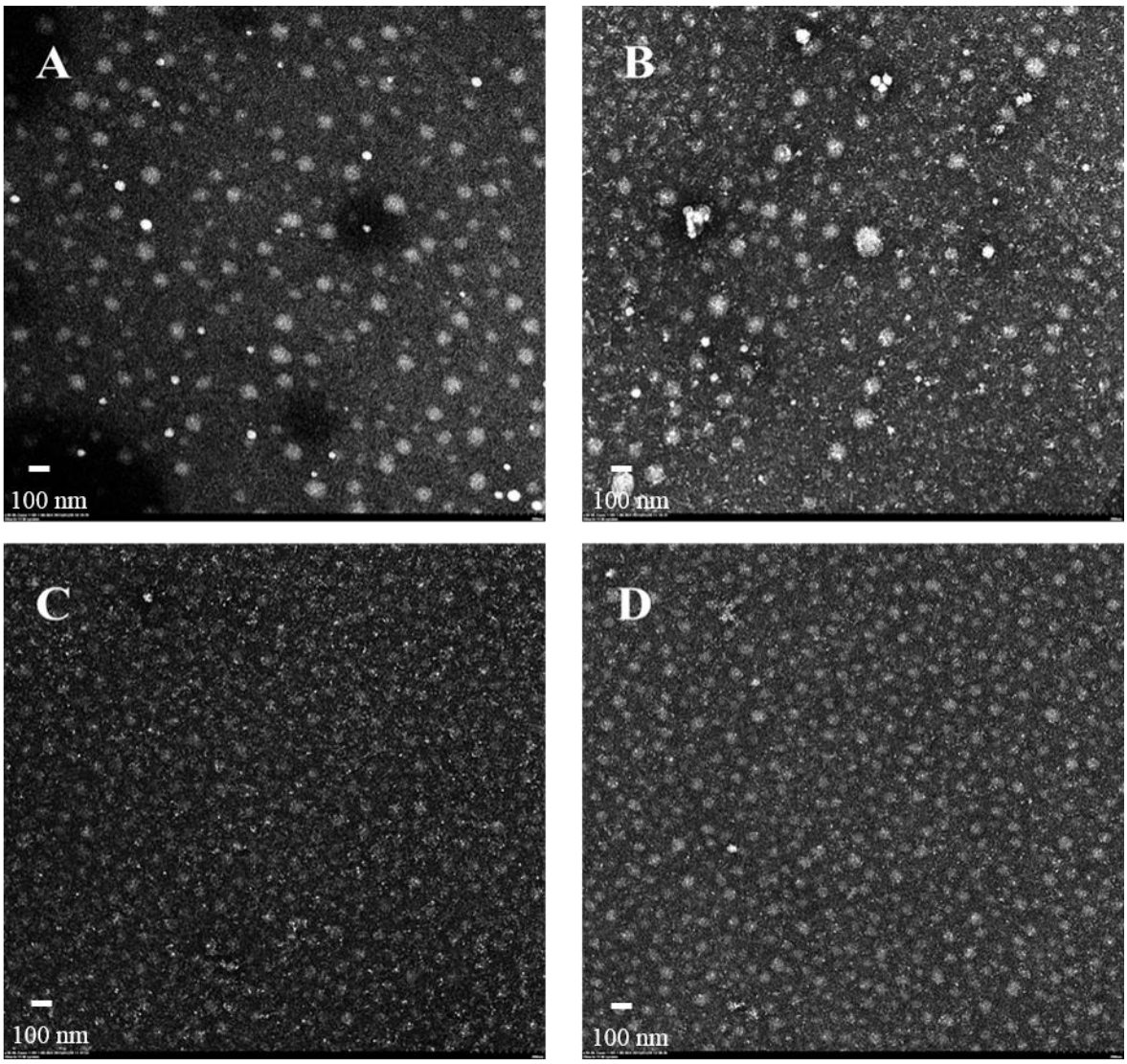

Figure 4. Transmission electron microscopy image (10,000×) of β-lactoglobulin (A), microcapsule of β-lactoglobulin-vitamin A (B), ultra-high pressure treated β-lactoglobulin (C), and microcapsule of ultra-high pressure treated β-lactoglobulin-vitamin A (D).

FT-IR spectroscopic analysis was used to investigate the binding interactions between vitamin A and β-Lg (Figure 5). Vitamin A was characterized by bands of 1700-1500 (C=O stretching vibrations) and 1300-1100 cm^{-1} (C-O stretching vibrations) (Figure 5A and 5B). β-Lg was characterized by protein amide I band at 1700-1600 cm^{-1} (mainly C=O stretch) and amide II band at 1541 cm^{-1} (C-N stretching coupled with N-H bending modes) (Michael and Heino 1986; Gunda et al. 1999). The intense bands observed in vitamin A disappeared in the microcapsule of β-Lg-vitamin A, suggesting vitamin A was incorporated in the hydrophobic central cavity of β-Lg. The spectrum of β-Lg-vitamin A microcapsule, compared with that of β-Lg, showed shifts from 1643 to 1644 cm^{-1} and from 1541 to 1536 cm^{-1}, due to vitamin A binding to proteins' C=O, C-N, and N-H groups (hydrophobic interaction) (Shpigelman et al. 2012). Wang et al. (2011) reported that the characteristic bands of garlic oil disappeared in the microcapsules of garlic oil and β-cyclodextrin due to formation of microcapsules. Shpigelman et al. (2012) also reported the characteristic bands of EGCG disappeared in β-Lg-EGCG nanovehicles due to formation of nanoencapsules. The same trend was found in the microcapsules of vitamin A using UHP treated β-Lg in this study.

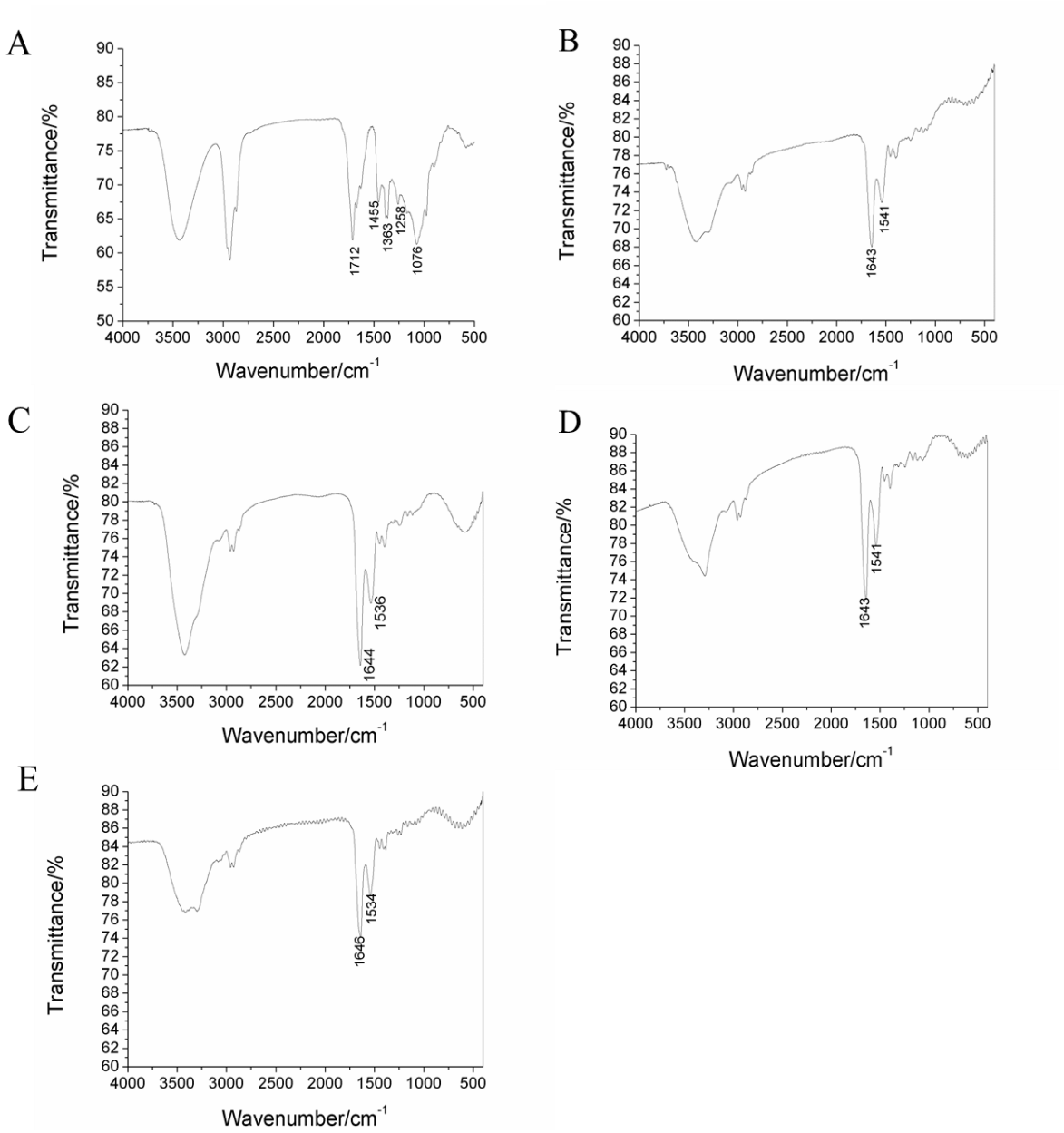

Figure 5. Fourier transform infrared spectra of vitamin A (A), β-lactoglobulin (B), microcapsule of β-lactoglobulin-vitamin A (C), ultra-high pressure treated β-lactoglobulin (D), and microcapsule of ultra-high pressure treated β-lactoglobulin-vitamin A (E).

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국문 초록

Vitamin A는 성장, 발달, 면역과 시력 등 생리 기능에 큰 영향을 주는 중요한 영양소이기 때문에 영양 강화제로 식품에 많이 첨가하고 있다. 그러나 vitamin A는 지용성이고 산소와 빛에 의해서 쉽게 산화되는 단점이 있다. 이러한 단점을 보완하기 위해서 식품공업에서 vitamin A를 미세캡슐화시킨다. β-Lactoglobulin(β-Lg)은 유청단백질의 주요 성분이고 외부에 수용성 성질을 가지고 있으며 내부에 cavity가 있어, 지용성 물질과 결합하는 능력이 있고, 특히 vitamin A와 친화도가 높아서 미세캡슐화할 때 좋은 피복재료로 사용할 수 있다. 그리고 β-Lg을 초고압 처리하면 vitamin A와의 결합능력을 향상시킬 수 있다. 따라서 이번 연구에서는 vitamin A를 미세캡슐화시키기 위하여 β-Lg을 피복물질로 사용하여 β-Lg-vitamin A의 미세캡슐화 조건을 최적화하였다. 미세캡슐의 microencapsulation efficiency(MEE)를 향상시키기 위하여 β-Lg을 초고압 처리하여 vitamin A와 미세캡슐화하였는데, 미세캡슐화하기 전에 β-Lg을 초고압 전처리하는 조건을 최적화하였다. 최적화한 미세캡슐의 구조를 transmission electron microscopy(TEM)와 and Fourier transform infrared(FT-IR) spectroscopy를 통하여 확인하였다.

β-Lg-vitamin A 미세캡슐화 조건을 최적화한 실험은 반응표면분석법을 이용하여 진행하였고, 몰 비율, 반응시간, pH, 반응온도의 4개 인자를 사용하여, 중심합성계획법으로 설계해서 MEE를 측정하였다. 실험 결과를 통하여 최적 조건을

예측하는 회귀식을 얻었고, 예측한 최적 캡슐화조건은 몰 비율 4.74:1, 반응시간 1.43 h, 반응온도 48.68℃, pH 6.87이었으며, 이 조건에서의 예측 MEE는 82.2%였고 실험하여 얻은 MEE는 81.5%였다. β-Lg의 초고압 전처리의 최적화 실험은 압력, 가압시간, 가압온도 세가지 인자를 사용하여 직교분석법을 이용해서 진행하였다. 실험에서 예측한 최적 초고압 전처리 조건은 압력 300 MPa, 가압시간 20 min, 가압온도 20℃였고, 이 조건에서 실험하여 얻은 MEE는 94.8%였다. β-Lg을 초고압 전처리하여 제조한 미세캡슐의 최대 MEE가 초고압 처리하지 않은 β-Lg을 실험하여 제조한 미세캡슐의 최대 MEE보다 약 13% 높았다. 최적화한 미세캡슐들은 TEM을 통하여 구형인 상태를 확인하였고, FT-IR을 통하여 vitamin A가 β-Lg 내부의 cavity에 들어가는 것을 확인하였다. 이번 실험을 통해 β-Lg-vitamin A의 최적 캡슐화 조건과 β-Lg의 초고압 전처리 최적 조건을 확립하였다고 생각한다. 그리고 최적 조건에서 제조한 미세캡슐이 성공적으로 형성된 것을 검증하였다.

주요어: β-lactoglobulin; vitamin A; microencapsulation efficiency; 초고압; 반응표면분석법; 직교분석법

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