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Formation of a novel coating material containing lutein and zeaxanthin via a Maillard reaction between bovine serum albumin and fucoidan

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ABSTRACT

The effective delivery of bioactive compounds has recently been receiving attention. In this study, a conjugate with BSA and fucoidan synthesized via the Maillard reaction was confirmed through electrophoresis, the o-phthalaldehyde assay, and through changes in absorbance. Two moles of fucoidan were glycated with one mole of BSA at 60 °C and 79% relative humidity for 4 days. The droplet coated with B-F conjugate remained stable during storage at 4 and 25 °C and slightly increased only at 55 °C however, the droplet coated with intact BSA and B/F mixture significantly increased. L/Z were degraded about 82, 79, and 36% for 4, 25, and 55 °C, respectively, regardless of the type of emulsifier. Although the conjugates could not prevent the degradation of lutein and zeaxanthin during storage, they improved the stability of the emulsion and showed 4.20-fold and 1.32-fold higher bioaccessibility than intact BSA and B/F mixtures, respectively.

1. Introduction

An emulsion is a mixture of two immiscible liquids, where one is dispersed as small droplets in the other. Oil-in-water (O/W) emulsions are one of the most widely consumed food products, in beverages, sauces, and creams, also in addition to being the most common delivery systems for drugs and other bioactive compounds. However, emulsions are thermodynamically unstable and are prone to form new droplets by coalescence or flocculation (McClements, 2015). To prevent the instability of emulsions, emulsifiers are often added, which are adsorbed at the interface and inhibit close contact between the droplets through electrostatic repulsion and/or steric hindrance (McClements, 2015).

Proteins are natural polymers composed of amino acids, and as such, are able to stabilize emulsions by minimizing the unfavourable interactions and maximizing the favourable ones through conformational changes at the oil-water interface (Evans, Ratcliffe, & Williams, 2013; McClements, 2004). However, proteins have limitations in industrial applications since they are prone to losing their emulsifying properties when exposed to environmental stress in the form of pH, temperature, ionic strength, among others. A protein's emulsifying ability and stability can be improved through the use of protein-polysaccharide electrostatic complexes and protein-polysaccharide conjugates synthesized via the Maillard reaction. These have received much attention in recent years as emulsifiers in the encapsulation of bioactive compounds (Anal, Shrestha, & Sadiq, 2019; Chang & McClements, 2016; Fan, Yi, Zhang, Wen, & Zhao, 2017; Gumus, Davidov-Pardo, & McClements, 2016; Liu, Ma, Gao, & McClements, 2017; Muhoza et al., 2018). The Maillard reaction, a non-enzymatic browning reaction, is a condensation reaction between the amino group of a protein and a reducing sugar. Maillard reaction conjugates maintain the characteristics of each component (Dickinson, 2009), leading to better functional properties such as heat stability (Kato, 2002; Zha, Dong, Rao, & Chen, 2019), antioxidant activity (Gumus et al., 2016; Nooshkam, Varidi, & Bashash, 2019; Zha et al., 2019), and digestive stability (Anal et al., 2019; Fan et al., 2017; Muhoza et al., 2018; Nooshkam & Varidi, 2020).

Fucoidans are anionic polysaccharides extracted from brown seaweed, such as kombu and wakame. They have complex structures, with different proportions of sugar linkages and sulphate substitution patterns that vary depending on the algal source; with the communality of consisting of α-1,3-linked-l-fucose-4-sulfate, a repeating sequence of alternating α(1 → 3) and possibly α(1 → 4) glycosidic bonds (Kim & Shin, 2015a, 2015b) Fucoidan has been attracting attention in the pharmaceutical and food industries due to its anti-inflammatory properties, biocompatibility, and biodegradability. Its sulphated groups attract the cationic amino group of proteins strongly (Kim & Shin, 2015b), leading to better stability in O/W emulsions with Bovine Serum Albumin (BSA) (Kim & Shin, 2009, 2016) and helping bioactive compounds reach the small intestine by inhibiting proteolysis and coalescence (Chang & McClements, 2015, 2016).

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The xanthophylls lutein and zeaxanthin (L/Z) are macular carotenoids that reduce the risk of eye diseases such as cataract and age-related macular degeneration (AMD). L/Z play functional roles in the retina not only as antioxidants protecting tissues from oxidative damage but also as photoprotective agents absorbing light of short wavelengths. Therefore, intake of L/Z above 10 mg per day is effective for maintaining eye health (Nwachukwu, Udenigwe, & Aluko, 2016). The macular pigment density is an indicator of the condition of the retina because hydroxyl groups of L/Z form hydrogen bonds with the polar group of ocular membranes, accumulating in the lens of the eye and the macula (Bone & Landrum, 1984; Sujak et al., 1999). Lutein is mainly obtained from marigold flowers (*Tagetes erecta L.*), but it has limitations in that its production is costly compared to that of microalgae in terms of cultivation area, time, and required nutrients (Lin, Lee, & Chang, 2015). Additionally, there are no abundant sources of zeaxanthin, although some vegetables and fruits such as collards, yellow corn, and wolfberries reportedly contain some amount of xanthophylls (Nwachukwu et al., 2016). In recent years, many studies have been conducted on L/Z co-production through microalgae by light-stress (Park, Lee, & Jin, 2013) or genetic treatment (Baek et al., 2018; Kim, Ahn, Jeon, & Jin, 2017).

Nevertheless, the use of L/Z in the food industry is challenging due to their low stability. These compounds are highly sensitive and easily degraded by light, high temperature, and acidic conditions (Boon, McClements, Weiss, & Decker, 2010; Steiner, McClements, & Davidov-Pardo, 2018), in addition to their low water-solubility that reduces their bioaccessibility. Colloidal delivery systems are considered to enhance solubility, stability, and bioaccessibility of bioactive compounds, increasing their efficacy and utilization in foods and beverages (Fan et al., 2017; Gumus et al., 2016; Nooshkam et al., 2019; Salvia-Trujillo, Sun, Um, Park, & McClements, 2015).

Fucoidan, in complex with proteins, is known to stabilize emulsions in storage and for digestion. However, to the best of our knowledge, there are no studies confirming that conjugated fucoidan can retain its properties to be used as carotenoid carrier. Therefore, the aim of this study was to investigate the performance of BSA-fucoidan (B-F) conjugates on the physical and chemical stability of L/Z-enriched emulsions and on the gastrointestinal tract compared to that of intact BSA and BSA/fucoidan (B/F) mixtures.

2. Materials and methods

2.1. Reagents and materials

Chlamydomonas reinhardtii was kindly provided by prof. Eon Seon Jin (Department of Life science, Hanyang University, Korea). BSA and digestive enzymes such as pepsin (P7012), pancreatin (P7547), bile extract (B8631), lipase (L3126) were purchased from Sigma-Aldrich. Fucoidan was provided by HarimBio Co., Ltd. All other reagents used were of analytical grade and were used as received. All solutions were prepared with deionized water.

2.2. L/Z preparation from Chlamydomonas reinhardtii

C. reinhardtii mutant was generated by CRISPR-Cas9 for lutein and zeaxanthin accumulation. *C. reinhardtii* was dispersed at 0.01 mg/mL in a mixture of hexane and isopropanol (3:2 v/v) and sonicated in an ultrasonic bath with a constant frequency of 40 kHz (Powersonics 505, Hwashin technology.). Samples were processed at 35.7 °C for 150 min and centrifuged at 3500*g* for 10 min. To collect the organic phase, the supernatant was mixed with 0.88% w/v KCl solution at the same volume, and the upper phase was separated and evaporated using a rotary evaporator. The extract was diluted 200 times with 100% acetone for HPLC analysis and as a result, 87% of total L/Z was extracted from *C. reinhardtii*.

2.3. Preparation of B-F conjugates

The B-F conjugates (B-F) were prepared under dry-heating conditions, as proposed previously (Kim & Shin, 2015a), with a slight modification. BSA and fucoidan were dissolved together at a molar ratio of 1:3.6 in deionized water overnight and then freeze-dried. The dried sample was incubated at 60 °C in a relative humidity of 79% using saturated KBr solution for 4 days. The incubated samples were then dissolved in deionized water (2% w/v) for 12 h and filtered using Whatman No. 2 to remove the protein aggregates. Following this, the filtered solution was lyophilized.

2.4. Confirmation of B-F conjugate formation

2.4.1. UV/vis spectroscopy

The samples were dissolved in deionized water at 0.1% (w/v) protein concentration. The absorbance of the solutions were measured at 294 and 420 nm (Nooshkam et al., 2019), as indicators of intermediate and final stages of the reaction, respectively, using a UV/visible spectrophotometer (Genesys 10S, ThermoFisher.).

2.4.2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in 4–10% gels following the method of Laemmli (1970). The samples were dissolved in deionized water at 20 μg/mL based on protein concentration and heated with sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol) at 95 °C for 5 min. An aliquot (15 μL) of the sample solution was loaded into the gel and separated at 150 V. Following electrophoresis, the gel was stained with Coomassie brilliant blue for protein detection and with periodic acid-Schiff (PAS) for glycoprotein detection (Kim & Shin, 2015a).

2.4.3. Measurement of the degree of glycation (DG)

The o-phthalaldehyde (OPA) assay was used to determine the number of fucoidan molecules attached to BSA by measuring the decrease in the amine group (Pan, Mu, Hu, Yao, & Jiang, 2006). The OPA reagent was prepared by mixing the following reagents: 40 mg OPA dissolved in 1.0 mL anhydrous ethanol, 25 mL of 100 mM sodium tetraborate buffer (pH 9.4), 0.1 mL β-mercaptoethanol, 2.5 mL of 20% (w/v) SDS solution, and 21.4 mL deionized water. Briefly, 2.7 mL of OPA reagent and 0.1 mL of sample solution (3.0 mg/mL in protein) were mixed together and incubated at room temperature for 1 min. The absorbance at 340 nm was immediately read on a UV/Visible spectrophotometer (Genesys 10S, ThermoFisher). L-leucine (1–5 mM) was used for construction of a calibration curve and the DG (%) was calculated using the following equation:

$$DG(\%) = \left(1 - \frac{aminegroupafterglycation\,(M)}{aminegroupbeforeglycation\,(M)}\right) \times 100$$

2.5. L/Z enriched O/W emulsion preparation

The oil phase was prepared by dispersing *C. reinhardtii* extract in corn oil (2% w/v), and the aqueous phase was prepared by dissolving the emulsifier in a buffer solution (0.4% w/v at protein concentration). Buffer solutions were made by dissolving 0.04% (w/v) sodium azide, as an antibacterial agent, in 5 mM phosphate buffer (pH 7.0). For preparing coarse oil-in-water emulsions, 20% oil phase and 80% aqueous phase were mixed at 13,600 rpm for 2 min in a high-speed homogenizer (Ultra-Turrax T25, IKA.) and subsequently sonicated at 20 kHz for 15 min using a probe type sonicator (VCX-750, Sonics & Materials.). The beaker was kept in iced water during homogenization to avoid temperature changes. The emulsion activity index (EAI) of each emulsifier was confirmed following the method described by Pearce and

Kinsella (1978) right after the O/W emulsion was prepared.

The final concentration of L/Z was 15 mg per 250 mL emulsion, which is its recommended daily intake in one serving of beverage for a beneficial effect on eye health. Corn oil was used as a carrier oil because previous studies have shown that it effectively increases the bioaccessibility of carotenoids (McClements, 2018).

2.6. Emulsion stability

To examine the stability of emulsions with respect to temperature changes, the emulsions were stored at different temperatures (4, 25, and 55 °C) for 7 days and were protected from light by wrapping the emulsion containers in aluminium foil. The change in the mean diameter of the droplets was measured to assess their physical stability of the emulsions. The mean droplet diameter, polydispersity index, and ζ-potential were measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments.). Emulsions were diluted 100 times with 5 mM phosphate buffer solution (pH 7) to avoid multiple scattering. All samples were measured at 25 °C.

The chemical stability of L/Z was assessed with the amount remaining in the emulsion. For total L/Z measurement, emulsions were mixed with 100% acetone in a ratio of 2:8 and the mixture was injected into the HPLC system.

2.7. In vitro gastrointestinal tract (GIT) simulation

In vitro digestion was carried out following the INFOGEST protocol with a slight modification (Minekus et al., 2014). NaCl replaces $NaHCO_3$ salts at the same molarity in simulated intestinal fluid (SIF) to avoid pH increase during the pH-stat experiment. Simulated salivary fluid (SSF) was mixed with the sample for consistency, but oral phase digestion was omitted since the liquid sample was kept in the mouth for a short time and did not contain carbohydrates. 5 mL of the sample were mixed with an equal volume of SSF (except α-amylase) and 10 mL of simulated gastric fluid (SGF) was added based on the final pepsin activity (2500 U/mL). The pH of the mixture was adjusted to 3 ± 0.05 using 1 N HCl or 1 N NaOH. The sample was placed in a 100 mL amber bottle and the headspace was flushed with nitrogen gas for 15 s to prevent the effects of oxygen and light. The mixture was incubated at 37 °C and 250 rpm for 2 h on a hotplate stirrer. For simulated intestinal digestion, an aliquot of 20 mL of sample from the gastric phase was mixed with 20 mL of SIF containing digestive enzymes, pancreatin (100 U/ml trypsin activity), bile extracts, and lipase (2000 U/ml). The pH of the samples was adjusted to 7 ± 0.05 with 1 N NaOH and maintained by the drop-wise addition of 0.25 N NaOH. The volume of NaOH added was recorded over 2 h and the percentage of free fatty acid (FFA) released from the digested lipids was calculated using the protocol described by Y. Li and McClements (2010). The amount of L/Z reaching the small intestine after gastric digestion was measured as described in Section 2.8 and calculated using the following equation:

$$Reachingrate(\%) = \frac{Cr}{Ci} \times 100$$

where Cr is the amount of L/Z reaching the small intestine, and Ci is the initial amount of L/Z in emulsion.

The L/Z bioaccessibility was evaluated according to the method described by Rodrigues, Mariutti, and Mercadante (2016). Briefly, an aliquot of the digest was centrifuged at 4000*g* at 25 °C for 30 min using a benchtop centrifuge (H-15FR, Kokusan Co. Ltd.). The micellar fraction, between the sediment and oil, was collected and mixed with 100% acetone at a concentration of 50% v/v. The mixture was then filtered using a 0.2 μm nylon syringe filter and injected into the HPLC system. The bioaccessibility was calculated as the percentage of solubilised L/Z in the micellar phase relative to the total input amount of the *in vitro* digestion system.

2.8. Quantification of L/Z

L/Z in the samples were quantified using Shimadzu Prominence system (Shimadzu Scientific Instruments, Inc.) with a C18 reversed-phase column (5 μm, 4.6 mm × 250 mm; Waters, Milford). The samples were mixed with 100% acetone, and the supernatant was filtered using a 0.2 μm nylon syringe filter and subjected to HPLC analysis. The mobile phase consisted of 0.1 M Tris-HCl (pH 8.0)-acetonitrile-methanol (14:84:2, v/v/v) for the first 15 min and acetonitrile-methanol (32:64, v/v) for the last 5 min. The injection volume was 20 μL and the flow rate was 1.2 mL/min. The column oven temperature was set to 40 °C, and L/Z were detected at 445 nm.

2.9. Statistical analysis

Each experiment was performed in triplicate and each data represents the mean value. A Significant difference between each treatment were analyzed by Duncan test with One-way ANOVA using SPSS 24.0 (SPSS Inc., Chicago.).

3. Results and discussion

3.1. B-F glycation via the Maillard reaction

In the Maillard reaction, the reducing aldehyde or ketone group of a sugar and the amino group of a protein form a covalent bond in a ratio of 1:1. The reaction is considered to progress in three stages: early, intermediate and final. In the early stage, the carbonyl group of the reducing sugar and the available amino group of protein form an *N*-substituted glycosylamine via a condensation reaction, coupled with the release of a water molecule. This is the only reversible reaction in the whole process. The unstable *N*-substituted glycosylamine rearranges to form Heyns rearrangement products (HRPs) from ketose sugars or Amadori rearrangement products (ARPs) from aldose sugars. HRPs and ARPs subsequently degrade to form furfural from pentose sugars or hydroxymethylfurfural from hexose sugars at pH ≤ 7 as well as reductones and fission products at pH > 7 in the intermediate stages. The amount of these products can be measured by their absorbance values at 294 nm (Nooshkam et al., 2019). In the final stage, melanoidins, which are nitrogen-containing brown-colored polymers, are formed as a result of condensation of the intermediate products, and their presence is confirmed by the absorbance values at 420 nm (Nooshkam et al., 2019). Fig. 1A presents the changes in absorbances at 294 and 420 nm. The significant increase in Abs 294 nm and Abs 420 nm indicates that the Maillard reaction between BSA and fucoidan occurred during dry-heating, resulting in the formation of intermediates and end products.

Fig. 1B. shows the SDS-PAGE results of native and heated BSA, heated fucoidan, B/F mixtures, and B-F conjugates. As mentioned above, fucoidan formed covalent bonds with BSA through the Maillard reaction, due to which the molecular weight of BSA changed after incubation. The molecular weight of BSA is approximately 66 kDa (Fig. 1B; lane 1), and dimers (133 kDa), trimers (200 kDa), and oligomers of BSA were seen to appear after incubation due to the formation of intermolecular disulfide bonds (lane 2). The bands of the B/F mixture presented features similar to those of native BSA and native fucoidan on the Coomassie blue- and PAS-stained gels, respectively. After dry-heating incubation, smeared bands were observed in lane 5 and 10, indicating the covalent coupling formed between BSA and fucoidan. These results are in agreement with those of previous studies (Fan et al., 2017; Kim & Shin, 2016). Additionally, the bands between stacking and separating gels also proved that fucoidan-glycated BSA was generated via the Maillard reaction (Kato, 2002).

The 1-alkylthio-2-alkylisoindoles, absorbing maximally at a wavelength of 340 nm, were formed by the reaction of the OPA reagents with amino groups of BSA. The glycation ratio depends on the molecular size

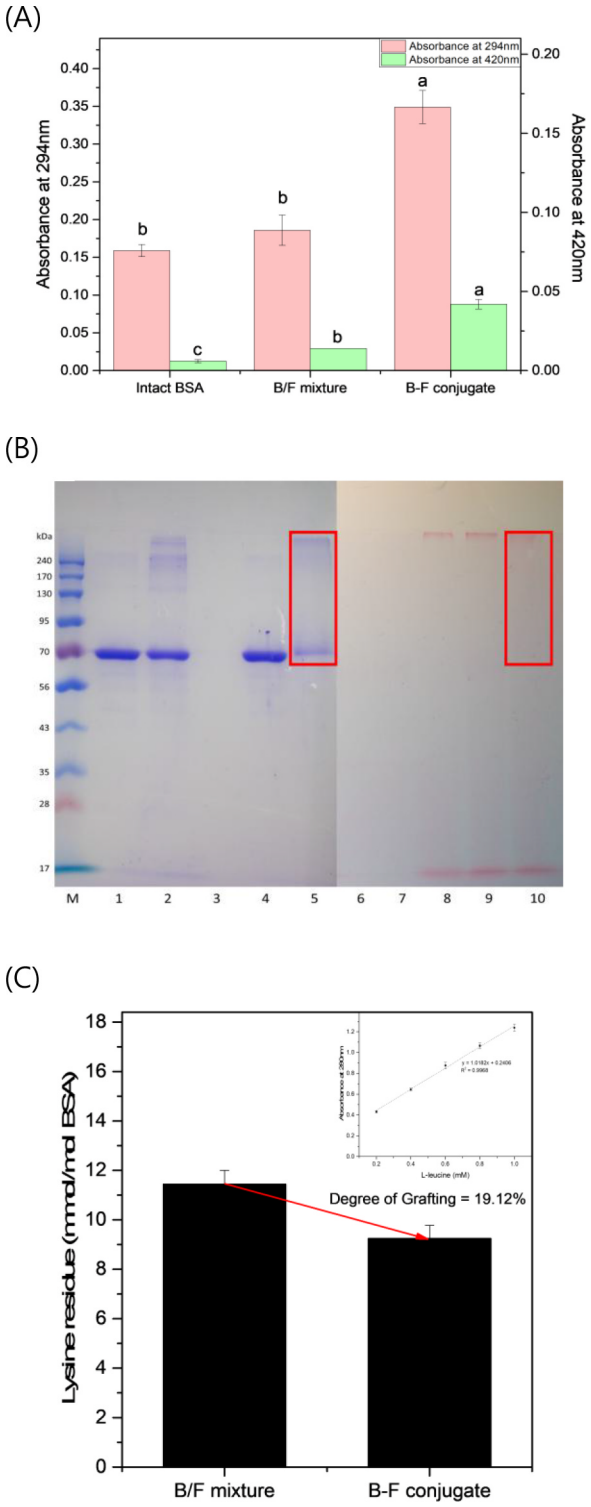

Fig. 1. Formation of Maillard reaction products (B-F conjugates). (A) Changes in absorbance at 294 and 420 nm (B) Coomassie brilliant blue staining for proteins (Lane 1–5) and with the Gelcode glycoprotein staining kit for carbohydrates (Lane 6–10). Lane M, molecular weight markers; lanes 1 and 6, intact BSA; lanes 2 and 7, heated BSA; lanes 3 and 8, heated fucoidan; lanes 4 and 9, BSA/fucoidan mixture; lanes 5 and 10, B-F conjugate. (C) Lysine residues before and after Maillard reaction, obtained with OPA reagent.

and the kind of polysaccharide (Kato, 2002). As shown in Fig. 1C, the remaining amino groups of BSA were reduced from approximately 12–10 mmol. Therefore, the number of fucoidan molecules attached to one BSA molecule was about 2 in this study, which was consistent with that of previous research (Kim & Shin, 2015a).

Table 1
Characteristics of emulsions stabilized with BSA, B/F mixture, and B-F conjugates right after formation.

	EAI (m^2/g)	Mean droplet diameter (nm)	PDI	z-potential (mv)
BSA	129.1 ± 4.0^b	2078.7 ± 998.4^a	0.184	−21.2 ± 6.6^b
B/F mixture	138.7 ± 5.1^a	411.1 ± 33.4^b	0.382	−59.2 ± 4.8^a
B-F conjugate	141.2 ± 2.5^a	304.4 ± 10.7^b	0.372	−60.7 ± 5.0^a

Different letters indicate significant difference in a row ($p < 0.05$).

3.2. Emulsion stability

During manufacturing and storage, beverages are subjected to various thermal treatments, such as mechanical heat generation and sterilization. Therefore, the influence of temperature on the stability of the emulsion and on L/Z loss were studied. Heating affects the stability of emulsions by various mechanisms: (i) it promotes aggregation between the lipid droplets with weak electrostatic repulsion due to increased frequency of droplet–droplet collision (ii) it can also cause aggregation since conformational changes in the proteins attached to the interface increase the hydrophobicity and/or disulfide bond formation between the droplets (McClements, 2015).

Table 1 shows the emulsion activity index (EAI), mean droplet diameter, and zeta-potential of the L/Z enriched emulsions stabilized with BSA, B/F mixtures, and B-F conjugates. The EAI was higher and the z-average was smaller in the presence of fucoidan. These observations might be attributed to the presence of a polysaccharide, which increases the hydrophilic-lipophilic ratio and forms a continuous thick layer at the O/W interface (Liu et al., 2017; Nooshkam et al., 2019). Additionally, the conjugates showed a slightly better emulsification capacity due to the conformational change in BSA. Kim and Shin (2016) found that the tertiary structure of BSA changed to a molten, globule-like state following heat treatment for 96 h, which led to the exposure of the non-polar groups of BSA. As the hydrophobic residues were exposed, the oil phase was properly dispersed in the aqueous phase. The zeta-potential value for L/Z enriched emulsion stabilized with BSA was −21.2 mV, while those of the B/F mixtures and B-F conjugates were −59.2 mV and −60.7 mV, respectively. These values could be mainly attributed to the adsorption of fucoidan onto the droplet surfaces, owing to its negatively charged characteristics (about −50 mV) (Chang & McClements, 2015, 2016; Kim & Shin, 2015b). In a brief, emulsions coated with B/F mixtures and B-F conjugates had similar characteristics, such as zeta-potential and particle size distribution at the beginning of storage. However, it was observed B-F conjugate-coated lipid droplets were stable regardless of temperature during storage, while the diameter of the droplets coated with BSA and B/F mixture increased from 2078.7 to 2855.7 nm and from 411.1 to 1720.3 nm at 55 °C, respectively (Fig. 2A). It is well known that intact BSA starts denaturing at 51.9 °C (Yamasaki, Yano, & Aoki, 1990), while B/F mixture does so at 56 °C (Kim & Shin, 2016). When proteins are denatured, they unfold and reveal residues, which makes it easier to form bonds. Based on these previous findings, the BSA and B/F mixtures were denatured during storage at 55 °C and protein aggregation occurred due to the formation of hydrophobic and disulfide bonds between the colliding droplets, which led to lipid droplet coalescence. However, Kim and Shin (2016) revealed the B-F conjugates started to denature at 91 °C with the DSC measurement. We could confirm that the non-denaturation of that B-F conjugates improved the stability of the emulsion. An increase in droplet diameters at 4 and 25 °C was only observed when the emulsions were stabilized with the B/F mixture, which was possibly attributed to the free fucoidan in the aqueous phase. When a large amount of free polymers are present in the aqueous phase, they form a bridge between the emulsion droplets due to electrostatic attraction (Chang &

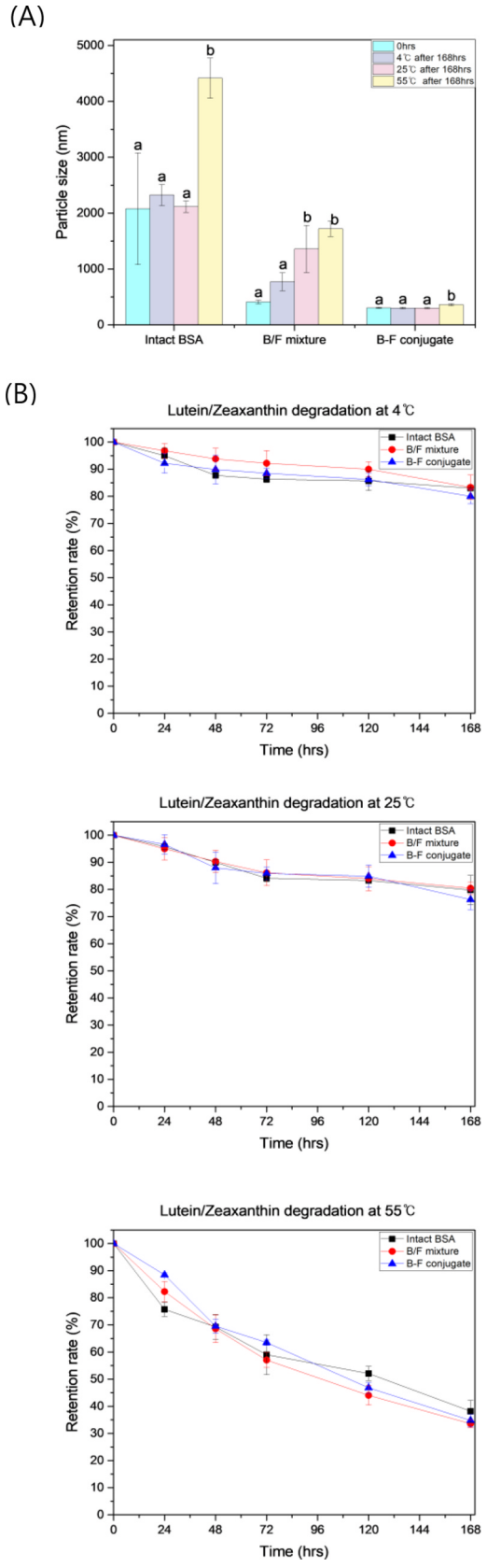

Fig. 2. (A) Changes in particle size at 4, 25, and 55 °C after 7 days. (B) Chemical stability of L/Z at 4, 25, and 55 °C during storage.

McClements, 2015). As a consequence, free fucoidan led to droplet coalescence, known as depletion flocculation. In contrast, since the covalently-bound fucoidan induced steric hinderance, the droplets coated with B-F conjugates were stable to aggregation under all storage circumstances. These results were in an agreement with those of Muhoza et al. (2018).

As shown in Fig. 2B, the L/Z degradation rates were dependent on the storage temperature. This phenomenon was also in an agreement with previous studies (Gumus et al., 2016; Teo, Lee, Goh, & Wolber, 2017). The degradation of carotenoids takes place through various mechanisms that depend on storage conditions. They include, among other, thermal degradation which occurs through the oxidation and propagation of the conjugated polyene chain of the carotenoids (Boon et al., 2010). In order to stop this chain reaction, antioxidants are needed. Generally, proteins can act as antioxidants and the Maillard reaction is well known to improve this property (M. Nooshkam et al., 2019). To be more specific, Maillard reaction products (MRPs), such as melanoidin, have a conjugate structure that terminates the oxidation reaction by removing free radicals through the acceptance of an electron and/or donation of hydrogen (Nooshkam & Varidi, 2020). However, as shown in Fig. 2B, B-F conjugate could not retard L/Z degradation during storage, and there was no difference in L/Z stability between the emulsifier studied. This is thought to be due to the lipid used as a carrier. In this study, not only carotenoid but also lipids could be oxidized by heat and produce reactive species, and the reactive species were scavenged by oxidizable products, the MRPs and the carotenoids. They could donate electron, which is called the HAT mechanism, and those with higher reducing power donated to reactive species more preferentially. The results of this study were in accordance with those previously published by Gumus et al. (2016) in regards to the glycosylation of sodium caseinate. Their results also showed that dextran-glycosylated sodium caseinate did not specifically prevent lutein oxidation. Although the antioxidant capacity of MRPs and carotenoids were not evaluated in this study, it could be infered that L/Z were preferentially oxidized as a result of their higher reducing power. As a result, Maillard reaction products were effective for the physical stability of emulsions, but ineffective for the thermal stability of L/Z. Therefore, in order to increase the stability of L/Z during storage, it seems to be necessary to add a substance having greater reducing power than carotenoid.

3.3. Gastro-intestinal digestion

A reason for the difficulty in delivering bioactive compounds is their possibility to degrade during digestion. Fucoidan is an anionic polysaccharide with sulfate groups, which are known to bind strongly to cationic patches of proteins, thereby improving digestive stability (Chang & McClements, 2015, 2016; Kim & Shin, 2015b). *In vitro* gastrointestinal digestion was used to compare the impact of the emulsifier on the stability of the emulsion during digestion. Previous studies have shown the protein-stabilized emulsions were unstable as a result of proteolysis and bridging-flocculation by pepsin, and weakened electrostatic repulsion by inorganic ions (e.g. Na^+, Cl^-, K^+, PO_4^{2-}, Ca^{2+}.) in the stomach (Chang & McClements, 2016; Nooshkam & Varidi, 2020). Fig. 3A shows the state of emulsion-stabilized BSA, B/F mixtures, and B-F conjugates after passing through the gastric phase. Only BSA, which encapsulated the lipid droplets, was completely proteolyzed, resulting in phase separation. In the presence of fucoidan, although creaming was observed, the lipid droplets were still well dispersed. These results were in accordance with those of previous reports, which showed the z-potential and droplet size of emulsions have an effect on gastric digestion and also suggested that highly anionic fucoidan molecules on the surface increased the steric and electrostatic repulsion between droplets (Chang & McClements, 2015, 2016). Noteworthy is the lipid droplets coated with conjugates were more stable than the mixtures. Gumus et al. (2016) suggested that the dextran moiety of conjugates produced

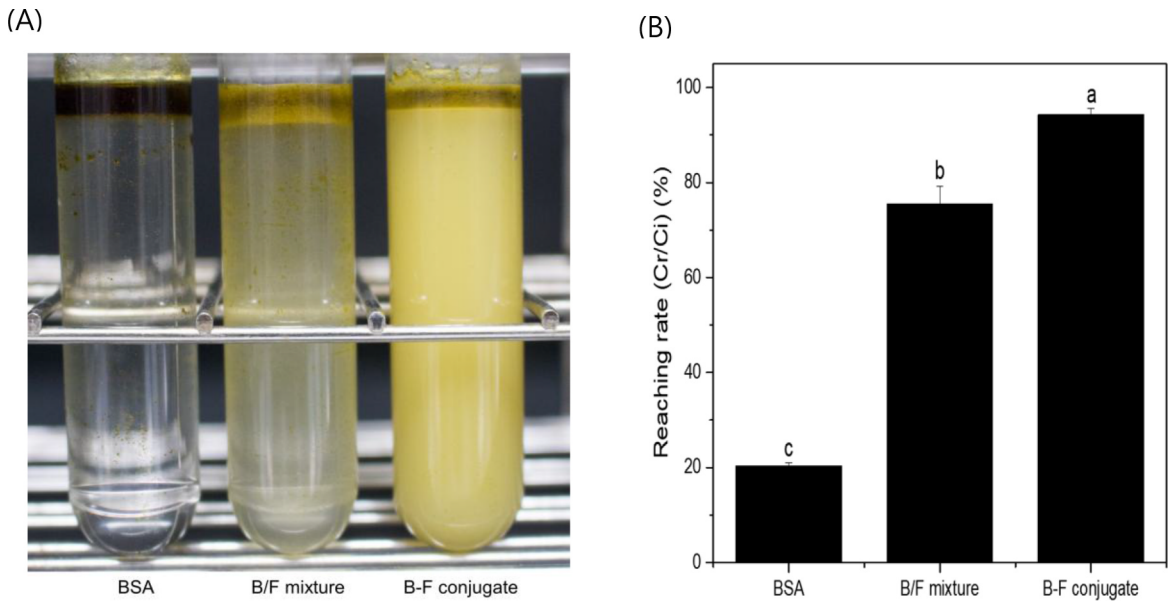

Fig. 3. State of emulsions stabilized with BSA, B/F mixture, and B-F conjugates. (A) Image after passing through gastric phase. (B) Reaching rate of L/Z to intestine.

a steric repulsion, prohibiting access of proteases that results in its stability in the gastric phase. The stability of the coating in the gastric phase practically helped L/Z reach the intestine without degradation. This was because the uncoated lipids were completely exposed to the acidic environment of the stomach and carotenoids are oxidized by protonation in the presence of acids (Boon et al., 2010). As a consequence, the amount of L/Z reaching the small intestine was 20.4%, 75.5%, and 94.3% for BSA, B/F mixture, and B-F conjugates, respectively (Fig. 3B).

Using a pH-stat method, a plot was drawn which showed the rate and extent of intestinal digestion of the delivered lipid droplet by lipase and pancreatin (Fig. 4). The rate of digestion depended on the emulsifier and the initial droplet size (Ma et al., 2019; McClements, 2018). As shown in Fig. 3A, the aggregation and coalescence of lipid droplets reduced the surface area, which decreased the extent of contact of the digestive enzymes, and caused it to float on the intestinal fluid (Ma et al., 2019). As a consequence, the lipid coated with BSA alone did not release free fatty acids during the first 4 min, after which digestion began slowly. Complete phase separation occurred in BSA-stabilized emulsions, inhibiting lipolysis in the intestinal phase. On the other hand, the lipid stabilized with B/F mixtures and B-F conjugates was well dispersed after gastric digestion, and had large enough surface area to react with digestive enzymes. And the emulsion stabilized with B-F conjugates, which retained the particles more stably during gastric digestion due to steric hindrance caused by bound fucoidan, was digested faster than the emulsion stabilized with B/F mixtures. As a result, after 3 h intestinal digestion, the extents of lipolysis were 59.8%, 79.3%, and 80.4% for native BSA, B/F mixture, and B-F conjugate encapsulations, respectively (Fig. 4).

The bioaccessibility was determined by calculating the concenctration of the amount finally dissolved in the micellar after gastrointestinal digestion compared to the initial input (Fig. 5). The bioaccessibility of L/Z correlated with both the amount of L/Z reaching the small intestine and the degree of lipid digestion which explains the release of the lipophilic bioactive compound trapped inside the lipid droplet (McClements, 2018). The extent of lipolysis was similar between the mixture and the conjugate, nevertheless the bioaccessibility was 14.6% and 19.3% in the mixture and conjugate, respectively. This was because, as shown in Fig. 3B, the reaching amount of L/Z encapsulated with the conjugate delivered to the intesine was 1.25-fold higher than the case of the mixture. Similarly, in case of BSA, the amount of L/Z that reached the intestine was one fifth and the lipolysis was three-quarters compared to the case of the conjugate, so the bioaccessibility was only 4.6%. Overall, both mixtures and conjugates

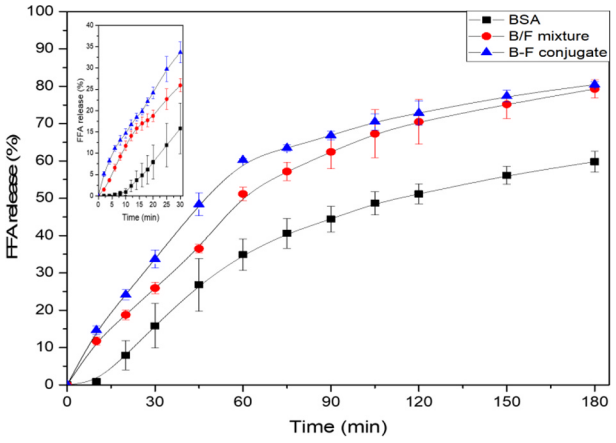

Fig. 4. Plot of free fatty acid (FFA) release (%) during the 3 h intestinal digestion.

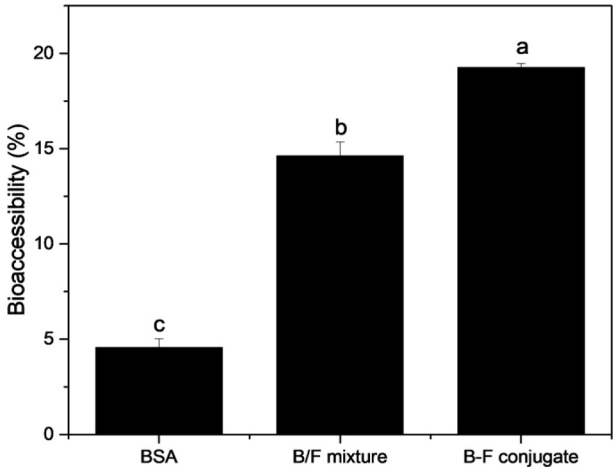

Fig. 5. Bioaccessibility of L/Z after gastro-intestinal digestion.

might be used as carriers because both were stable during digestion; however, comparatively, the conjugate appeared to be more suitable for delivery of L/Z than the mixture.

4. Conclusions

This study aimed to investigate the impact of BSA-fucoidan (B-F) conjugates, compared to BSA and BSA/fucoidan mixtures, on the physical and chemical stability and behaviour on the gastrointestinal tract of emulsions enriched with of L/Z. Conjugates were produced via Maillard reaction between BSA and fucoidan during dry-heating. This study confirmed that 2 mol of fucoidan formed covalent bonds with 1 mol of BSA. The products of this reaction effectively inhibited lipid droplets from aggregation during a 7 day storage period even at high temperatures of up to 55 ˚C. Furthermore, this conjugate improved the emulsion stability and the bioaccessibility of L/Z in gastro-intestinal digestion compared to intact BSA and a B/F mixture. This study suggests that the emulsifier produced with a glycated protein with fucoidan can be used as a carrier to deliver bioactive compounds effectively which, in consequence, could become an alternative for the development of functional beverages, among other nutraceutical products. Additionally, it is suggested to carry out further studies on this line of research to improve the chemical stability of L/Z during storage since the B-F conjugates did not prevent their degradation.

CRediT authorship contribution statement

Sunbin Kim: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration. **Weon-Sun Shin:** Validation, Resources, Data curation, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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