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The physicochemical properties of β-carotene emulsions stabilized by whey protein/octenyl succinic anhydride (OSA)-modified-starch complexes: Influence of OSA substitution degree of starch

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ABSTRACT

Complexes formed between heated whey protein isolate (HWPI) and octenyl succinic anhydride (OSA)-modified starches were prepared to stabilize β-carotene-containing oil-in-water emulsions. The zeta-potential, turbidity, particle size, and microstructure of the complexes were determined to evaluate the impact of the degree of substitution (DS) of OSA-modified starch on the complexes' structure. HWPI and OSA-modified starches with low DS values formed elongated complexes. With increasing DS, the particle size of the complexes reduced. In comparison to the emulsions stabilized by HWPI or OSA-modified starches, the emulsions stabilized by HWPI/OSAS complexes exhibited superior protection of β-carotene during storage under acidic conditions. When the DS of OSA-modified starch increased, the particle size of emulsions stabilized by the complexes decreased, with less droplet aggregation occurring. The physical stability of these emulsions against storage time, ions, and thermal process showed a positive relationship with the DS, while the β-carotene retention in the emulsions during storage showed a negative relationship with the DS. The greater physical stability of the complex-stabilized emulsions containing OSA-modified starch with a higher DS may be ascribed to enhanced electrostatic repulsion among oil droplets and the formation of a more rigid and denser surface structure in the presence of more OSA groups.

1. Introduction

Throughout the past decades, considerable research (Dickinson, 2008; Doublier et al., 2000; Goh et al., 2020; Niu et al., 2015; Patino & Pilosof, 2011; Schmitt & Turgeon, 2011; Ye, 2008) has been dedicated to understanding the protein-polysaccharide interactions, given their profound impact on food properties. Electrostatic complexes formed by these two macromolecules have attracted wide attention owing to their biological importance (Yao et al., 2016). Increasing interest has been generated in the use of protein-polysaccharide complexes for incorporation and protection of bioactive compounds, e.g., essential oils, flavors, probiotics, and nutraceuticals in foods (Devi et al., 2017; Du et al., 2022; Evans et al., 2013; Jain et al., 2015; Tan et al., 2023; Wijaya et al., 2019; Xiang et al., 2020; Xu et al., 2012; Yao et al., 2016).

Whey protein is widely utilized as an emulsifier, water-binding agent, gelling agent, and foaming agent in the food industry (Guo et al., 2014). Octenyl succinic anhydride (OSA)-modified starch is prepared by esterifying the hydroxyl groups of starch with OSA (Lin et al., 2018a; Sweedman et al., 2013). OSA-modified starch could be utilized as an emulsifier due to the presence of hydrophobic octenyl side chains and the hydroxyl groups (Torres et al., 2017). The incorporation of free carboxyl groups within OSA structures renders OSA-modified starch

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Abbreviations: HWPI, heated whey protein isolate; DS, degree of substitution; OSA, octenyl succinic anhydride; HWPI/OSAS, HWPI/OSA-modified starch; HWPI/OSAS1, complex between HWPI and OSAS1; HWPI/OSAS2, complex between HWPI and OSAS2; HWPI/OSAS3, complex between HWPI and OSAS3; HWPI/OSAS4, complex between HWPI and OSAS4.

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negatively charged under specific pH conditions, enabling electrostatic interactions with positively charged proteins (Wu et al., 2020).

Currently, extensive research has been conducted on the interactions between whey protein and non-starch polysaccharides, including pectin, Arabic gum, and carboxymethylcellulose (Jain et al., 2015; Koupantsis & Kiosseoglou, 2009; Salminen & Weiss, 2014; Yao et al., 2016; Zeeb et al., 2018). In contrast, the interaction between whey protein and OSA-modified starch has been the focus of only a few studies (Lin et al., 2021; Niu et al., 2023; Puerta-Gomez & Castell-Perez, 2016, 2017; Wu et al., 2020). Previously, we explored the complexation interactions between whey protein isolate (WPI) and OSA-modified starch under varying pH and protein/starch ratios (Wu et al., 2020). It was found that heated whey protein isolate (HWPI) exhibited significantly greater interaction with OSA-modified starches compared to unheated WPI. The optimal conditions for complexing HWPI with OSA-modified starch were found to be at pH 4.5 and a protein/starch ratio of 1/10. HWPI bound to OSA-modified starch mainly by electrostatic and hydrophobic interactions (Wu et al., 2020). Given that both WPI and OSA-modified starch serve as emulsifiers, it can be inferred that the complexes formed between them possess emulsifying capabilities. In another study, we successfully improved the stability and solubility of β-carotene by microencapsulating it within soluble complexes formed by HWPI and OSA-modified starch (Lin et al., 2021). Despite this success, the β-carotene within the soluble complex was not stable enough during storage under light. To improve the stability of lipophilic bioactive components, previous researchers have also tried to use protein-polysaccharide complex coacervates to encapsulate them in emulsions (Jain et al., 2015; Zhao et al., 2019). However, little information is available regarding the emulsifying capacity of complexes between HWPI and OSA-modified starches. A previous study reported that the complexes between unheated WPI and OSA-modified starch were effective for encapsulating *trans*-cinnamaldehyde in an emulsion (Puerta-Gomez & Castell-Perez, 2017). It was found that the entrapment efficiency of *trans*-cinnamaldehyde was positively related to the degree of substitution (DS) of OSA-modified starch. DS is a key structural characteristic of OSA-modified starch. Numerous studies have consistently reported that a higher DS contributes to increased emulsifying capacity and greater stability of emulsions stabilized by OSA-modified starch (Li et al., 2021; Lin et al., 2018a; Liu et al., 2018; Segura-Campos et al., 2008; Wang et al., 2010). Moreover, our recent study discovered that the DS of OSA-modified starch significantly influenced the complexation between HWPI and OSA-modified starch under pH 4.5 (Wu et al., 2020). A higher DS resulted in a smaller absorbance value and smaller particle of the complex, indicative of stronger electrostatic and hydrophobic interactions. These interactions effectively prevented further aggregation of WPI and resulted in the formation of smaller complexes (Wu et al., 2020). Therefore, we hypothesized that the DS of OSA-modified starch might impact the emulsification ability of the complex formed between HWPI and OSA-modified starch. However, to date, there are no reports addressing this aspect.

This study aimed to investigate the impact of DS of OSA-modified starch on both the structure and emulsification ability of the complex formed between HWPI and OSA-modified starch. β-carotene was selected as a representative nutraceutical. The stability of β-carotene emulsions coated by HWPI/OSA-modified starch (HWPI/OSAS) complex was examined under different environmental stresses (i.e., storage time, ionic strength, and temperature). This investigation aimed to shed light on the impact of DS of OSA-modified starches on the structural characteristics and emulsification performance of the complex, providing insights into its potential applications in preserving the stability of nutraceutical-loaded emulsions under various conditions.

2. Materials and methods

2.1. Materials

Corn oil was procured from Yonghui superstores (Hangzhou, China). WPI powder and native waxy maize starch were generously provided by Fonterra (Shanghai, China) and Ingredion (Auckland, New Zealand), respectively. OSA and β-carotene were purchased from Sigma-Aldrich (St. Louis, MO, USA). All additional chemicals were obtained from Sinopharm Chemical Reagent (Shanghai, China). Deionized water was utilized throughout experimental procedures.

2.2. Preparation of OSA-modified starch

According to the procedure outlined in our previous study (Lin et al., 2018a), four OSA-modified starches were synthesized through a 12 h acid hydrolysis of the waxy maize starch, followed by esterification with OSA at a concentration of 3%, 5%, 7%, and 9% relative to the dry starch weight. The obtained starches were named OSAS1, OSAS2, OSAS3, and OSAS4, respectively. The DS values of the starches were listed in Table 1, as measured using a titration method (Lin et al., 2018a). It's noteworthy that, although the US Food and Drug Administration stipulated in 1972 that the amount of OSA added to starch modification should not exceed 3% of the dry weight of starch, this study employed a broader range of OSA concentrations to achieve distinct DS values for investigative purposes.

2.3. Preparation and characterization of HWPI/OSAS complexes

2.3.1. Preparation

The preparation of HWPI/OSAS complexes followed the procedure detailed in our previous study (Wu et al., 2020). First, 5 g WPI were dissolved in 100 mL water overnight. Subsequently, the protein solution was heated at 90 °C with agitation for 20 min to generate the protein stock solution. In parallel, 20 g (dry weight) OSA-modified starch was dispersed in 100 mL water and then cooked at 95 °C with agitation for 20 min to generate the starch stock solution. Both polymer solutions were cooled in an ice bath for 5 min. The HWPI/OSAS complexes were obtained by mixing the protein solution and the starch solution at a protein/starch ratio of 1/10. A certain amount of water was added to adjust the concentrations of HWPI and OSA-modified starch in the mixture to 0.5%, and 5% (w/v), respectively. The pH of the mixture was set to 4.5 by adding 0.1 M HCl and 1.0 M NaOH. The obtained complexes between HWPI and OSAS1, OSAS2, OSAS3, and OSAS4 were named HWPI/OSAS1, HWPI/OSAS2, HWPI/OSAS3, and HWPI/OSAS4, respectively. Control samples, consisting of a 0.5% HWPI solution and four 5% OSA-modified starch solutions (OSAS1, OSAS2, OSAS3, and OSAS4 solution), were also prepared at pH 4.5. All samples were allowed to equilibrate at room temperature for 1 h before subsequent analysis.

2.3.2. Characterization

Consistent with our previous study (Wu et al., 2020), the turbidity values of the HWPI/OSAS complexes and the control solutions were evaluated by measuring the apparent absorbance at 515 nm using a

Table 1
DS values of OSA-modified starches.

Sample	OSA concentration(g/100 g starch)	DS
OSAS1	3	0.0170 ± 0.0028^{d}
OSAS2	5	0.0276 ± 0.0008^{c}
OSAS3	7	0.0354 ± 0.0023^{b}
OSAS4	9	0.0452 ± 0.0004^{a}

Note: Different superscript lowercase letters in the same column indicated significant differences ($P < 0.05$).

spectrophotometer (UV2100, Unico Instrument, Shanghai, China), with water employed as the reference.

The particle size and zeta-potential of the HWPI/OSAS complexes were measured without dilution using the Zetasizer Nano ZEN 5600 (Malvern Instruments Ltd, Worcestershire, UK).

Microstructural analysis of the HWPI/OSAS complexes was conducted using a light microscope (NE910, Ningbo Yongxin Optics, Ningbo, China). The WPI solution and the HWPI/OSAS complexes were stained with 10% fast green. Observations were carried out after placing a drop of the solution on a microscope slide and covering it with a coverslip.

2.4. *Preparation of β-carotene emulsions*

In the preparation process, 0.1% (w/w) β-carotene was dispersed in corn oil and agitated for 2 h. Subsequently, the oil phase was combined with the HWPI/OSAS complex solution or the control solutions at a ratio of 1/9 (w/w). The mixture was then homogenized at 20,000 rpm for 3 min using a high-speed homogenizer (Ultra-Turrax T18, IKA, Staufen, German). Following this, the coarse emulsion underwent further homogenization at a pressure of 50 MPa for 2 cycles using a microfluidizer (110P, Microfluidics, Westwood, MA, USA). To inhibit microorganism growth, the fine emulsions were treated with 0.02% sodium azide. Throughout the preparation, all the instruments and beakers were shielded with aluminum foil to prevent the samples from being exposed to light.

2.5. *Physicochemical stability of β-carotene emulsions*

2.5.1. *Storage stability*

Emulsions were stored in an incubator at 55 °C for 14 d. Throughout the storage period, samples were periodically withdrawn, and an appropriate amount of emulsion was taken for the measurement of particle size and β-carotene retention.

2.5.2. *Salt stability*

To examine the stability of the emulsions against ions, various concentrations of NaCl solutions (0.2 mL) were added to 5 mL of the fresh emulsions. The NaCl concentrations in the mixture were 0, 25, 50, 100, and 200 mM, respectively. The samples were then stood at room temperature for 17 h before measuring their particle size, zeta-potential, and microstructure.

2.5.3. *Thermal stability*

Fresh emulsions were subjected to heating at 60, 70, 80, and 90 °C for 30 min, respectively. The samples were subsequently cooled using an ice bath and stored at room temperature for 17 h before measuring their particle size and microstructure.

2.6. *Particle size of emulsions*

The particle size and particle size distribution of emulsions were determined using the Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). A refractive index of 1.46 was set for oil droplets and 1.33 was set for dispersion medium. An absorbance value of 0.001 was set for emulsion droplets.

2.7. *Zeta-potential of emulsions*

The zeta-potential of emulsions was determined by the Zetasizer Nano ZEN 5600 instrument. Before measurements, the emulsions were diluted in water or NaCl solutions (with the same concentration as the samples), and the pH was subsequently adjusted to 4.5.

2.8. *Microstructure of emulsions*

The microstructure of the fresh emulsions was examined by the NE910 microscope. For the emulsions treated with NaCl, or subjected to heating, observations were conducted using a confocal scanning laser microscope (Leica, Heidelberg, Germany). Prior to observation, samples were stained with Fast Green (with excitation at 633 nm) and Nile Red (with excitation at 488 nm).

2.9. *β-Carotene retention in emulsions during storage*

The extraction of β-carotene from emulsions was conducted following the procedure described in our previous study (Lin et al., 2018a), with minor adjustments. Briefly, a 0.1 g aliquot of emulsions was de-emulsified by vortexing with 1 mL dimethyl sulfoxide for 30 s. The resulting mixture was then treated with 5 mL n-hexane/dichloromethane (4/1, v/v) and vortexed for additional 30 s prior to centrifugation. The supernatant was analyzed at 450 nm with a spectrophotometer. β-Carotene content was measured by plotting absorbance versus concentration, and the retention of β-carotene was calculated using the formula below:

$$\text{Retention} = C_t/C_0 \times 100\% \tag{1}$$

where C_t and C_0 is the concentration of β-carotene in the emulsion on day t and day 0, respectively.

2.10. *Statistical analysis*

The results are present as the calculated means ± standard deviations. Statistical significances between means were assessed through a one-way analysis of variance, followed by Duncan's multiple-range test with a confidence level of 95%. The statistical analysis was conducted using the SPSS 19.0 software (IBM Inc, New York, NY, USA).

3. Results and discussion

3.1. *Influence of DS of OSA-modified starch on the structural properties of HWPI/OSAS complexes*

The zeta-potential of biopolymer solutions was determined (Table 2). At pH 4.5, the HWPI solution exhibited a positive charge, while all the OSA-modified starch solutions displayed a negative charge. With increasing DS, the magnitude of the zeta-potential of OSA-modified starch solutions increased, which was likely caused by an increasing number of carboxyl groups on the surface of starch micelles (Lin et al., 2018b). All the HWPI/OSAS complex solutions also showed negative charges, with each solution having a lower magnitude of zeta-potential compared to the corresponding starch solution (Table 2). This suggested the formation of electrostatic interactions between HWPI and

Table 2
The zeta-potential, turbidity, and particle size of the HWPI solution, OSA-modified starch solutions, and HWPI/OSAS complexes at pH 4.5.

Sample	Zeta-potential (mV)	Absorbance at 515 nm	Z-average diameter (nm)
HWPI	11.47 ± 1.81^{a}	1.461 ± 0.044^{c}	1818.7 ± 482.1^{c}
OSAS1	-3.10 ± 0.10^{c}	0.093 ± 0.003^{e}	117.3 ± 25.6^{d}
OSAS2	-3.82 ± 0.42^{cd}	0.080 ± 0.001^{e}	136.5 ± 47.3^{d}
OSAS3	-4.78 ± 0.30^{de}	0.065 ± 0.002^{ef}	94.7 ± 6.1^{d}
OSAS4	-5.59 ± 0.22^{e}	0.047 ± 0.002^{f}	64.5 ± 3.6^{d}
HWPI/OSAS1	-0.27 ± 0.04^{b}	2.102 ± 0.004^{a}	3866.7 ± 937.3^{a}
HWPI/OSAS2	-3.10 ± 0.21^{c}	2.103 ± 0.010^{a}	2604.7 ± 844.9^{b}
HWPI/OSAS3	-3.07 ± 0.19^{c}	1.832 ± 0.003^{b}	1153.3 ± 93.0^{c}
HWPI/OSAS4	-3.50 ± 0.16^{c}	0.214 ± 0.002^{d}	123.9 ± 1.9^{d}

Note: Different superscript lowercase letters in the same column indicated significant differences ($P < 0.05$).

OSA-modified starch at pH 4.5. Furthermore, as the DS increased, the magnitude of the zeta-potential of the complex solutions also increased. This observation implied that the electrostatic interactions between HWPI and OSA-modified starch became more pronounced with higher DS values.

Turbidity of the polymer solutions was determined using the apparent absorbance at 515 nm, as indicated in Table 2. The turbidity values of OSA-modified starch solutions at pH 4.5 decreased significantly ($P < 0.05$) with an increase in the DS, consistent with previous findings (Wu et al., 2020). At pH 4.5, the HWPI solution exhibited considerably higher turbidity compared to OSA-modified starch solutions, possibly due to its aggregation. Upon heat treatment, whey proteins unfolded, exposing more hydrophobic groups. The heated whey proteins aggregated at a pH (4.5) close to their pI (~5.0), driven by weak electrostatic attraction and strong hydrophobic interactions (Bryant & McClements, 2000; Wu et al., 2020). As the DS increased, the turbidity values of the HWPI/OSAS complex solutions decreased significantly ($P < 0.05$), following a similar trend to that of starch solutions. In comparison to HWPI alone, the mixture of HWPI and OSAS1, OSAS2, and OSAS3 exhibited higher absorbance values, while the mixture of HWPI and OSAS4 had a lower absorbance value. The high turbidity values of the mixtures of HWPI and OSAS1, OSAS2, and OSAS3 were attributed to the formation of insoluble complexes through complex coacervation, whereas the low turbidity of the mixture of HWPI and OSAS4 starch could be attributed to the formation of soluble complexes.

Table 2 shows that the Z-average diameter of the HWPI solution at pH 4.5 was approximately 1800 nm, indicating the formation of large aggregates of HWPI. As demonstrated in our previous study, OSA-modified starch has the ability to self-assemble into micelles at pH 4.5 (Lin et al., 2021). OSA-modified starches formed micelles, and the Z-average diameter of these micelles decreased with an increase in the DS values (Table 2). HWPI/OSAS complexes exhibited significantly larger particle size compared to the starch micelles, and their particle size decreased with an increase in DS. In comparison to the HWPI aggregates, the average particle size of the complex containing OSAS1 and OSAS2 was larger, while the average particle size values of the complexes containing OSAS3 and OSAS4 were lower. Generally, the particle size results were consistent with the turbidity results.

Fast green was added to the biopolymer solutions to stain protein and then observed under a light microscope. As shown in Fig. 1, at pH 4.5, many HWPI molecules clustered together, forming irregular structures with large size. This observation further supports the occurrence of mutual aggregation of HWPI molecules at pH 4.5, driven by electrostatic attraction and hydrophobic interaction. The mixture solutions of HWPI and OSAS1, OSAS2, and OSAS3 displayed evenly distributed elongated aggregates (Fig. 1), with the particle size of the aggregates decreasing as the DS increased. No aggregates were observed in the mixture of HWPI and OSAS4 (image not shown). For HWPI/OSAS4, the number mean diameter measured by the Zetasizer was approximately 20 nm, rendering it difficult to observe under the light microscope. As reported previously, the HWPI displayed as elongated particles at pH 7.0, and formed small aggregates below pH 6 before larger ones appeared at pH values close to their pI (Wu et al., 2020). During the pH adjustment, OSA-modified starch interacted with whey protein by electrostatic attraction and hydrophobic interaction, and the steric hindrance introduced by OSA-modified starch prevented further aggregation of whey protein. As the DS value of OSA-modified starch increased, electrostatic and hydrophobic interactions with HWPI were enhanced, resulting in an increased capacity to inhibit HWPI aggregation and the formation of a smaller complex (Wu et al., 2020).

3.2. Influence of DS of OSA-modified starch on the physical properties of β-carotene emulsion stabilized by HWPI/OSAS complexes

Table 3 shows that the emulsion stabilized by HWPI exhibited a positive charge, with the zeta-potential value approaching zero at pH 4.5 (close to pI of WPI). In contrast, the emulsions stabilized by OSA-modified starches and HWPI/OSAS complexes were negatively charged at this pH. The magnitudes of the zeta-potential increased with the DS, following the same trend observed for the magnitude of the zeta-potential of the HWPI/OSAS complexes and starch micelles (Table 1).

The particle size and particle size distribution of emulsions stabilized by HWPI, OSA-modified starches, and HWPI/OSAS complexes are shown in Table 3 and Fig. 2, respectively. The emulsion stabilized by HWPI exhibited the largest particle size, characterized by a bimodal particle size distribution. The four emulsions stabilized by OSA-modified

Table 3
The zeta-potential and particle size ($D_{4,3}$) of the emulsions stabilized by HWPI, OSA-modified starches, and HWPI/OSAS complexes.

Sample	Zeta-potential (mV)	$D_{4,3}$ (μm)
HWPI	4.03 ± 1.56^{a}	5.83 ± 0.71^{a}
OSAS1	-21.36 ± 2.15^{c}	0.21 ± 0.02^{b}
OSAS2	-27.87 ± 1.13^{d}	0.19 ± 0.01^{b}
OSAS3	-28.08 ± 2.23^{d}	0.21 ± 0.02^{b}
OSAS4	-30.26 ± 1.78^{e}	0.24 ± 0.01^{b}
HWPI/OSAS1	-18.33 ± 0.40^{b}	5.81 ± 0.99^{a}
HWPI/OSAS2	-19.07 ± 1.02^{b}	0.31 ± 0.01^{b}
HWPI/OSAS3	-21.58 ± 0.84^{c}	0.24 ± 0.01^{b}
HWPI/OSAS4	-22.73 ± 0.67^{c}	0.25 ± 0.03^{b}

Note: Different superscript lowercase letters in the same column indicated significant differences ($P < 0.05$).

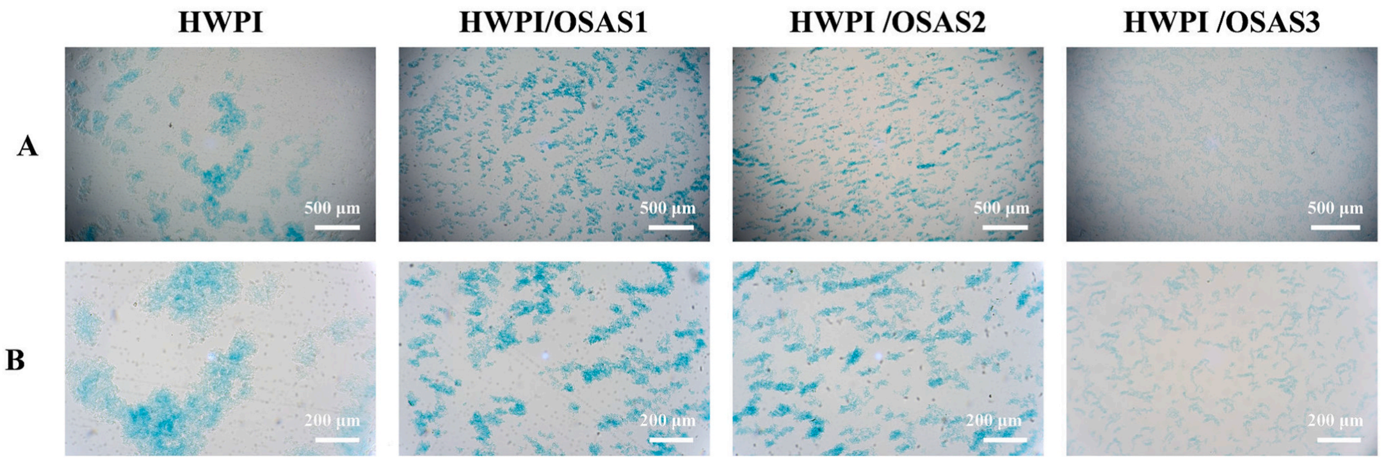

Fig. 1. Microstructure of the HWPI solution, and the HWPI/OSAS complexes: (A) magnification of 40 × ; (B) magnification of 100 × .

starches had similar $D_{4,3}$ values (approximately 0.2 μm) with monomodal distribution, consistent with our previous results (Lin et al., 2018a). Compared to the emulsion stabilized by HWPI, the emulsion stabilized by HWPI/OSAS1 had a similar average particle size (Table 3) but with a distinct particle size distribution (Fig. 2). As the DS of OSA-modified starch increased, a significant reduction in particle size was observed in the emulsions stabilized by HWPI/OSAS complexes. With the exception of the HWPI/OSAS1-stabilized emulsion, each HWPI/OSAS complex-stabilized emulsion had a particle size smaller than that of the HWPI-stabilized emulsion and slightly larger than that of the corresponding OSA-modified-starch-stabilized emulsion.

The microstructural images of the fresh emulsions are shown in Fig. 2. The HWPI emulsion exhibited extensive flocculation of small oil droplets, leading to the coalescence of some droplets into larger ones. Conversely, the emulsions stabilized by OSA-modified starches displayed a uniform distribution of small oil droplets. The emulsion stabilized by HWPI/OSAS1 displayed large flocs consisting of numerous small oil droplets, along with a few larger oil droplets located at the periphery of these flocs. Similarly, the emulsion stabilized by HWPI/OSAS2 displayed the formation of multiple flocs, although a few isolated small oil droplets were still present. Conversely, the emulsions stabilized by HWPI/OSAS3 and HWPI/OSAS4 demonstrated the presence of very small oil droplets that were uniformly distributed without any floc formation.

The analysis of particle size and microstructure revealed that HWPI exhibited a low emulsifying capacity, while OSA-modified starches demonstrated fairly high emulsifying capacities at pH 4.5. During homogenization, the large aggregates of HWPI molecules were unable to sufficiently cover the oil droplets interface, leading to bridging flocculation and some coalescence. Additionally, the surface of oil droplets in the HWPI-stabilized emulsion was close to electrically neutral, leading to electrostatic attraction between oil droplets and subsequent aggregation. Conversely, the OSA-modified-starch-stabilized emulsions exhibited good stability under acidic conditions, which could be because of the steric hindrance created by the branched amylopectin chains (Chanamai & McClements, 2002; Sweedman et al., 2013). Compared to HWPI, the HWPI/OSAS complexes exhibited significantly improved emulsifying capacity. Furthermore, their emulsifying capacity was enhanced when the DS of OSA-modified starch increased. Several factors may have contributed to this phenomenon. Firstly, an increase in the DS of OSA-modified starch led to a reduction in the particle size of the complexes. At pH 4.5, the insoluble complexes formed between HWPI and OSAS1 or OSAS2 were not able to completely cover the droplet interface during homogenization, resulting in flocculation and coalescence into larger oil droplets (Fig. 2). Conversely, the complexes formed between HWPI and OSAS3 or OSAS4 exhibited smaller particle size, allowing complete coverage of oil droplets during homogenization. Additionally, a higher DS value resulted in a greater abundance of hydrophilic carboxylate groups and hydrophobic alkenyl long chains in the OSA-modified starch, potentially enhancing the emulsifying capacity of the HWPI/OSAS complex. Moreover, an increase in DS led to an augmentation in the net charge on the surface of the oil droplets stabilized by the HWPI/OSAS complex (Table 3), thereby intensifying the electrostatic repulsion between them. As a consequence, the emulsion' stability against droplet flocculation and coalescence during homogenization enhanced with the increasing DS of the OSA-modified starch.

3.3. *Stability of β-carotene emulsions*

3.3.1. *Storage stability*

The physicochemical stabilities of emulsions were evaluated through an accelerated test at 55 °C for 14 d. After 1 d storage, the $D_{4,3}$ of the HPWI-stabilized emulsion increased markedly to 36.13 ± 4.41 μm (Fig. 3A), accompanied by phase separation (Fig. 3C). Subsequently, its $D_{4,3}$ gradually increased to 92.33 ± 13.24 μm at the end of storage. In contrast, the OSA-modified-starch-stabilized emulsions exhibited negligible variations in particle size and were free from phase separation throughout storage (Fig. 3C). On the other hand, emulsions stabilized by HWPI/OSAS1, HWPI/OSAS2, and HWPI/OSAS3 experienced a substantial increase in $D_{4,3}$ within the initial 2 days, with the magnitude of

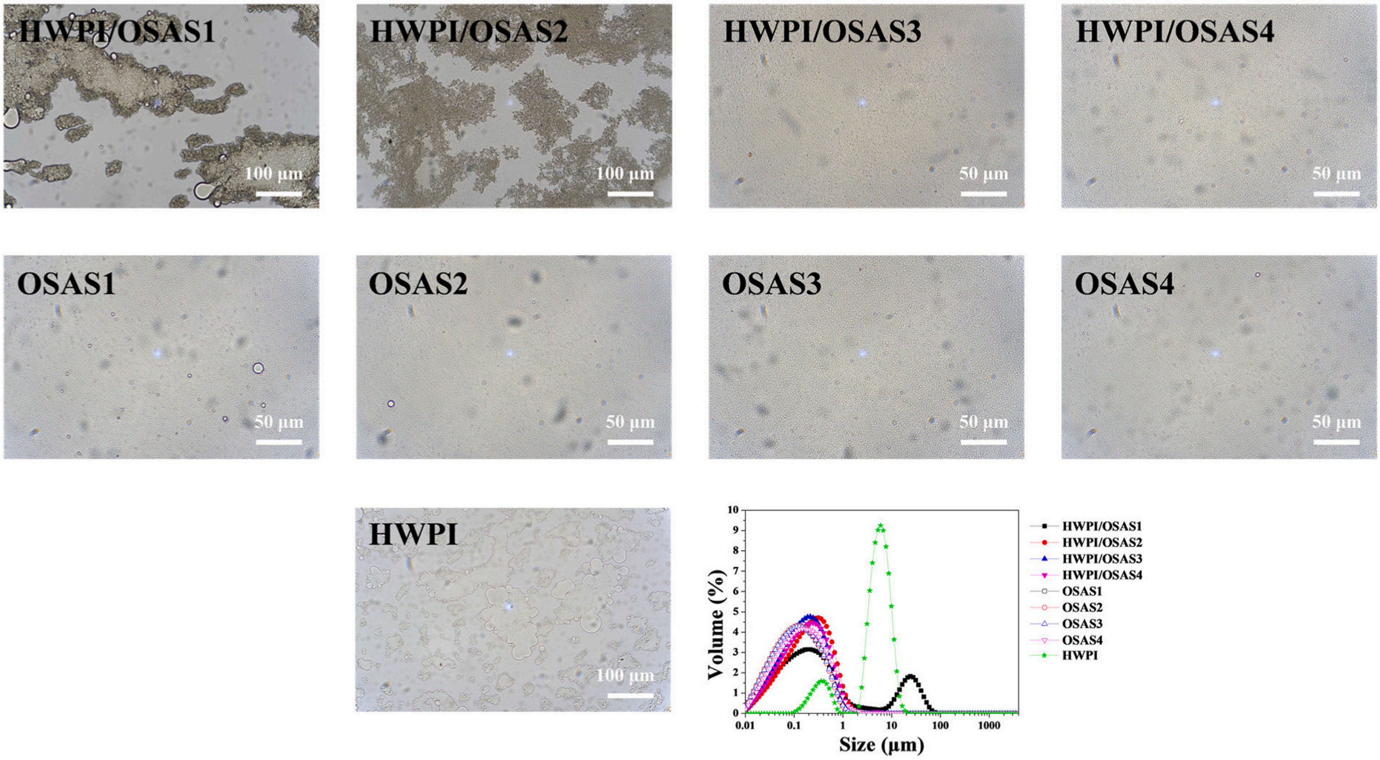

Fig. 2. Microstructure and particle size distribution of the fresh emulsions stabilized by HWPI/OSAS complexes, OSA-modified starches and HWPI.

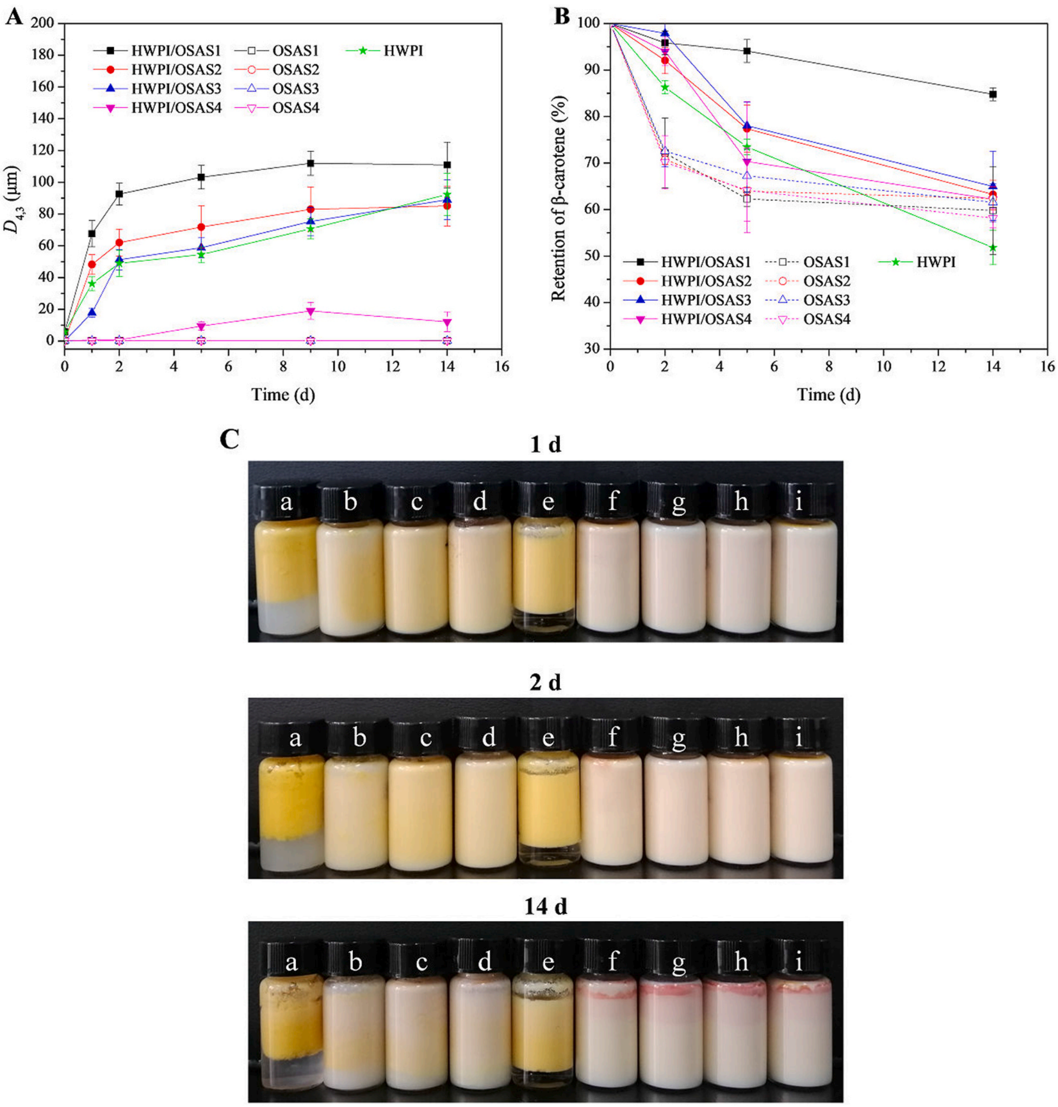

Fig. 3. The particle size (A), retention of β-carotene (B), and appearance (C) of the emulsions as a function of storage time at 55 °C: a-i in the appearance images present the emulsions stabilized by HWPI/OSAS1, HWPI/OSAS2, HWPI/OSAS3, HWPI/OSAS4, HWPI, OSAS1, OSAS2, OSAS3, and OSAS4, respectively.

this increase diminishing as the DS increased (Fig. 3A). Phase separation occurred in the HWPI/OSAS1-stabilized and HWPI/OSAS2-stabilized emulsions after 1 d storage, while a similar phenomenon occurred in the HWPI/OSAS3-stabilized emulsion after 2 d storage. Subsequently, these three emulsions experienced a gradual rise in particle size, with the $D_{4,3}$ values of the HWPI/OSAS1-stabilized and HWPI/OSAS2-stabilized emulsions remaining constant after storage for 9 d. However, the HWPI/OSAS3-stabilized emulsion exhibited a continuous rise in $D_{4,3}$ throughout the entire 14 d storage period. In the case of the HWPI/OSAS4-stabilized emulsion, there was a slight rise in particle size within the first 2 d, followed by a significant increase to 9.46 ± 2.56 μm after 5 d, maintaining a relatively constant size thereafter. Notably, the HWPI/OSAS4-stabilized emulsion did not exhibit noticeable phase separation during the 14 d storage period. The observed increase in particle size in the HWPI/OSAS-3-stabilized and HWPI/OSAS-4-stabilized emulsions during storage might be because of the depletion flocculation induced by unabsorbed complexes present in the aqueous phase. After a storage period of 14 d, the $D_{4,3}$ of the complex-stabilized emulsions reduced in the following order: HWPI/OSAS1 $>$ HWPI/OSAS2 $\geq$ HWPI/OSAS3 $>$ HWPI/OSAS4.

The findings regarding particle size and appearance suggested that the HWPI-stabilized emulsion exhibits low stability during storage. The rise in particle size might be attributed to the flocculation and coalescence of oil droplets at a pH close to the pI of WPI. In contrast, the OSA-modified-starch-stabilized emulsions exhibited substantial physical stability under acidic conditions, consistent with findings from a previous study (Chanamai & McClements, 2002). The primary mechanism behind this stability involves steric hindrance from the interfacial film formed by starch amylopectin, remaining unaffected by pH and temperature (Sweedman et al., 2013). Moreover, the HWPI/OSAS4-stabilized emulsion demonstrated superior physical stability during storage compared to the HWP-stabilized emulsion. Additionally, the physical stability of the emulsions stabilized by HWPI/OSAS complexes exhibited a positive correlation with the DS of OSA-modified starch. A higher DS value resulted in the enhanced hydrophobic interaction between the OSA-modified starch, oil droplets, and HWPI in the emulsions stabilized by the HWPI/OSAS complexes. This interaction potentially hindered the bridging flocculation between oil droplets. Furthermore, as shown in Fig. 1, the complex became smaller and denser with increasing DS, resulting in a more rigid and denser surface structure for complex-stabilized emulsions containing OSA-modified starch with a higher DS value. This modified structure provided enhanced stability against droplet flocculation and coalescence. Additionally, the net charge on the surface of complex-stabilized emulsions increased slightly with an increase in DS (Table 3), thereby conferring greater stability to the emulsion against aggregation during storage.

As shown in Fig. 3B, the β-carotene retention in emulsions exhibited a gradual decline over time. After storage for 2 days, the emulsions stabilized by HWPI/OSAS complexes retained over 90% of β-carotene, while those stabilized by OSA-modified starches and HWPI exhibited lower retention levels, dropping below 75% and 90%, respectively. The rapid degradation of β-carotene in OSA-modified-starch-stabilized emulsions during storage was probably attributed to their small particle size. Previous studies (Lee et al., 2011; Yi et al., 2014) have demonstrated that the emulsions with smaller particle size exhibit higher degradation of lipophilic components during storage, owing to the larger specific surface areas of oil droplets that facilitate the reaction between β-carotene and oxygen. It was found that DS had no significant influence on the retention of β-carotene in the OSA-modified-starch-stabilized emulsions, possibly due to the similar size of oil droplets. After 5 days of storage, complex-stabilized emulsions exhibited a reduction in the β-carotene retention in the following order: HWPI/OSAS1 > HWPI/OSAS2 ≈ HWPI/OSAS3 > HWPI/OSAS4. This suggested that as the DS increased, the retention of β-carotene in the complex-stabilized emulsions decreased, which was in contrast to the observed trend in the particle size of emulsions. Ultimately, the HWPI/OSAS1-stabilized emulsion retained the most β-carotene, while HWPI-stabilized emulsions retained the least. Each complex-stabilized emulsion exhibited relatively higher β-carotene retention compared to the corresponding OSA-modified-starch-stabilized emulsion. In comparison to the HWPI-stabilized emulsion, complex-stabilized emulsions displayed thicker and more compact interface layers. These characteristics proved advantageous in safeguarding β-carotene against oxidative

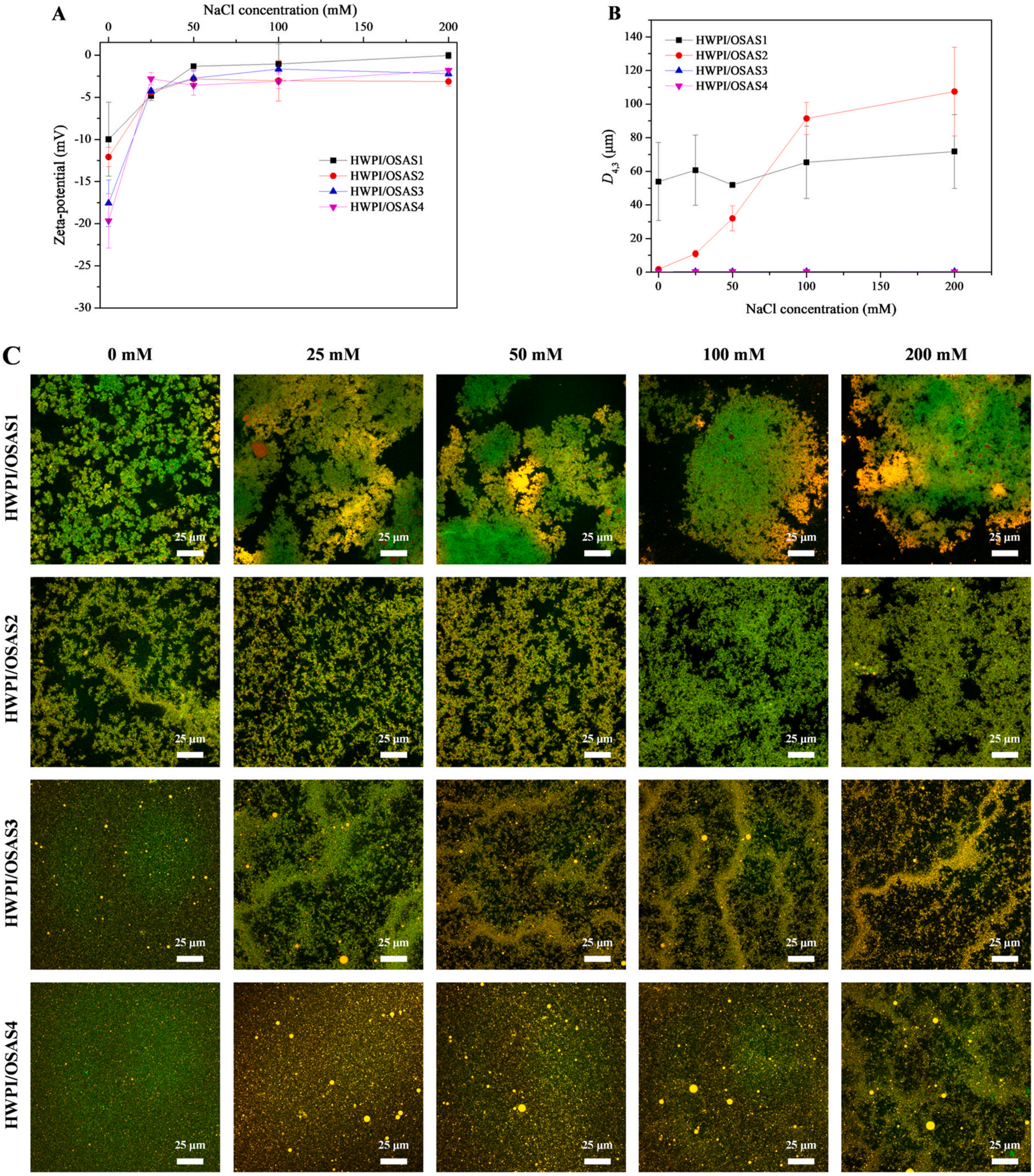

Fig. 4. The zeta-potential (A), particle size (B), and microstructure (C) of the emulsions stabilized by HWPI/OSAS complexes as a function of NaCl concentration.

degradation, consequently decelerating the degradation process of β-carotene. The HWPI/OSAS1-stabilized emulsion exhibited a higher retention of β-carotene compared to the other complex-stabilized emulsions. This phenomenon was probably attributed to its larger particle size and the formation of a three-dimensional network structure consisting of numerous flocs. These findings suggested that emulsions stabilized by HWPI/OSAS complexes offer superior protection for β-carotene during storage, compared to emulsions stabilized solely by HWPI or OSA-modified starches.

3.3.2. Salt stability

To investigate the impact of the DS of OSA-modified starch on the salt stability of emulsions stabilized by HWPI/OSAS complexes, the zeta-potential and particle size of emulsions were determined subsequent to the addition of NaCl. The magnitudes of zeta-potential of all emulsions exhibited a significant decrease as the salt concentration rose from 0 to 25 mM, and the values did not change as the salt concentration further increased to 200 mM (Fig. 4A). Compared to the initial emulsions (Fig. 2A), those stabilized by HWPI/OSAS1 and HWPI/OSAS2 exhibited an increase in particle size at room temperature, with this increase diminishing as the DS increased (Fig. 4B). Conversely, the emulsions stabilized by HWPI/OSAS3 and HWPI/OSAS4 maintained a relatively constant particle size. With the addition of NaCl, the emulsion stabilized by HWPI/OSAS1 experienced a slight, albeit statistically insignificant ($P > 0.05$), increase in particle size (Fig. 4B). The $D_{4,3}$ of the emulsion stabilized by HWPI/OSAS2 increased markedly from 1.73 ± 0.49 to 91.42 ± 9.61 μm as the salt concentration increased from 0.0 to 100 mM, plateauing at 200 mM NaCl. Conversely, the particle size of the emulsions stabilized by HWPI/OSAS3 and HWPI/OSAS4 remained relatively constant in the NaCl concentration range of 0–200 mM.

Fig. 4C illustrates changes in emulsion microstructure resulting from NaCl treatment. Oil droplets aggregated significantly in the emulsions stabilized by HWPI/OSAS1 and HWPI/OSAS2, which gradually diminished with increasing DS of OSA-modified starch. Conversely, the emulsion stabilized by HWPI/OSAS3 and HWPI/OSAS4 exhibited small and evenly distributed droplets without any aggregation. The addition of NaCl induced the formation of three-dimensional networks in the emulsions stabilized by HWPI/OSAS1 and HWPI/OSAS2, with tightness increasing proportionally to ion concentration. For HWPI/OSAS3-stabilized emulsions, the complex particles induced a typical depletion flocculation phenomenon, with microstructure remaining relatively stable despite variations in ion concentration. Conversely, the microstructure of the HWPI/OSAS4-stabilized emulsion remained mostly unchanged up to 100 mM NaCl, with only a limited emergence of larger oil droplets (~5 μm). However, depleted flocs appeared after the addition of 200 mM NaCl. The occurrence of depletion flocculation in the emulsions stabilized by HWPI/OSAS3 and HWPI/OSAS4 can be attributed to the existence of non-adsorbing complexes in the continuous phase, causing oil droplets to approach each other and facilitating floc formation. The introduction of NaCl expedited the process of depletion flocculation in these two emulsions.

Based on the aforementioned findings, it can be concluded that the salt stability of the complex-stabilized emulsion showed a positive relationship with the DS of OSA-modified starch. The introduction of NaCl induced a decrease in the surface charge of oil droplets, primarily due to electrostatic screening. As a result, the electrostatic repulsion between droplets reduced, thereby reducing their ability to counteract attractive forces and resulting in the aggregation of oil droplets and the formation of larger flocs (Lin et al., 2018b). At NaCl concentrations equal to or greater than 25 mM, the net charge values of all emulsions approached zero (Fig. 4A), indicating that the electrostatic repulsion alone was insufficient to stabilize the emulsions. It can be inferred that steric repulsion played a predominant role in emulsion stabilization. The difference in emulsion salt stability might be due to the differences in the steric repulsion formed by the complexes. As observed in Fig. 1, an increase in the DS of OSA-modified starch resulted in a smaller and denser complex. Consequently, higher DS values led to a more rigid and compact surface structure in the complex-stabilized emulsion, offering greater stability against the droplet flocculation and coalescence. A similar result was found in our recent study (Lin et al., 2018a); i.e., the salt stability of OSA-modified-starch-stabilized emulsion at low pH increased with an increase in the DS. The increase in stability was attributed to the formation of a more rigid and compact surface structure with a larger number of OSA groups (Lin et al., 2018a).

3.3.3. Thermal stability

Numerous studies have demonstrated that the WPI-stabilized emulsion exhibit low thermal stability as the pH approaches the pI of WPI (Demetriades et al., 1997; Dickinson & Parkinson, 2004; Setiowati et al., 2017). The present study aimed to examine whether the complexation with OSA-modified starch could enhance the thermal stability of WPI-stabilized emulsion at pH 4.5.

In Fig. 5A, it is evident that the emulsions stabilized by HWPI/OSAS1 and HWPI/OSAS2 exhibited an increase in $D_{4,3}$ as the temperature increased. However, the particle size of the emulsion stabilized by HWPI/OSAS3 kept fairly stable upon heating to 80 °C but experienced a slight increase to 1.23 ± 0.12 μm after heat treatment at 90 °C. For the emulsion stabilized by HWPI/OSAS4, heat treatment within the 60–90 °C range did not cause any significant changes in particle size. The morphologies of emulsions after heat treatment are shown in Fig. 5B. Extensive flocculation and coalescence happened in the emulsions stabilized by HWPI/OSAS1 and HWPI/OSAS2, leading to the formation of large irregular oil droplets after heating above 60 and 70 °C, respectively. For the emulsion stabilized by HWPI/OSAS3, depletion flocculation occurred after heating at 60–80 °C and large emulsion flocs formed after heating at 90 °C. The emulsion stabilized by HWPI/OSAS4 showed minimal changes in the microstructure upon heating at 60–90 °C. The microstructural results were in agreement with the particle size measurements, both indicated that the thermal stability of the complex-stabilized emulsions enhanced when the DS of OSA-modified starch increased. The primary mechanism responsible for the thermal stability of emulsions was the electrostatic and steric repulsion exerted by the complex at the droplet surface. A higher DS value of OSA-modified starch led to a stronger electrostatic repulsion between oil droplets. Moreover, a higher DS value favored the formation of a more rigid and compact surface structure by the complex, providing better stability against the aggregation of oil droplets. These two reasons explained why increasing the DS of OSA-modified starch contributed to greater thermal stability in complex-stabilized emulsions. The findings indicated that the complexation between HWPI and OSA-modified starch with a relatively high DS value had the potential to enhance the thermal stability of the HWPI-stabilized emulsion. However, the OSA-modified starch with the highest DS level in the present study was not allowed to be used in food by the FDA, which required further safety evaluation in the future.

4. Conclusion

In this study, complexes of HWPI and OSA-modified starch were developed to stabilize β-carotene emulsions. The impact of the DS of OSA-modified starch on the structure of the complex and the physicochemical characteristics of the complex-stabilized emulsions were investigated. Electrostatic interaction occurred between HWPI and OSA-modified starch at pH 4.5. The analysis of turbidity, particle size, and microstructure indicated that the interaction strength between HWPI and OSA-modified starch increased with increasing DS values, resulting in the formation of smaller complexes. The emulsifying capacity of HWPI/OSAS complexes improved when the DS of OSA-modified starch increased. With a higher DS value, the complex-stabilized emulsion exhibited a smaller particle size and enhanced physical stability against heating, ions, and storage time. However, the accelerated test results indicated a negative correlation between the DS of OSA-modified starch

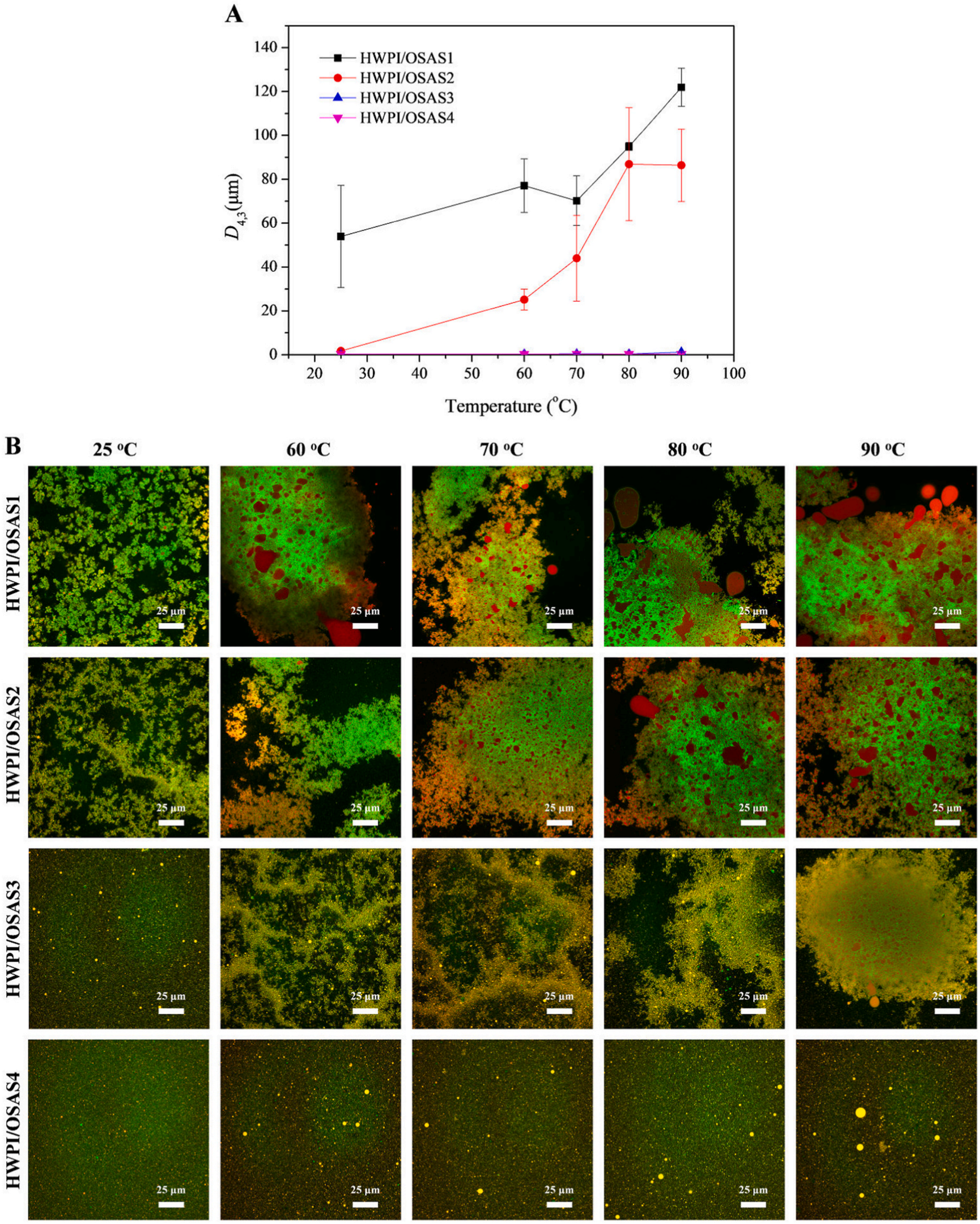

Fig. 5. The particle size (A) and microstructure (B) of the emulsions stabilized by HWPI/OSAS complexes as a function of temperature.

and the retention of β-carotene in the complex-stabilized emulsions. Lower DS values were associated with higher β-carotene retention, likely due to the smaller surface area of oil droplets and the formation of a three-dimensional network structure composed of flocs. In comparison to the emulsions stabilized solely by HWPI or OSA-modified starches, the emulsions stabilized by HWPI/OSAS complexes exhibited superior protection for β-carotene during storage under acidic conditions. The findings from this work are anticipated to have practical implications for utilizing the HWPI/OSAS complexes in the design of food emulsions with desirable physicochemical stability.

CRediT authorship contribution statement

Quanquan Lin: Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Xinyue Yang:** Investigation, Formal analysis, Data curation. **Yefei Liu:** Investigation, Formal analysis, Data curation. **Yujie Lu:** Investigation, Formal analysis, Data curation. **Weilin Liu:** Writing – review & editing, Methodology. **Jianzhong Han:** Supervision, Resources. **Harjinder Singh:** Writing – review & editing. **Aiqian Ye:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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