A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Improving productivity of starch nanocrystals (SNC) using sonication and characteristics of SNC-stabilized Pickering emulsions

초음파 처리를 통한 전분 나노결정의 생산성 향상과 전분 나노결정으로 안정화한 Pickering 에멀션의 특성에 관한 연구

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농학석사학위논문

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조 명 수

조명수의 석사 학위논문을 인준함

2017년 2월

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ABSTRACT

Pickering emulsions are an emulsion stabilized with solid particles instead of using chemical emulsifier. It has excellent storage stability compared to traditional emulsion. Recently, studies for stabilizing emulsions using food-grade particles such as protein and starch have been actively carried out. However, application of the emulsions to actual foods is restricted to ice cream, foam, and whipping cream because the size of the emulsions is too large to maintain the dispersed state. In this study, Pickering emulsion with nanoscale particle size and colloidal stability was developed using starch nanocrystals of 20-50 nm level. After acid hydrolysis, starch nanocrystals were separated by centrifugation, and microparticles were treated with ultra-sonication until the temperature at which starch nanocrystals were gelatinized to further separate starch nanocrystals, which were incompletely hydrolyzed during acid hydrolysis, the yield was improved. To increase the hydrophobicity of hydrophilic starch nanocrystals, OSA-modified starch nanocrystals were prepared by esterification of starch nanocrystals with octenyl succinic anhydride (OSA). Gellan gum and polydimethyl siloxane (PDMS) were used to confirm the dual wettability of OSA-modified starch nanocrystals to both aqueous phase and oil phase. Ultra-sonic treatment of

OSA-modified starch nanocrystals-stabilized Pickering emulsions reduced the emulsion particle size and increased the emulsion efficiency of OSA-modified starch nanocrystals. Then, Pickering emulsions which has excellent colloidal stability and storage stability was developed by varying the concentration of OSA-modified starch nanocrystals. The Pickering emulsions developed in this study can be used as a basis for research on the development of food and drug carriers by adding colloidal stability to existing Pickering emulsions.

Keywords: Pickering emulsions, colloidal stability, starch nanocrystals, particle-stabilized emulsions

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I. Introduction

Emulsions are a system consisting of dispersed droplets of one immiscible liquid in another immiscible liquid. This structure is important in many everyday products, such as food, creams, cosmetics, and pharmaceutics. Due to their inherent metastable state, these systems irremediably tend towards macroscopic phase separation which corresponds to their lower energy state. In order to kinetically stabilize emulsions and slow down their destruction, low molecular weight surface-active agents (surfactants) or macromolecules (such as proteins or polymers) are most commonly used as emulsifiers. Both are adsorbed at the interface, decrease the interfacial tension of the two phases, and increase steric hindrance and electrostatic repulsion between droplets, thereby decreasing the droplet size and increasing the stability of the emulsion against creaming but also against coalescence (Saari et al. 2016).

There is growing demand for natural nontoxic emulsifiers for stabilizing emulsions, which has led to an interest in new emulsion system s such as particle-stabilized emulsions. Particles have been successfully used to stabilize emulsions known as Pickering emulsions. Pickering emulsions was

first described by Pickering (1907) and Ramsden (1903) at the beginning of the 20th century. After one hundred years of being out of the spotlight, Pickering emulsions have attracted considerable research interest in the past decade when it comes to applications that use particles accepted for food and other life science applications (Rayner et al. 2014).

Some of the most interesting properties of Pickering emulsions involve the possibilities for completely blocking the process of Ostwald ripening, and forming an emulsion with long-term stability. The essentially irreversible anchoring and specific inter-particle interactions at the interface are the physical basis of the peculiar properties of the particle-stabilized emulsions. The particles mainly act as a physical barrier that prevents contact between droplets, and the adsorption of particles is irreversible once they attach at the interface, which are the main reasons for the high stability seen for these emulsions (Tcholakova et al. 2008; Dickinson 2010; Rayner et al. 2014; Wahlgren et al. 2014).

Particles as emulsion stabilizers have enabled formulators to widen the formulation design space and aid in the reduction or removal of small molecular weight surfactants from emulsion recipes. In some cases, reduced surfactant use is especially attractive as they have been under increasing scrutiny and regulatory control and are known to cause irritation in topical

formulations (Wahlgren et al. 2014). Much of the research into particle-stabilized emulsion systems until the past few years has been focused on using inorganic particles such as silica (Vignati et al. 2003), latex, clay (Bon et al. 2007; Teixeira et al. 2011), and titania (Song et al. 2009) as a result of their well-defined shape, availability in different sizes and at narrow size distributions, as well as the chemical tenability of their surfaces (Binks et al. 2005; Gonzenbach et al. 2006). While this work has revealed a great deal of information about the mechanisms by which particles stabilize emulsions, and defined the conditions necessary for improved stability in Pickering systems, such inorganic particles are limited in their relevance to applications requiring biocompatibility and biodegradability. Thus, in the past few years, there has been a shift toward studying materials of biological origin for the stabilization of emulsions with the goal of utilizing them in food and drug delivery applications (Dickinson 2010; Marku et al. 2012; Kargar et al. 2012; Liu and Tang. 2013)

Therefore, recently, environmentally benign particle emulsifiers have received reasonable attention due to their high biocompatibility and novel applications. It has been found that emulsions could be stabilized by proteins (Destribats et al. 2014), solid lipid nanoparticles (Gupta and Rousseau 2012), cellulose nanoparticles (Zoppe et al. 2012), chitin nanocrystals (Tzoumaki et

al. 2011), hydrophobic modified starch nanoparticles (Tan et al. 2012), and native starch granules (Li et al. 2013). Previous studies use environmentally benign particles to stabilize emulsion systems, but, in the majority of cases, these particles are not food-grade particles because particles are modified by esterification with non-food-grade hydrophobic materials. Also, some particles are too big to make emulsions having colloidal stability.

Starch is a natural, renewable, and biodegradable polymer produced by many plants as a source of stored energy. It is therefore a promising material because of its versatility, low price, availability, and numerous industrial applications including foods. It is the major carbohydrate reserve in plant tubers and seed endosperm, and it is found in plant roots, stalks, crop seeds, and staple crops such as rice, corn, wheat, tapioca, and potato (Buléon et al. 1998). The starch granule displays a multiscale structure as shown in Fig. 1. It consists of the (a) starch granule (2-100 μm), (b) growth rings (120-500 nm) composed of (d) blocklets (20-50 nm) made of (c) amorphous and crystalline lamellae (9 nm) containing (g) amylopectin, and (h) amylose chains (0.1-1 nm).

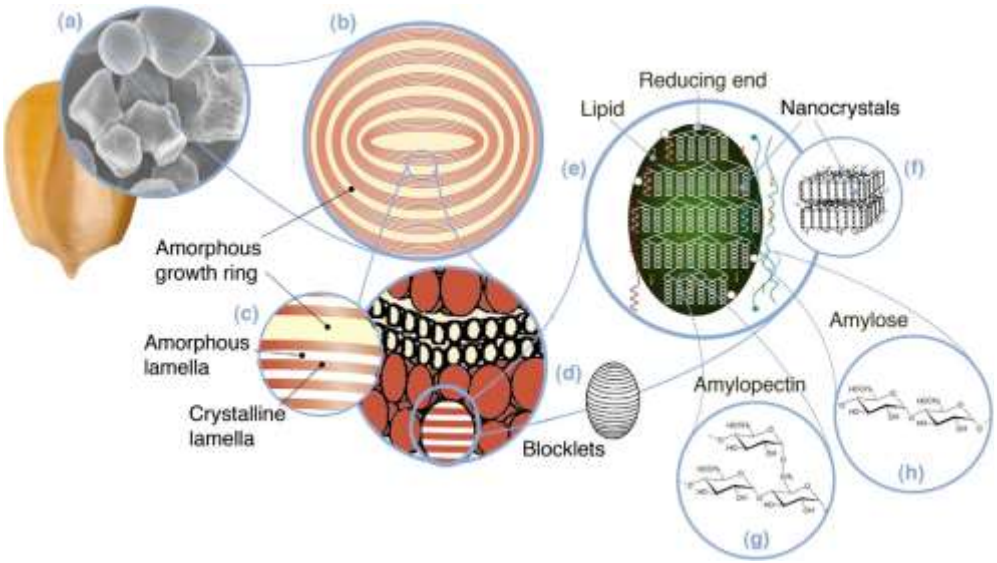

Figure 1. Starch multiscale structure: (a) starch granules from normal maize (30 μm), (b) amorphous and semi-crystalline growth rings (120-500 nm), (c) amorphous and crystalline lamellae (9 nm): magnified details of the semi-crystalline growth ring, (d) blocklets (20-50 nm) constituting unit of the growth rings, (e) amylopectin double helixes forming the crystalline lamellae of the blocklets, (f) nanocrystals: other representation of the crystalline lamellae called starch nanocrystals when separated by acid hydrolysis, (g) amylopectin's molecular structure, and (h) amylose's molecular structure (0.1-1 nm). Reproduced with some modification from (Le Corre et al. 2010).

The amorphous regions of starch are highly susceptible to hydrolysis and, under controlled conditions, may be dissolved leaving the rigid crystalline regions intact. The acid hydrolysis of native starch granules releases platelet-like nanoscale highly crystalline residues. As the size of a particle is decreasing down to the nanometer scale important changes occur. Both specific surface area and total surface energy increase. Moreover, starch nanoparticles display a highly reactive surface with plenty of hydroxyl groups (Dufresne 2014). But, the main limitation for the use of starch nanocrystals was the duration (40 days treatment) and the yield (0.5 wt%) of the HCl hydrolysis step (Battista et al. 1975). For the H_2SO_4 hydrolysis, response surface methodology was used to investigate the effect of five selected factors (temperature, acid concentration, starch concentration, hydrolysis duration, and stirring speed). The preparation of aqueous suspensions of starch nanocrystals was achieved after 5 days of 3.16 M H_2SO_4 hydrolysis at 40 °C, 100 rpm and with a starch concentration of 14.60 wt% with a yield of 15.7 wt% (Angellier et al. 2004). The H_2SO_4 hydrolysis required shorter time than the HCl hydrolysis, but the yield was still too low.

In this study, we use six kinds of native starches (maize, waxy maize, potato, waxy potato, rice, and waxy rice starch) to compare a yield of starch nanocrystals of each starches after acid hydrolysis using 3.16 M sulfuric acid

and an emulsifying ability of starch nanocrystals from each starches. And then, choosing one starch, waxy maize starch, has the highest yield and emulsifying ability. To increase the yield of starch nanocrystals, adding ultra-sonication step after getting starch nanocrystals of waxy maize starch (SNC) to break down aggregates and incompletely decomposed starch granules during acid hydrolysis. To increase the hydrophobicity of SNC, OSA-modified SNC (O-SNC) was made by synthesizing SNC with OSA through esterification. Moreover, developing O-SNC-stabilized Pickering emulsions after confirming SNC has partial dual wettability, leading to the spontaneous accumulation of particles at the oil-water interface. Furthermore, improving the emulsifying ability of O-SNC and reducing the droplet size of Pickering emulsions by using ultra-sonication and increasing the amount of O-SNC for stabilizing emulsions.

II. Materials and Methods

2.1. Chemicals and reagents

Waxy maize starch (N-WM) was a gift from Samyang Genex Inc. (Seoul, Korea). Waxy potato starch (N-WP) was gifted from Avebe (Veendam, Netherlands). Maize starch (N-M) and potato starch (N-P) were supplided from Tureban (Goyang, Korea). Waxy rice starch (N-WR) was obtained from Thai flour industry co. (Bangkok, Thailand). Tricaprylin (TC), Rice starch (N-R), gellan gum (phytagel), dimethylsulfoxide (DMSO) and 2-Octen-1-ylsuccinic anhydride (OSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sulfuric acid was purchased from Daejung (Siheung, Korea). Sylgard 184 curable silicone elastomer (polydimethylsiloxane, PDMS) was purchased from Dow Corning (Midland, MI, USA).

2.2. Starch nanocrystals preparation

Starch nanocrystals were prepared by acid hydrolysis. Starches (52.95 g) were mixed with 360 mL of a sulfuric acid solution (concentration of H_2SO_4 was 3.16 mol L^{-1}) and stirred 200 rpm for 6 days in 30 °C water bath. After acid hydrolysis, sulfuric acids were removed by centrifugation (29,000 rcf, 60

min) and collected pellet (hydrolyzates). And then, hydrolyzates were neutralized by 1 M sodium hydroxide solution followed by centrifugation (29,000 rcf, 60 min) to remove salts. Pellets re-dispersed in double-distilled water (DDW) and washed again by centrifugation (29,000 rcf, 60 min). After wash, pellets were re-dispersed in 0.02 wt% sodium azide solution, and then mild-centrifuged (500 rcf, 10 min) to get starch nanocrystal suspensions (supernatant). SNC suspensions were filtered by 0.7 μm filter (whatman glass fiber filters, GF/F) and then freeze-dried.

After mild-centrifugation, pellets have both unbroken starch granules and incompletely broken starch particles. To break down incompletely broken particles, collecting pellets and freeze-drying to get microstructural starch powder. This powder re-dispersed in DDW (15 wt%). Starch particles broken by ultra-sonication (sonication time from 100 s to 240 s, pulse 1 s on/off, and amplitude 60 %) in temperature-controlled jacked beaker (50 °C). After the sonication, suspensions mild-centrifuged (500 rcf, 10 min) to get starch nanocrystal suspensions (supernatant). Supernatants were filtered by 0.7 μm filter (whatman glass fiber filters, GF/F) and then freeze-dried.

Then, weighing the powdered starch nanocrystals to calculate the yield of starch nanocrystals (%)

$$\text{Yield of starch nanocrystals (\%)} = \frac{dry\ weight\ of\ starch\ nanocrystals}{dry\ weight\ of\ native\ starch\ before\ acid\ hydrolysis}$$

2.3. OSA Modifications

Starch nanocrystals from waxy maize starch (SNC) was suspended in distilled water (20 wt%) with stirring. Throughout OSA modification, the pH of the suspension was adjusted to 7.5-8.5 with a pH meter by adding 0.5 M sodium hydroxide aqueous solution. To this suspension, 3.0% OSA (wt% based on dried SNC) was added (diluted five times with absolute alcohol, v/v) slowly over 2 h. The reaction was carried out for 4 h. At the end of reaction, the pH of the starch suspension was adjusted to 6.5 using 0.1 M sulfuric acid. The resulting O-SNC was washed three times with water and then freeze-dried.

2.4. Determination of degree of substitution (DS)

Degree of substitution (DS) refers to the average number of the hydroxyl groups substituted per D-anhydroglucose unit in starch, the maximum possible DS is 3.0. DS of the O-SNC was determined using the method of Ren et al. (2016), some modification. O-SNC (0.2 g) was dissolved in 10 mL of DMSO by heating (50 °C, 20 min). After cooling to room temperature, 5-6 drops of phenolphthalein indicator were added. This solution

was titrated against 0.05 M standard sodium hydroxide aqueous solution until a permanent pale pink color was seen. The DS was calculated by using the following equation:

$$\mathrm{DS} = \frac{162 \times V \times M}{1000W - 266 \times V \times M}$$

where, V is the volume of sodium hydroxide aqueous solution used during titration, M is the molarity of NaOH aqueous solution, and W is the weight of SNC sample. Tests were performed in triplicate for each sample.

2.5. Dual wettability test

Dual wettability test of O-SNC was determined using the method of Paunov (2003) with some modification suitable for SNC. 2.0 wt% gellan solution was used as an aqueous phase. TC was used as an oil phase. PDMS (Sylgard 184 elastomer) was used in a ratio of 10:1 with respect to the curing agent. $\mathrm{SNC_{WM}}$ was used as a spreading particles. DDW was used as a spreading solvent to spread particles at the LCO-water interface. Gellan gum is a gel-forming polysaccharide consists of mono-saccharaides α-D-glucose, β-D-glucuronic acid, and α-L-rhamisnose in molar ratios of 2:1:1. At high temperatures, gellan polymers in aqueous solutions are in a disordered random coil state. When cooled to gelling temperature, this hydrocolloid forms double

helices which aggregate to firm junction zones. The presence of small amounts of cations (e.g., Na^{+}, Ca^{2+}) stabilizes the double helices and forms a 3D gel network. Gellan gum was initially dispersed as 2.0 wt% in DDW and heated to 95 ℃℃ in a water bath for 15 min to dissolve and hydrate. Bubbles were removed from the hot solution by centrifugation. The gelling temperature of 2.0 wt% gellan solution is in the range of 40-45 °C, but the setting of the gel does not happen instantaneously even if the solution is quickly cooled to room temperature. The spreading of SNC at the oil-water surface was done by injecting a sample of 20 μL of 0.5 wt% SNC suspension in DDW at the surface of gellan solution (kept in a Petri dish at a temperature about 50 °C). The system was then quickly cooled to 25 °C to set the gel. After 30 min, the oil phase was carefully removed and immediately replaced with PDMS. After curing the PDMS layer at room temperature for 72 h, it was peeled off the aqueous gel (together with the entrapped interfacial particles) and was additionally treated with hot distilled water to remove any gel residues from the PDMS surface. Then the PDMS-SNC samples were prepared for imaging with scanning electron microscopy (SEM) to measure partial wettability to both oil phase and aqueous phase.

2.6. Simple emulsification test

Oil-in-water emulsions were prepared by mixing 5 wt% lipid phase with 95 wt% aqueous phase (0.02 wt% sodium azide, 1.0 wt% SNC, i.e., a fifth of the lipid phase weight). 0.5 g of starch nanocrystals were re-dispersed in 47 g of 0.02 wt% sodium azide solution with stirring in a 100 ml beaker. 2.5 g of oil was added to the top. Then coarse oil-in-water emulsions were prepared by homogenizing the oil and aqueous phases together using a high-speed blender (Ultra-Turrax T25D, Ika Werke GmbH & Co., Staufen, Germany) at 8,000 rpm for 30 seconds and then at 11,000 rpm for 2 min 30 seconds.

2.7. O-SNC-stabilized Pickering emulsion preparation

TC was used as oil phase. 0.02 wt% sodium azide solution was used as aqueous phase. O-SNC was used as a Pickering-stabilizer and dispersed in aqueous phase before Pickering emulsion preparation. Oil-in-water Pickering emulsions were prepared by mixing 5 wt% oil phase with 95 wt% aqueous phase. O-SNC was re-dispersed in 0.02 wt% sodium azide solution with stirring in a 100 ml beaker. 2.5 g of oil was added to the top. Then coarse oil-in-water emulsions were prepared by homogenizing the oil and aqueous

phases together using a high-speed blender (Ultra-Turrax T25D, Ika Werke GmbH & Co., Staufen, Germany) at 8,000 rpm for 30 seconds and then at 11,000 rpm for 1 min 30 seconds. The droplet size was further reduced by ultra-sonication (VCX 750, Sonics & Materials Inc., Newtown, CT, USA) for 5 min at an amplitude of 60% and a duty cycle of 1 s. To prevent O-SNC crystalline destruction, ultra-sonication was conducted in a 4 °C jacketed beaker. To confirm the Pickering emulsions successfully maden, curcumin (1 mg/g tricaprylin) was added to oil phase.

2.8. Powder X-ray diffraction (XRD) analysis

The powder of N-WM, SNC, S-SNC, and O-SNC after freeze-drying were collected to analyze XRD patterns using an X-ray diffractometer (Bruker D8 Advance, Karlsruhe, Germany) with Cu-Kα radiation at λ=1.542 Å (40 kV, 40 mA). Results of wide-angle X-ray scattering (2θ = 5–40°, 0.2°/s) were obtained with a general area detector diffraction system.

2.9. Differential scanning calorimetry (DSC)

Gelatinization temperature of N-WM, hydrolyzate of waxy maize starch (H-WM), and SNC was determined using a differential scanning

calorimeter (Diamond DSC, PerkinElmer, Waltham, MA, USA). Starch samples were diluted 3-fold by DDW to gelatinize starch at gelatinization temperature. Starch suspension (25 mg) was placed in a hermetic aluminium pan, which was sealed and equilibrated at room temperature overnight prior to the measurements. An empty pan was used as a reference. The DSC scan started at 25 °C, increased by 5 °C min^{-1} to 95 °C.

2.10. Scanning electron microscopy (SEM)

The morphology of N-WM, H-WM, S-SNC and PDMS-SNC was observed by using a field emission scanning electron microscope (FE-SEM, SIGMA, Carl Zeiss, Oberkochen, Germany). Powdered samples were spread onto the carbon tape. Then a thin platinum conductive coating was used to reduce the charge effect by using low vacuum coater (EM ACE200, Leica, Wetzlar, Germany).

2.11. Light transmittance (%)

Before and after ultra-sonication, H-SNC suspensions (15 wt%) were introduced into a polystyrene cuvette (Ratiolab GmbH, Germany), and the light transmittance (%) of the suspension was determined by measuring the

absorbance at 640 nm using a Shimadzu UV-vis spectrophotometer (UV-1700, Kyoto, Japan). DDW was used as a reference.

2.12. Particle size and ζ-potential determination

2.12.1. Dynamic light scattering (DLS)

Mean particle diameters (hydrodynamic diameter, z-average) and particle size distribution (poly diversity index, PDI) of SNCs and SNC-stabilized Pickering emulsions were assessed using dynamic light scattering technique (DLS). Samples were analyzed by a zetasizer (Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK) using a helium-neon laser (λ = 633 nm).

2.12.2. Laser diffraction

The microstructural size of native starches was measured using a laser diffraction analyzer (S3500, Microtrac Inc., Montgomeryville, PA, USA) to figure out the size of H-SNC. Samples were stirred continuously throughout the measurements to ensure that they were homogeneous with the water flow option. The particle size was reported as the mean diameter in micrometers of the volume distribution, $\mathrm{MV} = \sum V_i d_i / \sum V_i$ (where V_i is the volume percent of particles of diameter d_i in the population).

2.13. Optical microscopy

A drop of O-SNC-stabilized Pickering emulsions was placed on a microscope slide and covered using a coverslip. The morphology of a cream layer and dispersed layer of O-SNC-stabilized Pickering emulsions was observed using a conventional optical microscope (DCM130, Hangzhou HauXin IC Technology INC., Hangzhou, China) equipped with a digital camera (DCM130E, BW Optics Co., Nanjing, China) and digital image processing software (ScopePhoto version 3.0, Hangzhou Scopetek Opto-Electric Co., Hangzhou, China).

2.14. Transmission electron microscopy (TEM)

The nanostructure of O-SNC-stabilized Pickering emulsions and O-SNC suspensions was observed using a transmission electron microscope (JEM1010, JEOL, Tokyo, Japan). First, samples were placed on a film-coated copper grid and negatively stained with a 1 % (w/v) aqueous solution of phosphotungstic acid for 30 s. And then, the overflow solution on the sample was wiped off by filter paper before drying for 10 min at room temperature before observation.

2.15. Statistical Analysis

All results were analyzed using Tukey's significant difference test with IBM SPSS Statistics version 21.0 (IBM Co., Armonk, NY, USA). Data represent the averages of at least three independent experiments or measurements.

III. Results and Discussion

3.1. Starch nanocrystals (SNC) preparation

The degree of acid hydrolysis (3.16 M H_2SO_4, 30 °C. 200 rpm) for the starches during the hydrolysis period (14 days) are presented in Figure 2. After 6 days, hydrolysis of six starches (N-M, N-WM, N-P, N-WP, N-R, N-WR) was almost finished. Regardless of starch origin, all starch samples showed fast hydrolysis in the early state up to 6 days, followed by relatively slow hydrolysis. That is, hydrolysis occurred in two phases. It was assumed that acid hydrolysis initially occurred mainly in the amorphous regions, followed by erosion of the crystalline regions of the starch granules. After 6 days, the degree of acid hydrolysis for different starches tested were 50.2, 62.3, 72.9, 77.5, 55.7, and 74.0% for N-M, N-WM, N-P, N-WP, N-R, and N-WR, respectively. It shows that A-type starches (N-M, N-WM) are less susceptible to acid hydrolysis than B-type ones (N-P, N-WP), which was in contrast to previous study (Robin et al. 1974). It might be due to the loose structure of N-P and N-WP. During the hydrolysis, N-P and N-WP might be more swollen than N-M and N-WM, so potato and waxy potato starches, a typical B-type

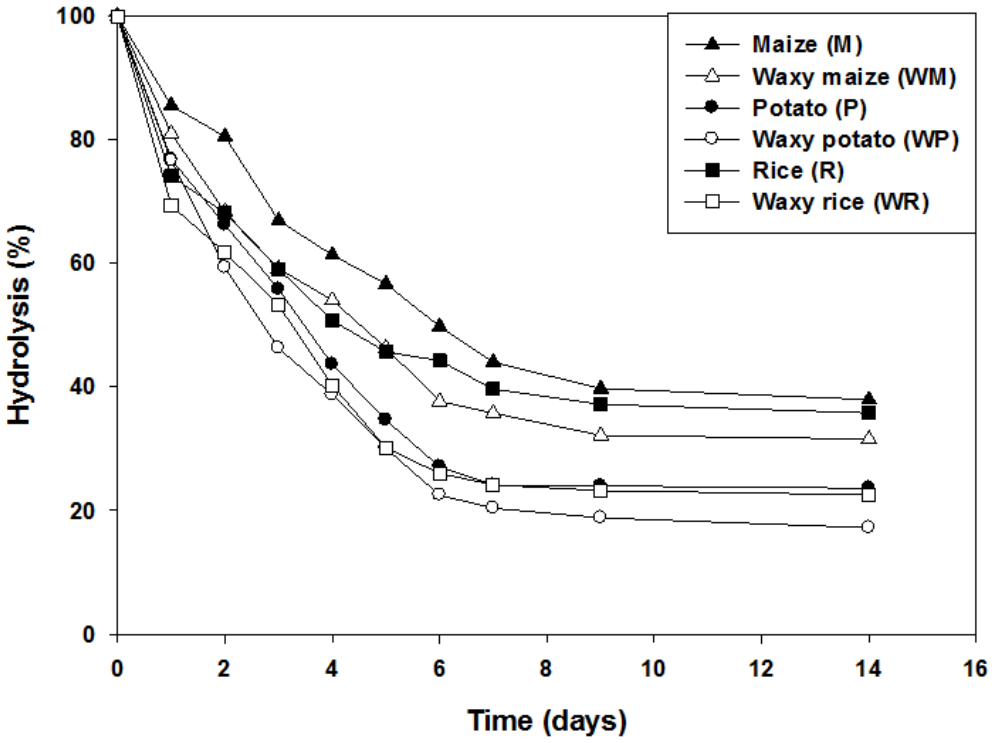

Figure 2. Acid hydrolysis profile of starches different in origin and amylose content in 3.16 M H_2SO_4 at 30 °C.

starches, was more susceptible to the hydrolysis than A-type maize and waxy maize starches. The extent of hydrolysis of N-R and N-WR, a C-type starch, was intermediate results because it consisted of a mixture of A- and B-type crystals and also had loose structure. All waxy starches were susceptible to hydrolysis than their non-waxy starches. It might be amylose (has long linear chain) in crystalline regions more readily crystallize than amylopectin (has short, highly branched chains) (Figure 1-(g), (h)).

Microstructural size of hydrolyzate from six starches after 6 days acid hydrolysis and nanostructural size of starch nanocrystals were shown in Figure 3. Although 6 days acid hydrolysis decomposed starch granules, still a lot of microns existed after acid hydrolysis (Figure 3-a) because some starch granules had resistance to acid hydrolysis. After mild centrifugation (500 rcf, 30 min) and vacuum filtration (0.7 μm pore size), all microns were successfully removed (Figure 3-b). Starch nanocrystals from waxy maize, potato, waxy potato, and waxy rice starches had similar nanostructural size on DLS data (80-100 nm). SNC from maize starch had monomodal size distribution, but had slightly big size (about 200 nm) than earlier four starches. SNC from rice starch had bimodal size distribution (120 nm and 800 nm). It had bigger size than filter pore size (700 nm) due to platelet-like morphology of SNC. After isolation by differential centrifugation and filtration, SNC

suspensions of waxy maize, potato, waxy potato and waxy rice starches were absolutely clear. On the other hand, SNC suspensions of maize and rice were not clear due to their relatively large particle size.

After filtration and freeze-drying, measuring powdered SNC weight to calculate the yield of acid hydrolysis. The yield of SNC for the six starches are presented in Figure 4. Except waxy rice, the yields of SNC were peaked at about 6 days. It was hard to get SNC of rice and waxy rice starches because these two starches entretallarsed together, so SNC of (waxy) rice starch was hard to isolate from assemblies composed of starch granules, SNCs and aggregated SNCs. In case of other 4 starches, after 6 days hydrolysis, the yield of SNC for different starches tested were 13.64, 15.12, 13.21, and 12.98 wt% for maize, waxy maize, potato, waxy potato, respectively. The yield of waxy maize starch was similar to previous optimized value, 15.7 wt% and the yield of other three starches were same or more than previous study. In our differential centrifugation steps, using very high speed (29,000 rcf) and very long time (60 min) than previous studies (500-5,000 rcf, 10-30 min) because SNC by using differential centrifugation was very difficult. SNC has very small size (20-50 nm), and continuously move vigorously, Brownian motion. While using under 5,000 rcf for differential centrifugation has revealed a great isolation of microstructural starch, such force are insufficient to isolate SNC.

And the longer the centrifugation time, the better. Higher centrifugal force and longer time were needed to separate SNC from water because SNC was easily re-dispersed in aqueous phase. Actually, at high centrifugal force (29,000 rcf, 60 min), the yield of SNC (15.12 wt%) was higher than that (11.38 wt%, data not shown) of low centrifugal force (5,000 rcf, 30 min). Also, the yield of SNC was high value at 5-7 days, and slowly decreased after 7 days, which suggested that hydrolysis occurred not only on the amorphous regions but on the crystalline regions of starch. So, prolonged acid hydrolysis was not desirable to obtain SNC.

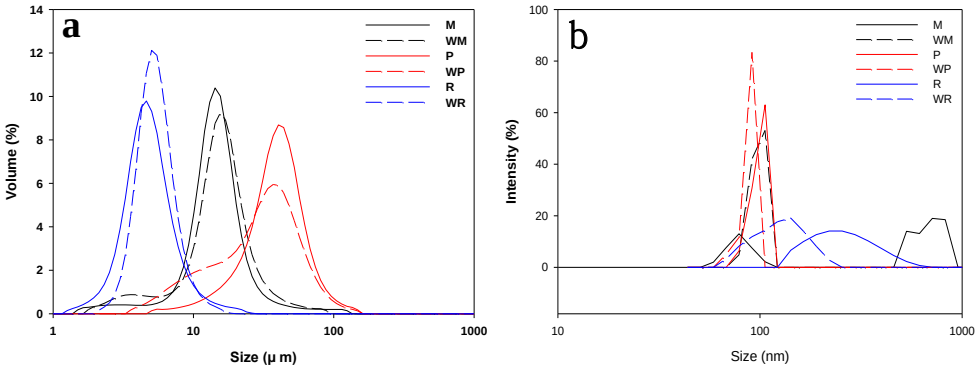

Figure 3. Microstructural size (LD) and nanostructural size (DLS) distributions of starch hydrolyzates, and starch nanocrystals. (a) LD data, after 6 days acid hydrolysis, before mild centrifugation (500 rcf, 30 min); (b) DLS data, after 6 days acid hydrolysis, after mild centrifugation (500 rcf, 30 min) and filtration. Abbreviation for starch samples: M, maize; WM, waxy maize P, potato; WP, waxy potato; R, rice; and WR, waxy rice.

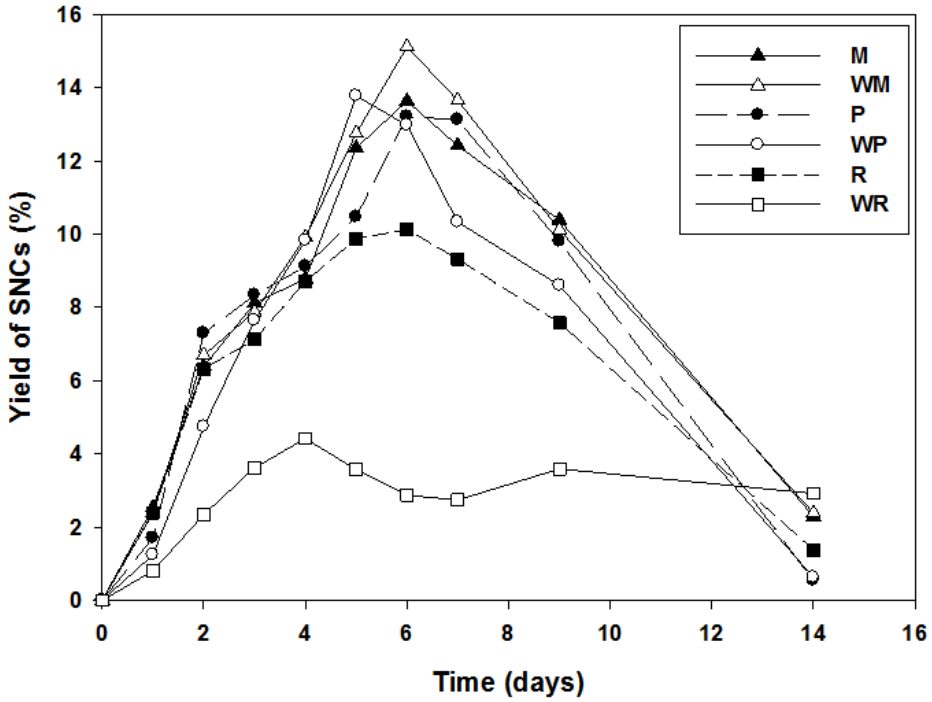

Figure 4. The yield of starch nanocrystals (SNC). Abbreviation: M, maize starch; WM, waxy maize starch; P, potato starch; WP, waxy potato starch; R, rice starch; and WR, waxy rice starch.

3.2. Simple emulsification activity test

To select suitable SNC as Pickering-stabilizer, simple emulsification test was executed for four SNCs that had small particle size (80-100 nm) because it was widely known that smaller size was more desirable to Pickering-stabilize. Oil-in-water emulsions were prepared by mixing 5 wt% lipid phase (TC) with 95 wt% aqueous phase (0.02 wt% sodium azide, 1.0 wt% SNC). Coarse oil-in-water emulsions were prepared by homogenizing the oil and aqueous phases together using a high-speed blender at 8,000 rpm for 30 seconds and then at 11,000 rpm for 2 min 30 seconds. Figure 5 shows relative emulsifying activity of the four starches (waxy maize, potato, and waxy potato). Emulsions stabilized by $SNC_{waxy\ maize}$ were prolonged more than 2 weeks against coalescence. On the other hand, Emulsions stabilized by SNC_{potato} and $SNC_{waxy\ potato}$ were rapidly coalescence just 1 days after emulsion preparation. Therefore, three factors (the yield, crystal size, and emulsifying ability) considered, waxy maize starch might be the best starch for Pickering emulsion.

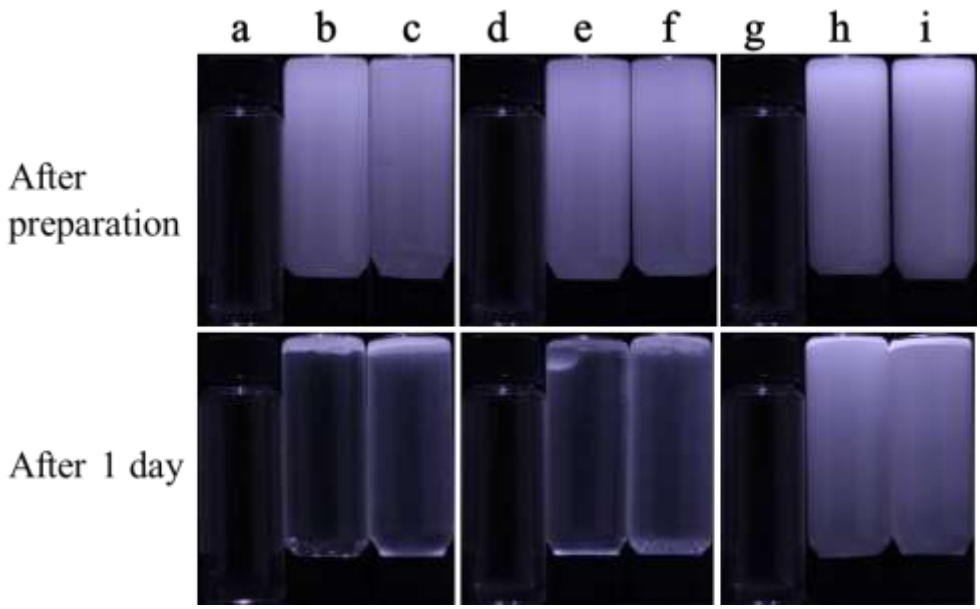

Figure 5. Visual stability of SNC-stabilized Pickering emulsions via simple emulsification activity test (5 wt% lipid phase with 95 wt% aqueous phase, homogenized at 8,000 rpm for 30 seconds and then at 11,000 rpm for 2 min 30 seconds). (a), (d), and (g) SNC was re-dispersed in DDW (1.06 wt%). Potato (a), waxy potato (d), and waxy maize (g) SNC; (b), (c), (e), (f), (h), and (i) SNC-stabilized Pickering emulsion. Using potato (b, c), waxy potato (e, f), and waxy maize (h, i) SNC. SNC:oil ratios of (b, e, h) were 1:10 and SNC:oil ratios of (c, f, i) were 1:5.

3.3. Enhancing the yield of SNC

SEM images of before and after 6 days acid hydrolysis are presented in Figure 6. Before and after acid hydrolysis, overall size of starch granules were decreased. Panels b-c in Figure 6 show incompletely decomposed starch granules after acid hydrolysis. Sharp-pointed structure in Fig. 6-b was 50-times magnified in Fig. 6-c. It made up of SNC (20-50 nm) and partially hydrolyzed amorphous regions (between SNCs). To break down this incompletely decomposed starches, ultra-sonication step was added.

Before adding ultra-sonication, measuring the thermal property of native waxy maize starch, hydrolyzates, and SNC by using differential scanning calorimetry (DSC) to identify on-set temperature (T_o) of starch gelatinization (Figure 7). T_o of native waxy maize starch, hydrolyzates, and SNC_{WM} were 60.9, 58.8, and 55.6 °C, respectively. Amorphous regions were partially removed during acid hydrolysis, so T_o was decreased after acid hydrolysis. T_o of SNC_{WM} was lowest because it had almost no amorphous regions. Therefore, in order to increase the yield of SNC_{WM} without harming SNC, the temperature of starch suspensions must below 55.6 °C during ultra-sonication step.

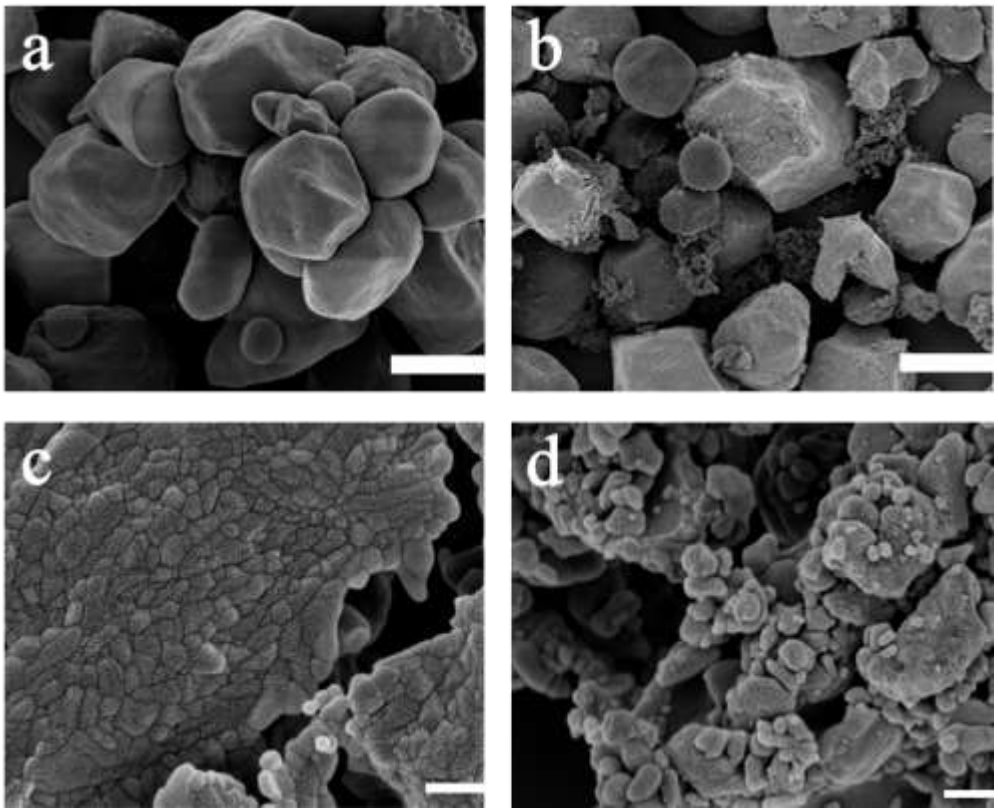

Figure 6. Scanning electron micrographs (SEM) images of N-WM and H-WM before and after ultra-sonication. (a) N-WM; (b), (c) H-WM; (d) S-SNC. Scale bar = 10 μm for (a) and (b); 200 nm for (c) and (d)**.**

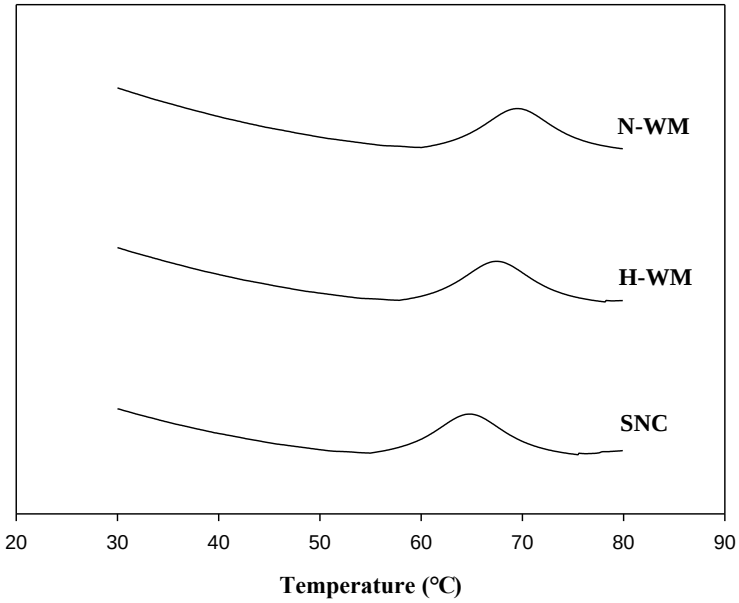

Figure 7. DSC thermograms of N-WM, H-WM, and SNC. Abbreviation: N-WM, native waxy maize starch; H-WM, waxy maize starch hydrolyzate after 6 days acid hydrolysis; SNC, starch nanocrystal from waxy maize starch.

In previous study, ultra-sonication step to hydrolyzate suspensions had no particle size reduction effect in the case of the temperature of suspensions was lower than 45 °C (duration time 300 s in a 25 °C jacketed beaker, end temperature was 44.8 °C, Figure 8-j). So, before applying ultra-sonication, starch hydrolyzate suspensions were stored in 50 °C jacketed beaker with stirring for 12 min to raise the temperature of suspensions to 45 °C.

Visual turbidity change of starch hydrolyzate suspensions (15 wt%) was shown in Figure 8, light transmittance (%) change of starch hydrolyzate suspensions (15 wt%) was shown in Figure 9, and particle size distribution change of starch hydrolyzate suspensions (15 wt%) before and after ultra-sonication by DLS measurements was shown in Figure 10. To prevent rapid temperature rise during ultra-sonication step, the suspensions were placed in a 45 °C jacketed beaker and stirred throughout the sonication. During ultra-sonication, the temperature of starch hydrolyzate suspensions gradually increased from 45 °C to about 55.6 °C after 160 seconds. When ultra-sonication was applied for longer than 160 seconds, starch gelatinization started and the light transmittance (%) of the suspensions increased rapidly (Figure 8 and Figure 9). Therefore, when the H-WM suspensions (15 wt%) was pre-heated to 45 °C and then ultra-sonicated for 160 seconds in a 45 °C jacketed beaker, most of the non-gelatinized SNC was successfully obtained. Figure 6-d shows the additional SNC obtained by adding optimal ultra-sonication (pre-heating to 45 °C and then ultra-sonicating 160

seconds in a 45 °C jacketed beaker) to the H-WM suspensions after 6 days acid hydrolysis using 3.16 M sulfuric acid. As the ultra-sonication was applied locally, there was a localized increase in temperature of more than 55.6 °C for a while, so some swollen particles were also observed (Figure 4-d). Finally, SNC added to starch nanocrystals obtained through ultra-sonication (S-SNC), and then the yield of SNC increased from 15.12 wt% to 22.36 wt%.

X-ray diffractograms of N-WM, H-WM, and SNC were shown in Figure 10. The specific XRD lines (3.84, 4.90, 5.19, and 5.85 nm with WAXS) for the A-type crystalline arrangements of N-WM were similar to the previous findings (Kim et al. 2013). During 6 days acid hydrolysis, amorphous regions of starch granules were hydrolyzed and some crystalline peaks 18.7° (4.75 nm), 31.8° (2.81 nm), 34.2° (2.63 nm), and 37.9° (2.37 nm) (2θ) of several SNC generated during acid hydrolysis began to appear. Before and after ultra-sonication to hydrolyzates, the specific XRD lines (2.37, 2.63, 2.80, 3.47, 3.75, 4.46, and 4.73 nm with WAXS) was almost same by X-ray diffractograms (Figure 10). So, Ultra-sonication to enhance the yield of SNC was effective and safe method.

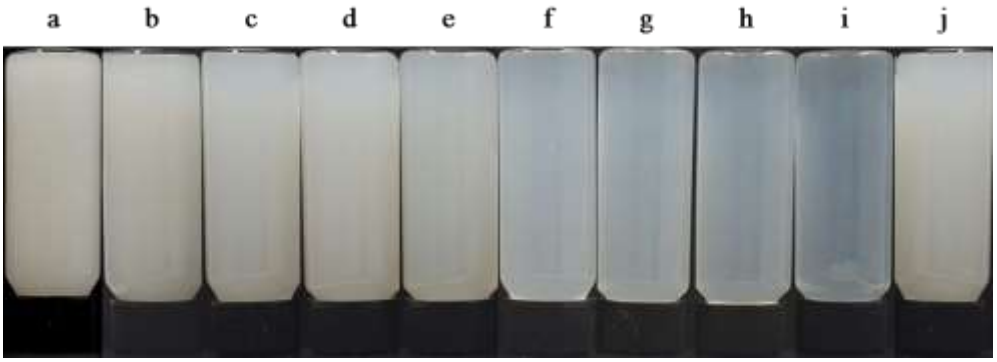

Figure 8. Visual turbidity changes of starch hydrolyzate suspensions (15 wt%) before and after ultra-sonication. Ultra-sonication in a 45 °C jacketed beaker duration times (a) 0, (b) 100 s, (c) 120 s, (d) 140 s, (e) 160 s, (f) 180 s, (g) 200 s, (h) 220 s, and (i) 240 s; Ultra-sonication in a 25 °C jacketed beaker duration times (j) 300 s.

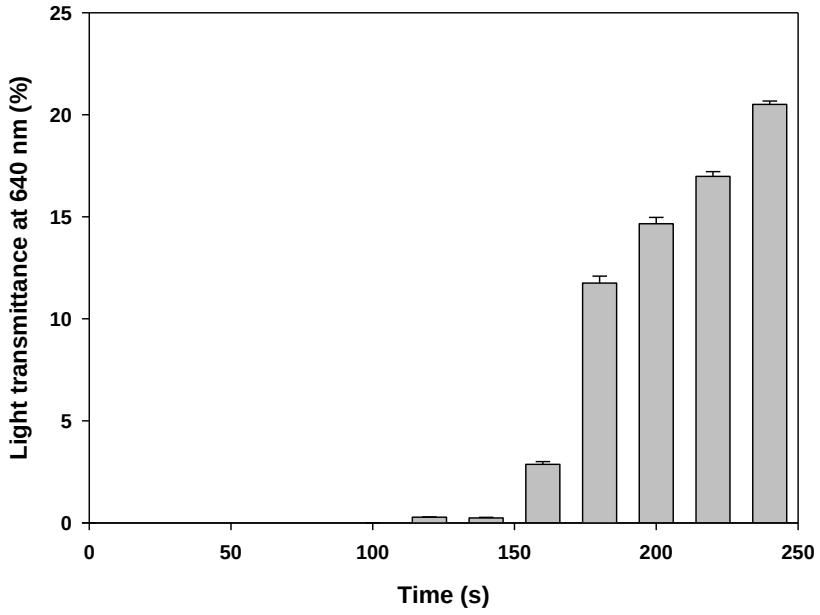

Figure 9. Effect of ultra-sonication duration times on light transmittance (%) of starch hydrolyzate suspensions (15 wt%).

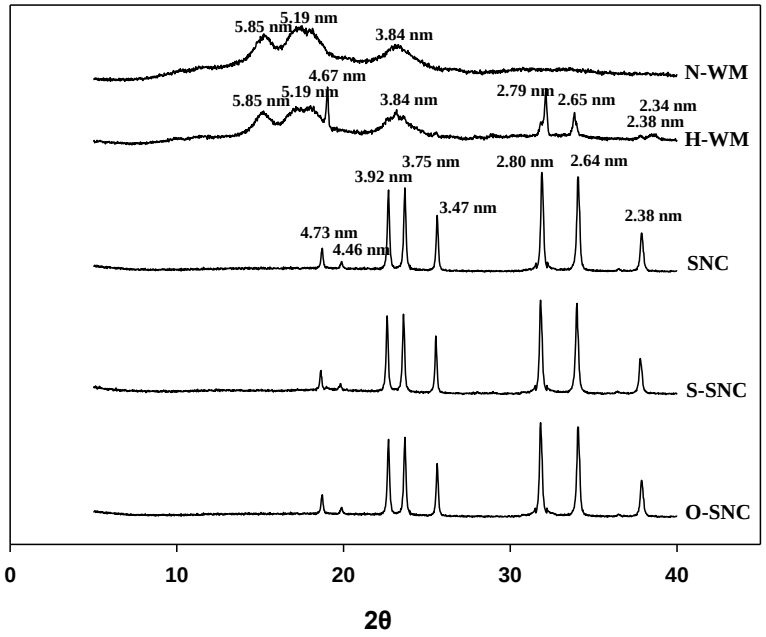

Figure 10. X-ray diffractograms of N-WM, H-WM, SNC, S-SNC, O-SNC.

Abbreviation: N-WM, native waxy maize starch; H-WM, waxy maize starch hydrolyzates after 6 days acid hydrolysis (3.16 M sulfuric acid, 30 °C); SNC, starch nanocrystal from waxy maize starch; S-SNC, SNC produced by ultrasonication to H-WM; O-SNC, OSA-modified SNC.

3.4. OSA-modification of SNC

OSA-starches have been used in a range of industrial applications, particularly as a food additive, for more than half a century. Moreover, usage restrictions of OSA-starches as food emulsifier, stabilizer, and thickener were lifted in 2013 by CODEX Alimentarius.

Previously, the simple emulsification activity test of SNC showed that SNC had emulsifying activity, but not sufficient. It might SNC had much more hydrophilic than hydrophobic. To increase the hydrophobicity of SNC, OSA-modified-SNC (O-SNC) was modified by synthesizing SNC with OSA through esterification in aqueous solutions (20 wt%). Using the most widely described synthesis pathway is a reaction in aqueous medium under mild alkaline conditions (pH 7.5-8.5 adjusted by sodium hydroxide solution). Slightly basic conditions help to reduce hydrogen bonding between starch chains by the formation of alkoxide functionalities with the starch OH groups, which consequently favors the diffusion of OSA molecules within the starches.

O-SNC were obtained from the esterification reaction between starch hydroxyl groups and octenyl succinic anhydride (Sweedman et al. 2013). A commonly used parameter in this regard is the degree of substitution, *DS*, which is the average number of octenyl succinate derivatives per glucose unit.

In this study, DS of O-SNC was about 0.013 which is an appropriate value for using in foods after 4 hours esterification reaction. The maximum level of OSA treatment for starch allowed in food industry is 3% (DS $\approx$ 0.02) .

X-ray diffractograms of SNC and O-SNC were shown in Figure 10. Before and after OSA modification of SNC, X-ray diffractograms showed peaks in the almost same distances. Therefore, OSA modification of SNC might not affect the crystalline structure of SNC.

3.5. Partial wettability of O-SNC to both aqueous phase and oil phase

When modified with OSA, the normally hydrophilic SNC gains a hydrophobic element in the form of octenyl groups, resulting in whole molecules with an amphiphilic character.

In order to confirm whether the hydrophobicity enhanced SNC was actually exist at the interface between aqueous phase and oil phase, 2% gellan solution and polydimethylsiloxane (PDMS) were used as trapper of particles at aqueous phase and oil phase interfaces to imaging SEM. Figure 11 shows schematic diagram of SEM imaging the PDMS-SNC_{WM} at the water-oil interface. To prevent gelatinization of SNC, temperatures were always under the T_o of SNC during gelation and curing of PDMS. PDMS-SNC was thin-coated with platinum to prevent charging effect.

An SEM images of the surfaces of PDMS-SNC after the gel trapping technique were shown in Figure 12. Although the extent to which SNC were embedded in PDMS was different because the degree of hydrophobicity of SNCs varies from individual particle to particle, SNC actually existed at the interface between the aqueous phase and oil phase. That is, SNC had partial wettability to both two phases.

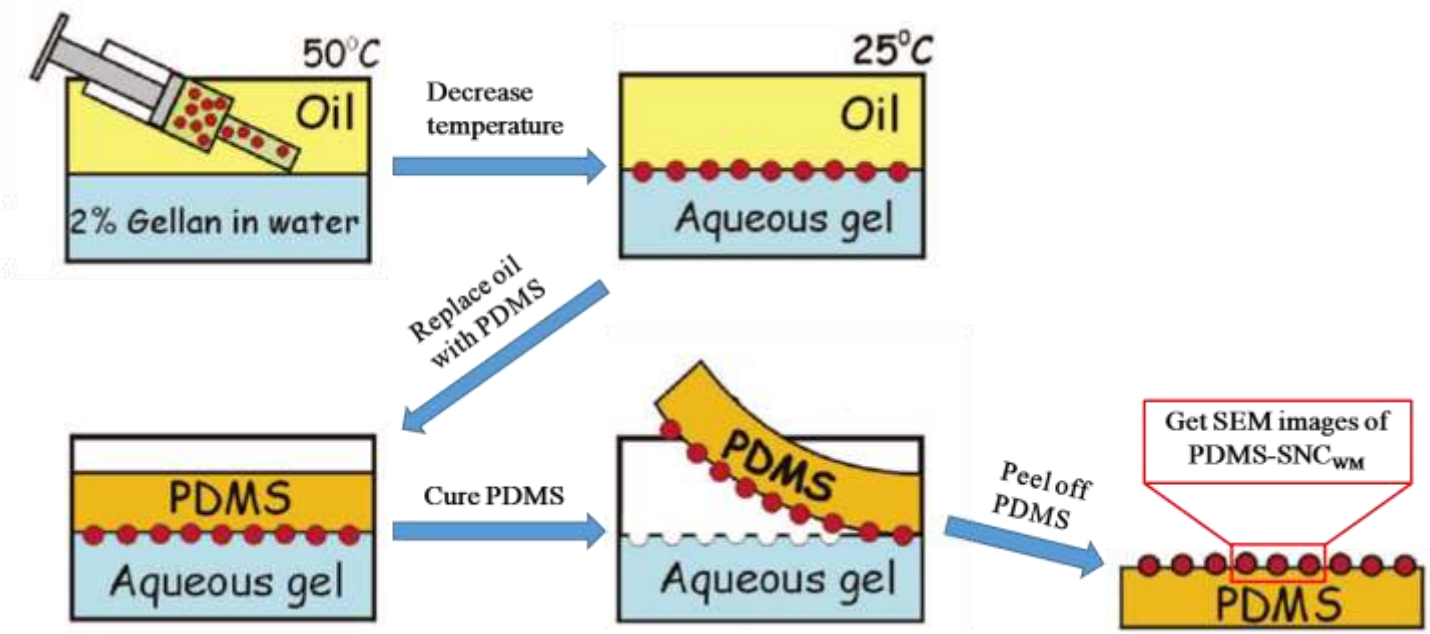

Figure 11. Schematic diagram of the gel trapping technique for determining partial wettability of SNC wettability to both aqueous phase and oil phase. Reproduced with some modification from (Paunov 2010).

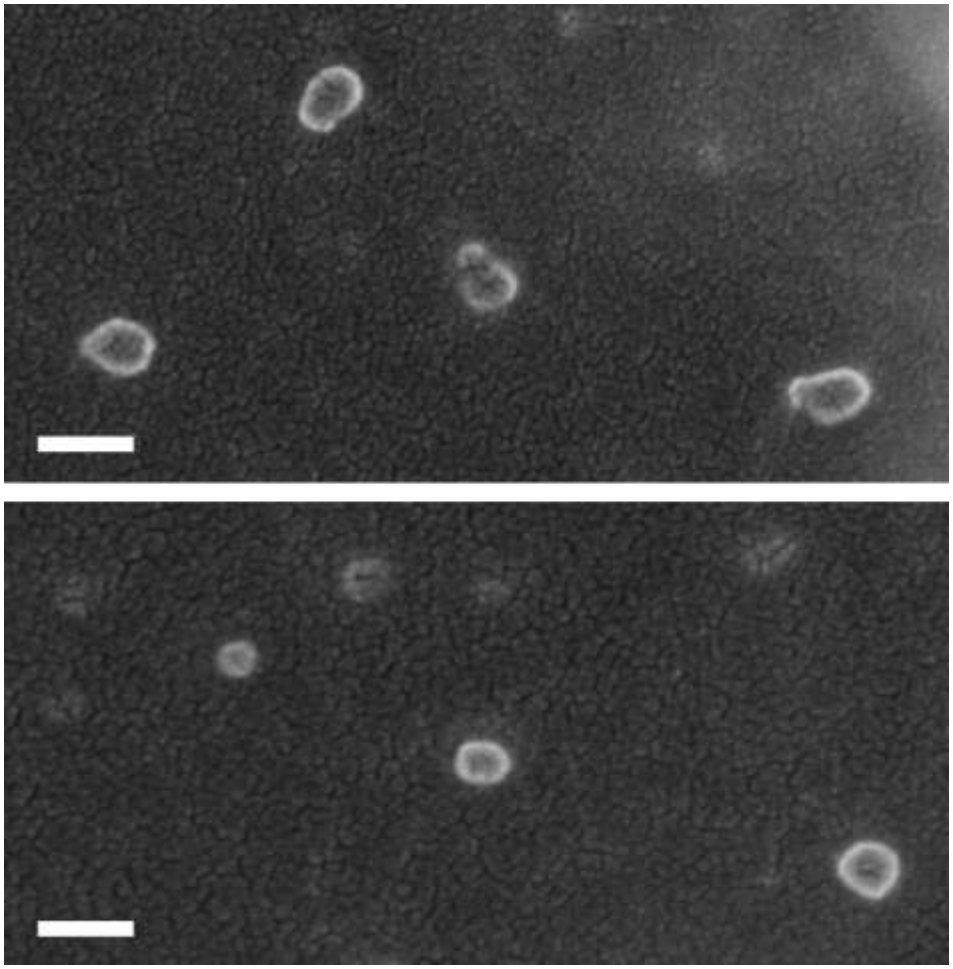

Figure 12. SEM images of the surfaces of PDMS-SNC after the gel trapping technique. Scale bar = 100 nm.

3.6. O-SNC-stabilized Pickering emulsions

Pickering emulsions was prepared by using O-SNC of high crystallinity with dual wettability previously isolated. When emulsions were prepared just using a high-speed blender, which is a commonly used method in previous studies, a slightly large droplet was observed (Figure 14) and non-emulsified oils were also observed (Figure 13-a) in dispersed layer of Pickering emulsions stabilized by O-SNC.

Therefore, ultra-sonication step was added to decrease the size of Pickering emulsion droplet and to increase the emulsifying ability of O-SNC. In order to minimize the crystalline destruction of the O-SNC, the sonication step was carried out in a 4 °C jacketed beaker. In order to focus on the effect of ultra-sonication, the amount of O-SNC was fixed at 71.6 mg (0.14 %, w/w emulsion systems), and the amount of oil was also fixed at 2.5 g (5.0 %, w/w emulsion system), and the experiment was performed with different duration times of ultra-sonication. Visual colloidal stability and emulsifying ability of O-SNC were shown in Figure 13 and effect of ultra-sonication on particle size distribution of Pickering emulsions stabilized by O-SNC was shown in Figure 14. When emulsions were prepared just by high-speed blender, a large amount of oil was not emulsified, but floated up and formed oil-layer. While applying

ultra-sonication for up to 5 min, the amount of oil-layer gradually decreased, but it did not decrease any further after ultra-sonication (Figure 13). In the case of droplet size, the size gradually decreased during the application of ultra-sonication for up to 3 min and the size did not decrease even if ultra-sonication was applied for more than 3 min. The power of ultra-sonication, 450 W, was not enough to separate the SNC from the interface of water and oil, but just to remove the aggregated O-SNC and act as the Pickering-stabilizer as a single particle. Ultra-sonication also served to split droplets that had not been stabilized in optimal conditions into the most stable size (about 500 nm) of Pickering emulsions, approximately 10 times the size of the Pickering-stabilizer (O-SNC, 20-50 nm). As a result, ultra-sonication (5 min) successfully reduced droplet size of the Pickering emulsions and improved emulsification ability of O-SNC.

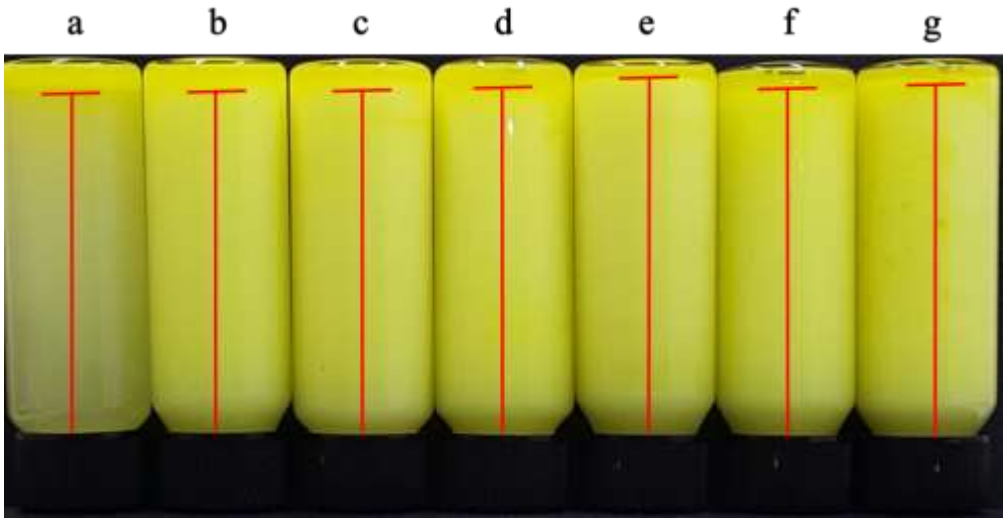

Figure 13. Visual colloidal stability of the Pickering emulsions and emulsifying ability of O-SNC. All samples consisted of 0.14 wt% O-SNC, 5.0 wt% tricaprylin, 94.86 wt% DDW. Ultra-sonication treating time variations (a) 0 min; (b) 1 min; (c) 2 min; (d) 3 min; (e) 5 min; (f) 7 min; (g) 10 min. All sample images were stored 7 days after emulsion preparation. Upper red line means oil-layer. Abbreviate: O-SNC, OSA-modified starch nanocrystal from waxy maize starch.

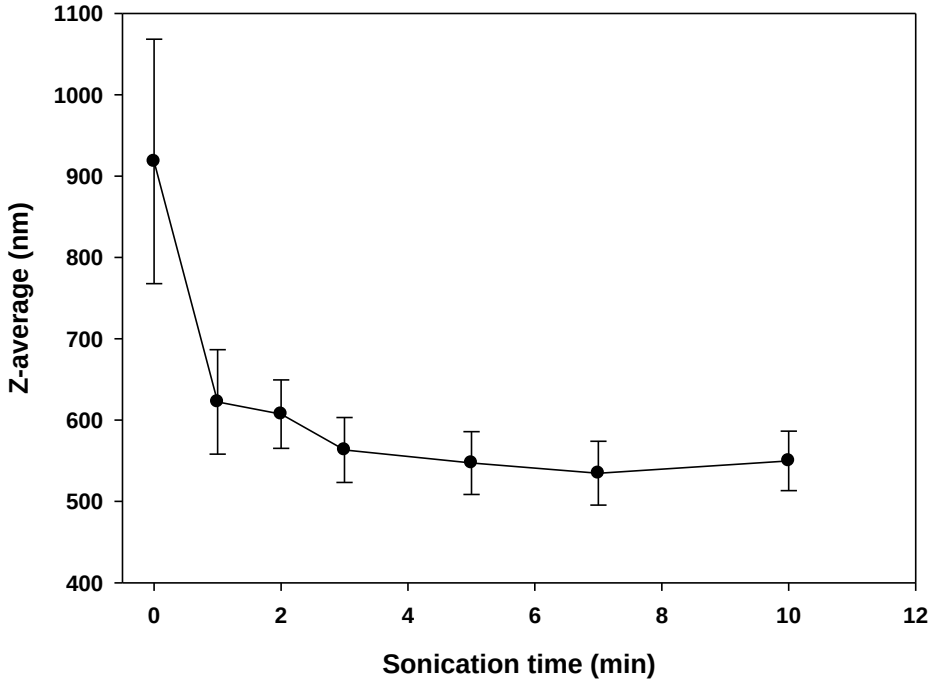

Figure 14. Effect of ultra-sonication time on particle size of Pickering emulsions stabilized by O-SNC. Abbreviate: O-SNC, OSA-modified starch nanocrystal from waxy maize starch.

In addition, to test the emulsifying capacity of O-SNC, the amount of oil (2.5 g) and ultra-sonication duration time (5 min) were fixed, and only the amount of O-SNC was controlled to confirm whether an oil-layer was formed and to confirm the correlation between the amount of O-SNC and a size of the Pickering emulsions. As the amount of O-SNC increased to about 40 mg, the size of the Pickering emulsions tended to decrease rapidly and at a higher O-SNC amount it decreased very slowly (Figure 15). In the case of the emulsifying capacity of O-SNC, the oil-layer gradually decreased until the amount of O-SNC was about 130 mg, and when more O-SNC was used, the Pickering emulsions was well dispersed with a stabilized cream layer on top without the oil layer. The cream layer was formed by the aggregation of imperfectly stabilized Pickering emulsions that were unavoidable in the Pickering emulsions manufacturing process.

Unlike previous studies that did not produce well-dispersed Pickering emulsions with colloidal stability due to their large size (about 10-100 μm), the Pickering emulsions, which was created by controlling the amount of O-SNC and the ultra-sonication treating time in this study, had colloidal stability

over 2 weeks due to their small size (about 530 nm) and stability against coalescence also over 2 weeks.

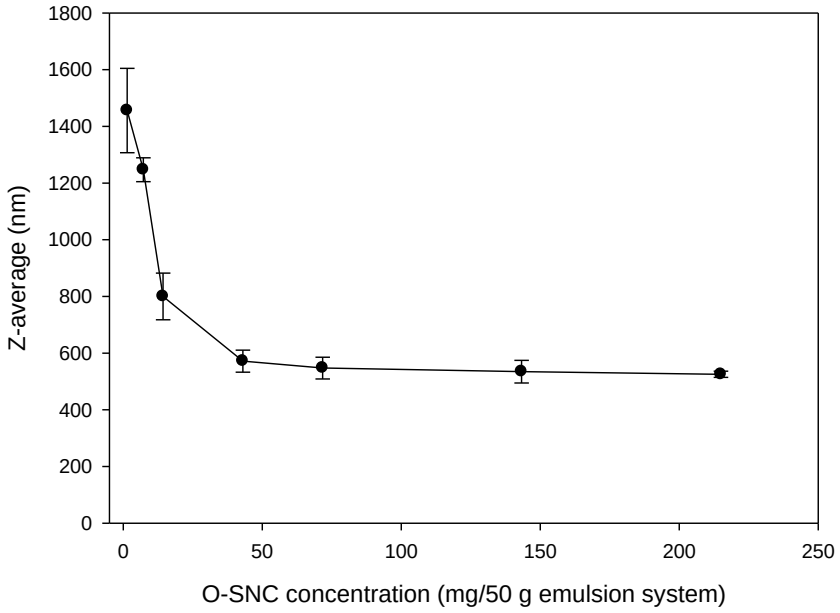

Figure 15. The correlation between the amount of O-SNC and a size of Pickering emulsions. Abbreviation: O-SNC, OSA-modified waxy maize starch nanocrystals.

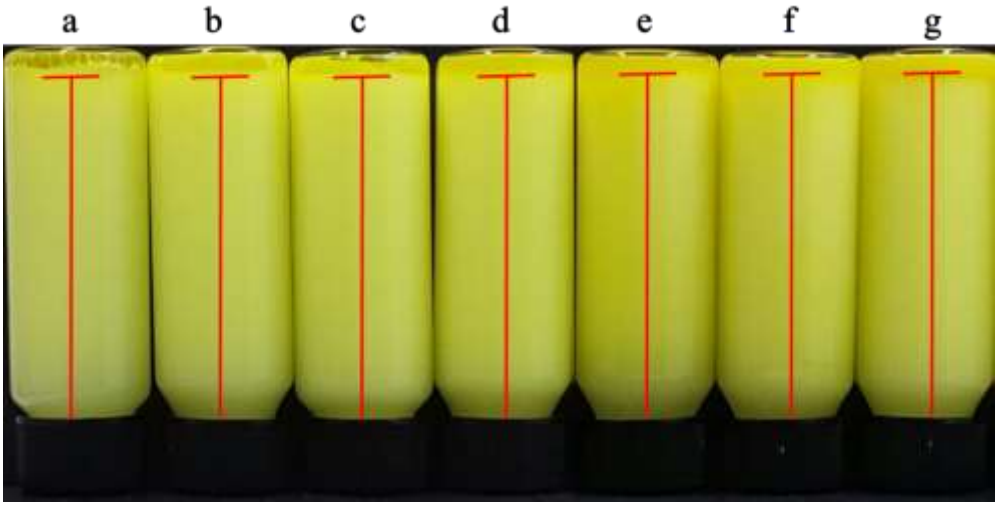

Figure 16. The correlation between the amount of O-SNC and a volume of dispersed Pickering emulsions. Abbreviation: O-SNC, OSA-modified waxy maize starch nanocrystals. All samples were 5 wt% oil phase with 95 wt% aqueous phase. Aqueous phase had O-SNC in different concentration (a) 1.43; (b) 7.17; (c)14.3; (d) 43.0; (e) 71.7; (f) 143.3; (g) 215 (mg/50 g emulsion system).

3.7. Morphologies of O-SNC-stabilized Pickering emulsions

Morphology of O-SNC-stabilized Pickering emulsions were observed by optical microscope shown in Figure 17 and TEM shown in Figure 18. In the cream layer of O-SNC-stabilized Pickering emulsions shown in Figure 17-a, unstable emulsions were tangled together and had slightly large droplet size (about 2-10 μm). On the other hand, in the dispersed layer of O-SNC-stabilized Pickering emulsions has small droplet size (small dots in Figure 17-b, about 500 nm) and well dispersed. During the measurement, some small droplets were continuously moving, Brownian motion, because of their nanoscale-particle size.

In addition, panel a in Figure 18 shows the nanostructural morphology of O-SNC-stabilized Pickering emulsions. The size of droplets observed in the TEM images were about 500 nm and the size of O-SNC observed in TEM images were about 20-50 nm shown in Figure 18-b.

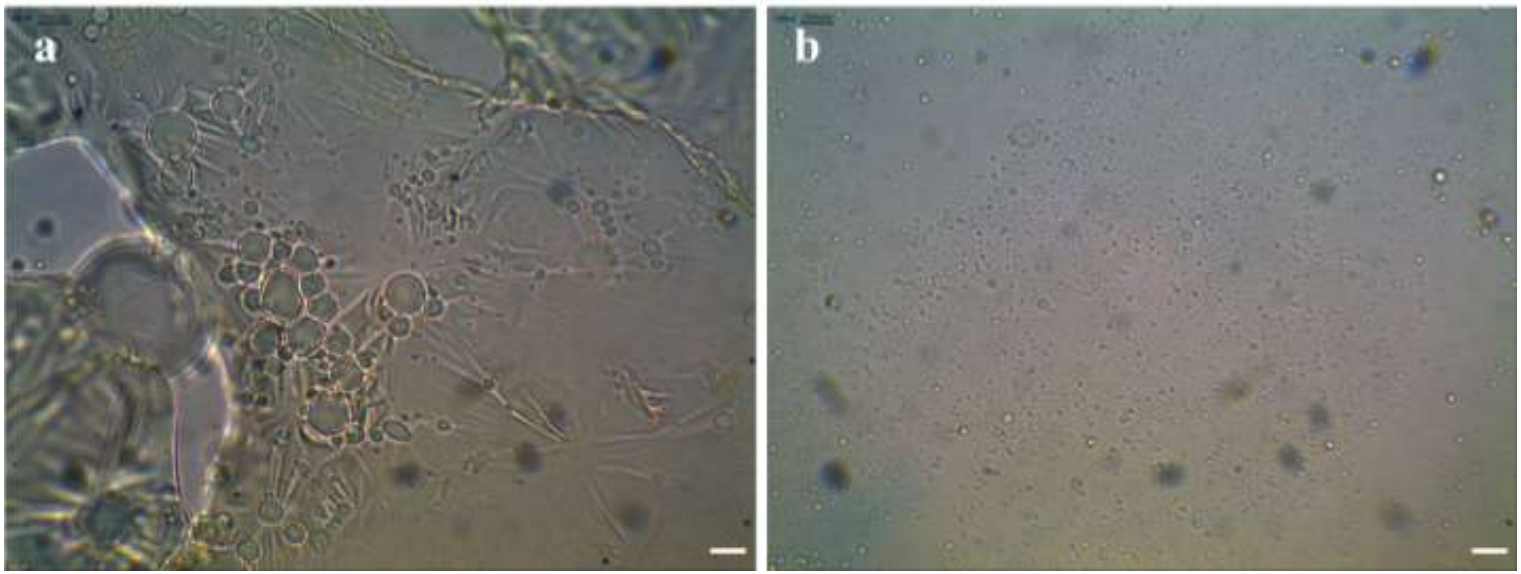

Figure 17. Optical microscope images of O-SNC-stabilized Pickering emulsions. Abbreviation: O-SNC, OSA-modified starch nanocrystals; a, optical microscope image of cream layer of O-SNC-stabilized Pickering emulsions; b, optical microscope image of dispersed layer of O-SNC-stabilized Pickering emulsions. Scale bar = 10 μm.

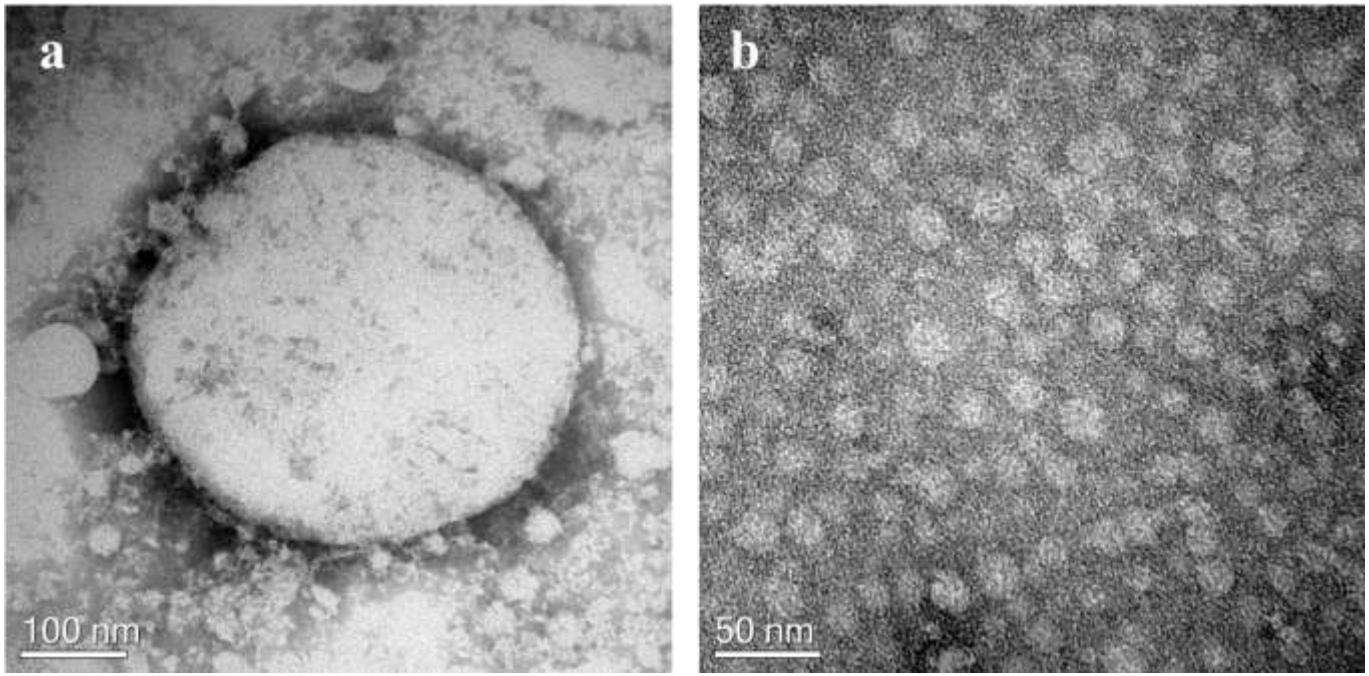

Figure 18. TEM images of O-SNC-stabilized Pickering emulsion and O-SNC. TEM images of O-SNC-stabilized Pickering emulsion (a) and O-SNC (b). Abbreviation: O-SNC, OSA-modified starch nanocrystals.

IV. CONCLUSIONS

In this study, the colloidal stable Pickering emulsions stabilized by food-grade starch based nanoparticles, OSA-modified waxy maize starch nanocrystals. Starch nanocrystals, difficult to isolate from native starch, was obtained by 3.16 M sulfuric acid hydrolysis for 6 days. The yield of starch nanocrystals was about 15.12 wt% (based on dry starch). This yield was rise sharply to about 22.36 wt% by breaking down a incompletely broken starch particles by using ultra-sonication (at 60% amplitude; duration times, 160 s; duty cycle, 1s on/1s off; in the 45 °C jacketed beaker). Esterification of starch nanocrystals with OSA was conducted in slightly basic aqueous media (20 wt% starch, pH 7.5-8.5). Degree of substitution was about 0.013. Dual wettability of OSA-starch nanocrystals to both aqueous phase and oil phase was confirmed by gel-trapping technique with PDMS. The best stable Pickering emulsions stabilized OSA-modified starch nanocrystals was obtained at starch nanocrystals-oil ratios (starch nanocrystals : tricaprylin = 1 : 20) and ultra-sonication duration time (5 min) in terms of emulsifying capacity of OSA-modified starch nanocrystals and emulsion droplet size. In addition, this Pickering emulsions had colloidal stability. In previous studies, almost food-grade particle stabilized Pickering emulsions had large size (dozens of

micrometer), so their applications were limited to foods like ice cream, mayonnaise, foam, and whipping cream. Furthermore, our Pickering emulsions with colloidal stability were also applied to the functional beverages for the delivery of lipophilic bioactive molecules.

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VI. 국문초록

Pickering 에멀션은 화학적 유화제를 사용하지 않는 대신 고체 입자를 이용해 안정화시킨 에멀션으로, 전통적인 에멀션에 비해 저장 안정성이 매우 뛰어나다. 최근에 식품 유래 입자인 단백질, 전분 등을 이용해 에멀션을 안정화하는 연구가 활발히 진행되고 있으나, 입자의 크기가 커 분산상태를 유지하지 못하므로 실제 식품에의 적용은 아이스크림이나 휘핑 크림 등에 국한된다. 본 연구에서는 20-50 nm 수준인 전분 나노결정을 이용하여 나노 수준의 입자크기와 교질 안정성을 갖는 Pickering 에멀션을 개발하였다. 산 가수분해 후에 전분 나노결정을 원심분리법으로 분리하고, 마이크로 입자에는 전분 나노결정이 호화되는 온도 직전까지 초음파를 처리해 가수분해 기간 동안 불완전하게 분리된 전분 나노결정을 추가로 분리해 전분 나노결정의 수율을 향상시켰다. 전분 나노결정의 소수성을 강화하기 위해 전분 나노결정에 옥테닐 호박산을 합성하여 옥테닐 호박산 전분 나노결정를 얻었고, 겔란 검과 실리콘을 이용해 양쪽 친매성을 확인하였다. 옥테닐 호박산 전분 나노결정을 이용하여

안정화한 Pickering 에멀션을 제조하고, 초음파 처리와 기름 대비 옥테닐 호박산 전분의 양 조절을 통하여 교질 안정성을 향상시켰다. 결론적으로, 본 연구에서 개발된 Pickering 에멀션은 기존 Pickering 에멀션의 저장 안정성에 교질 안정성까지 더해져 식품과 약품용 운반체 개발 등의 연구에 기초 역할을 할 수 있을 것이다.

핵심어: Pickering emulsion, 전분 나노결정, 교질 안정성

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