A THESIS FOR MASTER'S DEGREE OF ENGINEERING

Characteristics of O/W emulsion, protein-based particle and inclusion complex for the improvement of retinol stability and bioaccessibility

레티놀 안정성과 생체접근률 향상을 위한 수중유적형 에멀션, 단백질 기반 입자, 포접 복합체의 특성

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Abstract

Retinol is a fat-soluble vitamin, vitamin A, which not only exhibits anti-aging function, but also has useful physiological activities such as anti-cancer and anti-inflammation. However, it is difficult to apply to foods because of its low solubility in water and instability in UV and heat. Therefore, this study aimed to develop delivery systems to increase retinol stability and bioaccessibility. O/W emulsion, protein complex and cycloamylose were developed and optimized for retinol as delivery systems to improve its stability and bioaccessibility.

O/W emulsion, protein-based particle and cycloamylose, respectively, were compared in terms of retinol stability and bioaccessibility as functional of emulsifier type, coating agent type, and host material concentration. The stability of incorporated retinol was analyzed under UV irradiation and storage at different temperatures (4, 25, 40°C). Finally, bioaccessibility of retinol was estimated in each delivery system with the most stable conditions.

UV stability of retinol in the O/W emulsion was significantly improved at an oil concentration of 10 wt% or more. With anionic emulsifier, the storage stability of retinol was lowered due to interaction with a trace amount of metal ion present in the aqueous solution. When protein-based particles containing retinol were coated with

polysaccharide, the loading efficiency reached to more than 90%. Retinol was found to have the highest UV and pH stability in protein-based particles coated with pectin. Residual amount of retinol increased with cycloamylose during UV and storage test periods regardless of cycloamylose concentration used in this study. Finally, when using the delivery systems, the bioaccessibility of retinol was significantly improved to at least 50% to 80% depending on the delivery systems, compared to less than 20% for pure retinol.

This study showed that O/W emulsion, protein-based particle and cycloamylose were effective delivery systems that improved retinol stability and bioaccessibility by encapsulation. These delivery systems could be highly useful for the food and cosmetic industry who utilize functional ingredients such as retinol.

Key word: Retinol, O/W emulsion, Protein-based particle, Cycloamylose, Bioaccessibility

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1. Introduction

Vitamin A is a fat-soluble vitamin exemplary, and can be taken into animal foods and vegetable foods are absorbed by the body only when combined with the fat or oil. Vitamin A helps maintain the normal growth promoting visual function, regulation and reproduction of epithelial tissue proliferation and differentiation (Eskandar & Nasrin Ghouchi *et al*, 2009). Despite these many benefits, vitamin A, especially retinol, is sensitive to oxygen, heat, and light, and has low solubility in water. Retinol is a chemical name for vitamin A, also known as pure vitamin A. It is present in the intestinal mucous cells of the animal, and is abundant in greenish-yellow plants. It is also known to play an important role in maintaining the original function of epidermal cells, which is sometimes transformed into retinoic acid, the active form. So, the formulation of all-*trans*-retinol into dermal delivery systems is challenging due to poor water solubility and various instability pathways such as isomerisation to cis-isomers with reduced activity, molecular fragmentation, and photochemical and chemical oxidation (SM Loveday & H Singhds, 2008). Therefore, a formulation that can increase the chemical stability of retinol needs to be developed.

In order to efficiently deliver the fat-soluble vitamin, retinol, to the body, a delivery system with the ability to capture hydrophobic substances is needed. Delivery systems related to this are being studied in various ways

such as emulsion, liposome, SLN and cyclodextrin (Augustin & Hemar, 2009; McClements, Decker, & Weiss, 2007). The aim of this study was to compare the delivery system (O/W emulsion, protein-based particle and inclusion complex) for improving the stability of retinol. In the case of O/W emulsion, determine how retinol stability and bioaccessibility vary with emulsifier type, oil concentration, and oil type. Protein-based nanoparticles are identified by the type of stabilizer and the type of polysaccharides to find the optimal conditions to stabilize retinol. And then, we investigate the effect of temperature, UV, and pH stability of retinol depending on delivery systems in various conditions.

2. Objectives

The purposes of this studies are

- to optimize the conditions of production of retinol-loaded O/W emulsion
- to optimize the conditions of production of retinol-loaded protein-based particles
- to optimize the conditions of production of retinol-loaded cycloamylose
- to analyze the stability of retinol incorporated the prepared delivery system (O/W emulsion, Protein-based particles and Cycloamylose)
- to analyze the bioaccessibility of retinol incorporated the prepared delivery system.

3. Background and Literature Review

3.1. Efficacy and unstable properties of retinol

Vitamin A is a fat-soluble vitamin exemplary, and can be taken into animal foods and vegetable foods are absorbed by the body only when combined with the fat or oil. Retinol has been widely used for cosmetics and pharmaceutics because it has an effect on preventing wrinkles and skin aging. The deficiency of this compound causes roughness of the skin and degradation of mucous membranes. Given the many beneficial effects of retinol, formulation strategies have been used to overcome the decomposition problem. All-*trans*-retinol, with trans double bonds in the isoprenoid side chain, undergoes degradative reaction due to the characteristics of conjugated double bonds which result in the partial or total loss of vitamin A bioactivity. These reactions include isomerization from trans to cis isomers, molecular fragmentation and chemical oxidation. The double bonds in the polyene chain of retinoids can undergo cis-trans isomerization, especially at positions 9, 11 and 13 (von Lintig, Kiser, Golczak, & Palczewski, 2010). Therefore, it is necessary to other alternative which are safe from the external stresses and when put into the human body. Recently, many studies have been reported that retinol incorporation within various complexes. However, there are not many studies to compare how these various complexes affect the stability of retinol. In this study, we aimed to compare how O/W emulsion, protein-based particle, and cycloamylose increase retinol stability and bioaccessibility.

3.2. Types and composition of emulsion

3.2.1. Characteristics of O/W emulsion

The first retinol-loaded delivery system is O/W emulsion. An emulsion consists of two immiscible liquids, with one of the liquids dispersed as small spherical droplets in the other (Yoshida, Sekine, Matsuzaki, Yanaki, & Yamaguchi, 1999). At this time, the substances are generally water and oil, and the two dispersed states are called the general emulsion system. In most foods, the diameters of the droplets usually lie somewhere between 0.1 and 100㎛ (D. J. McClements, 2015). An emulsion in which oil is dispersed in water is called an oil-in-water (O/W) emulsion. When water is dispersed in oil, it is called a water-in-oil (W/O) emulsion. The substance that makes up the droplets in an emulsion is referred to as the dispersed, discontinuous, or internal phase, whereas the substance that makes up the surrounding liquid is called the continuous or external phase (D. J. McClements, 2015). In this study, Retinol is encapsulated in the oil droplet of O/W emulsion considering that most food base is water.

3.2.2. Characteristics of emulsifier

To make an emulsion, an emulsifier is required to emulsify the interface between water and oil. The most commonly used emulsifiers in the food industry are small-molecule surfactants, amphiphilic biopolymers and surface-active particulate matter. Ideally, an emulsifier should rapidly adsorb to the oil-water interface during homogenization, reduce the interfacial tension by an appreciable amount, and prevent droplet coalescence from occurring during homogenization. In addition, it is usually important that the emulsifier forms an interfacial membrane that prevent droplet aggregation (D. J. McClements, 2015). Emulsifiers are generally composed of a hydrophilic head and a hydrophobic tail, which are characterized by their parts. Particularly, they are classified into anionic, nonionic and cationic emulsifiers depending on the charge of the head part (Corradini, Curti, Meniqueti, Martins, Rubira, & Muniz, 2014; Dickinson & Stainsby, 1982; St. Angelo, Vercellotti, Jacks, & Legendre, 1996). In addition, the head and tail parts of the emulsifier determine the HLB. Hydrophile-lipophile balance (HLB) is a measure of the relative affinity of the emulsifier for aqueous and oil phase. For the continuous phase, use an emulsifier with a high HLB and for an oil, select a low HLB emulsifier. In addition, when adding an emulsifier, the amount of the critical micelle concentration (CMC) of the emulsifier to be used must be determined. Emulsifiers form micelles themselves in the aqueous phase above a certain concentration, the concentration at which CMC is called

(da G Miguel, Burrows, Formosinho, & Lindman, 2001). Therefore, if the emulsifier is added above the CMC, it will affect the properties of the emulsion, since no additional monomer is formed without the monomer. In this study, O/W emulsion was prepared by using nonionic emulsifier (Tween 20, Decaglycerin myristate) and anionic emulsifier (WPI) as an emulsifier to investigate the stability of retinol depending on the kind of emulsifier.

3.3. Composition of protein based nanoparticles

3.3.1. Hydrophobic characteristics of corn protein zein

The Second is the zein-based particles. In the past, zein was regarded as a byproduct of the corn industry and was thought of as a material of low value, not of importance. However, with the advancement of technology, it began to attract attention in various areas. Therefore, in modern times, zein and zein-based materials are regarded as more valuable materials (Corradini, Curti, Meniqueti, Martins, Rubira, & Muniz, 2014).

Because retinol is a water-insoluble substance, it is advantageous to use proteins rich in hydrophobic amino acids to encapsulate them. Zein is a prolamine storage protein from corn. Zein-based nanoparticles can be easily prepared by liquid-liquid dispersion method due to its unique solubility. The large proportion (>50%) of non-polar amino acids (leucine, proline, alanine, and phenylalanine) in zein makes it water insoluble. Protein molecules in the complex nanoparticles provide strong affinity to bioactives resulting in high encapsulation efficiency, meanwhile polysaccharide molecules serve as coating on the surface of protein core and thus contribute to better stability. These features make protein-polysaccharide complex nanoparticles better delivery systems than nanoparticles prepared from protein or polysaccharide alone (Matalanis, Jones, & McClements, 2011). Thus far, zein-based nanoparticles have been extensively studied for encapsulation and delivery of lipophilic nutrients and drugs, including essential oils and fat soluble vitamins.

3.3.2. Characteristics of sodium caseinate as a stabilizer

Sodium caseinate, a soluble mixture of several different caseins, is widely used as an ingredient in the food industry. It contains hydrophilic and hydrophobic groups in various sequences and proportions. Sodium caseinate has been reported to act as an emulsifier or stabilizer due to the combination of electrostatic and steric stabilization (Dickinson, 1997). It is a suitable candidate for use as a stabilizer in this study. Zein tends to aggregate at neutral pH because it has a pI value at pH 6.2. A stabilizer should be used during the precipitation process to prevent protein-based particles from aggregation. In this study, sodium caseinate is used to stabilize zein-based particles to make them more applicable for the development of retinol delivery system. Their stability as a function of pH and ionic strength, redispersibility after drying are investigated.

3.3.3. Characteristics of polysaccharide as a coating agent

Although this is a strategic improvement for zein nanoparticles as delivery vehicles, the stability of zein-sodium caseinate nanoparticles under gastrointestinal (GI) conditions is still a major obstacle for their future applications as oral delivery vehicles. Therefore, the addition of polysaccharide is necessary.

Polysaccharides are natural polymers that are widely used as functional ingredient for various food colloids formulations. Also, polysaccharides are the essential ingredients of any food colloid formulation mainly due to their ability to change product shelf life by varying food texture (Bandyopadhyay, Navid, Ghosh, Schnitzler, & Ray, 2011). Polysaccharides generally have hydrophilic properties and remain in the aqueous phase, thus helping to control aqueous phase rheology such as thickening, gelation and stabilizers. The formation and deformation of polysaccharide-protein complexes and their solubility depend on various factors like charge and nature of biopolymers, pH, ionic strength and temperature of the medium and even the presence of surfactant of the medium (Turgeon, Schmitt, & Sanchez, 2007; Xia & Dubin, 1994). When the pH of the medium decreases below the equivalent point (pI) of the existing protein, the net positive charge of the protein becomes noticeable, interacting with the negatively charged polysaccharide to form a stable electrostatic complex. On the same principle, if the solution pH is higher than the pI of protein, the net negative

charge of the protein tends to form a complex with the positively charged polysaccharide (Bandyopadhyay, Navid, Ghosh, Schnitzler, & Ray, 2011). Generally, when the pH of the solution is about the same as the protein pI, it forms a weaker complex because the surface charge of the protein is almost zero at that pH range. Carboxylate polysaccharides get deprotonated (become anionic) at a pH range higher than its pKa. This electrical charge on the back bone of protein or polysaccharide chain is responsible for electrostatic attraction or repulsion between them.

Although protein-polysaccharide complex nanoparticles have the potential to achieve desirable stability under GI conditions due to the polysaccharide coating, few studies have actually investigated and addressed this challenge. In this study, we examined the stability of retinol when three polysaccharides (pectin, sodium alginate and sodium carboxymethyl cellulose) were added to each retinol-zein particle using sodium caseinate as a stabilizer.

3.4. Characteristics of cyclic glucans

Most of the hydrophobic food components with low solubility in water are unstable at room temperature and have low bioavailability. In order to overcome these limitations, studies on inclusion complexes have been actively conducted for a long time. The most important characteristic of inclusion complexes is that a “host” component is able to admit “guest” components into its cavity without any covalent bonds being formed. Inclusion complex can change the solubility, stability or bioaccessibility of guest molecule. Representative hosts for inclusion complexes are cyclodextrin (CD) and cycloamylose (CA), which are glucose units.

CDs are cyclic oligosaccharides containing six (α-CD), seven (β-CD) and eight (γ-CD) glucopyranose units which are bound together by α-*1, 4* -linkages forming a truncated conical structure, which allows CDs to form host-guest inclusion complexes with guest molecules of different sizes (Barman, Barman, & Roy, 2018). The diameter and solubility of CD are determined by the number of glucose units constituting it. The inner cavity of which is hydrophobic, but the outer surface is hydrophilic. When hydrophobic materials bind to the interior of the host via hydrogen bonding, van der Waals, and hydrophobic interactions, they form inclusion complexes. Thus, CD is soluble in aqueous solution but can accommodate hydrophobic guest molecules in the central cavity with high specificity (Szejtli, 1998).

Cycloamylose (CA), also called large-ring CD, is a cyclic molecule consisting of α-*1, 4*-glucans. It has a broad degree of polymerization (DP) distribution that ranges from nine to a few hundred (Takaha, Yanase, Takata, Okada, & Smith, 1996). CA also can form inclusion complexes with various materials and has unique characteristics compared to small CD. Compared to CDs with DPs of 6, 7 and 8, CA is a relatively large molecule with DP17 or higher. Therefore, it is expected that the space for binding the guest component is wider than the CD and the solubility in water is high, so that the guest component is more stably incorporated in the CD. The formation of such complexes improves physical and chemical properties of the hydrophobic guest and, consequently, it has lead to an extensive application of these cyclic carbohydrate derivatives in various field (Brewster & Loftsson, 2007; Carrier, Miller, & Ahmed, 2007; Jordheim, Degobert, Diab, Peyrottes, Périgaud, Dumontet, et al., 2009; Loftsson & Duchêne, 2007).

In this study, CA was used as a major inclusion complex and the stability of retinol, a guest substance, was compared using β-CD of the same conditions.

3.5. Definition and necessity of bioaccessibility

The ingested foods are subjected to a series of digestion processes that are exposed to various conditions in the body. The ingested food is digested through the mouth, stomach, and small intestine. Until the nutrients in the food are absorbed, they are subject to biochemical effects (temperature and shear) and physical effects (pH, enzyme, bile salt and so on) in the body (Castenmiller & West, 1998).

The ingested foods are mixed with saliva under constant temperature and pH conditions in the mouth and affected by salivary enzymes and biopolymers such as mucin and amylase. As it passes through the mouth, it undergoes a rapid pH change and is mixed with gastric fluid with another enzyme, pepsin. In addition, the stomach's peristaltic motion in this process also causes considerable shear (Bourne, 2002; Schipper, Silletti, & Vingerhoeds, 2007). Finally, in the small intestine stage, it is affected by salt and lipase with pH change (Sarkar, Goh, Singh, & Singh, 2009).

Bioaccessibility is a concept that defines solubilized compounds in a form that can be absorbed in the small intestine (Benito & Miller, 1998). Therefore, knowing the bioaccessibility of nutrients in food can not accurately determine the amount of nutrients absorbed into the body, but it can be a predictor of absorption efficiency of the delivery system (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009; Schipper, Silletti, & Vingerhoeds, 2007).

In this study, in vitro digestion test was performed using a delivery system that optimizes the stability of retinol. Finally, we measured the bioaccessibility of retinol in each delivery system and estimated the amount absorbed into the body.

4. Materials and Methods

4.1. Materials

All-*trans*-retinol, tween 20, zein, sodium caseinate, pectin from apple, sodium carboxymethyl cellulose (CMC, average Mw ~90,000), sodium alginate, β-cyclodextrin hydrate, mucin, bile extract, pancreatin from porcine pancreas and alpha-amylase from porcine pancreas (13 units/mg) were purchased from the Sigma Aldrich Chemical Co. Whey protein isolate (WPI, Product code: 9500) was obtained from Protient, Inc. (St. Paul, MN, USA). Decaglycerin myristate was obtained from Mitsubishi - kagaku foods corp. (Shinba-koen, Minato-ku, Tokyo, Japan) Soybean oil and coconut oil was purchased from a local supermarket and used without further purification. Cycloamylose was purchased from the Ezaki glico Co. All other chemical were of analytical grade.

4.2. Methods

4.2.1. Preparation and characteristics of retinol-loaded O/W emulsion

4.2.1.1. Preparation of retinol-loaded O/W emulsion

O/W emulsion was compared according to the type of emulsifier and the concentration of oil. To add the same amount of retinol to the total emulsion, add 1.67% (w/w) to 0.3 g, 0.125% (w/w) to 4 g and 0.25% (w/w) of Retinol to 10g of soy bean oil, resulting in O/W emulsion (0.3, 4 and 10% oil emulsion), respectively. In order to completely dissolve, soybean oil containing retinol was sonicated (Ultrasonic cleaner-Powersonic 410, Hwashin, Seoul, Korea) for 15min. Three emulsifiers (Tween 20, Decaglycerin Myristate, and WPI) were used to determine the difference in retinol stability in the emulsion depending on the type of emulsifier. Emulsifier solution was prepared by dispersing Tween 20, Decaglycerin Myristate and WPI (0.6 wt.%, w/w) in 10mM phosphate buffer (pH 7) for 3hours with mild stirring as aqueous phase. A stock emulsion (0.3, 4 wt.% oil emulsion) was prepared by homogenizing soybean oil containing retinol and emulsifier solution using a high speed blender (ULTRA-TURRAX model T25 digital, IKA, Germany) for 1 min at 12,000rpm and then passing the solution through a microfluidizer (Picomax MN 250A, Micronox, Seongnam, Korea) three times at 0.5MPa to make the nano-size emulsion. O/W emulsion using WPI as an emulsifier prevents the growth of microorganisms by adding sodium azide

(0.25%, v/v) after this process. All samples are stored at 4℃.

To compare the UV stability of retinol in O/W emulsion, emulsion is prepared by different oil concentration. The oil concentration should be 0.1, 0.5, 1, 2, 4 and 10% and Decaglycerin Myristate used as the emulsifier. An oil phase was prepared by dissolving retinol (5, 1, 0.5, 0.25, 0.125 and 0.25%, w/w) in soybean oil to add the same amount of retinol to the total emulsion. The method of manufacturing the emulsion is the same as above. Measure the turbidity of the emulsion to determine if the turbidity affects the UV stability of the retinol in O/W emulsion. The turbidity is measured at 500 nm wavelength with a UV-spectrophotometer after diluting the emulsion 100-fold. Samples at each oil concentration are stored at 4 ° C and UV stability confirmed.

All-*trans*-retinol and Tween 20 was purchased from the Sigma Aldrich Chemical Co. Whey protein isolate (WPI, Product code: 9500) was obtained from Protient, Inc. (St. Paul, MN, USA). Decaglycerin myristate was obtained from Mitsubishi - kagaku foods corp. (Shinba-koen, Minato-ku, Tokyo, Japan) Soybean oil and coconut oil was purchased from a local supermarket and used without further purification. All other chemicals were of analytical grade.

4.2.1.2. Characteristic of retinol-loaded O/W emulsion

4.2.1.2.1. Particle size distribution and zeta potential analysis

Size, polydispersity index (the width of particle size distribution), and the zeta potential of emulsions in the absence and presence of nanoparticles were measured with dynamic light scattering. Prior to the measurements, the emulsions were diluted by pure water or 10mM phosphate buffer (pH 7). Measurements were performed in triplicate and each individual measurement was an average of 10 runs. All measurements were conducted at ambient temperature.

4.2.1.2.2. Turbidity analysis of O/W emulsion

Verify that there is a difference in turbidity between each sample with different emulsifier and oil concentration conditions and verify that the value affects the stability of retinol in the oil. Turbidity is determined by diluting the sample 5 times with 10 mM phosphate buffer (pH 7) and measuring absorbance (A_{500}) using a UV-spectrophotometer. Determine the turbidity of the emulsion by the measured absorbance value and compare it with the retinol stability results and verify that there is a correlation.

4.2.1.3. Stability of retinol in O/W emulsion

4.2.1.3.1. UV stability

The effects of UV light on the stability of retinol incorporated into O/W emulsion were investigated. The photo-stability of retinol was performed in a UVA irradiation chamber. 4mL of each emulsion sample was placed on a rotating plate at the same distance of about 10cm from the G8T5 UV lamp (8W, Philips, Poland) and irradiated with UV lamps for up to 24 h. The remaining concentration of retinol in emulsion were determined using spectrophotometric analysis at each time.

4.2.1.3.2. Storage stability at different temperature

The effects of temperature on the storage stability of retinol incorporated into O/W emulsion were investigated. Added 1 mL of the emulsion into the 1.5mL brown tube and stored at 4, 25, and 40 °C, respectively, to check the amount of retinol remaining over time. Allow to stand at room temperature for 10 minutes before taking samples to keep the temperature constant. To observe only the effect of temperature, we cut off the light and oxygen and discard the once used sample without reusing it. Retinol-loaded oil is blank. The remaining concentration of retinol in oil and emulsion were determined using spectrophotometric analysis at each day.

4.2.1.4. Retinol contents analysis in O/W emulsion

Retinol contents analysis was determined by method of Zhao et al (Zhao, Guan, Pan, Nitin, & Tikekar, 2015) with slight modification. To extract retinol and measure stability (retinol retention rate % at each point) in oil and emulsion, 100μL of each sample was mixed 1min using the vortex mixer with 1,900 μL of Methanol (20 times dilution). After that, the mixture was centrifuged at 14,000 rpm for 10min and filtered the supernatant using 0.45㎛ filter. The filtered supernatant was collected to measure the absorbance at 325 nm and same emulsion without retinol is blank. The absorbance was measured using UV-spectrophotometer (UV-1800 model, Shimadzu, Japan). The standard curve (R^2 = 0.998) was prepared by measuring the absorbance of known concentration of retinol in methanol.

4.2.1.5. Statistical analysis

All data were recorded as mean ± standard deviation and analyzed by SPSS for Window (version 24.0; SPSS inc., Chicago, IL, USA). The one-way ANOVA test followed by a Student-Newman-Keuls's multiple range tests were performed to identify statistical significances. A *p*-value <0.05 was considered to be statistically significant.

4.2.2. Preparation and characteristics of protein based nanoparticles

4.2.2.1. Preparation of retinol-loaded zein particles

4.2.2.1.1. Preparation of retinol-zein complex nanoparticles

Optimize the ratio of materials used to make protein-based particles. Determine the most stable conditions and make the particles as follows. 0.5% of zein was dissolve in 80% (v/v) aqueous ethanol solution using magnetic stirring at 500rpm for 30min. Add 0.005% (w/v) retinol powder to the prepared zein solution and further stir in dark for 10 minutes to make retinol-zein stock. 1% sodium caseinate is dissolved in citrate-phosphate buffer (pH 6) as a stabilizer and stirred overnight to prepare a stabilizer solution. 12.5 mL of the stabilizer solution is stirred at 500 rpm and 10 mL of retinol-zein stock is poured and stirred for 30 seconds. The ethanol contained in the resulting solution is removed using a rotary evaporator and the final volume is adjusted with citrate-phosphate buffer (pH 6).

4.2.2.1.2. Preparation of polysaccharide coated nanoparticles

Sodium alginate and sodium carboxymethyl cellulose are used to compare optimal polysaccharide conditions for coating reinol-zein complex nanoparticle (RZ). 0.5% polysaccharide is dissolved in distilled water. Mix 12.5 mL of polysaccharide solution at 500 rpm, pour 22.5 mL of RZ solution, and further stir for 30 seconds. The finished solution is lyophilized and stored as a powder.

4.2.2.2. Characteristics of retinol-loaded zein particles

4.2.2.2.1. Particle size distribution and zeta potential analysis

Size, polydispersity index (the width of particle size distribution), and the zeta potential of protein-based particles in the absence and presence of nanoparticles were measured with dynamic light scattering. Prior to the measurements, the protein-based particle solutions were diluted by citrate-phosphate buffer (pH 6). Measurements were performed in triplicate and each individual measurement was an average of 10 runs. All measurements were conducted at ambient temperature.

4.2.2.2.2. Particle yield and loading efficiency analysis

Freshly prepared protein colloidal dispersions are lyophilized and the weight of the powder is measured. Calculate the particle yield (%) using the following formula with the total weight of zein, sodium caseinate, polysaccharide, retinol, and the weight of dry powder added to make a colloidal dispersion. :

$$\text{Particle yield (\%)} = \frac{the\ weight\ of\ the\ freeze\ dried\ protein\ particles}{total\ input\ weight\ of\ zein,\ sodium\ caseinate,\ polysaccarides\ and\ retinol} \times 100\%$$

To calculate the retinol loading efficiency, the lyophilized sample was dissolved in DMSO at 0.1% and then stirred overnight in the dark. The resulting solution was centrifuged at 15,000 rpm for 30 min. Taking the supernatant and measuring the absorbance at 325 nm using a UV-spectrophotometer, the amount of retinol is calculated using the calibration curve of the standard solution (0-10 μg / ml free retinol in 90% DMSO). The retinol loading efficiency is calculated by substituting the amount of retinol in the following equation:

$$\text{Loading efficiency } (\%) = \frac{Retinol\ in\ nanoparticles}{Total\ retinol\ input} \times 100\%$$

4.2.2.2.3. Scanning Electron Microscopy (SEM)

Morphological structures of PN were observed by SEM (SUPRA 55VP, Oberkochen, Germany). Freshly prepared samples (Z, ZSc, ZScP and ZScP-R) are lyophilized and powdered. Then, Conductive carbon tape was attached to the stub used for the measurement. The prepared sample was fixed on a carbon tape and coated with a thin (<10 nm) conductive platinum layer using a sputter coater (EM ace200, Leica Mikrosysteme, Wetzlar, Germany). The morphology was visualized by a SEM at an acceleration voltage of 2 kV. Representative SEM images were reported.

4.2.2.2.4. Differential Scanning Calorimeter (DSC)

The thermal property of the dried samples were characterized using a differential scanning calorimeter (Q1000, TA Instruments, New Castle, UK). Each sample was placed in an aluminum pan at 7 mg and sealed. The aluminum pan was heated at 35 to 170 °C at a rate of 5 °C/min. An empty sealed aluminum pan was applied as the baseline. Nitrogen was used as the transfer gas at a flow rate of 50 mL/min.

4.2.2.2.5. Fourier transform infrared spectroscopy (FT-IR)

FTIR analysis was conducted to study the interactions between individual ingredients that make up protein-based particles. The vibration spectra of freeze-dried samples were recorded by TENSOR27 (Bruker, Germany). The FT-IR spectra were obtained by 32 acquisitions at 4 cm−1 resolution from the wavenumber of 400–4000 cm−1. The spectra were analyzed by OPUS software version 8.0. pure state retinol, zein, sodium caseinate and pectin were analyzed. physical mixtures of ingredients and nanoparticles were analyzed.

4.2.2.3. Stability of retinol in zein particles

4.2.2.3.1. pH stability

The protein has a unique isoelectric point at pH at which the net charge is zero, and the polysaccharide used to coat the retinol-zein complex particle has a pKa value because it has a charge. Therefore, it is important to check pH stability when making protein-based particles. To confirm pH stability, protein-based particles must first be made under stable conditions. Since the isoelectric point of sodium caseinate is pH 4.6, the samples containing sodium caseinate (ZSc, ZScP, ZScA and ZScC) are prepared at pH 6, and the other samples (Z and ZP) are prepared at pH 2 considering that the isoelectric point of zein is pH 6.2. Stable Retinol-Zein-Polysaccharide particles were diluted 5 times in citrate-phosphate buffer (pH 2, 4, 8) and analyzed for size distribution, zeta potential and aggregation and pH stability.

4.2.2.3.2. UV stability

The effects of UV light on the stability of retinol incorporated into protein-based particles were investigated. The photo-stability of retinol was performed in a UVA irradiation chamber. 4mL of each colloidal solution was placed on a rotating plate at the same distance of about 10cm from the G8T5 UV lamp (8W, Philips, Poland) and irradiated with UV lamps for up to 6 h. The remaining concentration of retinol in protein-based particles were determined using

spectrophotometric analysis at each time.

4.2.2.4. Retinol contents analysis in protein-based particles

To extract retinol and measure stability (retinol retention rate % at each point) in protein-based particles, 360 μL of each sample was mixed 1min using the vortex mixer with 1,440 μL of Methanol (5 times dilution). After that, the mixture was centrifuged at 12,000 rpm for 15min and filtered the supernatant using 0.45㎛ filter. The filtered supernatant was collected to measure the absorbance at 325 nm and same protein-based particle without retinol is blank. The absorbance was measured using UV-spectrophotometer (UV-1800 model, Shimadzu, Japan). The standard curve (R^2 = 0.998) was prepared by measuring the absorbance of known concentration of retinol in methanol.

4.2.2.5. Statistical analysis

All data were recorded as mean ± standard deviation and analyzed by SPSS for Window (version 24.0; SPSS inc., Chicago, IL, USA). The one-way ANOVA test followed by a Student-Newman-Keuls's multiple range tests were performed to identify statistical significances. A *p*-value <0.05 was considered to be statistically significant.

4.2.3. Preparation and characteristics of inclusion complexes of retinol and cyclic glucans

4.2.3.1. Preparation of retinol inclusion complexes

2% (w/v) of retinol is dissolved in 95% EtOH to prepare retinol stock. Retinol stock is added to each solution with 0.2, 0.5 and 1% (w/v) concentration of host component (CA, CD), and finally 0.1% (w/v) retinol is dissolved in 5% (v/v) EtOH. The guest-host mixture is mixed with a rotator for 24 hours at room temperature to reach the equilibrium. It is then filtered with a 0.45 μm syringe filter to remove the insoluble guest. The sample is frozen at -60°C for 24 hours and then dried in a freeze dryer (FD8508, ilSinBioBase Co.Ltd, Korea).

4.2.3.2. Characteristics of retinol inclusion complexes

4.2.3.2.1. Phase solubility

Measurements of phase solubility of the inclusion complexes are important since this is an index of changes in physicochemical properties of a compound upon inclusion (Del Valle, 2004). In this study, the concentration of CA was 0.5-30% (w/v) and the concentration of CD was 0.1-1.0% (w/v) because its solubility was lower than CA. Retinol concentration was fixed at 0.1% (w/v). The vials are mixed at constant conditions (room temperature, 24hours) until equilibrium is established. The solid drug is then removed by filtering and the solution assayed for the total concentration of retinol. The detailed method is the same as 4.2.3.1. Lipid solution was diluted 10-fold and absorbance was measured at 325 nm, the maximum absorbance of retinol. A Phase–solubility diagram is constructed by plotting the total molar concentration of retinol on the *y*-axis and the total molar concentration of CA or CD added on the *x*-axis.

4.2.3.2.2. Differential Scanning Calorimeter (DSC)

The thermal property of the dried samples were characterized using a differential scanning calorimeter (Q1000, TA Instruments, New Castle, UK). Each sample was placed in an aluminum pan at 7 mg and sealed. The aluminum pan was heated at 35 to 200 °C at a rate of 5 °C/min. An empty sealed aluminum pan was applied as the baseline. Nitrogen was used as the transfer gas at a flow rate of 50 mL/min.

4.2.3.2.3. Fourier transform infrared spectroscopy (FT-IR)

FTIR analysis was conducted to study the interactions between individual ingredients that make up inclusion complex. The vibration spectra of freeze-dried samples were recorded by TENSOR27 (Bruker, Germany). The FT-IR spectra were obtained by 32 acquisitions at 4 cm−1 resolution from the wavenumber of 400–4000 cm−1. The spectra were analyzed by OPUS software version 8.0. pure state retinol, cycloamylose, physical mixture and inclusion complex were analyzed.

4.2.3.2.4. Scanning electron microscope (SEM)

Morphological structures of inclusion complex were observed by SEM (SUPRA 55VP, Oberkochen, Germany). Freshly prepared samples (retinol, cycloamylose, physical mixture and inclusion complex) are lyophilized and powdered. Then, Conductive carbon tape was attached to the stub used for the measurement. The prepared sample was fixed on a carbon tape and coated with a thin (<10 nm) conductive platinum layer using a sputter coater (EM ace200, Leica Mikrosysteme, Wetzlar, Germany). The morphology was visualized by a SEM at an acceleration voltage of 2 kV. Representative SEM images were reported.

4.2.3.3. Stability of retinol in inclusion complexes

4.2.3.3.1. UV stability

The effects of UV light on the stability of retinol incorporated into inclusion complexes were investigated. The photo-stability of retinol was performed in a UVA irradiation chamber. 4mL of each colloidal solution was placed on a rotating plate at the same distance of about 10cm from the G8T5 UV lamp (8W, Philips, Poland) and irradiated with UV lamps for up to 6 h. The remaining concentration of retinol in protein-based particles were determined using spectrophotometric analysis at each time.

4.2.3.3.2. Storage stability at different temperature

The effects of temperature on the storage stability of retinol in inclusion complexes were investigated. In order to compare cyclic glucans of the same concentration, the concentration of the host molecule was fixed to 0.2% according to the result of the phase solubility. Added 1 mL of the inclusion complexes into the 1.5mL brown tube and stored at 4, 25, and 40 °C, respectively, to check the amount of retinol remaining over time. Allow to stand at room temperature for 10 minutes before taking samples to keep the temperature constant. To observe only the effect of temperature, we cut off the light and oxygen and discard the once used sample without reusing it. Retinol in 90% dmso is blank. The remaining concentration of retinol was determined using spectrophotometric analysis at each day.

4.2.4. Bioaccessibility analysis for encapsulated retinol

4.2.4.1. Sample preparation

In order to measure the bioaccessibility of retinol, we use the sample of the most optimized condition among the delivery systems that evaluated the stability of retinol. Each delivery system uses three types of samples for comparative analysis. The oil concentration of O/W emulsion was fixed at 10 wt% and three kinds of emulsifiers (Tween 20, Decaglycerin myristate and WPI) were used. In the case of protein-based particles, three samples were used depending on the presence or absence of sodium caseinate as a stabilizer and pectin as a coating agent. Finally, the inclusion complex used three samples with different concentrations of cycloamylose. Each delivery system contained retinol at the same concentration and all experiments were repeated three or more times.

4.2.4.2. *In vitro* digestibility test

Simulated saliva fluid (SSF), containing mucin, alpha-amylase (15 Units/mg) and various salts (NaCl, NH_4NO_3, KH_2PO_4, KCl, $K_3C_6H_5O_7$, $C_5H_3N_4O_3Na$, H_2NCONH_2 and $C_3H_5O_3Na$), was prepared. The samples (O/W emulsion, protein based particles and inclusion complex) were mixed with SSF at a 50:50 vol/vol ratio and adjusted to pH 6.8 using 6M NaOH. The oral phase mixture was incubated at 37°C for 10minutes with continuous agitation using stirrer water bath (HK-SWS20, HANKUK S&I Co., Korea).

Simulated gastric fluid (SGF), containing sodium chloride and 34% hydrochloric acid, was prepared. Mix the mixture through the oral phase and SGF at a ratio of 50:50 and adjust to pH 2.5 using 6M NaOH. The gastric phase mixture was incubated at 37°C for 2 hours with continuous agitation using stirrer water bath.

After incubation time, 30 mL of the mixture is adjusted to pH 7.0. Then add 3.5 mL bile salt (187.5 mg / 3.5 mL) dissolved in phosphate buffer and 1.5 mL salt stock containing CaCl 2 and NaCl. After adjusting the pH to 7.0 with NaOH, add freshly prepared pancreatin (187.5 mg / 2.5 mL) dissolved in phosphate buffer and incubate at 37 ° C for 2 hours with continuous agitation using a stirrer water bath.

4.2.4.3. Bioaccessibility analysis for encapsulated retinol

Bioaccessibility of retinol is determined after the retinol-loaded delivery system has passed the in vitro GIT model. After centrifugation (4000 rpm, 40 min) of 10 mL of the digestion through all steps, take 5 mL of the supernatant of the digest (SD). Add 5 mL of raw digests (RD) and SD sample to chloroform at 50:50 vol/vol ratio and let them voltexed sufficiently. All samples were centrifuged (1750 rpm, 10 min) and chloroform was recovered in the lower layer and the same procedure was repeated three times in total. Absorbance is measured at 325 nm using a UV-spectrophotometer to quantify the retinol contained in the recovered chloroform. The concentration of retinol is determined by the standard curve. The standard curve (R^2 = 0.999) was prepared by measuring the absorbance of known concentration of retinol in chloroform.

5. Result and Discussion

5.1. Characteristics and stability of retinol-loaded O/W emulsion

5.1.1. Characteristics of retinol-loaded O/W emulsion

5.1.1.1. Effect of emulsifier types in O/W emulsion

The O/W emulsions containing retinol were prepared with the different types of emulsifier. Tween 20, Decaglycerin myristate and WPI were used as emulsifiers when making emulsion containing retinol. The size distribution of retinol-loaded O/W emulsions with different emulsifiers is shown in **Figure 1A**. The size distribution of the uniform distribution of the droplets of the emulsion showed the same uniform peak, which means that the emulsion is in a stable state.

Since Tween 20 and Decaglycerin myristate are nonionic emulsifiers and WPI is an anionic emulsifier with negative charge, the absolute value of Zeta potential is expected to be larger when compared to the other two cases. As a result of measurement, Zeta value of Tween 20 was -10.3 ± 1.1, Decaglycerin myristate was -13.4 ± 1.41 and WPI was -44.6 ± 1.14 based on O/W emulsion with oil concentration of 4 wt% (**Fig. 1B**). The average size was the biggest when WPI was used as an emulsifier (218.3 nm), but no significant difference was found in the average size depending on the oil concentration (not shown). Since WPI is a globulized structure different from synthetic emulsifier, size of emulsion is larger than other emulsifier. Therefore, it can be seen that there is no significant difference in the stability depending on the emulsifier used in a fresh emulsion.

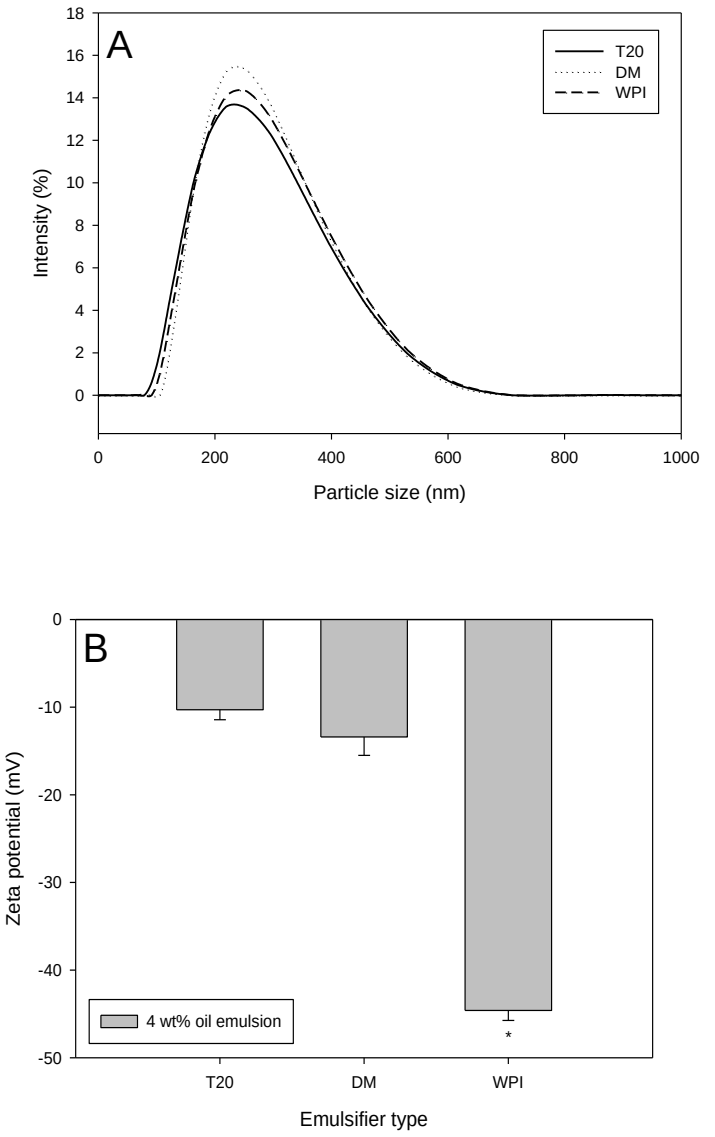


Figure 1. Size distribution(A) and zeta potential value(B) of retinol-loaded O/W emulsion using different types of emulsifier (T20, Tween20; DM, Decaglycerin myristate; WPI, Whey protein isolated).

5.1.1.2. Effect of oil concentration in O/W emulsion

Figure 2 and **table 1** shows the size and zeta of emulsion by concentration prepared to see if there is a difference in stability depending on the oil concentration.

Size is 210 nm on average and there is no significant difference according to oil concentration (**Table 1**). The size distribution shows that all the samples have a single peak, indicating that the emulsion is stable (not shown). What is notable is the change in the Zeta potential value. The zeta potential is also a measure of the stability of the emulsion. The larger the surface charge, the greater the zeta potential value, which means that the repulsion between particles is stronger. In case of using Tween 20 and Decaglycerin myristate as an emulsifier, the absolute value of zeta potential decreases as the concentration of oil increases. Since the same amount of retinol was added to the entire emulsion system, the density of retinol present in the oil becomes lower as the oil concentration of the O/W emulsion increases. Therefore, in order to investigate the change of emulsion characteristics according to the amount of retinol in the oil, the concentration of oil was fixed to 4 wt% and the amount of retinol was further dissolve and further experiments were conducted.

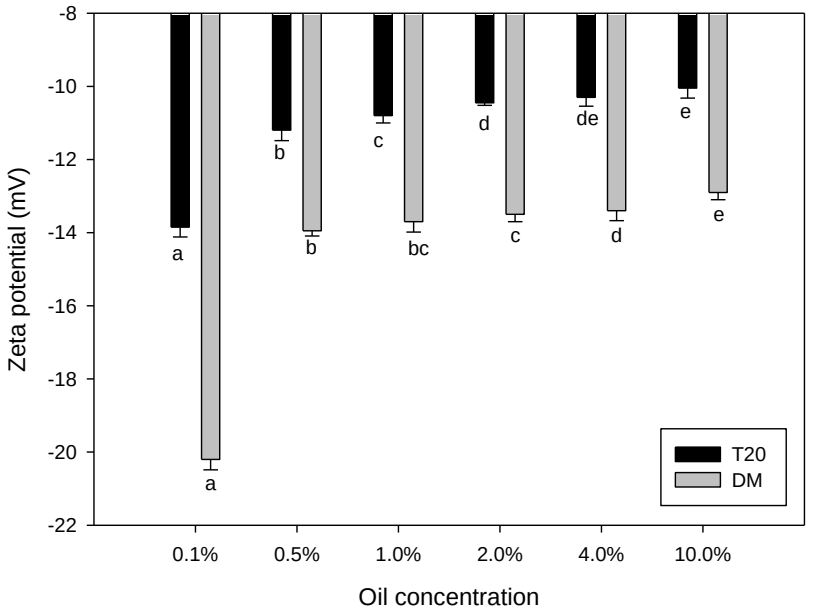


Figure2. Zeta potential (mV) of retinol-loaded O/W emulsion using different emulsifier (T20 and DM) with different concentration of oil (0.1, 0.5, 1, 2, 4 wt. % oil emulsion). Means followed by different letters in each samples are significantly different ($p<0.05$).

Oil concentration (%)

	0.1	0.5	1.0	2.0	4.0	10.0
T20	210.7±1.3	204.4±2.1	210.5±4.3	209.8±3.5	203.8±1.4	202.8±3.2
DM	218.5±2.4	220.2±5.0	211.1±1.7	220.2±3.1	212.5±1.8	218.1±4.3
WPI	200.1±0.9	202.1±1.3	200.3±0.7	201.7±1.2	199.9±2.2	201.2±1.2

Table 1. Average particle size of retinol-loaded O/W emulsion using different emulsifier with different concentration of oil (0.1, 0.5, 1.0, 2.0, 4.0 and 10wt. % oil emulsion).

As a result of comparing retinol contents of 1.25, 2.5, and 5 mg of retinol with fixed oil concentration, the absolute value of zeta potential increased as the amount of retinol increased in the oil (**Fig. 3**). Zeta potential is a measure of the charge on the surface of an oil droplet. It can be expected that the change in value is due to the fact that retinol is dispersed on the surface of the oil rather than dispersed in the oil, thus affecting the surface charge. In the case of α-Tocopherol, it was also found that when the oil is encapsulated in the lipid droplet of the O/W emulsion, the relatively hydrophilic part is in the continuous phase and the lipophilic part is in the oil and is distributed on the surface of the oil droplet (Silvestre, Chaiyasit, Brannan, McClements, & Decker, 2000). Retinol is also hydrophobic throughout its structure, but hydroxyl groups are present and can not be completely dispersed inside the oil. These results are also related to the degradation of retinol by the lipid oxidation of the emulsion at the later stage.

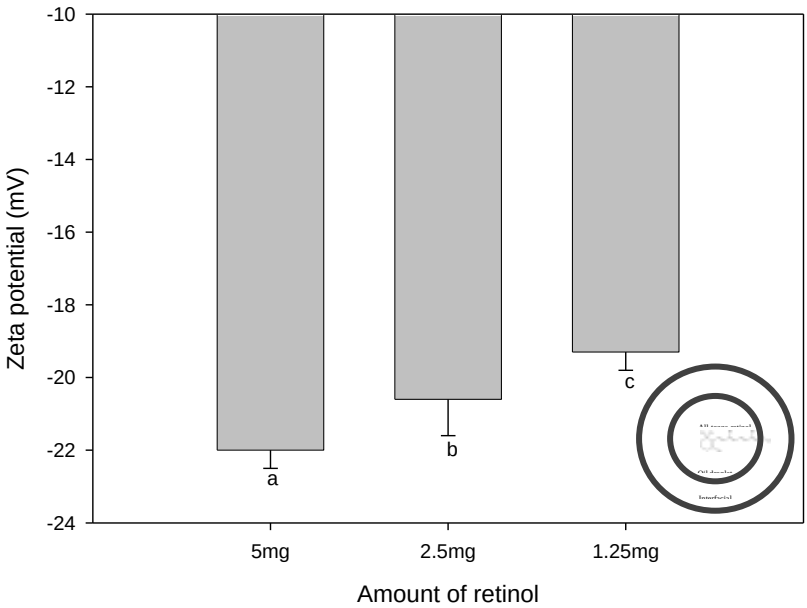


Figure 3. Zeta potential value of retinol-loaded O/W emulsion (4% oil emulsion using Tween20 as an emulsifier) when the amount of retinol contained in oil is different (1.25, 2.5 and 5mg in 4g oil). Means followed by different letters in each samples are significantly different (p<0.05).

5.1.2. Stability of retinol in O/W emulsion

5.1.2.1. UV stability

The UV stability of retinol (retention rate of retinol %) incorporated into freshly-made O/W emulsions was examined using UV chamber. After irradiating the sample and blank with UV light, confirm retinol residual amount at each time for 24 hours.

Comparing the UV stability of retinol in O/W emulsions (4wt% oil emulsion) with different emulsifiers, the stability of retinol was the highest in emulsion using DM and the lowest in emulsion using WPI as emulsifier (**Fig. 4**). However, all samples showed lower stability than bulk oil control.

Emulsions form oil droplets, therefore, a larger lipid surface area is exposed to the water phase and light than with bulk oil; thus, more light can penetrate into the emulsions. Furthermore, as previously mentioned, retinol is likely to be dispersed on the surface of the oil rather than entirely within the oil. Thus, the retinol that is contained within the emulsion systems is more easily reached and degraded by UV compared to retinol dispersed in bulk oil.

Eskandar et al. reported that despite the relatively low water solubility of retinol at room temperature and pH 7.3, retinol can move through the aqueous phase, thus, resulting in the fast partitioning of AR from the oil phase of the emulsions to the aqueous phase. They also suggested that this kind of distribution of AR could be limited by adding an interfacial barrier such as a silica-oleylamine complex at the oil-water interface (Eskandar, Simovic, & Prestidge, 2009). Cho

et al. prepared the Retinol 50C emulsion, which is co-stabilized with triblock copolymers. They suggested that the UV stability of retinol was improved by using triblock copolymers with a long PCL block length and low HLB value (Cho, Cho, Choi, & Cheong, 2012). These previous studies suggest that the structure of the oil-water interface and the interaction of retinol with the emulsifier at the interface may be another important factors to protect the retinol loaded in O/W emulsions from UV degradation.

When the emulsions with different oil concentrations were compared, the UV stability of retinol increased with increasing oil concentration (**Fig. 5**). As a result of measuring the turbidity of the emulsion, it was confirmed that it increased in proportion to the oil concentration (**Table 3**). As the oil concentration increases, the turbidity of the emulsion increases, which causes more UV light to be scattered within the emulsion. Thus, although the amount of retinol contained in each emulsion is the same, the effect of UV light on retinol in the oil is reduced. when comparing the UV stability of the retinol contained in the emulsions to that of the retinol dispersed in bulk oil, it was seen that at oil concentrations below 10 wt%, the retinol retention rate of the emulsions was lower than that of the bulk oil. However, at 10 wt% oil, the retinol retention rate of the emulsions became greater than that of the bulk oil.

Comparing the UV stability of retinol in O/W emulsions (10wt% oil emulsion) with different emulsifiers, all samples showed higher stability than bulk oil control (**Fig. 6**). In addition, it was confirmed that the higher the turbidity keep

retinol stable (**Table 4**). This result suggests that the O/W emulsions systems with low oil concentrations, irrespective of the emulsifier used (T20, DM, or WPI), are not effective at protecting retinol against UV; however, over a certain oil concentration, they become effective.

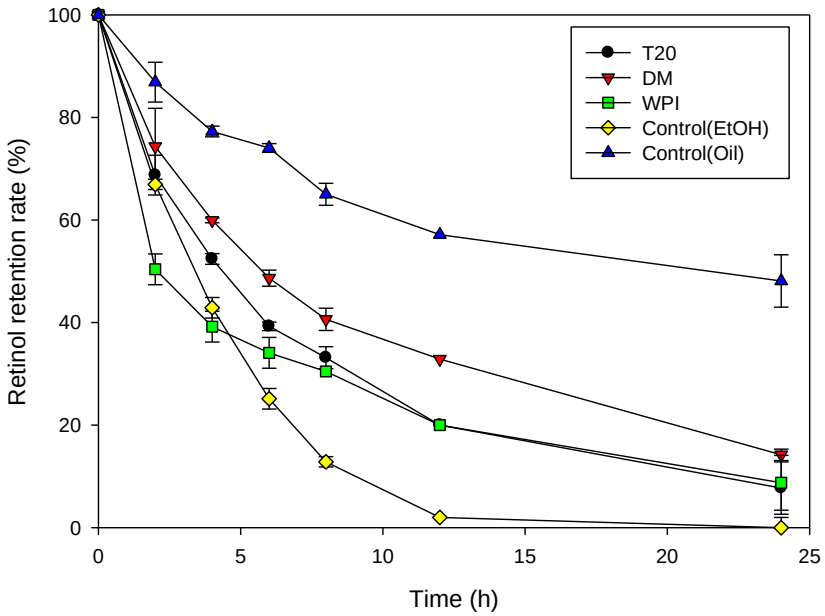


Figure 4. UV stability of retinol incorporated O/W emulsion (4 wt. % oil emulsion) with different types of emulsifier

Turbidity (A_{500})		
T20	DM	WPI
0.791±0.005	0.799±0.006	0.659±0.007*

Table 2. Turbidity (measured by 100-fold dilution) of O/W emulsion (4 wt. % oil emulsion) with different types of emulsifier (T20, Tween20; DM, Decaglycerin myristate; WPI, Whey protein isolated). Means followed by different letters in each concentration are significantly different (p<0.05).

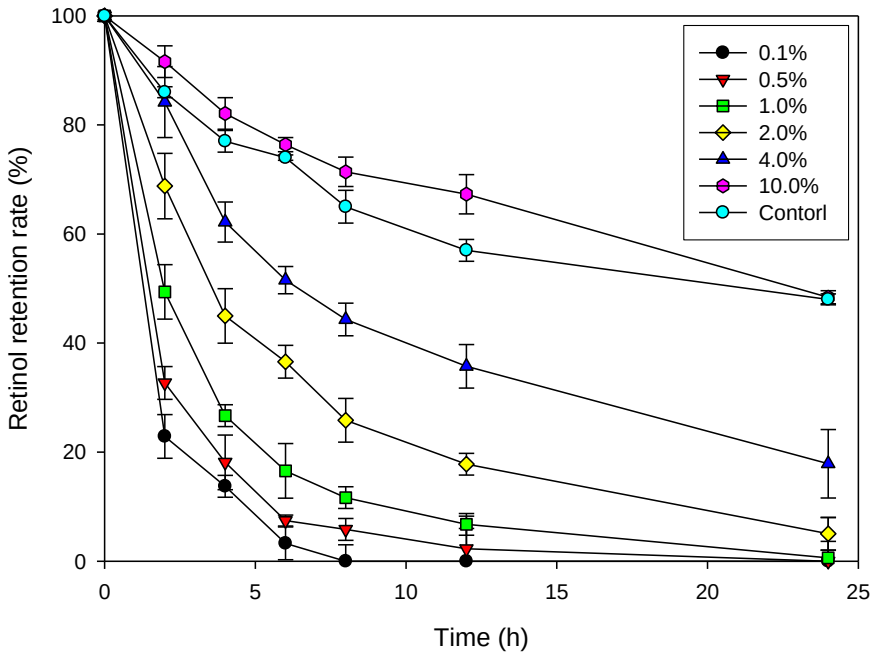


Figure 5. UV stability of retinol incorporated O/W emulsion using decaglycerin myristate as an emulsifier with different concentration of oil (0.1, 0.5, 1.0, 2.0, 4.0 and 10.0 wt %)

Oil concentration	**0.1%**	**0.5%**	**1.0%**	**2.0%**	**4.0%**	**10.0%**
Turbidity (A_{500})	0.069 ±0.002[a]	0.159 ±0.012[b]	0.344 ±0.014[c]	0.555 ±0.024[d]	0.799 ±0.006[e]	1.497 ±0.02[f]

Table 3. Turbidity (measured by 100-fold dilution) of O/W emulsion using decaglycerin myristate as an emulsifier with different concentration of oil (0.1, 0.5, 1.0, 2.0, 4.0 and 10.0 wt %). Means followed by different letters in each concentration are significantly different (p<0.05).

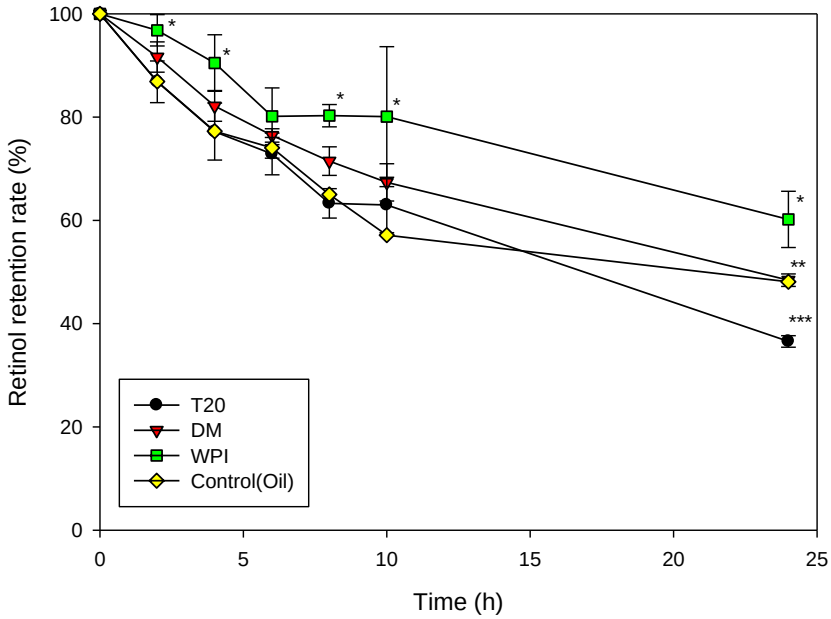


Figure 6. UV stability of retinol incorporated O/W emulsion (10 wt. % oil emulsion) with different types of emulsifier

Turbidity (A_{500})		
T20	DM	WPI
0.510 ± 0.037^{a}	0.718 ± 0.003^{b}	1.687 ± 0.016^{c}

Table 4. Turbidity (measured by 100-fold dilution) of O/W emulsion (4 wt. % oil emulsion) with different types of emulsifier (T20, Tween20; DM, Decaglycerin myristate; WPI, Whey protein isolated). Means followed by different letters in each concentration are significantly different ($p<0.05$).

5.1.2.2. Storage stability

To confirm storage stability of retinol, it is stored at 4, 25 and 40℃ for one week and retinol residue is checked daily to compare the stability of retinol in O/W emulsion. Before checking the stability of retinol, characterization of the emulsion was investigated to confirm the stability of the emulsion itself to the temperature.

When the storage temperature is changed, the stability of the O/W emulsion itself with different emulsifier and oil concentrations is shown in **Figure 7**. At 4℃ and 25℃, the change of size and zeta value was not significant regardless of oil concentration and type of emulsifier, so emulsion remained stable ($p < 0.05$). O/W emulsion with low oil concentration (0.3% oil), however, was unstable at 40℃ regardless of the type of emulsifier. The larger the zeta potential absolute value, the stronger the repulsive force between the oil particles is, and the coagulation does not occur. The O/W emulsion with the oil concentration of 0.3% shows a sharp decrease at 40°C (**Fig. 7B**). Instability of these samples can also be confirmed by average particle size. Unlike the 4% O/W emulsion, where the average size is kept constant regardless of temperature and emulsifier type, the 0.3% O/W emulsion increases rapidly at 40℃ (**Fig. 7A**) and its size distribution is not uniform (not shown). Unlike bulk oil, O/W emulsion is vulnerable to oxidation because it is in contact with water and oil. Also, as the oil concentration in the O/W emulsion increases, the unsaturated fatty acid migrates into the oil and the oxidation rate slows down (Coupland, Zhu, Wan, McClements, Nawar, & Chinachoti, 1996; D. McClements & Decker, 2000;

Osborn & Akoh, 2004) . Unsaturated fatty acid promotes the oxidation of emulsion, so the lower the oil concentration, the faster the oxidation occurs during storage. The decrease in the stability of the emulsion itself can be expected to have a great influence on the stability of retinol incorporated in the lipid droplet of O/W emulsion.

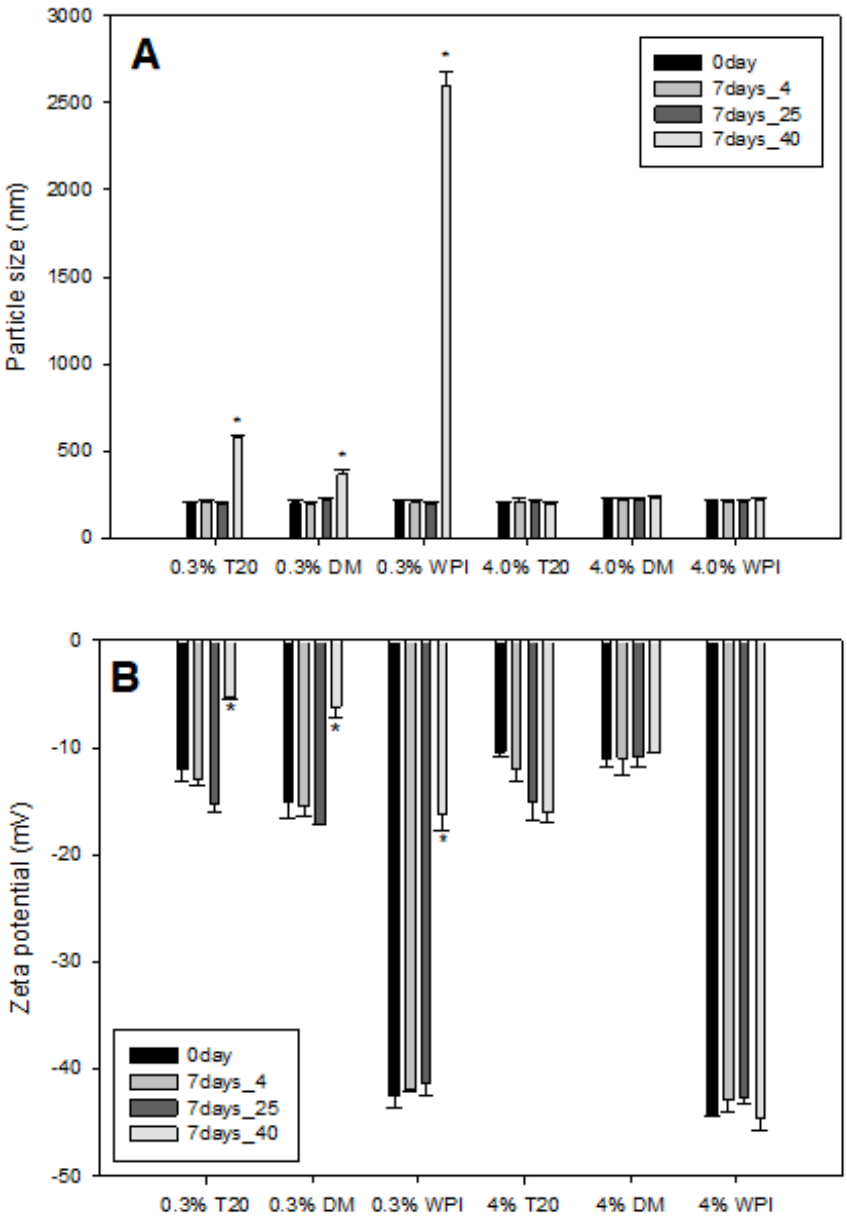


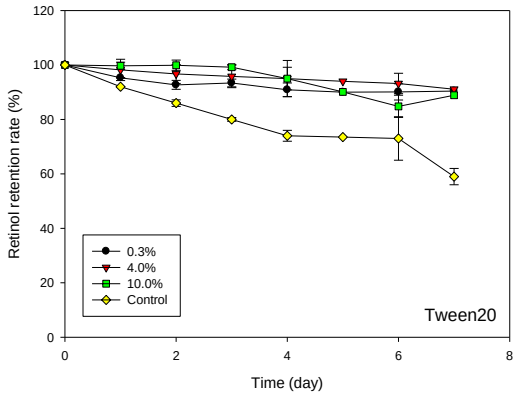
Figure 7. Changes in the particle size (A) and zeta potential value (B) of O/W emulsions stored at different temperature (4, 25 and 40℃) and for one week. Emulsions were fabricated with different oil concentrations (0.3, 4% oil emulsion) and different emulsifier.

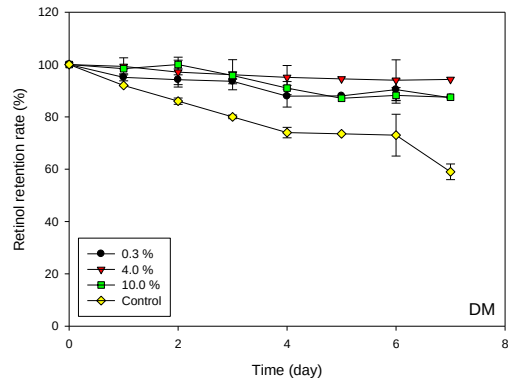
Figure 8 shows the retention rate (%) of retinol in the O/W emulsion depending on oil concentration, emulsifier type, and storage temperature. At the refrigeration temperature of 4℃, retinol remained almost constant when Tween20(T20) and Decaglycerin myristate(DM) were used as emulsifiers regardless of oil concentration (**Fig. 8A**). When WPI was used as an emulsifier, however, the amount of retinol was reduced in a manner similar to control (retinol in bulk oil) regardless of oil concentration. When the storage temperature was above 25℃, the effect of oil concentration was also observed (**Fig. 8B**). The residual amount of retinol was reduced to similar level when T20 and DM were used as emulsifiers in a relatively high oil concentration (4, 10 % oil emulsion), and was more stable at 25°C compared to control and at 40°C it was unstable (**Fig. 8C**). Remarkably, the O/W emulsion using WPI as an emulsifier decreased the retinol residue more rapidly as the oil concentration decreased, and was more unstable than the control in all cases where the storage temperature was above room temperature.

These results are related to the characteristics of the emulsion system. Emulsion is a thermodynamically unstable system whose stability is affected by physical and chemical factors. In this study, the storage temperature is a physical effect, which can lead to unstable conditions such as Ostwald ripening, creaming, flocculation, coalescence, partial coalescence and phase inversion (Rousseau, 2000). Additionally, lipid oxidation and hydrolysis in O/W emulsion can be a chemical factor affecting stability (D. J. McClements, 2015). Oxidation of lipid droplet in the O/W emulsion occurs mainly in the interfacial region, which is the

interface between the oil interior and the aqueous phase. Lipid oxidation of O/W emulsions is affected by several conditions (Fomuso, Corredig, & Akoh, 2002; Frankel, Satué-Gracia, Meyer, & German, 2002; Jacobsen, 1999; Mancuso, McClements, & Decker, 1999; Nuchi, McClements, & Decker, 2001). As a first factor, the lower the oil concentration, the greater the amount of radicals produced per droplet. This can be explained by the fact that at higher oil concentrations, the unsaturated fatty acid migrates to the inside of the oil droplet and therefore has less direct contact with the prooxidant present in the aqueous solution (Coupland, Zhu, Wan, McClements, Nawar, & Chinachoti, 1996; D. McClements & Decker, 2000; Osborn & Akoh, 2004). Therefore, as the oil concentration of the emulsion is lowered and the storage temperature is higher, the stability of the emulsion itself decreases and the retinol in the oil is also affected because lipid oxidation occurs well in the interfacial region of the oil droplet. Second, the type of emulsifier can be a factor affecting the lipid oxidation of the emulsion (Mancuso, McClements, & Decker, 1999; Nuchi, McClements, & Decker, 2001). Of the three emulsifiers used in this study, Tween 20 and Decaglycerin myristate are nonionic emulsifiers and WPI is anionic emulsifier. Previous studies have shown that anionic emulsifiers promote lipid oxidation of emulsions over other emulsifiers (Hu, McClements, & Decker, 2003; Mei, Decker, & McClements, 1998). Lipid oxidation rates were highest for negatively charged droplets, and were fairly similar for uncharged droplets and positively charged surface. Negatively charged droplets are easily reacted with trace amounts of metal ions present in the aqueous phase. A detailed

description can be found in relation to the next factor, pH. In addition, promoting oxidation by free radicals is one of the influencing factors because WPI itself has residual lipid (Donnelly, Decker, & McClements, 1998; Kinsella & Whitehead, 1989). The final factor depends on the pH of the aqueous solution and the trace amount of cations in it. The oxidation of the emulsion is affected by a trace amount of iron ions in the water. The iron ion has a lower solubility at pH 7, and ions that do not dissolve in the aqueous solution adhere to the surface of the oil droplet (Mancuso, McClements, & Decker, 1999; Zumdahl). Therefore, oxidation is promoted on the surface of oil droplet. Also, proteins such as WPI have inherent pI values, so the charge depends on the pH of the aqueous solution. The pH of the buffer used in this study is 7, which is higher than the pI value of WPI of 4.6, so that WPI has a negative charge (Mancuso, McClements, & Decker, 1999) According to the above results, retinol is distributed in the oil surface rather than dispersed in oil, so it is likely to be directly affected by oxidation. In order to confirm whether this interpretation is correct, EDTA which chelates the metal in the emulsion of the same condition using the WPI as the emulsifier was added.





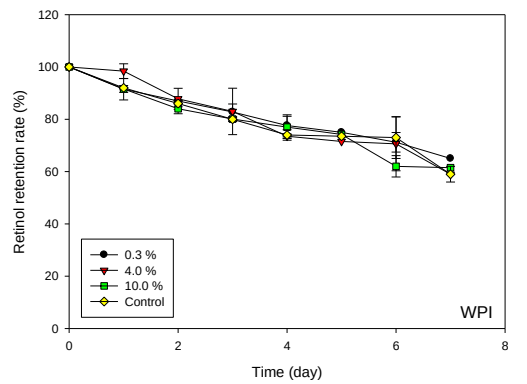
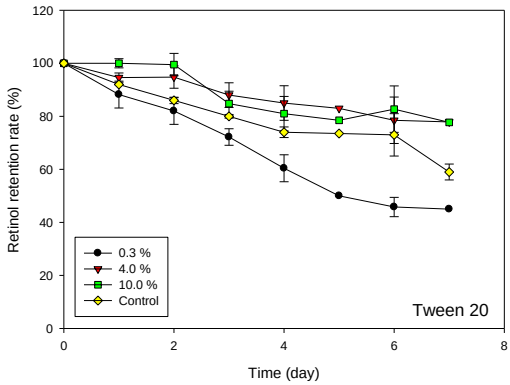
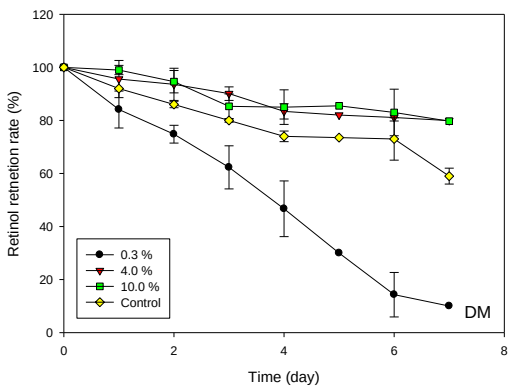


Figure 8A. Retention rate (%) of retinol in O/W emulsion according to oil concentration (0.3, 4 and 10%), type of emulsifier (Tween 20, Decaglycerin myristate and WPI) and storage temperature at 4℃.





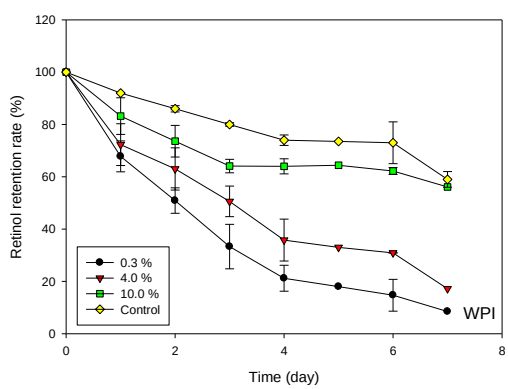
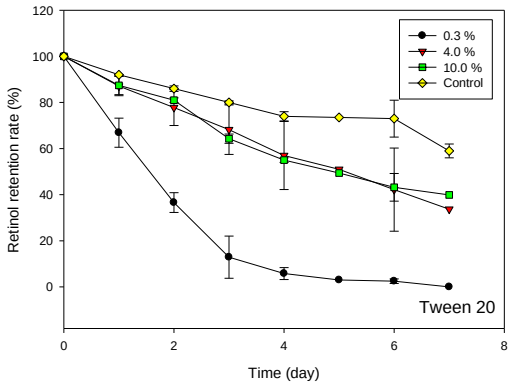
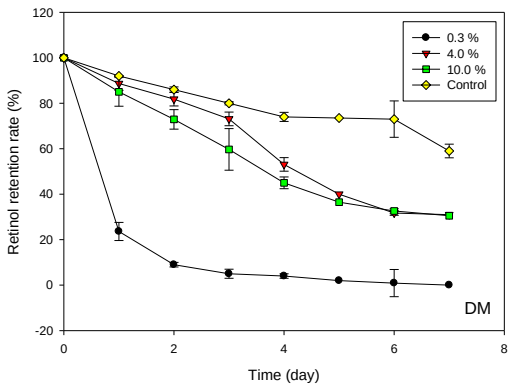


Figure 8B. Retention rate (%) of retinol in O/W emulsion according to oil concentration (0.3, 4 and 10%), type of emulsifier (Tween 20, Decaglycerin myristate and WPI) and storage temperature at 25℃.





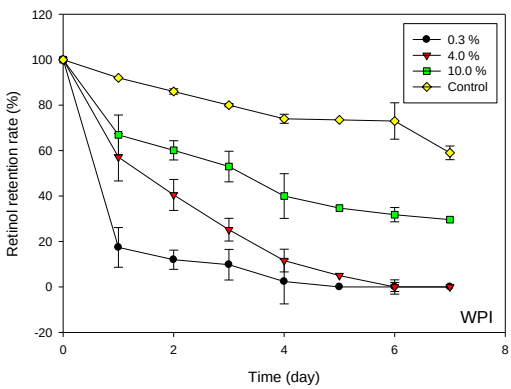
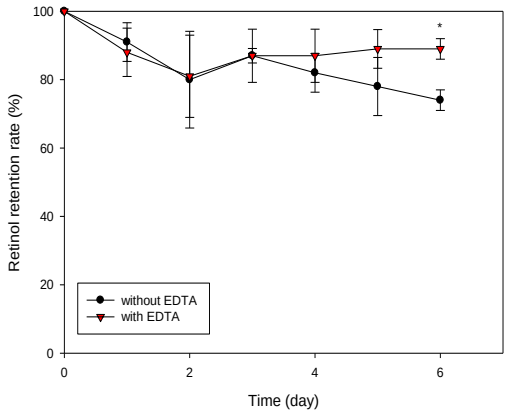
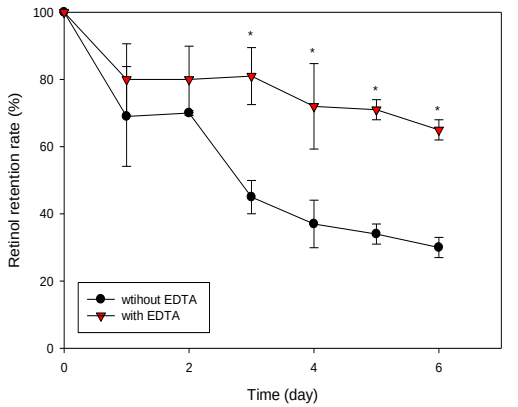


Figure 8C. Retention rate (%) of retinol in O/W emulsion according to oil concentration (0.3, 4 and 10%), type of emulsifier (Tween 20, Decaglycerin myristate and WPI) and storage temperature at 40℃

We investigated whether WPI, an anionic emulsifier with negative charge, reacted with trace amounts of metal present in the aqueous phase to affect retinol oxidation. Add 75mM EDTA to the retinol-loaded O/W emulsion prepared in the same condition as in **Figure 8** and allow to dissolve for 3 hours. The samples were stored at 4, 25, and 40℃ for one week in the same condition as in **Figure 8**, and the results of confirming the residual amount of retinol are shown in **Figure 9**. At the refrigerated temperatures, the retinol content in the sample with EDTA was better maintained after 6 days. At 25℃ and 40℃, the residual amount of retinol was higher in the sample containing EDTA and there are significant differences ($p < 0.05$). It was confirmed that WPI reacted with the metal ion present in the aqueous phase to affect retinol oxidation.

Finally, we examined whether the type of oil affects O/W emulsion oxidation. In this experiment, the oil used to make the O/W emulsion is soybean oil, which is a long chain triglyceride having about 55% linoleic acid which is vulnerable to oxidation. The effect of oxidation of soybean oil on the storage stability of retinol in O/W emulsion was investigated by using coconut oil composed of 95% Medium chain triglyceride (MCTs). MCTs provide less energy than triglycerides composed of mainly long-chain saturated fatty acids (LCTs) (Kupongsak & Sathitvorapojjana, 2017).





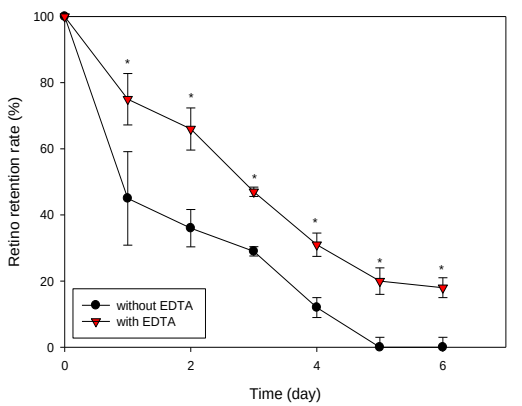


Figure 9. Retention rate (%) of retinol in O/W emulsion (soybean oil 4% w/w) using WPI as emulsifier with or without EDTA (from top to bottom; 4, 25 and 40℃)

Coconut oil was used to prepare an emulsion with the same conditions as the retinol-loaded O/W emulsion (4 wt% oil) shown in **Figure 10** to confirm the storage stability. As a result, the stability of retinol was higher when emulsion of the same condition was made using coconut oil. When WPI was used as an emulsifier, the emulsion of soybean oil remained similar to the control at 4℃ and remained at the retinol level lower than 25℃ at all times. On the other hand, the emulsion made of coconut oil has a higher residual amount than the control in all cases. These results are due to the composition of fatty acids depending on the kind of oil as mentioned above. The oxidation of oil is influenced by the composition and content of fatty acids. Oxidation is promoted as the number of double bonds increases. In other words, the higher the content of unsaturated fatty acids, the more easily the oxidation proceeds. Previously used soybean oil has about 15% saturated fatty acid and about 80% unsaturated fatty acid, while coconut oil has 9% unsaturated fatty acid (Kupongsak & Sathitvorapojjana, 2017). Therefore, it can be seen that the oxidation rate of oil during storage is different, which also affects the oxidation of retinol distributed on the surface of oil.

Therefore, it can be interpreted that the reason why retinol residual amount is lower than control when WPI is used as an emulsifier in Figure 9 is due to oxidation in the emulsion system. We confirmed that the stability of the emulsion was maintained by measuring the average size, size distribution and zeta potential of the emulsion before and after storage. Therefore, this result is not related to the stability of the emulsion. Regardless of the stability of the emulsion,

it is presumed that the oxidation of emulsion occurs during storage due to various factors that cause oxidation in the emulsion system (type of emulsifier, unsaturated fatty acid content of the oil, pH *etc*.), and retinol in the oil is affected by the degradation. It is not the main purpose of this study to investigate the oxidation mechanism of O/W emulsion according to the type of oil. Therefore, the study on this should be concretely carried out later.

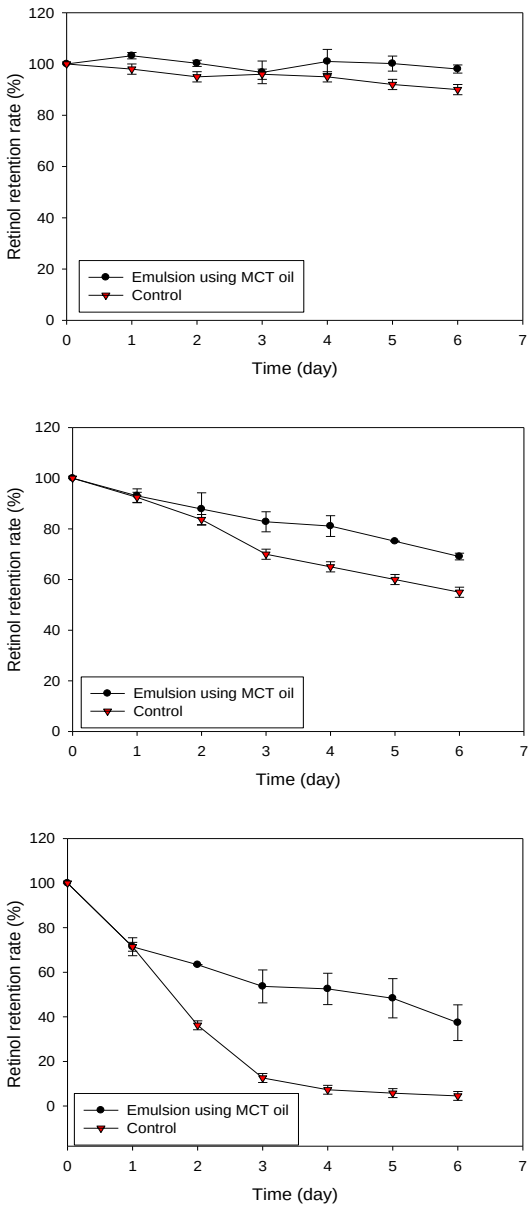


Figure 10. Retention rate (%) of retinol in O/W emulsion (4 wt% MCT oil) using WPI as an emulsifier according to storage temperature (left; 4, middle; 25 and right; 40℃).

5.2. Retinol-loaded protein based nanoparticles

5.2.1. Characteristics of retinol-loaded zein particles

5.2.1.1. Particle size distribution and zeta potential analysis

5.2.1.1.1. Effect of materials used in particle formation

To create a stable protein-based particle according to the ratio of the stabilizer and coating agent confirmed the particle size and zeta potential changes. To determine the effect of sodium caseinate as a stabilizer, particles were prepared by changing the ratio of zein to sodium caseinate without adding pectin (**Table 5.** SC1-6). To determine the effect of coating agent, the ratio of zein to sodium caseinate was fixed at 1: 1.25 and the ratio of pectin was changed (**Table 5.** P1-P6).

First, the particle size did not change significantly with increasing pectin ratio. On the other hand, in the case of SC samples, the particle size was significantly increased as the amount of zein was larger than that of sodium caseinate, and the size increased sharply when it was 1.25 times or more (**Fig.11** SC6). Zeta potential values remain constant without being influenced by sodium caseinate and pectin. Therefore, the ratio of zein, sodium caseinate and pectin was fixed at 1: 1.25: 1.25 to prevent the rapid increase of particle size.

	SC1	SC2	SC3	SC4	SC5	SC6	P1	P2	P3	P4	P5	P6
Zein	0.25	0.5	1	1.25	1.25	1.25	1	1	1	1	1	1
Sodium caseinate	1.25	1.25	1.25	1.25	1	0.5	1.25	1.25	1.25	1.25	1.25	1.25
Pectin	0	0	0	0	0	0	0	0.25	0.5	0.75	1	1.25

Table 5. Ratio of zein, sodium caseinate and pectin

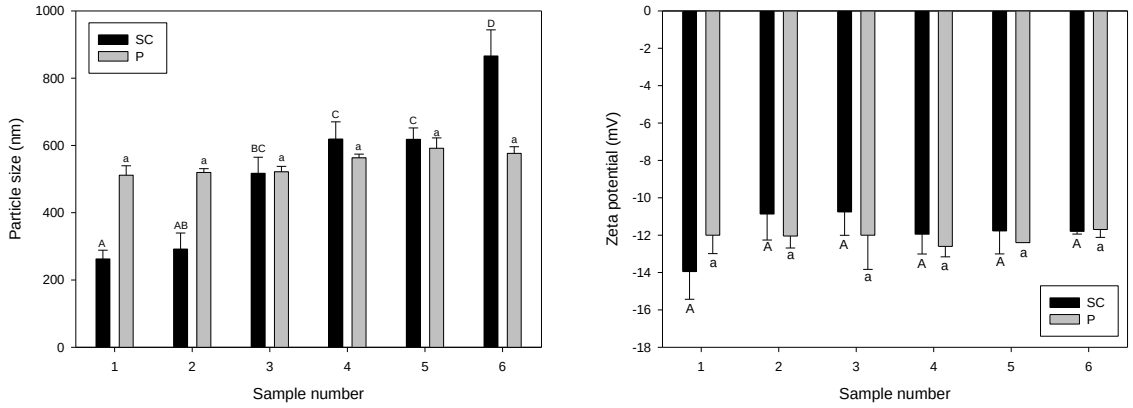
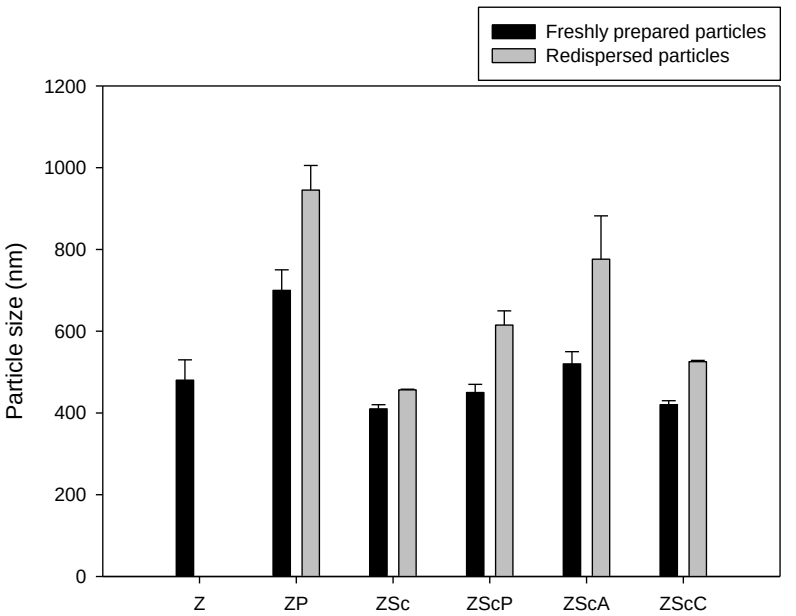


Figure 11. Effect of ratio of zein, sodium caseinate and pectin on particle size and zeta potential of protein based particles.

5.2.1.1.2. Effect of freeze drying

Figure 12 shows the particle size of zein-based particles coated with different polysaccharides before and after freeze-drying. The control of all samples was a plain zein particle (Z) and sample was coated with pectin without the stabilizer Sodium caseinate (ZP), with only the stabilizer and without the polysaccharide coating (ZSc), and with the polysaccharide in the presence of the stabilizer (pectin coating, ZScP; Sodium alginate coating, ZScA; Sodium carboxymethyl cellulose coating, ZScC). In the case of freshly prepared particles, the size of the sample coated with polysaccharide without the stabilizer (ZP) was the largest, and the samples coated with polysaccharide in the presence of the stabilizer (ZScP, ZScA and ZScC) were larger than the control but not significantly different depending on the type (p<0.05). These results suggest that sodium caseinate affects particle size. Previous studies have shown that adding more than a certain percentage of sodium caseinate to a zein based particle avoids aggregation of particles and increases stability (Luo, Teng, Wang, & Wang, 2013). Each sample was lyophilized and re-dispersed to measure its size. As a result, aggregation occurred only in plain zein particles (Z). All other samples increased in size when re-dispersed the particles. In the case of zeta potential, all of the coated samples have a negative charge because they use a sodium caseinate (pI 4.6) with a negative charge on the surface of zein (pI 6.2) with a positive charge at pH 6. The sample using zein alone was not redispersible and could not be measured. It can be seen that the polysaccharide coated the particles as the particle size increases with the addition of the polysaccharide and the

charge of the particle surface changes to negative. However, there was no difference in zeta potential value before and after freeze drying.



	Z	ZP	ZSc	ZScP	ZScA	ZScC
Freshly prepared nanoparticles	470.1 ±42.4^{AB}	700.8 ±56.6^{C}	407.5 ±10.6^{A}	462.5 ±17.7^{AB}	519.1 ±28.3^{B}	421.0 ±14.1^{A}
Re-dispersed nanoparticles	-	945.2 ±60.2^{d}	456.3 ±1.7^{a}	614.8 ±34.9^{b}	776.0 ±106.1^{c}	525.4 ±3.2^{ab}

Figure 12. The average size (nm) and zeta potential (mV) of freshly prepared particles and redispersed particles (Z, plain zein particles; ZP, Zein/Pectin particles; ZSc, Zein/Sodium caseinate; ZScP , Zein/Sodium caseinate/Pectin; ZScA, Zein/Sodium caseinate/Sodium alginate; ZScC, Zein/Sodium caseinate/Sodium carboxymethyl cellulose).

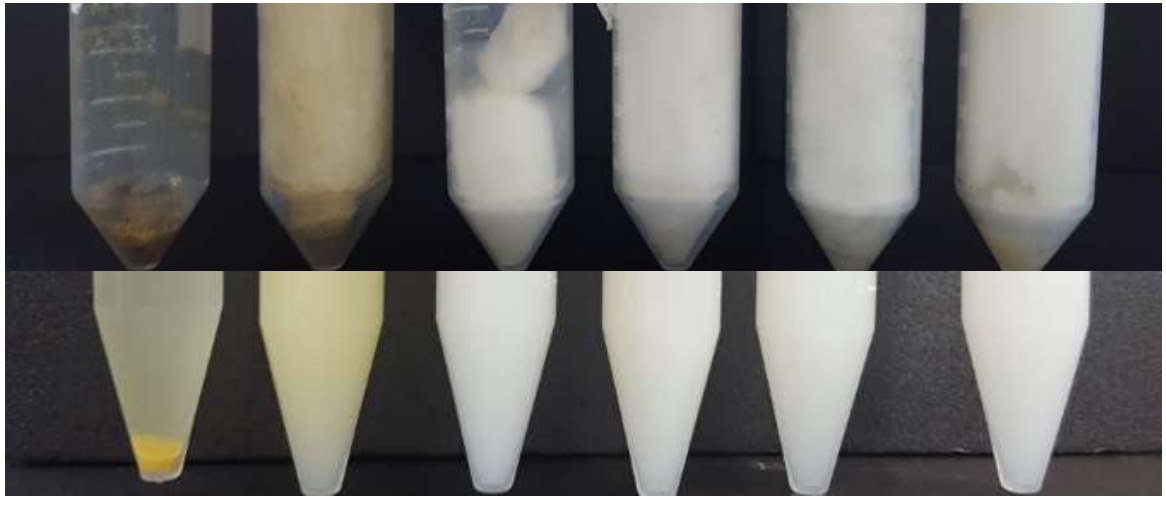


Figure 13. Image of lyophilized samples and redispersed colloid (From the left Z, ZP, ZSc, ZScP, ZScA and ZScC).

5.2.1.2. Particle yield and loading efficiency analysis

The particle yield (%) and retinol loading efficiency(%) were measured after lyophilization to characterize each sample. First, the samples with plain zein particles (Z) and pectin (ZP) were not uniformly formed after lyophilization. So these two samples were not measurable. These results suggest that sodium caseinate prevents aggregation of particles during zein based particle production. Previous studies have shown that sodium caseinate with a large portion of negatively charged groups adheres to the surface of zein based particles with a strong hydrophobic interaction to provide stability (Luo, Teng, Wang, & Wang, 2013). Particle yields were the lowest with ZSc at 84.2% and the samples containing polysaccharides (ZScP, ZScA and ZScC) were 92.6, 93.3 and 92.6%, respectively (**Table. 6**). The particle yield of the polysaccharide coated particles did not show any significant difference (p<0.05). Retinol loading efficiency of all samples was more than 90% and there was no difference within the error range (p<0.05). This means that when making zein-based particles, stabilizers alone are capable of retinol embedding, but that does not mean that the particles are stable. Both of these analyzes identify the characteristics of the particles, and the stability of the particles created reinforces the latter. Also, detailed comparisons of the differences in particle yields are mentioned in the section on morphological characteristics.

	Z	ZP	ZSc	ZScP	ZScA	ZScC
Particle yield (%)	-	-	84.2±1.3*	92.6±0.5	93.3±1	92.6±2.2
Retinol loading efficiency (%)	-	-	92.8±0.8	92.5±1.2	93.6±2.1	91.1±1.2

Table 6. particle yield (%) and retinol loading efficiency (%) of the lyophilized samples. Means followed by different letters in each samples are significantly different (p<0.05).

5.2.1.3. Scanning Electron Microscopy (SEM)

SEM images are shown in the **Figure 14** to identify the morphological characteristics of protein-based particles. A sample made using only zein and sodium caseinate, which are basic components for making protein-based particles, appears as an aggregate of small particles (**Fig 14B**). On the other hand, the sample coated with pectin has a core-shell structure, which clearly shows the formation of particles. Considering that the particle size of ZScP measured in 5.2.1.1. corresponds to about 700 nm, it can be seen that pectin is coated particle because it has a similar size in **Figure 14C**. This is supported by previous research that homogenous particles are formed when pectin is added, with or without sodium caseinate (Veneranda, Hu, Wang, Luo, Castro, & Madariaga, 2018). At this time, particles made only with zein and pectin are formed with core-shell structure by pectin, but the particle size is significantly larger than that with sodium caseinate added at about 800nm. (not shown). Retinol has unstable properties at room temperature, so it is difficult to measure SEM but it has some irregular shape (**Fig 14A**). An image of retinol encapsulated in a protein-based particle (ZScP) is shown in **Figure 14D**. First, no particles visible in the form of retinol at the same rate, which means retinol is encapsulated. Spherical particles were formed similarly to the ZScP particles. However, unlike ordinary ZScP particles, the size of particles containing retinol is noticeably reduced (about 400nm). This phenomenon is believed to be caused by the hydrophobic interaction between retinol and zein at the innermost part of the particle, pulling

the core part inward and reducing the overall particle size (Pan, Zhong, & Baek, 2013). The size of the particles encapsulating retinol was significantly reduced by measuring the particle size using dynamic light scattering (ZScP-700nm; ZScPR-440nm).

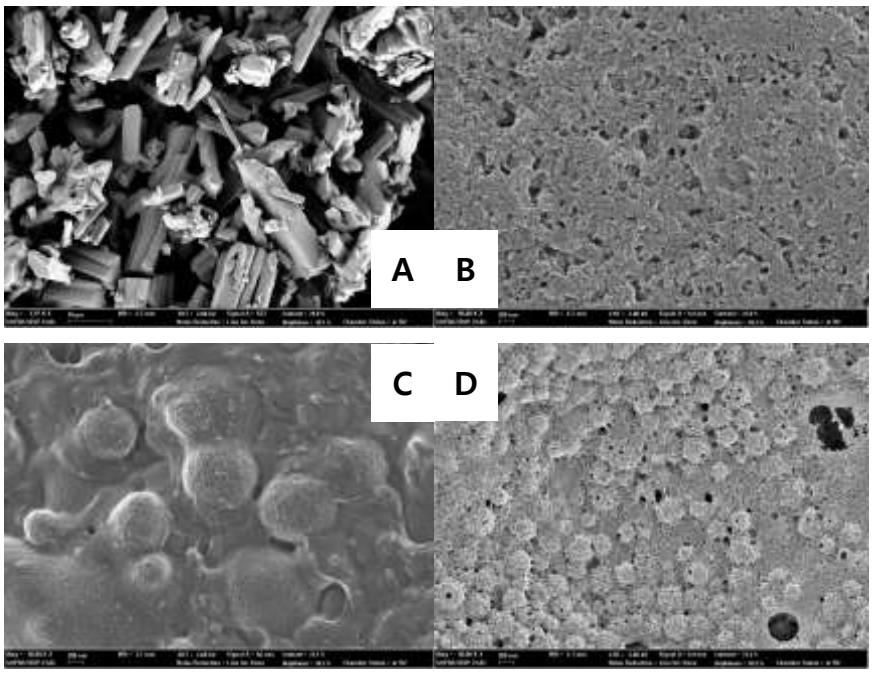


Figure 14. SEM image of retinol and nanoparticles. A, pure retinol; B, protein based particles using zein and sodium caseinate; C, protein based particles using zein, sodium caseinate and pectin; D, Retinol loaded protein particles using zein, sodium caseinate and pectin.

5.2.1.4. Differential Scanning Calorimeter (DSC)

The thermal properties of nanoparticles and physically mixed physical mixtures were analyzed (**Fig. 15**). Zein and pectin appeared broad without a specific peak. In the pure retinol, there was endothermic peak around 55℃, which has previously been attributed to melting of retinol crystals. The physical mixture also showed an endothermic peak near 55℃, which suggests that the retinol was in a crystalline form in the physical mixtures. Protein-based particles were coated with a pectin-coated ZScP sample. Unlike the physical mixture, peaks did not appear near the melting point of retinol. This means that retinol does not exist in a crystalline form but is encapsulated in a particle. Since the peaks of zein and pectin were not present, it was difficult to confirm whether the nanoparticle was properly coated with pectin. However, it was confirmed that retinol was encapsulated.

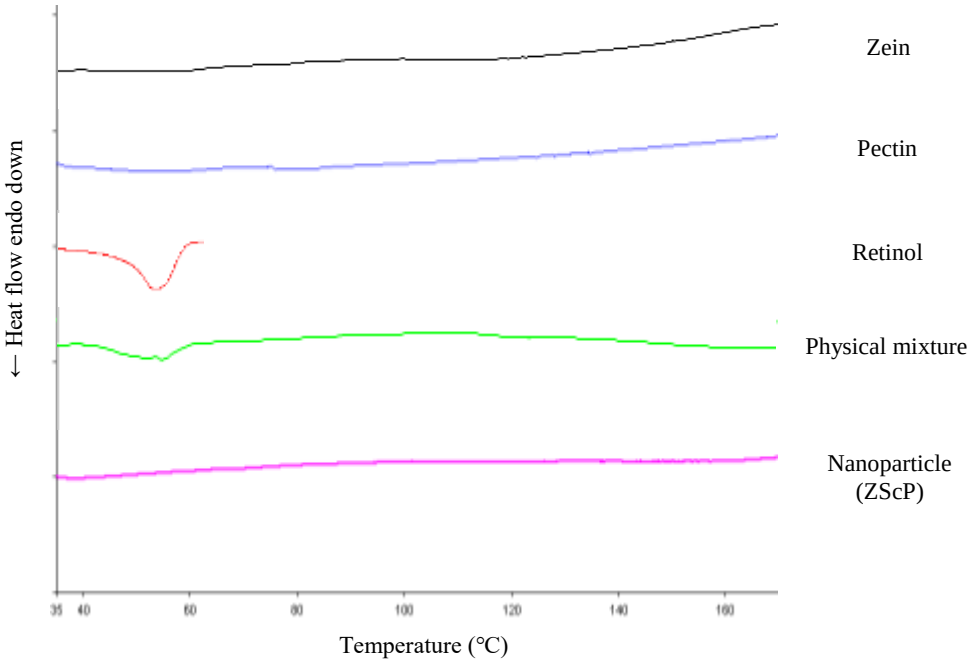


Figure 15. DSC curve of protein based particles and individual ingredients.

5.2.1.5. Fourier transform infrared spectroscopy (FT-IR)

The FTIR spectra of the protein-based particles, the individual ingredients used, and the physical mixture are shown in the **Figure 16**. In zein and sodium caseinate, a typical peak of the protein appears. The peak at 3272-3368 cm^{-1} indicates the stretching of hydrophilic O-H bond and the peak at 2961-2928 cm^{-1} indicates the stretching of hydrophobic C-H bond. It is a functional group showing the amphipathic nature of the protein. The peak at 1642-1631 cm^{-1} and 1516-1512 cm^{-1} indicate amide I and amide II, respectively, which also show the characteristics of the protein (Chang, Wang, Hu, & Luo, 2017; Farrell Jr, Wickham, Unruh, Qi, & Hoagland, 2001). Pectin has O-H and C-H stretching peaks (3368 and 2984 cm^{-1}) and has characteristic peaks of carboxylic polysaccharides(1735 and 1609 cm^{-1}) such as esterified carbonyl(C=O) and carboxylate ion(COO-) stretching bonds (Gnanasambandam & Proctor, 2000). The peak at 1014 cm^{-1} is C-O-H alcohol bonds of saturated carbon as well as to C-O stretching and C-O deformation. Retinol has O-H and C-H stretching peaks (3253 and 2928 cm^{-1}) and has a CH_3 bending peak at 1441 cm^{-1}. Characteristically, it represents a trans out-of-plane bend at 965 cm^{-1} (Kim, Hu, Jeong, Jun, Cho, & Jung, 2016). In the physical mixture, which is a mixture of individual ingredients, the peaks of the ingredients appear the same, but the peaks of the retinol loaded protein particles (ZScP-R) are shifted by the interactions between the compounds. In addition, the disappearance of the carboxylate ion (COO-) stretching peak in pectin is associated with the carboxylate group of pectin and the amide group of sodium caseinate (Chang,

Wang, Hu, & Luo, 2017). 2380-2327 cm^{-1} is the peak of C=O stretching vibrations of CO_2, so it can be ignored instead of the unique peak of the sample.

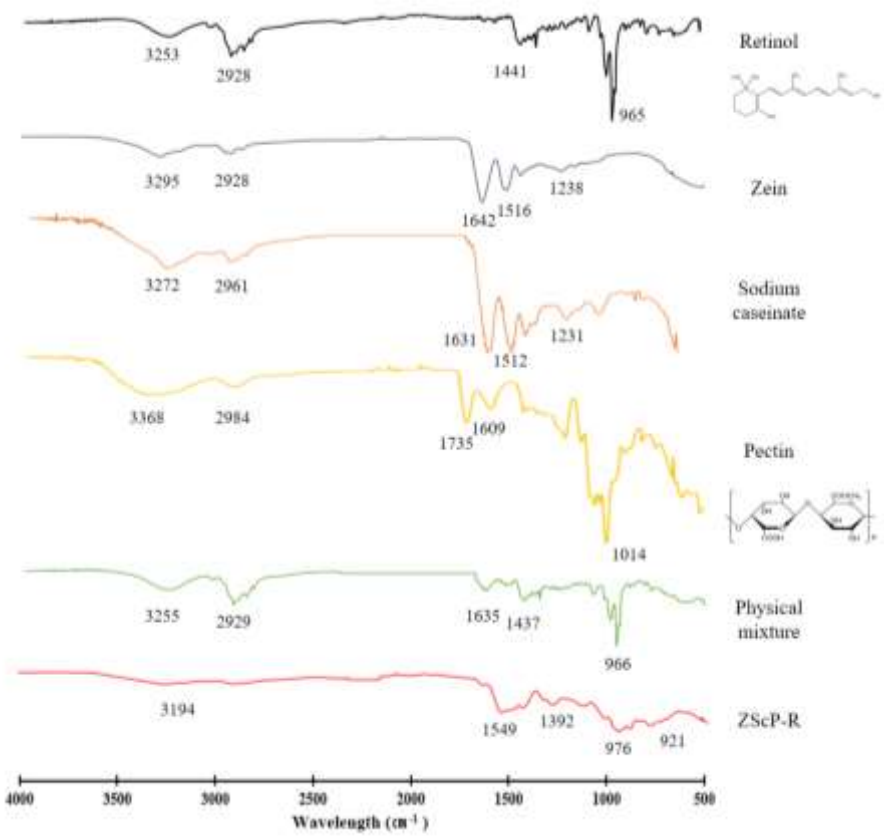


Figure 16. FT-IR spectrum of protein based particles and individual ingredients.

5.2.2. Stability of retinol in zein particles

5.2.2.1. pH stability

Figures 17, 18 and Table 7 show the stability of each sample in citrate-phosphate buffer (pH 2, 4, 6, and 8). The easiest way to confirm the pH stability was to use naked eye to check the occurrence of aggregation (**Fig. 17**). A sample made only of zein alone (Z) caused aggregation at pH 6 or above because the isoelectric point of zein is pH 6.2. On the other hand, the sample containing sodium caseinate as a stabilizer with zein (ZSc) was stable at the isoelectric point of zein but aggregation occurred near the isoelectric point (pH 4.6) of sodium caseinate. This means that sodium caseinate, not zein, is affected by the pH of the aqueous phase, so that the surface of zein particles is coated with sodium caseinate. In addition, the pH stability of the polysaccharide-loaded samples was also checked in order to improve the stability at all pH conditions. The samples coated with pectin (ZP, ZScP) did not aggregate at all pH, but the samples coated with sodium alginate (ZScA) and sodium carboxymethyl cellulose (ZScC) had aggregation at the isoelectric point of each polysaccharide (pH 2). The zeta potential was measured and compared according to pH to check the stability of the particles in more detail (**Fig. 18**).

Table 7 shows the particle size of each smaple according to pH. The particle size increased to such an extent that measurement was impossible under the condition of coagulation. There was no significant difference in the pH of the same sample ($p<0.05$). On the other hand, when comparing the average value of

each sample, the particle coated with pectin without sodium caseinate (ZP) was the largest, and the particle using only zein (Z) was the next largest. It appears that the size of the particles increases as the pectin attaches to the surface of the zein. On the other hand, the particles containing sodium caseinate (ZSc, ZScP, ZScA and ZScC) were smaller (about 500 nm) than the other particles and there was no significant difference between these four samples ($p<0.05$). Therefore, it can be seen that when making zein based particles, sodium caseinate acts to keep the particle size from being increased by preventing the increase of size by the polysaccharide coating.

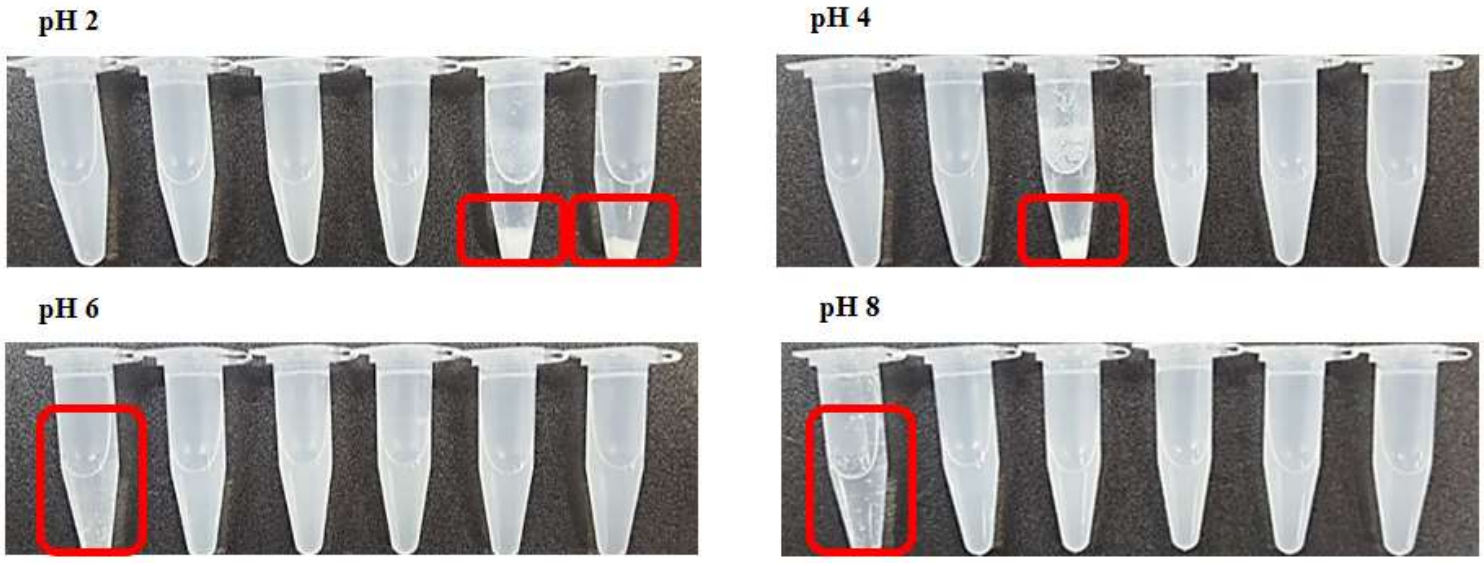


Figure 17. pH stability of sample (from left; Z, ZP, ZSc, ZScP, ZScA and ZScC) according to pH condition (pH 2, 4, 6 and 8)

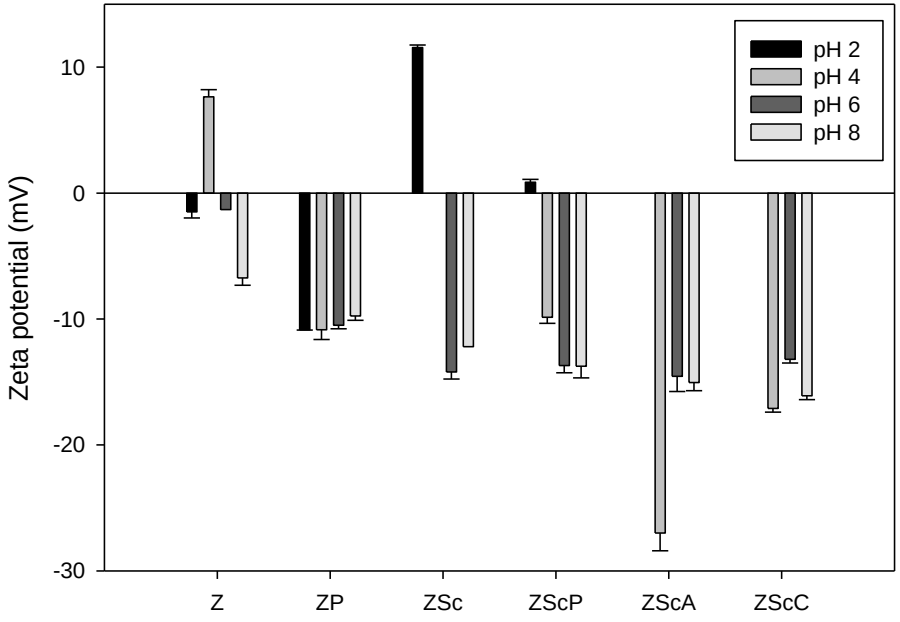


Figure 18. The zeta potential (mV) value of protein based particles according to pH condition.

	Z	ZP	ZSc	ZScP	ZScA	ZScC
pH 2	695±68.4	826±33.9	551.1±37.6	619.4±36.0	-	-
pH 4	644.4±15.7	833.05±66.0	-	416.55±12.9	553.65±5.9	411±10.6
pH 6	-	870.85±69.4	499.5±42.4	520.55±45.0	557.8±30.8	517.45±49.2
pH 8	-	865.15±34.6	463.05±27.7	515.4±35.6	495.5±53.8	502.9±20.1
Average	669.7±35.8*	848.8±22.5**	504.6±35.9	518.0±32.4	535.7±34.8	477.1±26.6

Table 7. The particle size (nm) of protein based particles according to pH condition.

- means that measurement is impossible.

Figure 19 shows the zeta potential values of ZScP particles and individual ingredients according to pH. Zein (Z) and sodium caseinate (Sc) exhibited zero charge (Z; pH 6.2, Sc; pH 4.6) at pH close to the isoelectric point due to their protein characteristics and aggregation occurred due to weak electrostatic interactions between particles. On the other hand, in the case of pectin, the charge was close to 0 near the pKa value due to the nature of the anionic polysaccharide, but no aggregation occurred on the colloid. When a pectin is added to a zein particle without a stabilizer (ZP), it changes in the same manner as the pectin charge depending on the pH. This means that without a stabilizer, the polysaccharide can be attached to the surface of the zein core by electrostatic interactions. It is noteworthy that sodium caseinate is added as a stabilizer and pectin is added to make core-shell structure particles (ZScP). In the case of this particle, the charge is similar to that of sodium caseinate when the pH is lower than pH 2, which is close to the pKa of pectin. When the pH is above 2, the charge is almost the same as that of pectin. This effect can be attributed to protonation of the carboxyl group (-COOH) on the polysaccharide molecules when the pH is reduced below their pKa value. Therefore, the polysaccharide molecules desorbed from the zein nanoparticle surfaces due to a reduction in electrostatic attraction. As a result, it can be seen that the core-shell structure based on zein is stable at pH where the anionic polysaccharide has a negative charge, ie at a pH higher than pKa. All three polysaccharides used in this study had the same results (Sodium alginate and Sodium carboxymethyl cellulose were not shown).

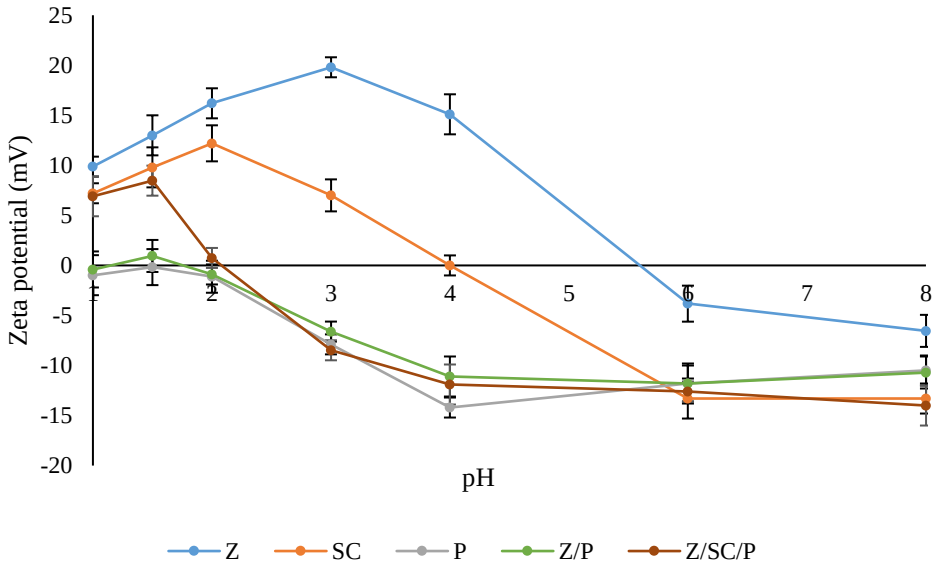


Figure 19. The zeta potential (mV) of protein based particles using pectin according to pH change

5.2.2.2. UV stability

Each sample was irradiated with UV to confirm the residual amount of retinol contained in the particles, and the stability against UV was confirmed (**Fig. 20**). As mentioned earlier, the most stable particles should be used, so Z and ZP without sodium caseinate are made at pH 2 and the remaining samples are at pH 6. As a result, When the UV irradiation time was within 2 hours, the retinol in the protein particle decreased sharply. Retinol was most rapidly degraded in plain zein particle (Z) that was not coated but all samples were more stable than control. The particles coated only with pectin without stabilizer (ZP) showed the next lowest result. The residual amount of retinol by UV irradiation was significantly higher in particle made of sodium caseinate and pectin (ZScP) ($p<0.05$). Z, ZP and ZScA showed aggregation after 2 hours of irradiation with UV and were unstable visually. Therefore, UV stability is found to be stable in the presence of stabilizers coated with pectin.

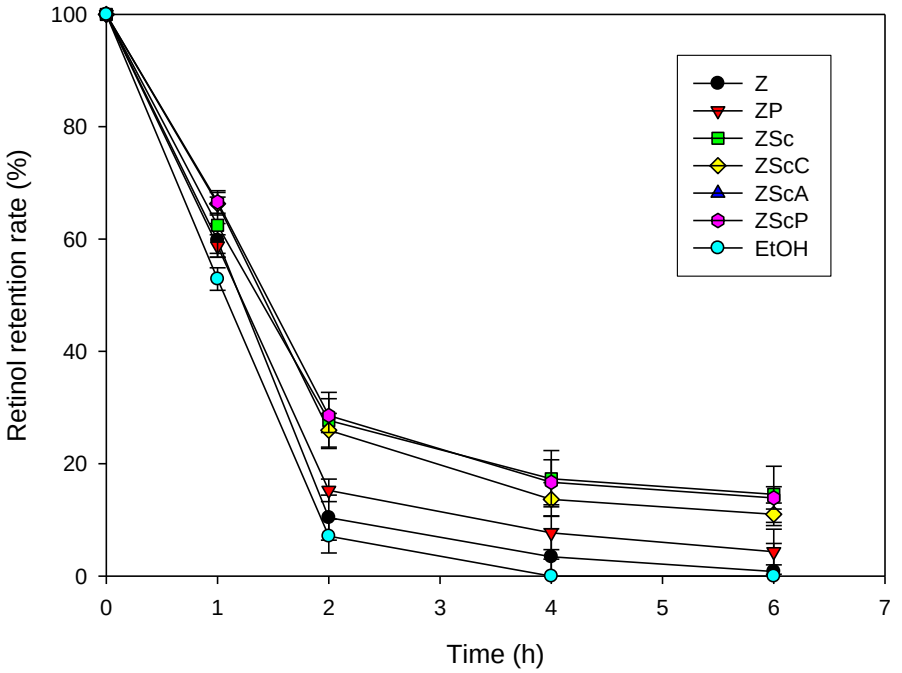


Figure 20. UVA stability of retinol in protein based particles (Z, plain zein particles; ZP, Zein/pectin; ZSc, Zein/Sodium caseinate; ZScP, Zein/Sodium caseinate/Pectin; ZScA, Zein/Sodium caseinate/Sodium alginate; ZScC, Zein/Sodium caseinate/sodium carboxymethyl cellulose)

5.3. Characteristics and stability of inclusion complexes of retinol and cyclic glucans

5.3.1. Characteristic of retinol inclusion complexes

5.3.1.1. Phase solubility

The phase solubility between retinol and cyclamate (CA) or cyclodextrin (CD) is shown in the **Figure 21**. When the amount of retinol is fixed and the concentration of host molecule is increased, the concentration of retinol increases linearly with increasing concentration of CA. On the other hand, when CD is 0.2%, the concentration of retinol is maximal and thereafter decreases. The phase solubility diagram can be divided into two types (A and B type). The A-type is a soluble inclusion complex and the B-type is a complex with a low solubility (Del Valle, 2004). The CA used in this experiment is A_L-type which is concentration-dependent when host and guest are combined 1: 1 in A-type. On the other hand, among the B-type CD, the CD is seen as a B_S-type in which the guest is increased in a concentration-dependent manner to a certain concentration of the host. In general, it is known that non-chemically modified β -CD exhibits B-type due to low solubility in water (Del Valle, 2004). The solubility of retinol gradually decreases, suggesting the formation of a less soluble complex, perhaps at a 1: 2 molar ratios. In other words, the β-CDs of two molecules encapsulate the retinol of one molecule. Based on these phase solubility results, we determined the concentration of the host molecule to be used for characterization and stability analysis of inclusion complex.

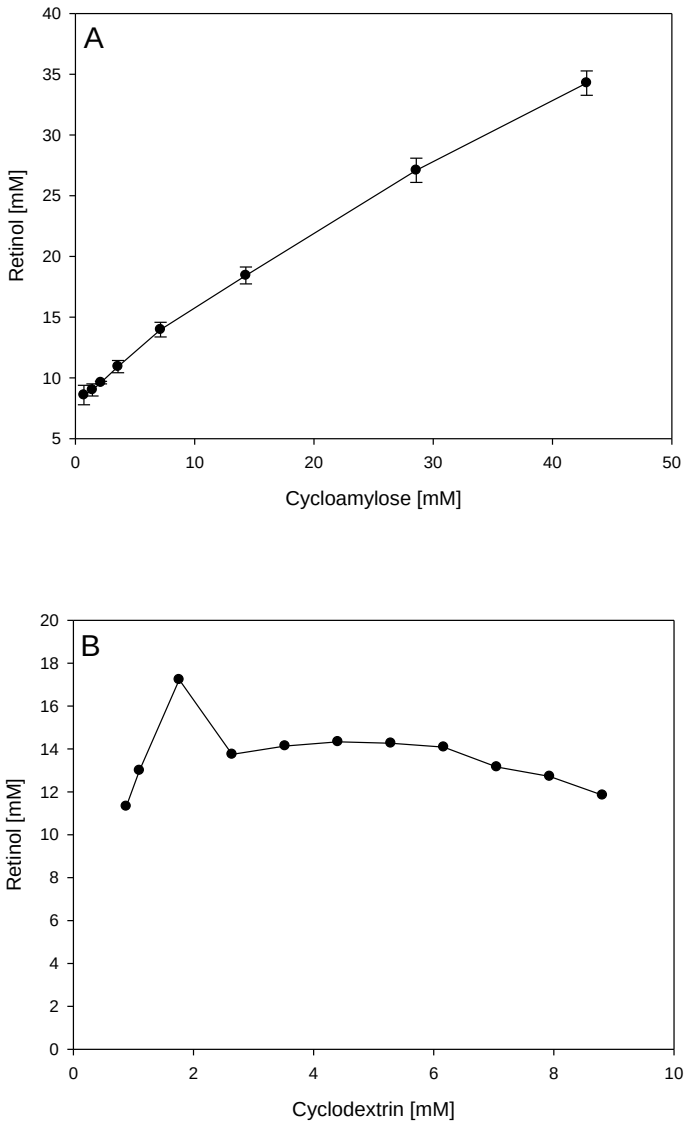


Figure 21. Phase-solubility of retinol in a mixture water/EtOH (95:5 v/v) containing increasing concentration of CA and CD, at ambient temperature.

5.3.1.2. Differential Scanning Calorimeter (DSC)

In order to identify inclusion complexes of cyclamylose (CA) and retinol, thermal characteristics were analyzed using a differential scanning calorimeter (**Fig. 22**). In the pure retinol, there was a endothermic peak around 55℃, which has previously been attributed to melting of retinol crystals. The physical mixture also showed an endothermic peak near 55 ° C, which suggests that the retinol was in a crystalline form in the physical mixtures. On the other hand, the inclusion complex did not show retinol peaks and the CA similar pattern was measured. This means that all of the added retinol was encapsulated inside CA.

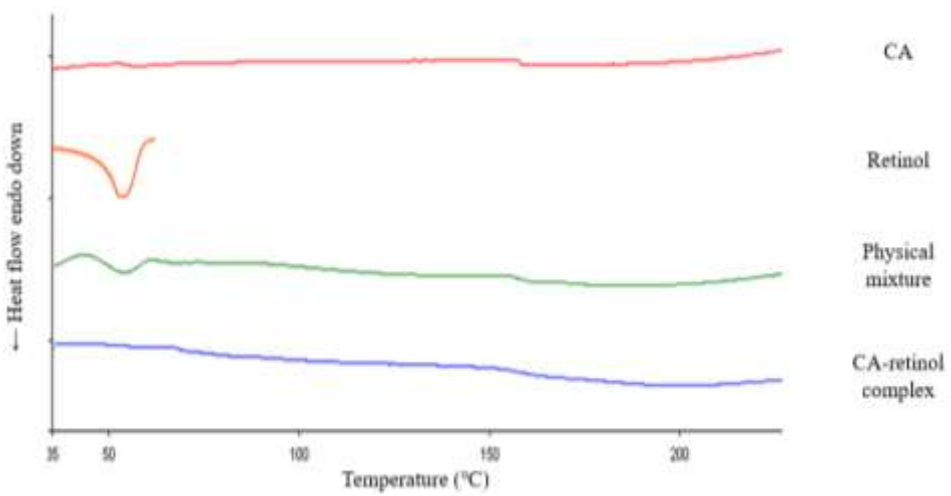


Figure 22. DSC curve of retinol inclusion complex

5.3.1.3. Scanning Electron Microscopy (SEM)

Morphological characteristics of cycloamylose (CA)-retinol inclusion complex were analyzed using SEM (**Fig. 23**). Figures 23A, B and C show the CA, retinol and physical mixture, respectively, at the same magnification (500X). CA showed a fraction type structure which is characteristic of polysaccharides. Retinol showed fraction smaller than CA and both materials were observed in physical mixture. Figure 23D, on the other hand, is a CA-retinol inclusion complex with a 30KX magnification. The size of the particles became much smaller and the shape changed into a perfect spherical shape. Experiments were done at different concentrations of retinol to determine if the particle shape was due to retinol. (**Fig. 24).**

In Figure 24 is 30 KX images and the inclusion complex has retinol concentrations of 0, 5, 10, 50, 100 and 200 ppm. As a result, the particle size was changed according to the concentration of retinol but the shapes were all the same. The particle size of CA was the smallest at about 800 nm ($p < 0.05$). The larger the retinol concentration in the CA, the larger the particle size (**Table 8**). Zeta potential showed a small absolute value when retinol was not added and there was no difference according to the amount of retinol added. This suggests that retinol embedded in the CA has a charge on the surface of the inclusion complex, but there is no charge change depending on its amount.

Next, I compared the three host molecules to see why the particle shape is spherical. **Figure 25** is a SEM image of particles lyophilized after dissolving CA,

CD (cyclodextrin), and MD (maltodextrin) in pure water and 5% EtOH, respectively. When three kinds of host materials are dissolved in water and dried, the same fraction type as polysaccharide is observed (A1-CA, B1-CD, C1-MD). On the other hand, when dissolved in 5% EtOH and dried, CA and MD are spherical particles. The solubility of water in the beta-CD used in this experiment is much lower than that of CA and MD, and the solubility is maximally increased in the aqueous mixture containing alcohol (Blackwood & Bucke, 2000; Chatjigakis, Donze, Coleman, & Cardot, 1992). On the other hand, CA and MD have low solubility to organic solvents in terms of structure. In this experiment, the host material is dissolved in water, and 95% EtOH is added to make a final concentration of 5%. At this time, water has more affinity with alcohol than host material, so CA or MD forms spherical shape and maximizes solubility. Therefore, the particle size and charge of the CA-retinol inclusion complex are influenced by the retinol contained inside, and its shape is due to the solubility of CA.

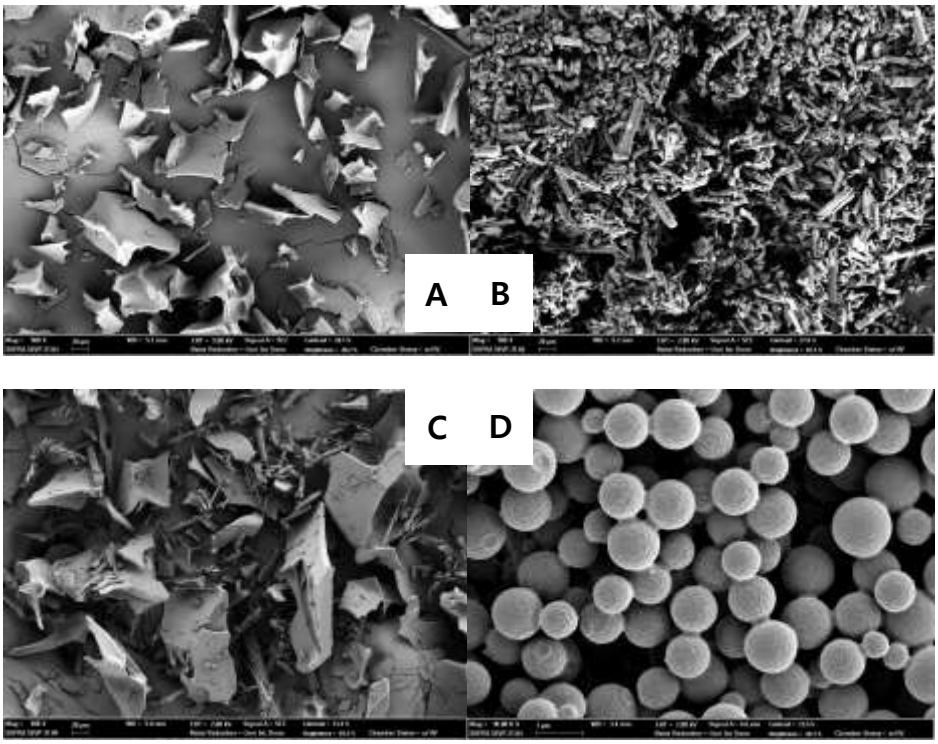


Figure 23. SEM image of retinol inclusion complex. A: cycloamylose; B: pure retinol; C: physical mixture of retinol-CA; D: retinol-CA inclusion complex.

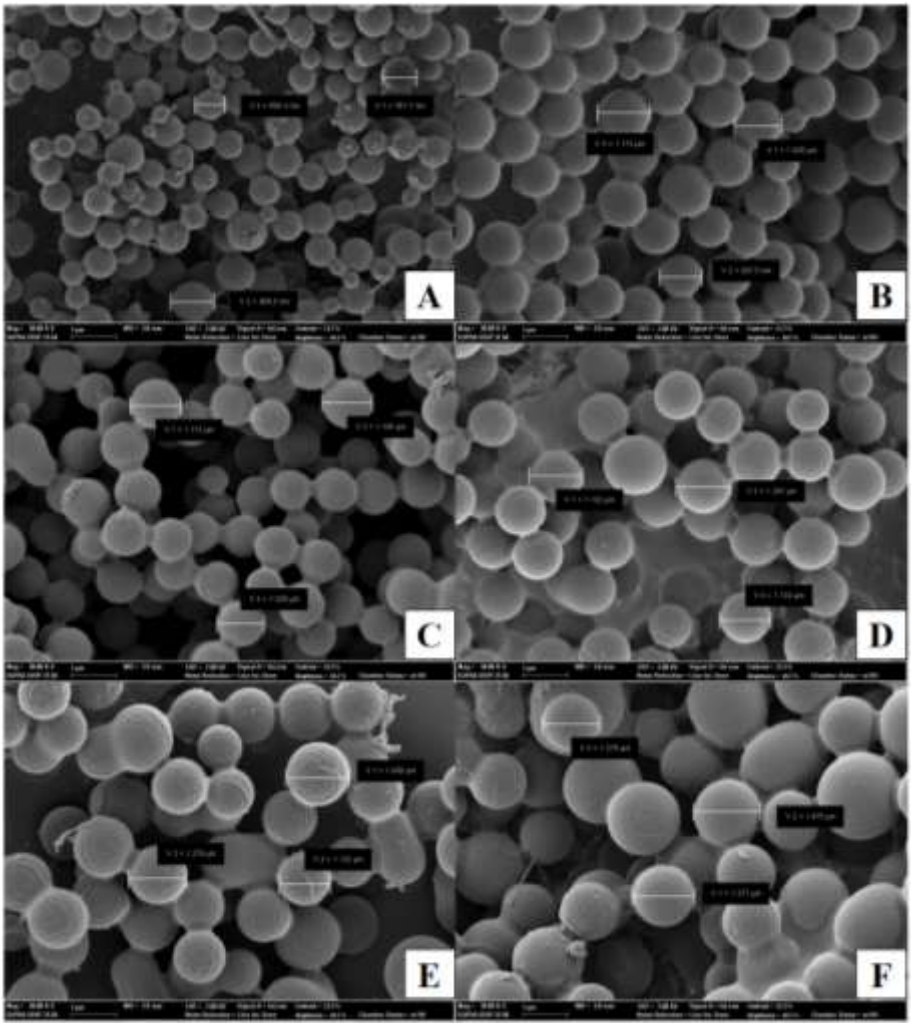


Figure 24. SEM image of retinol inclusion complex using different concentration of retinol (A: 0ppm; B: 5ppm; C:10ppm; D:50ppm; E:100ppm, F: 200ppm retinol)

Retinol concentration (ppm)	0	5	10	50	100	200
Particle size (nm)	794.3±158.8[a]	1048.2±117.8[b]	1074.0±42.7[b]	1172.0±35.1[bc]	1269.3±141.7[bc]	1377.0±98.0[c]
Zeta potential (mV)	-18.5±4.9*	-22.4±3.8	-23.9±3.9	-22.7±2.0	-30.1±3.2	-26.2±0.1

Table 8. Particle size and zeta potential of retinol-CA inclusion complex using different concentration of retinol.

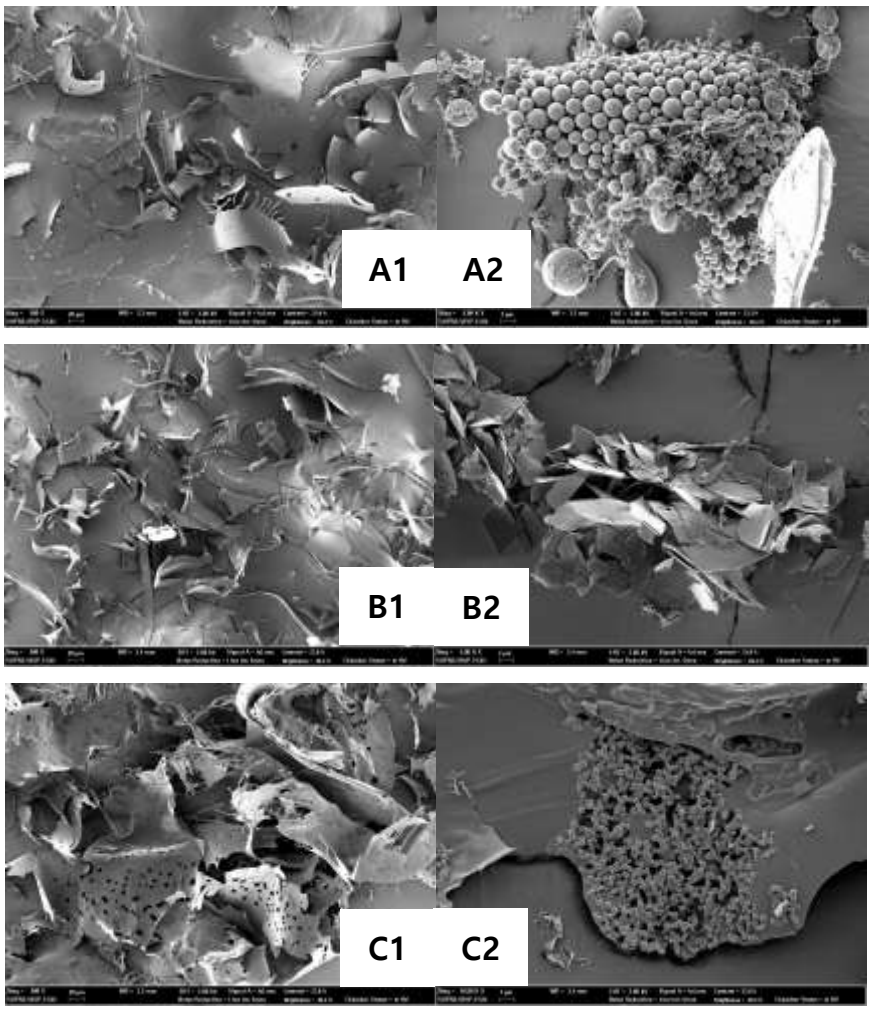


Figure 25. SEM image of different host material in different concentration of ethanol. A, cycloamylose; B, cyclodextrin; C, maltodextrin. 1 is host material in 0% ethanol and 2 is host material in 5% ethanol.

5.3.1.4. Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra of the inclusion complexes, the individual ingredients used, and the physical mixture are shown in the **Figure 26**. Retinol has O-H and C-H stretching peaks (3253 and 2928 cm^{-1}) and has a CH_3 bending peak at 1441 cm^{-1}. Characteristically, it represents a trans out-of-plane bend at 965 cm^{-1} (Kim, Hu, Jeong, Jun, Cho, & Jung, 2016). The most interesting signal of cycloamylose is wide band at 3349-3310 cm^{-1} due to O-H bonds stretching. Peak at 2927-2925 cm^{-1} means stretching vibrations of the bonds in -CH and -CH_2 groups. The peak at 1024-997 cm^{-1}, which represents a strong signal, is due to C-O-bonds stretching.

In the physical mixture, retinol and CA peaks were observed. Among them, trans-out-of-plane bend at 965 cm^{-1} of retinol was the strongest. On the other hand, the inclusion complex was the same as the peak of CA as a whole. It seems that retinol binds to CA cavity and conceals its structure. The peaks indicating O-H bond stretching and C-O-bond stretching were shifted to the right (from 3349 to 3332 cm^{-1} and from 1024 to 997 cm^{-1}).

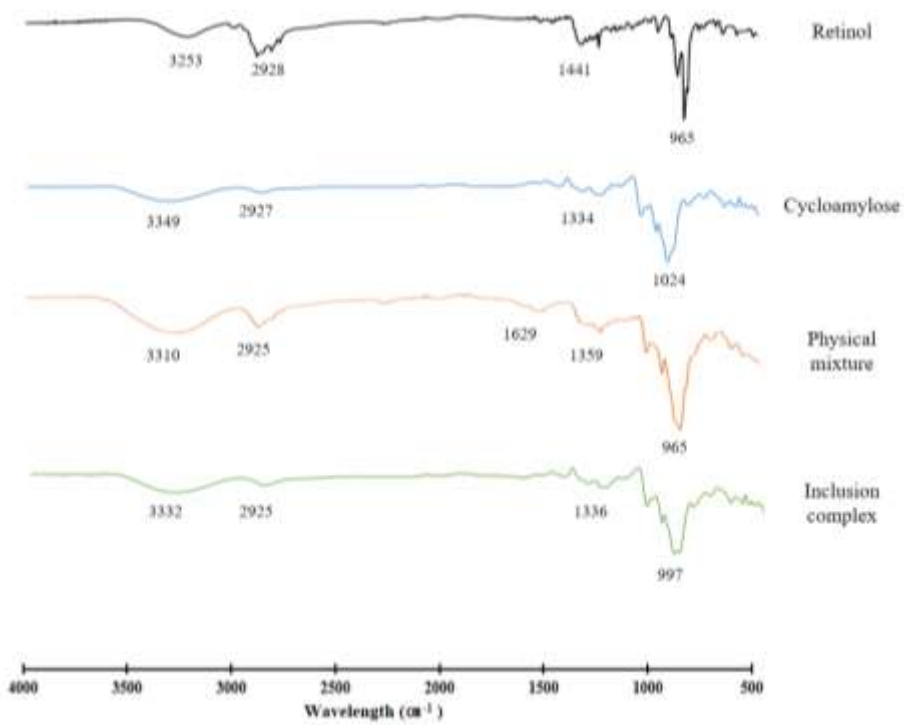


Figure 26. FT-IR spectrum of inclusion complex and individual ingredients

5.3.2. Stability of retinol in inclusion complex

5.3.2.1. UV stability

Each sample was irradiated with UVA to confirm the retention rate and residual amount of retinol contained in cycloamylose (CA) and cyclodextrin (CD) against UV was confirmed. CA showed a completely constant retinol retention rate regardless of its concentration (**Fig 27**A1). When the absolute value of retinol was compared, the amount of retinol was significantly increased as CA concentration increased ($p < 0.05$) (**Fig 27** B1). On the other hand, in the case of CD, the retention rate of retinol tended to decrease significantly as CD concentration increased (**Fig 27** B1). Comparing quantitative residuals, retinol content was the highest at 0.2% CD concentration and decreased at higher concentrations (0.5 and 1.0%) (**Fig 27** B2). These results can be explained in relation to the phase solubility mentioned in 5.3.1.1. When the same amount of retinol was encapsulated, the concentration of retinol was increased with increasing concentration of CA, whereas the retinol content was maximal when CD was 0.2%, and decreased at higher concentration (**Fig 21**-phase solubility). This result shows that CA is a complex of A-type, so that the host CA and the guest retinol are stable with 1: 1 binding. Therefore, as a result of UV stability analysis, although there is an absolute amount of retinol according to CA concentration, it is a stable complex, so there is no relative difference. CD-retinol inclusion, on the other hand, is a B-type complex, meaning that the host and

guest do not bind 1: 1 and are in an unstable state. Thus, in the case of CD, as with the phase solubility results, the absolute amount of retinol is most stable and stable when the CD concentration is 0.2%. At higher concentrations, CD and retinol form an unstable complex, so retinol is reduced faster in UV conditions and relative differences are also seen.

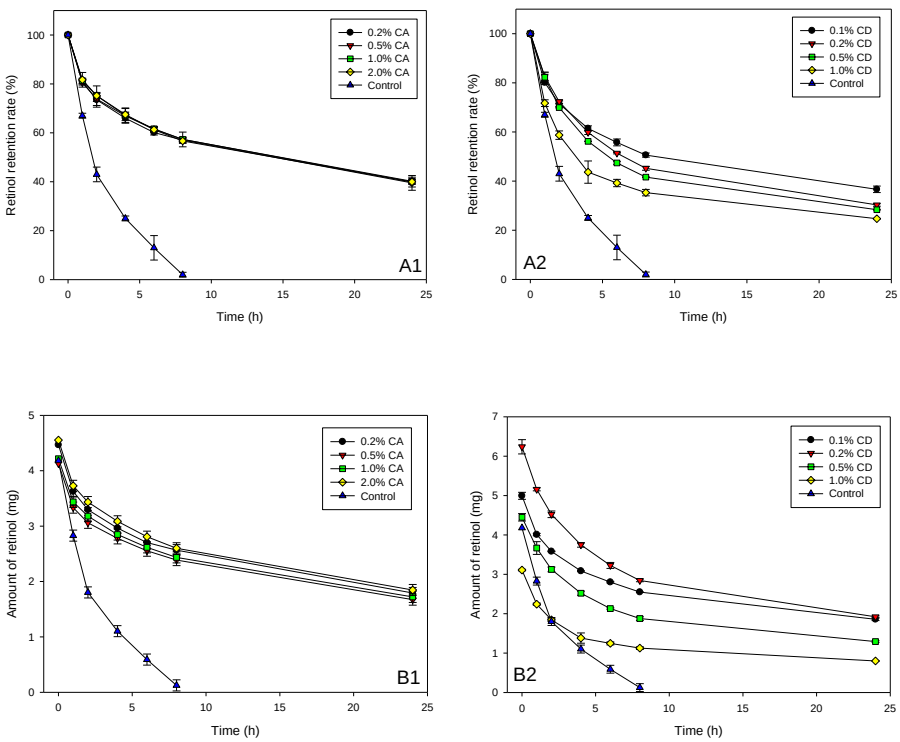
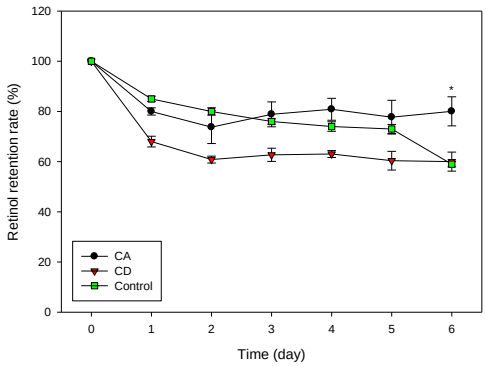
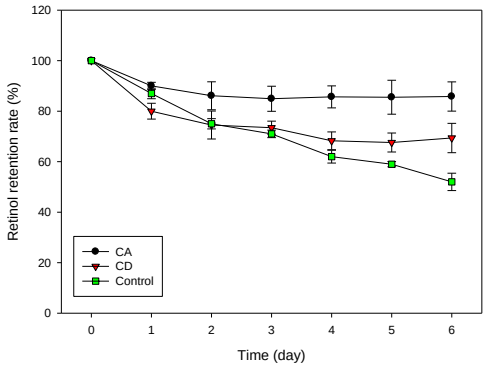


Figure 27. Photo stability of retinol in inclusion complex using different concentration of host materials.

5.3.2.2. Storage stability at different temperature

Figure 28 shows the storage stability of retinol inclusion complexes with host molecule concentration of 0.2 wt%. Regardless of storage temperature, residual amount of retinol was significantly higher when cycloamylose was used as a host molecule ($p < 0.05$). At 4°C, inclusion complex with cyclodextrin showed lower retinol residue than control, suggesting that cyclodextrin decreased solubility at refrigeration temperature. At room temperature, the residual amount of cyclodextrin is similar to that of control, but there is a significant difference from 5 days after. Pure retinol was 100% degraded for one week at 40°C, while all inclusion complexes were found to be more stable than control.





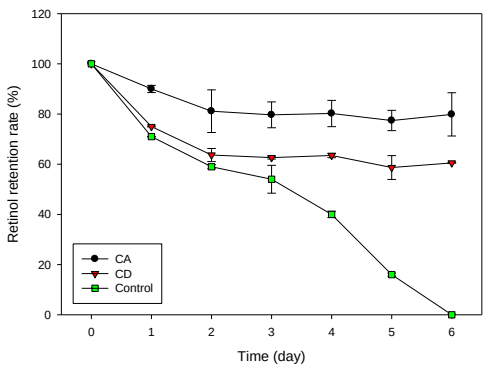


Figure 28. Retention rate (%) of retinol in inclusion complex according to host molecule (CA and CD) and storage temperature (from top to bottom; 4, 25 and 40℃)

5.4. Comparison of retinol stability using different delivery system

5.4.1. Stability of retinol in different delivery system

Previously, three delivery systems were used to confirm that retinol remained stable according to UV and storage temperature. Among them, the most stable conditions for each delivery system were confirmed. The O/W emulsion (10wt% oil emulsion) was prepared using a non-ionic emulsifier, tween20. Protein-based particles were coated by pectin. Cycloamylose was used in 0.2wt%, and each delivery system contained total 0.01wt% of retinol to compare the retention rate.

A comparison of UV stability of retinol showed that all delivery systems retained retinol more than control (retinol in ethanol) (**Figure 29**). O/W emulsion increased the retinol retention rate 40-fold. Protein-based particle increased 15-fold and cycloamylose increased the retinol retention rate 25-fold. Therefore, O/W emulsion is the most efficient delivery system to enhance the UV stability of retinol.

The comparison of the stability of retinol to storage temperature is shown in **Figure 30**. At 4 °C, both O/W emulsion and cycloamylose increased the retinol retention rate by about 1.4-fold compared to the control and increased about 1.6-fold at 25 °C. At 40 °C, retinol retention rate increased by 3-fold in O/W emulsion and 5-fold in cycloamylose. Therefore, when storage temperature is high, it is advantageous to use cycloamylose as delivery system.

Storage stability was analyzed in liquid phase. Protein-based particles were precipitated in a short time when they were dispersed in aqueous solution. For this reason, protein-based particles excluded from this analysis.

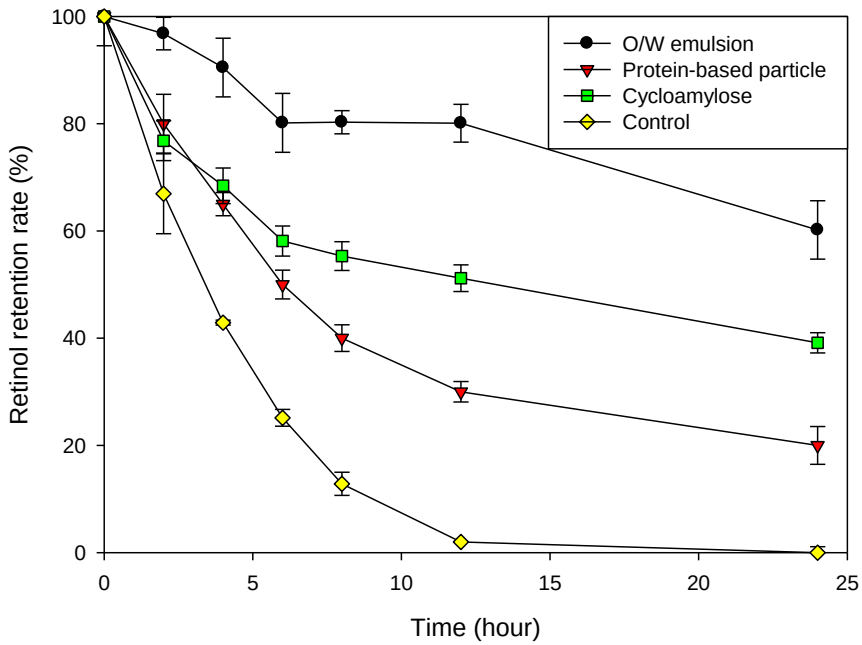


Figure 29. UV stability of retinol incorporated O/W emulsion, protein-based particle and cycloamylose

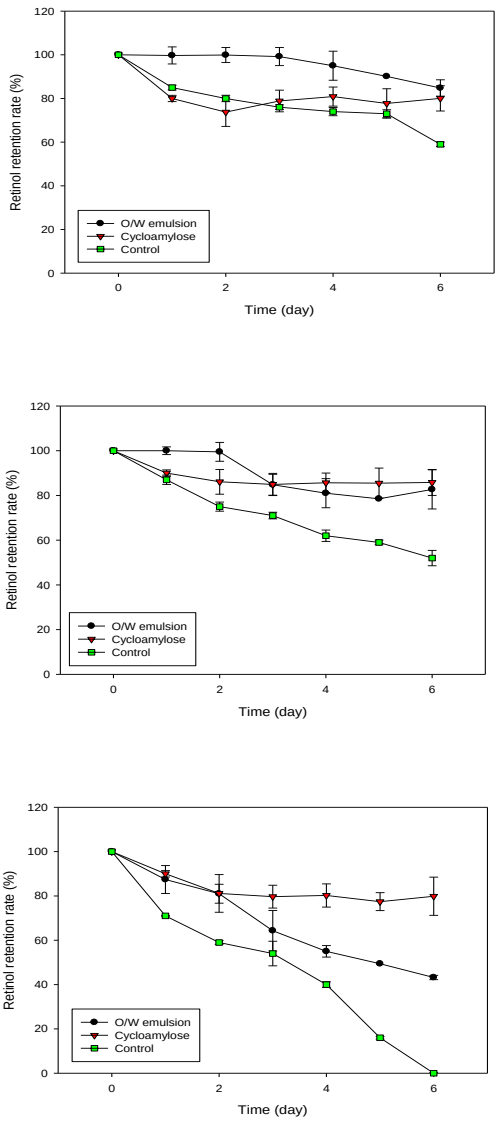


Figure 30. Storage stability of retinol incorporated O/W emulsion and cycloamylose at different temperature (top: 4°C, middle: 25°C, bottom: 40°C)

5.4.2. Bioaccessibility analysis of retinol in different delivery system

Before measuring the bioaccessibility of retinol in each delivery system, retinol retention rate was confirmed during the digestion phase (**Figure 31**). The digestion phase consists of oral, gastric and intestine phase.

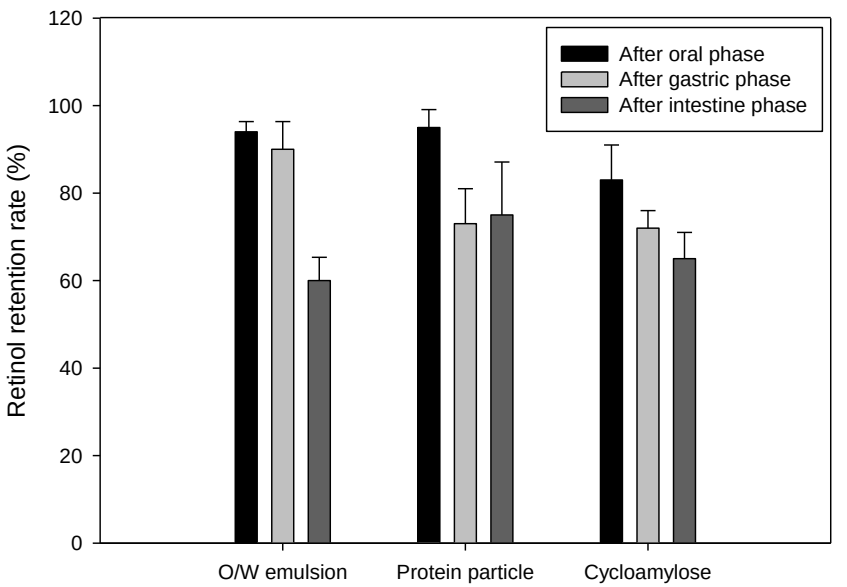


Figure 31. Retinol retention rate of each digestion phase using O/W emulsion, protein-based particle and cycloamylose

Retinol in the O/W emulsion did not differ from the initial amount even after the oral and gastric phases. However, after the intestine phase, the residual amount decreased sharply. The enzyme used in the intestine phase, pancreatin, contains a lipase that digests fat. Therefore, it seemed that oil was decomposed at this stage, and retinol which was present in oil was oxidized out of the aqueous phase, and the residual amount was rapidly decreased.

The retinol in the protein particles was similar to the initial amount even after the oral phase but decreased after the gastric phase. These results suggest that the protein degraded by pepsin used in the gastric phase. On the other hand, there was no change in the residual amount of retinol after intestine phase.

Retinol incorporated in cycloamylose decreased in the oral phase, unlike the previous two delivery systems. The α-amylase in the oral phase breaked down the α-1,4-glycosidic bond. So, it can decompose cycloamylose. In addition, the retinol retention rate decreased after the intestine phase, which suggests to be due to α-amylase in pancreatin.

The bioaccessibility of retinol in the delivery system optimized for retinol stability is shown in the **Figure 32**. O/W emulsion, protein-based particle and cycloamylose were prepared under three different conditions (**Table 9**) to compare the bioaccessibility of retinol. Pure retinol had the lowest bioaccessibility (30.2 ± 1.9%). The O/W emulsion showed no significant difference by emulsifier, and the average value was 52.2% (T20: 51.4 ± 5.3%, DM: 52.0 ± 5.0%, WPI: 53.1 ± 12.7%). Protein-based particles showed the

highest bioaccessibility of retinol (above 80%) when sodium caseinate was used and did not change significantly when pectin was added (ZSc: 82.6±12.9%, ZScP: 80.5±14.2%). On the other hand, when zein was used without stabilizer and coating agent (Z), it was 46.3%, which was higher than pure retinol but the difference was the least. When using the inclusion complex, the bioaccessibility of retinol was not significantly different by about 75% regardless of the cycloamylose concentration. (0.2%: 68.9±9.9%, 0.5%: 79.8±1.6%, 1.0%: 78.1±3.3%). Thus, using three delivery systems can increase the bioaccessibility of retinol, and protein-based particles were most effective.

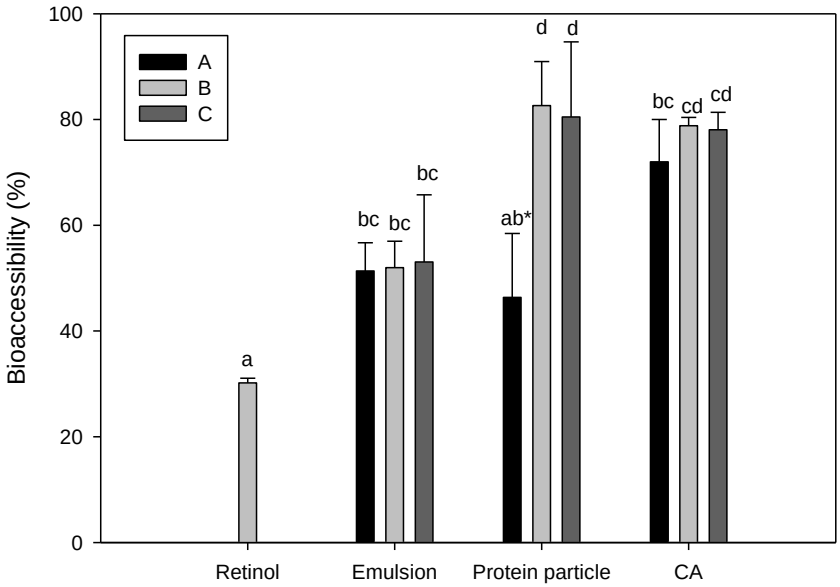


Figure 32. Bioaccessibility of retinol in delivery system (O/W emulsion, protein-based particles and cycloamylose)

	A	B	C
Type of emulsifier	T20	DM	WPI
Type of protein particle	Z	ZSc	ZScP
CA concentration	0.2%	0.5%	1.0%

Table 9. Conditions of each delivery system

6. Conclusions

1) Characteristics of retinol-loaded delivery system

- O/W emulsions can be applied to food only in liquid phase. Protein-based particles and cycloamylose can be used in both liquid and powder form. However, protein-based particles are very unstable due to precipitation in the liquid phase. Therefore, the powder state is advantageous when applied to foods.

2) Stability of retinol in delivery system

- During UV irradiation (12 h), the retinol retention rate was 80% within the O/W emulsion, 30% within the protein-based particle and 50% within the cycloamylose. Previous study using solid lipid nanoparticle (SLN) has shown that 40% of retinol remains (Semenova, Cooper, Wilson, & Converse, 2002). Therefore, O/W emulsion and cycloamylose can effectively protect retinol from UV. However, since protein-based particles were vulnerable to UV, they must block light when applied to foods.
- The stability of retinol according to storage temperature is as follows. In previous studies, 60-80% retinol remained at 4 °C, 40-60% at 25 °C and 20-40% at 40 °C (Ko & Lee, 2010; Lee, Lee, Kim, & Lim, 2005; Luo, Zhang, Whent, Yu, & Wang, 2011). On the other hand, retinol contained in O/W emulsion and cycloamylose

remained 80%. When stored at 40 °C, 40% retinol remained in the O/W emulsion and 80% remained in the cycloamylose. Therefore, retinol was protected similar to conventional system at refrigeration temperature, and O/W emulsion and cycloamylose were advantageous at room temperature. And at 40°C, cycloamylose most effectively protected retinol. Therefore, cycloamylose can be used for foods that require heating.

- Bioaccessibility of retinol increased 2-4 folds in each delivery system.

In conclusion, the solubility of retinol increased in all delivery systems, and stability and bioacessibility increased. Therefore, the delivery system of this study can help improve the stability of retinol in foods, cosmetics, and pharmaceuticals.

O/W emulsions in liquid form can be applied to beverages, when they are stored at low temperature (below 25 °C). Protein-based particles can be used as a powder form and relatively stable without UV exposure. Also, it can be used as a seasoning ingredient which is not heated because it is vulnerable to high temperature. Cycloamylose can be used as a liquid or powder forms, and relatively stable for high temperature application such as baking, heating and cooking.

7. Summary

Retinol, a fat-soluble vitamin A, has excellent efficacy in anti-aging, anti-cancer and anti-inflammation. However, it is unstable at room temperature and easily decomposed in UV, heat and pH, so it is difficult to use in food. Therefore, this study aims to develop a delivery system to improve the stability and bioaccessibility of retinol. O/W emulsion, protein-based particles, and cycloamylose to optimize the conditions for stabilizing retinol and analyze bioaccessibility through in vitro digestion.

O/W emulsion was prepared to increase the stability and solubility of fat soluble vitamin Retinol, which is easily oxidized by oxygen, heat and light and has low solubility in water. Comparison of the storage and UV stability of retinol-loaded O/W emulsions prepared with different emulsifiers (tween 20 (T20), decaglycerin myristate (DM) and whey protein isolated (WPI)) and oil concentrations (0.1, 0.3, 0.5, 1, 2, 4 and 10 wt%) saw. The average droplet size was about 200 nm, which did not show any significant difference depending on the type of emulsifier and the concentration of oil. Zeta potential, which measures surface charge, has the largest absolute value of WPI, anionic emulsifier, than the absolute values of T20 and DM, which are non-ionic emulsifiers. Note that when the same emulsifier is used, the absolute value of the zeta potential decreases as the oil concentration increases. Because the amount of retinol entering the entire system is fixed, the density of retinol in the oil decreases as the oil concentration increases. Thus, the decrease in the absolute

value of zeta potential means that retinol is distributed on the surface of the oil droplet and affects the charge of the particle.

Comparing the stability, the UV stability was more affected by the concentration of oil than the type of emulsifier. Since the turbidity increases as the oil concentration increases, the UV stability of retinol in the O/W emulsion depends on the turbidity. On the other hand, stability according to storage temperature was influenced not only by oil concentration but also by type of emulsifier. The reasons for the different factors that affect stability depend on the storage period. Light stability is stored for a relatively short period of time (24 hours), but storage stability is preserved for a long time (7 days) and the retinol residue is confirmed. The longer the storage time of the O/W emulsion is, the more the oxidation is affected in the emulsion. Factors that promote the oxidation of O/W emulsions include oil concentration, type of emulsifier, and pH. The lower the oil concentration, the more the oxidation by unsaturated fatty acids is promoted and the type of emulsifier is decreased by using WPI which is anionic emulsifier. This is because the negatively charged droplet easily combines with iron ions present in the aqueous phase at neutral pH to promote oxidation. To confirm this, retinol storage stability in O/W emulsion with the addition of EDTA under the same conditions was examined and the residual amount of retinol was increased up to 40%. This study provides important information for designing effective emulsion-based delivery systems for improving the stability of retinol.

Protein-based particles were used as a second delivery system to increase

retinol stability and solubility. Coat the protein-based particles with a stabilizer and a polysaccharide to make stable particles. The protein was made from the corn protein zein, which is a hydrophobic amino acid. Sodium casinate was used as a stabilizer and coated with polysaccharide (pectin, sodium alginate and sodium carboxymethyl cellulose).

The average size and zeta potential were measured to characterize the particles. In the case of particle size, the size does not change even with the addition of the stabilizer sodium caseinate, but when the polysaccharide is coated, the size of the particles increases and there is no difference in size depending on the type of polysaccharide. In all cases, particles with retinol were significantly reduced in size. This means that retinol and zein are bonded via hydrophobic interaction at the innermost part of the particle, thus reducing the overall size. The charge of a plain zein particle has a positive charge, but the particles coated with a stabilizer and a polysaccharide all have a negative charge. When the sample was lyophilized, particles that did not contain the stabilizer did not become a powder, and other samples were redispersed to increase particle size. It can be seen that freeze drying makes the particles unstable. On the other hand, there was no change in zeta potential value after freeze drying. Afterwards, we confirmed that protein-based particles contained retinol using DSC, FT-IR and SEM.

Particle yield (%) was higher in the sample containing stabilizer and polysaccharide (ZScP, ZScA and ZScC), and retinol loading efficiency (%) was more than 90% except for Z and ZP. Therefore, it can be seen that the coating

agent affects the particle yield and the stabilizer is the factor affecting the loading efficiency.

As a result of comparing the stability, the pH stability of the sample coated with pectin in the presence of the stabilizer (ZScP) was the most stable. The change of charge according to the pH range shows that charge of ZScP and pectin changes in a similar pattern, which means that zein particles are coated with pectin. It is noteworthy that at a pH lower than the pKa value of pectin, it changed similar to the charge of sodium caseinate, but at a higher pH it changes in a similar manner to that of pectin. Thus, it can be seen that particles coated with polysaccharide are most stable at pH above pKa. The UV stability of retinol was higher in the polysaccharide coated particles in the presence of the stabilizer (ZScP, ZScA and ZScC) than in the plain zein particles (Z), and there was no difference in the type of polysaccharide. However, when compared to the residual amount for the same time, the disadvantage is that the protein-based particle is 20% compared to 60% retinol remaining in the O/W emulsion.

The third delivery system is a retinol inclusion complex with cyclic glucan. Cyclic glucan types were compared between cycloamylose (CA) and cyclodextrin (CD). The retinol concentration was fixed and the phase solubility was confirmed by increasing the concentration of the host molecule. The solubility of retinol was the highest at 0.2 wt% of CD, and the solubility of retinol was increased as the concentration of CA increased. This means that CA and retinol are A_L-type binding at 1: 1 ratio and CD is B_s-type. Formation of retinol

inclusion complex was confirmed by DSC, FT-IR and SEM.

UV stability showed that the residual amount of retinol was higher in the inclusion complex using CA as a host molecule. CA did not affect the residual amount of retinol even when its concentration increased. However, when 0.2wt% of CD was used, retinol was most stable, which is also the highest value of phase solubility. Storage stability also showed higher residual retinol in inclusion complex using CA for all temperatures. In the case of CD, the storage stability at room temperature and 40 ℃ was more stable than control at 3 days, but it was rather unstable at 4 ℃. This is due to the characteristic of the CD itself, which at low temperatures has a low solubility of CD to water and therefore also affects the retinol contained inside.

The bioaccessibility of retinol was compared using optimized O/W emulsion, protein-based particle and cycloamylose. O/W emulsion (10wt% oil emulsion) was determined according to the type of emulsifier (tween 20, decaglycerin myristate and whey protein isolated), protein-based particles according to stabilizer and coating agent and inclusion complex according to cycloamylose concentration. O/W emulsion was more than 50% (T20: 51.4 ± 5.3%; DM: 52.0 ± 5.0%; WPI: 53.1 ± 12.7%). Protein-based particles (ZSc: 82.6 ± 12.9%, ZScP: 80.5 ± 14.2%) showed a value of more than 80% in the case of zein alone (46.3%). Inclusion complex showed about 70% retinol remained, which was not significantly different according to the concentration of cycloamylose (0.2%: 68.9 ± 9.9%; 0.5%: 79.8 ± 1.6%; 1.0%: 78.1 ± 3.3%). Considering that the

bioaccessibility of pure retinol is about 20%, it can be seen that all three delivery systems deliver retinol stably at the *in vitro* level.

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레티놀 안정성과 생체접근률 향상을 위한 수중유적형 에멀션, 단백질 기반 입자, 포접 복합체의 특성

박 희 수

국문초록

레티놀은 지용성 비타민인 비타민 A로, 항노화 기능을 나타낼 뿐만 아니라 항암, 항염증과 같은 유용한 생리활성을 갖는다. 하지만 물에 대한 낮은 용해도와 자외선 및 열에 쉽게 분해되기 때문 에 식품에 응용함에 제한이 따른다. 따라서, 본 연구는 레티놀의 안정성 및 생체접근률을 증가시킬 전달시스템 개발을 목적으로 한다. 전달시스템으로는 O/W emulsion, 단백질 복합체, 환형아밀로스를 개발하고 레티놀에 최적화 시켜 안정성 및 생체접근률을 확인한다.

전달시스템의 조건은 O/W emulsion의 경우 유화제와 오일농도에 따라, 단백질 복합체는 stabilizer와 coating agent에 따라, 마지막으로 환형아밀로스는 농도에 따라 분석한다. 안정성은 UV, pH, 온도에 따른 저장안정성을, 레티놀의 잔류량으로 확인한다.

O/W emulsion내의 레티놀은 10wt% 이상의 오일농도의 조건에서 UV에 안정하다 (24시간 조사 시, 80% 잔류). Negative charge를 갖는 유화제를 사용한 조건에서는 수용액 상에 존재하는 미량의

금속이온과의 상호작용으로 인해 저장 안정성이 낮아진다 (WPI 사용시, 20% 잔류). 단백질 복합체 내의 레티놀은 다당류로 코팅한 경우에 loading efficiency가 90% 이상으로 유의적으로 높게 나타났다. pectin으로 코팅한 단백질 복합체에서 레티놀의 UV, pH안정성이 가장 높았지만 잔류량이 약 20%에 그쳤다. 환형아밀로스 내의 레티놀은 phase solubility를 확인해본결과, 1:1의 비율로 결합하고, 환형아밀로스의 농도에 따라 UV 및 저장안정성의 차이는 나타나지 않았다 (p<0.05). UV 조사 시 레티놀의 잔류량은 약 50%였고, 온도에 따라서는 약 80% 이상이 잔류했다. 최종적으로, 레티놀의 생체접근률은 O/W emulsion에서 약 50%, 단백질 복합체와 환형아밀로스는 약 80% 이상으로 나타났다. 순수한 레티놀의 생체접근률이 20%에 불과한 것을 미루어보아 안정성 및 생체접근률이 크게 증가했음을 보여준다.

본 연구결과, O/W emulsion과 단백질 복합체 그리고 환형아밀로스는 레티놀을 포접하여, 안정성과 생체접근률을 향상시켜주는 전달시스템으로 효과적이라는 것을 알 수 있다. 이와 같은 결과에 따라, 각 전달시스템은 식품 및 화장품 산업에 활용도 높은 정보를 제공할 것으로 생각된다.

주요어: 레티놀, 수중유적형 에멀션, 단백질복합체, 환형아밀로스, 생체접근률