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Running Title (within 10 words)	Casein derivative-based delivery system for docosahexaenoic acids
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Abstract

Although omega-3 fatty acids including docosahexaenoic acid (DHA) contain various health-promoting effects, their poor aqueous solubility and stability make them difficult to be induced in dairy foods. The aims of this research were to manufacture casein derivative-based delivery system using acid-induced gelation method with glucono-σ-lactone and to investigate the effects of production variables, such as pH and charged amount of linoleic acid, on the physicochemical properties of delivery systems and oxidative stability of DHA during storage in model milk. Covalent modification with linoleic acid resulted in the production of casein derivatives with varying degrees of modification. As pH was reduced from 5.0 to 4.8 and the charged amount of linoleic acid was increased from 0 to 30%, an increase in particle size of casein derivative-based delivery systems was observed. The encapsulation efficiency of DHA was increased with decreased pH and increased charged amount of linoleic acid. The use of delivery system for DHA resulted in a decrease in the development of primary and secondary oxidation products. An increase in the degree of modification of casein derivatives with linoleic acid resulted in a decrease in in the formation of primary and secondary oxidation products than of free DHA indicating that delivery systems could enhance the oxidative stability of DHA during storage in model milk. In conclusions, casein derivatives can be an effective delivery system for DHA and charged amount of linoleic acid played a key role determining the physicochemical characteristics of delivery system and oxidative stability of DHA.

Keywords: sodium caseinate, casein derivative, delivery system, acid-induced gelation, docosahexaenoic acid

Introduction

As the consumer demand for reduced fat diet has been increasing, there has been a growing intake of low- and non-fat dairy foods (Boukid et al., 2021). However, low- and non-fat dairy foods may have textural defects, such as formation of week body and increased whey separation (Saleh et al., 2020). Moreover, a reduction in the fat in dairy foods leads to the deficiency of hydrophobic nutrients including omega-3 polyunsaturated fatty acid (PUFA) (Ziment and Livney, 2009). Omega-3 PUFA including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has various health benefits. It can decrease the risk of cardiovascular disease and high blood pressure (Ha et al., 2018; Nain et al., 2021). The oxidation of DHA and DHA-rich ingredients (e.g., fish oil) resulted in the formation of fish-like off-flavor and thereby reducing the food quality during storage in low- and non-fat dairy foods (O'Dwyer et al., 2012; Saga et al., 2013; Hwang et al., 2017). Moreover, high hydrophobicity of DHA with poor aqueous solubility makes it difficult to be applied in low- and non-fat foods. Therefore, it is challenged to develop new methods to overcome the limitations when applying DHA for foods. Delivery systems have been used to exceed those limitations by encapsuling and protecting DHA inside of delivery system.

Various studies have focused on the encapsulation of DHA and DHA-enriched ingredients (e.g., fish oil) in nano- and micro-sized delivery systems, which are nanoparticles (Zimet and Livney, 2009; Zimet et al., 2011; Ha et al., 2018), microparticles (Vaucher et al., 2019), nanoemulsions (Hwang et al., 2017), and microemulsions (Cortés et al., 2019).

Among various synthetic- and natural biopolymers, milk proteins including caseins have been used as delivery materials for the delivery of hydrophobic nutrients due to their high nutritional value and various functional properties, such as an ability to bind hydrophobic

nutrients, gel forming capacity, stabilization of emulsions, and antioxidant activity (Livney, 2010; Kimpel and Schmitt, 2015). Caseins are the main milk protein component accounting for about 80% of total milk proteins (Livney et al., 2010; Wang and Zhao, 2022). The use of glucono-σ-lactone (GDL) or starter culture reduces the pH of of milk and casein, which can lead to the production of acid gels (Lucey et al., 1997; Luo et al., 2015; Nag et al., 2011). The acid gelation is a way to develop delivery systems since the formation of acid-induced gels can efficiently protect and enhance the stability of probiotics (Nag et al., 2011), curcumin (Khanji et al., 2015) and polyphenols (Bayraktar et al., 2019).

It was reported that caseins can bind hydrophobic nutrients via hydrophobic interactions (Forrest et al., 2005; Semo et al., 2007; Zimet and Liveny, 2011). For the delivery of hydrophobic bioactive compounds including DHA, it is beneficial to enhance the hydrophobicity of delivery materials, which may efficiently encapsulate and protect hydrophobic bioactive compounds inside of delivery systems (Ha et al., 2013; Ha et al., 2018). In this study, we hypothesized that the formation of casein derivative by covalent modifications with hydrophobic fatty acids can increase the hydrophobicity of casein effectively enhancing the oxidative stability of DHA in aqueous-based delivery system. Therefore, it can reduce the formation of fishy-like off flavor during storage in dairy foods.

The aims of this study were to prepare hydrophobically modified casein derivatives and to investigate how production variables, such as degree of modification of casein derivatives and pH, affect the physicochemical characteristics of casein derivative-based delivery systems including DHA.

Materials and Methods

Chemicals and Reagents

Sodium caseinate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy succinimide (NHS), O-pthalaldehyde (OPA), sodium dodecyl sulfate (SDS), 2-mercaptoethanol (2-ME), 1-Anilinonaphthalene-8-Sulfonic Acid (ANS), glucono-σ-lactone (GDL) were obtained from Sigma-Aldrich (St. Louis, USA).

Preparation of casein derivatives

Hydrophobically-modified casein derivatives were produced by using EDC-mediated coupling reactions (Ha et al., 2013; Ha et al., 2018). EDC can induce covalent conjugations between the amino groups of sodium caseinate and carboxyl groups of linoleic acid while NHS was used to enhance the stability of reaction intermediate (O-acylisourea intermediate). Two hundred milligrams of sodium caseinate were dissolved in 20 mL of deionized water and mixed with various concentration of 10 mL of linoleic acid dispersed in ethanol. Linoleic acid concentration levels in ethanol were 0 mg/10mL, 17.9 mg/10 mL, 35.9 mg/10 mL, and 53.8 mg/10 mL, which correspond to the molar ratio (charged amount of linoleic acid) of 0, 10, 20, or 30% to sodium caseinate solution, respectively. Then, 5 mL of EDC and NHS dissolved in tetrahydrofuran was added to sodium caseinate/linoleic acid mixture followed by stirring for 12 h at room temperature (molar ratio of EDC:NHS:linoleic acid = 1:1:1). To remove unbound linoleic acid and residual reaction chemicals, caseinate/linoleic acid/EDC/NHS mixtures were

dialyzed against 50% ethanol and deionized water for 24 and 48 h using dialysis membrane (3.5 kDa molecular weight cut-off, Thermo Scientific, Rockford, USA) followed by freeze-drying.

Degree of modification

The degree of modification of casein derivatives, a number of linoleic acid bound to sodium caseinate, was determined by measuring the number of free amino groups using OPA reagent. Twenty-five milliliters of sodium tetraborate buffer (pH 9.5) was mixed with 2.5 mL OPA dissolved in methanol (16.4 mg/2.5 mL) followed by addition of 5 mL of 20% SDS to sodium tetraborate buffer/OPA mixture. Then, 400 μL of 2-ME was added to sodium tetraborate buffer/OPA/SDS mixture and final volume of mixture was adjusted to 100 mL with deionized water. To determine the number of amino groups, 50 μL of unmodified (standard) or hydrophobically-modified sodium caseinate was mixed with 1 mL of OPA reagent. Measurement of the number of amino acids groups at 340 nm was performed using a UV-VIS spectrophotometer (GENESYS10-S, Thermo Spectronic, Rochester, U.S.A).

$$\text{Degree of modification (\%)} = \frac{\text{Amino groups of sodium caseinate} - \text{amino groups of casein derivative}}{\text{Amino groups of sodium caseinate}} \times 100$$

Surface hydrophobicity of casein derivative

The surface hydrophobicity of casein derivatives with various charged amount of linoleic acid was assessed by using a procedure represented in Monahan et al. (1995) and Ha et al. (2013). ANS was used as a fluorescence probe for the hydrophobic affinity of protein surface.

Freeze-dried casein derivatives were dispersed in Tris-HCl buffer (pH 5.0) and diluted to selected concentration of 0.005, 0.01, 0.015, 0.02, and 0.25% (w/v) followed by adding 20 μL of 8 mM ANS dispersed in 0.1 M phosphate buffer (pH 7.4) to 3 mL of casein derivative solution. Fluorescent intensity of casein derivative/ANS mixture was evaluated at excitation wavelength of 390 nm and emission wavelength of 470 nm using a spectrofluorometer (Luminescence Spectrometer LS50 B, Perkin-Elmer Co, USA). The R value calculated by following equation was used as an indication of surface hydrophobicity of casein derivatives.

$$R = (F - F_0) / F_0$$

where F is the fluorescent intensity of casein derivatives with ANS and F_0 is the fluorescent intensity of ANS solution without casein derivatives.

Manufacture of casein derivative delivery system

Casein derivative delivery systems were developed by the use of a modified pH-induced gelation method with GDL (Nag et al., 2011). Five percent (w/v) of casein derivative solution was mixed with DHA dissolved in ethanol and 2% (w/v) GDL solution followed by stirring for 10 min. Final concentration of DHA in casein derivative solution was 25 mg/100 mL. To form delivery system, 10 mL of casein derivative solution including GDL and DHA was added to 40 mL of soybean oil (Sajo Haepyo Co., Korea). It was mixed thoroughly for 5 min in magnetic stirrer and then held for 60 or 70 min to allow to form gel matrix. After holding for 60 and 70 min, pH of sodium caseinates solution reached to 5.0 or 4.8, respectively and aqueous phase (sodium caseinate solution) was turned into micro-sized particles by acid-induced gelation. Casein derivative-based delivery systems formed in water phase were collected by

158 centrifugation at 15,000 g for 1 min and oils at top layer were discarded. After washing 3 times
159 with deionized water to remove residual oil, casein derivative delivery systems were collected
160 and then freeze-dried.

161

162 **Physicochemical properties of casein derivative delivery systems**

163 Dynamic light scattering measurement was done with a particle size analyzer (Zetasizer
164 Nano ZS, Marvern Instruments, UK) to assess the average particle size, distribution
165 (polydispersity index), and zeta potential values of casein derivative delivery system. It was
166 prepared with a 633 nm HeNe laser, operating at a scattering angle of 173º.

167

168 **Oxidative stability of DHA**

169 Oxidative stability of DHA during storage in model milk was determined by assessing
170 peroxide, *p*-anisidine value, and development of volatile compounds (Ha et al., 2018). Model
171 milk was prepared by adjusting pH of distilled water to 6.7 with 0.01 M HCl. Free DHA and
172 freeze-dried casein derivative-based delivery system containing DHA and free DHA were
173 added to model milk and stored at 4°C for 16 d. The development of hydroperoxide (primary
174 oxidation product) and aldehyde (secondary oxidation product) during storage in skim milk
175 was assessed by using peroxide (IDF method 74A, 1991) and *p*-anisidine value (AOCS method
176 CD 18–90 described by Kargar, et al., 2011), respectively. The formation of volatile compounds
177 during storage was measured by using a GC-mass spectrometry (GC MS-TQ8030, Shimadzu,
178 Kyoto, Japan). An internal standard (3-methyl-3-buten-1-ol, final concentration: 4 mmol/L) for
179 estimating the relative concentration of (E,E)-2,4-heptadienal was added to free DHA and

casein derivative delivery system containing DHA in model milk. A solid phase microextraction fiber (Supelco, Bellefonte, USA) was used to obtain volatile compounds from model milk containing DHA during storage. Chromatographic separation was achieved via a DB-5ms column (30m×0.25mm i.d.×0.25 μm film thickness, Agilent J&W Scientific, Folsom, USA). The relative concentrations of (E,E)-2,4-heptadienal were evaluated by following equation:

$$\text{Relative concentration} = \frac{\text{Peak area of volatile compound}}{\text{Peak area of internal standard}} \times \text{internal standard concentration}$$

Statistical analysis

All data were demonstrated as a mean of three replicates. The effects of production variables, such as degree of modification of casein derivative and pH, on the physicochemical characteristics of delivery system and oxidative stability of DHA were analyzed by one-way analysis of variance (ANOVA) with a statistical significance of $p<0.05$. Repeated-measures ANOVA was applied to assess the impacts of production variables, storage time, and their interactions on the oxidative stability of DHA during storage. The statistical analysis system (Version 9.1, SAS Institute Inc., USA) was used to conduct ANOVA.

Results and Discussion

Degree of modification and surface hydrophobicity of casein derivatives

To enhance the hydrophobicity of caseins, the hydrophobic fatty acid, linoleic acid, was covalently attached to caseins using EDC and NHS. When EDC without NHS was used, less than 2% of amino groups of casein was covalently linked with the carboxyl groups of linoleic acid (data not shown). It can be due to the poor stability of reaction intermediate that is acylisourea intermediate. On the other hand, when both EDC and NHS were used, the degree of modification, the number of amino acids of caseins bound to carboxyl groups of linoleic acid, was increased up to 23% (Fig. 1). The effects of charged amount of linoleic acid on the degree of modification of casein derivative were presented in Fig. 1. As the charged amount of linoleic acid was increased from 0 to 30%, an increase in the degree of modification from 0 to 23% was observed indicating that more linoleic acids were covalently attached to amino groups of caseins. Casein derivatives with increasing charged amount of linoleic acid had more linoleic acid residues on the surface of casein derivative. Those more hydrophobic sites on the surface of casein derivative led to an increase in the hydrophobic affinity for ANS resulting in increased surface hydrophobicity (Fig. 1B).

Physicochemical properties of casein derivative delivery system

222 The effects of pH on the physicochemical characteristics of casein derivative delivery
223 systems, such as particle size, polydispersity index, zeta-potential value and DHA
224 encapsulation efficiency, were presented in Fig. 2. There was an increase in the size of delivery
225 systems from 6.5 to 9.4 μm when pH was decreased from 5.0 to 4.8 (Fig. 2A). Since the
226 isoelectric pH of caseins was about 4.6, there are stronger aggregations between casein
227 derivative molecules at pH 4.8 through electrostatic attractions than that of pH 5.0
228 (McClements, 1999). It resulted in the formation of larger particles at pH 4.8 (McClements,
229 1999). No significant differences were observed in the polydispersity index values of delivery
230 systems formed at 4.8 and 5.0 (Fig. 2B). The negative surface charges of delivery systems was
231 significantly ($p<0.05$) increased with increased pH from 4.8 to 5.0 (Fig. 2C). Due to the
232 isoelectric pH of casein is about 4.6, sodium caseinate had more negative charges at pH 5.0
233 than pH 4.8, which can lead to the formation of sodium caseinate delivery systems with higher
234 negative charges at pH 5.0. Manufacturing pH did not significantly affect the encapsulation
235 efficiency of DHA (Fig. 2D). Since delivery system had much smaller size with higher negative
236 charges at pH 5.0 than pH 4.8, optimum pH was set at 5.0 for further experiments.

237 Fig. 3 revealed that increasing charged amount of linoleic acid significantly ($p<0.05$)
238 enhanced the size and DHA encapsulation efficiency of casein derivative-based delivery
239 systems. Since casein derivatives with higher charged amount of linoleic acid had more
240 hydrophobic linoleic acid moieties and higher surface hydrophobicity (Fig. 1), it could increase
241 hydrophobic attractions between casein derivative molecules and hydrophobic DHA. This can
242 lead to an increase in the size of delivery systems from 6.5 to 7.2 μm (Fig. 3C) and DHA
243 encapsulation efficiency from 85.7 to 88.5% (Fig. 3D).

244

245 **Oxidative stability of DHA during storage in model milk**

246 The effect of encapsulation of DHA in casein derivative-based delivery system on the
247 oxidative stability was presented in Fig. 4. Repeated-measures ANOVA results exhibited that
248 both treatment (encapsulation in delivery system) and storage time (15 d) had a significant
249 effect on peroxide value (Fig 4A), *p*-anisidine value (Fig 4B), and development of (E,E)-2,4,-
250 heptadienal (Fig 4C). A significant ($p<0.001$) increase in peroxide value, *p*-anisidine value, and
251 development of (E,E)-2,4,-heptadienal was observed with storage time. Polyunsaturated fatty
252 acids including DHA are easily oxidized resulting in the production of primary oxidation
253 product (hydroperoxide) and secondary oxidation products (aldehydes and (E,E)-2,4,-
254 heptadienal). During storage in model milk, noticeable increases in the primary and secondary
255 oxidation products were observed after 6-d storage and gradually increased to 15 d. However,
256 when DHA was encapsulated in casein derivative-based delivery system, peroxide value, *p*-
257 anisidine value, and development of (E,E)-2,4,-heptadienal, a major compound related to fishy-
258 like off-flavor and a potential marker for DHA autoxidation (Venkateshwarlu et al., 2004),were
259 significantly ($p<0.0001$) reduced compared with that of free (unencapsulated) DHA. It
260 indicates that the use of casein derivative-based delivery systems could efficiently protect DHA
261 from oxidative rancidity during storage. Caseins have been reported to increase the oxidative
262 stability of omega-3 polyunsaturated fatty acids (Gallaher et al., 2005; Nielsen and Jacobsen,
263 2009) due to their antioxidant activity by chelating potential metal pro-oxidants, especially iron.
264 Phosphoseryl groups in caseins are key components responsible for the intense binding
265 properties to transition metals (Vegarud et al., 2000; Diaz et al., 2003). Moreover, the formation
266 of delivery systems by acid-induced gelation may provide physical barrier for DHA, which can
267 contribute to shielding effects reducing the autoxidation of DHA.

Fig. 5. presents the impacts of manufacturing pH on the oxidiative stability of encapsulated DHA in casein delivery system. Repeated-measures ANOVA results exhibited that storage time significantly ($p<0.0001$) affected peroxide value, *p*-anisidine value, and formation of (E,E)-2,4,-heptadienal while there are no significant effects on treatment (pH). Although pH had a significant effect on the size and zeta-potential value of casein derivative-based delivery systems (Fig. 2), its protective effects from the oxidative rancidity of DHA were not significantly affected by altering pH.

Effects of charged amount of linoleic acid on the oxidative stability of encapsulated DHA were shown in Fig. 6. Similar to Figs 4 and 5, repeated measured ANOVA results revealed that storage time significantly ($p<0.0001$) affected the peroxide value (Fig 4A), *p*-anisidine value (Fig 4B), and development of (E,E)-2,4,-heptadienal indicating that the oxidation of DHA tended to be increased with storage time. The production of primary oxidation product (hydroperoxide) and secondary oxidation product (aldehydes) was markedly increased after 3-d storage while a significant increase on the fishy-like off-flavor compound, (E,E)-2,4,-heptadienal was found after 6-d storage (Fig. 6). Repeated-measures ANOVA presented that hydrophobically modification of sodium caseinate by covalent attachment of linoleic acid with various charged amount of linoleic acid had a significant effect on peroxide vale ($p<0.01$), *p*-anisidine value ($p<0.01$), and development of (E,E)-2,4,-heptadienal ($p<0.05$) during storage in model milk for 15 d (Fig. 6). After 16 d of storage, an increase in the charge amount of linoleic acid from 0 to 30% resulted in a reduction in the formation of (E,E)-2,4,-heptadienal from 2.1 to 1.4 μg/g. It indicates that increasing the number of hydrophobic linoleic acid residues attached to sodium caseinate by increasing charged amount of linoleic acid enhanced the hydrophobicity of casein derivative-based derivatives, which can lead to a reduction in the oxidative rancidity of DHA. An increase in hydrophobicity of sodium caseinate could enhance

292 the hydrophobic attractions between sodium caseinate molecules and DHA and may reduce the
293 mobility of DHA and diffusion of prooxidants resulting in a reduction of DHA oxidation
294 (Kellerby et al., 2006). Similar result was reported in a previous study for the hydrophobically
295 modified chitosan/*β*-lactoglobulin nanoparticles (Ha et al., 2018).
296

297 ## Conclusions

298 In conclusions, hydrophobically-modified casein derivatives with various degree of
299 modification were produced by modulating charged amount of linoleic acid. Casein derivative-
300 based delivery systems with a size ranging from 6.5 to 9.4 μm were successfully manufactured
301 using acid-induced gelation method with GDL. More than 85% of DHA was encapsulated in
302 casein derivative-based delivery system. During storage in model milk, encapsulated DHA in
303 casein derivative-based delivery system exhibited better oxidative stability than free
304 (unencapsulated) DHA indicating that casein derivative-based delivery system could efficiently
305 protect DHA against autoxidation. In addition, the charged amount of linoleic acid was a key
306 factors affecting the encapsulation efficiency and oxidative stability of DHA during storage in
307 skim milk. Our results suggest that casein derivative-based delivery systems have great
308 potential for the application of DHA to functional dairy foods.
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Figure Legends

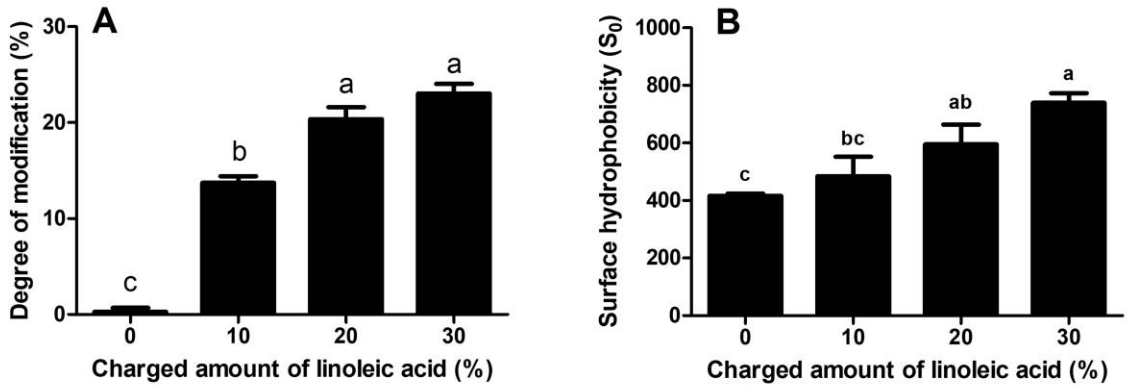
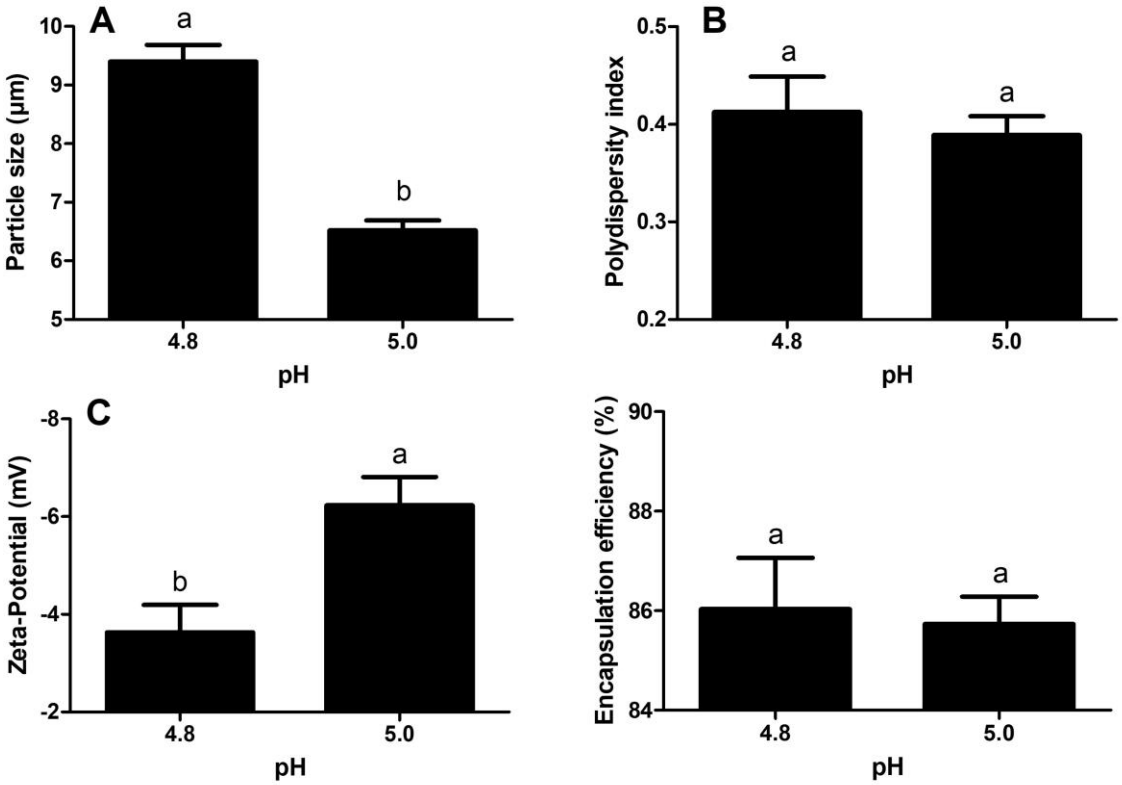


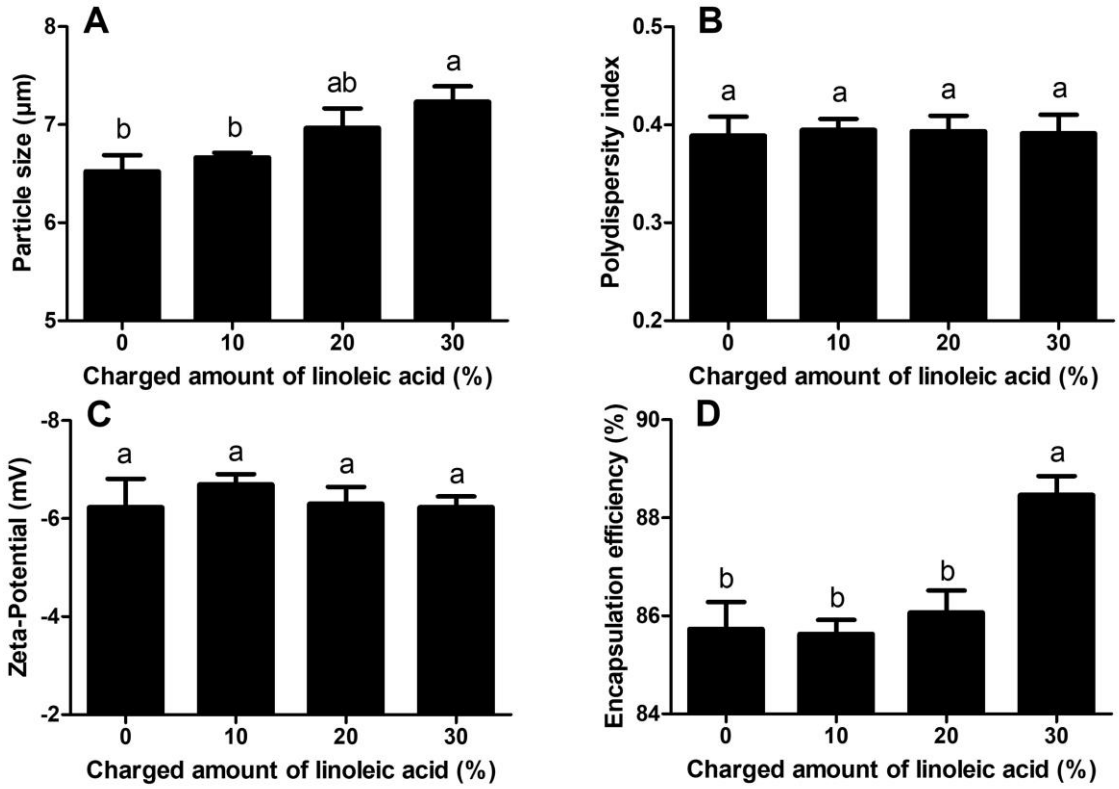
Fig. 1. Impacts of charged amount of linoleic acid on the degree of modification (A) and surface hydrophobicity (B) of casein derivatives. Different letters on each bar indicate significant ($p<0.05$) differences.



389

390 **Fig. 2.** Impacts of pH on the size (A), polydispersity index (B), and zeta-potential value (C)
391 of derivative-based delivery systems. Delivery systems were prepared with 5% (w/v) sodium
392 caseinate at pH 4.8 or 5.0. Different letters on each bar denote significant ($p<0.05$) differences.

393



394

395 **Fig. 3.** Impact of charged amount of linoleic acid on the size (A), polydispersity index (B),
396 zeta-potential value (C), and DHA encapsulation efficiency (D) of casein derivative-based
397 delivery systems. Delivery systems were prepared with 5% (w/v) casein derivatives at pH 5.0.
398 Different letters on each bar denote significant ($p<0.05$) differences.

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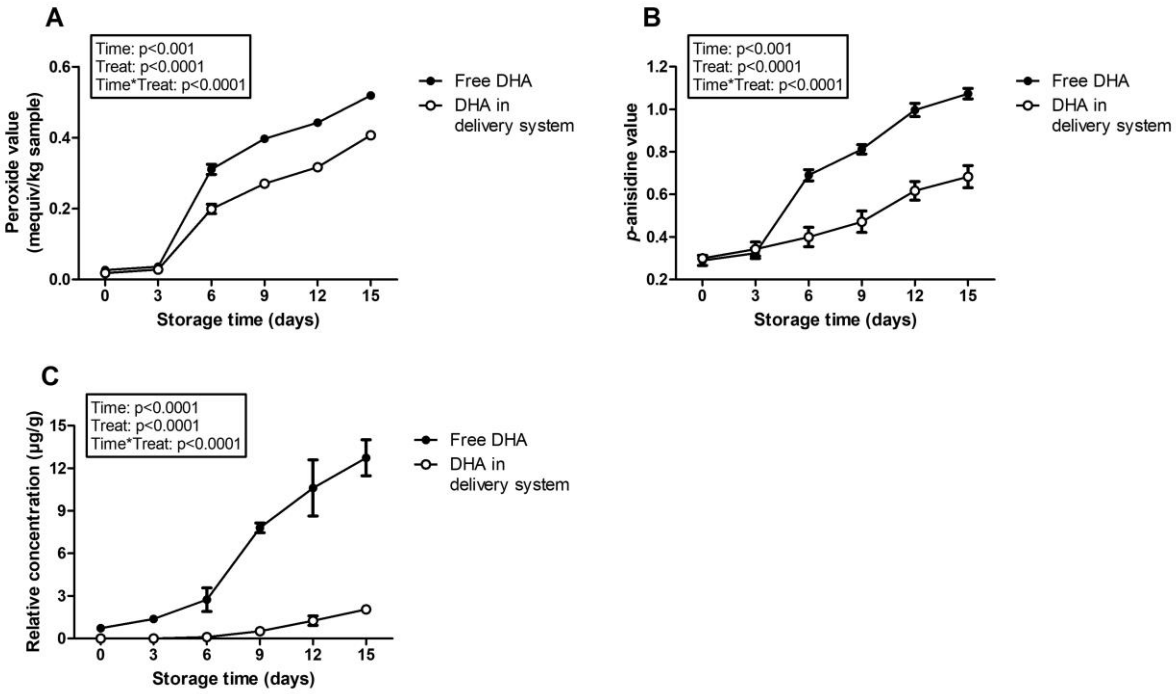
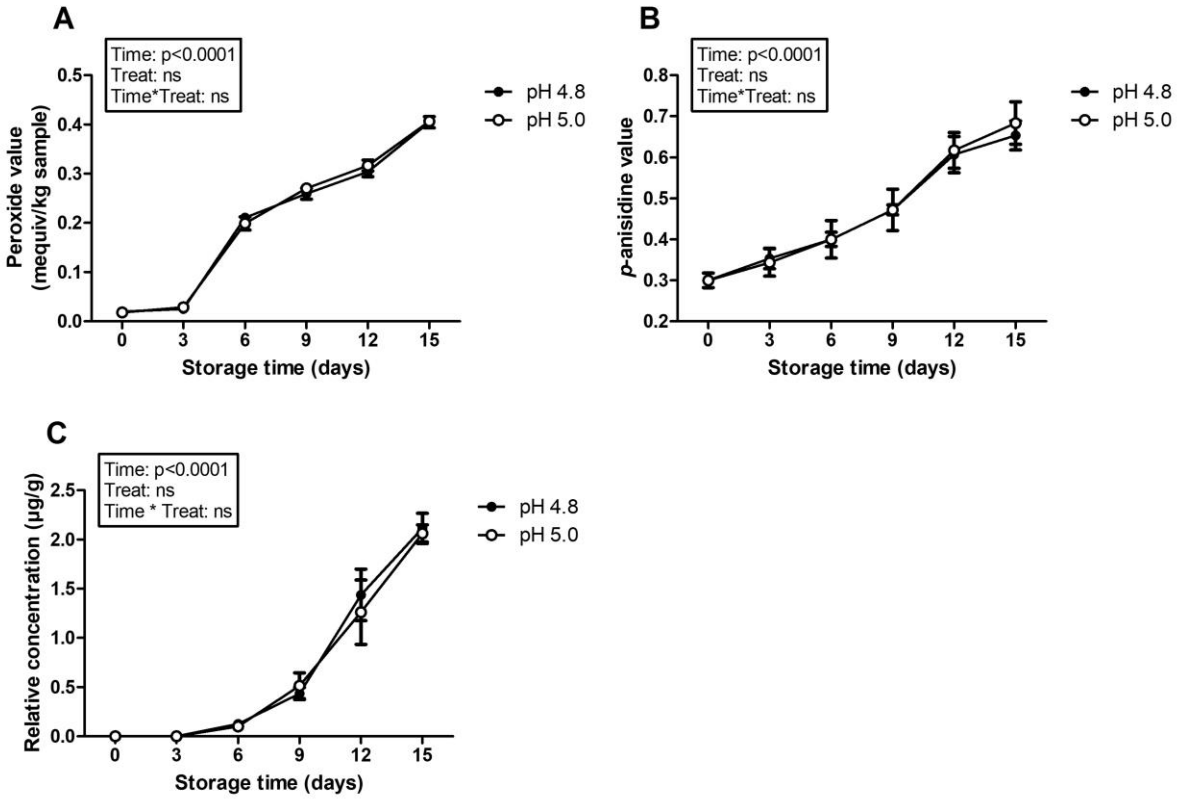


400

401 **Fig. 4.** Effects of docosahexaenoic acid (DHA) encapsulation in casein derivative-based
402 delivery system on the peroxide value (A), *p*-anisidine value (B), and production of (E,E)-2,4,-
403 heptadienal (C) during storage in model milk at 4°C for 15 d. The error bars on each point
404 denote the standard deviations of three replicates. Repeated-measures ANOVA was used to
405 analyze the impacts of DHA encapsulation in casein delivery system over time. Time, storage
406 time in d; treat, DHA encapsulation; time×treat, interaction between time and treat.

407



408

409 **Fig. 5.** Impacts of manufacturing pH on the peroxide value (A), *p*-anisidine value (B), and
410 development of (E,E)-2,4,-heptadienal (C) during storage of DHA-loaded casein derivative-
411 based delivery system in model milk at 4°C for 15 d. The error bars on each point denote the
412 standard deviations of three replicates. Repeated-measures ANOVA was used to analyze the
413 impacts of pH over time. Time, storage time in d; treat, manufacturing pH; time×treat,
414 interaction between time and treat.

415

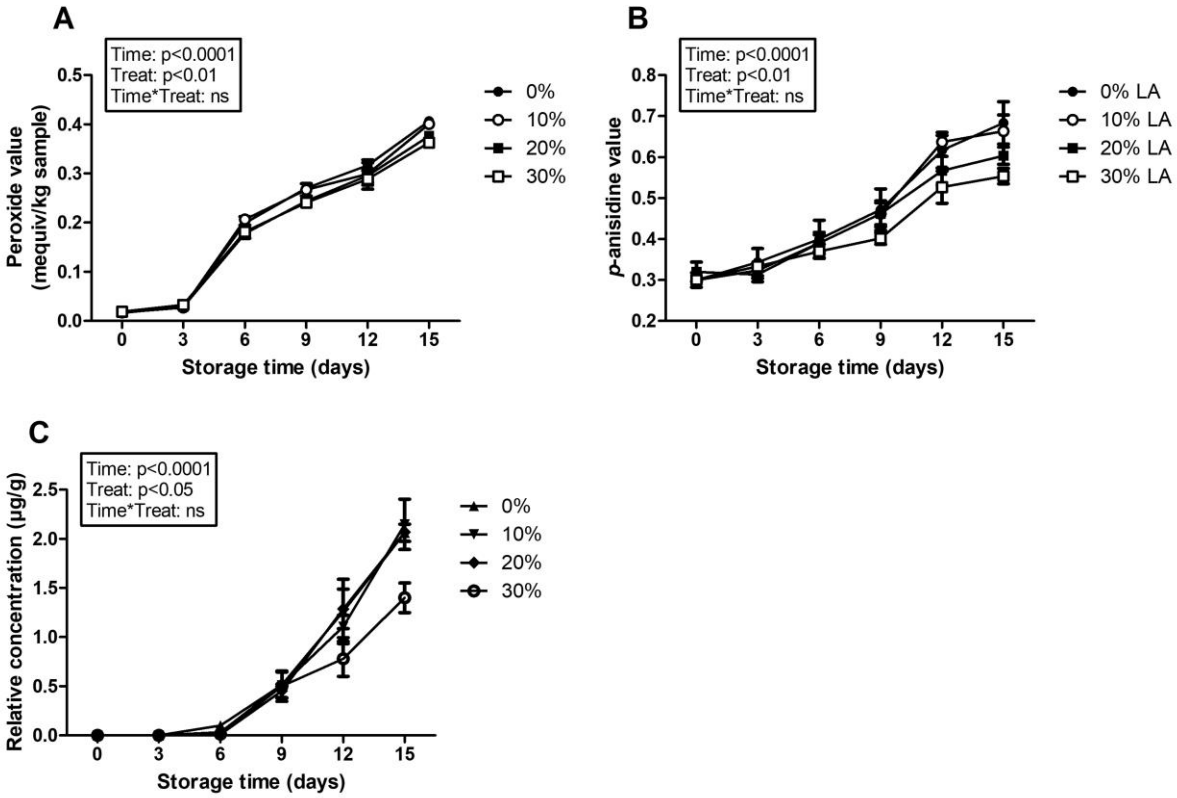


Fig. 6. Effects of charged amount of linoleic acid on the peroxide value (A), *p*-anisidine value (B), and development of (E,E)-2,4,-heptadienal (C) during storage of DHA-loaded casein derivative-based delivery system in model milk at 4°C for 15 d. The error bars on each point denote the standard deviations of three replicates. Repeated-measures ANOVA was used to analyze the impacts of charged amount of linoleic acid over time. Time, storage time in d; treat, charged amount of linoleic acid; time×treat, interaction between time and treat.